Understanding neuromuscular responses to pharmacological and exercise interventions: Manipulating motor unit plasticity in humans to improve muscle function.

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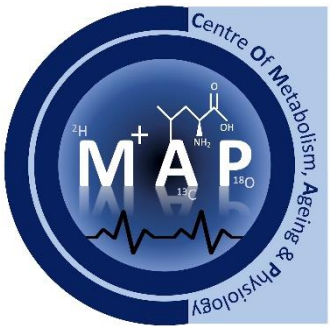

Supervisors

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Abstract

Sarcopenia, the loss of muscle mass and strength observed with ageing, presents a challenge to the growing ageing population and healthcare services. Motor unit (MU) numbers decrease with age but have remodelling potential, with the ability to rescue denervated fibres via axonal sprouting and reinnervation. Exercise interventions targeting the ageing musculoskeletal system, particularly resistance exercise training (RET), have showed promise in ameliorating declining function, however, blunted hypertrophic responses in the muscles of the older population demonstrate the need for further investigation of the neuromuscular system to develop optimal interventions.

In Chapter 2 of this thesis, intramuscular and high-density surface electromyography (EMG) were used to assess the effects of concentric (CON) and eccentric (ECC) loading on MU properties. Only ECC led to an increase in MU firing rate (FR) while changes to membrane excitability were observed with CON only. These findings demonstrates that MU features are altered following exercise-induced fatigue in an exercise modality dependant manner which is important when considering interventional strategies.

Chapter 3 further investigated physiological parameters associated with muscle fatigue, specifically the effects of an isometric fatiguing contraction on MU properties (via EMG), and muscle microvascular blood flow (MBF) using contrast enhanced ultrasound (CEUS). During the contraction, FR initially decreased before increasing in the later phase accompanied by increases in common synaptic input to motoneurons, and force variability. No change in MBF was observed, indicating this was not responsible for MU or functional changes with fatigue.

In Chapter 4, the effect of 6-weeks unilateral RET in older males on force control and underlying MU properties was assessed. RET resulted in increased strength (via 1-repetition maximum) and improved knee extensor force control. However, no changes in FR variability or common inputs to the vastus lateralis were observed, indicating an alternative mechanism is responsible for these beneficial effects of RET.

To follow on, in Chapter 5 the effect of 6-weeks unilateral RET on MU properties with or without administration of a mammalian target of rapamycin (mTOR) inhibitor (Rapamune) in older people was investigated. Rapamune did not prevent exercise-induced strength gains or MU remodelling but did prevent reductions in MU potential

size and neuromuscular junction transmission instability which was observed in the placebo group.

Overall, the findings of this work provide fundamental information on changes in MU properties during and in response to different acute and chronic exercise challenges in older adults, including RET with mTOR inhibition. This is important knowledge for developing future exercise interventions targeting sarcopenia.

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Abbreviations

1-RM – 1-repetition maximum

AA – amino acid

ACh – acetylcholine

AChR – acetylcholine receptor

AI – acoustic index

ANOVA – analysis of variance

ATP – adenosine triphosphate

AUC – area under the curve

BDNF – brain derived neurotrophic factor

BMI – body mass index

CEUS – contrast-enhanced ultrasound

CI – confidence interval

CNS – central nervous system

CON – concentric

CoV – coefficient of variation

CSA – cross sectional area

CST – cumulative spike train

DE – drug effect

DQEMG – decomposition based quantitative electromyography

DXA – dual-energy x-ray absorptiometry

ECC – eccentric

ECG – electrocardiogram

EET – endurance exercise training

EMG – electromyography

EWGSOP – European Working Group on Sarcopenia in Older People

FGFBP1 – fibroblast growth factor binding protein 1

FOXO – fork-head box O

FR – firing rate

FS – force steadiness

GDNF – glial cell line derived neurotrophic factor

GFAP – glial fibrillary acidic protein

GH – growth hormone

GSK3B – glycogen synthase kinase-3B

HD-sEMG – high-density surface electromyography

HIIT – high intensity interval training

iEMG – intramuscular electromyography

IGF – insulin-like growth factor

Int – interaction

IPAQ – international physical activity questionnaire

ISI – interspike interval

Lrp-4 - Low-density lipoprotein receptor-related protein 4

MBF – microvascular blood flow

MBV – microvascular blood volume

MFP – muscle fibre potential

MHC – myosin heavy chain

MPB – muscle protein breakdown

MPS – muscle protein synthesis

MRI – magnetic resonance imaging

mTOR – mechanistic target of rapamycin

mTORc1 – mechanistic target of rapamycin complex 1

MU – motor unit

MUP – motor unit potential

MUPT – motor unit potential train

MuSK – muscle specific kinase

MVC – maximum voluntary contraction

NCAM – neural cell adhesion molecule

NFM – near fibre motor unit potential

NMD – neuromechanical delay

NMES – neuromuscular electrical stimulation

NMJ – neuromuscular junction

PIC – persistent inward current

PSC – perisynaptic Schwann cell

QoL – quality of life

RET – resistance exercise training

RMS – root mean square

ROI – region of interest

SC – satellite cell

SD – standard deviation

SIL – silhouette

SPPB – short performance physical battery

SR – sarcoplasmic reticulum

TE – time effect

TrkB – tyrosine receptor kinase B

TUG – timed-up-and-go

VL – vastus latera

Chapter 1: Introduction

1.1 Implications of an ageing society

Over the next 10 years the population of the UK is expected to pass 70 million and in 2018 the proportion of people over the age of 65 rose to 18.3%, growing faster than any other age group (Office for National Statistics, 2019*a*). Life expectancy has simultaneously risen, with those age 65 in 2018 expected to live to 85 and 87 years for males and females respectively (Office for National Statistics, 2019*b*). However, morbidity levels have also increased resulting in a lower healthy life expectancy of 63 and 64 years for males and females respectively (Office for National Statistics, 2019*c*). To put this simply, older adults will experience greater time living with disability and/or dependency. Alongside increases in common age-related comorbidities such as type 2 diabetes and cardiovascular disease, frailty is a key age-associated condition which leads to increased falls and fractures and reduced independence and is also associated with all-cause mortality and morbidity (Yeung *et al.*, 2019). Ultimately, frailty which is largely driven by neuromuscular dysfunction, increases pressure and financial burden on healthcare services (i.e., the National Health Service (NHS) in the UK) with, for example, muscle weakness in older people estimated to cost an extra £2.5 billion to health and social care in the UK (Pinedo-Villanueva *et al.*, 2019).

1.2 Sarcopenia

Even without a diagnosis of frailty (or indeed, even pre-frailty) (Taylor *et al.*, 2023), a major risk factor for poorer outcomes and quality of life (QoL) is sarcopenia. Recently afforded an ICD-10 disease code, sarcopenia is known to have a detrimental impact on the functional independence of older individuals and their ability to carry out activities of daily living (Landi *et al.*, 2013). Sarcopenia is commonly defined as simply the age-related loss of muscle mass and function, and was originally used by Rosenberg in 1989 (Rosenberg, 1989). This age-related decline in muscle mass appears to be linear but is further accelerated after the age of 60 (Kyle *et al.*, 2001), with the lower limbs demonstrating a greater rate of decline (Janssen *et al.*, 2000; Shur *et al.*, 2021).

The European Working Group on Sarcopenia in Older People (EWGSOP) have recently updated their clinically used definition (from 2010) based on research evidence to: “a

muscle disease (muscle failure) rooted in adverse muscle changes that accrue across a lifetime"(Cruz-Jentoft *et al.*, 2010, 2019), and placed muscle function at the forefront of the criteria for sarcopenia diagnosis (compared to previous definitions placing greater focus on muscle mass (Rosenberg, 1989)). Despite this updated definition, variation in methods and cut-offs for diagnosis in clinical practice often lead to wide-ranging estimates of prevalence (i.e., ranging from 9-18% (Beaudart *et al.*, 2015)). As with frailty, sarcopenia also confers a higher risk of falls and fractures in older people (Yeung *et al.*, 2019) with consequences that affect QoL and independence. That diagnosis of the condition now focuses on low muscle strength as a key characteristic which reflects the observation that muscle strength decreases at a substantially faster rate than size (Mitchell *et al.*, 2012; Cruz-Jentoft *et al.*, 2019), highlighting the complex interplay of different features (beyond muscle size) that contribute to optimal muscle function.

At the level of the muscle, there are multiple features observed in the pathophysiology of sarcopenia affecting different components, contributing to both the loss of muscle size and strength. Human skeletal muscle is formed of individual fibres consisting of sarcomeres giving its striated appearance. Contractile proteins actin and myosin enable contraction to occur in the presence of ATP through the action of the sliding filament model (Frontera & Ochala, 2015). Human skeletal muscle is heterogeneous with multiple fibre types present within individual muscles with observable differences in biochemical and mechanical properties between the phenotypes (Schiaffino & Reggiani, 2011). Multiple different classification methods have been employed to distinguish these fibre types including myosin heavy chain (MHC) and troponin isoforms, force production, fibre size and metabolic properties (Galpin *et al.*, 2012; Lamboley *et al.*, 2014; Brocca *et al.*, 2017) with MHC isoforms most commonly used.

There are 3 main fibre types found in human skeletal muscle – type 1, type 2A and 2X. Type 1 fibres are slow twitch fibres with a greater oxidative capacity and higher mitochondrial content than type 2 fibres and are therefore suited to endurance. Type 2 fibres are fast twitch with type 2X being faster than 2A, both of which produce greater force and power than type 1 (Larsson *et al.*, 1991; Pette & Staron, 2001; Narici & Maffulli, 2010). With age, changes in both the size and number of all fibre types occurs leading to reduced muscle mass. Although collective data are mixed, generally speaking there is a reduction in size and number of fibres, but it is unclear if one type is more susceptible than others (McPhee *et al.*, 2018). The consequences of these changes results in the loss of muscle mass and strength giving rise to poorer function. Additionally, a greater number

of hybrid fibres co-expressing MHC isoforms with advancing age has been observed which may contribute to reduced specific force (D'Antona *et al.*, 2003; Kelly *et al.*, 2018).

In addition to changes within the muscle fibres, there are also changes in metabolism and the muscle environment which contribute to sarcopenia. These include anabolic resistance (Burd *et al.*, 2013), changes in protein breakdown and synthesis rates associated with changes in mechanistic target of rapamycin (mTOR) signalling (Francaux *et al.*, 2016), fewer satellite cells which are required for repair (Kadi *et al.*, 2004*a*), fat infiltration (myosteatosis) reducing muscle quality (Marcus *et al.*, 2010), lower levels of anabolic hormones such as testosterone and insulin-like growth factor-1 (IGF-1) and increased inflammation and oxidative damage (Boirie, 2009; Narici & Maffulli, 2010). These factors in combination contribute to the muscle fibre atrophy observed in sarcopenia making it a multifactorial condition however, the relative contributions of each are still not fully understood.

1.3 Neuromuscular ageing

In addition to structural changes within the muscle and its environment, neurological changes with advancing age can impact the whole motor unit (MU) (Figure 1.1). The term MU encompasses the alpha motor neuron and the muscle fibres it supplies. The multiple fibres innervated by the nerve have a homogenous phenotype and are activated simultaneously (Enoka, 1995*a*). Additionally, to ensure even force distribution, fibres forming part of the same MU are spread throughout the muscle to form a MU territory (Piasecki *et al.*, 2016*b*).

With sarcopenia and indeed often earlier in the ageing process, changes in MU size and number directly affect overall muscle function. For example, while the number of MU's declines with advancing age, these MUs are often larger, resulting in loss of fine motor control due to bigger MUs consisting of more muscle fibres (Piasecki *et al.*, 2016*c*). Further, denervated (orphaned) fibres which are more common with advancing age lead to fibre atrophy or loss, and although remodelling mechanisms exist to enable reinnervation through collateral sprouting this can also lead to fibre type grouping (Kelly *et al.*, 2018; Jones *et al.*, 2022) which has probable implications for force control. Additionally, changes in neural drive at the motor cortex level, neuromuscular junction (NMJ) instability and MU firing irregularity may all play a role in changes in neuromuscular function observed with ageing (Hunter *et al.*, 2016) (Figure 1.1).

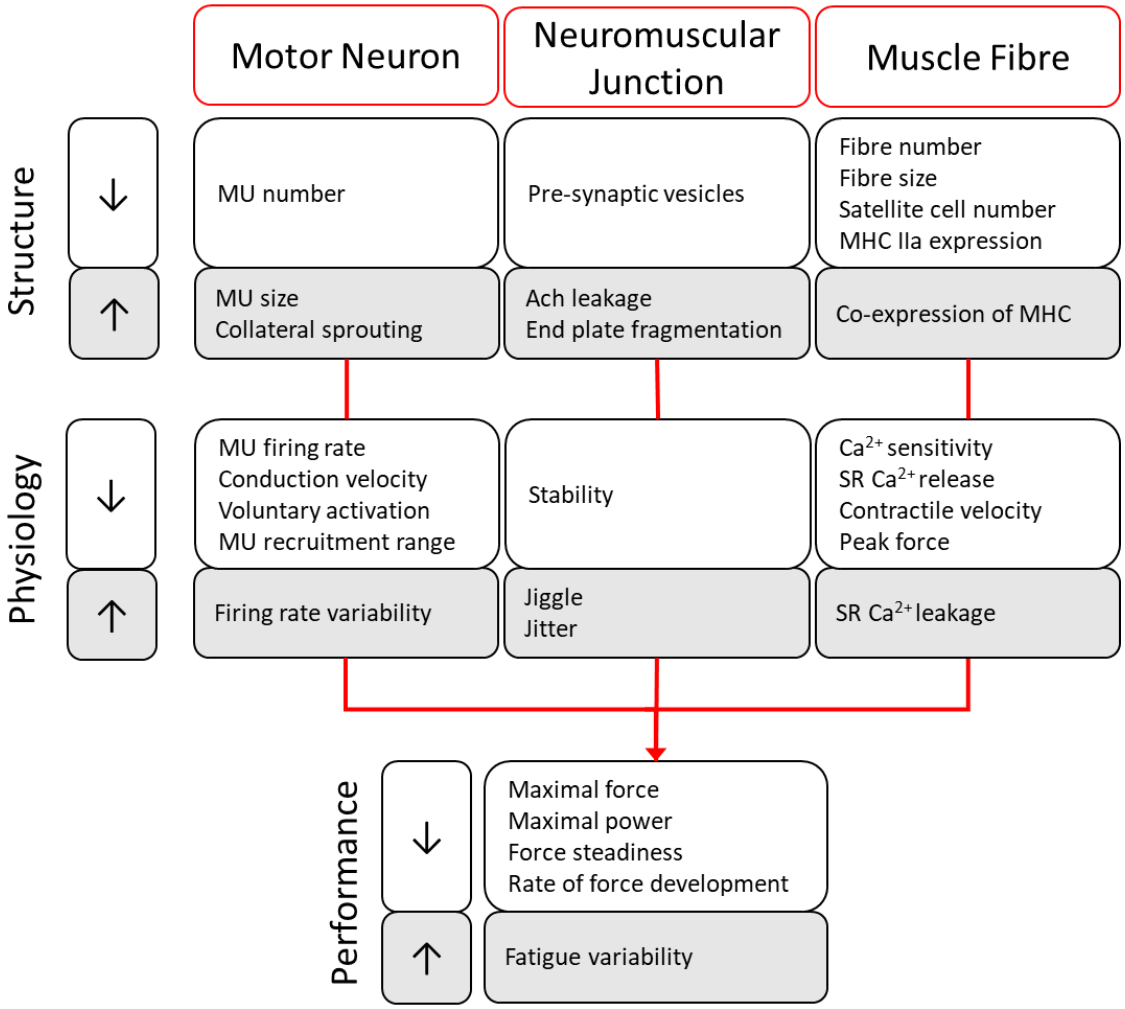


***Figure 1.1**: Summary of neuromuscular changes occurring with advancing age (adapted from Hunter et al., 2016).*

Abbreviations: Ach (acetylcholine), MHC (myosin heavy chain), MU (motor unit), SR (sarcoplasmic reticulum).

1.4 Studying the neuromuscular system

Multiple methods have been used to record the electrical activity of MUs enabling estimation of size, number, and structural and functional characteristics in a range of muscles. Surface electromyography (EMG) is commonly used as a simple non-invasive method of measuring the electrical activity of a sample of MUs during a range of contractions. The EMG technique detects MU potentials (MUPs) from action potentials propagating along muscle fibres of the same MU. These action potentials are generated by the reversal of the resting membrane potential because of a voltage-dependent increase in the permeability of the fibre membrane to sodium. This encompasses the exchange of sodium and potassium ions across the membrane when a received nerve

stimulus is sufficiently above activation threshold (Zwarts & Stegeman, 2003). The membrane potential must then be repolarised, with this often being the rate-limiting factor before the next stimulus can be received known as the refractory period (Hodgkin & Horowicz, 1960).

Building from this technique, high density surface EMG (HD-sEMG) enables sampling from a greater area of the muscle, with additional possibilities of recording conduction velocity and discharge patterns from across the MU (Stegeman *et al.*, 2012). Despite the development and notable recent advances of HD-sEMG, limitations of surface EMG methods include signal attenuation due to interference from subcutaneous tissue and the sampling of primarily superficial MUs (Nordander *et al.*, 2003). Intramuscular EMG (iEMG) involves a concentric needle electrode inserted directly into the muscle, so although this is more invasive than surface EMG, iEMG enables sampling of MUs directly in vivo and at different depths of the muscle to obtain information from a range of different MUs (Piasecki *et al.*, 2016*b*; Jones *et al.*, 2021). This technique also allows near fibre recordings to provide more detail of the contributions of those fibres closest to the recording electrode which is useful to identify changes with, for example, age or exercise (Stashuk, 1999*a*; Piasecki *et al.*, 2021*b*). Despite the limitations of both surface and iEMG methods they can be used in combination to allow in-depth examination of MUs and therefore improved insight into muscle function. Some MU properties such as FR and FR variability and can be assessed using both techniques and have been shown to be comparable between methods in validation studies across a range of muscles (Holobar *et al.*, 2010; Enoka, 2019).

1.5 Interventions targeting ageing muscle

As the proportion of older people in the population increases, the need for interventions to maintain musculoskeletal health into older age is more pressing than ever (Harvey *et al.*, 2022). Multiple interventions aiming to mitigate age-associated losses of muscle mass and strength to preserve function and independence have been employed and evaluated including those based on exercise (Beckwée *et al.*, 2019), nutrition (e.g., protein /amino acid supplementation (Breen & Phillips, 2011) and omega-3 (Bird *et al.*, 2021)), pharmacological compounds (hormones, growth factors, drug inhibitors (Borst, 2004; Martin-Montalvo *et al.*, 2013)) and neuromuscular electrical stimulation (NMES) (Dirks *et al.*, 2018; Guo *et al.*, 2021). Exercise is a key intervention for increasing and/or mitigating

losses of muscle mass and strength with advancing age, with multiple formats of exercise (e.g., high intensity interval training (HIIT), resistance exercise training (RET) and endurance exercise training (EET)) each eliciting distinct physiological adaptation (Hughes *et al.*, 2018) and enabling tailoring of programmes as required. Although losses of muscle mass and function with advancing age are seemingly inevitable, evidence from older masters athletes (>65 y) who undertake lifelong exercise both RET or EET, demonstrate a reduced rate of neuromuscular decline and maintenance of higher levels of physical function suggesting beneficial effects of maintaining activity (Piasecki *et al.*, 2019). With few studies exploring the impact of 'newer' exercise training formats (e.g., HIIT), both RET and EET interventions have been shown to slow the neuromuscular declines commonly observed in ageing and reduce the functional impact of these changes. However, challenges are present in promoting/facilitating exercise uptake and adherence in older adults, especially with RET (e.g., due to joint limitations) (Dismore *et al.*, 2020).

RET has been demonstrated to be an effective intervention for improving muscle strength and promoting MU plasticity in older people, but the expected hypertrophic responses are often blunted when compared with younger individuals (Greig *et al.*, 2011). It is not uncommon that small or no increases in muscle mass are observed in older adults despite full engagement in a supervised programme of RET (Phillips *et al.*, 2017). In order to address the concerns and physiological limitations of some older adults, lower loads are often used in older populations, and therefore lower absolute forces are generated. This results in a lower mechanical stimulus received by the muscle, possibly explaining the reduced responsiveness even when relative training intensity (i.e., % 1-repetition maximum (1-RM)) is matched to younger individuals. Higher doses have been shown to result in a better response to RET (Peterson *et al.*, 2011) and the intensity of training important for neural adaptations (Law *et al.*, 2016). Although challenges engaging older adults in RET do exist, RET has been demonstrated to be feasible and effective in numerous populations of older adults with different levels of ability (Steib *et al.*, 2010). Further, despite higher (current and lifelong) physical activity levels, older masters athletes still do lose muscle mass and strength due to neuromuscular changes, and MU number is not preserved. Although these losses cannot be wholly attributed to increased inactivity, exercise appears to ameliorate these losses to some extent (Piasecki *et al.*, 2016*a*, 2019).

These interventions are often dynamic involving multiple of the three muscle contraction types although the distinct effects of these on MU properties still remains unclear.

Isometric contractions are the production of force by a muscle with no change in muscle length or joint angle. Concentric contractions involve the production of force where the muscle shortens in length. Eccentric contractions are the production of force where the muscle lengthens. Different muscle effects between the contraction types have been identified which include neural input required, metabolic demand and fatigue and damage profiles (Kay *et al.*, 2000). These factors are important to consider during exercise interventions where neuromuscular limitations could be present in the population and one form of contraction type could be favourable.

1.6 Fatigue

A key factor influencing exercise capacity is fatigue which can be classified as acute or chronic (Wan *et al.*, 2017). Performance fatigue is the loss of force and/or power output from a muscle as a result of impaired contractile function and/or muscle activation (Enoka & Duchateau, 2016). It is a feature often seen acutely post exercise and is usually the cause of task failure (Hunter *et al.*, 2004). Fatigue also can be observed in chronic clinical conditions such as chronic obstructive pulmonary disease and cancer (Chaudhuri & Behan, 2004; Burtin *et al.*, 2012; Prinsen *et al.*, 2015) and with advancing age (Merletti *et al.*, 2002; Christie *et al.*, 2011). Acute fatigue or performance fatigue can be attributable to both central and peripheral factors each of which influence exercise/physical performance.

1.6.1 Central fatigue

Central fatigue refers to aspects occurring within the central nervous system (CNS) and includes processes in the motor cortex, neuronal transmission, and muscle activation (Enoka, 1995*b*). Muscle force generation is highly dependent upon effective modulation of MU discharge rate (Del Vecchio *et al.*, 2019*a*). Excitatory output signals from the somatic primary motor cortex (M1) in the human brain ultimately lead to muscle contraction following transduction through the motor pathway. These primary motor axons pass through the brainstem to the spinal cord down descending tracts, eventually synapsing to the interneuron and motor neuron circuitry, with both excitatory and inhibitory corticospinal inputs influencing synergistic muscle coordination (Gandevia, 1998). With the high levels of plasticity within the CNS and the neurons it contains, training can influence neurotransmission, structure and cognition (Carroll *et al.*, 2001;

Mueller, 2007); with previous research demonstrating increased motor cortex and corticospinal excitability following RET (Carroll *et al.*, 2002; Hortobágyi *et al.*, 2003; Leung *et al.*, 2015; Latella *et al.*, 2017). These adaptations in the CNS in response to exercise could help to attenuate muscle fatigue by improving agonist activation resulting in improved strength and functional ability of the muscle (Kennedy *et al.*, 2016).

1.6.2 Peripheral fatigue

Peripheral fatigue is the loss of force and/or power output due to changes in neuromuscular junction (NMJ) transmission and contractile properties of muscle fibres (Wan *et al.*, 2017) and includes factors such as metabolite accumulation and excitation-contraction coupling failure (Lanza *et al.*, 2006; Enoka & Duchateau, 2008). Muscle properties such as blood supply, fibre composition and metabolism can all influence muscle fatigability (Sjøgaard *et al.*, 1988; Clark *et al.*, 2005; Hunter, 2014). The muscle microvascular blood supply is important to prevent metabolite accumulation and for oxygen and nutrient delivery (Kusters & Barrett, 2016) and research has noted reductions in muscle perfusion with age compromising muscle function and increasing fatiguability (Wigmore *et al.*, 2004).

Despite declines in multiple aspects of neuromuscular function with age such as central drive (Merletti *et al.*, 2002), many studies have found increased fatigue resistance in older people when compared to younger individuals, particularly related to peripheral factors (Kent-Braun *et al.*, 2002; Lanza *et al.*, 2004). This can partly be attributed to a predominance of phenotypically fatigue resistant type 1 muscle fibres, but with the consequence of a reduced capacity for power generation, contributed to by a selective denervation of fast-twitch fibres with age (Lexell *et al.*, 1988; Deschenes, 2004). However, it has been suggested that contraction type can affect the extent of age-related fatigue resistance (Christie *et al.*, 2011).

The involvement of multiple physiological mechanisms and experimental triggers presents challenges in determining the precise cause of fatigue but factors including reduced skeletal muscle blood flow, metabolite accumulation and neural impairment have all been implicated (Enoka & Duchateau, 2008). Interventions to improve fatigue resistance of the muscle include RET, nutritional supplementation (Rawson *et al.*, 2011), and NMES (Sabatier *et al.*, 2006). RET has already demonstrated beneficial effects on muscle fatiguability with possible mechanisms including increased energy availability,

greater mitochondrial activity and improved capillarisation (Walker *et al.*, 2013). However, challenges may present where functional ability to perform RET is limited so further research into pharmacological interventions as an alternative for those most vulnerable to muscle mass and function losses is warranted.

1.7 Resistance exercise training in older age

1.7.1 Muscle size and physical function

The use of imaging techniques such as magnetic resonance imaging (MRI), dual-energy x-ray absorptiometry (DXA) and ultrasound have enabled the non-invasive study of skeletal muscle size and structure (Franchi *et al.*, 2018). Due to sarcopenia, older adults of both sexes have demonstrated smaller muscle cross sectional areas (CSA) than their younger counterparts in a variety of muscles and a decline with age (Häkkinen & Häkkinen, 1991; Frontera *et al.*, 2000; Klein *et al.*, 2002; Nilwik *et al.*, 2013). In response to RET, muscle hypertrophy is observed in healthy young people with additional increases in muscle thickness, fibre length and pennation angle (Brook *et al.*, 2015). In older adults, RET has also been shown to be somewhat effective in stimulating muscle hypertrophy (Flack *et al.*, 2016; Moro *et al.*, 2020; Quinlan *et al.*, 2021) however, they have been shown to be less responsive than younger individuals (Welle *et al.*, 1996; Brook *et al.*, 2016), with muscle blood supply, protein turnover and satellite cell (SC) function all postulated as having an influence (Kadi *et al.*, 2004*b*; Moro *et al.*, 2019).

The increases observed in muscle size in response to RET have also been shown to translate into improvements in muscle strength, power and physical function. In both young and older adults, muscle strength increases observed are often greater than those in muscle size indicating a key role of neural adaptations particularly in the early stages of RET (Folland & Williams, 2007). Clinically, older adults with limited muscle mass have also demonstrated improvements in basic functional tasks such as timed-up-and-go (TUG), functional reach and timed walks following RET (Papa *et al.*, 2017) therefore highlighting the beneficial effects of not only size, but also muscle function on maintaining functional independence (Runge *et al.*, 2004).

1.7.2 Fibre level and signalling

In order for muscle hypertrophy to occur and for strength increases to be observed in response to RET, metabolic and molecular adaptations are needed at the fibre level.

Muscle protein turnover which encompasses muscle protein synthesis (MPS) and muscle protein breakdown (MPB) is key in regulating muscle mass. In younger healthy adults, where muscle mass is maintained, a dynamic equilibrium of MPS and MPB across the diurnal cycle ensures net balance. This is achieved by postprandial periods where rates of MPS exceed MPB, interspersed with postabsorptive periods where MPB predominates. In response to RET, MPS rates increase to outweigh the rate of MPB which can be further facilitated by dietary essential amino acid (AA) intake (Kumar *et al.*, 2009; Burd *et al.*, 2009). The duration and intensity of RET can also influence the extent of MPS stimulation, with higher intensities and greater volume resulting in a greater MPS response (Kumar *et al.*, 2009; Burd *et al.*, 2010). However in older people, rates of MPS in response to both AA nutrition alone and AA + RET are lower than in younger individuals (Kumar *et al.*, 2009); an observation commonly referred to as anabolic resistance (Burd *et al.*, 2013).

MPS is triggered through stimulation of the mTOR signalling pathway (Figure 1.2). The protein kinase complex, mechanistic Target of Rapamycin Complex 1 (mTORc1) is a key regulator of protein synthesis and is highly responsive to muscle contraction. It is a serine/threonine kinase with the ability to sense intracellular changes in AAs and respond to mechanical stimuli (Laplante & Sabatini, 2012), as well as having an important role in regulating cell growth (Bodine *et al.*, 2001). The loss of mTOR signalling can result in muscle dystrophy, reduced lifespan and reduced metabolism (Zhang *et al.*, 2019). Paradoxically, the mTOR pathway becomes hyper-active in older age leading to impaired responsiveness to nutrition and exercise, and dysregulated autophagy (Francaux *et al.*, 2016). Furthermore, this hyper-activity has also been postulated to prevent the growth of new axonal branches needed for MU remodelling and deterioration of NMJ integrity which will be discussed in section 1.7.4 (Castets *et al.*, 2020; Ham *et al.*, 2020). However, mTOR inhibitors such as rapamycin have shown positive effects in counteracting the age-related loss of muscle mass, maintaining function and extending lifespan in murine models (Harrison *et al.*, 2009; Joseph *et al.*, 2019; Ham *et al.*, 2020). Considering all of these aspects together, protein supplementation and pharmacological interventions such as mTOR inhibitors, given in combination with RET, may enable increased levels of muscle anabolism by restoring the balance of mTOR signalling and therefore enabling more substantial beneficial responses to nutrition and RET in older populations.

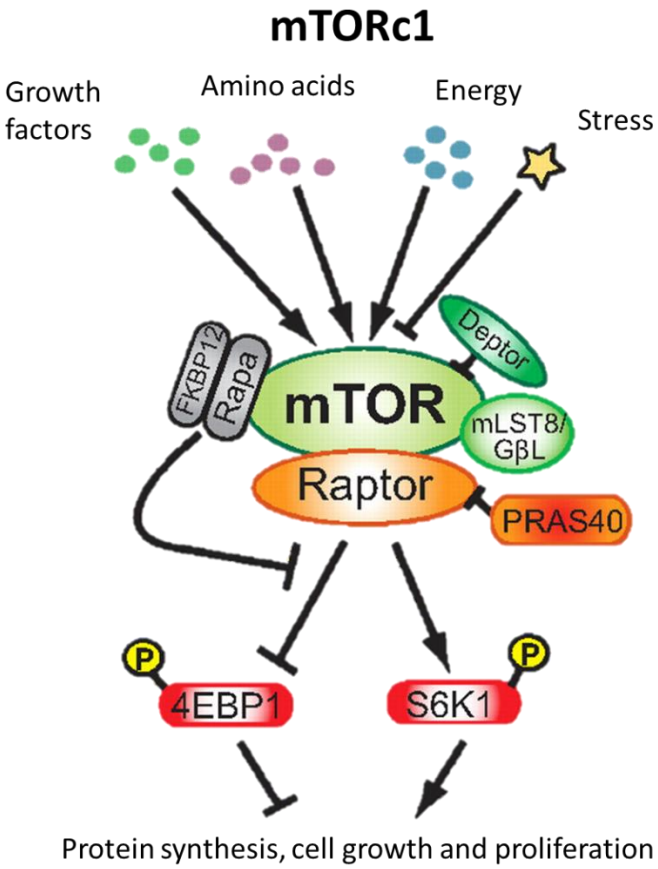


***Figure 1.2**: Schematic of the mechanistic target of rapamycin complex 1 (mTORc1) signalling pathway (adapted from Foster & Fingar, 2010).*

Abbreviations: mTOR (mechanistic target of rapamycin), Rapa (rapamycin), FKBP12 (FK – binding protein 12), P (phosphate) 4EBP1 (Eukaryotic translation initiation factor 4E-binding protein 1), S6K1 (Ribosomal protein S6 kinase beta-1), PRAS40 (proline-rich Akt substrate of 40 kDa), mLST8 (MTOR Associated Protein, LST8 Homolog), GβL (G protein beta subunit-like protein).

1.7.3 Motor unit properties

The *in-vivo* study of human MUs is possible via various applications of EMG, whereby action potentials from MU muscle fibres are sampled with regards to a number of signature parameters to generate imaging biomarkers (Piasecki *et al.*, 2018*a*; Del Vecchio *et al.*, 2020). Applications of these methods have helped establish, at least broadly speaking, that older age is associated with a decline in MU number but an initial increase in MU size (McNeil *et al.*, 2005; Piasecki *et al.*, 2016*b*). Denervation becomes more common with older age in a complex "cause-or-consequence" relationship with individual fibre dysfunction (Hepple & Rice, 2016; Anagnostou & Hepple, 2020), whereby if MU

expansion via rescue is lacking, fibre loss will be permanent (Aare *et al.*, 2016; Piasecki *et al.*, 2018*b*).

Human muscles contain multiple MUs and different numbers and types of MUs are recruited according to their properties in a task dependent manner. The study of MUs is important as it encompasses not only morphological alterations within the muscle fibre but also changes in muscle innervation and can be measured using EMG methods (Piasecki *et al.*, 2018*a*).

