Activity of group III-IV muscle afferents: Implication for the neuromuscular and cardiovascular responses to exercise in humans

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PhD 2023

Activity of group III-IV muscle afferents: Implication for the neuromuscular and cardiovascular responses to exercise in humans

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A thesis submitted in partial fulfilment of the requirements of Manchester Metropolitan University for the degree of Doctor of Philosophy

Department of Exercise and Sport Sciences

Manchester Metropolitan University

2023

“Fatti non foste a viver come bruti ma per seguir virtute e canoscenza.”

“Ye were not made that ye should live like beasts, but follow after virtue and the truth”

Dante Alighieri (1265-1321)

Divina commedia, Inferno, canto XXVI

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Acknowledgments.

I would love to thank you my supervisor Jamie McPhee for his enormous patience and excellent mentoring who really helped me every day to overcome and face with a positive attitude all the obstacles we encounter during this journey. I learned to be a sharper physiologist and a wiser person.

A big thank you to all my supervisors and collaborator team, especially to Arwel, Azmy, Johnny, Liam, and Tiago who helped, assisted, and guided me through different projects with great skills and positiveness. Thanks for teaching me essential techniques and specially to think outside the box and independently.

A special thanks go to Massimo and his team who host me in Verona and helped me through the most challenging period of my academic life, learning difficult techniques and work at high standards under pressure. I was really impressed by him and his team, and I will always use as a reference in my career development.

I want to also thank all the member of the sport tech team for being so helpful, flexible, and patient with me. Without them nothing was probably possible to achieve in such a short time and with so high standards and quality.

Last but not the least important, a big thanks to all the people in the office that just make these last three years so full of memories and positive vibes. I've made such great friends that I decided to make Manchester my home.

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(those not widely used in scientific literature are listed):

AGP	Age Graded Performance
ANOVA	Analysis of Variance
ASIC	Acid-sensing ion channel
AT	Anaerobic Threshold
ATP	Adenosine Tri-Phosphate
AUC	Area Under the Curve
BIA	Bio-impedance Analysis
BP	Blood Pressure
BMI	Body Mass Index
CFS	Chronic Fatigue Syndrome
CO	Cardiac Output
CPET	Cardiopulmonary Exercise Rest
DBP	Diastolic Blood Pressure
DXA	Dual-energy X-ray Absorptiometry
EBPR	Exaggerated Blood Pressure Response
EIMD	Exercise induced Muscle Damage
sEMG	Electromyography
EPHPP	Quality Assessment Tool for Quantitative Studies
EPR	Exercise Pressor Reflex
FBF	Femoral Blood Flow
FFM	Free Fat Mass
FM	Fat Mass

FMS	Fibromyalgia Syndrome
HG	Handgrip
HR	Heart rate
HR_{peak}	Peak Heart Rate
HRV	Heart Rate Variability
IE	Ischemic Exercise
LF/HF	Ratio between Low and high frequency of RR intervals
LVC	Leg Vascular Conductance
MAP	Mean Arterial Pressure
MA	Master Athletes
MM	Muscle Mass
MSNA	Muscle Sympathetic Nerve Activity
MVC	Maximal Voluntary Contraction
PEO	Post Exercise Occlusion
PECO	Post Exercise Cuff Occlusion
P2X4	Purinergic Receptor 4
Pi	Phosphate
PRISMA	Preferred Items for Systematic Reviews and Meta-Analyses
PROSPERO	International Prospective Register of Systematic Reviews
Qcsa	Quadriceps Cross Sectional Area
Qtw, pot	Resting Twitch Potentiated
RMSSD	root mean square of successive differences between normal heartbeats
RR	Intervals distance from each R heart peak
RPE	Rate of perceived exertion
SBP	Systolic Blood Pressure

SMD	Standardized Mean Difference
SPLM	Single Passive Leg Movement
SV	Stroke Volume
TI	Total Impulse (force)
TPR	Total peripheral Resistance
TPRV	Transient receptor potential vanilloid
TTE	Time To Exhaustion
VA	Voluntary Activation
VAS	Visual Analog Scale
VCO_2	Carbon Dioxide Volume Expired
VE	Ventilation
VO_2	Oxygen Uptake
$VO_{2\ max}$	Maximal oxygen uptake
$VO_{2\ peak}$	Peak oxygen uptake

List of original published work

(All the work listed below is bound at the end of this thesis)

Chapter 5: Zambolin F, Giuriato G, Laginestra FG, Ottaviani MM, Favaretto T, Calabria E, Duro-Ocana P, Bagley L, Faisal A, Peçanha T, McPhee JS, Venturelli M. Effects of nociceptive and mechanosensitive afferents sensitization on central and peripheral hemodynamics following exercise-induced muscle damage. J Appl Physiol (1985). 2022 Aug 18. PMID: 35981730.

Chapter 7: Zambolin F, McPhee JS, Duro-Ocana P, Ganse B, Bagley L, Faisal A. The association of elevated blood pressure during ischaemic exercise with sport performance in Master athletes with and without morbidity. Eur J Appl Physiol. 2022 Jan;122(1):211-221. Epub 2021 Oct 15. PMID: 34652528;

Chapter 8: Zambolin F, Duro-Ocana P, Faisal A, Bagley L, Gregory WJ, et al. (2022) Fibromyalgia and Chronic Fatigue Syndromes: A systematic review and meta-analysis of cardiorespiratory fitness and neuromuscular function compared with healthy individuals. PLOS ONE 17(10): e0276009. https://doi.org/10.1371/journal.pone.0276009

Papers in preparation:

Chapter 4: F. Zambolin, S. Pinner, J. McStravick, J.S. McPhee. Cardiovascular Responses and Metaboreflex Isolation following exercise-induced muscle damage.

Chapter 6: Zambolin F, Laginestra FG, Favaretto T, Giuriato G, Ottaviani MM, Duro-Ocana P, McPhee JS, Venturelli M. Activation of nociceptive and mechanosensitive skeletal

muscle fibres following exercise-induced muscle damaged impairs contralateral knee extensor exercise performance.

Abstract

Changes in activation of muscle III-IV afferents has shown to lead to impaired cardiovascular and neuromuscular responses to exercise. The mechanisms underlying these alterations has been attributed to changes in sensitivity of the muscle afferents that associated with muscle inflammation, ageing and pathological conditions. In the first three experimental chapter (chapter 4 to 6), we collected data from healthy moderately active young male performing an exercise induced muscle damage protocol (EIMD), that induced muscle inflammation and changes in muscle afferent activation (i.e., mechano and nociceptors) 48h post EIMD. These changes were associated to alterations in blood pressure regulation, cardiovascular responses, peripheral blood flow, neuromuscular function, and exercise performance. Following these experiments, we tested the association of blood pressure responses and exercise performance in a master athletes' cohort (Chapter 7), showing links between increased blood pressure responses and lower exercise performance. In the last chapter (chapter 8), we conducted a systematic review with meta-analysis to determine cardiorespiratory fitness and neuromuscular function of people with Chronic fatigue and Fibromyalgia syndromes compared to healthy individuals. From the 99 studies selected we found a large decreased in cardiorespiratory fitness, neuromuscular function, and fatigability within increased in perceived exertion in patients. In conclusion, the data collected showed the physiological relevance of the changes in muscle afferents activation, becoming relevant for future studies in chronic pain and fatigue conditions.

Chapter 1. Introduction

1.1. Human movement

Integrative function of body systems during movement helps to precisely control muscle actions and to ensure that movements are carried out at the required intensity and duration to match the task demands. This is achieved by intricate signalling from the central nervous system to skeletal muscles – and back again. It doesn't matter whether the task requires a fine, discreet action such as finger tapping, or whether the task is to run at high intensity for as long as possible: the instructions that orchestrate movements follow the same principles.

1.2. Instructing skeletal muscles to contract

Human movement starts from the compelling necessity of the organism to respond to the external environment. All voluntary human movements are pre-organised and controlled in the central nervous system. The motor cortex is the core of the execution of any human movement and in communication with other structures (such as the pre-motor cortex, the sensory motor cortex, the cerebellum) they finely control and recruit the skeletal muscles. Therefore, the idea to move comes from higher brain centres, which communicates with the motor control centre and appropriate signals are derived in upper motor neurones which are relayed along the central nervous system to reach the target peripheral motor units. From here, efferent signals travel along axons and reach the axon terminal which is the point of innervation of the neuromuscular junction with individual skeletal muscle fibres. The efferent signals from the CNS to skeletal muscles are precise to effectively match the skeletal muscle contractions with the required movements. All

these steps are possible through the transmission of action potentials that travel along the central nervous system and motoneurons to the neuromuscular junction allowing the release of the acetylcholine into the extracellular space that bind specific to receptors and initiate a cascade of events that leads muscle fibers contracting and using ATP to generate force and consequently movement.

1.3. Feedback to the brain from contracting muscles

The neural signalling is not one-way; rather, afferent signals are relayed in the opposite direction from muscles to the CNS. These include muscle spindles (group Ia), Golgi tendon organs (group Ib), secondary spindles (group II) and free nerve endings (group III-IV) which collectively sense absolute contractile tension, movements, and a range of other mechanical and metabolic factors. The sum of this sensory feedback informs the brain that movements are precise or that further refinement is needed. Moreover, these sensory feedbacks inform the CNS to the mechanical and metabolic perturbations present during movements, which in turn decide if the movement can continue or should be stopped because it is unsustainable for the homeostasis of body systems and risking overexertion or structural damage.

1.4. Interaction with other brain centres

Group III-IV muscle nerve afferent signals arriving at the brain can regulate responses of blood flow, respiration frequency, minute ventilation and blood pressure, connecting the

muscle and the central nervous system within the cardiovascular and cardiorespiratory centres. These afferents are stimulated by changes of muscle tension or metabolic perturbations of the muscle, becoming particularly active during exercise and being essential in regulating cardiorespiratory and cardiovascular responses, including forming the afferent arch of the exercise pressor reflex. Moreover, Group III-IV muscle afferents signal pain and fatigue during exercise providing information on effort and exertion to the higher brain centres and being responsible of the exercise tolerance limit.

1.5. Fatigue

With sustained movements during exercise, a further challenge of maintaining sufficient energy to continue to move must be overcome. This requires close interactions with not only neuro-muscular, but also cardiovascular and respiratory systems to regulate blood flow and supply of oxygen and nutrients to working muscles.

Very long duration exercise, or that performed at very high intensity can lead to fatigue. Fatigue is characterised by reduced muscular force, slowing of contractile velocity, reduced power output, poor coordination of movements and sensations of discomfort and very high effort, which together prevent exercise from continuing, or at least forces a significant reduction of voluntary effort.

Peripheral fatigue: relates to a biochemical impairment occurring at or distally to the neuromuscular junction, where the contractile properties of the muscle may be impaired. This includes disruption of contraction-excitation uncoupling, alterations in cross-bridge

function, changes in calcium actions and loss of intracellular stores. Moreover, decreased glycogen stores and pathologic alteration of the peripheral nerves may cause mitochondrial dysfunctions, acetylcholine depletion or motor conduction block inducing an earlier onset of fatigue and exercise performance deterioration.

Central fatigue: is defined as an impairment of the central motor drive that restricts motor unit recruitment and eventually causes discontinuation of exercise. Different studies have shown an inhibitory role of muscle III-IV afferents, that restrict motoneuronal output and locomotor muscle activation facilitating central fatigue. These changes are attributed to decrease in spinal excitability and motor cortex at the CNS level.

1.6. Fatigue syndromes

For some people, the perception of effort can be far more than the physiological indicators of effort from skeletal muscle and cardio-respiratory indicators. To these people, exercise is perceived as unpleasant and is terminated before muscle and cardio-respiratory systems reach their typical maximal function during exercise.

It is not fully clear why some individuals experience heightened perceptions of effort and therefore early onset of fatigue when exercising. It may be linked to altered transmission of afferent signals from contracting muscles and/or altered processing of signals arriving at, or originating from, the brain.

Functioning of group III-IV afferents may also change following injuries, trauma and inflammation, where a sensitisation (hyper- or hypo-sensitisation) may occur. These

alterations are present in different patients' populations but also during ageing and chronic conditions. Evidence from the literature shows that afferent sensitisation could lead to altered response to exercise. Indeed, peripheral sensitisation of muscle afferents may play a role in chronic fatigue and pain. Chronic exposure to repetitive stimuli from increased feedback arising from muscle afferents (i.e., hyperalgesic priming) induces changes at the central nervous system resulting in a central sensitisation (CNS level). Central sensitisation amplifies signals coming from the periphery, even when a peripheral sensitisation is no longer present, acting as a sort of protective mechanism for the entire organism. However, while some sensitisation may be short lasting, such as injuries, more chronic sensitisation can be debilitating, decreasing the threshold of tolerance for different afferent stimuli, especially regarding fatigue and pain.

1.7. Human models of aberrant signalling from muscle to brain

Different models have been proposed so far for the study of aberrant muscle afferent signalling:

- *Ischemia, post-exercise cuff occlusion, ischemic exercise:* During ischemic contraction the metabolic environment perturbates quickly reaching very high levels of chemical disturbances (including lower pH) and metabolites accumulation. This model has been used to isolate the metaboreflex activation from other reflexes present during muscle contraction and in absence of central command involvement.

- *Hypertonic saline:* Injections of hypertonic saline similarly acts to alter the metabolic environment, decreasing the intramuscular pH and causing sensations of pain. This model activates mainly metabosensitive afferents.
- *Pre-induced fatigue models:* these models have been implemented to increase activation of group III-IV afferents and to assess the entity of their pre-activation on the function of a remote limb.
- *Intrathecal injection of fentanyl* is implemented to reduce the activity of muscle afferents, by approximately 50-60%. This invasive procedure requires a team of anaesthetists but has been recently considered the gold standard for studying the involvement of muscle afferents in different settings.
- *Muscle damage:* Muscle damage has been used as a model to study muscle afferent sensitisation and mechanical hyperalgesia following immune mediated inflammation, post muscle damage. The sensitisation of the muscle afferent may cause increased or decreased physiological functions. This model however it has not been implemented extensively so far and will serve as a basis for this doctoral project to study the physiological responses following mechano- and metaboreceptors sensitisation. Indeed, EIMD it has been shown to be an interesting model to study peripheral sensitisation due to its low invasiveness in humans and tolerance by participants that usually fully recover after one week from the exercising protocol, without extensive impact on participant daily living activities. Moreover, it does not require invasive procedures or drug administrations.

Chapter 2. Literature Review

2.1. Neuromuscular system structure and function

A primary function of skeletal muscles is force production to enable movements, but they also contribute key roles in thermoregulation, paracrine and endocrine functions as well as metabolism. Skeletal muscles are enclosed by connective tissue known as the epimysium, which in some muscles forms a dense fascia. Each muscle is composed of individual muscle fibres which are bundled together into fascicular arrangements; each one being surrounded by connective tissue known as the perimysium and each muscle fibre is enclosed by a thinner layer of connective tissue known as the endomysium. The connective tissue of muscles was once thought to be secondary to the muscle fibres, but more recently its functional importance was recognised including elastic components that are fundamental to maintain the shape and length of a muscle after deformation (contraction, stretch, pressure) (1, 2). Each muscle fibre is composed of several subunits called myofibrils which consist of a chain of sarcomeres arranged both in series and in parallel. A sarcomere is the smallest functional unit of a skeletal muscle (approximately 2.5 micrometres length) and it is composed of molecular filaments of actin and myosin, which are interconnected and slide against one another to produce muscle contraction and relaxation (3). The thin actin filaments (actin) are anchored to the Z band that forms the structural and fixed component of the sarcomere across the longitudinal axis of the muscle. The myosin (thick filaments) terminates with a head that anchors to the actin filaments 'producing a power stroke' to permit muscle contractions. They are usually located between the two actin filaments (4). Another important molecule in muscle contraction is titin, a long-coiled filament that acts as a spring to return the sarcomere

back to its original length after stretch, contributing to the stiffness of a muscle at rest and during lengthening contractions (5).

Skeletal muscle contraction is initiated by an action potential that arrives via the alpha-motor neurone at the neuromuscular junction. The action potential triggers release of the neurotransmitter acetylcholine (ACh) from the terminal branches of the motor nerve which diffuses across the inter-synaptic space to the muscle cell membrane binding to specific receptor molecules. The ACh binding opens positive ions channels allowing Na+ to enter the muscle fibre causing depolarization of the cell membrane (the endplate potential). The endplate potential is propagated along the muscle fibre membrane, known as the sarcolemma, in both directions (propagating away from the nerve innervation point) eventually reaching transverse tubules (t-tubules) in close contact with the sarcoplasmic reticulum (SR). The SR stores calcium (Ca^2+) and releases this Ca2+ into the fibre (sarcoplasm) when stimulated by the arrival of the action potential. Ca2+ floods into the sarcoplasm and binds with troponin causing tropomyosin to unmask the binding sites which allow myosin filaments to anchor their associated actin filaments which initiates contraction.

The rowing movements of the myosin permit the pull of the actin filament and therefore, the shortening of the sarcomere. However, this is only possible via hydrolysis of adenosine triphosphate (ATP) that is bound with myosin heads, via an enzyme (ATPase) that splits ATP into adenosine diphosphate (ADP) and a phosphate group (P) liberating energy. ATP is present in the cytoplasm, and in resting muscle some of this is bound to the myosin head, which in the absence of Ca^{2+} cannot bind with the actin molecule (1, 6).

However, with the arrival of an action potential triggering release of Ca2+ from the SR, the myosin-actin binding process is followed by the release of P, causing the myosin head to pivot which moves the actin towards the centre of the sarcomere. This cycle continues if enough ATP is available and the sarcoplasmic Ca^{2+} levels remain high. When the sarcoplasmic calcium concentration diminishes after reuptake into the SR, the movements of the myosin heads and therefore contraction of the muscle, stops. The function of calcium reuptake is performed by a molecular calcium pump which transports the Ca^{2+} back into the SR.

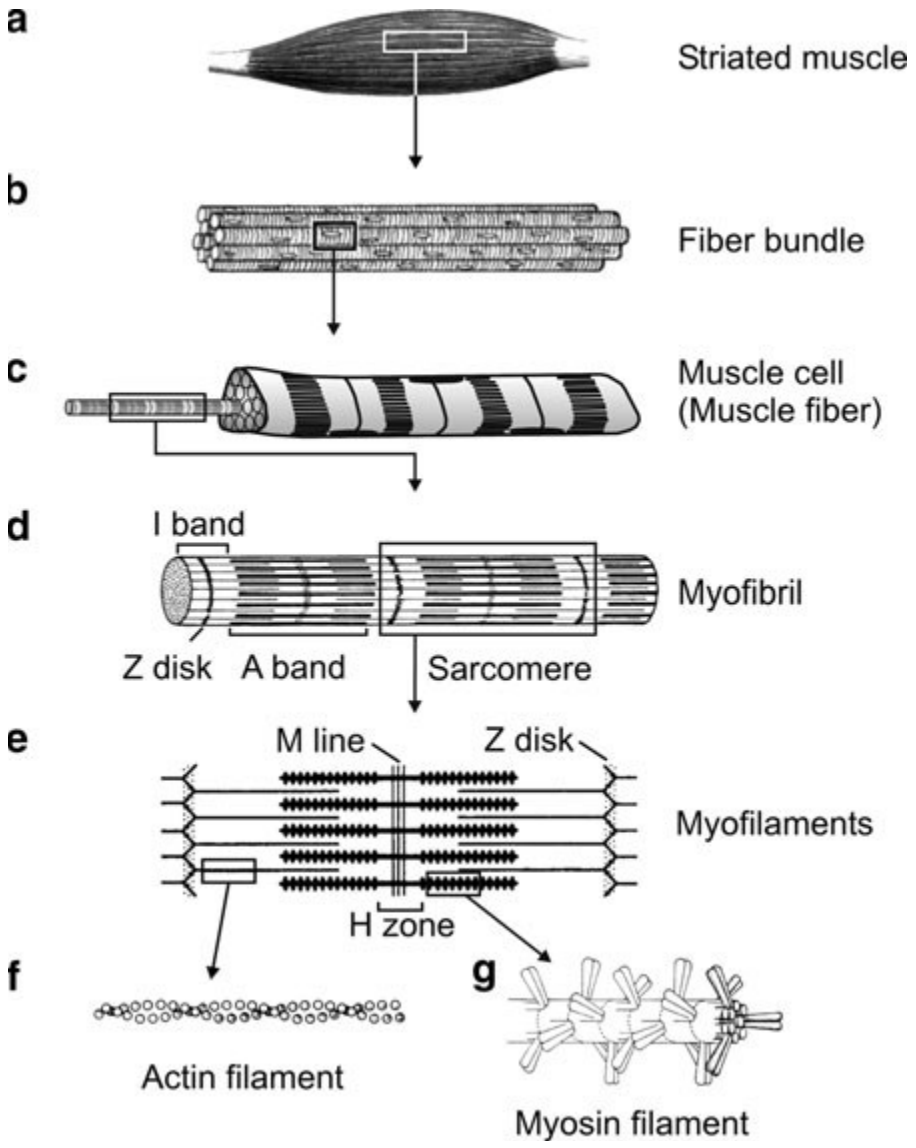


Figure 2.1 Skeletal muscle structure.

Reprinted from Mense et al (1)

Physiologically, a skeletal muscle can perform four types of contraction (1).

- *Concentric contraction.* This is characterized by shortening of the muscle length which generates muscle force.
- *Isotonic contraction*. This is defined as length change without change in the force exerted. Isotonic contractions are very rare in the normal environment, because almost

all movements are associated with a change in force. Pure isotonic contractions can be performed only on exercise machines which provide constant resistance through the range of movement. However due to the different muscle leverage even if the load is kept constant, the force exerted will vary.

- *Isometric contraction.* Isometric contractions are defined by an increase in force without length change. Isometric contractions are also rare during daily living activities. During Isometric contractions, the myosin heads pull at the actin filament developing force however the muscle is not shortening putting more tension on the insertion points and the elastic tissue components of the muscle.
- *Eccentric contraction*. Eccentric is defined as a lengthening of muscle by external forces, with the muscle resisting to the lengthening. Importantly in eccentric contractions, the force developed by the muscle is smaller than that causing the lengthening (otherwise the muscle would not be lengthened). However due to the additive involvement of titin and the elastic components, humans can exert more force in eccentric contractions compared with isometric or concentric contraction. Evidence suggest that this type of contraction in high volume may lead to the development of muscle damage and soreness (7).

Skeletal muscle is composed of two categories of types of muscle fibres: classically known form animal studies as “white” and “red” fibres (although not visibly different in colour in humans) with different characteristics and functions (8). The proportion of each fibre type varies between muscles and could also include fibres that combine both characteristics (hybrid). The white fibres are also named Type II fibres, and the red fibres named Type I

fibres due to the differing properties of the myosin heavy chain. Type II fibres are further divided into type IIx and IIa. IIa muscle fibres are intermediate fibres that exhibit characteristics of high aerobic capacity and high rate of contraction, while IIx fibres have lower aerobic capacity but very high rate of contraction (8).

2.2. Exercise Induced Muscle Damage (EIMD)

EIMD is particularly prevalent after unaccustomed eccentric contractions and is characterised by muscle weakness and soreness lasting several days (9). Following unaccustomed exercise, particularly that with eccentric contractions, there is evidence of possible injury to skeletal muscle fibers suggesting a disruption of the myofilament structures in some sarcomeres from biopsies samples (10) and possible loss of creatine kinase enzymes into the plasma, indicating damage to sarcolemma (11). These structural impairments are probably responsible for the temporary reductions in muscle force and delayed-onset soreness that can occur following eccentric exercise. The mechanisms underlying this injury are not known, although loss of intracellular Ca^{2+} homeostasis could play a primary role seen that administration of Ca^{2+} blockers can improved EIMD (12). Regardless of the cause(s), the initial and early events in the injury process are inflammation driven and immune-mediated related leading to macrophage action in remodelling and regeneration of the muscle function following several days after the initial exercise bout (13). Moreover, following an initial inflammation, muscle soreness occurs, and this has been associated with an increased sensitisation of mechano and nociceptive muscle afferents, causing mechanical hyperalgesia (14-19). Indeed, following

localised inflammation (20) there is also evidence of nerve microdamage (21) and increased abundance of metabolites that may in turn stimulate metabosensitive muscle afferents (16, 17, 19). Indeed, increased concentration of ATP, Lactate and H+ and deprotonated phosphate (Pi) have shown to increase metabosensensitive afferent activation producing an increased pressor response (22, 23) leading to sensation of pain and fatigue (24, 25), sensitisation of mechanoreceptors (26), mechanical hyperalgesia and reduced muscle strength (27). Therefore, the inflammatory status following EIMD has shown to sensitise muscle afferents (i.e., mechano- and nociceptive predominantly) (28-30). For all these reason previous research has implemented EIMD as a model for studying the effects of muscle injury and inflammation on peripheral sensitisation and muscle nerve afferent sensitivity (30).

2.3. Fatigue

There is a substantial literature on the influence of fatigue on human performance. The concept of fatigue however could be extended to a reduction in exercise-induced motor performance to a sensation of tiredness and weakness that accompany some clinical conditions (i.e., Chronic Fatigue Syndrome, Fibromyalgia, Multiple Sclerosis, Parkinson and others) (31). The first definition described fatigue as a progressive reduction of force or power exert by a muscle during exercise (32), however, this definition has been challenged to account for recent advances in the field (33). Indeed, different definitions were proposed in the last decades, distinguishing neuromuscular (i.e., peripheral, and central factors) from perceptive (i.e., homeostatic and psychological factors) components

of fatigue (34). Neuromuscular fatigue (i.e., performance fatigability) can be further divided into peripheral and central fatigue.

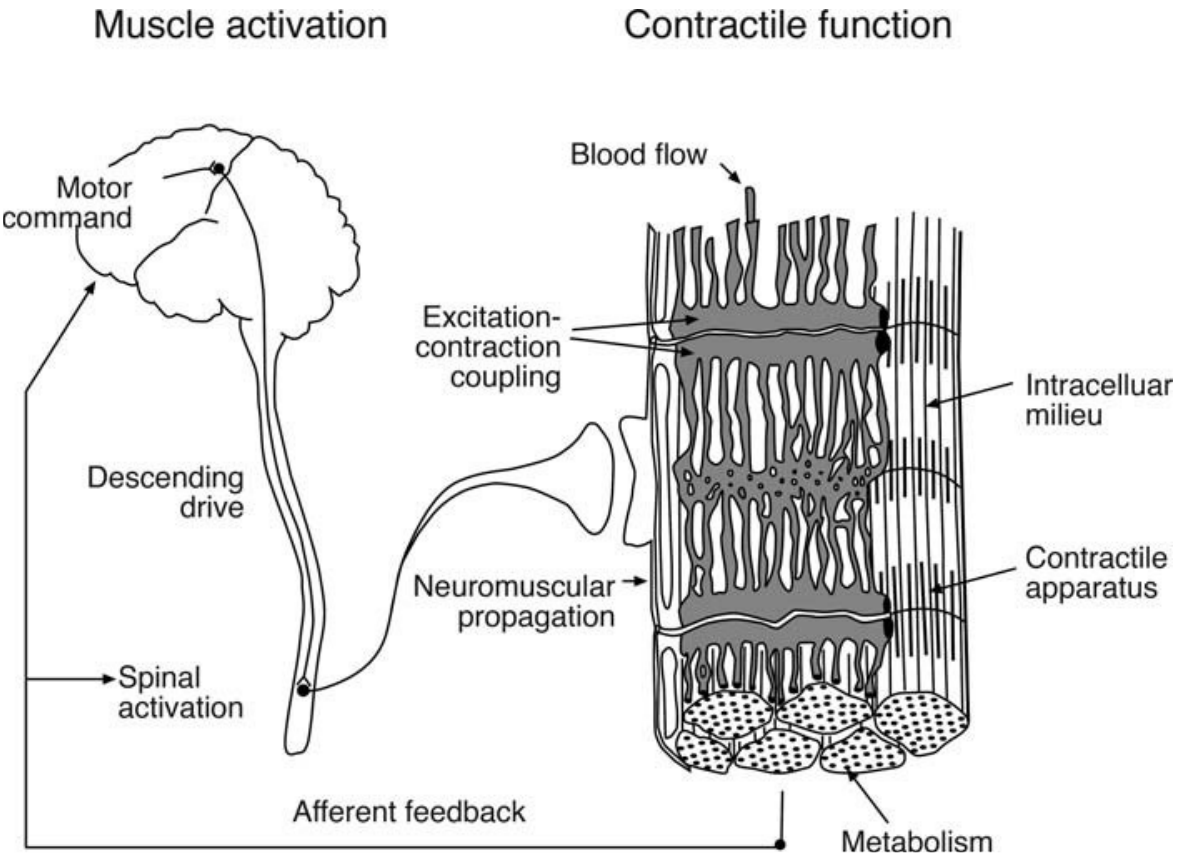


Figure 2.2 Central and peripheral component of fatigue.

Reprinted from Enoka (31)

Perceptive Fatigue

Homeostatic factors

Maintaining a stable environment in the human body is challenging, especially during stresses such as exercise, where physiological machinery of organs, tissues and cells can

be pushed to their limits. Homeostasis refers to the capacity of the human body to maintain this functioning despite these challenges. This is accomplished by balancing feedback and feedforward pathways, controlling energy expenditure, and protecting from overexertion. Metabolic stimuli arising from the muscle triggers the sensation of muscle fatigue from activation of muscle III-IV nerve afferents, especially group IV that are metabolically sensitive (25, 35). Increases in Pi, K+, lactate and ammonia with decreases in pH have been shown to trigger the activity of afferent sensations of fatigue and pain (25) and inducing increases in cardiovascular and cardiorespiratory responses (36). One current hypothesis suggests that fatigue can be seen as a complex of brain derived emotions and that central nervous system (CNS) structures (hypothalamus, insula, etc.) act as a "central governor" to limit energy utilization and avoid potential collapse (37).

Psychological factors

Psychological factors contributing to the perception of fatigue varies across different populations. For healthy participants, it may include expectations, familiarity, motivation, temporal and performance feedback, arousal, and mood (38). Different research has also advanced the role of perception of effort as a psychological factor contributing to exercise performance (37, 39) with a further link to the activity of III-IV muscle afferents (40). However, psychological factors are only occasionally acknowledged in studies on healthy individuals and this becomes even more impactful in clinical cohorts (31). It seems that

the level of fatigue in individuals with MS and Parkinson disease, is significantly related to the level of depression (33), as is also seen in end-stage kidney disease patients (41). Performance feedbacks are also important during an exercise task, and have been seen to greatly influence the tolerance of exercise in healthy individuals(42).

The Neuromuscular Fatigue:

Peripheral factors are related to a biochemical impairment occurring at or distally to the neuromuscular junction, where the contractile properties of the muscle may be impaired. This includes disruption of contraction-excitation uncoupling, alterations in cross-bridge function, changes in calcium conductance and loss of intracellular stores (43, 44). Moreover, decreased glycogen stores and pathological alteration of the peripheral nervous system may also play a role in mitochondrial dysfunction, acetylcholine depletion or motor conduction block (45-47), inducing an earlier onset of fatigue and exercise performance deterioration during activities of daily living.

Central factors have been extensively studied through transcranial magnetic stimulation and nerve stimulation via interpolated twitch techniques, revealing changes in motor cortex and spinal excitability following exercise-induce fatigue tasks (48). This suggests that these excitability factors play an important role on how fatigue develops, however this also appears to be task dependent (isometric single limb contraction vs whole-body exercise)(49). Central factors also play an important role in neurological disorders where structures of the central nervous system seems to be damaged or malfunctioning (33), for example the basal ganglia or frontal lobe structures (43). Moreover, studies on

multiple sclerosis have shown several correlates for self-reported fatigue and fatigability and cortical areas, especially in frontal lobes, thalamus or basal ganglia (27). Central fatigue has also been attributed to increase inhibition from activity of muscle III-IV afferents, that limit activation of the central motor drive, with reduction in motor unit recruitment (43, 50). Indeed, several studies have pointed out the role of muscle III-IV afferents as mediators between peripheral and central fatigue (51). Their role is to facilitate O_2 regulation through changes in haemodynamics and increasing ventilatory responses to exercise, slowing peripheral fatigue development during exercise. However, while delaying peripheral fatigue, they also restrict motoneuronal output and locomotor muscle activation, facilitating central fatigue (49). Exercise will stop when peripheral factors become unsustainable and reach the peripheral fatigue threshold (52). However. this model has been challenged recently showing that in several cases, exercise was stopped before attainment of a peripheral fatigue (53, 54). This may be due to other factors that may influence fatigue and its perception, such as teleo-anticipation and centrally acting performance modifiers (37). However, this last model has not been extensively investigated and presents some limitations. Another model proposed recently took into account all the sensory feedbacks arising from peripheral nerves in limiting exercise, called sensory tolerance limit (55). This model proposes that the sensory tolerance limit may be described as a global (i.e., not limited to a single muscle/muscle group) negative feedback loop leading to task failure when a finite level of stimulation is reached from sensory afferents originating in muscles that are directly (e.g., leg muscles during cycling) or indirectly (e.g., respiratory muscles during cycling) involved in the

exercise, and from corollary discharge associated with central motor command (Figure 2.3).

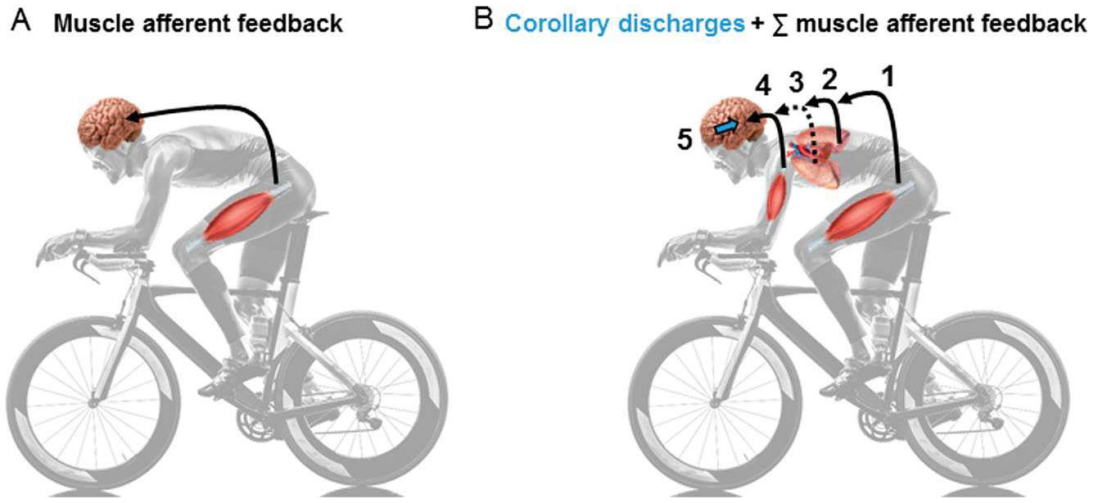


Figure 2.3 Peripheral fatigue and sensory tolerance limit.

Simplified schematic illustration of the 'critical threshold of peripheral fatigue' (A) and the 'sensory tolerance limit' (B). The critical threshold model proposes a large influence of muscle afferent feedback from locomotor muscles in regulating the degree of exercise-induced neuromuscular fatigue and exercise performance. The sensory tolerance limit is less specific and suggests that neural feedback from loco- motor muscles (1), respiratory muscles (2), possibly organs (3), remote muscles not directly involved in the exercise (4), and the corollary discharges associated with central command (5, blue arrow) are integrated within the brain and ultimately determine the magnitude of CMD. Reprinted from Hureau et al. (55).

2.4. Chronic Fatigue syndromes and Fibromyalgia

Fibromyalgia and Chronic Fatigue Syndrome (FMS, CFS) affect nearly 2% of the worldwide population, with higher prevalence in females than males (56, 57). CFS is mainly characterise by chronic fatigue symptoms lasting for period of at least 6 months and that can't be fully explained by an underlying medical condition (58). On the other hand, people living with Fibromyalgia also experience widespread musculoskeletal pain in the

absence of any structural or morphological abnormalities of skeletal muscle tissue (59-61). However, even if the main symptomatology is different, CFS and FMS conditions overlap substantially through the shared symptom of chronic fatigue. Chronic fatigue is described as an extreme tiredness ("overwhelming") at rest that debilitate daily living activities and is not improved by rest (62). Moreover, symptoms may exacerbate following intense or prolonged physical activity where a post-exertional malaise can take place (63-65) causing long-term kinesiophobia and sedentarism (66-68).

Unfortunately, the current FMS and CFS diagnosis lack of objective biomarkers, making this process very long and challenging for clinicians and patients (60, 69-71). Different authors suggested that a multitude of factors may be associated with FMS and CFS onset, such as psychological traits, post-traumatic stress disorders, post-viral infection, childhood experience and repetitive trauma (56, 72, 73).

Moreover, CFS and FMS pathophysiology is not fully understood (56, 74). Different hypothesis has been proposed during the years, suggesting a "sensitisation" of central and/or peripheral nervous systems to sensory stimuli (59, 75-78). Indeed, afferent signals originating in the periphery may be amplified ahead of processing in the central nervous system (79) leading to a hypersensitisation of typical somatosensory stimuli during everyday activities. However other theories suggested that the main drive may be a consequence of a central sensitisation that leads to 1) an amplification of the afferent signal arriving from the periphery (80, 81) or 2) decreased effectiveness of the descending-inhibition systems (60). However, despite previous research tried to address these problems, there is no clear explanation on pathophysiology of these syndromes,

and it remain still very challenging to address these components, and especially the origin and causes. For this reason, it has also been complicated to find an effective treatment in CFS and FMS syndromes, and still today no effective treatments are available for these patients (82, 83). However physical activity has been shown to be a promising too for improving pain, fatigue, quality of life and well-being (84-87). Unfortunately, it has not been so far postulated which training may be more beneficial, within the correct administration (I.e., intensity, frequency, time, type) especially due to the lack of knowledge of the correct exercise-dose responses for each patient and difficulties in training individualisation (88-93). Indeed, patients may experience extreme discomfort during exercise, with exacerbated perceptions of effort even at moderate exercise intensities (94, 95). Previous research found that this phenomenon could be ascribed to an aberrant somatosensory signalling that increase perceptions of effort at any given workload and make exercise unbearable (36, 96). From our recent work, we found a reduced cardiorespiratory and neuromuscular function with an increased perception of effort in FMS and CFS compared with healthy age matched controls (97), even when studies were controlled for study quality and participants were matched for the same level of physical activity. This may advance the hypothesis that the increased perception of effort may be a consequence of the increased afferent feedback arising from the working muscles, and not as an exclusively consequence of patient's deconditioning (59, 98). However, due to conflicting reports, heterogeneity of methodological approaches or few studies including relevant outcome data in the literature, this hypothesis remain un-answered.

2.5. Group III-IV muscle afferents and their contribution during exercise.

Anatomy of group III-IV muscle nerve afferents

The anatomy of muscle nerve afferents has been documented since the early twentieth century, although this knowledge is predominantly based on studies conducted using animals (usually cats, dogs and rodents). Indeed, very little is known about their anatomy in human skeletal muscle, particularly for the muscle III-IV afferents. Following the classification of Lloyds et al (99), skeletal muscle sensory nerves are divided into five types (Ia, Ib, II, III, IV) that respond specifically to different types of stimuli (i.e. chemical or mechanical). This classification is based on the diameter and degree of myelination that in turn determine the level of axonal conduction velocity, where the thicker the fibre and the myelin surrounding it, the faster the transmission of the conduct potentials (100). Therefore, group Ia and Ib which are thickly myelinated (12–20 μm) conduct the fastest impulses (72-120 m/s) and are primary composed by muscle spindle and Golgi tendon organs respectively. Group Ia fibres are positioned in "parallel" with the skeletal muscle whereas group Ib are positioned in "series" responding to muscle stretch and force tension respectively (1, 101). Group II afferents are also myelinated (6–12 μm), in cats they are usually referred to as "secondary spindles", situated in parallel with the muscle fibres, and usually stimulated by muscle stretch at conducting impulse speeds of 31-71 m/s. The main difference between group I and II muscle nerve afferents is that the first

are capable of signalling the rate of stretch, therefore they usually named as “dynamically sensitive” (102).

Group III afferents are also thinly myelinated and conduct impulses between 2.5 and 30 m/s. These fibres are also known as Aδ fibres, and their receptors are composed of free nerve endings. Group IV afferents (i.e., C-Fibres) on the other hand are unmyelinated and conduct impulses at slower rates (2.5 m/s) and similar to group III afferents they terminate with free nerve endings. Muscle III afferents are usually located in vessels (venules and lymph) or in the connective tissue (peritenonium and endoneurium), whereas group IV afferents are more consistently found in the interstitial spaces close to the blood and lymphatic vessels of tendons and muscles (103-105) .

Group III-IV afferents with endings on the skeletal muscle project to the spinal cord by the dorsal root, with synapses in the dorsal horn of the grey matter (102). First studies from Light and Perl (106) characterized the projection of III-IV afferents to the spinal cord. This group found connections to the laminae I and II, and these findings were confirmed from the following studies of Craig and Mense (107). From the dorsal horn connections lead to the central nervous system, interfacing with other pathways essential for the regulation of blood pressure, for example the ventrolateral medulla, hypothalamus, mid-pons, insula cingulate cortex and amygdala (102, 108, 109).

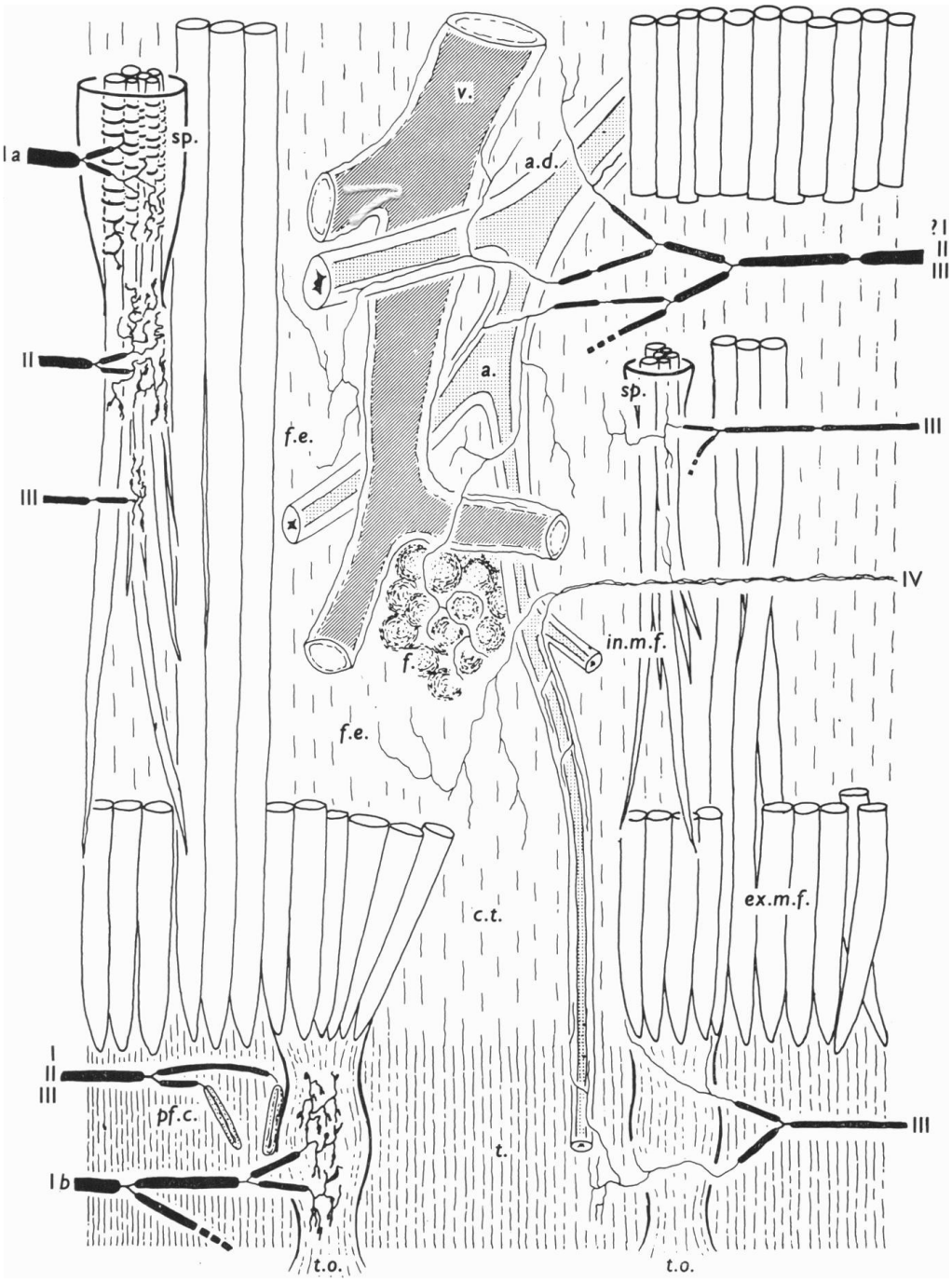


Figure 2.4 Sensory innervation scheme of mammalian skeletal muscle.

Abbreviations: (s.p.) = muscle spindle; (t.o.) = tendon organ; (pf.c.) = paciniform corpuscles; (f.e.) = free nerve endings; (in.m.f) intrafusal muscle fibres; (ex.m.f) = extrafusal muscle fibres; (t.) = tendon tissue at musculo-tendinous junctions; (a.d.) = the adventitia of arterioles (a.) and venules (v.); (f.) = fat cells; connective tissue (c.t.). As reported from Stacey et al (105).

Membrane Receptors for group III-IV Afferents

Group III-IV muscle afferents terminate in free nerve endings and possess different receptors that mediate different physiological functions, such as mechano- and metaboreflex in fatigue and especially nociception (1). As shown in figure 2.5, there are a large number of receptors that have been detected in the membrane of nociceptors and in a variety of tissues, even in muscle, that are relevant for pain and tenderness (110).

The main receptors and function are listed below:

1) *Bradykinin (BKN) receptors (B1 and B2):* In intact tissue, BKN excites nerve endings, following inflammation and low-threshold mechanosensitive endings.

2) Serotonin receptors (particularly 5-HT3): Serotonin is released from blood platelets during blood clotting, this is not believed to be sufficient to excite nociceptors directly but in turn they can be sensitized to the effects of BKN stimulation.

3) Prostaglandins, particularly prostaglandin E2 (PGE2): Prostaglandins (PGs) are usually released in the damaged/altered muscle by cyclooxygenase, sensitizing the membrane of the nociceptive endings.

4) Acid-sensing ion channels (ASICs): ASICs receptors involved different subunits that mainly respond to a pH reduction (proton sensitive), where the family ASIC1 and ASIC3 are the most important in transmitting muscle pain sensations with many pathological states in the muscle being associated with a reduction in pH. This is further evidenced by the chronic muscle pain associated with repetitive injections of acid solution that induce a long-lasting hyperalgesia in previous studies (111).

5) Purinergic receptors P2X3: This receptor's subtype responds to ATP and its derivatives. ATP is the most common noxious substances that is usually released from the tissues during trauma, damage and cell death (1).

6) Transient receptor potential receptor subtype 1 (TRPV1): this receptor is one of the most important for the induction of pain. These receptors are usually stimulated by capsaicin; however, these receptors are also sensitive to proton accumulation (reduced pH) and increases in temperature.

7) Tyrosine kinase A (TrkA) receptor: TrkA is a ligand of the nerve grown factor receptors (NGF) and is well known for its role in sensitizing nociceptors in the periphery and the central nervous system.

8) Glutamate receptors: Glutamate can induce mechanical sensitisation in the muscle and its attenuation with NMDA receptors agonist or ketamine can reduce pain pressure threshold (112).

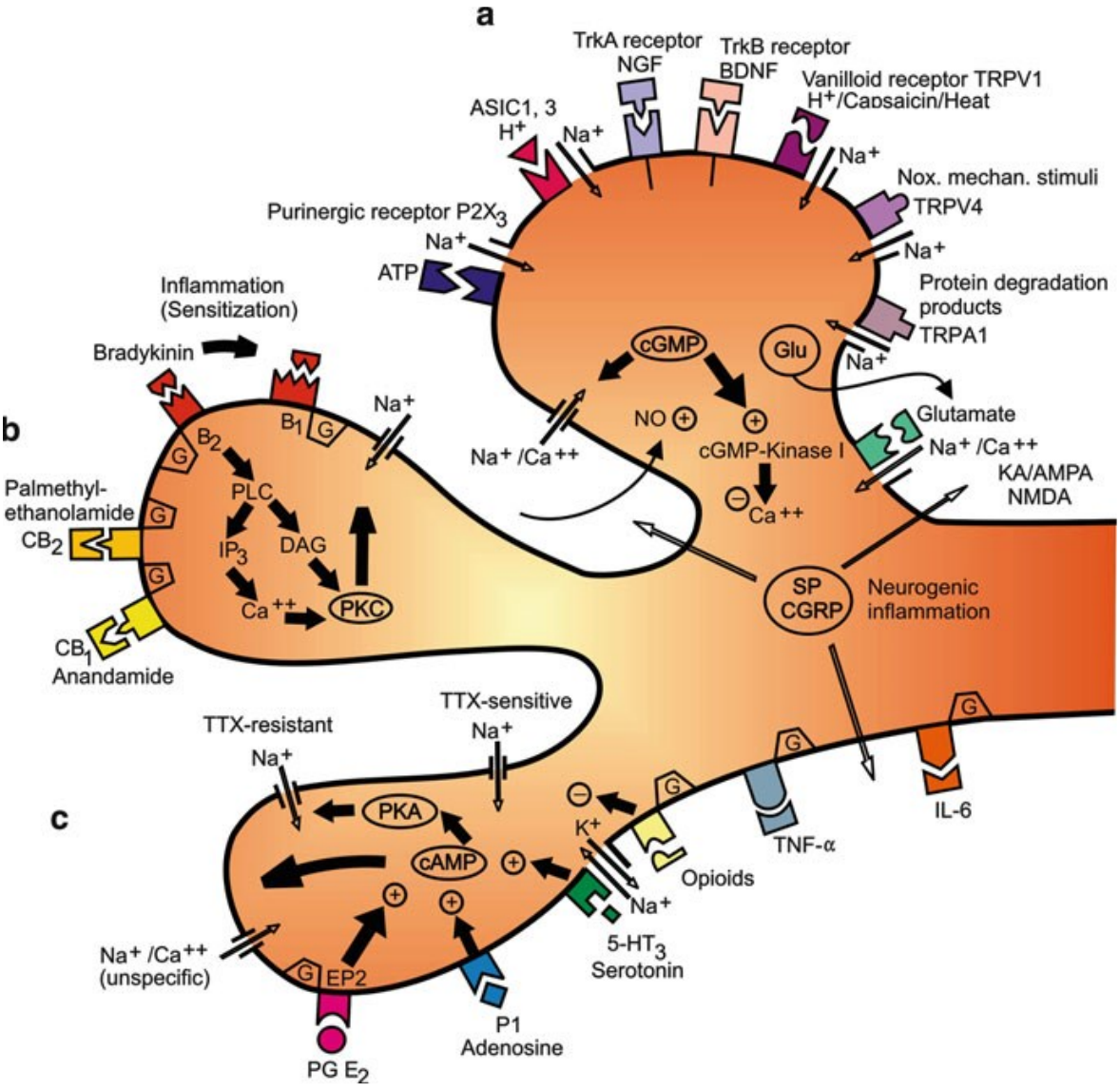


Figure 2.5 Membrane receptors of a nociceptive nerve ending.

Of particular importance for muscle. pain are the receptor molecules and processes shown in branches a, b and c. (a) There are two main receptors sensitive to H+ ions: acid-sensing ion channels (ASIC 1, 3) and the transient receptor potential subtype V1 (TRPV1). The purinergic receptor P2X3 binds ATP, a molecule that is present in each cell of the body but has a particularly high concentration in muscle cells. ATP could serve as a general pain signal because it is released by any cell damage. All these receptor molecules are ion channels which span the axonal membrane and are permeable to Na+ and other cations (Ca2+ and/or K+). (b) Shows the change of the bradykinin receptor B2 to B1. In intact tissue, bradykinin (BKN) excites or sensitizes the ending by acting on the B2 receptor; in inflamed tissue, it binds to the newly synthesized B1 receptor. BKN exerts its action not by opening an ion channel but by activating a G protein that regulates intracellular metabolic changes. These changes lead to an increased excitability of the ending

(sensitization). The change from B2 to B1 shows that even in the very periphery there are neuroplastic changes, i.e., functional, or morphological changes of neurons brought about by pathological tissue alterations. (c) In addition to BKN there are other sensitizing substances such as prostaglandin E2 (PGE2) and serotonin (5-HT) which likewise bind to specific membrane receptors. The receptors for 5-HT3 and PGE2 induce intracellular cascades of events that increase the sensitivity of the Na+ channels by activating protein kinase A (PKA). The larger ion currents that flow through the channel proteins of a sensitized ending render the ending more sensitive to external stimuli. Branch c also shows that tetrodotoxin(TTX)- resistant Na+ channels are present in the membrane of nociceptive endings. cAMP, cyclic adenosine monophosphate, a second messenger. Figures and description reported from Mense et al (1).

Physiology of Muscle Afferents

Muscle afferents have been mainly studied in animals implementing different stimuli (electrical direct nerve stimulation, injection of chemicals, hindlimbs muscle contraction, passive mechanical stretch of the muscle) (1, 102) and in humans (voluntary muscle contractions/ exercise, injection of chemicals, muscle electrical stimulation, post exercise occlusion and ischemia, stretching and passive leg movement) via study of the mechano- and metaboreflex and the exercise pressor reflex (113). These animal studies have yielded important information about the neuronal and autonomic contribution of muscle nerve afferents. Specifically, animal studies have repeatedly shown that only group III-IV afferents contribute to the cardiovascular and ventilatory functions, whereas stimulation of thickly myelinated (i.e., group I and II) muscle afferents exert minor or no contribution to fatigue pathways. This point is important to understand the following studies implemented in humans, and to distinguish the studies that activated predominantly muscle III-IV afferents rather than other muscle afferents.

In recent decades, studies of muscle afferents have become more plentiful in humans, confirming the role of group III-IV afferent in regulating cardiovascular, ventilatory and autonomic functions (36, 114, 115). This also translates into mechanisms of fatigue and pain in some clinical conditions, from Hypertension, Heart failure, COPD, two-type diabetes mellitus T2DM and chronic fatigue syndrome (59, 116-124), as well as athletes and endurance performance (49, 55, 125). Indeed, the importance of the muscle afferent has also been crucial to understand the onset and role of fatigue and pain in participants' exercise tolerance (51, 126, 127). This has been possible by some previous studies analysing the role of group III-IV afferents of perception of fatigue and pain at different levels of stimulation in mice and then in humans (25, 35, 128, 129). Thanks to all these studies, it has been possible to provide the basis of the complex role of afferent feedback into the exercise pressor reflex, fatigue, pain, and exercise tolerance during exercise, as well as dysregulation in different disease and clinical conditions. However, it remains to be explored the contributions of each single reflex (mechano vs metabo vs nociceptive) and their interaction in different physiological function that may lead to altered responses and pathophysiological complications. Especially in regard to possible interventions aiming to counterbalance re restore these deleterious effects.

Role of muscle afferents on cardiovascular and respiratory function

Group III-IV muscle afferents have been implicated in the increases in cardiovascular responses and autonomic response to different stimuli (exercise, passive muscle

contraction, electrical stimulation and chemicals injections) (102, 130). From the first animal studies it has been well documented that group III-IV afferents activation via direct *electrical stimulation* increased arterial pressure, heart rate, cardiac contractility, ventilation, and sympathetic discharge (131-136). However, there is still controversy on the role of group I and II on these functions reporting modest increases or no changes (102) in animals. In humans, involuntary contraction of skeletal muscle induced by percutaneous electrical stimulation has been used to evoke autonomic reflexes. The importance of this method is to bypass the central command; however, it remains difficult to discern mechano- and metaboreceptors involvement during the experiment (102, 113, 130). In addition, electrical stimulation may activate different proportions of muscle fibres, usually type II rather than type I (137), which are usually activated during exercise first (138). Recruitment is also synchronous in electrical stimulation and asynchronous during exercise (137), making it difficult to translate and compare electrical stimulation studies to exercise models (102). However, despite this limitation, electrical stimulation to involuntarily contract skeletal muscles has repeatedly shown in humans to increase mean arterial blood pressure, sympathetic drive, heart rate, cardiac output and ventilation (113, 139-143).

