

Dietary and Exercise Manipulation of Skeletal Muscle Function in Older Humans

By

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ABSTRACT

Background.

Although life expectancy is increasing, this often comes at the cost of declining health through an increased incidence of cancer, cardiovascular disease and arthritis in older age. In addition, a decline in muscular performance is commonly observed with increasing age, combining a loss of skeletal muscle ('sarcopenia'), a decrease in muscle oxidative capacity and a reduction in muscle strength. Research has shown that it is possible to arrest, or even reverse, the changes in muscle mass and oxidative capacity that occur with age. Two of the most successful strategies identified to date in this regard are exercise, in particular resistance-based training, and protein supplementation. We devised a series of four related studies to investigate and refine strategies for the prevention or mitigation of sarcopenia among the elderly.

Methods.

A strategic, integrated research program involving four independent but complementary studies was undertaken. Interventions investigated for their anti-sarcopenic effects included creatine (Cr) or whey-protein supplementation for a period of 6 weeks (Study 1), a 12-week resistance training program, either alone or in combination with whey-protein supplementation (Studies 2 and 3), and the use of a 4-week protein-rich meal replacement diet (Study 4). The effect of these strategies on improvement in muscle oxidative capacity, muscle protein content, muscle strength, muscular reduction-oxidation (redox) state, body composition and feelings of satiety was evaluated. All studies were randomized, double-blinded and placebo-controlled. The first three studies were performed in healthy male subjects aged 55 years and over, while Study 4 was carried out in obese, but otherwise healthy, male and female

participants aged 22–54 years. The number of participants who completed each trial ranged from 14 and 20. All participants were volunteers who received no inducement.

Results.

Supplementation with either Cr or whey-protein in isolation was found not to improve muscle oxidative capacity or muscle protein concentration (Study 1) compared with placebo. When combined with a 12-week resistance-training program, whey-protein supplementation offered improved body composition (highly significant decreases in fat mass and fat percentage and a highly significant increase in lean mass), and significantly improved muscle function, according to certain criteria (Study 2). However, the same effect was observed with a 12-week resistance-training program alone, suggesting that whey-protein supplementation offered no incremental benefit. By contrast, the combination of a 12-week resistance-training program and whey-protein supplementation was found to have no effect on muscular electrochemical properties, including plasma or tissue thiol or disulfide levels or plasma or tissue redox states (Study 3). Comprehensive refinement of the literature methods was required to successfully complete this investigation, providing a blueprint for further investigations of this kind. In the final study, a protein-enriched meal replacement was found to improve body composition by promoting an increase in lean mass and weight-loss exclusively from fat mass (Study 4). This was in contrast to the control meal replacement in which weight loss occurred from both fat mass and lean mass. The protein-enriched meal replacement diet was also found to induce a significant post-meal subjective satiety, suggesting that this intervention has potential in the control of sensations of hunger.

Conclusions.

A number of strategies investigated in this series of studies have been identified as offering benefit in the management of sarcopenia. In particular, the introduction of a 12-week resistance training program, with or without the addition of a whey-protein supplementation program, offers highly significant benefits in terms of body composition and certain muscle function indicators. In addition, a 4-week protein-enriched meal replacement diet delivered benefits in terms of weight loss and lean mass preservation as well as benefits resulting from increased satiety and improved hunger control. These factors all have an important role in the pathology and development of sarcopenia and other age-related declines in muscle performance.

DECLARATION

I, Graeme Smith, declare that the PhD Thesis entitled “Dietary and Exercise Manipulation of Skeletal Muscle Function in Older Humans” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. All work in this thesis was conducted by me with the exception of the measurement of MHC isoforms using SDS-PAGE, which were completed by doctoral student Deanna Horvath.

Graeme Smith

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I dedicate this thesis to my dearest friend and wife, Yukako. You are my everything.

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LIST OF ABBREVIATIONS

[]	concentration
Ach	acetylcholine
AChE	acetylcholinesterase
ADP	adenosine diphosphate
AI	adequate intake
ANT	adenine nucleotide translocator
AP-1	activator protein 1
ATP	adenosyl triphosphate
ATPase	adenosine triphosphatase
BMC	bone mineral content
BV	biological value
Ca²⁺	calcium
CAT	choline acetyltransferase
CCK	cholecystokinin
CK	creatine kinase
CO₂	carbon dioxide
coA	coenzyme A
CoQ10	coenzyme Q10
CPTI	carnitine palmitoyltransferase I
CPTII	carnitine palmitoyltransferase II
Cr	creatine
creaT	creatine transporter
CS	citrate synthase
CSA	cross-sectional area
CT	computerized tomography
CuSOD	copper-dependent superoxide dismutase
CyS	cysteine

CySS	cystine
DEXA	dual energy x-ray absorptiometry
DHPR	dihydropyridine receptor
DNA	deoxyribonucleic acid
EDL	extensor digitorun longus
E_h	redox potential
ELC	essential (myosin) light chain
ETC	electron transport chain
F₀	membrane-bound proton channel of the ATPase complex
F₀F₁	ATPase complex
F₁	catalytic subunit of the ATPase complex
FAD	flavin adenine dinucleotide
FADH₂	reduced flavin adenine dinucleotide
Fe-S	iron-sulfur
Fe²⁺	iron
FFM	fat free (body) mass
FM	fat mass
FMN	flavin mononucleotide
FSANZ	Food Standards Australia & New Zealand
GCL	glutamate cysteine ligase
GDH	glucose dehydrogenase
GDP	guanosine diphosphate
GLP-1	glucagon-like peptide-1
GLUT-1	glucose transporter-1
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GTP	guanosine triphosphate

H⁺	hydrogen ion
H₂O	water
H₂O₂	hydrogen peroxide
HIV	human immunodeficiency virus
IL-6	interleukin-6
K⁺	potassium
KDa	kilodalton
K_M	michaelis mention constant
LBM	lean body mass
LCD	light chain domain
LDH	lactate dehydrogenase
LMM	light meromyosin
MAPR	mitochondrial ATP production rate
MD	motor domain
Met	methionine
MHC	myosin heavy chain
mi-CK	mitochondrial creatine kinase
Mn²⁺	manganese
MnSOD	manganese superoxide dismutase
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
Na⁺	sodium
NAD	nicotinamide adenine dinucleotide
NADH⁺	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
O₂⁻	superoxide
O₂	oxygen

OGDH	oxoglutarate dehydrogenase
OH⁻	hydroxyl ion
OXPPOS	oxidative phosphorylation
PCr	phosphocreatine
PDH	phosphate dehydrogenase
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
PHOSPH	glycogen phosphorylase
P_i	inorganic phosphate
PYY	peptide tyrosine tyrosine
RDI	recommended daily intake
RLC	regulatory light chain
RNA	ribonucleic acid
ROS	radical oxygen species/reactive oxygen species
RyR	ryanodine receptor complex
SDH	succinate dehydrogenase
SDS-Page	sodium dodecylsulfate polyacrylamide gel electrophoresis
SERCA	sarcoplasmic reticulum
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
1-RM	one repetition maximum
TBW	total body water
TCr	total creatine
Tn-C	troponin-calcium
Tn-I	troponin-inhibitory
Tn-T	troponin-tropomyosin
TRX	thioredoxin
UQ	ubiquinone
UQH₂	reduced ubiquinone

VL	vastus lateralis
V_{max}	maximum velocity
VO₂	oxygen consumption
VO_{2max}	maximum oxygen consumption
VO_{2peak}	peak oxygen consumption
ZnSOD	zinc-containing superoxide dismutase
β-HAD	β-3-hydroxyacyl coenzyme A dehydrogenase
Δp	proton motive force
Ψ	membrane potential

Chapter 1

Introduction

1.0 Introduction

We are an aging population (De Flora, Quaglia et al. 2005)! Human life span has increased dramatically over the last few centuries, with life expectancy in Australia currently 76 years for males and 82 years for females (Australian Bureau of Statistics. 2001). According to the Australian Bureau of Statistics (ABS) population projection, the percentage of the Australian population aged 65 years or older and 85 years or older will increase by 125% and 250%, respectively, over the next 50 years (Australian Bureau of Statistics. 2000). In contrast, the population under 65 years of age will increase by just 27% (Australian Bureau of Statistics. 2000).

An increase in the average age of a population is a sign of societal success, whereby improvements in the quality of healthcare has led to the creation of a demographic group of elderly citizens (Bramstedt 2001). However, this increased lifespan appears to come at a cost, as the quality of life tends to decrease due to an increase in age-related pathologies such as cancer, cardiovascular disease and arthritis. Aged patients are also likely to require more frequent and more intensive critical care (Nagappan and Parkin 2003) compared with the younger population, and often have a comparatively poorer prognosis. The greater healthcare demands of the elderly create a cost burden on the individual as well as on society in general. Disability and loss of independence are a major public health care concern that cost governments billions of dollars per year (Janssen, Shepard et al. 2004).

Costly advances in curative medicine may be sufficient to address health issues in the geriatric population; however, it is equally essential to provide preventive healthcare interventions in order to prevent or reduce the occurrence of severe disabilities in the elderly (Romero Cabrera 2008). Hence, research is required

to attenuate the growing problem of disability in the aged population, not only to remove the burden on the public health care system but, most importantly, to improve the quality of life for the older individual.

Aging is associated with a progressive decrease in lean body mass (LBM) and increase in fat mass (FM) (Kyle, Genton et al. 2001) Most of the decrease in LBM comes from decreases in skeletal muscle (Cohn, Vartsky et al. 1980; Cohn, Vaswani et al. 1985), in a process known as *sarcopenia*.

Sarcopenia, in which muscle tissue integrity and functional capability are gradually diminished (Marzetti, Anne Lees et al. 2009), is one of the most significant problems associated with aging (Lynch 2004). The condition is characterized by decreased muscle strength (Navarro, Lopez-Cepero et al. 2001) and reduced endurance capacity (Rooyackers, Adey et al. 1996), which combine to impair muscle function and performance (Broadwin, Goodman-Gruen et al. 2001). Such changes lead to frailty (Roubenoff 2000), with an increased incidence of falls and impaired mobility in the older person (Carmeli, Coleman et al. 2002). Restriction of mobility, limitations of physical functions and the attendant disabilities brought on by sarcopenia can severely undermine the life quality of people in an aging population. Frailty and falls in the elderly can lead to disability (Broadwin, Goodman-Gruen et al. 2001), loss of independence and possible institutionalization of the individual.

Although sarcopenia is a progressively debilitating condition with a complex etiology, it can be arrested or even reversed by intervening with regimes of exercise and/or dietary supplementation. The role of exercise, in particular, in maintaining physical strength and metabolism is well known and has been extensively discussed and debated.

Exercise may allow the changes in muscle mass and oxidative capacity, as well as the free-radical damage, that occur with age to be slowed or even reversed. Exercise has been shown to increase muscle mass (Westterterp 2000), muscle oxidative capacity (Cartee 1994; Westterterp 2000) and antioxidant defenses against free-radical damage (Lands, Grey et al. 1999). Any intervention that is able to increase the rate of muscle protein synthesis could reverse losses in muscle mass and oxidative capacity, and restore muscle function. Importantly, it seems that resistance training, in particular, may play a crucial role in the prevention of sarcopenia (Johnston, De Lisio et al. 2008). Indeed, one study demonstrated that three months of high-intensity resistance exercise training significantly stimulated the *in vivo* rate of muscle protein synthesis in the vastus lateralis of individuals aged between 76 and 92 years (Yarasheski, Pak-Loduca et al. 1999). Regular resistance training, therefore, is thought to be a potentially valuable intervention in helping to prevent age-related loss of muscle mass and strength.

Unlike the use of exercise regimes, dietary interventions to mitigate sarcopenia have not been explored in great detail to date. The dietary approach, however, demands further attention because, if successful, dietary interventions could be implemented on a huge scale with total inclusion of all sarcopenia cases, irrespective of health status or co-morbidities.

In our laboratory, we have adopted various strategies to investigate the role of dietary interventions in altering muscle physiology. Recently, observations from our laboratory showed that both creatine (Cr) and whey-protein supplementation increased muscle strength (Cooke, Hayes et al. 2001) and muscle oxidative capacity (Smith, Hayes et al. 2001) in rat muscle. This has prompted consideration that Cr and

whey-protein supplementation may reverse, or at least arrest, the debilitating loss of muscle size and function that occurs with age in humans.

There are a number of physiological reasons why supplementation with Cr or whey protein may have a beneficial effect on sarcopenia. For example, it is thought that Cr and whey protein in the diet may cause an increase in protein synthesis by acting osmotically to cause the cell to swell. It has been shown that cell volume changes play an important role in regulating the metabolic activity of the cell, particularly in relation to protein synthesis (Haussinger 1996; Lang, Busch et al. 1998; Haussinger, Graf et al. 2001). Of interest to this research is the possibility that both Cr and whey-protein supplementation could increase, or maintain, muscle oxidative capacity in aging humans by increasing mitochondrial protein synthesis. However, the effect of creatine and whey-protein supplementation on skeletal muscle mitochondrial protein synthesis has not yet been investigated in aging humans.

Whey protein has also been shown to act as an effective and safe cysteine (Cys) donor for glutathione (a potent antioxidant) replenishment during glutathione depletion in immune deficiency states such as that seen with Human Immunodeficiency Virus (HIV), infection (Micke, Beeh et al. 2001) and cancer (Bounous 2000). Interestingly, at least one study has found increased tissue levels of glutathione and improved muscle strength after protein supplementation, suggesting that the improved performance was due to augmented antioxidant defenses (Lands, Grey et al. 1999). As mitochondria undergo substantial free-radical damage due to the production of radical oxygen species (ROS) (Beal 1998), an increase in antioxidant defenses would protect them from oxidative damage. If this is the case, then an increase in glutathione, after supplementation with whey protein, may increase muscle

oxidative capacity. Furthermore, it seems that whey-protein supplementation may be of more benefit to glycolytic muscle rather than oxidative muscle (Smith, Hayes et al. 2001). This is an important observation, as the decreases in fibre size with age in humans are predominantly in the glycolytic fast type IIX fibres (Essen-Gustavsson and Borges 1986; Kovanen and Suominen 1987; Carmeli and Reznick 1994). Therefore, it appears that whey protein may be particularly important in the treatment and intervention of sarcopenia.

Evidence available to date, therefore, suggests that resistance training and supplementation with whey protein may both have important roles in reducing age-related loss in muscle mass and strength, helping to restore muscle function in the elderly.

Finally, whilst sarcopenia can be found in non-obese individuals, the combination of obesity and sarcopenia in aging individuals poses a particularly serious health burden (Kennedy, Chokkalingham et al. 2004). In individuals of advanced age, these conditions restrict mobility of the individual and may impair performance of ordinary tasks of daily living (Davison, Ford et al. 2002; Zoico, Di Francesco et al. 2004).

Obesity-induced insulin insensitivity is instrumental in promoting muscle tissue wastage. Additionally, pro-inflammatory cytokines such as interleukin-6 (IL-6) are upregulated by morbid obesity, which can further cause progressive sarcopenia (Jensen 2008). Conversely, progressive loss of muscle strength and vigor resulting from sarcopenia can present difficulties in tackling obesity. Hence, we face an even more serious problem with the convergence of these two epidemics.

Since obesity and the accompanying sarcopenia are mutually complementary, it is necessary to arrive at strategies to combat both. A two-pronged strategy of exercise and diet may therefore be recommended across all age groups to both bring about fat loss and reverse sarcopenia.

The work described in this thesis is devoted to the development of exercise and supplementation strategies to arrest, or reverse, sarcopenia and sarcopenic obesity. Success in this endeavor will help mitigate the muscular degradation that accompanies the aging process and will contribute to the improvement of the quality of life among the elderly.

In the following review, the structure and function of the skeletal muscle and mitochondria that are pivotal in the condition of sarcopenia are described in detail. Also considered in depth are the underlying processes that lead to sarcopenia, and the theoretical reasons that interventions such as nutritional therapy and physical activity may have a positive effect on the condition.

The experimental sections of the thesis describe four studies conducted in our laboratory into the benefit of regimes of supplementation and exercise in groups of elderly volunteers in practice. These results demonstrate how the theoretical discussion of the underlying processes may be translated into real-world practice.

Chapter 2

Literature Review

2.0 Literature review

The literature review presented in Chapter 2 is intended to provide a comprehensive overview of muscle aging and the steps that can be taken to mitigate the deleterious consequences of this process.

The first part of the review focuses on the principal processes involved in muscle aging, and the effects that these have on the aging individual. A full knowledge of the structure and function of the healthy skeletal muscle is pivotal in understanding how the muscle ages, and this subject is considered in depth in Section 2. Many of the degenerative processes associated with aging can be attributed to the impact of ROS on healthy cells. As ROS are generated within the mitochondria, these organelles are therefore also pivotal in the aging process and are also discussed in some detail in Section 2.4.

Having considered the structure and function of healthy skeletal muscle and mitochondria, the review considers how some of the key functional parameters may be assessed. The efficiency of skeletal muscle movement can be measured in a number of ways, including an assessment of enzyme (ATPase) activity. Muscle length and speed of muscle contraction can be measured through use of isokinetic dynamometry, and muscular function can also be assessed by analysis of body composition.

The redox activity of a cell determines the impact of the ROS on the cell, and therefore methods for the accurate assessment of a cell's redox state are vital if we are to understand the aging process at a cellular level. The redox potential (E_h) of a cell can be calculated from the relative concentrations of GSH and GSSG using the Nernst

equation. This tells us whether a cell is in a pro-oxidative or pro-reductive state, and therefore how effectively it will be able to counteract the effects of ROS.

After the biochemistry of muscular aging, and available methods for the measurement of the different processes have been presented, the second part of the literature review considers possible interventions to arrest or reverse the loss of muscle function that is associated with aging. Key potential interventions include resistance training and supplementation with Cr or whey protein. Considerable evidence is presented for the effectiveness of both approaches on certain key parameters associated with aging.

2.1 Skeletal Muscle Structure

Skeletal muscle is composed of striated muscle fibres (myofibrils) with a diameter of about 10–100 μm and a length of up to 20 cm. A collection of muscle fibres come together to form a muscle fibre bundle, about 100–1000 μm in diameter (McComas 1996). The muscle fibre bundle is surrounded by a membrane called the *sarcolemma*, which encloses the myofibrils, sarcoplasm, cell nuclei, mitochondria, triglycerides, glycogen and various other organelles and cellular components (Figure 2.1a).

Each muscle contains hundreds of muscle fibres, each of which is divided into the different compartments that make up the *sarcomere*. The sarcomere is subdivided by Z-disks and is roughly 1.5–3.0 μm in length (McComas 1996). The different sections of the sarcomere can clearly be seen under a microscope (Figure 2.1b) with each muscle fibre containing alternating light and dark bands and lines, giving the muscle its striated appearance. The light and dark bands are made up of thin (light)

actin and thick (dark) myosin filaments. The actin filaments run through each Z-disk, with half of the actin filament on one side of the sarcomere and the other half on the other side of an adjacent sarcomere. Each side of the Z-disk is composed entirely of actin filaments in an area called the *I-band*. The area where the actin filaments cross over with the myosin filaments is referred to as the *A-band*, and the area composed entirely of the myosin filament is called the H-zone.

The myosin filaments are connected from the M-line to the Z-disk by a very large protein called titin/connectin (chemical formula $C_{132983}H_{211861}N_{36149}O_{40883}S_{693}$). One function of titin is to bind the thin filaments to the M-line, where they interact with the thick filaments (Figure 2.1b).

2.1.1 Contractile Proteins: Myosin and Actin

Myosins are a large family of motor proteins that are responsible for actin-based motility. In muscle cells, myosin II is responsible for producing the contractile force that induces skeletal movement. Myosin II comprises a long tail of intertwined paired strands, which consists of light meromyosin (LMM) and a flexible subfragment (C-terminal) (Figure 2.2). A neck region is located at the end of the myosin strands, containing a double head (N-terminal) (Huxley 1965). Each myosin head contains a motor domain that hydrolyzes adenosine triphosphate (ATP) and binds with actin.

The combination of the myosin neck and myosin head is known as the *myosin heavy chain* (MHC) (Craig and Woodhead 2006). It is the interaction of the myosin head with actin, the cross-bridge, that generates force in skeletal muscle.

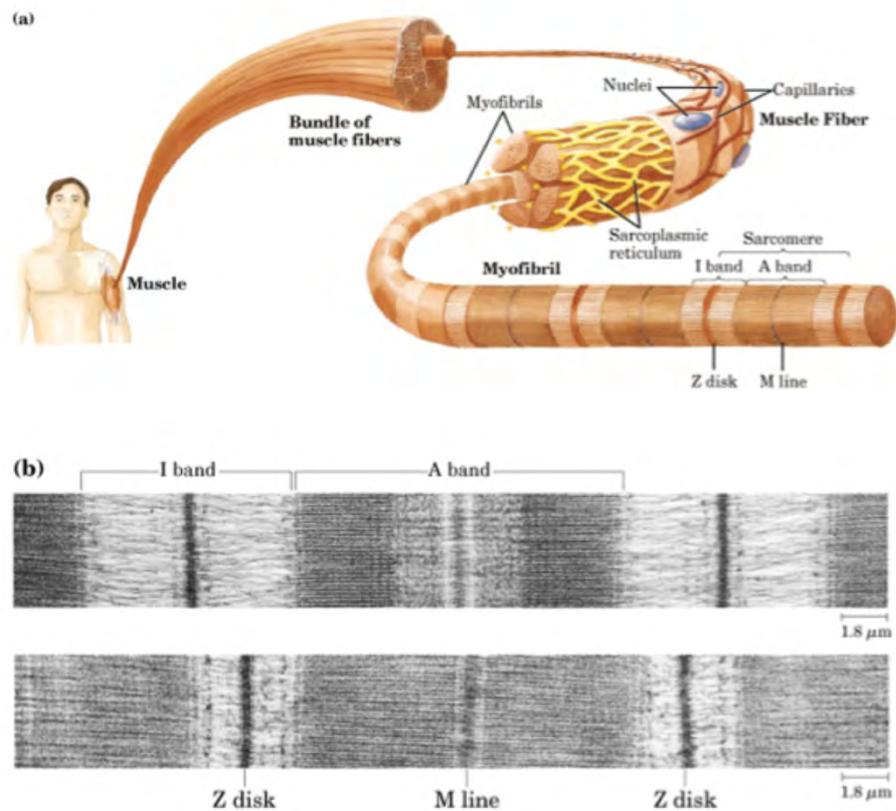


Figure 2.1: The Structure of Skeletal Muscle (a) The muscle fibre bundle, myofibrils, and their associated structures, (b) the sarcomere as seen under a light microscope.



Figure 2.2: The structure of myosin. Showing the two stranded (red and green) myosin tail (light meromyosin; LMM + flexible subfragment 2; S2), the myosin head (S1) with the motor domain (MD) involved in actin binding and the light chain domain (LCD) containing the different light chains (essential light chain; ELC, and regulatory light chain; RLC). Taken from (Craig and Woodhead 2006).

Skeletal muscle fibres can be broken down into two categories; type I (slow) and type II (fast), with type II fibres containing the subtypes IIa and IIb (Table 1). Muscle-fibre type can be delineated according to MHC isoforms based on the regulatory action of the myosin light chains (Pette and Spamer 1986; Pette and Staron 1990; Pette and Staron 2000). The different MHC isoforms differ in their functional and metabolic characteristics (Table 1). For example, the ATPase activity of some MHC isoforms is positively correlated with the speed of contraction, with MHC I having slow ATPase activity (thus referred to as slow twitch muscle) and MHC IIa and IIb having a much faster ATPase activity (thus being called fast-twitch muscle) (Barany, Barany et al. 1965). This classification of muscle fibre types is made possible through the use of histochemical techniques that assess the activity of myosin ATPase (Brooke and Kaiser 1970; Hamalainen and Pette 1993) and more recently through SDS-PAGE gel electrophoresis (d'Albis, Pantaloni et al. 1979) and Western blotting (Koga, Abe et al. 1987).

Table 1: Characteristics of Different Skeletal Muscle MHC Isoforms. Adapted from Close (Close 1972). NOTE: MHC IIb is referred to as MHC Ix in humans

Characteristic	MHC I Slow Oxidative	MHC IIa Fast Oxidative	MHC IIb Fast Glycolytic
Myosin ATPase	Low	High	High
Speed of contraction	Slow	Fast	Fast
Fatigue resistance	High	Moderate	Low
Oxidative capacity	High	High	Low
Mitochondrial density	High	High	Low
Myoglobin content	High	High	Low
Anaerobic enzyme content	Low	Moderate	High
Capillary density	High	High	Low
Glycogen	Low	Moderate	High
Fibre Diameter	Small	Medium	Large

The actin molecule consists of globular protein (G-actin) that gathers to form a chain of polymer beads called F-actin. Like the myosin molecule, the actin molecule also consists of two chains that are twisted around each other (Elzinga, Collins et al. 1973). The actin filament contains two regulatory proteins called tropomyosin and troponin (Figure 2.3).

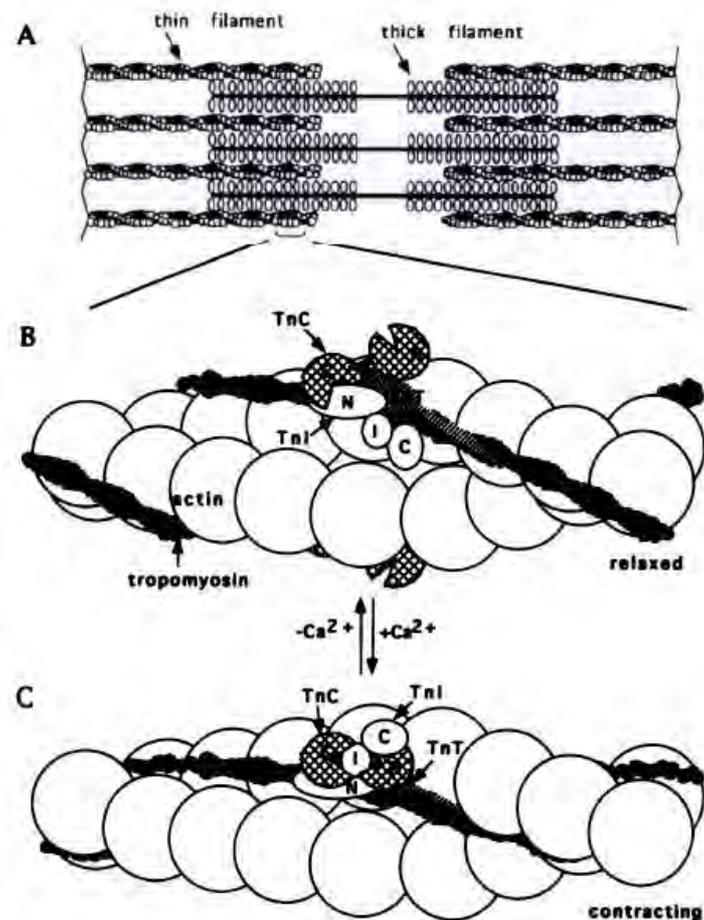


Figure 2.3: The structure and location of the actin filament. (A) location in the sarcomere with myosin (B) troponin-tropomyosin complex in relaxed state with tropomyosin covering the myosin binding site and (C) in the contracting state with Ca^{2+} bound to troponin C (Tn-C). Taken from (Farah and Reinach 1995)

Tropomyosin is an actin-binding protein and winds itself around the actin filament, whereas troponin is attached to tropomyosin and is made up of three subunits: 1) troponin-C (Tn-C), 2) troponin-T (Tn-T), and 3) troponin-I (Tn-I). These subunits, with tropomyosin, form the troponin-tropomyosin complex and act as regulatory proteins that block binding sites on the actin molecule that prevent cross-bridge formation and subsequent contraction (see Section 2.2.1) of the muscle when in the relaxed state (Huxley 1965).

2.1.2 Sarcoplasmic Reticulum Proteins

The sarcoplasmic reticulum (SR) is the endoplasmic reticulum of striated muscle. The SR is a specially organized tubular system that lies parallel to the myofibrils of the striated muscle and acts as a reservoir of calcium (Ca^{2+}) ions that forms a network throughout the surrounding sarcoplasm of the muscle fibre (Jorgensen, Shen et al. 1982). Transverse tubules (T-tubules) are located at the intersection between the A and I-bands and act as tunnel-like infoldings of the sarcolemma, running at right angles to the sarcomere directly into each muscle fibre. Running parallel on both sides of each T-tubule are fluid-filled sacs called *terminal cisternae*. The terminal cisternae are enlarged areas within the muscle cell that store the Ca^{2+} that gets released during excitation-contraction coupling (see Section 2.2.1).

The T-tubule and the terminal cisternae are collectively referred to as a *triad*. The triad is the functional unit of the SR that contains the dihydropyridine receptors (DHPR), which are the voltage-dependant Ca^{2+} channels responsible for controlling Ca^{2+} release. Action potentials arrive from the motor unit and travel into the muscle where the SR initiates the contraction of skeletal muscle by releasing Ca^{2+} from the terminal cisternae (see Section 2.2.1).

2.2 Skeletal Muscle Function

2.2.1 Excitation-Contraction Coupling

Excitation-contraction coupling involves a process whereby an electrical stimulus in the form of an action potential is converted into a biomechanical movement in the form of muscle contraction (Figure 2.4) (for review see Huxley HE, 1965).

Muscle contraction is initiated when a motor neuron fires and releases the neurotransmitter acetylcholine (ACh) into the neuromuscular junction where it crosses the synaptic cleft and binds to ACh receptors on the postsynaptic membrane located on the muscle being innervated (i.e. excitation). The binding of ACh to acetylcholine receptors on the postsynaptic membrane results in the opening of ion channels on the postsynaptic membrane allowing Na^+ to flow into the cell and for K^+ to flow out of the cell. This depolarizes the cell membrane and triggers an 'all-or-nothing' action potential response across the plasma membrane of the muscle cell that travels across the sarcolemma and into the muscle fibre via the T- tubules.

Once inside the muscle fibre, the action potential travels along the T-tubules and activates voltage sensitive DHPR receptors that subsequently activate ryanodine receptors (RyR) in the nearby SR, releasing Ca^{2+} into the sarcoplasm (muscle cytoplasm) from the SR.

When the muscle is relaxed, intracellular Ca^{2+} concentration is low (~50 nM), and the myosin heads cannot bind to actin because tropomyosin is blocking the actin-myosin binding sites. The release of Ca^{2+} from the SR (see above) increases intracellular Ca^{2+} concentration. At higher concentrations, Ca^{2+} binds to troponin C of

the troponin-tropomyosin complex and lifts the tropomyosin off and away from myosin binding site located on the actin filament, preparing it for cross-bridge formation and muscle contraction.

In the relaxed state the myosin head contains an ATPase molecule that hydrolyses ATP into adenosine diphosphate (ADP) and inorganic phosphate (P_i). The hydrolysis of ATP by myosin ATPase transfers energy into the myosin head and readies it for cross bridge cycling. Once the myosin binding site becomes available on the actin filament the myosin head spontaneously binds to the actin binding site forming a cross bridge.

Energy contained within the myosin head produces an inward “power stroke”, sliding the bound actin towards the centre of the sarcomere and shortening the muscle fibre (i.e. contraction). Immediately following this, the myosin head releases ADP and ATP once again binds to the ATP-binding site on the myosin head releasing it from the actin binding site. Starting the process all over again, myosin ATPase hydrolyses the ATP into ADP and P_i which transfers energy into the myosin head, returning it into its original upright position (Figure 2.4).

Following contraction, Ca^{2+} ATPase pumps located on the SR actively resequeter Ca^{2+} back in to the SR, restoring intracellular Ca^{2+} concentrations back to resting levels. Additionally, following the activation/excitation of the muscle fibre by the binding of ACh to the postsynaptic membrane, an enzyme called *acetylcholine esterase* (AChE), located in the synaptic cleft, breaks down the ACh bound to the ACh receptors of the postsynaptic membrane (i.e. muscle fibre). This results in repolarisation of the membrane, ending the generation and propagation of the action potential into the muscle fibre, closing the RyR located on the SR membrane.

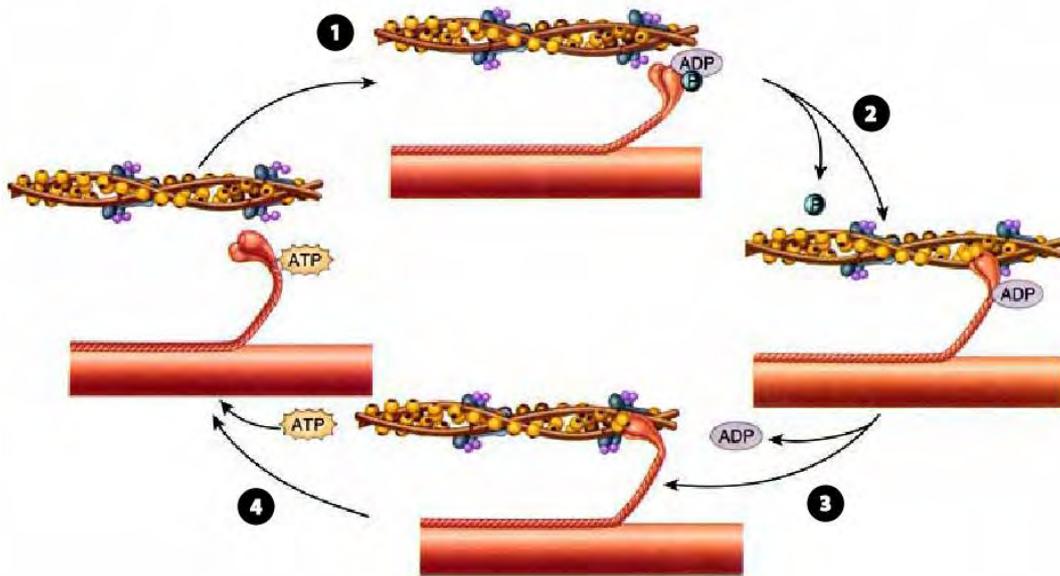


Figure 2.4: Excitation-contraction coupling as proposed by Huxley. 1. Relaxed state, 2. cross-bridge formation, 3. Shortening of muscle fibre, 4. Myosin head releases ADP (Huxley 1965)

2.3 Assessment of Skeletal Muscle Structure and Function

There are a number of ways that skeletal muscle structure and function can be measured. Muscle cross-sections cut from muscle obtained by biopsy can be stained using histochemical techniques to assess muscle structure, and can then be classified (MHC I, IIa and IIb) based on enzyme activity (ATPase), capillary density, oxidative capacity and other important structural and functional characteristics (see Table 1) (Close 1972; Pette and Staron 1990).

Barany et. al. (1965) demonstrated that the activity of myosin ATPase differed in slow MHC I and fast MHC II muscle fibres, and that this correlated closely with the speed of contraction giving the respective muscle fibres their names (Barany, Barany

et al. 1965). Likewise, a similar metabolic classification scheme was set up that measured the activity of various enzymes related to aerobic and anaerobic metabolism (see below), extending the classification scheme used by Barany et. al (1965) with names such as slow oxidative MHC I (aerobic), fast oxidative MHC IIa (aerobic) and fast glycolytic MHC IIb (anaerobic) (Barnard, Edgerton et al. 1971; Close 1972).

Analysis of body composition is a way to measure skeletal muscle mass. Body composition allows for the measurement of different body compartments based on the physical properties of the particular body compartment being measured. In the traditional two-compartment model of body composition analysis, the body is divided into fat mass (FM) and fat free mass (FFM), with the density of compartments assumed to be 0.9 g/cm^3 (Fidanza, Keys et al. 1953) and 1.1 g/cm^3 (Brozek, Grande et al. 1963; Withers, LaForgia et al. 1998), respectively. The masses of each of these components (FM and FFM) is then measured via whole-body immersion using the density of each compartment (Behnke AR 1942). The two-compartment model, however, assumes that the components that make up FFM (total body water, protein and bone mineral content) are present in constant proportions. This assumption does not always hold true because these components can be affected by age, sex, physical activity level and genetics (Brozek, Grande et al. 1963). Therefore, a three-compartment model is often used that measures FM, total body water (TBW) and bone mineral content (BMC), with lean mass measured by assuming a mineral-to-protein ratio of 0.35 (Withers, LaForgia et al. 1998).

The three-compartment model serves to eliminate any error associated with individual differences in hydration status. Dual-Energy X-ray Absorptiometry (DEXA) is a good example of the three-compartment model (Prior, Cureton et al.

1997; Andreoli, Scalzo et al. 2009), and is generally the preferred ‘gold standard’ reference method for measuring body composition. DEXA is also useful for measuring regional analysis of fat, bone mineral content and lean tissue (Prior, Cureton et al. 1997). Regional analysis of body composition and body composition changes allows for the detection of changes in regional areas such as the trunk and limbs that would otherwise be missed with generalized measurements (Atlantis, Martin et al. 2008). Atlantis et. al. (2008) analyzed 1200 Australian males between the ages of 35 and 81 years, and found that while whole body fat mass remained stable irrespective of age, fat mass increased viscerally (Atlantis, Martin et al. 2008). Further to this, age-related differences in whole-body fat mass percentage were mostly due to a reduction in lean mass, while differences in abdominal fat mass percentage were actually due to greater fat mass. Differences of this type can not be detected by fat mass percentage analysis alone.

Muscle function is dependent on muscle structure. For example, enzyme activity, muscle fibre type, and muscle fibre size can all influence speed of contraction (see Table 1) and hence the force produced by a muscle (Close 1972; McComas 1996). Additionally, the cross-sectional area (CSA) of the muscle is also positively correlated with muscle force (Ikai and Fukunaga 1968).

Muscle strength and function can be tested using a wide variety of techniques ranging from isometric measurements of a particular muscle at a particular length and angle (Medicine 2006) to dynamic measurements of muscle strength that mimic everyday activities such as standing up from a chair (Alexander, Schultz et al. 1997) or climbing a flight of stairs (Cavanagh, Mulfinger et al. 1997).

Muscle length and speed of muscle contraction is often measured using isokinetic dynamometry. Isokinetic dynamometry allows for the measurement of isokinetic, isometric and dynamic strength and power (Medicine 2006). Many studies have used isokinetic dynamometers (e.g cybex) to measure muscle strength (Aquino Mde, Leme et al. 2002; Tsourlou, Benik et al. 2006). Isokinetic dynamometry allows for the measurement of dynamic muscular strength with different muscle groups across the muscles full range of motion at variable speeds in both the concentric (shortening) and eccentric (lengthening) phases. For example, dynamic isokinetic strength can be measured for the large muscle groups of the lower body using knee extension (quadriceps; rectus femoris) and knee flexion (hamstrings; biceps femoris). The knee extensors and flexors are important muscles involved in everyday activities such as walking, running, jumping and other movements necessary for everyday functioning and the maintenance of physical independence. Isokinetic dynamometry also allows for the measurement of isometric muscle strength using similar muscle groups at different joint angles and lengths.

Whilst the measurements gained through isokinetic dynamometry can be very precise, the equipment required to collect these measurements is quite large and often expensive. Hence, more convenient and less expensive methods are often used.

One of these methods, that measure strength, is the one-repetition maximum (1-RM) test. The 1-RM method of measuring skeletal muscle function measures dynamic functional strength using either inexpensive machines or free weights and calculates the 1-RM, or a predicted 1-RM, whilst performing an exercise with one of the large muscle groups.

A predicted 1-RM can be calculated using a regression model (Willardson and Bressel 2004; Jandacka and Vaverka 2008) to predict the 1-RM from a submaximal 4-6-RM or 7-10-RM lift. The predicted 1-RM model is often used with novice lifters (Willardson and Bressel 2004) and aged subjects (de Vos, Singh et al. 2008) to minimize the risk of muscle soreness and serious injury.

Whilst the 1-RM is a valuable measurement to assess muscle strength and force in a functional environment using free weights, it lacks the specificity possible using isokinetic dynamometry. The traditional 1-RM and predicted 1-RM tests do not measure the velocity of the movement, and hence cannot measure the power output (force \times velocity) of a lift or number of lifts/repetitions whilst using free weights.

Previously, measurement of power output whilst lifting free weights was only achievable by using video equipment to capture velocity over distance and time (Howell, Gaughan et al. 2001). The use of video equipment is quite laborious and impractical for use as an effective research tool in everyday environments such as the gym. Real-time measurements in the gym are often more useful as they mimic normal, real-life training situations (i.e. multiple sets, different exercises, different location).

To overcome these limitations, a portable optical encoder containing a linear transducer to measure the power output of a particular movement was developed (Gymaware, see Chapter 3, Section 3.6.4). To measure concentric and eccentric mean and peak muscle power output whilst performing different exercises/movements a linear transducer is used that sends time and position data to a personal digital assistant via an infrared signal (Drinkwater, Galna et al. 2007). The optical encoder

can be easily moved from one exercise to another in a very short time (minutes), eliminating the need for large video equipment.

The direct measurement of strength associated with everyday activities (walking, stair climbing, sitting and standing) can also be used to assess muscle strength. Such measurements are especially useful in populations such as the elderly where loss of muscle strength and function is common. Loss of muscle strength and function in the elderly is often associated with fall risk and decreased independence (Tinetti, Speechley et al. 1988; Gehlsen and Whaley 1990). This will be discussed in greater detail in Section 2.8.

In summary, skeletal muscles, based on contractile proteins (myosin and actin) and sarcoplasmic reticulum proteins, are responsible for all skeletal movement. They function via signals received from central nervous system via the motor neuron to contract the muscle and induce movement. A part of the natural aging process is the degradation of skeletal muscle function, leading to impaired mobility and stability in the elderly adult. In order to mitigate the effects of skeletal muscle impairment that occurs with aging, it is necessary first to be able to accurately measure skeletal muscle structure and function and as discussed above, a number of techniques exist for this purpose, including histological assessment of muscle biopsies, analysis of body composition, isometric measurements, and dynamic measurements of muscle strength.

Having considered the physiological processes underlying skeletal muscular excitation and contraction, it is important to remember that the ability of the skeletal muscle to function is entirely dependent on the availability of ATP, the source of chemical energy in the muscles. It is of particular interest, therefore, to consider the

production of ATP, and the effect that this has on muscle function. The following sections will therefore consider the source of ATP in the body, the mitochondria, and the role of ATP itself.

2.4 Mitochondria: Structure and Function

Mitochondria are vital cell organelles that are responsible for carrying out various cellular functions necessary for survival. These functions include metabolism (energy synthesis), redox control, mineral (calcium) homeostasis, and other important biosynthetic pathways.

It is known that a number of structural and functional changes occur in the mitochondria as we age, including changes in mitochondria structure and number, changes in mitochondrial enzyme activity, changes in mitochondrial oxidative capacity, and changes in mitochondrial ATP production rate (MAPR). These changes have an important impact on muscle function and on physical degeneration with aging. The age-related changes to the mitochondria are considered in detail later, in Section 2.6.2. However, first we will consider how the mitochondria function in young, healthy individuals.

2.4.1 Mitochondrial Structure

Mitochondria are the oval shaped organelles that act as the power stations of the cell. Mitochondria are typically 1-2 μm in length and 0.5–1.0 μm in diameter (Figure 2.5). Mitochondria are found in almost all eukaryotic cells in large numbers (Scheffler 1999; Schaffer 2007). The number of mitochondria varies from cell to cell depending on the metabolic requirement of the cell and its specialization (Scheffler

1999). The number of mitochondria in the cell also changes with age (Short, Bigelow et al. 2005). This will be discussed in more detail in Section 2.6.2.1.

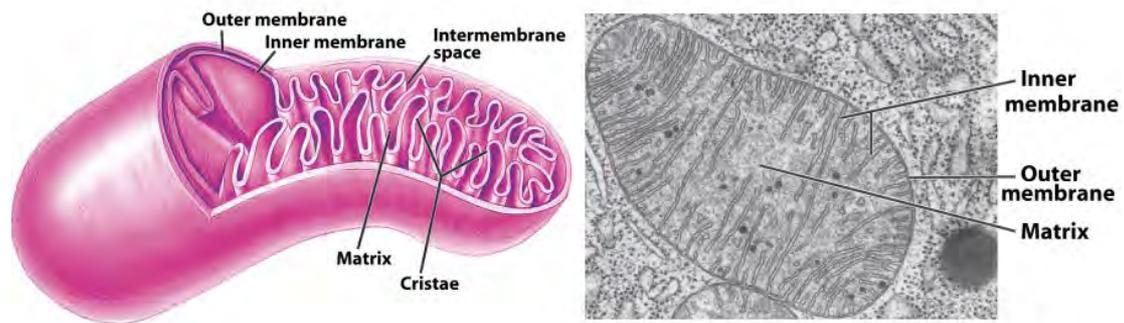


Figure 2.5: The basic structure of the mitochondria (a) showing a graphical representation of the various mitochondrial membranes and intermembranous spaces and (b) the actual structure of a mitochondrion as seen under a microscope (Palade 1953)

Originally, mitochondria were thought to be intracellular organelles that floated freely in the cytosol in vast numbers (Palade 1953). However, due to advances in electron tomography (Frey and Mannella 2000; Frey, Renken et al. 2002), fluorescent microscopy and the availability of very specific mitochondrial fluorescent probes (Griparic and van der Bliik 2001), we now have a better understanding of the three-dimensional interaction of mitochondria in living cells. Rather than acting as individual, free-floating structures in the cytosol, we now see that organelles appear to form an elaborate network of long tubules.

Mitochondria contain two membranes, an outer membrane and a folded inner membrane (crista) that encloses the intracellular mitochondrial matrix. The space

between the outer and inner mitochondrial membranes is called the *intermembranous space*. The folded inner membrane can vary from cell to cell depending on the oxidative demand. For example, the inner membrane surface area of heart mitochondria is very large and tightly packaged to support its high rate of oxidative phosphorylation (Schaffer 2007).

Mitochondrial membranes are not unlike other cell membranes in that they contain integral membrane proteins and phospholipids (Lehninger, Nelson et al. 1993), and are a good representation of the fluid mosaic model structure of a cell membrane (Singer and Nicolson 1972). The outer mitochondrial membrane contains porins, barrel proteins that cross the cellular membrane forming pores to make the outer membrane permeable to small molecules of less than 10 kDa. The outer membrane also contains enzymes involved in oxidation-reduction (*'redox'*) reactions as well as fatty acid metabolism (Lehninger, Nelson et al. 1993).

The inner mitochondrial membrane is highly sophisticated and contains all complexes of the electron transport chain (ETC), the ATP synthase complex and various other transport proteins (Scheffler 1999). Unlike the outer membrane, the inner mitochondrial membrane is impermeable to most molecules except O₂, CO₂ and H₂O, which can diffuse directly across the membrane (Scheffler 1999). The inner membrane is especially impermeable to ions such as H⁺ and Na⁺; hence separate pools of these ions are found in both the extracellular (cytoplasm) and intracellular (mitochondrial matrix) spaces (Sherratt 1991).

The inner membrane contains a high percentage (almost 75%) of protein (Scheffler 1999). The proteins of the inner mitochondrial membrane include specific transport proteins that transport substances such as pyruvate, ATP, ADP, glutamate,

malate and citrate (malate shuttle) and fatty acids across the membrane. It also contains the ETC proteins that comprise complexes I-IV (Figure 2.6) and ATP synthase (Hatefi 1985), sometimes referred to as complex V or F_0F_1 ATPase.

Not unlike the inner membrane, the innermost mitochondrial matrix is also very rich in proteins and contains many enzymes such as those from the Krebs cycle and the biochemical intermediates involved in the production of ATP.

One of the most important discoveries of the last century in the understanding of mitochondrial structure and function is that mitochondria are unique in comparison to most cells in that they contain their own DNA - mitochondrial DNA (mtDNA) (Nass and Nass 1963). According to the endosymbiosis theory, this is thought to be the result of two separate evolutionary origins, whereby the mitochondria evolved from aerobic prokaryotic bacteria living in symbiosis with an anaerobic eukaryotic host cell resulting in a cell/organism with two DNA (nuclear and mitochondrial) coding regions (Margulis 1975). It is also thought that the genome of primordial mitochondria was too small to sufficiently maintain a free-living organism and this was compensated for by the fusion of mtDNA and nuclear DNA (Schaffer 2007).

Mitochondrial DNA is circular and contains 37 genes; 13 genes that encode for peptides of the ETC and 24 genes that encode for ribonucleic acid (RNA; ribosomal RNA 2, transfer RNA 22) containing about 16,500 base pairs (Anderson, de Bruijn et al. 1982), which is used to synthesize certain mitochondrial proteins. However, most mitochondrial proteins are encoded for by nuclear DNA (Smeitink, van den Heuvel et al. 2001) (see Figure 2.6).

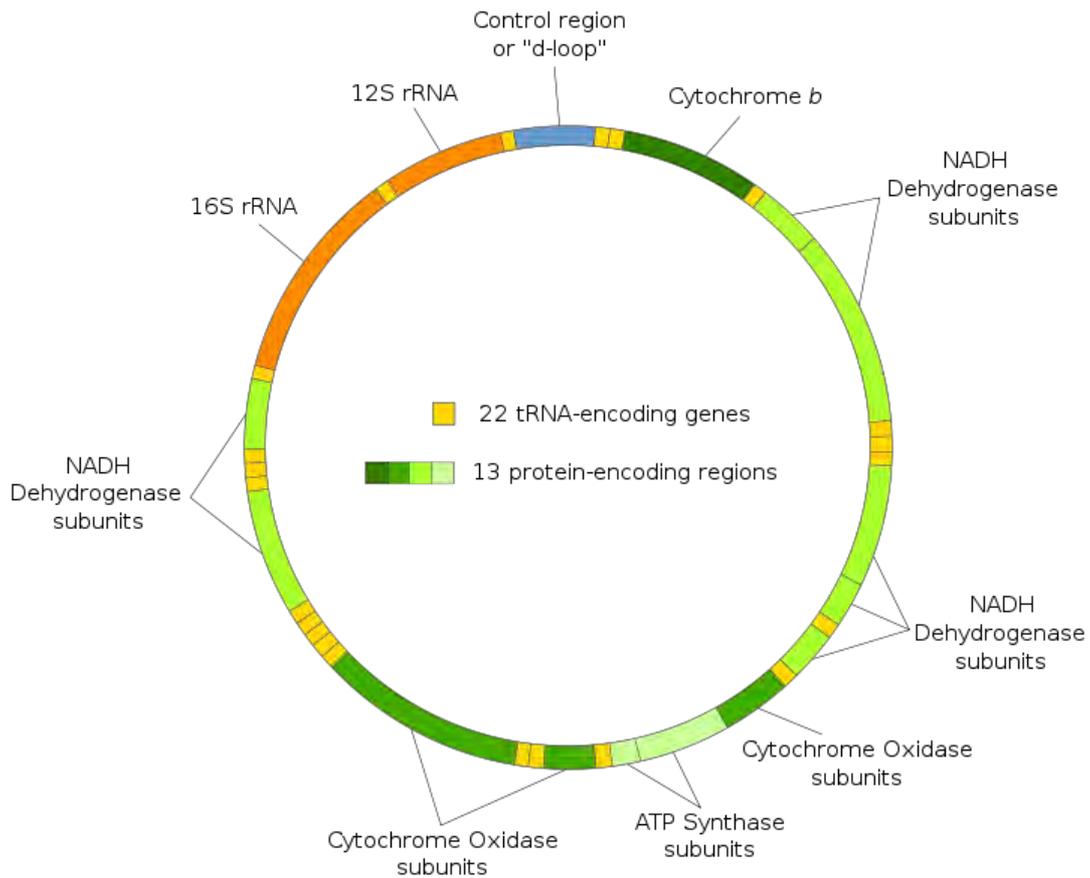


Figure 2.6: Structure of the mitochondrial genome

It is important to note that, due to the proximity of mtDNA to the ETC, mtDNA is particularly susceptible to reactive oxygen species (ROS) and therefore, also particularly susceptible to oxidative damage (Arnheim and Cortopassi 1992). It has been suggested that oxidative damage and mutations of mtDNA are directly responsible for many genetic diseases (myopathies) (Holt, Harding et al. 1988), with the accumulation of defects that comes from damage to mtDNA being a major contributor to the aging process (Arnheim and Cortopassi 1992; Cortopassi, Shibata et al. 1992; Alexeyev, Ledoux et al. 2004). This will be discussed in greater detail in Section 2.6.2.

2.4.2 Mitochondrial Function

Mitochondria are sometimes described as "cellular power plants" because they generate most of the cell's supply of ATP, used as a source of chemical energy. In addition, mitochondria are involved in numerous metabolic processes including the biosynthesis of amino acids, vitamin cofactors, fatty acids, and iron-sulfur clusters (Henchcliffe and Beal 2008). Mitochondria are also the main cellular source of free radicals; hence they are also integral to the oxidative stress response.

The principal oxidative processes that take place in the mitochondria include:

- Conversion of pyruvate to acetyl-coenzyme A (acetyl-coA)
- Conversion of acetyl-coA to CO₂, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (hydroquinone form) (FADH₂) and ATP via the Krebs cycle
- NADH and FADH₂ release electrons into the ETC to form ATP and H₂O via oxidative phosphorylation
- Control of oxidative phosphorylation via the phosphocreatine shuttle.

2.4.2.1 Metabolic Synthesis of ATP

2.4.2.1.1 Anaerobic Metabolism

In the absence of oxygen (anaerobic conditions), ATP can be produced from glucose stored within glycogen (in a process known as *glycogenolysis*) or from its freely available monosaccharide form, glucose (in a process known as *glycolysis*).

Glycogenolysis is the breakdown of glycogen to release the potential energy contained within glucosyl units. By contrast, glycolysis is the conversion of glucose

into glucose-6-phosphate (see Figure 2.7) via hexokinase before being degraded into two units of pyruvate.

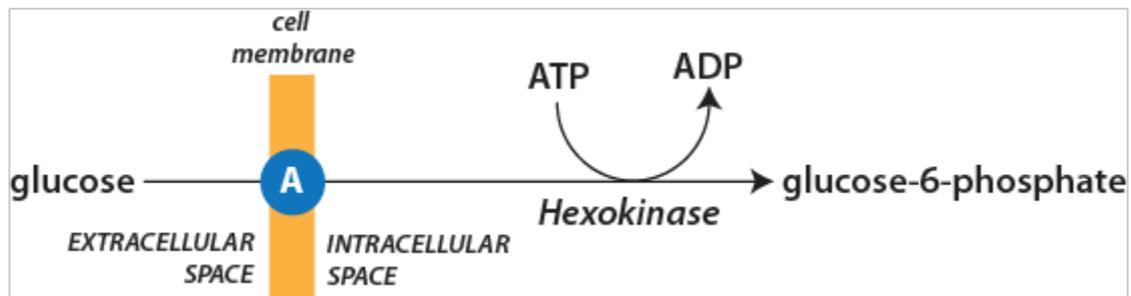


Figure 2.7: the transport of glucose across the cell membrane via GLUT-1 transporter (A) and conversion to glucose-6-phosphate via hexokinase in skeletal muscle.

Glycolysis involves ten steps whereby glucose is broken down into two molecules of pyruvate with two ATP and two $\text{NADH} + \text{H}^+$ moieties being produced in the process (Figure 2.8).

In the absence of oxygen, pyruvate is converted to lactic acid by the enzyme lactate dehydrogenase (LDH). The concentration of lactic acid or lactate in the muscle is therefore often used as a measure of the muscle's glycolytic rate (Bangsbo, Graham et al. 1992). In the presence of oxygen, pyruvate is converted into acetyl coA via the pyruvate dehydrogenase (PDH) reaction, which can then enter the Krebs cycle located in the mitochondrial matrix.

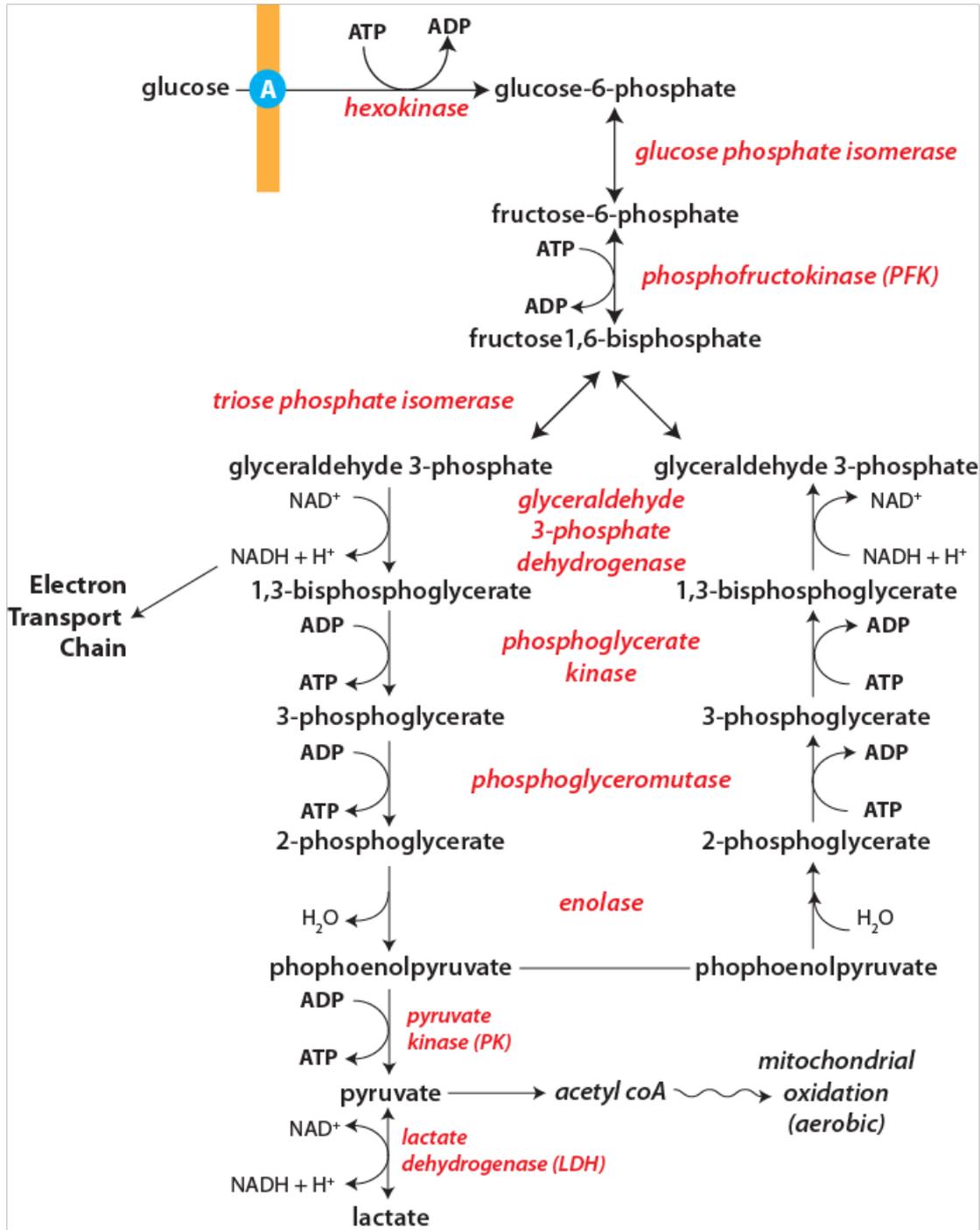


Figure 2.8: Anaerobic glycolysis and glycogenolysis

2.4.2.2 *The Krebs Cycle*

The Krebs cycle is a common catabolic pathway for the breakdown products of carbohydrate, amino acid and fat metabolism. The main function of the Krebs cycle is the oxidation of acetyl residues derived from the PDH reaction and fatty acid β -oxidation to produce the electron carriers NADH, FADH₂, CO₂ and ATP (Krebs and Johnson 1937).

The Krebs cycle is of central importance in the mitochondria of all living cells that use oxygen as part of cellular respiration. As mitochondrial respiratory function declines with age, an understanding of the Krebs cycle allows us to assess the impact of age-related changes to the cycle on cellular respiration. Furthermore, it is important that we understand the structural and functional changes that occur in the mitochondria as we age, including changes in mitochondrial enzyme activity, oxidative capacity and ATP production rate (MAPR). This knowledge provides an insight into the impact of aging on muscular structure and function.

There are eight enzyme intermediates involved in a total of ten steps in the cycle; starting from citrate and finishing at oxaloacetate (see Figure 2.9).

2.4.2.3 *Substrate Oxidation*

Living cells are reliant on a constant supply of energy yielding substrates (such as carbohydrates, proteins, and fat) that are supplied by the diet. These energy-yielding substrates often cannot be utilized directly and need to be first broken down into their simple forms by the digestive system.

