Skeletal muscle fatigue: Investigating the role of cytokines, ROS and mitochondrial function

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Abstract

Skeletal muscle dysfunction and increased rate of fatigue is observed in a number of disorders. An increase in systemic and/or local pro-inflammatory cytokines has been proposed to modulate the ability of skeletal muscle to generate force, alter mitochondrial bioenergetics and induce reactive oxygen species (ROS) generation in muscle. Evidence suggests cytokines such as Tumour necrosis factor-α (TNF-α) play a major role in modulating the function of muscle cells. Muscle contraction and fatigue is a multidimensional concept which can be defined through both physical and psychological concepts, thus systemic changes in cytokine levels may alter both central and peripheral fatigue. Increased levels of dietary polyphenols have been associated with a reduction in systemic inflammation and may therefore alleviate muscle dysfunction.

The aim of this work was to use cell-based studies to validate TNF-α induced cytokine production and examine the potential effects of targeted polyphenol interventions on cytokine release by skeletal muscle cells. Moreover, the aim of this study was to investigate in a human clinical population the role of cytokines in subjective fatigue and peripheral function of skeletal muscle. The human study focussed on patients with chronic fatigue syndrome (CFS), a condition associated with enhanced and chronic perceived fatigue. Patients with CFS report symptoms of post exertion malaise and general widespread fatigue in the outer limbs. The cellular mechanisms associated with perceived muscle weakness in CFS are poorly characterised. The hypothesis of this thesis was that altered circulating levels of cytokines and chemokines may play a major role in the pathogenesis of CFS.

A cell model of increased cytokine release through TNF-α exposure of muscle cells was validated. C2C12 myotubes treated with 25ng/ml TNF-α released increased levels of IL-6, CCL2, CCL5 and CXCL1. Pre-treatment with polyphenol compounds followed by TNF-α exposure showed that resveratrol attenuated TNF-induced release of IL-6, CCL2, CCL5 and CXCL1 cytokines from C2C12 myotubes. These data suggest that resveratrol may inhibit the actions of TNF-α, thus can potentially modulate local environment and potentially affecting muscle function. These findings are clinically relevant and have widespread implications for the understanding of diverse scientific areas, including the potential effect of polyphenol compounds on muscle function as well as inflammatory human muscle diseases in which cytokines may alter both central and peripheral muscle fatigue.

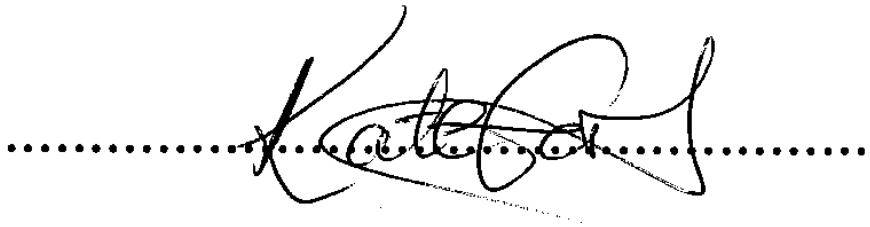
This study showed that maximum voluntary contractions (MVC) were significantly reduced in patients with CFS compared with Healthy Controls (HCs). No difference was seen in electrically stimulated muscle tetanic forces or fatigue or single muscle fibre force measurements between groups. There was no difference in circulating cytokine levels or cytokine gene expression within the muscle in CFS compared with HCs although there was a suggestion of an increase in IP-10 and CCL2. Muscle mitochondrial respiratory function and ROS generation were not

significantly different between patients with CFS and HCs although mitochondria in muscles of patients with CFS showed a blunted response to substrates compared with HCs. Overall these data suggest that patients with CFS do not show grossly altered muscle ROS generation, mitochondrial function or altered systemic levels of cytokines. Further investigations are required to determine the cause of reduced MVC and elevated perception of fatigue in patients with CFS and to examine the effect of stratification of these data from patients with CFS.

Doctor of Philosophy Declaration

I hereby declare that this dissertation is a record of work carried out in the Institute of Ageing and Chronic Disease at the University of Liverpool during the period from November 2012 to February 2016. The dissertation is original in content except where otherwise indicated.

February 2016



(Kate Elizabeth Earl)

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Dedication

I dedicate this work to my Grandma Ada Johnson.

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List of Abbreviations

3-NT – 3-nitrotyrosine

4-HNE – 4-hydroxynonenal

ADP – Adenosine Diphosphate

Ant A – Antimycin A

APS – Ammonium persulphate

ATP – Adenosine Triphosphate

BCA – Bicinchonic acid

BSA – Bovine Serum Albumin

CAT – Catalase

CCL – Chemokine Ligand

cDNA – Complementary deoxyribose nucleic acid

CCL2 – Chemokine ligand 2 (MCP-1)

CCL5 – Chemokine ligand 5 (RANTES)

CXCL1 – Chemokine (CXC motif) ligand 1 (KC)

CCR – Chemokine receptor

CFS – Chronic Fatigue Syndrome

CuZnSOD – Copper-zinc superoxide dismutase

DMEM – Dulbecco's Modified Eagle's Medium

DNA – Deoxyribose nucleic acid

DPBS – Dulbecco's phosphate buffered saline

ELISA – Enzyme-linked immunosorbent assay

eNOS - Endothelium nitric oxide synthase

FBS – Foetal Bovine Serum

GPX – Glutathione peroxidase

H_2O_2 – Hydrogen peroxide

HSP – Heat Shock Protein

HS – Horse Serum

IFN-γ – Interferon-gamma

IL-1β – Interleukin-1 Beta

IL-1ra – Interleukin-1 receptor agonist

IL-2 – Interkeukin-2

IL-4 – Interkeukin-4

IL-5 – Interkeukin-5

IL-6 – Interkeukin-6

IL-7 – Interleukin-7

IL-8 – Interkeukin-8

IL-10 – Interkeukin-10

IL-12 - Interkeukin-12

IL-13 – Interkeukin-13

IL-15 – Interkeukin-15

IL-17 – Interkeukin-17

iNOS – Inducible nitric oxide synthase

KC – Keratinocyte chemoattractant (CXCL1)

LPS – Lipopolysaccharide

MCP-1 – Monocyte chemotactic protein-1 (CCL2)

ME - Myalgic Encephalopathy

MnSOD – Manganese superoxide dismutase

NADH – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

NFκB – Nuclear Factor kappa B

nNOS Neuronal nitric oxide synthase

NO – Nitric oxide

O_{2}^{-} – Superoxide

PBS – Phosphate buffered saline

qPCR – Quantitative polymerase chain reaction

RANTES – Regulated upon activation normal T expressed & secreted (CCL5)

RNA – Ribose nucleic acid

ROS – Reactive oxygen species

RONS – Reactive oxygen and nitrogen species

SDS – Sodium dodecyl sulphate

SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

TBS – Tris Buffered Saline

TBST – Tris buffered saline tween

TEMED – NNN'N'-tetramethylethylene-diamine

TNF-α – Tumour necrosis factor alpha

CHAPTER One

Introduction

1.1 Skeletal muscle

Skeletal muscle is a diverse and plastic tissue displaying a highly organised and complex structure, which is matched to its principle function of force generation and locomotion. It is the most abundant tissue in the human body, supporting multiple biological functions; including the maintenance of core body temperature through the production of heat, as well as providing storage to macromolecules amino acids and carbohydrates. (Marieb and Hoehn, 2009). Muscle has the ability to exert numerous endocrine effects though the production and release of a wide-range of small peptides and proteins.

1.1.1 Skeletal muscle structure

Skeletal muscle is typically characterised as a striated and multi-nucleated tissue; which is attached to the skeleton by tendons and works under voluntary control from the peripheral nervous system. Skeletal muscle is the largest tissue in the human body making up 40% of the total body weight in men and 30% in women and is the largest supply of protein in body containing 50 – 75% % of the total body store (Janssen et al., 2000). Skeletal muscle mass is maintained through a fine balance between protein synthesis and degradation (Kandarian and Jackman, 2006). The continual turnover of muscle accounts for 30-50% of all protein turnover in the body. Factors such as mobility, physical activity and nutritional status play affect these processes (Henriksson, 1995, Gerdhem et al., 2003, Baxter-Jones et al., 2008, Mazis et al., 2009).

Skeletal muscle is characterised by a precise arrangement of muscle cells (also referred to as muscle fibres or myofibres) which is highly organised and conserved (Figure 1.1). The size and number of fibres is the main determinant for whole muscle size and function. Muscle fibres are bound together to form bundles termed fascicles; which collectively make up a whole muscle. Each fascicle is encapsulated by layer of connective tissue called a perimysium. The perimysium is an extension of the connective tissue which surrounds the entire muscle, termed the epimysium. Neuronal innervation is essential for skeletal muscle function and contraction. Each individual muscle fibre is innervated by a single branch from a motor neurone.

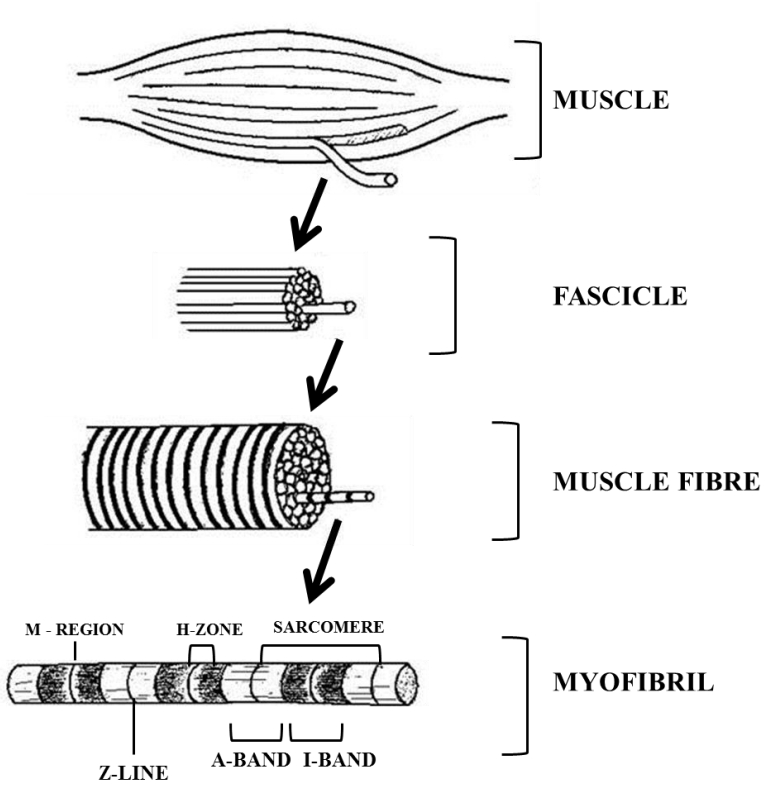


Figure 1.1 Schematic representation of the structure of skeletal muscle. A representation of the hierarchical organisation of skeletal muscle. Adapted from Lightfoot, 2011.

A single muscle cell is elongated and cylindrical in shape, multinucleated and in a post mitotic phase. Each fibre is surrounded by a layer of connective tissue called an endomysium as well as a plasma membrane called a sarcolemma. Muscle fibres appear striated by a highly organised internal arrangement of units called myofibrils embedded within the cytoplasm of the cell. The striated appearance of muscle is a result of alternating dark and light areas caused by the arrangement of contractile proteins (actin/myosin) units which comprise the sarcomere. Myofibrils are divided into segments called sarcomeres which contain thick and thin filaments which collectively work together to generate force. Thick filaments consist of myosin whilst thin filaments consist mainly of actin, tropomyosin and troponin. The most prominent striations are the A and I bands and the Z lines. A bands are made up of a combination of thick and thin filaments whilst I bands consist of thin filaments only. Z discs mark the borders which link and separate individual sarcomeres. Within the middle of the A band is a region when the thin myosin filaments do not

cross the thick actin filaments. This line is known as the H zone. The M line is situated within the H zone (Figure 1.2) (Mougios, 2006).

1.1.1.2 Skeletal muscle - fibre type

Human skeletal muscle consists of a heterogeneous collection of muscle fibre types. These can be broadly classified as oxidative (type I), fast oxidative (Type IIα) and fast glycolytic (IIβ and IIx) muscle fibres (Table 1.1). Each fibre type has its own unique properties which enables whole muscles to perform a magnitude of different contractile functions (Essen et al., 1975). Different muscles are made up of different compositions of fibres. Type I fibres are also known as slow twitch fibres. They are very resistant to fatigue and are capable of producing repeated low-level contractions. These fibres are type I myofibres are rich in mitochondria and able to produce large amounts of ATP through oxidative phosphorylation. Type IIα fibres are known as fast/oxidative/glycolytic, they produce fast, strong muscle contractions, although they are more prone to fatigue than type I fibres. These fibres contain a large number of mitochondria and manufacture ATP at a fast rate by utilising both aerobic and anaerobic metabolism. Type IIβ fibres are known as fast glycolytic fibres. They contain few mitochondria, producing ATP at a slow rate by anaerobic metabolism which is broken down very quickly. These fibres are able to produce short, fast bursts of power and rapid fatigue (Macintosh et al., 2006). Muscles are adaptive to changing demands; they can by alter their size or fibre type composition i.e. Type IIβ fibres can turn into type IIα fibres by resistance training. This is beneficial due to an increase in the muscle's ability to utilise ATP and fatigue more slowly. The impairments and disabilities seen in some patient groups suffering from deconditioning because of prolonged inactivity, limb immobilization, or muscle denervation may be partly the result of changes in fibre type composition.

	Type I Fibres	Type IIα Fibres	Type IIβ/IIX Fibres
Motor unit type	Slow/Oxidative	Fast/Ox&Gly	Fast/Glycolytic
Twitch Speed	Slow	Fast	Fast
Twitch force	Small	Medium	Large
Resistance to fatigue	High	Fairly high	Low
Oxidative capacity	High	Higher	Moderate

Table 1.1 Human skeletal muscle fibre type properties.

1.1.2 Muscle contraction

The contraction of skeletal muscle and force generation is a highly complex and specialised process, which focusses around two central components, actin and myosin filaments. As previously mentioned actin and myosin are the major contractile proteins involved in force production and constitute a large proportion of the thin and thick filaments respectively (Figure 1.2). The lengths of filaments do not alter during contraction, however, the intersection between them increases. This results in the shortening of the sarcomeres in proportion to the whole muscle. The basic mechanism thorough which a muscle fibre generates force was first proposed in 1954 as the sliding-filament model (Huxley and Niedergerke, 1954).

The contraction of skeletal muscle is initiated by the propagation of a neural impulse from the central nervous system to a motor neuron which innervates a number of muscle fibres. The neural impulse is propagated down an axon toward the neuromuscular junction. A neural impulse of sufficient magnitude to raise the membrane potential of the surrounding sarcolemma will cause the activation and opening of Na^+/K^+ channels. The influx of Na^+ across the fibre causes depolarisation and propagates an action potential. The outer plasma membrane of muscle fibres, the sarcolemma contains transverse tubules (T-tubules) which run perpendicular to the myofibril surface. These allow the action potential to transmit from the motor end plate across the sarcolemma resulting in depolarisation of the sarcoplasmic reticulum (SR). Depolarisation of the SR initiates the release of Ca^{2+}

though voltage-gated calcium channels into the cytosol. Calcium ions bind to troponin located on the thin actin filaments of the myofibrils. At rest, tropomyosin acts as a blocker between the myosin binding sites on the thin actin filaments. The binding of Ca^{2+} to troponin causes tropomyosin to alter its configuration (via allosteric modification) freeing up the myosin binding sites. The myosin heads are then free to bind to the binding sites on the thin actin filaments forming a cross bridge. ATP bound to the myosin head is utilised to produce ADP and inorganic phosphate (Pi) as the actin and myosin filaments slide past each other. This draws the Z-bands towards each other thus shortening the sarcomere and the I-band. The actin and myosin filaments therefore remain the same length throughout the contraction. The process of muscular contraction through the re-binding of myosin to its binding site can be repeated given there is a constant supply ATP and Ca^{2+} is still bound to the thin actin filaments through the presence of a neural impulse. When calcium ceases to be bind to troponin on the thin filament, tropomyosin is modified back to its previous state thus blocking the binding sites of myosin again. Myosin is unable to bind to the thin actin filament, and the contractions cease (Mougios, 2006).

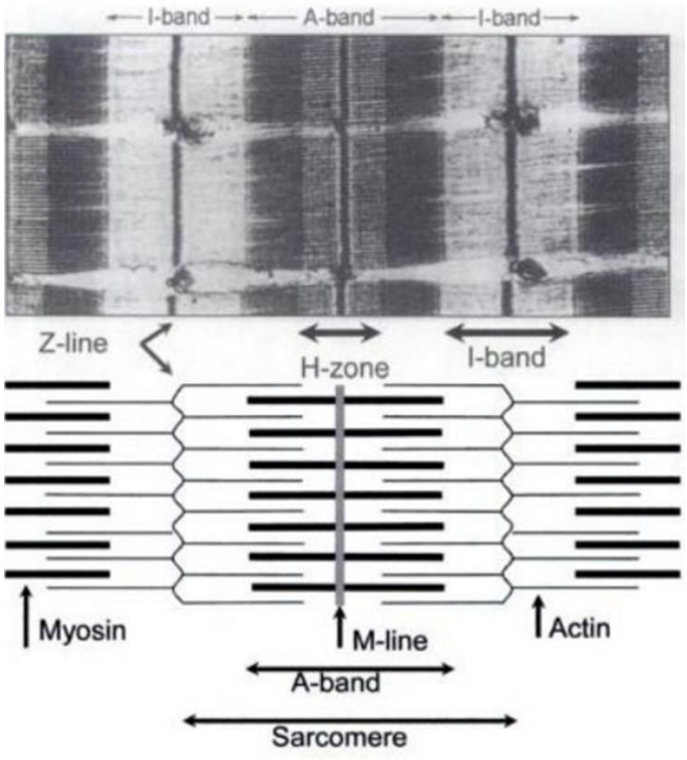


Figure 1.2 A longitudinal electron micrograph of several myofibrils (top) and a schematic representation of the overlapping arrangement between thick (myosin)

and thin (actin) filaments responsible for the characteristic striated appearance. Taken from (Macintosh et al., 2006).

1.2 The pathophysiology of fatigue

Fatigue is a common aspect of life, which affects the majority of individuals to varying degrees. The word "fatigue" can be used to describe the real or perceived reduction in physical or mental capability and performance (Macintosh et al., 2006). It can therefore be divided into either physiological and psychological fatigue or a combination of the two. The presence of fatigue can be the consequence of altered central or peripheral functioning in the body (Davis and Walsh, 2010). Despite extensive research having been undertaken, the exact aetiology of fatigue is often unclear. This is due, at least in-part, to the difficulty in making objective measurements in relation to everyday activity. Some of the more common causes of fatigue within the general population include inadequate sleep, physical and mental exertion, poor diet/nutritional deficiency, anaemia and altered thyroid function (Hublin et al., 1996, Hardy et al., 1997, Huskisson et al., 2007, Bager, 2014).

The extent to which fatigue impacts upon a healthy individual is generally of little significance and may be resolved through rest or cessation of fatigue-inducing activity. However, for a growing number of individuals, fatigue is becoming a more frequent problem. According to international studies, the prevalence of fatigue-related complaints ranged from 10-30% of patients visiting their general practitioners and within the wider population, the proportion of individuals reporting symptoms of fatigue rose to between 30-50% (Cullen et al., 2002, van't Leven et al., 2010). The overall incidence of all fatigue diagnoses made in the UK between 1990 and 2001 decreased by 44% taking the previous number of diagnoses from 87 per 100,000 patients to 49 (Gallagher et al., 2004). Other forms of fatigue related disease such as CFS and fibromyalgia have increased in their incidence (Table 1.2).

The occurrence of fatigue in the general population remains relatively unknown. At present the concept of "fatigue" holds no clear or widely accepted definition within scientific literature. This is largely due to the vast number of problems which arise when trying to study such a multidimensional concept. This is largely a result of the varying use of definitions and diagnosis for patients. Most studies investigating fatigue and chronic fatigue are also based on small and pre-selected samples of

patients giving rise to sample bias. The majority of analyses undertaken on muscle, in the context of muscle fatigue, are carried out within a laboratory setting.

Fatigue can affect individuals of all ages, race and sex. Numerous reports have suggested specific age groups may have an increased susceptibility to fatigue, however data between studies is inconclusive. The main consensus indicates fatigue is low in the childhood population below 11 years of age (Farmer et al., 2004). Despite being lower than adult figures, the prevalence of fatigue is still high amongst the adolescent population according to various epidemiological studies. Female adolescents were reportedly more affected by fatigue with a 3:1 gender ratio of fatigued girls to boys (ter Wolbeek et al., 2006).

Fatigue can present itself in people, with no defined somatic disease has been established. Prevalence rates may vary as a result of differing views of fatigue between health professionals (Hamilton et al., 2010). Symptoms of fatigue recorded can range between tiredness, weakness or exhaustion. As prevalence and severity increases, the importance of acknowledging fatigue as a serious ailment increases. Knowledge behind the mechanisms of fatigue and potential therapies to reduce it need to improve. This cannot be accomplished without first outlining a clear definition of fatigue. Individual research groups define fatigue relative to the setting in which it is being used to avoid confusion. This has led to inconsistencies and comparability issues with other papers. The work detailed in this thesis, focuses on muscle fatigue which is defined as an inability to maintain prescribed muscle force output and define it according.

1.2.1 Psychological fatigue

Psychological (mental) fatigue is a subjective concept. Common symptoms include somnolence, lethargy, reduced alertness, depression and impaired cognitive functioning. The majority of which cannot be objectively assessed in a clinical setting. Currently no quantifiable measures have been defined for psychological fatigue. Differentiating between psychological fatigue and other morbidities such as stress and depression can be difficult. As well as being an entity in itself, psychological fatigue is also an attributing factor for voluntary physical actions and can increase voluntary muscular fatigue (Marcora et al., 2009).

1.2.2 Muscle fatigue

Despite being extensively investigated; the cause of muscle fatigue is still relatively unclear in many situations. Muscular fatigue is generally a consequence of increased muscular activity; however, muscle fatigue can also be reported when little muscular activity has been undertaken. This may be triggered by psychological factors associated with perceived effort and the emotional response to activity (Marcora et al., 2009). The most common general description of muscular fatigue in scientific literature is the reversible decline of performance during activity" (Allen et al., 2008). A decrease in physical performance due to a failure to maintain the requested or expected power output may be due to a rise in real or perceived difficulty. In this thesis, muscular fatigue will be focused on the following definition; "failure to maintain the required or expected force" (Edwards, 1981). Muscular fatigue is a tangible concept and may be recognised more objectively through biological markers (Finsterer, 2012). This may be driven by the peripheral or central nervous system. Muscular fatigue is a reversible sensation. The rate of fatigue depends on the muscles employed and the fibre type composition of the muscle, the relative intensity of the exercise, and the type of muscle contraction whether it be intermittent or continuous (Macintosh et al., 2006). The fatigue profile of a muscle during exercise is influenced by the following factors; muscle fibre composition, neuromuscular characteristics, energy metabolite stores, buffering capacity, ionic regulation, capillarisation, and mitochondrial density (Costill et al., 1976, Amann and Calbet, 2008, McKenna et al., 2008). Figure 1.3 shows the decline in force of the quadriceps during sustained contraction as the muscle fatigues. The exact cause of skeletal muscle fatigue if often unknown and/or cannot be determined. This is due to the complexity of muscle contraction and the potential involvement of multiple sites of failure.

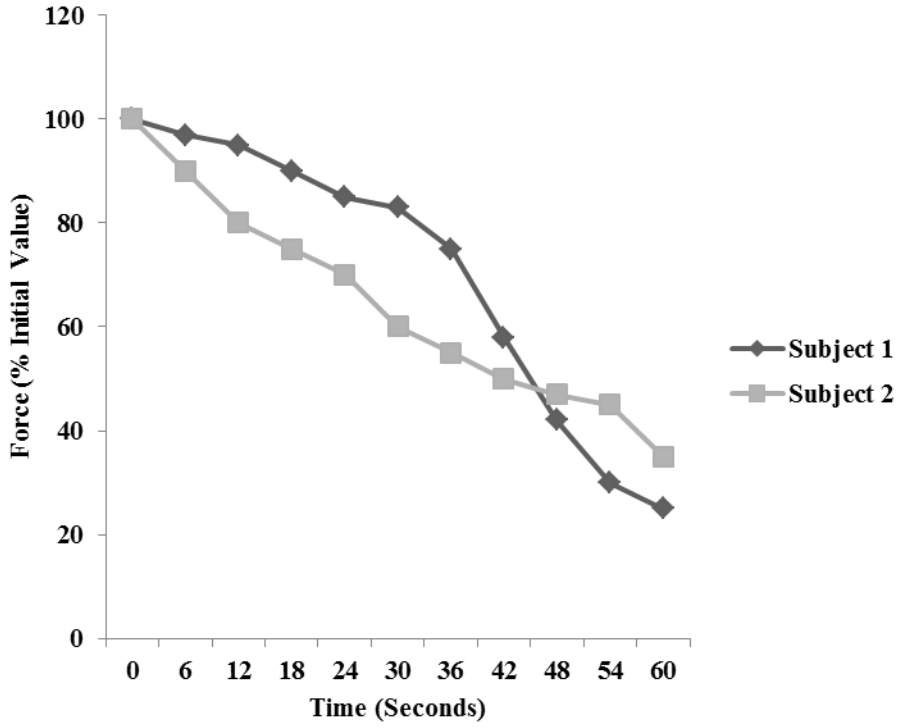


Figure 1.3 Loss of force during uninterrupted maximum voluntary contractions (MVC) of the quadriceps from two individuals. Subjects were asked to hold a MVC for 1 min. Force is expressed as a percentage of the initial value. (Adapted from Jones et al, 2009).

1.2.2.1 Mechanisms underlying skeletal muscle fatigue

Muscle contraction is a complex process under the voluntary control of the brain (Section 1.1). Figure 1.4 illustrates the chain of command leading to muscle contraction and force generation. A break in the chain at any point will reduce or prevent force production culminating in muscle fatigue. At the point marked on Figure 1.4 “electrical stimulation”, failures prior to this point suggests centrally driven fatigue whilst failures after this point are described as peripheral driven fatigue. In general fatigue is not caused by one single entity but rather it is the consequence of multiple factor occurring independently at numerous locations simultaneously in both the central and peripheral nervous system (Enoka and Stuart, 1992).

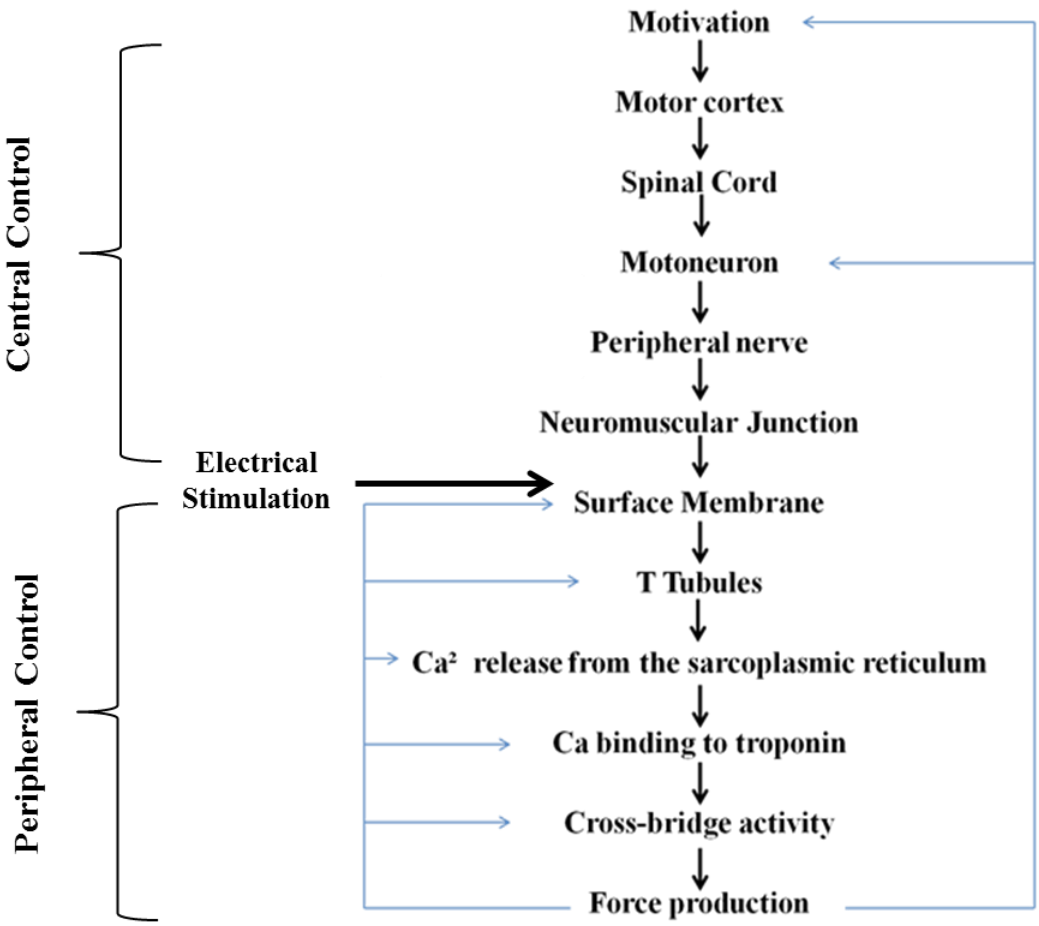


Figure 1.4 Schematic representation of the chain of command in muscle contraction leading from motivation, to the generation of force by cross bridges. Adapted from (Jones et al., 2004).

In this thesis, fatigue is defined by the most common general definition of muscular fatigue in scientific literature as "failure to maintain the required or expected force". Mechanisms behind a decrease in maximal contractile force include the unavailability of metabolic substrates, the accumulation of toxic products, impaired neuromuscular transmission, compromised calcium uptake and release and dysfunction in the actin-myosin cross-bridges (Allen et al., 2008). A failure of appropriate energy supply to muscles is a fundamental cause of fatigue and metabolic changes associated with energy supply have been associated with muscle fatigue including a reduction in pH (6-8), an increased concentration of inorganic phosphate (Pi), and modified levels of Reactive Oxygen Species (ROS) and cytokine molecules (Reid, 2001, Westerblad et al., 2002, Debold et al., 2008, Visser et al., 2002, Davis and Bailey, 1997).

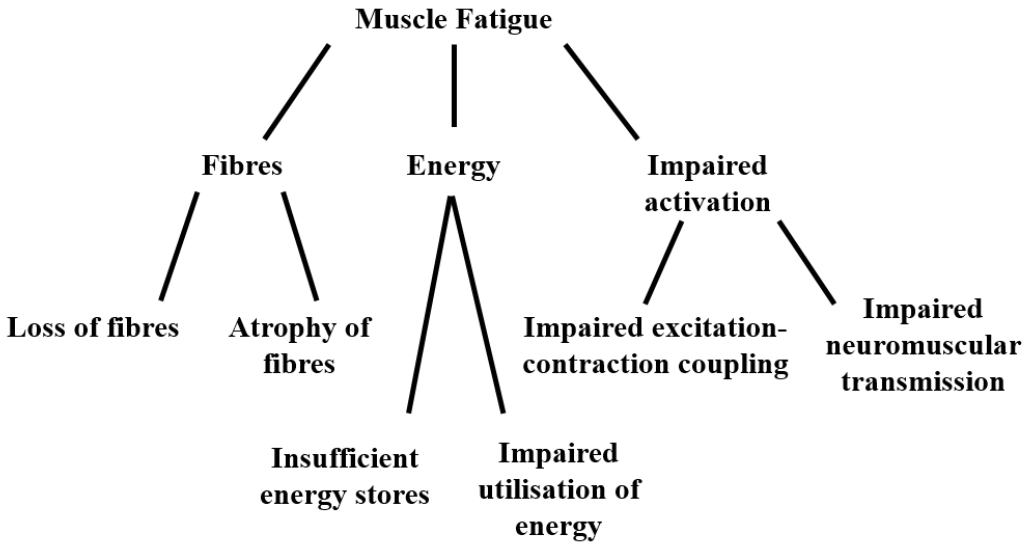


Figure 1.5 Practical scheme illustrating the contributing factors of muscle fatigue. Adapted from (Edwards, 1978).

1.3 Cytokines

Cytokines make up a large and diverse family of polypeptides, protein and glycoprotein molecules within the body. The term cytokine is derived from the Greek word *cyto* meaning cell and *kinos* meaning movement thus implying their role as cell signalling molecules which travel throughout the body (Murphy, 2011). Cytokines are small intracellular signalling molecules which have the ability to mediate a wide range of aspects of cell function throughout the body including eliciting cellular proliferation/differentiation.

The term cytokine encompasses interferons, the interleukins, the chemokine family, mesenchymal growth factors, the tumor necrosis factor family and adipokines. Cytokines were originally, thought to act only within the innate immune system acting to defend the host and aid recovery by eliciting flu-like symptoms. However, it is now clear that cytokines mediate an array of diverse effects in normal physiology and disease (Kelso, 1998, Dinarello, 2007).

Cytokines are produced by multiple cell types both within and outside of the immune system in response to various stimuli. Cytokines producing immune cells include monocytes, macrophages, B and T lymphocytes and NK (natural killer) cells (Cooper et al., 2001). Non-immune cells which have been identified as

cytokine producing cells include adipose, endothelial, fibroblast and muscle cells (Sironi et al., 1989, Pedersen, 2011, Apte, 1995, Hotamisligil et al., 1993). The majority of cytokines are produced from monocytes, macrophages and lymphocytes, in addition to microglia and astrocytes.

1.3.1 Impact of cytokines on skeletal muscle

Cytokines are known mediators of muscle function and physiology (Spate and Schulze, 2004). Low levels of both pro-inflammatory cytokines and anti-inflammatory cytokines are essential for muscle repair and maintenance (Arnold et al., 2007). Determining a physiological level at which cytokines may potentially be detrimental to muscular health is yet to be defined. The overall effect of cytokine levels is dependent on the type of cytokine produced, the source of the cytokine (local vs systemic), the availability of the cytokine to bind receptors, the expression of appropriate receptors, the length of exposure (chronic vs acute) and the response of the target cell. Raised levels of circulating cytokines are common in conditions including chronic obstructive pulmonary disease (COPD), chronic heart failure, cancer and sepsis (Tisdale, 1999, Bolton, 2000, Gan et al., 2004). Muscular abnormalities have been reported in the advanced stages of these conditions including altered muscle morphology, atrophy, and metabolism (Drexler et al., 1992, Anker et al., 1997).

A widely studied area of altered cytokine profiles is the effect on muscle mass. Cytokines have a profound role in the loss of muscle mass in many disease states (Fearon et al., 2006, Anker and Coats, 1998, Wagner, 2008) through activation of the ubiquitin proteasome pathway skeletal muscle and muscle atrophy (Li et al., 1998). Muscle atrophy is a debilitating consequence of several disease including advanced cancer, sepsis and diabetes. Muscle atrophy results from an imbalance between the rates of protein synthesis and degradation. Elevated levels of circulating pro-inflammatory cytokines activate muscle atrophy through elevated production of free radicals and activation of proteolysis through the ubiquitin proteasome pathway (Strassmann et al., 1992, Acharyya et al., 2004, Lang et al., 2007). This is characterised by a reduction in protein synthesis and an increase in protein degradation. This subsequently leads to weight loss, muscle wasting and a

reduction in muscle function as commonly reported in these patients (Lightfoot et al., 2009, Remels et al., 2013). Loss of muscle mass can lead to muscle weakness and potentially precipitate to impaired mobility. Loss of fibres and fibre atrophy is also accompanied by a depletion in mitochondrial oxidative enzymes leading to uncoupling of oxidative phosphorylation and reduced aerobic capacity. Reports have demonstrated a reduction in muscle mass and function is associated with increased morbidity and mortality rates (Evans, 2010).

Cytokines have been identified as potential modulators of contractile activity and force production independently of signalling pathways in which fibre morphology is altered (fibre atrophy and loss of fibres). Research has identified TNF-α's actions through binding to the TNFR1 receptor causes an increase in cytosolic antioxidant activity which can lead to a depression in force production. Increased oxidant levels have been shown to depress force through a reduction in myofibrillar function (Reid and Moylan, 2011).

1.3.1.1 TNF-α

Tumour necrosis factor–α (TNF-α) is one of the most widely researched cytokines in the context of muscle physiology. TNF-α is involved in regulating numerous diverse bodily functions including inflammation, autoimmunity, septic shock cell growth modulation, viral replication, and tumour genesis (Aggarwal et al., 2012). TNF-α plays a major role in a range of myo-pathologies, through its contribution to growth stimulation and inhibition as well as playing a major role in the immune response. Originally named cachectin due its first identified role as a stimulant of cachexia (Beutler et al., 1985), TNF-α is an acute phase pro-inflammatory protein secreted by macrophages in response to a wide range of immunological stimuli; these include other cytokines, bacteria (lipopolysaccharides), viruses and parasites (Janeway et al., 2001). The TNF-α gene is encoded on chromosome 6 in humans (Nedwin et al., 1985). TNF-α is primarily produced as a trans-membrane protein from which the soluble homotrimeric form of TNF-α is produced through proteolytic cleavage (Wajant et al., 2003). TNF-α functions as a trimer when bound to its receptor. Despite having the ability to bind to approximately 25 different receptors in the body (Idriss and Naismith, 2000), TNF-α has been shown to largely

signal through two distinct cell surface receptors, termed TNFR-1 and TNFR-2 (Smith et al., 1994). TNFR-1 is the most prevalent receptor in the body, present in nearly all cells in the body and extensively throughout the lymphoid system and skeletal muscle (Loetscher et al., 1990). Consequently, the majority of biological functions of TNF occur via is TNFR-1 (Chen and Goeddel, 2002). TNFR-1 can be fully activated by both the membrane-bound and soluble trimeric forms of TNF. TNFR2 is not as widely expressed to the same extent as TNFR1. TNFR2 is mainly found in immune cells including certain populations of lymphocytes, including T-regulatory cells (Ware et al., 1991), endothelial cells, microglia, neuron subtypes, oligodendrocytes cardiac myocytes, and human mesenchymal stem cells (Faustman and Davis, 2013). TNFR2 can be activated by the membrane-bound form of the TNF homotrimer.

TNF-α is proposed to be a mediator of contractile dysfunction which can occur dependently or independently of changes in protein turnover and muscle mass TNF-α induced muscle atrophy can occur through the impairment of muscle cell differentiation and can therefore result in muscle weakness (Langen et al., 2002). This commonly leads to a reduction in the capability of an individual to carry out everyday tasks. Experimental evidence shows that muscle-derived oxidants are essential mediators of TNF/TNFR1-induced muscle weakness/impaired muscle contraction. Intervention studies have revealed, the presence of ROS scavengers such as the SS-31 peptide reduce oxidant levels which in turn reduces contractile dysfunction and/or muscle atrophy. (Murrant and Reid, 2001, Reid, 2001, Li et al., 2005, Hardin et al., 2008, Reid and Moylan, 2011). Muscle weakness and impaired contractile function is also proposed to be a downstream consequence of muscle atrophy (Li et al., 2005). However, there is a growing body of evidence associating the actions of inflammatory molecules such as cytokines with impaired muscle function, fatigue and weakness separately to muscle atrophy through increased production and signalling of reactive oxygen species (ROS) (Reid and Moylan, 2011). Altered levels of pro and anti-inflammatory cytokines can impact upon both voluntary force production suggesting a potential effect on the central pathways leading to muscle contraction (McNicol et al., 2010). Given the identification of a wide range of cytokines directly expressed and secreted from skeletal muscle, the effect of both local and systemic levels of circulating cytokines need to be

considered when assessing the relationship between muscle function and cytokines (Wessely et al., 1997).

TNF-α is the most widely studied pro-inflammatory cytokine associated with muscle physiology. Increased levels of pro-inflammatory cytokine TNF-α have been associated with muscle atrophy and wasting in human and animal studies (Reid et al., 2002). Direct administration of TNF-α to mice was shown to increase muscle atrophy and reduce force contraction (Hardin et al., 2008).

1.3.1.2 IL-6

IL-6 is one of the most widely studied cytokines in inflammatory/autoimmune disease (Jones et al., 2011). Il-6 is elevated in a large number of inflammatory states and is a recognised target for therapeutic interventions e.g. the use of tocilizumab in Rheumatoid Arthritis (Maini et al., 2006). IL-6 is predominantly regarded as a pro-inflammatory cytokine although it has also been associated with anti-inflammatory activities and regeneration (Starkie et al., 2003). Reports show a significant increase in circulating levels of IL-6 following periods of acute exercise; a process which occurs independently of induced muscle cell damage (Ostrowski et al., 1998). Evidence suggests that this surge in IL-6 following exercise may play an anti-inflammatory role, as it can promote the inhibition of TNF-α production (Pedersen et al., 2003). IL-6 exerts its effects through the receptor complex IL-6 binding type I transmembrane glycoprotein termed IL-6R (Scheller et al., 2011). The effect of elevated "pro-inflammatory" cytokines such as IL-6 on skeletal muscle have generally been viewed as adverse however this is dependent on the time of exposure i.e acute vs chronic. IL-6 has been directly and indirectly associated with deleterious effects such as muscle catabolism and sarcopenia (Haddad et al., 2005), however, this is dependent on receptor binding and local/systemic levels. The presence of IL-6 within circulation is complex given the different effects locally or systemically and depending of the time of exposure.

1.3.1.3 CCL and CXCL family

Within the chemokine family are two major subtypes, otherwise known as the CC and CXC chemokines. CC and CXCL chemokines are typically small peptides, with the primary function of initiating inter-cellular chemotaxis. The relationship between circulating levels of CCL and CXCL chemokines and muscle physiology is poorly understood.

1.3.1.4 CCL2/MCP-1

CCL2, also referred to as monocyte chemoattractant protein-1 (MCP-1), is a member of the CC chemokine family. These small peptides are potent chemotactic factors for monocytes. CCL2 is produced by a number of different cell types, either constitutively or through stimuli such as oxidative stress, cytokines, or growth factors (Deshmane et al., 2009). CCL2 has been reported to play an integral role in muscle regeneration following injury (Warren et al., 2005). Further evidence showing cultured myoblasts (C2C12) exposed to CCL2 exhibit increased myoblast proliferative responses supports the role of CCL2 in muscle repair (Yahiaoui et al., 2008).

1.3.1.5 CCL5/RANTES

CCL5, also known as RANTES (regulated on activation, normal T cell expressed and secreted) acts as a potent chemoattractant for many cell types. CCL5 was originally identified as a T cell-specific chemokine, however it is now known to be expressed by a number of other cell types including epithelial cells, platelets, monocytes, NK cells and eosinophils (Crawford et al., 2011). CCL5 plays an important role in homing and migration of effector and memory T cells during acute infections. There is little evidence published on the role of CCL5 in the functioning and physiology of muscle. Reports have suggested a potential effect of CCL5 in the suppression of muscle regeneration (Kohno et al., 2011).

1.3.1.6 CXCL1/KC

The chemokine, keratinocyte chemoattractant (KC), also known as CXCL1 is a potent neutrophil chemoattractant. Keratinocytes, vascular endothelial cells, monocytes, and macrophages produce CXCL1 in potentially high levels in response

to a variety of endogenous stimuli, including platelet-derived growth factor, colony stimulating factor-1, TNF-α and microbial stimuli (Shea-Donohue et al., 2008). Relatively little is known about the regulation of CXCL1/KC expression or its role in inflammation. Whether elevated levels of CXCL1 play a role in the induction protective or pathologic response pathways is yet to be fully elucidated. CXCL1/KC has been observed to be strongly upregulated in a variety of *in vitro* and *in vivo* systems (Lira et al., 1994, Roche et al., 2007).

1.3.2 Muscle derived cytokines (myokines)

Muscle acts as a secretory organ producing an array of peptides including cytokines. IL-6 was the first myokine to be identified by (Ostrowski et al., 1998). The group demonstrated the ability of muscle to produce and secrete peptides/proteins into circulation and the endocrine effects of these molecules. The authors termed these secreted molecules "myokines" (Pedersen et al., 1998).

Muscle cells have the ability to synthesise the cytokines (myokines) IL-6, CCL5/RANTES, MCP-1/CCL2, KC/CXCL1 and secrete them into the surrounding milieu (Lightfoot et al., 2015). Skeletal muscle is now a recognised source of an array of cytokines in the body (Peake et al., 2015). Research has shown that resting healthy human muscles express cytokines in a fibre type specific manner (Plomgaard et al., 2005). The extent to which myokine production can impact upon circulating cytokine levels and muscle physiology is not yet fully understood. The impact of myokines may potentially be greater locally given the higher concentration at which they are present. The most widely characterised myokine is IL-6. Levels of IL-6 in the blood have been shown to increase in response to muscle contraction (Ostrowski et al., 1998).

Skeletal muscle constitutes a large proportion of the total body weight and protein content. An increase in myokine release has the potential to result in a major change in the overall concentration of systemic cytokines. Assessment of local production and systemic cytokine levels would aid researchers in determining the impact of myokines on circulating cytokine levels.

1.3.3 Cytokine induced NFκB activation

Nuclear factor kappa B (Nf-κB) is a complex of transcription factors (Li and Verma, 2002) ubiquitously expressed throughout the body in almost all cell types and tissues. The Nf-κB complex encompasses 5 related transcription factor subunits; p50, p52, RelA (p65), c-Rel and RelB (Moynagh, 2005, Hayden and Ghosh, 2011). A range of stimuli can activate NF-κB, including ROS (Reactive Oxygen Species), viral and bacterial antigens and cytokines including TNF-α (Ashall et al., 2009). NF-κB family members play an important role as a major regulator of inflammatory responses within innate and adaptive immunity (Vallabhapurapu and Karin, 2009, Hayden and Ghosh, 2008). Research initially focussed on NF-κB activation in the immune system however research interest has grown in other types including muscle. The activation of NF-κB in muscle has been associated with age-related sarcopenia, muscle weakness, muscle atrophy in diseases including heart disease, AIDS and cancer and inflammatory myopathies (Reid, 2001, Reid and Moylan, 2011, Jackman et al., 2013, Huang et al., 2014, Guttridge et al., 2000). NF-κB activation has been strongly linked to the effects exerted by TNF-α upon skeletal muscle dysfunction and wasting. The actions of TNF-α in stimulating muscle atrophy through a loss of muscle protein, reduction in fibre diameter and contractile dysfunction are dependent on Nf-κB activation and are mediated by ROS generation and the activation of redox-sensitive transcription factors (Jackson et al., 2002, Reid and Moylan, 2011, Morgan and Liu, 2011).

1.4 Reactive oxygen species produced by skeletal muscle

1.4.1 Reactive oxygen and nitrogen species

Free radicals are molecules containing one or more unpaired electrons which have the capability to exist independently (Radak, 2000). Molecules with unpaired electron are very unstable and reactive. The most widely studied include hydroxyl, superoxide, nitric oxide (NO) and peroxyl radical. The term reactive oxygen species (ROS) is a general term which encompasses both free radical (species which contain one or more unpaired electrons) and non-free radical species of molecular oxygen derived molecules. These include peroxynitrite, hypochlorous acid, hydrogen peroxide (H_2O_2), singlet oxygen, and ozone (O_3) which are not free radicals but are highly reactive oxidising agents and can easily lead to free radical reactions in living organisms (Halliwell, 2006). These molecules are highly reactive and are capable of oxidising many biological targets including protein, lipid and DNA. Similarly, the term RNS collectively describes free radical and non-free radical species in which nitrogen is at the reactive centre. Excessive levels of RONS as a result of increased production and/or a reduction in antioxidant defence systems and can lead to oxidative damage and cellular death. This can subsequently lead to impaired physiological functioning and contribute to the pathology of many chronic diseases (Zelko et al., 2002).

1.4.2 Skeletal muscle produces reactive oxygen species

It is now widely recognised that skeletal muscle is a constant source of ROS and RNS (collectively termed RONS) both at rest and during contractions (Barbieri and Sestili, 2012). Some of the first major studies to report ROS accumulation and potential effects of RONS in contracting muscle were published in the late 1970s and 1980s. Early studies were restricted by limitations in analytical techniques of RONS detection and focused on the quantification of oxidative damage as a marker of RONS levels. Since these early findings, research has advanced significantly in confirming the continual production of RONS from skeletal muscle cells and the importance of RONS in skeletal muscle maintenance and functioning. Skeletal muscle is known to produce a range of RONS. More recently, research has focused on identifying the multiple sources of RONS, factors affecting their production and

the role RONS plays in skeletal muscle physiology and function. RONS are produced from a number of sub-cellular locations in muscle.

RONS have been identified as essential components of normal muscle physiology and function (Westerblad and Allen, 2011) which play multiple regulatory roles including mediating cell signalling pathways, the regulation of gene expression through the activation of redox sensitive pathways and the modulation of force production. These molecules are produced during basal metabolic conditions and are required at low concentrations for many metabolic processes however production can be modulated in response to increased contractile activity, inactivity, foreign pathogens and inflammatory molecules including cytokines. Evidence has shown a link between excess free radical production and the pathogenesis of diseases. RONS production by skeletal muscle is proposed to contribute to disuse muscle atrophy (Powers et al., 2012).

RONS are produced from numerous sub-cellular compartments within skeletal muscle cells including mitochondrion, sarcoplasmic reticulum, transverse tubules, sarcolemma and the cytosol (Jackson, 2011). Original reports first identified mitochondria as the predominant site of cellular RONS production in skeletal muscle (Davies et al., 1982, Koren et al., 1983). RONS are produced during mitochondrial respiration and the formation of ATP. The final stage of this process is oxidative phosphorylation in which molecular oxygen is reduced to water. Secondary products of this process are intermediate ROS which can, in turn, be reduced to more reactive free radical species. More recent studies have identified alternative sources to be the major contributors of RONS. A number of different sources which contribute to RONS production within skeletal muscle have been identified. Non-mitochondrial sources of RONS include nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), xanthine oxidase, NADPH is the major source of RONS (superoxide) in skeletal muscle (Powers et al., 2010, Powers et al., 2011). These sources produce a number of different RONS either at rest and/or during contraction. The primary RONS produced by skeletal muscle are superoxide and NO during rest as well as during contraction (McArdle and Jackson, 2000).

The superoxide anion is an oxygen centred radical, produced through the one electron reduction of molecular oxygen (O_2). Production can occur at multiple locations within the muscle fibre, including the mitochondrion, sarcoplasmic reticulum, transverse tubules, sarcolemma and the cytosol. Superoxide is a comparatively stable ROS and has a relatively long half-life (Valko et al., 2007). Superoxide does not readily react with protein, lips or DNA molecules causing oxidative damage and is generally converted to secondary ROS such as hydroperoxyl radicals (HOO-) or hydrogen peroxide H_2O_2 by reacting with additional molecules, such as MnSOD/CuZnSOD and extracellular SOD. Superoxide conversion can occur directly or through catalysed enzyme or metal linked superoxide dismutase reactions (McCord and Fridovich, 1969).

Nitric oxide (NO) is a primary radical species in which a nitrogen is the central element. Production arises from the action of nitric oxide synthase (NOS) enzymes in converting L-arginine to citrulline. The reaction utilises NADPH as a co-factor (Droge, 2002). There are three isoforms of NOS in humans; these are neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). All three are present in skeletal muscle. nNOS and eNOS are constitutively expressed however iNOS is only expressed intermittently (Hussain et al., 1997). nNOS is localised to the plasma membrane of skeletal muscle fibres. eNOS expression was originally thought to be specific to endothelial cells however it is also expressed in the mitochondria of skeletal muscle cells response though HSP90. The majority of iNOS activity occurs under inflammatory conditions in skeletal muscle (Powers and Jackson, 2008). NO readily diffuses over cells, through the cytoplasm and plasma membranes and is generally unreactive to most biological materials however it does react readily with radical species as well as oxygen and water. NO readily reacts with oxygen to produce nitric dioxide (NO_2) as well as superoxide to produce peroxynitrite (ONNO-) (Halliwell, 1989).

1.4.3 Detecting and quantifying RONS

RONS (particularly free radical species of RONS) are highly reactive species and subsequently have very short half-lives. This makes accurate detection and quantification challenging. RONS have previously only been assessed indirectly in skeletal muscle through end products formed through RONS reacting with proteins, lipids and DNA (such as protein carbonyls, malondialdehyde (MDA) and 8-hydroxy-2'deoxyguaosine respectively). Recent developments in the technology used to detect RONS has enables researchers to not only identify free radical species produced by skeletal muscle but quantify production in real time. Detecting and quantifying RONS in skeletal muscle has been attempted through a number of techniques (Murrant and Reid, 2001). These methods including spectrophotometry, fluorescence, chemiluminescence and electron spin resonance have been developed to assess individual RONS or total levels (He et al., 2014, Dikalov and Harrison, 2014). *In vivo* measurements are preferable over *in vitro* analysis due to the risk of artificially heightened ROS generation during tissue processing and homogenisation (Palomero et al., 2008).

1.4.4 Regulation of RONS in skeletal muscle

RONS play an important role in regulating cell signalling pathways associated with skeletal muscle physiology and the adaptive response of muscle to physical inactivity or activity. The presence of RONS is necessary for the activation of redox sensitive transcription factors associated with regulation of cell signalling pathways associated with skeletal muscle physiology. Excess RONS can be detrimental, causing oxidative damage which may potentially be implicated in the process of ageing (Liochev, 2013) and the cause of many diseases including atherosclerosis, cancer, neural diseases and diabetes (Pham-Huy et al., 2008, Uttara et al., 2009). As a result of the importance in regulating RONS levels within skeletal muscle cells, the body has developed a number of systems in place which maintain redox homeostasis. Muscle fibres contain a network of defence mechanisms which aim to reduce elevated levels of RONS; these include the antioxidant defence systems and synthesis of heat shock proteins (HSPs) which aim to protect cells and aid recovery (Jackson, 2005). Within the current context antioxidants are broadly defined as any substance that delays or prevents the oxidation of a substrate (Valko et al., 2007).

Antioxidant defence systems aim to reduce excessive free RONS thus preventing oxidative damage. These systems can be grouped into enzymatic and non-enzymatic systems. Enzymatic regulators within skeletal muscle which directly aid the scavenging of oxidants includes the superoxide dismutase (CuZnSOD, MnSOD), catalase, thioredoxin reductases, glutathione peroxidases (GPX), peroxiredoxins and glutaredoxins (Halliwell, 2006). The activity level of these enzyme is associated with muscle fibre type. Expression of these enzymes is highest in type one oxidative fibres. Physical activity has been shown to increase the activity of these enzymes (Powers et al., 1994).

1.4.4.1 Superoxide dismutase

The superoxide dismutases (SODs) are part of the main regulatory antioxidant enzymes defence systems in the body. SODs catalyse the conversion of superoxide to hydrogen peroxide through the one electron dismutation (Jackson and McArdle, 2011). SOD is present in two isoforms within skeletal muscle; each is characterised by the metal ion it is bound to (Zelko et al., 2002). The copper-zinc isoform (CuZnSOD or SOD1) is primarily located within the cytosol and the intermembrane space of mitochondria. The manganese isoform (MnSOD or SOD2) is expressed in the mitochondrial matrix of skeletal muscle (Powers and Jackson, 2008). Muscles with a higher proportion of type 1 fibres reportedly have higher levels of SOD activity in their muscles (Radák, 2000). An additional isoform of SOD known as SOD 3 which is CuZn bound exists in within the extracellular fluid of numerous cells and tissues and the interstitial regions of tissue (Mates and Sanchez-Jimenez, 1999). SOD activity was first within the human plasma, lymph, ascites, and cerebrospinal fluids. SOD3 accounts for the majority of SOD activity in the tissues it is expressed in (Marklund et al., 1982).

1.4.4.2 Catalase

Catalase is another major endogenous antioxidant enzyme. Catalase is a heme enzyme (Requires Fe^{3+} at the enzymes active site) which catalyses the breakdown of hydrogen peroxide (H_2O_2) species to water (H_2O) and oxygen (O_2) (Zamocky and Koller, 1999). Catalase is predominantly found in the cytosolic compartment of the muscle fibres. Catalase activity alters in response to H_2O_2 production (Mates

and Sanchez-Jimenez, 1999). An increase in catalase protein expression would be indicative of muscle adaptations to raised H_2O_2 production.

1.4.4.3 Glutathione peroxidase

Glutathione peroxidase (GPX) is the final major antioxidant enzyme within skeletal muscle. It is a homotetramer which contains the element selenium at its active site in the form of selenocysteine. GPX catalyses the reduction of H_2O_2 to water and organic hydroperoxides to alcohols. The reduced form of glutathione (GSH) is utilised by GPX as an electron donor, resulting in the formation of oxidised glutathione disulphide (GSSG). Glutathione reductase acts to regenerate GSH (Brigelius-Flohe, 1999). There are five isoforms of GPX (GPX1-GPX5) in humans. Each isoform occupies a different cellular location.

GPX 1	Thyroid, liver, kidney, and pituitary gland
GPX 2	Thyroid, pituitary gland, central nervous system, brown adipose tissue
GPX 3	Skin, placenta and central nervous system
GPX 4	Expressed in all tissues.
GPX 5	Epididymis

Table 1.2: Expression of glutathione peroxidase (GPX) 1-5 in different tissues. (Brigelius-Flohe, 1999, Arthur, 2000)

1.4.4.4 Peroxiredoxins

Peroxiredoxins (PRXs) are an additional group of enzymes which contribute to cellular antioxidant defence in maintaining RONS homeostasis and preventing excess levels of RONS (Wood et al., 2003). PRXs are capable of reducing hydroperoxide. Six isoforms of PRX (PRX I – VI) are expressed in skeletal muscle. PRX I, II and VI are located in the cytosol, PRX III is in the mitochondria and PRX IV is present in the extracellular space (Powers and Jackson, 2008). PRX V is expressed in numerous cellular areas including the peroxisome, cytosol, mitochondria and nuclei (Rhee et al., 2005).

1.4.4.5 Non enzymatic antioxidants

An additional system of defence exists within the body; this includes non-enzymatic antioxidant systems including glutathione, uric acid, bilirubin and Coenzyme Q_{10} which are endogenously produced within the body. Glutathione is the most abundant non enzymatic anti-oxidant in the body and aids in the protection of cells from damage. Glutathione exists in both an oxidised (GSSG) and reduced (GSH) state. The reduced state (GSH) can scavenge free radicals providing direct protection from reactive oxygen species (ROS). The balance between GSH and GSSG maintains the redox environment of the cell. Alternatively, exogenous antioxidants can be obtained through dietary intake in the form of polyphenols, polyphenolic acids, carotenoids, and vitamins C and E (Roberts et al., 2007, Krinsky, 1998, Pandey and Rizvi, 2009).

1.4.5 RONS mediated cytokine release

Increased generation of RONS by muscle occurs in response to cytokines such as TNF-α (Langen et al., 2002). However, elevated RONS can also act as a stimulant for the production of pro-inflammatory cytokines. RONS activate numerous diverse downstream signalling pathways including mitogen-activated protein kinases (MAPKs). This can lead to the activation of transcription factors which modulate various stages of the inflammatory cascade including the production of IL-6, CCL2, CCL5, CXCL1 (Kosmidou et al., 2002, Lightfoot et al., 2015).

1.4.6 ROS and skeletal muscle function

RONS are continuously generated by skeletal muscle and low-to-moderate levels of oxidants are essential in the physiology of skeletal muscle as they are implicated in the control of gene expression, regulation of cell signalling pathways, and modulation-optimization of skeletal muscle force production (Powers et al., 2011). An excessive increase in RONS production coupled with a decline in antioxidant defence systems may lead to oxidative damage in skeletal muscle cells and potentially physiological dysfunction.

Production of RONS is augmented in skeletal muscle in response to numerous factors including altered inflammatory environment, local cytokine levels as well as contractile function (Jackson, 2005). Intense physical activity increases the productions of ROS (Powers and Jackson, 2008, Lamb and Westerblad, 2011). Endogenous ROS scavenging pathways, such as glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities are substantially up-regulated by exercise training as well as inflammation. Increased production and accumulation of RONS in skeletal muscle cells has been implicated in the mechanisms responsible for muscle fatigue (Allen et al., 2008). RONS have been implicated in process of oxidative damage of cellular proteins, DNA, and lipids and have thus been associated with muscle damage and muscle wasting observed in high intensity dynamic exercise, disuse, and various pathological conditions (Pellegrino et al., 2011).

Physical inactivity is also associated with increased ROS production in skeletal muscle (Powers et al., 2005, Zhang et al., 2007). The role of RONS has been

assessed in models of muscle disuse in which physical activity is greatly reduced and limbs are immobilised. Muscle disuse is characterised by muscle atrophy and reduced force generation. Evidence suggests an increase in radical species and a reduction in cellular defence systems (a reduction in the activity of antioxidant enzymes including catalase and GPX and HSPs) resulting increased oxidative damage contributes to the atrophy (Lawler et al., 2003, Pellegrino et al., 2011, Lawler et al., 2006).

1.5 Modulation of cytokine levels through polyphenols

1.5.1 Polyphenols and phenolic Acids

Polyphenols are a collection of naturally occurring organic chemicals which are found predominantly in plants. Polyphenols are found in a large number of foods and drinks including vegetables, fruits, red wine, and tea (Manach et al., 2004). The term polyphenol denotes the presence of multiple phenolic rings. A phenolic ring is a 6-carbon benzene ring with an attached hydroxyl (OH) group, also referred to as the hydroxyl functional group (Pandey and Rizvi, 2009). Researchers have identified thousands of different natural polyphenols; which are categorised according to the presence of phenolic structural units per molecule and are divided into several groups. The ability of polyphenols to interact with other compounds as well as their antioxidant capability and bioavailability are all derived from their intricate chemical structure (Scalbert and Williamson, 2000). Polyphenols are associated with anti-oxidant and anti-inflammatory effects within the body (Rahman et al., 2006). Increased consumption of dietary polyphenols has been linked with a number of health benefits including reduced risk of cardiovascular disease, cancer and many inflammatory based diseases (Arts et al, 2005, Riboli et al, 2003).

Polyphenols were first identified as exogenous antioxidants which could work within the body to scavenge free radicals; however, the antioxidant properties of these molecules are now thought to be elicited through secondary mechanisms of antioxidant prevention by the activation of signalling pathways associated with internal redox defence mechanisms. The exact mechanisms by which polyphenols

exert their biological effect are still not fully understood. The common action associated with antioxidant activity is hydrogen donation however it is now thought that the cellular effects associated with polyphenol consumption must be via an alternative mechanism.

The absorption of polyphenols and their bioavailability is affected by numerous factors during digestion, modifications by intestinal enzymes, ability to be transported through the gut lining, and metabolism to phenolic acids by the colonic microflora (Manach et al., 2004). Upon ingestion and during the process of digestion and absorption, polyphenols are often altered considerably. The metabolism of these substances naturally results in structural changes and a change in antioxidant potential.

1.5.2 Resveratrol

Resveratrol (trans-3,4',5-trihydroxystilbene) is a non-flavonoid polyphenolic compound with natural antioxidant properties. It is one of the most widely studied phenolic acids and has been associated with numerous health benefits including anti-inflammatory and anti-cancerous activities (King et al., 2006, Baur and Sinclair, 2006). Commonly found in raw foods such as red grapes, peanuts, cocoa, resveratrol is transferred during processing to the subsequent by-products; red wine, chocolate, peanut butter etc. Resveratrol is part of the stilbene subclass of non-flavonoid polyphenolics. It is a phytoalexin produced by higher plants in response to environmental stresses such as infection, nutrient deprivation and UV radiation. Two isomeric forms of resveratrol exist naturally. A free form in *cis* or *trans* configuration as well as a β-glucoconjugated form (Gulcin et al, 2010).

Resveratrol was first identified through epidemiological studies; a strong inverse relationship between diets high in resveratrol such as the Mediterranean Diet are associated with a significantly reduced risk of diseases such as cardiovascular disease, metabolic syndrome and cancer (Keys et al., 1986). Wine consumption was also inversely related to coronary disease associated mortality despite a high intake of saturate fat. This was first described by St Leger et al as the “French Paradox”. This led many researchers to investigate the properties of resveratrol further. The

majority of results shows beneficial effects and identified key pathways and targets of Resveratrol. *In vivo* studies have been less conclusive. The concentration of resveratrol used in *in vitro* studies was considerably higher than levels recorded plasma levels. Resveratrol has a low bioavailability and would therefore enter the systemic blood stream at considerably lower concentrations than those used in most resveratrol cell culture models. Due to the high number of hydroxyl groups on polyphenol compounds, polyphenols including resveratrol are broken down rapidly in the body to smaller metabolites and do not enter circulation.

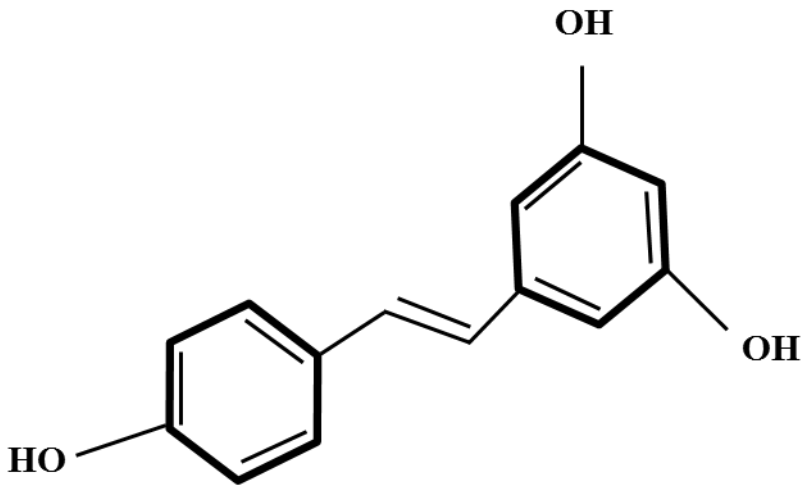


Figure 1.6 Basic structure of resveratrol.

1.5.3 Curcumin

Curcumin (diferferuloylmethane) is a natural diphenylheptanoid polyphenol found within the tropical spice Turmeric (*Curcuma Ionga* rhizomes) commonly cultivated in Southeast Asia. Curcumin is the primary active constituent responsible for Turmeric's vibrant yellow colour. Curcumin was first isolated almost two centuries ago, and its structure as diferuloylmethane was determined in 1910.

Curcumin has been shown to suppress the release of cytokines IL1β, IL8, TNFα, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) from PMA (*para*-Methoxyamphetamine) or LPS (lipopolysaccharide) stimulated peripheral blood monocytes (Abe et al., 1999). Similar studies have reported a reduction in the release of cytokines IL-1β and PMA-induced IL-6 expression from MH7A cells (a human synovial fibroblast cell

line) and RA-FLS, (fibroblast-like synoviocytes (FLS) derived from patients with rheumatoid arthritis (RA). Evidence suggests the mechanism through which curcumin may be mediating cytokine secretion maybe through the inhibition of transcriptional factor NF-kB (Singh and Aggarwal, 1995) (Kloesch et al., 2013).

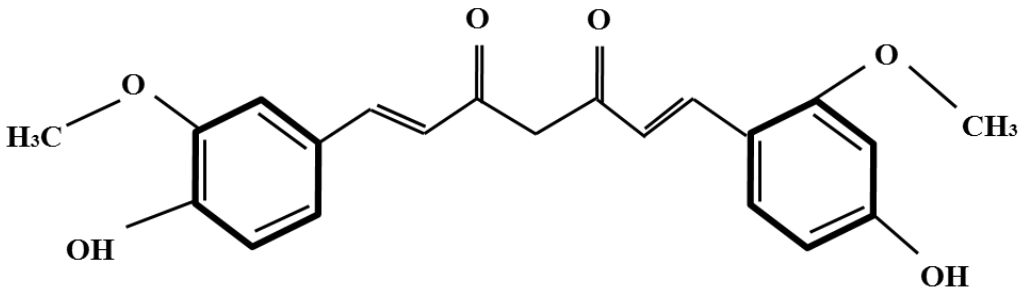


Figure 1.7 Basic structure of curcumin.