In the ageing population, increases in size (Frontera *et al.*, 2008; Ling *et al.*, 2009) and decreases in the number of MUs are observed, particularly with sarcopenia (McNeil *et al.*, 2005; Piasecki *et al.*, 2018*b*) which has consequences for motor control and appropriate force generation. As with RET, EET has also been demonstrated to prevent the loss of MUs with advancing age (Power *et al.*, 2010), demonstrating the possible beneficial effects of exercise in reducing some of the MU declines observed with ageing. Notably, exercise in older age may stimulate structural neural adaptations such as the formation of new axons (Tam & Gordon, 2003), with mounting evidence to suggest that older (>65 y) masters athletes are more successful at reinnervation of denervated fibres, theoretically creating an environment within the muscle (via chronic exercise) which enables axonal sprouting and NMJ formation (Piasecki *et al.*, 2019). Reflecting this, in comparison to a younger group, older masters athletes exhibit electrophysiological markers of larger MU size, as well as a more homogeneous distribution of measures of MUP size and complexity across the muscle depth (Piasecki *et al.*, 2019; Jones *et al.*, 2021). Other methodological approaches support this notion, with increased fibre type grouping (Zampieri *et al.*, 2015) and fewer histological markers of denervated fibres in older athletes (Sonjak *et al.*, 2019; Soendenbroe *et al.*, 2021) (Figure 1.3). However, the underlying mechanisms remain unclear due to the majority of the investigations on the effects ageing and exercise on MU plasticity in humans being cross-sectional in design.

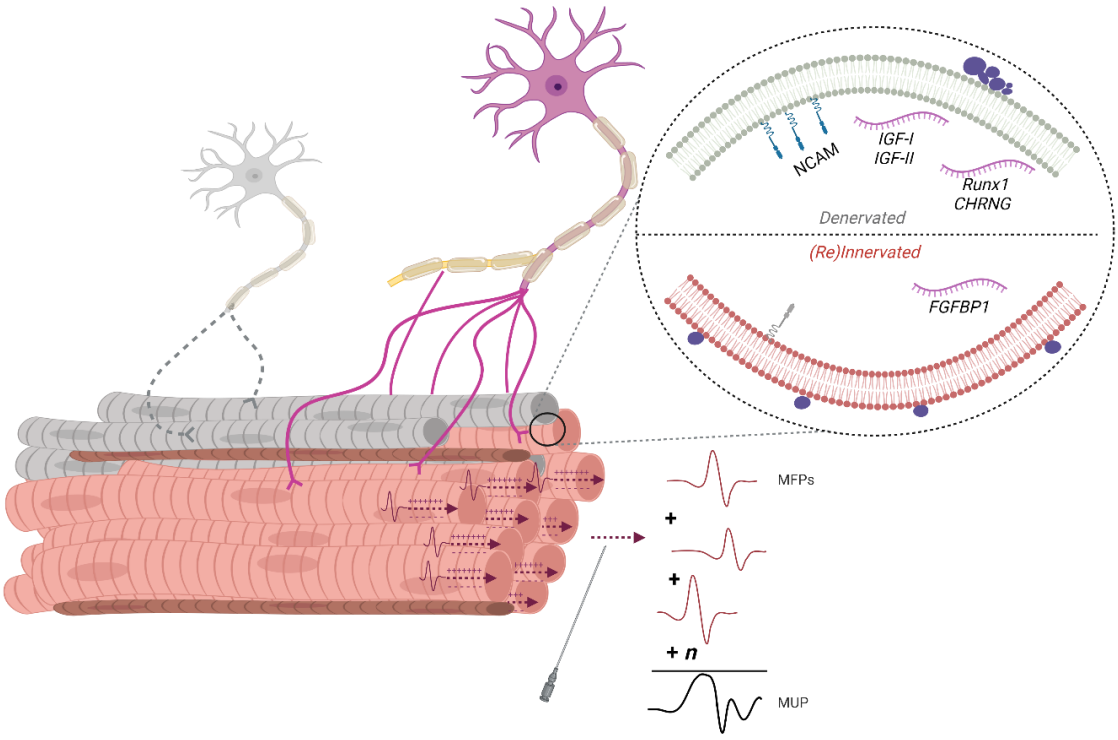


***Figure 1.3**: Summary of human evidence to support greater reinnervation capacity in highly exercised older muscle, largely generated from studies of masters athletes.*

A neuron from one motor unit reinnervates denervated fibres from another unit. Changes to the motor unit potential and signalling factors are shown. Changes following reinnervation include increased fibre type grouping, larger electrophysiological markers of motor unit size, fewer histological markers of denervation and reduced expression of denervation-related genes.

Abbreviations: CHRNG (acetylcholine γ subunit), FGFBP1 (fibroblast growth factor binding protein 1), IGF-I/II (insulin-like growth factor-I/II), MFP (muscle fibre potential), MUP (motor unit potential), NCAM (neural cell adhesion molecule).

Multiple characteristics assessed as methods for quantifying MU number and size have been demonstrated to change in response to exercise training in both young and older populations and with ageing. In comparison to a younger controls, older participants had a larger MUP area which is representative of a greater MU size. Furthermore, masters athletes had greater MUP area than old controls irrespective of training type (e.g., power or endurance) indicating greater fibre size or number of fibres within the unit possibly as a result of reinnervation (Piasecki *et al.*, 2019). As a result of EET throughout life, no differences in M-wave negative peak amplitude or surface MUP size were observed compared to sedentary individuals (Power *et al.*, 2010). However, greater MUP amplitudes have been observed in young endurance trained individuals compared to sedentary controls (Dimmick *et al.*, 2018), indicative of more contractile material in the

muscle suggesting a greater MU size. There is however limited research looking directly at the effect of RET on MU size and number, especially in the context of advancing age.

MUs within a muscle are generally recruited according to the Henneman size principle with smaller, slower units recruited first (Henneman, 1957). The combination of MUs recruited during a contraction contributes to increasing muscle force generation to meet task demand and a reduced recruitment threshold has been observed in response to RET (Del Vecchio *et al.*, 2019*a*). Similarly, EET also resulted in lower recruitment levels required to achieve same force when compared to a control group (Dimmick *et al.*, 2018). In a comparison between young EET and RET individuals, recruitment strategies differed, with RET individuals exhibiting a greater de-recruitment threshold, particularly at higher intensity contractions (Herda *et al.*, 2015).

In addition to changes in recruitment strategy, changes in coordination between the agonist and antagonist muscles, involving increased agonist muscle activation and reduced antagonist coactivation in maximal contractions, is also observed after RET. This adaptation helps to increase net force generation (Häkkinen *et al.*, 1998, 2001) through possible central mechanisms affecting motor cortex activation (Dal Maso *et al.*, 2012). With age, although lower agonist muscle activation and greater antagonist coactivation has been observed compared to younger individuals (Macaluso *et al.*, 2002; Hortobágyi *et al.*, 2009), older groups can respond to RET to improve overall muscle activation and reduce antagonist coactivation (Morse *et al.*, 2005; Cadore *et al.*, 2013*a*).

Another neural mechanism used to modulate force output of the muscle is rate coding which is the change in firing rate (FR) with stimulus intensity. Usually, as the force requirement from the muscle increases, MU FR increases to enable demand to be met (Enoka & Duchateau, 2017). With RET, multiple adaptations affect MU firing properties including increased discharge rate (Vila-Chã *et al.*, 2010; Del Vecchio *et al.*, 2019*a*), and increased maximum FR (Kamen & Knight, 2004). However, studies have also found no changes in average FR following 3-8 weeks of RET despite strength increases (Pucci *et al.*, 2006; Sterczala *et al.*, 2020). Contrastingly, following EET, research found no change in MU discharge rate following 2 weeks of training (Martinez-Valdes *et al.*, 2017*a*), but a decreased discharge rate following 6 weeks of training (Vila-Chã *et al.*, 2010). However, in a direct comparison between EET and RET participants, a greater mean FR was observed in those endurance trained (Herda *et al.*, 2015). This demonstrates that adaptations within the MU are exercise manner dependent. With advancing age, there is

generally a decrease in MU FR (Ling *et al.*, 2009), peak discharge rate and rate coding range (Barry *et al.*, 2007) however after RET by older people an increased FR has been observed (Kamen & Knight, 2004; Watanabe *et al.*, 2018). These changes in the MU, due to alterations in motor neuron input, have a cumulative effect to impact the overall function of the muscle which enables transfer of motor skills from RET and EET to more functionally relevant tasks, which is particularly important in an older population (Carroll *et al.*, 2001).

1.7.4 Motor unit remodelling

1.7.4.1 Axonal sprouting

Human alpha motor axons are the myelinated projections of a neuron responsible for conducting excitatory signals from upper motor neurons to initiate muscle contraction. The generation of motor neuron action potentials depends on the integration of synaptic inputs from descending pathways and afferent feedback from peripheral receptors. These nerves extend relatively long distances, and utilise protein-assisted mRNA transport along axons and localised translation within axons, to respond to external stimuli (Dalla Costa *et al.*, 2021). The process of MU remodelling involves sprouting of new axonal growth cones in existing neurons, from either the nodes of Ranvier, the nerve terminal, or the motor endplate, and the eventual formation of new connections with denervated muscle fibres (Tam & Gordon, 2003). This can be achieved through both the formation of new NMJs and reinnervation of existing postsynaptic targets (Rantanen *et al.*, 1995; Li *et al.*, 2018). This process is stimulated by neurotrophins in an autocrine and/or paracrine fashion, as outlined by work in animal models (English *et al.*, 2014; Rigoni & Negro, 2020), and is known to decrease with advancing age (Aare *et al.*, 2016). Although much of the sprouting processes described have been generated from models of nerve damage (e.g. full and partial nerve sectioning), it has been demonstrated that sprouting can also be initiated by muscle fibre inactivity (Tam & Gordon, 2003) - highlighting the importance of muscle-nerve communication.

During initial axonal sprouting, the neurotrophin, brain-derived neurotrophic factor (BDNF) is synthesized by motor neurons, local Schwann cells, and muscle fibres (Pradhan *et al.*, 2019). It then acts via tyrosine receptor kinase B (TrkB) signalling and p75 in neurons to upregulate production of proteins of multiple signalling pathways required for enhancing neuron excitability, synaptic strength, and axonal growth cone formation for

neurogenesis (Pradhan *et al.*, 2019) (Figure 1.4), which may be dysregulated in older age (Anisimova *et al.*, 2020). The BDNF-TrkB signalling pathway in motor axons is well established; blocking BDNF activity and BDNF-KO mice each result in reduced axon outgrowth (Zhang *et al.*, 2000; Wilhelm *et al.*, 2012), while TrkB agonists enhanced axon regeneration (English *et al.*, 2013). Circulating BDNF spatial specificity is partly controlled by calcium influx stimulating transcription of TrkB signalling components (McGregor & English, 2019) therefore may have a greater effect on reinnervation in active muscles. BDNF levels have been reported to decrease with older age (Ziegenhorn *et al.*, 2007), and reciprocally, to increase in response to acute exercise in older individuals (Nilsson *et al.*, 2020).

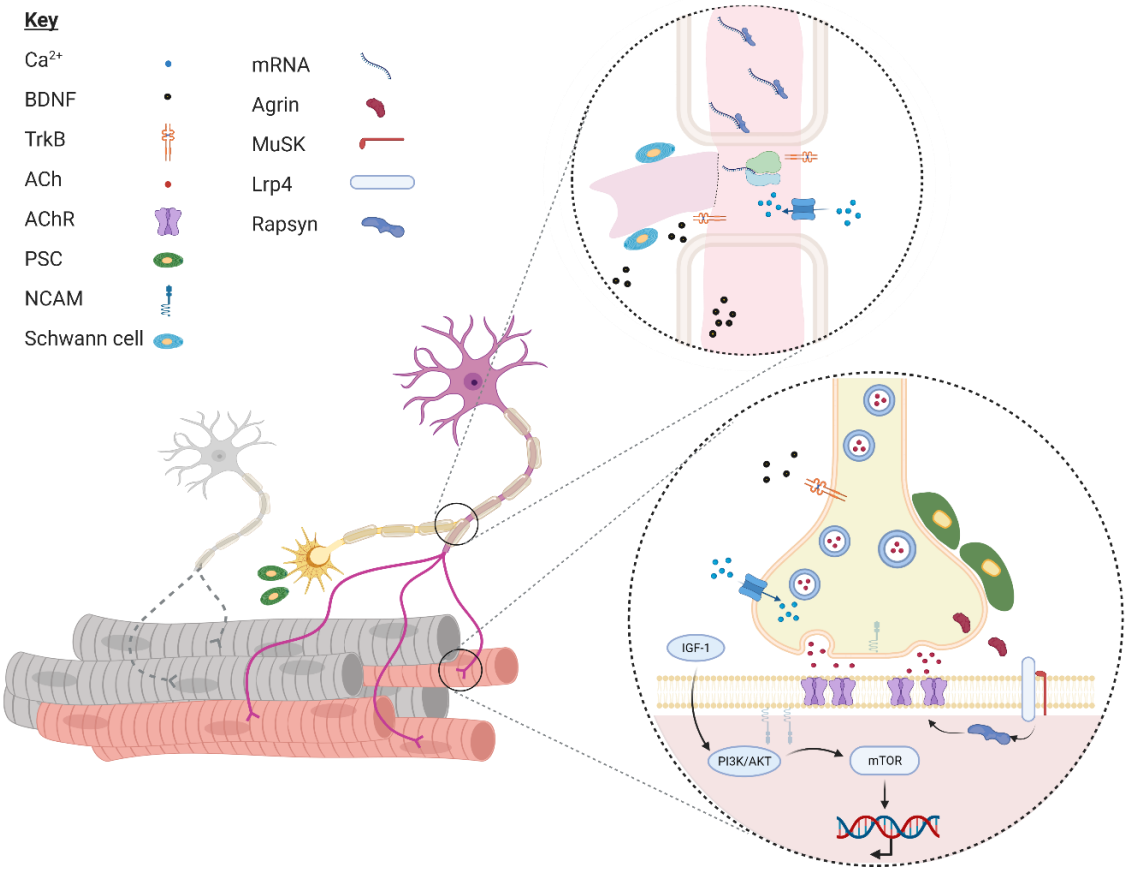


***Figure 1.4**: Motor unit expansion through reinnervation.*

The process of motor unit (MU) expansion to rescue denervated muscle fibres involves axonal branching from adjacent surviving motor neurons and the formation of new neuromuscular junctions (NMJ). Extending long distances, axons utilise protein-assisted mRNA transport and localised translation to alter the localised proteome to facilitate sprouting. This is mediated by a number of factors secreted by the motor neurons, muscle fibres and Schwann cells. Formation of new NMJs is largely mediated by the release of the neural agrin and the Lrp4-MuSK signalling complex.

Abbreviations: IGF-I (insulin-like growth factor-1), mTOR (mechanistic Target of Rapamycin), PI3K/AKT (phosphatidylinositol 3-kinase/protein kinase B), BDNF (brain-derived neurotrophic factor), TrkB (tyrosine receptor kinase B), ACh (acetylcholine), AChR (acetylcholine receptor), PSC (perisynaptic Schwann cell).

Following experimental nerve crush, insulin-like growth factors I and II (IGF-I, IGF-II) are upregulated in nerve and muscle, with both serving a number of neuroprotective roles in motor neurons (Sakowski & Feldman, 2012). IGF-I has demonstrated beneficial neurotrophic and myogenic effects (Rabinovsky *et al.*, 2003), while IGF-II is synthesised by inactive fibres in partially denervated muscles and acts as a sprouting stimulus via muscle-nerve cross-talk (Glazner & Ishii, 1995). Furthermore, greater expression of IGF-II and IGF-I receptors are associated with motor neurons less susceptible to degeneration in amyotrophic lateral sclerosis rodent models (Allodi *et al.*, 2016). As a primary downstream mediator of growth hormone (GH), circulating IGF-I responds to exercise yet outcomes from plasma measures are inconsistent, likely explained by the localised nature of its secretion and receptor binding (Birzniece, 2019). Similarly, the role of IGF and its signalling in ageing has produced contentious findings and the evidence supporting increased lifespan with reduced levels is on face value difficult to reconcile with the numerous neuroprotective effects, both peripherally and centrally (Bartke *et al.*, 2003).

Alongside being a key regulator of protein synthesis as discussed earlier, mTORc1 also plays a role in the maintenance of NMJ structure. It is clear the temporal regulation of mTORc1 following denervation is paramount, with potential mechanisms including increased synthesis of proteins required for structural NMJ stability (Ham *et al.*, 2020), minimisation of mitochondrial dysfunction via maintenance of autophagic flux (Baraldo *et al.*, 2020), and the promotion of nuclear import of denervation gene regulators (histone deacetylase HDAC4) (Castets *et al.*, 2019). As a potent stimulator of the mTOR pathway, IGF-1 has an important role in regulating nerve regeneration through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway as well as promoting muscle hypertrophy (Rabinovsky, 2004). Prolonged mTOR activation appears to impair acetylcholine receptor (AChR) clustering at the NMJ thereby limiting reinnervation (Castets *et al.*, 2019) but administration of an mTOR inhibitor – rapamycin, reverses age-related detrimental features at the NMJ (Ham *et al.*, 2020) and reduces expression of NMJ denervation markers (Joseph *et al.*, 2019). Collectively these findings support the use of pharmaceutical interventions targeting the age-related imbalance of mTOR to reduce

fibre denervation through maintenance of existing NMJ structures and/or improve reinnervation.

Perisynaptic Schwann cells (PSCs) are non-myelinated glial cells at the NMJ, sensitive to mechanical signals and synaptic transmission. As such, they may be capable of responding to denervation and chronic inactivity (Ko & Robitaille, 2015). Indeed, following denervation PSCs produce extensions to form bridges with axonal growth cones to guide sprouting to denervated fibres (Tam & Gordon, 2003; Darabid *et al.*, 2014). Exercise is thought to encourage sprouting through promoting calcium influx into nerve terminals and upregulating glial fibrillary acidic protein (GFAP) which is needed for maintaining strength and shape in the PSCs (Tam & Gordon, 2003). As a result of treadmill running EET, greater nerve terminal branching has been observed in the hindlimb muscles of young rats (Deschenes *et al.*, 2016) and enhanced axon regeneration in mice following peripheral nerve injury (Sabatier *et al.*, 2008). With chronic inactivity, often observed in older age, this calcium influx would be markedly lower and therefore less supportive of axonal sprouting. To date, methodological limitations have prevented research determining the effect of exercise on axonal sprouting in humans, and animal models of nerve sectioning may be a poor proxy for multiple fibre denervation in aged human muscle (Jones *et al.*, 2017).

Multiple markers of denervation and expression and transcription of certain genes are also shown to be upregulated with advancing age (Soendenbroe *et al.*, 2019). For example, *Runx1* and acetylcholine γ subunit (*CHRNG*) expression were shown to be upregulated with muscle denervation in older individuals, but to decrease in response to RET (Messi *et al.*, 2016). Neural cell adhesion molecule (NCAM) is a glycoprotein expressed on the surface of neurons and muscle cells and whose upregulation facilitates reinnervation, and as such has been utilised as a marker of individual fibre denervation (Messi *et al.*, 2016). Observed decreases of NCAM+ fibres were inversely proportional to increases in muscle strength following RET in older people (Messi *et al.*, 2016), and also increased in response to unloading in healthy young muscle (Monti *et al.*, 2021). Furthermore, in masters athletes lower levels of NCAM and upregulation of the reinnervation promoting fibroblast growth factor binding protein 1 (FGFBP1) were noted when compared to non-athletic age-matched controls (Sonjak *et al.*, 2019) (Figure 1.3). Although immunohistochemical data reveal little of MU function, collectively the data

generated by these methods are supportive of exercise attenuating age-related fibre loss via increased reinnervation.

1.7.4.2 Neuromuscular junction formation

The NMJ comprises three major components: the presynaptic axon terminal, the post synaptic muscle fibre, and the supporting PSCs. It is a specialised chemical synapse which transmits signals from motor neurons to postsynaptic nicotinic AChRs on the muscle fibre membrane to activate the release of calcium ions from the sarcoplasmic reticulum (SR) and stimulate contraction of sarcomeres. As such, it has been a focus of research interest in neuromuscular disorders and ageing. Like synapses found in other locations, they also demonstrate plasticity and undergo both morphological and physiological remodelling in response to exercise, and degradation in structure and function with age (Valdez *et al.*, 2010; Deschenes *et al.*, 2010; Deschenes, 2019). This can include declines in mitochondrial number and vesicles in the presynaptic terminal and increases in postsynaptic endplate area (Jang & Van Remmen, 2011).

NMJ formation is initiated in large part by the nerve-specific isoform of agrin and muscle-specific kinase (MuSK). Neural agrin released from the motor axon activates the Lrp4-MuSK complex on the post-synaptic fibre membrane, and the sequential activation of rapsyn which clusters AChR in the post-synaptic region (Tintignac *et al.*, 2015) (Figure 1.4). During development, multiple nerve branches innervate the same fibre and, as the NMJ develops, AChR clusters mature with retraction and synapse stabilisation occurring - stimulated by increased glial cell line-derived neurotrophic factor (GDNF) expression, which ensures a fibre is only innervated by a single motor nerve (Ham & Rüegg, 2018).

PSCs also have an important role in synaptic transmission and elimination during maturation, by providing structural support and phagocytically degrading redundant or degenerated axons (Alvarez-Suarez *et al.*, 2020). SC (myogenic stem cells) assist with muscle repair, remodelling and adaptation predominantly of the muscle fibre (Yin *et al.*, 2013), and in young mice a reduction in SC number contributes to impaired NMJ regeneration following denervation as a result of structural instability and declines in muscle force generating capacity (Liu *et al.*, 2015). With age, SC numbers decrease, but increase with both RET and EET, as measured by Pax7+, possibly contributing to improving NMJ function (Kadi *et al.*, 2004*a*; Mackey *et al.*, 2014; Moore *et al.*, 2018).

The ability to histochemically image the NMJ in animal models has provided a wealth of data. In healthy young rats, RET via weighted ladder climbing resulted in an expansion of the postsynaptic area identified by cytofluorescence, where AChR are located (Deschenes *et al.*, 2000, 2015) while EET training yields remodelling in the pre- and post-synaptic areas, including greater dispersion of acetylcholine (ACh) vesicles at the pre-, and AChR in post-synaptic terminals (Deschenes *et al.*, 2016). Research on the NMJ in aged animal models has also found NMJ fragmentation (Pannérec *et al.*, 2016), which resonates with aligned findings of MU plasticity in atrophy resistant/susceptible muscle groups in humans (Piasecki *et al.*, 2018*b*). Animal NMJs also have an age-specific response to exercise, with NMJ hypertrophy occurring in the young but lesser so in aged animals in response to RET (Krause Neto *et al.*, 2015). Similarly with EET, although fibre adaptations are observed, in older animals the ability of the NMJ to adapt to an exercise stimuli is significantly affected in both soleus and plantaris muscles in both a muscle and fibre type-specific manner (Deschenes *et al.*, 2011, 2016). However, fibre adaptations are still observed in older animals with increases in fibre area and alterations in fibre type composition to a greater percentage expression of type 1 fibres. Furthermore, there is variability in rat NMJ adaptations to EET that could be age-dependent, with both cellular and sub-cellular components of the neuromuscular signalling process displaying inconclusive findings; therefore further work is warranted (Deschenes *et al.*, 2020).

Direct structural imaging of the NMJ in humans is notoriously complex and often requires full cadaveric or post-amputation limbs (Boehm *et al.*, 2020). However, targeted biopsy techniques involving stimulation to produce a muscle twitch to locate the area of highest NMJ density have improved NMJ yield (Aubertin-Leheudre *et al.*, 2020). Unlike animal models, in muscles obtained from humans with peripheral vascular disease, no age-related differences in human NMJ postsynaptic area were found in the lower limb - and except for a modest increase in axon diameter - the NMJ remained structurally stable with advancing age (Jones *et al.*, 2017). Research in age-related changes in NMJs from intercostal muscles has demonstrated mixed results, with some reporting both larger and more complex post-synaptic regions (Wokke *et al.*, 1990), while others show no differences (Oda, 1984). However, comparisons of NMJ imaging across the few human studies are difficult due to different methodological considerations and muscles studied, while data exploring additional effects of in/activity in elderly humans are scarce. Finally,

the association of NMJ structure and nerve-muscle communication, or NMJ transmission instability, is not well defined.

1.7.4.3 Neuromuscular junction transmission

Briefly, NMJ transmission refers to the release of ACh from the motoneuron and binding to the synaptic region of the muscle fibre to initiate a muscle fibre action potential (MFP) and contraction. Aged rats have demonstrated a decline in NMJ transmission stability and reliability which correlated with declines in functional measures such as grip strength (Padilla *et al.*, 2021), and voluntary running exercise improved NMJ transmission stability in older mice (Chugh *et al.*, 2021). However, these beneficial effects of exercise on NMJ transmission are less clear in older humans (Deschenes *et al.*, 2011).

Functional NMJ transmission instability can be measured *in vivo* with iEMG using a statistic ('jiggle') representative of the variability in consecutive MUPs (Stålberg & Sonoo, 1994) or near fibre potentials (Piasecki *et al.*, 2021*b*). Longitudinal iEMG data on the effects of an exercise training intervention are unavailable, however, cross-sectional data highlight age-related increases in NMJ instability in lower limb muscles (Hourigan *et al.*, 2015; Piasecki *et al.*, 2016*c*, 2021*a*), which may reflect early stages of fibre denervation-reinnervation. In a direct comparison, older runners (80±5 y) had lower NMJ instability than age-matched inactive individuals (Power *et al.*, 2016), yet these values were still around 30-60% greater than healthy non-athletic, and athletic, individuals aged 5-10 years younger from two separate studies (Hourigan *et al.*, 2015; Piasecki *et al.*, 2016*a*). These electromyography methods require extensive operator input to establish reliable repeat occurrences of an individual MUP, which may partly explain the large variability across studies.

1.8 Summary

In summary, although there has been much investigation on the effects of RET and ageing on different aspects of human skeletal muscle, little is known about the impact on MU properties. Moreover, there is a lack of longitudinal research exploring these concepts in humans, with consequently little being known about: i) the functional outcomes of MU remodelling, ii) an identifiable time course of mal/adaptation, or iii) the underpinning mechanisms of these scenarios. Nevertheless, as the collective evidence suggests that both structural and functional alterations are physiologically possible, the peripheral

motor system thus represents a realistic target in human ageing which may involve voluntary exercise, pharmaceutical intervention, and/or NMES (Guo *et al.*, 2021).

1.9 Aims

Based on the existing literature outlined in the sections above, the aims of this work are: first, to determine the effects of different methods of fatigue on MU properties in order to identify possible neuromuscular causes and second, to investigate the effect of RET and pharmacological intervention in older people on MU properties.

This will be addressed through several experimental chapters each with distinct aims, including:

1. To assess the effect of concentric and eccentric loading on MU properties,
2. To assess MU properties during an isometric fatiguing contraction and the role of muscle microvascular blood flow,
3. To assess the effect of 6-weeks RET on force control and MU properties in older males,
4. To assess the effect of 6-weeks RET on MU properties in older males with or without oral supplementation of an mTOR inhibitor for 8 weeks.

Chapter 2: Exploring the acute adaptation of central and peripheral motor unit features to exercise-induced fatigue with different modes of loading.

2.1 Introduction

The motor unit (MU) is a key component of the motor system with control of muscle force output regulated by rate coding and MU recruitment (Enoka & Duchateau, 2017). The concept of fatigue is vast and encompasses several definitions, however that related to neuromuscular decrement is most accurately described as performance fatigue; the loss of force and/or power output from a muscle as a result of impaired contractile function and/or muscle activation (Enoka & Duchateau, 2016). Performance fatigue is a feature often seen acutely post exercise and is usually the cause of task failure (Hunter *et al.*, 2004). It is also observed in chronic clinical conditions (Chaudhuri & Behan, 2004; Burtin *et al.*, 2012; Prinsen *et al.*, 2015) and ageing (Merletti *et al.*, 2002; Christie *et al.*, 2011). Irrespective of the setting, the involvement of multiple physiological mechanisms and experimental triggers presents challenges in determining the cause (Enoka & Duchateau, 2008).

The muscle response to fatigue has been shown to be affected by the muscle contraction modality. For example, fatigue caused by both concentric (CON) and eccentric (ECC) contractions elicits reductions in muscle strength (Linnamo *et al.*, 2000) and muscle activation (Peñailillo *et al.*, 2013) yet the recovery profiles relating to damage are different (Souron *et al.*, 2018). Of the three commonly applied contraction types (isometric, CON and ECC) ECC "lengthening" contractions generate greater voluntary forces and appear less strenuous due to a lower metabolic cost and cardiovascular stress (Webber & Kriellaars, 1997; Overend *et al.*, 2000; Hody *et al.*, 2019). These features make eccentric training programs favourable for improving muscle mass and strength in both healthy adults and those with compromised musculoskeletal health (Roig *et al.*, 2009; Cook *et al.*, 2013).

The neural mechanisms influencing performance fatigue, including MU recruitment thresholds, firing rate (FR), and voluntary activation, appear to differ across exercise modalities (Kay *et al.*, 2000; Duchateau & Baudry, 2014) with some data showing an increase in MU FR following fatiguing exercise (Dartnall *et al.*, 2009; Piitulainen *et al.*, 2012), while others report a decrease (Kuchinad *et al.*, 2004; Adam & De Luca, 2005; Rubinstein & Kamen, 2005; Stock *et al.*, 2012; Contessa *et al.*, 2016). More recently, a direct comparison of contraction modalities showed a greater increase in MU FR following

CON, when compared to ECC contractions (Hirono *et al.*, 2022). These equivocal findings may be explained by a muscle specific effect or by differences in the way fatigue was induced, with reductions in maximal contraction following prolonged low-intensity activity occurring as a result of reduced muscle activation, and reductions following high-intensity activity attributable to impaired contractile function (Enoka & Duchateau, 2016).

High density surface EMG (HD-sEMG) records multiple MU potentials (MUPs) from a relatively large volume of muscle (Martinez-Valdes *et al.*, 2016) and is well placed to assess FR of multiple MUs during a single contraction (Negro *et al.*, 2016; Oliveira & Negro, 2021) and at larger contraction intensities (Del Vecchio *et al.*, 2018*a*). Additionally, the use of intramuscular EMG (iEMG) with concentric needles enables a more detailed view of MUPs via sampling without the limitations of signal attenuation through skin and subcutaneous tissue. iEMG recordings enable estimation of neuromuscular junction (NMJ) transmission instability and temporal dispersion across MU propagating action potentials (Piasecki *et al.*, 2021*b*) as well as MUP gradients reflecting ion exchange and membrane excitability. Thus, the combination of these techniques allows an overall assessment of central (properties relating to MU activation) and peripheral (properties affected by structure and function of the muscle fibres) MU adaptations to a given stimulus.

The aim of this study was to determine the muscle-specific response of vastus lateralis (VL) MU features following a functional stepping task employing bilateral CON and ECC movements performed to failure. It was hypothesised that strength and force steadiness would decline in both legs as a result of performance fatigue. Due to the higher metabolic demand of CON movements, it was hypothesised MU function would be more greatly affected by CON movements than ECC.

2.2 Methods

2.2.1 Participants

17 healthy recreationally active volunteers (9 female, 8 male) gave written informed consent to take part in this study which was approved by the University of Nottingham Faculty of Medicine & Health Sciences Research Ethics Committee (186-1812). The study was conducted in accordance with the Declaration of Helsinki except for registration in a database. Power calculations indicated a minimum of 10 participants to detect a within-subject difference of 20% with α of 0.05 and β of 0.85. Data is available for 12 participants

(6 female, 6 male) for iEMG, and 10 participants (5 female, 5 male) for HD-sEMG due to low yield of MUs at one or more time points and/or contraction levels. Exclusion criteria included a body mass index (BMI) <18 or >35kg/m^2, currently competing in sports at regional level or above, or a history of cardiovascular, respiratory, or neuromuscular disorders. All participants were asked to refrain from strenuous exercise 48 hours prior to assessment. All participants were medically screened prior to the study via a medical history questionnaire and a resting electrocardiogram (ECG).

2.2.1.1 Anthropometric measures

Body mass and height were measured using calibrated scales and stadiometry, respectively, and BMI calculated. Individual muscle cross sectional area (CSA) was recorded using ultrasound (MyLab, Esaote, Italy) from the mid-belly of VL of both legs. The mid-belly of VL was determined as the mid-point between the greater trochanter and the patella. Medial and proximal borders of the VL were identified from the points the aponeuroses intersected with vastus intermedius muscle. Three axial plane images were collected and subsequently analysed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA).

2.2.2 Experimental protocol

2.2.2.1 Fatigue protocol

To induce simultaneous CON and ECC fatigue in all participants, we employed a step up, step down protocol (Kostek *et al.*, 2007). Participants concentrically contracted the quadriceps by stepping up onto a 43.5 cm high bench with one leg, and stepping down with the opposing leg eccentrically, with each stepping contraction timed to a 3s metronome (Figure 2.1). Participants wore a weighted vest (initially 25% of body weight but increasing up to 40% depending on fitness and tolerance) during the intervention. The task was performed until self-ascribed exhaustion, which was indicated as 10 using a modified Borg scale. The average time to exhaustion was 53±12 minutes, with 60 s timed rest stop permitted when requested, for a maximum of 3 rest stops. All participants reported they were right-leg dominant, and the leg assigned to CON or ECC was randomised, as was the order in which the post-stepping assessments were conducted. All assessment procedures were performed immediately before, and post-testing began on the first limb within 5 minutes after completing the fatiguing protocol. All electrodes

remained in place secured by tape between pre- and post-testing, so reapplication was not required.

