THE EFFECT OF EXERCISE ON OXIDATIVE STRESS AND OTHER HEALTH MARKERS:
EXPLORING NEW TECHNOLOGY AND METHODOLOGY

by

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ABSTRACT

Physical activity is beneficial for health; physical activity has been linked to reduced risk of diseases associated with ageing and reduced risk of mortality. Whereas, oxidative stress is seen with ageing and diseases associated with ageing. Taking part in regular physical activity leads to adaptive response that enables the body's antioxidant defence to be better equipped to fight against oxidative stress, and indeed exercise training leads to lower level of oxidative stress. Exercise intensity seems to be one of key factors that determines the effectiveness of exercise.

This thesis contributes to the existing literature by being the first to investigate the effect of exercise on a marker of oxidative stress in the brain, an organ that becomes impaired (including oxidative damage) with ageing and diseases associated with ageing. The finding from this thesis suggests that brain glutathione (GSH) as measured by magnetic resonance spectroscopy (MRS) decreases following an acute bout of exercise in sedentary young men. In the periphery, high intensity interval exercise (HII) involving very high intensity intervals was found to elicit a greater oxidative stress response in comparison to continuous moderate intensity exercise. An unsupervised form of home-based exercise intervention in older adults was not successful in increasing physical activity level, which was objectively measured using an activity monitor. Thus, as would have been potentially expected, there was no improvement in markers of oxidative stress and other health markers in response to the exercise intervention.

The work presented in this thesis used novel approaches, through the application of emerging technologies, to study physical activity and its effects. Brain GSH was altered in response to exercise, in an exercise intensity dependent manner, and the brain seems to be protected

against hyperperfusion injury during the high intensity phase of the HII exercise. Observed changes in peripheral markers of oxidative stress (GSH, total antioxidant capacity and malondialdehyde) were also exercise intensity dependent. Objectively measured physical activity levels were not significantly increased by an unsupervised exercise intervention, potentially due to a lack of progressive goals based on adherence to physical activity.

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This thesis is dedicated to

my dad, Ratna Rai

and

my mom, Tasbira Rai.

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List of abbreviations

µl	Micro litre/s
ANOVA	Analysis of variance
AU	Arbitrary units
BSA	Bovine serum albumin
CBF	Cerebral blood flow
CSF	Cerebrospinal fluid
CV	Coefficient of variation
ELISA	Enzyme linked immunosorbent assay
FRAP	Ferric reducing ability of plasma
GM	Grey matter
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HII	High intensity interval exercise
H_2O_2	Hydrogen peroxide
HR	Heart rate
MEGA-PRESS	MEshcher-Garwood Point RESolved Spectroscopy
MCAv	Middle cerebral artery velocity

MDA	Malondialdehyde
MRS	Magnetic resonance spectroscopy
NAA	N-acetyl aspartate
ppm	Parts per million
PWD	People with dementia
ROS	Reactive oxygen species
RONS	Reactive oxygen and nitrogen species
TCD	Transcranial Doppler
$\dot{V}O_2max$	Maximal oxygen consumption
VOI	Volume of interest
WM	White matter

1 CHAPTER 1: General Introduction

1.1 SUMMARY

Oxidative stress is a state that can exist within a cell, tissue or organism, which describes an imbalance between oxidants and antioxidants. If the imbalance is severe enough cell damage can result. Oxidative stress has been implicated in ageing and with diseases associated with ageing, however oxidative stress can be perturbed by lifestyle. Regular exercise leads to many health benefits including reducing the risk for diseases, and some of these benefits have been associated with reduced oxidative stress. It is now generally accepted that unaccustomed exercise can result in oxidative stress, while regular physical activity leads to an adaptive response to resist against exercise or non-exercise induced oxidative stress. The level of oxidative stress in response to exercise is dependent on many factors, of which exercise intensity is one. Blood/plasma and muscle are the most frequently sampled tissues in studies that assess exercise induced oxidative stress. No previous human studies have looked at the effect of exercise-induced oxidative stress in the brain, an organ that becomes impaired with ageing and with diseases associated with ageing. Perhaps one contributing factor to decreased health with age is physical inactivity and it is well known that levels of physical activity reduce with age. Very few studies use objective measures to assess physical activity in free living conditions and most of these studies are cross-sectional in design and thus, there is a lack of information on the quality of physical activity in free-living older adults. This thesis comprises studies in which methods to monitor oxidative stress in the brain in response to exercise in sedentary young adults are developed, and technology to monitor physical activity in older adults is explored.

1.2 OXIDATIVE STRESS

1.2.1 Free radicals and oxidative stress

Free radicals are molecules with an unpaired electron in their outer orbit. Free radicals, first described by Moses Gomberg in 1900 (Gomberg, 1900), initially were not considered to be present in living organisms due to their high reactivity and short life span (Lushchak, 2014). In 1954, Commoner et al. (1954) provided the first proof that free radicals exist in living organisms. Following this, it was assumed that free radicals played deleterious role in biological systems, until the 1970s when studies reporting their important role in many functions of living organisms started to emerge (Babior et al., 1973, Furchgott and Zawadzki, 1980).

Most of the free radicals that are found in living organisms either are, or originate from, reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Cooper et al., 2002, Fisher-Wellman and Bloomer, 2009). ROS includes oxygen based radicals (such as superoxide, hydroxyl, alkoxyl, peroxyl and hydroperoxyl) as well as some derivatives of oxygen that do not contain unpaired electrons (such as hydrogen peroxide and lipid peroxides). RNS includes nitric oxide and derivatives of nitric oxide such as peroxynitrite. ROS and RNS are collectively referred to as reactive oxygen and nitrogen species (RONS).

The human body produces RONS as by-products of numerous physiological and biochemical processes, but primarily as a result of aerobic metabolism (Uttara et al., 2009). Oxygen is necessary for the life of aerobic organisms and acts as a terminal oxidant in the mitochondrial respiratory chain (Figure 1-1). In the mitochondrial electron transport chain, majority of the consumed oxygen (about 95%) undergoes the cytochrome oxidase-catalysed tetravalent

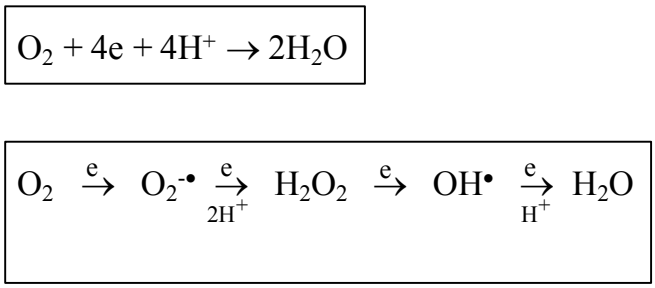


Figure 1-1Schematic representation of the tetravalent and monovalent reduction of molecular oxygen to water.

Top: The tetravalent reduction of molecular oxygen to water occurs efficiently during mitochondrial respiration. **Bottom:** Alternatively, monovalent reduction can occur which produces reactive intermediates such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$).

reduction to water (Comporti et al., 2008). However, a portion of oxygen can be reduced via a univalent pathway even under normal conditions, leading to the formation of reactive intermediates such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). RONS plays a crucial role in normal functioning, as RONS are involved in many functional activities of cells such as control of cell signalling pathways and regulation of gene expression (Powers et al., 2011, Uttara et al., 2009). However, overproduction of free radicals can cause oxidative damage to biomolecules which, if severe, can be toxic and may lead to cell death. The three main classes of biological macromolecules susceptible to free radical attack are lipids, proteins and nucleic acids (Beckman and Ames, 1998). An elaborate system of antioxidant defence is in place as a counterbalance to the reactivity of RONS. This finely regulated antioxidant system maintains a well-balanced production and elimination of RONS resulting in a steady-state RONS level within the cell. Oxidative stress (a concept introduced in 1980s (Sies, 1985)) is a state where there is an imbalance between antioxidants and oxidants in favour of the oxidants, leading to a disruption of redox signalling and/or molecular damage. This imbalance can be an effect of increased RONS production and/or depletion of antioxidants including inactivation of antioxidant enzymes.

Oxidative stress results in a wide spectrum of genetic, metabolic, and cellular responses (Davies, 2000). Oxidative stress may modulate gene expression and regulate cell signalling pathways, or it may stimulate cell growth or cause a protective temporary growth-arrest of cells (Davies, 2000). All of these effects could be regarded as beneficial, or at the very least non-detrimental. However, in certain circumstances oxidative stress can cause necrosis or cell death. Lushchak (2014) proposed a theoretical classification of oxidative stress according to its intensity as basal, low intensity, intermediate intensity and high intensity oxidative stress. At basal level, the effects of oxidative stress are so negligible that they cannot be observed by

methods routinely applied. It could be therefore suggested in this case that RONS and antioxidants are in balance. At low intensity oxidative stress, there is an adaptive response, suggesting RONS are able to overwhelm antioxidants to signal, but are not damaging at this level. At intermediate intensity, there is a combined response of adaption vs damage to cellular components, whereas, high intensity involves necrosis and apoptosis. This theoretical classification bears a striking similarity to the free radical hormesis theory, proposed by Radak et al. (2005). This theory is presented in section 1.5.2.1.

1.3 OXIDATIVE STRESS IN AGEING AND DISEASE

Ageing can be defined as the progressive accumulation of diverse, deleterious changes with time that increase the risk of disease and death (Harman, 2006). One of the most plausible and acceptable explanations for the mechanistic basis of ageing is the "free radical theory of ageing" which postulates that ageing is the sum of the free radical damage associated with suboptimal living condition plus that produced by the inborn ageing process (Harman, 2006). Oxidative stress is found to a limited extent in healthy ageing, and to a greater extent in diseases associated with ageing such as mild cognitive impairment and dementia (age is the greatest risk factor for a number of neurodegenerative diseases) (Mariani et al., 2005). Chronic oxidative stress has been suggested to play a primary or secondary role in the development of multiple acute and chronic human diseases (Dalle-Donne et al., 2006, Fisher-Wellman and Bloomer, 2009). Oxidative stress has been implicated in many degenerative diseases such as cancer, diabetes and neurodegenerative diseases (Barnham et al., 2004, Beckman and Ames, 1998) such as dementia and Alzheimer's disease. Decline in the antioxidant glutathione (GSH) levels and GSH metabolism has been linked to diseases associated with ageing (Lang et al., 1992, Maher, 2005, Puertas et al., 2012).

1.3.1 Neurodegenerative disease and oxidative stress

Several neurodegenerative diseases are associated with oxidative stress, and oxidative stress is thought to play an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease and multiple sclerosis (Butterfield et al., 2002). Alterations in glutathione homeostasis and glutathione-dependent enzymes have been implicated in the induction and progression of neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (Johnson et al., 2012). There is a large amount of compelling data demonstrating increased oxidative stress in Alzheimer's disease (the most common form of dementia) (Nunomura et al., 2001, Zhu et al., 2007, Bennett et al., 2009, Bonda et al., 2010, Sultana and Butterfield, 2010), with oxidative stress seen early in the disease (Nunomura et al., 2001). In fact, an increased level of oxidative stress is also seen in individuals with mild cognitive impairments, a clinical population who are at the highest risk to progress to Alzheimer's disease (Pratico et al., 2002, Sultana and Butterfield, 2010). Increased oxidative stress is an early event in AD, with studies showing that oxidative stress is detected prior to Aβ deposition and NFT Increased oxidative stress and decreased antioxidant capacity has been reported in AD patients compared to age matched controls (Aldred et al., 2010, Montine et al., 2002), whereas increased antioxidant levels have been linked to reduced risk of Alzheimer's disease (Mangialasche et al., 2010).

Brain impairments occur in neurodegenerative diseases associated with ageing such as dementia (Alzheimer's disease (most common form), Parkinson's disease). Indeed, similarly to the periphery (Lang et al., 1992, Puertas et al., 2012), lower GSH levels have been reported in the brain in these conditions (Gu et al., 1998, Mandal et al., 2012, Sofic et al., 1992) as well as in apparently healthy older adults (Emir et al., 2011).

1.4 ASSESSMENT OF OXDIATIVE STRESS

Studies generally report the presence of oxidative stress when one or more (ideally several) parameters reflecting disturbance in the redox balance are observed. Commonly used markers of oxidative stress include oxidatively modified lipids, proteins and nucleic acids and the level and/or activities of antioxidants such as GSH, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Lushchak, 2014). Using the activities of antioxidant enzymes as markers of oxidative stress is challenging due to the complexity of their response– depending on the intensity of oxidative stress, the activities of antioxidant enzymes may increase, decrease or remain unchanged. In accordance with the theory suggested by Luschak and others (Lushchak, 2014, Radak et al., 2005) enzymes are usually up-regulated at low RONS concentrations as an adaptive response and are decreased at higher RONS concentrations as they become depleted. An increase in RONS production on its own is also not a definitive marker of oxidative stress as an increase in RONS production does not necessarily define a pro-oxidant condition (Powers and Jackson, 2008). Therefore an ideal assessment of oxidative stress would include measurement of several oxidatively modified macromolecules along with assessment of antioxidant system (Lushchak, 2014).

1.4.1 Protein Oxidation

Proteins are major targets for RONS because of their high abundance in biological systems and indeed proteins scavenge the majority (50-75%) of produced RONS (Dalle-Donne et al., 2006). Modification of proteins by RONS leads to the formation of carbonyl groups into amino acid side chains. Most protein damage is irreparable and oxidative modification of the protein structure can lead to loss of structural function in the affected proteins (Levine and Stadtman, 2001). Protein carbonyls are one of the most stable of all of the adducts formed by interaction of RONS and biomolecules (Dalle-Donne et al., 2003). Carbonyl groups can be

measured by HPLC or ELISA procedures. Other commonly used measures for assessment of oxidised proteins include individual oxidised amino acids and nitrotyrosine.

1.4.2 Lipid Oxidation

Unsaturated lipids are particularly susceptible to oxidative modification. RONS can attack polyunsaturated fatty acids in the cell membrane leading to a chain of chemical reactions called lipid peroxidation. The process of lipid peroxidation is initiated by RONS attack on the double bond of unsaturated fatty acids which generates highly reactive lipid peroxy radicals which leads to a chain reaction of further attacks on other unsaturated fatty acids (Figure 1-2) (Barnham et al., 2004, Davies, 2000). Lipid peroxidation leads to the breakdown of lipids and the formation of various primary oxidation products such as conjugated dienes and lipid hydroperoxides, and secondary oxidation products such as malondialdehyde (MDA) and F2-isoprostanes. Lipid peroxidation is commonly assessed by measurement of thiobarbituric acid reactive substances (TBARS) as a measure of MDA, although this reaction is rather non-specific for MDA and measures many parameters in addition to lipid peroxidation (Janero, 1990). MDA can be measured by HPLC, spectrophotometry or spectrofluorescence (Urso and Clarkson, 2003). Other frequently used measures of lipid peroxidation includes assessment of end products such as isoprostanes, lipid hydroperoxides, conjugated dienes and oxidised low density lipoprotein. Assessment of F_2-isoprostanes is regarded as one the most reliable measure of lipid peroxidation (Dalle-Donne et al., 2006).

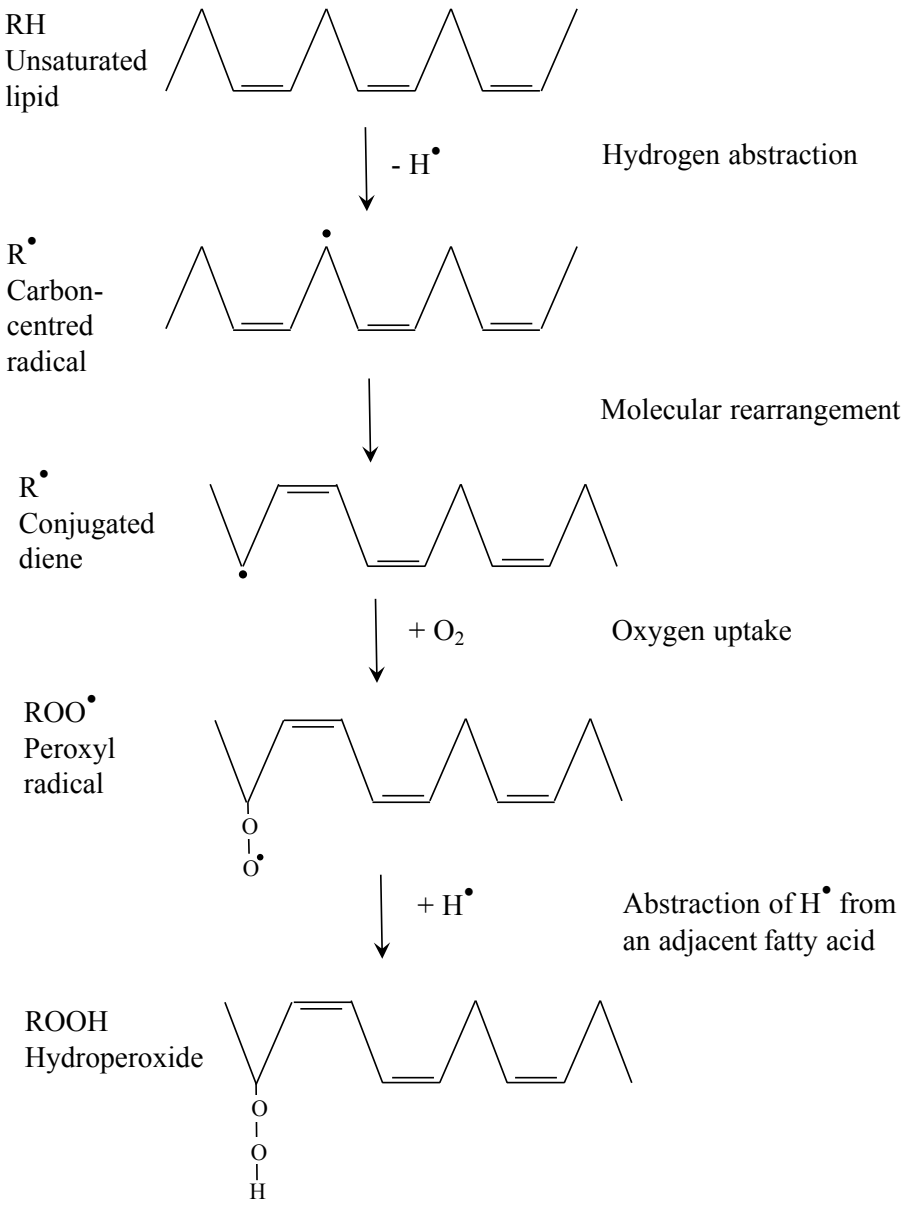


Figure 1-2 Mechanism of lipid peroxidation.

Figure adapted from (Gutteridge, 1995). The process of lipid peroxidation is initiated by RONS attack on the double bond of unsaturated fatty acids and once the process is initiated, it proceeds as a RONS-mediated chain reaction involving initiation, propagation and termination.

1.4.3 DNA oxidation

RONS can cause oxidative damage to both nuclear and mitochondrial DNA. Increased oxidation of DNA by RONS, especially by the hydroxyl radical, has been implicated in several neurodegenerative diseases (Lovell, Gabbita and Markesbery, 1999, Murata, Ohtsuka and Terayama, 2008, Zhang et al., 1999). Hydroxyl radicals can react with DNA in one of two ways i) by an addition reaction- where the hydroxyl radical binds to the DNA bases and ii) by directly removing a hydrogen atom from DNA resulting in DNA cleavage (Kawanishi, Hiraku and Oikawa, 2001, Cooke et al., 2003). Out of the four DNA bases, guanine is the most vulnerable to oxidation due to its low oxidation potential (Kawanishi et al., 2001). Guanine oxidation produces 8-hydroxy-2-deoxyguanosine (8-OHdG), which is the most common marker used to assess DNA modification (Finaud et al., 2006, Cooke et al., 2006). The oxidised nucleotide, 8-OHdG, is excreted via blood and urine (Finaud et al., 2006). Unlike oxidatively modified lipids and proteins which may be removed via normal turnover of the respective molecules, damage to DNA must be repaired (for example DNA repair by base excision repair enzymes) (Evans et al., 2004). Methods for the assessment of oxidative damage of DNA include HPLC, mass spectrometric methods and comet assay in urine and blood samples (Cooke et al., 2006).

1.4.4 Antioxidant defences

Human beings have developed a highly complex system of antioxidant defence, which work synergistically and in combination with each other, to protect biomolecules against damage from RONS. The antioxidant system includes endogenous (enzymatic and non-enzymatic) antioxidants such as GSH, SOD, GPx and catalase (CAT), as well as exogenous antioxidants (the diet being the main source) such as vitamin C, vitamin E, carotenoids and polyphenols. The most well studied, and perhaps most well-known non-enzymic antioxidants include

glutathione (GSH), vitamin C and vitamin E. The endogenous and exogenous antioxidants act interactively to maintain redox balance.

1.4.4.1 Glutathione

GSH, the most abundant antioxidant in the body, is a ubiquitous molecule present in almost all mammalian tissues, and is produced in all organs (the highest being in the liver). Its intracellular concentrations usually range from 1 to 10 mM and extracellular concentrations (such as in plasma) range from 2-20 μM (Giustarini et al., 2013). Apart from its role in antioxidant defences, GSH is involved in a multitude of cellular functions such as protein synthesis.

Figure 1-3 shows GSH redox cycle. GSH provides a first line of defence against ROS- it can scavenge free radicals and reduce hydrogen peroxide (H_2O_2). GSH reduces H_2O_2 using the enzyme GPx, during which GSH itself is oxidised to glutathione disulfide (GSSG). Glutathione reductase (GR) reduces GSSG back to GSH using an electron from NADPH. In order to maintain a constant intracellular GSH concentration, consumed GSH has to be replaced by resynthesis from its constituent amino acids.

Despite the substantial interest in the measurement of GSH and GSSG in blood, there is currently no 'gold standard' protocol for sample preparation and analysis in biological samples (Giustarini et al., 2013). In blood, GSH should be ideally measured in whole blood as GSH is mostly present inside the cell, and thus the concentration of GSH in plasma samples is very low. Methods used to measure GSH in samples include spectrophotometric

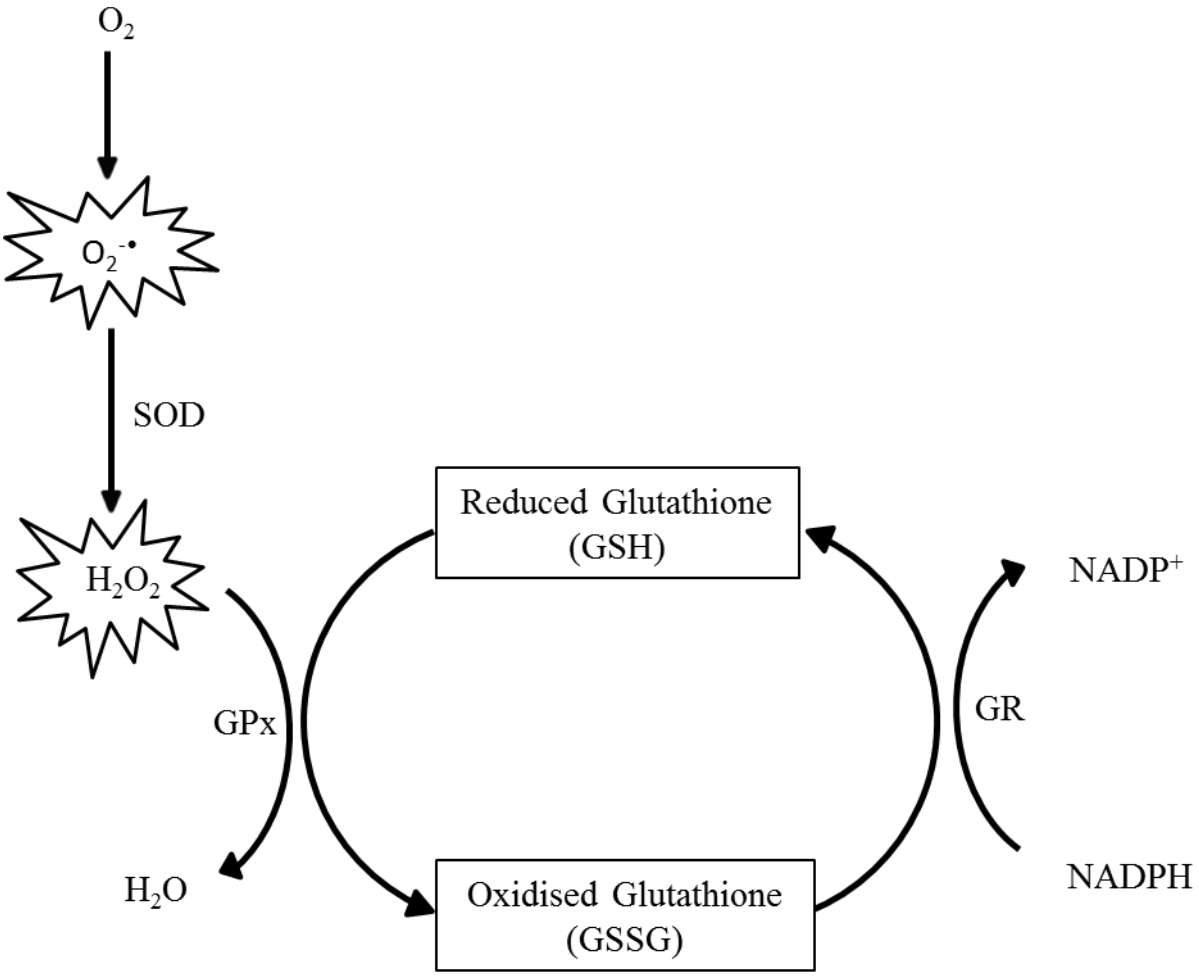


Figure 1-3 Glutathione redox cycle

Superoxide (O2-•) is converted into hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD). H_2O_2 is reduced to water (H_2O) catalysed by glutathione peroxidase (GPx), while glutathione (GSH) is oxidised to glutathione disulfide (GSSG). Gluathione reductase (GR) reduces GSSG into 2GSH using electrons from the oxidation of NADPH.

(Tietze, 1969), fluorometric and bioluminometric assays. One of the main concerns during sample manipulation is the auto-oxidation of thiols which may lead to overestimation of disulphides (i.e. GSH or GSSG). To prevent this, various agents such as N-ethylmaleimide (NEM) and 2-vinyl pyridine are used to block thiol groups. Only methods such as NMR are suitable for measuring GSH in intact cells or non-deprotenised samples. Nuclear magnetic resonance spectroscopy (MRS) is the only method that allows non-invasive *in vivo* assessment of GSH. The detection of GSH using MRS in the human brain *in vivo* was first described by Trabesinger et al. (1999) and this technique will be introduced in more detail in chapter 3 of this thesis.

1.4.4.2 Total antioxidant capacity

Total antioxidant capacity can be used as an indicator of oxidative stress. Methods to assess antioxidant capacity typically involve a tissue or a blood sample being added to a chemical free radical generation system *in vitro* and then the measurement of the ability of the sample to resist oxidative stress (e.g. resistance to lipid peroxidation) (Urso and Clarkson, 2003). Total antioxidant capacity assays include Trolox Equivalent Antioxidant Capacity (TEAC), Total Antioxidant Status (TAS), and Ferric Reducing Ability of Plasma (FRAP).

1.4.4.3 Antioxidant enzymes

Assessment of antioxidant enzyme activity is also used as a marker of oxidative stress; the most commonly enzymes assessed being SOD, GPx, GR and catalase. Ideally, assessment of antioxidant enzyme activity should be carried out in cell samples, as they are active inside the cell.

1.4.5 Measurement of free radicals

The methods, in order to assess oxidative stress, mentioned so far in this section 1.4 are all indirect measures of interaction of free radical (RONS) with biomolecules. Measuring free

radicals directly is difficult, primarily due to their short lifetime. Additionally, they are highly reactive and occur at low concentrations in living systems. A further limitation is that the probes or spin traps that are used to measure free radicals may perturb the biological system being investigated (Powers and Jackson, 2008). The most commonly used method to directly assess free radical production is electron spin resonance. Other less common methods include radiolysis and laser flashphotolysis.

1.5 CHANGES IN OXIDATIVE STRESS MARKERS IN RESPONSE TO EXERCISE

1.5.1 Overview

Dillard et al. (1978), in 1978, were the first to report that exercise can result in increased lipid peroxidation in humans, and a study by Davies et al. (1982) in rats provided the first evidence that skeletal muscle produces RONS and exercise can induce tissue damage as measured by marker of lipid peroxidation. Since then, an accumulating body of evidence supports the hypothesis that physical exercise has the potential to increase RONS production which can result in oxidative stress (Fisher-Wellman and Bloomer, 2009). In response to intense exercise, the body's antioxidant system may not be able to effectively remove the excess RONS produced (Kerksick and Willoughby, 2005). A single bout of exercise, dependent on factors including exercise intensity and duration, and fitness of the individual, will increase RONS production and stimulate adaptive changes, which if repeated via regular bouts of exercise can lead to decreased markers of oxidative damage and increased antioxidant capacity, both at rest and in response to physical activity (Camiletti-Moiron et al., 2013, Finaud et al., 2006, Radak et al., 2008, Radak et al., 2001a). Studies that investigate oxidative stress in response to exercise in humans have mostly assessed oxidative stress markers in blood and urine, and fewer studies have examined muscle tissue (Urso and Clarkson, 2003).

1.5.2 Oxidative stress markers in response to acute exercise

A single bout of unaccustomed exercise can elicit a transient increase in markers of oxidative stress, dependent on factors such as exercise intensity, exercise duration and participant's characteristics (Fisher-Wellman and Bloomer, 2009). Various studies in sedentary humans have shown that an acute bout of exercise can change markers of oxidative stress including protein carbonyls and lipid hydroperoxides in blood/plasma (Bloomer et al., 2007, Michailidis et al., 2007, Steinberg et al., 2007, Wadley et al., 2016a).

Studies in humans have shown an acute bout of exercise can temporarily decrease GSH in blood immediately post exercise which then returns back to baseline level (Elokda et al., 2010, Elokda et al., 2005, Laaksonen et al., 1999, Steinberg et al., 2007). Elokda et al. (2005) found that blood GSH decreased immediately after acute exercise in sedentary participants which then returned back to baseline level. Acute exercise has also shown to decrease GSH post-exercise in older adults as well as older adults with Parkinson's disease (Elokda et al., 2010). To assess exercise-induced oxidative stress by measuring antioxidant response on its own is challenging because exercise can result in decrease in antioxidants but the influx of antioxidants from secondary sources elsewhere in the body can result in transient increase. (Cooper et al., 2002). Changes in antioxidant levels could mean impaired antioxidant defences, but they do not necessarily indicate tissue damage (Cooper et al., 2002).

Exercise mode, intensity, and duration as well as participant's training status, age, dietary intake and gender, all can impact the extent of exercise-induced oxidative stress (Cooper et al., 2002, Fisher-Wellman and Bloomer, 2009). The differences across studies in the detected markers of oxidative stress in response to exercise therefore could be due these factors such as the type of exercise and the training status of the participants. Indeed, accumulating evidence seems to suggest that exercise intensity is a determinant of the magnitude of RONS formation

and thus the magnitude of oxidative stress in response to a single bout of exercise (Bailey et al., 2004, Goto et al., 2007, Lamprecht et al., 2008, Lovlin et al., 1987, Quindry et al., 2003, Wang and Huang, 2005). For instance, in a study of sedentary men assessing the response of acute bout of moderate or high intensity exercise (of the same duration) in blood lymphocytes reported greater decreases and increases in GSH level and lipid peroxidation respectively in response to high intensity exercise compared to moderate exercise (Wang and Huang, 2005).

One form of exercise that involves high exercise intensity is high intensity interval exercise (HII), which is gathering considerable interest as an alternative exercise method to improve health. One of the reasons for the emerging interest in this time-efficient form of exercise is to counter the reported perceived "lack of time" associated with traditional exercise guidelines (Gibala et al., 2012). There are various forms of HII, but generally it is comprised of repeated bouts of brief intermittent exercise of high intensity of ~85% to 95% or beyond maximal aerobic capacity (Lucas et al., 2015). Emerging evidence suggests that, compared to the traditional continuous moderate-intensity exercise, HII provides equivalent if not superior benefits for metabolic, cardiac and vascular adaptations (Lucas et al., 2015). A study carried previously in our lab, found that HII elicited oxidative stress response similar to those by both high-intensity and moderate-intensity exercise (Wadley et al., 2016a). This thesis examined HII further, by using a higher volume HII compared to the low volume HII used by Wadley et al. (2016a).

Timing of sampling should also be considered, as an increase in oxidative stress markers in blood and skeletal muscle may persist for and/or appear several days after exercise (Michailidis et al., 2007, Nikolaidis et al., 2008, Vollaard et al., 2005). Other factors that could impact the observed oxidative stress include the sensitivity and specificity of the biomarker used, and the type of tissue sampled (for example blood vs muscle sample) (Fisher-

Wellman and Bloomer, 2009), however the finding by Veskoukis et al. (2009) (in an animal model) suggests that markers of oxidative stress in blood, skeletal muscle, heart and liver are correlated. Nevertheless, markers of oxidative stress, as assessed by increased damage to lipids and proteins along with changes in antioxidant systems, in response to exercise have been well documented (Cooper et al., 2002).

1.5.2.1 Hormesis

The hormesis hypothesis suggests that biological systems respond positively to chemicals, toxins and radiation until a threshold is reached, whereby the system breaks down. Radak et al. (2005) extended the hormesis theory to the free radical generating effects of exercise, encompassing a low-dose stimulation or beneficial effect through to a high-dose inhibitory or toxic effect. The hormesis theory applied to exercise encompassing a bell shaped curve is shown in Figure 1-4. Production of RONS above the optimal threshold (which is currently an undefined level) may serve to overwhelm antioxidant defences, and thus may result in extensive oxidative damage (Fisher-Wellman and Bloomer, 2009).

1.5.3 Possible mechanisms of RONS production during exercise

There are numerous potential sites at cellular level and mechanism for RONS production in response to exercise include mitochondria, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, PLA_2 (phospholipase A_2)-dependent processes, and xanthine oxidase (as shown in Figure 1-5); although at present the precise role of each of these in exercise induced RONS production remains unclear (Powers and Jackson, 2008, Powers et al., 2011, Vollaard et al., 2005). Primary ROS generation in response to acute exercise can occur via mitochondrial respiration, and oxidase enzymatic activity (such as NADPH oxidase and xanthine oxidase) (Fisher-Wellman and Bloomer, 2009).

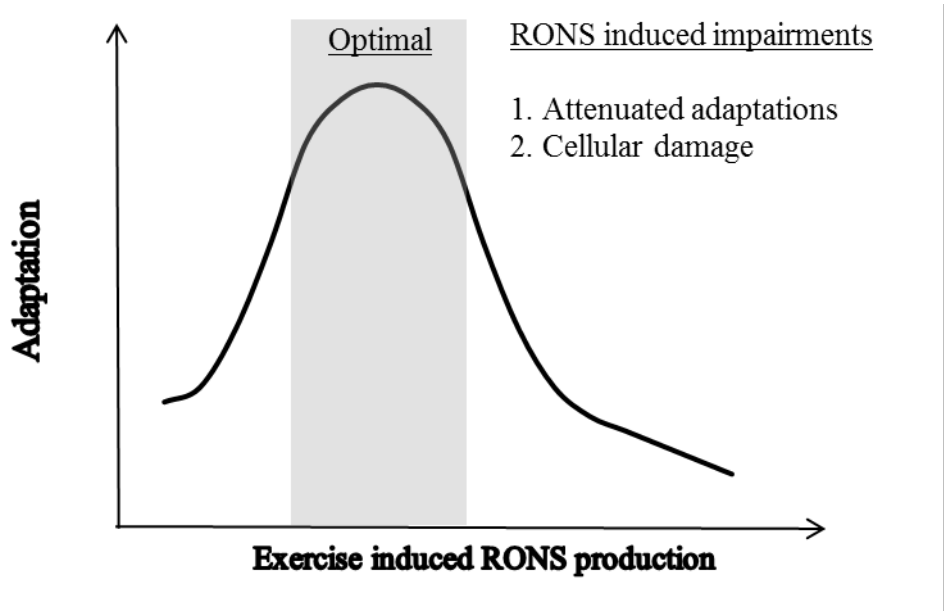


Figure 1-4 Hormesis theory to free radical generating effect of exercise

The theoretical model which extends the hormesis theory to the RONS generating effects of exercise, encompassing a bell shaped curve with a low-dose stimulation or beneficial effect through to a high-dose inhibitory or toxic effect.

