Evaluating Glutathione Peroxidase Activity as a Marker of Cognitive Impairment in Coronary Artery Disease Patients

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Coronary artery disease (CAD) patients are at risk for vascular cognitive impairment (VCI), yet mechanisms remain elusive. We hypothesized that glutathione peroxidase activity (GPx), which protects from oxidative damage, would be higher in CAD patients with cognitive impairment (n=36) compared to those without (n=84), and baseline GPx and change in GPx over time would be associated with changes in verbal memory. CAD patients were recruited from the Toronto Rehabilitation Institute Cardiac Rehab (TRI-CR) program. Cognitive performance and GPx activity were measured at baseline and 6-month timepoints. GPx was significantly elevated in those with cognitive impairment ($F_{1,119}=3.996, p=0.048$). While a 1 nmol/min/mL increase in GPx activity was associated with a decrease in verbal memory z-scores by 0.02 standard deviations (SD) over 6 months ($b=-0.02, df=184.65, p=0.004$), baseline GPx did not predict change in verbal memory. This research suggests that alterations in GPx activity may be associated with cognition in CAD patients.
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List of Abbreviations

ACE- Angiotensin converting enzyme
AD- Alzheimer’s Disease
ANCOVA- Analysis of covariance
ANOVA- Analysis of variance
BCA- bicinehoninic acid
BEST- Biomarkers, EndpointS and other Tools
BMI- Body Mass index
CABG- Coronary artery bypass graft
CAD- Coronary artery disease
CR- Cardiac rehabilitation
EDTA- Ethylenediaminetetraacetic acid
FDA- Food and Drug Administration
GPx- Glutathione Peroxidase
GR- Glutathione Reductase
GSH- Glutathione
GSSG- Glutathione disulfide
GST- Glutathione S-transferase
Max VO$_2$ peak- Maximal rate of oxygen consumption
MCI- Mild cognitive impairment
MI- myocardial infarction
MMSE- mini mental state exam
NAC- N-acetylcyesteine
NADPH- Nicotinamide adenine dinucleotide phosphate
NINDS-CSN- Neurological Disorders and Stroke-Canadian Stroke Network
PTCA-Percutaneous transluminal coronary angioplasty
SD-Standard Deviation
SOD-Superoxide dismutase
UHN-TRI-University Health Network-Toronto Rehabilitation Institute
VCIND-Vascular cognitive impairment-no dementia
VCI-Vascular cognitive impairment
VaD-Vascular Dementia
Chapter I

1 Chapter I Introduction

1.1 Statement of Problem

Coronary artery disease is a pressing healthcare and economic concern as the leading cause of mortality. Cognitive impairment is a significant yet under diagnosed symptom in CAD patients. It is a risk factor associated with negative health outcomes such as poor medical compliance, and increased rates of institutionalization and mortality (Conn, Taylor, and Miller, 1994; Ekman, Fagerberg, & Skoog, 2001; Takata et al., 2014; Tilvis et al., 2004). This clinical population is particularly at risk for vascular cognitive impairment-no dementia (VCIND), known as the prodromal condition for vascular cognitive impairment (VCI), in which cognitive deficits are attributable to cerebrovascular disease but these deficits not being severe enough to be classified as dementia. Interventions for cognitive impairment have the potential to mitigate associated adverse outcomes and improve the prognosis of this disease.

Previous studies have shown that controlling for vascular factors has not been successful in reducing the long-term risk of dementia (Peters et al., 2008). Exercise interventions, such as cardiac rehabilitation (CR) programs, have demonstrated promising efficacy in improving cognitive outcomes; however, results have been mixed in populations exhibiting mild stages of cognitive impairment (Gates, Fiatarone Singh, Sachdev, & Valenzuela, 2013; Verdelho et al., 2012; Young, Angevaren, Rusted, & Tabet, 2015; G. Zheng, Xia, Zhou, Tao, & Chen, 2016). Before successful interventions can be implemented to improve cognitive impairment, the etiology of these symptoms in CAD patients must be elucidated.

It has been established that the glutathione (GSH) antioxidant system is dysregulated in neurocognitive disorders and this can affect the progression from mild stages of cognitive impairment to severe dementia (Baldeiras, Santana, Proenca, & Garrucho, 2010). This could be particularly relevant for CAD patients due to the inflammatory, ischemic and pathological processes associated with this disease which promote oxidative stress (Gorelick et al., 2011; Pashkow, 2011). Glutathione peroxidase (GPx) is
the main enzyme in the GSH antioxidant system, catalyzing the reduction of oxidative stress products such as lipid peroxidation markers. GPx activity has been explored in neurocognitive disorders such as VaD, Alzheimer’s disease (AD) and its prodromal condition mild cognitive impairment (MCI). However, there has been limited research on the activity of this enzyme conducted in VCIND patients or in populations at risk for it, such as CAD patients. Our lab recently reported persistent oxidative stress in CAD patients with possible VCIND, as demonstrated by higher ratios of late stage to early stage lipid peroxidation markers, highlight the potential involvement of GPx in association with their cognitive impairment symptoms (Suridjan et al., 2017), hence providing rationale for this study.

Because of the increased risk of neurodegeneration in this population, this research is important because it investigates an easily accessible serum marker of oxidative stress as a biomarker of early cognitive changes. In addition, also suggests opportunities for the development of optimal therapeutic interventions and strategies to deal with these symptoms in prodromal populations.

1.2 Study Objectives and Hypotheses

The role of the GSH antioxidant system, particularly GPx activity, in association with cognition in CAD patients has not been explored. This study sought to assess if peripheral GPx activity was associated with verbal memory and executive function in CAD patients. The activity of this enzyme was explored as a cross-sectional marker of cognition, comparing possible VCIND patients with CAD patients who did not demonstrate symptoms of cognitive impairment at baseline. Furthermore, baseline GPx activity and change in GPx activity over a 6-month CR program were evaluated as prospective markers of change in verbal memory and executive function performance over time.

Primary Hypothesis

GPx activity will be higher in those with possible VCIND, compared to CAD control patients who did not demonstrate cognitive impairment at baseline.
**Rationale:** GPx activity has been shown to be dysregulated in AD and the prodromal condition MCI, compared to healthy controls (Shrag M et al., 2013). Various studies have reported that activity has been increased or decreased in AD and MCI patients (Balmus et al., 2017; Bermejo et al., 2008; Casado, Encarnacion Lopez-Fernandez, Concepcion Casado, & de La Torre, 2008; Ceballos-Picot et al., 1996; Cristalli, Arnal, Marra, de Alaniz, & Marra, 2012; Jeandel et al., 1989; Kharrazi et al., 2008; Kosenko et al., 2012; Krishnan & Rani, 2014; Martin-Aragon et al., 2009; Padurariu et al., 2010; Puertas et al., 2012; Rinaldi et al., 2003; Torres et al., 2011; Vural, Demirin, Kara, Eren, & Delibas, 2010); and a single study suggests that plasma GPx activity is increased in patients with VaD (Krishnan & Rani, 2014). GPx activity has been documented to be increased in CAD patients compared to healthy adults (Flores-Mateo et al., 2009). A study conducted in the same patient population as this study found that CAD patients with possible VCIND had a higher ratio of late stage to early stage lipid peroxidation markers (Suridjan et al., 2017). Based on the function of GPx in reducing lipid peroxidation products, it was hypothesized that plasma GPx activity would be increased in these patients in order to mitigate the accumulation of oxidative stress, compared to healthy controls.

**Secondary Hypothesis #1**

Baseline GPx activity will predict changes in verbal memory performance in CAD patients over a 6-month CR program.

**Rationale:** The neuropsychological profile associated with VCIND implicates verbal memory as a primary cognitive domain affected in this condition (M. Garrett & Coote, 2009; Gorelick et al., 2011; Vasquez & Zakzanis, 2015). This is further predicated by verbal memory being associated with cognitive impairment in CAD patients and cognitive decline over time (Eggermont et al., 2012; K. Garrett et al., 2004; Laukka, Macdonald, Fratiglioni, & Backman, 2012; Nyenhuis et al., 2004; Santiago et al., 2015; Silbert, Scott, Evered, Lewis, & Maruff, 2007; Vicario, Martinez, Baretto, Diaz Casale, & Nicolosi, 2005; L. Zheng et al., 2012). Prior to this study, baseline GPx activity has not...
been studied in association with change in cognition over time. Our laboratory previously reported results that persistent oxidative stress, evidenced by higher ratios of late stage to early stage lipid peroxidation markers, was associated with decreased improvement in verbal memory over 6 months of CR (Suridjan et al., 2017). Thus, it was hypothesized baseline GPx activity, indicative of the antioxidant response to oxidative stress, would predict changes in verbal memory over this time.

Secondary **Hypothesis #2**

| Change in GPx activity over time will be associated with changes in verbal memory performance in CAD patients over a 6-month CR program. |

**Rationale:** Similar to baseline GPx activity, changes in GPx activity over time have not been studied in association with changes in cognition over time in our population of interest. One study found that an increase in GPx activity was associated with a decline in memory performance (Revel et al., 2015). Thus, this study sought to explore whether similar changes in GPx activity over a 6-month CR program would be associated with changes in verbal memory.

**Exploratory Hypotheses**

- Baseline GPx activity will be associated with baseline executive function z-scores
- Baseline GPx activity will predict changes in executive function performance in CAD patients over a 6-month CR program
- Change in GPx activity over time will be associated with changes in verbal memory performance in CAD patients over a 6-month CR program

**Rationale:** Along with verbal memory, this study explored associations between GPx activity and executive function. Executive function is another cognitive domain most commonly affected in vascular cognitive impairment (Gorelick et al., 2011; Vasquez & Zakzanis, 2015). Exploring associations with this domain in addition to verbal memory would further contribute the consensus about whether GPx activity could be associated
with cognition overall. Based on similar rationale to the hypotheses surrounding verbal memory, GPx activity and executive function composite Z-scores at baseline were hypothesized to be correlated with each other. Baseline GPx activity was also hypothesized to be a predictor of change in executive function over the 6-month CR program. Moreover, it was hypothesized that there would be an association between changes in GPx activity and change in executive function over a 6-month CR program.

1.3 Review of Literature

1.3.1 Coronary Artery Disease and Cognitive Impairment

1.3.1.1 Clinical Characteristics of Coronary Artery Disease and Management

Coronary artery disease (CAD) is the primary cause of mortality worldwide, with treatments for CAD representing the leading costs in healthcare (Pollock & Jones, 2015). The World Health Organization (WHO) has reported that 10% of the global disease burden is due to cardiovascular disease, of which CAD and cerebrovascular events represent 64% of deaths (WHO, 2011). While mortality rates from CAD have declined in developed countries, CAD still represents a global concern in which standardized death rates are rising in developed countries (WHO, 2011). Overall, CAD is projected to still be the leading cause of death as of 2019 (Mathers & Loncar, 2006). Presence of CAD is associated with a lower quality of life and predictive of increased rates of hospitalization and disability (Sanchis-Gomar, Perez-Quilis, Leischik, & Lucia, 2016; Unsar, Sut, & Durna, 2007; WHO, 2011).