Injection of chemicals (i.e., algesic, metabolic by-product of muscular contraction) into the arterial hindlimb skeletal muscle give similar results to direct electrical stimulation in animals (102). The main noxious agents used in these studies have been potassium (144-146), capsaicin (147, 148), bradykinin (149, 150), lactic acid (151, 152), citrate and deprotonated sodium phosphate (22, 153). However, the use of noxious agent injection

in the skeletal muscle presents some disadvantages. While the main advantage is the ability to measure the concentration of the metabolite injected a priori, it is still impossible to measure the concentration within the interstitial fluids, making it difficult to match for similar concentrations during the contraction of muscle during exercise stress (102). Further studies in mice have also shown that there are multiple functionally distinct populations of group III and IV afferents that can be characterized by their chemosensitivity and neurochemical identity differentiating from metaboreceptors and metabo-nociceptors even if they project in a similar location of the spinal cord (35). This suggests that different subunit populations possibly have different functions (nociceptors vs metaboreceptors vs chemoreceptors vs heat receptors) that respond to different mixtures of metabolites and pH alterations (128). In humans, repetitive injections of a mixture of metabolites showed similar results, whereas at decreased pH levels, there was increased stimulation of III-IV afferents subunits that evoked sensations of fatigue and pain (25).

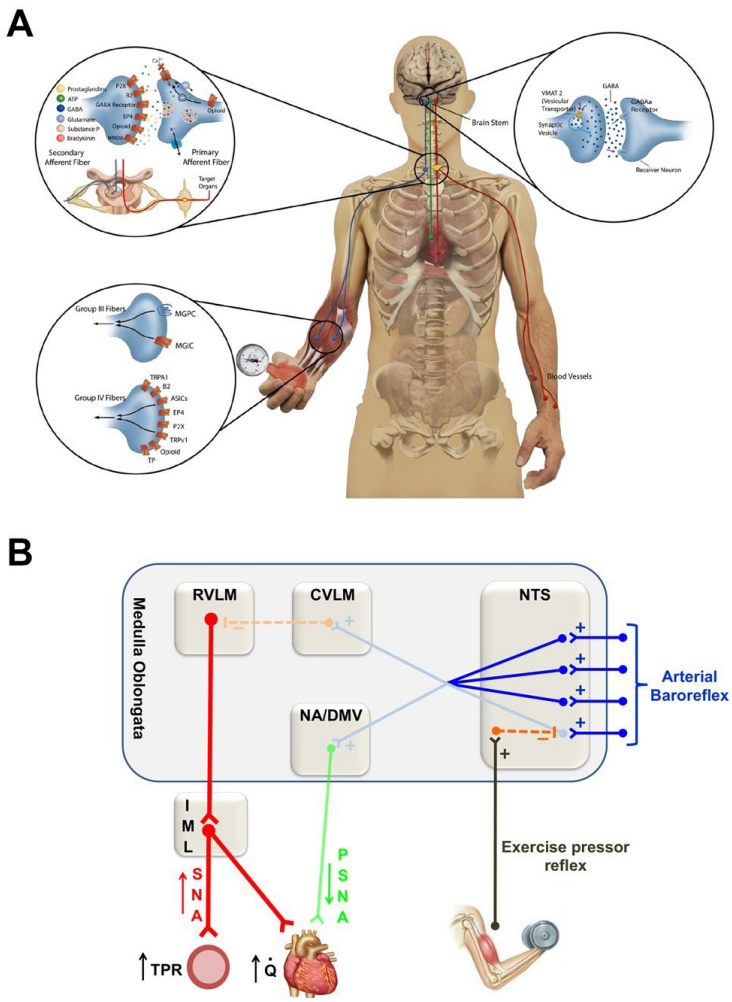


Figure 2.6 Schematic illustration during the activation of the exercise pressor reflex.

Emerging evidence suggests that activation of the exercise pressor reflex inhibits, via GABAergic interneurons, the baro-sensitive neurons in the nucleus of tractus solitarius (NTS), decreasing the activation of second-order neurons. The final consequence of the exercise pressor reflex activation is a decrease in parasympathetic nerve activity (PSNA) and an increase in sympathetic nerve activity (SNA) causing an increase in heart rate, cardiac output (Q̇), total peripheral resistance (TPR), and blood pressure during exercise. Figures and description reported from Teixeira et al (143)

Contraction of hindlimb skeletal muscle has been one of the most commonly used methods to investigate the reflexes associated with the sensory muscle nerve stimulation. Static contraction has been shown to increase arterial pressure, heart rate and minute

ventilation in animals (154-157), but the exercise pressor reflex has also shown to activate other important reflexes as increases in cardiac output (156), increases in cardiac contractility, activity of muscles responsible to dilate the upper ways (157), reducing the blood flow to the kidney with no effect on the brain, heart, liver and spleen (158). Importantly, static evoked contractions increase the α-adrenergic response, causing vasoconstriction to the coronary arteries, usually masked by a vasodilation induced by peripheral metabolite increases (159). This mechanism is called "functional sympatholisis" and may constitute a protective mechanism that optimizes muscle blood flow to the exercising muscles in spite of generalised vasoconstriction (113). Other studies have applied lidocaine to the dorsal roots, showing that this manoeuvre was able to block the impulses from group III and IV afferents, but not in group I and II, preventing the increases in heart rate and blood pressure normally seen during contraction (160). Another consideration is the differences in effects of muscle fibre type I (oxidative) and type II (mixed-anaerobic) on the exercise pressor reflex. For example, preliminary studies found that soleus muscle (higher concentration of type I muscle fibres) compared with gastrocnemius muscle (higher concentration of type II muscle fibres), exerts a lower pressor response, however differences in muscle mass may have been an important confounding factor in this study (161). Another important consideration is the role of the exercise pressor reflex which functions to supply blood to the exercising muscles, due to the increased demand from exercise. Indeed, during *ischemic contractions* where the supply of blood is reduced, the exercise pressor response is changed compared with non-ischemic contractions (160, 162, 163). In humans, important advances- in our knowledge

on the role of metabolite accumulation in activating cardiovascular and respiratory system was firstly proposed by Zuntz and Geppart in 1886. before being experimentally isolated and confirmed by Alam and Smirk (164). In this study, they demonstrated that blood pressure remained elevated after trapping metabolites with circulatory cuff occlusion immediately following static contraction, suggesting a role of metabo-sensitive sensory nerve endings. This experimental protocol and its findings have been replicated numerous times since then, to the point now where it represents the gold-standard in the study of the muscle chemoreflex (143). Muscle chemoreflexes have been shown to be directly proportional to the mass of the muscle being exercised (165) and the quantity of intramuscular inorganic phosphate accumulated, or the intensity of the exercise performed (22, 164, 166). This approach opens the possibility of studying metaboreflex activation without central command or mechanoreflex activation, however there are limitations which should be acknowledged when extrapolating these results to ischemic exercise(102). Muscle metaboreflex has also been studied by applying different levels of limb negative pressure, aiming to describe a possible threshold on the chemoreflex activation (167). All these studies show that the threshold for activation of chemoreflex is lower when involving large muscle mass.

Another important point is that chemoreflex and exercise pressor reflex (EPR) activation is closely related with metabolism. Studies have previously shown this relationship using ^{31}P nuclear magnetic resonance (NMR) spectroscopy, demonstrating that the hydrogen ions produced from muscle metabolism were correlated with mean arterial pressure, vascular resistance (exercising muscle) and sympathetic nerve activity (non-exercising

muscles) (168). Moreover, the importance of the correlation of dipronated phosphate with sympathetic nerve activation (22) has also been established, showing the level of metabolite accumulation and chemoreflex activation are related to one another.

Another way to study muscle afferent feedback is in the form of *mechanical "passive" stimulation of hindlimb muscle* with mainly two methods: 1) application of pressure to the muscle; 2) tendon stretch. The purpose of these methods is to isolate the response of mechanically sensitive afferents from the metabolically active whilst at rest (102). Application of pressure to the muscle in animals has shown to increase blood pressure and ventilation, with no effect on heart rate (151, 169, 170). Similar responses were found from tendon stretch, that increases ventilation, mean arterial pressure, heart rate and cardiac contractility (133), further suggesting that this was attributed to stretch tension mainly from III muscle afferents (151). Mechanical stimulation of the sensory nerve endings can also induce the activation of the exercise pressor reflex in humans. First, studies hypothesing the mechanoreflex involvement were performed during electrical muscle stimulation, where an increase heart rate was found after only 500 milliseconds from the start of the stimulation (141). The mechanisms of this suggest that this is attributed to a reduction in vagal tone, however muscle-heart reflex seems to occur even in participants paralyzed with curare-like drugs, suggesting that this mechanism is possibly driven by central command (171, 172). Passive leg movement of the legs is also another important method to assess the involvement of mechanoreflex in the autonomic nervous control (113, 173). One of the first studies implemented passive cycling of the legs by strapping subjects' feet to the pedals of a stationary tandem bicycle that was

cycled by a staff member, finding increases in heart rate, cardiac output, stroke volume, blood pressure and blood flow to the muscle (174, 175). Another method to passively stretch mechanoreceptors was through compression of both legs through compression garments (176, 177), showing increases in mean arterial blood pressure and heart rate, even when combined with concomitant handgrip exercises (178). However passive leg cycling, or limb compression have some limitations in that it is difficult to differentiate the origin of the mechanoreceptors stimulated (joint, skin, muscle)(102). Limb congestion, induced by ischemia has also been used to study mechanoreflex (179), suggesting that this increase in pressure may in turn sensitise mechanoreceptors, rather than stimulate metaboreceptors, as observed by 31-pNMR techniques, revealing no changes on the hydrogen ion or dipronated phosphate ion concentration in the muscle. Few studies have found an increase in ventilation in healthy humans (180-182).

Role of muscle afferents on neuromuscular components of fatigue

The role of muscle afferents on the neuromuscular components of fatigue has been studied applying different models to isolate the activation of the different fibres (mechano, metabo and nociceptors) individually or in combination. There are different models used so far for studying these mechanisms: 1) ischemia, post-exercise cuff occlusion, ischemic exercise; 2) hypertonic saline; 3) pre induced fatigue models 4) intrathecal injection of fentanyl. All these different studies were able to manipulate the activation of III-IV afferents and give important insights on the functioning of, and

relationship between, peripheral and central components of fatigue in different contexts, from healthy exercising humans through to pathological conditions and athlete populations.

Ischemia alone, Post Exercise Occlusion Models (PECO), or Ischemic Exercise (IE) present significant differences between each modality. The first is that ischemia alone may activate only pain related mechano- and nociceptors rather than pure metaboreceptors, given that the concentration of metabolites is not significantly elevated during ischemia alone (179). On the other hand, PECO has been used in the past decade to isolate the involvement of metaboreceptors (164). However, concerns were raised from this procedure, with recent studies (25, 35) suggesting that variations in the level of metabolites concentrations achieved may cause metaboreceptors to be activated alongside nociceptors that increase pain sensations. Ischemic Exercise (IE) instead exacerbates the activation of III-IV afferents, observing that during exercise there is the involvement of both mechano- and metaboreceptors with a concomitant block of metabolites wash-out, which causes a rapid increase in metabolites and rapid activation of metabo- and mechano-nociceptors (183).

The first studies to examine III-IV muscle afferents mainly used models of Ischemic exercise to understand the involvement of III-IV afferents on central and peripheral components of fatigue. Indeed, the pain experience during ischemia alone, PECO, and IE are different, where the first is lower and increases proportionally to the level of metabolites accumulated. First studies on the inhibitory effect of muscle III-IV afferents were conducted assessing voluntary activation (VA) through peripheral and transcranial

stimulation, showing that continuous discharge of muscle III-IV afferents following PECO maintain the reduced VA and MVC at the end of exercise, which is then recovered once the cuff is released (184). This demonstrates the pivotal role of III-IV afferent feedback in the absence of central command in the onset of central fatigue. Further studies were implemented following this protocol suggesting that activation of muscle nociceptors (through PECO) reduced the voluntary activation in different circumstances: 1) muscle antagonists actions reduced VA in muscle agonists (185); 2) distal muscle activation reduced the VA of proximal muscle (186) 3) muscle nociceptors reduced ipsilateral VA but not on contralateral limb (187); 4) remote muscles impair VA on remote exercising muscles (188). However recent evidence suggests that increasing experimental pain induced by blood flow restriction may also impair contralateral exercise performance increasing corticospinal excitability (127, 189). Ischemic exercise has been extensively studied through the exercise methodology known as “blood flow restriction” training. Indeed, this method also led to positive neuromuscular and physiological adaptations (190). However, it also raised concerns regarding the exaggerated exercise pressor reflex achieved, and potential for vascular damage (183) and accelerated neuromuscular fatigue (191). Nevertheless, this model of III-IV activation was one of the first implemented to study the neuromuscular components of fatigue (167) and provides important translations to conditions where there is an exaggerated EPR and muscle pain due to ischemia, such as Peripheral Artery Disease; hypertension and chronic fatigue syndrome) (190, 192).

Hypertonic Injection of Saline

Hypertonic injection of saline has been used as a model to study III-IV afferents activation, especially metaboreceptors and metabo-nociceptors. Studies utilising this model observed that hypertonic injection of saline is responsible for acidification of the muscle with increases in H+ and fall in pH (25, 193, 194). The intramuscular injection of hypertonic saline is well established and safe; under resting conditions it induces acute pain which is predominantly associated with group III-IV afferents activation with the main contribution coming from the IV group (195). However, saline is usually utilised to investigate pain-induced changes and not the neuromuscular components of fatigue (126). Preliminary studies showed that injections decrease the maximal voluntary contraction (196) and impair short term exercise performance (197). Recently, studies have emerged showing that hypertonic saline increases the intensity of pain during exercise, which results in a faster onset of exercise-induced fatigue (126). Two studies from the same research group found that the reduction in exercise performance during injection of hypertonic saline is related to an increase in central activation in the ipsilateral and contralateral limb (198, 199).

Pre induced fatigue models

Pre induced fatigue models use electrically induced or voluntary contractions to induce muscle fatigue before commencing an exercise bout of other muscle groups which were not fatigued. Emerging evidence implementing voluntary contraction to induce a priori fatigue, suggest that inhibitory feedback originating from a remote muscle group could

alter exercise performance and neuromuscular responses of the muscles being assessed, where it has been suggested that the afferent feedback limits the development of peripheral fatigue and compromises endurance exercise performance by inhibiting central motor drive (200). Similar to this study, a decrease in exercise performance was also found in the lower limb muscle after fatiguing arm cranking exercises (201-203), although none of these studies were able to establish mechanisms of the excitability and inhibition of corticospinal pathway. Recent studies showed no differences in corticospinal excitability and voluntary activation declines after pre-induced or concurrent rising pain. This is regardless of the origin/ mechanism modulating sensory afferent feedback suggesting that the limit of exercise is set by attainment of a sensory tolerance limit (127). Inducing fatigue through voluntary exercise is a potential source of bias when assessing limitation of central motor drive (CMD), since the voluntary activity per se requires the involvement of central command. Indeed, during a prior voluntary muscle contraction a "copy" of the neural signal (i.e., corollary discharge) is sent from the premotor areas to the sensory areas of the brain facilitating effort perception and potentially impairing exercise performance (96). On the other hand, using electrical stimulation to induce fatigue may bypass this system and in turn reduce this possible bias. A recent study from Laginestra et al (96), found that the crossover effect of central fatigue is mainly mediated by group III/IV muscle afferent feedback and suggests that impairments associated with central motor drive may only play a minor role in this phenomenon.

Lumbar intrathecal injection of fentanyl

When fentanyl (a μ-opioid receptor agonist) is applied intrathecally via injection into the spinal canal at the lumbar level, it attenuates the central projection of group III/IV afferents innervating locomotor muscle (i.e., legs) by approximately 55-60% (204) and without reducing the muscle force-generating capacity (central motor drive activation)(205). This technique has been able to untangle the role of III-IV muscle afferents in the development of contractile locomotor muscle fatigue (49). A number of studies since have observed that fatigue related group III-IV afferents facilitate intracortical inhibition, alter motor cortical and corticospinal excitability, thus increasing central fatigue and limiting exercise performance (206-208).

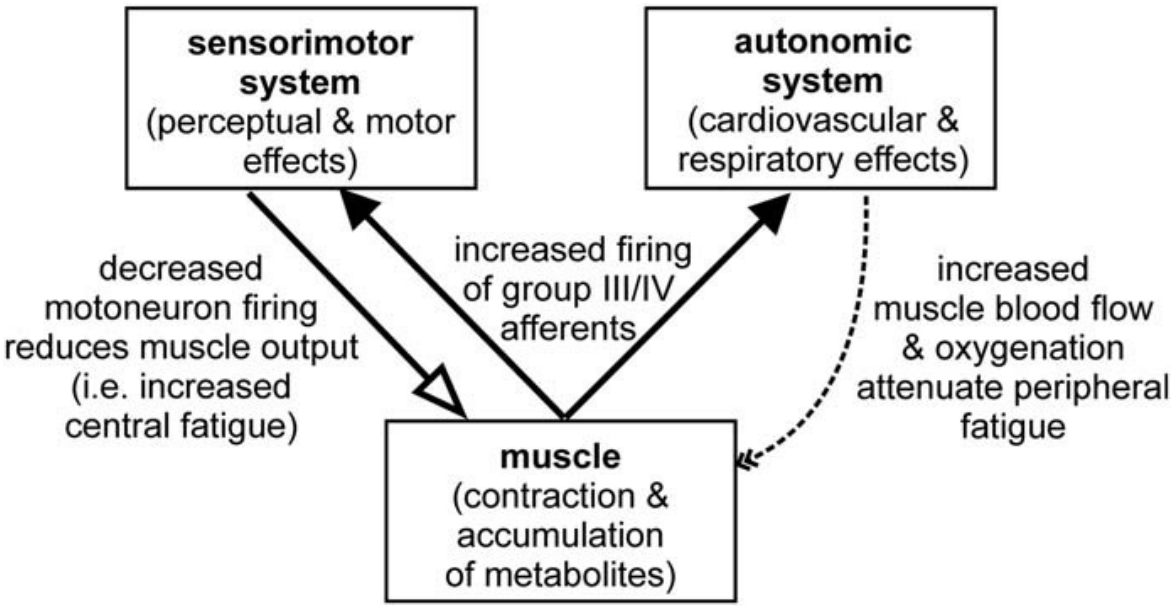


Figure 2.7 Group III/IV muscle afferents increase central fatigue but attenuate peripheral fatigue.

Firing of group III/IV muscle afferents increases during fatiguing contractions. During exercise, these afferents produce reflex increases in heart rate, blood pressure, and respiration to improve muscle blood flow and oxygenation. This slows the development of fatigue of the muscle itself (peripheral fatigue). At the same time, the afferent firing also leads to a reduction in voluntary neural drive to the muscle. That is, it

contributes to central fatigue. The precise pathway for this effect is not known. The afferents evoke sensations of muscle discomfort and fatigue, increase supraspinal fatigue, presynaptic ally inhibit Ia input to motoneurons, and have differing actions on different motoneuron pools. Figure and description reported from Taylor et al (184).

Role of muscle afferents on perception of fatigue

Rate of perceived exertion (RPE) is the most widely utilised scale for quantification of perceived exertion. Borg (209) initially developed the 15-point scale that ranged from 6 to 20 in order to have a linear and strong relationship between exercise intensities and heart rate. The Borg scale has been shown to be reliable and accurate to measure self-perceived exertion in different investigations and populations (210, 211). However, it presents some difficulties, due to this scale being a poor reflection of the more exponential growth of perceptual response as intensity increases (212). Moreover, it does not differentiate from which exponential growth of non-linear physiological responses it may be composed. For this reason, Borg developed a ratio scale to use in combination with the standard one, assessing accumulation of lactate and pulmonary ventilation (39). Despite this, it is still not clear how the brain interprets afferent feedback to assess the perception of exertion during exercise (39). The perception of effort could be generally classified in two components: 1) cardiopulmonary; and 2) peripheral factors. The first includes respiratory rate, minute ventilation, heart rate, and oxygen uptake while the second includes blood lactate level, mechanical strain, skin and core temperature (39). It has been suggested that the integration of these sensory cues may influence the perception of effort during exercise (213), and that further depends upon the exercise

modality, intensity and the environment, whereby a particular physiological cue may be markedly altered and become the overall mediator of the RPE (214). This is also in line with recent advances on the theory of fatigue during exercise, suggesting that the cessation of activities performed is dependent on the sensory tolerance limit, arising from the sensory feedback arising from the periphery (55). Previous studies initially hypothesized that muscle afferent feedback does not play a role on RPE (215), advancing the hypothesis that perception of effort during exercise is independent of afferent feedback from skeletal muscles, heart, and lungs. However, later studies suggested the muscle afferent feedback is important in the determination of an individual's rate of perceived exertion (40). Inducing pre-fatigue on the ipsilateral leg followed by an exercise bout on the contralateral leg increased the RPE leading to an early termination of exercise as the tolerance limit and fatigue had been reached (40). This phenomenon is also hypothesized to also involve limits from central motor command in addition to III-IV afferents due to the corollary discharge observed on the previous task. This problem was examined in a recent study of Laginestra et al (96) where the authors used an electrically induced pre fatiguing protocol to mitigate the intervention of central motor drive and the corollary discharge. These studies have shown the importance of muscle afferent feedback (III-IV afferents) on RPE, considering that these afferents are also responsible for the cardiovascular and cardiorespiratory function and exercise pressor reflex during exercise. Several studies and systematic reviews have also observed an increased RPE in chronic fatigue and fibromyalgia syndrome patients (216). This may be due to increased afferent sensitisation for any given intensity of exercise in these patients that lead to

premature exercise intolerance and lower attainment of cardiorespiratory fitness values (216, 217).

Activation of group III-IV muscle nerve afferents depending on sex

No sex differences in muscle metaboreflex activation in dogs were found (218). However, in humans, sex-differences were found in metaboreflex between premenopausal women and age matched men, where a decreased muscle sympathetic nerve activity (MSNA), blood pressure and ventilatory response was seen in women compared to men (219-222). Studies investigating the role of mechanoreflex are instead inconsistent, with some reporting attenuated or similar responses (223) to passive movement in premenopausal women. The main sex differences were attributed to alpha or beta-adrenergic sensitivity (224), nitric oxide synthase expression (225), baseline muscle strength (226) and estrogen level (227).

Increase activation of group III-IV muscle nerve afferents in ageing

The impact of ageing on the pressor response to exercise is also controversial, especially in humans, with studies showing enhanced (228-231), similar (232-234), or attenuated responses in older adults (235). Differences in these results have been attributed to differences in type of exercise implemented, exercise intensities, type of mechano- and metaboreflex activation, perhaps due to lack of sex matching in these studies. This is an

important point, as it may be that there is a different pressor response in older women (but not older men) compared to young counterparts, suggesting a role of nitric oxide and estrogen (236).

Increase activation of group III-IV muscle nerve afferents in clinical populations.

An abnormal exercise pressor reflex has been identified in a variety of chronic disease states because of an increased activation of muscle III-IV afferents. This could be important, because it can result in an exaggerated blood pressure response in abnormal sympathetic vasoconstriction that will limit blood flow redistribution to the working muscle. This mismatch between oxygen supply and demand may cause dyspnoea, early onset of fatigue, autonomic incompetence and aberrant pressor response, impairing exercise capacity and tolerance. This may have negative effects which may be due to an increased activity of muscle III-IV afferents in the following pathological states (143):

1) Heart failure: Piepoli et al. (237) was one of the first in demonstrating disproportionate and aberrant increases in cardiorespiratory and cardiovascular function in heart failure patients during metaboreflex activation with PECO in comparison with age matched controls. This disproportionate activation has been linked to the original concept of a ventricular disfunction (present in heart failure patients) that leads to reduction in skeletal muscle perfusion during exercise, increasing metabolite concentration and increasing cardiovascular and respiratory responses (238). Moreover, other successive studies confirm the alteration of exercise pressor reflex, blood flow perfusion,

hyperaemic response, and exercise intolerance in heart failure patients, mainly attributed to mechano- and metabo- sensitive muscle afferent activation (116, 122, 239, 240).

2) Hypertension: Regulation of blood pressure and the exercise pressor reflex is key for hypertensive patients, given that an exaggerated blood pressure response to exercise may increase the risk of acute cardiovascular and cerebrovascular events (241). This is particularly important for hypertensive patients who control their blood pressure at rest through medication but still exhibit an exaggerated blood pressure during exercise (242). These mechanisms have been attributed to an increased sensitivity of the exercise pressor reflex, especially the metaboreflex (242). Delaney et al (243) first demonstrated an increase metaboreflex response in hypertensive patients, through increases in MSNA and blood pressure response to isometric exercise. Recently, Barbosa et al found that following intrathecal administration of fentanyl (group III-IV afferent inhibitor), the exaggerated blood pressure response in hypertensive patients was normalized (244). These findings suggest that group III-IV afferents sensitivity concur via an upregulation of target genes as purinergic(245), mechanically gated ion channels and TRPV1, however an increased peripheral vasoconstriction and impaired vasodilatory signalling (246), within increased oxidative stress may also play an important role in this condition (247).

3) Diabetes: Both Type 1 and Type 2 diabetes display an increased exercise pressor reflex and abnormal pressor response to exercise (248-250). Blood pressure response and MSNA responses to PECO were found increased in patients with diabetes (type 2), suggesting that these response were correlated with the severity of the condition and the level of HBA1c (251). Moreover, Grotle et al found an increase cardiovascular response

following muscle contraction and tendon stretch, suggesting a potential role of mechanoreceptors in T2DM rats (252). The mechanisms underpinning these impairments remain to be seen, but recent studies found a that increases in TRPV1 channel and rats with hyperglycaemia (253, 254).

4) Peripheral arterial disease (PAD): Peripheral arterial disease is characterized by a lack of perfusion in the extremities caused by atherosclerosis in which intermittent claudication is the most common symptom in this disease (143). In animal models that simulate PAD, an exaggerated blood pressure response and exercise pressor reflex activation, that has been attributed to oxidative stress and dysregulation of different key receptors present on muscle III and IV afferents (EP4, TP, P2X, Bradykinin B2, ASICs, Opioid receptors ad GsMTx4 channels) has been observed (143). In humans, an increased blood pressure response during voluntary and electrically evoked muscle contraction has been seen (255), which was eased following revascularization of the coronary blood flow during exercise in PAD (256), hypothesing an involvement of blood flow supply following exercise in PAD.

5) Chronic obstructive pulmonary disease (COPD): few studies have so far examined the activation of the exercise pressor reflex in COPD. Bruce et al (257) found that metaboreflex activation significantly increased minute ventilation in patients with COPD, while no differences were found in blood pressure. On the other hand, no differences were found in COPD patients in response to metaboreflex isolation via PECO compared with age-matched healthy controls (258-260). To note the impact of III-IV afferents in exercise intolerance in COPD patients, attenuation of their activity through intrathecal

fentanyl injection showed an increased cardiorespiratory function and exercise performance (261).

6) *Obesity:* An exaggerated exercise pressor reflex in obesity is usually attributed to presence of comorbidities (i.e., hypertension) that may alter their pressure response (262). In fact, “pure” obese patients may display the “obesity paradox”, in being protected against obesity-related metabolic and cardiovascular complications. Indeed, Milia and colleagues (263) demonstrated that obesity per se is not responsible for the pressor response dysregulation and abnormal muscle metaboreflex and this becomes apparent only in patients with metabolic syndrome (263). Further studies found attenuated blood pressure response and metaboreflex activation in obese women (264, 265).

7) *Chronic fatigue Syndrome (CFS) and Fibromyalgia (FMS):* Few studies examine the effect of III-IV afferent activation in CFS and FMS. CFS is characterised by a long-lasting fatigue (>6months) after minimal exertion that could be not explained by an underlying medical condition (62). Only one study (266) found an increase in muscle afferents following exercise and PECO in CFS, but no studies have yet investigated the role of blood pressure response or MSNA activation in this condition. Similar to CFS, fibromyalgia syndrome has not been studied regarding this matter. FMS is characterized by a wide-spread pain across different muscle regions, that cannot be explained by an underlying medical condition(267). Only one study examines blood pressure response in FMS, finding similar blood pressure response to exercise compared with healthy age-sex matched controls (268). However, in this study no metaboreflex or mechanoreflex isolations were performed and blood pressure analysis were not implemented with continuous blood

pressure devices, making difficult any assumptions on the level of activation of group III-IV muscle nerve afferents.

Muscle afferents sensitisation

Muscle afferents sensitisation may occur following chemical alteration of the interstitial space of the muscle (102). For example, intra-arterial injection of bradykinin moderately increases the activity of both group III-IV muscle afferents during intermittent repetitive contraction (269). Moreover, arachidonic acid shows similar increases, but mainly for group III afferents (270, 271), while aspirin and indomethacin (decreased ability to synthetise prostaglandins) and dichloroacetate (decrease lactic acid) decreases the activation of group III and IV muscle nerve afferents (272). These findings are extremely important to understand the role of III-IV afferents sensitisation at a physiological level, mainly when inflammation or trauma can increase the concentrations of these metabolites. Models of muscle ischemia and muscle reperfusion have been also implemented to understand the role of group III-IV muscle afferents sensitisation in mice, finding a reduced grip strength, spontaneous pain behaviours and heat sensitivity (27, 273).

Models of muscle damage have been also broadly used to induce peripheral sensitisation of muscle afferents. The most common is eccentric exercise or protocol for exercise induced muscle damage. Several studies have found an increase in group III-IV muscle

afferents sensitivity using this model where mechanical hyperalgesia, heat hypersensitivity and decreased performance is observed (15, 16, 19, 29, 30).

Peripheral sensitisation of group III-IV muscle afferents could be ascribed to sensitisation of key receptors present at the synapses (1). Two main substances have been identified as key generators of muscle pain: adenosine triphosphate (ATP) and protons (H^+ ions) that antagonize the nerves once binding to the respective receptors located in the membrane of the nerve ending (110).

It has been hypothesized that peripheral sensitisation of mechanoreceptors (Aδ) of muscle nerve fibres can also occur through the interaction of metabolites produced during the contraction and become subsequently trapped in the muscle (274, 275). However, successive study from Fisher et al (276) did not find any mechanoreflex sensitisation following different levels of metaboreflex activation, showing that HR and blood pressure are independent. On the other side Cui et al (277) found that mechanoreceptors stimulation in healthy humans evokes significant increases in mean MSNA and BP when muscle metabolite concentrations are increased above a certain threshold. These differences have been ascribed to the different level of metabolites present in the muscle, the type of muscle utilised or the type of mechanoreflex stimulation performed. Indeed, recent studies from Venturelli et al (278) found marginal interaction from mechano and metaboreflex in central and peripheral haemodynamics with static or dynamic stretching of the quadriceps. So overall it's still difficult to determine the role of a mechanoreflex sensitisation following metaboreflex activation in

healthy humans suggesting to me that this needs to be understood before applying to clinical populations.

Role of muscle afferents on pain (from peripheral to central sensitisation)

Muscle pain is a major problem in the general population (60% to 85%) and seems to be one of the most common reasons for which patients consulted a doctor (279). Musculoskeletal pain underlies several rheumatologic and chronic disorders such as fibromyalgia, chronic back pain, arthritis, cancer, myalgias and chronic fatigue syndrome, however the aetiology and origin of the muscle pain is still not fully known. It may include pathophysiological and psychosocial factors that are specific and can vary between patients (110). Moreover, muscle pain is not recognised as a single entity, but as different types with different origins and mechanisms. An important difference has been made assessing and differentiating cutaneous and muscle pain from their perceptive differences (110). Peripheral mechanisms of pain involve nociceptors present in the muscle that are composed of free nerve endings and originate from group III-IV nerve afferents (280, 281). These afferents may be also become sensitised through endogenous inflammatory mediators such as BK and PGE2 (282). It has been further postulated that nervous impulses from muscle nociceptors into the spinal cord increase the excitability of posterior horns more than cutaneous nociceptors (283). Moreover, in one study, repetitive stimuli and persistent activation in experimental myositis in rats led to an increase in the number of neurons activated from the nerve afferents in the muscle (284).

Repetitive stimuli cause the so called “hyperalgesic priming” that cause a decrease of the activation threshold for the sensory neurons, causing allodynia and hyperalgesia (285). This spread of excitation is due in part to an increased excitability in the spinal cord neurons that leads to activation of NMDA (N-methyl-D-aspartate) receptors, substance P and NK1 (neurokinin 1) leading to central sensitisation. The repetition of this circuit in the long term creates a pathway that is suggested to change the plasticity of some brain areas and memorize in the brain, as protective mechanisms from future injuries (286). Central sensitisation is an amplification of the afferent signal in the central nervous system and may characterise different chronic condition as fibromyalgia and CFS/ME (81).

Chapter 3. Overall Thesis Aims

Overall thesis aims

The overarching aim of the work presented in this thesis was to assess changes in muscle nerve afferent sensitivity with neuromuscular and cardiovascular responses to exercise induced muscle damage, ageing, and chronic fatigue syndromes. This aim was addressed through 4 chapters each setting out the results of experimental or cross-sectional research and one chapter providing a systematic review with a meta-analysis of available original research. Each of these chapters had their own individual aims:

- *The first original chapter aimed* to characterise the neuromuscular and cardiovascular responses following an exercise induced muscle damage (EIMD) protocol. 31-P NMR (Nuclear Magnetic Resonance) Spectroscopy was used to assess alterations of muscle metabolism alongside measurements of changes in blood pressure with exercise.
- *The second original chapter aimed* to study the effect of mechano- and nociceptive afferent stimulation on cardiovascular and hyperaemic responses following single passive leg movement (sPLM) of remote muscle after EIMD.
- *The third original chapter aimed* to study the effect of mechano- and nociceptive afferent stimulation on exercise performance and the neuromuscular component of fatigue in remote muscle after EIMD.
- *The fourth original chapter aimed* to investigate the role of muscle afferents in the exercise pressor responses of Master Athletes participating at the World Master Track Cycling Championships (2019).

- *The fifth original chapter aimed* to investigate the cardiorespiratory fitness and neuromuscular function in Chronic Fatigue and Fibromyalgia Syndrome, and to identify possible underlying physiological processes associated with reduced physical performance of the patient groups.

Chapter 4. Experimental Study I

Sensitivity of group III-IV muscle nerve afferents following EIMD: Role of Metaboreceptors on cardiovascular function

What is known? In the previous chapter (I.e., Chapter 2) we collect and summarise evidence on the role of group III-IV muscle afferents in regulating cardiovascular and cardiorespiratory responses, presenting several models to isolate their activity. However, despite the previous model adopted, in this current experiment we implemented EIMD to induce muscle inflammation in and study the following changes in cardiovascular responses and metaboreflex activation.

What is going to be assessed? We hypothesized that muscle damage would have increased immune mediated inflammation and therefore activation of muscle nerve afferents, leading to abnormal cardiovascular responses and metaboreflex activation. Thus, the aim of this project is to identify how EIMD changes phosphate metabolism at rest and during exercise and if either any changes are associated with cardiovascular response to exercise and metaboreflex isolation.

The relationship between muscle metabolites measured with ^{31}P MRS with muscle function and the exercise pressor reflex after exercise-induced muscle damage

4.1. Introduction

Skeletal muscle myelinated type-III (Aδ) and unmyelinated type-IV (C-fiber) nerve afferents detect mechanical and metabolic stimuli (130, 164, 287). Their activity is proportional to contractile tension and the rate of metabolites accumulation to influence perceptions of effort, fatigue, and pain (25). These sensory neurons also orchestrate the afferent arch of the exercise pressor reflex (160) to influence respiratory, cardiac and vascular responses to exercise (288). However, their typical function can be altered in disease states (289) and also by exercise induced muscle damage (EIMD) (19, 30, 290).

EIMD is particularly prevalent after unaccustomed eccentric contractions and is characterised by muscle weakness and soreness lasting several days. The soreness occurs due to an increased sensitisation of mechano and nociceptive muscle afferents, causing mechanical hyperalgesia (14-19). This, in turn, is associated with localised inflammation (20), nerve microdamage (21) and increased abundance of metabolites that stimulate metabosensitive muscle afferents (16, 17, 19). Indeed, increased concentration of ATP, Lactate and H^+ and deprotonated phosphate (Pi) have shown to increase metabosensensitive afferent activation producing an increased in the pressor response (22, 23) leading to sensation of pain and fatigue (24, 25), sensitisation of mechanoreceptors (26), mechanical hyperalgesia and reduced muscle strength (27). Therefore, since the inflammatory status following EIMD has shown to sensitise muscle afferents (i.e., mechano- and nociceptive predominantly) (28-30), it is possible that the increased inflammation and metabolites abundance following EIMD may have in turn sensitise metabosensitive afferents resulting in an increased cardiovascular responses to

muscle contractions, including the pressor response. However, previous studies into the effect of EIMD on cardiovascular responses to exercise reported contrasting results. For example, Miles et al (1997) and Ray et al (1998) found similar blood pressure responses following EIMD compared with baseline conditions (291, 292), however differences between relative and absolute force values and exercise intensity make difficult to understand if a possible muscle afferent sensitisation may occurred.

Indeed, possible differences in the literature may be due to divergent methodological approach across studies including the muscle groups involved, or due to differences in exercise duration and intensity affecting metabolite stimulation of afferents. Moreover, no studies tried to isolate metaboreceptors activation from central command following post exercise cuff occlusion protocol (PECO). In fact, relatively little is known about the metaboreflex response to exercise after EIMD. Evidence from investigations with 31P magnetic resonance spectroscopy (31P MRS) shows EIMD increases resting inorganic phosphate concentrations, and this change is correlated with both muscle soreness at rest (293) and the rating of perceived exertion during exercise (294-296). It is therefore possible that metabolic changes measurable by 31P MRS during exercise and following EIMD not only associate with perceptions of pain and exercise effort, but also with the cardiovascular responses to exercise via afferent stimulatory pathways.

Therefore, the aim of the present study was to investigate whether changes of phosphorous metabolism are related to muscle function and cardiovascular responses to exercise at baseline and after EIMD. We hypothesised that increases in muscle inflammation and metabolites accumulation following EIMD would lead to muscle

afferent sensitisation and altered blood pressure responses to exercise and post-exercise cuff occlusion.

4.2. Methods

Participants

The study received ethical approval from the Faculty of Science and Engineering Research Ethics and Governance Committee (reference number: 37464) and conformed to the Declaration of Helsinki. The inclusion criteria were males or females aged 18 – 30 years and willing to abstain from caffeine and large meal consumption for 2 hours prior to participation, as well as alcohol usage and intense exercise for 2 days prior to any testing session. Exclusion criteria included: use of non-steroidal anti-inflammatory medication (NSAIDS) and presence of injury or medical conditions that prevented resistance exercise participation. Participants were also excluded for MRI scanning if they had a cardiac pacemaker; joint replacements; any surgery or injury that left ferrous metallic implants in the body; shrapnel or a bullet injury; ever suffered from epilepsy; a tattoo on the legs; were pregnant or had contraceptive diaphragm in situ.

Experimental Design

Participants visited the laboratory for a familiarization session, verbal explanation of the study procedures and to agree an appointment for the first (baseline) experimental session and the follow-up which took place 48 h after baseline (48 h EIMD). Assessments

at baseline and 48 h EIMD followed the same procedures, with the exception that the EIMD exercise protocol was completed only once, which was at the end of the baseline session (Fig 4.1).

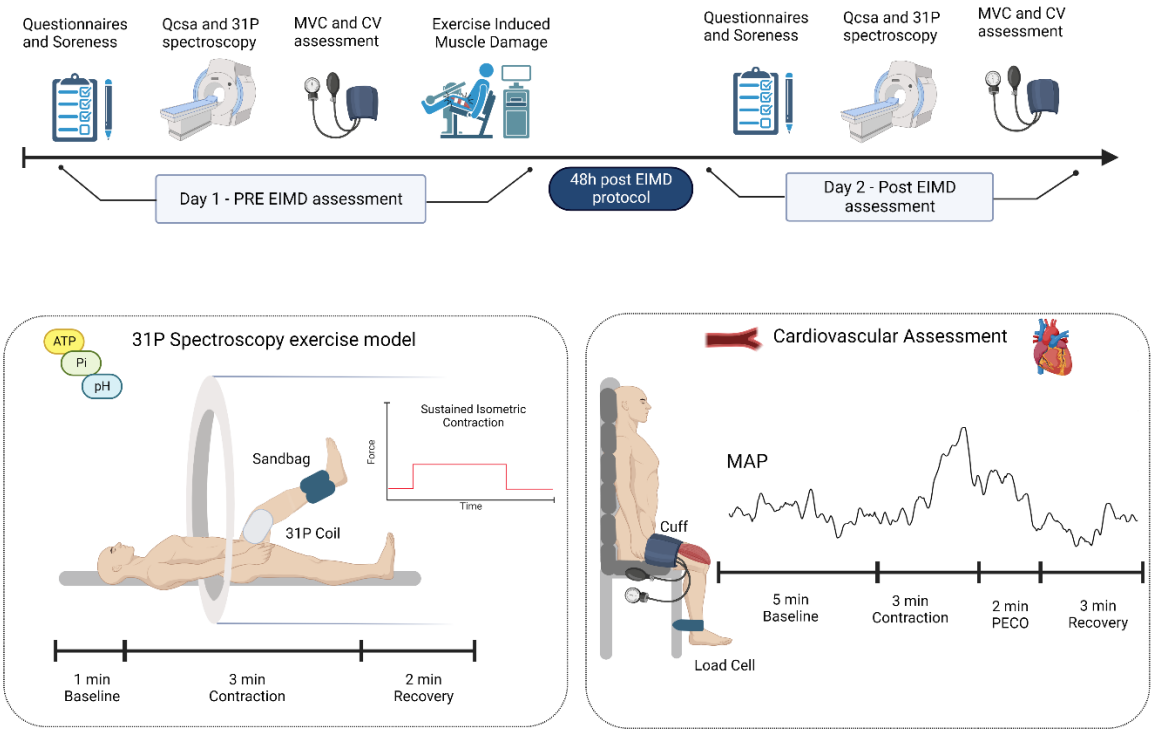


Figure 4.1 ***Experimental design and assessments.***

Qcsa = Quadriceps cross sectional area; MVC = Maximal Voluntary contraction; EIMD = Exercise induced muscle damage; = MAP = Mean arterial Pressure. Created with BioRender.com

Questionnaires and Soreness Assessment

A physical activity readiness questionnaire PAR-Q was completed and standing height and body mass were measured. Perceived muscle soreness of the knee extensors was

measured using a visual analogue scale (VAS_{SQ}) as participants held a single-leg squat with one knee flexed at 90˚ (297). The assessment was made by asking participants to mark a "X" along a 10 cm scale to indicate the level of soreness: from 0 meaning no muscle soreness to 10 meaning muscles are too sore to move (297, 298).

31P-NMR spectroscopy assessment

Twenty physically active, healthy volunteers (age 23.4 ± 4.0 years; mass 70.6 ± 13.4 kg; height 172.9 ± 10.8m; training hours 5.4 ± 3.3 (mean ±SD)) were eligible and provided written, informed consent to take part in the study. Participants lay supine and rested with legs fully extended and the thigh muscles positioned at the centre of the bore of the MRI scanner (3.0 Tesla , Magneton Vida, Siemens Healthcare GmbH, Erlangen, Germany). A flexible coil covered both thighs and transverse section T1-weighted images were acquired with 28 slices (0.5x0.5x7 mm voxels) centred on the widest point of the quadriceps (occurring approximately 3-5 cm proximal to the mid femur) (TR 800 ms, TE 12 ms).

Thereafter, a ^{31}P flexible coil (31P/1H Flex Coil 3T, RAPID Biomedical GmbH) was placed on the anterior thigh of one leg to acquire free induction decay signals with the knee raised to rest on a firm bolster 14.5 cm high (thus, flexing the hip to approximately 20˚) and the coil at the MRI isocentre to maximise signal-to-noise ratio (299). Acquisitions were collected sequentially every 8 seconds for a total of 6 min to capture rest, exercise, and recovery phases. The phases consisted of 1 min resting measurements, 3 min submaximal

steady state isometric contraction with a 3 Kg sandbag strapped firmly around the distal tibia close to the malleoli (adapted from (300)), and 2 min resting recovery. MRI data was acquired and processed using the Numaris/X software (v. VA31A, Siemens Healthcare GmbH, Erlangen, Germany). At the start and the end of the sustained contractions participants were asked to rate their perceived exertion (RPE) and pain sensations (PAIN) every minutes, respectively with the Borg Scale (301) and a numeric pain rating scale (p-NRS) (302).

Maximal Voluntary Contraction Assessment

Participants sat upright on a custom-made chair with hips and knees flexed at 90˚ and straps secured around the waist to minimise extraneous movements. Single leg maximal voluntary knee extension isometric contraction (MVC) was tested with the leg secured 2 cm proximal to the malleolus by an inextensible strap connected at the other end to a calibrated load cell. Force signals were amplified and recorded (PowerLab 16/30; ML880, ADInstruments, Bellavista, NSW, Australia) and real-time feedback of force was available on a computer monitor. A warm-up was provided consisting of six isometric contractions at 50-80% maximal effort. After a 1 min rest, participants performed three MVCs, each separated by 1 min rest. The highest external force value was accepted as participant's MVC (303). This procedure was repeated for the other leg.

Cardiovascular Assessment

Participants remained seated in the chair used for testing MVC and rested for 10 min, in which time the non-invasive blood pressure plethysmography for continuous blood pressure monitoring and a 3-leads ECG were connected to power lab (PowerLab 16/30; ML880, ADInstruments, Bellavista, NSW, Australia). Resting measurements were collected for 5-min before a rapid inflatable cuff (16 cm wide; . E. Hokanson, Inc. Bellevue, WA 98005 USA) positioned around the proximal thigh was inflated to 220-240 mm/Hg for 2 min. After 5 min rest, participants performed a sustained isometric knee extension for 180 s at 30% of the MVC. At the end of contraction, the cuff was rapidly inflated around the exercising thigh (220-240 mmHg) for post exercise cuff occlusion (PECO) and remained inflated for 2 min. PECO stops arterial and venous blood from entering/leaving the limb and therefore maintains the muscle metabolic environment in the exercise state. Participants rested for 3 min at the end of PECO (304).

Exercise induced muscle damage protocol

EIMD consisted of eccentric knee extensor contractions of one leg (EIMD leg), while the second leg remained rested and acted as control. A Kineo Multistation machine (Globus, Italy) was used providing isokinetic mode and enabling the eccentric load to be accurately and rapidly adjusted in relation to the concentric load. A warm-up was provided consisting of 10 isokinetic concentric leg extensions performed through the full range of motion. The maximal concentric and eccentric torques were then assessed so the EIMD exercise could be set proportional to the maximal eccentric torque. Participants then

completed repeated sets of 10 dynamic eccentric knee extensions in isotonic mode with the load set at 100% of the eccentric peak torque and participants were asked to give a maximal effort to oppose the load. The concentric load was set at 50% of concentric peak torque. At the end of each set of 10 repetitions, an MVC isometric force was tested, and exercise was terminated when MVC was reduced by 40% compared with starting values (9, 305).

Data Handling and Statistical Analysis.

Data collected through LabChart 8 software (ADInstruments, Bellavista, NSW, Australia) was imported into Microsoft Excel for calculation of the main variables. Cardiovascular outcomes were analysed for each given workload and presented as deltas were calculated subtracting the baselines values from the other phases. Cardiac output (CO) and stroke volume (SV) were calculated using the Labchart 8 Software using the non-invasive cardiac output function with Liljestrand equation. Intramuscular pH was calculated using the chemical shift of the Pi resonance in relation to the PCr peak, using Henderson-Hasselbalch equation (306). Delta Pi/PCr and pH were calculated subtracting the baseline values from the contraction (ΔPi/Pcr resting exercise).

A paired t-test was performed at baseline for control and EIMD legs within participants (table 1). The presence of muscle damage between control and EIMD leg was assessed with a two-way repeated measures ANOVA performed for MVC, Qcsa, VAS_{SQ}, Pi, PCr, Pi/PCr and pH values (two time: baseline and 48 h EIMD) and two group (Control and

EIMD legs). To compare control and EIMD leg responses to exercise in the MRI scanner, a three-way repeated measures ANOVA was performed (two time: baseline and 48 h EIMD), three workloads (rest, exercise, recovery) and two group (Control and EIMD legs) for Pi, PCr, pH, and PDE. Cardiovascular responses to exercise were assessed with a two-way repeated measures ANOVA for delta values of SBP, MAP, DBP, HR, CO, SV values with two within subject factors (Time: baseline and 48 h EIMD) and three phases (contraction, PECO, and recovery). Pearson's Product Moment Correlations were used to assess relationships between continuous variables.

4.3. Results

Participant characteristics are shown in Table 4.1. At baseline, there were no differences between the EIMD and control legs for MVC, Qcsa, resting muscle phosphorous measurements (Table 4.1).

Table 4.1. Participant characteristics at baseline

Male / Female (*n*)	**11 / 9**		
Age (years)	23.1 ± 3.9		
Height (cm)	171.6 ± 11.0		
Body mass (Kg)	69.9 ± 13.0		
BMI (Kg/m^2)	23.6 ± 2.4		
	EIMD leg	Control leg	p-value
Qcsa (cm^2)	78.9 ± 19.5	77.7 ± 17.3	.849
MVC (N)	634.6 ± 229.3	607.2 ± 207.6	.694

Abbreviations: BMI: body mass index; MVC: maximum voluntary contraction from isometric knee extension; CSA: cross sectional area of knee extensors. Data are mean ± standard deviation. P-value indicates results of t-test comparing EIMD and Control legs. *=p<0.05

Effect of EIMD on skeletal muscle function and metabolism

EIMD was apparent at 48 h from the significant time, leg, and time x leg interactions for Qcsa, MVC, RPE, PAIN and VAS_{SQ} (all $p<0.003$; Table 4.2). The EIMD leg showed 18±4% reduction of MVC, 2.80 ± 0.04% increase of Qcsa, 20.8±0.1% increase in RPE and 8267±1734% increase of VAS_{SQ} at 48 h compared with baseline (all $p<0.001$), while the control leg showed no significant changes from baseline for these measurements (Table 4.2).

Effect of EIMD on resting muscle Pi, PCr and pH

PCr, Pi, Pi/PCr and ATP_{γ} all showed significant effects of Time (all $p\leq0.010$). PCr values were marginally (-2.3%) lower at 48 h compared with baseline for both the EIMD and control legs (effect of time p=0.010; time x leg interaction p=0.446). A significant time x leg interaction for Pi, Pi/PCr and ATP_{γ} (all $p\leq0.002$) was found, as values for Pi and Pi/PCr were higher and ATP_{γ} was lower in the EIMD leg at 48 h compared with baseline, but control leg values for these measurements remained unchanged over 48 h (Figure 1). Resting muscle pH did not change significantly with time (baseline vs 48 h: p=0.236) and there was no significant time x leg interaction for pH (p=0.766). PDE, which has been used as a marker of membrane integrity, did not change over time (PDE: p=0.123), however there was a significant interaction effect (p=0.048), as values for the EIMD leg were 16% lower at 48 h than baseline, while values in control leg were unchanged.

Table 4.2 Indices of skeletal muscle damage and metabolic perturbation following EIMD.

Outcome measured	EIMD Leg		Control Leg		Effects (p-values)	
					Time	Group x time
	Pre	48 h post	Pre	48 h post		
MVC (N)	634.6 ± 229.3	504.0 ± 160.5	607.2 ± 207.6	611.6 ± 219.50	0.001	<0.001
Qcsa (cm2)	78.2 ± 5.4	82.6 ± 5.7	77.4 ± 5.1	76.8 ± 5.9	0.003	<0.001
VAS_{SQ} (mm)	2.3 ± 1.5	39.5 ± 27.3	1.7 ± 1.5	N/A	<0.001	N/A
RPE (6-20)	12.0 ± 2.2	14.3 ± 2.1	12.3 ± 2.2	12.5 ± 2.1	<0.001	0.002
Pi (a.u.)	118180.9 ± 24532.8	155573.0 ± 45280.2	120068.2 ± 23724.9	117199.5 ± 19291.2	0.005	0.002
PCr (a.u.)	946462.9 ± 158597.1	925302.2 ± 166964.5	951403.3 ± 150761.3	942478.3 ± 151946.3	0.010	0.446
Pi/PCr (a.u.)	12.7 ± 2.6	16.9 ± 3.8	12.7 ± 2.4	12.5 ± 1.6	0.001	<0.001
PDE (a.u.)	47830.4 ± 22390.1	40582.8 ± 20572.9	51133.6 ± 25086.7	50077.7 ± 23198.0	0.123	0.048
ATPγ (a.u.)	255882.8 ± 46200.4	229579.0 ± 44510.3	258856.7 ± 49386.5	261693.3 ± 42903.1	<0.001	<0.001
pH	7.11 ± 0.02	7.11 ± 0.02	7.11 ± 0.02	7.11 ± 0.02	0.201	0.568

Data are mean ± SD. Main effects are shown for time **(**Pre- and 48h post); group (EIMD vs Control leg) and the interaction of the two. Abbreviations: MVC: maximum voluntary contraction from isometric knee extension; CSA: cross sectional area of knee extensors; VAS_{SQ}: Squat visual analogue scale; Pi: inorganic phosphate; PCr: phosphor creatine.

4.4. Association of muscle function with muscle metabolite changes following EIMD

The EIMD changes in MVC, Qcsa and RPE from baseline to 48 h (i.e., delta (Δ) changes) were significantly associated with delta changes to resting muscle measurements of [Pi], Pi/PCr and ATPγ. Furthermore, ΔpH of the EIMD leg was inversely correlated with ΔRPE. Correlations for the Control leg were not significant (Table 4.3).

Table 4.3 Relationships between markers of muscle damage and metabolite changes

		EIMD leg			Control leg		
		ΔMVC	ΔQcsa	ΔRPE	ΔMVC	ΔQcsa	ΔRPE
ΔPi	r=	**-.631**	**.562**	**.832**	-.117	-.011	.169
	p=	**.005**	**.015**	**<.001**	.656	.966	.518
ΔPCr	r=	.371	-.383	-.233	.209	-.128	-.063
	p=	.129	.117	.351	.421	.625	.810
ΔPi/PCr	r=	**-.675**	**.598**	**.856**	-.168	.023	.185
	p=	**.002**	**.009**	**<.001**	.519	.930	.477
ΔpH	r=	.127	.210	**-.502**	.037	.365	-.159
	p=	.616	.402	**.034**	.887	.150	.543
ΔATPγ	r=	**.723**	**-.531**	**-.594**	-.027	.197	-.130
	p=	**.001**	**.023**	**.009**	.917	.450	-.620
ΔPDE	r=	.094	.272	-.014	.191	.135	-.194
	p=	.709	.275	.956	.464	.604	.456

Delta (Δ) values represent changes occurring from baseline to 48 hr measurements. Statistically significant relationships are highlighted with bold text. Abbreviations: Pi: inorganic phosphate; PCr: phosphor creatine.

Effect of EIMD on metabolite and pH responses to exercise

Results from the effect of EIMD on Pi, Pi/PCr, ATPγ and pH in response to exercise are shown in Figure 4.2. There were no significant correlations between baseline MVC and the delta from rest-to-exercise for ΔPi, ΔPi/PCr, ΔATPγ or ΔpH at baseline. This was also the case for measurements taken at the 48h time point.

Pi, PCr, Pi/PCr, ATPγ and pH all showed significant effects of Workload (all $p<0.001$). However, Workload x Time interactions as well as Workload x Leg interactions were not significant, indicating that EIMD and Control legs showed similar overall patterns of response to changes in workload at baseline and 48 hr later. Nevertheless, the values for the EIMD leg were raised compared to those of the control leg for Pi and Pi/PCr

proportional to the elevation reported above for the rested state. pH showed a significant effect of Workload ($p<0.001$), as values were lower during recovery than at rest or exercise with no workload x time interaction ($p=0.568$).