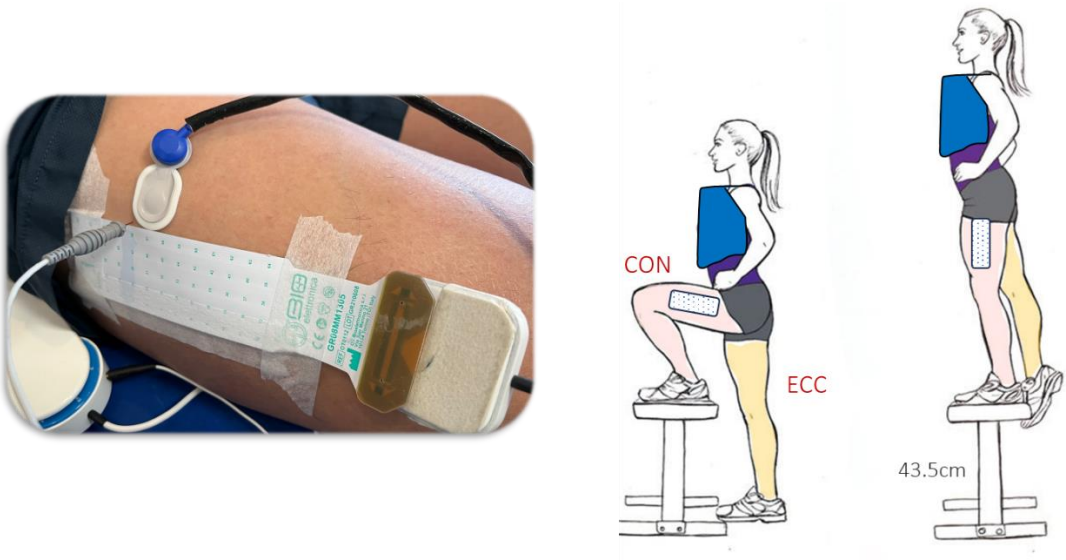


***Figure 2.1**: Schematic showing electrode placement for EMG recordings during pre and post assessments which remained in place during the step-up, step-down exercise protocol.*

2.2.2.2 Strength assessment

Knee extensor strength was assessed with participants sitting with hips and knees flexed at 90° and the leg securely fastened to a force transducer just above the ankle. To familiarize with the equipment and "warm-up" the muscle, participants performed a series of submaximal contractions. They were then instructed to perform a maximal isometric contraction, accompanied by verbal encouragement and visual feedback of force. This was repeated three times, with 60 s rest intervals. The best effort was taken as maximum voluntary isometric contraction force (MVC). After determining MVC, participants performed isometric voluntary contractions each lasting 12–15s, aiming to hold a target line set at 25% and 40% MVC displayed on a monitor, and rested for ~30s between contractions (Figure 2.2A). The coefficient of variation of the force trace (CoV force) represented force steadiness (FS). Voluntary contraction and signal recording was repeated until a minimum of six recordings at 25% and three at 40% had been obtained, alternating between 25% and 40% MVC. Post-intervention forces were normalised to the post-intervention MVC. The force signals were displayed in real-time using Spike2 software (v9.06, Cambridge Electronic Design, UK) and data collected simultaneously from the force transducer for offline analysis. HD-sEMG and iEMG were recorded simultaneously during each of these contractions as detailed below.

2.2.2.3 High-density surface EMG (HD-sEMG)

A semi-disposable adhesive monopolar HD-sEMG matrix (GR08MM1305, OT Bioelettronica, Torino, Italy) consisting of 64 x 8 mm spaced electrodes was positioned in the presumed direction of the fascicles on both (right and left) VLs and secured to the skin before the pre-fatigue assessments (Figure 2.1). This remained in situ until after the post-assessments. A reference cable (CPAT1, OT Bioelettronica) was attached around the ankle of the recording limb. The signal was amplified, sampled at 2000 Hz, band-pass filtered (10-500 Hz), and converted to digital data (16-bit analogue-digital converter, 3 dB bandwidth) using a Sessantaquattro (OT Bioelettronica) multi-channel amplifier. Data was visualised in real-time in OTBioLab+ software (v1.3.2, OT Bioelettronica) and stored for offline analysis.

2.2.2.4 Surface EMG (sEMG)

An active recording sEMG electrode (disposable self-adhering Ag-AgCl electrodes; 95 mm^2, Ambu Neuroline, Baltorpbakken, Ballerup, Denmark) was placed over the motor point located around the mid-thigh of the VL. A reference electrode was placed over the patella tendon and a common ground electrode for both surface and iEMG measurements placed over the patella. Surface EMG signals were bandpass filtered between 5 Hz and 5 kHz via CED 1902 amplifiers (Cambridge Electronics Design Ltd., Cambridge, United Kingdom), sampled at 10 kHz and digitized with a CED Micro 1401 data acquisition unit (Cambridge Electronic Design).

2.2.2.5 Intramuscular EMG (iEMG)

A 25 mm concentric needle electrode (74025-45/25 Neuroline; Ambu, Baltorpbakken, Ballerup, Denmark) was inserted directly above the HD-sEMG electrode. A voluntary, low force contraction was performed by the participant while the needle position was adjusted to ensure its tip was close to fibres belonging to active MUs (Stashuk, 1999*a*; Piasecki *et al.*, 2019). The participant then performed isometric voluntary contractions lasting 12–15 s, aiming to hold a target line set at 25% and 40% MVC as described above. The needle electrode was repositioned between contractions by combinations of rotating the bevel 180° and withdrawing it by 10–25 mm to sample MUs at a range of depths (Jones *et al.*, 2021). The procedure of needle positioning, voluntary contraction, and signal recording was repeated until a minimum of six recordings at 25% from varying

depths had been obtained to ensure sampling from a representative set of MUs. iEMG signals were amplified (D440, Digitimer, UK), acquired and bandpass filtered from 10 Hz to 10 kHz and sampled at 50 kHz (1401, Cambridge Electronic Design, UK). The force and EMG signals were displayed in real-time using Spike2 software (v9.06), and data were stored for off-line analysis (Figure 2.2A). The needle insertion site was recorded and matched from pre- to post-intervention.

2.2.3 Data analysis

2.2.3.1 HD-sEMG analysis

HD-sEMG data was analysed in MATLAB (v2019a, IBM) using custom written scripts to decompose and identify MUs using an extensively validated method (Negro *et al.*, 2016; Martinez-Valdes *et al.*, 2017*b*). HD-sEMG data from the two middle contractions (contraction 3 and 4 out of six recorded) at 25% and from contraction 2 and 3 at 40% MVC were analysed. The HD-sEMG recording electrodes remained in place during the intervention to improve the probability of sampling the same MUs. The accuracy of the decomposition for each MU was tested with the silhouette (SIL) measure which is a normalized accuracy index for sEMG decomposition (Negro *et al.*, 2016). Only MUPs with a SIL greater than 0.90 were included for further analysis. Subsequently, the decomposition accuracy was improved by manual editing of consecutive firings (Boccia *et al.*, 2019; Afsharipour *et al.*, 2020). Mean FR and the CoV of the interspike interval (CoV ISI - FR variability) from recruited MUs were calculated from the HD-sEMG signals from the sustained force section of the contractions. Following decomposition, individual MUs were tracked by a previously validated technique based on cross-correlation of single-differential 2D MUPs (Martinez-Valdes *et al.*, 2017*b*). In this procedure, matched MUPs between pre and post fatigue trials were visually inspected, and the two identified motor units were regarded as the same when they had a cross-correlation coefficient >0.80.

2.2.3.2 iEMG analysis

The procedures for recording and analysing individual MUPs have been described in detail previously (Piasecki *et al.*, 2016*c*, 2016*a*). Intramuscular signals from 25% contractions only were analysed using decomposition based quantitative electromyography (DQEMG) (Stashuk, 1999*b*). DQEMG was used to automatically extract MU potential trains (MUPTs) of separate MUs from an iEMG signal and calculate a MUP template for each extracted

MUPT. MUPTs composed of MUPs from more than one MU or with fewer than 20 MUPs were excluded. MUP templates of included MUPTs were visually inspected and markers corresponding to the onset, end, and positive and negative peaks adjusted where required. MUP duration, in ms, is the time interval between the MUP template onset and end, MUP area, in µVms, is the MUP template area between its onset and end. MUP amplitude, in mV, is the difference between the MUP template positive and negative peak values. MUP thickness is MUP area divided by MUP amplitude and describes the shape of the MUP template (Abdelmaseeh *et al.*, 2014). MUP complexity was assessed using the number of turns in the MUP template. A 'turn' was defined as a change in direction of the MUP template of at least 25 µV and indicates the level of temporal dispersion across individual muscle fibre contributions to a single MUP. MUP negative peak slope ratio was calculated as the absolute value of the rise of the MUP template negative peak, across the 500 µs interval before the negative peak, divided by the fall of MUP template negative peak, across the 500 µs interval after the peak (Figure 2.2B). While the MUP template negative peak rise and fall slopes will each be similarly influenced by the relative location of the recording electrode, their ratio can represent relative rates of ion exchange during the depolarisation and repolarisation phases of an action potential, respectively. A near fibre MUP (NFM) is calculated by applying a low pass, second-order differential filter to its corresponding MUP (MUP acceleration), which effectively reduces the recording area of the needle electrode, ensuring only the nearest fibres significantly contribute to the NFM and reducing interference from distant active fibres of other MUs (Stashuk, 1999*a*; Piasecki *et al.*, 2021*b*). All NFMs were visually inspected and those containing contamination from other NFMs were removed. NFM jiggle is a measure of the shape variability of consecutive NFMs of an MUPT expressed as a percentage of the NFM template total area (Piasecki *et al.*, 2021*b*) and is representative of NMJ transmission instability (Figure 2.2C). Example data are shown in Figure 2.2.

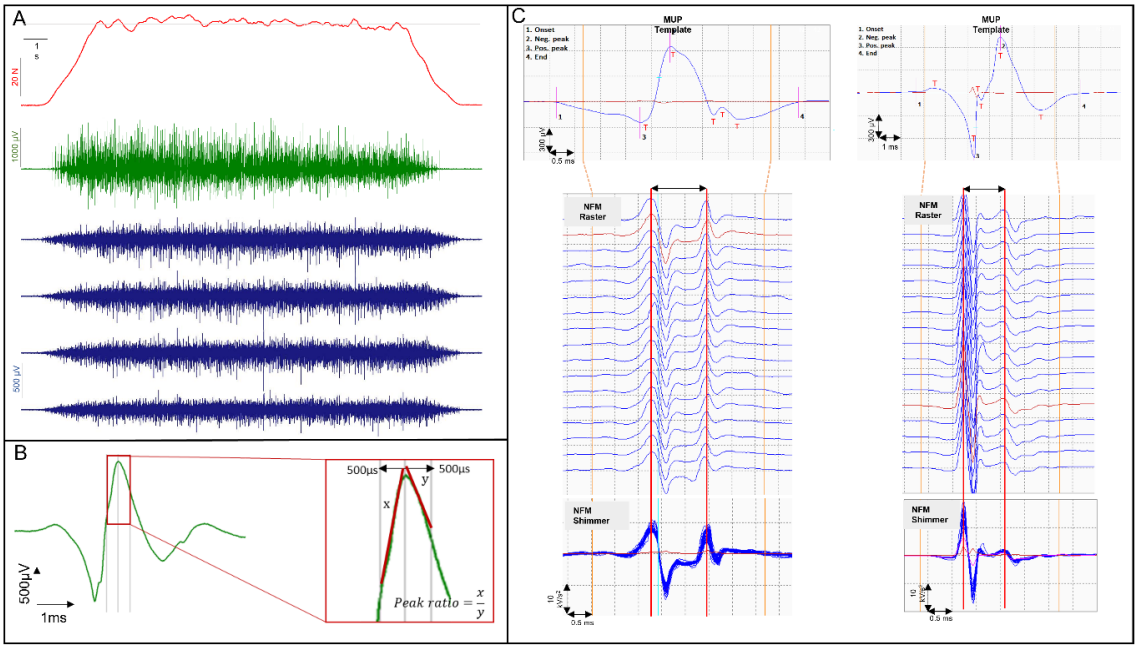


***Figure 2.2**: Example iEMG waveform analysis.*

A) Example force trace at 25% MVC (red) with corresponding iEMG (green) and subset of 4 HD-sEMG channels (blue). B) Representative MUP template indicating peak ratio measure. C) Representative MUP templates indicating MUP turns (T) and corresponding near-fibre MUPs (NFMs) shown in raster and shimmer plots.

Abbreviations; N; newtons, µV; microvolts, MUP; motor unit potential, NFM; near fibre motor unit potential, ms; milliseconds µs; microseconds.

2.2.3.3 Statistical analysis

Two-way repeated measures analysis of variance (ANOVA) with Sidak's post hoc analysis were performed in GraphPad Prism (v9.2, USA) to test for the effect of time and contraction modality on MVC and CoV force. Where no significant contraction modality x time interaction was found, main effects are reported. As multiple MUPs were recorded from each participant, multi-level mixed effects linear regression models were performed in StataSE (v15.0, StataCorp LLC, TX, USA) with contraction modality and time as factors and contraction x time interactions included in each model. In each model the first level (individual MUs) were clustered according to each participant to form the second level. This modelling framework is suitable for data of this nature as it incorporates all sampled MUs as opposed to only the mean values obtained from each participant, which preserves variability to a greater extent within and across participants simultaneously. The use of linear models is also suitable for data which is both normally and non-normally distributed. Regression coefficients (β) and 95% confidence intervals (CI) are reported

that indicate the magnitude and direction of the effects of interest. Significance was assumed if $p<0.05$.

2.3 Results

2.3.1 Participant characteristics

Participant characteristics (6M/6F) are presented in Table 2.1.

Table 2-1: Participant characteristics (n=12).

	Mean (SD)	
Age (years)	21 (0.6)	
Height (m)	1.74 (0.11)	
Weight (kg)	71.9 (13.5)	
BMI (kg/m^2)	23.7 (3.4)	
	CON	ECC
VL CSA (cm^2)	28.71 (11.41)	29.23 (12.32)

2.3.2 Effect of fatigue on the motor unit population

2.3.2.1 Functional properties

There was no significant contraction modality x time interaction ($p=0.742$) for MVC but there was a main effect of time ($p<0.0001$; Figure 2.3A), with MVC decreasing approximately 15.8% with CON (Pre vs Post: 473±143N vs 386±126N) and 20.6% with ECC (463±142N vs 363±109N).

No significant contraction modality x time interaction for FS was observed at 25% MVC but there was a main effect of time ($p=0.0016$; Figure 2.3B) with a 21.7% increase with CON (3.51±0.95% vs 4.09±0.95%) and a 48.8% increase with ECC (3.34±0.99% vs 5.04±1.96%). No significant contraction modality x time interaction for FS was observed at 40% MVC but a main effect of time was observed ($p=0.0038$; Figure 2.3C) with a 20.9% increase with CON (3.71±1.26% vs 4.22±1.51%) and a 56.1% increase with ECC (3.65±1.34% vs 5.45±2.38%).

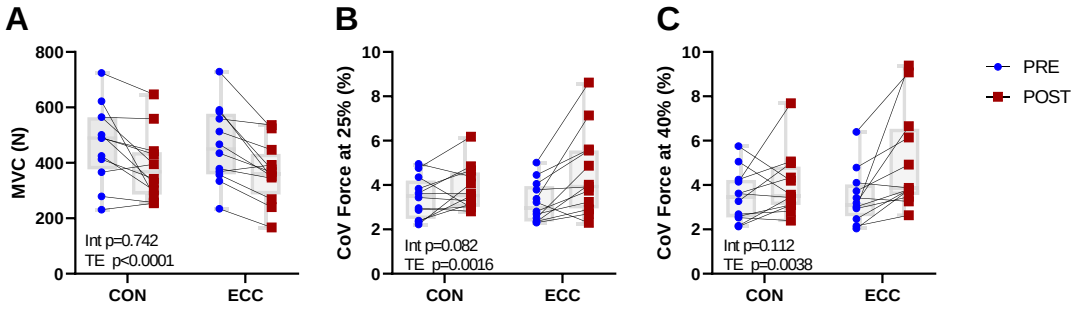


***Figure 2.3**: Maximum voluntary contraction and force steadiness results.*

Individual maximum voluntary contraction (MVC) (A) and force steadiness at 25% (B) and 40% MVC (C) before (PRE-blue) and after (POST-red) concentric (CON) and eccentric (ECC) exercise. Interaction (Int) and effect of time (TE) statistical results of the two-way ANOVAs are shown.

2.3.2.2 Motor unit discharge features

A total of 881 MUs were sampled with HD-sEMG, with a mean of 7±2 per 25% contraction per leg, and a mean of 6±2 per 40% contraction per leg. At 25% MVC there was a significant contraction modality x time interaction for FR ($p<0.001$), with no difference following CON ($\beta=0.34$, $p=0.112$) but a significant increase following ECC ($\beta=1.44$, $p<0.001$). Similarly, at 40% MVC there was a significant contraction modality x time interaction ($p<0.001$) for FR, with no significant change in the CON limb ($\beta=-0.55$, $p=0.072$), but an increase in the ECC limb ($\beta=1.62$, $p<0.001$) (Table 2.2, Figure 2.4A). There was no significant contraction modality x time interaction for FR variability at 25% MVC ($p=0.526$), although it was higher post exercise at 25% MVC (CON; $\beta=2.32$, $p<0.001$, ECC; $\beta=1.79$, $p=0.004$). At 40% MVC there was also no significant contraction modality x time interaction ($p=0.902$) for FR variability, with an increase in both the CON leg ($\beta=2.27$, $p=0.002$) and the ECC leg ($\beta=2.35$, $p=0.006$) (Table 2.2, Figure 2.4B).

Table 2-2: Group means, regression coefficient (β) and 95% confidence intervals (CI) for all HD-sEMG derived MU features (n=10).

	Mean (SD)		Regression coefficient (β)	Confidence Interval (CI)	Significance (p)
	PRE	**POST**			
Firing rate (Hz) – 25%					
CON	7.73(1.23)	8.00(1.53)	0.34	-0.08:0.75	p=0.112
ECC	7.71(1.02)	8.78(2.33)	**1.44**	**0.98: 1.90**	**p<0.001**
Firing rate (Hz) – 40%					
CON	8.99(1.84)	8.82(2.02)	-0.55	-1.15: 0.05	p=0.072
ECC	8.81(1.41)	10.1(4.08)	**1.62**	**0.91: 2.33**	**p<0.001**
FR Variability (%) – 25%					
CON	12.0(2.02)	13.6(2.20)	**2.32**	**1.22: 3.41**	**p<0.001**
ECC	12.7(2.36)	14.5(3.20)	**1.79**	**0.589: 2.99**	**p=0.004**
FR Variability (%) – 40%					
CON	13.8(2.16)	16.1(3.18)	**2.27**	**0.49: 4.05**	**p=0.002**
ECC	13.8(2.49)	15.5(5.02)	**2.35**	**0.67: 4.03**	**p=0.006**

Significant values shown in bold. Hz, hertz; CON, concentric; ECC, eccentric; FR, firing rate

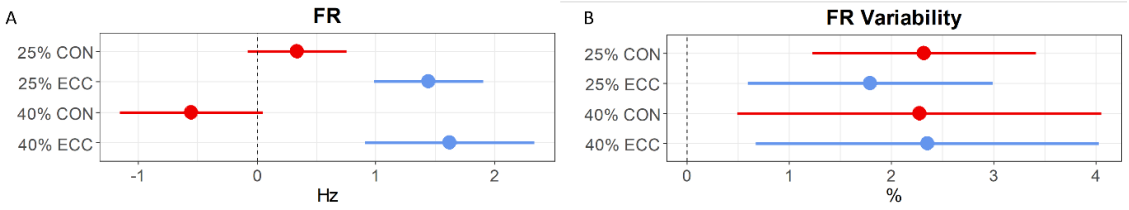


***Figure 2.4**: Firing rate and firing rate variability results.*

Motor unit firing rate (FR) (A) and FR variability (B) forest plots showing β and 95% confidence intervals at 25% (top) and 40% maximum voluntary contraction (MVC) (bottom) following concentric (CON) and eccentric (ECC) exercise. Statistical analyses are based on multilevel mixed effects linear models.

2.3.2.3 Tracked motor units

From HD-sEMG, 129 MUs (approx.15%) were tracked from pre to post fatiguing exercise across 10 participants. At 25% MVC there was no significant contraction modality x time interaction for FR (p=0.053), and no difference in FR was observed in the CON leg following fatigue (β=-0.15, p=0.725; Figure 2.5A). However, with ECC FR increased at 25% (β=1.12, p=0.025, Table 2.3, Figure 2.5A). There was a significant contraction modality x time interaction in FR at 40% MVC (p=0.011). There was no difference in FR in the CON

leg following fatigue (β=-0.27, p=0.471; Table 2.3; Figure 2.5B) however, as at 25%, with ECC FR increased at 40% MVC (β=1.17, p=0.006; Figure 2.5E). Additionally, in these tracked units, there was no significant contraction modality x time interaction for FR variability at 25% (p=0.663) with FR variability not significantly changing in CON (β=1.96, p=0.08) or ECC (β=1.21, p=0.352) following fatigue (Figure 2.5C). At 40% there was no significant contraction modality x time interaction (p=0.203) and FR variability increased in the CON leg (β =2.62, p=0.011) but did not change in the ECC leg (β=0.64, p=0.580; Table 2.3, Figure 2.5D, 2.5F).

Table 2-3: Regression coefficient (β) and 95% confidence intervals (CI) for all HD-sEMG derived MU features in tracked units (n=10).

	Regression coefficient (β)	Confidence Interval (CI)	Significance (p)
Firing rate (Hz) – 25%			
CON	-0.15	-0.98:0.68	p=0.725
ECC	**1.12**	**0.14: 2.09**	**p=0.025**
Firing rate (Hz) – 40%			
CON	-0.27	-1.01: 0.47	p=0.471
ECC	**1.17**	**0.34: 2.00**	**p=0.006**
FR Variability (%) – 25%			
CON	1.96	-0.23: 4.15	p=0.080
ECC	1.21	-1.34: 3.76	p=0.352
FR Variability (%) – 40%			
CON	**2.62**	**0.588: 4.65**	**p=0.011**
ECC	0.64	-1.63: 2.91	p=0.580

Significant values shown in bold. Hz, hertz; CON, concentric; ECC, eccentric; FR, firing rate.

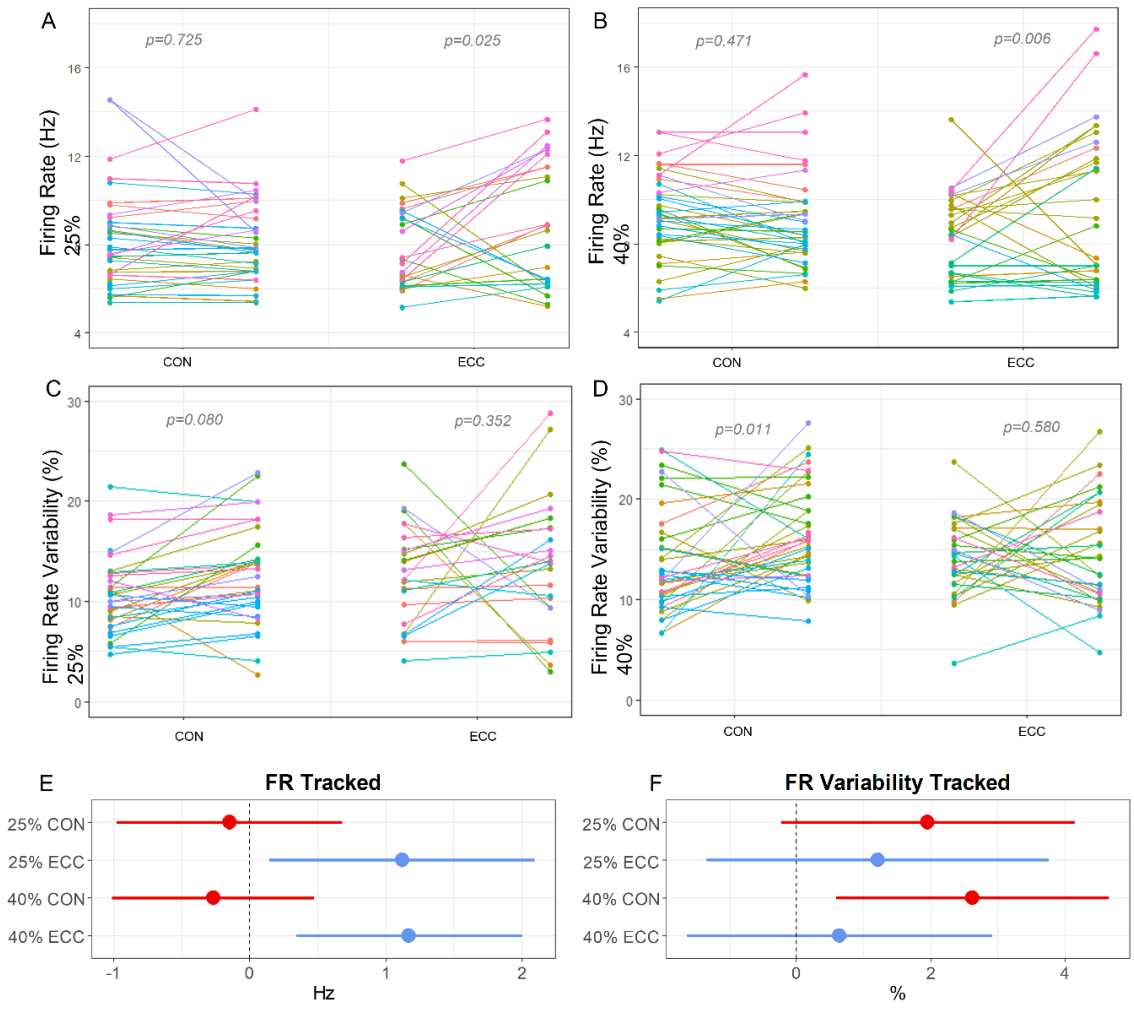


***Figure 2.5**: Motor unit results from tracked units.*

Motor unit firing rate (A) and firing rate variability (B) at 25% maximal voluntary contraction (MVC) of individual tracked motor units for before and after concentric (CON) and eccentric (ECC) exercise and motor unit firing rate (C) and firing rate variability (D) at 40% MVC. Points are colour coded for individual participants.

Motor unit firing rate (E) and firing rate variability (F) forest plots showing β and 95% confidence intervals for the change in CON and ECC exercise at both 25% (top) and 40% MVC (bottom) for the tracked MUs, from pre to post fatiguing exercise.

Statistical analyses are based on multi-level mixed effects linear models.

2.3.3 Peripheral motor unit adaptations

Multi-level mixed effects regression models showed there was no significant contraction modality x time interaction for MUP thickness (p=0.059) which did not differ in the CON (β=0.0611, p=0.130) or ECC leg (β=-0.0511, p=0.244) (Table 2.4, Figure 2.6A). No significant contraction modality x time interaction was observed for MUP complexity (p=0.58) with no significant change with either CON (β=-0.216, p=0.168) or ECC (β=-0.088, p=0.605; Table 2.4, Figure 2.6B). There was no significant contraction modality x time interaction for MUP negative peak slope ratio (p=0.319). With CON, MUP negative peak slope ratio significantly increased (β=0.411, p=0.018), however there was no change with ECC (β=0.159, p=0.392; Table 2.4, Figure 2.6C). Although there was no significant contraction modality x time interaction for NMJ transmission instability (p=0.244), it increased in both the CON (β=1.66, p=0.037) and ECC legs (β=3.08, p=0.001) (Table 2.4, Figure 2.6D).

Table 2-4: Group means, regression coefficient (β) and 95% confidence intervals (CI) for all iEMG derived motor unit and near fibre features (n=12).

		Mean (SD)		Regression coefficient (β)	Confidence Interval (CI)	Significance (p)
		PRE	POST			
MUP Thickness						
	CON	1.34(0.41)	1.34(0.46)	0.0611	-0.018: 0.140	p=0.130
	ECC	1.50(0.44)	1.48(0.57)	-0.0511	-0.137: 0.035	p=0.244
MUP Complexity						
	CON	4.1(1.0)	4.1(0.8)	-0.216	-0.523:0.091	p=0.168
	ECC	4.0(1.1)	3.9(1.0)	-0.088	-0.421:0.245	p=0.605
MUP Neg Peak Slope Ratio						
	CON	1.87(0.28)	2.14(0.53)	**0.411**	**0.072: 0.751**	**p=0.018**
	ECC	2.07(0.72)	1.97(0.28)	0.159	-0.204: 0.521	p=0.392
NMJ Transmission Instability (%)						
	CON	14.2(3.3)	16.0(4.7)	**1.66**	**0.10: 3.23**	**p=0.037**
	ECC	14.8(1.9)	17.2(3.3)	**3.08**	**1.29: 4.88**	**p=0.001**

Significant values shown in bold. MUP, motor unit potential; CON, concentric; ECC, eccentric; NMJ, neuromuscular junction.

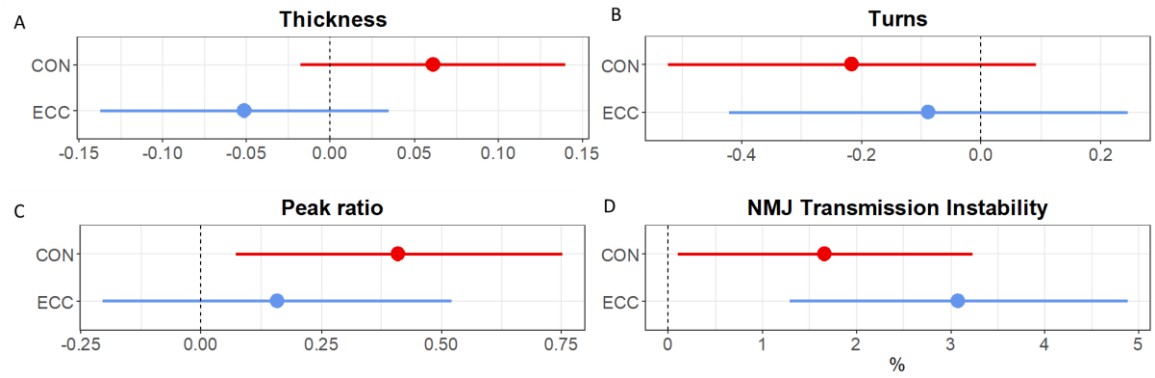


***Figure 2.6**: Results from iEMG measures.*

Motor unit potential (MUP) thickness (A), MUP turns (B), MUP negative peak slope ratio (C) and near fibre motor unit potential (NFM) features of neuromuscular junction (NMJ) transmission instability (D) forest plots showing β and 95% confidence intervals for the change after concentric (CON) and eccentric (ECC) exercise. Statistical analyses are based on multi-level mixed effects linear models.

2.4 Discussion

Here data is presented using intramuscular needle electrodes in combination with HD-sEMG to investigate muscle specific responses to a targeted fatiguing intervention and demonstrate that individual MU features are mediated by contraction type. Both ECC and CON resulted in similar reductions in isometric strength and FS which were matched by an increase in FR variability. The neural response differed across limbs within an individual at sub-maximal levels, with only ECC loading resulting in an increased FR during normalised force levels, and CON loading altering fibre membrane properties to a greater extent than ECC. These findings are important in understanding neuromuscular function following exercise-induced performance fatigue and highlight the influence of contraction type on these parameters.

The functional endurance-based intervention utilised in this study was selected to induce performance fatigue via limiting muscle activation as opposed to contractile function (Enoka & Duchateau, 2016), and the form of movement mimics that of ascending and descending stairs; a highly functional task with translational implications to ageing and disease. Both forms of contraction elicited similar decreases in maximal isometric strength, which supports similar previous findings in this muscle group (Molinari *et al.*, 2006). Neuromuscular control, as assessed by FS, at normalised force levels across limbs, deteriorated in both legs. The ability to hold a steady contraction with minimal fluctuation

requires effective neuromodulation of motor output (Enoka & Farina, 2021), and FR variability also increased following both CON and ECC, at both contraction intensities.

MU FR increased following ECC but did not change following CON, when assessed at 25% and 40% of MVC. Contrasting findings of FR have been observed during ECC and CON contractions in previous studies (Linnamo *et al.*, 2003; Altenburg *et al.*, 2008), but here we demonstrate different FR responses during an identical isometric task, after an ECC and CON fatiguing intervention. In a recent unilateral crossover study, tracked MUs increased their FR to a greater degree following CON when compared to ECC, again occurring in a contraction-level specific manner (Hirono *et al.*, 2022). However, these MU FR were calculated across a broad range of contraction levels during ramped contractions, making the studies difficult to compare, and fatigue was induced via machine-based exercise as opposed to the functional task applied here. Previous findings also show muscle twitch force reduced to a greater extent following ECC, when compared to CON contractions (Piitulainen *et al.*, 2011), and the marked increase in FR following ECC observed in the current study likely occurs as a central mechanism to compensate for declines in twitch force (Pincheira *et al.*, 2021). These findings are also supported by the observations of Piitulainen *et al.,* (Piitulainen *et al.*, 2012), where FR increased during sustained contractions following ECC exercise.

From the smaller sample of MUs tracked following fatigue, the differences in firing properties generally followed a similar pattern to the population results, showing an increase in FR in ECC but no significant change in CON. However, FR variability increased in CON at 40% MVC only following fatigue, but no statistical differences were observed at the lower intensity or after ECC in these tracked units. The percentage of MUs tracked is lower than previously reported and may reflect the methodological approach (e.g. HD-sEMG electrodes remaining in place during intervention), an alteration of MUP duration as a result of fatigue (McManus *et al.*, 2017) or it may reflect differences in MU populations recruited immediately post fatigue (Martinez-Valdes *et al.*, 2017*b*). Although previous research has found moderate to high level recruitment thresholds to decrease following concentric and eccentric fatiguing exercise (Hirono *et al.*, 2022), the relatively low proportion of MUs able to be tracked prevents robust reporting of this in the current study.

