Glutathione peroxidase in acute coronary syndromes

By

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Abstract

Glutathione peroxidase (GPx) and superoxide dismutase (SOD) are among the primary antioxidant enzymes that scavenge reactive oxygen species in the blood (ROS), thereby protecting against high levels of oxidative stress. The consequences of oxidative stress include cellular injury and tissue damage. High levels of oxidative stress have been implicated in the pathogenesis of acute coronary syndromes (ACS), however large clinical trials involving dietary antioxidant supplements have not shown a reduction in the rate of major adverse cardiovascular events (MACE).

In a cohort of 262 ACS patients we examined the relationship between GPx activity, SOD activity and MACE. Patients with MACE were found to have significantly lower levels of GPx activity than those without MACE, whereas SOD activity did not differ between the groups. Furthermore, dividing the patients into quartiles corresponding to levels of GPx activity demonstrated significantly higher rates of MACE in the lower quartile of GPx activity compared to the highest quartile.

Previous studies have demonstrated that deficiencies in GPx activity are associated with vascular dysfunction and platelet-dependent thrombosis, leading to the hypothesis that low levels of GPx activity would be associated with increased levels of platelet reactivity. In 51 ACS patients we did not observe a significant relationship between these two parameters, however we did demonstrate that increasing levels of GPx activity was associated with lower levels of ROS. ROS measures were based on the response of the platelets to addition of exogenous nitric oxide. Such an inverse relationship between GPx activity and levels of ROS is consistent with the view that GPx activity may play an important role in an ACS by reducing ROS-mediated damage, thereby protecting against MACE.

We examined levels of GPx activity, protein concentration and mRNA expression across populations of ACS patients, stable coronary disease patients and healthy subjects. Cardiovascular risk factors thought to influence levels of GPx activity were controlled for

in all three cohorts. These studies demonstrated that GPx activity, protein and mRNA levels were significantly elevated in the ACS patients compared to the stable coronary disease patients and healthy subjects. Oxidised low-density lipoprotein (oxLDL), a widely used marker of oxidative stress, was also significantly elevated in the ACS patients compared to the other two cohorts.

In a study examining the temporal changes in GPx activity in the acute phase of an ACS, GPx activity was found to be highly dynamic, with no consistent single time point that identified when peak activity occurred. In the majority of patients, levels of oxLDL were found to peak prior to, or at the same time, as peak GPx activity, suggesting that GPx activity was modulated by changes in oxidative stress.

In conclusion, the elevated levels of GPx activity observed in ACS patients were found to be highly dynamic throughout an ACS event. However those with lower levels of GPx activity have an increased risk of adverse clinical outcomes that may be due to an inadequate defence against levels of ROS. Whether these patients can be accurately identified and targeted with an appropriate therapeutic intervention warrants further investigation.

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We shall not cease from exploration, and the end of all our exploring
will be to arrive at the beginning, and know the place for the first time.
-T.S.Eliot

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Abbreviations

18S – 18S ribosomal ribonucleic acid

ACS – acute coronary syndromes

ADP – adenine diphosphate

AMI – acute myocardial infarction

ApoE-KO – apolipoprotein E knock out

ASAP – antioxidant supplementation in atherosclerosis prevention study

ATBC – alpha-tocopherol beta carotene cancer prevention study

BMI – body mass index

CABG – coronary artery bypass grafting

CAD – coronary artery disease

CAT – catalase

cGMP – cyclic guanosine monophosphate

CHAOS – cambridge heart antioxidant study

Ct – cycle threshold

CVA – cerebrovascular accident

CVD – cardiovascular disease

cDNA- complementary deoxyribonucleic acid

DNA – deoxyribonucleic acid

EC – endothelial cells

EDRF – endothelium relaxing factor

ELISA – enzyme-linked immunosorbent assay

eNOS – endothelial nitric oxide synthase

ETC – electron transport chain

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GI – gastrointestinal

GPx(s) – glutathione peroxidase(s)

GRACE – global registry of acute coronary events

GSH – glutathione

GSSG – reduced glutathione

H_2O - water

H$_2$O$_2$ – hydrogen peroxide

HOPE – heart outcomes prevention evaluation study

HPS – heart protection study

Hs-TnT – high-sensitivity troponin

KO – knock out

LDL – low-density lipoprotein

LOOH(s) – lipid hydroperoxidase(s)

LOX – lipooxygenases

MACE – major adverse cardiovascular events

MI – myocardial infarction

MMP(s) – matrix metalloproteinase protein(s)

mRNA – messenger ribonucleic acid

NADPH – nicotinamide adenine dinucleotide phosphate

NaNO$_2$ – sodium nitrite

NO – nitric oxide

NOS(s) – nitric oxide synthase(s)

NSAID – non-steroidal anti-inflammatory drug

NSTEMI – non-ST-segment elevation myocardial infarction

O$_2^-$ - superoxide radical

$^-$OH – hydroxyl radical

ONOO$^-$ – peroxynitrite

oxLDL – oxidised LDL

PBS – phosphate buffered saline

PCI – percutaneous coronary intervention

PPP – collaborative primary prevention project

RNA - ribonucleic acid

ROC - receiver operator curve

ROS – reactive oxygen species

Sec – selenocysteine

SD – standard deviation

SNO-Glu – *S*-nitrosothiol-glutathione

SOD – superoxide dismutase

SPACE – secondary prevention with antioxidants of CVD in endstage renal disease

STEMI – ST-segment elevation myocardial infarction

TF – tissue factor

UA – unstable angina

VSMC(s) – vascular smooth muscle cell(s)

XO – xanthine oxidase

Chapter 1 - Introduction

1.1   Oxidative stress overview

Reactive oxygen species (ROS) are some of the most important free radicals in biological systems.   As derivatives of oxygen, they are normal by-products of the oxidative respiration process, and under normal circumstances physiological levels are necessary for cell signalling and growth, which are essential to almost all the body's processes [1, 2].   ROS are highly reactive and include free radicals containing one or more unpaired electrons, such as the superoxide radical (O_2^-) and the hydroxyl radical (^-OH), as well as non-radical molecules such as hydrogen peroxide (H_2O_2) [3].   Primary sources of ROS in the vascular wall include the enzymatic systems of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidases, enzymes of the mitochondrial respiratory chain, and an uncoupled endothelial nitric oxide synthase (eNOS) [4, 5].

Under normal circumstances the physiological levels of intracellular ROS are maintained by various antioxidant systems participating in the *in vivo* redox homeostasis.   As humans, we produce endogenous antioxidants, and consume exogenous dietary antioxidants, that work to maintain homeostasis.   Endogenous antioxidants consist of enzymatic e.g glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) and non-enzymatic (e.g. glutathione) compounds.   Dietary antioxidants include vitamin E (tocopherols), vitamin C (ascorbic acid), and vitamin A (β-carotene) among others.   Antioxidants function by slowing or preventing the oxidation of other molecules by either removing free radical intermediates or inhibiting other oxidation reactions by being oxidised themselves [6].

Oxidative stress occurs when the homeostatic processes fail and ROS production exceeds the capacity of the antioxidant defence system, thus promoting cellular injury and tissue damage (Figure 1-1) [7].   Oxidative damage to DNA and protein content of cells, as well as lipid peroxidation of cellular membranes, calcium influx and mitochondrial swelling and lysis can all occur as a result of increased levels of ROS [8].   This disturbance in homeostasis has been implicated in the pathogenesis of various human diseases including those of the cardiovascular system.   In particular, one disease

widely purported to be associated with elevated oxidative stress is atherosclerotic cardiovascular disease.
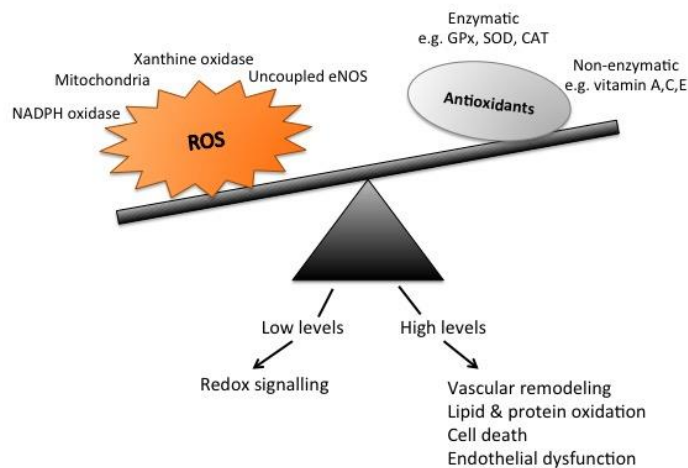
Figure 1-1 Schematic representation of *in vivo* redox homeostasis.

ROS levels are maintained by enzymatic and non-enzymatic antioxidants for homeostatic processes. ROS occurs from several different sources, and in oxidative stress conditions, the balance tips towards high levels of ROS, overwhelming the antioxidant defence system. This can lead to detrimental effects implicated in the pathogenesis of various disease states. GPx-glutathione peroxidase; SOD-superoxide dismutase; CAT-catalase. Figure modified from Tsutsui et al. 2011 with permission from The American Physiological Society [9].

1.2 Atherosclerosis

1.2.1 Burden of coronary artery disease

In New Zealand, Ministry of Health statistics show that cardiovascular disease (CVD) is the leading cause of death, accounting for 30% of all deaths annually [10]. Ischaemic heart disease, also known as coronary artery disease (CAD), is responsible for over 6000 (22%) of these deaths [11]. A high mortality and economic burden still exists despite the development of effective treatment strategies and improvements in both medical and invasive therapies [12-14]. Following initial presentation, these patients have an increased risk of repeat CVD events such as heart failure, stroke, myocardial infarction (MI) and death [15, 16].

1.2.2 Pathophysiology of coronary artery disease

Atherosclerosis is the underlying pathophysiology of CAD in the majority of cases [17]. Coronary atherosclerotic lesions are characterised by the adaptive intimal thickening of the artery as a result of the accumulation of lipids, immune cells, connective tissue elements and endothelial and smooth muscle cells [18]. Simple lesions, known as fatty streaks, can develop in childhood and adolescence [19], with the influence of a combination of genetic and environmental factors responsible for the progression of coronary atherosclerosis throughout adulthood [20]. These fatty streaks are subclinical, however the evolution to complex vulnerable plaques can manifest into clinical events such as MI or stroke [21].

1.2.2.1 Progression of the atherosclerotic lesion

The evolution of the fatty streak to a complex vulnerable plaque is depicted in Figure 1-2. The atherosclerotic lesion results from a complex interplay between circulating factors and various cell types in the vessel wall that create plaque which gradually impinges on the vessel lumen and impedes blood flow [3]. The atherosclerotic lesion core is formed through cholesterol deposits in macrophages creating a lipid-laden environment that attracts inflammatory and immunogenic cells. The subsequent disruption to the endothelium leads to the proliferation of vascular smooth muscle cells (VSMC) and increased collagen production that creates the fibrous cap that separates

the necrotic core from the circulation [18, 22]. The thick fibrous cap formed by the collagen-producing VSMC keeps the lesion structurally stable. The pro-inflammatory and pro-atherogenic environment surrounding the plaque promotes the release of cytokines and increases ROS generation, inducing the apoptotic death of VSMCs and stimulating the release of matrix-degrading enzymes that diminish the integrity of the fibrous cap [3]. Combined with the mechanical force of blood flow, plaque rupture can occur, exposing the core and activating platelet adhesion, thrombosis and vasospasm, manifesting into clinical coronary events such as an MI [23].
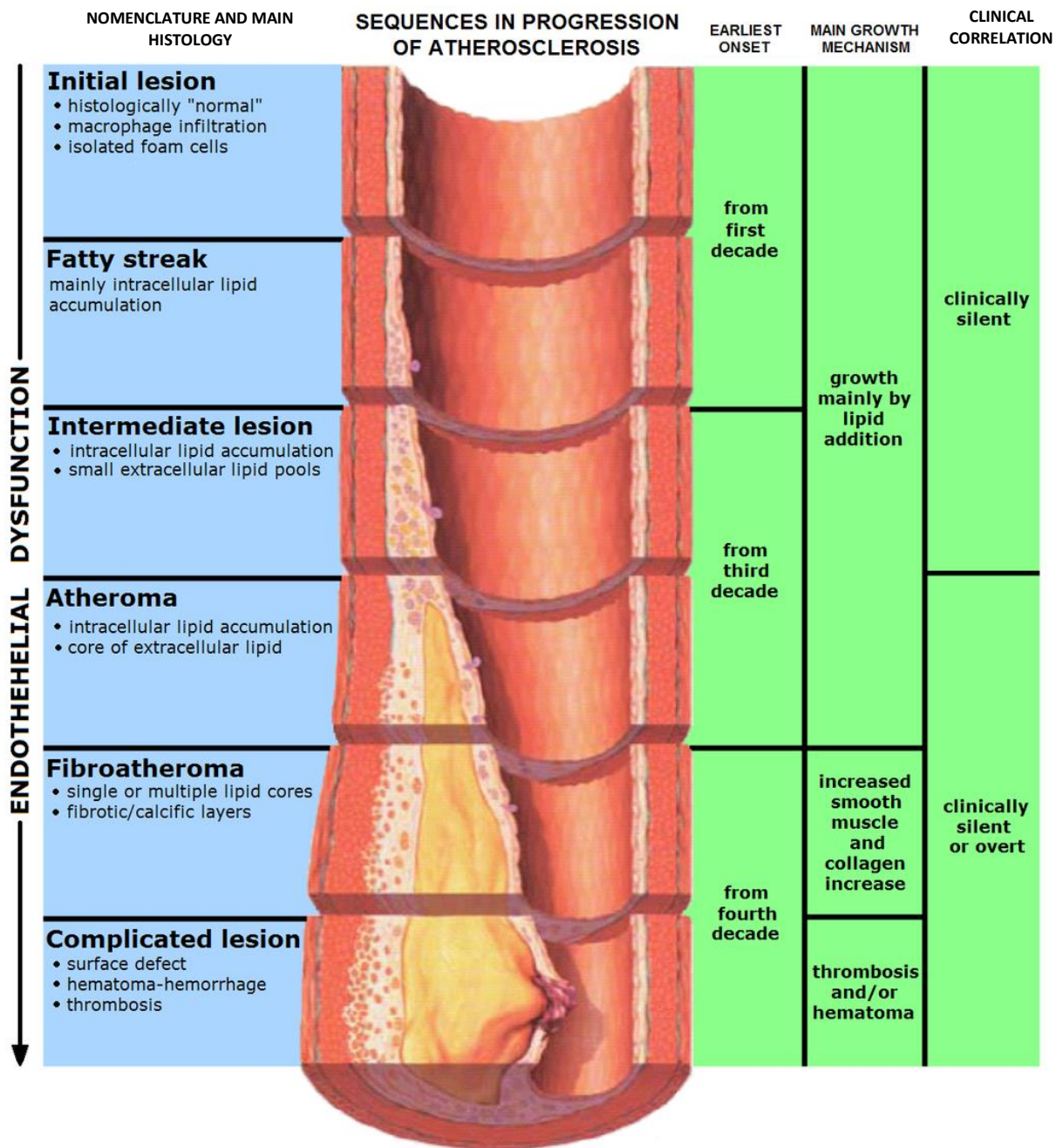
Figure 1-2 Progression of the atherosclerotic plaque.

Diagrammatic depiction of the sequence involved in the progression of atherosclerosis. Development of the lesion is shown along with the main histology findings associated with the onset of each stage according to earliest onset (patients' age), main growth mechanism and clinical manifestation. Increased endothelial dysfunction and lesion complexity are other factors associated with the progression of the atherosclerosis (not shown). Image reproduced with permission from Creative Commons Attribution-Share Alike 3.0 Unported.

1.2.3 Acute coronary syndromes

An acute coronary syndrome (ACS) is an unstable and potentially life-threating presentation of CAD [24]. The presence of an atherosclerotic lesion may obstruct blood flow and cause symptoms of angina pectoris, but rarely does this alone cause a fatal clinical event. Often ACS events are not accompanied by the preceding angina symptoms indicative of a high-grade stenosis. Manifestation of ischemia and infarction is the activation of vulnerable plaques, and ACS is nearly always caused by the formation of an occluding thrombus on the culprit atherosclerotic plaque [23, 25]. Thrombosis on the surface of a plaque occurs due to plaque rupture – identifiable in 60-70% of cases - and less commonly, plaque erosion [26]. Vulnerable plaques susceptible to rupturing are often those with a thin fibrous cap that tear, exposing the lipid core to arterial blood flow. The core area is highly thrombogenic, containing tissue factor (a potent pro-coagulant found in the core), collagen fragments and crystalline surfaces to accelerate coagulation. The thrombus initially forms in the plaque, distorting and expanding from within, and may extend into the arterial lumen [27]. The fibrous cap allows a barrier between the immuno- and thrombogenic rich lesion core and the coagulation factors found in the blood. The collagen-producing smooth muscle cells provide the tensile strength of the cap. The production of proteinases caused by the enhanced inflammatory environment local to the plaque breaks down collagen and inhibits replenishment from the VSMC. These enzymes belong to the matrix-metalloproteinase (MMP) family, with 3 of the human isoforms over-produced by macrophages in atherosclerotic plaques [28]. Rupture of the fibrous cap allows blood to come into contact with the necrotic core, allowing the action of tissue factor to cause thrombin generation and platelet activation and aggregation [29].

Autopsy studies show that the majority of deaths are caused by plaque rupture, with superficial plaque erosion accounting for 20-25% of fatal ACS cases [30, 31]. The mechanisms of plaque erosion have received much less attention and are less understood than those involved in plaque rupture. Apoptosis of the endothelial cells, induced by oxidative stress mechanisms, could contribute to desquamation and the production of tissue factor [32]. Endothelial cell death and tissue factor production

could propagate local thrombosis in the coronary arteries. From the growing body of evidence, it is now undisputed that plaque rupture or erosion in isolation is not enough to cause coronary events [33]. Indeed, rupture and erosion occur frequently, with subclinical symptoms, and are key factors in plaque progression, remodelling and subsequent luminal narrowing [30, 34]. Instead, it is not only the exposure of the plaque core to the blood, but the combination of other factors such as thrombogenicity of the plaque material, local flow dynamics and systemic thrombotic predisposition that lead to a perfect storm scenario resulting in ischemia and infarction [23].

1.2.3.1 Clinical treatment of ACS

ACS encompasses a spectrum of clinical conditions ranging from undifferentiated chest pain to unstable angina to non-ST-segment elevation myocardial infarction (NSTEMI) to ST-segment elevation myocardial infarction (STEMI) (Figure 1-3). The severity of myocardial ischaemia increases from UA through to STEMI, dictating the degree of injury to the myocardial cells. The subendocardial region is the first to be affected since this layer of the heart is farthest from the blood supply [35]. Injury to this area manifests itself on an electrocardiogram (ECG) as ST-segment depression and/or T-wave abnormalities (UA or NSTEMI) [13, 36]. Transmural injury manifests itself on an ECG as ST-segment elevation, and is generally considered to be a more severe event due to the larger area of infarction occurring (STEMI) [37, 38]. Management and treatment according to this spectrum dictates the appropriate treatment and allows for the best possible outcomes. The main goal is to reduce the amount of myocardial necrosis thus preserving left ventricular function. This allows for the prevention and management of major adverse cardiac events such as ventricular fibrillation, decompensated heart failure and cardiogenic shock [39]. Reperfusion therapy to restore coronary blood flow to the ischemic myocardium is the primary objective for ACS treatment to limit infarct size. Percutaneous coronary intervention (PCI) with stents and balloons is the treatment of choice and can restore coronary artery flow in >90% of patients under optimal circumstances. STEMI patients are advised to undergo PCI by a skilled provider in a well-equipped facility within 90 minutes of presenting to hospital, with fibronolytic therapy recommended if this time frame cannot be achieved. Adjunct therapies to manage both

the acute phase and chronic disease include: oxygen, nitroglycerin, analegesia, antiplatelet agents, hypertensive medications, anticoagulation and statin therapy [39].

Unstable angina (UA)	Non-ST segment Elevation Myocardial Infarction (NSTEMI)	ST segment Elevation Myocardial Infarction (STEMI)
Angina pain with at least one of the following features: • Is of new onset and severe • Occurs at rest or with minimal exertion • Pain is worsening in the severity and length of each episode (crescendo angina) • New ischemic ST segment changes not including ST elevation	Characterised by clinical features of unstable angina in addition to elevated cardiac markers: Troponin & CKMB. Cardiac markers are elevated as a result of myocardial necrosis.	Characterised by clinical features of myocardial infarction in addition to ST-segment elevation on a 12-lead ECG

Figure 1-3 Spectrum of Acute Coronary Syndromes (ACS).

Schematic table describing the clinical manifestation of ACS with arrows depicting the increase in the clinical severity of the ACS spectrum from UA to STEMI. Adapted from Boateng et al. [40] with permission from Elsiever.

1.3   Oxidative stress in atherogenesis and thrombosis

Atherogenesis is a complex, multifactorial process.   Several factors, such as dyslipidaemia, hypertension, diabetes, obesity, cigarette smoking and aging are established risk factors for CAD, with evidence that they are associated with high levels of oxidative stress [41]. As no single genetic marker or test accurately predicts cardiovascular death, research into the role of oxidative stress has highlighted its importance as a unifying mechanism across many vascular diseases [42]. ROS mediate various signalling pathways that underlie vascular inflammation in atherogenesis, from the initiation of fatty streak development through lesion progression to ultimate plaque rupture. Animal and human studies corroborate on the oxidative stress hypothesis of atherosclerosis. A summary of these studies are described in the following section.

1.3.1   The "oxidative modification hypothesis" of atherosclerosis

A general consensus exists that atherosclerosis represents a heightened state of oxidative stress, that can be characterised by lipid and protein oxidation in the vascular wall [3]. The ability of low-density lipoproteins (LDL) to transverse the subendothelial space of the arterial wall via binding to proteoglycans, and subsequently undergoing oxidation, forms the basis of the "oxidative modification hypothesis" of atherosclerosis first described by Steinberg et al. [43].   Recruitment of macrophages and their subsequent uptake of cholesterol in the form of oxidised LDL (oxLDL) are the main cellular events driving the formation of fatty streaks in the arterial endothelium. The formation of oxLDL renders it susceptible to macrophage uptake via the scavenger receptor pathway, transforming them into foam cells [44]. Foam cells are cytotoxic and promote the loss of endothelial integrity, a hallmark of atherosclerotic lesion formation. This concept is supported by considerable experimental evidence, demonstrated in multiple animal models, in which there is a defect in the scavenger receptor that protects against atherosclerosis, and results in a lower burden of disease [45, 46]. The oxidation process of LDL has a number of other pro-atherogenic properties, including the activation and recruitment of monocytes [47] and inflammatory/immune cells [48] into the arterial wall which sets the stage for the catalytic expansion of the atherosclerotic lesion and the full-blown spectrum of atherosclerosis [49]. OxLDL has

several biological effects: it is pro-inflammatory, it causes inhibition of endothelial nitric oxide synthase (eNOS), it promotes vasoconstriction and adhesion, it stimulates cytokines such as interleukin-1 and it increases platelet aggregation.   A schematic representation of the oxidative modification hypothesis is shown in Figure 1-4.

The ability of LDL to transit the subendothelial space and arrive back into the circulation means that a certain fraction exhibits an enhanced oxidised lipid content [50], and human plasma has been found to contain immunoreactivity towards epitopes generated from oxLDL [51]. However, the existence of oxLDL in the circulation has been controversial on the basis of potential artifacts that may occur during the *ex vivo* handling of plasma [52]. Nevertheless, several studies have shown that circulating levels of oxLDL epitopes can be used to distinguish between patients with and without evident atherosclerosis [53, 54], with increased circulating levels being associated with ACS [55]. No causal relation between oxLDL and atherosclerosis has been identified in the literature, and it remains unclear whether oxLDL levels are implicated in determining disease burden [52].
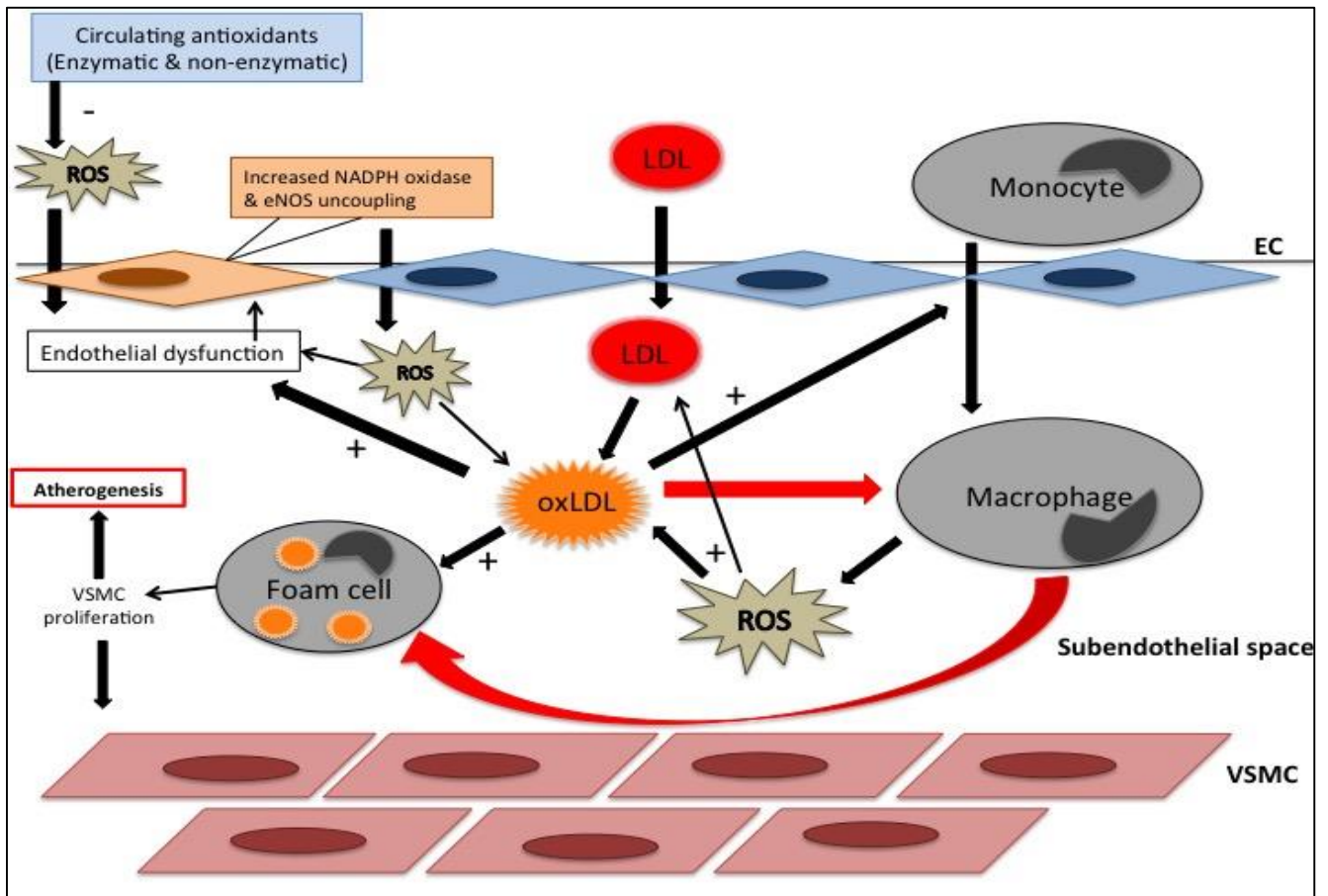
Figure 1-4 Oxidative modification hypothesis of atherosclerosis.

LDL is protected from oxidative modification in systemic circulation by the abundant endogenous circulating antioxidants. Once LDL passes into the subendothelial space and subsequently oxidised by reactive oxygen species (ROS) forming oxidised LDL (oxLDL). OxLDL is a chemoattractant to circulating monocytes and is transcytosed by them via scavenger receptor pathways, leading to formation of foam cells. Accumulation of foam cells and subsequent vascular smooth muscle cell (VSMC) proliferation is the hallmark of atherogenesis. The chain of events is amplified via a feed-forward mechanism, as oxLDL contributes to endothelial dysfunction, promoting further ROS formation via eNOS uncoupling and increased NADPH oxidase activity. Figure modified from Lee et al. 2012 Unported [56] with permission from Creative Commons Attribution-Share Alike 3.0 Unported.

Other abbreviations in the figure: EC – endothelial cells; eNOS – endothelial nitric oxide synthase; NADPH – nicotinamide adenine dinucleotide phosphate.

1.3.2 Production of reactive oxygen species

Oxidative modifications in the vessel wall are caused by several radical and non-radical oxidants, collectively called ROS. Free radicals can be defined as any molecule capable of independent existence that contains one or more unpaired electrons [57]. In biological systems, a variety of free radicals exist, and, if two radicals meet, they can join their unpaired electrons, which lead to non-radical products. ROS are a particularly destructive aspect of oxidative stress, and excess generation of ROS causes damage to proteins, lipids and DNA [41]. Consequences of this damage are associated with the pathological state of atherosclerosis, characterised by vascular remodelling, reperfusion injury, endothelial dysfunction, and plaque rupture [58]. Oxygen is the most abundant molecule in biological systems and can subsequently undergo enzymatic and non-enzymatic reduction to form the superoxide anion (O_2^-). An example of the detrimental effect this has in the vessel wall, is the fast reaction between superoxide anions with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a compound that promotes endothelial dysfunction and VSMC growth [59]. Similarly superoxide anions have the ability to form hydrogen peroxide (H_2O_2), arguably the most abundant non-radical species found in the vasculature. A chain of radical reactions produces highly reactive hydroxyl radicals capable of oxidative destruction of biomolecules. Lipid oxidation initiated by ROS molecules to generate lipid hydroperoxides (LOOH) is also a frequent occurrence in the vasculature [59], and is intricately linked to the pathophysiological relevance of atherosclerosis.

Under physiological conditions ROS can be generated *in vivo* by the NADPH oxidases, xanthine oxidase (XO), mitochondrial respiratory chain and nitric oxide synthases (NOS). A brief summary of the implications of dysregulation of these ROS sources on atherogenesis can be found below.

1.3.2.1 NADPH oxidases

NADPH oxidases are major sources of ROS in the vasculature, producing O_2^- from molecular oxygen using NADPH as the electron donor. An upregulation of NADPH oxidase subunits has been associated with the development of human CAD [60]. ROS

production from the p22phox subunit of NADPH oxidise has been reported to mediate the formation of oxLDL in atherosclerotic coronary arteries [61], supporting the notion that ROS plays a role in the pathogenesis of CAD.

1.3.2.2 Xanthine oxidase

XO donates electrons to molecular oxygen, producing O_2^- and H_2O_2, making it another primary source of O_2^- in atherosclerotic coronary arteries, alongside the NADPH oxidases [60]. Activity of XO is increased in human plaque [62], supporting the contribution of XO-derived ROS in the pathogenesis of CAD.

1.3.2.3 Mitochondria respiratory chain

Continuous ROS production occurs as a result of the electron transport chain (ETC) located in the mitochondrial membrane and is essential for energy production inside the cell [63]. The ETC is of central importance to atherogenesis. Dysfunctional mitochondrial ROS production has been linked to early atherosclerotic lesion development [64], and the site where electrons enter the ETC is reported to be critically involved in the oxidative burst observed during ischemia/reperfusion damage [65].

1.3.2.4 Nitric oxide synthases

Under physiological conditions, endothelial NOS (eNOS) is responsible for the production of NO that has an important vasoprotective function in the endothelium [66]. The uncoupling of eNOS (i.e uncoupling of O_2 reduction from NO synthesis) under pathological conditions associated with oxidative stress can render it dysfunctional such that it no longer produces NO, but produces superoxide instead. The result is significant endothelial dysfunction that plays an important role in the progression of CAD [67].

1.4   The antioxidant defence system

The vascular wall contains a variety of enzymes that can reduce the oxidative stress burden by acting as antioxidant defence systems. The classic antioxidant enzymes are both cell-associated and found in the extracellular matrix, whose function is to maintain the redox potential. Endogenous antioxidant systems protect against atherogenesis by virtue of their ability to scavenge ROS, facilitate endothelium-dependent vasorelaxation, inhibit inflammatory cell adhesion to endothelium, and alter vascular cellular responses, such as VSMC and endothelial cell apoptosis, VSMC proliferation, hypertrophy, and migration [68]. Enzymatic antioxidants principally include GPx, glutathione reductase and transferases, SOD, CAT and peroxiredoxins, with many of these antioxidants present in normal arteries.   Non-enzymatic antioxidants are another of the body's defence systems against oxidative stress.   These are generally found in the forms of the antioxidant vitamins ascorbic acid (vitamin C), α-tocopherol (the principal constituent of vitamin E), and β-carotene (pro-vitamin A). Compared with the in-depth knowledge of lipid changes in atherosclerosis, little is known of the accompanying changes to vascular wall antioxidants. Systematic studies on antioxidant changes at the various stages of CAD are not readily available. It is worth noting that the interpretation of changes to antioxidant defences is complicated because the same material is not normally analysed for the presence of oxidised biomolecules and oxidative tissue damage. For example, an increase in antioxidant defence is commonly interpreted as a biological response to increased oxidative stress without the knowledge of whether this is associated with an overall decrease or increase in oxidative tissue damage. A brief summary of the common enzymatic and non-enzymatic antioxidant systems and their association with atherogenesis can be found below.

1.4.1 Enzymatic antioxidants

1.4.1.1 Glutathione peroxidase

GPxs are a family selenocysteine (Sec)-containing antioxidant enzymes that use glutathione (GSH) as an obligate co-substrate in the reduction of H_2O_2 to water (H_2O):

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$$
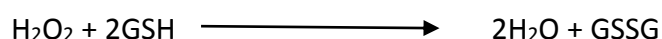
Not all GPxs (defined by homology), use GSH, nor do they all contain Sec at the catalytic site. Some of these enzymes are functionally identified as thioredoxin-dependent peroxidases containing a redox-active Cys in place of the Sec. There is a growing body of evidence that GPx enzymes play an important role in protecting against atherosclerosis. Mice deficient in GPx1 and GPx3 show anomalies in cardiac structure and function [69] and an increased pro-thrombotic state with vascular dysfunction [70]. Selected clinical studies suggest GPx is protective against adverse cardiovascular events [71] and deficiencies in this enzyme are associated with arterial thrombosis in human subjects [72]. More detail on the individual GPxs and their respective characteristics and role in CAD can be found in Section 1.7.