Mitochondria are considered the main source of RONS during normal respiration. During aerobic respiration in the mitochondria (in order to produce energy in the form of ATP), oxygen (O_2) is reduced to water (H_2O). However, it seems that mitochondria electron transport is not perfect, and one-electron reduction of O_2 to $O_2^{-\bullet}$ occurs (Beckman and Ames, 1998). Next, the dismutation of $O_2^{-\bullet}$ (both spontaneous and catalysed by SOD) yields H_2O_2, which subsequently may form hydroxyl radicals and other low-molecular weight oxidants located in the RONS cascade. It is a common assumption that increased mitochondrial oxygen consumption in response to exercise leads to increased RONS production; superoxide production in the mitochondria has been generally cited as the predominant source of RONS during exercise (Cooper et al., 2002, Di Meo and Venditti, 2001, Powers and Jackson, 2008, Vollaard et al., 2005). However, studies suggest that mitochondria may not be the dominant source of exercise-induced RONS production; for instance mitochondria produce more RONS in basal respiration compared to active respiration (Powers et al., 2011, Vollaard et al., 2005).

NADPH oxidase (located in several cellular compartments including sarcoplasmic reticulum, transverse tubules, and sarcolemma) produces superoxide by transferring electrons from NADPH to oxygen and is considered to contribute to exercise-induced RONS production (Powers et al., 2011). Superoxides have been reported to be released into extracellular spaces as the plasma membrane contains redox systems capable of electron transfer across the membrane (Powers et al., 2011). Activation of the enzyme PLA_2 can activate NADPH oxidase, stimulate RONS production in both the mitochondria and the cytosol, and release RONS into extracellular space (Powers et al., 2011), thus, an increase in RONS inside the cell

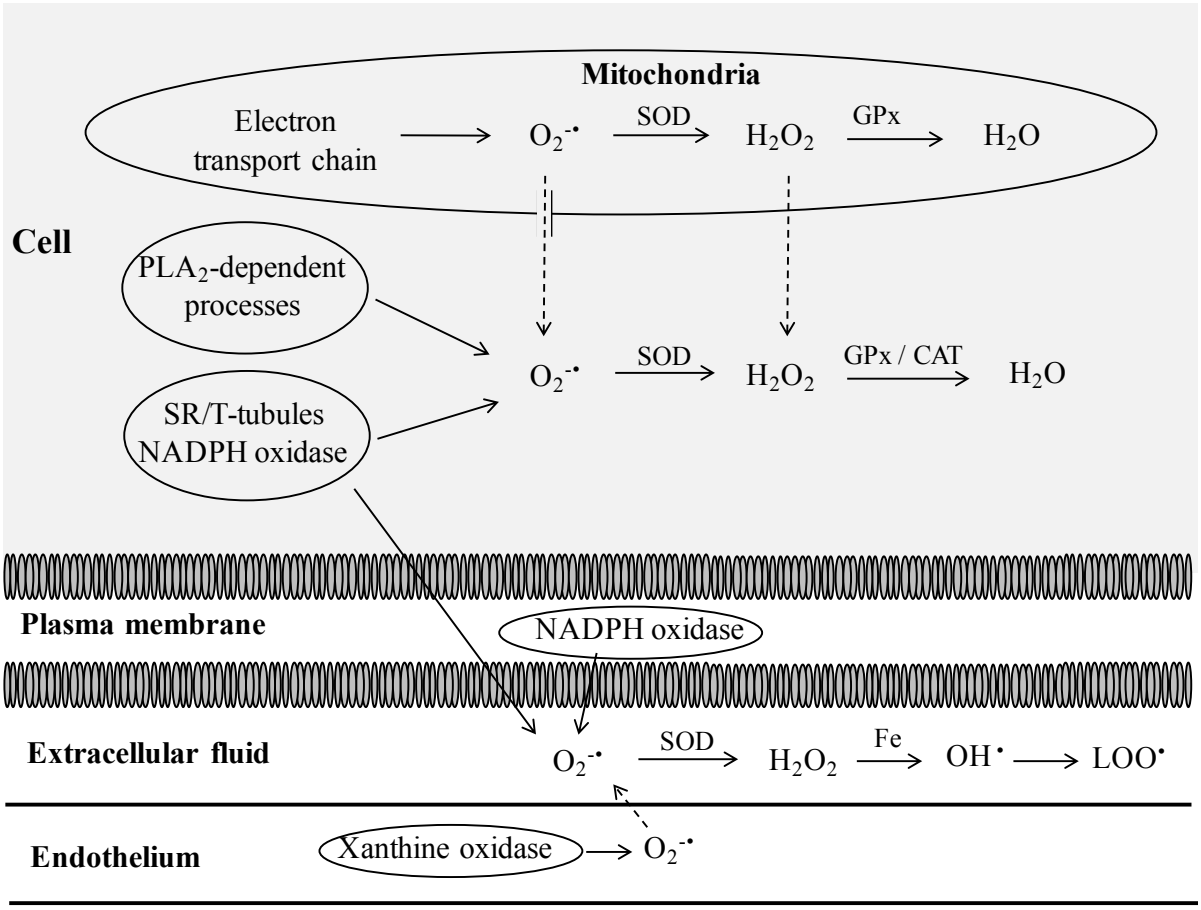


Figure 1-5 Sources of RONS at cellular level

Figure adapted from (Powers et al., 2011). Schematic diagram depicting the potential sites of RONS sources during / following exercise. SR/T-tubule – sarcoplasmic reticulum / transverse tubule, PLA_2 – phospholipase A_2, SOD – superoxide dismutase, GPx – glutathione peroxidase, CAT - catalase.

may also be detected outside of the cell. Xanthine oxidase generates superoxide and xanthine by oxidising hypoxanthine (Powers et al., 2011). It is especially involved in RONS production during anaerobic exercise such as at very high intensity exercise (Radak et al., 2013).

1.5.3.1 Ischaemia-reperfusion

An alternative mechanism for ROS production in response to exercise involves ischaemia-reperfusion (Cooper et al., 2002). Intense exercise causes transient tissue hypoxia in several organs as blood is directed away to increase blood supply in active skeletal muscles (Cooper et al., 2002), and this can result in activation of xanthine oxidase (Sachdev and Davies, 2008). Additionally, exercising at intensities above the V̇O2max can lead to hypoxia in muscle fibres (Cooper et al., 2002). On cessation of exercise, reperfusion occurs and re-oxygenation of these tissues can result in burst of superoxides and H_2O_2 (Sachdev and Davies, 2008).

1.5.4 Oxidative stress markers in response to exercise training: Adaptation

It seems paradoxical that although oxidative stress is linked to ageing and numerous pathologies (as discussed in section 1.3) while acute exercise stimulates oxidative stress but regular exercise decreases oxidative stress and is linked to numerous health benefits including reducing risk of mortality as well as reduced risk of these very pathologies (Powers et al., 2011). The first study to report that exercise-induced RONS production could be a stimulus for exercise training adaptation was in the 1980s by Davies et al. (1982), since which numerous studies have accumulated that support this finding (Powers et al., 2011). RONS can activate several different signalling pathways including the mitogen-activated protein kinase (MAPK) and nuclear factor κB (NFκB) pathways (Powers and Jackson, 2008). The activation of these pathways leads to adaption of cells to oxidative stress through upregulation of antioxidant enzymes such as SOD and catalase (Powers and Jackson, 2008). In addition Elokda and Nielsen (2007) reported that exercise training in humans lead to increased resting

GSH and GSH:GSSG ratio. This upregulation in the body's antioxidant defence system (Douris et al., 2009, Elokda and Nielsen, 2007, Fatouros et al., 2004), increases protection against oxidative stress during subsequent exercise training (Fatouros et al., 2004, Fisher-Wellman and Bloomer, 2009).

1.5.5 Exercise-induced oxidative stress: assessment in the brain

As discussed in section 1.3, oxidative stress has been implicated in ageing and neurodegenerative diseases such as Alzheimer's disease, and Parkinson's disease where the brain is the organ most affected by the disease. The brain is highly susceptible to oxidative stress and indeed the presence of oxidative stress in the brain has been demonstrated in these conditions. As exercise can act favourably to reduce oxidative stress, there is a need to investigate the effect of exercise on oxidative stress is the brain. However, to date, no study has investigated the effect of exercise on oxidative stress markers in the human brain. The underlying theme of the first two experimental chapters of this thesis was to assess antioxidant response in the brain.

1.5.6 Habitual physical activity

Physical activity is defined as any bodily movement produced by skeletal muscles that requires energy expenditure, whereas, exercise is a subset of physical activity that is planned, structured, and repetitive with the intention to improve or maintain physical fitness (Caspersen et al., 1985). Physical activity, whether exercise or non-exercise, positively contributes to overall health. Globally, a third of adults do not reach the recommended levels of physical activity- those in high income countries are more inactive, and also older adults are less active to younger adults (Hallal et al., 2012). Physical inactivity has been reported to be the fourth leading risk factor for non-communicable diseases (Hallal et al., 2012).

There is now strong evidence that links regular physical activity or exercise to higher cognitive function (Colcombe and Kramer, 2003, Lautenschlager et al., 2008), decreased cognitive decline (Sofi et al., 2011) and reduced risk of numerous diseases including the diseases associated with ageing such as Alzheimer's disease (Brown et al., 2013, Hamer and Chida, 2009), dementia (Hamer and Chida, 2009, Laurin et al., 2001), cancer (Warburton et al., 2006, Wen et al., 2011) and diabetes (Warburton et al., 2006). Physical activity or exercise is also linked to reduced mortality (Hupin et al., 2015, Warburton et al., 2006, Wen et al., 2011). The currently recommended physical activity level for adults is 150 min/week of moderate-intensity physical activity or 75 min/week of vigorous-intensity activity (Public Health England, 2014). The guidelines by the Public Health England on physical activity for older adults are: i) Those who participate in any amount of physical activity gain some health benefits. Guidelines go on to clarify that some physical activity is better than none, and more physical activity provides greater health benefits. ii) Physical activity should add up to at least 150 min/week of moderate-intensity activity. iii) Those who are already regularly active at moderate-intensity, comparable benefits can be achieved through 75 min/week of vigorous-intensity activity or a combination of moderate and vigorous activity. iv) Those who are at risk of falls should incorporate physical activity to improve balance and coordination on at least two days a week (Public Health England, 2014). However, it may be that even a lower dose than the recommendation is sufficient for attaining exercise benefits (Hupin et al., 2015, Lee et al., 2014, Wen et al., 2011). For example, Wen et al. (2011) found that a low-volume physical activity (90 min/week moderate-intensity) had significant health benefits for both adults (20-59 years) and older adults (≥60 years). In agreement, Hupin et al. (2015) also reported that low-volume physical activity reduced mortality in older adults, a demographic group that does not usually achieve the recommended dose (Hupin et al., 2015). Low-volume

physical activity has been shown to be effective also in attenuating oxidative stress in older adults. Low volume (100 min/week) exercise training in older adults has also been shown to decrease markers of oxidative damage and increased antioxidant defence (Takahashi et al., 2013).

1.5.6.1 Objective measure of habitual physical activity

Recommendations for low-intensity physical activities are missing from the current guidelines, and not as much is known about low-intensity physical activities, and whether they also have similar benefits to that in the recommended physical activity guidelines (Lee and Shiroma, 2014). Studies investigating the effects of habitual physical activity generally are limited in the estimation of physical activity by using self-report tools such as questionnaires or diaries. Such self-reports have issues of subjectivity and recall bias, and may also have problems associated with inaccuracy especially for estimation of housework and occupational physical activity (Hallal et al., 2010). Additionally, observational epidemiological studies, which also use self-reports, tend to limit their assessments to physical activities of moderate and vigorous intensities only, as self-reports are more reliable and valid for moderate to vigorous intensity activities compared to low-intensity activities (Lee and Shiroma, 2014). Thus current guidelines do not prescribe low-intensity physical activity. This is not because there is data to indicate non-benefit, but rather because there are very few existing datasets available (Lee and Shiroma, 2014).

Emerging technologies assessing measurement of body movements is a promising alternative objective method for future assessments of habitual physical activity, in particular accelerometry. These technologies allow estimation of the frequency, duration, and intensity of physical activity in free-living individuals. Additionally accelerometry records physical activity by accumulating data in periods shorter than 10 minutes, whereas self-report

assessments usually prompt the respondent to report activities lasting at least 10 minutes, thus the granularity of data is greater (Hallal et al., 2012, Hallal et al., 2010). The most commonly used physical activity monitors are pedometers and accelerometers, which can vary in sensitivity, cost, memory, and software capabilities. Pedometers are activity monitors which measure the number of steps that a person takes during ambulatory activity such as walking or running, and are attached by a waistband in the midline of the thigh on either of the leg (Berlin et al., 2006). Pedometers cannot identify intensity level as it does not take into account the intensity of vertical displacement (Berlin et al., 2006). Pedometers can be very accurate in individuals without any mobility impairments, however they can underestimate the steps in individuals with reduced mobility or impairments who may have slower gait speed, irregular and unsteady gait patterns (Berlin et al., 2006). Accelerometers are activity monitors that measure both the quantity and intensity of movement, and are capable of measuring the intensity, frequency, pattern, and duration of activity (Berlin et al., 2006). Accelerometers can be worn on the waist, wrist, or ankle. Actigraph is the most commonly used accelerometer in studies using this kind of technology (Murphy, 2009).

Although accelerometers provide objective measurements of physical activity, there are some limitations. Typical placement position of accelerometers are on the hip, and thus upper body movements are not measured (Lee and Shiroma, 2014), especially in those with mobility issues. These devices also cannot identify if a person is carrying weight, during which more energy is spent. There are several issues to take into account while measuring physical activity in older adults that are unique to this population, for instance the type and intensity of activities that they engage in are different to younger adults (Murphy, 2009). Currently there are studies that have used accelerometry to objectively measure physical activity, however most of these studies are cross-sectional, i.e. they have investigated the association of physical

activity with various health factors (Ekelund et al., 2009, Gennuso et al., 2013, Healy et al., 2008, Lee and Shiroma, 2014).

1.6 OVERVIEW OF THESIS

The aims of the thesis was to

1. develop an MRS method to measure GSH in the brain
2. investigate the effect of exercise on GSH in the brain in sedentary humans
3. assess oxidative stress in response to different exercise intensities in the context of peripheral oxidative stress and cerebral blood flow
4. investigate a device capable of objectively measuring habitual physical activity in sedentary older adults
5. assess the effect of low dose exercise (less than the recommended guideline) in older adults on markers of oxidative stress and other health markers

The study presented in **chapter three** details the steps in the method development for assessment of GSH in the brain using MRS. Then in **chapter four**, the effect of exericse on brain GSH in young sedentary participants was investigated, using two different types of acute exercise bouts that differed in exercise intensity. Other markers of oxidative stress were also assessed in the plasma/blood. Cerebral blood flow in response to these two different exericse bouts were also assessed. Finally, **chapter five** looked at the effect of an unsupervised exericse intervention in older adults on markers of oxidative stress as well as other health markers such as physical fitness. Physical activity was measured objectively using a commercially available activity monitor Basis Peak. The appropriatness of this acitivity monitor in this population was also assessed.

2 CHAPTER 2: General methods

2.1 SUPPLIERS

Glacial acetic acid, hydrochloric acid and multiwall plates were purchased from Fisher. 2,4-Dinitrophenylhydrazine was purchased from Camlab chemicals. The antibodies mouse IgE anti-dinitrophenyl and rat anti-mouse IgE conjugated horseradish peroxidase were purchased from Sigma and AbD Serotec respectively. All other chemicals and reagents were purchased from Sigma.

2.2 CHEMICAL ASSAYS

Absorbance values for all assays were measured using plate reader (Multiskan MS, LabSystems, Finland).

2.2.1 Total antioxidant capacity measurement using FRAP assay

Plasma total antioxidant capacity was measured using the ferric reducing ability of plasma (FRAP) assay as described by Benzie and Strain (1996) (inter and intra-assay coefficient of variation (CV) <3 % and <1 % respectively). Working reagent was made by mixing i) 30 ml acetate buffer (300 mM) at pH 3.6 (3.1 g $C_2H_3NaO_2$ in 16ml glacial acetic acid per litre of buffer solution), ii) 3 ml TPTZ (10 mM) (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and iii) 3 ml FeCl3.6H2O solution (20 mM). Samples (1:1 dilution) and standards (10 µl) were added to the respective wells of a multiwell plate, followed by addition of working reagent (300 µl). After incubating the sample and reagent mix for 8 minutes at room temperature, the absorbance values were measured at 650 nm. Standard curve of known concentrations of commercially available ascorbic acid (0-1,000 µM) was used to estimate the antioxidant power of the samples. The antioxidant power as measured by FRAP is double that of an ascorbic acid standard as the direct reaction of ascorbic acid gives a change in absorbance

double that of Fe (II) (Benzie and Strain, 1999). Mean intra-assay coefficient of variation (CV) was 5 %.

2.2.2 Lipid peroxidation measurement using MDA assay

Lipid peroxidation was measured in plasma samples using the malondialdehyde (MDA) assay. The basis of the method is the reaction of MDA with thiobarbituric acid (TBA) at low pH and high temperature (90-100 °C) to form a coloured complex, the MDA-TBA adduct, with maximal absorption at 532nm.

In brief, 100 µl samples were mixed with 100 µl trichloroacetic acid (410 mM in 33.3 mM H_2SO_4) and 800 µl the colour reagent (4.6 mM thiobarbituric acid, 1.74 M glacial acetic acid, and 0.67 µM butylated hydroxytoluene). After boiling vigorously in boiling water (100 °C) for one hour, the reaction was stopped by placing in ice bath for 10 minutes. Supernatant was read at 540 nm in multiwell plate. Standard curve of known concentrations of commercially available 1,1,3,3-tetramethoxypropane was used to estimate MDA levels in the samples.

2.2.3 Measurement of protein content using BCA method:

Plasma protein concentration was assessed using bicinchoninic acid (BCA) assay which is based on the method described by Smith et al. (1985). Standards (0 to 1 mg/ml) were prepared by diluting bovine serum albumin (BSA). Fresh working solution was prepared by mixing BCA solution (Sigma) with copper sulphate solution (4% w/v) in a 50:1 ratio. Samples (1:200 dilution) and standards (10 µl) were added to the respective wells in a multiwell plate. Working solution (200 µl) was added to each well. Following incubation at room temperature in the dark for 30 minutes, absorbance was measured at 540 nm. Mean intra-assay CV was 5%.

2.2.4 Protein oxidation measurement using ELISA method

Protein carbonyl concentration was measured in plasma samples using the enzyme-linked immunosorbent assay (ELISA) method that uses anti-dinitrophenyl (DNP) antibody for measuring total protein carbonyl groups, first described by Buss et al. (1997) which was further developed by Carty et al. (2000) (inter and intra-assay CV 5 % and 1 % respectively).

Standard preparation:

Standards for the assay were prepared by the method described by Buss et al. (1997). In brief, the standards were prepared by mixing varying proportions (0-100 %) of oxidised BSA (using 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)) with fully reduced BSA (using sodium borohydride) to produce standards of a range of carbonyl values. Carbonyl content was determined as nmol/mg protein using the molar absorption coefficient (ε_{360} = 22,000 M^{-1} cm^{-1}). Carbonyl values are expressed as nmol/mg protein. Standards were aliquoted and stored at -70 °C.

ELISA method:

Samples (diluted in coating buffer (50 mM sodium carbonate at pH 9.2) and standards (50 µl) were allowed to bind to the respective wells of multi-sorb plates (Nunc, Fisher Thermo Scientific) for 1 hour at room temperature. After washing (3x each wash) the plates with TBS wash buffer (6 g/L Tris HCl and 9 g/L NaCl with 0.05 % Tween-20), 2,4-dinitrophenylhydrazine (DNPH) (1 mM in 2 M HCl) was added and the plates were incubated for another 1 hour. Following another wash, the plates were incubated overnight at 4 °C with TBS with 0.1 % Tween-20 (200 µl) to block any non-specific binding. Primary antibody (mouse IgE anti-dinitrophenyl, 50 µl, 1:1000 dilution) was added following a wash with wash buffer, and incubated for 2 hour at 37 °C. Subsequently, the plate was again washed and

incubated for 1 hour with secondary antibody (anti-mouse IgE horse radish peroxidase conjugate, 50 µl, 1:5000 dilution) at room temperature. The plates were washed, to remove any excess unbound antibodies. Then, 50 µl substrate (10ml citrate phosphate buffer (citric acid and disodium hydrogen phosphate, 0.5 M at pH 5) containing 8 µl H_2O_2 and 2 mg o-phenyldiamine tablet) was added. After, 1 hour incubation in the dark at room temperature, the reaction was stopped with 2 M sulphuric acid and absorbance values were measured at 490 nm. Mean intra-assay CV was 13%.

2.2.5 Brain derived neurotropic factor

Plasma brain derived neurotropic factor (BDNF) was measured using a sandwich ELISA method with a commercially available kit (BDNF Quantikine ELISA kit, R&D systems, Minneapolis, USA) according to the manufacturer's instructions (inter and intra-assay CV 9 % and 5 % respectively). In brief standards and samples (1:1 dilution, 50 µl with 100 µl diluent) were added into the respective wells of the provided multiwell plate which is pre-coated with monoclonal antibody specific for human BDNF. During incubation the BDNF in the samples were allowed to bind to the antibody. Next, enzyme-linked antibody that is specific for BDNF was added. After a wash to removed unbound antibody, a substrate solution was added for colour development. A stop solution was added and the absorbance was read at 450 nm. Mean intra-assay CV was 8%.

2.3 CEREBRAL BLOOD FLOW MEASUREMENT

Blood flow velocity of the middle cerebral artery (MCAv) was measured with a 2-MHz pulse wave transcranial Doppler (TCD) ultrasound system (Compumedics Ltd, Germany). TCD is a non-invasive method that allows beat-to-beat measure of changes in blood flow velocity thus enabling measurement of transient change in cerebral blood flow (CBF). This method is based

on the detection of frequency shifts from insonated red blood cells. The middle cerebral artery was insoated via the temporal window above the zygomatic arch of the head. After a satisfactory signal was found, the probe was fixed in place with a headband. MCAv was acquired continuously using an analog-to-digital converter (Powerlab; ADInstruments) interfaced with a computer, and were subsequently analysed using commercially available software (Lab Chart, ADInstruments).

3 Chapter 3: Development of an MRS method to assess brain GSH

3.1 ABSTRACT

Magnetic resonance spectroscopy (MRS) provides a non-invasive technique to measure brain metabolites. Glutathione (GSH) is one of the most abundant antioxidants in the human body, however detection of GSH in the brain using MRS is challenging due to spectral overlap and the comparatively small concentration of GSH compared to other metabolites. Using the MEGA-PRESS technique, a method for GSH detection in the brain (anterior cingulate cortex) was optimised by conducting experiments both *in vitro* and *in vivo*. Oxidation of GSH phantoms confirmed the identity of reduced GSH, and suggested that oxidised GSH (GSSG) did not contribute to the detected GSH signal. Quantification was undertaken using the freely available software jMRUI. Repeated scanning showed reproducibility of the GSH signal over time, on the same day and also between different days.

3.2 INTRODUCTION

MRS is an emerging method to assess brain GSH. MRS provides biochemical information non-invasively, which becomes particularly useful if the region being investigated is difficult to access such as the brain.

The phenomenon of nuclear magnetic resonance occurs when an atomic nucleus with a magnetic moment (or spin) is placed in a magnetic field (B) can exhibit resonance behaviour. This magnetic resonance (MR) effect is governed by the Larmor equation

$$w = -\gamma B$$

where, w is the resultant resonance frequency, and γ is gyromagnetic ratio which is a nucleus specific constant.

Nuclei such as ^{1}H, ^{13}C and ^{31}P possess a magnetic moment which can be thought of as microscopic magnets. When placed in a magnetic field the spins become polarised, and are either parallel or antiparallel, longitudinal to the external magnetic field. The spins that are polarised parallel to the field are in a lower energy state, which is the preferred state.

In MRS, the region being assessed is placed in an external magnetic field. Then in order to detect signal, a radio frequency pulse (RF) (an electromagnetic wave) is applied (Stagg and Rothman, 2014), shown in Figure 3-1. The RF pulse has two effects on the protons (that occurs simultaneously and independently): i) it transits the protons to a higher level of energy (i.e. antiparallel) leading to decrease of longitudinal magnetisation and ii) it generates a new magnetisation in the transverse direction called transverse magnetisation (Schild, 1990). Then the RF pulse is switched off, which causes the system disrupted by the RF pulse to go back to

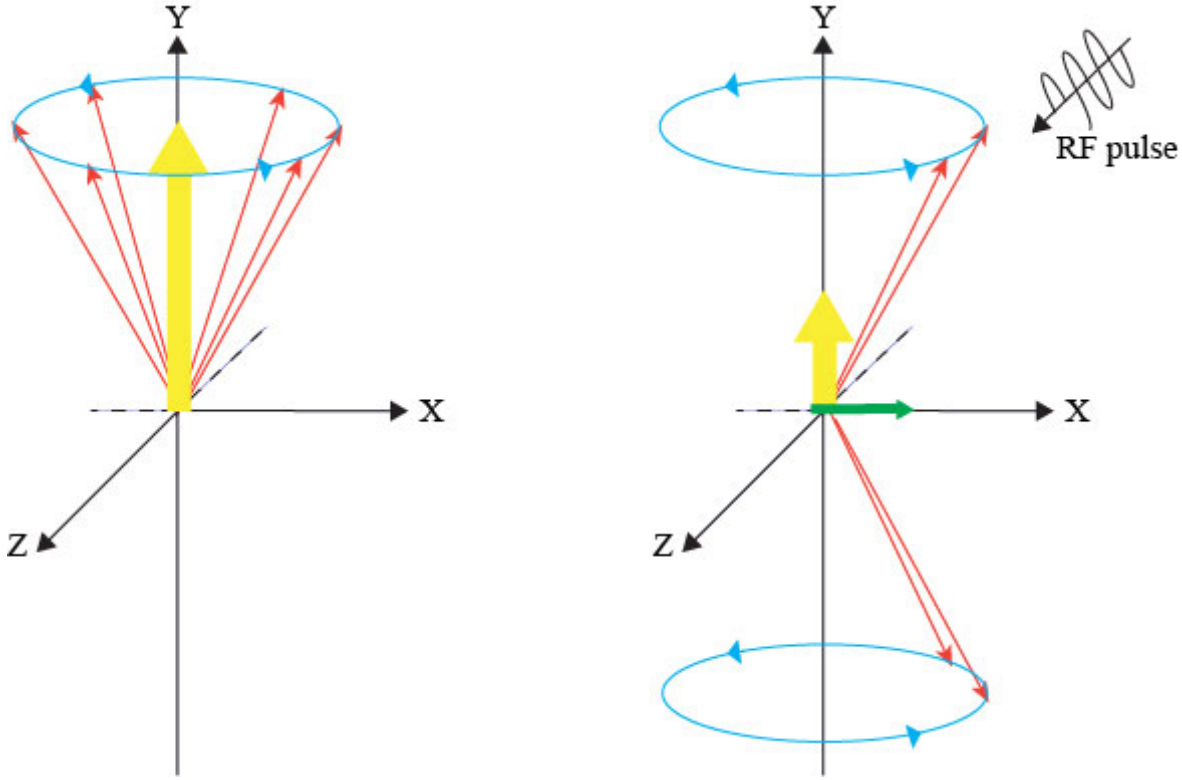


Figure 3-1 Magnetisation by the RF pulse

Right image: Magnetic field of protons longitudinal to the external magnetic field (longitudinal magnetisation- big yellow arrow) Left image: RF pulse is applied which causes i) decrease in longitudinal magnetisation (yellow arrow) and ii) Generation of a new transversal magnetisation (green arrow). (figure adapted from (Schild, 1990)).

the initial state. The longitudinal magnetisation increases again (longitudinal relaxation), described by time constant T_1. The transverse magnetisation starts to disappear, a process called transversal relaxation (described by the time constant T_2). During relaxation, signal is emitted– this free-induction decay (FID) response signal is detected by the scanner (Tognarelli et al., 2015). As MRS signal (a spectrum) is heavily influenced by electromagnetic noise, a number of spectra are obtained which are then averaged/added up in order to improve the signal quality (i.e. to improve signal to noise ratio) (Alger, 2010).

As water is the most prevalent hydrogen (^{1}H) containing compound in the human tissue, it contributes the largest part of the ^{1}H MR signal (Hutton et al., 2009). When a human is placed in the scanner, all of the body's nuclei of the same atom, say ^{1}H, do not exhibit the same resonance frequency, thus causing frequency shifts. This is because minute variations occur depending on the molecule that the atom is embedded in. These variations are based on two different effects: chemical shift and J-coupling, both rely on the molecules' chemical and geometric composition (Stagg and Rothman, 2014). Chemical shift is the result of the chemical environment around the atomic nucleus at hand. J-coupling refers to an interaction between non-identical nuclear spins within a molecule through the electrons of covalent bonds. These variations allow the discrimination of nuclei that belong to different molecular configuration.

Trabesinger et al. (1999) were the first to detect GSH *in vivo* in the human brain by MRS. This is the only method which allows non-invasive measurement of GSH (Mueller et al., 2001). It has been challenging to reliably measure GSH with MRS for two main reasons: i) the concentration of GSH is inherently low compared to other brain metabolites, and ii) GSH peak has spectral overlap with other neurotransmitters which are present in much greater concentrations such as creatine (Cr), γ-aminobutyric acid (GABA) and aspartate, in particular

the Cr peak. Apart from spectral overlap, problems of measuring brain GSH include complicated spectral patterns resulting from strong coupling between some of its hydrogen nuclei. Nevertheless, Holmay et al. (2013) showed that MRS is a method that is capable of detecting changes in GSH levels in the human brain; in this study the participants were given GSH precursor N-acetylcysteine intravenously. However, no previous studies have assessed whether MRS is sensitive enough to detect potential changes in GSH following a physiological stimulus such as exercise.

3.3 METHODS

3.3.1 MRS GSH

GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), and each peptide residue gives rise to a signal of particular resonance. The cysteinyl residue is most suitable for use in the detection of GSH using MRS, as the resonances of the glutamyl and glycine compounds are heavily overlapped by other metabolites. The cysteine β-CH_2 of GSH resonates at 2.95 ppm- other brain metabolites that resonate nearby include Cr (3.03 ppm), aspartate (2.82 ppm), GABA (3.01 ppm), and macromolecules (2.98 ppm) (Terpstra et al., 2003).

All *vivo* and *vitro* scans were undertaken using ^{1}H-MRS with a Philips 3.0 T Achieva scanner using MEGA-PRESS, as described below.

3.3.2 MEGA-PRESS

MEGA-PRESS (MEshcher-Garwood Point RESolved Spectroscopy) is a difference-edited MRS technique that allows separation of GSH signal from the overlapping signals from other metabolites. MEGA-PRESS utilises known couplings within the GSH molecule by editing the cysteinyl β-CH_2 protons of GSH resonating at 2.95 ppm, which are *J*-coupled to the cysteinyl α-CH at 4.56 ppm (Satoh and Yoshioka, 2006). An editing pulse was applied to GSH to

selectively refocus the evolution of J-coupling to the GSH spins. Two alternate datasets that differed in the treatment of the GSH spin systems were collected– signal for each type of treatment were collected in alternate order, one after the other. Signals for the different treatments were collected in such alternate fashion in order to minimise the effect arising from hardware instabilities or any subject movements. Signals collected with the application of the editing pulse is referred to as edit-on signals. Signals collected with the pulse applied elsewhere (thus allowing the J-coupling to evolve freely) is referred to as edit-off signals.

In order to measure GSH signal, during post-processing the edit-on signals were subtracted from the edit-off signals– this removed all the peaks leaving behind only those that were affected by the editing pulse (most of the peaks were not affected by the editing pulse). Unsuppressed water signal was acquired as a separate scan after the GSH scan– this water signal was used as tissue water for concentration reference of metabolites.

3.3.3 *In vitro* GSH measurement and assessment of potential GSSG contribution

Standards, or so called phantoms, were used to confirm the spectral pattern of GSH, and also to refine an optimal sequence using a MEGA-PRESS sequence. GSH (Sigma-Aldrich, G4251) solutions were prepared in a physiologically representative pH (pH 7.2) phosphate buffer condition. GSH phantoms (GSH concentration ranging from 14 to 70 mM) were made and also included other metabolites found in the brain– sodium azide (15 mM), sodium chloride (800 mM), sodium formate (200 mM), GABA (3 mM), N-Acetyl-L-aspartic acid (25 mM), Cr (20 mM), choline (Ch) (6 mM), myo-inositol (15 mM), glutamate (25 mM), glutamin (12 mM), lactate (10 mM), 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (2 mM), potassium phosphate monobasic (28 mM), and potassium phosphate dibasic (72 mM) at pH 7.2.

To assess the GSSG signal and potential overlap with the GSH signal in the MRS spectrum, an oxidised GSH phantom was made (GSH (30 mM), sodium formate (200 mM), potassium phosphate monobasic (30 mM), and potassium phosphate dibasic (70 mM) at pH 7.2) and was oxidised by the addition of hydrogen peroxide (H_2O_2) (30 mM). Scans were acquired before and after oxidation with H_2O_2.

Phantoms were stored and scanned in 250 mls short necked round bottom flasks. For scanning, the volume of interest (VOI) (30×30×20 mm) was placed at the centre of the flask. As the MRS signal can be influenced by pH and temperature, phantoms were stored at - 4 °C and were allowed to rest at room temperature before being scanned. Additionally, phantom solutions degrade over time, especially GSH, therefore various phantoms were made over the course of this method development study. Wherever possible, all scans assessing a particular phantom concentration or a scan parameter were scanned in one session.

3.3.4 Quantification of GSH using jMRUI

All of the MRS data were analysed by the author using the freely available software jMRUI (which uses java-based graphical user interface to analyse the time-domain MRS data). Spectral peaks and patterns provide a direct measure for the concentration of the metabolites, as the corresponding area of spectral peak is directly proportional to the number of nuclei that contribute to it and to the abundance of the resonating nucleus (Jansen et al., 2006, Stagg and Rothman, 2014). Data were exported from the scanner as *.SDAT and *.SPAR files and opened in jMRUI as a time series. The edit-on and edit-off spectra were 180° out of phase.

Chemical shifts were reported with reference to sodium formate singlet resonance at 8.4 ppm for the phantom data and to NAA singlet resonance at 2.01 ppm for the brain data. The water signal was removed using a HLSVD (Hankel Lanczos Squares Singular Value

Decomposition) filter. GSH was quantified by peak fitting using an algorithm called the advanced method for accurate, robust, and efficient spectral fitting (AMARES), after selecting the GSH region using an ER filter. In phantoms, GSH concentrations were estimated using tissue water as internal standard. For the brain data, GSH concentrations were estimated using tissue water as concentration standard as described by Gasparovic et al. (2006). The observed water signal in MRS comes from a combination of the grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF) water fractions, and each of these are weighted by different relaxation times (Gasparovic et al., 2006). It is necessary to modify the internal water concentration on the basis of the GM, WM and CSF content of the volume assessed, and the MRS water content of the grey and white matter fractions (Keevil et al., 1998).