CAD is characterized by the narrowing of arteries due to the buildup of atherosclerotic plaques, leading to the eventual restriction of oxygen to various tissues and the heart (Grech, 2003; Sanchis-Gomar et al., 2016). When plaque buildup accumulates to 50% diameter stenosis or more, this can result in unstable angina and complete arterial occlusion and can lead to a myocardial infarction (MI) (Grech, 2003). Plaques consist of fatty deposits, calcium deposits and scar tissue as a result of inflammatory cells (Kristensen, Ravn, & Falk, 1997). Rupture of a plaque can lead to a thrombotic response, from which dislodging of a thrombus results in a circulating mass called an embolus, which can occlude arteries resulting in an MI as well (Grech, 2003). The emergence and exacerbation of CAD is often associated with cardiovascular risk factors
such as hypercholesterolemia, obesity, smoking status, diabetes, hypertension, and a sedentary lifestyle (Grech, 2003). For the purposes of this thesis, CAD was defined as having a history of MI, coronary angiographic evidence of greater than 50% stenosis in at least one major coronary artery or a prior revascularization procedure.

CAD is managed by a combination of lifestyle modifications, pharmacological interventions and surgery (Pollock & Jones, 2015). Diet and exercise interventions are beneficial in improving outcomes in CAD patients and are recommended (Suzuki, Kohro, Hayashi, Yamazaki, & Nagai, 2012). Pharmacological management of CAD involves controlling high blood pressure with β-receptor antagonists, calcium channel blockers, diuretics, and/or angiotensin converting enzyme (ACE) inhibitors, with acetylsalicylic acid, and platelet inhibitors/anticoagulant agents preventing the likelihood of future cardiovascular incidents (Kovacic & Fuster, 2011). Risk factors such as high cholesterol and diabetes are controlled with 3-hydroxy-3-methylglutaryl-coenzyme reductase inhibitors (statins), and anti-diabetic medications, respectively (Kovacic & Fuster, 2011; Tardif, 2010). Furthermore, nitro-glycerine is prescribed for angina pectoris (Kovacic & Fuster, 2011; Tardif, 2010). Stenosis of arteries is corrected surgically through revascularization procedures such as a coronary artery bypass graft (CABG) or a percutaneous transluminal coronary angioplasty (PTCA) (Levine et al., 2011).

Lifestyle modifications increasing physical activity are widely known to be highly beneficial in reducing cardiovascular events (Suzuki et al., 2012; Williams, 2001). Hence CAD patients are recommended to attend a cardiac rehabilitation (CR) program. Comprehensive CR programs consist of regular medically supervised and unsupervised exercise classes and educational components, with the purpose of managing risk factors in addition to learning methods to make appropriate lifestyle modifications. CR has been demonstrated to be effective in managing CAD risk factors, reducing hospital admission and mortality rates following a cardiovascular incident (Anderson et al., 2016; Lee, Paffenbarger, & Hennekens, 1997; Thompson et al., 2003).

Cardiovascular risk factors are highly prevalent with estimations that 13% of Canadians have hypertension, 4.2% having diabetes, 52.5% being physically inactive, 26% are
current smokers, and 14.9% have a BMI>30 (Tanuseputro et al., 2003). With an increasingly aging population and the increasing prevalence of such risk factors, CAD continues to represent a pressing healthcare issue worldwide (Tanuseputro et al., 2003; Tardif, 2010).

1.3.1.2 Coronary Artery Disease and Cognitive Impairment

Cardiovascular disease is a known risk factor for developing dementia and influences the course of cognitive decline (Bleckwenn et al., 2017; Gorelick et al., 2011). It has been recorded that 35% of CAD patients may have cognitive impairment (Silbert et al., 2007); however, it is frequently under diagnosed in this population (Patridge et al., 2014). Cognitive impairment occurs when an individual exhibits changes in cognitive functions, such as having difficulty remembering, learning new things, concentrating or making decisions. Pathological indices and risk factors such as atherosclerotic plaques (Hofman et al., 1997), blood vessel wall thickness, hypertension (Vicario et al., 2005) and diabetes (Yaffe et al., 2004) have been associated with poor cognitive performance compared to healthy control participants (Ottens, Hendrikse, Nathoe, Biessels, and van Dijk (2017). Moreover, numerous studies have linked such indices with cognitive decline over time and progression to dementia (Gorelick et al., 2011; Hachinski et al., 2006; Hajjar, Schumpert, Hirth, Wieland, & Eleazer, 2002). With cognitive impairment symptoms being prevalent in CAD patients, and the recognition that vascular pathologies contribute to cognitive impairment, CAD patients are at risk for a condition known as vascular cognitive impairment-no dementia (VCIND) (Gorelick et al., 2011).

1.3.1.3 Vascular Cognitive Impairment-No Dementia

VCIND is considered the prodromal stage for vascular dementia (VaD), and a mild stage on the spectrum of VCI. It is defined as having mild cognitive deficits due to vascular reasons/cerebrovascular disease not severe enough to be classified as dementia. Along the spectrum of VCI, VCIND is most common amongst those aged 64-84 years old (Rockwood et al., 2000). The cognitive domains commonly affected in VCIND are memory, including verbal memory, and executive function K. Garrett et al. (2004); (Gorelick et al., 2011; Vasquez & Zakzanis, 2015). As such, impairments in these domains have been documented in CAD patients (Eggermont et al., 2012; K.
Garrett et al., 2004; Nyenhuis et al., 2004; Santiago et al., 2015; Silbert et al., 2007; Vicario et al., 2005; L. Zheng et al., 2012). Patients are classified as having probable VCIND when they demonstrate cognitive impairment and imaging evidence of cerebrovascular disease, in addition to a) a clear relationship in the severity/pattern of cognitive impairment and presence of diffuse, subcortical cerebrovascular disease pathology or b) a clear temporal relationship between a vascular event such as a stroke and onset of cognitive effects (Gorelick et al., 2011). Patients can be classified as having possible VCIND when their clinical symptoms suggest cognitive impairment and the presence vascular disease; however, neuroimaging is not available to substantiate the presence of cerebrovascular disease (Gorelick et al., 2011; Hachinski et al., 2006; Sachdev et al., 2014).

1.3.1.4 Therapeutic Interventions for Cognitive Impairment in CAD

Currently, there is no treatment approved by the Food and Drug Administration (FDA) for VCIND. Trials controlling for vascular risk factors have not demonstrated significance in reducing dementia risk (Peters et al., 2008). Pharmacological studies have demonstrated that cholinesterase inhibitors such as donepezil, galantamine, and rivastigmine, in addition to the N-methyl-d-aspartate antagonist memantine have demonstrated some efficacy in improving cognitive outcomes in VaD patients (Kavirajan & Schneider, 2007). However, the treatment of MCI with cholinesterase inhibitors appears to have very little therapeutic benefit (Fitzpatrick-Lewis, Warren, Ali, Sherifali, & Raina, 2015). A clinical trial did find rivastigmine to demonstrate some efficacy in improving one measure of executive function in VCIND patients; however, that study was limited by a small sample size (Narasimhalu et al., 2010). Furthermore, the safety profile associated with rivastigmine is not ideal (Raschetti, Albanese, Vanacore, & Maggini, 2007). A large prospective study including 639 participants has demonstrated that exercise may be efficacious in reducing the risk of cognitive impairment (Verdelho et al., 2012; G. Zheng et al., 2016). Moreover, a meta-analysis has shown that exercise can improve symptoms of cognitive impairment in MCI patients (G. Zheng et al., 2016). However, that finding was suggested to be interpreted with caution given the limitations of the included studies and small positive effect size.
Cognitive impairment is a worrisome symptom because there has been evidence to show poor cognition impedes beneficial outcomes from therapeutic interventions for CAD. The presence of cognitive impairment has been associated with poor medication compliance (Conn et al., 1994; Ekman et al., 2001). Furthermore, impaired verbal memory performance has shown to be predictive of CR incompletion (Swardfager et al., 2011). Cognitive impairment has also been associated with poorer outcomes associated with CAD such as longer length of hospital stays, increased readmission rates, and mortality (Ekman et al., 2001; Patridge et al., 2014; Takata et al., 2014; Tilvis et al., 2004).

1.3.2 Glutathione Peroxidase Activity as a Potential Marker of Cognition

1.3.2.1 Alterations in Antioxidant Status and Neurodegeneration

It has been established that oxidative stress is involved in the pathological processes associated with dementia and in prodromal conditions such as VCIND, resulting in elevated levels of lipid peroxidation products in these conditions (Gustaw-Rothenberg, Kowalczuk, & Stryjecka-Zimmer, 2010; Praticò & Sung, 2004; Shrag M et al., 2013; Suridjan et al., 2017). Persistent oxidative stress can cause cellular damage, mitochondrial dysfunction and impair the DNA repair, all of which propagate neuronal injury and apoptosis (Barnham et al., 2004; Santos et al., 2012). Moreover, it has been demonstrated that glutathione (GSH) levels, the main antioxidant in the brain, are decreased in the brains of patients with neuropsychiatric conditions increasing the vulnerability of neurons to oxidative damage (Gawryluk, Wang, Andreazza, Shao, & Young, 2011). Furthermore, oxidative stress is involved in and exacerbated through inflammation and ischemic processes active in cardiovascular disease, exemplifying why CAD patients are at risk for VCIND (Gorelick et al., 2011; Pashkow, 2011). AD patients with cardiovascular disease have exhibited greater levels of oxidative damage compared to AD patients and control patients, and a greater accumulation of oxidative damage over time (Hatanaka et al., 2015; Hatanaka et al., 2017). As such, alterations in the glutathione antioxidant system, which is the body’s main defense system against damage induced by oxidative stress, have been observed in neurodegenerative processes as well (Shrag M et al., 2013). With the current body of evidence available, elevating antioxidant levels as a therapeutic strategy to prevent cognitive decline for has
also been suggested (Pocernich & Butterfield, 2012). While the therapeutic benefits related to this strategy are yet to be elucidated, the glutathione antioxidant system, specifically glutathione peroxidase (GPx), has been implicated with cognitive impairment in neurodegenerative processes.

1.3.2.2 The Glutathione antioxidant system

The GSH antioxidant is the body’s primary defence, protecting against the damage from the accumulation of oxidative stress products. GSH is an endogenous tripeptide consisting of glycine, glutamate and cysteine. Within cells, the cytosolic concentration ranges from 1-10 mM while plasma concentrations are in the micromolar range (Meister, 1988). GSH is maintained at lower concentrations in plasma because it is required for metabolism by various cell types (Sies & Graf, 1985). It contains a sulphydryl group which is important in reduction and conjugation reactions to eliminate reactive oxidative stress products such as lipid peroxidation products and xenobiotic compounds (Forman, Zhang, & Rinna, 2009). In participating in a spontaneous reaction, 2 GSH molecules become oxidized to form glutathione disulfide (GSSG). Reactive oxygen species, such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) can be spontaneously reduced by reacting with the sulphydryl group (-SH) of GSH in cells. The main enzyme in the GSH antioxidant system, glutathione peroxidase (GPx) can catalyze this process as well.