1.5.4 EGCG

Epigallocatechin-3-gallate (EGCG), the most abundant catechin component in green tea. It is the major polyphenolic constituent found in green tea (Bettuzzi et al., 2006; Demeule et al., 2002). More than 50% of the mass of this catechin combination is composed of EGCG and a vast body of scientific research suggests that EGCG (and other catechins) is responsible for the majority of the potential health benefits attributed to green tea consumption (Nagle et al., 2006). Further research has identified potent antioxidant, chemopreventive and anti-tumour activity from EGCG (Tipoe et al, 2007). The effect of EGCG exposure on cytokine release has been investigated in a number of cell lines (with the exception of skeletal muscle) providing mixed results.

Treatment of corneal epithelial cells (HCEpiC) with EGCG reduced IL-1β induced cytokine release of Il-6, IL-8, GM-CSF and MCP-1 in a dose dependent manner (3-30μM) (Cavet et al, 2011). Primary white blood cells (WBC) stimulated with the T-cell mitogen concanavalin A followed by EGCG treatment showed reduced gene expression of TNF-α, however there was no effect on the expression pattern of IL-6 and IL-1β (Sehm et al, 2005). EGCG inhibited PMA (phorbol 12-myristate 13-

acetate) induced TNF-α, IL-6 and IL-8 expression and production in human mast cell line (HMC-1) (Shin et al, 2007).

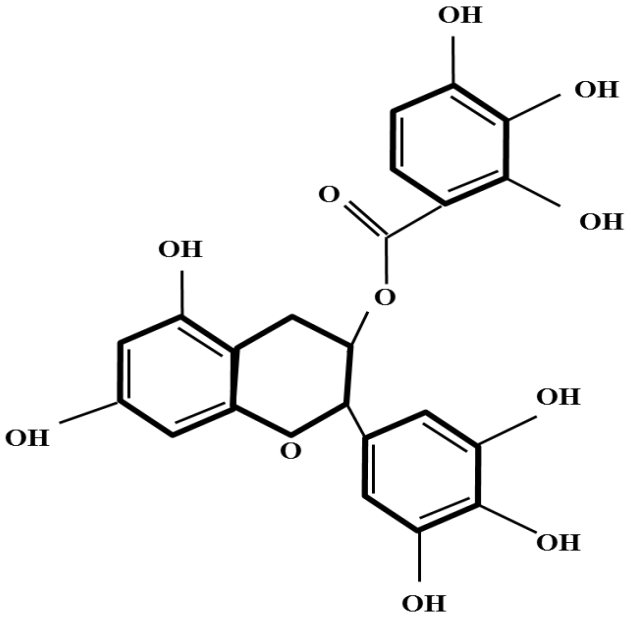


Figure 1.8 Basic structure of EGCG.

1.6 Chronic Fatigue Syndrome as a complex model of fatigue

1.6.1 The pathophysiology of chronic fatigue syndrome

Chronic fatigue syndrome (CFS) as it has been known in the UK since the 1950s, is a clinically accepted condition now referred to in Britain (Hutchinson et al., 2002). Historically, the earliest mention of CFS dates back to the 19th century under the names neurasthenia, neauromyasthenia, and Beard's disease, in which patients described symptoms of extreme fatigue, headache, and irritability (Beard, 1989). CFS also referred to as or Myalgic Encephalopathy (ME), Myalgic Encephalomyelitis (ME), Iceland disease, Effort Syndrome, post-viral fatigue syndrome (PVFS) and chronic fatigue immune dysfunction syndrome (CFIDS) (White and Burtch, 1954, Lindan, 1956, Stokes et al., 1988, Defreitas et al., 1991) is a severely debilitating disease characterised by extreme and disabling fatigue. Worldwide prevalence of CFS is unknown due to inconsistencies in epidemiological data on CFS. This may be a consequence of the changing case-definitions and titles for the condition between different populations. Two US community-based surveys, found the prevalence of 0.23% and 0.42% (Jason et al., 1999, Reyes et al., 2003), whereas a British primary care study, using the same case-definition, found a prevalence of 2.6% (Wessely et al., 1997). This is contrary to two more recent US and UK studies which found prevalence at 0.4–1% and 0.2-0.5% respectively (Devanur et al, 2006, (Nacul et al., 2011). Prevalence in the UK and United States has been estimated over 240,000 and 800,000 patients respectively. Figures are often inconsistent, due to a lack of understanding of the disease and imprecise diagnosis methodology. Despite minor changes in diagnostic criteria, the incidence of patients entering NHS patients care for CFS is rising (Buchwald et al., 1995). Table 1.3 reveals completed triaged referrals from the Royal Liverpool and Broadgreen University Hospital Trust CFS Services. These figures support previous evidence of an increase in referrals of CFS. This is likely to reflect the changing attitude of medical staff towards fatigue and CFS and perception within the public.

CFS affects both male and female individuals of all ages. Evidence suggests that CFS is more prevalent in adults and females (Jason et al., 1999); (Reyes et al., 2003). Research from Reyes and Colleagues (2003) revealed that the mean age of

sufferers with CFS within their study cohort of 3528 individuals was 47 years. Eighty percent of individuals within the cohort were women. There are discrepancies in the literature regarding a specific age group most affect or pre-disposed to the disease. Adults suffering from general fatigue are believed to hold the same risk of developing CFS as adults (Farmer et al., 2004). The Centres for Disease Control and Prevention (CDC) in America has reported the highest incidence of CFS in individuals aged between 40 – 50 years (CDC, 2013). A study by Dowsett et al (1990) reported the majority of individuals within their cohort (75%) developed CFS earlier on in life between adolescence and their mid-forties of which the ratio of women to men was 3:1 (Dowsett et al., 1990). CFS is also found in children and young adults. Incidence rates across adolescents (aged 11-15 years) have been reported as high as 0.5% (Rimes et al., 2007).

YEAR	NUMBER OF CASES
2011	268
2012	328
2013	384
2014	442
2015	536

Table 1.3 Completed triaged referrals from the Royal Liverpool and Broadgreen University Hospital Trust CFS Services. Part completed referrals returned to GPs not included. Provided by C. Foster - Service Manager, Chronic Fatigue Services. Unpublished data. (2016).

The pathophysiological cause of this disease is yet to be fully defined. There is a lack of evidence within current literature to confirm any specific biological abnormalities which may underlie CFS. This is not to say that there are no objective abnormalities. This has been demonstrated when comparing CFS individuals with healthy subjects and/or patients with other chronic illness where fatigue is a caused. CFS is a medical syndrome and will therefore have numerous causal factors (Afari and Buchwald, 2003). This makes identifying the disease very difficult. CFS has been linked with other diseases such as fibromyalgia, depression/anxiety, infectious mononucleosis, lyme disease and sleep disorders (Griffith and Zarrouf, 2008, Patrick et al., 2015, Bourke, 2015, Ferre, 2016). There are a broad range of symptoms associated with CFS. The main symptom of CFS is severe

fatigue/exhaustion which can be triggered by minimal/ no activity. Additional symptoms commonly associated with CFS include general malaise/flu-like symptoms, muscular and joint pain, sleep disturbances, headaches, gastric disturbances and cognitive impairments such as short term memory loss, poor concentration and mental confusion. Further reports have described symptoms including intolerance to noise and light, sensory overload, difficulties with balance, dizziness, disordered temperature control, restless legs and muscle twitching.

At present, there is no treatment for CFS. Current therapies offered to CFS sufferers by the National Health Service (NHS) in England include cognitive behavioural therapy (CBT) and graded exercise therapy (GET). Both have been shown to improve both primary and secondary outcomes of CFS (White et al., 2011).

1.6.2 Diagnosing chronic fatigue syndrome

The characteristics of populations with CFS differ greatly depending on the diagnostic criteria utilised and the procedure by which cases are determined. A number of terms and case definitions have been produced for CFS (Table 1.3). The creation of the term Chronic Fatigue Syndrome originated from Holmes et al (1988) which thus led to the formation of the Holmes Case Criteria (Holmes et al., 1988). There have been numerous case definitions since then. A recent review of case definitions identified 20 different case definitions in published studies of which 13 had been assessed in 1 or more validation studies (Brurberg et al., 2014). There are currently 5 major different case definitions available to define CFS. The diagnostic case definition for CFS produced by the American Centre for Disease Control and Prevention (CDC) in 1994 (Fukuda et al., 1994) has been identified as the most widely used around the world (Brurberg et al., 2014). Guidelines within the UK were produced by the National Institute for Care and Excellence (NICE) in 2007. For an individual to be diagnosed with CFS, they must have had persistent/recurrent fatigue for at least six months as well as four of the following symptoms; difficulty sleeping or insomnia, muscle or joint pain without inflammation, headaches, painful lymph nodes that are not enlarged, sore throat, poor mental function (difficulty in thinking), symptoms getting worse after physical or mental exertion, feeling unwell

or having flu-like symptoms, dizziness or nausea or heart palpitations (without heart disease).

Diagnosing CFS is often a case of excluding other causes of fatigue such as an underlying illness e.g. multiple sclerosis, hypothyroidism or depression. Diagnosis is subject to the physicians own views which can be highly confounding (Bayliss et al., 2014). The issue of diagnosis is often a controversial topic between sufferers and physicians.

Diagnostic Criteria	**Case Definition and Author**	**Syndrome Title**
American Centre for Disease Control (CDC)	Holmes case definition (Holmes et al., 1988) Fukuda case definition (Fukuda et al., 1994)	Chronic Fatigue Syndrome (CFS)
The Oxford criteria	The Oxford criteria	Chronic Fatigue Syndrome Post inflectional viral syndrome (PIFS)
Nice Guidelines	Chronic fatigue syndrome/myalgic encephalomyelitis (or encephalopathy): diagnosis and management in adults and children (Turnbull et al., 2007)	Chronic Fatigue Syndrome/Myalgic encephalomyelitis (or encephalopathy)
Canadian Clinical Case Definition	(Carruthers et al., 2003, Carruthers, 2007)	Myalgic encephalomyelitis/ Chronic Fatigue Syndrome

Table 1.4 The main diagnostic criteria, case definitions and syndrome titles for CFS.

1.6.3 Aetiologies of chronic fatigue syndrome

The primary cause of CFS remains elusive. Many theories for the pathophysiology of CFS have been proposed. Previous assumptions, based loosely on the presence of symptoms, suggested an acute viral infection or a psychiatric disorder. Recent investigators have postulated that CFS is now more likely to be of a complex and multifactorial aetiology. Reports suggest there may be elements which predispose an individual to develop CFS, it is unclear why some individuals go to suffer from CFS and some do not. Numerous factors including endocrine, neuroendocrine, psychosocial, and potentially immunological have all been associated with facilitating the physiological response of CFS and the course/duration of the illness. Despite the demonstration of abnormalities across these and other domains, such findings remain largely isolated observations, with the interactions and relationships among them unexplored. However, the potential link behind these symptoms and the cause of CFS remains unknown.

Alternative terms for CFS such as Post Viral Syndrome (PVS) and ME have been created based on from terminology describing the suspected cause of the disease. For example, PVS arose from the high incidence of CSF/ME sufferers reporting a previous viral infection prior to the onset of the disease. Lyme disease, the Human Herpes Virus family including the Epstein-Bar virus (Glandular fever) (Manian, 1994). Myalgic Encephalitis (ME) is a neurological disease characterised by inflammation of the brain and spinal cord. Despite being technically incorrect, the term myalgic encephalopathy is the most widely used alongside CFS.

Patterns of risk factors associated with CFS have been hard to determine from current literature, due to differences within sample populations and recruitment. Associations have been made between lifestyles choices and risk of CFS suggesting a higher use of anti-depressants, sleeping-pills, painkillers and antibiotics within this population (Jones et al., 2003). Research has suggested, early onset of severe and inexplicable fatigue during adolescent and even childhood may be a predictor of CFS in later life (Huibers et al., 2004). The use of different diagnostic criteria between studies has led to the formation of heterogeneous CFS populations and contributed to the high output of contradictory findings. This has made determining the pathology of the disease troublesome. The Fukuda case definition report

suggested the understanding that a heterogeneous group was being identified using its methodology and the potential need for sub-grouping within CFS, (Fukuda et al., 1994). This has been re-iterated in a recent review (Jason et al., 2005).

There is a growing body of research which suggest that infections and immunological dysfunction including perturbed cytokine levels may be a key contributing factor in the development and/or maintenance of CFS and its related symptoms, with the possibility of an interacting genetic and psychosocial factors. The current study will focus on immunological disturbances focussing on cytokine dysregulation and the muscle fatigue in patients with CFS.

1.6.4 Treatment of chronic fatigue syndrome

To date, there is no cure or direct treatment for CFS. Some of the symptoms associated with the disease such as myalgia or sleep disturbances, can be alleviated through painkillers or sleeping medication respectively. The NHS offers two forms of treatment which aim to alleviate the symptoms associated with CFS; Cognitive behavioural therapy (CBT) and graded exercise therapy. Recommendations are based on the 2007 NICE guidelines for the diagnosis and management of CFS. CBT aims to help patients cope with the mental stress of the condition of suffering with CFS. Many patients with CFS suffer from anxiety, stress and depression as a consequence of the disease. Additionally, CBT aims to change the way in which you approach daily talks in life and pace yourself. The use of CBT does not suggest that CFS is a psychological condition. CBT is used in a wide range of patient groups including severely fatigued cancer survivor in which it has been shown as an effective treatment (Gielissen et al., 2006). The main outcome measures of CBT include improved functional status and a reduction in fatigue scores (Deale et al., 1997, Prins et al., 2001). The mechanisms through which CBT influences CFS and other conditions are unclear (Wiborg et al., 2010). Graded exercise therapy is a controlled exercise programme. It uses a combination of aerobic exercises which raise an individual's heart rate. The therapy aims to gradually increase how long an individual can carry out a physical activity. There are some conflicting data regarding the use of GET. There is evidence which suggest GET can worsen CFS in a cohort of patients (Lapp, 1997) whilst other reports propose physical

deconditioning is not contributing factor in the is a progression/maintenance of CFS and therefore GET is ineffective. The overall consensus is GET is an effective treatment for CSF/ME and has a positive effect on patient physical functioning, fatigue, self-perceived general health and sleep quality (Larun et al., 2015).

Additional therapy which is not included in the NHS treatment programme for CFS includes pacing. Pacing is a process in which people are encouraged to make gradual increases in both mental and physical activity levels depending on stage, severity and variability of their illness. Pacing encourages patients to maintain a constant level of activity without causing over exertion (and a relapse). It is a flexible approach which is tailored specifically to an individual's needs. This process is fully supported by the ME Association charity (Shepherd, 2015)

Muscular and join pain is a widespread problem amongst patients with CFS (Krzeczkowska et al., 2015). Painkillers may be taken to relieve the patients from muscular aches and pains they may be experiencing. Self-medicating amongst patients is high. A case-control study reported >90% of Patients with CFS to have consumed at least one drug or supplement in the two weeks prior to the screening. The average intake of drugs/supplements was 5.8 per person (Boneva et al., 2009). Further research has reported patients with CFS were significantly more likely to use antidepressants, sedatives, muscle relaxants, and anti-acids, antihistamines and cold/sinus medications than were HCs.

When left untreated, the estimated number of patients with CFS who make a recovery is <10% (Nisenbaum et al., 2003). The likeliness of a patient recovering is thought to be greater when the duration of illness is less than 15 months prior to treatment (van der Werf et al., 2002).

1.6.5 Impaired muscle function in patients with chronic fatigue syndrome

There has been a large amount of interest in the objective determination of muscle function in patients with CFS given the obvious symptoms of muscle weakness and fatigue, post-exertional malaise and a general feeling of weak muscles in the limbs of these patients (Friedberg et al., 2000). The sensation of fatigue which CFS patients describe has still not been clearly defined. In this study, fatigue is defined

objectively as “failure to maintain the required or expected force”. Additional symptoms of muscle pain (fibromyalgia), “restless, muscle twitching legs” also feature highly in the list of ailments commonly reported in CFS (Fukuda et al., 1994). This may suggest an abnormality within the muscles which is contributing to impaired physical functioning.

Patients with CFS commonly self-report high scores of impaired muscle function and perceived effort and exertion during physical activity (Fulcher and White, 2000). CFS report symptoms of extreme periods of physical fatigue/exhaustion both post exercise (Edwards et al., 1977) as well as post mental stimuli and an inability to perform physical tasks previously performs with ease (Marcora et al., 2009). Muscle function has been extensively investigated in these patients however results are still not full conclusive. Studies examining involuntary and voluntary force production have produced mixed results. Maximal voluntary force measurements alone have been assessed in a collection of studies. Evidence supporting the hypothesis of normal maximal isometric strength in unfatigued muscle of patients with CFS has been reported by (Lloyd et al., 1991, Gibson et al., 1993, Stokes et al., 1988) From these reports, a reduction was identified (which did not reach significance) in maximum voluntary force generation in some patients with CFS (Kent-Braun et al., 1993). A significant reduction in MVC was identified by (Schillings et al., 2004). Twitch interpolation on top of maximal isometric force measurements indicated submaximal effort in a minority of subjects suggesting suboptimal effort or impaired central motor drive given the increase in force (Stokes et al., 1988, Lloyd et al., 1991, Gibson et al., 1993). mechanisms responsible for the patients with CFS inability to perform maximal voluntary contractions (Schillings et al., 2004) may be due to insufficient central or peripheral activation from the nerve. Patients with CFS generally reported high levels of fatigue and post-exertional fatigue following low intensity activities. To date, general everyday activity and sub-maximal contractile function has not been assessed in patients with CFS.

The effect of repeated contractile function (muscle fatigue) on force production has also been assessed in patients with CFS. Further analysis from (Kent-Braun et al., 1993) revealed, following a four minute fatiguing protocol of sustained maximal contraction, the reduction in central activation was significantly greater in CFS

subjects than HCs. These results are supported by additional studies assessing central activation in CFS (Schillings et al., 2004). Despite no difference in markers of contractile muscle function, the perception of exertion, effort and fatigue was greater in patients with CFS (Gibson et al., 1993). Recovery of maximal isometric strength was significantly impaired after endurance sequence testing in the patients in comparison with control subjects. Strength, force decline and recovery measurements following repeated maximal isometric contractions in Patients with CFS revealed no significant contractile abnormality in these patients (Lloyd et al., 1988). To date there have been no investigations into muscle fatigue following repeated involuntary submaximal isometric contractions (in the absence of volition) which would aid researchers in confirming whether reduced central activation was present in CFS. As previously mentioned in Section 1.3.2.3, physical activity and muscle function occurs through a combination of signalling pathways in the central and peripheral nervous system. Skeletal muscle contraction in under voluntary control and is reliant on a motivation factor.

1.6.6 Muscle physiology in chronic fatigue syndrome

Several studies have investigated whether patients with CFS have altered muscle physiology which may potentially be as a result of the disease or a contributing factor in the disease pathology. Early light and electron microscope studies examining muscle biopsies from patients with CFS have revealed there are no major abnormalities in muscle physiology with regards to fibre type content, fibre size (markers of fibre atrophy/hypertrophy (Edwards et al., 1993).

Patients with a severe form of CFS are often forced to bed rest and unable to weight bear for relatively long periods of time. A reduction in physical activity and mechanical loading commonly results in a number of morphological changes within the muscle with the end results of increased muscle atrophy and a muscle weakness (Chambers et al., 2009). Muscle unloading reduces electromyographic activity and causes muscle atrophy and significant decreases in capillarisation and oxidative enzymes activity (Bogdanis, 2012). A reduction in protein synthesis combined with acceleration in protein degradation leads to a reduction in muscle mass (Thomason and Booth, 1989). Behan et al demonstrated atrophy of type II fibres in muscle

biopsies taken from patients with CFS (Behan et al., 1991). These findings have not been uniformly reported from all studies (Lane et al., 1998).

The overlap with symptoms associated with the common-cold/flu and other viral infections lead many researchers to hypothesis there may be the presence of a persistent infection in the muscles. Previous reports suggested the presence of enteroviral RNA within 21% of the muscles of patients with CFS in their study (in 20 out of 96 patients) (Archard *et al.,* 1988), and the persistence of enteroviral antigen in the serum of 51% of patients with CFS (44 of 87 patients) (Yousef *et al.,* 1988). A more recent investigation by polymerase chain reaction of enteroviral infection in CSF/ME patients revealed no quantifiable amounts of enteroviral RNA (McArdle et al., 1996).

1.6.7 Cytokine dysregulation in patients with chronic fatigue syndrome

Perturbed circulatory cytokine levels (plasma or serum) have been proposed to be an important component in the pathophysiology of CFS. Numerous symptoms commonly reported in patients with CFS are linked to the flu-like symptoms and inflammatory processes (e.g. swollen and tender lymph nodes, sore throat, myalgia, and arthralgia). This has led many researchers to hypothesise that cytokine dysregulation (e.g. increased levels of pro-inflammatory cytokines and/or decreased anti-inflammatory cytokines) may be a key contributing factor in the pathophysiology of the illness (Lorusso et al., 2009). Alternatively, cytokine dysregulation may be a consequence of the illness and thus a product of the CFS symptom complex. Thus, disease duration may play an important role in the analysis of cytokine levels in patients with CFS because for this reason (Hornig et al., 2015). At present there is no clear consensus as to the presence and degree to which altered cytokine profiles are present in patients with CFS, despite several investigations into circulating cytokine levels. There are an array of behavioural and symptomatic similarities CFS and symptoms of the common cold/flu. The collection of symptoms common experience during the common cold/flu have described as “sickness behaviour” by (Morris et al., 2013). These observations have directed researchers to hypothesise the role of immunological dysfunction (i.e. – altered cytokine profiles) in causing/maintaining the disease. Potential sites/sources

of dysregulated cytokine production may be immune and non-immune cell types including macrophages and/or skeletal muscle cells which may in turn affect local and/or systemic cytokine levels.. Studies investigating altered cytokine profiles in patients with CFS reveal mixed results. There are reports which suggest alterations in systemic cytokine expression in CFS and an equal number which do not.

Potential reasoning may be the sensitive nature of these signalling molecules which is subject to change in response to factors such as physical activity, time of day, stress and diet (Krueger et al., 2001, Esposito et al., 2004, Mozaffarian et al., 2004). Sample processing and detection methods can also have a large impact on the level of these molecules detected (Zhou et al., 2010). Cytokines circulate at very low levels and therefore high sensitivity methods are required for reliable detection. Making comparison between studies utilising different assays is very difficult (Banks, 2000). CFS may potentially be associated with subtler alterations of cytokine networks rather than individual cytokines. Cytokine levels in adolescent patients with CFS have not been as extensively investigated. A recent study published by Wyller et al investigating adolescents (ages 12-18 years) with CFS found no difference in cytokine levels between CFS and HCs (Wyller et al., 2015).

1.6.8 Elevated levels of RONS and oxidative stress in muscle of patients with chronic fatigue syndrome

Excess production of ROS from skeletal muscle cells, combined with insufficient antioxidant capacity, results in an accumulation of these species and oxidative stress. Substantial oxidative stress can lead to cellular damage, impaired physiological functioning and cell death. In more recent years, there has been a change in the focus of CFS research towards subtler metabolic changes such as mitochondrial function and subsequently perturbed ROS homeostasis in an attempt to explain the symptoms present in patients with CFS. Markers of oxidative damage and altered levels of antioxidant enzymes have been assessed between patients with CFS and HCs in muscle and blood samples. Evidence which suggests ROS are elevated in patients with CFS (Pall, 2000, Pall, 2003) includes the assessment of markers of oxidative stress including oxidative damage to lipids (malondialdehyde (MDA), isoprostane and thiobarbuturic), proteins (protein carbonyls) and DNA (8-

hydroxyguanosine). Increased blood markers of oxidative stress and decreased antioxidant defences have been reported in patients with CFS. Lower plasma concentrations of Vitamin E (known to have antioxidant properties) and low-density lipoproteins (LDL), higher LDL thiobarbituric acid reactive substances (TBARS). These levels were shown to correlate with symptoms of musculoskeletal health reported by Patients with CFS (Vecchiet et al., 2003). Increased oxidative damage to DNA and lipids and increased activity of the antioxidant enzymes catalase, glutathione peroxidase, and transferase, and increases in total glutathione plasma levels were reported in muscle of patients with CFS (Fulle et al., 2000). These data suggests oxidative damage in muscle from patients with CFS is not due to the decline in antioxidant enzymes given the significantly higher levels in CFS patients reported above but an increase in the generation of ROS. Additionally, evidence has reported patients with CFS have significantly increased levels of isoprostanes and oxidised low-density lipoproteins in the muscle. CFS symptoms (total symptom score, joint pain, and post-exertional malaise) were found to correlate with isoprostane levels (Kennedy et al., 2005). Reports also reveals higher levels of MDA (malondialdehyde) in patients with CFS than age and sex match HCs (Richards et al., 2000). Further experimental evidence shows urinary excretion of 8-hydroxy-deoxyguanosine (8-OhdG), a marker of oxidative damage to DNA was higher in patients with CFS and depression (Maes et al., 2009). No difference was reported between patients with CFS and HCs.

One study has investigated oxidative stress in skeletal muscle of patients with CFS. Data revealed increased levels of 8-hydroxyguanosine and MDA as well as increased levels of antioxidant enzymes catalase, GPX and increases in total glutathione plasma levels (Fulle et al., 2000). These data suggest an increase in oxidative stress in patients with CFS although the study only contained six patients with CFS vs. age and sex matched controls.

Despite a relatively large number of studies investigating oxidative stress in CFS, there is very little/no existence of any overlapping data and so comparable markers between studies investigating elevated ROS and oxidative stress in patients with CFS. In addition there have not been any direct measures of ROS production from *in situ* permeabilised muscle fibres.

1.6.9 Altered muscle mitochondrial function in patients with chronic fatigue syndrome

Fatigability and post-exertion malaise remain fundamentally at the centre of CFS (Fukuda et al., 1994). These symptoms are associated with energy metabolism and so potentially mitochondrial function. There are numerous similarities between CFS and disorders involving mitochondrial dysfunction as shown in Table 1.6. Muscle impairments such as muscle weakness, cramps and pain are all present in mitochondrial dysfunction and CFS. Mitochondrial dysfunction has been associated with irregular immuno-inflammatory pathway signalling resulting in elevated activation of the NF-κB and perturbed cytokine profiles. Increased levels of pro-inflammatory cytokines, such as interleukin-1 and tumour necrosis factor-α may alter mitochondrial respiration, the activities of the electron transport chain and mitochondrial membrane potential, increase mitochondrial membrane permeability, interfere with ATP production and cause mitochondrial shutdown (Lopez-Armada et al., 2006, Lopez-Armada et al., 2013, Doll et al., 2015).

Mitochondrial function has been investigated in muscle cells and white blood cells in CFS patients (Booth et al., 2012). Within muscle, data suggests abnormalities in mitochondrial function the form of reduced ATP production, impaired oxidative phosphorylation, mitochondrial degeneration and elevated ROS production in CFS (McCully et al., 1996, Booth et al., 2012). Evidence has suggested the presence of structural abnormalities in muscle mitochondria of patients with CFS including the fusion and branching of mitochondrial cristae (Behan et al., 1991, Plioplys and Plioplys, 1995) which may impact on functional capacity. These differences in mitochondrial function between CFS patients and HCs are yet to be fully established or confirmed in studies with larger sample sizes. Such deficiencies may contribute to the sensation of overwhelming fatigue and post-exertional malaise which these patients suffer from. Evidence in this area is limited and requires further investigation. Excessive levels of RONS causes oxidative and nitrosative damage which can potentially lead to cellular damage (Zelko et al., 2002). This can in turn cause impaired electron transport chain functioning resulting in a reduction in the production of ATP (Tiwari et al., 2002). Reduced ATP synthesis causes a deficiency in oxidative phosphorylation and can subsequently lead to mitochondrial

dysfunction (Kirkinezos and Moraes, 2001). Impaired oxidative phosphorylation alone can lead to increase production of RONS thus creating a cycle self-propagating dysfunction (Wei et al., 1998).

The quality and study design of a large proportion of the investigations on CFS and oxidative stress are relatively weak containing few objective markers of ROS production, oxidative damage and mitochondrial function. There is lack of primary evidence to support a large number of conclusions (Maes et al., 2007).

Organ System	Symptoms in Mitochondrial Disorders	Symptoms in CFS
Muscle	• Weakness • Cramps • Pain (Fibromyalgia) • Hypotonia	Yes Yes Yes
Brain	• Migraine • Dementia • Neuropsychiatric disorders	Yes Neurocognitive disorders Depression
Nervous System	• Neuropathic pain and weakness • Neuropathic gastrointestinal problems (gastroesophageal reflux, constipation) • Fainting, absent or excessive sweating, aberrant temperature regulation	Yes Yes Yes
Heart	• Cardiomyopathy	
Liver	• Hypoglycaemia • Gluconeogenic defects • Non-alcoholic liver disease • Liver failure	
General/ Systemic	• Fatigue • Exercise Intolerance • Respiratory	Yes Yes

Table 1.5 Signs, symptoms, and diseases associated with mitochondrial dysfunction. Adapted from (Cohen and Gold, 2001, Morris and Maes, 2014).

1.7 Summary

In summary, perturbed levels of cytokines such as TNF-α are known to influence of muscle function and physiology. An increase in systemic pro-inflammatory cytokines can modulate the ability of skeletal muscle to generate force, alter mitochondrial bioenergetics and induce reactive oxygen species (ROS) generation in muscle. Cytokines are produced by a number of cells including skeletal muscle and research suggests a potential role of antioxidants such as naturally occurring dietary polyphenols in the modulation of cytokine production. Patients with CFS suffering from perceived increases in fatigue may have altered muscle function which may be, at least in part, the consequence of altered levels of systemic cytokines and/or altered mitochondrial function and oxidative stress. Reports to date are inconclusive regarding the role of altered cytokine levels in patients with CFS, the production of cytokines by muscle, muscle mitochondrial function and/or oxidative stress in muscle.

1.8 Hypothesis

We hypothesise that:

- Treatment of C2C12 myotubes with TNF-α will result in the release of pro-inflammatory cytokines and this can be altered by pre-treatment of myotubes with polyphenols.
- Patients with CFS show evidence of fatigue and impaired muscle force generation.
- Altered muscle function seen in patients with CFS is associated with modified levels of cytokines either systemically or locally to muscle.
- Altered muscle function and altered cytokine levels in patients with CFS will be associated with altered mitochondrial ROS generation and respiratory function.

1.9 Aims

The main aims of this thesis were to:

- Establish a comprehensive, non-damaging model of TNF-α treatment of C2C12 myotubes and determine the effect of pre-treatment of C2C12 myotubes with resveratrol, curcumin or EGCG on TNF-α induced cytokine release.
- Use Chronic Fatigue Syndrome as a potential model of fatigue and determine muscle function and fatigue (central and peripheral) in a patient cohort reporting increased fatigue by questionnaire analysis, compared with matched Healthy Controls (HCs).
- Investigate plasma cytokine levels and cytokine gene expression in skeletal muscle of patients with CFS and compared with matched HCs.
- Assess mitochondrial content and respiratory function *in situ* in permeabilised muscle fibre bundles isolated from skeletal muscle of patients with CFS and HCs and characterise mitochondrial ROS production in muscle from patients with CFS compared with HCs *in situ* in permeabilised muscle fibre bundles
- Assess markers of oxidative damage in skeletal muscle of patients with CFS compare with HCs.

Chapter Two

Methods

Chapter 2: Methods

2.1 Cell culture techniques

2 .1.1 Tissue culture material

Plasticware was obtained from Sarstedt, Germany and Fisher Scientific UK. Ltd, Loughborough, UK. All cell culture procedures were carried out in aseptic conditions in a laminar flow hood with sterilised plasticware, reagents and media.

- Dulbeco's Modified Eagles Medium DMEM (Sigma Aldrich, Dorset, UK)
- L-Glutamine (Lonza, UK)
- Penicillin/Streptomycin (Sigma Aldrich, Dorset, UK)
- Fetal calf serum (FCS) (Life Technologies, UK)
- Horse Serum (Sigma Aldrich, Dorset, UK)

2.1.2 Cell culture lines

C2C12 cells were derived from an existing mouse myoblast cell line. These cells were originally obtained from the thigh muscle of a healthy wild type C3H mouse (Yaffe and Saxel, 1977).

2.1.3 Basic culture techniques of muscle cells

Cell growth media consisted of 10% Fetal Bovine Serum (FBS) (v/v) in Dulbecco's Modified Eagle's medium (DMEM) containing 0.45% (w/v) glucose supplemented with 2mM L-glutamine (Sigma Aldrich, Dorset, UK), 50 i.u penicillin and 50µg/ml streptomycin (Sigma Aldrich, Dorset, UK). C2C12 myoblasts were grown in T25cm^2 or T75cm^2 flasks at 5% CO_2 saturation at 37°C in a humidified environment and media was replaced daily. Upon reaching 60-70% confluence, cells were passaged. Cells were washed with Dulbecco's Phosphate Buffered Saline (PBS) without Calcium and Magnesium and sub-cultured using 0.25% (w/v) trypsin, 0.025% (w/v) EDTA in Dulbecco's PBS into 9.5cm^2 6 well plates (Costar, UK) prior to treatment.

Upon reaching 80% confluence, myoblast differentiation was induced by changing the media to 2% horse serum (HS) in DMEM containing 0.45% (w/v) glucose

supplemented with 2mM glutamine over a 6 day period. Media was replaced daily. The change in cellular media promotes the formation of myotubes through the fusion of myoblasts. Development of myoblasts was continually assessed by light microscopy throughout the 6 day period.

2.1.4 Treatment of cells

- Tumour Necrosis Factor-Alpha TNF-α (murine) endoxin-free (R&D Systems, UK)
- Dimethyl Sulphoxide (DMSO) (Sigma Aldrich, Dorset, UK)
- Resveratrol (Sigma Aldrich, Dorset, UK)
- Epigallocatechin Gallate (EGCG) (Sigma Aldrich, Dorset, UK)
- Curcumin (Sigma Aldrich, Dorset, UK)
- Dulbecco's Phosphate Buffered Saline (PBS) (Sigma Aldrich, Dorset, UK)

At 6 days post differentiaton C2C12 myotubes were exposed to carrier free recombinant TNF-α (5 or 25ng/ml) for 3, 8 and 24 hours (Alvarez et al., 2002, Lightfoot et al., 2015). Thirty microlitre aliquots of 50µg/ml TNF-α were stored at -80°C prior to analysis. Following treatment cell culture media was removed and stored for cytokine analysis by Luminex®. Figure 2.1 illustrates the time course of TNF-α treatment. Cell viability was assessed using a LIVE/DEAD assay (Invitrogen, UK).

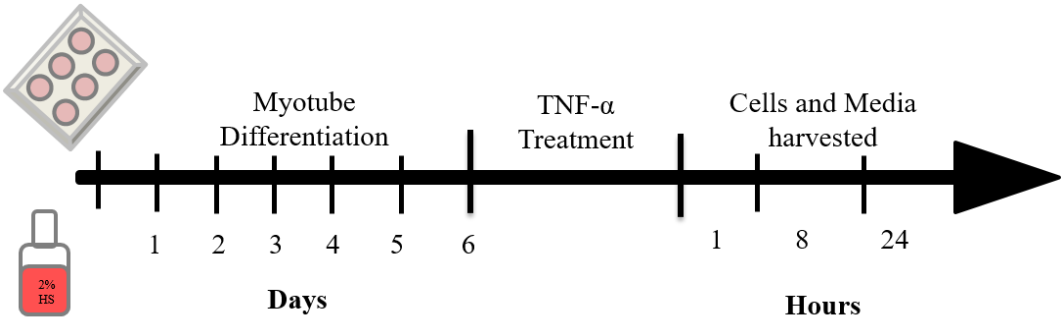


Figure 2.1 Time course of TNF-α treatments to determine the effect on TNF-α induced cytokine release from C2C12 myotubes.

For polyphenol treatments myotubes were exposed to either curcumin, EGCG or resveratrol (1 or 10μM) dissolved in DMSO for 24 hours (Howells et al., 2007). Control wells of cells were treated with DMSO only to elucidate whether the DMSo alone was exerting an effect on the cells. Following treatment, media were discarded and replaced with fresh differentiation media containing carrier free recombinant TNF-α (25ng/ml) for 24 hours. The time course of polyphenol pre-treatment and TNF-α treatment shown below. Cell culture media was aspirated and stored at -80°C for future cytokine analysis by Luminex® and cells were harvested in DPBS on ice and centrifuged at 14,000g, 4°C for 10 minutes. Cell pellets were stored at -80°C for analysis by western blotting, qPCR and Luminex®. Cells were used immediately for a LIVE/DEAD viability assay (Invitrogen, UK).

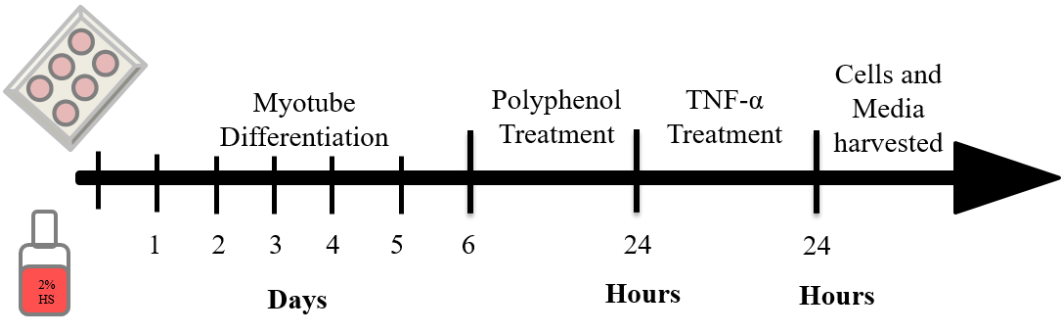


Figure 2.2 The time course of polyphenol and TNF-α treatments for studies of the effect of polyphenols on TNF-α induced cytokine release from C2C12 myotubes.

2.1.5 LIVE/DEAD Viability Assay

A commercially available Live/Dead Assay Cell Imaging Kit was used to assess cell viability (Invitrogen, UK).

- Calcein AM ester in DMSO (Invitrogen, Paisley, UK).
- Ethidium homodimer-1 (Invitrogen, Paisley, UK).
- Dulbecco's Phosphate Buffered Saline (PBS) without CaCl/MgCl (Sigma Aldrich, Dorset, UK).

The kit contained two fluorescent dyes, calcein AM solution and Ethidium homodimer, which detect both living and necrotic cells respectively. Non-fluorescent cell-permeant calcein AM ester is enzymatically cleaved by esterases to calcein within the cytosol and retained within viable cells. Ethidium homodimer is a cell-impermeant nuclear fluorochrome and consequently only enters cells with a compromised membrane and intercalates between nucleotide bases. Ethidium homodimer has been widely used to identify necrotic cells in culture due to its high affinity for DNA (Markovits et al., 1979). This assay provides a strong indication of necrotic cell death/cytotoxicity.

C2C12 myotubes were cultured in 9.5cm² 6 well plates (Costar, UK) and treated with a polyphenol (curcumin, EGCG or resveratrol) only and/or TNF-α at 6 days post-differentiation (Section 2.1). Control C2C12 myotubes and myotubes treated cells were exposed to LIVE/DEAD assay solution comprised of 4mM calcein-AM ester and 2mM ethidium homodimer-1 in DPBS for 15 minutes at room temperature, protected from light.

Confocal laser microscopy was used to image the cells. Calcein and ethidum was visualised using a fluorescein and a rhodamine optical filter respectively. Live cells produce a strong, even green fluorescence (excitation/emission 488 nm/515 nm). Dead cells produce a nuclear red fluorescence (excitation/emission 570 nm/602 nm).

2.2 Recruitment of patients with Chronic Fatigue Syndrome and Healthy Controls

2.2.1 Recruitment of patients with Chronic Fatigue Syndrome

The study recruited 95 patients with CFS. Subjects with newly diagnosed CFS were recruited through the local Merseyside CFS services. Following a referral from their local general practitioner (GP) individual cases are reviewed by consultant physicians from the Royal Liverpool University Hospital. Patients diagnosed with CFS entered the Liverpool CFS specialist outpatient NHS Service at Broadgreen Hospital. In exceptional cases, patients were fast-tracked through to Broadgreen CFS Services with diagnosis by their GP only. All subjects were newly diagnosed by clinicians in accordance to the Oxford Criteria (1990) and recommended NICE guidelines (2007).

Prior to their first appointment session at the CFS specialist outpatient NHS Service all patients received the study participant information sheet allowing them more than 24 hours to consider the study. The study team attended an introductory session provided to newly diagnosed CFS sufferers. A short presentation was given at the end of the session to remind patients about the study. Patients were invited to consent into the study, complete all the necessary study material and questionnaires and provide a blood sample after the talk. A schematic flow diagram of the study design and subject recruitment is shown in Figure 2.3.

Main inclusion criteria for CFS subjects:

- Age range including 18-55 years
- There should be a definitive onset i.e. it is not lifelong
- Fatigue must be the main symptom
- The fatigue should be severe, disabling and affect both physical and mental functioning i.e. significant impairment of short term memory or concentration
- The fatigue must be present for at least 6 months, during which it has to have been present for more than 50 % of the time
- All subjects must be able to provide written informed consent

Other symptoms may be present;

- Myalgia
- Sore throat
- Tender lymph glands in the neck region or under the armpits
- Headaches of a new type, pattern and severity
- Malaise following effort that lasts more than 24 hours
- Pains in several joints occurring without swelling or redness
- Mood and sleep disturbance
- Palpitations without heart disease
- Dizziness, with or without nausea

2.2.2 Recruitment of Healthy Controls

Ninety-five age and sex matched healthy controls (HCs) were recruited within the Merseyside area through recruitment posters. Volunteers who contacted the study team were provided with the participant information forms. Volunteers needed to consider the study information for a minimum of 24 hours before consenting into the study.

Main inclusion criteria for healthy controls:

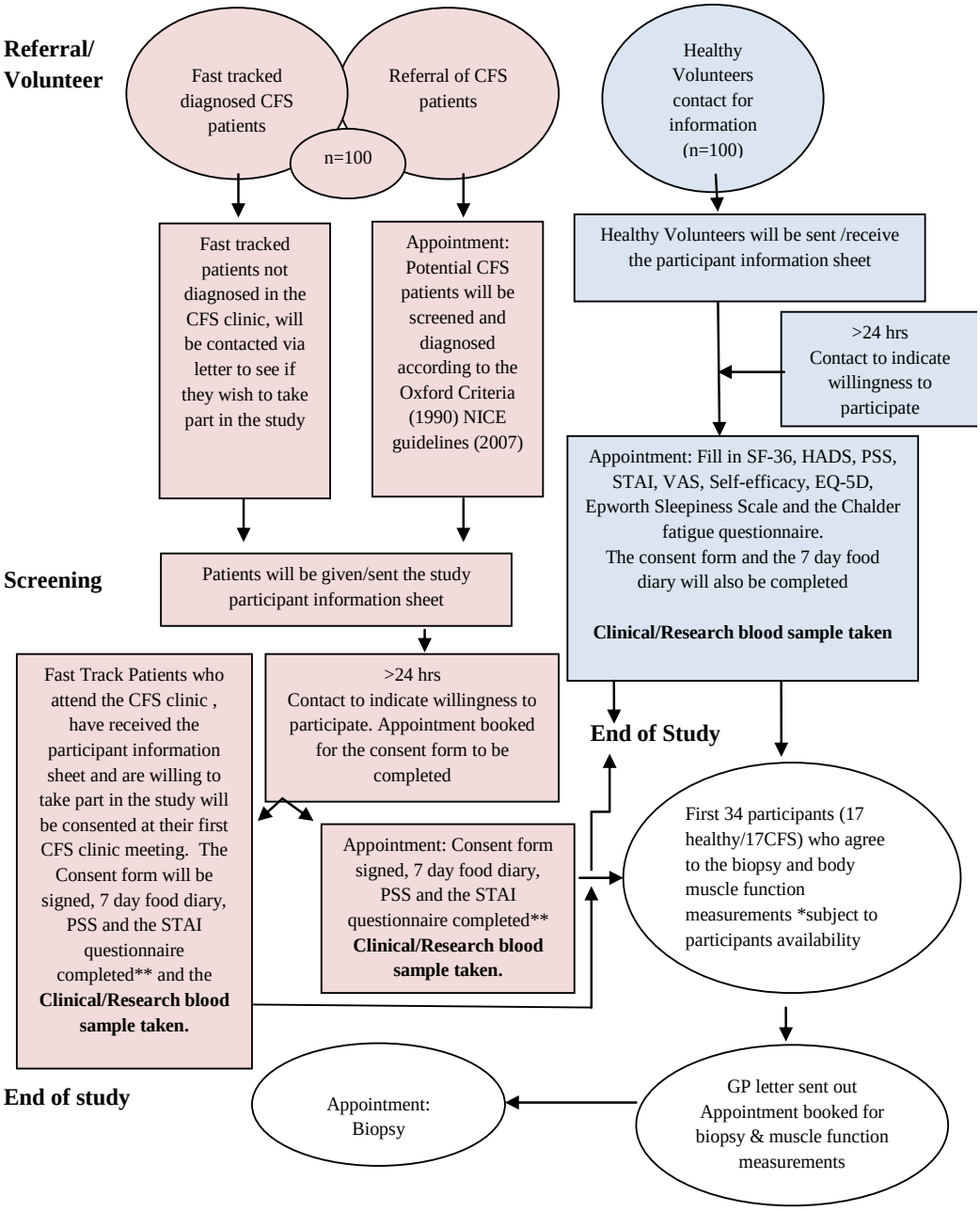
- Age limit 18-55 years
- All subjects must be able to provide written informed consent
- Subjects needed to be in good health, without any inflammatory based disease

Main exclusion criteria for CFS subjects and healthy controls:

- Individuals with a history of substance misuse
- Smokers

Through consenting into the study, all participants agreed to the provision of a blood sample and completion of a 7-day food and activity diary and study questionnaires. During consent, participants granted the study team access to their

medical notes to obtain subjects medical history. Patients could additionally consent into further study investigations by provision of a muscle biopsy and the completion of muscle function testing.



**Any information from questionnaires that are already collected by the Royal Liverpool and Broadgreen Hospital NHS CFS staff, from CFS patients, which we have proposed to also collect (Chalder Fatigue questionnaire, SF-36, HADS, EQ-5D, VSR, Epworth Sleepiness scale, Self-Efficacy Scale) will not be given to patients by the research team but, the information will be collected from the NHS in order to avoid duplication.

Figure 2.3 Schematic flow diagram of the human fatigue study design illustrating the recruitment of patients with CFS and HCs.

2.3 Health questionnaire assessment

CFS patients entering the NHS services were required to complete a series of health questionnaires in their CFS Initial Postal Assessment Pack. Participants granted permission for study team members to access these questionnaires during consent. The assessment pack contained the Chalder Fatigue Questionnaire, Visual Analogue Pain Rating Scale, Hospital Anxiety and Depression Score, Self-Efficacy Scale, The Short Form (36) Health Survey (SF-36), EQ-5D, Epworth Sleepiness Scale. Questionnaires are described in detail in Section 5.3.2. Questionnaires are displayed in Section 8.5. Healthy controls were provided with all questionnaires by the study team upon consent into the study. NHS questionnaires were scored by the study team or NHS nurses from Broadgreen hospital NHS services or the study team. University questionnaires were scored by the study team.

2.4 Blood collection

All blood collection tubes were supplied from Sarstedt AG & Co, Germany.

- 4 X 4.7ml Brown Cap S-Monovette Serum Collection Tubes
- 2 X Pink Cap S-Monovette Plasma Collection Tubes
- 1 X S-Monovette 2.7 ml, K3 EDTA Full Blood Count Tubes
- 1 X S-Monovette 3 ml Clotting Tubes

All blood samples were taken from patient and controls by trained individuals registered in the study log. Blood was drawn from the right/left arm using a butterfly needle.

2.4.1 Serum processing

Serum was collected in brown cap 4.7ml blood collection tubes (no additive), incubated for at least 30 minutes to induce clotting, and then processed within 3hrs of collection. Samples were centrifuged at 1500g for 15 minutes at 4°C. Supernatant was aspirated from the samples band stored at -80°C for future analysis.

2.4.2 Isolation of plasma from human blood

Plasma was collected in S-Monovette 7.5ml K_2EDTA gel pink capped blood collection tubes (Sarstedt AG & Co, Germany). Blood was stored on ice until they

were ready to be spun. All samples were processed within 3hrs of collection. Samples were centrifuged at 1500g for 15 minutes at 4°C. Supernatant was aspirated from the samples band stored at -80°C for future analysis.

2.4.3 Clotting Screen and Full Blood Count for muscle biopsy participants

Additional bloods were drawn from subjects willing to donate a muscle biopsy. Blood was collected in S-Monovette 2.7ml K3 EDTA, red cap collection tubes for full blood count analysis and S-Monovette 3ml green cap collection tubes for clotting screen. Samples were taken to Liverpool Royal Hospital to be processed by the Haematology department. The clotting screen involves a collection of tests which aim to detect potential problems with a person's blood coagulation / clotting mechanism. Prothrombin time (PT) is a blood test that measures the time it takes the blood to clot. The APTT, in contrast to the PT, measures the activity of the intrinsic and common pathways of coagulation. The APTT measures the formation of a complex formed from various plasma clotting factors which converts prothrombin to thrombin and the subsequent formation of the fibrin clot. These tests were used to determine whether a person has an increased risk of bleeding during the biopsy. The Full Blood Count included a group of tests designed to investigate markers of blood cell profile, including red blood count, platelet and haemoglobin levels.

	Platelets (x10^9/L)	**Prothromin (secs)**	**APTT Partial Thromboplastin Time (secs)**
Healthy Range	**150 - 400**	**9.0 - 13.0**	**25.0 - 36.0**

Table 2.1 Healthy ranges for platelet count and clotting screen from blood samples according to NHS guidelines.

2.5 Muscle biopsy procedure

- TSK Acecut Biopsy System (TSK Laboratory, Oisterwijk, Netherlands)
- 2% Lidocaine
- Scalpel
- Sterile wipes/dressings pack

A percutaneous needle biopsy was taken from newly diagnosed patients with CFS and healthy volunteers providing their FBC and clotting screening was within normal laboratory range. Clotting Screen - Prothrombin (seconds). A fully trained physician carried out the procedure. An aseptic Acecut automatic Biopsy system – Needle 22mm was used for each procedure. Biopsies were taken from vastus lateralis of the right leg. Subjects lay supine on a padded table with the thigh exposed. The leg was positioned in a relaxed manner, with the knee fully extended, thus placing the vastus lateralis in a shortened position. The area was shaven and sterilized with swabs pre-soaked with a topical antiseptic, (chlorhexidine gluconate) prior to the procedure. The skin and thigh were both anaesthetised with 4ml 2% lidocaine. A needle was inserted approximately horizontal to the skin into the dermis, 100 µl of lidocaine was infiltrated. The needle was withdrawn and then advanced into the subcutaneous tissue. After 2-3 minutes' post anaesthetic, the biopsy site was probed to ensure the patient could not feel anything. A small 1cm incision was made through the skin, subcutaneous tissues and fascia using a scalpel. The biopsy needle was advanced into the skeletal muscle through an incision in the skin, subcutaneous tissue, and fascia. Three passes of muscle were collected from the muscle from different regions. Pressure was applied directly to biopsy site with sterile gauze. Once haemostasis was achieved, to the wound was cleaned and steri-strips were applied (Lacomis, 2000, Owens et al., 2015).

2.6 Plasma cytokine analysis

2.6.1 Bio-Plex multi-bead cytokine analysis

- Bio-Plex Pro™ Cytokine Reagent Kit (Bio-Rad, Hercules, USA)
- Assay Buffer (PBS-0.05% Tween 20, pH 7.4, 0.1% BSA, 0.01% NaN_3)
- Wash Buffer (PBS-0.05% Tween 20, pH 7.4)
- Streptavidin-phycoerythrin fluorophore
- 96-well nano-pore filter membrane microplate or 96-well flat bottom microplate (Invitrogen/ Bio-Rad, Hercules, USA)
- Bioplex® single-plex (mouse or human) cytokine coupled magnetic beads and detection antibodies

All buffers, standards, coupled beads and samples were brought up to room temperature prior to use. A vacuum pressure or magnetic plate washing system was utilised to aspirate liquid from each well of the filter plate during a wash. During a plate wash, 200µl of wash buffer was added to each well. The plate was either placed onto the vacuum filtration platform and pressure (a pressure of 1-3mmHg) was applied ensuring the plate was tightly sealed down pulled liquid in the wells through. Alternatively, the plate was placed on a magnetic plate station and inverted allowing the wash buffer to drain out whilst the beads are retained.

A 96-well filter plate was initially pre-wetted with 200µl of assay buffer followed by 50µl of diluted coupled magnetic beads. Standards were made using Bio-Plex Pro™ mouse cytokine standards (Bio-Rad, Hertfordshire, UK) and were loaded onto the plate along with the samples. These were incubated under set conditions: 30 min under constant agitation, protecting the plate from light. The plate was washed 3 times before samples and standards were incubated with a complementary detection antibody for 30 minutes under agitation. 3 further wash steps were performed using the vacuum filtration or a magnetic plate station. The penultimate step involved the addition of Streptavidin-phycoerythrin fluorophore to each well. Samples and standards were incubated for 10 minutes in darkness under agitation (300rpm). Samples and standards underwent a final wash with 100µl of assay buffer before being analysed using the Bioplex® 200 platform (Bio-Rad Hercules, USA).

2.6.1 Cytokine analysis by ELISA

- Human TNF-α Quantikine ELISA (R&D Systems, UK).
- Human TNF-α Microplate, conjugate and standard
- Assay Dilutent (RD1F - Buffered protein base with preservatives)
- Wash Buffer (Concentrate & ddH_2O)

Levels of TNF-α in plasma samples was analysed using a commercial TNF-α ELISA. Plasma was obtained using the plasma isolation protocol as previously described in Section 2.2.4.2. The kit provided a 96-well microplate pre-coated with human TNF-α monoclonal antibody.

50µL of assay diluent was added to each well followed by 200µL of standard, sample or control per well. The plate was covered and incubated for 2hours at room temperature. Each well was aspirated and washed a total of four times. 200µL of Human TNF-α conjugate was added to each well and incubated for 2 hours at room temperature. A second set of washes was completed before 200µL of substrate solution was added to each well. The plate was incubated for 20minutes at room temperature. The final step of the protocol involved the addition of 50µL of stop solution. Optical density of each well was determined at 540 nm using a Flurostar Optima (BMG Labtech, Aylesbury, UK).

2.7 Quantification and analysis of proteins in human tissue and cells

2.7.1 Preparation of human biopsy tissue samples

- RIPA Buffer - 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM EDTA, 2.5 mM sodium deoxycholate, 1:1000 protease inhibitor cocktail (Sigma-Aldrich).

Prior to analysis, muscle was ground under liquid nitrogen. The resulting powder was placed in to an eppendorf with 200µl of RIPA buffer and homogenised (Sartorius BBI Systems, Model POTTER S, Germany). Samples were then centrifuged at 10,000 g for 10 minutes at 4°C (Eppendorf Centrifuge 5402, London, U.K.) and the supernatants were stored at –80°C until further analysis.

2.7.2 Preparation of C2C12 cells

- Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma Aldrich, Dorset, UK).
- 1% Sodium dodecyl sulphate (SDS) (Sigma Aldrich, Dorset, UK).
- Protease Inhibitor -1mM iodoacetimide, 1mM benzithonium chloride, 5.7mM Phenylmethylsulphonyl fluoride (Sigma Aldrich, Dorset, UK).

Cells were harvested from the 6-well tissue culture plates in 2ml PBS solution (without $MgCl_2$ or NaCl) using sterile cell lifters (Corning Incorporated, USA). Cells were centrifuges at 10,000g for 10 minutes at 4°C to form a pellet. The supernatant was removed and cell pellets were re-suspended in 100µl of 1% sodium dodecyl sulphate (SDS) containing protease inhibitors (1/1000) to prevent protein degradation. Samples were stored at -80°C until further analysis.

2.7.3 Bradford assay

The Bradford assay was used to determine total protein content of each human muscle sample according to the manufacturer's protocol.

- Bovine Serum Albumin (BSA) – Protein Standard
- Bradford Reagent (Sigma Aldrich, Dorset, UK)

The principle of the Bradford assay is based on the binding of Coomassie blue dye to proteins (Bradford, 1976). Under neutral conditions, the dye exits in a green form. However, when the dye binds to protein, it is converted to a stable un-protonated blue form (Compton and Jones, 1985). It is this blue protein-dye form that is detected at 595nm in the assay using a spectrophotometer.

A set of standards ranging from 25-500µg/ml were made from a 2mg/ml stock solution of bovine serum albumin (BSA) with dH_2O. Samples were diluted in a 1:20 dilution with dH_2O. 200µl of Bradford reagent was added to each well and mixed well. The absorbance of both the samples and standards was measured using a microplate spectrophotometer (Bio-Tek instruments, Vermont, USA) at 570nm. The protein content of each sample was calculated using the standard curve.

2.7.4 Bicinchoninic Acid (BCA) Assay

The BCA is a reliable, sensitive assay used to assess the total protein content of C2C12 cells.

- Reagent A: Bicinchoninic acid (BCA) solution consisted of 160mM $NaCO_3.H_2O$, 25mM BCA-Na, 7mM Na_2 tartrate & 0.95% $NaHCO_3$.
- Reagent B: 160mM $CuSO_4.5H_2O$
- Bovine serum albumin (BSA) 1mg/ml.
- Reagent C was comprised of 12.5ml of Reagent A to 250µl of Reagent B.

The principle of the bicinchoninic acid (BCA) protein assay depends on the formation of a Cu^{2+} protein complex. This is followed by reduction of the Cu^{2+} to Cu^{+}. The amount of reduction is proportional to the amount of protein present. BCA forms a purple-blue complex with Cu^{+} (Smith et al., 1985).

A 1mg/ml stock solution of BSA was utilised to prepare a series of standards ranging from 50-1000µg/ml. Samples were analysed against these standards. A total volume of 20µl of samples and standards were added to a 96-well microplate before 180µL of Reagent C was added to each well. The microplate was incubated for 30 minutes at 50°C. A microplate reader was used to read the absorbance of both the samples and standards at 570nm. Protein content was determined by using the standard curve.

2.8 Gel Electrophoresis

2.8.1 Preparation of gels

- 12% Acrylamide Solution (40% Acrylamide Solution, 26% resolving buffer and 34% ddH_2O
- 4% Acrylamide Solution (13% Acrylamide Solution, 25% stacking buffer, 61% ddH_2O
- Stock Acrylamide Solution: 30% Acrylamide, 0.8% methylene bisacrylamide stabilised solution (37.5:1 ratio) (Protogel, National Diagnostics, USA)
- Stock Resolving Buffer: 0.375M Tris-HCl, 0.1% SDS, pH 8.8. (Protogel, National Diagnostics, USA)
- Stock Stacking Buffer: 0.125M Tris-HCl, 0.1% SDS, pH 6.8. (Protogel, National Diagnostics, USA)
- NNN'N –Tetramethylethylene (TEMED), (Sigma Aldrich, Dorset, UK)
- 10% (W/V) Ammonium Persulphate (APS), (Sigma Aldrich, Dorset, UK)

Gels were cast in 2mm sections between 2 x (8x10cm) glass plates. 10ml of 12%gel was prepared for each gel as detailed above. The polymerisation of each gel required 100µl of 10% APS solution and 10µl TEMED to catalyse the reaction. A 4% stacking gel was prepared and cast on top of the 12% gel. 10ml of 4% gel required 100µl 10% APS and 25µl TEMED. A comb was inserted into the 1-1.5cm of stacking gel to form the wells for protein loading.

2.8.2 Protein Electrophoresis

- Running buffer -10X Tris/Glycine/SDS (0.25M Tris base, 1.92M glycine and 1% (w/v) SDS (National Diagnostics, Georgia, USA)
- Lamelli loading buffer (2X) – 0.5M Tris-HCl, pH 6.8, 4.4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and bromophenol blue in ddH_2O

Fifty micrograms of total protein was aliquoted from each sample and combined with an equal volume of Lamelli loading buffer. Samples were boiled for five minute to ensure all proteins were denatured and to help break up any complexes. Samples were left to cool to room temperature for 15 minutes before being loaded

into the wells in the 4% stacking gel. Gels were placed into an electrophoresis tank filled with 1X running buffer in ddH_2O. Proteins were run through the 4% gel at a constant current of 20mA per gel until they reached the 12% gel. Proteins were separated by electrophoresis across the 12% gel at 30mA per gel.

2.8.3 Western Blotting

- Anode I Buffer – 0.3M Tris in 20% methanol solution, pH 10.4
- Anode II Buffer – 25mM Tris in 20% methanol solution, pH 10.4
- Cathode Buffer – 40mM 6-amino-n-hexanoic acid in 20% methanol solution, pH 7.6

Gels were removed from the electrophoresis tanks and separated from the glass plates. The gel was placed on top of a nitrocellulose membrane over two layers of Whatman No°1 filter paper which had been previously soaked in Anode I and II. A third layer of filter paper which had been pre-soaked in cathode was laid on top (Figure 2.4). Proteins were transferred on to the nitrocellulose membrane by a Multiphore Continuous Blotting System (Pharmacia, Uppsala, Sweden). A constant current of 45mA per gel was applied for 90 minutes. Graphite electrodes facilitated the electrical current between the layers of filter paper, allowing the transfer of the separated proteins from the gels to the nitrocellulose membrane. Confirmation that the proteins had been transferred homogenously was achieved through ponceau staining of the nitrocellulose membrane.

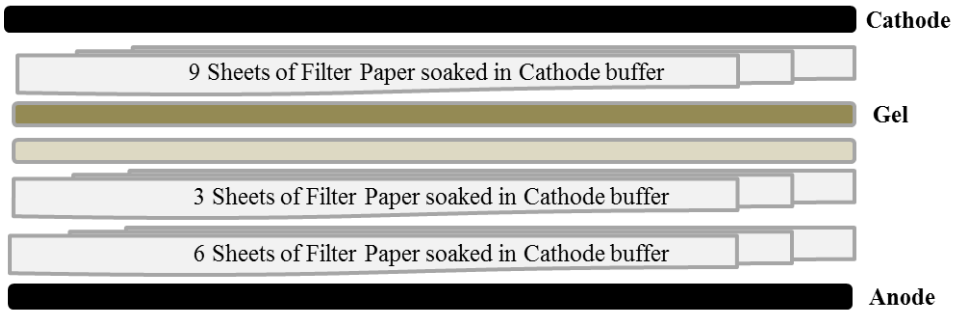


Figure 2.4 Schematic diagram of western blotting, arrangement of gel and nitrocellulose membrane and filter paper between the two electrodes for efficient protein transfer.

2.8.4 Membrane probing for specific proteins

- Blocking Solution – 5% (w/v) powdered milk in TBS-Tween.
- 10X TBS solution (1L) – 24.23g Tris-HCl, 80.06g NaCl, in ddH_2O. pH 7.6.
- TBS-Tween Solution (1L) – 1X TBS in ddH_2O, 0.05% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20).
- SuperSignal West Dura Chemiluminescence Kit (Thermo Fisher Scientific, UK)

Membranes were incubated for 60 minutes in a 5% milk solution to prevent non-specific binding between the membrane and the primary antibody followed by 3x 5 minute washes in TBST. Membranes were cut according to the size of proteins being analysed. Sections of blots were analysed for the presence of Catalase, IκBα, MnSOD and CuZnSOD using antibodies raised against these proteins (Enzo Life Sciences, Exeter, UK) (Sigma Aldrich, Dorset, UK). Antibodies were prepared in 10mls of 3% (w/v) powdered milk in TBS-Tween and incubated with the appropriate section of membrane overnight at 4°C on an agitator. Membranes were washed in TBS-Tween 3x 10 minutes before being incubated with a second species specific peroxidise conjugated secondary antibody relevant to the primary antibody of interest for 60 minutes under agitation at room temperature. Following this, membranes underwent 3x 10 minutes washes in TBS-Tween. The membrane was exposed under a chemiluminescent agent using SuperSignal West Dura Kit (Thermo Fisher Scientific) before being placed between two sheets of acetate. It was then placed under a Bio-Rad Chemi-doc XRS system with QuantityOne software (Bio-Rad, Hercules, USA).

Protein levels were quantified by assessing the intensity of each band using densitometry. Pixel saturation was avoided by ensuring linear intensity during the exposure.

Antibody	Company	Cat Number	Species	Dilution
GAPDH	Abcam	Ab8245	Mouse	1:5000
SOD1	Enzo Life Sciences	ADI-SOD-100-F	Rabbit	1:1000
SOD2	Enzo Life Sciences	ADI-SOD-111-F	Rabbit	1:1000
nNOS	Abcam	Ab76067	Rabbit	1:1000
eNOS	Abcam	Ab76198	Mouse	1:1000
iNOS	Abcam	Ab49999	Mouse	1:1000
PRX3	Abcam	Ab16751	Mouse	1:1000
4-HNE	Abcam	Ab46545	Rabbit	1:1000
3-NT	Cayman	189542	Rabbit	1:1000
Prot. Carbonyls	Cell Biolabs, Inc.	STA-308	Rabbit	1:1000
Catalase	Sigma	C 0979	Mouse	1:1000
HSP 10	Abcam	Ab53106-100	Mouse	1:1000
HSP 25	Enzo Life Sciences	ADI-SPA-801	Rabbit	1:1000
IκBα	Abcam	Ab32518	Rabbit	1:1000

Table 2.2 Antibodies used for western blotting. Company details - Abcam, Cambridge, UK. Sigma Aldrich, Dorset, UK. Cell Biolabs, Inc. San Diego, USA. Enzo Life Sciences, Exeter, UK.

2.9 Assessing markers of oxidative damage in muscle biopsy tissue

2.9.1 Assessing changes in the 3-nitrotyrosine (3-NT) content of proteins

Previous research has indicated that the 3-nitrotyrosine (3-NT) content of the major muscle protein, carbonic anhydrase III (CAIII), is a relatively sensitive marker of muscle oxidative stress (Vasilaki et al., 2007). Peroxynitrite was assessed via changes in the level of nitration of tyrosine using the techniques described by (Vasilaki et al., 2007).

Skeletal muscles were ground under liquid nitrogen as described in Section 2.4 and total cellular protein (50μg) was separated on 1D SDS-PAGE followed by western blotting as described in Section 2.5. A mouse monoclonal antibody was used to assess the content of 3-NT (Cayman Chemical Co., Ann Arbor, Michigan, USA). Bands were visualized using a Bio-Rad Chemi-Doc System (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The density of bands quantification of the CAIII band was undertaken, and the protein content was normalized to the GAPDH content of the same sample. Comparisons were made between samples on the same gel.

2.9.2 Assessing changes in protein oxidation

Protein carbonyls were analysed as a marker of protein oxidation in muscle tissue. Proteins (50μg) were separated on 1D SDS-PAGE and transferred on a PVDF membrane.