The T_1-weighted images were segmented using SPM8 (Wellcome Trust Centre for Neuroimaging, University College London, UK) to determine the percentage of GM, WM and CSF in each VOI. The concentration of GSH was calculated as follows,

$$\text{Corrected GSH concentration (mM/kg brain tissue)} = (f_{GM} * R_{H2O\_GM} + f_{WM} * R_{H2O\_WM} + f_{CSF} * R_{H2O\_CSF}) * (S_{GSH\_obs} / S_{H20\_obs}) * [1/(1- f_{CSF})] * (1/ R_{GSH}) * [H_2O] * (\#H_{H2O} / \#H_{GSH}) * 1000 \quad \text{Equation 1}$$

where,

S_{GSH_obs} is observed GSH signal, S_{H20_obs} is observed water signal, $[H_2O]$ is molal concentration of MR visible water in metabolite solution of the parenchyma (= 55.51), $\#H_{GSH}$ is the number of proton that give rise to GSH peak (= 2), and $\#H_{H2O}$ is the number of proton water protons (= 2).

R_{H2O} is relaxation attenuation factor:

$$R_{H2O\_GM} = \exp[-TE/T2_{H20\_GM}]* [1 - \exp (-TR/T1_{H2O\_GM})] \quad \text{Equation 2}$$

$$R_{H2O\_WM} = \exp[-TE/T2_{H20\_WM}]* [1 - \exp (-TR/T1_{H2O\_WM})] \quad \text{Equation 3}$$

$$R_{H2O\_CSF} = \exp[-TE/T2_{H20\_CSF}]* [1 - \exp (-TR/T1_{H2O\_CSF})] \quad \text{Equation 4}$$

$$R_{GSH} = \exp[-TE/T2_{GSH}]* [1 - \exp (-TR/T1_{GSH})] \quad \text{Equation 5}$$

where, $T2_{H20\_GM}$ = 0.1 s, $T2_{H20\_WM}$ = 0.07 s, $T2_{H20\_CSF}$ = 0.5 s, $T2_{GSH}$ =0.062 s, $T1_{H20\_GM}$ = 1.65 s, $T1_{H20\_WM}$ = 1.1 s, $T1_{H20\_CSF}$ = 4.16 s, and $T1_{GSH}$ =0.397 s. These relaxation times were estimated from various published studies from other labs (Emir et al., 2011, Ethofer et al., 2003, Minati et al., 2010).

f_{GM}, f_{WM}, and f_{CSF} are the fractions of water attributable to GM, WM, and CSF respectively. Assuming that the relative densities of MR-visible water in GM, WM, and CSF are 0.78, 0.65, and 0.97 respectively:

$$f_{GM} = [\ f_{GM\_vol}*0.78\ ] / [\ (f_{GM\_vol}*0.78) + (f_{WM\_vol}*0.65) + (f_{CSF\_vol}*0.97)\ ] \quad \text{Equation 6}$$

$$f_{WM} = [f_{WM}\_vol*0.65] / [(f_{GM\_vol}*0.78) + (f_{WM\_vol}*0.65) + (f_{CSF\_vol}*0.97)\ ] \quad \text{Equation 7}$$

$$f_{CSF} = [f_{CSF\_vol}*0.97] / [(f_{GM\_vol}*0.78) + (f_{WM\_vol}*0.65) + (f_{CSF\_vol}*0.97)\ ] \quad \text{Equation 8}$$

where f_{GM_vol}, f_{GM_vol}, and f_{GM_vol} are the GM, WM, and CSF volumes respectively determined by segmentation.

3.3.5 Optimisation of method *in vitro*

The parameters for the MEGA-PRESS method were optimised *in vitro* using a phantom of concentration 70mM GSH. Time of echo was optimised for detection of GSH by assessing the GSH signal at time of echo of 92, 110, 130 and 145 ms. Pulse duration was optimised by assessing GSH signal at pulse durations of 30, 40 and 49 ms.

3.3.6 Method development for GSH detection in the human brain

The MEGA-PRESS method was then optimised to detect GSH in the brain in one healthy male participant (aged 23 years). The study was approved by University of Birmingham ethics committee, and written consent form was obtained from the participant before participation. MRS scans were acquired with the participant in the supine position. The participant was asked to remain fasted for at least 4 hours and refrain from exercise for 24 hours prior to each visit. The region of interest was set in the anterior cingulate cortex with VOI (30×30 × 20 mm) (as shown in Figure 3-2). In order to optimise the MEGA-PRESS parameters, different scans were acquired using different excitation positions (4.40, 4.45, and 4.56 ppm) of the selective refocusing pulse and different echo times (112, 120 and 130 ms) while keeping all other parameters the same. All scans for each parameter being assessed were acquired on the same day in the same scanning session.

3.3.7 Brain GSH through 6 hours

In order to assess the stability of brain GSH over time, two young healthy males (aged 19 and 36 years) were scanned four times (using the optimised MEGA-PRESS method) on the same day over a 6 hour period. Participants were scanned four times during the 6 hours (at approximately 0, 2, 3 and 5 hours from the first scan) starting from early afternoon (13:00 and 14:30 for the two participants respectively). The second and the third scans were undertaken in the same scanning session, i.e. the participant did not come out of the scanner between these two scans. The participants were asked not to move throughout the scanning sessions. When the participants were outside the scanner in between the scanning sessions (between the first and second GSH scan, and the third and fourth scan), the participants were asked to sit quietly in the waiting room at BUIC. The participants usually worked on their laptop or read.

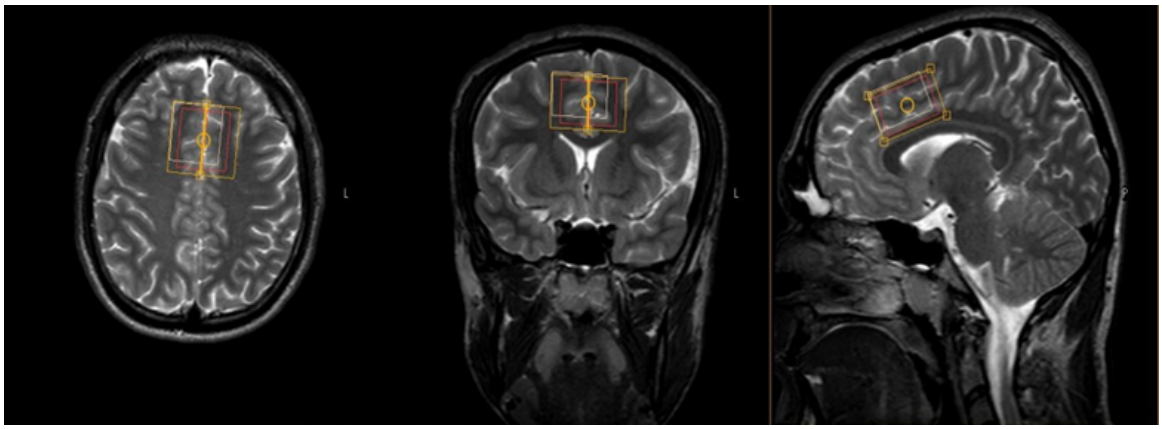


Figure 3-2 Placement of voxel at region of interest in the brain.

Representative T1 weighed MRI images of a participant brain illustrating the voxel placement at the region of interest in the anterior cingulate cortex with volume of interest (VOI) 30×30 × 20 mm. Boxes representing the VOI at the axial, frontal, and sagittal view from left to right.

The participants were asked to arrive fasted for at least 4 hours (no food or drink apart from water) and refrain from exercise for 24 hours prior to visit.

3.4 RESULTS

3.4.1 Declaration of ownership

Due to the regulations surrounding operation of the scanner, all of the phantom MRS scans were undertaken by Dr Alimul Chowdhury, in the presence of the author of this thesis. All of the *in vivo* scans were carried out by Dr Chowdhury or Dr Renate Reniers in the presence of the author of this thesis. All the data analysis presented herein was undertaken by the author.

3.4.2 *In vitro* GSH measurement, and assessment of potential GSSG contribution

GSH produced a single or double positive signal peaks at 2.95 ppm (2.97 and 2.92 ppm) dependent on the GSH concentration (as seen in Figure 3-3); higher concentrations produced double peaks. A positive correlation was found between the GSH concentration of phantoms and the GSH signal measured by the MRS method, as shown in figures A and B in Figure 3-4. Repeating the scan of a 70 mM GSH phantom on the same day gave a coefficient of variation (CV) of 2 %.

Oxidation of GSH phantom by addition of H_2O_2 resulted in decreased GSH signal at 2.95 ppm, and generation of a new peak at 3.24 ppm, proposed as GSSG, as shown in Figure 3-5.

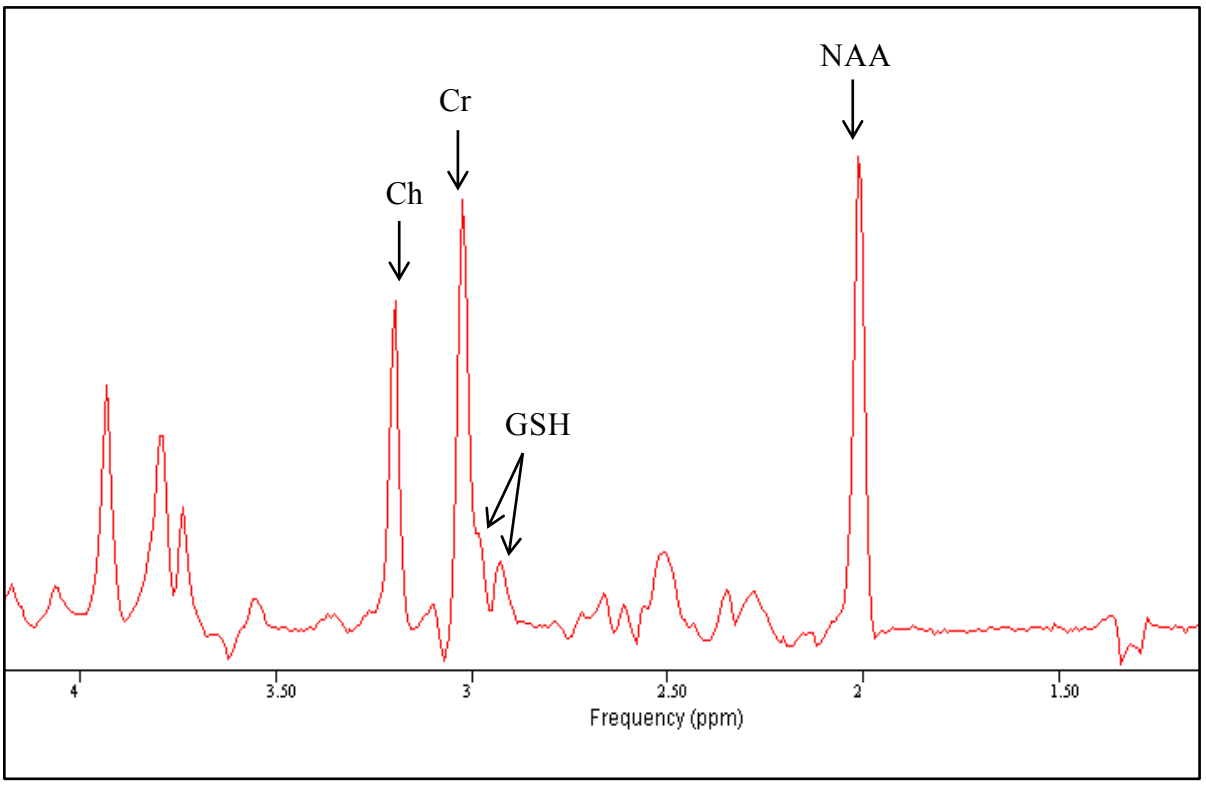


Figure 3-3 *In vitro* measurement of GSH in phantoms

A spectrum acquired in the absence of the editing pulse (edit-off spectrum). Arrows pointing to the respective metabolites: Ch, choline at 3.19 ppm; Cr, creatine at 3.02 ppm; GSH, glutathione at 2.92 and 2.97 ppm; and NAA, N-aceylaspartate at 2.01 ppm. Doublet GSH signal peak seen at 2.92 and 2.97 ppm.

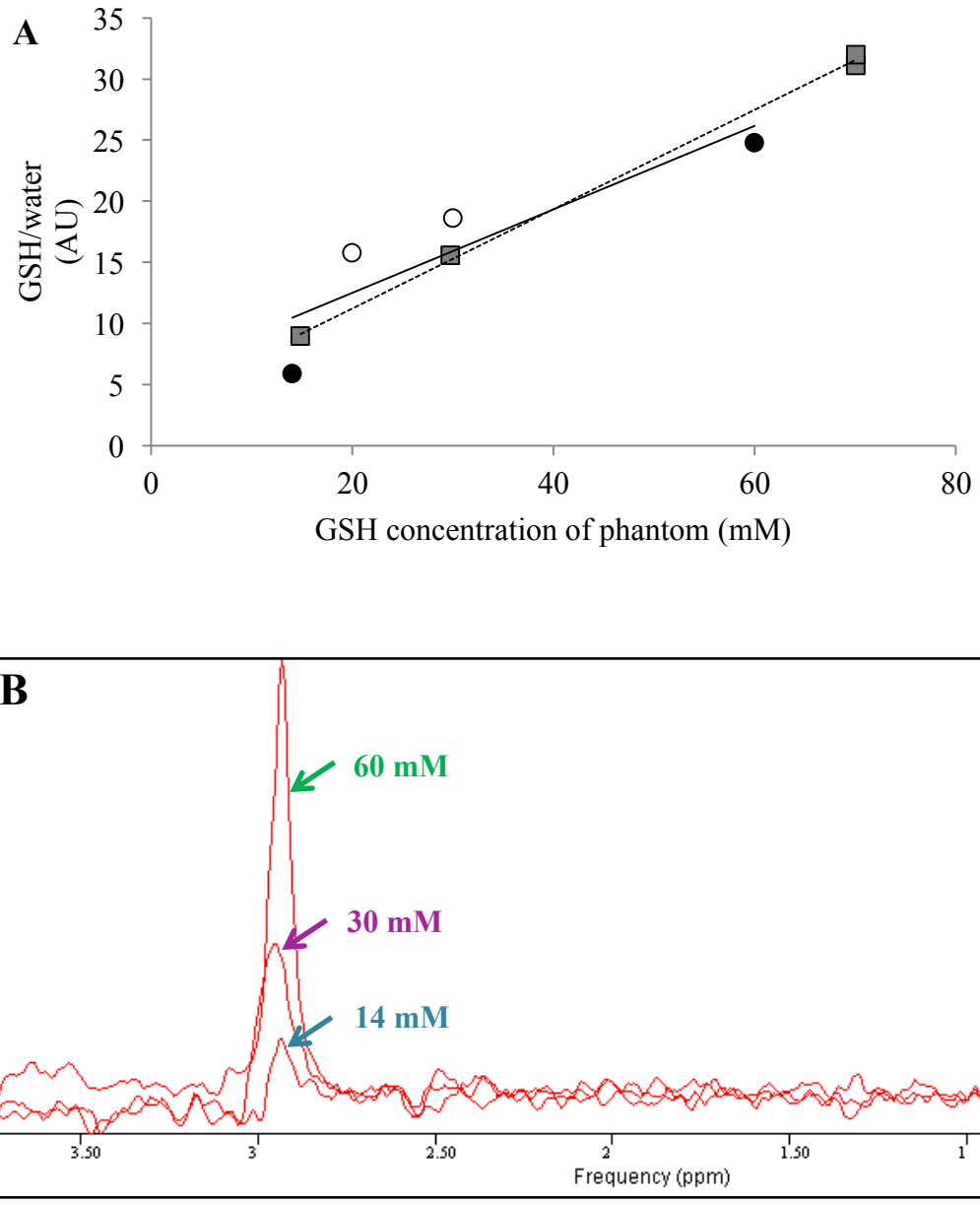


Figure 3-4 Measurement of GSH signal from phantoms of various GSH concentrations.

A) Graph showing positive correlation between the GSH concentration in the phantoms and the GSH signal measured by MRS method. Open and closed circled phantoms were scanned using the same method parameters, but were prepared and scanned separately (months apart). *Squared phantoms* data were scanned using different scan parameters to that of circled phantoms. **B)** Superimposed spectra of edited GSH signals (i.e. edit-on and edit-off spectra subtracted) of three phantoms with GSH concentrations of 14 mM (blue arrow), 30 mM (purple arrow) and 60 mM (green arrow).

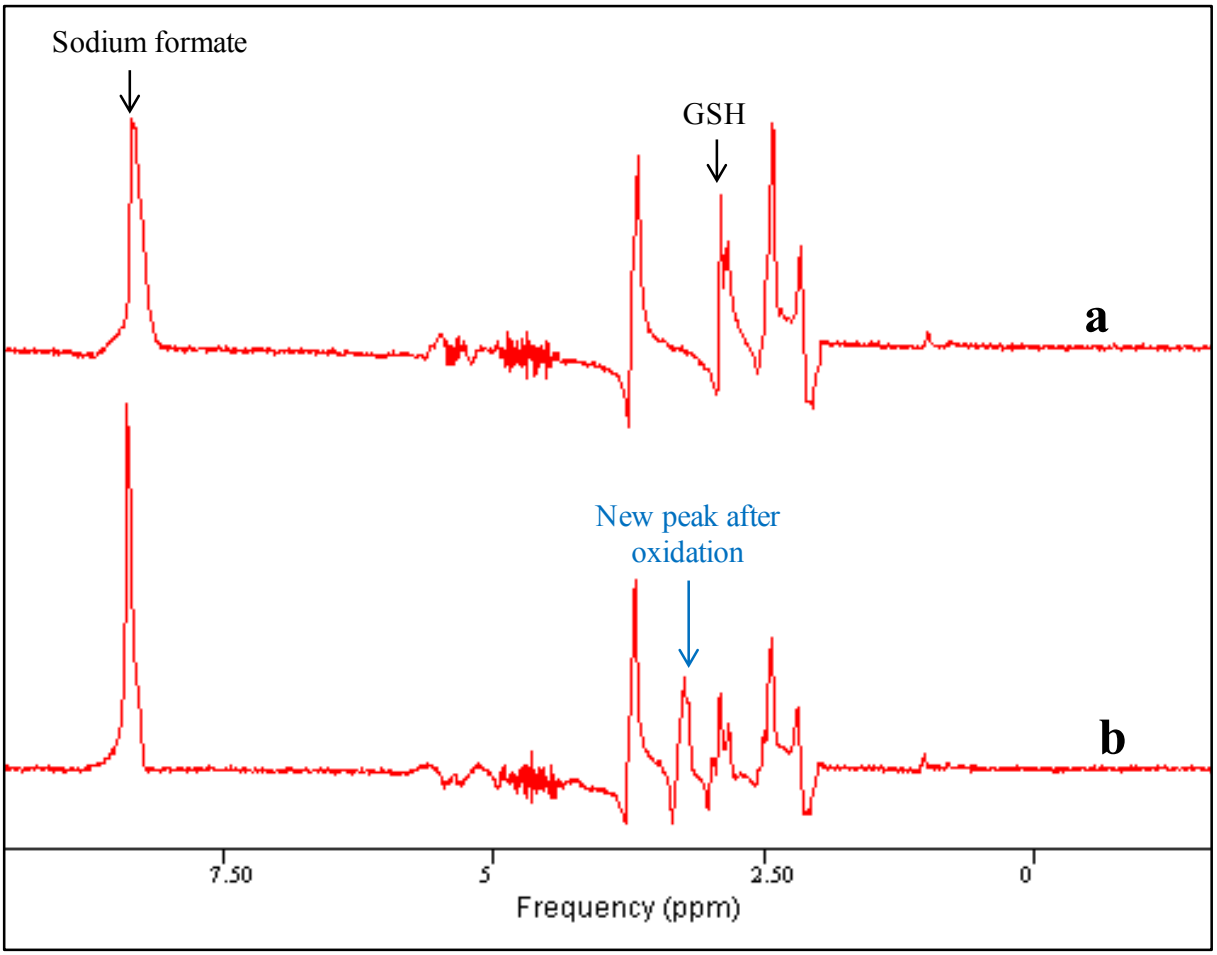


Figure 3-5 Oxidation of GSH by addition of H_2O_2.

A 30 mM GSH phantom before **(a)** and after **(b)** oxidation by addition of H_2O_2. On oxidation, the GSH peak was decreased and a new peak was observed at 3.24 ppm.

3.4.3 Quantification of GSH using jMRUI

Other metabolites, other than GSH, were quantified from the edit-off spectra (all the edit-off spectra of a scan were summed). Metabolites such as Cr and Ch could be quantified automatically using the peak fitting algorithm AMARES in jMRUI as shown in a 30 mM GSH phantom Figure 3-6 (AMARES was set for 35 peaks). In higher GSH concentration phantoms as in Figure 3-6, AMARES was able to automatically pick and quantify the GSH peaks in the edit-off spectra. However, in phantoms with low GSH concentrations at around 14 mM (which is closer to physiological concentrations), AMARES did not pick the GSH peak as the size of the peak was very small and the peak also overlapped with the Cr peak (data not shown).

In order to quantify GSH, the edit-on spectra were subtracted from the edit-off spectra as shown in Figure 3-7. Subtraction resulted in the removal of most metabolites, leaving GSH and some other residues. The GSH peak was selected manually using ER filter and was quantified using AMARES (AMARES was set to 1 peak).

3.4.1 Optimisation of method *in vitro*

In order to refine the MEGA-PRESS method for better GSH detection, parameters (time of echo and pulse duration) of the MRS sequence were assessed, shown in Figure 3-8. An optimal MRS sequence for GSH detection was therefore developed, as shown in Figure 3-9 B. This sequence was superior in suppressing the GSH peak at 2.95 ppm compared to the pilot sequence Figure 3-9 A. This optimised sequence was then used to acquire brain MRS data for the next chapter (chapter 4).

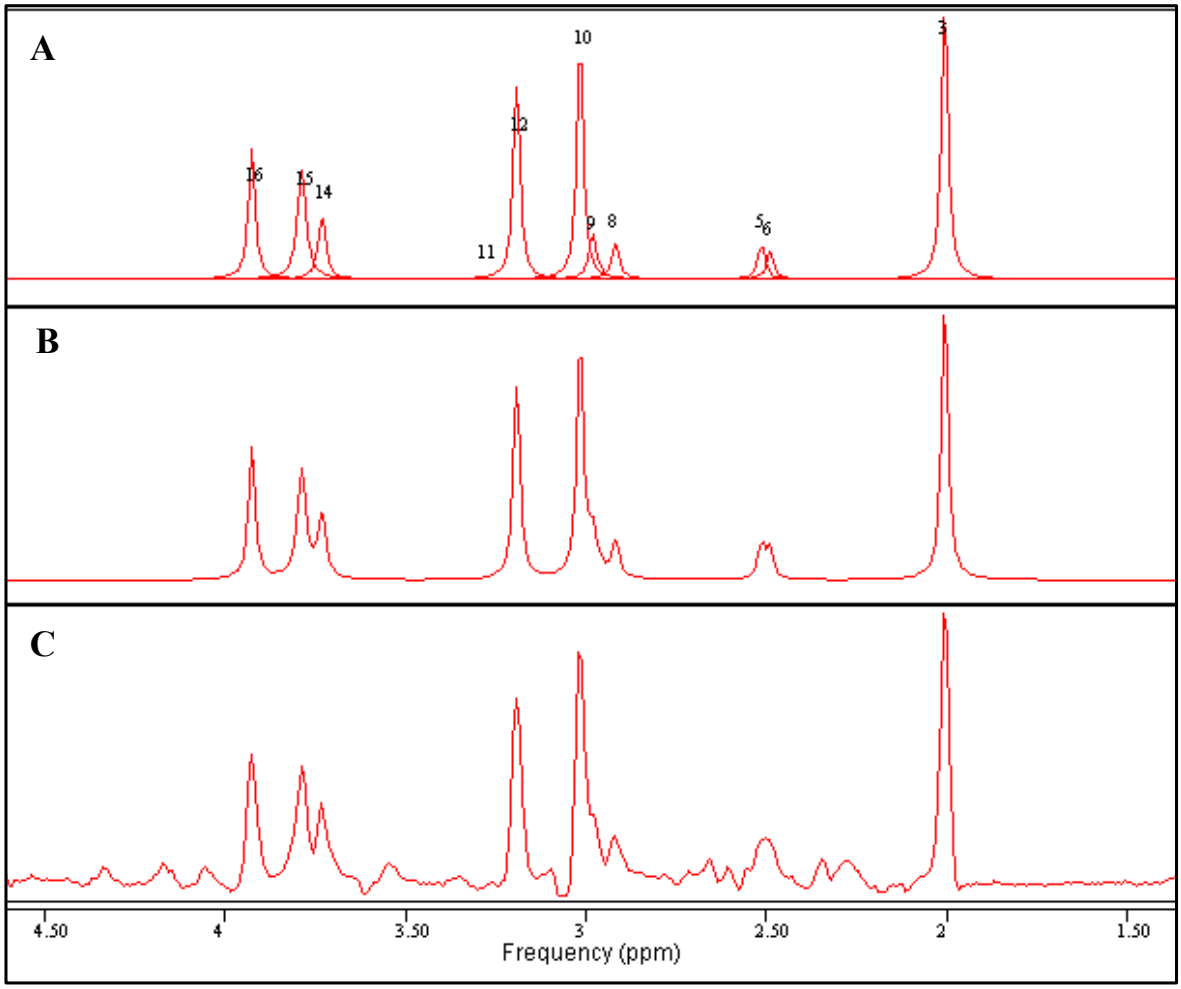


Figure 3-6 Peak fitting using AMARES

A) Metabolite peak estimation: Automatic peak fitting in jMRUI using the algorithm AMARES to quantify major metabolites such as N-aceylaspartate (peak numbered 3), creatine (peak numbered 10) and choline (peak numbered 12) in a spectrum obtained from a phantom containing 30 mM GSH. GSH peaks are numbered 8 and 9. **B)** Panel showing estimate fit of the original spectrum **C)** Panel showing original spectrum (edit-off spectrum).

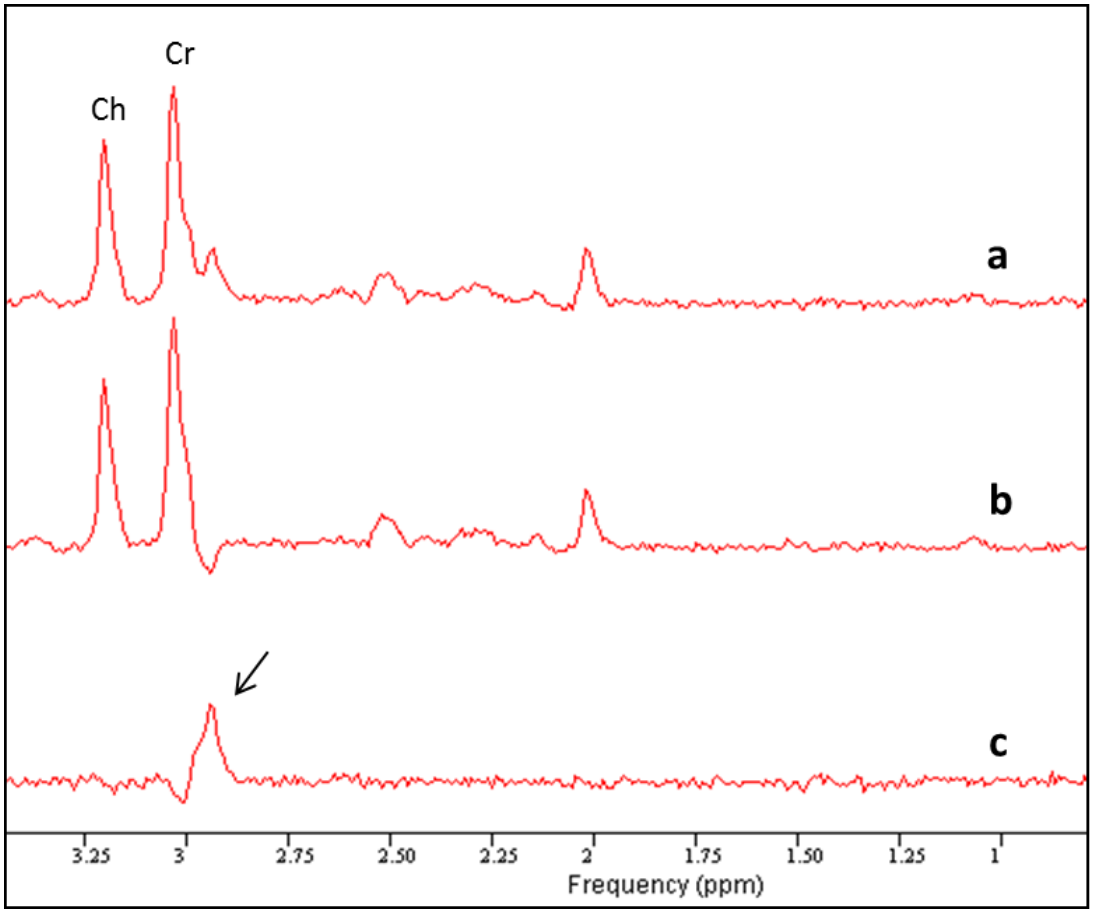


Figure 3-7 *In vitro* GSH quantification

GSH editing in a 20mM GSH phantom in spectra acquired using the optimised sequence parameters: **(a)** unsuppressed frequency spectrum (edit-off spectrum); **(b)** spectrum with MEGA-suppression (edit-on spectrum); **(c)** edited spectrum obtained on subtraction of spectrum (b) from (a). Arrow pointing to the GSH signal obtained on subtraction. Ch, choline ; Cr, creatine

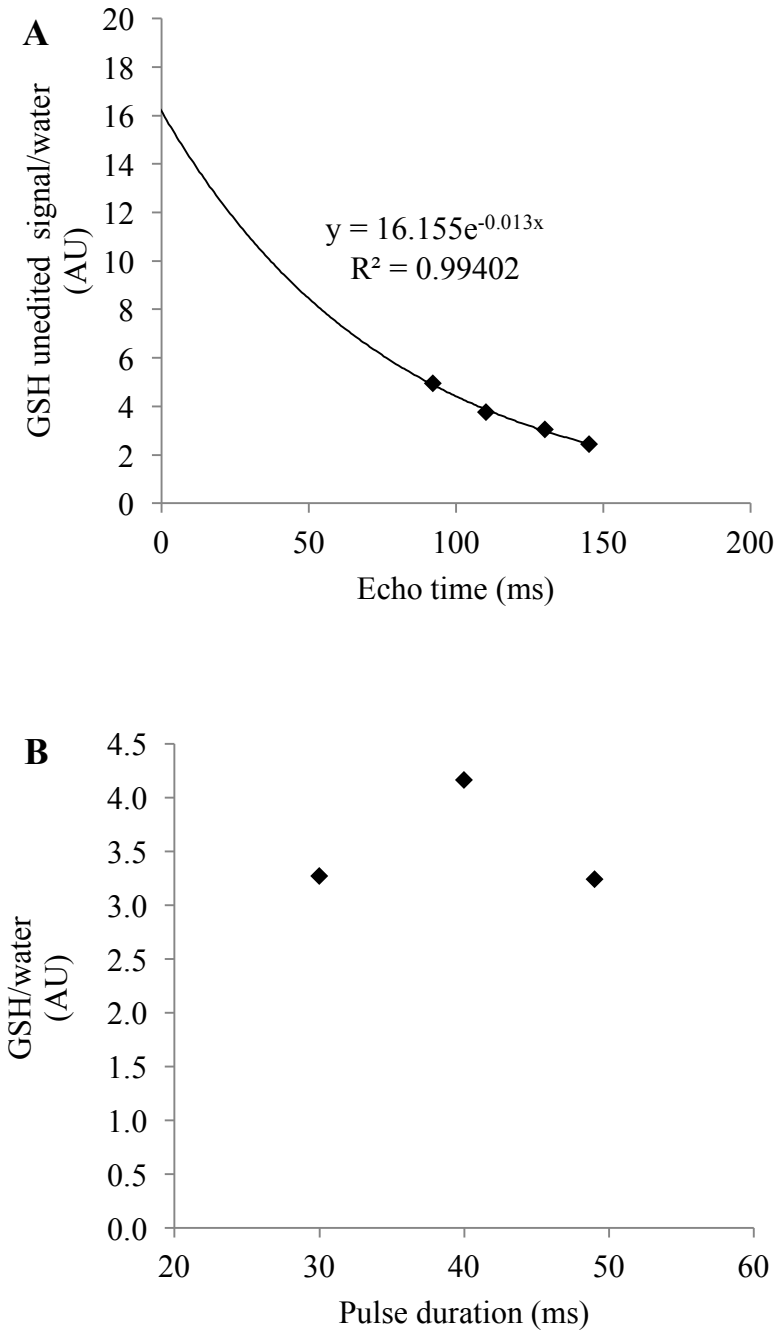


Figure 3-8 MRS sequence development *in vitro*

A) Graph showing exponential curve fit as a function of echo time used and GSH signal obtained using the MEGA-PRESS method. **B)** Graph showing relationship between pulse duration used and GSH signal obtained.

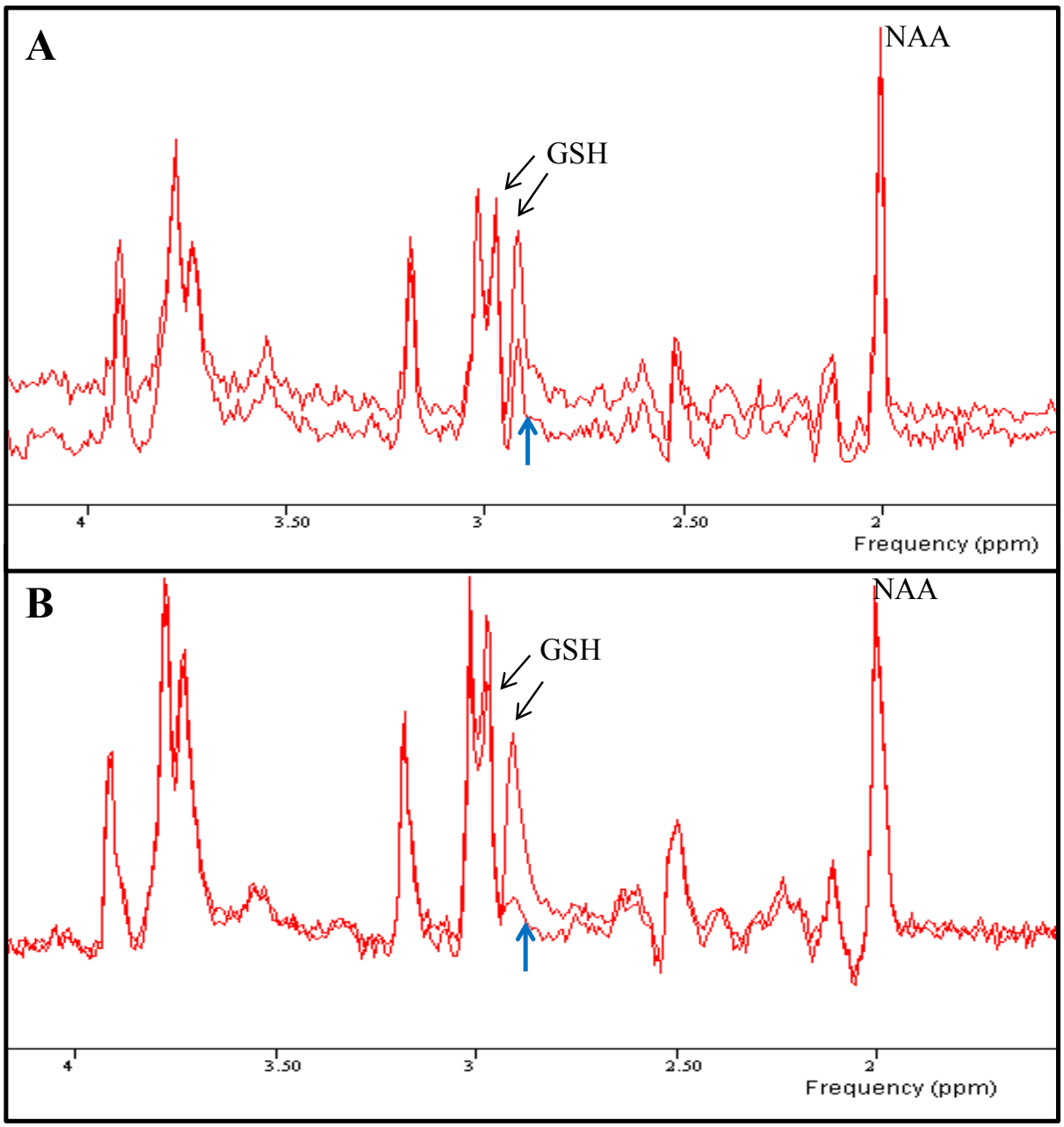


Figure 3-9 Comparison of developed MRS sequence

Spectra obtained using pilot sequence **(A)** and the developed sequence **(B)** in a phantom containing 60 mM GSH. Unsuppressed frequency spectrum (edit-off) and suppressed spectrum (edit-on) are superimposed. Thin arrows pointing to the double GSH peaks obtained in the unsuppressed edit-off spectrum. Thick arrows (blue) pointing to suppressed GSH peak obtained in the suppressed edit-on spectrum. Developed sequence (B) is superior in suppressing GSH compared to the pilot sequence (A). NAA, N-aceylaspartate; GSH, gluathione

3.4.2 Method development for GSH detection in the human brain

MRS sequence development for optimal detection of GSH in the brain was undertaken as described in section 3.3.6 section. The highest GSH signal was obtained with excitation at 4.56 ppm (Figure 3-10). Repeating the scan at 4.56 ppm excitation on a different day gave a CV of 17 %. The level of GSH signal measured across the various time of echo were similar- CV% was 4 % (shown in Figure 3-10).