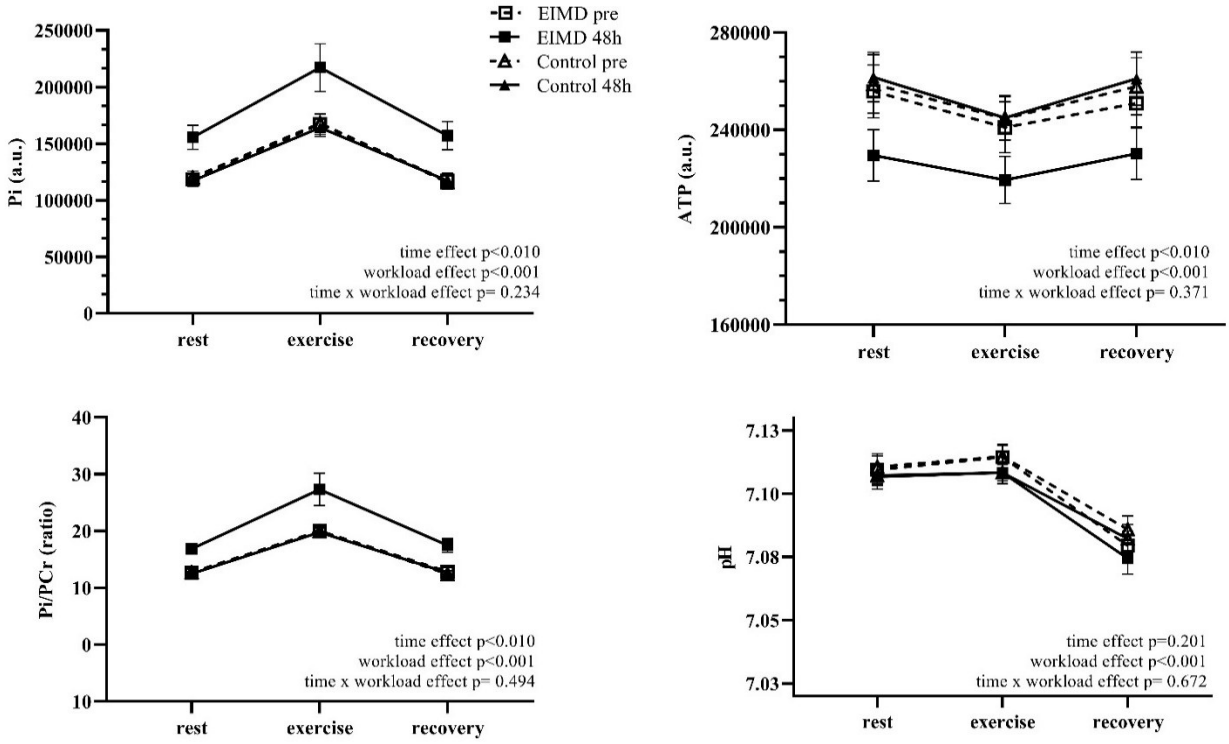


Figure 4.2 Phosphate and ATPγ and pH results following EIMD.

Abbreviations: Pi: inorganic phosphate; PCr: phospho creatine; Data are reported as mean ± SEM.

Cardiovascular Responses after EIMD.

Indices of cardiovascular function were collected in the rested state at baseline and 48 h post EIMD and there were no significant differences between these time points (Table 4.4). Values for MAP, HR, SV and CO changed significantly with changes through contraction to PECO and recovery phases (all $p<0.001$; Figure 4.3). There were no

significant effects of time for any of the cardiovascular measurements during any of the phases. However, a significant time x phase interaction was found for MAP responses ($p=0.021$, Fig 4.3) as blood pressure recovered more slowly from PECO to recovery phases at 48 h after EIMD compared with baseline. ΔHR, SV and CO did not show significant time x phase interactions (all $p>0.05$).

At baseline, the increases of Pi and Pi/PCr from rest to end-exercise were significantly correlated with the increase in MAP from rest to end-exercise (Pi: $r=0.415$, $p=0.01$; Pi/PCr: $r=0.495$, $p=0.03$). However, this was not the case 48 h later in the damaged muscle (Pi: $r=0.296$, $p=0.892$; Pi/PCr: $r=0.296$, $p=0.784$). However, at baseline the change in ATP_γ from rest to end-exercise was not significantly correlated with the increase in MAP from rest to exercise ($r=-0.156$, $p=0.537$), but at 48 h the change in ATP_γ from rest to end-exercise was significantly correlated with the increase in MAP from rest to end-exercise ($r=0.588$, $p=0.013$).

Table 4.4 Cardiovascular Responses at baseline before and after EIMD

Variables	baseline	48 h post EIMD
SBP (mmHg)	139.3 ± 19.0	134.9 ± 18.4
DBP (mmHg)	82.1 ± 13.8	82.4 ± 11.1
MAP (mmHg)	98.3 ± 14.9	97.1 ± 12.5
Heart Rate (bpm)	78.1 ± 10.7	75.9 ± 10.7
Cardiac Output (L/min)	3.1 ± 0.6	2.8 ± 0.53
Stroke Volume (mL)	39.6 ± 6.5	36.6 ± 5.3

Data are mean ± SD. Abbreviations: MAP: mean arterial pressure; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure.

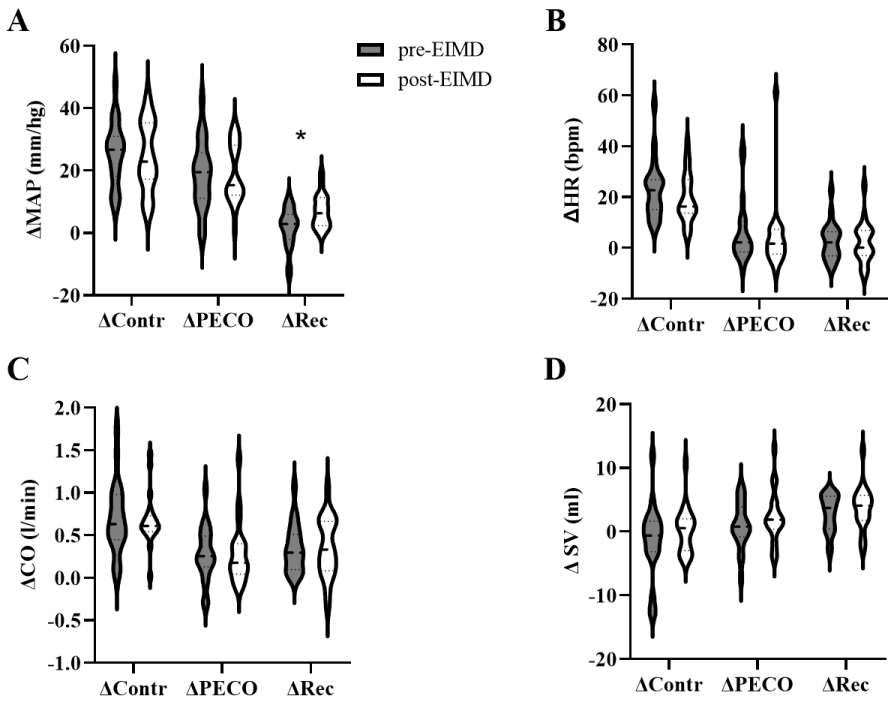


Figure 4.3 Delta MAP and central Haemodynamics following EIMD.

Abbreviations: ΔContr = delta contraction; ΔPECO = delta post-exercise cuff occlusion; ΔRec = delta recovery; MAP = Mean arterial Pressure; HR= Heart Rate; CO= Cardiac Output; SV = Stroke Volume. Data are reported as violin plot.

4.5. Discussion

Skeletal muscles were damaged by eccentric exercise and ^{31}P MRS was used to determine the relationship between changes to phosphorous energy metabolism, muscle function and cardiovascular responses to exercise. We found that the metabolic disturbance of damaged muscles was associated with muscle functional outcomes of weakness, swelling and perceived exertion. We also found changes to the relationship between muscle metabolites and the pressor response when damaged muscles were exercised. This may

reflect changes in muscle afferent sensitivity or just a disruption in the metabolic environment following EIMD. However, the MAP remained similarly elevated during exercise and throughout PECO when mechanoreceptor and central command activity had ceased, suggesting that metaboreceptors where not sensitised and a more complex regulation of the pressor response when damaged muscles are exercised

Skeletal muscle function

The participants in this study were young, recreationally active men and women who were not regularly engaged in competitive sports or resistance training. Skeletal muscles are prone to damage during unaccustomed eccentric exercise (9) and to this end we observed 18% lower MVC at 48 h, which was similar to past studies (305, 307, 308), an increase in Qcsa indicating localised swelling (309, 310) and muscle soreness (291, 311). The muscle damage after eccentric contractions occurs as sarcomeres attempting to shorten are forcibly lengthened causing high tension (20) affecting extracellular structures, the sarcolemma and myofibrils (312), but it remains unclear how this affects the muscle metabolic environment. By applying 31P MRS, we found a 34% increase of the resting muscle Pi/PCr ratio, 10% decrease of ATP_{γ} and 15% decrease of PDE in damaged muscles. The control leg, which was not eccentrically exercised, did not change compared with baseline. The altered Pi/PCr, Pi, ATP_{γ} correlated with changes to MVC, Qcsa and perceived exertion. Although mean pH did not differ at rest between baseline and 48 h in the EIMD leg, it was nevertheless inversely correlated with changes to perceived exertion as a lower pH was associated with greater perception of effort. These findings show greater disturbance of resting muscle metabolism with more extensive EIMD.

The elevated Pi/PCr ratio was not caused by changes in PCr, which reduced only 2.3%, but was instead primarily due to the 34% elevated Pi relative to PCr. Others also found elevated Pi/PCr in damaged muscles at rest (313-315). It is not fully clear why resting Pi is raised in damaged muscles. One possibility is that it is linked to the reduction of available creatine kinase after leakage into the circulation (316), but this seems unlikely since the PCr levels and responses to exercise were normal. Instead, a more likely explanation is an increased resting energy cost associated with the repair of damaged tissues and the increased passive muscle tension caused by elevated resting sarcoplasmic Ca^{2+} (309, 310). This energy turnover may also explain why ATP levels were reduced 10% in resting damaged muscles, although ATP leakage and Pi movement into the sarcolemma through damaged membranes cannot be ruled out. Damaged membranes are permeable after EIMD, even to large enzymes including release of creatine kinase (317) and movement of albumin into damaged fibres (312). PDE has been used as a marker of membrane damage (299, 318) and we found a 15% reduction of PDE levels in the EIMD leg, with no changes in the control leg. PDE changes signify membrane phospholipid turnover (319) and several studies reported higher PDE in muscle diseases (299, 318, 320). While this might seem to contradict our findings, a possible explanation is that EIMD rapidly causes membrane damage which is typically repaired within a few days, compared with the slower acting chronic impairments of muscle diseases.

The reduced ATP_γ levels at rest after EIMD is similar to (293) who found a reduction of approximately 7-10% at 24-48h validated against a calibrated phantom. Although several studies reported altered glycolytic and mitochondrial processes (293, 321, 322), this was

unlikely to be the cause of lower resting ATP_{γ} in our study because muscles generally have very high reserves for energy production.

We showed that damaged muscles transitioning from rest to exercise follow similar relative changes for Pi, PCr, Pi/PCr, ATP_{γ} and pH compared with undamaged muscles, except that the concentration of Pi, and thus the Pi/PCr ratio, were elevated for all workloads roughly proportional to their elevations at rest. Control and EIMD legs both showed similar 4-5% further decrease of ATP_{γ} during exercise compared with their respective resting levels. The 15% lower resting ATP in the damaged leg and the further 5% reduction occurring during exercise would likely reduce ATP availability for cross bridge function and Na/K+ pumps which would accelerate the onset of fatigue during prolonged higher intensity exercise. However, we could not confirm this from our work because a higher intensity exercise was not included. The raised Pi of resting and active muscles would add to the muscle susceptibility to fatigue, as Pi can directly interfere with cross bridge function (323) and enter the sarcoplasmic reticulum forming calcium-phosphate to limit calcium release into the sarcoplasm (324).

Cardiovascular function

Despite the clear muscle deficits of EIMD, the cardiovascular responses at rest and to exercise were unchanged from their baseline responses. In line with these results, Ray et al (291) found similar HR and MAP responses to 3 min isometric knee extension with the same absolute workload (when tested 2 days after EIMD), hypothesising no changes in the central command or baroreflex activation during isometric exercise following EIMD.

However, due to changes in resting contralateral forearm vascular resistance they suggested a possible increase in sympathetic outflow from increased activity of muscle afferents following EIMD. Similar to these findings, Miles et al (292) reported unchanged HR and MAP (when tested 48 h after EIMD) during isometric elbow flexors contraction, although participants exercised at lower absolute exercise intensity in the EIMD condition. The authors hypothesized that similar blood pressure responses at a lower absolute exercise intensity (but same relative) would have possibly been ascribed to increased activation of muscle afferents following EIMD (292). To account for this, we implemented a PECO protocol to assess the involvement of metaboreflex activation in blood pressure response following EIMD in the absence of central command and mechanoreflex activation. We found no difference in MAP between exercise and PECO phases, and no differences in delta metabolite responses to contraction (Pi, PCr, ATP_{γ}), suggesting no metaboreflex sensitisation following EIMD (325). However, even if the cardiovascular and MAP responses to exercise and PECO did not differ with EIMD this does not mean that the mechanisms regulating muscle afferents activation in the exercise pressor response were not altered. Indeed, at baseline we found that the increase in Pi and Pi/PCr occurring for transitions from rest to exercise were correlated with the MAP response which reveals the close link between energy stress and MAP. Similar to our findings, previous studies also showed associations between Pi and pH with muscle pain, perceived exertion and MAP in healthy individuals at baseline (326-328). However, the relationship between Pi and MAP was not evident after EIMD, suggesting that the sensitivity of afferents to stimulating metabolites might have changed with EIMD. Indeed

muscle III-IV afferents are activated by ATP and H+ (24, 25) and associated with intracellular decreases in pH and increases in di-protonated phosphate H2PO4 (22, 166, 329). In line with these findings, patients with multiple sclerosis displayed weaker correlations between MAP and Pi or pH compared with healthy controls (328).

Conversely, ATP_{γ} occurring for transitions from rest to exercise was not correlated with the MAP response at baseline, but it was positively correlated 48 h later when muscles were damaged. ATP usually increase following the immune-inflammatory process (330) where it showed to binds to nociceptive purinergic signalling pathway (331) and sensitise muscle mechanoreceptors (26). ATP may bind to muscle nerve afferents in the damaged muscles, through the purinergic receptors, altering sympathetic and cardiovascular responses (26). Indeed, blockade of P2- receptors was associated with decreased metaboreflex (14) and mechanoreflex activation (332), but also its increases in gene expression were associated with mechano- and nociceptive sensitisation following EIMD (290). Previous research showed that ATP may be released from nerve endings and skeletal muscle during electrical stimulation and voluntary muscle contractions (333, 334) where it could regulate cardiovascular function and muscle afferent activity (335, 336). High tension eccentric exercise can cause mechanoreceptors to experience microdamage due to acute compression axonopathy and immune mediated inflammation (21) which has been previously implicated in the aberrant cardiorespiratory and vascular responses to exercise after EIMD (337, 338). Moreover, in our study participants performed isometric exercise contraction at similar relative exercise intensity but at a lower absolute force. In healthy exercising muscles exercising at lower absolute force generate a lower

mechanical tension with reduced central command and mechanoreceptors activation, leading to lower BP responses (339, 340). However, in our study, we found similar BP response at lower mechanical tension (i.e., higher BP response per unit of force produced) suggesting the possible role of heightened mechanoreceptors response. Indeed, mechanoreceptors were sensitised by EIMD leading to mechanical hyperalgesia in mice (15-17) and in humans (20, 341).

Limitations

The same absolute exercise load of 3 Kg was used for all participants and since the MVC values varied between participants it follows that the relative exercise load was different between participants. However, there were no significant correlations between baseline MVC and the delta from rest-to-exercise for ΔPi, ΔPCr, ΔPi/PCr and ΔpH. This was also the case for measurements taken at the 48-h time point.

Practical Applications and implications

The results of the current investigation could be helpful to identify the role of peripheral sensitisation on the blood pressure regulation and cardiovascular responses to exercise following muscle injuries or increased peripheral inflammation. An increased exercise pressor reflex may lead to an impairment in muscle blood flow to the exercising muscle resulting in lower exercise tolerance and performance, explaining the exaggerated perception of effort during exercise experienced during the isometric task. These findings

may also inform trainers and coaches on training load management following muscle soreness and avoid further strain to athletes experiencing EIMD during the following training sessions.

4.6. Conclusion

EIMD seems to induce neuromuscular impairment and soreness associated with cardiovascular and blood pressure impairment, especially during exercise recovery, and possibly linked with muscle nerve afferents sensitisation. Metaboreflex remain unchanged suggesting that other factors may be linked to the altered blood pressure response found after EIMD (i.e., mechano- and nociceptors, central command, NO-availability). However, more research is still needed to untangle the role of muscle afferent reflexes in cardiovascular Reponses following EIMD.

Chapter 5. Experimental Study II

Sensitivity of group III-IV muscle nerve afferents following EIMD: Role of Mechano- and Nociceptor activation on cardiovascular function

What is known? In the previous chapter (I.e., Chapter 4) we underlined the role of muscle metabolism and cardiovascular responses following EIMD, showing that group III-IV muscle afferents may have become sensitive to metabolites produced following muscle damage inducing cardiovascular abnormalities. However, these abnormalities were not attributed to increased activity of metaboreceptors, suggesting that other factors may have been involved.

What is going to be assessed? We used EIMD to induce sensitisation of muscle nerve afferents in the muscles and static stretching to activate mechanoreceptors while assessing central and peripheral hemodynamic and vascular responsiveness to single passive leg movement executed in remote muscles. We hypothesise that sensitisation of muscle afferents would result in a concurrent reduction of peripheral circulation and vascular responsiveness to single passive leg movement executed in remote muscles.

This work has been published as: Zambolin F, Giuriato G, Laginestra FG, Ottaviani MM, Favaretto T, Calabria E, Duro-Ocana P, Bagley L, Faisal A, Peçanha T, McPhee JS, Venturelli M. Effects of nociceptive and mechanosensitive afferents sensitization on central and peripheral hemodynamics following exercise-induced muscle damage. J Appl Physiol (1985). 2022 Aug 18. doi: 10.1152/japplphysiol.00302.2022. Epub ahead of print. PMID: 35981730.

Effects of nociceptive and mechanosensitive afferents sensitization on central and peripheral haemodynamics following exercise-induced muscle damage.

5.1. Introduction

Peripheral group III-IV muscle nerve afferents regulate cardiorespiratory response to exercise, composing the afferent arch of the exercise pressor reflex (EPR) (160). Thinly myelinated (group III), and unmyelinated (group IV) muscle afferents mainly respond to mechanical and metabolic stimuli respectively and their activation regulate the increase in mean arterial pressure (MAP), heart rate (HR) and limb blood flow during exercise (49, 125). However, these afferents show polymodal characteristics being sensitive also to other stimuli, such as thermal and nociceptive (35, 269). Despite the exact mechanisms are not fully understood, it has been postulated that the alteration of the muscle chemical milieu after exercise induced muscle damage (EIMD) (13), due to increase metabolites production and accumulation (i.e., prostaglandins, lactate, protons), could sensitize and or increase muscle nerve afferents and related nociceptor activity (25, 35, 270). Indeed, inflammation following muscle damage may cause mechanical hyperalgesia (tenderness and movement induced-pain) (15-17) better known as delayed onset of muscle soreness (DOMS) (342, 343) that is suggested to be linked to an increased activity and sensitization of Aδ- (III) and C-fiber (IV) nerve endings (29, 30). Indeed, inflammation, injuries or muscle damage events (as EIMD) are thought to induce afferent nerve sensitization with related increases in expression of purinergic 2X receptor (P2X), acid sensing-ion channel receptor (ASIC), transient receptors potential vanilloid channels receptors (TRPV) (15-17). These receptors are usually present on mechano and metabo sensitive muscle III-IV afferents (1, 128) which seem to be involved in chronic pain condition (344, 345), heart failure (346) and mechanical hyperalgesia following EIMD (17). Specifically, P2X receptors have been

suggested as responsible for the increased sympathetic nerve activity (SNA) to mechanical deformation in heart-failure rats (347). Mechanical hyperalgesia from peripheral nerve afferents sensitization following EIMD is linked with autonomic nervous system and may cause an increased sympathetic activity (348), that in turn has been suggested as one of the potential mechanisms of impaired vascular function and increased cardiovascular responses following EIMD (291, 349-351). Considering activation of muscle mechanoreceptors with static or dynamic muscle stretching has been shown to activate mechanoreflex (352-355), it is likely that the activity of the sensitized mechanoreceptors would be heightened increasing pain sensations (17, 30, 356) and thereby reducing limb blood flow and vascular hyperemia~~,~~ even in remote muscle. For instance, sensitization of mechanosensitive afferents and heightened mechanoreflex responses seems to augment peripheral vasoconstriction in patients with heart failure showing decreased vascular responsiveness following passive leg movement (PLM) (239, 357). Mechanoreflex hypersensitivity plays a significant role in cardiovascular diseases where it may lead to dysregulated cardiovascular responses and possible exercise intolerance (116, 123). Different studies have also suggested that mechanoreflex sensitivity may be altered in pain-related diseases where small fiber neuropathy (358, 359) and mechanical hyperalgesia is present (360). This may help to explain the abnormal cardiovascular responses to exercise in pain-related diseases (304) which deserves further attention.

Yet, no studies have described the singular and combined effects of mechano- and nociceptors sensitization on peripheral and central haemodynamics. Therefore, the aim of this study was to determine the separated and combined effects of mechanoreflex

activation and nociceptive stimulus following exercise induced muscle damage on central and peripheral hemodynamic and vascular responsiveness to single passive leg movement executed in remote muscles. We hypothesized that only the combined sensitization of mechano- and nociceptors would result in an autonomic-mediated increase in central haemodynamics and a concurrent reduction of peripheral circulation and vascular responsiveness to single passive leg movement executed in remote muscles.

5.2. Methods

Participant's characteristics. Eight healthy, non-smoking, active male volunteers (age: 24.2 ± 2.2 yrs.; body mass 72.4 ± 10.1 kg; and height 179.3 ± 7.7 cm; means ± SD) took part in the study. Participants self-reported moderate levels of physical activity (3.0 ± 0.5 hours of training per week) with no specific experience of strength training exercise. All procedures conformed to the Declaration of Helsinki standards and were approved by the ethical committee of the University of Verona (CARP) acceptance number (n.14 R2/2021). The participants gave written, informed consent before their participation after full explanation of the purpose and experimental procedures of the study. The participants reported to the laboratory in the morning (8–9 AM) in a fasted state. They were asked to abstain from consuming caffeine 24h and heavy exercise for 48h. Participants were also abstaining from consuming before and 24h after each visits any vitamin supplements, high content vitamin C food, alcohol, or pain medications.

Experimental Design. After a first familiarization visit, the participants reported to the laboratory for 5 distinctive lab visits, in which they performed one of the four experimental sessions in different days: (Control (CTRL); Stretching (ST); DOMS, DOMS with stretching (DOMS+ST)) and the EIMD protocol (Fig 1). Before each of these conditions a blood sample was taken from each participant. The CTRL session consisted of a single passive leg movement (sPLM; described in further details below) of the dominant leg while the contralateral leg remained resting fully extended. ST consisted of the same sPLM on the dominant leg, but with the application of concomitant static stretching-protocol (described in further detail below) in the contralateral leg. DOMS condition consisted of the same sPLM test on the dominant leg while the contralateral leg was resting fully extended after having previously performed the EIMD protocol. DOMS+ST conditions consisted of the same sPLM on the dominant leg while in the contralateral leg a static stretching-protocol was applied after previous application of EIMD protocol. The order of both experiments (CTRL vs ST and DOMS vs DOMS +ST) was randomized and counterbalanced between participants (Fig. 5.1). Each session was performed in a separate day with DOMS and DOMS+ST conditions performed randomly and counterbalanced at 24 or 48h after the EIMD protocol.

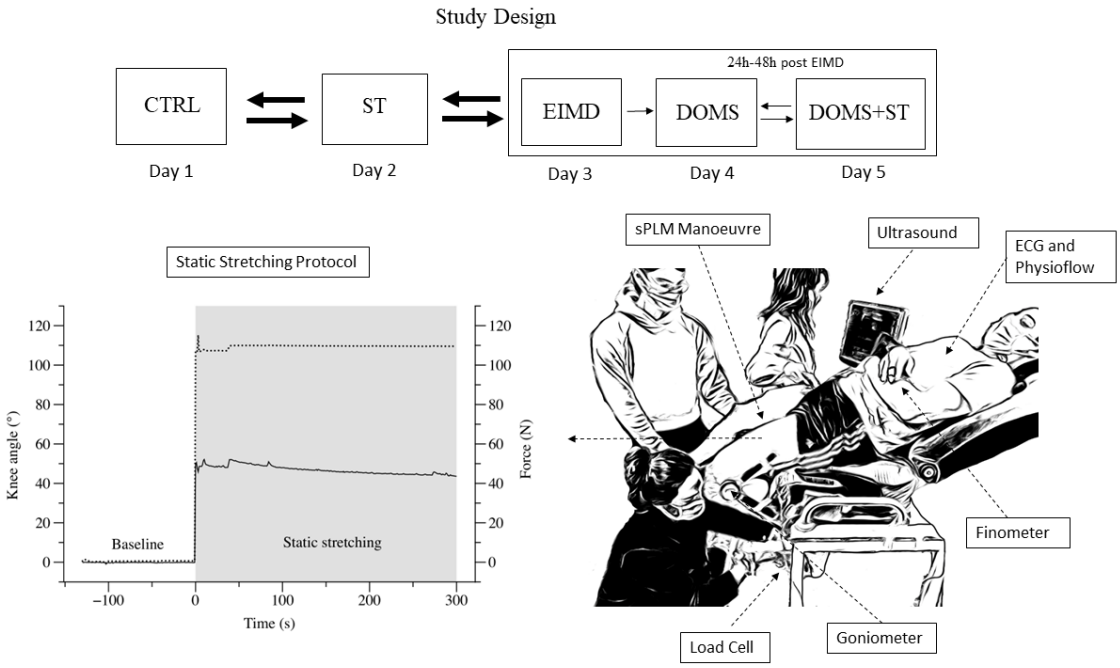


Figure 5.1 Study design and experimental procedures.

On the top center the study design and randomization procedure. On the bottom left is represented with an outlined lined the force registered in the load cell applied to the stretched leg, while the solid line represents the knee angle measured with the electrical goniometer during stretching protocol. On the bottom right is represented the experimental procedures during the different sessions. Abbreviations: Control Condition (CTRL); Stretching condition (ST); DOMS condition (DOMS); and DOMS with stretching condition (DOMS+ST); Exercise Induced Muscle Damage (EIMD).

Experimental protocol.

Blood sampling

The subjects were asked to avoid vigorous physical activity in the 24 h before blood sampling and to fasten from the evening meal until the morning, when samples were obtained. Blood samples were collected before the start of each condition. Blood was sampled from antecubital vein of each subject using a 21-gauge needle. To preserve RNA quality and integrity 3 mL of blood have been collected directly into TEMPUS Blood RNA

tubes (ABI, Foster City, CA, USA) containing 6 mL Applied BioSystems RNA stabilization reagent. These samples were immediately frozen at -20°C. A 6 mL K3-EDTA Vacuette was used for hematology.

Hematological testing

All the samples were processed for routine hematological testing immediately after collection (<15min) on the same Sysmex XN-1000 hematology analyzer (Sysmex, USA) using standard local procedures at GB Rossi Hospital, Verona, Italy. The parameters tested included red blood cells count (RBC), white blood cells (WBC) count, and WBC differential, including lymphocytes, monocytes, neutrophils, eosinophils, basophils and large unstained cells, platelet count, mean platelet volume. The instrument was calibrated against appropriate proprietary reference standard material and verified with the use of proprietary controls.

Total RNA preparation

Total RNA was isolated from the blood samples using Tempus Spin RNA Isolation Kit (Applied Biosystems) as previously described (361). Quality of the purified RNA from was verified on an Agilent® 2100 Bioanalyzer (Agilent Technologies, CA); RNA concentrations were determined using a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, DE).

Quantitative real time PCR

For quantitative Real Time-PCR assays, total RNA was characterized by electrophoresis (Agilent 2100 Bioanalyzer, CA). 400 ng of RNA was converted to cDNA using random primers and Superscript III (Invitrogen, CA). Amplification was carried out in using SYBR green chemistry (Fast SYBR green master mix Applied Biosystems) and a standard 2-step protocol. The coefficients of variation for gene expression assays triplicates were 0.5 ± 0.2%; min-max [0.1-1.6] on average for all genes analyzed. The primers specific for each gene are reported below. Identity of the amplicons was confirmed by their dissociation profiles and gel analysis. Quantitative PCR experiments were performed in triplicate for each sample. The data were normalized against *Gapdh* housekeeping gene.

Primer's list:

P2X4

F: TCCGTCTTGGCAAAATAGTG
R: AGGTTGCAGTCCCAGTTGAC

IL1B

F: CTGTCCTGCGTGTTGAAAGA
R: TGAAGACAAATCGCTTTTCCA

IL10

F: TGCTGGAGGACTTTAAGGGTTA
R: GGGTCTTGGTTCTCAGCTTG

ASIC3

F: TTCTGGAACCGACAGCACTC
R: GAGGGGTGGGAGGTCTGG

TRPV1

F: AACTGGACCACCTGGAACAC
R: GCCTGAAACTCTGCTTGACC

GAPDH-6

F: CAGCCTCAAGATCATCAGCA
R: GTCTTCTGGGTGGCAGTGAT

Static stretching protocol and range of movement assessment. During the familiarization visit participant's maximal knee flexion range of movement (ROM) was assessed on the non-dominant limb with the participants in the supine position. All assessments were conducted by the same operator, who moved the participant's non-dominant joint through a 50° range of motion (knee extension) until reaching the point of tolerable maximal flexion where subjective tension-discomfort was rated using Visual Analog Scale (VAS) and Pain Numeric rating scales (P-NRS) (302). VAS was used to self-reported perception of stretching intensity, using a 100-mm scale in which participants rated their perception of stretching intensity from zero (no stretch at all) to ten (maximal stretch as possible). Moreover, subjective feeling of pain was recorded with P-NRS scale, rating the pain arising from the stretching protocol from zero (no pain at all) to ten (pain as bad as it could be). Static stretching consisted of passive single knee flexion of 6 minutes which spanned the 5 minutes of baseline, sPLM maneuver and 1 minute recovery on the contralateral leg to the point of maximal flexion at rest and lasting the entire duration of

the time of the sPLM protocol. During stretching protocol, knee joint angle was continuously recorded using a biaxial electro goniometer (Twin axial Goniometer TN1750/ST ADI Instruments Systems, Oxford, UK). During the entire stretching protocol, the knee extensors were stretched to the same range of motion obtained during the initial ROM assessment (362) which was kept identical for all stretching conditions (ST and DOMS+ST respectively). An adjustable load cell was also fixed on the participant's non-dominant ankle and held during the experiment by the same operator measuring the force applied from the flexed stretched non-dominant leg during the entire protocol (Fig. 1).

Exercise induced muscle damage protocol. A warm-up of 10 non-dominant single leg isokinetic knee extensions and knee flexions were carried out through the full test range of motion, ensuring a progressive increase in effort, using an isokinetic dynamometer (Cybex, division of Lumex Inc., Ronkonkoma, NY, USA). A single leg maximal isometric voluntary contraction (MVIC) of the non-dominant limb was assessed prior to the EIMD protocol, where participants were instructed to exert the maximal voluntary isometric contraction during a leg extension movement at 90°. The EIMD protocol, consisted of several blocks of 3 sets of 12 maximal voluntary eccentric single knee extensions of the non-dominant limb with 30 seconds of recovery. Following each block, a MVIC was performed to determine the loss of muscle strength from baseline. Exercise was stopped once MVIC was reduced by 40% or more from baseline (9). The eccentric phase of the contractions was performed at an angular velocity of $90°\cdot s^{-1}$. The concentric phase was

performed sub-maximally at an angular velocity of $90°\cdot s^{-1}$ to minimize fatigue and enhance eccentric damage (363). To ensure the presence of DOMS after EIMD protocol a series of tests were carried out before the starting of each condition (311). Pain Pressure Threshold (PPTS) and indirect measurements of muscle damage were used to assess the entity of DOMS after EIMD (364). PPTS were assessed to underline mechanical hyperalgesia following EIMD using a mark placed on different points of the non-dominant quadriceps using a mechanical pressure algometer (Hilitand, NK-100 Force Gauge, USA) which were standardize between participants and marked to being kept similar between condition (365). Indirect measures of muscle damage were assessed trough MVIC using a Biodex dynamometer (Biodex, Shirley, NY, USA) (297) and a 100-mm visual analog scale (VAS) anchored on the left edge of the scale with the phrase "no pain or soreness" and on the right edge "worst pain/soreness imaginable"(305). Participants were asked to rate their pain-related soreness during their ~~for~~ daily living activities (VAS_{DA})(311) and after performing a squat at approximate 90 degrees of knee angle (VAS_{SQ}) (297).

Single Passive Leg Movement Test. SPLM was implemented as a testing procedure for assessing vascular function during all sessions. Participants remained rested in the upright-seated position for 20 min before the start of data collection and remained in this position throughout the study. The sPLM protocol consisted of 5 min of resting baseline data collection followed by one passive knee flexion and extension, which took 1 s, after which the leg was maintained fully extended for the remaining 59s of post movement data collection (366).

Leg blood flow and leg vascular conductance. Measurements of arterial blood velocity and vessel diameter were performed in the common femoral artery of the dominant leg (i.e., passively moved leg), distal to the inguinal ligament and proximal to the deep and superficial femoral bifurcation with a Logiq-7 ultrasound Doppler system (General Electric Medical Systems, Milwaukee, WI) (366). The ultrasound Doppler system was equipped with a 12- to 14-MHz linear array transducer. Artery diameter was determined at a 90° angle along the central axis of the scanned area. Mean blood velocity (Vmean) was measured using the same probe utilizing a frequency of 5 MHz Measurements of Vmean were obtained with the probe positioned to maintain an insonation angle of 60°, and the sample volume was centered and maximized according to vessel size. Utilizing arterial diameter and Vmean, femoral blood flow (FBF) was calculated second by second as:

$$FBF = V_{mean} \times \pi \times \left(\frac{Vessel\ Diameter}{2}\right)^2 \times 60$$

where FBF is in milliliters per minute. All scanning and blinded analyses were performed by experienced and skilled sonographers.

Autonomic and central haemodynamics. HR was assessed using a 3-leads electrocardiogram. Beat-by-beat arterial pressure was determined by finger plethysmography on the non-dominant hand (Finapress model 2300; Ohmeda,

Englewood, CO, USA). Automatic calibration was turned off during data collection. The photoplethysmography cuff of the finger pressure device was placed on the third finger of the left hand. The subject's arm was supported by an armrest to avoid arm and finger movement. The Finometer signal was calibrated utilizing the procedure indicated by the manufacturer. All the signals were amplified and recorded trough the Power Lab System (PowerLab 16/30; ML880, ADInstruments, Bellavista, NSW, Australia). A non-invasive thoracic impedance cardiograph (Physio Flow®, Manatec, Strasbourg, France) was used to measure heart rate (HR) and estimate stroke volume (SV).

Data Collection and Analysis. V_{mean} of the femoral artery blood was analyzed for 30 s at baseline and for 60 s during the sPLM test. Before analysis, all hemodynamic data were smoothed using a 3-s rolling average (355). As the response to sPLM is transient and varies between individuals, a peak response was determined for all variables on an individual basis. Maximal absolute peak (peak), relative change calculated as the peak minus the baseline (Δpeak) and area under the curve (AUC) were determined after normalization for baseline for all variables for each subject as the summed response for 60 sec (367) for each subject in all measured variables (368). Mean arterial blood pressure (MAP) was calculated as (1/3 SBP + 2/3 DBP). Leg vascular conductance (LVC) was calculated as FBF/MAP. Cardiac output (CO) was calculated as stroke volume * HR (369). MAP, R-R peaks traces, and knee joint angle and force output were A/D converted using LabChart Pro software (LabChart Pro 8, with HRV Module, ADInstruments, Bellavista, NSW, Australia).

Delta for singular effects were calculated deducting CTRL values for all outcomes from ST and DOMS respectively. Delta for interaction were calculated summing previously singular delta ST and delta DOMS and compared with delta between CTRL and DOMS+ST condition. The interaction mode (hypo-additive, hyper-additive or additive) was defined as reported by Wan and colleagues (370). Briefly, hyper-additive, additive or hypo-additive effects refers to an observed response that during the synergic activation of the reflexes is respectively larger, equal or smaller than the sum of the response evoked by each reflex alone (370).

HRV analysis calculations were performed for the 300 seconds at baseline before the sPLM maneuver using (LabChart Pro 8, with HRV Module, ADInstruments, Bellavista, NSW, Australia). RR intervals trace was checked and edited for artifact by visual inspection (371). Root mean squared of successive intervals (RMSSD) was calculated as an index of HRV from the RRi series. Frequency domain analysis for HRV were performed through spectral decomposition of the RRi signal using Fast Fourier Transform via Welch's method with Hanning window in 256 sample segments with 50% overlap (371). Low frequency (LF, 0.04 – 0.15 Hz) and high frequency (HF, 0.15 – 1.0 Hz) were calculated as integrals under the respective power spectral density curve, LF/HF was calculated as the ratio between the low and high frequency power (372).

Statistical Analysis. Normal distribution of the data was assessed with a Shapiro-Wilk test. Student's paired t-test was implemented to determine differences between VAS pain and VAS stretching intensity within stretching measurements (ROM and Force) during ST and

DOMS+ST data. One-way repeated measures ANOVA with Tukey-B post-hoc analysis was implemented for gene expressions (P2X4, ASIC3, TRPV1, IL1β, IL10), baselines and sPLM maneuver outcomes for central and peripheral haemodynamics(FBF, MAP, LVC, CO, SV, HR and AUC) within and outcomes of autonomic responses. A Bonferroni-Holm correction was performed for deltas interaction for all peripheral and central haemodynamics outcomes (370). Pearson single correlation analysis were implemented between autonomic responses values of resting and delta peak for FBF and LVC, across all conditions. A sample size of eight participants was selected to ensure a statistical power higher than 0.80 with a type 1 error <0.05 to detect ~15-20% in FBF (main outcome) under stretching conditions (368). All data were analyzed using a statistical software package Graph Pad Prism v.9 (GraphPad Software, San Diego, California USA). Data are presented as mean ± standard deviation (SD) and considered significant when $p<0.05$.

5.3. Results

All participants took part in the study and completed all sessions without reporting any position or postural discomfort during the stretching procedures. Participants were set at the same knee angle during ST and DOMS+ST condition (54.9 ± 4.9 vs 55.1 ± 4.7 degrees; $p>0.05$), and the force detected were similar between conditions (67.1± 22.2 vs 77.4 ± 24.5 N for ST and DOMS+ST, respectively; $p>0.05$). VAS for stretching intensity were similar between participants and between the two stretching conditions (7.1 ± 1.3 vs 8.1 ± 1.6 cm for ST and DOMS+ST, respectively; $p>0.05$). VAS for pain intensity was higher for DOMS+ST compared with ST (7.0 ± 2.6 cm vs. 2.6 ± 1.9, $p<0.05$).

Direct and Indirect measures of DOMS. Results and comparison for DOMS are reported in Table 5.1. Mean leg extensor MVC, PPTS, VAS_{DA} and VAS_{SQ} were not different between CTRL and ST conditions ($p>0.05$). However, MVC decreased significantly in DOMS and DOMS+ST compared with CTRL and ST condition (all $p<0.05$). PPTS decreased significantly in DOMS and DOMS+ST compared with CTRL and ST condition (all $p<0.05$). VAS_{DA} was increased in DOMS and DOMS+ST compared with CTRL conditions ($p<0.05$). VAS_{DA} also increased significantly from ST condition to DOMS and DOMS+ST ($p<0.05$). VAS_{SQ} increased significantly in DOMS and DOMS+ST compared with CTRL and ST conditions respectively (all $p<0.05$).

Table 5.1 Direct and Indirect measurements of DOMS.

Variable	**CTRL**	**ST**	**DOMS**	**DOMS+ST**
MVC (N)	686 ± 121	682 ± 130	422 ± 170*	432 ± 197*
PPTS (kg)	6.05 ± 1.30	5.89 ± 1.46	3.99 ± 1.19*	3.92 ± 1.49*
VAS daily activities (mm)	0.41 ± 0.27	0.67 ± 0.51	53.88 ± 28.11*	54.25 ± 27.37*
VAS squat (mm)	0.49 ± 0.36	0.70 ±0.28	46.50 ± 28.07*	51.00 ± 20.45*

Data are presented as mean ± standard deviation. MVC = Maximal Voluntary Contraction; PPTS = Pain Pressure Thresholds; VAS = Visual Analog Scale; CTRL = Control Condition; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition; *$p<0.05$ respect to CTRL.

Blood Cell Count and Gene expression. All blood test results and comparison for gene expression across conditions are reported in Table 5.2. We found P2X4 expression significantly upregulated in DOMS and DOMS+ST condition (all $p<0.05$). The changes in the expression levels of TRPV1 and ASIC3 follow a similar pattern, although variations reach statistical significance only for TRPV1 in DOMS+ST ($p<0.05$). IL1B gene showed a sustained increase in expression levels in the responses for DOMS and DOMS+ST condition (all $p<0.05$). No relevant variation was displayed by IL10 gene. No changes in white blood cells, monocytes, lymphocytes and neutrophils and platelets were found between conditions however red blood cells count statistically decreased in DOMS and DOMS+ST condition compared with baseline ($p<0.05$).

Resting Measurements. All results and comparison for central and peripheral haemodynamics at rest are reported in Table 5.2. Resting FBF and LVC were significantly decreased in DOMS+ST condition (all $p<0.05$) compared to CTRL. Resting MAP, HR, CO and SV were increased in DOMS+ST (all and ST condition compared to CTRL (all $p<0.05$). Resting MAP, HR and SV were increased in ST condition compared to DOMS ($p<0.05$). Moreover, resting MAP, HR, CO, and SV were increased in DOMS+ST compared to DOMS (all $p<0.05$). Resting MAP and SV increased in DOMS+ST compared with ST (all $p<0.05$). FBF, LVC, HR, MAP, CO, SV were not different between CTRL and DOMS conditions. A Statistical difference was also found for RMSSD, HF and HF/LF ratio between CTRL and DOMS+ST condition ($p<0.05$).

Table 5.2 Resting peripheral and central haemodynamics with autonomic responses and blood gene expression.

Variable	**CTRL**	**DOMS**	**ST**	**DOMS+ST**
FBF (ml/min)	316 ± 80	249 ± 135	246 ± 106	198 ± 72§
LVC (ml/min/mmHg)	3.5 ± 1.0	2.6 ± 0.8	2.5 ± 1.1	1.8 ± 0.3§
MAP (mmhg)	92 ± 3	92 ± 4	107 ± 5§†	117 ± 3 $^{\S\dagger *}$
Heart Rate (bpm)	68 ± 3	60 ± 2	81 ± 4 §†	99 ± 4 $^{\S\dagger *}$
Cardiac Output (l/min)	5.9 ± 0.6	5.8± 0.7	8.0 ± 0.8§	10.7-± 0.9§†
Stroke Volume (ml)	88 ± 7	91 ± 6	99 ± 6 §†	109 ± 5$^{\S\dagger *}$
RMSSD (ms)	52 ± 21	38 ± 25	41 ± 18	31 ± 16§
LF/HF (ms^2)	1.7 ± 0.7	2.5 ± 1.2	2.7 ± 1.2	2.9 ± 1.2§
P2X4 (FC)	0.9 ± 0.1	1.4 ± 0.4§	0.8 ± 0.2	1.5 ± 0.6§
ASIC3 (FC)	0.9 ± 0.1	1.4 ± 1.1	0.9 ± 0.2	1.4 ± 0.5
TRPV1 (FC)	0.8 ± 0.1	1.5 ± 0.6	0.9 ± 0.2	1.5 ± 0.4§
IL1β (FC)	0.8 ± 0.2	1.7 ± 0.5§	1.0 ± 0.4	1.6 ± 0.4§
IL10 (FC)	0.9 ± 0.2	1.3 ± 0.8	1.3 ± 0.5	1.3 ± 0.5
RBC ($10^{12}cell*L^{-1}$)	5.0 ± 2.7	4.7 ± 2.4$^{\S *}$	4.9 ± 1.9	4.7 ± 1.8$^{\S *}$
WBC ($10^{9}cell*L^{-1}$)	6.8 ± 2.2	6.8 ± 2.4	6.8 ± 2.6	6.8 ± 2.5
PLT ($10^{9}cell*L^{-1}$)	2.8 ± 0.2	2.8 ± 0.4	2.4 ± 0.6	2.5 ± 0.5
LYMPH ($10^{9}cell*L^{-1}$)	2.8 ± 0.1	2.8 ± 0.4	2.4 ± 0.3	2.5 ± 0.5
MONO ($10^{9}cell*L^{-1}$)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.1
NEU ($10^{9}cell*L^{-1}$)	4.0 ± 0.7	3.9 ± 1.4	4.1 ± 1.1	3.7 ± 1.2

Data are presented as mean ± standard deviation. FBF= Femoral Blood Flow; LVC= Leg Vascular Conductance; bpm= beat per minute; MAP= mean arterial pressure; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition; RMSSD = Root mean squared of successive differences; LF/HF = ratio between Low frequency and high frequency of the heart rate variability; P2X4 = purinergic-2X4 receptor; ASIC3 = acid sensing ion channel 3*;*

TRPV1 = transient receptor potential cation channel subfamily V member 1; IL1β = interleukin 1β ; IL10 = interleukin 10; RBC = red blood cell count; WBC = white blood cells counts; PLT = Platelet; LYMPH = lymphocytes; MONO = Monocytes ; NEU = Neutrophil; § p<0.05 respect to CTRL, †p<0.05 respect to DOMS. *p<0.05 compared to ST.

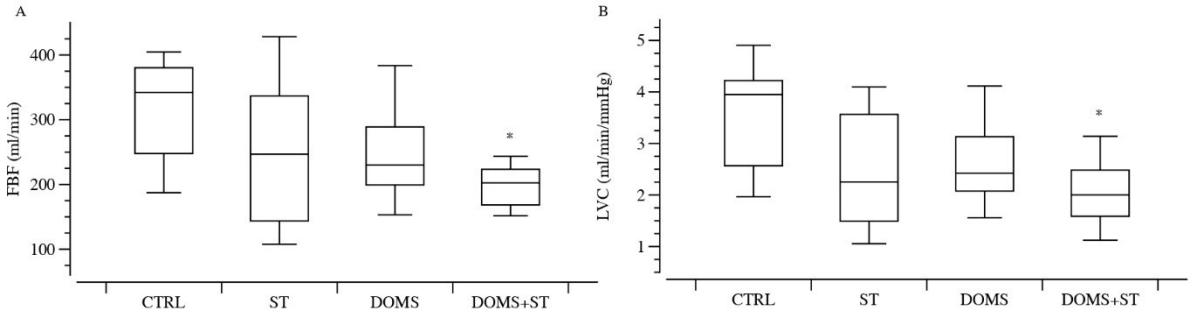


Figure 5.2 Changes in peripheral hemodynamic responses at rest

Abbreviations: Control condition (CTRL), Stretching condition (ST), DOMS condition (DOMS) and DOMS with stretching condition (DOMS+ST) respectively. A: Femoral Blood Flow (FBF); B: Leg Vascular Conductance (LVC); *significantly different from CTRL.

Central and Peripheral Haemodynamics during sPLM. All results and comparison are reported in Table 5.3. ΔPeak FBF and LVC significantly decreased in DOMS+ST conditions (all p<0.05) compared with CTRL. FBF and LVC AUC decreased significantly from CTRL to ST and DOMS+ST conditions (all p<0.05) (Table 5.3). Δpeak for MAP, SV, HR, and CO within respectively AUC were not statistically different between all conditions (all p>0.05) (Table 5.3).

Table 5.3 Peripheral and central haemodynamics during sPLM.

Variable	**CTRL**		**DOMS**		**ST**		**DOMS+ST**	
	Δ Peak	AUC	Δ Peak	AUC	Δ Peak	AUC	Δ Peak	AUC
FBF (ml/min)	802 ± 250	1322 ±377	545 ± 228	1113 ± 542	423 ± 202	956 ± 327	390 ± 146§	745 ± 192§
LVC (ml/min/mm Hg)	8.6 ± 3.6	15 ± 5.1	6.1 ± 3.3	11.8 ± 6.6	4.4 ± 1.8	9.7 ± 3.4	2.7 ± 1.1§	6.9 ± 1.7§
MAP (mmhg)	-0.2 ± 0.1	0.1 ± 0.1	-0.2 ± 0.1	0.1 ± 0.2	-0.2 ± 0.1	0.1 ± 0.1	-0.2 ± 0.1	0.1 ± 0.1
Heart Rate (bpm)	2.0 ± 0.7	1.0 ± 0.3	2.1 ± 0.5	1.0 ± 0.4	1.9 ±1.2	0.9 ± 0.6	1.8 ±1.2	0.9 ± 0.6
Cardiac Output (l/min)	0.2 ± 0.1	0.19 ± 0.04	0.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
Stroke Volume (ml)	0.6 ± 0.2	0.3 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	0.6 ± 0.3	0.3 ± 0.2	0.5 ± 0.4	0.3 ± 0.2

Data are presented as mean ± standard deviation. FBF = Femoral Blood Flow; LVC = Leg Vascular Conductance; MAP = mean arterial pressure; Δpeak = delta peak; AUC = area under the curve; CTRL = Control Condition; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS + ST = delayed onset muscle soreness with stretching, condition; §=$p<0.05$ respect to CTRL; *=$p<0.05$ respect to ST.

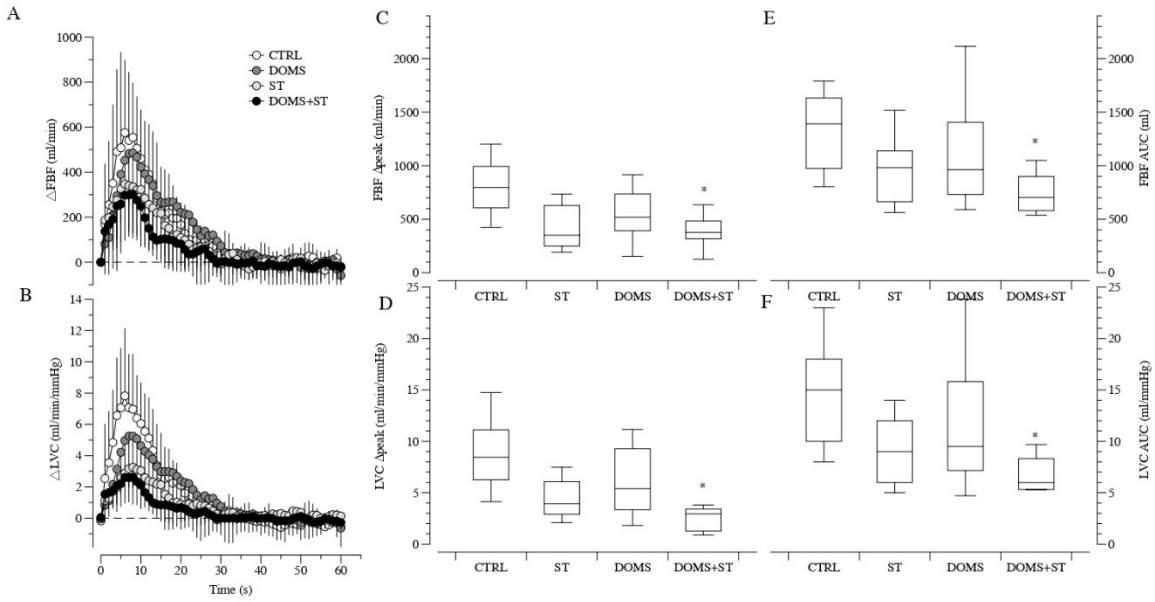


Figure 5.3 Changes in central and peripheral haemodynamics responses to sPLM maneuver.

A: Femoral Blood Flow Haemodynamics (FBF); B: Leg Vascular Conductance Haemodynamics (LVC); C: Peak ΔFBF; D: Peak ΔLVC; E: FBF Area Under the Curve (AUC); F: LVC Area Under the Curve (AUC); *significantly different from CTRL vs DOMS+ST.

Delta Interaction.

All results and comparison are reported in Table 5.4. The delta interaction (ΔDOMS+ΔST vs ΔDOMS+ST) showed no statistical differences in peripheral haemodynamics at rest (FBF, LVC) central haemodynamics(CO, SV) and autonomic responses (RMSSD and LF/HF ratio), resulting in an additive effect of DOMS+ST condition compared with the combination of the singular effects from ST and DOMS condition respectively. However, differences were found in resting HR, CO and MAP in ΔDOMS+ST compared with ΔDOMS+ΔST, showing hyper-additive effect for these parameters ($p<0.05$). No differences were found in vascular responsiveness outcome for the delta interaction.

Table 5.4 Effects of singular and combined reflex effects

Variable	**ΔST**	**ΔDOMS**	**ΔDOMS+ ΔST**	**ΔDOMS+ST**	**Effect**
LVC (ml/min/mmHg)	-1.1 ± 1.3	-0.9 ± 1.0	-2.0 ± 2.2	-1.7 ± 0.9	Additive
FBF (ml/min)	-68 ± 133	-70 ± 92	-138 ± 210	-1.7 ± 0.9	Additive
MAP (mmhg)	13.5 ± 1.8	-3.0 ± 7.4	10.4 ± 8.8	31.8 ± 2.9*	Hyper-additive
Heart Rate (bpm)	15.5 ± 2.4	0.0 ± 3.6	15.5 ± 4.4	25.8 ± 3.6*	Hyper-additive
Cardiac Output (l/min)	-2.2-± 0.5	-0.3-± 1.0	-2.4-± 0.9	-5.0-± 0.8*	Hyper-Additive
Stroke Volume (ml)	12.5 ± 8.7	9.1 ± 15.1	21.7 ± 18.3	22.8 ± 9.0	Additive
RMSSD (ms)	-10.3 ± 6.0	-13.6 ± 16.3	-23.8 ± 17.1	-20.9 ± 14.1	Additive
LF/HF (ms^2)	0.8 ± 0.8	0.9 ± 1.5	1.8 ± 1.4	1.2 ± 0.9	Additive
FBF Δpeak (ml/min)	-378 ± 288	-257 ± 263	-636 ± 494	-411 ± 281	Additive
LVC Δpeak (ml/min)	-4.4 ± 3.2	-2.7 ± 3.4	-7.1 ± 5.7	-6.09 ± 3.64	Additive
MAP Δpeak (mmhg)	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	Additive
Heart Rate Δpeak (bpm)	0.0 ± 0.5	0.1 ± 0.9	0.1 ± 1.2	0.2 ± 1.0	Additive
Cardiac Output Δpeak (l/min)	0.0 ± 0.1	0.0 ± 0.2	0.0 ± 0.2	0.0 ± 0.2	Additive
Stroke Volume Δpeak (ml)	0.0 ± 0.2	0.0 ± 0.3	0.0 ± 0.4	0.0 ± 0.3	Additive
FBF AUC (ml)	-367 ± 334	-210 ± 493	-578 ± 735	-579 ± 335	Additive
LVC AUC (ml/mmhg)	-5.1 ± 3.6	-3.1 ± 5.4	-8.2 ± 7.7	-7.7 ± 4.1	Additive
MAP AUC (mmhg)	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	Additive
Heart Rate AUC (bpm)	0.0 ± 0.5	0.1 ± 0.4	0.0 ± 0.6	0.1 ± 0.5	Additive
Cardiac Output AUC (l)	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	Additive
Stroke Volume AUC (ml)	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	Additive

Data are presented as mean ± standard deviation. FBF= Femoral Blood Flow; LVC= Leg Vascular Conductance; bpm= beat per minute; MAP= mean arterial pressure; AUC = area under the curve; Δpeak = delta peak; ΔDOMS= delta between delayed onset muscle soreness and control conditions; ΔST= delta between stretching and control conditions; ΔDOMS +ΔST = delta delayed onset muscle soreness summed with delta stretching; ΔDOMS +ST= delta between DOMS+ST and control conditions; RMSSD = Root mean squared of successive differences; LF/HF = ratio between Low frequency and high frequency of the heart rate variability; *=$p<0.05$ compared to ΔDOMS+ ΔST.