2.9.2.1 Derivatization

Following the electroblotting step (Section 2.8.2), PVDF membranes were immersed in 100% methanol for 15 seconds, and then allowed to dry at room temperature for 5 minutes. Membranes were equilibrated in TBS containing 20% methanol for 5 minutes and were washed in 2N HCl for 5 minutes. Membranes were incubated with 1X DNPH solution (a solution used to derivatize the carbonyl groups) for exactly 5 minutes and were washed three times in 2N HCl, 5 minutes each time, followed by five minute washes in 100% methanol.

2.9.2.2 Immunoblotting

Membranes were blocked and probed for 2 hours using a freshly diluted 1:1000 Rabbit Anti- DNP antibody (Cell Biolabs, San Diego, CA, USA).

2.9.3 Assessing changes in lipid peroxidation

Lipid peroxides are unstable indicators of oxidative stress that decompose to form more complex and reactive compounds such as Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), natural bi-products of lipid peroxidation (Sakellariou et al., 2013). Lipid peroxidation was assessed via changes in 4-HNE protein conjugates. Both markers were assessed using the same protocol as for 3-NT (Section 2.11.1). Rabbit Anti-MDA primary antibodies (Cell Biolabs, San Diego, CA, USA) and rabbit anti 4-HNE (Abcam, Cambridge, UK) were used.

2.10 RNA extraction, purification and cDNA synthesis

2.10.1 RNA Extraction

- TRI Reagent (Sigma Aldrich, Dorset, UK)
- Isopropanol (Sigma Aldrich, Dorset, UK)
- Chloroform (Sigma Aldrich, Dorset, UK)
- Ethanol (75%)
- RNase-free H_2O (Sigma Aldrich, Dorset, UK)

Supernatant were aspirated and the cell pellets (Section 2.7.1 and 2.7.2) were re-suspended in 200µl of Tri reagent and centrifuged at 12,000g for 10 minutes in 4°C to remove insoluble material. Samples were left for 5 minutes at room temperature before 200µl of chloroform was added to the supernatant. Samples were then agitated for 15 seconds at room temperature before standing at room temperature for 15 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C causing 3 phase separations of the lysate. A lower red phase containing organic protein, a middle DNA interphase and a colourless upper RNA phase containing aqueous residue is produced. The upper RNA phase was removed from each sample and aspirated into a new tube.

Samples were vortexed with 500µl of isopropanol and left to stand at room temperature for 10 minutes. Samples were centrifuged at 12000rpm for 10 minutes at 4°C. Aspirated supernatant from the samples was discarded from each sample leaving a pellet of RNA. Pellets were washed with 1ml of 75% ethanol and centrifuged at again at 12,000g for 5 minutes at 4°C. The supernatant was aspirated and discarded. Pellets were air dried at room temperature for 5 minutes. The RNA pellet was re-suspended in 100µl DNase/RNase free ddH_2O pipetting the solution repeatedly to break it up. Samples were incubated at 55°C for 15 minutes before being stored at -80°C for future analysis.

2.10.2 Purification and DNase treatment of total RNA

All RNA samples were purified and DNase-treated using the RNeasy MinElute cleanup-kit (Qiagen) as described below.

- Buffer RLT (lysis buffer)
- Buffer RPE (washing buffer)
- RNase- Free water
- RNeasy MinElute Spin Columns
- Collection tubes (1.5 & 2ml)

The total volume of RNA samples was adjusted to 100µl with RNase-free water. Three hundred and fifty microliters RLT buffer and 250µL 100% ethanol were added to the samples and mixed well by pipetting. Samples were transferred to an RNeasy MinElute Spin column which was placed in a 2mL collection tube. Columns were centrifuged for 15sec at 8,000g. The flow-through was discarded and the RNeasy MinElute Spin columns were placed in a new 2ml collection tubes. Five hundred microliters RPE buffer was added before centrifuging the spin columns for 15sec at 8,000g to wash the column membrane. The flow-through was again discarded. Five hundred microliters of 80% Ethanol was added to the RNeasy MinElute spin column and samples were centrifuged for 2min at 8,000g to wash the spin column membrane. The collection tubes were discarded. RNeasy MinElute Spin columns were placed in a new 2mL collection tubes and centrifuged at 20,800g for 5 min. The collection tubes were discarded. The spin column was placed in a new collection tube (1.5mL) and 14µL of RNase-free water was added directly to the centre of the spin column membrane. Columns were centrifuged for 1 minute at full speed 20,800g to elute the RNA.

2.10.3 RNA quantification

RNA was quantified using a Nanodrop 2000 (Thermo Scientific, Massachusetts, USA). 1µl RNase free ddH_2O was initially used to calibrate the system. One microlitre of sample was pipetted onto the measurement pedestal of the nanodrop and quantified. The purity of RNA was assessed though the ratio of absorbance at 260/280. RNA absorbs at 260nm whilst protein, phenol and other contaminants absorb strongly at or close to 280 nm. A ratio of ~2.0 is generally accepted as "pure" for RNA. If the 260/280 ratio is considerably lower, it may indicative of protein, phenol or other contaminants being present. The 260/230 ratio is a secondary measure of nucleic acid purity. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm suggesting phenol contamination or excess guanidine.

2.10.4 Complimentary DNA (cDNA) synthesis

First-strand cDNA was generated from the purified RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hertfordshire, UK).

- 5 x iScript reaction mix
- iScript reverse transcriptase
- Nuclease-free water

cDNA synthesis was carried out by combining 4µL of 5 x iScript reaction mix and 1µL of iScript reverse transcriptase to create a reaction master mix. One microgram sample of template RNA was combined with 5µL of master mix in DNase-RNase free eppendorfs. Nuclease free water was added to each sample to bring the total volume of each reaction mixture up to 20µL. Separate reaction mixes were prepared in which template RNA was combined with the 5x iScript reaction mix and nuclease free water only with a total volume of 20 µL. Samples were assessed for the presence of genomic DNA. Each reaction mix was incubated in a PCR thermal cycler (Bio Gene Rapid cycler, Idaho Technology, Idaho Falls, USA) using the following reaction protocol: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, 5 minutes at 4°C. Newly synthesised cDNA was diluted 1:100 into new

DNase-RNase free eppendorfs with nuclease-free water. Samples were stored in -20°C until further analysis.

2.11 qPCR

- SensiMix SYBR & Fluorescein Kit (Bioline, UK).
- cDNA Sample
- Forward and Reverse Primer

Twelve and a half microliters of qPCR SensiMix containing SYBR green fluorescence was combined with 2µL of forward and 2µL of reverse primer formed the PCR reaction master mix within each well of a 96-well plate. 8.5µL of cDNA sample was added to each master mix in triplicate. The plate was sealed and run under conditions to suit the primers being analysed (primer sets are described in methodology of results chapters). The PCR reaction was carried out using a PCR thermal cycler (Bio Gene Rapid cycler, Idaho Technology, Idaho Falls, USA). The specificity of the PCR products was determined by melt curve analysis and agarose gel electrophoresis.

Fluorescence detected by the intercalation of SYBR green in double stranded DNA was quantified during each cycle allowing the threshold of each gene to be obtained (Ct value). The delta-delta Ct ($\Delta\Delta^{Ct}$) method was used to quantify changes in gene expression between the control and experimental samples (Livak and Schmittgen, 2001). Genes of interest were standardised against a housekeeping gene. Data were expressed as fold change.

Primer Name (ID)	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size (BP)
TNF- α	CCCAGGGACC TCTCTCTAATC	ATGGGCTACA GGCTTGTCACT	360
IL-6	GGTACATCCT CGACGGCATCT	GTGCCTCTTTG CTGCTTTCAC	81
KC (CXC1)	ATGGCCCGCGCTGCT CTCTC	GTTGGATTTGTCACT GTTCAG	344
RANTES (CCL5)	GCTGTCATCCTCATT GCTACTG	TGGTGTAGAAATACT CCTTGATGTG	175
MCP-1 (CCL2)	CCCCAGTCACCTGCT GTTAT	TGGAATCCTGAACCC ACTTC	171
Eotaxin 1	AGGAGAATCACCAGT GGCAAAT	GCACAGATATCCTTG GCCAGTT	451
MIP-1 α	CCCGGTGTCATCTTC CTAAC	GTGTGGCTGTTTGGC AACAAC	236
MIP-1 β	AGCTTCCTCGCAACT TTGTG	AGTCCTGAGTATGGA GGAGA	241
IL-8	CCAGGAAGAAACCAC CGGA	GAAATCAGGAAGGCT GCCAAG	92

Table 2.3 Sequences of the specific primers used for q-PCR.

Primer Name (ID)	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size (BP)
GAPDH	TGAAGGTCGGAGTC AACGGATTTGGT	CATGTGGGCCATGAGG TCCACCAC	983
B-Actin	ATTGGCAATGAGCG GTTCC	GGTAGTTTCGTGGATG CCACA	87
B2M	GAGTATGCCTGCCG TGTG	AATCCAAATGCGGCAT CT	110

Table 2.4 Sequences of the specific primers used for q-PCR amplification of housekeeping genes.

2.12 Preparation of human muscle samples for analysis of mitochondrial respiration, ROS production and mitochondrial membrane potential

2.12.1 Preparation of permeabilised muscle fibre bundles

Skeletal muscle samples were obtained from the VL muscle using Acecut needle biopsy performed while the subjects were under local anaesthesia. Approximately 80 mg of muscle was collected.

- Saponin (50µg/ml) in Buffer A
- Buffer A
- Buffer B
- Buffer Z

Muscle biopsy samples were immediately placed into ice-cold stabilizing buffer A composed of 2.77 mM CaK_2 ethylene glycol*bis*-(2-aminoethylether)- *N*,*N*,*N*=,*N*=-tetraacetic acid (EGTA), 7.23mMK_2 EGTA, 6.56mM $MgCl_2$, 0.5mM dithiothreitol (DTT), 50mM 2-(*N*-morpholino) ethanesulfonic acid potassium salt (KMES), 20 mM imidazol, 20 mM taurine, 5.3 mM Na_2 ATP, and 15 mM phosphocreatine, pH 7.3 at 4°C. Visible fat and connective tissue were both removed and the muscle was manually teased into small fibre bundles. Once the mechanical separation into fibre bundles was completed, fibre bundles were transferred into buffer A supplemented with 0.05 mg/ml saponin for 30 min at low rocking speed to selectively permeabilise the sarcolemma (Gouspillou et al., 2014).

Fibre bundles prepared for respiration analyses were then washed 3 times 10 min in buffer B, composed of 2.77 mM CaK_2 EGTA, 7.23 mM K_2EGTA, 1.38 mM $MgCl_2$, 3.0 mM K2HPO_4, 0.5 mM DTT, 20mM imidazole, 100 mM K-MES, and 20 mM taurine, pH 7.3 at 4°C, supplemented with fatty acid-free bovine serum albumin (BSA: 2 mg/ml).

Fibre bundles prepared for ROS production analyses were then washed 3 times for 10 min in buffer Z, composed of 110 mM K-MES, 35 mM KCl, 1 mM EGTA, 3 mM $MgCl_2$, and 10 mM K_2HPO4, pH 7.3 at 4°C, supplemented with BSA (5 mg/ml).

2.12.2 Mitochondrial respiration

- Glutamate (5mM) and Malate (5mM) (G/M)
- ADP (2mM)
- Buffer B (See Section 2.9.1 for details)

An oxygen electrode measurement systems from Hansatech Instruments Ltd, King's Lynn, UK was utilised to assess mitochondrial respiration (Clark et al., 1956). Within the instrument, a S1/MINI Clark type electrode is contained at the bottom of a water-jacketed respiratory chamber (Walke, 1987). The temperature of the chamber was set at 37 °C and sealed with a plunger. Oxygen dissolves in the chamber of the liquid-phase system and is detected polarographically by the electrode. Four hundred and fifty microliters of buffer B (2.77mM CaK_2 EGTA, 7.23mM K_2EGTA, 1.38mM $MgCl_2$, 3.0mM K_2HPO_4, 0.5mM DTT, 20mM imidazole, 100mM K-MES, and 20mM taurine, pH 7.3 at 4°C) was added to the chamber alongside an electromagnetic stirrer bar to mix its contents. Following a period of stabilisation 10µL of 10mM glutamate + 5mM malate was added into the oxytherm chamber. After 3 minutes, the permeabilised myofibre bundles (14-16mg wet weight) were added into the chamber. After a further three minutes 2mM ADP was added into the chamber. The bundles were left until all the O_2 within the chamber had been utilised or the muscle had run out of substrates. After respiration measurements were completed, bundles were removed and placed in liquid nitrogen and stored at -80°C for protein quantification. Analysis of the oxygraph results is subject to interpretation. Analysis of the output was completed by 2 individuals blinded. Respiratory control index (RCI) was calculated by dividing state 3 by state 4 respiration and the efficiency of oxidative phosphorylation was determined by calculating the ratio of ATP amount to consumed O_2 during state 3 respiration (P:O ratio) (Gnaiger et al., 2000).

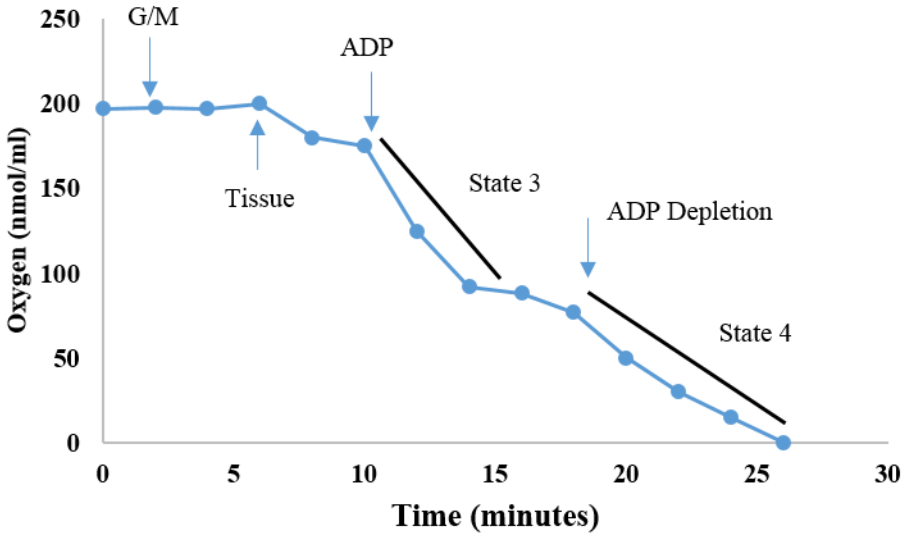


Figure 2.5 Schematic diagram of a typical oxygraph output. G/M (Glutamate/Malate), ATP (Adenosine Triphosphate).

2.13 Analysis of mitochondrial ROS production in skeletal muscle

2.13.1 Mitochondrial H_2O_2 generation – Amplex Red Assay

- Hydrogen Peroxide Solution (Sigma Aldrich, Dorset, UK)
- Amplex Red Reagent (Life Technologies, UK)
- Horse Radish Peroxidase (HRP) (Sigma Aldrich, Dorset, UK)
- Superoxide Dismutase from bovine erythrocytes (Sigma Aldrich, Dorset, UK)
- Buffer Z (Recipe – See Section 2.9)
- Glutamate/Malate (5mM) (Sigma Aldrich, Dorset, UK)
- Succinate (0.5M) (Sigma Aldrich, Dorset, UK)
- Rotenone (1mM) (Sigma Aldrich, Dorset, UK)
- Antimycin Alpha (Sigma Aldrich, Dorset, UK)

Mitochondrial hydrogen peroxide release was measured using Amplex Red- HRP (Molecular Probes, Eugene, OR, USA) as previously described (Muller et al., 2004, Vasilaki et al., 2006). Rate of ROS production was detected by measuring the rate of resorufin red formation at an excitation/emission wavelength of 571/585 nm.

Resorufin red is formed through the hydrogen peroxide-dependent oxidation of the non-fluorescent Amplex Red compound. This process is catalysed by horseradish peroxidase (HRP). Resorufin is formed by the 1:1 reaction of H2O2 and Amplex Red. The assay only detects hydrogen peroxide which has been released from the mitochondria. This is a result of size constraints which restricts HRP from entering the mitochondria. To preventing any interaction between superoxide molecules and HRP, CuZnSOD was added to ensure superoxide was converted to hydrogen peroxide.

At the onset of the experiments a standard curve was generated using successive amounts of H_2O_2. Each permeabilised myofibre bundles, prepared as described previously, were added to a single well within a 96 well microplate containing l00μL Amplex Red solution (19.44mM Amplex Red, 5U/ml HRP, 37.5U/μL SOD in Buffer Z). Samples were incubated in the dark at 37°C for 5 minutes. Substrates were added as follows Glutamate/Malate (10 + 5 mM), succinate (10 mM), ADP (100M), and Antimycin Alpha (10 μM). H_2O_2 production was measured using a Flurostar Optima (BMG Labtech). The increase in fluorescence produced a slope; this converted to the rate of hydrogen peroxide production with a standard curve. At the end of the ROS measurements, muscle fibre bundles were placed in liquid N2 and stored at -80°C. H_2O_2 production was expressed as picomoles per minute per protein content of the sample.

Well	1	2	3	4	5	6	7	8	9	10	11	12
	Amplex Red Solution Only		Bundle of Fibres Only		Bundles + Glutamate Malate		Bundles + Succinate		Bundles + Succinate Rotenone		Bundles + Antimycin A	

Table 2.5 Representative row from a 96 well plate illustrating the Amplex Red assay preparation.

2.13.2 Mitochondrial superoxide generation. fluorescence-based method to measure mitosox red oxidation

- MitoSOX Red (Invitrogen, California, USA)

MitoSOX Red (Invitrogen, California, USA)

To monitor changes in mitochondrial superoxide, a MitoSOX Red mitochondrial superoxide indicator (Mito-HE) probe was utilised. MitoSOX Red is a derivative of dihydroethidium and is a highly sensitive mitochondrial superoxide indicator which exhibits fluorescence upon oxidation by O_2^- (superoxide) (Robinson et al., 2006). The reaction between superoxide and MitoSOX Red generates a highly specific red fluorescent product, 2-hydroxyethidium (2-OH-Mito-E^+) (Zielonka and Kalyanaraman, 2010). Isolated fibres from the VL muscle were loaded with 250nM MitoSOX Red (Invitrogen, California, USA) for 30min as previously described (Sakellariou et al., 2013, Pearson et al., 2015). Fibres were maintained in buffer Z containing MitoSOX Red (20nM) during the experimental period. Fluorescence was quantified at an excitation/emission wavelength of 405/605nm.

Fluorescence images were obtained using a C1 confocal laser scanning microscope (Nikon, Surrey, UK) equipped with a 405nm excitation diode laser, a 488nm excitation argon laser, and a 543nm excitation helium-neon laser. Emission fluorescence was detected through a set of 450/35, 515/30, and 605/15 emission filters. Using 10x magnification, fluorescence images were captured and analysed with the EZC1 V.3.9 (12bit) acquisition software. Individual images acquired from each section were merged into a single high-resolution image using Adobe photoshop CS5.

2.14 Mitochondrial membrane potential

In order to assess potential changes in mitochondrial membrane potential ($\Delta\Psi m$) in intact mitochondria of isolated VL fibres, fibres were loaded with a florescent dye, tetramethylrhodamine, methyl ester (TMRM), as previously described in (Irwin et al., 2003). Changes in TMRM fluorescence of isolated skeletal muscle fibres were

assessed by loading fibres with 30nM TMRM for 30 minutes and 20nM TMRM for 30 minutes (Invitrogen, California, USA). Fluorescence was detected at an excitation/emission wavelength of 543/605nm. Changes in ΔΨm were determined in the presence of oxidative phosphorylation inhibitors; oligomycin (2.5μM) and FCCP (4μM). Images obtained using a C1 confocal laser scanning microscope (Nikon, Surrey, UK) as described above in Section 6.2.6.

2.15 Analysis of mitochondrial content in skeletal muscle

Citrate Synthase Assay

- MitoCheck Citrate Synthase Activity Assay Kit (Cayman Chemical Michigan, USA)

Citrate synthase is a mitochondrial enzyme, which is commonly used as a normalization factor for mitochondrial protein (Wiegand and Remington, 1986, Lopez-Lluch et al., 2006). It can also be used as a biomarker for mitochondrial content in a tissue homogenate. The study used a MitoCheck Citrate Synthase Activity Assay Kit (Cayman Chemical Michigan, USA) to determine mitochondrial content in muscle homogenates from CFS and control subjects. Product protocol was followed accordingly. The assay measured the production of SH-CoA by monitoring the absorbance of citrate synthase developing reagent.

The condensation of dicarboxylate oxaloacetate and acetyl CoA to the tricarboxylate citrate is catalysed by the enzyme citrate synthase. It is within this reaction that carbon molecules (as acetyl CoA) obtained from pyruvate oxidation are fed into the tricarboxylic acid (TCA or citric acid) cycle.

Samples of tissue homogenate were diluted 1:200 in assay buffer. Fifty microliters of 2% Citrate Acetyle Co-A Reagent, 2% Citrate Synthase Developer Reagent in Assay buffer was added to each well of a 96-well plate with 30μl of sample. A positive control was also run on the plate to ensure the kit is functioning correctly. Twenty microliters of 4% citrate synthase oxaloacetate reagent in assay buffer was added to each well. The plate was immediately placed in the microplate spectrophotometer (Bio-Tek instruments, Vermont, USA). Absorbance was measured at 412nm.

2.16 *Ex vivo* single muscle fibre analysis

Single fibre force analysis was undertaken by Dr Adam Lightfoot. Single muscle fibres isolated from the VL muscle were maintained in ice-cold stabilizing buffer A composed of 2.77 mM CaK_2 ethylene glycol*bis*-(2-aminoethylether)-*N*,*N*,*N*=,*N*=-tetraacetic acid (EGTA), 7.23mMK_2 EGTA, 6.56mM$MgCl_2$, 0.5mM dithiothreitol (DTT), 50mM 2-(*N*-morpholino) ethanesulfonic acid potassium salt (KMES), 20 mM imidazole, 20 mM taurine, 5.3 mM Na_2 ATP, and 15 mM phosphocreatine, pH 7.3 at 4°C (Gouspillou et al., 2014). Fibre bundles were transferred into relax solution for 15 minutes on ice. Fibres were mounted onto an 802D permeabilised fibre apparatus (Aurora Scientific, Canada). Skinned myofibres were attached to insect pins affixed to a 403A (5mM) force transducer and 312C length controller, using ultra-fine nylon thread (see Figure 2.7). Fibres were maintained in relax solution throughout assembly. Sarcomere length (SL) was measured using 900B Video Sarcomere Length (VSL) software (Aurora Scientific, Canada). SL was adjusted on each fibre to 2.4-2.6μm, fibre diameter was measured at four intervals along the length, and a circular circumference assumed for the basis of CSA calculation. Fibres were maximally activated in Ca^{2+} activating solution (pCa 4.5), containing in addition to Ca^{2+}, (in mM) 5.3 MgATP, 1 free Mg^{2+}, 20 imidazole, 7 EGTA, 19.6 PCr, and 64 KCl (pH 7.0 at 4°C). Peak force was recorded and normalised to fibre CSA (Degens et al., 2010). This approach allows force generated by sarcomere proteins to be explored independent of fibre number, innervation, ATP levels and calcium release (Sartori et al., 2013).

Figure 2.6 Representative image of a skinned isolated myofibre from *VL* muscle, attached to a force transducer and high-speed length controller. Scale bar, 400μm.

2.17 *In Vivo* Muscle function testing

Muscle Function testing was completed in the Research Institute for Sport and Exercise Science at Liverpool John Moores University in collaboration with Dr Graeme Close and Daniel Owens.

2.17.1 Assessing muscle function

Muscle function was assessed using a Biodex isometric dynamometer (Biodex Medical Systems Inc. Shirley, NY, USA), previously validated for its use in reliable assessment of muscle function variables related to force production, (Drouin et al., 2004). Participants were seated as per the manufacturer's guidelines with a 90^{o} flexion of the hip and non-extendable straps crossing the chest and abdomen and across the quadriceps to maximise isolation of the target muscle group. Information collected from the dynamometer was visualized and analysed using AcqKnowledge v.3.7.2 software (BIOPAC systems Inc. Santa Barbara, CA) (Owens et al., 2014).

2.17.2 Assessing maximal voluntary contraction

Once seated in the dynamometer as described, the angle of knee extension was set at 80^{o} (assuming 0^{o} as full extension). Participants were instructed to give 5 submaximal voluntary isometric contractions, increasing in intensity before providing a maximal voluntary contraction, recorded in newton meters (nm). In an attempt to ensure maximal contractions were obtained, verbal encouragement was given to all participants and bio-feedback made available through projection of the force trace for the participants to view. The study investigated difference in peak force during MVC between subjects as previously executed in CFS research (Paul et al., 1999). Peak force is the highest level of force achieved during an isometric contraction. This generally occurs during the first 2 seconds of contraction.

2.17.3 Involuntary contraction

For the involuntary stimulation of the knee extensors, two 3 x 5 inch (8 x 13 cm) oval self-adhesive stimulating electrodes (Chattanooga, DJO Global, CA, USA) were placed approximately 2 inches above the knee joint across the *vastus medialis* (VM) and proximal to the acetabulofemoral joint across the *vastus lateralis* (VL) and *rectus femoris* (RM). Participants were required to contract the quadriceps to

make the muscle body more apparent prior to electrode placement to minimise antagonist co-activation, although this was not monitored with electromyography during stimulation. Stimulation was applied through the surface electrodes, delivered via a BIOPAC systems MP100 stimulator (BIOPAC systems Inc., Santa Barbara, CA).

2.17.4 Force frequency relationship

An electrical stimulus was first delivered at 100 Hz with amplitude (mA) to elicit 30% of the participants MVC force. Following this, at the determined amplitude, 1 second trains of 50, 20 and 10 Hz and a single 1 Hz twitch were delivered in a random order to generate a force frequency relationship.

2.17.5 Fatigue protocol

Fatigue resistance was determined from two minutes repeated stimulation with 30 Hz, 1 sec trains interspersed by 1 sec relaxation (total of 60 evoked contractions equating to 60 seconds of total stimulation). Fatigue index was calculated as the percentage force loss from the initial recorded contraction to the final recorded contraction.

2.15 Statistics

Data are presented as mean ± SEM for each experiment. Single comparisons between two experimental conditions at a time point were undertaken using the unpaired Student's t test. For multiple comparisons analyses, One-way ANOVA followed by the post hoc LSD test was used. A P-value <0.05 was considered to be statistically significant with the exception of the Bio-Plex Luminex® cytokine analysis in which multiple comparisons were made in one experimental procedure. There is a greater probability of significance in multiple analysis assays. This was accounted for using the Bonferroni method of correction. SPSS 18 was used to analyse all data.

Chapter Three

Resveratrol attenuates TNF-α induced cytokine release from C2C12 myotubes

3.1 Introduction

Research over the last two decades has clearly demonstrated the ability of skeletal muscle to act to as an endocrine organ, releasing an array of different peptides into systemic circulation (Ostrowski et al., 1998, Pedersen, 2011, Iizuka et al., 2014, Lightfoot et al., 2015). These proteins mediate a broad range of processes including cell signalling pathways as well as critical aspects of cell behaviour, including differentiation, proliferation and survival (Nathan and Sporn, 1991). More specifically, skeletal muscle has been shown to produce an array of protein and polypeptide cytokine molecules, collectively termed myokines. As previously described in Section 1.3.1, the term cytokine includes a large family of polypeptides and proteins. This includes interleukins, chemokines and interferons, members of the tumour necrosis factor group, transforming growth factors as well as growth and colony-stimulating factors (Dinarello, 2007). Cytokines play a crucial role in the cell communication throughout the body. They are secreted by cells, and through their respective receptors play a regulatory role as intercellular messengers in both a local environment or systemically (Zhang and An, 2007). Pedersen and colleagues were among the first to identify the production and secretion of cytokines from skeletal muscle (Ostrowski et al., 1998, Pedersen et al., 1998). These authors termed muscle secreted cytokines as myokines. Evidence has shown that myokine production is augmented during periods of disease as well as during contractile activity (Benatti and Pedersen, 2015). Figure 3.1 shows skeletal muscle is a secretary organ expressing an array of cytokines.

The first cytokine to be identified as a product of skeletal muscle cells was IL-6, a pro-inflammatory cytokine secreted by muscle in response to exercises independent of muscle damage and released from muscle cells *in vitro* in response to inflammatory stimuli (Ostrowski et al., 1999, Nagaraju et al., 1998, Ostrowski et al., 1998). In contrast, IL-6 has also been shown to have an anti-inflammatory effects including the inhibition of endotoxin-induced tumour necrosis factor-α (TNF-α) secretion (Keller et al., 2006). The following cytokines have been reported as being produced and secreted by skeletal muscle: TNF-α, IL-6, IL-8, IL-15, IL-18, CXCL1, CCL2, CCL5 and brain-derived neurotropic factor (BDNF) (Pedersen, 2012). The precise function and expression pattern of these cytokines varies in response to changing conditions. Elevated myokine release can occur after a wide

variety of stimuli, ranging from inflammatory cytokines, infection and endoplasmic reticulum stress (Lightfoot et al., 2009, Schulte et al., 2013). Knowledge in this area is still relatively sparse and unclear.

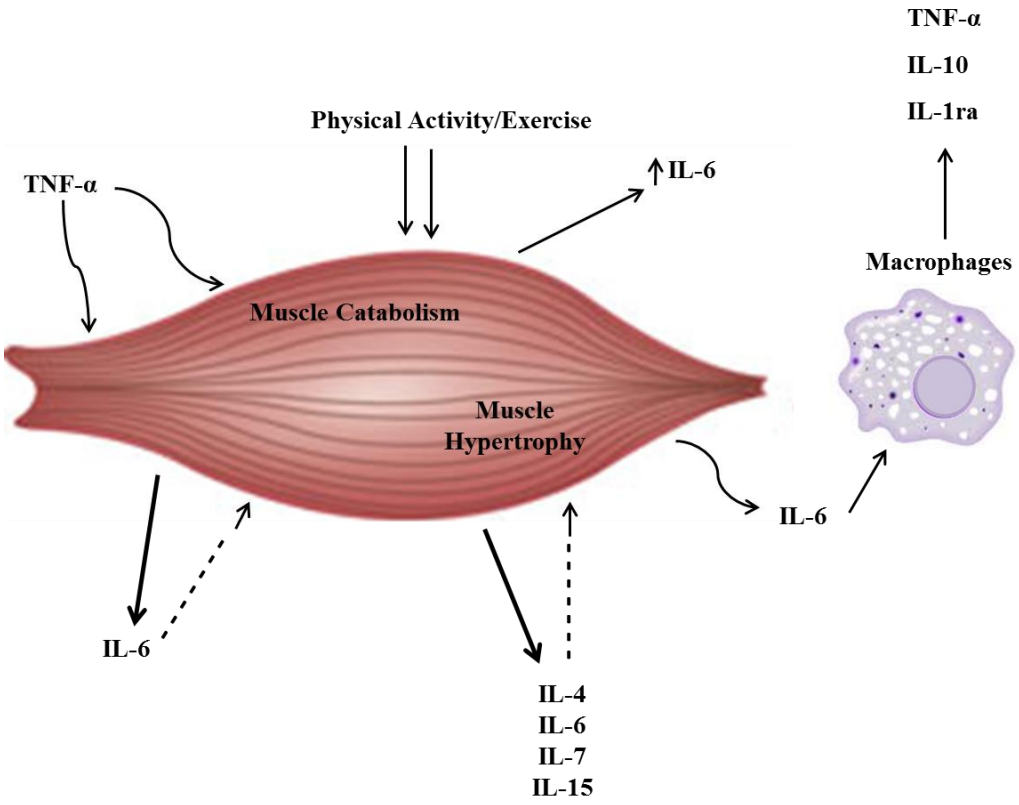


Figure 3.1 Schematic diagram showing muscle as a secretory organ. Adapted from (Benatti and Pedersen, 2015). Potential sources of TNF-α include macrophages, endothelial cells, adipose tissue, mast cells and fibroblasts.

A growing body of evidence is emerging which details the extent to which cytokines are involved in the regulation of skeletal muscle through the mediation of muscle satellite cell activation, proliferation, and differentiation as well as muscle atrophy (Kurek et al., 1998). Elevated systemic cytokine levels perturb normal skeletal muscle physiology and function (Spate and Schulze, 2004, Toth et al., 2006, Reid and Moylan, 2011). For instance, research has shown that IL-6 knock-out mice have delayed accretion of muscle mass during muscle recovery from disuse atrophy (Washington et al., 2011).

The presence of raised systemic levels of cytokines is a common feature of many chronic diseases including cancer, sepsis, myositis and COPD (Gan et al., 2004, McMillan, 2013, Lightfoot et al., 2015). Muscle atrophy, weakness and fatigue are

commonly reported features in such diseases as chronic heart failure, cancer, AIDS and ageing (Engelen et al., 2000, Spate and Schulze, 2004).

There is evidence to suggest that diseases in which muscle fatigue is a prominent feature are characterised by elevated systemic TNF-α (Moss et al., 1999). TNF-α is the most widely studied and prominently linked cytokine to muscle pathophysiology. TNF-α is a potent, multifunctional, pro-inflammatory cytokine, associated with numerous diverse physiological and pathological roles. TNF-α was initially associated with muscle dysfunction and fatigue and has since been identified as a key mediator of muscle function, affecting muscle growth and differentiation as well as cell death and muscle atrophy (Reid and Li, 2001, Reid and Li, 2001). Muscle catabolism is commonly found in a wide range of patients suffering from diseases where cytokine levels are raised including sepsis and chronic obstructive pulmonary disease (COPD) is attributed to elevated levels of circulating TNF-α (Tisdale, 1999, Farber and Mannix, 2000). Evidence suggests that TNF-α mediated skeletal muscle wasting occurs through activation of the NF-κB pathway in muscle cells (Li et al., 1998, Guttridge et al., 2000, Cai et al., 2004). Previous work from our research group has demonstrated that C2C12 myotubes when exposed to increased levels of TNF-α, release significantly greater levels of cytokines than control, untreated cells (Lightfoot et al., 2015). Moreover, evidence suggests that TNF-α induced cytokine secretion is, at least in part, mediated through increased RONS production given the increase in mitochondrial superoxide production from myotubes (Reid and Moylan, 2011).

Thus, muscle is a source of inflammatory cytokines of which TNF-α is a major regulator of cytokine release which can in turn modify muscle function. Additional studies have reported lipopolysaccharide (LPS), IL-1α, IL-1β and IFN-γ as potential modulators of cytokine release from muscle (Nagaraju et al., 1998, Lappas et al., 2004). Evidence suggests patients suffering from diseases characterised by fatigue such as patients with CFS may have perturbed cytokine profiles. Intervention studies aiming to counteract raised systemic cytokine levels seen in some disease groups have taken several approaches; these include anti-TNF therapy, and dietary interventions (Charles et al., 1999, Cunningham-Rundles et al., 2005). Naturally occurring polyphenol and polyphenolic compounds in our diet have been suggested to play a role in reducing pro-inflammatory cytokine levels

and scavenge free radical species. There is a need to investigate the effect of these polyphenolic substances on muscle myokine release and muscle function.

Polyphenols are naturally occurring compounds found in fruits, vegetables and grains (Section 1.4), produced by the plants defence mechanism in response to stresses. Over 8000 polyphenolic compounds have currently been identified. These can be classified into 4 four main groups; phenolic acids, flavonoids, stilbenes and lignans (Spencer et al., 2008). A number of polyphenols including curcumin, resveratrol and EGCG have been identified as potent anti-inflammatory compounds (Gonzalez et al., 2011).

Curcumin (diferferuloylmethane) is a natural diphenylheptanoid polyphenol, derived from the plant *Curcuma longa* and the primary active constituent of Turmeric. Curcumin has been used in medical practice as an anti-inflammatory in many Asian countries for centuries (Ammon and Wahl, 1991). Curcumin inhibits the release of a number of cytokines from numerous cell types including monocytes and fibroblasts (Abe et al., 1999, Kloesch et al., 2013). There have not been any studies investigating the effect of curcumin on cytokine release by skeletal muscle cells. However, reports from human airway smooth muscle cells (HASMC) showed a concentration-dependent reduction in IL-17–induced IL-8 production (Wuyts et al., 2005). The study identified NF-κB play as essential in IL-17-induced IL-8 production in HASMC *in vitro.*

Epigallocatechin-3-O-gallate (EGCG) is one of the major polyphenols found in green tea. Epidemiological studies continue to suggest that there may be significant health benefits associated with drinking green tea (Saito et al., 2015). Further research has identified potent antioxidant, chemopreventive and anti-tumour activity from EGCG. EGCG pre-treatment prevented atrophy in C2C12 myotubes exposed to increased levels of TNF-α, by reducing protein synthesis and increase in protein degradation (Mirza et al., 2014). *In vivo* studies have shown that EGCG to improves muscle function in dystrophic mdx5Cv mice (Dorchies et al., 2006) and reduces contractile dysfunction in unloaded skeletal muscle (Ota et al., 2011).

Resveratrol (trans-3,4',5-trihydroxystilbene) is a non-flavonoid polyphenolic compound with natural antioxidant properties. Resveratrol is one of the most widely studied phenolic acids and has been associated with numerous health benefits

including anti-inflammatory and antioxidant activities. Both *in vivo* and *in vitro* studies using nutritionally relevant concentrations of resveratrol demonstrated the ability of resveratrol to inhibit inflammatory processes, including activation of NF-κB and the expression inflammatory genes in human monocyte (THP-1) and macrophage (U937 and RAW 264.7 cells) cell lines (Tsai et al., 1999, Holmes-McNary and Baldwin, 2000, Yamamoto and Gaynor, 2001). Analysis of cellular cytokine release revealed that pre-treatment with resveratrol reduced the secretion of CCL2, IL-6 and IL-8 by HASMCs stimulated with LTA (lipoteichoic acid) (Knobloch et al., 2014).

There are no published findings regarding the effect of curcumin, resveratrol or EGCG on cytokine release by muscle cells.

3.1.2 Hypothesis

Treatment of C2C12 myotubes with TNF-α results in cytokine release and this can be altered by pre-treatment of cells with polyphenols.

3.1.3 Aims

The current chapter aims to:

- Validate and expand a model of TNF-α treatment of murine C2C12 myotubes to induce release of cytokines without significant cell death.
- Determine the effect of a range of concentrations of TNF-α exposure for up to 24 hours on cytokine release from C2C12 myotubes.
- Identify an appropriate concentration of resveratrol, curcumin and EGCG which does not result in death of murine C2C12 myotubes.
- Determine the effect of pre-treatment of C2C12 myotubes with resveratrol, curcumin or EGCG on TNF-α induced cytokine release.

3.2 Methods

3.2.1 Establishment of a suitable time course and concentration of TNF-α treatment in C2C12 myotubes

Murine C2C12 myoblasts (Yaffe and Saxel, 1977) were grown in 6 well plates tissue culture (Costar, UK). Myoblasts were seeded at $1x10^5$ cells/well in 10% FBS, DMEM as described in Section 2.1. Upon reaching 60-70% confluence, cells were differentiated into myotubes in growth media supplemented with 2% HS over a 6-day period; cellular differentiation was assessed periodically over the duration. Following 6 days in differentiation media, myotubes were treated with 1, 5 and 25ng/ml endotoxin free TNF-α (murine) (Research and Development Systems, Oxford, UK) and myotubes were examined at 3, 8 and 24 hours.

3.2.2 Choice of concentration of TNF-α

The choice of TNF-α concentrations (as shown in Table 3.1) used in this study was consistent with previous work from our research group which reported an increase in cytokine release from C2C12 myotubes at 3 hours following exposure to 25ng/ml TNF-α for 3 hours (Lightfoot et al., 2015).

Time Point	**TNF-α Treatment**			
3 hrs	**Control**	**1ng/ml**	**5ng/ml**	**25ng/ml**
8hrs				
24hrs				

Table 3.1 Cell Treatments - Time course and concentration of TNF-α treatment to C2C12 myotubes at 6-days following the addition of differentiation media.

3.2.3 Assessment of C2C12 myotube viability in response to TNF-α

Cellular viability was assessed using light-microscopy to identify any gross changes in cell morphology at 3, 8 and 24 hours post TNF-α treatment. A LIVE/DEAD assay (Invitrogen, UK) was carried out, as described in Section 2.1.5 to assess cellular necrosis in a semi-quantitative manner. Fluorescent confocal microscopy was used to visualise cell death. Observed changes in the % of necrotic cells were used as marker of cell viability.

3.2.4 The effect of TNF-α on cytokine release from C2C12 myotubes

The release of cytokines from C2C12 myotubes was assessed using bead based multiplex technology. A Bioplex® 200 Luminex immunoassay was used to analyse cell culture media and myotube homogenates for the presence of cytokines (Bio-Rad, Hercules, USA) as described in Section 2.3. At each respective time point, cyokine levels released into the cell culture media from TNF-α treated myotubes was compared with media from untreated control cells. Cytokine levels in cell culture media was quantified at 3, 8 or 24 hours from myotubes exposed to 1ng/ml, 5ng/ml or 25ng/ml TNF-α.

3.2.4 The effect of TNF-α on cytokine levels in C2C12 myotubes

Cytokine content of C2C12 myotubes was assessed using bead based multiplex technology. A Bioplex® 200 Luminex immunoassay was used to analyse homogenised cell samples re-suspended in PBS. Intracellular cytokine levels were normalised to total cellular protein, quantified using the BCA protein assay as described in Section 2.4.3.

3.2.5 Establishment of a suitable time course and concentration of polyphenol treatment in C2C12 myotubes

At six days following the addition of differentiating media, C2C12 myotubes were exposed to single polyphenol treatment (1 or 10μM curcumin, EGCG or resveratrol dissolved in DMSO) for 24 hours (Howells et al., 2007). DMSO was used as a vehicle carrier for all polyphenols due to the hydrophobic nature of EGCG and curcumin. Resveratrol can be dissolved in water or DMSO, however, to maintain uniformity across all treatment groups; DMSO was used for all three polyphenols. Polyphenol containing media was discarded and replaced with fresh differentiation

media containing carrier free recombinant TNF-α (5 or 25ng/ml) for 24 hours. Media was aspirated and stored for future analysis of cytokine content by Luminex® and cells were harvested in DPBS on ice and centrifuged at 14,000g, 4°C for 10 minutes. Cell pellets were stored at -80°C for analysis by western blotting, qPCR and Luminex®.

3.2.6 Choice and concentrations of polyphenols

The choice of polyphenol concentrations used in our study were 1µM and 10µM. This was in agreement with previous work from our research group investigating the effect of polyphenols and polyphenolic acids on immune cells (Ford et al., 2013) and *in vitro* polyphenol interventions (Wuyts et al., 2005). In this study, we used concentrations which were within the reported physiological range (Howells et al., 2007, Nicholson et al., 2010). Similar concentrations to those used in this study have been reported to reduce the release of pro-inflammatory cytokines through the down regulation of inflammatory signalling pathways (Gupta et al., 2014).

3.2.7 Pre-treatment of C2C12 myotubes with curcumin, EGCG or resveratrol prior to treatment with TNF-α

At six days following the addition of differentiating media, C2C12 myotubes were treated with 1 or 10µM curcumin, resveratrol or ECGC (Epigallocatechin-3-O-gallate) for 24 hours prior to treatment with TNF-α. Cells and cell media were harvested 24 hours after treatment with TNF-α.

3.3 Results

3.3.1 The effects of TNF-α on cytokines released by C2C12 myotubes

All concentrations of TNF-α induced a significant release of the cytokines IL-6, CXCL1 (KC), CCL2 (MCP-1) and CCL5 (RANTES) into cell culture media when compared with control wells.

Results showed a general trend in cyokine release in reponse to TNF-α treatments (Figures 3.1 to 3.4) whereby the increase in cytokine release appeared to be doses and time dependent. Data demonstrated that muscles released IL-6, CXCl1, CCL2 and CCl5 in response to treatment with TNF-α in C2C12 cells (Figure 3.2 – 3.5).

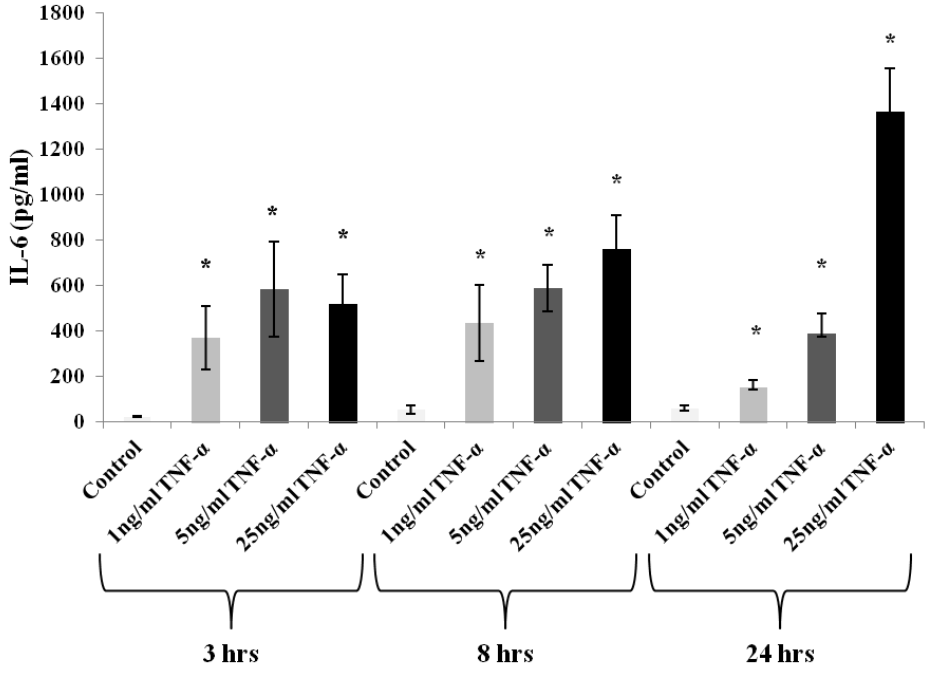


Figure 3.2 Level of IL-6 in cell cuture media from C2C12 myotubes at 3, 8, 24 hours following treatment with 1, 5 and 25ng/ml of TNF-α. Data are presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated controls for the same time point.

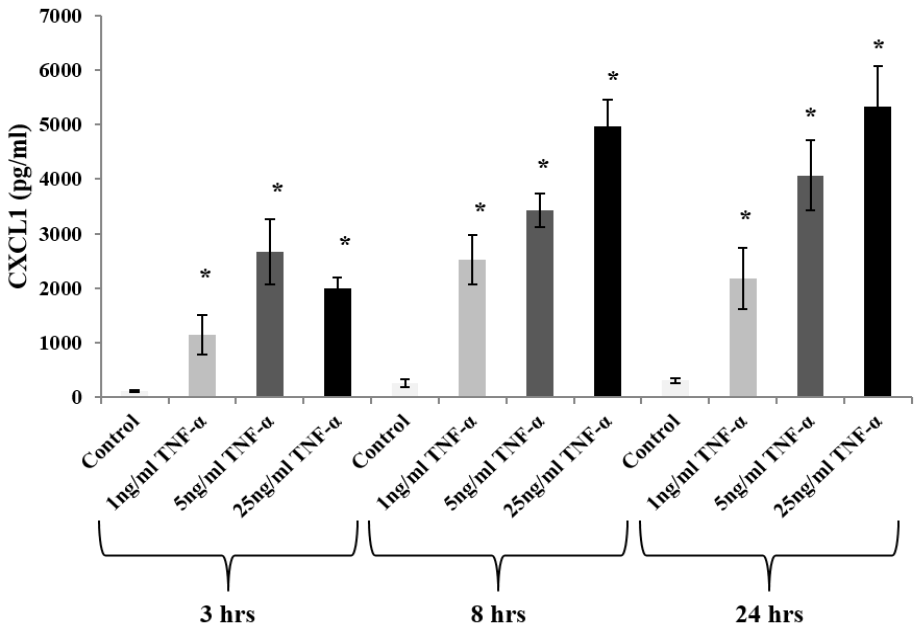


Figure 3.3 Level of CXCL1 (KC) in cell cuture media from C2C12 myotubes at 3, 8, 24 hours following treatment with 1, 5 and 25ng/ml of TNF-α. Data are presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated controls for the same time point.

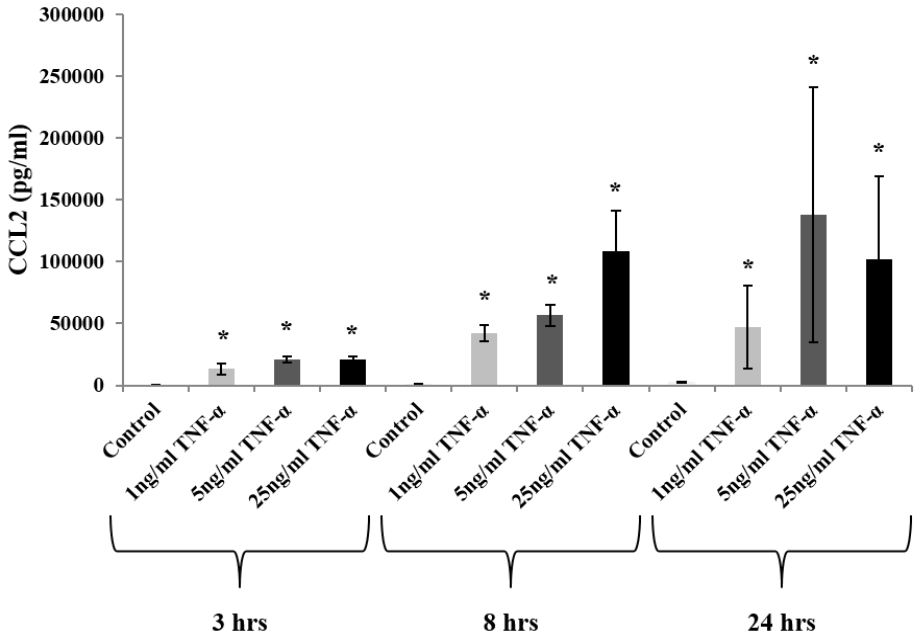


Figure 3.4 Level of CCL2 (MCP-1) in cell cuture media from C2C12 myotubes at 3, 8, 24 hours following treatment with 1, 5 and 25ng/ml of TNF-α. Data are presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated controls for the same time point.

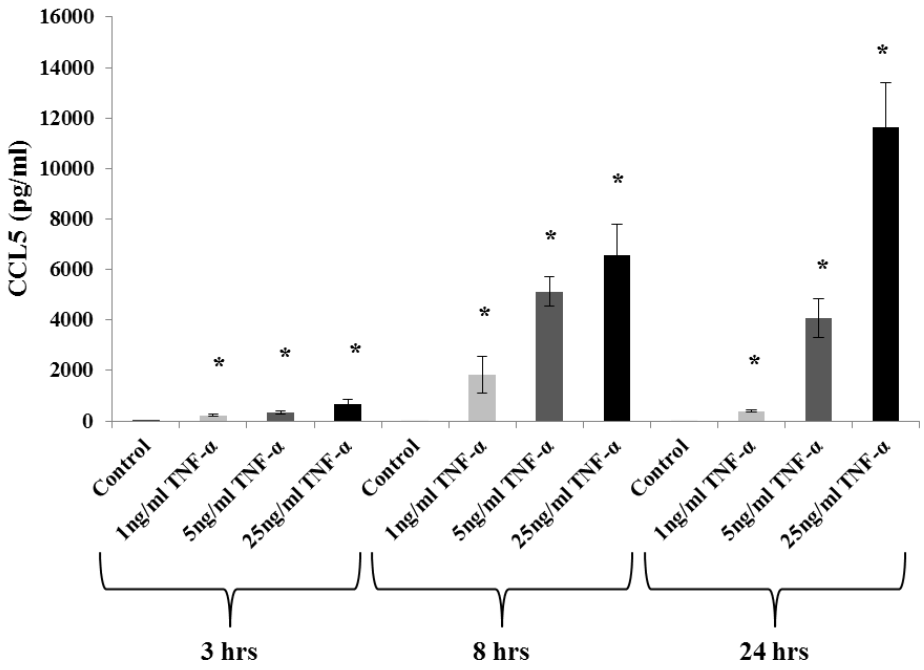


Figure 3.5 Level of CCL5 (RANTES) in cell cuture media from C2C12 myotubes at 3, 8, 24 hours following treatment with 1, 5 and 25ng/ml of TNF-α. Data are presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated controls for the same timepoint.

3.3.2 The effect of polyphenol treatment alone on the release of cytokines from C2C12 myotubes

10μM Polyphenol Treatments

C2C12 myotubes exposed to 10μM resveratrol alone for 24 hours did not elicit an increase in secretion of IL-6, CXCL1 (KC), CCL2 (MCP-1) and CCL5 (RANTES) into cell culture media (Figures 3.6 to 3.9). Media harvested from C2C12 myotubes treated with 10μM curcumin or 10μM EGCG at 24 hours had secreted significantly higher levels of IL-6, CXCL1 and CCL5 into the media compared with untreated or DMSO treated control cells. Media collected at 24 hours from DMSO (polyphenol vehicle carrier) treated cells revealed no difference in cytokine levels to media collected from untreated control cells.

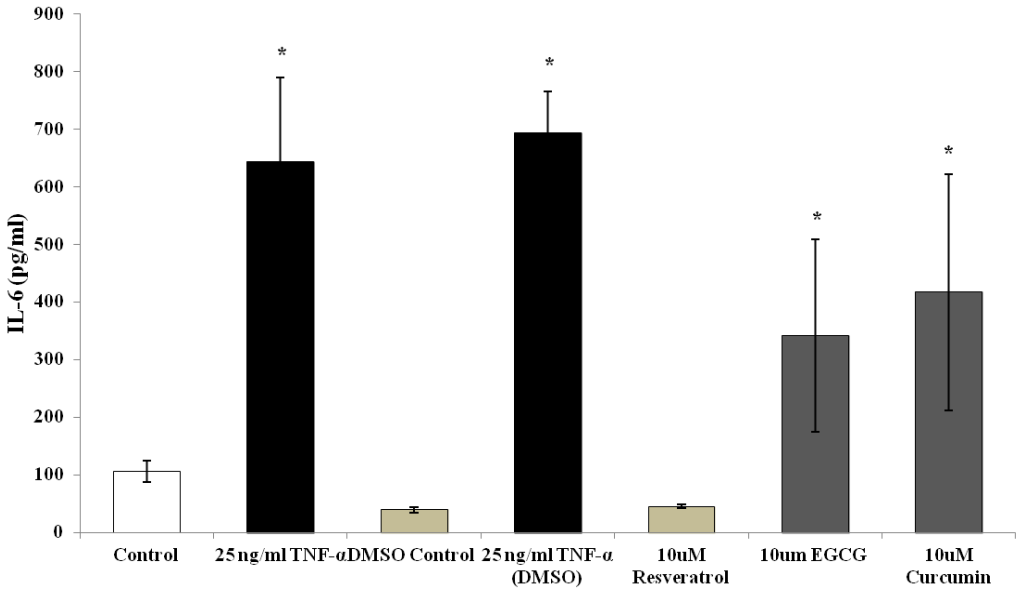


Figure 3.6 Levels of IL-6 in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α and 10μM resveratrol, 10μM EGCG, 10μM curcumin. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

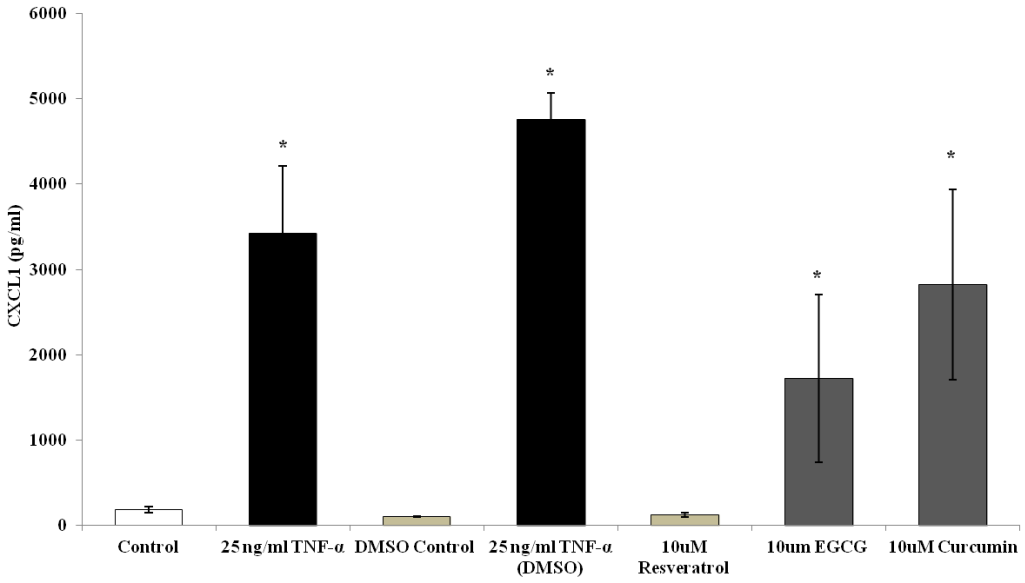


Figure 3.7 Levels of CXCL1 (KC) in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α and 10μM resveratrol, 10μM EGCG, 10μM curcumin. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

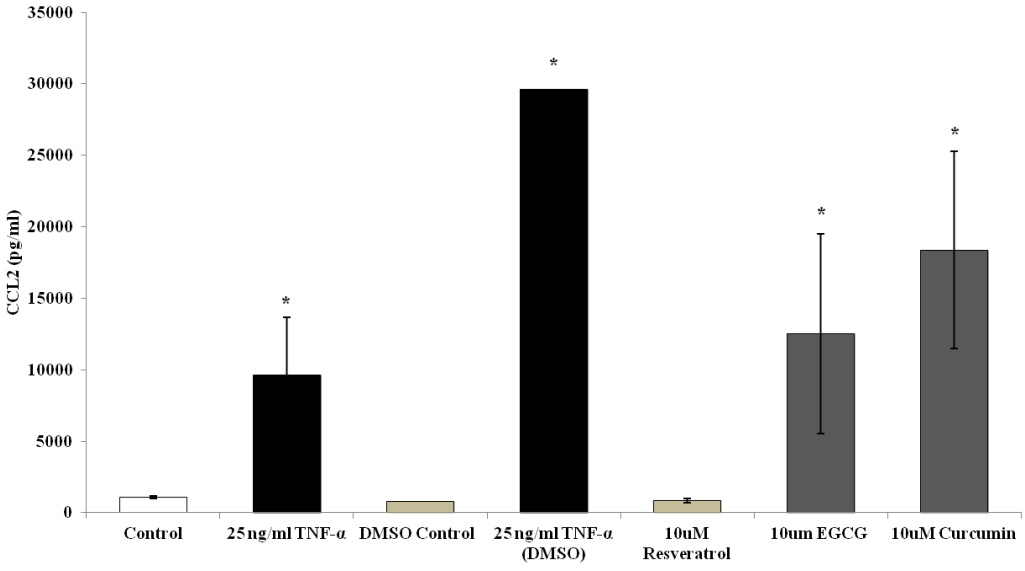


Figure 3.8 Levels of CCL2 (MCP-1) in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α and 10μM resveratrol, 10μM EGCG, 10μM curcumin. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

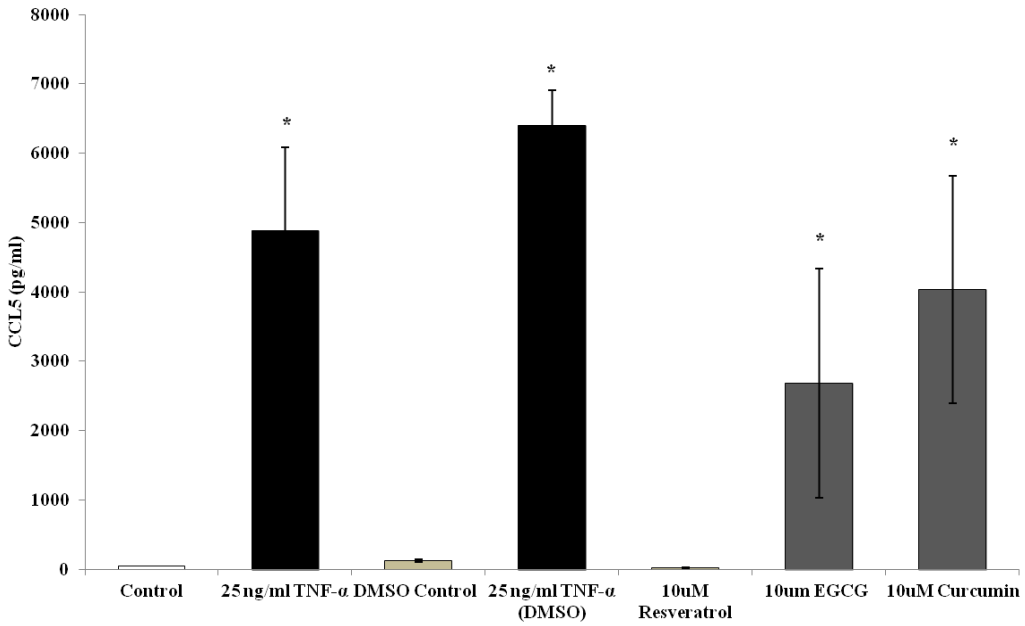


Figure 3.9 Levels of CCL5 (RANTES) in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α and 10µM resveratrol, 10µM EGCG, 10µM curcumin. Data presented as mean ± SEM (n=6) *$P \leq 0.05$ compared with untreated control cells.

1µM Polyphenol Treatments

There was no difference in the cytokine levels in media of Il-6, CXCL1, CCL2 and CCL5 harvested at 24 hours from cells treated with 1µM polyphenols compared with cytokine levels in media from control, untreated wells (Figures 3.10 to 3.13).

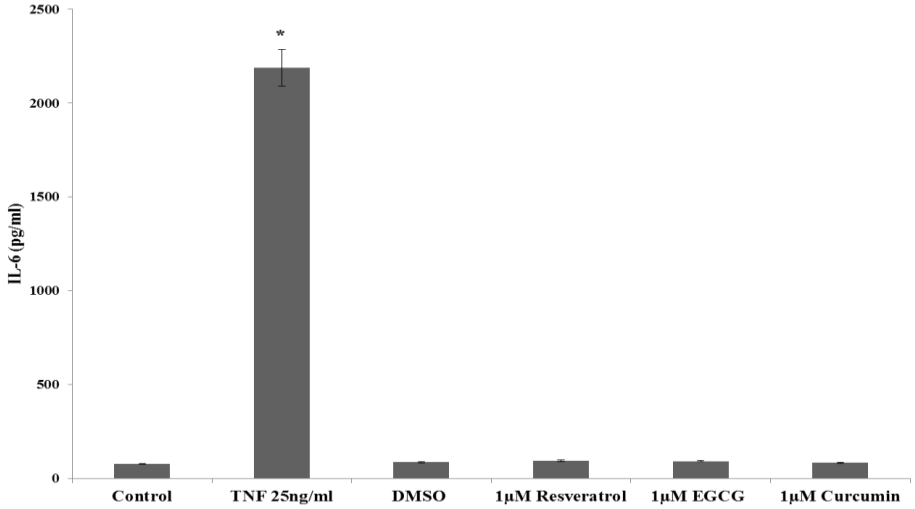


Figure 3.10 Levels of IL-6 in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α, DMSO , 1μM resveratrol, 1μM EGCG, 1μM curcumin. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

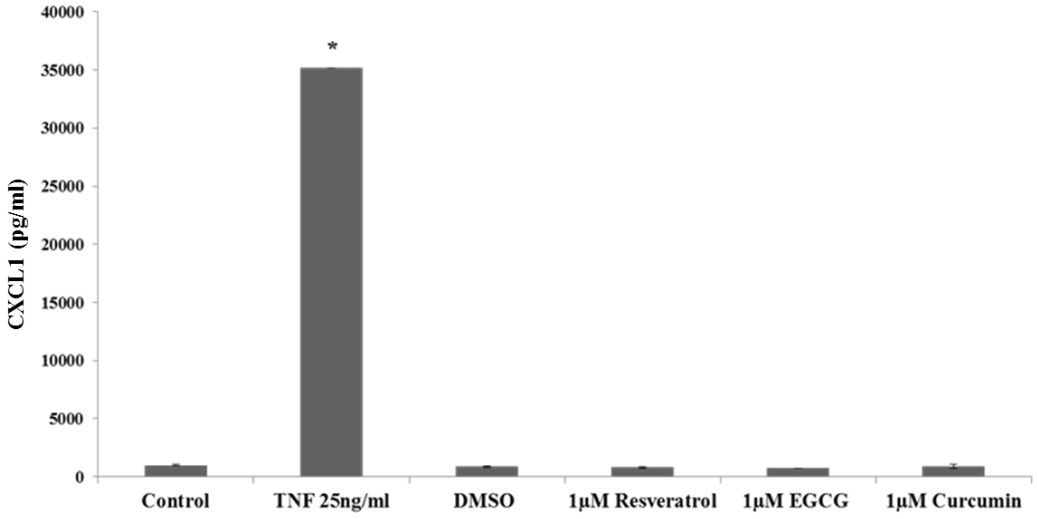


Figure 3.11 Levels of CXCL1 (KC) in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α, DMSO and 1μM Resveratrol, 1μM EGCG, 1μM Curcumin. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

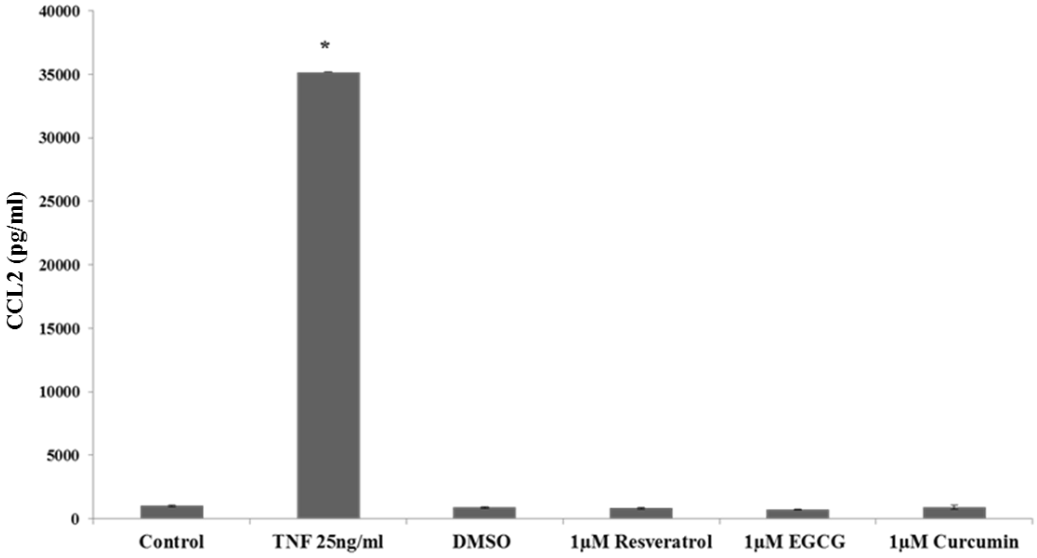


Figure 3.12 Levels of CCL2 (MCP-1) in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α, DMSO and 10μM resveratrol, 10μM EGCG, 10μM curcumin. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

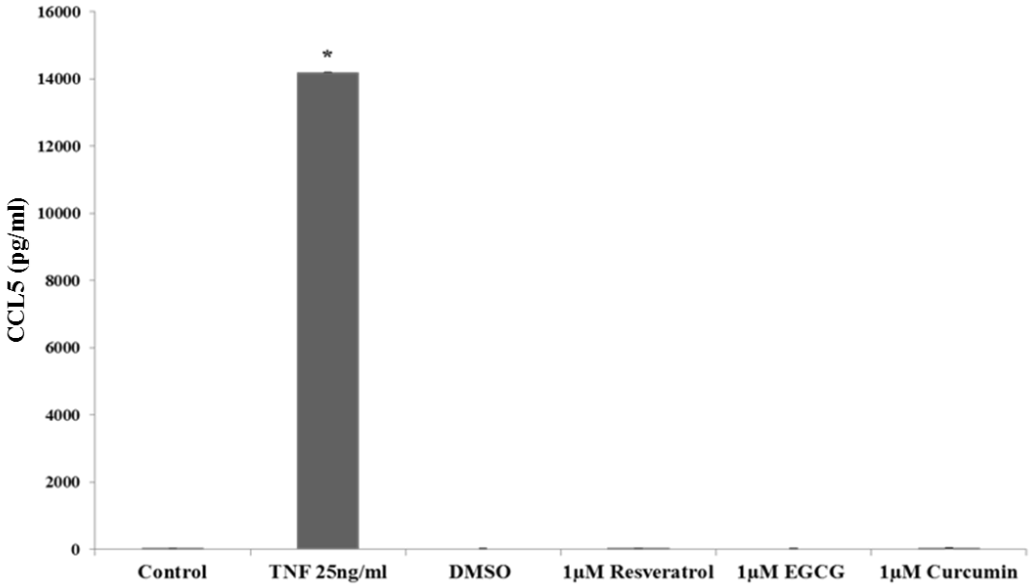


Figure 3.13 Levels of CCL5 (RANTES) in cell culture media from C2C12 myotubes at 24 hours following 25ng/ml of TNF-α, DMSO and 10µM resveratrol, 10µM EGCG, 10µM curcumin. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

3.3.3 The effect of 1µM curcumin and 1µM EGCG pre-treatment on TNF-α induced cytokine release from C2C12 myotubes

Release of IL-6, CXCL1, CCL2 and CCL5 was significantly greater from C2C12 myotubes at 24 hours following treatment of cells with 25ng/ml TNF-α in comparison with cytokine release from untreated control cells. Treatment of C2C12 myotubes with the polyphenols curcumin (1µM) or EGCG (1µM) had no effect on the release of IL-6, CXCL, CCL2 and CCL5 from C2C12 myotubes at 24 hours compared with untreated control cells (Figures 3.14 to 3.17). Prior treatment of cells with polyphenols curcumin or EGCG had no effect on TNF-α induced release of IL-6, CXCL1, CCL2 and CCL5 release from C2C12 myotubes with the exception of EGCG which significantly reduced CXCL media content (P=0.04) (Figures 3.14 to 3.17).

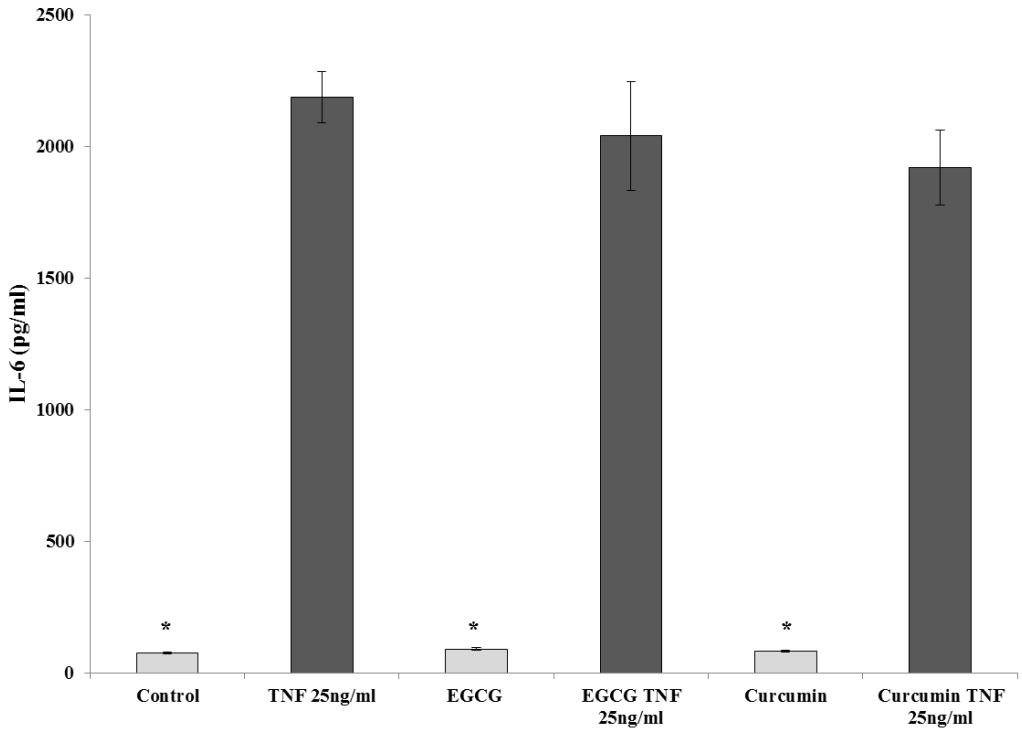


Figure 3.14 Levels of IL-6 in cell culture media from C2C12 myotubes at 24 hours following treatment with 25ng/ml of TNF-α, 1µM EGCG or 1µM curcumin for 24 hours and culture media from C2C12 myotubes at 24 hours following treatment with 1µM EGCG or 1µM curcumin pre-treatment for 24 hours followed by 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with with 25ng/ml of TNF-α.

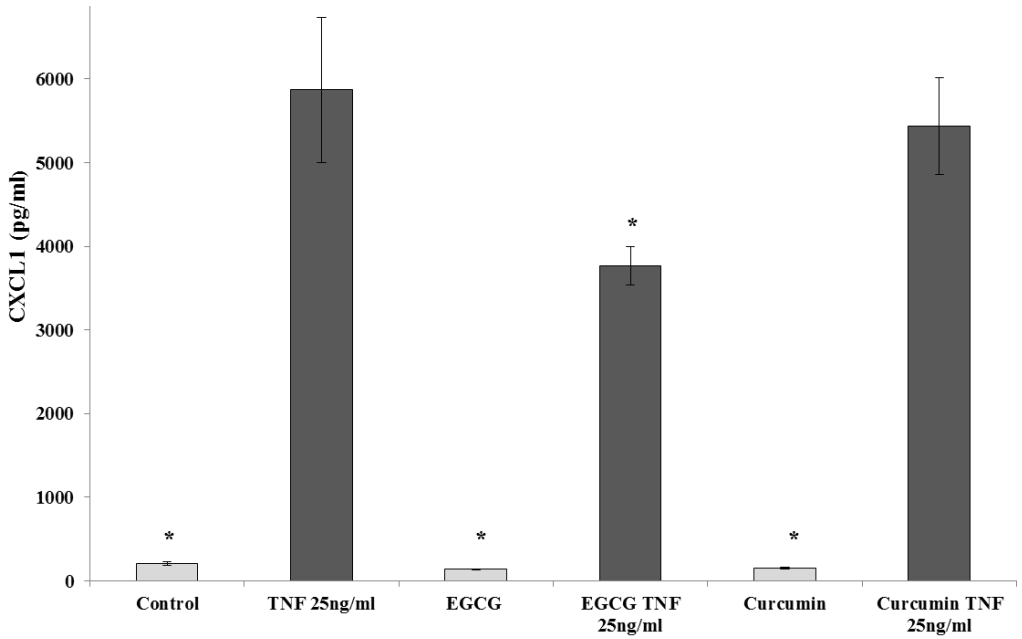


Figure 3.15 Levels of CXCL1 (KC) in cell culture media from C2C12 myotubes at 24 hours following treatment with 25ng/ml of TNF-α, 1μM EGCG or 1μM curcumin for 24 hours and culture media from C2C12 myotubes at 24 hours following treatment with 1μM EGCG or 1μM curcumin pre-treatment for 24 hours followed by 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with with 25ng/ml of TNF-α.

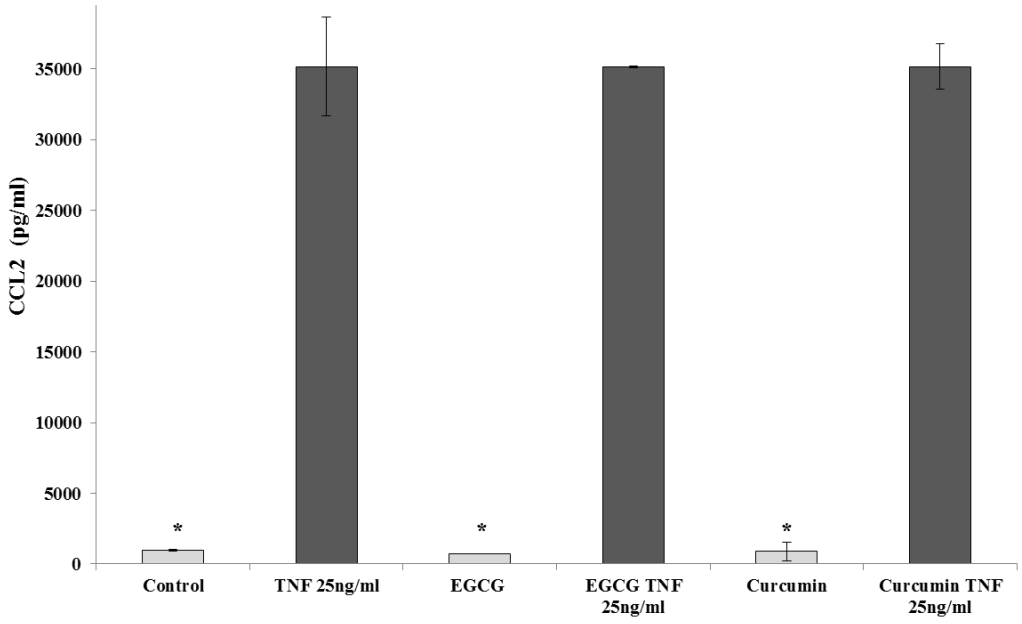


Figure 3.16 Levels of CCL2 (MCP-1) in cell culture media from C2C12 myotubes at 24 hours following treatment with 25ng/ml of TNF-α, 1μM EGCG or 1μM curcumin for 24 hours and culture media from C2C12 myotubes at 24 hours following treatment with 1μM EGCG or 1μM curcumin pre-treatment for 24 hours followed by 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with with 25ng/ml of TNF-α.

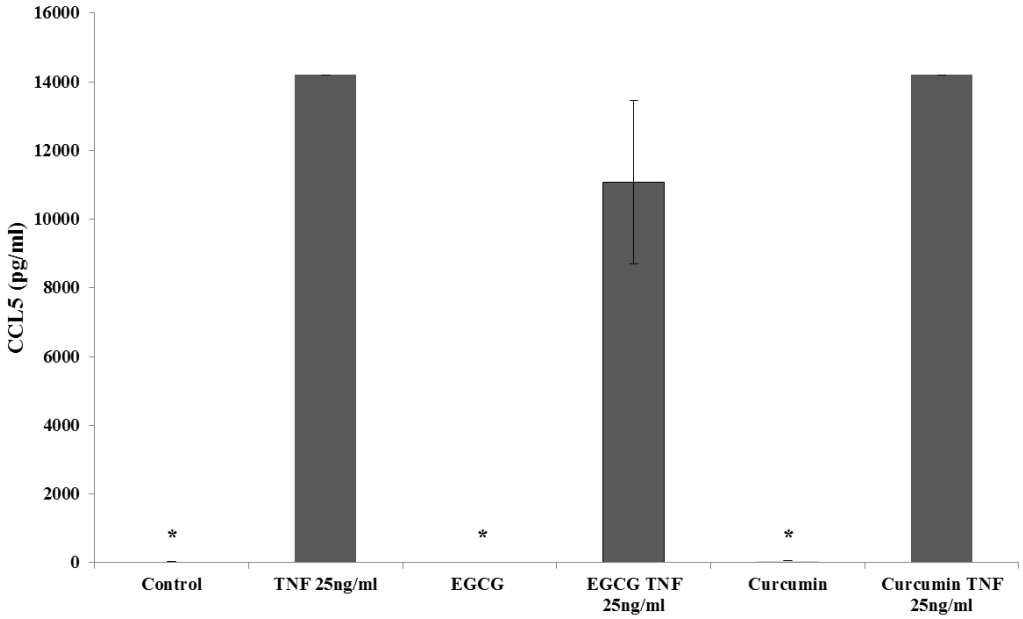


Figure 3.17 Levels of CCL5 (RANTES) in cell culture media from C2C12 myotubes at 24 hours following treatment with 25ng/ml of TNF-α, 1μM EGCG or 1μM curcumin for 24 hours and culture media from C2C12 myotubes at 24 hours following treatment with 1μM EGCG or 1μM curcumin pre-treatment for 24 hours followed by 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with with 25ng/ml of TNF-α.

3.3.4 The effect of resveratrol on TNF-α induced cytokine release from C2C12 myotubes

Media concentrations of IL-6, CXCL1 and CCL5 were significantly lower from cells pre-treated with 1μM resveratrol for 24 hours prior to treatment with 25ng/ml of TNF-α when compared with cells treated with 25ng/ml TNF-α (Figure 3.18 to 3.21). Prior treatment of cells with 1μM resveratrol had no effect on TNF-α induced MCP-1 release from C2C12 myotubes.

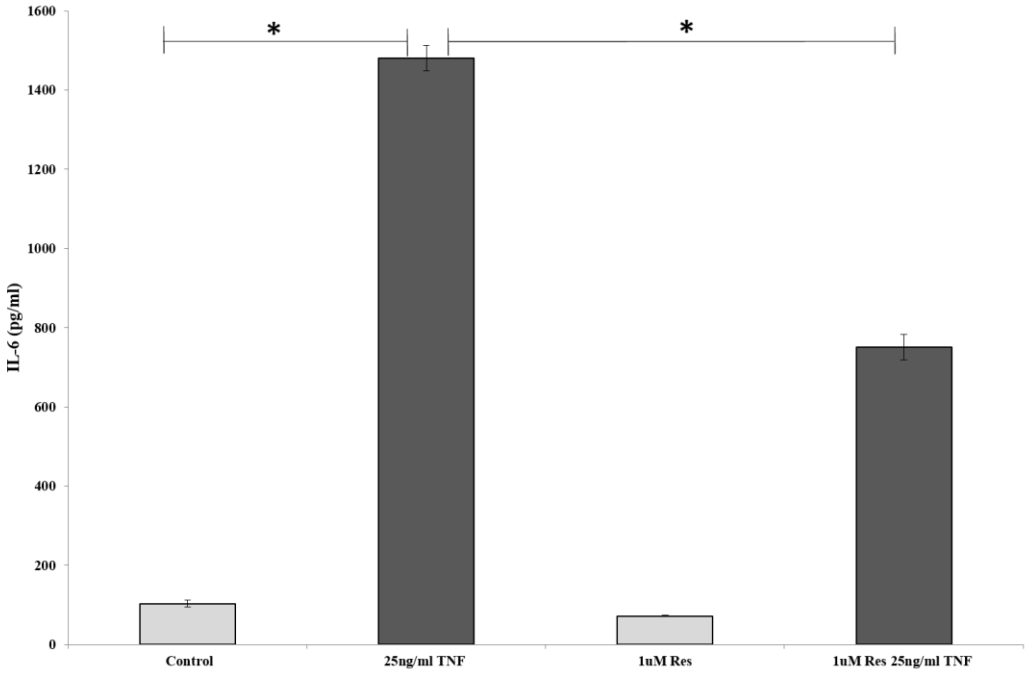


Figure 3.18 Levels of of IL-6 in cell culture media from C2C12 myotubes at 24 hours following a treatment with 1µM resveratrol for 24 hours prior to treatment of 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) $p \leq 0.05$.

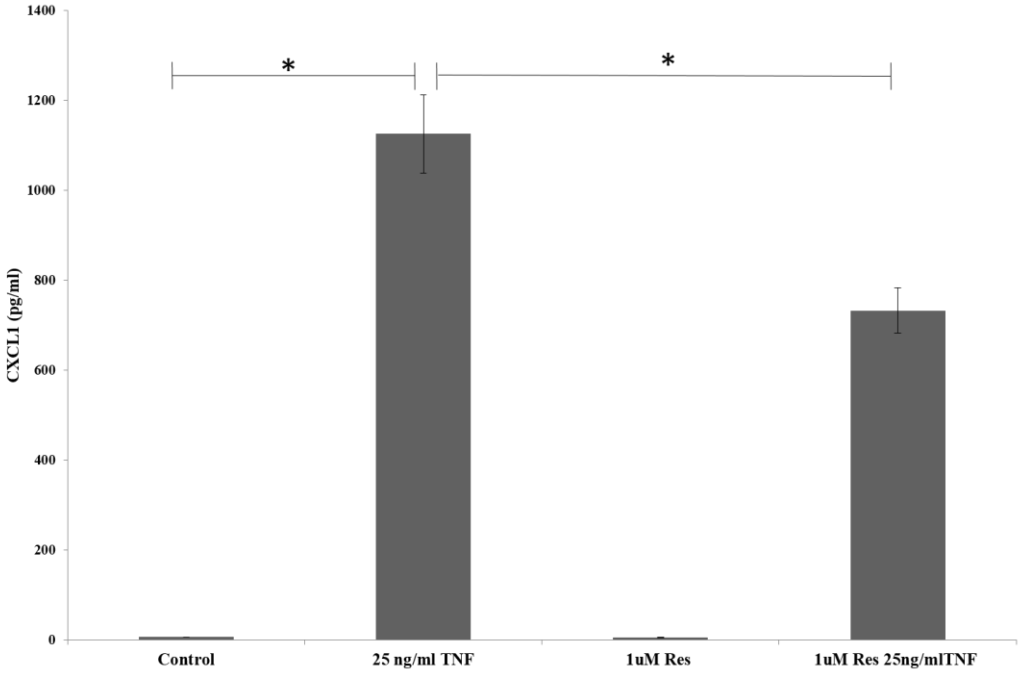


Figure 3.19 Levels of of CXCL1 (KC) in cell culture media from C2C12 myotubes at 24 hours following a treatment with 1µM resveratrol for 24 hours prior to treatment of 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) $p \leq 0.05$.

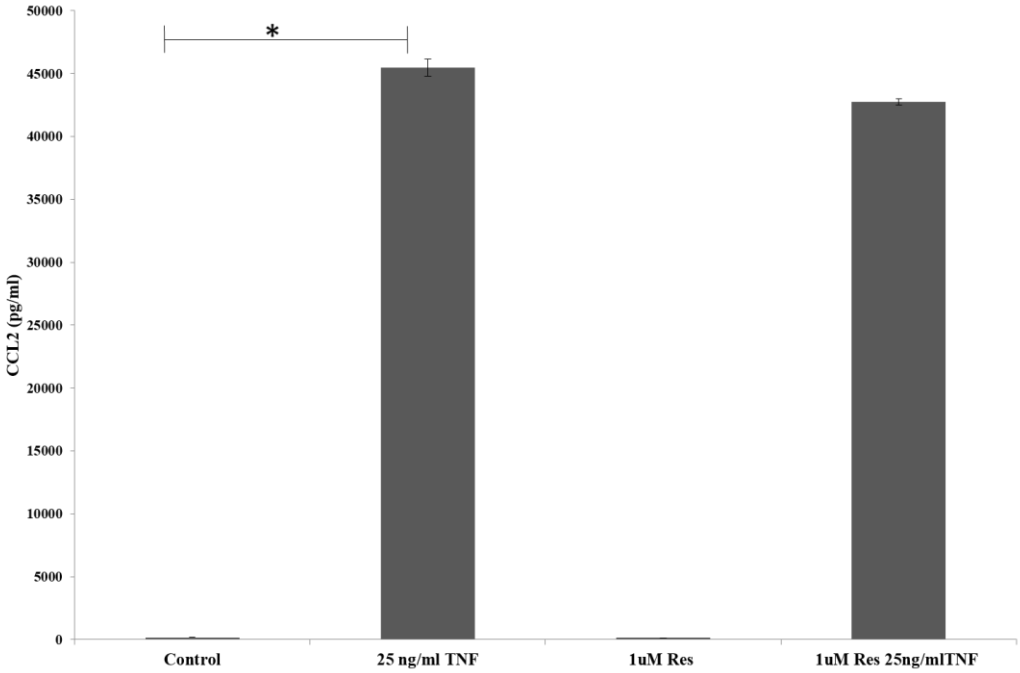


Figure 3.20 Levels of of CCL2 (MCP-1) in cell culture media from C2C12 myotubes at 24 hours following a treatment with 1μM resveratrol for 24 hours prior to treatment of 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) $p \leq 0.05$.

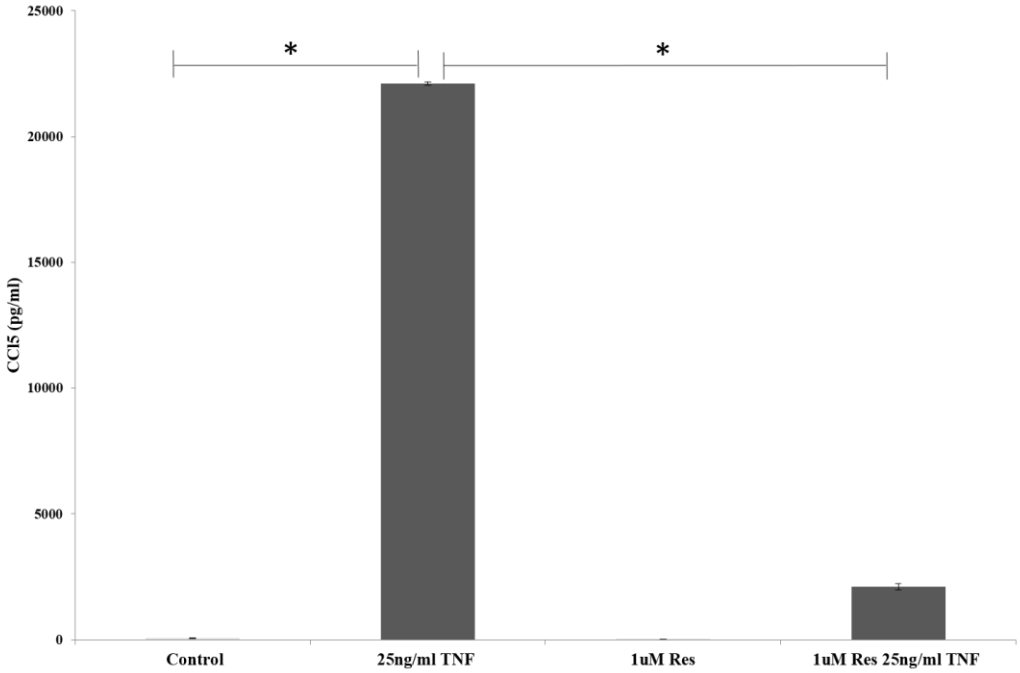


Figure 3.21 Levels of of CCL5 (RANTES) in cell culture media from C2C12 myotubes at 24 hours following a treatment with 1μM resveratrol for 24 hours prior to treatment of 25ng/ml of TNF-α for 24 hours. Data presented as mean ± SEM (n=6) $p \leq 0.05$.

3.3.5 The effect of resveratrol pre-treatment on the viability of TNF-α treated C2C12 myotubes

Little cell death was seen between control C2C12 myotubes and myotubes treated with 25ng/ml of TNF-α for 24 hours, 1µM resveratrol for 24 hours, or 1µM resveratrol for 24 hours followed by a treatment of 25ng/ml of TNF-α for 24 hours (Figure 3.21).

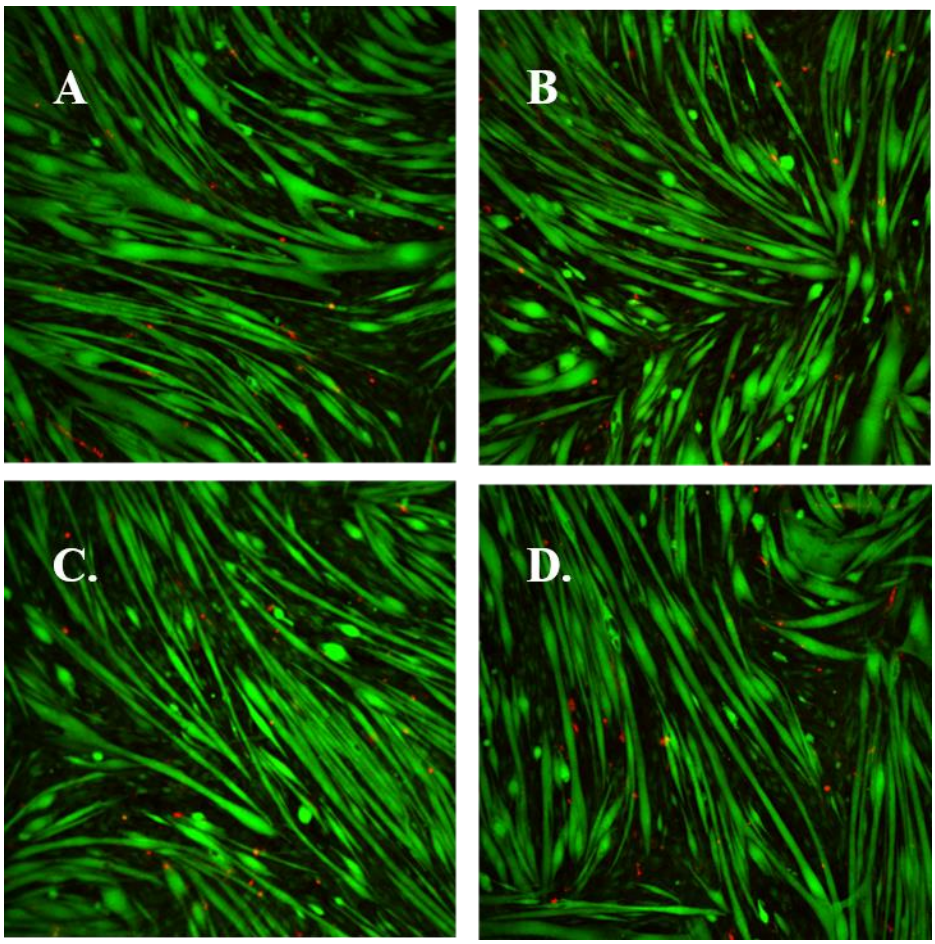
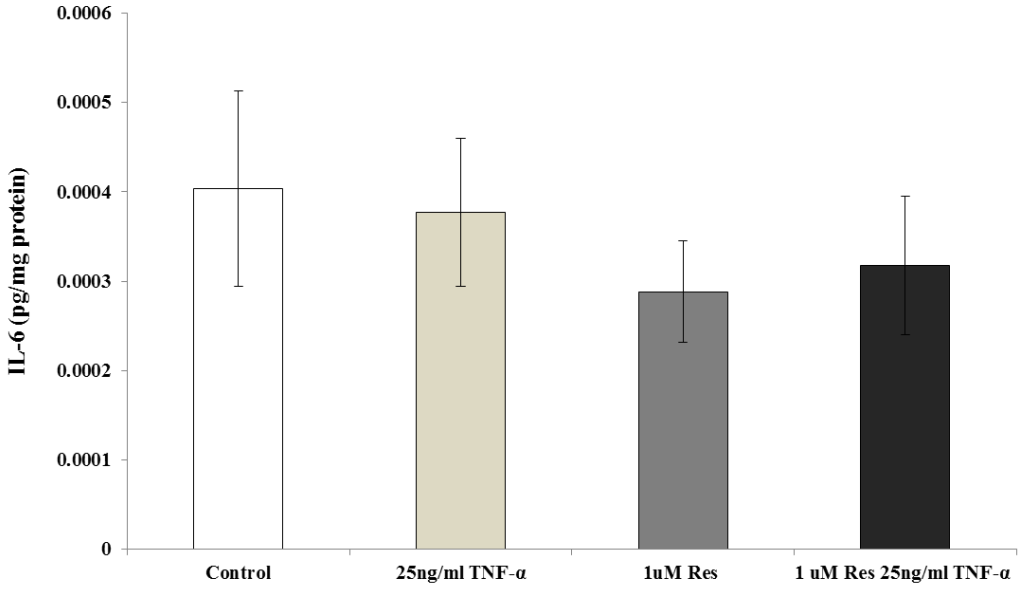


Figure 3.22 Representative LIVE/DEAD images of C2C12 myotubes. (**A**) Untreated C2C12 myotubes, (**B**) myotubes at 24 hours following treatment with 25ng/ml of TNF-α, (**C**) mytoubes at 24 hours following treatment with 1µM resveratrol and (**D**) mytoubes at 24 hours follwing treatment with 1µM resveratrol for 24 hours followed by a treatment of 25ng/ml of TNF-α for a further 24 hours. Calcein-AM ester enters live cells staining them green while ethidium enters necrotic cells and stains red. Representative confocal microsopy image for each treatment.

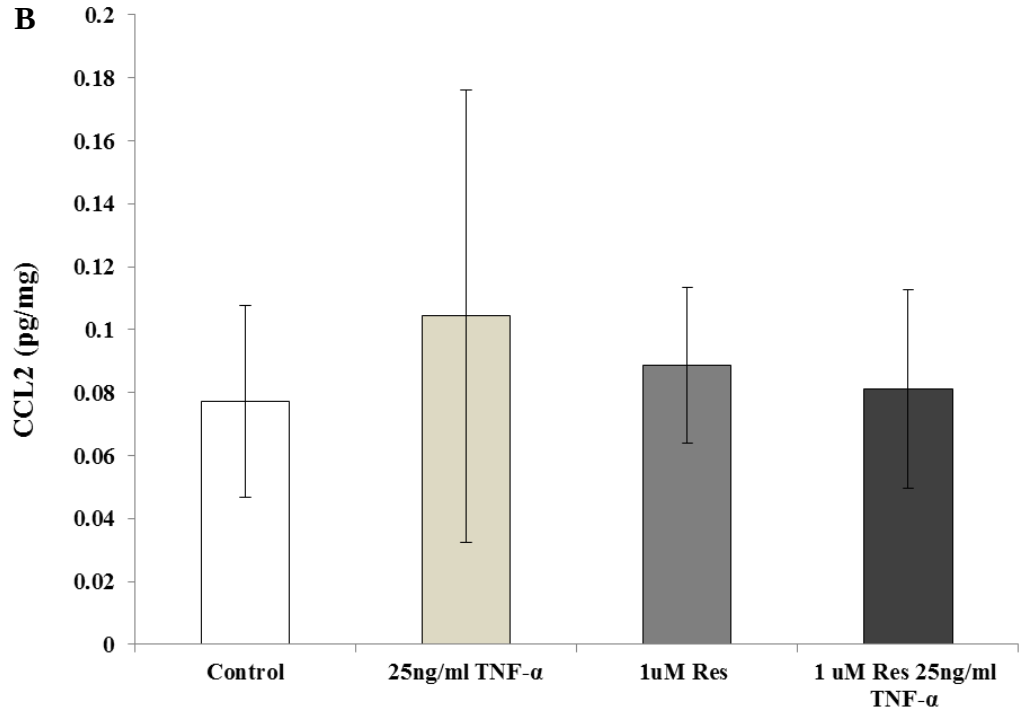
3.3.6 Intracellular cytokine content of C2C12 myotubes pre-treated with 1µM resveratrol for 24 hours followed by a 25ng/ml TNF-α for 24 hours

Data revealed no difference in the level of IL-6, CCL2 and CCL5 between treatment groups (Figure 3.23). No detectable levels of KC were reported in any groups. All replicates for each treatment were out of range; i.e. less than the lowest standard.

A



B



C

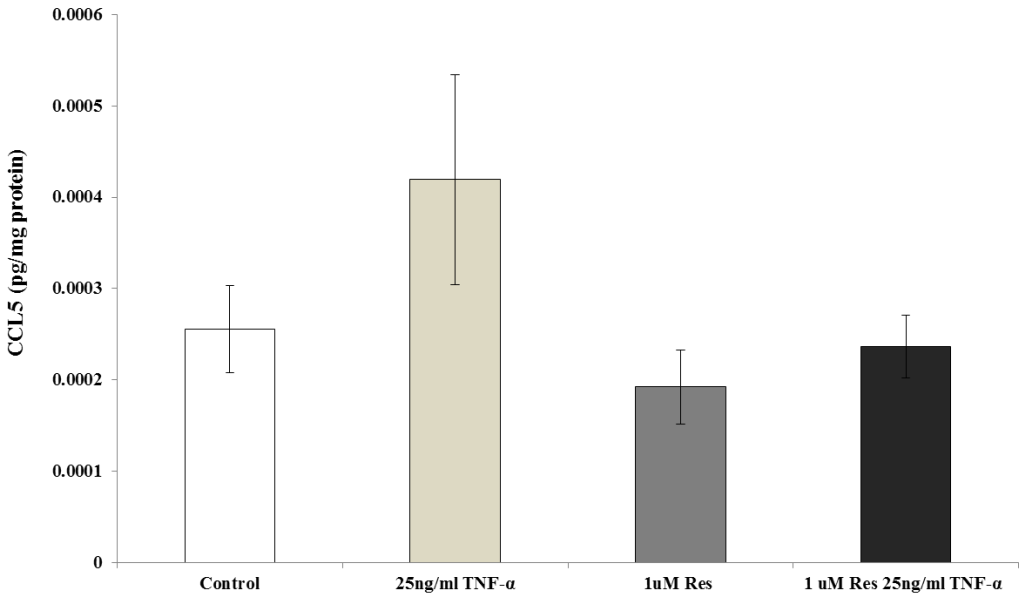


Figure 3.23 Levels of IL-6 (**A**), CCL2 (MCP-1) (**B**) and CCL5 (RANTES) (**C**) in C2C12 myotubes at 24 hours following exposure to TNF-α (25ng/ml) for 24 hours, 1uM resveratrol, or combined individual treatments. Data were normalised to total cellular protein and presented as mean ± SEM (n=6) *p≤0.05 compared with control cells.

3.4 Discussion

The work presented in this chapter describes the effect of polyphenol pre-treatment on TNF-α induced cytokine release from C2C12 myotubes. Initial work focussed on the development of a suitable treatment course of TNF-α on C2C12 myotubes to stimulate the release of pro-inflammatory cytokines which could then be used to assess the effect of polyphenols on TNF-α induced cytokine release.

Since the early work identifying muscle as a source of cytokines (Pedersen et al., 1998), large gene-array screening has been used to determine the expression of inflammatory genes in skeletal muscle (Bhatnagar et al., 2010). Previous work from our group revealed an increase in the release of IL-6, CXCL-1 (KC), CCL2 (MCP-1) and CCL5 (RANTES) from C2C12 myotubes at 3 hours following treatment of myotubes 25ng/ml TNF-α (Lightfoot et al., 2015). This chapter has confirmed and expanded these observations, to demonstrate that C2C12 myotubes treated with 1, 5 and 25ng/ml TNF-α for 3, 8 or 24 hours all showed an increase in the release of IL-6, CXCL-1 (KC), CCL2 (MCP-1) and CCL5 (RANTES) into cell media while cell viability was maintained.

This chapter examined the effects of polyphenol pre-treatments on TNF-α induced cytokine release by muscles. The study investigated the effects of 1μM and 10μM curcumin, EGCG and resveratrol on TNF-α mediated cytokine release from C2C12 myotubes. Polyphenols were chosen following a literature search identifying these polyphenols as they are associated with the modulation of cytokines *in vitro* (Jobin et al., 1999, Lacomis, 2000, Conover et al., 2006). Treatment of cells with concentrations of 10μM curcumin, EGCG or resveratrol had no effects on TNF-α induced cytokine release with curcumin and EGCG treatments alone causing an increase in cytokine release suggesting a pro-inflammatory effect of these polyphenols. These polyphenols may be potentially stressors of the cells exerting a pro-oxidant effect thus increasing cytokine release. Potential reasons behind these effects of curcumin and EGCG on cytokine release from muscle may relate to the differences in chemical structure. The study identified 1μM resveratrol as a modulator of TNF-α induced cytokine release with a reduction in the release of IL-6, CCL2 (MCP-1) and CCL5 (RANTES) from C2C12 myotubes. Neither TNF-α or 1μM resveratrol treatments caused any gross or apparent effects on myotube viability. Intracellular levels of cytokines were unchanged in C2C12 myotubes treated with TNF-α or myotubes pre-treated with resveratrol prior to TNF-α treatment in comparison to control mytoubes. These data suggests that the cytokines produced by cells in response to TNF-α are rapidly exported from the cells. A potential mechanism by which this concentration of resveratrol (1μM) exerts these effects may be through the down regulation of inflammatory regulators. Pro-inflammatory cytokines and chemokines are under the transcriptional control of the ubiquitous inducible factor named nuclear factor-κB (NF-κB) (Chen et al., 1999). This is supported by data showing that pre-treatment of myotubes with sodium salicyate (as an inhibitor NF-kB activation) prevented the increase in cytokine release mediated by TNF-α (Lightfoot et al., 2015). Data from the current study suggests that the reduction in media levels of cytokine from myotubes pre-treated with resveratrol was not a result of impaired mechanisms associated with golgi-mediated release of peptides from myotubes as there was not an accumulation of intracellular cytokines. These data are supported by other work which has assessed the effects of pre-treatment of mytotubes with Brefeldin A (BFA) prior to treatment with TNF-α. BFA indirectly inhibits protein transport from the endoplasmic

reticulum to the golgi apparatus and pre-treatment also resulted in reduced release of cytokines from TNF-α treated myotubes.

The mechanisms of cytokine production from skeletal muscle are poorly understood and understanding of the physiological relevance of cytokine release by muscle also remains unclear. Skeletal muscle is the largest organ system and protein store in the human body. The generation of skeletal muscle cytokines may potentially have a significant impact upon systemic inflammation (Raj et al., 2005, Lightfoot, 2011).

Studies show an association between reduced muscle function and fatigue and elevated cytokine levels (Gan et al., 2004, McMillan, 2013). Raised local and systemic levels of pro-inflammatory cytokines may act as inhibitory modulators of muscle force production, particularly Tumour Necrosis Factor-α (TNF-α) through a cascade of events including raised levels of ROS (Reid and Moylan, 2011).

The clinical relevance of studies that examine the effects of polyphenols such as EGCG, resveratrol and curcumin on *in vitro* cell cultures is limiting. The work in this chapter highlights resveratrol as a potential modulator of cytokine release and supports previous epidemiological studies which suggest that increased levels of dietary polyphenols are associated with reduced systemic inflammation and we propose that this may in turn alleviate muscle dysfunction. Caution should be taken in the interpretation of studies assessing the direct effect of polyphenols on cells *in vitro.* Unlike many polyphenols, resveratrol has been detected in plasma or serum following oral administration at a level of ≤ 2% (Goldberg et al., 2003, Wenzel and Somoza, 2005).

The next stage of this work would be to identify the mechanisms through which resveratrol attenuates TNF-α induced cytokine release. Reports have shown that C2C12 myotubes treated with TNF-α have increased levels of ROS production (Lacerda et al., 2006). As mentioned in Section 1.5, there are 5 main sources of ROS production within the cell: cyclo-oxygenase-2 (COX-2), xanthine oxidase, reduced nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate oxidase system (NADPH oxidase), nitric oxide synthase and mitochondrial ROS, however the exact source of ROS in TNF-α treated cells is yet to be fully characterised although evidence suggests that TNF-α may alter

mitochondrial electron transport chain function resulting in increased mitochondrial ROS production (McLean et al., 2013).

Evidence for a role of mitochondrial ROS production in the mechanisms by which TNF-α mediates its effects comes from studies where prior SS31 treatment resulted in the attenuation of TNF-α induced cytokine release from C2C12 myotubes (Lightfoot et al., 2015). SS31 is an antioxidant peptide that accumulates on the inner mitochondrial membrane thus targeting mitochondrial ROS production. From these and the current data we hypothesis that the effects of resveratrol in attenuating TNF-α mediated cytokine release from muscle cells may be due to the potential antioxidant properties of this compound. However further work is needed to confirm this.

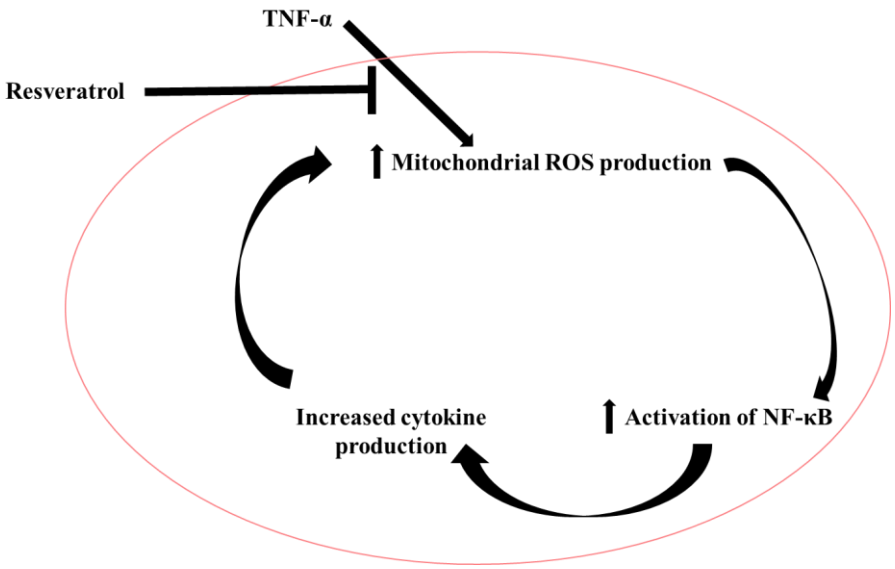


Figure 3.24 Schematic diagram illustrating the potential mechanism through which resveratrol exerts effects.

3.5 Summary

This study successfully:

- Validated and expanded the effect of TNF-α treatments on cytokine release from murine C2C12 myotubes.
- Assessed the effects of varying concentrations of TNF-α exposure for up to 24 hours on cytokine release from C2C12 myotubes.
- Developed a suitable model of resveratrol, curcumin and EGCG polyphenol treatments on C2C12 myotubes.
- Identified the polyphenol resveratrol as capable of altering the effects of TNF-α on skeletal muscle by attenuating TNF-α induced cytokine release.

In summary, resveratrol might be helpful in understanding conditions where elevated cytokines may be associated with aspects of muscle dysfunction and fatigue such as chronic fatigue syndrome (CFS).

Chapter Four

Skeletal muscle function in patients with CFS

4.1 Introduction

Patients with chronic fatigue syndrome (CFS) are a highly heterogeneous group of sufferers reporting complex and multifactorial aetiologies which manifest in similar symptoms. Sufferers of CFS describe a wealth of different symptoms and potential causes/triggers with each case of CFS being unique (Section 1.6). A common factor between all patients is the symptom of recurrent or persistent fatigue/exhaustion (including descriptions of both mental/cognitive fatigue and outer limb/muscular sensations of fatigue) at rest which is exacerbated by physical exertion and exercise resulting in severe post-exertional fatigue. This is the only compulsory symptom for diagnosis within the majority of CFS diagnostic criteria/guidelines (Holmes et al., 1988, Fukuda et al., 1994, Sharpe et al., 1991). The use of the term fatigue by patients with CFS is a generalised perception of their health or well-being, which is potentially unrelated to any objective markers of impaired physical health; such as compromised contractile function and fatigue within the muscle. The range of symptoms exhibited by patients with CFS may plausibly be associated with altered skeletal muscle function; these symptoms include restless legs, muscle twitching, muscle pain (myalgia) and the overall sensation of reduced functional capacity (Vercoulen et al., 1994, Afari and Buchwald, 2003). The relationship between increased sensation of fatigue in patients with CFS and the functioning and skeletal muscle physiology has not yet been fully elucidated. Inconsistencies are commonly found in a large proportion of studies into CFS and this includes there are discrepancies in the data from muscle function and physical activity assessments in these patients (Lloyd et al., 1988, Stokes et al., 1988, Lloyd et al., 1991, Gibson et al., 1993, Paul et al., 1999).

The sensation of weakness and fatigue is typical following vigorous exercise in all individuals regardless of health (Boerio et al., 2005). The decline in muscle contractile force during repeated contractions was originally thought to be a result of muscle cell dysfunction (peripheral fatigue) only, however there is also evidence to suggest a relationship between central impairment (central motor drive and force output) in healthy individuals which occurs from feedback from fatiguing muscles to the brain (Amann et al., 2008).

The process which leads to muscle contraction includes a combination of central and peripheral signalling (full schematic representation of the chain of command in muscle contraction shown in Figure 1.2.2, (Kent-Braun, 1999). A failure at any point in the process can lead to a decline in force generation or failure to elicit muscle contraction (Westerblad et al., 1991, Allen et al., 2008). The causes of fatigue may be classified as central or peripheral (Gandevia, 2001). Central fatigue is due to failure of neural drive and may result from lack of motivation, from impaired firing frequency at or proximal to the anterior grey column, or from impaired recruitment of motor units. Peripheral fatigue results from failure at or beyond the neuromuscular junction. These include factors such as metabolic depletion, the accumulation of intramuscular metabolites, excitation contraction coupling failure (Kent-Braun, 1999). The rate of fatigue varies between different muscle groups, as a result of fibre type composition, mitochondrial density and motor neuron innervation (Enoka and Duchateau, 2008).

A lack of formal definition for general fatigue hinders researchers and leads to the formation of study specific definitions resulting in a lack of comparability between studies (Aaronson et al., 1999). The current work has focussed on muscle fatigue which is defined as a failure to maintain a required force or output of power during sustained or repeated muscle contractions (Allen et al., 2008). Functional assessment using a combination of voluntary contractile assessment and involuntary contractile force (using percutaneous electrostimulation) allow researchers to assess contractile properties of skeletal muscle more closely and helps decipher between reductions in central and peripheral driven force (Edwards et al., 1977b).

A high proportion of patients with CFS report extreme fatigue/exhaustion particularly in the arms and legs both at rest and following physical activity (Hickie et al., 2006). The functional assessment of muscle contraction in patients with CFS (PVFS, PVF and Effort Syndrome) has been recurrent since the 1980's (Lloyd et al., 1988, Stokes et al., 1988, Vos-Vromans et al., 2013). These studies have reported mixed results as to whether skeletal muscle function is altered/impaired in CFS patients.

A study (Gibson et al., 1993) in which voluntary and involuntary (stimulated contraction) muscle function of the quadriceps femoris was assessed by dynamometry revealed both patients with CFS and HCs were able to produce maximal contractile force. There was no significant difference in MVC force between patients with CFS and HCs. A minority of patients with CFS required twitch interpolation to reach maximal force. Following a voluntary incremental exercise test until exhaustion using a cycle ergometer, five minutes post exercise, MVC force was lower in both groups with no significant difference in MVC force reduction between patients with CFS and HCs. The ability to reproduce pre-exercise MVC force was recovered in both groups at 24 hours post-exercise. Patients with CFS had higher perceived exertion scores during the incremental exercise test (Gibson et al., 1993). Further evidence supporting a potential central limiting factor in muscle function in patients with CFS is present by (Stokes et al., 1988). These authors reported no difference in contractile properties of muscle in patients (classified is the study as sufferers of "Effort Syndrome" - patients with a history of excessive general and muscular fatigue but no evidence of neuromuscular disease on clinical, biochemical, histological, or histochemical examinations). Voluntary maximal isometric strength measurements of the quadriceps femoris in the patient cohort fell below that of the authors' expected normal range except in a minority of subjects who had evidence of impaired central motor drive (Edwards et al., 1977b). A voluntary resistance exercise to exhaustion test using a cycle ergometer revealed an inability of some patients with Effort Syndrome to reach their predicted maximum heart rate. These data correlated with the inability of individuals to reach their MVCs. Involuntary muscle function of the *adductor pollicis* was assessed through direct electrical stimulation of the motor nerve (stimulation in the absence of volition). This showed no differences in the contractile performance and relaxation rates between patients and control subjects suggesting a central impairment in MVC forces (Stokes et al., 1988). Direct electrical stimulation of the motor nerve rather than direction stimulation of the muscle allows measurement of an impairment in nerve function. These data also suggest the absence of any failures in contractile elements of the muscle and suggested potential central impairments in muscle function.

There have been reports of significantly reduced voluntary maximal force in the quadriceps of patients with CFS (Paul et al., 1999). However, twitch interpolation testing was not included in this study. Further investigation by these authors included a fatiguing protocol in which force for all patients with CFS and HCs fell during the 18 maximal voluntary contractions. Results revealed no differences in absolute changes in peak force or when expressed as a percentage of the initial MVC between the patients and control groups. Subjects were also assessed at 24 hours following the fatigue protocol; absolute changes in force were still significantly lower that initial MVC forces in the CFS participants, suggesting an inability for patients to recover at the same rate as healthy individuals (Paul et al., 1999).

Subtle differences in study outcome may potentially be a consequence of the heterogeneity amongst patients with CFS. There may be differences in perceived activity effort. There is disparity between studies with regard to the muscle (groups) investigated; to date there have been investigations on a number of muscles including the *anterior tibialis*, *quadriceps* and elbow flexor muscle. Different muscle groups fatigue at varying rates. The *adductor pollicis* predominantly composed of type 1 muscle fibres in contrast to the quadriceps which is a combination of both fibre types (Round et al., 1984). Skeletal muscle physiology also varies between men and women and across age groups. Age and sex matching is an important confounder which needs to be considered. For example; age related changes in muscle include a reduction in muscle fibre number and fibre cross sectional area as well as a reduction in whole muscle and single fibre force production (Narici and Maganaris, 2006, Nilwik et al., 2013). Muscle physiology and function is highly adaptable to factors such as nutritional status and exercise as well as changes in inflammatory and redox environment. To date, involuntary muscle contractile force of the quadriceps muscle has not been assessed during a fatigue protocol in patients with CFS. This may help elucidate the role of central and peripheral contributors in muscle function in patients with CFS.

A major issue regarding muscle functional assessment is the effect of physical status on the majority of objective measures used in muscle contraction. Despite using activity matched or sedentary controls, fitness level may still be significantly different to patients CFS. It is questionable as to whether changes in muscle

function reported in some studies is a consequence or the disease or a secondary outcome resulting from reduced physical activity. A number of studies assessing habitual physical activity reported lower activity levels in patients with CFS in comparison with HCs (Vercoulen et al., 1997). Periods of activity were reportedly less intense and were followed by longer episodes of rest (van der Werf et al., 2000). However, there is also evidence to suggest there are no differences in habitual physical activity between patients with CFS and age and sex matched healthy individuals (Evering et al., 2011).

Underlying questions remain surrounding the physical fitness of patients with CFS and whether the condition leads to reduced activity and increased sedentary behaviour leading to the deconditioning of numerous health parameters including the condition of muscle. A number of studies have investigated physical fitness in patients with CFS which in part covers muscle health and function. Robust evidence confirms the effects of prolonged bed rest on reduced physical work capacity, muscle disuse and musculoskeletal deconditioning (Convertino et al., 1997). However, the evidence to suggest that deconditioning is a perpetuating factor in CFS remains inconclusive. There is evidence for (Fulcher and White, 2000, De Becker et al., 2000) and against (Bazelmans et al., 2001) this hypothesis. Despite a lack of robust evidence to suggest a physical ailment or physical deconditioning in patients with CFS, physical activity intervention studies are used in CFS therapy. Research has focussed on graded exercise therapy (GET) and physical pacing as a therapeutic option for patients with CFS (Larun et al., 2015). The evidence to support the use of GET and pacing in CFS is mixed given the lack of a formal identification of contractile impairments in patients.

The complex nature of muscle contractile function in different muscle groups has resulted in a multifaceted, unclear picture of muscle function in CFS. Mixed reports surround both MVC and muscle fatigue with inconsistent data. Further studies are warranted to clarify if CFS in associated with altered skeletal muscle function.

4.1.2 Hypothesis

Patients with CFS show greater evidence of fatigue and impaired muscle force generation compared with age and sex matched healthy controls.

4.1.3 Aims

The current chapter aims to determine:

- Maximal voluntary isometric force generation of the quadricep muscle in patients with CFS compared with HCs.
- Force frequency relationship using percutaneous electrical stimulation of the quadriceps muscle in patients with CFS compared with HCs.
- Percentage force reduction during a muscle fatigue protocol in patients with CFS compared with HCs.
- Maximal Ca^{2+} activated force of single fibres from the *VL* muscle.

4.2 Methods

4.2.1 Patient and HC recruitment

Subjects with CFS were recruited through the Merseyside CFS services. All subjects were newly diagnosed by clinicians in accordance the Oxford Criteria (1990) and recommended NICE guidelines (2007). 95 age and sex matched HCs were recruited within the Merseyside area through recruitment posters. The study recruited subjects between 18 and 55 years old, non-ambulatory and non-smoking. See Section 2.2 for full details of inclusion and exclusion criteria.

Following the provision of a blood sample for cytokine analysis and completion of questionnaire assessment, subjects were invited to complete an additional, optional component of the study which involved muscle function testing. Sixteen HC and 16 patients with CFS agreed to participate in the muscle function testing (study population characteristics are shown in Table 4.1). HCs subjects were matched according to the age and sex of CFS patients prior to the completion of the testing.

	HC	**CFS**
Sample Acquisition	16	16
Women	13	13
Men	3	3
Age	36.2 ± 2	36.4 ± 2.6
Height (m)	1.7 ± 0.02	1.7 ± 0.02
Weight (kg)	69.1 ± 2.6	63.6 ± 4.3
BMI (kg/m²)	23.9 ± 0.7	23 ± 1.4
SF-36 Score	29.9 ± 0.1	17 ± 1*****

Table 4.1 - Baseline characteristics of study participants completing muscle function testing. Height reported in meters (m) and weight reported in kilograms (kg). Short form health survey (SF-36) for physical functioning. Data shown as average ± SEM. *****$p \leq 0.05$ compared with HCs.

4.2.2 Questionnaire assessment

The Short Form Health Survey (SF-36) (Version 1.0 - physical functioning section) was used to assess functional status and well-being/quality of life. The SF-36 is a self-reporting, standardised questionnaire widely used in research (Stewart et al., 1988). The full questionnaire is presented in thesis appendix (Figure 8.19). Patients with CFS and HCs completed the 10 scaled questions in assessment. Low scores are indicative of greater disability.

4.2.3 Muscle function testing

Muscle function was assessed using isometric dynamometry and percutaneous isometric electromyostimulation using a Biodex isometric dynamometer (Biodex Medical Systems Inc. Shirley, NY, USA) (described in Section 2.10). Application of these methods have been validated for their use in reliable assessment of force production and muscle function (Drouin et al., 2004). Participants were seated with a 90° flexion of the hip and non-extendable straps crossing the chest and abdomen and across the quadriceps to maximise isolation of the target muscle groups.

4.2.3.1 Maximal voluntary contraction

Once seated in the dynamometer (Section 4.3.3), the angle of knee extension was set at 80^{o} (assuming 0^{o} as full extension). Participants were instructed to give 5 submaximal voluntary isometric contractions, increasing in intensity, before providing a maximal voluntary contraction, recorded in newton meters (nm). Verbal encouragement was given to all participants and bio-feedback made available through projection of the force trace for the participants to view.

4.2.3.2 Percutaneous isometric electromyostimulation

Percutaneous stimulation was applied through surface electrodes, was delivered via a BIOPAC systems MP100 stimulator (BIOPAC systems Inc., Santa Barbara, CA, USA) and knee extension forces were recorded with a tension compression load cell (Tedea-Huntleigh, Vishay Precision Group Inc. Malvern, PA, USA) connected

to a non-extendable strap attached to the lower leg, approximately 2–3 inches above the ankle (malleolus). Information collected from the force transducer was visualized and analysed using AcqKnowledge v.3.7.2 software (BIOPAC systems Inc. Santa Barbara, CA, USA) (Owens et al., 2014).

4.2.3.3 Force frequency relationship

An electrical stimulus was first delivered at 100 Hz with an amplitude (mA) to elicit 30% of the participants individual MVC force. Following this, at the determined amplitude, 1 second trains of 50, 20 and 10 Hz and a single 1 Hz twitch were delivered in a random order to generate a force frequency relationship.

4.2.3.4 Fatigue protocol

For the determination of fatigue resistance, contractions were evoked by 20 Hz, 200 ms square wave pulses, previously defined to be representative of normal voluntary human motor neuron firing rates (Biglandritchie et al., 1979) and thus physiologically valid when used to assess the fatigue resistance of human skeletal muscle. For every session, the position of the participant in the isometric chair (Lido Active, Loredan, Davis, CA, USA) was standardised in accordance with previously published guidelines (Morton et al., 2005). Two 3 × 5 inch (8 × 13 cm) oval self-adhesive stimulating electrodes (Chattanooga, DJO Global, CA, USA) were placed approximately 2 inches above the knee joint across the *vastus medialis* (*VM*) and proximal to the acetabulofemoral joint across the *vastus lateralis* (*VL*). Participants were required to fully extend and contract the quadriceps to make the muscle body more apparent prior to electrode placement to minimise antagonist co-activation, although this was not monitored with electromyography during stimulation. Fatigue resistance was determined from 2 min repeated stimulation with 20 Hz, 200 ms square wave pulses every 750ms (total of 160 evoked contractions equating to 32 seconds of total stimulation). Fatigue index was calculated as absolute force loss from the first stimulated contraction to the final stimulated contraction.

4.2.4 Biopsy procedure

Of these 16 patients and HCs, 11 completed a muscle biopsy procedure. HCs were invited to biopsy according to the age and sex of CFS biopsy subjects. A biopsy was taken from the *Vastus lateralis* (*VL*) muscle of patients with CFS and HCs (Section 2.2.5). An Acecut automatic Biopsy system (TSK Laboratory, Oisterwijk, Netherlands) was used for each procedure. The leg area was sterilised with a topical antiseptic, (povidone-iodine or chlorhexidine gluconate). The skin and thigh area were both anaesthetised with 4ml 2% lidocaine. Three biopsies were collected from the muscle from different areas of the *VL* muscle.

	HC	**CFS**
Sample Acquisition (n)	11	11
Women (n)	9	9
Men (n)	2	2
Age (Average ± SEM)	35.8 ± 3.4	35.8 ± 3.5
SF-36*	30 ± 0	16.2 ± 1.4

Table 4.2 Baseline characteristics of study participants that undertook a muscle biopsy (SF-36 - Short Form Health Survey - Physical Activity). *P≤0.05.

Muscle biopsy samples were immediately placed into ice-cold stabilizing buffer A composed of 2.77mM CaK_2 ethylene glycol*bis*-(2-aminoethylether)- *N*,*N*,*N*=,*N*=-tetraacetic acid (EGTA), 7.23mMK_2 EGTA, 6.56mM$MgCl_2$, 0.5mMdithiothreitol (DTT), 50mM 2-(*N*-morpholino)ethanesulfonic acid potassium salt (KMES), 20mM imidazol, 20mM taurine, 4.3mM Na_2 ATP, and 15 mM phosphocreatine, pH 7.3 at 4°C.

4.2.6 *Ex vivo* single muscle fibre analysis

Single muscle fibres isolated from the *vastus lateralis* (*VL*) muscle were maintained in ice-cold relax solution, containing 4.5mM MgATP, 1 free Mg^{2+}, 10mM imidazole, 2mM EGTA, and 100mM KCl (pH 7.0 at 4°C) (Degens et al., 2010). Fibres were treated with 50μg/ml saponin (in relax solution) for 15 minutes on ice and then mounted onto an 802D permeabilised fibre apparatus (Aurora Scientific, Canada). Skinned myofibres were attached to insect pins and fixed to a 403A (5mM) force transducer and 312C length controller, using ultrafine nylon thread and maintained in relax solution throughout assembly. Sarcomere length (SL) was measured using 900B Video Sarcomere Length (VSL) software (Aurora Scientific, Canada). SL was adjusted on each fibre to 2.4-2.6μm, fibre diameter was measured at four intervals along the length, and a circular circumference assumed for the basis of CSA calculation. Fibres were maximally activated in Ca^{2+} activating solution (pCa 4.5), containing in addition to Ca^{2+}, 4.3mM MgATP, 1 free Mg^{2+}, 20mM imidazole, 7mM EGTA, 19.6mM PCr, and 64mM KCl (pH 7.0 at 4°C). Peak force was recorded and normalised to fibre CSA (Degens et al., 2010).

4.3 Results

4.3.1 Clinical characteristics

A cohort of 16 CFS patients and 16 HCs from the main study cohort consented to take part in additional muscle function testing. Testing was available to all subjects in the main study cohort. 16 HCs and 16 patients with CFS completed the muscle function testing. Both groups did not differ significantly in age, height or weight (BMI) (Table 4.1). Both groups contained a higher proportion of women; which is characteristic of the main study cohort group. Patients with CFS and HCs had a mean age of (36.4 ± 2.6 and 36.2 ± 2 years, respectively). The mean height of the patients was 1.7 ± 0.02m and 1.7 ± 0.02m for the control group, mean weights were 63.6 ± 4.3 kg and 69.1 ± 2.1kg, respectively (Table 4.1).

4.3.2 Physical functioning assessment

The physical functioning status of all 16 subjects was assessed using the SF-36 activity questionnaire. The SF-36 questionnaire performs well in assessing functional status and well-being/quality of life in patients with CFS (Buchwald et al., 1996). The assessment contains 10 questions. High scores are indicative of superior health and less body pain. Control subjects obtained higher scores than the patients with CFS (mean 29.9 ± 0.01 vs. 17 ± 1; $P < 0.05$) indicating impaired physical functioning in the CFS patient group (Table 4.1).

4.3.3 Maximal voluntary contractile force

Maximal voluntary force measurements were assessed in all 32 subjects using an isokinetic dynamometer. The final analyses on peak torque production included 31 subjects; results from one CFS subject were excluded due to extremely poor mental motivation. The subject was unable to elicit a maximal voluntary contraction and force was > 2 SD away from the group average. Subjects were encouraged to provide >2 attempts at eliciting maximal voluntary force contraction. Isometric maximal voluntary contractile force was lower in subjects with CFS compared to HCs (p= 0.02) as shown in Figure 4.1.

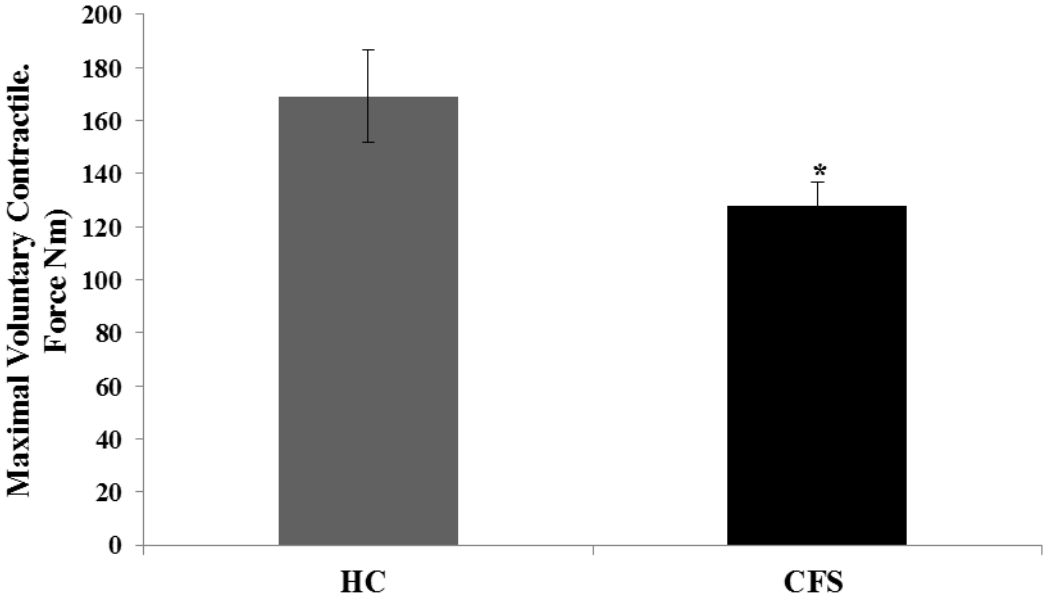


Figure 4.1 - Maximal voluntary isometric force (nm) of the right knee extensors in HCs (n=16) and patients with CFS (n=15). P≤0.05.

4.3.1.2 Electrically stimulated force frequency relationship

Following MVC, all subjects underwent involuntary muscle contraction analysis. Quadricep muscle was stimulated to produce a force 30% equivalent to each MVC at 1, 10, 20, 50 and 100Hz. The relation between force (relative to 30% MVC) and stimulus frequency over the frequency range from 1 to 100 Hz is shown in the force frequency curve below (Figure 4.2). There was no difference in force produced between patients with CFS and HCs across any of the stimulation frequencies.

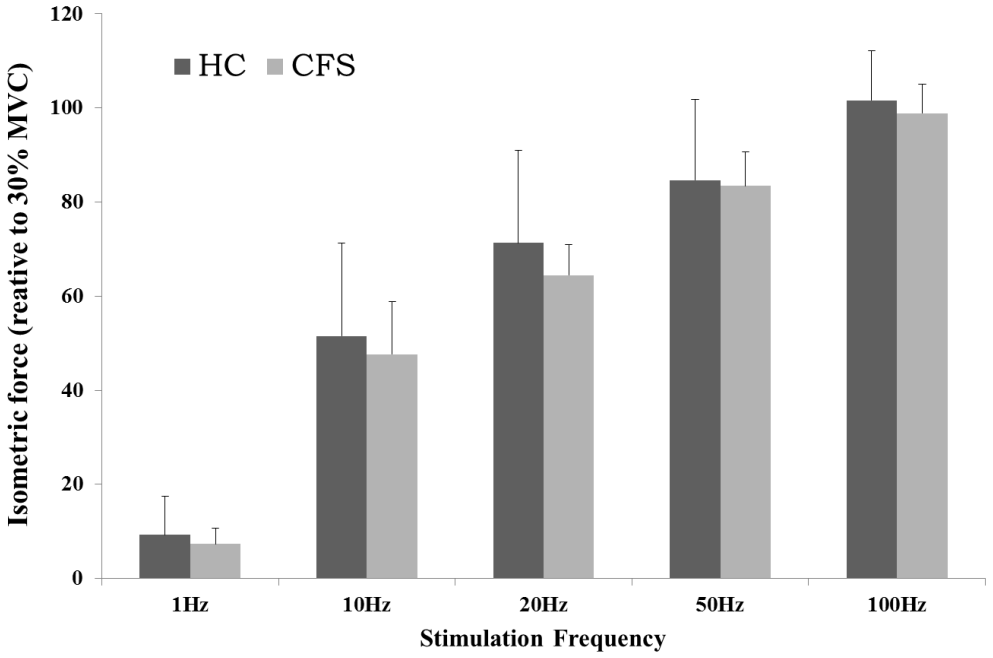


Figure 4.2 Electrically stimulated force frequency relationship of the right knee extensors in HCs (n=16) and patients with CFS (n=15). The intensity of the applied current was set at an amplitude to elicit 30% of the participants MVC force when stimulated at 100Hz.

4.3.1.3 Fatigue index

The study further investigated muscle fatigue in CFS subjects and HCs. A fatigue index was created by assessing the % loss in force over a 2 minute repeated contractile protocol. Results revealed no difference between groups in the percentage loss of force over the 2 minute fatiguing protocol (Figure 4.3). Data from three subjects were excluded; 1 control and 2 CFS patients due to errors in the execution of the protocol.

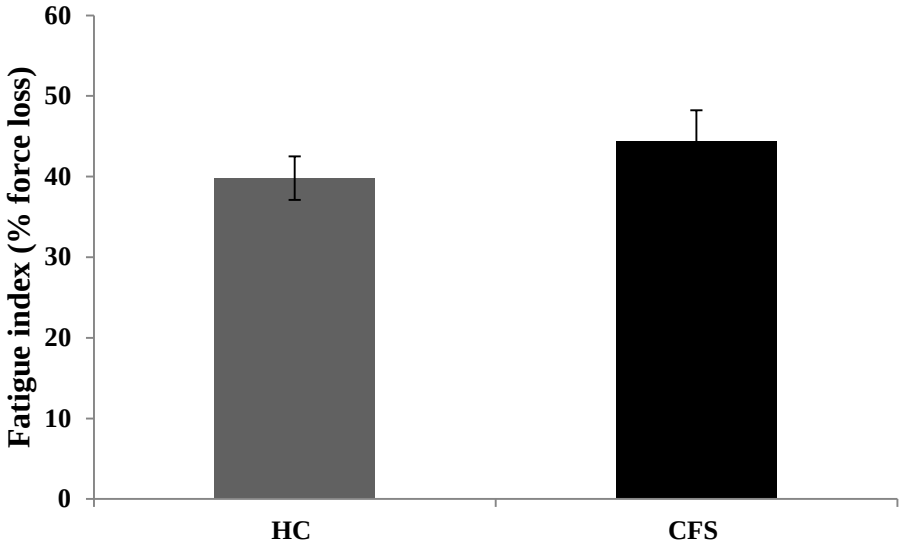


Figure 4.3 Fatigue index of the right knee extensors in HCs and patients with CFS when subjected to 120 seconds of repeated 1 second, 30 Hz trains interspersed with 1 second rest. The intensity of the applied current was set to an amplitude which would elicit 30% of the participant's MVC force when stimulated at 100Hz.

4.3.2 Single muscle fibre cross sectional area, force and specific analysis

Finally, to evaluate force generation at the single-fibre level, *ex-vivo* muscle force of single isolated skinned fibres from *VL* muscle was measured. Cross sectional area (CSA) did not significantly differ (p=0.07) between patients with CFS and HCs (Figure 4.5). Maximal Ca^{2+} activated force (absolute force) was not significantly different between patients with CFS and HCs (Figure 4.4). Comparable results were observed when maximal Ca^{2+} activated force was normalised to fibre CSA (Figure 4.6).

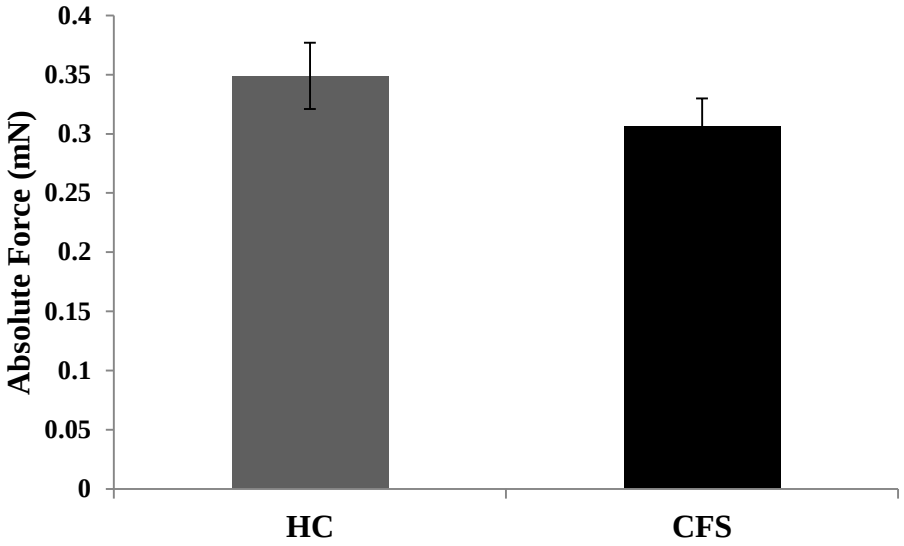


Figure 4.4 Maximum Ca^{2+} activated tetanic force of isolated single muscle fibres from patients with CFS and HCs (n=11). Data presented as mean ± SEM.

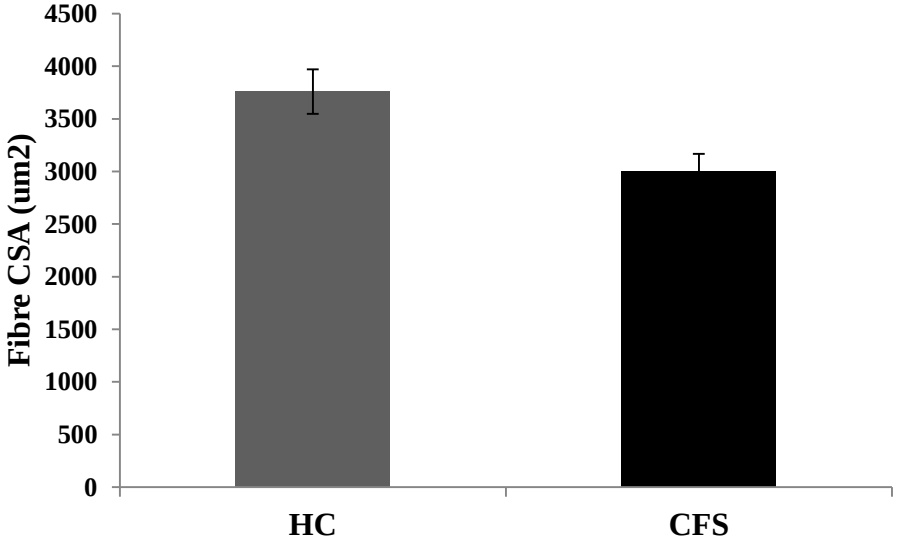


Figure 4.5 Single fibre cross-sectional area (CSA) of the *vastus lateralis* muscle in HCs and patients with CFS (n=11). Data presented as mean ± SEM.

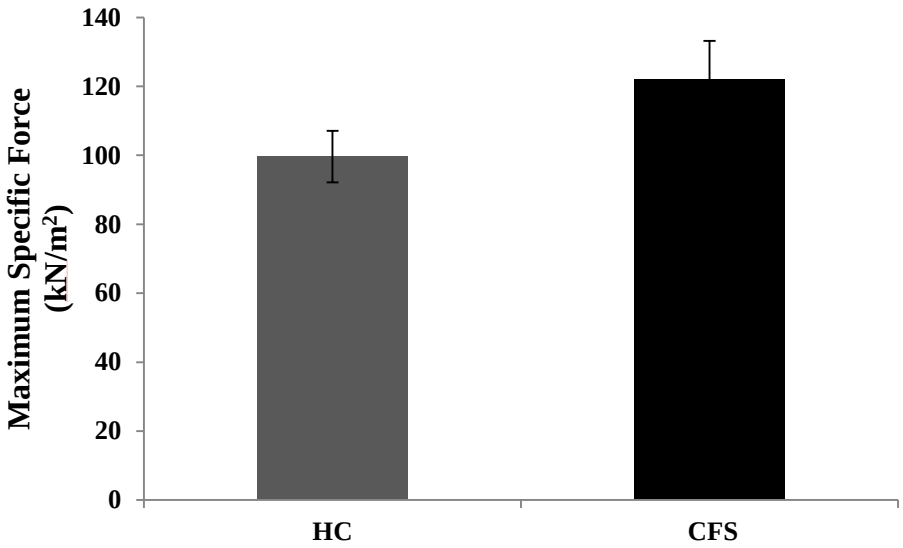


Figure 4.6 Maximum Ca^{2+} activated force in isolated single muscle fibres normalised to fibre cross-sectional area in HCs and patients with CFS (n=11). Data presented as mean ± SEM.

4.4 Discussion

This study investigated functional characteristics of the quadriceps muscle in patients with CFS. Patients with CFS reported lower SF-36 scores suggesting individuals are limited in performing physical tasks as a consequence of their health. Healthy subjects scored highly which suggests no limitations in physical functioning with individuals able to perform all activities including the most vigorous.

Previous studies have assessed contractile function in a range of different muscle groups. Major differences between muscle groups with regards to fibre typing, fatigue resistance, mitochondrial density etc. can often make comparison between studies difficult. Results from the current study show that patients with CFS have significantly lower MVC force of the quadriceps in comparison with HCs (Figure 4.1). This supports previous work (Stokes et al., 1988, Paul et al., 1999). Physiological characterisation of fatigue as assessed by percutaneous electromyostimulation revealed no difference between groups (Figure 4.2). The fatiguing of muscle through involuntary contraction of the quadriceps does not appear to have been investigated before in CFS. The advantage of percutaneous electrostimulaton is the removal of volition. Results revealed no difference in muscle fatigue between patients and HCs (Figure 4.3). In other studies, similar testing of muscle fatigue using repeated voluntary maximal isometric contractions and incremental exercise testing (cycle ergometer) until exhaustion also found no difference between patients with CFS and HCs (Gibson et al., 1993, Paul et al., 1999). This is supported by single fibre specific force analysis; this approach allowed force generated by sarcomeric proteins to be assessed independently of fibre number, innervation, ATP levels and calcium release (Sartori et al., 2013). This study also revealed no difference between patients with CFS and HCs suggesting no inherent dysfunction in actin-myosin cross bridge interactions.

CFS is a heterogeneous group of individuals with individual triggers (including a range of physical and psychological traumas) and symptom profiles. Variation of CFS diagnostic criteria and populations is widespread in CFS research. This undoubtedly contributes to failings to obtain uniformity across findings. Potential reasoning behind the difference in MVC data between studies may arise from

suboptimal effort and motivation from patients in groups who have significantly lower MVCs (Stokes et al., 1988, Schillings et al., 2004). Many researchers use the addition of percutaneous electromyostimulation (twitch interpolation) which superimposes a stimulus at maximal voluntary contraction and aids in the differentiation between central and peripheral deficiencies. The development of any additional force during twitch interpolation enables researchers to decipher whether muscles are capable of producing a maximal contraction or if "effort" is submaximal. The benefits of electrical stimulation include assessing muscle function independently of volition as shown in previous studies suggesting sub-maximal effort or a central impairment in CFS subjects (Stokes et al., 1988, Schillings et al., 2004). A previous study (Gibson et al., 1993) in which voluntary and involuntary (stimulated contraction) muscle function of the *quadriceps femoris* was assessed by dynamometry for both voluntary and stimulated contractions in 12 patients with CFS and 12 age, sex and physical activity matched controls revealed that some subjects were able to produce maximum force however submaximal effort was reported in 5 of the 12 patients with CFS when assessed following twitch interpolation. Unfortunately, this could not be tested in the current cohort of patients with CFS due to the time necessary to familiarise the patients with the twitch interpolation technique.

The majority of muscle function testing uses MVC as an objective marker in determining the potential pathophysiology of fatigue in CFS. This type of maximal exercise is not generally common to everyday life in these patients (Noonan and Dean, 2000). Repetitive, submaximal exercise is more effective in imitating the demands of everyday activities on skeletal muscles. Increasing evidence points to altered central processing and an increased perception of fatigue during physical activity in these patients (Schillings et al., 2004).

Involuntary force analysis removes any effects of volition on contractile function. A lack of difference in force produced at different frequencies suggests no difference in the recruitments of motor units between patients with CFS and HCs. Average isometric force was slightly lower in the patients with CFS due to the lower MVC forces. Direct stimulation of the muscle does not allow for nerve function to be assessed. The 2 minute fatiguing protocol used in this study showed a similar

reduction in muscle force across both HCs and patients with CFS suggesting no difference in fatigue resistance between these groups.

Patients with CFS report extreme feelings of fatigue after minor physical activity as well as an inability to perform tasks that were previously achieved with ease. This is supported by data showing that patients with CFS reported significantly lower physical functioning in the SF-36 score. It would therefore be plausible that these individuals have reduced physical activity and a more sedentary life style. Further investigation into muscle function in patients with CFS needs to account for any variations in muscle physiology and function between subjects who are able to continue everyday activities and physical functioning compared with those who have reduced their physical activity, are immobilised or those who have non-specific underlying muscular myopathies which may be causing their symptoms. Despite proposals for these measures in the early 1990's, little research has been undertaken taking these issues into consideration (Edwards et al., 1993). In general, the relationship between CFS and habitual physical activity levels is unclear. Physical activity is an important consideration in patients with CFS given the evidence surrounding the effects of prolonged periods of physical inactivity on altered on muscle function and physiology. Muscle disuse is characterised by a reduction in contractile function and induces changes including a reduction in muscle fibre size and protein content through increased rates of degradation and reduced protein rates of protein synthesis. To try and reduce the inclusion of patients suffering from gross muscle disuse, patients who were non-ambulatory were excluded from the current study. Some reports have shown evidence of muscle atrophy in patients with CFS reporting a reduction in fibre number and cross-sectional area (Powers et al., 2005) however there is little evidence to support the presence of muscle atrophy across individuals with CFS (Lane et al., 1998).

The study assessed physical function through the use of the SF-36 score. Physical function relates to habitual physical tasks which are generally undertaken in everyday life (dressing, bathing, etc.). This is different to physical activity and exercise which focusses on defined periods of physical exertion such as walking, cycling, running. The SF-36 score is a good marker of health related function status in patients with CFS however it cannot be used to differentiate between the severities of fatigue and cannot be used to differentiate between physical activity

status in subjects (Buchwald et al., 1996). As expected, patients with CFS were found to have impaired physical functioning as a consequence of their health.

Within the large majority of studies assessing CFS there are often basic errors in study design. Recruitment of a comparable control group taking into consideration for age, gender, body weight, height and current physical activity level have been mixed. A recent systematic review has underlined the uncertainty in this area (Nijs et al., 2011). The current study addressed factors including age, gender, body weight and height.

A single exercise test may be insufficient to assess muscle function in CFS patients given previous evidence demonstrating functional impairment in participants only following repeated exercise testing. A recent study found no significant differences between control and CFS patients during the first exercise protocol. However, during a second repeated exercise protocol CFS patients achieved significantly lower values for oxygen consumption and workload at peak exercise and at the anaerobic threshold (Snell et al., 2013). There is a lack of studies investigating muscle function fatigue and recovery. Given the presence of post-exertional fatigue symptoms in all patients with CFS, further investigations into this area are necessary.

One of the limitations of the current study was that post-exertional fatigue was not assessed in the subjects. A study in which subjects performed 18 consecutive isometric contractions using a 50% duty cycle (10 seconds contraction/10 seconds rest) with a 1 minute rest interval between each contraction and showed no difference in absolute changes in peak force or when expressed as a % of the initial MVC between the patients and controls groups (Paul et al., 1999). Peak force for all subjects fell during the 18 contractions indicating fatigue which was still present 24 hours following the fatigue protocol. Absolute changes in peak force were significantly lower in patients with CFS in comparison with controls. Normalised absolute values of MVC at 24 hours following exercise were significantly lower in the patient group but not in the control group.

Further work could include a larger study cohort to allow for greater stratification of patients with CFS regarding disease severity and physical activity. This would enable researchers to stratify and subgroup patients according to more objective

marks of physical status and disease severity whilst maintaining a strong sample size. Given the extent to which activity status can influence muscle strength and physiology, this may be key in establishing the relationship between CFS and muscle function which may be otherwise masked by collectively analysing all patients in one group. Additionally, the use of transcranial magnetic stimulation would aid researchers confirm whether there is a central or peripheral deficit in this patient cohort.

One of the major therapies used in CFS management is graded exercise therapy (GET) (Edmonds et al., 2004). Patients are encouraged to participate in a range of aerobic exercises such as walking, swimming, cycling or dancing. Exercise intensity is dependent on the severity of the CFS. Results from a systematic review reveal that patients with CFS who undertook exercise therapy feel less fatigued than control participants, have improved physical functioning, sleep and mood (Larun et al., 2015). Additional scores of resting systolic blood pressure and net blood lactate were investigated in a randomised control trial of graded exercise therapy. Following the GET intervention, subjects reported improved scores (Wallman et al., 2004). There was a high dropout rate in some studies which suggests GET may not be suitable for all patients with CFS (Powell et al., 2001). Experts within CFS research have called for the use of pacing therapy rather than GET to improve patient outcome to ensure all severities can be included however this is the topic of much debate (White et al., 2011, Shepherd, 2015). There is evidence to suggest patients suffering from either psychological illness or physical myopathies, benefit from physical exercise has numerous benefits in health related outcomes (Oldervoll et al., 2003, Cicek et al., 2015, Lightfoot and Cooper, 2016). The general benefits of exercise may result in health related improvements in patients rather than any effect of improving any muscle ailments.

Data presented in this chapter support previous findings that the symptoms of fatigue experienced in CFS may be a result of suboptimal motivation and/or impaired central processing during muscle contraction and not as a result of peripheral abnormalities. Potential peripheral abnormalities may still exist which are more subtle in patients with CFS and therefore do not cause a significant reduction in objective measures of force production, but may elicit the symptoms described by CFS patients. Additionally, peripheral causes of impaired maximal

voluntary contraction may exist surrounding the function of neuromuscular junctions.

4.5 Summary

This study successfully identified:

- Patients with CFS have a reduced maximal voluntary contractile (MVC) force in comparison with HCs.
- Normal force frequency relationship of the quadriceps in patients with CFS compared with HCs.
- No difference in fatigue in patients with CFS compared with HCs.
- No difference in *VL* muscle single fibre force generation in patients with CFS compared with HCs suggesting no defect in actin-myosin cross bridge interactions.

Further investigations are required to understand the cause of reduced MVC in patients with CFS and whether there may be perturbed systemic and/or local cytokine levels, skeletal muscle redox homeostasis and mitochondrial function which may potentially contribute to a deficit in peripheral neural function.

Chapter Five

Plasma cytokine levels and expression of cytokine genes in skeletal muscle of patients with CFS

5.1 Introduction

Chronic Fatigue Syndrome (CFS) is a debilitating condition which is characterised by an overwhelming sensation of mental and physical fatigue (NICE, 2010). Full details of the pathophysiology of CFS are described in Section 1.6.1. CFS reportedly affects 0.3 – 2.5% of the population depending on the criteria used to diagnose patients (Prins et al., 2006). The diagnosis of patients with CFS occurs through the assessment of primary symptoms and the exclusion of other illness or diseases. Multiple diagnostic criteria exist for CFS; as described in Section 1.6.2. The process through which CFS diagnostic criteria was developed was a result of there being no direct, well-characterised biomarkers for CFS.

Since its early origins, CFS has been proposed to be linked with immune dysfunction and viral infections such as glandular fever and Lyme disease (Bansal et al., 2012). There have been several similar syndromes described under different names, such as post vial syndrome (PVS) and chronic fatigue immune dysfunction syndrome (CFIDS) (Archer, 1987, Uchida, 1992). One prospective cohort study followed patients infected with Epstein-Barr virus (glandular fever), Ross River virus (epidemic polyarthritis) and Coxiella burnetti (Q fever) and reported that 11% of individuals suffered from prolonged illness symptoms of 6 months post-infection. Patients displayed symptoms such as disabling fatigue, musculoskeletal pain, mood disturbances and impaired cognitive function (Hickie et al., 2006); all of which are commonly present in individuals with CFS. Additional symptoms commonly reported by patients with CFS include sore throat, aches and pain (fibromyalgia), headaches, tender lymph nodes and neurocognitive disorders, disturbed sleep and disrupted thermoregulation (Fukuda et al., 1994). Thus, a large number of the symptoms associated with CFS are similar to those associated with flu and/or the common cold. This set of "flu-like" symptoms has been termed the "sickness behaviour/response" (Morris et al., 2013). The "sickness response" is associated with acute infections/injury and the symptoms presented by the patients are thought to be mediated through increased expression of a range of pro-inflammatory cytokines (Dantzer, 2001, Konsman et al., 2002). The presentation of flu-like symptoms at the onset of CFS accompanied by evidence showing a high re-occurrence of bacterial and viral infections in patients with CFS (Chia and Chia,

2008) has led many researchers to associate CFS with altered immune response and perturbed circulating cytokine profiles (Buchwald et al., 1996).

A detailed overview of the role and function of cytokines and chemokines is described in Section 1.3. In brief, cytokines are small intercellular signalling molecules, typically polypeptides or proteins, which have the ability to mediate a wide-range of cellular functions throughout the body (Kelso, 1998). Cytokines are associated with immune regulation and host response to trauma, injury and infection (Zhang and An, 2007). Cytokines are intricately involved in a number of physiological processes, such as tissue homeostasis, cellular relocation and cell cycle regulation; proliferation and differentiation (Austin et al., 1992, Fujigaki et al., 2001). Cytokines are produced and secreted by multiple cell types from both within and outside of the immune system. Cytokines have the ability to signal to both immune and non-immune cells locally and systemically. Cytokine producing immune cells include monocytes, macrophages, B and T lymphocytes and NK (natural killer) cells (Cooper et al., 2001). Non-immune cells which have been identified as cytokine producing cells in response to various stimuli include endothelial, fibroblasts, adipose tissue and skeletal muscle (Sironi et al., 1989, Hotamisligil et al., 1993, Murwani and Armati, 1998, Pedersen et al., 1998). Evidence has shown cytokine levels fluctuate in response to various factors including, sleep, physical exercise, stress and anxiety. Gender specific differences are also present. Thus, serum cytokines levels vary during different stages of the menstrual cycle, pregnancy and between pre and post-menopausal women suggesting an association between hormone levels and cytokines (Verthelyi and Klinman, 2000). Given the increased prevalence of CFS in females, there may be gender specific changes in cytokines levels in this cohort of patients.

A number of studies have been published examining altered cytokine levels in serum/plasma from patients with CFS (Patarca et al., 1994, Swanink et al., 1996, Lattie et al., 2012, Khaiboullina et al., 2015, Hornig et al., 2015). Despite such studies, results remain inconclusive. This is demonstrated in a recent systematic review, aimed at collectively assessing whether patients with CFS have altered circulating cytokine levels, from the results of 38 individual investigations (Blundell et al., 2015). The study identified one potential cytokine, TGF-β to be modified in patients with CFS.

A study investigating immune function in patients with CFS revealed reduced Natural Killer (NK) cell cytotoxic activity is one of the most predominant and consistent outcomes of immunological studies in CFS (Hardcastle et al., 2015). Cytokines, IFN-γ, macrophage colony-stimulating factor (M-CSF), IL-10 and IL-13 are all produced by NK cells an innate immune response (specifically CD56bright NK cells) (Fauriat et al., 2010).

Chapter 3 provided evidence that skeletal muscle can produce and secrete a number of cytokines. Although, the full expression profile of cytokines by skeletal muscle is unknown (Pedersen, 2011, Lightfoot et al., 2015). Skeletal muscle is the largest store of protein in the body and so it is reasonable to hypothesise that muscle could be a significant contributor to the overall systemic pool of cytokines in certain conditions. However, the direct assessment of muscle as a secretory organ *in vivo* is methodologically difficult; there is no robust way of directly examining the ability of muscle to release cytokines. Arteriovenous measures of cytokines have been used to assess acute cytokine release post-exercise, but this process is complicated by the presence of other cells and tissues, such as adipose tissue and endothelium (Korsheninnikova et al., 2002).

Systemic and local raised levels of inflammatory cytokines can affect both muscle function and physiology in a number of ways. Diseases characterised by increased levels of pro-inflammatory and reduced levels of anti-inflammatory cytokines can drive muscle atrophy through a reduction in protein synthesis and degradation resulting in impaired contractile function. This is thought to occur via ROS mediated signalling pathways (Reid and Moylan, 2011).

It is unknown whether there is a link between muscle dysfunction and the flu-like symptoms exhibited in patients with CFS. To date, there has been no research in the area of muscle cytokine production in patients with CFS despite the widespread interest in cytokines and muscle function. This is the first study to assess both systemic cytokine levels and cytokine gene expression in muscles of patients with CFS.

5.2 Hypothesis

Low MVC seen in patients with CFS is potentially due to modified levels of cytokines either systemically resulting in increased mental fatigue or through local peripheral effects on the neuromuscular tissue.

5.3 Aims

The current chapter aims to:

- Investigate plasma cytokine levels in patients with CFS and compared with those of matched HCs.
- Investigate plasma cytokine levels in female patients with CFS and compared with those of matched HCs.
- Examine cytokine gene expression in skeletal muscle of patients with CFS compared with those of matched healthy controls.

5.3 Methods

5.3.1 Recruitment of patients and HCs

Full details of the inclusion and exclusion criteria for the study are described in Section 2.2. In brief, 95 subjects with CFS were recruited through the Royal Liverpool and Broadgreen University Hospital NHS Trust Chronic Fatigue Syndrome services. All subjects were diagnosed by clinicians from the Royal Liverpool and Broadgreen University Hospital NHS Trust in accordance with the Oxford Criteria (1990) and recommended NICE guidelines (2007). Ninety-five age and sex matched healthy controls were recruited from within the Merseyside area. The study did not recruit subjects over the age of 55 years. Research has reported a natural increase in pro-inflammatory cytokines with age which are significantly greater in persons ages >55years (Alvarez-Rodriguez et al., 2012). Fifty-five years of age was deemed an appropriate cut off to ensure age related increments in cytokine levels would not mask any findings associated with CFS. Individuals were excluded from the study if they smoked, had a history of substance misuse, or had any underlying inflammatory-based medical conditions such a cold/flu, infection, arthritis, diabetes, heart disease etc (These factors were reported at the subjects own discretion). Upon study consent, CFS subjects provided members of the research team access to their NHS medical history records. CFS symptoms were assessed upon diagnosis.

	HC	**CFS**
Women	63	63
Men	32	32
Age (Years)	34 ± 1	38 ± 1
Height (m)	1.7 ± 0.01	1.7 ± 0.01
Weight (kg)	71.8 ± 1.2	73 ± 1.5
BMI (kg/m²)	24.5 ± 0.3	25.9 ± 0.4
Illness Duration (Months)	0	37 ± 4.9 (6-276)

Table 5.1: Baseline characteristics of all study participants. Average ± SEM.

5.3.2 Health questionnaire assessment

Patients were obliged to complete a series of health questionnaires (As described in Section 2.2). The study included the Chalder Fatigue Questionnaire, Hospital Anxiety and Depression Score, The Short Form (36) Health Survey (SF-36) physical functioning section and Epworth Sleepiness Scale. These questionnaires were included in the NHS CFS assessment pack and were then used in the study. Questionnaires were anonymously scored by nurses in the CFS services, Royal Liverpool and Broadgreen University Hospital NHS Trust. Questionnaires completed by healthy controls and all non-NHS questionnaires were assigned a subject code and anonymously scored.

Chalder Fatigue Questionnaire

The Chalder Fatigue Questionnaire is an effective measure of fatigue in patients with CFS. It is comprised of 11 questions associated with fatigue, i.e. Do you lack of energy? The questionnaire asks if the feature is less of a problem than usual, no more than usual, more than usual, much more than usual (Appendix - Figure 8.15). The total sum of the CFQ is quantified (e.g. the sum total of all answers in the CFQ) using continuous (Likert Scoring) which allocates 0, 1, 2 and 3 to the answers. Scores can range from 0 – 33 with the higher the score representing a greater level of fatigue.

Short Form Health Survey 36 (SF-36) – Physical functioning assessment

As previously described in Section 5.3.2, the Short Form Health Survey (SF-36) (Version 1.0) physical functioning section was used to assess functional status and well-being/quality of life. Low scores are indicative of greater disability. The full questionnaire is displayed in thesis appendix (Figure 8.19). The SF-36 questionnaire is a suitable, reliable, and valid measure of physical functioning and health status in patients with CFS (Garratt et al., 1993).

Epworth Sleepiness Scale

One of the most widely used scales to assess subjective sleepiness is the Epworth Sleepiness Scale (Neu et al., 2008). The scale contains eight questions designed to assess daytime sleepiness (Figure 8.21). Scores above 10 are commonly interpreted as increased daytime sleepiness and a high risk of dozing (Johns, 1991). Scores greater than 16 suggest individuals are "dangerously" sleepy.

Hospital Anxiety and Depression Questionnaire (HADS)

The Hospital Anxiety and Depression (HADS) questionnaire contains 14 questions split equally to assess anxiety and depression. The score HADS has been shown to perform well in screening a range of patient groups as well as the general population the symptom severity and cases of anxiety disorders and depression. (Bjelland et al., 2002). The HADS questionnaire grades anxiety and depression as mild for individuals who score between 8-10, moderate for scores between 11-14 and severe for scores between 15-21. The full questionnaire is displayed in thesis appendix (Figure 8.18).

5.3.3 Blood donation

Blood was collected for full blood count (FBC) analysis, clotting screen analysis and plasma isolation. Following collection, samples for FBC and clotting blood was kept at room temperature whilst plasma samples were placed immediately on ice. FBC and clotting samples were analysed by the Department of Haematology at the Royal Liverpool University Hospital whilst samples for plasma isolation were processed within the Institute of Ageing and Chronic Disease, University of Liverpool. All samples were processed within 3 hours of collection.

5.3.3.1 Isolation of plasma from human blood

Plasma was isolated from blood samples by centrifugation at 1500g for 15 minutes at 4°C. The plasma was aspirated from the monovettes into cryovials and stored at -80°C for future analyses.

5.3.4 Bioplex multi-bead cytokine analysis of plasma samples

The concentration of cytokines in isolated plasma samples was determined using Bioplex® multi-bead analysis as described in Section 2.6. A multi-plex panel of 27 markers of inflammation including cytokines from the TNF family proteins, IFN family proteins, CCL chemokines, CXCL chemokines and growth factors; a full list is detailed in Table 5.2. Samples were analysed across three separate plates. Inter-plate variation was assessed and corrected for by running a randomly selected group of samples across all three luminex plates.

TNF-α	**IL-15**
IL-1β	**IL-17**
IL-1ra	**IFN-γ**
IL-2	**CCL2/MCP-1 (MCAF)**
IL-4	**CCL5/RANTES**
IL-5	**CCL3/MIP-1α**
IL-6	**CCL4/MIP-1β**
IL-7	**CXCL10/IP-10**
IL-8	**Eotaxin**
IL-9	**GM-CSF**
IL-8	**G-CSF**
IL-9	**PDGF-BB**
IL-10	**FGF Basic**
IL-12	**VEGF**
IL-13	

Table 5.2 List of analytes of Bioplex® Pro Human Cytokine Assay – 27-Plex.

5.3.5 Cytokine analysis by ELISA

- Human TNF-α Quantikine ELISA (R&D Systems, UK).
- Human TNF-α Microplate, Conjugate and Standard
- Assay Dilutent (RD1F - Buffered protein base with preservatives)
- Wash Buffer (Concentrate & ddH_2O)

Levels of TNF-α in plasma samples was analysed using a commercial TNF-α ELISA as previously described in Section 2.6.2. The kit provided a 96-well microplate pre-coated with human TNF-α monoclonal antibody. Following the addition of 50µL of assay diluent and 200µL of standard, sample or control to each well, the plate was covered and incubated for 2 hours at room temperature. Each well was aspirated and washed a total of four times. Two hundred microliters of human TNF-α conjugate was added to each well and incubated for 2 hours at room temperature. A second set of washes was competed before 200µL of substrate solution was added to each well. The plate was incubated for 20 minutes at room temperature. The final step of the protocol involved the addition of 50µL of stop solution. Optical density of each well was determined at 540 nm using a Flurostar Optima (BMG Labtech, Aylesbury, UK.

5.3.7 Muscle biopsy procedure

Full details of the biopsy procedure are described in Section 2.2.5. A percutaneous biopsy was performed on the *vastus lateralis* muscle of patients with CFS and healthy controls, providing their FBC and clotting screening was within the normal laboratory range. Clotting Screen - Prothrombin (seconds).

	HC	CFS
Sample Acquisition (n)	11	11
Women (n)	9	9
Men (n)	2	2
Age (Average ± SEM)	35.8 ± 3.4	35.8 ± 3.5
SF-36*	30 ± 0	16.2 ± 1.4

Table 5.3 Baseline characteristics of biopsy study participants. Average ± SEM.

	HC	CFS
Prothrombin (secs)	11.6 ± 0.2	11.6 ± 0.05
APTT (secs)	30.7 ± 0.6	31.2 ± 0.2
Platelets (x10^9/L)	239.4 ± 7.5	240.4 ± 3.8

Table 5.4 Clotting factors from study participants. APPT (Activated Partial Thromboplastin Time). Seconds (Secs). Average ± SEM.

5.3.8 RNA isolation and qPCR analysis of cytokine expression in *VL* muscle

The method for RNA extraction and qPCR is detailed in Section 2.10. Briefly, RNA from muscle was isolated using Tri Reagent (Qiagen, Sussex, UK). First-strand cDNA was synthesised using the iScript cDNA synthesis kit (Bio-Rad, Hertfordshire, UK). The primers for real-time PCR analyses used in this study are described in Table 4.3. GAPDH, B-Actin and Beta-2-microglobulin (B2M) were used as housekeeping (reference) genes. A range of cytokine targets were selected based upon findings from the literature and from the multiplex cytokine analysis of human plasma samples in the study.

5.4 Results

5.4.1 Study population demographics

As previously described in Section 4.3, the main study recruited 95 patients with CFS and 95 healthy controls. Recruitment took place over 1 year from April 2014 to April 2015. 63 patients with CFS were female; healthy controls were matched ± 3 years of the patients with CFS. The average age within the group of patients with CFS was 38 ± 1 years and healthy controls was 34 ± 1. There was no significant difference between the average weight and height of the CFS and HC recruits and subsequently there was no difference in BMI between the two groups (Table 5.1). The average illness duration in the CFS group was 36 months ± 4.8 (Ranging from 6-276 months).

5.4.2 Cohort characteristics of patients with CFS and HCs

5.4.2.1 Questionnaire Analysis

Health questionnaire scores are shown in Table 5.5. The Chalder Fatigue Questionnaire (CFQ) is highly regarded as an effective and valid measure of fatigue in patients with CFS. Patients with CFS had significantly higher CFQ scores than HC ($p \leq 0.05$). Subjective sleepiness was assessed using the Epworth Sleepiness Scale (ESS) (Neu et al., 2008). On average patients with CFS scored significantly higher than HCs in the study with the majority scoring greater than 10 on the questionnaire scale ($p \leq 0.05$). This indicates patients with CFS are excessively sleepy and may have a dangerously high risk of falling asleep during day-to-day tasks. Patients with CFS had significantly higher scores of both anxiety and depression as reported by the HADS ($p \leq 0.05$). Physical functioning was assessed using the SF-36 health survey and showed patients with CFS scored significantly higher scores than HC ($p \leq 0.05$) suggesting reduced physical functioning in patients with CFS.

	HC	CFS	*P*
Chalder Fatigue Score	10.2 ± 0.36	28 ± 0.39	$p \leq 0.05$
SF-36 Score	29.6 ± 0.36	17.6 ± 0.48	$p \leq 0.05$
Epworth Sleepiness Scale (ESS)	4.5 ± 0.22	10.8 ± 0.37	$p \leq 0.05$
HADS Anxiety	5.2 ± 0.14	10.4 ± 0.49	$p \leq 0.05$
HADS Depression	2.1 ± 0.4	9.8 ± 0.49	$p \leq 0.05$

Table 5.5 Health Questionnaire Scores from Patients with CFS and HCs.

5.4.2.2 Symptom assessment in patient cohort

Upon diagnosis, medical staff assessed symptoms of patients with CFS. A comprehensive list of symptoms commonly reported in patients with CFS is listed in the NHS diagnostic pack (Appendix).

The current investigation was focussed upon serum and muscle cytokine levels and the association with loss of muscle function. Symptoms associated with perturbed cytokine levels and signs of the illness/common cold flu included sore throat, malaise/flu like systems and painful lymph nodes. 29/95 recruited subjects reported malaise and flu-like symptoms whilst 17/95 reported sore throats and 7/95 painful lymph nodes. 100% of patients reported post-exertional malaise, whilst 42/95 suffered from muscle pain (Table 5.6).

	CFS (%)		CFS (%)
Sleep disturbances	64	**Post exertional malaise**	100
Muscle pain	42	**Cognitive dysfunction**	29
Joint pain	33	**General malaise/flu-like**	29
Headaches	35	**Dizziness**	25
Painful lymph nodes	7	**Nausea**	17
Sore Throat	17	**Palpitations**	8

Table 5.6 Symptom assessment in patients with CFS. Data presented as % of study population with symptom.

5.4.3 Full blood count assessment in patients with CFS and HCs

Results from a full blood count (FBC) analysis were examined to determine markers of anaemia (haemoglobin and haematocrit) to confirm patients with CFS were not suffering from anaemia (a potential causes of fatigue). The FBC provided information regarding patient clotting profile (platelet count). An adequate platelet count was required to ensure blood would clot successfully following the biopsy procedure. A full blood count was undertaken in all patients willing to donate a muscle biopsy. Results from the full blood count revealed no significant differences in haemoglobin, red blood count, haematocrit and platelets between patients with CFS and HCs.

Inflammatory blood measures included white blood cell (WBC) counts of neutrophil granulocytes, lymphocytes, monocytes and eosinophil granulocytes. Raised levels of neutrophil granulocytes may indicate bacterial infection or be a sign of acute viral infection and are part of the body's first line of defence. There was no difference in neutrophil count between patients with CFS and HCs. Lymphocytes levels are raised in response to invading bacteria, viruses, and toxins and increase during viral infections such as glandular fever. Lymphocyte levels were similar in both cohorts. Monocytes count increases in a range of conditions, such as bacterial infection or tuberculosis. Eosinophil granulocytes are raised in parasitic infections, asthma, and during allergic reactions. Eosinophil levels were slightly higher in patients with CFS than HCs however the difference did not reach statistical significance. A high number of eosinophils (eosinophilia) are linked to allergic diseases (asthma, hay fever,

eczema) and some infections. The average number of basophils in the blood was significantly lower in patients with CFS than the HC group. Raised basophil secretion is associated with allergic reactions.

	HC	**CFS**	***p***
Haemoglobin (g/L)	136.89 ± 1.53	138.92 ± 1.6	0.45
Red Blood Count (x10^{12}/L)	4.39 ± 0.1	4.55 ± 0.1	0.22
Haematocrit (L/L)	0.44 ± 0.03	0.41 ± 0.0	0.27
MCV (fL)	89.19 ± 0.58	89.56 ± 0.6	0.71
MCH (pg)	30.54 ± 0.24	30.6 ± 0.2	0.8
Platelets (x10^{9}/L)	237.89 ± 5.93	238.2 ± 7.5	0.98
WBC (x10^{9}/L)	6.99 ± 0.3	7.1 ± 0.4	0.9
Neutrophils (x10^{9}/L)	4.36 ± 0.24	4.3 ± 0.3	0.9
Lymphocytes (x10^{9}/L)	1.94 ± 0.08	2.0 ± 0.1	0.7
Monocytes (x10^{9}/L)	0.53 ± 0.02	0.5 ± 0.03	0.99
Eosinophils (x10^{9}/L)	0.14 ± 0.01	0.2 ± 0.02	0.06
Basophils (x10^{9}/L)	0.06 ± 0.01	0.04 ± 0.007	0.03

Table 5.7 Full blood count analysis – markers of anaemia, clotting, and inflammation. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), white blood cell (WBC). Data presented as average ± SEM. $p \leq 0.05$

5.4.4 Plasma levels of cytokines in patients with CFS and HCs

The multiplex assay utilised in this study examined 27 key cytokines and chemokines including TNF-α, a wide range of interleukins, growth factors and chemokines in plasma samples. Analytes IL-2, IL-15, GM-CSF and MCP-1 (MCAF) were excluded from analysis. Since >90% of samples reported values less than the bottom value on the standard curve in both study groups. Data revealed no significant differences between the CFS patient group and the HC group for any of the analytes (Figures 5.1-5.20) as average plasma levels (mean ± SEM) for patients with CFS and HCs and box plots as median (the box depicts the middle 50th percent of the distribution. Outliers which are between 1.5 and 3 interquartile ranges and those >3 interquartile ranges from the nearest edge of the 50th percentile box are symbolised by circles and stars respectively. Results for growth factor analytes are (VEGF, PDGF-bb and FGF-basic) found in the thesis appendix. A more sensitive assay of cytokine detection was performed for plasma levels of TNF-α. Data revealed no significant difference between patients with CFS and HCs Figures 5.1-5.21.

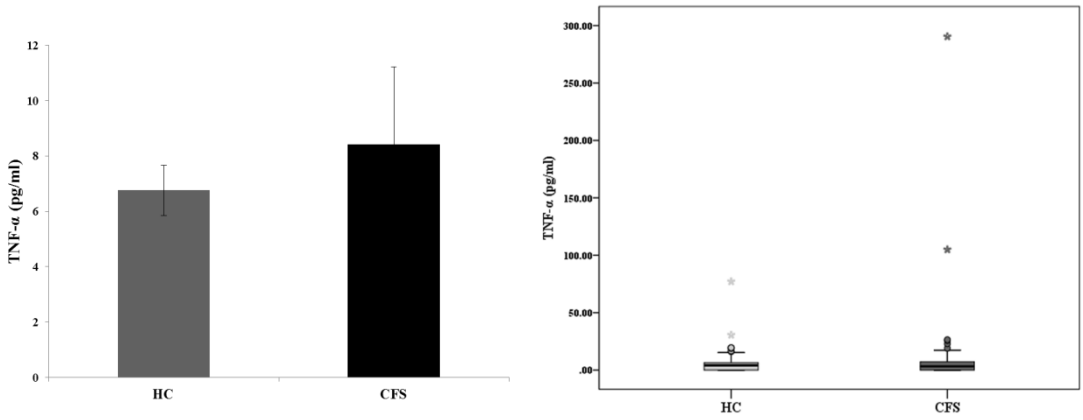


Figure 5.1 Plasma levels of TNF-α in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

Interleukins

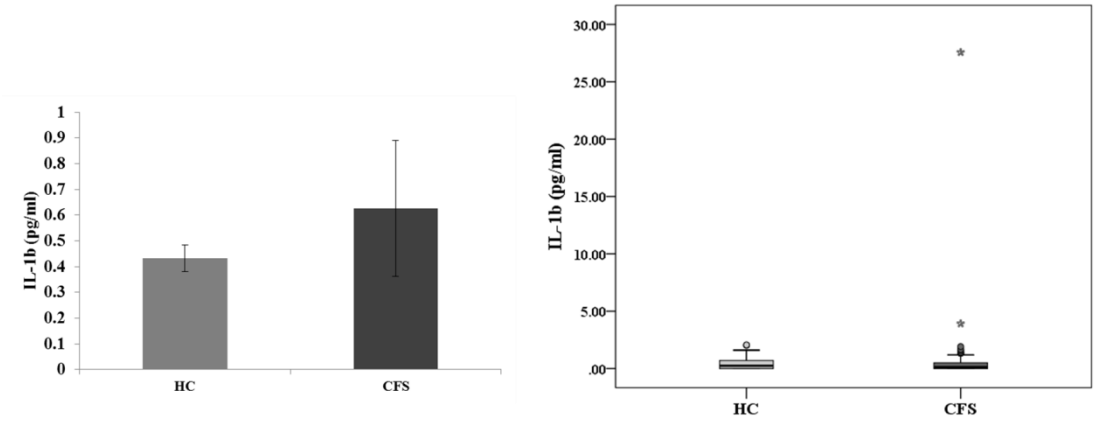


Figure 5.2 Plasma levels of IL-1β in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

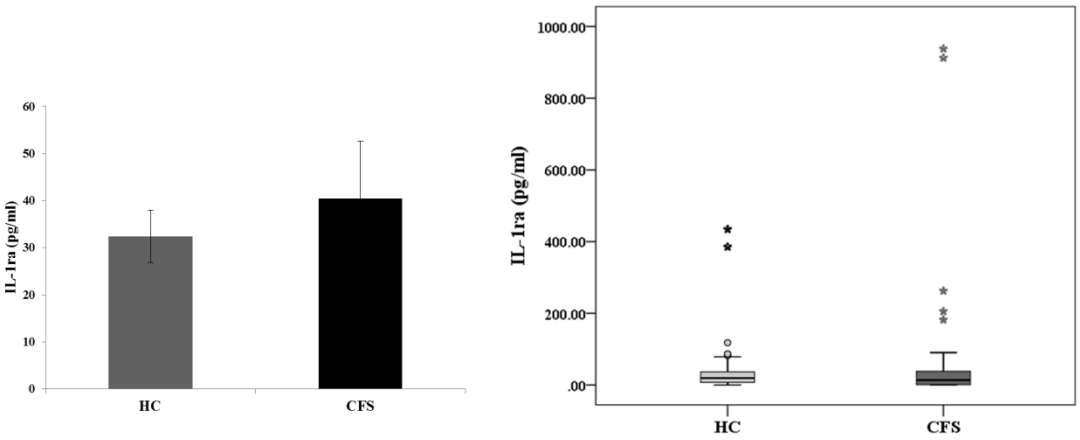


Figure 5.3 Plasma levels of IL-1ra in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

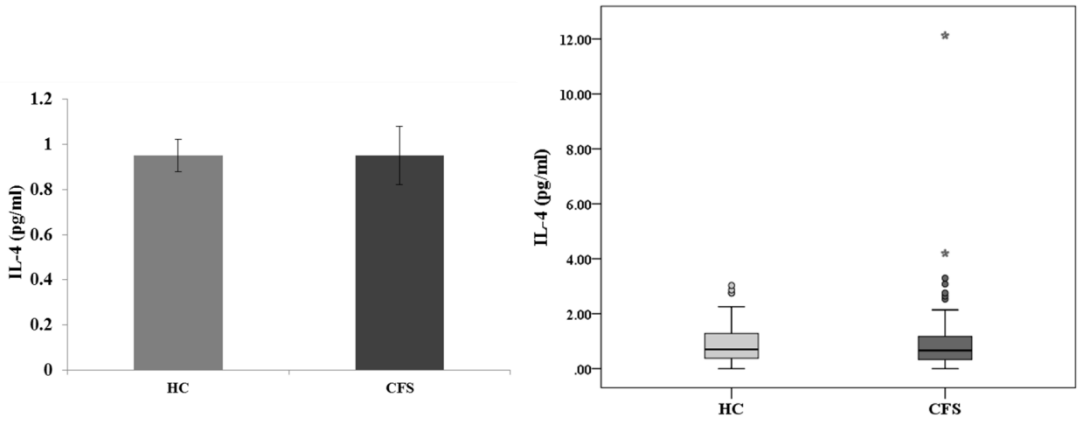


Figure 5.4 Plasma levels of IL-4 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

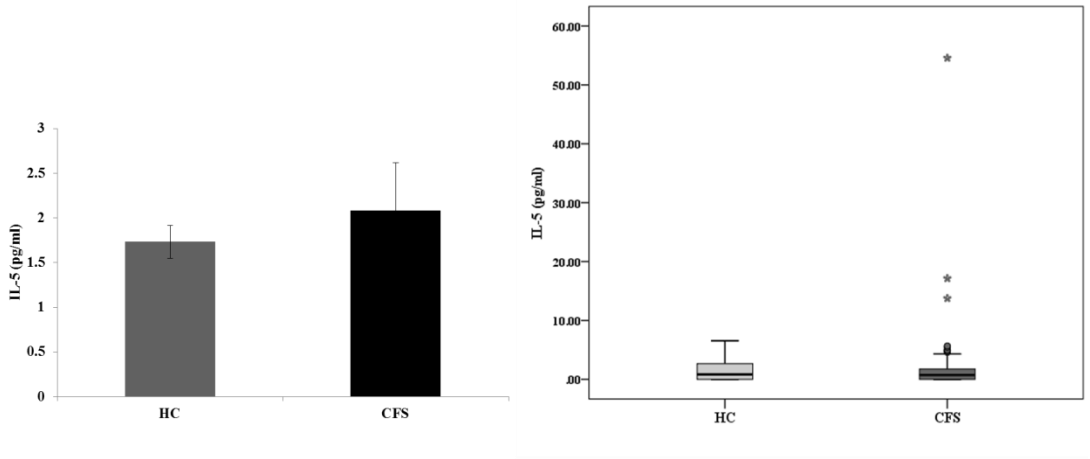


Figure 5.5 Plasma levels of IL-5 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

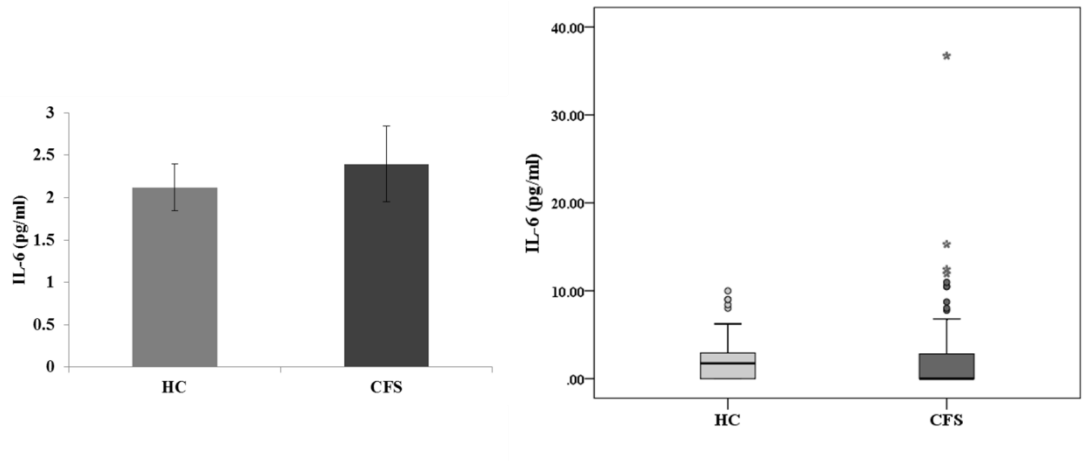


Figure 5.6 Plasma levels of IL-6 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

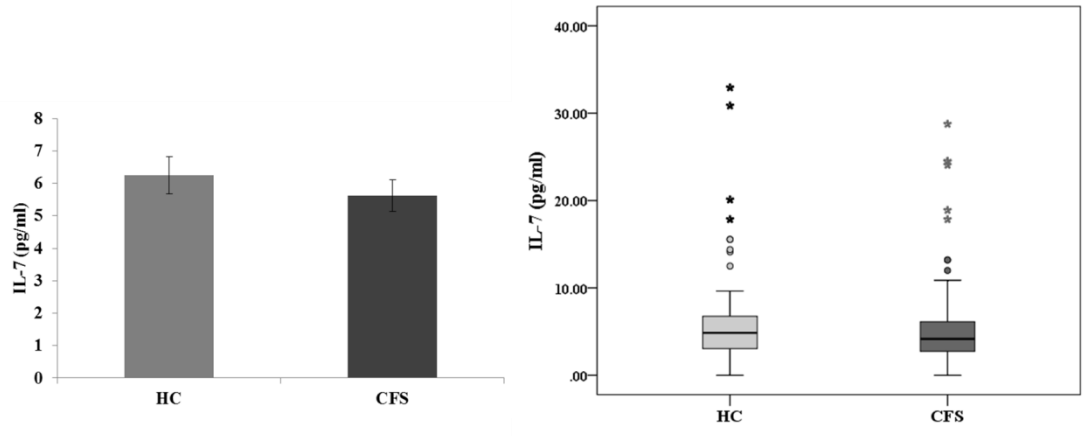


Figure 5.7 Plasma levels of IL-7 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

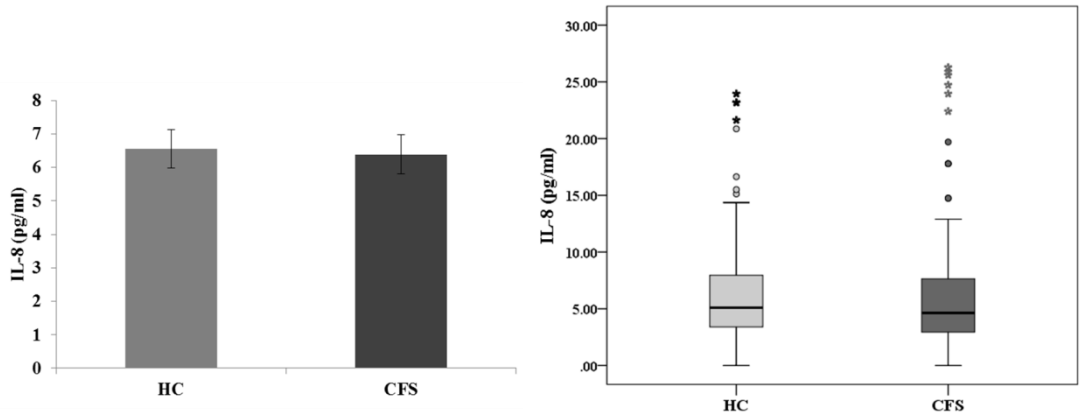


Figure 5.8 Plasma levels of IL-8 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

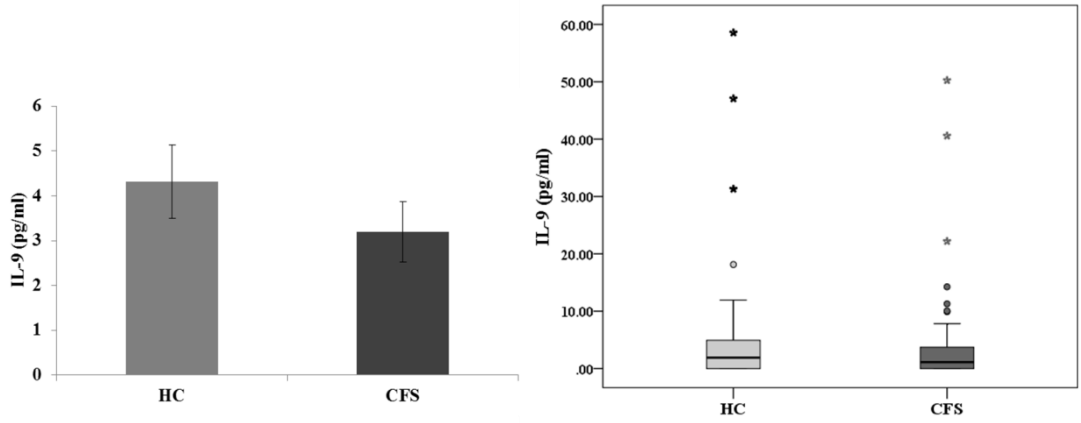


Figure 5.9 Plasma levels of IL-9 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

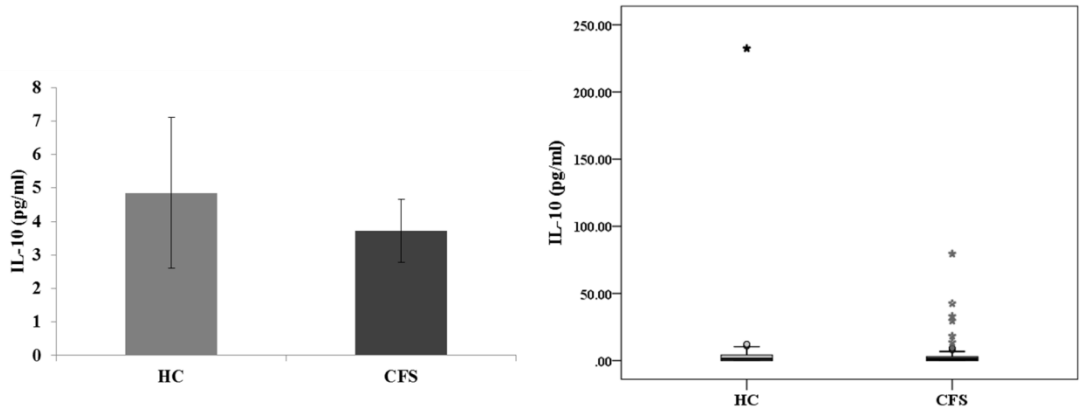


Figure 5.10 Plasma levels of IL-10 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

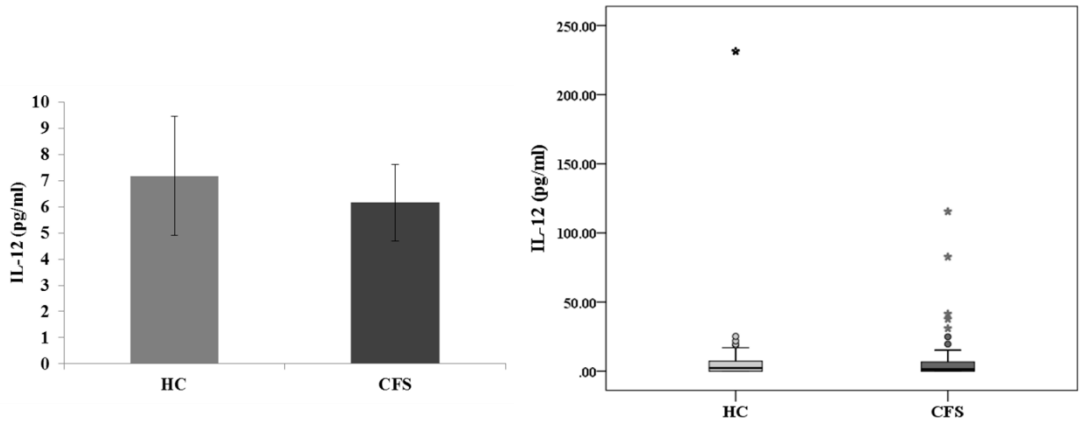


Figure 5.11 Plasma levels of IL-12 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

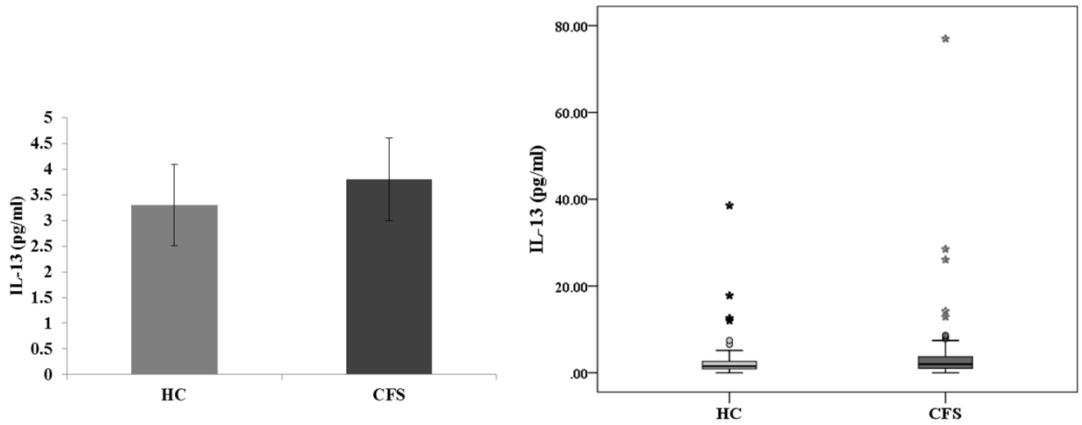


Figure 5.12 Plasma levels of IL-13 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

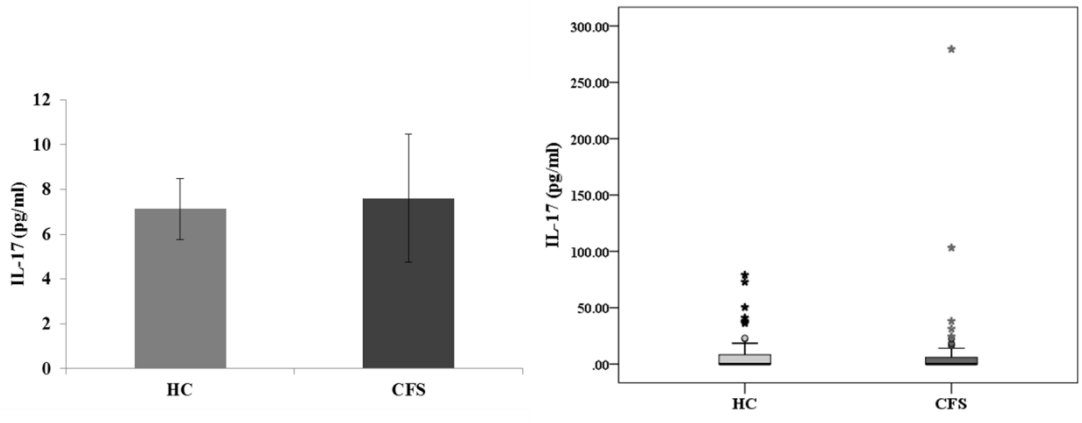


Figure 5.13 Plasma levels of IL-17 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

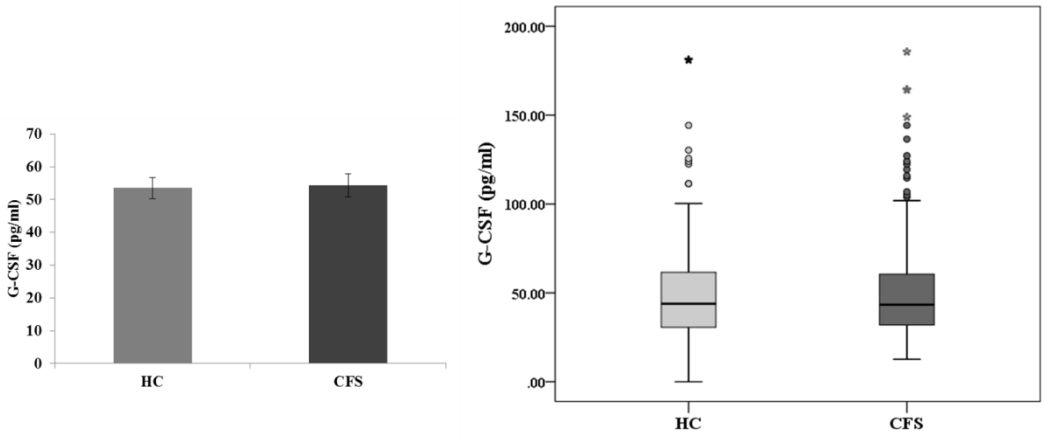


Figure 5.14 Plasma levels of G-CSF (Granulocyte-Colony Stimulating Factor) in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

CC Chemokines

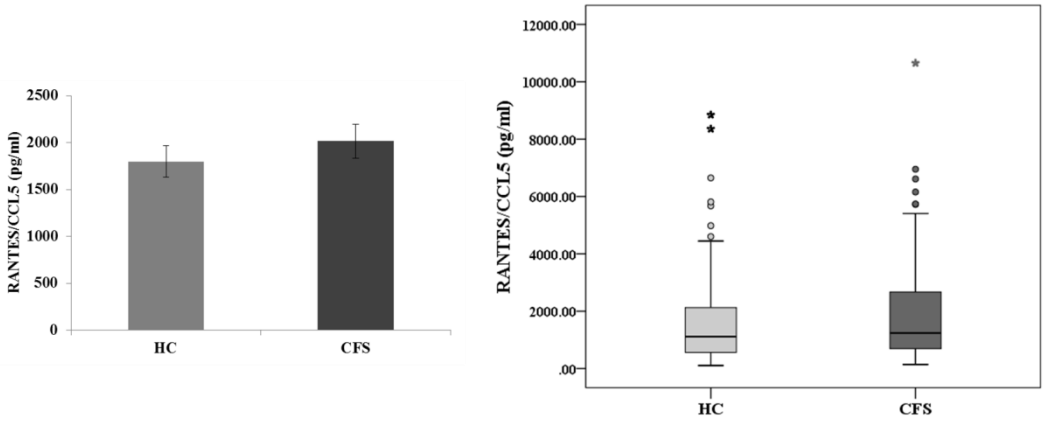


Figure 5.15 Plasma levels of RANTES/CCL5 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM). Distribution of data shown in box plots as median ± interquartile ranges (n=95).

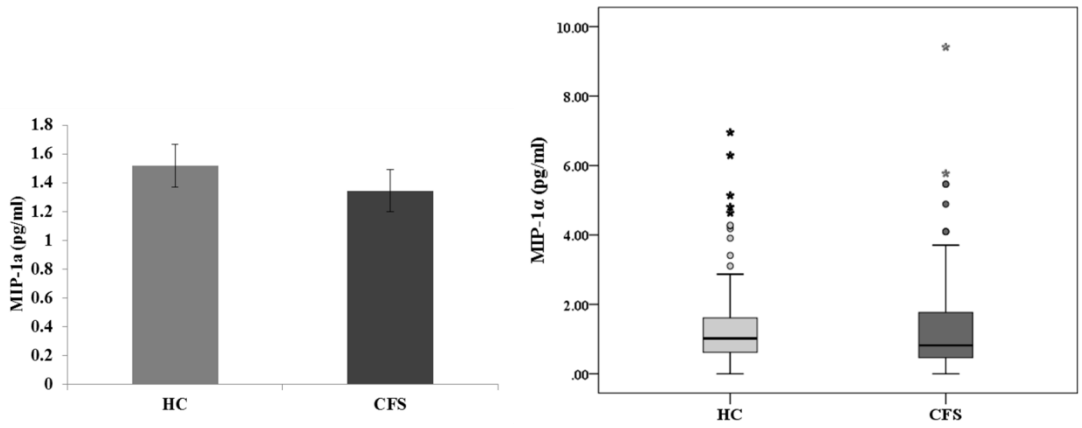


Figure 5.16 Plasma levels of MIP-1α/CCL3 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM). Distribution of data shown in box plots as median ± interquartile ranges (n=95).

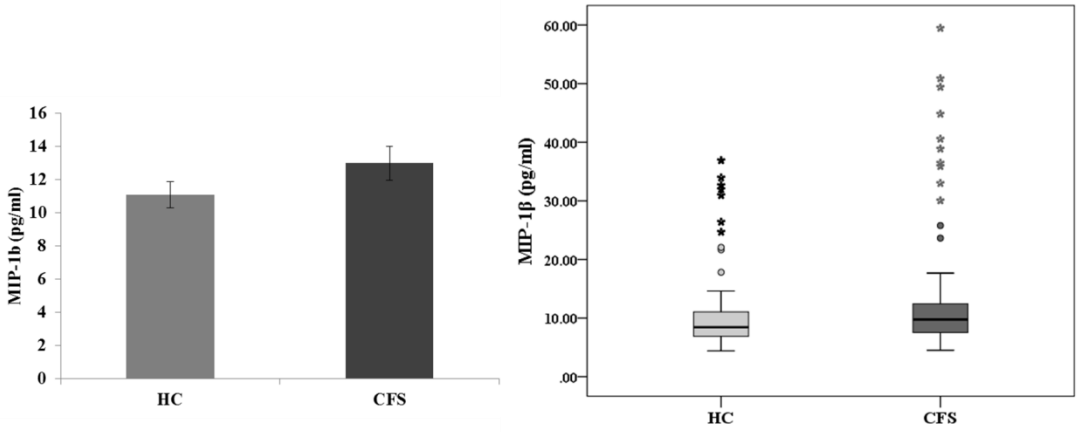


Figure 5.17 Plasma levels of MIP-1β/CCL4 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM). Distribution of data shown in box plots as median ± interquartile ranges (n=95).

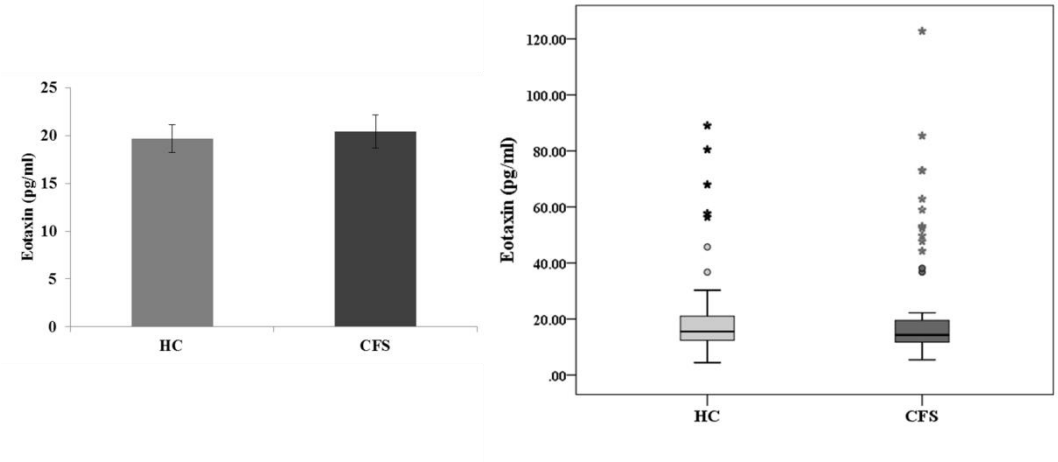


Figure 5.18 Plasma levels of Eotaxin/CCL11 in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

<u>CXCL Chemokines</u>

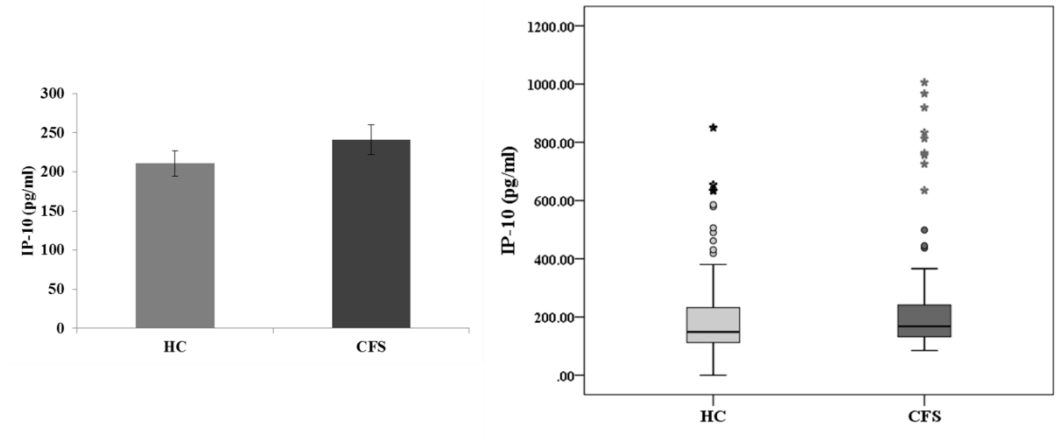


Figure 5.19 Plasma levels of IP-10 (CXCL10) in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

Interferons

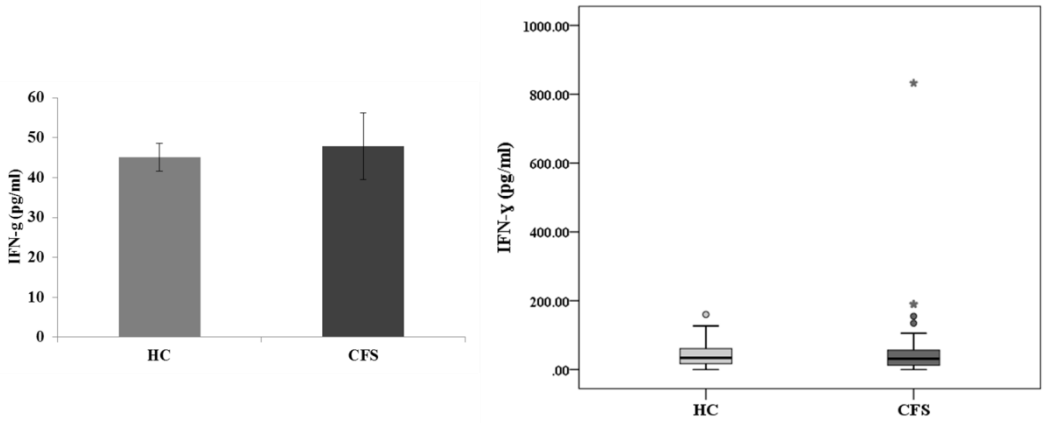


Figure 5.20 Plasma levels of IFN-γ in patients with CFS compared with HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots as median ± interquartile ranges (n=95).

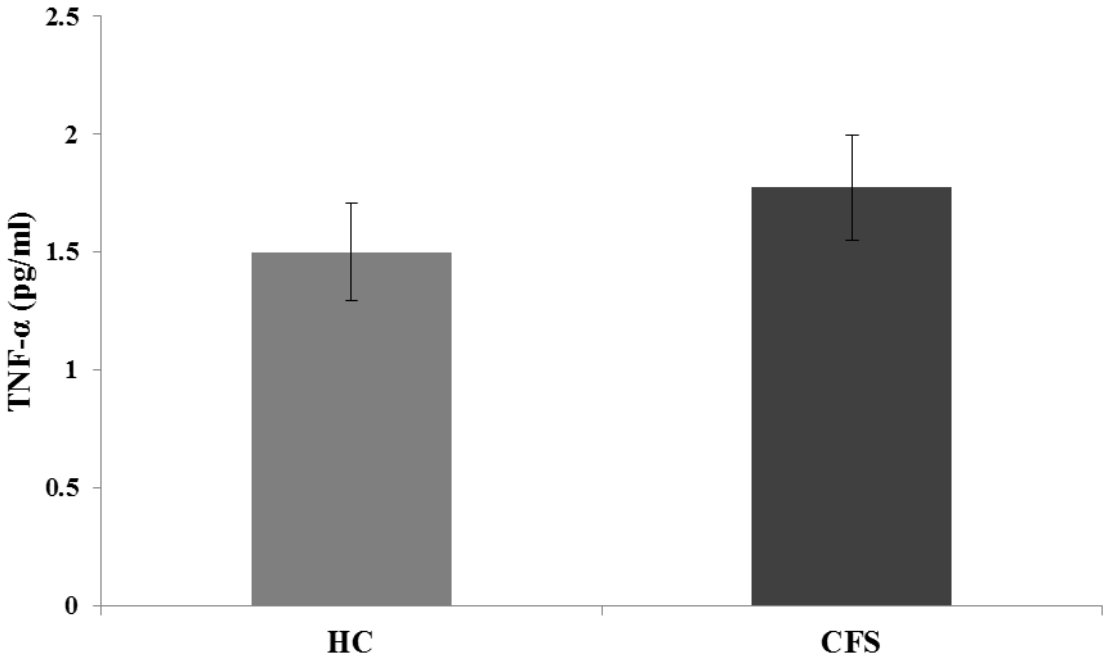


Figure 5.21 Plasma concentration of circulating TNF-α in patients with CFS and HCs (detected through ELISA). Average ± SEM. (n=95)

5.4.4.2 Plasma cytokine levels in female patients with CFS

Due to potential differences in plasma cytokine values between sexes. Data for female patients with CFS and HCs are presented below. CFS is more predominant in female sufferers. The current study included ~70% females. Data revealed no significant changes between female patients with CFS and HCs as shown in (Table 5.10). Additionally, there was no difference between cytokine levels between male CFS sufferers and male HCs.

Cytokine	Female CFS	Female HCs	P Value
PDGF-bb	61.81±12.28	66.56 ± 10.83	0.77
IL-1b	0.37 ± 0.06	0.29 ± 0.05	0.30
IL-1ra	36.94 ± 14.59	25.11 ± 6.41	0.46
IL-4	0.83 ± 0.09	0.81 ± 0.09	0.89
IL-5	1.44 ± 0.27	1.6 ± 0.14	0.36
IL-6	1.88 ± 0.38	1.44 ± 0.27	0.34
IL-7	5.60 ± 0.64	5.69 ± 0/64	0.93
IL-8	6.61 ± 0.78	5.49 ± 0.70	0.28
IL-9	3.09 ± 0.78	3.08 ± 0.91	0.99
IL-10	2.58 ± 0.63	5.48 ± 3.73	0.44
IL-12	4.29 ± 0.99	6.96 ± 3.72	0.48
IL-13	3.02 ± 0.60	2.54 ± 0.67	0.59
IL-17	5.74 ± 1.85	5.64 ± 1.36	0.97
Eotaxin	19.55 ± 1.93	18.87 ± 1.82	0.80
FGF basic	13.16 ± 1.76	11.64 ± 1.75	0.54
G-CSF	54.42 ± 4.88	50.44 ± 4.27	0.54
GM-CSF	27.64 ± 2.81	30.94 ± 2.49	0.87
Hu IFN-g	41.22 ± 4.85	37.50 ± 4.41	0.57
IP-10	259.25 ± 26.87	203.58 ± 18.84	0.09
MIP-1a	1.43 ± 0.18	1.47 ± 0.20	0.88
MIP-1b	12.70 ± 1.23	10.66 ± 0.87	0.18
RANTES	2108.00 ± 267.65	1608.10 ± 230.99	0.16
TNF-a	5.01 ± 0.57	3.0 ± 0.41	0.01
VEGF	1.15 ± 0.48	0.79 ± 0.26	0.51

Table 5.8 Plasma cytokine levels in female patients with CFS compared with female HCs. Data are presented as average ± SEM (n=63).

5.4.5 Expression of cytokines in skeletal muscle of patients with CFS

5.4.5.1 Housekeeping genes

The expression of three housekeeping genes, B-Actin, GAPDH and B2M was examined in skeletal muscle biopsies from patients with CFS and HCs. All housekeeping genes showed stable levels of expression across both cohorts of samples.

All samples were normalised to a single gene; B-Actin. Relative mRNA expression of the three housekeeping genes from all samples is displayed in the appendix in Figures 8.1 to 8.3 respectively. Achieving stable levels of expression for housekeeping genes in muscle can be difficult (Thomas et al., 2014).

5.4.5.2 Cytokine (myokines) expression in skeletal muscle from patients with CFS and HCs

The cytokine genes measured are reported in Table 5.3. Gene targets for qPCR analysis were based on data from the multiplex cytokine analyses and current literature. Quantitative qPCR analysis was performed and demonstrated that the gene expression of all cytokine targets was not significantly different between patients with CFS and HCs (Figure 5.22). However, CCL2 and IP-10 appeared to be raised in a significant proportion of patients with CFS compared with HCs however, this did not reach significance. Expression of MIP-1β (CCL4) and CXCL1 (KC) were not detected in skeletal muscle.

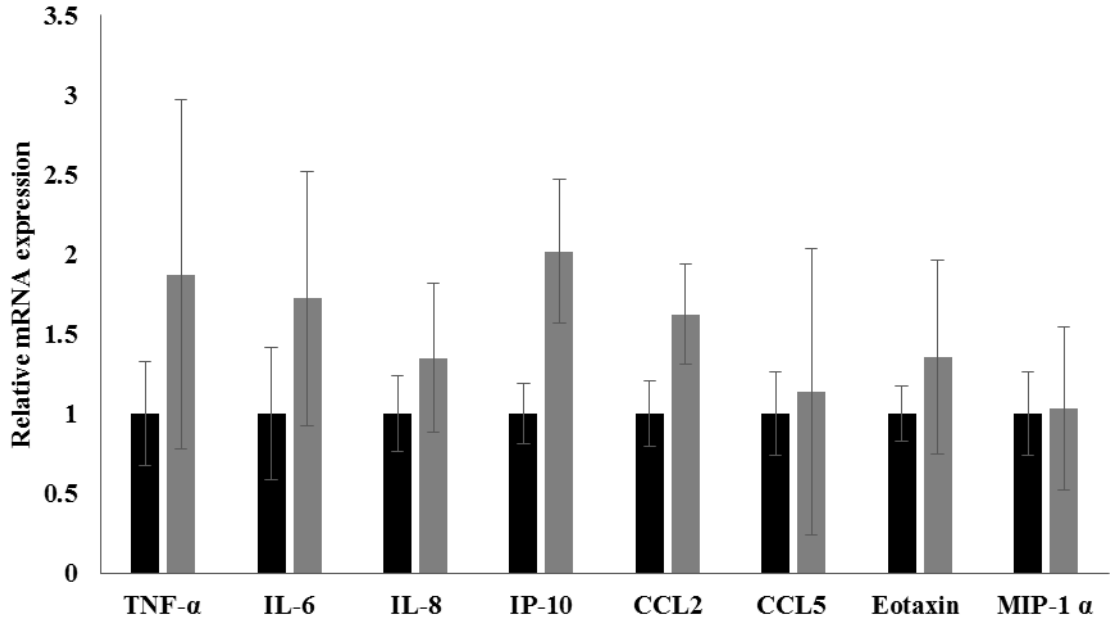


Figure 5.22 Relative mRNA expression for TNF-α, IL-6, IL-8, CXCL10 (IP-10), CCL2 (MCP-1), CCL5 (RANTES), Eotaxin 1 and MIP-1α(CCL3). Data are presented as mean ± standard error of the mean (SEM) n=11. Black bars represent HCs and grey bars represent patients with CFS.

5.5 Discussion

The current study recruited 95 patients with CFS and 95 HCs, and is one of the largest cross-sectional studies to have been conducted in patients with CFS investigating plasma cytokine levels. Within the current study >70% patients with CFS were female. This is supported by recent reports confirming females are the predominant sufferers (Bested and Marshall, 2015), however, the syndrome can affect both men and women. The study was open to participants aged 18-55 years of age across this age bracket, with the youngest participant at 18 years and 54 being the oldest. The study encompassed a wide age range reflecting CFS affecting people of all ages. However, recruitment was excluded for people >55 years of age due to the natural increase in pro-inflammatory cytokines with age (Pedersen et al., 2000) as evidence suggests that cytokine levels are influenced by age and sex (Kleiner 2013). The study had age and sex matched HCs to remove any confounding effects. The mean age of patients with CFS and HCs was 38±1 years and 35±1 years respectively. Patients were age matched to a healthy control because of natural variations between males and females across different age groups (Larsson et al., 2015). This ensured that any age/sex related differences in cytokines were accounted for.

Given the open diagnostic criteria of CFS and lack of objective markers to define patients, CFS cohorts are naturally more heterogeneous than other patient groups. The characteristics of this large CFS cohort are, in general, typical of those within the wider CFS community with the exception of bed ridden patients. The patient cohort was characterised regarding age, sex, disease duration, disease symptoms, mental health, physical functioning, and sleep health. The use of health questionnaires is beneficial in detecting general differences between patients with CFS and HCs; however the lack of depth and simplicity of these assessments prevents discrimination between patients (potential subgroups) and accurate determination of disease severity.

As expected, patients with CFS scored higher in subjective fatigue questionnaires in comparison to healthy control individuals. However, scores were lower among both patients with CFS and HCs compared with more recent studies of adults (Cella and Chalder, 2010). This might be partly explained from the exclusion of the most disabled patients (i.e. bedridden) in the present study and study design (Loge et al., 1998). The use of the Chalder Fatigue Questionnaire does not allow for stratification across the CFS cohort. A more detailed perceived fatigue scale would have allowed the study to differentiate between perceived fatigue level in patients with CFS and grade subjects

accordingly (Jason et al., 2011). Perceived fatigue severity may act as a significant differentiator between different subgroups of patients.

Anxiety and depression scores reported in the HADS questionnaire were high in patients with CFS in comparison with health controls. Approximately 50% of the patients with CFS within this cohort of CFS patients scored >10 in the HADS questionnaire. Both depression and anxiety (mental health) have been proposed as potential modulators of systemic cytokine levels (Gadek-Michalska et al., 2013). Thus, there is evidence to suggest both individuals with high levels of anxiety and/or depression may have raised cytokine profiles independently of disease (Reichenberg et al., 2001, Miller et al., 2009). Further analysis examining sub-groups of patients with raised anxiety and depression scores (using the HADS questionnaires) and their respective cytokine profiles would be interesting to determine any potential effects of anxiety/depression.

On average patients with CFS in this study are at a greater risk of dozing/falling asleep during the day time than HCs. This may be as a result of inadequate night sleep or reduced quality of sleep. Sixty five patients with CFS subjects in this study reported sleep disturbances during diagnosis. Sleep disturbances are a common complaint across CFS populations (Jackson and Bruck, 2012). Systemic cytokines levels may also be perturbed in patients with sleep disorders (Alberti et al., 2003) and healthy participants deprived of sleep.

The study reported symptoms at diagnosis as reported in the NHS CFS service introductory assessment. These included, sleep disturbances, muscle pain, joint pain, headaches, painful lymph nodes, sore throat, post-exertional malaise, cognitive dysfunction, dizziness, nausea, palpitations and general malaise/flu like symptoms. The most predominant of symptoms included post exertional malaise and sleep disturbances (100% and 65% respectively). Patients within the CFS community report a much larger number of different symptoms than other patients groups. Symptoms assessed were limited by the NHS CFS assessment format. The grouping of symptoms i.e. – influenza like symptoms, sore throat would be beneficial and aid stratification and the identification of potential subgroups of patients.

Common traits between CFS and diseases associated with altered cytokine profiles (e.g. viral infections such as the Epstein Barr virus, Lyme disease) has led many researchers to propose altered immune function in the form of altered cytokine levels may be a significant contributor to the pathogenesis of CFS. Despite some published

findings reporting perturbed cytokine levels in patients with CFS, there has been very little agreement between results (Patarca et al., 1994, Swanink et al., 1996, Lattie et al., 2012, Hornig et al., 2015). Cytokines and chemokines are integral components of the immune system which regulate many of the immune system responses and can drive the inflammatory process. The balance between pro and anti-inflammatory cytokines is crucial in an effective immune response. In this and other ways, cytokines aid the body in fighting infection and dysregulation can have major consequences.

The findings of the current investigation into plasma cytokine levels in patients with CFS in comparison to HCs revealed no significant differences in mean concentration of any of the of the 27 analytes assessed. Additional analysis revealed no significant differences in plasma cytokine levels in females alone with CFS compared with HCs or in newly diagnosed CFS subjects compared to long term CFS sufferers of the disease. A full list of plasma cytokine analytes is shown in Table 5.2. This study challenges the widely viewed hypothesis that patients with CFS have perturbed cytokine levels. However, it does contribute to a growing discussion that CFS is a collective term encompassing a wide-range of disorders, in which cytokine disturbances may affect one or more subgroups of patients. Due to the heterogeneity of CFS, collectively pooling all these disorders together might potentially mask any subgroups. The current study did not include assessment of Transforming Growth Factor β, which was identified in a comprehensive systematic review to be elevated in patients with CFS (Blundell et al., 2015).

Previous investigations examining altered cytokine levels in CFS have reported inconsistent findings. For example, individual cytokines may be reported as raised and reduced in separate studies investigating cytokine profiles in patients with CFS and HCs. Patients with CFS may have more subtle alterations in cytokine networks rather than individual cytokines (Banks, 2000). A recent systematic review, investigating the available literature on modified cytokine profiles in patients with CFS identified one cytokine, TGF-β (Transforming growth factor) which was significantly elevated in patients with CFS in five out of the eight studies included in the review and this requires further exploration. Moreover, this study did not report any other cytokines to be abnormal. The review acknowledges the limitations of a shortage of high quality published papers on CFS and cytokines. The review also encompassed studies which used a wide range of different techniques to assess cytokine levels. A number of studies included potential selection bias, due to patients only being recruited from one

source (main a tertiary health care setting). This highlights further issues in CFS recruitment as the more severe sufferers (bedbound) of CFS are commonly excluded.

The findings of the study described in this chapter are supported by a recently published cross-sectional study investigating adolescents with CFS (Wyller et al., 2015). The study used a number of the same analyses including the same multiplex cytokine assay used in this study (The Bio-Plex Human Cytokine 27-Plex Panel) and the Chalder Fatigue Questionnaires. In a similar manner to the current study, data from (Whyler et al, 2015) showed that adolescent patients with CFS and HCs have similar circulating levels of all cytokines measured with no statistical differences between any markers studies. The study showed that patients with CFS also recorded significantly higher scores in the Chalder Questionnaire ($p < 0.001$). It is worth noting that (Whyller et al, 2015) did not formally match patients and controls with regards to variables such as age and body-mass index which may have concealed group differences.

The current study also investigated whether cytokine expression from skeletal muscle was significantly altered in patients with CFS in comparison with HCs. Expression of IP-10 and CCL2 appears to be moderately higher in patients with CFS compared to HCs, however, these data are not statistically significant. Data from Chapter 3 provided evidence that CCL2 is a cytokine that can be produced by skeletal muscle. Increased CCL2 production has been associated with muscle regeneration. IP-10 is also reportedly produced by skeletal muscle and has been shown to amplify T-cell tissue infiltration (Crescioli et al., 2012). Elevated levels of IP-10 have been found in the muscle of individuals suffering from inflammatory myopathies (De Paepe et al., 2005). The presence of elevated cytokines in muscle cells may be consequence of T-cell infiltration rather than increased production (Deyhle et al., 2015). A limitation of the current study was the study of cytokine content within the muscle through the analysis of mRNA levels. Data from Chapter 3 suggests that when cytokines are produced by skeletal muscle cells then they are rapidly exported, thus potentially having a greater effect on the local cytokine environment. In addition, analysis of a muscle biopsy does not allow for the identification of the source of mRNA and care must be taken in the interpretation of the evidence of modified cell content of the biopsy, such as the presence of connective tissue, immune, adipose or endothelial cells.

Skeletal muscle is one of the largest organs in the body and secretes a large number of proteins into systemic circulation. It is unknown whether the ability of skeletal muscle to produce and secrete cytokines can significantly contribute to systemic cytokine

levels in healthy individuals or during disease. Local changes in cytokine levels in and surrounding the muscle may not be reflected by changes in systemic levels of cytokines. Cytokine targets for analysis of mRNA expression in muscle biopsy samples were chosen according to previous data (Chapter 3) confirming the production of these cytokines in muscle (Chapter 3) and published data. Analysis into the gene expression of cytokines from muscle biopsies taken from patients with CFS and HCs is a novel investigation and has not been undertaken before. These data revealed no differences between any targets. Some of the targets assessed have not been previously confirmed in skeletal muscle. The infiltration of cytokine producing immune cells in muscle occurs particularly during injury and is common in diseases such as inflammatory idiopathic myopathies (IIM) (Lepidi et al., 1998, Civatte et al., 2005, Lightfoot et al., 2015). Muscle biopsies taken from our participant biopsies, may reflect contributions from intracellular and interstitial sources of cytokines and not all expression may be directly from the muscle.

Conflicting results between studies reporting changes or no differences in cytokines in CFS may be due to patient related variables such as the patient diagnostic criteria used, disease duration, the state of the CFS upon entrance into the study (relapse/remission cycle), the level of physical activity, stress, diet and sleep pattern and the time of day that blood sampling occurred as well as methodological differences. The current study attempted to control for a number these variables and if this is was not possible, to record differences between which could be used for sub-group analyses. The average illness duration in the CFS group was 36 months ± 4.8 (Total range: 0 – 276 months). The study originally aimed to recruit only newly diagnosed patients with CFS through the Liverpool and Broadgreen green NHS CFS services. However, logistical reasons in trying to screen out anyone who had already been through an NHS services and had been re-diagnosed with CFS preventing us from maintaining this aim. Hence the large discrepancies in the range of illness duration. The majority of studies published on serum/plasma cytokine levels in patients with CFS have used a wide range of different study designs and methodological techniques i.e. – direct serum/plasma analysis, immunoassay analysis of *in vitro* culture supernatants of stimulated or unstimulated cultures of whole blood, separated mononuclear gene expression in mononuclear cells and quantitative flow cytometry of intracellular protein cells (Gupta et al., 1997). Thus, the detection of altered cytokine levels, reported between studies has been highly variable. These inconsistencies may reflect variations in laboratory methods such as the processing and storage of serum and plasma samples. This is often dissimilar

between studies or study reports fail to report these details. Varying factors include the method of cytokine analysis (immunoassay or bioassay), CFS diagnostic criteria, as well as the timing of sample collection during the course of the illness. Additionally, the vast majority of research to date investigating altered cytokine profiles in patients with CFS has been conducted on small sample sizes.

The current study addresses systemic levels of cytokines (within plasma) and localised expression of cytokines from muscles within patients with CFS under basal conditions only. Patients with CFS may have potentially altered levels of cytokines locally or systemically in response to various stimuli given the fluctuating symptoms of CFS and bouts of high or low fatigue. Additional stresses which may significantly alter cytokine levels in patients with CFS only may include altered activity level (physical or mental), altered dietary and sleep patterns.

A large proportion of studies used different methods of analysis. Many pro-inflammatory cytokines are heavily influenced by pre-analytical factors and circulate at very low levels, requiring high sensitivity methods for reliable detection and making comparison between studies utilizing different assays difficult. More recently, published studies have utilised similar if not the same methods for assessing cytokine levels in plasma or serum which makes comparisons between studies easier. New multiplex assays such as the Bioplex® Luminex Assay used in the current study allow for a large number of cytokines to be assesses at once using small quantities of plasma. This is a highly accurate and sensitive technique for cytokine analysis thus strengthening the current data set. To determine whether changes were evident in cytokines present in very low levels in patients with CFS, the study used a very sensitive ELISA for analysis of plasma levels of TNF-α. Data demonstrated a non-significant increase in mean plasma TNF-content in patients with CFS in a similar manner to luminex analyses.

Studies have also assessed cytokine levels in cerebrospinal fluid of patients with CFS. To date, there are the only two investigations examining cytokine levels in cerebrospinal fluid of patients with CFS. Both utilised a multiplex bead technology (The Bio-Plex Human Cytokine 27-Plex Panel or the reagent set Beadlyte 48-011 human 22-plexcytokine detection system). However, the sample size was low in both studies and patients were not age and sex matched to controls. Cerebrospinal fluid from a cohort of 18 patients with CFS revealed lower levels of the pro-inflammatory cytokine IL-10 in comparison to 5 healthy control samples (Marshall-Gradisnik et al.,

2014, Peterson et al., 2015). A study from (Natelson et al, 2015) revealed that levels of granulocyte-macrophage colony-stimulating factor were lower in patients than controls, levels of IL-8 were higher in patients with sudden, influenza-like onset compared with patients with gradual onset of illness or controls and IL-10 levels were higher in the patients with abnormal spinal fluids than in those with normal fluid or controls (Natelson et al., 2005). This may suggest altered cytokine levels between patients with CFS and HCs is specifically localised to cerebrospinal fluid.

5.6 Summary

This study successfully identified:

- No significant differences in plasma cytokine levels between patients with CFS and HCs.
- No significant difference in cytokine mRNA expression in skeletal muscle of patients with CFS compared with those of matched healthy controls. A potential suggestion of increased IP-10 and CCl2 expression warrants furthers investigation.

Chapter Six

Mitochondrial function and redox homeostasis in skeletal muscle of patients with CFS

6.1.1 Introduction

One of the primary functions of skeletal muscle mitochondria is the production of ATP (adenosine triphosphate) through oxidative phosphorylation within the inner mitochondrial membrane (Balaban, 1990). ATP is the principal form of energy within all biological systems. In addition to the role of energy production, mitochondria are involved in neurotransmitter synthesis, calcium homeostasis, apoptosis, insulin secretion, heat production, the regulation of cellular substrate levels, pyrimidine and lipid synthesis and the production of reactive oxygen and nitrogen species (RONS) (Duchen, 2004, Pieczenik and Neustadt, 2007). The consequences of mitochondrial dysfunction can be profound and are often incompatible with life. Mechanisms underlying mitochondrial-related diseases have previously focussed on the production of ATP (Wallace, 1992, DiMauro and Schon, 2003). However there is also evidence to suggest a role of RONS induced oxidative damage and mitochondrial DNA damage in mitochondrial dysfunction (Pieczenik and Neustadt, 2007). Mitochondrial dysfunction can be of primary origin through inherited mutations in mitochondrial DNA or in nuclear DNA which codes for mitochondrial components. Secondary origins of mitochondrial dysfunction are acquired through life from external sources impacting on mitochondrial DNA, physiology and function and include environmental or pharmacologic toxins (Cohen and Gold, 2001). Additional factors associated with altered mitochondrial function include elevated RONS and oxidative stress, insufficient substrate levels and a reduction in intermediary metabolism, and metabolic acidosis (Kucharczyk et al., 2009). Oxidative phosphorylation and ATP production are integral components of human metabolism, however these processes are a source of reactive oxygen and nitrogen species (See Section 1.5 for full details). Mitochondria are both a source and target of increased free radial species and oxidative and nitrosative stress. These dynamic organelles have the ability to respond to changing demands in energy production and environmental stresses (Kotiadis et al., 2014).

Mitochondrial dysfunction has been reported in a wide range of diseases/clinical conditions including cancer, neurodegenerative disorders and cardiovascular disease (Ballinger, 2005, Lin and Beal, 2006, Boland et al., 2013) as well as in premature ageing and age-related dysfunctions (Lin and Beal, 2006, Valko et al., 2007). Increased sensation of fatigue has been shown to occur in the absence of impaired objective markers of peripheral muscle function in severely fatigued cancer survivors (Prinsen et al., 2015). This suggests a potential underlying physiological mechanism of fatigue which may apply to additional syndromes.

Mitochondrial dysfunction has been proposed as a potential contributor to CFS (Wong et al., 1992, Kuratsune et al., 1994, Plioplys and Plioplys, 1995, Booth et al., 2012, Morris and Maes, 2014). There are many symptoms which overlap between patients with CFS and mitochondrial dysfunction (Section 1.6.5 - Table 1.6). These include muscle cramps and weakness and neuropathic pain. The main primary characteristics of CFS which is shared with mitochondrial dysfunction is a persistent, debilitating fatigue accompanied by post-exertional malaise (Christley et al., 2012). A recent cross-sectional survey assessing symptom severity of perceived fatigue in patients with mitochondrial disease and patients with CFS reported comparably high Fatigue Impact Scale (FIS) scores in patient groups when compared with HC scores (Gorman et al., 2015). In the absence of any other underlying diseases, these symptoms may be associated with a more subtle defect in numerous body systems, however, (Filler et al., 2014) reported a strong association between fatigue and a failing in energy metabolism and mitochondrial dysfunction in CFS. Dysfunctions in the structure of mitochondria, levels of mitochondrial enzymes, oxidative/nitrosative stress and mitochondrial energy metabolism (ATP production), were investigated as potential contributors to fatigue. Evidence to support perturbed mitochondrial function in CFS is currently inconclusive (Vermeulen et al., 2010). Support for the hypothesis of mitochondrial dysfunction in CFS is limited to few primary studies with relatively small sample size. For example, only two patients with CFS (post viral relapsing myalgia) and 13 HCs were included in the study in which muscle histology and respirational capacity from biopsy samples was investigated (Byrne et al., 1985). The study identified type II fibre atrophy and mitochondrial respiration analysis *in vitro* revealed a slight reduction in State 3 respiration rates. However, data from studies with a small sample size can often be misleading and/or misinterpreted.

Markers of mitochondrial function has previously been investigated in white blood cells and in muscle cells of patients with CFS in a number of studies (Edwards et al., 1993, Kurup and Kurup, 2003, Behan et al., 1991, Behan et al., 1997). However, the outcome of some studies is not authenticated due to the use of non-standardised or validated methods such as the "ATP profile" as illustrated in studies from (Myhill et al., 2009, Booth et al., 2012). This Chapter focuses on various aspects of mitochondrial function in skeletal muscle biopsies from patients with CFS. Some evidence has suggested the presence of structural abnormalities in muscle mitochondria of CFS (Behan et al., 1991, Plioplys and Plioplys, 1995) which may impact on functional capacity. These data suggest that impaired mitochondrial function in CFS may be

linked to a reduction in oxidative metabolism in muscle (McCully et al., 1996), reduced ATP production and/or elevated ROS production (Booth et al., 2012). These differences in mitochondrial function between patients with CFS and HCs are yet to be fully elucidated and confirmed and additional studies suggest no difference in mitochondrial function in biopsies of skeletal muscle from patients with CFS (Smits et al., 2011).

RONS have typically been associated with oxidative/nitrosative stress and cellular dysfunction (Sohal and Weindruch, 1996). Increased production and excess exposure of cells to RONS can be detrimental, however, research has now demonstrated the importance of RONS in muscle cell signalling or the effect of excess RONS in disrupting normal ROS signalling pathways. (Powers et al., 2010). It is essential for skeletal muscle to maintain redox homeostasis within cells given the role of RONS in normal skeletal muscle physiology and function. Some evidence suggests RONS are elevated in plasma from patients with CFS and consequently induces oxidative damage to lipids, proteins and DNA. Protein carbonyls, isoprostane, 8-hydroxyguanosine, thiobarbuturic and malondialdehyde are all markers of oxidative damage and there is some evidence for increased levels in plasma of patients with CFS (Vecchiet et al., 2003).

Mitochondrial dysfunction can arise from a number of factors including abnormalities in inflammatory pathways involving elevated pro-inflammatory cytokines (Samavati et al., 2008), excessive production and reduced removal of RONS as well as elevated NF-κB activation (Li et al., 1999, Garrabou et al., 2012). Reports suggesting increased activation of immune-inflammatory pathways in CFS may support the potential presence of mitochondrial dysfunction given the relationship between immune-inflammatory pathways, ROS signalling and mitochondrial function (Naik and Dixit, 2011, Morris and Maes, 2014). Research suggests that altered cytokine levels can disrupt normal mitobioenergetics, thus affecting mitochondrial function and thereby increase oxidative stress (Lopez-Armada et al., 2013). However, there is also evidence to suggest patients with muscle weakness may have decreased muscle function without any signs of a pro-inflammatory environment within the quadriceps. These data report high levels of oxidative stress in the muscle of clinically and weight stable patients with COPD (Barreiro et al., 2008).

It is plausible that patients with CFS may experience increased perception of fatigue and impairments in muscle function with or without known pathological changes in the muscle. Data shown in Chapter 4 (Figure 4.1) demonstrated that patients with CFS

have reduced MVC forces in comparison to HCs. Further investigation to elucidate whether there is an association between reduced MVC forces and altered muscle mitochondrial redox homeostasis and function using state of the art approaches/techniques to ensure accurate analysis.

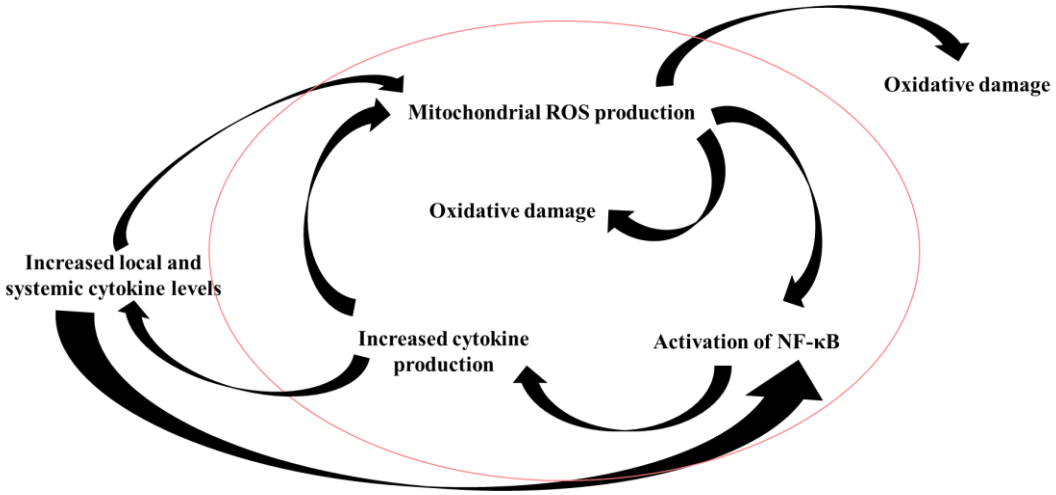


Figure 6.1 Schematic diagram illustrating the potential vicious cycle of ROS production and cytokines levels in skeletal muscle.

6.1.2 Hypothesis

Mitochondrial ROS generation and respiratory function is altered in skeletal muscle of patients with CFS compared with HCs.

6.1.3 Aims

The current chapter aims to:

- Assess mitochondrial content and respiratory function *in situ* in permeabilised muscle fibre bundles isolated from skeletal muscle of patients with CFS and HCs.
- Characterise mitochondrial ROS production in skeletal muscle from patients with CFS by directly assessing mitochondrial H_2O_2 and superoxide production *in situ* in permeabilised muscle fibre bundles from skeletal muscle biopsies.
- Assess markers of oxidative damage and the expression of redox proteins involved in RONS regulation in skeletal muscle from patients with CFS and HCs.

6.2 Methods

6.2.1 Study secruitment

Subjects with CFS were recruited through the Royal Liverpool and Broadgreen University NHS Trust CFS services. All subjects were newly diagnosed by clinicians in accordance the Oxford Criteria (1990) and recommended NICE guidelines (2007) (See Section 2.2 for full details of study recruitment and inclusion and exclusion criteria). 11 Patients with CFS (9 women and 2 males) and 11 healthy age and sex matched HCs (9 women and 2 males) provided muscle biopsies (Table 6.1).

	HC	**CFS**
Sample Acquisition (n)	11	11
Women (n)	9	9
Men (n)	2	2
Age (Average ± SEM)	35.8 ± 3.4	35.8 ± 3.5
SF-36*	30 ± 0	16.2 ± 1.4

Table 6.1 Baseline characteristics of study participants who undertook a muscle biopsy (SF-36 - Short Form Health Survey - Physical Activity). *$p \leq 0.05$.

6.2.3 Biopsy Procedure

A biopsy was taken from the *vastus lateralis* muscle of patients with CFS and HCs (Section 2.2.5). An Acecut automatic Biopsy system (TSK Laboratory, Oisterwijk, Netherlands) was used for each procedure. In brief, the thigh area was sterilized with a topical antiseptic, (povidone-iodine or chlorhexidine gluconate). The skin and thigh area were both anaesthetised with 4ml 2% lidocaine. A small 1cm incision was made through the skin, subcutaneous tissues and fascia using a scalpel. Three biopsies were collected from the muscle from different regions within 2-3 cm of the biopsy incision.

6.2.4 Preparation of muscle sample for analysis of mitochondrial ROS production and respiration function

Muscle biopsy samples were immediately placed into ice-cold stabilizing Buffer A composed of 2.77mM CaK_2 ethylene glycol*bis*-(2-aminoethylether)- *N*,*N*,*N*=,*N*=-tetraacetic acid (EGTA), 7.23mMK_2 EGTA, 6.56mM $MgCl_2$, 0.5mM dithiothreitol (DTT), 50mM 2-(*N*-morpholino)ethanesulfonic acid potassium salt (KMES), 20mM imidazol, 20mM taurine, 5.3mM Na_2 ATP, and 15 mM phosphocreatine, pH 7.3 at 4°C. Muscle biopsy samples were manually teased into small fibre bundles and isolated fibres. Fibre bundles were permeabilised for 30 minutes in Buffer A supplemented with 0.05mg/ml saponin on ice (Gouspillou et al., 2014). The use of this technique to analyse intact skeletal muscle mitochondria *in situ* has been thoroughly validated and described (Anderson et al., 2009, Picard et al., 2010, Anderson et al., 2007). *In situ* permeabilisation of fibres aids the preservation of mitochondria morphology and structural interactions and is reflective of the functioning of all mitochondria within the muscle fibre (Kuznetsov et al., 2008, Picard et al., 2010).

Fibre bundles required for respiration analyses were washed 3 x 10 minutes in buffer B, composed of 2.77mM CaK_2 EGTA, 7.23mM K_2 EGTA, 1.38mM $MgCl_2$, 3.0mM K_2HPO_4, 0.5mM DTT, 20mM imidazole, 100mM K-MES, and 20mM taurine, pH 7.3 at 4°C, supplemented with fatty acid-free bovine serum albumin (BSA) (2mg/ml) (Gouspillou et al., 2014).

Fibre bundles required for H_2O_2 production analyses were washed 3 x 10 minutes in buffer Z, composed of 110 mM K-MES, 35 mM KCl, 1 mM EGTA, 3 mM $MgCl_2$, and 10 mM K_2HPO_4, pH 7.3 at 4°C, supplemented with BSA (5 mg/ml) (Gouspillou et al., 2014).

6.2.5 Mitochondrial H_2O_2 generation

To assess H_2O_2 release from permeabilised fibre bundles, an Amplex Red assay was used as previously described (Gouspillou et al., 2014, Picard et al., 2010). Permeabilised myofibre bundles (10 bundles), prepared as described previously (Section 6.2.4), were added to single wells within a 96 well microplate containing l00µL Amplex Red solution (19.44mM Amplex Red, 5U/ml HRP, 37.5U/µL SOD in 50ml Buffer Z). Samples were incubated at 37°C for 5 minutes. Substrates/inhibitors were added as follows: Glutamate/Malate (10mM + 5mM), Succinate (S, 10mM), Rotenone (R, 1µM) and Antimycin A (AA, 10 µM) to activate different mitochondrial

complexes. H_2O_2 production was measured using a Flurostar Optima Fluorimeter (BMG Labtech, Aylesbury, UK). At the end of the H_2O_2 measurements, bundles were placed in liquid N_2 and stored at -80°C. H_2O_2 production was expressed as picomoles per minute per unit protein content.

6.2.6 Mitochondrial superoxide generation. Fluorescence-based methods to measure MitoSOX Red oxidation

To monitor changes in mitochondrial superoxide, a MitoSOX Red mitochondrial superoxide indicator (MitoSOX Red) probe was utilised. MitoSOX Red is a derivative of dihydroethidium. It is a highly sensitive mitochondrial superoxide indicator and exhibits fluorescence upon oxidation by superoxide (Robinson et al., 2006). The reaction between superoxide and MitoSOX Red generates a highly specific fluorescent product, 2-hydroxyethidium (2-OH-Mito-E^+) (Zielonka and Kalyanaraman, 2010). Isolated fibres from the *VL* muscle were loaded with 250nM MitoSOX Red (Invitrogen, California, USA) for 30 minutes as previously described (Sakellariou et al., 2013, Pearson et al., 2015). Fibres were maintained in buffer Z containing MitoSOX Red (20nM) during the experimental period. Fluorescence was quantified at an excitation/emission wavelength of 405/605nm.

Fluorescence images were obtained using a C1 confocal laser scanning microscope (Nikon Instruments Europe BV, Surrey, UK) equipped with a 405nm excitation diode laser and a 605/15 emission filter. Using 60x magnification, fluorescence images were captured and analysed with the EZC1 V.3.9 (12bit) acquisition software.

6.2.7 Mitochondrial membrane potential

In order to assess potential changes in mitochondrial membrane potential ($\Delta\Psi m$) in intact mitochondria of isolated VL fibres, fibres were loaded with a florescent dye, tetramethylrhodamine, methyl ester (TMRM), as previously described (Irwin et al., 2003). Changes in TMRM fluorescence of isolated skeletal muscle fibres were assessed by loading fibres with 30nM TMRM (Invitrogen, California, USA). Fluorescence was detected at an excitation/emission wavelength of 543/605nm. Changes in $\Delta\Psi m$ were determined in the presence of oxidative phosphorylation inhibitors; oligomycin (2.5μM) and FCCP (4μM). Images were obtained and analysed using a C1 confocal laser scanning microscope (Nikon Instruments Europe BV, Surrey, UK).

6.2.8 Mitochondrial respiration

Changes in mitochondrial O_2 consumption were assessed with the use of the Oxytherm oxygen electrode control unit (Hansatech Instruments, Norfolk, UK) following permeabilisation of fibre bundle (15-17mg wet weight) as described above. Four hundred and fifty microliters of Buffer B containing, (in mM) 100 K-MES, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 imidazole, 0.5 DTT, 20 taurine, 3 K_2HPO_4, and 1.38 $MgCl_2$-$6H_2O$ (pH 7.3 at 4°C), supplemented with 2mg/ml BSA was added to the chamber and was constantly stirred. Following a period of stabilisation 10µL of 10mM glutamate + 5mM malate was added into the oxytherm chamber. After 3 minutes, the permeabilised myofibre bundles (14-16 mg wet weight) were added into the chamber. After a further three minutes, 2mM ADP was added into the chamber. The bundles were left until all the O_2 within the chamber had been utilised or the muscle had used up all of the substrates. After respiration measurements were completed, fibre bundles were removed and placed in liquid N_2 and stored at -80°C for protein quantification by Bradford assay (Section 2.4.1).

6.2.9 Analysis of mitochondrial content in skeletal muscle - citrate synthase assay

Citrate synthase activity can be used as a biomarker for mitochondrial content in a tissue homogenate. Citrate synthase activity was assessed using the Mitocheck Citrate Synthase Activity Assay Kit (Cayman Chemical Co., Michigan, USA). In brief, muscle homogenates (1:200) were added to each well of a 96-well plate in addition to the following reagents provided in the kit: 2% Acetyle CoA Reagent, 2% Developer Reagent and 4% Oxaloacetate Reagent in the provided assay buffer. The assay measured the production of SH-CoA by monitoring the absorbance of citrate synthase developing reagent at 412nm. A 96-well plate format was used and absorbance was detected using a microplate spectrophotometer (Bio-Tek instruments, Vermont, USA).

6.2.10 Western blotting of muscle proteins

Protein content was assessed by western blot analysis (Full details of western blotting described in Section 2.5). In brief, 20µg of total protein was loaded and separated by electrophoresis across an 8-15% polyacrylamide gel with a 4% stacking gel. Proteins were transferred onto a nitrocellulose membrane using a Multiphore Continuous

Blotting System (Pharmacia, Uppsala, Sweden). Ponceau-S staining was used to visualise the membrane, ensure the correct transfer of proteins and used as a loading controls against the protein of interest. Nitrocellulose membranes were blocked for 1 hour at room temperature in 5% milk TBS Tween (Tris-Buffered Saline with Tween-20) before being incubated overnight with primary antibodies (See Table 6.3 for detailed list of antibodies). Membranes were washed in TBST (3 x 5 minutes) and incubated with secondary antibody for 1 hour with a horseradish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG (Cell Signalling, Hitchin, UK). Peroxidase activity was detected using an ECL kit. Band intensities were analysed using NIH Image J Software.

6.2.11 Analysis of 3-nitrotrosine (3-NT) content of muscle

Changes in 3-nitrotyrosine (3-NT) content were assessed in muscle biopsies as described in Section 2.6.1. Previous studies have shown that 3-NT levels are a sensitive marker of muscle oxidative stress (Vasilaki et al., 2007, Sakellariou et al., 2011). In brief, 20µg of total protein was separated by a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. The content of 3-NT was analysed by using a rabbit monoclonal antibody according to manufacturer's instructions (Cayman Chemical Co., Michigan, USA) and the bands were visualized using a Bio-Rad Chemi-Doc System (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Densitometric quantification of bands was undertaken and the protein content was normalised to ponceau stains. Comparisons were made between samples on the same gel/western blot.

6.2.12 Determination of protein oxidation and lipid peroxidation

Changes in muscle protein oxidation and lipid peroxidation were assessed in muscle samples as previously described (Section 2.6). Protein oxidation was assessed via changes in protein carbonyl content of the muscle. Proteins (20µg) were separated by 12% polyacrylamide gel and transferred onto a PVDF membrane. Proteins were derivatised and membranes were blocked and incubated for 2 hours using an anti-DNP antibody (Cell Biolabs, San Diego, CA, USA). Membranes were incubated with a secondary HRP conjugated antibody for 1 hr before visualisation with ECL. Lipid peroxidation was assessed via changes in 4-hydroxynonenal (4-HNE) protein conjugates. Proteins were separated by 12% polyacrylamide gel and transferred to a

nitrocellulose membrane as detailed in Section 2.8. Membranes were probed with anti 4-HNE primary antibody (Abcam, Cambridge, UK). Membranes were then washed (3 x 5 minutes) and incubated for 1 hour incubation with a secondary HRP conjugated antibody. Bands were visualised using a Bio-Rad Chemi-Doc System (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

6.3 Results

6.3.1 Mitochondrial content of skeletal muscle

Mitochondrial content of muscle homogenates from patients with CFS and HCs was assessed using a citrate synthase activity kit. A reduction in citrate synthase activity may be associated with lower mitochondrial content. Analyses revealed no difference in citrate synthase activity between patients with CFS and HCs indicating that CFS was not associated with altered mitochondrial content (Figure 6.2).

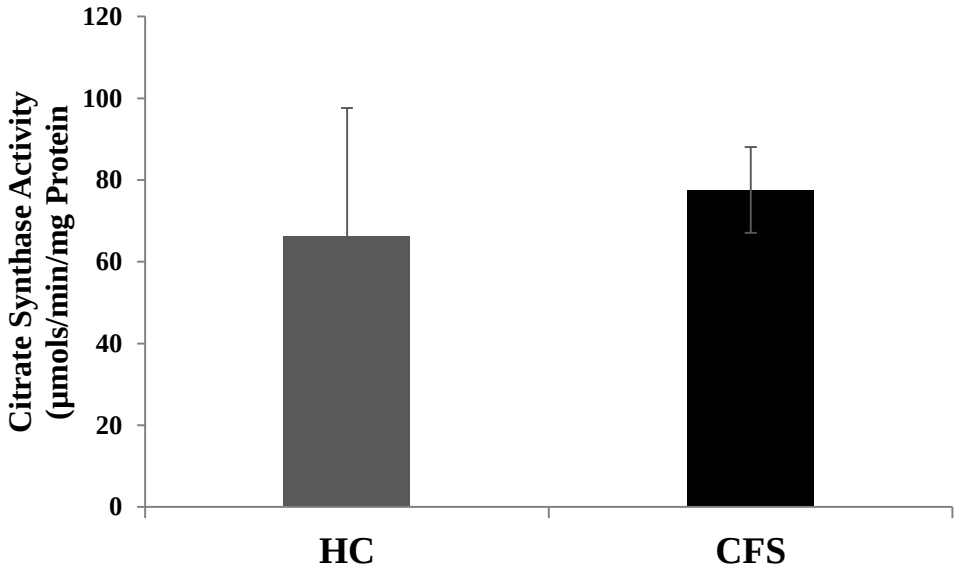


Figure 6.2 Citrate synthase activity of muscle homogenates expressed as µmols/min/mg protein in patients with CFS and HCs (n=11). Data presented as mean ± SEM.

6.3.2 Mitochondrial ROS generation

6.3.2.1 Mitochondrial H_2O_2 generation under state I respiration

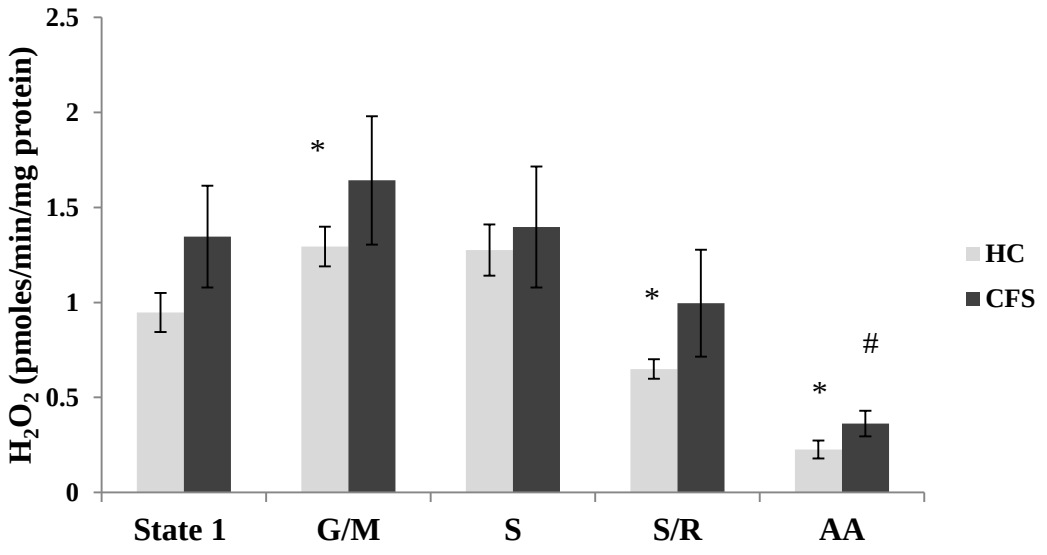
To assess whether mitochondria in muscle of patients with CFS produce elevated levels of mitochondrial ROS under state I respiration, H_2O_2 efflux was measured in permeabilised myofibres from *vastus lateralis* biopsy samples. There was no significant difference between average mitochondrial H_2O_2 emissions from permeabilised fibre bundles during state 1 respiration in data normalised to protein content of the muscle samples or citrate synthase activity (Figure 6.3 a and b).

6.3.2.2 Mitochondrial H_2O_2 generation by skeletal muscle in response to ETC substrates/inhibitors

Figure 6.4. shows the changes in H_2O_2 generated by skeletal muscle in response to ETC complex substrates, and/or inhibitors. Following the addition of complex I substrates, Glutamate/Malate (G/M), intact mitochondria in muscle bundles of HCs showed a significant increase in H_2O_2 emission (P=0.03) in comparison with H_2O_2 emission during state I respiration, whereas mitochondria from CFS bundles showed no significant increase. Following the addition of succinate, a complex II substrate, mitochondrial H_2O_2 emission from HCs was higher in comparison to state I respiration, however, this was not significant (P=0.07). Mitochondria from CFS bundles showed no detectable changes in response to succinate. Succinate induced increments in H_2O_2 emission from HC bundles was abolished by complex I inhibitor rotenone indicating complex-I dependent superoxide production in response to succinate. Moreover, there was no effect of S/R on H_2O_2 emission from bundles within patients with CFS. Thus, patients with CFS may have altered sensitivity in response to complex substrates/inhibitors, particularly G/M.

Both patients with CFS and HCs revealed a significant reduction in H_2O_2 emission from bundles exposed to antimycin alpha (AA), a complex III inhibitor compared to state I respiration. Overall these data suggest that there are no significant gross changes in mitochondrial H_2O_2 production between the CFS and HCs.

a)



b)

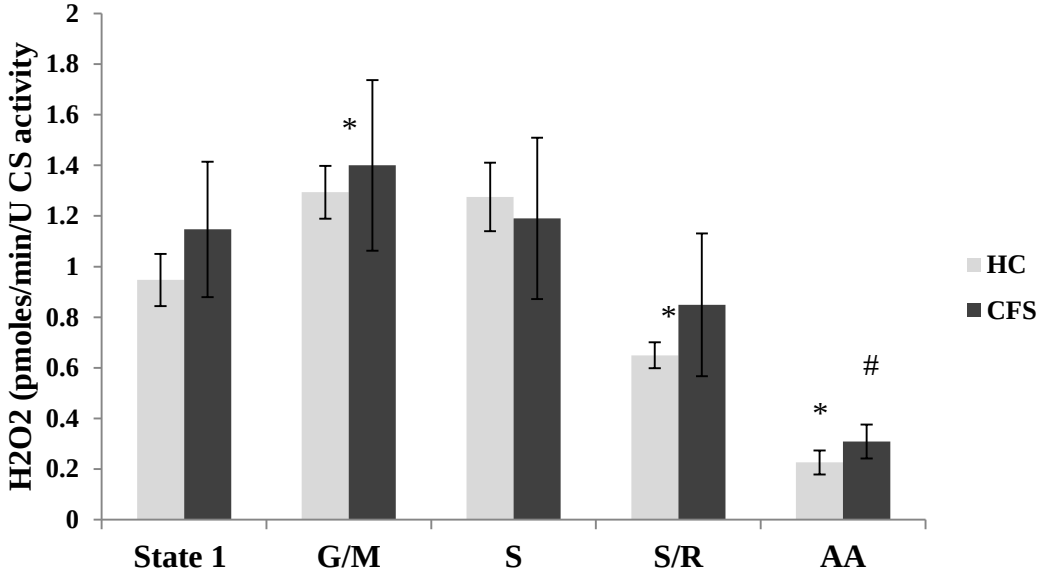


Figure 6.3 Generation of mitochondrial hydrogen peroxide (H_2O_2) assessed in permeabilised fibre bundles prepared from *vastus lateralis* muscle of patients with CFS and HCs a) Normalised to protein content of the muscle. b) Normalised to citrate synthase activity in musce. Mitochondrial substrates and inhibitors; Glutamate/Malate (G/M, 10mM + 5mM), Succinate (S, 10mM), Rotenone (R, 1μM) and Antimycin A (AA, 10 μM). * $p<0.05$ Refers to comparisons made between H_2O_2 emission for HCs during state I respiration and following the addition of the respective substrate/inhibitors. # $P<0.05$ Comparisons made between H_2O_2 emission from CFS during state I respiration and following the addition of the respective substrate/inhibitors.

6.3.2.3 Superoxide Production

Representative images of single isolated fibres under bright field, following loading with MitoSOX Red, and a merged image is shown in Figure 6.4. Levels of superoxide did not differ between patients with CFS and HCs over a period of 1 hour as indicated by changes in MitoSOX Red (Figure 6.5). These data support the previous findings regarding no differences in H_2O_2 production between patients with CFS and HCs (Figure 6.3) suggesting no differences in ROS generation in skeletal muscle mitochondria between patients with CFS and HCs.

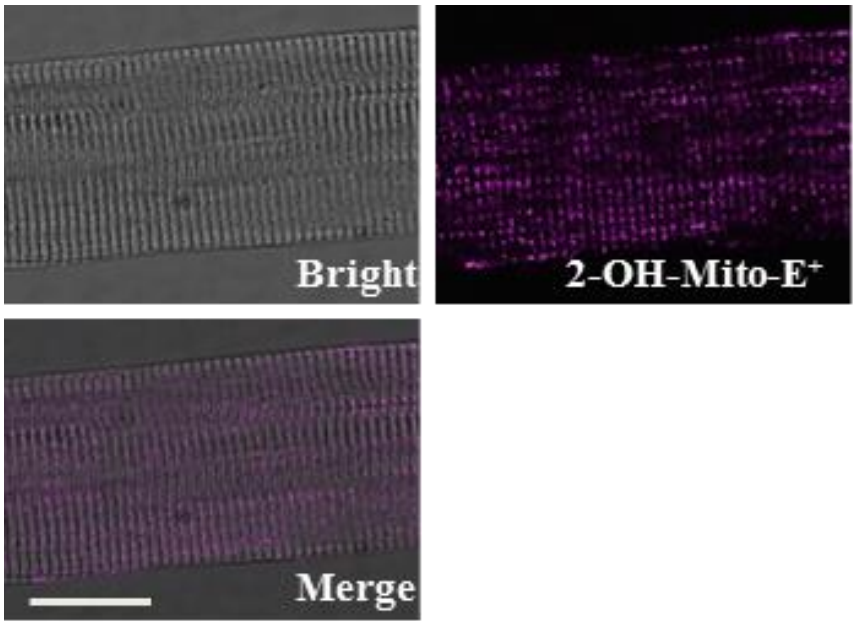


Figure 6.4 Representative images of a single fibre isolated from the VL muscle under bright field, fluorescent image following loading with MitoSOX Red (20nM, Purple), and a merged image as indicated and analysed by confocal microscopy. 60x original magnification. Scale bar, 25μm.

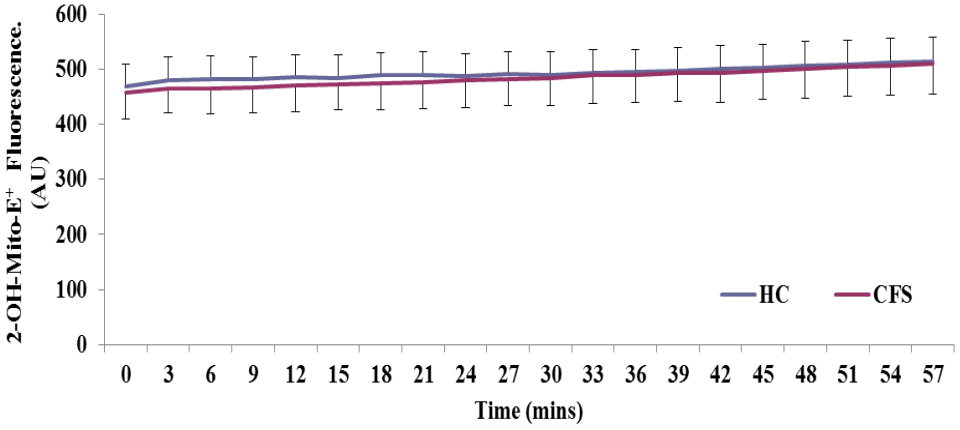


Figure 6.5 MitoSOX Red fluorescence from permeabilised fibres from the *VL* muscle in patients with CFS and HCs. Indicator of superoxide production from permeabilised fibres. Data presented as mean ± SEM. HCs error bars presented in plus direction. CFS error bars presented in minus direction (n=11).

6.3.3 Mitochondrial membrane potential

Mitochondrial membrane potential was assessed in isolated muscle fibres from the *vastus lateralis* using a TMRM cationic fluorophore probe as described in Section 2.13.2. A representative image of an isolated fibre loaded with TMRM is shown in Figure 6.6. Data revealed no differences in TMRM fluorescence with or without the addition of the oxidative phosphorylation inhibitors (oligomycin, Olm) and carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) between patients with CFS and HCs, suggesting that skeletal muscle mitochondrial of patients with CFS do not show altered mitochondrial membrane potential (Figure 6.7).

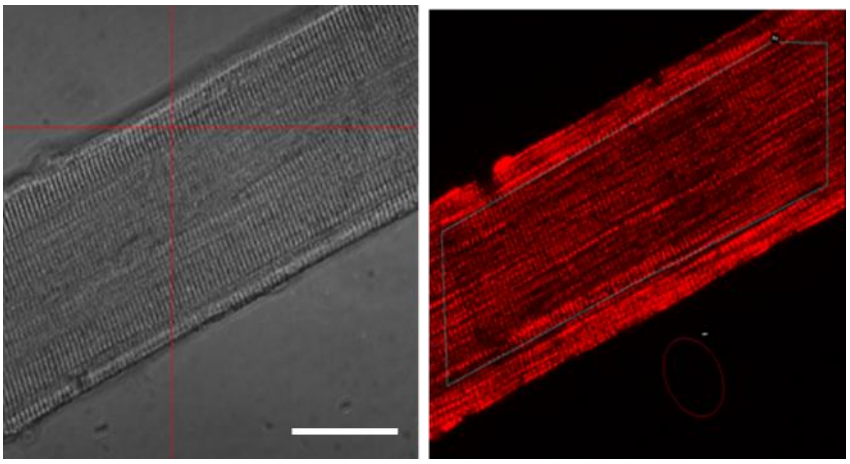


Figure 6.6 Representative confocal images of an isolated fibre showing TMRM fluorescence. 60x original magnification. Scale bar, 25μm.

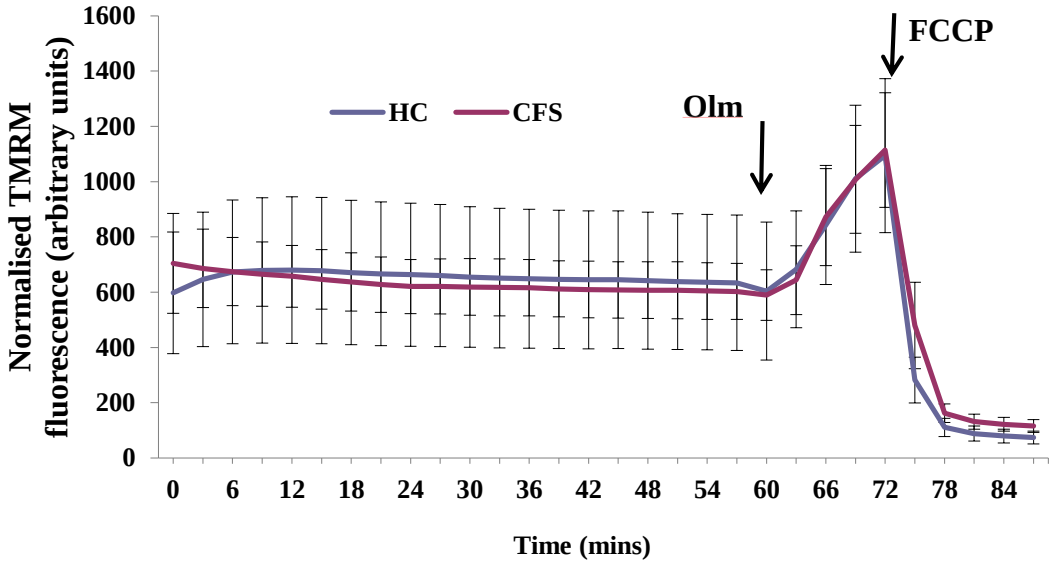


Figure 6.7 Measurement of mitochondrial membrane potential (ΔΨm) in intact mitochondria of isolated VL fibres from patients with CFS and HCs, assessed by

changes in TMRM (30nM) fluorescence in response to oligomycin (Olm, 2.5μM) and the protonophore carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP, 4μM), added at the indicated time points. n=12 fibres, 11 subjects/group.

6.3.4 Mitochondrial respiration in permeabilised fibre bundles

Respiratory function of muscle mitochondria was assessed in HCs and patients with CFS by assessing the respiratory control index (RCI, marker of O_2 consumption) and the ratio between state III and state IV respiration. The RCI was not different between patients with CFS and HCs (See Figure 6.8). The P:O ratio is another marker of respiratory function, which represents the amount of ATP produced by the reduction of one oxygen atom through the electron transport chain (Nicholls and Ferguson, 2002). The P:O ratio was also not significantly altered in patients with CFS compared with HCs however data does suggest ~38% increase in the mean ratio of patients with CFS (Figure 6.9). These data indicate no differences in respiratory function between patients with CFS and HCs.

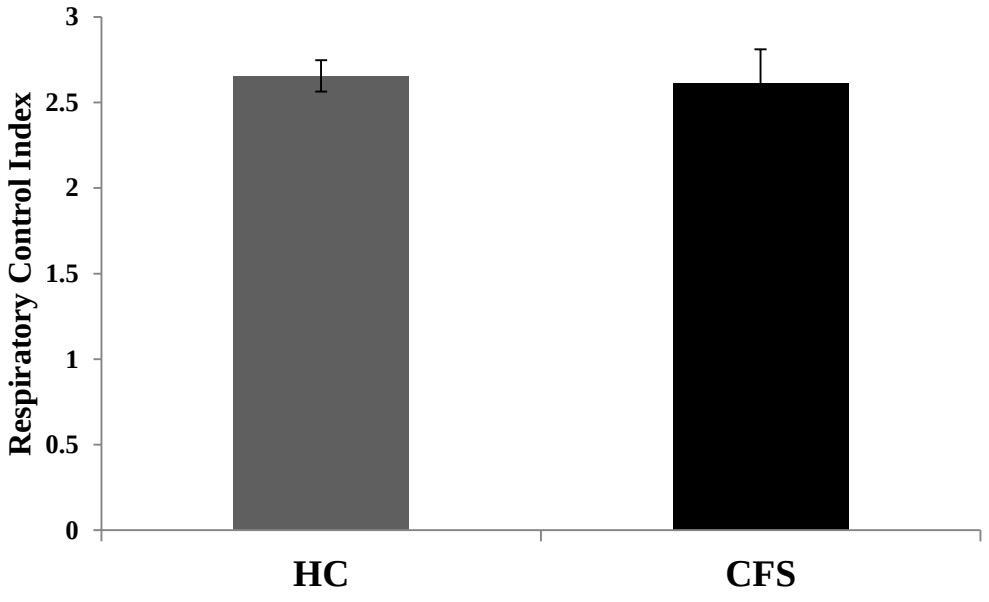


Figure 6.8 Respiratory control index (RCI) of intact mitochondria in permeabilised myofibres prepared from *vastus lateralis* (*VL*) from patients with CFS and HCs. (n=11). Data presented as mean ± SEM.

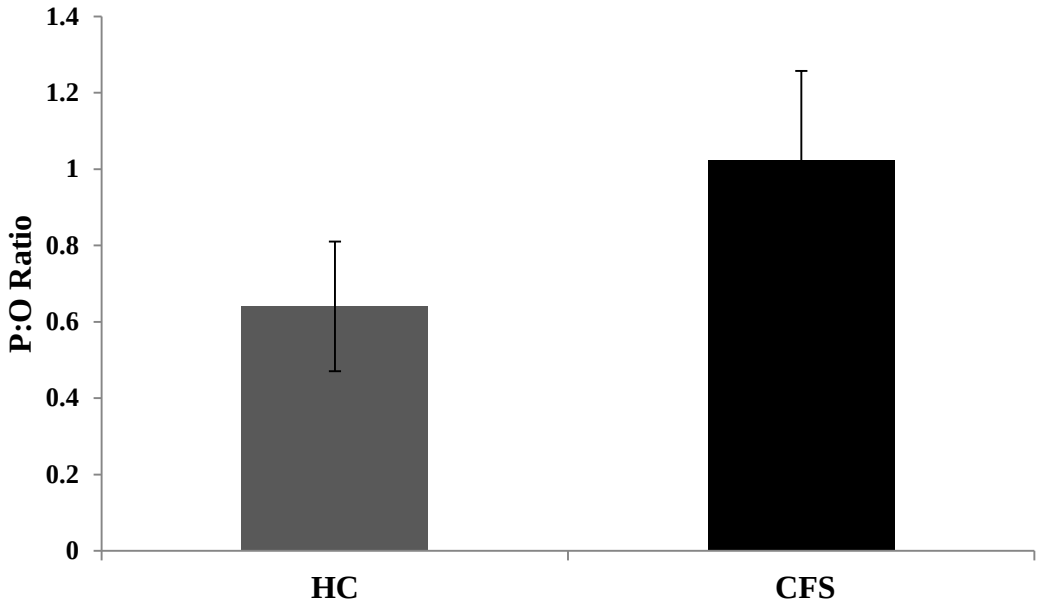


Figure 6.9 The P:O Ratio of ATP formed against oxygen utilised of intact mitochondria in permeabilised myofibres prepared from *VL* from patients with CFS and HCs (n=11). Data presented as mean ± SEM.

6.3.4 Oxidative damage and expression of redox-regulatory proteins in skeletal muscle of patients with CFS

To determine the overall changes in redox homeostasis in skeletal muscle of patients with CFS, indicators of redox status including markers of protein oxidation, lipid peroxidation and protein nitration and the expression of RONS regulatory proteins were examined.

6.3.4.1 Markers of oxidative damage in skeletal muscle

Protein carbonyl content is a marker of protein oxidation and data revealed no significant differences in protein carbonyls between patients with CFS and HCs (Figure 6.10). A representative protein carbonyl blot is shown in Figure 6.11. To determine the extent of lipid peroxidation, 4-HNE (4-hydroxynonenal) protein conjugates were assessed in skeletal muscle lysates. Similarly, to protein oxidation, lipid oxidation was not altered between HCs and patients with CFS (Figure 6.11). An additional marker of oxidative stress is the levels of protein nitration. This was determined through the assessment of 3-nitrotyrosine (3-NT) muscle content. The study found no difference between muscle 3-NT content between HCs and patients with CFS as shown in Figure 6.12. A representative western blot of 3-NT content is shown in Figure 6.12.

Overall, these data demonstrate a lack of any gross changes in oxidative status and demonstrate that skeletal muscle of patients with CFS does not exhibit grossly altered redox homeostasis compared with HCs.

6.3.4.1.1 Protein oxidation

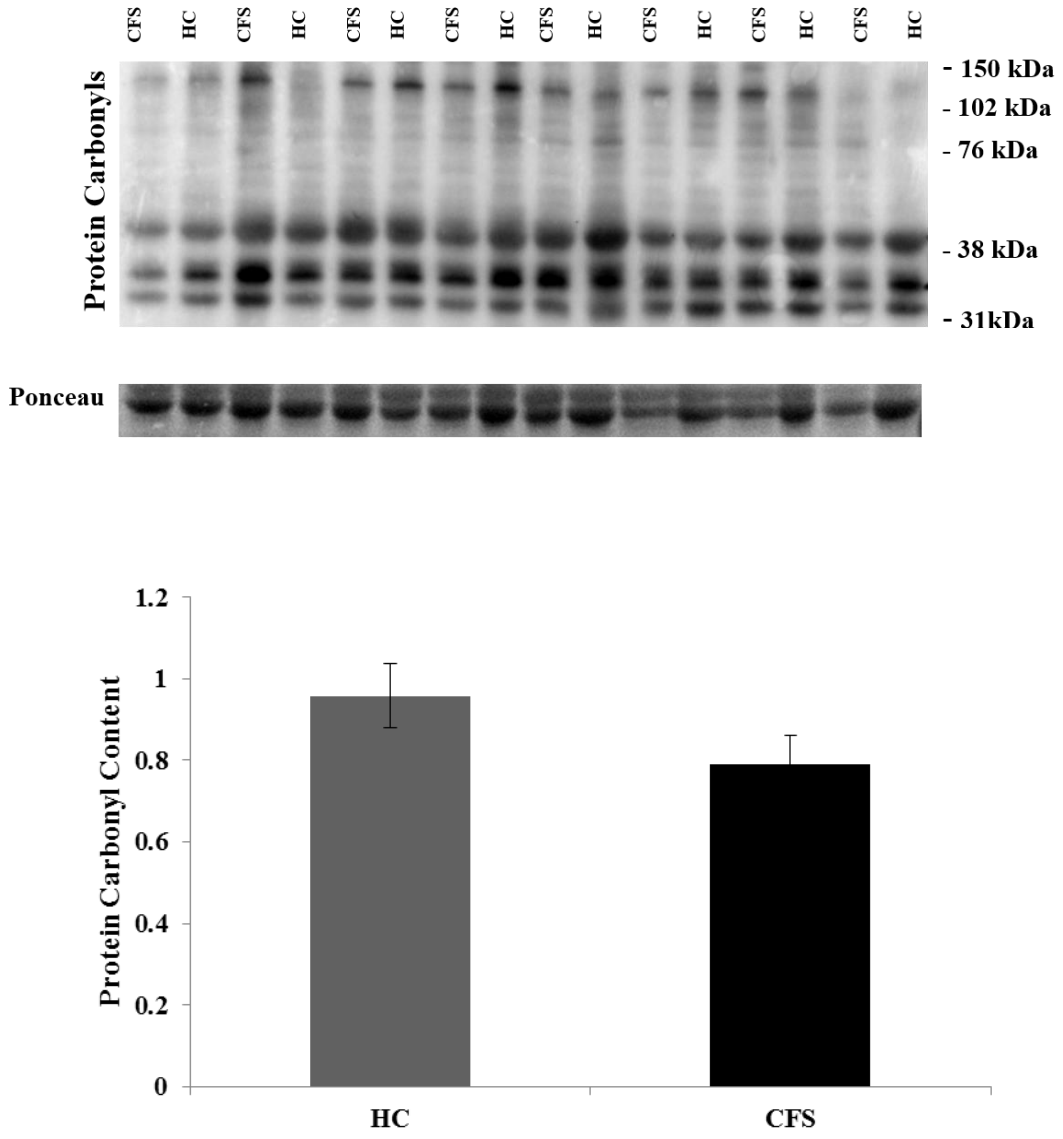
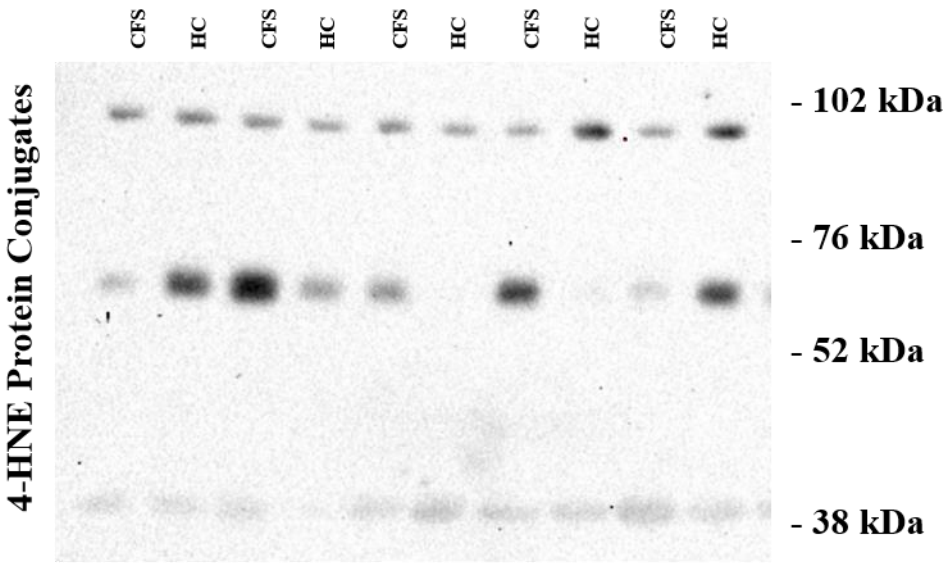
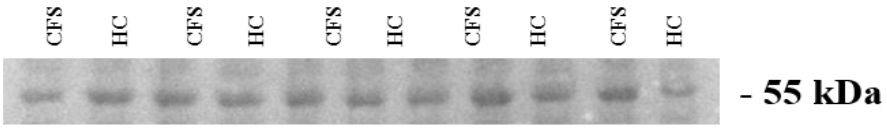


Figure 6.10 Representative western blot of protein carbonyl content in *VL* muscle of patients with CFS and HCs and densitometric quantification of the blot. n=8. Data presented as mean ± SEM. p= 0.12

6.3.4.1.2 Lipid peroxidation



Ponceau



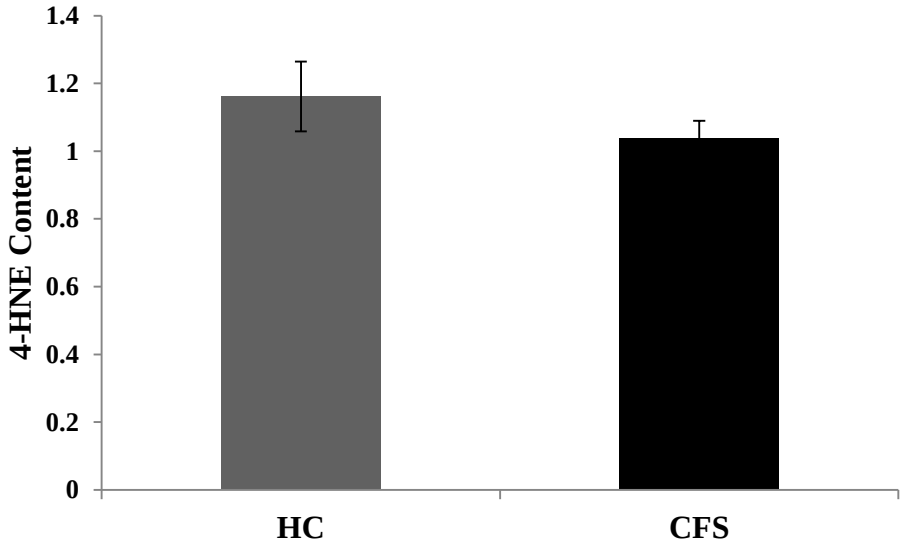


Figure 6.11 Representative western blot of 4-hydroxynonenal protein adducts (4-HNE) content in *VL* muscle of patients with CFS and HCs and densitometric quantification of the blot. n=8. Data presented as mean ± SEM. p=0.30

6.3.4.1.3 3-Nitrotyrosine

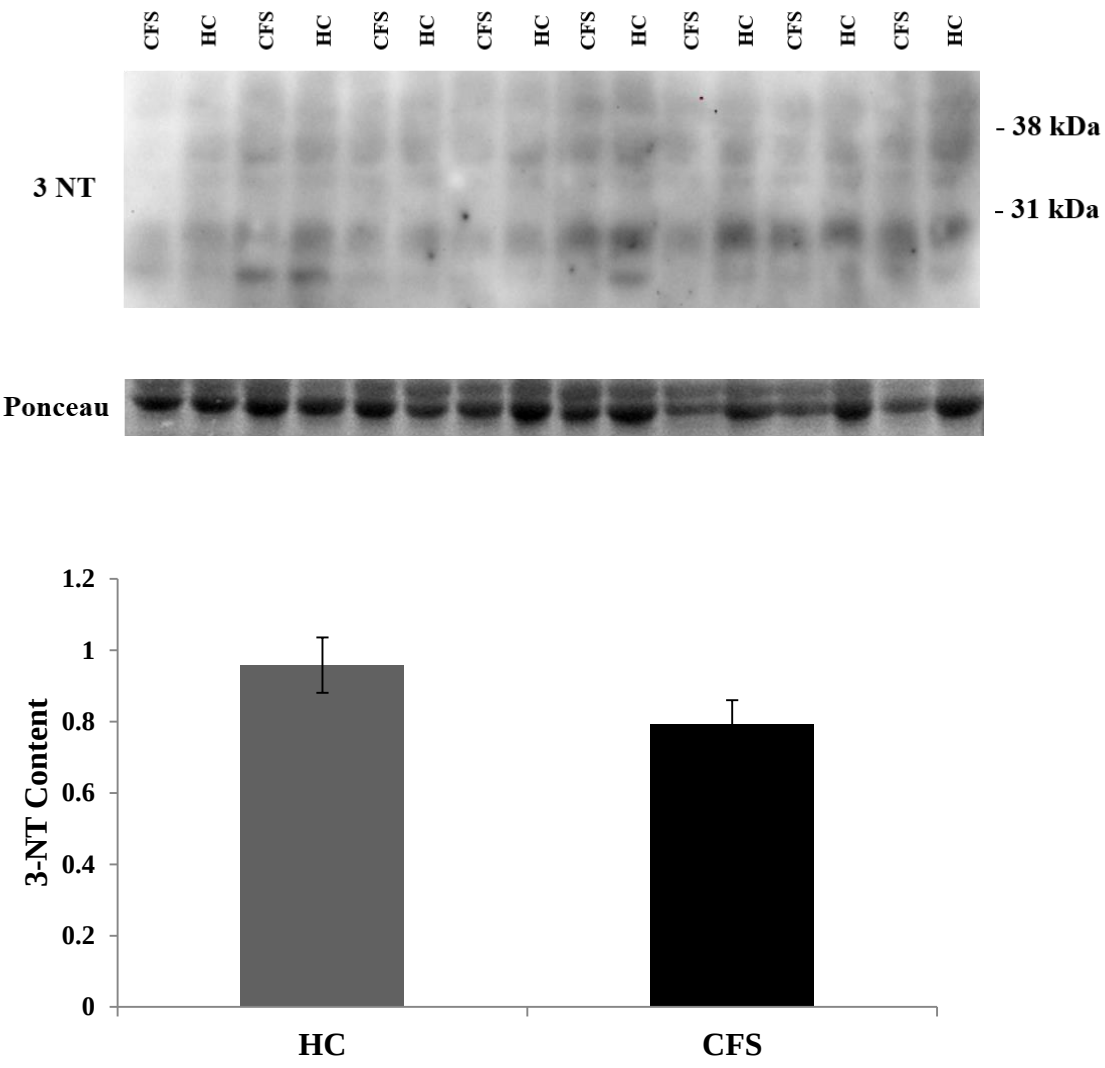


Figure 6.12 Representative western blot of 3-nitrotyrosine (3-NT) content in *VL* muscle of patients with CFS and HCs and densitometric quantification of the blot. n=8. Data presented as mean ± SEM. $p = 0.13$

6.3.5 RONS regulatory enzymes and HSPs in skeletal muscle

To further determine whether patients with CFS have altered redox homeostasis, the expression of RONS regulatory enzymes in skeletal muscle was also determined. Changes in RONS levels may lead to adaptations in the expression of proteins involved in antioxidant defences. The study measured the expression of RONS regulatory enzymes and proteins including; superoxide dismutase (SOD) isoforms (SOD I and SOD II), H_2O_2 reducing enzymes including glutathione peroxidase 1, catalase and PRXIII, nitric oxide synthase (NOS) isoenzymes (iNOS, eNOS, nNOS), H_2O_2 reducing enzymes including glutathione peroxidase 1, catalase and PRXIII, redox proteins involved in the thioredoxin-peroxiredoxin system and heat shock proteins which have been shown to provide protection against increased RONS production (Jackson, 2005) .

6.3.5.1 SOD protein content

Figure 6.13 shows a representative western blot of SOD I and SOD II from skeletal muscle of patients with CFS and HCs. Figure 6.14 and 6.15 demonstrates the quantification of these blots by densitometry. Neither SOD I or SOD II content was altered in muscles of patients with CFS in comparison with HCs.

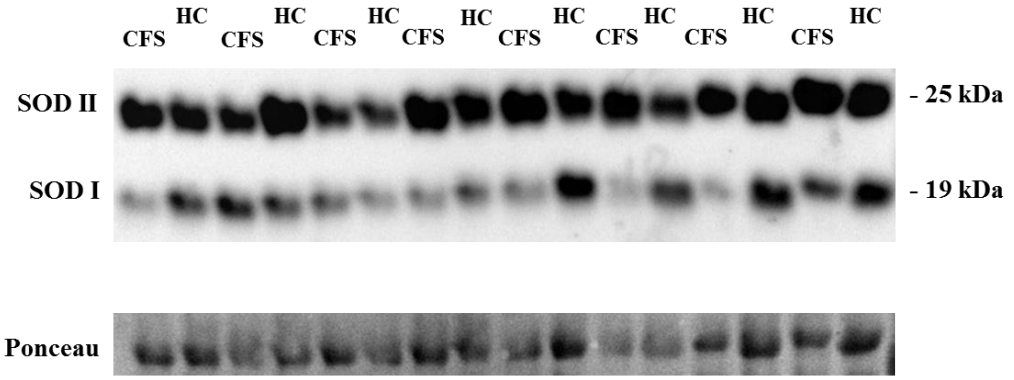


Figure 6.13 Representative western blots of proteins SOD1 and SOD2 in the *VL* muscle of patients with CFS and HCs.

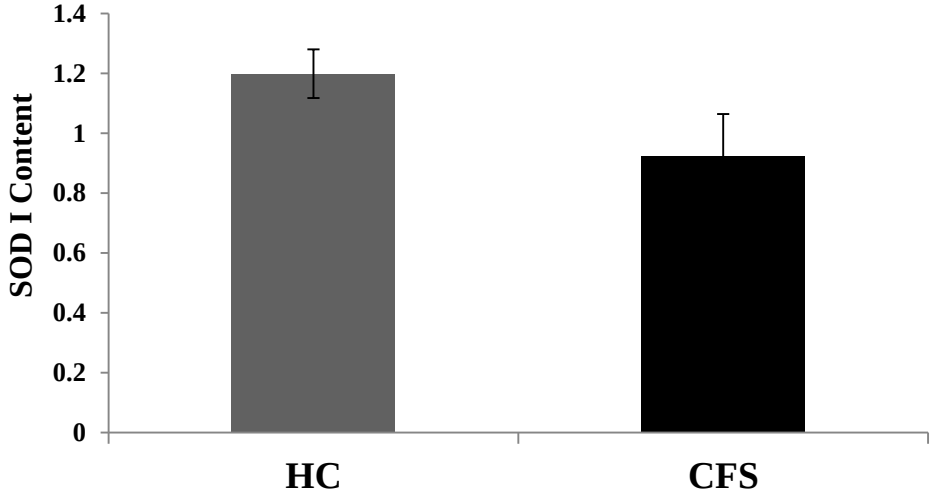


Figure 6.14 Densitometric quantification of the blot for SOD1 protein content of skeletal muscle of patients with CFS and HCs. n=8. Data presented as mean ± SEM. *p*= 0.11

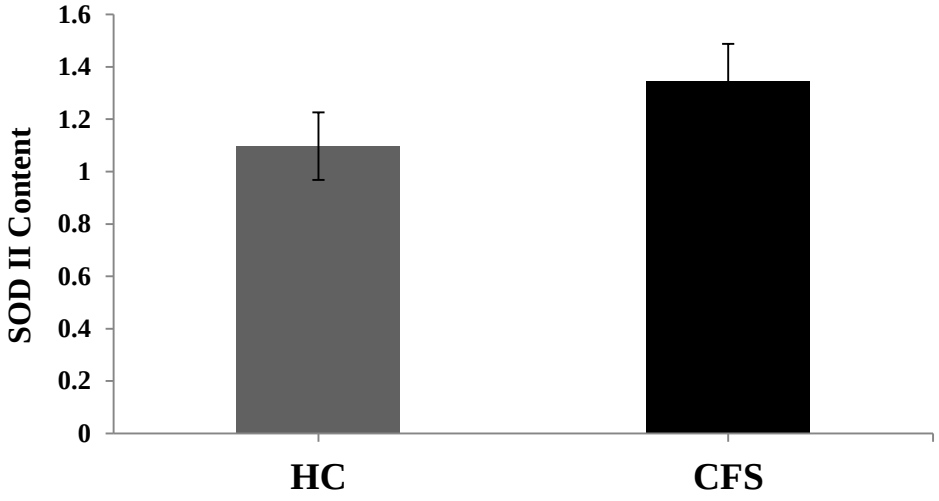


Figure 6.15 Densitometric quantification of the blot for SOD2 protein content of skeletal muscle of patients with CFS and HCs. n=8. Data presented as mean ± SEM. *p*= 0.22

6.3.5.2 Catalase protein content

Protein levels of catalase were not significantly different in muscle from patients with CFS in comparison to HCs (Figure 6.16). A representative blot is shown in Figure 6.16.

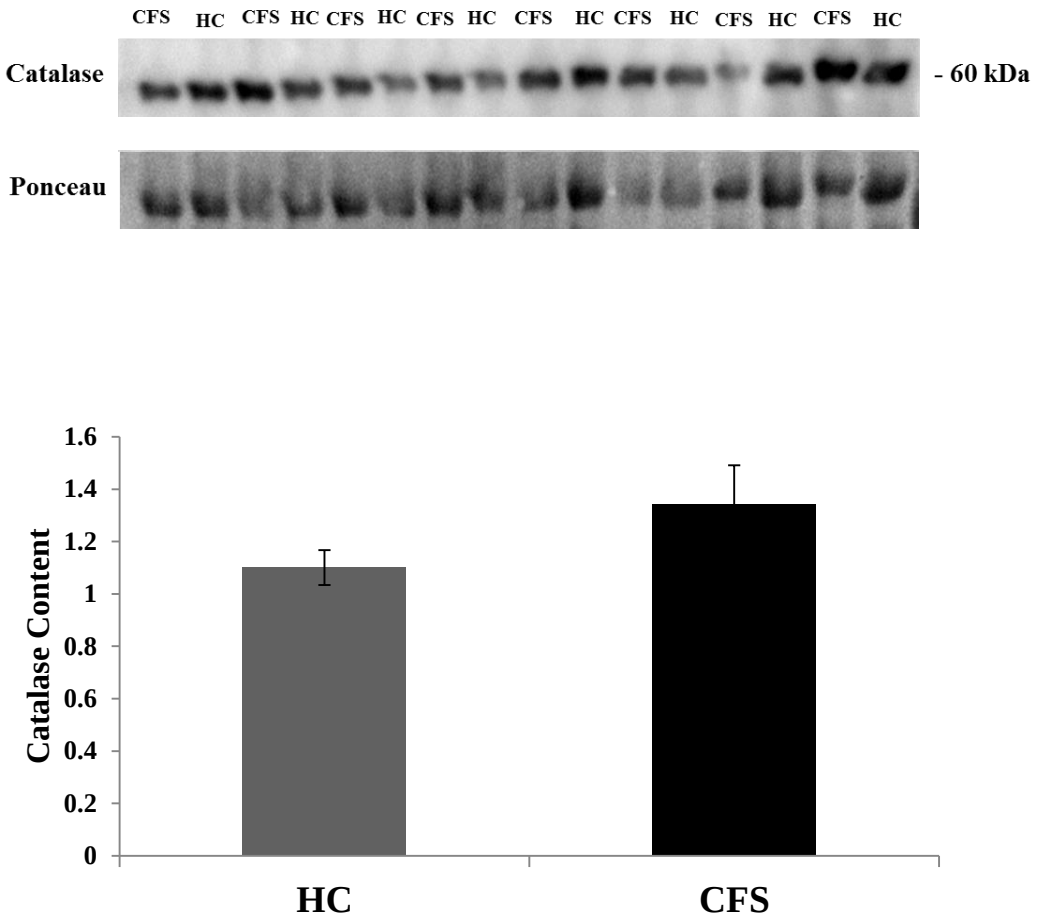


Figure 6.16 Representative western blots of catalase protein in *VL* muscles of patients with CFS and HCs and densitometric quantification of the blot. n=8. Data presented as mean ± SEM. p= 0.89

6.3.5.3 NOS protein content

Changes in redox homeostasis may induce changes in the expression of redox regulatory proteins including NOS enzymes. Protein expression of all three isoforms of NOS; neuronal nitric oxide synthase (nNOS), endothelium nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS)) were examined by western blot analysis. Analysis revealed no significant differences in the content of any NOS proteins between CFS and HCs (Figures 6.17 – 6.20).

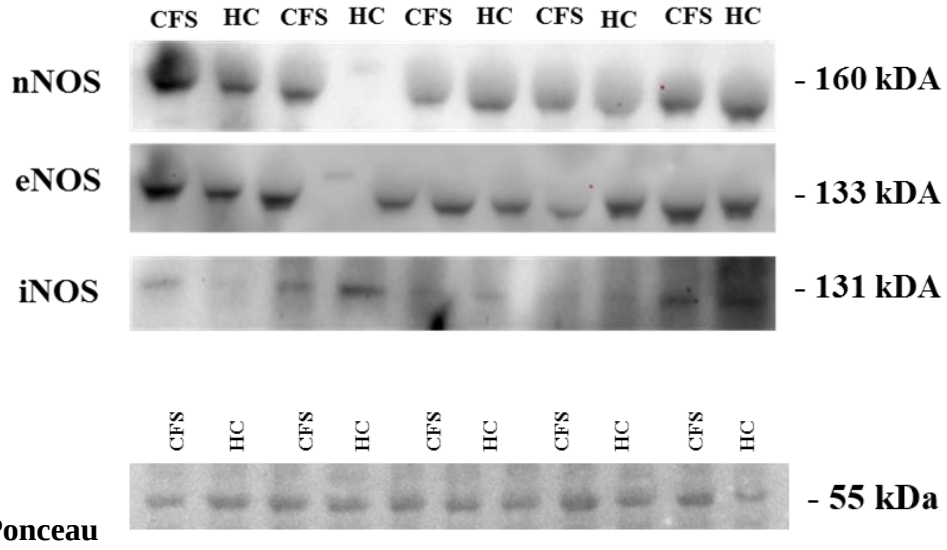


Figure 6.17 Representative western blots of neuronal nitric oxide synthase (nNOS), endothelium nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) protein in *VL* muscles of patients with CFS and HCs.

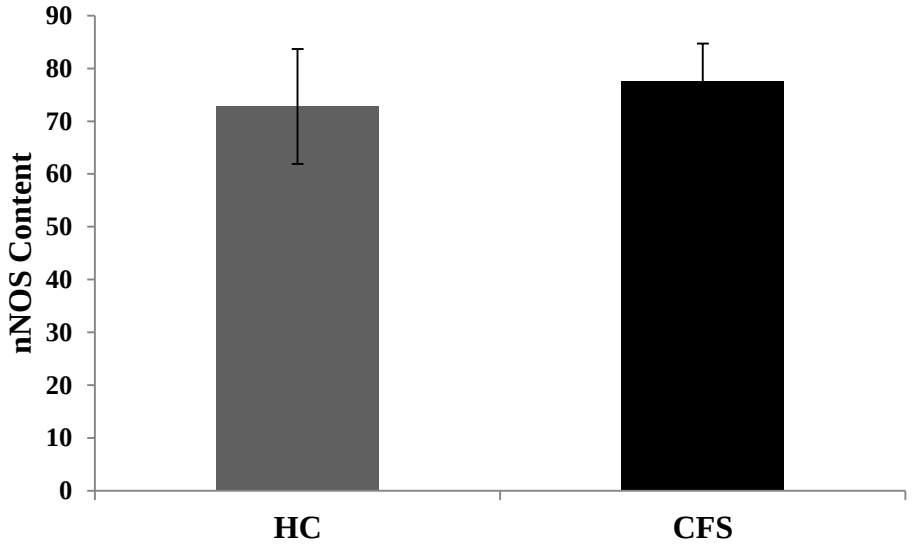


Figure 6.18 Densitometric quantification of the blot for nNOS protein. n=8. Data presented as mean ± SEM. p= 0.72

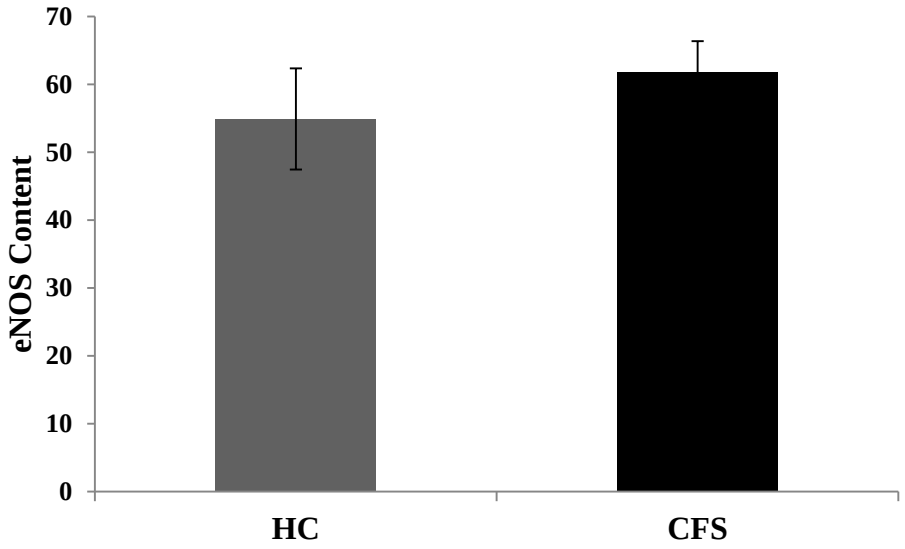


Figure 6.19 Densitometric quantification of the blot for eNOS protein. n=8. Data presented as mean ± SEM. *p*= 0.44

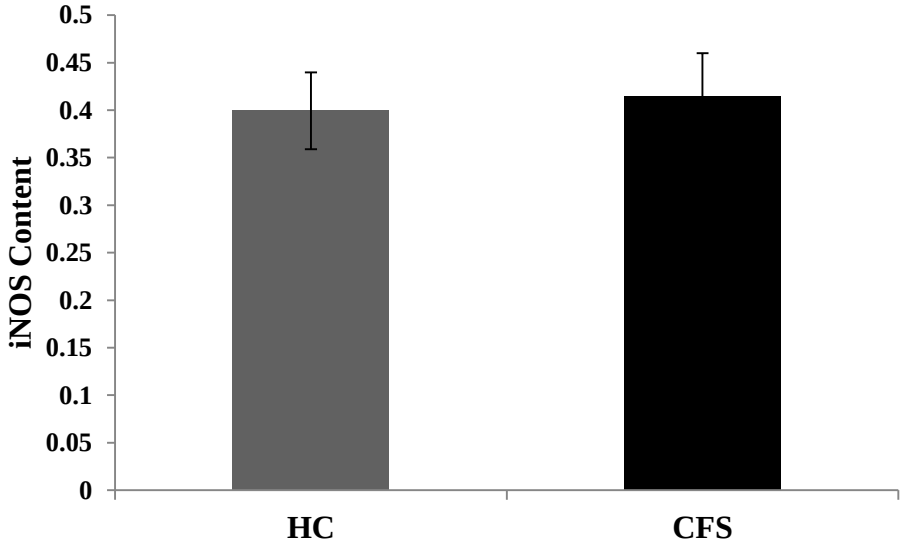


Figure 6.20 Densitometric quantification of the blot for iNOS protein. n=8. Data presented as mean ± SEM. *p*= 0.85

6.3.5.4 PRX protein content

As previously mentioned in Section 1.5.4, PRX proteins are mainly involved in the degradation of H_2O_2.with the exception of PRXV which is also implicated in the reduction of peroxynitrite. Western blot probed for PRX III (mitochondrial specific isoform) did not reveal any differences in PRXIII muscle content between patients with CFS and HCs as shown in Figure 6.21 which suggests no significant changes in H_2O_2 within the mitochondrial organelles of patients with CFS and HCs.

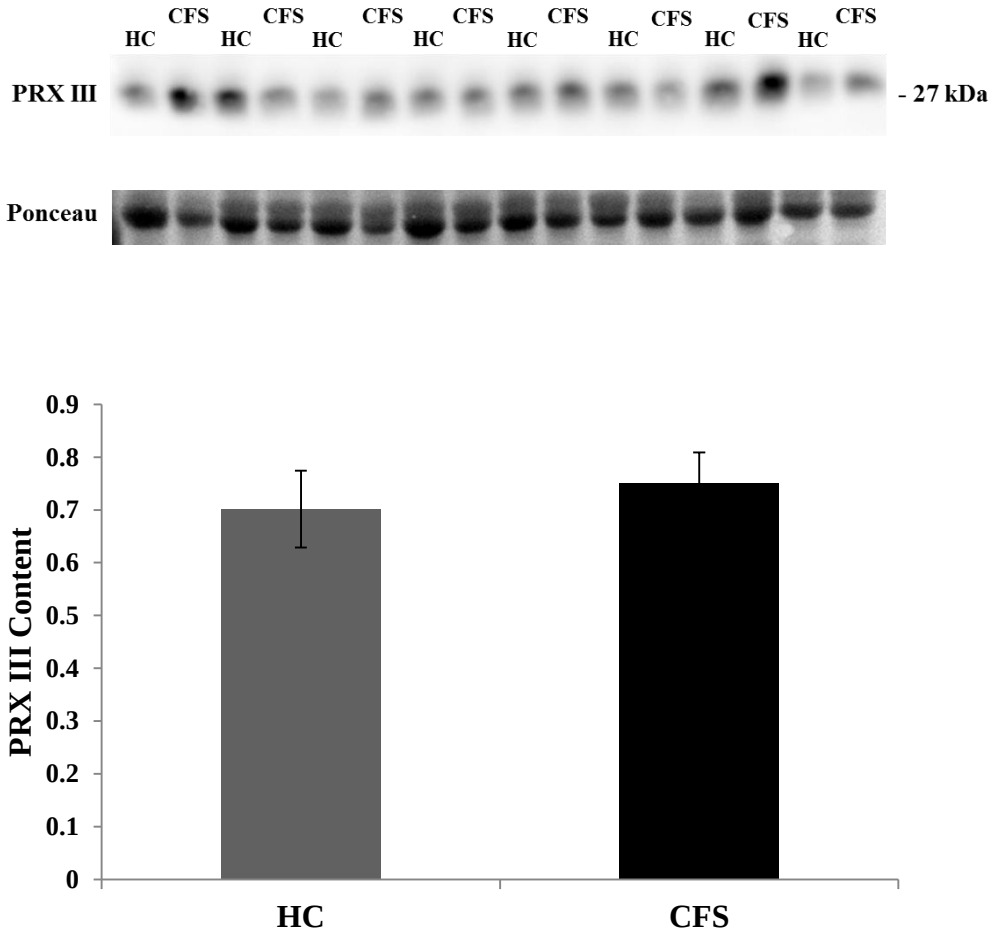


Figure 6.21 Representative western blots of PRX III protein in *VL* muscles of patients with CFS and HC recruits and densitometric quantification of the blot. n=8. Data presented as mean ± SEM. p= 0.73

6.3.5.5 Changes in HSP content in skeletal muscle

Skeletal muscle from patients with CFS showed no changes in either HSP 10 or HSP 25 content in comparison to muscle from HCs as shown in Figures 6.22, 6.23 and 6.24.

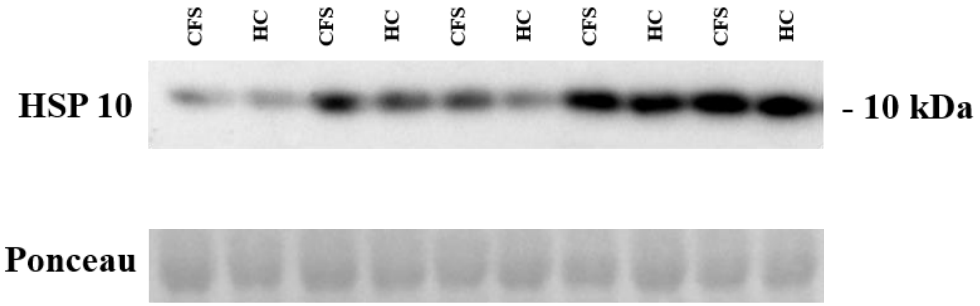


Figure 6.22 Representative western blots of HSP 10 and HSP 25 proteins in *VL* muscles of CFS and HC recruits and densitometric quantification of the blots.

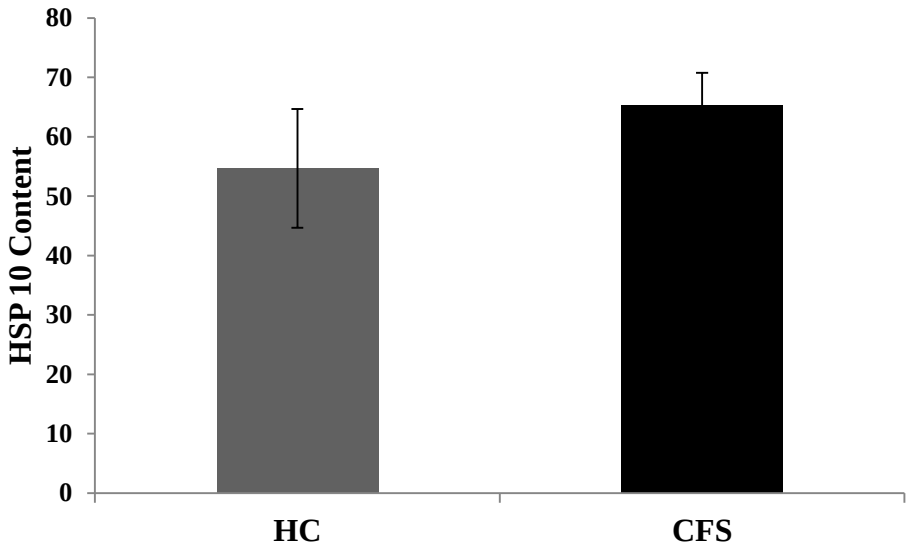


Figure 6.23 Densitometric quantification of the blot for HSP 10 protein. n=8. Data presented as mean ± SEM. *p*= 0.37

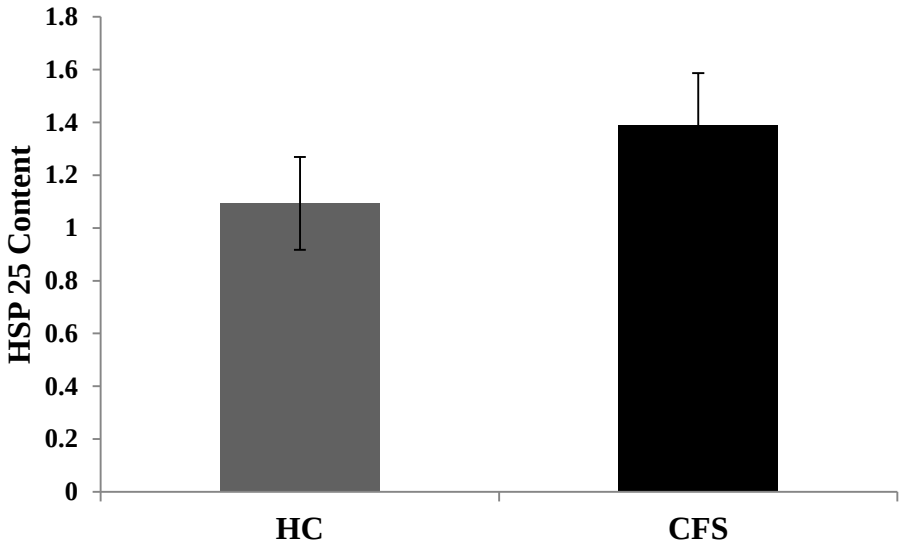


Figure 6.24 Densitometric quantification of the blot for HSP 25 protein. n=8. Data presented as mean ± SEM. *p*= 0.28

6.3.5.6 Changes in IκBα content of skeletal muscle

Analysis of IκBα content of skeletal muscle did not reveal any differences in muscle content between patients with CFS and HCs as shown in Figure 6.25 and 6.26 suggesting no gross activation of NFκB canonical pathway.

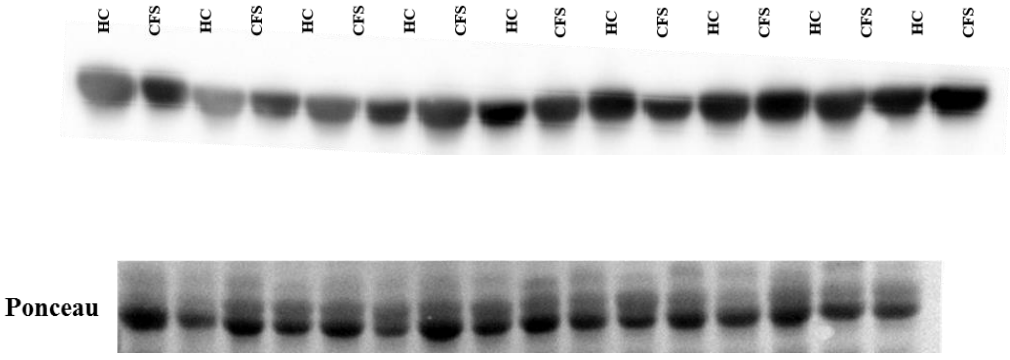


Figure 6.25 Representative western blots of IκBα protein in *VL* muscles of patients with CFS and HC recruits.

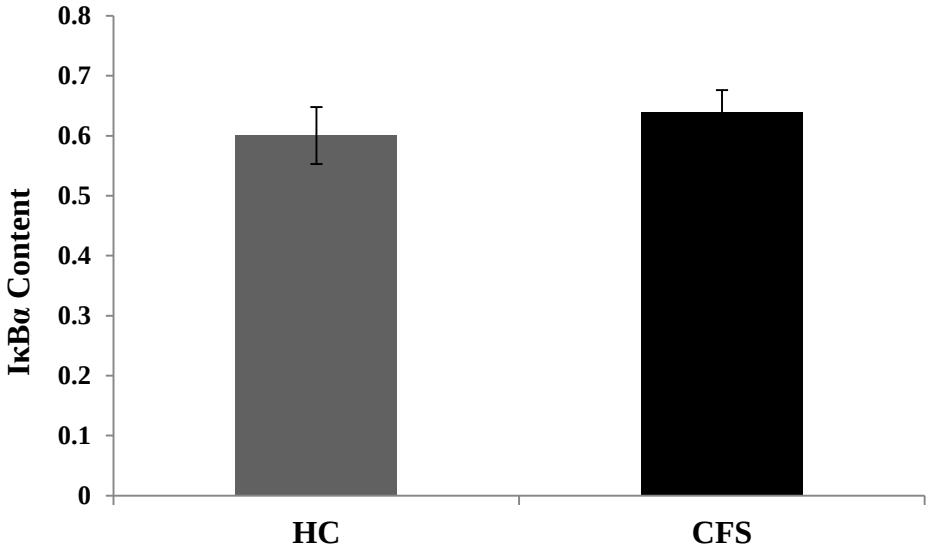


Figure 6.26 Densitometric quantification of the blot of IκBα protein in *VL* muscles of patients with CFS and HC recruits**.** n=8. Data presented as mean ± SEM. *p*= 0.66

6.4 Discussion

Patients with CFS exhibit a number wealth of symptoms of which a large proportion are also reported in individuals with mitochondrial dysfunction (Table 1.6). The rationale to support the hypothesis of mitochondrial dysfunction and modified ROS generation in patients with CFS is plausible yet limited by a lack of primary evidence. The majority of published literature on this subject includes CFS and mitochondrial dysfunction hypothesis papers, intervention studies and reviews which have very little primary evidence supporting these hypothesis e.g. (Booth et al., 2012, Castro-Marrero et al., 2013, Morris and Maes, 2012, Nicolson, 2005). Conclusions are often drawn from poorly designed studies in which the number of participants is exceptionally low (e.g. – inclusion of 2 patients with CFS) and a lack of appropriate matching to HCs e.g.(Byrne et al., 1985). There are concerns with regards to the interpretation of primary data regarding mitochondrial function in a number of reviews and the use of non-validated mitochondrial function tests ("ATP profile" tests) in assessing patients with CFS as demonstrated in studies such as (Myhill et al., 2009, Meeus et al., 2013). To overcome these limitations, this study aimed to determine whether CFS is associated with muscle mitochondrial dysfunction and redox dysregulation.

In this study, mitochondrial function and redox homeostasis were assessed in permeabilised muscle fibre bundles from patients with CFS and HCs. The use of fibre bundles and isolated skeletal muscle fibres are likely to reflect the situation in muscle *in vivo*. Many studies have utilised mechanically isolated mitochondria from skeletal muscle to study mitochondrial function and ROS production. Isolation of mitochondria can severely affect structural integrity and potentially provide inaccurate results (Picard et al., 2011). The technique of *in situ* analysis is advantageous over experiments using mechanical isolation of mitochondria, by maintaining the mitochondria in their native intracellular and systemic environment, structure is preserved and structural interactions with other sub-cellular compartments are not disrupted (Picard et al., 2010).

Perturbed mitochondrial ROS production from skeletal muscle is a complex process which contributes to oxidative damage in a wide range of muscle pathologies (Lin and Beal, 2006, Eckhardt et al., 2007, Prinsen et al., 2015). Altered ROS levels can disturb cellular redox homeostasis thus impacting on a vast number of redox sensitive signalling pathways (Powers et al., 2010). Despite previous evidence suggesting elevated ROS production through the presence of cumulative oxidative damage and a

subsequent decline in mitochondrial function in patients with CFS, this area of research remains poorly understood (Richards et al., 2000, Castro-Marrero et al., 2013). The current study did not identify any differences in mitochondrial ROS generation as assessed though H_2O_2 and superoxide production in intact mitochondria of skeletal muscle fibres from patients with CFS and HCs. This is the first study to directly measure mitochondrial ROS production in skeletal muscle *in situ,* utilising permeabilised muscle fibres in patients with CFS. Data did reveal mitochondrial in muscles of patients with CFS have potentially altered sensitivity to ETC substrates. Evidence from our research group has identified a reduction in substrate sensitivity in aged mice which is reversed following a treatment with a ROS scavenging peptide SS-31 (unpublished data). To date, there is no published evidence on the effect of training/exercise on mitochondrial ETC substrate sensitivity and H_2O_2 production.

To further assesses the muscle redox environment, markers of oxidative damage and the expression of RONS regulatory proteins were assessed. Oxidative damage in skeletal muscle of patients with CFS and HCs was determined through the assessment of changes in protein oxidation, lipid peroxidation and protein nitration. Skeletal muscle of patients with CFS did not show any gross changes in protein carbonyls, 4-HNE and 3-NT content compared to HCs. Previous analyses examining protein carbonyl content of skeletal muscle reported similar results (Fulle et al., 2000). These data therefore demonstrate no evidence of elevated levels of oxidative damage in patients with CFS. To further assess whether the redox sensitive protective systems were altered in skeletal muscle CFS, the current study also examined the expression of a wide range of proteins including superoxide dismutase (SOD) isoforms (SOD I and SOD II), H_2O_2 reducing enzymes catalase and PRXIII, nitric oxide synthase (NOS) isoenzymes and heat shock proteins which have been shown to provide protection against increased RONS production. Protein content of all markers did not differ between patients with CFS and HCs, again suggesting that the redox status of muscle cells in patients with CFS is relatively normal.

Studies suggest that perturbed ROS homeostasis resultant of cumulative oxidative damage can lead to a reduction in mitochondrial number and function within a wide range of pathologies. Given the lack of evidence to suggest patients with CFS have altered redox homeostasis is not surprising that the mitochondrial function analysis in skeletal muscle also showed no differences between patients with CFS and HCs.

The reliability of a large proportion of evidence surrounding elevated oxidative damage and mitochondrial dysfunction in patients with CFS is unclear. There is often a lack of primary evidence or direct markers used to support claims in a large number of reviews (Myhill et al., 2009). A large number of statements made by Maes and Myhill et al are often supported with reviews from the same author which include hypothesis papers and additional reviews rather than primary based studies (Maes et al., 2006, Maes, 2009, Maes and Twisk, 2010, Maes, 2011, Maes et al., 2012, Maes, 2013).

The majority of studies examining mitochondrial function in muscle have utilised mechanically isolated mitochondria from skeletal muscle to study mitochondrial function and ROS production. Isolation of mitohondria can severely affect structural integrity and provide inaccurate results. Evidence has shown that the use of isolated mitochondria to study mitochondrial ROS and function can provide artificially high levels of ROS production (exacerbated response) as the isolation process can alter ROS (Picard et al., 2010). Additional errors have occurred in the normalisation of data assessing mitochondrial function. Previous data which has reported mitochondrial dysfunction in patients with CFS as a result of reduced ATP production has since been questioned due to the lack of normalisation of ATP production e.g. protein content of muscle or citrate synthase activity which acts as a fundamental marker of mitochondrial content. The current study did not find a significant difference in citrate synthase activity between patients with CFS and HCs. Previous studies assessing citrate synthase in skeletal muscle of patients with CFS have reported reductions in activity (McArdle et al., 1996, Smits et al., 2011). Evidence suggesting reduced oxidative phosphorylation is based upon data revealing a lower Vmax (VO_2 max) in patients with CFS (McCully et al., 1996). However, Vmax is a poor secondary measure of oxidative phosphorylation which may be a result of reduced physical fitness rather than linked directly to CFS (Tabata et al., 1996). A number of parameters commonly assessed during investigations into metabolic abnormalities in CFS are drastically affected by physical activity status (Blomstrand et al., 1997). Given the increased perception of fatigue in CFS patients and evidence suggesting an increase in sedentary behaviour and muscle disuse, there is a risk of confounding factors affecting parameters and the misinterpretation of results.

Overall, the data indicate that mitochondrial ROS generation (H_2O_2 and superoxide), oxidative damage (protein oxidation, lipid peroxidation and protein nitration) and the expression of RONS regulatory proteins are not altered in patients with CFS.

Moreover, mitochondrial function assessed via changes in mitochondrial membrane potential and respiration were no altered in patients with CFS compared with HCs. Overall the data of this study suggest that changes in mitochondrial function and redox homeostasis are not major contributing factors to the pathophysiology or symptoms associated with CFS.

6.5 Summary

This study successfully identified:

- No difference in mitochondrial content or respiratory function *in situ* in permeabilised fibres isolated from skeletal muscle between patients with CFS and HCs.
- No differences in ROS production in skeletal muscle between patients with CFS and HCs.
- Potential sub-group of patients have reduced mitochondrial ETC sensitivity to complex substrates and inhibitors.
- No differences in markers of oxidative damage or the expression of redox proteins involved in RONS regulation in skeletal muscle from patients with CFS and HCs,

Chapter Seven

General discussion and future directions

7.1 Summary of Findings

The current study identified a number of major findings:

1. Skeletal muscle releases cytokines including IL-6, CXCL1, CCL2 and CCL5 in response to TNF-α treatment.
2. Resveratrol modulated TNF-α induced cytokine release from C2C12 myotubes, providing evidence of a potential nutritional therapy.
3. Patients with chronic fatigue demonstrated reduced maximal voluntary but a normal stimulated involuntary force production suggesting centrally driven mechanisms of fatigue in this cohort.
4. Force loss of the quadriceps following repeated percutaneous stimulated isometric contractions was similar between patients with CFS and age and sex matched HCs.
5. Plasma cytokine levels were not altered in patients with CFS compared with HCs and expression of cytokines in the *vastus lateralis* muscle, revealed no significant difference between CFS patients and HCs. Expression of IP-10 and CCL2 appear to be marginally raised in patients with CFS.
6. Muscle mitochondrial content and ROS production (Hydrogen peroxide and superoxide production) were not altered between patients with CFS and HCs. Thus, there was no major evidence of gross differences in oxidative damage and/or RONS regulatory proteins in patients with CFS.
7. ETC substrate sensitivity to Glutamate/Malate and Succinate appears to be slightly reduced in patients with CFS compared with HCs.
8. Mitochondrial respiration is not altered in patients with CFS compared with HCs.

7.2 General Discussion

Raised systemic levels of pro-inflammatory cytokines including TNF-α have been reported in a wide range of conditions including COPD, cachexia, chronic heart failure, and sepsis (Tisdale, 1999, Bolton, 2000, Gan et al., 2004). Patients suffering from such conditions report a wide range of co-morbidities including chronic fatigue. Increased systemic levels of TNF-α have also been associated with muscle dysfunction and fatigue *in vivo* mouse animal studies (Reid and Moylan, 2011).

The initial findings of the current study included the validation of a comprehensive, non-damaging model of cytokine treatment on muscle *in vitro* with the aim of mimicking increased exposure of muscle cells to circulating TNF-α. TNF-α was successfully validated as a modulator of cytokine release from muscle cells and confirms the role of muscle as an endocrine organ and its ability to produce and secrete cytokines, particularly IL-6, CXCL-1 (KC), CCL2 (MCP-1) and CCL5 (RANTES) confirming previous findings from (Lightfoot, 2011a, Lightfoot et al., 2015). TNF-α induced cytokine release from muscle may in turn add to the overall systemic levels inflammatory cytokines in a number of conditions. Thus, cytokine release from muscle may potentially be a significant contributor to the overall systemic cytokine profile in patients with elevated levels of circulating pro-inflammatory such as TNF-α creating a viscous cycle. The study investigated the role of polyphenols such as resveratrol, curcumin and EGCG on modulating the release of cytokines by muscle cells (Rahman et al., 2006). This preliminary cell work aimed to elucidate the potential therapeutic effects of polyphenols on cytokine release from skeletal muscle with the theory that it may be a potential therapeutic intervention in patients suffering from perturbed cytokine induced fatigue and muscle dysfunction. Previous literature has an association between perturbed cytokine levels in patients with CFS and the commonly reported impaired muscle dysfunction, weakness and fatigue, however the current study successfully dispels these theories. The study identified resveratrol as a modulator of TNF-α induced cytokine release, suggesting that resveratrol may be a potentially therapeutic compound in diseases characterised by raised pro-inflammatory cytokines such as TNF-α. Despite not being applicable to the results from the current CFS study, these data are useful in the approach to alternative diseases characterised by increased systemic levels of TNF-α and may potentially alleviate cytokine induced muscle dysfunction and fatigue.

Previous evidence has suggested that the condition CFS may be linked to altered circulating cytokine levels. The primary complaint/characteristic of CFS is an

overwhelming and debilitating sensation of fatigue (physical and mental exhaustion) which persists regardless of rest but often worsens following physical/mental exertion (Christley et al., 2012). Patients with CFS are a viable model of fatigue and potentially muscle dysfunction given the elevated scores of fatigue and reduced MVC force reported in this study. The reduction in MVC force and lack of changes in involuntary force, fatigue and single fibre force generation suggests impaired force generation is likely due impaired motivation and/or possibly impairments in the nervous system (potentially at the motor neurone). Among the findings observed in this study, expression of IP-10 within skeletal muscle of patients with CFS appears to be marginally raised compared with that of HCs. This is the first study to directly assess expression of cytokines in skeletal muscle in patients with CFS. The majority of cytokine targets are known to be directly produced from muscle (TNF-α, IL-6, CCL5, CCL2, CXCL1, IL-8). The detection of low relative expression levels from all targets may be due to low expression within muscle and/or the rapid export of cytokines from cells. A number of targets are not confirmed as muscle producing cytokines (TNF-α, Eotaxin 1, MIP-1 α), mRNA expression detected may be from connective, adipose or white blood cells rather than muscle (Wong et al., 2012).

The study did not find any differences between plasma of patients with CFS and HCs for any of the 27 cytokines assessed (Chapter 4). This is supported by a recent review compiling data from a number of CFS cytokine studies which found no differences in any cytokine targets in patients with CFS compared with HCs (Blundell et al., 2015). Previous differences between data sets has been attributed to differences in diagnostic criteria used, low sample size and differences in study technique used to assess cytokine levels. The current study aimed to fully elucidate the cytokine profile of patients with CFS and examine whether it was altered in comparison to HCs using a large cohort of individuals diagnosed through one central CFS services ensuring diagnostic uniformity. Additionally, the study examined sub-groups of data from female subjects only with CFS and found no significant differences compared with HCs suggesting cytokine levels in CFS are not associated with hormonal regulation. Disease duration and cytokine levels were also assessed in the CFS patient cohort. These data did not reveal any difference between more newly diagnosed patients (<12 months since the onset of fatigue) and those with long term CFS (suffering for >12 months). These data contradict previously published findings (Hornig et al., 2015). Further assessment of the distribution of cytokine levels between individuals would aid in the identification of potential sub-groups with altered cytokine profiles in

comparison to HCs. These could include disease severity as previously shown by (Hornig et al., 2015). To accurately determine the significance of a potentially altered cytokine or chemokine profile within disease states, it is important to first identify the physiological range within a healthy population. A "healthy" physiological range of these molecules has not yet been determined. Variation in cytokine detection and sample processing has resulted in large variations between data sets. A new technique of cytokine analysis using the multiplex bead analysis is a leading strength of this study given the sensitivity and accuracy of the assay (Agalliu et al., 2013). A number of studies in both adults and children with CFS have been conducted using these systems in both blood and cerebrospinal fluid strengthening comparisons between studies (Natelson et al., 2005, Stringer et al., 2013).

Further investigation into the production of ROS and markers of oxidative damage in skeletal muscle revealed no differences in mitochondrial content, respiratory function or mitochondrial redox changes as assessed though H_2O_2 and superoxide production in intact mitochondrial of skeletal muscle fibres from patients with CFS and HCs. However, data suggests patients with CFS may have decreased sensitivity to ETC substrates. The current study investigated mitochondrial ROS production and function *in situ*, utilizing permeabilised muscle fibres, isolated from skeletal muscle. This technique is advantageous over experiments using mechanical isolation of mitochondria as the mitochondrial remain intact and able to maintain their interactions with other subcellular compartments. No difference in oxidative damage in the *VL* muscle of CFS/ME subjects and HCs was confirmed through protein carbonyl, 4-HNE and 3-NT content. ROS regulatory proteins including superoxide dismutase (SOD) isoforms (SOD I and SOD II), catalase and PRXIII, nitric oxide synthase (NOS) isoenzymes and heat shock proteins were all similar between patients with CFS and HCs.

7.3 General problems facing CFS research

There has been a considerable amount of research undertaken in the field of CFS. Despite a wide coverage of investigations across various fields of science with regards to the pathology and pathophysiology of CFS, little progress has been made. The CFS population is highly heterogeneous and varied; which, combined with an absence of objective markers to formally diagnose patients and the presence of multiple diagnostic criteria (i.e. American Centre for Disease Control (CDC) 1994 definition (Fukuda et al., 1994), NICE guidelines (2007), the "Oxford Criteria" (1991) and the

"Canadian Guidelines") and names (Chronic Fatigue Syndrome, Myalgic Encephalopathy, Myalgic Encephalitis, Post viral syndrome, Post-infectious Fatigue Syndrome, Effort Syndrome, and more recently Systemic Exertion Intolerance Disease). This has created confusion when defining patient cohorts and sometimes prevented direct comparison between studies. In turn this has made identifying potential differences to HCs very difficult and hindered progress in this area.

The history of CFS has been plagued with scepticism from both health professionals and the general public, poor media coverage and inadequate quality of studies being undertaken/published across some areas of CFS research. This may potentially stem from the continual publication of negative data, low sample sizes and an often biased approach resulting in the creation of positive data based on tenuous associations. A resurgence in interest and funding in the 00's from bodies such as the MRC (Medical Research Council) lead to the creation of the CFS Research Advisory Group (2003), a new collaborative workshop between the MRC and Action for ME (The UK's leading charity for CSF/ME) and the formation of the MRC CFS Expert Group in 2008 in a bid to encourage and initiate "new high-quality research into CFS".

Recent advances in CFS research have seen the launch of the CFS Biobank which aims to encourage new collaborations between various field experts and CFS research. Studies have been recently published within the field of autonomic dysfunction, sleep, immune dysfunction (including cytokine levels) within CFS and genotyping of patients in the CFS wide genome database. A consistently low sample size across a large number of studies reduces the ability of data to be stratified and the potential identification of CFS subgroups. The need for sub-group analysis has been slowly progressed to the top of CFS priorities and a recognised entity (Jason et al., 2005, Twisk, 2014). 2016 sees the launch of the ME Association "Grand Challenge" which aims to collaboratively recruit ≥10,000 patients with CFS for detailed phenotyping and genetic analyses.

Within many human studies, a large problem remains in the recruitment of a suitable appropriately matched control group taking into consideration study outcomes and confounding factors which may impact these. In the current study, a large number of markers were investigated. Controls were comparably matched for age and gender; two of the major factors which affect the scientific data/outcome.

The stigma surrounding CFS has led to often a failing in the reporting of negative data, which is something that is endemic across all aspects of scientific research. In contrast, the findings reported in this thesis are very important, and will aid future researchers

in focussing CFS research towards identifying the exact pathophysiology behind the condition and symptoms associated with it.

7.4 Study strengths and limitations

Resveratrol attenuates TNF-α induced cytokine release from C2C12 myotubes

This study validated and expanded upon a pre-existing model of TNF-α induced cytokine release from C2C12 mytoubes (Lightfoot et al, 2015). As an *in vitro* study, there are the obvious limitations of applicability to *in vivo.* Using a muscle cell line allowed cytokine release to be assessed directly from muscle without any confounding cell types such as immune cells infiltrating. However, cells were observed outside of their normal environment and have reduced functional ability (lack of innervation and contractile function). The effects of polyphenols on TNF-α induced cytokine release from muscle cells *in vitro* has not been investigated before. The study used physiologically relevant doses of polyphenol treatments according to previous literature and demonstrated resveratrol as a potential therapy for patients with pathologically elevated cytokines levels.

Patients with CFS as a model of Fatigue

The overall design of this study is strong in comparison to a large amount of CFS research studies to date. The main body of the study included 95 patients with CFS and 95 HCs. This is one of the largest cross sectional studies to investigate cytokine levels in patients with CFS from one cohort. All subjects entered the study via the CFS service from the Royal Liverpool and Broadgreen University Hospitals NHS Trust. Accurate diagnosis/acceptance of referrals of CFS was left to the discretion of one unit of health care professions within the Royal Liverpool and Broadgreen University Hospitals NHS Trust, CFS services using the Oxford (1990) diagnostic criteria of CFS and recommended NICE guidelines (2007). Given the disparity between many health care professions, this study is strengthened by this aspect of uniformity across all patients entering the CFS services and therefore the possibility of entering the study. The study would have benefitted from a more rigorous, inclusion/exclusion criteria for CFS subjects in addition to the CFS diagnosis. No further investigation was undertaken to assess whether subjects had any co-existing morbidities or illnesses which may affect markers known to influence cytokines, ROS and oxidative stress. Logistical issues within study design meant the study team were not able to pursue these matters further during consent into the study.

The study was strengthened with the exclusion of smokers and individuals with a history of substance abuse (informed by subjects at their own discretion) as both these factors directly affect systemic cytokine levels and the production of RONS and subsequently increased oxidative stress (McCrea et al., 1994, Morrow et al., 1995). The study would have benefitted from a stronger exclusion criteria for HCs. Further control matching with regards to detailed analysis of habitual physical activity/ physical fitness would have been beneficial.

There are fundamental issues regarding the recruitment of CFS patients into research studies given the lack of diagnostic markers, multiple diagnostic criteria, stigma from health care professionals and a lack of CFS specialist centres within the NHS. Inconsistencies in CFS have been largely attributed to the case definition utilised and the varying approaches to diagnosis (Christley et al., 2012).

As previously mentioned, there are no biomarkers or tools to confirm CFS. Diagnosis is made using one of many case definitions and the exclusion of any other medical disorders associated with CFS symptoms which has subsequently created a poor, error prone, inefficient and slow diagnosis process. The current study utilised the Oxford Criteria (1990) and recommended NICE guidelines (2007) which are in accordance with the National Institute for Health and Clinical Excellence, UK (NHS) through which recruitment took place. These case definitions have both been extensively researched and tested (Sharpe et al., 1991, Baker et al., 2007). There is a lack of data regarding the validation of the NICE guidelines (2007) from external studies which may potentially be due to its relatively recent creation. The Oxford criteria/NICE guidelines are not as extensively used as other diagnostic criteria. This may limit potential comparisons between this study and those using an alternative criterion. The CDC-1994/Fukuda is reportedly the most frequently applied case definition (Brurberg et al., 2014).

All complaints and ailments associated with CFS are subjective to the individual. The use of health questionnaires is common within diagnostic clinics and research however; the power of their applications is limited. There have been attempts to create objective diagnostic markers in CFS (Fletcher et al., 2010) however the lack of consistency between data from different studies has prevented this.

Within the current study, a wide range of techniques were utilised to assess markers of i) systemic cytokine levels, ii) skeletal cytokine production iii) skeletal muscle function, iv) *in vivo* skeletal muscle mitochondrial respiration, v) skeletal muscle redox

homeostasis vi) *in vivo* skeletal muscle ROS generation. The experiments included sensitive, accurate, novel techniques.

7.5 Future directions

7.5.1 Mechanisms through which resveratrol attenuates TNF-α induced cytokine release.

The current study identified resveratrol as a modulator of TNF-α induced cytokine release from C2C12 myotubes. Further work investigating this effect in additional cell lines such as a human muscle cell line or an immune cell line would be beneficial in assessing whether the effect of resveratrol can be translated to humans and whether it is specific to certain cell types. Additionally, the study of mechanisms by which resveratrol attenuated TNF-α induced cytokine release is warranted. Current data suggested that resveratrol acts through redox sensitive pathways including the activation of transcriptional factors SIRT1 and NRF2 with increased production of glutathione and an up regulation of antioxidant systems including the SODs and catalase (Li et al., 2016).

7.5.2 Stratification of CFS patients

CFS encompasses a wide range of individuals. Each case is unique with sufferers reporting their own particular set of symptoms, potential triggers and disease patterns. Many researchers have suggested CFS is a broad spectrum disorder or syndrome which encompasses many subgroups of diseases. Within many disease areas there are overlapping triggers/symptoms. The causes and mechanisms underpinning these symptom profiles are different. By grouping all patients with CFS together, potential differences to HCs may be masked. Improved phenotyping, stratification and subtyping of patients is essential in CFS in providing valuable new insights into the pathology of the disease and associated symptoms and enable the development of more effective, better targeted treatments. Subgrouping of patients will require the recruitment of a much larger number. A new collaborative approach which spans the potential disciplines of research which CFS may cover is essential (immunology, neurology/neurobiology, psychology, sociology, physiology etc.). The phenotyping of subjects needs to occur through objective measurements including triggers and symptoms (e.g. medical history – glandular fever, measures of cognitive function, sleep, inflammation, psychological profile, physical function, pain, etc.). There is a

large overlap between CFS and fibromyalgia (chronic widespread musculoskeletal pain). Evidence suggests that 43-70% of CFS patients also suffer from fibromyalgia (Bradley et al., 2000, Ciccone and Natelson, 2003, Goldenberg et al., 1990). More objective markers of differentiation between these two disorders are also needed to help stratify patients. The similarities between these two cohorts should be utilised

Symptoms associated with CFS can be grouped under endocrine, cognitive, psychological, cardiovascular, immune, gastrointestinal, neurological and motor-sensory to aid grouping. In order to stratify and subgroup patient cohorts whilst maintaining a large enough sample size, studies will require even greater total recruitment numbers. The new ME collaborative "Grande Challenge" aims to tackle these issues (recruiting >10,000 subjects). Recruiting a large enough population will also allow for subjects to be diagnosed across multiple diagnostic criteria removing a major source of heterogeneity.

Findings within the current study including potentially raised levels of the cytokine IP-10 and CCL2 in muscle and altered mitochondrial ETC sensitivity to different substrates may be fully elucidated through the stratification of subjects. These differences may be specific to a cohort of individuals diagnosed with CFS and potentially masked in the current study.

7.5.3 Determining the cause of reduced MVC in patients with CFS

The current study revealed patients with CFS have reduced MVC in comparison to HCs. Additional experiments of percutaneous electromyostimulation did not reveal any differences in force generation between these two groups. The study did not find any peripheral impairments in the muscle function of patients with CFS with additional investigations into isolated single fibre force production revealing no difference. Patients with CFS displayed comparable redox homeostasis and mitochondrial function to HCs supporting the theory that peripheral muscle function is not altered. These data suggest a more central deficit in force production in patients with CFS. A natural progression of this work would include the use of transcranial magnetic stimulation to stimulate the motor cortex and/or direct stimulation of the peripheral motor nerve. This area of research is yet to be addressed in patients with CFS.

7.5.4 Physical activity, skeletal muscle cytokine release and fatigue in patients with CFS

In the current study, cytokine responses to exercise and other stimuli were not assessed. Normal levels at rest do not rule out abnormal response patterns, however, a recent review did not find evidence of such abnormalities in CFS (Nijs et al.,2014). Despite these limitations the current results are generally in support of similar, physiological levels of plasma cytokines in patients with CFS and in HCs.

Increased subjective fatigue and impaired physical function has generated a large interest in skeletal muscle function in patients with CFS. As presented in the current work, impaired central processes appear to be the major cause of decreased voluntary force production in a large proportion of CFS patients. The cause of post exertional malaise and extreme bouts of fatigue following physical or mental activity remains unclear in CFS. Further investigation is necessary to elucidate the cause of post exertional malaise also commonly known by patients as "payback" and whether changes in mitochondrial respiration, redox homeostasis or cytokine levels play a role in such changes. It has been suggested that CFS patients have altered cytokine levels in response to exercise in comparison to HCs (Nijs et al., 2014). This would include a similar set of experiments to those completed in the current study assessing mitochondrial function, ROS production (redox homesostasis) and local/systemic cytokine levels.

Additionally, some evidence suggests that CFS individuals have elevated RONS and oxidative damage following physical exercise compared with HCs (Jammes et al., 2005, Jammes et al., 2009). Given the post exertional malaise experienced by all patients with CFS and current understanding of exercise and elevated ROS production; there is plausible rational to hypothesis that patients with CFS may have elevated RONS production during increased physical exercise resulting in elevated oxidative damage and the symptoms of post-exercise malaise. This may explain potential differences in studies reporting redox homeostasis in CFS subjects as previous physical activity in the prior 24 hours to analysis is rarely reported.

7.5.5 Interventions

Despite a lack of understanding and knowledge surrounding the pathophysiology of CFS and its associated symptoms, there have been numerous intervention studies. These include nutritional, immunological, behavioural, pharmacological and complimentary therapies (such as homeopathy, osteopathy and massage therapy) (Hobday et al., 2008).

Non-pharmacological behavioural interventions/treatments have been a strong focus for CFS research proving promising results. The NHS, UK has enrolled cognitive behavioural therapy (CBT) as part of its treatment programme for CFS to relieve symptoms associated with the disease as well as graded exercise therapy (GET). CBT and GET have been shown to improve a wide range of psychological and physical markers in patients with CFS. Exercise as a therapeutic intervention has been widely described to be a very successful non-pharmacological means of treatment in several muscle pathologies, such as myositis (Lightfoot & Cooper, 2016). Specifically, periods of endurance training reduced disease status and induced long-term physiological improvements in patients with myositis (Alemo Munters et al., 2013, Munters et al., 2016, Schmidt and Wonneberger, 2014)

A number of research groups have begun more extreme intervention studies in patients with CFS without sufficient evidence to support study rational. Recently, interventions studies in CFS have begun with Rituximab (a B-lymphocyte (B-cell) depleting monoclonal anti-CD20 antibody) (Pescovitz et al., 2009). Rituximab targets a protein called CD20, which is found on the surface of white blood cells called B-cells, however patients do not appear to experience a change in their symptoms/fatigue severity for several months. Phase three clinical trials are currently underway using Rituximab in patients with CFS. Early investigations included a double-blind, placebo-controlled phase 2 study in which subjects received rituximab (500 mg/m^2) or saline twice with two weeks apart, with follow-up for 12 months. Sixty-seven percent (10/15) of patients reported major or moderate responses with improvements in self-reported fatigue scores (Fluge et al., 2011). A second single-centre, open-label, one-armed phase 2 study with no randomization has recently been completed. Twenty-nine patients were exposed to rituximab (500 mg/m^2) twice, two weeks apart, followed by maintenance rituximab infusions after 3, 6, 10 and 15 months and follow-up for 36 months. The major limitation of this work was that it did not include a placebo group and the open-label design is flawed (Fluge et al., 2015). Phase 3 trials are currently underway. The extreme effects of Rituximab should ensure health professionals act with caution. If CFS is a condition containing more than one sub-group of disease/disorders, the response of all CFS patients may not be uniform. Given the lack of objective markers to differentiate between patients with CFS, it may be hard to determine who will benefit from this drug.

In summary, CFS research should focus its attention on determining the pathophysiology of CFS and CFS related symptoms by determining differences in

objective measures between CFS subjects and HCs. Intervention trials in patients with CFS should be carefully monitored and the potential existence of subgroups within the CFS cohort should be noted, as dissimilar groups may respond to interventions differently. The use of drugs such as Rituximab should be cautioned given the adverse effects these drugs may elicit.

Chapter Eight

Appendix

8.1 Plasma levels of growth factor cytokines in patients with CFS and HCs

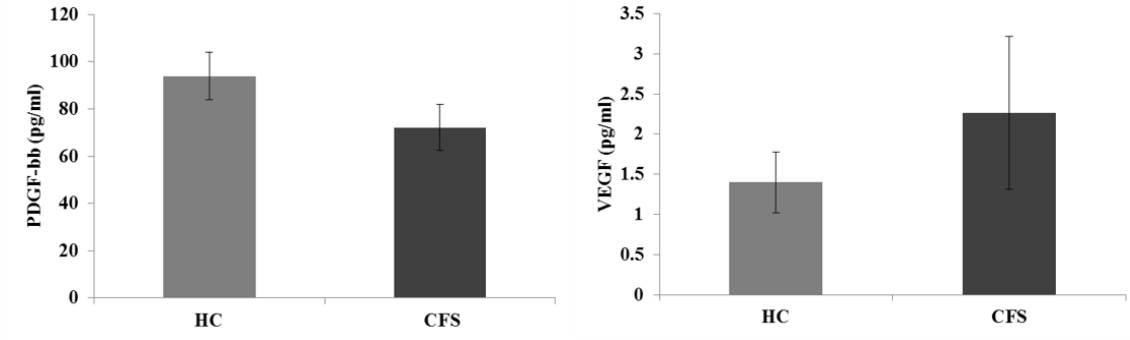


Figure 8.1 Plasma levels of PDGF and VEGF in patients with CFS and HCs. Data are presented as mean ± standard error of the mean (SEM) (n=95).

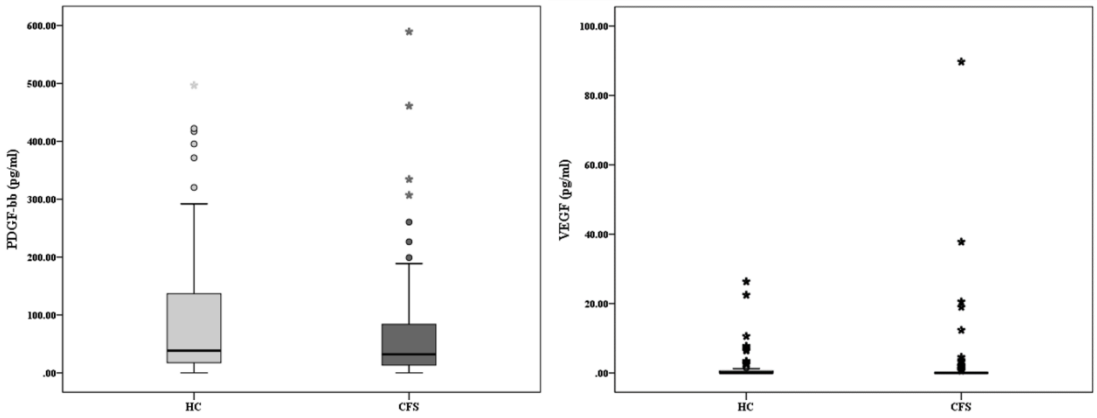


Figure 8.2 Box plots of PDGF and VEGF in patients with CFS and HCs. Data are presented as mean ± standard error of the mean (SEM) (n=95).

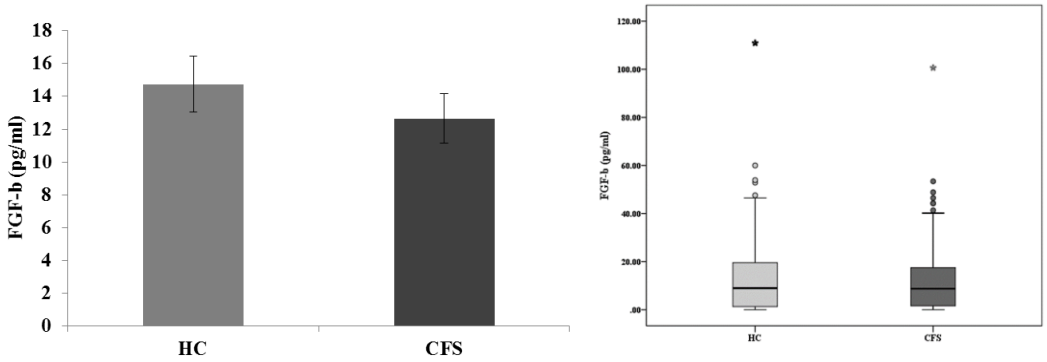


Figure 8.3 Plasma levels of FGF-b (basic) in patients with CFS and HCs. Data are presented as mean ± standard error of the mean (SEM) and box plots (n=95).

8.2 qPCR – Housekeeping gene analysis

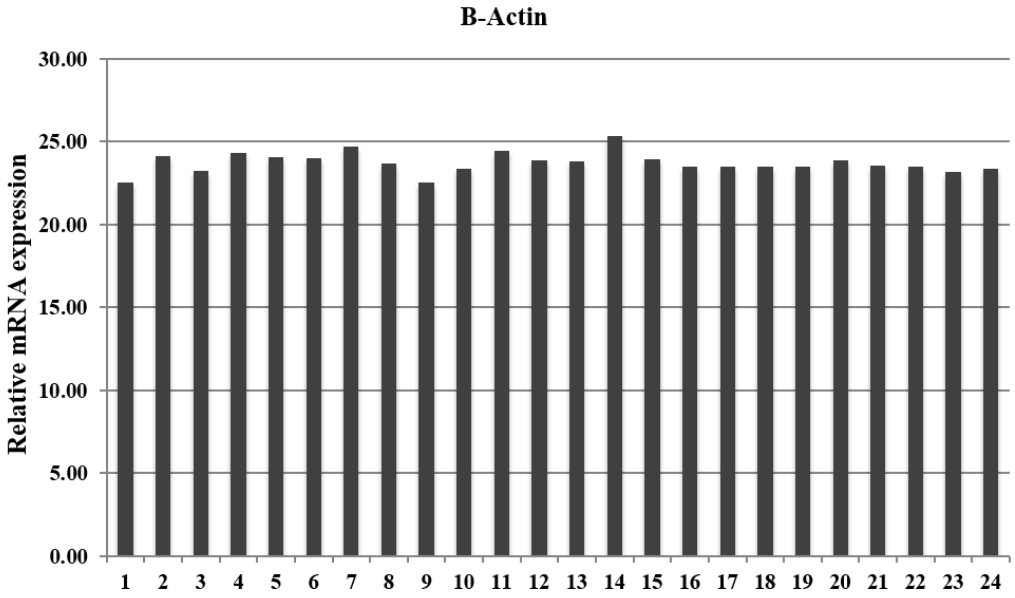


Figure 8.4 Relative mRNA expression of housekeeping gene B-Actin from muscle biopsies. Data presented at mean ± SEM.

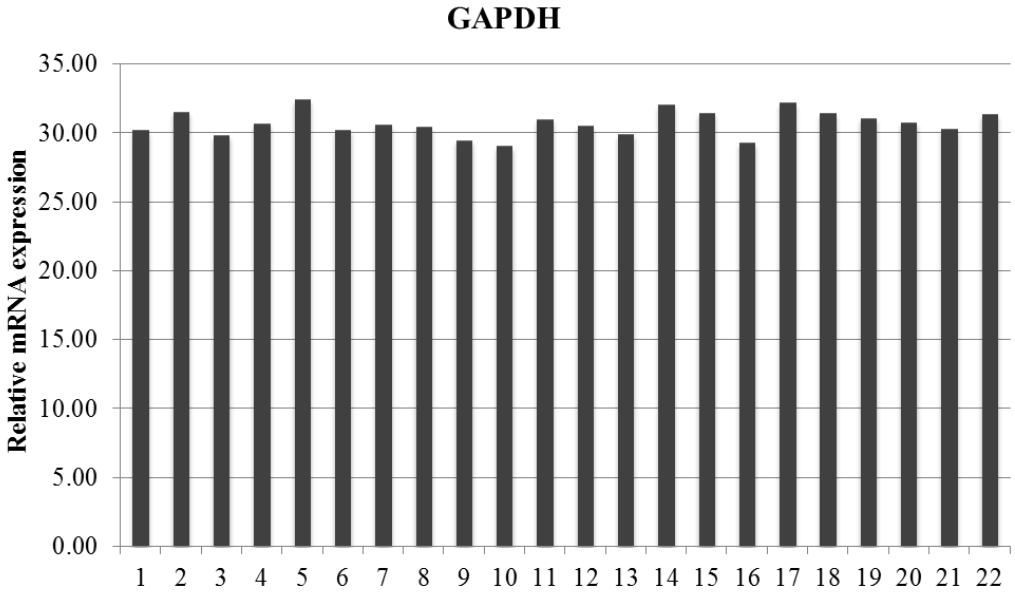


Figure 8.5 Relative mRNA expression of housekeeping gene GAPDH from muscle biopsies. Data presented at mean ± SEM.

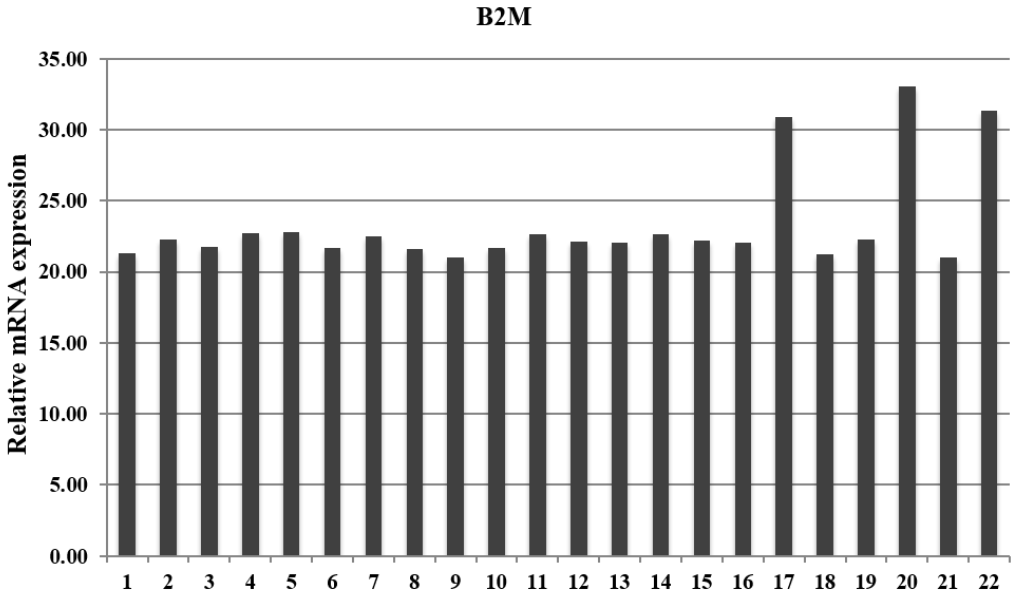


Figure 8.6 Relative mRNA expression of housekeeping gene B2M from muscle biopsies. Data presented at mean ± SEM.

8.3 Maximal Voluntary Contraction (MVC) force vs. plasma cytokine levels in patients with CFS and HCs

Interleukins

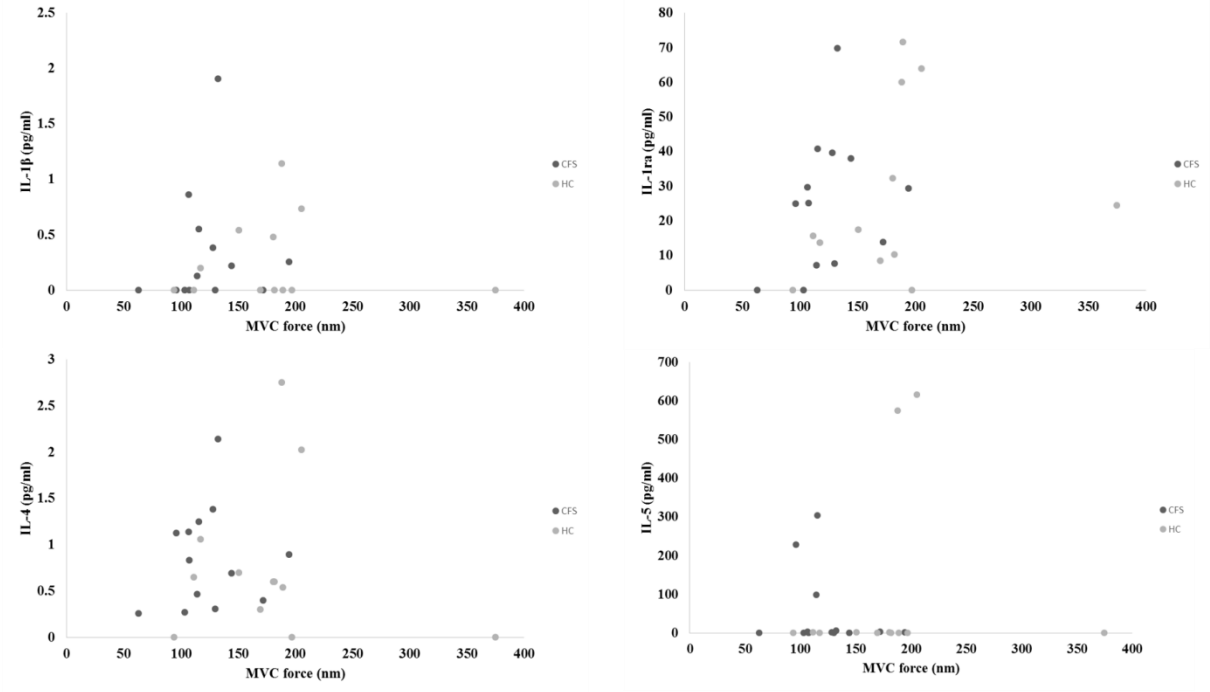


Figure 8.7 Correlations analyses between the MVC force and plasma cytokine levels (IL-1β, IL-1ra, IL-4, IL-5) in patients with CFS in comparison with HCs.

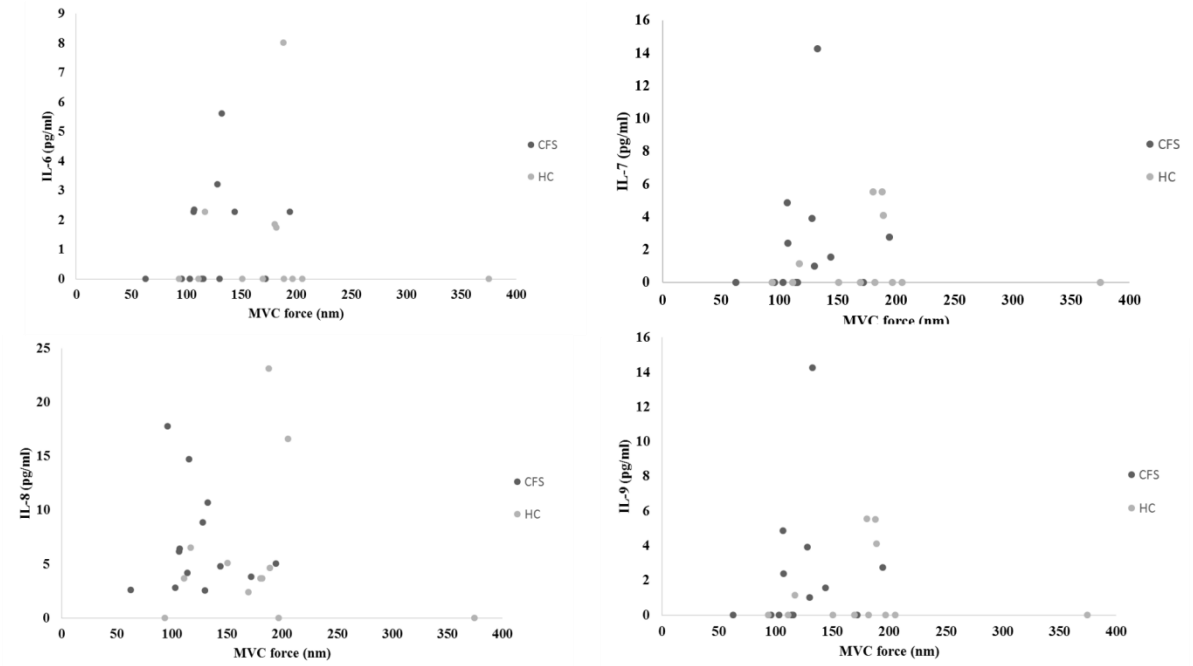


Figure 8.8 Correlations analyses between the MVC force and plasma cytokine levels (IL-6, IL-7, IL-8, IL-9) in patients with CFS in comparison with HCs.

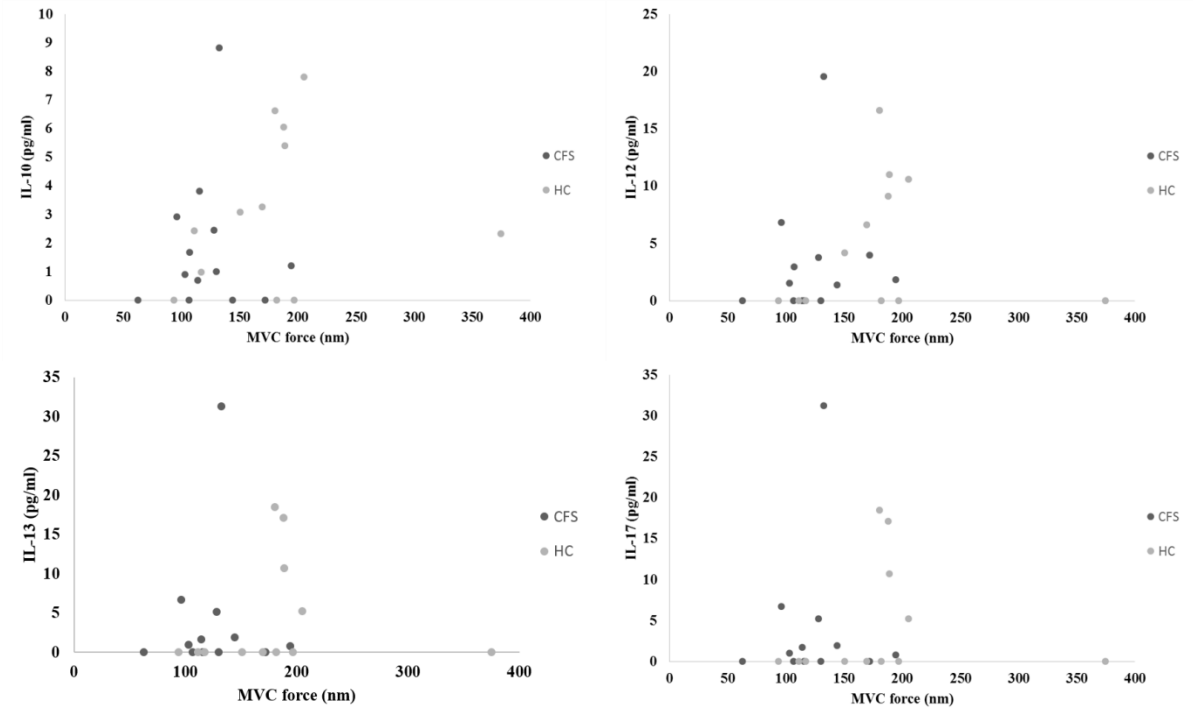


Figure 8.9 Correlations analyses between the MVC force and plasma cytokine levels (IL-10, IL-12, IL-13, IL-17) in patients with CFS in comparison with HCs.

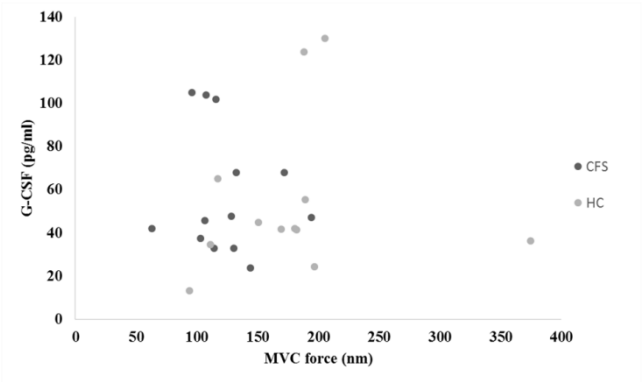


Figure 8.10 Correlations analyses between the MVC force and plasma cytokine levels (G-CSF) in patients with CFS in comparison with HCs.

CC Chemokines

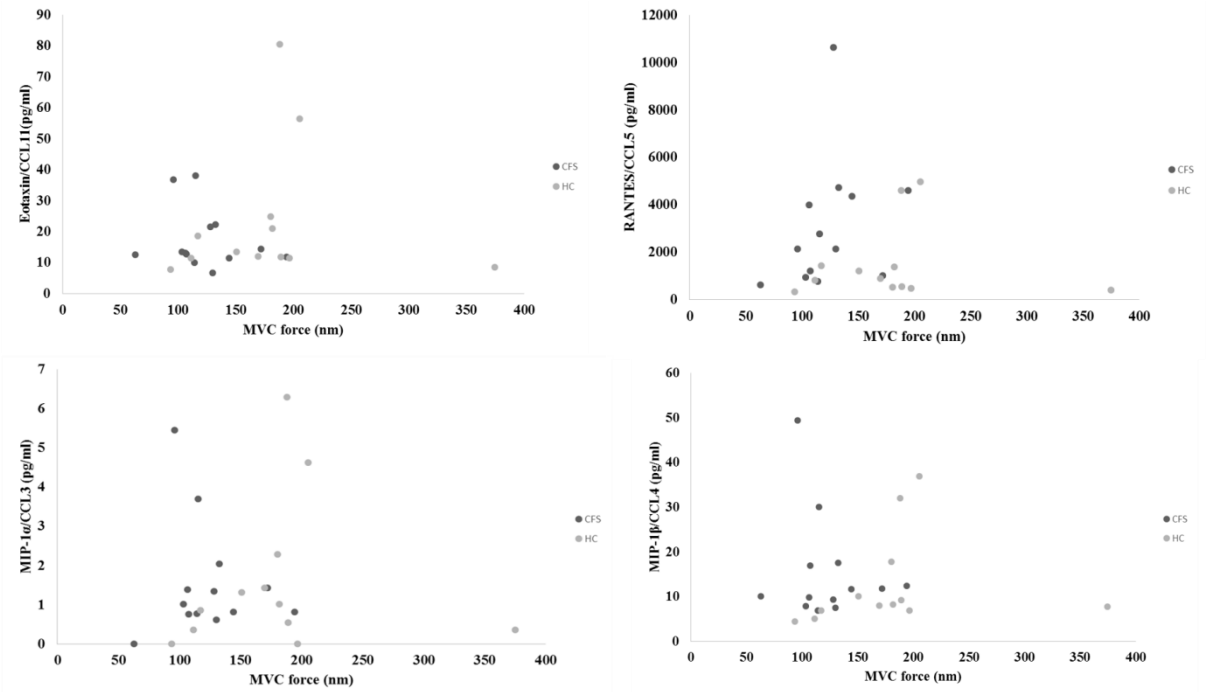


Figure 8.11 Correlations analyses between the MVC force and plasma cytokine levels (Eotaxin/CCL11, RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4) in patients with CFS in comparison with HCs.

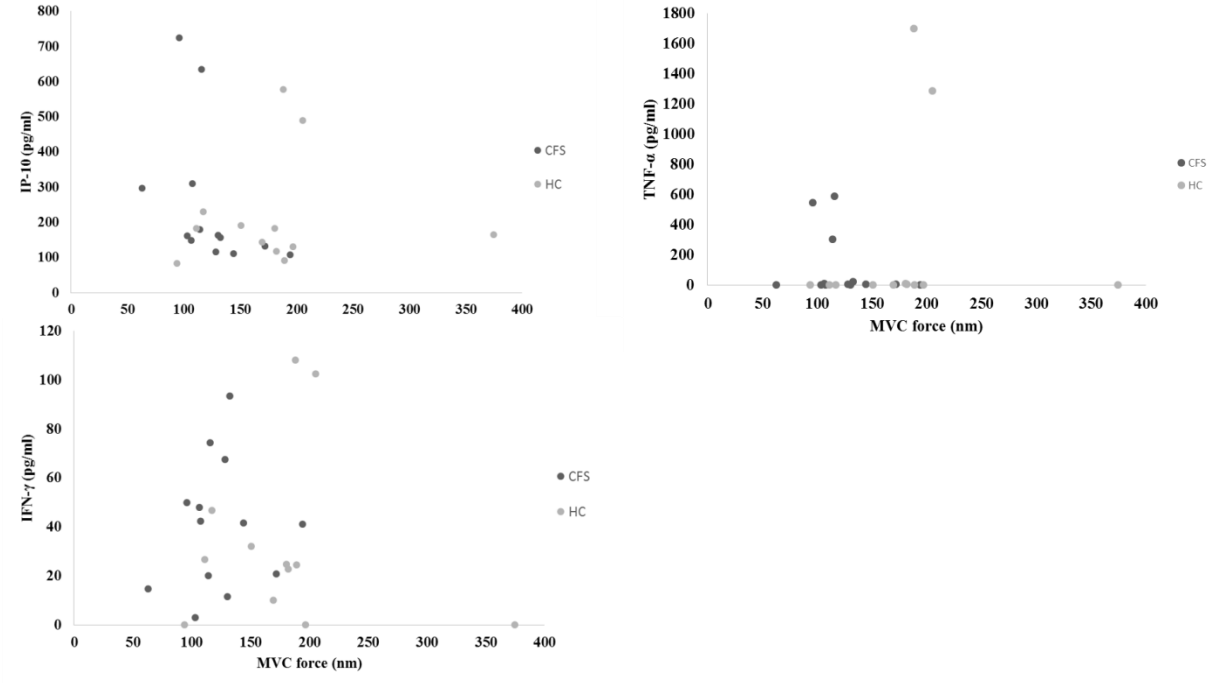


Figure 8.12 Correlations analyses between the MVC force and plasma cytokine levels (IP-10, TNF-α and IFN-γ) in Patients with CFS in comparison with HCs.

8.4 Percentage force reduction of the quadriceps during fatigue protocol vs. plasma cytokine levels in patients with CFS and HCs

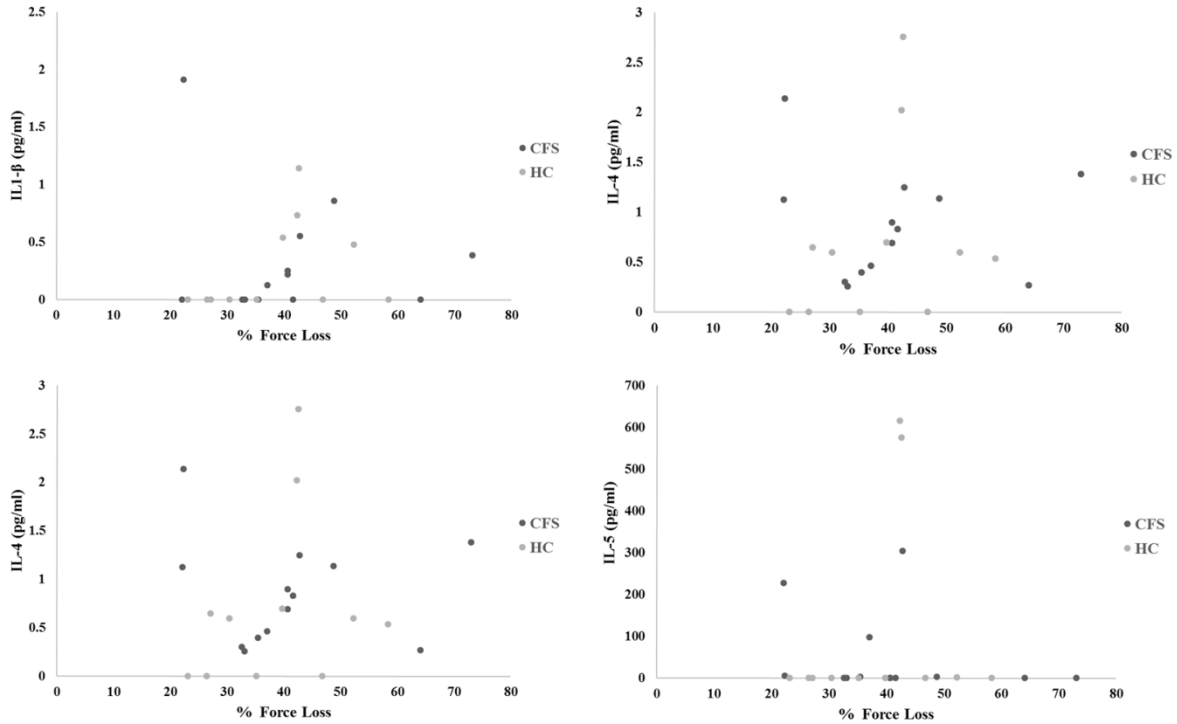


Figure 8.13 Correlations analyses between the % force loss and plasma cytokine levels (IL-1β, IL-1ra, IL-4, IL-5) in patients with CFS in comparison with HCs.

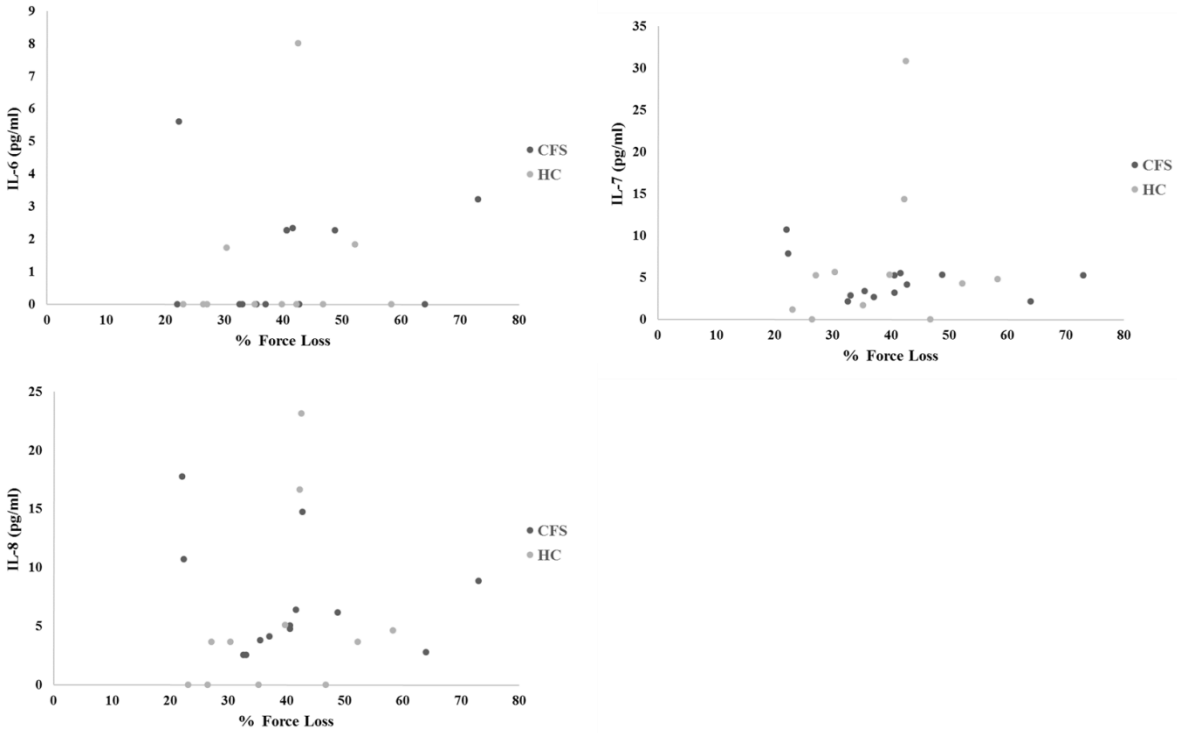


Figure 8.14 Correlations analyses between the % force loss and plasma cytokine levels (IL-6, IL-7, IL-8, IL-9) in patients with CFS in comparison with HCs.

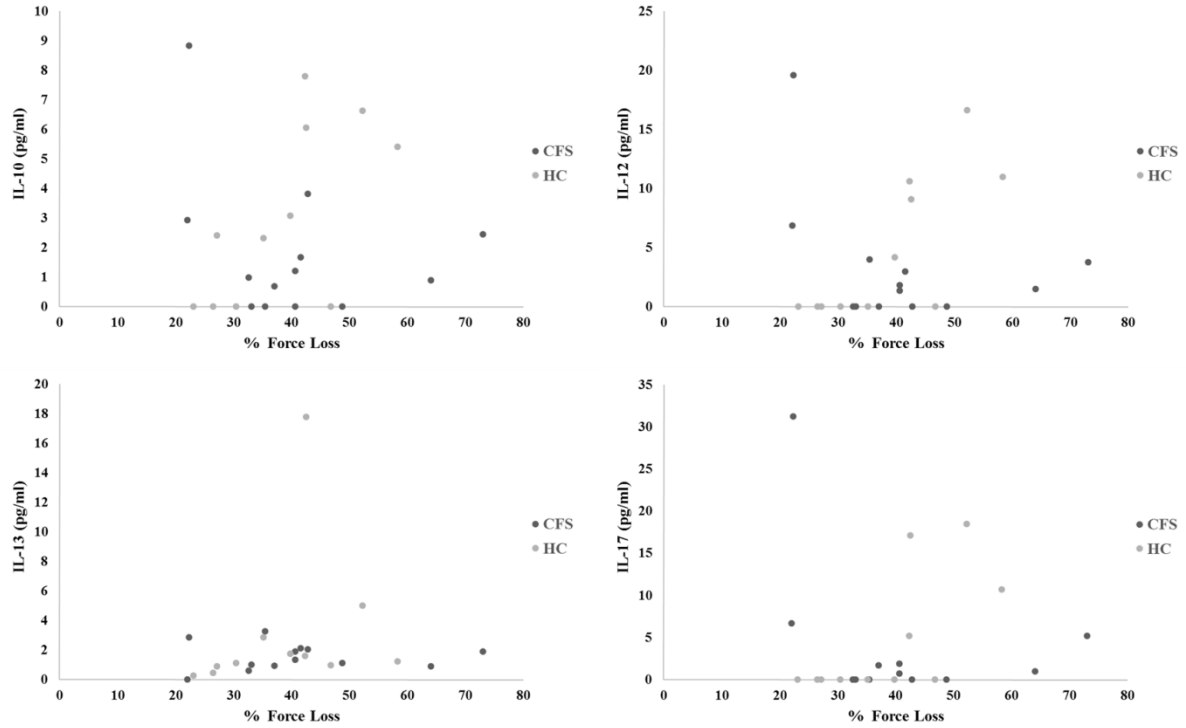


Figure 8.15 Correlations analyses between the % force loss and plasma cytokine levels (IL-10, IL-12, IL-13, IL-17) in patients with CFS in comparison with HCs.

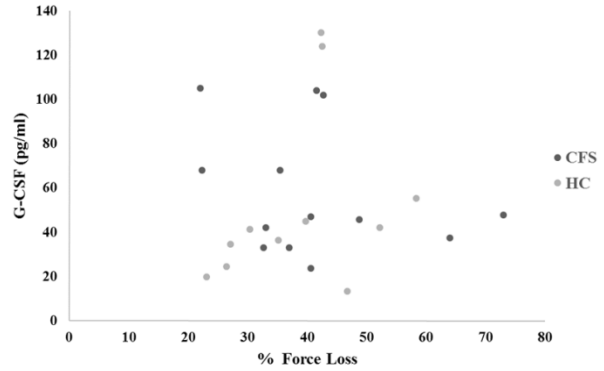


Figure 8.16 Correlations analyses between the % force loss and plasma cytokine levels (G-CSF) in patients with CFS in comparison with HCs.

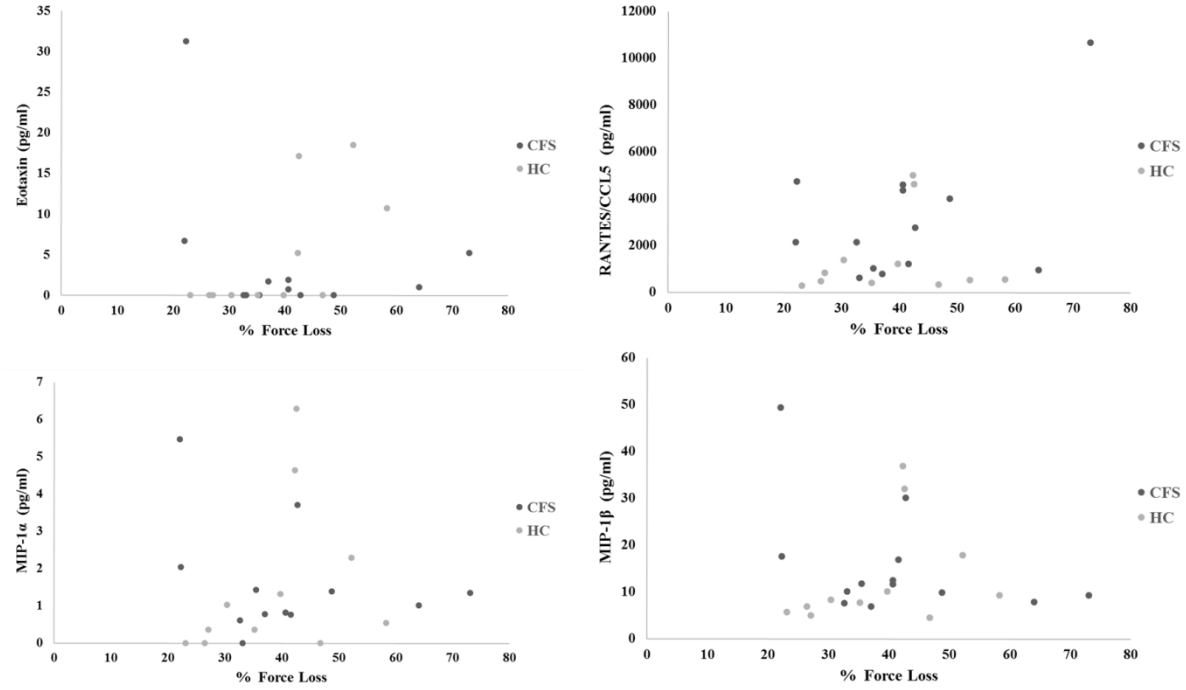


Figure 8.17 Correlations analyses between the % force loss and plasma cytokine levels (Eotaxin/CCL11, RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4) in patients with CFS in comparison with HCs.

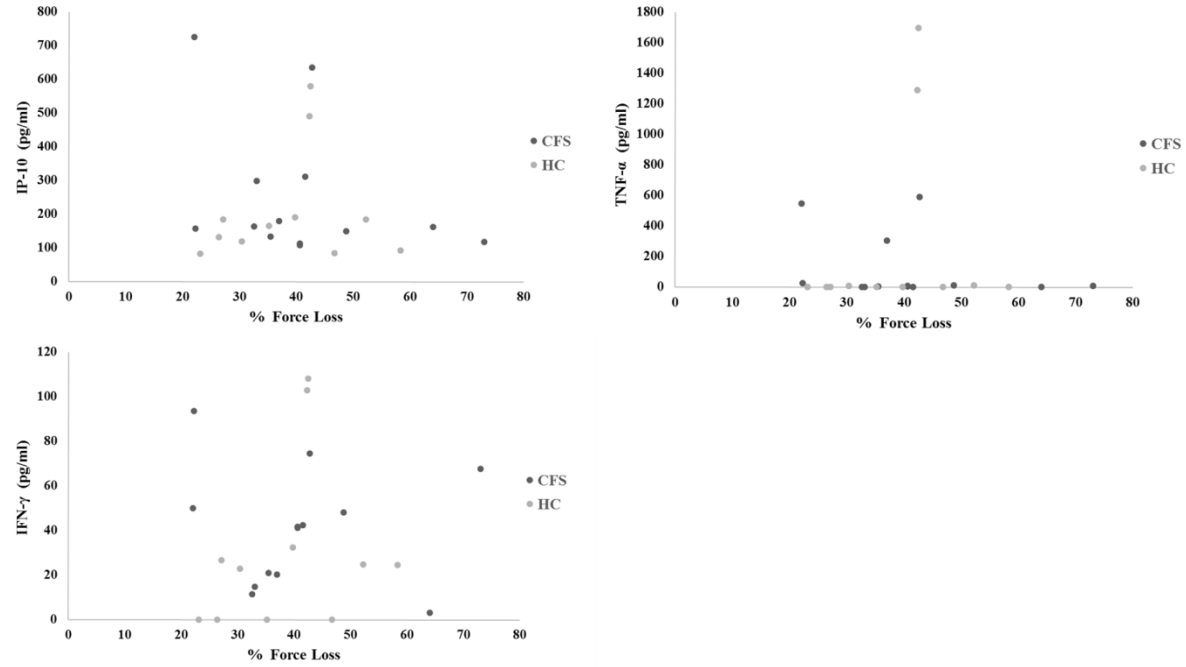


Figure 8.18 Correlations analyses between the % force loss and plasma cytokine levels (IP-10, TNF-α and IFN-γ) in patients with CFS in comparison with HCs.

8.5 Health Questionnaires

ID Code: Flag 1

1 - Pre 5 - 24 mth
2 - 6 wk 6 - Misc
3 - 6 mth 7 - D/C
4 - 12 mth

CFS/NHS/ADULT - *Specialist help for ME* NHS

Chalder Fatigue Questionnaire

Today's Date / /

We would like to know more about any problems you have had with feeling tired, weak or lacking in energy in the last month. Please answer ALL the questions by crossing the answer that applies to you most closely. **If you have been feeling tired for a long while, then compare yourself to how you felt when you were last well**

Please cross only one box in each line

		Less than usual	No more than usual	More than usual	Much more than usual
1.1	Do you have problems with tiredness?	1 ☐	2 ☐	3 ☐	4 ☐
1.2	Do you need to rest more?	1 ☐	2 ☐	3 ☐	4 ☐
1.3	Do you feel sleepy or drowsy?	1 ☐	2 ☐	3 ☐	4 ☐
1.4	Do you have problems starting things?	1 ☐	2 ☐	3 ☐	4 ☐
1.5	Do you lack energy?	1 ☐	2 ☐	3 ☐	4 ☐
1.6	Do you have less strength in your muscles?	1 ☐	2 ☐	3 ☐	4 ☐
1.7	Do you feel weak?	1 ☐	2 ☐	3 ☐	4 ☐
1.8	Do you have difficulty concentrating?	1 ☐	2 ☐	3 ☐	4 ☐
1.9	Do you make slips of the tongue when speaking?	1 ☐	2 ☐	3 ☐	4 ☐
1.10	Do you have problems thinking clearly?	1 ☐	2 ☐	3 ☐	4 ☐
		Better than usual	No worse than usual	Worse than usual	Much worse than usual
1.11	How is your memory?	1 ☐	2 ☐	3 ☐	4 ☐

V2.0

Figure 8.19 Chalder Fatigue Questionnaire.

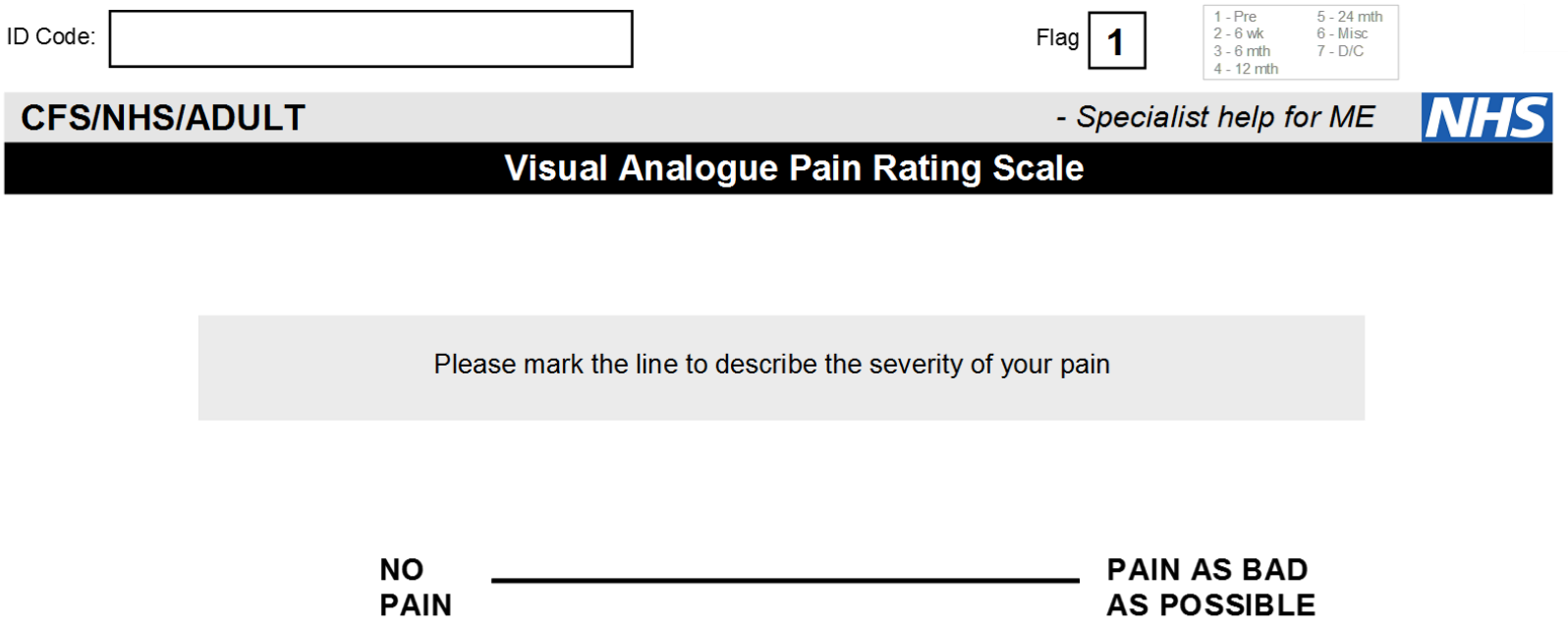


Figure 8.20 Visual Analogue Pain Rating Scale.

1214148461 ID Code: Flag

1 - Pre 5 - 24 mth
2 - 6 wk 6 - Misc
3 - 6 mth 7 - D/C
4 - 12 mth

CFS/NHS/ *- Specialist help for ME*

Self Efficacy Scale

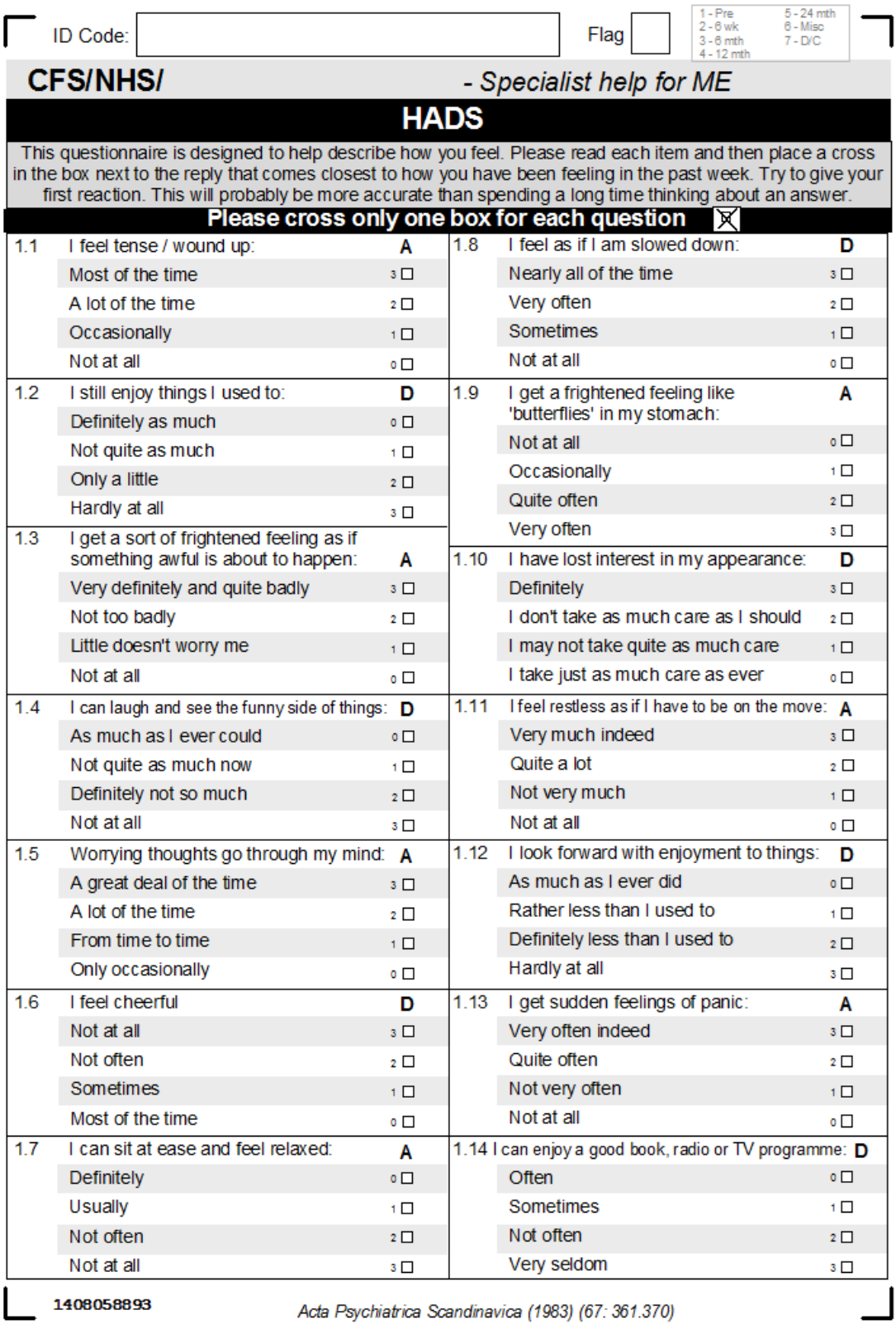
Today's Date / /

We would like to know how confident you are in doing certain activities. For each of the following questions, please choose the number that corresponds to how confidently you can do these tasks regularly at the present time.

Please cross only one box on each line

		Not at all confident 1	2	3	4	5	6	7	8	9	Completely confident 10
	Example										
1.1	How confident are you that you can keep the **fatigue** caused by your disease from interfering with the things you want to do?	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6 ☐	7 ☐	8 ☐	9 ☐	10 ☐
1.2	How confident are you that you can keep the **physical discomfort** or **pain** of your disease from interfering with the things you want to do?	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6 ☐	7 ☐	8 ☐	9 ☐	10 ☐
1.3	How confident are you that you can keep the **emotional distress** caused by your disease from interfering with the things you want to do?	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6 ☐	7 ☐	8 ☐	9 ☐	10 ☐
1.4	How confident are you that you can keep any **other symptoms** or **health problems** from interfering with the things you want to do?	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6 ☐	7 ☐	8 ☐	9 ☐	10 ☐
1.5	How confident are you that you can do the **different tasks** and **activities** needed to manage your health condition so as to reduce your need to see a doctor?	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6 ☐	7 ☐	8 ☐	9 ☐	10 ☐
1.6	How confident are you that you can do things **other** than just taking medication to reduce how much your illness affects your everyday life?	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6 ☐	7 ☐	8 ☐	9 ☐	10 ☐

Figure 8.21 Self Efficacy Scale.



ID Code: Flag

1 - Pre 2 - 6 wk 3 - 6 mth 4 - 12 mth 5 - 24 mth 6 - Misc 7 - D/C

CFS/NHS/ *- Specialist help for ME*

HADS

This questionnaire is designed to help describe how you feel. Please read each item and then place a cross in the box next to the reply that comes closest to how you have been feeling in the past week. Try to give your first reaction. This will probably be more accurate than spending a long time thinking about an answer.

Please cross only one box for each question

1.1	I feel tense / wound up:	A	1.8	I feel as if I am slowed down:	D
	Most of the time	3 ☐		Nearly all of the time	3 ☐
	A lot of the time	2 ☐		Very often	2 ☐
	Occasionally	1 ☐		Sometimes	1 ☐
	Not at all	0 ☐		Not at all	0 ☐
1.2	I still enjoy things I used to:	D	1.9	I get a frightened feeling like 'butterflies' in my stomach:	A
	Definitely as much	0 ☐		Not at all	0 ☐
	Not quite as much	1 ☐		Occasionally	1 ☐
	Only a little	2 ☐		Quite often	2 ☐
	Hardly at all	3 ☐		Very often	3 ☐
1.3	I get a sort of frightened feeling as if something awful is about to happen:	A	1.10	I have lost interest in my appearance:	D
	Very definitely and quite badly	3 ☐		Definitely	3 ☐
	Not too badly	2 ☐		I don't take as much care as I should	2 ☐
	Little doesn't worry me	1 ☐		I may not take quite as much care	1 ☐
	Not at all	0 ☐		I take just as much care as ever	0 ☐
1.4	I can laugh and see the funny side of things:	D	1.11	I feel restless as if I have to be on the move:	A
	As much as I ever could	0 ☐		Very much indeed	3 ☐
	Not quite as much now	1 ☐		Quite a lot	2 ☐
	Definitely not so much	2 ☐		Not very much	1 ☐
	Not at all	3 ☐		Not at all	0 ☐
1.5	Worrying thoughts go through my mind:	A	1.12	I look forward with enjoyment to things:	D
	A great deal of the time	3 ☐		As much as I ever did	0 ☐
	A lot of the time	2 ☐		Rather less than I used to	1 ☐
	From time to time	1 ☐		Definitely less than I used to	2 ☐
	Only occasionally	0 ☐		Hardly at all	3 ☐
1.6	I feel cheerful	D	1.13	I get sudden feelings of panic:	A
	Not at all	3 ☐		Very often indeed	3 ☐
	Not often	2 ☐		Quite often	2 ☐
	Sometimes	1 ☐		Not very often	1 ☐
	Most of the time	0 ☐		Not at all	0 ☐
1.7	I can sit at ease and feel relaxed:	A	1.14	I can enjoy a good book, radio or TV programme:	D
	Definitely	0 ☐		Often	0 ☐
	Usually	1 ☐		Sometimes	1 ☐
	Not often	2 ☐		Not often	2 ☐
	Not at all	3 ☐		Very seldom	3 ☐

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Acta Psychiatrica Scandinavica (1983) (67: 361.370)

Figure 8.22 Hospital Anxiety and Depression Score Questionnaire (HADS).

ID Code: [ ] Flag: **1**

1 - Pre, 2 - 6 wk, 3 - 6 mth, 4 - 12 mth, 5 - 24 mth, 6 - Misc, 7 - D/C

CFS/NHS/ADULT *- Specialist help for ME* NHS

SF-36

The following questions are about ACTIVITIES you might do during a typical day. **Does your health now limit you in these activities?** If so, how much?

Please cross only one box in each line ☒

		Yes, limited a lot	Yes, limited a little	No, not limited at all
1.1	Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	1 ☐	2 ☐	3 ☐
1.2	Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	1 ☐	2 ☐	3 ☐
1.3	Lifting or carrying groceries	1 ☐	2 ☐	3 ☐
1.4	Climbing several flights of stairs	1 ☐	2 ☐	3 ☐
1.5	Climbing one flight of stairs	1 ☐	2 ☐	3 ☐
1.6	Bending, kneeling, or stooping	1 ☐	2 ☐	3 ☐
1.7	Walking more than a mile	1 ☐	2 ☐	3 ☐
1.8	Walking half a mile	1 ☐	2 ☐	3 ☐
1.9	Walking one hundred yards	1 ☐	2 ☐	3 ☐
1.10	Bathing or dressing yourself	1 ☐	2 ☐	3 ☐

Figure 8.23 Short Form Health Survey 36 (SF-36) – Physical Functioning.

ID Code: Flag: 1

1 - Pre 5 - 24 mth
2 - 6 wk 6 - Misc
3 - 6 mth 7 - D/C
4 - 12 mth

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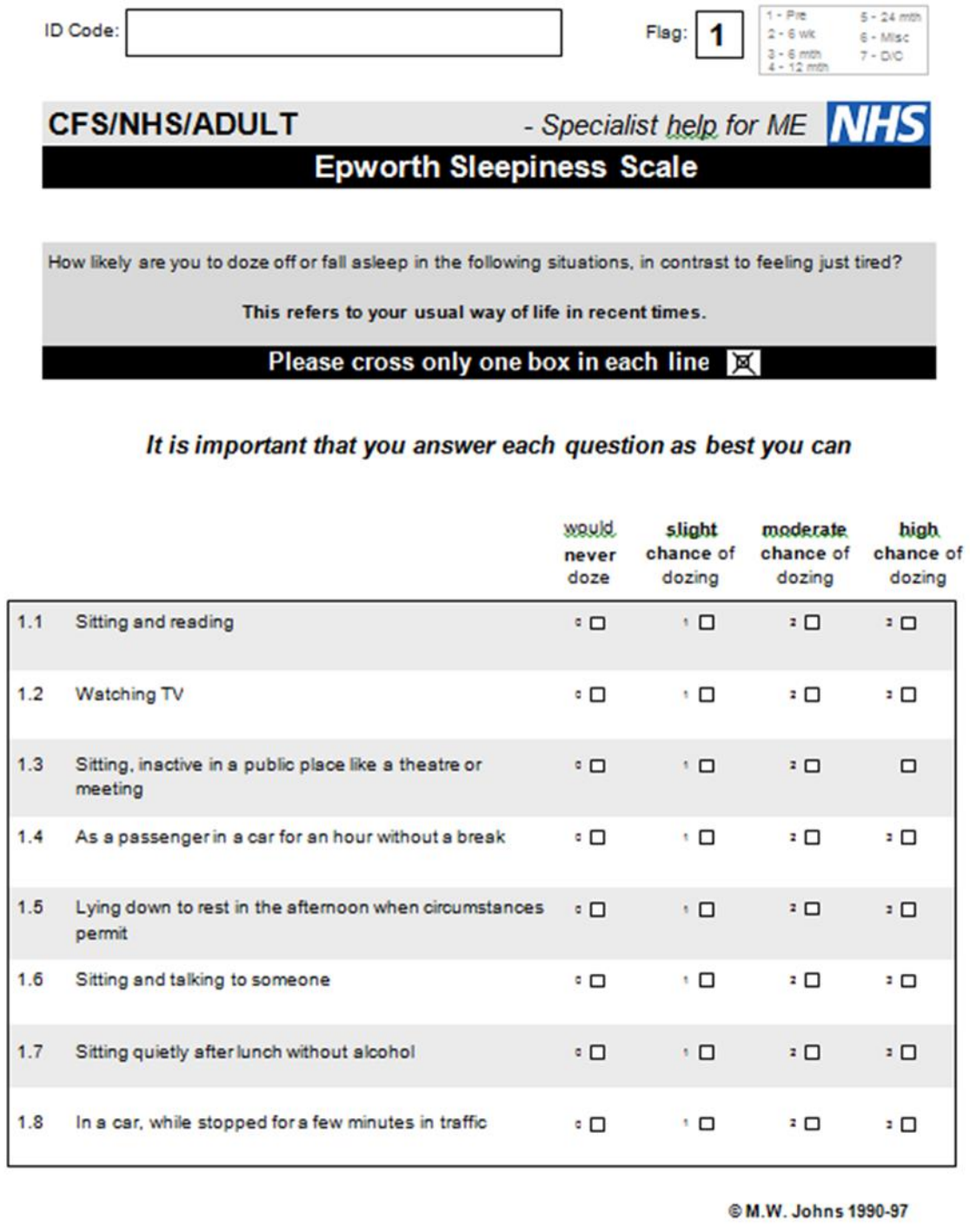
EQ-5D™

By placing a tick in one box in each group below, please indicate which statements describe your own health state today

1.1 Mobility	
I have no problems in walking about	☐ 1
I have some problems in walking about	☐ 2
I am confined to bed	☐ 3
1.2 Self-Care	
I have no problems with self-care	☐ 1
I have some problems with washing or dressing myself	☐ 2
I am unable to wash and dress myself	☐ 3
1.3 Usual Activities *(e.g. work, studies, housework, families or leisure)*	
I have no problems with performing my usual activities	☐ 1
I have some problems with performing my usual activities	☐ 2
I am unable to perform my usual activities	☐ 3
1.4 Pain/Discomfort	
I have no pain or discomfort	☐ 1
I have moderate pain or discomfort	☐ 2
I have extreme pain or discomfort	☐ 3
1.5 Anxiety/Depression	
I am not anxious or depressed	☐ 1
I am moderately anxious or depressed	☐ 2
I am extremely anxious or depressed	☐ 3

Figure 8.24 European Quality of Life Questionnaire (EQ-5D).



ID Code: Flag: 1

1 - Pre 5 - 24 mth
2 - 6 wk 6 - Misc
3 - 6 mth 7 - D/C
4 - 12 mth

CFS/NHS/ADULT - *Specialist help for ME* NHS

Epworth Sleepiness Scale

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired?

This refers to your usual way of life in recent times.

Please cross only one box in each line

It is important that you answer each question as best you can

		would never doze	slight chance of dozing	moderate chance of dozing	high chance of dozing
1.1	Sitting and reading	0 ☐	1 ☐	2 ☐	3 ☐
1.2	Watching TV	0 ☐	1 ☐	2 ☐	3 ☐
1.3	Sitting, inactive in a public place like a theatre or meeting	0 ☐	1 ☐	2 ☐	☐
1.4	As a passenger in a car for an hour without a break	0 ☐	1 ☐	2 ☐	3 ☐
1.5	Lying down to rest in the afternoon when circumstances permit	0 ☐	1 ☐	2 ☐	3 ☐
1.6	Sitting and talking to someone	0 ☐	1 ☐	2 ☐	3 ☐
1.7	Sitting quietly after lunch without alcohol	0 ☐	1 ☐	2 ☐	3 ☐
1.8	In a car, while stopped for a few minutes in traffic	0 ☐	1 ☐	2 ☐	3 ☐

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Figure 8.25 Epworth Sleepiness Scale.

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