1.4.1.2 Superoxide dismutase

Three isoforms of SOD exist in mammals. Copper-zinc SOD (Cu-Zn-SOD, SOD1) is a soluble enzyme localised to the cytosol. Mitochondrial SOD (Mn-SOD, SOD2) is expressed in the mitochondrial matrix and extracellular SOD (EC-SOD, SOD3) is found in the extracellular space. All three isoforms catalyse the dismutation of superoxide into oxygen and H_2O_2, thereby serving an important antioxidant function.

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$
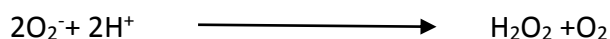
SOD3 is abundantly present in the vascular wall and is synthesised in atherosclerotic lesions by smooth muscle cells and macrophages [73]. Thus, in the vessel wall, SOD3 expression may play a critical role in regulating the vascular redox state by preventing deleterious effects of superoxide and superoxide-mediated inactivation of NO.

However the functional significance of SOD3 activity in the development of atherosclerosis is still unclear. Murine models show that SOD3 expression and activity is markedly increased in macrophage-rich atherosclerotic lesions [74], and that SOD inhibition increases lipid peroxidation [75], indicating a potential protective role. However, overexpression of SOD increases, rather than decrease, lesion formation [76]. Studies carried out in a mouse model of atherosclerosis (apolipoprotein E knock-out mice) have demonstrated that a genetic deletion of SOD3 paradoxically causes a slight reduction in atherosclerotic lesions after a 1-month atherogenic diet, while having no effect after 3 months on the atherogenic diet, or after 8 months on a standard diet [77]. Human studies have not been any more conclusive, with a large study conducted by Blankenberg et al. in 643 CAD patients showing no significant association with SOD activity and CAD progression [71].

1.4.1.3 Catalase

CAT functions by metabolising H_2O_2 produced from the dismutation of O_2^-. It directly decomposes H_2O_2 to water and molecular oxygen:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$
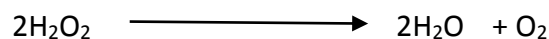
Overexpression of CAT reduces the vascular cell response to oxidised lipids [78, 79] and retards atherosclerosis in apoE-KO mice [80]. However a study in humans has shown that vascular and endothelial cells lack CAT activity [81], suggesting that CAT isn't the most important antioxidant shield in atherosclerotic plaque in humans.

1.4.2   Non-enzymatic antioxidants

1.4.2.1   Vitamin A

Collectively, the three active forms of the fat-soluble vitamin A are called retinoids, with the most important one being β-carotene which has high antioxidant properties [82, 83]. It plays a key role in quenching free radical reactions, particularly those involved in singlet oxygen species. This prevents lipid peroxidation and potential oxidative damage to DNA, both key determinants in the atherosclerotic process. β-carotene has been studied extensively in large clinical trials for its antioxidant effect in prevention of atherosclerosis [84]. For an in-depth review refer to Section 1.6.

1.4.2.2   Vitamin C

Vitamin C is a water-soluble substance, with ascorbic acid being one of the two biologically active forms. Ascorbate's biological role is related to its reducing capability, in which it is readily oxidised to dehydroascorbate. As a hydrogen donor, vitamin C can inactivate free radicals before they exert damaging effects on lipids or proteins [85]. Vitamin C has been used as an antioxidant agent in large clinical trials to determine its effect on atherosclerosis [86]. For an in-depth review refer to Section 1.6.

1.4.2.3   Vitamin E

Vitamin E plays an important protective role against free radical damage by being a hydrogen donor, preventing the oxidation of other molecules. Importantly it prevents the oxidation of polyunsaturated fats in cell membranes. When fatty acids are damaged, lipid peroxides are produced which can alter the function of cell membranes and causes damage to metabolic pathways [85]. It also has the ability to enhance the bioactivity of NO, inhibit smooth muscle proliferation and limit platelet aggregation [3]. The form that accounts for 90% of the vitamin activity in tissues is α-tocopherol, and has been studied in large clinical trials for its antioxidant effect in prevention of atherosclerosis [84]. For an in-depth review refer to Section 1.6.

1.5 The antioxidant paradox

There is an undeniable wealth of data demonstrating that atherosclerosis and subsequent cardiovascular events are associated with a number of oxidative events ranging from LDL oxidation to the production of ROS. Increased levels of oxidative stress biomarkers have been shown to predict CVD diseases [87-89]; therefore targeting this system has been an obvious focus of investigation. The 'antioxidant paradox' refers to the observation that despite this knowledge about oxidative stress being involved in CVD, giving large doses of dietary antioxidant supplements to human subjects to prevent or limit atherosclerosis and its clinical events, has, in most cases, demonstrated little or no therapeutic effect [90]. There remains today no consensus that antioxidant supplementation of patients at risk of, or suffering from, atherosclerosis is able to prevent or ameliorate the disease process.

However, with the abundant pathophysiological, epidemiologic, and mechanistic data that support the value of antioxidant therapies, it is critical to evaluate both the information supporting the need for antioxidants, as well as examine the clinical trials to understand why they have not demonstrated the expected results. This will allow for new research questions to be raised and strategies to be developed that could target potential biomarkers to help explain the antioxidant paradox.

1.6 Antioxidant Clinical Trials

1.6.1 Prospective cohort studies:

Epidemiological and population studies have demonstrated that consumption of a diet rich in antioxidants is associated with a reduced mortality by CVD [91], and that plasma antioxidant levels reflect this benefit [92, 93]. A number of other observational studies that employed long-term follow-up evaluation on >100,000 individuals also support the observation that high levels of dietary antioxidants are cardioprotective. In the Health Professionals Follow-Up Study involving 40,000 males with a 4-year follow-up, an inverse relationship was observed between vitamin A and vitamin E intake and risk of CAD [94]. Similarly, in the Nurse's Health Study involving 87,000 female nurses with an 8-year follow-up, vitamin E dietary supplements were found to beneficial in reducing

the risk of CAD [95]. Results from observational studies are not always so clear-cut; another population study of 35,000 postmenopausal women showed a significant reduction in CVD risk with an increase in dietary, but not supplemental vitamin E [96]. In yet another study, the Scottish Heart Health Study, intake of dietary vitamin E was recorded in 8,000 men and women who were followed for 9 years found that higher levels of vitamin E intake had no benefit for lowering CAD risk. However there was an inverse association between levels of vitamin A and vitamin C and risk of CAD in men but not women [97]. This is in contrast to the study that reported a similar inverse relationship with vitamin A and C with CAD risk, only it was in their woman's group compared to their men [98].

With the data from the observational studies suggesting individuals who increase their levels of antioxidants via diet or supplements may benefit from a decreased risk of CVD, large-scale randomised controlled trials were carried out to determine whether the disease could be modulated via antioxidant therapy. However when investigating the benefits of antioxidants used, such as vitamin E, C and β-carotene, the evidence for linking supplementation to prevent or reduce CVD is inconsistent. A discussion of the clinical trials can be found below, with a summary in Table 1-1 located at the end of Section 1.6.4.

1.6.2   Randomized controlled studies: primary preventions

A wide range of prospective studies have been undertaken that compare antioxidant treatment with placebo for the prevention of CVD. Where the primary aim was prevention of CVD, the major clinical trials reported no significant benefit of vitamin E on the traditional end-points (MI, CVD and stroke). Two such examples of these trials are the Finnish Alpha-Tocopherol Beta Carotene Cancer Prevention Study (ATBC) [99] and the Collaborative Primary Prevention Project (PPP) [100], with study populations of 29,000 and 4,500 respectively. Risk factors were reported for all subjects and these trials used a low-dose and high-dose vitamin E vs placebo regimen. The ATBC reported no benefit on the prevalence of fatal or nonfatal MI, but a small, yet significant, increase in haemorrhagic stroke was observed. The importance of this is yet to be determined,

as this correlation was not seen in several secondary prevention studies (Section 1.6.3). The PPP study similarly showed no effect on cardiovascular deaths by vitamin E, although this study was halted early as it confirmed a protective effect by aspirin that was considered the primary study end-point. In addition to the traditional clinical end points, other studies have used carotid intima-to-media thickness as a marker of on-going atherosclerotic disease, although a limitation of this parameter must be noted, in that it only correlates weakly when disease is measured by angiography [101]. Studies that used this measure reported no decrease by vitamin E or vitamin C supplementation after a follow-up of 3 years [86], although one study reported a borderline disease-promoting effect from α-tocopherol supplements [102]. The conclusions from these studies were that vitamin E supplements failed to slow or inhibit the progression of intima-to-media thickness in healthy men and women at low risk for CVD.

1.6.3 Randomised controlled studies: secondary preventions

In the studies that investigate the effect of vitamin E supplementation in patients with pre-existing cardiovascular disease, there are two studies that appear to show favourable results. In the Cambridge Heart Antioxidant Study (CHAOS), vitamin E supplementation was observed to decrease the risk of nonfatal acute MI (AMI), however they did report a non-significant small increase in total mortality in the treatment group [103]. Similarly, in a smaller study, the Secondary Prevention with Antioxidants of Cardiovascular Disease in Endstage renal disease (SPACE), reported that in haemodialysis patients, vitamin E supplementation resulted in a significant decrease in AMI [104]. This raises the question of whether vitamin E supplementation will only show a benefit in some populations, for example those with renal failure who may demonstrate increased oxidative stress, or those with a certain level of chronic coronary disease. Both these studies had a relatively short follow-up of 1.4 years. The positive results reported from these two studies are inconsistent with other studies that report no significant benefit with vitamin E supplementation. In the sub-group of the ATBC trial, no benefit was reported in cardiovascular events of this secondary prevention study [105]. Similarly, in the large GISSI Prevenzione trial in which 11,000 patients with a recent history of MI were given vitamin E supplementation for 3 years, no decrease

was found in cardiovascular death, nonfatal MI or stroke [106]. The large Heart Outcomes Prevention Evaluation (HOPE) study investigated patients with CVD and diabetes and another risk factor, and showed no benefit with vitamin E supplementation [107, 108], which is at odds with the CHAOS study despite similar supplementation regimes and a longer follow up (4.5yr vs 1.4yr respectively).

1.6.4   Vitamin E in combination with other antioxidants

Vitamin E has also been studied in combination with other antioxidants for its effect on cardiovascular health in both primary and secondary prevention trials. The ASAP study looked at the effect of vitamin E combined with vitamin C and reported that it significantly reduced the intima-to-media progression rates in men [86]. THE ATBC study reported no significant benefit on coronary events when vitamin E and β-carotene were supplemented in men as a secondary prevention strategy [105]. The large Heart Protection Study (HPS) with over 20,000 patients examined the benefit of antioxidant combination (vitamin E, C and β-carotene) on secondary prevention. Although the blood antioxidant levels were raised with this treatment regimen, no significant reductions in the mortality from, or incidence of, any type of vascular disease, or other major outcome was found [109]. Several meta-analyses of these randomised, controlled trials have been carried out that support the overall finding that vitamin E therapy does not reduce the risk of cardiovascular events [110-113].

Table 1-1 Summary of randomised controlled clinical trials for antioxidant therapy

Study (yr), [ref]	Subjects				Treatment				
	No. of pts	Sex	Age	Characteristics	Dose	Duration (yrs)	Prevention		Study Outcome
Vitamin E									
ATBC (1994) [99]	29133	M	50-69	Smokers, no medical history	50 mg	6.1	Primary	Adverse	Increase in haemorrhagic stroke
PPP (2001) [100]	4495	M/F	64	At risk of CVD	300 mg (synthetic)	3.6	Primary	Null	No effect on MI/CVD death/stroke
HOPE (2000) [108]	9541	M/F	≥55	High CVD risk	400 IU (natural)	4.5	Primary & Secondary	Null	No effect on MI/CVD death/stroke
CHAOS (1996) [103]	2002	M/F	62	Coronary disease	800 vs 400 IU	1.4	Secondary	Beneficial	Decreased non-fatal acute MI
ATBC (1997) [105]	1862	M	50-69	Smokers who had a MI	50 IU	5.3	Secondary	Beneficial	Reduction in non-fatal MI occurrence
GISSI (1999) [106]	11324	M/F		Post-MI	300 mg (synthetic)	3.5	Secondary	Null	No effect on MI/CVD death/stroke
Micro-HOPE (2002) [107]	3654	M/F	65	Diabetics	400 IU (natural)	4.5	Secondary	Null	No effect on MI/CVD death/stroke
CHAOS (1996) [103]	2002	M/F	62	Coronary disease	800 vs 400 IU	1.4	Secondary	Beneficial	Decreased non-fatal acute MI
SPACE (2000) [104]	196	M/F	40-75	Haemodialysis patients	800 IU	2	Secondary	Beneficial	Decreases acute MI + PVD + UA
β-carotene									
ATBC (1994) [99]	29133	M	50-69	Smokers with no medical history	20 mg	6.1	Primary	Adverse	Increase in overall mortality. More deaths due to: IHD,

Trial	N	Sex	Age	Population	Dose	Duration	Prevention	Effect	Outcome
									haemorrhagic & ischemic stroke
ATBC (1998) [84]	27271	M	50-69	Smokers with no medical history	20 mg	6.1	Primary	Null	No effect on: all coronary cases, nonfatal MI, fatal CHD
Antioxidant cocktails									
ATBC (1998) [84]	27271	M	50-69	Smokers with no medical history	50 mg vit E + 20 mg β-carotene	6.1	Primary	Null	No effect on: all coronary cases, nonfatal + fatal MI
ASAP (2000) [86]	520	M/F	45-69	Elevated cholesterol levels	182 mg d-α-tocopherol + 500 mg vit C	3	Secondary	Beneficial	Progression of intima-media thickness reduced from placebo in men, but not female
HPS (2002) [109]	20536	M/F	40-80	High CVD risk	600 mg vit E, 250 mg vit C, 20 mg β-carotene	5	Secondary	Null	No effect on CVD mortality
Fang et al (2002) [114]	40	M/F	≥18	After cardiac transplant	500 mg vit C + 400 IU vit E	1	Secondary	Beneficial	No increase in intimal index in treatment group vs placebo group

Trial names: ATBC – alpha-tocopherol, beta-carotene cancer prevention study; ASAP – antioxidant supplementation in atherosclerosis prevention study; CHAOS – Cambridge heart antioxidant study; HOPE – heart outcomes prevention evaluation study; HPS – heart protection study; PPP – collaborative primary prevention project; SPACE - Secondary prevention with Antioxidants of Cardiovascular Disease in Endstage renal disease; GISSI – GISSI prevenzione trial.

Abbreviations: CHD – coronary heart disease; CVD – cardiovascular disease; F - female; IHD- ischaemic heart disease; IU – international units; M – male; MI – myocardial infarction; PVD – peripheral vascular disease; UA – unstable angina; vit – vitamin.

Table adapted from Katsiki et al. 2009 [115] with permission from Elsiever.

1.6.5 Why have the antioxidant clinical trials failed?

There are many speculative reasons as to why the results from the clinical trials do not support the putative role of antioxidants in the protection against atherosclerosis. There is strong experimental evidence to support the conclusion that oxidative stress plays a central mechanism by which clinical risk factors lead to vascular damage and atherosclerosis. The concept was that the prevention of oxidative stress induced by CVD risk factors would prevent the formation of endothelial dysfunction and the eventual manifestation of plaque rupture and thrombosis seen in the acute setting of CAD [58].

First and foremost, it must be questioned whether the right drug or combination of drugs have been used in these trials. The fact is, the requisite fundamental understanding of the relevant oxidants in atherosclerosis is not yet fully appreciated, and therefore the appropriate antioxidant treatment has most likely not been tested yet. This is important to consider given that most of the clinical trials have used species that are only effective at scavenging a certain type of oxidant [3]. This design flaw may potentially mean that only some, but not all, relevant oxidizing species implicated in atherosclerosis are metabolised. Whether the antioxidant agents are selected on the basis of their availability is another unknown, and it may be that they are not ideal or equivalent to the endogenous antioxidants. When examining the trials that use vitamin E, it is concerning that some report a pro-oxidant effect [116], and that high-dose supplementation was associated with an increased mortality [117, 118]. Therefore, trials of vitamin E alone could be causing more harm than good. Even if vitamin E is the correct antioxidant agent to use, it is possible that the correct form of vitamin E has not been chosen. Cheap, easily available, synthetic vitamin E is mostly used throughout the trials; however natural vitamin E is different from the synthetic form and is composed of 8 different isoforms that may render it more potent. Additionally, vitamin E may require a co-antioxidants to prevent oxLDL formation [119].

Another possibility that needs to be evaluated is that although the appropriate drug may have been used, were the appropriate doses and/or treatment durations sufficient. An important consideration when determining optimal therapy duration is that animal

models tend to show effective treatment with antioxidants in the earliest forms of atherosclerotic lesion formation [120]. Even those trials with follow-up lasting years may be too short to observe any benefit. The primary and secondary prevention studies used subjects with 40-50 years' worth of oxidative stress in the endothelium, and it may be unrealistic to assume, for example, that vitamin E supplementation can alter this within a 1-5 year period. Dose regimens of particular antioxidant agents also remain controversial. The most effective doses are not known, and doses above 400 UI/day have been linked to increases in mortality [118]. Another factor to consider is that individual variability in response to vitamin supplements has been proposed to affect results [121]. Therefore administering the same dose of vitamin E to everybody will not lead to the same effect in all individuals.

This leads to another explanation for the failure of the antioxidant clinical trials in that simply the wrong study population were investigated. First, as highlighted above, it may be that intervention in adults is too late in the atherosclerotic process to see a difference. An alternative consideration is that not all patients may require antioxidant therapy. Instead of treating everyone with antioxidant therapy, as has been done to date in the clinical trials, it is potentially more beneficial to target the population who are proven to be deficient in either antioxidants or have an elevated oxidative stress threshold. This could explain why those trials that have seen benefit of antioxidant therapy, did so in a select population, such as subjects with renal failure [103] or having had post-cardiac transplantation [114].

1.6.6   Antioxidants as a biomarker in coronary artery disease

The lack of benefit seen in clinical trials and the subsequent 'antioxidant paradox' does not disprove the central role of oxidative stress in atherosclerosis. Rather these trials prove that better design is required for future antioxidant intervention studies. Areas that need to be addressed are the determination of the best antioxidant species at the right doses, and the determination of the correct study population for treatment, as well as what the optimal duration of treatment should be. To address these areas, a strategy that has been highlighted in the literature to the attention of trial designers, is to direct their focus on enhancing the expression and/or activity of the endogenous antioxidant

defence systems and examine whether these systems are affected throughout the different stages of CAD.

The principal limitation in this field of research is the lack of understanding of the connection between oxidative stress and CVD. Atherosclerosis is a multifactorial disease, and oxidative stress may be the predominant effector of pathology in only a subset of patients. The lack of proven markers for oxidative stress, which could help identify a subset of a population that can benefit from antioxidant supplementation, and the complexity of the redox reactions, are among some of the factors that are responsible for the mixed outcomes in the use of antioxidants for the prevention of CVD. Current methods rely on indirect measurements to assess oxidative stress, such as lipid and protein oxidation levels [6]. There is limited evidence that these reflect oxidative stress *in vivo*, and the lack of standardisation of many of these methods potentially explains different outcomes in the same population group. An example of this is the assessment of oxLDL as a predictor for CVD outcomes. In the same cohort, Tsimikas et al. [122] looked at 5-yr outcomes, Keichl et al. [123] at 10-yr outcomes, and Mayr et al. [124] at 5-yr outcomes. Tsimikas et al. and Keichl et al. found oxLDL predictive of CVD; however Mayr et al. did not find any association, despite reporting similar end-points. Nevertheless, oxLDL has been the most common oxidative biomarker used to investigate CVD progression. Due to their highly reactive nature ROS molecules usually do not lead to the release of stable by-products, and combined with their relatively short half-lives, measurement presents a significant challenge to researchers. It is widely accepted that measurement of systemic markers of oxidative stress in the circulation offers limited information about the true redox state of the individual in the vasculature [56]. Due to the highly contentious argument on which is the most suitable biomarker of oxidative stress, the evidence for antioxidants to be used as a potential surrogate marker for oxidative stress is emerging. The implications of using the antioxidant defence system as a measure for risk prediction and/or oxidative stress in CAD is an important area of research, and one that needs to be closely examined.

A strong piece of evidence supporting the need for this line of research comes from the results published by a recent meta-analysis [125]. The 42 case-control and 3 cohort

studies analysed suggested that low activity levels of the endogenous antioxidant systems, GPx, SOD and CAT were associated with a higher risk of CAD. Further experimental studies have demonstrated that deficiencies in GPx activity promote a pro-thrombotic state and vascular dysfunction [70], with clinical evidence suggesting that GPx activity has potential as a biomarker for predicting future cardiovascular events in a CAD population [71]. These findings have prompted renewed interest in the GPx antioxidant system in anticipation that it may have clinical relevance in oxidant-mediated progression of CAD. The following section will provide an in-depth overview of the GPx enzyme and current evidence supporting its role in CAD.

1.7   Glutathione peroxidase overview

Glutathione peroxidases (GPxs) constitute a family of phylogenetically related antioxidant enzymes, as evidenced by sequence homology. GPx was the first selenoenzyme discovered in mammals [126, 127], now this family has grown to include more than 700 members spread over all living organisms, from prokaryotes to humans [128]. In mammals, GPxs constitute a primary defence mechanism against oxidative damage. Along with SOD and CAT, GPxs exert their antioxidant properties in order to maintain the redox potential in the body. Oxidative stress occurs as a consequence of an imbalance between the levels of oxidants and antioxidants, tipped in favour of the former [7]. An oxidative challenge or a loss of antioxidants alone does not account for oxidative stress; it is when there is increased formation of oxidants accompanied by a loss of antioxidants and/or accumulation of oxidised forms of the antioxidants, that oxidative stress is approached [3]. Disturbances in normal cellular redox homeostasis contribute to the susceptibility and/or pathology in many common and complex human diseases, such as CAD.

1.7.1   Structural and mechanistic aspects of GPx

For decades, GPxs have been known to catalyse the reduction of H_2O_2 and organic hydroperoxides to water and the corresponding alcohols, typically using glutathione (GSH) as the reductant [129].

In mammals, up to 8 different isoforms of GPx have been described. GPx1, 2, 3, 4 and in humans, GPx6, are selenoproteins, containing a selenocysteine (Sec) at their catalytic center [130]. The Sec moiety enables a fast reaction with the hydroperoxide substrates and a fast reducibility of GSH as the reductant. The remaining variants have a redox-active cysteine residue in place of the Sec moiety, and therefore are functionally identified as thioredoxin-dependent peroxidases [131]. As for many other selenoproteins, the availability of selenium represents the single most important factor for biosynthesis of GPxs, but the individual GPx isoenzymes respond to selenium depletion differently. GPx-1 undergoes a particularly rapid decrease of enzymatic activity, protein expression and mRNA stability in cultured cells and animals when

switched to selenium deficiency [132]. Each isoform is postulated to exhibit tissue-specific expression and different substrate specificity. They are divided into classes based on their primary sequence, substrate specificity and subcellular location. GPxs are predominately located intracellularly, the exception for this being the secreted plasma isoform GPx3. Cytosolic GPx1 and phospholipid hydroperoxide GPx4 are ubiquitously expressed. GPx2 is an epithelium-specific gastrointestinal protein, and GPx6 is found in the olfactory epithelium and embryonic tissues [133, 134]. Only GPx1, 3 and 4 have been partially functionally characterized. Discussion of the individual isoforms can be found below.

1.7.2   GPx1

The first GPx to be identified, GPx1 was discovered by Mills et al. in 1957. It was described as a selenoprotein that is able to protect haemoglobin from oxidative degradation in red blood cells [135]. GPx1 is the most abundant member of the antioxidant family, and is a crucial antioxidant enzyme involved in preventing the harmful accumulation of intracellular H_2O_2. GPx1 is a ubiquitous enzyme, present in the cytosol [127] and mitochondria [136] of mammalian cells – including in the endothelium [137] and erythrocytes [138]. It metabolizes H_2O_2 and some organic hydroperoxides. Studies from genetically modified mice where there is a deficiency in GPx1 are developmentally normal and show no increased oxidative stress or sensitivity to hyperoxia compared with wild-type mice [139]. This indicates that either other proteins may compensate for the lack of GPx1, or that GPx1 plays a limited role under physiologic conditions. In contrast, under acute oxidative stress conditions, GPx1 KO mice did not survive irrespective of selenium supplementation. This is in comparison to selenium-supplemented wild-type mice that survived [140, 141]. These findings show that GPx1 has a primary antioxidant function and cannot be replaced by another selenoprotein to maintain protection from generalised oxidative stress.

1.7.3 GPx2

GPx2 is a homotetramer and another cytoplasmic antioxidant found originally in cells of rodent gastro-intestinal (GI) tract. It was subsequently identified in human cells throughout the GI tract. It is an epithelial-specific enzyme that is highly expressed in the intestine of mammals and humans as well as the liver of humans [138, 142]. Enzymatically, it is postulated that GPx2 is similar to GPx1, and the different gene expression patterns are thought to make GPx2 an essential protector against gut pathogens and inflammation. However, since the protein has not been purified so far, neither substrate preferences nor kinetic constants have been reported [131].

1.7.4 GPx3

Also similar to GPx1, GPx3 is a tetramer and is enzymatically comparable to GPx1 [143]. However, the physical and kinetic properties are distinct from the other isoforms. GPx3 is a glycosylated protein secreted from the cell. It exhibits tissue-specific expression, with mRNA expressed in the proximal convoluted tubule of the kidney and released into the plasma via the basolateral membrane of the cell [144, 145]. GPx3 mRNA has also been found expressed in the heart, liver and breast [146]. In human plasma, GPx3 is the most important ROS-detoxifying selenoenzyme, catalysing the reduction of extracellular H_2O_2 and lipid hydroperoxides [147]. It can also accept thioredoxin as well as GSH as a reducing co-substrate [148]. GPx3 is widely used as a marker for selenium status [134]. Transcriptional regulation of GPx3 has largely remained uncharacterized. The possibility of enhancing GPx3 expression is highly variable between cell types and tissues therefore general regulatory mechanisms cannot be postulated [147]. GPx3 can prevent hydroperoxide-mediated activation of lipoxygenases (LOX), thereby preventing undue inflammatory responses which would otherwise occur by LOX activation [147]. GPx3 helps to maintain the vascular bioavailability of NO, a major vasorelaxant and inhibitor of platelet function [149].

1.7.5 GPx4

This selenoenzyme is a monomer, that, unlike other GPxs, can reduce phospholipid- and cholesterol-hydroperoxides directly, using electrons from GSH as well as from other protein thiols in mammalian cells [150]. It is located in cytosolic, mitochondrial and sperm nuclear isoforms, and has differential tissue distribution. A systemic knockout of the GPx4 gene is lethal in mouse models, with GPx4+/- mice more sensitive to oxidative stress [151]. This demonstrates an essential role for GPx4 as an antioxidant in mammalian cells, although the particular reason for its unique essentiality remains to be determined [147].

1.7.6 GPx5

The Cys variants of GPx include the epididymis-specific GPx5 [152, 153]. Its closet homologue is GPx; however, the kinetics and substrate specificities remain to be investigated. The putative function of GPx5 in fertility is that it regulates oxidants in the epididymis since it has a restricted expression and secretion in this tissue [154].

1.7.7 GPx6

GPx6 is a newly discovered GPx, located in olfactory epithelium and embryonic tissue [155]. It is a selenoprotein in humans, but a CysGPx in other species. So far, GPx6 has not been purified and kinetic analyses do not exist. Knowledge about the characteristics of this GPx is very limited.

1.7.8 GPx7 and GPx8

Both the CysGPx, GPx7 and GPx8 have putative roles as antioxidants, although neither has been purified to date. GPx7 was identified in the lumen of the endoplasmic reticulum [156], and GPx8 was described as a membrane protein in the endoplasmic reticulum (ER) [157]. Because of their location, these enzymes have been hypothesised to play a role in protein folding, since disulphide formation is a redox-dependent process

that takes place in the ER of eukaryotic cells [157]. More detailed information on GPx7 and GPx8 is not available.

1.7.9 Experimental implications for GPx in cardiovascular disease

Experimental studies have highlighted the protective properties of the GPx system against oxidative stress in the setting of CVD. Animal models with GPx1 deficiencies show anomalies in the structure and function of the vessels and heart [69] and increases in the level of lipid oxidation [78]. Overexpression of GPx1 improves ventricular remodelling after an MI [158]. Mice deficient in GPx3 enter a pro-thrombotic state with vascular dysfunction that promotes platelet-dependent arterial thrombosis [70]. Long-term selenium deficiency results in markedly decreased GPx activity in the arterial wall and in the heart of rats, accompanied by enhanced levels of lipid peroxides, and clear oxidative damage in the tissues. Sodium selenite supplementation restores GPx activity and decreases the formation of lipid hydroperoxides [159]. Ischemic animal models fed a hyperlipidemic diet show increases in selenium- dependent GPx activity [160]. GPx2 knock-out mouse models do not develop any obvious phenotype under unstressed conditions; however, studies indicate that it has important anti-inflammatory properties since deficiencies in GPx2 expression are linked to inflammatory bowel disease [147]. Together these experimental studies illustrate the importance of this key antioxidant enzyme in the functional and structural responses of the mammalian cardiovascular system.

1.7.10 Clinical relevance of the GPx enzymes

With experimental studies suggesting a protective role of GPx in CVD, translational studies have been carried out to determine whether GPx is a factor in contributing CVD risk in humans. Supportive evidence includes the finding that GPx1 activity is decreased or absent in carotid atherosclerotic plaques, and that the lack of GPx1 activity in atherosclerotic lesions appears to be associated with the development of more severe lesions in humans [161]. Decreased GPx3 activity is found in patients with a history of arterial thrombosis and stroke syndromes [72]. It has been postulated that GPx3

potentiates the inhibition of platelet function by NO by maintaining low levels of ROS. Selenium is an essential cofactor for the activity of the seleno-GPxs, but the role of selenium in CVD is controversial. Low serum selenium levels may be a risk factor for CVD; however epidemiologic studies have been inconclusive, and prospective, controlled trials of selenium supplementation report inconsistent results [162]. Keshan's disease is a cardiomyopathy primarily due to dietary deficiency in selenium. A link has been suggested between a polymorphic gene variation of GPx1 that results in decreased activity and the development of Keshans disease in a regional selenium-deficient area of China [163].

The association of GPx activity and risk of CAD is not completely understood to date. It is postulated that low levels of GPx activity are associated with an increased risk of CAD events, but the literature on this topic is divergent. A recent meta-analysis examined 32 case-control studies and 2 prospective studies and concluded that GPx activity levels are protective against CAD outcomes [125]. However the results are not as clear-cut as they appear. Most of the studies examined were small case-control studies and lacked adjustment for factors such as smoking, age, diet and other conditions known to be affected by oxidative stress. More rigorous adjustments for cardiovascular risk factors are needed to ensure that the association between antioxidant activity and CAD is robust. Additionally the majority of the studies examined were carried out in patients who had a chronic CAD. This includes The Atherogene Study, the largest study to examine the association between GPx1 activity and CAD outcomes. In 636 stable coronary patients, an inverse association was shown between levels of GPx1 activity and risk of CAD events [71].

Consequently, due to the divergent reports from the current literature, the effects of GPx activity on CAD outcomes in the acute setting have been poorly evaluated. On study has reported contrasting results to the above studies, where high levels of plasma-borne GPx activity was associated with worse clinical outcomes in 137 ACS patients [70]. During an acute event there is an increase in ROS production and antioxidants have ability to change rapidly. The magnitude and the time course of these changes can be affected by the severity of the acute event, by therapeutic interventions, and by the

development of complications. Even in the studies that measured GPx activity in stable CAD, activity may be affected by the use of some medications (e.g. statin use) or lifestyle (e.g. smoking). High-quality prospective studies evaluating the association between GPx activity and CAD endpoints in an acute population are warranted.

1.8   Overall aims

This thesis explores the variability of the plasma levels of GPx activity and its prognostic value in determining clinical risk in an ACS population. Investigation of the relationship between GPx activity and levels of platelet reactivity and ROS, both factors implicated in ACS, are studied in an ACS population. How levels of the GPx system are affected in different stages of CAD are examined by comparison of ACS patients to stable coronary disease patients and healthy subjects. Corresponding changes in oxidative stress levels are also examined. Finally, the temporal changes of GPx activity are studied during the acute phase of a STEMI, along with corresponding changes in oxidative stress levels. This allows for the determination of the dynamic nature of GPx activity present during an ACS event. The results from this thesis will help determine the suitability of GPx as a potential biomarker in an ACS population.

Therefore the aims of the thesis are:
- To examine the variance that exists in GPx activity in an ACS population
- To determine whether GPx activity is predictive of major adverse cardiovascular outcomes in an ACS population
- To examine the relationship between GPx activity and levels of platelet reactivity and ROS in an ACS population
- To determine how levels of GPx activity, protein concentration and mRNA expression changes in ACS patients compared to stable coronary disease patients and healthy subjects
- To determine how levels of oxLDL change in ACS patients compared to stable coronary disease patients and healthy subjects
- To explore the temporal changes in GPx activity and oxLDL over the acute phase of a STEMI

Chapter 2 -   Glutathione peroxidase and major adverse cardiovascular events in acute coronary syndromes

2.1 Introduction

ROS are maintained at physiological levels by antioxidant enzymes such as GPx and SOD, to keep a balanced redox state. Experimental studies have demonstrated that deficiencies in plasma-borne GPx activity have resulted in abnormal vascular and cardiac structure and function [69], promoting a pro-thrombotic state in mice [70]. Additionally overexpression of extracellular SOD has been shown to improve endothelial dysfunction in mice [164] and reduce myocardial infarct size in rabbits [165].