The simultaneous use of iEMG enabled the collection of more detailed information on peripheral MU parameters following differing loading strategies. MUP thickness describes

the shape and size of a MUP and no changes were observed following either exercise modality. The number of turns of a MUP is an assessment of MUP complexity and reflects increased temporal dispersion across MU propagating action potentials. It is applied in clinical settings to investigate myopathic and neuropathic conditions alongside other MU parameters (De Carvalho *et al.*, 2014; Allen *et al.*, 2015), and increases acutely following limb immobilisation (Inns *et al.*, 2022; Sarto *et al.*, 2022). However, similar to MUP thickness, complexity did not change following CON or ECC exercise. NFM jiggle is a measure of the variability of temporal dispersion across MU propagating action potentials occurring primarily due to NMJ transmission variability (Piasecki *et al.*, 2021*b*), and this increased following both contraction modes. Although identifying pre- and post-synaptic NMJ dysregulation in response to fatigue is extremely difficult in humans, physiological plausibility exists relative to both sites; data from animal models has shown a depletion of synaptic vesicles following prolonged stimulation mimicking fatigue (Wu & Betz, 1998), and increased exposure to acetylcholine (ACh) can result in AChR desensitisation (Magleby & Pallotta, 1981). These distinct differences in NFM jiggle have also been observed across age (Hourigan *et al.*, 2015; Piasecki *et al.*, 2021*a*) and in disease (Allen *et al.*, 2015), however as a result of MU remodelling functionally affecting the NMJ (Jones *et al.*, 2022) which is unlikely to have occurred acutely in the current study. Notably, NMJ transmission instability increased at a lower absolute force (normalised to 25% post-fatigue MVC), which is the opposite to the expected trend of increasing with larger forces (Guo *et al.*, 2022).

The MUP negative peak slope ratio quantifies the relationship between the rise and fall slopes of the negative peak of the MUP template, reflecting the ratio of ionic exchange rates across the depolarization and repolarization phases of the action potential, respectively. This ratio increased following CON exercise only, which was explained by a shorter *rise* (depolarisation) time relative to the *fall* time (repolarisation), the latter of which is achieved via the efflux of intracellular K^+. Repeated activation of skeletal muscle results in a *net* efflux of K^+, and thus an increase in extracellular K^+ concentration in fatigued muscle (Allen *et al.*, 2008; Fortune & Lowery, 2009). In this fatigued state, K^+ is required to move up its concentration gradient and may explain the slowing of repolarisation (relative to depolarisation) occurring in the limb with higher metabolic cost (the CON leg).

Collectively, these findings of adaptation of MU features following fatiguing contractions as a direct result of contraction modalities has implications for interventional strategies

intended to target neural input and MU function. This knowledge may influence the contraction type employed for training depending on the requirements of the targeted population. This may also have translational relevance in understanding conditions such as sarcopenia and cancer where exercise tolerance due to muscle fatigue and weakness present a challenge in strength training (Gault & Willems, 2013).

2.4.1 Limitations

The fatiguing exercise protocol employed here required CON and ECC contractions performed to self-reported exhaustion, however it is not possible to directly quantify the contribution of each contraction type to total exhaustion or the bilateral influence of central fatigue. In addition, the cross-talk effects from other quadriceps muscles could not be accounted for therefore compensation activity from other muscles in both contraction types could be masking possible differences in strength and MU properties following fatigue. MU features were explored up to 40% of MVC only, and further contraction-level specific adaptations may be present at higher contractions where MUs with MU fibre type proportions may differ. Here, possible muscle damage effects were not being investigated and therefore changes to contractile properties were not assessed. The possible influence of sex on these parameters is unclear and we did not control for hormonal status in females. However, whilst this may be viewed as a mechanistic limitation, the full exclusion of females in studies of this nature would be more limiting and previous work has found no differences in neuromuscular recruitment strategies between sexes (Guo *et al.*, 2022). Finally, this study was conducted in young participants only therefore the translation of results to older adults and disease cohorts may be altered and requires investigation.

2.4.2 Conclusions

The current findings highlight how MU features respond to fatigue in a contraction dependent manner, and how despite similar reductions in strength after fatiguing exercise, MU FR responded differently following CON and ECC contractions. The instability of NMJ transmission increased following both exercise modes, however, markers of fibre membrane excitability were altered following the more metabolically demanding CON loading only, which likely reflects an accumulation of extracellular K^+ and a slowing of fibre repolarisation. Differences in the MU response to CON and ECC induced

fatigue could have relevance for optimising exercise training protocols by including only specific contraction types, particularly in populations with specific musculoskeletal limitations or injury.

Chapter 3: Examining the impact of skeletal muscle fatigue on motor unit properties and microvascular blood flow.

3.1 Introduction

As outlined in the previous chapters, performance fatigue is commonly defined as the loss of force and/or power output from a muscle as a result of impaired contractile function and/or muscle activation affecting performance (Enoka & Duchateau, 2016). Generating submaximal forces for a prolonged period of time is necessary for multiple tasks including walking and standing, and therefore it is an important factor in both exercise performance and the performance of activities of daily living. Increased muscle fatiguability is present in a number of disease cohorts which can contribute to functional impairment and subsequently impact recovery and quality of life (Kent-Braun *et al.*, 2012).

The motor unit (MU) plays a key role in regulating force production through MU recruitment and rate coding (Enoka & Duchateau, 2017). With the onset of fatigue, a combination of central and peripheral factors are altered resulting in a decline in force output characteristic of muscle fatigue affecting exercise performance. Previous research has found declines in firing rate (FR) (Calder *et al.*, 2008), a reduction in recruitment thresholds (McManus *et al.*, 2015) and slowing of conduction velocity (Lowery *et al.*, 2002) as a result of fatigue, particularly with isometric contractions as reviewed previously in Chapter 2. Using a combination of high density surface electromyography (HD-sEMG) and intramuscular EMG (iEMG) to record MU potential (MUPs) during a fatiguing contraction allows tracking of individual MUPs across the whole contraction (Martinez-Valdes *et al.*, 2020) and also detailed study of parameters reflective of neuromuscular junction (NMJ) activity without signal attenuation being a factor, as demonstrated in Chapter 2.

Decreases in the median frequency of the EMG power spectrum have often been used to detect muscle fatigue (Georgakis *et al.*, 2003) however, these spectral properties are heavily influenced by fibre distance from the detection point and MU recruitment limiting validity (Farina, 2006). Advances in surface EMG signal decomposition using pattern recognition enable the recording of individual MUP trains allowing a greater yield of information about specific MU firing patterns and recruitment. Analysis of these signals in the frequency domain to generate coherence measures within different frequency bands, allow quantification of MU synchronisation and study of motor neuron connectivity enabling a greater understanding of neural drive and common synaptic input

(Dideriksen *et al.*, 2018). The common synaptic input within different frequency bands is associated with force control, afferent feedback or contraction strength dependent on activity within a specific band (Alix-Fages *et al.*, 2023). Previous research has found increases in MU synchronisation and common drive during isometric fatiguing contractions however, many of these study the muscles of the hand (Hwang *et al.*, 2020; Liu *et al.*, 2021). Common neural drive is important for accurate control of muscle force output with low frequency components of common synaptic input influencing motor control (Farina & Negro, 2015) and it could be sensitive to fatigue induced changes modifying afferent feedback loops and affecting the descending pathway (Gandevia, 2001).

Isometric contractions, where the muscle produces a force with little change in fascicle length, are widely used for the study of muscle function, particularly those with EMG applications. However during an isometric contraction, microvascular blood flow (MBF) can be restricted due to anatomical occlusion and increases in intramuscular pressure (McNeil *et al.*, 2015). Muscle blood flow is important for its contractile and metabolic functions including the delivery of oxygen and nutrients which are required for aerobic respiration to generate energy for contraction and for the removal of by-products, as well as delivery of other factors for physiological processes such as cell signalling, protein synthesis and inflammation (Kusters & Barrett, 2016). Changes in muscle blood flow have frequently been linked to muscle fatigue and task failure (Sjøgaard *et al.*, 1988; Clark *et al.*, 2005), with associated mechanisms responsible for this suggested to include accumulation of metabolites such as lactate and potassium ions affecting afferent feedback, haemoglobin desaturation and reduced substrate delivery (Lanza *et al.*, 2006; Enoka & Duchateau, 2008).

Despite both MU properties and MBF being suggested as contributing factors to fatigue, there is currently limited research simultaneously investigating muscle MBF and its relationship with specific MU properties reflecting MU size, MUP shape and FR; all of which are known to impact muscle function (Piasecki *et al.*, 2018*b*). Indeed, it is known that blood flow is important for the delivery of neurotrophins required for plasticity, repair and regeneration of the motor nerves for MU remodelling to prevent muscle atrophy and fibre loss (Cheng *et al.*, 2012; English *et al.*, 2014). However, this is primarily based on measures of limb or large vessel blood flow, and as such the relationship between muscle MBF and MU properties is unclear.

Therefore, the aims of this study were to determine the effects of an isometric fatiguing contraction on MU properties, alongside the effects on MBF using contrast enhanced ultrasound (CEUS).

3.2 **Methods**

3.2.1 Participants

8 healthy young volunteers (3 female; 31±6yrs) gave written informed consent to participate in the pilot study approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (516-2003 CEUS-MRI), with participant characteristics summarised in Table 3.1. Before enrolment on to the study, a screening visit was attended by all participants where medical history, height and weight, electrocardiogram (ECG) and blood pressure were recorded to confirm suitability for the study. All participants were asked to refrain from exercise for 48 hours prior to each assessment visit. The menstrual cycle was not controlled for in female participants, but this has previously been shown not to affect microvascular function (Williams *et al.*, 2020) or neuromuscular performance, having no effect on FR at this force level (Piasecki *et al.*, 2023).

3.2.1.1 Anthropometric measures

Body mass and height were measured using calibrated scales and stadiometry, respectively, and body mass index (BMI) calculated. Vastus lateralis (VL) cross sectional area (CSA) and muscle thickness were recorded using ultrasound (MyLab, Esaote, Italy) from the right VL using previously described protocols (Inns *et al.*, 2022) and as described in section 2.2.1.1. In brief, for the assessment of CSA the mid-belly of VL was determined as the mid-point between the greater trochanter and the patella. Medial and proximal borders of the VL were identified from the points the aponeuroses intersected with vastus intermedius muscle. Three axial plane images were collected and subsequently analysed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA). Muscle thickness was determined as the distance between the superficial and deep aponeurosis taken from 3 points across the muscle and an average calculated.

3.2.2 Experimental study day protocol

Participants attended the research laboratory at ~0900h following an overnight fast (from midnight with water *ad libitum*). Right knee extensor strength was assessed with participants sitting on a custom-built chair with hips and knees flexed at 90° and the leg securely fastened to a force transducer 41.8±2.8 cm (Mean±SD) below the centre of the knee joint (i.e., above the medial malleolus). Participants performed a maximal voluntary isometric contraction (MVC), accompanied by verbal encouragement and visual feedback of force on a computer screen. 90 s after determining the MVC, the participant performed an isometric voluntary fatiguing contraction lasting 3 min, aiming to hold a target line set at 30% MVC shown on a computer monitor. Participants then rested for 90 s before performing another MVC (Figure 3.1). The force signals were displayed in real-time using Spike2 software (v9.06, Cambridge Electronic Design, UK) and data collected simultaneously from the force transducer, HD-sEMG and iEMG for offline analysis.

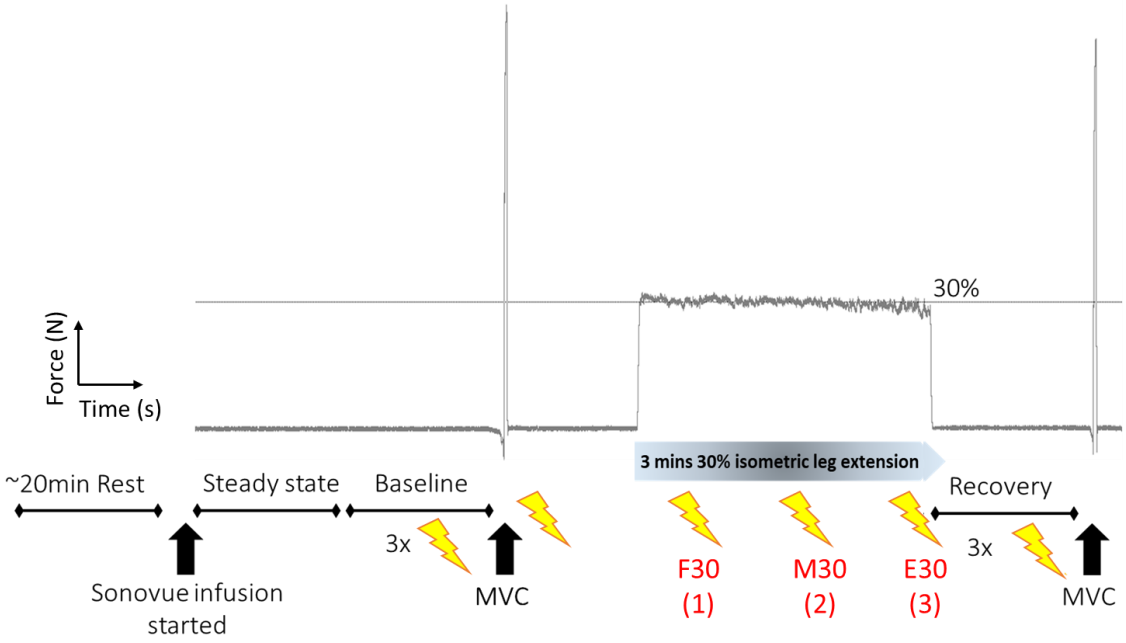


***Figure 3.1**: Schematic of the experimental protocol.*

Schematic demonstrating points of contrast bubble destruction ("flash" = lightning bolt) followed by 30s captures and corresponding force trace showing maximum voluntary contractions (MVCs) before and after a fatiguing contraction at 30% MVC.

3.2.2.1 High-density surface electromyography (HD-sEMG)

A semi-disposable adhesive monopolar HD-sEMG matrix (GR08MM1305, OT Bioelettronica, Torino, Italy) consisting of 64 x 8 mm spaced electrodes was positioned at the angle of the fibres on the right VL and secured to the skin 3 min before the first MVC. A reference cable (CPAT1, OT Bioelettronica) was attached around the ankle of the recording limb. The data was sampled at 2000 Hz and the signal was amplified using Sessantaquattro (OT Bioelettronica) and visualised in real-time in OTBioLab+ (v1.3.2, OT Bioelettronica) and stored for offline analysis.

3.2.2.2 Intramuscular EMG (iEMG)

A 25 mm concentric needle electrode (74025-45/25 Neuroline; Ambu, Denmark) was inserted adjacent to the recording HD-sEMG surface electrode with a semi-adhesive ground electrode positioned on the patella (Ambu). The needle was positioned 30 s before the start of the fatiguing contraction and remained in the same position throughout the 3 min contraction before being removed. iEMG signals were bandpass filtered from 10 to 10 kHz and sampled at 50 kHz. The force and EMG signals were displayed in real-time using Spike2 software (v9.06) and data were stored for off-line analysis.

3.2.2.3 Contrast enhanced ultrasound (CEUS)

A cannula was inserted into the vein of the preferred arm before a velcro-secured custom-made probe holder was fixed around the right thigh with ultrasound gel on the skin and an L9-3 mHz probe (Philips Healthcare, USA) in situ (Figure 3.2). This was followed by a 20-minute rest period to ensure a fixed period of rest prior to baseline measurements and to allow pH and temperature equilibrium between the skin and the probes. After this, Sonovue™ contrast agent (Bracco, Milan, Italy) was infused at 2ml/min for 1 minute and then 1ml/min for 30 sec to achieve systemic steady state (Mitchell *et al.*, 2013), with a continued infusion at 1ml/min for the duration of the measurements (10-min total). To determine baseline microvascular blood volume (MBV), three, 30-second capture-flash cycles were recorded using a Philips iU22 ultrasound machine (Philips Healthcare, USA) to form a Sonovue™ replenishment curve for the portion of VL under the probe. Next, an MVC, a 3 min submaximal isometric leg extension, 90 s recovery and repeat MVC were

performed with the right leg for the remaining infusion time (~5 minutes), with consecutive 30-second capture-flash cycles throughout (Figure 3.1).

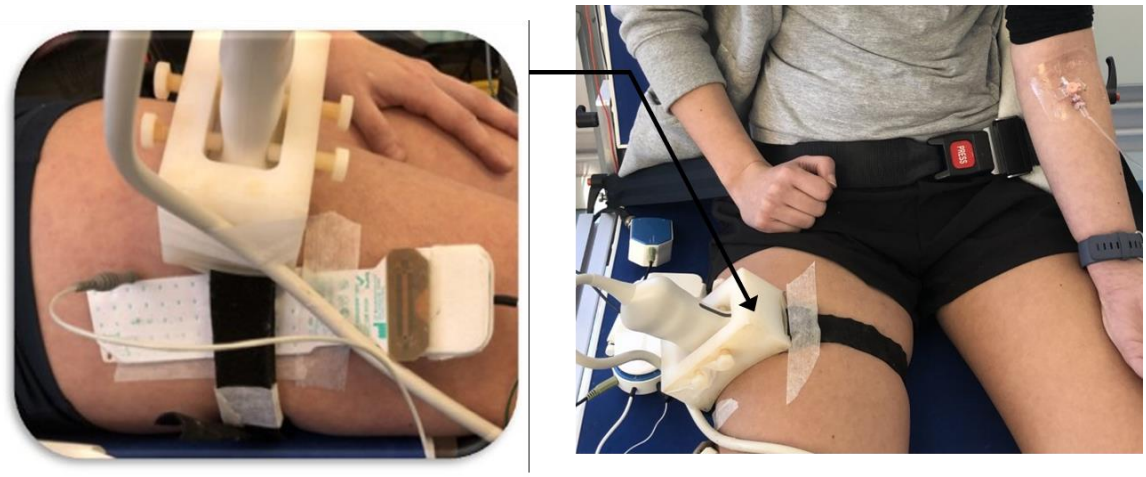


***Figure 3.2**: Participant set-up in a custom chair with the right leg secured to a force transducer.*

Ultrasound probe and high-density surface and intramuscular needle electrode positioning on the right vastus lateralis and cannula location in the left arm for Sonovue™ contrast infusion are shown.

3.2.3 Data analysis

3.2.3.1 HD-sEMG analysis

HD-sEMG data was analysed using the DEMUSE tool in MATLAB (v2019a, IBM) to decompose and identify MUs using an extensively validated method (Holobar *et al.*, 2014; Francic & Holobar, 2021; Hug *et al.*, 2021*a*). Briefly, the data was decomposed from 20s of the recording 10s from the end of the contraction using a blind source separation method using Convolution Kernel Compensation algorithm to identify MUs through estimating MU filters to generate a spike train (Holobar & Zazula, 2007). Firings for each MUP were manually inspected and edited through optimising and reapplying the MU filter and including appropriate firings and removing unreliable units with a pulse-to-noise ratio below 30dB (Holobar *et al.*, 2014). For this process, the MU filter was expanded over 30s segments of the contraction starting at the end of the contraction and working to the start with 15s overlapping sections (Martinez-Valdes *et al.*, 2020) and recalculated to identify spikes to generate the firing pattern (Figure 3.3). Once cleaned, MU properties for each MU were extracted in MATLAB. 1 male participant was excluded from this analysis due to only having 2 MUs identified during the contraction.

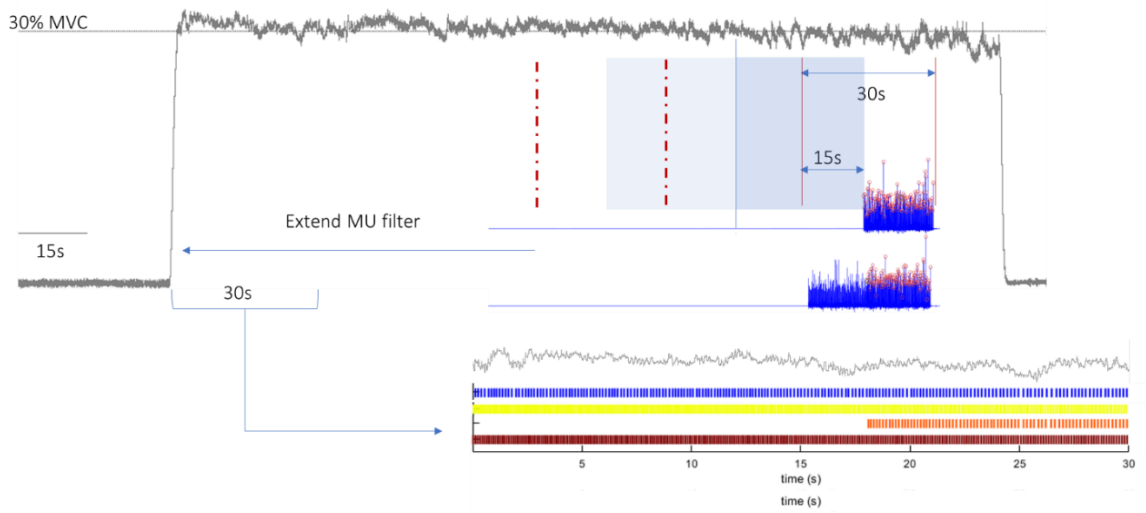


***Figure 3.3**: Force trace of the sustained contraction demonstrating MU FR analysis.*

A 20s segment was decomposed 10s from the end of the contraction and firings manually cleaned to optimise the MU filter. This was then expanded in 15s overlapping segments across the whole contraction. Representative MU spike trains for a 30s segment are shown following cleaning.

Mean FR, the coefficient of variation for the interspike interval (CoV ISI - FR variability), the coefficient of variation of force (CoV force) and root mean square amplitude (RMS) from recruited MUs were calculated from the HD-sEMG signals from the first 30s, middle 30s and end 30s of the plateau force section of the contraction. The RMS obtained from the sustained submaximal contractions was averaged over all channels of the electrode grid for each muscle. Cumulative spike trains (CST) were calculated as the sum of discharge times from the identified MUs and filtered using a 400ms Hanning window and represents the neural drive to the MU pool (Hug *et al.*, 2021*b*). Mean and CoV of the CST were calculated over the first, middle and end 30 s time periods for each participant (Figure 3.6).

To assess common drive to the MUs, the magnitude-squared coherence was calculated using the Welch's averaged periodogram with nonoverlapping windows of 1 s separately across the first, middle and end 30 s segments (Alix-Fages *et al.*, 2023). Two equally sized CSTs calculated from three MUs randomly selected from the identified MUs were used as the level of coherence depends on the number of MUs considered in the analysis (Farina & Negro, 2015). All unique combinations of three MUs were tested up to a maximum of 60 random permutations and the pooled coherence of these permutations was used for further analysis. The mean coherence within four different bandwidths (Delta: 0–5 Hz, Alpha: 5-12 Hz, Beta: 15-30 Hz, Piper: 40-50 Hz) were assessed to investigate different

features of neural input (Figure 3.8A). Coherence values were transformed to a standard z-score using the equation:

$$Z\ score = \sqrt{2L} \times \operatorname{atanh}\sqrt{C}$$

where C is coherence and L is the number of time segments used for the coherence analysis (e.g., for 30 s, L = 30, as the analysis was performed on 30 windows of 1 s) (Del Vecchio *et al.*, 2019*c*; Avrillon *et al.*, 2021). Coherence was considered significant when the z score was greater than the 95% confidence limit.

3.2.3.2 iEMG analysis

The procedures for recording and analysing individual MUPs have been described in detail previously (Piasecki *et al.*, 2016*c*, 2016*a*) and in section 2.2.3.2. Intramuscular EMG signals were analysed using decomposition based quantitative electromyography (DQEMG) (Stashuk, 1999*b*) used to automatically identify MUPs. Individual MUPs from MU potential trains (MUPTs) of separate MUs were identified from the iEMG signal. MUPTs that were composed of MUPs from more than one MU or had fewer than 20 MUPs were excluded. All MUP templates were visually inspected, and markers adjusted corresponding to the onset, end and positive and negative peaks of the waveforms where required. 2 participants (1 male, 1 female) were excluded from the iEMG analysis because not all timepoints had MUs of sufficient quality.

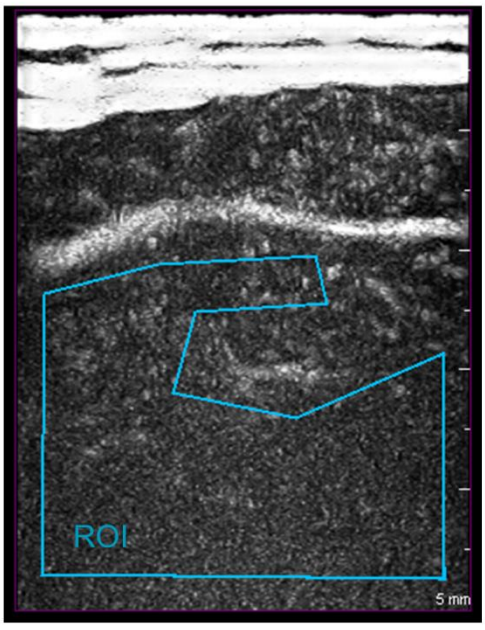
MUP duration, in ms, is the time interval between the MUP template onset and end, MUP area, in µVms, is the MUP template area between its onset and end. MUP amplitude, in mV, is the difference between the MUP template positive and negative peak values. MUP thickness is MUP area divided by MUP amplitude and describes the shape of the MUP template (Abdelmaseeh *et al.*, 2014). The MUP complexity was assessed using the number of turns in the MUP template. A 'turn' was defined as a change in direction of the waveform of at least 25 µV. The number of turns in the MUP templates indicates the level of temporal dispersion across individual muscle fibre contributions to a single MUP.

A near fibre MUP (NFM) is defined as the acceleration of its corresponding MUP and calculated by applying a second-order, low-pass differentiator to the MUP reducing the recording area of the needle electrode, ensuring only the closest fibres significantly contribute to the NFM and reducing interference from distant active fibres of other MUs

(Stashuk, 1999*a*; Piasecki *et al.*, 2021*b*). NFM jiggle is a measure of the shape variability of consecutive NFMs of an MUPT expressed as a percentage of the total NFM area.

3.2.3.3 CEUS analysis

CEUS video recordings were exported to Q-lab quantification software (Philips Healthcare, USA), where regions of interest (ROI) were selected from the recorded ultrasound image to minimise signal contribution from connective tissue and large rapid-filling vessels (Figure 3.4). From the selected ROI, the mean background acoustic index (AI) from tissue echogenicity (where all microbubbles were destroyed (Sjøberg *et al.*, 2011; Mitchell *et al.*, 2013)) was calculated from the period directly following a flash (~0.5 s) and subtracted from all subsequent values from recordings occurring during microbubble replenishment. These values were plotted as a replenishment curve and used to calculate the microvascular flow parameter of MBV using a one phase association non-linear regression in GraphPad Prism (v9.2, USA) to obtain an A (MBV) value (Figure 4). Measurement of the concentration of microbubbles at steady state provided an estimation of MBV from microvascular cross-sectional area and this was determined from the point of plateau from the replenishment curve after a high MI flash. An average of 3 resting MBV values taken over a 90 s period were used to calculate the baseline MBV.



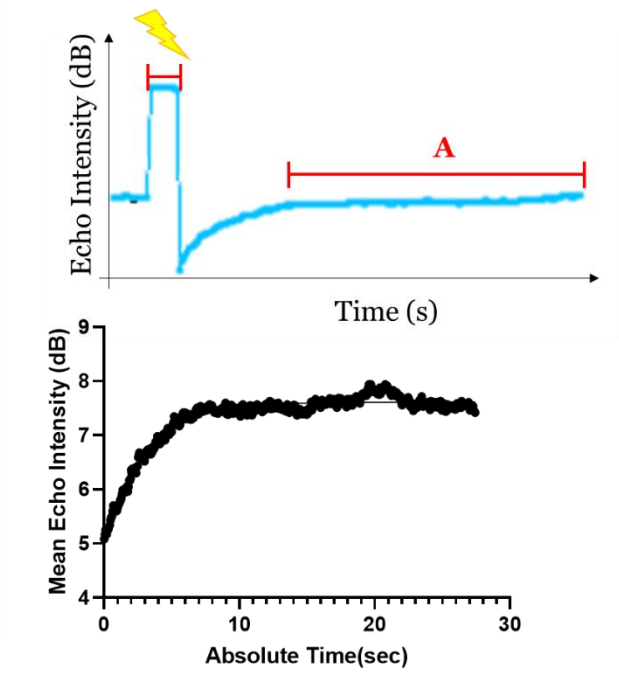


***Figure 3.4**: Region of interest and replenishment curve from CEUS analysis.*

Representative region of interest (ROI) avoiding rapid filling vessels and connective tissue from the ultrasound image. Replenishment curve from the ROI obtained following bubble destruction showing the plateau region (A) representing microvascular blood volume (MBV).

3.2.3.4 Statistical analysis

The Shapiro-Wilk test was used to assess normality with all data being normally distributed except for CoV CST, MUP thickness and NFM Jiggle. Paired t-tests were performed to test for the effect of fatigue on MVC and one-way repeated measures ANOVAs with Tukey's post-hoc analysis were performed to test for the effect of fatigue on force steadiness (FS), MBV and MU coherence during the contraction (GraphPad Prism). Multi-level mixed-effects linear regression models were performed in RStudio (v 2022.07.2 Build 576, PBC, USA) using the lme4 package (v 1.1-23) for each group to test for differences before and after fatigue in MU parameters while accounting for within-subject variability. These tests are suitable for both normally and non-normally distributed data. Additionally, regression coefficients and 97.5% confidence intervals (CI) are reported. Finally, linear regressions were performed to determine relationships between changes in MBV, FR, MVC and CoV force. Significance was assumed if $p<0.05$.

3.3 **Results**

3.3.1 Participant characteristics

The mean age of the participants was 31±6 years (Table 3.1), with other anthropometric characteristics also presented in Table 3.1. There were 5 males and 3 females.

Table 3-1: Participant characteristics (n=8).

	Mean (SD)
Age (years)	31 (6)
Height (m)	1.74 (0.09)
Weight (kg)	73.4 (14.8)
BMI (kg/m^2)	24.0 (3.6)
VL CSA (cm^2)	26.4 (9.9)
VL Thickness (cm)	2.5 (0.6)

3.3.2 Functional properties

MVC significantly decreased after the isometric fatiguing contraction (Pre vs Post: 471±165N vs. 424±136N, p=0.016; Figure 3.5A). CoV force significantly increased between F30 and E30 (p=0.013) and between M30 and E30 (p=0.014), but there was no significant difference between F30 and M30 (p=0.462; Figure 3.5B).

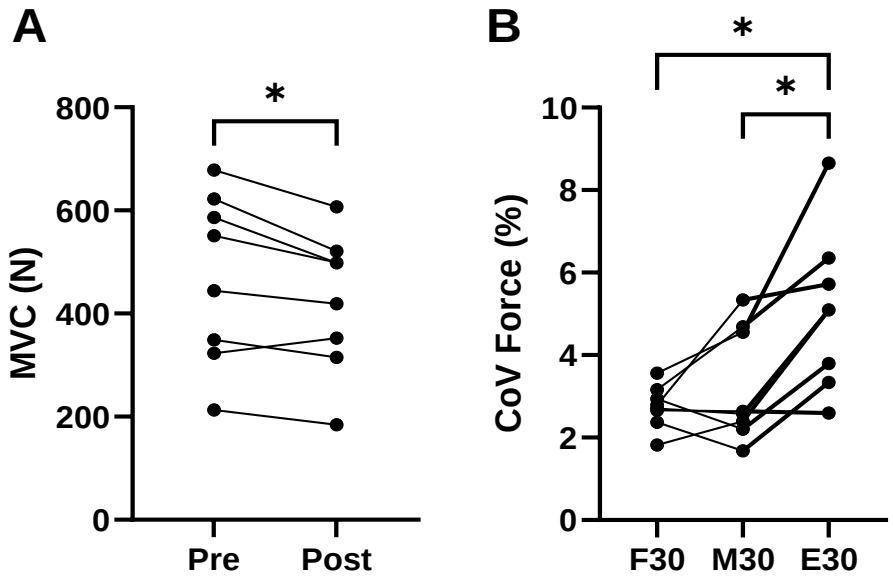


Figure 3.5*: Maximum voluntary contraction and force steadiness results.*

*A. Individual maximum voluntary contraction (MVC) before and after the fatiguing contraction. B. Individual force steadiness during the first 30 seconds (F30), middle 30 s (M30) and end 30 s (E30) (n=8). *= p<0.05.*

3.3.3 HD-sEMG parameters

An average of 9 MUPs were identified per person (n=7) at each time point. RMS did not change from F30 to M30 (β=-0.22, p=0.715) but did increase from M30 to E30 (β=5.58, p<0.001) and was greater than F30 (β=5.80, p<0.001) (Table 3.2, Figure 3.7A). FR significantly decreased between F30 and M30 (β=-1.66, p<0.001) but increased between the M30 and E30 (β=0.66, p=0.015), although remaining lower than F30 (β=-1.00, p<0.001) (Table 3.2, Figure 3.7B). Similarly mean CST also followed this pattern, initially decreasing (β=-0.012, p<0.001) before increasing (β=0.002, p=0.006) to a lower value that at the start of the contraction (β=-0.010, p<0.001) (Table 3.2, Figure 3.7D). There was no

change in CoV ISI at any time point during the contraction (Table 3.2, Figure 3.7C) but CoV CST decreased between F30 and M30 (β=-0.0074, p=0.0007) and F30 and E30 (β=-0.0093, p<0.001) but did not significantly change between M30 and E30 (β=-0.002, p=0.035) (Table 3.2, Figure 3.7E).

Coherence in the delta band (0-5Hz) progressively increased across the contraction between F30 and E30 (β=1.262, p=0.022) but did not significantly change between F30 and M30 (β=0.573, p=0.257) or M30 and E30 (β=0.689, p=0.178) (Figure 3.8B, Table 3.2). In the alpha band (5-12Hz) coherence did not change between F30 and M30 (β=0.212, p=0.501) but increased between M30 and E30 (β=0.695, p=0.042) and between F30 and E30 (β=0.907, p=0.012) (Figure 3.8C, Table 3.2). Coherence in the beta band (15-30Hz) did not change between F30 and M30 (β=-0.122, p=0.581) or E30 compared to both F30 (β=0.379, p=0.102) and M30 (β=0.257, p=0.253) (Figure 3.8D, Table 3.2). Similarly, in the piper band (40-50Hz) coherence did not change between F30 and M30 (β=-0.058, p=0.806) between M30 and E30 (β=0.116, p=0.628) or F30 and E30 (β=0.174, p=0.469) (Figure 3.8E, Table 3.2).