After assessing the results from *in vivo* and *in vitro* experiments, the acquisition parameters for the MRS MEGA-PRESS sequence for data collection were set as follows: NSA = 8, dynamics = 128, echo time = 130 ms, TR = 1800 ms, pulse duration = 40 ms, phase cycles = 16, water suppression = vapour, water NSA = 32. It took 45 minutes to run the scan with this sequence, excluding the initial preparation time such as the structural scan. Figure 3-11 shows GSH editing in spectra acquired in a participant using this optimised sequence parameters.

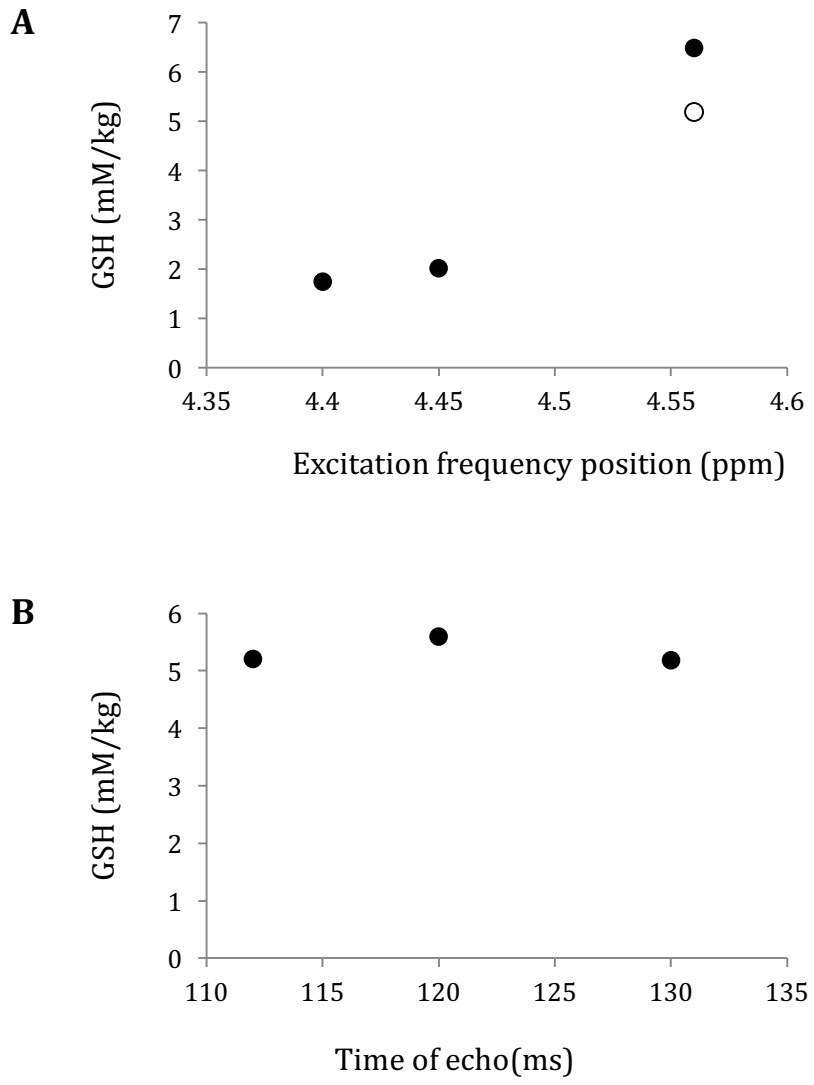


Figure 3-10 MRS method development *in vivo*

A) Graph showing the detection of GSH at various excitation frequency position of the selective 180° pulse for cysteine residue while keeping other experimental parameters the same. Closed circles data were collected on the same day, while open circle was collected on a different day. **B)** Graph showing the detection of GSH using different time of echo while keeping other experimental parameters the same. All scans for each parameter were acquired on the same day. All data were collected from a young male participant.

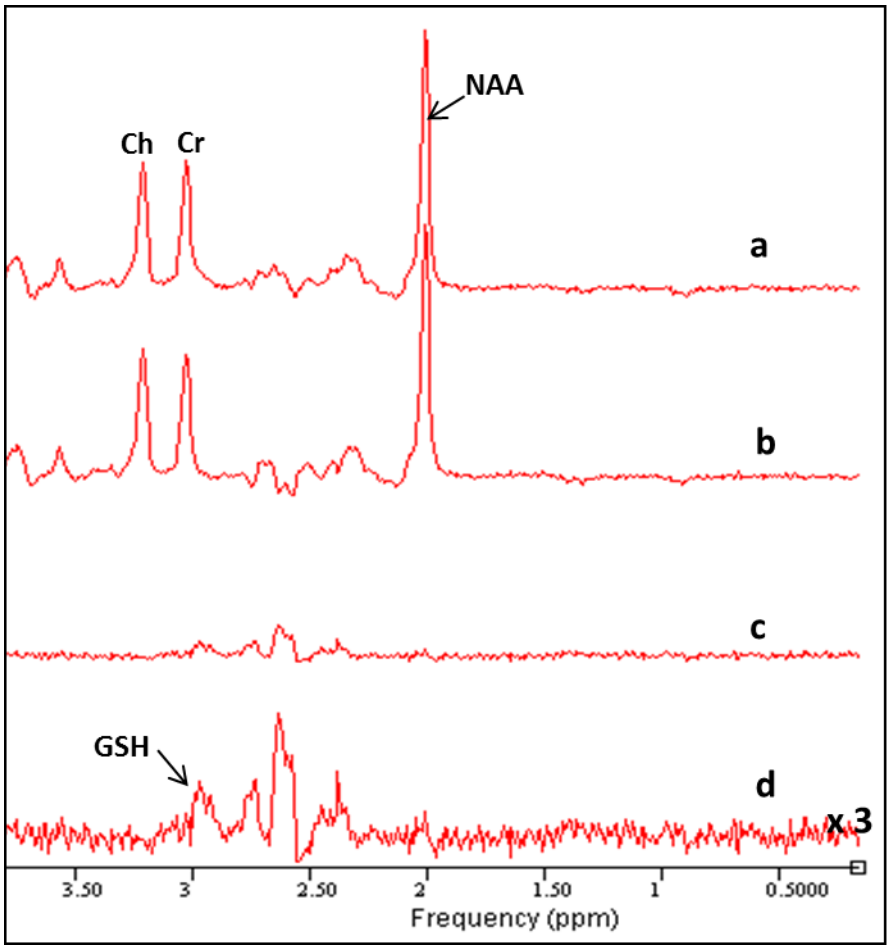


Figure 3-11 *In vivo* GSH quantification

(a) Unsuppressed frequency spectrum (edit-off); **(b)** suppressed spectrum (edit-on); **(c)** edited spectrum derived by subtraction of spectrum (b) from (a); **(d)** spectrum (c) multiplied by a factor of three. Arrow pointing to the GSH signal obtained on subtraction. Data was acquired in participant using the final optimised sequence parameters. Ch, choline; Cr, creatine; and NAA, N-aceylaspartate

3.4.3 Brain GSH through 6 hours

Figure 3-12A shows the mean brain GSH level over a 6 hour (at 0, 2, 3, and 5 hours from the first scan) period of two participants. Scans at time 2 hour and 3 hour were acquired in the same scanning session (i.e. the participant did not come out of the scanner in between these two scans). As can be seen in Figure 3-12A the GSH concentration did not change across time except the 3rd scan in one of the participants (closed circles). The CV for the four scans were 19 % (open circles) and 74 % (closed circles) respectively. The CV% for NAA/water for the two participants were 7% (open circles) and 26% (closed circles) respectively. Assessment of the NAA/water signal (shown in Figure 3-12B) detected an increased NAA/water at the 3rd scan in one of the participants (closed circles), which suggests that this was a 'noisy' scan. The concentration of other metabolites such as Cr and Ch also showed similar pattern of CV (data not shown). Removing this noisy scan, the average CV% for GSH concentration was 22%.

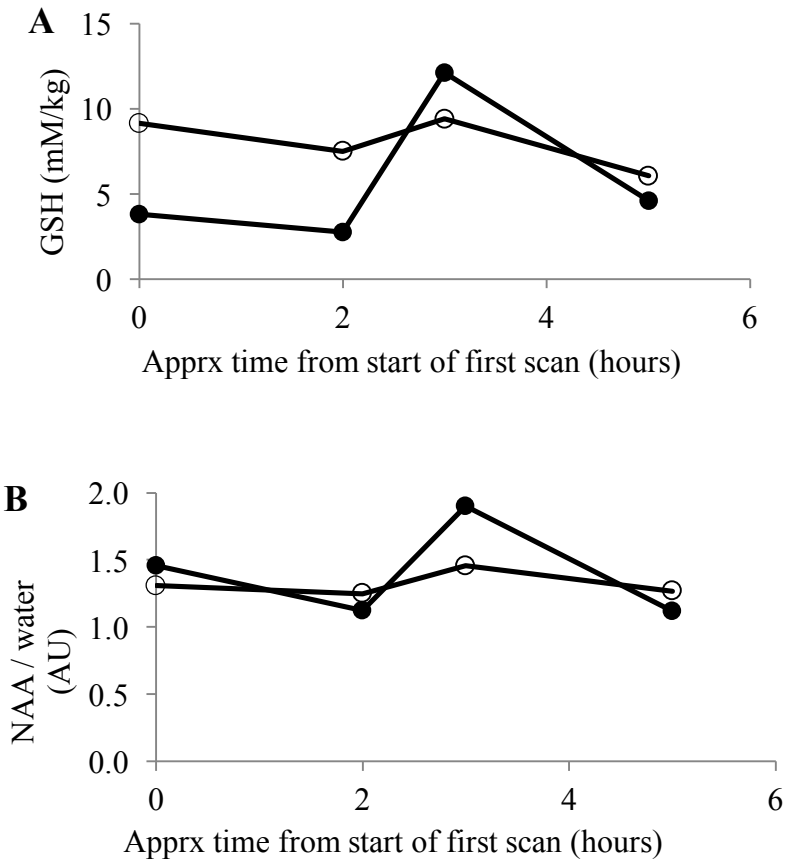


Figure 3-12 Brain GSH through 6 hours

A) Brain GSH through 6 hours in two participants. **B)** NAA/water signal of the two participants. The 3rd scan of the closed circle participant seems to be a noisy scan.

3.5 DISCUSSION

Using the MEGA-PRESS technique, measurement of GSH was optimised firstly using *in vitro* experiments. The measured GSH signal positively correlated to the GSH concentrations in the phantoms, which confirmed that the observed signal was GSH and that GSH could be successfully quantified using this technique. The results of the experiments presented herein suggest that the detected GSSG signal did not contribute to the detected GSH signal. This is largely in agreement to that observed by others at similar GSH concentrations (Satoh and Yoshioka, 2006, Terpstra et al., 2006). Oxidation of the GSH phantom with H_2O_2 resulted in decreased GSH signal at 2.95 ppm and the appearance of a new peak at 3.24 ppm in the spectrum, which suggested that GSH has been oxidised to GSSG. In agreement, Satoh and Yoshioka (2006), also using MEGA-PRESS, reported that the signal from GSSG was distinctive to that from GSH and that under normal conditions (GSH:GSSG ratio = 100:1) the GSSG signals were negligible, while at condition of strong oxidative stress (GSH:GSSG ratio = 10:1) the GSH signal was prominent and the GSSG signal was faint. While at GSH:GSSG ratio of 1:1, they found that the GSSG signal was pronounced and interfered with the GSH signal– however, this is a very extreme condition where neurons cannot survive (Satoh and Yoshioka, 2006). Therefore, it can be concluded that GSSG did not contribute to the GSH signal detected in the human participants that were scanned in the experiments described herein.

The MEGA-PRESS method for GSH detection in the brain was optimised in a male participant, by assessing various MEGA-PRESS parameters (time of echo and excitation position of the selective refocusing pulse). It was found that 4.56 ppm was the best excitation position of the selective refocusing of 180° pulse for the cysteine residue. This is in contrast to Mandal et al. (2012), who also used MEGA-PRESS and found that 4.4 ppm was the best

position and that 4.56 ppm gave the most inferior GSH spectra. The difference in the findings may be explained by variations in the settings and the methodology (including scan parameters such as time of echo and time of relaxation) used between the two studies.

The data presented herein suggests that brain GSH measured by MRS is reproducible using the method described herein. *In vitro* measurement of GSH in phantoms on two separate occasions on the same day gave a CV of 2 %. Repeat measurement of brain GSH on a different day and on the same day gave CV of 17 % and 22 % respectively. This CV of 22 % of brain GSH measured over 6 hour period indicate that brain GSH is stable over this period. Of the two participants scanned in this time course experiment, GSH levels in one participant were stable across 6 hours (CV 19 %), whereas in the other participant brain GSH appear to increase at the 3rd scan thus giving an overall CV 74 %. Other metabolite concentration (with reference to water) showed similar patterns in the respective participants and CV i.e. increased concentration at scan 3. It is highly likely that this increase observed in the 3rd scan in one of the participant is due to noise arising from scanner instability, physiologic motion (such as cardiac and respiratory activity) or both (Jansen et al., 2006, Lange et al., 2012). Although the measurement of brain GSH using the MRS method is sensitive to noise, the data presented suggests that brain GSH can be successfully measured repeatedly using this method. The data indicate that brain GSH is stable with time. Terpstra et al. (2011) have also reported brain GSH levels to be stable over a 24 hour period.

3.5.1 Post-processing and signal quantification

GSH concentration was quantified using jMRUI software. One of the limitations during quantification was that the GSH peak was selected manually before quantification by AMARES, which could lead to subjective bias. To avoid subjective bias as much as possible, all data were analysed together without referring to the experimental condition. Using

AMARES fitting for quantification in jMRUI requires some prior knowledge and some degree of manual input. Spectral quantification methods that use predefined models of spectral peak fitting such as LCModel have previously been used for quantifying GSH (Opstad et al., 2003, Terpstra et al., 2003, Terpstra et al., 2011, Wood et al., 2009). In comparison to LCModel software, jMRUI requires user interaction. Although LCModel is potentially better for the analysis of ^{1}H-MRS spectra (Mullins et al., 2014), relatively little work has been undertaken in measuring GSH using LCModel. The majority of work using this method of metabolite quantification has been undertaken in GABA quantification. Quantification of GABA acquired using MEGA-PRESS also has similar issues (as with GSH) of spectral overlap with other larger metabolites. GABA quantification has various predefined spectral peak fitting models such as LCModel, TARQUIN, and QUEST (in jMRUI), as well as custom-written GABA-MRS analysis software (Gannet) (Mullins et al., 2014). A study which assessed the reliability of these various quantification methods reported that the reproducibility of metabolite quantification (for GABA and also Glx: glumate+glutamine) was similar across AMARES (in jMRUI), Gannet, LCModel, and TARQUIN (O'Gorman et al., 2011). This suggests that jMRUI could have similar efficiency for GSH quantification as LCModel.

3.5.2 Tissue water as concentration reference

Tissue water was used as the concentration reference for the GSH data presented herein. External referencing (such as by simultaneously scanning known concentration of phantoms) may be prone to system instability, whereas the water and metabolite signal comes from the same voxel scanned and are acquired essentially in the same way (Mullins et al., 2014). The advantage of using tissue water as reference is that it removes potential sources of error that needs to be considered when using external concentration reference (Gasparovic et al., 2006).

Additionally, a large multicentre study which compared between external reference placement and internal water reference methods reported that the internal water reference gave better precision and inter-laboratory reproducibility (Keevil et al., 1998). Furthermore, in the method developed herein, the internal water concentration of GM and WM were taken into account.

3.6 CONCLUSION

An optimised MEGA-PRESS method was developed for the detection of GSH in the brain. The findings presented here suggest that GSSG does not contribute to the GSH signal as detected by this method. Repeated scanning within and between days indicated reproducibility of GSH quantification.

4 CHAPTER 4: Effect of acute exercise on markers of oxidative stress in the brain and the blood

4.1 ABSTRACT

The brain is highly susceptible to oxidative stress due to its high metabolic demand. In this study, the effect of acute exercise bouts on brain glutathione (GSH) in humans was measured for the first time. Brain GSH was measured using magnetic resonance spectroscopy (MRS), examining an anterior cingulate cortex voxel. To add context to these measures, GSH levels and other markers of oxidative stress were also assessed in the periphery (in blood) at three time points [pre-, immediately post- and post (~1 hour)- exercise]. In a cross-over design, young healthy sedentary males (n = 9) undertook a bout of continuous moderate exercise (65% workload max for 40 mins) and a high intensity interval (HII) exercise bout (200% workload max for 30 s with 4 mins recovery * 4 cycles) on separate days. In addition, in a time-course study, brain GSH was monitored for 6 hours in response to the moderate exercise trial (n = 4 exercise). Moderate exercise caused a significant decrease in brain GSH from 8.24 ± 1.59 mM/kg to 4.74 ± 1.13 mM/kg (n = 7, p = 0.002). Blood GSH levels increased immediately post-HII exercise, 580 ± 101μM to 692 ± 102 μM (p = 0.006). Total antioxidant capacity significantly increased post (~1 hour)-HII exercise from 1,125 ± 187 μM to 1,351 ± 152 μM (p = 0.003). Measure of lipid peroxidation (MDA levels) decreased post (~1 hour)-HII compared to immediately post-HII (11.6 ± 3.8 μM from 17.1 ± 9.9 μM, p = 0.01). The finding from this small cohort study suggests that an acute exercise caused a decrease in brain GSH, however statistical assessment is challenging due to the small sample size and the limitation of the MRS method. In blood, HII elicited a greater oxidative stress response compared to moderate exercise bout.

4.2 INTRODUCTION

The human brain is highly susceptible to oxidative stress as it consumes approximately 20 % of the oxygen utilised by the body although it only constitutes 2 % of the body weight. It has a high iron content that can catalyse ROS generation (Dringen, 2000). GSH is an antioxidant whose depletion can contribute to oxidative stress, and oxidative stress has been identified in the pathogenesis of various diseases affecting the brain such as Alzheimer's disease and Parkinson's disease (Aoyama and Nakaki, 2013). Decreased brain GSH, measured by MRS, has been reported in ageing (Emir et al., 2011) and in diseases such as Alzheimer's Disease (Mandal et al., 2012), epilepsy (Mueller et al., 2001) and schizophrenia (Do et al., 2000). Thus, a therapeutic strategy to increase the concentration of brain GSH, would therefore be desirable in these disorders. No therapeutic drugs are available for increasing brain GSH levels at present (Aoyama and Nakaki, 2013).

The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: i) the first and the rate limiting step is the formation of γ-glutamylcysteine from glutamate and cysteine, and then ii) formation of GSH from γ-glutamylcysteine and glycine. The highest levels of GSH are found in the liver, and approximately 1/3 to 1/2 of this GSH in the liver serves as a cysteine reservoir that can be released into the blood as necessary. The majority of GSH produced by the liver does not reach the brain due to the brain blood barrier (BBB) where it can penetrate only poorly by passive diffusion. Cysteine is also unable to penetrate the BBB, however, the disulfide form with two cysteines called cystine is capable of passing the BBB into the CSF via the transporter LAT1. The inability of most cell types to effectively import intact GSH suggests *de novo* GSH biosynthesis is necessary to maintain neuronal GSH levels. GSH synthesis in brain cells follows the same pathway as in other

tissues (Dringen, 2000). Cell-to-cell metabolic interaction occurs in the brain- with astrocytes providing precursor amino acids to neighbouring neurons necessary for GSH biosynthesis.

Export of intact GSH from blood into the brain is unlikely to influence GSH levels in the brain (Zeevalk et al., 2008) and notably, blood makes up only around 3% of the volume in the brain (Holmay et al., 2013). Over the last decades numerous studies in humans have shown that acute exercise alters peripheral redox status including that of GSH system (Fisher-Wellman and Bloomer, 2009), however, to assess this in the brain is challenging due to difficulty in accessing the brain and in acquiring samples from the brain. In rodents, exercise training has been shown to increase brain GSH levels (Ohkuwa et al., 1997). MRS is an emerging technique to measure brain GSH. Although this method has its limitations, especially in its ability to measure transient changes in brain metabolites, recently a small proof-of-concept pharmacodynamics study reported measurement of increased brain GSH using MRS method in humans (Holmay et al., 2013).

Radak et al. (2005) extended the hormesis theory to the free radical generating effects of exercise, encompassing a low-dose stimulation or beneficial effect through to a high-dose inhibitory or toxic effect. Hormesis relating to free radical generation can be affected by parameters such as exercise duration, intensity and frequency in addition to training status. Therefore, assessing the effect of each exercise parameter on radical species may be a crucial step in determining the 'best' exercise strategy for optimising brain structure and function (Lucas et al., 2015). Two epidemiological studies reported exercise intensity to be more important than exercise duration in preventing cardiovascular disease (Tanasescu et al., 2002, Wisloff et al., 2006), and there is evidence to suggest that exercise intensity is a determinant of the magnitude of free radical formation in the bloodstream (Bailey et al., 2004, Lamprecht et al., 2008, Quindry et al., 2003, Wang and Huang, 2005).

HII exercise involves repeatedly exercising at a high intensity for between 30 seconds and 2 minutes, with each high intensity interval separated by 1-5 minutes of recovery consisting of rest or low intensity exercise. HII involves substantially lower time commitment and reduced total exercise volume, and some people suggest this form of exercise is more enjoyable compared to longer sessions of moderate-intensity continuous exercise (Gibala et al., 2012). Emerging evidence indicates that HII provides equivalent if not indeed superior metabolic (Burgomaster et al., 2008, Little et al., 2010, Molmen-Hansen et al., 2012, Sijie et al., 2012, Trapp et al., 2008) and cardiac (Currie et al., 2013, Guiraud et al., 2012b, Lalande et al., 2010, Weston et al., 2014, Wisloff et al., 2007) adaptions. None of the previous studies have looked at the effect of HII in the brain.

The maintenance of adequate cerebral blood flow (CBF) to the metabolically active brain is critical to maintain a constant supply of oxygen and nutrients (Murrell et al., 2013). Exercise exerts a different response in the cerebral vasculature compared to that in the peripheral vasculature (Ogoh and Ainslie, 2009). During low to moderate intensity exercise there is an elevation in CBF, up to an exercise intensity of around 60% of maximal oxygen uptake, after which CBF decreases despite the cerebral metabolic demand. During higher intensity exercise, the changes in global CBF are not matched with brain neuronal activity and metabolism (Ogoh and Ainslie, 2009). Even though HII has been shown to have superior, if not equivalent, adaptation benefit to cardiovascular health as mentioned previously, no previous study has looked at CBF in response to HII exercise. Assessing HII induced CBF response has the potential to reveal i) the underlying mechanism of improved cardiovascular and metabolic benefits of HII compared to continuous exercise, and ii) the mechanisms linking the periphery and the brain.

The aim of the study was to investigate the effect of an acute bout of HII exercise versus continuous (steady state) moderate exercise on GSH in the brain. To give context, markers of oxidative stress and BDNF (the most abundant growth factor in the brain) were assessed in the blood. CBF was measured during the two types of exercise to investigate any linking mechanism between the periphery and the brain in response to the exercise bouts. To our knowledge, this is the first study to assess the effect of exercise on brain GSH. Also, this is the first study to look at the effect of HII on CBF.

4.3 METHODS

4.3.1 Participants

Healthy males who did not undertake in more than 2 hours of moderate exercise per week were recruited to this study. Nine participants were recruited to the cross-over study, and an additional four participants were recruited to the time-course study. Participants had not taken vitamin supplements for at least six weeks prior to recruitment and were healthy as assessed by a general health questionnaire. Participants, who were scanned (n = 8), also had to be free from any contraindication to be able to undertake an MRI scan, such as having metal implants. All participants gave written informed consent. The study was approved by the University of Birmingham ethics committee.

4.3.2 Cross-over exercise study

Participants (n = 9) visited the laboratory to undertake exercise having been asked to fast for at least 4 hours prior to each visit. Participants were also asked to refrain from exercising and drinking alcohol 24 hours prior to the visits. All 3 visits were separated by at least 3 days. All participants were assessed for peripheral markers of oxidative stress in response to the two

exercise bouts, but only four participants were assessed for brain GSH due to costs. The MRS scans were started between 17:00 and 19:30.

A schematic diagram of the study protocol is presented in Figure 4-1. For the first visit, participants were accompanied to the Birmingham University Imaging centre (BUIC) (around 10 minutes walk from the exercise laboratory), where they undertook an MRS scan (baseline scan) to assess brain GSH. After the scan, the participants returned to the exercise laboratory to carry out a graded exercise test (max test) on a cycle ergometer by cycling to volitional exhaustion. Maximal oxygen consumption ($\dot{V}O_2max$) and maximum work load were calculated from this test.

During the second and the third visit, the participants took part in either HII exercise or a continuous moderate exercise (the sequence of the exercise trial was counterbalanced between participants). Following arrival to the lab, an indwelling catheter was inserted into an antecubital vein in the forearm, which was kept patent by flushing regularly with saline. A pre-exercise blood sample was taken. Blood flow velocity of the middle cerebral artery (MCAv) was then assessed by transcranial Doppler (TCD) for 3 minutes at rest while sitting on a chair. The participant then began the exercise trial. Following the exercise bout, a blood sample was immediately taken (immediately post-exercise sample) by the catheter. The participant then walked to BUIC for a post-exercise MRS scan for brain GSH measurement. A final blood sample (post-exercise sample) was taken by venepuncture ~1 hour after end of exercise.

4.3.3 Time course study: the effect of exercise on brain GSH across a time-course

To investigate brain GSH levels 4 hours post-acute exercise, an additional four participants were tested. The participants were scanned four times over a period of 6-7 hours (at

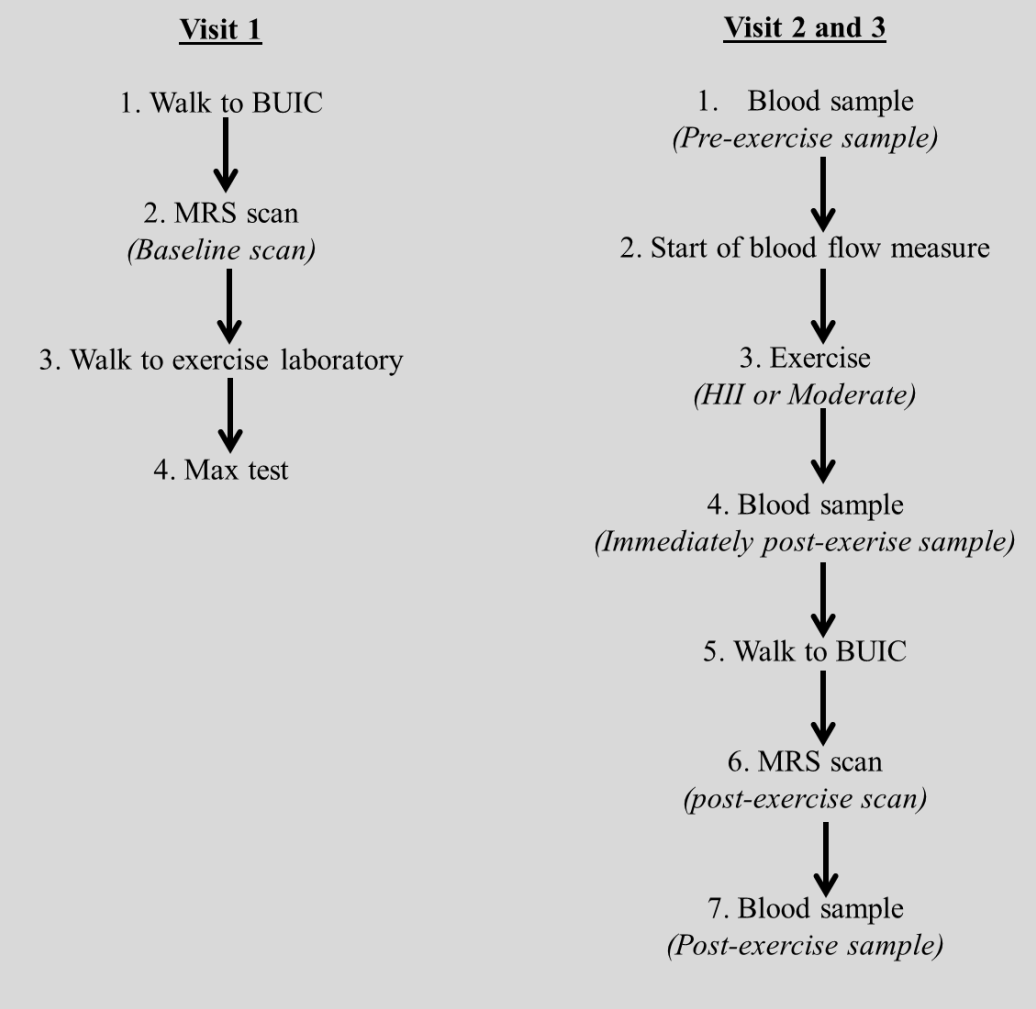


Figure 4-1 Schematic diagram of cross-over exercise study

BUIC, Birmingham University Imaging Centre; MRS, magnetic resonance spectroscopy; and HII exercise, high intensity interval exercise

approximately 0, 2, 3 and 5 hours from the first scan), starting in the afternoon between 13:00 to 17:00. A schematic diagram is presented in Figure 4-2. Participants took part in continuous moderate exercise as described in the "Exercise trials" in 4.3.4 after completing the first scan. Immediately after the end of the exercise, participants were taken to the scanner for the second scan. The second and the third scans were undertaken in the same scanning session, i.e. the participant did not come out of the scanner between these two scans. The participants were asked to come fasted for at least four hours prior to the visit. When the participants were outside the scanner in between the scans, the participants were free to do anything as long as they did not take part in strenuous physical activity (participants usually used/worked on their electronic or smart devices).

4.3.3.1 Effect of acute moderate exercise on brain GSH

Overall 8 participants were scanned post-moderate exercise, four took part in the cross-over study (baseline scan having been done prior on a different day) and four participants in the time-course study (baseline scan carried out on the same day before starting exercise). The brain GSH results from these 8 data sets with their respective control/baseline measurement were assessed for statistical difference.

4.3.4 Exercise trials

All exercise tests were performed on a cycle ergometer (Lode Excalibur Sport, Netherlands). Max test (incremental exercise test starting at 60 W and increasing by 35 W every 3 minutes until exhaustion) was undertaken to calculate individual $\dot{V}O_2max$, during which breath-by-breath measurements (Oxycon Pro, Jaegar, Germany) were recorded. Heart rate was monitored (using Polar, Finland) throughout the bout, and rating of perceived exertion (RPE) (Borg, 1973) recorded during the final minute of each stage. Maximal workload was

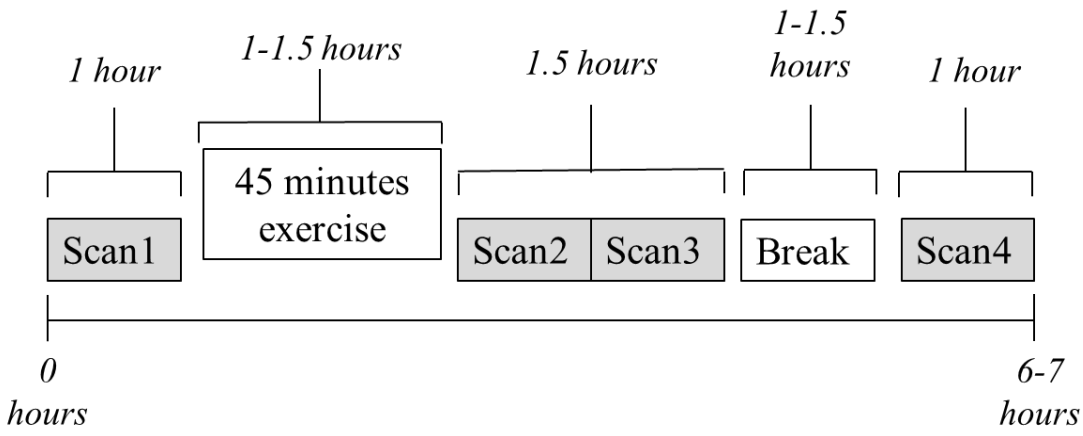


Figure 4-2 Schematic diagram of the time course study

calculated as

Workload max = last completed work load + (time spent in the final non-completed work rate × work rate increment)

Schematic representation of the HII and the continuous moderate exercise trials are shown in Figure 4-3. The HII exercise trial consisted of cycling at 200 % workload max for 30 seconds with 4 minutes recovery time with 4 cycles (18 minutes total time). The continuous moderate exercise trial consisted of continuous cycling at 65 % workload max for 20 minutes and then at 55 % workload max for last 20 minutes (total 40 minutes) [the work load was decreased in the last 20 minutes to match the oxygen consumption in the first 20 minutes (Hagberg, Mullin and Nagle, 1978)]. Both exercise bouts were preceded with a warm up (3 minutes) and followed by a cool down (2 minutes for continuous moderate bout) cycling at 40 % workload max.

4.3.5 Measurement of brain GSH

GSH was measured in the brain at anterior cingulate cortex using MRS method as described in chapter 3. In brief, acquisition parameters for the MEGA-PRESS were as follows: NSA = 8, dynamics = 128, echo time = 130 ms, TR = 1800 ms, pulse duration = 40 ms, phase cycles = 16, water suppression = vapour, water NSA = 32.

4.3.5.1 Analysis: Quantification of brain GSH

Brain GSH quantification from the MRS scans were undertaken as described in chapter 3.