GPx is the main enzyme responsible for reducing reactive oxygen species and lipid peroxidation products (Forman et al, 2010). It is a tetrameric, selenium dependent enzyme with each subunit containing a selenocysteine residue in the active site (Arthur, 2000). The molecular weight of each subunit is approximately 23-25 kDa (Arthur, 2000). The most abundantly expressed GPx isoform is GPx-1 located in most cells including the brain while the GPx isoform expressed in plasma is GPx-3 (Arthur, 2000). It requires GSH as a cofactor, employing the –SH group to reduce lipid peroxides to their corresponding alcohols, and H$_2$O$_2$ to water. The reaction catalyzed by this enzyme is illustrated below.

$$2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS-SG} + 2 \text{H}_2\text{O}$$
Glutathione reductase (GR) functions in recycling GSSG back to GSH such that it can function as a cofactor for GPx once again. This enzyme oxidizes the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) during the reaction, illustrated below (Forman et al., 2009).

\[
GSSG + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+
\]

An additional enzyme in the glutathione antioxidant system is glutathione S-transferase (GST). GST is able to conjugate GSH to cysteine residues in proteins to form disulphide bonds or facilitate conjugation of GSH to xenobiotic and endogenous compounds, such that they are more water-soluble and are able to excreted from the body (Espinosa-Diez et al., 2015). GST activity in plasma is low and has been noted as unreliable to measure from such samples (Hayes, Bouchier, & Beckett, 1991). Another antioxidant enzyme with an isoform present in plasma is superoxide dismutase (SOD), which catalyzes the dismutation of \(\text{O}_2^-\) to \(\text{H}_2\text{O}_2\) (McCord & Fridovich, 2014).

The process of lipid peroxidation product formation and corresponding GSH antioxidant enzyme defenses are illustrated in figure 1. Essentially, when a reactive oxygen species such as a superoxide molecule (\(\text{O}_2^-\)) is produced near a membrane, lipids can become oxidized initiating a chain reaction to create a lipid radical (\(\text{L}^-\)). Lipid radicals can react with oxygen to produce a lipid peroxide radical (\(\text{LOO}^-\)). Further reactions with lipid molecules generate lipid peroxide (LOOH) products, damaging lipid membranes and producing by-products. Peripheral levels of oxidative stress products and glutathione peroxidase activity have been previously reported in numerous studies and have been considered informative indicators of the overall oxidative status of an entire organism (Shrag M et al., 2013).
Figure 1. Mechanism of the initiation of lipid peroxidation products and corresponding GSH antioxidant system defences. Essentially, when a reactive oxygen species such as a superoxide molecular (O₂•) is produced near a membrane, lipids can become oxidized initiating a chain reaction to create a lipid radical (L•), from which can react with oxygen to produce a lipid peroxide radical (LOO•). Further reactions with lipid molecules generate lipid peroxide (LOOH) products; thus, damaging lipid membranes.
1.3.2.3 Previous Associations between Glutathione Peroxidase and Coronary Artery Disease

Given the objectives of this thesis, it was of interest whether GPx activity is affected in CAD and there has been literature documenting previous associations between GPx and CAD. A study found that low erythrocyte GPx activity appears to be predictive of cardiovascular events in individuals with suspected CAD (Blankenberg et al., 2003). Furthermore, a meta-analysis found that peripheral GPx activity was increased by 1 standard deviation in patients with coronary heart disease based on 42 case-control studies and 3 prospective studies (Flores-Mateo et al., 2009).

1.3.2.4 Prior Evidence for Glutathione Peroxidase as a Marker of Cognition

The FDA-National Institutes of Health (FDA-NIH) biomarker working group has established harmonized definitions for various types of biomarkers, defined as a characteristic that is objectively measured and evaluated as an indicator of normal or pathogenic biological processes or as a biological response to a therapeutic intervention. Biomarkers can be diagnostic, predictive, and responsive in nature. A biomarker is diagnostic if it can differentiate individuals with a certain condition. Predictive biomarkers describe markers that can help identify individuals likely to experience a particular outcome compared to similar individuals who do not have the same biomarker. Finally, a monitoring biomarker is measured serially to assess the status of a condition for evidence of or effect of an external influence. Essentially, a monitoring biomarker can be interpreted as a marker used to measure a change in condition. There has been literature to show GPx has been evaluated as a diagnostic biomarker for cognitive performance in dementia and MCI patients. Moreover, there has been some evidence indicating that GPx may demonstrate characteristics of a predictive and monitoring biomarker in similar patient populations, in addition to healthy older adults.

There has been evidence supporting peripheral GPx activity as a marker for differentiating between severities of cognitive states. Krishnan and Rani (2014) found that plasma GPx activity was higher in patients with AD and VaD patients compared to healthy controls and this was accompanied by a significant decrease in GSH levels. Additional studies have found similar results in which AD patients had significantly
higher plasma GPx activity compared to controls (Ceballos-Picot et al., 1996; Martin-Aragon et al., 2009). Conversely, in another cohort of AD patients, plasma GPx activity was decreased compared to age-matched controls (Puertas et al., 2012). This was accompanied by a decrease in plasma GSH levels (Puertas et al., 2012). Rinaldi et al. (2003) found plasma GPx activity to be similarly decreased in AD patients and MCI patients; whereas other studies (Perrin et al., 1990; Tabet, Mantle, Walker, & Orrell, 2001) found that plasma GPx was not significantly different between dementia patients and controls. Serum GPx activity has been shown to be lower in MCI patients compared to healthy controls and diminished to a greater extent in AD patients (Balmus et al., 2017). Another study found serum GPx activity to similarly decreased in MCI patients and AD patients (Padurariu et al., 2010).

GPx activity has also been measured in erythrocytes. Its activity in erythrocytes has been documented to be decreased in AD and VaD patients compared to age-matched controls (Bermejo et al., 2008; Casado et al., 2008; Jeandel et al., 1989; Kharrazi et al., 2008; Kosenko et al., 2012; Vural et al., 2010); whereas, another study found erythrocyte GPx activity to be higher in AD patients compared to healthy controls and patients diagnosed with MCI (Torres et al., 2011). Studies measuring GPx in red blood cells have found that activity was more impaired in AD and VaD patients compared to MCI patients and MCI patients compared to healthy controls, implying a progressive decline in GPx dysfunction with severity of cognitive impairment (Bermejo et al., 2008; Cristalli et al., 2012; Torres et al., 2011). However, Ceballos-Picot et al. (1996), Fernandes et al. (1999), Bourdel-Marchasson et al. (2001) and Perrin et al. (1990) found no difference in erythrocyte GPx activity between AD patients and age-matched controls.

Both plasma and erythrocyte GPx activities are indicators of overall antioxidant system activity. Peripheral GPx activity can also be inferred from plasma GSH levels as this molecule is its cofactor. Studies have found that lower GSH levels have been associated with neurogenerative disorders indicating that GPx activity may be increased and consuming this molecule as a cofactor leading to reduced baseline levels (Gironi et al., 2011). Based on the literature, it appears that overall GPx activity is altered in
various states of cognitive impairment compared to healthy, age-matched controls. Its activity has yet to be characterized in patients with possible VCIND.

Studies have not directly explored GPx activity as a predictive marker of cognition. However, few studies have implicated GSH levels, the cofactor for GPx, as a predictive marker of cognitive impairment. A longitudinal study demonstrated that a decrease in reduced GSH over time is a strong predictor of progression to AD from MCI (Baldeiras et al., 2010). That study showed that there was no difference in the level of the oxidative stress markers measured at baseline between patients who had stable MCI over time versus those who developed AD, and lipid peroxidation markers increased over time in both groups but did not differ significantly. Recently, Suridjan et al. (2017) found that higher lipid peroxidation products at baseline was predictive of decreased improvement in executive function and verbal memory in response to a 6-month exercise program. It was also found that lower baseline GSH levels were associated with a decline in executive function in older healthy adults over 4 years (Hajjar et al., 2018). As such, given that GPx functions in reducing lipid peroxidation products and oxidizes GSH as a cofactor diminishing its levels, it is plausible that higher GPx activity at baseline, to counteract the accumulation of these species, may predict improvement in executive function and verbal memory over time.

Similarly, while changes in GPx have not been associated with changes in cognition, evidence from studies investigating GSH with cognitive outcomes provides a rationale for implicating GPx as a potential monitoring biomarker. McCaddon et al. (2003) were also able to demonstrate that a decrease in plasma GSH levels over time was associated with a significant decrease in Mini Mental State Exam (MMSE) scores. Revel et al. (2015) found that a 100 International Unit increase in GPx activity over 6 months was significantly associated with an average loss of 1.19 points on the MMSE over six months in 97 patients with various types and stages of neurodegenerative disease such as AD, VaD and MCI. Paradoxically, this study also found that a 100 μmol/L increase in GSH was associated with an average loss of 1.80 points over six months. This is paradoxical because an increase in GPx activity would suggest a decrease in GSH levels based on the mechanism of this enzyme. A longitudinal decline in GSH over 4 years was associated with a faster decline in executive function in healthy older adults.
as well (Hajjar et al., 2018). Overall, the results from those studies suggest that an increase in GPx activity over time, which would allow for the decline of its cofactor GSH, would be associated with a decrease in cognitive performance over time.

To our knowledge, GPx activity cannot be measured in the brain \textit{in vivo}. However, there have been few studies exploring GSH levels using magnetic resonance spectroscopy. Duffy et al. (2014) reported that MCI patients had elevated GSH levels in the anterior and posterior cingulate and this was related to poorer memory and executive function performance compared to control subjects. This was attributed to a compensatory response in increasing antioxidant levels in brain areas to circumvent oxidative stress, which have been related to cognitive impairment. Somewhat conversely, another study found that GSH was decreased in the frontal cortex and hippocampus MCI patients compared to healthy controls and more decreased in AD patients, correlating with declines in cognitive function (Mandal, Saharan, Tripathi, & Murari, 2015).

Evidently, research surrounding GPx as a marker for cognitive performance is highly variable and this may be rooted in the measure of its activity in patients with varying degrees of cognitive impairment and disease etiology. However, this indicates that the antioxidant system may indeed be impaired in a neurodegenerative state and may be associated with cognitive impairment in mild stages of cognitive impairment due to vascular reasons. Therefore, the antioxidant system, particularly GPx activity, remains an important marker to be studied in milder stages of cognitive important, especially in conditions in which limited research has been conducted such as in VCIND or patients with CAD at risk for dementia.
2. Chapter II Materials and Methods

2.1 Study Design

This study assessed associations between cognitive performance and GPx activity in CAD patients. A cross-sectional analysis was employed comparing differences in GPx activity between possible VCIND patients and CAD patients without cognitive impairment, who served as controls at baseline. A longitudinal study was then used to measure associations between GSH antioxidant enzyme activities and cognitive performance in CAD patients over the course of a 24-week CR program. Patients diagnosed with CAD were recruited from the UHN TRI Cardiac Rehabilitation Centre. Neurocognitive assessments were conducted and plasma samples were collected at baseline and 6 months. GPx activity was subsequently measured in plasma using a GPx assay.