Although previous studies have attempted to determine the relationship between levels of antioxidant activity and clinical cardiovascular complications, no consensus has yet been reached. This is due to studies publishing contradictory results, and the fact that divergent reports exist depending on whether chronic [71] or acute coronary populations [166] are examined. This makes it difficult to compare findings, and therefore it has not been determined whether GPx and SOD activity can act as a biomarker to predict clinical risk in CAD.

The pro-thrombotic and pro-inflammatory state of ACS patients causes them to be at an increased risk of major adverse cardiovascular events (MACE). One study has suggested that GPx activity is a surrogate marker for the level of oxidative stress in ACS patients, with higher levels associated with a higher rate of MACE [166]. However, this finding is at odds with a recent meta-analysis demonstrating that the majority of clinical studies find an inverse relationship between antioxidant activity and CAD outcomes [125]. To address this discrepancy in the literature this study aimed to examine the variance in GPx and SOD activity in a large ACS population. Combined with the experimental studies that suggest GPx and SOD activity are protective against oxidant-mediated damage, and the meta-analysis results showing an inverse association with CAD outcomes, we hypothesise that low levels of GPx and SOD activity will be associated with higher rate of MACE in an ACS population, therefore identifying a subset of patients at increased risk of adverse clinical events occurring.

Thus the aims of this study were:

- To examine the variance in GPx and SOD activity in an ACS population

- To determine whether this variance in activity levels is associated with the rate of MACE in an ACS population.

2.2 Methods

2.2.1 Study population

A group of 262 patients presenting to Wellington Regional Hospital between January 2012 and October 2012 diagnosed with an ACS and undergoing an invasive treatment (coronary angiography ± PCI) were eligible for inclusion in the study. ACS was defined by a cardiologist where symptoms suggestive of myocardial ischemia lasting longer than 10 min with either troponin elevation or ≥1 mm of new ST-segment deviation or T wave inversion on an ECG in at least 2 contiguous leads was observed, consistent with the current universal definition of ACS [24]. Patients were adequately pre-treated with dual anti-platelet therapy defined as chronic therapy with aspirin (≥75 mg/day) and clopidogrel (≥75 mg/day) and/or loading with aspirin ≥300 mg and clopidogrel ≥300 mg at least 6 hrs prior to enrolment. Exclusion criteria included a platelet count of less than 100×10^9/L, a known platelet function disorder, administration of a thrombolytic agent within 24 hr of enrolment or administration of a glycoprotein IIb/IIIa receptor antagonist within a week prior to enrolment. This study was reviewed and approved by the Lower South Ethics Committee (LRS/11/09/035) (Appendix 1). Participation was voluntary, and each patient gave informed written consent at the time of recruitment.

2.2.2 Data collection

Patient demographics, clinical characteristics, medications and clinical management were collected prospectively from review of the medical records and cardiac catheterisation database. Cardiology registrars collected procedural variables and in-hospital outcomes. Cardiology research nurses collected follow-up data with telephone calls at 30 days and 1 year. Where necessary, a cardiologist performed a review of case notes and the appropriate general practitioner contacted to further classify clinical outcomes.

2.2.3   End points and definitions

The primary endpoint was a composite of major adverse cardiovascular events (MACE) including death, nonfatal MI, nonfatal ischaemic stroke, stent thrombosis and new heart failure presentation.

2.2.3.1   Major adverse cardiac event (MACE)

Death encompassed all-cause mortality including cardiovascular death. Acute MI was defined using the third universal definition of myocardial infarction [24]. This included the detection of a significant high sensitivity troponin (hs-TnT) rise and the presence of symptoms suggestive of myocardial ischaemia, new or presumed new ST-segment-T wave changes or new left bundle branch block, development of new pathological Q waves, or imaging showing new loss of myocardial tissue. Evaluation of definite stent thrombosis was performed according to the Academic Research Consortium criteria [167]. Acute ischaemic cerebrovascular accident (CVA) was defined as signs of CVA confirmed with imaging studies.

2.2.3.2   GRACE score

The Global Registry of Acute Coronary Events (GRACE) is a prospectively studied scoring system to risk stratify patients with diagnosed ACS to estimate their in-hospital and 6-month to 3-year mortality [168]. The score is calculated using the following clinical factors; age, heart rate, systolic blood pressure, creatinine, heart failure class, ST-segment deviation, troponin level and cardiac arrest at index presentation to determine the mortality risk post-ACS discharge. All patients had a 6-month GRACE score calculated using Microsoft Excel software (Microsoft Corporation; Washington, USA).

2.2.4   Blood collection

Blood samples were collected from ACS patients within a mean time of 2-4 days post-presentation to hospital. Whole blood samples were collected into tubes anti-coagulated with sodium citrate (0.109M, BD Vacutainer; New Jersey, USA) from a peripheral vein using a 21-gauge needle performed myself before angiography or by the interventional cardiologist in the cardiac catheterisation laboratory from the arterial sheath immediately after insertion and prior to heparin administration. Citrated whole

blood was centrifuged (1500 g, 12 min, 4°C), to separate the plasma from the cellular components. Aliquots of plasma were stored at -80°C for subsequent analysis of antioxidant activity measurements.

2.2.5 Antioxidant activity measurement

2.2.5.1 Glutathione peroxidase activity assay

GPx activity kits (Enzo Lifesciences; New York, USA) were used as per manufacturer's instructions using a colourimetric-based assay. The experimental protocol was based on a coupled reaction of the GPx reaction with the reduction of oxidised glutathione by glutathione reductase using NADPH. The oxidation of NADPH to NADP+ accompanies a decrease in absorbance at 340 nm that is proportional to total GPx activity found in the plasma sample.

$$2GSH + 2H_2O_2 \xrightarrow{\boxed{GPx}} GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{\boxed{GR}} 2GSH + NADP^+$$
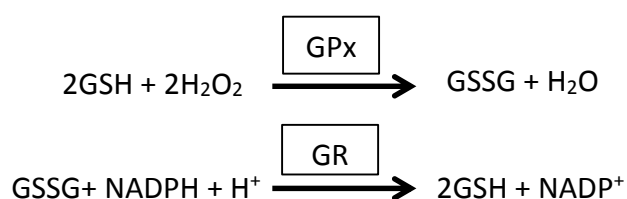
Briefly, plasma samples were aliquoted in triplicate into 96-well microtitre plates containing 1x Assay Buffer. GPx supplied in the kit served as a positive control and a set of wells containing 1x Assay Buffer in place of GPx or sample served as a negative background control. These wells were assayed in triplicate alongside the plasma samples. A 10x Reaction Mix containing glutathione reductase and GSH + NADPH was added to each well, followed by addition of Cumene Hydroperoxide to initiate the reaction. The rate of transformation of NADPH to NADP+ was measured by absorbance readings at 340 nm every minute for 10 mins in a microplate reader (VersaMax™, Molecular Devices; California, USA). GPx activity was defined as nanomoles of NADPH consumed per minute and expressed as units per mL of plasma. The intra-assay coefficient of variance was 7.3%, and the inter-assay coefficient of variance was 9.9%.

2.2.5.2 Superoxide dismutase activity assay

SOD activity was measured in a similar manner to GPx activity using a coupled reaction with xanthine oxidase, which generates the superoxide anion from oxygen. A superoxide radical reduces the dye, WST-1, to a coloured product that absorbs light at 450 nm. SOD scavenges superoxide anions, thereby reducing the rate of formation of the coloured dye product, WST-1-formazan. Reduction in the appearance of WST-1-formazan is proportional to the SOD activity present in the sample.

Xanthine $2O_2$ WST-1 Formazan

XOD

H_2O_2 + uric acid   WST-1

$2O_2^-$

SOD

$O_2 + H_2O_2$
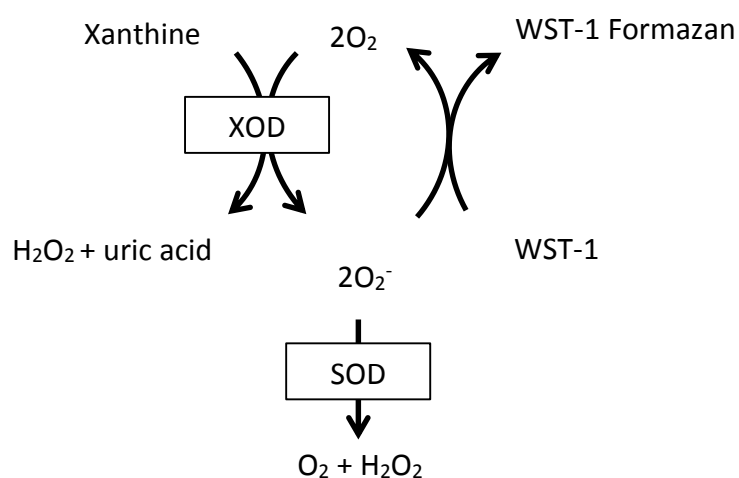
Briefly, plasma extracted from whole blood samples was aliquoted in triplicate into 96-well microtitre plates containing 1X SOD Buffer. A concentration range of SOD standards was prepared spanning from 10 U to 0.1 U per well, and these standards were run in triplicate alongside the plasma samples. A Master Mix containing WST and xanthine oxidase was added to each well, followed by the addition of Xanthine Solution to initiate the reaction. Absorbance was measured at 450 nm using a microtitre plate reader (VersaMax™), and subsequent data analysis reported units of SOD activity expressed as per mL of plasma. Intra-assay coefficient of variance was 8.6%, and inter-assay coefficient of variance was 10.2%.

2.2.6 Statistical Analysis

Continuous variables were reported as the mean ± standard deviation (SD), and categorical variables were reported as frequencies and percentages. Statistical tests to compare continuous variables with the rate of MACE were carried out using a Student's unpaired *t*-test and categorical variables were analysed using chi-square tests. Relationships between continuous parameters were determined by the Pearson's correlation coefficient. A receiver operator curve was used to examine the relationship between enzyme activity levels and MACE. GPx activity was divided into quartiles in order to examine the rate of MACE using a linear-by-linear association test. Differences in values corresponding to $p<0.05$ were taken as statistically significant. All statistical analyses were carried out in either GraphPad Prism Software v.06 (GraphPad Software Inc; California, USA) or SPSS v.22 (IBM; Armonk, NY).

2.3 Results

2.3.1 Baseline characteristics

The demographic data and clinical characteristics of the 262 ACS patients are summarised in Table 2-1. The study population was 69% male, had a mean age of 63 years and a mean BMI of 29.5. The clinical presentation was STEMI in 23%, NSTEMI in 71% and UA in 7%. The mean Grace Score on admission was 100. The clinical management of the study group was as follows: PCI in 50%, coronary artery bypass grafting (CABG) in 14% with the remaining 34% being medically managed.

2.3.2 Patient outcome

At 1 year follow up, 34 (13%) patients experienced MACE. This included 10 deaths (3.8%) all from cardiovascular causes (1 CVA, 9 from cardiac causes). Nonfatal MI occurred in 11 patients (4.2%), and ischemic CVA in 4 patients (1.5%). The rate of stent thrombosis was relatively low, occurring in 2 patients (0.8%) during the follow-up period. A further 7 patients (2.7%) were admitted with acute heart failure presentations.

Comparing the patients with MACE to the patients without MACE (Table 2-1), MACE patients were older, more likely to have a history of hypertension, dyslipidaemia, diabetes and renal dysfunction, and have higher GRACE Scores.

Table 2-1 Baseline characteristics of the ACS study population.

Characteristic	ACS patients (n=262)	MACE Group (n=34)	No MACE Group (n=228)	p value
Male	180 (69)	26 (76)	154 (68)	0.29[a]
Age (years)	63 ± 10	66 ± 11	62 ± 10	0.02[b]
Body Mass Index	29.5 ± 5.8	29.9 ± 7.2	29.5 ± 5.5	0.64[b]
Risk Factors				
Hypertension	175 (67)	31 (91)	144 (63)	0.001[a]
Dyslipidaemia	182 (70)	31 (94)	151 (66)	0.003[a]
Diabetes	66 (25)	18 (52)	48 (21)	0.001[a]
Current Smoker	54 (21)	8 (25)	46 (20)	0.65[a]
Renal Dysfunction	17 (7)	6 (17)	11 (5)	0.005[a]
Clinical Presentation				
STEMI	59 (23)	4 (12)	55 (24)	
NSTEMI	185 (71)	28 (82)	157 (69)	0.24[a]
Unstable Angina	18 (7)	2 (6)	16 (7)	
Grace Score	100 (24)	111 (27)	99 (24)	0.006[b]
Clinical Management				
Medical Management	95 (36)	15 (44)	83 (36)	
PCI	131 (50)	12 (35)	119 (52)	0.12[a]
CABG	35 (14)	7 (21)	26 (11)	

[a] chi-square test [b] Student's unpaired t-test,. Continuous variables are expressed as mean ± SD, categorical variables are expressed as frequencies and (percentages).

2.3.3 Antioxidant activity

GPx activity in the ACS population ranged from 30.4 to 180 U/mL, with a mean ± SD of 123 ± 32 U/mL. MACE patients were found to have significantly lower GPx activity compared to patients who did not experience MACE (p<0.05) (Table 2-2). GPx activity was found to be significantly lower in diabetic patients (115 ± 32 U/mL) compared to non-diabetic patients (126 ± 32 U/mL) (p<0.05), and significantly lower in males (120 ± 33.5 U/mL) compared to females (129 ± 29 U/mL) (p<0.05). Remaining cardiovascular risk factors did not appear to influence levels of GPx activity.

SOD activity in the ACS population ranged from 8.2 to 725 U/mL, with a mean ± SD of 67.3 ± 82.5 U/mL. There were no significant differences detected between patients who experienced MACE, compared to those who did not (Table 2-2). No correlation between GPx and SOD activity was detected.

Table 2-2 GPx and SOD activity in ACS patients.

Antioxidant Enzymes	ACS patients (n=262)	MACE Group (n=34)	No MACE Group (n=228)	p value
GPx (mean ± SD)	123 ± 32.4	112 ± 33.5	125 ± 32.0	0.03
SOD (mean ± SD)	67.3 ± 82.5	72.1 ± 80.6	67.0 ± 83.0	0.80

Student's unpaired *t*-test was used to detect differences in GPx and SOD activity in patients who had MACE, compared to those that did not.

2.3.4 Predictive value of GPx activity for MACE

To assess the predictive value of GPx activity at identifying patients at increased risk of MACE, a receiver operator curve (ROC) analysis was conducted. GPx activity was found to be a moderate predictor of MACE with an area under the curve of 0.62 (0.57-0.67, SEM) that was significantly different from 0.5 (p=0.02) (Figure 2-1). However, the ROC curve demonstrated the lack of a single cut-point that would indicate an optimal level of GPx activity that was predictive of MACE risk.
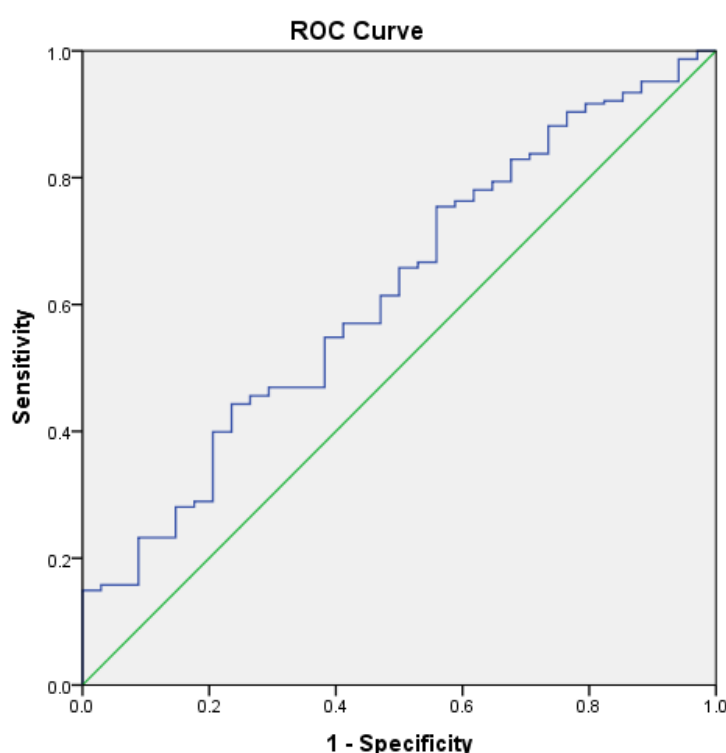
Figure 2-1 ROC curve analysis of GPx activity and MACE.

The ROC curve is a graph of sensitivity (y-axis) vs. 1-specficity (x-axis). Although the area under the curve was significantly different (0.62, p<0.05), a single cut-off value corresponding to maximum sensitivity with a high sensitivity could not be determined for GPx activity and prediction of MACE.

When the MACE rate was examined by quartiles of GPx activity, a significant decrease of MACE was demonstrated across the four quartiles (p<0.05). Event rate for patients in the lowest quartile of GPx activity (19.6%) was approximately 2.5 times higher than that for the patients in the upper quartile of GPx activity (7.9%) (Figure 2-2).
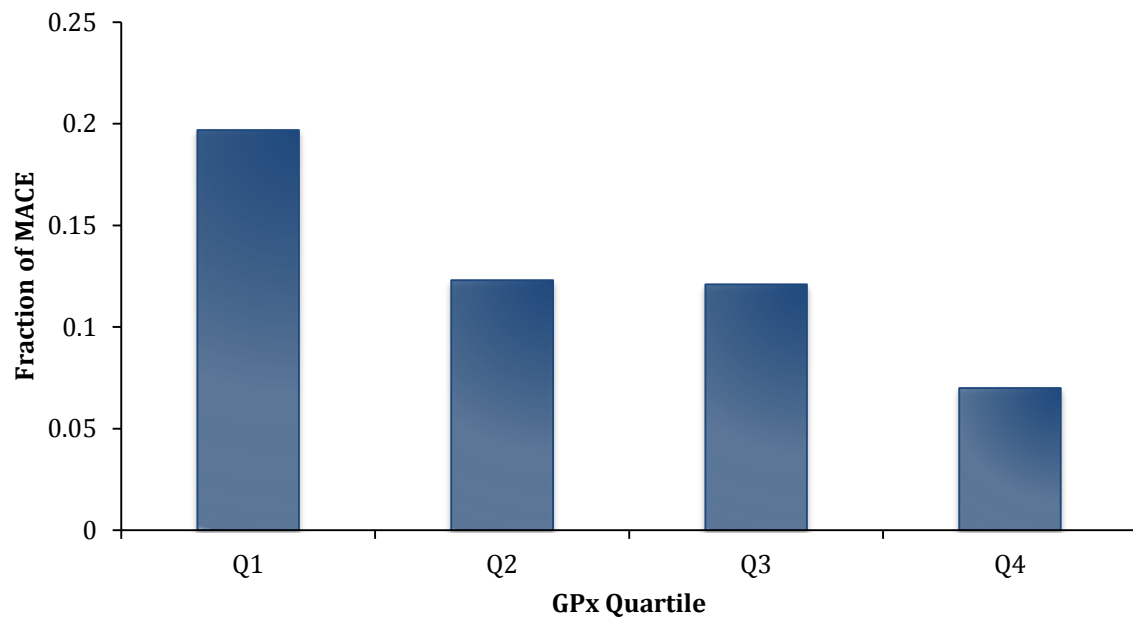
Figure 2-2 MACE rate by GPx quartile.

The fraction of MACE rate as observed by GPx activity quartile. MACE rate significantly decreased across the GPx quartiles, with the highest fraction of MACE occurring in the lowest quartile of GPx activity. The lowest MACE fraction occurred in the upper quartile of GPx activity. Data plotted from all 262 ACS patients, p<0.05 Linear-by Linear association.

2.4   Discussion

To date, this is the largest prospective cohort study we are aware of that examines the relationship between GPx and SOD activity levels and the occurrence of MACE in patients with ACS. We found significantly lower levels of GPx activity in patients who experienced MACE compared to those who did not. Our ROC curve analysis demonstrated a modest, but significant relationship between GPx activity and MACE, although a clear cut-point in which GPx activity predicts risk could not be defined. The MACE rate in patients found in the lower quartile of GPx activity was 19.6% compared to 7.9% found in patients in the upper quartile. We did observe significantly lower levels of GPx activity in both diabetic patients and in male patients. It is possible that these factors may contribute to the inverse relationship observed between GPx activity and MACE, however this study was underpowered to examine these factors in a multivariate model. No significant relationship was observed between SOD activity and MACE rate in this ACS population.

The GPx enzyme is well established as an antioxidant defence mechanism against oxidative stress. Its importance as a potential anti-atherogenic enzyme has been highlighted in the growing body of literature, with both experimental and translational studies demonstrating supportive evidence for its ability to protect against oxidative stress damage in a cardiovascular setting. In animal models, reduced GPx expression has been shown to increase cell-mediated oxidation of LDL [78], with heterozygous mutations of the GPx1 gene promoting endothelial dysfunction and structural and functional anomalies of the heart [69], effects presumed to be caused by increased oxidative stress. Supportive evidence from translational studies includes the finding that GPx activity is decreased or absent in carotid artery lesions, with the lack of GPx activity associated with more severe lesion progression [161]. Further examples of glutathione pathways being important in preventing vascular injury include a study by Voetsch et al [149] who reports polymorphisms that are present within the promoter region of plasma-borne GPx resulting in decreased activity, and that impaired GPx defence is associated with increased risk of premature ischemic strokes [72].

From a clinical perspective, it is therefore postulated that a defence system such as GPx will play a protective role in CAD, potentially lending itself as a prognostic marker for risk prediction in CAD patients. However, as highlighted by a recent meta-analysis [125], the relationship between GPx activity and CAD is not well-understood, with divergent study results demonstrating this relationship is not to be as straightforward as initially hypothesised. Overall, the meta-analysis carried out by Flores-Mateo et al. concluded that lower activity levels of antioxidant enzymes such as GPx and SOD were associated with increased CAD risk. However the limitations arising from the study highlight the fact that this message is not consistent throughout the individual studies and that the association is difficult to interpret in mechanistic or etiologic terms. The majority of studies examined were small case-control populations consisting of both chronic and acute coronary disease patients.

Although our observation that lower levels of GPx activity are associated with a higher rate of MACE is consistent with reports from chronic coronary population studies, it is in direct contrast to the study by Garcia-Pinilla et al. [166]. In this study carried out in 137 ACS patients, Garcia-Pinilla et al. reported that the 2-year MACE rate was significantly higher in patients whose GPx activity was above the 50th percentile, compared with those below. There are a number of potentially important differences between our two studies that merit discussion. Our study collected blood samples prior to angiography, whereas Garcia-Pinilla et al. collected blood following angiography. It is likely that GPx activity levels are highly dynamic during an ACS presentation, but the nature of that change and whether factors arising from the angiography procedure e.g. revascularisation, affect activity levels are not well-understood. Our study population was similar in age and gender ratio to Garcia-Pinilla et al., but we report a substantially lower rate of diabetes (25% vs 37%) and proportion of smokers (21% vs 64%). While diabetes was related to the occurrence of MACE in our study, and smoking was related to MACE in the Garcia-Pinilla et al. study, the literature reports divergent results on how either is related to GPx activity [169, 170]. Although diabetes was associated with GPx activity and with the occurrence of MACE in our study, we were not powered to examine this relationship in a multivariate model. Therefore it is possible that the presence of diabetes contributes to the inverse relationship between GPx activity and MACE.

Although our cohort is substantially larger, our follow-up period is 1 year rather than 2, therefore our event rate is significantly lower.

A common limitation that both studies have is the fact that no measurement was made that could be directly related to a baseline oxidative stress value. Because of this, it is difficult to exactly determine if high levels of GPx activity is a response to a greater oxidative stress load experienced by patients, and therefore an indirect measure of an adverse risk profile as suggested by Garcia-Pinilla et al. Or, the alternative is that high levels of GPx activity reflect a greater ability to defend against oxidative stress, and therefore is a protective marker associated with better clinical outcomes, as suggested by our study.

As an antioxidant enzyme, the assumption is that it is a protective molecule which can prevent or alleviate oxidant-induced damage, therefore could have prognostic value. It is plausible to suggest that patients with lower levels of GPx activity have a reduced defence against ROS, making them susceptible to oxidative-stress mediated damage in the vasculature after an ACS event. To explore this hypothesis further it is first central to understand how both the oxidative and antioxidant systems interact with each other, and how they change over the different stages of CAD. If GPx activity was to be utilised as a risk marker it is essential to understand how dynamic the activity is over the course of an ACS event. It is not ideal to have a biomarker that is highly dependent on sampling time. Our results are assessed from blood collection taken at a mean time of 2-4 days post-presentation to hospital. The variability in the timing of assessment of GPx activity in respect to the onset of an ACS has added an unknown quantity of within-study variability to our results. The magnitude and the time course of antioxidant enzyme changes can be affected by the severity of the acute event [171] and subsequent therapeutic intervention such as reperfusion [172]. Closer examination of the dynamic change in GPx activity during an ACS event will be crucial to understand how it is affected by sampling time and interventional strategies such as revascularisation.

Vitamin supplements of dietary antioxidants have been largely ineffective in preventing the complications of atherosclerotic disease [121]. There are various speculative explanations for these findings, but the most crucial is that we still do not fully

understand what drives the variance in the antioxidant system and how this translate to clinical practice. The present study demonstrates that there is a large variance in GPx activity following an ACS event. Whether this variance exists throughout the progression of CAD, or whether it is induced by an ACS event is largely unknown. Understanding what drives this variance, and how it relates to measures of oxidative stress will be crucial to understanding how therapeutic strategies can be designed. Currently we demonstrate that ACS patients with low levels of GPx activity are at an increased risk of MACE. The transfer of this knowledge to a clinical setting potentially means that GPx activity could identify a population that would benefit from targeted antioxidant therapy. It is possible that the large clinical trials with antioxidant therapies have been too broad in terms of population heterogeneity. In an acute population at least, subjects with high GPx activity might profit less from antioxidant supplementation than patients with lower concentrations of this enzyme. What the antioxidant supplemental strategy should be in this population remains to be determined.

SOD activity is another antioxidant defence system thought to have a protective effect in a cardiovascular setting. The meta-analysis published by Flores-Mateo et al. [125] reported that the majority of studies found an inverse association between SOD activity and CAD risk, however not all studies were statistically significant [71]. Our study did not observe a significant relationship between SOD activity and rate of MACE. However we did observe a considerable variation in levels of SOD activity between the ACS patients, and it is possible that our study was underpowered to detect differences in this population due to this large variance. Additionally SOD has been shown to be most active in the vessel wall and sampling the circulating SOD may not be the best representative of its antioxidant abilities [71, 173]. It is feasible that the protective effect of GPx activity on lipid peroxidation arises from the detoxification of H_2O_2 produced by SOD enzyme as well as converting lipid hydroperoxides to non-toxic alcohols, therefore making it the primary plasma enzyme in protecting against increased free radical production in ACS.

A common limitation in both SOD and GPx activity assays is that they are not designed to differentiate between the isoforms for each enzyme found in the plasma. Although GPx3 and SOD3 are reported to be the predominant isoforms in the plasma, there is an

inability to determine if other isoforms have been secreted from cellular locations and are contributing to total activity. This study was designed to capture the global activity present in the circulating plasma, however we cannot determine what proportion each isoform is contributing to the total activity measured in our plasma samples.

2.5 Conclusion

This study demonstrates a significant inverse relationship between GPx activity and rate of MACE in ACS patients. These findings suggest that patients with lower levels of GPx activity are at increased risk of developing adverse clinical events within 1-yr following an ACS event, possibly due to a decreased defence against oxidant-mediated damage. What factors may influence this variability in GPx activity in an ACS population will be further examined in this thesis.

Chapter 3 -   Relationship between glutathione peroxidase and platelet reactivity in acute coronary syndromes

3.1 Introduction

In the previous study we observed that low levels of plasma GPx activity was associated with increased rate of MACE. There is mechanistic evidence that indicates deficiencies in GPx activity is related to enhanced platelet function and an enhanced ROS flux [70]. Excessive platelet reactivity is correlated with worse clinical outcomes in an ACS population [174], and high levels of ROS are produced during myocardial ischaemia [41]. ROS are known to rapidly consume nitric oxide (NO), a vasodilator released from the endothelium and an important inhibitor of platelet activation [175]. Therefore we hypothesise that low levels of GPx activity will be associated with high platelet reactivity, and a high level of ROS in an ACS population. We suggest that high platelet reactivity and ROS levels due to low GPx activity may account for the increased MACE we see in the previous chapter.

Experimental studies have demonstrated GPx activity potentiates the inhibition of platelet activation by NO in healthy volunteers [176]. A case study examining familial arterial thrombosis found decreased levels of plasma GPx activity, an increased level of H_2O_2, and an attenuation of platelet inhibitory response to NO [72]. The authors deduce that it is the interaction with ROS that reduces the bioavailability of NO leading to a thrombotic state. Addition of exogenous GPx led to a restoration of platelet inhibition by NO. Further experimental studies have demonstrated an association between deficiencies in plasma GPx activity (GPx3) and enhanced platelet reactivity [70]. Impaired endothelial function, an enhanced ROS flux and larger stroke size greater stroke infarct volumes in the GPx3 knock-out mice were also observed. The authors concluded that a GPx deficiency promotes platelet-dependent thrombosis due to an impaired ROS metabolism and an impaired platelet inhibition mechanism.

In order to test our hypothesis we utilised the Multiplate system, a measure of platelet function, to quantify platelet reactivity. To examine whether GPx activity is associated with high levels of ROS we adapted the methodology described from Freedman et al. [72, 176]. This allowed us to develop a test that measured both platelet reactivity and gave an indirect measure of ROS in a single test.

The principle of this method is based on the consumption of NO by ROS. By measuring platelet function in whole blood samples with and without the addition of exogenous NO, it was possible to quantify the level of NO-mediated platelet inhibition. Based on the principle that ROS rapidly inactivates NO, high levels of ROS would be associated with a rapid consumption of NO, and a lower level of NO-mediated platelet inhibition. In contrast, where levels of ROS are low, NO would be able to have a greater effect on inhibiting platelet function. In this way, the percentage (%) of inhibition of platelet aggregation by NO acts as an inverse marker for ROS.

Therefore this study aimed to:

- To examine the relationship of GPx activity and platelet reactivity in ACS patients
- To develop a methodology that allows an indirect measure of ROS based on NO-mediated platelet inhibition
- To examine the relationship between ROS levels, GPx activity and platelet reactivity in ACS patients

3.2 Methods

3.2.1 Study population

From the recruited cohort described in Section 2.2.1, a subset of 51 consecutive ACS patients was further examined for GPx activity, platelet reactivity and ROS measurements. This study was conducted under the same ethical approval as Chapter 2 (LRS/11/05/035) (Appendix 1).

3.2.2 Data collection

Patient demographics, clinical characteristics and clinical management were previously collected as described in Section 2.2.2.

3.2.3 Blood collection

Whole blood samples were collected into tubes anticoagulated with hirudin (25 µg/ml, Dynabyte; Munich, Germany) or sodium citrate (0.109 M, BD Vacutainer) from a peripheral vein using a 21 gauge needle performed myself before angiography, or by an interventional cardiologist in the cardiac catheterisation laboratory from the arterial sheath immediately after catheter insertion and prior to administration of heparin. Plasma was extracted from citrated whole blood by centrifugation at 1500 g for 12 min at 4˚C, and stored at -80˚C for GPx activity measurements.

3.2.4 Preparation of *S*-nitrosothiol

The endothelium-derived relaxing factor (EDRF) congener, a naturally occurring thiol adduct of NO, *S*-nitrosoglutathione (SNO-Glu) was prepared by reacting equimolar concentrations of freshly prepared solutions of glutathione with sodium nitrite ($NaNO_2$) at an acidic pH, as previously described [177]. Due to the unstable nature of the *S*-nitrosothiol, SNO-Glu was prepared within 10 min of use, kept at 4˚C and diluted as necessary into aqueous buffer immediately before addition to the Multiplate analyser assay system.

3.2.5   Platelet function testing

Platelet reactivity was assessed using whole blood multiple electrode impedance platelet aggregometry with the Multiplate analyser (Dynabyte; Munich, Germany) as previously described by our laboratory [178]. An illustration of the Multiplate is depicted in Figure 3.1.

Figure 3-1   Multiplate analyser system.