4.3.6 Markers of oxidative stress and other biomarkers in blood

Blood samples (6 ml) were collected in EDTA vacutainers. For GSH analysis, blood samples were immediately aliquoted and frozen in liquid nitrogen. The remaining blood was placed on

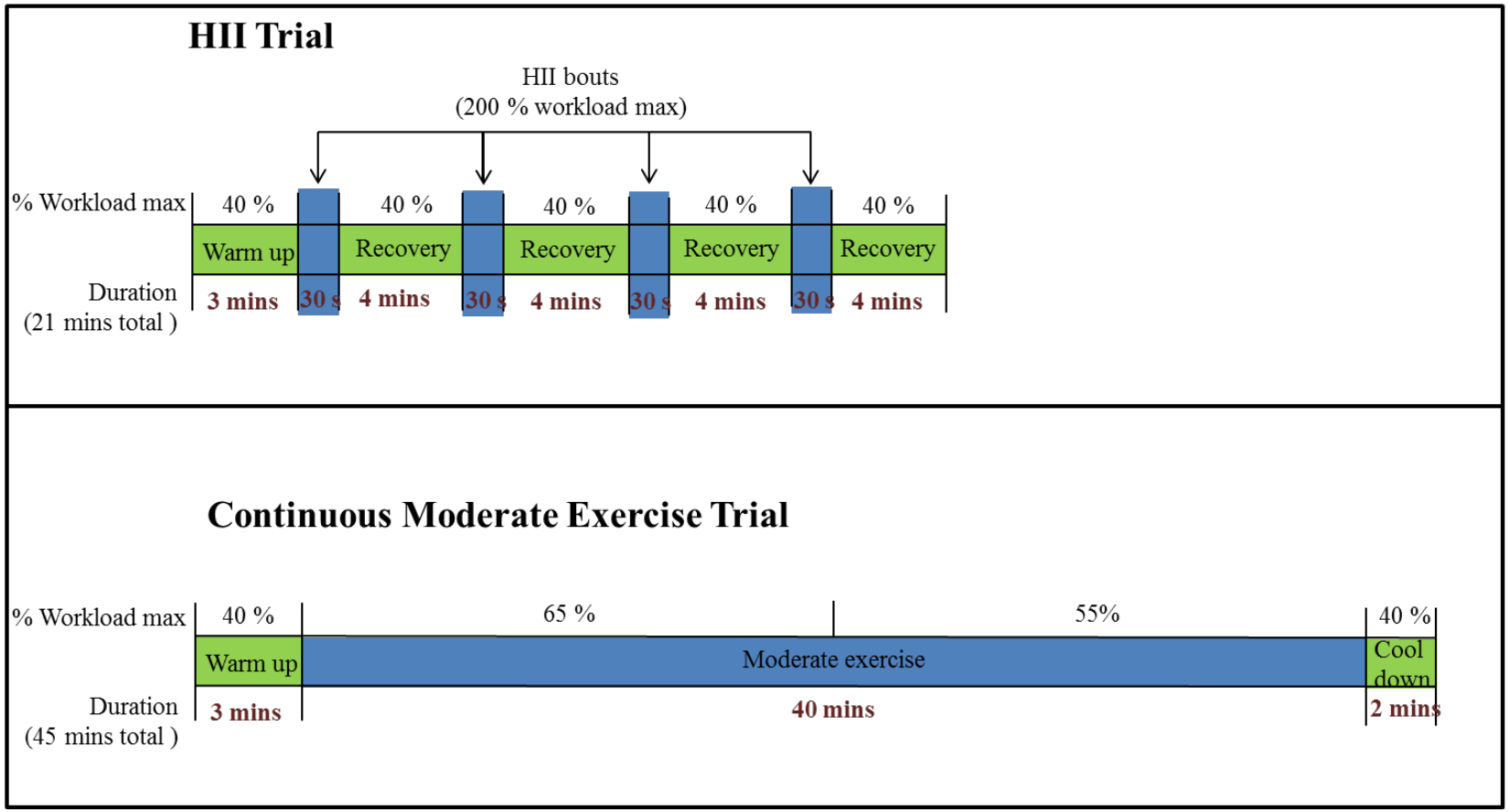


Figure 4-3 Schematic diagram of the two exercise bouts

Figure showing schematic diagram of HII and continuous moderate exercise trials. Maximum workload of individual participants were calculated from the max test, which was then used to calculate the workload for various phases of the exercise trial as shown in the figure.

ice until processing for plasma samples by centrifugation at 2,800 rpm for 15 minutes at 4 °C. The samples were then stored at -70 °C.

4.3.6.1 Glutathione

GSH in whole blood was measured using a commercially available kit (GSH-Glo Glutathione Assay V6911, Promega, USA) according to the manufacturer's instructions. The assay quantifies GSH using bioluminescence, based on the conversion of luciferin derivative into luciferin in the presence of GSH, catalysed by glutathione S-transferase. In brief, whole blood was first lysed with tricine. Then, the supernatant was transferred to multiwell plate with luciferin-NT substrate and glutathione s-transferase. After incubation with luciferin detection reagent, the luminescence was read. Mean intra-assay CV was 1 %.

4.3.6.2 Total antioxidant capacity

Total antioxidant capacity in plasma samples was determined by the FRAP assay as described in the general methods chapter (section 2.2.1).

4.3.6.3 Lipid peroxidation measurement

4.3.6.3.1 Malondialdehyde (MDA) measurement

Lipid peroxidation in plasma samples was determined by the MDA assay as described in the general methods chapter (section 2.2.2).

4.3.6.3.2 8-isoprostane

Additionally, lipid peroxidation was measured in plasma by determining 8-isprostane level using a commercially available kit (8-Isoprostane EIA kit 516351, Cayman Chemical, USA). This competitive ELISA is based on the competition between 8-isoprostane and 8-isoprostane tracer for 8-isoprostane-specific binding (to an antiserum). In brief, sample (1:1 dilution) and standards were incubated with tracer and antiserum in respective wells of the multiwell plate that is pre-coated with antibody. After 18 hours incubation, the wells were washed to remove

any unbound reagents. Colour was developed using Ellman's reagent and the absorbance was read. The absorbance is inversely proportional to the amount of 8-isoprostane in the samples/standards. Mean intra-assay CV was 8 %.

4.3.6.4 Protein carbonyl

Protein carbonyl was measured in plasma samples as described in the general methods chapter (section 2.2.4).

4.3.6.5 Brain derived neurotropic factor:

BDNF was measured in plasma samples as described in the general methods chapter (section 2.2.5).

4.3.6.6 Cerebral blood flow measure during exercise

Cerebral blood flow (CBF) was assessed in response to the two exercise trials in the 4 participants who took part in the cross over study, in section 4.3.2. MCAv was assessed using TCD as described in general methods chapter (section 2.3). Participants breathed through a respiratory mask attached to a Y-shaped two-way non-rebreathing valve. Breath-by-breath pressure of end-tidal CO_2 ($PETCO_2$) was sampled from the mask and measured using a gas analyser. Cerebrovascular hemodynamics (MCAv) and respiratory variable were acquired continuously using an analog-to-digital converter (Powerlab; ADInstruments) interfaced with a computer, and were subsequently analysed using commercially available software (Lab Chart, ADInstruments).

4.3.7 Statistical Analysis

Data were inspected for normal distribution using the Shapiro-Wilk test. Data which were not normally distributed were either transformed for normal distribution prior to statistical analyses (for example FRAP) or were tested using appropriate non-parametric tests. Analysis on the effect of exercise bouts on brain GSH were assessed using one-way repeated measure

analyses of variance (ANOVA). Analyses on the effect of two exercise trials (HII and moderate exercise trial) at each of the three time points (pre-, immediately post- and post (~1 hour)- exercise) on markers of oxidative stress and BDNF levels in the blood/plasma were performed using a two-way repeated measure ANOVA. Where appropriate, post hoc analyses were conducted to explore the main effects in more detail. Statistical significance was accepted at the $p<0.05$ level. Data were analysed using SPSS 22.0 statistical package for Windows (SPSS Inc, USA). Data are presented as means ± standard deviation of the mean (SD), unless otherwise stated.

4.4 RESULTS

4.4.1 Declaration of ownership

Due to the regulations surrounding operation of the scanner, all of the MRS scans were carried out by Dr Alimul Chowdhury or Dr Renate Reniers in the presence of the author of this thesis. All the data analysis presented herein was carried out by the author.

4.4.2 Participants

Table 4.1 shows the participant demographics.

4.4.3 Effect of exercise on brain GSH

4.4.3.1 Effect of HII and moderate exercise on brain GSH

The result in Figure 4-4 shows brain GSH post-exercise (HII and moderate bout) compared to control/baseline GSH concentration (without any exercise). One-way repeated measure ANOVA showed that exercise caused a significant difference in brain GSH $F(2, 6) = 29.19$, $p < 0.01$. Bonferroni post-hoc analysis showed a significant decrease in GSH level post-moderate exercise compared to baseline, 9.15 ± 1.45 mM/kg to 4.68 ± 1.47 mM/kg ($p = 0.031$), and also this post-moderate GSH decrease was significantly lower compared to post-

Table 4.1 Participant characteristics

Characteristics	Cross-over Study (n = 9)	Time course study (n = 4)	Both studies combined (n = 13)
Age (years)	25 ± 4	23 ± 5	25 ± 5
Measured V̇O2max ($ml.kg^{-1}.min^{-1}$)	40 ± 8	42 ± 7	41 ± 7

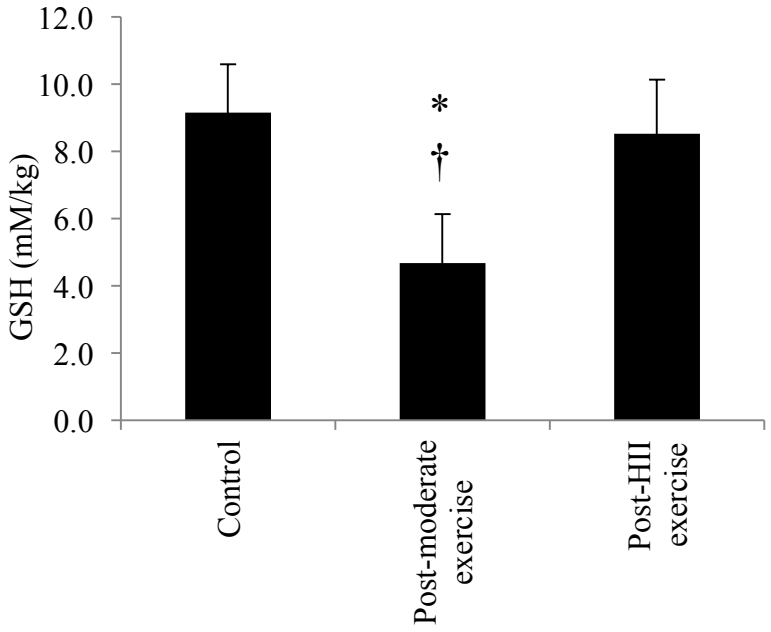


Figure 4-4 Effect of HII and moderate exercise on brain GSH

Brain GSH levels were measured post-exercise (HII or moderate exercise) and compared with control/baseline (without exercise) measurement, carried out on separate days (n = 4). Pairwise comparisons: * indicates significant difference relative to control and † indicates significant difference compared to post-HII GSH concentration.

HII GSH, 8.52 ± 1.61 (p = 0.031).

4.4.3.2 Monitoring the effect of exercise on brain GSH over 4 hours post-exercise

Figure 4-5 A shows the time course of GSH measured over six hours, including four hours post-exercise (n = 3). Out of 4 participants assessed, one participant was removed due to noisy signal; the NAA/water signal CV % was 31 % (NAA/water signal CV % for other participants were less than 14 %). GSH was measured before exercise and at 3 different times post-exercise. Figure 4-5 B shows additional data from the time course study (presented in Chapter 3 section 3.4.6) where the participants did not do any exercise.

4.4.3.3 Effect of acute moderate exercise on brain GSH level

Data from the 7 participants who were scanned post-moderate exercise (n = 3 time-course study with control and post-exercise scans undertaken on the same day; and n = 4 cross-over study with control and post-exercise scans undertaken on different days) were tested for statistical difference in brain GSH (Figure 4-6). Paired sample t-test showed that there was a significant decrease in GSH following moderate exercise from 8.24 ± 1.59 mM/kg to 4.74 ± 1.13 mM/kg, t (6) = 5.24, p = 0.002. There was no difference in baseline GSH levels between the two study designs (independent t-test of the baseline levels was not significant, p = 0.074).

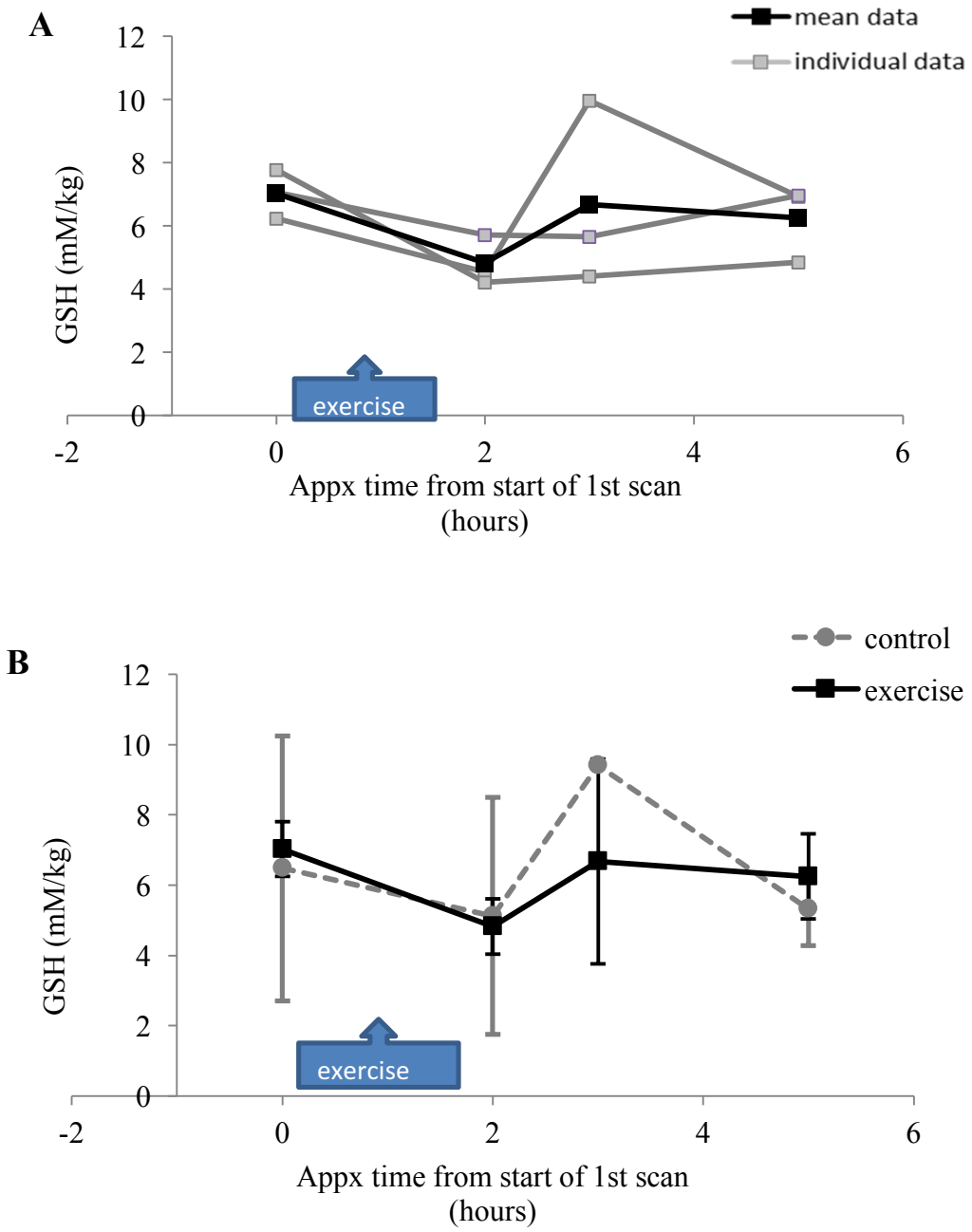


Figure 4-5 Monitoring the effect of exercise on brain GSH through 6 hours

A) Brain GSH was monitored for 6 hours in response to an acute moderate exercise (n=3). Exercise was undertaken after the first scan, after which the participant immediately went back to the scanner for the second scan. The black and the grey line represents mean data and individual data respectively. **B)** Mean data of participant who took part in moderate exercise (from graph A, n = 3) and control participants who did not exercise (data from chapter 3, n = 2). One noisy scan (3rd scan) has been removed from one of the control participant.

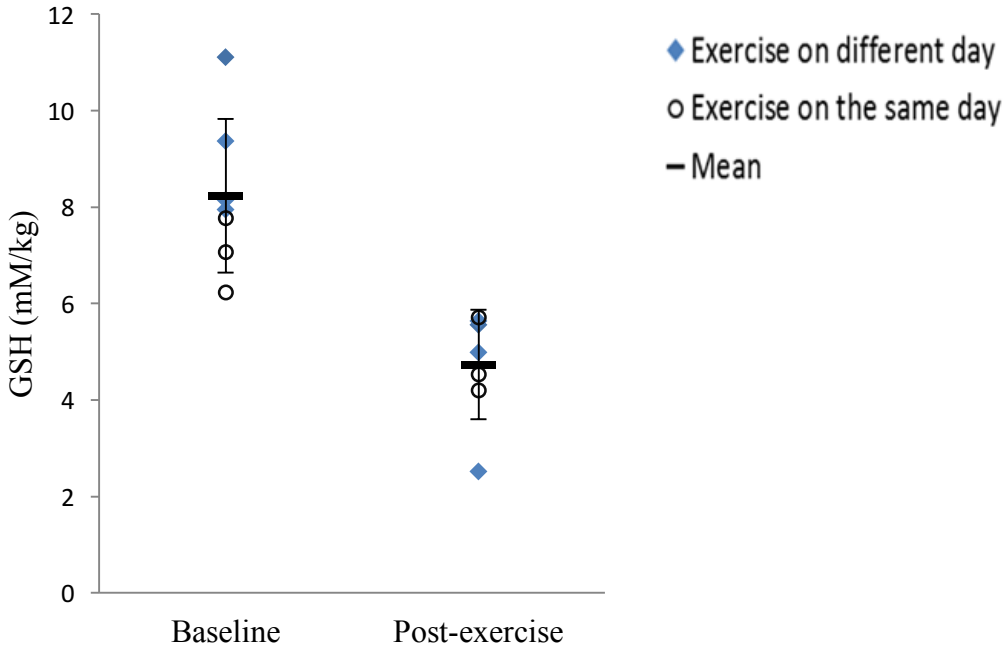


Figure 4-6 Brain glutathione in response to moderate exercise

Brain GSH was measured at baseline (without exercise) and after moderate exercise (post-exercise) (n = 7). Three participants (open circles) undertook the exercise on the same day after the baseline measurement. Four participants (closed diamonds) undertook the exercise on a different day after the baseline GSH measurement. Horizontal line represents the mean of all participants. There was significant decrease in GSH concentration from control to post-exercise ($p < 0.01$).

4.4.4 Markers of oxidative stress and other biomarkers in blood of the cross-over study

4.4.4.1 GSH in whole blood

GSH was assessed in whole blood [pre-, immediately post- and post (~1 hour)- exercise] in response to the two different exercise bouts (n = 8) (Figure 4-7). A two-way repeated measure ANOVA showed that there was a significant main effect of the type of exercise trial (moderate and HII) on GSH level, $F(1, 7) = 14.8$, $p = 0.006$. Similarly, there was significant main effect of time on GSH level, $F(2, 14) = 7.0$, $p = 0.008$, and contrasts revealed that the significant difference was between pre- and immediately post- exercise GSH levels ($p = 0.004$). There was a significant interaction effect between the type of exercise and the time point on GSH, $F(2, 14) = 9.22$, $p = 0.003$. Contrasts revealed a significant difference between pre- and immediately post- exercise comparing the two exercise trials, $F(1, 7) = 31.4$, $p = 0.001$.

Comparison of the two exercise trials at each time points revealed a significant difference in GSH levels at immediately post-exercise (692 ± 102 μM for HII and 598 ± 90 μM for moderate exercise), $F(1, 7) = 20.1$, $p = 0.003$. Whereas, there was no significant difference at pre-exercise ($p = 0.63$) and a borderline significant difference at post (1~ hour)- exercise (0.05) in GSH levels. Further analysis of the individual exercise trial (moderate and HII) using one-way ANOVA revealed that a significant increase in GSH level occurred at immediately post- HII exercise from the pre-HII level (692 ± 102 μM from 580 ± 101μM), $p = 0.006$.

4.4.4.2 Total antioxidant capacity

Figure 4-8 shows total antioxidant capacity in plasma [pre-, immediately post- and post (~1 hour)- exercise] in response to the two different exercise bouts (n = 8). As the data was not

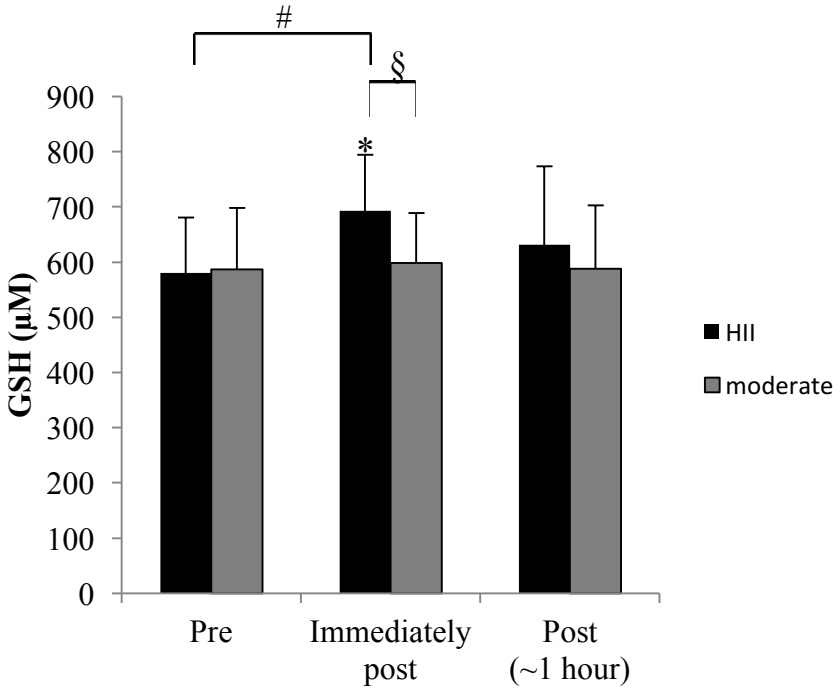


Figure 4-7 GSH in periphery

GSH in whole blood was measured in response to the two exercise bouts (n = 8). # indicates significant difference between pre and immediately post levels comparing the two exercise bouts (HII and moderate exercise). Pairwise comparisons: § indicates significant difference between the two exercise bouts and * indicates significant difference from pre-HII.

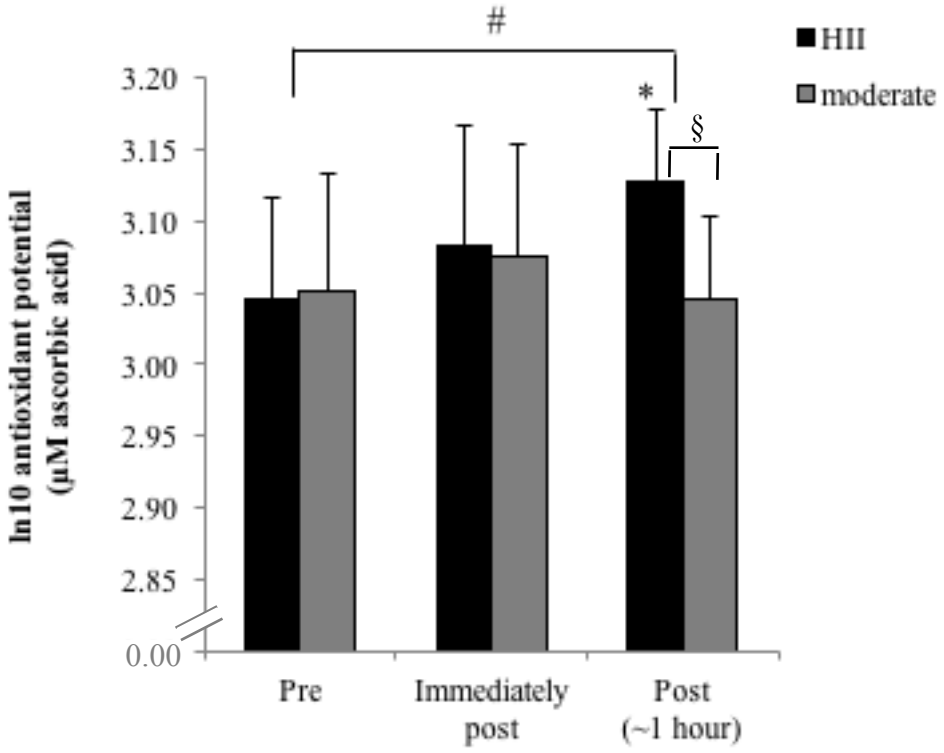


Figure 4-8 Total antioxidant capacity

Mean plasma total antioxidant capacity pre-, immediately post- and post (~1 hour)- exercise (n = 8). Data was ln10 transformed for normal distribution. # indicates significant difference between pre and post levels comparing the two exercise bouts (HII and moderate exercise). Pairwise comparisons: § indicates significant difference between the two exercise bouts and * indicates significant differences at the post (~1 hour)-exercise, relative to pre-exercise for the HII trial ($p < 0.05$).

normally distributed, data was normalised by ln10 transformation. A two-way repeated measure ANOVA showed that there was a non-significant main effect of the type of exercise trial on antioxidant capacity, F (1, 7) = 4.21, p = 0.079. Whereas, there was a significant main effect of time, F (2, 14) = 5.3, p = 0.02, and contrast revealed that the significant difference was between pre- and post (~1 hour)- exercise (p = 0.02). There was a significant interaction effect between the type of exercise and the time point on antioxidant capacity, F (1.1, 8.08) = 6.3, p = 0.03. Contrasts revealed a significant difference between pre and post antioxidant capacity comparing the two exercise trials, F (1, 7) = 9.18, p = 0.02.

Comparison of the two exercise trials at each time points revealed a significant difference in total antioxidant capacity at post (1~ hour)- exercise (1351 ± 152 μM for HII and 1118 ± 146 μM for moderate exercise), F (1, 7) = 25.7, p = 0.001. Comparison of total antioxidant capacity between the two exercise conditions at pre- and immediately post- exercise were not significant (p = 0.78 and p = 0.76 respectively). Further analysis of the individual exercise bouts using one-way ANOVA revealed that a significant increase in antioxidant capacity occurred post (~1 hour)-HII relative to pre-HII (1351 ± 152 μM from 1125 ± 187 μM) (p = 0.003).

4.4.4.3 Lipid peroxidation

4.4.4.3.1 Malondialdehyde levels

The result in Figure 4-9 A shows MDA levels (reciprocal transformation) in response to the two exercise bouts (moderate and HII) at the three time points: pre-, immediately post- and post (~1 hour)- exercise (n = 8). As data was not normally distributed, data was normalised by reciprocal transformation. Two-way repeated measure ANOVA showed no significant main effect of the type of exercise bout on MDA levels F (1, 7) = 0.35, p = 0.57. There was a significant main effect of time, F (2, 14) = 5.58, p = 0.01, however contrasts did not revealed

a significant difference in MDA levels when comparing immediately post-exercise and post (~1 hour)-exercise with pre-exercise levels. There was a significant interaction effect between the type of exercise bout and the time point on MDA levels, $F(2, 14) = 5.27$, $p = 0.02$. Further analysis using contrasts did not show any significant difference in MDA levels between pre-exercise level with either immediately post- or post (~1 hour)- levels comparing the two exercise trials, $F(1, 7) = 4.87$, $p = 0.06$ and $F(1, 7) = 1.85$, $p = 0.22$ respectively.

Comparison of the two exercise trials at each time points did not show any significant difference in MDA levels at any of the 3 time points; $p = 0.27$, $p = 0.14$ and $p = 0.06$ at pre-exercise, immediately post-exercise and post (~1 hour)-exercise respectively. Analysis of the individual exercise bouts using one-way ANOVA (Bonferroni) revealed that there was a significant decrease in MDA levels post (~1 hour)-HII compared to immediately post-HII ($p = 0.01$), to 11.6 ± 3.8 from 17.1 ± 9.9 μM.

4.4.4.3.2 8-Isoprostanes

8-Isoprostanes levels in response to exercise are presented in Figure 4-9 B (n =7). Two-way repeated measure ANOVA produced no significant main effect of the type of exercise bout on isoprostane levels $F(1, 6) = 2.61$, $p = 0.16$. Similarly, no significant main effect of time was observed in the isoprostane levels, $F(2, 12) = 1.42$, $p = 0.28$. There was no significant interaction effect between the type of exercise bout and the time point on isoprostane levels, $F(1.04, 6.26) = 2.64$, $p = 0.15$.

Comparison of the two exercise trials at each time points did not show any significant difference in isoprostane levels at any of the 3 time points; $p = 0.85$, $p = 0.28$ and $p = 0.09$ at pre-exercise, immediately post-exercise and post (~1 hour)-exercise respectively. Analysis of the individual exercise bouts using one-way ANOVA (Bonferroni) did not show any significant difference due to any of the exercise bout.

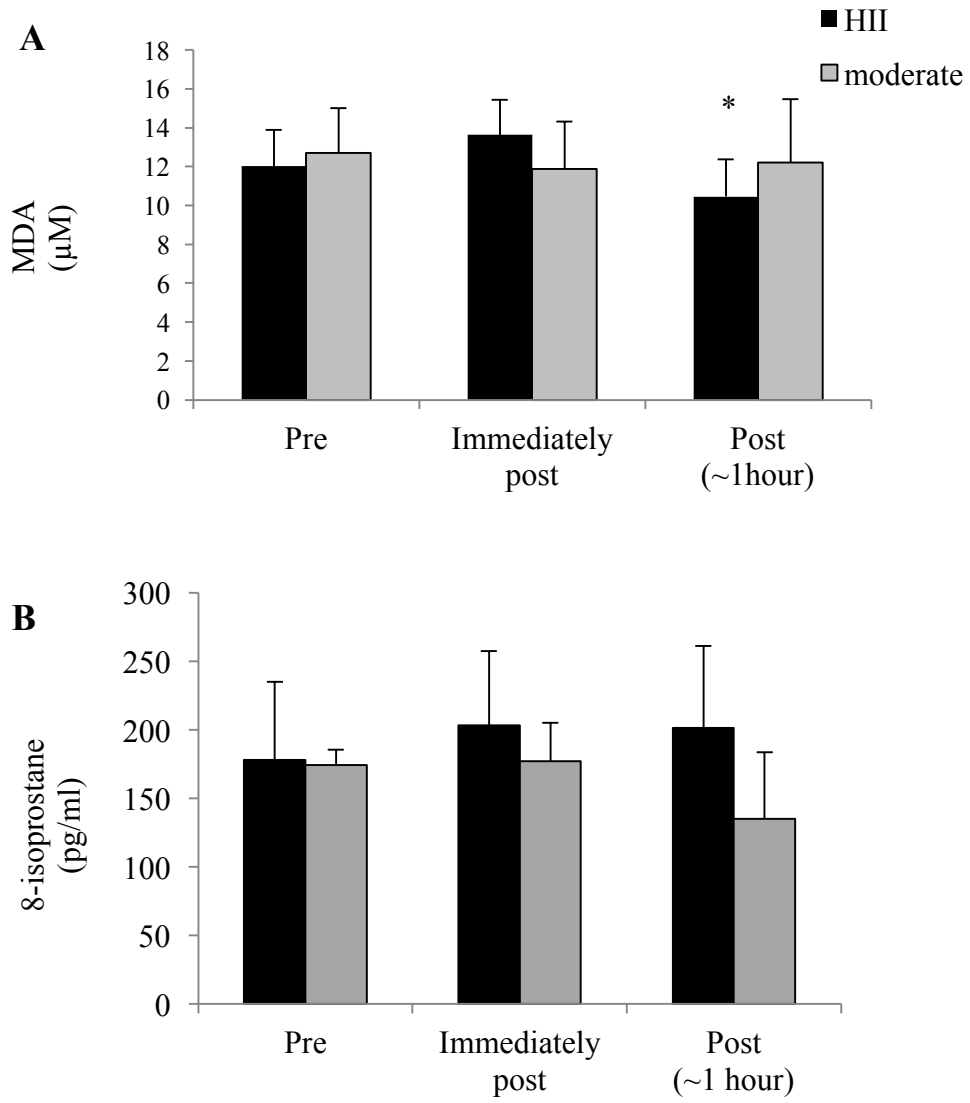


Figure 4-9 Lipid peroxidation

Lipid peroxidation was estimated by measuring MDA and 8-isoprostane in plasma samples at pre-, immediately post- and post (~1 hour)-exercise in response to the two types of exercise. **A)** Mean MDA levels (n = 8). **B)** 8-Isoprostane (n = 7) levels.
Pairwise comparisons: * indicates significant difference between immediately post- and post (~1 hour)-HII ($p < 0.05$).

4.4.4.4 Protein carbonyl

Changes in protein carbonyls in response to the HII and the moderate trial are shown in Figure 4-10 (reciprocal transformed) (n=8). As data was not normally distributed, data was normalised using reciprocal transformation. A two-way repeated measure ANOVA showed no significant difference in protein carbonyl between the two types of exercise, $F(1, 7) = 0.15$, $p = 0.71$. Whereas, there was a significant main effect of time in protein carbonyl levels, $F(2, 14) = 5.02$, $p = 0.02$, and contrasts revealed that the significant difference was between pre-and immediately post-exercise levels ($p = 0.01$). There was no significant interaction between time and type of exercise (HII and moderate trial) on protein carbonyl, $F(2, 14) = 0.62$, $p = 0.55$.

Comparison of the two exercise trials at each time points did not show any significant differences in protein carbonyl levels at any of the 3 time points; $p = 0.36$, $p = 0.72$ and $p = 0.44$ at pre-exercise, immediately post-exercise and post (~1 hour)-exercise respectively. Further analysis of the individual exercise bouts using one-way ANOVA revealed that a significant change in protein carbonyl levels in response to moderate trial, $F(2, 14) = 3.97$, $p = 0.04$. Post hoc comparison (Bonferroni) revealed significant decrease in protein carbonyl levels occurred at immediately post- from pre-exercise level in response to moderate exercise trial ($p = 0.05$).

4.4.4.5 Brain derived neurotropic factor

Changes in plasma BDNF in response to the two types of exercise trials (n = 9) are shown in Figure 4-11 (squared root transformed). As data was not normally distributed, data was normalised using squared root transformation. A two-way repeated ANOVA showed no significant difference in BDNF levels between the two types of exercise, $F(1, 8) < 0.001$, $p = 0.99$. Whereas, there was a significant main effect of time, $F(2, 16) = 7.66$, $p = 0.005$; and

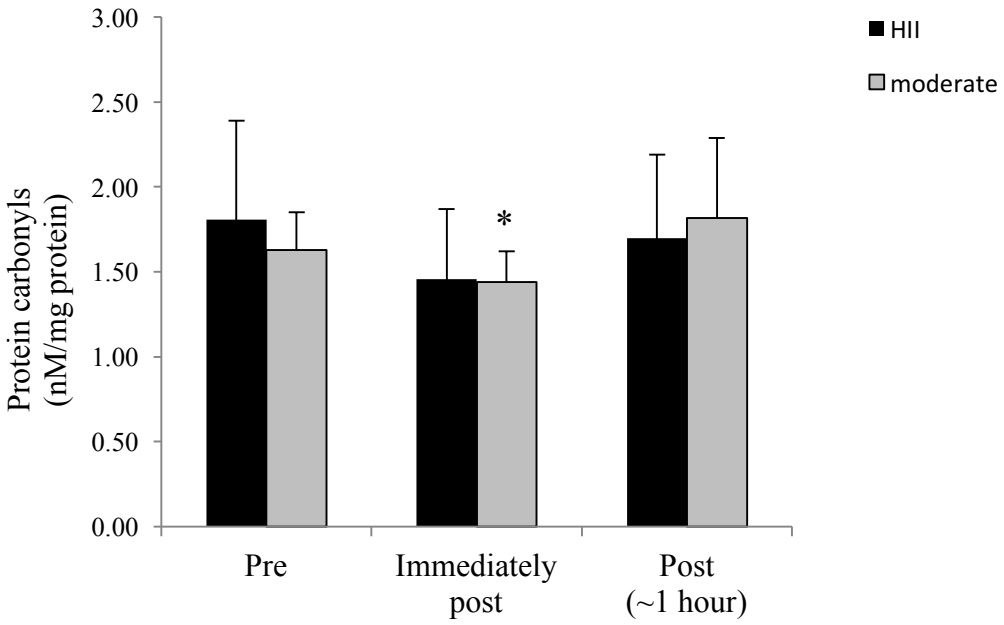


Figure 4-10 Protein carbonyl

Protein carbonyl levels in response to the two exercise trials (n = 8). Pairwise comparison: * indicates significant difference to pre-exercise value in response to moderate exercise trial.