2.2 Cardiac Rehabilitation Program

This study included patients enrolled in the University Health Network Toronto Rehabilitation Institute (UHN-TRI) CR program. Patients were referred by a physician to the 24-week program, as part of the Ontario Health Insurance Plan (Hamm & Kavanagh, 2000). The program consisted of a weekly 30-minute group education sessions and 60-minute supervised exercise class in which patients perform stretches, warm-up exercises, aerobic exercises such as walking or jogging and resistance training with hand-held weights. Patients were advised to exercise 4 more times during the week at home which include 1-2 resistance training sessions and were required to keep an exercise diary. Cardiopulmonary fitness was assessed by exercise stress tests at entry, midpoint, and upon completion of CR, in which the maximal rate of oxygen consumption measurement (VO_{2 peak}) was obtained. Exercise stress tests were conducted on a cycle ergometer (Ergoline 800 EL). Work-rate was increased every minute by 16.7 Watts until maximal exertion was reached by the patient, at which ventilatory capacity measurements resulted in the VO_{2 peak}. Measurements were calibrated for each patient throughout the stress test (Hamm & Kavanagh, 2000). For the purposes of this study, VO_{2 peak} values were obtained from the entry and 6-month
timepoint upon completion of CR, as a reliable measure of cardiovascular fitness (Hawkins & Wiswell, 2003).

### 2.3 Inclusion/Exclusion Criteria

Patients were recruited from the UHN TRI CR centre. Cognitive assessment data and plasma samples for this study were obtained from the previous study: The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease, which was approved by the research ethics boards of UHN Toronto Rehab and Sunnybrook Health Sciences Centre (primary trial site) [Appendix A]. All patients provided, written informed consent [Appendix B].

All patients in the study were diagnosed with CAD. CAD diagnosis was based on having a previous hospitalization for at least 1 acute myocardial infarction (MI), coronary angiographic evidence of ≥ 50% blockage in at least 1 major coronary artery or a previous revascularization procedure (coronary artery bypass graft or stent), as documented in their medical records. Patients who provided consent were contacted by a researcher associated with the Heart-Mind Connection study and were provided details of the study in person or over the phone.

#### 2.3.1 Inclusion Criteria

Patients were included in the study if they met the following inclusion criteria:

- were between the ages of 45 and 80 years
- spoke and understood the English language
- stable CAD defined as having no hospitalization events, such as acute MI, unstable angina, congestive heart failure, ventricular arrhythmias, coronary revascularization, or Canadian Cardiovascular Society Class 4 angina, in the last 4 weeks

#### 2.3.2 Exclusion Criteria

Patients were excluded from the study if they met any of the following exclusion criteria:
- Presence of significant acute medical illness (uncontrolled hypothyroidism, autoimmune disorders, sepsis, drug overdose, severely disrupted liver/kidney/lung function)

- A current neurological condition (Parkinson’s disease, any diagnosis of dementia including AD, Huntington’s chorea, history of epilepsy, subdural hematoma, traumatic brain injury, clinical stroke, progressive supranuclear paralysis)

- Killip Class III or IV states (classification system for people who have experienced an MI, indicating a higher risk of mortality)

- Canadian Cardiovascular Society Class 4 angina

- Use of medications affecting cognition including hypnotics, antipsychotics, antidepressants, and anticholinergic medication

- Diagnosis of major psychiatric condition including schizophrenia or bipolar disorder

- Significant cognitive impairment (demonstrated by having a MMSE score of <24)

- Diagnosis or any current Axis I disorder other than depression, anxiety, phobias or nicotine abuse/dependence

**2.4 Demographics and Medical History**

Upon entry into the study, demographic information, medical history, comorbidities, concomitant medications, cardiovascular health indices and other relevant information were obtained from TRI’s electronic medical records and from patient interviews. Demographic information included age, sex, ethnicity, marital status, years of education and smoking history. Cardiopulmonary fitness and health indices included height, weight, VO₂ peak, body mass index (BMI), body fat percentage, and peripheral lipid concentrations.

**2.5 Neurocognitive Assessments**

Cognitive assessments were chosen from the National Institute of Neurological Disorders and Stroke-Canadian Stroke Network (NINDS-CSN) 30-min standardized battery of tests, a battery validated to detect cognitive impairment across the spectrum of vascular cognitive impairment (Hachinski et al., 2006). The cognitive domains of executive function and memory were assessed given these domains are mainly affected
in the neurocognitive profile of VCIND (Hachinski et al., 2006; Vasquez & Zakzanis, 2015).

2.5.1 Verbal Memory

The California Verbal Learning Test-II (CVLT-II) evaluated verbal memory in these patients (Woods, Delis, Scott, Kramer, & Holdnack, 2006). This test is comprised of 5 verbal learning trials (having participants recall a list of words read to them immediately before), a short delay free recall trial (in which participants are asked to recall the list of words after being read a list of distractor words), and long delay free recall trial (being asked to recall the original list of words following a 20 minute delay). Raw scores are converted to Z-scores based on population norms. The CVLT-II has good test-retest reliability and has been demonstrated to be sensitive in detecting cognitive impairment in patients with cardiovascular issues (Dickson, Tkacs, & Riegel, 2007; Hachinski et al., 2006; Swardfager et al., 2010; Woods et al., 2006).

2.5.2 Executive Function

Executive function was evaluated using the assessments: Trails Making Test B, Controlled Oral Word Association Test (COWAT) and Category Fluency Test. These assessments have been validated as measures of executive function (Barry, Bates, & Labouvie, 2008; Gaudino, Geisler, & Squires, 1995; Malek-Ahmadi, Small, & Raj, 2011). The Trails Making Test B involves the participant drawing a continuous line connecting numbers and letters in ascending, alternating order as accurately as they can, and as quickly as possible (Gaudino et al., 1995). For the COWAT, participants were asked to list as many words that begin with a letter beginning to a certain letter of the alphabet within 1 minute, excluding proper nouns and variations of the same word in which the suffix was changed (Barry et al., 2008). Similarly, the category fluency test involves having the participant listing as many words that belong to a certain category, in this case being “animals”, within one minute (Barry et al., 2008).

2.5.3 Classification of Vascular Cognitive Impairment-No Dementia

Patients were classified as having possible VCIND based on demonstrating cognitive impairment in either domains of executive function or verbal memory, performing at
least 1 standard deviation below the population norm based on their composite z-scores in those domains (Gorelick et al., 2011; Hachinski et al., 2006).

### 2.6 Glutathione Peroxidase Assay

The analysis of plasma samples from these patients with a GPx assay was approved [Appendix C]. GPx activity was measured in plasma using a GPx Assay kit (Cayman 703102), according to manufacturer’s instructions. Study participants had fasted for 12 hours prior to having blood drawn from the antecubital vein at a standardized time for both baseline and 6-month time points. Blood samples were centrifuged at 1000g for 10 minutes (Model 614B, The Drucker Company) from which the plasma supernatant was aliquoted and stored at -80°C until analysis.

This assay measures GPx activity indirectly by coupling the activity of this enzyme with GR. GPx activity results in the production of oxidized glutathione, which must be restored to a reduced state by GR which uses NADPH as a cofactor. Oxidation of NADPH results in a decrease in absorbance at 340 nm, which indicates an increase in GPx activity.

Plasma samples were thawed on ice for 2.5-3 hours. Samples were then diluted 2-fold using the GPx Sample Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mg/ml bicinchoninic acid (BCA)) supplied in the assay kits. The assay plates were set up such that 3 wells were designated as background (non-enzymatic) wells, 3 wells were positive control wells, and the remaining wells were sample wells run in triplicate. 70 μL of Assay buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA), 50 μL of Co-substrate Mixture (lyophilized GSH and GR reconstituted in Assay buffer) and 50 uL of NADPH (lyophilized NADPH reconstituted in Assay buffer) was added to the background wells. 50 μL of Assay Buffer, 50 μl of Co-substrate Mixture, 50 μl NADPH, and 20 μL of diluted control GPx (bovine erythrocyte GPx diluted 1:100 in Sample Buffer) were added to the positive control wells. 50 μL of Assay Buffer, 50 μL of Co-substrate mixture, 50 μL of NADPH were added to each sample well in addition to 20 μL of plasma samples. To initiate the reactions, 20 μL of Cumene Hydroperoxide were added to all the wells and the plate
was gently shaken. Absorbance at 340 nm was read every minute for 8 minutes at room temperature on a Biotek Synergy H1 Hybrid plate reader.

Change in absorbance per minute (ΔA$_{340}$/min) was measured by calculating the slope of the absorbance values plotted as a function of time and taking the absolute value. The rate of change of absorbance over time for the non-enzymatic wells was then subtracted from the sample values. This value was then inputted into the following equation to determine glutathione peroxidase enzyme activity: GPx activity = (ΔA$_{340}$/min)/(0.00373 μM$^{-1}$) x 0.19 ml/0.02 mL x Sample dilution factor. Units of GPx activity was reported in nmol/min/ml.

2.7 Statistical Analyses

All statistical analyses were performed on the IBM SPSS Statistics (version 20; Armonk, NY) software and were considered significant at a two-tailed p < 0.05. Demographics and associations between clinical factors and VCIND groups were assessed using Chi-square tests and analysis of variance (ANOVA) and reported in terms of means and standard deviations (SD) for continuous variables and percentages for categorical variables. Linear mixed model analyses were conducted to identify associations between VO$_2$peak and verbal memory or executive function over 6 months to determine if changes in cognitive performance were associated with increasing physical fitness. Similarly, linear mixed model analyses were also conducted to identify associations between VO$_2$peak and GPx activity to assess whether GPx activity was changing with increasing fitness. In these analyses, the dependent variables were either verbal memory, GPx activity or executive function; fixed effects included VO$_2$peak, in addition to the a priori covariates selected for the dependent variable in question as described in section 2.7.4. Random effects were represented by Patient ID, a variable designated to identify each unique participant. Baseline and 6-month time points were included in the analysis and the repeated covariance type was unstructured.

2.7.1 Testing Primary Hypothesis

To assess GPx activity as a diagnostic marker of cognitive performance, CAD patients were dichotomized into two groups: possible VCIND patients or CAD control patients
based on cognitive performance as previously described in section 2.5.3. An analysis of covariance was performed to compare GPx activity between groups, with the *a priori* covariates being age, smoking status, and whether the participant was taking antioxidant/multivitamin supplementation. As a sub-analysis, the association with GPx activity and verbal memory composite z-scores at baseline was evaluated in a linear regression, controlling for sex, BMI, VO$_{2\text{peak}}$, and years of education.

2.7.2 Testing Secondary Hypothesis 1

Baseline GPx activity was assessed as a predictor of verbal memory performance over 24 weeks of CR using a linear mixed effects analysis. A linear mixed effects model was chosen because this model has been recommended as a flexible approach for repeated measure analyses and does not omit cases with missing values (Bolker et al., 2009). This approach accounts for both fixed and random effects. The present analysis inputted the following fixed effects in the model: baseline GPx activity, in addition to the following covariates: sex, total years of education, max VO$_{2\text{peak}}$, and BMI. Random effects were represented by Patient ID. Baseline and 6-month time points were included in the analysis and the repeated covariance type was unstructured.