Single correlation analysis.

RMSSD was inversely correlated with increases in HR ($r=-0.46$; $p<0.05$), MAP ($r=0.44$; $p<0.05$) and CO ($r=0.40$; $p<0.05$) whereas as LF/HF was found positively correlated with increases in HR ($r=0.44$; $p<0.05$), MAP ($r=0.38$; $p<0.05$) and CO ($r=0.40$; $p<0.05$). Moreover, ASIC3 was inversely correlated with RMSSD ($r=-0.49$; $p<0.01$) and positively correlated with LF/HF ratio ($r=0.58$; $p<0.01$). TRPV1 was found inversely correlated with RMSSD ($r=-0.37$; $p<0.05$) within LF/HF ratio ($r=-0.37$; $p<0.05$). P2X4, ASIC3 and TRPV1 correlated with MVC ($r=-0.72$; $r=-0.51$; $r=-0.72$; all $p<0.01$); VAS_{DA} ($r=0.67$; $r=0.57$; $r=0.78$; all $p<0.01$) and VAS_{SQ} ($r=0.71$; $r=0.55$; $r=0.72$; all $p<0.01$). PPTS correlated with P2X4 and TRPV1 ($r=-0.38$; $r=-0.36$; all $p<0.05$).

5.4. Discussion

This is the first study investigating the singular and combined effects of mechano- and nociceptor activation on central and peripheral haemodynamics and vascular responsiveness. We found that only the combination of mechanoreflex and nociceptor activation promotes greater changes on central and peripheral haemodynamics at rest with reduced vagal activity (reduced RMSSD) and concomitant increases of sympathetic drive (increased LF/HF ratio) (Fig 5.2; Table 5.2), that inversely and positively correlate with HR, CO, and MAP respectively. Moreover, when both the mechanoreflex and nociceptors were activated, the contralateral leg exhibited a reduction in leg blood flow and vascular responsiveness to sPLM in remote muscles (Fig 5.3). These results, suggest

that the stimulation of mechano- and nociceptive afferents trigger increases in central haemodynamics mediated by vagal tone suppression and increase in sympathetic drive (hyper-additive and additive effects). The sympathetic gain is also responsible, at least in part, for the reduction in resting limb blood flow and decreased vascular responsiveness with additive effects (Fig 5.4). Interestingly, the singular stimulation of the mechano- and nociceptive afferents via passive static stretching of the skeletal muscle or the DOMS resulted in negligible changes of the peripheral circulation. Furthermore, genes associated with pain and mechanoreceptors activity (P2X4, TRPV1), with marker of increases inflammation (IL1β), increases following EIMD suggesting a possible mechano- and nociceptive sensitization of nerve endings afferents. Therefore, in agreement with our initial hypothesis, only the sensitization of both mechano- and nociceptors resulted in autonomic-mediated increase in central haemodynamics and a concurrent reduction of peripheral circulation and vascular responsiveness in remote skeletal muscles.

Exercise induced muscle damage and stretching as a model to study mechanical hyperalgesia and mechanoreflex sensitization.

In our experiment we applied EIMD to induce DOMS and mechanical hyperalgesia, with concomitant application of static stretching in the damaged muscle to activate mechanosensitive and nociceptive afferents (Fig. 5.4). Previous animal studies reported an increased mechanical sensitization of Aδ and C-fibers and concomitant mechanical hyperalgesia following EIMD (15, 16, 30). Moreover, other studies revealed a mechanical sensitization of large mechanical fibers in humans (29, 341) after EIMD. In line with these

reports, we found an increased mechanical hyperalgesia (from reduced PPTs), with increased self-reported pain during DOMS+ST condition compared with ST alone, suggesting an increased mechano- and nociceptive activation. Moreover, we found an increased gene expression in P2X4 channel, and a positive trend in ASIC3 and TRV1 in DOMS and DOMS+ST. These data are in line with previous investigations on chronic pain, and mechanical hyperalgesia in different patient's population (344, 347) and exercise induced muscle damage (16, 17, 30, 373). From the single correlation analysis, we found that these genes correlated with markers of EIMD (PPTS, VAS and MVC), suggesting that the experience of the soreness and hyperalgesia following EIMD was correlated with the higher gene expression.

Evidence of mechano- and nociceptors activation on the peripheral and central haemodynamics at rest.

Vascular function within peripheral haemodynamics has been previously found to be impaired after EIMD (349). Indeed, despite the big impact EIMD has on muscle function, it also seems to impair the cardiovascular system, particularly endothelial and microvascular function (374) with increases in arterial stiffness (349). These impairments have been linked to the increased inflammatory response following EIMD in the damaged limb (349). Although several studies found an impaired vascular function in the skeletal muscle directly affected by DOMS, limited studies investigated the possible cross-over effects of DOMS on remote skeletal muscle (291, 351). In the current study we did not find reduced blood flow in the contralateral leg at rest during DOMS compared with CTRL

condition, despite higher IL1β levels. Our findings are in line with Caldwell et al. (351) who found that systemic vascular function measured with FMD, was not affected following EIMD in a remote healthy muscle. However, some studies found a close relationship between pro-inflammatory cytokines and decreased vascular function (375-377). Moreover, studies on healthy volunteers following vaccine-induced inflammation found decrease vascular function (decreased FMD and increased arterial stiffness) (378, 379) while in the aging population, this effect was not appreciable (380). The discrepancies between these studies have also been attributed to different levels of systemic inflammation following influenza-vaccine administration (380). This could have been the case in our current study where the level of inflammation following EIMD may have not been sufficient to cause a level of systemic inflammation necessary to induce systemic vascular function impairment (351). Unfortunately, we can not completely rule out the effect of inflammation to systemic vascular function impairments, so future studies in healthy humans should more extensively monitor inflammation (i.e., TNF-α activity) during DOMS, to elucidate this relationship. Looking at the stretching condition, no significant changes in peripheral haemodynamics were found although an overall reduction in FBF and LVC compared with CTRL. Reduction of peripheral haemodynamics was previously found in the contralateral (resting) leg following static stretching protocol, suggesting an increased sympathetic-mediated vasoconstriction from activation of mechanoreceptors as a potential mechanism for this attenuation (368). The entity of these changes however may differ between the current study due to the distinct stretching protocol implemented, which may have led to different mechanoreflex

activation and peripheral haemodynamics changes. Interestingly, one of the major findings of the current study is a decreased FBF and LVC at rest in DOMS+ST conditions. In fact, the singular and distinctive effect of mechanoreflex (ST condition) or nociceptive (DOMS condition) reflexes stimulation seems to not be sufficient to alter peripheral haemodynamics (Table 5.4). Recently, studies of cardiovascular reflexes have brought attention to the importance of the individual and interactive relationships between cardiovascular reflexes, suggesting hypo-additive, hyper-additive, or additive effects on peripheral and central haemodynamics(370). Reduction of peripheral and central haemodynamics in DOMS+ST condition may have been a consequence of a hypo-additive effect (370) after mechanoreflex and nociceptive activation due to nerve peripheral sensitization.

Indeed, we found increases in blood gene expression for P2X4, and TRPV1 that have been linked to cardiovascular function (381), with P2X4 receptors be linked to nociceptive and mechanoreflex sensitization (373, 382). Purinergic receptors seem to act as mediators in peripheral vasoconstriction by ATP released from sympathetic nerve activation (383), their sensitization after EIMD may have led to an increase sympathetic nerve activation. So, it may be concluded that the stimulation of mechano- and nociceptive reflexes leads to additive effects in reducing peripheral haemodynamics at rest compared with the singular effects alone.

Regarding changes on central haemodynamics, previous studies suggest a possible alteration following EIMD where an increased blood pressure and HR responses was

found during isometric exercise (291, 292). Interestingly, resting HR and MAP appeared to not be impacted by EIMD at baseline (292, 349, 384). In line with these studies, we did not find any differences in central haemodynamics at rest between CTRL and DOMS conditions. On the other hand, central haemodynamics increased in ST conditions compared with CTRL, as previously reported, following static and dynamic quadriceps stretching protocols (353, 354, 362). This has been suggested by different authors to be linked to increase parasympathetic withdrawal after the onset of muscle stretch (385, 386) combined with an increased vascular resistance within the stretched limb. This mechanism has been attributed to the muscle lengthening that increase activation of perivascular sympathetic nerves, or cell to cell signaling, resulting in norepinephrine release (387, 388), with increased vasculature resistance and decreased blood flow (389). Furthermore, HR, MAP and SV were significantly different in DOMS+ST conditions compared to all conditions, moreover for delta's interaction for resting HR and MAP showed hyper-additive effect of mechano- and nociceptors sensitization on central haemodynamics.

Evidence of mechano- and nociceptors activation on sPLM-induced hyperemia.

Previous studies have shown that EIMD may cause a reduction in vascular hyperemia in the leg impacted by DOMS (350). However, no differences were found in the systemic vascular responsiveness in a remote muscle (brachial artery) 48h post-EIMD (351). In line with this result, we did not find any statistical difference in sPLM-induced hyperemia in DOMS condition in a remote skeletal muscle, suggesting that systemic inflammation

following EIMD did not exert a sufficient effect to decrease systemic vascular function, as suggested in previous model of inflammation (375-377). Interestingly, the major finding of the current study was a decreased vascular responsiveness (FBF and LVC peaks within related AUCs) following sPLM in DOMS+ST condition (Fig. 5.3). These results could be explained by an increased systemic vasoconstriction, following mechanoreceptors sensitization (Fig. 5.4). For instance, previous studies revealed that heightened mechanoreflex sensitivity seems to augment peripheral sympathetic vasoconstriction in response to PLM (239, 357). Moreover, a recent study reported a reduced LVC after superimposed PLM during concomitant exercise executed in different muscles (390), underlining the role that sympathetic vasoconstriction has in attenuating the PLM-induced hyperemia. Indeed, previous investigators have reported an increased sympathetic activity was linked to suppressed vasodilatory responses following exercise or negative pressure stimulation (391), linking the role of sympathetic drive in reducing vascular vasodilatory responses. Despite singular effects of mechano- and nociceptive reflexes were not sufficient to alter vascular responsiveness following sPLM, we found an additive effects in DOMS+ST condition without differences in central haemodynamics response. However, we can not completely exclude that inflammation following EIMD had exert a possible role in decrease vascular responsiveness. Changes in central haemodynamics are usually associated to continues PLM, rather than sPLM, where only a single limb movement is performed, avoiding increases in mechanoreflex activation and central haemodynamics, that is usually associated with continues PLM (366).

Mechanisms of mechanoreflex and nociceptor activation as mediators of peripheral and central haemodynamics alterations at rest and during sPLM.

The intensity and modality of static stretching has suggested to play an important role on mechanoreflex activation and central haemodynamics responses (352). Indeed, constant angle at low/to moderate intensity static stretching have shown to activate mechanoreflex only in the early phase of the stretching protocol increasing slightly the central haemodynamics (277, 353, 362) showing that mechanical tension play an important role on maintaining mechanoreflex discharge. For these reasons in our protocol, we decided to adopt a high intensity static stretching protocol to maintain mechanoreflex activation within higher parasympathetic withdrawal that in turn has elicited strong increasing in resting central haemodynamics. This effect was amplified during DOMS+ST protocol, presumably due to an increased stiffness of the muscle (increased in mechanoreceptors discharge) and increased nociceptive activity coupled with higher rating of self-reported perceived pain and P2X4, TRPV1 gene expression following EIMD.

Although no difference was found in HR, MAP, SV, and CO at rest between CTRL and DOMS conditions, increased central haemodynamics in ST was detected (278, 353, 354) with further increased in DOMS+ST conditions, suggesting an increased parasympathetic withdrawal from heightened mechanoreflex activation. Indeed, previous study has reported a decreased RMSSD in subjects with low flexibility following stretching, finding an impaired sympatho-vagal balance, and increased parasympathetic withdrawal (392). Moreover, in line with this hypothesis, we found a reduced HRV (i.e., RMSSD) during

DOMS+ST condition, with significant correlation in increased HR, CO and MAP, linking a suppression of vagal tone as the main drive to the increased central haemodynamics (385, 386). Decrease in RMSSD was also correlated with increases in ASIC3 and TRPV1 possibly linking increase in parasympathetic withdrawal and mechanical hyperalgesia.

In the current studies we also recorded an increased LF/HF ratio in DOMS+ST, and a positive correlation between LF/HF ratio and increases in central haemodynamics. LF/HF ratio has been proposed as metric to measure sympatho-vagal balance (393) and reflecting an increase in sympathetic activity (372). This result could be explained by an increased stimulation of nociceptors following EIMD due to afferents sensitization. Thus, seen that muscle afferents has been reported to become sensitized after EIMD due to increase inflammation (394) that in turns increases in sympathetic mediated pain activity (348), and seen that LF/HF has shown to be correlated with an increased inflammatory response (395), it may be hypothesize that sympathetic mediated pain from increased nociceptors sensitization may have led to increases in LF/HF ratio during DOMS+ST condition, within concomitant increases in central haemodynamics. However, critiques were raised on the LF frequency and increased LF/HF ratio as a marker of increased sympathetic nerve activity (396), so future studies are needed to confirm its validity as "sympathetic biomarker". From these results it could be hypothesized that the singular effects of mechano- and nociceptive stimulation are not sufficient to elicit strong changes in peripheral haemodynamics and vascular responsiveness while their combination exert an additive effect in DOMS+ST condition. Indeed, increased sympathetically mediated-pain activity, coupled with increased parasympathetic withdrawal, from heightened

nociceptors and mechanoreflex activation, resulted in 1) increased resting central haemodynamics (hyper-additive and additive effect); 2) reduced resting peripheral haemodynamics (additive effect); 3) reduced sPLM-induced hyperemia (additive effect).

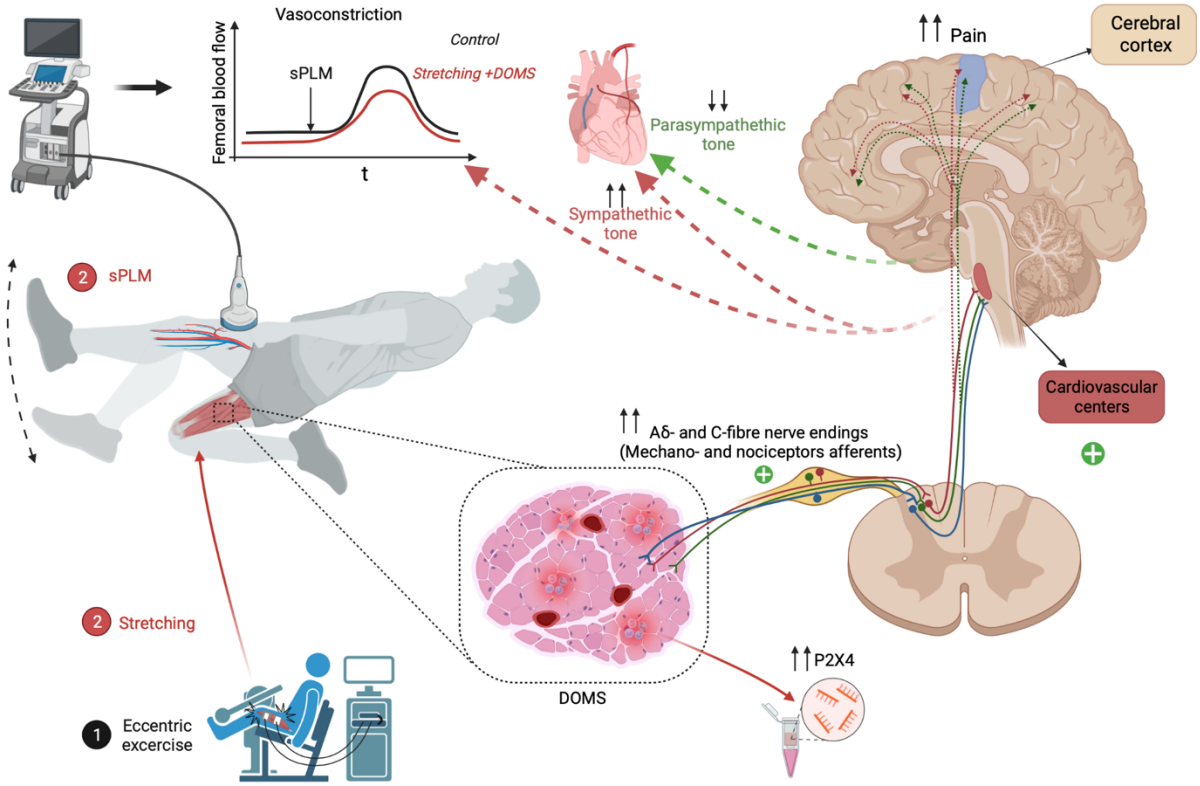


Figure 5.4 Physiological Mechanisms underpinning mechano and nociceptors sensitisation following EIMD. After performing the exercise-induced muscle damage protocol (1) a state of muscle inflammation was initiated in the non-dominant limb that in turn sensitize muscle nerve afferents within nociceptors gene expression (P2X4) present on the Aδ and C-fibers nerve endings, causing delayed onset muscle soreness (DOMS). Furthermore, static stretching protocol (2) was applied to the sensitized muscle to activate the mechanoreflex. These combining effects resulted in an increased activation of the muscle nerve afferents and nociceptors, leading to heightened responses from the cardiovascular centers and concomitant decreased of vagal activity and increase in sympathetic tone, leading to systemic vasoconstriction. The increased activation of the muscle nerve afferents induced a decrease in femoral blood flow at rest (top left) and blunted vasodilation response following sPLM in DOMS+ST condition compared with CTRL.

Implication for research and clinical practice.

Our study highlights the role of mechanoreflex sensitisation on the autonomic nervous system and blood flow regulation in a remote non affected muscle. The current results may shed light on possible mechanisms of increase cardiovascular response and exercise intolerance for people experiencing DOMS. Indeed, previous research has highlighted the cardiovascular and neuromuscular impairments following EIMD (9, 292), and proposed the increased muscle nerve afferents activity as a potential mechanism underlining these impairments (394). Moreover increase in muscle nerve afferent feedback from mechanoreceptors activity has been suggested as a possible cause of elevated cardiorespiratory responses and hyperpnea during exercises after EIMD (337), leading to exaggerated ventilation at the onset of exercise. Similar results were found recently in patients with chronic fatigue, were an increased VE/VO_2, VE/VCO_2 and tidal volume were found (397). Interestingly these outcomes were previously associated with group III-IV muscle nerve afferents activation (398). Indeed, muscle nerve afferents sensitisation seem to play a significant role in cardiovascular diseases, such as heart failure, where a mechanoreflex sensitisation led to dysregulated cardiovascular and cardiorespiratory responses and exercise intolerance (124). More recently, studies have also suggested that mechanoreflex sensitivity may be altered in diseases such as Rheumatoid Arthritis, Myofascial Pain Syndromes and Fibromyalgia (358, 359, 399). From all these evidence it seems that nerve afferent sensitisation is responsible of the dysregulation of different physiological mechanisms linked to exercise intolerance and performance (49). Future

research should focus on the role that muscle peripheral sensitisation may have in exercise intolerance and find targeted intervention to restore its correct functioning

5.5. Conclusions

All these findings, suggest that only the combined effects of sensitized mechanosensitive and nociceptive fibers led to a parasympathetic withdrawal within possible increase in sympathetic drive, increasing central haemodynamics with concomitant decreasing in blood flow at rest and reduced vascular responsiveness to sPLM in a contralateral non-damaged limb. These findings may help to underline the additive interactive effects of mechano- and nociceptors sensitization on blood flow supply in remote muscle, improving the existing knowledge on the effects of a heightened mechano- and nociceptors feedback in pain-related diseases and syndromes.

Limitations:

One possible limitation of the present study is the lack of direct sympathetic measures as usually assessed trough muscle nerve sympathetic activity (MSNA). However, this technique is extremely difficult to implement in a such experimental study design, so we decided to use HRV as a surrogate of autonomic nervous system activity. Another possible limitation is represented by the white blood cells (WBC) gene expression analysis. Despite it is very well known about the interaction of immune-system and nerve afferents sensitization (373) and that previous studies adopted WBC gene expression for studying

chronic conditions and nerve afferents (344, 345), this technique may lack of specificity. Indeed, it may be difficult to define in which region of the human body (e.g., skeletal muscle) there was an increased expression of these receptors. However, seen that the musculoskeletal system was more impacted from EIMD we may infer that most of the inflammation and related sensitization was located on the damaged limb and not elsewhere.

Practical Applications and implications

The results of the current investigation could be helpful to identify the role of peripheral sensitisation on the blood pressure regulation and cardiovascular response to sPLM following muscle injuries or increased peripheral inflammation. Increased mechanoreceptors activity shows to lead to an impairment in muscle blood flow to the contralateral muscle that may result in a lower exercise tolerance and performance once exercising. Moreover, the physiological implication of mechanoreceptors sensitisation following a model of muscle injury and inflammation could inform future studies on the pathophysiological mechanism underlying similar conditions affected by peripheral sensitisation and target effective intervention to improve muscle blood flow, sympathetic responses, and blood pressure regulation and as a consequence perception of pain and effort during exercise or daily living activities.

Contributions

All the authors played a role in the content and writing of the manuscript. In addition, M.V was the principal investigator; M.V, F.Z., and J.S.M. had input into the original idea, study design, and conduct of the study. F.Z., M.V., G.G., T.F., M.M.O. collected the data; F.Z., M.V., F.G.L., and P.D.O. performed data analysis and statistics, and F.Z., F.G.L., E.C. prepared it for presentation. F.Z. and M.V wrote the manuscript. J.S.M., M.V. T.P., A.F, L.B, E.C. and P.D.O reviewed the manuscript.

Acknowledgements

We would also like to thank all participants that took part in this project with a special thanks to L. Sacco, D. Sartorio, O. Modica and E. Pujatti for their contribution in the project, the technical support during the data collection and help during the experimental set up.

Fundings:

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 801604 for F.Z. and P.D.O. This project was also partially supported by the Brain Research Foundation Verona Onlus and by the Italian Ministry of Research and University (MIUR) 5-year special funding to strengthen and enhance excellence in research and teaching (https://www.miur.gov.it/dipartimenti-di-eccellenza).

Chapter 6. Experimental Study III

Sensitivity of group III-IV muscle nerve afferents following EIMD: Role of Mechano- and Nociceptor's activation on Neuromuscular Fatigue

What is known? In the previous chapter (I.e., Chapter 5) we underlined the single and combined activation of mechano- and nociceptors on central and peripheral haemodynamics following EIMD. We showed that afferent sensitisation reduced blood flow on remote muscles, inducing an exaggerated cardiovascular response and parasympathetic withdrawal. Increased activation of group III-IV afferents has been linked to decreased exercise performance and exercise intolerance; however, no studies had yet underlined the activity of mechanoreceptors before and after afferent sensitisation on exercise performance. We also interrogate the possible reason of an exercise performance reduction (I.e., Central and Peripheral Fatigue).

What is going to be assessed? We used an EIMD protocol to induce sensitisation of muscle nerve afferents and static stretching to activate mechanoreceptors while assessing central and peripheral component of fatigue and exercise performance during a time to exhaustion task in remote muscles. We hypothesise that sensitisation of muscle afferents would result in an increased in central fatigue and reduced exercise performance.

Activation of nociceptive and mechanosensitive skeletal muscle fibres following exercise-induced muscle damaged impairs contralateral knee extensor exercise performance.

6.1. Introduction

Neuromuscular fatigue has been defined as the progressive reduction of force or power exerted by a muscle during an exercise task (32). Different factors contribute to the development of neuromuscular fatigue and can be ascribed to a) an impairment of the neural drive to the muscle (central fatigue), or b) a biochemical impairment occurring at or distally to the neuromuscular junction (i.e., peripheral fatigue) (43). Central and peripheral components of neuromuscular fatigue are interconnected and mutually regulated by the activity of group III-IV muscle afferents (49). Group III-IV muscle afferents respond to mechanical (mechanoreceptors), metabolic (metaboreceptors), and partially to nociceptive (nociceptors) stimuli (1, 25, 35, 400), playing an important role in regulating cardiovascular, respiratory, and neuromuscular function during exercise (36, 49, 401). Increasing evidence suggests that the activation of metabo- and nociceptors have a deleterious effect on exercise performance, facilitating the development of central fatigue and reducing voluntary activation of the exercising muscles (186-188, 198, 199). While activation of metabo- and nociceptors subunits have been extensively studied (50, 127, 187, 199), very little is known about the effects of mechanoreceptors activation alone on the central and peripheral component of fatigue. Mechanoreceptors have been studied by applying static or dynamic muscle stretching in humans (402), showing increased central haemodynamics, muscle sympathetic nerve activity (276, 385, 403, 404) and reduced exercise performance (405, 406). Moreover, previous studies underlined possible anatomical and functional overlap between mechano- and muscle nociceptors (1, 102), suggesting their possible interactions once sensitised (270).

Following muscle injury or unaccustomed eccentric exercises a mechano- and nociceptors sensitisation may occur (17, 30), resulting in mechanical hyperalgesia at 24-48 hours: the so-called delayed onset of muscle soreness (DOMS) (13, 295). Previous research showed that DOMS may

also affect exercise performance (296, 407), neuromuscular (408), and vascular function (351) in the limb performing EIMD or even in a rested contralateral limb (409). However, no studies have yet investigated the combined effects of mechano- and nociceptors sensitisation on exercise performance with the central and peripheral components of fatigue in a remote non-damaged exercising muscle. We expected that when the muscle that previously performed the EIMD protocol would be stretched, the concomitant activation of mechano- and nociceptive subunits would increase pain sensations (356, 410) with greater central drive inhibition to the contralateral muscle, leading to reduced exercise performance. Therefore, the aim of this study was to evaluate the separated and combined effects of increasing mechanical and nociceptive afferent feedback following exercise-induced muscle damage on exercise performance with related central and peripheral components of neuromuscular fatigue in remote muscles. We hypothesized that only simultaneous stimulation of mechano- and nociceptors sensitization would result in extensive increases in central fatigue, leading to a decreased exercise performance.

6.2. Methods

Participant's characteristics. Eight male volunteers (age: 24 ± 2 years.; body mass 72 ± 10 kg; and height 179 ± 8 cm) took part in the study. Participants were healthy, non-smokers, and without any ongoing conditions or comorbidity. Participants were moderately active (3.0 ± 0.5 hours of training per week) with no specific experience of strength training exercise. All procedures conformed to the Declaration of Helsinki standards and were approved by the ethical committee of the University of Verona (CARP) acceptance number (n.14 R2/2021). The participants gave written, informed consent before their participation after full explanation of the purpose and experimental procedures of the study. The participants reported to the laboratory in the morning

(8–9 AM) in a fasted state. They were instructed to refrain from taking caffeine for 24 hours before the test and to report to the laboratory without engaging in any sort of strenuous physical activity in the preceding 48 hours. Participants were also restricted from eating any vitamin supplements, high-vitamin C foods, alcohol, or pain medicines within 24 hours of each visit.

Experimental Design. The experimental design of this study consisted of a total of seven distinct laboratory visits. Two familiarization visits were performed to ensure that participants were familiar with the time to exhaustion (TTE) task before starting the intervention. Thereafter, the participants reported to the laboratory for 5 distinctive lab visits, in which they performed one of the four experimental sessions in different days: (Control (CTRL); Stretching (ST); DOMS, DOMS with stretching (DOMS+ST)) and the EIMD protocol. The control condition (CTRL) consisted of a TTE (described in further detail below) performed on the dominant leg while the contralateral was resting fully extended. ST consisted of the same TTE on the dominant leg, but with the concomitant static stretching protocol (described in further detail below) on the contralateral leg. DOMS condition consisted of the same TTE test on the dominant leg while the contralateral leg was resting fully extended after having previously performed the EIMD protocol. DOMS+ST conditions consisted of the same TTE on the dominant leg, while in the contralateral leg, a static stretching protocol was applied after the previous EIMD protocol. The order of each condition (CTRL vs ST and DOMS vs DOMS+ST) was randomized and counterbalanced between participants. Each session was performed on a separate day with DOMS and DOMS+ST conditions performed randomly and counterbalanced at 24 or 48h after the EIMD protocol (Figure 6.1).

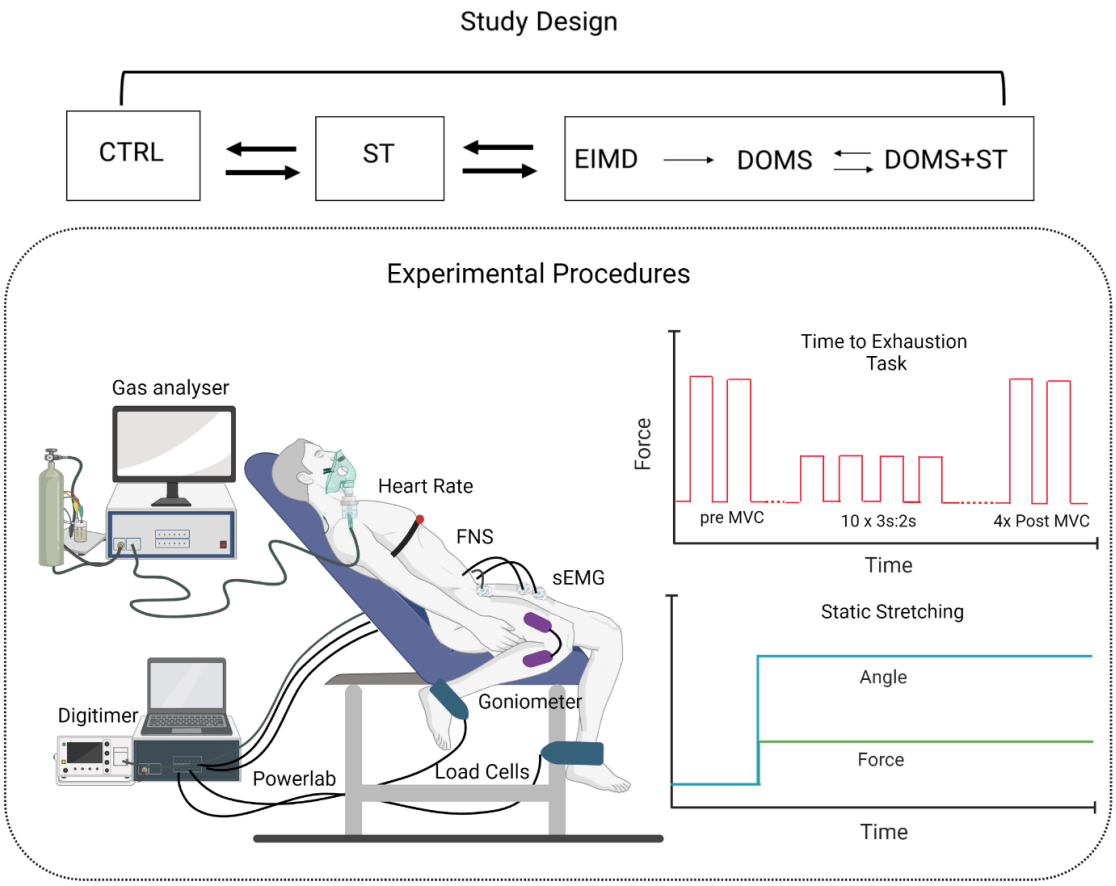


Figure 6.1 Study design and protocols.

Abbreviations: CTRL = Control Condition; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition; FNS = femoral nerve stimulation; sEMG = surface electromyography; s= seconds. Created with BioRender.com

Surface electromyography. Vastus lateralis (VL) EMG were continuously recorded with a dual bioamplifier (ML135; ADInstruments, Bellavista, NSW, Australia). On the VL, two surface Ag/AgCl electrodes (PG10C; Fiab, Vicchio, Italy) were attached to the skin with a 20-mm interelectrode distance. The electrodes were placed longitudinally, in line with the underlying muscle fibres arrangement, at two-thirds of the distance between the anterior iliac spine and the lateral part of the patella. The reference electrode was placed on the patellar tendon. Before the application of

the electrodes, the skin was shaved, abraded with sandpaper, and finally cleansed with an alcohol swab to minimize impedance. The electrode location was marked on the skin with indelible ink to maintain placement constant across visits. The raw EMG signal was acquired at 2-kHz sampling frequency and stored for offline analysis. Acquisition of the EMG data was done using a computer-based data acquisition and analysis system (hardware: PowerLab 16/30 (ML880; ADInstruments), and software: LabChart8 (ADInstruments). EMG data were analyzed with a custom-built MATLAB routine (MATLAB 2020b; Mathworks, Natick, MA). Briefly, the raw EMG signal was band-pass filtered (10–450 Hz) with a fourth-order finite impulse response filter and full-wave rectified. Afterward, a 250-ms baseline was detected between each knee extension, and contraction onset was defined as the point when the signal deviated by 3 SD from baseline. The same algorithm was applied to find contraction offset. For each muscle contraction, average root mean square of the EMG signal (EMGRMS) for the VL muscle was calculated and normalized by the maximum EMG_{RMS} obtained during the last three maximal voluntary contraction (MVC) performed during the neuromuscular assessment. Maximum EMG_{RMS} was calculated from a 500-ms window preceding the superimposed electrical stimulation. Successively, data contained in the last 20 s of each minute were averaged together (411).

Femoral nerve stimulation.

The motor nerve was stimulated with the anode placed between the greater trochanter and the iliac crest and the cathode placed over the femoral nerve in the femoral triangle via a constant current electrical stimulator (Digitimer DS7AH, Welwyn Garden City, United Kingdom). Electrical stimuli were delivered through rectangular (50x90 mm) self-adhesive electrodes (Myotrode Plus, Globus G0465). The evoked twitch force was measured by a force transducer (model UU2; DaCell,

Korea) previously calibrated and connected to a custom-made chair through a noncompliant strap placed around the ankle (96). The participants were seated with a 90° knee flexion with the hip extended at 40-45°. The output from the load cell was amplified (INT2-L, London Electronics Limited, Sandy Bedfordshire, United Kingdom), and recorded at a sampling rate of 2 kHz. Once the electrodes were in place, stimulation intensity was increased by 25-mA increments until the size of the evoked twitch and compound muscle action potential measured from the vastus medialis electrode (M-wave) demonstrated no further increase (*i.e.*, amplitude of maximal M-wave; M_{max}). Stimulation intensity was set at 125% of this value and was kept constant throughout the experimental session.

To evaluate quadriceps neuromuscular function, we measured potentiated twitch force ($Q_{tw,pot}$) 2-s after a 3-s MVC of the knee extensors and repeated this procedure four times. These MVCs were separated by at least 30 s of rest. Voluntary activation (VA) was assessed using the interpolated twitch technique by comparing the force produced during a single superimposed twitch on the MVC with the potentiated single twitch delivered 2 s afterwards.

Time to exhaustion exercise task (TTE)

Participants were seated in a comfortable handmade chair with the hip extended at 40-45^{o} while the knee angle of the dominant limb fixed at 90^{o}. TTE consisted of blocks of ten repeated isometric leg extensor contractions at 45% of MVC (3s of contraction on 2s of recovery), followed by an isometric 3s MVC. During the MVC, an imposed electrical stimulation twitch was delivered over the femoral nerve followed by a second electrical stimulation 2s after the MVC. Blocks were performed until participants reached exhaustion, which was defined as when participants were not able to continue to match the exercise intensity for two consecutive attempts.

Cardiorespiratory Responses

Pulmonary gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$,) were measured breath-by-breath with a metabolic cart (Quark b^2, Cosmed, Italy). Moreover, heart rate was measured during the TTE task through a chest strap heart rate monitor (H10 sensor, Polar, Finland). Before each session, after an appropriate warm-up of the instrument, the gas analyser and the turbine flowmeter were calibrated according to the instructions of the manufacturer. The data were averaged over the last 30s of each minute.

Perceptual responses

Rate of perceived exertion (RPE) and subjective feeling of pain (PAIN) were measured through the 6-20 Borg Scale (412) and a 1-10 numeric rating scale (p-NRS) (302) at the end of each minute of the TTE until the end of the protocol.

Static stretching protocol

During the familiarization visit participant's maximal knee flexion range of movement (ROM) was assessed on the non-dominant limb with the similar position of the TTE trial. All assessments were conducted by the same operator, who moved the participant's non-dominant joint through a 50° range of motion (knee extension) until reaching the point of tolerable maximal flexion, where subjective tension-discomfort was rated using Visual Analog Scale (VAS) and P-NRS scale (302). VAS was used to self-report perception of stretching intensity, using a 100-mm scale in which participants rated their perception of stretching intensity from zero (no stretch at all) to ten (maximal stretch as possible). Moreover, subjective feeling of pain was recorded with P-NRS scale, rating the pain arising from the stretching protocol from zero (no pain at all) to ten (pain as bad as it could be). Static stretching consisted of passive single knee flexion lasting throughout the

trial. During stretching protocol, knee joint angle was continuously recorded using a biaxial electro goniometer (Twin axial Goniometer TN1750/ST ADI Instruments Systems, Oxford, UK). During the entire stretching protocol, the knee extensors were stretched to the same range of motion obtained during the initial ROM assessment (362), which was kept identical for all stretching conditions (ST and DOMS+ST, respectively). An adjustable load cell was also fixed on the participant's non-dominant ankle and held during the experiment by a trained operator to measure the force applied from the flexed stretched non-dominant leg during the entire protocol as previously described (413).

Exercise Induce Muscle damage (EIMD) protocol

A warm-up of 10 non-dominant single leg isokinetic knee extensions and knee flexions were carried out through the full test range of motion, ensuring a progressive increase in effort, using an isokinetic dynamometer (Cybex, division of Lumex Inc., Ronkonkoma, NY, USA). A single leg maximal isometric voluntary contraction of the non-dominant leg was assessed prior to the EIMD protocol, where participants were instructed to exert the maximal voluntary isometric contraction during a leg extension movement at 90°. The EIMD protocol, consisted of several blocks of 3 sets of 12 maximal voluntary eccentric single knee extensions of the non-dominant limb and 30s of recovery followed by a post block assessment of the maximal force. Exercise was stopped once all participants reached a reduction of >40% of the maximal force from baseline (9). The eccentric phase of the contractions was performed at an angular velocity of $90°\cdot s^{-1}$. The concentric phase was performed sub-maximally at an angular velocity of $90°\cdot s^{-1}$ to minimize fatigue and enhance eccentric-load-related muscle damage (363).

Direct and Indirect Measure of muscle Damage

All direct and indirect measures of delayed onset of muscle soreness (MVC, PPTS, $VAS_{DA,}$ VAS_{SQ}) were assessed on the non-dominant limb before each trial for each condition. Full rest was given before starting the trial. Indirect measures of muscle damage were assessed through MVC using a isokinetic dynamometer (Cybex, division of Lumex Inc., Ronkonkoma, NY, USA) (297) and a 100-mm visual analog scale (VAS) anchored on the left edge of the scale with the phrase "no pain or soreness" and on the right edge "worst pain/soreness imaginable"(305). Participants were asked to rate their pain-related soreness after performing a squat at approximate 90 degrees of knee angle (VAS_{SQ}) (297, 298). Pain Pressure thresholds (PPTs) were assessed using a marker placed on a standardized point of the non-dominant quadriceps on the first day of testing to ensure reliable measurements between testing sessions (365). To determine PPTs a standardized mechanical pressure algometer was used (Hilitand, NK-100 Force Gauge, USA). The same operator used the algometer at a gradual force to the marked spot on the participant's quadriceps until the participant verbally informed the researcher when the sensation of pressure became painful and the PPTs were recorded (364, 365).

Data analysis

Voluntary activation was calculated as [1-(superimposed twitch force/$Q_{tw,pot}$) · 100]. Peak force was calculated as the highest value reached for every $Q_{tw,pot}$. Deltas between pre and post neuromuscular function values before and after each TTE were calculated as the difference between pre and post values averaged across the four repetitions. Total Impulse was calculated as the force integral of the time to exhaustion task using the integral function of the force signal channel with the Labchart software (LabChart 8, ADInstruments, Bellavista, NSW, Australia). Peak values from the cardiorespiratory assessments were averaged across the last minute of the TTE .

Delta for single effects were calculated deducting CTRL values for all outcomes from ST and DOMS respectively.

Statistical analysis

Normal distribution of the data was assessed with a Shapiro-Wilk test. If the sphericity assumption was violated, the Greenhouse–Geisser correction coefficient was reported. One-way repeated measures ANOVA with Tukey-B post-hoc analysis was implemented for time to exhaustion and time integral, pre-TTE and deltas for neuromuscular function outcomes. A two-way repeated measures ANOVA was performed for neuromuscular and cardiorespiratory outcomes. When a significant time x condition interaction was found, pairwise differences were identified using Bonferroni post hoc test correction for multiple comparisons. All data were analyzed using a statistical software package Graph Pad Prism v.9 (GraphPad Software, San Diego, California USA). Data are presented as mean ± standard deviation (SD) and considered significant when $p<0.05$.

6.3. Results

Static stretching protocol

All participants completed all sessions without reporting any postural discomfort during the stretching procedures. Participants were set at the same range of movement during ST and DOMS+ST condition (54.9 ± 4.9 vs 55.1 ± 4.7 degrees), however the force detected from the load cell between conditions was similar during DOMS+ST compared with ST condition (67.1± 22.2 vs 77.4 ± 24.5 N; $p>0.05$). VAS for stretching intensity was similar between participants and between the two stretching conditions (7.1 ± 1.3 vs 8.1 ± 1.6 cm) for ST and DOMS+ST, respectively. Pain

intensity rated with VAS was higher for DOMS+ST compared with ST (2.6 ± 1.9 vs 7.0 ± 2.6 cm, $p<0.05$) before the starting of the experimental procedure.

Direct and Indirect measures of DOMS

Data from direct and indirect measure of DOMS are reported in table 6.1. MVC for the EIMD leg decreased significantly across conditions ($F=12.94$, $p<0.01$), and particularly in DOMS ($p= p<0.01$, $p=0.03$) and DOMS+ST ($p=0.01$, $p=0.04$) compared with CTRL and ST condition, respectively. PPTS decreased significantly between conditions ($F=11.45$, $p<0.01$), particularly in DOMS (all $p<0.01$) and DOMS+ST ($p<0.01$, $p=0.02$) compared with CTRL and ST condition, respectively. VAS_{SQ} was statistically different across conditions ($F=9.89$, $p<0.01$) and increased significantly in DOMS (both $p=0.02$) and DOMS+ST (all $p<0.01$) compared with CTRL and ST conditions respectively.

Exercise Performance and Neuromuscular Function Assessment

Data from the exercise performance test and neuromuscular function assessment are reported in table 6.1. TTE was significantly different between conditions ($F=12.83$; $p<0.01$) and reduced in ST and DOMS+ST condition compared with CTRL (All $p<0.01$) respectively. TTE Integral was significantly different between conditions ($F=7.084$; $p<0.01$) and reduced in ST and DOMS+ST condition respectively (both $p=0.02$). No difference between Pre-TTE VA, $Q_{tw,pot}$ was found ($F= 2.58$; $p=0.08$; $F= 2.01$; $p=0.15$; $F=1.56$; $p=0.45$) respectively. However, pre-TTE MVC was statistically reduced between conditions ($F=4.65$, $p=0.04$) showing a significant reduction in DOMS+ST condition compared with CTRL ($p=0.03$). Exercise-induced change in ΔMVC was found significantly different between conditions ($F =11.48$, $p<0.01$) within DOMS and DOMS+ST conditions significantly different to CTRL condition ($p=0.02$; $p<0.01$) respectively. Significant differences were found across conditions for exercise-induced change in $\Delta Q_{tw,pot}$ ($F=4.24$, $p=0.03$)

and ΔVA (F=3.94; p=0.04) with DOMS+ST condition significantly different from CTRL (p=0.04), figure 6.2.

Table 6.1. Direct and Indirect measures of DOMS within neuromuscular assessment outcomes before and after TTE.

Variable	CTRL	ST	DOMS	DOMS+ST
MVC EIMD limb (N)	685.3 ± 120.8	681.9 ± 130.1	419.5 ± 170.3**	411.1 ± 196.9**
PPTS (kg)	6.05 ± 1.3	5.89 ± 1.4	3.99 ± 1.2**	3.92 ± 1.5**
VAS squat (mm)	0.4 ± 0.3	0.7 ±0.2	46.5 ± 28.1**	51.0 ± 20.4**
Time TTE (s)	332.4 ± 87.5	259.3 ± 114.2*	286.1 ± 109.4	233.0 ± 97.2**
Total Impulse (N*s)	66931 ± 20197	50850 ± 24387*	54894 ± 17967	49817 ± 23757**
Pre-TTE MVC (N)	707 ± 134.3	626 ± 135.9	656 ± 72.8	617 ± 95.9*
Pre-TTE $Q_{tw,\ pot}$ ***(N)***	296 ± 41.1	268 ± 47.0	288 ± 15.2	297 ± 65.8
Pre-TTE **VA** ***(%)***	92 ± 5.9	88 ± 6.5	87 ± 11.1	85 ± 6.6
Delta MVC (%)	29.9 ± 6.6	21.5 ± 6.8*	22.1 ± 5.3	15.0 ± 6.9**
Delta VA (%)	0.1 ± 7.0	3.8 ± 8.6	3.2 ± 6.6	9.4 ± 5.9*
Delta $Q_{tw,\ pot}$ ***(%)***	37.4 ± 12.8	25.3 ± 10.3	24.8 ± 16.7	22.6 ± 15.1*

Abbreviations: MVC = Maximal Voluntary Contraction; PPTS = Pain Pressure Thresholds; VAS = Visual Analog Scale; TTE = time to exhaustion; VA = Voluntary activation; Qtw,pot = resting potentiated twitch; CTRL = Control Condition; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition; *p<0.05; **p<0.01; ***p<0.001 respect to CON, †p<0.05 respect to ST. Data are presented as mean ± standard deviation.

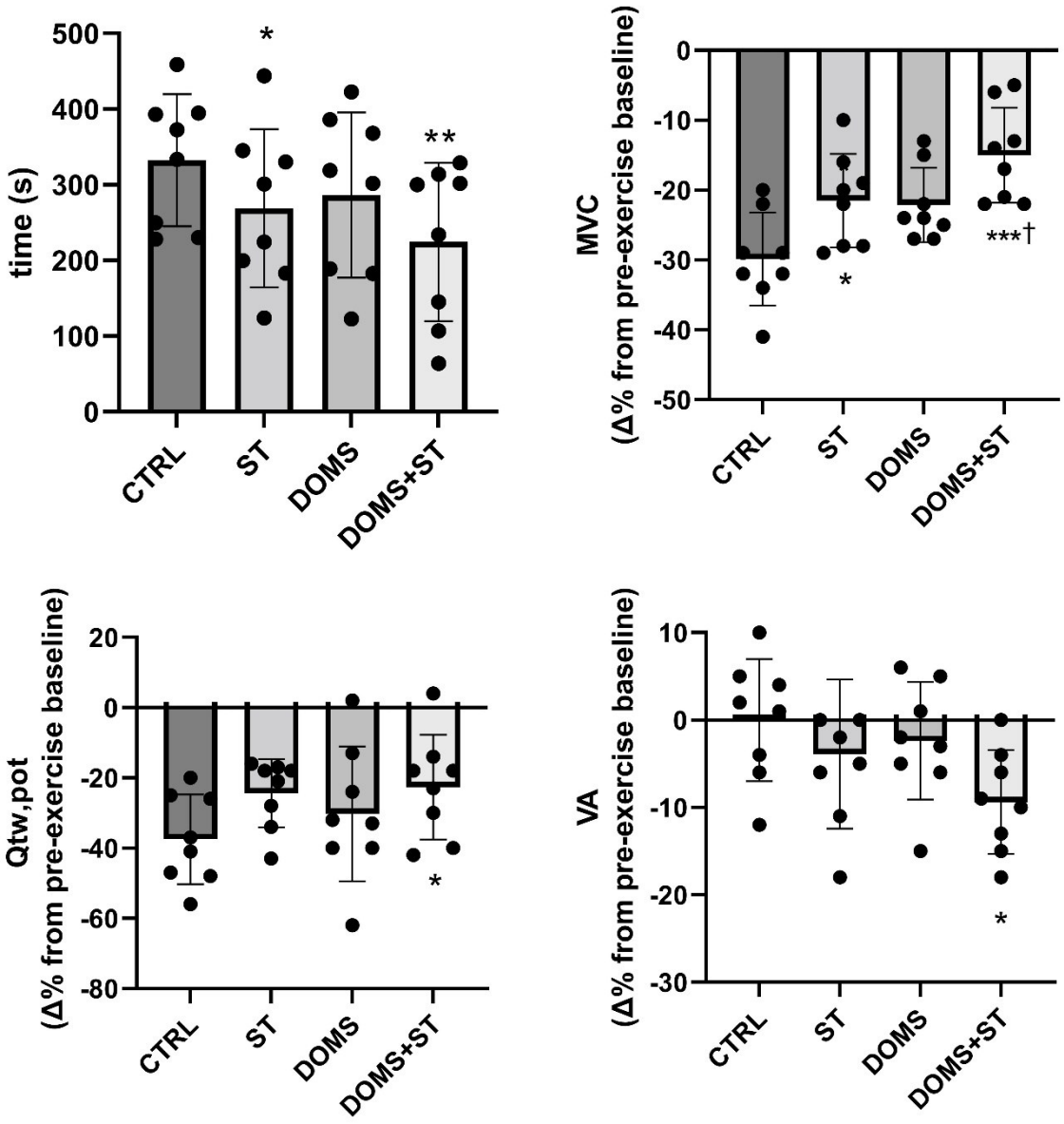


Figure 6.2 Neuromuscular assessment before and after the time to exhaustion task.

Abbreviations: MVC = Maximal Voluntary Contraction; TTE = time to exhaustion; VA = Voluntary activation; Qtw, pot = resting potentiated twitch; CTRL = Control Condition; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition; *p<0.05; **p<0.01; ***p<0.001 respect to CON, †p<0.05 respect to ST. Data are presented as mean ± standard deviation.

Cardiorespiratory Variables and Neuromuscular function Assessment during TTE.

Data from the cardiorespiratory variables during TTE are reported in figure 3. A significant interaction for time was found for $V \cdot O_2$, $V \cdot CO_2$ and HR (all $p<0.0001$). Significant effects for condition ($F=3.19$; $p=0.03$; $F=3.92$; $p=0.03$) and time x condition interaction was found for $V \cdot O_2$ and HR ($F=3.21$; $p=0.04$; $F=4.32$; $p=0.02$). Data from the neuromuscular function assessment during TTE are reported in figure 4. A significant interaction for time was found for MVC, VA, $Q_{tw,pot}$, EMG_{RMS} during the TTE tasks (all $p<0.01$), however no significant condition or time x condition interaction was found for VA and $Q_{tw,pot}$ (all $p>0.05$) during the TTE task. A significant condition ($F=3.04$; $p<0.01$ and $F=2.09$; $p=0.03$) and time x condition interaction was found for MVC expressed as percentage ($F=2.74$; $p<0.01$). A significant interaction for time ($F=369.4$, $p<0.01$; $F=465.4$, $p<0.01$), condition ($F=234.4$, $p<0.01$; $F=325.4$, $p<0.01$) and time x condition ($F=3.971$, $p<0.01$; $F=3.475$, $p<0.01$) was found for RPE and PAIN respectively during the TTE task.

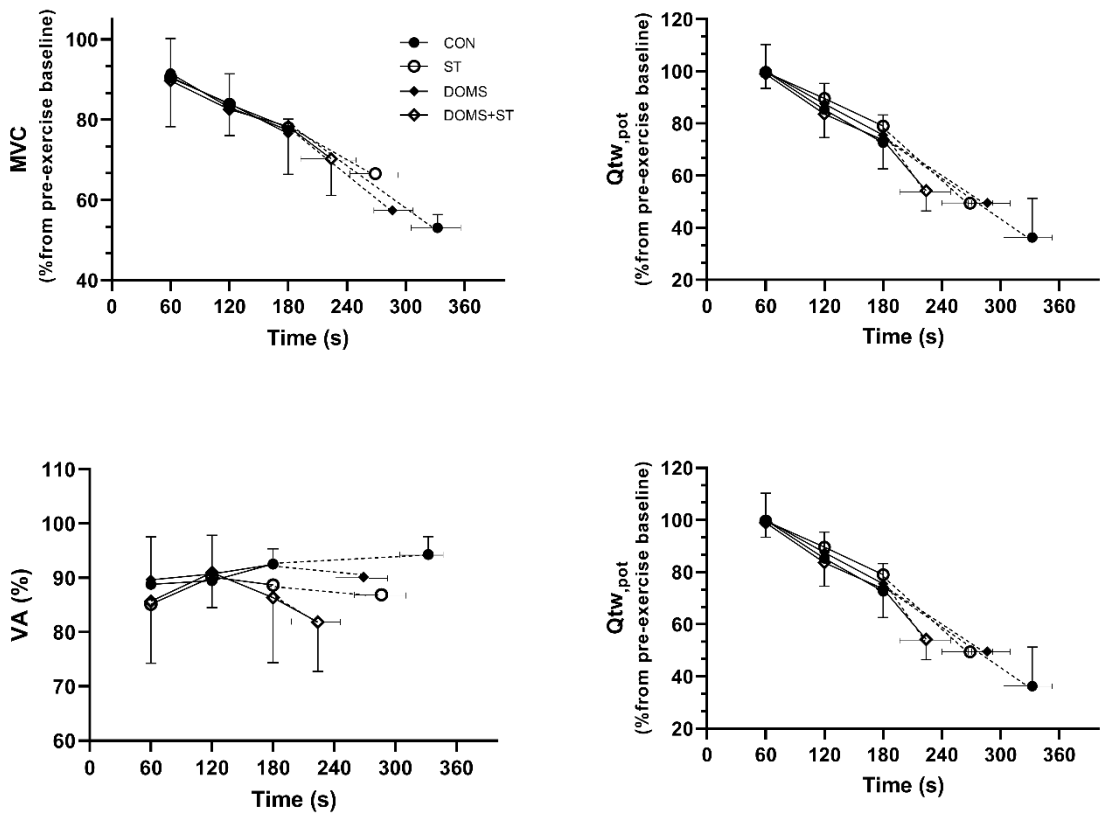


Figure 6.3 *Neuromuscular function during TTE.*

*Abbreviations: MVC = Maximal Voluntary Contraction; TTE = time to exhaustion; VA = Voluntary activation; Qtw, pot = resting potentiated twitch CTRL = Control Condition; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition; *p<0.05; respect to CON after post-hoc t-test with holm Bonferroni correction. Data are presented as mean ± standard deviation.*

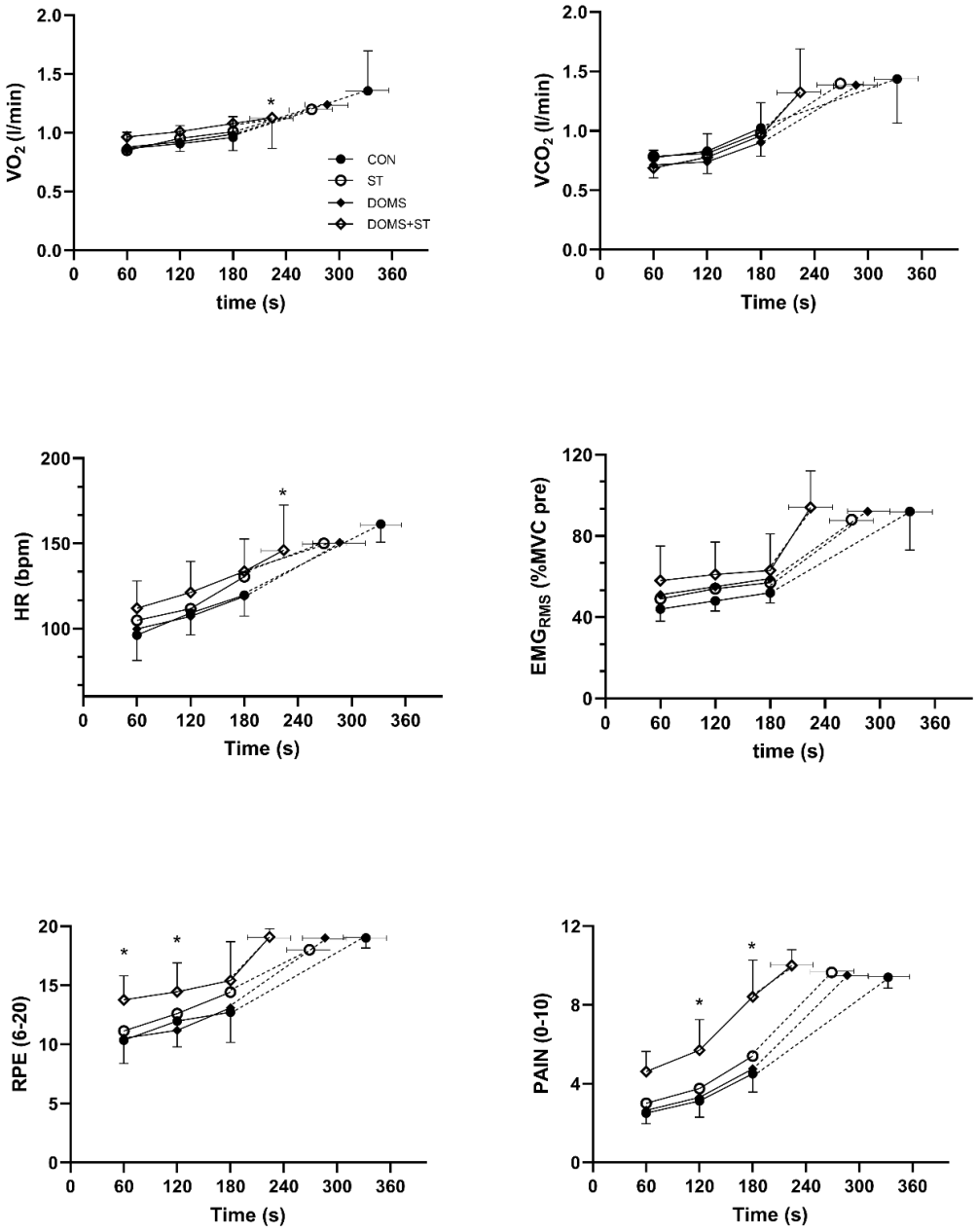


Figure 6.4 Cardiorespiratory function, Muscle Activation and Perceptual responses during TTE.