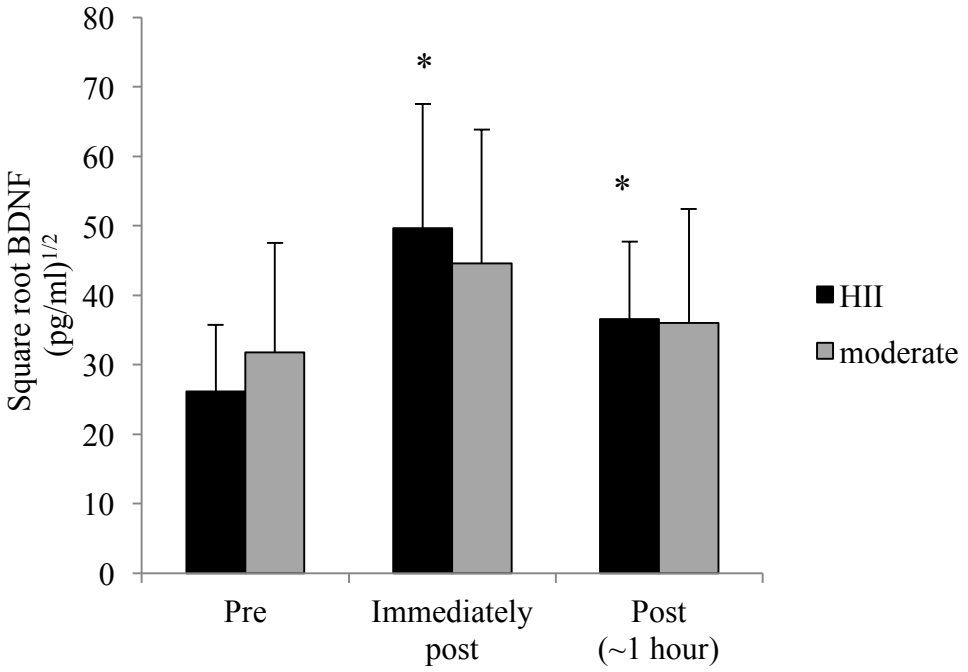


Figure 4-11 BDNF levels

Plasma BDNF levels in response to the HII and the moderate exercise trial (n = 9). Data was squared root transformed for normal distribution. Pairwise comparisons: * indicates significant difference compared to pre-exercise levels in response to HII trial.

contrasts revealed that the significant difference in BDNF levels was between pre- and immediately post-exercise ($p = 0.006$). There was no significant interaction in BDNF levels between time and type of exercise (HII and moderate trial), $F(2, 16) = 1.77$, $p = 0.20$.

Comparison of the two exercise trials at each time points did not show any significant differences in BDNF levels at any of the 3 time points; $p = 0.21$, $p = 0.15$ and $p = 0.89$ at pre-exercise, immediately post-exercise and post (~1 hour)-exercise respectively. Further analysis of the individual exercise bouts using one-way ANOVA revealed that a significant change in BDNF levels in response to HII trial, $F(2, 16) = 10.96$, $p = 0.001$. Post hoc comparison (Bonferroni) revealed significant increase in BDNF levels occurred at immediately post- (2748 ± 1924 pg/ml) and post (~1 hour)- (1448 ± 888 pg/ml) compared to pre-exercise (765 ± 471 pg/ml) BDNF levels in response to HII trial ($p = 0.02$ and $p = 0.03$ respectively).

4.4.5 Cerebral blood flow in response to the two exercise trials

Figure 4-12 shows the changes in MCAv in response to the two exercise trials in 3 participants (one participant was removed because of incomplete data of the whole trial due to poor TCD signal) who undertook the cross-over study. Data was analysed by dividing each of the exercise trial into 4 parts (i.e. 1st $1/4^{th}$, 2^{nd} $1/4^{th}$, 3^{rd} $1/4^{th}$ and 4^{th} $1/4^{th}$ of the exercise trial). Overall the MCAv in response to the two exercise bout seems to be similar across the exercise bouts.

Figure 4-13 shows MCAv changes during the high intensity phases of the HII bout (10 s increments) along with warm up/ recovery before the high intensity phase (1 minute average) and after the high intensity phase (10 s increments for 60 s after the high intensity phase). One-way repeated measures ANOVA was carried out on the MCAv response of all 14 individual high intensity phases of the four participants (2 high intensity phases of one

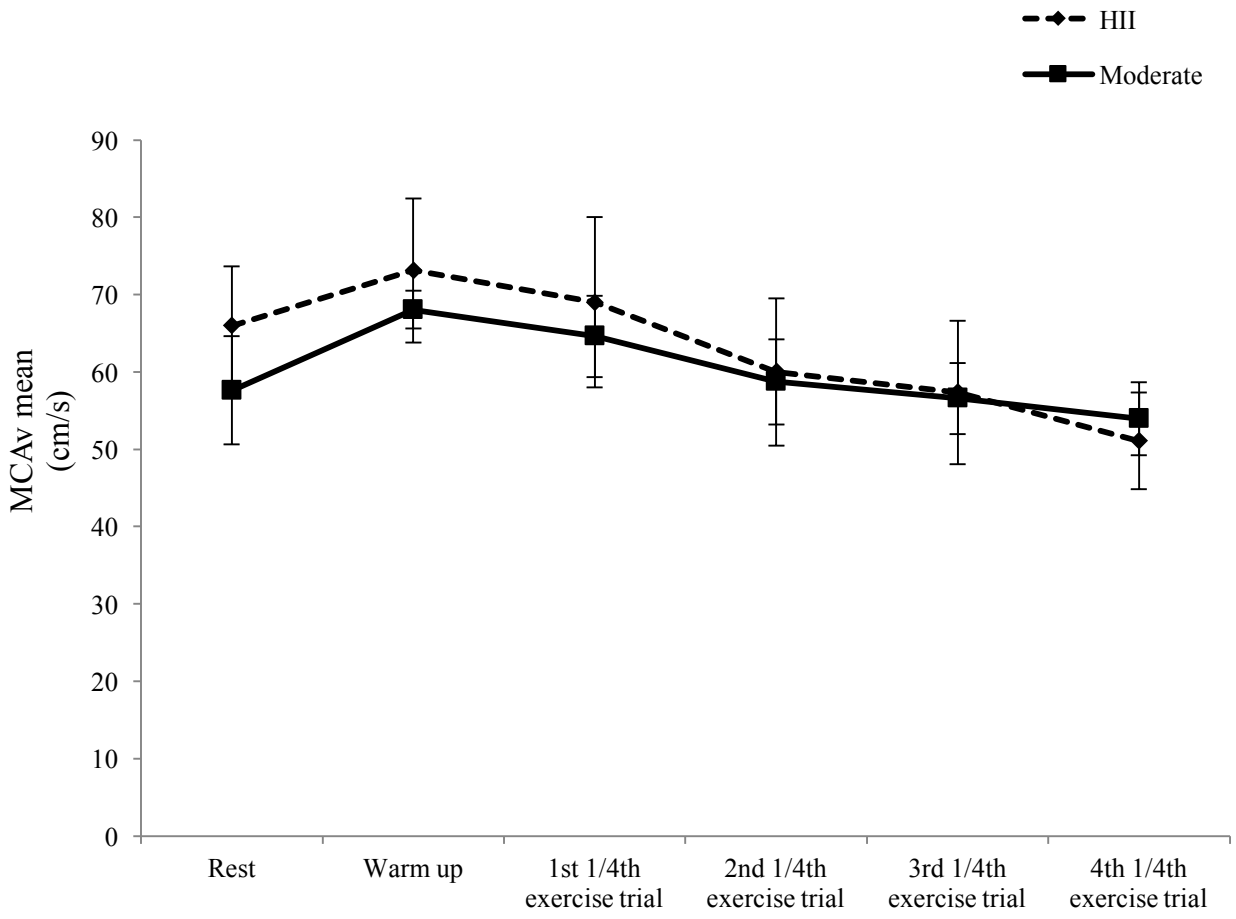


Figure 4-12 MCAv changes in response to the two exercise trials

Mean of MCAv during the two exercise trials (HII and moderate bout) divided into 4 parts each (n=3).

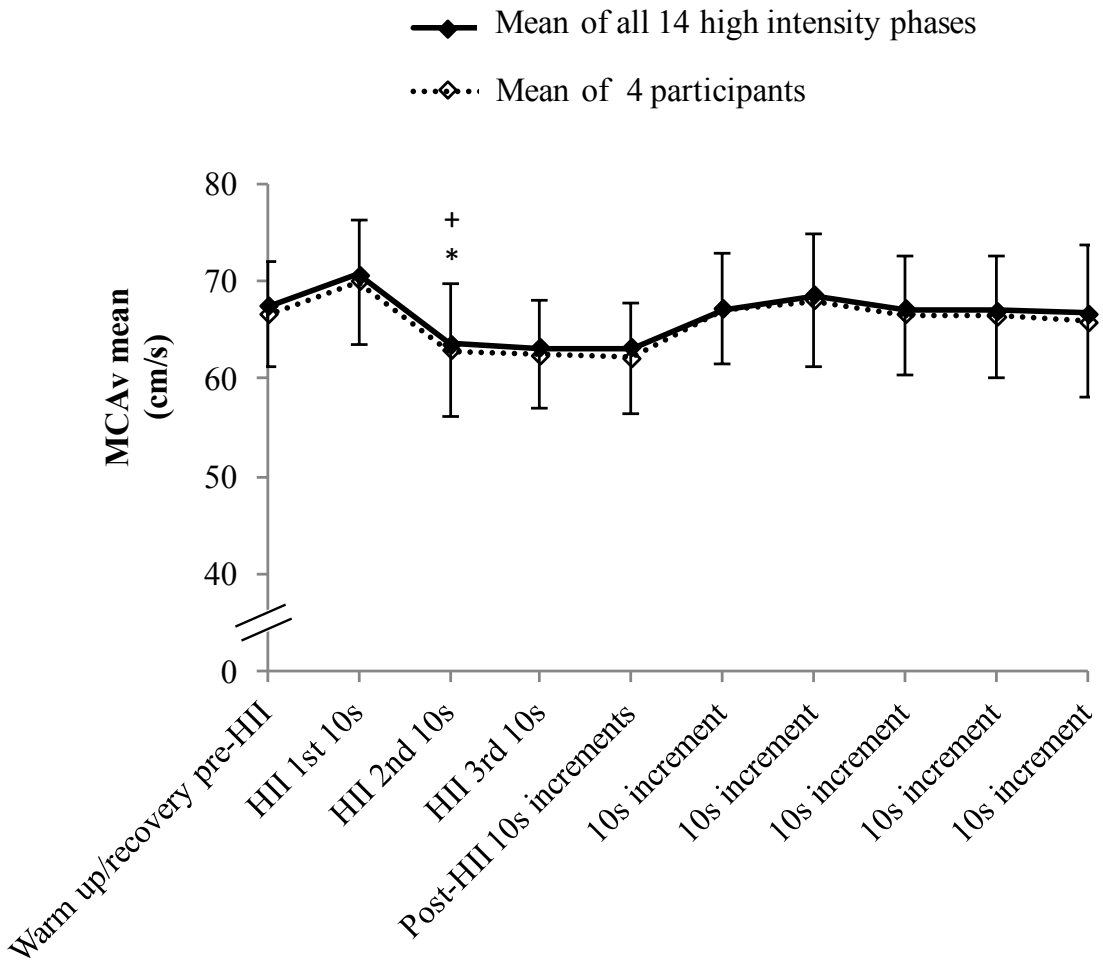


Figure 4-13 MCAv changes during high intensity phase of HII bout

MCAv changes during high intensity phases (10s increments) were compared with warm up/ recovery before the high intensity phases (1 minute average) and after the high intensity phases (10 s increments for 60 s after the high intensity phase). Solid line represents mean of all 14 individual high intensity phases of all the four participants (2 high intensity phases of one participant were not included due to poor TCD signal). The dotted line (represents mean of the four participants individually averaged high intensity phases data. Pairwise comparisons: * and + indicates significant difference from 1st 10 s of high intensity phase (HII 1st 10s in figure) in the 14 individual high intensity phases data and the four participant's individually meaned data respectively ($p<0.05$).

participant were not included due to poor TCD signal) (solid line in figure). There was a significant effect of HII bouts on MCAv $F(9, 117) = 7.80$, $p < 0.001$. Post-hoc tests using Bonferroni corrections revealed that compared to first 10 s of the high intensity phase (HII 1st 10s in figure), there was a significant decrease in MCAv during the second 10 s of the high intensity phase (HII 2nd 10s in figure) ($p = 0.003$).

All four high intensity phases of the HII trial of each participant ($n = 4$) were individually averaged (with the exception of one participant whose 2 high intensity phases were not included due to poor signal) and then the mean of all four participants are presented as dotted lines in Figure 4-13. One-way repeated measure ANOVA showed a significant effect of HII exercise on MCAv $F(9, 27) = 8.90$, $p < 0.001$. Post-hoc tests using Bonferroni corrections revealed that there was a significant decrease in MCAv during the second 10 s of the high intensity phase (HII 2nd 10s in figure) ($p = 0.04$) compared to the first 10s of the high intensity phase (HII 2nd 10s in figure). Time series of MCAv and $PETCO_2$ during a high intensity phase of a HII bout is shown in Figure 4-14.

4.5 DISCUSSION

In this small cohort study, an acute bout of moderate exercise was found to decrease brain GSH, an effect that was not seen in response to HII. In contrast, GSH in the periphery was increased immediately post-exercise in response to the HII bout whereas there was no significant change in blood GSH in response to moderate exercise. Total antioxidant capacity and marker of lipid peroxidation were significantly altered post (~1 hour)-exercise in response to HII bout, while moderate exercise elicited a decrease in protein carbonyl immediately post exercise. The findings of this study suggest that exercise induced changes in markers of oxidative stress are exercise intensity dependent, and that the redox response in the brain is

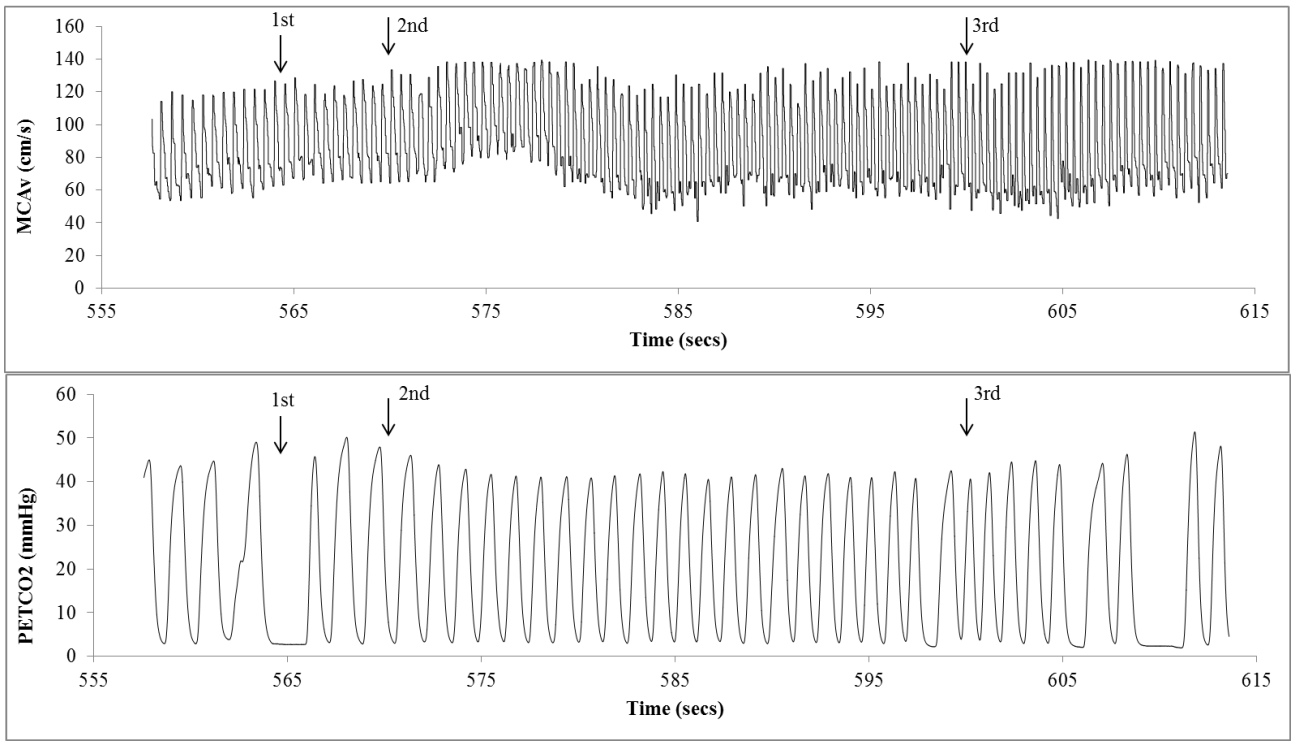


Figure 4-14 Time series of cerebral blood flow during a high intensity phase of HII exercise

Time series of MCAv and $PETCO_2$ is shown during one of the high intensity phases (out of four) in one of the participant. The first, second and third arrow indicates pre 5 seconds before the high intensity phase, start of the high intensity phase and end of the high intensity phase respectively.

probably independent to that in the periphery.

4.5.1 Effect of exercise on brain GSH and CBF

The result from this small cohort study suggests that an acute bout of moderate exercise elicits a decrease in brain GSH. Additionally, it seems that a moderate exercise bout had a larger effect on brain GSH than HII exercise. However, these results are difficult to interpret due to the small sample size and the sensitivity of the MRS method in measuring brain GSH. For example movement or motion due to cardiac and respiratory activity can have a large effect on MRS quantification (Felblinger et al., 1998, Jansen et al., 2006). The finding from this study suggests that the sensitivity of MRS with current available technology limits the ability to detect potential changes in brain GSH in response to exercise. A study involving large number of individuals is needed to confirm the potential exercise-induced change in brain GSH. A power analysis indicated that 46 participants would be required to observe a 0.80 power (i.e. $1-\beta = 0.8$) with $\alpha = 0.05$ and partial $\eta^2 = 0.1$.

GSH has been reported to have diurnal variation with highest level in the morning and lowest level in the afternoon/evening. In humans, some studies have reported diurnal variation of GSH in plasma (lowest level early afternoon) (Blanco et al., 2007, Samuelsson et al., 2011) and bone marrow (lowest level in the evening) (Bellamy et al., 1988, Smaaland et al., 2002), whereas another study reported no diurnal variation of GSH in the skeletal muscle (Luo et al., 1995). Such a diurnal variation is unlikely to have a significant effect on the brain GSH measured within the study design of the study presented in this chapter. The scans for the cross-over study were started in the late afternoon/evening between 17:00 and 19:30. Whereas the scans for the time course study (lasting for around 6-7 hours) were started between 13:00 and 17:00. Furthermore Terpstra et al. (2011) reported no change in brain GSH level

measured over a 24 hour period in healthy individuals, suggesting there is no diurnal variation in brain GSH.

There is a wealth of evidence to show that exercise-induced increases in oxidative stress are key to adaptive processes (Jackson, 1999, Radak et al., 2008, Ristow et al., 2009). The GSH system has been shown to have an adaptive response to exercise training: Elokda and Nielsen (2007) found exercise training in sedentary humans increased resting blood GSH. Exercise training can act as a stimulus to increase synthesis of endogenous antioxidants like GSH (Elokda and Nielsen, 2007, Nyberg et al., 2014) and antioxidant enzymes such as glutathione peroxidase (Bogdanis et al., 2013, Elosua et al., 2003, Takahashi et al., 2013). These adaptations in response to exercise are well documented in muscle (Hellsten et al., 1996, Powers et al., 1999), and occur in response to repeated exercise training. Although no studies have established that brain antioxidant levels are higher in trained humans, there is enough evidence from whole body studies to suggest that exercise could help maintain redox balance in the brain. Exercise training has been shown to decrease protein oxidation (reactive carbonyl derivatives) in rat brain (Ogonovszky et al., 2005). A review by Camiletti-Moiron et al. (2013) found that moderate aerobic exercise training in rodents promoted the antioxidant capacity of brain, while high-intensity or aerobic-exhausted exercise training could deteriorate the antioxidant response. The authors of the review did not find any relevant studies in humans but they hypothesised that aerobic moderate training is the most appropriate exercise to positively enhance the brain antioxidant response. The results presented herein are in agreement with this hypothesis, as moderate exercise, rather than HII exercise, caused a greater decrease in brain GSH in all of the participants tested.

As GSH levels were only assessed at the anterior cingulate cortex, there is a possibility that exercise could be altering brain GSH levels at other brain regions in a different way. In rats, it

has been reported that different brain regions contained different levels of GSH (Somani et al., 1995). The anterior cingulate cortex was selected in this study as the activation of this region is linked to the physical fitness of individuals (Colcombe et al., 2004) and also this region is detrimentally impacted by ageing and dementia (Chapman et al., 2013). Furthermore, exercise training has been shown to increase resting CBF in the anterior cingulate region (Chapman et al., 2013).

CBF during the two exercise trials was assessed by measuring MCAv. The MCAv in response to the two exercise bouts seemed to be similar when comparing the exercise trials as a whole. However, looking at MCAv changes specifically during the high intensity phase of the HII exercise showed that there is an instantaneous decrease in MCAv after the initial increase. The CBF seems to be protective at high intensity exercise; the brain seems to be protected against hyperperfusion during the 30 seconds of high intensity phase of the HII bout probably due to exercise-induced hyperventilation hypocapnic effect, as suggested by the significant decrease in MCAv compared to the initial increase seen during the short 30 seconds high intensity phase. Exercising above ~60% V̇O2max induces a hyperventilation induced hypocapnia and the subsequent cerebral vasoconstriction reduces CBF towards the resting value; this constrictive effect may serve as a neuroprotective response to prevent BBB disruption and hyperfusion injury (Lucas et al., 2015).

4.5.2 Effect of exercise on markers of oxidative stress and BDNF in the blood

The effect of exercise on markers of oxidative stress in the periphery seems to be dependent on the type of exercise bout. The HII bout elicited a transient increase in blood GSH immediately post-exercise. Turner et al. (2011) also reported an increase in GSH level post ultraendurance exercise along with increase in other markers of oxidative stress including lipid peroxidation, oxidative DNA damage, and protein carbonylation. Similarly other studies

have also found increases in blood GSH post-acute exercise (Hessel et al., 2000, Ji et al., 1993, Subudhi et al., 2003). This transient increase seen in blood is probably due to hepatic efflux of GSH (Anderson et al., 1980, Lu et al., 1990) in order to compensate for the ROS production during exercise. However, not all studies report increased GSH following exercise. Fisher-Wellman and Bloomer (2009) reviewed several studies that either report a decrease or no change in blood/plasma GSH levels during or at the end of exercise. These differences across studies could well be explained by training status of the participants, and duration, mode and intensity of exercise.

Measures of total antioxidant capacity and lipid peroxidation in plasma were respectively increased and decreased post-exercise in response to the HII bout. Whereas, moderate exercise elicited a decrease in protein carbonyl immediately post exercise. In contrast to the finding of this study, Wadley et al. (2016a) reported a similar oxidative stress response due to HII compared to continuous moderate exercise. This difference in finding is probably due to the difference in exercise bout; Wadley et al. (2016a) used a low volume HII of 90 % ẆO2max whereas the study presented herein used high volume HII of 200 % ẆO2max. Periods of intensive exercise can cause temporary ischemia and hypoxia in certain regions of the body and this can result in activation of xanthine oxidase (Sachdev and Davies, 2008). On reperfusion, reintroduction of oxygen can result in burst of superoxide and H_2O_2 which would be expected to result in further oxidation including lipid peroxidation and glutathione oxidation (Sachdev and Davies, 2008). The repeated cycle of high intensity phase during the HII trial probably could also be one of the contributing factors for higher oxidative stress response seen in the study presented here. The increase in total antioxidant capacity in plasma after HII exercise as assessed by FRAP assay is probably due to efflux of vitamin C from the adrenal glands into the circulation (Gleeson et al., 1987) and purine nucleotide degradation

leading to uric acid production (Green and Fraser, 1988). Uric acid is a main contributor to the antioxidant capacity measured by this assay (Cao and Prior, 1998) and the concentration of uric acid in plasma has been shown to be dependent on exericse intensity (Green and Fraser, 1988).

Exercise of sufficient intensity and duration can cause acute oxidative stress; various factors drive the direction of the response of oxidative stress to exercise including participant physiology (such as training status), and the intensity, duration and type of exercise (Wadley et al., 2016b). For instance, many studies have reported acute increases protein carbonyl in response to exercise, while some studies have found decrease in protein carybonyl (Wadley et al., 2016b), including the study presented herein. Wadley et al. (2016b) suggest that exercise may stimulate the clearance of plasma protein carbonyl present at baseline, whereas simultaneously increasing ROS production that facilitates the formation of new protein carbonyl groups. The balance between the formation and the clearance processes (probably involving 20S proteasome system) following exercise likely explains the increase, decrease or no change in plasma protein carbonyl level in response to exercise reported across studies (Wadley et al., 2016b).

Plasma BDNF increased in response to HII exercise but not moderate exercise. This finding is in agreement with the study by Ferris et al. (2007), who reported that peripheral BDNF levels increased in response to exercise and this increase was correlated with exercise intensity. BDNF is the most abundant growth factor in the brain and is often thought to be a link between exercise and improved brain health. A review by (Knaepen et al., 2010) indicates that acute aerobic exercise causes a transient increase in basal peripheral BDNF concentration, and thus could be an important modulator of the beneficial effects of exercise for the brain. BDNF levels are increased not only in the brain and plasma due to exercise, but also in skeletal

muscle during contraction (Pedersen et al, 2009). However, BDNF produced in muscle is probably not released into the circulation (Pedersen et al, 2009).

4.5.3 Comparing the response between the periphery and the brain

The effect of exercise on markers of oxidative stress was dependent on the intensity of the exercise bout in both the brain and the periphery. However, it seems that the effect in the brain may be independent to the periphery, as the oxidative stress response to exercise intensity in the blood and the brain showed contrasting responses. Only the moderate trial induced a decrease in brain GSH, whereas in the periphery the HII trial was the most effective in inducing changes in oxidative stress markers. As introduced earlier, the majority of GSH produced in the liver does not reach the brain due to the BBB, suggesting *de novo* GSH biosynthesis in the brain.

Exercise training studies in rats have suggested that brain and blood GSH are related, although studies report differences in their findings (Itoh et al., 1998, Ohkuwa et al., 1997). For example, Ohkuwa et al. (1997) observed that moderate exercise training increased both brain and plasma GSH levels in rats, whereas Itoh et al. (1998) did not find any effect of training on both plasma and brain GSH levels. Although contradictory in the direction of change, both studies indicate that similar oxidative stress responses occur both in the periphery and the brain. However, the study presented herein assessed the effect of acute exercise only, and thus it is possible that repeated bouts of exercise are necessary for whole body (brain and periphery) changes to occur. Holmay et al. (2013) observed maximal changes in brain GSH levels after around 30 minutes delay relative to the redox change seen in blood in response to intravenous infusion of N-acetylcysteine. They observed a rapid decrease in blood GSH levels, while the brain GSH levels remained elevated until the end of measurement at 2 hours from infusion suggesting different environment/mechanism in the

brain and the blood. It is accepted that the contribution from the vasculature to the GSH levels measured in the brain using the MRS method is negligible (Holmay et al., 2013, Terpstra et al., 2011).

4.5.4 Strengths and limitations of the study

This study included a novel approach in assessing redox change in the brain in response to an acute exercise bout using MRS methodology and is also the first to investigate the effect of exercise on a marker of oxidative stress in the brain. However, the small sample size of the study and the limited sensitivity of MRS method means that the results should be interpreted with caution. Another strength of this study was that CBF was assessed during the exercise trials which may provide more insight into mechanism potentially relating to the exercise-induced response seen in the brain and the periphery.

4.6 CONCLUSION

The results presented herein suggest that an acute bout of exercise can induce a change in brain GSH. If the mechanisms of adaptation are the same in the brain as they are in other tissues, then such response may contribute to brain health. However, due to the small sample size and the limitation of the MRS method, this data should be evaluated with caution. Additional studies are required, using this methodology with larger numbers of participants, to assess the effect of exercise on brain GSH. A bout of HII exercise consisting of short phases of a very high intensity exercise elicited a greater oxidative stress in the periphery compared to moderate exercise.

5 CHAPTER 5: The effect of a bespoke home based physical activity intervention on markers of oxidative stress and markers of general health in older adults

5.1 ABSTRACT

Home-based unsupervised exercise training has been shown to improve health in older adults. Assessing adherence, especially using a self-report format, in an unsupervised exercise intervention is problematic. The aim of the study was to assess the effect of an unsupervised home-based exercise intervention in community dwelling sedentary older adults. Markers of oxidative stress and measures of health were assessed, including cerebral hemodynamics and cognitive function. Physical activity was measured continuously using Basis Peak, an activity monitoring device. A secondary aim of the study was to test the appropriateness of the Basis Peak as a tool to assess physical activity in this population. The intervention failed to increase the level of physical activity in all the participants. No significant changes were observed in markers of oxidative stress or other measures of health in response to the exercise intervention. Progressive goals, based on adherence measurement, are potentially important for the success of an unsupervised exercise intervention. Basis Peak may not be the optimal tool for the assessment of physical activity in sedentary older adults.

5.2 INTRODUCTION

Ageing is associated with cognitive decline (Deary et al., 2009), diminished cerebral haemodynamics (Demirkaya et al., 2008, Krejza et al., 1999) and reduced physical function (Kuh, 2007). Physical activity has been shown to improve cognitive function in older adults who are healthy, with cognitive impairments and also those with diseases/conditions associated with ageing such as dementia (Brown et al., 2013, Heyn et al., 2004, Kramer and Erickson, 2007). Physical activity can improve quality of life (Conn et al., 2009, Netz et al., 2005, Rejeski and Mihalko, 2001) and increase physical function (Chin et al., 2008, Gine-Garriga et al., 2014) in older adults. Global cerebral blood flow (CBF) declines gradually with ageing (Demirkaya et al., 2008, Grolimund and Seiler, 1988, Krejza et al., 1999), whereas CBF has been shown to be elevated with higher aerobic fitness across the adult lifespan (Ainslie et al., 2008, Bailey et al., 2013). The potential mechanisms behind this increased CBF may be increased grey matter volumes (Colcombe et al., 2006) and more endothelium-dependent vasodilation (Green et al., 2004).

The current recommended physical activity level for adults is a minimum of 150 min/week of moderate-intensity exercise (equivalent to approximately 1000 kcal/week) (Haskell et al., 2007). The guidelines by the Public Health England on physical activity for older adults are: i) Those who participate in any amount of physical activity gain some health benefits. Guidelines go on to clarify that some physical activity is better than none, and more physical activity provides greater health benefits. ii) Physical activity should add up to at least 150 min/week of moderate-intensity activity. iii) Those who are already regularly active at moderate-intensity, comparable benefits can be achieved through 75 min/week of vigorous-intensity activity or a combination of moderate and vigorous activity. iv) Those who are at risk of falls should incorporate physical activity to improve balance and coordination on at

least two days a week (Public Health England, 2014). A volume of exercise that is about half of 1000 kcal/week may be sufficient, particularly for people who are frail and elderly (Warburton et al., 2006). A recent cohort study found that 90 min/week of moderate intensity exercise is effective in reducing mortality and extending life expectancy (Wen et al., 2011). Similarly, Takahashi et al. (2013) showed that 100 min/week walking exercise intervention in older adults had a protective role against oxidative stress including increased antioxidant potential.

For older adults living in the community, being more physically active has the potential to extend home-dwelling and delay moving into a care home or hospital. Most of the exercise intervention studies in older adults are undertaken in supervised form usually in structured group sessions, and are undertaken at facilities. Only few studies have investigated unsupervised home-based exercise intervention under free-living conditions, and the assessment of adherence to the prescribed exercise in these studies are through self-report measures, which have limitations such as subjective bias and being reliant on memory. The increasing development of physical activity monitoring devices such as heart rate monitors, accelerometers and pedometers allows physical activity in free-living conditions to be objectively measured with greater confidence (Colley et al., 2008).

The aim of this study was to assess the effect of increasing physical activity by 10 % (based on energy expenditure) in older adults receiving care in a bespoke home-based exercise intervention. Markers of oxidative stress as well other measures of health including physical function, cognition, cerebral haemodynamics, and quality of life were assessed. A secondary aim of the study was to test the appropriateness of the Basis Peak (a commercially available activity sensor) as a tool to assess physical activity in this population who are generally sedentary.

5.3 METHODS

5.3.1 Study design

A physical activity intervention tailored to each participant was developed aiming to increase their current physical activity level by 10 % over a period of 4-6 weeks, based on weekly energy expenditure. A schematic diagram of the study design and the measures assessed is outlined in Figure 5-1. Ethical approval for the study was obtained from the NRES Committee Nottingham1.

5.3.2 Participants

Participants (60 years and above) were all older adults receiving care in their own homes by a care provider, Care Companions Ltd, located in Sutton Coldfield, UK. Clients of Care Companions were given participant information by the carer and asked if they were interested in taking part in this study. Those that expressed an interest were visited by the researcher, and if appropriate, were recruited for the study. All participants provided written consent before taking part. Four people with dementia (PWD) and four people without dementia were recruited. Presence of dementia was self-reported in the general health questionnaire as well as through the participant's carers.