2.7.3 Testing Secondary Hypothesis 2

Change in GPx activity was assessed as a monitoring marker of change in verbal memory performance over 24 weeks of CR using a linear mixed model. The following fixed effects were included in the model: GPx activity, in addition to the following covariates: age, sex, total years of education, max VO$_{2\text{peak}}$ and BMI. Baseline and 6-month time points were included in the analysis and the repeated covariance type was unstructured.

2.7.4 Testing Exploratory Hypothesis

The present study also sought to explore associations between GPx activity and the cognitive domain of executive function. This was achieved by associating GPx activity and executive function composite Z-scores at baseline using a linear regression. Baseline GPx activity was also evaluated as a predictor of change in executive function over the 6-month CR program using similar linear mixed model analyses described
previously. Moreover, the association between changes in GPx activity and change in executive function over time was evaluated using a linear mixed model as described previously. The covariates sex, total years of education, max VO\textsubscript{2 peak} and BMI were included in all of these analyses.

**2.7.5 Selection of Covariates**

The following covariates were selected \textit{a priori}. The covariates age, smoking history, and antioxidant supplementation were selected as covariates for the primary hypothesis because these factors have been shown to be associated with GPx activity. The covariates sex, BMI, VO\textsubscript{2 peak} and years of education have been shown to be associated with performance in verbal memory and executive function.

\textit{Age}

It has been reported that a one-year increase in age was associated with a 2.9 \textmu{mol}/min/L decrease in GPx activity in elderly women. This remained the case when controlling for coronary disease, disease, selenium levels, and hemoglobin (Espinoza et al., 2008). Evidence for GPx activity decreasing with age has been corroborated further in AD patients (Ceballos-Picot et al., 1996). Age has been controlled for in previous studies investigating GPx activity in non-geriatric populations as well (Zeni-Graiff et al., 2017). As such, this factor was controlled for when comparing GPx activity between groups for testing the primary hypothesis.

\textit{Smoking History}

In this study patients were asked about their smoking history: whether they had ever smoked, had quit smoking, or were currently smoking. Smoking has been thought to be a contributing factor for GPx activity, because it exposes the body to free radicals, reducing agents and pro-oxidants. Subsequently, this has been shown to affect GPx activity and antioxidant imbalance (Yildiz, Kayaoglu, & Aksoy, 2002). Therefore, smoking history was included as a relevant covariate because previous studies have controlled for the effects of smoking when measuring oxidative stress in individuals with AD with and without cerebrovascular disease, or including it as a covariate (Da Silva et al., 2018; Hatanaka et al., 2015).
Antioxidant Supplementation

In this cohort, 36% of participants were taking antioxidant and/or vitamin supplementation. Such supplementation reduces peripheral levels of oxidative stress, affecting the activity levels and expression of antioxidant enzymes (Mates, Perez-Gomez, & Nunez de Castro, 1999). Animal studies have also demonstrated that long-term supplementation reduces oxidative stress markers (Kolosova, Shcheglova, Sergeeva, & Loskutova, 2006; Siedlak et al., 2009). Furthermore, oxidized GSH levels were lower in AD patients treated with vitamin E (Lloret et al., 2009). Therefore, this factor was included as a covariate when analyzing GPx activity.

Sex

Sex is a covariate that has been controlled in studies measuring cognitive outcomes (Hatanaka et al., 2015; Saleem et al., 2013; Santiago et al., 2015; Suridjan et al., 2017). There have been demonstrated sex differences in verbal memory (Sundermann et al., 2016). The nature of cardiovascular disease also differs between the sexes; for example, men are more likely to experience a heart attack, which is a clinical issue demonstrated to be predictive of cognitive impairment (Silbert et al., 2007). Therefore, this variable was accounted for in this study.

BMI

BMI is a factor that has been associated with cognitive function (Momtaz, Haron, Hamid, Ibrahim, & Tanjani, 2018; Saleem et al., 2013; Steenbergen & Colzato, 2017; Suridjan et al., 2017). It was found that adults aged 45 years and older with a BMI classified as overweight or obese were more likely to develop severe cognitive decline and obesity may increase the rate of cognitive decline (Kim, Kim, & Park, 2016; Kirton & Dotson, 2016). This factor was controlled for because the patients enrolled in this study had a wide range of BMI values given their cardiovascular issues and this may have potentially confounded results measuring cognitive outcomes.

Max VO$_2$ peak
CAD patients in this study were participating in an exercise program. Aerobic exercise is known to have positive impacts on cognition and physical fitness has been associated with cognitive performance (Hwang, Castelli, & Gonzalez-Lima, 2017; Swardfager et al., 2010). As such, the VO$_2$$_{\text{peak}}$ as a reliable marker of fitness, was controlled for when measuring cognitive outcomes in these patients (Hawkins & Wiswell, 2003).

*Years of Education*

Years of education is known to be a contributing factor to cognitive performance. Education level has also been shown to a predictor of cognition (Ottens et al., 2017). Previous have also controlled for years of education in their analysis of cognitive performance (Saleem et al., 2013; Yaffe et al., 2004).

**2.8 Power calculation**

The power associated with this study was calculated using the software G*Power 3.1, based on the primary hypothesis that GPx activity will be higher in patients with possible VCIND compared to patients with cardiovascular disease who did not demonstrate cognitive impairment. No previous studies have compared GPx activity between these populations. Based on a post hoc power analysis to detect a medium effect size ($f=0.25$) with an alpha of 0.05, a total sample size of 120 yields a power (1-$\beta$) of 0.77.
Chapter III

3. Chapter III Results

3.1 Patient Recruitment

Between February 2012 and November 2015, 1563 patients entered CR of whom 933 patients had evidence of CAD and 555 patients agreed to contacted for research purposes. Overall, 300 patients provided consent to be screened for the study. Of those 159 patients met exclusion criteria and another 21 patients either withdrew consent, had scheduling conflicts, or missed study visits. Therefore, 120 patients were included in this study. The inclusion flow diagram is illustrated in Figure 2.
Figure 2. Patient inclusion process.

3.2 Demographics and Clinical Characteristics

Demographics and clinical factors in those with and without possible VCIND at baseline are described in Table 1. Participants were aged 64±6 years, mostly male (84%), Caucasian (83%), with on average 16±3 years of education. Demographic characteristics did not differ between groups, except for total years of education in which CAD controls did have on average an extra year of education. Baseline body composition, lipid profile, cardiopulmonary fitness parameters, vascular risk factors, and comorbidities did not differ between groups. A significantly greater proportion of CAD control patients were taking omega-3 supplements. For the resting physiology parameters, only resting diastolic pressure was significantly lower in CAD controls.