Image recreated from Multiplate educational material
http://www.cobas.com/home/product/hemostasis-testing/multiplate-analyzer.html

Briefly, blood samples were tested within 30 min of collection. Hirudin anticoagulated whole blood was diluted 1:1 with 0.9% NaCl solution to give a final volume of 600 μL. Each test cuvette possessed a Teflon-coated magnetic stirring bar (Figure 3-2A) and samples were stirred for 3 min at 37˚C, after which 20 μL of 0.2 mM ADP (adenosine diphosphate, Dynabyte) was added to give a final concentration of 6.5 μM. Addition of ADP stimulates platelets to aggregate to the paired electrodes, impeding the current between them (Figure 3-2B&C).
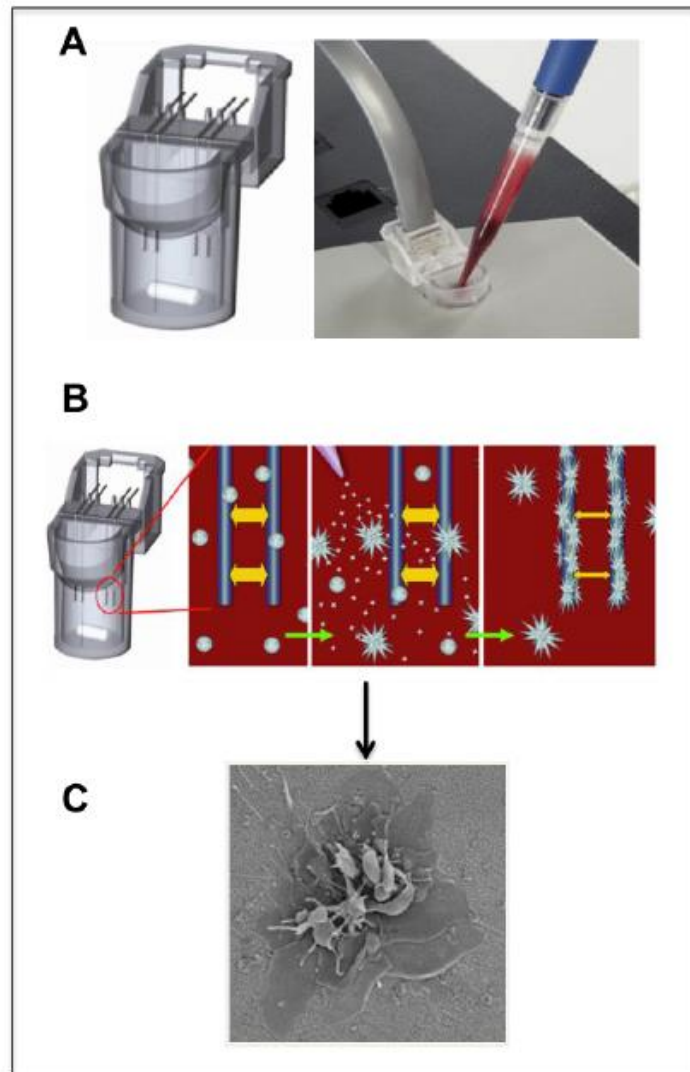
Figure 3-2 Multiplate electrode impedance platelet aggregometry

Saline and whole blood were added to a test cuvette using an automated pipette (A). Paired electrodes are present in each test cuvette and with addition of ADP, a platelet agonist, platelets are activated and aggregates are formed on electrodes, which impedes the current (B). A scanning electron microscopy image of platelet aggregates on the electrode surface (C). The images are recreated from the Multiplate educational material, sourced from http://www.multiplate.net/en/detection.php.

Platelet aggregation was then continually recorded for 6 min. The increase of impedance due to the attachment of platelets to the paired electrodes was detected for each sensor unit separately and transformed to arbitrary units (AU) that are plotted against time. Aggregation values are quantified as area under the aggregation curve expressed as aggregation units x minutes (AU·min).

For the experiments to assess the effect of SNO-Glu on platelet aggregation and give a ROS measurement, two test cuvettes were run simultaneously on the Multiplate analyser. One test cuvette contained the diluted whole blood and was incubated with just ADP (6.5 μM) to give a platelet reactivity measure. The second cuvette was incubated with the SNO-Glu compound (6.5 μM) for 1 min before platelet aggregation was stimulated with ADP (6.5 μM). A representative example from an individual patient showing platelet impedance aggregometry with and without the addition of SNO-Glu is shown in Figure 3-3. The difference in the areas under the curve from the two tests is expressed as % inhibition of platelet aggregation, and is the inverse representation of the amount of ROS in the blood sample.
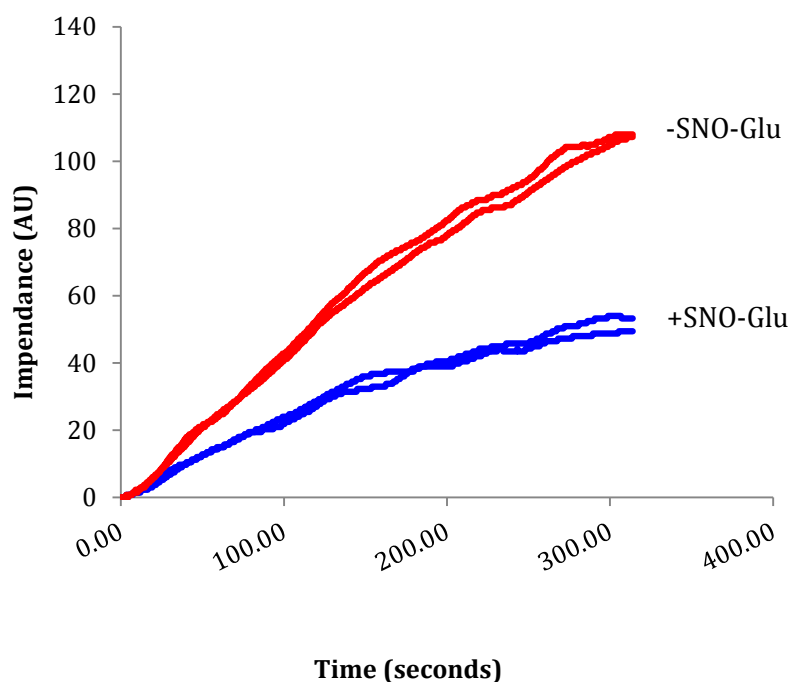
Figure 3-3 Platelet impedance aggregometry with and without SNO-Glu.

An individual representation of platelet impedance aggregometry in two test cuvettes containing paired electrodes from an ACS patient. Whole blood from a patient was measured for platelet reactivity (AU) after ADP stimulation (6.5 µM) using the Multiplate analyser. The red line represents platelet reactivity from one cuvette after ADP alone, and the blue line represents platelet reactivity in the second cuvette after incubation with SNO-Glu (6.5 µM) for 1 min followed by ADP stimulation. The difference between the areas under the curve for the two cuvettes (without SNO-Glu - with SNO-Glu) was calculated and expressed as % inhibition of platelet aggregation.

3.2.6 Pilot study – Platelet aggregation: Effects of SNO-Glu & GPx activity

To ensure the % change in platelet inhibition we observe by SNO-Glu is through the action of NO, and therefore can be a representative of ROS levels, we replicated a further methodology proposed by Freedman et al. GPx activity has been shown to potentiate the SNO-Glu mediated platelet inhibition of platelet aggregation [176]. The concept is that addition of GPx will help scavenge ROS, preventing it from activating the platelet or from destroying NO. Either way, it will preserve the bioavailability of SNO-Glu therefore we will observe a greater inhibition of platelet aggregation. Freedman et al. have demonstrated this concept; therefore

we sought to carry out a pilot study to measure platelet reactivity in 6 healthy individuals where whole blood was incubated with ADP and SNO-Glu in addition to the GPx enzyme.

As described above, whole blood was incubated with and without SNO-Glu, followed by ADP stimulation in two separate test cuvettes, with subsequent platelet aggregation recorded for 6 min. In a third cuvette GPx (1 U/mL, Enzo Lifesciences) was incubated with stirred whole blood for 30 s before stimulation with ADP and subsequent recording of platelet aggregation. In a fourth cuvette, GPx and SNO-Glu was sequentially added, followed by ADP stimulation and subsequent recording of platelet aggregation. A control test cuvette was run in parallel where only phosphate buffered saline (1x PBS) was added, followed by ADP-stimulated platelet aggregation. This was to ensure the differences in platelet aggregation were due to the different treatments, and not confounding factors. Results were expressed as platelet reactivity aggregation units (AU).

3.2.7   Glutathione peroxidase activity assay

Plasma extracted from sodium citrated whole blood was assayed for GPx activity as previously described in Section 2.2.5.1. GPx activity was defined as nanomoles of NADPH consumed per minute and expressed as units per mL of plasma. Intra-assay coefficient of variance was 7.3% and inter-assay coefficient of variance was 9.9%.

3.2.8   Statistical analysis

Continuous variables were reported as the mean ± SD and categorical variables as frequencies and percentages. Relationships between continuous parameters were analysed using the Pearson's correlation coefficient test. Statistical tests to compare means between continuous variables were carried out using a one-way ANOVA with Tukey's multiple comparison test. Differences corresponding to values of $p < 0.05$ were taken as statistically significant. All statistical analyses were carried out in either GraphPad Prism Software v.06 or SPSS v.22.

3.3 Results

3.3.1 Pilot study - Platelet aggregation: effects of SNO-Glu and GPx activity

Addition of SNO-Glu significantly inhibited platelet aggregation compared to ADP treatment alone ($p<0.05$). The addition of GPx potentiates the level of inhibition of platelet aggregation mediated by SNO-Glu compared to ADP alone ($p<0.0001$) and ADP + SNO-Glu ($p<0.05$) (Figure 3-4A). Addition of GPx alone was not found to be significantly different from ADP treatment alone ($p=0.48$), the small study population and level of variance may account for why differences in this treatment was not statistically significant. Figure 3-5B shows a representative example of platelet reactivity in response to each of the treatments from one study participant.
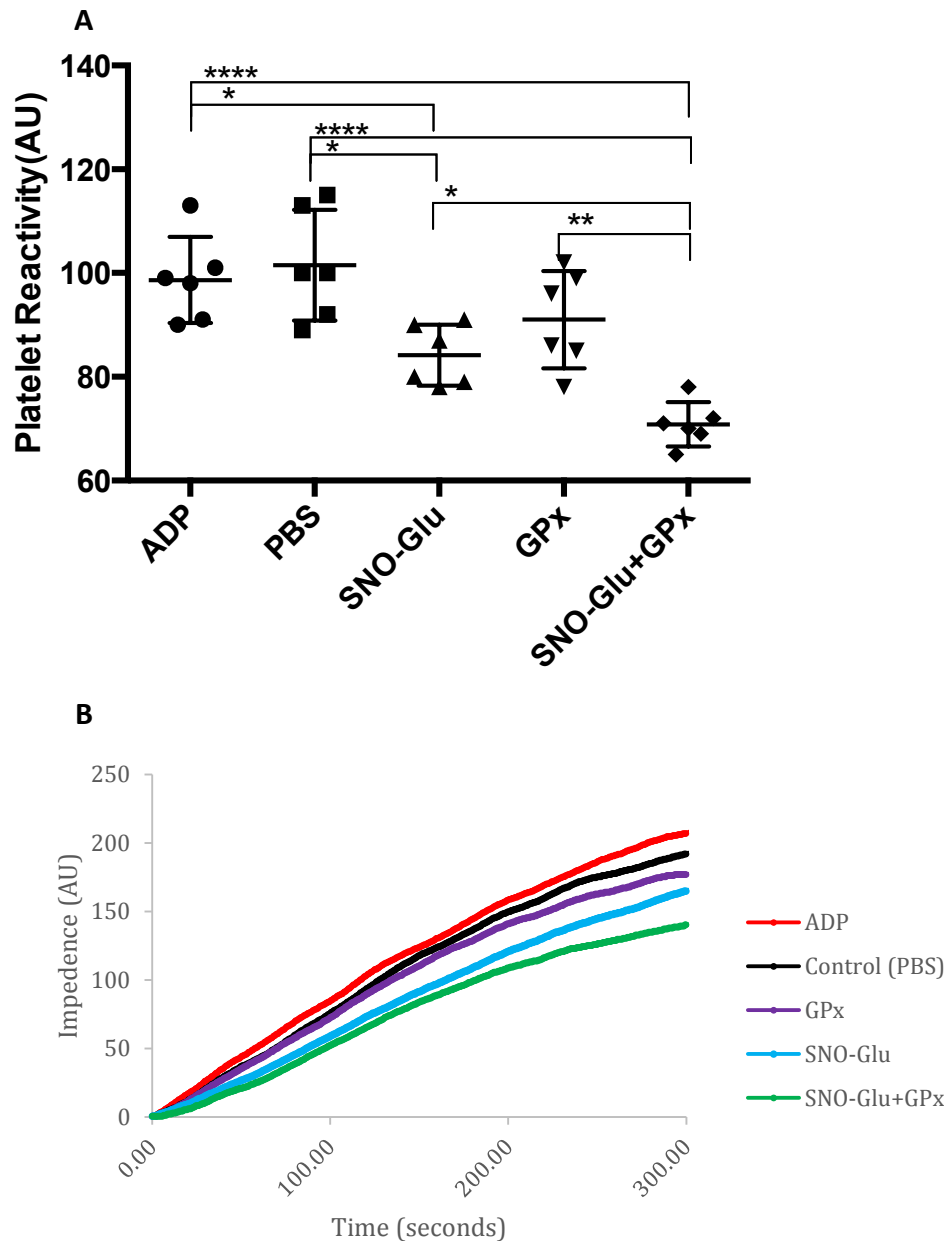
Figure 3-4 Effect of GPx activity on platelet aggregation.

(A) Addition of GPx (1U/mL) to stirred whole blood for 30 s, followed by addition of SNO-Glu (6.5 μM) for 1 min was carried out before platelet aggregation was induced by addition of ADP (6.5 uM). Platelet reactivity was measured for 6 min and plotted as AU. SNO-Glu and GPx were carried out as single treatments in separate cuvettes followed by ADP stimulation for comparison. Controls included 20 μL of 1x PBS followed by ADP stimulation and ADP stimulation alone in separate cuvettes. Data plotted is platelet reactivity (AU) from 6 healthy individuals as mean ± SD. *p<0.05, **p<0.01, ****p<0.0001, one-way ANOVA with Tukey's multiple comparisons test was used to detect differences. (B) A representative example of a healthy individual showing the difference in impedance platelet aggregometry (AU) when GPx was added to SNO-Glu + ADP (green) compared to SNO-Glu + ADP (blue), GPx + ADP (purple), PBS + ADP (black) and ADP alone (red).

3.3.2 ACS population – Platelet reactivity, GPx activity & ROS

3.3.2.1 Baseline characteristics

Baseline demographics and clinical characteristics of the study population are presented in Table 3-1. The study population was 68.6% male, had a mean age of 60.7 years and a mean BMI of 31.7. Of the 51 ACS patients, 25.5% presented with STEMI and 74.5% with NSTEMI. No patients presented with UA in this study population.

3.3.2.2 Glutathione peroxidase, platelet reactivity and ROS levels

GPx activity from the study population is shown in Figure 3-5A. The mean GPx activity was 108 ± 32 U/mL. Platelet reactivity from the study population is shown in Figure 3-5B. The mean platelet reactivity was 45 ± 21 AU. The % inhibition of platelet aggregation in response to SNO-Glu was calculated for each patient, and is shown in Figure 3-5C. The mean % inhibition was 39%. The inverse of these values corresponds to the arbitrary ROS levels, as a larger shift in % inhibition represents greater NO-mediated platelet inhibition, suggesting a lower level of ROS is present. ROS levels are shown in Figure 3-5D. The mean ROS level was 61 AU.

Table 3-1 Baseline characteristics of the ACS study population

Characteristic	ACS patients (n=51)
Male	35 (68.6)
Age (years)	60.7 ± 9.8
Body mass index	31.7 ± 6.0
Risk Factors	
Hypertension	30 (58.8)
Dyslipidaemia	36 (70.6)
Diabetes	11 (21.6)
Current Smoker	11 (21.6)
Clinical Presentation	
STEMI	13 (25.5)
NSTEMI	38 (74.5)

Continuous variables are expressed as mean ± SD , categorical variables as frequencies (percentages)
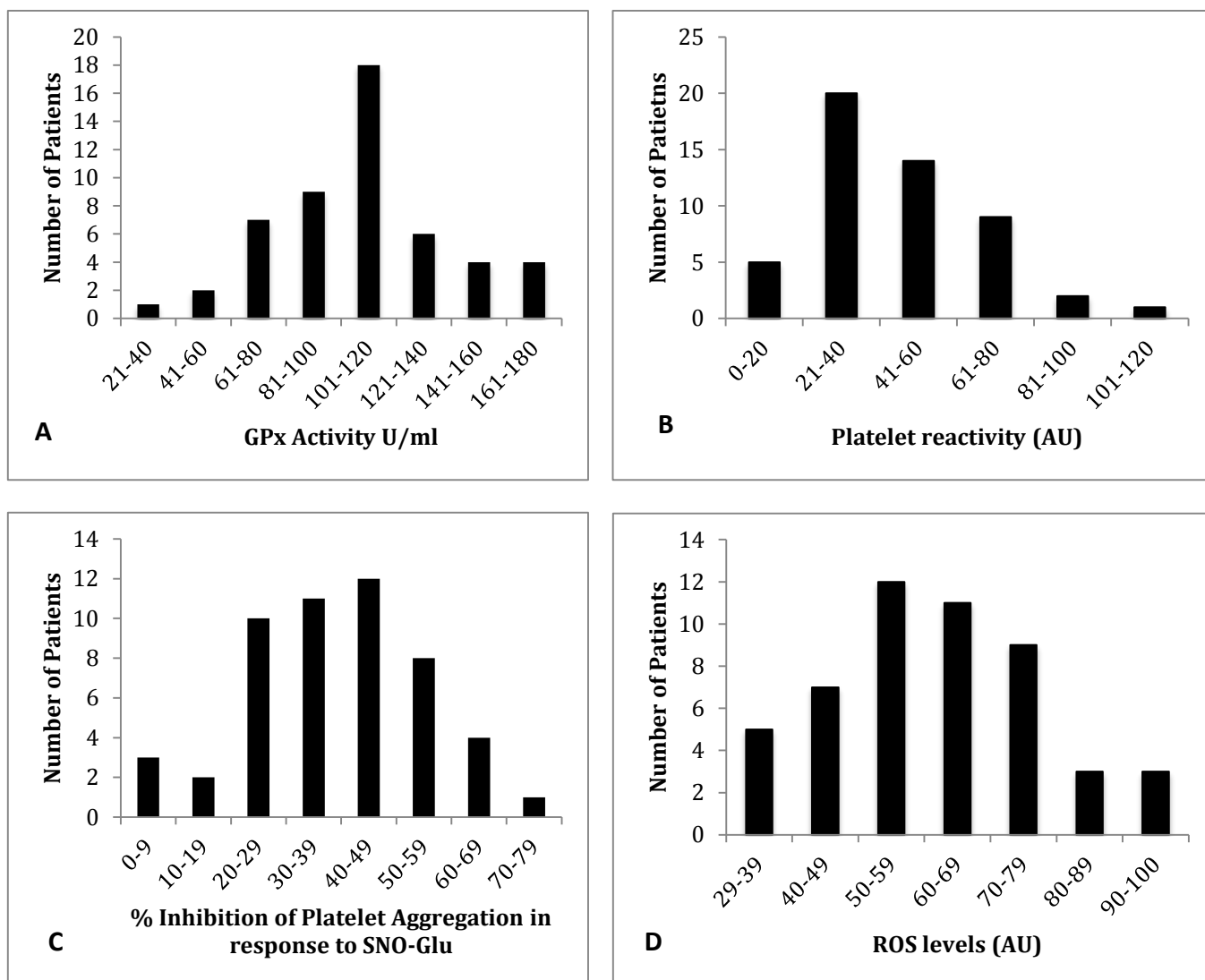
Figure 3-5 GPx activity, platelet reactivity, SNO-Glu and ROS levels.

In 51 ACS patients the following measures are displayed A) GPx activity had a mean of 108 ± 32 U/mL, range of 32-180 U/mL, B) platelet reactivity had a mean of 44 ± 21 AU, range of 11-108 AU C) % inhibition of platelet aggregation mediated by SNO-Glu had a mean of 39 ± 16 %, range of 0-70 %, with inverse of these values corresponding to D) ROS levels that had a mean of 61 ± 16 AU, range of 29-100 AU.

No significant correlation was observed between GPx activity and absolute levels of platelet reactivity (Figure 3-6A).   Again, a non-significant relationship was observed between % inhibition of platelet aggregation in response to SNO-Glu, and the absolute level of platelet reactivity (Figure 3-6B).

A

B
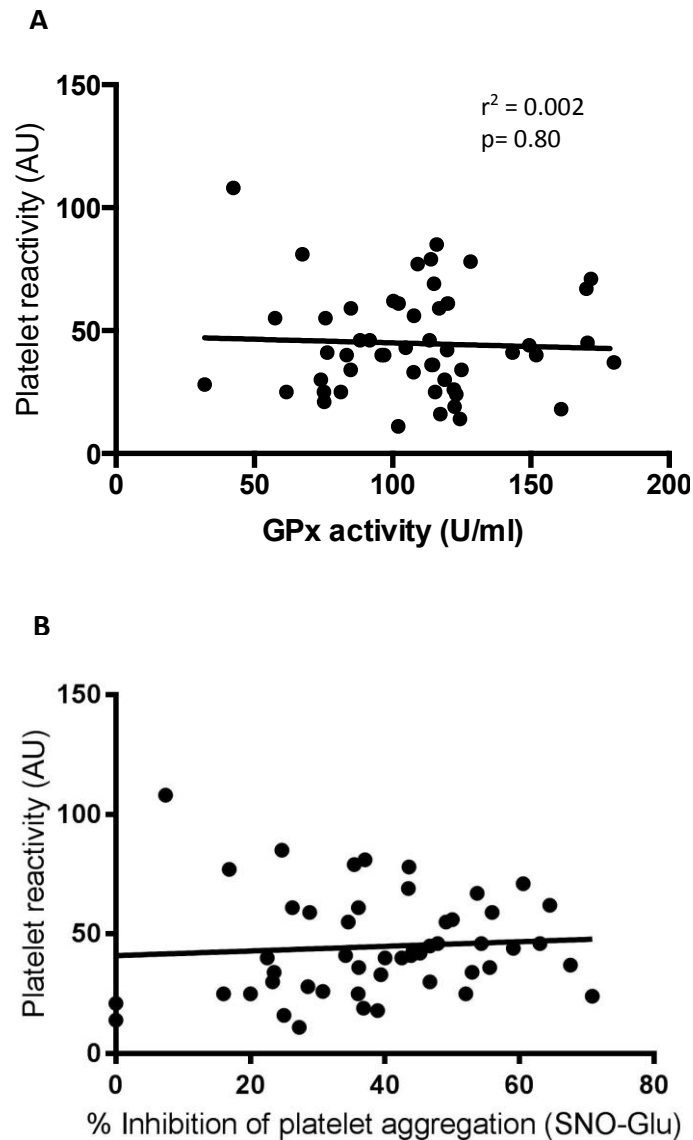
Figure 3-6 Correlation between platelet reactivity, GPx activity and SNO-Glu.

A) Platelet reactivity (AU) was not significantly correlated with GPx activity (U/mL), r^2=0.002, p=0.8, nor was B) platelet reactivity significantly correlated with % inhibition of platelet aggregation mediated by SNO-Glu, r^2=0.007,p=0.6, Data plotted for all 51 ACS patients with Pearson's correlation coefficient test.

A significant correlation was observed between GPx activity and the % inhibition of platelet aggregation in response to SNO-Glu (Figure 3-7A) (p<0.05). This suggests that high levels of GPx activity are associated with a larger shift in inhibition of platelet aggregation mediated by SNO-Glu, which is indicative of lower levels of ROS. Therefore high levels of GPx activity are correlated with low levels of ROS (Figure 3-7B) (p<0.05).
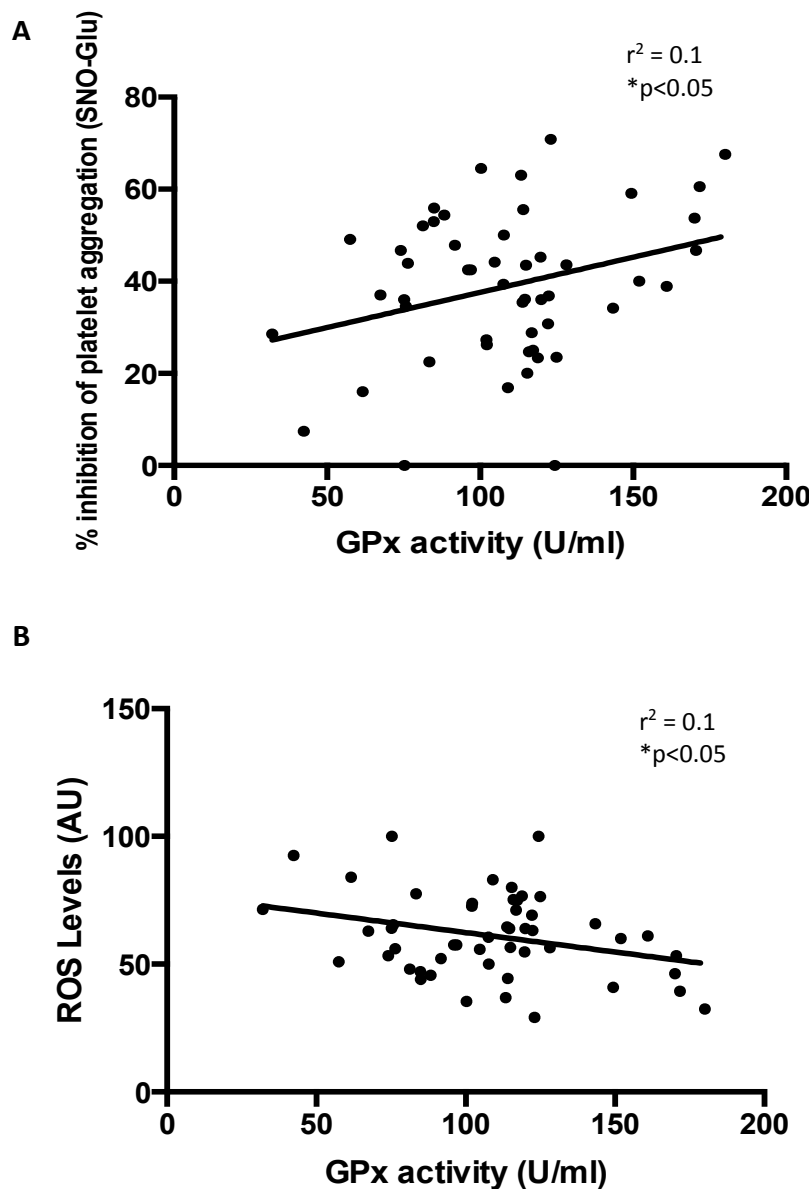
Figure 3-7 Correlation between GPx activity and ROS levels.

A) GPx activity (U/mL) was significantly correlated with the level of % inhibition of platelet aggregation mediated by SNO-Glu, r^2=0.1, *p<0.05. % Inhibition is the inverse marker of ROS, therefore B) GPx activity was significantly inversely correlated with ROS levels (AU) r^2=0.1, *p<0.05, Pearson's correlation coefficient test.

3.4   Discussion

This study aimed to examine the relationship between levels of GPx activity, platelet reactivity and ROS levels in an ACS population.   ROS levels were interpreted as the inverse values corresponding to SNO-Glu-mediated inhibition of platelet aggregation.   High levels of inhibition were suggested to reflect low levels of ROS in the blood, whilst lower levels of inhibition were suggested to reflect high levels of ROS in the blood. Absolute levels of platelet reactivity were not found to correlate with GPx activity, nor with levels of ROS. This suggests that neither GPx activity nor ROS levels are significant determinants of platelet reactivity in an ACS population. GPx activity was found to significantly correlate with ROS levels, with high levels of GPx activity associated with low levels of ROS. This is consistent with the theory from the previous chapter that high levels of GPx activity may play an important protective role in an ACS population by reducing ROS-mediated damage.

Previous research has demonstrated that deficiencies in GPx activity is associated with a pro-thrombotic state [70], and is implicated in platelet-dependent arterial thrombosis [72]. GPx activity has also been shown to preserve the bioavailability of NO [72], therefore sustaining platelet inhibitory mechanisms. Additionally aberrant NO signalling has been associated with an increased risk of an MI [179]. Therefore we suggested that low levels of GPx activity may be associated with a higher level of platelet reactivity in an ACS population. This may have contributed to the increased MACE we observed in the ACS population from the previous chapter. However we did not observe a significant association between GPx activity and platelet reactivity in this ACS cohort, suggesting that these two parameters are independent of each other.

A possible explanation for the lack of relationship between GPx activity and platelet reactivity that we observed is the use of antiplatelet therapy in these patients. All patients were on dual antiplatelet therapy consisting of clopidogrel and aspirin as part of their routine clinical management.   The residual platelet reactivity on treatment with these agents will be determined by a range of factors including patient co-morbidities and genetic variants [180, 181]. It is probable that these factors may be more significant than the influence GPx may have on NO-mediated platelet inhibition for determining platelet reactivity levels.

It is well known that NO has the ability to critically affect platelet activation, but that NO is also inactivated upon interaction with ROS in the vasculature, leading to an impairment in its vascular dilatory and platelet inhibitory function [182]. We utilised the inhibitory nature of NO by adding the exogenous NO donor, S-nitrosoglutathione (SNO-Glu), a stable conjugate of endothelium derived NO. S-nitrothiols, such as SNO-Glu, are naturally occurring thiol adducts of NO, and exhibit properties of NO, such as inhibiting platelet function and elevating intracellular cyclic guanosine monophosphate (cGMP) [72, 183]. By measuring platelet reactivity in our ACS study population, we were able to examine the level of inhibition by SNO-Glu on the level of platelet aggregation induced by ADP. The difference was expressed as % inhibition of platelet aggregation. Because SNO-Glu will be inactivated by high levels of ROS as demonstrated by Freedman et al. [72], we suggest that high levels of inhibition of will be due to low levels of ROS.

We observed a considerable variability in ROS levels calculated by this method, and due to the knowledge that ROS levels have the ability to activate platelets [184], we hypothesised that ROS levels would be correlated with levels of platelet reactivity in the ACS patients. However, similar to the correlation between platelet reactivity and GPx activity, we did not observe a significant relationship between platelet reactivity and ROS levels in this ACS cohort. A possible explanation for this relationship is again, that any effects of ROS on platelet reactivity are relatively small compared to the other factors driving variability in response to clopidogrel and aspirin.

Our results did demonstrate a significant inverse correlation between levels of GPx activity and ROS. This suggests that high levels of GPx are associated with lower levels of ROS in an ACS population. This is consistent with the view from our previous chapter where GPx may have a protective role in an ACS population by mediating oxidant damage, and may contribute to the relationship seen between low levels of GPx activity and a higher rate of MACE. However we have not established whether this is a causative relationship. On the basis that ACS patients demonstrate considerable variability in GPx activity levels from the previous chapter, we suggest that patients have a differential capacity to respond to levels of oxidative

stress during an ACS event that may contribute to clinical risk. However where this variability originates from and what is driving it remains undetermined.

We observed an increased level of platelet inhibition after addition of GPx to SNO-Glu + ADP-stimulated platelets, and we suggest that this is due to a decrease in ROS levels caused by GPx activity. Therefore this allows SNO-Glu to inhibit platelet aggregation to a greater extent, than SNO-Glu treatment alone. We suggest this demonstrates using SNO-Glu as a marker for ROS is an effective method in this study. These assay conditions have been described previously by Freedman et al [176], where it was suggested that GPx activity preserves the function of SNO-Glu by scavenging ROS and lipid hydroperoixdes thereby preventing the inactivation of NO. A further study confirms this concept in human studies where the addition of exogenous GPx normalised the platelets attenuated response to SNO-Glu [72].

3.4.1   Limitations

ACS represents a dynamic, rapidly evolving clinical condition. This study was designed to examine the relationship between GPx activity, platelet reactivity and ROS levels. It is likely that all three of these parameters will change rapidly during the time course of an ACS event, and may all be influenced by therapeutic interventions. Our study has looked at these using a snapshot approach, taking samples at a single, non-standardised time point from each individual. While this does allow us to examine the correlations that exist between these measures, this approach does not allow us to explore the dynamics of the system, and how this may influence the associations.

While we have examined the relationships between GPx activity, platelet reactivity and ROS levels, this has been carried out in patients receiving antiplatelet therapy. The influence that clopidogrel and aspirin have on platelet function may obscure relationships between these factors that may be different under other circumstances. Furthermore, due to the SNO-Glu method being dependent on platelet function it is only suitable for comparing ROS levels within an ACS population. It would have limited use for comparing across other coronary populations who have different antiplatelet medication profiles.

While we can provide robust, verified measures of GPx activity and platelet function through commercially available products that are widely used, there are significant limitations when choosing a marker for ROS in a clinical setting. There are numerous species of ROS, most of which are volatile and have short half-lives. This makes accurate and reproducible measures of ROS a challenge. Because of this, there is a lack of widely used gold standard marker to quantify total ROS in a clinical setting. We utilised a platelet function methodology based on the principal of NO consumption by ROS to determine ROS levels in whole blood. Although further validation studies are warranted to ensure this is an accurate representation of ROS in the blood, this measure has promising clinical application due to its simple, time-effective method. Variation in sample timing and preparation may introduce unknown variability in ROS measures, therefore blood sampling was standardized and strict time protocols were adhered to in order to minimise these limitations.

3.5   Conclusion

In conclusion we demonstrate that GPx activity and ROS levels are not correlated with levels of platelet reactivity in ACS patients. However low levels of GPx activity were found to be significantly associated with high levels of ROS. This suggests that ACS patients may have a differential capacity in response to ROS levels, and that high levels may protect against ROS-mediated damage. However where this variability originates from and what is causing it, remains to be determined.

Chapter 4 - Comparison of the glutathione peroxidase system in healthy subjects, stable coronary artery disease and acute coronary syndromes

4.1 Introduction

In Chapter 2, we reported a considerable variation in levels of GPx activity in ACS patients, with lower levels associated with an increased rate of MACE at 1 year. We do not know whether GPx activity changes in response to an ACS event, and whether the variability we have observed is the product of a differential capacity to respond to an acute injury. The alternative is that GPx activity may not differ with coronary disease progression, and the variability may occur regardless of disease state. Previous literature on how GPx changes in an ACS population relative to healthy controls and patients with stable CAD has given conflicting results, with reports of increases [185], decreases [186] and no change [187] all featuring. Resolving these contradictory reports regarding how the GPx enzyme differs in coronary disease progression is important for understanding its clinical significance.

Understanding if there is variability between coronary disease states is not well defined; a common limitation among the studies is the lack of control for CVD risk factors that may influence the level of GPx activity. Diabetes and gender may affect GPx activity levels, as seen in Chapter 2, and reported in previous studies [188, 189]. In addition, smoking, heart failure and arrhythmias such as atrial fibrillation may also affect GPx levels [170, 190, 191]. In order to clarify the differences in GPx levels in disease progression, it is necessary to control for these factors. In addition, previous work has been mainly focused on examining GPx activity, with little or no information on corresponding changes in protein and mRNA expression levels. By examining activity, protein and mRNA levels, a thorough understanding of the production, activity and variation of this antioxidant enzyme is possible.