Abbreviations: VO_2 = Oxygen consumption; RPE = Rate of Perceived Exertion; HR = Heart Rate; VE = Ventilation, VCO_2 = carbon dioxide production CTRL = Control Condition; DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition; *p<0.05; respect to CON after post-hoc t-test with holm Bonferroni correction. Data are presented as mean ± standard deviation

6.4. Discussion

To the best of our knowledge, this is the first study to assess the role of single and combined effects of mechano- and nociceptors activation on exercise performance in healthy young participants. We also incorporated measures of cardiorespiratory and neuromuscular function during the time to exhaustion task to understand the possible physiological mechanism underpinning the reduction in exercise performance. The main findings of this study are: 1) single activation of mechanoreceptors (i.e., ST condition) caused a decrease in exercise performance and 2) Activation of mechano- and nociceptors following EIMD (i.e., DOMS+ST) extensively decreased exercise performance with concomitant decrease in voluntary activation. These results were in line with our initial hypothesis, showing that an increased activation of mechano- and nociceptors would have led to an increased in central fatigue (i.e., reduction in ΔVA) and an early attainment of the exercise sensory limit, reducing overall exercise performance.

While previous studies found impaired or similar neuromuscular function following application of static stretching protocols on the same, remote or antagonist muscle (414-418), no studies have yet investigated the effects of concomitant static stretching during a TTE in a contralateral leg. In our study we found no difference at rest in neuromuscular function and force production while a significant reduction of performance was found following the TTE task. The absence of neuromuscular impairments at rest could be ascribed to the type and intensity of protocol utilised (419). Indeed previous study found that contralateral intermittent (45s on 15off) static stretching were able to elicit a reduction in MVC and VA (stretching maximal tolerance of 80%) (420). However, in our study we implement a continuous protocol at moderate intensity (i.e., VAS: 7 out of 10cm) with no experienced and or reported pain. This is important because continuous protocol rather than intermittent showed an increase stretch tolerance effect (421), due to a

decreased nociceptive nerve endings sensitivity (422) and decreased inhibition effect from muscle spindle activation (423, 424). In fact, previous research found no H-reflex inhibition on contralateral leg muscles during continuous static stretching protocol in the ipsilateral limb (425).

Despite no differences were found at rest, we found significant changes in exercise performance (lower TTE and total impulse) following a TTE isometric task in ST condition. Previous research found that reduction in the force generating capacity may be attribute to alteration in the afferent feedback by type Ia, type-II (muscle spindles) (426), type III (mechanoreceptors), and type-IV (metabo-/nociceptors) fibres (278, 354), due to an increasing activation of the central nervous inhibitory system leading to a reduced spinal reflex excitability (415, 418). Seen that we adopted a continuous stretching protocol we hypothesise that muscle spindle and nociceptive nerve endings were not activated (i.e., stretch tolerance effect) while instead other type of receptors would have been involved (i.e., group III muscle afferents). Muscle III-IV afferents (i.e., mechano and metabo receptors) have been showed to play an important role in the regulation of neuromuscular system, showing that their joint activation was affecting subsequent exercise performance on the contralateral leg (96, 200). However, in our experiment we found for the first time a reduced exercise performance while stimulating predominantly Aδ -mechanoreceptors fibres (group III fibres). This is an important finding showing that single activation of III afferents exert a powerful suppressive stimulus sufficient to alter exercise performance even in a remote contralateral muscle. Our second and most important finding is deriving from the combined activation of mechano- and nociceptors, following EIMD immune-mediated afferent sensitisation (i.e., DOMS+ST condition). Increase sensitisation of mechano and nociceptors showed to exert a more extensive inhibitory effect than the activation of mechano and nociceptors alone, reducing significantly VA (i.e., increase in central fatigue) in the DOMS+ST condition. Indeed increased activation of muscle nerve afferents have been previously suggested to decrease the voluntary

drive (206, 207) and exercise performance (49, 427, 428). Moreover, we found a substantial increase in RPE and overall perception of pain from mechano- and nociceptors activation. In line with our hypothesis several studies confirmed the pivoting role of heightened nociceptor activation in increasing central fatigue, perception of effort and decreasing overall performance (126, 127, 198, 199). However, while these studies found reduced exercise performance following metabo-nociceptors activation in both ipsi- and contralateral limb, in our study we activated exclusively mechano and nociceptors with no metabosensitive interaction, demonstrating for the first time the role of mechano and nociceptors sensitisation in reducing exercise performance and voluntary activation with an early attainment of the sensory tolerance limit.

The sensory tolerance limit has been firstly described as a hypothetical "threshold" from which continuing the exercise/task was no longer tolerated because becoming 'unattractive' (43). This concept was further expanded by Hureau et colleagues (55) where the authors suggested that "the sensory tolerance limit may be described as a global negative feedback loop leading to task failure when a finite level of stimulation is reached from sensory afferents originating in muscles that are directly or indirectly involved in the exercise". Indeed, in our experiment, we demonstrate that by increasing the level of stimulation (single vs combined mechano and nociceptors activation) of the muscle nerve afferent (i.e., negative feedback loop), there is an increased inhibition in VA and concomitant decreased in exercise performance, (fig. 6.5). Moreover, this results were reflected by the reduced VO_2 and HR peak, showing a premature stop and reduced exercise tolerance during DOMS+ST, characteristics that are usually found in different clinical conditions when the rate of perceived exertion is heightened compared with the physiological demand (97, 429-431).

To summarise, The physiological mechanism underlying these results could be possibly ascribed to: 1) Increases in central fatigue limiting the ability of recruit available motor unit during the

exercising task; 2) increases in rate of perceived effort and pain that reduced the willingness of the participants to continue in the exercising task facilitating the early attainment of the sensory tolerance limit.

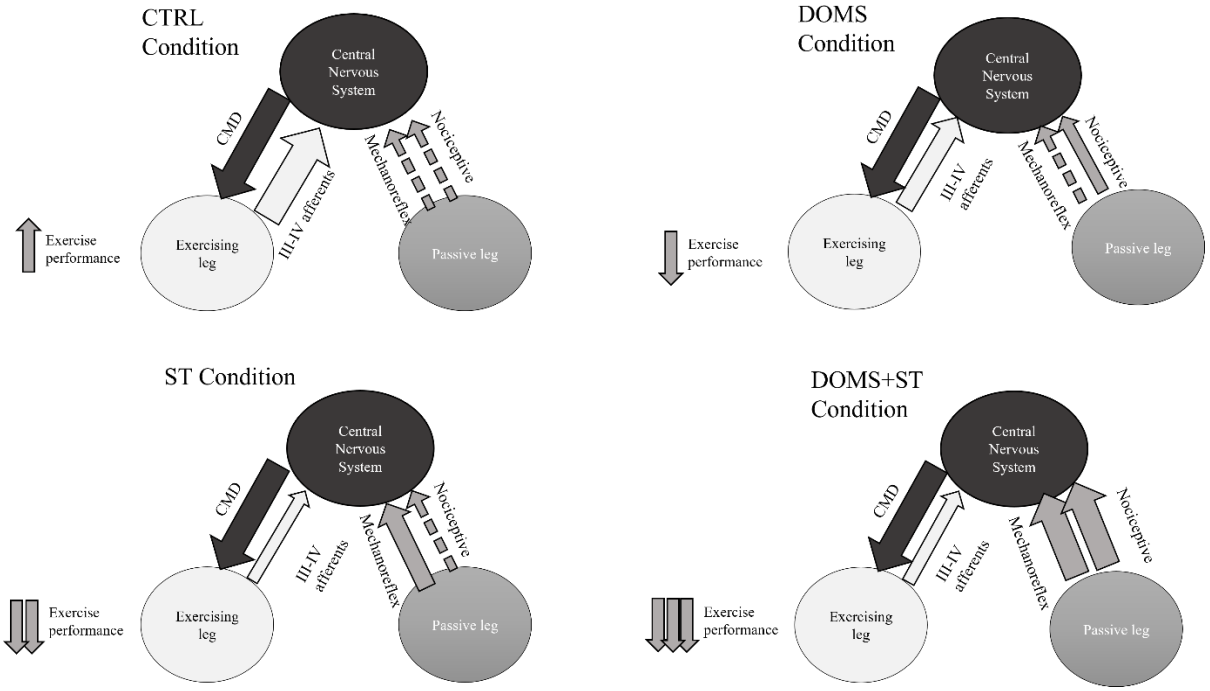


Figure 6.5 Potential mechanisms of mechano and nociceptive reflexes activation during a TTE.

CTRL= Control condition DOMS= delayed onset muscle soreness condition; ST= Stretching condition; DOMS +ST = delayed onset muscle soreness with stretching, condition CMD = Central Motor Drive. During the control condition the exercise tolerance limit was influenced only by the feedback from the exercising leg due the lack of mechanoreflex and nociceptive afferent feedback from the passive leg that overall contribute to a greater performance compared with the other condition. During DOMS and Stretching condition instead due to an increased nociceptive and mechanoreflex activation respectively on the passive leg, the overall performance was reduced due to an early attainment of the exercise tolerance limit. During DOMS+ST condition display the lower performance due to the increased pain and RPE arising from the sensitisation of mechano and nociceptive receptors that lead to an early attainment of exercise tolerance limit.

6.5. Conclusion

All these findings together suggest that the combined effects of sensitised mechanosensitive and nociceptive fibres led to an impaired neuromuscular function, exercise performance and early attainment of the sensory tolerance limit. These findings may help to underline the interactive effects of mechano- and nociceptors sensitization on exercise performance in remote muscle, with possible translation in clinical conditions affected by peripheral nerve muscle afferent sensitisation.

Contributions

All the authors played a role in the content and writing of the manuscript. In addition, M.V was the principal investigator; M.V, F.Z., F.S. and J.S.M. had input into the original idea, study design, and conduct of the study. F.Z., M.V., G.G., T.F., M.M.O. collected the data; F.Z., M.V., F.G.L., and P.D.O. performed data analysis and statistics, and F.Z., F.G.L. prepared it for presentation. F.Z. and M.V wrote the manuscript. All authors reviewed the manuscript before submission.

Fundings:

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 801604 for F.Z. and P.D.O. This project was also partially supported by the Brain Research Foundation Verona Onlus and by the Italian Ministry of Research and University (MIUR) 5-year special funding to strengthen and enhance excellence in research and teaching (https://www.miur.gov.it/dipartimenti-di-eccellenza).

Chapter 7. Experimental Study IV

Sensitivity of group III-IV muscle nerve afferents in ageing: Role of chronic training exposure on the exercise pressor reflex

What is known? In the previous chapter (i.e., Chapter 4-6) we investigated the effects of muscle afferent sensitisation on cardiovascular, cardiorespiratory, and neuromuscular function in a controlled experimental environment. During ageing, activation of group III-IV muscle afferents seems to be altered, with contrasting results in the literature. Moreover, exercise has been proposed as a mechanism to counteract these effects and restore their functionality.

What is going to be assessed? For these reasons, we decided to assess the effect of ageing and chronic exposure of training in a master athlete population and compared it with a healthy age matched control. Moreover, we correlate changes of the cardiovascular response to exercise with sport performance. We hypothesized that increases in blood pressure responses were correlated with impairment on blood pressure regulation leading to lower performance.

This work has been published as: Zambolin F, McPhee JS, Duro-Ocana P, Ganse B, Bagley L, Faisal A. The association of elevated blood pressure during ischaemic exercise with sport performance in Master athletes with and without morbidity. Eur J Appl Physiol. 2022 Jan;122(1):211-221. doi: 10.1007/s00421-021-04828-9. Epub 2021 Oct 15. PMID: 34652528; PMCID: PMC8748359.

The association of elevated blood pressure during ischemic exercise with sport performance in Master cyclists with and without comorbidities

7.1. Introduction

Elevated exercise blood pressure (BP) is a prevalent risk factor for cardiovascular (CV) diseases in sedentary individuals (432) and elite athletes (433). Regular moderate exercise can maintain healthy vascular function (434-436), and reduce the risk of hypertension (437, 438). However, there is a controversy about the impact of lengthy and intense training on cardiovascular health and the incidence of hypertension (439-444). Master athletes provide an opportunity to study vascular function and BP responses of exceptionally active individuals, since they typically train regularly and compete in athletic events at very high intensity (445). Middle-aged and elderly master athletes show improved vascular function with optimized blood flow and BP regulation at rest and during exercise (435). However, some vulnerable master athletes are at risk of developing adverse cardiovascular comorbidities (441). Indeed, a prevalence of 10% with established respiratory and cardiovascular diseases was found in a master athletes' cohort (446). These comorbidities are often connected to an impaired BP regulation during exercise despite a normotensive status at rest (447), indicating an early state of hypertensive disease.

The increase in BP during exercise is regulated through a feed-forward central command mechanism (288) and affected by the baroreflex (448), and the exercise pressor reflex (EPR) (160) feedback mechanisms. Activation of muscle afferents (III-IV) by mechanical and metabolic stimuli evoke EPR which increases sympathetic outflow to the heart and resistance vessels (400, 449-451). The magnitude of EPR during fatiguing isometric

exercise increases with aging and cardiovascular comorbidities (243, 452). Endurance training showed blunted EPR (453, 454) and long-term endurance athletes exhibit lower EPR than untrained individuals (455). However, susceptible master athletes are at risk of an exaggerated BP response during exercise (456). An elevated BP during a progressive maximal cardiopulmonary exercise test (CPET) was associated with a decreased exercise capacity in elite young athletes with autonomic dysfunction (457). However, the connection of an elevated exercise BP with sport performance is not well documented. Forearm occlusion, ischemic isometric exercise and post-exercise circulatory occlusion have shown to elevate the muscle III/IV afferent feedback and exaggerate the BP response (458-460). While these interventions induce minimum changes in muscle metabolism (i.e., O_2 stores and phosphocreatine) during occlusion and fully return to base line state with 5 minutes of recovery (329, 461, 462). Therefore, the aim of the present study was to examine the BP response to a fatiguing occlusion protocol in Master cyclists presenting with- and without comorbidities and its impact on their sport performance during World Master Track Cycling Championships. Our hypothesis was that Master athletes with comorbidities would experience an exaggerated EPR and BP response as a reaction to a fatiguing occlusion protocol compared to Master athletes without comorbidities, and that these athletes would have a lower performance during the competition.

7.2. Methods

Participants:

Forty-eight non-smokers (F:13) master athletes competing at the 2019 World Master Track Cycling Championships in Manchester (UK) were recruited to participate in this study along with a healthy non-athletic control group (N:10). All participants completed a general health questionnaire and provided detailed medical history and use of medications. MA were divided into two matched groups (N:24), healthy MA or comorbidities MA based on the presence or history of cardiovascular diseases (N:9), respiratory diseases (N:4) or any other pathology (N:11) that could possibly alter BP at rest and during exercise (463-467). Reported comorbidities were hypertension, hypotension, blood clot, iliac arterial occlusive disease, thrombosis, myocarditis and cardiac arrythmias (Atrial fibrillation), asthma, hypothyroidisms, history of breast, skin and prostate cancer, cox-arthritis, glaucoma, post traumatic syndrome disorder. Details of medications The healthy and comorbidities MA groups were matched for age, sex, self-reported weekly training volume over the last year of training before competition (average: 11h/week), and handgrip maximum voluntary contraction (MVC) (48.3 ± 10.3; 44.2 ± 12 kg, $p>0.05$, respectively). Subsequently, the healthy MA group was divided in a median age (M:57 and F:48 years) to sub-groups of middle-aged MA (48.9 ± 9.2 years, N:12) and old MA (65.8 ± 10.2 years, N:12) and were compared with a middle-age none-athlete controls (48.3 ± 8.3 years, N:10). This study was approved by Manchester Metropolitan University Ethics committee (Approval ID: 11704). Participants provided written, informed consent and were requested to avoid caffeine intake for 12 hours prior to participation in the study.

Experimental Design and Procedures:

All participants completed a fatiguing occlusion protocol in one session at least 24h prior to or following participation in the competition. Following 15 mins of comfortable upright sitting, participants performed 3 handgrip maximal voluntary contractions (MVC) with a one-minute recovery period between contractions. MVC was assessed with a grip force transducer attached to data acquisition system (Power lab - ADI Instruments Systems, Oxford, UK) and calculated as the average of one-sec peak strength in the best two trials. The fatiguing occlusion protocol was adapted from previous studies on BP response and metaboreceptors activity to ischemia (243, 447, 460, 468). It included 5min of resting, 5min of forearm occlusion including 1min of isometric contraction at 40% MVC and 5min recovery (Fig. 7.1). The protocol was applied in an upright position using the dominant arm extended at the heart level. Circulatory occlusion of the brachial artery distal to the elbow was achieved by rapid inflation of a standard blood pressure cuff to 220∼250mmHg (Elite BFR Occlusion Cuffs, © The Occlusion Cuff). Exercise ischemia was assessed by the absence of the radial artery pulse during the occlusion protocol (190, 469), and the occlusion period was terminated by rapid deflation of the occlusion cuff. Beat-by-beat BP was measured throughout the testing protocol using finger plethysmography (Human NIBP nano, ADI Instruments Systems, Oxford, UK). Finger plethysmography has been shown to be a valid and reliable tool for assessing blood pressure during hemodynamic changes (470). Two participants fainted during the fatiguing occlusion protocol, and they have been excluded from the study. Participants

were asked to rate the intensity of pain-discomfort using a 0-10 Numeric Rating Scale (471), throughout the 5min fatiguing occlusion protocol (at 2, 3 and 5mins).

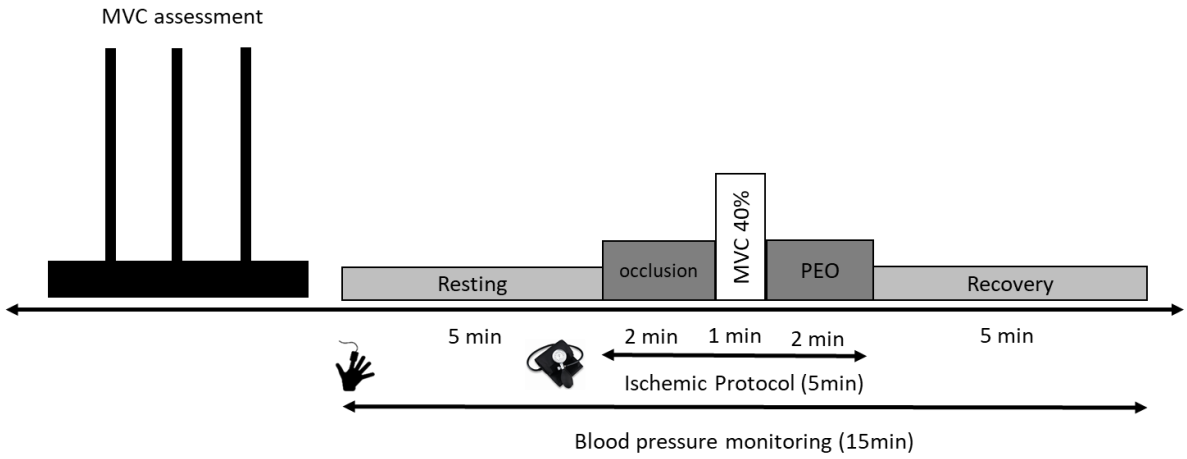


Figure 7.1 Experimental Procedures of the ischaemic exercise protocol

Experimental testing protocol on top and a representative recordings of blood pressure using finger plethysmography at the bottom. Abbreviations: MVC maximal voluntary contraction, PEO post-exercise occlusion

Data analysis

Systolic blood pressure (SBP), mean arterial pressure (MAP) and diastolic blood pressure (DBP) values were calculated as a mean of each phase of the fatiguing occlusion protocol (occlusion, ischemic exercise (MVC40% under occlusion), post-exercise occlusion (PEO)), and at the last min of resting and recovery periods before and after the fatiguing occlusion protocol (Fig. 1). Delta BP among phases of the testing protocol were calculated as the differences between BP means. Beat-by-beat SBP and MAP dynamics were assessed by slope analysis throughout ischemic isometric exercise, PEO phases and during the first

2mins of recovery after cuff release. Age-graded performance (AGP) was calculated the performance in the best event of a person of a given age as a percentage of the world record at that age. Age graded performance (AGP) analysis compared race performances in the MA cohort, within different age groups and races, to corresponding age-group world records for each event (472). Master athletes were competing in different events (200m – 500m – 2000m), and only the best result of each participant in the championship was included in the analysis. Official race results were obtained from the 2019 World Master Track Cycling Championships website.

Statistical analysis

All variables met the parametric assumption for normality distribution and the homogeneity of variance using Shapiro-Wilk and Leaven's tests. A sample size of 24 was estimated to provide 80% power to detect differences in BP responses between Master cyclists with or without comorbidities, based on a SD of one unit, α of 0.05, and a two-tailed test of significance. An unpaired T-test was performed for all the participant's characteristics, pain, AGP, BP slopes between healthy and comorbidities master athletes' groups. A one-way repeated measure ANOVA with Bonferroni post-hoc analysis was performed to examine differences in mean and delta SBP, MAP, DBP responses at different phases of the testing protocol between healthy MA and comorbidities MA, as well as between old and middle age MA, and healthy middle-age controls. A simple linear regression analysis was performed on the MA cohort to examine any correlation between the raising slope of SBP and MAP during ischemic isometric handgrip contraction and AGP.

All the statistical analysis were performed using the GraphPad Prism 8 statistical analysis software. Data are reported as mean ±SD with statistically significant $p<0.05$.

7.3. Results

Comorbidities effect in Master athletes:

The healthy and comorbidities MA groups were of similar age, height, weight, MVC, and weekly training volume (Table 7.1). Both groups showed similar changes in BP responses (mean, delta and slope) (Fig. 7.2, Tables E1, E2 - Online Supplement) and reported similar pain discomfort during the 3 phases of the fatiguing occlusion protocol (Table E3 - Online Supplement). There were no significant differences in AGP between healthy and comorbidities MA groups (90.13 ± 4.26 and 90.98 ± 5.33%, $p>0.05$).

An inverse correlation was found between the slope of increased SBP and MAP during ischemic exercise and AGP ($r = 0.50$, $r = 0.46$, $p<0.05$ for both- Fig.7.3a,b).

Table 7.1 Subject characteristics in healthy and comorbidities Master athletes

Group	Healthy MA (24)	Comorbidities MA (24)
Age (y)	57.2 ± 12.6	60.7 ± 12.5
Height (cm)	172.5 ± 6.9	169.4 ± 6.3
Weight (kg)	75.7 ± 11.3	76.8 ± 12.7
BMI (kg/m^2)	25.3 ± 2.7	25.3 ± 3.4
Training (h/week)	11.1 ± 4.6	9.8 ± 3.3
MVC 40% (kg)	19.5 ± 4.3	17.7 ± 4.8
Resting SBP (mmHg)	131.3 ± 12.6	133.1 ± 12.7
Resting MAP (mmHg)	94.7 ± 7.8	94.4 ± 7.8
Resting DBP (mmHg)	77.1 ± 7.3	77.5 ± 7.7

Values are means ± SD. Abbreviations: BMI= body mass index; DBP= diastolic blood pressure; MAP= mean arterial pressure; MVC= maximal voluntary contraction; SBP= systolic blood pressure.

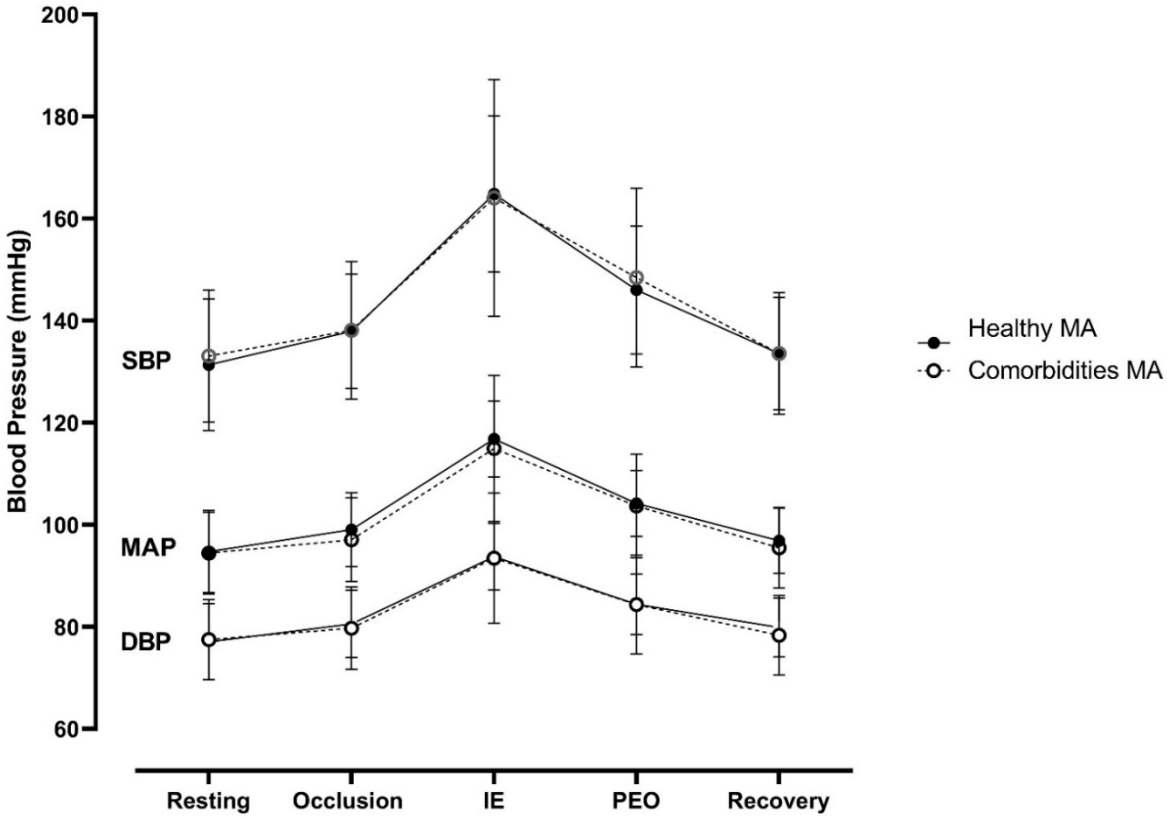


Figure 7.2 Blood Pressure responses in healthy and Master cyclists with morbidity groups at resting, occlusion, ischaemic exercise (IE), postexercise occlusion (PEO), and recovery. Values are means ± SE.

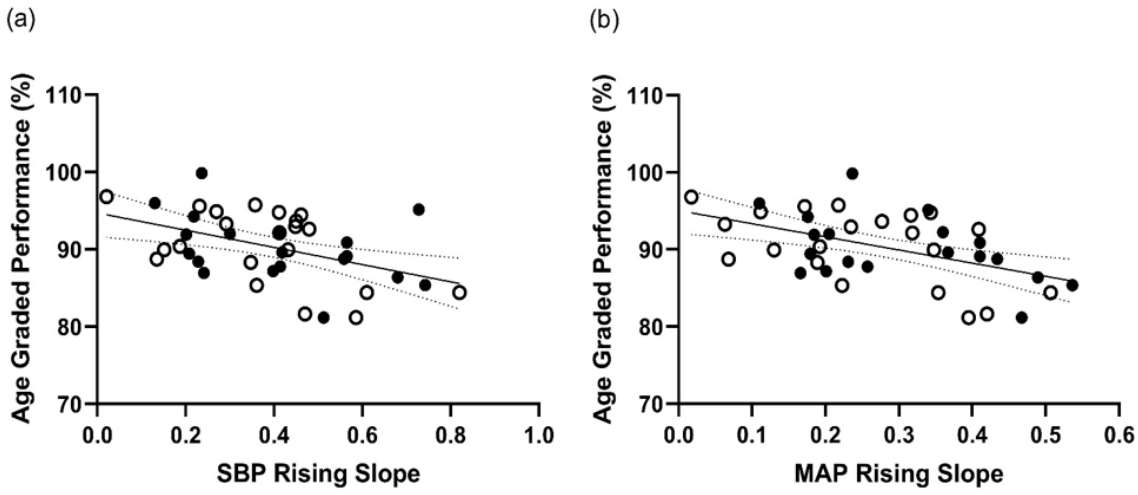


Figure 7.3 Results from the linear regression analysis

Linear regression of age-graded performance and the slopes of SBP rises (a) and MAP rises slope (b) during ischaemic handgrip isometric exercise in Master athletes cohort [healthy (solid circles) and Master athletes with morbidity (open circles)]

Aging and training effect in healthy Master athletes:

Based on the inclusion criteria of the healthy non-athletes control group, there was a significant difference in age with old MA and significant difference in weekly training volume with both middle-age and old MA (Table 7.2). Old and middle-aged MA showed similar changes in SBP, MAP, DBP at rest, occlusion, ischemic exercise, PEO and recovery phases ($p>0.05$ for all, Fig. 7.4; Table E4. E5 – Online Supplement). There were no differences between MA groups and middle-age healthy controls in the rise slope of SBP or MAP during ischemic exercise. However, compared to the healthy middle-age group, both old and middle-aged MA groups showed a steeper decrease in SBP and MAP slopes over 1 or 2 mins of PEO (i.e., SBP 1 min: 0.38 ± 0.23, 0.35 ± 0.20 vs. 0.15 ± 0.10, $p<0.05$ for

both) with larger delta (Table E5 - Online Supplement). There was a faster BP recovery from the fatiguing occlusion protocol in the MA groups with a steeper decrease in SBP during 1 and 2 mins of recovery compared to the healthy middle-age controls ($p<0.05$, Fig. 7.4; Table E5 - Online Supplement). Moreover, the middle-age healthy MA group showed a lower SBP at the end of recovery phase compared to the middle-age healthy controls (132.3 ± 8.2 vs. 143.0 ± 14.1 mmHg, $p<0.05$, Fig. 7.4, Table E4 – Online Supplement). All groups reported similar pain discomfort during the 3 phases of the fatiguing occlusion protocol (Table E6 - Online Supplement).

Table 7.2 Subject characteristics in middle-age and old MA and middle-age non-athlete controls

Group	Middle-age Non-athlete Controls (10)	Middle-aged Master Athletes (12)	Old Master Athletes (12)
Age (y)	48.3 ± 8.3	48.9 ± 9.1	65.8± 10.2*
Height (cm)	172.4 ± 6.4	175.5 ± 7.4	169.8 ± 6.1
Weight (kg)	77.2 ± 9.6	77.4 ± 11.3	70.2 ± 11.2
BMI (kg/m^2)	25.9 ± 3.0	25.0 ± 2.3	23.8 ± 3.2
Training (h/week)	3.7 ± 1.5*	10.5 ± 2.8	11.6 ± 6.2
MVC (kg)	40.4 ± 14.8	49.7 ± 11.3	46.9 ± 10.9
Resting SBP (mmHg)	136.9 ± 11.6	129.4 ± 8.4	133.2 ± 16.4
Resting MAP (mmHg)	98.6 ± 11.59	94.9 ± 6.4	94.5 ± 9.7
Resting DBP (mmHg)	79.4 ± 11.9	77.5 ± 6.1	76.6 ± 8.8

Values are means ± SD; *p <0.05 old MA vs. middle-aged MA vs and none-athlete controls.

Abbreviations: BMI= body mass index; DBP= diastolic blood pressure; MAP= mean arterial pressure; MVC= maximal voluntary contraction; SBP= systolic blood pressure.

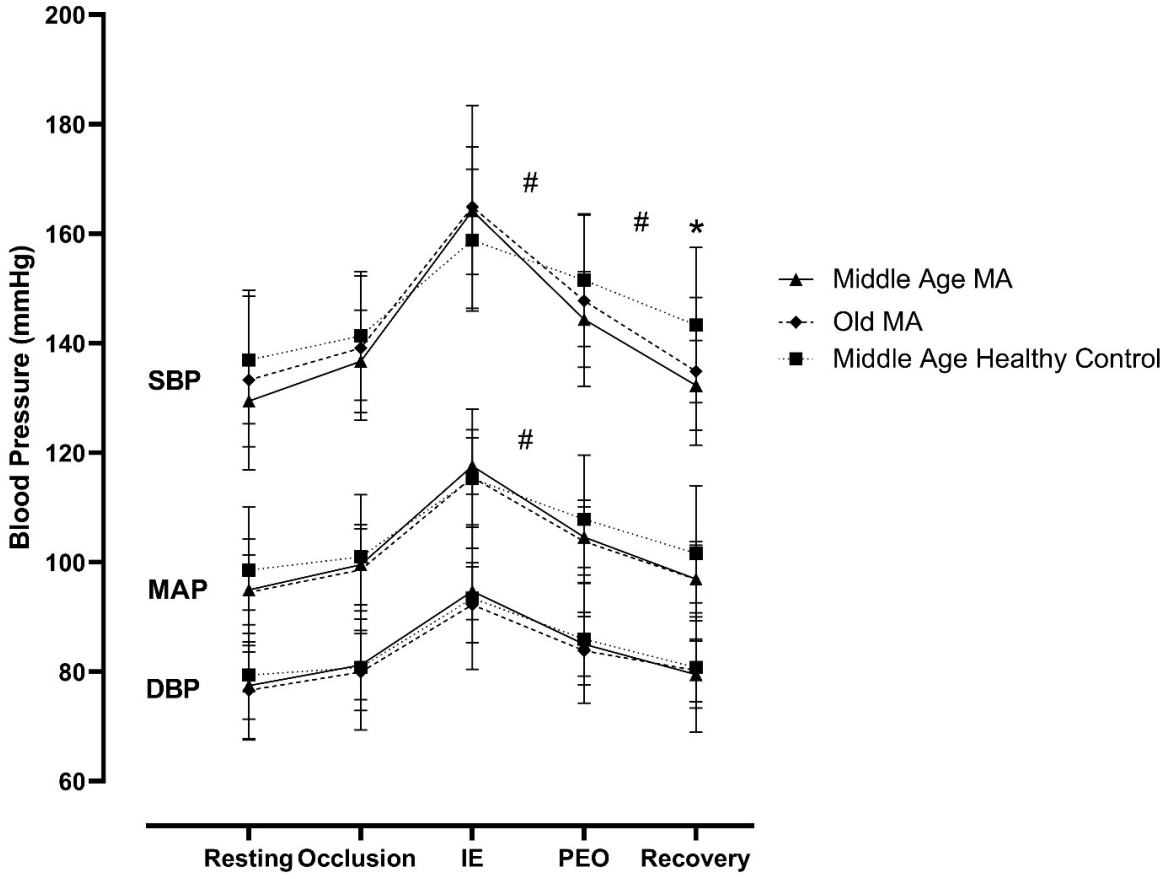


Figure 7.4 Blood Pressure responses in healthy middle-aged and older Master cyclists' groups and healthy middle-aged non-athletic controls at rest

Description: Blood Pressure responses in healthy middle-aged and older Master cyclists' groups and healthy middle-aged non-athletic controls at rest, occlusion, ischaemic exercise (IE), post-exercise occlusion (PEO), and recovery. Values are means ± SE. *$p < 0.05$ for middle-aged MA vs. non-athlete controls; #$p < 0.05$ for middle-age and old MA vs. non-athlete controls in SBP and MAP slopes.

7.4. Discussion:

To the best of our knowledge, this is the first study of exercise-related BP dynamics in master cyclists with and without comorbidities during an international competition. The main findings of this study are as follows: 1) In contrast to our hypothesis, Master cyclists

with underlying comorbidities showed similar BP responses at rest, occlusion, ischemic exercise, post ischemic exercise and recovery as healthy master cyclists, 2) the slope of SBP rise during ischemic isometric exercise was inversely correlated with AGP during the cyclist competition, and 3) old MA showed similar BP responses compared to healthy middle-age non-athletes, and 4) both old and middle-age MA showed a faster BP recovery during PEO and following fatiguing occlusion protocol compared to middle-age healthy controls.

Impact of underlying comorbidities on BP regulation in MA:

Our findings associate athletic training with improved BP regulation under occlusion, ischemic exercise and during recovery after exercise occlusion, regardless of underlying comorbidities in our MA participants. The link between altered BP responses to exercise and underlying comorbidities has been studied in detail amongst the general population (473, 474) and patient groups (118, 243, 432, 475, 476), but less is known about BP responses for athletic populations (444). Master athletes are characterised by very high levels of exercise training and they typically present with fewer underlying comorbidities compared with non-athletic individuals of the same age (445). However, long-term strenuous exercise could potentially lead to cardiovascular dysfunction (477, 478) and masked hypertension with altered cardiac function being a highlighted risk among master athletes (479).

The increases of blood pressure during isometric exercise are linked to the activation of peripheral mechano- and metabo-receptors (groups III and IV afferents) in musculature which activate central sympathetic outflow driving generalised arterial constriction (160, 287, 288). Cardiovascular diseases are characterized by altered functionality of peripheral or central influences on sympathetic outflow, which is associated with exaggerated increases BP and vascular resistance during exercise (119, 246, 480). Overactivation of sympathetic outflow and pressor responses during post-exercise ischemia, mediated by muscle metaboreflex, was linked to an increased BP in hypertensive individuals (243). However, Currie et al. reported similar arterial stiffness and sympathetic reactivity during isometric handgrip exercise and post-exercise muscle ischemia in healthy endurance MA with and without exaggerated BP to graded dynamic exercise (447). A proportion of the master cyclists in our study reported underlying medical conditions, albeit controlled and without any symptoms that interrupted their ability to compete in world championship events. Despite the presence of comorbidities, we did not find differences in BP responses during occlusion, IE or during post-exercise cessation compared to master cyclists free from comorbidities (Fig. 7.2). Therefore, one might conclude a possible protective effect of long-term intense exercise on BP regulation even in the presence of co-morbidities within elite master athletes.

While an exaggerated exercise BP in MA is considered a compensatory mechanism to maintain adequate perfusion for active muscles (447), increased BP during dynamic exercise was associated with lower exercise capacity in hypertensive patients (481-483) and elite young athletes (457). Among over 200,000 recreational to elite skiers, including

8% with comorbidities, higher performance in a Nordic skiing race at the Vasaloppet Swedish competition was strongly associated with a lower incidence of hypertension (444). In master cyclists, we found an inverse correlation between the rise in BP during ischemic isometric exercise and AGP regardless of the underlying comorbidities in our MA groups (Fig. 5.3). In approximately 10% with hypertensive resting BP of our MA population, exaggerated blood BP responses during IE (above 190 and 210 mmHg for women and men, respectively) allied to lower AGP in the world cycling competition.

Impact of age and training on BP regulation:

Age-associated changes in vascular structure and loss of central arteries elasticity (484-487), and chronic elevation of muscle sympathetic nerve activity (MSNA) (488, 489) are key determinants for impaired BP responses in sedentary aging. In contrast, endurance master athletes (cyclists, runners and swimmers, triathletes) show improved vascular function compared to sedentary healthy age-matched controls (490). This includes increased conduit artery cross-sectional area (435) higher arterial compliance (491-493), less hypertrophy of the arterial wall (490), lower arterial stiffness (485, 494), enhanced endothelial function and improved blood flow circulation (435) to optimize blood flow and BP regulation at rest and during exercise (435). Exaggerated BP responses (EBPR) with aging were reported during dynamic exercise (459, 495), but still controversial during isometric exercise (451, 496, 497). Our results showed that BP responses were comparable in old MA, middle-aged MA and middle-age healthy non-athletic controls during occlusion and IE (Fig. 4). It can be assumed that the stimulus of the mechano- or

metabo-receptors was similar for all groups. This may decrease the aging effect on the contribution of muscle III/IV afferents to stimulate BP under ischemic or isometric exercise conditions (451). The impact of lifetime training on vascular sympathetic activity in master athletes remains elusive and showed inconsistent findings of higher (488, 498) or no changes (489) in resting MSNA compared to healthy untrained counterparts. However, it is noteworthy that increased BP during occlusion or ischemic exercise does not necessarily follow elevated vascular sympathetic activity at rest (499).

The accelerated decrease of BP during PEO in middle-age and old masters cyclists compared to middle-age healthy non-athletic controls (Fig. 7.4.) may suggest robust reactivation of cardiac parasympathetic tone and increased cardiac baroreflex sensitivity in our masters cyclists following the Inhibition of central command and removal of mechano-reflex stimulation (500-502), causing a rapid reduction of BP despite sustained high MSNA due to muscle metaboreflex activation (503-506). Moreover, the accelerated recovery of BP in the master cyclists post-fatiguing occlusion protocol could be related to enhanced vasodilatory capacity (498, 507) and/or increased cardiovagal baroreflex sensitivity (508), that lead to a rapid decrease of BP. However, further and detailed measurement should be implemented in the future to clarify the contribution of chronic training on the interaction between muscle metaboreflex with arterial and cardiac baroreflex to the neural control of BP during PEO and recovery in MA population.

Study limitation

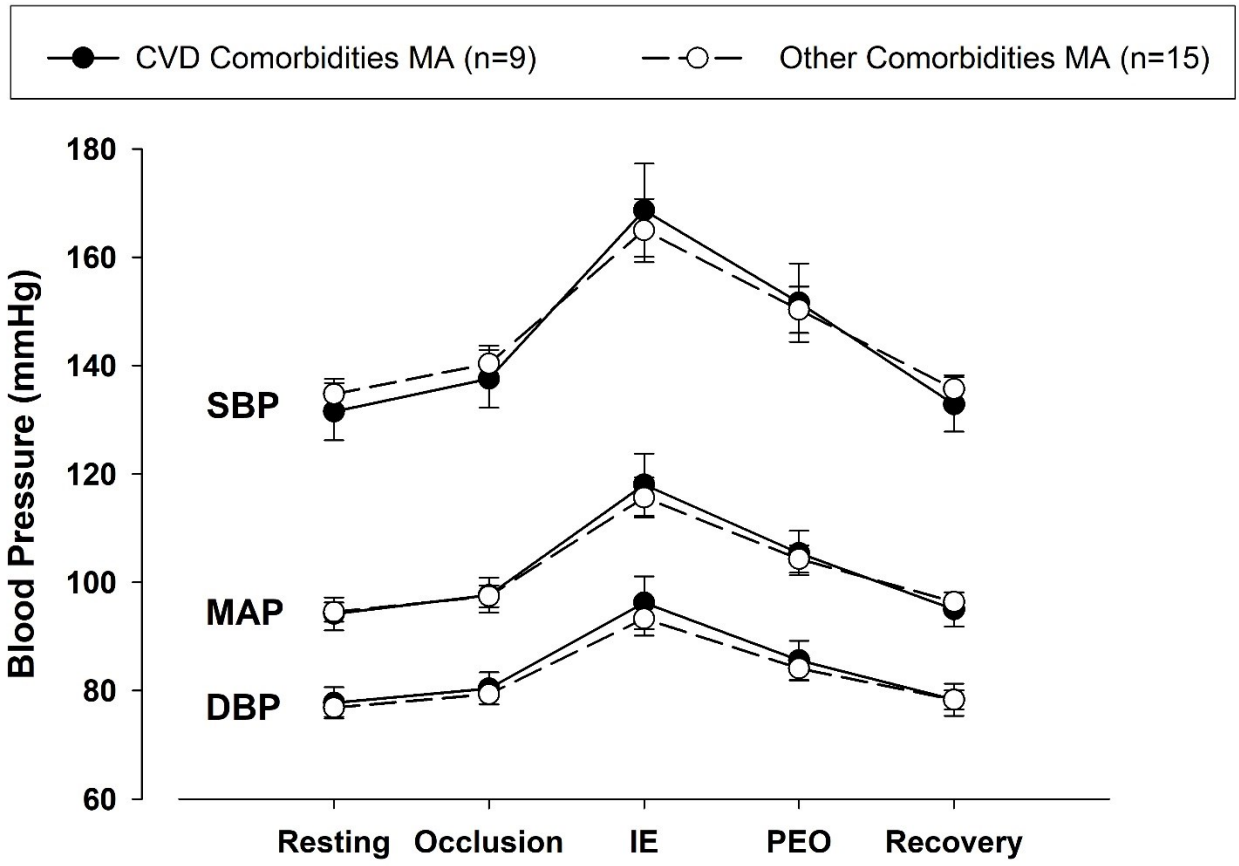
A possible limitation of this study was that the data from Master cyclists were collected during World Championships, which may have elevated BP responses due to anticipation, excitement, dehydration, or prior exercise. However, steps were taken to avoid strenuous exercise prior to testing and to ensure hydration before testing. Additionally, there were no differences in baseline BP in Master cyclists compared to middle-age healthy non-athletic controls who did the measurements in a quiet laboratory setting. Our main finding of similar BP responses between healthy MA and those with underlying comorbidities is likely to be influenced by medication. The comorbidities MA group has been screened and their medications were eligible to be used during international events (434, 509). More invasive techniques of microneurography were not possible due to testing constraints, but these would have provided greater insights into the possible mechanisms influencing BP responses during IE, PEO and recovery. In addition, only 25% of the athletes were female due to lower participation of females in the events compared to male athletes. More female athletes should be studied in the future as endurance training showed only higher resting MSNA mainly in women Master athletes (488).

7.5. Conclusion

These findings of accelerated blood pressure recovery after cessation of ischaemic hand grip exercise in master cyclists associate long-term athletic training with improved blood pressure dynamics regardless of age or underlying comorbidities of Master athletes. Middle-aged and older Master cyclists showed accelerated BP recovery after cessation of ischaemic hand grip exercise compared with non-athletic middle-aged adults. This

response was similar between master cyclists with and without underlying comorbidities. Exaggerated BP in Master cyclists during ischemic exercise was associated with lower AGP during the World Master Track Cycling Championships.

Supporting information:



Fig_E1. Representing blood pressure response in Master athletes with cardiovascular and other comorbidities

Table E1. List of Medications

Medication	Number of Patients
Anti hypertensive	1
Anti-GERD	3
Statins	2
Aspirin	2
Thyroid replacement therapy	3
SABA	4
ICS	1

GERD, gastroesophageal reflux disease; SABA, short-acting β2 agonist; ICS, inhaled corticosteroids.

Table E2. Blood Pressure Responses in Healthy and Comorbidities Master Athletes Groups

Group	Healthy MA (24)	Comorbidities MA (24)
SBP Resting (mmHg)	131.3 ± 12.6	133.1 ± 12.7
SBP Occlusion (mmHg)	137.9 ± 11.0	138.1 ± 13.2
SBP Ischemic Exercise (mmHg)	164.8 ± 15.0	164.1 ± 22.7
SBP Post Exercise Occlusion (mmHg)	146.0 ± 12.2	148.5 ± 17.1
SBP Recovery (mmHg)	133.6 ± 10.8	133.6 ± 11.7
MAP Resting (mmHg)	94.7 ± 7.9	94.4 ± 7.9
MAP Occlusion (mmHg)	99.1 ± 7.1	97.1 ± 8.0
MAP Ischemic Exercise (mmHg)	116.8 ± 7.3	114.9 ± 14.0
MAP Post Exercise Occlusion (mmHg)	104.2 ± 6.3	103.7 ± 9.9
MAP Recovery (mmHg)	96.9 ± 6.3	95.5 ± 7.7
DBP Resting (mmHg)	77.1 ± 7.3	77.5 ± 7.7
DBP Occlusion (mmHg)	80.6 ± 6.4	79.7 ± 7.9
DBP Ischemic Exercise (mmHg)	93.8 ± 6.4	93.4 ± 12.5
DBP Post Exercise Occlusion (mmHg)	84.4 ± 5.8	84.4 ± 9.5
DBP Recovery (mmHg)	79.9 ± 5.7	78.3 ± 7.7
SBP Delta Rest/Occl (mmhg)	6.5 ± 3.9	5.1 ± 6.1

SBP Delta Occl/IE (mmHg)	26.9 ± 9.5	25.9 ± 12.3
SBP Delta IE/PEO (mmHg)	18.8 ± 10.9	15.6 ± 9.8
SBP Delta PEO/Recovery (mmHg)	12.5 ± 6.3	14.9 ± 10.9
MAP Delta Rest/Occl (mmhg)	4.3 ± 2.8	2.6 ± 3.1
MAP Delta Occl/IE (mmHg)	17.8 ± 5.3	17.9 ± 8.6
MAP Delta IE/PEO (mmHg)	12.6 ± 5.6	11.3 ± 6.3
MAP Delta PEO/Recovery (mmHg)	7.3 ± 3.6	8.2 ± 6.5
DBP Delta Rest/Occl (mmhg)	3.5 ± 2.7	2.2 ± 2.4
DBP Delta Occl/IE (mmHg)	13.2 ± 4.3	13.7 ± 7.2
DBP Delta IE/PEO (mmHg)	9.3 ± 4.1	9.1 ± 5.6
DBP Delta PEO/Recovery (mmHg)	4.6 ± 3.0	6.0 ± 4.8

Values are means ± SD; groups did not differ significantly for any of the variables (p>0.05).

Table E3. Blood Pressure Slope Analysis in Healthy and Comorbidities Master Athletes Groups

Group	Healthy MA (24)	Comorbidities MA (24)
SBP Slope HG	0.41 ± 0.18	0.39 ± 0.17
SBP Slope 1 PEO	-0.37± 0.21	-0.26 ± 0.17
SBP Slope 2 PEO	-0.14 ± 0.09	-0.10 ± 0.06
SBP Slope 1 Recovery	-0.18 ± 0.09	-0.15 ± 0.22
SBP Slope 2 Recovery	-0.09 ± 0.07	-0.09 ± 0.10
MAP Slope HG	0.31 ± 0.12	0.27 ± 0.13
MAP Slope 1 PEO	-0.20 ± 0.15	-0.16 ± 0.10
MAP Slope 2 PEO	-0.04 ± 0.07	-0.04 ± 0.06
MAP Slope 1 Recovery	-0.07 ± 0.08	-0.08 ± 0.13
MAP Slope 2 Recovery	-0.02 ± 0.06	-0.03 ± 0.06

Values are means ± SD; groups did not differ significantly for any of the variables (p>0.05).

Table E4. Pain Responses in Healthy and Comorbidities Master Athletes Groups

Group	Healthy MA (24)	Comorbidities MA (24)
Pain – Discomfort Occlusion	1.7 ± 1.3	1.7 ± 1.1
Pain – Discomfort IE	2.9± 2.0	3.6 ± 1.4
Pain – Discomfort PEO	4.7 ± 2.4	4.9 ± 1.6

Values are means ± SD; groups did not differ significantly for any of the variables (p>0/05)

Table E5. Blood Pressure Responses in Middle-age MA, Old MA and Middle-age- Non-athlete Controls

Group	Middle-age Non-athlete Controls (10)	Middle-age Master Athletes (12)	Old Master Athletes (12)
SBP Resting (mmHg)	136.9 ± 11.6	129.5 ± 8.4	133.3 ± 16.4
SBP occlusion (mmHg)	141.4 ± 11.8	136.7 ± 9.3	139.1 ± 13.2
SBP Ischemic Exercise (mmHg)	158.8 ± 13.0	164.2 ± 11.6	164.9 ± 18.5
SBP Post Exercise Occlusion (mmHg)	151.5 ± 12.1	144.4 ± 8.8	147.7 ± 15.7
SBP Recovery (mmHg)	143.0 ± 14.1*	132.3 ± 8.2	134.8± 13.5
MAP Resting (mmHg)	98.6 ± 11.6	94.9 ± 6.4	94.5 ± 9.7
MAP occlusion (mmHg)	101.0 ± 11.4	99.5 ± 7.3	98.6 ± 7.5
MAP Ischemic Exercise (mmHg)	115.2 ± 12.7	117.6 ± 5.1	115.5 ± 8.7
MAP Post Exercise Occlusion (mmHg)	107.8 ± 11.7	104.6 ± 5.5	103.8 ± 7.5
MAP Recovery (mmHg)	101.6 ± 12.3	96.9 ± 6.2	96.9 ± 6.9
DBP Resting (mmHg)	79.4 ± 11.9	77.5 ± 6.1	76.6 ± 8.8
DBP occlusion (mmHg)	80.8 ± 11.4	81.2 ± 6.4	80.0 ± 7.0
DBP Ischemic Exercise (mmHg)	93.4 ± 13.0	94.7 ± 5.2	92.2 ± 6.9
DBP Post Exercise Occlusion (mmHg)	86.0 ± 11.7	85.0 ± 5.8	83.8 ± 6.2
DBP Recovery (mmHg)	80.8 ± 11.8	79.5 ± 6.2	80.2 ± 5.7
SBP Delta Rest/Occl (mmhg)	4.4 ± 3.9	7.3 ± 4.1	5.8 ± 4.0
SBP Delta Occl/IE (mmHg)	17.5 ± 3.4*	27.5 ± 10.0	25.8 ± 8.9

SBP Delta IE/PEO (mmHg)	7.3 ± 3.5*	19.9 ± 11.3	17.2 ± 10.0
SBP Delta PEO/Recovery (mmHg)	8.5 ± 5.2	12.1 ± 7.0	12.9 ± 6.0
MAP Delta Rest/Occl (mmhg)	2.4 ± 2.1	4.6 ± 2.8	4.1 ± 3.0
MAP Delta Occl/IE (mmHg)	14.3 ± 4.1	18.1 ± 6.4	16.9 ± 3.4
MAP Delta IE/PEO (mmHg)	7.4 ± 3.3*	13.0 ± 5.8	11.7 ± 4.6
MAP Delta PEO/Recovery (mmHg)	6.2 ± 2.9	7.7 ± 3.9	6.9 ± 3.4
DBP Delta Rest/Occl (mmhg)	1.4 ± 1.4	3.7 ± 2.7	3.3 ± 2.9
DBP Delta Occl/IE (mmHg)	12.7 ± 4.7	13.5 ± 5.2	12.3 ± 3.3
DBP Delta IE/PEO (mmHg)	7.5 ± 3.6	8.7 ± 4.9	6.9 ± 4.1
DBP Delta PEO/Recovery (mmHg)	5.2 ± 2.3	5.5 ± 2.8	3.6 ± 3.0

*Values are means ± SD; *p <0.05 Middle-age MA vs Old MA vs Control.*

Table E6. BP Slope Analysis in Middle-Age and Old MA and Middle-Age Non-Athlete Controls

Group	Middle-age Non-athlete Controls (10)	Middle-age Master Athletes (12)	Old Master Athletes (12)
SBP Slope HG	0.31 ± 0.09	0.39 ± 0.21	0.43 ± 0.17
SBP Slope 1 PEO	-0.15 ± 0.10#	-0.35 ± 0.20	-0.38 ± 0.23
SBP Slope 2 PEO	-0.05 ± 0.07#	-0.14 ± 0.09	-0.13 ± 0.10
SBP Slope 1 Recovery	-0.06 ± 0.13#	-0.17 ± 0.08	-0.20 ± 0.11
SBP Slope 2 Recovery	-0.01 ± 0.06#	-0.09 ± 0.09	-0.09 ± 0.05
MAP Slope HG	0.27 ± 0.12	0.28 ± 0.13	0.34 ± 0.12
MAP Slope 1 PEO	-0.06 ± 0.04#	-0.22 ± 0.13	-0.21 ± 0.14
MAP Slope 2 PEO	-0.02 ± 0.03#	-0.06 ± 0.05	-0.06 ± 0.05
MAP Slope 1 Recovery	-0.06 ± 0.05	-0.09 ± 0.04	-0.09 ± 0.07
MAP Slope 2 Recovery	-0.02 ± 0.01	-0.04 ± 0.6	-0.04 ± 0.02

Values are means ± SD; # p <0.05 Middle-age MA vs Old MA vs Control.