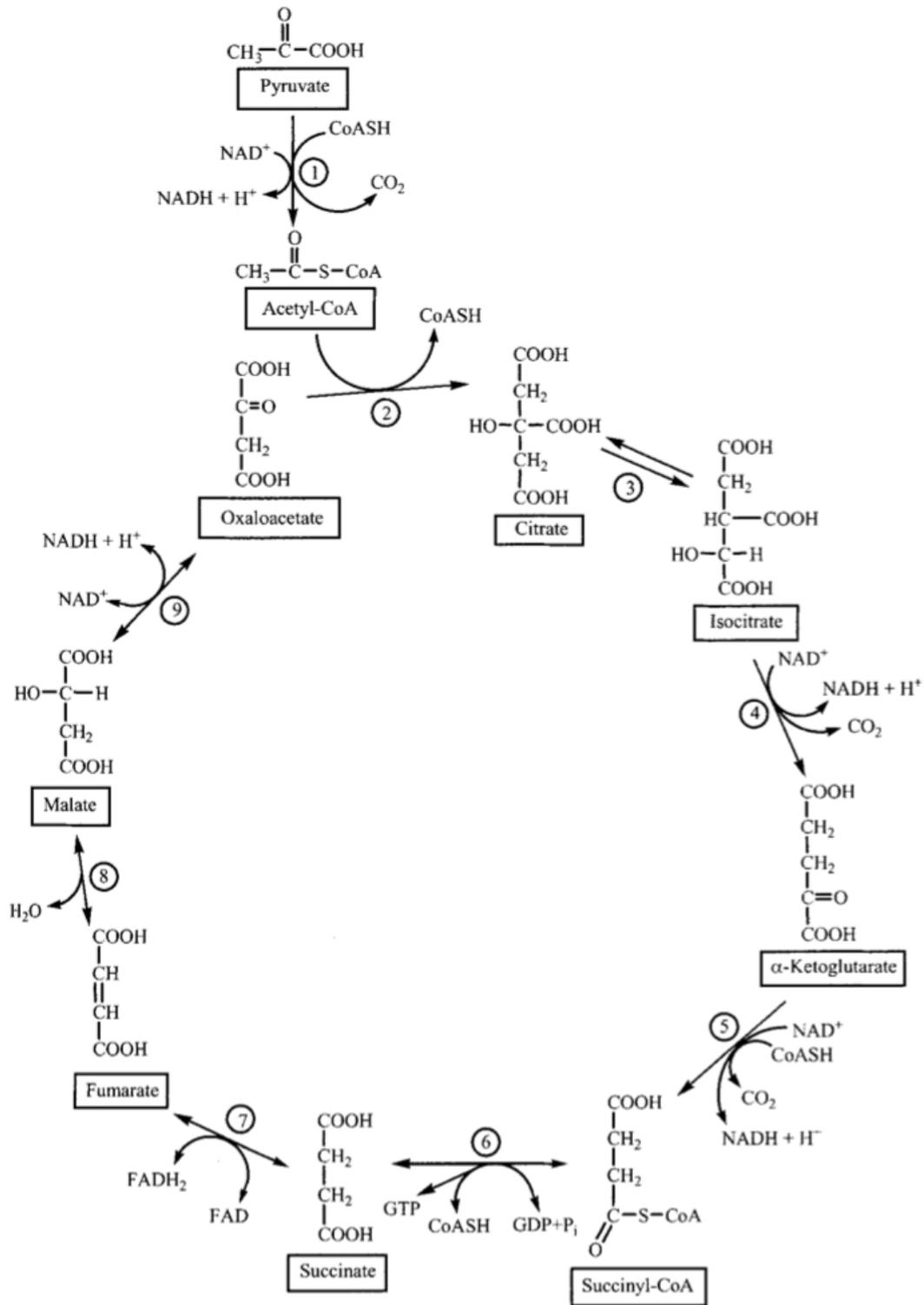


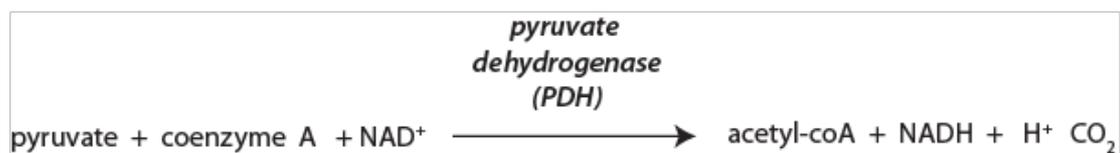
Figure 2.9: The Krebs cycle (taken from Figure 5.4 page 83, Medical Biochemistry: Human Metabolism in Health and Disease)

One of the major roles of the mitochondria is to convert energy-yielding substrates, via oxidative degradation, into smaller molecules which can be further transformed into the energy equivalents, NADH and FADH₂, via the Krebs cycle and β-oxidation (Scheffler 1999). NADH and FADH₂ release electrons that are carried along protein complexes (complexes I-IV), setting up an electrochemical gradient that drives the oxidative phosphorylation of ADP to produce ATP and H₂O (Scheffler 1999) (see Section 2.4.1).

2.4.2.3.1 Carbohydrates

Glucose enters the blood stream where it is taken up by the cell and converted into energy or stored as glycogen. Before glucose can be converted into energy, it must first be converted into pyruvate by anaerobic metabolism (see Section 2.4.2.1).

Pyruvate dehydrogenase (PDH) catalyses the decarboxylation of pyruvate (in the presence of coenzyme A) into acetyl-coA, NADH, H⁺ and CO₂ (Lehninger, Nelson et al. 1993; Scheffler 1999), according to the equation shown below (Denton and Halestrap 1979; Scheffler 1999):



Under aerobic conditions, the acetyl-coA produced in the above reaction enters the Krebs cycle (see Section 2.4.2.2).

2.4.2.3.2 Amino Acids

Like pyruvate, amino acids can also be degraded into acetyl-coA and, hence, enter the Krebs cycle. Additionally, amino acids can also enter the Krebs cycle at different intermediate steps (α -ketoglutarate, succinyl-coA, fumarate, and oxaloacetate) via a process called transamination (Goodman and Lowenstein 1977).

2.4.2.3.3 Fats

After entering the blood stream and entering the cell, fatty acids are made available in the cytosol before being activated by binding to coA (Scheffler 1999).

The activation process can be shown as follows:



Following activation, a shuttle system assists in transportation of fatty acids across the mitochondrial membrane (outer and inner membranes) and into the mitochondria, as shown in Figure 2.10 (McGarry and Foster 1980; Zammit 1983); (Groot, Scholte et al. 1976; Scheffler 1999).

Once activated, the breakdown of the fatty acids continues in the mitochondrial matrix via a series of oxidative reactions referred to as β -oxidation. β -oxidation involves the oxidative removal of two carbon (C_2) units from the β -carbon position of the fatty acid/acetyl-CoA; hence the name β -oxidation.

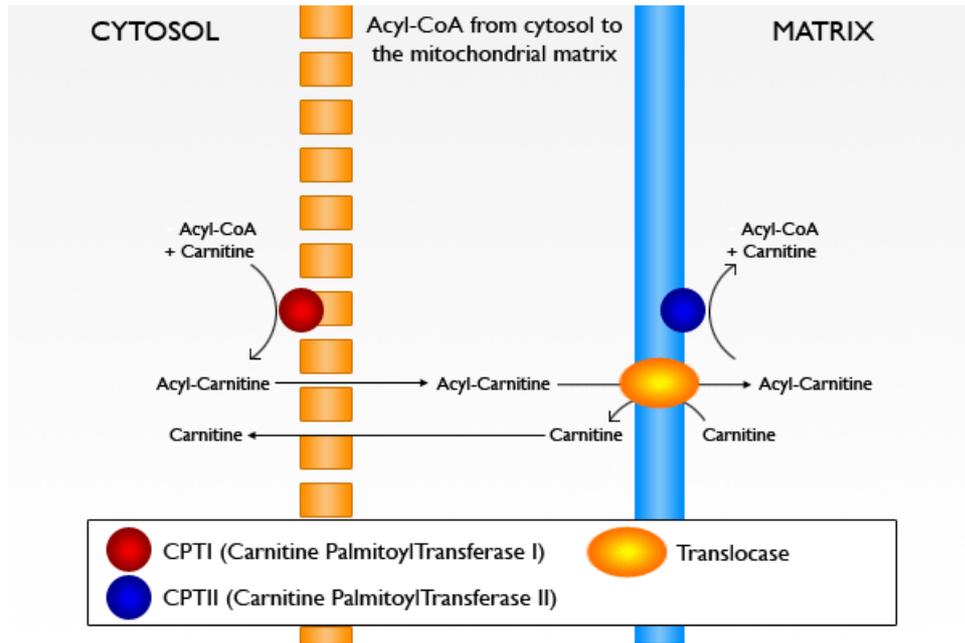


Figure 2.10: Carnitine palitoyltransferase shuttles

The two-carbon β -oxidation of fatty acids involves four steps (dehydrogenation, hydration, oxidation and thiolysis) (Schulz 1991). The four steps are summarized in Figure 2.11.

2.4.2.4 Aerobic Metabolism

Under aerobic conditions, most of the ATP is generated via oxidative phosphorylation (see Section 2.4.2.4.1). The pyruvate molecules produced in glycolysis are transported in the mitochondrial matrix where they are converted to acetyl-coA molecules via the PDH complex. The acetyl-coA molecules are then transferred into the Krebs (or citric acid) cycle where they are oxidized to form CO₂.

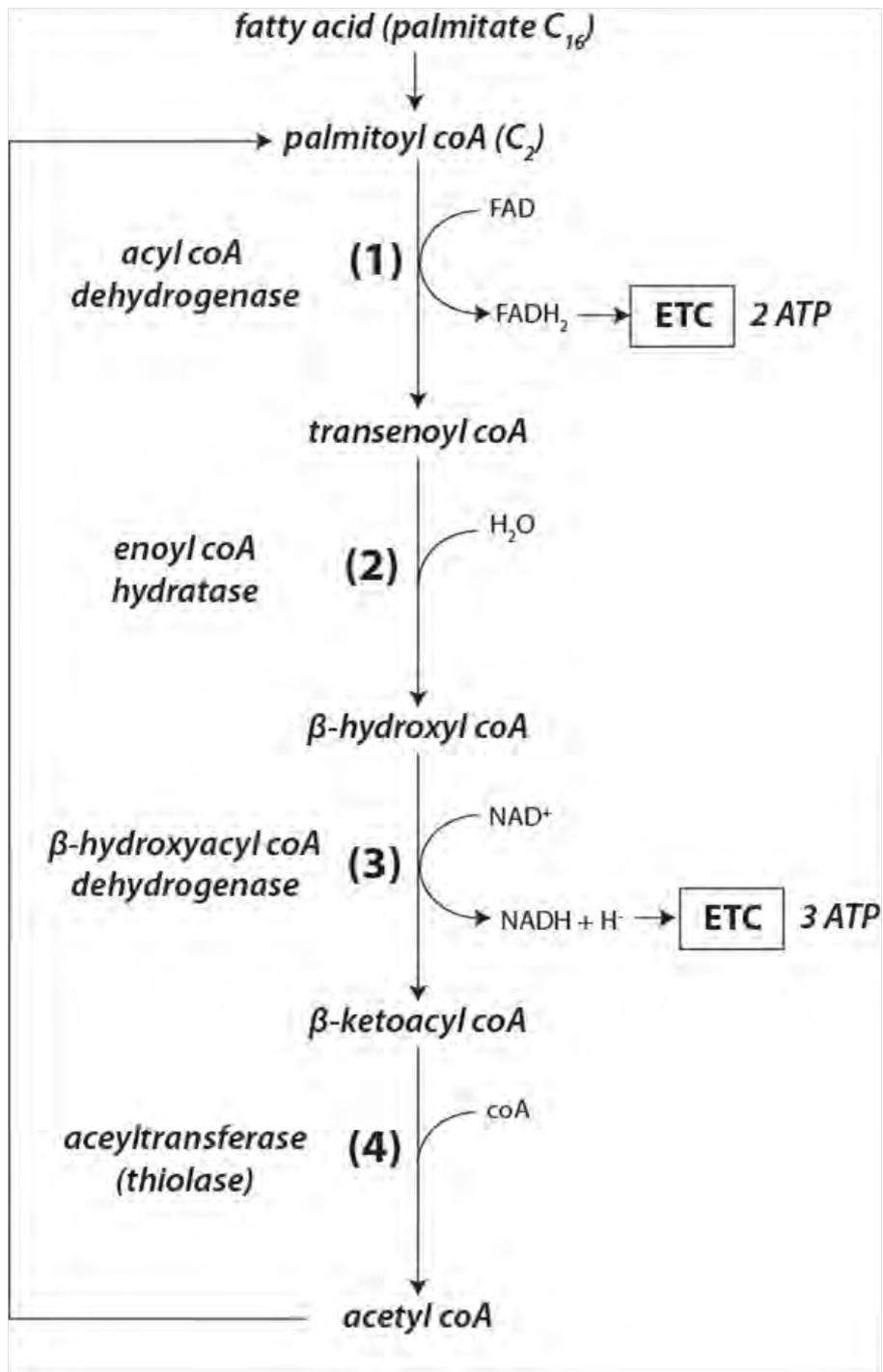


Figure 2.11: β -oxidation of fatty acids (palmitate). Adapted from (Gropper, Smith et al. 2005) p152

In anaerobic metabolism, NADH and FADH₂ cannot be reoxidized. This increases the NADH concentration, inhibiting ATP synthesis and the PDH reaction. Hence, the cell becomes reliant on ATP synthesis from glucose via glycolysis (see above). In aerobic metabolism, however, reoxidation of NADH and FADH₂ occurs via the coenzymes FAD and NAD, which form part of the PDH enzyme complex.

The NADH and FADH₂ produced in this reaction (see reaction above) can be used to release electrons into the ETC driving the synthesis of ATP via a process called oxidative phosphorylation.

2.4.2.4.1 Oxidative Phosphorylation and the Electron Transport Chain

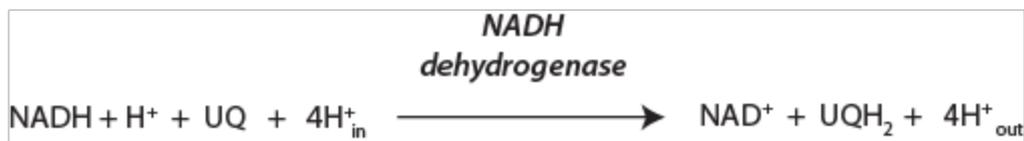
The process of oxidative phosphorylation is highly exergonic and involves a series of redox reactions whereby electrons are transferred from reduced coenzymes such as NADH and FADH₂ to O₂-producing H₂O (Scheffler 1999). According to the chemiosmotic theory, the energy released from these redox reactions is used to establish an electrochemical gradient that drives the synthesis of ATP via ATP synthase (see Section 2.4.2.4.2).

The enzymes of the Krebs cycle (except SDH) and β -oxidation are located within the mitochondrial matrix. The NADH + H⁺ and FADH₂ produced in the Krebs cycle and β -oxidation are oxidized by dehydrogenase enzymes to release H⁺ and electrons.

Electrons are carried along the ETC. The protein complexes that form the ETC are integral proteins located in the inner mitochondrial membrane and include complex I (NADH dehydrogenase), complex III (cytochrome c reductase) and complex IV (cytochrome c oxidase). Complex II (succinate dehydrogenase) is also

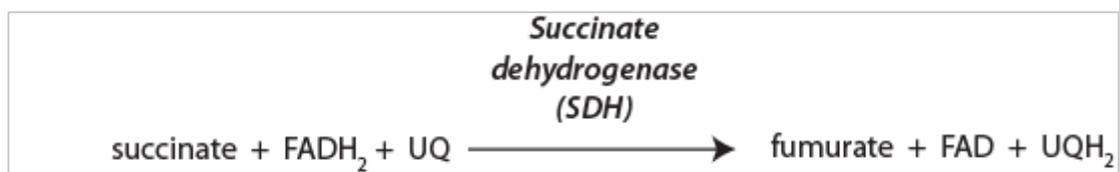
located in the inner mitochondrial membrane; however it is not generally considered part of the ETC as it only serves to channel electrons into the ETC by removing them from succinate (see Section 2.4.2.4.1) and transferring them onto ubiquinol (UQ) via FADH₂ (Weiss, Friedrich et al. 1991).

Complex I catalyzes the transfer of two electrons from NADH + H⁺ as shown in the following equation (Weiss, Friedrich et al. 1991):

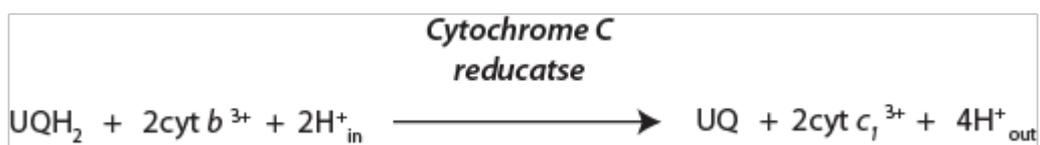


The above reaction generates a proton gradient across the membrane, which is coupled to an electron gradient to form an electrochemical gradient that drives ATP synthesis.

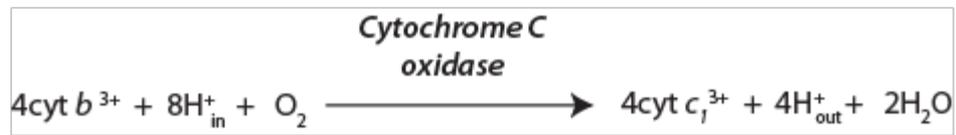
Complex II catalyses the transfer of electrons from succinate to FADH₂ (in the Krebs cycle), as shown in the following reaction (Hatefi 1985):



Complex III acts according to the equation shown below (Hatefi 1985):



Finally, complex IV catalyses the final transfer of electrons from cytochrome c to molecular O₂, to form H₂O (Hatefi 1985). The equation for this reaction is as follows:



The formation of H₂O from O₂ drags more protons across the membrane, once again contributing to the proton gradient which is coupled to, and drives, the synthesis of ATP via ATP synthase (Mitchell and Moyle 1967). ATP synthase (also called F₀F₁ ATPase or complex V) catalyses the oxidative phosphorylation of ADP to P_i to form ATP (Hatefi 1985) (see Figure 2.12).

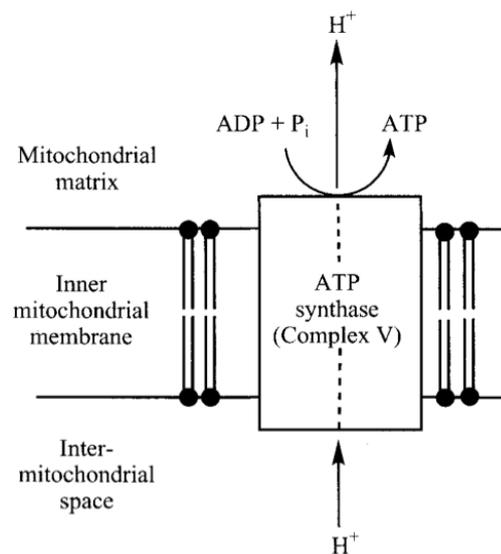


Figure 2.12: ATP synthase. A schematic diagram showing the production of ATP via ATP synthase that occurs as a result of H⁺ ions being pumped back into the mitochondrial matrix against their concentration gradient. Taken from Medical Biochemistry: Human Metabolism In Health and Disease, figure 6.4 page 94 (D. Rosenthal and H. Glew 2009).

2.4.2.4.2 ATP synthesis

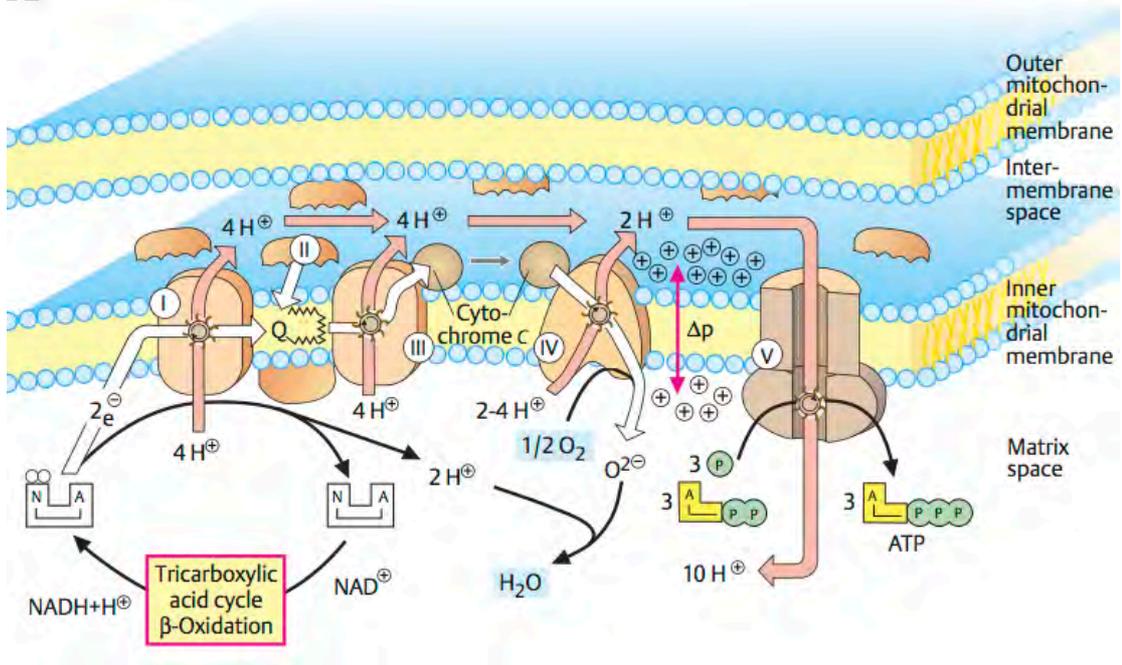
The energy released from the flow of electrons through complexes I, III and IV is used to pump protons from within the mitochondrial matrix and into the intermembranous space (Mitchell 1961; Mitchell and Moyle 1967). When the positively charged protons are pumped from within the mitochondrial matrix, a net negative charge (or potential; Ψ) results in the mitochondrial matrix. This sets up an electrical gradient across the inner mitochondrial membrane. The negative membrane potential (Ψ) and chemical gradient contributes to the proton motive force (Δp). The amount of energy stored within the proton gradient is dependent on the difference between the concentration of protons in the mitochondrial matrix and in the intermembranous space.

The fundamental step in this process is the passive diffusion of protons back into the mitochondrial matrix from the intermembranous space along their chemical gradient via ATP synthase (F_0F_1 ATPase). The proton-transporting channel of ATP synthase (F_0) utilizes the energy stored in the electrochemical gradient to drive the oxidative phosphorylation of ADP and P_i to ATP via the catalytic unit (F_1) of ATP synthase (Mitchell 1961) (Figure 2.13).

2.4.2.4.3 The Phosphocreatine Shuttle

The traditional model for control of ATP synthesis via oxidative phosphorylation has been postulated to be regulated through feedback of cytosolic ADP and inorganic phosphate (P_i) (Chance and Williams 1955). The mechanism by which this feedback occurs in isolated mitochondria appears to be muscle specific. Although ADP acts to regulate oxidative metabolism, the supply of ADP for oxidative

A



B

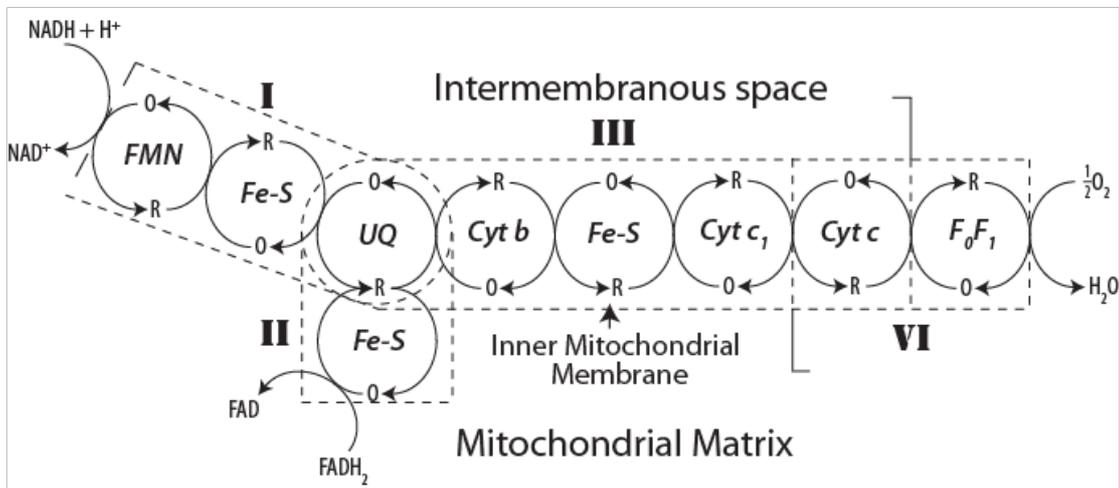


Figure 2.13: The electron transport chain. A) The electron transport chain (ETC) (complexes I-IV) of the inner mitochondrial membrane. Taken from (Koolman and Röhm 2005) p141, B) a schematic diagram of the redox reactions that take place in the ETC. Adapted from (Gropper, Smith et al. 2005) figure 3.16 p67.

phosphorylation is different. For example, in glycolytic muscle, ADP diffuses directly across the outer mitochondrial membrane into the mitochondrial matrix via an adenine nucleotide translocase (ANT, see Figure 2.14) where the Michaelis-Menton constant, (K_m) of mitochondrial respiration for ADP is low (Kuznetsov, Tiivel et al. 1996). K_m is equal to the substrate concentration at which the velocity of the reaction is at half its maximum (V_{max}). K_m provides a measure of the affinity of an enzyme, or enzyme system, for its substrate. The lower the K_m , the greater the affinity of an enzyme or enzyme complex for its substrate.

In contrast, the mechanism by which control of respiration occurs in oxidative muscles is somewhat different. The permeability of outer mitochondrial membrane to adenine nucleotides, such as ADP, in oxidative muscle *in vivo*, is low (Saks, Khuchua et al. 1994). Unlike glycolytic muscle, the apparent K_m of mitochondrial respiration for ADP in oxidative muscle is initially high but decreases dramatically in the presence of Cr (Kuznetsov, Tiivel et al. 1996). Hence, the control of mitochondrial respiration in oxidative muscle is less reliant on cytosolic ADP and is influenced more by intracellular Cr concentration.

This process is explained by the so-called '*phosphocreatine shuttle*' (Figure 2.14), first proposed by Bessman and Savabi (1988).

In the first part of the shuttle, phosphocreatine (PCr) is consumed in the myofibrillar compartment by the creatine kinase (CK) reaction in rephosphorylating ADP produced by myosin ATPase during muscle contraction. This causes an increase in intracellular Cr concentration.

In the second part of the shuttle, the Cr diffuses toward the mitochondria within the muscle where it is rephosphorylated to PCr by mitochondrial CK (mi-CK), attached to the outside of the inner mitochondria membrane, using ATP produced in the mitochondria as the substrate. This increases the concentration of ADP within the mitochondria, thereby stimulating oxidative phosphorylation. Therefore, the ‘phosphocreatine shuttle’ model hypothesizes that it is the ADP produced in the mitochondria by the mi-CK that controls mitochondrial respiration. Hence the control of respiration in oxidative muscles is quite different to that in glycolytic muscles, and appears to be under the control of mi-CK.

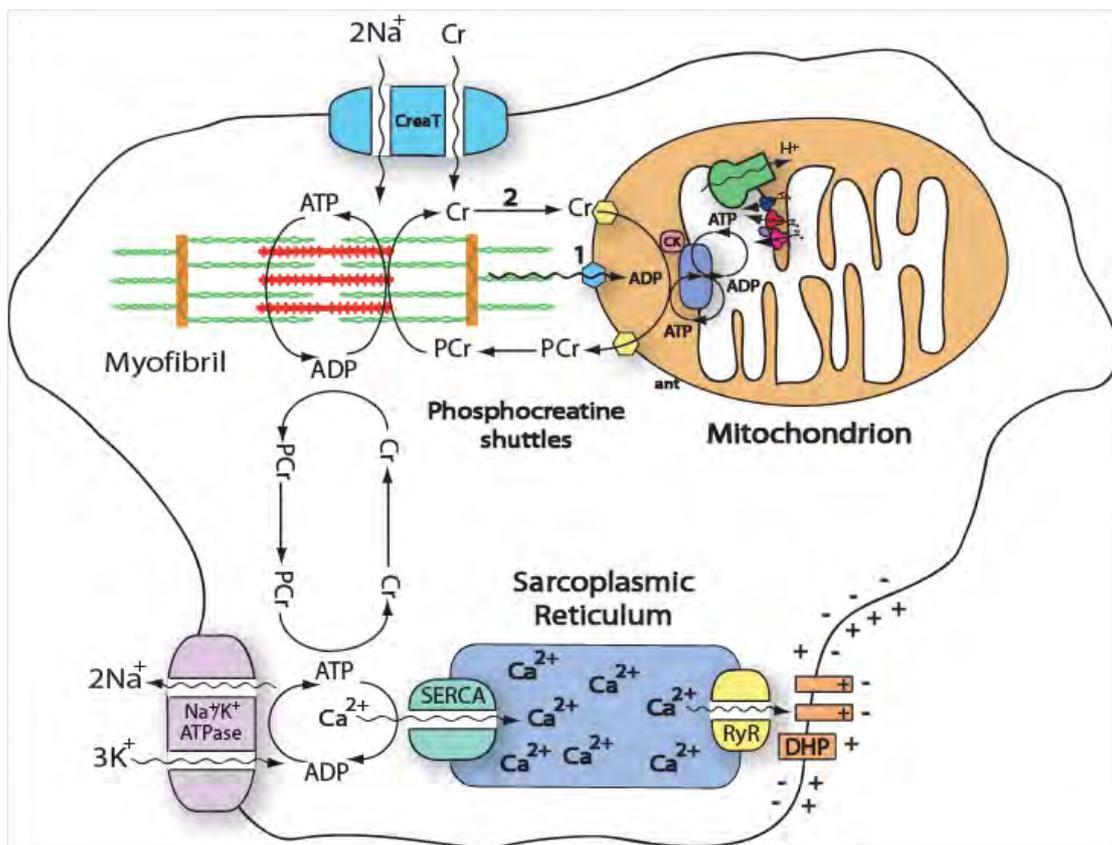


Figure 2.14: The phosphocreatine shuttle

This model has been interpreted by others (Wallimann, Wyss et al. 1992; Guerrero-Ontiveros and Wallimann 1998; Saks, Kongas et al. 2000) as indicating a possible mechanism whereby increased intracellular Cr levels could increase oxidative phosphorylation in oxidative muscle by decreasing the apparent K_m of mitochondrial respiration for ADP. Increasing the intracellular Cr concentration is possible by supplementing the diet with Cr monohydrate. This will be discussed in more detail in Section 2.9.2.

2.4.3 Assessment of Mitochondrial Function

As mitochondrial function changes as we age, it is important that we have tools at our disposal to measure accurately different aspects of mitochondrial function and follow the changes through the aging process.

There are a number of methods that can be employed to measure mitochondrial function, such as determination of oxidative enzyme activity, mitochondrial respiration, or mitochondrial ATP production rate (MAPR).

Muscle fibre type is also an indicator of the oxidative capacity of the muscle. For example, type I oxidative fibres, have a high oxidative capacity due to their high mitochondrial density, compared with that of glycolytic fibres (Jackman and Willis 1996). Glycolytic fibres, however, have a much lower mitochondrial density and hence oxidative capacity (Jackman and Willis 1996) (see Table 1). Therefore, histochemical staining of muscle sections to determine muscle fibre type can also assess the oxidative capacity of muscle. However this is not a very quantitative method in comparison to other methods discussed below.

2.4.3.1 Mitochondrial preparation

Until recently, most of the research on mitochondria and mitochondrial dysfunction was conducted using mitochondrial tissue prepared from animals (Saks, Kapelko et al. 1989; Wibom, Lundin et al. 1990). This is because it was not always possible to obtain sufficiently large (>1 g of muscle) from human tissue to effectively measure mitochondrial function without some form of invasive surgery. To overcome the complications involved with open surgery biopsies, Rasmussen and colleagues (1997) developed a needle biopsy method to isolate mitochondria from only a small amount of skeletal muscle (25–100 mg) that can successfully be used to measure mitochondrial enzymatic activity and ATP synthesis (Rasmussen, Andersen et al. 1997) (see Chapter 3, Section 3.9.1)

2.4.3.2 Oxidative Enzymes

A number of studies have used the activities of various enzymes involved in oxidative metabolism as a measure of mitochondrial function (Blomstrand, Radegran et al. 1997; Wang, Williams et al. 1999; Carey, Williams et al. 2000). Enzymes measured in such studies include the Krebs cycle enzymes, citrate synthase (CS) (Blomstrand, Radegran et al. 1997; Wang, Williams et al. 1999; Carey, Williams et al. 2000), succinate dehydrogenase (SDH) (Blomstrand, Radegran et al. 1997), oxoglutarate dehydrogenase (OGDH) (Blomstrand, Radegran et al. 1997; Wang, Williams et al. 1999), as well as β -hydroxyacyl-coA dehydrogenase (β -HAD) (Wang, Williams et al. 1999), an enzyme involved in the β -oxidation of fatty acids.

The activity of ETC complexes (complexes I-IV) can also be measured spectrophotometrically/fluorometrically by isolating the respiratory enzyme of

interest in mitochondrial preparations and quantifying the activity of specific enzymes. For example, rotenone is a potent inhibitor of respiratory complex I (NADH dehydrogenase) and is often used to determine the activity of specific NADH oxidation reactions that occur through complex I (Chretien, Bourgeron et al. 1990; Wibom, Lundin et al. 1990).

Whilst these methods are useful in assessing mitochondrial oxidative capacity, they tend to be limited because they measure specific mitochondrial processes rather than overall mitochondrial function.

2.4.3.3 Oxygen Consumption

Other studies have used the determination of oxygen consumption as a measure of mitochondrial function (Chance and Williams 1955). This method assesses the rate of ADP-stimulated oxygen consumption either in whole tissues (Chance and Williams 1955) or in isolated mitochondria (Guerrero-Ontiveros and Wallimann 1998). The usefulness of this method is also limited because the electron transfer may not be completely coupled to the synthesis of ATP, as protons may leak back into the mitochondrial matrix across the inner membrane, rather than through ATP synthase. This process is termed 'proton leak' (Mitchell and Moyle 1967), and has been shown to occur in a number of tissues including skeletal muscle (Rolfe and Brand 1996).

The use of mitochondrial oxygen consumption as a measure of mitochondrial function may therefore be limited by the fact that not all the ATP produced is coupled to electron transport and oxygen consumption. This is quite likely in a number of

disease states, and may also be true in the elderly as inefficiencies and mtDNA changes begin to occur.

2.4.3.4 Mitochondrial ATP Production Rate (MAPR)

Other measures of aerobic metabolism have been based on the luminometric measurement of light emitted by a firefly luciferase reagent to measure ATP production (Lemasters and Hackenbrock 1973). The bioluminescent properties of firefly luciferase have been widely used in the measurement of ATP content in various tissues (Strehler and Totter 1952; Lemasters and Hackenbrock 1973; Lundin, Richardsson et al. 1976) including skeletal muscle (Wibom, Lundin et al. 1990; Wibom, Hultman et al. 1992; Wang, Williams et al. 1999) because the intensity of the light emitted (measured bioluminometrically at 560 nm) is proportional to the amount of ATP in the tissue (Wibom and Hultman 1990; Wibom, Lundin et al. 1990) (see Figure 3.9 in Chapter 3)

The MAPR method used by Wibom et al. (1992) is a reliable index of muscle oxidative capacity, as it is not concerned with oxygen consumption and thus proton leak, but rather the direct measurement of ATP production by the mitochondria. In addition, the use of different substrate combinations to measure MAPR provides a good reflection of ATP-production rates from different metabolic pathways representing carbohydrate, fat and amino acid metabolism (Wibom, Lundin et al. 1990).

When the different methods available for the assessment of mitochondrial function, and hence muscle oxidative capacity, are compared (oxidative enzyme activity, oxygen consumption as a measure of mitochondrial respiration, and MAPR),

it becomes clear that MAPR is the most reliable and effective method with the fewest drawbacks. It is for this reason that MAPR was chosen as the method for measurement of muscle oxidative capacity in two of the studies reported herein.

2.4.4 Mitochondria: The Double Edged Sword

Mitochondria are the primary intracellular site of oxygen consumption. However, it is estimated that approximately 1–2% of this oxygen is naturally converted into the ROS superoxide (O_2^-) as a result of leakage that occurs as a by-product of normal metabolism (Sohal and Weindruch 1996). Superoxide can also be generated by UV radiation from the sun, xanthine oxidase, lipoxygenases as well as from leakage of electrons from cytochrome P450 reductase (Kamata and Hirata 1999).

ROS play an important role in cell signaling (Kamata and Hirata 1999; Droge 2002; Jackson 2005; Jackson 2008; Jackson 2008), however ROS can cause significant cellular damage in times of stress (a process known as *oxidative stress*), especially when ROS production by the cell is significantly increased (Kong and Davison 1980; Jaeschke 1995; Droge 2002). Thus, the mitochondria, which are the source of many ROS, may be considered a ‘double-edged sword’ – fulfilling vital biochemical roles, while generating potentially destructive ROS.

The primary ROS produced in the mitochondria are O_2^- (superoxide radical), H_2O_2 (hydrogen peroxide), and $OH\cdot$ (hydroxyl radical) (Droge 2002). In general, the superoxide radical is not all that reactive and is formed when O_2 is reduced by another electron to form O_2^- , often via a leak occurring at complex I and complex III in the respiratory chain (Turrens 2003). The majority of the superoxide produced via

complexes I and III is converted into H_2O_2 by superoxide dismutases (SODs) (Beyer 1990; Turrens 2003). Superoxide can also be released into the inner-mitochondrial membrane; however it is usually quickly re-oxidized into O_2 by cytochrome *c* (Turrens 2003) and then subsequently oxidized via complex IV, contributing to overall ATP production.

Inhibitors of complex III, such as antimycin A, can also contribute to the production of superoxide by blocking the transfer of electrons to cytochrome *c*, which can leak onto O_2 to form the superoxide radical.

Hydrogen peroxide (H_2O_2), whilst not technically a ROS because it does not have any unpaired electrons, can diffuse throughout cells and cause damage by reacting with reduced metals such as iron (Fe^{2+}) to form the highly reactive hydroxyl radical (OH^\cdot). H_2O_2 is produced by a SOD that catalyzes the conversion of superoxide into H_2O_2 from O_2 . Extracellular and cytosolic SOD is sometimes referred to as CuSOD and ZnSOD, because it is functionally dependant on copper and zinc (Tainer, Getzoff et al. 1983), respectively, whereas mitochondrial SOD is often called MnSOD because it is dependant on manganese (Borgstahl, Parge et al. 1992). Hence, these minerals (Cu, Zn, and Mn) play an important biological role as cofactors that assist in the quenching of ROS.

H_2O_2 can also be processed by glutathione peroxidase (GPx) whereby there is complete reduction of O_2 by the addition of four electrons to yield two molecules of water (H_2O) – a stable compound that is no longer referred to as a ROS (Figure 2.15).

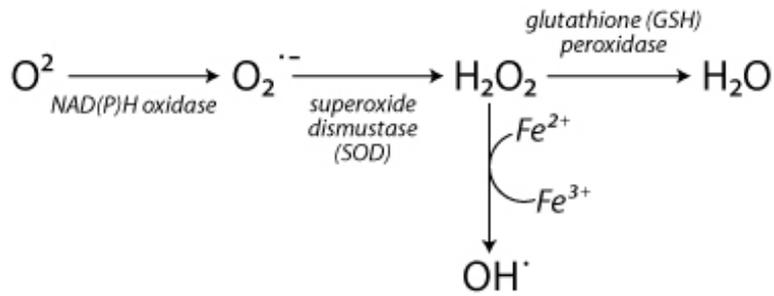


Figure 2.15: Production of reactive oxygen species (ROS) from oxygen

As organelles, mitochondria are unique in that they contain their own DNA genome (Cottrell and Turnbull 2000; McKenzie, Bua et al. 2002), with their proteins encoded for by both nuclear DNA as well as by mitochondrial DNA (mtDNA). However, mtDNA does not appear to have an efficient protection and repair mechanism when compared with nuclear DNA (Beckman and Ames 1998). Additionally, mtDNA appears to be more prone to oxidative damage than nuclear DNA due to its proximity to the source of ROS (Stevnsner, Thorslund et al. 2002; Shokolenko, Venediktova et al. 2009).

Some of the damage caused by ROS is irreversible, and over time there can be accumulation of damaged cells and mutated DNA (Mecocci, Fano et al. 1999) that changes the cells structure, affecting cell function. In this regard, ROS-associated damage to cell organelles such as the mitochondria is likely to be an important factor contributing to a reduced muscle oxidative capacity and function with age. In fact, it has been shown that the number of mtDNA mutations and deletions increase with age (McKenzie, Bua et al. 2002) (see Section 2.4.4).

Mutations and deletions can decrease the functional size of the mtDNA genome (McKenzie, Bua et al. 2002). If this smaller functional genome were replicated, it would result in either the decreased production of desired products or the increased production of dysfunctional proteins. This effect would be especially relevant in post-mitotic cells, such as skeletal muscle. Such changes are thought to be a possible mechanism underlying decreases in protein metabolism with age (Rooyackers, Adey et al. 1996; Conley, Jubrias et al. 2000). However, the effects of increasing ROS-associated damage on muscle oxidative capacity with age have not been widely researched to date. This will be discussed in more detail in Section 2.6.2.3.3.

2.4.4.1 Mitochondrial Antioxidant Systems

The harmful effects of ROS are generally mitigated by the different antioxidant systems present in the mitochondria. To protect themselves from the damaging effects of ROS, mitochondria have evolved to possess elaborate defense mechanisms that can be classified into two major categories:

- Antioxidant enzyme systems such as Mn²⁺-dependent superoxide dismutase (MnSOD), copper/zinc (CuZnSOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT), and
- Non-enzymatic water and lipid soluble radical scavengers that include vitamin C, Vitamin E (tocopherols), flavonoids, caretenoids, ubiquinol (CoQ10) and glutathione (GSH).

The SOD metalloenzymes convert the superoxide to water following a series of enzymatic reactions (Fridovich 1995). The role of cytochrome *c* is also considered

vital in this process. The electrons that leak out of the ETC react with cytochrome *c* to reduce it while regenerating oxygen in the process (Butler, Jayson et al. 1975).

Non-enzymatic, hydrophilic radical scavengers such as ascorbate (vitamin C) and glutathione (GSH) as well as lipophilic scavengers such as tocopherols, flavonoids, carotenoids, and ubiquinol (CoQ10) have also been shown to act as antioxidants within the mitochondria (for review see Beckman (1998)). The inner membrane of the mitochondria also contains vitamin E, a known antioxidant that prevents free-radical-mediated chain reactions (Ham and Liebler 1995). Coenzyme Q, which is considered a source of superoxide when partially reduced, can also function as an antioxidant when fully reduced (Beyer 1990). Additionally, the mitochondria have a variety of DNA-repairing enzymes that help in the correction of errors caused due to oxidative damage (Turrens 2003).

Although a balance exists between ROS formation and antioxidants under normal circumstances, the antioxidant defenses can become insufficient in several pathological conditions. This often leads to oxidative stress, which can result in apoptosis and cell death. An in-depth discussion of all these systems is beyond the scope of this review. The main focus of the discussion relating to mitochondrial antioxidant defenses will concern the role of glutathione and the glutathione antioxidant enzyme system, and their interaction with ROS.

2.4.4.2 Cell Redox State and its Significance

All living cells are involved in reduction-oxidation (*redox*) activities that play an essential role in cellular functions such as mitochondrial respiration and ATP generation. The term “redox state” simply implies that some cells in some states are oxidative and hence are considered to be in a pro-oxidative state, and vice versa.

The primary factor governing ROS production is the redox state of the respiratory chain (Lambert and Brand 2004; Brookes 2005). Under normal circumstances, the electrons formed during oxidation reactions are transported through the redox carriers of the respiratory chain to molecular oxygen, which acts as the final electron acceptor in the process. However, electron leakage can occur during this process, and this results in the formation of ROS such as superoxide or hydroxyl anions (Bugger and Abel 2008).

The transfer of the electrons in the respiratory chain is dependent upon the membrane potential across the inner mitochondrial membrane, which needs to be at an optimum level. Increases in the membrane potential or the redox potential can lead to increased formation of ROS (Korshunov, Skulachev et al. 1997).

ROS production varies depending on the respiratory state of the cell. The various respiratory states can be explained as follows:

State 1 respiration: establishing a proton gradient

In the mitochondria, energy released from the flow of electrons down the ETC is converted into a trans-membrane, electrochemical proton gradient and into a membrane potential. These proton gradients generate the chemiosmotic potential, also known as the *proton motive force* (see also Section 2.4.2.4.2). The potential energy contained within this gradient, in turn, is used for the synthesis of ATP by oxidative phosphorylation. It is important to note that pumping protons across the membrane creates both a chemical gradient and an electrical gradient (since protons are charged), and the resulting proton gradient is referred to as an electrochemical gradient.

State 2 respiration: dissipation of proton gradient

The gradient developed in state 1 respiration is dissipated via the ATP synthase complex, which is responsible for ATP synthesis.

State 3 respiration: reduction

In the absence of ADP, the movement of H^+ ceases and the concentration of H^+ increase. This causes the electron flow to slow down and the respiratory chain to become more reduced (state 4).

State 4 respiration: Increase in O_2 concentration

Finally, the steady-state concentration of O_2 increases. Hence, it is widely thought that the mitochondria only produce ROS in state 4. It should be noted, however, that the addition of ADP and pyruvate (complex I substrate) demonstrates that complex I still produces ROS in state 3. It has been suggested that proton leakage is lower in state 3 compared with leakage in state 4 respiration based on the lower reduction of the ETC complexes in state 3 respiration in comparison to state 4 respiration (Beckman and Ames 1998).

The consequence of this is that the production of ROS is higher in state 4 respiration as well as in all conditions wherein the respiratory components are substantially in the reduced form. Therefore, processes or conditions that hamper the electron transfer in the respiratory chain indirectly enhance the production of ROS (Lenaz 2001).

Finally, whilst complexes I and III in the respiratory chain are considered as main sites of superoxide radical production, other enzymes complexes such as

complex II, glycerol-1-phosphate dehydrogenase, and dihydroorotate dehydrogenase are also noted to have a role in the formation of ROS (Lenaz 2001).

2.4.4.3 Assessment of Redox Status

The redox-state of a cell is regulated and determined by thiol systems such as the glutathione-SH (GSH):glutathione disulfide (GSSG) system (Jones 2002; Ji 2008; Jones 2008) and the thioredoxin (Trx)-system (Jones 2005; 2008) (Figure 2.16).

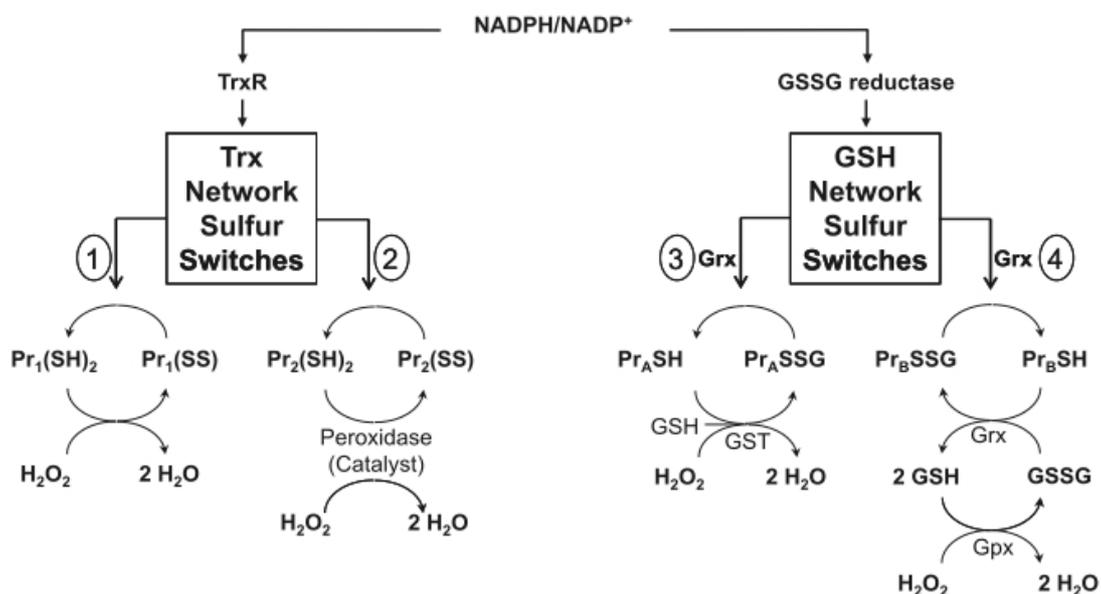


Figure 2.16: Redox systems. A schematic diagram showing the glutathione and thioredoxin redox systems. Taken from Jones (2008)

Glutathione (GSH) is a small three-amino acid-long peptide consisting of the amino acids glutamate, cysteine, and glycine (hence its alternative name γ -glutamylcysteinylglycine). GSH is a non-enzymatic cellular antioxidant that plays a pivotal role in biological antioxidant systems. The GSH/GSSG system is generally regarded as the primary redox buffering system in most cellular compartments due to

its low redox potential and relatively high abundance. The vast majority of GSH is in the cytosol (>90%), with the rest being found in the mitochondria, the nucleus and the endoplasmic reticulum (Banjeree 2008). Most of the cellular GSH exists in the reduced state, with the typical GSH:GSSG ratio being >10:1 (Banjeree 2008). In the endoplasmic reticulum, however, where disulfide bonds in newly synthesized proteins need to be formed, the conditions are significantly more oxidizing, with GSH:GSSG ratios as low as 1:1 (Banjeree 2008).

Thioredoxin is another redox system that is involved in the regulation of the redox state of the cell (Jones 2008). Thioredoxin is a protein of about 12 KDa that is present in all living cells from archaeobacteria to mammals (Banjeree 2008). In the reduced dithiol-form, Trx-(SH)₂ can reduce disulfide bonds in other proteins. The resulting disulfide form of Trx (Trx-S₂) can be reduced to Trx-(SH)₂ by the FAD-containing enzyme thioredoxin reductase in the presence of NADPH as electron donor. The Trx-system has a significant impact on the reducing environment of the cytosol (Jones 2008).

It has also been shown that other cellular components are involved in controlling the redox state of the cell. Many enzymes like proteases, caspases, phosphatases, and kinases contain an oxidation-sensitive cysteine (Cys)-residue in their active centre. Alterations in the oxidative state of the environment of these enzymes can have a significant impact on their function and essentially work as an on-off switch for these proteins (Jones 2008). Thioethers in methionine (Met) are another example of an oxidation sensitive residue present in many biologically active proteins. Hence, the oxidation of both Cys and Met-residues can provide on-off switches for protein activity. An example of this is shown by the loss of activity of the

α 1-antitrypsin inhibitor following the oxidation of a its Met residue (Carp, Miller et al. 1982). Many receptors in the plasma membrane of the mammalian cells contain conserved Cys-rich regions that are sensitive to allosteric changes through changes in the redox balance of their immediate environment.

As discussed, the GSH redox system and other redox systems have the ability to reduce ROS that have been produced by the cell. In the GSH redox system, GSH is used as the substrate, with its thiol group accepting an electron from H_2O_2 , decreasing the formation of the hydroxyl radical and minimizing oxidative damage (Mills 1957). In the process, GSH is converted to its oxidized form, glutathione disulfide (GSSG). The GSSG formed in this reaction can subsequently be reduced back to GSH via glutathione reductase and NADPH, setting up a redox cycle that can be used as an indirect measurement for the oxidative state of the cell (see Figure 2.16). Direct measurement of ROS is often difficult because ROS are quickly quenched by cellular antioxidants. Thus measurements of antioxidants are made, with higher levels interpreted as providing the cell with a better ability to remove ROS and hence, a likely reduction in oxidative damage.

Quantification of the GSH and GSSG redox pool considers the stoichiometric oxidation of two GSH molecules per GSSG, which is determined by the redox potential of the environment (E_h). E_h is calculated according to the Nernst equation, using the ratio of GSH/GSSG (i.e. donor/acceptor), and is often used to provide a reliable measure of the cell's redox state (Jones 2002; Ji 2008).

The Nernst equation is as follows:

$$E_h = E_o + 30 \log ([GSSG] / [GSH]^2)$$

or, alternatively

$$E_h = E_o + 30 \log ([acceptor] / [donor]^2)$$

Where E_o in cells is -264 mV at pH 7.4 and the E_h is a measure of the redox couple's ability to either accept or donate electrons. Couples that have a more negative E_h value have a greater reducing ability. In other words, more negative redox values represent systems that are less oxidized (i.e. more reduced).

It is important to note that to avoid errors associated with the measurement of GSH and GSSH in plasma, blood preservation solutions need to be considered to avoid hemolysis (as red blood cells have their own antioxidant systems) and minimize oxidation and the breakdown of GSH by γ -glutamyltranspeptidase (Jones, Carlson et al. 1998) Additionally, oxidation of thiols can occur rapidly with tissue disruptions. Therefore, it is preferred that samples are snap-frozen in liquid nitrogen shortly after being excised. These methods will be discussed in more detail in chapter 3.

Redox states can be measured in a number of biological media, including the tissue, whole blood or plasma. The outcome usually varies according to the medium selected. Assessment of the intracellular redox state can be determined by measurement of GSH and GSSG levels in the tissue, to determine the E_h for the redox couple GSH/GSSH.

Studies of the GSH/GSSG redox state in human plasma show that this pool is oxidized relative to tissue values. Measurements of E_h for the GSH/GSSG couple in

tissues provide evidence that this redox may be an important characteristic of cell growth and may function in the control of fundamental cell processes, including control of gene expression, cell proliferation, and execution of apoptosis (Jones 2002).

One study reported herein (Chapter 6) focuses on methods for the measurement of tissue GSH and GSSG levels for assessment of the intracellular GSH/GSSG redox in order to assess the effects of supplementation and resistance training in the elderly on the redox state.

2.5 Summary of the Structure and Function of Skeletal Muscle and Mitochondria

In young, healthy adults, movement is controlled by the skeletal muscles which extend and contract according to signals received from the central nervous system via the neurotransmitter, acetylcholine (ACh). The binding of ACh to the receptor of the skeletal muscle triggers a cascade of events, which causes the muscle to contract. The effectiveness of this system in a given individual can be assessed in a number of ways, including the measurement of enzyme (ATPase) activity. Muscle length and speed of muscle contraction can also be measured non-invasively through use of isokinetic dynamometry. In addition, muscular function can also be assessed by analysis of body composition.

The mitochondria are organelles found in most cells of the body and are responsible for carrying out various essential cellular functions. Mitochondria play a key role in electron transport and oxidative phosphorylation, but are also the main cellular source of ROS. Many of the degenerative processes associated with aging can be attributed to the impact of ROS on healthy cells; hence the mitochondria are

heavily implicated in the aging process. The redox activity of a cell determines the impact of the ROS on the cell, and therefore methods for the accurate assessment of a cell's redox state are vital if we are to understand the aging process at a cellular level. The redox potential (E_h) of a cell can be calculated from the relative concentrations of GSH and GSSG using the Nernst equation. This tells us whether a cell is in a pro-oxidative or pro-reductive state, and therefore how effectively it will be able to counteract the effects of ROS.

Having considered the structure and function of both skeletal muscle and the mitochondria at some length, the following sections of this literature review will investigate how these systems change as the body ages, and will consider strategies to mitigate the effects of aging on these key biochemical processes.

2.6 Aging and Skeletal Muscle Structure and Function

Aging can be defined as: “*deteriorative changes with time during post maturational life that underlie an increasing vulnerability to challenges, decreasing the ability of the organism to survive*” (Masoro 1995). Whilst aging is not always associated with disease, the incidence of disease generally increases over time as we age (Caruso, Lio et al. 2004).

Aging is associated with structural and functional changes that increase vulnerability to disease and compromise an organism's ability to adapt to environmental stresses (Harman 1956; Walford 1964). This is especially true for post-mitotic cells, such as myoblasts (progenitor cells that give rise to new skeletal muscle cells) (Grounds 1998). Myoblasts lack the ability to reproduce and hence are more likely to acquire an accumulation of damage over time that may contribute to a

progressive loss of skeletal muscle function and ability to thrive as we move toward old age (60+ years).

Sarcopenia is the gradual progressive loss of muscle mass and strength associated with aging (for review see Welle (2002) and Ryall et al. (2008)). A full discussion of the factors influencing sarcopenia is given in Section 2.8. In general, there is a 30–50% decrease in muscle mass between the ages of 40 and 80 years, or about 10% per decade from the age of 40 years (Lexell, Taylor et al. 1988; Akima, Kano et al. 2001).

Decreases in muscle mass and the associated functional changes can have a devastating affect on an individual's health in old age, resulting in possible loss of independence and an increased risk of falls (Roubenoff 2000). Additionally, the age-associated changes in the structure and function of skeletal muscle can result in metabolic dysfunction that may increase the risk of diseases such as type 2 diabetes (Karakelides and Nair 2005) as well as obesity (Roubenoff 2004). Hence, it is important to gain a greater understanding of the underlying mechanisms involved so that we can intervene before damage occurs. Of utmost importance, however, is not the preservation of muscle mass *per se* but rather the preservation of an individual's health, independence and overall quality of life. However, maintaining and even improving lean muscle mass is a key strategy to achieving this.

Whilst abundantly apparent that changes at the molecular level contribute to the age-associated decrease in muscle structure and function, an in-depth discussion about this topic is beyond the scope of this review. The main focus of this review will be on the age-related changes that occur to muscle physiology and biochemistry.

2.6.1 Contractile structure and function changes in skeletal muscle due to aging

Sarcopenia is associated with a deterioration in muscle structure that is subsequently associated with a deterioration in muscle function (Trappe 2009). These changes are thought to result from multiple factors at the physiological/biochemical level, including changes in:

- neuromuscular structure and function (Ansved and Larsson 1990; Lexell 1997; Cederna, Asato et al. 2001; Faulkner, Larkin et al. 2007)
- muscle-fibre type, cross-sectional area and number (Akima, Kano et al. 2001; Faulkner, Larkin et al. 2007),
- structural and functional properties of myosin and actin (Larsson, Li et al. 1997; D'Antona, Pellegrino et al. 2003), including myosin ATPase (Prochniewicz, Thomas et al. 2005) ,
- mitochondrial density, protein content and enzyme activity (Rooyackers, Adey et al. 1996; Conley, Jubrias et al. 2000; Short, Bigelow et al. 2005),
- mitochondrial DNA abundance, mutation and accumulation (Melov, Shoffner et al. 1995; Lee, Weindruch et al. 1997; Welle, Bhatt et al. 2003), and
- concentration of ROS, antioxidant defenses and cell redox state (Beckman and Ames 1998; Jones 2006; Humphreys, Martin et al. 2007; Rizvi and Maurya 2007).

The first part of this discussion will focus on the structural and functional changes with age that occur specifically to the contractile apparatus, followed by a discussion of age-related changes in mitochondrial structure and function.

2.6.1.1 Neuromuscular function

As we have already seen in Section 2.1, a motor unit is a single α -motor neuron plus all the skeletal muscle fibres that it innervates. The motor neuron initiates excitation-contraction coupling by transmitting action potentials, thus activating all the muscle fibres that it innervates. All the muscle fibres innervated by a single motor neuron are of one particular fibre type; therefore the motor unit and the muscle fibres contained within that motor unit are named similarly (slow-twitch unit/fibre or fast-twitch unit/fibre).

Skeletal muscles consist of a collection of motor units that activate the muscle in a graded fashion that is dependant on the force requirement of the muscle. The ability to recruit muscle fibres in a graded fashion is essential in controlling and coordinating movement. In comparison to the fast-twitch or type II motor units, slow-twitch or type I motor units take longer to reach peak twitch tension as well as taking longer to relax. Additionally, slow-twitch motor units are a lot smaller and therefore, based on their size, innervate a smaller number of muscle fibres. As a result, slow-twitch motor units produce a lot less force in comparison to the larger fast-twitch or type II motor units that innervate a greater number of muscle fibres.

Differences in motor units and the characteristics of the associated muscle fibres are also accompanied by differences in energy metabolism, substrate use and fatigability (for characteristics of different muscle fibre type see Table 1).

Aging is associated with a slowing of muscle contraction (Larsson, Li et al. 1997), which can often be seen in the slow moving, unstable and frail older individual. The slowing of muscle contraction tends to occur before the onset of age-

related changes in muscle mass (i.e. sarcopenia) (Akima, Kano et al. 2001), indicating the involvement of other intrinsic factors not associated with changes in muscle size.

The neuromuscular junction is thought to play an important role in contributing to age-related changes in muscle function, as its structural and functional integrity is compromised in the elderly. Aging is associated with a motor unit remodeling whereby there is a decrease in the number of motor units (preferentially fast motor units) and an abandonment/denervation of the associated muscle fibres (Degens 2007).

Loss in the number of motor units is a significant contributor to the loss of muscle fibres associated with aging (Ansved and Larsson 1990; Cederna, Asato et al. 2001). It is thought that the decrease in physical activity associated with aging results in a decrease in motor unit activity (i.e. disuse syndrome), initiating the motor unit remodeling process. Denervation of the muscle fibres results in muscle fibre atrophy, which over time (if the muscle fibre does not become reinnervated) results in muscle fibre loss (Degens and Alway 2006). Fibre atrophy and loss are thought to be two fundamental factors contributing to the progressive loss of muscle mass and strength we see with age.

Abandoned muscle fibres are reinnervated by near-by motor neurons. This compensates by increasing the force generating capacity of the remaining motor units by increasing the number of fibres innervated by each unit (Kadhiresan, Hassett et al. 1996). In other words, the remaining units attempt to compensate in an attempt to maintain the same force-generating capacity of the muscle *in lieu* of a loss in motor unit number. The remaining fibres that are not reinnervated undergo denervation atrophy and death.

There is a preferential loss of the larger fast-twitch motor units with aging, and it is the slow-twitch motor units that are often left to compensate. This decreases the overall force-generating capacity of the muscle as well changing other important metabolic characteristics of the muscle. Denervation of single fibres independent of the motor unit is also possible (see Faulkner (2007) for review).

The preferential loss of fast-twitch motor units over slow-twitch units appears to be associated with the manner in which the muscle is activated and used as we age (Rosenheimer and Smith 1985). Physical inactivity and a decrease in the intensity of movement have been suggested as explanations for the decreases in fast-twitch motor unit recruitment resulting in motor unit remodeling (Degens and Alway 2006). However, physical inactivity cannot entirely account for the loss of muscle function as we age because decreases in neuromuscular function with age are also observed in very active athletes (Faulkner, Larkin et al. 2007), suggesting that an increase in physical activity may not be sufficient to completely offset the age-related changes in muscle structure and function. Hence, intrinsic factors, such as changes in the systemic environment, changes in protein structure and oxidative stress, may also be involved.

2.6.1.2 Muscle Fibre Type, CSA, Fibre Number

A change in skeletal muscle mass can result from 1) change in muscle volume (fibre length and CSA) and 2) change in the number of fibres present in the muscle (Lexell, Taylor et al. 1988; Lexell 1995).

As adults, muscle volume changes as a result of hypertrophy or atrophy (increased or decreased fibre size, respectively). Whilst some might argue that hyperplasia (increased fibre number) occurs (Antonio and Gonyea 1993), this does

not appear to be the case in human skeletal muscle in general (Tesch and Larsson 1982; Larsson and Tesch 1986) due to the post-mitotic nature of myoblasts. Apparent hyperplasia is more likely to be associated with hypertrophy and subsequent splitting of existing fibres. If the number of fibres does not increase, then changes in the mass of a given muscle must result primarily from a change in the CSA of the individual's fibres and/or a decrease (i.e. loss) in the number of fibres. Both a change in the CSA of an individual's fibres and a decrease in the number of fibres have been observed with aging (Lexell, Taylor et al. 1988).