Table 1. Patient demographics and clinical characteristics between VCIND groups at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>Possible VCIND</th>
<th>CAD controls</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=36</td>
<td>N=84</td>
<td></td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>62.6 ± 6.5</td>
<td>64.4 ± 6.3</td>
<td>0.166</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>89%</td>
<td>82%</td>
<td>0.262</td>
</tr>
<tr>
<td>Total years of education</td>
<td>15 ± 4.3</td>
<td>16 ± 2.9</td>
<td>0.011</td>
</tr>
<tr>
<td>Ethnicity, Caucasian</td>
<td>75%</td>
<td>87%</td>
<td>0.093</td>
</tr>
<tr>
<td>Body Composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>172.6 ± 8.1</td>
<td>171.6 ± 7.8</td>
<td>0.534</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.2 ± 14.4</td>
<td>86.5 ± 17.6</td>
<td>0.930</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.9 ± 4.5</td>
<td>29.3 ± 5.4</td>
<td>0.744</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>99.3 ± 10.2</td>
<td>99.1 ± 13.0</td>
<td>0.955</td>
</tr>
<tr>
<td></td>
<td>105.6</td>
<td>10.8</td>
<td>105.9</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>Hip circumference</td>
<td>105.6</td>
<td>10.8</td>
<td>105.9</td>
</tr>
<tr>
<td>% Body fat</td>
<td>29.3</td>
<td>8.0</td>
<td>32.7</td>
</tr>
<tr>
<td>Lipid Profile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>1.6</td>
<td>0.7</td>
<td>1.6</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.3</td>
<td>0.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>3.5</td>
<td>0.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Cardiopulmonary Fitness Parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting heart rate (bpm)</td>
<td>71.3</td>
<td>13.5</td>
<td>68.3</td>
</tr>
<tr>
<td>Resting systolic blood pressure (mmHg)</td>
<td>129.9</td>
<td>23.5</td>
<td>123.2</td>
</tr>
<tr>
<td>Resting Diastolic blood pressure (mmHg)</td>
<td>81.6</td>
<td>10.1</td>
<td>75.2</td>
</tr>
<tr>
<td>Maximum heart rate (bpm)</td>
<td>122.7</td>
<td>22.4</td>
<td>121.5</td>
</tr>
<tr>
<td>Maximum systolic blood pressure</td>
<td>168.4</td>
<td>27.5</td>
<td>170.9</td>
</tr>
<tr>
<td>Maximum diastolic blood pressure</td>
<td>81.2</td>
<td>11.5</td>
<td>77.2</td>
</tr>
<tr>
<td>Peak oxygen consumption (VO2peak) mL/kg/min</td>
<td>20.4</td>
<td>5.6</td>
<td>21.1</td>
</tr>
<tr>
<td>Vascular Risk factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>97%</td>
<td></td>
<td>93%</td>
</tr>
<tr>
<td>Diabetes</td>
<td>14%</td>
<td></td>
<td>18%</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>100%</td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>Variable</td>
<td>Outcome 1</td>
<td>Outcome 2</td>
<td>p-value</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>BMI &gt;=30</td>
<td>33%</td>
<td>42%</td>
<td>0.258</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>58%</td>
<td>63%</td>
<td>0.385</td>
</tr>
<tr>
<td>Number of Vascular Risk Factors</td>
<td>3.0</td>
<td>0.8</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>Cardiac History</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>14%</td>
<td>15%</td>
<td>0.533</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>53%</td>
<td>46%</td>
<td>0.330</td>
</tr>
<tr>
<td>Coronary artery bypass graft surgery</td>
<td>33%</td>
<td>33%</td>
<td>0.587</td>
</tr>
<tr>
<td>PTCA stent procedure</td>
<td>61%</td>
<td>65%</td>
<td>0.399</td>
</tr>
<tr>
<td>Valvular heart disease</td>
<td>6%</td>
<td>6%</td>
<td>0.649</td>
</tr>
<tr>
<td>Aortic heart disease</td>
<td>2%</td>
<td>1%</td>
<td>0.515</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>3%</td>
<td>0%</td>
<td>0.300</td>
</tr>
<tr>
<td>Pacemaker procedure</td>
<td>11%</td>
<td>2%</td>
<td>0.065</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>0%</td>
<td>4%</td>
<td>0.339</td>
</tr>
<tr>
<td>Conduction deficit</td>
<td>0%</td>
<td>4%</td>
<td>0.339</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>3%</td>
<td>4%</td>
<td>0.652</td>
</tr>
<tr>
<td>Angioplasty</td>
<td>8%</td>
<td>7%</td>
<td>0.542</td>
</tr>
<tr>
<td>Triple A repair</td>
<td>0%</td>
<td>1%</td>
<td>0.700</td>
</tr>
<tr>
<td>Atrial Fibrillation</td>
<td>0%</td>
<td>5%</td>
<td>0.235</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>3%</td>
<td>1%</td>
<td>0.512</td>
</tr>
<tr>
<td>Cardiac Arrest</td>
<td>6%</td>
<td>4%</td>
<td>0.474</td>
</tr>
<tr>
<td>Angina</td>
<td>6%</td>
<td>8%</td>
<td>0.458</td>
</tr>
<tr>
<td>Peripheral vascular</td>
<td>0%</td>
<td>3.2%</td>
<td>0.339</td>
</tr>
<tr>
<td>disease</td>
<td>2020</td>
<td>2021</td>
<td>p-value</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------</td>
<td>------</td>
<td>---------</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>0%</td>
<td>4%</td>
<td>0.339</td>
</tr>
<tr>
<td>Chronic Obstructive Pulmonary Disorder</td>
<td>3%</td>
<td>7%</td>
<td>0.322</td>
</tr>
<tr>
<td>Cancer in the past</td>
<td>6%</td>
<td>10%</td>
<td>0.375</td>
</tr>
<tr>
<td>Asthma</td>
<td>7.4%</td>
<td>7.5%</td>
<td>0.673</td>
</tr>
<tr>
<td>Renal disease</td>
<td>3%</td>
<td>0%</td>
<td>0.300</td>
</tr>
<tr>
<td><strong>Concomitant Medications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiarrhythmic</td>
<td>3%</td>
<td>4%</td>
<td>0.652</td>
</tr>
<tr>
<td>Beta blocker</td>
<td>81%</td>
<td>80%</td>
<td>0.567</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>11%</td>
<td>15%</td>
<td>0.376</td>
</tr>
<tr>
<td>Diuretic</td>
<td>11%</td>
<td>18%</td>
<td>0.262</td>
</tr>
<tr>
<td>Vitamins/Antioxidants</td>
<td>22%</td>
<td>33%</td>
<td>0.159</td>
</tr>
<tr>
<td>Gout</td>
<td>8%</td>
<td>4%</td>
<td>0.252</td>
</tr>
<tr>
<td>Antidiabetic</td>
<td>11%</td>
<td>15%</td>
<td>0.376</td>
</tr>
<tr>
<td>Insulin</td>
<td>0%</td>
<td>5%</td>
<td>0.235</td>
</tr>
<tr>
<td>Gliptin DPP-4</td>
<td>6%</td>
<td>4%</td>
<td>0.474</td>
</tr>
<tr>
<td>Thiazolidinedione</td>
<td>0%</td>
<td>0%</td>
<td>-</td>
</tr>
<tr>
<td>Biguanide</td>
<td>11%</td>
<td>13%</td>
<td>0.512</td>
</tr>
<tr>
<td>Potassium supplement use</td>
<td>0%</td>
<td>4%</td>
<td>0.339</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0%</td>
<td>1%</td>
<td>0.700</td>
</tr>
<tr>
<td>Asthma</td>
<td>11%</td>
<td>10%</td>
<td>0.512</td>
</tr>
<tr>
<td>Anti-inflammatory medication</td>
<td>89%</td>
<td>87%</td>
<td>0.512</td>
</tr>
<tr>
<td>Platelet inhibitor</td>
<td>100%</td>
<td>95%</td>
<td>0.235</td>
</tr>
<tr>
<td><strong>Lipid-lowering agents/Statins</strong></td>
<td>100%</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------</td>
<td>------</td>
<td>---</td>
</tr>
<tr>
<td>Hormone replacement therapy</td>
<td>0%</td>
<td>1%</td>
<td>0.700</td>
</tr>
<tr>
<td>Gastric acid inhibitor (proton pump inhibitors)</td>
<td>25%</td>
<td>36%</td>
<td>0.175</td>
</tr>
<tr>
<td>Thyroid medication</td>
<td>8%</td>
<td>10%</td>
<td>0.570</td>
</tr>
<tr>
<td>Vasodilator use</td>
<td>39%</td>
<td>45%</td>
<td>0.330</td>
</tr>
<tr>
<td>Anticoagulant use</td>
<td>6%</td>
<td>5%</td>
<td>0.584</td>
</tr>
<tr>
<td>Antidepressant/anxiolytic use</td>
<td>6%</td>
<td>4%</td>
<td>0.474</td>
</tr>
<tr>
<td>Antidepressant</td>
<td>0%</td>
<td>0%</td>
<td>-</td>
</tr>
<tr>
<td>Anxiolytic</td>
<td>6%</td>
<td>4%</td>
<td>0.474</td>
</tr>
<tr>
<td>Omega-3 supplementation</td>
<td>9%</td>
<td>30%</td>
<td>0.017</td>
</tr>
</tbody>
</table>

**Abbreviations:** BMI: body mass index, bpm: beats per minute, CAD: coronary artery disease, HDL: high-density lipoprotein, LDL: low-density lipoprotein, mmHg: milliliters of mercury, PTCA: percutaneous transluminal coronary angioplasty, SD: standard deviation, VCIND: vascular cognitive impairment-no dementia.

### 3.2.1 Cognitive Performance

Cognitive performance at baseline and 6-month time points are displayed in Table 2. Possible VCIND patients were significantly more cognitively impaired at both time points.

**Table 2. Cognitive Performance at baseline and 6-months**

<table>
<thead>
<tr>
<th>Cognitive domain</th>
<th>Baseline</th>
<th>Significance</th>
<th>6-months</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VCIND</td>
<td>CAD controls</td>
<td></td>
<td>VCIND</td>
</tr>
<tr>
<td>Verbal memory composite Z-score</td>
<td>-0.94±0.96</td>
<td>0.40±0.71</td>
<td><strong>P&lt;0.001</strong></td>
<td>-0.72±1.07</td>
</tr>
<tr>
<td>Executive function composite Z-score</td>
<td>-0.99±0.73</td>
<td>0.42±0.78</td>
<td><strong>P&lt;0.001</strong></td>
<td>-0.94±0.72</td>
</tr>
</tbody>
</table>
3.2.2 Associations between physical fitness and cognitive performance over Cardiac Rehab

An increase in physical fitness over CR was not significantly associated with improvements in verbal memory ($b[SD]=0.0015[0.0088]$, $p=0.861$) or executive function ($b=0.0092[0.0076]$, $p=0.226$). When including VCIND status as a covariate, CAD patients without VCIND had significantly improved in executive function ($b[SE] = 1.38[0.15]$, $p<0.005$) and verbal memory ($b[SE] = 1.30[0.15]$, $p<0.005$) with improved fitness.

3.2.3 Associations between physical fitness and GPx activity over CR

GPx activity was increasing over time ($b[SE] = 1.62[0.81]$, $p<0.048$). An increase in physical fitness over CR was not significantly associated with an increase in GPx activity over CR ($p=0.104$) in an adjusted model including the additional covariates: age, antioxidant supplementation, and smoking history ($p=0.09$).

3.3 Analyses to Test Hypotheses

3.3.1 Primary Hypothesis: Glutathione Peroxidase activity as a diagnostic marker of VCIND

GPx activity was significantly higher in patients with possible VCIND compared to CAD patients who did not demonstrate cognitive impairment ($F_{1,119}=4.00$, $p=0.048$), as noted in table 3, and illustrated in Figure 3. The mean GPx activity for possible VCIND patients was 62.45 nmol/ml/min (SD: 13.05) and 58.30 nmol/ml/min (SD: 11.67) for CAD controls.

Table 3. Coefficients of an ANCOVA detecting differences in GPx activity between possible VCIND patients and CAD control patients.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>F</th>
<th>p-value (significance at $p&lt;0.05^*$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>4</td>
<td>1.30</td>
<td>0.276</td>
</tr>
<tr>
<td>Intercept</td>
<td>1</td>
<td>23.87</td>
<td>$&lt;0.005^*$</td>
</tr>
<tr>
<td>VCIND status</td>
<td>1</td>
<td>4.00</td>
<td>0.048*</td>
</tr>
<tr>
<td>Cigarette smoking history (y/n)</td>
<td>1</td>
<td>0.17</td>
<td>0.679</td>
</tr>
<tr>
<td>Antioxidant/vitamin supplementation</td>
<td>1</td>
<td>1.39</td>
<td>0.241</td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>0.06</td>
<td>0.805</td>
</tr>
</tbody>
</table>
Abbreviations: df: degrees of freedom; y/n: yes/no. Covariates included in the model: age, smoking history (yes or no), and whether they were taking antioxidant/vitamin supplementation.

Figure 3. Mean GPx activity in VCIND patients compared to CAD controls. Activity was higher in possible VCIND patients, controlling for age, antioxidant/vitamin use and cigarette smoking history. The adjusted mean GPx activity for possible VCIND patients was 60.99 ± 2.12 nmol/ml/min, and the adjusted mean for CAD controls was 56.19 ± 1.42 nmol/ml/min.

Upon further exploration, higher GPx activity was significantly associated with poorer verbal memory performance at baseline (β=-0.182, p=0.048) when controlling for the covariates: max VO₂ peak, sex, total years of education, and BMI (Figure 4). In this model, years of education was also significantly associated with verbal memory scores at baseline (β=-0.182, p=0.042).
3.3.2 Secondary Hypothesis #1: Baseline GPx activity as a predictive marker of verbal memory

The estimates of fixed effects for the linear mixed model assessing GPx as a predictive marker of verbal memory are displayed in Table 4. Baseline glutathione peroxidase did not predict change in verbal memory performance over CR, when controlling for sex, BMI, max VO₂ peak, and total years of education. Higher years of education was associated with an increase in verbal memory over 6 months.

Table 4. Estimates of fixed effects of a Linear Mixed Model describing whether baseline GPx activity predicts change in verbal memory over time. Covariates include: age, sex, BMI, max VO₂, and years of education

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>df</th>
<th>t</th>
<th>Significance (p&lt;0.05)</th>
</tr>
</thead>
</table>

Figure 4. Higher GPx activity is significantly associated with poorer verbal memory performance at baseline in CAD patients. Covariates included in this model include: sex, BMI, max VO₂ peak, and years of education.

Post-hoc analysis: Upon comparison of the baseline characteristics of both groups, it was found that a significantly greater proportion of CAD control patients were taking omega-3 supplements. When controlling for this as an additional covariate, GPx activity was still significantly higher in possible VCIND patients (F=6.549, df=1, p=0.012).
Post-hoc analysis: In a post-hoc analysis with VCIND added as an additional predictor, baseline GPx activity did not predict changes in verbal memory over time ($p = 0.677$). VCIND status was significantly associated with verbal memory performance over 6 months ($p < 0.001$). Specifically, possible VCIND patients demonstrated a decline in verbal memory by 1.22 SD over 6 months.