Since we previously assayed GPx activity from the plasma, and GPx3 is the predominant isoform reported to be present in the plasma [145] and implicated in thrombosis [70, 72], levels of the GPx3 protein were the main focus in this study. Whole blood was used to determine levels of GPx mRNA expression, as levels have been shown to be comparable to those isolated from the major tissues [192] e.g. heart, liver and kidney. As well as GPx3, GPx1 and GPx4 were measured for comparison, as all three isoforms have been implicated in CAD [70, 71, 193]. Since GPx2 is restricted to the gastrointestinal tract, and GPx5-GPx8 have only

putative roles or have not yet been characterised (Section 1.7.6-8), they were excluded from analysis.

Thus, the aims of this study were to investigate the following parameters in healthy subjects, stable CAD patients and ACS patients:

- To examine the levels of GPx activity and GPx3 protein concentration.
- To examine GPx1, 3 and 4 mRNA expression levels.
- To examine the levels of oxLDL in order to relate the GPx measures to an oxidative stress marker.

4.2 Methods

4.2.1 Study population

Three patient groups were included to investigate the GPx system and oxidative stress status. All patients in the healthy and stable CAD groups provided informed written consent, and the study was reviewed and approved by the Central Health and Disability Ethics Committee (13/CEN/29) (Appendix 1). Similarly, a subset of ACS patients from a larger ongoing study from our laboratory group provided informed written consent that received ethics approval from the Lower South Ethics Committee (LRS/11/09/035) (Appendix 1).

4.2.1.1 Healthy volunteers

Healthy volunteers were recruited using poster advertisements and word of mouth. Twenty volunteers were age-matched and gender-matched to the cohort of 20 ACS patients. Criteria of the study dictated that volunteers had no known cardiovascular disease, or other acute illness in the preceding six weeks. Other exclusion criteria included diabetes, smoking, pregnancy, and those who were on regular cardiovascular or immune-modulating medication or had been treated with antiplatelet therapies or non-steroidal anti-inflammatory drugs (NSAID) within the last 7 days.

4.2.1.2 Stable coronary disease patients

Stable coronary patients were recruited through Wellington Hospital cardiology outpatient clinics. Twenty stable CAD patients were age-matched and gender-matched to the cohort of 20 ACS patients. Criteria of the study dictated that patients have confirmed coronary disease by either angiographic evidence of a greater than 70% stenosis in a significant coronary artery and be receiving chronic aspirin therapy (100 mg/day). Exclusion criteria included diabetes, pregnancy, smoking, and acute illness in the preceding six weeks.

4.2.1.3 Acute coronary syndrome patients

Patients presenting to Wellington Regional Hospital with ACS and undergoing an invasive approach (coronary angiography ± PCI) adequately pre-treated with dual antiplatelet medications were eligible for inclusion. ACS was defined as having symptoms suggestive of

ischaemia lasting > 10 min in duration coupled with either a troponin elevation or new 1 mm ST-segment deviation or T wave inversion in at least 2 contiguous leads [24]. Adequate pre-treatment with antiplatelet medications was defined as chronic therapy with aspirin (≥75 mg) and clopidogrel (≥75 mg) or loading with aspirin ≥300 mg at least 2 hours before or clopidogrel ≥300 mg at least 6 hours before enrolment. Exclusion criteria included a platelet count less than 100 x 10^9/L, known platelet function disorder, administration of a fibrinolytic agent within 24 hr of enrolment and administration of a glycoprotein IIb/IIIa receptor antagonist within a week prior to enrolment. Additional exclusion criteria included smoking, diabetes, pregnancy, or an age outside the range of 45-65 years old.

4.2.2   Data collection and blood sampling

Patient demographics, clinical characteristics, medications including antiplatelet therapy and clinical management were collected prospectively from review of the medical records and cardiac catheterisation database. Blood samples were collected from ACS patients within a mean time of 2-4 days post-presentation to hospital. Samples were collected into tubes anticoagulated with sodium citrate (0.109M, BD Vacutainer) from a peripheral vein using a 21-gauge needle before angiography or alternatively in the cardiac catheterisation laboratory from the arterial sheath immediately after catheter insertion and prior to administration of heparin. Blood samples from stable CAD patients and healthy subjects were collected from the Clinical Measurement Unit at Wellington Hospital. Citrated whole blood was centrifuged at 1500 *g* for 12 min at 4°C to extract plasma for antioxidant activity measurement. Aliquots were stored at -80˚C until required for analysis.

4.2.3   Glutathione peroxidase system

4.2.3.1   GPx activity assay

Glutathione peroxidase activity assay kits (Enzo Lifesciences) were carried out as described in Section 2.2.5.1. GPx activity was defined as nanomoles of NADPH consumed per minute, and expressed as units per mL of plasma. Intra-assay coefficient of variance was 9.9%, and inter-assay coefficient of variance was 8.1%.

4.2.3.2 GPx3 protein ELISA kit

GPx3 has been the predominant isoform reported to be present in human plasma, therefore a sandwich GPx3 (human) ELISA Kit[TM] (Adipogen International, CA, USA) was used to detect GPx3 protein levels in the plasma as per manufacturer's instructions. Plasma samples were diluted 1:1000 with 1x ELISA buffer and measured in duplicate.   Human GPx3 standard supplied by the manufacturer was used to construct a protein standard curve. The standard protein concentrate (64 ng/mL) was reconstituted with 1 mL deionised water and 2-fold serial dilutions made with 1x ELISA buffer to generate a seven-point standard curve (0-32 ng/mL). 100 µL of standards and samples were added to 96-well plates pre-coated with a specific polyclonal antibody to GPx3, and incubated for 1 hr at 37˚C. The standards and samples were discarded and the plate washed three times with 1x Wash Buffer. A 100 µL volume of the Detection Antibody, a biotinylated polyclonal antibody specific to GPx3, was added to each well and incubated for 1 hr at 37˚C. After the antibody solution was removed and the plates washed three times with 1x Wash Buffer, 100 µL of horse radish peroxidase labelled streptavidin (STREP-HRP, 1:200) was added and incubated for 1 hr at 37˚C. The STREP-HRP solution was discarded and plates washed five times with 1x Wash Buffer. After the final wash, 100 µL of TMB Substrate Solution was added to the wells, and the colour allowed to develop for 20 min at room temperature. The colour reaction was stopped by addition of 100 µL of the acidic Stop Solution to each well. The optical density of the plate was measured at 450 nm on microplate reader (VersaMax™) and a standard curve was generated on Microsoft Excel software to determine the sample concentrations of GPx3 protein.   Intra-assay coefficient of variance was 2.5% and inter-assay coefficient of variance was 7.2%.

4.2.3.3 Quantitative real-time PCR (qPCR): GPx mRNA expression

4.2.3.3.1 RNA extraction and cDNA synthesis

Total RNA from whole blood was extracted using the QIAamp® RNA blood mini kit (Qiagen; Hilden, Germany) following the manufacturer's instructions. In detail, 1 mL of citrated whole blood was incubated with 5 mL of Buffer EL for 15 min on ice to ensure erythrocyte lysis. Leukocytes were pelleted by centrifugation at 400 x g at 4˚C for 10 min, and resuspended with a further 2 mL of Buffer EL. The pellet was recovered by another centrifugation spin at 400 x g at 4˚C for 10 min. A volume of 600 µL of Buffer RLT was used to resuspend the pellet

by thorough vortexing for 1 min. The lysate was transferred to a QIAshredder spin column and centrifuged at maximum speed (16,000 x g) for 2 min at room temperature to homogenize the lysate pellet.   Precipitation of the homogenized lysate was carried out by mixing with 600 μL of 70% ethanol to adjust binding conditions. The sample was transferred to a QIAamp spin column and centrifuged at 10,000 x g at room temperature for 15 s to allow selective binding of RNA to the silica-based membrane in the spin column. After washing away contaminants by centrifuging 350 μL Buffer RW1 through the spin column at 10,000 x g at RT for 15 s, 80 μL of DNase Buffer containing 10 μL of Dnase I stock solution was added to the column and incubated at room temeprature for 15 min. A further wash step with Buffer RW1 was carried out. Buffer RPE (500 μL) containing 99% ethanol was used to wash the spin column by centrifugation at maximum speed for 3 min. Traces of ethanol were removed by a second centrifugation of the empty spin column at maximum speed for 1 min. The QIAamp spin column was transferred to a 1.5 mL microcentrifuge tube and 30 μL of RNase-free water was used to elute the RNA bound to the column by centrifugation at 12,000 x g for 1 min. This step was repeated to ensure all RNA was eluted. RNA was quantified using a Bioanalyzer 2100 (Agilent Technologies Inc; Santa Clara, USA). Aliquots of RNA were stored at -80˚C until required for complementary DNA (cDNA) synthesis as described below.

The synthesis of cDNA from total RNA was performed using the SuperScript® ViLo™ cDNA Synthesis Kit (Invitrogen; Auckland, NZ) following the manufacturer's instructions. In detail, 10 ng of the RNA sample made up to a final volume of 10 μL was added to 4 μL of 5x VILO reaction mix, 2 μL of 10x SuperScript® ViLo™ enzyme and made up to a total volume of 20 μL with 4 μL of RNAse-free distilled water. The reaction was mixed by thorough vortexing, and the samples were incubated in the Corbett Rotor-Gene 6000 machine (model no. 11754-050; Corbett Life Science; Sydney, Australia) under the following conditions: 25˚C for 10 min, 42˚C for 120 min, 85˚C for 5 min. The samples were removed immediately following cDNA synthesis and stored at -20˚C until required for qPCR experiments (described below).

4.2.3.3.2 Real-time qPCR with SYBR Green chemistry

Gene expression was quantified on the Corbett Rotor-Gene 6000 machine using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies). SYBR® Green dye detects PCR products by binding to double-stranded DNA formed during DNA polymerase amplification of

the target sequence. The result is an increase in fluorescence intensity proportional to the amount of PCR product produced. Each reaction contained a set of optimised concentrations of the appropriate forward and reverse primers (Invitrogen; Auckland, NZ), SYBR® Green master mix (Agilent Technologies), and cDNA template, and was made up to a total volume of 52 µL with UltraPure dH$_2$O (Invitrogen). This reaction was mixed by vortex before being pipetted in duplicate 25 µL aliquots into 0.1 mL strip eppendorfs. Each qPCR experiment contained a calibrator control, a no template control and an RNA control. The calibrator control contained RNA extracted from a healthy subject used for all qPCR optimization experiments, and was included in every experiment to enable normalisation against differences in the qPCR reactions. The thermal cycle conditions were as follows: 10 min at 95˚C, then 40 repeats of 15 s at 95˚C and 60 s at 60˚C. To check primer specificity, a final melt step was added that involved heating the samples from 60˚C to 95˚C, with 5 s incubations for each 0.5˚C rise in temperature. As the temperature rises, double-stranded DNA will denature at a specific temperature depending on the sequence, which results in the release of the intercalated SYBR green and therefore a reduction in the fluorescent signal. The Corbett Rotor-Gene Q Series Software v2.3.1 then plots the negative first derivative of the change in fluorescence as a function of temperature, and specific PCR products will produce distinct peaks in the graph. Single peaks indicate a single product, whereas a diffuse peak in the lower temperature range indicates other products such as primer dimers, or genomic DNA contamination. Melt curve analysis was also carried out on negative controls to ensure there was no contamination.

4.2.3.3.3 Primer optimisation and efficiency testing

To determine gene expression levels of GPx1, GPx3 and GPx4, specific primer sequences were chosen from the literature and ordered through Invitrogen, along with reference genes glyceraldehye-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (18S) which were designed using the Beacon Designer™ software v8.0 (Premier Biosoft International; California, USA). The sequences of each forward and reverse oligonucleotide primer set are listed in Table 4-1.

The optimal concentration of the forward and reverse primers for each gene was determined individually by titration of the primer concentrations between 50 nM and 300 nM. The

combination of primer concentrations that gave the lowest cycle threshold (Ct) value without compromising on endpoint fluorescence was chosen. The optimal primer concentrations for each gene are shown in Table 4-2.

Table 4-1 Oligonucleotide sequences.

Primer Target/Ref	NCBI Accession No.	Forward (F)/ Reverse R)	Primer Sequence 5'-3'
GPx1 [194]	NM_000581	F	GCAACCAGTTTGGGCATCAG
		R	CGTTCACCTCGCACTTCTCG
GPx3 [195]	NM-002084	F	CATCCCCTTCAAGCAGTATGC
		R	GCCCGTCAGGCCCTCAGTAG
GPx4 [194]	NM_001039848	F	TGGGAAATGCCATCAAGTGG
		R	GGTCCTTCTCTATCACCAGGGG
GAPDH	NM_001289745	F	CGGATTTGGTCGTATTGG
Beacon Designer™		R	GGTGGAATCATATTGGAACAT
18S	M10098	F	GACAGGATTGACAGATTGATAG
Beacon Designer™		R	AATCGCTCCACCAACTAA

Oligonucleotide sequences for forward and reverse primers used in SYBR Green qPCR reactions, including reference source and NCBI accession numbers, for each gene investigated.

Table 4-2 Optimal final concentrations (nM) of forward and reverse primers.

Gene	Forward Primer	Reverse Primer
GPx1	100	100
GPx3	100	200
GPx4	200	200
GAPDH	300	200
18S	200	300

Primer efficiencies were calculated for each primer set. Determining the reaction efficiency is critical for data interpretation, as primer sets with poor efficiency will have poor sensitivity. A cDNA dilution series was prepared, with each successive dilution containing half the concentration of cDNA. Mean Ct values were plotted against the log of the concentration of the template, and the reaction efficiency calculated by the Rotor-Gene Q Series Software. R^2 values show the fit of the trendline to the data, and the efficiency is related to the slope (m):

$$E=(10-1/m)-1$$

$$E=1=100\%$$

If the reaction had 100% efficiency, the Ct value for each successive dilution would differ by 1.0, as Ct values are on a logarithmic scale. Ideally primer efficiencies should be approximately 100%, as less indicates the presence of primer dimers, and greater than 100% indicates the presence of non-specific products. The primer efficiencies for each of the genes are presented in Figure 4-1. Primer efficiencies were calculated from the slope of the line and are: GPx1 100%, GPx3 98%, GPx4 103%, GAPDH 100%, 18S 95%.

GPx1

r²=0.99451, E=100%

Cycling A.Green (Page 1):
R=0.99725
R^2=0.99451
M=-3.313
B=32.813
Efficiency=1.00

Ct

Concentration

GPx3

r²=0.99264, E=98%

Cycling A.Green (Page 1):
R=0.99264
R^2=0.98534
M=-3.377
B=36.241
Efficiency=0.98

Ct

Concentration

GPx4

r²=0.99281, E=103%

Cycling A.Green (Page 1):
R=0.99640
R^2=0.99281
M=-3.263
B=31.870
Efficiency=1.03

Ct

Concentration

GAPDH

r²=0.99747, E=100%

Cycling A.Green (Page 1):
R=0.99874
R^2=0.99747
M=-3.314
B=27.302
Efficiency=1.00

Ct

Concentration

18S

r²=0.98987, E=94%

Cycling A.Green (Page 1):
R=0.99492
R^2=0.98987
M=-3.461
B=33.944
Efficiency=0.94

Ct

Concentration

Figure 4-1 Primer Efficiencies.

cDNA template dilutions were amplified in duplicate and the average Ct value was plotted against the log of the concentration for each primer set. R^2 values show the line of best fit, and the slope was used to calculate the efficiencies (E) for each primer set.

Two technical replicates were performed on all samples, with each replicate measuring all samples in duplicate. The mean Ct value of each duplicate was used for analysis. Any duplicates with Ct values differing by more than 0.5 were not used in the final data. Validation of the two reference genes, GAPDH and 18S, was carried out by plotting the mean Ct values in GraphPad Prism 6 Software and demonstrate a significant correlation (p<0.0001) (Figure 4-2). The gene abundance of GAPDH was a closer match to the target genes, therefore GAPDH was used as the reference gene for further analysis of target gene expression.
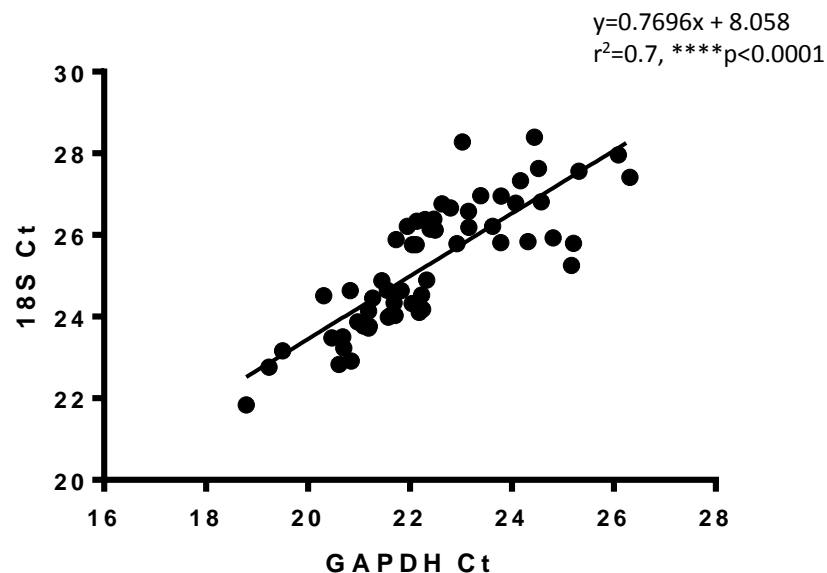
Figure 4-2 Linear regression of mean Ct values for GAPDH vs 18S.

Pearson's correlation test showed a significant relationship, r^2 value = 0.7, ****p<0.0001.

Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ as described previously by Livak et al. [196]. Each sample had its gene expression normalised to the reference gene (GAPDH), then further corrected for the internal calibrator control, and the result obtained is the relative fold increase (or decrease) of the target gene. Normalising the expression of the target gene to that of the reference gene compensates for any difference in the amount of RNA added to the reverse transcription reactions, and using an internal calibrator control corrects for inter-assay differences.

For each sample and calibrator control, the Ct value for GAPDH was subtracted from the Ct value of the gene (Gpx1 or GPx3 or GPx4) to give the ΔCt value. For each gene, the average ΔCt of the calibrator control was calculated and subtracted from the ΔCt values for each sample to give the $\Delta\Delta$Ct. The $\Delta\Delta$Ct values were converted to linear values by applying the formula $2^{(-\Delta\Delta Ct)}$. This formula gives a positive change in linear values as mRNA levels increase. The average $2^{(-\Delta\Delta Ct)}$ was calculated for the two technical replicates carried out to give the mean relative change in gene expression corrected to the calibrator control and normalised to GAPDH. Statistical analysis was performed using the GraphPad Prism 6 software program with a one-way ANOVA with Tukey's multiple comparisons test to detect mRNA expression differences between the patient cohorts. Intra- and inter-assay coefficient of variance values were calculated based on mean Ct values for each gene using a two-way ANOVA. Intra- and inter-assay coefficient of variances are as follows, respectively: GPx1 (1.7% & 0.9%), GPx3 (0.3% & 0.9%), GPx4 (0.4% & 0.9%) and GAPDH (0.4% & 0.3%).

4.2.4 Oxidised LDL ELISA

The amount of oxLDL present in plasma samples was measured using a sandwich ELISA in accordance with the manufacturer's guidelines (Oxidized LDL ELISA, Mercodia; Uppsala, Sweden).   The solid phase two-site enzyme immunoassay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. Briefly, all reagents were brought to room temperature and the lyophilized human oxLDL calibrators were reconstituted in redistilled water and used to construct a calibrator curve for each assay. Concentrations ranged from 1.24 – 20.1 mU/L. A 25 µL sample, calibrator or control was added to a 96-well plate that was pre-coated with the specific murine monoclonal antibody, mAb-4E6 that targets oxLDL. Plasma samples were diluted 1/6561 using Sample Buffer 1X solution and all samples were tested in duplicate. The plate was sealed and incubated at room temperature for 2 hr while shaking at 800 rpm. The plate was washed and incubated for 1 hr at room temperature while shaking with the detector antibody, peroxidase-conjugated anti-human apolipoprotein B antibody that recognises the oxLDL bound to the solid phase. The bound conjugate was detected after washing of the plate by addition of Substrate TMB (3,3', 5,5'-tetramethylbenzidine) and incubation for 15 min in the dark at room temperature. The reaction was stopped by addition of acid (Stop Solution) to give a colourimetric endpoint that was read spectrophotometrically at 450 nm (VersaMax™). The concentration of oxLDL in the samples was determined by computerised data reduction of the absorbance for the calibrators' vs the concentration using a cubic spline regression in GraphPad Prism 6 software. Intra-assay coefficient of variance was 3.9% and inter-assay coefficient of variance was 9.9%.

4.2.5 Statistical analysis

Based on the observed level of GPx activity in Chapter 2 (mean ± SD, 123 ± 32.4 U/mL) we determined that to have an 80% chance of detecting a change of ± 30% across the three groups at p≤0.05 required 19 subjects per group. We therefore designed the study to have 20 patients per group. Continuous variables were reported as mean ± SD, and categorical variables were reported as frequencies and percentages. Statistical tests for continuous variables were performed using Student's *t*-test and one-way ANOVA with Tukey's multiple comparisons test, and chi-square or Fisher Exact tests for the categorical variables. Relationships between parameters were determined by Pearson's correlation coefficient

tests. Differences in values corresponding to $p < 0.05$ were taken as statistically significant. All statistical analyses were carried out in either GraphPad Prism Software v.06 or SPSS v.22.

4.3 Results

4.3.1 Baseline characteristics

All three cohorts were age- and gender-matched. Baseline characteristics are presented in Table 4-3 and displays differences found between the cohorts. The ACS patients had a significantly higher BMI than the stable CAD and healthy populations ($p<0.001$). In the stable CAD cohort, 13 patients (65%) had hypertension and 16 patients (80%) had dyslipidaemia, whilst 9 ACS patients (45%) presented with hypertension and 14 (70%) with dyslipidaemia on index admission. Table 4-4 lists the cardiovascular medications that the stable CAD and ACS patients were on at the time of enrolment in the study.

Table 4-3 Baseline characteristics of patient groups.

	Healthy Group N=20	Stable Group N=20	ACS Group N=20
Male	15 (75)	17 (85)	16 (80)
Age (years)	57.1 ± 6.5	59.4 ± 5.0	59.0 ± 6.6
BMI	24.1 ± 2.8	28.0 ± 4.8***	30 ± 5.1***
Ethnicity			
European	20 (100)	20 (100)	18 (90)
Other	0 (0)	0 (0)	2 (20)
Cardiovascular Risk Factors			
Prior MI	0	13 (65)****	4 (20)*##
Prior PCI	0	11 (55)****	4 (20)*#
Prior CABG	0	9 (45)***	1 (5)##
Prior Stroke	0	3 (15)	1 (5)
Hypertension	0	13 (65)****	9 (45)***
Dyslipidaemia	0	16 (80)****	14 (70)****

Continuous variables expressed as mean ± SD, categorical variables expressed as frequencies (percentages). *denotes significance in relation to healthy group, #denotes significance in relation to stable group, *#$p<0.05$, ##$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Table 4-4 Cardiovascular medication profile of patient groups.

	Healthy Group N=20	Stable Group N=20	ACS Group N=20
ACE Inhibitor	0	7 (35)	9 (45)
Beta-Blocker	0	15 (75)	14 (70)
Ca Channel Antagonist	0	3 (15)	3 (15)
Statin	0	16 (80)	18 (90)
Nitrates	0	3 (15)	1 (5)

Categorical variables expressed as frequencies (percentages).

Abbreviations: ACE – angiotension converting enzyme.

4.3.2 Glutathione peroxidase measurements

GPx activity was significantly higher in ACS patients (109 ± 7.7 U/mL) compared to activity levels in the stable CAD patients (95.2 ± 16.4 U/mL, p<0.01) and healthy subjects (87.6 ± 8.3 U/mL, p<0.001) (Figure 4-3). No difference was detected between the stable CAD patients and healthy volunteers.

GPx3 protein concentrations were significantly higher in ACS patients (21.6 ± 9.5 µg/mL) compared to the stable CAD patients (16.5 ± 2.8 µg/mL, p<0.05) and healthy subjects (16.3 ± 5.3 µg/mL, p<0.05) (Figure 4-4). No difference was detected between the stable CAD patients and healthy volunteers.

When examining the relationship between GPx activity and GPx3 protein levels, no significant correlation between the two measures was found in ACS patients (r^2=0.02, p=0.56) or stable CAD patients (r^2=0.02, p=0.58). Although not statistically significant, there appeared to be a positive correlation between GPx activity and GPx3 protein levels in the healthy cohort (r^2=0.17, p=0.071).

Figure 4-3 Levels of GPx activity

Levels of GPx activity (U/mL) was measured in healthy subjects, stable CAD patients and ACS patients. Data is plotted for all 20 patients in each cohort as mean ± SD, and one-way ANOVA with Tukey's multiple comparison test was used to compare differences, **p<0.01, ****p<0.0001. Healthy subjects are represented as Healthy, stable CAD patients as Stable and ACS patients as Acute.
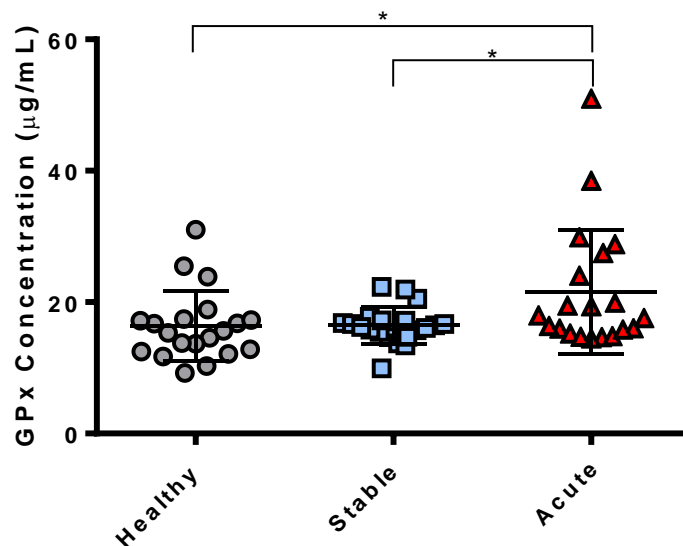
Figure 4-4 Levels of GPx3 protein concentration

Levels of GPx3 protein concentration (µg/mL) was measured in healthy subjects, stable CAD patients and ACS patients. Data is plotted for all 20 patients in each cohort as mean ± SD, and one-way ANOVA with Tukey's multiple comparison test was used to compare differences, *p<0.05. Healthy subjects are presented as Healthy, stable CAD patients as Stable and ACS patients as Acute.

4.3.2.1 qPCR

4.3.2.1.1 Trace images and melt curve analysis

Figure 4-5 shows representative trace images of the amplification data for GPx1, GPx3, GPx4 and GAPDH. The corresponding melt-curve analysis conducted after amplification for each primer set is shown under each trace image. Distinct single peaks can be seen for GPx1 (82.5˚C), GPx3 (82.35˚C), GPx4 (82.60˚C) and GAPDH (79.85˚C). Negative control samples containing H_2O or RNA in place of cDNA template were run with each experiment, and melt-curve analysis, as expected, showed no specific products for these samples (Figure 4-5).

4.3.2.1.2 GPx mRNA expression levels

GPx1, GPx3 and GPx4 mRNA expressions were measured from whole blood samples collected from 19 healthy volunteers, 20 stable CAD patients and 20 ACS patients. One sample from a healthy volunteer could not be used for qPCR analysis due to degradation, and it was therefore excluded from analysis.

GPx1 mRNA expression was significantly higher in ACS patients, with a 1.6 fold increase compared to healthy controls ($p<0.0001$). No difference was detected between ACS and stable CAD patients (1.3 vs 1.1 fold change, respectively, $p=0.12$). Stable CAD patients had a 1.4 fold increase in GPx1 mRNA levels when compared to healthy controls ($p<0.01$) (Figure 4-6A).

GPx3 mRNA expression was significantly higher in ACS patients, with a 1.9 fold increase when compared to stable CAD patients ($p<0.01$). Although the difference was not significant, the fold change in GPx3 expression was higher in ACS patients compared to healthy controls (2.6 vs 1.8 fold change, respectively, $p=0.09$). No difference was detected between the stable CAD and healthy cohorts in GPx3 mRNA expression (1.4 vs 1.8 fold change, respectively, $p=0.42$) (Figure 4-6B).

GPx4 mRNA expression was significantly higher in ACS patients, with a 1.4 fold increase compared to both stable CAD patients and healthy controls ($p<0.01$). No significant

differences were detected between the stable CAD and the healthy cohorts (1.02 vs 1.03 fold change, respectively, p=0.99) (Figure 4-6C)

Pearson's correlation tests did not demonstrate any significant relationships between GPx3 mRNA and GPx3 concentration levels in ACS patients (r^2=0.01, p=0.65), stable CAD patients (r^2=0.06, p=0.31) and the healthy controls (r^2=0.04, p=0.43). Again no significant correlations were observed when comparing GPx3 mRNA and GPx activity levels in ACS patients (r^2=0.02, p=0.55), stable CAD patients (r^2=0.04, p=0.42) and healthy controls (r^2=0.003, p=0.83)
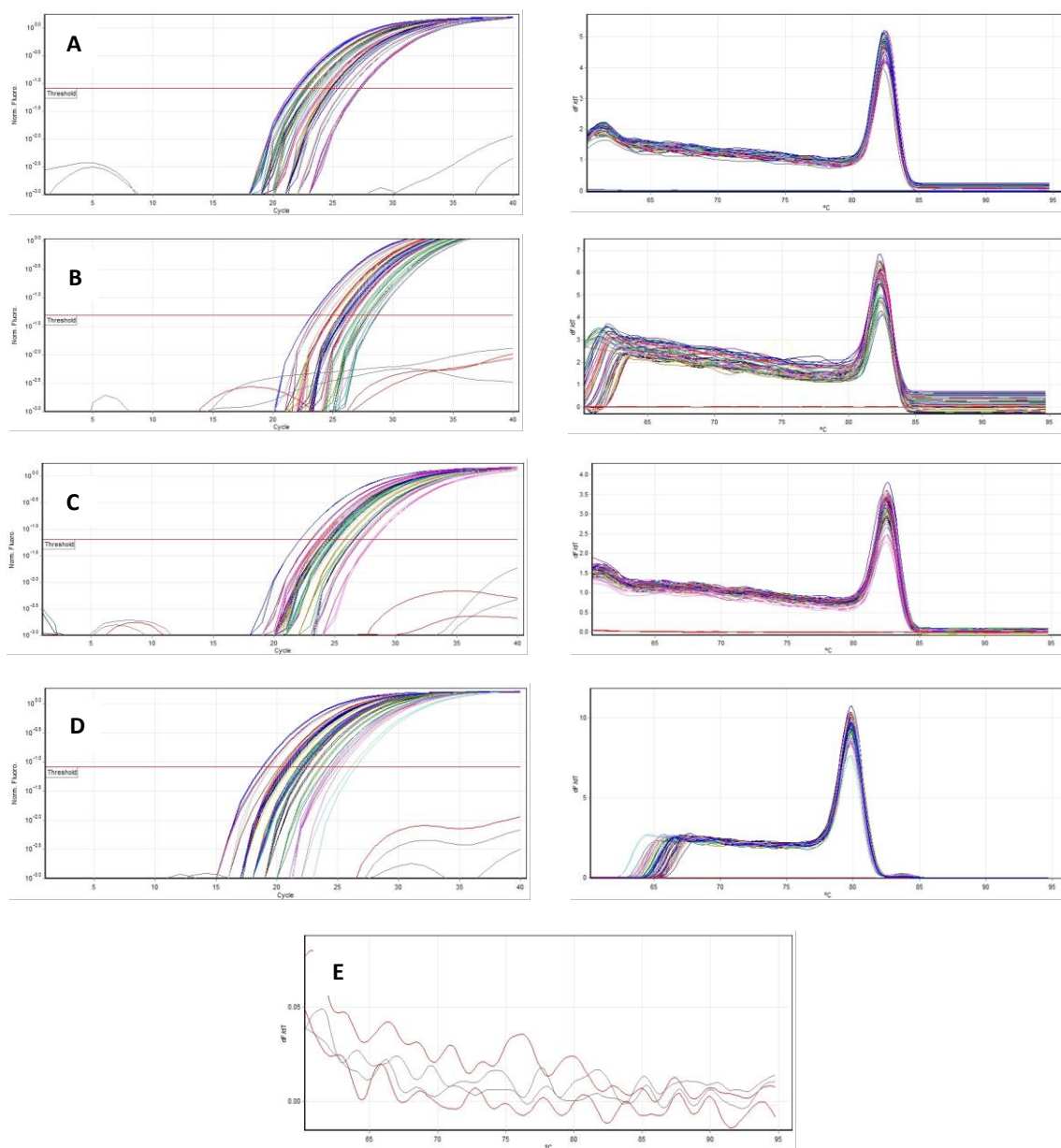
Figure 4-5 Representative data from real-time PCR experiments.