Table E7 Pain Responses in Middle-Age and Old MA and Middle-Age Non-Athlete Controls

Group	Middle-age Non-athlete Controls (10)	Middle-age Master Athletes (12)	Old Master Athletes (12)
Pain – Discomfort Occlusion	1.7 ± 1.6	1.6 ± 1.5	1.6 ± 1.3
Pain – Discomfort IE	2.0± 2.3	2.5 ± 2.1	2.5 ±2.7
Pain – Discomfort PEO	3.9 ± 2.5	3.9 ± 2.4	4.2 ± 2.9

Values are means ± SD; groups did not differ significantly for any of the variables (p>0.05).

Chapter 8. Experimental Study V

Sensitivity of group III-IV nerve afferents: Implications on the reduced cardiorespiratory and neuromuscular function in Chronic Syndromes

What is known? In the previous chapter (i.e., Chapter 4-6) we investigated the effects of muscle afferent sensitisation on cardiovascular, cardiorespiratory, and neuromuscular function in a controlled experimental environment. During Fibromyalgia and Chronic fatigue syndrome an increase of rate of perceived exertion and pain during exercise has been found suggesting a link between an increased muscle afferent activity to exercise tolerance, reduced exercise performance, lower cardiorespiratory fitness, and neuromuscular function. However, despite several studies are present in the literature, a clear picture of all these impairments and entity is missing.

What is going to be assessed? For these reasons, we decided to carry an extensive systematic review and meta-analysis on the cardiorespiratory fitness and neuromuscular component in fibromyalgia and chronic fatigue syndromes, to quantify the entity of these changes and to assess if study quality was affecting the outcomes. Moreover, we performed several subgroup analyses, for comparing results between the two conditions and controlling for physical activity and pharmacological wash out, as possible co-funding factors across studies.

This work has been published as: Zambolin F, Duro-Ocana P, Faisal A, Bagley L, Gregory WJ, et al. (2022) Fibromyalgia and Chronic Fatigue Syndromes: A systematic review and meta-analysis of cardiorespiratory fitness and neuromuscular function compared with healthy individuals. PLOS ONE 17(10): e0276009. https://doi.org/10.1371/journal.pone.0276009

Fibromyalgia and Chronic Fatigue Syndromes: a systematic review and meta-analysis of cardiorespiratory fitness and neuromuscular function compared with healthy individuals.

8.1. Introduction

The incidence of Fibromyalgia Syndrome and Chronic Fatigue Syndrome (FMS, CFS) is estimated to be 1.0 – 2.7% of the worldwide population, with 2 – 3 times higher prevalence for females than males (56, 57, 510-512). Both syndromes are difficult to identify, and diagnosis usually follows recommendations of 2010 FMS diagnostic criteria and the 2015 IOM diagnostic criteria for ME/CFS (62, 267). However, these criteria are not entirely objective (60, 69-71) and concerns remain about their implementation (513). This leads to delayed diagnosis which impacts on quality of life for those affected (70) before clinical management plans can be implemented.

CFS and FMS conditions overlap substantially through the shared symptom of chronic fatigue which occurs in the absence of intense or prolonged physical activity and is not necessarily ameliorated by rest (63). FMS also causes widespread musculoskeletal pain in the absence of any structural or morphological abnormalities of skeletal muscle tissue from histological and imaging analysis (59-61).

The origin and pathophysiology of these syndromes are not fully understood (56, 74). They may arise due to “sensitisation” of central and/or peripheral nervous systems to sensory stimuli (59, 75-78): afferent signals originating in the periphery may be amplified ahead of processing in the central nervous system (79) leading to a hypersensitisation of typical somatosensory stimuli during everyday activities. If this were the case, then physical activity should aggravate symptoms, which is consistent with patient reports of discomfort during exercise (94, 95), reduced exercise tolerance (216, 217, 514) and relatively low habitual physical activity levels (515, 516). Aberrant somatosensory signalling may reduce cardiorespiratory fitness and neuro-muscular

function and increase perceptions of effort to levels excessive for the respective physiological strain, but this remains unclear due to conflicting reports, heterogeneity of methodological approaches or few studies including relevant outcome data in the literature. Previous reviews investigated cardiorespiratory fitness and neuromuscular function in CFS and FMS , finding that peak oxygen consumption was reduced while muscle strength and rate of perceived exertion were increased (216, 512, 517, 518). However, the possible underlying physiological processes of the lower physical function remain unclear.

The aim of this systematic review and meta-analysis was to determine physical performance of people living with CFS and FMS compared with healthy controls, and to identify possible underlying physiological processes associated with reduced physical performance of the patient groups. Physical performance was classified into two main components: cardiorespiratory fitness and neuromuscular function. Cardiorespiratory fitness was taken as the peak rate of oxygen uptake (VO_2peak: measured during incremental exercise) and anaerobic threshold. Secondary indicators were collected where available to understand possible causes of reduced cardiorespiratory fitness, including peak lactate measurements, peak heart rates and ratings of perceived exertion (RPE). Neuromuscular function was characterised as maximal voluntary contraction (MVC), performance fatigability (519), voluntary activation (the ability to fully activate available motor units during MVC), alongside measures of skeletal muscle mass, and rate of perceived exertion. Performance fatigability is defined as an acute decline in motor performance caused by an exercise-induced reduction in force or power of the involved muscles (33).

8.2. Methods

The present systematic review is reported in accordance with the Preferred Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (520). Methods of the analysis and inclusion criteria were pre-specified and registered on the International Prospective Register of Systematic Reviews (PROSPERO: protocol-CRD42020184108). Full ethical approval was given from the Science and Engineering Research Ethics and Governance Committee at Manchester Metropolitan University (reference number 23820).

Eligibility Criteria

Studies were included if they were observational or interventional designs providing data for patients with diagnosed CFS or FMS. Participants needed to be aged 18 years or older and studies needed to provide results for healthy controls. For interventional studies only the baseline data were included.

VO_2peak, ventilatory threshold and/or lactate measurements were classified as cardiorespiratory fitness outcomes. Peak heart rate and RPE during incremental exercise were also recorded to indicate whether incremental exercise tests were terminated before the estimated peak heart rate was achieved. MVC, fatigability, voluntary activation, muscle mass or volume were recorded as neuromuscular outcomes. Fat mass and RPE during fatigue tests were also recorded.

Studies were excluded if presenting results from animal models, if they included participants aged younger than 18 years, if they were not peer reviewed or not written in English language.

Search Strategy

PubMed, Medline, CINAHL, AMED, Cochrane Central Register of Controlled Trials (CENTRAL), and PEDro databases were searched using keywords and medical subject headings structured in a PICO framework (S4 File). Initial literature searches were conducted between April - June 2020 and updated then updated to June 2022 .

Selection Process

Search results were collated in referencing software (EndNote X9 – Clarivate Analytics) and shared between two independent researchers (FZ and PDO). Records were removed if titles and abstracts clearly showed they were not eligible. Full texts of the remaining records were screened by the two independent researchers (FZ and PDO). Disagreements about whether a study should be included were resolved by discussion with a third researcher (JM).

Data Items and Collection Process

Included studies were read in full and the relevant data were entered to a Microsoft© Excel spreadsheet. The following data were extracted from each included study: type

of diagnosis (CFS and/or FMS) publication details, sample size, subjects' characteristics (age-sex), diagnostic criteria, participants' matching type, outcome assessments and details of test protocols.

If data from the studies were not available, or were incomplete, they were not included for further analysis. Where data were reported only in a figure format without any possibility to precisely extract their values, the corresponding author of that study was contacted for the numerical data. If the data could not be provided or no answer was received, the study was excluded from further analysis.

Risk of bias assessments

Risk of Bias was assessed using the Quality Assessment Tool for Quantitative studies (EPHPP) (521) covering six domains: 1) selection bias (representation of the target population), 2) study design, 3) confounding factors, 4) blinding, 5) data collection method and, 6) withdrawal. Components 2 and 4 were excluded since they relate primarily to intervention studies (522). Studies were evaluated for components 1, 3, 5 and 6 as strong, moderate, or weak depending on the number of parts evaluated as weaker (i.e., two or more weak evaluations was recorded as a low study quality; only one, or no weak points was recorded as high study quality).

A risk of bias tool from Nijs et al (512) was applied to improve the quality ratings of the confounding domains of the EPHPP tool across eight domains: 1) presence of a priori power calculations; 2) controls comparable for age; 3) gender ; 4) body height or weight; 5) physical activity level; 6) presence of sedentary subjects; 7) blinded

assessments; and 8) medications wash-out prior to the tests. If reaching a score lower than 50% on the specific tool from Nijs (512), the confounding domain was rated as weak. This process helped to define specific cofounding factors as applied in previous reviews(512, 517).

Sensitivity analysis (523) was completed to examine possible differences in outcomes based on the risk of bias and a further subgroup analysis was conducted on the low risk of bias studies if heterogeneity was still present (I^2>40%)

Data Synthesis.

Continuous data were pooled using a random-effect meta-analysis using the inverse variance method, with the measure of effect between patient and control groups being standardized mean differences (SMD) and 95% confidence intervals. Effect size thresholds were considered at 0.2, 0.5 and 0.8 for small, moderate and large effect sizes, respectively (522). Where results were stratified by sex, the male and female samples were combined to derive a single effect (524). Combined means and standard deviations for Wåhlén et al (525), Sargent et al.(526) and Vermeulen et al(527, 528) were calculated using the method described in the Cochrane Handbook(524). Studies reporting the median and interquartile range or minimum and maximum range were transformed into mean and SD from the Microsoft© Excel spreadsheet tool provided by Wan et al(529). Heterogeneity was classified in four domains: 1) might not be important (I^2 = 0% - 40%), 2) may represent moderate heterogeneity (I^2 = 30% - 60%), 3) may represent substantial heterogeneity (I^2 = 50% - 70%), or 4) considerable heterogeneity (I^2 = 70 – 100%) using the criteria proposed by Higgins et al(530).

Initially all the samples for each outcome were included within a single model, but if heterogeneity was considered important (I^2 >40%) further subgroup analysis was conducted on the potential source of heterogeneity. All meta-analyses and subgroup analyses were performed using RevMan 5.4 software. The a priori level of significant difference was set at $p<0.05$. Pooled data are presented as Cohen's d standardized mean difference (SMD) with 95% confidence intervals. Our primary approach was to combine results of patients with CFS and FMS for comparison to healthy controls, given the overlap that is present between the two syndromes (531-533) and the small sample sizes. This approach was also implemented in a previous systematic review with meta-analysis (534). However, we have also provided a subgroup analysis differentiating studies from FMS and CFS for each available outcome measurement (Fig 8.3 and 8.4. and S3).

8.3. Results

Selection of Studies

A total of 7984 records were identified and 99 of those met the inclusion criteria (Fig 8.1.). From the 99 studies, 40 of them reported cardiorespiratory fitness outcomes, 28 reported muscle function outcomes, 10 reported body composition outcomes and the remaining 21 studies included outcomes of cardiorespiratory fitness and muscle function and body composition. Summary of overall results are reported in figure 8.2.

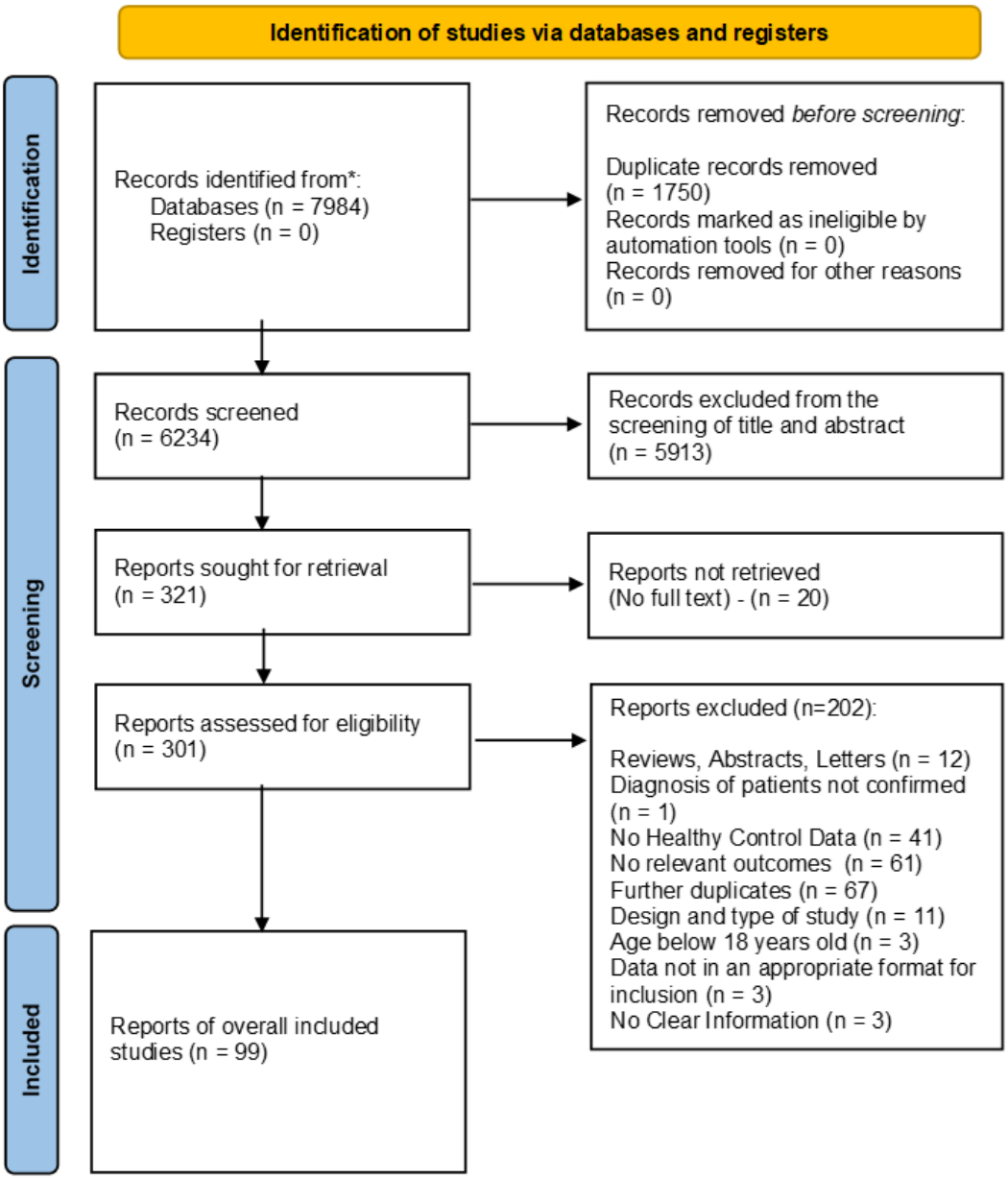


Figure 8.1 The 2020 prisma flow diagram.

Flow diagram for selection and inclusion of studies.

Cardiorespiratory fitness

VO2peak

Data from 52 studies showed a significant difference between patient and control groups, with a large effect size (SMD=-0.86, 95%CI=-1.04 to -0.69) revealing lower VO_2 peak values for patients. Heterogeneity was classified as substantial (I^2= 80%).

Studies with lower risk of bias included 875 patients and 643 healthy controls (I^2= 51%), again revealing lower VO_2peak values for patients compared with healthy controls but with a moderate effect size (SMD=-0.61; 95%CI =-0.77, -0.46).

Anaerobic Threshold (AT)

The 16 included studies showed that patients had significantly lower AT values than controls with a large effect size (SMD-0.86, 95%CI=-1.12 to -0.60). Heterogeneity was classified as substantial (I^2= 57%).

Studies with low risk of bias included 412 patients and 214 healthy controls (I^2= 66%). They also showed lower AT for patients compared with healthy controls with large effect size (SMD=-0.80; 95%CI =-1.15, -0.46).

Peak lactate

Data from 11 studies showed that patients had significantly lower peak lactate values than controls (Z=3.44, p=0.00001), with a large effect size (SMD=-0.84, 95%CI=-1.33 to -0.36). Heterogeneity was classified as substantial (I^2= 80%).

Studies with low risk of bias included 82 patients and 81 healthy controls (I^2= 41%). They also showed lower peak lactate for patients, but with small effect size (Z=1.50, p=0.13; ES=-0.27; 95%CI =-0.62, 0.08).

Peak Heart Rate

Thirty-nine studies showed that patients had significantly lower peak HR than controls at the end of incremental exercise, even when normalized to age. There was a moderate-high effect size (SMD=-0.64, 95%CI=-0.77 to -0.50) and heterogeneity was classified as substantial (I^2= 58%).

Studies with low risk of bias included 1025 patients and 813 healthy controls (I^2= 26%). They also showed lower peak HR for patients, but with large effect size (SMD=-0.57; 95%CI =-0.70, -0.44).

Neuromuscular function

Maximal Voluntary Contraction (MVC)

Data from 42 studies showed significant difference between groups, revealing lower MVC values for patients and a large effect size (SMD=-0.93, 95%CI=-1.12 to -0.75). Heterogeneity was classified as substantial (I^2= 89%).

Studies with low risk of bias included 864 patients and 667 healthy controls (I^2= 39%). They showed a moderate decrease of MVC for patients (SMD=-0.63; 95%CI =-0.78, -0.49).

Fatigability

Fifteen studies were included, showing that patients were more fatigable than controls with a large effect size (SMD=-0.88, 95%CI=-1.19 to -0.57). Heterogeneity was classified as substantial (I^2= 84%).

Studies with low risk of bias included 135 patients and 85 healthy controls (I^2= 70%). They also showed patients to be more fatigable than controls with moderate effect size; (SMD=-0.47; 95%CI =-0.77, -0.18).

Voluntary activation

Seven studies considered voluntary activation, providing data of 145 patients and 147 healthy controls. Patients had lower voluntary activation than controls with moderate effect size (SMD=-0.34, 95%CI=-0.70 to 0.03). Heterogeneity was classified as moderate (I^2= 54%). Further sub-group analysis was not completed due to the low number of available studies.

Muscle Mass

Data from 11 studies showed similar muscle mass for patients and controls and small effect size (SMD=-0.14, 95%CI=-0.30 to 0.02). Heterogeneity was classified as substantial (I^2= 70%).

Studies with low risk of bias included 173 patients and 171 healthy controls (I^2= 0%). They showed no difference in muscle mass for patients compared to healthy controls (SMD= -0.00; 95%CI =-0.21, 0.21).

Muscle Volume

Four studies considered thigh muscle volume (quadriceps, hamstrings), providing data of 63 patients and 58 healthy controls. Patients had lower muscle volume than controls (Z=2.96, p=0.003), with moderate effect size (SMD=-0.56, 95%CI=-0.92 to -0.19). There was no indication of heterogeneity (I^2= 0%). Further sub-group analysis was not completed due to the low number of available studies.

Fat Mass

Data from 16 studies showed higher fat mass for patients compared to controls with moderate effect size (SMD 0.36, 95%CI=0.22 to 0.50). Heterogeneity was classified as moderate (I^2= 55%).

Studies grouped as low risk of bias included 184 patients and 182 healthy controls (I^2= 0%). They showed similar fat mass for patients and controls with small effect size (SMD=0.21; 95%CI =0.00, 0.41).

Perception indicators

Rate of Perceived Exertion (RPE)

Mean and Peak RPE outcomes were available for 29 studies covering cardiopulmonary fitness and muscle function. RPE was significantly higher for patients than controls for the given workload, with moderately large effect size (SMD 0.84, 95%CI=0.60, 1.08). Heterogeneity was classified as substantial (I^2= 77.0%).

Studies with low risk of bias included 330 patients and 336 healthy controls (I^2= 53%). They showed higher RPE for patients than controls and a large effect size (SMD=1.06; 95%CI =0.81, 1.31).

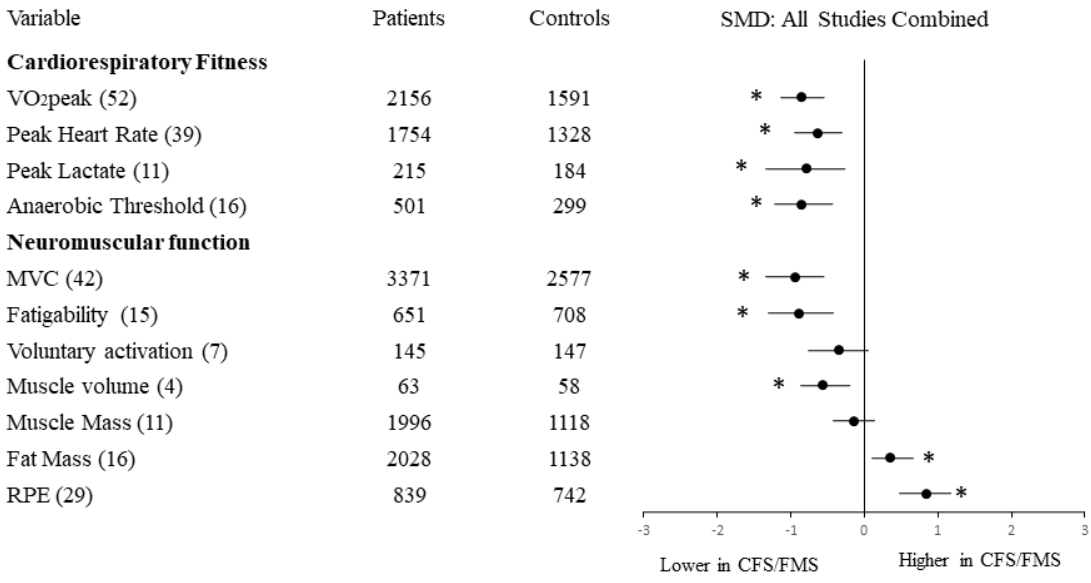


Figure *8.2* Effect Size of differences in cardiorespiratory and neuromuscular outcomes in CFS and FMS compared with healthy controls. Numbers in brackets indicate the number of studies reporting each variable. Abbreviations: MVC: Maximal Voluntary Contraction; RPE: rating of perceived exertion. *p<0.05 for overall effect size.

Comparison of results for FMS and CFS

Figures 3 and 4 present the results for FMS and CFS separately. Sub-group analysis was performed to determine whether, compared with their respective healthy controls, the functional reductions of FMS were different from those of CFS. The results showed no significant differences between the two (FMS and CFS) for cardiorespiratory fitness or neuromuscular function outcomes, except for MVC which was relatively lower for FMS than CFS (p=0.04).

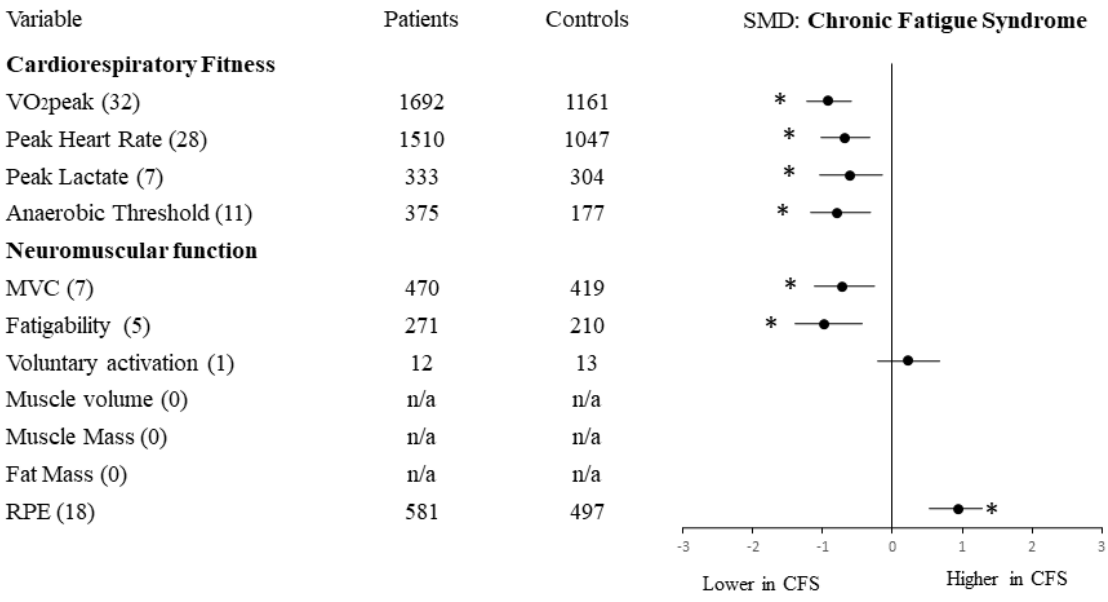


Figure 8.3 Effect Size of differences in cardiorespiratory and neuromuscular outcomes in CFS compared with healthy controls.

Numbers in brackets indicate the number of studies reporting each variable. Abbreviations: MVC: Maximal Voluntary Contraction; RPE: rating of perceived exertion. *p<0.05 for overall effect size.

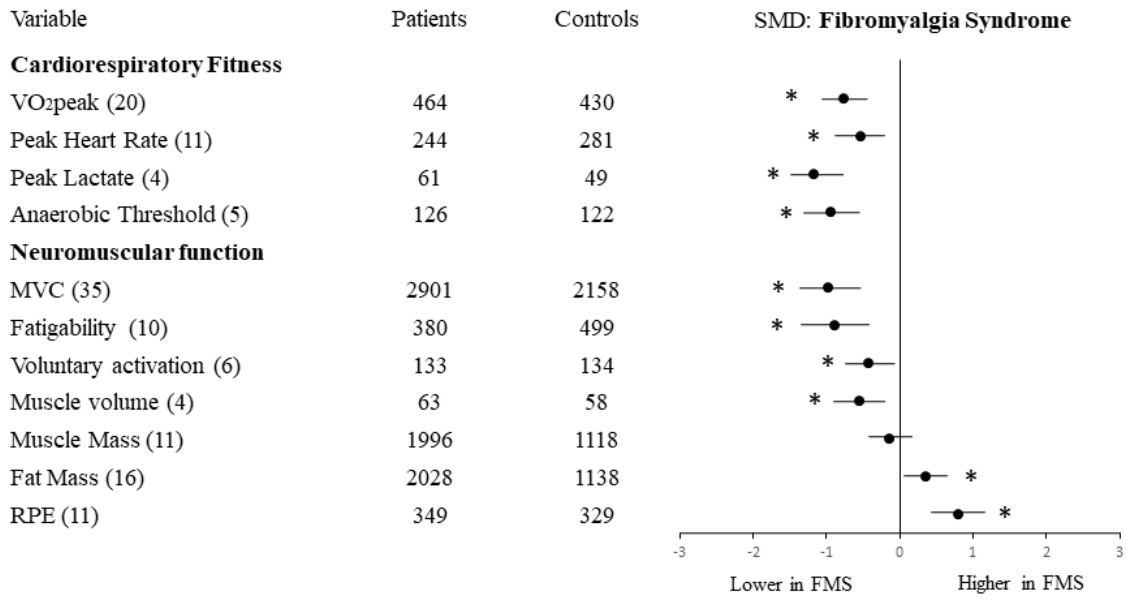


Figure 8.4 Effect Size of differences in cardiorespiratory and neuromuscular outcomes in FMS compared with healthy controls.

Numbers in brackets indicate the number of studies reporting each variable. Abbreviations: MVC: Maximal Voluntary Contraction; RPE: rating of perceived exertion. *p<0.05 for overall effect size.

8.4. Discussion

Summary of the main findings

This systematic review and meta-analysis included 99 studies detailing results of CFS and/or FMS patients compared with their healthy counterparts. The results showed lower VO_2peak, AT, and MVC for patients compared with controls. We further sought to understand the underlying causes of their lower physical function by exploring the possibility of patients having lower muscle mass, reduced ability (or willingness) to fully activate motor pathways, or increased perceptions of effort during exercise. The available evidence showed a discordance of perceived to physical exertion as

exercising patients reported higher ratings of perceived exertion compared with controls for a given workload or heart rate. Patients also had lower voluntary activation and fatigued more quickly than controls.

Our sub-group analysis showed negligible differences between CFS and FMS for their relative reductions of cardiorespiratory or neuromuscular outcomes, except for a lower MVC in FMS compared with CFS (S3). However, few studies of CFS reported skeletal muscle mass, fat mass or voluntary activation so conclusions on these outcomes are limited.

This systematic review and meta-analysis provide new insights into the possible underlying determinants of reduced cardiorespiratory and muscle function of patients with CFS and FMS. Previous reviews (216, 512, 518, 535) reported physical function in CFS and/or FMS. Of these, Franklin et al (535) performed a meta-analysis of 32 studies reporting VO_2peak outcomes in CFS, and Gaudrealt et al (518) considered cardiorespiratory fitness of FMS with a narrative review: both reported lower cardiorespiratory fitness of patient groups. More recently, Barhorst et al (216) reported higher RPE during exercise for patients with CFS and FMS, alongside lower heart rates at volitional exhaustion compared with healthy controls. We are aware of only one previous systematic review of muscle function impairments in CFS (512), but no others considering FMS or including wider aspects of muscle function such as fatigability and VA outcomes.

Cardiorespiratory fitness

Some studies utilised indirect assessments of VO_2peak by extrapolating data points obtained from submaximal exercise testing, but indirect assessments have greater errors of estimates and accordingly the reported results varied considerably from one study to the next. Direct tests of VO_2peak are more accurate and require participants to continue to exercise through progressively increasing intensities until volitional exhaustion. From the reviewed literature it was clear that few patients satisfied the criteria (ACSM guidelines (536)) for achieving a true VO_2max and they terminated exercise at lower peak workloads than controls.

The AT was usually estimated during tests of VO_2peak, and it represents the threshold beyond 'steady-state' at which glycolytic rates rise. Indicators of AT include blood lactate above 4 mmol/L or the gas exchange or ventilatory thresholds identified from the non-linear relationship of VCO_2 to VO_2 (537, 538). Regardless of study quality, risk of bias or methodological approaches, the results were consistent with those of VO_2peak, where values for AT were lower for patients than controls. This suggests the impairments affecting cardiorespiratory fitness of patients are not only evident during very intense activity where high motivation is required, but they are also evident during moderate intensity activities.

Since VO_2peak and AT are highly adaptable: decreasing with periods of inactivity and increasing with periods of exercise training, it may be speculated that values for both were lower for patients than controls due to habitual sedentary lifestyles (512, 539) or pharmacological effects on the patient groups (512). However, this conclusion is not fully supported by the available data, given the other indicators that patients were not able to exercise at the high intensities required to achieve VO_2peak. These

included lower peak heart rates and peak blood lactate levels during exercise for FMS and CFS compared with healthy controls. To account for possible confounding effects, some studies matched groups for habitual physical activity levels or included a pharmacological wash-out period prior to participation. A more detailed look at these studies revealed that patients had lower VO_2peak and MVC than controls even after controlling for physical activity levels or after pharmacological wash-out (S3).

Muscle function

Different assessments of MVC were applied across the different studies, with some using isometric and others dynamic concentric contractions of upper body or lower body muscle groups. These methodological differences did not change the outcome of lower muscle strength expressed as MVC for patients compared with healthy controls. Force production, being a primary function of skeletal muscle, is determined by the total muscle mass (or, more precisely the physiological cross-sectional area) and the level of voluntary activation which represents the ability to activate all available motor units (540). Muscle mass estimated by dual-energy x-ray absorptiometry (DXA) or bioimpedance analysis (BIA) was similar for patients and controls. However, the more accurate approach(541) to estimating muscle size by magnetic resonance imaging showed lower muscle volume for patients than controls. Voluntary activation was lower for patients than controls, suggesting that patients were unable or unwilling to produce a true maximal force contraction. Lower voluntary activation may indicate deficits of central motor pathways through the motor cortex, upper motor neurons and peripheral motor neurons(43). Such neural

deficits are possible, given the known changes of central and peripheral sensitization in FMS and CFS patients (60, 80, 542) that may affect motor output.

Our results showed that FMS and CFS patients fatigued more quickly during exercise than their healthy counterparts, and this remained the case after considering only those studies with lowest risk of bias. Notably, as was the case for other measurements of muscle function, fatigability (expressed as performance deterioration)(519) was measured in different ways across studies. The lack of consistency across studies makes it difficult to determine how skeletal muscle fatigue relates to the experience of generalized fatigue, which is a primary manifestation of CFS, and FMS that requires further investigation (33).

The accelerated onset of exercise-related fatigue alongside the perceptions of generalized fatigue highlights the different ways in which fatigue is experienced by FMS and CFS patients. There are some possible overlaps with interaction effects linked to sensitized afferent pathways that exaggerate mechano- and metabo- receptor activity, or brain regions receiving and interpreting information from distal body parts. This concept of heightened afferent feedback could contribute not only to generalized and exercise-related fatigue but also to the relative muscular weakness of patients (59). It may also contribute to heightened rates of perceived exertion and pain during exercise reported by patients (216, 534).

Implications for research and practice

Chronic fatigue is a primary feature of CFS and FMS. It can impact greatly on those affected by reducing their social and economic interactions. Alongside the generalized fatigue, our findings reveal the extent of reduced physical function of patients which occurs in excess of that expected from sedentary living. The reasons for the generalized fatigue and reduced physical function remain largely unknown. Future studies should aim to understand what causes the heightened perceptions of effort during exercise and how this relates to fatigue. Possible mechanisms may include sensitized peripheral afferents, including the III-IV muscle afferents (55, 59, 398), and/or regions of the central nervous system that receive those afferent signals.

If effective therapies are not applied, the individuals with CFS and FMS will continue to experience a poorer quality of life and will be at greater risk of inactivity-related poor-health conditions. To this end, a recent study showed increased incidence of skeletal muscle weakness and low muscle mass in relatively young populations of people with FMS placing them at greater risk of sarcopenia (543). The most effective way of improving cardiorespiratory fitness and muscle mass/function is regular intense exercise and there is evidence that exercise may be effective for individuals with CFS and FMS (90, 92, 544, 545). Future studies should investigate whether regular intense exercise familiarizes patients to afferent signals from exercising limbs to improve exercise tolerance by reducing the heightened perceptions of effort and fatigue. However, the benefits of training will depend on a person's commitment to training and the fact that CFS and FMS patients experience heighted perceived exertion may reduce their tolerance of the higher intensity workloads which are most effective for improving physical fitness (546).

8.5. Conclusions

Overall, our results demonstrate lower cardiorespiratory fitness and muscle function of individuals with CFS, and FMS compared with healthy controls. There were indications of dysregulated neuro-muscular interactions including heightened perceptions of effort, reduced ability to activate the available musculature during exercise and reduced tolerance of exercise. Future work should investigate whether impairments of the nervous system cause these changes.

Financial Disclosures

This project has received funding from the European Union's Horizon 2020 research and innovation programmed under the Marie Skłodowska-Curie grant agreement No 801604 for F.Z. and P.D.O. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

All the authors played a role in the content and writing of the manuscript. In addition, J.S.M. was the principal investigator; F.Z., P.D.O., J.S.M, A.F., A.W.J., L.B, and W.G had input into the original idea, study design, and conduct of the study. F.Z., P.D.O., and J.S.M collected the data; F.Z. and P.D.O. and A.W.J performed data analysis, and F.Z. and J.S.M. prepared it for presentation. A.F. F.Z. and J.S.M wrote the manuscript. J.S.M., L.B., A.W.J, and W.G. reviewed the manuscript

8.6. Supporting information

S1 File. Risk of Bias Table:

Study Characteristics						*EPHPP Quality Assessment*		
Year	Name	Type	Selection of Bias	data collection	Drop Out	*Confounders*	*Total*	*High/Low Risk*
2015	Aerenhouts(547)	CFS	2	2	3	3	3	High
2017	Acosta -Manzano(548)	FMS	2	2	3	3	3	High
2020	Andretta(549)	FMS	2	1	1	2	2	Low
2010	Aparicio(550)	FMS	2	2	3	3	3	High
2011	Aparicio(551)	FMS	2	2	3	3	3	High
2013	Aparicio(552)	FMS	2	2	3	3	3	High
2015	Aparicio(553)	FMS	2	3	3	3	3	High
2013	Bachasson(554)	FMS	2	2	3	3	3	High
2014	Balbaloglu(555)	FMS	2	3	3	3	3	High
2013	Bardal(514)	FMS	1	1	3	3	3	High
2015	Bardal(556)	FMS	2	2	2	2	1	Low
2001	Bazelmans(557)	CFS	2	1	2	2	1	Low
1998	Blackwood(558)	CFS	2	2	3	3	3	High
1999	Borman(559)	FMS	1	1	3	2	2	Low
2021	Berardi(560)	FMS	2	2	2	2	2	Low
2019	Bouquet(561)	CFS	1	2	2	1	1	Low
2018	Ceron Lorente(562)	FMS	2	2	3	3	3	High
2003	Cook(563)	CFS	1	2	3	1	2	Low
2006	Cook(564)	CFS/FMS	2	2	1	2	1	Low
2012	Cook(565)	CFS/FMS	1	1	2	1	1	Low
2022	Cook(397)	CFS	2	1	2	1	1	Low
2011	Da Cunha(566)	FMS	2	2	3	2	2	Low
2000	De Becker(567)	CFS	3	3	1	2	3	High
2007	Dinler(568)	FMS	3	2	3	3	3	High
2009	Dinler(569)	FMS	2	2	3	3	3	High
2001	Elert(570)	FMS	2	2	3	3	3	High
2002	Farquhar(571)	CFS	2	3	2	3	3	High

2000	Fulcher(572)	CFS	2	2	1	2	1	Low
2005	Gallagher(573)	CFS	1	2	1	1	1	Low
2003	Georgiades(574)	CFS	2	2	3	2	2	Low
2016	Gerdle(575)	FMS	1	1	1	2	1	Low
2013	Gerdle(576)	FMS	2	2	3	3	3	High
2008	Giske(577)	FMS	2	2	3	2	2	Low
2012	Goes(578)	FMS	2	1	1	1	1	Low
2016	Goes(579)	FMS	1	1	1	2	1	Low
2015	Gomez -Cabello(580)	FMS	2	2	3	3	3	High
2020	Hodges(581)	CFS	2	1	2	3	3	High
2010	Hsieh(582)	FMS	1	2	3	3	3	High
2013	Ickmans(583)	CFS	2	3	3	1	3	High
2014	Icksman(584)	FMS	1	2	3	1	2	Low
2001	Inbar(585)	CFS	2	2	3	2	2	Low
2005	Jammes(586)	CFS	2	2	3	2	2	Low
2007	Javierre(587)	CFS	3	2	3	3	3	High
2021	Jäkel (588)	CFS	2	2	2	2	2	Low
2022	Kapuczinski (543)	FMS	3	3	2	3	3	High
2012	Klaver - Krol(589)	FMS	2	2	3	2	2	Low
2015	Koklu(590)	FMS	2	1	3	3	3	High
2015	Latorre Roman(591)	FMS	2	2	3	3	3	High
2018	Larsson(592)	FMS	2	2	3	3	3	High
2014	Lee(593)	FMS	2	2	3	3	3	High
1991	Lloyd(594)	CFS	2	2	1	2	1	Low
2006	Lowe(595)	FMS	2	1	3	1	2	Low
2003	Lund(515)	FMS	2	2	3	2	2	Low
2010	Maquet(596)	CFS/FMS	2	2	3	3	3	High
2002	Maquet(597)	FMS	2	2	3	3	3	High
2005	Maquet(598)	FMS	3	2	3	3	3	High
1990	Mengshoel(599)	FMS	3	2	3	3	3	High
1995	Mengshoel(600)	FMS	2	2	3	2	2	Low
2018	Nacul(601)	CFS	2	2	3	3	3	High
2019	Nelson(602)	CFS	1	1	3	1	1	Low
2010	Nijs(603)	CFS	1	2	3	2	2	Low
1993	Nordenskiöld(604)	FMS	3	2	3	3	3	High

1994	Nørregaard(605)	FMS	2	3	3	2	3	High
1995	Nørregaard (606)	FMS	3	1	3	2	3	High
2017	Van Oosterwijck(607)	CFS	1	2	2	1	1	Low
2021	Van Oosterwijck (608)	CFS	3	2	2	3	3	High
2017	Paiva(609)	FMS	2	2	3	3	3	High
2006	Panton(610)	FMS	2	2	3	2	2	Low
2008	Patrick -Neary(611)	CFS	1	2	3	2	2	Low
1999	Paul (612)	CFS	1	2	3	2	2	Low
2017	Pieroni-Andrade(613)	FMS	2	2	3	3	3	High
1990	Riley(614)	CFS	2	2	3	3	3	High
1998	Rowbottom(615)	CFS	2	2	3	2	2	Low
1999	Sacco(616)	CFS	2	1	3	2	2	Low
2004	Sahin(617)	FMS	2	2	3	2	2	Low
2020	Salaffi(618)	FMS	2	2	3	3	3	High
2002	Sargent(526)	CFS	1	1	1	1	1	Low
2015	Segura(619)	FMS	2	2	3	3	3	High
2019	Sempere-Rubio (620)	FMS	2	2	3	3	3	High
2016	Sener(621)	FMS	2	2	3	2	2	Low
2004	Siemionow(622)	CFS	2	1	3	2	2	Low
1994	Simms(623)	FMS	2	2	3	2	2	Low
1996	Sisto(624)	CFS	1	1	3	1	2	Low
2013	Srikuea(625)	FMS	2	2	3	2	2	Low
2005	Staud(626)	FMS	2	2	3	3	3	High
2013	Strahler(627)	CFS	2	3	3	2	3	High
2010	Suarez(628)	CFS	2	3	3	2	3	High
2020	Tavares(629)	FMS	1	2	3	2	2	Low
2015	Umeda(94)	FMS	2	1	2	2	2	Low
2003	Valim(630)	FMS	2	2	2	2	1	Low
2008	Valkeinen(631)	FMS	2	2	3	3	3	High
1992	Van Denderen(632)	FMS	2	3	3	3	3	High
2007	Van Ness(633)	CFS	2	3	3	3	3	High
2010	Vermeulen(527)	CFS	1	2	2	2	1	Low
2014	Vermeulen(634)	CFS	3	1	3	2	2	Low
2018	Villafaina(635)	FMS	2	2	3	3	3	High
2016	Vincent(636)	FMS	2	3	2	3	3	High

2022	Wåhlén (525)	FMS	2	2	1	2	2	Low
2004	Wallman(637)	CFS	2	2	3	1	2	Low

Table 1. Risk of Bias Table. Low = Low risk of Bias; High = High risk of Bias

S2 File. Characteristics of the studies. Descriptive tables of all the studies included in the systematic review and meta-analysis

Table 2. Study characteristics for the CPET studies included in the Systematic Review and Meta-analysis for chronic fatigue syndrome.

Year	Name	Type	Diagn.	test	type	Testing protocol	RPE	HR	AT	Lactate
2015	Aerenhouts	CFS	CDC	VE	C	Cycling, test started at 60w with 30w increased every minute until exhaustion or participant pedalling rate sunk below 55rpm				
2001	Bazelmans	CFS	CDC	VE	C	Cycling: Workload was increased every minute in step of 10% of estimated maximal workload, from 10 to 30%	At 3min	Peak		
1998	Blackwood	CFS	Not specified	VE	T	Treadmill: Standardize Bruce Protocol	At 85% HRmax			
2019	Bouquet	CFS	Canadian 2003	VE	C	Cycling: Workload was increased 15 watts per minute until volitional fatigue.		Peak	VT	
2003	Cook	CFS	CDC	M	T	Treadmill: exercise stage was 3 min, and the exercise test began at 67 m·min1 and no incline. For stage 2 of exercise, the treadmill speed was increased to 94 m·min1. For the remaining stages, speed was kept constant, and intensity was	At GET	Peak	GET	

						increased by raising the incline of the treadmill by 2% at each stage until the end of the test.					
2006	Cook	CFS	CDC	M	C	Cycling: increased by 5W every 20 seconds	mean	Peak			
2012	Cook	CFS	CDC	S	C	Cycling: Constant Intensity at 40% VO2peak for 20minutes	mean	Mean			
2022	Cook	CFS	Not known	VE	C	3min unloaded warm-up. Exercise testing from 0 Watts with linear increases of 5 Watts every 20 seconds (15 Watts/min) until volitional exhaustion at 60-70rpm.	Mean	Peak			Peak
2000	De Becker	CFS	CDC	S/M	C	Cycling: starting at 10w and increasing 10w each minute		Peak			
2002	Farquhar	CFS	CDC	VE	C	Cycling: 2 min stages, workload increase of 25-30watts		Peak			
2000	Fulcher	CFS	Oxford	VE	T	Treadmill: 5kph with 2.5% of gradient increase every 2minutes		Peak			
2005	Gallagher	CFS	Oxford	VE	T	Treadmill: 5kph with 2.5% of gradient increase every 2minutes		Peak			
2003	Georgiades	CFS	CDC	VE	C	Cycling: incrementation rate for individual subjects varied between 3 and 10w/min		Peak			Peak
2020	Hodges	CFS	ICC	VE/ M	C	Cycling: Starting at 15w and increase 15w/min	At VT	Peak	RER		
2013	Icksman	CFS	CDC	VE	C	Cycling: start from 60w and increase by 30w every minute		Peak			

2001	Inbar	CFS	CDC	M	T	Treadmill: Modified balke protocol - constant speed of 2.0-3.5mph while the slope is elevated by 2% every minute		Peak	GET	
2005	Jammes	CFS	symptoms for CFS	VE	C	Cycling: workload started at 20W and increased every minute of 20w until subjects stop pedalling			VT	
2007	Javierre	CFS	CDC	VE	C	Cycling: increase of 12.5 watt every one minute until exhaustion	Peak			
2019	Nelson	CFS	CDC	M	C	Cycling: 5minutes at 40w (male) and 30w (female) and then 5w increased every 20sec until exhaustion	at VT	Peak	VT	
2010	Nijs	CFS	CDC	S	C	Cycling: Submaximal Aerobic Power Index test				Peak
2017	Van Oosterwijck	CFS	CDC	S	C	Cycling: Submaximal Aerobic Power Index test		Peak		
2008	Neary	CFS	CDC	I	C	Cycling: at 60 W for a period of 2 min, followed by a work rate increase of 25 W every 2 min until exhaustion		Peak		
1990	Riley	CFS	CDC	VE	T	Treadmill: Modified Bruce Protocol	Peak	Peak		Peak
1998	Rowbottom	CFS	Komaroff	VE	T	Treadmill: Modified Bruce protocol	mean	Peak		
2002	Sargent	CFS	CDC	M	C	Cycling: 25w every 2 minute until the subject was not able to maintain the power output		Peak	LT	Peak
1996	Sisto	CFS	CDC	M	T	Treadmill: 3.5mph with the decline increase of 2% every minute	at GET	Peak	GET	

2013	Strahler	CFS	CDC	VE	C	Cycling: Started at 50 W for men and 30 W for women, respectively, with 40 W increments every 3 min until the subject was no longer able to continue or until predicted maximum heart rate (85% of 220bpm)	Peak	Peak	
2010	Suarez	CFS	CDC	VE	C	Cycling: 20w every minute until exhaustion	Peak	Peak	Peak
2007	Van Ness	CFS	CDC	VE	C/T	Cycling or treadmill: Bruce treadmill or 10w/min ramping protocol			AT (not specified)
2021	Van Oosterwijck	CFS	CDCP	VE	C	Cycling: Submaximal Aerobic Power Index test	Peak	Peak	
2010	Vermeulen	CFS	CDC	VE	C	Cycling: RAMP protocol based on gender and history of physical examination, Weight and height		Peak	AT (not specified)
2014	Vermeulen	CFS	CDC	VE	C	Cycling: RAMP protocol based on gender and history of physical examination, Weight and height		Peak	AT (not specified)
2004	Wallman	CFS	CDC	S	C	Cycling: Aerobic Power Index test - Increased of 25w every minute until reaching 75%of age predicted target HR	At peak workload		3 min after the test

Abbreviations: FMS = Fibromyalgia Syndrome, ACR = American College of Rheumatology, VE= Voluntary Exhaustion, M= Maximal, S = Submaximal, I = Indirect, C= Cycling, T = Treadmill, HG = Handgrip, RPE = Rate of Perceived Exertion, HR = Heart Rate, AT = Anaerobic Threshold, VT = Ventilatory Threshold, GET = Gas Exchange Threshold.

Table 3. Study characteristics for the CPET studies included in the Systematic Review and Meta-analysis for Fibromyalgia.

Year	*Name*	*Type*	*Diagn.*	*Test*	*Type*	*Testing protocol*	*RPE*	*HR*	*VT*	*Lactate*
2013	*Bachassons*	*FMS*	*ACR*	*VE*	*C*	*Cycling:15w initial power and increase of 15w/min for FMS and initial power at 30w increase of 30w/min for control group*	*At 75 or 50%*	*Peak*		*Peak*
2014	*Balbaloglu*	*FMS*	*ACR*	*VE*	*T*	*Treadmill: Modified Bruce Protocol*		*Peak*		
2013	*Bardal*	*FMS*	*ACR*	*VE*	*C*	*Cycling: Stepwise Increase of 15w/min*	*Peak*	*Peak*	*At 4mmol*	*Peak*
2015	Bardal	FMS	ACR	S	C	Cycling: a stepwise increase in workload (10 W/min) until blood lactate concentration (bLa) reached > 5 mmol/l.	mean	Mean		
2021	Berardi	FMS	Not Known	Strength	Elbow Flexors	Submaximal intermittent isometric and concentric muscle contractions matched for intensity (20% of maximal	mean			
2006	Cook	FMS	ACR 1990	M	C	Cycling: 3min warm up at 20w, then 5w increase every 20sec until exhaustion	mean	Peak		
2012	Cook	FMS	ACR 1990	S	C	Cycling: Constant Intensity at 40% VO2peak for 20minutes	mean	Mean		
2011	Da Cunha	FMS	ACR	VE	T	Treadmill: A modified Balke treadmill maximal exercise test		Peak		
2007	Dinler	FMS	ACR	VE	T	Treadmill: modified Bruce multistage protocol				
2009	Dinler	FMS	ACR	VE	T	Treadmill: Standard Bruce multistage protocol	Mean			

2012	Gerdle	FMS	Not Known	I	C	Cycling: Astrand Indirect Protocol				
2015	Gomez-Cabello	FMS	Not Known	VE	T	Treadmill: Fernhall protocol 1996				
2010	Hsieh	FMS	ACR	VE	C	Cycling: starting from 0 watt, adding increment of 10-15w/min	peak	Peak	VT	
2003	Lund	FMS	ACR 1990	VE	C	Cycling: Starting with two steady state submaximal levels of 6 min each [20 and 40 W 30 and 60 W], participants were		Peak	VE/VO2	
1990	Mengshoel	FMS	Yunus	I	C	Cycling: Indirect and submaximal Astrand protocol				
1995	Mengshoel	FMS	ACR	I	C	Cycling: Indirect and submaximal Astrand protocol	at 6min			
1994	Norregard	FMS	ACR	VE	C	Cycling: initial load of 40 watts was used with stepwise increments every 3 min using the following	At 6 min		At 2mmol (W)	Peak
2017	Pieroni - Andrade	FMS	ACR	VE	C	Cycling: Incremental Protocol, with increment proposed by Wasserman based on age, weight, and height		Peak	VT (ml/kg/min)	
2016	Sener	FMS	ACR	I	C	Cycling: Indirect and submaximal Astrand protocol				
1994	Simms	FMS	ACR	VE	C	Cycling: 1-minute stages of 15W increases, beginning at 0 W.	at peak	Peak		
2013	Srikuea	FMS	ACR	strength	Knee Extensor	6 sets of 12 isometric contractions with each set followed by MVIC as described above. Incremental intensity from 20% to	mean			
2005	Staud	FMS	ACR	Strength	HG	Sustained handgrip exercise at 30% of MVC for 90sec	End			

2002	Valim	FMS	ACR	VE	T	Treadmill: a 3 km/h load, with a 1 km/h increase every minute up to 7 km/h, after that, a 2.5% slope inclination increase up to	Peak	AT (%)	
2008	Valkeinen	FMS	ACR	VE	C	Cycling: 3min at 50w initial load, then 20w every 2min increases	Peak		Peak
1992	Van Denderen	FMS	Yunus	VE	C	Cycling: Starting with 5O Watts. the workload was raised every three minutes by 30 Watts	Peak		
2016	Vincent	FMS	ACR	VE	C	Cycling: Incremental until reaching VO2max or patients too fatigued (not specified the increment)	Peak		

Abbreviations: FMS = Fibromyalgia Syndrome, ACR = American College of Rheumatology, VE= Voluntary Exhaustion, M= Maximal, S = Submaximal, I = Indirect, C= Cycling, T = Treadmill, HG = Handgrip, RPE = Rate of Perceived Exertion, HR = Heart Rate, AT = Anaerobic Threshold, VT = Ventilatory Threshold, GET = Gas Exchange Threshold.

Table 4. Study characteristics for the Strength Assessment studies included in the Meta-analysis for Fibromyalgia and Chronic Fatigue Syndrome.