5.3.3 Assessment of physical activity

5.3.3.1 Comparison and selection of activity monitoring systems

In December 2014 the following activity monitoring devices were available, and capable of monitoring physical activity in a form that would be acceptable to this participant group: Basis Peak (Basis, 2015 [online]) (BASIS an Intel Company, San Francisco, USA), Fitbit Flex (Fitbit Inc., San Francisco, USA), Jawbone Up (Jawbone, San Francisco, USA), Mio Alpha [Mio Global (Physical Enterprises Inc.), Vancouver, Canada], Nike+ Fuelband SE

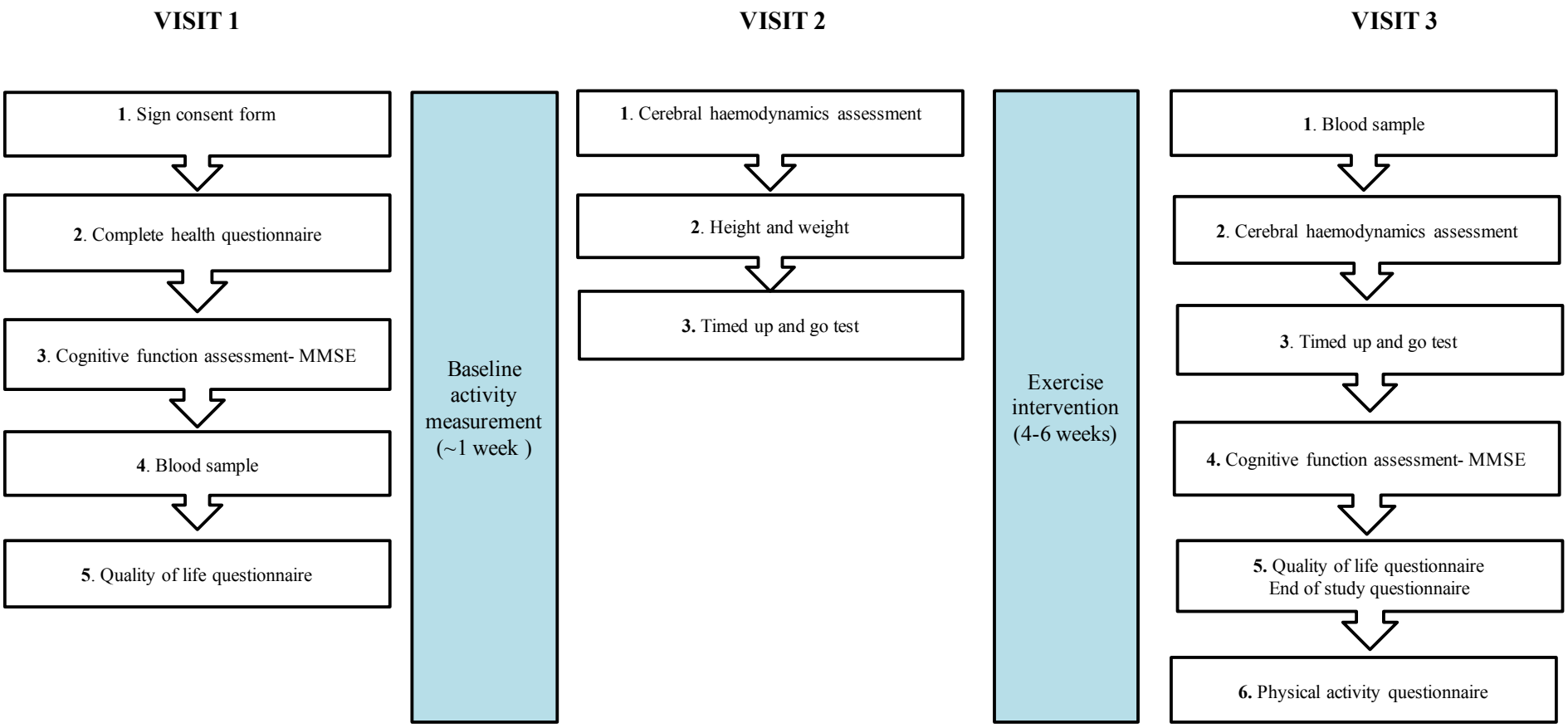


Figure 5-1 A schematic diagram of the study protocol

(Nike Inc., Oregon, USA), Polar FT60 (Polar Electro, Kempele, Finland), and Withings Pulse Ox (Withings, Issy-les-Moulineaux, France). Of these devices, the Basis peak, Fitbit flex and the Withings pulse ox were tested by the research team. The Basis Peak, became available commercially in the UK in January 2015, and was selected for the study. More information on the selection of the device for the study is presented in section 5.4.4.

5.3.3.2 Measurement of physical activity

Physical activity was measured objectively using Basis Peak. This device, which is in a wrist watch format, measures accelerometry, heart rate, skin temperature, and electrodermal activity (as indicator of perspiration). Participants were asked to wear this water resistant activity monitor on the wrist, and to keep it on all the time including when sleeping at night. Participants and carers (if applicable) were instructed to charge the activity monitor every three days (the Basis Peak activity watch has a battery life of between 3-5 days depending on usage). The activity monitor is designed to synchronise data with an iPhone (iOS) or Android smartphone via a wireless Bluetooth connection using Basis app which communicates via the internet with the database held by Basis. Data from the activity monitors were synced by the researcher every two weeks on average. Separate user accounts were created for the different watches and were updated with respective participant's age, height and weight.

Physical activity was also assessed at the end of the exercise intervention using modified version of 'Physical Activity Scale for the Elderly (PASE)' questionnaire (appendix I). The main modifications to the questionnaire were as follows. i) The original PASE questionnaire was modified in language, away from US English to UK English, and examples were modified so that they were more relevant to the UK population. ii) Instead of having individual questions for varying intensities/forms of recreational/sports activities, in the modified version the participant was asked to list all recreational/sports activities. iii) An

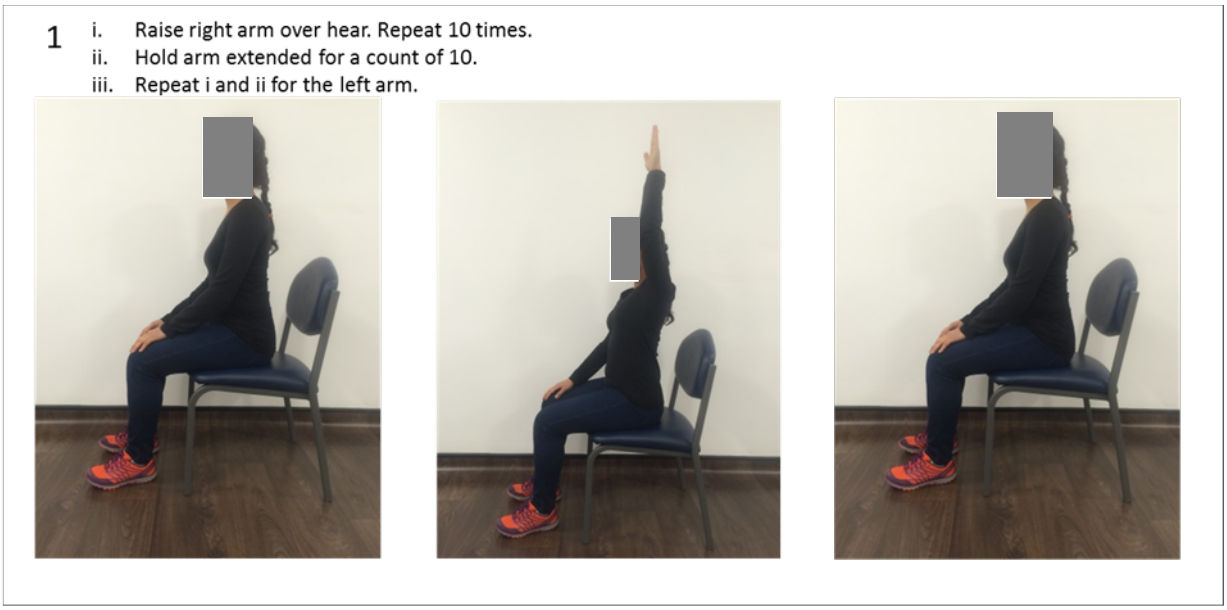
additional question was added to ask if the participants had stairs in their house, and if so, how often they used them. The scoring method of the original PASE was used for scoring this modified version.

5.3.3.3 Basis Peak data analysis

The raw sensor data from Basis Peak activity monitor are provided in minute-to-minute format in CSV (Comma-Separated Value) form. This data was analysed using MATLAB 8.5 and Statistics and Machine Learning Toolbox 10.0, The MathWorks, Inc., Natick, Massachusetts, United States.

5.3.4 Physical activity intervention

Instructions to increase an individuals' physical activity level were provided according to the participant's habitual physical activity (using the Basis peak) and his/her physical capability. Every effort was made to adapt the exercise training to integrate it with the lifestyle of the participant. Opportunities to increase the physical activity of the participants were identified following discussions with the participants and family members/carers. For instance, if a participant already walked regularly, he/she was advised to gradually increase the distance and pace of their current level. Participant who used to do gardening were encouraged to do more gardening activities. Participants without mobility problems and who had stairs in their house were asked to climb up the stairs an additional 5 times in their daily routine. Chair based exercises were undertaken in a supervised session on the first occasion (with the participant and the carer together). The carers and participants were asked to integrate the chair based exercises into their daily routine, for example they were advised to do them in the morning after they woke up and in the evening before going to bed so that this exercise would become part of their daily routine. Written instructions with figures were provided for the chair based exercise (example shown in Figure 5-2). The full instructions for the chair based



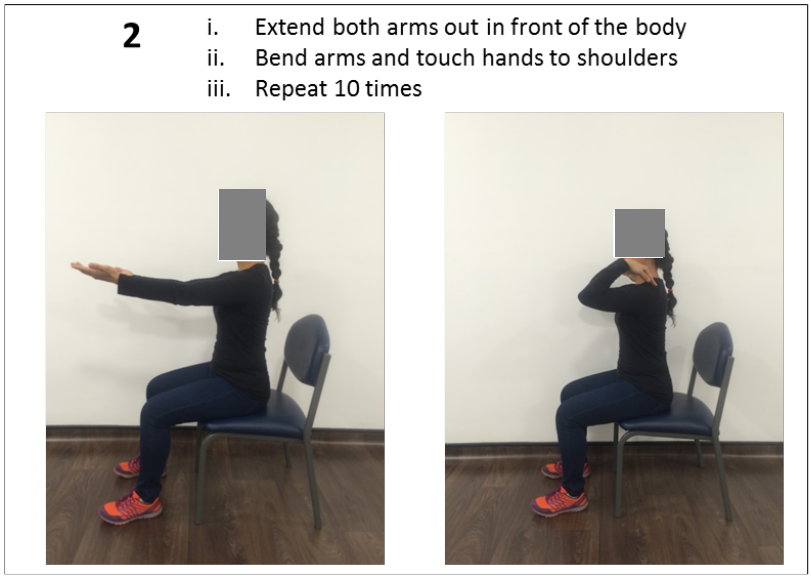


Figure 5-2 Example of chair based exercise

exercise is presented in Appendix II. This type of exercise was especially encouraged in participants with mobility issues.

Participants were seen every 2 weeks during the study to discuss progress and to receive encouragement. During these visits, discussions would be carried out with the participants and the carer regarding the activities that they have been doing to increase their physical activity level and their frequencies. Participants would be acknowledged for their efforts and be encouraged to increase their activities further.

5.3.5 Markers of oxidative stress in blood

Blood samples were obtained from the antecubital vein using venepuncture. Blood samples (6 ml) were collected in EDTA vacutainers. The blood was immediately centrifuged in a portable centrifuge for 15 minutes. This was immediately followed by separation of plasma samples which were then aliquoted and placed on ice (around 3 hours) before being stored at -70° C. Markers of oxidative stress in plasma (malondialdehyde (MDA) levels, total antioxidant capacity levels and protein carbonyl) were assessed as described in chapter 2.

5.3.6 Cerebrovascular measures

Five participants were assessed for cerebrovascular measures before and after the exercise intervention. Three participants were not assessed due to participant availability. CBF velocity was measured from the middle cerebral artery (MCA) using TCD as described in chapter 2 2.3. Blood pressure (BP) data was collected simultaneously using Portapres (Finapres Medical Systems, Netherlands), allowing acquisition of synchronised data of beat-to-beat variability in BP and MCAv (MCA velocity). BP was measured in the finger by photoplethysmography (Finapres, Netherlands). The hand with the Portapres cuff was held steadily in place at heart level. Both MCAv and BP were acquired continuously using an

analog-to-digital converter (Powerlab; AD Instruments) interfaced with a computer, and were subsequently analysed using commercially available software (Lab Chart, AD Instruments).

To enhance the magnitude of alterations in BP and CBF, and to mimic such changes in daily life associated with changes in body posture, changes in BP and CBF were induced by sit-to-stand manoeuvre. Participants were instructed to sit for 5 minutes and then stand for 2 minutes which was repeated twice. BP and CBF were further monitored for 3 minutes at the end of the second stand. Physical assistance during standing up was provided to aid the performance of these manoeuvres as well as verbal instructions.

Resting MCAv was calculated from 60 s data, one minute prior to the first stand manoeuvre. To evaluate the CBF response to acute posture change in the single sit-stand protocol, the differences between the sitting value (averaged over a period of 20 s) and the value at the nadir of BP (average of 5 values surrounding the nadir) for each sit-to-stand manoeuvre was calculated.

5.3.7 Other outcome measures: cognition, quality of life, and physical function

Cognitive function was assessed using the Mini Mental State Examination (MMSE) (Folstein et al., 1975), which takes approximately 10 minutes to administer. Quality of life was assessed using Dementia Quality of Life measure (DEMQoL) questionnaire (Smith et al., 2007). Physical function was assessed using the 'timed up and go' test (TUG) (Podsiadlo and Richardson, 1991), which is a widely used clinical performance-based measure of lower extremity function, mobility and fall risk. For the TUG, subjects are asked to stand from a standard chair, walk a distance of 3 meter at a comfortable pace, turn, walk back and sit down. The time to complete the task was measured. At the end of the intervention, participants' attitude towards taking part in the study were evaluated using an End of Study questionnaire.

5.3.8 Statistical analysis

Statistical significance was accepted at the $p<0.05$ level. Data were analysed using SPSS statistical package for Windows (SPSS Inc, USA). Paired sample t-test was used to test differences between baseline and intervention period, while independent t-test was used to test for differences between PWD and the non-dementia group. Relationships were determined using Pearson Product Moment Correlations. Data, that was not-normally distributed, was either transformed for normal distribution, or an equivalent non-parametric test was carried out. Data are presented as means ± standard deviation of the mean (SD), unless otherwise stated.

5.4 RESULTS

5.4.1 Declaration of ownership

In this chapter, comparison and selection of activity monitoring system was undertaken in collaboration with Dr Tim Collins and Dr Sandra Woolley, Lecturers in health technology, School of Electrical and electronic engineering, University of Birmingham. The expertise and advice of Dr Tim Collins was sought for the analysis of the activity monitor data. The author was responsible for running of the study and all further data analysis.

5.4.2 Declaration of competing interests

No financial support was received from Basis an Intel Company for the purpose of this study.

5.4.3 Participants

Participant characteristics are presented in Table 5-1. There was no significant difference in the age ($t(6) = 1.56$, $p = 0.17$), BMI (body mass index) ($t(6) = 2.21$, $p = 0.07$) and mean heart rate measured ($t(6) = 0.03$, $p = 0.07$) between PWD and the non-demented group. There was equal ratio of males and females in both groups.

Table 5-1 Participant characteristics

Participant Characteristics	**All participants (n = 8)**	**PWD (n = 4)**	**Non-demented (n = 4)**
Age (years)	78 ± 8	82 ± 4	74 ± 9
Height (cm)	160 ± 10	163 ± 8	158 ± 12
Weight (kg)	72 ± 18	85 ± 11	60 ± 16
BMI (kg/m^2)	28 ± 7	32 ± 7	24 ± 4
Mean HR measured	76.1 ± 5.5	72.8 ± 4.5	79.5 ± 4.4
Gender ratio (m/f)	4/4	2/2	2/2

Mean HR measured- mean heart rate measured by the Basis Peak activity monitor from all the HR data collected from a participant, BMI- body mass index, PWD- people with dementia

5.4.4 Comparison and selection of activity monitoring system

Out of the seven activity monitoring systems (Basis Peak, Fitbit Flex, Jawbone Up, Mio Alpha, Nike+ Fuelband SE, Polar FT60, and Withings Pulse Ox) assessed (summarised in Table 5-2), all were of wrist watch formats with the exception of Withings Pulse Ox which could optionally be clipped on to clothing including belt or pocket.

First, activity monitors that did not measure heart rate were eliminated. The Withings Pulse Ox could measure heart rate but not continually; in order to measure heart rate it was required for the wearer to remove the device from the wrist strap or clip and put their finger over an optical sensor on the back. Furthermore, the device required the wearer to turn on and off the setting for sleep tracking. Thus the Withings was eliminated. The Mio Alpha was dismissed as it did not measure activity monitoring data apart from heart rate. The Polar FT60 required a separate heart rate sensor mounted on a chest strap, so was eliminated. The device that met criteria of measuring activity data as well as heart rate was the Basis Peak.

5.4.5 Change in physical activity

5.4.5.1 Analysis of activity data from Basis Peak

Wear time of the watch was estimated as the time the activity monitor recorded heart rate, as the activity monitor automatically records heart rate when it is in contact with skin. The minimum wear time for each day was set at 75 % which amounts to 18 hours a day, with the exception of one participant whose minimum wear time was set at 50 % (which amounts to 12 hours). This participant had low wear time (data shown in Figure 5-5) and the participant's carer stated that the participant would often take off the watch and forget to put it back on. The mean of the activity measures of each day were calculated. Then the mean of each day

Table 5-2 Comparison of monitoring systems

Device Manufacturer - Product	Activity monitoring	Heart rate monitoring	Raw data access
Basis - Peak	Yes	Yes	Yes
Fitbit - Flex	Yes	No	Yes
Jawbone - Up24	Yes	No	No**
Mio - Alpha	No	Yes	Yes
Nike - Fuelband	Yes	No	No**
Polar – FT60	Yes	Yes*	Yes
Withings – Pulse Ox	Yes	Yes	Yes

*Requires separate chest-strap sensor for heart rate measurement
** Raw data access not supported by the manufacturer

Table 5-3 Assessment of physical activity

	All participants (n = 8)			PWD (n = 4)			Non-demented (n = 4)			
	Baseline	Intervention	p value	Baseline	Intervention	p value	Baseline	Intervention	p value	p value [a]
Total calories (kcal) per day	2296 ± 344	2388 ± 367	0.37	2206 ± 287	2292 ± 107	0.44	2387 ± 413	2483 ± 527	0.63	0.50
Steps per day	2355 ± 2478	2291 ± 2478	1.00	366 ± 342	433 ± 315	0.07	4343 ± 1902	4148 ± 2242	0.61	0.01*
% HR > 100 per day (bpm)	8.1 ± 8.1	7.6 ± 8.3	0.26	3.9 ± 3.0	2.5 ± 1.5	0.19	12.4 ± 9.9	12.8 ± 9.4	0.70	0.15
% HR > mean HR plus 10% mean HR (bpm)	21.8 ± 6.9	23.5 ± 7.2	0.14	18.9 ± 6.6	19.4 ± 6.6	0.65	24.6 ± 6.8	27.5 ± 5.8	0.19	0.28
Number of days	8 ± 3	29 ± 8		9 ± 3	36 ± 10		6 ± 4	31 ± 7		

p value- comparison between baseline and intervention period (paired t-test)
p value [a]- comparison between PWD and non-demented participants at baseline (independent t-test).
* significant difference between PWD and non-demented participants at baseline
HR- heart rate, bpm- beats per min, PWD- people with dementia

(which were within the cut off wear time) were used for analysis for change in physical activity.

5.4.5.2 Activity data from Basis Peak

The Table 5-3 shows various measures that were used to assess the physical activity by changes between the baseline and the intervention period. There was no significant difference in any of these measures (average calorie per day, total calorie per day, steps per day, % of the day heart rate was above 100 bpm, and % of the day heart rate was above mean plus 10 % of the mean heart rate) between the baseline and intervention period in the non-demented group, PWD group, or all participants as a whole. However, the increase in steps/day post-intervention for the PWD group approached significance ($p = 0.07$).

The colour scale figures in Figure 5-3 and Figure 5-4 shows activity details (calorie/minute, steps, and heart rate) of PWD and a non-demented participant respectively (the colour scales for both the participants are of the same scales). Visually it is quite clear how the non-demented participant shown in Figure 5-4 is more active than the PWD in Figure 5-3.

5.4.5.2.1 Assessment of activity data from Basis Peak for the aimed 10 % increase

The measures recorded by Basis Peak to assess physical activity were evaluated for success of increasing physical by 10 % above the baseline period. This data is presented in Table 5-4. Three participants' activity increased up to or above the 10 % aimed increase based upon calorie measure, three participants based upon step count, and four based upon heart rate measure (% of the day heart rate was above mean plus 10 % of the mean heart rate). However, these measures identified different participants. For example, 2 out of the 3 participants that increased calorie count were not those which were identified as having increased step count.

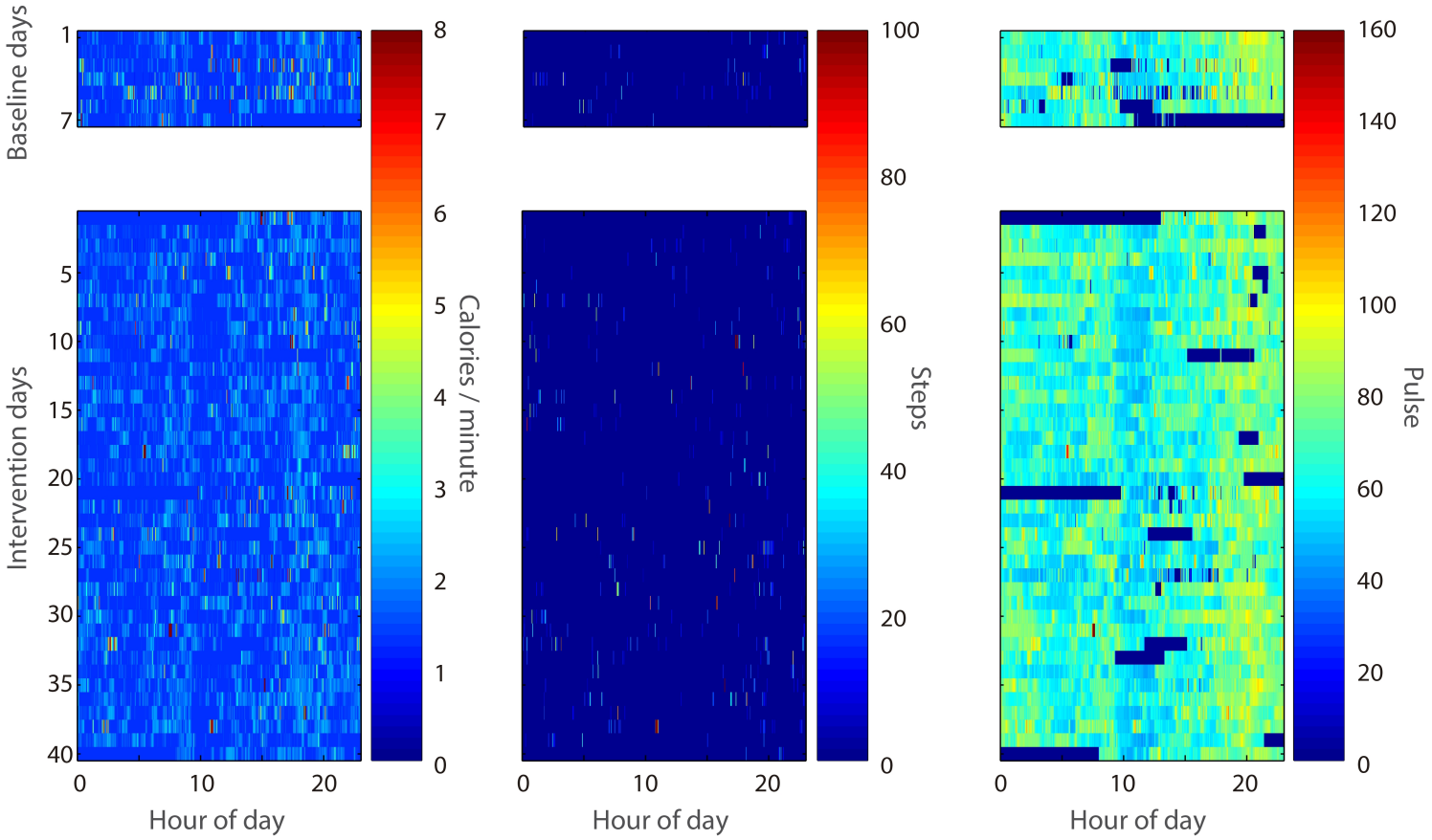


Figure 5-3 Activity measurements of a PWD participant

Left figures: calorie/minute (0-8), middle figures: steps (0-100), and right figure: heart rate/pulse (0-160) with its colour scale next to the figure. Top and the bottom figures showing the baseline and intervention period respectively. Each data point corresponds to average 3 minutes data.

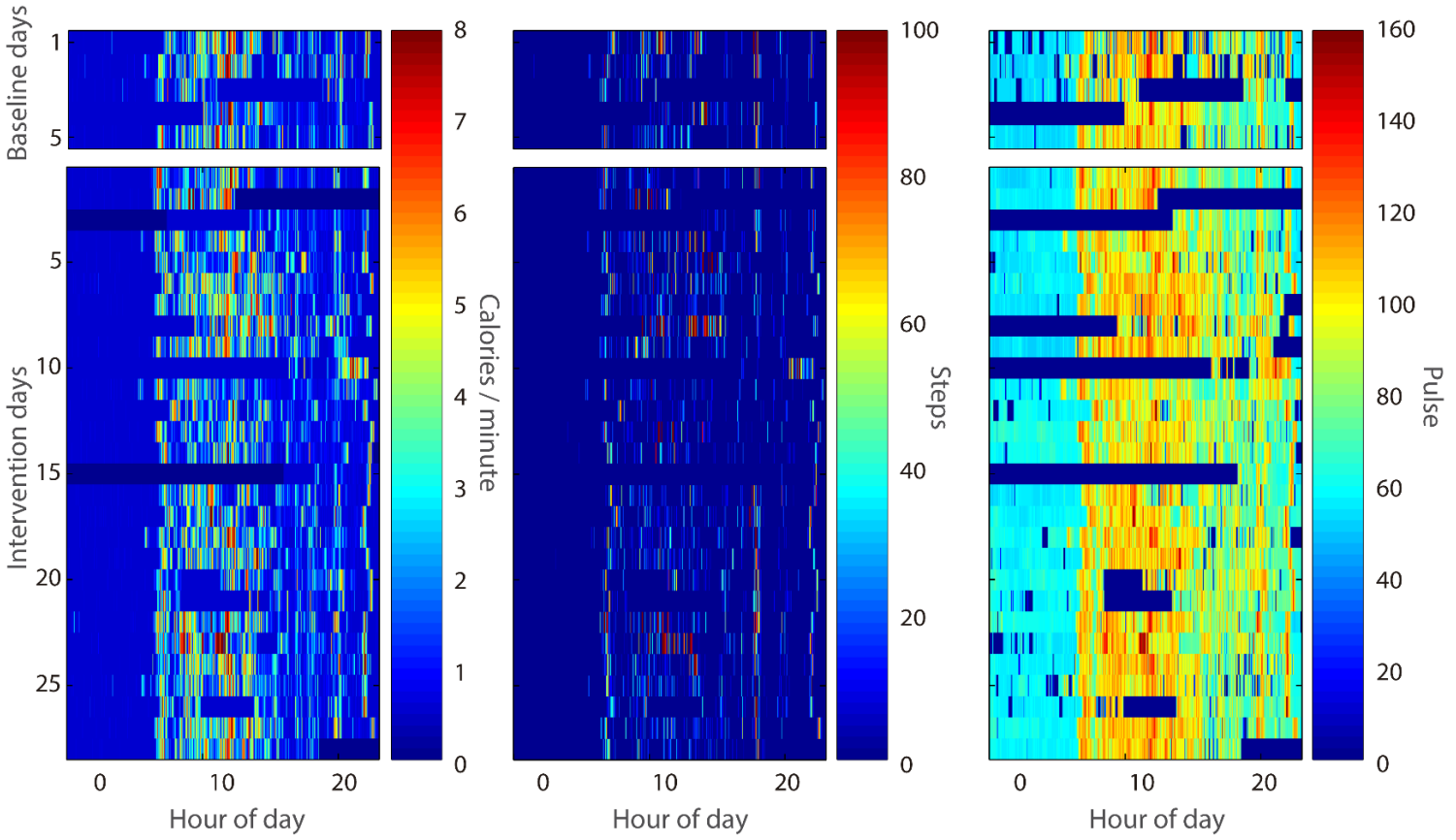


Figure 5-4 Activity measurements of a non-demented participant.

Left figures: calorie/minute (0-8), middle figures: steps (0-100), and right figure: heart rate/pulse (0-160) with its colour scale next to the figure. Top and the bottom figures showing the baseline and intervention period respectively. Each data point corresponds to average 3 minutes data

Table 5-4 Aimed 10 % increase in activity

Calorie			**Steps**			**% HR > mean HR plus 10% mean HR (bpm)**		
Baseline	Aimed 10% increase	Intervention	Baseline	Aimed 10% increase	Intervention	Baseline	Aimed 10% increase	Intervention
1874	*2061*	***2142***	*879*	*967*	*903*	*19.53*	*21.49*	*20.28*
2419	*2660*	*2341*	*182*	*200*	***272***	*27.78*	*30.56*	*27.23*
2472	*2719*	*2387*	*216*	*237*	*234*	*16.09*	*17.70*	***18.92***
2059	*2264*	***2299***	*188*	*206*	***324***	*12.37*	*13.61*	*11.13*
2345	2580	**2990**	6806	7487	6472	16.10	17.71	**22.98**
1928	2120	1859	3112	3423	2418	26.78	29.46	25.27
2341	2576	2243	2608	2868	2047	32.29	35.52	**35.96**
2933	3226	2843	4847	5331	**5656**	23.18	25.49	**25.96**

Each row represents measures for a particular participant. The top four rows (in italics) are participants in PWD group and the bottom four in the non-demented group.
Measures that were raised up to or above the 10 % aimed increase during the intervention period are highlighted in grey and in bold font.
HR- heart rate

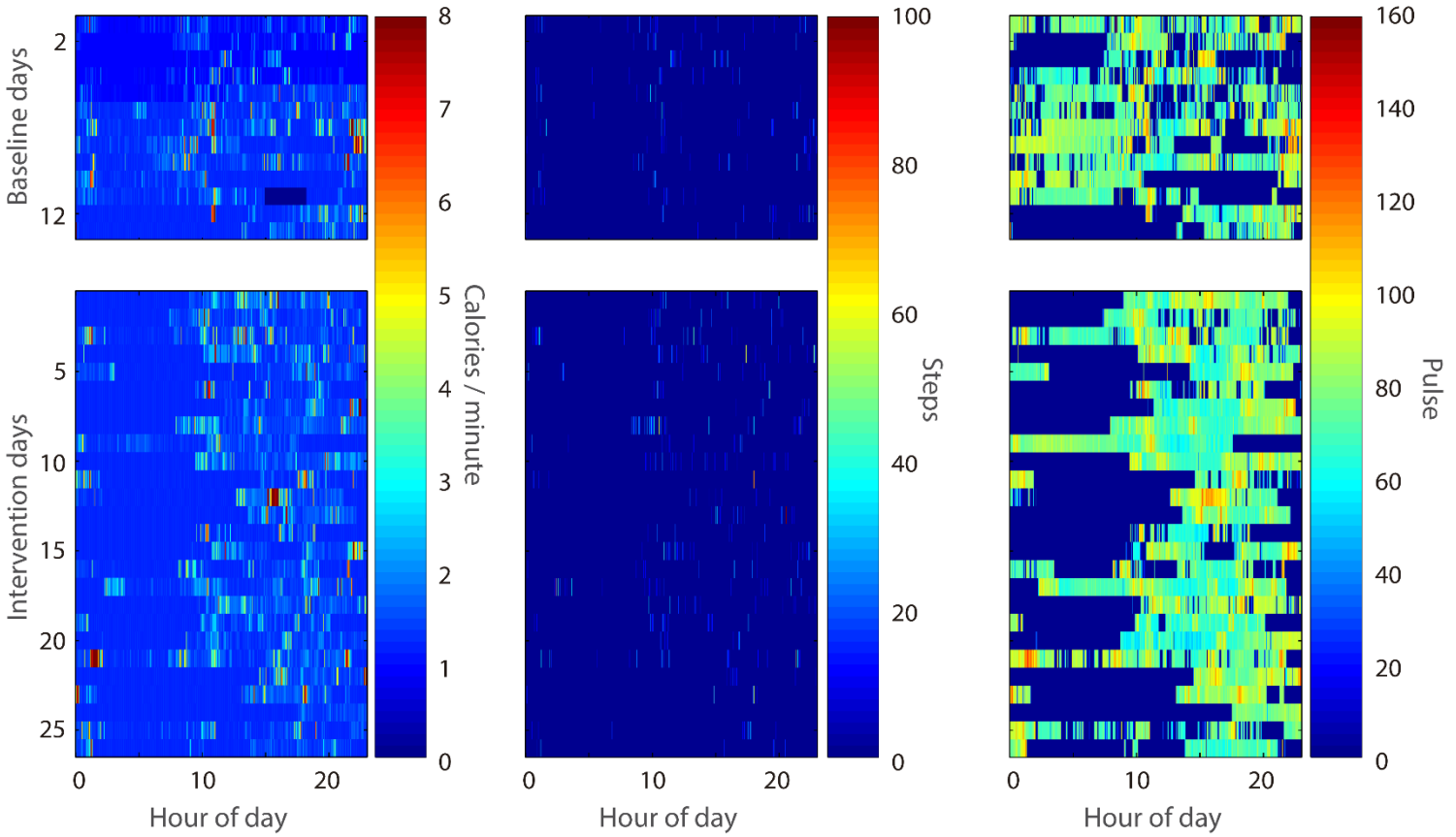


Figure 5-5 Activity measurements of the participant with the lowest activity monitor wear time.

Left figures: calorie/minute (0-8), middle figures: steps (0-100), and right figure: heart rate/pulse (0-160) with its colour scale next to the figure. Top and the bottom figures showing the baseline and intervention period respectively. Each data point corresponds to average 3 minutes data.

5.4.5.3 Basis Peak activity monitor retention

The mean wear time of all the participants (n = 8) was 81 ± 14 %. The wear time for the PWD group (n = 4) and the non-demented group (n = 4) were 73 ± 15 % and 89 ± 7 % respectively. There was no significant difference in the wear time between the PWD and the non-demented group t (6) = -2.01, p = 0.09. The lowest wear time was 57 % for a PWD participant, shown in Figure 5-5.

5.4.5.4 Measurement using physical activity questionnaire

The mean physical activity questionnaire scores for the PWD and non-demented groups were 18 ± 20 and 107 ± 59 respectively, and this was significantly different t (6) = -2.83, p = 0.03. The relationship between the scores from the physical activity questionnaires (taken post intervention) and the data from the basis peak was assessed. Pearson correlation showed a significant positive correlation between the physical activity questionnaire score and the steps during the intervention period (lg10 transformed for normal distribution) measured by the activity monitor (r = 0.88, n = 8, p < 0.01) (Figure 5-6). The questionnaire scores were not correlated with calorie (p = 0.08), % of the day heart rate was above 100 bpm (p = 0.20), or % of the day heart rate was above mean plus 10% of the mean heart rate (p = 0.57).

5.4.6 Markers of oxidative stress in blood

Markers of oxidative stress in plasma before and after the exercise intervention are shown in Figure 5-7. There were no significant changes in any of the markers before and after the intervention: MDA levels [t (5) = 0.05, p = 0.66], total antioxidant capacity levels [t (5) = -0.45, p = 0.67], and protein carbonyls [Z = -0.67, p = 0.50].