As discussed in the previous section, the loss of motor units is a major contributor to the loss of muscle fibres as we age. Similarly, change in muscle CSA is thought to be the major contributing factor associated with age-related changes in muscle strength.

Whilst there has been substantial research into the relationship between changes in peak force with changes in CSA with age, these studies are limited by the use of rough estimates of changes in muscle size (i.e. mean CSA calculated by measuring the area of fibres taken from muscle biopsies) (Frontera, Hughes et al. 1991) and/or the use of a narrow range of age groups (Young, Stokes et al. 1985).

To resolve this issue, Akima *et al.* (Akima, Kano et al. 2001) used a high-resolution imaging technique (MRI) in conjunction with strength measurements to age-measure changes in muscle function in 164 subjects divided into five age groups; 20s (20–39 years), 40s (40–49 years), 50s (50–59 years), 60s (60–69 years), and 70s (70–84 years). Muscle peak torque and force in the knee extensors and flexors (i.e. quadriceps and biceps femoris) was inversely related to age in both men and women at all velocities. However, a decrease in isometric strength with age was only seen in

men but not women (Akima, Kano et al. 2001), suggesting that changes in muscle function in men may also involve other factors. For example, it has been shown that testosterone decreases with age (Starka, Pospisilova et al. 2009) and this would thus have a bigger effect in men than women, who have much lower levels of testosterone compared with men.

As with the preferential loss in fast-twitch motor units observed with age, there is also a preferential loss and change in the CSA of fast-twitch muscle fibres, with no real changes to slow-twitch fibres (Lexell, Taylor et al. 1988). Whilst the loss of muscle fibres appears to be an inevitable part of the aging process, the rate of loss appears to depend partly on the intensity and regularity of physical activity (Brach, Simonsick et al. 2004; Ryall, Schertzer et al. 2008). Physical activity affects the CSA of muscle fibres and hence may offset the age-related decrease in muscle size somewhat by maintaining or increasing (hypertrophy) the CSA of the remaining fibres. The effects of physical activity on muscle structure and function will be discussed in more detail in Section 2.9.1 of this review.

2.6.1.3 Myosin and Actin Filaments

The myosin and actin filaments are contractile proteins that play a fundamental role in muscle contraction, as described previously (see Section 2.1.1). To measure age-related changes in muscle function specifically related to myosin and actin, permeabilized muscle fibres are often used (Larsson, Li et al. 1997; D'Antona, Pellegrino et al. 2003; Prochniewicz, Thomas et al. 2005). Permeabilization of skeletal muscle fibres isolates the myosin and actin proteins by removing components associated with the excitation-contraction coupling system (Degens and Larsson 2007). Isolating the myosin actin filaments removes possible confounding factors in

the muscle, such as changes in protein phosphorylation, that may also associated with aging (Gannon, Staunton et al. 2008).

The myosin heavy chain content and maximum shortening velocity and force of slow oxidative (type I) and fast oxidative (type IIa) fibres was found to be lower in the vastus lateralis in elderly subjects (>70 years) when compared with young controls (<31 years) (Larsson, Li et al. 1997; D'Antona, Pellegrino et al. 2003), suggesting an age-related change in the structure and function of contractile filaments.

Degens (2007) also found a decrease in muscle shortening velocity that was not explained by changes in muscle fibre-type composition, suggesting that a change in muscle shortening velocity may be associated with the filaments and not simply the change in muscle fibre-type composition commonly seen with age. Additionally, age-related decreases in myosin ATPase activity have also been suggested with changes in the interaction between the myosin and actin filaments, possibly explaining some of the losses in muscle force production with age (Prochniewicz, Thomas et al. 2005). Therefore, the decrease in muscle mass and strength that occurs with age may not only be associated with changes in whole muscle or muscle fibre type transitions, but may also be related to functional changes in actin-myosin complex and the overall quality of the muscle.

2.6.2 Structural and Functional Changes in Skeletal Muscle Mitochondria due to Aging

Mitochondrial respiratory function declines with age (Wei 1998). A number of structural and functional changes in mitochondria occur, including changes in mitochondrial structure and number, changes in mitochondrial enzyme activity,

changes in mitochondrial oxidative capacity (VO_2max) and changes in mitochondrial ATP production rate (MAPR).

2.6.2.1 Changes in Mitochondrial Structure and Content

Changes in mitochondrial structure and content are considered to be significant contributors to the changes in mitochondrial function with progressing age (Rooyackers, Adey et al. 1996; Balagopal, Rooyackers et al. 1997; Short and Nair 1999; Short, Vittone et al. 2003). The major age-related structural changes in mitochondria include breakdown of the mitochondria and a decrease in the number of healthy functional mitochondria (with a concomitant decline in ATP production), reduced energy-dependent protein synthesis, and fewer specialized cellular functions (Melov, Shoffner et al. 1995; Lee, Weindruch et al. 1997; Barazzoni, Short et al. 2000; Welle, Bhatt et al. 2003).

Numerous studies have shown that the rate of synthesis of mixed muscle proteins, myosin heavy chains, and mitochondrial proteins, in human skeletal muscles decrease with increasing age (Rooyackers, Adey et al. 1996; Balagopal, Rooyackers et al. 1997; Short and Nair 1999). Furthermore, a reduction in the activity of oxidative enzymes such as citrate synthase and the content of mRNA transcripts that encode for mitochondrial proteins has also been shown to occur (Short, Vittone et al. 2003). Therefore, muscle function is altered as a result of a reduction in the synthesis and activity of specific proteins. Additionally, there appears to be a decrease in mtDNA abundance and an increase in the number of mtDNA mutations in the mitochondria, and this has been proposed as a cause of mitochondrial dysfunction in elderly individuals (Melov, Shoffner et al. 1995; Lee, Weindruch et al. 1997; Barazzoni, Short et al. 2000; Welle, Bhatt et al. 2003).

As discussed in Section 2.4.2, if mtDNA abundance decreases with age, this would decrease the transcription and translation of important, key mitochondrial proteins. Decreased mtDNA abundance has been reported to be associated with lower content of mRNA transcripts that encode mitochondrial proteins (Short, Bigelow et al. 2005). The mtDNA decrease seen with age might also result from an increase in oxidative stress and oxidative damage (Shigenaga, Hagen et al. 1994).

Due to the proximity of the DNA structures to the source of oxidative stress (i.e. where the ROS are produced in the mitochondria), oxidative damage is reported to be three-times higher in mtDNA than in nuclear DNA. (Stevnsner, Thorslund et al. 2002; Shokolenko, Venediktova et al. 2009)

2.6.2.2 Mitochondrial Function

The major functional role of the mitochondria is ATP generation, and it still remains to be resolved whether MAPR in skeletal muscle declines with age in humans. Previous studies that attempted to address this question are not in agreement; reporting that MAPR is either unchanged (Taylor, Kemp et al. 1997; Chretien, Gallego et al. 1998; Petersen, Befroy et al. 2003; Rasmussen, Krustrup et al. 2003) or declines (Conley, Esselman et al. 2000; Tonkonogi, Fernstrom et al. 2003) with age.

A recent study, however, found that MAPR declined with age in a well-characterized group of healthy adults when expressed either per unit of muscle mass or after normalization for mitochondrial protein (Short, Bigelow et al. 2005). This study in healthy men and women between 18 and 87 years of age noted a continuous decline in the mitochondrial capacity for oxidative phosphorylation (ATP production) with advancing age in skeletal muscle. It was concluded that the decline in MAPR in older muscles was due to the combined effect of reduction in the mitochondrial

content and the functional alteration in the existing mitochondrial population indicating that the gradual MAPR decline in aged muscles might be the result of reduced mitochondrial content. MAPR was also closely associated with maximal oxygen consumption (VO_2max), which is the body's maximum capacity to transport and utilize oxygen during an incremental exercise, even after adjusting for differences in leg lean mass. This suggests that muscle mitochondrial function is a determinant of VO_2max in untrained individuals and may contribute to the decline in VO_2max seen with advancing age (Short, Bigelow et al. 2005).

Therefore, just as structural changes occur in the contractile proteins and contractile function, many structural and functional changes also occur in skeletal muscle mitochondria with age.

It has also been proposed that aging results in progressive damage to mitochondria due to an increase in ROS production, leading to deterioration in the functional capacity of the ETC which is essentially linked with decreased ATP synthesis (Dirks and Leeuwenburgh 2002).

Direct, *in vivo* measurements of mitochondrial function showed that reduced mitochondrial content and significant functional impairment occurs even in the absence of disease states in older subjects (Conley, Jubrias et al. 2007). A recent study by Conley et al. (2007) measured the oxidative capacity of isolated mitochondria taken from the vastus lateralis (VL) muscle and found that the mitochondrial capacity of the aged group was half that of the adult group, yet the mitochondrial content dropped by only 25%. This greater loss in mitochondrial function than content points to a 30% reduction in the capacity of each mitochondrion to generate ATP. The individuals studied were all carefully screened for a majority of common ailments and

disorders and were all physically active. Hence, changes in mitochondrial function appear to represent a normal, physiological phenomenon of aging rather than the outcome of some pathological state (Conley, Jubrias et al. 2007).

2.6.2.3 Proposed Mechanisms Contributing to Skeletal Muscle Mitochondrial Dysfunction

There are at least several potential mechanisms by which aging might contribute to skeletal muscle mitochondrial dysfunction. These include uncoupling of oxidative phosphorylation (Conley, Jubrias et al. 2007), free-radical production and damage to mitochondrial DNA (Beckman and Ames 1998), impact of ROS and oxidative stress (Kong and Davison 1980; Jaeschke 1995; Droge 2002), as well as a decrease in antioxidant content and antioxidant system function (Wei and Lee 2002; Droge 2005).

2.6.2.3.1 Uncoupling of Oxidative Phosphorylation

It has been proposed that when protons re-enter the mitochondrial matrix without contributing to ATP production (*mitochondrial uncoupling*), potentially due to oxidative damage from ROS, they may act as initiators of the cell death pathway that leads to the irreversible loss of muscle fibres (sarcopenia) in old age (Echtay, Pakay et al. 2005). Hence, uncoupling may play a significant role in increased mitochondrial dysfunction with age. Indeed, it is interesting to note that only fast-twitch muscles tend to show uncoupling in mitochondria (Amara, Shankland et al. 2007) and it is the fast-twitch fibres that are the most susceptible to the aging process.

It should be noted, however, that the nature and impact of this uncoupling evidently changes with age. In adult muscle, mitochondrial coupling is usually maintained under physiological regulation, although uncoupling in elderly muscle

seems to be associated with unregulated proton leak, possibly because of oxidative damage to the inner mitochondria membrane (Conley, Jubrias et al. 2007). The end result of the age-related uncoupling is cellular ATP depletion, which is associated with the activation of the first steps of cell death leading to sarcopenia (Conley, Jubrias et al. 2007).

2.6.2.3.2 Free Radical Production and Damage to Mitochondrial DNA

The free-radical damage theory of aging was first proposed by Denham Harman over 50 years ago (Harman 1956). ROS are free radicals that contain an oxygen atom. ROS can cause damage to cells and are thought to accelerate the progression of cancer, cardiovascular disease, arthritis and many other diseases (Valko, Leibfritz et al. 2007). In a state of oxidative stress, ROS can also cause significant damage to myofibrils which has been shown to contribute to age-associated changes in muscle mass and function (Rooyackers, Adey et al. 1996; Short and Nair 1999).

The mitochondria is both a major source of ROS production (Sohal and Weindruch 1996) and a primary area of ROS-induced damage (Lenaz 2001). As discussed in section 2.4.4, enhanced production of ROS and the accumulation of mtDNA mutations in the mitochondria of post-mitotic cells is thought to be another major contributory factor to the human aging process (Wei and Lee 2002). The mitochondrial free-radical theory of aging proposes that the cause of mitochondrial dysfunction is damage resulting from ROS generation (Beckman and Ames 1998). This theory is a commonly held view that argues that mitochondrial defects in aging and disease are the result of mutations in mitochondrial DNA (mtDNA) (Beckman and Ames 1998).

2.6.2.3.3 Reactive Oxygen Species (ROS) and Oxidative Stress

As discussed in Section 2.4, mitochondria produce ROS as a by-product of normal metabolism (Sohal and Weindruch 1996) that plays an important role in cell signaling (Kamata and Hirata 1999; Droge 2002; Jackson 2005; Jackson 2008; Jackson 2008). However, in times of elevated stress, ROS can cause significant amount of damage to mitochondrial proteins (Friguet, Bulteau et al. 2008; Ikawa and Yoneda 2009) and mtDNA (Kong and Davison 1980; Jaeschke 1995; Droge 2002) (see Figure 2.17).

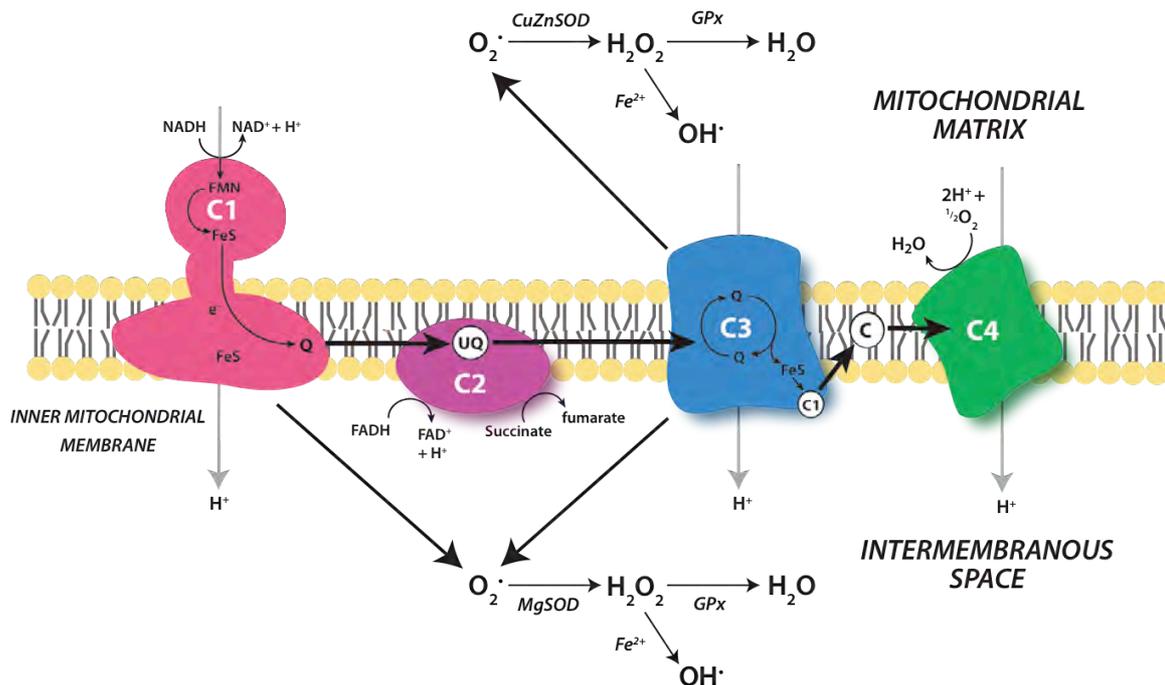


Figure 2.17: Production of ROS in the mitochondria [adapted from Figueiredo et al. (2008)]

It is now generally accepted that the aging-associated decline in mitochondrial function can result in enhanced production of ROS in mitochondria that appears to occur as a result of electron leakage from the respiratory chain (Beckman and Ames 1998).

As noted, a significant loss of skeletal muscle mass and function that occurs can have a dramatic impact on the quality of life of older individuals. The processes underlying this loss of mass and function are unknown, but a chronic increase in cellular superoxide (O_2^-) has been implicated in contributing to age-associated mitochondrial dysfunction (Wei and Lee 2002).

Mitochondria are a major cellular site for superoxide generation at complexes I and III of the ETC (Turrens 2003). Superoxide is converted to hydrogen peroxide (H_2O_2) within the mitochondrial matrix in a reaction catalyzed by Mn-superoxide dismutase (MnSOD). However, some of the superoxide produced at complex III is also partially released into the intermembranous space of mitochondria, which has recently been recognized as having a copper and zinc superoxide dismutase (CuZnSOD) (Jackson 2006) (see Figure 2.17). Removal of this CuZnSOD is thought to lead to a phenotype of enhanced loss of skeletal muscle mass and function due to aging (Jackson 2006). However, it is not yet established whether this is the result of enzymatic loss in the cytosol or if the intermembranous space has some role in this aspect.

Following the conversion of superoxide to hydrogen peroxide, hydrogen peroxide is then converted to water by glutathione peroxidase (GPx), or alternatively by catalase (CAT).

Coupled to the increase in ROS production seen with age, the functional capacity of these antioxidant enzymes and the amount of small-molecular-weight antioxidants present in the blood and tissues have also been shown to decrease in the elderly (Wei and Lee 2002). Hence, there is an age-dependent increase in ROS that remains unaffected by the cellular defense mechanisms, creating a state of oxidative stress that can potentially damage various cellular structures such as lipids, proteins and nucleic acids.

2.6.2.3.4 Oxidative Stress

Oxidative stress is defined as an imbalance between the cellular antioxidant defense systems and the production of ROS, ultimately leading to oxidative damage of cellular macromolecules (Beckman and Ames 1998).

As discussed in Section 2.4.4, under normal physiological conditions, a small percentage of the oxygen consumed by the mitochondria is converted to superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (O^\bullet), and other ROS. Within a certain concentration range, ROS play an important role in regulating many cellular functions as well as acting as an important secondary messenger that activates specific transcription factors such as NF- κ B and AP-1 (Ji 2008). Beyond this, an excess production of ROS can be harmful to cells. In fact, ROS can modify the biological activity of enzymes, intracellular signaling events, and damage biological macromolecules (Beckman and Ames 1998).

2.7 Aging and Redox Regulation of Skeletal Muscle function

Oxidative stress has been a major focus of research into aging processes for the last few decades. A component of oxidative stress research that has attracted a lot

of attention in the last few years is free-radical damage to cells that occurs as a result of ROS production and changes in cellular redox state (Jones 2008). Free radicals in biological systems are rapidly converted into non-radical oxidants that can shift the redox-balance of the cell and can alter the functions of proteins like transcription factors, enzymes, transporters, signal-transducers, structural proteins and others.

2.7.1 Redox Biochemistry and Cell Signaling

Redox biochemistry and the assessment of cell redox state were discussed in Section 2.4.4.2. In summary, redox signaling in the cell occurs through ROS. An imbalance between cellular antioxidant defense systems and the production of ROS is known as oxidative stress, a process which ultimately leads to oxidative damage of cellular macromolecules.

The redox state of a cell is determined and regulated by thiol systems such as the GSH/GSSG system and the thioredoxin (Trx)-system in the cell. These redox systems have the ability to reduce levels of ROS within the cell.

The GSH/GSSG system is generally regarded as the primary redox buffering system in most cellular compartments due to its low redox potential and relatively high abundance. In this redox system, GSH is oxidized to GSSG by accepting an electron from hydrogen peroxide (a ROS), thus minimizing ROS-induced oxidative damage. The GSSG formed in this way is subsequently reduced back into GSH via glutathione reductase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), setting up a redox cycle that can be used as an indirect measurement for the oxidative state of the cell.

As well as the GSH/GSSG redox system, useful information about the level of oxidative stress can be ascertained from the cysteine/cystine (Cys/CySS) redox pair. Cys/CySS ratios in humans usually display a more oxidized state than GSH/GSSG ratios.

2.7.2 Glutathione Redox State and Disease

The GSH redox system is one of the most important systems for the homeostasis of the redox balance in the cell. It is the most abundant redox-system in the cells and can reach concentrations ranging from 0.5–10 mM (Banjeree 2008).

Many human diseases show an altered GSH/GSSG ratio and/or alterations in plasma or cellular GSH contents. For example, in atherosclerosis, inflammation of the blood vessel wall leads to oxidative stress inside the cells of the endothelial lining of the vessel. Changes in the GSSG concentrations in the plasma have also been detected, signifying that the increased oxidative stress is associated with changes in the redox state of the environment (Kondo, Hirose et al. 2009). Other examples where changes in the redox state of the cell and oxidative stress have been implicated include type 2 diabetes (Whiting, Kalansooriya et al. 2008), metabolic syndrome (Giral, Jacob et al. 2008), and lupus erythematosus (Tewthanom, Janwityanuchit et al. 2008). Decreased GSH plasma levels have also been found in patients with preeclampsia (Kaur, Mishra et al. 2008), non-alcoholic hepatic steatosis (Machado, Ravasco et al. 2008), gastrointestinal tumors (Scibior, Skrzycki et al. 2008), and with rheumatoid arthritis (Seven, Guzel et al. 2008).

Age-related changes in the redox state of the GSH/GSSG system (i.e. an increased concentration of the oxidized GSSG with a concomitant decrease in GSH concentration) also correlate very well with the increased mitochondrial production of

ROS with age, as previously discussed (see Section 2.6.2.3.1). Whilst the increased concentration of ROS has been implicated in contributing to changes in redox state (Jones 2006; Rebrin and Sohal 2008), changes in the concentration of GSH and Cys have also been suggested (Dröge 2005; Guayerbas, Puerto et al. 2005)

2.7.3 Age-Related Changes in Glutathione and Cysteine Concentrations

There is ample evidence that the serum GSH/GSSG ratio in humans declines with age (Kretzschmar and Muller 1993; Michelet, Gueguen et al. 1995; Piccinini, Minetti et al. 1995; Yang, Chou et al. 1995; Rondanelli, Melzi d'Eril et al. 1997; Paolisso, Tagliamonte et al. 1998; Samiec, Drews-Botsch et al. 1998; Erden-Inal, Sunal et al. 2002; Jones, Mody et al. 2002; Gil, Siems et al. 2006; Jones 2006; Rizvi and Maurya 2007; Rizvi and Maurya 2007; Rizvi and Maurya 2008). As the concentration of ROS increases with age, so too would the activity of antioxidant systems that are used to quench these ROS.

The intracellular GSH/GSSG ratio shifts toward a more oxidizing state with increasing age as the relative amount of GSSG rises (Rebrin, Kamzalov et al. 2003; Rebrin and Sohal 2008). In humans, aging is associated with a decrease in the glutathione concentration in whole blood (Erden-Inal, Sunal et al. 2002) as well as mononuclear cells such as lymphocytes (Hernanz, Fernandez-Vivancos et al. 2000).

A decrease in the concentration of both Cys and GSH offers indirect evidence that there is an increase in oxidative stress. This indirect evidence is valuable as it is often difficult to directly measure the concentration of ROS because they are quickly quenched by antioxidants and/or reduced by endogenous antioxidant systems such as the GSH antioxidant system. Additionally, a decrease in the concentration of Cys and

GSH would decrease the ability of the cells to deal with the increase in ROS resulting in a state of oxidative stress.

Similar to the GSH/GSSG redox pair, the cysteine/cystine (Cys/CySS) redox pair can also provide useful information about the level of oxidative stress present. However, in the case of Cys/CySS redox pair, it is referring to the level of oxidative stress in the plasma. Cys/CySS ratios in humans usually display a more oxidized state than GSH/GSSG ratios (Jones, Mody et al. 2002). The redox state of the Cys/CySS pool is linked to the redox state of the GSH/GSSG pool because the GSH released from cells can react with the cysteine in the plasma to produce the disulfide CySSG. Therefore, it is difficult to determine whether the changes in the Cys/CySS redox state are the result of oxidative stress in the plasma or within the cell.

Interestingly, there appears to be an age-related shift in the plasma cysteine/cystine redox state in humans between the third and ninth decade of life that is also accompanied by a decrease in plasma glutathione (Droge 2005). A similar age-related decline also occurs with muscle mass from the fourth decade onwards (Lexell, Taylor et al. 1988; Akima, Kano et al. 2001) suggesting that changes in plasma and cellular redox status and muscle mass may be linked possibly through cell signaling cascades that are regulated by the redox state of the cell.

2.7.4 Proposed Mechanism of Age-Related Oxidizing Shift in Glutathione Redox State

As discussed in Section 2.4.4.3, an increase in ROS concentration can affect the GSH/GSSG balance in the cell. Additionally, an increased usage of GSH without a similar increase in the rate of its production and/or reclamation will lead to a decreased

GSH/GSSG ratio. Therefore, an age-related decrease in the GSH/GSSG ratio could also be caused by a decrease in GSH synthesis (Rebrin and Sohal 2008).

The rate-limiting step in GSH synthesis is the ligation of Cys to glutamate that is catalyzed by glutamate Cys ligase (GCL). Hence, GSH synthesis is dependent on Cys supply and the affinity of GCL for its substrates, glutamate and Cys.

Cys is not an essential amino acid, which means it can be synthesized in the body. However, as only one of two naturally occurring sulfur-containing amino acids, its biosynthesis is dependent on methionine supply, the other sulfur containing amino acid. Methionine is an essential amino acid in humans, which means it has to be supplied by nutrition and cannot be synthesized in the body.

If the diet is deficient in Cys and methionine, a Cys deficiency may occur and this can lead to serious consequences for GSH biosynthesis and for the redox balance in general. A decrease in the synthesis of GSH can lead to an accumulation of oxidation byproducts in cells (i.e. free radical damage), that correlates closely with aging rates and decreased life spans in several animal models (Sohal and Weindruch 1996; Rebrin, Kamzalov et al. 2003; Jones 2008; Rebrin and Sohal 2008).

Supplementation with Cys as oxidation resistant N-acetyl-Cys (NAC), which can easily be transformed to Cys in the body, has been shown to at least partly reduce the effects of aging in different systems (De La Fuente, Miquel et al. 2002; Guayerbas, Puerto et al. 2002; Miquel 2002; Grattagliano, Portincasa et al. 2004; Guayerbas, Puerto et al. 2004; Guayerbas, Puerto et al. 2005; Morrison, Coleman et al. 2005; Nicoletti, Marino et al. 2005; Muscari, Bonafe et al. 2006; Chan and Shea 2007; Kanwar and Nehru 2007; Arranz, Fernandez et al. 2008; Bagh, Maiti et al. 2008). Cognitive function has been shown to be improved in aged mice and rats after dietary supplementation

with NAC (Nicoletti, Marino et al. 2005; Chan and Shea 2007; Kanwar and Nehru 2007). Additionally, immune function improvements have been detected in postmenopausal women (Arranz, Fernandez et al. 2008). A similar improvement in immune function was shown in a model of prematurely aging mice, and this was thought to protect the mice from mitochondrial damage (De La Fuente, Miquel et al. 2002).

Whey protein is another rich source of Cys that aids in the synthesis of glutathione (Zavorsky, Kubow et al. 2007; Balbis, Patriarca et al. 2009). Hence, whey protein/Cys supplementation might possibly attenuate the age-related changes in GSH/GSSG redox state by increasing the biosynthesis of GSH.

In summary, the finely tuned cellular redox state becomes more oxidizing with increasing age, which increases oxidative stress. This leads ultimately to reduced muscular oxidative capacity and oxidative damage. Previous studies have shown that the cellular redox equilibrium can to some extent be restored by supplementation with Cys, readily available in whey protein. Given that whey protein has also been shown to be a powerful anabolic agent and is well tolerated (Hayes and Cribb 2008), it may be a good nutritional strategy to offset the effects of aging. This will be discussed further in Section 2.9.2.2.

2.8 Behavioral Changes, Functional Impairment and Physical Disability Associated with Aging

As discussed in Section 2.6, a prominent feature of aging is a gradual decline of muscle mass, known as sarcopenia (reviewed in Thompson (2009) and in Ryall et al. (2008)), which may set the stage for injury resulting from instability and falls, with a debilitating effect on the quality of life.

Sarcopenia shows a complex etiology (Ryall, Schertzer et al. 2008). The factors that influence sarcopenia can be classified as:

- Intrinsic Factors
- Systemic biology factors
- Decreased physical activity
- Dietary factors

2.8.1 Intrinsic Factors

Intrinsic factors such as reduction in the levels of myogenic regulatory factors, intracellular levels of calcium and regulation of Notch signaling pathways are thought to contribute to sarcopenia (Ryall, Schertzer et al. 2008).

2.8.2 Systemic Biology Factors

Extrinsic factors such as depletion of IGF-1, testosterone and inhibitory effects of cytokines like IL-6 have been known to play a role in the progression of sarcopenia (reviewed in Thompson (2009) and in Ryall et al. (2008)); see also Srinivas-Shankar and Wu (2009)).

2.8.3 Decreased Physical Activity

Lifestyle factors such as physical activity and diet also influence sarcopenia. Sarcopenia is intrinsically linked to muscle activity. It seems, therefore, that inclusion of physical activity should reverse the process of sarcopenia of aging. Although inclusion of at least 30 minutes of physical activity does improve the functional capacity in adults aged 40-60 years (Lemura, von Duvillard et al. 2000; Brach, Simonsick et al. 2004), this does not result in total reversal of sarcopenia. It is surmised that other factors like oxidative stress and apoptosis in muscles may also contribute to sarcopenia of aging (Combaret, Dardevet et al. 2009).

Muscle tissue also shows infiltration of fat cells with increasing age. This infiltration of the tissue is thought to diminish the force generating capacity of muscles (Walston, Hadley et al. 2006). Lack of physical activity or exercise results in an increase in the total body fat mass in individuals of 30 years of age and above. An interesting observation to note is that the infiltrating fat cells are thought to be similar to visceral fat (Walston, Hadley et al. 2006) indicating that an expanding waistline with increasing age can accelerate aging by triggering the onset of sarcopenia as well.

2.8.4 Dietary Factors

It is known that appetite decreases with age and can potentially result in nutritional deficiencies (Bales and Ritchie 2002). The anabolic response of muscles also declines with age with a concomitant increase in proteolysis in muscles (Ryall, Schertzer et al. 2008). Aging also results in reduced production of proteins like myosin heavy chain. Concomitantly, the ubiquitin-proteasome pathway, which degrades mistranslated proteins during synthesis, also gets down-regulated (Thompson 2009). Together, these intracellular changes contribute towards loss of proteins in aging muscles.

2.9 Interventions

It has been shown that the changes that occur naturally with age, deterioration in muscle mass and oxidative capacity, as well as free radical damage, can be slowed or even reversed. For example, in elderly individuals, exercise has been shown to increase muscle mass (Carmeli and Reznick 1994), increase muscle oxidative capacity (Carmeli and Reznick 1994; Westerterp 2000) and increase antioxidant defenses against free radical damage (Cartee 1994; Johnston, De Lisio et al. 2008). Unfortunately, other problems associated with aging, such as cardiovascular disease

and arthritis, can make exercise difficult. Hence, in cases where exercise is not possible, other interventional approaches are required.

Decreases in muscle cell volume and muscle protein synthesis may contribute to the decrease in muscle mass and oxidative capacity with age in human skeletal muscle. Therefore, interventions that can reverse the decrease in cell volume and/or rates of muscle protein synthesis could reverse any associated losses in muscle mass and oxidative capacity, therefore restoring muscle function. Additionally, maintenance of muscle cell volume and/or protein synthesis could prevent the associated disabilities that come with increased frailty in the aged, and bring about a restoration of an individual's independence.

Of interest to the current research are two interventions that show promise in offsetting age-related decreases in muscle structure and function. These interventions include physical activity in the form of resistance training, and dietary supplementation with creatine monohydrate (Cr) or whey protein. The latter intervention is likely to alter cell volume and protein synthesis and may also act to preserve muscle mass and improve oxidative capacity through its ability to increase the muscle antioxidant defenses and therefore minimize any free-radical damage to cells.

2.9.1 Physical Activity

Studies suggest that physical exercise and training, especially resistance training, may play a crucial role in the prevention of sarcopenia and the other age-related pathophysiological states such as coronary heart disease, type 2 diabetes, hypertension, osteoporosis and obesity (Lortie, Simoneau et al. 1985; Phillips, Green et al. 1996). Endurance training has been shown to increase mitochondrial density as

well as the activity of oxidative enzymes (Lortie, Simoneau et al. 1985; Phillips, Green et al. 1996) that are thought to result from an increase in mitochondrial biogenesis (Gollnick, Riedy et al. 1985). However, it is important to remember that whilst aerobic exercise generally improves cardiovascular function and aerobic capacity, it is sometimes contraindicated in the aged population as a result of cardio-respiratory complications and disease (ACSM 1994). In contrast, heavy resistance strength training is thought to result in muscle adaptation that could offset changes to muscle function seen with age.

The adaptive responses to endurance training and strength training have been shown to differ. Prolonged endurance training increases the metabolism of the cells, inducing transformation from fast-twitch (type II) to slow-twitch (type I) fibres and leads to the development of more mitochondria within the cell. In contrast, strength training induces changes that lead to muscular hypertrophy and increased maximal strength and power of the muscle (Putman, Xu et al. 2004).

When comparing strength increases after strength or endurance training, or a combination of both, strength training increases muscle strength the most, followed by combination training, which is more effective in increasing strength, lean muscle mass, and muscular CSA than endurance training alone. Hence strength training might be more appropriate to counteract age-related losses in muscle mass and strength than endurance training (Putman, Xu et al. 2004). Additionally, the use of training techniques that have little rest in-between sets, as is the case with circuit training, provides endurance benefits in addition to the resistance training effect (Carey, Williams et al. 2000; Williams, Carey et al. 2007)

Resistance training brings about various physiological changes within the nervous system that play an important role in the development of muscle strength.

These changes have been collectively termed as “neural adaptations” and consist of a number of factors, such as selective activation of motor units, increased neural drive, synchronization, selective activation of muscles, increased rate coding (frequency), increased reflex potential and increased recruitment of motor units (Aagaard, Simonsen et al. 2002). It should also be noted that part of the ‘training effect’ and enhanced ability to perform heavy resistance strength training is due to an increased ability to coordinate other muscle groups involved in the movement, especially those which stabilize the body (Rutherford and Jones 1986).

As discussed in Section 2.6.1.1, aging affects motor units, in particular the motor neurons located in the lower body, with older persons showing diverse loss of motor activities, ranging from mildly reduced muscle strength and mass and decreased speed and dexterity to considerable motor impairment with concomitant disability (Roubenoff 2000).

Studies have shown that it is the reduced activation of these motor units that eventually leads to a progressive loss of the muscle fibres innervated by these neurons (Ansved and Larsson 1990; Cederna, Asato et al. 2001). This reduction in functioning motor units is particularly evident after the age of 60 years, resulting in consistent denervation and reinnervation of muscles and, eventually, reduction in muscle volume and strength (Lexell 1997).

It should be noted that while these studies show some strong evidence of progressive loss of motor neurons due to aging, they do not suggest a possible role or involvement of physical activity/inactivity in these degenerative changes. However, recently it has been hypothesized that changes in the neuromuscular system may play

an important role in the onset of sarcopenia and that physical activity can significantly alter the course of age-related motor decline (Buchman, Boyle et al. 2007).

Buchman and others examined whether exercise in later life was related to motor function in elderly subjects (Buchman, Boyle et al. 2007). They found that increased levels of physical activity were associated with a relatively slower rate of motor decline among elderly subjects. It was shown that each additional hour of physical exercise at baseline caused a significant reduction of about 5% in the rate of decline of global motor function, suggesting that the link between physical activity and motor decline is partly due to the impact of physical activity/inactivity on motor unit activation. Physical activity, therefore, can be considered as a modifiable risk factor that may help slow the decline in motor function that occurs with aging and therefore may be able to delay any adverse health outcomes associated with motor function decline.

One of the most widely observed effects of regular resistance training is increased muscle mass and size. Hence, regular resistance training is thought to be a possible intervention in helping prevent age-related loss in muscle mass and strength (Johnston, De Lisio et al. 2008).

Even among older adults, heavy resistance strength training has been shown to increase muscle mass, muscle strength, and anatomical CSA (for review see (Singh 2004), although the increases were not as pronounced as those seen in younger adults (Welle, Totterman et al. 1996; Kosek 2006; Folland and Williams 2007). While some studies suggest that the lower strength improvement is proportional to the smaller muscle size in older adults (Welle, Totterman et al. 1996), others claim that the

difference seen between young and old in response to heavy resistance strength training is due to a lower hypertrophic response training in the elderly (Kosek 2006).

One of the reasons for this observation may be associated with the intensity at which the training is conducted. For example, the change in functional capacity in adults between 46 and 90 years of age is significantly different when training is done at high intensities (>80% of $VO_2\text{max}$) as opposed to medium intensity training (60–75% of $VO_2\text{max}$) (Lemura, von Duvillard et al. 2000).

Several studies have outlined a possible association between muscle atrophy (i.e sarcopenia) in the elderly and a reduced rate of muscle protein synthesis (Dupont-Versteegden 2005; Degens and Alway 2006). It also appears that muscle contractile and mitochondrial protein synthesis rates reduce with advancing age, eventually resulting in reduced muscle volume, strength, and functional capacity (Rooyackers, Adey et al. 1996; Balagopal, Rooyackers et al. 1997).

Yarasheski's group demonstrated that three months of high-intensity resistance exercise training significantly stimulated the *in vivo* rate of muscle protein synthesis in the vastus lateralis of individuals aged 76–92 years (Yarasheski, Pak-Loduca et al. 1999). There have been several possible explanations for this resistance training-based increase in muscle protein synthesis in old age. It has been hypothesized that an increase in protein synthesis following resistance training acts as a protective response against increased rate of muscle protein wasting, which coincidentally, is common in elderly persons. Another explanation is that it is the rate of “mixed” muscle protein synthesis (including major protein types found in muscle cells, e.g. sarcoplasmic, mitochondrial and enzymatic proteins) that is significantly faster than that of contractile proteins (actin and myosin), causing an “overall” increase in muscle protein synthesis. A number of other studies have also shown that

resistance exercise leads to increased strength, fat-free mass, and rate of myofibrillar protein synthesis in middle-aged, older, and physically frail older adults (Yarasheski, Pak-Loduca et al. 1999; Candow and Chilibeck 2008; de Vos, Singh et al. 2008).

Changes in muscle mass and strength, possibly as a result of heavy resistance strength training, leads to physical improvements such as increased walking speed and stair climbing (Frontera, Hughes et al. 2000), and increased independence, even among frail elderly adults, in normal daily activities.

Other additional benefits from regular resistance training include improved bone health and concomitant reduction in risk for osteoporosis (ACSM 1995; Williams, Higgins et al. 2002); improved postural stability, thereby reducing the risk of falling and associated injuries and fractures (ACSM 1998; Liu and Latham 2009); and increased flexibility and range of motion (ACSM 1998; Chodzko-Zajko, Proctor et al. 2009).

Thus, while participation in physical activity may not elicit increases in the traditional markers of physiological performance and fitness in older adults (e.g., VO_2 max, mitochondrial oxidative capacity, body composition), it does reduce disease risk factors and increase functional capacity. Thus, regular exercise and physical activity contributes to a healthier, independent lifestyle; greatly improving the functional capacity and quality of life.

2.9.2 Nutritional Therapy/Supplementation

Dietary supplements and ergogenic aids have been made popular by athletes in the belief that they will provide a competitive advantage. Although the effectiveness of many of these dietary supplements is not supported by scientific proof, a few supplements have been shown to enhance muscular performance.

Importantly, these may also contribute to enhancing muscle performance in situations such as sarcopenia where muscle function has been affected. Two such supplements are creatine and whey protein.

2.9.2.1 Creatine Monohydrate

Creatine (methylguanidine-acetic acid) is a naturally occurring guanidino compound found in muscle in both the non-phosphorylated (Cr) and phosphorylated (PCr) forms (Harris, Hultman et al. 1974). Synthesis of Cr occurs in the liver, kidney, and pancreas from its precursors, glycine and arginine (Delvin 1992). As synthesis does not occur in muscle, Cr is transported in the blood stream and taken up by the muscle through a specific Na⁺-dependant Cr transporter (Fitch, Shields et al. 1968) called '*CreaT*'.

The existence of a specific transporter on the muscle cell membrane suggests that muscle cell levels of Cr could be influenced by Cr availability. Indeed, a study by Harris et al. (1992) was the first to demonstrate that supplementation of the diet with Cr at 20–30 g (taken in four to six 5 g doses) per day for five to seven days increased the skeletal muscle TCr by about 20%, with ~20–30% of this increase accounted for by PCr (Harris, Soderlund et al. 1992).

Numerous subsequent studies have confirmed the results of this early study, showing that dietary supplementation with Cr in a similar regime increases muscle TCr and PCr levels (Greenhaff, Bodin et al. 1994; Hultman, Soderlund et al. 1996; Vandenberghe, Goris et al. 1997). PCr and Cr stores play an important role in the regulation of skeletal muscle energy, metabolism and ATP homeostasis (Bessman and Savabi 1988; Wilson 1994; Brannon, Adams et al. 1997; Saks, Kongas et al. 2000) via the CK reaction shown in below:



Hence, it has been suggested that increasing muscle stores of Cr might influence muscle metabolism and performance. In fact, Cr supplementation has been shown to significantly improve performance in a variety of activities (Harris, Hultman et al. 1974; Sipila, Rapola et al. 1981; Bessman and Savabi 1988; Birch, Noble et al. 1994; Greenhaff, Bodin et al. 1994; Earnest, Snell et al. 1995; Casey, Constantin-Teodosiu et al. 1996; Brannon, Adams et al. 1997; Vandenberghe, Goris et al. 1997; Dangott, Schultz et al. 2000), particularly those involving repeated bouts of high intensity exercise (Birch, Noble et al. 1994). Reported improvements in performance with Cr supplementation have been ascribed to higher resting PCr levels and/or increased PCr resynthesis after repeated bouts of high-intensity exercise (Greenhaff, Bodin et al. 1994).

The combination of both higher resting PCr and increased PCr resynthesis between bouts should maximize the availability of PCr in the muscle, thus increasing ATP buffering capacity in muscles. Clearly, this should be of great benefit to the muscle, as decreased PCr availability, and hence a diminished ability to rephosphorylate ADP to ATP, is thought to be implicated in the loss in force output (i.e. fatigue), during high-intensity exercise (Hultman, Bergstrom et al. 1967).

Of particular interest to the current study, however, is the potential for Cr supplementation to enhance the muscle oxidative capacity and ADP-stimulated MAPR. The mechanism whereby Cr supplementation could increase muscle oxidative capacity is not immediately apparent, although it is known that Cr supplementation increases PCr resynthesis (Greenhaff, Bodin et al. 1994). The authors of this study suggest that the increased post-exercise PCr resynthesis is the result of increased post-exercise levels of Cr causing an increased flux through CK reaction (Greenhaff, Bodin et al.

1994). However, this is not likely as PCr resynthesis is a highly oxidative process reliant on ATP supply from oxidative phosphorylation in the mitochondria. The reliance of PCr resynthesis on the oxidative processes is demonstrated by the fact that post-exercise PCr resynthesis in ischaemic muscle is absent (Yamada, Kikuchi et al. 1993; Conley, Blei et al. 1997; Conley, Kushmerick et al. 1998). Therefore, increased post-exercise PCr resynthesis after Cr supplementation must be due to increased rates of oxidative phosphorylation. An increased oxidative capacity of the muscle, then, is most likely to be the result of an increase in V_{\max} (the maximum velocity of an enzyme catalyzed reaction) or a decrease in K_m for ADP.

Brannon (1997) found significant increases in CS activity (a Krebs cycle enzyme) as a result of Cr supplementation in rat soleus but not plantaris muscle. Significantly, Cr supplementation also increased performance in exercise likely to involve significant contribution from oxidative pathways for energy provision. Likewise, Guerrero-Ontiveros (1998) showed that Cr supplementation also produced a significant increase in the activity of the oxidative enzyme SDH, in both rat soleus muscle homogenates and isolated mitochondria.

Conversely, some studies appear to contradict the claim that Cr supplementation increases muscle oxidative capacity (Vandenbergh, Goris et al. 1997; Finn, Ebert et al. 2001). For example, Finn (2001) found no increase in CS activity after Cr supplementation. Additionally, Vandenburgh (1997) found no change in post-exercise PCr resynthesis after Cr supplementation.

However, no studies to date have examined the effects of Cr supplementation on MAPR, which would provide an overall view of ATP production from a range of

metabolic pathways. Hence, the mechanisms by which Cr supplementation may affect muscle oxidative capacity remain unresolved.

Another mechanism that may cause an increase in PCr resynthesis after Cr supplementation is a decrease in the K_m of mitochondrial respiration for ADP, especially in oxidative muscles. Currently, there is no research that has used MAPR to investigate this mechanism, so the possibility remains plausible.

Cr supplementation in humans is associated with an increase in body weight that is usually attributed to an increase in water retention (Balsom, Soderlund et al. 1995). Increases in muscle total creatine (TCr) increases the solute concentration inside the cell, thus increasing the osmotic flow of water into the cell, causing a state of hyperhydration resulting in swelling of the cell. It has been suggested that this swelling acts as a possible anabolic signal, stimulating an increase in protein synthesis (Haussinger 1996; Lang, Busch et al. 1998; Haussinger, Graf et al. 2001).

In support of this theory, one study demonstrated that Cr supplementation increased *in vivo* protein synthesis in muscle cell cultures (Ingwall, Weiner et al. 1974). Unfortunately, these authors did not measure cell volume and therefore no correlation between cell volume and protein synthesis could be made (Ingwall, Weiner et al. 1974). Additionally, the changes in muscle cell protein synthesis in this study were global and the authors did not measure protein synthesis from the different muscle fractions, i.e. contractile elements, mitochondria, or sarcoplasmic reticulum. If the increase in protein synthesis was isolated to the contractile elements only (causing an increase in muscle fibre size), this may be detrimental to aerobic metabolism, as the diffusion distance for substrates would be increased, hence the oxidative capacity of the cell decreased. Conversely, if protein synthesis was global and resulted in an increase in other proteins within the cell such as enzymes, enzyme complexes and

mitochondria, then the aerobic capacity would most definitely be increased. Such a suggestion potentially explains how Cr supplementation may increase muscle oxidative capacity. This may be achieved through an increase in cell volume, which subsequently increases the protein synthesis of oxidative apparatus, such as the mitochondria. However, the direct effect of Cr supplementation in this regard has not yet been studied. Additionally, one recent study found that Cr had direct, but mild, antioxidant properties (Lawler, Barnes et al. 2002), although further research is needed to establish this effect.

Therefore, if Cr does indeed have the ability to increase mitochondrial protein synthesis and/or antioxidant defenses within the muscle, then this would provide a very effective and safe remedy for the decreases in muscle mass and oxidative capacity seen with age.

2.9.2.2 Whey Protein

Whey protein has a high biological value (BV) score compared with other proteins such as casein, beef and fish (Renner 1983). BV is a measure of the protein's ability to retain nitrogen in the muscle (Colgan 1993), and a positive nitrogen balance is associated with muscle anabolism. Therefore, the high BV of whey protein renders it a valuable dietary source of protein.

Whey protein has been shown to increase muscle mass (Lands, Grey et al. 1999; Cribb 2000; Burke, Chilibeck et al. 2001; Hayes and Cribb 2008) and protein synthesis (Poullain, Cezard et al. 1989; Tipton, Elliott et al. 2004), and may, therefore, also have a benefit in restoring muscle function in the elderly. However, there appears to be no research in the literature assessing the effects of whey-protein supplementation on muscle oxidative capacity.

A number of studies have indicated that supplementation with whey protein increases muscle mass and protein accrual. For example, a study in resistance-trained males found that whey-protein supplementation resulted in significantly greater muscle hypertrophy than supplementation with carbohydrate only, leading to significant improvements in muscle strength (Cribb, Williams et al. 2007).

A study of whey-protein ingestion conducted specifically in elderly persons found that ingestion improved skeletal muscle protein accrual through mechanisms that are beyond those attributed to its essential amino acid content (Katsanos, Chinkes et al. 2008).

In a similar fashion to Cr, the amino acid glutamine, contained in high amounts in whey protein, influences muscle cell volume (Haussinger 1996; Low, Taylor et al. 1996; Haussinger, Graf et al. 2001) and thus protein synthesis. Therefore, it is possible that whey-protein supplementation might increase muscle oxidative capacity in a similar fashion to that suggested with Cr supplementation. In support of this, results from our laboratory showed significant increases in MAPR and CS activity in rat extensor digitorum longus (EDL, predominately, glycolytic type IIB) muscle following whey-protein supplementation (Smith, Hayes et al. 2001).

Whey protein has also been shown to act as an effective and safe Cys donor for glutathione replenishment during glutathione depletion in immune deficiency states such as that seen with HIV infection (Micke, Beeh et al. 2001) and cancer (Bounous 2000). Interestingly, however, at least one study has found increased tissue levels of glutathione and improved muscle strength after protein supplementation, suggesting that the improved performance is due to augmented antioxidant defenses (Lands, Grey et al. 1999).

As discussed in previous sections, mitochondria undergo substantial free-radical damage due to the production of ROS (Beal 1998). Therefore, an increase in antioxidant defenses would be beneficial to the mitochondria and protect them from oxidative damage. If so, an increase in glutathione following whey-protein supplementation may increase muscle oxidative capacity. Results from our laboratory demonstrate that an effect occurs predominantly in glycolytic rather than oxidative muscle (Smith, Hayes et al. 2001). This might be explained by evidence that shows that mitochondria in oxidative fibres, such as the soleus, are less prone to oxidative damage compared with glycolytic fibres (Lawler and Demaree 2001). Therefore, whey-protein supplementation appears to offer greater benefit to glycolytic muscles. This is important as the decreases in fibre size with age in humans are predominantly in the glycolytic fast type IIX fibres (Essen-Gustavsson and Borges 1986; Kovanen and Suominen 1987; Carmeli and Reznick 1994). Therefore, it appears that whey protein would be particularly important in the management of sarcopenia

As discussed in Section 2.9, the decrease in muscle mass and oxidative capacity observed with age in human skeletal muscle may be a result of decreases in muscle cell volume and muscle protein synthesis. Therefore, reversal of the decrease in cell volume and/or rates of muscle protein synthesis could be essential in restoring muscle function. Additionally, maintenance of muscle cell volume and/or protein synthesis could prevent the associated disabilities that come with increased frailty in the aged, and bring about a restoration of an individual's independence.

Supplementation with whey-protein is likely to alter cell volume and protein synthesis and may also act to preserve muscle mass and improve oxidative capacity

through its ability to increase the muscle antioxidant defenses and therefore minimize any free-radical damage to cells.

Although supplementation with whey protein seems likely to be a good approach in the mitigation of age-related muscle deterioration, a number of gaps exist in the literature in this regard. In particular, it is not currently clear whether whey supplementation actually increases protein levels within the muscles or directly increases muscle oxidative capacity of isolated skeletal muscles. Furthermore, no studies to date have investigated the effects of whey protein on muscle oxidative capacity.

2.10 The Convergence of Two Epidemics: Sarcopenic Obesity

Aging and other associated medical conditions are predicted to become significant medical concerns in many countries, especially in aging populations in developed countries (Buckley 2001).

Obesity in people across all age groups, including in the elderly, is on the rise, and is a significant health concern worldwide (Chumlea and Sun 2004). Estimates have suggested that by 2010, approximately 33–39% of people in the United States over 60 years of age will be obese (Zamboni, Mazzali et al. 2008). This further complicates the problems associated with sarcopenia, as obesity puts a severe strain on the mobility of the individual and, when combined with decreasing muscle strength, it can lead to severe disability. We have evidence also that in obesity, especially in diabetic obese individuals, skeletal muscle mitochondria are smaller when compared to lean individuals (Kelley, He et al. 2002).

Obesity is associated with disorders that confer morbidity and increased mortality (Pi-Sunyer 1993; Must, Spadano et al. 1999). Metabolic syndrome is a good example of the metabolic dysregulation that can occur in overweight or obese adults as a result of long-term diabetic and cardiovascular irregularities (Peytremann-Bridevaux and Santos-Eggimann 2008). Other obesity-associated co-morbidities include osteoarthritis, sleep apnea and cancer (Chau, Cho et al. 2008). Moreover, obesity also contributes to sarcopenia in elderly people (Roubenoff 2004; Zamboni, Mazzali et al. 2008).

2.10.1 Pathogenesis of Sarcopenic Obesity

Sarcopenic obesity, as the name suggests, has a dual etiology. The gradual decline in muscle strength with age results in decreased physical activity, which, if dietary habits are not changed, leads to excess energy intake. The consequent obesity can also result in low-grade inflammation, since adipose tissue can promote the production of pro-inflammatory cytokines. (Schrager, Metter et al. 2007; Zamboni, Mazzali et al. 2008)

Loss of structural integrity and change in the quality of muscle fibres with age is the principal cause of sarcopenia. However, obesity also leads to infiltration of fat cells directly into skeletal muscles (Zamboni, Mazzali et al. 2008). CT scans of skeletal muscles of sarcopenic obese individuals show significant intra-muscular fat, which results from infiltration of fat into the muscle, leading to greater loss of structural integrity. Loss of muscle function can also be gauged in terms of reduction in muscle mitochondrial oxidative and phosphorylation potential (OXPHOS), as discussed in a review by Chanséaume and Morio (2009). In this review, the authors have looked at evidence to suggest that reduction in muscle mitochondrial OXPHOS can lead to

altered gene expression leading to sarcopenic obesity. Interestingly, the authors also list nutrition as a factor that promotes sarcopenic obesity. Excess sugar intake and diet-induced obesity has been linked to the down regulation of genes involved in OXPHOS activities (Chanseaume and Morio 2009). Although, these changes are more prominent in oxidative muscles than glycolytic ones, the association between diet-induced obesity and sarcopenia is noteworthy.

Given the dual etiology of sarcopenic obesity, strategies to combat it need to involve both the reduction of excess energy intake as well as the build-up muscle strength and quality (see Chapter 7).

2.10.2 Dietary Interventions

A commonly used and easily accessible dieting approach is the use of meal replacements. Meal replacements control a proportion of the diet with a calorie controlled liquid meal (i.e. shake) or food (pre-prepared meal or bar). In some cases, depending on the weight of the individual, and the level of dietary control needed, all meals in the diet are replaced with meal replacements. Without medical supervision, complete dietary replacement can be dangerous, and have resulted in deaths in some cases (Wadden, Stunkard et al. 1983; Wadden, Stunkard et al. 1983).

2.10.3 Meal Replacements

The large majority of meal-replacement programs in Australia are offered direct to the consumer through pharmacy retailers, often without any supervision. This can be dangerous as the general approach is to market the product based on the amount of weight lost in the shortest amount of time (i.e. the biggest loser wins).

Currently available meal replacements provide the bare minimum in calorific and nutritional content as required by the food standards code for Australia and New Zealand (FSANZ 2.9.3) for meal replacement products (FSANZ 2007). The calorific content of these meal replacements has been kept to the minimum to promote the greatest weight loss possible. However this has been achieved at the unnecessary expense of the meal's nutritional value, which puts the consumer at risk of not meeting their daily nutritional requirements.

A recent 2009 paper published in *Nutrition & Dietetics* assessed the nutritional value of 17 formulated meal replacements sold in Australian pharmacies, and found that, whilst these products are compliant with food standard (FSANZ 2.9.3) for formulated meal replacement, the large majority were nutrient deficient (Collins, Jones et al. 2009). For example, the Tony Ferguson Weight Loss program did not meet the nutritional requirements for folate, magnesium or calcium, and did not meet the requirement for thiamine for men and iron for women (Collins, Jones et al. 2009). Importantly, this survey noted that programs that promoted the use of shakes for total diet replacement did not meet the recommended daily intake (RDI) for either macro- or micronutrients in the diet. For example, when the Optifast vanilla shake was recommended as a total meal replacement, it did not meet the RDI or adequate intake (AI) levels for protein, calcium, phosphorus, potassium or magnesium for men, even when consumed with the recommended two cups of low-starch vegetables (Collins, Jones et al. 2009).

Interestingly, calorific restriction (abstinence from overeating but intake of a nutritionally balanced diet) is thought to slow the aging process (Weindruch 1996; Holloszy and Fontana 2007). It is believed that calorific restriction can increase the

life span of individuals by preventing immunosenescence (Pahlavani 2000) and minimizing free radical damage (Seo, Hofer et al. 2006). Although this strategy has been proven in rodents (Weindruch 1996), there is only preliminary evidence to support the theory in humans (Hollooszy and Fontana 2007).

2.10.4 Dietary Protein and Obesity

The possible benefits of whey protein supplementation in preventing age-related decreases in muscle mass were discussed in Section 2.9.2.2. A higher protein diet may also assist in dealing with the obesity by 1) preserving lean muscle mass (Jean, Rome et al. 2001; Lejeune, Kovacs et al. 2005), 2) increasing satiety and controlling appetite (Westerterp-Plantenga, Rolland et al. 1999; Lejeune, Westerterp et al. 2006), and 3) increasing diet-induced thermogenesis (Dulloo and Jacquet 1999; Stock 1999).

Protein is an essential molecular building block of all cells and molecules that integrate intracellular and extracellular functions such as enzymes and peptide hormones (World Health Organisation 2007). The RDI for protein is 1.0 g/kg of body weight (NHMRC 1991). Whilst it is stated that RDI levels support the nutrient requirements of healthy persons, the daily protein requirements for elderly individuals may be higher due to poor absorption via the gastrointestinal tract, decreased dietary intakes and increased levels of protein degradation (Hayes and Cribb 2008). Additionally, the RDI levels for protein may not meet the requirements of persons in hypercatabolic states (induced by surgery, infection or trauma, for example) where a negative energy balance exists, and considerable lean tissue wasting occurs, requiring additional protein fortification.

Increasing plasma amino acid concentration, particularly in branch-chained amino acids, has been shown to decrease muscle turnover (Laviano, Muscaritoli et al. 2005). Given that a hypocaloric diet designed to promote weight loss can be considered metabolically equivalent to a wasting condition, then an increase in the daily requirement for protein would assist in minimizing lean body mass loss.

Protein has also been shown to induce a greater degree of post-meal satiety compared with carbohydrate and fat (Stubbs and Whybrow 2004; Bowen, Noakes et al. 2007), particularly in dosages exceeding 40 g per serving (Bowen, Noakes et al. 2007). It has been hypothesized that protein-induced satiety could be due to alterations in the secretion of gut neuropeptides, in which an increase in the gut hormones cholecystikinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) decreases food intake and promotes satiety while an increase in the gut hormone ghrelin increases hunger and promotes food intake (Lenard and Berthoud 2008).

The presence of protein and fat in the small intestine (duodenum) stimulates the release of CCK, which causes the release of digestive enzymes and bile from the pancreas and gallbladder, and has been shown to inhibit food intake (Lenard and Berthoud 2008). Whey protein was shown to increase the duration of the post-meal elevation of CCK when compared with an isocaloric pre-load of glucose (Bowen, Noakes et al. 2007). Additionally, there was a strong correlation between whey or casein and post-meal CCK release, with appetite ratings as reported on a post-meal hedonic scale (Bowen, Noakes et al. 2006).

Another important gut hormone and inhibitor of food intake is GLP-1. Like that of CCK, GLP-1 secretion is dependent on the presence of nutrients in the small intestine. The effects of GLP-1 appear to be related to the chronic intake of higher

dietary protein (Lejeune, Westerterp et al. 2006), and not just to the acute post-meal effect of dietary protein (Smeets, Soenen et al. 2008).

Hall (2003) showed that the energy intake from an *ad libitum* buffet meal was significantly less 90 minutes after a liquid preload containing 48 g of whey protein compared with an equivalent liquid preload containing casein. The decreased energy intake in the whey group was shown to be associated with the release of CCK and GLP-1, demonstrating a greater satiation effect of whey protein compared with casein.

Whey protein also produces a prolonged suppression of ghrelin, which has been shown to decrease food intake, compared with fructose- and glucose-based beverages (Bowen, Noakes et al. 2007). Interestingly, the prolonged satiety effect of the whey-protein beverage was removed when 50% of the whey protein was replaced with fructose. This suggests that the satiety effect may be related to the amount of protein ingested (Bowen, Noakes et al. 2007) and not simply the energy intake. It has been suggested that an elevated concentration of circulating amino acids, which cannot be channeled into protein synthesis, may serve as a satiety signal regulating food intake. Indeed amino acids, such as leucine, have been shown to inhibit central mechanisms related to hunger (Morrison, Xi et al. 2007).

Hence, it is proposed that a nutrition program that promotes weight loss through calorific restriction while suppressing hunger and increasing satiety between meals may actually help regulate body weight whilst preserving lean muscle mass.

2.11 Literature Review Summary

Mitochondria are vital cell organelles that are responsible for carrying out various cellular functions necessary for survival. A number of structural and

functional changes occur in the mitochondria with age, including changes in mitochondria structure and number, changes in mitochondrial enzyme activity, changes in mitochondrial oxidative capacity, and changes in mitochondrial ATP production rate (MAPR). These changes have an important impact on muscle function and on physical degeneration with aging.

The efficiency of skeletal muscle movement can be measured in a number of ways, including an assessment of enzyme (ATPase) activity. Muscle length and speed of muscle contraction can be measured through use of isokinetic dynamometry, and muscular function can also be assessed by analysis of body composition.

The redox activity of a cell determines the impact of damaging ROS on the cell, and therefore methods for the accurate assessment of a cell's redox state are vital if we are to understand the aging process at a cellular level. The redox potential (E_h) of a cell can be calculated from the relative concentrations of GSH and GSSG using the Nernst equation. This tells us whether a cell is in a pro-oxidative or pro-reductive state, and therefore how effectively it will be able to counteract the effects of ROS.

Available evidence suggests that key potential interventions to arrest or reverse the loss of muscle function that is associated with aging include resistance training and supplementation with Cr or whey protein.

2.12 Study Aims & Hypotheses

The current research project was implemented following careful consideration of the evidence presented in this literature review. The interventions of supplementation and resistance training, and the methods used to measure the effects of these interventions, were selected based on the available data discussed above.

The aims of this research are to investigate possible underlying mechanisms that lead to the age-related decrease in muscle function (muscle strength and muscle oxidative capacity) in humans. In particular, the research focuses on the potential benefits of interventions such as resistance training, with or without additional dietary Cr or whey-protein supplementation, to offset the effects of aging on muscle function. In light of a newly identified association of sarcopenia and obesity, we also investigate the effects of two different meal replacement diets on weight loss and lean mass preservation in obese individuals. The complete body of research presented herein comprises four distinct studies, which can be summarized as follows:

Study 1: The Effects of Creatine and/or Whey-Protein Supplementation on Muscle Oxidative Capacity with Aging in Humans (Chapter 4)

The first study investigates whole body aerobic capacity, body composition, mitochondrial ATP production rate (MAPR), citrate synthase and mitochondrial protein content in aged male subjects. The study also investigates the response to supplementation with Cr monohydrate, whey protein or placebo in these subjects.