	Study Characteristics					Type	Type	VA	Fatigability
year	name	syndr.	Diagnosis	Sex	Matched	test	Muscle Action	type	type
2015	Aerenhouts	CFS	CDC - Fukuda	F	age - BMI	MVC	Handgrip		
1998	Blackwood	CFS	CDC- Fukuda	F/M	Age-sex	MVC	Handgrip		
2021	Berardi	Not Known	Physician	F	Age-Sex-BMI-PA	MVC	HG/elbow flexors	ITT	MVC decline

2000	Fulcher	CFS	CDC - Fukuda	F/M	Age sex-BMI	MVC	Leg extension	ITT (data not reported)	
2013	Icksman	CFS	CDC - Fukuda	F	Age sex and BMI	MVC	Handgrip		
2014	Icksman	FMS	CDC - Fukuda	F	Age sex and BMI	MVC	Handgrip		
1991	Lloyd	CFS	Lloyd et al 1988	F/M	age-BMI	MVC	Elbow flexion	ITT (endurance sequence test)	
2010	Maquet	CFS	CDC – Fukuda	F	Age sex and BMI	Submaximal Isometric Contraction	Arm Abduction		Time to exhaustion
2018	Nacul	CFS	CDC	-	None	MVC	Handgrip		
1999	Paul	CFS	CDC - Fukuda	F/M	age sex-BMI	MVC-Torque	Leg extension		fatigue index (%MVC)
1999	Sacco	CFS	CDC - Fukuda	F/M	age-sex-BM	MVC (N)	Elbow flexor		endurance time
2004	Siemionow	CFS	CDC - Fukuda	F/M	age-sex-BMI	MVC (N)	Handgrip		
2010	Aparicio	FMS	ACR	M	Age Sex BMI	Isometric MAX	Handgrip		
2011	Aparicio	FMS	ACR	F	Age Sex BMI	Isometric MAX	Handgrip		
2013	Aparicio	FMS	ACR	F	Age Sex BMI	Isometric MAX	Handgrip		
2013	Aparicio	FMS	ACR	F	Age Sex BMI	Isometric MAX	Handgrip		
2015	Aparicio	FMS	ACR	F	Age Sex BMI	Isometric MAX	Handgrip		
2013	Bachassons	FMS	ACR	F	Age Sex BMI	MVC	Leg extension		

1999	Borman	FMS	ACR	F	age and sex	isokinetic (PT)	Lex extension/flexors		Endurance ratio
2018	Ceron Lorente	FMS	ACR	?	Age – BMI	MVC	Leg extension		
2001	Elert	FMS	ACR	F	Age -Sex BMI	Isokinetic (PT)	Shoulder Abduction		
2012	Ge	FMS	ACR	F	Age – Sex - BMI		Shoulder Abduction		
2013	Gerdle	FMS	ACR	F	Sex- BMI	MVC and submaximal Isometric Contraction	Handgrip		Endurance/Time
2016	Gerdle	FMS	ACR	F	age and sex	MVC	Elbow Flexion		
2016	Gerdle	FMS	ACR	F	age and sex	MVC	Leg extension		
2012	Goes	FMS	ACR	F	Age-sex-BMI-PA	MVC - Torque	Leg extension		
2016	Goes	FMS	ACR	F	Age-sex-BMI	MVC	plantar flexion and dorsiflexion	CAR	
2008	Giske	FMS	ACR	F	age sex and weight	MVC	Quadriceps		
2021	Jäkel	CFS	CCC	F/M	Age-Sex	MVC	Quadriceps/HG		Fatigue Ratio
2015	Gomez-Cabello	FMS	ACR	F	Age -Sex	MVC	Leg extension and Handgrip		
2022	Kapuczinski	CFS	ACR	F	Sex	MVC	Handgrip		
2015	Koklu	FMS	ACR	F	Age-sex-BMI	MVC	Handgrip		

2018	Larsson	FMS	ACR	F	Age – sex	MVC	Leg extension/ Elbow flexion/ Handgrip		
2015	La Torre Roman	FMS	ACR	F/M	Age	MVC	Handgrip		
2014	Lee	FMS	ACR	F/M	BMI	MVC	Handgrip		
2012	Klaver – Kol	FMS	ACR	F/M	Age sex and BMI	MVC	Elbow flexor		
2002	Maquet	FMS	ACR	F	Age -Sex – BMI	MVC/ Isokinetic (PT)	Leg (extensors/flexors)		NCW
2005	Maquet	FMS	ACR	F	Age sex and BMI	Isokinetic (PT)	Leg (extensors/flexors)		NCW
2010	Maquet	FMS	ACR	F	Age – Sex – BMI	Submaximal Isometric contraction			Endurance/Time
1990	Mengshoel	FMS	Yunus	F	Age – Sex	MVC, Dynamic, Static (paper)	Handgrip		n. contraction in 30 sec/ time (s)
1993	Nordenskiöld	FMS	ACR	F	Sex Matched	Sustained MVC (10sec)	Handgrip		
1994	NØrregard	FMS	ACR	F	age-sex-BMI	MVC	Leg extension	VA	Endurance time (min)
1995	NØrregard	FMS	ACR	F	Age-sex-BMI	MVC	Leg extension	VA	

2006	Panton	FMS	Not mentioned	F	age-sex-BMI	MVC	Handgrip		
2004	Sahin	FMS	ACR	F	age sex-BMI	MVC	Handgrip		
2020	Salaffi	FMS	ACR	F	Age-sex-BMI	sustained MVC (30sec)	Handgrip		
2019	Sempere-Rubio	FMS	ACR	F	Age – Sex	MVC	Handgrip, Elbow flexion		
2016	Sener	FMS	ACR	F	age-sex-BMI	MVC	Handgrip		
1994	Simms	FMS	ACR	F	age-sex-BMI	MVC	Trapezius/Tibialis		
2013	Srikuea	FMS	ACR	F	Age-sex-BMI	MVC	Knee extensors	CAR	loss of strength
2020	Tavares	FMS	ACR	F	Age-sex-BMI	Isokinetic (PT)	Knee (extensor /flexor)		fatigue index
2015	Umeda	FMS	ACR	F	Age -sex	MVC	Handgrip		
2008	Valkeinen	FMS	ACR	F	Age-sex-BMI	MVC	Leg extension		
2018	Villafaina	FMS	ACR	F	Age – Sex -BMI	MVC	Handgrip		
2022	Wåhlén	FMS	ACR	F	Age – Sex	Maximal Tests	Leg extension/elbow flexors/HG		

Abbreviations: FMS = Fibromyalgia Syndrome, CFS = Chronic Fatigue Syndrome, ACR = American College of Rheumatology, F =Female, M= Male, BMI = Body Mass Index, ITT= Interpolated twitch technique, CAR = Central Activation Ratio, NCW = Normalized Cumulative Work – PA = Physical activity CCC- Canadian Consensus Criteria.

Table 5. Study characteristics for the Body Composition studies included in the Meta-analysis for Fibromyalgia and Chronic Fatigue Syndrome.

	Study Characteristics					Test	Muscle Volume	Muscle mass	Fat Mass
year	name	Syndrome	Diagnosis	Sex	Matched	type	Type	type	type
2017	Acosta-Manzano	FMS	ACR	F	Age	BIA			Fat Mass (%)
2020	Andretta	FMS	ACR	F	Age-BMI	DEXA		Lean Mass (%)	Fat Mass (%)
2013	Aparicio	FMS	ACR	F	Age -BMI	BIA		Muscle Mass (kg)	Fat Mass (%)
2015	Aparicio	FMS	ACR	F	Age – Sex -BMI	BIA		Muscle Mass (kg)	Fat Mass (%)
2013	Bachassons	FMS	ACR	F	Age – Sex -BMI	Skinfolds – Truncated cone calculation	Thigh		Fat Mass (%)
2021	Berardi	FMS	Not Known	F	Age- sex-BMI-PA				
2012	Gerdle	FMS	ACR	F	Age – Sex -BMI	MRI	Thigh		Subcutaneous Fat
2015	Gomez-Cabello	FMS	ACR	F	Age – Sex	DEXA		Lean Muscle Mass (kg)	Fat mass (kg)
2022	Kapuczinski	FMS	ACR	F	Sex	BIA		Lean Mass (kg)	Fat mass (kg)
2015	La Torre-Roman	FMS	ACR	F/M	Age	BIA		Muscle Mass (kg)	
2006	Lowe	FMS	ACR	F	Age-Sex-BMI	BIA		Fat Free Weight (kg)	Fat Mass (%)
1994	Norregard	FMS	ACR	F	Age-Sex-BMI	MRI		Fat free area (cm^2)	
1995	Norregard	FMS	ACR	F	Age-Sex-BMI	MRI	Sum-max Area (cm^2)		
2017	Paiva	FMS	ACR	F	Age-Sex-BMI	DEXA			Fat Mass (kg)

2014	Segura	FMS	ACR	F	Age	BIA	Muscle Mass (kg)	Fat Mass (%)
2016	Sener	FMS	ACR	F	Age-Sex-BMI	BIA		Fat Mass (%)
2013	Srikuea	FMS	ACR	F	Age-Sex-BMI	DEXA	Lean Muscle Mass (kg)	Fat Mass (%)
2016	Vincent	FMS	ACR	F	Sex-BMI	DEXA		Fat Mass (%)

Abbreviations: FMS = Fibromyalgia Syndrome, ACR = American College of Rheumatology, F =Female, BMI = Body Mass Index, BIA = Bio-Electrical Impedance Analysis, DEXA = Dual-energy X-ray absorptiometry

List of the tables' abbreviations:

Abbreviations: FMS = Fibromyalgia Syndrome, CFS = Chronic Fatigue Syndrome; ACR = American College of Rheumatology, VE= Voluntary Exhaustion, M= Maximal, S = Submaximal, I = Indirect, C= Cycling, T = Treadmill, HG = Handgrip, RPE = Rate of Perceived Exertion, HR = Heart Rate, AT = Anaerobic Threshold, VT = Ventilatory Threshold, GET = Gas Exchange Threshold, F =Female, M= Male, BMI = Body Mass Index, ITT= Interpolated twitch technique, CAR = Central Activation Ratio, NCW = Normalized Cumulative Work – PA = Physical activity CCC- Canadian Consensus Criteria. BMI = Body Mass Index, BIA = Bio-Electrical Impedance Analysis, DEXA = Dual-energy X-ray absorptiometry

S3 File. **Statistical and Sensitivity Analysis.** RevMan full graphs and sensitivity analysis for all the outcomes included in the systematic review and meta-analysis.

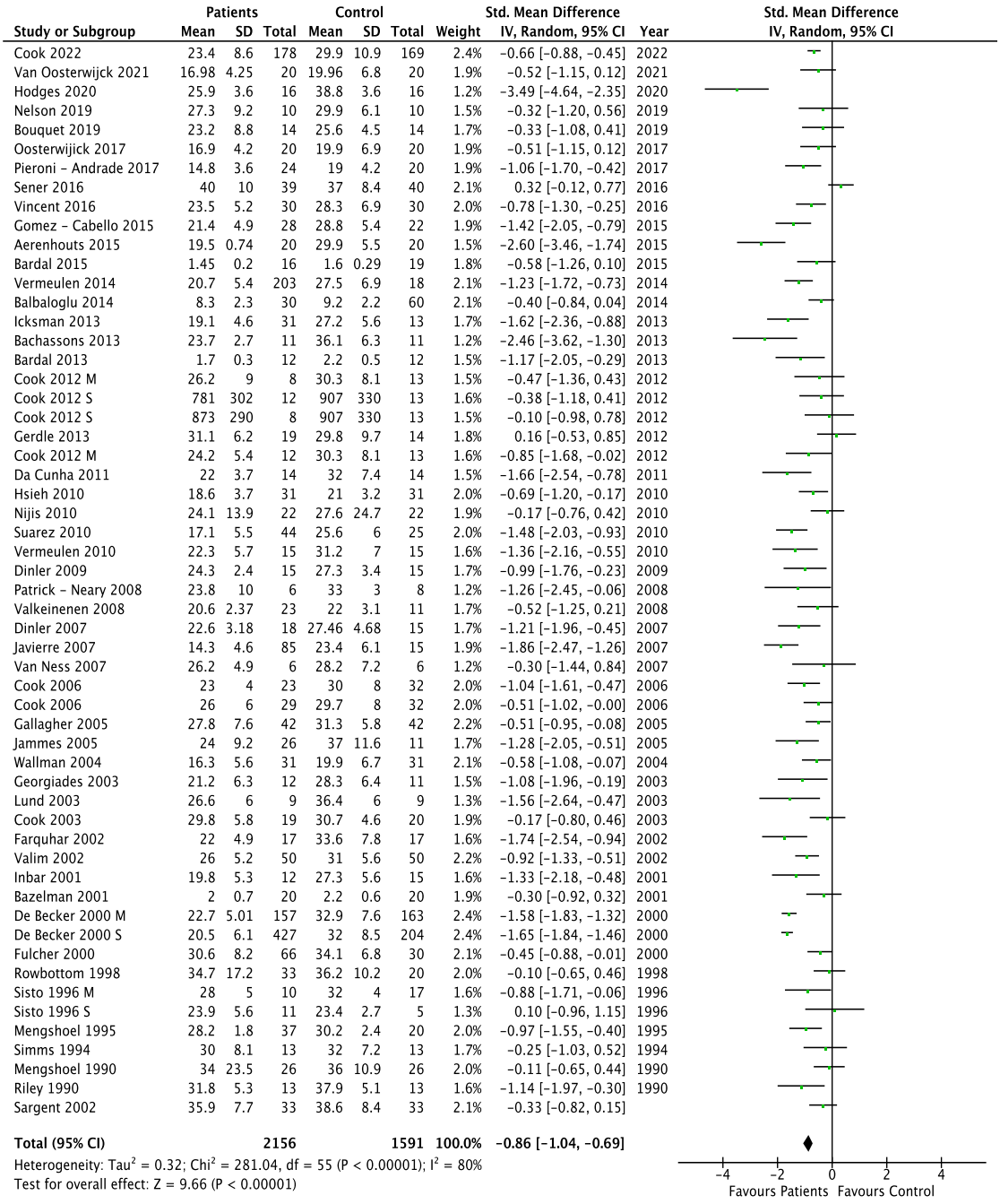


Figure A. Peak Oxygen Consumption (VO_2 Peak) values for all studies included in the analysis.

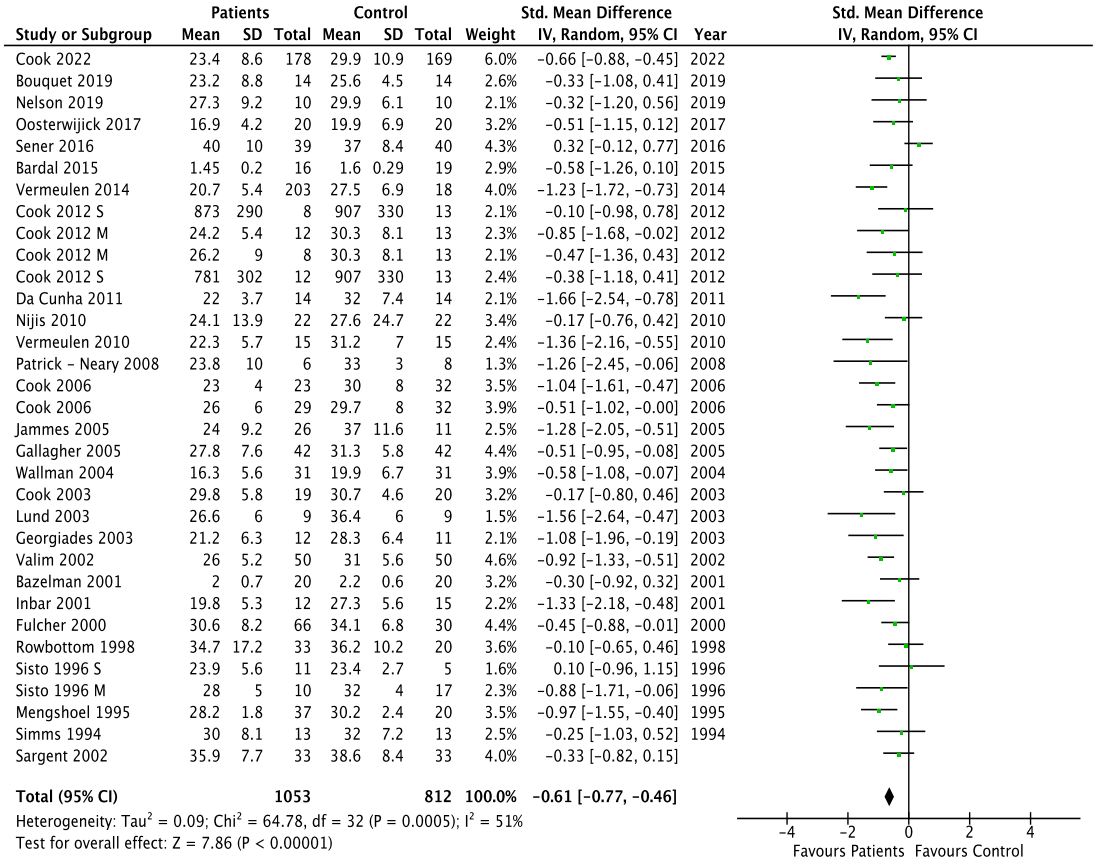


Figure A1. Peak Oxygen Consumption (VO_2 Peak) values after sensitivity analysis.

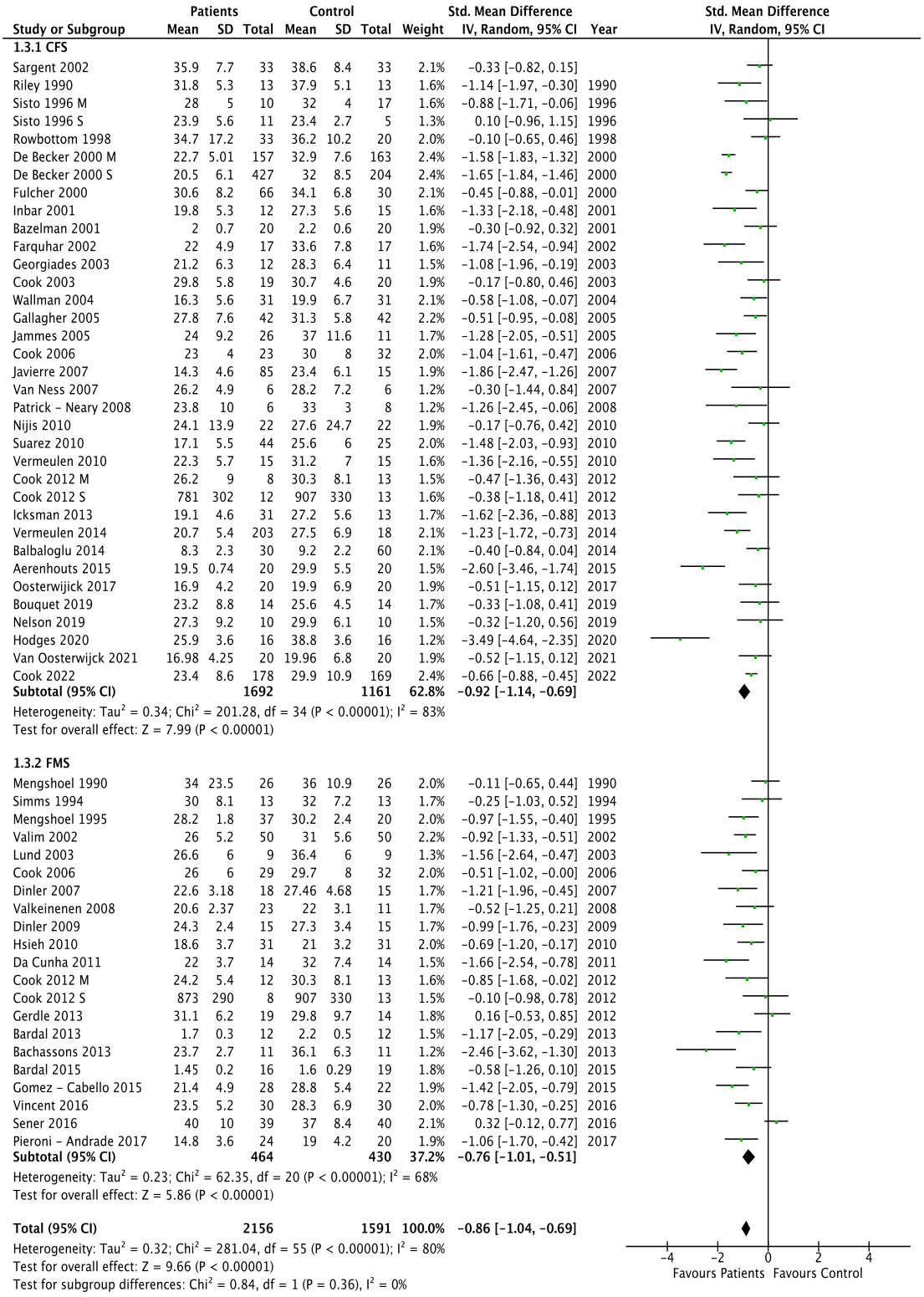


Figure A2. Peak Oxygen Consumption (VO_2 Peak) values between CFS and FMS

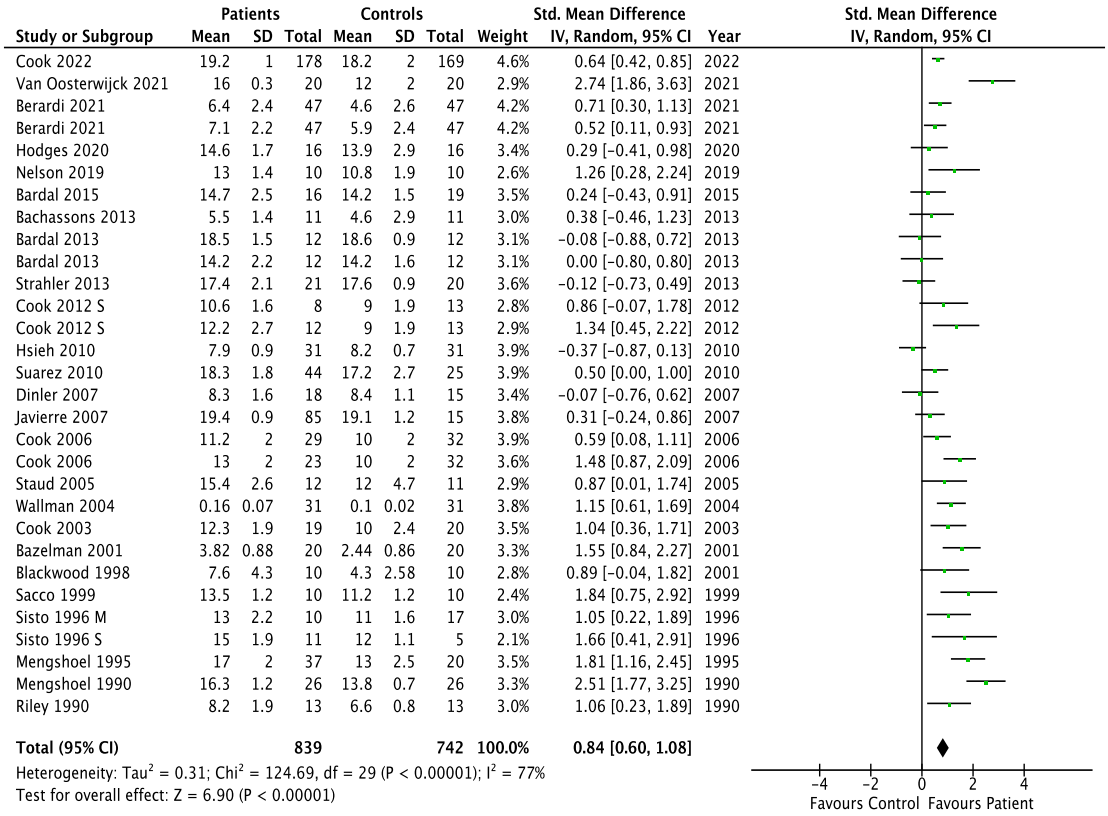


Figure B. Rate of Perceived Exertion (RPE) values for all studies included in the analysis.

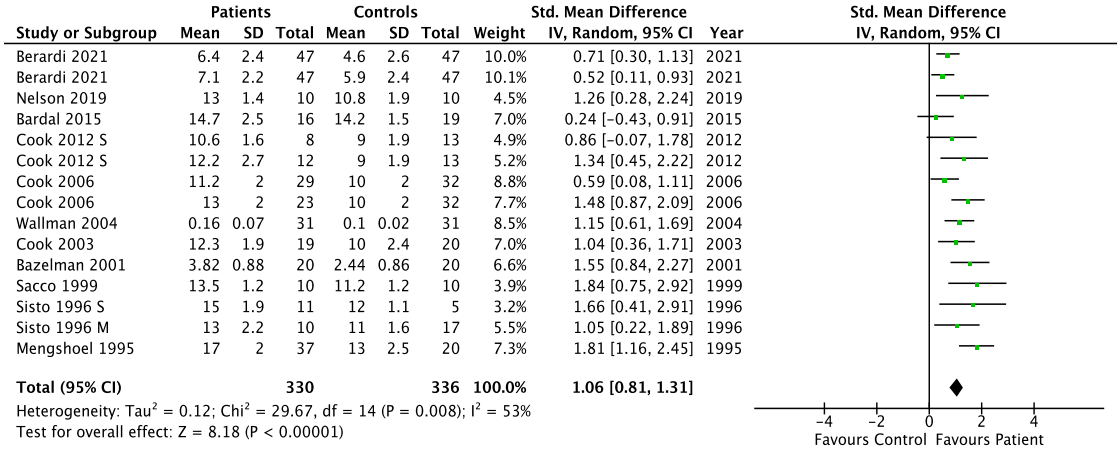


Figure B1. Rate of Perceived Exertion (RPE) values for all studies after sensitivity analysis.

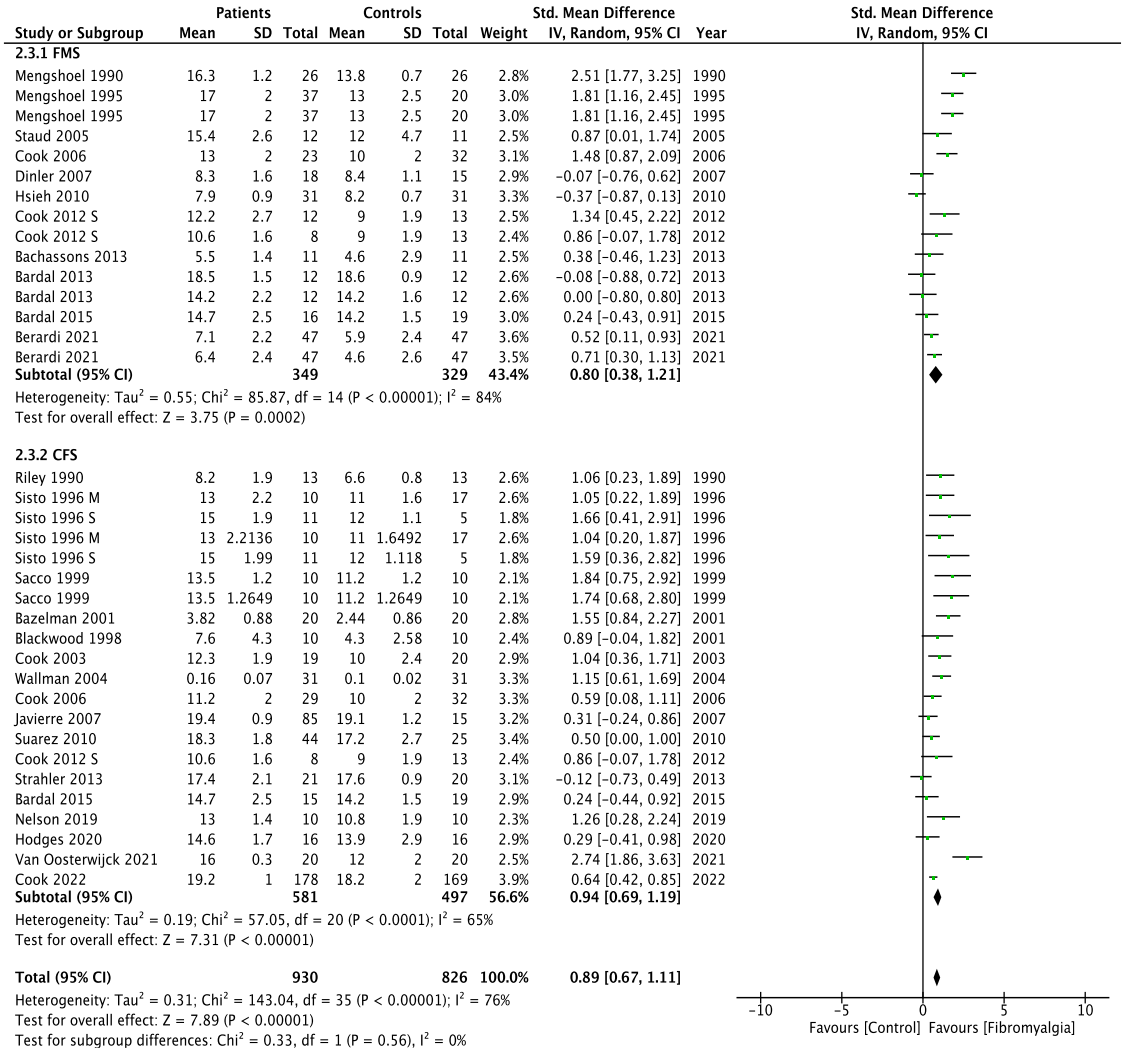


Figure B2. Rate of Perceived Exertion (RPE) values for all studies between CFS and FMS patients.

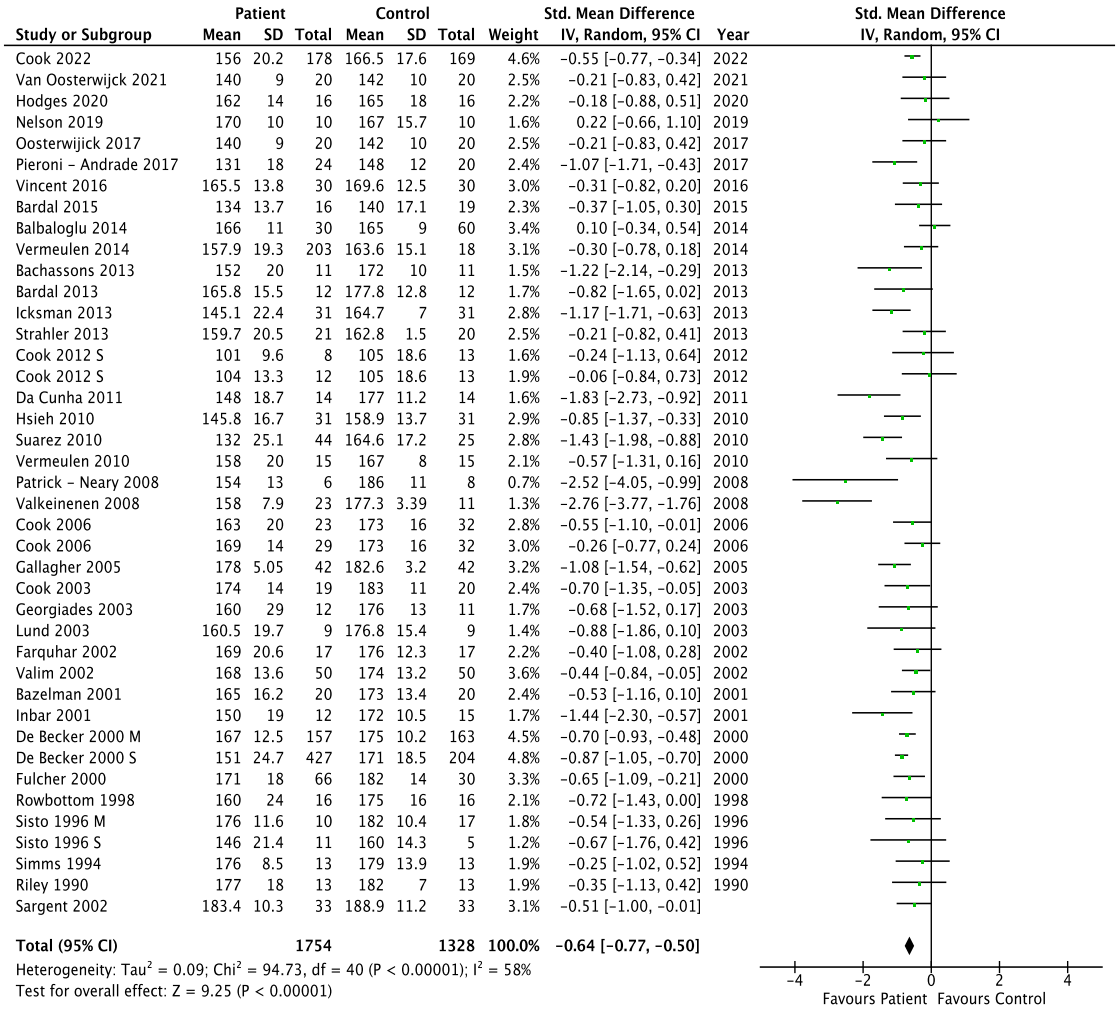


Figure C. Peak Heart Rate (HR) values for all studies included in the analysis.

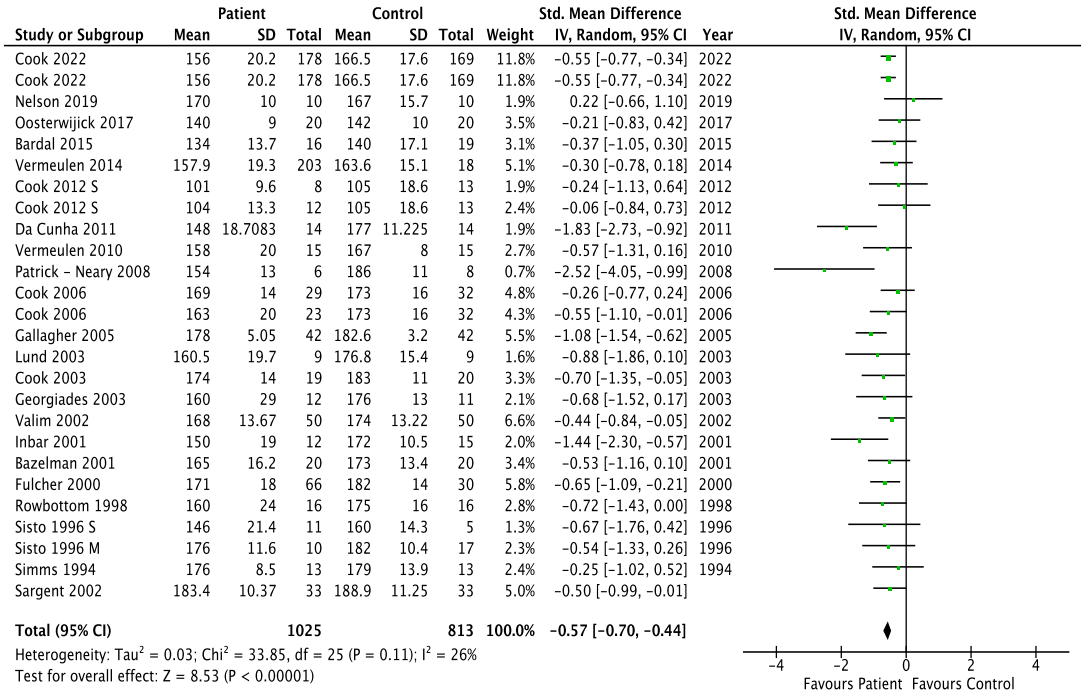


Figure C1. Peak Heart Rate (HR) values for all studies after sensitivity analysis.

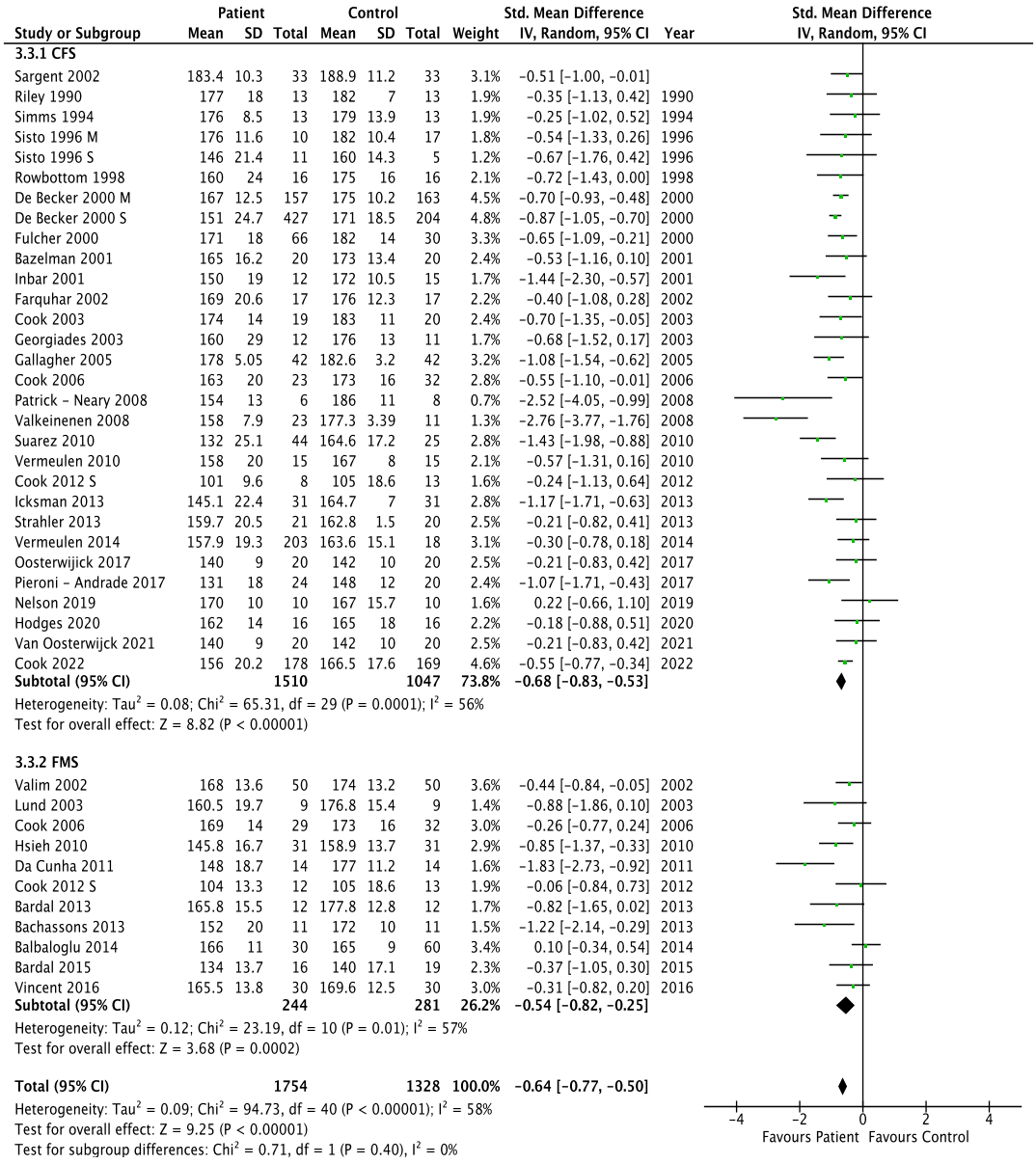


Figure C2. Peak Heart Rate (HR) values for all studies between CFS and FMS patients.

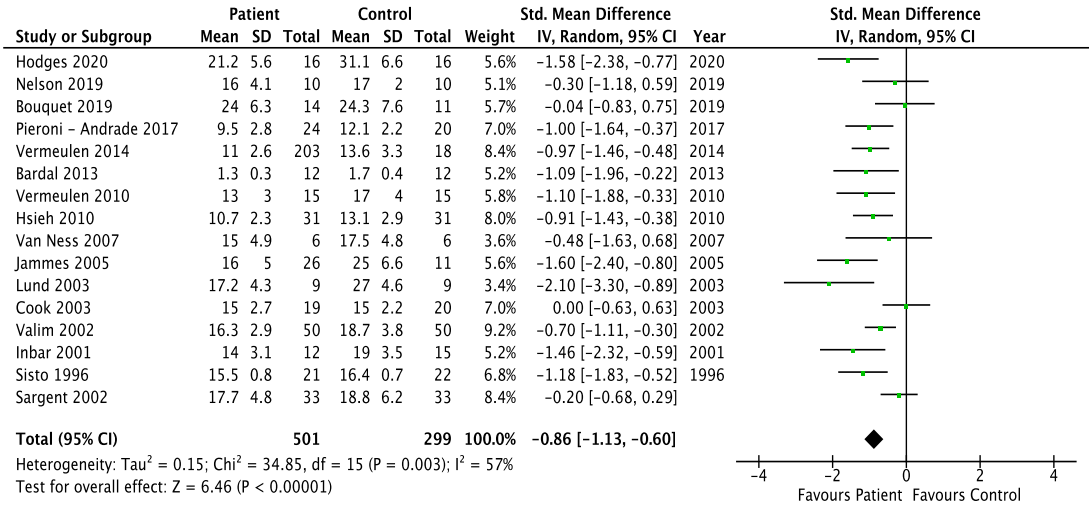


Figure D. Anaerobic Threshold (AT) values for all studies included in the analysis.

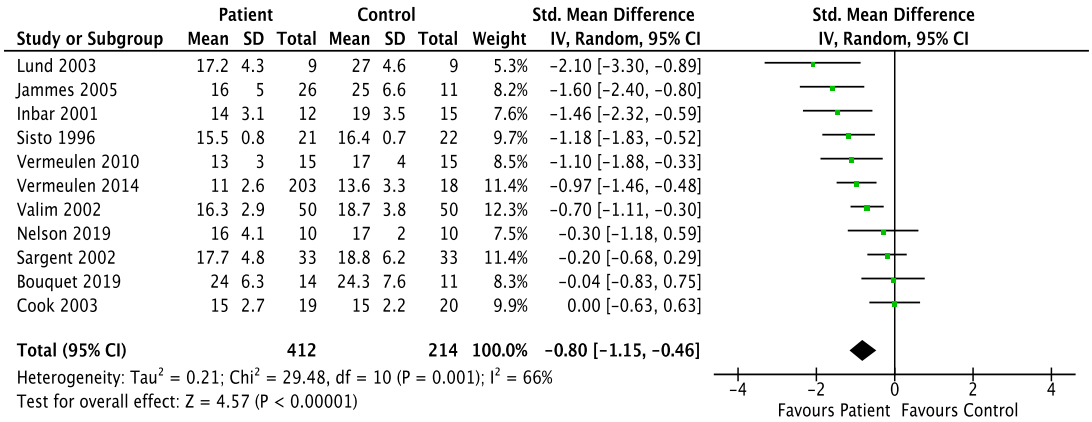


Figure D1. Anaerobic Threshold (AT) values for all studies after sensitivity analysis.

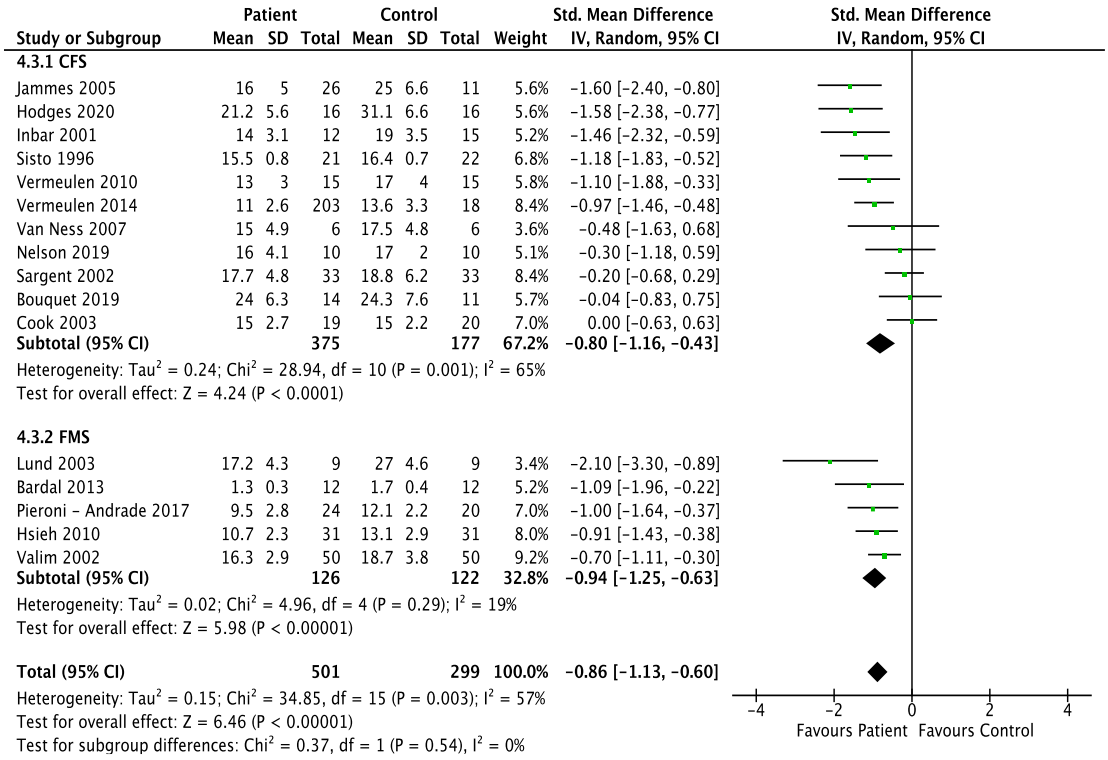


Figure D2. Anaerobic Threshold (AT) values for all studies between FMS and CFS

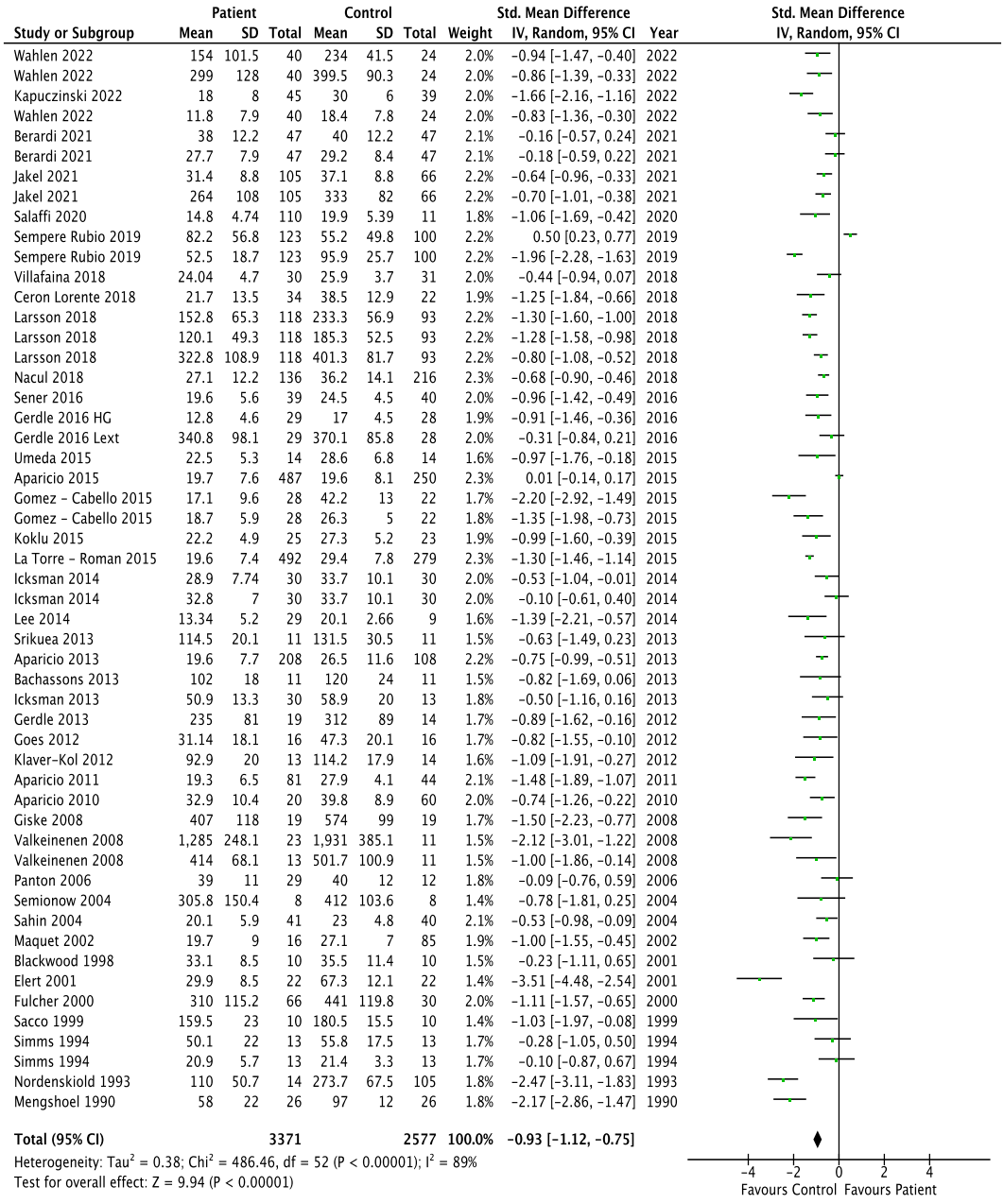


Figure E. Maximal Voluntary Contraction (MVC) values for all studies included in the analysis.

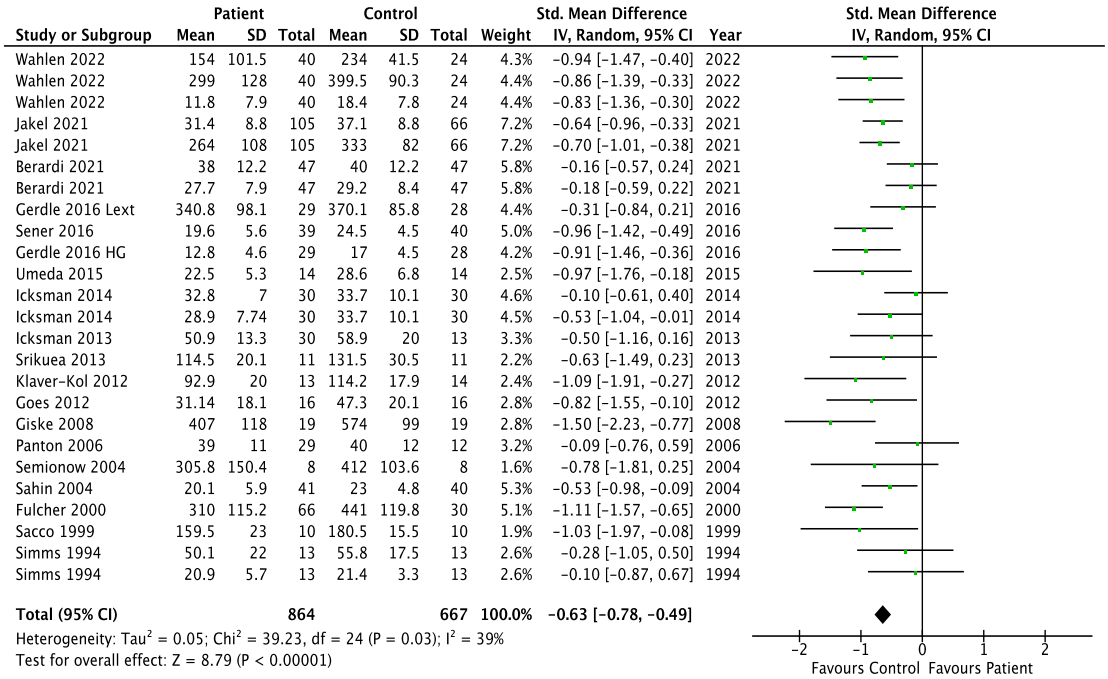


Figure E1. Maximal Voluntary Contraction (MVC) values for all studies after sensitivity analysis.

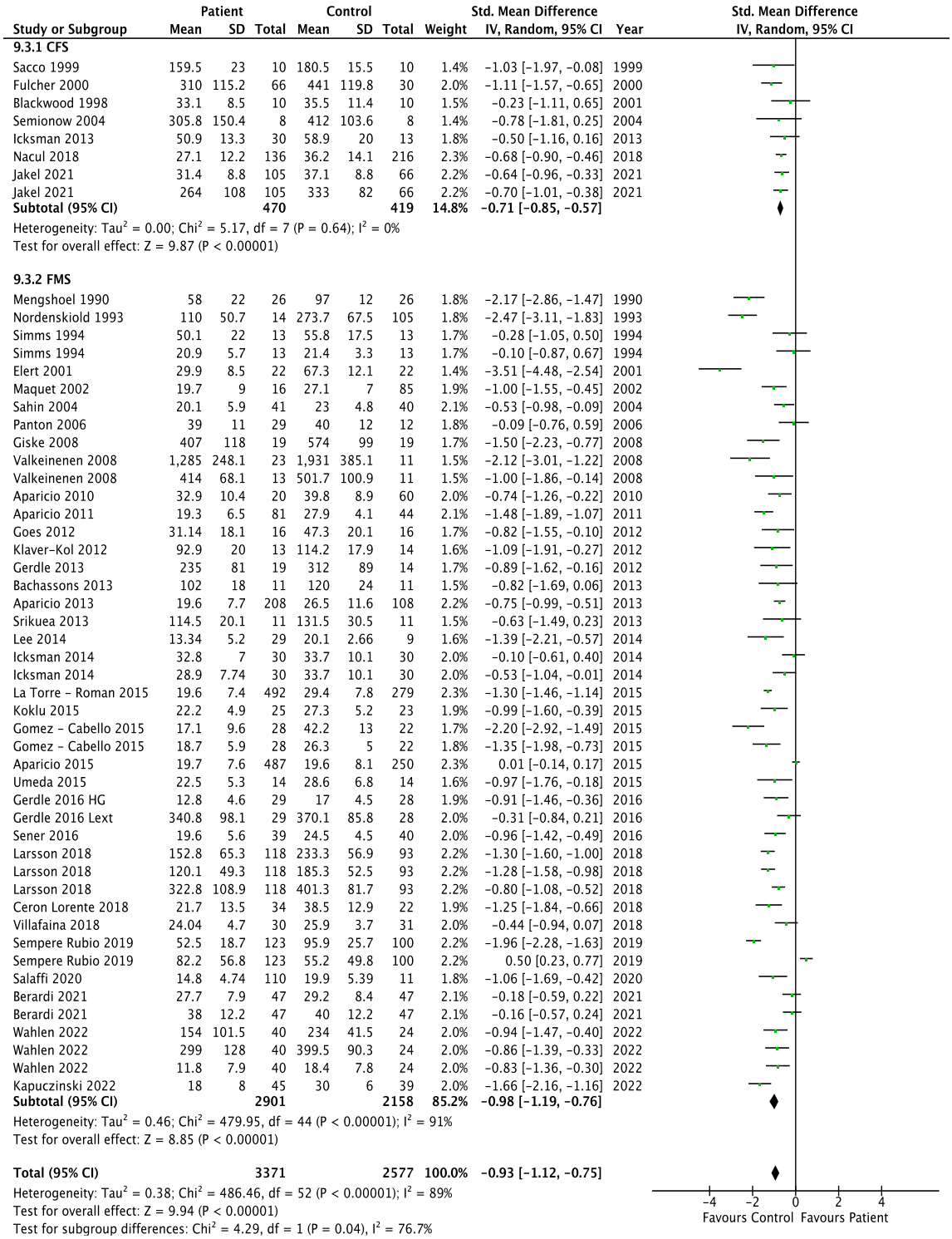


Figure E2. Maximal Voluntary Contraction (MVC) values for all CFS and FMS patients.

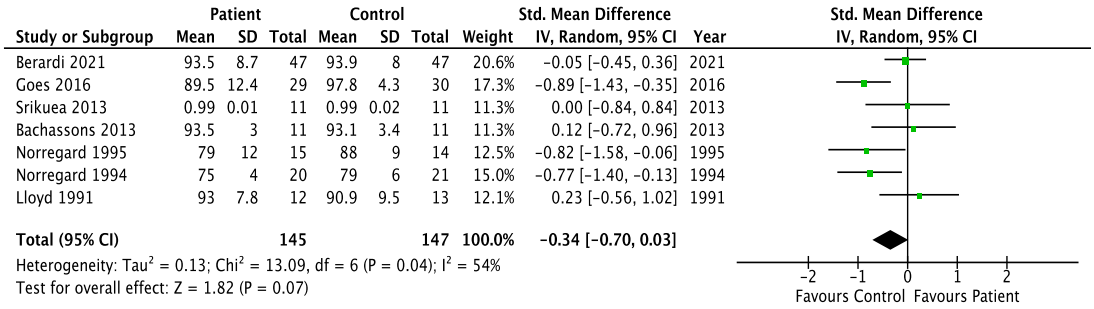


Figure F. Voluntary Activation values for all studies included in the analysis.