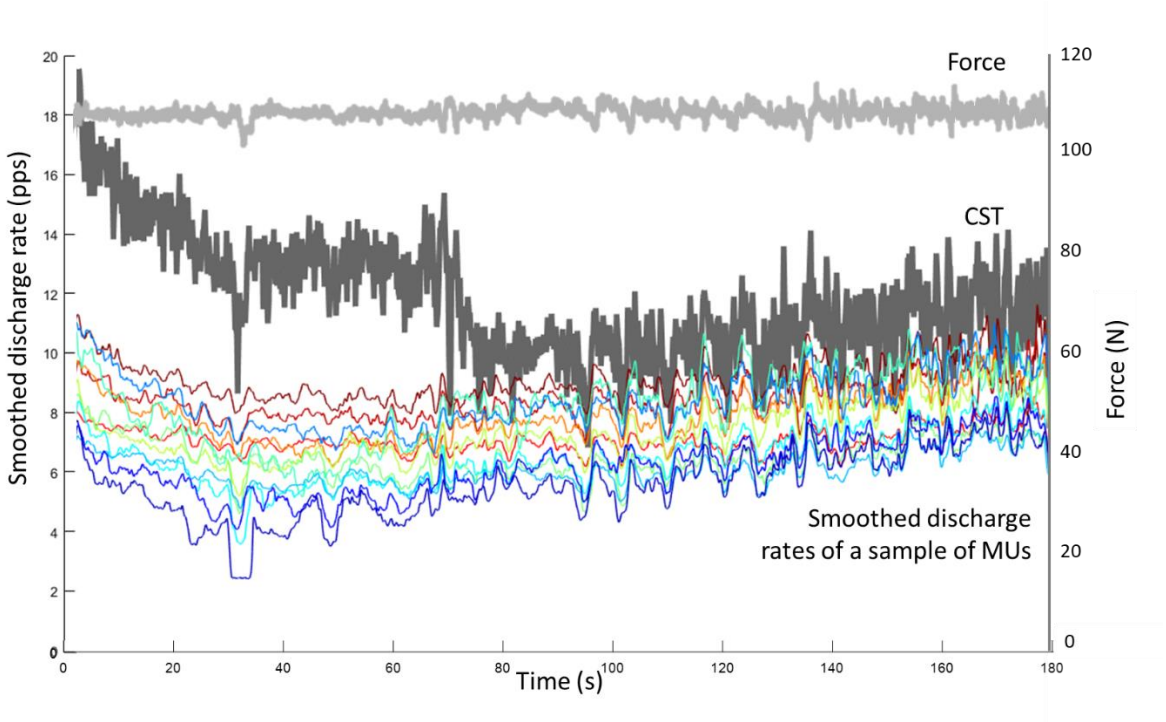


Figure 3.6: *Force, cumulative spike train and smoothed discharge rates from a sample of motor units.*

Representative force trace (light-grey), cumulative spike train (CST, dark-grey) and smoothed firing rates of identified from a sample of MUs for one participant across the whole 3-min contraction.

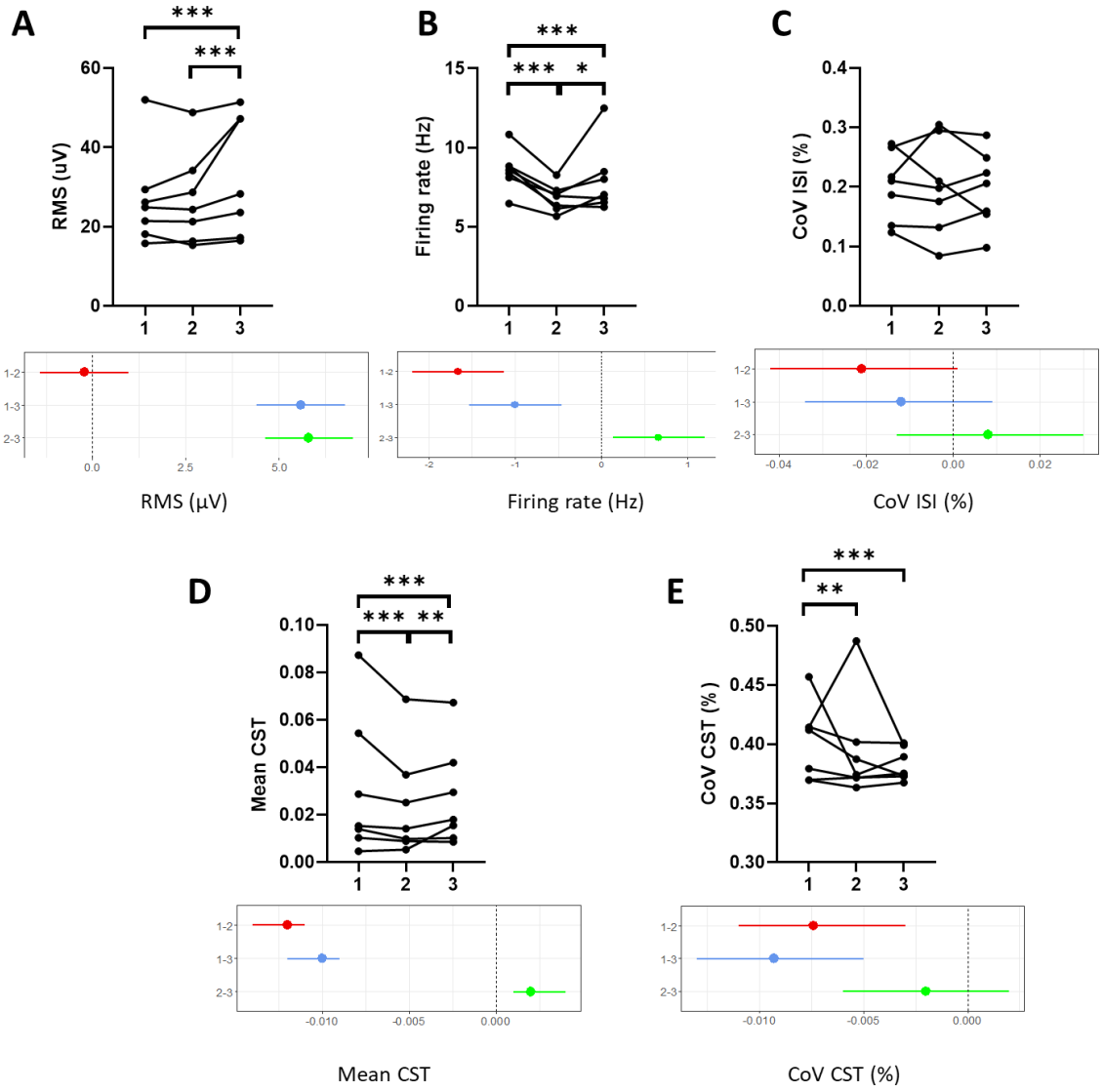


***Figure 3.7**: Individual means at each time point and forest plots for HD-sEMG parameters.*

*HD-sEMG parameters (n=7) of RMS (A), Firing rate (B), Firing rate variability (C), Mean CST (D) and CoV CST (E). Statistical analyses are based on multi-level mixed effects linear models to account for data clustering. *=p<0.05, ** p<0.01, *** p<0.001.*

Table 3-2:Regression coefficient (β) and 95% confidence intervals (CI) for all HD-sEMG derived MU features (n=7).

		Regression coefficient (β)	Confidence Interval (CI)	Significance (p)
RMS (µV)				
	1-2	-0.22	-1.404:0.963	p=0.715
	1-3	**5.58**	**4.398:6.764**	**p<0.0001**
	2-3	**5.80**	**4.618:6.985**	**p<0.0001**
Firing rate (Hz)				
	1-2	**-1.66**	**-2.195:-1.128**	**p<0.0001**
	1-3	**-1.00**	**-1.539:-0.461**	**p<0.0001**
	2-3	**0.66**	**0.126:1.198**	**p=0.015**
CoV ISI (%)				
	1-2	-0.021	-0.042:0.001	p=0.170
	1-3	-0.012	-0.034:0.009	p=0.528
	2-3	0.008	-0.013:0.03	p=0.528
Mean CST				
	1-2	**-0.012**	**-0.014:-0.011**	**p<0.0001**
	1-3	**-0.010**	**-0.012:-0.009**	**p<0.0001**
	2-3	**0.002**	**0.001:0.004**	**p=0.006**
CoV CST (%)				
	1-2	**-0.0074**	**-0.011:-0.003**	**p=0.0007**
	1-3	**-0.0093**	**-0.013:-0.005**	**p<0.0001**
	2-3	-0.0020	-0.006:0.002	p=0.348
Coherence – 0-5Hz				
	1-2	0.573	-0.37:1.517	p=0.257
	1-3	**1.262**	**0.318:2.206**	**p=0.022**
	2-3	0.689	-0.255:1.632	p=0.178
Coherence – 5-12Hz				
	1-2	0.212	-0.388:0.812	p=0.501
	1-3	**0.907**	**0.307:1.507**	**p=0.012**
	2-3	**0.695**	**0.095:1.295**	**p=0.042**
Coherence – 15-30Hz				
	1-2	0.122	-0.298:0.542	p=0.581
	1-3	0.379	-0.041:0.799	p=0.102
	2-3	0.257	-0.163:0.677	p=0.253
Coherence – 40-50Hz				
	1-2	-0.058	-0.513:0.397	p=0.806
	1-3	0.116	-0.34:0.571	p=0.628
	2-3	0.174	-0.281:0.629	p=0.469

Significant values shown in bold. Timepoints represented as 1, F30; 2, M30 and 3, E30. Abbreviations: Hz, hertz; RMS, root mean square; FR, firing rate; CoV, coefficient of variation; ISI, inter-spike interval; CST, cumulative spike train.

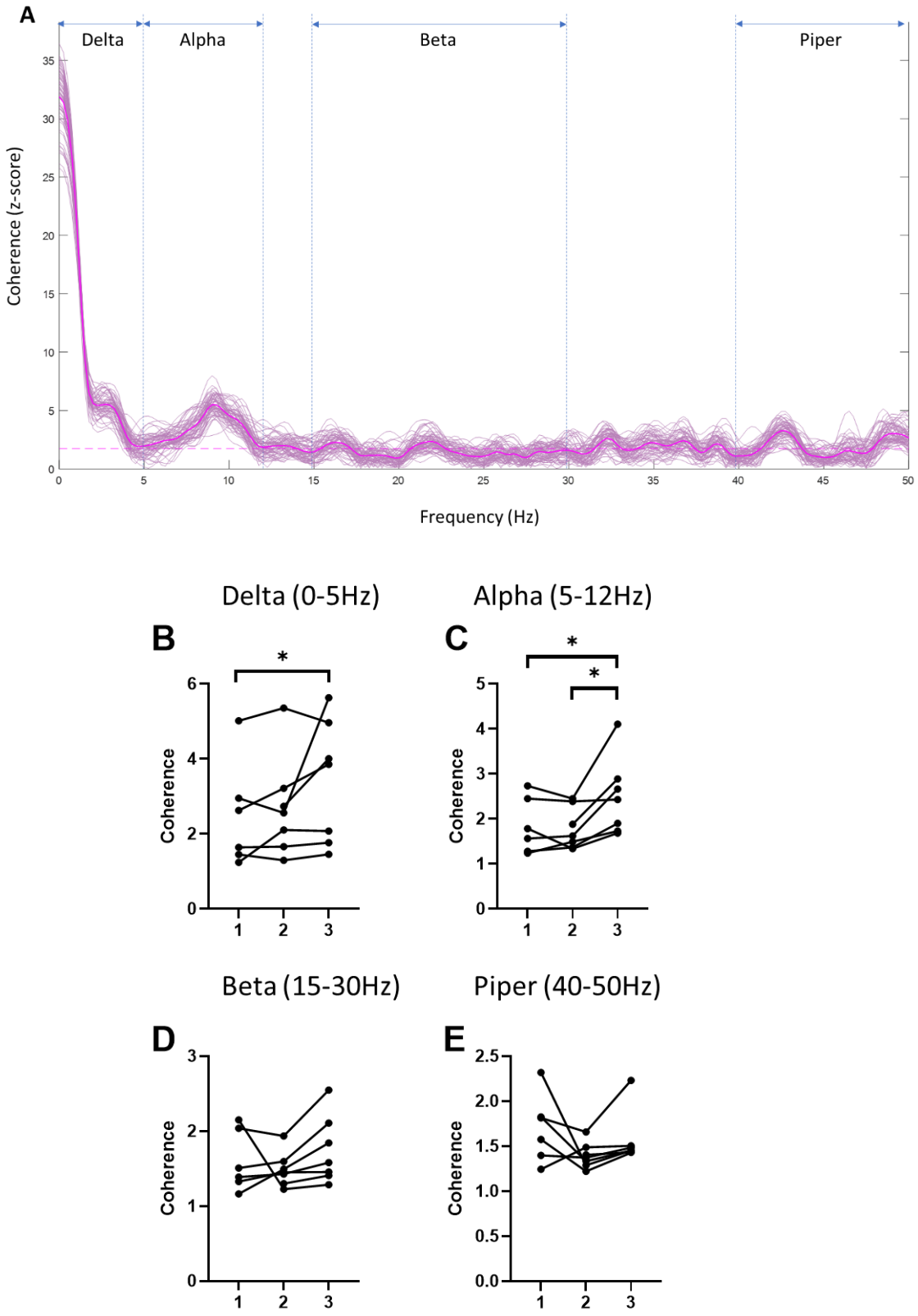


***Figure 3.8**: Motor unit coherence results.*

*Representative plot of coherence during the fatiguing contraction indicating the frequency bands reported and significance level (A) and individual means for the delta (B), alpha (C), beta (D) and piper (E) frequency bands at each time point (n=7). *= p<0.05.*

3.3.4 iEMG parameters

An average of 10, 10 and 8 MUPs were identified per person during F30, M30 and E30 respectively. MUP thickness increased from F30 to M30 (β=0.102, p=0.009) and from F30 to E30 (β=0.144, p=0.0003), but did not significantly increase from the M30 to E30 (β=0.043, p=0.268) (Table 3.3, Figure 3.9A). There was no change in MUP complexity (Figure 3.9B) or NMJ transmission instability (Table 3.3, Figure 3.9C) during the contraction.

Table 3-3: Regression coefficient (β) and 95% confidence intervals (CI) for all iEMG derived MU features (n=6).

		Regression coefficient (β)	Confidence Interval (CI)	Significance (p)
MUP Thickness				
	1-2	**0.102**	**0.031:0.172**	**p=0.009**
	1-3	**0.144**	**0.071:0.218**	**p=0.0003**
	2-3	0.043	-0.033:0.118	p=0.268
MUP Complexity				
	1-2	0.634	-0.028:1.307	p=0.181
	1-3	0.162	-0.536:0.860	p=0.649
	2-3	-0.477	-1.197:0.242	p=0.387
NMJ Transmission Instability (%)				
	1-2	-1.27	-3.818:1.269	P=0.652
	1-3	0.791	-1.87:3.452	P=0.652
	2-3	2.07	-0.678:4.808	P=0.420

Significant values shown in bold. MUP, motor unit potential; NMJ, neuromuscular junction.

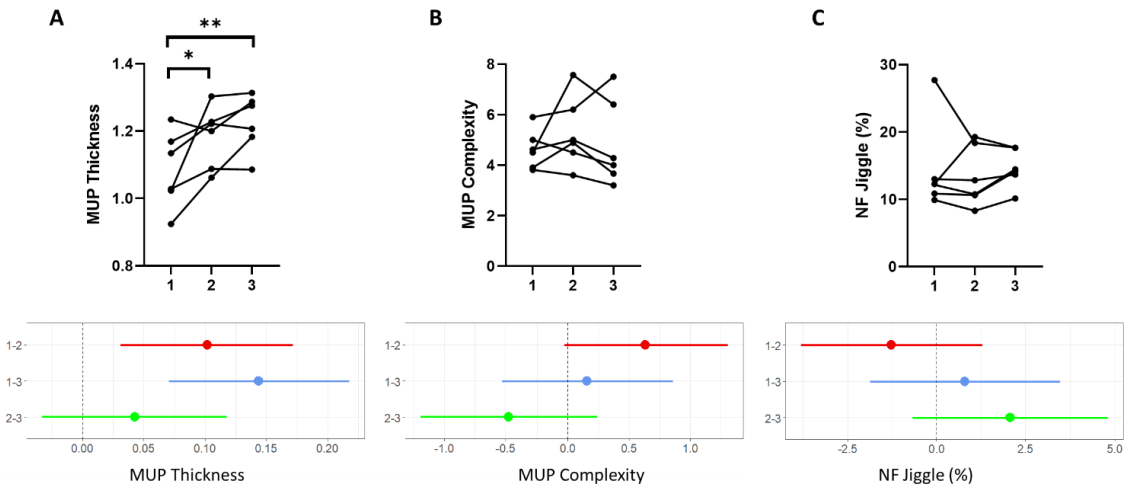


***Figure 3.9**: Results from iEMG.*

*Individual means and forest plots for intramuscular electromyography (iEMG) parameters (n=6) of MUP thickness (A), MUP complexity (B) and NF jiggle (C). *=indicates p<0.01, ** indicates p<0.001.*

3.3.5 Microvascular blood flow

No significant difference was observed in MBV from baseline to F30 or during the contraction (Baseline: 6.073±0.533, F30: 7.206±1.642, M30: 7.503±1.619, E30:7.649±1.946; p=0.076; Figure 3.10).

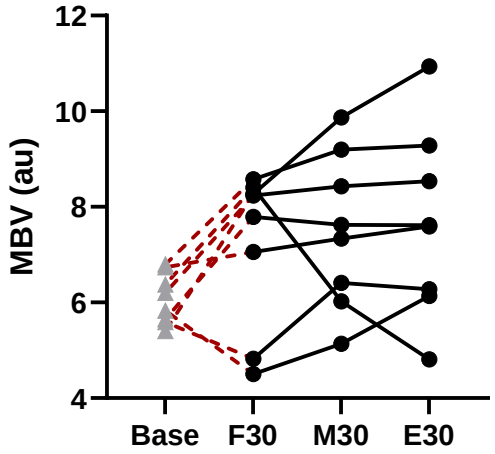


***Figure 3.10**: Individual microvascular blood volume (MBV) at baseline, during the first 30 seconds (F30), middle 30 s (M30) and end 30 s (E30) of the fatiguing contraction (n=8).*

3.3.6 Relationships between motor unit properties and mircrovascular blood volume with performance

No significant correlation was observed between the change in MBV and change in mean FR during the first and last 30s of the contraction (r=0.32, p=0.44, Figure 3.11A). No correlation was observed between the change in FR and the change in MVC (r=0.67, p=0.06, Figure 3.11B), and there was no correlation with the change in CoV force (r=0.14, p=0.73, Figure 3.11C) Additionally, no significant correlation was observed between the change in MBV and either the change in MVC (r=0.08, p=0.84, Figure 3.11D) or the change in CoV force (r=0.03, p=0.94, Figure 3.11E).

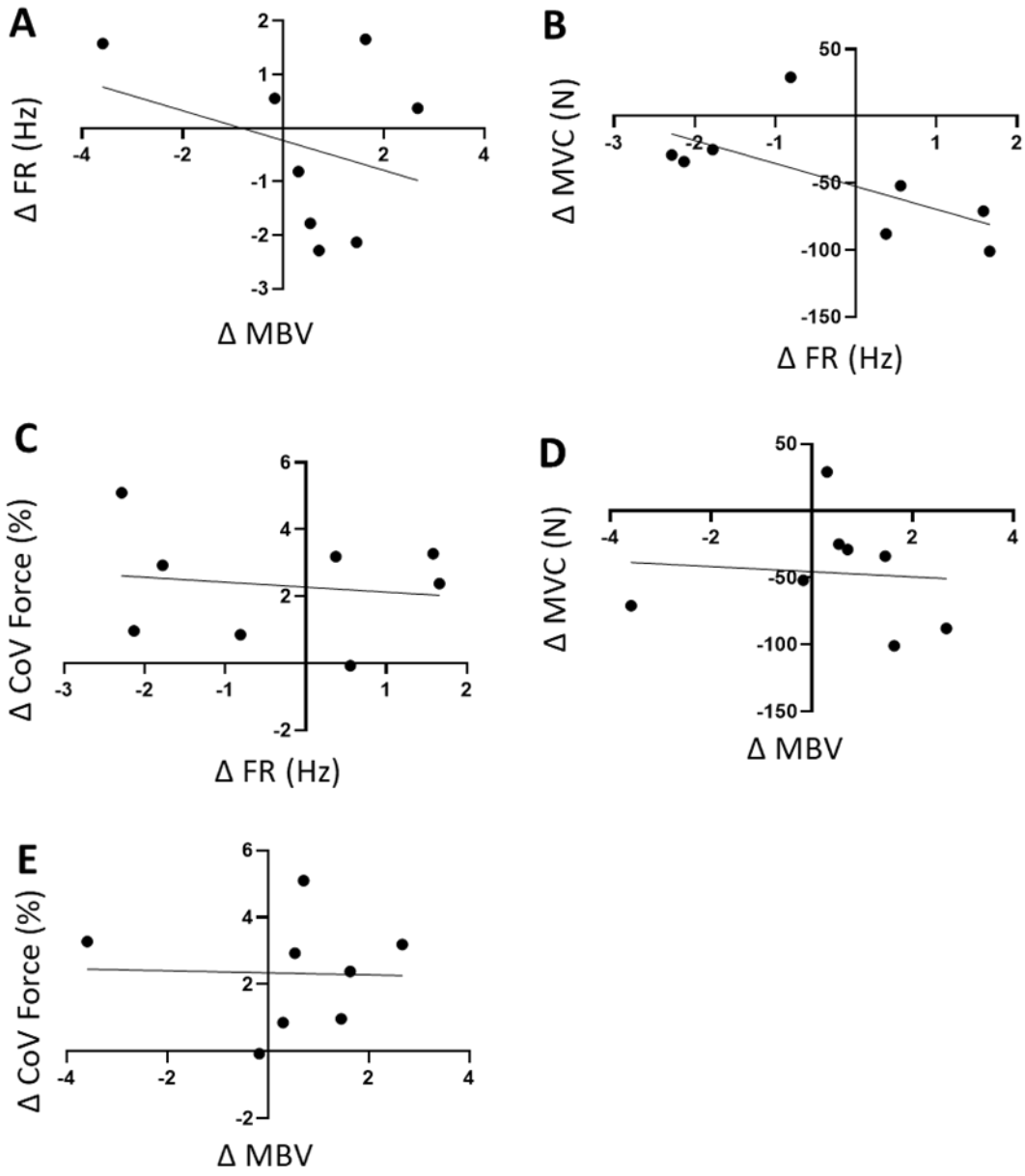


Figure 3.11*: Correlations of motor unit properties, function and microvascular blood volume.*

*Correlations (n=8) between the change in microvascular blood volume (MBV) and change in firing rate (FR) (A), change in FR and change in maximum voluntary contraction (MVC) (B), change in FR and change in coefficient of variation of force (CoV force) (**C**), change in MBV and change in MVC (**D**), change in MBV and change in CoV force (**E**).*

3.4 Discussion

Here we demonstrate a functional decline during a 3-min isometric fatiguing contraction that initiated marked changes to MU properties with no change to MBF. As a result of fatigue demonstrated by the reduction in voluntary force output after the sustained contraction, FS declined during the contraction with muscle activity increasing in the later stage. FR and therefore CST initially decreased before increasing again in the latter half of the contraction. No changes were observed in FR variability, MUP complexity or NMJ transmission instability but MU coherence increased in the delta and alpha frequency bands across the contraction, representing increases in common synaptic input. That MBV did not change during the isometric contraction indicates possible occlusion further contributing to fatigue. Additionally, observed changes in MU properties and MBV showed no correlation in this fatiguing task indicating distinct mechanisms in the contribution to muscle fatigue.

MVC declined following the 3-min submaximal fatiguing contraction as expected, with this strength decrease confirming that fatigue was induced and the development of fatigue during the contraction was evident in the declining control and ability to maintain the 30% target force which has also been observed previously (Martinez-Valdes *et al.*, 2020). The task became perceivably more difficult due to the increase in relative force needing to be produced to maintain the target nearer the end of the contraction as fatigue developed. This is further supported by an increase in overall muscle activity in the later stage as represented by the increase in EMG RMS.

During the contraction, FR initially decreased between the start and the middle 30 s before increasing again but to a lower rate than at the start. This supports previous findings (Adam & De Luca, 2005; Mettler & Griffin, 2016) including from the VL by Martinez-Valdes *et al.,* who concluded there was a breakpoint where FR and CoV force began to increase and the maximum number of MUs were recruited approximately 80 s from the start of contraction which could be used to predict the time to task failure (Martinez-Valdes *et al.*, 2020). These FR responses suggest the strategy of increasing recruitment of different MUs is responsible for maintaining force output at the beginning of the contraction but then later in the contraction increasing FR becomes the dominant method of maintaining the required force level. This is partly supported by initial changes in the peripheral MU properties of MUP thickness recorded by iEMG, which increased in size between the first and middle 30 s as larger MUs were recruited. Although not significant ($p=0.06$) there was weak negative correlation of the change in FR with loss of

strength indicating that individuals with a greater FR at the end of the contraction than the start exhibited greater levels of fatigue. Therefore, the extent of FR increase during a sustained contraction could be an indicator of muscle fatigue, and future studies should aim to explore this hypothesis.

We found no difference in FR variability during the contraction which has previously been shown to increase as FS declined with muscle fatigue (Enoka *et al.*, 2003; Moritz *et al.*, 2005). This could be because the contraction was limited to 3 minutes and not until task failure and therefore levels of fatigue were variable between individuals. Previous work only found changes in CoV ISI at >80% of the full contraction time (Martinez-Valdes *et al.*, 2020). This could also explain why no changes were observed in NMJ transmission instability and MUP complexity, but they are more likely to be affected following training protocols, muscle damage or in chronic conditions where reinnervation could occur rather than over this acute time period.

The CST is the sum of all active MU spike trains and represents the net neural drive to the MUs (Dideriksen *et al.*, 2012). Mean CST showed a decrease between the start and middle of the contraction before increasing although to a lower level than the start. The variability in the CST is associated with fluctuations in force (Dideriksen *et al.*, 2012) and this decreased between the start and middle of the contraction however there was no change between the middle and end of the contraction observed despite an increase in force variability. This could be because the force deviations were not great enough to have a significant impact on an already variable measure and is also reflective of the constant FR variability of the contributing units to the CST over this time period.

Coherence represents the correlation between two signals at given frequencies (Maillet *et al.*, 2022) allowing investigation of common drive to MUs and further analysis of the different frequency bands enables the study of different aspects of neural drive (Dideriksen *et al.*, 2018; Alix-Fages *et al.*, 2023). Increases in common drive were observed represented by the delta increase across the contraction. This is consistent with previous findings that during a fatiguing task reduced common synaptic input represented by reduced coherence between muscles is associated with more changes in neural drive and flexible coordination strategies which results in better force control (Rossato *et al.*, 2022). This explains the decline in FS observed in the later part of the contraction as delta coherence continued to increase and as such common drive. Increases observed in the alpha band of MU coherence only occurred between the first and end 30s and middle

and end 30s of the contraction demonstrating increased common synaptic input and descending cortical drive needed to increase force generation as fatigue develops but no changes occurred in the beta band during the contraction. Previous research has also found increases in the delta, alpha and beta bands following a submaximal isometric fatiguing contractions of other limb muscles (Semmler *et al.*, 2013; Castronovo *et al.*, 2015; McManus *et al.*, 2016). MU coherence in the piper band did not change during the contraction. As this represents the cortical drive influencing force generation in stronger contractions such as MVCs, this is unlikely to have changed during a 30% contraction despite the increased effort needed to maintain this force level. However, in the beta and piper bands a greater number of MUs are needed to accurately estimate coherence due to the non-linear relationship between the synaptic input and output signal at higher frequencies (Farina *et al.*, 2016). Additionally, the increases in common drive occurred in conjunction with decreases in force control with fatigue which suggests greater MU synchronisation or common noise influencing force variability with these oscillations in common synaptic input previously suggested as a reason for impaired force steadiness (Yao *et al.*, 2000; Farina & Negro, 2015; Pereira *et al.*, 2019).

Using CEUS to directly assess tissue perfusion, no change in MBV was observed during the contraction which could indicate occlusion caused by the isometric contraction. The lack of increased blood flow commonly observed during exercise can result in reduced oxygen delivery for aerobic respiration further contributing to the build-up of metabolites within the muscle. This could lead to increases in afferent feedback which would contribute to the increases observed in alpha band MU coherence reflective of common synaptic input associated with afferent feedback in the later part of the contraction (Kent-Braun, 1999; Alix-Fages *et al.*, 2023). This may also affect contractile properties by limiting calcium ion release and sensitivity required for cross-bridge cycling and thereby contributing to fatigue (Allen *et al.*, 2008). CEUS has been shown to be a repeatable and reliable measure for recording MBV when recorded across sessions 2-weeks apart both at rest, during and after contractile activity (Jones *et al.*, 2023) and has previously been used to investigate skeletal muscle microvascular function in response to exercise (Krix *et al.*, 2010; Hildebrandt *et al.*, 2017) and disease (Amarteifio *et al.*, 2011). However, this technique has not yet been used during fatiguing contractions generally because it relies on minimal probe movement to maintain the ROI so other methods such as near infra-red spectroscopy are favoured (Jones & Phillips, 2023).

Despite both MU properties and MBV being factors and possible mechanisms responsible for fatigue, no correlation was found with functional performance outcomes and there was no correlation between MBV and FR. This supports previous findings in older healthy males where no correlation was observed (Murphy *et al.*, 2019). However, in stroke patients the change in blood flow was found to be directly proportional to the change in MU FR indicating possible common neural pathways involved in both FR modulation and blood flow control damaged by stroke lesions (Murphy *et al.*, 2019). Therefore, it is important to advance understanding of the causes of fatigue as a component of disease which may enable future identification of targets to improve performance and recovery.

3.4.1 Limitations

Although using a combination of EMG methods enabled us to measure both central and peripheral MU properties as in Chapter 2, the nature of this contraction where relative force production increased towards the end resulted in poor EMG quality due to superimposition of MUP templates complicating decomposition. A lower yield of MUs particularly in female participants meant lower numbers of participants included and the inability to conduct valid analysis of any sex differences in fatiguability previously observed (Ansdell *et al.*, 2017). Inclusion of both sexes in the study is important however, this may have affected findings as different strategies in both neuromuscular and microvascular control may play a role in making females more fatigue resistant so this should be investigated in further work.

Additionally, the changes reported in this study may be an underestimation of the response to fatigue because the length of the submaximal contraction was limited by constraints of using CEUS and therefore not performed to task failure. Additionally, the post contraction MVC was measured after 90s rest to enable the measurement of MBF in the recovery phase therefore there is the possibility of a greater strength decrease being observed if performed immediately following the contraction.

3.4.2 Conclusions

During a submaximal isometric contraction initially the neuromuscular strategy to maintain force is MU recruitment then an increase in FR. The increases in common drive required to oppose fatigue lead to the declines observed in force control which were further amplified by increases in afferent feedback due to occlusion preventing increases

in MBF. Understanding the neuromuscular mechanisms involved in fatigue enables identification of possible targets to reduce fatigue for both improving athletic performance and in recovery and rehabilitation for conditions with muscle fatigue as a component.

Chapter 4: Exploring the impact of 6-weeks resistance exercise training on force control in older males.

The participants and data included in Chapter 4 and Chapter 5 have been collected as part of a larger study which is still ongoing. This study is investigating the effects of 16-week mTOR inhibition on aged human muscle including muscle protein metabolism, structure and function including proteostasis measures using stable isotope tracer techniques from collected biological samples, cell signalling and MRI imaging alongside measures reported in the following chapters. As a result, the sample sizes reported are smaller than power calculations performed for the study (based on muscle protein synthesis - 8 per group) and not all measured outcomes are reported due to ongoing data collection and analysis. Some of this future analysis is further discussed in section 6.3.

4.1 Introduction

Sarcopenia has detrimental impact on the functional independence of older individuals and their ability to carry out activities of daily living (Landi *et al.*, 2013). Exercise is a key intervention for increasing muscle mass and strength, particularly resistance exercise training (RET). As mentioned in Chapter 1, a wide body of research has demonstrated increases in muscle strength with RET, with initial changes observed attributed to neural mechanisms followed by structural and size adaptations (Degens *et al.*, 2009). However, with age, although still present, the hypertrophic responses to RET are blunted, postulated to be a result of a combination of factors including altered protein turnover (Brook *et al.*, 2016; Quinlan *et al.*, 2021).

Following RET, even with blunted hypertrophic adaptation, multiple functional improvements have been reported making it a valuable intervention for rehabilitation and improving strength in older adults (Peterson *et al.*, 2010). These improvements include balance, gait speed, timed-up-and-go (TUG) and grip strength, all of which are frequently used for clinical assessment of physical function such as fitness for surgery and hospital discharge (Kwak *et al.*, 2016). However, most of these improvements are based on strength and/or power, yet in older adults, reductions in force control are also strongly associated with declines in daily functional tasks, specifically those associated with balance and dexterity (Keogh *et al.*, 2019). In addition, reduced ability to control force production is also a debilitating feature of multiple clinical conditions such as stroke and

Parkinson's disease (Lodha *et al.*, 2010; Rose *et al.*, 2013), many of which are associated with advancing age.