It is hypothesized that six weeks of supplementation with Cr and whey protein would achieve beneficial effects in terms of body composition, muscle oxidative capacity (measured by MAPR), mitochondrial function (measured by MAPR and CS activity), and muscle protein content in healthy aged individuals when compared with placebo.

Study 2: The Effect of Whey-Protein Supplementation and Resistance Training on Muscle Protein Content and Muscle Strength with Aging in Humans (Chapter 5)

The second study investigates body composition, muscle strength (functional and isolated), muscle force and velocity (muscle power), mitochondrial ATP production rate (MAPR), citrate synthase, histochemistry and mitochondrial protein content in aged male subjects. This study also investigates the response to supplementation with whey protein or placebo and 12-weeks of resistance training (three times per week) in these subjects.

It is hypothesized that a 12-week resistance-training program in combination with whey-protein supplementation would achieve beneficial effects in terms of body composition, muscle strength, muscle oxidative capacity (measured by MAPR), mitochondrial function (measured by MAPR and CS activity), and muscle protein content when compared with a resistance-training program alone.

Study 3: The Effect of Whey-Protein Supplementation and Resistance Training on Intracellular Redox Status with Aging in Humans (including Optimization of Methods)

The third study can be separated into two parts. The first part of the third study involves the optimization and further development of methods to measure intracellular redox status by direct measurement of thiol and disulfide concentrations. The development and refinement of these methods allowed the investigation of intracellular redox status in aged skeletal muscle following dietary supplementation with whey protein or placebo and 12-weeks of resistance training in aged male subjects. Hence, the second part of the third study investigates plasma (extracellular) redox status in aged male subjects in response to supplementation with whey protein or placebo and 12-weeks of resistance training.

It is hypothesized that a 12-week resistance-training program in combination with whey-protein supplementation would arrest age-related changes to the cellular redox processes, in terms of plasma or tissue thiol or disulfide levels or plasma or tissue redox states.

Study 4: The Effect of Two Meal Replacement Diets on Lean-Mass Preservation and Subjective Satiety in Obese Males and Females (Chapter 7)

The fourth study investigated the effects of two different meal replacement diets on lean-mass preservation and subjective satiety over a 4-week long period in obese males and females.

It is hypothesized that a 4-week protein-enriched meal replacement shake and diet program would promote weight loss and lean mass preservation in overweight and obese adult participants, safely preventing the onset and progression of sarcopenic obesity.

2.13 Contribution to Knowledge and Significance to the Wider Community

Although the decreases in muscle function (muscle strength and muscle oxidative capacity) with age are well documented, the mechanism(s) that underlie such changes are not fully defined. Therefore, research at a cellular level is needed to identify possible initiators of these age-related changes. We anticipate that the findings of this research will contribute to the body of knowledge aimed at reversing or preventing sarcopenia. It is hoped that this research will allow a safe and alternative treatment regimen to be developed to reverse or prevent sarcopenia and help to maintain the functional integrity of the muscle in the aging individual.

Chapter 3

Experimental Methods

3 Experimental Methods

3.1 Participants

A total of 37 participants were recruited for Studies 1 ($n=20$) and 2 ($n=17$). All participants were healthy male volunteers between 50 and 74 years of age mainly of Australian and European decent from the Western Suburbs region in Melbourne, Australia.

Participants were recruited from a variety of sources. Initially, participants were recruited from a database of aged participants (>55 years of age) who had previously been contacted and who had indicated that they would be interested in participating in research Studies. This database was compiled and maintained by Dr. Andrew Williams, previously of the Exercise Metabolism Unit where all Studies were conducted. Additional participants were recruited from a series of articles published in local newspapers and by using the Victoria University global staff email system. Study 3 was an extension of Study 2, and conducted in the same population.

A total of 22 participants were recruited for Study 4. All participants were healthy male and female volunteers aged between 22–54 years, also mainly of Australian and European decent from the Western Suburbs region in Melbourne, Australia. Participants were recruited by public advertisement in a local newspaper. All participants were required to complete a detailed medical questionnaire.

The participants were not paid to be involved in the studies. However, reimbursement was provided to cover any medical expenses that occurred as a result of having to gain prior medical consent to take part. For study 4, and a month's

supply of the treatment meal replacement was offered to all volunteers who successfully completed the studies.

The Human Research Ethics Committee of Victoria University of Technology approved all procedures and protocols conducted at Victoria University during each of the Studies.

3.2 Diet and Supplementation

Apart from the intake of control or treatment products, each participant was instructed to maintain normal dietary intake throughout both Studies 1 and 2/3. In Study 4, participants were assigned to a specific meal replacement shake and diet program. For Studies 1 and 2/3, participants were asked to complete a 24-hour food recall exercise on two week days and one weekend day during the week prior to commencement of the study. In Study 4, food intake was recorded in a participant diary that was given to each participant prior to the commencement of the study.

Dietary analyses were completed by using Foodworks 2007 software (version 6; xyris software, Australia) to determine daily energy, protein, carbohydrate and fat content of the diet for all studies.

In Studies 1 and 2/3, after taking baseline measurements participants were matched for body mass index (BMI) using height and weight measurements and then randomly assigned to one of the supplements below in a double-blind fashion.

- *Placebo (Study 1 and 2):* A carbohydrate (CHO) supplement containing dextrose
- *Creatine + CHO (Study 1 only):* Creatine supplementation was given at a loading dosage (7 g creatine, 3 times per day) for the first week (acute phase). Such a loading dose has previously been shown to successfully increase intramuscular

TCr levels by roughly 30% (Harris, Soderlund et al. 1992; Hultman, Soderlund et al. 1996). Following this one-week loading regime, participants consumed 2 g creatine per day (maintenance dose) for the remainder of the study. Creatine appears to be more effective when carbohydrate (such as dextrose) is added to the supplement dose (Tarnopolsky, Parise et al. 2001). The addition of carbohydrate to creatine supplements potentiates muscle-building effects because of the insulin-stimulating effect of the carbohydrate (Tarnopolsky, Parise et al. 2001). Insulin secretion enhances nutrient uptake and inhibits protein breakdown within muscle (Tarnopolsky, Parise et al. 2001). Therefore carbohydrate (in the form of dextrose, as in the placebo group) was added to the creatine supplement group as well as acting as a control.

- *Whey protein (Study 1 and 2/3):* Whey protein (AST Sport Science, VP2™, Evergreen, Colorado, USA) was given at a dosage of 1.5 g/kg/day. This dosage of whey protein has previously been shown in our laboratory to produce a significant increase in lean body mass and strength in resistance-trained young men, compared with aged matched controls using an equivalent dosage of casein (Cribb and Hayes 2006). After initial body weighing and dosage calculation, participants were shown the exact amount of supplement to be consumed on a daily basis.

In Studies 1 and 2/3, all supplements were colour- and flavour- matched, and provided to participants in identical, unmarked containers. To ensure that the studies were double blinded, Dr. Alan Hayes coded the supplements before they were distributed to the participants. Participants were advised to measure out their assigned dosages using scoops that were provided with the supplements. Participants were asked to mix the supplement in water and to consume it in divided dosages three times per day in the morning, afternoon and evening, with or without food.

Participants were asked to return their empty containers as a measure of compliance. The whey, creatine and carbohydrate employed for this study are commercially available supplements and were provided by AST Sport Science Evergreen, Colorado, USA.

In Study 4, participants were randomly assigned to one of the following supplement groups in double-blind fashion.

- *Control*: A representative of a commonly available meal replacement product (Tony Ferguson Weightloss program™ Meal Replacement Shakes, Tony Ferguson, Australia).
- *Treatment*: A high-protein modified meal replacement

The macronutrient composition of the control and whey protein meal replacements is shown in Table 3.1.

Table 3.1: Macronutrient composition of meal replacement shakes and diet.

Macronutrient composition	Control	Treatment
Energy (kcal)	203	221
Weight (g)	52	62
Protein (g) (%energy)	17 (34)	41 (74)
Carbohydrates (g) (%energy)	27.9 (55)	12 (22)
Fat (g) (%energy)	2.2 (10)	0.8 (3)

The ingredients for each meal replacement were flavoured (vanilla, chocolate, or espresso) and dry powder blended by a professional flavour house (FlavourMakers, Braeside, VIC, Australia) and then packed and labeled (e.g. product A flavour ‘X’) into sachets by a professional downpacker (Manfields, Braeside, VIC, Australia)

according to the required weight specifications for each of the meal replacements (see Table 3.1). In both the control and treatment groups, participants were given a choice of the three meal replacement flavours to choose from for ease of use. All supplements were colour- and flavour-matched and provided to the participants in identical, unmarked sachets that only contained a randomized code and flavour for blinding purposes. Participants were advised to mix the supplement in 250–300 mL water and to consume it according to the directions outlined in their meal replacement program (Table 3.2). All ingredients were sourced and provided by FlavourMakers, Braeside, Australia. All packing supplies were sourced and provided by Mansfields, Braeside, Australia.

Both meal replacement program groups were hypocaloric and designed to deliver approximately 1300 kcal per day for both groups. Participants in both meal replacement groups were advised to consume two meal replacements per day in addition to meals and snacks as outlined in meal replacement diet program booklet (see Appendix A & B). Participants in each group were given a list of allowable and non-allowable food groups that was to be used to help with their food choice (see Appendix A & B). The control group was asked to choose from a wide variety of foods, whereas the treatment group was asked to choose foods that were higher in protein. Participants were also given a sample meal plan and recipes (Appendix A & B) with the treatment group's meal plan recommending an extra serving of protein (Table 3.2).

The meal plans (Table 3.2) were as follows:

- *Control*: two meal replacements per day (breakfast and lunch) and one meal plus snacks
- *Whey protein*: two meal replacements per day (breakfast and afternoon snack) plus two meals (lunch and dinner)

Table 3.2: Sample meal plans for each of the meal replacement groups.

Control sample meal plan		Treatment group sample meal plan	
Meal		Meal	
Breakfast	Meal Replacement (shake)	Breakfast	Meal Replacement (shake)
Snack	Small serve of fruit	Snack	-
Lunch	Meal Replacement (shake)	Lunch	Salad or steamed vegetables + serve of protein
Afternoon Snack	Vegetable sticks and dips	Afternoon Snack	Meal Replacement (shake)
Dinner	Salad or steamed vegetables + serve of protein	Dinner	Salad or steamed vegetables + serve of protein
Desert/snack	Diet jelly	Desert/snack	-

3.3 Resistance Training Protocol

The 12-week resistance training program (used in study 2/3) was prescribed and based on the Living Longer Living Stronger Program provided by the Council of the Aging (COTA) through selected fitness centers in Melbourne and Victoria. The training program involved three supervised exercise sessions per week on two different campuses of Victoria University (Footscray and Werribee).

Two weeks prior to the commencement of the resistance-training program, all participants took part in a familiarization program. The main aim of this program was to familiarize participants with the different resistance exercises using weight-training machines and also to teach them to perform the 1-repetition maximum (1RM) test. This program also aimed to familiarize participants with the correct technique to be used in the exercises for the 12-week resistance-training program.

Following familiarization and baseline testing, participants were assigned to take a dietary supplement or placebo that they then consumed during the 12-week resistance-training program. Participants were assigned to work in pairs and allocated to one of the training locations; Footscray campus (n=10) or Werribee campus (n=7) based on their preferred location and for participant convenience. Training sessions commenced with a 5- to 10-minute warm up using a stationary exercise bike. Sessions included some flexibility work and four core exercises: leg press, leg extensions, lat pull down and bench press. These exercises were chosen because they are compound movements that involve the larger muscle groups of the upper body (lat pull down and bench press) and lower body (leg press). A range of optional exercises were also included, and participants

performed four to six exercises at each supervised session (Table 3.3). Participants completed two sets of 8 to 10 repetitions of each exercise with roughly a 3 mins rest between sets. The 12-week training program used a progressive overload system. With each exercise, participants started by lifting 8 repetitions ((approximately 80% of their baseline 1-RM) to failure. Following this, participants were encouraged to increase the resistance of the exercise once they reached the upper repetition range (i.e 10 repetitions) and to increase the weight to a weight that they could only lift for 8 repetitions to failure. This progressive overload cycle was continued throughout the 12-week resistance-training program.

Table 3.3: 12-Week Resistance Training Program.

12-week training protocol: 2 sets of 8 to 10 repetitions*		
Monday	Wednesday	Friday
Leg press	Leg press	Leg press
Leg extension	Leg extension	Leg extension
Lat pull down	Lat pull down	Lat pull down
Bench press	Bench press	Bench press
Hamstring curl	Bicep curl	Abdominal crunch
Shoulder press	Tricep push down	Lower back hyperextensions

*each session included a 5–10 minute warm up session before and after training plus flexibility exercises.

3.4 Body Composition testing

3.4.1 Dual Emission X-ray Absorptiometry (DEXA)

Whole-body composition measurements were determined using a Hologic QDR-4500 dual energy X-ray absorptiometry (DEXA; Figures 3.1 and 3.2) with the Hologic version 7, REV F software (Waltham,MA).



Figure 3.1: Dual Energy X-ray Absorptiometry (DEXA; Hologic QDR-4500)

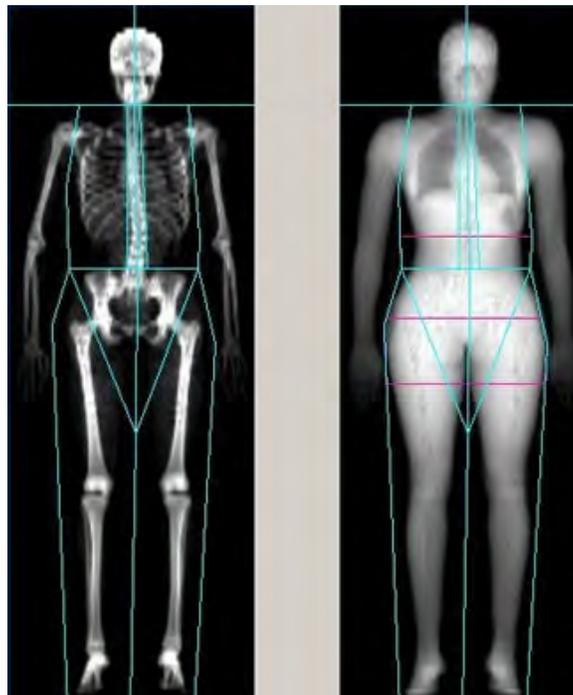


Figure 3.2: Measurement of Segmental Body Composition using Dual Energy X-ray Absorptiometry (DEXA; Hologic QDR-4500)

All scans were performed on the same apparatus, located at Victoria University's Biomechanics Laboratory, by the same licensed DEXA operator.

Quality control calibration procedures were performed on a spine and step phantom prior to each testing session according to procedures previously described (Ellis and Shypailo 1998). Participants were positioned exactly in the manner as recommended by the manufacturer to ensure reproducibility of position for subsequent scans. The DEXA performs by scanning all regions of the body to determine the amount of bone mass, fat mass and lean body mass within each region. Following the scan, Hologic software was used to assess the composition of specific regions of the body (Figure 3.2). Body fat percent is calculated by the software, dividing the amount of measured fat mass by total scanned mass (sum of bone mass, fat mass and lean mass). Participants were scanned at the same time of the day and asked to eat and drink the same amount of food before the scan (if not fasted) to maintain consistency between tests. For longitudinal studies in which small changes in body composition are to be detected, whole body scanning with this instrument has been shown to be accurate and reliable (precision errors 0.8–2.8%) (Prior, Cureton et al. 1997; Ellis and Shypailo 1998).

3.4.2 Bioelectrical-Impedance Analysis (BIA)

While DEXA is generally regarded as the gold standard in body composition measurements and provides highly accurate and reproducible measurements, practicality of use for a large number of participants is limited. Also, since DEXA involves the use of radiation, it should not be used for multiple measurements in a short space of time. Thus, as Study 4 required body composition testing once a week for four weeks, DEXA was

deemed to be inappropriate due to concerns over radiation levels. Alternatives to DEXA that are practical and safe include bioelectrical impedance analysis (BIA), and other estimates such as the use of skin folds. As BIA is considered the next best practical method after DEXA, this method was selected for Study 4. BIA can be used for quick, safe measurement of body composition (total body weight, lean mass, fat mass, body water, and bone mineral content). However, BIA values should be interpreted with caution. BIA can be quite variable, with 95% confidence intervals for the prediction of four-compartment percentage body fat (BF) from BIA being in the region of $\pm 5\%$ BF (Jebb, Cole et al. 2000; LaForgia, Gunn et al. 2008).

Body composition measurements using BIA were taken at baseline and then every week during the four-week trial with a seven-day interval between measurements. Participants attended each session at the same time in a fasted state wearing the same light clothing that they wore at the last session. Participants were asked to refrain from alcohol consumption and strenuous activity for 24 hours before each visit. Participants were also asked to refrain from consuming caffeine on the morning of the test, and to eat their last standard meal no later than 10 hours before their visit and to fast thereafter (water permitted) until body composition measurements were taken the next day. Importantly, because estimates of fat free mass (FFM) are derived from total body water (TBW) measurements in BIA testing, participants were also asked to consume 500 mL water on the morning before the visit to ensure adequate hydration.

The weight and height of the participants were measured at the first session. BMI was calculated as weight (kg) divided by height squared (m^2). Body composition was then measured using BIA. BIA was measured using a TANITA BC-558 MA instrument

(Tanita Corporation of America Inc., US) which provides a readout for fat mass (FM), fat-free mass (FFM), fat percentage, bone mass, body water content and total body weight (TBW) using manufacturers' algorithms. With this instrumentation, impedance is measured using four separate electrodes with the participant standing onto the scales and holding hand grips that are attached to the side of the scales. The system base has two stainless-steel rectangular foot-pad electrodes fastened to a metal platform set on force transducers for weight measurement. Each of the extremity hand-grips also contains electrodes fastened to them. For measurement of body composition, a signal is passed through each of the electrodes and impedance across the participant's tissues is measured.

The participant's age, gender, and height were entered into the instrument. Participants were then asked to stand barefoot on the metal footplates of the instrument while holding the handles for ~1 min. The computer software then uses the measured impedance, the programmed participant's sex and height, and the measured weight to calculate body composition estimates based on manufacturers' algorithms.

In addition, waist and hip measures were calculated by measuring the waist at its narrowest and the hips at their widest point using a measuring tape. The waist-to-hip ratio was calculated by dividing the waist measurement (cm) by the hip measurement (cm). These measurements correlate well with laboratory-based measures (CT and MRI) of abdominal adiposity (Lapidus, Bengtsson et al. 1984; Lean, Han et al. 1995). Waist circumference (WC) in particular is thought to be a more reliable measure of obesity-associated comorbidity risk than the commonly used body mass index (BMI) (Lofgren, Herron et al. 2004; Brenner, Tepylo et al. 2010).

3.5 Measurement of Aerobic Capacity

Peak oxygen consumption (VO_2 peak) was determined in a graded cycle exercise test to volitional exhaustion using procedures in common use in Victoria University's Exercise Physiology Unit. The test began with a 3-min warm-up exercise performed at very low intensity. The test commenced with a workload of 20 W and was increased by 20 W/min until a respiratory exchange ratio (RER) of 1.0 was reached. Workload was then increased by 10 W/min until volitional exhaustion or until the participant reached a perceived exertion level of 17 (very hard) on the Borg 6–20 scale of perceived exertion (Borg 1970). During the test, the participants' ECG output was monitored continuously, and respiratory gases collected continuously for gas analysis and $\text{VO}_{2\text{max}}$ determination at 1-min intervals during the test. Measurements were made every 30 s of VO_2 (OM-11 Medical Gas Analyzer, Beckman, CA, USA), carbon dioxide production (VCO_2) (LB2 Medical Gas Analyzer, Beckman, CA, USA), and ventilation (V_E [BTPS], 47304A respiratory transducer with Fleisch pneumotach, Hewlett Packard, USA). Respiratory gas analyzers were calibrated using a two-point calibration procedure involving room air and gas cylinders containing β -gases in the range of 12–15% O_2 and 4–5% CO_2 . β -gases had been previously checked against an α -standard gas. A 3-L calibration syringe was used to calibrate respiratory volumes. A qualified medical physician was present during all tests to monitor ECG continuously (EK43 Multiscriptor 12 lead ECG, Hellige, Belgium) and participants' perception of exercise-related symptoms was monitored via the Borg scale (Borg 1970).

3.6 Measurement of Muscle Strength

All muscle testing was conducted at Victoria University. Isokinetic and isometric strength testing took place in Victoria University's Exercise Physiology Unit. Functional strength measures were conducted in the Victoria University's fitness and Aquatic Center, Footscray Park campus. Prior to all muscle strength tests, participants underwent a familiarization session of the testing equipment.

3.6.1 Isometric and Isokinetic Strength

Isometric and isokinetic muscle strength were examined by voluntary isometric and isokinetic knee extension using a Cybex dynamometer (Cybex International Inc. Ronkonkoma, New York; Figure 3.3). Unlike free weight strength testing, these tests require minimal time to complete and cause minimal fatigue from repetitive testing, while combined they reflect the general contractile properties of the muscles used (Hakkinen 1993; Clarkson and Sayers 1999; Warren, Lowe et al. 1999; Raastad and Hallen 2000).

Participants were asked to confirm leg dominance before all strength measurements were performed on their dominant leg. All participants were seated during the testing protocols, and the dynamometer axis of rotation was orientated in alignment with their knee. The point of contact between the participant's lower leg and the padded lever arm was at a fixed distance relative to the participant's lower limb length, slightly above their ankle. All positions, padded lever arm placement, seat height and position, and lever arm height and position were recorded during the baseline testing for each participant and used with subsequent measurements to maintain consistency.



Figure 3.3: Isolated Strength Measurements as Performed on the Cybex Dynamometer (Cybex International Inc. Ronkonkoma, New York). Taken from Cooke (2005) with permission.

All participants were encouraged verbally throughout the testing procedures, and instructed to place their hands on the sidebars, and to work as hard and as fast as possible against the resistance of the dynamometer.

Measurements of isokinetic knee extension and flexion torque were performed at $60^{\circ}/s$ ($1.57 \text{ rad}/s$) velocity torque in one continuous kicking motion. The range of motion for knee extension and flexion was from 90° to 0° and 0° to 120° , respectively. Contact between the participant's lower leg and the padded lever arm was at a fixed distance relative to the participant's lower limb length, slightly above their ankle (Figure 3.4).

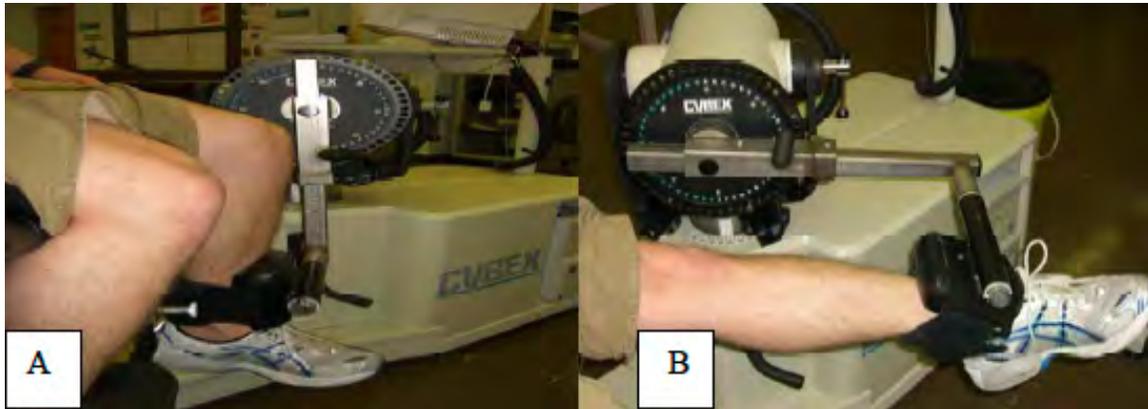


Figure 3.4: Range of Movement for the Knee Extension from (A) 90° to (B) 0°.
Taken from Cooke (2005) with permission.

Participants started in a seated position with their knee aligned with the axis of the dynamometer lever arm and their leg in a resting 90° position. Participants then extended their leg against the resistance provided by the lever arm strapped above their ankle as quickly and forcibly as possible. At the top of the range of motion (~0°), participants lowered their leg against the resistance provided by the lever arm, as quickly and forcibly as possible, until the end of their range of motion (~120°). The participants repeated the knee extension/flexion kicking motion for three continuous repetitions.

Following the measurement of isokinetic knee extension, participants performed three maximal isometric knee extension contractions at a knee angle of 60° (0° = full knee extension) and a duration of 5 s. A 20-s rest was allocated between each isometric contraction, while a 60-s rest was allowed between the isokinetic and isometric force measurements.

3.6.2 Functional Strength

Functional strength was used to measure changes in strength associated with exercises and movements used in real world everyday situations such as walking, climbing and moving from a seated position to a standing position.

Functional muscle strength was measured with three different functional exercises in which the large muscle groups that are important in daily activities were assessed: the 10-m walk test (10 MWT), the 14-stair climb test and the 30-s sit-to-stand-test (STS), as previously described (Brose, Parise et al. 2003).

The 10MWT required participants to walk a distance of 10 m up to a marked turn point at which the participants made a turn and walked 10 m back to the starting point. The 10MWT was first conducted at normal walking pace and then as quickly as possible both without the use of external aids.

The timed stair climb required participants to walk up 14 stairs without the use of railings. Again, the stair climb was first conducted at normal pace and then as quickly as possible, on both occasions without the use of external aids and with a rest of 60 s between tests.

The 30-s sit-to-stand test required participants to move from a sitting position to a standing position in a controlled fashion repeatedly for 30 s as quickly as possible with arms folded in front of their chests, on a firm, armless chair placed against a wall. Uncontrolled and partial stands were not included in the count.

Participants completed each of the functional tests as part of an initial familiarization trial during the recruitment phase. The functional tasks were all measured by the same evaluator and were timed to the nearest 0.1 s through use of an electronic stopwatch.

3.6.3 1-Repetition Maximum (1RM)

Strength assessments consisted of the maximal weight that could be lifted once in two weight training exercises; machine bench press and horizontal leg press.

There is an inverse relationship between the amount of weight that can be lifted and the number of repetitions that the weight can be lifted; and this relationship is often used to determine an individual's maximum strength or 1RM (Baechle TR 2000). The 1RM is the weight that can be lifted, using the correct technique, for one repetition of a given exercise. It can also be defined as the maximum weight that can be lifted for a number of repetitions (Mayhew, Prinster et al. 1995).

The 1RM testing protocol and exercise execution guidelines that were followed have been previously documented (Baechle TR 2000). The participant's maximal lift was determined within no more than five single repetition attempts following three progressively heavier warm-up sets. Participants were required to lift each weight successfully before attempting a heavier weight. Each exercise was completed before the next attempt and in the same order. Each 1RM strength assessment was performed at least 5 to 7 days apart from any other exercise or test that might affect muscle strength during the training/supplementation program.

All strength tests were completed at Victoria University's Sports and Aquatic Center, Footscray Park.

In cases when the 1RM could not be achieved, a calculation of the 1RM from a 2–5 repetitions to failure (RTF) exercise was performed (Mayhew, Prinster et al. 1995).

3.6.4 Measurement of Muscle Power (Muscle Force x Muscle Velocity) using Optical Encoder

Measurement of concentric and eccentric mean and peak muscle power output was performed in real time while participants performed three different exercises (leg press, lat pull down and bicep curl), allowing for the assessment of mean and peak power output in different muscle groups (legs, back and biceps) using the Gymaware optical encoder (Kinetic Performance Technology, Canberra, Australia).

Gymaware is a commercially available device that consists of a floor unit, comprising a spring-powered retractable cord that passes around a pulley mechanically coupled to an optical encoder (Figure 3.5 and 3.6). The free end of the cord (the end not attached to floor unit) was attached to a barbell or moving resistance.

The floor unit was positioned on the floor directly under the movement of the barbell or resistance. The microprocessor in the floor unit calculated velocity and distance of the barbell or resistance from the spinning movement of the pulley (1 pulse approximately every 3 mm of load displacement, with each displacement value time stamped with a 1-ms resolution). Time and position data, generated at a maximum of 25 Hz, were sent via a fixed wire from the floor unit to an infrared transceiver (Figure 3.6).

The infrared transceiver sent the time and position data by infrared signal to a personal digital assistant (PDA, Tungsten-e; Palm, Milpitas, CA). The mass of the barbell or resistance (entered into the PDA by the operator) and the entire displacement (mm) for the movement was used to calculate the mean values for power.

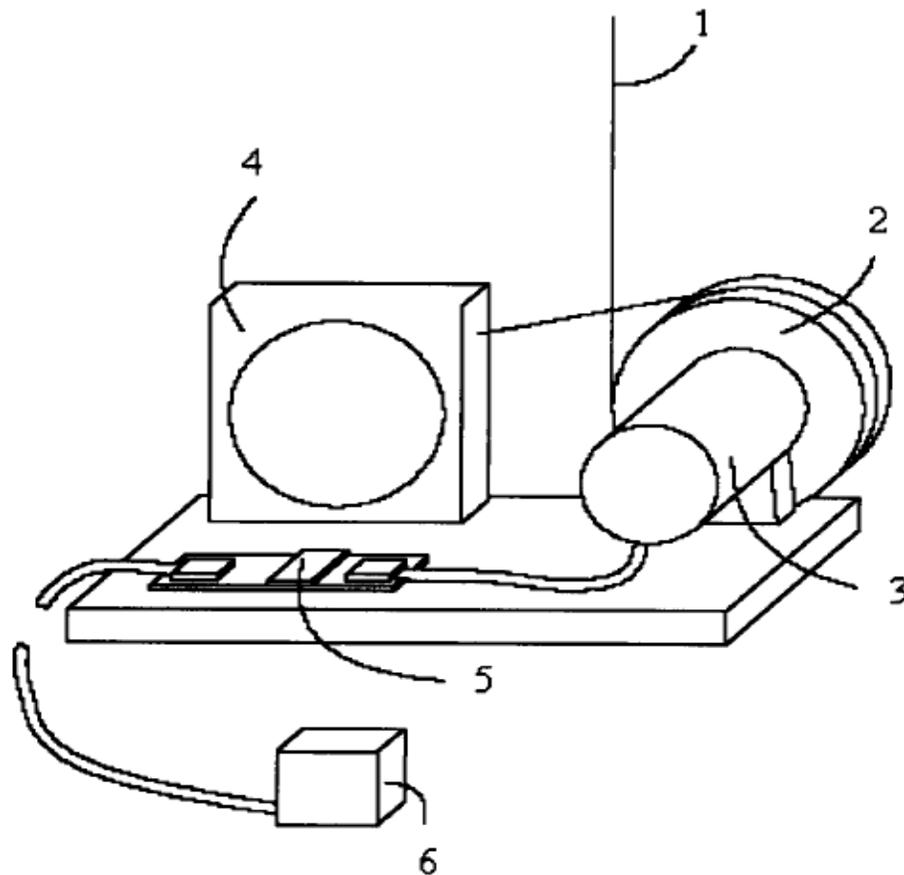


Figure 3.5: The Gymaware Optical Encoder Floor Unit. 1 indicates the retractor cord that attaches to the external load. 2 shows the pulley around which the retractor cord wraps. 3 indicates the optical encoder that sends a pulse top the microprocessor every 3 mm of rotation. 4 shows the retractable cord assembly that stores the retractor cord when the device is in the retracted position. 5 indicates the microprocessor and interface circuit that translates pulse information into position and velocity data. 6 indicates the infrared transceiver that communicates with the personal digital assistant to store data.

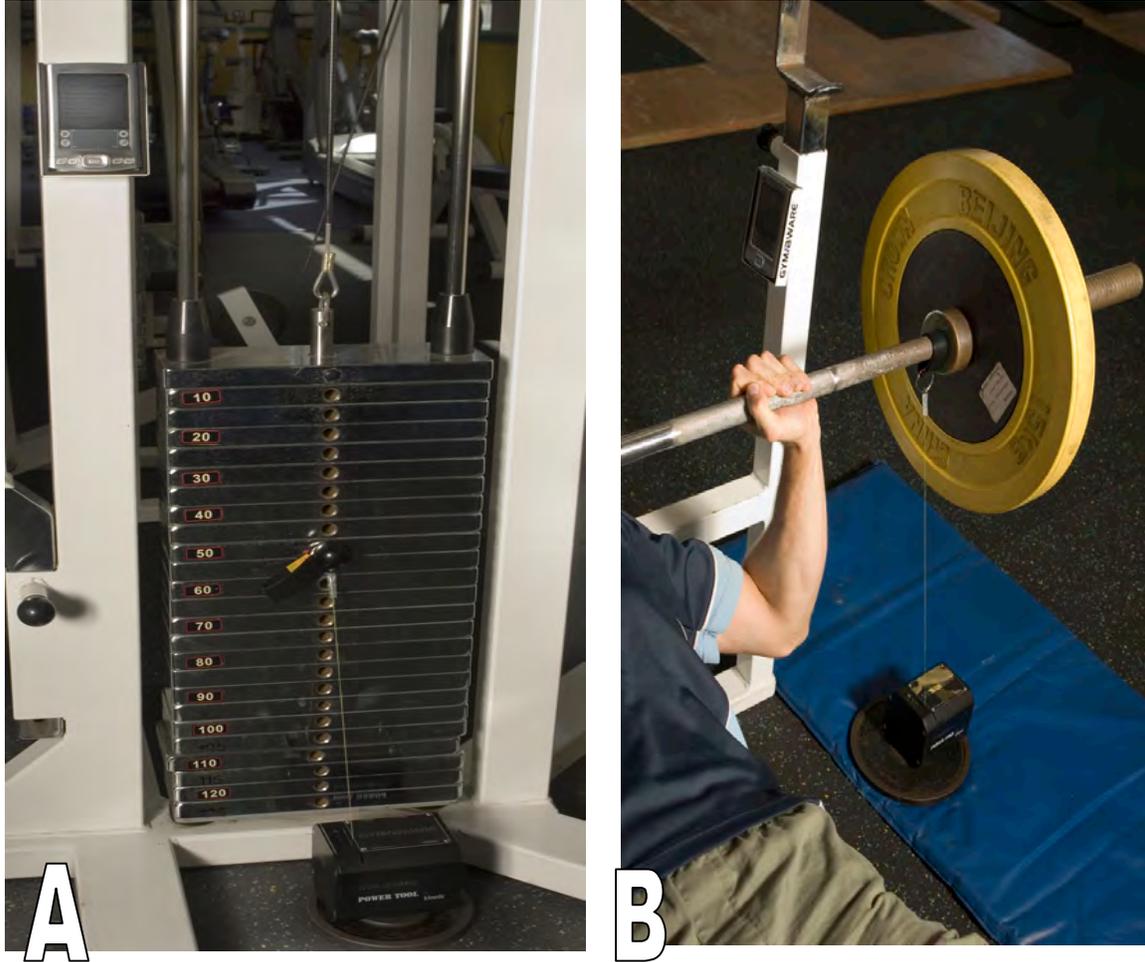


Figure 3.6: The Gymaware Set-up on a Weight Stack (left) and Free Barbell Bench Press (right). The Gymaware cord connected to the floor unit is attached to either the weight stack (A) or barbell (B). The floor unit is wired to an infrared transceiver, which sends the time and position data to the personal digital assistant via an infrared signal.

To measure changes in power output that occurred during the training program participants' performed two sets of 8 to 10 repetitions of leg press, lat pull down or arm curls while being continuously monitored using the optical encoder. Each exercise (leg press, lat pull down or arm curl) was measured only once per week and on the same day of each week (Table 3.4) during the 12-week resistance training program. All repetitions were measured on the PDA for analysis of changes in kinetics over the set and training protocol.

Table 3.4: Schedule for the measurement of mean and peak power output.

Measurement of mean and peak power out protocol: Weekly Schedule		
Monday	Wednesday	Friday
Leg press	Bench press	Arm curls

After the data were collected from the Gymaware optical encoder, they were exported back to the PDA. The PDA was then synchronized with a laptop connected to the Internet, and the exercise data uploaded to the Gymaware website (<https://gymaware.kinetic.com.au>) using a personalized log-in and password. Once uploaded, the peak power and maximal strength for each repetition were quantified.

The Gymaware optical encoder has previously been shown to be an accurate and reliable measure of kinetics during free-weight resistance training movements (standard error of estimate [*SEE*] for power, 3.6 -14.4W; coefficient of variation (CV) 1.0 – 3.0%; correlation, 0.97 – 1.0) (Drinkwater, Galna et al. 2007).

3.7 Blood Sampling

Venous blood (approximately 5 mL) was removed from the antecubital fossa vein using a 23G needle and 5 ml syringe at baseline, 6 weeks (Study 1 endpoint, Study 2 mid-point), and at 12 weeks (Study 2 end-point). The blood was immediately placed into a heparinized tube (BD, Franklin Lakes, NJ) and gently inverted and rolled several times, and used for the analysis of plasma glutathione (see below).

3.8 Measurement of Plasma Redox Status

Plasma thiols (reduced glutathione [GSH], cysteine [Cys]) and disulfides (oxidized glutathione [GSSG], cysteine [CySS]) were measured as previously described (Jones, Carlson et al. 1998).

3.8.1 Solutions

All chemicals were purchased from Sigma Aldrich (St. Louis, MO). The composition of preservation solution (solution A) and perchloric acid (solution B) is detailed in Tables 3.5 and 3.6.

3.8.2 Blood Preparation

At each sampling point (0, 6 and 12 weeks), 0.5 ml blood was added into an Eppendorf tube labeled “A”, which contained a preservation solution of 100 mM serine-borate (pH 8.5) containing (per ml) 0.5 mg sodium heparin, 1 mg BPDS, 2 mg iodoacetic acid, 8mL 100 mM boric acid and 2mL 100 mM sodium tetraborate.

Table 3.5 Preservation Solution A.

Solution	Chemical	Volume
A	100mM Boric Acid stock (boric acid 0.62g/100mL)	8 mL
	100mM Sodium tetraborate stock (sodium tetraborate 3.81g/100mL)	2 mL
	L-serine	105 mg
	Sodium Heparin	5 mg
	Bathophenanthroline dislphonate sodium salt (BPDS)	10 mg
	Iodoacetic acid	20 mg

The preservation solution was used to prevent coagulation and to inhibit degradation and breakdown of the thiols and disulfides found in blood plasma (Reed, Babson et al. 1980; Lash and Jones 1985; Smith, Hansen et al. 1993).

Tube A was gently inverted twice to mix the blood with the preservation solution and then centrifuged in a microcentrifuge (Eppendorf 5415C microcentrifuge, Hamburg, Germany) at 15,000 rpm for 30 s to remove red blood cells. A 200 μ L aliquot of the supernatant from tube A was then transferred to an Eppendorf tube labeled “B”, which contained 0.2 mL perchloric acid solution containing a 10- μ M γ -glutamylglutamate (γ -GluGlu) internal standard. The perchloric acid solution (Table 3.6) was made with a solution of 10% (w/v) perchloric acid containing 0.2 M boric acid and 10 mM γ -Glu-Glu (Table 3.6). The Eppendorf tube B was gently inverted twice to mix the contents and then stored at -80°C for the analysis of plasma thiols and disulphides. All tubes were analyzed within two months of processing.

Table 3.6: Preservation Solution B.

Solution	Chemical	Volume
B	Boric Acid γ -Glutamylglutamate (γ -GluGlu) 70% Perchloric acid add H ₂ O up to	6.6 g 1.4 mg 71 mL 500 mL

3.8.3 Derivatization

The derivatization process is outlined in Figure 3.7. ‘B’ tubes were spun for 2 min in a microcentrifuge to pellet the protein. A 300 μ L aliquot of each supernatant was transferred to a fresh microcentrifuge tube (‘tube C’) and 60 μ L 40 mM iodoacetic acid

solution was added to each. The pH was adjusted to 9.0 ± 0.2 with a KOH/tetraborate solution (approximately 200 μL). The KOH/tetraborate solution was prepared by adding 5.6 g KOH to a plastic bottle containing 50 g $\text{K}_2\text{B}_4\text{O}_7 \cdot 4\text{H}_2\text{O}$ and 100 mL of water. The KOH/tetraborate solution was mixed and allowed to stand overnight at room temperature before removing the solution from the remaining precipitate.

Following the addition of the KOH/tetraborate solution, about 3 min was allowed for complete precipitation of potassium perchlorate. The pH of some of the samples was checked to verify that they were in the correct range ($\text{pH } 9.0 \pm 0.2$). After 25 min, 300 μL dansyl chloride solution was added to tube C, and the samples were mixed and placed in the dark at room temperature for 16 to 26 hours. The dansyl chloride solution was prepared fresh on the day of analysis by dissolving 200 mg dansyl chloride in 10 mL acetone. After overnight incubation, 500 μL chloroform was added to each tube to extract the unreacted dansyl chloride. Samples were stored in the presence of both the perchlorate precipitate and the chloroform layer at $0-4^\circ$ until analysis using High Performance Liquid Chromatography (HPLC).

3.8.4 HPLC Analysis and Conditions

Before HPLC analysis, samples were centrifuged for 2 min in a microcentrifuge to allow for the transfer of an aliquot of the upper (aqueous) layer to the HPLC autosampler.

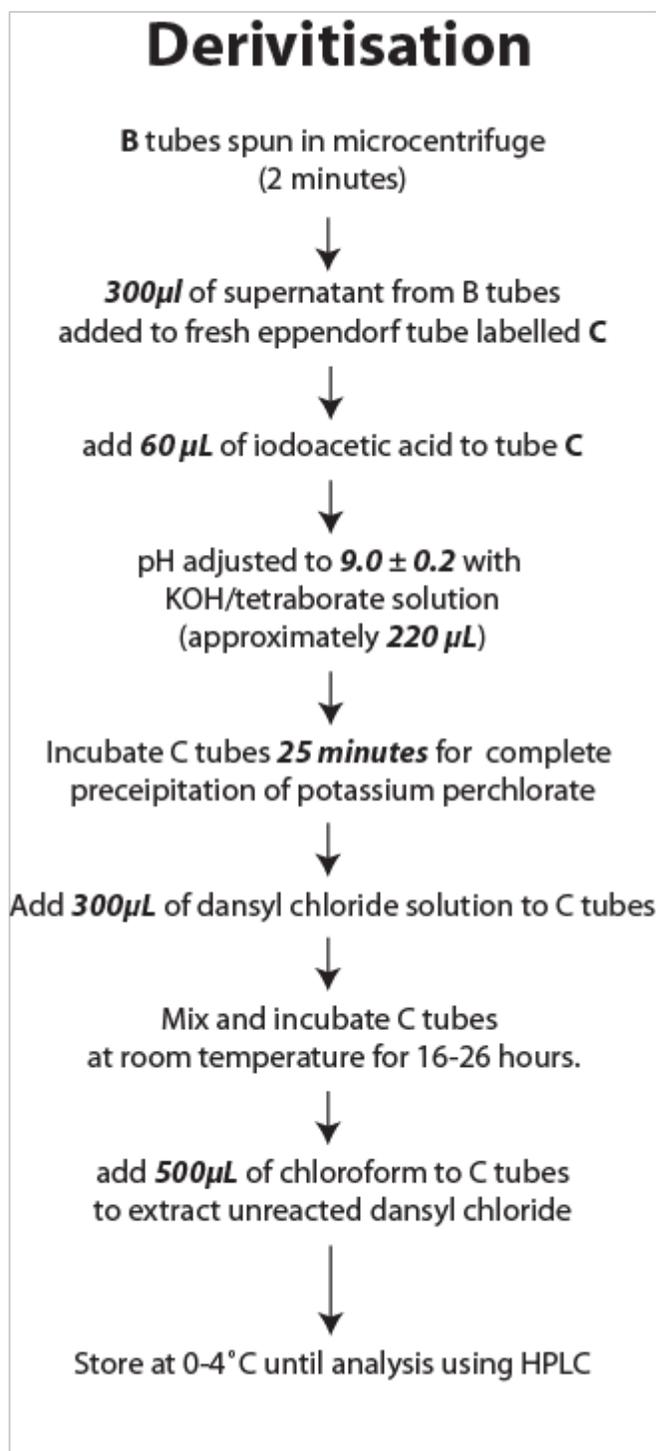


Figure 3.7: Derivatization of Plasma Thiols/Disulfides.

The HPLC separation of the danylated adducts was achieved on a 3-aminopropyl column (25 × 4.6 mm ID, 5- μ m particle size; Custom LC, Houston) and followed by fluorimetric detection at an excitation wavelength of 305–395 nm and an emission wavelength of 510–650 nm. The system consisted of a Waters 600 LC pump system, a Hitachi F-1000 fluorescence detector, and a Waters 717Plus sample processor (Waters, Milford, MA). Elution solvent A consisted of 80% (v/v) methanol/water and elution solvent B was an acetate-buffered (pH 4.6) methanol solution prepared by mixing 640 mL methanol, 200 mL acetate stock, 125 mL glacial acetic acid and 50mL water.

The acetate stock was prepared by mixing 272g sodium acetate trihydrate, 122 mL H₂O and 378 mL glacial acetic acid. Both solvents were filtered with a 0.45- μ m pore size filter prior to use.

The HPLC conditions were set to allow simultaneous measurement of thiols (GSH and CyS) and disulfides (GSSG and CySS). The elution program is detailed in Table 3.7. The flow rate was 1.0 ml/min, the injection volume was 20 μ L and the total run time was 60 min, with a 12 minute equilibration time between runs.

Initial solvent conditions were 80% A, 20% B run at 1 mL /min for 10 min. A linear gradient to 20% A, 80% B was run over the period from 10 to 30 min. From 30 to 46 min, the conditions were maintained at 20% A, 80% B and then returned to 80% A, 20% B from 46 to 48 min (Table 3.7). Equilibration time between samples was approximately 12 min, giving a run time per sample of 60 min.

Table 3.7: Elution Program.

Time (min)	% A	% B
0	80	20
10	20	80
20	20	80
46	80	20

3.9 Muscle Samples and Analysis

3.9.1 Muscle Biopsies of Vastus Lateralis

All muscle samples were obtained from the vastus lateralis via needle biopsy using a sterile Bergstrom muscle biopsy needle (5 mm x 101 mm length; Stille, Stockholm, Sweden). After local anesthesia (1% xylocaine), a small incision of the skin was made (approximately 10 cm), and a muscle sample (100–300mg) was obtained from the vastus lateralis muscle using the percutaneous needle biopsy technique modified for suction (Evans, Phinney et al. 1982). Roughly 30–50mg of the muscle was placed immediately into a labeled cryule and stored in ice, before the measurement of mitochondrial ATP production rate (MAPR) and citrate synthase. Another 30–80mg of the muscle was placed immediately into a labeled cryule, and snap frozen in liquid nitrogen for intracellular thiols/disulfide and protein analysis. The remaining tissue was mounted using Tissue-Tek medium after the fibres were orientated in a longitudinal direction and snap frozen in isopentane, pre-cooled in liquid nitrogen and then stored at -80°C for histochemical analysis. Biopsies were obtained at Victoria University’s Exercise Physiology Laboratory and all muscle analyses were completed at Victoria University’s Exercise Metabolism Unit.

3.9.2 Measurement of Mitochondrial ATP Production Rate (MAPR)

3.9.2.1 Solutions

All chemicals were purchased from Sigma, BDH or Roche, unless otherwise stated. With the exception of palmitoylcarnitine, which was prepared with 0.5% BSA, all solutions were prepared with milliQ double distilled, deionized water. The composition of experimental solutions (Solutions A, B & C) is detailed in Tables 3.8–3.10.

3.9.2.2 Mitochondrial Preparation

The homogenizing solutions for the preparation of isolated mitochondria and the isolation procedures were prepared as described (Wibom, Lundin et al. 1990). Fresh muscle was placed on a Petri dish over ice, dissected free of fat and connective tissue, cut into small pieces and weighed. The muscle sample was put into 1 mL of pre-cooled homogenizing solution (solution A) and homogenized carefully using a glass-on-glass mortar and pestle (Kontes, New Jersey). Solution A contained tris(hydroxymethyl) amino methane (50 mM), KCl (100 mM), MgCl₂ (5 mM), ATP (1.8 mM), EDTA (1 mM), at pH 7.2 (Table 3.8).

The homogenized muscle was centrifuged (Biofuge 28RS, Heraeus Sepatech GmbH, West Germany) at 650 g at 4 °C for 3 min. The supernatant was then removed and transferred to a new Eppendorf tube and the pellet was discarded. The supernatant was then centrifuged again at 15000 g at 4 °C for a further 3 min. On this occasion, the supernatant was discarded and the pellet was re-suspended in 1 mL homogenizing solution (solution A) using a fine-bore glass pasteur pipette and then centrifuged again at 15000 g at 4 °C for a further 3 min. Finally, the pellet from the

centrifugation, which contained the mitochondria, was suspended in 200 μL solution B to produce the mitochondrial suspension (MS). Solution B contained sucrose (180 mM), KH_2PO_4 (35 mM), Mg acetate (5 mM), EDTA (1 mM) at pH 7.5 (Table 3.9). A 100 μL aliquot of the MS was diluted with 400 μL solution B (1:5 diluted mitochondrial suspension).

Next, 10 μL of the 1:5 diluted mitochondrial suspension was further diluted with 490 μL (1:250 diluted mitochondrial suspension) of the ATP-monitoring reagent (AMR) solution. AMR was prepared by diluting the commercially provided firefly luciferase solution (FL-AAM, Sigma) 12.5-fold with solution C, containing tetra-Na pyrophosphate (0.05 mM), Mg acetate (0.5 mM) bovine serum albumin (BSA; 1 mg/mL), sucrose (180 mM), KH_2PO_4 (5 mM), EDTA (1 mM) at pH 7.5 (Table 3.10). The final mitochondrial suspension used for MAPR was a 1:250-dilution of the mitochondria in the original muscle. The suspension was placed on ice ready for MAPR analysis. The remaining 100 μL undiluted mitochondrial suspension was used for determination of citrate synthase activity. The steps of the mitochondrial preparation are shown in Figure 3.8.

3.9.2.3 Preparation of Solutions for MAPR

Reaction cocktails that mimic various metabolic pathways fueling mitochondrial ATP production were prepared by adding 40 μM ADP (Boehringer Mannheim, Germany) plus the AMR solution and the various substrate combinations to a kimble tube (Table 3.11) to produce a final volume of 1mL.

Table 3.8: Composition of Homogenization Solution (A) for MAPR.

	MW	Stock Concentration (M)	Final Concentration (M)	In Volume (100mL)	
				Volume (mL)	Mass (g)
KCl	74.55	1.0	100	5	0.7455
TRIS	121.14	1.0	50	10	0.6057
MgCl ₂ ·6H ₂ O	203.3	1.0	5.0	0.5	0.1017
ATP	605.2	0.1	1.8	1.8	0.1089
EDTA	372.24	0.1	1.0	1	0.0372

pH to 7.2

Table 3.9: Composition of Mitochondrial Solution (B) for MAPR.

	MW	Stock Concentration (M)	Final Concentration (M)	In Volume (100mL)	
				Volume (mL)	Mass (g)
Sucrose	342.3	1.0	180	18	6.1615
KH ₂ PO ₄	136.1	1.0	35	3.5	0.4763
Mg Acetate	214.1	1.0	5.0	0.5	0.1073
EDTA	372.2	0.1	1.0	1	0.0372

pH to 7.5 with KOH

Table 3.10: Composition of ATP-Monitoring Reagent (AMR) Solution (C) for MAPR.

	MW	Stock Concentration (M)	Final Concentration (M)	In Volume (100mL)	
				Volume (mL)	Mass (g)
Na ₄ P ₂ O ₇ ·10H ₂ O	446	0.001	1	0.1	0.466
Mg Acetate	214.48	0.5	10	2	0.2145
BSA			1mg/mL		0.1
Sucrose	342.3	1.0	180.0	18	6.1614
KH ₂ PO ₄	136.09	1.0	35.0	3.5	0.4763
EDTA	372.24	0.1	1	1	0.03372

pH to 7.5 with KOH

MAPR mitochondrial preparation for MAPR

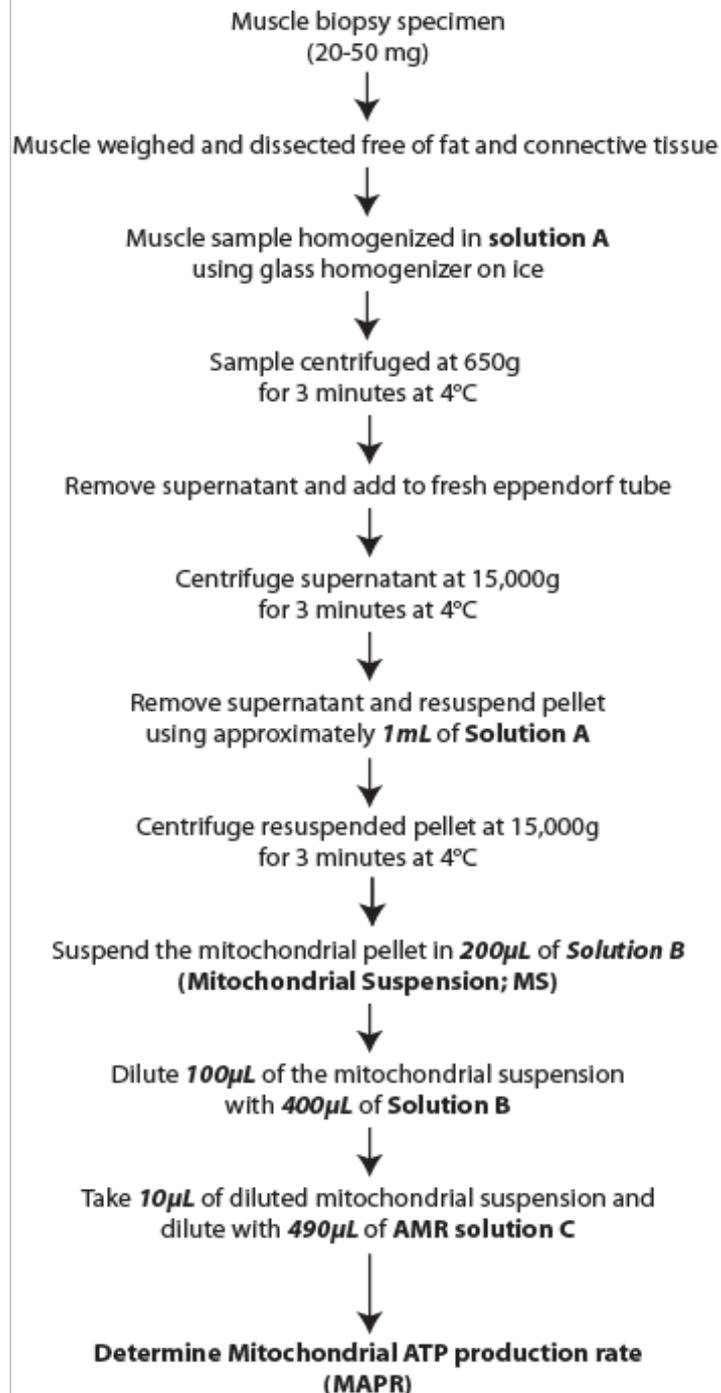


Figure 3.8: Preparation of Mitochondrial Suspension for Measurement of Mitochondrial ATP Production Rate.

Table 3.11: MAPR Reaction Cocktail composition (volume per muscle sample) and their Associated Metabolic Pathways.

Substrate Cocktail	Substrates	Volume (μL)
P+M Carbohydrate metabolism	Pyruvate 1mM Malate 1mM	25 10
PC+M Fat Metabolism	Palmitoyl-L-Carnitine 5 μM Malate 1mM	25 10
α-KG protein Metabolism	α -ketoglutarate 10mM	35
S+R Complex II Metabolism	Succinate 20mM Rotenone 100 μM	10 25
PPKM Carbohydrate + Fat + Protein metabolism	Pyruvate 1mM Palmitoyl-L-Carnitine 5 μM α -ketoglutarate 10mM Malate 1mM	25 35 10 10
Blank	MilliQ H ₂ O	35

The substrate combinations were as follows: 1.0 mM pyruvate + 1.0 mM malate (P + M), 0.005 mM palmitoyl-L-carnitine + 1.0 mM malate (PC + M), 10 mM α -ketoglutarate (α -kg), 20 mM succinate + 0.1 mM rotenone (S + R) and 1.0 mM pyruvate + 0.005 mM palmitoyl-L-carnitine + 10 mM α -ketoglutarate + 1.0 mM malate (PPKM). A blank was also prepared containing 25 μL H₂O, ADP and the AMR solution but no substrates, in which ATP production may be due to adenylate kinase reaction and other non-specific reactions (Wibom, Lundin et al. 1990).

3.9.2.4 Preparation of Solutions for ADP-stimulated MAPR

Additionally, 2.5 μM , 5 μM , 10 μM , 20 μM and 40 μM ADP solutions were added to an additional set of tubes containing the substrate combination PPKM. These tubes were then added to different set of wells in the same 96-well plate and used for the measurement of ADP-stimulated MAPR at various ADP concentrations.

An additional blank was prepared containing the five different concentration of ADP but no substrate and added to another set of wells to measure any residual ATP production from the adenylate kinase reaction and other non-specific reactions.

3.9.2.5 Measurement of Mitochondrial ATP Production Rate (MAPR)

MAPR was determined bioluminometrically according to the methods of Wibom et al. (1991) utilizing the firefly luciferase reaction, in which the proportion of emitted light is proportional to the ATP concentration (Figure 3.9).

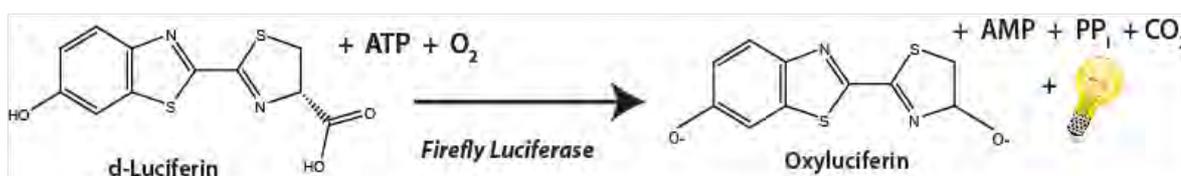


Figure 3.9: Firefly Luciferase Reaction.

A 200 μL aliquot of cocktail containing the various substrates shown in Table 3.11 and a blank were added to a 96-well plate and incubated for 5 min at 25 $^{\circ}\text{C}$ in a luminescence spectrometer (Fluoroskan Ascent FL, Finland). After incubation, baseline measurements for each of the substrate combinations were taken, followed by the addition of 20 μL of a 2 μM ATP standard (FL-AAS, Sigma), which was added to the wells as an internal ATP standard, to allow for the quantification of ATP production.

Following this, 10 μL aliquots of mitochondrial suspension were added to the wells, and light emission was measured in the luminescence spectrometer chamber

(25°C). All measurements were made in triplicate and were completed within 1.5 h of the muscle excision. The final volume in the wells for MAPR was 230 µL. Values for MAPR were calculated and expressed as mmol/min/kg muscle.

3.9.3 Citrate Synthase Activity and Calculation of Mitochondrial Yield

The activity of CS in whole muscle and mitochondrial suspensions was determined spectrophotometrically (Ultraspec II LKB biochrom, Cambridge, UK) using PowerLab® software (Chart V4, PowerLab/410, AD Instruments) as previously described (Srere 1969). Citrate synthase was measured under the following reaction (Figure 3.10):

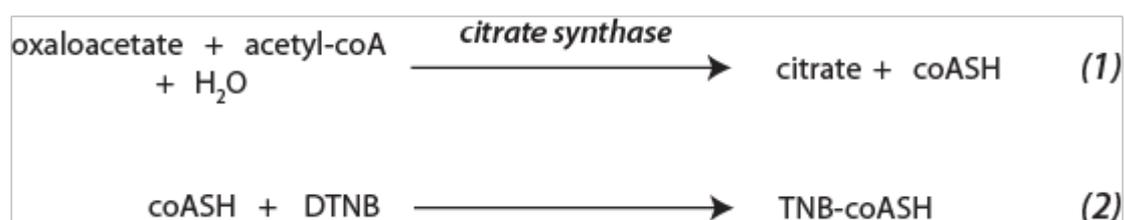


Figure 3.10: Calculation of Citrate Synthase (CS) Activity.

When reacted with coASH (reaction 2, Figure 3.10), DNTB (5,5'-dithiobis(2-nitrobenzoic acid)) forms a fluorescent yellow product, TNB-coASH, that can be measured at an absorbance of 412 nm using a spectrophotometer.

CS is an enzyme that is found only within the mitochondria. Therefore CS activity can be used as an indicator of mitochondrial content within a given muscle sample. Many mitochondria are lost in the extraction process. CS can be used to determine the yield of mitochondria by determining the total muscle CS (CS_{total}) and

CS activity in the mitochondrial suspension before (CS_{before}) and after (CS_{after}) disruption of the mitochondrial membrane. The difference between the CS_{before} and CS_{after} in the mitochondrial suspension indicates the number of intact mitochondria in the suspension.

3.9.3.1 Solutions

CS_{after} and CS_{total} samples were homogenized in CS homogenizing buffer containing 175 mM KCl and 2 mM EDTA (pH 7.4). The homogenizing solution used to treat CS_{after} samples also contained 1% Triton-X. Samples were monitored in solution containing 70 mM tris-HCl buffer (pH 8.3; containing tris[hydroxymethyl] aminomethane and tris[hydroxymethyl] aminomethane hydrochloride), 0.1 mM 5,5-dithiobis-2-nitrobenzoate (DTNB), 0.45 mM acetyl coenzyme A and 0.5 mM freshly prepared oxaloacetic acid.

3.9.3.2 Determination of CS Activity and Mitochondrial Yield

A 50 μL aliquot of MS was added to a cryule containing 150 μL solution B, and labeled CS_{before} . The CS_{before} sample contained intact mitochondria and acted as a measure of CS from disrupted mitochondria. Another 50 μL aliquot of MS was then added into a cryule with 150 μL of a CS homogenizing solution containing Triton X-100 (0.05% vol/vol), 50 mM KH_2PO_4 and 1 mM EDTA, pH 7.5 (1:100 w/v), and labeled CS_{after} . The CS_{after} sample contained fractured mitochondria and acted as a measure of the CS activity contained within the final MS.