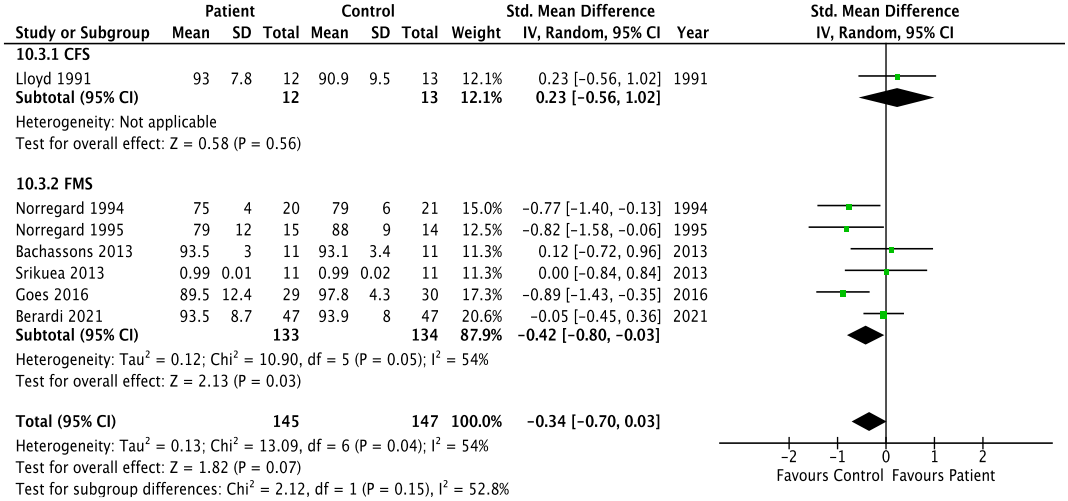


Figure F1. Voluntary Activation values for CFS and FMS patients

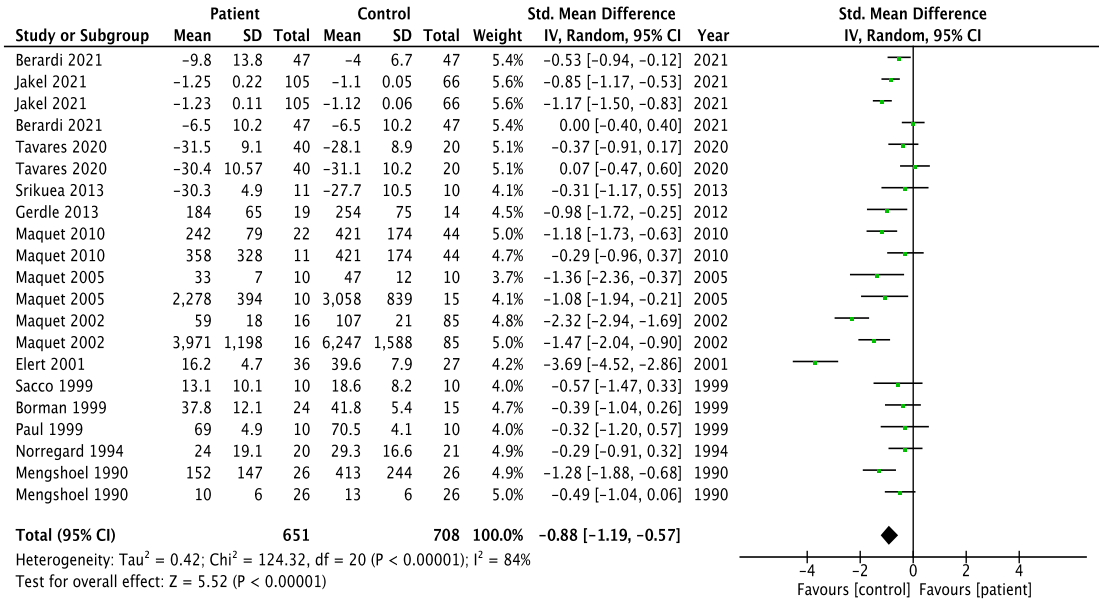


Figure G. Fatigability values for all studies included in the analysis.

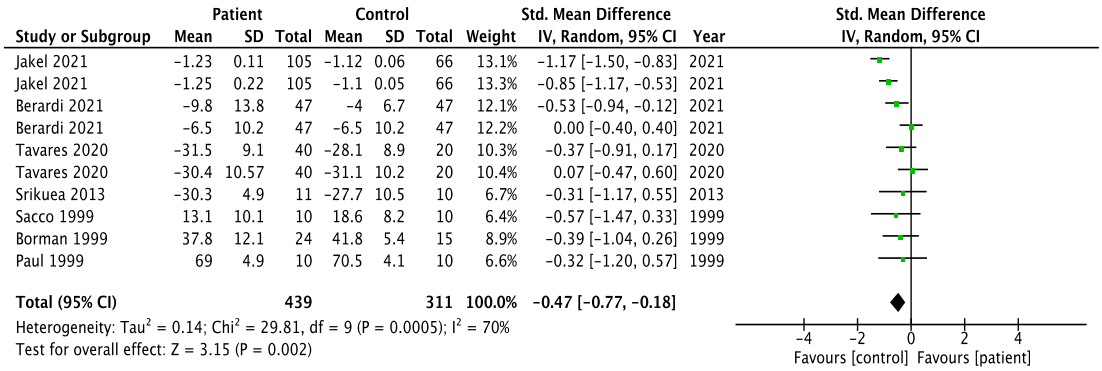


Figure G1. Fatigability values for all studies after sensitivity analysis.

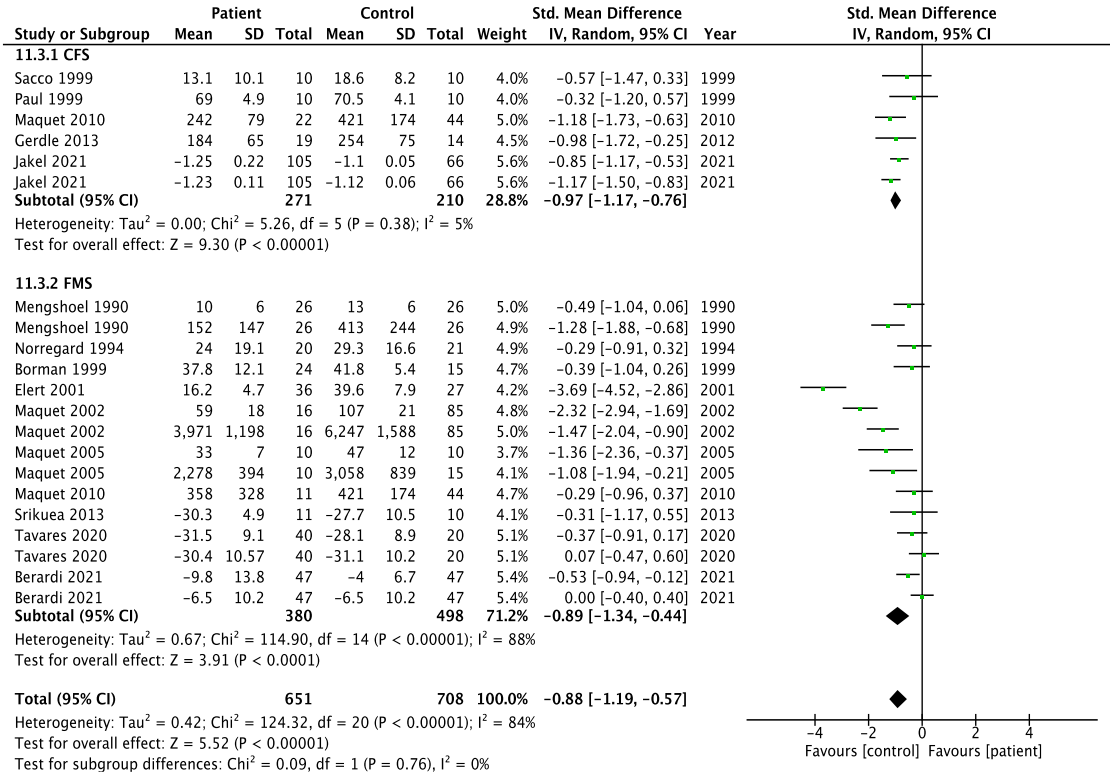


Figure G2. Fatigability values between CFS and FMS patients

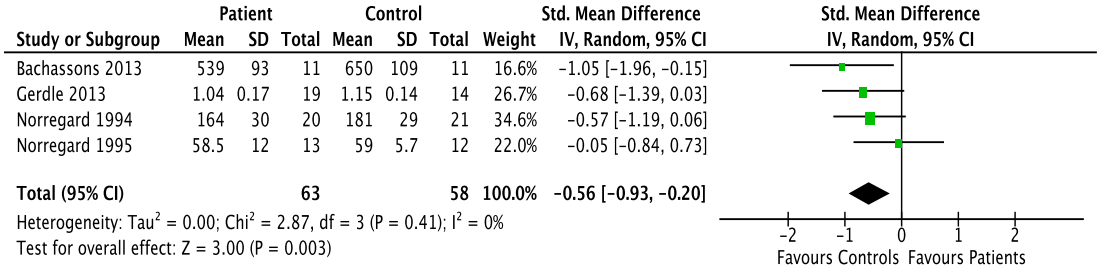


Figure H. Muscle Volume values for all studies included in the analysis.

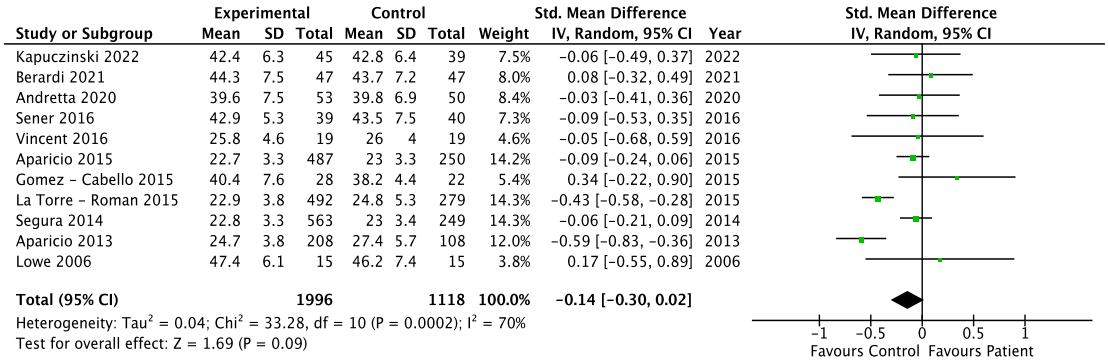


Figure I. Muscle Mass values for all studies included in the analysis.

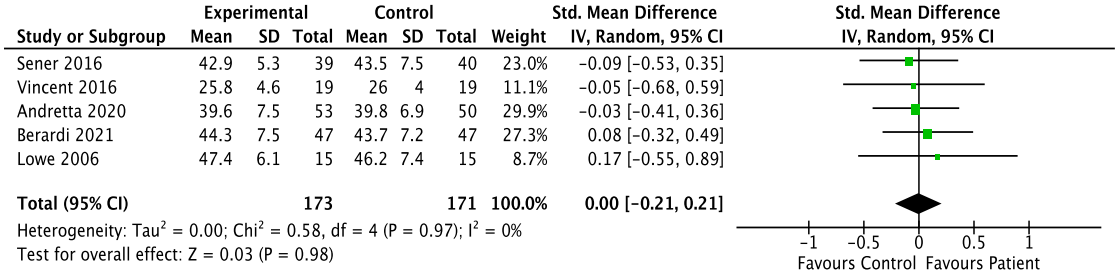


Figure I1. Muscle Mass values after sensitivity analysis for all studies included in the analysis.

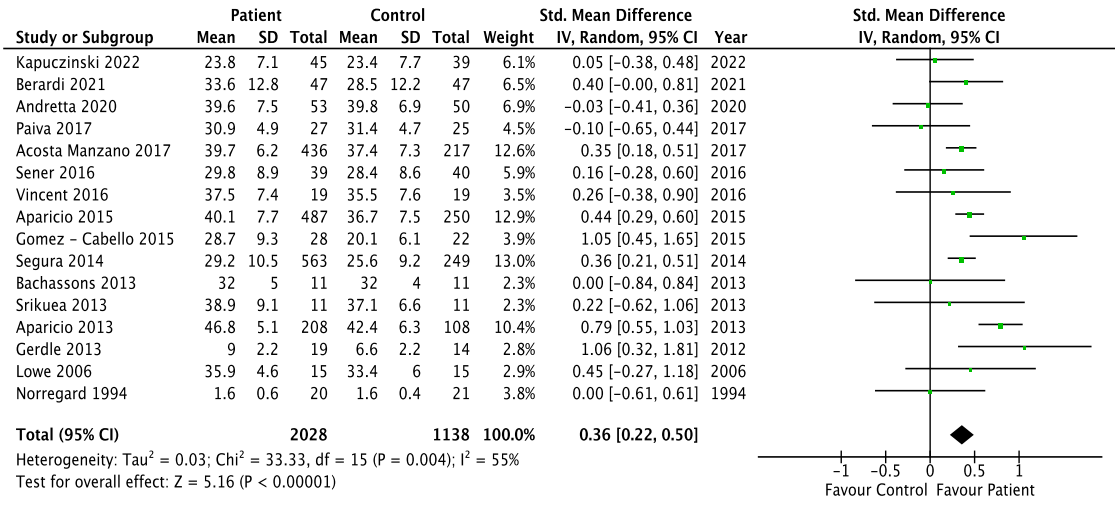


Figure L. Fat Mass values for all studies included in the analysis.

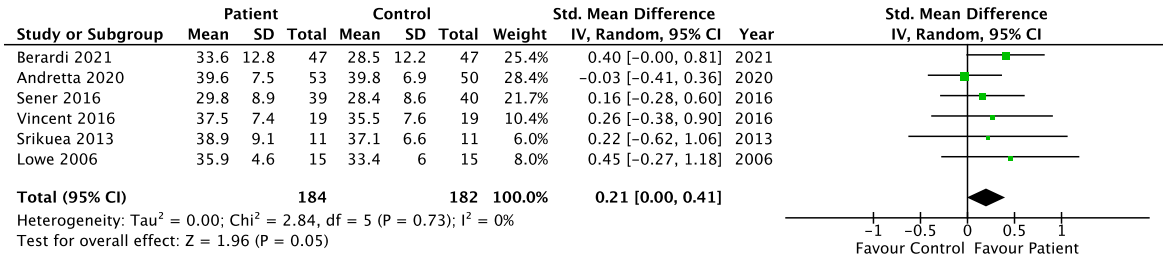


Figure L1. Fat Mass values for all studies after sensitivity analysis.

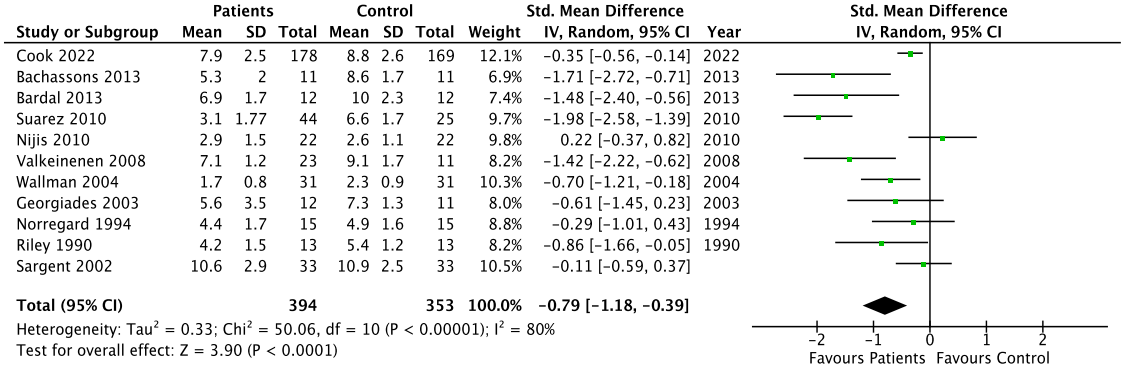


Figure M. Lactate values for all studies included in the analysis.

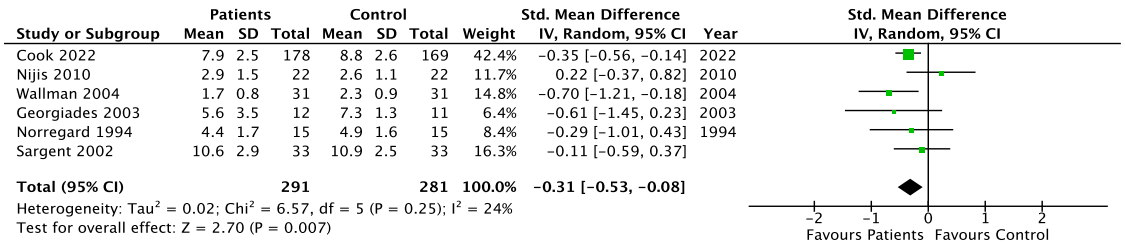


Figure M1. Lactate values for all studies after sensitivity analysis.

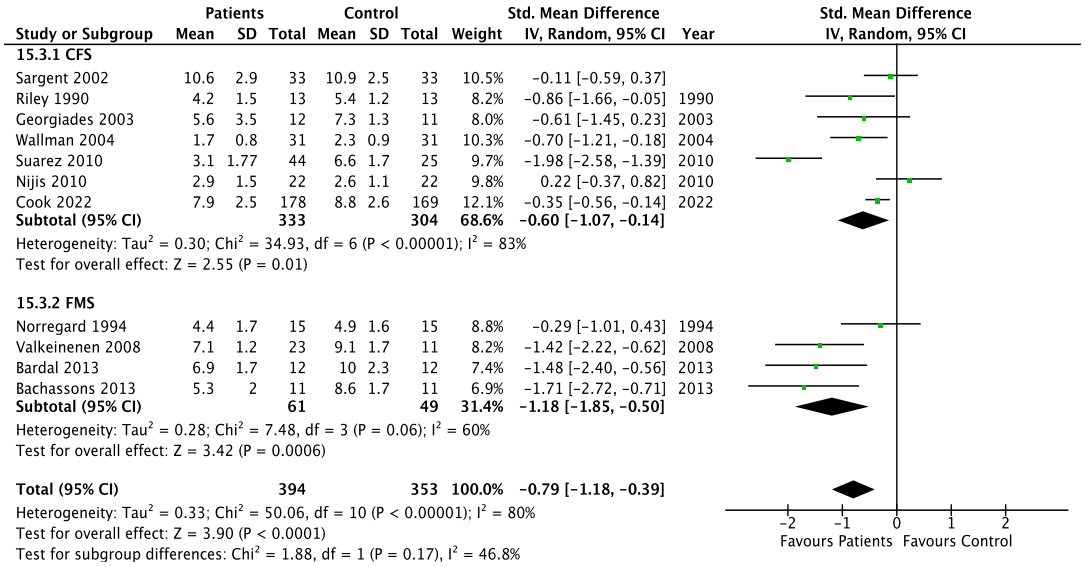


Figure M2. Lactate values in CFS and FMS patients

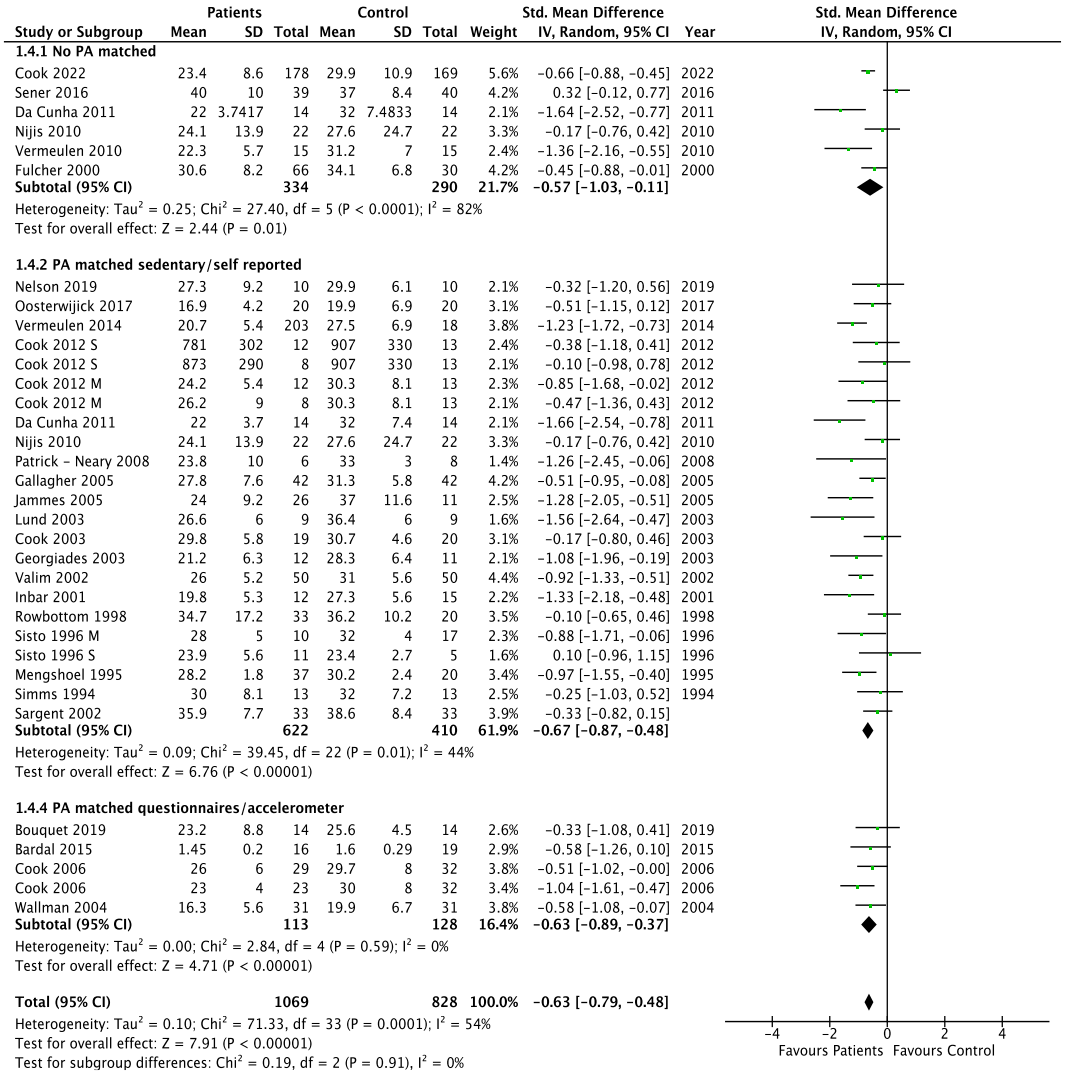


Figure N. Sensitivity analysis for low risk of bias studies based on matched physical activity levels (only VO2 Outcome)

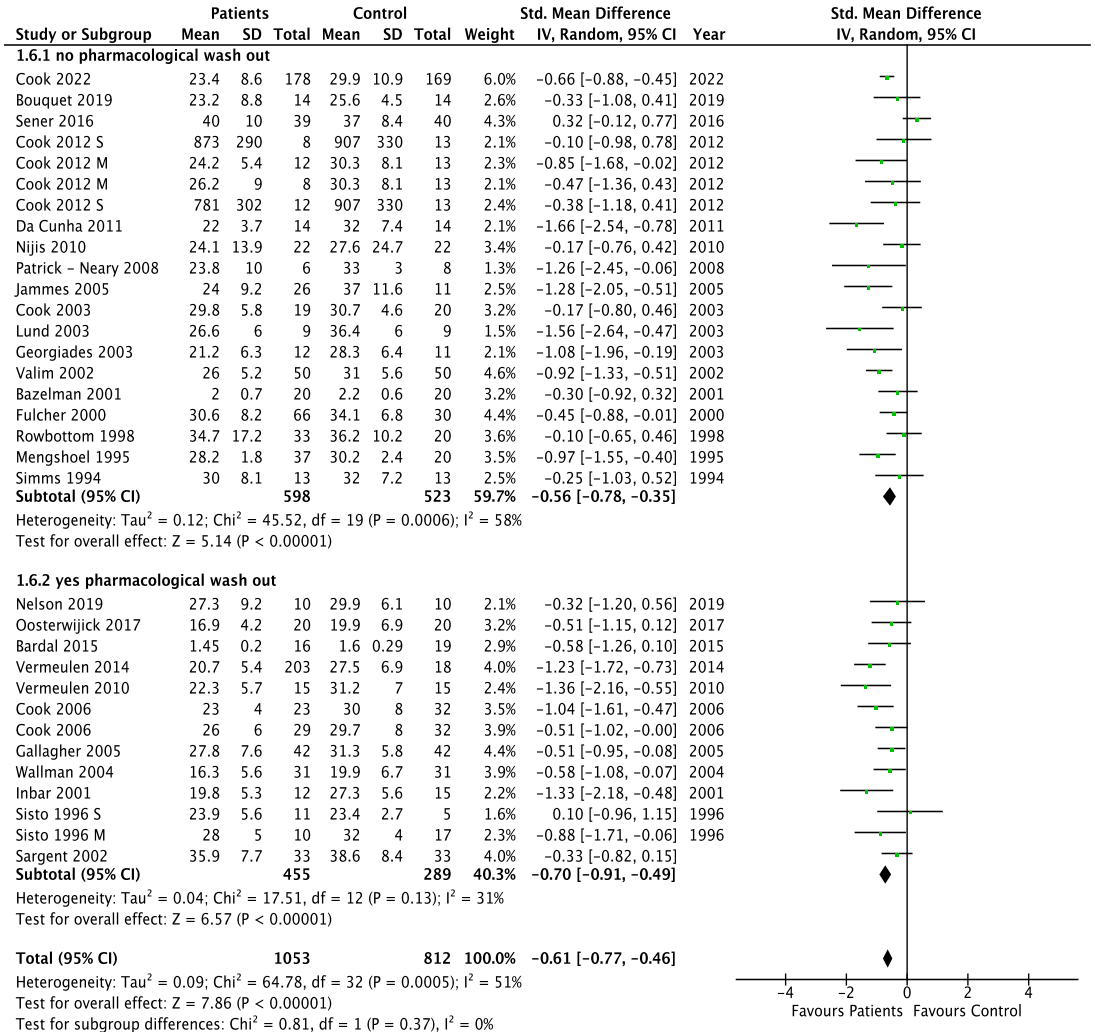


Figure 0. Sensitivity analysis for low risk of bias studies based on presence of pharmacological wash-out prior to the test levels (only VO2 outcome)

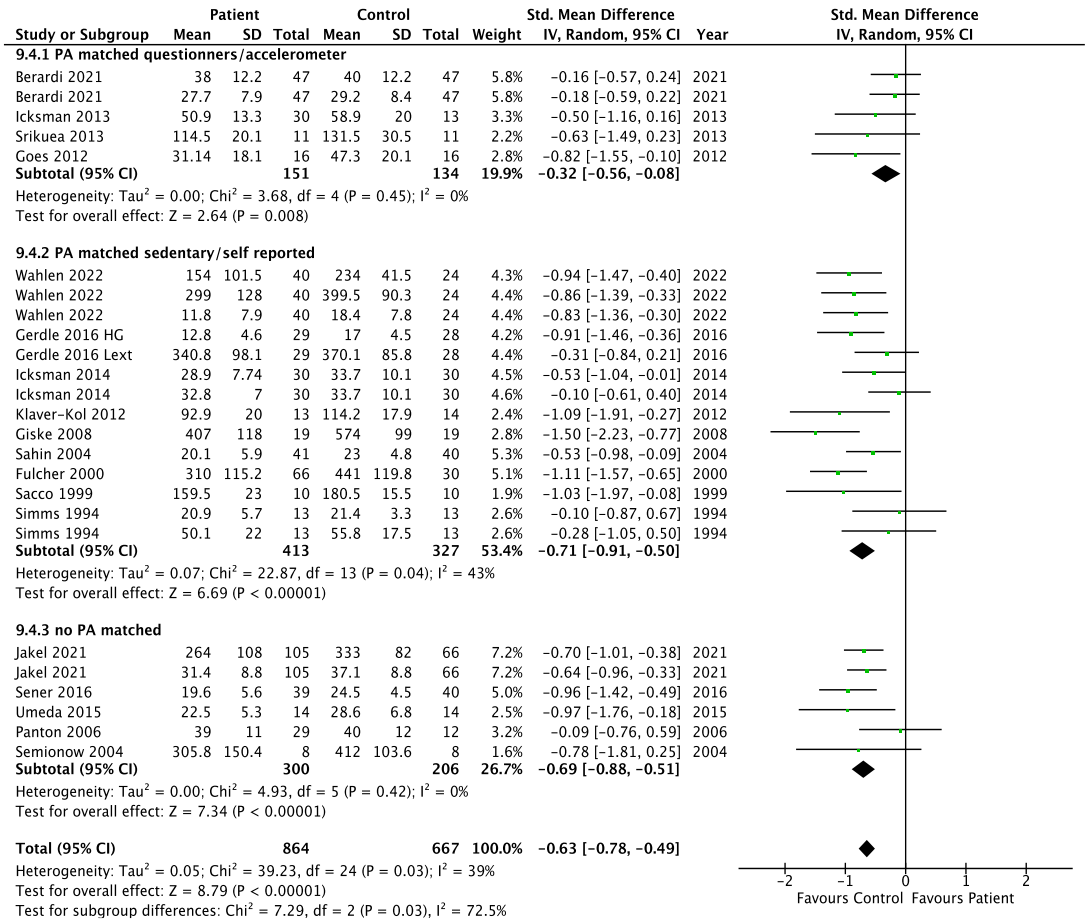


Figure P. Sub-group analysis for low risk of bias studies based on matched physical activity levels (only MVC Outcome)

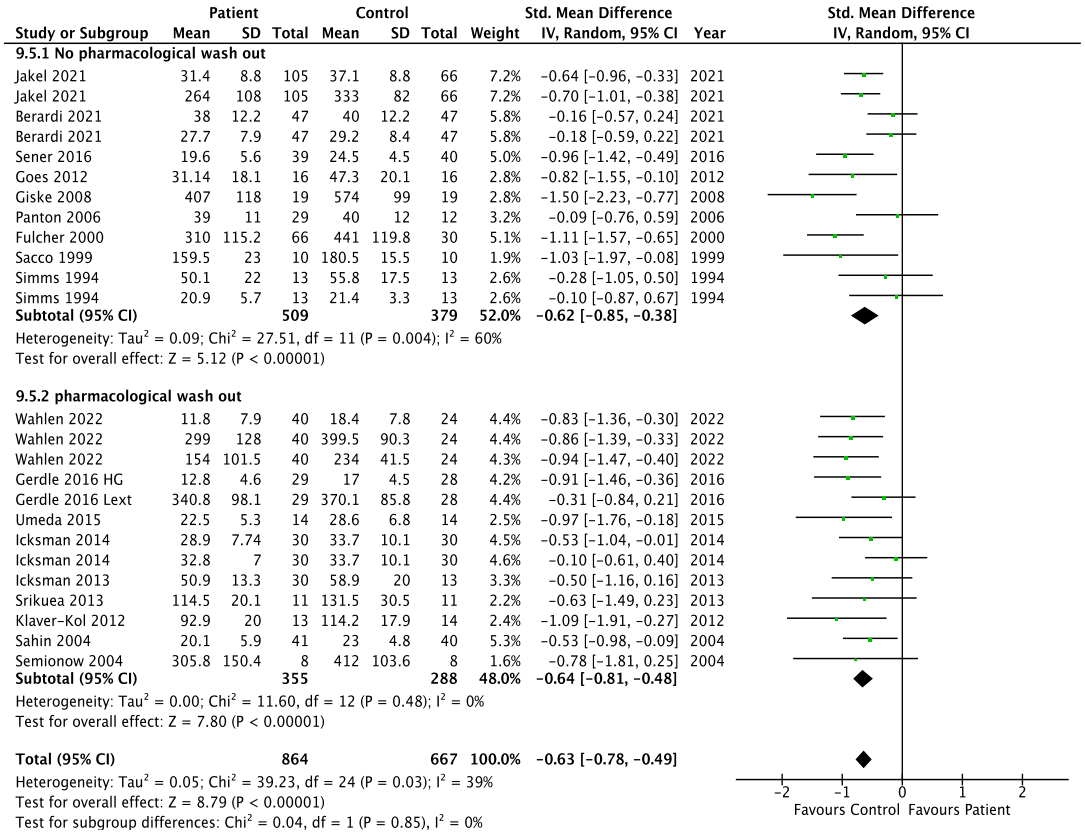


Figure Q. Sub-group analysis for low risk of bias studies based on presence of pharmacological wash-out prior to the test levels (only MVC outcome)

S4 File. **Full Research Strategy.**

(FT= Full Text; MH= Mesh terms)

Search *Term*	Field
Fibromyalgia	MH
Fatigue Syndrome, Chronic	MH
Fibromyalgia	FT
Chronic Fatigue syndrome	FT
Myalgic Encephalomyelitis	FT
All population combined with OR	
Exercise test	MH
Physical fitness	FT
Functional capacity	FT
Exercise performance *	FT
Aerobic power	FT
Peak power *	FT
Aerobic fitness	FT
Heart Rate *	FT
Aerobic assessment	FT
Oxygen uptake N2 maximum OR peak	FT
Threshold N2 Anaerobic OR Ventilatory OR lactate	FT
Cardiorespiratory Fitness	FT
Aerobic Capacity	FT
Lactate	FT
Rate of perceived exertion OR RPE	FT
All Aerobic Assessment outcomes combined with OR	
Muscle strength	MH
Muscle strength dynamometer	MH
Muscle strength	FT
Muscle strength dynamometer	FT
Muscle Strength Assessment	FT
Maximal Voluntary Contraction	FT
Voluntary Activation	FT
Fatigability	FT
Endurance *	FT
Maximal strength	FT
Handgrip assessment	FT
Handgrip test	FT
Rate of Torque	FT
Peak Torque	FT
All Strength outcomes combined with OR	
Body composition	MH
Body Mass Index	FT
Muscle volume	FT
Muscle cross-sectional area	FT
Mass N2 lean body OR free fat OR fat OR muscle	FT
All body composition outcomes combined with OR	

ALL Outcomes combine with AND Fibromyalgia OR Chronic Fatigue	

Research strategy PUBMED.

Search ((((((((((((((((((((Exercise test[MeSH Terms]) OR Exercise test) OR Physical fitness) OR Functional capacity) OR Exercise performance *) OR Aerobic power) OR Peak power *) OR Aerobic fitness) OR Heart Rate *) OR Aerobic assessment) OR ((Oxygen uptake N2 maximum OR peak))) OR ((Threshold N2 Anaerobic OR Ventilatory OR lactate))) OR Cardiorespiratory Fitness) OR Aerobic Capacity) OR Lactate) OR (Rate of perceived exertion OR RPE))))) OR (((((Body composition[MeSH Terms]) OR Body composition) OR Muscle volume) OR Muscle cross-sectional area) OR ((Mass N2 lean body OR free fat OR fat OR muscle)))) OR ((((((((((((Muscle strength[MeSH Terms]) OR Muscle strength) OR Muscle strength dynamometer) OR Muscle strength dynamometer[MeSH Terms]) OR Muscle Strength Assessment) OR Maximal Voluntary Contraction) OR Voluntary Activation) OR Fatigability) OR Endurance *) OR Maximal strength) OR Handgrip assessment) OR Handgrip test) OR Rate of Torque) OR Peak Torque)) AND ((((((fibromyalgia[MeSH Terms]) OR chronic fatigue syndrome) OR chronic fatigue syndrome[MeSH Terms]) OR fibromyalgia) OR Myalgic Encephalomyelitis) OR Myalgic Encephalomyelitis[MeSH Terms]) Sort by: Best Match

Research strategy Cochrane (CENTRAL)

ID Search Hits

#1 MeSH descriptor: [Fibromyalgia] explode all trees

#2 Fibromyalgia

#3 MeSH descriptor: [Fatigue Syndrome, Chronic] 4 tree(s) exploded

#4 Chronic fatigue syndrome

#5 Myalgic Encephalomyelitis

#6 #1 OR #2 OR #3 OR #4 OR #5

#7 MeSH descriptor: [Exercise Test] explode all trees

#8 exercise test

#9 Physical fitness

#10 Functional capacity

#11 Exercise performance

#12 Aerobic power

#13 Peak power

#14 Aerobic fitness

#15 Heart Rate

#16 Aerobic assessment

#17 Oxygen uptake N2 maximum OR peak

#18 Threshold N2 Anaerobic OR Ventilatory OR lactate

#19 Cardiorespiratory Fitness

#20 Aerobic Capacity

#21 Lactate

#22 Rate of perceived exertion OR RPE

#23 #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #20 OR #21 OR #22

#24 Muscle strength

#25 MeSH descriptor: [Muscle Strength] explode all trees

#26 MeSH descriptor: [Muscle Strength Dynamometer] explode all trees

#27 Muscle strength dynamometer

#28 Muscle Strength Assessment

#29 Maximal Voluntary Contraction

#30 Voluntary Activation

#31 Fatigability

#32 Endurance

#33 Maximal strength

#34 Handgrip assessment

#35 Handgrip test

#36 Rate of Torque

#37 Peak Torque

#38 #24 OR #25 OR #26 OR #27 OR #28 OR #29 OR #30 OR #31 OR #32 OR #33 OR #34 OR #35 OR #36 OR #37 36590

#39 Body composition

#40 Body Mass Index

#41 MeSH descriptor: [Body Composition] explode all trees

#42 Muscle volume

#43 Muscle cross-sectional area

#44 Mass N2 lean body OR free fat OR fat OR muscle

#45 #39 OR #40 OR #41 OR #42 OR #43 OR #44

#46 #23 OR #38 OR #45

#47 #6 AND #46

Research strategy PEDRO

Research strategy for PEDRO was performed using only the following words: Fibromyalgia and Chronic fatigue Syndrome separately. The results thereafter were combined in Endnote and duplicates if present removed.

Research Strategy for EBSCOhost: Medline, AMED, CINAHL:

The research strategy was performed trough EBSCOhost database for Medline, AMED and CINAHL databases as reported in the following page (below).

Chapter 9. General discussion

Overall thesis aims

The overarching aim of the work presented in this thesis was to assess changes in muscle nerve afferent sensitivity and its physiological responses to exercise induced muscle damage, ageing, and clinical population

This aim was addressed through 4 chapters each setting out the results of experimental or cross-sectional research and one chapter providing a systematic review and meta-analysis of available original research. Each of these chapters had their own individual aims.

The first original chapter aimed to characterise the cardiovascular and neuromuscular responses following an exercise induced muscle damage (EIMD) protocol. 31-P NMR (Nuclear Magnetic Resonance) Spectroscopy was used to assess alterations of muscle metabolism and investigate links with blood pressure responses to exercise.

The second original chapter aimed to study the effect of mechano- and nociceptive afferent stimulation on cardiovascular and hyperaemic responses following single passive leg movement (sPLM) of remote muscle.

The third original chapter aimed to study the effect of mechano- and nociceptive afferent sensitisation on exercise performance and the neuromuscular component of fatigue in remote muscle.

The fourth original chapter aimed to investigate the role of muscle afferents in the blood pressure responses of Master Athletes participating at the World Master Track Cycling Championships (2019). We further investigated the effect of comorbidities,

ageing, level of fitness and exercise performance on blood pressure responses and recovery.

The fifth original chapter aimed to investigate the cardiorespiratory fitness and neuromuscular function in Chronic Fatigue and Fibromyalgia Syndrome, and to identify possible underlying physiological processes associated with reduced physical performance of the patient groups.

Overview of the results

Chapter 4 - Cardiovascular Responses following EIMD: implication on metaboreflex isolation. Exercise induced muscle damage did not alter blood pressure responses (e.g., MAP) and metaboreflex sensitivity to isometric exercise at matched relative intensities. However, the cardiovascular response to exercise was no longer correlated with phosphate metabolism, suggesting a changed mechanism of blood pressure regulation during isometric exercise, following EIMD.

Chapter 5 - Cardiovascular Responses following EIMD: implication on mechano- and nociceptive reflexes. Exercise induced muscle damage caused an increase in mechano- and nociceptors sensitivity, resulting in a decreased peripheral haemodynamic response at rest (e.g., low FBF (Femoral Blood Flow) and LVC (Leg Vascular Conductance)) with exaggerated central cardiovascular responses (e.g., MAP, CO, HR (Heart Rate,), SV (Stroke Volume)). Moreover, increases in mechanoreceptors and nociceptors sensitivity caused a decreased hyperaemic response following single passive leg movement (e.g., ΔLVC and AUC (Area Under the Curve)) showing reduced blood flow supply to remote muscles (290).

Chapter 6 – Neuromuscular fatigue following EIMD: implication on mechano- and nociceptive reflex isolation reflexes. Increase in mechanoreceptors and nociceptors sensitivity was shown to decreased exercise performance with an impaired neuromuscular function at baseline and following a time to exhaustion isometric task. These findings have been linked to an increase in central fatigue (e.g., decrease in voluntary activation), and/or early attainment of the sensory exercise tolerance limit.

Chapter 7 – The association of elevated blood pressure during ischaemic exercise with sport performance in Master athletes with and without morbidity. Long-term athletic training was associated with improved blood pressure recovery following blood flow occlusion exercise regardless of age or reported morbidity. The slope in blood pressure rise was associated with lower AGP in the master athletes competing at the World Master Track Cycling Championships (428).

Chapter 8 – Fibromyalgia and Chronic Fatigue Syndromes: a systematic review and meta-analysis of cardiorespiratory fitness and neuromuscular function compared with healthy individuals. An extensive systematic review and metanalysis was completed to quantify the cardiorespiratory and neuromuscular function in FMS (Fibromyalgia Syndrome) and CFS (chronic fatigue syndrome) compared with healthy individuals. We found that Fibromyalgia and Chronic fatigue syndromes exhibit lower cardiorespiratory fitness, fatigability, and neuromuscular function with increased rate of perceived exertion and pain during exercise, even when controlling for study quality, level of physical activity and pharmacological wash-out. (97).

Overall flow across experimental chapters.

Muscle afferent Sensitivity could be ascribed to a change in sensitivity of the muscle afferent, showing increased or decreased sensitisation of muscle nerve afferents -, metabo- and nociceptive) (117). The changes in sensitivity could lead to different physiological responses at cardiovascular, cardiorespiratory, and neuromuscular levels, since muscle nerve afferents are polymodal and respond to multiple stimuli when activated (27, 35, 102). Therefore, we decided to study their function using an integrative approach that involved the acquisition of several physiological signals during different challenges (exercise, muscle stretching, post-exercise occlusion) to differentiate and isolate their contributions at different level (cardiovascular, cardiorespiratory, neuromuscular). We investigated the role of muscle afferents following muscle damage and inflammation, showing that this model may have altered their sensitivity and in turn modified several physiological functions mirroring physiological impairments founded in ageing, metabolic and cardiovascular conditions with chronic pain and fatigue syndromes (fig 9.1).

The mechanisms behind these disfunctions may be ascribed to changes in muscle afferent sensitivity as previously found in mice models (17-19, 30). Following EIMD we found altered cardiovascular responses in the exercising damaged leg despite no changes in metabolites concentration during muscle contractions. We initially hypothesized that these differences were linked to increased metabolites that were stimulating muscle metaboreceptors, however following metaboreceptors isolation no changes in blood pressure responses were found. This led to the hypothesis that other factors rather than metaboreceptors may have been involved (i.e., Chapter 4).

For this reason, we linked these results with our second experimental study in which we induced muscle damage to induce muscle inflammation and afferent sensitisation, and we focused on testing the cardiovascular response following mechano- and nociceptive activation (290). We found that once stimulated mechanoreceptors and nociceptors increased autonomic functioning leading to parasympathetic withdrawal (i.e., increases in sympathetic activity) with blood pressure and central haemodynamic responses (I.e., HR, CO, SV). Moreover, we found decreased peripheral haemodynamics (FBF and LVC) at rest and hyperaemic responses to single passive leg movement in a remote muscle, suggesting that mechano- and nociceptors sensitisation were acting systemically to reduce cardiovascular function in a remote healthy muscle (i.e., Chapter 5).

Following these results, we decided to implement the same study design to establish if this increased sensitivity led to decreased exercise performance. The rationale was based on the assumptions that an increased activation of muscle afferents throughout their previous sensitisation would have led to increases in sympathetic activity, cardiovascular responses, perception of pain and fatigue and central fatigue, resulting in an early attainment of the exercise tolerance limit and reduced time to exhaustion. For the first time we found that of mechano- and nociceptors activation following EIMD caused an extensive decrease in exercise performance due to an increased central fatigue and reduced voluntary activation on the exercising muscle. Moreover, we found concomitant increases in perception of effort and pain suggesting an early attainment of the exercise tolerance limit reflecting the lower exercise performance and lower peak heart rate and oxygen consumption achieved during mechano- and nociceptors sensitisation condition (i.e., Chapter 6).

Since the exaggerated blood pressure and exercise intolerance has been extensively reported in ageing and master athlete's cohort and possible related CV comorbidities (118, 119, 241, 456, 638-640) we decided to investigate blood pressure responses in a master athletes cohort participating at the World Master Track Cycling Championships 2019 and comparing these responses with age-matched healthy controls (I.e., Chapter 7). Interestingly, we found that increases in blood pressure responses (BP rising slopes) were associated with lower exercise performance in Master athletes, suggesting that an increased sensitivity of muscle III-IV afferents may play a role in exercise performance in this population. Surprisingly, we found no effect of age or comorbidities in blood pressure response in the Master Athletes' cohort while age-matched sedentary controls exhibited lower blood pressure recovery. This finding may suggest that chronic exposure to exercise had an important effect preserve blood pressure responses and recovery with ageing and across a different incidence of comorbidities (428).

Lastly, we conducted a systematic review and metanalysis on cardiorespiratory fitness and neuromuscular function in Fibromyalgia and Chronic Fatigue Syndrome compared with healthy controls. From analysis of 99 studies, we found that these two conditions of FMS and CFS showed an increased rate of perceived exertion and pain during exercise, increased fatigability, lower cardiorespiratory fitness, and neuromuscular function. However, despite several studies present in the literature, a clear picture of all these impairments was missing. Moreover, most studies were often at elevated risk of bias and not controlling for important co-founding factors (sex, age, level of physical activity, wash out, testing protocol), making it difficult to evaluate and compare the quality and applicability of the data arising from the literature. For these reasons, we

decided to carry an extensive systematic review and meta-analysis on the cardiorespiratory fitness and neuromuscular function in fibromyalgia and chronic fatigue syndromes, to quantify the entity of these changes and to assess if study quality and other co-founding factors were affecting the outcomes. Indeed, we performed several subgroup analyses, matching for similar physical activity level and pharmacological wash out, as co-funding factors across studies. We collected evidence from *9853* participants (5808 patients; 4405 healthy controls). We performed a random effects meta-analysis showing lower cardiorespiratory fitness (VO_2max, anaerobic threshold, peak lactate) and neuromuscular function (MVC, fatigability, voluntary activation, muscle volume, muscle mass, rate of perceived exertion) in CFS and FMS compared to controls: all with moderate to high effect sizes even when controlling for study quality, level of physical activity or pharmacological wash-out (97). These are important points because they suggest that study quality, deconditioning or medication are not the only direct influences of the reported lower physical function found in FMS and CFS studies, and other factors could play a pivotal role in driving these dysfunctions. Seeing that several studies reported heightened perceptions of effort, reduced ability to activate the available musculature during exercise and reduced tolerance of exercise, we advanced the hypothesis that peripheral muscle afferent may be involved. Indeed, previous research has found that RPE (Rate of Perceived Exertion) is linked to increased activation of muscle nerve afferents (40).

All these findings highlight the importance of the sensitization of group III-IV afferents in cardiovascular, cardiorespiratory, and neuromuscular function. Seen that their involvement may affect different key physiological functions in chronic conditions

where pain and fatigue may also play a key role in impacting the quality of life and exercise therapies.

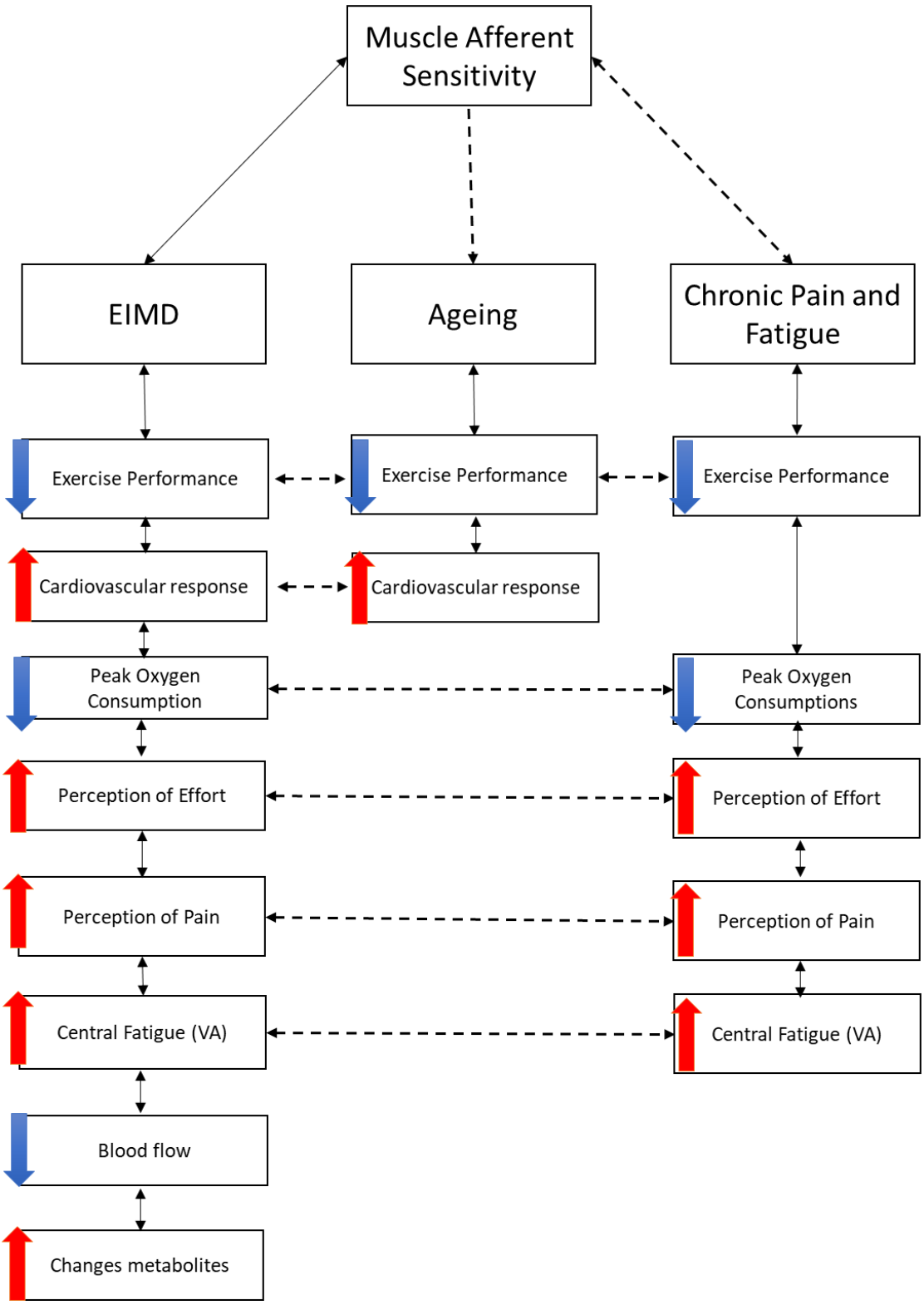


Figure 9.1 Experimental chapter flow

Blue arrows represent a decreased response while red arrow an increased response of related outcomes. Dotted arrows represent weak correlations or associations while full lines represent stronger correlation and associations between the outcomes.

Main limitations.

The main limitation of our experimental approach could be attributed to the difficulty of measuring changes in gene expression linked with peripheral sensitisation of muscle nerve afferents. Previous study analyses gene related expression of muscle nerve afferents with biopsies (346, 641), or blood samples (344, 642), however, seen that nerve afferents are located outside the sarcolemma it is difficult to associated changes in these results specifically with muscle afferent sensitisation.

Moreover, no direct measure of autonomic nervous system was implemented to quantify the activation of the sympathetic nervous systema via microneurography (i.e., muscle sympathetic nerve activity) and understand the role of mechano- or metaboreflex sensitisation. This is due to the invasiveness and complexity of the technique especially when involving population with chronic pain or during repeated measure study design or non-laboratory-based investigations.

Another limitation of the present approach was the absence of blood pressure or heart rate monitoring during the MRI (Magnetic Resonance Imaging) exercising protocol (chapter 4). This would have benefitted the study thanks to the possibility of associate contemporary changes in muscle metabolism and blood pressure and cardiovascular responses during the exercise task. Indeed, all metaboreflex studies based the assumption on similar rate of metabolites accumulation when a relative intensity is set between participants. However, fibre composition and fatigability difference may differ between different subgroup population influencing the variations across participants taking part in metaboreflex isolation studies (328). We

overcome this limitation assessing the BP responses to isometric exercises outside the scanner in the same day, however changes in body position, contraction angle, instrumentation, may have influenced the absolute values of the findings.

A further limitation could be represented by the single passive leg movement test. Despite a good reliability when the same operator performs the task (366), it would have been helpful to use a more standardize protocol (I.e., Cybex dynamometer) to set the right amount of force and frequency of stimulation to minimize variability responses between participants and across different condition, to have the same level of mechanoreflex activation.

Experimental options.

Seen the extensive literature present on muscle III-IV afferents we decided to translate animal studies to human studies in the most complete and less invasive way possible. However, future studies are needed to characterise muscle afferents in humans and their activity across populations, especially the molecular basis and expression following different stressor (exercise, experimental pain model, drugs) to quantify changes occurring in the muscle peripheral nerve endings. Yet, animal models are the most effective way to study muscle afferent plasticity and adaptation to external stressors, however the translation of this results could be always complex and limited compared with humans' participants and especially in patients' populations.

We hope that with advancing of imaging techniques there will new emerging technological advances that will allow to study these adaptations in humans avoiding invasive and painful techniques.

Practical application and implications

The results emerging from the different experimental chapters showed the importance of the correct functioning of muscle nerve afferents, especially on regulating cardiovascular, cardiorespiratory, and neuromuscular function during exercise. Their alteration has shown to led to increased perception of effort and exercise intolerance, showing the importance of their role in different chronic conditions. These investigations could be helpful to identify the role of peripheral sensitisation on the blood pressure regulation and cardiovascular response to exercise showing the importance of metaboreceptors and mechanoreceptors plasticity and adaptation following muscle injuries or increased peripheral inflammation. Indeed, despite no changes in muscle metaboreceptors activity (Chapter 4), an increased mechanoreceptors activity leads to an impairment in muscle blood flow to the contralateral muscle (Chapter 5) resulting in lower exercise tolerance and performance once exercising (Chapter 6). Moreover, higlined the physiological implication of mechanoreceptors sensitisation following a model of muscle injury and inflammation could inform future studies on the pathophysiological mechanism underlying similar conditions affected by peripheral sensitisation and target effective intervention to improve muscle blood flow, sympathetic responses, and blood pressure regulation and as a consequence perception of pain and effort during exercise. Moreover, these results could be translated to condition where chronic fatigue is a primary feature, as for example CFS and FMS. Indeed, peripheral sensitisation could be a possible mechanism underlying the increased perception of effort and pain during exercise and the lower neuromuscular and cardiorespiratory function we found (Chapter 8). It can impact greatly on those affected by reducing

their social and economic interactions. Alongside the generalized fatigue, our findings reveal the extent of reduced physical function of patients which occurs in excess of that expected from sedentary living. Finally, BP regulation is key in the healthy aging process showing that regular exercise contribute to the preservation of the exercise pressor reflex in a Master Athletes cohort with and without underlying comorbidities (Chapter 7).

Future directions

Regarding future studies, we would continue to investigate the role of muscle afferent in chronic pain and fatigue conditions. Specifically, we would attempt to isolate the role of mechano-, metabo- and nociceptors contributions to cardiovascular, cardiorespiratory, and neuromuscular system with a particular focus on exercise intolerance in these populations. We would implement similar procedures utilised in the past years, improving current limitations and knowledge. We hypothesise that the neurogenic pain experienced by chronic pain and fatigue conditions is a result of peripheral sensitisation that enhance the pain and a fatigue response at rest and during exercise. Finally, once identify the possible origin and physiological impairments, we will aim to differentiate the physiological consequences of peripheral and/or central sensitisation in chronic pain conditions and to establish an effective treatment to re-establish the correct functioning of these pathway, restoring their activity and possibly reducing pain amplification.

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