### 3.3.3 Secondary hypothesis #2: GPx activity as a monitoring biomarker of verbal memory

The estimates of fixed effects for this model are displayed in Table 5. An increase in GPx activity was significantly associated with a decline in verbal memory performance over 6 months when controlling for sex, BMI, max VO$_2$ peak and total years of education. Specifically, a 1 nmol/min/mL increase in glutathione peroxidase activity is associated with a 0.02 SD decrease in verbal memory z-scores over 6 months (Figure 5). Higher years of education was also significantly associated with a significant improvement in verbal memory over 6 months.

Table 5. Estimates of fixed effects of a Linear Mixed Model describing associations between change GPx activity and change in verbal memory over time.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>df</th>
<th>t</th>
<th>Significance ($p&lt;0.05$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.53</td>
<td>0.88</td>
<td>143.46</td>
<td>0.60</td>
<td>0.548</td>
</tr>
<tr>
<td>Sex=male</td>
<td>-0.32</td>
<td>0.24</td>
<td>116.32</td>
<td>-1.31</td>
<td>0.192</td>
</tr>
<tr>
<td>Sex=female</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total years of education</td>
<td>0.07</td>
<td>0.03</td>
<td>117.94</td>
<td>2.84</td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td>Max VO2</td>
<td>-0.0007</td>
<td>0.01</td>
<td>132.23</td>
<td>-0.08</td>
<td>0.939</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.01</td>
<td>0.02</td>
<td>133.80</td>
<td>-0.83</td>
<td>0.411</td>
</tr>
<tr>
<td>GPx</td>
<td>-0.02</td>
<td>0.01</td>
<td>184.65</td>
<td>-2.93</td>
<td><strong>0.004</strong></td>
</tr>
</tbody>
</table>

Abbreviations: df: degrees of freedom. Covariates included in the model: sex, total years of education, max VO$_2$ peak, and BMI.
Abbreviations: df: degrees of freedom. Covariates included in the model: sex, total years of education, max VO$_2$ peak, and BMI.

Figure 5. Changes in GPx activity significantly predicts changes in verbal memory in CAD patients over a 6 month CR program. Covariates included sex, BMI, VO$_2$ peak and years of education.

**Post-hoc analyses:** Post-hoc analyses revealed that VCIND was not a significant predictor of changes in verbal memory over 6 months and the addition of this predictor in the model attenuated the significance of changes in GPx activity being associated with changes in verbal memory (p = 0.06). VCIND status was significantly associated with verbal memory performance over 6 months (p < 0.001). Specifically, possible VCIND patients demonstrated a decline in verbal memory by 1.19 SD over 6 months in this model.

3.3.4 Testing Exploratory Hypotheses: associations between GPx and executive function

In exploring associations between GPx activity and executive function, GPx activity was not significantly associated with executive function composite scores at baseline ($\beta=-0.144, p=0.108$). Furthermore, baseline glutathione peroxidase did not predict change in executive function over the course of CR ($p=0.117$), when controlling for sex, BMI, max VO$_2$ peak, and total years of education in a linear mixed model analysis. The estimates of
fixed effects for this model are displayed in Table 6. However, an increase in GPx activity over time was significantly associated with a decline executive function over time (b= -0.01, p=0.027), controlling for sex, BMI, max VO2 peak and total years of education. The estimates of fixed effects for this model are displayed in Table 7. Specifically, A 1 nmol/min/mL increase in glutathione peroxidase activity is associated with a 0.01 SD decrease in executive function z-scores over 6 months (Figure 6). A higher level of education at baseline was also significantly associated with an improvement in executive function over time.

Table 6. Estimates of fixed effects of a Linear Mixed Model evaluating whether baseline GPx activity predicts change GPx activity and change in executive function over time. The following covariates were included in the model: age, sex, BMI, max VO2 peak, and total years of education.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>df</th>
<th>t</th>
<th>Significance (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.56</td>
<td>0.86</td>
<td>134.03</td>
<td>-1.82</td>
<td>0.071</td>
</tr>
<tr>
<td>Sex=male</td>
<td>-0.32</td>
<td>0.24</td>
<td>117.62</td>
<td>-1.36</td>
<td>0.176</td>
</tr>
<tr>
<td>Sex=female</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total years of education</td>
<td>0.09</td>
<td>0.03</td>
<td>118.20</td>
<td>3.53</td>
<td>0.001</td>
</tr>
<tr>
<td>Max VO2</td>
<td>0.01</td>
<td>0.01</td>
<td>124.21</td>
<td>1.41</td>
<td>0.162</td>
</tr>
<tr>
<td>BMI</td>
<td>0.02</td>
<td>0.02</td>
<td>140.90</td>
<td>1.38</td>
<td>0.171</td>
</tr>
<tr>
<td>BL GPX</td>
<td>-0.01</td>
<td>0.01</td>
<td>112.41</td>
<td>-1.32</td>
<td>0.190</td>
</tr>
</tbody>
</table>

Abbreviations: df: degrees of freedom. Covariates included in the model: sex, total years of education, max VO2 peak, and BMI.

Table 7. Estimates of fixed effects of a Linear Mixed Model describing associations between change in GPx activity and change in executive function over time. The following covariates were included in the model: age, sex, BMI, VO2 peak, and years of education.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>df</th>
<th>t</th>
<th>Significance (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.42</td>
<td>0.84</td>
<td>148.38</td>
<td>-1.69</td>
<td>0.093</td>
</tr>
<tr>
<td>Sex=0</td>
<td>-0.32</td>
<td>0.24</td>
<td>117.56</td>
<td>-1.34</td>
<td>0.182</td>
</tr>
<tr>
<td>Sex=1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total years of education</td>
<td>0.09</td>
<td>0.03</td>
<td>118.70</td>
<td>3.58</td>
<td>0.001</td>
</tr>
<tr>
<td>Max VO2</td>
<td>0.01</td>
<td>0.01</td>
<td>116.59</td>
<td>1.46</td>
<td>.147</td>
</tr>
<tr>
<td>BMI</td>
<td>0.02</td>
<td>0.02</td>
<td>140.56</td>
<td>1.39</td>
<td>0.168</td>
</tr>
<tr>
<td>GPx</td>
<td>-0.01</td>
<td>0.01</td>
<td>185.58</td>
<td>-2.23</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Abbreviations: df: degrees of freedom. Covariates included in the model: sex, total years of education, max VO2 peak, and BMI.
Figure 6. Changes in GPx activity significantly predict changes in executive function in CAD patients over a 6 month CR program. Covariates included sex, BMI, max VO$_2$ peak and years of education.
Chapter IV

4. Chapter IV Discussion

4.1 Study Findings and Interpretations

4.1.1 Primary Hypothesis

The present study investigated whether GPx activity was different between possible VCIND patients and CAD control patients who did not demonstrate cognitive impairment, essentially evaluating GPx activity as a diagnostic marker for cognitive impairment. GPx activity has differed between patients of varying neurodegenerative status, such as in MCI, AD and VaD patients, compared to healthy controls. However, GPx activity has not been compared between patients with possible VCIND patients and a control population before. Based on previous findings in CAD patients with possible VCIND having a higher ratio of late stage to early stage lipid peroxidation markers (Suridjan et al., 2017), it was hypothesized that plasma GPx activity would be increased in these patients to mitigate the accumulation of oxidative stress. Consistent with the hypothesis, GPx activity was higher these patients. Moreover, higher baseline GPx activity was significantly associated with poorer baseline verbal memory performance. Unsurprisingly, the covariate ‘years of education’ was significantly associated with verbal memory scores as baseline.

4.1.2 Interpretation of Primary Hypothesis

While the majority of studies have found that GPx activity is decreased in MCI and AD patients, its activity was hypothesized to be increased in this population based on GPx activity previously documented to be higher in CAD patients compared to control patients (Flores-Mateo et al., 2009), and on reported results in this specific patient sample in which possible VCIND patients had a higher ratio of late stage to early stage lipid peroxidation markers (Suridjan et al., 2017). Therefore, this hypothesis was based on the rationale that the peripheral antioxidant system may be responding to the persistent oxidative stress being observed. This is in line with a compensatory response by the GSH antioxidant system seen in early stages of disease in response to oxidative stress (Lubrano & Balzan, 2015), but is being exhibited to a greater extent in possible
VCIND patients consistent with their persistent levels of oxidative stress. This hypothesis was further supported with the finding that higher baseline GPx activity was associated with poorer verbal memory performance.

The FDA defines diagnostic biomarkers as measures that can be used to identify disease subtypes. In this study, GPx activity was higher in possible VCIND patients by approximately 4.80 nmol/mL/min, which corresponds to an effect size (Cohen’s d) of 2.66. GPx activity has been evaluated as a marker of cognitive performance before in reference to various control groups, which is typical in testing diagnostic biomarkers. The variability in sample types, disease states, and severity of cognitive decline across studies have not resulted in a specific range of plasma GPx activity which can be characterized as physiologically normal. One study conducted in Valencia, Spain reported that a reference point for plasma GPx activity in a healthy population could be 196-477 U/L (Alegria et al., 1996). However, those values have not been validated and are limited to a specific population. Overall, there is a general consensus that standard plasma GPx values are unknown. More research needs to be conducted to determine cut off points for characterizing high GPx activity indicative of cognitive impairment in CAD patients. As such, the clinical sensitivity associated with GPx activity as a diagnostic biomarker remains uncertain. GPx activity should be measured in a larger population of possible VCIND patients and compared to CAD controls, in addition to healthy controls to further validate this finding, in order to further the consensus on whether using GPx activity as a marker of cognitive impairment is reliable.

Something to be noted is that significantly more CAD controls were taking omega-3 supplements, which has been shown to increase GPx activity in \textit{in vitro} and animal studies (Joulain, Prigent, Nemoz, & Lagarde, 1994; Patten, Brocardo, & Christie, 2013). In \textit{post-hoc} analyses, when omega-3 supplementation was included as a covariate GPx activity was still significantly greater in possible VCIND patients. However, the difference in GPx activities potentially may have been greater had these groups been homogenous in the proportion of subjects taking these supplements.

The results outlined suggest that the GSH antioxidant status may be impaired in possible VCIND patients. Thus, contributing to the body of literature highlighting that
there is an antioxidant imbalance with cognitive impairment and neurodegenerative states.

4.1.3 Secondary Hypotheses

This study attempted to explore the potential GPx activity had as a novel predictive and monitoring biomarker for cognitive outcomes in CAD patients. Given some evidence that baseline GSH and lipid peroxidation levels have been predictive of cognitive outcomes, it was hypothesized that GPx values may function similarly in predicting verbal memory performance over time in response to an exercise intervention. Contrary to what was hypothesized, baseline GPx activity did not predict change in verbal memory in CAD patients over 6 months. When VCIND status was included in the statistical model, possible VCIND patients demonstrated a decline in verbal memory by 1.22 SD over 6 months; however, this was not associated with baseline GPx activity values.

Interestingly, an increase in GPx activity over 6 months was significantly associated with verbal memory decline over the same span of time. Specifically, a 1-unit increase in GPx activity was associated with a decrease in verbal memory scores by 0.02 SD. Having completed more years of education was significantly predictive of an improvement in verbal memory over 6 months.