(A-D) Representative trace images in left column (X axis 'Norm Fluoro', Y axis 'cycle') and corresponding melt curve profiles in right column (X axis 'dF/dT', Y axis '°C') for GPx1 (A), GPx3 (B), GPx4 (C) and GAPDH (D). All samples were run in duplicate, and duplicates of RNA and H_2O negative controls (samples that stay below the threshold line in the trace image) were run with every experiment. The melt curves show a single distinct peak for each gene, indicating a single specific PCR product. (E) Representative melt curve profile analysis for negative controls show no specific PCR product.
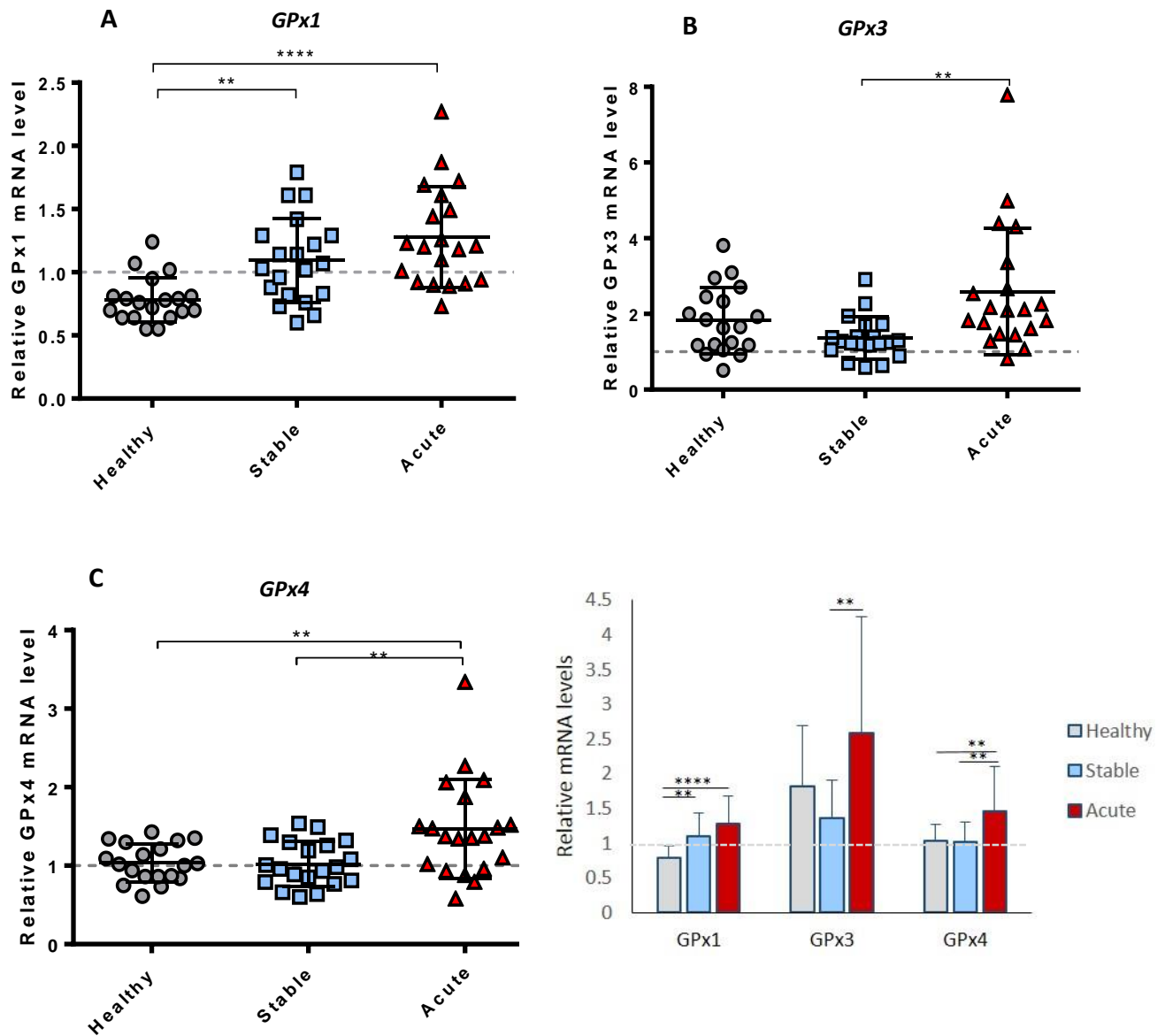
Figure 4-6 GPx 1, 3 and 4 mRNA expression levels

Levels of GPx1 (A), GPx3 (B) and GPx4 (C) mRNA expression were measured in healthy subjects, stable CAD patients and ACS patients. D) Bar graph displays all three isoforms on the same y axis to compare the magnitude of change in each isoform for each cohort. mRNA expression is presented as arbitrary values expressed as fold change relative to GAPDH and normalised to calibrator control. Fold change is relative to the value 1.0. Data is plotted for 19 healthy subjects and 20 stable CAD and ACS patients as mean ± SD. One-way ANOVA with Tukey's multiple comparison test was used to compare differences, $*p<0.05$, $**p<0.01$, $****p<0.0001$. Healthy subjects are presented as Healthy, stable CAD patients as Stable and ACS patients as Acute.

4.3.3   Oxidative stress biomarkers

OxLDL was significantly higher in the ACS patients (61.9 ± 22.2 U/L) compared to the stable CAD (47.8 ± 10.4 U/L, p<0.05) and healthy cohorts (48.9 ± 11.9 U/L, p<0.05) (Figure 4-7). No significant difference was detected between the stable CAD and the healthy cohorts.
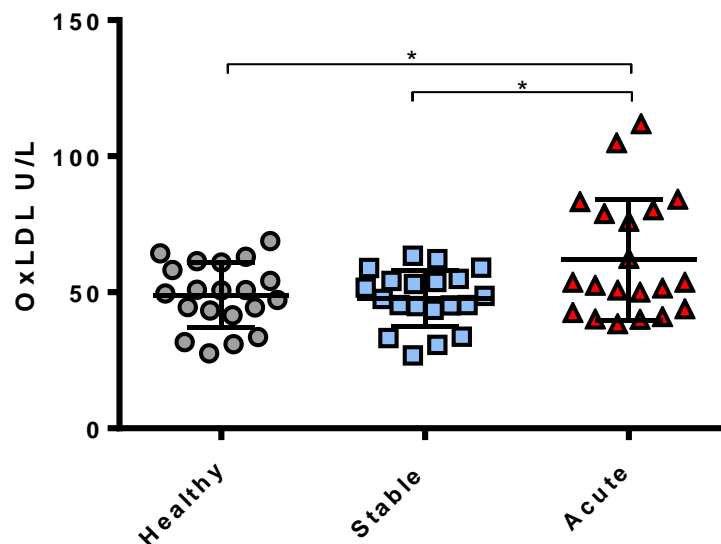
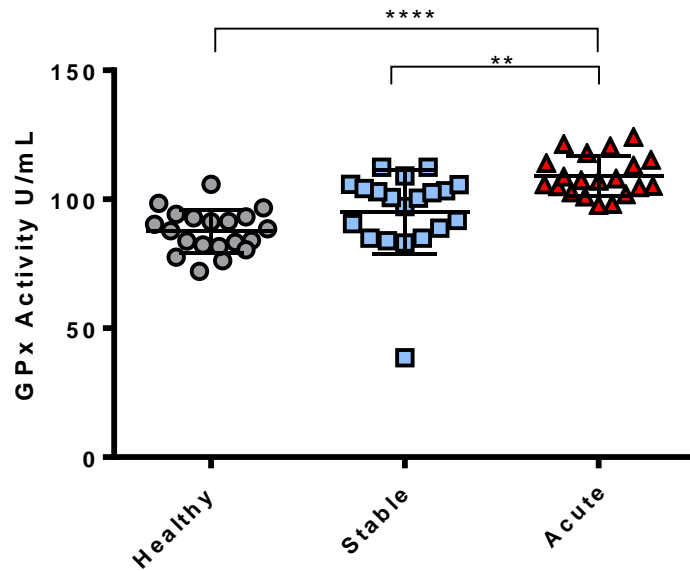**Figure 4-7 Levels of oxLDL**

Levels of oxLDL (U/L) were measured in healthy subjects, stable CAD patients and ACS patients. Data is plotted for all 20 patients in each cohort as mean ± SD, and one-way ANOVA with Tukey's multiple comparison test was used to compare differences, *p<0.05. Healthy subjects are presented as Healthy, stable CAD patients as Stable and ACS patients as Acute.

4.3.4   Correlation between GPx and oxidative stress biomarkers

Pearson's correlation tests did not demonstrate any significant relationships between GPx activity and oxLDL levels in ACS patients (r^2=0.02, p=0.51), stable CAD patients (r^2=0.07, p=0.26) and the healthy controls (r^2=0.02, p=0.55).). Again no significant correlations were observed when comparing GPx3 concentration and oxLDL levels in ACS patients (r^2=0.03, p=0.47), stable CAD patients (r^2=0.03, p=0.49) and the healthy controls (r^2=0.03, p=0.47).

4.4 Discussion

This study demonstrated that ACS patients had higher levels of GPx activity, with corresponding increases seen in both protein and mRNA expression levels, when compared to stable CAD patients and healthy controls. Levels of oxLDL were also seen to be higher in ACS patients relative to the stable CAD patients and healthy controls. Therefore these results suggest that the ACS cohort experience higher levels of oxidative stress, with a corresponding systemic increase in the GPx system. It is unclear whether the relationship between increased oxLDL and the increased levels of the GPx system is a causal relationship or an associative one.

4.4.1 GPx system

4.4.1.1 GPx activity

The increase in GPx activity we observed in this ACS cohort suggests that the GPx enzyme may be influenced during the acute event. No difference was detected in GPx activity between the stable CAD patients and healthy controls, suggesting lower levels of GPx activity are found in healthy subjects and CAD patients who are clinically stable. Our results are consistent with studies published by Kok et al. [185] and Bor et al. [197] who report increased levels of GPx activity in ACS patients. Although these studies utilised erythrocyte lysates to measure GPx activity, a positive correlation has been reported between erythrocyte-GPx and plasma-GPx activity [198], allowing us to compare our results to these other studies. Similarly Zachara et al. [171] found that plasma GPx activity was significantly higher at day 2 post-ACS compared to healthy controls.

However, there are other studies that have reported no difference in GPx activity between ACS patients and healthy subjects [187, 199], and some studies that have reported that GPx activity is lower in ACS patients when compared to healthy subjects [186, 200]. It is not clear what accounts for these existing differences in the literature. Different studies utilise different methodologies in terms of when samples are taken in the course of an acute event, the type of ACS patients recruited, how samples are handled and whether GPx activity is examined in plasma, cell fractions, or whole blood. It is not known how these factors may influence measurement of GPx. In addition, patient factors are not always adequately controlled.

Factors such as age, gender, smoking and diabetes, all of which may influence both oxidative stress levels and GPx activity [169, 189, 198, 201], have been variably controlled for in the literature. Table 4-5 provides a summary of the studies examined in this discussion.

Since the cause of variability in GPx activity between patients is not well understood, this adds to the challenge of understanding the role of GPx in coronary disease progression. The influence that age, gender, lifestyle and environmental factors have on GPx activity is not well established. GPx activity is thought to decrease with age [198], smoking [170], and diabetes [202] and is reported to be higher in women [189]. Without controlling for these factors, it is possible that some of the differences in GPx activity seen in previous studies may be due to confounding factors rather than an accurate reflection of the progression of CAD. Therefore this may contribute to the variable results as depicted in Table 4-5. The current study has controlled for cardiovascular risk factors that have been reported to influence levels of GPx activity in order to minimise possible confounding factors.

Table 4-5 Summary of factors used in GPx activity studies

First author, Year, Reference	Enzyme/population	Biological source	Diabetes	Age	Gender	Smoking	Conclusion
Kok et al, 1989 [185]	GPx/AMI & healthy	Erythrocyte	x	✓	✓	x	GPx higher in AMI vs healthy.
Jayakumari et al, 1992 [203]	GPx, SOD, CAT & LPx in UA, SA & healthy	Erythrocyte	x	✓	✓	x	GPx & CAT unchanged. SOD lower in SA vs healthy, higher in UA vs SA & healthy. LPx higher in SA & UA vs healthy.
Akyol et al, 1993 [186]	GPx in AMI, UA & healthy	Serum	✓	x	x	NS	Higher GPx in AMI vs UA, both lower than healthy.
Bor et al, 1999 [197]	GPx in AMI vs healthy	Erythrocyte & plasma	NS	✓	NS	NS	Ery GPx higher in AMI vs healthy, no change in plasma GPx
Dusinovic et al, 1998 [187]	GPx in AMI vs healthy	Erythrocyte	NS	NS	NS	NS	No significant change in GPx.
Dwivedi et al, 2006 [204]	Whole blood SOD, CAT & GR in AMI vs healthy	Whole blood	NS	✓	✓	NS	Lower antioxidants in AMI vs healthy.
Zachara et al, 2001 [171]	GPx in AMI vs healthy	Erythrocyte & plasma	NS	✓	✓	NS	Plasma GPx higher in AMI at day 2 vs healthy, no change in ery GPX
Dubois-Rande et al, 1994 [199]	GPx in UA , SA & healthy	Plasma	NS	✓	NS	NS	No significant change in GPx.
Lafont et al, 1996 [200]	GPx in AMI vs healthy	Erythrocyte	x	x	x	x	GPx lower in AMI vs healthy
Gunaldi et al, 2012 [205]	GPx in AMI vs healthy	Serum	✓	x	x	NS	Non-significant rise in GPx in AMI vs healthy
Cheng et al, 2009 [206]	GPx in AMI vs healthy	Erythrocyte	x	✓	✓	x	GPx lower in AMI vs healthy
Motghare et al, 2001 [207]	GPx & LPx in AMI, SA & healthy, whole blood	Whole blood	✓	✓	✓	x	GPx lower in AMI vs healthy, LPx higher in AMI vs healthy. No change in SA vs healthy.

*NS - not stated by study ; ✓- controlled factor; x - uncontrolled factor. Abbreviations: AMI -acute myocardial infarction; Ery - erythrocyte; GPx - glutathione peroxidase; LPx - lipid peroxides; UA - unstable angina; SOD - superoxide dismutase; CAT - catalase; SA - stable angina; GR - glutathione reductase;

4.4.1.2 GPx protein

The studies on GPx listed in Table 4-5 have all focused on quantifying the level of activity of GPx. There is little or no attention to the changes in the corresponding GPx protein levels in coronary disease progression that can be seen in the wider clinical literature. We utilised a commercially available ELISA kit (Adipogen®) that provided a polyclonal antibody specific for GPx3, and detected a significant increase in this protein in the ACS cohort, with no differences detected between the stable CAD and healthy cohorts. While this implies some level of coupling between protein and activity levels, within each cohort we did not observe a significant correlation between GPx3 protein and GPx activity levels in the plasma. We did detect a non-significant correlation in the healthy cohort (p=0.07). Overall this implies the coupling is relatively weak. However this was not surprising, as GPx requires several secondary enzymes and cofactors [208] that influence the performance of the protein as an active enzyme [209]. It was beyond the scope of this study to monitor these other enzymes and cofactors, such as glutathione and selenium, but such information in future studies may help explain the extent of changes seen in GPx activity. In addition, it is possible that GPx3 is not the only isoform present within the plasma accounting for total GPx activity [210].

4.4.1.3 GPx mRNA

We observed higher levels of mRNA for each of the GPx isoforms in ACS patients relative to stable CAD patients and healthy controls, although these differences were not statistically significant in all cases. Levels of GPx3 mRNA showed the greatest variance of the three isoforms, and this limited our ability to detect GPx3 differences between groups. What accounts for this variance cannot be determined from this study. It may be that the study is underpowered to look for differences in these populations, as the cohorts are a moderate size of 20 subjects (study power was to determine differences in GPx activity rather than mRNA levels, see Section 4.2.5). This may account for why a significant difference was detected only between ACS patients and the stable CAD patients, and not between ACS patients and healthy subjects.

What accounts for the differential upregulation seen across the GPx isoforms cannot be determined from the results of this study. The extent of transcriptional and translational

regulation of the GPx enzymes in the progression of CAD has not yet been fully determined. As these GPx isoforms are selenoproteins, their biosynthesis is dependent on the availability of selenium that is incorporated into the growing polypeptide chain [134]. Consequently, selenium is an essential cofactor for optimal GPx activity [211]. As mentioned above, GPx cofactors such as selenium were not measured in this study, however it would be interesting to determine the role they play in the level of GPx upregulation seen in this study. Transcription binding sites and promoter regions that are modulated through oxygen tension and levels of hypoxia have been described in GPx1 and GPx3 mRNA [209, 212] and may account for the increased GPx mRNA seen in ACS patients who present with greater levels of oxidative stress.

Taken together, the mRNA data suggest that several isoforms of the GPx enzyme are upregulated in an ACS population. A recent study carried out in a mouse model suggests that GPx1 may contribute a small proportion to the total plasma activity [210]. Since our methodology for determining GPx activity does not allow us to differentiate between GPx isoforms, it is not possible to determine which isoform is contributing the activity measured in human plasma in this study. Although the literature reports that GPx3 is the predominant isoform responsible for ROS reduction in the plasma [145], it may be possible that several isoforms may be secreted into the circulation. Further studies to corroborate what isoforms contribute to overall activity levels, both in the plasma and in the erythrocyte would be both interesting and relevant to our understanding of the intricacies of the GPx system.

4.4.2 Oxidative stress status in coronary disease progression

This study demonstrated that ACS patients have significantly higher levels of oxLDL compared to stable CAD patients and healthy controls. As oxLDL is a biomarker for the level of oxidative stress, it suggests that there is an increase in the pro-oxidant environment that promotes oxLDL formation. There are a number of studies that consistently demonstrates protein and lipid oxidation biomarkers [53, 55, 213-215] and other ROS markers [216] are increased in ACS.

The stable CAD patients did not exhibit elevated levels of oxLDL compared to the healthy controls. This result is consistent with some studies that report no difference between these two groups [53, 215]; however an equal number of studies report a significant increase in oxLDL in their stable CAD cohorts versus healthy controls [217, 218]. Similar to the reports with GPx activity, it is not clear what the cause of this difference between the two sets of studies is. Medical therapy for CVD, such as statins, have been reported to indirectly lower oxLDL levels by reducing levels of oxLDL constituents, LDL-C [219] and apolipoprotein B [220]. However this is a routine medication that the majority of patients take in all studies, therefore it cannot fully explain the discrepancies. We have applied rigorous controls for conditions known to possess high levels of oxidative stress, such as diabetes [169], smoking [201] and heart failure [221]. Although this may not fully explain the discrepancy in the literature, it is possible that the absence of these risk factors have contributed to the lower levels seen in our stable CAD patients who are clinically stable.

It is well accepted that an MI represents an acute oxidative stress state [55]; however we cannot determine if the elevated levels of oxidative stress are causing the increase seen in GPx in this study. Although a causative link has been reported previously in the literature, in which ROS is the driving force behind the changes in GPx activity, most of these studies rely on surrogate lipid or protein oxidation markers for measuring oxidative stress [213]. This is due to the challenge ROS molecules present when quantified in a clinical setting. ROS have an evanescent nature, and their short half-lives make them good signalling molecules but confounds their measurement in the circulation by standard approaches [222]. Instead the focus is normally on measuring stable markers in the circulation that may reflect systemic

oxidative stress. A consequence of excessive ROS *in vivo* is the modification of the LDL particle that generates oxLDL [43]. OxLDL is a frequently used marker for levels of oxidative stress and it has been implicated in *de novo* GSH synthesis [223], the obligatory cofactor for GPx activity. Accordingly GPx activity is thought to indirectly lower oxLDL levels by consumption of H_2O_2 therefore minimising the level of oxidative modifications on the LDL compound [78]. Whilst there is evidence that these two factors are associated, how dynamic these changes are throughout an ACS event cannot be determined from this study.

Taken together, increased GPx mRNA, protein and activity all support the view that the GPx system is upregulated in an ACS event. This is consistent with the view that antioxidant defence systems will tend to respond to some form of acute challenge [63]. In the context of an ACS event, this challenge could encompass an increase in ROS production [224] as well as the increase in the overall pro-inflammatory environment associated with an MI [225]. In the present study, we have not examined what factors are leading to the change in the GPx system in ACS, or when these changes occur, but such studies would be strongly considered in future investigations.

4.4.3 Limitations

This study was designed to examine changes in the plasma measures of GPx and the corresponding oxidative stress levels. It is possible that some calculations were underpowered to detect differences between the groups due to the moderate study size.

Although we employed frequently used commercially available kits for analytical detection of our markers, these methods still have limitations. All the methods used to assess oxLDL and GPx are indirect, and there is conflicting evidence about how well these circulating markers reflect oxidative stress *in vivo* [56]. Analytical differences between laboratories make it difficult to compare the results obtained in different studies. Although most laboratories use methods based on the same methodological principles, the specific assay, conditions with regard to concentration of reagents, recording time, and temperature differ. Sample handling is also extremely important for accurate measuring of oxidative stress. For example, if samples are not immediately centrifuged at 4˚C after collection and stored appropriately, lipids have been shown to auto-oxidise [226].

We controlled for multiple cardiovascular risk factors that may influence oxidative stress status in our three cohorts. This may partially explain why our results differ from some previous studies. Other factors, such as the type of ACS recruited and the timing of blood samples may introduce an unknown variance to measures of GPx and oxLDL. For example, in our study, we collected blood within a mean time of 2-3 days post-MI. Another study collected blood from patients who were admitted with a history of 5 hours of chest pain [207]. Intra-patient variance has been reported [227], but an exact quantification of oxidative stress is not yet known in an ACS population. Further investigation into the dynamics of GPx and oxLDL are warranted to understand how they are affected throughout the course of an ACS event, and whether an optimal sampling time can be identified.

4.5 Conclusion

In conclusion, this study has demonstrated that patients with ACS have higher levels of plasma-borne GPx, and multiple isoforms of GPx are correspondingly upregulated. In turn, patients with ACS have higher levels of oxidative stress, as demonstrated by increased oxLDL levels. We conclude that ACS patients have an increased oxidant/antioxidant status that is distinct from stable CAD patients and healthy subjects.

Chapter 5 -   The dynamics of glutathione peroxidase and oxidised low-density lipoprotein during ST-segment elevation myocardial infarction (STEMI)

5.1 Introduction

Our results from Chapter 4 demonstrate that the GPx system is increased in ACS patients compared to stable CAD patients and healthy controls. However, what has not been examined is the time course over which this change occurs. In both Chapter 2 and Chapter 4 single samples for the measurement of GPx activity were collected prior to angiography. This was after a mean time of 2-4 days of patients presenting to hospital.

How dynamic the changes in GPx activity are over the immediate course of an ACS is an important area of investigation. There is limited data on the dynamics of GPx activity between index admission and throughout the development of an ACS. One study reports an increase in GPx activity [171], whilst another reports no change [200] following reperfusion therapy and throughout the hospital admission. It is possible that some of the variation in GPx activity observed in Chapter 2 may have been due to differences in the timing of the sampling. Whether this is an important contributor, and how this may influence the relationship between measured activity and clinical outcomes is not known.   The present study was therefore designed as an exploratory investigation into the temporal changes of GPx activity that occur throughout an ACS event.

Along with examining the temporal changes in GPx activity, oxLDL levels were measured. This allows for the examination of corresponding changes in measures of oxidative stress in ACS patients. Based on the theory that GPx is a defensive antioxidant enzyme, and therefore driven by changes in pro-oxidant levels, we hypothesise that similar temporal changes will occur in these two parameters.

Thus, the aims of this study were:
- To investigate the temporal changes in GPx activity and oxLDL levels in a STEMI cohort.
- To compare whether the temporal changes of GPx activity and oxLDL display a similar pattern.

5.2   Methods

5.2.1   Study population

Twenty patients presenting to Wellington Regional Hospital with STEMI within 12 hours of symptom onset between January 2015 and May 2015 were eligible for inclusion in this study. STEMI was defined as symptoms consistent with myocardial ischaemia lasting longer than 15 min, and ECG changes demonstrating new ST-segment elevation at the J point in two contiguous leads with the following cut points: ≥0.1 mV in all leads other than V_2-V_3, where the following cut points apply: ≥0.2 mV in men ≥40 years, ≥0.25 mV in men <40 years or, ≥0.15 mV in women as per the third universal definition of myocardial infarction [24]. Exclusion criteria consisted of inability to give informed consent, cardiogenic shock and/or referral for emergency coronary artery bypass grafting (CABG). This study was reviewed and approved by the Northern B Health and Disability Ethics Committee (14/NTB/198) (Appendix 1). Participation was voluntary, and each patient provided written informed consent at the time of recruitment.

5.2.2   Data collection

Baseline demographics, clinical characteristics and procedural variables were collected prospectively from review of the medical records and cardiac catheterisation database. GRACE scores were calculated as previously described in Section 2.2.3.2.

5.2.3   Blood collection

Blood collection was sampled at empirical time points. The first two blood samples were collected immediately prior to angiography (Pre) and immediately following revascularisation (Post) directly from the arterial sheath. The rationale was to collect measurements as close to the STEMI onset as possible following admission. This allowed for further blood samples to be collected from a peripheral vein using a 21-gauge needle at the following time intervals post angiography: 6 hr, 12 hr, 24 hr, 48 hr and 72 hr, or up until the time of discharge. A follow-up blood sample was collected when the patient returned for their cardiology outpatient clinic visit between 6-12 weeks post-hospital discharge. The rationale was to collect measurements at a time remote from the event in order to assess the levels when the

patient was clinically stable. Whole blood samples were collected into tubes anticoagulated with sodium citrate (0.109 M, BD Vacutainer). Citrated whole blood was centrifuged at 1500 g for 12 min at 4°C to extract plasma. Plasma was aliquoted and stored at -80°C for subsequent analysis of GPx activity and oxLDL levels.

5.2.4 GPx activity and oxLDL measurements

Glutathione peroxidase activity assay kits (Enzo Lifesciences) were used to calculate GPx activity in the plasma samples as described in Section 2.2.5.1. GPx activity was defined as nanomoles of NADPH consumed per minute and expressed as units per mL plasma. The intra-assay coefficient of variance was 7.8%, and the inter-assay coefficient of variance was 10.7%.

OxLDL ELISA kits (Mercodia) were used to calculate oxLDL levels in the plasma samples as described in Section 4.2.4. Concentration of oxLDL was determined by the cubic spline regression curve and expressed as units per mL plasma. The intra-assay coefficient of variance was 5.8%, and the inter-assay coefficient of variance was 10.4%.

For ease of comparisons, values for GPx activity and oxLDL were normalised to the pre-angiography time point and expressed as a percentage increase or decrease respective to this baseline.

5.2.5 Statistical analysis

Continuous variables were reported as mean ± SD, and categorical variables were reported as frequencies and percentages. Statistical tests to compare GPx activity and oxLDL over the different time points were carried out using either a one-way ANOVA with Tukey's multiple comparisons or a Student's paired t-test. Chi-square test and Student's t-test were used to compare categorical and continuous variables respectively. Differences in values corresponding to $p < 0.05$ were considered statistically significant. All statistical analyses were carried out in either GraphPad Prism Software v.06 or SPSS v.22.

5.3 Results

5.3.1 Demographics and clinical characteristics

Of the 20 study patients that were enrolled, 4 were excluded on the basis that blood samples after 24 hr could not be obtained. Of these 4 patients, 2 were discharged back to regional hospitals, 1 had extremely poor venous access, and 1 withdrew consent. The demographics and clinical characteristics of the remaining 16 patients are shown in Table 5-1. The study population was found to be predominantly male (81%), with an average age of 61.5 years, and a BMI of 27.2. Dyslipidaemia was diagnosed in 81.3% of patients, 50% had hypertension and 18.8% of patients were current smokers.

5.3.2 Angiographic characteristics

The angiographic characteristics of the 16 study patients are presented in Table 5-2. Angiographic success was obtained in all patients. The mean peak value for high sensitivity-troponin (hs-TnT) was 4669 ng/L. The mean time between symptom onset of ischaemia and collection of the first blood sample (Pre) was 230 min.

Table 5-1 Demographics and clinical characteristics of study population.

Patient #	Age, years	Gender M=1, F=0	BMI	Prior MI	Family Hx	HTN	Dyslip	AF	Stroke	PVD	Current Smoker	GRACE Scores
Mean±SD	61.5±13.5		27.2±3.7									108±28
Sum, (%)		13 (81.3)		1 (6.3)	5 (31.3)	8 (50)	13(81.3)	1 (6.3)	2 (12.5)	1 (6.3)	3 (18.8)	
1	79	1	22.0	0	0	1	1	0	1	0	0	135
2	53	1	24.5	0	0	0	1	0	0	0	0	102
3	49	1	25.2	0	1	0	1	0	0	0	1	96
4	59	1	33.6	0	0	1	0	0	0	0	0	98
5	74	1	24.8	0	0	1	1	0	1	0	0	129
6	54	1	21.4	0	1	0	1	0	0	0	0	89
7	81	1	24.5	0	0	1	1	1	0	1	0	158
8	59	1	22.3	1	0	0	1	0	0	0	0	133
9	70	0	30.9	0	0	1	1	0	0	0	0	120
10	58	0	27.9	0	1	1	1	0	0	0	0	106
11	65	1	29.4	0	1	0	1	0	0	0	0	114
13	49	1	31.9	0	0	0	1	0	0	0	1	70
14	77	1	26.4	0	0	1	0	0	0	0	0	141
17	48	1	29.3	0	0	0	1	0	0	0	0	76
19	34	1	30.3	0	1	0	1	0	0	0	1	55
20	75	0	29.8	0	0	1	1	0	0	0	0	119

Abbreviations: BMI – body mass index; MI – myocardial infarction; Hx – history; HTN – hypertension; dyslip – dyslipidaemia; AF – atrial fibrillation; PVD – peripheral vascular disease; GRACE – global registry of acute coronary events.
The value of 1 implies 'yes' whereas the value of 0 implies 'no'.

Table 5-2 Angiographic characteristics of study population.

Patient #	Peak hs-TnT (ng/L)	Culprit lesion				Culprit lesion stenosis	ACC/AHA lesion classification				# stents		Angio-graphic success	Symptom onset – (Pre) sample (mins)
		LM	LAD	Cx	RCA		A	B1	B2	C	BMS	DES		
Mean ± SD	4669±3055													232 ± 140
Sum, (%)		0 (0)	9 (56.3)	3 (18.8)	5 (31.3)		0 (0)	7 (43.8)	9 (56.3)	0 (0)	1	21	16(100)	
1	5684	0	1	0	0	95	0	1	0	0	0	1	1	140
2	11298	0	1	0	0	100	0	1	0	0	0	1	1	170
3	6514	0	0	1	0	100	0	1	0	0	0	1	1	100
4	3157	0	1	0	0	100	0	1	0	0	0	1	1	100
5	6080	0	0	0	1	95	0	1	0	0	1	0	1	125
6	3533	0	0	0	1	100	0	0	1	0	0	2	1	180
7	8326	0	1	0	0	100	0	0	1	0	0	3	1	228
8	2675	0	0	1	0	100	0	0	1	0	0	1	1	135
9	298	0	0	1	0	90	0	0	1	0	0	1	1	265
10	149	0	0	0	1	100	0	0	1	0	0	1	1	190
11	7979	0	1	0	0	100	0	1	0	0	0	1	1	510
13	1978	0	1	0	0	95	0	0	1	0	0	2	1	120
14	3958	0	1	0	0	90	0	1	0	0	0	2	1	310
17	6970	0	1	0	0	100	0	0	1	0	0	1	1	123
19	3090	0	0	0	1	100	0	0	1	0	0	1	1	495
20	3017	0	1	0	1	100	0	0	1	0	0	2	1	302

Abbreviations: hs-TnT - high-sensitivity troponin; LM – left main artery; LAD – left ascending artery; Cx – circumflex artery; RCA – right coronary artery; ACC – American College of Cardiology; AHA – American Heart Association; BMS – bare metal stent; DES – drug eluting stent.
*The value of 1 implies 'yes' whereas the value of 0 implies 'no'.

5.3.3   Patterns of GPx activity over a 72-hour time period

Of the 16 study patients, we observed that there were two distinct patterns of GPx activity over the of 72-hr period following hospital admission with STEMI. The first pattern was that GPx activity appeared to peak early in the admission period, with 8 patients (50%) exhibiting higher levels before the 24 hr time point. Figure 5-1A & B shows two representative examples of patients who demonstrated an early peak of GPx activity. The corresponding oxLDL measures are shown as well.

In 7 patients (43.8%) we observed a late peak of GPx activity with higher levels occurring after the 24 hr time point. Figure 5-2A & B show two representative examples of patients who demonstrated a late peak of GPx activity. The corresponding oxLDL measures are shown as well.

The remaining 1 patient was unable to be classified due to the unclear pattern of their GPx activity. This patient did not appear to have a distinct peak that clearly categorised them into either an early or a late group – (graph not shown).

A closer examination of these two groups, shows that the patients who were classified into the early peak group, had significantly higher GPx activity immediately post PCI (Post time point), compared to subsequent time points. This is shown in Figure 5-3. GPx activity was found to be significantly higher at the Post time point compared to subsequent activity levels found at 24 hr ($p<0.01$), 48 hr ($p<0.05$), 72 hr ($p<0.01$) and follow-up ($p<0.05$).

Examination of the patients who were classified as the late peak group shows they had significantly higher GPx activity after the 24 hr time point. This is shown in Figure 5-4. GPx activity was found to be significantly higher at the 48 hr time point compared to the activity levels found at Pre ($p<0.01$), Post ($p<0.05$), 6 hr ($p<0.01$), 12 hr ($p<0.05$), 24 hr ($p<0.05$) and follow-up ($p<0.05$) times.

The descriptive statistics for the patients who have been classified into either an early peak or a late peak of GPx activity are shown in Table 5-3. Levels of peak high-sensitivity troponin

(hs-TnT) were significantly higher in patients who were classified as having an early peak of GPx activity compared to the hs-TnT levels found in those classified as having a late peak (p<0.05) (Figure 5-5). Remaining cardiovascular risk factors did not appear to influence whether GPx activity corresponded to levels defined as an early peak or a late peak.

5.3.4 Comparison of GPx and oxLDL measures at Pre vs F/U times

Of the 16 patients, 9 (56.3%) had follow-up (F/U) measures taken at a time remote from their index study admission (6-12 weeks post-hospital discharge). Both GPx activity (p<0.01) and oxLDL levels (p<0.05) were found to be significantly lower at the F/U time point compared to the corresponding levels found at the pre-angiography (Pre) time point (Figure 5-6A & B respectively).