5.4.7 Cerebrovascular measures

There was no significant change in sitting MCAv mean following the exercise intervention (n = 5, baseline: 44 ± 8 cm s^{-1} and intervention: 43 ± 9 cm s^{-1}) t (4) = 0.44, p = 0.68. Out of the 5

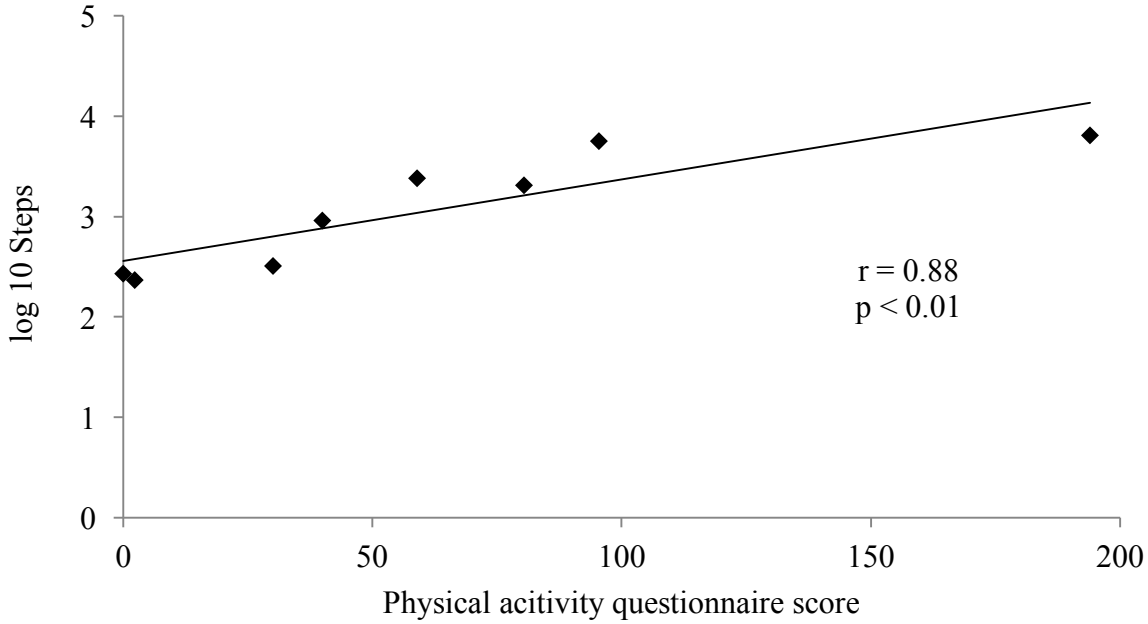


Figure 5-6 Pearson's correlation graph of physical activity questionnaire score with log 10 steps.

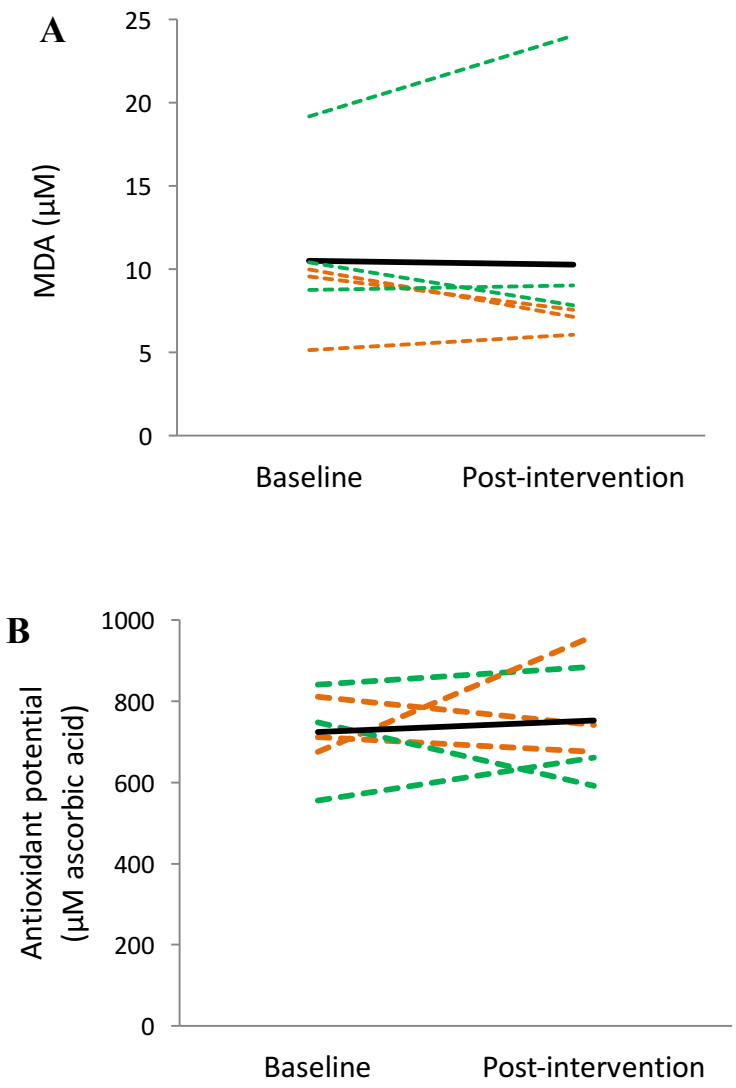


Figure 5-7 Markers of oxidative stress

A) MDA levels, **B)** total antioxidant capacity, and **C)** protein carbonyl in plasma at baseline and after intervention ($p = 0.66$, $p = 0.75$and $p = 0.50$ respectively). The dotted lines (**green** - non-demented individual, **brown** – PWD participant) represent an individual participant, and the black solid line represents the mean of all participants ($n = 6$).

participants assessed for cerebrovascular measures during the sit-to-stand manoeuvre, clean/good cerebrovascular resistance (CVR) [mean arterial pressure/blood flow velocity (mm Hg/ cm s^{-1})] data was obtained from only two participants for both the pre and post intervention (due to loss of BP signal during the sit-to-stand manoeuvre, especially in participants with mobility issues). Therefore, changes in CVR before and after the intervention could not be assessed statistically in this study. The data for the cerebral blood flow response to the manoeuvre is presented in Figure 5-8. The time series of arterial pressure and blood flow velocity is shown in Figure 5-9.

5.4.8 Other outcome measures: cognition, quality of life, and physical function

Table 5-5 shows measures of cognition, quality of life, and physical function as measured by MMSE, DEMQoL and TUG respectively before and after the exercise intervention. There was a non-significant improvement in MMSE and TUG after the exercise intervention in all the groups. Similarly, there was no significant change in DEMQoL. There were no significant differences in the MMSE and DEMQoL scores between the PWD and non-demented group at baseline. TUG was significantly better in non-demented group compared to PWD group at baseline. One participant in the non-demented group did not take part in the MMSE test, as the participant's first language was not English.

5.4.9 Participant's perspective (End of study questionnaire)

Five (PWD/non-demented group = 2/3) out of the eight participants thought that they had managed to increase their physical activity to the 10 % during the intervention period when asked in the end of study questionnaire (according to energy expenditure data from Basis Peak, only 3 participants had achieved the 10 % increase). The participants responded that they would like to maintain the increased physical activity level after the end of the study. The

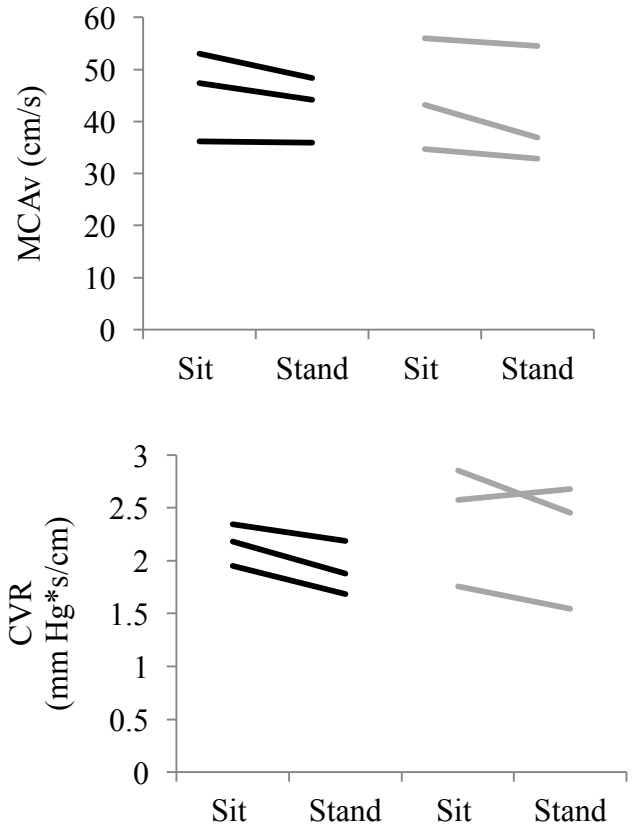


Figure 5-8 Cerebral blood flow response to single step change in blood pressure

Cerebral blood flow-velocity (MCAv) changes (top) and CVR index (bottom) induced by sit-to-stand manoeuvre (n=4). Left (black lines) and right side (grey lines) are pre- and post-exercise intervention respectively.

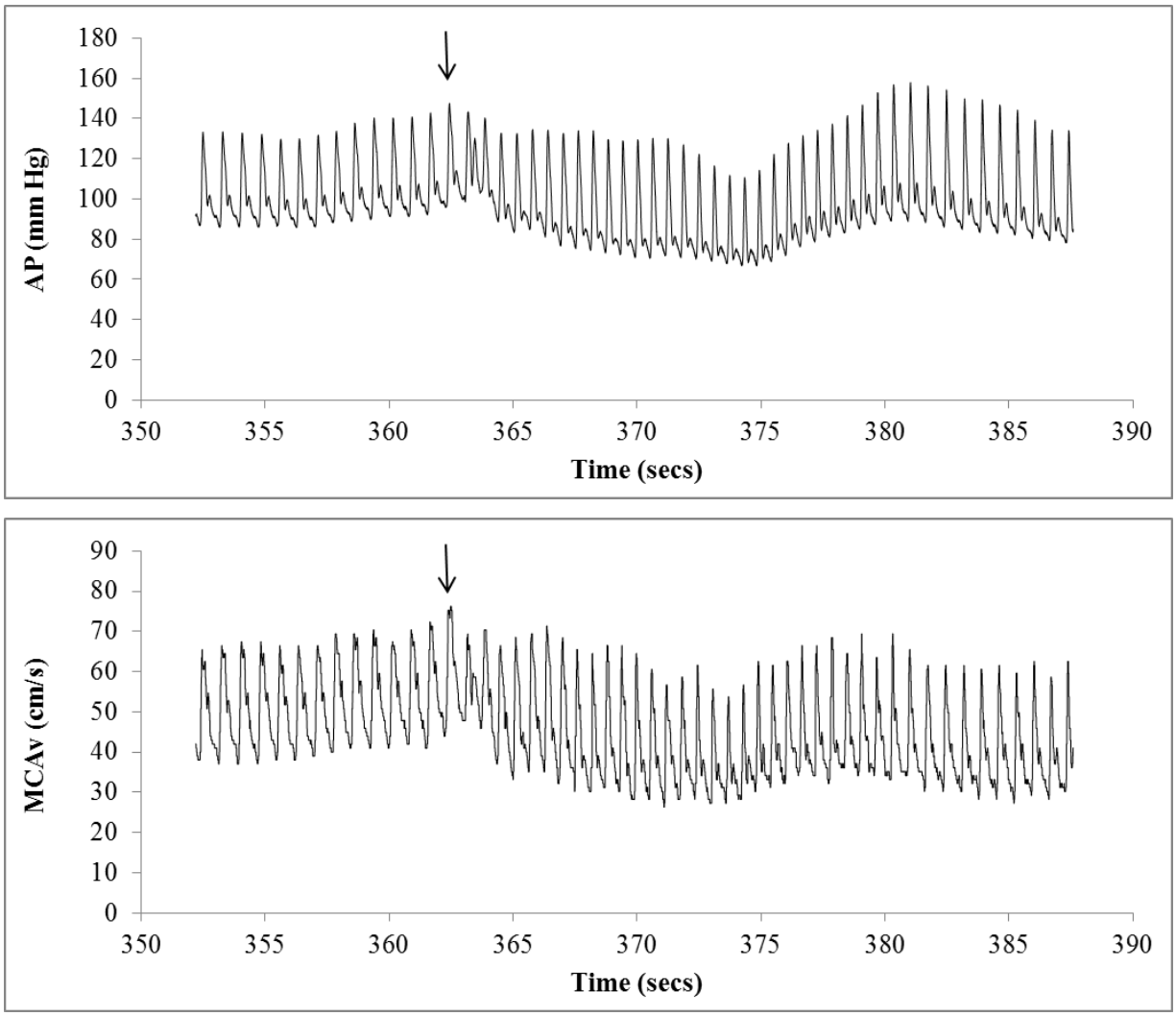


Figure 5-9 Time series of cerebral blood flow response to single step change in blood pressure

Time series of arterial pressure (AP) and MCAv before and after posture change (stand from sit) for a participant. The beginning of the posture change is indicated by the arrow.

Table 5-5 Other outcome measures: cognition, quality of life, and physical function

	All participants (n = 8)			PWD (n = 4)			Non-demented (n = 4)			
	Baseline	Intervention	p value	Baseline	Intervention	p value	Baseline	Intervention	p value	p value [a]
MMSE*	25.6 ± 4.2	26.1 ± 4.6	0.48	24.3 ± 5.1	24.5 ± 5.6	0.86	27.3 ± 2.5	28.3 ± 2.1	0.22	0.38
DEMQoL	95.5 ± 10.0	98.4 ± 10.8	0.57	94.5 ± 8.9	90.5 ± 9.5	0.37	96.5 ± 12.4	106.3 ± 3.9	0.30	0.80
TUG	21.1 ± 14.3	19.3 ± 14.4	0.14	31.1 ± 13.7	29.1 ± 14.8	0.43	11.1 ± 4.7	9.6 ±3.8	0.06	0.03 $^{\phi}$

p value- comparison between baseline and intervention period (paired t-test)
p value [a]- comparison between PWD and non-demented participants at baseline (independent t-test).
$^{\phi}$ - significant difference between PWD and non-demented participants at baseline
* - MMSE only had 3 participants in the non-demented group and thus there were only 7 participants overall
PWD- people with dementia

participants responded that the activity monitor was easy and comfortable to wear, with the exception of one participant who found it uncomfortable at night. This participant commented "it was ok during the day, but was difficult sleeping with it during the night".

5.5 DISCUSSION

The exercise intervention was not successful in increasing the physical activity (assessed by energy expenditure) of all of the participants. As would be potentially expected when considering this failure to achieve the desired increase in physical activity level, no significant changes were observed in measures of oxidative stress, nor other measures of health including CBF, quality of life, cognition, and physical function. The findings from the study suggest the delivery of the physical activity intervention was not successful. The Basis Peak identified different participants as having achieved a 10 % increase in physical activity dependent on which outcome was used to measure physical activity.

The unsupervised home-based exercise training design in this study was not successful in increasing the physical activity of all participants, however previous studies have shown that unsupervised exercise interventions can increase physical activity levels (Hillsdon et al., 1995). Numerous studies, including randomised controlled trails, have found home-based exercise training to be effective in improving the respective study measures (such as balance and physical function) (Ashburn et al., 2007, Campbell et al., 1999, Donat and Ozcan, 2007, Gardner et al., 2011, Jette et al., 1996, Li et al., 2015, Marshall et al., 2001, Mock et al., 2001, Moorcroft et al., 2004, Olney et al., 2006, Schwartz, 2000, Segal et al., 2001, van Diest et al., 2016). In addition, unsupervised exercise training has been successful in older adults both living both in the community (Campbell et al., 1999, Jette et al., 1996, Li et al., 2015, van Diest et al., 2016) and nursing homes (Donat and Ozcan, 2007).

Unsupervised home-based exercise training has several advantages to supervised exercise training. Compared to supervised exercise training, unsupervised exercised training is cost effective and not labour intensive. More importantly, participants may adapt better to unsupervised home exercise sessions rather than to supervised sessions in a lab or facility based environment. This was an important consideration for the participants of the current study, as they were home based and received care visits at home. Although participants were free-living, and independent in their own homes, they did not often leave the house, and almost never alone, thus exercise that could be undertaken in the home, or walking close to home with their carer or family member was important. Facility based group exercise interventions may impact adherence due to time constrains and barriers in getting to the facility. Some studies have found supervised exercise training to be superior to unsupervised home-based training (Donat and Ozcan, 2007, Olney et al., 2006), whereas, other studies have found that unsupervised home based exercise intervention resulted in larger improvement in physical functioning compared to supervised facility based training (Gardner et al., 2011, Segal et al., 2001). Nevertheless, transferring an exercise training programme to the community and sustaining it, is a challenge that needs to be met, whether that programme be supervised or unsupervised.

A review looking at studies of physical activity promotion, found that unsupervised exercise interventions are most effective with personalised instructions, continued support/contact, exercise of a moderate intensity, not dependent on attendance at a facility, and an exercise programme that can easily fit into an existing lifestyle (Hillsdon et al., 1995). The study design of the current study met these recommendations. However, there are several potential factors that may have contributed to the failure of the current intervention in achieving the aimed increase in physical activity. Although the researcher was in contact with the

participants during the intervention period to check on the study progress and to motivate the participants, the data on physical activity undertaken was not analysed until the end of study, which meant that progress goals were not based on objective measures taken from the activity monitor. Adherence facilitation is important; a study (Duncan and Pozehl, 2002) looking at adherence to unsupervised exercise following completion of a supervised exercise programme found that exercise adherence was higher in those who received adherence facilitation compared to control group who did not receive facilitation (facilitation involved graphic depiction of each patient's exercise goals in comparison to each patients' exercise participation). Although the current study was undertaken in collaboration with a care provider, the limited time that the carers spent with some of the participants (for example some participants received regular and multiple short visits daily) may not have been adequate to facilitate the integration of regular exercise into their daily routine.

Basis peak (with its ability to monitor heart rate continually) was the most suitable activity monitor to meet the requirements of the study, at the time of assessment of potential activity monitors for this study. Ability to measure heart rate continually was a priority for selection of the activity monitor to be used. This was because the population group of our study were expected to be sedentary with some mobility issues, so monitoring heart rate would allow alternative measure of physical activity to the step count measure. Motion sensors such as accelerometers and pedometers are mainly limited to ambulatory activities (Crouter et al., 2004). Although the use of heart rate for estimating energy expenditure during exercise has limitations: i.e. factors such as stress, hydration level, gender, temperature and humidity can effect heart rate (Crouter et al., 2004), it is considered to be a valid measure to quantify exercise energy expenditure (Colley et al., 2008, Hiilloskorpi et al., 2003). Heart rate is linearly related to oxygen uptake for dynamic activities involving large muscle groups, so it

can provide a reasonable estimate of energy expenditure during exercise, and could be valuable tool for quantifying intensity of exercise bouts (Crouter et al., 2004).

Basis Peak, as a tool to assess physical activity in older adults, had both its pros and cons. The step count measured by the Basis Peak was positively correlated to the physical activity questionnaire score, which suggests that the activity monitor has the capability to accurately assess step count in this population. The wrist watch format (and displaying time) of the Basis Peak activity monitor is a familiar format for participants who are not accustomed to activity monitoring compared to activity monitors with other wear locations such as waist, ankle or thigh. Additionally, it is waterproof, which enables wearers to keep the device on at all times including when showering, thus minimising the chances of participants forgetting to put the device back on. This is perhaps particularly beneficial for participants with memory problems. This is supported by the high wear time in this study; 81 % of the total time on average in all participants and 73 % of the total time in the PWD group. One of the main drawbacks of the Basis Peak activity monitor is its short battery life. If it ran out of battery, it had to be synced with its app in order to sync the time on the activity monitor. Data, from the period when time on the activity monitor was not synced, cannot be acquired as Basis Peak produces data in relation to time. Assessing measures obtained from Basis Peak to check if participants were successful in meeting the aimed 10 % increase in physical activity level showed that different measures identified different participants to have achieved the 10 % increase (3 participants increased energy expenditure over 10 % during the intervention period). Thus this monitor may not be appropriate for people who are very sedentary. Additionally, acquiring activity measures with it for periods longer than couple of days (as in this study) would require monitoring that the battery is charged regularly. Additionally, the memory capacity of the activity monitor was only around two weeks (depending on usage).

There was no significant improvement in any of the blood biomarkers of oxidative stress, or markers of health measured in the study presented herein. Previous studies, in healthy older adults as well as PWD, have shown that exercise intervention training can improve measures of oxidative stress (Takahashi et al., 2013), as well as the measures of quality of life (Liu-Ambrose et al., 2005, Williams and Tappen, 2008), cognition (Colcombe and Kramer, 2003, Kemoun et al., 2010, Lautenschlager et al., 2008), and physical function (Kalapotharakos et al., 2010, Kemoun et al., 2010, Rolland et al., 2007). However, as this study was not successful in achieving the aimed increase in physical activity level, it is not surprising that no significant improvements were seen in these measures after the exercise intervention. Similarly, no changes in CBF were observed following the exercise intervention. Despite higher aerobic fitness being linked to higher CBF across the adult lifespan (Ainslie et al., 2008, Bailey et al., 2013), human studies have not found exercise training to increase CBF in young and old individuals (Murrell et al., 2013, Tomoto et al., 2015) or in stroke patients (Ivey et al., 2011). We could not assess cerebral autoregulation statistically due to problems acquiring clean BP data from our small sample size. Some of the participants had mobility issues which affected the BP signal during standing and during sit and stand manoeuvres.

The level of physical activity of the PWD group was significantly lower compared to the non-demented group, both when assessed by the physical activity questionnaire and the step count measured by the Basis Peak. The PWD group also had impaired performance in physical function, assessed by TUG, compared to the non-demented group. In our small sample sized study, due to problems in acquiring samples and measurements from all participants, it was not appropriate to run statistical tests on markers of oxidative stress and CBF to compare between the PWD and the non-demented participants.

One of the main strengths of the study presented herein was in the measurement of physical activity of the participants: i) the objective measurement of physical activity and ii) continuous measurement of physical activity throughout the day during the baseline and intervention period. No previous studies were found that undertook an unsupervised exercise intervention measuring adherence with an activity monitor continuously through the day. With unsupervised exercise training, adherence is not easily monitored, and is usually done by self-report in form of diaries, charts or questionnaires. These subjective measures of adherence have the problem of overestimation (Conway et al., 2002). Whereas, the main limitation of the study was the small sample size, which means that the findings of the study should be interpreted with caution.

5.6 CONCLUSION

The home-based unsupervised physical activity intervention presented herein, failed to increase the level of physical activity, assessed by energy expenditure, in sedentary older adults. No changes were found in markers of oxidative stress or other health measures assessed in response to the exercise intervention. Progressive goals, based on adherence measurement, are potentially important for the success of an unsupervised exercise intervention. Basis Peak may not be the optimal tool for assessing physical activity in sedentary older adults, especially for long term assessment.

6 CHAPTER 6: General Discussion

6.1 Summary

Brain GSH is a potential marker of redox status and MRS represents an important non-invasive methodology to monitor its changes. In a small cohort of sedentary young men, an acute bout of moderate exercise caused a decrease in brain GSH. In the periphery, HII, an exercise involving a very high intensity, was found to elicit a greater oxidative stress response when compared to moderate intensity exercise in the traditionally prescribed form of continuous steady state. The CBF to the brain seemed to be protected against hyperperfusion during the high intensity phase of the HII exercise. An unsupervised exercise intervention in older adults was not successful in increasing the physical activity level of all the participants to the aimed 10 % increase, which was objectively measured using an activity monitor. There were no significant changes in markers of oxidative stress or other health measures in response to this exercise intervention. The effectiveness of objectively measuring habitual physical activity level in this participant group using the wrist-worn activity monitor was assessed.

Optimising exercise to target the ageing brain has the potential to prevent diseases associated with ageing including dementia, but although the benefits of exercise for the brain are becoming increasingly apparent, its mechanisms are still poorly understood (Lucas et al., 2015). There is clear evidence that exercise can play a significant role in both the prevention as well as treatment of diseases and conditions associated with ageing such as dementia, cancer and diabetes (Hamer and Chida, 2009, Heyn et al., 2004, Lee et al., 2012). With no curable treatment for many of these diseases at present, there is a necessity to focus on exercise as a modifiable risk factor, which would help reduce the ever-increasing economic burden associated to these conditions.

Exercise or physical activity of any intensity is probably beneficial. However, there is not enough data regarding low intensity exercise to verify this (Lee and Shiroma, 2014). There is a need to investigate if low intensity physical activities are also capable of inducing similar benefits as moderate to vigorous intensity physical activities, especially in older population who are likely to be restricted in their physical capabilities. If indeed they are, their beneficial effects are probably lower compared to higher intensity activities as studies (Bailey et al., 2004, Goto et al., 2007, Guiraud et al., 2012a, Swain and Franklin, 2006, Wang and Huang, 2005) (including that presented in chapter 4) have shown exercise intensity to be correlated to the measurement assessed including oxidative stress (although chronic exercise of high intensity can be detrimental, an area that this thesis has not covered). What is certain is that the intensity of exercise contributes to the extent of the resulting exercise effect whether it be oxidative stress other exercise induced effects.

6.2 Effect of acute exercise in the brain

The effect of acute exercise on brain GSH was investigated using an MRS method in sedentary young men. In chapter 3 an MRS method was optimised to detect and quantify GSH in the brain. Then, in chapter 4, brain GSH in response to two types of exercise, HII and continuous moderate exercise, were assessed using this optimised MRS method. The results presented suggested that brain GSH decreased in response to an acute bout of continuous moderate intensity exercise, an effect that was not seen with HII. If indeed an acute bout of exercise of moderate intensity is capable of inducing redox changes in the brain, an organ that is highly susceptible to oxidative stress, it is plausible that it may lead to an adaptive response to protect the brain against oxidative stress. This is in agreement with the findings reported in rodent studies that aerobic moderate exercise may promote a protective antioxidant function of the brain (Camiletti-Moiron et al., 2013). This is the first study to investigate the effect of exercise on one of the markers of oxidative stress in the human brain.

Additionally, CBF to the brain during the two types of exercise was assessed by measuring MCAv using TCD, a method which is capable of yielding beat-by-beat measures of CBF. Looking at each of the individual responses to the exercise bouts, the change in MCAv was similar, however, looking specifically at the high intensity phase of HII trial showed that there was a significant decrease in MCAv after an initial increase. Previous studies have shown that CBF increases with exercise until ~60 % $\dot{V}O2max$ after which hyperventilation-induced hypocapnia reduces CBF towards resting value (Ogoh and Ainslie, 2009). Indeed, at the start of the high intensity phase of HII, the MCA_V increased which then rapidly declined within seconds. This instantaneous decrease in MCAv indicates that there is a very finely tuned mechanism of CBF which protects the brain against hyperperfusion and thus it damaging consequences such as BBB disruption.

6.3 Effect of exercise on oxidative stress in the periphery

In addition to brain GSH, chapter 4 investigated the effect of the two types of exercise on markers of oxidative stress in the blood. There was a difference in the oxidative stress response in the blood between the two types of exercise, with HII eliciting greater changes in oxidative stress markers. This was in contrast to the finding in the brain where moderate exercise seemed to have elicited a significant decrease in brain GSH, compared to HII. This suggests that the HII used in this study elicited a stronger oxidative stress in the periphery then moderate exercise trial. This is in agreement with various studies that have reported that higher intensity exercise results in greater oxidative stress compared to moderate intensity (Goto et al., 2007, Quindry et al., 2003, Wang and Huang, 2005). In contrast to the finding in chapter 4, a recent study by Wadley et al. (2016a) reported that HII elicited a similar oxidative stress response compared to moderate exercise in plasma. However, Wadley et al. (2016a) used a comparatively lower intensity profile in the HII, to the study presented in this thesis, and indeed the variety of HII forms used in studies make it difficult to directly compare results. A schematic diagram of existing literature regarding exercise intensity and oxidative stress together with the findings from chapter 4 is presented in Figure 6-1.

6.4 Unsupervised exercise intervention in older adults

In chapter 5, a low dose unsupervised home-based exercise intervention was prescribed in a group of older adults. The intervention was unsuccessful in increasing the physical activity, assessed by energy expenditure, of all the participants to the aimed 10 % increase from their baseline level. Thus as would be potentially expected, the intervention did not alter markers of oxidative stress or other markers of health such as physical function. Although the design of

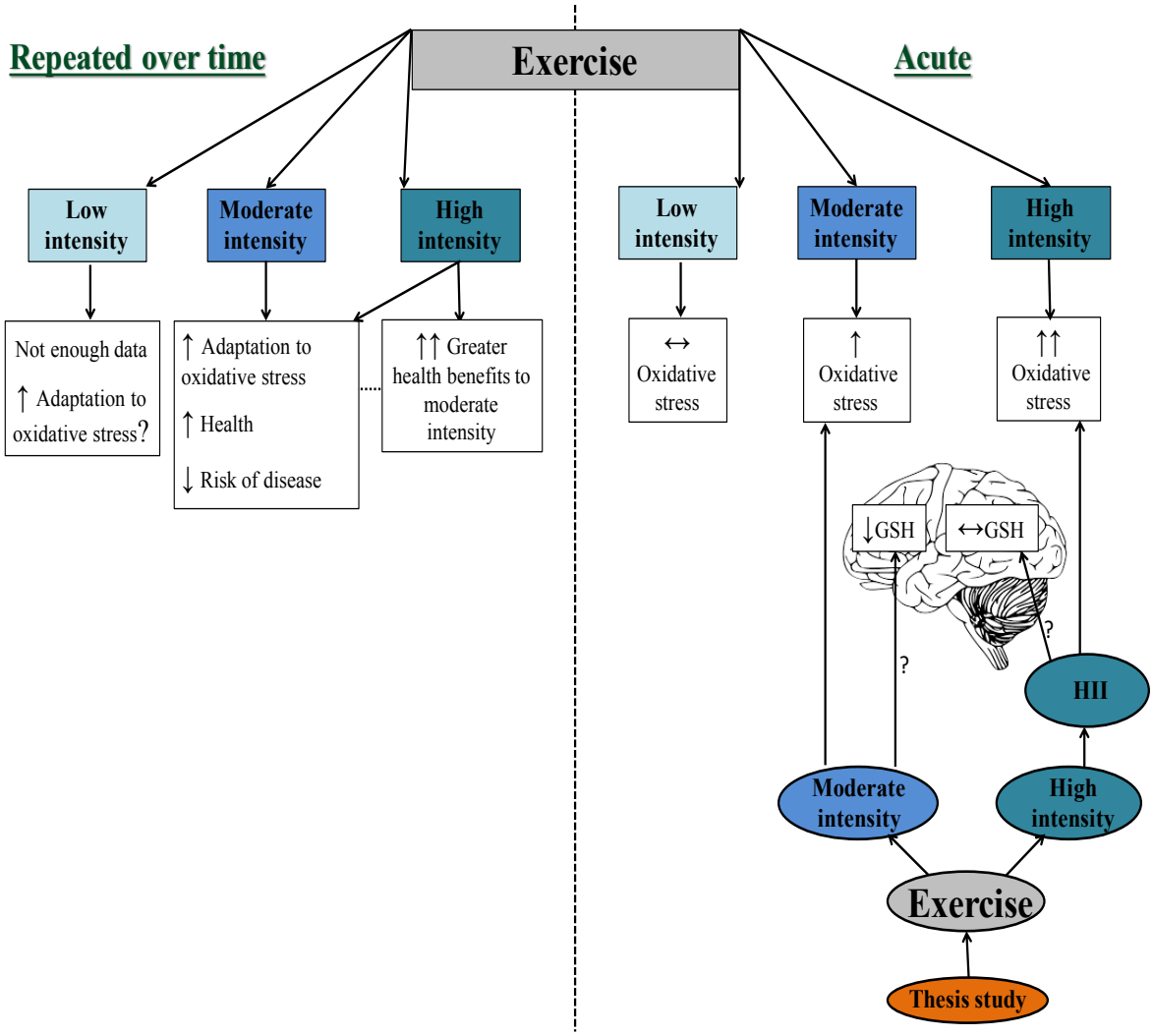


Figure 6-1 Exercise intensity and oxidative stress: existing literature plus thesis finding

Schematic diagram showing the effect of regular and acute exercise according to exercise intensity. Findings from existing literature is presented along with the finding of the study presented in chapter 4. HII, high intensity interval exercise; GSH, glutathione

the exercise intervention included factors found to be effective for unsupervised exercise training including personalised instructions, continued support/contact, not dependent on attendance of a facility, and a programme that can fit into an existing lifestyle as recommended by Hillsdon et al. (1995), the intervention was not successful probably as it lacked progressive goals and adequate facilitation.

6.5 Basis Peak as a tool to assess habitual physical activity in older adults

A secondary aim of the study presented in chapter 5 was to assess the appropriateness of the activity monitor, Basis Peak, to measure habitual physical activity in older adults. Basis Peak was chosen from the range of available activity monitors as it was capable of measuring continuous heart rate along with other activity measures, whilst not being invasive or unusual to wear. The adherence to wearing Basis Peak was good, although Basis Peak had its limitations for wear, as with any other activity monitor. Basis Peak, in the form of wrist watch, was able to collect a wealth of information through 24 hours.

There are certain issues to take into account while measuring physical activity in older adults using such activity monitors. The physical fitness and the type of activities differ between older and younger adults. One of the criteria for the selection of activity monitor was ability to measure heart rate continually. As some of the participants of this study would be expected to have mobility issues as well as being sedentary and this would allow alternative assessment of physical activity. It was found that scores from the physical activity questionnaire were correlated to the steps counts measured by Basis Peak, but not to other measures including heart rate. This may be due to limitations with the device finding heart rate if not worn tight enough, or may be due to the very small increases seen with low intensity activity such as arm raises or sit to stand movements. The device is perhaps most successful in detecting the

magnitude of increase seen when running or cycling is initiated. Overall, Basis Peak is able to provide very informative data on physical activity but with its current features it was not an optical activity monitor to assess physical activity in this population.

6.6 Strengths and limitations

There were many novel investigations carried out in the studies presented in this thesis. GSH, one of the markers that is generally used to assess oxidative stress, was measured for the first time in the brain in response to acute exercise. This was the first work to measure HII induced changes in CBF, even though there is an increasing interest in this form of exercise as an alternative to improve health. In the study in older adults, the exercise intervention in free living condition was measured objectively using an activity monitor –Basis Peak. The sample size of the studies presented is perhaps the main limitations of the studies presented.

6.7 Future directions

Brain is an import organ to target for exercise-induced benefits, however, it is a difficult organ to access. With advancement of technologies available to access various measures in the brain, more studies are starting to investigate the exercise-induced effects in the brain. In order to further validate the finding presented herein that an acute bout of moderate exercise can decrease brain GSH, the effect of acute exercise on brain GSH should be assessed on a larger sample size, especially due to the limitations in the sensitivity of MRS method with current technology. The findings from chapter 4 suggest that the brain might be protected against sudden high intensity exercise induced hyperfusion injury, at least in apparently healthy young adults. Exercise is not risk free, especially during very high intensity exercise. Previous studies including a meta-analysis review reported that HII can be safe and that risk of cardiovascular event with HII is low even though HII has ~5 times higher risk of acute

adverse cardiovascular event compared to continuous moderate exercise (Lucas et al., 2015, Rognmo et al., 2012, Weston et al., 2014). A next step would be to look into elderly population- in order to investigate if HII induced effects on measures of blood flow and oxidative stress in the brain are similar to that in young individuals.

In the periphery, it is evident that higher intensity exercise induces a greater oxidative stress response (as observed in the study presented in chapter 4) that would potentially lead to better adaptive response. Older adults, especially those with mobility issues, could potentially take part in higher intensity exercise in a suitable format to suit their condition, in order to assess the exercise induced responses due to varying form of exercise intensity.

6.8 Concluding remarks

The work presented in this thesis has enhanced our understanding of exercise and oxidative stress, and physical activity in older adults with the following novel findings:

1. Brain GSH seems to be decreased in response to acute exercise bout, but further investigation is required to confirm this finding.
2. A HII bout, involving very high intensity exercise phases, elicited a greater oxidative stress response in blood/plasma compared to a continuous moderate exercise.
3. During the high intensity phase of HII, there was a rapid significant decrease in the MCAv after the initial increase, which suggests the brain is protected against hyperperfusion.
4. An activity monitor, Basis Peak, has limitations in its capability to measure physical activity in older adults.

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