Overall, GPx activity was increasing over time in CAD patients, confirming what has previously been reported about GPx activity in these patients (Flores-Mateo et al., 2009). It was established that an increase in enzyme activity was not associated with an increase in fitness.

4.1.4 Interpretation of Secondary Hypotheses

Studies showing improvement in cognitive performance from exercise have been mixed and this has been speculated to be because of underlying differences in levels of oxidative stress (Suridjan et al., 2017). Exercise overall, has been shown to increase antioxidant capabilities in individuals over time regardless of health status (de Sousa et al., 2017). In this study, an increase in physical fitness over CR was not significantly associated with improvements in either verbal memory or executive function. However,
when including VCIND as a covariate, CAD patients without cognitive impairment had significantly improved in their cognitive assessments over the 6 months. This suggests that CAD patients with cognitive impairment may not receive optimal benefits associated with CR, as demonstrated previously (Swardfager et al., 2010).

The FDA-NIH Biomarkers, EndpointS and other Tools (BEST) resource has stated that diagnostic biomarkers that identify disease types can be useful when the resulting classifications can be used as predictive markers. While the primary hypothesis for this study in which GPx activity would be higher in possible VCIND patients was confirmed, this study could not establish the predictive ability of GPx activity for changes in verbal memory over 6-months. While baseline GPx activity may be indicative of a cross-sectional state of cognitive impairment, such as VCIND status, it may be that it is not telling of changes in cognition over time.

While the results from this analysis yielded negative results, the potential for GPx activity as a predictive biomarker of a verbal memory performance in response to an exercise intervention should not be discounted. The FDA-NIH BEST resource states that for a biomarker to be predictive of an intervention’s effect, there needs to be comparison to a control treatment. If this study had measured GPx activity in CAD patients who did not participate in CR, there might have been sufficient evidence to suggest that GPx activity could be predictive of whether CR would be effective in improving verbal memory performance.

An increase in GPx activity being significantly associated with a decline in verbal memory over 6-months, suggests that GPx activity may serve as a monitoring biomarker for changes in this cognitive domain in CR. A monitoring biomarker, as defined by the FDA-NIH BEST resource, is any biomarker measured serially over time to assess for evidence of the effects of an intervention. Therefore, diagnostic and predictive biomarkers can also be monitoring biomarkers when they are measured repeatedly. The BEST resource clarifies that the magnitude of the observed changes must be interpreted relative to the expected variation in the biomarker values. As it was mentioned previously that there are no standardized values for what characterizes normal GPx activity; changes in GPx activity were interpreted in reference to the
observed differences between possible VCIND patients and CAD control patients in this study. Seeing how a 1 nmol/min/mL increase in GPx activity was associated with a 0.02 SD decrease in verbal memory z-scores over the course of CR, and possible VCIND patients differed from CAD control patients in GPx activity by 4.80 nmol/mL/min while control patients performed better on average by 1.34 z-scores at baseline, it is possible that a 1 nmol/mL/min increase in GPx activity over 6 months is clinically meaningful. However, a 1 nmol/mL/min difference in activity may also be a result of natural variability in GPx activity. Greater increases in GPx activity over time may be indicative of greater declines in verbal memory over time.

It was hypothesized that GPx activity would increase over time in response to the persistent oxidative stress observed in possible VCIND patients and cognitively impaired populations in general. This raises the question of why this increase in activity is not sufficiently mitigating oxidative stress subsequently resulting in an improvement in cognitive performance in these CAD patients. It appears that the GSH antioxidant system may be dysfunctional where imbalance with the production of oxidative stress reduces its ability to maintain a balanced redox state.

A systematic review on the effect of N-acetylcysteine (NAC), a GSH precursor, on human cognition revealed that NAC treatment could significantly improve cognition (Skvarc et al., 2017). A double-blind, placebo-controlled study investigating NAC in probable AD patients showed that GPx activity was not significantly different between both groups at the end of the trial (Adair, Knoefel, & Morgan, 2001). However, there is little known about the role GPx could play as a marker for cognition in response to NAC in MCI populations (Adair et al., 2001). In studies investigating the therapeutic implications of NAC on similar outcomes in milder stages of cognitive impairment, GPx could potentially serve as a marker based on the results of this study. It may be especially pertinent as a marker of cognitive impairment in CAD populations given previous evidence that its activity is elevated in these patients (Flores-Mateo et al., 2009). Moreover, such clinical trials could continue to further evaluate GPx activity as a biomarker.
4.1.5 Exploratory Hypothesis

Baseline GPx activity was not associated with executive function scores at baseline, nor did baseline GPx activity predict change in executive function over time. However, changes in GPx activity over time was associated with a decrease in executive function scores over 6-months. The observed change in executive function over time was not as great as the association between increasing GPx activity and decline in verbal memory performance over six months. As expected, in these analyses having completed more years of education was significantly associated with improvement in executive function over time.

4.1.6 Interpretation of Exploratory Hypothesis

We wanted to explore the association between GPx and executive function in this population because this cognitive domain is characteristic of vascular cognitive impairment as well (Gorelick et al., 2011). However, because these patients could not be confirmed as having cognitive impairment due to vascular reasons, we explored memory in the main analyses, as this is the cognitive domain salient to most neurocognitive disorders.

Based on these results, it appears that GPx activity could serve as a monitoring biomarker in CAD patients in association with cognitive impairment: changes in its activity over time demonstrated significant associations with verbal memory and executive function. Both domains of which are of interest for dementia, particularly VaD. Measuring changes in the activity of the antioxidant system over time may be more indicative of long-term effects. Furthermore, a 6-month timeline may be a reference point to determine which patients are at risk for decreased improvement to exercise and whether antioxidant supplementation may be required.

4.2 Limitations and Recommendations for Future Studies

In testing the primary hypothesis, a limitation of this study was that patients could not be diagnosed with VCIND in that magnetic resonance imaging was not conducted to confirm the presence of underlying cerebrovascular disease. Conducting an MRI allows for the detection of white matter hyperintensities or small brain infarcts, which are
normally associated with classifying cognitive impairment due to vascular reasons (Gorelick et al., 2011). Classifying patients as having possible VCIND represents a clinically useful and convenient option, given that obtaining an MRI for cognitive impairment is an expensive procedure and may not be an accessible diagnostic procedure. For the purposes of this study evaluating glutathione peroxidase activity as a diagnostic marker differentiating between different cognitive impairment states, the results may have been more robust if neuroimaging was available to more closely link cognitive impairment to cerebrovascular changes. However, the patients enrolled in this study had a longstanding history of CAD and no history of neurodegenerative disease; hence, it is very likely that cognitive performance could be attributed to vascular reasons. Furthermore, establishing cognitive impairment through solely cognitive assessments is clinically relevant especially to CAD patients enrolled in a CR program, as it is not readily accessible for patients to receive a scan.

GPx is a selenium-dependent enzyme (Arthur, 2000). This study did not measure or control for selenium levels or intake. This may have been a relevant consideration because dietary intake has been suggested to promote optimal glutathione peroxidase activity (Arthur, 2000). However, selenium levels have shown not to differ between healthy controls, AD patients and VaD patients in a previous study (Krishnan & Rani, 2014). The primary purpose of this study was establishing the role of GPx and whether its functionality was associated with cognitive impairment and it is likely that selenium levels would not have differed significantly between the groups in this study considering how homogenous this sample was.

In terms of the measurement of antioxidant capacities, this study was not able to measure GPx levels via Western blot analysis. This would allow for a measure of GPx protein expression and ascertain whether the abundance of this enzyme is related to its activity, cognitive status, or it may have provided a measure that could be controlled for since the GPx assay measures its activity indirectly. Furthermore, while the purpose of this study was evaluating GPx activity as a marker of cognition, a limitation that remains is that GSH itself was not measured, as GSH is a cofactor for this enzyme and may have informed GPx activity or provided a greater understanding of antioxidant imbalance in this population (Arthur, 2000). While it would have been interesting to
determine is protein expression levels differed with varying cognitive performance, this study was investigating the activity of GPx given previous results in this population associating higher ratios of late stage to early stage lipid peroxidation markers with cognitive performance (Suridjan et al., 2017). As such, the activity of the enzyme responsible for reducing such products was studied and deemed informative. Future studies may be able to measure GPx protein expression as an outcome of interest.

This study measured the activity of a GPx isoform expressed in plasma. It could be argued that it may have been more advantageous to measure GPx activity in erythrocyte samples since this isoform (GPx-1) is the same as the isoform expressed in the brain (Arthur, 2000). However, GPx activity as well as other oxidative stress markers have been measured in numerous studies and as such, can be interpreted as an informative indicator of an individual's overall antioxidant status.

The majority of the study sample was male and Caucasian. As such, results from this study must be interpreted with caution when applied to the general public. This is pertinent to keep in mind as the prevalence and presentation of cardiovascular disease varies across ethnicities and sexes (Maas & Appelman, 2010). For example, cardiovascular disease is highly prevalent in South Asian populations compared to Chinese populations; while African Americans have low rates of heart disease, this population has the least favourable risk profile (Chiu, Austin, Manuel, & Tu, 2010). The research conducted in this thesis would not substantially contribute to discerning the prevalence of cognitive impairment as a symptom of CAD across different ethnicities or sexes. However, this study is representative of the CR population and as a proof of concept study investigating GPx activity in association with cognition in this population for the first time, it was advantageous to have a homogenous sample.

Future studies should be sufficiently powered such that inferences from the data can be made confidently. They should also measure peripheral GSH levels in addition to the activities of the other enzymes in the overall antioxidant system such as GR, GST, and SOD. As described before, the activity of these enzymes been reported in various neurodegenerative diseases, but limited research has been conducted in various stages of vascular cognitive impairment. Furthermore, future studies may benefit from obtaining
erythrocyte samples in addition to plasma samples to measure the activities of these enzymes. This is such that GR and GST activity can be measured as well as this enzyme is not reliably measured in plasma (Chang, van der Hoeven, & Haddox, 1978; Hayes et al., 1991).

4.3 Conclusions

In conclusion, this study found that plasma GPx activity was higher in patients with possible VCIND compared to CAD control patients who did not demonstrate symptoms of cognitive impairment. Furthermore, a change in GPx activity was associated with a decline in verbal memory and executive function over 6 months. However, baseline GPx activity was not predictive of change in either verbal memory or executive function. These results highlight that the GSH antioxidant system is altered in CAD patients who demonstrate mild cognitive impairment as possible VCIND patients, contributing to the body of research surrounding the role of the GSH antioxidant and neurocognitive impairment. More research needs to be conducted to validate these findings and investigate the reliability and sensitivity of GPx as a marker for cognitive impairment as either a diagnostic and monitoring biomarker for cognition. The clinical implications for this research involve potentially using GPx as a marker for response to antioxidant supplementation such as NAC. Research into the role of the GSH antioxidant system in informing the antioxidant status of individuals with various forms of vascular cognitive impairment, especially in those who are deemed possible VCIND patients as the most prevalent form, may aid in the administration of personalized or precision medicine in the future.
References


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Appendices

Appendix A: Research Ethics Board Approval
Appendix B: Study Consent Form
Appendix C: Letter of Approval for Sample Analysis