5.3.5 Relationship between GPx activity and oxLDL levels

The relationship between GPx activity and oxLDL appears to be complex. In 12 of the 15 patients (80%), levels of oxLDL were found to be at their highest either at the same time (Figure 5-1A for representative example), or prior to (Figure 5-2A & B for representative examples) the highest level of GPx activity. However in 3 of the 15 patients (20%), this was not observed. Instead, oxLDL levels were at their highest at time points after the highest peak level of GPx activity (Figure 5-1B for representative example).
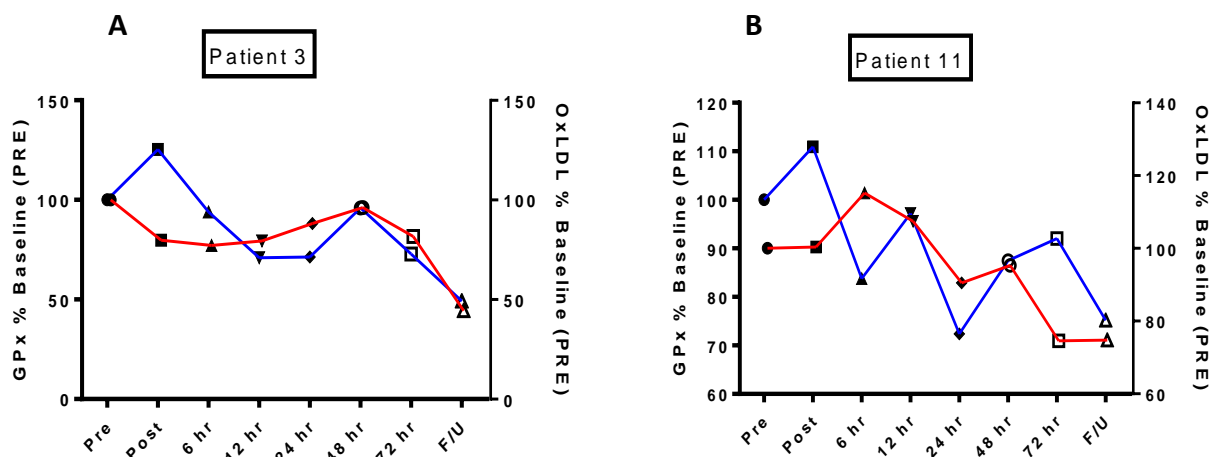
Figure 5-1 Representative graphs from ACS patients with an early peak of GPx activity.

(A-B) Individual graphs from study patients representing an early peak in GPx activity (blue line), with the corresponding measures for oxLDL (red line) shown over the acute phase of a STEMI and the subsequent follow-up measure. Data is normalised to the respective pre-angiography measure (Pre), and expressed as a % increase or decrease respective to this baseline.
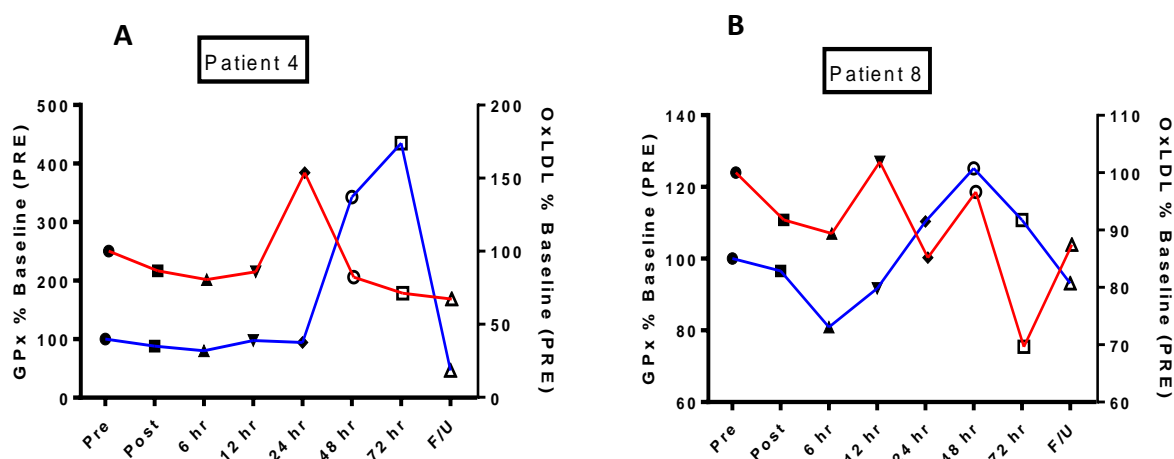
Figure 5-2 Representative graphs from ACS patients with a late peak of GPx activity.

(A-B) Individual graphs from study patients representing a late peak in GPx activity (blue line), with the corresponding measures for oxLDL (red line) shown over the acute phase of a STEMI and the subsequent follow-up measure. Data is normalised to the respective pre-angiography measure (Pre), and expressed as a % increase or decrease respective to this baseline.
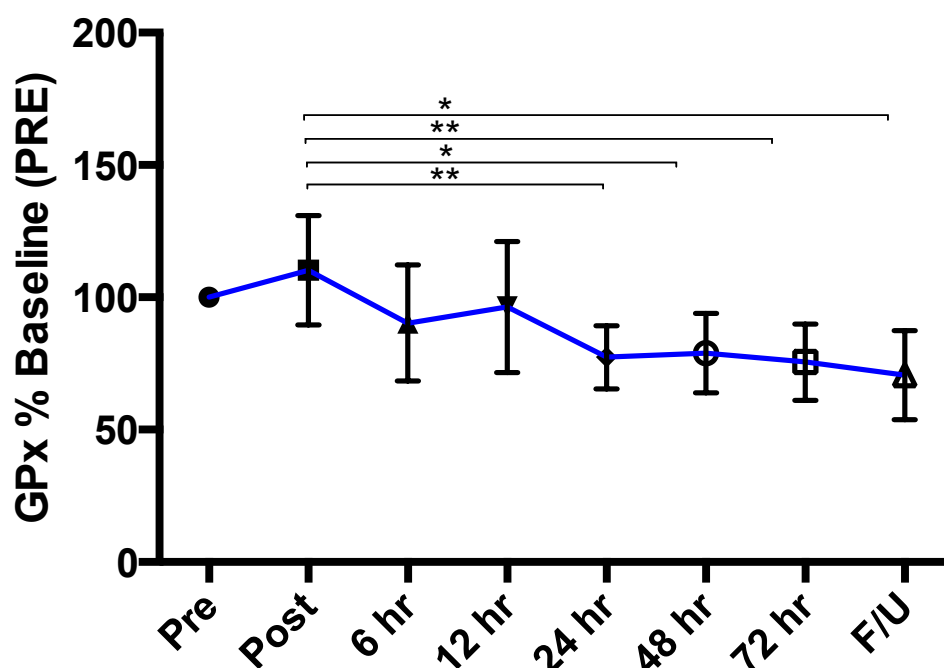
	Pre	Post	6hr	12hr	24hr	48hr	72hr	F/U
Mean	100	110	90.3	96.4	77.4	79.0	75.5	70.6
Std. Deviation	0.0	20.7	21.9	24.8	11.9	15.0	14.5	16.8

Figure 5-3 ACS patients with an early peak of GPx activity

GPx activity was measured pre-angiography (Pre), post-angiography (Post) and subsequently at 6 hr, 12 hr, 48 hr, 72 hr and at a follow-up (F/U) time point. Eight patients exhibited GPx activity that was higher before the 24 hr time point and therefore classified as having an early peak. GPx activity was found to be significantly higher at the Post time point compared to activity levels found at 24 hr, 48 hr, 72 hr and (F/U). GPx activity at each time point was normalised to the respective measure taken at the pre-angiography time (Pre) and expressed as a % increase or decrease respective to this baseline. Data is plotted for all eight study patients and presented as mean ± SD, *$p<0.05$, **$p<0.01$, one-way ANOVA, Tukey's multiple comparisons test.
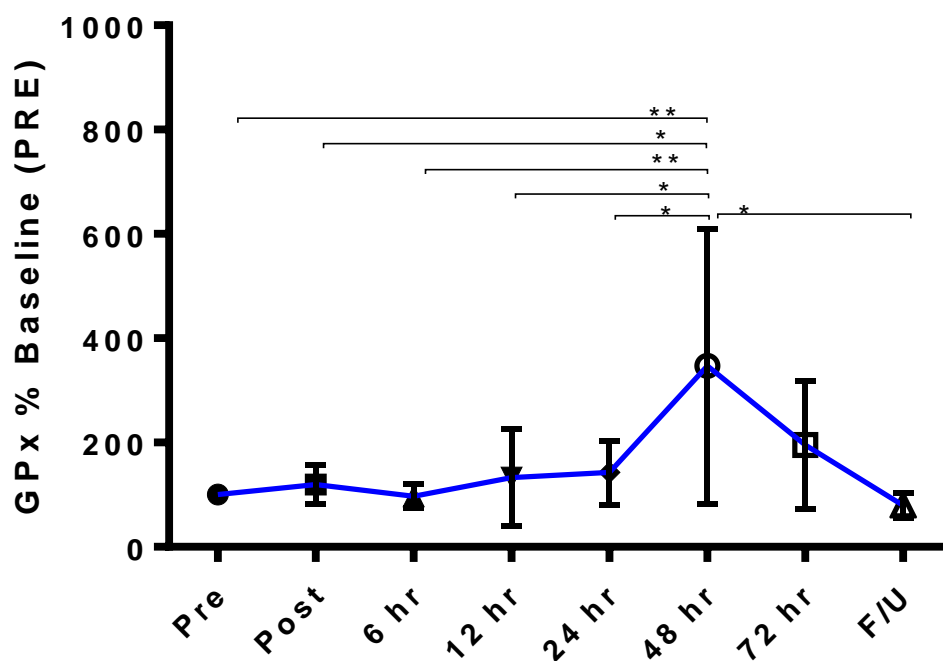
	Pre	Post	6hr	12hr	24hr	48hr	72hr	F/U
Mean	100	119	97.4	133	143	347	196	79.6
Std. Deviation	0.0	36.9	23.0	93.0	61.4	264	122	23.4

Figure 5-4 ACS patients with a late peak of GPx activity

GPx activity was measured pre-angiography (Pre), post-angiography (Post) and subsequently at 6 hr, 12 hr, 48 hr, 72 hr and at a follow-up (F/U) time point. Seven patients exhibited GPx activity that was higher after the 24 hr time point and classified as having a late peak. GPx activity was found to be significantly higher at the 48 hr time point compared to activity levels found at Pre, Post, 6 hr, 12 hr, 24 hr and F/U. GPx activity at each time point was normalised to the respective measure taken at the pre-angiography time (Pre) and expressed as a % increase or decrease respective to this baseline. Data is plotted for all seven study patients and presented as mean ± SD, *p<0.05, **p<0.01, one-way ANOVA, Tukey's multiple comparisons test.

Table 5-3 Characteristics of the early vs late peak groups of GPx activity

Variable	Early peak group (n=8)	Late peak group (n=7)	p value
Age (years)	65 ± 15	58 ± 13	0.34[a]
BMI	27 ± 3.3	27 ± 4.6	0.99[a]
GRACE Scores	114 ± 31	104 ± 27	0.55[a]
Peak hs-TnT (ng/L)	5553 ± 2338	2712 ± 2031	0.03[a]
Symptom onset – Pre sample, (mins)	252 ± 150	244 ± 150	0.82 [a]
Male	7 (88)	5 (71)	0.44[b]
Dyslipidaemia	7 (88)	6 (86)	0.92[b]
Hypertension	4 (50)	4 (57)	0.60[c]
Stroke	1 (13)	1 (14)	0.73[c]
Family History of CAD	2 (25)	3 (43)	0.43[c]
Current Smoker	2 (25)	1 (14)	0.27[c]

Abbreviations: BMI – body mass index; GRACE – global registry of acute coronary events; hs-TnT – high-sensitivity troponin.
[a]Student's t-test, [b]chi-square test, [c]Fisher's exact test, continuous variables expressed as mean ± SD, categorical variables expressed as frequencies (percentages).
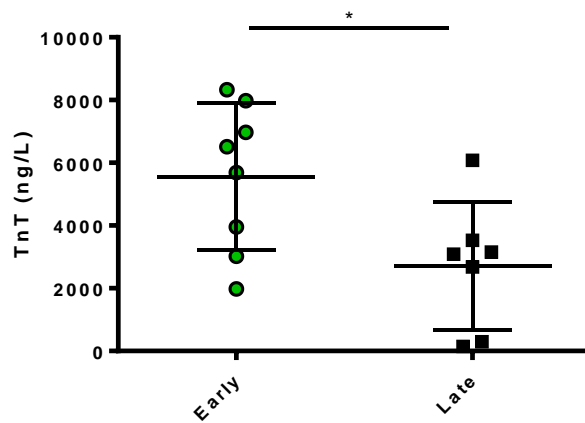
Figure 5-5 Troponin levels in ACS patients

The peak troponin (TnT, ng/L) levels were recorded for each patient. Patients were classified as either having an early peak of GPx activity (Early), or a late peak of GPx activity (Late). Data is plotted for all 16 patients as mean ± SD, *p<0.05, Students unpaired *t*-test.
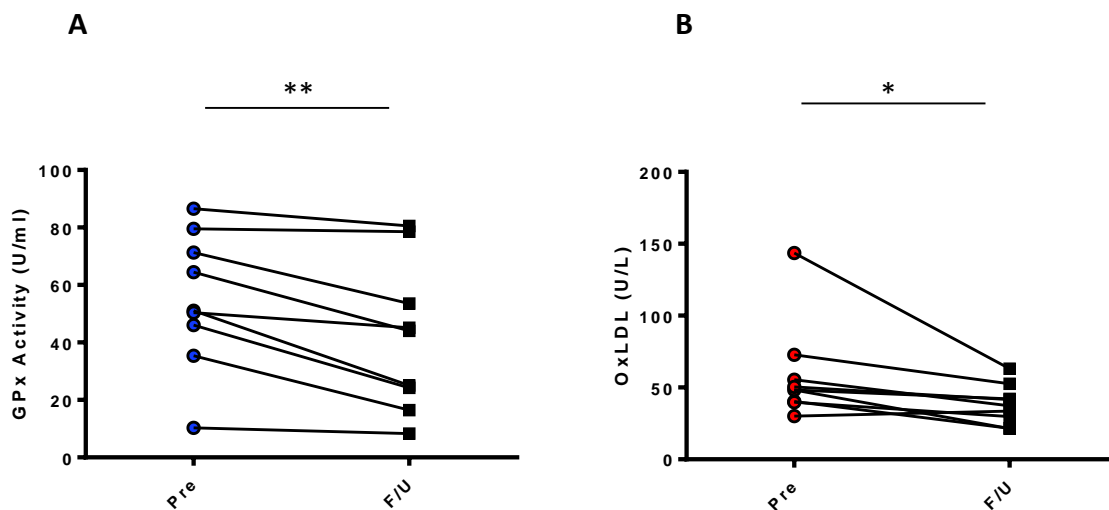
Figure 5-6 GPx activity and oxLDL levels at follow-up vs pre-angiography

(A) GPx activity (U/mL) and (B) oxLDL (U/L) were measured in nine study patients at follow-up (F/U) time points remote from the initial admission (6-12 weeks post-hospital discharge). These values were compared to the values calculated at the pre-angiography (Pre) time point. Data is plotted for all 9 study patients, *p<0.05, **p<0.01, Student's paired *t*-test.

5.4   Discussion

In this study we examined the temporal changes that occur in GPx activity over the acute phase of a STEMI. Corresponding measures of oxLDL were taken to assess whether the fluctuation in GPx activity was reflected in this biomarker of oxidative stress. The results demonstrate that GPx activity is highly dynamic in STEMI patients, and that two distinct patterns of activity could be identified. These were classified as an early peak group and a late peak group. Patients in an early peak group of GPx activity were found to have significantly higher levels of hs-TnT compared to those patients in a late peak group of GPx activity. Further observations concluded that oxLDL was found to be at its highest level either at the same time, or prior to, the GPx activity peak in the majority of patients.

Our observation of two distinct patterns of change in GPx activity following a STEMI was not expected. From this study cohort, 15 of the 16 patients could be characterised into either having an early peak of GPx activity or a late peak of GPx activity, with one patient not demonstrating any obvious GPx peak. There has been no previous description of two distinct time courses of change in GPx activity prior to this, and it is unclear why there are two different patterns of response. We observed that those patients in the early peak group had their highest level of GPx activity recorded within the first 24 hours of the study, whilst those patients in the late peak group had their highest level of GPx activity recorded after 24 hours, making the two groups significantly different from one another.

Patients in an early peak group were found to have higher levels of hs-TnT than a late peak group. This suggests that these patients in an early peak group experienced a larger myocardial infarct. Troponin is a surrogate marker of infarct size that correlates moderately to strongly with cardiac MRI-derived parameters [228]. The size of infarct is important as it correlates with factors such as left ventricular dysfunction [229], and is important for predicting MACE risk in ACS patients.

The magnitude of change in GPx activity seen over time was different between the early and late peak groups. Those in the early peak group had a mean GPx activity that was 10% higher than the pre-angiography value. In contrast those in the late peak group had a mean GPx

activity that was 247% higher than the pre angiography value. We do not know why the magnitude is so different between these two groups. These changes have been calculated with reference to the initial measurement. This measure most likely does not reflect the true baseline of the patient, and it is possible that the patients with a larger infarction had an earlier peak in GPx activity and/or in oxLDL which our initial measure did not capture. Alternatively, it is possible that patients with a larger infarction are not able to increase levels of GPx activity to the same extent as those with a smaller infarct. Although a mechanism cannot be determined for this hypothesis from these studies, it can be speculated that patients with a larger MI may have a greater shift in the net balance of their redox state, predisposing them to greater ROS-mediated damage. This hypothesis would need further investigation, but if this were found to be the case it could partially explain the inverse relationship between GPx activity and MACE observed in Chapter 2.

The relationship between GPx activity and oxLDL appears to be quite complex. Since the scale and magnitude of each measure have an unknown influence on the other, it is difficult to draw conclusions about the potential relationship. In the majority of patients we observed that oxLDL reached its highest level either at the same time point, or prior to, the time point that GPx activity peaked; however, this was not consistent for all patients. Three patients had high levels of oxLDL at time points after GPx activity was reached. Nevertheless, it appears that in the majority of patients the increase in oxidative stress, reflected by the peak oxLDL measures, may be influencing the subsequent increase in GPx activity. However it is entirely possible that the temporal increases seen in oxLDL and GPx are independent of each other and are being driven by other factors that have not been investigated in this study.

Previous studies examining the temporal changes in GPx activity have been limited and have largely focused on patients receiving thrombolytic therapy. To the best of our knowledge, this is the largest study to examine the temporal changes of GPx activity and oxLDL in an ACS cohort undergoing primary PCI. We chose to conduct this study in a cohort of STEMI patients as this allowed us to minimise the time between symptom onset and initial blood collection. Patients presented to the angiography suite less than 12 hours post-symptom onset, and this meant that blood samples could be taken before and after PCI as well as over the course of the hospital admission.

Previous studies examining the temporal change in GPx activity after thrombolytic therapy have yielded variable results. Consistent with our findings, Zachara et al [171] and Beard et al [172] showed a significant rise at 48 hr, and Pucheu et al [230] and Muzkova et al [213] observed a significant rise post thrombolytic therapy. Other studies did not detect any significant differences in GPx activity over the course of an acute event [186, 199, 200]. Previous studies have also examined the temporal changes in oxidative stress over the course of an acute event, using a range of lipid and protein oxidation markers. Those that looked at oxLDL levels found a temporal increase following reperfusion [215, 231] with a decrease back to levels comparable to healthy controls at the time of discharge [214]. The increase in oxLDL is thought to be modulated by an oxidative burst that causes increased ROS production during myocardial ischaemia and subsequently released into the plasma from the injured plaque site [232, 233].

Both GPx activity and oxLDL were found to be significantly lower at the follow-up time point when compared to the pre-angiography measure. At this time point the patients are no longer in the acute stage of an MI, and are more likely to represent the levels seen chronically in stable CAD. The significant difference we detected between these two time points supports the results seen in Chapter 4, in which stable CAD patients had significantly lower levels of both GPx activity and oxLDL compared to the ACS patients. This result is also in accordance with previous literature on ACS patients, in which both GPx activity [186] and oxLDL [214] were reported to decrease back to levels comparable to healthy controls within 30 days of discharge and before the point of hospital discharge.

It has been suggested that GPx activity may be used as a biomarker to predict clinical prognosis [71, 166]. Our results suggest that utilising GPx activity as biomarker may be problematic. Because of the large variability in the level of GPx activity throughout the time course of an ACS, the result obtained will be strongly influenced by sampling time. If the pattern of response was consistent across all patients then it would be possible to estimate when peak levels occur and sample at that time. However as our results demonstrate, there are at least two distinct time points when GPx activity levels peak. This means there is an inability to select a set time point for optimal measurement. Furthermore, the clinical

significance of the two patterns we observe in this study is unknown, and therefore interpreting GPx activity as a biomarker remains challenging. Possibly measuring GPx activity at a time remote from the acute event may yield more consistent levels, but the clinical value of this would need further investigation.

5.4.1 Limitations

The ability to acquire a baseline measure prior to an ACS event occurring in human studies is, of course, not possible. In an effort to overcome this limitation, we selected a sample time that was as close to an ACS admission as logistically possible, followed by a sample time that was remote from the acute event. This allowed us to examine measurements of GPx activity and oxLDL that were likely to represent a level of a more chronic oxidant/antioxidant state and compare them to measures taken throughout an acute setting.

Due to the exploratory and observational nature of the present study, it was not possible to investigate whether there was a causal relationship between GPx activity and levels of oxidative stress. Although we do see similar patterns of temporal change between GPx activity and oxLDL, we are limited to assumptions that oxLDL represents the level of oxidative stress that may be influencing the level of GPx activity in our ACS cohort. Further investigations into the mechanisms that modulate GPx activity in elevated levels of oxidative stress in ACS patients are necessary to determine how it is influenced during an ACS event.

Due to the short follow-up period, it was not possible to examine the relationship between GPx activity, oxLDL levels and clinical events. It would be interesting to determine whether the two patterns of response in GPx activity have a relationship with different clinical outcomes in a larger, and a longer duration study.

Finally, there were issues with three patients being discharged or transferred to regional hospitals early on during the study period. In one case, a patient withdrew consent due to the intrusive nature of repeated blood sampling. Consequently this affected the final numbers in our study population for analysis of GPx activity and oxLDL measurements.

5.5 Conclusion

In conclusion, GPx activity demonstrates considerable variability in the acute phase of an ACS, with two patterns of response identified. Consistent with our findings from Chapter 4, the levels of GPx activity during an ACS admission are significantly higher than those taken at a time remote from the occurrence of an acute event. The temporal changes in oxLDL appear to peak either at the same time, or prior to, the peak in GPx activity in the majority of ACS patients. This finding raises the possibility that GPx activity is driven by changes in oxidative stress levels.

Chapter 6 -   Summary and future directions

6.1   Summary

In this thesis, the first aim was to examine the variability of GPx and SOD activity in an ACS population, and to determine whether this variability was associated with MACE at a 1-year follow-up. To the best of our knowledge, this is the largest prospective cohort study to examine the relationship between GPx and SOD activity and the occurrence of MACE in ACS patients. This study demonstrated a significant inverse relationship between GPx activity and the rate of MACE; however SOD activity was not associated with MACE rate. These findings suggest that patients with lower levels of GPx may have reduced antioxidant defence, increasing their risk for oxidant-mediated damage and predisposing them to MACE. ROC curve analysis found GPx to be a moderate predictor of MACE (AUC=0.62). However the ROC curve did not identify a single cutoff value for GPx activity that predicted the occurrence of MACE, making its application as a biomarker in ACS patients challenging.

The second study examined the hypothesis that low levels of GPx activity may be associated with high levels of platelet reactivity and ROS, therefore exploring a possible mechanism contributing to the higher rate of MACE experienced by a subset of patients. Although GPx activity was not correlated with platelet reactivity in this study, this is possibly due to GPx only having a small effect in the setting of ACS, in which there are other more significant determinants of platelet reactivity overriding any effect of GPx activity.

Increasing levels of GPx activity were found to be associated with a larger shift of inhibition of platelet aggregation mediated by SNO-Glu. A large inhibition of platelet aggregation suggests a lower level of ROS is present due to SNO-Glu's inhibitory properties being less impaired. The inverse relationship observed between GPx activity and ROS levels is consistent with the hypothesis from Chapter 2 in that GPx activity plays a protective role in an ACS population by decreasing ROS-mediated damage that may predispose patients to MACE occurrence.

In the third study, we observed ACS patients to have higher levels of GPx activity and higher GPx3 protein concentration, with corresponding increases in GPx1, GPx3 and GPx4 mRNA expression levels. This compared to patients with stable CAD and healthy subjects, in which no statistically significant differences were detected in the GPx system. Additionally, levels

of oxLDL were found to be higher in ACS patients compared to patients with stable CAD and healthy subjects. Taken together, this study demonstrated that both the GPx system and levels of oxidative stress are elevated in ACS. Whether this level is chronically elevated throughout the ACS could not be determined from this study.

In the fourth set of experiments, GPx activity proved to be highly dynamic in a STEMI population, with no single consistent time point at which activity peaked. Two distinct patterns of activity were observed, with some patients exhibiting an early peak in GPx activity (before 24 hr), whilst others exhibited a late peak (after 24 hr). Patients with an early peak of GPx were found to have a smaller magnitude of change in their activity levels compared to those patients with a late peak of GPx. Furthermore, patients with an early peak of GPx activity had higher peak levels of troponin, suggesting a larger myocardial infarction. The relationship between levels of GPx activity and oxLDL appeared to be quite complex. However, in the majority of STEMI patients, peak levels of oxLDL occurred prior to, or at the same time, as the peak GPx activity level. This suggests that the levels of GPx activity are modulated by increases in oxidative stress. The clinical significance of the two response patterns of GPx activity requires further investigation.

6.2 Limitations

Throughout this thesis, there were unavoidable limitations that may have influenced our findings. Our study populations were of a moderate size, and in some instances this reduced our ability to investigate relationships between GPx, oxidative stress measures, clinical factors and outcomes. However, with time constraints of the thesis and factoring for follow-up time allowances, recruiting larger patient cohorts was not possible.

One particular limitation that is still prevalent in the current literature is the lack of a standardised marker for ROS that reflects the levels of oxidative stress *in vivo*. This thesis utilised both an indirect surrogate marker for ROS levels, SNO-Glu, and the frequently used oxidative stress marker, oxLDL. However, neither measure are direct indicators of the oxidant environment in the vasculature therefore have limitations when quantifying oxidative stress levels. A novel SNO-Glu oxidative status assay was developed in this research project. SNO-

Glu is based on platelet function measures, therefore highly dependent on the type of antiplatelet medication patients are treated with. The variability of response to antiplatelet agents adds an additional unknown variance to the SNO-Glu assay that would need to be investigated more thoroughly. Nonetheless, the SNO-Glu assay does have reasonable potential to be a useful measure for quantifying ROS in a clinical setting.

OxLDL is a frequently used marker of oxidative stress that is widely published throughout the literature. However it is a single marker representing the extent of lipid and protein oxidation. There is conflict in the literature surrounding its accuracy for quantifying the extent of oxidative stress in the vasculature and therefore a lack of understanding in its role in CAD progression [6, 232, 234]. Additionally, statin medication has been reported to variably affect the levels of oxLDL measured in CAD patients [235]. This thesis highlights that there still remains a clear need for an appropriate and accurate oxidative stress measure that can be sampled from the peripheral circulation and applied in a clinical setting.

GPx activity is widely quantified using commercially available colourimetric activity kits. Although the particular kit used in our study was thoroughly validated and showed a high reproducibility, colourimetric analysis still has limitations. Interferences with absorbance readings can arise from sample turbidity, bubbles in the samples, and similar colours from interfering substances adding an unknown variance. Care must be taken when performing these tests to ensure precision and reproducibility in the results. The measure of GPx activity is an indirect method, with the oxidation of NADPH to NAD^+ accompanied by a decrease in absorbance at 340 nm that is proportional to the level of GPx activity. A direct measure of GPx activity would be more reliable; however such a method is not yet available commercially.

The biochemical assays used in this thesis were commercially sourced from reputable biomedical companies and their use has been widely published in the literature. The aim of this thesis was not to develop new measurement tools for oxidative stress and the GPx enzyme. Instead, it was to use the appropriate pre-existing tools and apply them in a clinical context.

One of the strengths of this thesis has been the recruitment of a high-risk patient cohort. Each cohort has been characterised in considerable detail, and as described in Chapter 2, carefully and thoroughly followed up.

6.3 Clinical implications and future directions

The findings from this thesis have several clinical implications and raise further avenues for investigation. In addition, some of our observations may further explain why there has been such discrepancy in the literature examining the relationship between GPx activity and the risk of adverse clinical events.

First, we observe that there is a distinct increase in the GPx system in ACS patients compared to stable CAD patients and healthy subjects. Additionally there is a corresponding increase in oxLDL levels in ACS patients compared to the stable CAD patients and healthy subjects. This highlights the importance of identifying and distinguishing the stage of coronary artery disease of patients when studying the effects of GPx activity as the levels appear to be influenced by the disease state.

Second, we identified that variability in levels of GPx activity between ACS patients was associated with the rate of MACE at 1-yr. When examining the temporal changes in GPx activity over the acute phase of a STEMI we demonstrated that there was also significant variability both within and between patients. The dynamic nature of the GPx activity observed meant that no consistent time point at which peak GPx activity occurred could be identified. The marked variation in GPx activity that occurred with the different sampling times in those with ACS may help explain the divergent findings regarding the relationship between GPx activity and the risk of adverse clinical events, as GPx activity will be strongly influenced by sampling time. As discussed in Chapter 5, there are only a handful of studies that have examined GPx activity over the time period similar to our design, with the majority carried out in patients who received thrombolytic agents. This is in contrast to our study cohort who received primary PCI within 12 hours of ischaemic symptom onset. It is possible the different reperfusion treatments contribute to the variable reports in the literature,

however further investigation would be needed to thoroughly answer this question. Additionally, the clinical significance of the novel finding of two distinct patterns of response we observed in GPx activity reported in Chapter 5 requires further investigation.

What is driving the type of response as well the magnitude of response in GPx activity over an ACS event is unknown. We demonstrate significantly higher levels of troponin in patients with an early peak of GPx activity, suggesting these patients experience a larger myocardial infarction. The magnitude of change in GPx activity at this time point was lower than the change observed in a late peak of GPx activity. It is possible that ACS patients with lower levels of GPx activity experience a bigger infarct, therefore contributing to the relationship we observed between low levels of GPx activity and a higher rate of MACE. Further investigations into this relationship would help to answer this hypothesis.

Although we currently do not understand the cause of the variability in GPx levels, we noted that both GPx activity and oxLDL are elevated in those with ACS. The temporal changes in oxLDL appear to coincide with the temporal changes in GPx activity in the majority of patients, suggesting that GPx activity may be responding to changes in oxidative stress. However the proportional changes that occur between these two parameters in an ACS event, or whether there is a causal relationship, cannot be determined from our studies. Determining the proportional change is important as oxidative stress is essentially the change in the redox balance that predisposes the patient to oxidative damage. So although we have demonstrated that both pro-oxidant and antioxidant measures are higher in an ACS event, from our oxLDL and GPx measures respectively, we cannot determine what the net redox balance between the two systems is in those with ACS. Essentially our ROS marker from Chapter 3 can be used as a representation for the redox balance. We demonstrated that high levels of ROS were correlated with lower levels of GPx activity in ACS patients, and suggested that this may partially contribute to the higher rates of MACE observed in this group in Chapter 2. Even though all the ACS patients from the Chapter 2 study cohort may have had increased levels of GPx activity in response to the event, it is feasible to suggest that some ACS patients do not up-regulate their GPx activity in response to an oxidative challenge to the same extent as others. Thus the net change in redox balance may differ within a population.

As our results demonstrated, it is those patients with low levels of GPx activity that are most at risk of MACE. If there were indeed a group of patients in which GPx activity does not increase adequately during an ACS event, either due to infarct size, or to an as yet unestablished factor, then potentially this group of patients may benefit from an intervention strategy, such as supplemental antioxidant therapy. We suggest that GPx activity has potential to identify this population who may benefit from targeted therapy. However the standardisation of measuring GPx activity as a biomarker remains problematic. The Atherogene study prospectively studied GPx activity in a large population with suspected chronic coronary artery disease. This study concluded that GPx activity was predictive of CVD events after a 5-yr follow-up [71], therefore it is possible that measurement of GPx activity at a time remote from an acute event may have merit. In a clinically stable population, GPx activity may be more constant and exhibit less variance. However, this may limit the ability to intervene in an ACS population with low levels of GPx activity who exhibit a higher risk of poorer outcomes. Further investigation into the dynamics of GPx activity in a stable coronary disease population, and the clinical impact it has, is warranted to establish GPx as a risk biomarker or as a tool to direct intervention in individual patients.

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Appendix 1 – Health and Disability Ethics Committee Information

**Health
and
Disability
Ethics
Committees**

**Lower South Regional Ethics
Committee**
Ministry of Health PO Box 5013 1 The
Terrace Wellington
Phone: (04) 816 2646 Email:
lowersouth_ethicscommittee@moh.g
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16 December 2011

Dr Scott Harding
Capital and Coast District Health Board
Cardiology Department
Wellington Hospital
Private Bag 7902
Wellington

Dear Dr Harding

Re: Ethics ref: **LRS/11/09/035**   (please quote in all correspondence)

Study title: Platelet reactivity and adverse outcomes in patients undergoing coronary angiography

Investigators: Dr Scott Harding, Dr Alexander Sasse, Associate Professor Peter Larsen, Ms Anne Camille La Flamme, Mr Michael Chen-xu

Approved site: Wellington Hospital - Capital and Coast District Health Board

This study was given ethical approval by the Lower South Regional Ethics Committee on **16 December 2011**. A list of members of the Committee is attached.