The samples for CS_{total} determination were prepared in CS homogenizing solution using an electric homogenizer (OMNI International S/N TH-1276, Warrenton, USA) and homogenizing the muscle sample put aside in the preparation

of the MS. The CS_{total} muscle sample was homogenized twice for 10 s at medium speed with a 15-s rest in between, and then finally for 5 s at high speed. Samples were stored on ice during the 15-s rest periods. The CS_{total} sample contained fractured mitochondria and acted as a measure of the CS activity from the original muscle sample. Both the CS_{total} and CS_{after} samples were put through a freeze-thaw fracture cycle three times to further disrupt the mitochondrial membrane. ATP production was corrected for the proportion of intact (i.e. functional) mitochondria brought through the extraction process, relative to the total mitochondrial content of the muscle to calculate mitochondrial yield. The formula used to calculate mitochondrial yield is shown below (Figure 3.11):

$$\text{Mitochondrial Yield (\%)} = \frac{CS_{after} - CS_{before}}{CS_{total}} \times 100$$

Figure 3.11: Calculation of Mitochondrial Yield.

3.10 Histochemistry

3.10.1 Fibre Typing

The mounted biopsy samples were serially sectioned (10 μm thick) on a cryostat microtome at $-20\text{ }^{\circ}\text{C}$ (Microm GMBH D-6900 500, Heidelberg, Germany) and placed on a microscope slide. Staining for ATPase was used to classify muscle fibre types I, IIa and IIx based on the methods of Brooke and Kaiser (1970). Muscle fibre types were determined using the myofibrillar ATPase method as described by

Hammilanen and Pette (1993). Histochemistry also allows for fibre areas to be determined.

Sections were pre-incubated at low pH, and stained for myosin ATPase activity. Once stained, the muscle sections were photographed and counted to determine the percentage of particular fibre types using AIS® software. Fibres were classified into type I, type IIA, intermediate, or type IIB according to myofibrillar staining patterns. Preincubation at pH 4.54 along with the standard preincubations of 4.3 and 4.6 was included to enable a clear differentiation of the type-II subgroups (Dubowitz 1985). Baseline and endpoint cross-sections were assayed simultaneously.

3.11 Fibre Typing using SDS-PAGE

Histochemistry is useful for the measurement of myofibrillar ATPase activity, but does not allow for measurement of MHC protein expression in the muscle. Fibre typing using SDS-PAGE is an accurate way to assess MHC protein expression, and allows an assessment of skeletal muscle responses to chronic changes in neuromuscular activity. This method was therefore used for increased accuracy and to provide a comparison to correlate against the results obtained histochemically.

MHC isoform (MHC_i) composition were determined according to methods outlined by Kemp, Blazev et. al (2009), with modifications made for the measurements of MHC_i in human muscle.

3.11.1 Muscle Homogenization

Frozen human muscle (approximately 5–10 mg) was manually homogenized on ice using a glass/glass homogenizer with approximately six volumes of relaxing solution (~1-mL volume to 1 g wet muscle). The relaxing solution comprised 90 mM

HEPES, 126 mM K⁺, 36 mM Na⁺, 1 mM Mg²⁺, 50 mM EGTA, 8 mM ATP, and 10 mM CrP (pH 7.10).

3.11.2 Analysis of MHC_i using SDS-PAGE

Separation of myosin heavy chain isoform in muscle homogenates (6 µL per well) was based on the glycine-SDS-PAGE method of Talmadge and Roy (1993). Stacking gels consisted of 30% glycerol, 4% acrylamide-*N*, *N*'-methylene-bis-acrylamide (bis) (50:1), 70 mM Tris (pH 6.7), 4 mM EDTA, and 0.4% sodium dodecyl sulfate (SDS). Separation gels consisted of 30% glycerol, 8% acrylamide-bis (50:1), 0.2 M Tris (pH 8.8), 1 M glycine, and 0.4% SDS. Stock solutions were used for the preparation of gel constituents. Polymerization was initiated using 0.05% *N,N,N',N'*-tetramethylethylenediamine and 0.1% ammonium persulfate. An MHC_i marker containing MHC I, MHC IIa, MHC IIb and MHC IIc isoforms was made from sternomastoid (SM), extensor digitorum longus (EDL) and soleus (SOL), using the methods of Bortolotto et al. (1999). This was applied to the gels to enable identification of the individual MHC isoforms in each sample when stained for visualization (Figure 3.12). Electrophoresis was subsequently carried out at 5°C for 27 h using a constant voltage of 150 V.

3.11.3 Protein visualization and Quantitation on SDS-polyacrylamide Gels

Coomassie brilliant blue (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA) was used to stain the myosin heavy chain isoforms in the muscle homogenate samples. A Molecular Dynamics Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, USA) was used to carry out volumetric quantitation of electrophoretic bands. A background correction was made using the object average

method (ImageQuaNT V5.2 software, Molecular Dynamics), to provide a measure of each MHC isoform/band (Figure 3.12).

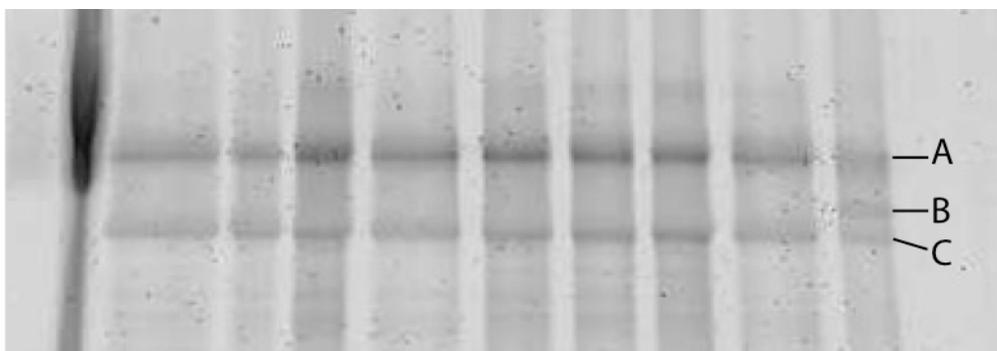


Figure 3.12: MHC isoform identification using SDS-PAGE. MHC identified using a mixed muscle mouse marker containing MHC type I (A), MHC type IID (B) and MHC IIA (C) isoforms.

3.12 Muscle Protein

The protocol for muscle protein concentration determination was based on the methods of Bradford (1976). A Bradford Protein Assay kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA) was used for this procedure.

Total and contractile proteins were isolated using literature methods (Beitzel et al. (2004). Approximately 10 mg tissue was homogenized on ice in a glass tissue grinder (Kontes, New Jersey) using a buffer solution of 50 mM KCl, 10 mM KH_2PO_4 , 2 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 mM EDTA and 2 mM DTT (1:50 wet weight:volume). Crude homogenates (200 μL) were removed and later used for determination of total protein concentration. The residual homogenate was centrifuged at 5°C and 1000 g for 10 min (Haeraeus Sepatech Biofuge, USA). The supernatant was discarded, and the contractile protein homogenate was suspended on ice once again in buffer solution.

Prior to determination of MAPR, mitochondrial proteins were extracted from the mitochondrial suspension, snap frozen in liquid nitrogen and stored at -80 °C.

For analysis of protein content, the amount of protein in standard protein solutions prepared from a bovine serum albumin (BSA) stock solution (20 mg/mL) was first determined. The concentration of the standard solutions increased in 0.1 mg/mL increments from 0.1 to 1.2 mg/mL. Bradford Assay dye was prepared by diluting one part dye reagent concentrate with four parts distilled deionized water, and then running through Whatman #1 filter paper (Whatman International Ltd, Maidstone, England) to remove particulates.

All blanks, standards and samples (40 μ L) were added to diluted reagent dye (2 mL) in glass kimble tubes, and incubated at room temperature for 10 min. Following incubation, the contents of the kimble tube were transferred to disposable UV-visible cuvettes for analysis by UV-visible spectrophotometer (UV-1700 Pharma Spec; Shimadzu, Jiangsu China) at 550 nm against reagent blank.

3.13 Measurement of Intracellular Redox Status

3.13.1 Solutions

Tissue samples were homogenized in cryule tubes containing 1 mL of 6.5% (wt/vol) salicylasilicylic acid (SSA) solution and approximately 500 mg 1.0 mm zirconia/silica beads (Biospec Products. Inc.) using a bench top fast-prep homogenizer (BIO101/Savant FastPrep FP120) at a setting of 6.0 for 40 s. Homogenized samples were placed immediately on ice for 5 min and then homogenized a second time at the same setting to ensure complete disruption of the muscle cell membrane and then centrifuged (Biofuge28RS, HERAEUS Sepatech) at 12,000 g for 15 min at 4°C. The acid extracts were then split into portions of thiols (GSH), disulphides (GSSG) and total thiols (GSH and GSSG).

3.13.2 Measurement of Thiols, Disulfides and Total Thiols.

For the measurement of thiols (GSH), a portion of 100- μ L SSA extracted sample was neutralized with approximately 6 mg NaHCO_3 powder and mixed with 100 μ L of monobromobimane (8 mM in acetonitrile). The reaction was allowed to run for 10 min in the dark at room temperature and stopped by the addition of 10 μ L of 90% (w/v) SSA (Figure 3.13).

For the measurement of disulphides (GSSG), a second portion of 100 μ L SSA extracted sample was neutralized with approximately 6 mg of NaHCO_3 powder. N-ethylmaleimide (NEM) was added to the neutralized sample at a final concentration of 10 mM, then the sample was incubated at room temperature for 5 min. Excess NEM was destroyed using 475 μ L of a 3 M potassium phosphate buffer (pH 13) to adjust the pH for alkaline hydrolysis (final concentration pH 11.5–11.8). After 25 min incubation for the hydrolysis of NEM, the samples were neutralized by the addition of 20 μ L 90% (wt/vol) SSA. GSSG was then reduced by incubating the samples in 10 μ L of 50 mM DTT for 30 min and then derivatized with 100 μ L monobromobimane (20 mM) in the dark for 10 minutes. Reactions were stopped by acidifying with 10 μ L 90% (w/v) SSA (Figure 3.13). Total thiols were measured as their thiol equivalents (e.g., total GSH). A third portion of 100 μ L SSA extracted sample was neutralized with approximately 6 mg NaHCO_3 powder and then treated with 10 μ L DTT (50 mM) for 30 min, followed by derivatization for 10 min. Reactions were stopped with 10 μ L 90% (w/v) SSA (Figure 3.13).

3.13.3 HPLC conditions

The HPLC separation of the thiol-bimane adducts was achieved on a reverse-phase Supelcosil C-18 octadecylsilyl silica column (150 \times 4.6 mm ID, 3- μ m particle

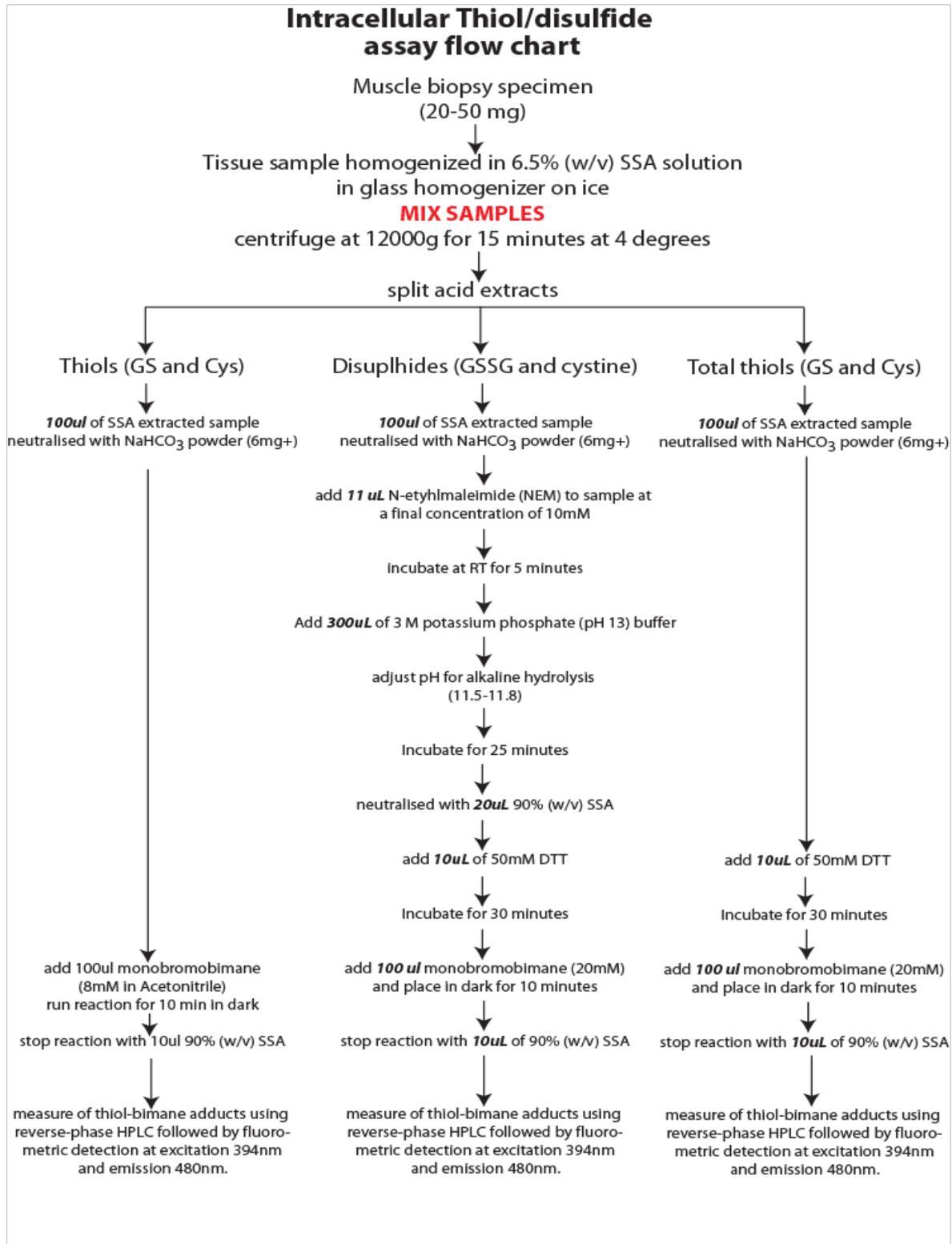


Figure 3.13: Derivatisation of Thiols, Disulfides and Total Thiols.

size) and followed by fluorimetric detection at an excitation wavelength of 394 nm and an emission wavelength of 480 nm (Figure 3.14). The system consisted of a Waters 600 LC pump system, a Waters 717 Plus sample processor (Waters, Milford, MA), and a Hitachi F-1000 fluorescence detector.

Elution solvent A was MilliQ water, Elution solvent B was acetonitrile and Elution solvent C contained 0.25% acetic acid and 0.25 % perchloric acid in MilliQ water, adjusted to pH 3.71 with sodium hydroxide (Table 3.12). The elution program is detailed in Table 3.12 below. The flow rate was 1.2 ml/min, the injection volume was 25ul and the total run time was 20 minutes.

The monobromobimane derivatives of GSH and Cys in each portion of derivatized samples were separated by HPLC and quantified on the basis of peak areas and compared with the authentic GSH and other thiol standards.

Table 3.12: Elution Program.

Time (min)	% A	% B	% C
0	15	5	80
7.2	15	5	80
7.6	25	75	0
12.6	25	75	0
12.7	15	5	80

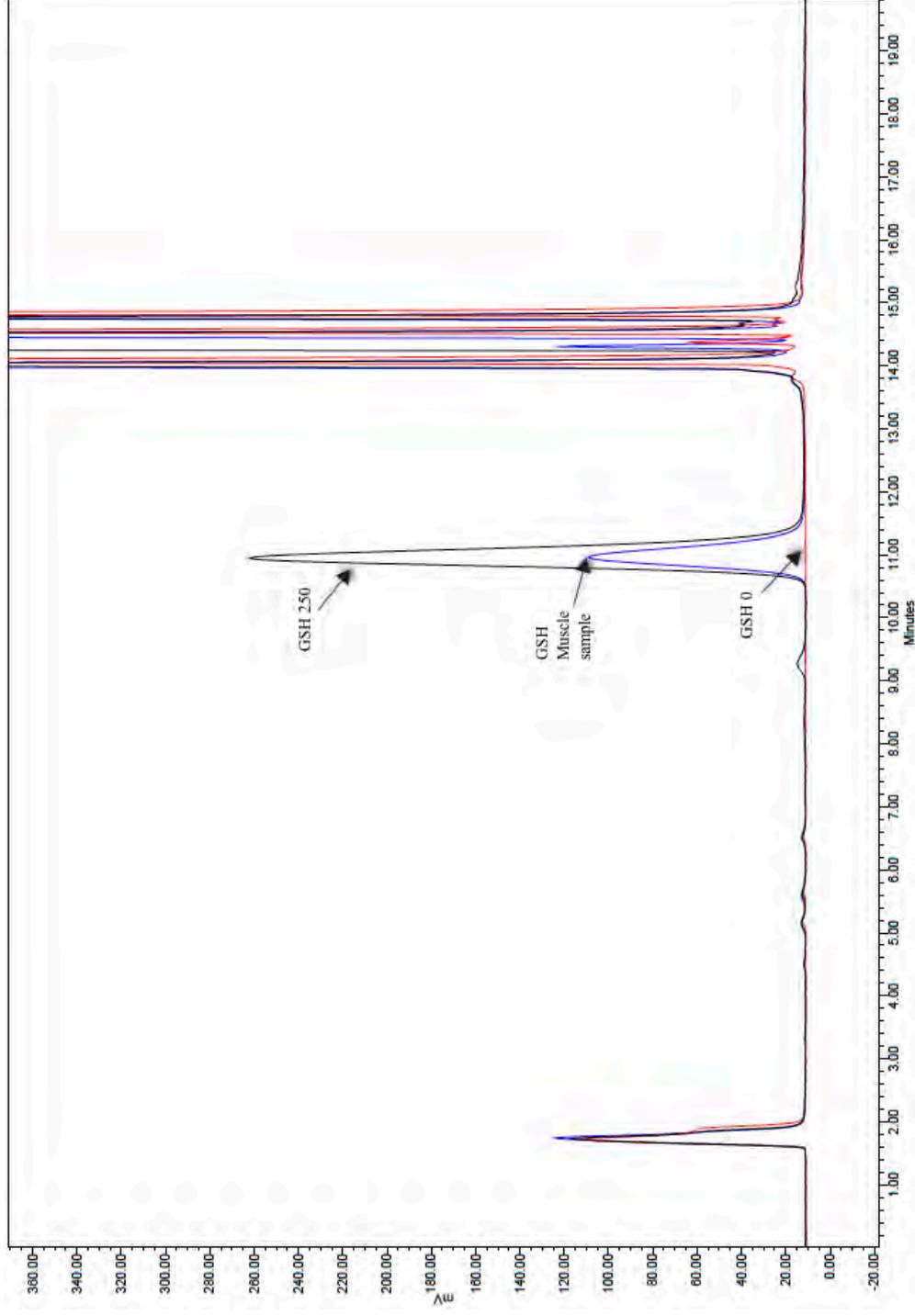


Figure 3.14: Typical HPLC chromatogram of muscle tissue with identification of GSH standards and muscle sample. Included in the above figure are two GSH standards (0 μ M and 250 μ M; orange and black) plus sample derivitised from muscle (blue).

3.14 Statistical Analysis

Statistical analyses were carried out using Prism 5 for Mac OSX (GraphPad Software Inc. USA). Differences in baseline characteristics between groups were compared using a one-way analysis of variance (ANOVA) when 3 groups were present and unpaired Student's t-tests when less than 3 groups were present. A two-way repeated measures analysis of variance with treatment as the between-group factor and time (three time points of 0, 6 and 12 weeks) as the within-group factor was used to test the effects of the treatment. When an interaction effect was identified, group differences were identified using a Bonferroni post-hoc analysis. When an almost significant result was achieved ($p < 0.10$) and the direction of change was different, a post-hoc analysis using an unpaired Student's t-test on the difference between before and after measurements (i.e delta change) was used to test the effect of the treatment. A p-value of < 0.05 was considered statistically significant.

Chapter 4

The Effects of Creatine or Whey
Protein Supplementation on Muscle
Oxidative Capacity in Older Humans

4.1 Introduction

Aging and muscle function are closely interlinked. Loss of muscle mass and functional ability, a condition known as sarcopenia, is a hallmark of aging. Muscle fibres, although they are post-mitotic tissues, show remarkable changes in structure and function with aging. In addition to structural changes, changes in muscle physiology, especially in muscle mitochondria, are an important feature of aging. Mitochondrial oxidative capacity declines with aging (reviewed in Johannsen and Ravussin (2009)). Peterson and Shulman have argued that a decline in mitochondrial oxidative capacity can lead to intracellular accumulation of lipids, a phenomenon which is also responsible for insulin insensitivity of muscle cells (Petersen and Shulman 2006).

Aging in muscles is also characterized by decrease in protein synthesis in the myofibres as well as in muscle mitochondria (Karakelides and Nair 2005). Protein and mRNA levels of myosin heavy chains decline, as well as the muscle proportion of type-2 muscle fibres (Karakelides and Nair 2005). Further to this, a reduction in the activity of oxidative enzymes such as citrate synthase (CS) and the content mRNA transcripts that encode mitochondrial proteins has also been shown to occur (Short, Vittone et al. 2003). Resting levels of adenosine triphosphate (ATP) and phosphocreatine (PCr) also decline in aged muscles (Muscarello, Calderara et al. 1992; Pastoris, Dossena et al. 1994).

Although sarcopenia is a progressively debilitating condition with a complex etiology, it is thought that it may be possible to arrest or even reverse the condition with interventions such as resistance training and dietary supplementation. The role of exercise, in particular, in maintaining physical strength and a healthy metabolism is

well known and has been extensively discussed and debated (see Chapter 2, Section 2.9.1). The effects of resistance training on physical strength in aged males will be discussed in more detail in Chapter 5.

Dietary interventions to address sarcopenia, on the other hand, have not been explored in great detail. This approach demands further attention because, if successful, dietary interventions can be implemented on a huge scale to achieve total inclusion of all sarcopenia cases, irrespective of their health status or co-morbidities. The proposed mechanisms by which dietary supplementation might offset these age-related changes was discussed in detail in Section 2.9.2.

Briefly, supplementation with different dietary proteins and amino acids has been shown to bring about reversal of sarcopenia in skeletal muscles (Boirie 2009; Kim, Wilson et al. 2010). It is also important to note that skeletal muscles show a decline in post-prandial anabolism and that dietary habits and taste preferences in elderly people may also lead to nutritional deficiencies (Kim, Wilson et al. 2010), thereby promoting sarcopenia. Hence, an increase in protein intake, both qualitative and quantitative (Boirie 2009) may help to reverse sarcopenia. Although these studies indicate that dietary supplementation of proteins and amino acids may serve to reverse sarcopenia, the role of dietary supplementation at the cellular level remains poorly understood.

It has also been suggested that creatine (Cr) supplementation might have the potential to enhance the muscle oxidative capacity and adenosine diphosphate (ADP)-stimulated mitochondrial ATP production rate (MAPR). It has been shown that Cr supplementation increases phosphocreatine resynthesis (Greenhaff, Bodin et al. 1994) and we argue that this must be due to increased rate of oxidative phosphorylation (see

Section 2.4.2.4.3). Brannon (1997) found significant increases in CS activity (a Krebs cycle enzyme) as a result of Cr supplementation in rat soleus but not plantaris muscle. Additionally, Guerrero-Ontiveros (1998) showed that Cr supplementation also produced a significant increase in the activity of the oxidative enzyme succinate dehydrogenase (SDH), in both rat soleus muscle homogenates and isolated mitochondria. Conversely, however, some studies do not support the claim that Cr supplementation increases muscle oxidative capacity (Vandenberghe, Goris et al. 1997; Finn, Ebert et al. 2001). For example, Finn et al. (2001) found no increase in post-muscle CS activity after Cr supplementation, while Vandenburghe et al. (1997) found no change in post-exercise PCr resynthesis after Cr supplementation.

It is noticeable that no studies have examined the effects of Cr supplementation on MAPR, which provides an overall view of ATP production from a range of metabolic pathways. Hence, the mechanisms by which Cr supplementation affect muscle oxidative capacity remains unresolved.

Therefore, evidence suggests that dietary supplementation with Cr and whey protein may play an important role in offsetting the age-related changes in muscle oxidative capacity. However this has only been shown in animals and not humans. If Cr and whey protein are, in fact, found to have the ability to increase muscle oxidative capacity by increasing mitochondrial protein synthesis and/or antioxidant defenses within the muscle, then this would provide a very effective and safe treatment for the decreases in muscle mass and oxidative capacity seen with age.

Thus, the following study was conducted to compare the effects of 6 weeks of supplementation with Cr and whey protein compared with placebo on muscle oxidative capacity in aging humans.

It was hypothesized that six weeks of supplementation with Cr and whey protein would achieve beneficial effects in terms of body composition, muscle oxidative capacity (measured by MAPR), mitochondrial function (measured by MAPR and CS activity), and muscle protein content in healthy aged individuals when compared with placebo.

4.2 Methods

4.2.1 Participants

A total of 20 healthy aged male participants (aged 55+ years) volunteered for the study. All participants were required to complete a detailed medical questionnaire and all gave written informed consent before commencing the study. Additionally, in order to meet the selection criteria for the study, participants (a) had not taken any ergogenic supplements for a six-month period prior to the commencement of the study, and (b) agreed not to ingest ergogenic supplements, other than that given as a part of the study, for the duration of study.

4.2.2 Diet and Supplementation

Each participant was instructed to maintain normal dietary intake throughout the study. To achieve a baseline understanding of the participants' diet, participants were asked to complete a 24-hour food recall on two-week days and one weekend day during the week prior to the commencement of the study. Dietary analyses were completed by using Foodworks 2007 software (Version 6; xyris software, Australia) software to determine daily energy, protein, carbohydrate and fat content of the diet.

Participants were randomly assigned to one of three supplemental groups (placebo, creatine or whey protein) in double-blind fashion as outlined in methods (section 3.2).

All supplements were colour- and flavour-matched, and provided to the participants in identical, unmarked containers. Participants were advised to mix the supplement in water and to consume it in divided dosages three times per day. Participants were asked to return their empty containers as a measure of compliance. The whey, Cr and carbohydrate used for this study are commercially available supplements and were provided by AST Sport Science Evergreen, Colorado, USA.

4.2.3 Body Composition

Whole-body composition measurements were determined using a Hologic QDR-4500 dual energy X-ray absorptiometry (DXA; Figure 3.1) with the Hologic version V7 software (Waltham,MA), according to methods outlined in Chapter 3, Section 3.4.1.

4.2.4 Measurement of Aerobic Capacity (VO_2 peak)

VO_2 peak was determined according to the methods outline in Chapter 3, Section 3.5.

4.2.5 Muscle Biopsy

All muscle samples were obtained from the vastus lateralis via needle biopsy using a sterile Bergstrom muscle biopsy needle (5mm x 101 mm length; Stille, Stockholm, Sweden) according to methods as outlined in Chapter 3, Section 3.9.1. Roughly 30–50 mg of the muscle was immediately placed into a labeled cryule and stored in ice before the measurement of MAPR and CS. Another 20–30 mg of the

muscle was immediately placed into a labeled cryule and snap frozen in liquid nitrogen for analysis of muscle proteins. The remaining tissue was mounted using Tissue-Tek medium after the fibres were orientated in a longitudinal direction and snap frozen in isopentane, pre-cooled in liquid nitrogen and then stored at -80 °C for histochemical analysis.

4.2.6 Measurement of MAPR, ADP-stimulated MAPR and CS

MAPR, ADP-stimulated MAPR and CS activity were measured according to the methods outlined in Chapter 3, Sections 3.9.2 and 3.9.3.

4.2.7 Measure of Muscle Protein Content

Total and contractile protein levels were determined according to the methods of Bradford (1976) as outlined in Chapter 3, Section 3.12.

4.2.8 Histochemistry

Muscle fibre type composition using histochemical techniques was determined according to methods outlined in Chapter 3, Section 3.10.

4.2.9 Analysis of MHC isoforms with SDS-PAGE

Myosin heavy chain (MHC) composition using SDS-PAGE was determined according to methods outlined in Chapter 3, Section 3.11.

4.2.10 Statistics

Statistical analyses were carried out using Prism 5 for Mac OSX (GraphPad Software Inc. USA) as outlined in Chapter 3, Section 3.14.

4.3 Results

4.3.1 Baseline Characteristics

Differences in baseline characteristics between groups were compared using one-way analysis of variance (ANOVA). At baseline there were no significant differences in age, height, VO₂peak, or body composition between the groups prior to the supplementation program (Table 4.1).

Table 4.1: Baseline Characteristics. Values are means \pm SD.

Characteristics	Control (n=6)	Creatine (n=7)	Whey Protein (n=7)
Age (yr)	61 \pm 4.6	60 \pm 7.0	61 \pm 4.7
Height (cm)	181 \pm 10.8	179 \pm 5.9	176 \pm 2.7
VO ₂ peak (ml.kg ⁻¹ .min ⁻¹)	36 \pm 10.8	34 \pm 9.0	33 \pm 8.1
Body Mass (kg)	86.1 \pm 17.8	82.1 \pm 13.5	75.3 \pm 5.3
Fat Mass (g)	20.1 \pm 7.7	20.0 \pm 5.3	15.4 \pm 5.5
Lean Mass (g)	63.2 \pm 10.6	59.3 \pm 8.1	57.3 \pm 3.0
Fat %	23 \pm 5	24 \pm 3	20 \pm 6
Bone Mineral (g)	2.8 \pm 0.2	2.8 \pm 0.2	2.6 \pm 0.2

4.3.2 Body Composition (DEXA)

Body composition measurements as measured by DEXA are presented in Figure 4.1 A-D. All groups showed a significant increase ($p < 0.05$) in total body weight and fat mass following the 6-week supplementation period, however no treatment or treatment plus time interaction effects were detected. There were no significant differences in lean mass, fat percentage or bone mineral content (BMC) detected between treatments following six weeks of dietary supplementation..

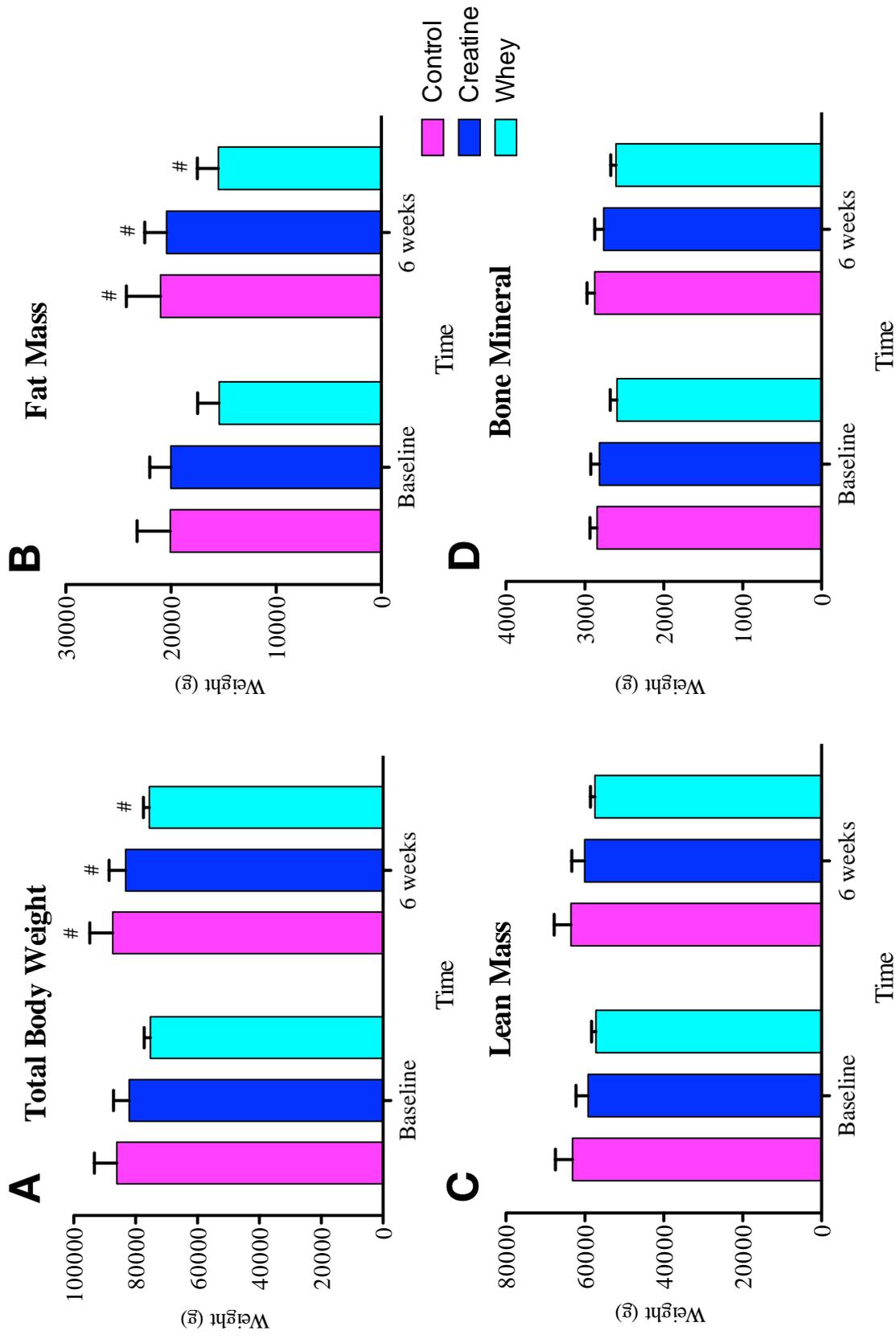


Figure 4.1: Body Composition (DEXA) changes for (A) Total Body Weight, (B) Fat Mass, (C) Lean Mass and (D) Bone Mineral Content (BMC) in aged males. Values are means \pm SE. [#]Significant time effect for total body weight and fat mass ($p < 0.05$), no treatment or treatment plus time interaction effects were detected. Controls ($n=6$), creatine supplemented ($n=7$) and whey protein supplemented ($n=7$).

4.3.3 Measurement of Aerobic Capacity (VO_2 peak)

VO_2 peak results are shown in Figure 4.2. There was no significant difference in VO_2 peak between groups or across time following six weeks of dietary supplementation. Not all participants completed VO_2 peak testing. Only complete datasets were included in the final analysis. Control (n=3), Creatine (n=3), and Whey Protein (n=3).

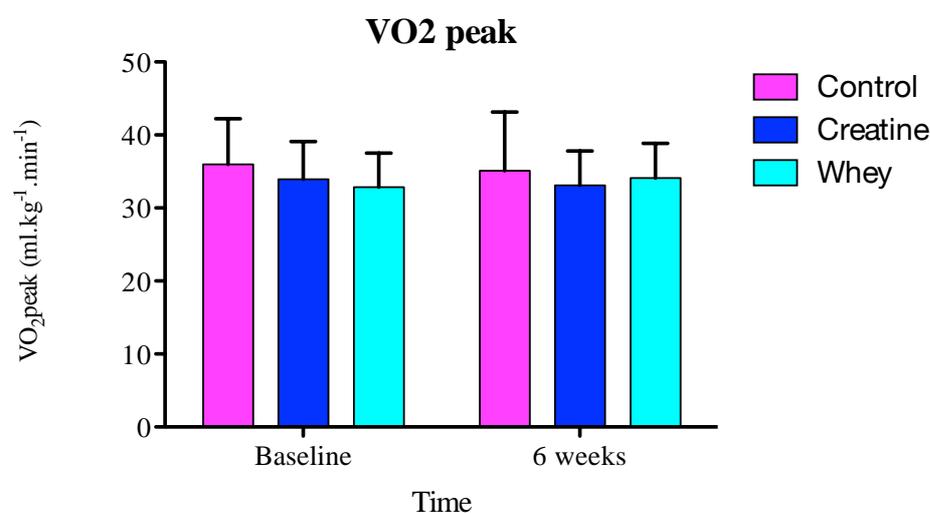


Figure 4.2: VO_2 peak in aged males. Values are means \pm SE. There was no significant difference in VO_2 peak between groups or across time following six weeks of dietary supplementation. Controls (n= 3), creatine supplement (n=3) and whey protein supplemented (n=3).

4.3.4 Dietary Analysis

The average of a three-day 24-hour dietary recall for energy (kcal), protein (g), carbohydrates (g) and fat (g) taken before the commencement of study are shown in Table 4.2. There were no significant differences in energy, protein, carbohydrate or fat intake identified between treatment groups (Table 4.2).

Table 4.2: Baseline Dietary Assessment. Values are means \pm SD of a three-day recall (two week days and one weekend day) conducted over a seven-day period before the commencement of trial. These values do not include dietary supplements.

Characteristics	Control (n=6)		Creatine (n=7)		Whey Protein (n=7)	
	per day	per kg/day	per day	per kg/day	per day	per kg/day
Energy (kcal)	1889 \pm 75	22 \pm 4.8	1936 \pm 496	27 \pm 10	1998 \pm 196	26 \pm 4.0
Protein (g)	109 \pm 24	1.2 \pm 0.1	105 \pm 23	1.4 \pm 0.5	92 \pm 24	1.2 \pm 0.3
Fat (g)	59 \pm 18	0.7 \pm 0.3	69 \pm 20	1.0 \pm 0.4	64 \pm 11	0.8 \pm 0.2
Carbohydrates (g)	193 \pm 33	2.3 \pm 0.8	202 \pm 68	2.8 \pm 1.3	196 \pm 50	2.6 \pm 0.8

4.3.5 Mitochondrial ATP Production Rate (MAPR)

Mitochondrial ATP production rates (MAPR) using the different substrate combinations are shown in Figures 4.3 – 4.5. All groups showed a significant decrease ($p < 0.05$) in MAPR with time using the substrates palmitoylcarnitine and malate (PC+M; fat metabolism), succinate and rotenone (S+R; complex II metabolism) and pyruvate, palmitoyl-carnitine, alpha-ketoglutarate and malate (PPKM; all substrates). Use of the substrates pyruvate and malate (P+M; carbohydrate metabolism) and alpha-ketoglutarate (alpha-KG; protein metabolism) did not quite reach statistical significance ($p = 0.09$ and $p = 0.05$, respectively). However, there were no significant differences in MAPR between groups or across time following six weeks of dietary supplementation.

4.3.6 Citrate Synthase (CS) and Mitochondrial Yield

CS activity and mitochondrial yield results are shown in Figure 4.5B and Table 4.3. There was no significant difference in before, after or total CS activity or in mitochondrial yield between groups or across time following six weeks of dietary supplementation.

4.3.7 ADP-Stimulated MAPR

ADP-stimulated MAPR results are shown in Table 4.4. All groups showed a highly significant increase ($p < 0.001$) in MAPR with increasing concentrations of ADP following the 6-week supplementation period, however there was no significant difference in ADP-stimulated MAPR between groups or across time following six weeks of dietary supplementation.

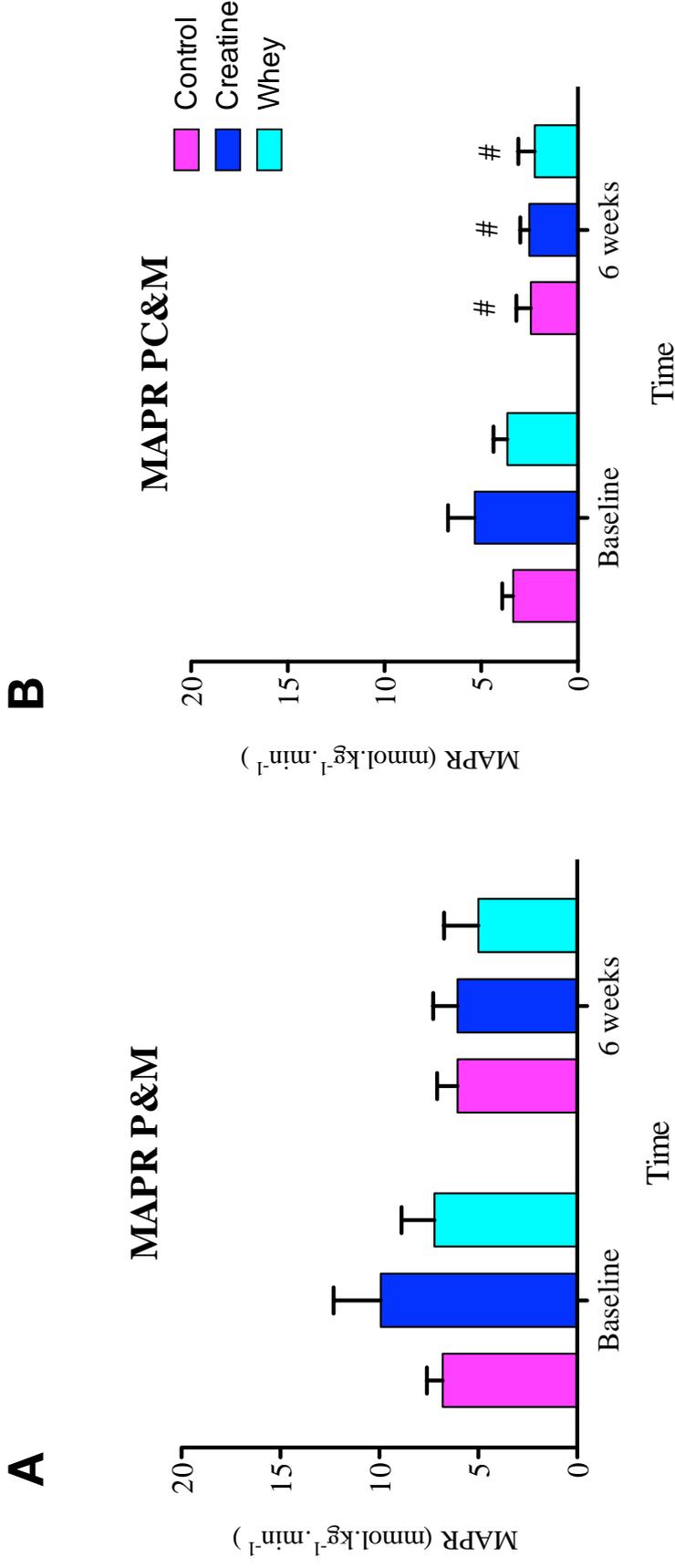


Figure 4.3: Mitochondrial ATP production Rate (MAPR) under (A) pyruvate and malate (P+M; carbohydrate metabolism) and (B) palmitoylcarnitine and malate (PC+M; fat metabolism) in aged males. Values are means \pm SE. # Significant time effect for substrates palmitoylcarnitine and malate (PC+M; fat metabolism; $p < 0.05$), no treatment or treatment plus time interaction effects were detected. Use of the substrates pyruvate and malate (P+M; carbohydrate metabolism) did not quite reach statistical significance ($p = 0.09$). Controls ($n = 5$), creatine supplement ($n = 7$) and whey protein supplemented ($n = 6$).

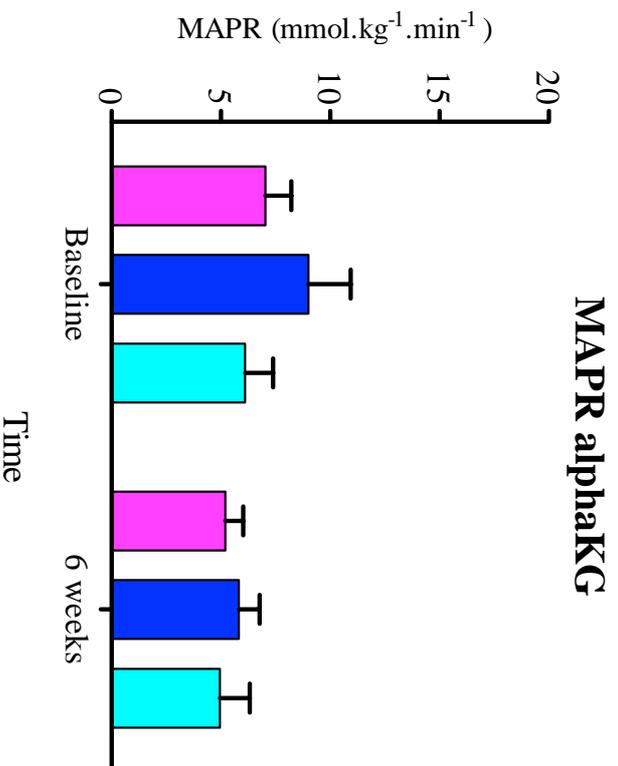
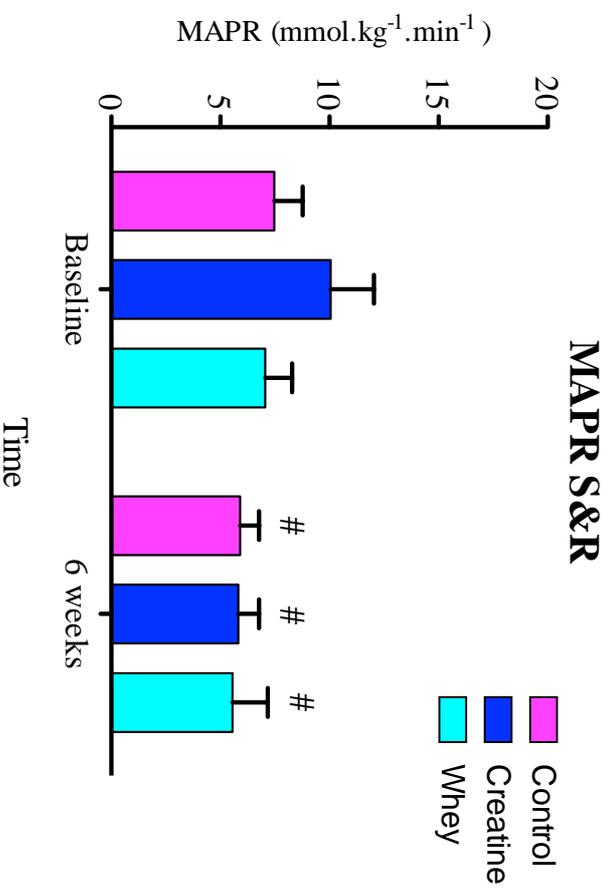
A**B**

Figure 4.4: Mitochondrial ATP production Rate (MAPPR) under (A) alpha-ketoglutarate (alpha-KG; protein metabolism) and (B) succinate and rotenone (S+R; complex II metabolism) in aged males. Values are means \pm SE. #Significant time effect for substrates succinate and rotenone (S+R; complex II metabolism; $p < 0.05$), no treatment or treatment plus time interaction effects were detected. Use of the substrates alpha-ketoglutarate (alpha-KG; protein metabolism) did not quite reach statistical significance ($p = 0.05$). Controls ($n = 5$), creatine supplement ($n = 7$) and whey protein supplemented ($n = 6$).

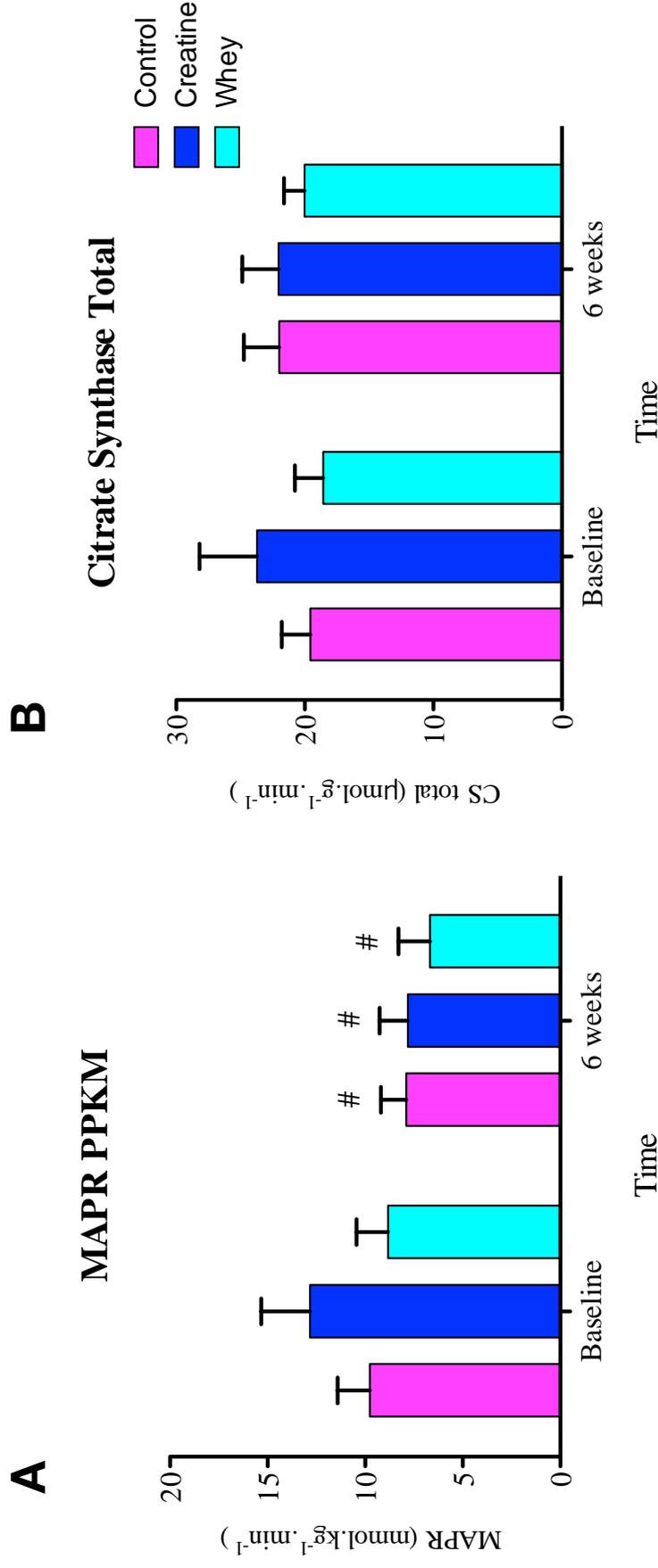


Figure 4.5: Mitochondrial ATP production Rate (MAPR) under (A) palmitoyl-carnitine, alpha-ketoglutarate and malate (PPKM; all substrates) and (B) citrate synthase (CS) activity in aged males. Values are means \pm SE. [#]Significant time effect for substrates palmitoyl-carnitine, alpha-ketoglutarate and malate (PPKM; all substrates; $p < 0.05$), no treatment or treatment plus time interaction effects were detected. Use of the substrates alpha-ketoglutarate (alpha-KG; protein metabolism) did not quite reach statistical significance ($p = 0.05$). There was no significant difference in total CS activity between groups or across time following six weeks of dietary supplementation. Controls ($n = 5$), creatine supplement ($n = 7$) and whey protein supplemented ($n = 5$).

Table 4.3: Citrate Synthase and Mitochondrial yield. Values are means \pm SD.

	Control (n=6)		Creatine (n=7)		Whey Protein (n=7)	
	0 weeks	12 week	0 weeks	12 week	0 weeks	12 week
CS before ($\mu\text{mol.g}^{-1}.\text{min}^{-1}$)	0.26 \pm 0.13	0.27 \pm 0.88	0.37 \pm 0.24	0.37 \pm 0.19	0.23 \pm 0.12	0.31 \pm 0.20
CS after ($\mu\text{mol.g}^{-1}.\text{min}^{-1}$)	4.41 \pm 3.82	5.19 \pm 2.98	5.92 \pm 4.00	5.51 \pm 2.77	2.75 \pm 1.49	4.86 \pm 3.48
CS total ($\mu\text{mol.g}^{-1}.\text{min}^{-1}$)	19.56 \pm 5.44	22.00 \pm 6.78	23.73 \pm 11.84	22.07 \pm 7.47	16.94 \pm 4.18	20.03 \pm 3.93
Mitochondrial yield (%)	20.19 \pm 14.10	21.44 \pm 9.12	19.76 \pm 5.59	22.52 \pm 10.15	14.02 \pm 6.91	19.16 \pm 11.30

Table 4.4: ADP-stimulated MAPR at different ADP concentrations. Values are means \pm SD for ADP-stimulated MAPR at different concentrations of ADP. ^{##}Highly significant treatment effect for increasing concentrations of ADP.

	Control (n=6)		Creatine (n=7)		Whey Protein (n=7)	
	Baseline	6 weeks	Baseline	6 weeks	Baseline	6 weeks
ADP 2.5 μ M (mmol.kg ⁻¹ .min ⁻¹)	5.82 \pm 4.18	6.84 \pm 2.46	8.66 \pm 3.99	7.78 \pm 4.28	5.17 \pm 3.10	5.35 \pm 3.19
ADP 5.0 μ M (mmol.kg ⁻¹ .min ⁻¹)	10.43 \pm 6.17	12.13 \pm 4.78	16.39 \pm 8.82	13.58 \pm 8.85	9.50 \pm 4.86	10.51 \pm 5.82
ADP 10.0 μ M (mmol.kg ⁻¹ .min ⁻¹)	17.72 \pm 10.36	16.62 \pm 5.84	27.48 \pm 16.71	21.46 \pm 14.36	14.78 \pm 7.50	13.85 \pm 7.99
ADP 20.0 μ M (mmol.kg ⁻¹ .min ⁻¹)	22.11 \pm 13.85	16.53 \pm 12.07	38.16 \pm 22.56	22.90 \pm 21.34	20.58 \pm 13.11	20.94 \pm 12.65
ADP 40.0 μ M (mmol.kg ⁻¹ .min ⁻¹)	24.43 \pm 16.38 ^{##}	25.38 \pm 10.90 ^{##}	43.63 \pm 30.25 ^{##}	30.34 \pm 22.49 ^{##}	21.37 \pm 10.80 ^{##}	22.96 \pm 14.51 ^{##}

4.3.8 Muscle Protein Content

Total and contractile protein content results are shown in Table 4.5. No significant differences were identified between the groups and across time for total or contractile protein content (Table 4.5). However, post-hoc analysis using an unpaired Student's t-test on the difference between before and after contractile muscle protein content revealed an almost significant ($p=0.06$) increase in contractile proteins in the Cr supplement group (+27.42 mg/g) when compared with the control group (+7.77 mg/g; Table 4.5).

4.3.9 Histochemistry

The muscle sections that were intended for histochemistry analysis were found to be damaged by the presence of ice crystals. Unfortunately, the extent of the damage was too great to allow for staining and viewing of muscle fiber type and muscle fiber area according to the original research plan.

4.3.10 Myosin Heavy Chains (MHC) isoform analysis using SDS-PAGE

Myosin Heavy Chain isoform results are shown in Table 4.6. The muscle weight of some of the samples obtained did not allow for reliable measurement in this analysis, therefore the sample size is lower in this assessment than in most other assessments (Table 4.6).

No significant differences were identified between the groups and across time for myosin heavy chain isoforms (Table 4.6).

Table 4.5: Total and contractile protein content. Values are means \pm SD for total and contractile protein content.

Protein concentration (mg/g)	Control (n=6)		Creatine (n=7)		Whey Protein (n=7)	
	Baseline	6 weeks	Baseline	6 weeks	Baseline	6 weeks
Total Protein	65.35 \pm 12.93	85.53 \pm 24.58	62.07 \pm 18.95	109.14 \pm 61.33	66.30 \pm 17.74	82.76 \pm 22.50
Contractile Protein	57.45 \pm 17.80	65.22 \pm 21.59	44.52 \pm 12.75	71.94 \pm 11.58	60.84 \pm 20.39	65.50 \pm 17.78

Table 4.6: Myosin Heavy Chain isoforms. Values are means \pm SD for MHC isoform percentage.

MHC isoform (%)	Control (n=4)		Creatine (n=4)		Whey Protein (n=5)	
	Baseline	6 weeks	Baseline	6 weeks	Baseline	6 weeks
MHC type I	23.65 \pm 2.1	39.47 \pm 6.3	26.39 \pm 3.1	39.55 \pm 3.4	27.84 \pm 6.5	29.78 \pm 3.1
MHC type II	76.36 \pm 2.1	60.53 \pm 6.3	73.62 \pm 3.1	61.89 \pm 4.1	72.16 \pm 6.5	70.22 \pm 3.1

Note: Type IIA and IIX fibers were combined in this analysis because of poor delineation on the gels between the two types of band

4.4 Discussion

In the present study, we have investigated the role of Cr and whey-protein supplementation in augmenting muscle oxidative capacity. In order to assess muscle function we chose MAPR as a marker for oxidative capacity, and CS activity as an indicator for increase in mitochondrial function/number. Of particular interest to the current study was the potential for Cr supplementation to enhance the muscle oxidative capacity and ADP-stimulated MAPR.

We found that supplementation with either Cr or whey protein offered no significant benefits in terms of body composition, muscle oxidative capacity or muscle protein content.

We felt it was reasonable to suppose that Cr and whey-protein supplementation would act on MAPR, although this has not explicitly been shown previously in the literature. Both Cr and glutamine (available in whey-protein) are known to increase the hydration status of the cell, stimulating an increase in protein synthesis. For example, ingestion of whey protein has previously been shown to improve muscle protein synthesis in elderly individuals (Katsanos, Chinkes et al. 2008) while glutamine (present in high amounts in whey protein) has been shown to increase muscle cell volume (Haussinger 1996; Low, Taylor et al. 1996; Haussinger, Graf et al. 2001) and protein synthesis. Indeed, unpublished data from our laboratory have shown significant increases in MAPR and CS activity in rat muscle following whey-protein supplementation (Smith, Hayes et al. 2001). Whey protein has also been shown to enhance glutathione levels and augment antioxidant defenses in immune deficiency states (Lands, Grey et al. 1999; Bounous 2000). As improved antioxidant defenses would protect mitochondria from oxidative damage, then an increase in

glutathione may also increase muscle oxidative capacity, as reflected in changes in MAPR.

4.4.1 MAPR

MAPR values were assessed according to the methods described in Chapter 3. In order to ensure that our MAPR findings are plausible, and that any interpretation of these values is meaningful, we compared our results against those previously reported in the literature (Wibom and Hultman 1990; Williams, Carey et al. 2007). Although our results are a little higher than the previously reported values, our findings are comparable and broadly in line with the literature values, and we are satisfied that the assessment of MAPR values in our study is valid.

An increase in MAPR, which is essentially an increase in V_{\max} for oxidative phosphorylation, is one way in which the rate of oxidative phosphorylation of the muscle might be altered.

Surprisingly, and contrary to our hypothesis, Cr and whey supplementation did not result in an increase in MAPR (Figures 4.3 – 4.5) after the six-week period of supplementation compared with the control group. However, a new and novel finding of this study was the highly significant increase ($p < 0.01$) in MAPR with increasing ADP concentration in all groups following the 6-week supplementation period. There were no significant differences in ADP-stimulated MAPR between the different supplemental groups following 6 weeks of dietary supplementation. These results indicate changes in mitochondrial sensitivity (i.e. K_m) for ADP at various concentrations. Whilst K_m of oxidative respiration for ADP was relatively unchanged following supplementation with creatine or whey, increasing concentrations of ADP were shown to increase MAPR in the vastus lateralis muscle of aged males.

4.4.2 Effects of Cr supplementation

The role of Cr supplementation in stimulating new protein synthesis in muscles undergoing experimental atrophy has been previously established (Hespel, Op't Eijnde et al. 2001), although the effect occurred after 10 weeks of supplementation and in a younger population. In a further study in younger participants (Deldicque et al 2008), five days of Cr supplementation increased the expression of certain targeted genes, although it did not enhance anabolic signaling.

The relative youth of the participants in these two studies may explain why an effect of Cr supplementation was observed in these studies but not in ours. As we have already seen, muscle protein synthesis declines with age, and this general decline may mask any benefit of short-term Cr supplementation. In addition, this difference in effect according to the age group may be the result of a lower resting PCr in the aged population (Smith, Montain et al. 1998). The lower baseline levels of PCr in aged muscle may also be partly responsible for decreases in muscle oxidative capacity, mitochondrial ATP production as well as skeletal muscle size, as discussed in Chapter 2, Section 2.6.1.

It has generally been shown that fast twitch fibres show the greatest response to Cr supplementation (Syrotuik and Bell 2004) thus given that these fibres atrophy and/or transition to type-I fibres as we age, this may limit the effect of Cr supplementation in older people. Unfortunately, Cr levels were not measured in the current study and thus we can only assume that there was loading into the muscle cell. However, the Cr-loading protocol used in this study has been used in previously-reported studies and has been shown to effectively increase muscle PCr levels in both young (Harris, Soderlund et al. 1992; Hultman, Soderlund et al. 1996) and old (> 50yrs) subjects (Smith, Montain et al. 1998). We are confident, therefore, that use of

this literature Cr-loading protocol would also have resulted in effective muscle Cr loading in our own study.

The lack of a resistance-training protocol in this study may also have contributed to the absence of a stimulation of oxidative phosphorylation and muscle protein synthesis following Cr supplementation (Paddon-Jones, Borsheim et al. 2004). Furthermore, Cr supplementation has not previously been shown to provide any additional benefit on top of normal training in healthy but aged subjects (Bermon, Venembre et al. 1998; Eijnde, Van Leemputte et al. 2003; Bembien, Witten et al. 2010). This may be because any slight incremental benefit due to the Cr-supplementation is masked by the greater benefit of the resistance training.

Post-hoc analysis between the before and after measurements revealed an almost significant increase in contractile protein content in the Cr supplemented group compared with control and whey supplemented groups. This is particularly interesting as the Cr supplement group tended to have higher MAPR values at baseline, and the greatest decrease in MAPR was observed in this group. An increase in the amount of contractile proteins together with increased volume of contractile fibres would effectively bring about a net dilution of intracellular enzymes and their substrates, which might explain why MAPR, CS activities and ATP production all decreased. It is also possible that the six-week time point represents the incipient anabolic phase and that the time period used in this study is too short to observe the net anabolic effect of Cr and whey supplementation.

A final important point to consider is the exact Cr dosage administered in each case, and its effect on the subjects' body weight. In order to attempt to correct for variations in Cr dosage, we performed a number of correlation tests between the Cr dosage and changes in lean mass, changes in fat mass, change in P+M MAPR, and

total CS. We found no correlation in any such tests and calculated r^2 values to be <0.1 in almost every case. We conclude, therefore, that less than 10% of the variation in our findings can be explained by variations in the Cr dose, and we are therefore satisfied with the validity of our conclusions.

4.4.3 Effects of whey-protein supplementation

Throughout the six-week trial period, overall body composition remained the same between control and supplemented groups. Total protein and contractile protein are also comparable between the control and whey supplemented groups (Table 4.5).