Motor unit (MU) function underlies these changes by modulating muscle force production through alterations in MU firing rate (FR) and recruitment (Enoka & Duchateau, 2017). There has been much investigation of the effects of RET and ageing on muscle strength and function, including some on the positive effects of RET on force control (Carroll *et al.*, 2001). Previous work has found improvements in force steadiness (FS) following different training methods in young cohorts (Kobayashi *et al.*, 2014; Ely *et al.*, 2022). Despite this, the effects on MU properties are unclear despite neural changes preceding muscle size increases and playing a role in force control (Enoka & Farina, 2021).

As reviewed in Chapter 1, exercise in older age stimulates MU remodelling through the release of neurotrophins, enabling expansion of MU size through reinnervation of denervated muscle fibres (Jones *et al.*, 2022). Although this remodelling process prevents the loss of muscle fibres which would usually contribute to strength declines, the resultant increase in MU size and possible fibre type grouping reduces fine motor control and increase the shape variability and complexity of the MU potentials (MUPs) (Piasecki *et al.*, 2016*c*; Kelly *et al.*, 2018). MU FR variability has previously been identified as a key determinant of muscle force control, with greater FR variability associated with poorer FS, particularly in older age (Tracy *et al.*, 2005), although it has the potential to be improved with training (Ely *et al.*, 2022). The recent advancement of high-density surface electromyography (HD-sEMG) enables further understanding of the neural mechanisms underlying these changes through simultaneous sampling of multiple MUs, and analysis of the cumulative spike trains (CST) and intramuscular coherence within specific frequency bands – a measure representing different aspects of common synaptic input (Alix-Fages *et al.*, 2023).

There is currently a lack of longitudinal research studying MU properties in humans and consequently little is known about the functional outcomes of MU remodelling, time course of adaptation, or the underpinning mechanisms, but previous evidence suggests structural and functional alterations are physiologically possible. Therefore, the aims of this study were to determine the effects of RET on force control and the underlying neuromuscular properties following a 6-week training period.

4.2 Methods

4.2.1 Participants

6 healthy older male volunteers (66±6 yrs) gave written informed consent to participate in the clinical trial (NCT05414292) approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (FMHS 90-0820). This subset of participants formed the control group in the study detailed in Chapter 5 therefore screening information gathered is based on these requirements. Before enrolling into the study, a screening visit was attended by all participants where a fasted blood sample (for standard clinical chemistry (U&E, LFT, TFT, coagulation, lipids, glucose and insulin)), medical history, height and weight, electrocardiogram (ECG) and blood pressure were collected/recorded to confirm suitability for the study (more information in Appendix 8.3). Body mass and height were measured using calibrated scales and stadiometry, respectively, and body mass index (BMI) was calculated. Exclusion criteria included a body mass index (BMI) <18 or >35kg/m^2, or a history of cardiovascular, respiratory, or neuromuscular disorders. Participants were instructed to maintain their usual diet and exercise throughout the study which was characterised using a 4-day diet diary and short-form international physical activity questionnaire (IPAQ), respectively for the purpose of the larger study and therefore outcomes are not reported here.

4.2.2 Experimental protocol

Participants attended for baseline testing of the vastus lateralis (VL) of both legs before commencing a 6-week fully-supervised unilateral RET (leg extension) protocol (Figure 4.1). A period of 2-weeks followed baseline testing where the participant maintained their usual exercise and dietary habits at home before beginning the 6-week RET protocol. Unilateral resistance training was performed 3 times per week for 6 weeks, fully supervised by a member of the research team. Participants performed 6 sets of 8 repetitions of leg extensions at 75% 1-repetition maximum (1-RM) on a free-standing machine (Leisure Lines, UK). 5 participants trained the right leg and 1 trained the left leg due to pre-existing knee problems.

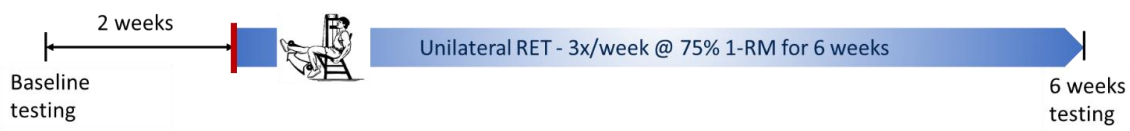


***Figure 4.1**: Schematic diagram summarising the experimental protocol timeline.*

4.2.3 Assessments

4.2.3.1 Functional assessment

At the baseline visit the Short Performance Physical Battery (SPPB) was performed which consisted of a side-by-side stand, semi-tandem stand, and tandem stand (each for 10 s), 4m gait speed assessment and timed 5 chair stands. Points were assigned based on the performance of each task to assess functional independence as previously described with a maximum possible score of 12 (Guralnik *et al.*, 1994). Unilateral balance was assessed on the trained leg only using a pressure plate (Footscan, RSscan, UK). Displacement of the centre of pressure (COP) representing stability was measured using the distance travelled during a 30s balance on one leg with the opposite leg lifted at least 15 cm above the ground. All functional assessments were repeated following 6-weeks RET.

4.2.3.2 Ultrasound scans

Individual muscle cross sectional area (CSA) was recorded using ultrasound (MyLab, Esaote, Italy) from the mid-belly of VL of both legs. As described in the previous chapters, the mid-belly of VL was determined as the mid-point between the greater trochanter and the patella. Medial and proximal borders of the VL were identified from the points the aponeuroses intersected with vastus intermedius muscle. Three axial plane images were collected and subsequently analysed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA) as previously reported (Inns *et al.*, 2022). Ultrasound scans were made at baseline and repeated following 6-weeks RET.

4.2.3.3 1-repetition maximum (1-RM) strength assessment

Strength was assessed by calculating the 1-RM for a single leg extension using a free-standing machine (Leisure Lines). After a warm-up and familiarisation, weight was added to the machine and the participant attempted 1-RM until maximal exertion was reached. The weight was adjusted for a maximum of 5 attempts. 75% of the recorded 1-RM was used as the training load during the 6-week RET and was assessed at baseline, 3-weeks, and 6-weeks to ensure the training load was maintained.

4.2.3.4 Isometric strength assessment

Bilateral isometric knee extensor strength was assessed by participants sitting with their hips and knees flexed at 90° and the leg (to be tested) securely fastened to a force

transducer just above the ankle. To familiarize participants with the equipment and to allow a "warm-up", participants performed a series of submaximal isometric contractions prior to the strength assessment. Participants were then instructed to perform a maximal isometric contraction, accompanied by verbal encouragement and visual feedback of force on a computer screen. This was repeated three times, with 60 s rest intervals and best effort taken as maximum voluntary isometric contraction force (MVC).

4.2.3.5 Force tracking assessment

Following determination of the isometric MVC, participants completed a series of steady and complex isometric contractions where they followed a target line visually displayed on a computer screen (Figure 4.2). For the steady contractions, participants performed isometric voluntary contractions lasting 12–15 s, aiming to hold a target line set at 10%, 25% or 40% MVC. Following a familiarisation at each contraction level, a minimum of 4 contractions at 10% and 25% and 2 at 40% were collected alternating between force levels (Figure 4.2). The complex contractions consisted of a 10 s ascending phase to 25% MVC, a 30 s sine wave containing 8 oscillations at an amplitude of ±4% (i.e. oscillating between 21% and 29% MVC), and a 10 s descending phase to relaxation (Figure 4.2). Participants were instructed to trace the displayed line by isometrically contracting and relaxing to control the force output and had one practice familiarisation attempt before completing the task.

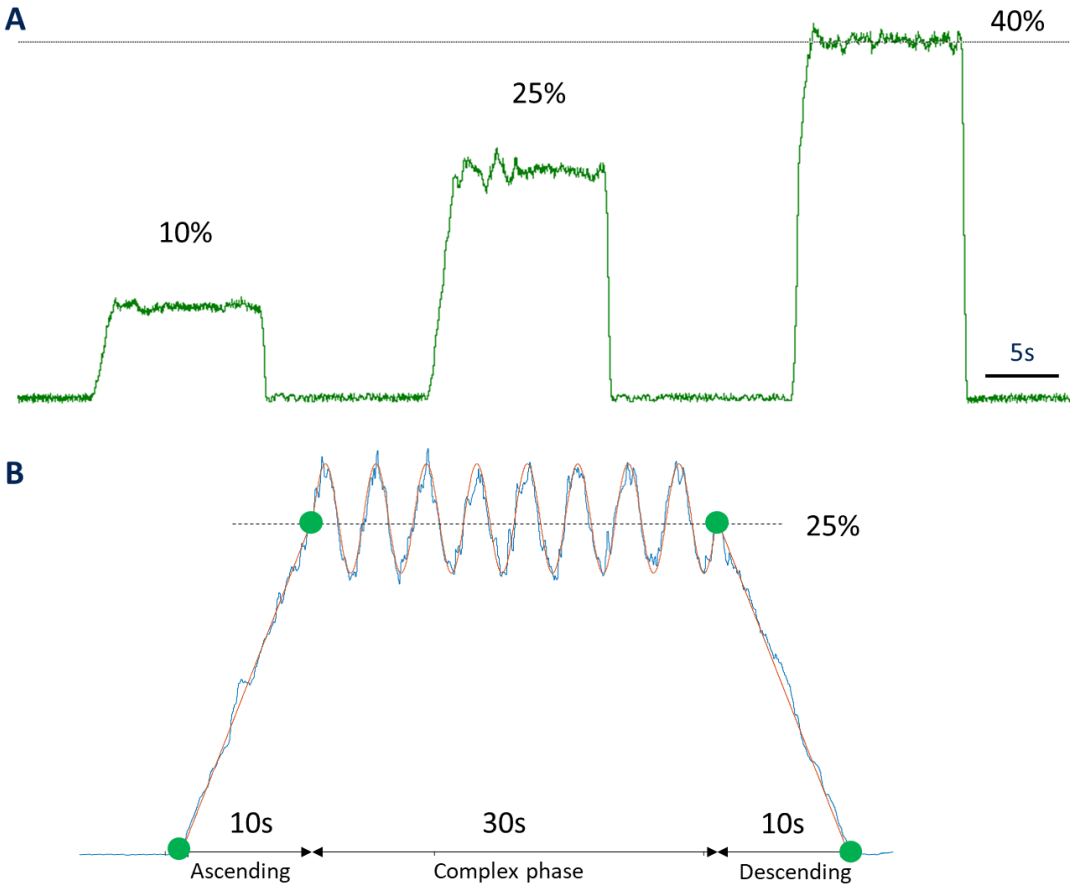


***Figure 4.2**: Force tracking tasks.*

Simple force tracking tasks of 12s isometric contractions at 10%, 25% and 40% of MVC (A) and complex force tracking tasks at 25% MVC (B) with the start and end of each phase marked by the green dots.

4.2.3.6 HD-sEMG

During the force tracking contractions HD-sEMG was recorded simultaneously. As described in Chapter 2 and 3, a semi-disposable adhesive monopolar HD-sEMG matrix (GR08MM1305, OT Bioelettronica, Torino, Italy) consisting of 64 x 8 mm spaced electrodes was positioned in the presumed direction of the fascicles on the VL and secured to the skin (Figure 4.3A). A reference cable (CPAT1, OT Bioelettronica) was attached around the ankle of the recording limb. The signal was amplified, sampled at 2000 Hz, band-pass filtered (10-500 Hz), and converted to digital data (16-bit analogue-digital converter, 3 dB bandwidth) using a Sessantaquattro (OT Bioelettronica) multi-channel amplifier. Data was visualised in real-time in OTBioLab+ (v1.3.2, OT Bioelettronica) and stored for offline analysis (Figure 4.3B).

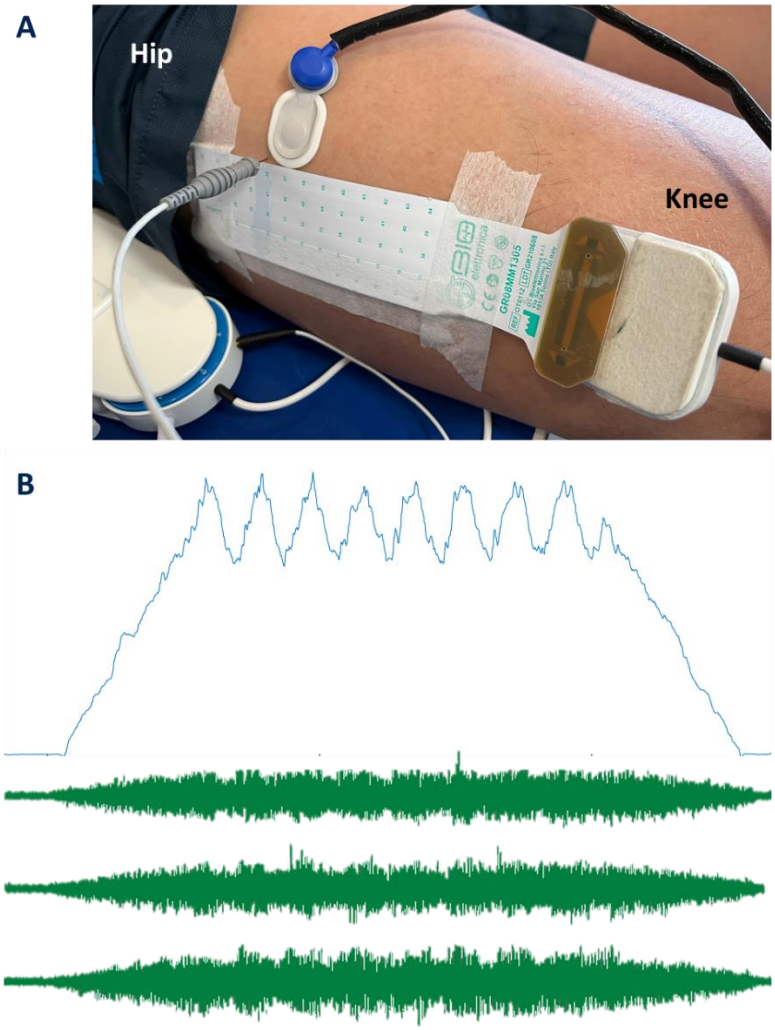


***Figure 4.3**: Electrode placement and representative raw data traces.*

HD-sEMG 64-channel electrode placement on the vastus lateralis (A) and representative force trace during the complex tracking task with a sample of corresponding raw EMG signals (B).

4.2.4 Data Analysis

4.2.4.1 Force steadiness

FS of steady contractions was calculated from the coefficient of variation of the steady section of the force line (CoV force) recorded in Spike2 (v9.06, Cambridge Electronic Design, UK). For complex contractions, the area under the curve (AUC) was calculated for the ascending, complex and descending phases separately in MATLAB (v2019a, IBM). Following data exportation from OT BioLab+ and selection of the data of interest, the acquired trace was subtracted from the requested path and rectified then the AUC was calculated.

4.2.4.2 HD-sEMG analysis

HD-sEMG data was analysed using the DEMUSE tool in MATLAB to decompose and identify MUs using an extensively validated method (Holobar *et al.*, 2014; Francic & Holobar, 2021; Hug *et al.*, 2021*a*) and previously described in section 3.2.3.1. Briefly, the data was decomposed from each complete contraction using a blind source separation method using Convolution Kernel Compensation algorithm to identify MUs through estimating MU filters to generate a spike train (Holobar & Zazula, 2007). Firings for each MUP were manually inspected and edited through optimising and reapplying the MU filter and including appropriate firings and removing unreliable units with a pulse-to-noise ratio below 30dB (Holobar *et al.*, 2014) (Figure 4.4). Once cleaned, MU properties for each MU were extracted in MATLAB and the coefficient of variation for the inter-spike interval (CoV ISI) representing FR variability was taken.

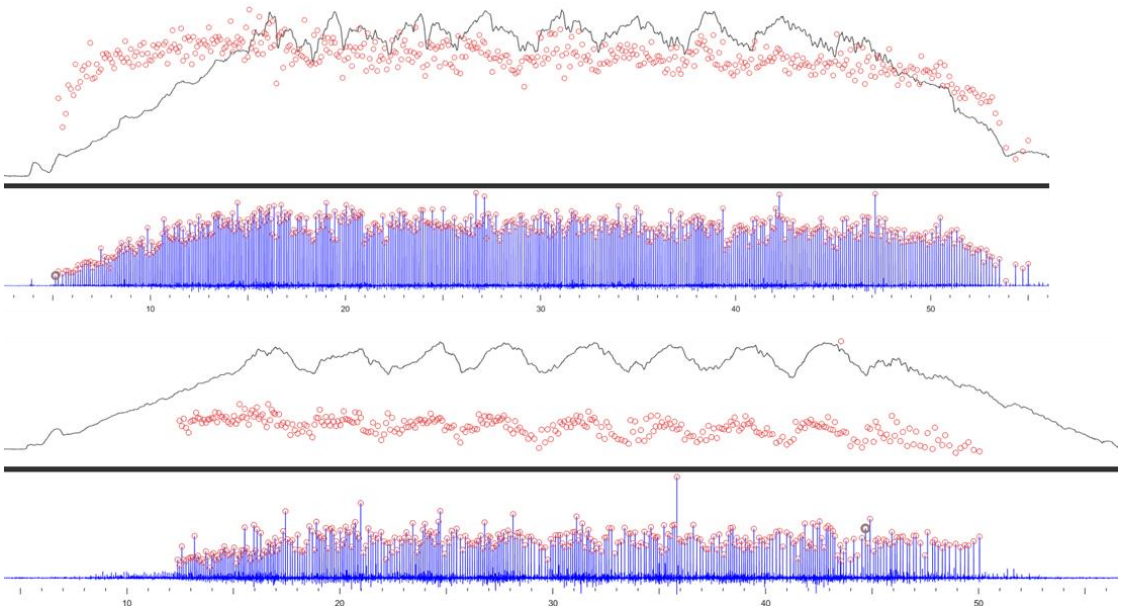


Figure 4.4*: Representative force traces of complex contractions showing sample firings of two different identified MUs after decomposition.*

To assess common drive to the MUs, the magnitude-squared coherence was calculated using the Welch's averaged periodogram with non-overlapping windows of 1 s (Alix-Fages *et al.*, 2023) as similarly described in section 3.2.3.1. Cumulative spike trains (CST) were calculated as the sum of discharge times from the identified MUs. Two equally sized CSTs calculated from three MUs randomly selected from the identified MUs were used as the level of coherence depends on the number of MUs considered in the analysis (Farina &

Negro, 2015). All unique combinations of three MUs were tested up to a maximum of 60 random permutations and the pooled coherence of these permutations was used for further analysis. The mean coherence within the Delta bandwidth of 0–5 Hz was assessed to investigate the features of neural input associated with force control. Coherence values were transformed to a standard z-score using the equation:

$$Z\ score = \sqrt{2L} \times \text{atanh}\sqrt{C}$$

where C is coherence and L is the number of time segments used for the coherence analysis as reported in Chapter 3 (Del Vecchio *et al.*, 2019*c*; Avrillon *et al.*, 2021).

To assess similarities in CST and force signal fluctuation, signals were first filtered using a low-pass 4th order zero-phase Butterworth, 2 Hz and then high-pass filter 4th order zero-phase Butterworth, 0.75 Hz. Then the cross correlation between the CST and force traces for each participant over the length of the complex contraction was calculated to obtain a cross-correlation coefficient assessing the similarities in any fluctuations (Martinez-Valdes *et al.*, 2022).

4.2.4.3 Statistical analysis

All data presented are for the trained leg only. The Shapiro-Wilk test was used to assess normality as this is better suited for smaller sample sizes, with data being normally distributed. Students paired t-tests were performed in GraphPad Prism (v9.2, USA) to test the effect of RET on strength, FS, MU coherence and CST-Force cross correlation. As multiple MUPs were recorded from each participant, multi-level mixed effects linear regression models were performed with time as a factor for CoV ISI in RStudio (v 2022.07.2 Build 576, PBC, USA) using the lme4 package (v 1.1-23). In each model the first level (individual MUs) were clustered according to each participant to form the second level. Significance was assumed if $p<0.05$.

4.3 Results

4.3.1 Participant characteristics

Participant characteristics are presented in Table 4.1. All participants were male and confirmed to be functionally independent with SPPB scores of 11±1 at the start of the study out of a maximum of 12.

Table 4-1: Participant characteristics (n=6).

		Mean (SD)
Age (years)		65.8 (6.2)
Height (m)		1.76 (0.07)
Weight (kg)		81.0 (12.6)
BMI (kg/m^2)		26.2 (3.2)
VL CSA (cm^2)	**R**	23.9 (5.8)
	L	22.4 (4.3)

4.3.2 Balance

There was no significant change in the displacement of COP following RET (1116±691mm vs. 1237±1207mm, p=0.68; Figure 4.5A).

4.3.3 1-Repetition maximum

There was a significant increase of 25.4±9.8% in 1-RM following 6-weeks RET (43.8±8.2kg vs. 54.9±8.8kg, p=0.001; Figure 4.5B).

4.3.4 Maximum voluntary contraction

There was no significant change in MVC after 6-weeks RET (393±110N vs. 437±139N, p=0.182; Figure 4.5C).

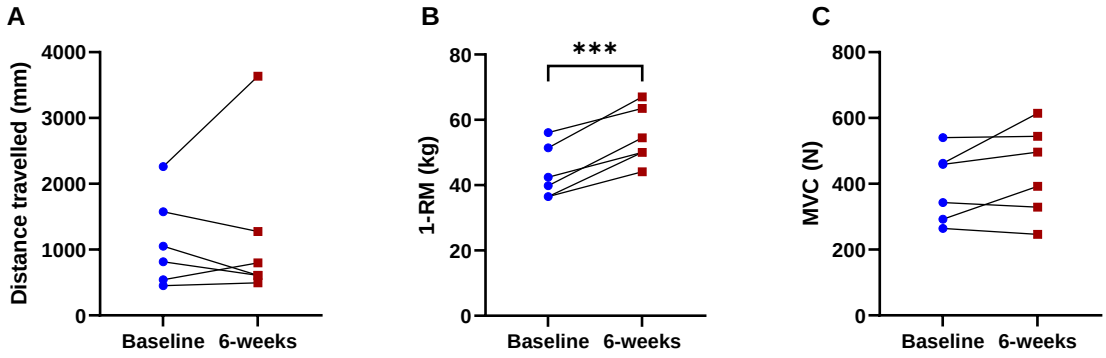


***Figure 4.5**: Balance and muscle strength results.*

*Individual data for distance travelled (A), one-repetition maximum (1-RM) (B) and maximal voluntary contraction (MVC) (C) at baseline and following 6-weeks resistance exercise training (RET). ***p=0.001 vs. baseline.*

4.3.5 Force steadiness

There was no significant difference in CoV force during the steady contractions following 6-weeks RET at any contraction level (Table 4.2 and Figure 4.6).

Table 4-2: Coefficient of variation (CoV) of force (±SD) at different contraction intensities (n=6).

Intensity	Baseline	6-weeks	P-value
10%	5.77±0.96%	5.18±1.34%	p=0.20
25%	3.85±1.43%	3.11±0.72%	p=0.29
40%	3.11±0.84%	2.95±0.73%	p=0.36

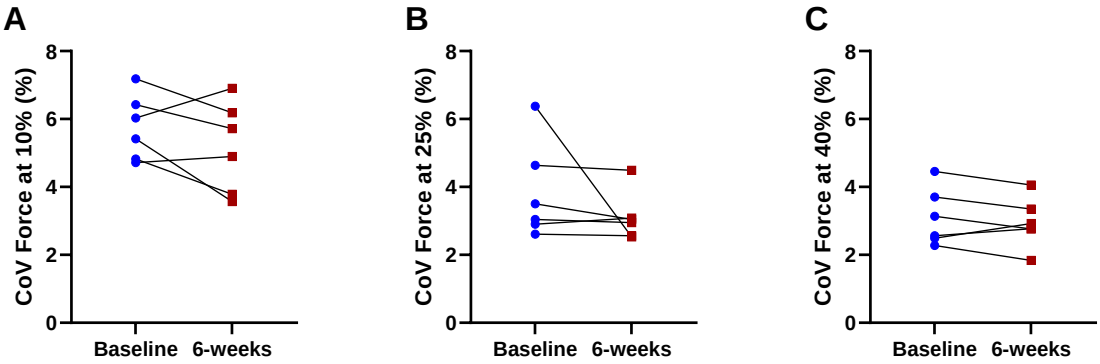


***Figure 4.6**: Simple force steadiness results at 10%, 25% and 40% maximum voluntary contraction.*

Individual values for force coefficient of variation (CoV) at 10% maximum voluntary contraction (MVC) (A), 25% MVC (B) and 40% MVC (C) at baseline and following 6-weeks resistance exercise training (RET).

4.3.6 Complex force steadiness

There was no significant change in force control during the ascending phase of the complex tracking task after RET (AUC: 7.67±1.85Ns vs. 6.17±2.70Ns, p=0.19; Figure 4.7A). There was however a significant decrease in force variability during the complex phase following RET (32.03±7.49Ns vs. 23.94±7.58Ns, p=0.0372; Figure 4.7B). Force variability also significantly decreased during the descending phase of the complex tracking task after RET (13.41±5.10Ns vs. 7.32±2.14Ns, p=0.0181; Figure 4.7C).

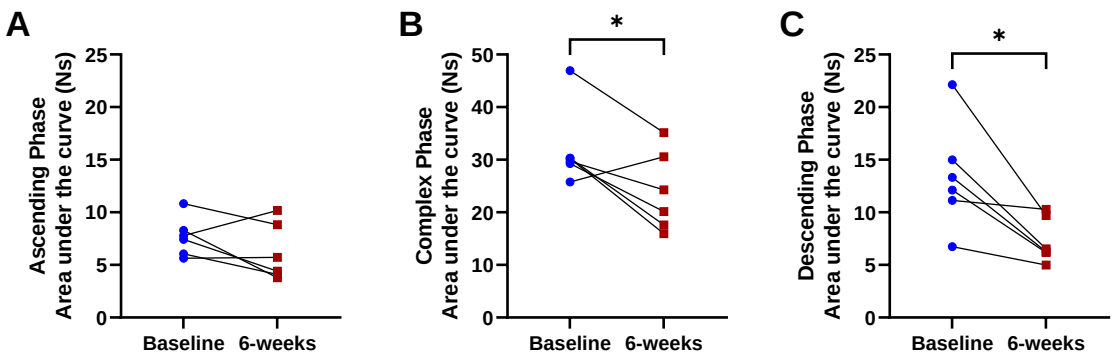


***Figure 4.7**: Complex force steadiness results in each phase.*

*Individual area under the curve for the ascending (A), complex (B) and descending (C) phases of a complex force tracking task at baseline and following 6-weeks RET. *p<0.05 vs. baseline.*

4.3.7 Motor unit properties

Despite changes in FS in the complex and descending parts of the tracking task, no differences were observed in CoV ISI between baseline and after 6-weeks RET in either the complex phase (0.22±0.04% vs 0.23±0.05%, p=0.15; Figure 4.8A) or the descending phase (0.27±0.03% vs 0.29±0.04%, p=0.41; Figure 4.8B). There were also no differences observed in MU coherence at 0-5Hz in either the complex (4.06±0.46 vs. 3.54±0.72, p=0.16; Figure 4.9A) or descending phases (4.65±0.72 vs. 4.14±0.89, p=0.40; Figure 4.9B). Cross-correlation coefficients between CST and force showed no difference between baseline and 6-weeks RET (0.63±0.11 vs 0.60±0.08; Figure 4.10).

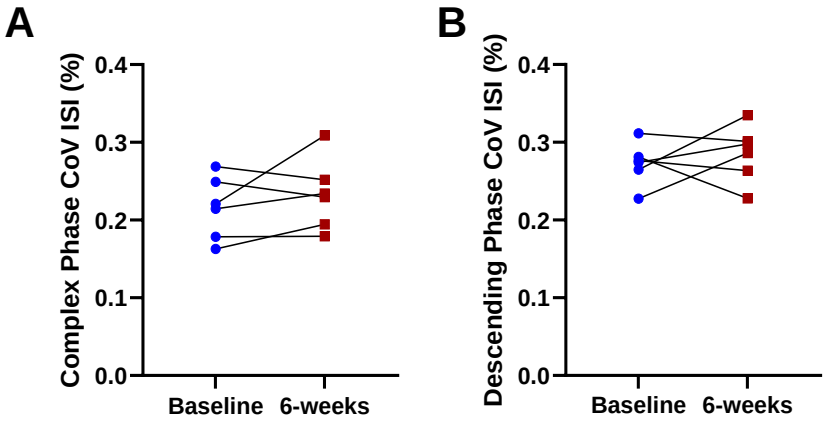


***Figure 4.8**: Participant means of coefficient of variation (CoV) ISI during the complex (A) and descending (B) phases of a complex force tracking task at baseline and following 6-weeks RET.*

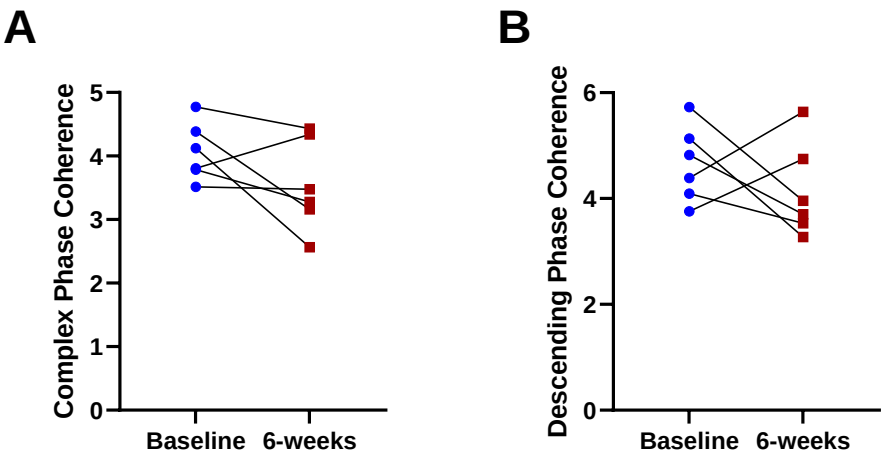


***Figure 4.9**: Participant means of delta band coherence (0-5 Hz) during the complex (A) and descending (B) phases of a complex force tracking task at baseline and following 6-weeks RET.*

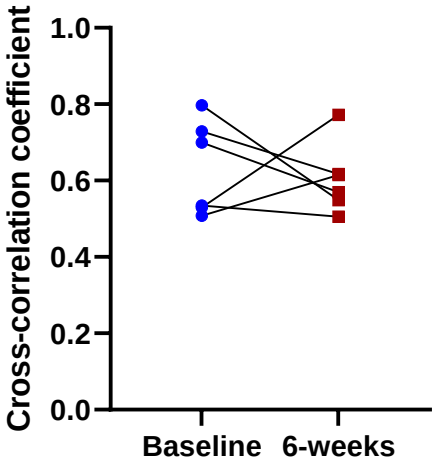


***Figure 4.10**: Individual cross-correlation coefficients between CST and force at baseline and after 6-weeks RET.*

4.4 Discussion

Here we demonstrate that 6-weeks of RET leads to improvements in complex force tracking without changing MU FR variability or common synaptic input. Although no changes were observed in balance or isometric MVC, there was an increase in strength represented by 1-RM. RET did not lead to improvements in force control during steady contractions at any contraction intensity. During the complex task there were significant improvements in both the complex and descending phases however, alterations in MU FR variability and low frequency components of neural drive were not responsible for this.

RET is a recommended form of exercise for increasing muscle strength particularly in older people (Mcleod *et al.*, 2019). Muscle strength measured by 1-RM increased following our RET intervention which was expected and has frequently been observed (Peterson *et al.*, 2010) as a result of neural adaptations and muscle hypertrophy (Schoenfeld, 2010; Škarabot *et al.*, 2021). However, when assessed by isometric MVC this did not change. This is most likely because the 1-RM assessment was similar to the leg extension RET modality and therefore also likely represents some improvement due to movement familiarity or learning effect. Additionally, no significant changes in single leg balance were observed, this is possibly because the task was limited to 30 s and therefore too short to find an improvement in performance in this healthy cohort who did not present with impaired balance prior to the intervention period. Mixed findings have been reported in previous research as to the effects of RET on balance, with both improvements (Gonzalez *et al.*, 2014) and no effects observed (Orr *et al.*, 2008); with this variation attributed to the different methods of assessing balance and with a likely ceiling effect in healthy older individuals that has previously been reported (Freund *et al.*, 2019).

The ability to maintain a constant force in a stable manner has been associated with motor function (Seynnes *et al.*, 2005; Davis *et al.*, 2020). It is usual for fluctuations in force output to occur around an average value, determined by MU properties of the recruited population and oscillations in neural drive (Enoka & Farina, 2021). However, large deviations in force indicative of instability are frequently observed in those with a greater likelihood of falls and pathological states (Carville *et al.*, 2007; Hyngstrom *et al.*, 2014; Davis *et al.*, 2020). In this study, no changes in force steadiness in the simple tracking task were observed at any contraction intensity. As all the participants in this study were healthy and recreationally active, any improvement following RET was likely to be minimal during non-fatiguing contractions at submaximal forces as has been noted previously, specifically with isometric contractions (Tracy *et al.*, 2004; Beck *et al.*, 2011). A constant force production is maintained through changing MU FR via altering neural drive and MU recruitment and with RET influencing these properties and MU remodelling (Del Vecchio *et al.*, 2019*a*; Jones *et al.*, 2022), it is feasible that RET could improve FS.

Complex force tracking requires a greater level of force control compared to steady contractions, requiring specific afferent feedback to vary neural input which is modulated by common synaptic input to the MU pool (Laine *et al.*, 2014). Here we show an improvement in complex force control following RET specifically during the complex and descending phases of the contraction. By separating the complex contraction into

different phases this enabled us to investigate the effects of RET on MU control during both recruitment and derecruitment phases. Following RET there was no change in control during the ascending phase of the contraction but a significant improvement in the descending phase which indicates possible different effects of RET on recruitment and derecruitment strategies of MUs, where it is more challenging to maintain force control in descending phases (Guo *et al.*, 2023). A possible mechanism for the improvements in the descending phase could be due to increases in persistent inward current (PIC) amplitude and modulation which have previously been observed following RET (Orssatto *et al.*, 2023) allowing greater responsiveness to motor neuron ionotropic inputs enabling better control of derecruitment (Heckman *et al.*, 2005).