Approved Documents

- National Application Form (with amendments)
- Signed Part 4: Declaration for Dr Scott Harding, dated 17 August 2011
- Completed Form A for Wellington Hospital, CCDHB dated 23 August 2011
- Part 5: Use of Human Tissue
- Part 6: Genetic Section
- Participant Information Sheet - Platelet reactivity and adverse outcomes in patients undergoing coronary angiography: Version 2, dated 25 September 2011
- Participant Consent Form - Platelet reactivity and adverse outcomes in patients undergoing coronary angiography: Version 2, dated 25 September 2011
- Participant Information Sheet - Platelet reactivity and adverse outcomes in patients undergoing coronary angiography - Genetic Sub-Study: Version 2, dated 25 September 2011
- Participant Consent Form - Platelet reactivity and adverse outcomes in patients undergoing coronary angiography - Genetic Analysis Sub-Study: Version 2, dated 25 September 2011
- Platelet reactivity and adverse outcomes in patients undergoing coronary angiography - 30 Day Follow-Up Data Form
- Platelet reactivity and adverse outcomes in patients undergoing coronary angiography - 12-Month Follow-Up Data Form
- Evidence of Maori Consultation - letter dated 27 November 2011 signed by Jack Rikihana on behalf of the Research Advisory Group - Maori

This approval is valid until **16 December 2016**, provided that Annual Progress Reports are submitted (see below).

Access to ACC

For the purposes of section 32 of the Accident Compensation Act 2001, the Committee is satisfied that this study is not being conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the trial is being carried out. Participants injured as a result of treatment received in this trial will therefore be eligible to be considered for compensation in respect of those injuries under the ACC scheme.

Amendments and Protocol Deviations

All significant amendments to this proposal must receive prior approval from the Committee. Significant amendments include (but are not limited to) changes to:

— the researcher responsible for the conduct of the study at a study site

— the addition of an extra study site

— the design or duration of the study

— the method of recruitment

— information sheets and informed consent procedures.

Significant deviations from the approved protocol must be reported to the Committee as soon as possible.

Annual Progress Reports and Final Reports

The first Annual Progress Report for this study is due to the Committee by **16 December 2012**. The Annual Report Form that should be used is available at www.ethicscommittees.health.govt.nz. Please note that if you do not provide a progress report by this date, ethical approval may be withdrawn.

A Final Report is also required at the conclusion of the study. The Final Report Form is also available at www.ethicscommittees.health.govt.nz.

Requirements for the Reporting of Serious Adverse Events (SAEs)

SAEs occurring in this study must be individually reported to the Committee within 7-15 days only where they:

— are *unexpected* because they are not outlined in the investigator's brochure, and

— are not defined study end-points (e.g. death or hospitalisation), and

— occur in patients located in New Zealand, and

— if the study involves blinding, result in a decision to break the study code.

There is no requirement for the individual reporting to ethics committees of SAEs that do not meet all of these criteria. However, if your study is overseen by a data monitoring committee, copies of its letters of recommendation to the Principal Investigator should be forwarded to the Committee as soon as possible.

Please see www.ethicscommittees.health.govt.nz for more information on the reporting of SAEs, and to download the SAE Report Form.

Statement of compliance

The committee is constituted in accordance with its Terms of Reference. It complies with the *Operational Standard for Ethics Committees* and the principles of international good clinical practice.

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The committee is approved by the Health Research Council's Ethics Committee for the purposes of section 25(1)(c) of the Health Research Council Act 1990.

We wish you all the best with your study.

Yours sincerely

Kirsten Forrest
Administrator
Lower South Ethics Committee
Email: LowerSouth_ethicscommittee@MOH.govt.nz

INFORMATION SHEET

Platelet reactivity and adverse outcomes in patients undergoing coronary angiography

You are invited to take part in a study that is investigating how effective two drugs we commonly use to limit blood clot formation, aspirin and clopidogrel, are in patients who have an acute coronary syndrome (heart attack). We are interested in learning more about why these drugs are not as effective in some patients as they are in others, and examining whether having a less effective response to these drugs is associated with an increased risk of adverse events following coronary angiography. This study has received ethical approval from the Central Region Human Ethics Committee.

Principal Investigator
<u>Dr Scott Harding</u>, Cardiologist, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No. ███████████
E-mail: ████████████████████

How are people selected for this study, and who will select them?
Patients who are undergoing coronary angiography after a heart attack will be invited to participate in this study by Ana Holley or Michael Chen-Xu.

What will happen during the study?
If you are happy to participate in the study you will have blood taken from you before you have your coronary angiogram. This blood will be taken by inserting a needle into a vein on your arm. When we do this we will be taking 30ml (about 6 teaspoons) in volume. Your blood will be tested for platelet function (how the blood clots) and serum will be extracted. The serum will be tested stored for later analysis of biological markers that will tell us about the state of your immune system. Any residual serum will be destroyed following this. Prior to tissue being disposed of (using the standard Hospital process) a Maori blessing will be performed.

With your permission, data from this study may be used in future related studies, which have been given ethical approval from a Health & Disability Ethics Committee.

As samples of human tissue will be taken during this study, there may be cultural issues associated with storing tissue that need to be discussed with your family/whanau. Some Iwi disagree with storage of human tissue citing whakapapa and advise their people to consult prior to participation in research where this occurs. To avoid problems at a later stage, we suggest your family/whanau is involved with you at all stages of the research. However, we also acknowledge that individuals have the right to choose to participate.

Confidentiality
All data will be kept confidential. You will not be personally identified in any reports or publications that are developed throughout the course of this research. The data will appear grouped and include no markings that can be traced back to you.

Participants Rights
You do not have to accept this invitation to participate in this research.
 If you agree to participate you have the right to:
 1. Ask any questions about the study anytime during your participation
 2. Withdraw from the study at anytime without any affect on your future health care/continuing health care at any time

Participation in this study will be stopped should the investigators of this study feel it is not in your best interests to continue.

What are the potential inconveniences of the study?
Insertion of a needle into a vein can result in some swelling around the area, and this area may be a little tender after the needle is removed. It is possible that you could get an infection due to insertion of the needle, although this is not common and we will take standard precautions to minimise this risk.

What are the benefits of this study?
This study will provide significant knowledge about how well anti-clotting drugs are working in patients who have had a heart attack.

There will be no direct benefit to you from participating in this study.

Costs for the study
It will not cost anything to take part in this study.

Family or Whanau support
You may have a friend, family or whanau support present.

For Maori Health Support please contact Whanau Care Services on 04 3855 999 ext ███

If you have any questions or concerns about your rights as a participant in this research study you can contact an independent health and disability advocate. This is a free service provided under the Health and Disability Commissioner Act.
Telephone: (NZ wide) 0800 555 050
Free Fax (NZ wide): 0800 2787 7678 (0800 2 SUPPORT)
Email (NZ wide): advocacy@hdc.org.nz

Results
A written report of the study will be available to participants on request.

Compensation
In the unlikely event of a physical injury as a result of your participation in this study, you will be covered by the accident compensation legislation with its limitations. If you have any

questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

Please feel free to contact the Study Investigators if you have any questions about this study. Their contact details are listed above.

Statement of Approval
This project has been given ethical approval by the Lower South Regional Ethics Committee. If you have any concerns about the ethics of this study, please contact the Lower South Regional Ethics Committee by email lowersouth_ethicscommittee@moh.govt.nz.

Co-investigators
Working with Dr Harding is the research team as follows:

Dr Alexander Sasse, Cardiologist, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No. ███████
Email: █████████████████

Assoc Prof Peter Larsen Clinical Physiologist, University of Otago, Wellington
Work Phone ██████████ extn ████
E-mail: ████████████████

Assoc. Prof Anne La Flamme, Senior Lecturer, School of Biological Sciences, Victoria University of Wellington
Work Ph: ██████████
E-mail: ████████████████

Michael Chen-Xu, MBChB Student University of Otago, Wellington
Work Phone ██████████ extn ████
E-mail: █████████████████

Ana Holley PhD Student, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No: ███████
E-mail: ████████████████

Participant Consent Form

Platelet reactivity and adverse outcomes in patients undergoing coronary angiography

- I have read and I understand the information sheet dated 22nd August 2011 for volunteers taking part in the study designed to study platelet reactivity in coronary angiography. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

- I have had the opportunity to use whānau support or a friend to help me ask questions and understand the study.

- I understand that taking part in this study is voluntary (my choice), and that I may withdraw from the study at any time, and this will in no way affect my future health care/continuing health care.

- I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study or in any subsequent study that may use the aggregated results.

- I understand the compensation provisions for this study.

- I have had time to consider whether to take part in the study.

- I know who to contact if I have any side effects from the study.

- I consent to the use of my data for future related studies, which have been given ethical approval from a Health & Disability Ethics Committee.

I wish to receive a copy of the results.   Yes ☐   No ☐

If yes, please include a postal or e-mail address to which you would like the summary report to be sent when the study has been completed.

I _____ hereby consent to take part in this study.

Date:

Signature:

Full names of researchers:

Contact phone number for researchers:

Project explained by:

Project role:

Signature:

Date:

Platelet reactivity and adverse outcomes in patients undergoing coronary angiography – Genetic Sub-Study

Principal Investigator
Dr Scott Harding, Cardiologist, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No. ████████
E-mail: ████████████████

Introduction
You have already agreed to take part in the main research study, and have signed the consent form. In the main study we will investigate the relationship between high on treatment residual platelet reactivity and rise in cardiac enzymes during coronary angiography. In addition, we will examine the factors associated with an increased risk of high on treatment platelet reactivity in acute coronary syndromes

We do not know exactly why some individuals are more likely to respond to the antiplatelet medications than other individuals. However, it has been suggested that there may be a genetic reason for the differences in response to the drugs.

Purpose of the Sub-Study
The purpose of this study is to find out to what extend the differences in response to clopidogrel are due to genes, which are found in your DNA. It has been suggested that some people have an alteration in the gene that metabolises clopidogrel, and that this could contribute to the variable response to this drug. By studying the DNA from your blood samples, researchers can try to understand how much of the high on treatment platelet reactivity is due to genetics.

Explanation of Study
This study involves obtaining a single blood sample for DNA 10 ml (about 2 teaspoons) which will be used for research purposes only. The blood sample will be taken at the same time that you require blood samples for the main research study. Your blood will be taken to the Clinical Research Laboratory in Wellington Hospital, where DNA will be extracted from your blood. This DNA will be stored in the Laboratory under strict supervision in a limited access facility.

As samples of human tissue will be taken during this study, there may be cultural issues associated with storing tissue that need to be discussed with your family/whanau. Some Iwi disagree with storage of human tissue citing whakapapa and advise their people to consult prior to participation in research where this occurs. To avoid problems at a later stage, we suggest your family/whanau is involved with you at all stages of the research. However, we also acknowledge that individuals have the right to choose to participate.

The DNA samples will be used to study various genetic causes for how patients may respond to clopidogrel and aspirin. The DNA samples will be stored to provide a resource for future studies that we will conduct on the genes responsible for how these drugs are processed by the body, how the drugs work, and other pathways that the drugs may interact with. Your blood cells will not be made to grow forever in the laboratory. Any future use of the genetic samples outside of this project will first be reviewed by a Health & Disability Ethics Committee.

The DNA samples will be stored for future testing for up to 10 years from the time the sample is taken, or until the sample is gone. When this 10 year period ends, any remaining DNA will be destroyed, and the destruction will be documented.

If any future testing is performed using your sample, and is within the scope of this consent form, no additional informed consent will be obtained from you, and you will not be notified.

Disclosure of Research Results

Many genetic studies are exploratory research studies, and may not yield information that will be clinically useful to patients for some time. Therefore, no information obtained from these studies will be disclosed to you, your family or your doctors. Research from this sub-study will not become part of your medical records.

Risks of Participation

The blood sample will be taken at the same time as a routine blood test is obtained, and therefore will not represent an additional risk for you. The amount of blood taken is small, and will not be harmful.

Benefits of Participation

You will receive no direct benefit from participating in this study. However, patients treated in the future with these drugs may benefit from information learned from your participation.

Confidentiality

All identifying information collected in this study will be kept strictly confidential. If any publication arises from this research, you will not be identified in by name. It is possible that if people who are not involved in this research knew your genetic information, this may cause problems with your employment or insurance. The chance that taking part in this research will harm you is very small.

To protect your privacy we will only use a subject number, instead of your name, on your blood sample. Your name will not be disclosed outside of the research clinic, where your information will be treated as strictly confidential. No genetic information will be entered into your medical record.

Participants Rights

You do not have to accept this invitation to participate in this research.

If you agree to participate you have the right to ask any questions about the study anytime during your participation.

Termination of Participation

If, at any time, you should choose to withdraw from participation in this study, all DNA samples from you will be removed from storage and destroyed. However, data obtained from your DNA prior to withdrawal of consent will not be deleted.

Costs for the study

It will not cost anything to take part in this study.

Family or Whanau support

You may have a friend, family or whanau support present.

For Maori Health Support please contact Whanau Care Services on 04 3855 999 ext ███

If you have any questions or concerns about your rights as a participant in this research study

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you can contact an independent health and disability advocate. This is a free service provided under the Health and Disability Commissioner Act.
Telephone: (NZ wide) 0800 555 050
Free Fax (NZ wide): 0800 2787 7678 (0800 2 SUPPORT)
Email (NZ wide): advocacy@hdc.org.nz

Results
A written report of the study will be available to participants on request.

Compensation
In the unlikely event of a physical injury as a result of your participation in this study, you will be covered by the accident compensation legislation with its limitations. If you have any questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.

If you have any questions about ACC, contact your nearest ACC office or the investigator. Please feel free to contact the Study Investigators if you have any questions about this study. Their contact details are listed below.

<u>Statement of Approval</u>
This project has been given ethical approval by the Lower South Regional Ethics Committee. If you have any concerns about the ethics of this study, please contact the Lower South Regional Ethics Committee by email <u>lowersouth_ethicscommittee@moh.govt.nz</u>.

Co-investigators
Working with Dr Harding is the research team as follows:

<u>Dr Alexander Sasse</u>, Cardiologist, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No. █████████
Email: ███████████████

<u>Assoc Prof Peter Larsen</u> Clinical Physiologist, University of Otago, Wellington
Work Phone ███████ extn ███
E-mail: ███████████

<u>Assoc. Prof Anne La Flamme</u>, Senior Lecturer, School of Biological Sciences, Victoria University of Wellington
Work Ph: ██████
E-mail: ███████████

<u>Michael Chen-Xu</u>, MBChB Student University of Otago, Wellington
Work Phone ███████ extn ███
E-mail: █████████████

<u>Ana Holley</u> PhD Student, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No: ██████
E-mail: ██████████

Participant Consent Form

Platelet reactivity and adverse outcomes in patients undergoing coronary angiography – Genetic Analysis Sub Study

- I have read and I understand the information sheet dated 22nd August 2011 for volunteers taking part in the sub-study designed to investigate genetic variants associated with high platelet reactivity. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

- I have had the opportunity to use whānau support or a friend to help me ask questions and understand the study.

- I understand that taking part in this study is voluntary (my choice), and that I may withdraw from the study at any time, and this will in no way affect my future health care/continuing health care.

- I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study or in any subsequent study that may use the aggregated results.

- I understand the compensation provisions for this study.

- I have had time to consider whether to take part in the study.

- I know who to contact if I have any side effects from the study.

- I consent to the use of my data for future related studies, which have been given ethical approval from a Health & Disability Ethics Committee.

I give consent to have my blood stored for analysis of DNA for this study, and understand that following 10 years any remaining sample will be destroyed:

Yes ☐   No ☐

I wish to receive a copy of the results.   Yes ☐   No ☐

If yes, please include a postal or e-mail address to which you would like the summary report to be sent when the study has been completed.

_____
_____
_____
_____

I _____ hereby consent to take part in this study.

Date:

Signature:

Full names of researchers:

Contact phone number for researchers:

Project explained by:

Project role:

Signature:

Date:

**Health
and
Disability
Ethics
Committees**

07 March 2013

Dr Scott Harding
Cardiology
Research
Wellington Hospital
Newtown/Wellington
6023

Dear Dr Harding

Re:	**Ethics ref:**	**13/CEN/29**
	Study title:	Investigation of the glutathione peroxidase system in stable coronary disease and healthy controls

I am pleased to advise that this application has been _approved_ by the Central Health and Disability Ethics Committee. This decision was made through the HDEC-Expedited Review pathway.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the Central Health and Disability Ethics Committee is required.

Standard conditions:

· Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.

· Before the study commences at *a given* locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the *Standard Operating Procedures for Health and Disability Ethics Committees* (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

Participant access to ACC
The Central Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC)

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Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,

Helen Walker
Chairperson
Central Health and Disability Ethics Committee

Encl: appendix A: documents submitted
        appendix B: statement of compliance and list of members

PARTICIPANT INFORMATION SHEET

Investigation of the glutathione peroxidise system in stable coronary disease and healthy subjects

Principal Investigator
Dr Scott Harding, Cardiologist, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No. ████████
E-mail: ██████████████████

You are invited to take part in a study that is investigating the levels of antioxidants in the blood stream. Oxidative stress is involved in the onset and progression of atherosclerosis (the build up of fatty deposits in the blood vessels), angina and heart attacks. Antioxidants in the blood stream such as glutathione peroxidase (GPx) provide a protective defence against high levels of oxidative stress and may slow the progression of atherosclerosis. We aim to investigate whether levels of GPx and other antioxidants vary in healthy volunteers and those with stable coronary disease. We will also aim to investigate how such variations are related to measures of oxidative stress and disease state.

How are people selected for this study, and who will select them?

Patients who have confirmed stable coronary disease will be invited to partake in this study. Healthy volunteers will also be invited to participate and will be invited to partake in this study through poster advertisement distributed throughout Wellington Hospital and Otago Medical School.

What will happen during the study?

If you are happy to participate in this study then you will have a single blood test. This blood will be taken by inserting a needle into a vein in your arm. When we do this we will be taking 30 mL (about 6 teaspoons) in volume. Your blood will be tested for platelet function (how the blood clots) and various components of the blood will be tested for GPx and markers of oxidative stress and inflammation. Any residual blood or components of blood will be destroyed following this. Prior to tissue being disposed of, (using the standard Hospital process) a Maori blessing will be performed.

As samples of human tissue will be taken during this study, there may be cultural issues associated with storing tissue that need to be discussed with your family/whanau. Some Iwi disagree with storage of human tissue citing whakapapa and advise their people to consult prior to participation in research where this occurs. To avoid problems at a later stage, we suggest your family/whanau is

involved with you at all stages of the research. However, we also acknowledge that individuals have the right to choose to participate.

Confidentiality

All data will be kept confidential. You will not be personally identified in any reports or publications that are developed throughout the course of this research. The data will appear grouped and include no markings that can be traced back to you.

Participant Rights

You do not have to accept this invitation to participate in this research. If you agree to participate you have the right to:

1. Ask any questions about the study anytime during your participation
2. Withdraw from the study at anytime without any affect on your future health care/continuing health care at anytime.

Participation in this study will be stopped should the investigators of this study feel it not in your best interests to continue.

What are the potential inconveniences of this study?

Insertion of a needle into a vein can result in some swelling around the area, and this area may be a little tender after the needle is removed. It is possible that you could get an infection due to insertion of the needle. However this is very rare and we will take standard precautions to minimise this risk.

What are the benefits of this study?

This study will provide significant knowledge about the variation in the GPx system and oxidative stress in a healthy population, and those with stable coronary artery disease. We believe that those with low levels of GPx will have higher levels of oxidative stress. If this is confirmed then the effect of targeted antioxidant therapies in those with low levels of GPx could be explored.

There will be no direct benefit to you from participating in this study.

Costs for the study

It will not cost anything to take part in the study.

Family or Whanau support

You may have a friend, family or whanau support present.

For Maori Health Support please contact Whanau Care Services:
Trish Heuser - Speciality Clinical Nurse Cardiology 04 3855 999 ext ████

If you any questions or concerns about your rights as a participant in this research study you can contact an independent health and disability advocate. This is a free service provided under the Health and Disability Commissioner Act:
Telephone: (NZ wide) 0800 555 050

Free Fax: (NZ wide) 0800 2787 7678 (0800 2 SUPPORT)
Email: (NZ wide) advocacy@hdc.org.nz

Results

A written report of the study will be available to participants on request.

Compensation

In the unlikely event of a physical injury as a result of participation in this study, you will be covered by the accident compensation legislation with its limitations. If you have any questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

Please feel free to contact the Study Investigators if you have any questions about this study. Their contact details are listed above and below.

Statement of Approval

This project is awaiting ethical approval by the Central Health and Disability Ethics Committee.

Co-investigators
Working with Dr Harding is the research team as follows:

Assoc. Prof Peter Larsen, Clinical Physiologist, University of Otago, Wellington
Work phone ▬▬▬▬▬ extn ▬▬
E-mail: ▬▬▬▬▬▬▬▬▬

Prof John Miller, Physiologist, Victoria University of Wellington
Work phone ▬▬▬▬
Email: ▬▬▬▬▬▬▬

Ana Holley, PhD Student, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone ▬▬▬▬
Email: ▬▬▬▬▬▬▬

Lisa Johnston, PhD Student, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone ▬▬▬▬
Email: ▬▬▬▬▬▬▬

Richard Portch, Masters Student, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone ▬▬▬▬
Email: ▬▬▬▬▬▬▬▬

Participant Consent Form

Investigation of the glutathione peroxidase system in stable coronary disease and healthy subjects

- **Request for interpreter:**   **Circle one**

English	I wish to have an interpreter	Yes	No
Deaf	I wish to have a NZ sign language interpreter	Yes	No
Māori	E hiahia ana ahau ki tetahi kaiwhaka Māori/kaiwhaka pakeha korero	Ae	Kao
Cook Island Māori	Ka inangaro au i tetai tangata uri reo	Ae	Kare
Fijian	Au gadreva me dua e vakadewa vosa vei au	Io	Sega
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu	E	Nakai
Sāmoan	Ou te mana'o ia i ai se fa'amatala upu	Ioe	Leai
Tokelaun	Ko au e fofou ki he tino ke fakaliliu te gagana Peletania ki na gagana o na motu o te Pahefika	Ioe	Leai
Tongan	Oku ou fiema'u ha fakatonulea	Io	Ikai

- I have read and I understand the information sheet dated 11th February 2013 for volunteers taking part in the study designed to study the glutathione peroxidase system in atherosclerosis. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.
- I have had the opportunity to use whanau support or a friend to help me ask questions and understand the study.
- I understand that taking part in this study is voluntary (my choice), and that I may withdraw from the study at any time, and this will no way affect my future health care/continuing health care.
- I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.
- I understand the compensation provisions for this study.
- I have had time to consider whether to take part in the study.
- I know who to contact if I have any side effects from the study.

I wish to receive a copy of the results.　　　Yes ☐　　　No ☐

I _____ (Participant's full name) hereby consent to take part in this study and have received a copy of the signed consent form for my own records.

_____　　　　　　　　　　　　　_____

Participant's　　　　　　　　　　　　　　　　　　　　　　　　Date

_____

Investigator/Researcher who explained

_____　　　　　　　　　　　　　_____

Investigator/Researcher Signature　　　　　　　　　　　　　　Date

If applicable:

Printed name of Interpreter
_____

Signature of Interpreter　　　　　　　　　　Date

_____　　　　　　　_____

---

10 December 2014

Dr Scott Harding
Cardiology Research
Wellington Hospital
Newtown/Wellington 6023

Dear Dr Harding

Re:	**Ethics ref:**	**14/NTB/198**
	Study title:	Investigation of the Dynamic Relationship of Antioxidant Enzymes in Myocardial infarction.

I am pleased to advise that this application has been _approved_ by the Northern B Health and Disability Ethics Committee. This decision was made through the HDEC-Full Review pathway.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the
Northern B Health and Disability Ethics Committee is required.

Standard conditions:

·   Before the study commences at _any_ locality in New Zealand, all relevant regulatory approvals must be obtained.

·   Before the study commences at _a given_ locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the _Standard Operating Procedures for Health and Disability Ethics Committees_ (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

Your next progress report is due by 10 December 2015.

<u>Participant access to ACC</u>

The Northern B Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC).

Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,

███████████

Mrs Raewyn Sporle
Chairperson
Northern B Health and Disability Ethics Committee

Encl: appendix A: documents submitted
        appendix B: statement of compliance and list of members

PARTICIPANT INFORMATION SHEET

The DREAM Study

Dynamic **R**elationship of antioxidant **E**nzymes in **A**cute **M**yocardial infarction

Principal Investigator

Dr Scott Harding, Cardiologist, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No. ▮▮▮▮▮▮▮   E-mail: ▮▮▮▮▮▮▮▮▮▮▮▮▮

You are invited to take part in a study that is investigating the levels of oxidative stress in the blood stream. High levels of oxidative stress are found in patients experiencing heart attacks, and are known to cause damage to the blood vessels. The antioxidant enzyme glutathione peroxidase (GPx) is known to protect against oxidative stress and may slow the progression of damage. We aim to investigate how GPx and oxidative stress increase during a heart attack, as this is not well understood.

Who is selected and what will happen in this study?

Patients who present to Wellington Hospital diagnosed with a ST-segment elevation myocardial infarction (STEMI), commonly known as a heart attack, will be invited by one of the study investigators to participate in this study. If you are happy to take part, we will initially take a 10 mL blood sample prior to and immediately after your angiogram. This will be done through a sheath that is inserted for the procedure. After the procedure, you will be given the full information sheet. You will then be able to ask further questions before continuing in this study. If you choose to participate in this study, your blood will be tested for GPx activity and markers of oxidative stress. If you choose to not participate further at this time, then any blood samples will be destroyed. None of your personal information will be used.

Confidentiality

All data will be kept confidential. You will not be personally identified in any reports or publications throughout the course of this research. The data will appear grouped with no markings that can be traced back to you.

Participant Rights

You do not have to accept this invitation to participate in this research. If you agree to participate, you have the right to:

1. Ask any questions about the study anytime during your participation

2. Withdraw from the study at anytime without any affect on your future healthcare/continuing health care at anytime.

Participation in this study will be stopped should the study investigators feel it not in your best interests to continue.

Participant Consent Form

"The DREAM Study"

- **Request for interpreter:**   **Circle one**

English	I wish to have an interpreter	Yes	No
Deaf	I wish to have a NZ sign language interpreter	Yes	No
Māori	E hiahia ana ahau ki tetahi kaiwhaka Māori/kaiwhaka pakeha korero	Ae	Kao
Cook Island Māori	Ka inangaro au i tetai tangata uri reo	Ae	Kare
Fijian	Au gadreva me dua e vakadewa vosa vei au	Io	Sega
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu	E	Nakai
Sāmoan	Ou te mana'o ia i ai se fa'amatala upu	Ioe	Leai
Tokelaun	Ko au e fofou ki he tino ke fakaliliu te gagana Peletania ki na gagana o na motu o te Pahefika	Ioe	Leai
Tongan	Oku ou fiema'u ha fakatonulea	Io	Ikai

- I have read and I understand the information sheet dated 9th December 2014 for volunteers taking part in the study designed to study the glutathione peroxidase system.

- I understand that taking part in this study is voluntary (my choice), and that I may withdraw from the study at any time, and this will no way affect my future health care/continuing health care.

- I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.

I _____ (Participant's full name) hereby consent to take part in this study and have received a copy of the signed consent form for my own records.

_____   _____

Participant's Signature   Date

_____

Investigator/Researcher who explained

_____   _____

Investigator/Researcher Signature   Date

INFORMATION SHEET

The DREAM Study

Dynamic **R**elationship of antioxidant **E**nzymes in **A**cute **M**yocardial infarction

Principal Investigator
Dr Scott Harding, Cardiologist, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital
Work phone No. ▮▮▮▮▮▮▮
E-mail: ▮▮▮▮▮▮▮▮▮▮▮▮▮▮

YAn abbreviated consent has been given to you prior to your angiogram due to the time constraints at the beginning of the procedure. You now have the opportunity to discuss this study fully and decide whether or not you wish to participate. If you no longer wish to participate in this study then all samples and clinical data collected at this point will be destroyed with no affect on your provision of healthcare.

This study is investigating the levels of oxidative stress in the blood stream. High levels of oxidative stress are found in patients experiencing heart attacks and this is known to cause damage to the blood vessels. Antioxidants in the blood stream, such as glutathione peroxidase (GPx), provide a protective defense against high levels of oxidative stress and may slow the progression of damage. We have previously shown that a low level of GPx increases the risk of worse outcomes after a heart attack. What is not clear is how the levels of these enzymes change over time in a person experiencing a heart attack. We aim to investigate how levels of GPx and markers of oxidative stress increase during a heart attack.

How are people selected for this study, and who will select them?

Patients who present to the Cardiology Angiography Suite who have been diagnosed as experiencing ST-segment elevation myocardial infarction, commonly know as a heart attack, will be invited by a study investigator to participate in this study.

What will happen during the study?

If you are happy to participate in this study then you will have up to 7 blood samples taken from you over a period of up to 72 hours during your admission. A further blood sample will be taken 6 weeks later when you attend your follow-up appointment in Cardiology Outpatients. The blood will be taken by inserting a needle into a vein in your arm. When we do this we will be taking 10 mL (2 teaspoonful) in volume. Your blood will be tested for GPx activity and markers for oxidative stress. Any residual blood or components of blood will be destroyed following this. Prior to tissue being disposed of, (using the standard Hospital process) a Maori blessing will be performed. We will also collect information about your medical history and treatment during your hospital admission.

As samples of human tissue will be taken during this study, there may be cultural issues associated with storing tissue that need to be discussed with your family/whanau. Some Iwi disagree with storage of human tissue citing whakapapa and advise their people to consult prior to participation in research where this occurs. To avoid problems at a later stage, we invite you to involve your family/whanau at all stages of the research. However, we also acknowledge that individuals have the right to choose to participate.

Confidentiality

All data will be kept confidential. You will not be personally identified in any reports or publications that are developed throughout the course of this research. The data will appear grouped and include no markings that can be traced back to you.

Participant Rights

You do not have to accept this invitation to participate in this research. If you agree to participate you have the right to:

1. Ask any questions about the study anytime during your participation

2. Withdraw from the study at anytime without any affect on your future healthcare/continuing health care at anytime.

Participation in this study will be stopped should the investigators of this study feel it not in your best interests to continue.

What are the potential inconveniences of this study?

Insertion of a needle into a vein can result in some swelling around the area. This area may be a little tender after the needle is removed. It is possible that you could get an infection due to insertion of the needle. However this is very rare and we will take standard precautions to minimise this risk.

What are the benefits of this study?

This study will provide significant knowledge about the upregulation and variation of the GPx system and oxidative stress over a time period where a person is undergoing a heart attack. We believe these systems are very dynamic and that the ability of the GPx system to increase will influence the levels of oxidative stress. Understanding the dynamic relationship of these biomarkers will help us to identify those patients who have low levels of GPx and who may benefit from the effect of targeted antioxidant therapies in the future.

There will be no direct benefit to you from participating in this study.

Costs for the study

It will not cost anything to take part in the study.

Family or Whanau support

You may have a friend, family or whanau support present.

or Maori Health Support please contact Whanau Care Services: Trish Heuser - Specialty Clinical Nurse Cardiology 04 3855 999 ext ████

If you any questions or concerns about your rights as a participant in this research study you can contact an independent health and disability advocate. This is a free service provided under the Health and Disability Commissioner Act: Telephone: (NZ wide) 0800 555 050

Free Fax: (NZ wide) 0800 2787 7678 (0800 2 SUPPORT) Email: (NZ wide) advocacy@hdc.org.nz

Results

A written report of the study will be available to participants on request.

Compensation

In the unlikely event of a physical injury as a result of participation in this study, you may be covered by the accident compensation legislation with its limitations. If you have any questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

Please feel free to contact the Study Investigators if you have any questions about this study. Their contact details are listed above and below.

Statement of Approval

This project is awaiting ethical approval by the Northern B Health and Disability Ethics Committee.

Co-investigators

Working with Dr Harding is the research team as follows:

Assoc. Prof Peter Larsen, Clinical Physiologist, University of Otago, Wellington, Work phone ██ ████████████████ E-mail: ██████████████████

Prof John Miller, Physiologist, Victoria University of Wellington, Work phone 04 463 6082 Email: ████████████████████

Sarah Fairley, Interventional Cardiology Fellow, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital, Work Phone ██████████ Email: ██████████████████████

Ana Holley, PhD Student, Cardiac Care Unit, Capital & Coast LTD, Wellington Hospital, Work phone ██████████ Email: ████████████████████

Participant Consent Form

Investigation into the dynamics of the glutathione peroxidase system in STEMI patients

- **Request for interpreter:**   **Circle one**

English	I wish to have an interpreter	Yes	No
Deaf	I wish to have a NZ sign language interpreter	Yes	No
Māori	E hiahia ana ahau ki tetahi kaiwhaka Māori/kaiwhaka pakeha korero	Ae	Kao
Cook Island Māori	Ka inangaro au i tetai tangata uri reo	Ae	Kare
Fijian	Au gadreva me dua e vakadewa vosa vei au	Io	Sega
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu	E	Nakai
Sāmoan	Ou te mana'o ia i ai se fa'amatala upu	Ioe	Leai
Tokelaun	Ko au e fofou ki he tino ke fakaliliu te gagana Peletania ki na gagana o na motu o te Pahefika	Ioe	Leai
Tongan	Oku ou fiema'u ha fakatonulea	Io	Ikai

- I have read and I understand the information sheet dated 9th December 2014 for volunteers taking part in the study designed to study the glutathione peroxidase system in STEMI patients. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.
- I have had the opportunity to use whanau support or a friend to help me ask questions and understand the study.
- I understand that taking part in this study is voluntary (my choice), and that I may withdraw from the study at any time, and this will no way affect my future health care/continuing health care.
- I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.
- I understand the compensation provisions for this study.
- I have had time to consider whether to take part in the study.
- I know who to contact if I have any side effects from the study.

I wish to receive a copy of the results.   Yes ☐   No ☐

I _____ (Participant's full name) hereby consent to take part in this study and have received a copy of the signed consent form for my own records.

_____   _____

Participant's   Date

_____

Investigator/Researcher who explained

_____   _____

Investigator/Researcher Signature   Date

| If applicable: |
| Printed name of Interpreter |
| _____ |
| Signature of Interpreter   Date |
| _____   _____ |