This effect appears to be in contrast with the earlier findings of Katsanos (2008), who reported that greater muscle protein accrual following whey-protein supplementation. However, in the Katsanos study, the whey protein was administered as a single 15 g bolus with muscle protein accrual measured immediately after the ingestion of the whey protein, compared with the six-week oral regimen of 1.5 g/kg/day of whey protein employed in our study that was ingested in divided dosage over an entire day. Furthermore, the Katsanos study evaluated protein synthesis and accretion compared with pure protein content which was assessed in the present study. These factors may help explain the apparent differences between these two studies.

The lack of effect on muscle protein content with whey-protein supplementation observed in the current study may also be explained by the absence of a resistance-training program in this study. As in the case of Cr supplementation as described previously, a training stimulus is known to enhance any potential benefit of whey-protein supplementation on muscle protein content (Burke, Chilibeck et al.

2001; Kerksick, Rasmussen et al. 2007). The effect of a concomitant resistance-training program with dietary supplementation is investigated in the next chapter.

Whey protein supplementation (with and without Cr supplementation) has been shown to increase protein synthesis in participants who have undergone resistance training (Burke, Chilibeck et al. 2001; Cribb, Williams et al. 2007; Kerksick, Rasmussen et al. 2007). Thus, our results may reflect the lack of the extra stimulus of training; a point that is addressed in the next chapter.

The protein content within the muscle would be expected to increase according to the functional demand. For example, unpublished data from our laboratory revealed a preferential increase in mitochondrial proteins in the diaphragm and gastronemius muscles of six-week old dystrophic mice following supplementation with whey protein and with a combination of Cr plus whey protein (Rybalka 2007). Rybalka hypothesized that whilst the total protein content was not increased following supplementation, specific protein degradation and synthesis pathways were being 'switched on' to meet the functional demand of the muscle. This suggests that, in the case of muscular dystrophy, protein synthesis is skewed towards maintaining energy metabolism and cell survival rather than increasing the synthesis of muscle proteins associated with muscle function and structure (Rybalka 2007).

Finally, it is thought that anabolic resistance to dietary amino acids may contribute to muscle wasting in the elderly. Anabolic resistance has previously been used to explain a blunting of the effect of resistance training and protein supplementation in aging individuals (Cuthbertson, Smith et al. 2005; Kumar, Atherton et al. 2009; Rennie 2009), although to the best of our knowledge, this has not previously been investigated with creatine in aged muscle. However, this may be a further explanation of the lack of effect demonstrated in this study.

Although the sample sizes were small, the observations from the MHC analysis warrant some discussion. Whilst a shift from type II to type I fibers following the 6-week supplemental period appeared to occur in our study, in reality it is highly unlikely that such a shift would be possible in such a short period of supplementation, particularly given the lack of exercise training involved.

The unusual results found in our study may be explained by the fact that our method had not been optimized for human muscle, and only previously optimized for mouse muscle (Kemp, Blazev et al. 2009). In addition, our methods used a MHC isoform marker from a mouse model, which may not have been compatible with the human muscle samples we used.

4.5 Conclusions

The purpose of this study was to assess the benefit of Cr and whey-protein supplementation on muscle oxidative capacity in aged males. Supplementation with either Cr or whey-protein offered no benefit in terms of body composition, muscle oxidative capacity or muscle protein content.

Interestingly, and contrary to our original hypothesis, muscle oxidative capacity appeared to decrease after six weeks of dietary supplementation. This is thought to have occurred as a result of a change in cell volume. Changes in cell volume have been shown to increase protein synthesis through a negative feedback process that corrects changes in the nuclear-to-cytoplasmic ratio and hence minimizes the dilution of intracellular proteins, enzymes and associated processes. It is suggested that the six-week time point represents an incipient anabolic phase and that the time period used in this study may be too short.

The findings of this study draw us to the conclusion that a six-week supplementation program with either Cr or whey protein does not increase muscle oxidative capacity in aged males. This may be due to lack of a stimulus, such as resistance training, or to the length of the current study. Hence, protocols invoking supplementation and resistance training for periods of greater than six weeks are warranted. This approach will be discussed in the next chapter.

Chapter 5

The Effect of Whey-Protein Supplementation and Resistance Training on Muscle Protein Content and Muscle Strength in Older Humans

5.1 Introduction

As we have seen in previous chapters, the aging process is associated with a loss of skeletal muscle ('sarcopenia'), a decrease in muscle oxidative capacity and a reduction in muscle strength (Cohn, Vartsky et al. 1980; Cohn, Vaswani et al. 1985). These factors combine in the elderly to impair muscle function and performance, resulting in greater frailty and an increased risk of falls (Roubenoff 2000; Carmeli, Coleman et al. 2002).

Research has shown that it is possible to slow, or even reverse, the changes in muscle mass and oxidative capacity, as well as the free-radical damage, that occur with age. Two effective interventions have been identified to date which appear to mitigate the effects of sarcopenia: exercise and dietary supplementation.

Exercise has been shown to increase muscle mass (Westterterp 2000), muscle oxidative capacity (Cartee 1994; Westterterp 2000) and antioxidant defenses against free-radical damage (Lands, Grey et al. 1999). Importantly, it seems that resistance training, in particular, may play a crucial role in the prevention of sarcopenia (Johnston, De Lisio et al. 2008). Regular resistance training, therefore, is thought to be a possible intervention in helping prevent age-related loss in muscle mass and strength (Johnston, De Lisio et al. 2008). Indeed, resistance training has been shown to be effective in increasing muscle mass and strength in even the very old (Singh 2004)

Dietary supplementation with whey protein has been shown to increase muscle mass (Lands, Grey et al. 1999; Cribb 2000; Burke, Chilibeck et al. 2001; Hayes and Cribb 2008) and protein synthesis (Poullain, Cezard et al. 1989) and may, therefore, also have a benefit in restoring muscle function in the elderly. Whey

protein has also been shown to enhance glutathione levels and augment antioxidant defenses in immune deficiency states (Lands, Grey et al. 1999; Bounous 2000), suggesting that increased glutathione levels from whey-protein supplementation may also increase muscle oxidative capacity. Furthermore, evidence suggests that whey-protein supplementation may be of greatest benefit in the glycolytic muscles (Smith, Hayes et al. 2001), an important consideration as these are the type of muscle that predominantly decrease in size with age (Essen-Gustavsson and Borges 1986; Kovanen and Suominen 1987; Carmeli and Reznick 1994).

Although dietary supplementation and resistance training have individually been shown to have important roles in reducing age-related loss in muscle mass and strength, it is interesting to consider the impact of the two interventions in combination. From the evidence already discussed in the Literature Review (Chapter 2), it may be expected that whey protein supplementation in combination with a training program may augment the effects of the training. This, therefore, was the rationale for the present study; to investigate the possible benefit of a resistance-training program, with additional concomitant whey-protein supplementation, in arresting the debilitating loss of muscle size and function that occurs with age. It is hoped that this research may provide a safe and alternative treatment that helps to maintain the functional integrity of the muscle in the aging human. This research may also be of benefit in the current state of understanding about how aging effects the human muscle, as well helping the afflicted individuals better understand their pathologies.

It was hypothesized that a 12-week resistance-training program in combination with whey-protein supplementation would achieve beneficial effects in terms of body composition, muscle strength, muscle oxidative capacity (measured by

MAPR), mitochondrial function (measured by MAPR and CS activity), and muscle protein content when compared with a resistance-training program alone.

5.2 Methods

5.2.1 Participants

A total of 17 healthy aged male participants (aged 55+ years) volunteered for the study. All participants were required to complete a detailed medical questionnaire and gave written informed consent before commencing the study. Additionally, to meet the selection criteria for the study, participants (a) had not taken any ergogenic supplements for a six-month period prior to the commencement of the study, (b) had not taken part in a heavy resistance training program for a six-month period prior to the commencement of the study, and (c) agreed not to ingest ergogenic supplements, other than that given as a part of the study, for the duration of study.

5.2.2 Diet and Supplementation

Each participant was instructed to maintain a normal diet throughout study. To get a baseline understanding of the participants' diet, participants were asked to complete a 24-hour food recall exercise on two-week days and one weekend day during the week prior to commencement of the study. Dietary analyses were completed using Foodworks 2007 software (Version 6; xyris software, Australia) to determine daily dietary energy, protein, carbohydrate and fat content.

Participants were randomly assigned to one of two supplemental groups (placebo + resistance training [RT] or whey protein + resistance training [RTPr]) in double-blind fashion as outlined in methods (section 3.2).

All supplements were colour- and flavour-matched, and provided to the participants in identical, unmarked containers. Participants were advised to mix the supplement in water and to consume it in divided dosages three times per day. Participants were asked to return their empty containers as a measure of compliance. The whey protein used for this study is a commercially available supplement and was provided by AST Sport Science Evergreen, Colorado, USA.

5.2.3 Resistance Training Protocol

The 12-week resistance-training program was prescribed and based on the Living Longer Living Stronger Program provided by the Council of the Aging (COTA) as outlined in Chapter 3, Section 3.3 of the methods.

5.2.4 Body Composition

Whole-body composition measurements were determined using a Hologic QDR-4500 dual energy X-ray absorptiometry (DEXA) with the Hologic version V 7, REV F software (Waltham, MA) according to methods outlined in Chapter 3, Section 3.4.1.

5.2.5 Muscle Biopsy

All muscle samples were obtained from the vastus lateralis via needle biopsy using a sterile Bergstrom muscle biopsy needle (5 mm x 101 mm length; Stille, Stockholm, Sweden) according to methods as outlined in Chapter 3, Section 3.9.1. Roughly 30–50 mg muscle was immediately placed into a labeled cryule and stored in ice before measurement of MAPR and CS. Another 30–80 mg muscle was immediately placed into a labeled cryule and snap frozen in liquid nitrogen for analysis of intracellular thiols/disulfides (see Chapter 6) and muscle proteins. The

remaining tissue was mounted using Tissue-Tek medium after the fibres were orientated in a longitudinal direction and snap frozen in isopentane, pre-cooled in liquid nitrogen and then stored at -80°C for histochemical analysis.

5.2.6 Measurement of Muscle Strength

5.2.6.1 Isometric and Isokinetic Strength

Isometric and isokinetic muscle strength were examined by voluntary isometric and isokinetic knee extension and isokinetic knee flexion using a Cybex dynamometer (Cybex International Inc. Ronkonkoma, New York) as outlined in Chapter 3, Section 3.6.1.

5.2.6.2 Functional Strength

Functional muscle strength was measured with three different functional exercises in which the large muscle groups that are important in daily activities were assessed: the 10-meter walk test (10 MWT), the 14-stair climb test and the 30-s sit-to-stand test (STS) according to the methods of Brose (2003), as outlined in Chapter 3, Section 3.6.2.

5.2.6.3 1-Repetition Maximum (1RM)

Strength assessments consisted of the maximal weight that could be lifted once (1RM) in two weight-training exercises: machine bench press and horizontal leg press. The 1RM was measured according the methods of Baechle (2000), as outlined in Chapter 3, Section 3.6.3.

As it is not always easy or safe to secure 1RM performances in older individuals, if the 1RM could not be achieved, a calculation of the 1RM from a 2–5

repetitions to failure (RTF) test was made using a predicted 1RM formula devised by Mayhew (1995), as outlined in Chapter 3, Section 3.6.3.

5.2.6.4 Measurement of Muscle Power using Optical Encoder

Measurement of concentric and eccentric mean and peak muscle power output was performed in real-time while participants performed three different exercises (leg press, lat pull-down and bicep curl) allowing for the assessment of mean and peak power output in different muscle groups (legs, back and biceps) using the Gymaware optical encoder, as outlined in Chapter 3, Section 3.6.4.

5.2.7 Measurement of MAPR and citrate synthase (CS)

Mitochondrial ATP production rate (MAPR) and citrate synthase (CS) activity were measured according to the methods outlined in Chapter 3, Sections 3.9.2 and 3.9.3.

5.2.8 Measurement of Muscle Protein Content

Total, contractile and mitochondrial protein content were determined according to the methods of Bradford (1976), as outlined in Chapter 3, Section 3.12.

5.2.9 Histochemistry

Muscle fibre type composition using histochemical techniques was determined according to methods outlined in Chapter 3, Section 3.10.

5.2.10 Analysis of MHC isoforms with SDS-PAGE

Myosin heavy chain (MHC) composition using SDS-PAGE was determined according to methods outlined in Chapter 3, Section 3.11.

5.2.11 Statistical Analysis

Statistical analyses were carried out using Prism 5 for Mac OSX (GraphPad Software Inc. USA) as outlined in Chapter 3, Section 3.14.

5.3 Results

5.3.1 Baseline Characteristics

A total of 17 healthy aged male participants (aged 55+ years) volunteered for the study. One participant had to withdraw from the study due to problems with blood sugar response that was thought to be related to ingestion of the dietary supplement. This left a total of 16 healthy, aged males who participated fully in the study. Seven participants were randomized to the RTPr group and nine to the RT group.

At baseline there were no significant differences in age, height, body composition or 1RM (leg press and bench press) between the groups prior to the supplementation and 12-week resistance-training program (Table 5.1).

5.3.2 Dietary Analysis

The average of the three-day, 24-hour dietary recall exercise for energy (kcal), protein (g), carbohydrates (g) and fat (g) taken before the commencement of study are shown in Table 5.2. In the RTPr group there was a significantly higher total energy intake ($p=0.017$) and carbohydrate intake ($p=0.021$), when measured per kilogram of body weight, than the RT group at baseline (Table 5.2). There were no significant differences in protein or fat intake at baseline between groups (Table 5.2).

Table 5.1: Baseline Characteristics. Values are mean \pm SD.

Characteristic	RT (n=9)	RTPr (n=7)
Age (yr)	61.8 \pm 6.0	64 \pm 3.9
Height (cm)	174.6 \pm 7.7	176.3 \pm 4.3
Body mass (kg)	81.5 \pm 12.5	75.9 \pm 8.1
Fat mass (kg)	20.1 \pm 6.1	16.0 \pm 6.4
Lean mass (kg)	56.6 \pm 5.1	57.1 \pm 2.6
Fat %	24.3 \pm 5.0	20.5 \pm 6.5
Bone mineral (kg)	2.7 \pm 0.4	2.8 \pm 0.3
1RM leg press (kg)	129 \pm 27	129 \pm 25
1RM bench press (kg)	61 \pm 25	56 \pm 20

Table 5.2: Baseline Dietary Assessment. Values are means \pm SD of a three-day recall (two week days and one weekend day) conducted over a seven-day period before the commencement of trial. These values do not include dietary supplements. *Significant difference in average daily energy and carbohydrate intake (kg/day; $p < 0.05$) between groups. §not all participants completed baseline dietary assessments.

Characteristics[§]	RT (n=7)		RTPr (n=4)	
	per day	per kg/day	per day	per kg/day
Energy (kcal)	1728 \pm 333	22 \pm 2.8	2132 \pm 239	29 \pm 5.5*
Protein (g)	81 \pm 9.5	1.0 \pm 0.13	89 \pm 23	1.2 \pm 0.4
Fat (g)	60 \pm 11	0.76 \pm 0.15	52 \pm 22	0.77 \pm 0.31
Carbohydrates (g)	193 \pm 64	2.4 \pm 0.54	272 \pm 51	3.7 \pm 1.1*

5.3.3 Body Composition (DEXA)

Body composition measurements are presented in Figure 5.1 A-D. Both RT and RTPr groups showed a highly significant decrease ($p < 0.0001$) in fat mass (Figure 5.1 B), as well as a highly significant increase ($p < 0.0001$) in lean mass (Figure 5.1 C) following the training program. However, no treatment or treatment-plus-time interaction effects were detected. No significant differences in total bodyweight or bone mineral content (BMC) were detected between the RT and RTPr groups as a result of 12 weeks of dietary supplementation or resistance training (Figure 5.1 A-D).

5.3.4 Isometric and Isokinetic Muscle Strength

Isometric muscle strength measurements for knee extension and isokinetic knee extension and flexion are shown in Table 5.3. Both the RT and RTPr groups showed a highly significant increase ($p < 0.01$) in isometric and isokinetic knee extension and isokinetic knee flexion following the training program (Table 5.3). No treatment or treatment-plus-time interaction effect was detected. However, a significant increase in isokinetic knee flexion strength ($p = 0.005$) was observed in the RT group when compared to the RTPr group following 12-weeks of dietary supplementation and resistance training (Table 5.3).

5.3.5 Functional Strength

Functional strength results, as determined across three different functional movement tests (10 MWT, 14-stair climb, 30-s STS test) at both normal and fast pace, are presented in Tables 5.4 – 5.6.

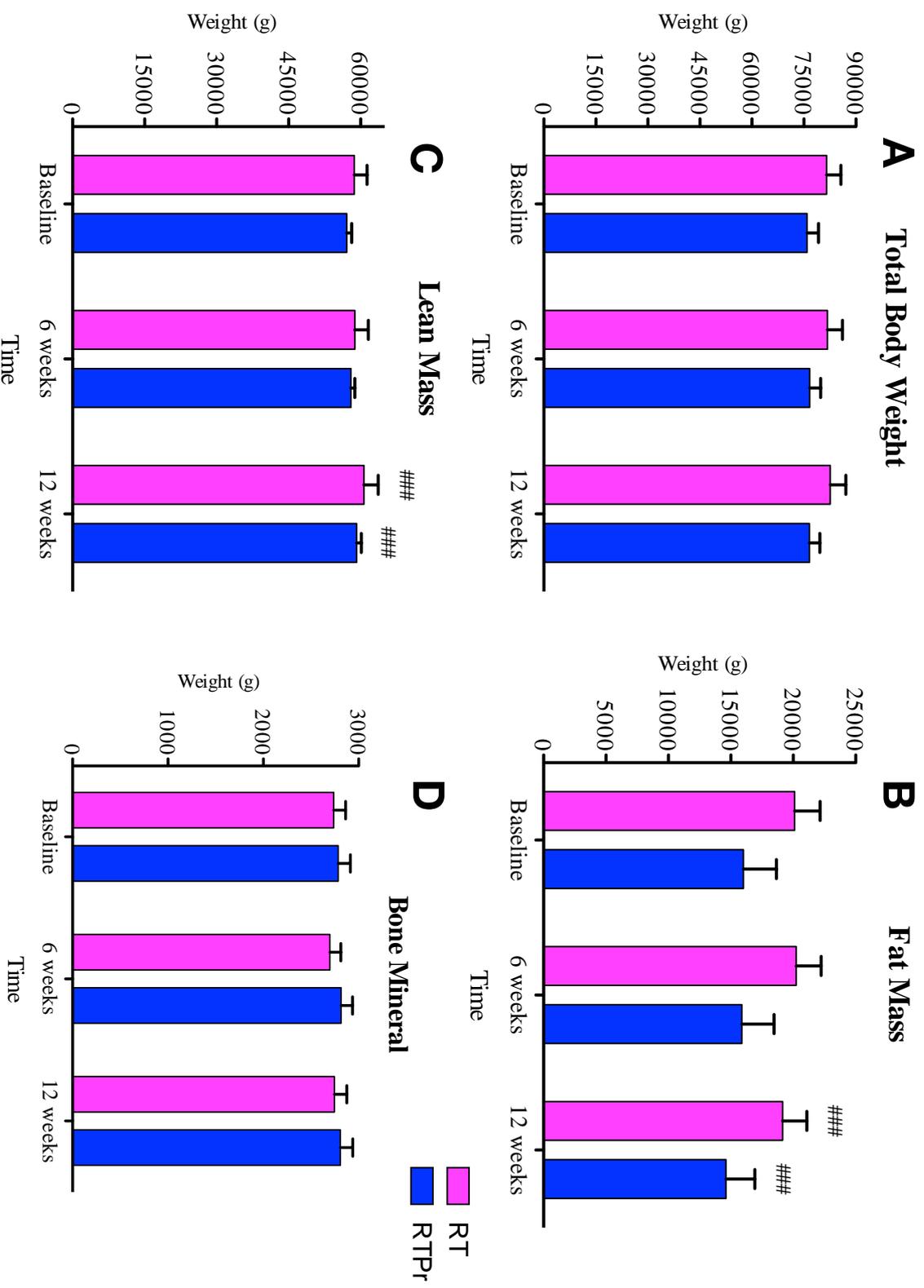


Figure 5.1: Body Composition (DEXA) changes for (A) Total Body Weight, (B) Fat Mass, (C) Lean Mass and (D) Bone Mineral Content (BMC) in aged males. Values are means \pm SE. ### Highly significant time effect for fat mass and lean mass ($p < 0.0001$) following the resistance-training program, no treatment or treatment plus time interaction effects were detected. RT (n=9) and RTPr (n=7).

Table 5.3: 1RM Tests for Isometric Strength, isokinetic extension (EXT) and flexion (FLX) in aged males. Values are mean \pm SD. # significant time effect for isokinetic knee extension (EXT) ($p < 0.01$)^{##} Highly significant time effect for isometric and isokinetic knee flexion ($p < 0.001$). *Significant increase in isokinetic knee flexion strength was observed in the RT group ($p < 0.05$) when compared to the RTPr group. ϕ significant difference between baseline values using unpaired t-test ($p < 0.05$). \S one participant in the RTPr group was not able to complete the cybex testing due to the increased risk associated pre-existing injury. Units for isometric and isokinetic strength measures is Nm.

Time point ^{\\$}	RT (n=9)			RTPr (n=6)		
	Isometric	Isokinetic (EXT)	Isokinetic (FLX)	Isometric	Isokinetic (EXT)	Isokinetic (FLX)
0 weeks	207 \pm 59	162 \pm 35	89 \pm 16	190 \pm 20	154 \pm 27	108 \pm 5 ϕ
6 weeks	214 \pm 58	172 \pm 29	103 \pm 13	211 \pm 33	173 \pm 32	111 \pm 6
12 weeks	233 \pm 63 ^{##}	176 \pm 33 [#]	115 \pm 13 ^{##*}	216 \pm 45 ^{##}	166 \pm 32 [#]	111 \pm 6

Table 5.4: Functional Strength Measures in Aged males using a 10-meter walk test.Values are mean \pm SD.

Timepoint	RT (n=9)		RTPr (n=7)	
	Normal (s)	Fast (s)	Normal (s)	Fast (s)
0 weeks	21.4 \pm 2.0	15.3 \pm 1.3	20.7 \pm 2.9	14.4 \pm 1.0
6 weeks	21.5 \pm 3.0	15.2 \pm 1.5	22.5 \pm 1.3	14.3 \pm 1.7
12 weeks	21.0 \pm 1.2	14.4 \pm 1.5	20.9 \pm 1.8	14.4 \pm 1.2

Table 5.5: Functional Strength Measures in Aged Males using a 14-step stair climb test. Values are mean \pm SD.

Timepoint	RT (n=9)		RTPr (n=7)	
	Normal (s)	Fast (s)	Normal (s)	Fast (s)
0 weeks	6.8 \pm 0.7	4.8 \pm 0.3	7.3 \pm 0.6	4.3 \pm 0.3
6 weeks	6.8 \pm 0.4	4.9 \pm 0.7	7.0 \pm 0.5	4.7 \pm 0.7
12 weeks	6.6 \pm 0.6	4.3 \pm 0.8	6.9 \pm 0.8	4.5 \pm 0.6

Table 5.6: Functional Strength Measures in Aged Males using a sit to stand test.

Values are mean \pm SD. Values are given as the number of sit-to stands completed in a 30-second time period. ^{##}Highly significant time effect for sit-to stand test ($p < 0.001$), no treatment or treatment plus time effects were detected.

Timepoint	RT (n=9)	RTPr (n=7)
0 weeks	17.4 \pm 4.6	21.4 \pm 7.9
6 weeks	22.4 \pm 7.4	26.0 \pm 5.5
12 weeks	23.5 \pm 7.8 ^{##}	27.6 \pm 8.3 ^{##}

Both the RT and RTPr groups showed a highly significant increase ($p < 0.0001$) in the sit-to-stand test (Table 5.6). However, no other significant differences were detected between the groups or over time in the functional strength measures (Tables 5.4 – 5.6).

5.3.6 1-Repetition Maximum (1RM)

One repetition maximum results for leg press and bench press are shown in Table 5.7. Both the RT and RTPr groups showed a highly significant increase ($p < 0.0001$) in leg press and bench press 1RM following the training program (Table 5.7). However no treatment or treatment-plus-time interaction effect was detected.

5.3.7 Measurement of Muscle Power using Optical Encoder

Concentric mean and peak muscle power, force and velocity results are presented in Figures 5.2 – 5.4 A - D. Both the RT and RTPr groups showed a significant increase ($p < 0.05$) in leg press concentric mean power and concentric peak force and lat pull down concentric peak force (Figure 5.3 and 5.4) following the training program. Additionally, both the RT and RTPr groups showed a significant decrease ($p < 0.05$) in lat pull down concentric peak velocity (Figure 5.4) following the training program. However no treatment or treatment-plus-time interaction effect was detected with the exception of a significant increased concentric peak force with the leg press ($p = 0.032$) observed in the RT group when compared to the RTPr group following 12-weeks of dietary supplementation and resistance training (Figure 5.3 D). No significant differences were observed in the bicep curl parameter between the groups or over time.

Table 5.7: 1RM Tests for Leg Press and Bench Press in Aged Males. Values are mean \pm SD. [#]Highly significant time effect for 1-RM Leg Press and 1-RM Bench Press ($p < 0.001$), no treatment or treatment plus time effects were detected. [§]two participants in the RT group were not able to complete the 1RM testing due to the increased risk associated pre-existing injury.

Timepoint [§]	RT (n=7)		RTPr (n=7)	
	1RM Leg Press (kg)	1RM Bench Press (kg)	1RM Leg Press (kg)	1RM Bench Press (kg)
0 weeks	129 \pm 27	61 \pm 25	129 \pm 25	56 \pm 20
6 weeks	148 \pm 47	68 \pm 27	147 \pm 30	63 \pm 21
12 weeks	186 \pm 43 ^{##}	76 \pm 22 ^{##}	157 \pm 25 ^{##}	70 \pm 26 ^{##}

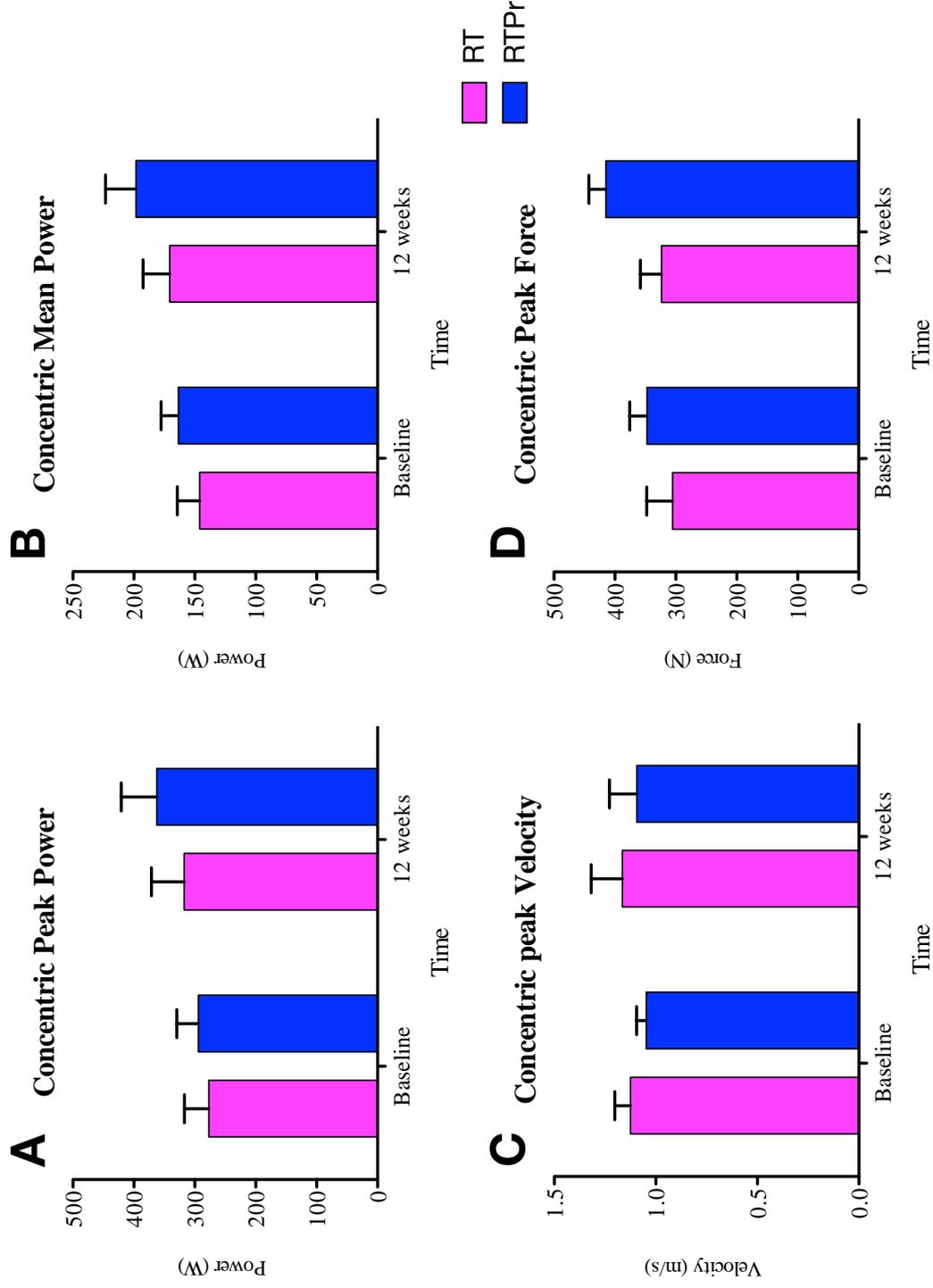


Figure 5.2: Changes in bicep (A) concentric peak power, (B) concentric mean power, (C) concentric peak velocity and (D) concentric peak force in aged males. One participant in the control group was not able to complete the bench press testing due pre-existing injury. RT (n=8) and RTPr (n=7).

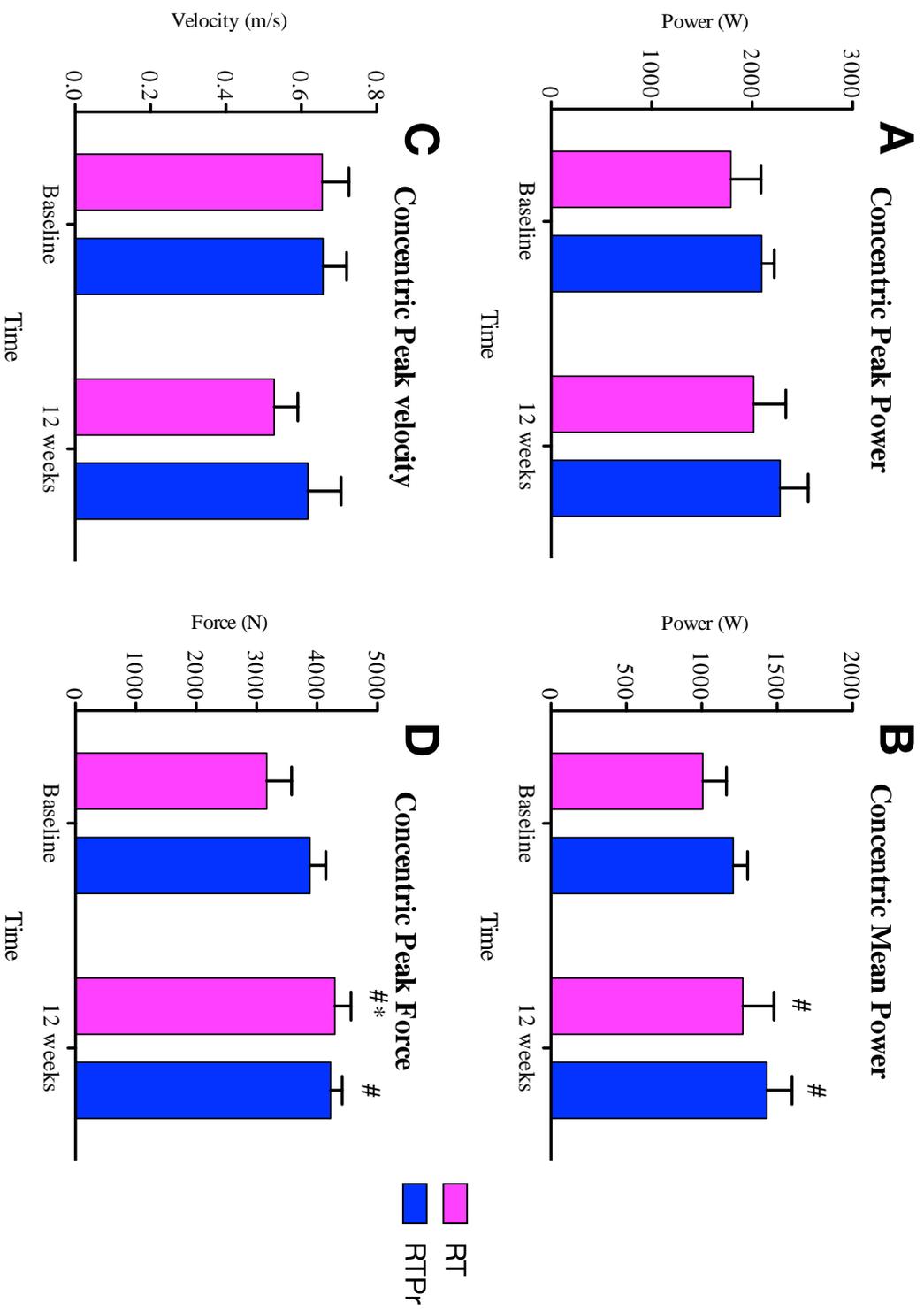


Figure 5.3: Changes in leg press (A) concentric peak power, (B) concentric mean power, (C) concentric peak velocity and (D) concentric peak force in aged males. Values are means \pm SE. # Significant time effect for concentric mean power and concentric peak force ($p < 0.05$). * Significant increase in concentric peak force ($p < 0.05$). One participant in the control group was not able to complete the leg press testing due pre-existing injury. RT ($n=8$) and RTPr ($n=7$).

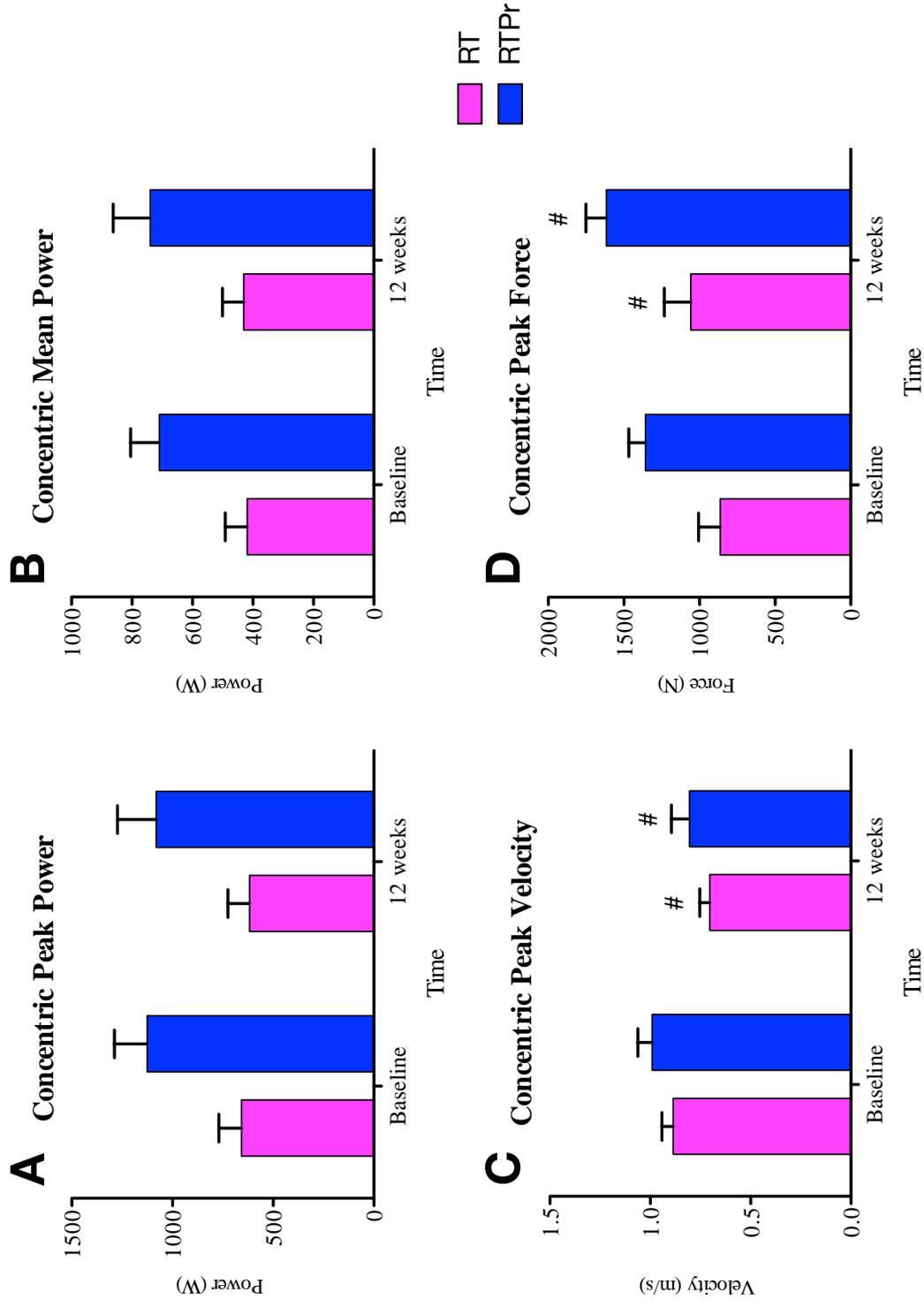


Figure 5.4: Changes in lat pull down (A) concentric peak power, (B) concentric mean power, (C) concentric peak velocity and (D) concentric peak force in aged males. Values are means \pm SE. # Significant time effect for concentric peak velocity and concentric peak force ($p < 0.05$), no treatment or treatment plus time interaction effects were detected. RT (n=9) and RTPr (n=7).

5.3.8 Mitochondrial ATP Production Rate (MAPR) and Citrate Synthase (CS)

The appropriate quantity of ADP was not added to the reaction mixture at baseline due to an error in experimental procedure. Because ADP is an important metabolic substrate required to stimulate oxidative metabolism, this meant that the necessary reaction did not proceed. We were not able to repeat the experiment using the snap frozen muscle, as MAPR assessment is only valid on fresh muscle samples. As we were not able to measure baseline MAPR, it was decided not to proceed with MAPR measurements at 6 and 12 weeks because, without the baseline measurements, these values would not have a useful comparison point at the beginning.

Thus, we are not able to comment on changes associated with muscle oxidative capacity via MAPR following dietary supplementation and 12-weeks of resistance training. However, we did achieve successful measurement of total citrate synthase (CS) activity at the key time points, which is an alternative measure of oxidative enzyme activity. Total CS activity results are shown in Table 3.8. There was no significant difference in total CS activity between groups or over time following 12-weeks of dietary supplementation and resistance training.

5.3.9 Muscle Protein Content

Total, contractile and mitochondrial protein content results are shown in Table 5.8. No significant differences were identified between the groups or over time for muscle protein content across all three measures (Table 5.8). Post-hoc analysis using an unpaired Student's t-test on the change from before to after for total muscle protein content revealed that there was a significant difference in total protein content ($p=0.047$) in the RTPr group compared to the RT group following 12 weeks of dietary

supplementation and resistance training (Table 5.8). Surprisingly, levels of total protein decreased by 18% in the RT group, but increased by 25% in the RTPr group.

Table 5.8: Total Protein, Contractile Protein, Mitochondrial Protein and Total CS Activity in Aged Males. Values are mean \pm SD. *Significant difference between change in total protein content ($p < 0.05$) observed using an unpaired students t-test.

Protein	RT (n=9)		RTPr (n=7)	
	0 weeks	12 weeks	0 weeks	12 weeks
Total (mg/g)	59.5 \pm 13.1	48.6 \pm 23.5	52.2 \pm 3.3	65.6 \pm 9.0*
Contractile (mg/g)	56.6 \pm 16.3	58.8 \pm 4.2	52.6 \pm 19.5	64.8 \pm 9.3
Mitochondrial (mg/g)	13.1 \pm 4.3	11.4 \pm 2.1	11.2 \pm 3.8	11.9 \pm 0.6
CS total ($\mu\text{mol.g}^{-1}.\text{min}^{-1}$)	21.52 \pm 4.5	17.39 \pm 4.3	23.59 \pm 8.1	19.47 \pm 6.4

5.3.10 Histochemistry

As discussed in section 4.3.9 in the previous chapter, the muscle sections that were intended for histochemistry analysis were found to be damaged by the presence of ice crystals. Unfortunately, the extent of the damage was too great to allow for staining and viewing of muscle fibre type and muscle fibre area according to the original research plan.

5.3.11 Myosin Heavy Chains (MHC) isoform analysis using SDS-PAGE

Myosin Heavy Chain isoform results are shown in Table 5.9. As described in Section 4.3.10, the muscle weight of some of the samples obtained did not allow for reliable measurement in this analysis, therefore the sample size is lower in this assessment than in most other assessments (Table 5.9).

No significant differences were identified between the groups and across time for myosin heavy chain isoforms (Table 5.9).

Table 5.9: Myosin Heavy Chain isoforms. Values are means \pm SD for MHC isoform percentage.

MHC isoform (%)	Control (n=3)		Whey Protein (n=3)	
	Baseline	12 weeks	Baseline	12 weeks
MHC type I	48.10 \pm 13.4	47.20 \pm 6.3	48.00 \pm 15.6	47.07 \pm 14.3
MHC type II	51.90 \pm 13.4	52.80 \pm 6.3	52.00 \pm 15.3	52.93 \pm 14.3

Note: Type IIA and IIX fibers were combined in this analysis because of poor delineation on the gels between the two types of band

5.4 Discussion

The study was designed to allow us to evaluate the benefit of resistance training alone compared with the combination of resistance training and whey-protein supplementation on muscle function in a group of elderly male participants. As expected, a statistically significant reduction in fat mass and fat percentage was observed, and a significant increase in lean mass in both groups over the 12-week program of dietary supplementation and resistance training. The lack of a reduction in total body weight in either group over time may be explained by the increase in muscle mass occurring in parallel with decreasing fat mass, due to the implementation of the training program.

However, interestingly, supplementation with whey-protein in addition to a 12-week resistance-training program was found to have no effect on body composition or muscle strength. The lack of difference in body composition may possibly be explained by the higher baseline energy intake in the RTPr group which limited the ability of the whey protein supplement to induce its typical response of increasing lean mass and decreasing fat mass, given that the overall energy expenditure of the resistance training session would be the same for both groups. In any case, the lack of difference between the two groups clearly indicates that the

improvement in body composition over the course of the study can be ascribed to the effects of the resistance-training program and not whey-protein supplementation.

Muscle strength was assessed in this study through evaluation of the four key parameters: isometric and isokinetic knee extension and flexion; functional strength; 1RM and muscle power. As expected, isometric and isokinetic knee extension and isokinetic knee flexion were increased in both groups ($p < 0.01$), following the 12-week resistance-training program. The RTPr group did not improve to a greater extent than the RT group in any parameter, while, surprisingly, the RT group revealed a greater improvement in isokinetic knee flexion over the course of the study compared with the RTPr group ($p = 0.005$). This apparent anomaly may be explained by the fact that the RTPr group had a higher starting isokinetic flexion strength than the RT group (108 ± 5 nm compared with 89 ± 16 nm, respectively, $p = 0.02$), and may therefore have been fitter at the start of the study. It is a common observation when a new diet and exercise regime is introduced, that an 'easy' gain may be seen rapidly in subjects who have a lower existing fitness level. While the 'overload principle of strength training' would suggest that even the fitter individuals should have been expected to make gains, the fact that both groups were following the same training program suggests that the "fitter" RTPr group may not have been training at the same intensity and therefore may not have been overloading their muscles. This would result in a reduced effect from the training compared with the RT group.

The suggested difference in baseline fitness levels cannot be demonstrated unequivocally, as activity levels prior to the start of the study were not measured, and all participants were selected on the basis of having 'normal' levels of activity. However, the greater initial fitness of the RTPr group is supported by the fact that

while this group had an average higher baseline calorie consumption (Table 5.2), it had a lower average baseline bodyweight and fat mass (Table 5.1) and similar lean mass to the RT group (Table 5.1), suggesting a greater level of activity among this group. Certainly, a greater level of fitness among the RTPr group at baseline would explain the greater improvement observed in the RT group in the isokinetic knee flexion test.

In terms of functional strength, another rather surprising finding was that no significant difference in the functional strength measures using the 10-meter walk or 14-stair climb was observed over the course of the 12-week training program. Both groups however showed a highly significantly improved performance in the 30-s sit-to-stand test over the course of the program ($p < 0.0001$). The absence of any improvement over the course of the training program in the 10-meter walk or 14-stair climb tests, especially with significant overall strength increases, may be understood when one considers the nature of the participants enrolled in this trial. The mean age of the patients was 61.8 ± 6 years in the RT group and 64.0 ± 3.9 years in the RTPr group, suggesting that although mature, the participants were not exceptionally elderly or frail. Hence, the participants in this study are likely to have been fairly healthy and mobile at baseline, and therefore capable of achieving a good result in these tests at baseline. The observation of particular interest is that both groups greatly improved in the 30-s sit-to-stand test during the training program. The sit-to-stand movement uses the large muscles of the lower body (the gluteal muscles and biceps femoris), which are precisely those muscles that suffer most as a result of aging. It is therefore interesting to note that the leg press exercise, conducted as part of a resistance training program, is likely to have a positive benefit on muscle strength in aging patients. Uses of other measures are only likely to be useful when there is

already impairment in their abilities, although maintaining ‘normal’ function cannot be underestimated as we age.

The significant increase in leg press and bench press 1RM observed in both groups was expected due to the impact of the 12-week resistance-training program. As these particular exercises formed a part of the training program itself, unlike some of the functional measures also assessed in this study, this finding is unsurprising (demonstrating the principle of specificity). Again, no difference was observed between the two groups in leg press and bench press 1RM, suggesting that the benefit is due to the training program and not the whey-protein supplementation.

The increase in strength observed in both groups following 12 weeks of resistance training is of real benefit to these aged individuals because decrease in muscle strength is one of the known effects of aging. Surprisingly, a near-significant increase in the 1RM for leg press in the RT group compared with the RTPr group was observed ($p=0.061$), suggesting that RT patients did marginally better in this test by the end of the study. This apparent anomaly may potentially be explained by differences in the equipment supplied at the two locations used for the training program. Only one subject from the RT group trained at Campus A (Werribee) while the rest trained at Campus B (Footscray). It is possible, therefore, that the leg press machines used at Campus B may have offered slightly less resistance than those used at Campus A, resulting in apparently slightly better results for the participants trained at Campus B. This highlights the importance of maintaining consistency when controlling for the many possible influencing factors.

The final measurement of muscle strength, muscle power as assessed by optical encoder, did not reveal any improvement following the 12-week resistance-

training program, however, there was a significant difference ($p=0.032$) in concentric peak force with the leg press in the RT group when compared to the RTPr group. Again, it seems likely that both groups achieved good results in all these measures at baseline, particularly in the RTPr group, and therefore improvement following 12 weeks of training may have been difficult to detect, although the RT group appears to have improved slightly more than the RTPr group. The difference in leg press concentric peak force may be explained in a similar manner to the differences between RT and RTPr groups in isokinetic flexion by the fact that, whilst not significant ($p=0.19$), the RTPr group had an 18% higher starting concentric peak force with the leg press than the RT group (3886 ± 520 N compared with 3170 ± 821 N, respectively), providing greater potential for the RT group to improve more rapidly and with apparently greater ease over the course of the resistance-training program.

Interestingly, participants in the RTPr group appeared to have a small, but not statistically significant, tendency for a higher bone mineral content than the RT group ($p=0.09$). From this we tentatively suggest that whey protein may have some beneficial effect on bone composition, suggesting a potential area of study for the future.

As well as a loss of muscle strength, elderly individuals often experience a reduction in muscle oxidative capacity, which can have a detrimental effect on muscle function. Two markers of muscle oxidative capacity were considered in this study; CS activity and protein content (total, contractile and mitochondrial). While no difference was found between the two groups in terms of CS activity, both groups experienced an improvement in CS activity during the course of the 12-week study. We had hoped also to measure MAPR to further investigate changes in muscle oxidative capacity

during the study and provide further evidence to support the CS activity findings. Unfortunately, due to an experimental error, the MAPR assessment could not be completed, and we can therefore not draw comparisons between these findings and the CS activity observations.

No differences were identified between groups or over time in total, contractile or mitochondrial protein content. However, further post hoc analysis using an unpaired Student's t-test indicated a significant increase in the RTPr group compared to the RT group ($p=0.047$), although participants were not effectively matched ($p=0.78$). As there were no changes in contractile or mitochondrial protein levels, the observed change in protein total protein content is likely to have been a random observation based on incomplete subject matching.

It should be stated that all individuals were untrained at the beginning of the study, and therefore the effects of the resistance-training program are measured from a low baseline expertise in performing the required exercises. The effects of the first 6 to 12 weeks of the new resistance-training program are therefore likely to differ to the effects that would be observed after 12 weeks of training. In particular, the changes expected in the first weeks of a training program would be expected to be largely neuromuscular and not changes in actual muscle mass.

The two treatment groups were well balanced in terms of age, height, body composition and 1RM at the start of the study, indicating that the randomization procedures employed were effective. Interestingly, the RTPr group had a significantly higher self-reported daily energy intake prior to the study compared with the RT group, which was accounted for by a higher carbohydrate intake in this group.

In any case, as the objective of this study was to investigate the effect of supplementing the diet with additional supplementary protein, the dietary parameter of principal concern was prior protein intake. No baseline difference was found between the two groups in this important parameter. Furthermore, baseline total fat consumption was also the same in both groups. As participants were requested to continue in their normal dietary habits during the course of the study, we can assume that the dietary protein intake would continue to be the same between the two groups, with the only difference in ingested protein being due to consumption of whey protein in one group. We can therefore be sure that the dietary protein intake was the same across both groups before and during the study, and it is unlikely that the greater carbohydrate intake in the RTPr group would affect the ability of participants to respond to resistance training. A further purpose of the dietary analysis was to ensure that all participants were receiving sufficient protein (i.e. the recommended daily intake of 1g/kg of body weight) prior to the study, and this was also confirmed (see Table 5.2).

The lack of a benefit in terms of muscle protein content and muscle strength following whey-protein supplementation observed in this study is surprising and warrants further examination. Previous studies have reported a significant increase in lean mass in male participants who have received whey-protein supplements (Burke, Chilibeck et al. 2001; Cribb, Williams et al. 2007). For example, in a study by Burke et al (2001), male subjects receiving whey-protein supplements in addition to resistance training experienced greater increases in lean muscle mass than participants of resistance training alone. Similarly, in a further study, supplementation with creatine/carbohydrate, whey protein, and creatine/whey protein resulted in significantly greater 1RM strength improvements and muscle hypertrophy compared

with carbohydrate supplementation only (Cribb, Williams et al. 2007). However, the participants involved in these studies were younger and fitter than the population under examination in the present research (subjects in the Burke trial were aged between 18 and 31 years, while the participants in the Cribb study were male recreational body-builders in their early twenties). This suggests that youth and relative fitness may be an important consideration in these studies.

Two further studies conducted in older male subjects (between 48 and 72 years), by contrast, also failed to find any incremental benefit of whey-protein supplementation over the observed benefit of resistance training (Eliot 2008, Bemben 2010). In particular, the study by Bemben and colleagues has many parallels with the work presented here (Bemben, Witten et al. 2010). In the Bemben study, the effects of creatine and protein supplementation on strength gains following a traditional resistance training program was assessed in 42 men aged 48-72 years. The researchers found that while strength and lean body mass increased significantly in each group, there were no significant group effects or group X trial interactions. These findings taken together draw us towards the conclusion that the additional benefit of whey-protein supplementation is more apparent in younger individuals. Indeed, studies suggest that the response to whey-protein supplementation may differ in the elderly because the rate of muscle protein synthesis is lower in aged males due to decreased testosterone production (Bhasin, Woodhouse et al. 2001) and myosin heavy chain protein synthesis (Balagopal, Rooyackers et al. 1997). Eliot et al. (2008) go on to suggest that the supplementation dose that is effective in young male subjects may not be sufficient to cause an effect in the older male.

Finally, it is known that nutrition in elderly people can be compromised by the fact that absorption of amino acids and sugars declines with age (Ferraris 1997). These observations all combine to suggest that the dosage of whey-protein used in these studies, while sufficient to induce a noticeable effect in young subjects, may have been insufficient to create a response in the elderly male participants, which brings into question whether the protein target requirements set for the elderly are high enough.

A further important consideration here is the role of the classic ‘training effect’, which may account for much of the response observed, potentially masking some of the effects of whey-protein supplementation. Contractile activity stimulates mammalian target of rapamycin (mTOR) and other protein synthesis pathways (Hulmi, Tannerstedt et al. 2009), increasing the effectiveness of resistance training and protein supplementation in young trained individuals (Deldicque, Theisen et al. 2005; Hulmi, Tannerstedt et al. 2009; Moore, Atherton et al. 2010). However, the effects of resistance training and protein supplementation on mTOR activity have so far not been studied in the elderly, although contraction-mediated mTOR activation in skeletal muscle appears to be attenuated with age (Parkington, LeBrasseur et al. 2004; Funai, Parkington et al. 2006). It is difficult, therefore, to quantify the effect of mTOR in the elderly. In addition, there is a strong neural learning component associated with the first few months of a new training program, during which time it is not easy to differentiate a real improvement in strength from an increasing skill in lifting weights. This, again, may mask any effect of whey-protein supplementation. It is possible, therefore, that an effect of whey-protein supplementation may have been observed if the training period had been prolonged or if subjects had been pretrained prior to the start of the study.

Another consideration which was not evaluated in this study is the effect of timing of supplement ingestion. In a study by Cribb and Hayes (2006), the timing of supplement ingestion was found to have a significant effect on the effectiveness of a training program. Supplementation before and after each workout resulted in a significantly greater improvement in 1-RM strength and body composition compared with supplementation outside of the pre- and post-workout time frames. As timing of supplementation ingestion was not recorded during this study, it is possible that this may explain the lack of effect observed.

While several important and interesting conclusions may be drawn from this work, the study does raise interesting questions for future investigation. In particular, it would be interesting to include patients of an even greater age or frailty than those studied here, so that improvements due to the resistance-training program in some of the functional parameters measured may have been more apparent. An investigation into the effects of these interventions in a more elderly population may be the focus of further research in the future. It would also be interesting in future studies to perform the MAPR assessment that was originally planned here. This would provide further insight into the potential benefit of whey-protein supplementation on muscle oxidative capacity. Finally, a future study would be designed to account for the possible effect of supplementation timing on the effectiveness of the resistance-training program.

5.5 Conclusions

Although a 12-week resistance-training program provided a significant benefit to participants in terms of decreased fat mass and fat percentage as well as increased lean mass, the addition of whey-protein supplementation offered no incremental

benefit over resistance training alone in terms of either body composition or muscle strength.

Resistance training, with or without additional whey-protein supplementation, offered a significant benefit in terms of isometric and isokinetic knee extension and isokinetic knee flexion, 30-s sit-to-stand test, and leg press and bench press 1RM, but not in the 10-minute walk test, 14-stair climb or any assessment of muscle power. In terms of muscle oxidative capacity, no difference was found between the two groups in CS activity, or in total, contractile or mitochondrial protein content.

These findings draw us to the conclusion that a 12-week resistance-training program achieves benefits in terms of muscle function in elderly individuals with sarcopenia. However, the inclusion of additional dietary whey protein, in the form of powdered whey protein, appears to offer no additional benefit over resistance training alone. This finding may be explained by variations in the timing of supplement ingestion as well as the differential response to whey protein supplementation seen between young and old subjects.

Chapter 6

The Effect of Whey Protein
Supplementation and Resistance Training
on Plasma and Tissue Redox Status in
Older Humans [including optimization of
intracellular redox status methods]

6.1 Introduction

The reduction-oxidation ('redox') processes that occur in biological cells are discussed in Section 2.7.1. The redox state of a cell is determined and regulated by thiol systems including the glutathione (GSH) /glutathione disulfide (GSSG) system (Jones 2002; Ji 2008; Jones 2008), which has the ability to reduce levels of reactive oxygen species (ROS) within the cell.

The GSH/GSSG system is generally regarded as the primary redox buffering system in most cellular compartments due to its low redox potential and relatively high abundance. In this redox system, GSH is oxidized to GSSG by accepting an electron from hydrogen peroxide, thus minimizing ROS-induced oxidative damage (Mills 1957). The GSSG formed in this way is subsequently reduced back into GSH via glutathione reductase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), setting up a redox cycle that can be used as an indirect measurement for the oxidative state of the cell.

As well as the GSH/GSSG redox system, useful information about the level of oxidative stress can be ascertained from the cysteine/cystine (Cys/CySS) redox pair. The redox state of the Cys/CySS pool is linked to the redox state of the GSH/GSSG pool because the GSH released from cells can react with the Cys in the plasma to produce the disulfide CySS. Therefore, it can be difficult to determine whether the changes in the Cys/CySS redox state are the result of oxidative stress in the plasma or within the cell.

In humans, aging is associated with a decrease in the GSH concentration both in whole blood (Erden-Inal, Sunal et al. 2002) and in mononuclear cells such as lymphocytes (Hernanz, Fernandez-Vivancos et al. 2000). GSH synthesis is known to

be dependent on the presence of the amino acids Cys and methionine, and, as Cys levels have also been found to decrease with age (Hernanz, Fernandez-Vivancos et al. 2000; Dröge 2005; Jones 2006), the reduction in GSH levels is exacerbated. The intracellular GSSG/GSH ratio therefore shifts toward a more oxidizing state with increasing age as the relative amount of GSSG rises (Rebrin, Kamzalov et al. 2003; Rebrin and Sohal 2008).

A decrease in the synthesis of GSH can lead to an accumulation of oxidation byproducts in cells. This is a clear sign of free radical damage, caused by an accumulation of ROS in the cells with increasing age. (Sohal and Weindruch 1996; Rebrin, Kamzalov et al. 2003; Jones 2008; Rebrin and Sohal 2008).

Supplementation with Cys as oxidation-resistant N-acetyl-Cys, which is subsequently transformed to Cys in the body, has been shown to at least partly reduce the effects of aging in different systems (De La Fuente, Miquel et al. 2002; Guayerbas, Puerto et al. 2002; Miquel 2002; Grattagliano, Portincasa et al. 2004; Guayerbas, Puerto et al. 2004; Guayerbas, Puerto et al. 2005; Morrison, Coleman et al. 2005; Nicoletti, Marino et al. 2005; Muscari, Bonafe et al. 2006; Chan and Shea 2007; Kanwar and Nehru 2007; Arranz, Fernandez et al. 2008; Bagh, Maiti et al. 2008). Whey protein, another rich source of Cys that aids in the synthesis of GSH (Zavorsky, Kubow et al. 2007; Balbis, Patriarca et al. 2009), has been shown to replenish glutathione in immune deficiency states such as Human Immunodeficiency Virus (HIV) infection (Micke, Beeh et al. 2001) and cancer (Bounous 2000). Hence, whey protein/Cys supplementation might possibly attenuate the age-related changes in GSH/GSSG redox state by increasing the biosynthesis of GSH.

It is also possible that physical activity may be able to decrease oxidative stress caused through aging by minimizing changes to the GSH/GSSG state by increasing

antioxidant defenses as a result of the muscle adapting to its environment. For example, it has been found that chronic exercise improves skeletal muscle oxidative capacity due to adaptive responses in the muscle that result in an up regulation of antioxidant defense systems that play a crucial role of maintaining intracellular GSH/GSSH homeostasis (Ji 2008). Therefore, physical activity has been suggested as a possible intervention to offset the age-related changes in muscle redox status and associated ROS-induced damage (Jackson 2005).

As considerable evidence suggests that a combination of whey-protein supplementation and resistance training may attenuate the effects of aging on the redox state, we decided to study the effect of these two interventions on the redox states of a group of elderly male subjects. In particular, we wished to determine how a 12-week resistance-training program, with or without additional whey-protein supplementation, would affect both the tissue and plasma thiol (GSH and Cys) and disulfide (GSSG and CySS) levels and plasma and tissue redox states among this aged male population.

6.1.1 Measurement of Redox States

Redox states can be measured in a number of biological media, including the tissue, whole blood or plasma. The outcome usually varies according to the medium selected. Assessment of a redox state can be determined by measurement of GSH, GSSG, Cys and CySS to determine the reduction potentials (Eh) for the redox couples GSH/GSSH and Cys/CySS. However, although a number of assay methods are available, many are problematic, and achieving the necessary sensitivity can be a challenge (Jones and Liang 2009).

We initially intended to follow literature methods for the determination of tissue and plasma GSH, GSSG, Cys and CySS levels. Indeed, our plasma assays were conducted according to the previously published methodology of Jones (Jones, Carlson et al. 1998), and allowed assessment of the Cys/CySS redox state (although not the GSH/GSSG redox state). However, we were not able to replicate the methods for the determination of intracellular GSH and GSSG using reverse phase HPLC outlined by Luo et al. (Luo, Hammarqvist et al. 1995; Luo, Hammarqvist et al. 1998). We therefore redeveloped and then optimized this method by refining some of the steps previously described. The redevelopment and optimization of the method for determining intracellular glutathione is discussed.

It was hypothesized that a 12-week resistance-training program in combination with whey-protein supplementation would arrest age-related changes to the cellular redox processes, in terms of plasma or tissue thiol or disulfide levels or plasma or tissue redox states.

6.2 Methods

The subjects used in this study have been previously described in Section 5.1.1 of Chapter 5. The diet and supplementation, resistance training protocol and body composition measurements have also been previously described in Chapter 5, Sections 5.2.2 – 5.2.4.

6.2.1 Muscle Biopsy

All muscle samples were obtained from the vastus lateralis via needle biopsy using a sterile Bergstrom muscle biopsy needle (5 mm x 101 mm length; Stille, Stockholm, Sweden) according to methods as outlined in Section 3.9.1. Roughly, 30–

80 mg muscle was immediately placed into a labeled cryule and snap frozen in liquid nitrogen for analysis of intracellular thiols/disulfides.