There was also an improvement in force steadiness during the complex phase following 6-weeks RET supporting earlier findings following low intensity RET (Kobayashi *et al.*, 2014) and specific force control training (Ely *et al.*, 2022). These improvements could be as a result of a learning effect although an attempt to mitigate this was employed by providing an identical practice contraction immediately before the analysed contraction at both visits. A voluntary contraction involving variable force production has greater translation to daily tasks requiring motor coordination and accurate force control during a dynamic movement (Ely *et al.*, 2022), therefore is a useful assessment of muscle function particularly in older people.

Reductions in FR variability have previously been associated with improvements in force control (Moritz *et al.*, 2005; Enoka & Farina, 2021; Ely *et al.*, 2022) however, despite improvements in complex force control no changes were observed in FR variability in this study. This could be because of identification of different MU populations at the two timepoints due to changes in recruitment, but also agrees with previous findings of no change (Beck *et al.*, 2011) and poor correlation of FR variability with force variability (Negro *et al.*, 2009). Previous work has demonstrated that specific low intensity training of complex tracking tasks in young people improves force control as a result of reductions in FR variability during the complex phase (Ely *et al.*, 2022). The more specific nature of this training compared to RET which is aimed at increasing muscle strength could be the reason for the varying neuromuscular effects observed and suggests differing strategies for improving FS.

Intramuscular coherence in the delta band (0-5Hz) represents the common drive related to the voluntary control of force and reflects force variability (Negro *et al.*, 2009; Farina

& Negro, 2015; Alix-Fages *et al.*, 2023). Despite improvements in force control in both the complex and descending phases there were no changes in coherence following RET indicating no net change in combined excitatory and inhibitory inputs to the motor neurons, therefore suggesting common drive was not modulating variations in force. This could be because the other muscles part of the quadriceps group were contributing so intermuscular coherence could be studied in further work to look at the coordination between these muscles. However, it has also been suggested that oscillations in neural drive below 0.5Hz most strongly contribute to force variability (Moon *et al.*, 2014) and therefore the wider frequency range of 0-5 Hz could account for no changes observed in this study. Increases in coherence and common drive have been associated with declines in force control (Contessa *et al.*, 2009; Castronovo *et al.*, 2015) and are present in pathological tremor (Gallego *et al.*, 2015). Previous research has also found significant associations between variability in common synaptic input and force variability (Negro *et al.*, 2009; Feeney *et al.*, 2018).

By using HD-sEMG during these complex contractions the calculation of the time lag between the MU recruitment obtained from the CST and force production known as neuromechanical delay (NMD) is also possible although not conducted in this thesis. Mean NMD values of 150-200 ms have previously been observed in the tibialis anterior muscle with neural drive preceding force production and cross-correlations between these measures have shown the CST could predict 62-88% of force changes (Del Vecchio *et al.*, 2018*b*; Martinez-Valdes *et al.*, 2022) but this has not yet been investigated following an intervention. Here, cross-correlation analysis was performed and showed that the neural drive could predict >60% of the force fluctuations and demonstrated no changes in this relationship following RET. However, due to the synergy of VL activity within the quadriceps muscle group to force production, the contribution of VL alone to overall force control is complex to determine. However, this provides further insight and understanding of central and peripheral MU properties involved in the control of force production (Begovic *et al.*, 2014; Del Vecchio *et al.*, 2018*b*). With FR variability and common synaptic input seemingly not responsible for improvements in force control this suggests other strategies could be involved including reduced coactivation of antagonists, altered MU recruitment and changes in NMD or peripheral adaptations of the muscle fibres, which were not assessed in this study.

4.4.1 Limitations

One of the main limitations of this study is the low number of participants therefore limiting the statistical power and validity of the findings and resulting in only males being included. As the participants in this study were all recreationally active it is possible that greater changes would be observed in a group not habitually active. With an average age of 66, the findings may not be the same in those older (i.e., 80+) where RET may have greater benefit. It is also possible that a greater period of RET such as 12-weeks or more would have more noticeable effects as is observed with muscle strength and size increases (Folland & Williams, 2007). Despite no changes in MU properties observed here following RET, as contractions were only up to a maximum of 40% MVC it is possible that RET may have a greater impact on higher threshold units and therefore may have been observed from contractions over 50% MVC.

4.4.2 Conclusions

6-weeks RET is an effective intervention for increasing muscle strength in older males and appears to have benefits for force control during complex tasks. However, these improvements are not explained by alterations in MU FR variability or common synaptic input and therefore the responsible mechanisms require further exploration. These findings support the effectiveness of RET as a useful intervention in older adults for increasing muscle strength and may also improve other aspects of function such as control which would be valuable for combatting sarcopenia and maintaining independence in daily life.

Chapter 5: Investigating the effects of an mTOR inhibitor on neuromuscular function in older males after 6-weeks resistance exercise training.

5.1 Introduction

Sarcopenia is defined as the age-related loss of muscle mass and function (Cruz-Jentoft *et al.*, 2019). Traumatic events such as fractures in older age resulting in immobilisation and/or bed rest can accelerate sarcopenia. This adds to age-related muscle catabolism presenting further functional limitations to exercise, which is commonly accepted as a protective intervention (English & Paddon-Jones, 2010). Therefore, alternative interventions in addition to exercise may be required to combat the muscle loss observed in ageing.

Contributing factors to sarcopenia include altered muscle protein turnover and denervation of muscle fibres, both regulated by the mechanistic target of rapamycin (mTOR) signalling pathway particularly mTOR complex 1 (mTORc1). mTOR is a serine/threonine kinase which is able to sense intracellular changes in amino acids and is responsive to mechanical stimuli (Laplante & Sabatini, 2012). The loss of mTOR signalling can result in muscle dystrophy, reduced lifespan and reduced metabolism (Zhang *et al.*, 2019). Paradoxically, the mTOR pathway becomes hyper-active in older age leading to impaired responsiveness to nutrition and exercise and dysregulated autophagy (Francaux *et al.*, 2016), in addition to preventing growth of new axonal branches needed for motor unit (MU) remodelling and deterioration of neuromuscular junction (NMJ) integrity (Castets *et al.*, 2020; Ham *et al.*, 2020). Therefore, maintaining the balance of this pathway provides a possible therapeutic target for drug interventions preventing muscle loss.

As reported in previous chapters, resistance exercise training (RET) is an effective intervention for improving muscle size and strength in both younger and older adults. RET has previously been shown to stimulate muscle hypertrophy (Moro *et al.*, 2020), increase muscle protein synthesis (MPS) rates (Kumar *et al.*, 2009) and alter MU properties including firing rates (FR) and recruitment thresholds (Del Vecchio *et al.*, 2019*a*). The increases in muscle strength have been demonstrated to precede increases in muscle size with this discordance attributed partly to neural factors (Degens *et al.*, 2009). RET along with protein intake have been demonstrated to stimulate mTOR signalling through protein phosphorylation resulting in increased protein synthesis contributing to muscle

hypertrophy (Dreyer *et al.*, 2010; Moore *et al.*, 2011; Brook *et al.*, 2015). However, inhibition of mTOR has been shown to block MPS increases usually observed directly following high intensity contractions (Drummond *et al.*, 2009) possibly preventing gains in muscle mass and function usually associated with RET.

In pre-clinical animal models, the use of pharmaceutical interventions including metformin, resveratrol and rapamycin have demonstrated positive effects on lifespan and attenuation of age-related healthspan declines (Valenzano *et al.*, 2006; Martin-Montalvo *et al.*, 2013; Bitto *et al.*, 2016). Rapamycin (sirolimus) is an allosteric mTOR inhibitor which binds directly to mTORc1. In mice, rapamycin or rapalog (rapamycin derivative) treatment has been shown to extend life span (Harrison *et al.*, 2009), counteract muscle loss (Joseph *et al.*, 2019) and maintain muscle function (Ham *et al.*, 2020). Rapamycin treatment has also demonstrated positive effects on the NMJ through increasing acetylcholine receptor (AChR) density and promoting axonal sprouting through gene upregulation (Baraldo *et al.*, 2020; Ham *et al.*, 2020). In humans, rapamycin is routinely used for its potent immunosuppressive properties which is achieved through binding to FKBP12 inhibiting mTOR and further development is underway for repurposing for use in clinical conditions and ageing (Yoo *et al.*, 2017). However, in humans the muscle biology consequences of rapamycin administration are unknown. Therefore, the aims of this study were to determine the effects of a low-dose mTOR inhibitor on muscle size, strength and neuromuscular function following 6-weeks of unilateral RET.

5.2 Methods

5.2.1 Participants

13 healthy older male volunteers (64±6 yrs) gave written informed consent to participate in the clinical trial (NCT05414292) approved by the University of Nottingham Medical School Research Ethics Committee (90-0820). Before enrolling, a screening visit was attended by all participants where a fasted blood sample, medical history, height and weight, electrocardiogram (ECG) and blood pressure were recorded to confirm suitability for the study as it involved exercise and drug administration (Appendix 8.3). Body mass and height were measured using calibrated scales and stadiometry, respectively, and body mass index (BMI) was calculated. Exclusion criteria included a body mass index (BMI) <18 or >35kg/m^2, or a history of cardiovascular, respiratory, or neuromuscular disorders (Appendix 8.4). Participants were instructed to maintain their usual diet and exercise

throughout the study which was characterised with a 4-day diet diary and international physical activity questionnaire (IPAQ) to account for any influence on muscle strength and metabolism parameters although outcomes are not reported in this thesis.

5.2.2 Experimental Protocol

Participants attended a baseline testing visit involving multiple tests of muscle mass, strength and function (Figure 5.1). This included ultrasound of the vastus lateralis (VL) of both legs, strength measured by 1 repetition maximum (1-RM) and maximum voluntary contraction (MVC) and intramuscular electromyography (iEMG) of both legs. These measures are further explained below. Participants were then randomly allocated (sealedenvelope.com) to take 1 tablet daily of either an mTOR inhibitor – Rapamune (1mg/day, Pfizer, Belgium) or a placebo tablet. A period of 2 weeks where the participant only took the given tablets to check for tolerability preceded a 6-week RET protocol. Participants were monitored for immunosuppression and general health throughout as outlined in Appendix 4. At the mid-point in the training period (3-weeks) and on completion (6-weeks), all baseline tests were repeated (Figure 5.1).

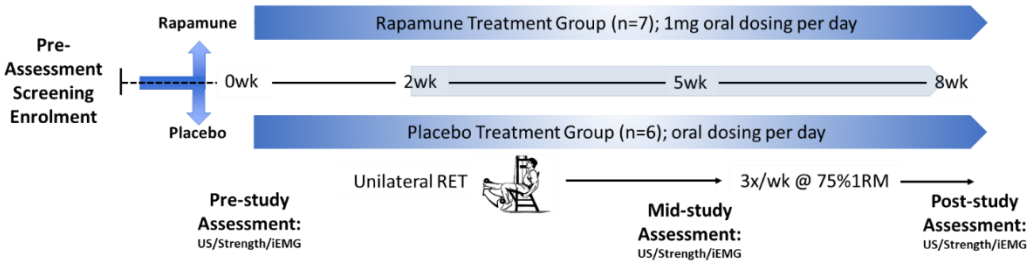


***Figure 5.1**: Schematic diagram summarising the experimental protocol timeline.*

5.2.2.1 Ultrasound Scans

Using the same method as reported in the previous chapters, individual muscle cross sectional area (CSA) was recorded using ultrasound (MyLab, Esaote, Italy) from the mid-belly of VL of both legs at baseline and following 3 and 6-weeks of RET. The mid-belly of VL was determined as the mid-point between the greater trochanter and the patella. Medial and proximal borders of the VL were identified from the points the aponeuroses intersected with vastus intermedius muscle. Three axial plane images were collected and

subsequently analysed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA) as previously reported (Inns *et al.*, 2022).

5.2.2.2 Strength Assessment

Knee extensor strength of both legs was assessed with participants sitting with hips and knees flexed at 90° and the leg securely fastened to a force transducer ~39 cm below the centre of the knee joint. To familiarize with the equipment and to "warm-up" the muscle, participants performed a series of submaximal contractions. Participants were then instructed to perform a maximal isometric contraction, accompanied by verbal encouragement and visual feedback of force on a computer screen. This was repeated three times, with 60 s rest intervals and the best effort taken as the MVC.

5.2.2.3 Intramuscular EMG

After determining the MVCs, a 25 mm concentric needle electrode (74025-45/25 Neuroline; Ambu) was inserted directly above a HD-sEMG electrode (Figure 5.2A). A voluntary, low force contraction was performed by the participant while the needle position was adjusted to ensure its tip was close to fibres belonging to active MUs (Stashuk, 1999*a*; Piasecki *et al.*, 2019). The participant then performed isometric voluntary contractions lasting 12–15 s, aiming to hold a target line set at 10%, 25% and 40% MVC (Figure 5.2B). The needle electrode was repositioned between contractions by combinations of rotating the bevel 180° and withdrawing it by 10–25 mm to sample MUs at a range of depths (Jones *et al.*, 2021). The procedure of needle positioning, voluntary contraction, and signal recording was repeated until a minimum of four recordings at 25% from varying depths had been obtained to ensure sampling from a representative set of MUs. iEMG signals were amplified (D440, Digitimer, UK), acquired and bandpass filtered from 10 Hz to 10 kHz and sampled at 50 kHz (1401, Cambridge Electronic Design, UK). The force and EMG signals were displayed in real-time using Spike2 software (v9.06), and data were stored for off-line analysis. All assessments were completed on one leg before moving to the other and the order the legs were tested in was randomised.

5.2.2.4 1-RM Strength Assessment

Strength was assessed by calculating the 1-RM of both legs separately. After a warm-up and familiarisation with the leg extension exercise, weight was added to the machine

(Leisure Lines, UK) and the participant attempted one repetition until maximal exertion was reached. The weight was adjusted for a maximum of 5 attempts. 1-RM was assessed at baseline, 3-weeks and 6-weeks to ensure at training load of 75% of 1-RM was maintained.

5.2.3 Training Protocol

Unilateral resistance training was performed 3 times per week for 6 weeks supervised by a member of the study team. Participants performed 6 sets consisting of 8 repetitions of single leg extension at 75% of 1RM (as determined at the baseline assessment) on a leg extension machine (Leisure Lines). 11 participants trained the right leg and 2 trained the left leg due to pre-existing knee problems. During the training period there was a reassessment of strength after 3 weeks to ensure that training load was maintained.

5.2.4 Data Analysis

5.2.4.1 iEMG analysis

The procedures for recording and analysing individual MU potentials (MUPs) have been described in detail previously (Piasecki *et al.*, 2016*c*, 2016*a*) and in section 2.2.3.2. Intramuscular signals from 25% contractions on the trained leg only were analysed using decomposition based quantitative electromyography (DQEMG) (Stashuk, 1999*b*). DQEMG was used to automatically extract MU potential trains (MUPTs) of separate MUs from an iEMG signal and calculate a MUP template for each extracted MUPT. MUPTs composed of MUPs from more than one MU or with fewer than 20 MUPs were excluded. MUP templates of included MUPTs were visually inspected and markers corresponding to the onset, end, and positive and negative peaks adjusted where required. FR variability was defined as the coefficient of variation of the inter-spike interval (CoV ISI). MUP duration, in ms, is the time interval between the MUP template onset and end, MUP area, in µVms, is the MUP template area between its onset and end. MUP amplitude, in mV, is the difference between the MUP template positive and negative peak values. MUP thickness is MUP area divided by MUP amplitude and describes the shape of the MUP template (Abdelmaseeh *et al.*, 2014). MUP complexity was assessed using the number of phases and turns in the MUP template. A phase was defined as a crossing of the MUP template above or below the baseline. A 'turn' was defined as a change in direction of the MUP template of at least 25 µV and indicates the level of temporal dispersion across

individual muscle fibre contributions to a single MUP. A near fibre MUP (NFM) is calculated by applying a low pass, second-order differential filter to its corresponding MUP, which effectively reduces the recording area of the needle electrode, ensuring only the nearest fibres significantly contribute to the NFM and reducing interference from distant active fibres of other MUs (Stashuk, 1999*a*; Piasecki *et al.*, 2021*b*). All NFMs were visually inspected and those containing contamination from other NFMs were removed. NFM jiggle is a measure of the shape variability of consecutive NFMs of an MUPT expressed as a percentage of the NFM template total area (Piasecki *et al.*, 2021*b*) and is representative of NMJ transmission instability. Example data are shown in Figure 5.2C.

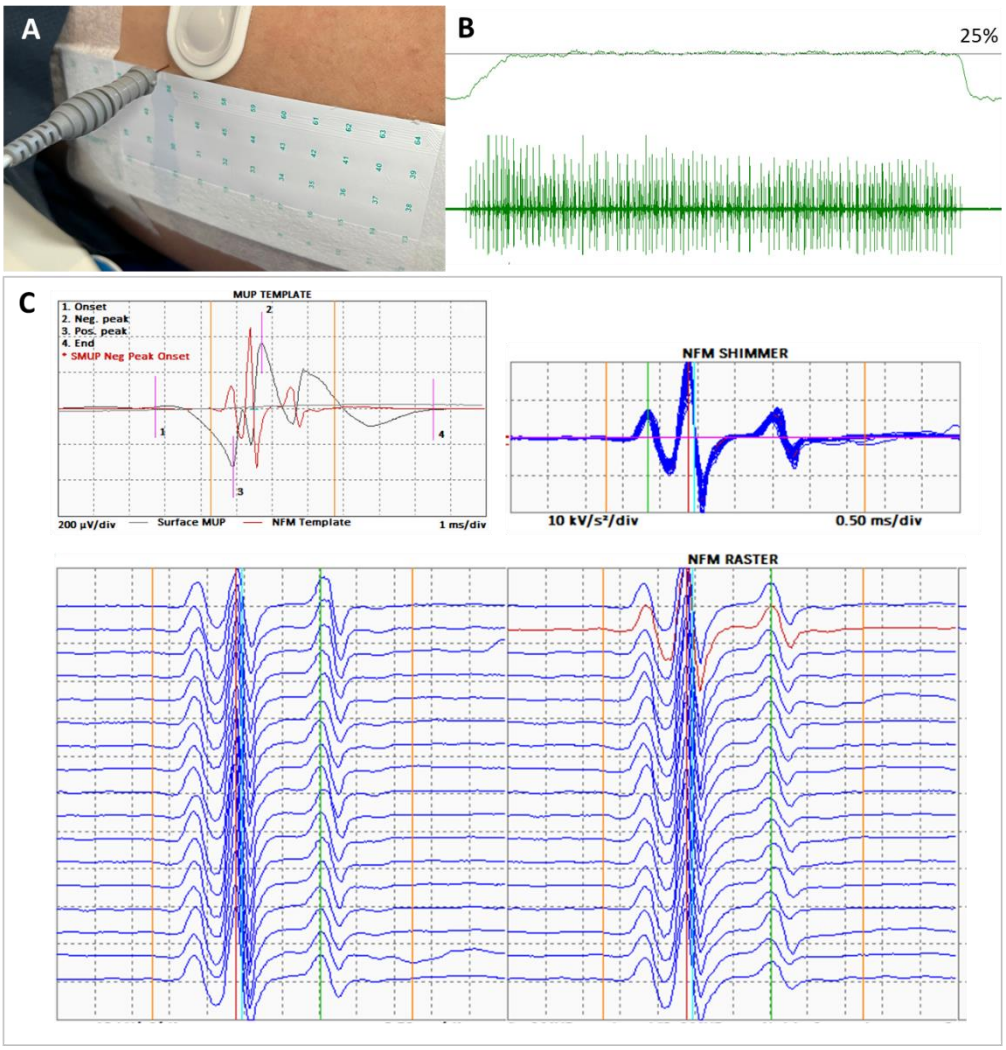


***Figure 5.2**: A. Image of the needle positioning in the mid-belly of VL for iEMG. B. Representative 12s contraction at 25% MVC with the force trace at the top and corresponding raw iEMG signal below. C. Example MUP template, NFM shimmer plot and NFM raster plot to demonstrate calculated MU properties.*

5.2.4.2 Statistical Analysis

The Shapiro-Wilk test was used to assess normality with data being normally distributed. Paired t-tests were performed to assess differences between participant characteristics at baseline. Two-way repeated measures analysis of variance (ANOVAs) were performed in GraphPad Prism (v9.2, USA) to assess time x treatment interactions and to compare the effects of the drug intervention (vs. placebo) before and after 6 weeks of unilateral RET for parameters of CSA, 1-RM, and MVC on the trained leg only. Where no significant interaction was found, main effects are reported otherwise Sidak's post hoc analysis was performed to correct for multiple comparisons. This method assumes each comparison is independent from the others and has more power than the Bonferroni correction method. As multiple MUPs were recorded from each participant, multi-level mixed effects linear regression models were performed in StataSE (v15.0, StataCorp LLC, TX, USA) with treatment and time as factors and interactions included in each model. Again, only data from the trained leg is reported. In each model the first level (individual MUs) were clustered according to each participant to form the second level. Where a significant interaction was present, post-hoc linear models were performed on individual time points and treatments separately. Regression coefficients (β) and 95% confidence intervals (CI) are reported that indicate the magnitude and direction of the effects of interest. Significance was assumed if $p<0.05$.

5.3 Results

5.3.1 Participant characteristics

Participant characteristics are presented in Table 5.1. All participants were male with 7 taking Rapamune and 6 taking a placebo tablet.

Table 5-1: Participant mean (SD) characteristics (n=13).

	Rapamune (n=7)	**Placebo (n=6)**	**P-value**
Age (years)	61.7 (5.3)	65.8 (6.2)	0.258
Height (m)	1.80 (0.07)	1.76 (0.07)	0.308
Weight (kg)	85.6 (7.3)	81.0 (12.6)	0.364
BMI (kg/m^2)	26.4 (1.1)	26.2 (3.2)	0.703

5.3.2 CSA

There was no significant time x drug interaction for VL CSA (p=0.8741) nor was there a main effect of time (p=0.0697) or drug (p=0.4931; Figure 5.3A) with only a 1% and 9% increase for those on Rapamune and the placebo respectively (Rapamune (Baseline vs 6-weeks): 27.0±3.8cm^2 vs 27.3±3.7cm^2; Placebo: 24.3±6.0cm^2 vs 26.4±5.1cm^2).

5.3.3 1-RM

There was no significant time x drug interaction for 1-RM (p=0.9470) but there was a main effect of time (p<0.0001; Figure 5.3B) with a 25% increase for both those on Rapamune and the placebo (Rapamune: 45.4±12.0kg vs 56.7±14.6kg; Placebo: 43.8±8.2kg vs 54.9±8.8kg).

5.3.4 MVC

No significant time x drug interactions for MVC were observed (p=0.9629) but there was a main effect of time (p=0.0444; Figure 5.3C) with a 12% and 11% increase for those on Rapamune and the placebo respectively (Rapamune: 349±73N vs 391±81N; Placebo: 393±110N vs 437±139N).

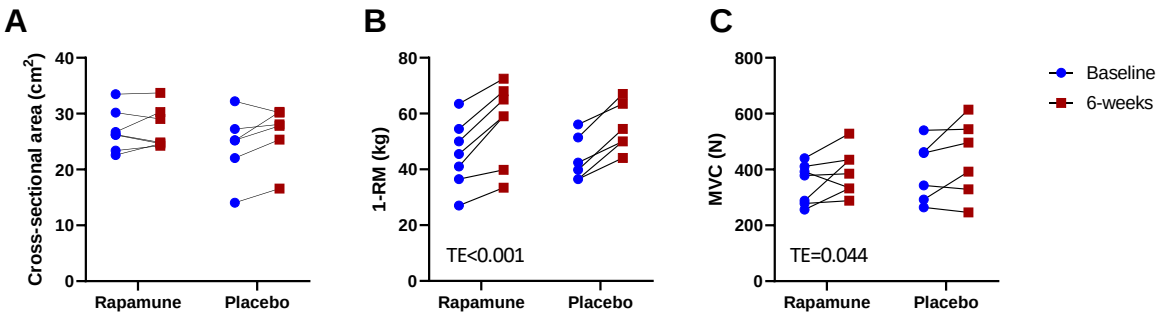


***Figure 5.3**: Individual VL CSA (A), 1-RM (B) and MVC (C) at baseline and following 6-weeks RET in the Rapamune and placebo groups. TE: time effect.*

5.3.5 MU properties

In total 418 MUs were identified at baseline and 457 MUs after 6-weeks RET with an average 8±2 units per person per contraction. There was no significant difference between groups in FR following RET (Rapamune: 7.88±1.12Hz vs 7.90±0.93Hz; Placebo: 8.22±0.39 vs 8.62±1.04Hz; p=0.075; Figure 5.4A). There were also no significant differences observed in CoV ISI between groups (Rapamune: 9.10±1.03% vs 8.99±1.57%; Placebo: 9.24±1.30% vs 9.28±1.01%; p=0.785; Figure 5.4B).

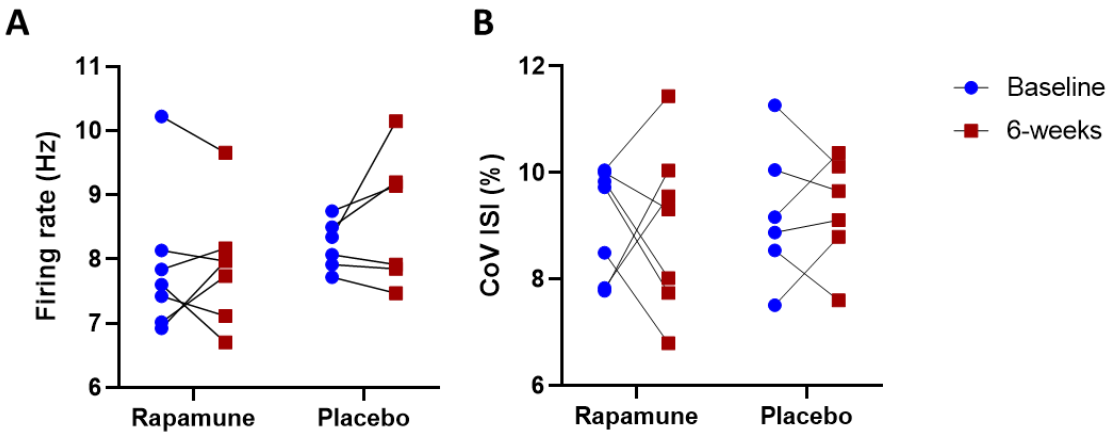


***Figure 5.4**: Participant means of firing rate (A) and firing rate variability (B) at baseline and following 6-weeks RET in the Rapamune and placebo groups.*

Statistics are based on multilevel models.

For parameters representing MUP shape, there was a significant time x drug interaction for MUP duration (p<0.001) but there was no significant main effect of time (p=0.327) or drug (p=0.173) (Placebo: 9.1±2.9ms vs 7.4±0.9ms; Rapamune: 8.4±1.0ms vs 8.6±0.9ms; Figure 5.5A; Table 5.2). Post-hoc analysis showed a significant decrease in MUP duration following RET in the placebo group only (p<0.001) There was a significant time x drug interaction for MUP area (p<0.001) but there was no effect of time (p=0.298). A significant drug effect indicates MUP area was greater in the placebo group (1123±544µVms vs 850±348 µVms) than the Rapamune group (704±145 µVms vs 649±130 µVms; p=0.026; Figure 5.5B; Table 5.2). Post-hoc analysis showed a significant decrease in MUP area following RET in the placebo group only (p<0.001). Similarly, there was a significant time x drug interaction for MUP thickness (p<0.001) but no effect of time (p=0.177). A significant drug effect indicates MUP thickness decreased to a greater extent in the placebo group (1.54±0.52 vs 1.29±0.07) than the Rapamune group (1.37±0.17 vs

1.32±0.12; p=0.023; Figure 5.5C; Table 5.2). Post-hoc analysis showed a significant decrease in MUP thickness following RET in the placebo group only (p<0.001).

Table 5-2: Group means, regression coefficient (β) and 95% confidence intervals (CI) for iEMG derived motor unit and near fibre features where an interaction was present.

	Regression coefficient (β)	Confidence Interval (CI)	Significance (p)
MUP Duration (ms)			
Rapamune	0.134	-0.134:0.402	p=0.328
Placebo	**-1.03**	**-1.35:-0.71**	**p<0.001**
MUP Area (μV.ms)			
Rapamune	37.3	-14.4:89.0	p=0.158
Placebo	**-206**	**-288:-124**	**p<0.001**
MUP Thickness			
Rapamune	-0.023	-0.049:0.003	p=0.089
Placebo	**-0.157**	**-0.198:-0.115**	**p<0.001**
NMJ Transmission Instability (%)			
Rapamune	-0.113	-1.07:0.847	p=0.817
Placebo	**-1.89**	**-3.25:-0.535**	**p=0.006**

Significant values shown in bold. MUP, motor unit potential; NMJ, neuromuscular junction.

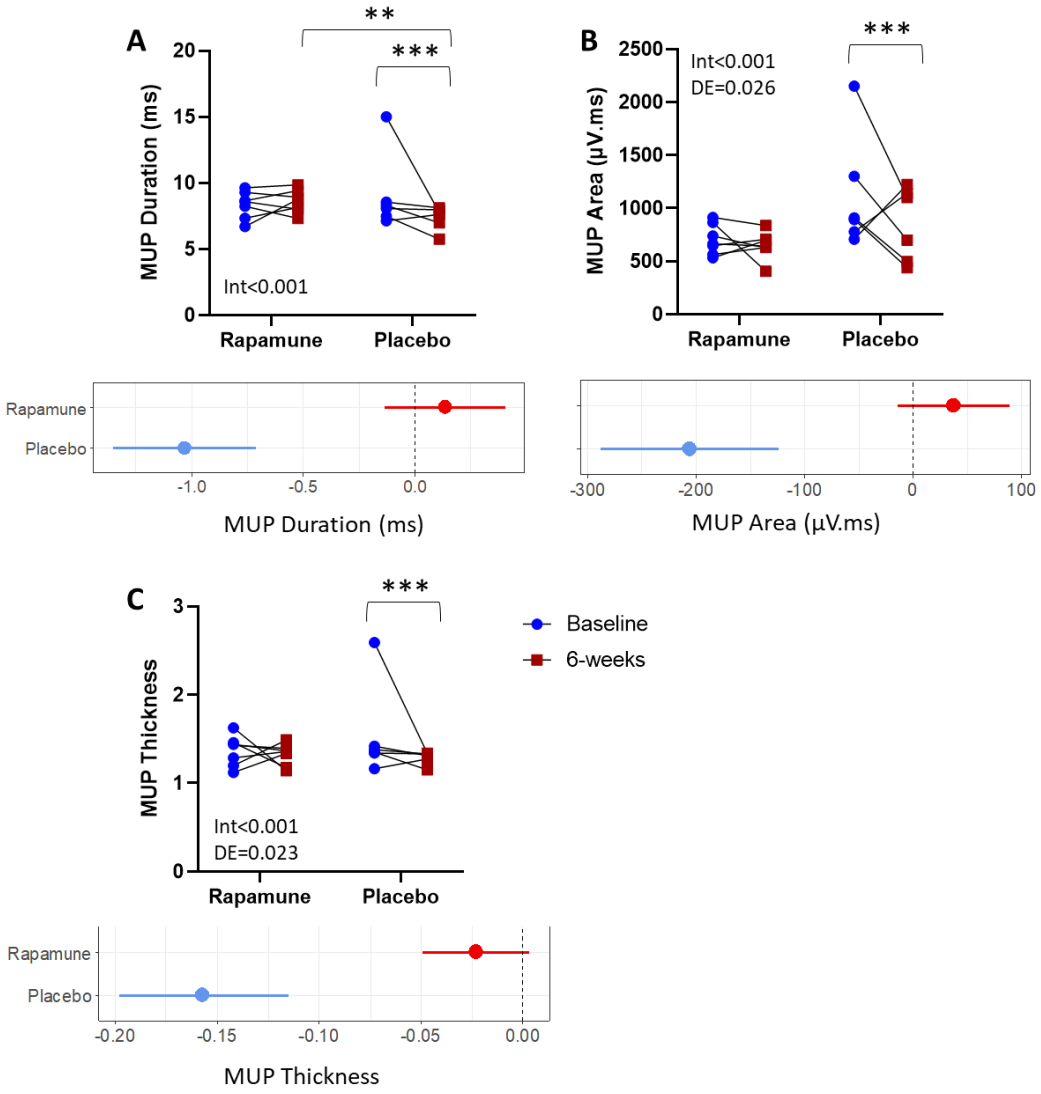


***Figure 5.5**: Participant means of MUP duration (A), MUP area (B) and MUP thickness (C) at baseline and following 6-weeks RET in the Rapamune and placebo groups.*

*** represents p<0.01 and *** represents p<0.001 with statistics based on multilevel models. Int: interaction, TE: time effect, DE: drug effect.*

For parameters representing MUP complexity, there were no significant time x drug interactions for MUP phases (p=0.837) or MUP turns (p=0.673). There was a main effect of time (p=0.045) and drug (p<0.001) for phases with an increase in the trained leg for both those on Rapamune and the placebo (Rapamune: 4.3±0.5 vs 4.5±0.4; Placebo: 3.7±0.4 vs 3.9±0.4; Figure 5.6A). There was also a main effect of time (p=0.036) and drug (p=0.002) for turns with an increase in the trained leg for both those on Rapamune and the placebo (Rapamune: 4.7±0.8 vs 5.2±0.5; Placebo: 3.9±0.7 vs 4.4±0.6; Figure 5.6B).

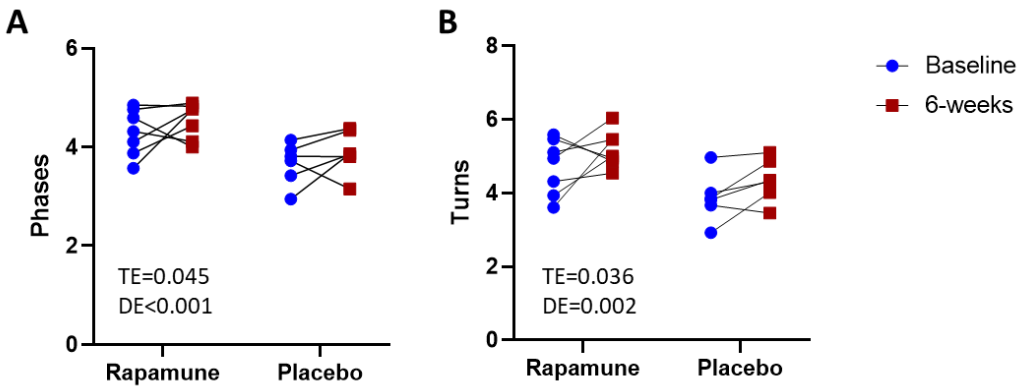


***Figure 5.6**: Participant means of MUP phases (A) and turns (B) at baseline and following 6-weeks RET in the Rapamune and placebo groups.*

Statistics are based on multilevel models. TE: time effect, DE: drug effect.

There was a significant time x drug interaction for the measure of NMJ transmission instability, NFM jiggle (p=0.033) however there were no significant main effects of time (p=0.833) or drug (p=0.099) (Placebo:17.6±6.3% vs 12.6±2.2%; Rapamune:14.0±2.0% vs 14.1±4.4%; Figure 5.7; Table 5.2). Post-hoc testing revealed a decrease in NFM Jiggle in following RET in the placebo group only (p=0.006).

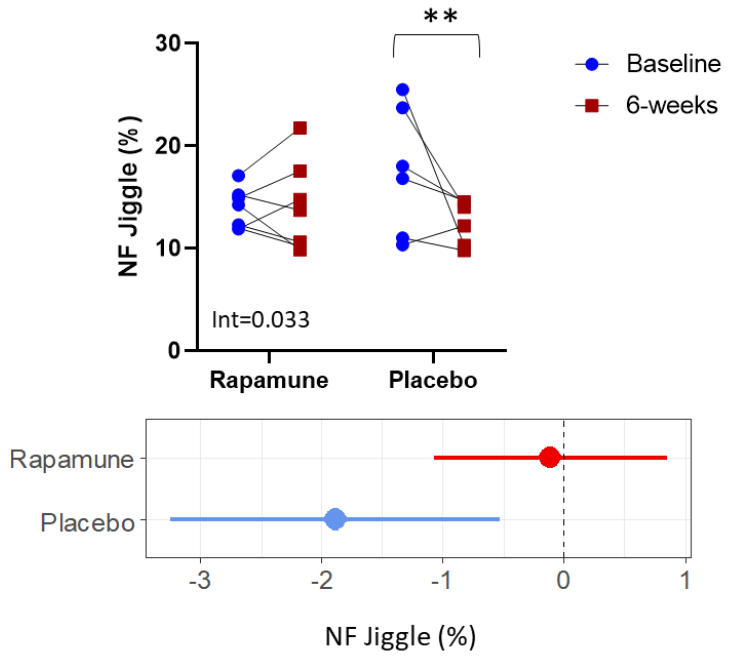


***Figure 5.7**: Participant means and forest plot for near fibre jiggle.*

*Participant means of near fibre (NF) jiggle representing neuromuscular junction transmission instability at baseline and following 6-weeks RET in the Rapamune and placebo groups with a forest plot showing β and 95% confidence intervals for the change in both Rapamune and placebo group following RET. ** represents p<0.01 with statistics based on multilevel models. Int: interaction effect.*

5.4 Discussion

Here we demonstrate that chronic low dose rapamycin does not inhibit RET induced strength gains in the human VL. Following 6-weeks of RET, increases in muscle strength were observed in both Rapamune and placebo groups with no change in muscle size observed in either group, indicating a possible role of neural adaptations. Following this, MU properties were investigated and no changes in FR in either group were observed despite the rise of the absolute force of 25% contractions. In the placebo group only, reductions in MUP size and NFM jiggle were observed indicating some influence of rapamycin administration. An increase in MUP complexity following RET observed in both groups is indicative of exercise-induced MU remodelling occurring and not being prevented by mTOR inhibition.

An increase in muscle strength as measured by both 1-RM and MVC was observed in both the Rapamune and placebo groups. It has been well documented that RET improves muscle strength in the older population (Mcleod *et al.*, 2019) and despite previous findings that mTOR inhibition can prevent strength gains (Drummond *et al.*, 2009) the low dose given did not blunt the response to exercise stimuli. Although mTOR appears to play a crucial role in mechanically-induced MPS (Hornberger *et al.*, 2004), alternative pathways also influenced by RET can enable improvement of strength despite mTOR inhibition including immune and hormonal responses, Akt inhibition of glycogen synthase kinase-3B (GSK3B) enabling translation initiation and protein synthesis, and inhibition of fork-head box O (FOXO) to prevent to production of ubiquitin ligases thereby reducing protein breakdown (Spiering *et al.*, 2008). However, no changes in VL CSA were observed following 6-weeks RET in either the Rapamune or placebo group indicating changes in muscle strength preceded changes in muscle size. This supports findings that initial increases in muscle strength following strength training particularly in the first 6-weeks are predominantly a result of neural factors rather than hypertrophy (Folland & Williams, 2007).

There was no change in MU FR observed in either group following RET when contractions were performed at the same relative force of 25% MVC. This finding is in agreement with previous work where no changes were observed at higher or lower intensity contractions (Kamen & Knight, 2004; Sterczala *et al.*, 2020) however, some previous research assessing MU FR has also found increases in FR following RET measured using both iEMG and surface EMG at medium level contractions (25-35%) (Vila-Chã *et al.*, 2010; Del Vecchio *et al.*, 2019*b*). No changes in FR variability were observed in either group following RET but

as this measure is more strongly associated with improvements in force control rather than strength (Ely *et al.*, 2022), this would not be expected to be directly affected as supported by the findings of Chapter 4. There have been few studies assessing FR variability following strength training but one study found a decrease in variability following 6-weeks of RET but this was more strongly associated with an improvement in FS (Vila-Chã & Falla, 2016).

The measures of MUP duration, area and thickness are indicative of MU size. In the placebo group a decrease in MU size was observed with significant reductions in MUP duration, area and thickness following training but there was no change in the Rapamune group. Although MU size would be expected to increase following RET either as a result of fibre hypertrophy or more frequently reinnervation increasing fibre number within the unit (Piasecki *et al.*, 2018*b*), the decrease observed in the placebo group could be because smaller MUs are recruited or faster, more efficient ion exchange across the membrane occurs shortening MUP duration. There may also be changes to the recruitment thresholds which were not measured here such as higher and later recruitment levels of larger units which could be observed at higher contraction levels e.g. >40%. This opposes previous findings measured indirectly with surface EMG that MUP size increases following RET (Pope *et al.*, 2016; Jenkins *et al.*, 2021) but this is limited by confounding factors of attenuation influencing sEMG signal. The extent of MUP increases may also depend on the recruitment threshold of the MU and muscle hypertrophy. In contrast to the placebo group, no changes were observed in any estimates of MU size in the Rapamune group. This could indicate mTOR inhibition is preventing RET-induced changes however, there was a smaller sample size in the placebo group therefore subject variability may account for the differences observed between groups.

Following RET there was an increase in MUP complexity in both groups as indicated by the number of MUP phases and turns with the numbers of both measures being greater in the Rapamune group compared to placebo. An increase in complexity demonstrates greater heterogeneity in the fibres comprising the MU largely due to temporal dispersion but also fibre diameter, spatial arrangement and conduction velocity (Abdelmaseeh *et al.*, 2014) suggesting MU remodelling through reinnervation has occurred following exercise training and is not prevented by Rapamune administration. This reinnervation of denervated fibres has benefits in preventing the loss of MUs and decreases in MU size due to fibre loss which lead to muscle atrophy observed in sarcopenia. mTOR has previously been reported to be important in the synthesis of new proteins where axonal

injury has occurred (Terenzio *et al.*, 2018) therefore inhibition would be expected to prevent this process occurring however, in older people where mTOR is hyperactive dampening its activity is clearly beneficial. The results from this study also support findings that exercise stimulates axonal sprouting via increases in brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) (Jones *et al.*, 2022). In particular, masters athletes demonstrate greater levels of remodelling which has beneficial effects on muscle function (Piasecki *et al.*, 2019) whereas contrastingly a feature of sarcopenia is the inability to increase the MU size by rescue of denervated fibres (Piasecki *et al.*, 2018*b*).

In the placebo group there was a decrease in NMJ transmission instability as measured by NFM jiggle with no change in the Rapamune group. This supports findings from animal models that exercise improves transmission at the NMJ, reduces fragmentation at postsynaptic sites and denervation markers (Deschenes *et al.*, 2000; Valdez *et al.*, 2010), as well as NFM jiggle data obtained from masters athletes (Power *et al.*, 2016). No change in the Rapamune group could be an early indicator that mTOR inhibition is preventing adaptations at the NMJ as this is known to induce increases in denervation markers such as NCAM (Baraldo *et al.*, 2020). However, combined with exercise the frequently observed age-related decline in NMJ function as has been previously shown (Ham *et al.*, 2020) appears to be attenuated but NMJ adaptations to exercise could be blunted.

5.4.1 Limitations

Although the positioning of the electrodes was attempted to be maintained across both visits facilitated by measurements of position, measures for estimating MU size in particular can be influenced by those MUs closest to the recording electrode therefore changes in location or muscle morphology as a result of training could result in different MUs being sampled (Abdelmaseeh *et al.*, 2014). As there was no familiarisation session before the baseline strength measures were recorded it is possible there was a learning effect and an increase in voluntary effort affecting excitatory input and force output however, this is unlikely to result in the significant changes in neuromuscular function observed. Although none of the participants were classed as masters athletes or competing in sports at high level, they were recreationally active therefore greater differences may have been observed following training in a more sedentary group. A larger sample size and more participants in the older age range in both the Rapamune

and placebo groups would also enable a smaller impact of subject variability on the findings and results applicable to much later in life. Additionally, this study also only included older male participants therefore the possible benefits of RET and mTOR inhibition for postmenopausal women where both methods could increase protective oestrogen levels activating anabolic signalling within muscles (Gharahdaghi *et al.*, 2021), remain unknown.

5.4.2 Conclusions

Chronic low-dose administration over 8 weeks of the mTOR inhibitor rapamycin does not inhibit gains in muscle strength following 6-weeks of RET. With no differences in the outcome of RET on muscle strength between the groups and only small differences with Rapamune use on MU function and remodelling, this means low-dose rapamycin administration did not completely inhibit all mTOR signalling required for muscle maintenance and strength gains.

Further work from this study will investigate the protein turnover and mTOR signalling involved in the response to RET from muscle biopsies obtained during the study to compare the Rapamune and placebo groups following up to 14-weeks of RET. This will further elucidate the possible beneficial effects of mTOR inhibition on muscle mass and function in the ageing population. The use of Rapamune clinically in the older population particularly in those unable to exercise to maintain muscle function may have therapeutic potential to extend healthspan following further exploration of optimal dosage.

Chapter 6: General Discussion

6.1 Summary

The aims of this work were to first determine the effects of different methods of fatigue on MU properties in order to identify possible neuromuscular causes and second, to investigate the effect of RET and pharmacological intervention in older people on MU properties. Following a review of the literature in Chapter 1, it was identified that previous findings on the effects of fatigue and RET on MU properties were inconclusive, causes of fatigue were yet to be fully elucidated, and data on the use of Rapamune in humans was lacking and its effects on muscle function and as a therapeutic intervention for sarcopenia were unknown.

Chapter 2 aimed to determine the effects of CON and ECC loading on MU properties using iEMG and HD-sEMG. MU properties responded to performance fatigue in a contraction-dependent manner with MU FR responding opposingly with CON and ECC loading. One possible reason for this is the greater metabolic cost of CON compared to ECC (Hody *et al.*, 2019) resulting in suppression of FR as a result of increased afferent feedback. Additionally, both forms of fatigue resulted in reduced FS and increased NMJ transmission instability as expected with fatigue. These differences should be accounted for when employing exercise interventions particularly in groups with musculoskeletal limitations.

In Chapter 3 the aim was to determine the effect of a sustained isometric contraction on MU properties and any relationship with MBF measured simultaneously using CEUS. During the contraction, as force control declined there were significant changes in MU firing properties with initial decreases in FR and later increases, possibly when the maximum number of MUs were recruited. Additionally, an increase in common synaptic input was one of the potential mechanisms underlying the increased force variation. However, no change in MBF was observed during the contraction and there appeared to be no direct relationship with changes in MU properties or functional performance thereby demonstrating the need for greater understanding into the mechanisms of fatigue to enable improvements in exercise performance and recovery.

Chapter 4 aimed to determine the effects of 6-weeks unilateral RET on muscle strength and function in older males. RET increased 1-RM as expected (Mcleod *et al.*, 2019) and also improved force control during a complex tracking task. However, changes in MU properties and common drive were unable to explain these improvements. Common synaptic input in the delta frequency band of MU coherence is reflective of force

variability (Alix-Fages *et al.*, 2023), therefore this may have been expected to decrease with the observed improvements in force control following RET. This demonstrates that although RET is beneficial for improving muscle strength in older age, the underlying neural mechanisms involved in these adaptations require further investigation.

Following on from the work in Chapter 4, Chapter 5 aimed to determine the effect of the same 6-weeks RET protocol on MU properties in older males with or without oral administration of the mTOR inhibitor Rapamune. As in the previous chapter, there was an increase in muscle strength but not size in both groups, indicating that neural adaptations were predominantly behind these strength increases. Differences in MUP size were observed between the treatment and placebo groups following RET, with decreases occurring in the placebo group only. MUP complexity increased in both groups following RET and was greater in those taking Rapamune. These adaptations to training in both groups show Rapamune does not prevent improvements in muscle function in response to RET by inhibiting mTOR, so could potentially alter protein metabolism without compromising muscle strength.

Overall, this research demonstrates that both acute and chronic forms of exercise employing different types of contraction directly affect MU properties which initiate task-dependent alterations in muscle function. In older people, RET has a beneficial effect on muscle strength and neuromuscular function which is not prevented by administration of an mTOR inhibitor. This provides options for both exercise and pharmacological therapeutic interventions targeting sarcopenia in the future, particularly for those unable to exercise with recommended sufficient loads to obtain the necessary stimulus to the muscle to prevent decline.

6.2 Clinical Applications

With a rise in sedentary behaviour and an increasing older population, musculoskeletal problems are becoming more prevalent in society, having multiple consequences on population health (Ingram & Symmons, 2018) and placing a significant strain on health and social care services (Pinedo-Villanueva *et al.*, 2019). Promoting exercise in older age can help to improve muscle mass and functional performance therefore mitigating falls risk, and levels of frailty and disability within the population (Cadore *et al.*, 2013*b*). Additionally, exercise can benefit older adults requiring clinical treatment for numerous age-associated conditions (e.g., cancer, arthritis), with pre- and/or rehabilitation shown

to improve, for example surgery survival rates, improved recovery times and better return to normal activities (Hoogeboom *et al.*, 2014).

Fatigue is a factor affecting exercise performance and tolerability and is present in many chronic clinical conditions such as heart failure and chronic obstructive pulmonary disease (Burtin *et al.*, 2012). A key finding of this work is that MU properties, which play a key role in force production, respond depending on the contraction mode employed. Additionally, the findings of this work indicate that MBF did not influence MU properties, nor did it correlate with functional outcomes. Understanding the physiological factors resulting in fatigue and ultimately task failure is important to improve therapeutic exercise interventions and physical performance, particularly in conditions where muscle fatigue limits exercise tolerance or where blood flow to the muscle (a potential factor in fatigue (Clark *et al.*, 2005)) could be restricted such as diabetes and peripheral artery disease (Duerschmied *et al.*, 2009; Groen *et al.*, 2014).

RET likely presents a more potent stimulus when targeting ageing muscle compared to endurance exercise (based on hypertrophic adaptation and strength gains (Hughes *et al.*, 2018)), and has been demonstrated to be both feasible and effective in multiple aged populations with different levels of ability (Steib *et al.*, 2010). Here we present improvements in muscle strength and complex force control following 6-weeks RET in older males. Despite increases in muscle strength, no change in muscle size was observed but MU properties also did not fully explain the observed training improvements so further investigation into the mechanisms is warranted.

Of note, recent advances in the literature contest the commonly held notion that high-intensity (i.e., heavy) RET is required to elicit improvement in muscle mass and strength, with low-load RET showing improvements in strength albeit lower than heavy loads, but equal increases in muscle size (Schoenfeld *et al.*, 2017). Despite this, older adults still show anabolic (Burd *et al.*, 2013) and adaptive (Phillips *et al.*, 2017) blunting in response to RET such that alternative or adjuvant interventions may be required to enhance the muscle 'health' (i.e., structure and function). Pharmacological drug interventions such as Rapamune may provide an option for those unable to exercise or achieve appropriate loading (i.e., those who are immobile or in an intensive treatment unit). Here we demonstrate that Rapamune did not prevent strength gains from RET or stop increases in MUP complexity indicative of fibre reinnervation. Therefore, although Rapamune demonstrates some therapeutic potential and does not appear to impede functional

adaptation, further investigation is required for optimisation and validation of this intervention.

6.3 Limitations and Future Considerations

One of the main limitations of this work is that the sample sizes may be viewed as low, and although this may limit the validity of any conclusions, it provides a solid basis for future investigations. This will be addressed in the near future, with an increase in sample size to expand upon current findings. This also means that sex differences in response to both fatigue and RET are still to be investigated, with potential differences in both fatiguability and MU recruitment strategies (Ansdell *et al.*, 2017; Guo *et al.*, 2022) as discussed throughout the thesis. In addition, the vastus lateralis muscle was used in all experimental chapters because as one of the quadriceps this muscle is functionally important but highly susceptible to loss of strength and mass with ageing although it also has the potential for neuromuscular remodelling (Piasecki *et al.*, 2016*c*). However, the findings may differ in muscles such as tibialis anterior, gastrocnemius or biceps brachii where physical function and fibre compositions differ.

The findings of Chapters 2 and 3 on the causes of fatigue remain inconclusive with no specific biomarker such as blood flow or MU feature responsible and therefore further work is required. With the different contraction types performed particularly ECC there is also scope to investigate the effects of acute muscle damage following fatiguing exercise to determine the effects on MU properties and the possible impact this could have on interventions. In Chapter 3, it is also plausible that differences may have been observed if the isometric task was performed to full exhaustion/task failure and there may also be unquantified sex differences in these data which need further exploration. Additionally, both of these chapters investigated changes in young participants therefore the conclusions may not be the same if performed in older adults therefore future work should be performed in later age groups where physiological declines may have occurred with ageing.

As a result of limitations imposed by the COVID pandemic Chapter 4 and 5 are taken from an ongoing study and changes were made to lower the age range of included participants (from 65y minimum to 55y) to allow continuation of testing in line with local restrictions. As a result, the effects on the studied population may be less reflective of physiological changes in older group age 70y+.

In Chapters 4 and 5, responses of MU properties to 6-weeks RET were presented but the temporality of adaptations to strength training within this window are still unknown, as are responses over a longer period of time e.g., 12 weeks. Future analysis of HD-sEMG data collected during this study will allow more detailed analysis of underlying PICs and changes to neural input in response to RET. By performing unilateral RET and taking bilateral measures this also enables future exploration of any cross-education effects (Fimland *et al.*, 2009) in MU properties which may have important implications in unilateral injury or medical conditions such as stroke.

As the effects of Rapamune on human muscle have not yet been studied there are multiple aspects requiring further investigation which will occur during this ongoing study. Firstly, the effect of the drug on muscle protein turnover and total whole body contractile mass can be studied using stable isotope tracer techniques (Wilkinson *et al.*, 2018) and is important for assessing its potential impact in sarcopenia and the translation of previous animal work (Joseph *et al.*, 2019) to humans. In addition, the mechanism of action and effects on cellular processes through inhibition of mTOR signalling also requires more research and, in order to ensure safety and effectiveness if used therapeutically, further research is needed on the optimal dose also considering the impact this may have on the immune system given the immunosuppressive nature of the drug (Yoo *et al.*, 2017).

The clinical impacts of all the findings in this thesis need further investigation to determine the true impact of exercise interventions on MU features and muscle function in distinct disease cohorts and the potential benefit of Rapamune in slowing the age-associated losses of muscle mass and function in vulnerable groups.

6.4 Conclusions

In conclusion, this thesis aimed to i) investigate the effects of different exercise-induced methods of fatigue on MU properties and potentially associated physiological parameters (e.g., MBF) and ii) determine the effect of RET and pharmacological intervention on MU properties and muscle function in older people. Both acute exercise-induced fatigue and chronic adaptations to RET resulted in alterations to both central and peripheral MU features, which were accompanied by changes in muscle function, including force production. Further work is needed to understand the mechanisms of fatigue and the effects of Rapamune with RET on muscle metabolism, structure and function. With an

increasing ageing population, this work has potential to make a valuable contribution to future interventions targeting the loss of muscle mass and strength observed with age, therefore contributing to increasing healthspan, improving quality of life and reducing future healthcare costs.

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Chapter 8: Appendices

8.1 Publications

Guo Y, **Jones EJ**, Skarabot J, Inns TB, Stashuk DW, Phillips BE, Atherton PJ, Piasecki M (2023). Sex disparities in age-related neuromuscular decline: unveiling female susceptibility from early to late elderly. [PrePrint, Under review]

Guo Y, **Jones EJ**, Skarabot J, Inns TB, Stashuk DW, Phillips BE, Atherton PJ, Piasecki M (2024). Common synaptic inputs and persistent inward currents of vastus lateralis motor units are reduced in older age. *Geroscience* [Accepted]

Piasecki J, Guo Y, **Jones EJ**, Phillips BE, Stashuk DW, Atherton PJ, Piasecki M (2023). Menstrual cycle associated alteration of vastus lateralis motor unit function. *Sports Medicine* **9**(1): p1-14

Jones EJ and Phillips BE (2023) Methodologies to quantify skeletal muscle blood flow/perfusion**.** In *Neuromuscular Assessments of Form and Function* (p299-315). New York, NY: Springer US

Jones EJ, Guo Y, Martinez-Valdes E, Negro F, Stashuk DW, Atherton PJ, Phillips BE, Piasecki M (2023) Acute adaptation of central and peripheral motor unit features to exercise induced fatigue differs with concentric and eccentric loading. *Experimental Physiology.* p1-11 [selected as Editors pick]

Jones EJ, Atherton PJ, Piasecki M, Phillips BE (2023) Contrast-enhanced ultrasound repeatability for the measurement of skeletal muscle microvascular blood flow. *Experimental Physiology.* **108**(4): p549-553

Jones EJ, Chiou SY, Atherton PJ, Phillips BE, Piasecki M (2022) Ageing and exercise-induced motor unit remodelling. *Journal of Physiology*. **600**(8): p1839-1849

Guo Y, **Jones EJ**, Inns TB, Stashuk DW, Wilkinson DJ, Smith K, Piasecki J, Atherton PJ, Phillips BE, Piasecki M (2022) Neuromuscular recruitment strategies of the vastus lateralis according to sex. *Acta Physiologica.* **235**(2): e13803.

Ely I, **Jones EJ**, Inns TB, Dooley S, Miller SBJ, Stashuk DW, Atherton PJ, Phillips BE, Piasecki M (2022) Training-induced improvements in knee extensor force accuracy are associated with reduced vastus lateralis motor unit firing variability. *Experimental Physiology.* **107**(9): p1061-1070.

Jones EJ, Piasecki J, Ireland A, Atherton PJ, Phillips BE, McPhee JS, Piasecki M. (2021) Lifelong exercise results in more homogeneous motor unit characteristics across deep and superficial areas of vastus lateralis. *Geroscience*. **43**(4): p1555-1565

8.2 Conference presentations

Jones E.J, Piasecki M, Guo Y, Phillips B.E, Smith K, Wilkinson D.J, Atherton P.J. Chronic oral administration of the mTOR inhibitor rapamycin to older people, is safe, does not perturb white blood cell counts, and does not limit resistance exercise-induced muscle strength gains. International Translational Sarcopenia Research Conference 2023, Newcastle [Oral, Prize winner]

Jones E.J, Guo Y, Smith K, Wilkinson D.J, Phillips B.E, Atherton P.J, Piasecki M. Complex force control is improved following 6-weeks resistance training in older males independent of motor unit firing variability. Physiology 2023, Harrogate [Oral]

Jones E.J, Piasecki M, Guo Y, Phillips B.E, Smith K, Wilkinson D.J, Atherton P.J. Chronic oral administration of the mTOR inhibitor rapamycin to older people, is safe, does not perturb white blood cell counts, and does not limit resistance exercise-induced muscle strength gains. CIMA-CMAR Joint Conference 2023, Birmingham [Oral, Prize winner]

Jones E.J, Piasecki M, Guo Y, Phillips B.E, Smith K, Wilkinson D.J, Atherton P.J. Chronic oral administration of the mTOR inhibitor rapamycin to older people, is safe, does not perturb white blood cell counts, and does not limit resistance exercise-induced muscle strength gains. International Conference on Frailty and Sarcopenia Research 2023, Toulouse. [Poster]

Jones EJ, Guo Y, Atherton PJ, Phillips BE, Piasecki M. Motor unit firing rate variability and microvascular blood flow as mediators of neuromuscular control. Europhysiology 2022, Copenhagen. [Oral]

Invited speaker – Motor unit function in muscle fatigue and damage. Biomedical Basis of Elite Performance 2022, Nottingham

Jones EJ, Martinez-Valdes E, Negro F, McCormick D, Atherton PJ, Phillips BE, Piasecki M. Tracking of individual motor units following concentric and eccentric exercise-

induced fatigue reveals contraction-type specific changes in discharge properties. Experimental Biology 2021, Virtual. [ePoster]

Jones EJ, Martinez-Valdes E, Negro F, McCormick D, Atherton PJ, Phillips BE, Piasecki M. Motor unit discharge properties following concentric and eccentric exercise-induced fatigue are dependent upon contraction type. Integrative Physiology of Exercise, American Physiological Society 2020, Virtual. [Oral]

Jones EJ, Piasecki J, Ireland A, Atherton PJ, Phillips BE, McPhee JS, Piasecki M. Lifelong exercise results in more homogeneous motor unit characteristics across deep and superficial areas of vastus lateralis. Future Physiology 2020, Virtual. [ePoster]

8.3 Screening parameters

Depending on the study the screening visit can include collection of a fasted blood sample (for standard clinical chemistry profiles (FBC, U&E's, LFTs, TFTs, coagulation, lipids, glucose)), obtaining a medical history, height and weight measures, an electrocardiogram (ECG) and a blood pressure assessment to confirm suitability for the study. The information obtained from the screening session was checked by a clinician to ensure it is safe for the participant to take part. The maximum duration between screening and starting the study was 3 months, although the preferred time frame was within 6 weeks. The reference ranges provided by the pathology lab and acceptable parameters for each blood test are included in Table 8.1. A medical history was taken to ensure none of the exclusion criteria were met and to obtain any additional health information relevant to the study to assist with clinical decision making. BMI was calculated to ensure this fell between 18 and 35 to ensure the population recruited was healthy and did not have any confounding metabolic phenotypes. As the studies involve exercise, some to fatigue and exhaustion and RET, ECGs were recorded to check for any unknown cardiac electrical problems such as atrial fibrillation and bundle branch block which could be more prevalent in older people. Blood pressure was measured 3 times and was required to be below 160/100 due to the expected increases during exercise being a risk with uncontrolled hypertension.

Table 8-1: Blood tests performed with recommended reference ranges.

Test	Reason	Reference range
Full blood count (FBC)	Test all blood cells including for immune function	WBC - 4-10 10*9/L RBC – 4.5-5.5 10*12/L Haemoglobin – 130-180 g/L Platelets – 150-400 10*9/L
Urea and electrolytes (U&E)	Test kidney function and electrolyte imbalance	Urea – 2.5-7.8 mmol/L Creatinine – 59-104 umol/L
Liver function tests (LFT)	Test liver function	Bilirubin – 0-21 umol/L Albumin – 35-50 g/L Alkaline Phosphatase – 40-170 [iU]/L
Thyroid function tests (TFT)	Test thyroid function which may have metabolic implications.	TSH – 0.3-5.5 mlU/L
Lipids	Test for high cholesterol.	Triglyceride – 0.5-2.0 mmol/L HDL Cholesterol – 0.94-1.48 mmol/L
Fasting Glucose	Test for diabetes	3.5-6.0 mmol/L
Coagulation	Check appropriate clotting for studies where a biopsy will be taken.	INR – 0.9-1.1

8.4 Exclusion criteria and safety monitoring (Chapter 4 and 5)

Due to the possible administration of rapamycin which is an immunosuppressant participants used in Chapter 4 and 5 who were part of this larger study were checked against strict exclusion criteria and were monitored for safety throughout the study. The exclusion criteria and safety protocols are outlined below.

The participant may not enter the study if ANY of the following apply:

- A BMI <18 or >35 kg/m^2
- Active cardiovascular, cerebrovascular or respiratory disease: e.g. uncontrolled hypertension (BP > 160/100), angina, heart failure (class III/IV), arrhythmia, right to left cardiac shunt, recent cardiac event, COPD, pulmonary hypertension or recent stroke
- Any metabolic disease
- Clotting dysfunction
- A history of, or current neurological or musculoskeletal conditions (e.g. epilepsy)

- Having taken part in a research study in the last 3 months involving invasive procedures or an inconvenience allowance
- Contraindications to MRI scanning including claustrophobia, pacemaker, metal implants etc. which was assessed through an MRI safety screening questionnaire.
- Contraindications to the use of Rapamycin

Eligibility for this study against all these criteria was be determined by a clinician taking into account all individual results.

Safety was monitored throughout the study with weekly blood samples (FBC, U&E's, LFTs, TFTs and lipids)), with particular interest in white blood cell (WBC) count as an indicator of immune function due to the immunosuppressive nature of the drug. On analysis of the participants in Chapter 5 no significant difference (p=0.655) was observed in WBC count between baseline and week 8 in either the Rapamune or placebo group (Figure 8.1) following a two-way ANOVA.

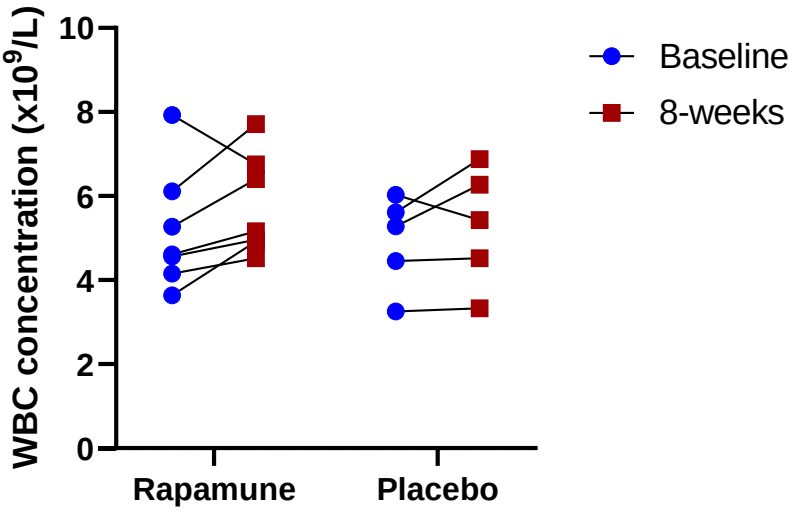

Figure 8.1: *Individual white blood cell counts at baseline and 8 weeks later in both the Rapamune and placebo groups.*

A lower dose of 1mg per day was administered compared to that used for immunosuppressive purposes to avoid detrimental effects and the bioavailability of rapamycin in the blood was assessed to ensure it was active in the body once taken (Figure 8.2). This was assessed by liquid chromatography mass spectrometry and a significant increase of sirolimus in the blood was observed in the Rapamune group only ($p<0.0001$) from baseline to 8 weeks of administration.

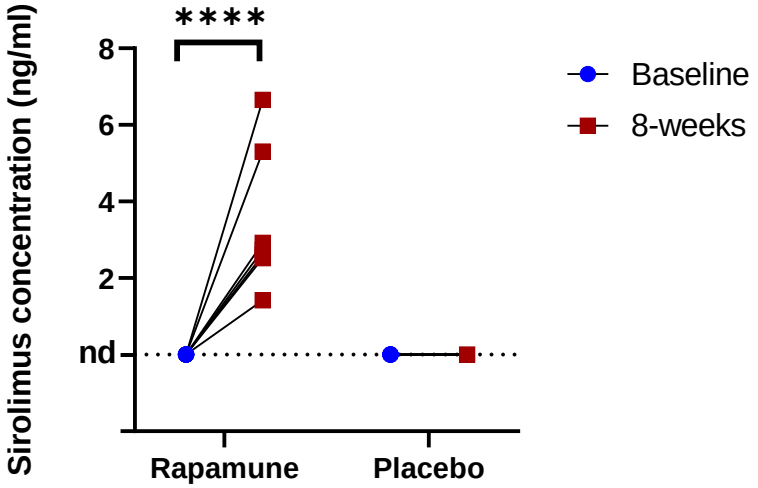

Figure 8.2: *Individual sirolimus blood concentrations at baseline and 8 weeks later in both the Rapamune and placebo groups.*

Abbreviations: nd (non-detectable).

In the case of a participant becoming unwell while taking part in the study, they were advised to stop taking the tablet (irrespective of drug or placebo) for the time of illness. If required, the study clinician will advise on further tests and continuation of the study depending on severity of the illness. For example, in cases where a biopsy site may become infected this would be assessed by the clinician and antibiotics prescribed where necessary. The participant would stop taking the study tablet during this time until the infection has cleared. Adverse event reporting will be followed as per usual ethical procedures.