6.2.2 Measurement of Plasma Redox Status

Plasma thiols (reduced glutathione [GSH], cysteine [Cys]), disulfides (oxidized glutathione [GSSG], cysteine [CySS]) and redox status were measured as previously outlined by Jones (Jones, Carlson et al. 1998) according to methods outlined in Section 3.8. Unfortunately, the assay was not sufficiently sensitive to measure the oxidized form of glutathione (GSSG), and therefore we were unable to derive the GSH/GSSH redox state. In all other respects, the literature method was accurately replicated, and we were able to derive the Cys/CySS redox state successfully.

6.2.3 Measurement of Tissue Redox Status

Muscle tissue thiols (reduced glutathione [GSH], cysteine [Cys]), disulfides (oxidized glutathione [GSSG], cysteine [CySS]) and redox status were measured using an optimized version of the methods previously outlined by Luo (Luo, Hammarqvist et al. 1995; Luo, Hammarqvist et al. 1998) as shown in Chapter 3, Section 3.13.

6.2.4 Statistical Analysis

Statistical analyses were carried out using Prism 5 for Mac OSX (GraphPad Software Inc. USA) as outlined in Chapter 3, Section 3.14. For measurement of plasma and tissue thiol, disulfide and redox states, only subjects with complete data sets (0, 6 and 12 weeks) were included (RT n=5, RTPr n=4).

6.3 Results

6.3.1 Baseline Characteristics

A total of 17 healthy aged male subjects (aged 55+ years) volunteered for the study. One subject had to withdraw from the study due to problems related to ingestion of the dietary supplement. This left a total of 16 healthy, aged males who participated fully in the study. Seven subjects were randomized to the RTPr group and 9 to the RT group. In some subjects (RT group n=4, RTPr group n=3) insufficient muscle tissue was retrieved for complete analysis of tissue redox status across all time points. Hence, this left a total of 9 healthy, aged males (RT group n=5, RTPr group n=4) that were included in the final analysis of plasma and tissue redox state.

At baseline there were no significant differences in age, height, body composition, plasma thiol and disulfide levels (Cys and CySS), plasma CySS/Cys redox state, tissue thiol and disulfide levels (GSH and GSSG) or tissue GSSG/GSH redox state. However, there was a significantly higher ($p=0.01$) plasma reduced glutathione (GSH) and a near-significant higher ($p=0.09$) plasma CySS in the RTPr group when compared to the RT group at baseline (Table 6.1). An analysis of covariance (ANCOVA) was used to test whether the difference in baseline plasma GSH had an effect on the final statistical analysis however no significant effect was found.

6.3.2 Optimization of Intracellular Redox Status Methods

We initially planned to measure muscle tissue thiols (reduced glutathione [GSH], cysteine [Cys]), disulfides (oxidized glutathione [GSSG], cysteine [CySS]) and redox status using the methods previously outlined by Luo (Luo, Hammarqvist et al. 1995; Luo, Hammarqvist et al. 1998). However, on attempting to replicate these

methods, we encountered a number of practical difficulties, which meant that the analysis could not proceed as initially planned.

Table 6.1: Baseline characteristics (mean \pm SD). For measurement of plasma and tissue thiols, disulfides and redox states: RT, n=5; RTPr, n=4. Plasma Cys, CySS and GSH values are expressed in μ M. For tissue GSH and GSSG, units are expressed as mmol/kg wet wt. Redox state for both plasma and tissue are in mV. *p<0.05 between groups

Characteristic	RT	RTPr
Age (yrs)	61.8 \pm 6.0	64 \pm 3.9
Height (cm)	174.6 \pm 7.7	176.3 \pm 4.3
Body Mass (kg)	81.5 \pm 12.5	75.9 \pm 8.1
Plasma Cysteine (Cys)	7.9 \pm 2.1	9.5 \pm 0.7
Plasma Cystine (CySS)	48.8 \pm 11.6	65.3 \pm 14.0
Plasma reduced glutathione (GSH)	0.96 \pm 0.13	1.28 \pm 0.72*
Plasma CySS/Cys redox	-74.4 \pm 1.8	-74.4 \pm 1.0
Tissue reduced glutathione (GSH)	0.59 \pm 0.1	0.60 \pm 0.1
Tissue oxidized glutathione (GSSG)	0.028 \pm 0.005	0.032 \pm 0.006
Tissue GSSG/GSH redox	-384.2 \pm 7.0	-384.9 \pm 3.8

Replication of the literature methods was also almost certainly compounded by the lack of clearly defined steps in the analytical process published. Finding a resolution to these difficulties ultimately required us to develop an entirely revised and optimized experimental procedure, a process that took close to two years to complete. Full details of the optimization of the methodology, and the difficulties, which required us to take this action, are provided below.

6.3.2.1 Attempt to Replicate Literature Methodology

We initially used the HPLC methods outlined by Luo (Luo, Hammarqvist et al. 1995), and analyzed samples on an equivalent column to that described. However, we discovered that GSH and GSSG could not be identified in the chromatogram trace. Furthermore, we could not detect any discernable differences between varying concentrations of GSH or GSSG from chromatograms of standard solutions. Importantly, many peaks on the chromatogram showed relative concentration changes, making it impossible to identify GSH and GSSG (Figure 6.1).

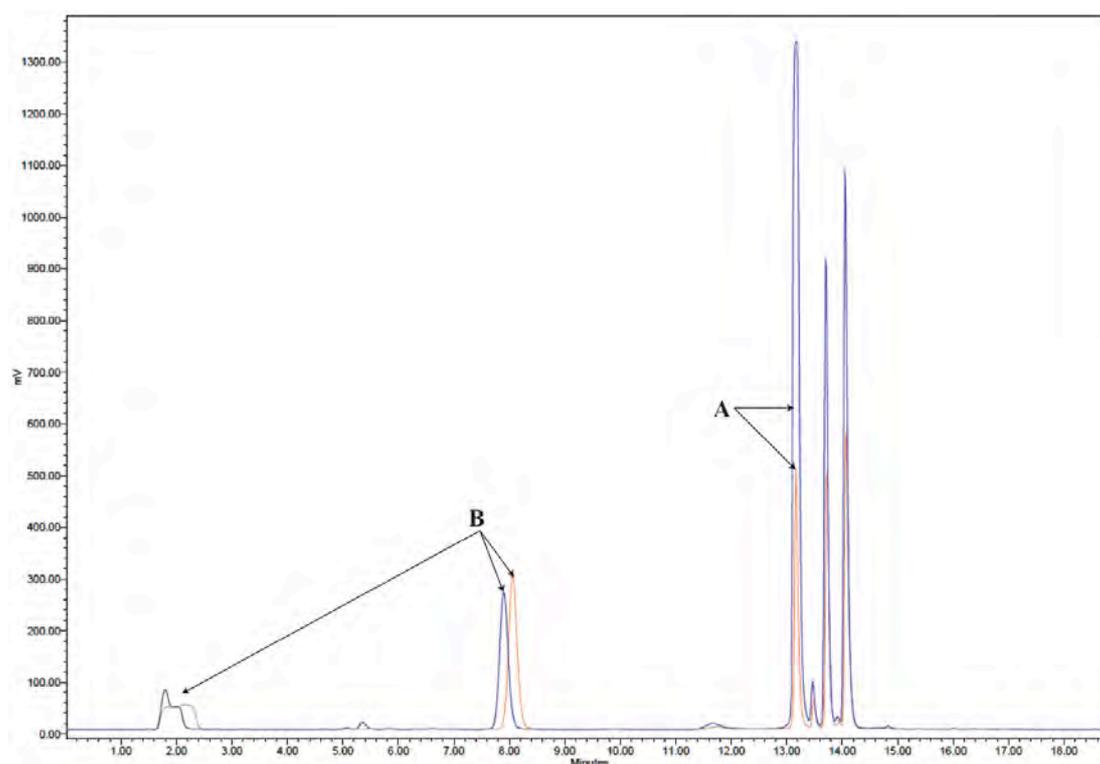


Figure 6.1: Initial HPLC chromatogram of muscle tissue with identification of GSH standards and muscle sample using the methods outlined by Luo (1995). A) Multiple peaks showing a 2:1 ratio not allowing for the identification of GSH standards, which were also in a 2:1 ratio. B) shift in elution time between standards. (orange=50 μ M and blue=100 μ M).

Therefore, before we proceeded further with the literature HPLC methods, we considered it important to establish whether the standard and sample preparation method outlined by Luo was effective. It was important to identify which peak in the chromatographic trace was in fact GSH. We therefore switched from high performance liquid chromatography (HPLC) to liquid chromatography-mass spectrophotometry (LC-MS) for specific detection and identification of GSH.

6.3.2.2 Identification of GSH using LC-MS

Our first task was to establish whether GSH was actually present and to identify the GSH peak using LC-MS.

As LC-MS requires a volatile eluent system, the perchlorate buffer system described in the literature method is not suitable for LC-MS. Therefore, we adapted the GSH HPLC methods outlined in the literature to include an ammonium formate (volatile) buffer system. Using this system, a derivatized GSH was identified as the peak eluting at approximately 3 min.

The LC-MS separation of the thiol-bimane adducts was achieved on a reversed-phase Waters Sunfire C-18 octadecylsilyl silica column (150 × 4.6 mm ID, 3.5- μ m particle size) followed by electrospray mass spectrometry detection in *positive mode*. The system consisted of a Waters 600 LC pump system, a Waters 717 Plus sample processor (Waters, Milford, MA), and a Micromass Platform II MS detector.

Elution solvent A was MilliQ water, Elution solvent B was acetonitrile and Elution solvent C was 100 mM ammonium formate adjusted to pH 3.75 with formic

acid. The elution program is detailed in Table 6.2 below. The flow rate was 1.0 ml/min, the injection volume was 25ul and the total run time was 30 minutes.

The monobromobimane derivatives of GSH and Cys in each portion of derivatized samples were separated by HPLC and quantified on the basis of peak areas and compared with the authentic GSH and other thiol standards.

Table 6.2: Elution program.

Time (min)	% A	% B	% C
0	50	10	40
2	50	10	40
15	18	42	40
15.1	15	85	0
20.1	15	85	0
20.2	50	10	40

The mass of derivatized GSH is 497.5, and this was detected as 498.5 in positive-mode MS. Following the identification of our peak using LC-MS we reverted back from the UV detector used with LC-MS to the fluorescence detector used with HPLC to see if the compound could be measured using the volatile ammonium formate buffer system on HPLC. We found that the volatile ammonium formate buffer system was amenable to HPLC with the fluorescence detection (Figure 6.2).

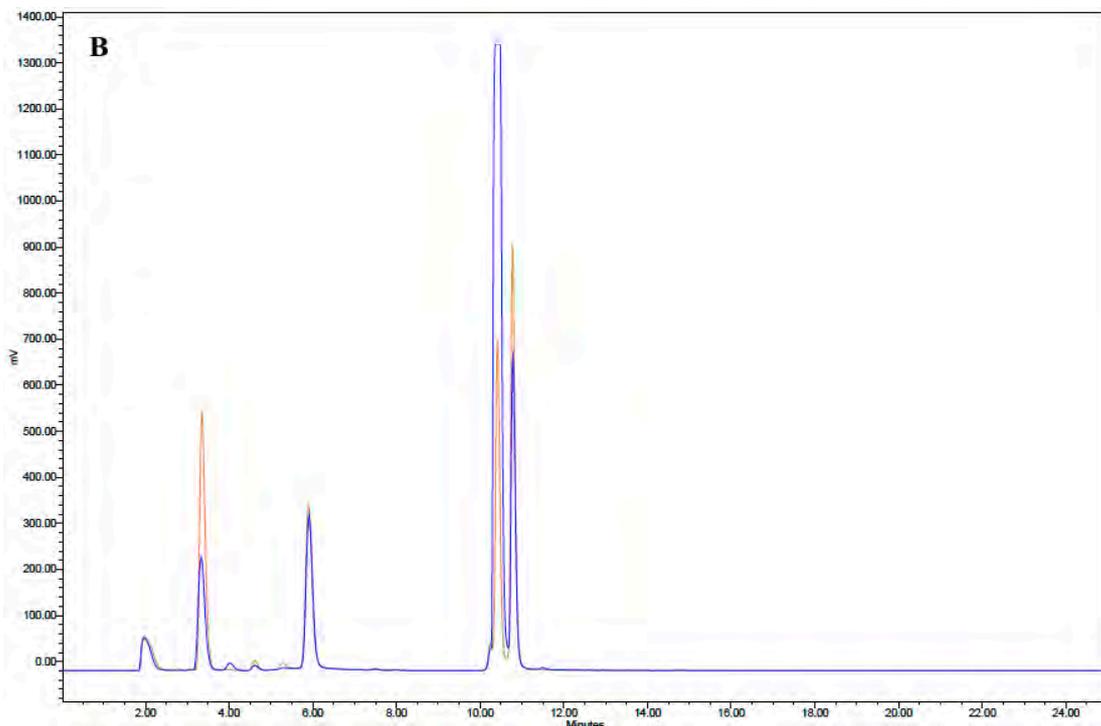
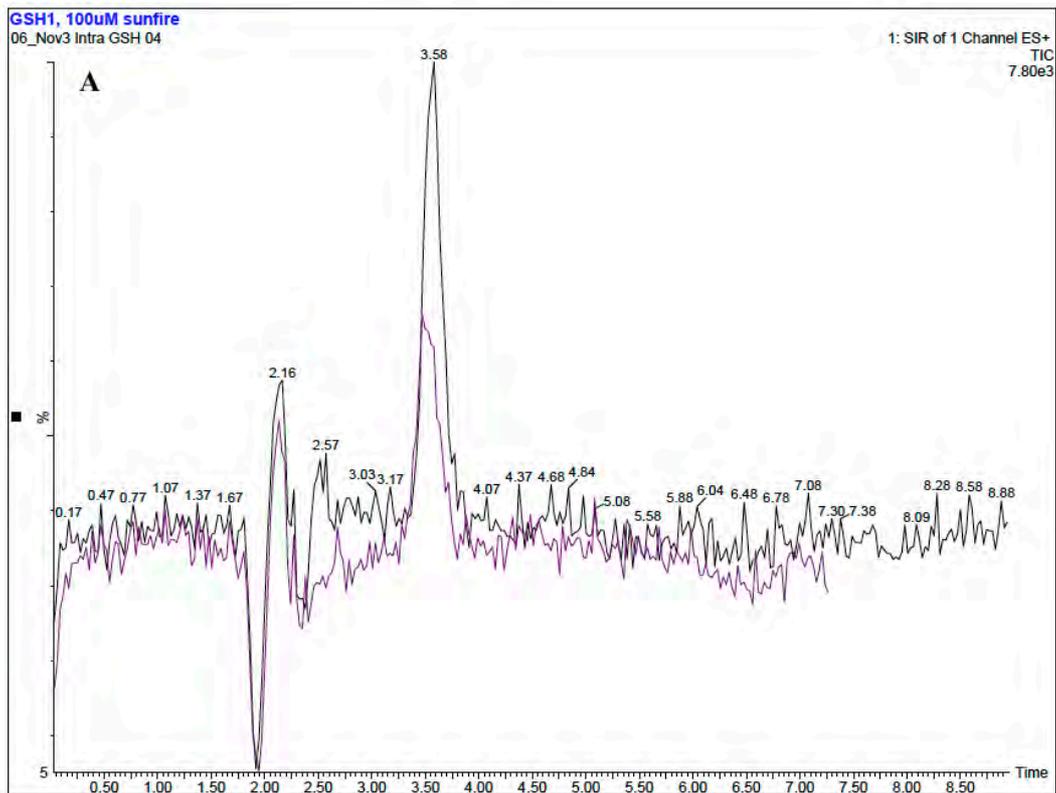


Figure 6.2: Chromatogram of muscle tissue with identification of GSH standards using A) LC-MS and B) HPLC. Note the presence of the GSH eluting at approximately 3 minutes in both the LC-MS and HPLC chromatograms. Included in figure B are two GSH standards; 50µM (blue) and 100µM (orange).

6.3.2.2 Identification of GSH and associated Thiols/Disulphides using Fluorescence

Following the successful detection of GSH using fluorescence, we turned our attention to identifying intracellular Cys. When trying to analyze cysteine/cysteine using an ammonium buffer system, it was found that 5 μL injections of the standard resulted in poor peak shape, rendering the results uninterpretable. When the injection volume was reduced to 3 μL the peak shape was found to improve, suggesting a problem in the matrix of the standard. Using a small, 3 μL injection volume we were able to identify the cysteine peak (Figure 6.3).

The methodology that we found to be successful differed from the published methodology in a number of ways. Firstly, the literature method did not provide the injection volume used. In our studies, injection volume was found to be an important factor, with a 5 μL injection volume resulting in a poor peak shape. Furthermore, because of the small volumes we found to be necessary, our samples were not filtered following the addition of SSA, in contrast to the literature method. Finally, evidence from preliminary cysteine analysis indicated that the samples needed to be pH-adjusted by dilution into the ammonium formate (pH 3.71) before injection. Once again, this requirement was not described in the literature.

After we had successfully resolved the cysteine and GSH peaks such that they were sufficiently separated to allow for analysis, we turned our attention to the detection of GSSG. We rapidly identified problems with the preparation procedure of GSSG as outlined in the literature. We first needed to ensure that the instrumental method was robust by testing the GSH and cysteine peaks. We know that GSSG, once derivatized, would give the same peak as GSH since GSSG is first cleaved during the

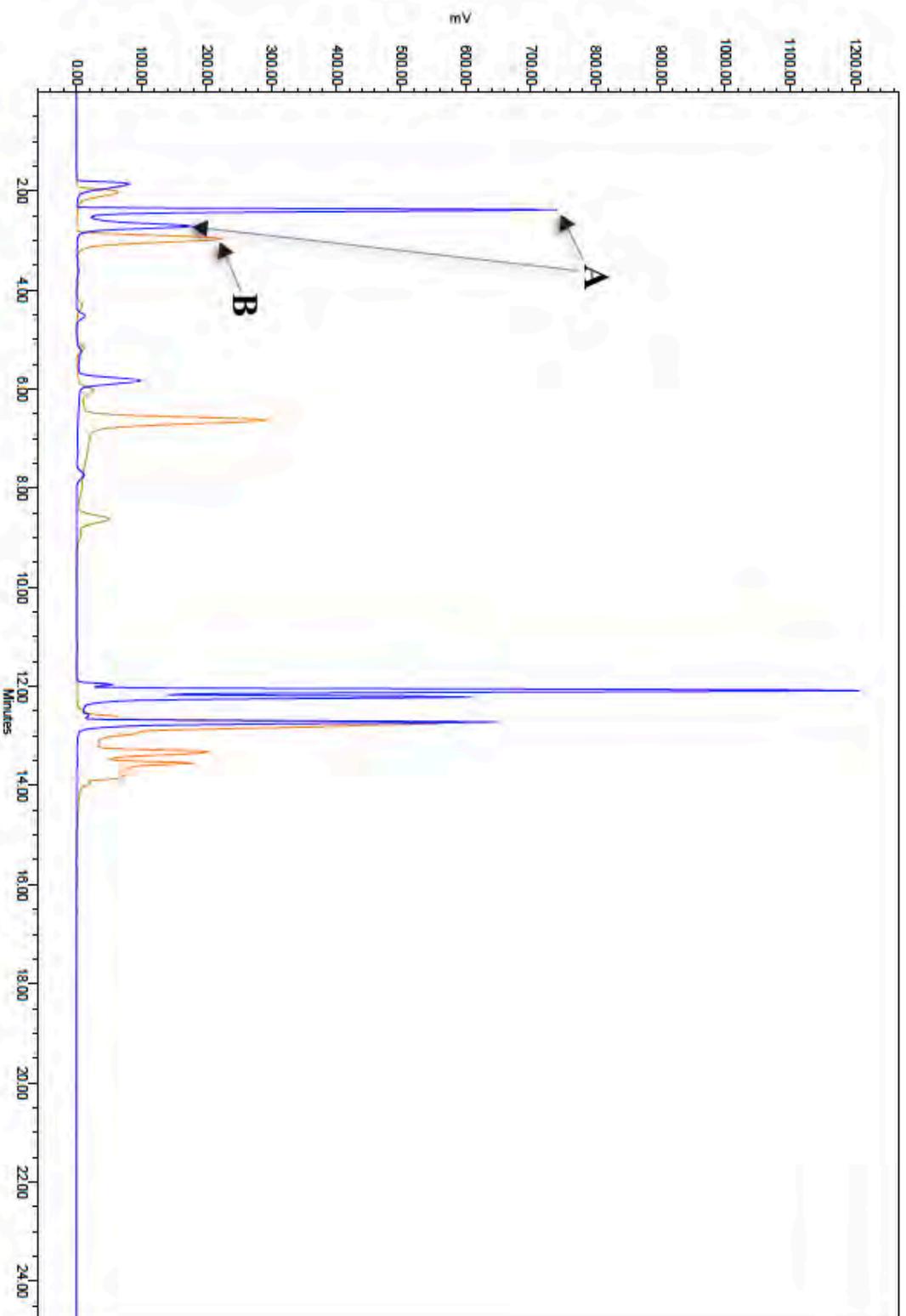


Figure 6.3: HPLC chromatogram of standard preparation with identification of cysteine (Cys) standard (100 μ M). A) Peak splitting of cysteine (Cys) chromatograph using injection volume of 5 μ L, shown in blue. B) Modified peak shape and no peak splitting using injection volume of 3 μ L, shown in orange.

derivatization process to give two molecules of GSH, which are then derivatized with monobromobimane (mBBr).

Having established that the analytical method was robust, we decided to investigate the sample preparation method for GSSG more closely as GSSG was not identified in any of the standards as prepared using the literature method. It was felt that the literature sample preparation procedure had failed since peaks resulting from varying concentrations of GSSG could not be interpreted.

Several steps in the literature method for the preparation of GSSG were not clearly defined, which may have affected the derivatization procedure. Therefore, an upscale model of the derivatization procedure was created to allow for pH measurement and a more defined volume addition at each step, resulting in a more robust and clearly defined method. Considerable refinement was required with the GSH and GSSG standards to achieve the desired consistency and linearity. The specific changes are described below:

1. The literature description of the homogenization of muscle tissue states simply that the pH of the supernatant should be adjusted to neutrality with 'excess' NaHCO_3 powder and the sample derivatized directly. In order to quantify the 'excess' of NaHCO_3 required to neutralize our 100 μL SSA-soluble fraction, we performed the following calculation: 10ml of SSA solution required 0.6 g of NaHCO_3 to neutralize the sample, equating to approximately 6 mg of NaHCO_3 to neutralize a 100 μL sample of SSA solution.

2. We refined the measurement of disulfides described in the literature by calculating the exact quantity of 3M potassium phosphate buffer (pH13) required to adjust the sample for alkaline hydrolysis (pH 11.5-11.8) as 300 μ L. We also found that in the derivatization process for disulfides, 20 μ L (not 10 μ L) of 90% (w/v) SSA solution was required for neutralization following the hydrolysis of *N*-ethylmaleimide (NEM).
3. The sodium *N*-ethylmorpholine required by the literature methodology was not available to us, and we used a non-sodium form instead. Further, as the non-sodium *N*-ethylmorpholine would not dissolve the mBBr fluorescent label, acetonitrile was subsequently used in its place.
4. Finally, we altered the chromatographic conditions described for HPLC to suit the equipment used in the following way:

	Literature method	Current method
Elution solvent A	% aqueous acetonitrile (v/v) containing 0.25% acetic and perchloric acid, final pH adjusted to 3.71 with sodium hydroxide	MilliQ water
Elution Solvent B	75% acetonitrile (v/v)	acetonitrile
Elution solvent C	none	0.25% acetic acid and 0.25% perchloric acid in milli Q water, adjusted to pH 3.71 with sodium hydroxide
Elution program	100% solvent A for 11 minutes, followed by 100% solvent B for 5 minutes to elute matrix interference, returning to solvent A for re-equilibration for 9 minutes.	Shown in Table 6.3. The flow rate was 1.2 ml/min, the injection volume was 25 μ l and the total run time was 20 minutes.

After making all the changes described and achieving satisfactory results, we returned to the original literature method (excluding our modifications), and found considerable noise on the chromatographic trace. This provided further justification for the modifications undertaken.

After we had successfully modified the methods for the ammonium formate system on LC-MS and then successfully transferred this onto HPLC, we then applied our modifications to the original acetic acid/perchloric acid method outlined in the literature (step 1 - 4 on previous page), and obtained extremely satisfactory chromatographic results (Figure 6.4). This further confirmed that the original, published method required significant refinement in order to successfully identify GSH and GSSG peaks. We reduced the acetonitrile concentration from the 7% quoted in the literature to 5% in order to achieve later elution of GSH and to separate this from co-eluting peaks and interferences (see Table 6.3). When we finally ran the samples according to all the refinements, the chromatographic results were highly satisfactory (Figure 6.4).

Table 6.3: Elution Program.

Time (min)	% A	% B	% C
0	15	5	80
7.2	15	5	80
7.6	25	75	0
12.6	25	75	0
12.7	15	5	80

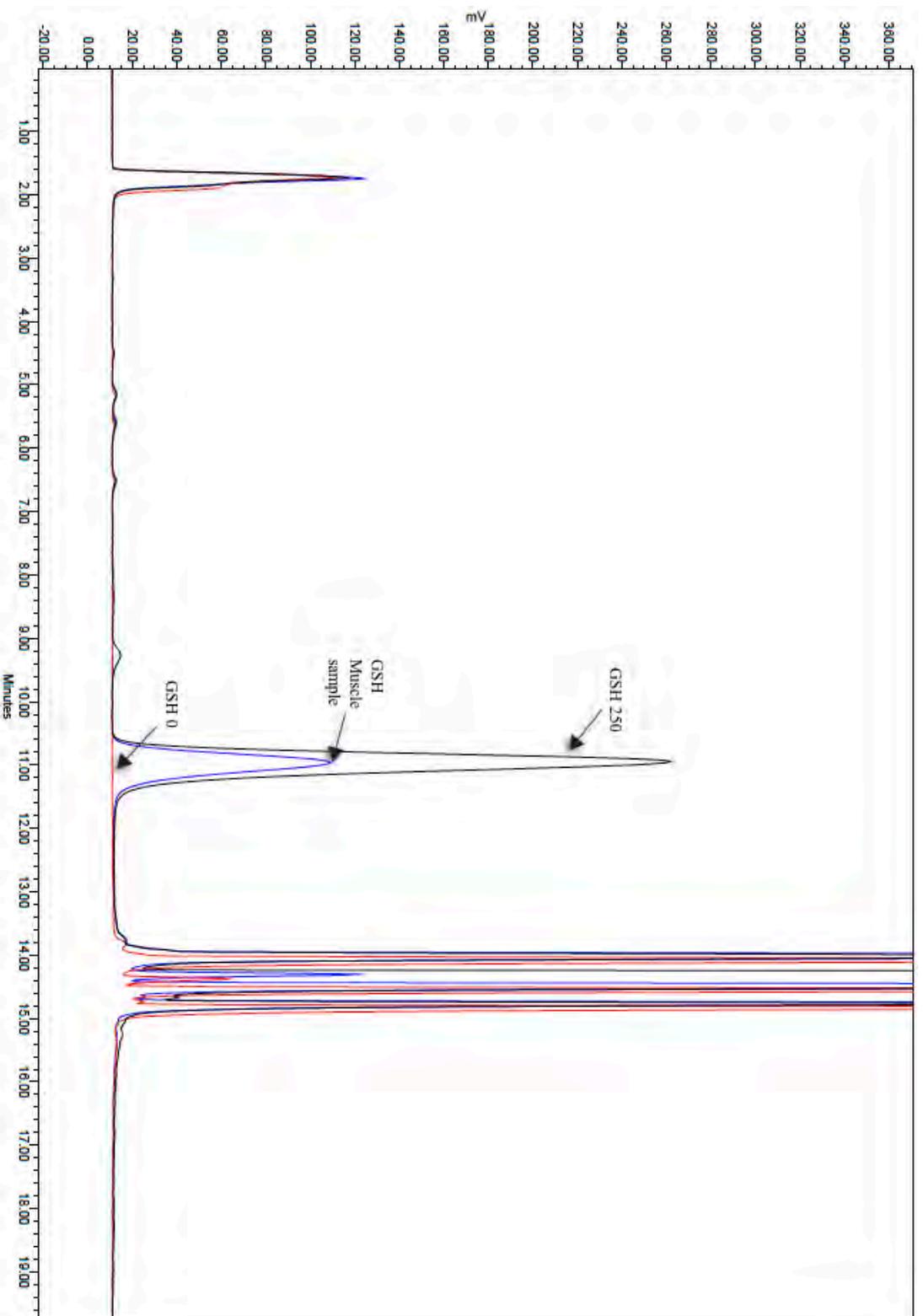


Figure 6.4: Typical HPLC chromatogram of muscle tissue with identification of GSH standards and muscle sample. Included in the above figure are two GSH standards (0 μ M and 250 μ M; orange and black) plus sample derivitised from muscle (blue).

6.3.3 Plasma Thiols (Cys and GSH), Disulfide (CySS) and CySS/Cys redox state

Plasma GSH, Cys and CySS levels and Cys/CySS redox state results are shown in Table 6.4. There was no significant difference in plasma GSH, Cys or CySS levels or CySS/Cys redox state between groups or over time following 12-weeks of dietary supplementation and resistance training.

In both the RT and RTPr groups there was a tendency for an increase in plasma thiol (cysteine; $p=0.11$) and plasma disulfide (cystine; $p=0.10$; Table 6.4) following the training program. Additionally, whilst not significant, in the RT group, the plasma Cys/CySS redox state becomes less negative after the first six weeks of the study, and then falls down again below the baseline level by week 12 (see Table 6.4).

6.3.4 Tissue Thiol (GSH), Disulfide (GSSG) and GSSG/GSH Redox State

No Cys or CySS peaks were identified in the HPLC chromatogram, suggesting a lack of Cys and CySS in the muscle samples. Tissue thiol (GSH), disulfide (GSSG) and GSSG/GSH redox state results are shown in Table 6.4. There was no significant difference in GSH, GSSG or GSSG/GSH redox state between groups or over time following 12-weeks of dietary supplementation and resistance training. Additionally, whilst not significant, in the RT group, the tissue GSH levels decline compared with baseline during the first six weeks, which are then restored to a level greater than baseline by the end of the study (see Table 6.4).

Table 6.4: Thiol and disulfide levels and redox states in plasma and tissue (mean \pm SD). Φ significant difference between baseline values using unpaired t-test ($p < 0.05$). For measurement of plasma and tissue thiols, disulfides and redox states RT n=5, RTPr, n=4. For plasma Cys, CySS and GSH values are expressed in μ M. For tissue GSH and GSSG unit are expressed as mmol/kg wet wt. Redox state for both plasma and tissue are in mV. $^{\#}$ p=0.11, $^{\#\#}$ p=0.10

Measurement	RT			RTPr		
	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
Plasma Cysteine (Cys)	7.9 \pm 2.1	8.2 \pm 2.1	13.1 \pm 3.8 [#]	9.5 \pm 0.7	10.8 \pm 4.6	18.9 \pm 7.4 [#]
Plasma Cystine (CySS)	48.8 \pm 11.6	56.2 \pm 2.6	80.9 \pm 11.8 ^{##}	65.3 \pm 14.0	65.6 \pm 13.4	91.4 \pm 17.3 ^{##}
Plasma Reduced Glutathione (GSH)	0.96 \pm 0.13	1.27 \pm 0.21	1.30 \pm 0.18	1.28 \pm 0.17 Φ	1.25 \pm 0.23	1.45 \pm 0.34
Plasma CySS/Cys redox	-74.4 \pm 1.8	-71.7 \pm 5.8	-78.7 \pm 7.4	-74.4 \pm 1.0	-75.9 \pm 7.9	-85.7 \pm 10.8
Tissue Reduced Glutathione (GSH)	0.59 \pm 0.12	0.56 \pm 0.08	0.59 \pm 0.12	0.60 \pm 0.05	0.65 \pm 0.09	0.64 \pm 0.05
Tissue Oxidized Glutathione (GSSG)	0.028 \pm 0.005	0.035 \pm 0.011	0.033 \pm 0.008	0.032 \pm 0.005	0.033 \pm 0.004	0.029 \pm 0.005
Tissue GSSG/GSH redox state	-386.3 \pm 4.3	-383.1 \pm 5.4	-384.5 \pm 7.1	-385.9 \pm 3.2	-387.1 \pm 4.5	-388.5 \pm 2.7

6.4 Discussion

This study is the first to directly investigate the potential benefit of a resistance-training program and whey-protein supplementation in arresting the changes to the cellular redox state that are normally associated with aging.

No differences were found either between the two treatment groups, or over time, in terms of plasma levels of thiols and disulfides and plasma CySS/Cys redox state, or in terms of tissue levels of thiol and disulfide and tissue GSSG/GSH redox state. The lack of any effect on plasma Cys or GSH levels through the addition of whey protein is particularly surprising, as the addition of dietary protein would certainly be expected to increase plasma Cys levels (Bounous 2000; Micke, Beeh et al. 2001).

There are several possible explanations for the observed lack of effect on the cellular redox states. One possibility could be poor subject compliance. Some participants may have failed to follow the instructions for consuming the supplements accurately, or there may have been inconsistent time delays between dosing and sampling. Alternatively, this finding could be due to day-to-day variability in the measurements. A study by Blanco and co-workers reported that Cys/CySS and GSH/GSSG redox states in human plasma undergo diurnal variation in a pattern indicating that meal intake has an important effect on these antioxidant systems (Blanco et al. 2007). Furthermore, they showed that the homeostatic regulation of Cys and GSH pools declines with age. This variation could alter sensitivity to oxidative stress within the time-frame of a day as a function of the timing and the quantity and quality of food intake.

As discussed in Chapter 5, the subjects included in this study were untrained individuals. It is possible, therefore, that any effect of whey-protein supplementation in terms of either functional strength or on the underlying physiological processes may have been hidden by the greater changes due to the impact of the new resistance training program. Also as discussed in Chapter 5, the timing of supplement ingestion may be important, as previous studies have shown supplement timing to have a significant effect on the effectiveness of a training program (Cribb and Hayes 2006). As supplement timing was not controlled or recorded in this study, it is not possible to account for this possible effect here.

However, if the lack of effect on cellular redox states is, in fact, a genuine outcome, then it may explain the absence of any benefit of whey-protein supplementation on other measures evaluated in this study, including oxidative capacity and muscle strength, as outlined in Chapter 5. In fact, a recent study published by Bemben et al. found that, whilst resistance training significantly increased muscular strength and muscle mass in middle-aged and older men, there was no additional benefit from creatine and/or protein supplementation (Bemben, Witten et al. 2010).

The possible reasons for the apparent lack of effect of whey-protein supplementation have been described previously. As described in Chapter 5, this may be because the rate of muscle protein synthesis is lower in aged males due to decreased testosterone production and myosin heavy chain protein synthesis (Eliot 2008, Bemben 2010). Finally, as discussed in Chapter 4, nutrition in the elderly can be compromised by the fact that absorption of amino acids and sugars declines with age (Ferraris 1997). These observations all combine to suggest that the dosage of

whey-protein used in these studies may have been insufficient to create a response in the elder male participants.

The two treatment groups (RT and RTPr) were well balanced at baseline in terms of age, height, body composition, and tissue thiols, disulphides and GSSH/GSH redox state, indicating that the randomization procedures employed were broadly effective. Interestingly, the RTPr group had significantly higher plasma GSH levels than the RT group at baseline.

The higher GSH levels would suggest a greater antioxidant capacity in the plasma at baseline, suggesting that the redox state of the RTPr group may have been less compromised than that of the RT group at the start of the study. This is consistent with the suggestion made in Chapter 5 that the subjects in the RTPr group had a higher baseline level of fitness than the RT group (Section 5.4). The higher GSH levels in the plasma would not necessarily be expected to be matched by higher levels in the tissue due to the complex nature of the thiol/disulfide interactions between plasma and tissue.

A greater plasma GSH at baseline suggests at first glance a greater reducing/quenching ability in the plasma. However, the greater plasma GSH may have also been accompanied by a greater GSSG in the plasma. If this were the case, then the GSH/GSSG redox state of the plasma would not be different and would simply represent an overall greater baseline concentration of both GSH and GSSG in the RTPr group. Unfortunately the plasma methods were not sufficiently sensitive to measure plasma GSSG which, in healthy adults, is found in very low concentrations (0.14 μM) in comparison to plasma GSH (2.80 μM) (Jones, Carlson et al. 2000). To support the suggestion that there was no difference in the redox state of the plasma, an

almost significantly higher amount of plasma CySS was revealed in the RTPr group at baseline compared with the RT group ($p=0.09$). This initially suggests a pro-oxidative state in the plasma. However, as Cys levels were also slightly elevated in the RTPr group, this observation merely seems to suggest a greater abundance of both Cys and CySS in the plasma of the protein group. The lack of any difference in oxidative stress is further supported by the identical values for the Cys/CySS redox state found in both groups.

An interesting observation can be made relating to the plasma CySS/Cys redox data. In the RT group, the plasma redox state was observed to become less negative after the first six weeks on the program, before returning to a value that was more negative than baseline by week 12. A less negative redox state suggests a more oxidized environment with a poorer reducing ability. It may be, therefore, that the initial six weeks of training causes an increase in oxidative stress followed by an adaptive response, which restored reducing ability by week 12. Interestingly, the drop in plasma redox state following the first 6 weeks of training does not occur in the RTPr group, with this group maintaining a similar redox state over the first six weeks, followed by change in the redox state similar to that seen in the RT group.

The absence of such a temporary drop in the RTPr group may be the result of the whey protein supplementation, as changes in the plasma Cys/CySS redox state closely mirror changes in plasma Cys. Plasma Cys values are affected by food intake, with the effect noticeable approximately 3 h after a meal (Blanco, Ziegler et al. 2007). As whey is a known Cys precursor, if a whey-containing meal was consumed (as happened three times a day in the RTPr group), then this might explain why no drop was observed in the plasma Cys/CySS redox state in the RTPr group.

The effect may also be related to the complex interaction between plasma and intracellular thiols and disulfides. This is supported by the observation that a similar pattern is observed in tissue GSH levels in the RT group, which decrease during the first six weeks and then rebounds by week 12. Tissue GSH levels in the RTPr group do not follow this pattern. We therefore speculate that the tissue GSH may be responsible for the rebound in plasma redox state. In other words, when the plasma environment experiences a state of oxidative stress, the higher concentration of GSH found in the cell is used to counter the oxidative stress state in the plasma. A similar observation is made with the tissue GSSH/GSH redox state, in which a small dip is observed at week 6 in the RT group, suggesting oxidative stress, followed by a rebound that might be explained by muscle adaptation.

Since the RTPr group did not experience this drop in Cys/CySS or GSSG/GSH redox state, it may be surmised that this group adapt more quickly. However, no significant improvement in muscle performance was observed in the RTPr group. It is possible, therefore, that the lower oxidative redox state seen with the RTPr group prevents adaptations, which provides a further possible explanation for the lack of change in muscle performance following whey-protein supplementation.

Indeed it has been shown that exercise-induced adaptations may be mitigated by antioxidant therapy (for review see Jackson (2008)). Therefore, it is possible that the effect of the whey-protein supplement in increasing post-absorptive Cys levels and decreasing plasma Cys/CySS redox to a more reduced state may have achieved an overall antioxidant effect. This would have had the effect of quenching ROS, therefore inhibiting the adaptive response.

The overall antioxidant effect achieved through reduction of the Cys/CySS redox state may provide a further possible explanation for the lack of improvement in muscle function following whey-protein supplementation observed in this study. As reported in Jackson's review (2008), the potential roles of ROS as inducers of adaptive responses to contractions also indicate that antioxidants might be capable of suppressing these adaptive responses. In a study of the effects of supplementation with either vitamin E or β -carotene on the stress response of muscle to exercise Jackson and co-workers found that these lipid-soluble antioxidants also reduced the responses of skeletal muscle to the oxidative stress of contractile activity (Jackson, Khassaf et al. 2004). The finding that oral antioxidant supplements may suppress some adaptive responses to contractions in muscle has received support from subsequent studies in other laboratories using cell culture (Silveira, Pilegaard et al. 2006), animal (Gomez-Cabrera, Borrás et al. 2005) and human (Gomez-Cabrera, Martínez et al. 2006) studies. These studies suggest that caution in the use of high-dose antioxidant supplementation in exercise training programs should be adopted, as clearly some level of ROS production and presence is required for adaptive responses.

The absence of Cys and CySS peaks on the tissue chromatogram was another surprising result. To confirm the lack of Cys and CySS in the muscle sample, we spiked a muscle sample with GSH, GSSG, Cys and CySS standards and compared it to the normal muscle tissue sample. While the normal tissue sample revealed only peaks for GSH and GSSG, the spiked sample also revealed both Cys and CySS peaks. This confirmed the absence of Cys and CySS in the study muscle samples. This surprising finding may potentially be explained by poor Cys and CySS extraction from the muscle sample or other problems connected with the analytical technique.

This apparent absence of Cys and CySS in the muscle samples of aged individuals is a finding that requires further investigation.

One interesting and useful aspect of this study, which emerged unexpectedly from this work, was the refinement of the methods used for the assay of the tissue thiols and disulfides that we have reported. We identified several weaknesses in the existing methodology, both in terms of points of detail and also in its applicability to different equipment types. The redevelopment of these methods was not a trivial task, requiring considerable time and effort. However, we are satisfied that the time devoted to this purpose was fully justified as, to our knowledge, no other methods existed for the direct measurement of intracellular GSH, GSSG and total GSH at the time when we conducted this assay. A number of assay kits that are currently available on the market for the measurement of cell redox state do not measure GSH directly. Instead, these kits tend to estimate the GSH value by subtracting free GSSG from total GSH. Such an assessment was not sufficient for our purposes, as we required detailed information concerning the redox state of the cell, which is a balance between GSH and GSSG.

It was essential that we devise a method to accurately and sensitively measure the intracellular redox state to enable us to derive meaningful results from this study. The modified assay method described at length in this chapter was the result of months of painstaking work. However, this work was justified in enabling us to measure the redox potentials in this study. We are confident that not only did this modified method enable us to complete this study, but that it will also be applicable in many future studies and will help to elucidate further precise details of biological redox reactions.

While several important and interesting conclusions may be drawn from this work, the study does raise a number of questions, which should be addressed in future research. In particular, the lack of significant results makes interpretation of the study somewhat difficult. It is possible that the changes which were observed and which are described above may be the result of poor subject matching and/or of variability within groups because of the low subject numbers. In particular, repeating the study with a larger number of subjects may enable some of the findings to reach significance. It would certainly be interesting to repeat the study with larger groups of subjects, and possibly with a higher average age. It would also be interesting to further develop the adapted methods for the measurement of the redox states. In particular, identification of GSH and GSSG on LC-MS using the ammonium formate buffer system may be worth investigating further as LC-MS offers better selectivity and potentially better sensitivity on more modern instruments that were used herein, especially LC-MS-MS. This would be especially important with compounds that are found in low concentrations and in complex matrices.

6.5 Conclusions

Neither a 12-week resistance training program on its own, nor in combination with whey protein dietary supplementation, altered the plasma or tissue thiol or disulfide levels or plasma or tissue redox states in a significant manner. A number of potential reasons are explored to explain this surprising result, including the potentially overwhelming effects of introducing a resistance-training program in an untrained older population, and the lack of control over the timing of whey-protein supplementation. However, if this lack of effect is a genuine outcome, then it may explain the absence of any benefit of whey-protein supplementation on other

measures evaluated in this study, including oxidative capacity and muscle strength as described previously.

An interesting observation was noted in terms of the plasma CySS/Cys redox state, tissue GSH levels and GSSH/GSH redox state in the RT group. In each case, the value observed after six weeks was slightly reduced relative to baseline, before being restored beyond baseline levels by week 12. This suggests the initial six weeks of training causes an increase in oxidative stress followed by an adaptive response, which restores reducing ability by week 12.

As an important part of this study, we also conducted a redevelopment and refinement of the methodology for the measurement of tissue thiol and disulfide levels in order to be able to calculate the GSSH/GSH redox state accurately. This new methodology will be applicable for many similar studies in the future.

Chapter 7

The Effect of Two Meal Replacement
Diets on Lean Mass Preservation and
Subjective Satiety in Obese Males and
Females

7.1 Introduction

Sarcopenia is a gradually debilitating condition that is potentiated by obesity (Roubenoff 2004). Although sarcopenia can also be found in non-obese individuals, the combination of obesity and sarcopenia in aging individuals creates a particular health burden (Kennedy, Chokkalingham et al. 2004). In individuals of advanced age, both obesity and sarcopenia restrict mobility of the individual and both are likely to introduce disabilities in performing ordinary tasks of daily living (Davison, Ford et al. 2002; Zoico, Di Francesco et al. 2004).

Since obesity and the accompanying sarcopenia are mutually complementary, it is necessary to arrive at strategies to combat both. As discussed in Chapter 2, Sections 2.9.1 and 2.9.2, a two-pronged strategy of exercise and diet appears the ideal approach to achieve fat loss and reverse sarcopenia. This approach also addresses the problem of obesity. However, in some cases the elderly may experience considerable difficulties in maintaining a regular exercise schedule. A nutrition-based approach therefore, that does not rely on a comprehensive exercise regime, may be the most practicable strategy to protect against sarcopenia in the particularly elderly and frail.

Nutrition plays an important role in both obesity and sarcopenia, and is a consideration that can often be overlooked. In a recent paper of malnutrition in the elderly, it was reported that 43% men and 21% women in 14 low-level aged care facilities in Melbourne, Australia studied were sarcopenic, while micronutrients such as calcium, magnesium, folate, zinc and dietary fibre were found to be lacking in the diet of these individuals (Woods, Walker et al. 2009). Sarcopenic obesity (defined as appendicular skeletal muscle mass/stature squared $<7.26 \text{ kg/m}^2$ for men and $<5.45 \text{ kg/m}^2$ for women plus body fat $>28\%$ for men and $>40\%$ for women (Baumgartner,

Wayne et al. 2004)) was also prevalent in this study group (14% of men and 12% of women, respectively). Although the mean age of the people included in the study was 86 years, the individuals selected were ambulatory and did not show overt signs of malnutrition.

A common and easily accessible dieting approach is to use meal replacements. Meal replacements control a proportion of the diet (typically 50%) through calorie controlled liquid meals (i.e shakes) or food (pre-prepared meals or bars). In some cases, depending on the weight of the individual and the level of dietary control needed, all meals in the diet are replaced with meal replacements. This approach, however, needs to be conducted under medical supervision as complete dietary replacement can be dangerous and has resulted in deaths (Wadden, Stunkard et al. 1983; Wadden, Stunkard et al. 1983).

Currently available meal replacement offerings provide the bare minimum in calorific and nutritional content as required by the food standards code for meal replacement products (FSANZ 2007). This puts the consumer at risk of not meeting their daily nutritional requirements.

As discussed in Chapter 5, nutrition in elderly people can also be compromised by the fact that absorption of amino acids and sugars declines with age (reviewed in Ferraris (1997)). The reduced absorptive capabilities coupled with restrictive diets may result in malnutrition in the elderly. Hence, restrictive diets alone, without proper nutrition, may not prove to be a comprehensive strategy for offsetting sarcopenia.

A moderate increase in the total dietary intake of protein has been recommended in the elderly to delay the development of sarcopenia (Paddon-Jones

and Rasmussen 2009). A nutrition program that promotes weight loss through calorific restriction while suppressing hunger and increasing satiety between meals may actually help regulate body weight whilst preserving lean muscle mass in the absence of exercise. With this principle in mind, a study was designed to test the effects of nutrition using a protein-enriched meal replacement diet on body composition and post-meal satiety in obese individuals.

It was hypothesized that a 4-week protein-enriched meal replacement shake and diet program would promote weight loss and lean mass preservation in overweight and obese adult participants, safely preventing the onset and progression of sarcopenic obesity.

7.2 Methods

7.2.1 Participants

A total of 22 male and female participants aged 22–54 years (41 ± 2 years; males $n=2$; and females, $n=20$) volunteered for the study. All participants were overweight or obese, but were otherwise healthy with no underlying health concerns.

Because there is a potential risk of exacerbating muscle wasting in aged individuals whilst on a diet, young healthy individuals rather than sarcopenic patients were used in the study. The use of young, healthy volunteers allows us to test the hypothesis that lean mass is preserved on a hypocaloric diet without any concerns for the health and safety of the participants.

Participants were recruited by public advertisement in a local newspaper. Participants were required to complete a detailed medical questionnaire. Additionally, in order to meet the selection criteria for the study, participants had to (a) have a BMI

>27 kg/m² and (b) have had a stable body weight for at least three months prior to commencing the study and (c) not to have been on another restrictive diet program for a six-month period prior to the commencement of the study. Exclusion criteria included (a) illnesses or medications that would affect appetite, fat absorption and/or blood glucose levels (b) smoking (c) complete vegetarians (i.e no animal products, excluding dairy and egg) and (d) available for a weekly measurements during the four-week trial. Seven individuals did not meet the inclusion criteria (medications that would affect appetite, fat absorption and/or blood glucose levels n=3, full vegetarian n=2, and smokers n=2). One subject withdrew during the first week of the study due to problems adhering to the meal replacement diet, leaving a total of 14 healthy participants (male n=2, female n=12) who completed the study.

The participants were not paid to be involved in the study. However; reimbursement was given to cover any medical expenses that occurred through having to gain prior medical consent to take part in the study. A month's supply of the treatment meal replacement was also offered to all volunteers who successfully completed the trial.

7.2.2 Study Design

This was a four-week trial. Participants were randomly assigned to receive a commonly available meal replacement product ('control' group) or a high-protein modified meal replacement ('treatment' group).

Both meal replacement programs groups were advised to consume two meal replacements per day in addition to meals and snacks as outlined in meal replacement diet program booklet (see Appendix A & B).

The dietary regime in this study was deliberately left more flexible than would be allowed in a fully controlled laboratory trial. Apart from the recommendations given in individual diet programs, participants were permitted to complete the study without weighing food or conforming to a strict diet. In a more controlled laboratory-style study, participants would be given scales and asked to weigh all food consumed in order to enforce strict control to calorie consumption.

It was decided to allow greater freedom in choice and quantity of food consumed to try to reflect a real-life situation and to generate results that have meaning in the real world. The regime followed in this study allows us to measure scientifically what might be found in a typical, non-laboratory environment.

7.2.3 Meal Replacements and Diet

The macronutrient composition of the control and high-protein meal replacements is shown in Table 7.1.

Table 7.1: Macronutrient composition of meal replacement shakes and diet of control (n=7) and treatment (n=7) groups.

Meal replacement shake	Control	Treatment
Energy (kcal)	203	221
Weight (g)	52	62
Protein (g) (% energy)	17 (34)	41 (74)
Carbohydrates (g) (% energy)	27.9 (55)	12 (22)
Fat (g) (% energy)	2.2 (10)	0.8 (3)

The ingredients for each meal replacement were flavoured and dry powder blended before being labeled and randomized for blinding purposes as outlined in Chapter 3, Section 3.2. Participants were advised to mix the supplement in water and

to consumer it according to the directions outlined in their meal replacement program (see Appendix A & B).

Participants in each group were given a list of allowable and non-allowable foods groups that was to be used to help with food choice (see Appendix A & B). The control group was asked to choose from a wide variety of foods, whereas the treatment group was asked to choose foods that were higher in protein content. Participants were also given a sample meal plan and suggested recipes (see Table 7.2; Appendix A & B). The meal plan of the treatment groups recommended an extra serving of protein.

The meal plans were as follows:

- *Control*: Two meal replacements per day (breakfast and lunch) and one meal plus snacks
- *High Protein*: Two meal replacements per day (breakfast and afternoon snack) plus two meals (lunch and dinner)

Dietary analyses were completed using Foodworks 2009 software (version 6; xyris software, Australia) to determine daily dietary energy, protein, carbohydrate and fat content.

7.2.4 Body Composition

Body composition measurements were determined using a TANITA BC-558 MA instrument (Tanita Corporation of America Inc., US), according to methods outlined in Section 3.4.2.

In this study, body composition measurements were conducted using bioelectrical impedance analysis (BIA). As body composition testing was required once a week for four weeks, the gold-standard method, DEXA, was deemed to be

inappropriate due to concerns over the levels of radiation that would be required. BIA is considered the next best method after DEXA, and can be used for quick, safe measurement of body composition (total body weight, lean mass, fat mass, body water, and bone mineral content). However, BIA values should be interpreted with caution. BIA can be quite variable, with 95% confidence intervals for the prediction of four-compartment percentage body fat (BF) from BIA being in the region of $\pm 5\%$ BF (Jebb, Cole et al. 2000; LaForgia, Gunn et al. 2008).

In one study looking at males and females participants across a wide range of ages (37-81 years old), BMI levels (normal weight [$18.5-24.9\text{kg/m}^2$], overweight [$\geq 25\text{kg/m}^2$] and obese [$\geq 30\text{kg/m}^2$]) and physical activity groups (low physical activity and high physical activity), BIA devices provided on average 2–6% lower values for FM% in normal BMI men, in women in all BMI categories, and in both genders in both low and high physical groups when compared to DEXA (Völgyi, Tylavsky et al. 2008).

7.2.5 Satiety Measurements

Measurement of subjective satiety was performed in real time every 30 minutes for a 4-hour period immediately following consumption of the morning meal replacement shake. This was performed using an adapted version of a method previously described by Kruse (2001).

To measure the difference in subjective satiety, a set of four sub-sensations were used. The sub-sensations used were:

- ‘very full’, defined to participants as a state where you feel a strong aversion to food. Mild nausea may be felt at this point.

- ‘full’, defined to participants as a pleasant feeling of satisfaction where you do not have an appetite or feel hungry.
- ‘hungry’, defined to participants as a state where an urge or desire to eat occurs without any physical signs of hunger such as stomach rumbling.
- ‘very hungry’, defined to participants as a state of strong hunger that is associated with physiological signs of hunger such as stomach rumbling.

The change in subjective satiety was recorded over a 4-hour period and marked in a table provided in the participants’ diary (see Appendix A & B). Each participant was given a 20-min tutorial regarding the various subjective satiety states to ensure that they understood each term and understood how to record these sensations in their participant diary.

7.2.6 Statistics

Statistical analyses were carried out using Prism 5 for Mac OSX (GraphPad Software Inc. USA). Differences in baseline characteristics between groups were compared using unpaired Student’s t-tests. A two-way repeated measures analysis of variance (ANOVA) with treatment as the between-subjects factor and time (5 time points of 0, 1, 2, 3, and 4 weeks) and the within-subjects factor was used to test the effects of the treatment. When an interaction effect was identified, group differences were identified using a Bonferroni post-hoc analysis. When an almost significant result was achieved ($p < 0.10$) and the direction of change was different, a post-hoc analysis using an unpaired Student’s t-test on the difference between before and after measurements (i.e delta change) was used to test the effect of the treatment. A p-value of < 0.05 was considered statistically significant.

Table 7.2: Sample meal plans for each of the meal replacement groups.

Control group sample meal plan		Treatment group sample meal plan	
Breakfast	Meal replacement (shake)	Breakfast	Meal replacement (shake)
Snack	Small serving of fruit	Snack	-
Lunch	Meal replacement (shake)	Lunch	Salad or steamed vegetables + serving of protein
Afternoon snack	Vegetable sticks and dips	Afternoon snack	Meal replacement (shake)
Dinner	Salad or steamed vegetables + serving of protein	Dinner	Salad or steamed vegetables + serving of protein
Desert/snack	Diet jelly	Desert/snack	-

7.3 Results

7.3.1 Baseline Characteristics

At baseline there were no significant differences in age, height, body composition or waist and hip circumference between the groups prior to the 4-week meal replacement diet. The average BMI of the subjects in the two groups was in excess of 30 kg/m², meeting the protocol requirements for the enrolment of obese participants (Table 7.3).

7.3.2 Body Composition (BIA)

Body composition measurements as measured by BIA are presented in Figure 7.1 A - D. Both control and high protein groups showed a highly significant decrease ($p < 0.0001$) in body weight (Figure 7.1A) and fat mass (Figure 7.1B) following the 4-week meal replacement diet.

Table 7.3: Baseline Characteristics. Values are means \pm SD (range).

Characteristics	Control (n=7)	Treatment (n=7)
Age (yrs)	41 \pm 13 (22 – 53)	42 \pm 9 (31 – 52)
Height (cm)	165 \pm 0.1 (160 – 174)	170 \pm 0.1 (160 – 185)
Body weight (kg)	96.1 \pm 15.9 (67.7 – 119.3)	94.1 \pm 21.8 (71.4 – 138.6)
BMI (kg/m ²)	35 \pm 5.0 (31.6 – 46.6)	33 \pm 5.5 (26.9 – 44.2)
Fat mass (kg)	40.4 \pm 11.2 (26.21 – 41.66)	40.4 \pm 15.4 (24.49 – 71.66)
Lean mass (kg)	56.6 \pm 10.3 (47.9 – 75.5)	51.6 \pm 9.5 (44.4 – 67)
Waist circumference (cm)*	101.0 \pm 14.9 (89.5 – 127)	100.5 \pm 16.2 (77 – 127)
Hip circumference (cm)*	124.7 \pm 11.4 (112.3 – 145)	122.3 \pm 13.4 (110.5 – 148)
Waist-to-hip ratio*	0.81 \pm 0.1 (0.75 – 0.88)	0.82 \pm 0.1 (0.70 – 0.89)

*only females included in this data (control n=6, treatment n=6).

There were no significant differences in body mass index (BMI), total body weight, fat mass, fat percentage or body water content detected between groups or across time following the 4-week meal replacement diet. However, a significant group \times time interaction ($p=0.012$) was observed for lean mass following the 4-week meal replacement diet. This difference in lean mass demonstrated a significant preservation of lean mass in the treatment group when compared with the control group, as lean mass significantly decreased (-2.7kg) in the control group. Additionally, there was an almost significant ($p=0.087$) decrease in fat percentage in the treatment group when compared with the control group. Both control and high protein groups showed a highly significant decrease ($p<0.0001$) in waist circumference (Figure 7.2A) and hip circumference (Figure 7.2B) following the 4-week meal replacement diet. However, there were no significant differences in waist or hip circumference or in waist-to-hip ratio detected between groups or across time following the 4-week meal replacement diet (Figure 7.2 A - C).

7.3.3 Dietary Analysis

A 4-week dietary analysis for energy (kcal), protein (g), carbohydrates (g) and fat (g) was conducted using the participants' diet program diaries (Table 7.4). There was a significant difference in protein intake ($p<0.001$) and carbohydrate intake ($p<0.05$) between the control and treatment group following the 4-week meal replacement diet. There were no significant differences in fat or total energy intake between groups or across time following the 4-week meal replacement diet.

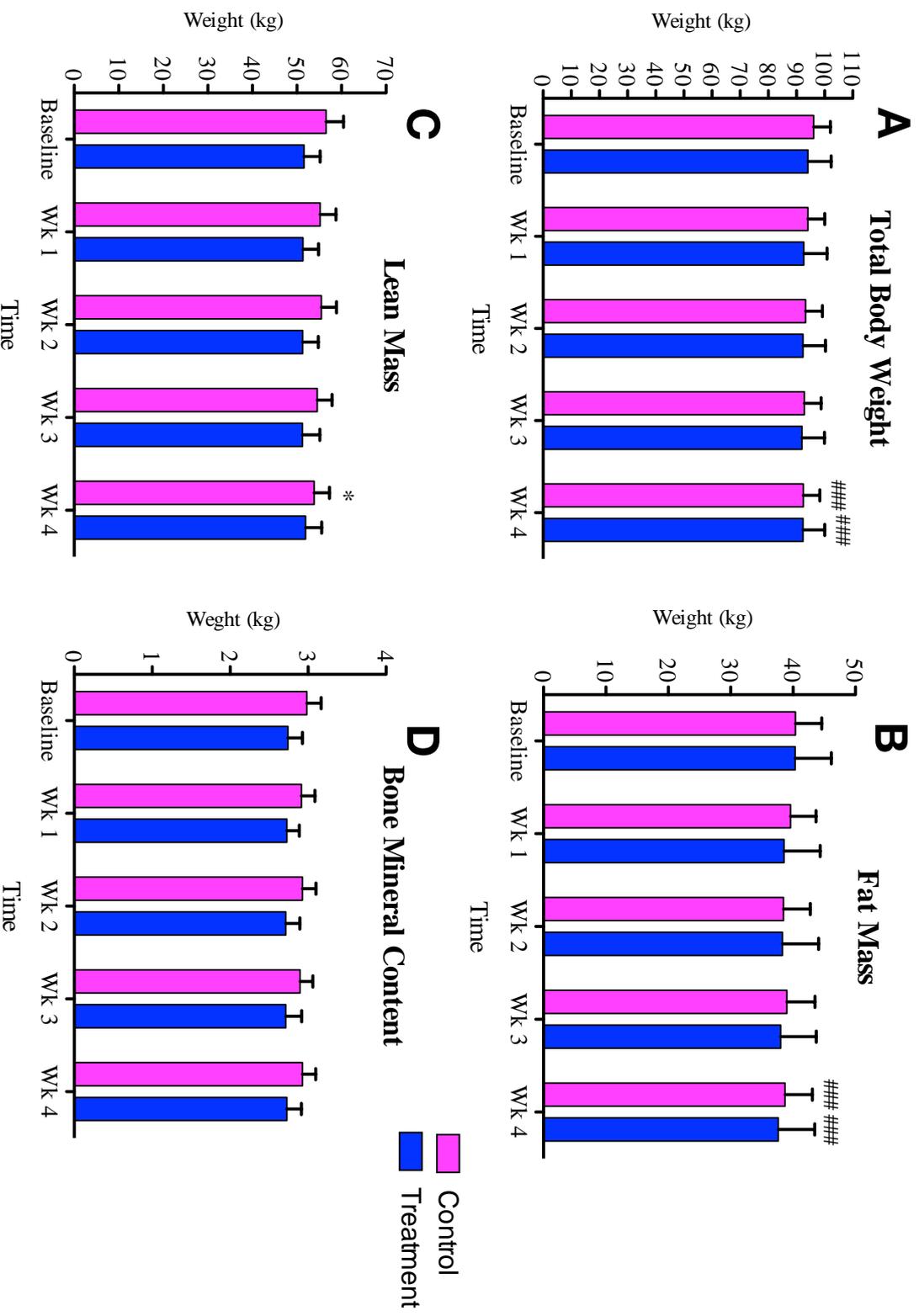


Figure 7.1: Body Composition (BIA) changes for (A) Total Body Weight, (B) Fat Mass, (C) Lean Mass and (D) Bone Mineral Content (BMC) in overweight and obese males and females. Values are means \pm SE. ^{###} Highly significant time effect for total body weight and fat mass ($p < 0.0001$) following the 4-week meal replacement diet, no treatment or treatment plus time interaction effects for total body weight, fat mass and bone mineral content were detected. ^{*} Significant decrease in lean mass ($p < 0.05$). Controls ($n = 7$) and High Protein supplemented ($n = 7$).

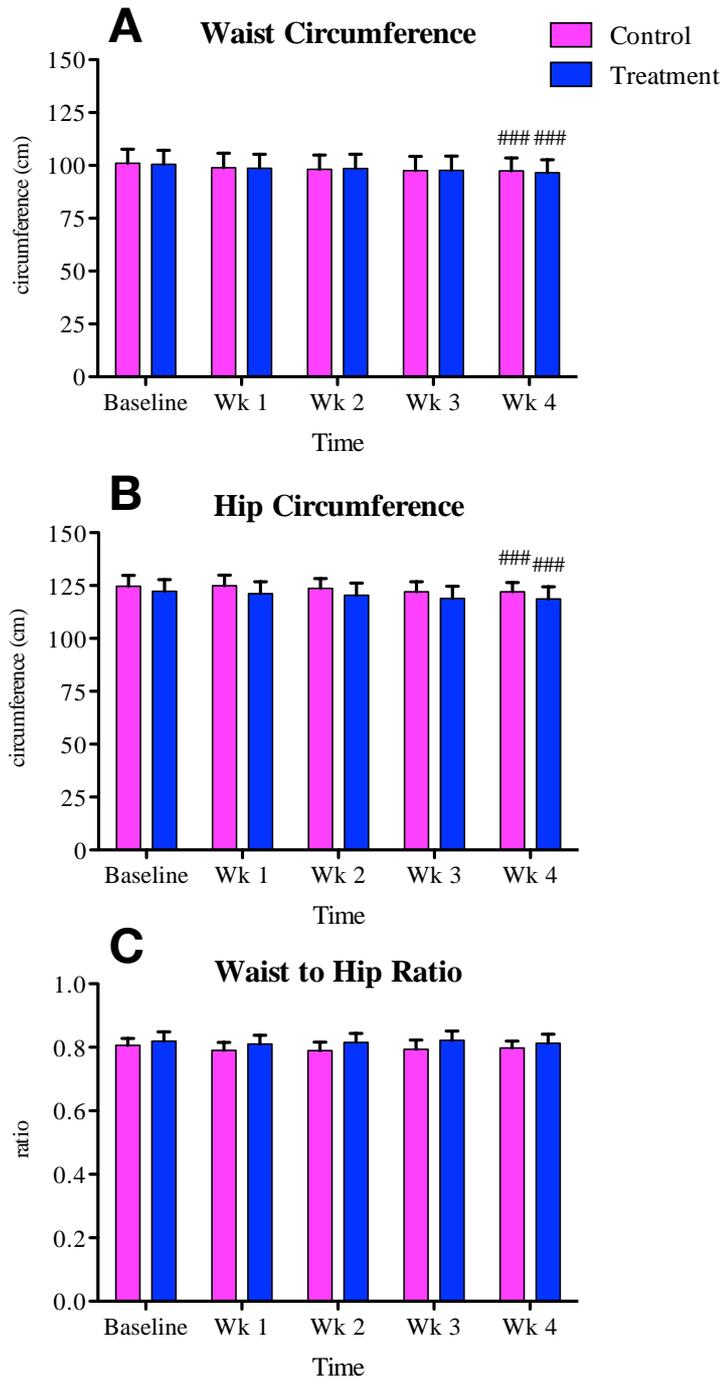


Figure 7.2: Body Composition changes for (A) Waist circumference, (B) Hip circumference, and (C) Wait-to-Hip ratio in overweight and obese females. Values are means \pm SE. ### Highly significant time effect for waist circumference and hip circumference ($p < 0.0001$) following the 4-week meal replacement diet, no treatment or treatment plus time interaction effects for waist circumference, hip circumference and waist-to-hip ratio were detected. Controls ($n=6$) and High Protein supplemented ($n=6$).

Table 7.4: Dietary Assessment. Values are means \pm SD of the average daily consumption averaged out over the 4-week trial period. § includes consumption of meal replacement shake * $p < 0.05$, *** $p < 0.0001$.

Macronutrient composition	Control (n=7)	Treatment (n=7)
Meal replacement diet [§]		
Energy (kcal)	1057 \pm 247	999 \pm 99
Protein (g)	77.8 \pm 11.6	135.0 \pm 8.5***
Carbohydrates (g)	116.5 \pm 47.5	47.1 \pm 5.7 *
Fat (g)	24.6 \pm 7.2	27.4 \pm 5.7

7.3.4 Subjective Satiety

Measurements of subjective satiety were taken every half hour for a 4-hour period immediately after the consumption of the meal replacement shake. Results are shown in Figure 7.3. There was a highly significant difference ($p < 0.01$) in post-meal subjective satiety over a 4-hour period at the 90, 180, 240 minute time points and a significant difference ($p < 0.05$) in post-meal subjective satiety over a 4-hour period at the 120, 150 and 210 minute time points between control and treatment groups.

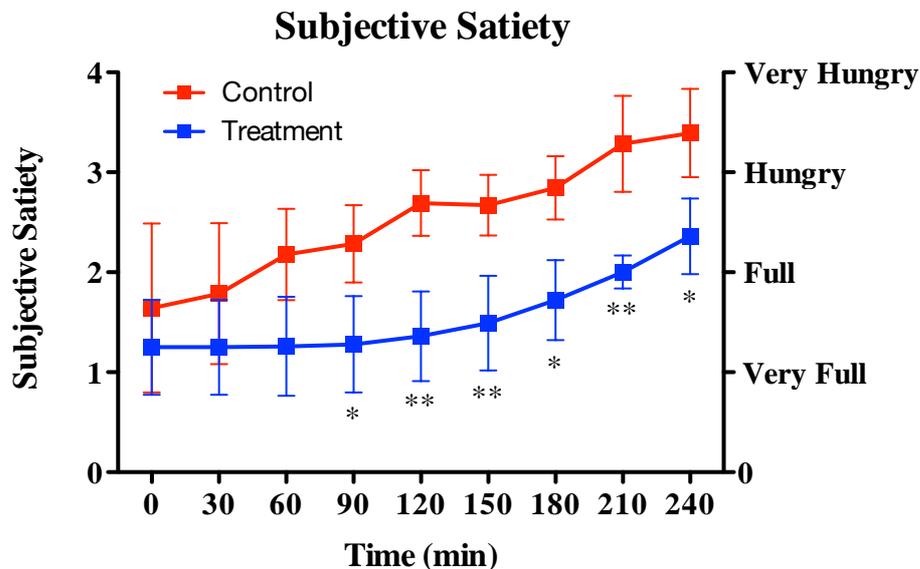


Figure 7.3: Subjective satiety measured over a 4 hour period following the consumption of a meal replacement product (MRP). Values are \pm SE. *Significant difference in post-meal subjective satiety at 90, 180 and 240 minutes ($p < 0.05$). **Highly significant difference in post-meal subjective satiety at 120, 150 and 210 minutes ($p < 0.01$). Not all subjects completed subjective satiety questionnaires. Only complete datasets were used. Controls (n=4) and treatment (n=4).

7.4 Discussion

This study was designed to evaluate the benefits of a protein-enriched meal replacement shake and diet compared with a standard meal replacement shake and diet on weight loss and lean mass preservation in overweight and obese adult participants. The main focus was on the benefit of this regime in loss of fat mass during the study.

There was no significant difference in the amount of weight lost between the two groups over the 4-week trial period. However, weight loss in the control group occurred as a result of losing both fat mass (-1.6 kg) and lean mass (-2.7 kg) in contrast to the treatment group in which the weight loss came exclusively from fat mass (-2.7 kg), with a small increase in lean mass (+0.4 kg). Hence, dietary supplementation with increased protein seems to directly affect body composition in the desired fashion, as individuals in the treatment group lost fat mass selectively while preserving lean mass. This confirms in a sedentary, obese population what has previously been observed in athletes and young individuals; namely that a higher dietary protein intake can alter the balance between fat and lean mass (Cribb, Williams et al. 2007). These results are significant as they raise the possibility that a more prolonged intervention may not only help to reduce body weight and fat mass, but may actually help to reduce the overall impact of sarcopenia. Although these results were found in normal aged adults, they are relevant to older adults with sarcopenia, suggesting that a protein-enriched meal replacement program may be a suitable strategy to combat the combination of sarcopenia and obesity (sarcopenic obesity). It would be interesting to note in future research whether similar diet plans

can restore lean muscle mass in individuals diagnosed with pre-existent sarcopenia and/or sarcopenic obesity.

It was decided to conduct this initial study in younger adults (aged 22–54 years) rather than aged individuals from a position of duty of care for the participants involved. We were concerned that introducing a hypocaloric diet to aged participants with sarcopenia might cause further dangerous lean mass losses in these individuals. This concern was borne out in practice in the study, as those participants who received the protein-supplemented meal replacement diet displayed a preservation of lean mass, while those on the control program demonstrated a loss of lean mass during the course of the study (Figure 7.1). Such a loss of lean mass may have posed a serious health risk to elderly patients. Thus, we feel justified in our decision to conduct this study in younger participants. This also highlights the danger of simply focusing on loss of weight when using a meal replacement program, as any loss of lean mass would ultimately be counter productive for maintenance of any weight loss.

The two meal replacement shakes (control and treatment) were well balanced in terms of the caloric content; however they did differ in relation to their protein and carbohydrate content (Table 7.1). The protein content of the treatment shake was set deliberately higher, not only to increase the intake of protein in the treatment group but also to induce a state of acute post-meal satiety following consumption of the shake. It has been shown that an acute high protein meal (~60% content) induces a greater feeling of satiety compared with a meal containing 10-20% protein (Stubbs, van Wyk et al. 1996; Crovetti, Porrini et al. 1998). The current study supports this notion, as the higher protein meal replacement group demonstrated a significantly lower hunger score 90 – 240 minutes after consuming their meal replacement shakes.

The percentage of total calories that came from protein in the treatment shake was 74% compared with 33% in the control group. The difference in carbohydrate content between control and treatment groups was a result of matching the caloric value of both groups given the extra protein in the treatment group.

No baseline differences were found between the two groups. In both groups, the participants were obese, with the average body weight being 94 kg with the average BMI greater than 30 kg/m². The fact that the baseline characteristics were well matched in both groups is important since the observed changes can therefore not be attributed to inherent metabolic differences.

Participants in both groups were asked to maintain a diet diary during the 4-week trial period whereby the total intake of energy, carbohydrates, proteins and fats was recorded over a 4-week period (Table 7.4). As expected, both groups differed in their protein ($p < 0.001$) and carbohydrate intake ($p < 0.05$). Participants in the treatment group showed a significant increase in their protein intake and slightly reduced carbohydrate intake compared with the control group (Table 7.4). This is clearly attributable to the fact that the meal-replacement shake is protein-enriched. Additionally, the treatment group was also asked to consume an extra serving of protein with their meals each day. We presume that the carbohydrate content of the treatment group was displaced by the increase in dietary protein, as the total energy intake did not differ between groups.

An interesting observation in the current study was the self-reported increase in satiety by individuals who received the protein-enriched meal replacement shake. This feature is important since it might be used as a tool to prevent overeating and snacking between meals. Protein-enriched meals provide satiety for longer periods of

time compared with the normal meals containing mainly carbohydrates and fat (Stubbs and Whybrow 2004; Bowen, Noakes et al. 2007) and may help to diminish snack cravings between meals.

Interestingly, however, although participants in the protein-enriched meal replacement group reported greater levels of satiety, the overall energy intakes of the two groups were not different. This is somewhat surprising, as one might have expected the group with the greater satiety levels to have lower energy consumption. There are several possible reasons for this interesting observation. One factor may be the difference in the number of main meals consumed by the two groups (two main meals in the treatment group versus one main meal in the control group). As the energy content of these meals were not controlled in the same way as the meal replacements, it is possible that the additional meal in the treatment group was of higher caloric value and increased the overall energy intake. It is also possible that the post-meal feeling of satiety experienced by the treatment group was only transient and did not affect energy intake of the subsequent meal consumed 3-4 hours later (Westerterp-Plantenga, Nieuwenhuizen et al. 2009)

In a similar study to ours, Bowen et al. (2007) suggested that the similar energy intake between treatment groups may reflect a sensation of emptiness owing to the liquid form of the preloads and/or the low energy content relative to the timing of next meal which resulted in a similar energy intake between groups.

In a review by Westerterp-Plantenga and Nieuwenhuizen et al. (2007), they discuss that the satiating power of a high-protein meal is affected by the timing in

relation to internal amino acid profiles. Therefore, the similar intake in both groups may be related to the timing of the meal replacement shakes.

Finally, in an earlier study by Westerterp-Plantenga and colleagues, it was shown that satiety is heavily influenced by dietary induced thermogenesis (DIT). Indeed, it seems that DIT may over-ride energy intake in terms of sensations of satiety (Westerterp-Plantenga, Rolland et al. 1999).

Whatever the explanation for this observation, it should be noted that the increase in post-meal satiety following the consumption of the higher protein meal replacement shake would make the diet a lot easier to maintain. The control group reached a state of subjective hunger at the 150-minute (2.5-hour) mark (Figure 7.3). This fits the traditional pattern of eating seen in Western society, in which we commonly eat breakfast at 8am, have morning tea at 10.30am followed by lunch at 1pm, afternoon tea at 3.30pm and dinner at 6pm. Often an evening snack is consumed at 8.30pm. Overall, eating every 2.5 hours as a result of feeling hungry can lead to an individual consuming up to six 'meals' each day. In fact, diet plans do typically allow for six meals (inclusive of two meal replacements and snacks) that are spread out evenly throughout the day. The concept behind these plans is to encourage 'grazing'; that is to eat small quantities of food with each meal, but to eat more often to maintain energy levels. However, portion size control and food choice is often very difficult to control in individuals who have a problem controlling energy intake and/or have a strong emotional relationship with food. Similarly, if junk food is consumed for even half of these meals, it results in a very unhealthy dietary pattern.

Hence, it can be argued that increasing post-meal satiety, by increasing the protein content of meals, could be used to increase the time gap between meals, presumably decreasing the number of meals consumed, thus minimizing the risk of over eating. Importantly, based on the satiety response, which can also be looked upon as a subjective measure describing the absence of hunger, a protein-enriched diet would deliver its effects passively, with an individual spending less time thinking about food because they are less hungry.

This is in contrast to what we see with commonly available diets that require individuals to actively count the calories that they consume. Calorie counting can be very time consuming and this may explain why most of these diets have high attrition rates. Additionally, individuals on commonly available low-calorie diets are often asked to satisfy their desire to eat with ‘fillers’ such as sugar free lollies and diet jellies that contain minimal, if any, nutrition. Although this may satisfy the desire to eat, the underlying hunger is not satisfied, making it even harder to adhere to these diets. Recently it was shown using brain scans that individuals who had missed breakfast responded a lot more favorably to energy-dense foods (cakes and deep fried foods) than to foods that were less energy dense (salads and breakfast cereals) suggesting that we are hardwired to maintain a set caloric intake when we actively decrease caloric intake. Conversely, those who had eaten breakfast responded equally to energy-dense and non-energy dense foods (Goldstone, de Hernandez et al. 2009). It should be noted that the same would not apply with the satiety response, as satiety tends to be associated with a physiological response to food involving a decrease in subjective hunger and does not involve an active decrease in food intake regardless of whether an individual is hungry or not.

7.5 Conclusion

This study found that a protein-enriched meal replacement diet was successfully able to preserve lean mass in overweight or slightly obese individuals. Weight loss came exclusively from fat mass (-2.7 kg), with a small increase in lean mass (+0.4 kg); in contrast to a standard meal-replacement diet in which weight loss occurred both from fat mass (-1.6 kg) and lean mass (-2.7 kg).

In terms of subjective satiety, we found that a protein-enriched meal replacement diet induced a significant ($p < 0.05$) increase in post-meal subjective satiety over a four-hour period at the 90, 120, 150, 180, 210, and 240-minute time points in the treatment group compared with the control group.

Therefore, the findings of this study draw us to the conclusion that a 4-week protein-enriched meal replacement diet achieves benefits in terms of weight loss and lean mass preservation, as well as benefits associated with satiety and hunger control in obese adults. As such, maintenance of such a diet in the long term may be expected to reduce body fat, improve body composition and reduce sarcopenia in elderly individuals. Further research, particularly among the elderly, needs to be undertaken to verify this conclusion and to examine long-term compliance.

Chapter 8

Limitations and Future Directions

8.1 Limitations

This research program was designed to be comprehensive and wide-ranging. We designed the studies so that they would be accurate, meaningful and reproducible. In addition, we tried to imitate the real-world situation as far as possible so that our findings would have practical application in everyday life. In the most part, we are confident that we achieved these aims satisfactorily.

However, as with any clinical research program, there are certain limitations that can be identified in this research. We hope to address a number of these limitations in future studies, but are also aware that considerable research in this field is also being carried out elsewhere which will also add to our knowledge in this area.

One major limitation of the studies conducted in the present work is the lack of a power analysis conducted to determine the appropriate sample sizes. Unfortunately, adherence to a rigorous power analysis was not possible for practical reasons, as the studies were conducted at a single unit and were limited by the number of eligible subjects practically available. The invasive nature of some of the procedures limited our pool of eligible subjects still further.

A retrospective power analysis was performed using the gymaware data used to calculate power in the Study 2 (chapter 5) suggested that a total of 30 participants would be optimally required. We calculated that a population of 10 participants should have revealed an impact of whey protein that was the same as the standard deviation of the difference in the response of matched pairs, which would have provided valuable data. Unfortunately, the variation in the effects (hence deviation of difference) was much greater than anticipated, possibly due variations in baseline

fitness levels, resulting in considerable variability in response. This had the effect of reducing the power still further.

Prior to the study, we had anticipated that the original study target should have been attainable with a sample size of 8. This was based on published MAPR data (Williams, Carey et al. 2007) that showed training increased P+M MAPR from 3.78 to 4.91 with a SD of 0.95. A sample size of 8 would have provided an acceptable power of 0.8. We therefore felt confident in the statistical validity of our study, even with the limited number of participants available for recruitment.

Due to the nature of this research program, consisting of four independent and sequential studies, we were able to address certain limitations identified early in the program during later studies. For example, the lack of benefit from supplementation with either Cr or whey-protein in isolation in terms of body composition, muscle oxidative capacity or muscle protein concentration compared with placebo found in Study 1 contrasted with previous research. We considered that this surprising finding may have been due to a lack of stimulus, through the provision of a resistance-training program, for example, or the insufficient length of the study. We were able to address these limitations in Studies 2 and 3 by doubling the length of the study and introducing a 12-week resistance-training program into the protocol.

In Studies 1 and 2, we decided to perform both histochemical analysis and fiber typing using SDS-PAGE for measurement of MHC muscular protein expression. We reasoned that performing both analyses would increase accuracy and provide comparative data for correlation. According to the MHC isoform analysis in study 1 (Chapter 4), however, an unlikely sized shift from type II to type I fibers was observed. This apparently anomalous finding is thought to be the result of the applied

method not being full optimized for human muscle hence, preventing us from drawing conclusions in this regard. Future studies should focus on appropriate optimization of the techniques involved in order to investigate this aspect of the research further.

We had originally planned in Study 2 to investigate three markers of muscle oxidative capacity, citrate synthase (CS) activity, protein content and mitochondrial ATP production rate (MAPR). Unfortunately, while we were able to show an improvement in CS activity in both groups, with no differences between resistance training plus whey-protein supplementation compared with resistance training alone, an experimental error meant that we were unable to measure MAPR. This meant that we were unable to further investigate changes in muscle oxidative capacity, a key objective of this study, or to provide further evidence to support the CS activity findings. As a result, whether there is a specific effect on MAPR in elderly people with and/or without resistance training is still unknown and thus MAPR measurements should be integrated into future research studies.

Although a number of important and interesting conclusions may be drawn from Study 3, the lack of significant results makes interpretation of the study somewhat difficult. It would be interesting to repeat the study with larger groups of subjects in order to try to improve subject matching and reduce variability within groups. This may allow differences between groups to reach statistical significance, enabling better interpretation. As mentioned in Chapter 6, it would also be interesting to further develop the adapted methods developed for the measurement of the redox states. In particular, it would be worth investigating further the identification of GSH on LC-MS or LC-M-MS using the ammonium formate buffer system. This limitation notwithstanding, the refinement in the literature methods that we undertook as part of

this research enabled us to complete a far greater range of analysis than would have been otherwise possible.

Study 4 may be scrutinized for a perceived lack of control in the dietary regime enforced during the study. This study was deliberately designed in this way, allowing greater freedom in choice and quantity of food consumed to try to reflect a real-life situation and to generate results that have meaning in the real world. However, we took this decision in the knowledge that there would be greater variability in the results than would be the case if we had chosen to follow strict laboratory conditions. This was a calculated choice, and we feel vindicated in our decision because significant results were obtained that we are now able to interpret with the confidence of knowing that they are more likely to be replicated.

Some wider limitations of the research program in general should be acknowledged. We decided to enroll participants from the age of 55 years upwards for reasons of caution and safety. However, this meant that the average age of the first three studies tended to be around 60 to 64 years. While this population may be considered aging, some would argue that it could not be described as 'elderly'. Now that we have established the safety of the 12-week resistance training program and supplementation regimes in this population, it would be interesting to go on and investigate similar schemes in populations with an average age of 70 years, or even higher. An investigation into the effects of these interventions in a more elderly population, that are much more likely to display the effects of sarcopenia, may be the focus of further research in the future.

In summary, the body of research reported herein has provided much important information regarding successful strategies to prevent or manage sarcopenia

and other signs of aging in the human muscle. However, this research has also shown that strategies that might appear to be effective in younger individuals are likely to need tailoring for older groups, due to the myriad of changes occurring as we age.

8.2 Future Directions

In order to address the limitations described above, we propose a similar series of studies to be conducted in the future, but with a number of important modifications.

Study 1: The Effect of Whey-Protein Supplementation and Resistance Training on Muscle Protein Content and Muscle Strength with Aging in Humans.

This is a refined version of Study 2 (presented as Chapter 5 herein). As the previous study may have been underpowered, the new study will recruit sufficient subjects to achieve a minimum statistical power of 0.80. In order to more accurately reflect the population of interest (elderly individuals with advancing sarcopenia), an older patient population will be recruited, with a minimum age no less than 65 years. In addition, in order to eliminate any variation arising due to the lack of control over the timing of supplementation, it will be required that protein be ingested immediately before and after training using the protocols as outlined by Cribb and Hayes (2006). Finally, the new study will involve the same 12-week resistance training protocol as described in Chapter 5 to provide contrasting data with those gathered from a new Study 2, as described below.

Measurements to be performed in the new Study 1 include:

- Protein concentration (total, contractile and mitochondrial),
- Histochemistry
- Refined SDS-PAGE method
- MAPR

- Citrate synthase
- Various muscle strength measures.

Study 2: The Effect of Whey-Protein Supplementation and Functional Training on Muscle Protein Content and Muscle Strength with Aging in Humans.

This is a second refined version of Study 2 (presented as Chapter 5 herein), involving a different training method. For the reasons described above, a minimum statistical power of 0.80 will be achieved through recruitment of sufficient subjects, the minimum age will be no less than 65 years, and protein will be ingested immediately before and after training using the protocols as outlined by Cribb and Hayes (2006). This study will differ from the new Study 1 described above in that the 12-week functional training protocol will involve supervised group personal training sessions that focus on functional movements three times per week. The aim of functional training will be to train the participants using a collection of movements that mimic everyday movements and that focus on developing a better connection between the physical body and cognitive awareness of the body. Training sessions will involve the uses of interactive games as well as using resistance devices such as therabands, medicine balls and free weights.

Measurements to be performed in the new Study 2 include:

- Protein concentration (total, contractile and mitochondrial)
- Histochemistry
- Refined SDS-PAGE method
- MAPR
- Citrate synthase
- Various functional strength measures.

Study 3: The Effect of Two Meal Replacement Diets on Lean-Mass Preservation and Subjective Satiety in Sarcopenic Overweight and Obese Aged Males and Females.

This is a refined version of Study 4 (presented as Chapter 7 herein). This study will ensure statistical validity by recruiting sufficient number of subjects to achieve a minimum statistical power of 0.80. In order to more closely mimic the population of interest, the study will recruit an older group of subjects with sarcopenia (low muscle mass [>2 SD below the mean of that found in young adults of same sex and ethnic background] plus low muscle function [strength and performance] with a BMI of greater than 27kg/m^2). However, in order to more closely assess the effect of the dietary programs than could be achieved in the present study, a greater control over the diet will be achieved by using two meal replacement shakes and two pre-prepared and packed meals per day with a defined macronutrient composition and caloric content. As the full extent of the effect in the current study could not be assessed over the longer term, the new Study 3 will be extended to 12 weeks. Finally, BIA measurements were used in the present study to measure body composition for practical reasons in preference to the gold standard, DXA. Although the data generated in this way are useful, BIA can be quite variable and its results should be interpreted with caution. Therefore, the new study will revert to the use of DXA analysis at 0, 4, 8, and 12 weeks for improved data quality.

Measurements to be performed in the new Study 3 include:

- Body composition using DXA and subjective satiety

Chapter 9

Conclusions

CONCLUSIONS

The results described herein are the outcome of a strategic, integrated research program involving four independent but complementary studies. The focus of the research was to establish strategies to mitigate the effects of aging on human muscle, including the loss of skeletal muscle ('sarcopenia'), decrease in muscle oxidative capacity and reduction in muscle strength that is commonly observed. These factors typically combine in the elderly to increase frailty and exacerbate the risk of falls and subsequent disability. Considerable research has been conducted in this area previously, with the two most effective strategies to arrest the effects of sarcopenia having been identified as exercise (in particular, resistance training), and regular consumption of a protein supplement. Our research program was designed to provide further insights into the use of resistance training and protein supplementation in the mitigation of sarcopenia and other negative age-related effects on muscle function.

We designed a series of four related studies investigating specific but complementary aspects in the management and prevention of sarcopenia. The first study was designed to compare the effects of 6 weeks of supplementation with Cr and whey protein compared with placebo on muscle oxidative capacity in aging males (aged 55+ years). The second and third studies were conducted across the same group of aging male participants (aged 55+) and investigated the effects of a 12-week resistance-training program either alone or in combination with whey-protein supplementation. Study 2 evaluated the effects of these interventions in arresting the debilitating loss of muscle size and function that occurs with age, while Study 3 investigated the effect of these regimes on tissue and plasma thiol (GSH and Cys) and disulfide (GSSG and CySS) levels and plasma and tissue redox states among this aged

male population. The final study was designed to test the effects of nutrition using a protein-enriched meal replacement diet on body composition and post-meal satiety.

This research program was carefully designed to provide maximum insight into the effects on muscle aging and sarcopenia of both resistance training and protein supplementation under a variety of conditions and circumstances. We aimed to replicate true-to-life situations as far as possible so that our findings would have maximum real-world significance.

Study 1 revealed that supplementation with either Cr or whey-protein in isolation did not improve body composition, muscle oxidative capacity or muscle protein concentration compared with placebo. This was somewhat surprising, as the literature and our own previous results had led us to anticipate an increase in all three of these parameters. Interestingly, muscle oxidative capacity appeared to decrease slightly (but non-significantly) after six weeks of dietary supplementation, possibly as a result of changes in cell volume. We considered that the six-week time frame of this study allowed us only to consider the incipient phase of the process, suggesting that the duration of this study may have been too short to observe a true effect. We also considered that the lack of response may have been due to the absence of a stimulus, such as resistance training. We therefore methodically addressed both of these potential contributing factors in the Studies 2 and 3.

Study 2 revealed that, when combined with a 12-week resistance-training program, whey-protein supplementation offered improved body composition (highly significant decreases in fat mass and fat percentage and a highly significant increase in lean mass), and significantly improved muscle function, according to certain criteria (isometric and isokinetic knee extension and isokinetic knee flexion, 30-s sit-

to-stand test, and leg press and bench press 1-repetition maximum testing). However, the same effect was observed with the 12-week resistance-training program alone, suggesting that whey-protein supplementation offered no incremental benefit. Interestingly, resistance training alone appeared to offer greater benefit than the whey-protein plus resistance training in terms of improved isokinetic flexion and 1RM leg press. However, these apparently anomalous findings may potentially be explained by the apparent greater fitness of the whey-protein group at baseline, providing less opportunity for improvement in this group. Even though the overload principle of strength training would dictate that even the fitter individuals should have made gains in fitness, it is possible that their higher level of fitness meant that if they may have been training at a lower intensity and therefore not effectively overloading their muscles.

Study 3 revealed that neither a 12-week resistance-training program on its own, nor in combination with whey-protein dietary supplementation, altered the plasma or tissue thiol or disulfide levels or plasma or tissue redox states. If this is a genuine outcome, it may explain the absence of any benefit of whey-protein supplementation on other measures evaluated in this study, including oxidative capacity and muscle strength as described above.

Study 4 demonstrated that a 4-week protein-enriched meal replacement diet offers advantages in terms of weight loss and lean mass preservation, as well as benefits associated with satiety and hunger control in obese individuals. As such, maintenance of such a diet in the long term may be expected to reduce body fat, improve body composition and reduce sarcopenia in elderly individuals.

As an important part of this research, we also conducted a refinement of the literature methodology for the measurement of tissue thiol and disulfide levels in order to be able to calculate the GSSH/GSH redox state accurately in Study 3. This new methodology will be available as a blue-print for further research of a similar nature in the future.

We have explored a number reasons for the observed lack of any incremental effect of whey-protein supplementation over and above the benefit of resistance training on the parameters assessed in Studies 2 and 3. One key explanation may be the lack of previous training in the population studied. This means that the introduction of a resistance training program would have caused a marked and rapid change in both the strength and redox activity of the subjects involved, which may have been large enough to mask any effect of the whey-protein supplementation. However, the population in this study was deliberately selected in order to closely match the real-world sarcopenic population, who in practice are likely to present with a similarly low level of training. Another possible reason for the lack of additional benefit of whey-protein supplementation in these two studies is the lack of control over timing of supplementation. Again, this is intended to mimic the real-world situation. A final consideration is the bioavailability of the protein ingested. Insufficient absorption of the protein may have led to lower than optimal blood protein concentrations, which may have reduced the impact of the supplementation.

In conclusion, a number of strategies investigated in this research program have been identified as offering benefit in the prevention and management of sarcopenia. In particular, the introduction of a 12-week resistance training program, with or without the addition of a whey-protein supplementation program, offers

highly significant benefits in terms of body composition and certain muscle function indicators. In addition, a 4-week protein-enriched meal replacement diet delivered benefits in terms of weight loss and lean mass preservation as well as potential advantages resulting from increased satiety and improved hunger control. We suggest, therefore, that implementation of a resistance-training program would be an important intervention for the prevention and management of sarcopenia among elderly individuals, and that the concomitant introduction of a protein-enriched meal replacement diet may provide further benefits, particularly in terms of body composition and hunger management.

Chapter 10

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Appendices

Appendix A

Control Meal Replacement Diary and Guidebook

Introduction

Hi and welcome to your 4 week weight loss trial. Keeping a diary of everything that you eat and drink is a very important and powerful tool to help you reach your weight loss goals. Additionally, it is a very useful tool in gaining a greater understanding about the functional aspects of your diet. Keeping a diary also tends to make you more accountable and more likely to stay on your program. It's very useful when you are having problems with your diet. You/we can quickly review your diary and program and identify changes that need to be made. So, please use your food diary on a daily basis and keep it with you wherever you go. I wish you every success during your 4 week trial period.

Instructions

This food diary is designed to be your companion while you are on the 4 week trial. The diary has been set up for a 4 week period, which is a reasonable time for you to get a greater understanding of, as well as learning how to control, your diet. Importantly, it will help us gain a greater scientific understanding of the 4 week diet program that you have been placed on.

Write everything down: Keep your diary with you all day every day, and write down everything that you eat and drink and at what time you ate or drank these items. Write down everything, even if you happen to slip up and eat the wrong thing – please write it down!

What time? It is very important that you record the time that you eat and drink every item. This will help you/us identify any habits that may be contributing to your weight gain. It will also help you/us understand a lot more about functional components of your diet (e.g. does the food you satisfy your hunger? and if so, for how long?)

Record as you go: Please do not wait until a later time to record what you ate or drank or depend on your memory to write down everything at the end of the day. Record the information as you go.

Be specific: Please include as much detail as possible about what you eat and drink. This includes recording information about extras such as sauces or gravies on pasta and meat dishes. Quite often, it is the hidden extras that overtime results in weight gain (e.g. carbonara sauces, milk in café lattes and so on). Every little bit of information counts.

Record amounts: Please record details about serving size. If you eat a serving of food estimate the size (e.g. palm size serving of protein, 1 medium sized salad) or if possible, include the weight of the item (e.g. 150g chicken breast without skin). With salad items and vegetables, please record the amount (e.g. 1 cup of spinach, 3 sliced mushrooms, ½ cup steamed broccoli, 1 tomato)

Example of Measurement of Safety Table.

DIET PLAN

- Two meal replacement shakes per day
- One serving of protein (size of palm)
- **MINIMUM** of three cups of vegetables including salad vegetables
- Two pieces of low GI fruit
- Two litres of water – other allowed liquid are in addition to two litres of water
- Dietary fibre – once per day
- Limit caffeine

FOODS TO AVOID

- Grains/cereals/legumes
- Pasta/rice/noodles
- Bread/pastries/biscuits
- Potato, sweet potato, corn
- Chocolate, lollies, ice-cream
- Fruit juice, nuts, dairy

ALLOWABLE EXTRAS

- Lollies, mints, or gum – needs to be sugar free with maximum of 3-6 per day
- Diet jelly – unlimited
- Milk – half cup low fat or skim milk may be used in hot beverages each day
- Fruit – consume 2 serves of low GI fruit from list
- Treats – one treat option may be consumed twice per week
- Condiments – may use a small amount of sauces, dressings, gray or mayonnaise each day.
- Herbs and spices – use fresh herbs where possible.

Example Daily Meal Plan

MEAL	ON WAKING	250ml water + Dietary fibre
1	BREAKFAST 8.00am	Meal replacement shake 1 with 200ml water
2	MORN. TEA 10.30am	2 Grapes (low GI fruit) 2 glass of water
3	LUNCH 12 noon	Meal replacement shake 2 with 200ml water Steamed vegetables 2 glasses water
4	ARVO. TEA 3.00pm	Vegetable sticks and dip or Apple (Low GI fruit) 1 glass water
5	DINNER 6.30-7pm	Palm-size serve of protein with Salad or vegetables 1 glass water
6	DESERT	Diet jelly 1 glass of water

Meal Replacement Product

Directions: To mix the meal replacement product please use the shaker that we provided*.

1. Add the required amount of water to the shaker.
2. Empty the contents of the meal replacement sachet into the shaker.
3. Place the lid on the shaker and shake vigorously until the all the powder is evenly mixed into the water.
4. Serve and drink immediately.

*If you have a blender/ blender or

Protein

All serves of protein are 120g for females and 220g for males.
This is traditional referred to as a "palm sized" portion of protein

Note: Lean = all fat trimmed where possible and <15% fat in minces

- **Lean Red meat** – Beef (eg. steak, lean mince), Veal, Lamb
- **Lean Pork and game**
- **Poultry** – Chicken and Turkey (Skin removed and breast meat)
- **Fresh fish**
- **Tinned fish** (Salmon or tuna) packed in water (not brine or oil)
- **Seafood** – prawns, oysters and crab (females 1 cup, males 1 ½ cups)
- **Eggs (large)** – females: 2 eggs, Males: 2 eggs + ½ listed serve protein
- **Cottage cheese (low fat)** females: 150g, Males: 200g
- **Ricotta Cheese (low fat) fresh or smooth packed** – females: 150g, Males 200g
- **Tofu (Firm)** – Females: 150g, Males: 250g
- **Soy Sausages** – females: 2 sausages, Males 3 sausages

FRUIT (ALL SERVES MUST BE LOW GI FRUITS)

Fruit	Quantity per serve	GI
Apple	1 medium	38
Apricot (dried)	10 halves	30
Banana (small, not too ripe)	1 small	52
Blackberries	25	15
Blueberries	25 + ½ other fruit serve	15
Cherries	20 small to medium	22
Fruit cocktail	Small, size equiv to one fruit	Less than 55
Grapefruit	1 medium	25
Grapes	20 small to medium	46
Kiwi fruit	1 large to small	47-58
Mandarin	2 medium	Less than 55
Mango	1 very small or half medium	51
Nectarine	1 large or 2 small	42
Orange	1 medium	42
Passion fruit	2 large + ½ other fruit serve	Less than 55
Peach	1 large or 2 small	42
Pear	1 small to medium	38
Prunes	5 large	29
Raspberries	25 + ½ other fruit serve	40
Strawberries	20 medium to large	40

VEGETABLES (UNLIMITED)

Alfalfa sprouts	Celery	Rocket
Artichoke**	Chives	Shallots
Asparagus	Choko	Silver beet
Avocado*	Cucumber	Snow peas
Bamboo shoots	Eggplant	Spinach
Beans (yellow, green)	Lettuce	Spring onion
Bean sprouts	Endive	Squash
Bok Choy	Fennel	Tomato
Broccoli	Leek	Vegetable greens
Broccoliini	Mung bean sprouts	Watercress
Brussel sprouts	Mushroom	Witlof
Cabbage (white/red)	Okra**	Zucchini
Capsicum (red, green, yellow)	Onion	
Cauliflower	Radish	

Limit to ½ medium or ½ small per day

**Limit to ½ cup

VEGETABLES (LIMITED)

Vegetable	Allowed quantity
Baby corn	½ cup
Beetroot	4 large slices
Carrot	½ medium or 1 small
Celeriac	½ cup, diced
Peas*	½ cup
Pumpkin	½ cup, diced

* Limited to 3 times per week

Beverages

WATER

2 litres of water per day (approx. 8 glasses)

SWEETENED BEVERAGES

Avoid all sweetened beverages such as cordials, soft drinks and juices however you may have the following artificially sweetened beverages as long as they contain less than 1g CHO per 100mL

- *Diet cordial*
- *Flavoured water*
- *Soft drinks – must be diet and caffeine free.*
- *Maximum of 3 cups per day. NOT to replace your water intake*
- *Sparkling mineral water – maximum of 3 cups per day.*
- *NOT to replace your water intake*

HOT BEVERAGES

These beverages do NOT contribute to your water intake. You may add a dash of low fat or skim milk (to maximum of half cup per day) and artificial sweeteners may be used in place of sugar or honey.

- *Coffee – 1 cup per day of regular coffee*
 - *Decaffeinated coffee – maximum of 3 cups per day*
 - *Green Tea – maximum of 5 cups per day*
 - *Herbal teas – unlimited amounts and consumption*
- DOES contribute to daily water intake*
- *Decaffeinated tea – unlimited*

ALCOHOL

You may choose an option below twice per week, as a treat option.

- *Wine (dry red or white) – 1 small (120 ml) glass*
- *Spirits – 1 nip/shot (30mL)*
- *Beer (Pure Blonde or Hahn Super Dry low CHO beers) – 1 bottle (330-355ml)*

Measurement of Satiety

Satiety is defined as the condition of being full or gratified beyond the point of satisfaction.

To measure post-meal satiety we will be asking you to note how you feel following your morning meal replacement shake. To do this you will be asked to indicate a change in your perception every 30 minutes over a 3-4 hour period before your consume your next meal.

The usual measurement to determine your perception of food intake is based on a rating system. The rating system that will be use is as follows:

Question: How do you feel? Please circle one of the four selections below.

1. **Very Full**
2. **Full**
3. **Hungry**
4. **Very Hungry**

The above states are defined as follows:

1. **Very Full:** This is state where you feel strong aversion to food. Food is the last thing on your mind.
2. **Full:** This is a pleasant feeling of satisfaction. You do not have an appetite or feel hungry
3. **Hungry:** a strong wish, urge or desire to eat without any physical signs such as stomach rumbling.
4. **Very Hungry:** this is a very strong form of hunger/appetite where your stomach starts to rumble.

Safety Table.

Please fill in the below table with a number rating how you feel immediately following the consumption of your meal replacement shake and every 30 minutes thereafter until you consume your next meal.

Question: How do you feel?

Time (minutes)	Rating (example)	Rating	Rating
0	1		
30	1		
60 (1 hour)	2		
90	2		
120 (2 hours)	2		
150	2		
180 (3 hours)	3		
210	3		
240 (4 hours)	4		

Troubleshooting Guide

SCENARIO 1:

I've been called out to a lunch meeting or invited out to a restaurant for dinner. How can I stick to my diet plan in this situation?

Solution: This is not an uncommon real world situation for most.

There is no need to be concerned if this should occur. All you need to do is to understand that there is a list of allowable and unallowable foods on your diet. It is no different to having certain likes and dislikes for certain food items.

ACCORDING TO YOUR LIST:

- Palm size serve of protein (Chicken, Fish or beef)
- Low Carbohydrates
- No high fat dressings

HENCE, YOU SHOULD STILL QUITE A BIT TO CHOOSE FROM.

BELOW ARE SOME EXAMPLES:

1. Chicken salad
2. Lamb salad

Basically, anything that contains protein on a bed of salad or vegetables.

It is important that you ask for any fatty items to be served "on the side" as restaurant often add dressings and other fatty items in higher than normal. Also asked for other items such as high fat cheeses, additional carbohydrates (such as croutons) and fatty meats (eg. bacon, salami, sausage) to be either excluded or added as a side dish. If you follow these guidelines you can still eat a meal that conforms to your dietary guidelines whilst enjoying the social outing at the same time.

SCENARIO 2:

You're out and about, perhaps your shopping with friends, or running a handful of errands, nonetheless you're busy and haven't had time to sit down and prepare a meal. What can you do in this situation?

This is another very common real world situation. This situation is similar to scenario 1 in that you just need to understand what foods are allowed and what foods are not allowed. Please refer to your guide for this.

Most fast food chains are located in shopping centers and food halls. Additionally, you will also find independent food outlets in food halls at shopping centers that you can still get a healthy meal at that will also conform to your diet plan.

FOR EXAMPLE:

Subway: Instead of getting a sub that includes a bread roll, you can have a large salad with a portion of protein in the form of chicken or turkey. Be sure not to include any dressing or cheese with the salad because these are high fat items that do not fit your diet plan. Subways are located in most suburbs and shopping centers and are open from early morning until late.

Ali Baba Kebabs: (in most food halls at shopping centers)

Like subway, you can purchase a salad with a portion of protein in the form of lean chicken or lamb. Once again, be sure to ask for no cheese or dressing/topping.

McDonalds, KFC and Red Rooster: your only healthy option at these fast food stores in a large cup of water and even that's questionable. Hence, NOT AN OPTION.

If you're stuck in this situation, call me on 0403 028 535.

SCENARIO 3:

You are out and have either forgotten to take you shake or do not have the sachet with you. What do I do?

Solution: If this should happen, and you felt hungry whilst you were out, you could have a meal instead of the shake. In this situation, you would swap the time that you have your meal replacement shake. For example, if you were meant to have the meal replacement at lunch but were stuck and had to consume a meal then please consume the meal replacement at dinner time. Hence, it's just a straight swap. The meal must conform to the requirements set out in your program. Please refer to your list of allowable and non-allowable foods. Additionally, if you are also stuck with what you can eat in this situation, refer to scenarios 1 and 2 and follow the solution provided in these scenarios. It is IMPORTANT that you note everything in the diary especially situations such as this. It is also important that you stick to your diet plan and do not swap and change the location of the meal replacements or meals unless you are caught in an unavoidable situation.

SCENARIO 4:

You have forgotten to take your supplements or dietary fiber.

What do you do?

Solution: If this should happen, just note it in your diary as "missed supplements" and/or "missed dietary fiber" and wait until you have to take the supplements/dietary fiber again. Please do not take a double dose.

Week 1 Day 1 Date: *Friday 8th May 2009*

Time: <i>6.00am</i>	Dietary Fibre	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Dietary fibre</i>
Time: <i>8.00am</i>	Breakfast: Meal Replacement in 200mL water	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Shake and 200mL water</i>
Time: <i>10.30am</i>	Morning tea	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Small handful of blueberries</i>
Time: <i>12.00 noon</i>	Lunch: Meal Replacement in 200mL water	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Shake and 200mL water</i>
Time: <i>3.00pm</i>	Afternoon tea	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Apple</i>
Time: <i>7.00pm</i>	Dinner: Serving of Protein on Salad or vegetable	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Fish and salad</i>
Time: <i>7.30pm</i>	Desert	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Diet jelly</i>
Bed Time: <i>9.30pm</i>			

Safety Table.

Please fill in the below table with a number rating how you feel immediately following the consumption of your meal replacement shake and every 30 minutes thereafter until you consume your next meal.

Question: How did you feel today?

Time (minutes)	Rating	Rating
0		1
30		1
60 (1 hour)		2
90		2
120 (2 hours)		2
150		3
180 (3 hours)		3
210		3
240 (4 hours)		4

How Are You Feeling?

Mood:	Rating
1 = Extremely Happy, 2 = Happy, 3 = Normal, 4 = Depressed	2
Energy:	Rating
1 = Energetic/Alert, 2 = Normal, 3 = Tired, 4 = Exhausted/Fatigued	2

Exercise Done?

<i>30 min walk</i>
<i>30 min aerobics</i>

Daily Comments:

Week 1 Day 1 Date:

Time:	Dietary Fibre	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:	
Time:	Breakfast: Meal Replacement in 200mL water	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:	
Time:	Morning tea	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:	
Time:	Lunch: Meal Replacement in 200mL water	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:	
Time:	Afternoon tea	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:	
Time:	Dinner: Serving of Protein on Salad or vegetable	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:	
Time:	Desert	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:	
Bed Time:				

Safety Table.

Please fill in the below table with a number rating how you feel immediately following the consumption of your meal replacement shake and every 30 minutes thereafter until you consume your next meal.

Question: How did you feel today?

Time (minutes)	Rating	Rating
0		
30		
60 (1 hour)		
90		
120 (2 hours)		
150		
180 (3 hours)		
210		
240 (4 hours)		

How Are You Feeling?

Mood: 1 = Extremely Happy, 2 = Happy, 3 = Normal, 4 = Depressed

Energy: 1 = Energetic/Alert, 2 = Normal, 3 = Tired, 4 = Exhausted/Fatigued

Exercise Done?

Daily Comments:

Appendix B

Treatment Meal Replacement Diary and Guidebook

Instructions

This food diary is designed to be your companion while you are on the 4 week trial. The diary has been set up for a 4 week period, which is a reasonable time for you to get a greater understanding of, as well as learning how to control, your diet. Importantly, it will help us gain a greater scientific understanding of the 4 week diet program that you have been placed on.

Write everything down: Keep your diary with you all day every day, and write down everything that you eat and drink and at what time you ate or drank these items. Write down everything, even if you happen to slip up and eat the wrong thing – please write it down!

What time? It is very important that you record the time that you eat and drink every item. This will help you/us identify any habits that may be contributing to your weight gain. It will also help you/us understand a lot more about functional components of your diet (e.g. does the food you satisfy your hunger? and if so, for how long?)

Record as you go: Please do not wait until a later time to record what you ate or drank or depend on your memory to write down everything at the end of the day. Record the information as you go.

Be specific: Please include as much detail as possible about what you eat and drink. This includes recording information about extras such as sauces or gravies on pasta and meat dishes. Quite often, it is the hidden extras that overtime results in weight gain (e.g. carbonara sauces, milk in café lattes and so on). Every little bit of information counts.

Record amounts: Please record details about serving size. If you eat a serving of food estimate the size (e.g. palm size serving of protein, 1 medium sized salad) or if possible, include the weight of the item (e.g. 150g chicken breast without skin). With salad items and vegetables, please record the amount (e.g. 1 cup of spinach, 3 sliced mushrooms, ½ cup steamed broccoli, 1 tomato)

Introduction

Hi and welcome to your 4 week weight loss trial. Keeping a diary of everything that you eat and drink is a very important and powerful tool to help you reach your weight loss goals. Additionally, it is a very useful tool in gaining a greater understanding about the functional aspects of your diet. Keeping a diary also tends to make you more accountable and more likely to stay on your program. It's very useful when you are having problems with your diet. You/we can quickly review your diary and program and identify changes that need to be made. So, please use your food diary on a daily basis and keep it with you wherever you go. I wish you every success during your 4 week trial period.

Example of Measurement of Safety Table.

DIET PLAN

- Two meal replacements shakes per day
- Two servings of protein (size of palm)
- MINIMUM of three cups of vegetables including salad vegetables
- Two litres of water – other allowed liquid are in addition to two litres of water
- Dietary fibre – twice per day
- 1 Tablespoon (15mL) of fresh essential oil such as extra virgin olive oil per day on vegetables or salad Oil must be used fresh and not heated.
- Black Coffee only (short and long machiatto allowed): no more than 3-4 cups per day

FOODS TO AVOID

- Grains/cereals/legumes
- Pasta/rice/noodles
- Bread/pastries/biscuits
- Potato, sweet potato, corn
- Chocolate, lollies, ice-cream
- Fruit (berries are allowed), fruit juices

ALLOWABLE EXTRAS

- Mints, or gum – needs to be sugar free with maximum of 3-6 per day
- Milk – half cup low fat or skim milk may be used in hot beverages each day
- Savoury Condiments – may use a small amount of low fat sauces, dressings, gravy or mayonnaise each day. Be conscious of sugar and salt (sodium) content.
- Herbs and spices – use fresh herbs where possible.

Example Daily Meal Plan

MEAL	ON WAKING	
	FIBRE	250ml water + Dietary fibre
1	BREAKFAST 8am	Meal replacement shake 1 with 300ml water 1 glass water Black coffee
2	LUNCH 11.30-12	Salad or Steamed vegetables + Protein With essential Oil 2 glass of water
	FIBRE	250ml water + Dietary fibre
3	ARVO SNACK 2.30-3pm	Meal replacement shake 2 with 300ml water Black coffee
4	DINNER 6.30-7pm	Palm-size serve of protein with Salad Or steamed veg + essential Oil 1 glass of water

NOTE: if you become unbearably hungry at some stage during the day you can have a small snack.
SNACK: small handful of nuts and/or 50-75g of blueberries.

Meal Replacement Product

Directions: To mix the meal replacement product please use the shaker that we provided*.

1. Add the required amount of water to the shaker.
2. Empty the contents of the meal replacement sachet into the shaker.
3. Place the lid on the shaker and shake vigorously until the all the powder is evenly mixed into the water.

4. Serve and drink immediately

* If you have a barmix blender or something similar, you can use this to blend the powder into the water rather than using the shaker

Protein

All serves of protein are 120g for females and 220g for males. This is traditional referred to as a "palm sized" portion of protein

Note: Lean = all fat trimmed where possible and <15% fat in minces

- **Lean Red meat** – Beef (eg: steak, lean mince), Veal, Lamb
- **Lean Pork and game**
- **Poultry** – Chicken and Turkey (Skin removed and breast meat)
- **Fresh fish**
- **Tinned fish** (Salmon or tuna) packed in water (not brine or oil)
- **Seafood** – prawns, oysters and crab (females: 1 cup, males: 1 ½ cups)
- **Eggs (large)** – females: 2 eggs, Males: 2 eggs + ½ listed serve protein
- **Feta Cheese (low fat)** female: 150g, Males 200g
- **Cottage cheese (low fat)** females: 150g, Males: 200g
- **Ricotta Cheese (low fat)** fresh or smooth packed – females: 150g, Males 200g
- **Tofu (Firm)** – Females: 150g, Males: 250g
- **Soy Sausages** – females: 2 sausages, Males 3 sausages

Alfalfa sprouts	Celery	Rocket
Artichoke**	Chives	Shallots
Asparagus	Choko	Silver beet
Avocado*	Cucumber	Snow peas
Bamboo shoots	Eggplant	Spinach
Beans (yellow, green)	Lettuce	Spring onion
Bea sprouts	Endive	Squash
Bok Choy	Fennel	Tomato
Broccoli	Leek	Vegetable greens
Broccolini	Mung bean sprouts	Watercress
Brussel sprouts	Mushroom	Witlof
Cabbage (white/red)	Okra**	Zucchini
Capiscum (red/green, yellow)	Onion	
Cauliflower	Radish	

*Limit to ½ medium or ½ small per day

**Limit to ½ cup

Vegetable	Allowed quantity
Baby corn	½ cup
Beetroot	4 large slices
Carrot	½ medium or 1 small
Celeriac	½ cup, diced
Peas*	½ cup
Pumpkin	½ cup, diced

* Limited to 3 times per week

Oils and Snack Option

OILS

One to two serves per day of one of the below (do not heat);

- **Extra Virgin Olive oil:** 1 tablespoon on salads and vegetables as dressing, or
 - **Medium chain triglycerides (MCT):** 1 tablespoon on salads and vegetables as dressing
- You can find this oil at health food stores

Snack Option (only if needed)

NUTS CHOOSE ONE SERVE (SMALL HANDFUL) PER DAY:

- **Almonds**
- **Macadamias**
- **Walnuts**
- **Pecans**
- **Brazil Nuts**

FRUIT CHOOSE ONE SERVE (SMALL HANDFUL) PER DAY:

(choose frozen variety for convenience)

- **Blueberries**
- **Blackberries**
- **Strawberries**

Beverages

WATER

2 litres of water per day (approx. 8 glasses)

SWEETENED BEVERAGES

Avoid all sweetened beverages such as cordials, soft drinks and juices however you may have the following artificially sweetened beverages as long as they contain less than 1g carbohydrate per 100mL

- *Soft drinks – must be diet. Maximum of 3 cups per day. NOT to replace your water intake*
- *Sparkling mineral water – maximum of 3 cups per day. NOT to replace your water intake*

HOT BEVERAGES

These beverages do NOT contribute to your water intake. You may add a dash of low fat or skim milk (to maximum of half cup per day) and artificial sweeteners may be used in place of sugar or honey.

- *Green Tea – maximum of 5 cups per day*
- *Herbal teas – unlimited amounts and consumption DOES contribute to daily water intake*
- *Decaffeinated tea – unlimited*

ALCOHOL

You may choose an option below twice per week, as a treat option.

- *Wine (dry red or white) – 1 small (120 ml) glass*
- *Spirits – 1 nip/shot (30mL)*
- *Beer (Pure Blonde or Hahn Super Dry low CHO beers) – 1 bottle (330-355ml)*

Measurement of Satiety

Satiety is defined as the condition of being full or gratified beyond the point of satisfaction.

To measure post-meal satiety we will be asking you to note how you feel following your morning meal replacement shake. To do this you will be asked to indicate a change in your perception every 30 minutes over a 3-4 hour period before you consume your next meal.

The usual measurement to determine your perception of food intake is based on a rating system. The rating system that will be use is as follows:

Question: How do you feel? Please circle one of the four selections below.

1. **Very Full**
2. **Full**
3. **Hungry**
4. **Very Hungry**

The above states are defined as follows:

1. **Very Full:** This is state where you feel strong aversion to food. Food is the last thing on your mind.
2. **Full:** This is a pleasant feeling of satisfaction. You do not have an appetite or feel hungry
3. **Hungry:** a strong wish, urge or desire to eat without any physical signs such as stomach rumbling.
4. **Very Hungry:** this is a very strong form of hunger/appetite where your stomach starts to rumble.

Safety Table.

Please fill in the below table with a number rating how you feel immediately following the consumption of your meal replacement shake and every 30 minutes thereafter until you consume your next meal.

Question: How do you feel?

Time (minutes)	Rating (example)	Rating	Rating
0	1		
30	1		
60 (1 hour)	2		
90	2		
120 (2 hours)	2		
150	2		
180 (3 hours)	3		
210	3		
240 (4 hours)	4		

Troubleshooting Guide

SCENARIO 1:

I've been called out to a lunch meeting or invited out to a restaurant for dinner. How can I stick to my diet plan in this situation?

Solution: This is not an uncommon real world situation for most.

There is no need to be concerned if this should occur. All you need to do is to understand that there is a list of allowable and unallowable foods on your diet. It is no different to having certain likes and dislikes for certain food items.

ACCORDING TO YOUR LIST:

- *Palm size serve of protein (Chicken, Fish or beef)*
- *Low Carbohydrates*
- *No high fat dressings*

HENCE, YOU SHOULD STILL CHOOSE A BIT TO CHOOSE FROM.

BELOW ARE SOME EXAMPLES:

1. *Chicken salad*
2. *Lamb salad*

Basically, anything that contains protein on a bed of salad or vegetables. It is important that you ask for any fatty items to be served "on the side" as restaurant often add dressings and other fatty items in higher than normal. Also asked for other items such as high fat cheeses, additional carbohydrates (such as croutons) and fatty meats (eg. bacon, salami, sausage) to be either excluded or added as a side dish. If you follow these guidelines you can still eat a meal that conforms to your dietary guidelines whilst enjoying the social outing at the same time.

SCENARIO 2:

You're out and about, perhaps your shopping with friends, or running a handful of errands, nonetheless you're busy and haven't had time to sit down and prepare a meal. What can you do in this situation?

This is another very common real world situation. This situation is similar to scenario 1 in that you just need to understand what foods are allowed and what foods are not allowed. Please refer to your guide for this.

Most fast food chains are located in shopping centers and food halls. Additionally, you will also find independent food outlets in food halls at shopping centers that you can still get a healthy meal at that will also conform to your diet plan.

FOR EXAMPLE:

Subway: Instead of getting a sub that includes a bread roll, you can have a large salad with a portion of protein in the form of chicken or turkey. Be sure not to include any dressing or cheese with the salad because these are high fat items that do not fit your diet plan. Subways are located in most suburbs and shopping centers and are open from early morning until late.

All Baba Kebabs: (in most food halls at shopping centers)

Like subway, you can purchase a salad with a portion of protein in the form of lean chicken or lamb. Once again, be sure to ask for no cheese or dressing/topping.

McDonalds, KFC and Red Rooster: your only healthy option at these fast food stores in a large cup of water and even that's questionable. Hence, NOT AN OPTION.

If you're stuck in this situation, call me on 0403 028 535.

SCENARIO 3:

You are out and have either forgotten to take you shake or do not have the sachet with you. What do I do?

Solution: If this should happen, and you felt hungry whilst you were out, you could have a meal instead of the shake. In this situation, you would swap the time that you have your meal replacement shake. For example, if you were meant to have the meal replacement at lunch but were stuck and had to consume a meal then please consume the meal replacement at dinner time. Hence, it's just a straight swap. The meal must conform to the requirements set out in your program. Please refer to your list of allowable and non-allowable foods. Additionally, if you are also stuck with what you can eat in this situation, refer to scenarios 1 and 2 and follow the solution provided in these scenarios. It is IMPORTANT that you note everything in the diary especially situations such as this. It is also important that you stick to your diet plan and do not swap and change the location of the meal replacements or meals unless you are caught in an unavoidable situation.

SCENARIO 4:

You have forgotten to take your supplements or dietary fiber. What do you do?

Solution: if this should happen, just note it in your diary as "missed supplements" and/or "missed dietary fiber" and wait until you have to take the supplements/dietary fiber again. Please do not take a double dose.

Week 1 Day 1 Date:

Time: <i>6:00am</i>	Dietary Fibre	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Dietary fibre</i>
Time: <i>8:00am</i>	Breakfast: Meal Replacement In 300mL water. Take your dose of vitamins	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Shake in 300mL water plus vitamins</i>
Time: <i>10:30am</i>	Lunch: Serving of Protein on Salad or vegetables	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Small handful of blueberries</i>
Time: <i>12:30 noon</i>	Dietary Fibre: This is taken 30-60 minutes before meal replacement like the morning shake	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Chicken & Salad Essential oil</i>
Time: <i>2:00pm</i>	Afternoon tea: Meal Replacement In 300mL water. Take your dose of vitamins	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Dietary fibre</i>
Time: <i>3:00pm</i>	Snack option: (only if needed)	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Shake in 300mL water plus vitamins</i>
Time: <i>7:00pm</i>	Dinner: Serving of Protein on Salad or vegetables	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown: <i>Fish and salad</i>
Bed Time: <i>9:30pm</i>			

Safety Table.

Please fill in the below table with a number rating how you feel immediately following the consumption of your meal replacement shake and every 30 minutes thereafter until you consume your next meal.

Question: How did you feel today?

Time (minutes)	Rating	Rating
0	1	1
30	1	1
60 (1 hour)	2	2
90	2	2
120 (2 hours)	2	2
150	3	3
180 (3 hours)	3	3
210	3	3
240 (4 hours)	4	4

How Are You Feeling?

Mood: 1 = Extremely Happy, 2 = Happy, 3 = Normal, 4 = Depressed

Energy: 1 = Energetic/alert, 2 = Normal, 3 = Tired, 4 = Exhausted/fatigued

2

2

Exercise Done?

30 min walk

30 min aerobics

Daily Comments:

Week 1 Day 1 Date:

Time:	Dietary Fibre	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:
Time:	Breakfast: Meal Replacement in 300mL water. Take your dose of vitamins	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:
Time:	Lunch: Serving of Protein on Salad or vegetables	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:
Time:	Dietary Fibre: This is taken 30-60 minutes before meal replacement like the morning shake	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:
Time:	Afternoon tea: Meal Replacement in 300mL water. Take your dose of vitamins	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:
Time:	Snack option: (only if needed)	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:
Time:	Dinner: Serving of Protein on Salad or vegetables	<input type="checkbox"/> YES <input type="checkbox"/> NO	Meal Breakdown:
Bed Time:			

Safety Table.

Please fill in the below table with a number rating from you feel immediately following the consumption of your meal replacement shake and every 30 minutes thereafter until you consume your next meal.

Question: How did you feel today?

Time (minutes)	Rating	Rating
0		
30		
60 (1 hour)		
90		
120 (2 hours)		
150		
180 (3 hours)		
210		
240 (4 hours)		

How Are You Feeling?

How Are You Feeling?	Rating
Mood: 1 = Extremely Happy, 2 = Happy, 3 = Normal, 4 = Depressed	
Energy: 1 = Energetic/Alert, 2 = Normal, 3 = Tired, 4 = Exhausted/Fatigued	

Exercise Done?

Daily Comments:

