Monitoring markers of oxidative stress in acute coronary syndrome.

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Abstract

It is estimated that there are around 80,000 hospital admissions in the UK each year due to acute coronary syndrome (ACS). The term ACS refers to clinical presentation characterised by chest pain, which may be due to an acute myocardial infarction (AMI) or unstable angina. AMI (or heart attack) arises due to cardiovascular disease (CVD), in which the blood supply to the heart muscle is decreased leading to ischemia and ultimately myocardial death. Thus, diagnosing AMI in a timely manner is essential.

Currently, high-sensitive cardiac troponin (hs-cTn) is the gold standard biomarker for AMI diagnosis, since hs-cTn is released by myocardial cells immediately following an AMI. Whilst hs-cTn has high sensitivity and specificity for diagnosing AMI, there are limitations. For example, hs-cTn at diagnosis does not predict readmission. There are also challenges with diagnosing certain demographics i.e., young females. Moreover, hs-cTn levels at diagnosis have no prognostic value for patient readmissions following percutaneous coronary intervention (PCI). Given the negative impact associated with AMI readmissions, identifying novel biomarkers that can predictive is attractive.

Since AMI is caused by ischemia, oxidative stress in a prominent pathological feature. During acute and chronic oxidative stress, biomarkers reflecting this such as thioredoxin (TRX), thioredoxin reductase (TRXr), peroxiredoxin-2 (PRDX-2) and peroxiredoxin-4 (PRDX-4) may be elevated. Therefore, evaluating these in AMI patients at diagnosis and during recovery may allow predictions regarding prognosis e.g., readmission probability. Therefore, the aim of this study was to evaluate TRX, TRXr, PRDX-2 and PRDX-4 in AMI patients at diagnosis and follow-up.

A total of 145 participants were recruited into this study, which included 80 AMI patients along with 65 healthy donor controls. Blood plasma was subsequently analysed by ELISA for TRX, TRXr, PRDX-2 and PRDX-4. The data presented illustrate for the first time that, healthy volunteers had significantly lower plasma levels of PRDX-4, TRX and TRXr compared with the AMI cohort ($p<0.05$), with females being significantly lower overall ($p<0.05$). Receiver operator curve analysis revealed that all four biomarkers could correctly predict an AMI in 4/5 cases, as determined by the area under the curve >0.80 discriminative for AMI. Stratification of patients according to biomarker concentration and culprit lesion during PCI demonstrated that, plasma TRX >13.40 ng/ml at screening was associated with a higher readmission risk ($p=0.009$), whereas patients with plasma TRXr >2.00 ng/ml had significantly lower risk of readmission overall ($p<0.05$). For TRXr, this was particularly apparent for patients who received PCI to the left anterior descending artery (LAD). Finally, PRDX-2 >30.60 ng/ml at first follow-up (1-3 months) was associated with an increased risk of readmission ($p=0.009$) and was most apparent when culprit lesion was the LAD. This information may inform clinical outcome which in turn may highlight strategies to improve ACS readmission rates in England, e.g., recombinant PRDX-2 therapy for when culprit lesion during PCI is the LAD. Taken together, the findings of this study could significantly benefit the diagnosis and risk stratification of AMI, as well as inform clinical decisions.

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Abbreviation

ACS	Acute Coronary Syndrome
AE	Adverse Event
AKD	Acute Kidney Disease
ALT	Aspartate transaminase
AMI	Acute Myocardial Infarction
CABG	Coronary Artery Bypass Graft
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CKD	Chronic Kidney Disease
cMy BP-C	Cardiac Myocin Binding Protein-C
Cr	Creatinine
CT	Computed Tomography
cTn	Cardiac Troponins
CVA	Cerebral Vascular Accident
CVD	Cardiovascular Disease
CV	Curriculum Vitae
DAPT	Dual Anti-Platelet Therapy
DM	Diabetes Mellitus
EC	Ethics Committee
ECG	Electrocardiogram
ED	Emergency Department
EDTA	Ethylenediaminetetraacetic Acid
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
EOS	End of study

FU	Follow-Up
FBC	Full Blood Count
GCP	Good Clinical Practice
Gender	Chromosomal Sex at birth i.e., xx = Female or xy = Male.
GRX	Glutaredoxin
HbA1c	Glycated haemoglobin A1C
HBS	Hanks Balancing Salt
HDL-C	High Density Lipoprotein Cholesterol
HF	Heart Failure
HS-cTnT	High-Sensitive cardiac troponin
HRA	Health Research Approval
ICA	Invasive Coronary Angiography
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
IHD	Ischemic Heart Disease
IRAS	Integrated Research Application System
ISF	Investigators Site File
Kg	Kilogram (s)
LDL-C	Low-Density Lipoprotein Cholesterol
LOD	Limit of detection
LVEF	Left Ventricular Ejection Fraction
MACE	Major Adverse Cardiac Event
MLs	Milliliters
MI	Myocardial Infarction
MTA	Material Transport Agreement
NICE	National Institute for Health and Care Excellence
NSTEMI	Non-ST-segment Elevated Myocardial Infarction
NT-pro BNP	Natriuretic peptide
PCI	Percutaneous Coronary Intervention
PI	Principal Investigator
PRDX (PRXD)	Peroxiredoxin

REC	Research Ethics Committee
redox	Reduction-Oxidation
R&D	Research & Development
ROC	Receiver Operator Curve
ROS	Reactive Oxygen Species
SAE	Serious Adverse Event
Sample-1	Baseline
Sample-2	Follow-up 1 (1-2 months post baseline)
Sample-3	Follow-up 2 (6 months post baseline)
SEX	Chromosomal Gender at birth i.e., xx = Female or xy = Male.
SOD	Super Oxide Dismutase
SOP	Standard Operational Procedure
STEMI	ST-Segment Elevated Myocardial Infarction
TRX	Thioredoxin
TRXr	Thioredoxin-Reductase
URN	Upper Range of Normal
VEGF	Vascular Endothelial Growth Factors
WAHT	Worcestershire Acute Hospitals Trust

Chapter 1 – Introduction

1. Introduction

1.1 Coronary artery disease (CAD).

Coronary artery disease (CAD) is a chronic condition. During CAD pathogenesis, atherosclerotic plaques progressively build up in the vasculature which can lead to occlusion of the coronary artery, limiting blood supply to the myocardium and potentially causing Acute Myocardial Infarction (AMI) (Nable *et al.,* 2012; White *et al.,* 2019). Coronary artery disease or coronary heart disease (CHD) as it can be referred as, is the most common cause of mortality worldwide according to the American Heart Association and is a major cause of morbidity worldwide (AHA, 2014; White *et al.,* 2019). It is estimated that cardiovascular morbidity and mortality occurs in 7 million individuals annually (Piepoli *et al*., 2016; Bhatt *et al.*, 2022) and has been the leading cause of death since the 2000's (Dabrowski *et al.,* 2022).

Acute Myocardial Infarction (AMI) is a subcategory of CAD and typically presents with chest pain (NICE 2020). Patients with suggestive symptoms of AMI account for approximately 10% of all emergency department admissions (Twerenbold *et al.*, 2018). Most AMI's (also referred to as a heart attack), are due to atherosclerotic plaques that rupture into the blood stream and cause blocking to the coronary arteries leading to heart muscle necrosis. In 2012 it was estimated that around 13% of deaths in England were because of AMI brought on by CAD, (ONS 2012) updated in 2021 as 6.6 million equating to 12.1% (Office for National Statistics 2021). United States figures from the American Heart Association (ASA) reported a prevalence of CHD in adults over 20 years of age as 7.0%, with an associated death rate within a year of AMI of 26% in men and 19% of women aged 45 years or older (AHA 2014). These statistics highlight the burden CAD has on healthcare.

Acute coronary syndrome (ACS) refers to the presenting symptoms such as chest pain brought on by an AMI or unstable angina (non-MI), thus it is important to differentiate the precise cause of ACS following the onset of the chest pain (Body *et al*., 2011). Elevated ST interval (STEMI) on an electrocardiogram (ECG) is indicative of AMI, however in ~40% of cases, ST elevation on ECG is not associated with AMI (NSTEMI) (Bardaji *et al*., 2019).

Therefore, molecules released by the damaged myocardium as a result of an AMI, known as 'cardiac biomarkers', are a central factor in ACS diagnosis to indicate AMI has occurred (Gard *et al*., 2017)

Currently, diagnosis of AMI is by clinical symptoms, predominantly crushing chest pain, pain radiating down arm or in jaw together with changes on an ECG, blood analysis for cardiac markers such as Troponin-T (TnT); the gold-standard marker recommended by National Institute for Health and Care Excellence (NICE 2013). Wildi *et al.*, (2017) claimed that, although 10% of patients symptomatic for AMI presented to the Emergency Department (ED), only 10-20% are ultimately diagnosed as AMI. However, discharge is not possible for the majority of symptomatic patients until AMI is ruled out (Apple *et al*., 2017; Schønemann-Lund *et al*., 2015; Thiele *et al*., 2021, ESC 2020). Thus, a timely diagnosis of the cause of chest pain is paramount. During chest pain diagnosis, it is especially important to rule in or out AMI (ischaemic chest pain at rest with cardiomyocyte necrosis) or Unstable Angina (UA) (ischaemic chest pain at rest or minimal exertion without cardiomyocyte necrosis) (Mueller- Hennessen, 2017), since prolonged loss of blood supply to the cardiac muscle (myocardium) can be detrimental and lead to death without early revascularization. Death from AMI are highest within the first few hours; therefore, early diagnosis is critical (Thygesen *et al.,* 2010). As previously stated, the damage to the myocardium that occurs during AMI leads to the release of cardiac biomarkers, such as TnT into blood. Therefore, the presence of TnT in the blood stream indicates that an AMI has occurred (Zhelev *et al*., 2015). However, TnT has little clinical utility in the assessment of sequences leading up to the event, since its release and subsequent detection means that the AMI has already happened, and the myocardium is to some extent already damaged.

1.2 Cardiac biomarkers during Acute Myocardial Infarction (AMI).

During an AMI, there is disruption of the cardiac myocyte cell membrane (Jaffe, A., Morrow, 2018). When enough of the myocytes have been affected, usually due to cell death (myocyte necrosis), TnT release occurs, which can be detected in serum blood samples. In fact, there are three troponin (Tn) isoforms (Collinson, Pathology and Wing, 2006). These include, 1) Troponin-C (TnC) which is expressed in cardiac and skeletal muscle, 2) Troponin-I which is cardiac specific and referred to as cTnI, and 3) TnT which is also cardiac specific and referred to as cTnT. The cTnI and cTnT proteins are produced by different genes (Chin *et al.*, 2014; Collinson *et al.*, 2020). Therefore, both cTnT and cTnI are routinely measured for AMI diagnosis (May *et al*., 2014).

Questions relating to the validity of cTnT have been controversial for many years, due to substantial evidence documenting the release of cTnT from skeletal muscle stress (Shi *et al.*, 2006; Schmid *et al.,* 2018). This controversy is highlighted further by Mair, *et al.,* (2018), who

summarise alternative evidence for cTnT release in other cardiac associated disorders, such as chronic heart failure, atrial pacing and even in highly fit endurance athletes. In these examples, the release of cTnT has occurred in the absence of myocyte necrosis. Yet, despite these pathophysiological controverses, cardiac troponin testing remains the standard practice of diagnosis of AMI (Nice 2015; Apple *et al*., 2017).

Conventional cTn assays were less sensitive than newer assays. Increasing the laboratory detection limit has greatly improved the clinical utility of cTnT / cTnI, now often referred to as 'high-sensitive cardiac troponins' (hs-cTn). As such, hs-cTnT and hs-cTnI have replaced CK-MB as the gold-standard, because of their myocardial tissue specificity and laboratory detection limit (Apple *et al*., 2017). The development of hs-cTn was driven by the need for faster triage of patients with chest pain presentation, to avoid unnecessary hospital admissions and speed up diagnosis (Zhelev *et al*., 2015; Pettersson *et al.,* 2018).

Diagnosis of ACS is differentiated by a rise and fall (or delta) of hs-cTn (Twerenbold *et al.*, 2018). Whereas chronic myocardial injury is determined when persistently high concentrations hs-cTn are detected (Bardaji *et al*., 2019). The hs-cTn diagnostic cut-off aka upper limit of normal (ULN), is determined when test results present at least one level above a predetermined 99th percentile of a healthy reference population (Body *et al.*, 2015; Jaffe *et al.*, 2018). However, determining the 99th percentile varies between laboratorians, clinicians and scientists who use these assays (Apple *et al*., 2017). In 2014, the National Institute of clinical Excellence (NICE., 2014) issued guidance on the clinical application of the hs-cTn assay to rule-out AMI, thus allowing local and national policies to be based upon. They concluded that hs-cTn's concentrations should be determined by meeting two criteria:

1) The ULN **is derived** from apparent healthy individuals 'enrolled in studies' designed by clinical/ scientific investigators (Sandoval *et al*., 2014), whereby the total coefficient of variation (imprecision) at the 99th percentile of this healthy reference population, should be 10% or less.

2) Measurable concentrations must be above the limit of detection and below the 99th percentile and should be at least 50% of the reference population (Apple *et l.*, 2015).

Since methods for detecting hs-cTn are sensitive, where some amount can be detected in non-cardiac individuals, differential diagnoses need to be considered if the test results are borderline. In this instance, the 99th percentile ULN will have various concentration ranges in accordance with different demographic groups, such as sex and age (Park *et al.*, 2017). Using

apparent healthy individuals for determining the 99th percentile, as outlined in criteria (1) above, is much debated (Ungerer, *et al*., 2016; Apple *et al*., 2017). However, consistency in defining what constitutes the healthy reference population is apparent and includes: non-cardiac individuals, age matched and sex matched individuals, in order to gain at least the 50% reference population. To this end, research is ongoing, especially when considering sex- specific cut offs applied for diagnostic purposes using hs-cTn. However, Wildi *et al.*, 2017 concluded that the concentration difference between both sex-specific cut offs is so small it is unlikely to statistically improve diagnosis of major adverse cardiac evens (MACE). But the conclusions made regarding sex specific cut of values remain contentious, and are currently under investigation, with the results of a widely anticipated randomised Canadian study anticipated for December 2023 (Humphries, 2020).

Clinical decisions with other assessment algorithms will remain necessary in patients classified as low risk, as the hs-cTn biomarker approach for all ACS is unsuitable, for example, when diagnosing unstable angina, which due to the nature of the aetiology has no myocardial necrosis (Hollander *et al*., 2016; Möckel *et al.*, 2015). However, as research by Humphries (2020) states, there remains issues using this approach, including, “persistent under- diagnosis, under treatment and high risk of adverse outcomes, especially in younger females compared to male counterparts”. Therefore, other more robust predictive algorithms will always be welcome for consideration, in particular with regards to females presenting in emergency departments. Currently, ‘our local policy’ based on (NICE 2015) is for serial hs-cTn sampling every 3 hours from admission or onset of chest pain. Assessment of the results are that, if two hs-cTn serum samples are >20ng/ml, then an ACS likely. Thus, our local approach may have numerous limitations, as outlined above.

A disadvantage is that hs-cTn is not released immediately following the onset of chest pain during ACS, hence why current local guidelines taken from NICE (2015) are to repeat sampling from the onset of chest pain, ideally every three hours until hs-cTn is detected (Thokala *et al.*, 2012; Raskovalova *et al.*, 2014). At the point of detection, if the chest pain is due to ACS, then extensive cardiac damage has already occurred due to the resulting AMI. This therefore limits the use of hs-cTn in predicting whether chest pain is due to an AMI ‘or’ unstable angina (Shah *et al.*, 2018; Boeddinghaus *et al.*, 2015; Marshall and Bangert, 2008). Moreover, alternative cardiac conditions, such as cardiac inflammatory response to severe illness, rather than acute coronary syndrome (Nice, 2020) can mimic AMI, which may result in elevated hs-cTn (Sieweke *et al.*, 2016), thus limiting the specificity of hs-cTn for ACS diagnosis. To overcome this limitation, alternative diagnostic tests must be considered.

Historically, various cardiac markers have been explored, such as myoglobin and CK-MB. However similar limitations exist with these biomarkers as outlined in Figure 1.1 (Anderson *et al*., 2007). The use of hs-cTn does have advantages over myoglobin and CK-MB, including a log serum half-life and improved clinical utility, with sensitivity and specificity values of 90.2% and 95.7% respectively for cTnI (Chang *et al.,* 1998).

There has been interest in alternative biomarkers that could be potentially more sensitive to myocardial necrosis, such as copeptin, which can have results available in 20 -30 minutes. Copeptin is a marker of endogenous stress including early MI and has value early-on to rule out MI when used with hs-cTn (Beri *et al.,* 2017). Copeptin was shown to have peak elevated levels within 4 hours, whereas hs-cTn levels peak 12 hours after symptom onset (Raskovalova *et al.*, 2014). In the 2014 study by Raskovalova and colleagues, the authors aimed to determine diagnostic accuracy of copeptin, but concluded further studies were required in combination with hs-cTn to evaluate the full clinical utility. Thus, whilst hs-cTn remains the gold-standard for diagnosing ACS, along with ECG measurements, alternative methods need to be established that can increase the speed of diagnosis as well as reliably predict whether hospital admissions for chest pain are due to ACS / AMI. This is particularly important for NSTEMI ACS patients i.e., those with non-elevated ST ECG interval, where chest pain could be attributed to stable angina (Mueller 2013; Raskovalova *et al.*, 2014).

One suggestion may be to evaluate markers of ACS pathogenesis, such as oxidative stress which is a biochemical feature ACS, see section 1.4 (Lubrano *et al.,* 2919)

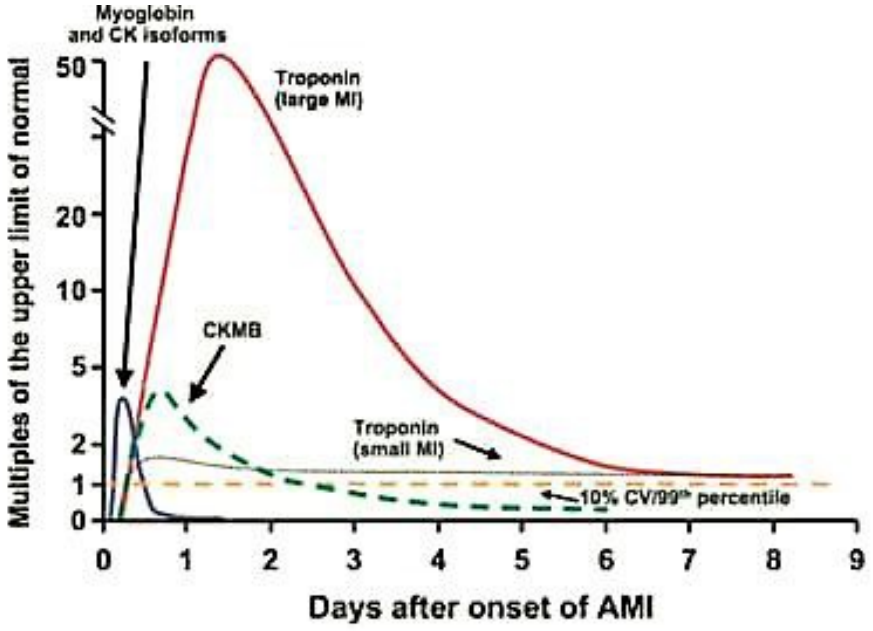


Figure 1.1: Release and detection limit of several cardiac markers following MI. Figure illustrates the detection limit and time frame of several cardiac markers following MI (AMI). Data shown include; myoglobin, CK isoforms, CKMB and troponin (c-Tn). It is noted that the 'large' troponin subunit (c-TnT) illustrated the greatest magnitude of release and remains elevated for the longest time <6 days. All markers are present after the AMI and not before. Figure taken from http://emedicine.medscape.com/article/155919-workup.

1.3 Limitations with current biomarkers.

As outlined in section 1.2, there are challenges associated with the use of hs-cTn, with respect to ACS diagnosis, which include, **1)** hs-cTn levels only increase after the AMI has occurred, therefore likely significant damage to the myocardium has already occurred (Thokala *et al.*, 2012; Raskovalova *et al.*, 2014), **2)** determination of the ULN can be challenging, which may pose problems for diagnosing certain demographics i.e., younger females (Ungerer *et al*., 2016; Apple *et al*., 2017; Humphries *et al*., 2020) and **3)** differentiating NSTEMI ACS patients, for whom their symptoms may be attributed to chronic coronary syndromes such as, stable angina (Shah *et al.*, 2018; Boeddinghaus *et al.*, 2015; Marshall and Bangert 2008). Further to these shortcomings, serum concentration levels of hs-cTn concentration does not accurately predict whether a patient is likely to have a secondary ACS event. A 2012 study evaluated the prognostic outcome of cTnT concentration levels in 1177 cardiac patients, with a mean age of 68 years (Gerber *et al*., 2012). The study stratified cardiac patients according to cTnT serum concentration, with patients grouped in the lower (< 0.22 ng/ml, n=396), middle (0.23-117 ng/ml, n=388) or upper (> 1.18 ng/ml, n=393) cTnT tertials.

Gerber *et al.* 2012 found that patients in the upper tertial for cTnT had an overall increased risk of death (~30%), compared with ~25% risk for the middle tertial group. A similar trend was observed with heart failure as the end point. However, there was no distinction between the upper and middle tertial cardiac patients with regards to predicting a secondary AMI. More recently, a 2021 study evaluating 30,173 cardiac patients, with a mean age of 70 years concluded that the maximum level of hs-cTn was found to be most predictive of AMI, as a reflection of myocardium necrosis, with peak hs-cTn indicating a negative clinical prognosis, regardless of the cause of the cardiac event (Fan *et al.,* 2021). However, this study did not evaluate whether hs-cTn was predictive of a secondary event, which may be more important for patients who fall in the middle quartile range.

Unpublished audit data from authors at our local Trust note that around 7-12% of AMI patients experience a secondary readmission event. Patients with ACS often experience non-specific pain after discharge (Chen *et al.,* 2021), which is increased following a Percutaneous

Coronary Intervention (PCI), which taken together are associated with increased unplanned readmission for angina and non-specific chest pain within 30-days of index PCI (Sykes *et al.*, 2020). Evaluation of 30-day readmission rates is limited. However, Kwok *et al.,* (2019a) analysed a national database of between 2010 and 2014, which reported that patients with a primary cardiac discharge had an 8.6% un-planned 30-day cardiac readmission. The study concluded that non-specific chest pain may not be a benign condition, as readmissions for a serious cardiovascular events occurred in 3% of patients within 180 days. (Kwok *et al*., 2019a). Similar findings from a UK based study conducted between 2012 and 2014, where a single tertiary hospital retrospectively reviewed the National Audit Project register of AMI admissions, concluded that 50% readmission were for cardiac aetiology, with common causes included ACS (17.1%), stable angina (11.6%), and heart failure (9.8%). The study concluded that chest pain is the most frequent cause of readmission, and interventions to reduce noncardiac chest pain admissions are needed (Kwok *et al*., 2019). Furthermore, Kwok *et al*., (2019a) went on to deduce that the rates of early unplanned readmissions occur for 1 in 12 admissions for nonspecific chest pain, with noncardiac causes being the most common reason. Unplanned readmission rates are often non-specific cardiac chest pain, however a secondary event in 27.6 % occurred between day 0 and day 7 (Kwok et al., (2019b). However, re-presenting patients with non-specific chest pain following hospital discharge may become a safe and cost-effective approach (Potezny *et al*., 2018; Moore *et al*., 2016). But this requires randomised trials to evaluate whether post-MI angina (new or persistent), is associated with higher likelihood of readmission (Doll *et al.,* 2016). Although after a PCI, the 30-day readmission is rarely due to a PCI complication or a re-infarction (Wasfy *et al.,* 2014), all indicating a demand for cost effect predictive tools or guidelines with regards to readmissions.

Taken together, this has a burden on the health care providers, since CVD related healthcare costs alone in England amount to an estimated £7.4 billion per year, with annual costs to the wider economy being an estimated £15.8 billion (OHID., 2022). Moreover, an estimated 7% of AMI hospitalisation results in death (Moy *et al.*, 2015). Thus, it is not surprising that chest pain, anxiety, and readmissions represent adverse outcomes for patients following an AMI (Baghaei *et al.,* 2021).

It has long been known that having an AMI has psychological implications (Affleck *et al*., 1987). As previously stated, patients with ACS often experience non-specific pain after discharge (Chen *et al*., 2021). Moreover, elevated levels of anxiety at baseline are predictive of readmission (Iles-Smith *et al*., 2015) and depressive disorders increase the risk of re-

hospitalization after an AMI (Myers et al., 2011; Reese *et al.,* 2012; Wang *et al*., 2022). In a systematic review completed by Bunker *et al.,* (2003), which evaluated the psychological risk factors between the development and progression of CHD or occurrence of acute events, it was concluded that psychosocial risk factors have implications for public health policies, which in turn informs research. Statistics from recent studies also support the notion with regards to the psychological impact surrounding patients who survive AMI, and the risk of recurrence (Wang *et al*., 2022).

Therefore, there is a clear rationale for determining those at risk of readmission. It must also be noted that, incidents of early unplanned readmission increase with greater comorbidity burden (Kwok *et al*., 2018a). Furthermore, sex differences have also been observed for cardiovascular causes of readmission rates (Kwok *et al*., 2018b). Interestingly, women who undergo PCI are at higher risk of adverse outcomes compared with men (Lundbäck *et al*., 2017). This observation is particularly evident among black female patients (Hess *et al.,* 2017). Despite a higher prevalence of readmission associated with increased depressive symptoms in women, mortality was not increased when compared to male readmissions (Parashar *et al*., 2009). It must also be noted that the psychological factors of a second AMI have a large impact on the partners of patients following AMI, with a high prevalence of sexual dysfunction (Tandeter *et al.,* 2000). Thus, the perceived stress, anxiety and psychological influences must exercise the capacity to improve quality of life in general, all of which may be improved with better predictive tools and clinical guidelines.

A further shortcoming with using hs-cTn is that, whilst it is indicative of myocardial damage / necrosis, and possibly extent, it does not predict the coronary artery lesion that has caused the AMI in the first place (Iftikhar *et al.,* 2022). Certain lesions carry a worse prognosis, for example left main (LM) coronary artery lesions are associated with significant myocardial necrosis, see figure 1.2 for an overview of cardiac anatomy (Iftikhar *et al.,* 2022). This may be important, since treatment involves percutaneous coronary intervention (PCI), where a stent is introduced to the occluded artery to increase blood flow to the myocardium, therefore minimising myocardial damage / necrosis (Giacoppo *et al*., 2017). The lesion that caused the AMI may therefore be indicative of patient outcome, i.e., the left anterior descending artery (LAD) infarctions have a higher mortality compared to right coronary artery (RCA) (Entezarjou *et al.,* 2018). The LAD in lay terms is often called the ‘widow maker’ as the occlusion stops blood to left side of heart (Steele 2021). LAD infarctions are particularly associated with increased heart failure, strokes and death; however, the culprit vessel does not influence the one-year

mortality if patients survived 30 days after the AMI (Entezarjou *et al*., 2018).

Therefore, there is a need for improved diagnostic biomarkers, that can help resolve some of the shortcomings with hs-cTn as outlined above i.e., the impact of cardiac lesion.

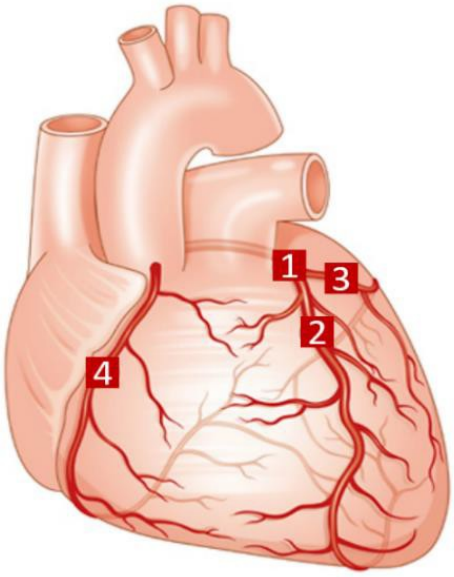


Figure 1.2: General anatomy of coronary arteries indicating lesions. The anatomy shows, 1) Left Main (LM), 2) Left anterior descending artery (LAD), 3) Circumflex artery (CX) and 4) Right Coronary Artery (RCA). Figure adapted from: https://www.medicoverhospitals.in/procedures/lmca

These cardiac lesions (Figure 1.2), result in occlusion of the arteries supplying blood to the myocardium reducing oxygen supply, causing metabolic adaptations occur inside the myocytes, leading to the generation of reactive oxygen species (ROS) and subsequent oxidative stress (Dubois-Deruy *et al.,* 2020; Madamanchi *et al.,* 2005). Given that ROS and oxidative stress are central mediators in the pathogenesis of CVD, investigation into oxidative stress as potential predictive biomarkers for ACS / AMI represent an attractive proposition.

1.4 Metabolic adaptations, ROS and oxidative stress.

The term oxidative stress refers to a cellular situation where the generation of ROS (or reactive nitrogen species, RNS) occurs at a level beyond which the cellular antioxidants can operate (Preiser., 2012). There are many types of ROS, which include free radicals (molecules with an unpaired electron) such as superoxide, peroxynitrite, hydroxyl radical, hydrogen peroxide (Das *et al*., 2014). These ROS are generated through normal cellular metabolism and play an important role in normal cell function (Ray *et al*., 2012; Das *et al*., 2014). During normal cell function, ROS levels are removed / neutralised to unreactive molecules, through action of the cellular antioxidant mechanisms (Das *et al.,* 2014). Cellular antioxidants are diverse, and include molecules ingested as part of a healthy diet e.g., plant polyphenols, but also include

small peptides such as glutathione and enzymes, which are coded for by the genome (Das *et al.,* 2014). Expression of these antioxidant enzymes become upregulated in response to ROS by the transcription factor NrF2, and include enzymes such as glutamate-cysteine ligase, which functions to increase the cellular pool of glutathione, as well as enzymes of the thioredoxin system, which function to reverse the damage caused by ROS to important cellular proteins (Tonelli *et al*., 2018), and catalase, which removes ROS via a haem moiety (Nandi *et al.*, 2019). However, under certain pathological conditions, such as those mediated by hypoxia (oxygen starvation), the generation of ROS may out-compete their removal by the antioxidant defence systems leading to an oxidative stress (Liguori., *et al* 2018; Görlach *et al*., 2015).

A key mediator in this process is the mitochondria, which requires good oxygen supply for normal metabolic function. During hypoxia, the supply of oxygen (O_2) to the mitochondria is reduced, decreasing flux through the electron transport chain increasing electron leakage, which leads to an overproduction in of ROS, in particular superoxide and hydrogen peroxide, see Figure 1.3 (Hamanaka *et al.,* 2009).

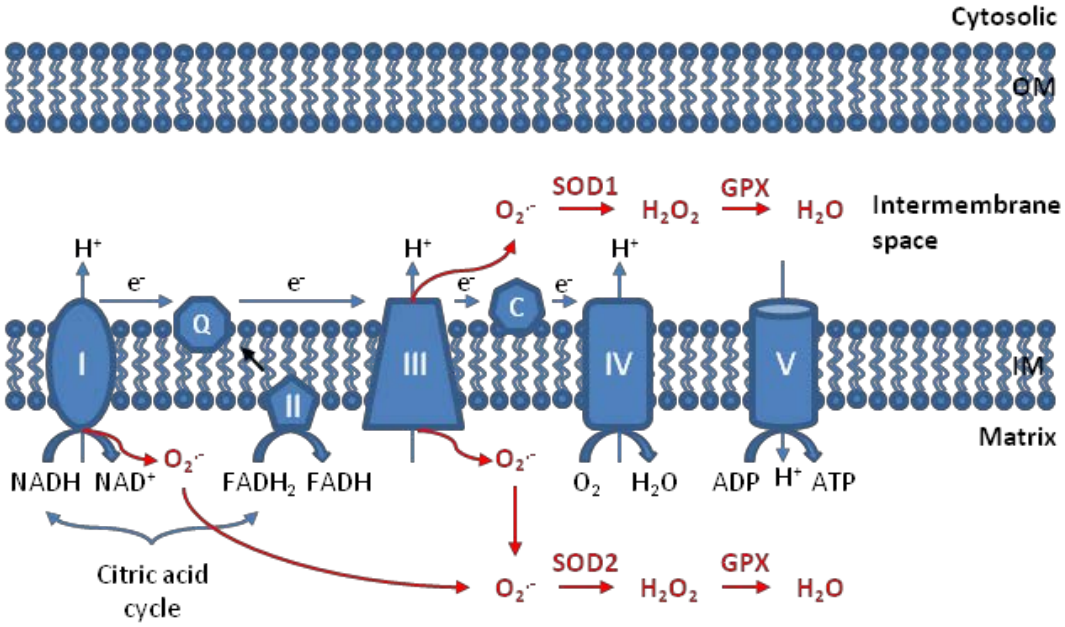


Figure 1.3: The importance of the mitochondrial electron transport chain in the generation of ROS. The figure shows that electron leakage from the electron transport chain, which may be increased during hypoxic conditions, reacts with molecular oxygen (O_2) forming the superoxide free radial (O_2) which is metabolised by superoxide dismutase-2 (SOD2) into hydrogen peroxide (H_2O_2). Glutathione peroxidase (GPX) converts H_2O_2 to water (H_2O). Under hypoxic conditions, the generation of $O_2^{\cdot-}$ and H_2O_2 occurs at a quicker rate than SOD2 and GPX can function, which leads to an oxidative stress. Image taken from Fang *et al*., (2013)

Reactive oxygen species (ROS) is a broad term, encompassing a plethora of highly reactive molecules that, if left unchecked cause damage to cellular components, including lipid membranes, nucleic acid (DNA and RNA) as well as proteins (structural and enzymes) (Ray *et al*.,

2012). A key feature of all ROS is that the damaged caused by them is through a process known as 'oxidation', whereby the ROS in question strips electrons away from the afore mentioned cellular components, modifying their structure and / or biological activity (Sies 2019). For example, certain enzymes become oxidised by ROS which causes them to alter their activity. An important example here is the impact that ROS have in CVD, where ROS are shown to mediate apoptosis signalling kinase-1 activation, leading to myocyte death (Senoner *et al*., 2019).

The various ROS react at different rates, with some ROS considered more damaging than others (Finosh *et al.,* 2013). Certain ROS have a very short half-life, which is indicative of their reactivity. For example, superoxide (O_2) has a half-life of just 1 millionth of a second, but hydrogen peroxide (H_2O_2) is relatively stable (Finosh, *et al*., 2013). Catalase for example can remove H_2O_2 by a 2-step redox reaction, rapidly decreasing the half-life (George 1947; Ivancich *et al*., 1997). Figure 1.4. illustrates the various types of ROS (and RNS) generated by cells, along with the type of cellular damage caused if left unchecked.

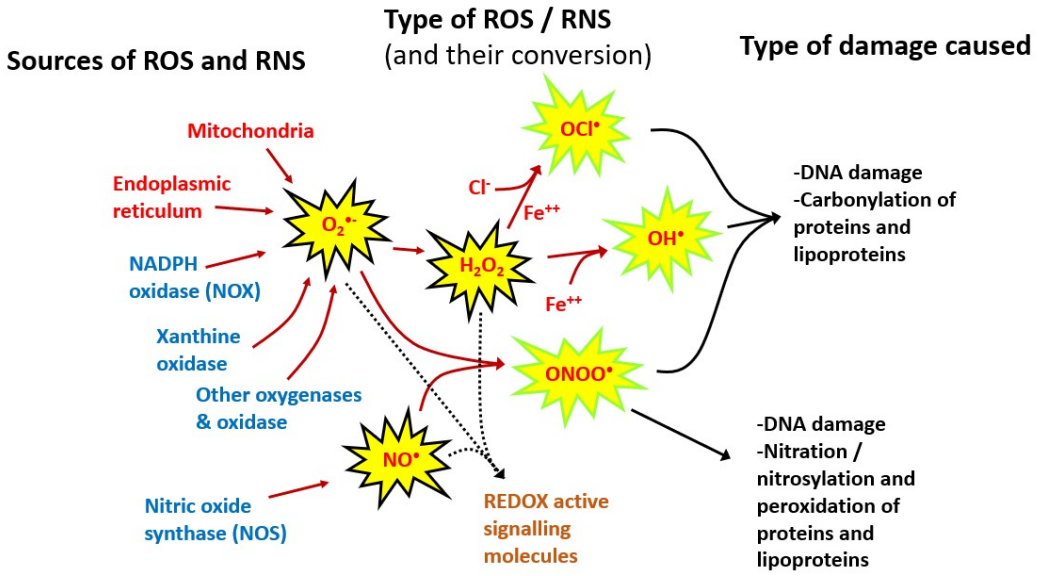


Figure 1.4: ROS and RNS generated by the cell and the damage caused. The mitochondria and endoplasmic reticulum are involved in continuous superoxide ($O_2^{\cdot-}$) generation through normal metabolic processes. However, $O_2^{\cdot-}$, can be generated enzymatically on demand e.g., via NOX. $O_2^{\cdot-}$ has a very short half-life and is rapidly converted to the more stable hydrogen peroxide (H_2O_2) by superoxide dismutase. Both O_2 and H_2O_2 are considered useful to the cell, particularly for normal cell function i.e., REDOX signalling. However, H_2O_2 can be converted to highly reactive and damaging ROS, such as the hypochlorite radical ($OCl^{\cdot}$) and the hydroxyl radical ($OH^{\cdot}$). Side reactions of ROS with nitric oxide (an RNS), leads to the formation of the highly reactive and damaging peroxynitrite radical ($ONOO^{\cdot}$). (Finosh, *et al*., 2013).

To summarise, the generation of ROS is complex, with various side reactions that convert useful ROS, such as superoxide and hydrogen peroxide, into more damaging ROS such as the hydroxyl radical. Under normal physiological and healthy conditions, the generation of ROS is

counterbalance by their removal by the antioxidants, e.g., catalase. When the antioxidant capacity of the cell is overwhelmed, an oxidative stress occurs, which is a pathological feature of numerous chronic health conditions, including CVD, neurodegeneration and cancer (Liou, *et al.,* 2010; Liu. *et al.,* 2017; Panth, *et al.,* 2016). Thus, the antioxidant enzyme systems within the cell have an important role to play. The key antioxidant enzymes focused on herein and their mechanism will be highlighted below.

1.5 The peroxiredoxins.

The peroxiredoxins are a family of antioxidant enzymes, containing 6 subgroups (PRDX1–6) (Bolduc *et al.,* 2021). Their function in the cell is well characterised and, as the name suggests, is to catalyse the reduction of hydrogen peroxide to water (Rhee *et al.,* 2012). The reducing power for PRDX activity comes from an active site Cysteine, that provides elections to hydrogen peroxide to split the molecule, thus the hydrogen peroxide is now reduced (Bolduc *et al.,* 2021). However, since the PRDX has donated electrons in this reaction, it becomes oxidised as a result. Therefore, this type or reaction is known as a REDOX reaction and is a common feature of many antioxidant enzymes in the cell (Hoyle *et al.,* 2015). Figure 1.5 illustrates the PRDX antioxidant mechanism of action for the reduction of hydrogen peroxide.

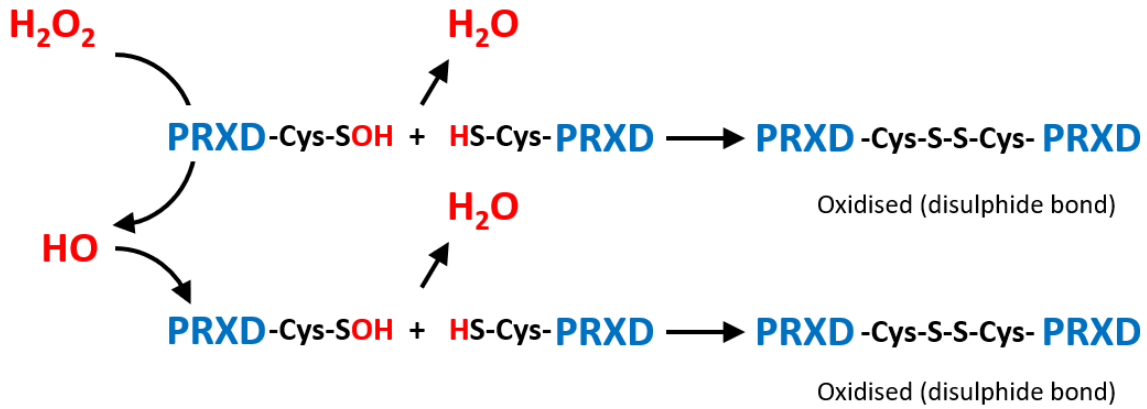


Figure 1.5: Basic antioxidant mechanism of the peroxiredoxins. Peroxiredoxin (PRDX) catalyses the reduction of hydrogen peroxide (H_2O_2) in the cell, converting it to water (H_2O). PRDX mediates this reaction via an active site cysteine (Cys) which contains a thiol group (SH). Electrons are transferred to H_2O_2 splitting the molecule, resulting the oxidation of the Cys-SH to form a sulphenic acid (SOH) intermediate, which is reduce by a second PRDX Cys-SH, releasing H_2O which results in the formation of a disulphide bond between the 2 Cys. Both Cys are now oxidised, which need reducing again for PRDX to continue function.

Interestingly, the subcellular location of the PRDX enzymes differ, with PRDX-1, PRDX-2 and PRDX-6 located in the cytoplasm, PRDX-5 found throughout the cell, PRDX-3 is mitochondria and PRDX-4 are located in the endoplasmic reticulum (Rhee *et al.,* 2012). Given that PRDX-4 has a signal peptide, this subtype it was once believed to be secreted by cells, with studies identifying PRDX-4 in the circulation of both healthy individuals following exercise as well as individuals with peripheral arterial disease (Wadley *et al*., 2019; Eter *et al.,* 2014). However, subsequent studies indicate that PRDX-4 is retained in the endoplasmic reticulum where it promotes protein folding, and lipid biogenesis (Elko *et al*., 2021), therefore cellular release is most likely a result of normal turnover.

Studies have demonstrated that some PRDXs are highly inducible following an increase in cellular ROS, as highlighted in particular by PRDX-6 in neonates and in adult lung tissue (Schremmer *et al.,* 2007). Other physiological responses for the PRDXs include a role in the inflammatory response (Davignon *et al.,* 2014). It is also noted that PRDX-1 plays a major role in cholesterol homeostasis, by regulating hydrogen peroxide excesses in macrophages, thus reducing foam cell formation (fatty deposits) and the formation of atherosclerotic plaques (Jeong *et al.,* 2018). This particular example specifically highlights the link between PRDX-1 and CVD. Interestingly, PRDX-2 is expressed by most cells in the body and 'appears' to have a role in the pathogenesis of CVD (Jeong *et al.,* 2021), as outlined in more detail in section 1.6.

Since the PRDXs become oxidised during the catalytic removal of hydrogen peroxide, they must be returned to their 'reduced state' if they are to continue functioning. As Figure 1.6 illustrates, the reduction of the PRDXs is mediated by the action of the thioredoxins (TRXs) and thioredoxin-reductases (TRXr's) (Stancill *et al.,* 2021; Lee *et al.,* 2013).

1.6 The thioredoxins and reductases.

Like the PRDXs, the TRXs belong to a family of enzymes., which includeTRX1 and TRX2 (Mohammadi *et al*., 2019). As the name suggests, the thioredoxins 'reduce thiol groups. Thiols are sulfhydryl (SH), and notably the functional Cysteine amino acid in the active site of the PRDXs contains a 'thiol group' (SH). It is this thiol group, as illustrated in figure 1.5 above, that is responsible for reducing hydrogen peroxide to water, however the thiol becomes oxidised forming a disulphide bond with a neighbouring Cysteine during the process (Hoyle *et al*., 2015). The role of the TRXs is to reduce these disulphide bonds back to the 'reduced' thiol form, see Figure 1.6. Thus, the TRXs ultimately play an important role in the removal of hydrogen peroxide, as part of the wider antioxidant network (Lee *et al.,* 2013).

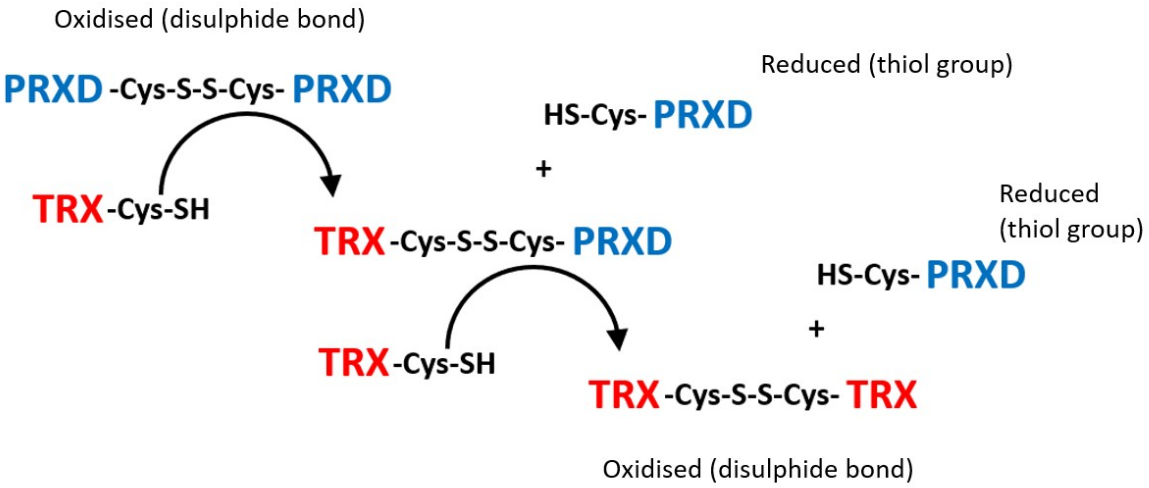


Figure 1.6: Reduction of oxidised peroxiredoxin by thioredoxin. Oxidised peroxiredoxin (PRDX) is returned to its reduced state by the catalytic action of thioredoxin (TRX). TRX mediates this mechanism via an active site cysteine (Cys). The thiol (SH) of the TRX active site Cys (Cys-SH) attacks the PRDX disulphide bond in a thiol-disulphide exchange, breaking the disulphide bond and liberating reduced PRDX (Cys-SH). The process is repeated for the mixed TRX- PRDX disulphide, resulting in a TRX disulphide bond. TRX is now oxidised and will need reducing again for TRX to continue function.

The TRX enzymes are able to reduce disulphide bonds, such as those formed between the PRDXs during hydrogen peroxide reduction, because they too contain Cysteines amino acids in their active site. Interestingly, there are 2 Cysteines (C) in the TRX active site that mediate this process, which are separated by 2 amino acids denoted by 'X', arranged in what is known as a CXXC motif (Lee *et al.,* 2013). Thus, during the reduction of the PRDX disulphide bond, a disulphide bond forms between the Cysteines of the TRX active site, in what is known as a 'thiol-disulphide exchange redox reaction' (Yi *et al.,* 2016). The PRDX is now reduced, however the TRX becomes oxidised in the process, Figure 1.6., which now must be returned to the reduced state again to allow continued TRX catalytic function. Hence, it is the role of the thioredoxin-reductases (TRXr) to reduce oxidised TRX. The TRXr functions like PRDX and TRX via active site Cysteines (Saccoccia *et al.,* 2014). The oxidised TRXr is subsequently reduced again by the final electron donor and ubiquitous cellular cofactor nicotinamide dinucleotide phosphate (NADPH) (Whayne *et al.,* 2015). A full metabolic pathway involving the reduction of hydrogen peroxide by PRDX, TRX and TRXr is summarised in Figure 1.7.

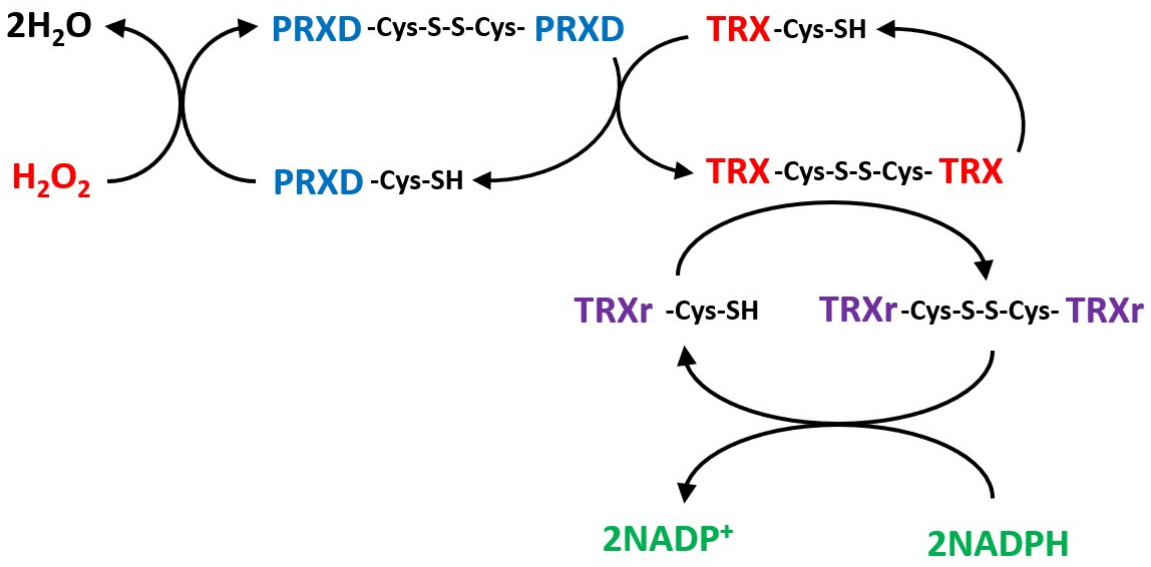


Figure 1.7: The full antioxidant catalytic cycle of peroxiredoxin and thioredoxin. Hydrogen peroxide (H_2O_2) in the cell is reduced to water (H_2O) by the action of peroxiredoxin (PRDX). The PRDX becomes oxidised in the process and is reduced by the action of thioredoxin (TRX), which becomes oxidised in the process. TRX is then returned to the reduced from by the action of thioredoxin-reductase (TXRr), which becomes oxidised in the process. TXRr is returned to the reduced form through a reduction involving the ubiquitous co-factor nicotinamide adenine dinucleotide phosphate (NADPH). Regeneration of NADPH is subsequently mediated by the pentose-phosphate pathway. Regeneration of NADPH is subsequently mediated by the pentose-phosphate pathway (Chen *et al.*, 2019)

Thus, to summarise, the removal of hydrogen peroxide by the PRDX enzymes is highly complex, involving the simultaneous action of TRX and TRXr. Therefore, it is conservable that, any disruption / dysregulation in the function of any of these 3 enzymes, may result in the accumulation of hydrogen peroxide causing an oxidative stress.

1.7 TRX, TRXr and PRDX, as biomarkers of oxidative stress.

Many studies demonstrate increased serum levels of PRDX, including PRDX-2 and PRDX-4 in response to diseases, where oxidative stress is a pathogenic feature (El-Gendy *et al.,* 2020; Abbasi *et al*., 2012). Oxidative stress is a central mediator in patients with β-thalassaemia, leading to haemolytic disease and iron overload syndrome, with PRDX-2 serum levels showing a positive correlation with serum iron levels (r=0.718, p=0.004), indicating the extent erythrocyte damage (El-Gendy *et al*., 2020). Interestingly, for β-thalassaemia patients, serum PRDX-2 levels were significantly lower than the control cohort, possibly indicating less PRDX-2 mediated antioxidant defence for these patients, which may exacerbate oxidative damage to the erythrocyte thus contributing to haemolysis (El-Gendy *et al*., 2020). However, this study was correlative and the exact mechanism mediating cellular release of the PRDX-2 remains unknown. Similar work in other disease states associated with oxidative stress have found

correlations between serum levels of PRDX-4 and disease progression. Historically, PRDX-4 has received more attention given that it is restricted to the endoplasmic reticulum, an organelle network associated with the secretory pathway in cells (Rhee *et al*., 2012; Barlowe *et al*., 2013). In 2014, the results from a large study involving PRDX-4 serum analysis from 1161 patients with type-2 diabetes was published (Gerrits *et al.,* 2014). Oxidative stress is a pathological feature of type-2 diabetes, as substantiated recently in a study by Oguntibeju, *et al*., (2019). Interestingly, the authors formally demonstrate an independent association with serum PRDX-4 levels and increased risk of mortality caused by CVD in type-2 diabetic patients (Oguntibeju *et al*., 2019).

As illustrated in figure 1.6, PRDX feeds into a larger antioxidant network involving TRX and TRXr. Therefore, by convention it makes sense that serum TRX / TRXr may also be representative of an underlying oxidative stress in respective disease states. For example, in patients with hepatitis-C, serum TRX levels were found to increase with the degree of liver fibrosis, indicating oxidative stress (Sumida *et al*., 2000). More recently, increases in serum TRX levels are shown to be a prognostic indicator in patients with sepsis (Li *et al.,* 2021). In the sepsis study, the authors evaluated serum TRX levels at hospital admission along with various markers of inflammation, including interleukin-6, concluding that early increase in serum TRX predicts 28-day mortality rate for patients in intensive care (Li *et al.,* 2021). Similarly, TRXr has also been investigated, with a recent study large-scale multicentre study demonstrating that plasma TXRr enzymatic activity has clinical utility for the diagnosis of non-small cell lung carcinoma (NSCLC), discriminating between metastatic and non-metastatic tumours (Ye *et al.,* 2019). This study is of particular interest, since oxidative stress is a pathogenic feature of NSCLC (Ilonen *et al*., 2008; Zabłocka-Słowińska *et al.,* 2019; Ito, *et al*., 2012). Taken together, this section highlights the importance of PRDX, TRX and TRXr as potential biomarkers in various diseases linked with oxidative stress. These biomarkers may therefore be important in CVD and AMI also.

It is now well accepted that oxidative stress plays a major role in the development of atherosclerosis (Turan *et al.*, 2015; Nandi et al., 2019)**.** Atherosclerosis is associated with CVD as discussed in section 1.7 and as atherosclerosis increases mortality and morbidity (Benjamin *et al*., 2018), PRDX-1 is known to be a major contributor to the maintenance of lipophagic flux and cholesterol homeostasis by regulating excessive H_2O_2 in macrophages and reducing foam cell formation and atherosclerosis (Jeong *et al*., 2017). Moreover, pre-clinical trials have evidenced that PRDX-2 has a beneficial effect in CVD, as it negatively regulates H_2O_2 generation and thrombosis formation by platelet and vascular smooth muscle cells (Jang *et al*., 2015),

suggesting that PRDX-2 could have protective benefits to the CVS, thus could represent a future potential treatment strategy (Jeong *et al.,* 2021).

Atherosclerosis represents a heightened state of oxidative stress (Soejima *et al*., 2003). Interestingly, plasma TRX concentrations have been documented to be higher in AMI patients, which may potentially predict failed reperfusion. Studies looking at cardiac drugs that stimulate nitric oxide production as well as ROS scavenging by the introducing the drug 'resveratrol', found that vascular endothelial growth factor (VEGF) was induced, which promote the growth of new blood vessels. Moreover, downstream signalling of VEGF has been linked to the regulation of TRX, leading to decreased infarct size and increased left ventricular function 4 days after infarct (Lipson *et al*., 2014; Chapman *et al*., 2017). Plasma concentrations TRX have already received some attention in literature for AMI (Rubio *et al*., 2013), as well as unstable / stable angina following chest pain (Hokamaki *et al,* 2005), and CVD resulting from atherosclerotic risk factors leading to ischemic events (Lubrano *et al.,* 2019., Pastori and Carnevale 2014). In a seminal study, Soejima *et al.*, (2003) concluded that plasma TRX levels in AMI patients decreased 12 hours without further change thereafter, however remained high in stable exertional angina, thus, giving substance for further research into the clinical utility of plasma TRX as a potential biomarker for readmission predictions.

The TRX biomarker of oxidative stress is a good starting point, however other markers (as previously outlined) are yet to be explored in much detail, including TRXr, PRDX-2 and PRDX-4. Moreover, there is no knowledge as to whether any of these biomarkers, including TRX may have clinical application for ACS readmission rates. Research in this context is especially limited with respect to the clinical setting because of financial constraints (Chung *et al.,* 2013). The TRX, TRXr, PRDX-2 and PRDX-4 enzymes are likely released during an AMI /chronic stress or ischemia due to cellular death / damage, where in the context of heart disease has not previously been researched for clinical utility. During myocyte oxidative stress, the contractile proteins may themselves become oxidised leading to potential contractile dysfunction and heart disease (Steinberg 2013), where increased turnover of PRDX-2, PRDX-4, TRX and TRXr to counter these effects may result in raised plasma levels. Ultimately, the antioxidant systems become overwhelmed, which leads to myocardial necrosis (Mueller 2013), and the subsequent release of cellular components e.g., hs-cTn as well as other proteins such as TRX, TRXr, PRDX-2 and PRDX-4. Thus, a clinical trial comparing these biomarkers at same time point i.e., during routine diagnostic sampling, may determine the relevance for intervention in patient care as a potential predictor tool.

1.8 Oxidative stress in cardiovascular disease and ACS.

As briefly stated, previously generation of oxidative stress is a key feature of coronary artery disease (CAD) pathogenesis (Chung *et al*., 2013). In this context, oxidative stress is produced in the myocardium due to the poor oxygen supply (ischaemia) during atherosclerosis (Burgoyne *et al.,* 2013), which occurs due to decreased mitochondrial flux, which leads to the generation of ROS as outlined in Figure 1.3. Seminal research has shown that this ROS may actually be released from the heart muscle and modify certain blood components (Hicks *et al.,* 1993). Ischemic reperfusion itself also causes an increase in ROS, which exacerbates the 'oxidative stress' and further induces cardiomyocyte death (Liang *et al.,* 2014). A study by Liang *et al*., (2014) found that loading doses of Rosuvastatin before the PCI reduced myocardial damage through inhibition of oxidative stress, as measured by the amount of superoxide dismutase (SOD) activity. Cardiovascular disease often precedes contributing risk factors such as diabetes mellitus, hypertension, hypercholesteremia, age, obesity, and smoking, all of which have been demonstrated to increase oxidative stress (Pastori *et al.,* 2014). During an AMI, various enzymes that modulate intracellular redox balances are secreted in response to cardiomyocyte oxidative stress, potentially modulating systemic inflammation. Increased levels of TRX have been reported in patients following AMI (Soejima, 2003), however it is not known whether these increased TRX levels have clinical utility i.e., can predict a readmission or lesion. To date, there are no studies which have monitored plasma TRX levels from time of event, to through the recovery period i.e., follow- up sampling. Thus, there is a gap in the knowledge in this regard, given the relevance of TRX in AMI. Given that plasma TRXr, PRDX-2, and PRDX-4 are also elevated in the context of oxidative stress, no study has evaluated the potential clinical utility of these biomarker collectively in the context of ACS, at point of diagnosis of an AMI and follow-up. Thus, further gaps in the knowledge are identified.

1.9 Aim and objectives

Following review of the above literature it is clear that early diagnosis of ACS is essential for the most effective treatment strategy for patients admitted with AMI. This is particularly important for NSTEMI patients as clinical symptoms are not as easy to diagnose. The hs-cTn is limited at the very early stages of pathogenesis and only rise once an ACS has developed and myocardial damage has occurred (Marshall and Bangert, 2008; NICE 2013; Nice 2015; Boeddinghaus *et al*., 2015; Apple 2017). Furthermore, hs-cTn does not predict readmission rate, or the lesion which caused the ACS in the first place, since some lesions carry a worse prognosis. Taking everything together, the plasma biomarkers of oxidative stress e.g., TRX, TRXr

PRDX-2 and PRDX-4, may therefore represent an interesting prospect for potential early detection, indication of potential readmission rates as well as the impact of cardiac lesion type and subsequent PCI, which may be associated with greater ischaemia and subsequent oxidative stress. Thus, this project seeks to evaluate these biomarkers in ACS from the onset of chest pain, and various time-points following the event i.e., 1-3 month and 6 months following the AMI. Understanding how these biomarkers change through the disease / recover course may thus help predict readmissions. Therefore, this is a unique opportunity to gain further knowledge and insight.

The literature reviewed provides the justification for the need to monitor these enzymes in ACS, specifically TRX, TRXr, but also for exploring the lesser studied PRDX-2 and PRDX-4. As the upper limited of normal is unknown for these biomarkers, healthy volunteers will be required for baseline assessment. It is anticipated it will be evident that during and after an AMI, plasma levels of these biomarkers will be elevated, which taken together with follow-up sampling, this information may predict readmission rate and / or cardiac lesion. To investigate this aim, the following objectives are identified:

a) To clarify the mean plasma concentrations for TRX, TRXr, PRDX-2 and PRDX-4 for healthy volunteers, stratified based on sex and age, which will be used as baseline measurements for ACS comparison as well as clinical utility evaluation, since the 'healthy population' are identified as 'true negatives' (specificity).

b) To evaluate the plasma concentrations levels of TRX, TRXr, PRDX-2 and PRDX-4 for ACS patients stratified based on age and sex at initial diagnosis / screening and follow-up. Clinical utility may subsequently be evaluated, as the 'ACS patients' represent the 'true positives' (sensitivity).

c) Monitor the concentration level of TRX, TRXr, PRDX-2 and PRDX-4 through ACS patient follow-up sampling, in order to evaluate whether these biomarkers may be predictive of an ACS readmission.

d) Evaluate whether TRX, TRXr, PRDX-2 and PRDX-4 may predict readmission based on ACS patient stratified according to PCI.

Thus, to address the aim and objectives, the clinical observation needs careful design to include ACS patients at point of diagnosis and follow-up for first year of the AMI, along with a healthy aged-matched cohort. The plasma levels of TRX, TRXr, PRDX-2 and PRDX-4 will be evaluated by ELISA, however this will need careful optimisation in order to establish a detection limit <5 ng/ml. Subsequent data analysis must include evaluation of clinical utility as well as analysis that predicts patient outcome.

Chapter 2 – Methodology.

2 Trial design and recruitment.

2.1 Methodological approach.

A quantitative method of data collection was selected to answer research questions and to evaluate the validity of the biomarkers of oxidative stress. A quantitative observational approach was justified to make statistical inferences regarding the population of interest. No textual data was required to answer the research questions; therefore, no open-ended questions were addressed. Participant information from questionnaires were closed question and coded, for example, male (1) female (2). This system was used for other demographic information, such as, medication, cardiac lesions, smoking history etc., as comparative to the standard care blood samples and blood samples obtained for the study. A collection of blood plasma samples was analysed for the study participants and was directly compared to current standard care diagnostic assays. However, due to several confounding variables that could potentially affect the levels of the oxidative stress biomarkers (TRX, TRXr PRDX-2 and PRDX-4), a demographic questionnaire was also chosen to ensure that data from the point of care of all participants was collected, whether non-cardiac or ACS. Methods predicted to analyse data ensured a robust cross-referencing and comparison between all stratified groups to evaluate the clinical relevance of the oxidative stress biomarker.

All data coded was entered to a Microsoft Excel database to allow descriptive statistical data analysis. All inferential and predictive statistical analysis was subsequently carried out using SPSS v28 (IBM) as described later.

2.2 Study design.

This research herein is a quantitative single site cohort study, recruiting cardiac participants from and sponsored by Worcestershire Acute NHS hospital trust (WAHT) (Appendix A).

All participants after gaining informed consent had a plasma blood sample collected (Appendix B) and completed a short demographic questionnaire (Appendix C and D). All ACS patients admitted to WAHT were evaluated for Troponin-T high-sensitive (hs-cTn) conducive with standard diagnostic protocols. These assays were performed by a local clinical laboratory

as part of routine care, with diagnosis as per hospital protocol for ACS using the >0.20ng/L as a positive biomarker for ACS (WAHT, 2015). This provided a definitive variable and inclusion criteria for which arm participants were stratified (Section 2.6). All ACS participants were followed up at 1-3 months and then at 6 months, before a medical review at 12 months. Healthy volunteers had a single visit only.

2.3 Trial method strategy and procedures.

The study population included patients admitted with ACS who met the inclusion criteria and were given an informed choice if they would like to participate. Written informed consent from patients was obtained before any study specific activities were conducted. Patients invited to participate were given a Patient Information Sheet (PIS) (Appendix E) to read and take away with them, and they had the opportunity to discuss and have questions answered before they agreed to participate. The PIS informed the participants the purpose of the study, what would happen if they took part and detailed information about the conduct of the study (Appendix E). Participants were given the opportunity to ask questions if there was anything that was not clear of if they needed more information. Participants were allowed to take time to decidewhetheror not they wished to take part.

If during the acute phase it was not possible to obtain full consent due to ACS stability or imminent procedures such as Percutaneous Coronary Intervention (PCI), willing participants could express verbal permission following the researcher reading verbal consent sheet to them (Appendix F). If it was deemed that they fully understood and gave permission, the sheet was signed / dated and placed in their medical notes. Full consent was then obtained in accordance with the Worcestershire Acute Hospitals Standard Operating Procedure (SOP) for obtaining informed consent for research study patients (SOP - WAT 01V1.0) when the participant became stable.

Blood plasma was collected from participants for oxidative stress biomarker analysis at point of hospital admission or clinic appointment following consent. This permitted stratification into one of two study arms according to hs-cTn (see section 2.6).

Inclusion eligibility was designed to allow recruitment of a wide range of participants at risk of ACS (ST Elevated Myocardial Infarction and Non-ST Elevated Myocardial Infarction) verses non-ACS / healthy volunteers. All participants were given a Participation Information Leaflet (PIL) before gaining written informed consent in accordance with ICH- GCP (Good Clinical Practice (2016).

All acute patients had routine hs-cTn assessment, and these results were obtained from a local diagnostic laboratory as previously stated. ACS was confirmed if blood hs-cTn level was >0.20ng/L as per Worcestershire Acute Hospitals Chest pain pathway (WAHT, 2015), which informed stratification (section 2.6). A small percentage of patients that were discharged were invited to participate as 'post-Myocardial Infarction' (Appendix G). A copy of the patient information leaflet and signed consent form was electronically stored on the WAHT electronic records (EZ notes), and a GP letter was sent internally (Appendix I). The recorded data was always kept in a secure location within Cardiology Research Department, locked and only accessible by authorised staff.

All participants from the ACS arm were recruited from study sites approved by the Ethics Committee and Local Research and Development Department. The control group participants consisted of colleagues from Worcestershire Hospitals and the University of Worcester. Following preliminary recruitment, further ethical approval was sought to increase the number 'healthy volunteers' from the Blood Transfusion Service to match cohorts. (Appendix H). It was necessary to use the Blood Transfusion Service for participant recruitment to match the female / male ratio in the 'healthy' and 'ACS' cohorts respectively.

2.4 Clinical population details.

Patients presenting with chest pain at Worcestershire Acute Hospital Trust (WAHT) on ward rounds were identified via the hospital admission system or consultant referrals. Potential participants were approached for eligibility at the time of their presentation to hospital or at outpatient clinics. All patients chronologically presenting with chest pain were eligible for inclusion based on clinical presentation and protocol design. The sampling was not to benefit or hinder any ACS cases that could've been missed.

Owing to the urgent nature of the admissions with ACS, information was provided as soon as clinically stable to discuss. Participants were also approached when attending rehabilitation classes within a few weeks following their admission, so again this was a chance for any patients that have been missed to have the opportunity to participate.

All patients were informed that participation in the study was completely voluntary and that their standard care would not alter if they wished to decline.

Healthy volunteers were approached verbally at WAHT via word of mouth. Participants from the University of Worcester were approached via email invite from the Head of Research, allowing potential volunteers to come forward after consideration. Healthy volunteers from the Blood Transfusion Service were approached separately, where blood was drawn for this study oxidative stress biomarker analysis following the Blood Transfusion own standard operation procedure.

2.5 Sample size calculation.

Using previous data obtained by our group from a preclinical model setting, it was determined that the various oxidative stress biomarkers (TRX and TRXr PRDX-2, PRDX-4) collectively had a standardised difference of 0.59, based on a target difference of 0.49 and standard deviation of 0.83. Therefore, a target sample size of 120 patients was required for $p<0.05$ and a power of 0.9 (in accordance with Whitley and Ball, 2002).

A total excess of 120 participants were therefore required to be recruited into this study to include Arm 1 (n=40), Arm 2 (n=40) and Healthy Cohort (n=40), from which resulted in a total of over 280 target blood samples i.e., ACS patients x point-of-diagnosis + follow-ups and Healthy Cohort x single sample. The sample size selected also accounted for statistical calculations in the case of unequal sized groups, should this had been the case at the end of the study (Al-Eid *et al*., 2019).

In this way, measuring the various plasma biomarkers of oxidative stress (TRX, TRXr and PRDX-2, PRDX-4) allowed for a direct comparison with the standard care biomarker used in diagnostics among all ACS participants recruited. This was irrespective of participant compliance; it was anticipated that the admission data would be able to assist in determining whether plasma biomarkers of oxidative stress level had impact on disease course and / or patient prognosis. The end point data was collected at a readmission event or captured at final follow-up at 12 the month medical review.

2.6 Sampling strategy for data collection.

Any patients ≥18 years old admitted with suspected ACS were screened for eligibility as per the researcher's availability. As previously stated, standard medical care was not affected upon inclusion into the study.

2.6.1 Sampling strategy to determine Arm-1 and Arm 2.

ACS patients (ST-elevated MI, non- ST elevated MI) patient admitted into hospital that were eligible to participate following informed consent were stratified into one of two groups (Arm-1 or Arm-2, see below) as defined by the standard care hs-cTn time (Figure 2.1) and described below.

2.6.2 ACS group Arm-1

- Patients who have had a Myocardial Infarction within 12 months of screening.
- Included patients as per WAHT ACS pathway with confirmed diagnosis of AMI e.g., with elevated hs-cTn >0.20ng/L (WAHT, 2018). Negative: both <20ng/L MI: Change in serial hs-cTn >10ng/L with one result > 20ng/L within last 12 months.

2.6.3 ACS group Arm-2

- Patients who have had a Myocardial Infarction screening and study drug obtained within 24 hours as hs-cTn of or within 24 hours (acute group).
- Included patients as per WAHT ACS pathway with confirmed diagnosis of AMI e.g., ACS diagnosed on clinical symptoms with for elevated hs-cTn >0.20ng/L, (WAHT, 2018).

2.6.4 Control group - healthy cohort

- No medical history /diagnosed cardiac events. An attempt to equal weighting of gender (sex) were attempted and age match of the ACS cohort.

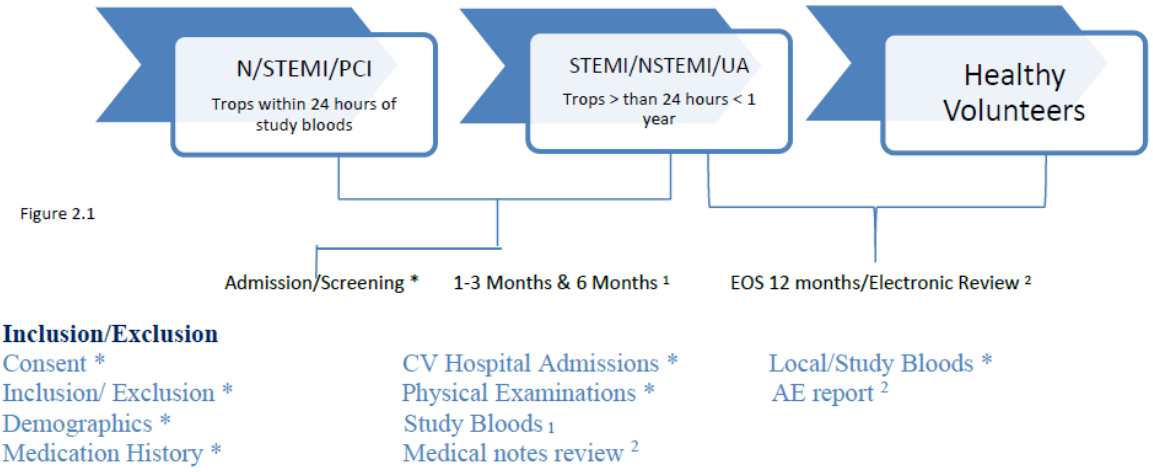


Figure 2.1

Figure 2.1. Schematic illustrating participant recruitment and stratification. Diagnosis criteria for suspected ACS was confirmed with at least one of following from standard care. **1)** hs-cTn on Admission. MI: Change in serial hs-cTn >10ng/L with one result > 20ng/L. Negative: both <20ng/L. **2)** ECG abnormalities - e.g., ST depression >0.5mm documented from standard care.

2.7 Inclusion criteria – ACS group.

As stated previously, inclusion eligibility was designed to allow recruitment of a wide range of participants at risk of ACS (ST Elevated Myocardial Infarction / Non-ST Elevated

Myocardial Infarction) verses non-ACS / healthy volunteers. All participants were given a Participation Information Leaflet (PIL) (Appendix E) and obtained written informed consent in accordance with Good Clinical Practice (ICH- GCP, 2016).

2.8 Exclusion criteria – ACS group.

- Aged <18 years.
- Unstable Angina / NSTEMI / STEMI complicated by trauma, Gastrointestinal bleeding.
- Co-morbidities with life expectancy less than 12 months.
- Known liver or renal disease prior to admission.
- Inability to consent.

2.9 Obtaining informed consent.

As discussed in Section 2.3, potentially eligible patients who were willing to take part in the study were asked to sign an approved written informed consent form. Consents were obtained in accordance with the WAHT Standard Operation Procedure Version 1 (10/10/2016).

At point of consent, the purpose of the study was explained to the participants, who were provided with a Patient Information Sheet (PIL) to read and to answer any questions they may have had. As guided by ICH-GCP, an appropriate time was given for participants to consent from admission to discharge. Capacity understanding was determined by me and appropriate time was considered based on the individual clinical evaluation. If discharged, then participation was still possible but the participant was stratified to a different group. The PIL explained the aim of the study, potential risks and all study related procedures (Appendix E). It was emphasised that the study is completely voluntary, and 'they' were free to withdraw at any stage without prejudice to standard care. No study related procedures were undertaken until full consent was obtained, unless verbal consent was given for unstable participants.

All clinical data required to confirm eligibility was considered as 'standard care' e.g., ECG's, hs-cTn results, and were not classed as study related procedures. Once written informed consent was obtained a copy was given to the participant, and a copy scanned in their medical notes, with the original kept onsite in file along with a copy of the PIL as previously stated.

In addition to blood plasma the study collected data on health and treatment; which did not present any additional risk to the participant, other than the ones related to taking blood samples, and did alter the standard procedure of care. It is noted that taking blood may cause the participant to feel faint, may cause bruising, pain or bleeding from the puncture site, but it was anticipated that this will be minimal as researcher was responsible for taking bloods and

an experienced phlebotomist. Thus, any of the afore mentioned risks were minimised as much as possible and standard trust policies were adhered to. All participants had a sample of blood taken into an EDTA blood test vacutainer for plasma biomarker of oxidative stress analysis. Approximately 4mls of peripheral venous blood was acquired for each participant. For admissions, the blood samples were collected as soon as possible after stabilisation of the ACS admission. This was followed by 1-3 month and 6 months participant matched blood sampling.

Participants for the healthy control group were approached for a single appointment and blood sample only. Interview questionnaires were completed by / with the participants, which included demographical and lifestyle indicators for comparatives to the various blood plasma biomarkers of oxidative stress. This was important ascertain since certain lifestyle choices may affect the biomarkers of oxidative stress, as described later).

2.10 Ethical approval.

Before commencing the study approval from the Health Research Authority and Local ethical approval was obtained.

The Health Research Authority consisted of a full protocol review followed by a face-to-face board meeting. All paperwork which formed the Health Research Authority approval is included in the appendix, where version two is displayed. This denotes board change requests, (see Appendix R) relating to further Information requested. The board consisted of a chair, professional representants including a GP, Physiotherapist and a lay person.

2.11 Ethical considerations and approvals.

Multiple ethical considerations were addressed for the recruitment of participants into this research study, since the initial introduction to the study on admission will potentially be during episodes of chest pain or during an AMI. As a researcher it was forefront to always have the welfare of the patient's best intentions to prevent non-maleficence. No treatment would be given to the patient that would cause unnecessary harm, but equally no delay in treatment could occur that could cause potentially more harm or damage to the heart tissue by delay. Assessing whether the patient had decision making capacity due to the pain, fear and being diagnosed was paramount. If assessed as having capacity, then a verbal consent would be obtained and documented in the medical notes and full consent obtained when the patient stable. The principal of beneficence is underpinned as 'researcher' and 'autonomous practitioner' was always for the best interest of the patient. Therefore, for this study, delay of

treatment was main ethical consideration. For the patient, participation is a complete altruistic act as the study will not directly benefit them but potential benefit others in the future.

Ethical issues surrounding the sensitive nature of being admitted during what is potentially a life changing or even life limiting event was always sensitively approached during initial assessment of capacity to consent. It was anticipated that the role of the researcher being visibility part of the clinical team in treating them for an acute episode, would hopefully develop a confident relationship between the researcher and participant, as ongoing relationships can be formed during the research process. Moreover, it was anticipated that the extra contact to a health care professional, with access to the team, would further benefit the patient participating.

However, working closely with other teams such as 'Cardiac Rehabilitation' ensures all patients recruited (or not as that case may be) benefit from this interaction, ensuring no bias in clinical practice. Moreover, study recruitment was randomly selected on admission and stratification was based on clinical symptoms and hs-cTn results. Therefore, there was no bias cohesion or prioritisation to any patients admitted, justifying the study design and collection methods from an ethical standpoint.

As previously stated, this study collected data on health and treatment; therefore, it does not present any additional risk other than the ones related to taking blood samples, and it did not alter the standard procedure of care. Bloods collection for the study where possible, was performed during the standard diagnostic procedure.

2.11.1 Other non-clinical ethical issues considered included the follow-up appointment and blood collection visits. Here, free parking was made available to all participants who made extra visits for purposes of the study to minimise the burden resulting from this visit. No other reimbursement was available. Participants had contact numbers for the Cardiology Department where after the study completion they were advised to ring for results but would not be routinely contacted.

2.11.2 The study was conducted in accordance with Good Clinical Practice (GCP). This is a pre-requirement for my position as the researcher and can be evidence by certification.

2.11.3 Ethical approval was submitted via the Integrated Research Application System (IRAS) IRAS Version 5.4.2 – reference 17/NS/0032. 189016, Full IRAS Draft (Appendix Q).

2.11.4 Favourable ethical opinion was obtained from Solihull HRA (Appendix S) followed by

local R&D approval, which was obtained prior to commencement of any study related procedures (Appendix T).

2.11.5 Protocol amendments were submitted during study and approved before implementation. Amendments submitted to local R&D for approval prior to implementation was submitted to recruit Healthy Cohort from blood transfusion service, and rational will be discussed later (Appendix U).

2.11.6 A summary report at the end of the study was presented to the Research Ethics committee with the year of completion.

During the study any substantial modifications to the clinical protocol would have been submitted for reapproval.

2.12 Confidentiality.

All data will be treated in the strictest of confidence.

Only the researcher and members of the Cardiology Research Department had access to patient information, with all data being kept in a secured locked / keypad-controlled room. Only Consent forms and patient screening log contained identifiable data, which was kept in secure site file.

A patient screening log was maintained for the duration of the study to enable participant contact (Appendix V). This was destroyed at the end of study and not archived with the Investigators Site File and participants files.

All identifiable information from data sets / clinical samples that were transferred to the University of Worcester, were be removed and unique number identifiers added. This system was used on all documents and clinical samples shipped. Likewise, all patient data was anonymised. Each patient entering the study was given a case specific unique number. This number replaces the use of any personal identifiable data and was used on laboratory specimens, participant questionnaires and data bases.

A site file and list of case specific study numbers for patients was kept, and this list did not leave the Worcestershire Acute Hospital site. Any published results / data will not contain any personal data that could allow identification of participant.

The Cardiology Research team had access to the personal data of participants during the study but are governed by the same data protection and Caldicott principles. All Cardiology

Research team staff are trained in Good Clinical Practice (GCP).

2.13 Data collection – methods.

Patients were screened, consented and recruited in the Coronary Care Unit or the Cardiac Catheterisation laboratory. Most of the patients stratified to Arm-2 (see section 2.6), were recruited from the Cardiac Catheter laboratory during their AMI. However, as long as the routine hs-cTn blood samples were obtained within 24 hours of the oxidative stress study blood samples, participants were included in this arm.

If greater than the 24 hours they were stratified to Arm-1. As only a single site was used for capturing the data for the ACS groups, it was simplified for continuity of the hs-cTn result. WAHT use the Abbott Architect high-sensitive Troponin T, the 99th percentile values for hs-cTn, where cut-off values are the same for men and women.

Data was collected following informed consent and a questionnaire completed as described in Figure 2.1. As previously stated, data was collected from routinely available standard care hospital records, including, base line demographic data, co-morbidities for exclusion criteria, standard care blood test results and Percutaneous Coronary Intervention (PCI) results. The questionnaire approach method assisted with the interpretation of the plasm biomarkers of oxidative stress (i.e., smoking can affect serum oxidative stress levels), whilst keeping the inclusion criteria as wide as possible. A study by Millett *et al*., (2018) identified that myocardial infarction is more prevalent in women than men that have high known risk factors such as hypertension, smoking and diabetes. The data used in the study by Millet *et al*., (2018) was obtained from the UK biobank, where all United Kingdom data on adult admissions are included. However, it is widely accepted overall, that men are at greater risk of myocardial infarction than pre-menopausal women (Pallarito *et al.,* 2019; Kmietowicz 2018).

Smoking regardless of sex is a high-risk factor for contributing to myocardial infarctions but a study has indicated that for individuals using e-cigarettes (vapes) daily, were 1.8 times as likely to have an AMI, but also may have an impact on oxidative stress (Tobore 2019; Onojighofia 2019). Furthermore, dual use (smoking and vape) is shown to increase AMI risk however, the authors acknowledged limitations of not knowing timings between smoking and the AMI event. Thus, ‘smoking’ and ‘vape’ use were added to questionnaire to aid data interpretation with regards to ACS and oxidative stress. Healthy volunteers also completed a questionnaire to obtain a base line indication of same risk factors.

The questionnaire also documented factors already known to increase the oxidative stress. For example, it is known some clinical conditions such as renal and hepatic disease may affect hs-cTn results and therefore may also influence changes in serum oxidative stress biomarkers. (Mbagaya *et al.,* 2015; Defilippi *et al.,* 2018; Corte *et al.,* 2014) uric acid results (Lazzeri 2015). Thus, due to the nature of the participant selection process, not all renal or hepatic disease was detectable until after recruitment, therefore it was documented on the questionnaire to facilitate later data interpretation.

Chronic Kidney disease (CKD) was an exclusion factor if known prior to recruitment, as studies demonstrate that hs-cTn results are challenging to interpret with these comorbidities (Defilippi *et al.,* 2018; *Wu et al.,* 2008). Interestingly, Li *et a*l (2019) found raised hs-cTn levels in CKD patients without evidence of AMI symptoms. However, these have been refuted following studies on patients receiving dialysis and, the overall consensus is that that 2 biomarkers provide similar diagnostic and prognosis in all patients (Defilippi *et al.,* 2018, Twerenbold *et al.,* 2018). Since it was unknown at the developmental stage of the study what the effects on plasma biomarkers of oxidative stress are for CKD patients, they were excluded from the study where possible.

The same impertinencies occur with heart failure patients. Following the inclusion criteria process, participants that had a history or developed heart failure during admission following a STEMI were included. Where possible natriuretic peptide (NT-proBNP) was be obtained. Echocardiograms are not routine until the patient has stabilised and are usually routinely carried out prior to discharge; therefore, it was impossible to determine reduced ejection fractions on assessment of AMI. This data was highlighted after the laboratory measurement of the plasma markers of oxidative stress.

2.14 Time points of data collection.

For ACS / non-ACS patients an initial blood sample was taken for plasma oxidative stress biomarker analysis at point of entry into the study i.e., at diagnosis of within the specified timeframe as previously stated. Follow-up blood sampling was taken at 1-3 months and 6 months (+/-7 days) following initial admission at Worcestershire Acute Hospital outpatient clinics. (See Table 2.1)

All appointments were made at 4 weeks post admission. If participants did not attend

within 12 weeks, the subsequent follow-up sample will be taken at the 6-month visit. It was preferable to gain samples at 4 weeks, since it is known that following myocardial necrosis or percutaneous coronary intervention, hs-cTn can continue to rise for 2 weeks, indicating ongoing myocardial damage which may also impact on oxidative stress biomarkers (Möckel *et al*., 2015; Liebetrau *et al.*, 2013; Mueller-Hennessen *et al*., 2017; Katus *et al*., 1992). It would have been beneficial to bring participants back within this time frame, however, patients are not allowed to drive for 1-2 weeks following a NSTEMI and 4 weeks after a STEMI. Therefore, to allow for continuity the 4 weeks follow-up was established. Final data collection after the end of the study was by medical notes only. This was at 12 months + 30 days. For all time-to-event analysis, the actual date of event was sought and factored into the data analysis accordingly.

2.15 Types of data collected.

As outlined previously, the participants admitted following ACS will all have had a hs- cTn result as part of standard care, which was used for screening / stratification and for subsequent comparison with the serum biomarkers of oxidative stress.

For participants recruited as 'outpatients', the hs-cTn will be sought within 12 months of recruitment. Other recorded parameters were Aspartate Transaminase (ALT) and Estimated Glomerular filtration Rate (eGFR) from local laboratory to assist in inclusion criteria (hepatic / renal), creatinine, total cholesterol, low-density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL).

Table 2.1: Assessment schedule of activities

Visit	Screening	1-3 & 6 Mths	12 Mths
Informed consent	**X**		
Inclusion/Exclusion	**X**		
Study bloods (4-8 mls)	**X**	**X**	
Medication	**X**		
Smoking & Alcohol	**X**		
Questionnaire	**X**		
Hospital Admissions review	**X**	**X**	**X**

2.16 Sample shipping and storage.

Blood sample specimens for subsequent oxidative stress biomarker analysis were stored locally in -20 ^{o}C freezer and a temperature log was maintained throughout (Appendix J). Sample 'practice runs' using dry ice was conducted prior to recruitment on a sample blood, to check the defrost time and stability of biomarkers. Samples were shipped on the day of collection or fortnightly to the University of Worcester (UoW) to a -80 ^{o}C freezer in accordance with biomedical transfer regulations Material Transfer Agreement (Appendix K) between the University of Worcester and Worcestershire Acute Hospitals (WAHT). All samples were treated the same in confidence that the short-term storage did not affecting stability of biomarkers when frozen at -20 ^{o}C or below (Jansen *et a*l., 2013). When samples were stored locally at WAHT the temperature log was maintained manually, however this was logged digitally at UoW as an integrated feature on the freezer.

The sample collection and storage policy were as follows: -

2.16.1 Complete the dangerous goods training as samples shipped on dry ice. By law, any person who handles dangerous goods to be transported by a public carrier must follow specific regulations and must have proof of training. Dangerous Goods training at www.mayomedicallaboratories.com for classifying, packaging, labelling and transporting specimens (Appendix L) for transfer label and certification kept in main site file as per index section 10.3 (Appendix M).

2.16.2 All consenting participants had 4mls of blood phlebotomised into an Ethylenediaminetetraacetic Acid (EDTA) BD vacutainer, Lavender top. Only 'in date' vacutainers were used to draw blood. Vacutainers contain EDTA to prevent coagulation prior to centrifugation. All samples were kept refrigerated or cold packed to be centrifuged within an hour of collection.

2.16.3 Centrifugation separates the whole blood by density, with blood cells sinking to the bottom and the plasma accumulating at the top. The centrifuge used for the study was a Kestrel laboratory centrifuge (calibrated yearly and certificates in site file). Bloods were centrifuged as per Section 2.21 and stored (Appendix N). The plasma (liquid part of the blood containing serum + blood clotting factors) was evenly distributed into four cryovials and frozen (-20^{o}C) for shipment to University of Worcester (See picture A), and defrosted once into 5 aliquots pre-assay to ensure all were kept under exact same conditions with no variability to ensure stability. All recorded as per section 2.16

Picture A: Plasma blood samples storage in cryovials and aliquots.

2.17 Trial retention.

All participants received an appointment card and parking permit in the post a few weeks prior to their follow-up appointment detailing appointment and location. A week before the appointment the participant was contacted by telephone to confirm time and attendance where possible. This approach was adopted to increase retention in the study.

Appointment management was a significant for obtaining data / results for the study, so every effort was made to keep participants in appointment windows. An enrolment and appointment tracker log were completed for all participants recruited (Appendix P), reminder appointment cards were sent one week preceding each follow-up appointment to maximise attendance and any missed appointments were followed-up with a telephone call.

It must be reiterated that; study participants were free to withdraw from the study at any time without reason. If a participant chose to withdraw, no adjustments for missing data were performed. All available data is presented herein. For time-to-event analyses, participant medical notes were confirmed from study site only.

2.18 Data collection for analysis.

The following data was collected regarding participant clinic events and readmissions during the course of follow-up verified at end of the study. All was entered into an excel data base ready for analysis, detailed as data base key in Appendix O.

Death

- Date, time or circumstances of death recorded.

ACS readmissions

- Myocardial Infarction
- Location of Myocardial Infarction of PCI
- Repeat PCI
- Unstable Angina

Other

- TIA/CVA
- Cancer
- General Surgery

2.19 End of study.

The end of study was 12 months after recruitment, equal to or up to one month after their coronary event or if a participant passed away. The end of study visit was a medical review of local hospital system. End points were categorised as 'no admissions', 'cardiovascular disease' defined by ACS admission, 'Cerebral Vascular Accident (CVA)', 'cancer' or 'other' non-cardiac admissions.

2.20 Blood sampling.

Participants recruited into the study were required to donate a blood sample for subsequent oxidative stress biomarker analysis. For this, non-fasting blood samples were taken at screening and scheduled visit at 1-3 months and 6 months for all Acute Coronary Syndrome (ACS) participants and at screening only for Healthy cohort. A total of 4 ml venous blood was drawn into a lavender EDTA vacutainer tube (BD). Following blood draw, the sample was gently inverted and left to stand for 30 minutes.

2.21 Blood plasma and erythrocyte isolation.

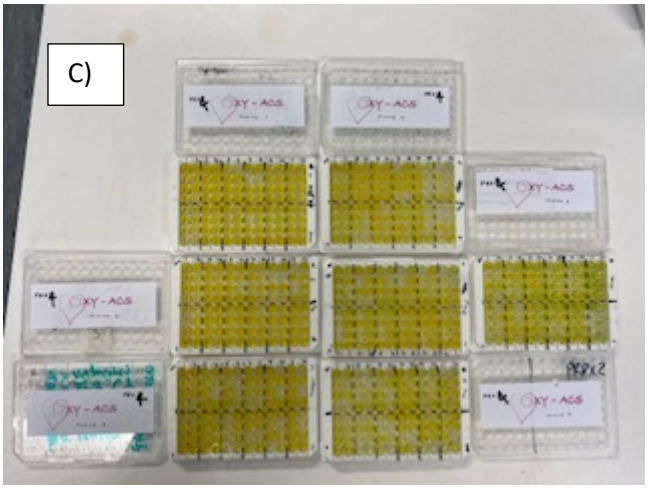
Separation of venous blood into plasma and cellular fractions was done by centrifugation. Each blood sample was centrifuged at 491 x g for 5 min using a Kestrel MSE1191 centrifuge (MES, East Sussex, UK). Following centrifugation haematocrit levels (packed cell volume) was determined by marking and measuring the erythrocyte and plasma layers on the tube respectively. This was done for each blood sample and recorded in Laboratory notebook 0127. Following this the plasma was aspirated and added to a fresh centrifuge tube and kept on ice. The remaining erythrocyte fraction was washed in a volume of Hank's Balanced Salt Solution (HBS) (Sigma-Aldrich, Dorset, UK) to the marked level of original plasma and inverted a few times to gently mix. This was followed by centrifugation at 491 x g for 5 min and the HBS removed to waste (VirkonTM). The erythrocyte wash step was repeated twice. After the second wash the erythrocytes were suspended in sterile phosphate buffered saline (PBS) adjusted to

contain 40% v/v glycerol (Sigma-Aldrich, Dorset, UK) up to the marked level of the original plasma line. The erythrocyte suspension was aliquoted into cryovials for storage at -80°C.

The previously harvested blood plasma fraction was centrifuged at 2,000 x g for 15 minutes to deplete platelets and other contaminating cellular material e.g., leukocytes, before aliquoting to cryovial and storage at -80°C until the analysis was carried out. The plasma aliquots were kept at -20°C at WAHT until transfer to UoW for long term storage at -80°C. It was ensured that the samples were frozen at -20 °C for maximum of 2 weeks to ensure consistency in handling all samples. Research on different oxidative stress biomarkers shows that the enzymatic activities of rat plasma enzymes are not affected by storage at-20 °C (Bortolin *et al.*,2016), nonetheless, transfer to -80 °C was consistent and rapid.

2.22 Blood plasma oxidative stress biomarkers analysis

A total number of 151 participants were recruited into this study, each plasma sample donated ('healthy donor control', 'diagnostic ACS' and 'ACS follow-up') was analysed for TRX, TRXr, PRDX-2 and PRDX-4, by ELISA (See picture B and C). A total of 3,456 ELISAs were subsequently performed.



Picture B and C: Demonstrates the plates set up methodically, each biomarker took seven complete plates to assay.

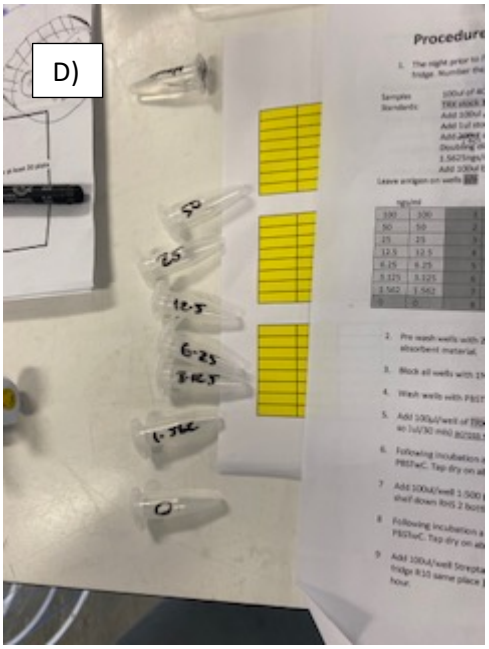
2.23 Quantitative ELISA for TRX, TRXr, PRDX-2 and PRDX-4.

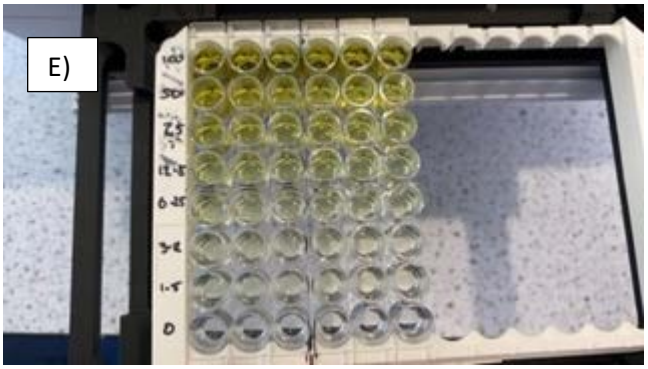
The plasma concentration of each biomarker was determined from a standard curve against commercially available recombinant human protein (Table 2.2).

Table 2.2. Details of recombinant protein and antibodies used for the quantitative ELISAs

Antigen	Recombinant Protein	Primary Antibody	Secondary Antibody
TRX	AbCam #ab51064	AbCam Rabbit polyclonal (IgG) biotin #ab26320	AbCam Goat anti-rabbit IgG (biotin)
TRXr	AbCam #ab168011	AbCam Rabbit monoclonal (IgG) biotin #ab124954	AbCam Goat anti-rabbit IgG (biotin)
PRDX-2	AbCam (Cambridge, UK) #ab85331	AbCam Rabbit monoclonal (IgG) biotin #ab133481	AbCam Goat anti-rabbit IgG (biotin)
PRDX-4	AbCam #ab93947	AbCam Rabbit monoclonal (IgG) biotin #ab184167	AbCam Goat anti-rabbit IgG (biotin)

Each antigen ELISA was optimised using a checkerboard titration prior for use in the evaluation of participant plasma. See Picture D, which demonstrates the preparation of dilution at different ratio's for PRDX-2.





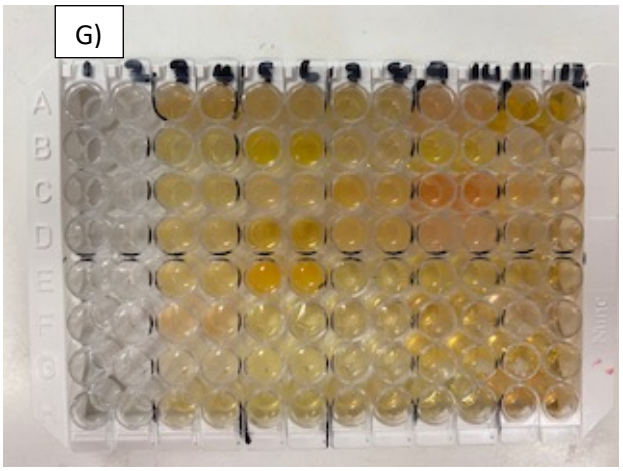
Picture D, E and F: Picture D, demonstrates the preparation of dilution series at different ratio's Picture E and F. Demonstrates the checkerboard titration used to optimize. Row A having highest concentration and row H no antigen and served as background control, using a primary antibody 1:1000, 1:2000 and 1:4000 with Secondary 1:5000 and 1:10000. The standard curve can be found in Appendix Y, PRDX-2 1:2000 dilution of the primary antibody (5 µl in 10 ml) with a 1:5000 dilution of the anti-rabbit biotin (2 µl in 10 ml) provided the optimal ratio and used for procedure.

Table 2.3 shows the optimal antibody dilution factors for each antigen to give best sensitivity (<5 ng/ml) as determined from the standard curve (Appendix W, X, Y and Z).

Table 2.3. Optimised antibody dilution factors for quantitative ELISA

Antigen	Primary Antibody	Secondary Antibody	Streptavidin-HRP, SLS (Nottingham, UK) #RPN10512ML
TRX	1 in 10,000	1 in 500	1 in 8,000
TRXr	1 in 1,000	1 in 5,000	1 in 8,000
PRDX-2	1 in 2,000	1 in 5,000	1 in 8,000
PRDX-4	1 in 2,500	1 in 4,00	1 in 8,000

Each biomarker of oxidative stress was quantified against a standard curve using the respective recombinant antigen at the following concentrations diluted in PBS; 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.125 ng/ml, 1.5625 ng/ml and 0 ng/ml. A 96-well Greiner ELISA plate (M6562, Sigma-Aldrich, Dorset, UK) was loaded with 100 µl the standard curve in duplicate and 100 µl of participant plasma in duplicate and incubated overnight at 4°C to ensure binding (Picture G).

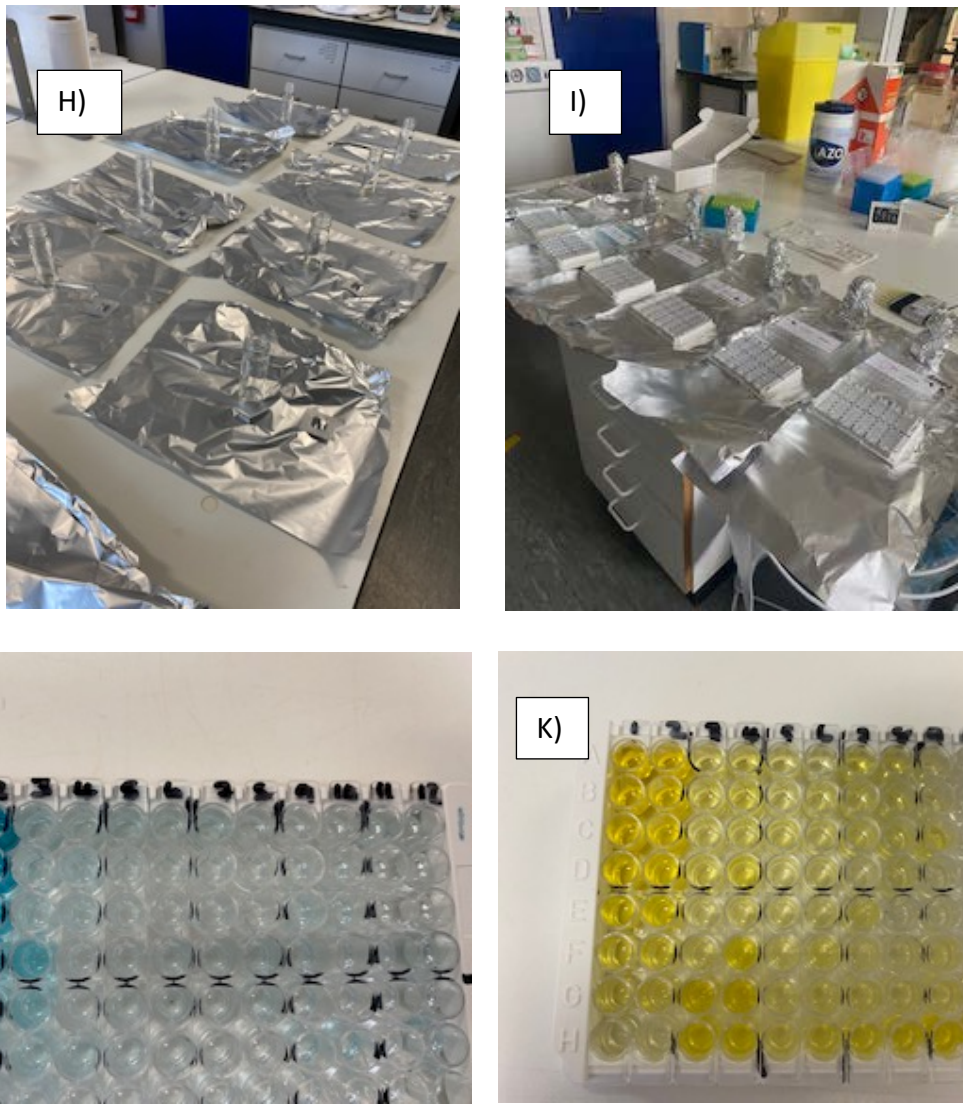


Picture E: 96-well Greiner ELISA. Plate loaded with 100 µl the standard curve in duplicate and 100 µl of participant plasma in duplicate and incubated overnight.

Each well was washed x 3 in 200 µl PBS adjusted to contain 0.1% w/v casein and 0.05% v/v Tween-20 (PBSTwC). 200 µl of block solution (PBS adjusted to contain 1% w/v casein) was added to each well and incubated for 30 minutes at room temperature (RT). Following this, each well was washed x 3 in 200 µl PBSTwC before the respective concentration of primary antibody was added (see Table 2.3) diluted in PBSTwC and incubated for 1 hour at RT. Following this, each well was washed x 3 in 200 µl PBSTwC before the respective concentration of secondary antibody was added (see Table 2.3) diluted in PBSTwC and incubated for 1 hour at

RT. Following this, each well was washed x 3 in 200 µl PBSTwC before the respective concentration of streptavidin-HRP was added (see Table 2.3) diluted in PBSTwC and incubated for 1 hour at RT. Following this each well was washed for a final time with 3 x PBSTwC before the addition of 100 µl TMB (3,3',5,5' tetramethylbenzidine) solution (1 TMB tablet, Fisher Scientific, Loughborough, UK., and 1 Phosphate Citrate Buffer tablet, Sigma-Aldrich in 100 ml dH_2O).

The plate was wrapped in foil to protect from light and incubated for 30 - 45 minutes at RT with gentle agitation (Picture H and I). The reaction was stopped by the addition of 50 µl 1.5M sulphuric acid, see picture J before and K after sulphuric acid.



Picture F, G, H, I: F) and G) show foil preparation before adding 100 µl tetramethylbenzidine (TMB) completed rapid process due to light sensitivity (picture H) shows the reaction and picture I) post 50 µl 1.5M sulphuric acid when reaction is stopped.

The ELISA plate absorbances were measured at λ450 nm using a Multiskan Ascent ELISA plate reader (Thermo-Labsystems, Cheshire, UK) See picture D example of results. Participant biomarker of oxidative stress (TRX TRXr PRDX-2 and PRDX-4) concentration was determined from the standard curve.

2.24 Data analysis

To answer the research questions, various data analysis approaches were adopted, which included a full descriptive statistical analysis, inferential statistical analysis and predictive statistical analysis.

2.24.1 **Descriptive statistics** - Shapiro-Wilks normality test was used for all data presented with respect to participant characteristics, as well as the blood plasma results for TRX, TRXr, PRDX-2 and PRDX-4. If the data passed the normality test ($\alpha<0.05$). A large p-value indicates the data set is normally distributed. T-Tests and other parametric tests e.g., ANOVA were performed. If the data failed the normality test i.e., due to low number following stratification, equivalent non-parametric analysis was carried out e.g., Mann-Whitney and Kruskal-Wallis.

2.24.2 **Two-way mixed ANOVA** - was conducted to evaluate the impact of sex (male / female) with respect to the plasma concentrations of TRX, TRXr, PRDX-2 and PRDX-4. Since the sample population cohorts included a healthy population, along with ACS Arm-1 and Arm-2, a two-way mixed ANOVA was selected as this would determine interaction between participant 'sex' and population cohort.

2.24.2.1. Assumptions In order to perform the two-way mixed ANOVA, eight assumptions were determined, as outlined below.

- **Assumption #1:** The dependent variable is the average plasma concentration of the four biomarkers TRX, TRXr, PRDX-2 and PRDX-4.
- **Assumption #2:** This assumption is the 'between-subject factor' which consists of two dichotomous independent variables, each of which has two levels: Male vs Females are independently measured on a nominal scale and split into two or more categorical independent variable groups i.e., healthy cohort, ACS Arm-1 and ACS Arm-2.
- **Assumption #3:** The categorical variable within the subject group is nominal to reflect the three groups, healthy cohort is the 'control group' along with ACS split into the two ACS groups, Arm-1 and ACS Arm-2.
- **Assumption #4:** For the two-way ANOVA the following were considered.
  - **a.** To meet assumption #4, it is required to have no significant outliers. Outliers are data points within data that do not follow the usual pattern, thus causing problems with

generalising the population. Initially data was assessed by 'Boxplots' created in SPSS to observe the values of each cell. As evidence of outliers when running ANOVA an alternative method to clarify normality and outliers was analysed via studentized residues in repeated measurements. The outliers deemed as studentized residuals were greater than ± 3 the standard deviations.

b. The distribution of the dependent variant for sample-1 (screening). **Healthy cohort** (n=65), for TRX (n=65), for TRXr, PRDX-2 and PRDX-4 (n=64). **ACS Arm-1** (n=36), for TRX, TRXr (n=36), for PRDX-2 (n=35) and for PRDX-4 (n=24). **ACS Arm-2** (n=44), for TRX (n=44), for TRXr (n=42), for PRDX-2 and PRDX-4 (n=44).

The distribution of the dependent variant for sample-2 (first follow-up). **Arm-1** for TRX (n=29), for TRXr and PRDX-2 (n=30) and for PRDX-4 (n=22). **Arm-2** for TRX (n=29) TRX, for TRXr and PRDX-2 (n=30 and for PRDX-4 (n=28).

The distribution of the dependent variant for sample-3 (second follow-up). **Arm-1** for TRX(n=23), and for TRXr, PRDX-2 and PRDX-4 (n=22). **Arm-2** for TRX (n=22), for TRXr (n=23), for PRDX-2 (n=24), and for PRDX-4 (n=23).

c. There was an assumption of homogeneity between the factor groups. The healthy cohort (n=65) consisted of male (n=32) and female (n=33). For the ACS cohort overall homogeneity was not biased. There was no control over gender as determined by acute status of admissions (n=80) of which there was male (n=59) vs female (n=21). Arm-1 (n=36) consisted of males (n=27) and female (n=9), and Arm-2 (n=44) consisted of male (n=32) and female (n=12).

d. 2.24.2.2 Outcome of outliers

Having established that the outliers were genuine, no appropriate reason to reject them was not ideal, since this would result in violating assumption #4 of the two-way mixed ANOVA. Outliers may impose a negative effect on the data distribution, thus consideration was given appropriately, where it was agreed that rather than removing them, they would be replaced with the second largest result. In this way participant data inclusion is maintained at the high end of the plasma concentration levels, which is advantageous. However, a disadvantage is that it forces the data to fit a normal distribution better. Thus, altering the genuine data would introduce bias to the original data set (Ghosh and Vogt., 2012). When considering downstream statistical analysis, the ANOVA is considered relatively 'robust' to deviations from normally distributed data (Maxwell and Delaney., 2004). Therefore, this provides the opportunity to use an ANOVA if the sample size is not too small, which is the case here for all four biomarkers analysed, across all cohorts and sexes.

When assessing the outliers for the healthy cohort, it was noted that one participant was an outlier for all biomarkers evaluated. It was therefore reasonable to consider excluding this participant from the data analysis. Even though a representative cross section of the healthy population was sought, this particular participant may have an unknown underlying clinical complication. Thus, removal of this data set is appropriate. For the ACS cohort, all participants

have a baseline of events equal to meet the eligibility criteria for inclusion, therefore removing any single data points considered as outliers was not generally appropriate.

The data set for healthy volunteers followed a normal distribution for both males and females. However, in the ACS cohort the data was skewed more in male participants than female. To correct this skew by applying transformation was not possible, therefore it was accepted that the data for ACS cohort in general was normally distributed. Wilcox (2012) noted that transformation can also expose outliers on the non-skewed side, as well as potentially introduce more outliers. Furthermore, it was noted that when removing outliers from the ACS cohort, the threshold altered.

Taking everything together, it was decided that the subsequent analysis by a two-way mixed ANOVA for all biomarkers would be conducted following an assessment of which, and how many assumptions (Section 2.24.2) may be violated, for each data set 'with' or 'Excluding' the outliers included (Table 2.4).

2.24.2.3 Assumptions met or violated including and excluding outliers between healthy and ACS cohorts

Table 2.4. Determining violations 'including' and 'without' outliers.

Assumption	TRX	TRX (O)	TRX -R	TRX-R (O)	PRDX-2	PRDX-2 (O)	PRDX -4
#1 Dependent Variables Biomarkers	✓	✓	✓	✓	✓	✓	✓
#2 Independent Variables Between Subject Factors	✓*	✓*	✓*	✓*	✓*	✓*	✓*
#3 Categorical Variable	✓	✓	✓	✓	✓	✓	✓
#4a Significant outliers	✗	✓	✓1	✓	✓	✓	✓
#5 Assumption of Normality (Shapiro-Wilk)	✓	✓	✓	✓	✓	✓	✓
#6 Homogeneity of variances (Levene test of Equality of Variance)	✓	✓	✓	✓	✓	✓	✓
#7 Homogeneity of covariance (Box Test)	✗	✓	✓	✓	✓	✓	✓
#8 Sphericity (Mauchly's Test of Sphericity) Tests of Within-Subjects Effects	✓ ✓	✓ ✓	✓ ✓	✓ ✓	✓ ✗	✓ ✗	✗ ✓

Table 2.4

Key	
✓	Assumption Met outliers
✓	Assumption Met Partially
(O)	Outliers removed based on Studentized data in Sample 1,2,3
✓1	1 Studentized outlier from all groups, sample 1,2,3
✓*	Assumption accepted - Gender not met
✕	Assumption violated

- **Assumption #5:** Normality was determined by the Shapiro-Wilk's test for each cell of the design to determine if the assumptions were met or violated. Residual analysis was performed to test for the assumption, outliers were assessed by inspection, initially by using a boxplot. Q-Q plots were used to test the studentized residuals of normality to determine whether this assumption met or violated, a dual approach ensured accurate data.

- **Assumption #6:** The variance of the dependent variables were equal as in assumption #4 and #5, to enable statistical significance testing in the two-way ANOVA. Equality of variance was assessed by Levene's test to determine whether this assumption met or violated.

- **Assumption #7:** Homogeneity of covariances is required for a two-way ANOVA. A 'Box test' of equalities of covariance matrices informed whether this assumption had been violated.

- **Assumption #8:** A Mauchly's Test of Sphericity was used to evaluate sphericity where repeated measurements between groups were measured rather than variance within each group. This was important, since violation can invalidate results.

2.24.3 **Receiver Operator Curve (ROC)** analysis was performed to establish the sensitivity and specificity values for each of the plasma biomarkers for TRX, TRXr, PRDX-2 and PRDX-4 of oxidative stress in ACS patients. If probability is determined to be less than 0.5, then it can be concluded that no ACS event had occurred. Correct ACS cases were based on the predictive cut-off value of ≥0.5 (≥50%). Thus, each participant with a predicted probability greater than or equal to 0.5 would be classified as having an ACS event, whereas those below this threshold would be classified as a healthy donor. A 'Receiver Operating Characteristics' was initially conducted to consider all cut-off points in the data sets, in order to evaluate how the cut-off points, alter specificity and sensitivity. This information was subsequently used to determine substantial distinctions in the probabilities of observations in either cohort, thus defining the threshold in order to confidently predict outcome. All biomarkers were processed/coded the same for the positive ACS cases.

This analysis was based on sensitivity of the biomarker vs the false positive rate (1-specificity), where the area under the curve indicates the degree of clinical utility. The rationale for running this binary logistic regression was to establish clinical utility for each biomarker to estimate the probability of event prediction, in this instance estimate whether an ACS event has occurred.

To clarify, the term 'sensitivity' refers to the detection limit of the biomarker and measures the incidence of true positive results in participants known to have an ACS event. The term 'specificity' refers to the detection limits with respect to the healthy cohort and measures the incidence of true negative results in healthy participants who did not have an ACS event. The 1-specificiy is the 'false positive rate', i.e., the probability of a 'true negative' testing positive for an ACS event (Zou et al., 2001).

2.24.3.1 In order to run the logistic regression ROC curve, the following assumptions need to be met:

- **Assumption #1:** The dependent variable was Acute Coronary Syndrome, dichotomous by splitting into healthy cohort or ACS group.
- **Assumption #2:** The independent variable is the four independent biomarkers TRX, TRXr PRDX-2 and PRDX-4, with hs-Tn and continuous independent variables of age and BMI.
- **Assumption #3:** Refers to the independence of the observation within the dichotomous dependent variables. It was assumed that there were no relationships between the healthy and ACS cohorts or male and female, which could not be placed in both groups.
- **Assumption #4:** Binominal logistic regression relies on maximum likelihood estimate (MLE) and thus requires a recommendation of 15-50 cases. This assumption met these requirements since all participants at 145 were included. Missing cases were taken into account where there was no sample available or an ng/ml reading as zero. This was most evident for TRXr. For each biomarker, the number of data sets including was TRX (n= 145), TRXr (n=75) PRDX-2(n=143) and PRDX-4 (n=122) and hs-Tn (n = 66)
- **Assumption #5:** Requires meeting a linear relationship between the independent variables identified in assumption #3. Linearity of the continuous variables with respect to the logit of the dependent variable was therefore assessed via the Box-Tidwell (1962) procedure. If required, a Bonferroni correction was applied using all eight terms in the model (0.05 ÷ 8 = 0.00625) resulting in statistical significance being accepted when $p<0.00625$ (Tabachnick & Fidell, 2014; Laerd Statistics (2017). Based on this assessment, all continuous independent variables (TRX, TRXr, PRDX- 2/4, HS-Trop T, BMI and Age) were each found to be linearly related to the logit of the dependent variable.
- **Assumption #6:** Is not violating multicollinearity between the two or more independent variables, as this leads to difficulties deciphering which independent variable contributes to the variance of the dependent variable.

- **Assumption #7:** There should ideally be no significant outliers as these unusual points can alter the regression lines. The 'case wise list' was reviewed for all biomarkers, participants with a standardised residual (reported on ZResid) greater than ± 2 standard deviations of the mean. There was one participant (108) in all biomarkers that had a standardized residual with a value of TRX 3.488, TRXr 3.015, PRDX-2 3.460, PRDX-4 3.876, standard deviations of the mean, which was kept in the analysis. This one participant outlier was inspected and removal was not deemed as justified, as same participant was consistent through all biomarkers and therefore the data presented was deemed accurate with no errors.

The sensitivity and specificity were determined for each of the biomarkers analysed. The cut of values of probability (≥0.50) were used as a predive threshold for a positive ACS event.

The independent variables are included in the calculations for TRX, TRXr, PRDX-2 and PRDX-4, for which respective percentage 'accuracy in classification' or ACS (correct predictions / total predictions made) was determined as over 70% for each biomarker.

2.24.4 **Kaplan - Meier** - To address the aim and objectives i.e., whether TRX, TRXr, PRDX-2 and PRDX-4 plasma concentrations may be indicative of an ACS event and/or predict a second event, the Kaplan-Meier analysis was systematically performed for all four biomarkers. Predictive statistical analysis was undertaken using a Kapan-Meier method utilised to estimate the probability of readmission rates for the ACS cohort. Kaplan – Meier 'time-to-event' was analysed on all readmission rates from PCI. The endpoint for this analysis was determined as a second ACS event or cardiac admission and was determined by troponin levels. Readmissions that were non-cardiac such as CVA / medical / surgical were be censored. (Appendix AA). All data was collected over 12 months or 365 days plus 30. Censoring in this way removed bias of patients who died before a recurrence See Appendix AA for included ACS events, events censored and the timing. Limitations are that there was only the two assumptions, Arm-1 and Arm-2 of ACS admission based on upper, median and lower quartile of biomarkers means. Initially, this was performed for the 'blood sample-1' percentile concentrations. To recap, these percentile concentrations were calculated as <25% percentile, the inter-percentile range and >75% percentile.

Additional analysis was conducted from all ACS patient Percutaneous Cardiac Intervention (PCI), in order to evaluate the impact of 'cardiac lesion' on biomarker level and patient outcome.

2.24.4.1 In order to perform the Kaplan-Meier analysis, six assumptions were made.

- **Assumption#1:** The event status consists of two mutually exclusive events of readmission. Events of interest included ACS admission (n=24) and Non-ACS Admissions (n=11). No bias was added within the independence of censoring. Censoring was conducted for all first admissions not associated as ACS admission, including cardiovascular accident (n=2) as both had ACS admission prior, cancer diagnosis (n=5) and other admissions (n=19) which included surgery, respiratory and anaemia admission. No-Admissions recorded were the end of the study (n=45) right censored.
- **Assumption#2:** The time-to-event was clearly defined in each group as index event time of Percutaneous Coronary Intervention (PCI). This assumption is the time-to-event or censorship (survival time) and was precisely measured in days from PCI date to first admission date. The starting point to avoid secular trends was assessed to be definitive. Initially it was planned to be the date of screening (consent), however this presented with 'left censored data' bias, as it did not observe the actual time to readmission, only the time from screening to readmission rate. The date of PCI was used for both ACS Arm (n=80). Breaking data down, inclusion was (n=36) for Arm-1, with (n=15) readmissions and (n=5) censored, verses (n=44) for Arm-2 with (n=20) readmission and (n=6) censored.
- **Assumption#3:** To ensure that ACS Arm-1 participants were not subjected to left-censoring, the dates for Arm-1 were based on the Assumption #2's dates, rather than consent date. To recap, ACS Arm-1 participant inclusion was within 12 months of an AMI event, whereas Arm-2 was within 24 hours of the AMI. Therefore, using the index PCI date, rather than screening or hs-cTn provides an unbiased date, up to a readmission without violating the assumption by left-censoring.
  Subsequent analysis for all four plasma biomarkers was computed from <25% percentile, median >25%~<75% Percentile and >75% percentile concentration ranges.
- **Assumption#4:** There was an independence of censoring all events. Admissions for non-ACS readmissions were censored identically in both groups (See appendix BB). All cases censored were admissions for non-cardiac diagnoses for first admission. All deaths (n=3) resulting from ACS deaths with an ACS admission preceding death, thus survival was not included in the readmission censoring.
  a. For the assumption of independent censoring, withdrawn participant data was consented to be included.
  b. Participants were followed up during the study period of 365+30 days. Day zero was classified as the date of index PCI event to allow conformity within each group. Arm-1 was less represented (n=36), out of the (n=14) participants readmitted, (n=10) were recruited within the index hospital admission. The remaining participants (n=4) had no readmissions until data captured after consent or where censored.
  c. All medical notes were reviewed at 12months plus 30 days (395 days following consent).
- **Assumption#5:** During the recruitment and follow-up stage, there was so secular trends in treatment of ACS events or treatment protocol changes. ACS participants were all treated to the exact same protocol. There were no changes in local policies or treatments throughout the study.

- **Assumption#6:** There was similar numbers in events in both Arm-1 and Arm-2 required for the Kaplan-Meier analysis, so this assumption was met. A similar percentage of censored cases was present in the Arm-1 (35.7%) and Arm-2 (30.0%) intervention groups (Table 3.82). However, the various Kaplan-Meier analysis performed presented different issues, primarily due to the study design not being 'event driven' and therefore all survival data was captured at 395 days.

The Kaplan-Meier analysis was carried based on various comparisons / stratifications for each of the plasma biomarkers. Since readmission rates was the principal end point central to addressing the aim / objectives of the Kaplan-Meier analysis was conducted to determine probability of time to readmission, based on biomarker stratification. Since the study was not designed as 'event driven', the points of interest were an ACS event (n=34) and readmission events censored as (n=23). All participants survival status was determined at the end of study. Finally, the four plasma biomarkers were analysed with respect to PCI stratification, to determine whether cardiac lesion was predictive of ACS readmission.

All analyses followed the assumptions as outlined above and reporting was dependent on results. Statistical significance was accepted at $p<0.05$. To increase the statistical power of the Kaplan-Meier using multiple comparisons, a Bonferroni correction was accepted to declare that the level of statistical significance (α-level) compensated with Bonferroni for multiple comparisons was 0.05 divided by 3. Therefore, the level of statistical significance was 0.05 / 3 = $p\leq0.0167$. This was reported where appropriate. The Bonferroni correction was used to reduce the chances of type 1 errors, i.e., false positive results, by using three comparisons in multiple pairwise tests as performed on each single set of data (Napierala, 2012). However, the sample size was relatively small, which can impose penalties (VanderWeele et, al, 2019), and potentially increase the 'false negatives' as an unfortunate by-product of correcting the multiple comparison (McDonald 2014). Therefore, suitable caution was applied to all results displayed, which were ultimately compared with other statistical analyses to not misinterpret the clinical significance.

The results of these analyses are described subsequently in Chapter 3 parts (a) TRX, (b) TRXr, (c) PRDX-2 and (d) PRDX-4. All values are presented as mean ± standard deviation and displayed throughout results. Statistical significance was accepted at the $p<0.05$ level.

All statistical analysis was carried out using SPSS v29 (IBM), where significant result for each pair is reported as pairwise p-values determined at the $p<0.05$ significance level.

2.25 Archive.

All patient consent forms will be archived for 15 years in the Cardiology Research archive facilities. Identifiable data containing personal data for follow-up appointments will be kept for 12 months.

2.26 Reporting, authority publication and notification of results.

The study protocol forms part of this thesis and will be submitted to the research degrees board. The protocol and study results will also be submitted for peer review publication in a cardiac specific Q1 journal.

The main outcomes will hopefully lead to further research. Joint ownership of any data arising from the study resides with Worcestershire Acute NHS Trust and the University of Worcester.

Participants will not formally be notified of results; however, they are aware that they can contact myself at the end of the study for general results, but not individual test results.

Chapter 3:

3.0 Statistical analysis characteristics.

A total of 151 participants were recruited from a single NHS Acute Hospital site and from a one-day visit to the blood transfusion service (n=151). Following censoring the results data presented are from a total sample group of (n=145). Initial descriptive statistical analyses were performed for participant characteristics, along with each plasma biomarker (TRX, TRXr, PRDX-2 and PRDX-4) to establish data spread. The characteristics/ demographics for all participants at baseline and respective clinical presentation for the ACS cohort are shown in Table 3.0. The data presented in Table 3.0 is following stratification into three groups, Healthy Volunteers (n=65), ACS Arm-1 (n= 36), and ACS Arm-2 (n=44).

Table 3.0 Participant characteristics and clinical presentation of ACS patients at baseline. Data presented are mean ± SD.

Characteristic	Healthy Cohort (N = 65)	ACS Arm-1 (N = 36)	ACS Arm-2 (N = 44)
Age — yr*	48.9 ± 17.9	66.8 ±	67.5 ± 8.7
Male — no. (%)	32 (54%)	27 (75%)	32 (73%)
Female — no. (%)	33 (51%)	9 (25%)	12 (27%)
Body-mass index†*	27.2 ± 3.9	28.90 ± 4.0	27.2 ± 4.6
Family history of CAD — no. (%)	18 (28%)	13 (36%)	19 (43%)
Arterial hypertension — no. (%)	10 (15%)	19 (52%)	22 (50%)
Diabetes mellitus — no. (%)	12 (18%)	10 (28%)	13 (27%)
Lipid Profile — no. (%) TC>4.0 mmol/L	~	20 (56%)	32 (72%)
Total Cholesterol mmol/L *	~	4.18 ± 1.7	4.48 ± 1.0
LDL*	~	2.43 ± 0.87	2.53 ± 1.16
HDL*	~	1.14 ± 0.30	1.27 ± 0.41
Creatine (62 - 106 mmol/L*	~	85.8 ± 22.2	85.0 ± 26.8
ALT (0-40iu/L)*	~	30.0 ± 17.9	24.8 ± 11.1
Cancer — no. (%)	0 (0%)	4 (11%)	6 (14%)
Smoking — no./total no. (%)			
Previous	26 (40%)	12 (33%)	11 (25%)
Current	26 (40%)	22 (61%)	26 (59%)
Never	35 (54%)	14 (39%)	17 (39%)
Unknown	2 (3%)	0 (0%)	1 (2%)

Chronic Pulmonary Disease — no. (%)	1 (2%)	2 (6%)	6 (14%)
Myocardial infarction — no. (%)	~		
Angina — no. (%)	~	3 (8%)	2 (4%)
NSTEMI — no. (%)	~	16 (44%)	25 (57%)
STEMI — no. (%)	~	17 (47%)	15 (34%)
Percutaneous Coronary Intervention — no. (%)	~		
Right Coronary Artery (RCA)	~	20 (56%)	19 (43%)
Circumflex	~	6 (17%)	5 (11%)
Left Anterior Descending (LAD)	~	10 (28%)	20 (45%)
Medication			
Single/Dual Antiplatelet Therapy	2 (3%)	28 (78%)	36 (82%)
Anticoagulation	1 (2%)	6 (17%)	8 (18%)
β Blockers	3 (5%)	32 (89%)	42 (95%)
Ace Inhibitors	5 (8%)	29 (81%)	43 (98%)
Statins	3 (5%)	34 (94%)	40 (91%)

***** Plus–minus values are means ± SD. Data on race/ethnic group are not reported due to small no.

† The body-mass index is the weight in kilograms divided by the square of the height in meters.

~ Healthy Cohort - excluded previous coronary disease.

A participant breakdown in terms of sex (Male, 1 or Female, 2) is presented in Table 3.1. A total of 6 participants were censored at screening and thus excluded from the data analysis. No blood samples were obtained for these participants, which left a total of 145 participants for subsequent analysis.

Table 3.1 Total consented participants ACS verses healthy cohort, with respect to males versus female.

		Value Label	N
Sex	1	Male	91
	2	Female	54
ACS V Healthy Cohort	1	Health Volunteers	65
	2	ACS ARM-1 & 2	80

The Arm-1 ACS group represents those that had suffered an ACS event within ≤ 12 months at time of screening (n=36). For Arm-1, blood plasma samples were obtained at screening point for subsequent TRX, TRXr, PRDX-2 and PRDX-4 analysis (n=36) and at first follow-up (n=28), at which point 1 participant had died and 5 participants declined. Blood plasma samples were also obtained at second follow-up (n=23) at which point a further

participant had died and 10 declined.

The Arm-2 ACS group represents those that had suffered an ACS event within ≤24 hours of standard care (n=44). For Arm-2, blood plasma samples were obtained at time of screening for subsequent TRX, TRXr and PRDX-2 and PRDX-4 analysis as well as first (n=30) and second (n=22) follow-up, at which point (n=14) and (n=22) had declined respectively. One participant had died after verbal consent was given; however, the blood plasma sample was discarded since participant passed away before full consent obtained. In the laboratory, it was intended that each plasma biomarker would be analysed for each participant, however, due to some technical difficulties and assay optimisation, there were a 'few instances' where there was insufficient plasma available to analyse each biomarker. The total number of each plasma biomarker analysed in the laboratory are clearly indicated in the subsequent analysis.

3.1 Kaplan-Meier ACS Arm-1 verses Arm-2 participants.

As the study design was not 'event driven' and survival for all participants (n=80) was known at day 395, statistical significance was observed for some of the biomarkers evaluated (see next sections). It was important to establish whether there were any differences between ACS participants recruited into Arm-1 compared with Arm-2. The case numbers are displayed in Table 3.1.1 as number of events of interest i.e., 'readmission with an ACS event' (n=34) and 'readmission events censored as non-cardiac events of origin' (n=23).

Table 3.1.1 Participant readmission and censor figures between Arm-1 and Arm-2.

Table 3.1.1

			Censored	
Intervention Arm 1, Arm 2	Total N	N of Events	N	Percent
Arm 1	14	9	5	35.7%
Arm 2	20	14	6	30.0%
Overall	34	23	11	32.4%

To establish some intuition regarding the readmission rates between Arm-1 and Arm-2, Kaplan-Meier analysis was performed comparing the two groups. Both Arms were treated with identical medical intervention, but screening and inclusion was at different timepoints, therefore as outlined in assumption #3, point of PCI date was used as Day 0. Table 3.1.2 surmises the participant data included in this analysis.

3.2 Kaplan-Meier readmission table.

Table 3.1.2. Survival analysis indicates readmission rates ACS and censored between Arm-1 and Arm-2.

Table 3.1.2

Arm-1, Arm-2		ID	Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
					Estimate	Std. Error		
Arm-1	1	17	6.000	ACS Admission	.929	.069	1	13
	2	20	29.000	ACS Admission	.857	.094	2	12
	3	55	62.000	ACS Admission	.786	.110	3	11
	4	98	69.000	ACS Admission	.714	.121	4	10
	5	71	165.000	Censored	.	.	4	9
	6	35	183.000	ACS Admission	.635	.131	5	8
	7	11	230.000	ACS Admission	.556	.136	6	7
	8	61	258.000	ACS Admission	.476	.138	7	6
	9	78	278.000	Censored	.	.	7	5
	10	48	312.000	Censored	.	.	7	4
	11	73	313.000	Censored	.	.	7	3
	12	39	342.000	ACS Admission	.317	.159	8	2
	13	19	374.000	ACS Admission	.159	.138	9	1
	14	38	482.000	Censored	.	.	9	0
Arm-2	1	105	4.000	ACS Admission	.950	.049	1	19
	2	9	6.000	Censored	.	.	1	18
	3	107	11.000	ACS Admission	.897	.069	2	17
	4	114	14.000	ACS Admission	.844	.083	3	16
	5	56	30.000	ACS Admission	.792	.093	4	15

	6	115	31.000	ACS Admission	.739	.101	5	14
	7	89	32.000	ACS Admission	.686	.106	6	13
	8	42	43.000	Censored	.	.	6	12
	9	100	43.000	Censored	.	.	6	11
	10	123	44.000	ACS Admission	.624	.113	7	10
	11	32	57.000	ACS Admission	.561	.118	8	9
	12	40	90.000	Censored	.	.	8	8
	13	119	93.000	ACS Admission	.491	.122	9	7
	14	97	102.000	ACS Admission	.421	.123	10	6
	15	86	205.000	ACS Admission	.351	.121	11	5
	16	112	231.000	ACS Admission	.281	.115	12	4
	17	43	241.000	Censored	.	.	12	3
	18	94	269.000	ACS Admission	.187	.108	13	2
	19	124	281.000	Censored	.	.	13	1
	20	41	360.000	ACS Admission	.000	.000	14	0

The data presented in Table 3.1.3 shows the mean and median time to readmission and associated statistics for Arm-1 and Arm-2. The data indicate that participants in Arm-1 had a greater time to ACS of 258.0 days (95% CI; 130.1, 385.3), compared with Arm-2 who 93.0 days (95% CI; 16.1, 169.9).

Table 3.1.3. Mean and median readmission time in days for Arm-1 and Arm-2.

Table 3.1.3

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
Intervention Arm 1, Arm 2	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Arm 1	255.270	44.687	167.684	342.855	258.000	64.970	130.658	385.342
Arm 2	149.537	33.656	83.571	215.502	93.000	39.236	16.098	169.902
Overall	199.648	29.118	142.576	256.719	205.000	105.145	.000	411.083

a. Estimation is limited to the largest survival time if it is censored.

Next, to determine the power of the statistical test between readmission rates, a log rank test (Mantel, 1966) was selected, which compares the weighting difference between the observed number of events and the expected number of events at each time point. For this test the null hypothesis is that 'there is no difference in the overall distribution between groups in the population'. A summary of the results is presented in Table 3.1.4.

Table 3.1.4. Overall comparison of readmission distribution between Arm-1 and Arm-2.

Table 3.1.4

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	2.984	1	.084

Test of equality of survival distributions for the different levels of Arm-1, Arm-2.

The data presented in Table 3.1.4 confirms there was no statistical significance, $\chi 2(2) = 2.984$, p=0.084. Therefore, it cannot be concluded that the readmission distribution is different between Arm-1 and Arm-2.

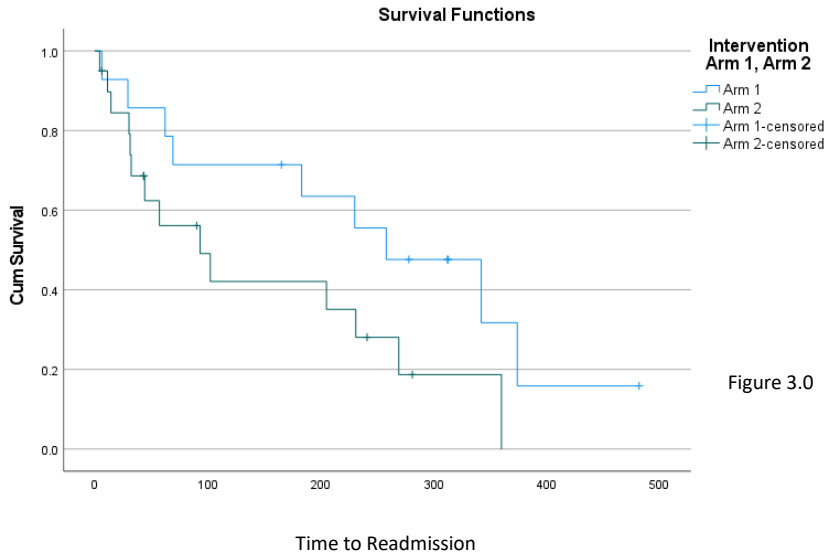


Figure 3.0

Figure 3.0. Readmission analysis on Arm 1 and Arm 2. To determine whether prediction of participant outcome (overall survival) could be determined between ACS readmission rates, all non-ACS readmission rates were censored. Kaplan-Meier analysis was performed and stratified according to Inclusion Criteria at baseline [hs-TnT]. **1)** Blue line represents Arm-1. **2)** Green line represents Arm-2. $\chi 2$ analysis, p>0.05.

Figure 3.0 illustrates the time to readmission for Arm-1 and Arm-2 ACS participants. Although the median time to readmission was greater for Arm-1 participants (Table 3.1.4), the overall readmission rate was not statistically different between Arm-1 and Arm-2 participants (p=0.084, Table 3.1.4).

Chapter 3a – Thioredoxin (TRX)

3.3 Brief introduction to the plasma thioredoxin (TRX) analysis.

As outlined in Chapter 1, the generation of reactive oxygen species (ROS) is a complex molecular process that if left unchecked can cause cellular damage (Section 1.4). Oxidation happens in cells due to the imbalance of the production of ROS and the availability of antioxidants or free radical scavengers (Das et al., 2014). Under normal physiological conditions, redox homeostasis is maintained in the cell, which is mediated 'in part' by the action of various antioxidant enzymes such as thioredoxin (TRX). As described in Section 1.6, TRX is small redox-regulating protein, which plays a crucial role in maintaining cellular redox homeostasis and cell survival by reversing the cysteine thiol oxidation caused by ROS (Saccoccia et al., 2014). Previous studies have indicated that changes blood [plasma or serum] concentrations of TRX may be indicative of an oxidative stress, and is linked to progression and patient outcome in numerous conditions such as cardiovascular disease (Whayne et al., 2015; Mongardon 2013). Thus, the following analysis as described in this chapter was conducted to determine if there are any changes in plasma [TRX] between a healthy cohort of participants and those following an AMI / during an ACS event. As described in chapter 2, the ACS patients and healthy cohort were recruited at WAHT. Participant plasma samples were subsequently evaluated using an optimised ELISA for TRX (Section 2.23., Table 2.2 and Appendix W). The data collected was subsequently analysed using various statistical methods as described in chapter 2. This chapter presents the results of this analysis is a logical order to ultimately evaluate the clinical utility of TRX in the context of ACS and establish whether TRX could reliably predict ACS patient outcome. In this case the time to event endpoint was an ACS readmission.

Objectives:

a) To clarify the mean plasma concentrations for TRX for healthy volunteers, stratified based on sex and age., which will be used as baseline measurements for ACS comparison.

b) To evaluate the plasma concentrations levels of TRX for ACS patients stratified based on age and sex at initial diagnosis / screening and follow-up. Clinical utility may subsequently be evaluated.

c) Monitor the concentration level of TRX through ACS patient follow-up sampling, in order to evaluate whether this biomarker may be predictive of an ACS readmission.

d) Evaluate whether TRX may predict readmission based on ACS patient stratified according to PCI.

3.4 Basic descriptive statistic of TRX.

To determine the mean plasma TRX for the 'healthy cohort', any healthy volunteers with a medical history of diabetes mellitus (n=5), hypertension (n=10), family history of cardiovascular conditions (n=19), or inflammatory disorders (n=1) were removed from the analysis. This equated to a healthy volunteer population with no medical conditions (n=38). This population had a slightly lower plasma [TRX] mean of 10.42 ng/ml ± 9.11 ng/ml, compared with the healthy cohort as a whole 10.80 ng/ml ± 7.82 ng/ml (n=56). Stratification of the ACS participants cohort into Arm-1 (n=36) and Arm-2 (n=44) revealed plasma [TRX] levels of 12.83 ng/ml ± 11.14 ng/ml and 11.99 ng/ml ± 5.53 ng/ml respectively. Next the 'smokers' were removed from the healthy cohort analyses, which resulted in a further drop in mean plasma TRX concentration to 10.34 ng/ml ± 10.45 ng/ml (n=27). The same convention was applied to the ACS participants, which resulted in mean plasma TRX concentrations of 11.52 ng/ml ± 6.15 ng/ml for Arm-1 (n=5) and 12.63 ng/ml ± 4.24 ng/ml for Arm-2 (n=8). Statistical analysis showed that these TRX values remained significantly higher for ACS Arm-1 non-smokers and ACS Arm-2 non-smokers, compared with healthy volunteer non-smokers ($p<0.05$), although the number of non-smokers who suffered an ACS was low. Taken together, the data indicate that smoking status has little impact on mean plasma TRX levels once an ACS has occurred.

Overall, males in the healthy cohort (n=32) had a mean plasma TRX concentration of 12.63 ng/ml ± 9.87 ng/ml, compared with a female (n=33) value of 9.02 ng/ml ± 4.64 ng/ml ($p=0.0007$), indicating that, for healthy males, the mean plasma TRX is higher.

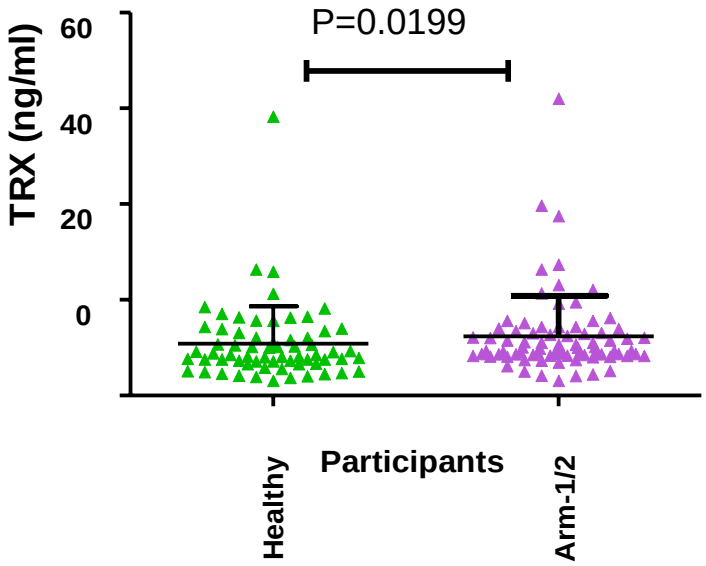


Figure 3.1

Figure 3.1. Overall blood plasma TRX levels between healthy and ACS participants cohorts. The data presented illustrate the spread of data between all healthy (n=65) and all ACS (n=80) plasma TRX levels (ng/ml). For healthy volunteers the mean TRX concentration was 10.8±7.8 ng/ml. For ACS the mean TRX concentration was 12.4±8.5 ng/ml. Mann-Whitney test confirmed an over a significant difference in mean, $p<0.001$.

The mean [TRX] for Healthy Volunteers (HV) was 10.80ng/ml ± 7.82 (p=<0.0001) versus the ACS group (n=80) 12.37ng/ml ± 8.47ng/ml (p=0.0001) Data was statistically significant demonstrated on a Mann-Whitney U test (p=0.0199). Figure 3.1 and table 3.2.

Table 3.2. Descriptive statistics for plasma TRX (ng/ml) concentration

Dependent Variable: TRX mean Sample 1

ACS V Healthy Cohort	Sex	Mean	Std. Deviation	N
Healthy Volunteer	Male	12.631	9.8700	32
	Female	9.024	4.6439	33
	Total	10.800	7.8277	65
ACS Arm 1 and Arm 2	Male	11.937	8.6242	59
	Female	13.586	8.1265	21
	Total	12.370	8.4769	80
Total	Male	12.181	9.0331	91
	Female	10.798	6.5559	54
	Total	11.666	8.2017	145

3.5 Basic TRX Analysis for ACS Arm-1 (ACS event ≥12 months from screening).

Given the significant differences in plasma TRX levels observed between males and females for the healthy cohort, it was next important to evaluate this effect with respect to the ACS participants. For the Arm-1 ACS participants, males (n=27) had a mean plasma TRX concentration of 12.47 ng/ml ± 11.10 ng/ml, compared to females (n=9) 13.91 ng/ml ± 11.87 ng/ml (p=0.0066), indicating for ACS Arm-1 at least, females were higher. The effects of smoking status were next evaluated for Arm-1 ACS, where non-smokers or ex-smokes ≥12 months (n=26) had a mean plasma TRX concentration of 12.74 ng/ml ± 11.31 ng/ml compared to smokers/vapers (n=10) which was significantly higher at 13.07ng/ml ± 11.31 ng/ml (p<0.0033). Interestingly, Arm-1 ACS participants with a family history of ACS (n=13) had a mean TRX plasma level of 16.32 ng/ml ± 1 5.38 ng/ml, compared to those without a reported family history of ACS (n=23) 10.87 ng/ml ± 7.57 ng/ml (p<0.001).

For Arm-1 ACS the primary endpoint (readmission) occurred in (n=15) 41.6%, of which ACS admission (n=10) 27.7% was the cause. Comparing plasma TRX concentrations at follow-

up appointments was challenging for those participants who were readmitted for causes other than ACS. However, mean TRX plasma concentrations for the readmission caused by a second ACS were 9.36ng/ml ± 4.38ng/ml (on readmission, n=10) compared to 8.65 ng/ml ± 3.90 ng/ml (at first follow-up, n=9, p>0.05).

3.6 Basic TRX analysis for ACS Arm-2 (ACS ≤ 24 hours at screening).

The same analysis was carried out for ACS Arm-2 participants (n=44), who had their ACS event within 24 hours of their hs-cTn result. Here, the male cohort (n=32) had a mean plasma TRX concentration of 11.72 ng/ml ± 5.93 ng/ml, compared to females (n=12) which was 13.34 ng/ml ± 4.18 ng/ml (p=0.0261). This finding agrees with Arm-1, whereby female ACS participants has a significantly higher mean plasma TRX concentration, compared with male ACS participants. As with Arm-1, the Arm-2 participants were stratified according to smoking status. The non-smokers or ex-smokers ≥12 months (n=30) had a mean plasma TRX concentration of 12.62 ng/ml ± 6.34 ng/ml, compared to smokers/vapers (n=15) 10.35 ng/ml ± 3.04 ng/ml (p>0.05).

For the Arm-2 ACS participants, the primary endpoint (readmission) occurred in (n=20) 45.4%, of which readmission due to a second ACS accounted for (n= 14) 31.8% cases overall. The mean plasma TRX concentration upon readmission was 11.68 ng/ml ± 4.09 ng/ml, compared with the first and second follow-up samples, which were 8.7 ng/ml ± 0.917 ng/ml (n=8) and 18.32 ng/ml ± 7.14 ng/ml (n=6) respectively. In spite of this large increase in [TRX] at second follow-up, these data did not reach significance when compared to the mean readmission concentration, due to the large variation in the data and small 'n' (p>0.05).

Combining the Arm-1 and Arm-2 ACS data, the primary endpoint (readmissions) occurred in (n=35) 44% of participants. Where a second ACS was the cause, this was observed in (n=24) 30% of all readmissions. The mean plasma TRX concentration for all ACS participants readmitted with a second ACS event was 10.44 ng/ml ± 4.33 ng/ml, compared with 8.44 ng/ml ± 2.98 ng/ml and 15.40 ng/ml ± 6.00 ng/ml at first (n=17) and second (n=12) follow-up respectively. Taken together, the difference in mean compared with the readmission plasma TRX concentration was significantly higher for the second follow-up (p=0.405) Table 3.3.

Table 3.3: Descriptive statistics for [TRX] in (ng/ml) biomarkers for readmission rates.

ACS Readmissions		ACS Admission	Std. Deviation	Non-ACS Readmission	Other Admissio	Std. Deviation	Sig
Baseline	(n=24)	**10.44**	4.33	(n=11)	**15.74**	14.59	
Follow-up 1	(n=17)	**8.44**	2.98	(n=11)	**10.06**	3.81	0.1466
Follow-up 2	(n=12)	**15.40**	6.00	(n=9)	**10.57**	5.96	0.0343

3.7 Age comparisons at screening TRX for all participant groups.

For healthy Volunteers at time of screening, the under 55's (n=36) had a mean plasma TRX concentration of 8.82 ng/ml ± 4.71 ng/ml compared, compared to over 55's (n=29) who had a mean concentration of 13.26 ng/ml ± 10.05 ng/ml ($p<0.0001$). For the Arm-1 ACS participants aged under 55 (n=6) at time of screening had a mean plasma TRX concentration of 13.77 ng/ml ± 14.00 compared, compared with over 55's (n=30) which was 12.65 ng/ ml ± 10.76. For Arm-2 ACS participants, the under 55's (n=11) had a mean plasma TRX concentration of 11.32 ng/ml ± 4.33 ng/ml compared with over 55's (n=33) which had a mean of 12.22 ng/ml ± 5.91 ng/ml (Figure 3.2).

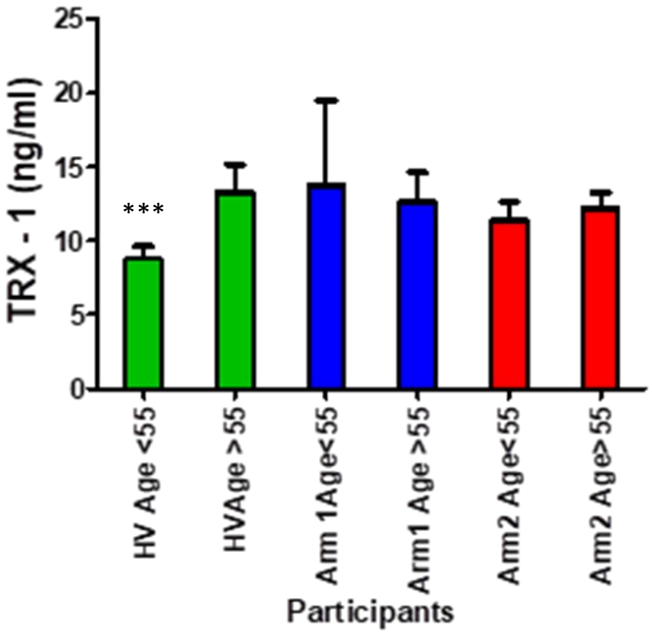


Figure 3.2

Figure 3.2. Age stratified blood plasma TRX levels between healthy and ACS participant cohorts. The data presented illustrate blood plasma TRX levels (ng/ml) following age stratification; <55 years or >55 years for **a)** healthy in green (<55 years, n=36, >55 years n=29), **b)** ACS Arm-1 in blue (<55 years n=6, >55 n=30) and **c)** ACS Arm-2 in red (>55 years n=11, >55 years n=33). *** $p<0.0001$.

3.8 Two-way mixed ANOVA.

To follow on from the descriptive statistical analysis presented above, it was next important to evaluate the impact of sex (male / female) with respect to the plasma concentrations of TRX. Since the sample population cohorts included a healthy population, along with ACS Arm-1 and Arm-2, a two-way mixed ANOVA was selected, as this would determine interaction between participant 'sex' and population cohort.

Initially an assessment of outliers using Boxplots and Studentized Residuals was conducted. All plasma biomarkers of were methodically assessed. Outliers in SSPS are classified as data points more than 1.5 box-lengths away from their box and depicted by a circular dot, whereas extreme outliers are illustrated with an asterisk (*). For outliers as determined using the studentized method, discrimination was based on values +3 / -3 standard deviations from the mean.

3.9 Assessment of outliers for thioredoxin (TRX).

For Sample-1 (Baseline blood taken at screening) for the healthy cohort (n=65), the Boxplot data presented in Figure 3.3a indicated that there was one extreme outlier (data point 144) and two outliers (data points 37 and 84). The extreme outlier (144) corresponded to a healthy male, with no known identifiable data to explain the raised [TRX] significantly beyond the mean. The ELISA readings were assessed in duplicate and equal, where both readings agreed. On review of the other two healthy cohort outliers, the data was recorded accurate and the duplicated ELISA test results were equal.

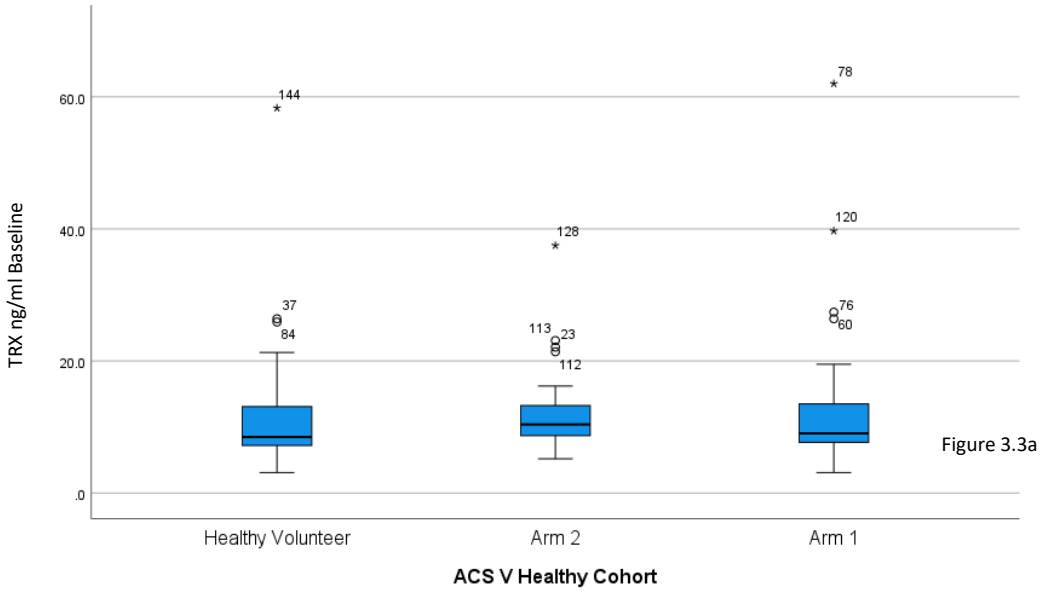


Figure 3.3a

Figure 3.3a. Evaluation of 'outliers' for blood plasma TRX sample-1 (Baseline). The box and whisker plots are presented for blood plasma TRX (ng/ml), along with 'outliers' for healthy volunteers, (n=80), ACS Arm-1 (n=36) and ACS Arm-2 (n=44). *Extreme outlier.

As illustrated in Figure 3.3a, for ACS Arm-1 (n=36) and Arm2 (n=44) there were three extreme outliers, (data points 78, 120, 128). Interestingly each of these extreme outliers corresponded to NSTEMI AMI. Furthermore, both 78 and 120 received PCI to RCA. For each of the extreme outliers, the ELISA readings were assessed in duplicate, which were equivalent in value respectively. Data point 128 was a PCI to LAD, of the three extreme outliers one participant went on to be readmitted for a non-ACS condition. For the non-extreme outliers, one of the five went on to have an ACS readmission.

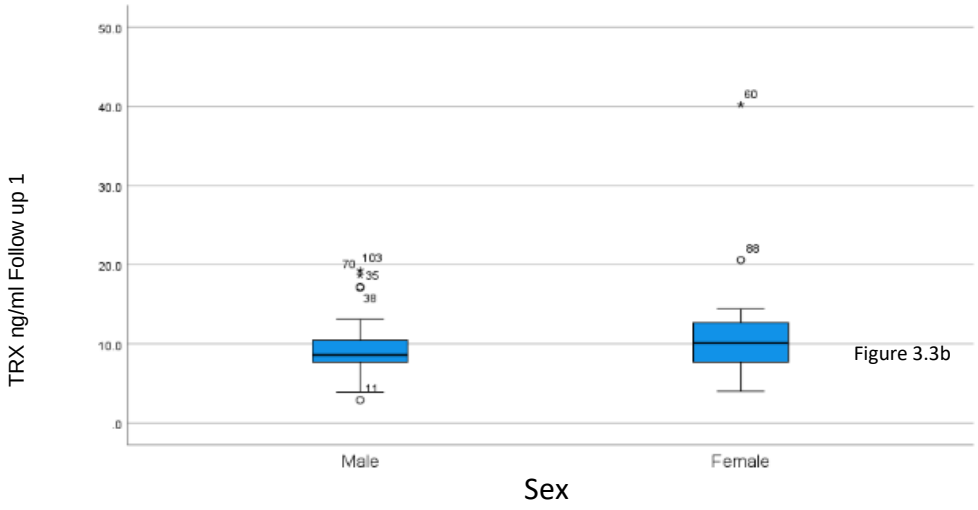


Figure 3.3b. Evaluation of 'outliers' for blood plasma TRX sample-2 (Follow-up 1). The box and whisker plots are presented for blood plasma TRX (ng/ml) along with 'outliers' ACS Arm-1 Male (n=27) Female (n=9) and ACS Arm-2 Male (n=32) Female (n=12). *Extreme outlier.

Extreme outliers increased at first follow-up sample-2 as illustrated in (Figure 3.3b). Data points 35 and 70 were both male from Arm-1 (n=27) and 103 Arm-2 (n=32). Data point 60 was female (n=9) from Arm-1. Each of the duplicate ELISA results for these extreme outliers were equivalent in respective value. Furthermore, extreme outlier data point 35 went on to have an ACS readmission. For the non-extreme outliers, interestingly one of the three with lower extreme went on to have an ACS readmission.

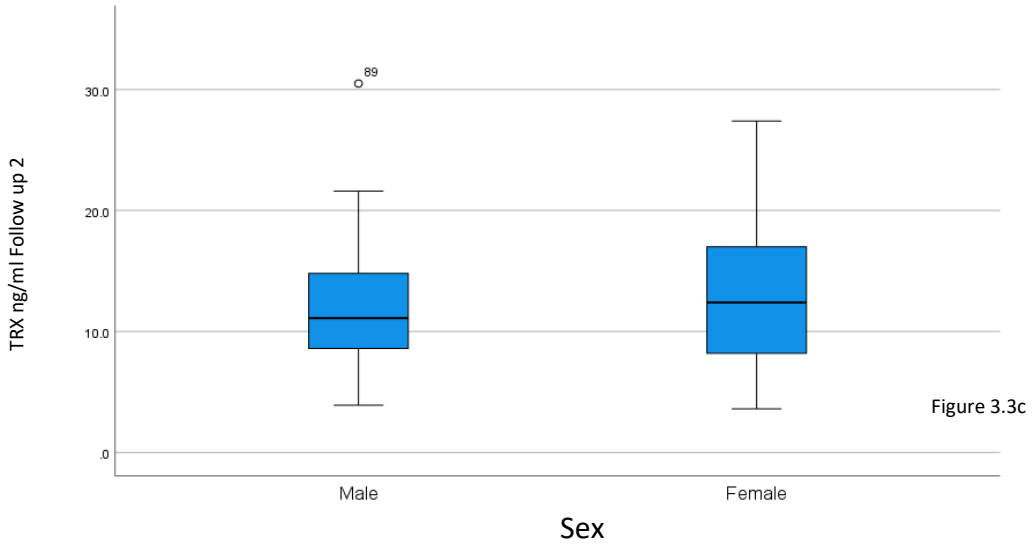


Figure 3.3c. Evaluation of 'outliers' for blood plasma TRX sample-3 (Follow-up 2). The box and whisker plots are presented for blood plasma TRX (ng/ml along with 'outliers' ACS Arm-1 Male (n=27) Female (n=9) and ACS Arm-2 Male (n=32) Female (n=12). ° outlier.

As illustrated Figure 3.3c resulted in no extreme outliers. Follow-up 2 (sample-3) resulted in one male (n=27) outlier from Arm-2 (n=44), the participant at baseline had a NSTEMI and PCI to LAD and had an ACS readmitted between Follow-up 1 (sample-2) and Follow-up 2 (sample-3).

3.10 Test of normality with outliers TRX sample 1, 2 and 3 including outliers.

Next a Shapiro Wilk's Test was conducted to determine whether the data fitted a normal distribution for blood plasma TRX. This test was initially conducted with the 'outliers' included. The data are presented in Table 3.4 for sample-1 (a), sample-2 (b) and sample-3 (c). The data in Table 3.4 shows that, plasma TRX concentration did not fit a normal distribution for all bloods taken at sample-1 ($p<0.001$), sample-2 ($p<0.001$) and for males at sample-3 ($p<0.01$). However, for females at blood sample-3, the plasma TRX concentration did fit a normal distribution ($p>0.05$).

Table 3.4a, b and c: Test of normality with outliers thioredoxin for blood sample-1 (a), sample-2 (b) and sample-3 (c).

(a) Test of Normality

	ACS V Healthy Cohort	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
TRX mean Sample-1 Baseline	Healthy Volunteer	.182	65	<.001	.666	65	<.001
	Arm-2	.195	44	<.001	.733	44	<.001
	Arm-1	.277	36	<.001	.650	36	<.001

a. Lilliefors Significance Correction

(b) Test of Normality

Table 3.4b

	Sex	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
TRX mean Sample-2 Follow-up 1	Male	.172	43	.003	.882	43	<.001
	Female	.263	15	.006	.714	15	<.001

a. Lilliefors Significance Correction

(c) Test of Normality

Table 3.4c

	Sex	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
TRX mean Sample-3 Follow-up 2	Male	.124	33	.200*	.903	33	.007
	Female	.116	12	.200*	.965	12	.857

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

To further confirm which participant data sets were 'outliers', studentized evaluation was performed. Here outliers are confirmed if the data point is ± 3 of standard deviations of the mean. This analysis confirmed that there was one outlier for in blood sample-1 (data point, 60) which was 3.85 standard deviations of the mean. For sample-2, the same data point (60) was identified as an outlier, which was 4.48 standard deviations of the mean. For sample-3 there was one outlier (data point, 89) which was 3.02 standard deviation of the mean. This data point was also noted on the boxplot (Figure 3.3c). Taking everything together along with the boxplots presented in Figure 3.3, if an outlier data point was found to be consistent between the two analyses (Boxplot and studentized evaluation), it was removed form subsequent analysis.

3.11 Test of normality excluding outliers thioredoxin sample 1, 2 and 3.

The Shapiro Wilks Test for normality was reconducted for the plasma TRX data, following the removal of the outliers as described above. The data presented in Table 3.5a and 3.5b illustrate an improvement in data sets which now fit a normal distribution, to include blood sample-2 (Follow-up 1) female participants ($p>0.05$) and blood sample-3 (Follow-up 2) male participants ($p>0.05$).

Table 3.5 ab. Test of normality excluding outliers thioredoxin sample 1, 2 and 3

(a) Tests of Normality

	ACS V Healthy Cohort	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
TRX mean Sample 1 Baseline	Healthy Volunteer	.137	64	.004	.900	64	<.001
	Arm-2	.154	43	.012	.861	43	<.001
	Arm-1	.166	33	.022	.884	33	.002

a. Lilliefors Significance Correction

(b) Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Sex	Statistic	df	Sig.	Statistic	df	Sig.
TRX mean Sample 2 Follow-up 1	Male	.204	29	.003	.849	29	<.001
	Female	.169	10	.200*	.928	10	.432
TRX mean Sample 3 Follow-up 2	Male	.146	29	.115	.957	29	.271
	Female	.118	10	.200*	.967	10	.858

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

3.12 Thioredoxin (TRX) Two-way mixed ANOVA.

A two-way mixed ANOVA was subsequently performed for plasma TRX concentration to establish whether there were interactions between the healthy cohort, the ACS cohort (Arm-1 and Arm-2) and sex (male and female). A summary of the data analysed in provided in Table 3.6.

Table 3.6 Case summary figures used for [TRX] blood plasma between ACS verses healthy cohort participants and sex.

Table 3.6

		Cases					
	ACS V Healthy Cohort	Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
TRX mean Sample-1	Healthy Volunteer	65	100.0%	0	0.0%	65	100.0%
	Arm-2	44	100.0%	0	0.0%	44	100.0%
	Arm-1	36	100.0%	0	0.0%	36	100.0%

		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
TRX mean Sample-1	Male	93	100.0%	0	0.0%	93	100.0%
	Female	52	100.0%	0	0.0%	52	100.0%

		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
TRX mean Sample-2	Male	43	46.2%	50	53.8%	93	100.0%
	Female	15	28.8%	37	71.2%	52	100.0%

		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
TRX mean Sample-3	Male	33	35.5%	60	64.5%	93	100.0%
	Female	12	23.1%	40	76.9%	52	100.0%

Initially, homogeneity of variance analysis was performed by the Levene's test for homogeneity of variance for TRX. The data presented in Table 3.7 confirmed that for the TRX data assessed for sample-1 (screening) and sample-3 (second follow-up) displayed equal variance across all analytical methods i.e., mean, median, median adjusted and trimmed mean

(p>0.05). However, this was not the case for sample-2 (first follow-up), whereby all data displayed equal variance, except for the mean (p=0.003).

Table 3.7. Levene's test of equality of dependent variable for

		Levene Statistic	df1	df2	Sig.
TRX mean Sample 1	Based on Mean	2.647	3	38	.063
	Based on Median	1.067	3	38	.374
	Based on Median and with adjusted df	1.067	3	14.530	.393
	Based on trimmed mean	2.355	3	38	.087
TRX mean Sample 2	Based on Mean	5.679	3	38	.003
	Based on Median	2.808	3	38	.053
	Based on Median and with adjusted df	2.808	3	11.023	.089
	Based on trimmed mean	5.079	3	38	.005
TRX mean Sample 3	Based on Mean	1.290	3	38	.292
	Based on Median	1.176	3	38	.332
	Based on Median and with adjusted df	1.176	3	30.426	.335
	Based on trimmed mean	1.219	3	38	.316

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

Table 3.28

Since downstream statistical analysis involes multivariate analysis, the Box's test of 'equality of covariance matrices' was next conducted. This test indicates whether two or more covariance matrices are homogenous. For the TRX homogeneity of covariance the null hypothesis was rejected, signifying that the covariances were not homogenous (p<0.001), (Table 3.8).

Table 3.8. The Box Test for homogeneity of equalities covariances.

Box's M	64.263
F	2.856
df1	18
df2	1339.079
Sig.	<.001

Tests the null hypothesis that the observed covariance matrices of the dependent variables are equal across groups. Design: Intercept + ARM + Gender + ARM * Gender Within Subjects Design: Time

Following this, the two-way mixed ANOVA was performed for TRX. Which evaluated the difference between male and female subjects for the health and ACS (Arm-1 and Arm-2) cohorts (Figure 3.4). The ANOVAs revealed that there was no statistical significance between means (p=0.237).

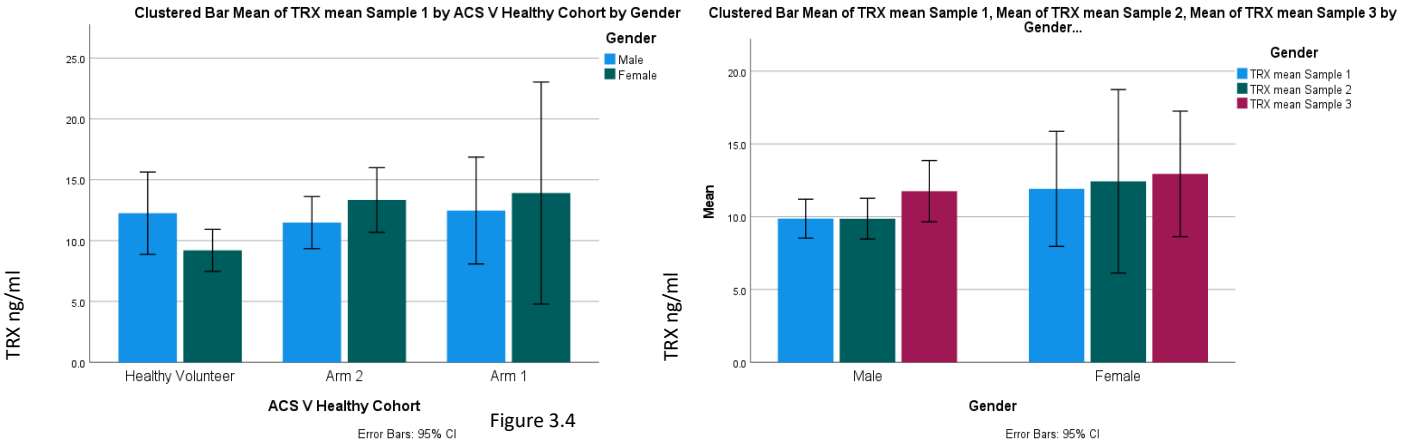


Figure 3.4

Figure 3.4. Gender stratified blood plasma TRX levels between healthy and ACS participant cohorts. The data presented illustrate mean plasma TRX levels between meals (light blue) and females (teal) for healthy volunteers and ACS Arm-1 and Arm-2 cohorts (left). The plot on the right shows the comparison between males and females at screening (sample-1), first follow-up (sample-2) and second follow-up (sample-3). Data presented are mean ± 95% CI, and analysed by two-way ANOVA Table 3.9 with Mauchly's test specificity for interaction p>0.05.

A Mauchly's Test of Sphericity was next performed to confirm the TRX ANOVA findings. This particular test evaluates sphericity in the data as appose to variance and is required to satisfy Assumption #8 Section 2.24.2.1). The results are presented in Table 3.9.

Table 3.9. Mauchly's Test of sphericity between gender and healthy and ACS cohort.

Measure: Thioredoxin

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b] Greenhouse-Geisser	Huynh-Feldt	Lower-
Time	.925	2.875	2	.237	.930	1.000	.50

Table 3.9

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept + ARM + Gender + ARM * Gender

Within Subjects Design: Time

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

The Mauchly's test of sphericity indicated that the assumption of sphericity was met for the two-way interaction (p=0.237). This finding indicates that the relationship between the different pairs of conditions is similar (i.e., males vs females, healthy vs ACS and sample time). Therefore, there is no evidence to suggest that plasma TRX concentration level differs between males and females, for healthy and ACS cohorts. The is also no difference in plasma TRX concentration between sample time and sex.

To evaluate the interaction between male vs female and healthy vs ACS, a Mauchly's multiple comparison 'within-subjects' test was performed, where individual participants are compared with themselves over time. Here the means were evaluated with respect to 'time' i.e., the point at which the blood sample was taken (sample-1, sample-2 and sample-3). The data presented in Table 3.10 illustrates that there was a statistically significant interaction between sample time and ACS Arm (p=0.016), indicating the plasma TRX concentration depends on the point at which the blood sample was taken. The data presented in Figure 3.4 shows that the mean data for males and females is not parallel, which is driven by the change in plasma [TRX] for females. Table 3.10 shows that there was no significant main effect on blood sample time vs gender or blood sample time vs ACS Arm vs Gender (p>0.05).

Table 3.10. Multiple comparisons of blood plasma sample TRX levels within gender, healthy and ACS cohorts.

Measure: Thioredoxin Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Time	Sphericity Assumed	41.409	2	20.705	.990	.376	.025
	Greenhouse-Geisser	41.409	1.861	22.253	.990	.372	.025
	Huynh-Feldt	41.409	2.000	20.705	.990	.376	.025
	Lower-bound	41.409	1.000	41.409	.990	.326	.025
Time * ARM	Sphericity Assumed	183.025	2	91.513	4.374	.016	.103
	Greenhouse-Geisser	183.025	1.861	98.355	4.374	.018	.103
	Huynh-Feldt	183.025	2.000	91.513	4.374	.016	.103
	Lower-bound	183.025	1.000	183.025	4.374	.043	.103

Time * Gender	Sphericity Assumed	8.348	2	4.174	.200	.820	.005
	Greenhouse-Geisser	8.348	1.861	4.486	.200	.804	.005
	Huynh-Feldt	8.348	2.000	4.174	.200	.820	.005
	Lower-bound	8.348	1.000	8.348	.200	.658	.005
Time * ARM * Gender	Sphericity Assumed	67.972	2	33.986	1.625	.204	.041
	Greenhouse-Geisser	67.972	1.861	36.527	1.625	.206	.041
	Huynh-Feldt	67.972	2.000	33.986	1.625	.204	.041
	Lower-bound	67.972	1.000	67.972	1.625	.210	.041
Error (Time)	Sphericity Assumed	1589.936	76	20.920			
	Greenhouse-Geisser	1589.936	70.713	22.484			
	Huynh-Feldt	1589.936	76.000	20.920			
	Lower-bound	1589.936	38.000	41.840			

Table 3.10

Next the interaction between male vs female and healthy vs ACS, was made by a Mauchly's multiple comparison 'between-subjects' test, where participant groups were compared over time. This type of test is more susceptible to individual participant variation. The results presented in Table 3.10 show that there was no significant interaction between ACS Arm and gender ($p>0.05$). Taken together, these multiple comparison tests indicate that, for females the time at which the blood sample was taken has an impact on plasma TRX level, however there are no overall significant differences between males and females over time.

Table 3.11. Multiple comparisons of blood plasma sample TRX between healthy and ACS cohorts and gender.

Measure: Thioredoxin

Transformed Variable: Average	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	13518.218	1	13518.218	294.728	<.001	.886
ARM	52.461	1	52.461	1.144	.292	.029
Gender	96.059	1	96.059	2.094	.156	.052
ARM * Gender	149.420	1	149.420	3.258	.079	.079
Error	1742.935	38	45.867			

Table 3.11

As a final analytical step, a Tukey's Honestly Significant Difference (HSD) test was performed for plasma TRX concentration, which compares the ANOVA means based on the studentized data range. The data presented in Table 3.12 shows that there was no statistically significant interaction between the ACS and healthy cohort, with respect to plasma TRX concentration ($p>0.05$). The pairwise comparison illustrates that there is no significant

difference between plasma TRX concentration for healthy volunteer's vs ACS Arm-1 and Arms-2 (Table 3.12, p>0.05).

Table 3.12. Tukey Multiple comparisons of blood plasma TRX levels between healthy and ACS cohorts

Dependent Variable: TRX mean Sample 1
Tukey HSD

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Healthy Volunteer	Arm 2	-1.191	1.6045	.739	-4.992	2.610
	Arm 1	-2.033	1.7075	.461	-6.079	2.012
Arm 2	Healthy Volunteer	1.191	1.6045	.739	-2.610	4.992
	Arm 1	-.842	1.8471	.892	-5.218	3.533
Arm 1	Healthy Volunteer	2.033	1.7075	.461	-2.012	6.079
	Arm 2	.842	1.8471	.892	-3.533	5.218

Based on observed means.
The error term is Mean Square(Error) = 67.551.

Table 3.12

Table 3.13. Multiple comparisons of blood plasma sample TRX levels between gender and ACS cohorts.

Dependent Variable: TRX mean Sample 2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	289.413[a]	3	96.471	3.711	.017	.171
Intercept	5228.488	1	5228.488	201.107	<.001	.788
ARM	176.079	1	176.079	6.773	.012	.111
Gender	96.272	1	96.272	3.703	.060	.064
ARM * Gender	130.526	1	130.526	5.021	.029	.085
Error	1403.921	54	25.999			
Total	7597.800	58				
Corrected Total	1693.334	57				

a. R Squared = .171 (Adjusted R Squared = .125)

Table 3.13

The Tukey's multiple comparison between-subjects test (Table 3.13) shows that there was no significant difference between plasma TRX concentration and sex (p>0.05). However, there was a significant difference for plasma TRX concentration between ACS Arm (p=0.012 , supporting the Mauchly's data presented in Table 3.9. The Tukey's test also illustrated a significant difference between ACS Arm vs sex (p=0.029). Given these differences, a multiple comparisons test was performed on all ACS participants (male and female) for Arm-1 and Arm-2, to establish whether there were any significant differences between these two recruitment strategies.

The data presented in Table 3.14 show that there were no significant differences in plasma TRX concentrations between ACS Arm ($p>0.05$). Taken together, the TRX two-way mixed ANOVA highlighted an interaction between ACS Arm and blood sample time (Muchly test), which was driven by the female cohort. This is highlighted by differences in plasma TRX concentration between males and females (Tukey test).

Table 3.14. Pairwise comparisons of blood plasma sample 2 TRX levels in ACS cohorts

Dependent Variable: TRX mean Sample 2

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Arm 2	Arm 1	-3.986*	1.532	.012	-7.057	-.915
Arm 1	Arm 2	3.986*	1.532	.012	.915	7.057

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 3.14

Data filed – Thesis Two-way TRX.spv (Document1) Output and Thesis Two-way TRX. DataSet1) Data-IBM SPSS Statistics Data Editor.

3.13 Thioredoxin (TRX) Receiver Operator Curve (ROC).

Following the analysis presented above, which illustrates changes in the plasma biomarkers, Thioredoxin (TRX) for ACS participants a Receiver Operating Curve (ROC) analysis was next carried out. This was completed to ascertain the probability of event prediction, in this instance estimate whether an ACS event has occurred.

Sensitivity (true positives) for TRX for blood taken at screening (sample-1) was determined as 81.3%, whereas specificity (true negatives) for TRX was determined as 60% (Table 3.15). The efficiency of TRX is calculated as (81.3%+60%) / (81.3%+60%+18.7%+40%) = 70.65%. In other words, blood plasma TRX predicts a correct diagnosis 70.65% of the time.

Table 3.15 Percentage accuracy in classification for TRX biomarker.

Table 3.15

Observed			Predicted: Presence of Heart Disease ALL/ACS		
			Healthy Cohort	ACS Cohort	Percentage Correct
Step 1	Presence of Heart Disease ALL/ACS	Healthy Cohort	39	26	60.0
		ACS Cohort	15	65	81.3
	Overall Percentage				71.7

a. The cut value is .500

The positive predictive value (percentage correctly predicted) for plasma TRX concentration at screening, which relates to 'observed characteristics' compared to 'case predictive characteristics' is 100 x (65 ÷ (26 + 65)) = 71.4%. This means that 71.4% of ACS cases are correctly predicted by evaluating plasma TRX concentration at screening. The negative predictive value, which relates to cases 'without the observed characteristics' compared to 'cases predicted not having the disease characteristic' is 100 x (39 ÷ (39 + 15)) = 72.2%. This means that 72.2% of non-ACS cases are correctly predicted by evaluating plasma TRX concentration at screening.

3.14 ROC Curve thioredoxin (TRX).

3.5 illustrates the ROC curve was analysis for TRX. Data included was plasma TRX concentrations at screening (sample-1) for the ACS cohort (n=80) and the healthy cohort (n=65). The area under the curve was determined as 0.819 (95% CI = 0.752 to 0.886).

Table 3.16. Area under the curve analysis for blood plasma TRX.

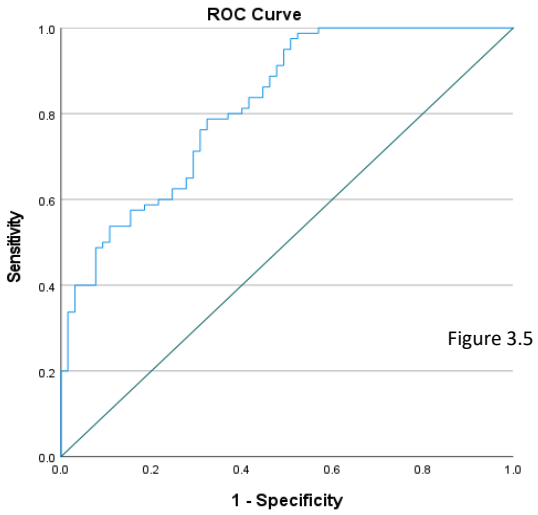


Figure 3.5

Area under the curve

Test Result Variable(s): Predicted probability TRX

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval Lower Bound	Asymptotic 95% Confidence Interval Upper Bound
.819	.034	.000	.752	.886

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Figure 3.5 and Table 3.16 Receiver Operator Curve (ROC) analysis for blood plasma [TRX]. The data presented illustrate the clinical utility of blood plasma TRX for the diagnosis of ACS. Blood plasma TRX concentrations for the healthy donor cohort i.e., 'true negatives' (specificity) was plotted with the ACS cohorts i.e., 'true positives' (sensitivity). The area under the curve was determined as 0.819 with a 95% CI or 0.752 to 0.888. This indicates that blood plasma TRX alone may predict a correct ACS diagnosis in 81.9% of cases.

Data filed – ROC TRX Thesis.spv (Document1) Output and ROC All Biomarkers. Sav (DataSet1) Data-IBM SPSS Statistics Data

3.15 Kaplan-Meier.

The ROC analysis presented above demonstrates clinical utility for each of the plasma biomarkers for ACS diagnosis, as determined by area under the curve. This was substantiated by specificity, sensitivity, and efficiency calculations, which showed that TRX biomarker was able to predict a correct result i.e., determine a 'true positive' and 'true negative' in >81% of cases. Therefore, to take this analysis further, it was next important to evaluate whether TRX, could reliably predict ACS participant outcomes, in this case the time to event endpoint was an ACS readmission.

3.16 Logistic regression predictions.

Using binomial logistic regression to predict if cases can be correctly predicted from the independent variables, it was then analysed which independent variable contributed and its statistical significance.

The variables in the equation below show each independent variable and statistical significance. The odds ratio ("Exp B" column) was used to predict the probability of an event occurring. Odds Ratio of each independent variable recorded below in tables A, B, C, D along with the confidence Intervals, showing the change in log odds occurring for one-unit change in independent variable, keeping the other independent variables constant.

Table 3.17. Logistic regression predicting likelihood of ACS event based on age, BMI, gender and thioredoxin.

Table 3.17		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	Age	.083	.016	27.278	1	<.001	1.087	1.054	1.122
	BMI	.090	.053	2.909	1	.088	1.095	.987	1.214
	Gender (1)	.706	.425	2.762	1	.096	2.027	.881	4.662
	TRX	.003	.023	.012	1	.913	1.003	.958	1.049
	Constant	-7.722	1.945	15.770	1	<.001	.000		

a. Variable(s) entered on step 1: Age, BMI, Gender, TRX mean Sample-1.

The statistical significance for TRX illustrates that Age (p<0.001) added significantly to the predictions model. Whereas BMI (p=0.088) and Gender (p=0.96) did not. For TRX, males had 2.02 (95% CI, 0.881 to 4.662) times higher odds to exhibit ACS than females (Table 3.17).

Having satisfied the robustness of the plasma biomarkers TRX a non-parametric Kaplan-Meier analysis was next performed to determine if these biomarkers impacted on participant prognosis and were able to predict the probability of an ACS readmission following stratification.

3.17 Kaplan-Meier all ACS participants thioredoxin (TRX).

The ACS cohort (n=80) displayed events and censoring at sample-1 (screening blood sample) as displayed in Table 3.18. The sample-1 [TRX] quartile means were subsequently calculated.

Table 3.18. Readmission analysis based on blood plasma [TRX] ng/ml at less than 25%, median and greater than 75% percentile as the cut-off values.

Table 3.18

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 8.42 25% percentile	19	6	13	68.4%
Median >8.42 ~ < 13.40	41	15	26	63.4%
> 13.40 75% percentile	20	3	17	85.0%
Overall	80	24	56	70.0%

For blood plasma analysis at sample-1 the ACS participants (n=80) were stratified according to TRX concentration. There were 19 participants in the <25% percentile (TRX <8.42 ng/ml), however 13 were censored (68.4%). For the 25%-75% inter-percentile range (TRX >8.42 ng/ml~<13.40 ng/ml), there were 41 participants, however 26 were censored (63.4%). Finally, there were 20 participants in the >75% percentile (TRX >13.40 ng/ml), however 17 were censored (85%). Taken together, a total of 70% ACS participants were censored (Table 3.63). For sample-1 and sample-2 there were no statistically significant findings, however for sample-3 (6 months from index event in all participants) there was a statistically significant difference in the readmission rate between the >75% TRX concentration stratified ACS participants, compared with the <25% TRX concentration, $\chi 2(1) = 6.892$, $p=0.009$ (Table 3.19).

Table 3.19. Multiple pairwise comparisons for blood plasma [TRX] ng/ml as the cut off values in sample 3.

Table 3.19

	Intervention Sample 3 ACS 25% ~ 75% percentile	< 8.42 25% percentile		Median >8.42 ~ < 13.40		> 13.40 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 8.42 25% percentile			2.577	.108	6.892	.009
	Median >8.42 ~ < 13.40	2.577	.108			2.377	.123
	> 13.40 75% percentile	6.892	.009	2.377	.123		

Figure 3.6 illustrates a visual representation of the data presented in Table 3.19, highlighting that there was a significant reduction in readmissions due to a second ACS event for participants who had a plasma TRX concentration of <8.42 ng/ml at sample-3.

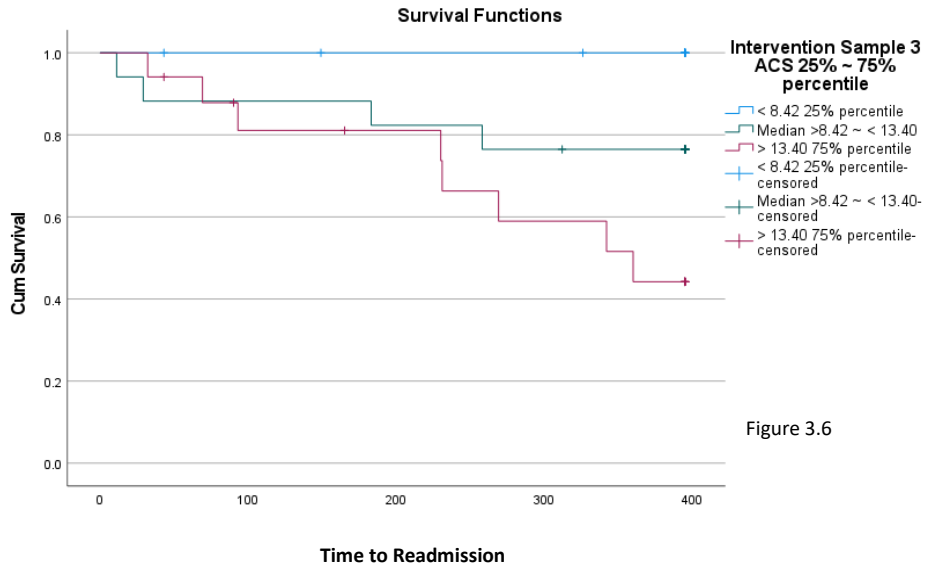


Figure 3.6

Figure 3.6. To determine whether blood plasma TRX (ng/ml) could predict participant outcome (overall survival without ACS readmission), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRX]. **1)** Blue line represents the 25% percentile, TRX <8.42 ng/ml. **2)** Green line represents 25%-75% percentile, TRX >8.42 ~ <13.40 ng.ml. **3)** Red line represented the 75% percentile, TRX >13.40 ng/ml. χ^2 analysis for 25% percentile vs 75% percentile = 6.89, p=0.009.

Data Filed - ALL KM Sample 1-2 TRX.sav [DataSet1] and ALL KM Sample 1-3 TRX.SPV [Document5] IBM SPSS Statistics Output.

3.18 Kaplan-Meier for plasma thioredoxin (TRX) concentration percentiles.

To address the aim and objectives i.e., whether TRX plasma concentrations may be indicative of an ACS event and/or predict a second event, the Kaplan-Meier analysis was systematically performed for TRX. Initially, this was performed for the 'blood sample-1' TRX percentile concentrations. To recap, these percentile concentrations were calculated as <8.42ng/ml for the <25% percentile (n=9), >8.42 ng/ml~<13.40 ng/ml for the inter-percentile range (n=19) and >13.40 ng/ml for the >75% percentile (n=7). Summarised data are presented in Table 3.20.

Table 3.20. Readmission analysis based on percentage of admissions and censored cases of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma TRX ng/ml as the cut-off values.

Table 3.20

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 8.42 25% percentile	9	6	3	33.3%
Median >8.42 ~ < 13.40	19	15	4	21.1%
> 13.40 75% percentile	7	3	4	57.1%
Overall	35	24	11	31.4%

The participants in the inter-percentile range for plasma TRX concentration had the lowest time to readmission of 69 days (95% CI, 45.1 to 638.1 days). The time to readmission values for the <25% and >75% plasma TRX concentration participants were much higher at 342 and 231 days respectively, see Table 3.21.

Table 3.21. Readmission analysis in days for sample 1 blood plasma TRX ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.21

Sample-1 ACS 25%~75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
<8.42 25% percentile	262.117	56.585	151.211	373.023	342.000	151.050	45.942	638.058
Median >8.42~<13.40	143.692	31.977	81.018	206.367	69.000	38.660	.000	144.773
>13.40 75% percentile	183.389	47.639	90.017	276.760	231.000	202.000	.000	626.920
Overall	194.609	28.693	138.370	250.847	205.000	86.812	34.849	375.151

a. Estimation is limited to the largest survival time if it is censored.

Assumption #4 (Section 2.24.4.1) states that for time to event statistics, there is similar censoring. The percentage of censored cases present in the <25 % percentile was 33.3%, compared to 21.1% and 57.1% for the inter-percentile and >75% percentile groups. Based on this, it is clear that the censoring of the ACS groups was not similar. It must be noted that the failure to meet this assumption can lead to incorrect interpretation or rejecting the null hypothesis incorrectly (Norušis, 2012). Figure 3.7 provides a visual representation of the data presented in Table 3.21. The plot shows a small interaction i.e., crossing of survival curves. However, in general those, participants with inter-percentile plasma TRX concentrations levels (>8.24~13.40 ng/ml) have a greater incidence of readmission than those with low concentrations.

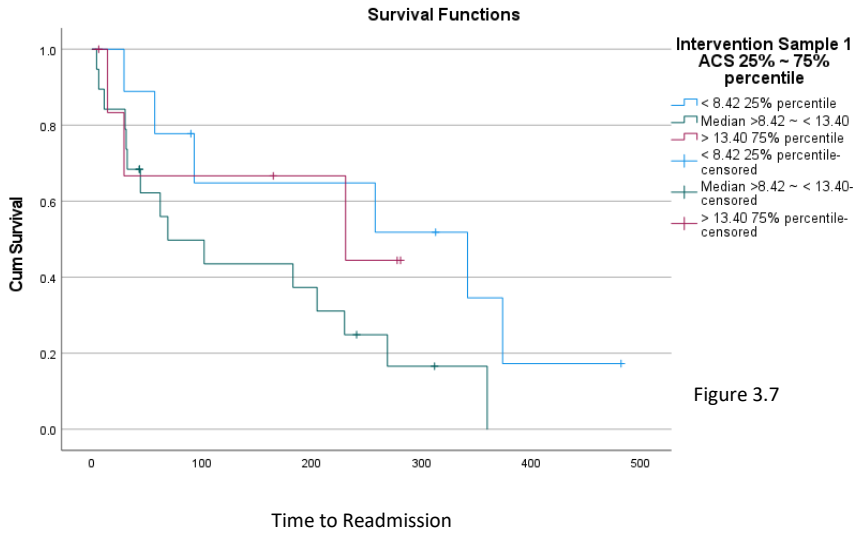


Figure 3.7

Figure 3.7. Readmission analysis using various blood plasma [TRX] ng/ml cut-off values of baseline sample 1. To determine whether blood plasma TRX (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRX]. **1)** Blue line represents the 25% percentile, TRX <8.42 ng/ml. **2)** Green line represents 25%-75% percentile, TRX >8.42 ~ <13.40 ng.ml. **3)** Red line represented the 75% percentile, TRX >13.40 ng/ml. $\chi 2$ analysis, $p>0.05$.

To follow-on from this, a log rank test was conducted, which showed that there were no statistical differences in the admission rate for the three plasma TRX concentrations, $\chi 2(2) = 3.837$, $p=0.147$ (Table 3.22).

Table 3.22. Multiple pairwise comparisons for admission rates using blood plasma [TRX] ng/ml cut off values at baseline.

Table 3.22

	Intervention Sample 1 ACS 25% ~ 75% percentile	< 8.42 25% percentile		Median >8.42 ~ < 13.40		> 13.40 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 8.42 25% percentile			3.288	.070	.155	.694
	Median >8.42 ~ < 13.40	3.288	.070			1.139	.286
	> 13.40 75% percentile	.155	.694	1.139	.286		

The data summarised in Table 3.22 shows that there was no significant difference in the admission distributions for the median concentrations of TRX for the <25% percentile concentration, $\chi2(1) = 3.288$, $p=0.070$, the >75% TRX concentration $\chi2(1) = 1.139$, $p=0.286$ or the >75% ~ <25% inter-percentile range, $\chi2(1) = 0.155$, $p=0.694$.

Next the same analysis was conducted on plasma TRX for blood sample-2 (first follow-up). The data presented in Table 3.23 shows the percentage of censored cases present in the <25% percentile (25.0% ACS participants), the inter-percentile range (36.4% ACS participants) and >75% percentile (66.7% ACS participants). These data illustrate that the proportion of censored groups was not similar.

Table 3.23. Readmission analysis at less than 25%, median and greater than 75% percentile of blood plasma TRX ng/ml as the cut-off values at sample 2.

Table 3.23

Intervention Sample 2 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 8.42 25% percentile	12	9	3	25.0%
Median >8.42 ~ < 13.40	11	7	4	36.4%
> 13.40 75% percentile	3	1	2	66.7%
Overall	26	17	9	34.6%

Participants with the inter-percentile plasma TRX concentration had a median time to readmission of 258 days (95% CI, 193.9 to 322.2 days). The <25% plasma TRX concentration group had a median readmission time of 93.0 days (95% CI, 40.0 to 146.0) days compared to >75% TRX plasma concentration group which was 183 days. The confidence interval was not able for the >75% group due to n=1, where participants censored was n=2. See Table 3.24 for full summary.

Table 3.24. Readmission analysis in days for sample 2 blood plasma [TRX] ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.24

Intervention Sample 2 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 8.42 25% percentile	159.766	45.415	70.753	248.780	93.000	27.058	39.966	146.034
Median >8.42 ~ < 13.40	247.752	39.571	170.193	325.311	258.000	32.748	193.814	322.186
> 13.40 75% percentile	332.500	105.712	125.304	539.696	183.000	.	.	.
Overall	223.605	31.535	161.797	285.412	231.000	36.262	159.926	302.074

a. Estimation is limited to the largest survival time if it is censored.

Figure 3.8 provides a visual representation of this data, highlighting that the <25% plasma TRX group display a reduction in ACS readmission time, compared with those in the inter-percentile range.

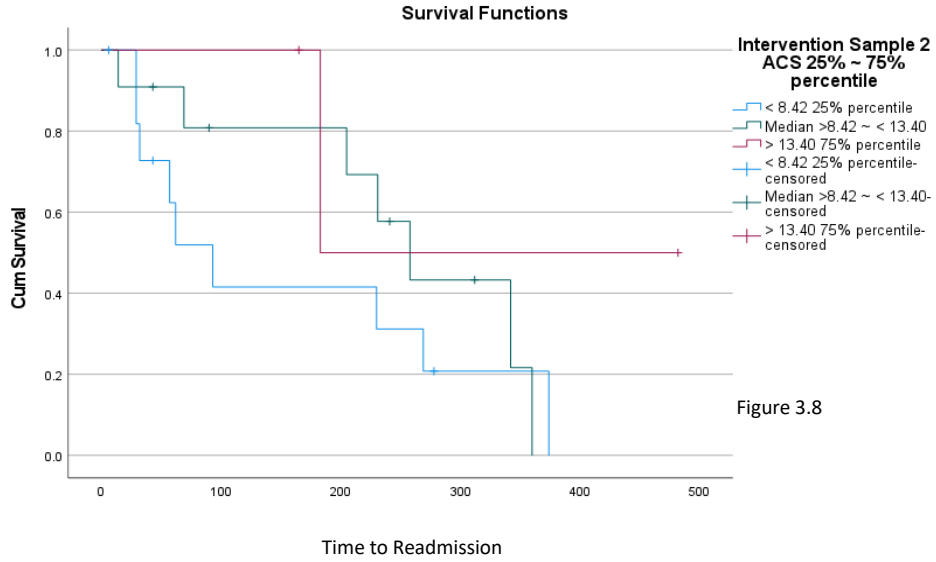


Figure 3.8

Figure 3.8. Readmission analysis using various blood plasma [TRX] ng/ml cut-off values of baseline sample 2. To determine whether blood plasma TRX (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRX]. **1)** Blue line represents the 25% percentile, TRX <8.42 ng/ml. **2)** Green line represents 25%-75% percentile, TRX >8.42 ~ <13.40 ng.ml. **3)** Red line represented the 75% percentile, TRX >13.40 ng/ml. $\chi 2$ analysis, p>0.05.

The survival readmission distributions for the three plasma TRX concentrations based on sample-2 were not statistically significant, p>0.05 (Table 3.25), as determined by Log rank pairwise comparison.

Table 3.25 The survival readmission distributions for healthy and ACS cohort of sample 2 blood plasma [TRX] ng/ml levels.

Table 3.25	Intervention Sample 2 ACS 25% ~ 75% percentile	< 8.42 25% percentile		Median >8.42 ~ < 13.40		> 13.40 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 8.42 25% percentile			.633	.426	1.956	.162
	Median >8.42 ~ < 13.40	.633	.426			.700	.403
	> 13.40 75% percentile	1.956	.162	.700	.403		

Finally, analysis was performed for plasma TRX, based on blood collected at Sample-3 (2^{nd} follow-up). The percent of censored ACS cases present in the <25 % plasma TRX concentration was 100%, compared with the inter-percentile range (20.0%) and >75 % (27.3%). Thus, the censored groups were not similar (Table 3.26).

Table 3.26. Readmission analysis of sample 3 percentage of admissions and censored cases at less than 25%, median and greater than 75% percentile of blood plasma [TRX] ng/ml as the cut-off values at baseline.

Table 3.26

Intervention Sample 3 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 8.42 25% percentile	3	0	3	100.0%
Median >8.42 ~ < 13.40	5	4	1	20.0%
> 13.40 75% percentile	11	8	3	27.3%
Overall	19	12	7	36.8%

The data presented in Figure 3.9 show that the ACS participants in the <25% plasma TRX concentration were not readmitted (n=3), compared with the inter-percentile and >75% participants. However, these data did not reach significance, (p>0.05).

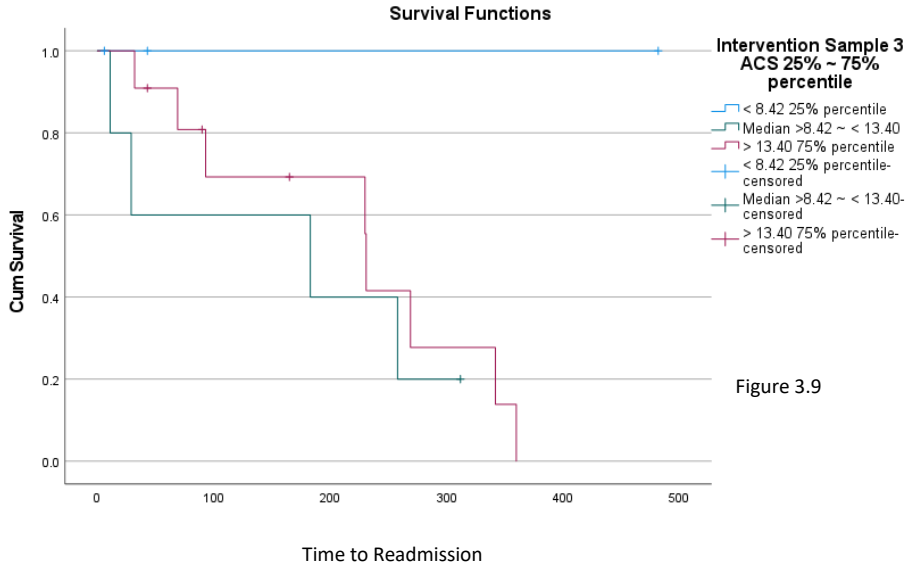


Figure 3.9

Figure 3.9. Readmission analysis using various blood plasma [TRX] ng/ml cut-off values of baseline sample 3. To determine whether blood plasma TRX (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRX]. **1)** Blue line represents the 25% percentile, TRX <8.42 ng/ml. **2)** Green line represents 25%-75% percentile, TRX >8.42 ~ <13.40 ng.ml. **3)** Red line represented the 75% percentile, TRX >13.40 ng/ml. $\chi 2$ analysis, p>0.05.

Table 3.27. Overall comparison of readmission distribution between Arm-1 and Arm-2.

Table 3.27

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	3.309	2	.191

Test of equality of survival distributions for the different levels of Sample-3 ACS 25%~75% percentile.

Table 3.27 illustrates the Log rank pairwise comparisons for plasma TRX at blood sample-3, between Arm-1 and Arm-2 ACS participants. The data did not reach statistical significance different, $\chi 2(2) = 3.309$, $p=0.191$.

Table 3.28 The readmission distributions for the healthy and ACS cohort of sample 3 blood plasma TRX ng/ml levels.

Table 3.28

	Intervention Sample 3 ACS 25% ~ 75% percentile	< 8.42 25% percentile		Median >8.42 ~ < 13.40		> 13.40 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 8.42 25% percentile			1.729	.189	2.596	.107
	Median >8.42 ~ < 13.40	1.729	.189			.497	.481
	> 13.40 75% percentile	2.596	.107	.497	.481		

The pairwise comparison data presented in Table 3.28 shows that there was no significant difference in readmissions between each of the plasma TRX concentration groups ($p>0.05$).

Data filed-SPPS KM sample1-3 TRX SC.spv [DataSet3]data and KM Sample 1-3 TRX SC.spv [document1]

3.19 Kaplan-Meier for lesions of Percutaneous Coronary Intervention.

The final set of analyses was to establish whether the TRX plasma biomarkers had any predictive value for ACS readmission with respect to ACS percutaneous coronary intervention (PCI) i.e., Right Coronary Artery (RCA), circumflex or Left Anterior Descending (LAD). ACS participants for blood sample-1 were subsequently stratified according to lesion of PCI as outlined in Table 3.29.

Table 3.29. Readmission analysis based on lesion of ACS event that resulted in baseline PCI

Table 3.29

Lesion of PCI	Total N	N of Events	Censored	
			N	Percent
RCA	39	13	26	66.7%
Circumflex	11	3	8	72.7%
LAD	30	8	22	73.3%
Overall	80	24	56	70.0%

Overall (n=80) ACS participants were included in this analysis, of these participants the PCI event is broken down as follows: RCA (n=39), Circumflex (n=11) and LAD artery (n=30), see Table 3.29.

TRX was reviewed with respect to the lesion of PCI event. As previously described, participants were further stratified according to plasma biomarker concentration i.e., <25%, inter-percentile range (>25% ~ <75%) and >75%. As previously stated, the design was non-event driven and all participant survival status was known at end of study. This limited the events of interest to (n=35) of all ACS admissions.

3.20 Thioredoxin Kaplan-Meier - readmission relating to Acute Myocardial Infarction lesion.

Initially, the impact of PCI lesion with respect to plasma TRX concentration was evaluated. This related to n=18 participants who received PCI to the RCA, n=5 for circumflex and n=13 for LAD. The full breakdown is presented in Table 3.30.

Table 3.30. Readmission analysis based on lesion of PCI at baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma TRX ng/ml cut-off values.

Table 3.30

Lesion of PCI	TRX Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored N	Censored Percent
RCA	< 8.42 25% percentile	5	4	1	20.0%
	Median >8.42 ~ < 13.40	10	7	3	30.0%
	> 13.40 75% percentile	3	2	1	33.3%
	Overall	18	13	5	27.8%
Circumflex	< 8.42 25% percentile	2	1	1	50.0%
	Median >8.42 ~ < 13.40	3	2	1	33.3%
	Overall	5	3	2	40.0%
LAD	< 8.42 25% percentile	2	1	1	50.0%
	Median >8.42 ~ < 13.40	6	6	0	0.0%
	> 13.40 75% percentile	4	1	3	75.0%
	Overall	12	8	4	33.3%
Overall	Overall	35	24	11	31.4%

The data indicate that ACS participants who received a PCI to RCA, had a greater readmissions rate for all concentrations of TRX, however the overall percentage was less when compared with circumflex and LAD. Table 3.110 shows that ACS participants who received a PCI to LAD had a median time of 93 days to readmission (95% CI, 36.9 to 149 days). For RCA participants, the time to readmission was longer at 230 days (95% CI, 42.9 to 417 days). Circumflex lesions had a median readmission time of 342 days; however, it was not possible to determine the 95% CI due to the low participant number (n=5).

Table 3.31. Readmission analysis based on lesion of ACS event that resulted in baseline PCI of [TRX] ng/ml levels as the cut-off values less than 25%, median and greater than 75% percentile.

Table 3.31

Means and Medians for Survival Time

Lesion of PCI	TRX Sample 1 ACS 25% ~ 75% percentile	Mean[a] Estimate	Mean[a] Std. Error	Mean[a] 95% CI Lower Bound	Mean[a] 95% CI Upper Bound	Median Estimate	Median Std. Error	Median 95% CI Lower Bound	Median 95% CI Upper Bound
RCA	< 8.42 25% percentile	177.400	57.023	65.635	289.165	258.000	159.960	.000	571.522
	Median >8.42 ~ < 13.40	188.683	51.585	87.577	289.789	205.000	119.358	.000	438.942
	> 13.40 75% percentile	163.667	77.750	11.277	316.056	231.000	.000	.	.
	Overall	181.025	33.069	116.209	245.841	230.000	95.422	42.973	417.027
Circumflex	< 8.42 25% percentile	342.000	.000	342.000	342.000	342.000	.	.	.
	Median >8.42 ~ < 13.40	132.333	58.505	17.664	247.003	183.000	.000	.	.
	Overall	237.400	70.660	98.906	375.894	342.000	.000	.	.
LAD	< 8.42 25% percentile	138.500	32.173	75.440	201.560	93.000	.	.	.
	Median >8.42 ~ < 13.40	86.667	38.917	10.390	162.943	44.000	22.658	.000	88.409
	> 13.40 75% percentile	214.250	57.807	100.948	327.552	.	.	.	.
	Overall	145.250	33.096	80.382	210.118	93.000	28.579	36.985	149.015
Overall	Overall	180.623	24.123	133.343	227.903	205.000	98.943	11.072	398.928

a. Estimation is limited to the largest survival time if it is censored.

The data presented in Figure 3.10 gives a visual representation of PCI lesion with respect to stratified plasma TRX concentrations (data also summarised in Table 3.31). As shown in Figure 3.10, whilst there were some interesting trends in the time to readmission based on stratified TRX concentration and lesion of PCI, there was no statistical significance.

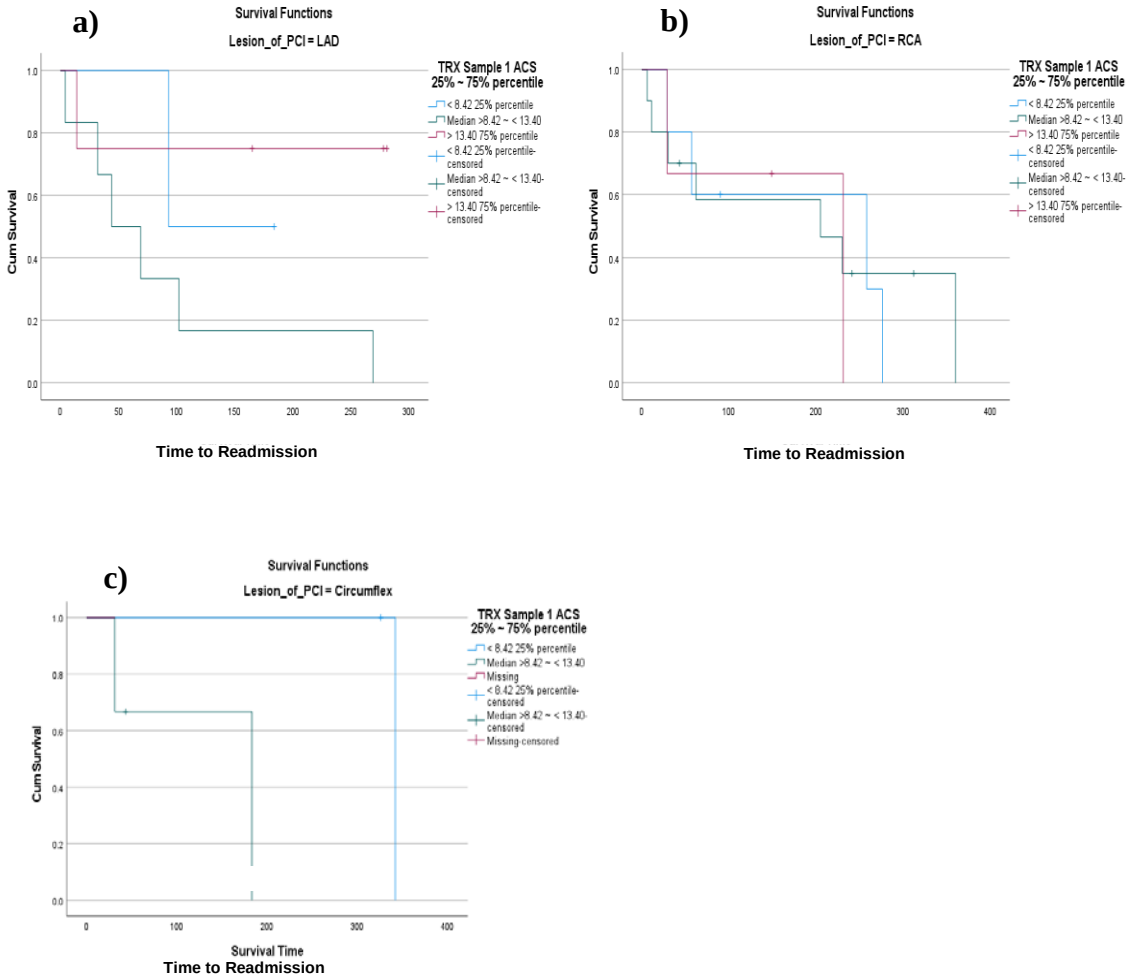


Figure 3.10. ACS Readmission analysis based on lesion of PCI of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [TRX] ng/ml cut-off values. [TRX]. **a)** Represents PCI to LAD, **b)** represents PCI to RCA and **c)** represents circumflex PCI. Blue line represents the 25% percentile, TRX <8.42 ng/ml. Green line represents 25%-75% percentile, TRX >8.42 ~ <13.40 ng.ml. Red line represented the 75% percentile, TRX >13.40 ng/ml. $\chi^{2\ analysis}$ p>0.05

Log rank pairwise between the three stratified TRX concentrations and lesion of PCI did not show significance (p>0.05).

3.21 Brief discussion of the main thioredoxin (TRX) findings.

The data presented in this chapter showed an overall increase in mean plasma TRX concentration for ACS participants (12.4 ng/ml) compared to the healthy cohort (10.8 ng/ml). Following censoring, the healthy cohort population without any underlying health conditions had a mean [TRX] as 10.30 ng/ml, which may be a good starting point as a predictor for an upper limit of normal (ULN). To the best of knowledge at the time of writing, this is the first study of its kind to evaluate [TRX] in a supposedly healthy cohort in this way. However, other studies have evaluated [TRX] in healthy participants to understand how plasma values change during stress exercise (Wadley et al., 2019). The values reported in this chapter are higher than those reported by Wadley et al., which evaluated younger individuals (median 29 years), and could be attributed to increases age related in oxidative stress (Tan *et al.*, 2018). There was also an identified difference between sexes, where healthy females displayed the largest difference between the in ACS cohort (13.5 ng/ml) and healthy population (9.02 ng/ml). Thus, this finding may be important for in the future for female AMI patients, who may be 'under-diagnosed' using the current hc-cTn 'gold-standard' biomarker (Shah *et al.*, 2015a). Interestingly, for the healthy cohort where inclusion numbers were relatively matched, the under 55's (n=36) had a mean plasma TRX concentration of 8.82 ng/ml compared to over 55's (n=29) who had a mean concentration of 13.26 ng/ml, an observation which again may be explained by an increase in age associated oxidative stress (Tan et al., 2018). For the ACS cohort the numbers include were less equal, but a similar trend of levels was observed for the 'under 55' (13.77 ng/ml for Arm-1 vs 11.32 ng/ml for Arm-2), compared with 12.65 ng/ml vs Arm-2 12.22 ng/ml for the over 55's respectively.

Statistical analysis showed that these [TRX] values remained significantly higher for ACS Arm-1 non-smokers and ACS Arm-2 non-smokers, compared with healthy volunteer non-smokers ($p<0.05$), although the number of non-smokers who suffered an ACS was low. Taken together, the data indicate that smoking status has little impact on mean plasma TRX levels once an ACS has occurred.

The ROC showed plasma TRX concentrations at baseline screening for the ACS cohort (n=80) and the healthy cohort (n=65) had an area under the curve determined as 81% thus, providing confidence in the biomarkers analysed would positively predict 4 in 5 ACS events. Combining the Arm-1 and Arm-2 ACS data, the primary endpoint (readmissions) occurred in

(n=35) 44% of participants. Where a second ACS was the cause, this was observed in (n=24) 30% of all readmissions. The mean plasma TRX concentration for all ACS participants readmitted with a second ACS event was 10.44 ng/ml, compared with 15.40 ng/ml at second follow-up (n=12). To the best of knowledge, this study is the first to monitor ACS patients in this way. This observed increase in plasma TRX concentration may be indicative of further damage to the cardiac muscle, an observation which has been demonstrated for skeletal muscle damage (Akbarpour Beni *et al.,* 2021). Therefore, this observation indicates a rationale for monitoring ACS patient recovery. Future research to evaluate the extent of any ongoing cardiac muscle damage in this context is warranted.

To investigate the impact of plasma [TRX] on ACS readmission rates, Kaplan-Meier analysis was conducted to determine probability of 'time-to-readmission', based on biomarker stratification. Initial admissions were recorded in days from PCI (Appendix AA) and any admission that was not due to an ACS admission was censored. The ACS participants (n=80) were thus stratified according to TRX concentration at this point. There were 19 participants in the <25% percentile (TRX <8.42 ng/ml), however 13 were censored (68.4%) for non-ACS readmissions. For the 25%-75% median range (TRX >8.42 ng/ml~<13.40 ng/ml), there were 41 participants, however 26 were censored (63.4%). Finally, there were 20 participants in the >75% percentile (TRX >13.40 ng/ml), however 17 were censored (85%). The data show that ACS patients within the >75% Percentile at baseline had an increased risk of readmission. Moreover, this increased risk was also notable in participants presenting with AMI to LAD. Given the negative prognosis associated with AMI due to LAD (Dadjoo *et al*., 2013), evaluating plasma [TRX] at ACS screening may therefore be clinically relevant for risk stratifying patients at point of diagnosis.

In conclusion, the data presented in this chapter highlight differences between plasma [TRX] for healthy individuals and ACS patients, which is particularly pronounced in females. Plasma [TRX] levels could reliably predict a correct diagnosis in ~80% cases and may indicate further cardiac muscle damage at follow-up. Moreover, a plasma [TRX] at diagnosis >13.40 ng/ml is associated with an increased risk of ACS readmission, particularly for those patients presenting with AMI to LAD.

Chapter 3b – Thioredoxin-Reductase (TRXr)

3.22 Brief introduction to plasma thioredoxin-reductase (TRXr) analysis.

Chapter 3a evaluated the plasma levels of thioredoxin-reductase (TRXr) in ACS patients compared with a healthy cohort. As outlined in section 1.6., TRX forms part of a wider intracellular antioxidant system. Oxidative stress occurs when reactive oxygen species (ROS) generation exceeds the capacity of the cellular antioxidants, which is a pathological feature linked to many disorders, including cardiovascular disease (Moris et al., 2017; Liou *et al.,* 2010; Singh *et al.*, 2019). A crucial target of ROS is the 'thiol groups' (SH) located at the terminus of the amino acid cysteine (Brosnan *et al.,* 2006). Proteins containing 'solvent accessible' cysteines may therefore be targeted by ROS, creating sulphenic acid (SOH), sulfinic acid (SO_2H) and sulphonic acid (SO_3H) respectively (Paulsen et al., 2013). If 2 cysteines are situated in proximity and are in the correct orientation, ROS mediated oxidation may lead to the formation of 'intra' or 'extra' molecular disulphide bonds (Paulsen et al., 2013). These oxidised cysteines may alter the structure and / or function of proteins in cells, the implications of which are important in the pathogenesis of heart disease (Herrero-Galán *et al*., 2022). These protein disulphide bonds are subsequently reduced by the action of TRX (chapter 3a), which mediates this via a thiol-disulphide exchange reaction (Section 1.6). The 'oxidised' TRX is subsequently reduced by the action of thioredoxin reductase (TRXr), which requires NADPH as a redox cofactor (Ahsan *et al*., 2009). Cellular damage has been linked to the release of TRXr and subsequent rise in plasma [TRXr] (Sun *et al*., 2014). Therefore, the following analysis as described in this chapter was conducted to determine if there are any changes in plasma [TRXr] between a healthy cohort of participants and those following an AMI / during an ACS event. As described in chapter 2, the ACS patients and healthy cohort were recruited at WAHT. Participant plasma samples were subsequently evaluated using an optimised ELISA for TRXr (Section 2.23., Table 2.2 and Appendix X). The data collected was subsequently analysed using various statistical methods as described in chapter 2. This chapter presents the results of this analysis is a logical order to ultimately evaluate the clinical utility of [TRXr] in the context of ACS and establish whether [TRXr] could reliably predict ACS patient outcome. In this case the time to event endpoint was an ACS readmission.

Objectives:

a) To clarify the mean plasma concentrations for TRXr for healthy volunteers, stratified based on sex and age., which will be used as baseline measurements for ACS comparison.

b) To evaluate the plasma concentrations levels of TRXr for ACS patients stratified based on age and sex at initial diagnosis / screening and follow-up. Clinical utility may subsequently be evaluated.

c) Monitor the concentration level of TRXr through ACS patient follow-up sampling, in order to evaluate whether this biomarker may be predictive of an ACS readmission.

d) Evaluate whether [TRXr] may predict readmission based on ACS patient stratified according to PCI.

3.23 Basic descriptive statistics of TRXr.

To determine the mean plasma [TRXr] for the 'healthy cohort, removal of healthy volunteers with a medical history of diabetes mellitus (n=5), hypertension (n=10), family history of cardiovascular conditions (n=19), or inflammatory disorders (n=1) was carried out. This equated to Healthy Volunteers with no medical conditions (n=38). This population had a slightly higher mean plasma TRXr concentration 0.71 ng/ml ± 1.44 ng/ml, compared with the healthy volunteers as a whole. Stratification of the cohort into Arm-1 (n=36) and Arm-2 (n=44) revealed plasma TRXr concentration levels of 1.38 ng/ml ± 1.53 ng/ml and 0.94 ng/ml ± 1.24 ng/ml respectively. Next the 'smokers' were removed from the healthy cohort analyses, which resulted in little change in mean plasma TRXr concentrations 0.78 ng/ml ± 1.65 ng/ml (n=27). The same convention was applied to the ACS participants, which resulted in mean plasma TRXr concentrations of 1.54 ng/ml ± 2.09 ng/ml for Arm-1 (n=5) and 1.57 ng/ml ± 1.94 ng/ml for Arm-2 ($p=0.0267$, n=7). These data show that, non-smokers presenting with ACS had significantly higher levels of plasma TRXr compared with non-smokers healthy volunteers, with no other medical history. Taking everything together, the mean [TRXr] for all healthy Volunteers 0.63 ng/ml ± 1.25 ng/ml, compared with all ACS admissions 1.14 ng/ml ± 1.39 ng/ml ($p<0.05$, Figure 3.11).

Overall, males in the healthy cohort (n=32) had a mean plasma TRXr concentration of

0.80 ng/ml ± 1.54 ng/ml, compared with females (n=33) 0.46 ng/ml ± 0.88 ng/ml.

The plasma TRXr concentrations for all healthy volunteers was 0.63 ng/ml ± 1.25 ng/ml compared to 1.144 ng/ml ± 1.39 ng/ml (p=0.0001) for all ACS participants. Data was statistically significant demonstrated on a Mann-Whitney U test (p=0.0008). Figure 3.11 and Table 3.32 for breakdown.

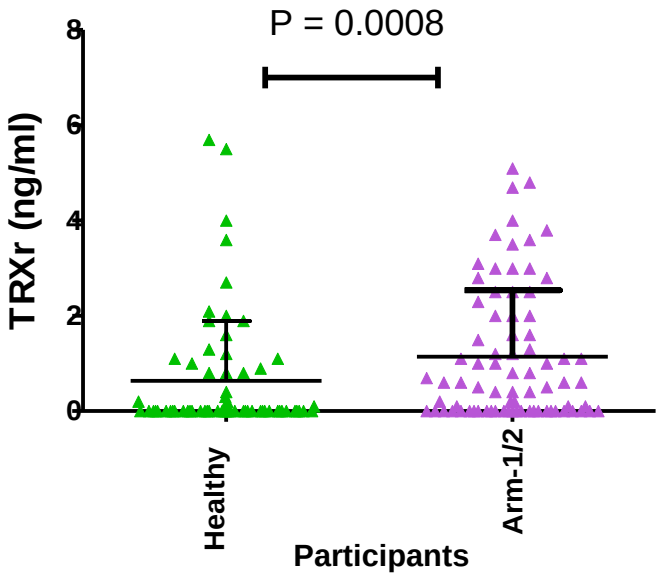


Figure 3.11

Figure 3.11 Overall blood plasma TRXr levels between healthy and ACS participant cohorts. The data presented illustrate the spread of data between all healthy (n=65) and all ACS (n=78) plasma TRXr levels (ng/ml). For healthy the mean TRXr concentration was 0.6±1.2 ng/ml. For ACS the mean TRXr concentration was 1.1±1.3 ng/ml.

Table 3.32. Descriptive statistics for plasma TRXr concentration

ACS V Healthy Cohort	Sex	Mean	Std. Deviation	N
Healthy Volunteer	Male	.803	1.5447	32
	Female	.470	.8823	33
	Total	.634	1.2543	65
ACS Arm-1 and Arm-2	Male	.984	1.2715	57
	Female	1.576	1.6401	21
	Total	1.144	1.3944	78
Total	Male	.919	1.3700	89
	Female	.900	1.3347	54
	Total	.912	1.3521	143

3.24 Basic TRXr analysis for ACS Arm-1.

It was next important to evaluate the plasma TRXr levels between males and female ACS participants. For the Arm-1 ACS participants, males (n=27) had a mean plasma TRXr concentration of 1.26 ng/ml ± 1.4 ng/ml, compared to females (n=9) [TRXr] 1.7 ng/ml ± 1.7 ng/ml ($p>0.05$). The effects of smoking status were next evaluated for Arm-1 ACS, where non-smokers or ex-smokes ≥12 months (n=26) had a mean plasma TRXr concentration of 1.21 ng/ml ± 1.56 ng/ml compared to smokers/vapers (n=10) 1.80 ng/ml ± 1.41 ng/ml ($p>0.05$).

To recap, for Arm-1 ACS the primary endpoint (readmission) occurred in (n=15) 41.6%, of which ACS admission (n= 10) 27.7% was the cause. The mean plasma TRXr concentrations at readmission for Arm-1 ACS participants due to a second ACS event was 0.97 ng/ml ± 1.45 ng/ml ($p<0.05$) when compared to primary admission (n=10).

3.25 Basic TRXr analysis for ACS Arm-2.

The same analysis was carried out for ACS Arm-2 participants (n=44), who had their ACS event within 24 hours of their hs-cTn result. Here the male cohort (n=32) had a mean plasma [TRXr] of 0.68 ng/ml ± 0.98 ng/ml compared to Females (n=12) 1.46 ng/ml ± 1.64 ng/ml ($p>0.05$). As with Arm-1, the Arm-2 participants were stratified according to smoking status.

For the non-smokers or ex-smokers ≥12 months (n=30), the mean plasma [TRXr] was 0.86 ng/ml ± 1.32 ng/ml compared to smokers/vapers (n=15), who had a [TRXr] mean of 1.19 ng/ml ± 1.24 ng/ml.

To reiterate, for the Arm-2 ACS participants the primary endpoint of readmission occurred in (n=20) 45.4%, of which readmission due to a second ACS accounted for (n= 14) 31.8% cases. The mean plasma [TRXr] upon readmission was 0.94 ng/ml ± 1.24 ng/ml, compared with the first and second follow-up samples, which were 0.90 ng/ml ± 0.99 ng/ml and 1.18 ng/ml ± 1.83 ng/ml respectively ($p>0.05$).

Combining the Arm-1 and Arm-2 ACS data, readmissions that were due to a second ACS event occurred in 30% (n= 24) of participants. For these participants, the mean plasma TRXr concentration at baseline was 0.81 ng/ml ± 2.07 ng/ml, compared with 0.77 ng/ml ± 0.84 ng/ml (n=18) and 1.20 ng/ml ± 1.87 ng/ml (n=9) at first and second follow-up respectively. See table 3.33 for full summary.

Table 3.33 Descriptive statistics for [TRXr] (ng/ml) biomarkers for readmission rates

ACS Readmission		TRXr (ng/ml)	SD	Non-ACS Readmission	TRXr (ng/ml)	SD	Significance
Baseline	(n=24)	**0.81**	2.07	(n=11)	**2.07**	1.57	0.0007
Follow-up 1	(n=17)	**0.77**	0.84	(n=11)	**1.20**	1.87	0.0025
Follow-up 2	(n=12)	**1.36**	1.69	(n=9)	**0.94**	1.40	0.0164

3.26 Age comparisons at screening TRXr.

Healthy volunteers at time of screening under 55 (n=36) had a mean plasma TRXr concentration of 0.41 ng/ml ± 0.86 ng/ml (n=29), compared with the over 55 mean of 0.90 ng/ml ± 1.59 ng/ml. For Arm-1 ACS participants, the mean plasma TRXr concentration was 1.20 ng/ml ± 1.49 ng/ml for under 55 (n=6), compared with over 55, which were 1.41 ng/ml ± 1.56 ng/ml (n=30). For Arm-2 ACS participants, the under 55's (n=11) had a mean plasma TRXr concentration of 1.10 ng/ml ± 1.23 ng/ml (n=33), compared with over 55's (n=33) which had a mean of 0.88 ng/ml ± 1.26 ng/ml (p=<0.0001), Figure 3.12.

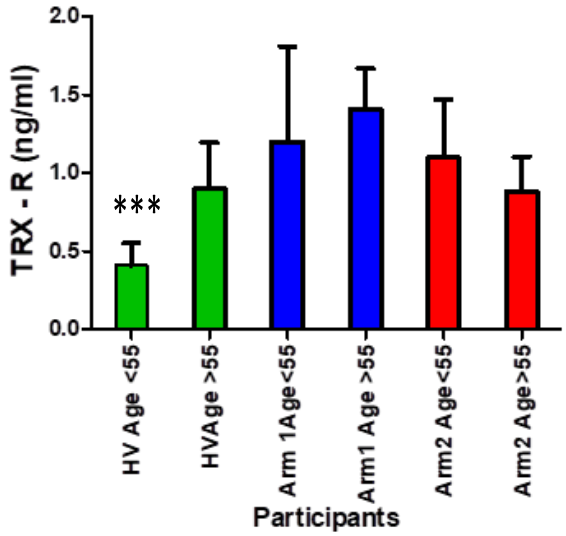


Figure 3.12

Figure 3.12 Age stratified blood plasma TRXr levels between healthy and ACS participant cohorts. The data presented illustrate blood plasma TRXr levels (ng/ml) following age stratification; <55 years or >55 years for **a)** healthy in green (<55 years, n=36, >55 years n=29), **b)** ACS Arm-1 in blue (<55 years n=6, >55 n=30) and **c)** ACS Arm-2 in red (>55 years n=11, >55 years n=33). ***p<0.0001.

3.27 Two-way mixed ANOVA.

To follow on from the descriptive statistical analysis presented above, it was next important to evaluate the impact of sex (male / female) with respect to the plasma concentrations of TRXr. Since the sample population cohorts included a healthy population, along with ACS Arm-1 and Arm-2, a two-way mixed ANOVA was selected, as this would determine interaction between participant 'sex' and population cohort.

3.28 Assessment of outliers for thioredoxin-reductase (TRXr).

Assaying the [TRXr] was sensitive at very low concentrations, with majority of the reading as 0.0 across both arms, but more prevalent in the ACS cohort.

For Sample-1 (Baseline blood taken at screening) for the healthy cohort (n=65), the Boxplot data presented in Figure 3.13b indicated that there was four extreme outlier (data point 64, 137, 139, 144) all had equal high duplicate results, no known past medical history or reasons to identify higher reading [TRXr]. The two outliers (data point 29, 109), was accurate with duplicate equally raised ELISA results, both were smokers and both diabetic. The extreme outlier (144) corresponded to a healthy male, with no known identifiable data to explain the raised [TRXr] significantly beyond the mean.

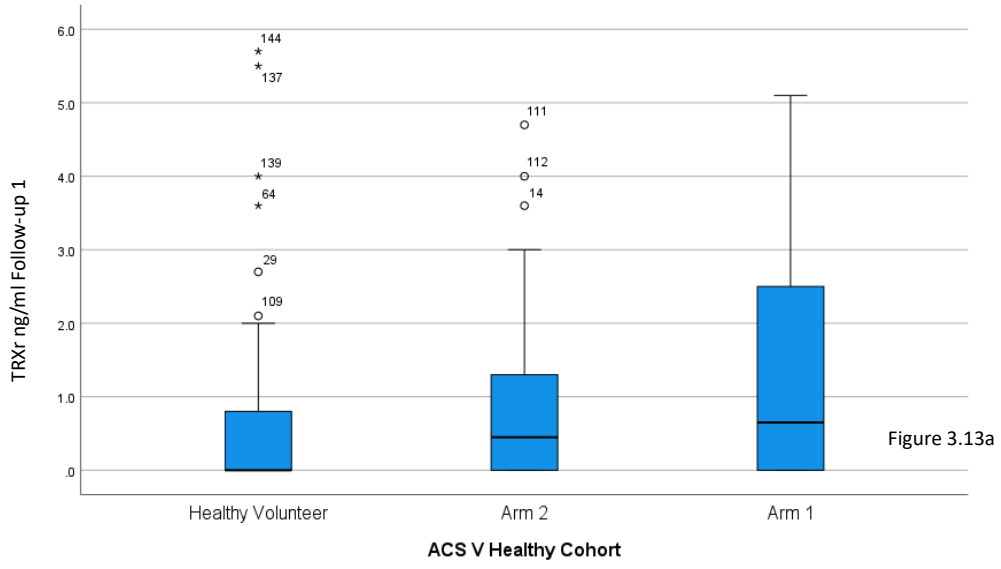


Figure 3.13a

Figure 3.13a. Evaluation of 'outliers' for blood plasma [TRXr] Sample-1 (Baseline). The box and whisker plots are presented for blood plasma TRXr (ng/ml), along with 'outliers' for healthy volunteers (n=80), ACS Arm-1 (n=36) and ACS Arm-2 (n=44). *Extreme outlier.

Two of these non-extreme outliers corresponded to a STEMI AMI (data point 14 and 112). Furthermore, both 14 and 111 received PCI to RCA and 112 to LAD. For each of the outliers, the ELISA readings were assessed in duplicate, which were equivalent in value respectively. Interestingly of the three non-extreme outliers' data point 111 went on to be readmitted for an ACS condition.

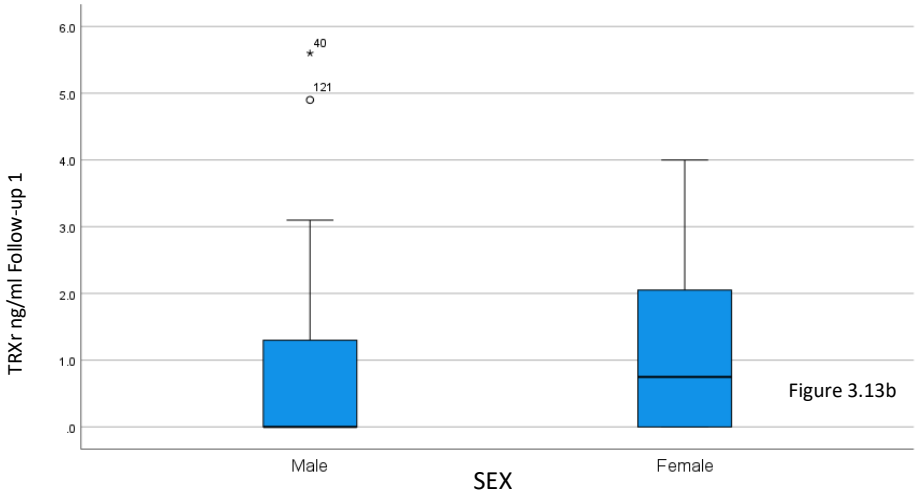


Figure 3.13b Evaluation of 'outliers' for blood plasma [TRXr] Sample-2 (Follow-up 1). The box and whisker plots are presented for blood plasma TRXr (ng/ml) along with 'outliers' ACS Arm-1 Male (n=27) Female (n=9) and ACS Arm-2 Male (n=32) Female (n=12) *Extreme outlier.

Only one extreme outlies was found at first follow-up (sample-2) (data point 40) and one non- extreme (Data point 121) as illustrated in (Figure 3.10b) Data points 40 and 121 were both male (n=32) from Arm-2 (n=32). Furthermore, extreme outlier data point 40 went on to have a non-ACS readmission.

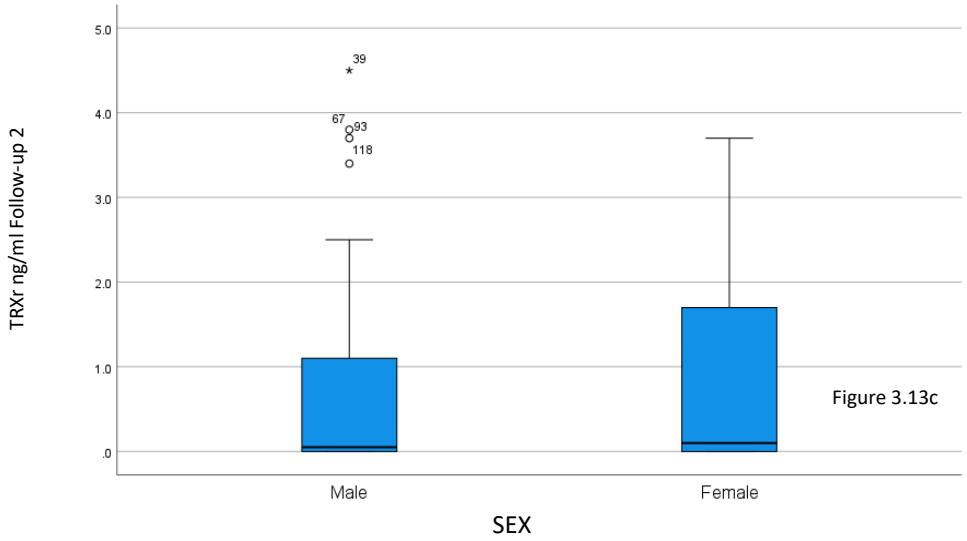


Figure 3.13c. Evaluation of 'outliers' for blood plasma [TRXr] Sample 3 (Follow-up 2). The box and whisker plots are presented for blood plasma TRXr (ng/ml) along with 'outliers' ACS Arm-1 Male (n=27) Female (n=9) and ACS Arm-2 Male (n=32) Female (n=12). * Extreme outlier.

As illustrated in Figure 3.13c, for ACS Arm-1 (n=36) and Arm2 (n=44) there was one extreme outliers, (data points 39) and three non- extreme outliers (data point 67, 93, 118) Interestingly the extreme outliers went on to be readmitted for an ACS condition. For the non-extreme outliers, none of the three went on to have any readmissions.

3.29 Test of normality with outliers TRXr sample 1, 2 and 3 including outliers.

Next a Shapiro Wilk's Test was conducted to determine whether the data fitted a normal distribution for blood plasma [TRXr]. This test was initially conducted with the 'outliers' included. The data are presented in Table 3.34 for sample-1 (a), sample-2 (b) and sample-3 (c). The data in Table 3.34 shows that, plasma TRXr concentration did fit a normal distribution for bloods taken at sample-1 (Baseline) and sample-2 (Follow-up 1) for females ($p>0.05$), but did not fit normal distribution for sample 3 ($p<0.001$).

Table 3.34a, b and c: Test of normality with outliers thioredoxin-reductase for blood sample-1 (a), Sample-2/3 (b).

(a)Test of Normality

Table 3.34a

	ACS V Healthy Cohort	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
TRX-RED mean Sample-1 (Baseline)	Healthy Volunteer	.324	65	<.001	.580	65	<.001
	Arm-2	.225	42	<.001	.775	42	<.001
	Arm-1	.199	36	<.001	.843	36	<.001

a. Lilliefors Significance Correction

(b)Test of Normality

Table 3.34b

	Sex	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
TRX-RED mean Sample-1 (Baseline)	Male	.254	41	<.001	.738	41	<.001
	Female	.202	12	.191	.820	12	.016
TRX-RED mean Sample-2 Follow-up 1	Male	.317	41	<.001	.642	41	<.001
	Female	.222	12	.107	.846	12	.033
TRX-RED mean Sample-3 Follow-up 2	Male	.253	41	<.001	.717	41	<.001
	Female	.306	12	.003	.697	12	<.001

a. Lilliefors Significance Correction

To further confirm which participant data sets were 'outliers', studentized evaluation was performed. Here outliers are confirmed if the data point is ± 3 of standard deviations of the mean. This analysis confirmed that there was no outlier for in blood sample-1 (baseline) or Sample 3 (Follow-up 2). For sample-2 (Follow-up 1), the same data point (40) was identified as an outlier, which was 3.57 standard deviations of the mean. This data point was also noted on the boxplot (Figure 3.13b). Taking everything together along with the boxplots presented in Figure 3.13, if an outlier data point was found to be consistent between the two analyses (Boxplot and studentized evaluation), it was removed form subsequent analysis.

3.27 Test of normality excluding outliers thioredoxin-reductase sample 1, 2 and 3.

The Shapiro Wilks Test for normality was reconducted for the plasma TRXr data, following the removal of the outliers as described above. The data presented in Table 3.35a illustrate an improvement in data sets which now fit a normal distribution, to include blood sample-1 and 2 (Baseline and Follow-up 1) for female participants ($p>0.05$) however did not fit a normal distribution for all bloods taken at sample-3 (Follow-up 2), ($p<0.001$).

Table 3.35 a. Test of normality excluding outliers thioredoxin-reductase (baseline, Follow-up 1 and 2)

Table 3.35a

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Sex	Statistic	df	Sig.	Statistic	df	Sig.
TRX-RED mean Sample 1 Baseline	Male	.241	38	<.001	.756	38	<.001
	Female	.202	12	.191	.820	12	.016
TRX-RED mean Sample 2 Follow-up 1	Male	.356	38	<.001	.661	38	<.001
	Female	.222	12	.107	.846	12	.033
TRX-RED mean Sample 3 Follow-up 2	Male	.275	38	<.001	.682	38	<.001
	Female	.306	12	.003	.697	12	<.001

a. Lilliefors Significance Correction

3.28 Thioredoxin-reductase (TRXr) Two-Way mixed ANOVA.

A two-way mixed ANOVA was subsequently performed for plasma TRXr concentration to establish whether there were interactions between the healthy cohort, the ACS cohort (Arm-1 and Arm-2) and sex (male and female). A summary of the data analysed in provided in Table 3.36.

Table 3.36. Case summary figures used for [TRXr] blood plasma between ACS verses healthy cohort participants and Sex.

		Cases					
		Valid		Missing		Total	
	ACS V Healthy Cohort	N	Percent	N	Percent	N	Percent
TRX-RED mean Sample 1	Healthy Volunteer	65	100.0%	0	0.0%	65	100.0%
	Arm 2	42	95.5%	2	4.5%	44	100.0%
	Arm 1	36	100.0%	0	0.0%	36	100.0%

		Valid		Missin		Tota	
		N	Percent	N	Percent	N	Percent
TRX-RED mean Sample-2	Male	42	45.2%	51	54.8%	93	100.0%
	Female	12	23.1%	40	76.9%	52	100.0%
TRX-RED mean Sample-3	Male	42	45.2%	51	54.8%	93	100.0%
	Female	12	23.1%	40	76.9%	52	100.0%

Initially, homogeneity of variance analysis was performed by the Levene's test for homogeneity of variance for TRXr. The data presented in Table 3.37 confirmed that for the TRX data assessed for sample-1 (screening) and sample-3 (second follow-up) displayed equal variance across all analytical methods i.e., mean, median, median adjusted and trimmed mean ($p=0.05$). However, this was not the case for sample-2 (first follow-up), displayed equal variance across all analytical methods i.e., mean, median, median adjusted and trimmed mean ($p>0.05$).

Table 3.37. Levene's test of equality of dependent variable for [TRXr].

		Levene Statistic	df1	df2	Sig.
TRX-RED mean Sample 1	Based on Mean	3.556	3	40	.023
	Based on Median	2.338	3	40	.088
	Based on Median and with adjusted df	2.338	3	33.853	.091
	Based on trimmed mean	3.507	3	40	.024
TRX-RED mean Sample 2	Based on Mean	.781	3	40	.511
	Based on Median	.215	3	40	.885
	Based on Median and with adjusted df	.215	3	26.679	.885
	Based on trimmed mean	.613	3	40	.611
TRX-RED mean Sample 3	Based on Mean	3.045	3	40	.040
	Based on Median	2.508	3	40	.073
	Based on Median and with adjusted df	2.508	3	31.641	.077
	Based on trimmed mean	2.760	3	40	.055

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

Table 3.37

Since downstream statistical analysis involes multivariate analysis, the Box's test of 'equality of covariance matrices' was next conducted. This test indicates whether two or more covariance matrices are homogenous. For the TRXr homogeneity of covariance the null hypothesis was rejected, signifying that the covariances were not homogenous (p=05), see Table 3.38.

Table 3.38. The Box Test for homogeneity of equalities covariances.

Box's M	35.309
F	1.574
df1	18
df2	1303.827
Sig.	.059

Tests the null hypothesis that the observed covariance matrices of the dependent variables are equal across groups.

a. Design: Intercept + ARM + Gender + ARM * Gender Within Subjects Design: Time

Following this, the two-way mixed ANOVA was performed for [TRXr]. Which evaluated the difference between male and female subjects for the health and ACS (Arm-1 and Arm-2) cohorts (Figure 3.14). The ANOVAs revealed that there was no statistical significance between means (p=0.282).

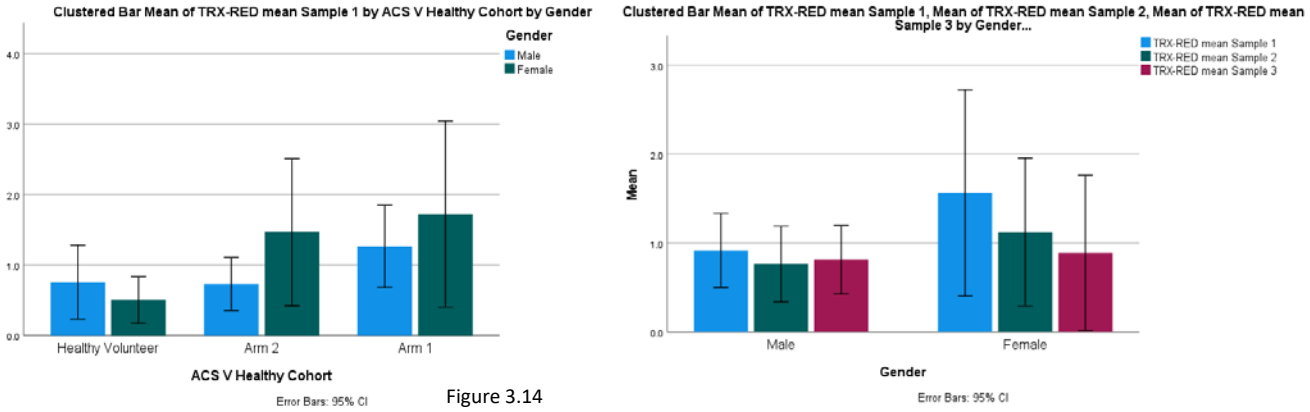


Figure 3.14

Figure 3.14. Gender stratified blood plasma TRXr levels between healthy and ACS participant cohorts. The data presented illustrate mean plasma TRXr levels between meals (light blue) and females (teal) for healthy volunteers and ACS Arm-1 and Arm-2 cohorts (left). The plot on the right shows the comparison between males and females at screening (sample 1), first follow-up (sample-2) and second follow-up (sample 3). Data presented are mean ± 95% CI, and analysed by two-way ANOVA Table 3.39 with Mauchly's test specificity for interaction p>0.05.

Table 3.39. Mauchly's Test of sphericity between gender, Healthy and ACS cohort.

Measure: Thioredoxin_Reductase

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b] Greenhouse-Geisser	Epsilon[b] Huynh-Feldt	Epsilon[b] Lower-bound
Time	.937	2.533	2	.282	.941	1.000	.500

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Table 3.39

A Mauchly's Test of Sphericity was next performed to confirm the TRXr ANOVA findings. This particular test evaluates sphericity in the data as appose to variance and is required to satisfy assumption #8 (Section 2.24.2.1). The results are presented in Table 3.39.

To evaluate the interaction between male vs female and healthy vs ACS, a Mauchly's multiple comparison 'within-subjects' test was performed, where individual participants are compared with themselves over time. Here the means were evaluated with respect to 'time' i.e., the point at which the blood sample was taken (sample-1, sample-2 and sample-3). The data presented in Table 3.40 illustrates that there was no statistically significant interaction between sample time and ACS Arm ($p>0.05$), indicating the plasma TRXr concentration depends on the point at which the blood sample was taken. The data presented in Figure 3.14 shows that the mean data for males and females is not parallel, which is driven by the change in plasma TRXr for females but did not reach significance. Table 3.40 shows that there was no significant main effect on blood sample time vs gender or blood sample time vs ACS Arm vs gender ($p>0.05$).

Table 3.40. Multiple comparisons of blood plasma sample TRXr levels within ACS cohorts.

Measure: Thioredoxin_Reductase

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Time	Sphericity Assumed	2.746	2	1.373	.702	.498	.017
	Greenhouse-Geisser	2.746	1.882	1.459	.702	.490	.017
	Huynh-Feldt	2.746	2.000	1.373	.702	.498	.017
	Lower-bound	2.746	1.000	2.746	.702	.407	.017
Time * ARM	Sphericity Assumed	1.673	2	.836	.428	.653	.011
	Greenhouse-Geisser	1.673	1.882	.889	.428	.641	.011
	Huynh-Feldt	1.673	2.000	.836	.428	.653	.011
	Lower-bound	1.673	1.000	1.673	.428	.517	.011
Time * Gender	Sphericity Assumed	1.538	2	.769	.394	.676	.010
	Greenhouse-Geisser	1.538	1.882	.818	.394	.663	.010
	Huynh-Feldt	1.538	2.000	.769	.394	.676	.010
	Lower-bound	1.538	1.000	1.538	.394	.534	.010
Time * ARM * Gender	Sphericity Assumed	9.875	2	4.937	2.526	.086	.059
	Greenhouse-Geisser	9.875	1.882	5.248	2.526	.090	.059
	Huynh-Feldt	9.875	2.000	4.937	2.526	.086	.059
	Lower-bound	9.875	1.000	9.875	2.526	.120	.059
Error(Time)	Sphericity Assumed	156.349	80	1.954			
	Greenhouse-Geisser	156.349	75.266	2.077			
	Huynh-Feldt	156.349	80.000	1.954			
	Lower-bound	156.349	40.000	3.909			

Table 3.40

Table 3.41. Multiple comparisons of blood plasma sample [TRXr] between Healthy and ACS cohorts and gender.

Dependent Variable: TRX-RED mean Sample 1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	20.242[a]	5	4.048	2.360	.043	.079
Intercept	126.508	1	126.508	73.743	<.001	.350
ARM	15.776	2	7.888	4.598	.012	.063
Gender	2.770	1	2.770	1.615	.206	.012
ARM * Gender	6.044	2	3.022	1.762	.176	.025
Error	235.028	137	1.716			
Total	373.270	143				
Corrected Total	255.270	142				

a. R Squared = .079 (Adjusted R Squared = .046)

Table 3.41

As a final analytical step, a Tukey's Honestly Significant Difference (HSD) test was performed for plasma TRXr concentration, which compares the ANOVA means based on the studentized data range. The data presented in Table 3.42 shows that there was statistically significant interaction between the Arm and gender, with respect to plasma TRXr concentration (p=0.05). The pairwise comparison illustrates that there is no significant difference between plasma TRXr concentration for healthy volunteer's vs ACS Arm-2 (Table 3.42, p>0.05).

Table 3.42. Tukey Multiple comparisons of blood plasma sample 1 TRXr levels between Healthy and ACS cohorts.

Dependent Variable: TRX-RED mean Sample 1

Tukey HSD

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Healthy Volunteer	Arm 2	-.305	.2609	.475	-.923	.314
	Arm 1	-.745*	.2737	.020	-1.393	-.096
Arm 2	Healthy Volunteer	.305	.2609	.475	-.314	.923
	Arm 1	-.440	.2984	.306	-1.147	.267
Arm 1	Healthy Volunteer	.745*	.2737	.020	.096	1.393
	Arm 2	.440	.2984	.306	-.267	1.147

Based on observed means.
The error term is Mean Square(Error) = 1.726.

*. The mean difference is significant at the .05 level.

The Tukey's multiple comparison between-subjects test (Table 3.42) shows that there was significant difference between plasma TRXr concentration and Arm-1 (p=0.020).

Data filed – Thesis Two-way TRX-RED.spv (Document1) Output & Thesis Two-way TRX RED.sav DataSet1) Data.

3.29 Thioredoxin Reductase (TRXr) Receiver Operator Curve (ROC).

Following the analysis presented above, which illustrates changes in the plasma biomarkers, thioredoxin-reductase (TRXr) for ACS participants a Receiver Operating Curve (ROC) analysis was next carried out. This was completed to ascertain the probability of event prediction, in this instance estimate whether an ACS event has occurred.

Sensitivity (true positives) for TRXr for blood taken at screening (sample-1) was correctly predicted in 78.2%. Specificity, percentage of all observed as Healthy Cohort (i.e., true negatives) correctively predicted as 61.5% (See Table 3.43). The efficiency of TRXr is calculated as (78.2%+61.5%) / (78.2%+61.5%+21.8%+38.5%) = 69.85%. In other words, blood plasma TRXr predicts a correct diagnosis 69.85% of the time.

Table 3.43 Percentage accuracy in classification TRXr biomarker.

Table 3.43

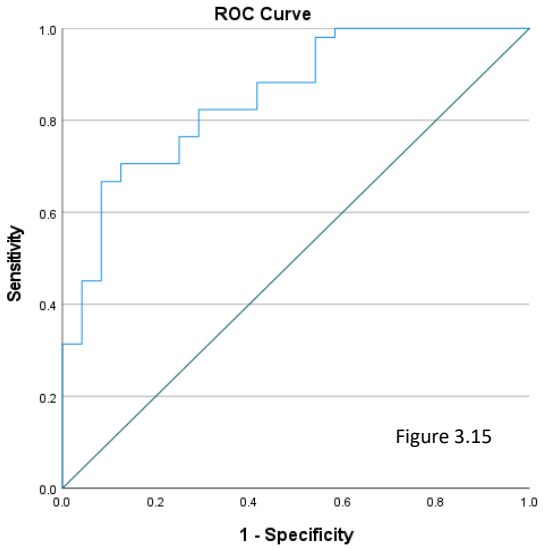
			Predicted		
			Presence of Heart Disease ALL/ACS		
	Observed		Healthy Cohort	ACS Cohort	Percentage Correct
Step 1	Presence of Heart Disease ALL/ACS	Healthy Cohort	40	25	61.5
		ACS Cohort	17	61	78.2
	Overall Percentage				70.6

a. The cut value is .500

The positive predictive value (percentage correctly predicted) for plasma TRXr concentration at screening, which relates to 'observed characteristics' compared to 'case predictive characteristics' is 100 x (61 ÷ (25 + 61)) = 70.1%. This means that 70.1% of ACS cases are correctly predicted by evaluating plasma TRXr concentration at screening. The negative predictive value, which relates to cases 'without the observed characteristics' compared to 'cases predicted not having the disease characteristic' is 100 x (40 ÷ (40 + 17)) = 70.1%. This means that 70.1% of non-ACS cases are correctly predicted by evaluating plasma TRXr concentration at screening.

Figure 3.15 illustrates the ROC curve was analysis for TRXr. Data included was plasma TRXr concentrations at screening (sample-1) for the ACS cohort (n=80) and the healthy cohort (n=65). The area under the curve was determined as 0.850 (95% CI = 0.761 to 0.940).

Table 3.44. Area under the curve analysis for blood plasma TRXr.



Area Under the Curve

Test Result Variable(s): Predicted probability TRX RED

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Lower	Asymptotic 95% Confidence Upper Bound
.850	.046	.000	.761	.940

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

Figure 3.15 and Table 3.44 Receiver operator curve (ROC) analysis for blood plasma TRXr. The data presented illustrate the clinical utility of blood plasma TRXr for the diagnosis of ACS. Blood plasma TRXr concentrations for the healthy donor cohort i.e., 'true negatives' (specificity) was plotted with the ACS cohorts i.e., 'true positives' (sensitivity). The area under the curve was determined as 0.850 with a 95% CI or 0.761 to 0.940. This indicates that blood plasma TRXr alone may predict a correct ACS diagnosis in 85% of cases

Data filed – ROC TRX RED Thesis.spv (Document1) Output and ROC All Biomarkers. Sav (DataSet1) Data-IBM SPSS Statistics Data

3.30 Kaplan-Meier.

The ROC analysis presented above demonstrates clinical utility for each of the plasma biomarkers for ACS diagnosis, as determined by area under the curve. This was substantiated by specificity, sensitivity, and efficiency calculations, which showed that TRX biomarker was able to predict a correct result i.e., determine a 'true positive' and 'true negative' in >85% of cases. Therefore, to take this analysis further, it was next important to evaluate whether [PRDX-2], could reliably predict ACS participant outcomes, in this case the time to event endpoint was an ACS readmission.

3.31 Logistic regression predictions.

Using binomial logistic regression to predict if cases can be correctly predicted from the independent variables, it was then analysed which independent variable contributed and its statistical significance.

The variables in the equation below show each independent variable and statistical significance. The odds ratio ("Exp B" column) was used to predict the probability of an event occurring. Odds Ratio of each independent variable recorded below in tables A, B, C, D along with the confidence Intervals, showing the change in log odds occurring for one-unit change in independent variable, keeping the other independent variables constant.

Table 3.45. Logistic regression predicting likelihood of ACS event based on age, BMI, gender and thioredoxin-reductase.

Table 3.45

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	Age	.082	.016	25.437	1	<.001	1.085	1.051	1.120
	BMI	.089	.053	2.884	1	.089	1.093	.986	1.212
	Gender (1)	.740	.432	2.927	1	.087	2.095	.898	4.889
	Thioredoxin Red	.243	.160	2.296	1	.130	1.275	.931	1.747
	Constant	-7.818	1.958	15.948	1	<.001	.000		

a. Variable(s) entered on step 1: Age, BMI, Gender, Thioredoxin-reductase mean.

The statistical significance in respect to TRXr found that Age ($p<0.001$) added significantly to the predictions model. However, BMI ($p=0.089$) and gender ($p=0.87$) did not. For TRXr, males had 2.09 (95% CI, 0.898 to 4.889) times higher odds to exhibit ACS than females (See Table 3.45).

Having satisfied the robustness of the plasma biomarkers TRXr a non-parametric Kaplan-Meier analysis was next performed to determine if these biomarkers impacted on participant prognosis and were able to predict the probability of an ACS readmission following stratification.

3.32 Kaplan-Meier all ACS participants thioredoxin-reductase (TRXr).

The ACS cohort (n=80) displayed events and censoring at sample-1 (screening blood sample) as displayed in Table 3.46. The sample-1 TRXr quartile means were subsequently calculated).

Table 3.46. Readmission analysis based on blood plasma TRXr ng/ml at less than 25%, median and greater than 75% percentile as the cut-off values.

Table 3.46

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 0.00 25% percentile	27	10	17	63.0%
Median >0.1 ~ < 1.9 (0.6)	30	10	20	66.7%
> 2.00 75% percentile	21	3	18	85.7%
Overall	78	23	55	70.5%

For blood plasma analysis at sample-1 the ACS participants (n=80) were stratified according to TRXr concentration. There were 27 participants in the <25% percentile (TRXr <0.00 ng/ml), however 17 were censored (63.0%). For the 25%-75% inter-percentile range (TRXr >0.00 ng/ml~<1.90 ng/ml), there were 30 participants, however 20 were censored (66.7%). Finally, there were 21 participants in the >75% percentile (TRXr >2.00 ng/ml), however 18 were censored (85.7%). Taken together, a total of 70% ACS participants were censored (Table 3.46). For sample-1, sample-2 and sample-3 there were no statistically significant findings in the readmission rate between the concentration of ACS stratified participants (Table 3.47).

Table 3.47. Multiple pairwise comparisons for blood plasma [TRXr] ng/ml cut off values in sample 2.

Pairwise Comparisons

Table 3.47	Intervention Sample 2 ACS 25% ~ 75% percentile	< 0.00 25% percentile		Median >0.1 ~ < 1.9(0.6)		> 2.00 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 0.00 25% percentile			1.129	.288	.813	.367
	Median >0.1 ~ < 1.9(0.6)	1.129	.288			2.724	.099
	> 2.00 75% percentile	.813	.367	2.724	.099		

Figure 3.16 illustrates the data presented in Table 3.47, highlighting higher concentrations of TRXr (>2.0 ng/ml) at sample-2 was associated with in increased median time to readmission, however there was no overall significant differences in readmissions due to a second ACS event at sample-2, in spite of the data trend observed.

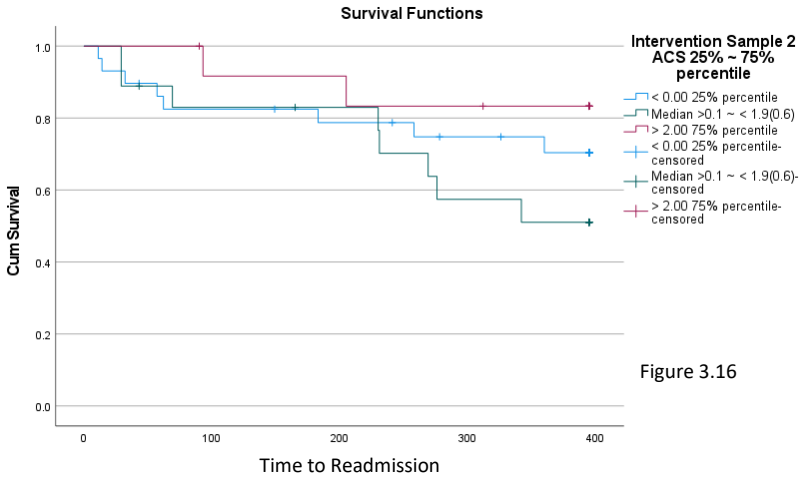


Figure 3.16

Figure 3.16. To determine whether blood plasma [TRXr] (ng/ml) could predict participant outcome (overall survival without ACS readmission), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRXr]. **1)** Blue line represents the 25% percentile, TRXr <0.00 ng/ml. **2)** Green line represents 25%-75% percentile, TRXr > 0.1 ~ <1.9 ng.ml. **3)** Red line represented the 75% percentile, TRXr >2.00 ng/ml. χ^2 analysis p=>0.05.

Data filed - ALL KM Sample1-3 TRX-RED.SAV [DataSET2] and ALL KM Sample1-3 TRX-RED.SPV [Document5] IBM SPSS Statistics Output.

3.36 Kaplan-Meier for plasma thioredoxin-reductase (TRXr) concentration percentiles.

To address the aim and objectives TRXr plasma concentrations were then analysed to determine if indicative of an ACS event and/or predict a second event, the Kaplan-Meier analysis was systematically performed for TRXr. Initially, this was performed for the 'blood

sample-1' TRXr percentile concentrations. To recap, these percentile concentrations were calculated as <0.00 ng/ml for the <25% percentile (n=12), >0.1 ng/ml~<1.9 ng/ml for the inter-percentile range (n=12) and >2.00 ng/ml for the >75% percentile (n=7). Summarised data are presented in Table 3.48.

Table 3.48. Readmission analysis based on percentage of admissions and censored cases of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [TRXr] ng/ml as the cut-off values.

Table 3.48

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 0.00 25% percentile	12	11	1	8.3%
Median >0.1 ~ < 1.9 (0.6)	12	9	3	25.0%
> 2.00 75% percentile	10	3	7	70.0%
Overall	34	23	11	32.4%

The participants in the inter-percentile range for plasma TRXr concentration had the lowest time to readmission of 57 days (95% CI, 0.0 to 146.5 days). The time to readmission values for the <25% and median plasma TRXr concentration participants were much similar at 57 and 69 days respectively, see Table 3.48. The confidence interval was not able for the >75% group due to n=10, where participants censored was n=7. See Table 3.49 for full summary.

Table 3.49. Readmission analysis in days for baseline blood plasma TRX ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.49

Intervention Sample 1 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 0.00 25% percentile	159.033	46.145	68.589	249.478	57.000	45.663	.000	146.500
Median >0.1 ~ < 1.9 (0.6)	107.083	27.285	53.605	160.561	69.000	9.899	49.597	88.403
> 2.00 75% percentile	385.250	44.668	297.701	472.799	.	.	.	.
Overall	199.617	29.123	142.535	256.699	205.000	105.145	.000	411.083

a. Estimation is limited to the largest survival time if it is censored.

Assumption #4 (Section 2.24.4.1) states that for time to event statistics, there is similar censoring. The percentage of censored cases present in the <25 % percentile was 8.3%, compared to 25.0% and 70.0 % for the inter-percentile and >75% percentile groups. Based on this, it is clear that the censoring of the groups was not similar.

Figure 3.17 provides a visual representation of the data presented in Table 3.49. The plot shows a small interaction i.e., crossing of survival curves with low and median (<0.0 ~ >0.1 ng/ml) plasma concentrations respectively. However, in general those, participants with higher plasma TRXr concentrations (>2.00 ng/ml) have a reduced incidence of readmission than those with low or inter-percentile concentrations.

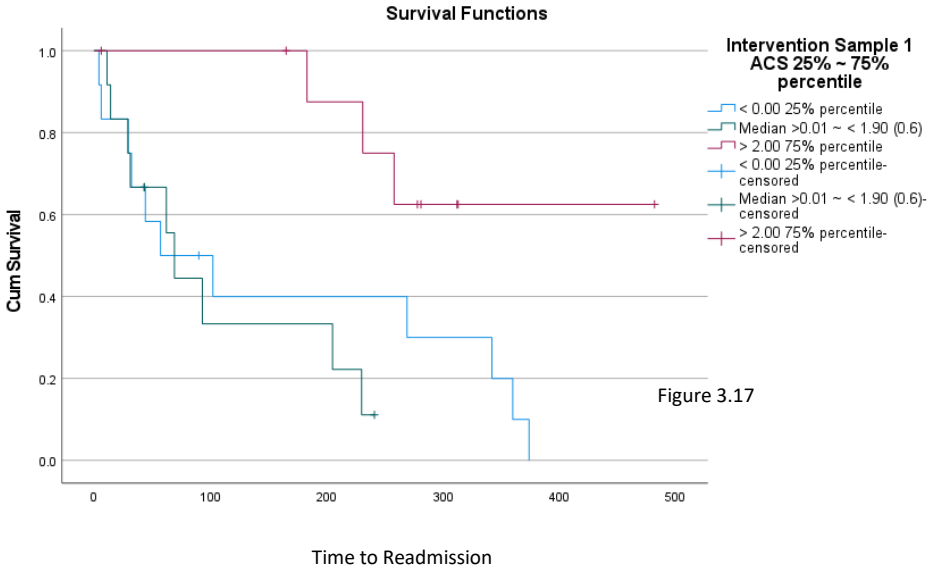


Figure 3.17

Figure 3.17. Readmission analysis using various blood plasma TRXr ng/ml cut-off values of baseline Sample 1. To determine whether blood plasma TRXr (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRXr]. **1)** Blue line represents the 25% percentile, TRXr <0.00 ng/ml. **2)** Green line represents 25%-75% percentile, TRXr > 0.1 ~ <1.9 ng.ml. **3)** Red line represented the 75% percentile, TRXr >2.00 ng/ml. χ^2 analysis between 3 healthy and ACS cohort concentrations = 9.31, p=0.010.

To follow-on from this, a log rank test was conducted, which showed that there were statistical differences in the admission rate for the three plasma TRXr concentrations, χ2(2) = 9.312, p = 0.010. (Table 3.50).

Table 3.50. Overall comparison of readmission distribution between Arm-1 and Arm-2.

Table 3.50

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	9.312	2	.010

Test of equality of survival distributions for the different levels of healthy and ACS cohort Sample-1 ACS 25%~75% percentile.

The data summarised in Table 3.51 shows that there was significant difference in the admission distributions for the low and high concentrations of TRXr χ2(1) =5.263, p=0.022 and

between the >75% ~ <25% inter-percentile range, χ2(1) =10.018, p=0.002.

Table 3.51 The survival distributions for the three healthy and ACS cohort for sample 1 blood plasma TRXr ng/ml levels.

Table 3.51

	Intervention Sample 1 ACS 25% ~ 75% percentile	< 0.00 25% percentile		Median >0.1 ~ < 1.9 (0.6)		> 2.00 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 0.00 25% percentile			.585	.444	5.263	.022
	Median >0.1 ~ < 1.9 (0.6)	.585	.444			10.018	.002
	> 2.00 75% percentile	5.263	.022	10.018	.002		

Next the same analysis was conducted on plasma [TRXr] for blood sample-2 (first follow-up). The data presented in Table 3.52 shows the percentage of censored cases present in the <25% percentile (38.5% ACS participants), the inter-percentile range (20.0% ACS participants) and >75% percentile (50.0% ACS participants). These data illustrate that the proportion of censored groups was not similar.

Table 3.52. Readmission analysis at less than 25%, median and greater than 75% percentile of blood plasma [TRXr] ng/ml as the cut-off values at sample 2.

Table 3.52

Intervention Sample 2 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 0.00 25% percentile	13	8	5	38.5%
Median >0.1 ~ < 1.9(0.6)	10	8	2	20.0%
> 2.00 75% percentile	4	2	2	50.0%
Overall	27	18	9	33.3%

Participants with the inter-percentile plasma TRXr concentration had a median time to readmission of 231.0 days (95% CI, 27.7 to 434.3). The <25% plasma TRXr concentration group had a median readmission time of 183.0 days (95% CI, 0.00 to 456.7) days compared to >75% TRXr plasma concentration group which was 205 days (95% CI, 25.7 to 384.2) days. Table 3.53 for full summary.

Table 3.53. Readmission analysis in days for baseline blood plasma TRXr ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.53

Intervention Sample 2 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 0.00 25% percentile	211.305	53.947	105.569	317.041	183.000	139.635	.000	456.684
Median >0.1 ~ < 1.9(0.6)	211.994	44.143	125.475	298.514	231.000	103.708	27.732	434.268
> 2.00 75% percentile	203.333	51.623	102.152	304.515	205.000	91.448	25.763	384.237
Overall	215.428	31.364	153.954	276.901	230.000	34.637	162.111	297.889

a. Estimation is limited to the largest survival time if it is censored.

Figure 3.18 provides a visual representation of this data in Table 3.53, highlighting that the <25% plasma [TRXr] group display a reduction in ACS readmission time, compared with those in the inter-percentile range.

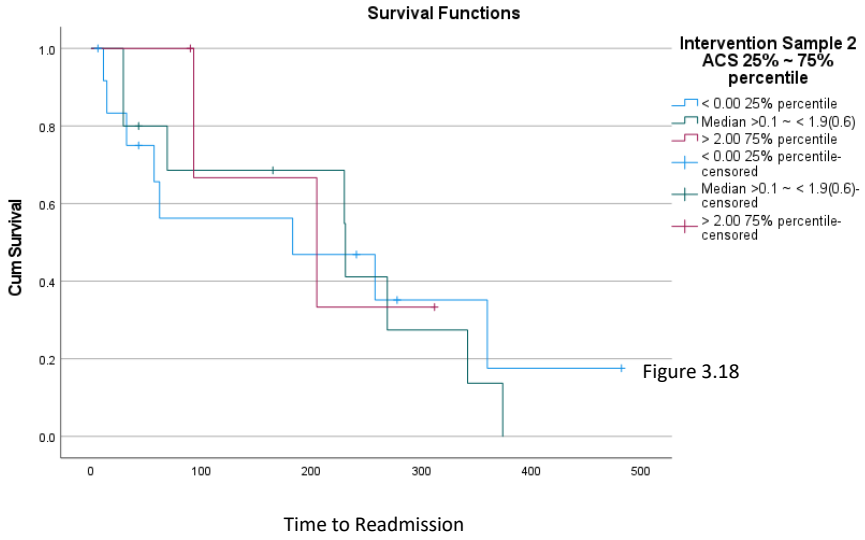


Figure 3.18

Figure 3.18. Readmission analysis using various blood plasma [TRXr] ng/ml cut-off values of baseline sample 2. To determine whether blood plasma TRXr (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRXr]. **1)** Blue line represents the 25% percentile, TRXr <0.00 ng/ml. **2)** Green line represents 25%-75% percentile, TRXr > 0.1 ~ <1.9 ng.ml. **3)** Red line represented the 75% percentile, TRXr >2.00 ng/ml. χ^2 analysis, p=>0.05.

The log rank (Table 3.54) test did not determine any significant differences in the readmission rates for the three plasma TRXr concentrations $\chi 2(2) = .159$, p=0.923.

Table 3.54. Overall comparison of readmission distribution between Arm-1 and Arm-2.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.159	2	.923

Test of equality of survival distributions for the different levels of Sample-2 ACS 25% ~ 75% percentile.

Finally, analysis was performed for plasma [TRXr], based on blood collected at Sample-3 (2nd follow-up). The percent of censored ACS cases present in the <25 % plasma TRXr concentration (37%), compared with the inter-percentile range (50.0%) and >75 % (20.0%). Thus, the censored groups were not similar (Table 3.55).

Table 3.55. Readmission analysis at less than 25%, median and greater than 75% percentile of blood plasma [TRXr] ng/ml as the cut-off values at sample 3.

Table 3.55

Intervention Sample 3 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 0.00 25% percentile	8	5	3	37.5%
Median >0.1 ~ < 1.9 (0.6)	6	3	3	50.0%
> 2.00 75% percentile	5	4	1	20.0%
Overall	19	12	7	36.8%

Participants with the inter-percentile plasma TRXr concentration had a median time to readmission of 230 days (95% CI, 96.4 to 363.6) days. The <25% plasma TRXr concentration group had a median readmission time of 258 days (95% CI,12.5 to 503) days compared to >75% TRXr plasma concentration group which was 269 days.

Table 3.56. Readmission analysis in days for baseline blood plasma TRXr ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.56

Intervention Sample 3 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 0.00 25% percentile	243.821	63.806	118.762	368.881	258.000	125.250	12.509	503.491
Median >0.1 ~ < 1.9 (0.6)	207.133	39.500	129.714	284.553	230.000	68.148	96.429	363.571
> 2.00 75% percentile	207.700	65.230	79.850	335.550	269.000	140.065	.000	543.527
Overall	228.576	36.637	156.768	300.384	231.000	42.997	146.725	315.275

a. Estimation is limited to the largest survival time if it is censored.

Figure 3.19 provides a visual representation of this data, highlighting that the <25% plasma TRX group display a reduction in ACS readmission time, compared with those in the inter-percentile range.

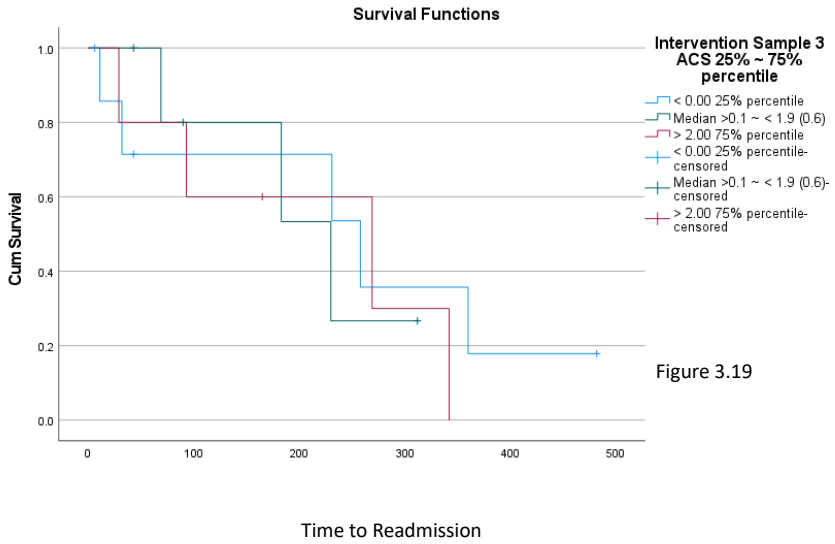


Figure 3.19

Figure 3.19. Readmission analysis using various blood plasma [TRXr] ng/ml cut-off values of baseline sample 3. To determine whether blood plasma TRXr (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [TRXr]. **1)** Blue line represents the 25% percentile, TRXr <0.00 ng/ml. **2)** Green line represents 25%-75% percentile, TRXr > 0.1 ~ <1.9 ng.ml. **3)** Red line represented the 75% percentile, TRXr >2.00 ng/ml. χ^2 analysis, p=>0.05.

The log rank pairwise comparison between each of the plasma TRXr concentration groups did not show significant difference in readmissions (p>0.05).

Data filed-KMSsample123TRX-red SC.spv [document 10] output KM Sample1-3 TRX-RED.sav. [DataSet2]data

3.37 Kaplan-Meier for lesions of Percutaneous Coronary Intervention.

The final set of analyses was to establish whether the [TRXr] plasma biomarkers had any predictive value for ACS readmission with respect to ACS percutaneous coronary intervention (PCI) i.e., Right Coronary Artery (RCA), circumflex or Left Anterior Descending (LAD). ACS participants for blood sample-1 were subsequently stratified according to lesion of PCI as outlined in Table 3.57.

Table 3.57. Readmission analysis based on lesion of ACS event that resulted in baseline PCI

Table 3.57

Lesion of PCI	Total N	N of Events	Censored	
			N	Percent
RCA	39	13	26	66.7%
Circumflex	11	3	8	72.7%
LAD	30	8	22	73.3%
Overall	80	24	56	70.0%

Overall (n=80) ACS participants were included in this analysis, of these participants the PCI event is broken down as follows: RCA (n=39), Circumflex (n=11) and LAD artery (n=30), see Table 3.57.

[TRXr] was reviewed with respect to the lesion of PCI event. As previously described, participants were further stratified according to plasma biomarker concentration i.e., <25%, inter-percentile range (>25% ~ <75%) and >75%. As previously stated, the design was non-event driven and all participant survival status was known at end of study. This limited the events of interest to (n=35) of all ACS admissions.

3.36 Thioredoxin-reductase – Kaplan-Meier readmission relating to Acute Myocardial Infarction lesion.

Initially, the impact of PCI lesion with respect to plasma TRXr concentration was evaluated. This related to n=17 participants who received a PCI to RCA, n=5 for circumflex and n=12 for LAD. The full breakdown is presented in Table 3.58

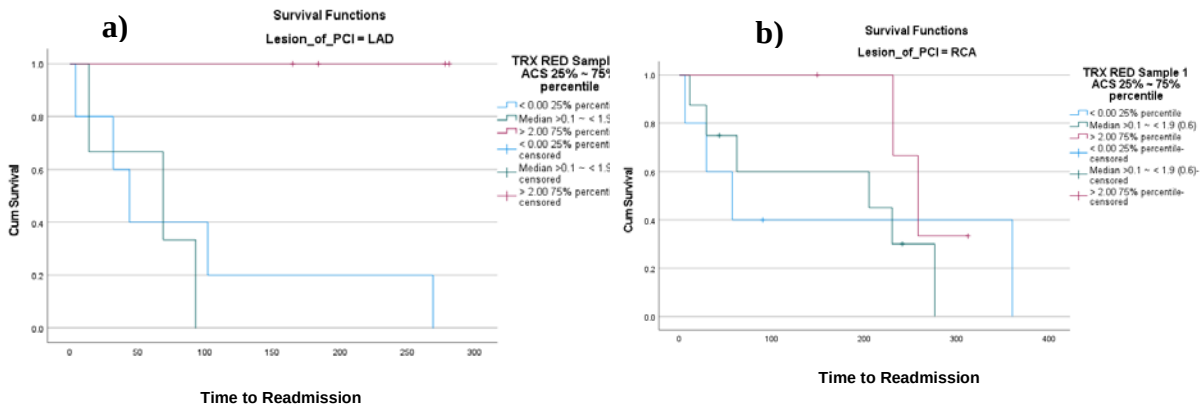
Table 3.58. ACS Readmission analysis based on lesion of PCI at baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [TRXr] ng/ml cut-off values.

Table 3.58

Lesion of PCI	TRX RED Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored N	Censored Percent
RCA	< 0.00 25% percentile	5	4	1	20.0%
	Median >0.1 ~ < 1.9 (0.6)	8	6	2	25.0%
	> 2.00 75% percentile	4	2	2	50.0%
	Overall	17	12	5	29.4%
Circumflex	< 0.00 25% percentile	1	1	0	0.0%
	Median >0.1 ~ < 1.9 (0.6)	2	1	1	50.0%
	> 2.00 75% percentile	2	1	1	50.0%
	Overall	5	3	2	40.0%
LAD	< 0.00 25% percentile	5	5	0	0.0%
	Median >0.1 ~ < 1.9 (0.6)	3	3	0	0.0%
	> 2.00 75% percentile	4	0	4	100.0%
	Overall	12	8	4	33.3%
Overall	Overall	34	23	11	32.4%

The confidence interval was not computed due the unequal censoring percentage, so means and medians times were not computed.

The data presented in Figure 3.20 gives a visual representation of PCI lesion with respect to stratified plasma TRXr concentrations. As shown in Figure 3.20, whilst there were some interesting trends in the time to readmission based on stratified TRXr concentration and lesion of PCI.



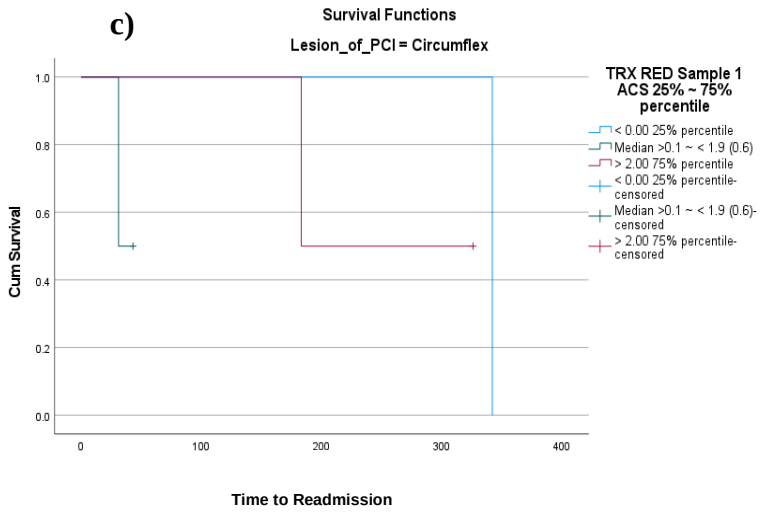


Figure 3.20. ACS Readmission analysis based on lesion of PCI of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [TRXr] ng/ml cut-off values. a) Represents PCI to LAD, **b)** represents PCI to RCA and **c)** represents circumflex PCI. [TRXr]. Blue line represents the 25% percentile, TRXr <0.00 ng/ml. Green line represents 25%-75% percentile, TRXr > 0.1 ~ <1.9 ng.ml. Red line represented the 75% percentile, TRXr >2.00 ng/ml.χ^2 analysis between 3 healthy and ACS cohort groups concentrations = 4.78, p=0.02. χ^2 analysis for 25% percentile vs 75% percentile = 4.64, p=0.031 and χ^2 analysis for median vs 75% percentile = 6.87, p=0.09.

The Log rank pairwise between the three stratified TRXr concentrations and lesion PCI did show statistical significance χ2(2) = 4.787, p=0.029 (Table 3.59).

Table 3.59. Overall Comparison of readmission distribution between lesion of PCI of baseline.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	4.787	1	.029

Table 3.59

The vector of trend weights is -1, 0, 1. This is the default.[a]

a. Adjusted for Lesion of PCI .

Log rank pairwise between the three stratified TRXr concentrations and lesions of PCI was conducted, which showed statistically significant TRXr plasma concentrations of TRXr >75 and <25% percentile concentrations, χ2(1) =4.645, p=0.031. TRXr plasma concentrations were also significant between inter-percentile ranges and > 75% TRXr concentrations χ2(1) =6.873, p=0.009 (Table 3.60).

Table 3.60 The readmission distribution between lesion of PCI of baseline bloods healthy and ACS cohort groups, blood plasma TRXr ng/ml levels.

Table 3.60	TRX RED Sample 1 ACS 25% ~ 75% percentile	< 0.00 25% percentile		Median >0.1 ~ < 1.9 (0.6)		> 2.00 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 0.00 25% percentile			.293	.588	4.645	.031
	Median >0.1 ~ < 1.9 (0.6)	.293	.588			6.873	.009
	> 2.00 75% percentile	4.645	.031	6.873	.009		

a. Adjusted for Lesion of PCI .

3.39 Brief discussion of thioredoxin-reductase (TRXr) findings.

The data presented in this chapter showed a significant overall increase in mean plasma TRXr concentration for ACS participants (1.14 ng/ml) compared to the healthy cohort (0.63 ng/ml), see Figure 3.11 (p=0.0008). To the best of knowledge at time of writing, this is the first study of its kind to report this, illustrating those changes in plasma [TRXr] correlate with an ACS event. Interestingly, a recent study examining plasma TRXr in the context of non-small cell lung carcinoma (NSCLC), demonstrated an increase in patient plasma TRXr enzymatic activity (15.66 U/L) compared with health donor controls (2.05 U/L) (Ye *et al.,* 2019). Whilst the units of measurement differ from the results presented herein, there is nonetheless an upwards trend demonstrated in the NSCLC patients, a disease which is characterized by oxidative stress (Ilonen *et al*., 2008; Zhao *et al*., 2020).

Following censoring, the healthy cohort population without any underlying health conditions had a mean [TRXr] as 0.78 ng/ml. This concentration may therefore be a good starting point as a predictor for an upper limit of normal (ULN). Unlike TRX, where a significant difference was identified between male and female ACS patients (chapter 3a), this was not the case for TRXr. However, there was an identifiable trend, where male plasma TRXr is around 2-fold lower than that of females at screening (Figure 3.14). As discussed later, this trend may be important, where the data presented herein demonstrates that higher plasma [TRXr] is associated with a better patient outcome (Figures 3.17 and 3.20). However, as recent research indicates in a study evaluating 362 male and 167 female AMI patients, there appears to be no significant overall improvement in patient outcome between males and females (Wilkosz *et al*., 2021).

Like TRX (chapter 3a), age had an impact on plasma [TRXr]. For the healthy cohort where

inclusion numbers were relatively matched, the under 55's (n=36) had a mean plasma TRXr concentration of 0.41 ng/ml compared to over 55's (n=29) who had a mean concentration of 0.90 ng/ml, an observation which again may be explained by an increase in age associated oxidative stress (Tan *et al.*, 2018). For the ACS cohort the numbers included were less equal, but a similar trend of levels was observed for the 'under 55' (1.20 ng/ml for Arm-1 vs 1.10 ng/ml for Arm-2), compared with 1.41 ng/ml vs Arm-2 0.88 ng/ml for the over 55's respectively. Interestingly, the statistical analysis showed that plasma TRXr values remained slightly higher for ACS Arm non-smokers, compared with healthy volunteer non-smokers ($p<0.05$). This finding may be explained by an *in vitro* study which demonstrated that cigarette smoke inhibited TRXr activity (Zhang *et al.*, 2016). Therefore, 'smokers' may have less TRXr activity, which in turn may have a negative impact on the overall antioxidant capacity of the cell. This further supports the data presented in Figure 3.17, which associates higher TRXr levels with a positive patient outcome. Although the number of non-smokers who suffered an ACS was low, the data show that non-smokers presenting with ACS had significantly higher levels of plasma TRXr compared with non-smokers healthy volunteers, with no other medical history. Interestingly, smokers who present with an ACS have an increased risk of readmission within 1 year following the primary event (Sia et al., 2021), which may 'in part' be due to the reduction in TRXr activity.

The ROC analysis evaluated plasma TRXr concentrations at baseline screening for the ACS cohort (n=80) and the healthy cohort (n=65) and had an area under the curve determined as 85%. Like TRX, this finding provides confidence for the clinical utility of TRXr on the basis that that plasma [TRXr] would correctly predict an ACS event in >4 in 5 cases (Figure 3.15). Combining the Arm-1 and Arm-2 ACS data, the primary endpoint (readmissions) occurred in (n=24) 30% of participants. Where a second ACS was the cause, this was observed in (n=24) 30% of all readmissions. The data show that the mean base line TRXr concentration was 0.81 ng/ml. However, the mean plasma TRXr concentration for all ACS participants readmitted with a second ACS event was 0.77 ng/ml, compared with 1.20 ng/ml at second follow-up (n=9).

To further investigate the impact of plasma [TRXr] on ACS readmission rates, Kaplan-Meier analysis was conducted to determine probability of 'time-to-readmission', based on biomarker stratification. Initial admissions were recorded in days from PCI (Appendix AA) and any admission that was not due to an ACS admission was censored. The ACS participants (n=80) were thus stratified according to TRXr concentration at this point. There were 27 participants

in the <25% percentile (TRXr <0.00 ng/ml), however 17 were censored (63.0%) for non-ACS readmissions. For the 25%-75% median range (TRXr >0.00 ng/ml~<1.90 ng/ml), there were 30 participants, however 20 were censored (66.7%). Finally, there were 21 participants in the >75% percentile (TRXr >2.00 ng/ml), however 18 were censored (85.7%). The data show that ACS patients within the <25% percentile at baseline had an increased risk of readmission (Figure 3.17). The data presented in Figure 3.20 show that this observation is most apparent for patients presented with an AMI to LAD. Taken together, these data show that plasma [TRXr] <1.90 ng/ml at diagnosis is associated with an increased risk of readmission, particularly for LAD PCI patient. As outlined in chapter 1 (Section 1.6), TRXr is the final enzyme in this particular antioxidant metabolic pathway, as depicted in scheme 1:

Scheme 1:

H_2O_2 → PRDX → TRX → **TRXr**

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electron flow

Therefore, it might be speculated that that, if TRXr concentration is high in cardiac myocytes, there is an enhanced reductive capacity for the removal of ROS, thus minimizing the impact of oxidative stress which may benefit myocyte healing. This may in turn impact positively on readmission rates, particularly for LAD PCI patients. Given the negative prognosis associated with AMI due to LAD (Dadjoo *et al*., 2013), evaluating plasma [TRXr] at ACS screening may therefore be clinically relevant for risk stratifying patients at point of diagnosis.

In conclusion, the data presented in this chapter highlights the differences between plasma [TRXr] for healthy individuals and ACS patients. Smokers were shown to have reduced levels of TRXr, which may be due to the impact of cigarette smoking on TRXr activity. Importantly, plasma [TRXr] levels could reliably predict a correct diagnosis in ~85% cases. Moreover, a low plasma TRXr concentration at baseline is associated with an increased risk of ACS readmission, particularly for those patients presenting with AMI to LAD. Therefore, evaluating TRXr at diagnosis may provide clinical value for risk stratifying patients.

Chapter 3c – Peroxiredoxin-2 (PRDX-2)

3.40 Brief introduction to plasma peroxiredoxin-2 (PRDX-2) analysis.

Previous analyses (chapter 3a and 3b) highlighted a role for TRX and TRXr in predicting ACS patient outcome. The data presented here seeks to evaluate PRDX-2 in this way. Reactive oxygen species specifically hydrogen peroxide (H_2O_2) is generated during cardiac ischaemia, which causes damage to the myocardium (Bae et al., 2016; Slezak *et al.*, 1995). PRDX-2 belongs to a large family of cysteine dependent antioxidant enzymes, that catalyse the removal of H_2O_2 in cells (Perkins *et al*., 2015). Studies have indicated that PRDX-2 (along with TRX) may be released by cells under oxidative stress, which in turn may modify cell receptors enhancing the inflammatory response, which is an important feature of ACS pathogenesis (Salzano *et al.,* 2014; Ong *et al.*, 2018). Thus, the following analysis as described in this chapter was conducted to determine if there are any changes in plasma [PRDX-2] between a healthy cohort of participants and those following an AMI / during an ACS event. As described in chapter 2, the ACS patients and healthy cohort were recruited at WAHT. Participant plasma samples were subsequently evaluated using an optimised ELISA for PRDX-2 (Section 2.23., Table 2.2 and Appendix Y). The data collected was subsequently analysed using various statistical methods as described in chapter 2. This chapter presents the results of this analysis is a logical order to ultimately evaluate the clinical utility of [PRDX-2] in the context of ACS and establish whether [PRDX-2] could reliably predict ACS patient outcome. In this case the time to event endpoint was an ACS readmission.

Objectives:

a) To clarify the mean plasma concentrations for PRDX-2 for healthy volunteers, stratified based on sex and age., which will be used as baseline measurements for ACS comparison.
b) To evaluate the plasma concentrations levels of PRDX-2 for ACS patients stratified based on age and sex at initial diagnosis / screening and follow-up. Clinical utility may subsequently be evaluated.
c) Monitor the concentration level of PRDX-2 through ACS patient follow-up sampling,

in order to evaluate whether this biomarker may be predictive of an ACS readmission.

d) Evaluate whether [PRDX-2] may predict readmission based on ACS patient stratified according to PCI.

3.41 Basic description statistics of PRDX-2.

To determine the mean plasma [PRDX-2] for the 'healthy cohort, removal of healthy volunteers with a medical history of diabetes mellitus (n=5), hypertension (n=10), family history of cardiovascular conditions (n=19), or inflammatory disorders (n=1) was carried out. This equated to Healthy Volunteers with no medical conditions (n=37). This population had a slightly higher mean plasma PRDX-2 concentration of 26.18 ng/ml ± 9.80 ng/ml, compared with the healthy volunteers as a whole.

Stratification of the cohort into Arm-1 (n=35) and Arm-2 (n=44) revealed plasma PRDX-2 concentration levels of 25.13 ng/ml ± 8.63 ng/ml and 24.7 ng/ml ± 8.86 ng/ml respectively. Next the 'smokers' were removed from the healthy cohort analyses, which resulted in little change in mean plasma PRDX-2 concentrations 27.20 ng/ml ± 9.92 ng/ml (n=26). The same convention was applied to the ACS participants, which resulted in mean plasma PRDX-2 concentrations of 25.76 ng/ml ± 4.61 ng/ml for Arm-1 (n=5) and 28.39 ng/ml ± 12.49 ng/ml for Arm-2 (n=8).

The data show that, non-smokers presenting with ACS had higher mean levels of plasma [PRDX-2] compared with non-smokers healthy volunteers, with no other medical history. Taking everything together, the mean PRDX-2 for all healthy volunteers 24.98 ng/ml ± 1.26 ng/ml, compared with all ACS admissions 24.92 ng/ml ± 1.39 ng/ml (Figure 3.21).

Overall, males in the healthy cohort (n= 32) had a mean plasma PRDX-2 concentration of 26.65 ng/ml ± 8.75 ng/ml, compared with females (n=33) 23.42 ng/ml ± 8.71 ng/ml.

The plasma PRDX-2 concentration for Healthy Volunteers (HV) was (n= 64) 24.98 ng/ml ± 1.26 ng/ml (p=<0.0032) compared to 24.92 ng/ml ± 1.39 ng/ml (p=0.0513) for all ACS participants. Data was not statistically significant on a Mann-Whitney U test. (p=<0.4355) Figure 3.21 and Table 3.61.

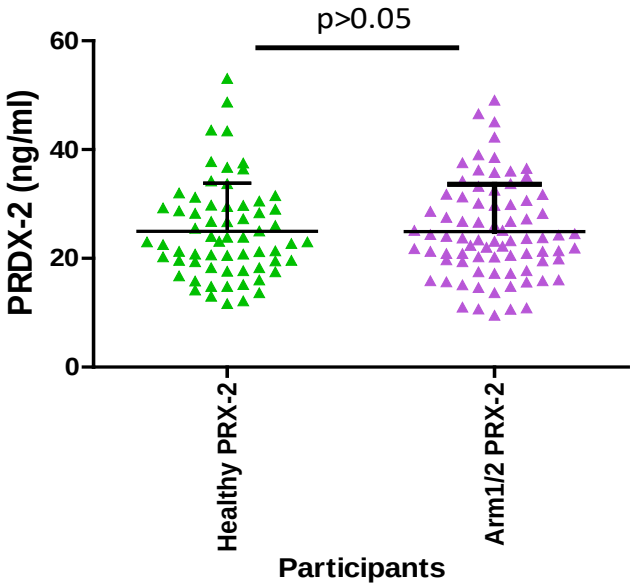


Figure 3.21 Overall blood plasma [PRDX-2] levels between healthy and ACS participant cohorts. The data presented illustrate the spread of data between all healthy (n=64) and all ACS (n=79) plasma PRDX-2 levels (ng/ml). For healthy the mean PRDX-2 concentration was 24.9±8.8 ng/ml. For ACS the mean PRDX-2 concentration was 24.9±8.7 ng/ml. A Mann-Whitney U test confirmed an over a significant difference in mean, p>0.05.

Table 3.61. Healthy cohort and ACS patient PRDX-2 (ng/ml) descriptive statistics.

		PRDX-2		
ACS V Healthy Cohort	Sex	Mean	Std. Deviation	N
Healthy Volunteer	Male	26.652	8.7566	31
	Female	23.418	8.7123	33
	Total	24.984	8.8159	64
ACS Arm-1 and Arm-2	Male	25.302	9.3447	58
	Female	23.852	6.7249	21
	Total	24.916	8.7077	79
Total	Male	25.772	9.1170	89
	Female	23.587	7.9335	54
	Total	24.947	8.7254	143

3.42 Basic PRDX-2 analysis for ACS Arm-1.

It was next important to evaluate the plasma PRDX-2 levels between males and female ACS participants. For the Arm-1 ACS participants, males (n=26) had a mean plasma PRDX-2 concentration of 25.71 ng/ml ± 9.68 ng/ml, compared to females (n=9) PRDX-2

23.48 ng/ml ± 4.43 ng/ml. The effects of smoking status were next evaluated for Arm-1 ACS, where non- smokers or ex-smokes ≥12 months (n=26) had a mean plasma PRDX-2 concentration of 24.82 ng/ml ± 8.56 ng/ml compared to smokers/vapers (n=9) 26.01 ng/ml ± 9.25 ng/ml (p>0.05). To recap, for Arm-1 ACS the primary endpoint (readmission) occurred in (n=15) 41.6%, of which ACS admission (n=10) 27.7% was the cause. The mean plasma PRDX-2 concentrations at readmission for Arm-1 ACS participants due to a second ACS event was 30.60 ng/ml ± 12.27 ng/ml (p>0.05) when compared to primary admission (n=10).

3.43 Basic PRDX-2 analysis for ACS Arm-2.

The same analysis was carried out for ACS Arm-2 participants (n=44), who had their ACS event within 24 hours of their hs-cTn result. Here the male cohort (n=32) had a mean plasma PRDX-2 of mean 24.97 ng/ml ± 9.20 ng/ml compared to Females (n=12) 24.13 ng/ml ± 8.22 ng/ml (p>0.05). As with Arm-1, the Arm-2 participants were stratified according to smoking status. For the non-smokers or ex-smokers ≥12 months (n=29), the mean plasma PRDX-2 was 24.94 ng/ml ± 9.01 ng/ml compared to smokers/vapers (n=15), who had a PRDX- 2 mean of 24.36 ng/ml ± 8.85 ng/ml.

To reiterate, for the Arm-2 ACS participants the primary endpoint (readmission) occurred in (n=20) 45.4%, of which readmission due to a second ACS accounted for (n= 14) 31.8% cases. The mean plasma PRDX-2 upon readmission was 25.59 ng/ml ± 6.61ng/ml, compared with the first and second follow-up samples, which were 21.56 ng/ml ± 11.59 ng/ml and 30.46 ng/ml ± 8.38 ng/ml respectively (p>0.05).

Combining the Arm-1 and Arm-2 ACS data, readmissions that were readmitted with a second ACS event occurred in 30% (n=23) of participants. For these participants, the mean plasma PRDX-2 concentration at baseline was 27.31 ng/ml ± 9.65 ng/ml, compared with 26.07 ng/ml ± 11.98 ng/ml (n=18) and 27.65 ng/ml ± 9.85 ng/ml (n=13) at first and second follow- up respectively. See table 3.62 for full summary.

Table 3.62 Descriptive statistics [PRDX-2] (ng/ml) biomarkers for readmission rates.

ACS Readmission		PRDX- (ng/ml	SD	Non-ACS Readmission	PRDX- (ng/ml)	SD	Significance
Baseline	(n=23)	**27.31**	9.65	(n=11)	**24.14**	8.34	
Follow-up 1	(n=18)	**26.07**	11.98	(n=9)	**20.32**	8.62	0.0072
Follow-up 2	(n=13)	**27.65**	23.04	(n=7)	**23.04**	6.51	0.5804

3.44 Age comparisons at screening [PDXD-2] for all participant groups.

Healthy volunteers at time of screening under 55 (n=35) had a mean plasma PRDX-2 concentration of 24.11 ng/ml ± 8.99 ng/ml (n=29), compared with the over 55 mean of 26.03 ng/ml ± 8.63 ng/ml. For Arm-1 ACS participants, the mean plasma PRDX-2 concentration was 22.94 ng/ml ± 7.16 ng/ml for under 55 (n=5), compared with over 55, which were 25.50 ng/ml ± 8.90ng/ml (n=30). For Arm-2 ACS participants, the under 55's (n=11) had a mean plasma PRDX-2 concentration of 23.15 ng/ml ± 7.42 ng/ml (n=33), compared with over 55's (n=33) which had a mean of 25.27 ng/ml ± 9.33 ng/ml (p>0.05), Figure 3.22.

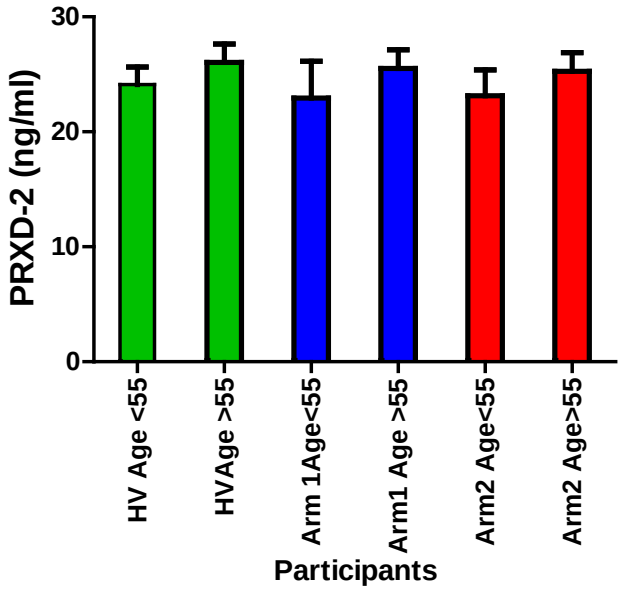


Figure 3.22 Age stratified blood plasma PRDX-2 levels between healthy and ACS participant cohorts. The data presented illustrate blood plasma PRDX-2 levels (ng/ml) following age stratification; <55 years or >55 years for a) healthy in green (<55 years, n=35, >55 years n=29), b) ACS Arm-1 in blue (<55 years n=5, >55 n=30) and c) ACS Arm-2 in red (>55 years n=11, >55 years n=33).

3.45 Two-way mixed ANOVA.

To follow on from the descriptive statistical analysis presented above, it was next important to evaluate the impact of sex (male / female) with respect to the plasma concentrations of PRDX-2. Since the sample population cohorts included a healthy population, along with ACS Arm-1 and Arm-2, a two-way mixed ANOVA was selected, as this would determine interaction between participant 'sex' and population cohort.

3.46 Assessment of outliers for peroxiredoxin-2 (PRDX-2).

For Sample-1 (Baseline) for the healthy cohort (n=65), the Boxplot data presented in Figure 3.23a indicated that there was no extreme outlier and four outliers (data points 5, 17, 97 and 134).

As shown in Figure 3.23a two non-extreme outliers (data point 17 and 97) Interestingly each of these ARM-1 (n=44) non-extreme outliers corresponded to STEMI AMI. Furthermore, both 17 and 97 had ACS readmission.

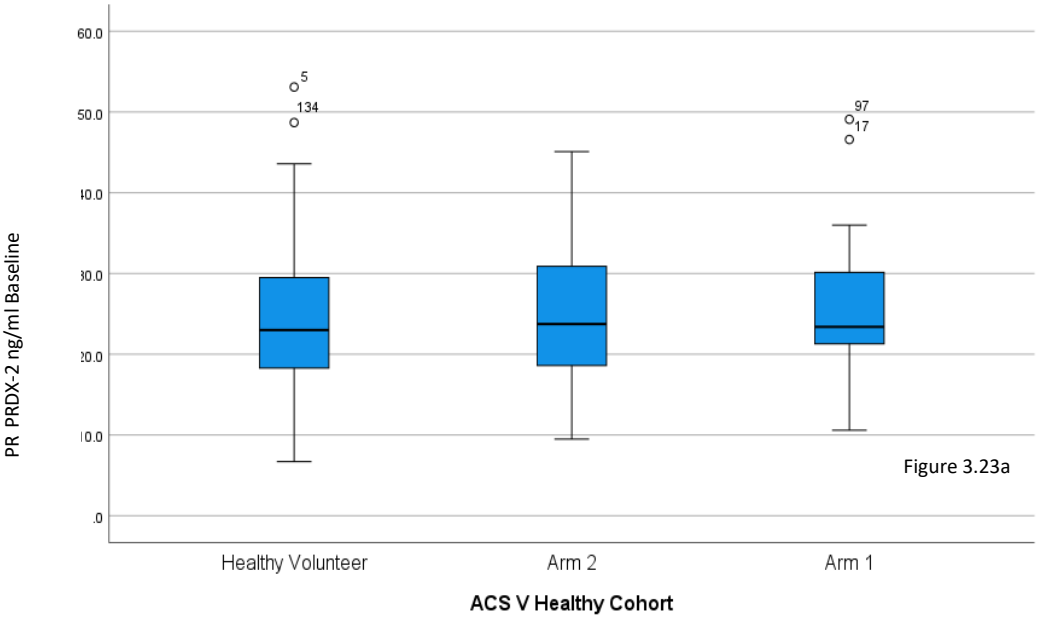


Figure 3.23a. Evaluation of 'outliers' for blood plasma PRDX-2 Sample 1 (Baseline). The box and whisker plots are presented for blood plasma PRDX-2 (ng/ml), along with 'outliers' for healthy volunteers (n=80), ACS Arm-1 (n=36) and ACS Arm-2 (n=44). °Outlier.

As illustrated in (Figure 3.11b) only one non-extreme outlier was identified (data points 14), in sample-2 (follow-up 1), data point 14 was male (n=32) from Arm-2 (n=44) who had

baseline STEMI with PCI to RCA and did not go on to have any ACS readmissions.

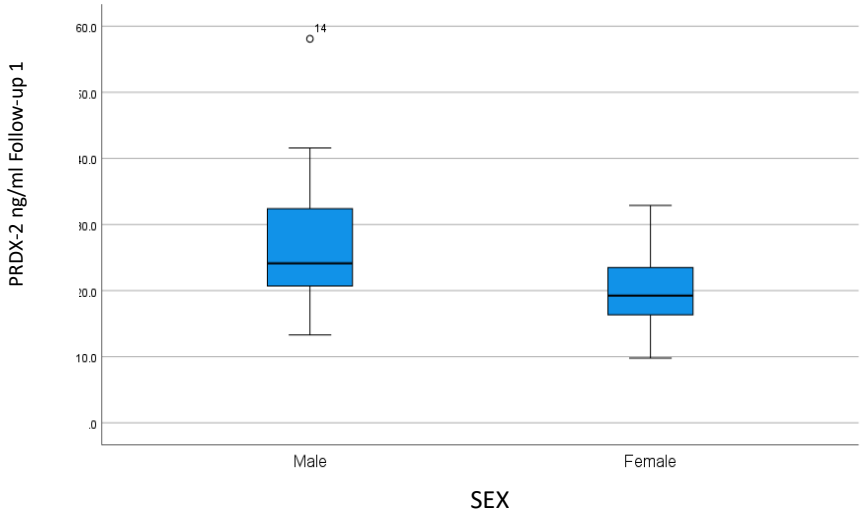


Figure 3.23b

Figure 3.23b. Evaluation of 'outliers' for blood plasma PRDX-2 Sample 2 (Follow-up 1). The box and whisker plots are presented for blood plasma PRDX-2 (ng/ml), along with 'outliers' for healthy volunteers (n=80), ACS Arm-1 (n=36) and ACS Arm-2 (n=44). ºOutlier.

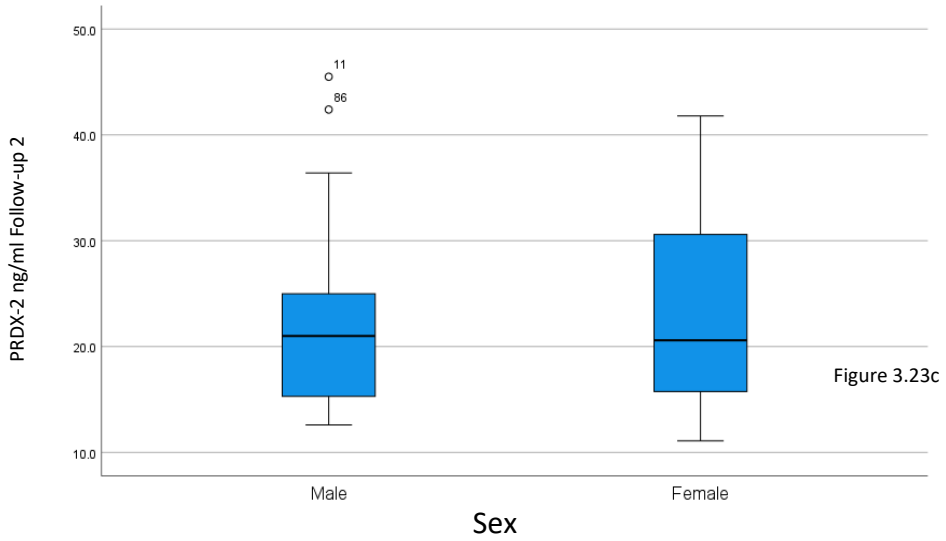


Figure 3.23c

Figure 3.23c. Evaluation of 'outliers' for blood plasma PRDX-2 sample 2 (Follow-up 1). The box and whisker plots are presented for blood plasma PRDX-2 (ng/ml), along with 'outliers' for healthy volunteers (n=80), ACS Arm-1 (n=36) and ACS Arm-2 (n=44) ºOutlier.

As illustrated, Figure 3.23c resulted in no extreme outliers. Follow-up 2 (sample-3) resulted in two male (n=27) outlier from Arm-1 (n=36) and one Arm-2 (n=44), interestingly both of these non-extreme outliers corresponded to UA, received PCI to RCA and both readmitted with ACS conditions.

3.47 Test of normality with outliers PRDX-2 sample 1, 2 and 3 including outliers.

Next a Shapiro Wilk's Test was conducted to determine whether the data fitted a normal distribution for blood plasma [PRDX-2]. This test was initially conducted with the 'outliers' included. The data are presented in Table 3.63 for sample-1 (a), sample-2 (b) and sample-3 (c). The data shows that, plasma PRDX-2 concentration was normal distribution for all bloods taken at sample 1 ($p>0.05$), for females at sample-2 and sample-3 ($p>0.05$).

Table 3.63 a: Test of normality with outliers peroxiredoxin-2 for blood sample-1, 2 and 3.

(a) test of normality

Kolmogorov-Smirnov[a] Table 3.63a					Shapiro-Wilk		
	Sex	Statisti	df	Sig.	Statisti	df	Sig.
PRX-2 mean Sample 1 Baseline	Male	.074	34	.200*	.990	34	.987
	Fema	.236	12	.063	.916	12	.252
PRX-2 mean Sample 2 Follow-up 1	Male	.152	34	.044	.886	34	.002
	Fema	.166	12	.200*	.945	12	.565
PRX-2 mean Sample 3 Follow-up 2	Male	.136	34	.112	.891	34	.003
	Fema	.202	12	.188	.890	12	.119

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

3.48 Test of normality excluding outliers peroxiredoxin-2 sample 1, 2 and 3

The Shapiro Wilks Test for normality was reconducted for the plasma PRDX-2 data, following the removal of the outliers as described above. The data presented in Table 3.64a and 3.64b equal data sets which fit a normal distribution, to include all blood samples participants ($p>0.05$) with the exception of sample-3 (Follow-up 2) male participants ($p<0.01$).

Table 3.64a and b: Test of normality with outliers Peroxiredoxin-2 for blood sample-1, 2 and 3 (a), healthy cohort and ACS (b)

(a)Test of Normality

Table 3.64a

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Gender	Statistic	df	Sig.	Statistic	df	Sig.
PRDX-2 mean Sample 1 Baseline	Male	.108	29	.200*	.971	29	.578
	Female	.236	12	.063	.916	12	.252
PRDX-2 mean Sample 2 Follow-up 1	Male	.143	29	.136	.961	29	.349
	Female	.166	12	.200*	.945	12	.565
PRDX-2 mean Sample 3 Follow-up 2	Male	.158	29	.063	.916	29	.024
	Female	.202	12	.188	.890	12	.119

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

(b)Test of Normality

Table 3.64b

	ACS V Healthy Cohort	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
PRDX-2 mean Sample 1 Baseline	Healthy Volunteer	.100	65	.172	.955	65	.019
	Arm-2	.122	44	.096	.967	44	.238
	Arm-1	.113	35	.200*	.945	35	.078

*. This is a lower bound of the true significance. a. Lilliefors Significance Correction

3.49 Peroxiredoxin-2 (PRDX-2) Two-way mixed ANOVA

A two-way mixed ANOVA was subsequently performed for plasma PRDX-2 concentration to establish whether there were interactions between the healthy cohort, the ACS cohort (Arm-1 and Arm-2) and sex (male and female). A summary of the data analysed in provided in Table 3.65.

Table 3.65 Case summary figures used for [PRDX-2] blood plasma between ACS verses healthy cohort participants and sex.

Cases

ACS V Healthy		Valid		Missing		Total	
	Cohort	N	Percent	N	Percent	N	Percent
PRDX-2 mean Sample-1	Healthy Volunteer	65	100.0%	0	0.0%	65	100.0%
	Arm-2	44	100.0%	0	0.0%	44	100.0%
	Arm-1	36	100.0%	0	0.0%	36	100.0%

		Valid		Missing		Total	
	Gender	N	Percent	N	Percent	N	Percent
PRDX-2 mean Sample-1	Male	93	100.0%	0	0.0%	93	100.0%
	Female	52	100.0%	0	0.0%	52	100.0%
		N	Percent	N	Percent	N	Percent
PRDX-2 mean Sample-2	Male	34	36.6%	59	63.4%	93	100.0%
	Female	12	23.1%	40	76.9%	52	100.0%
PRDX-2 mean Sample-3	Male	34	36.6%	59	63.4%	93	100.0%
	Female	12	23.1%	40	76.9%	52	100.0%

Initially, homogeneity of variance analysis was performed by the Levene's test for homogeneity of variance for PRDX-2. The data presented in Table 3.66 confirmed that for the PRDX-2 data assessed for sample-1 (screening) and sample-3 (second follow-up) displayed equal variance across all analytical methods i.e., mean, median, median adjusted and trimmed mean ($p>0.05$).

Table 3.66. Levene's Test of equality of dependent variable for [PRDX -2].

		Levene Statistic	df1	df2	Sig.
PRX-2 mean Sample 1	Based on Mean	1.351	3	42	.271
	Based on Median	1.246	3	42	.305
	Based on Median and with adjusted df	1.246	3	36.960	.307
	Based on trimmed mean	1.330	3	42	.277
PRX-2 mean Sample 2	Based on Mean	1.408	3	42	.254
	Based on Median	1.007	3	42	.399
	Based on Median and with adjusted df	1.007	3	26.700	.405
	Based on trimmed mean	1.156	3	42	.338
PRX-2 mean Sample 3	Based on Mean	.556	3	42	.647
	Based on Median	.428	3	42	.734
	Based on Median and with adjusted df	.428	3	39.642	.734
	Based on trimmed mean	.554	3	42	.648

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

Since downstream statistical analysis involves multivariate analysis, the Box's test of 'equality of covariance matrices' was next conducted. This test indicates whether two or more covariance matrices are homogenous. For the PRDX-2 homogeneity of covariance the null hypothesis was rejected, signifying that the covariances were not homogenous (p=0.005), see Table 3.67.

Table 3.67. The Box Test for homogeneity of equalities covariances.

Box's M	46.492
F	2.078
df1	18
df2	1273.793
Sig.	.005

Tests the null hypothesis that the observed covariance matrices of the dependent variables are equal across groups.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

Following this, the two-way mixed ANOVA was performed for PRDX-2. Which evaluated the difference between male and female subjects for the health and ACS (Arm-1 and Arm-2) cohorts (Figure 3.15). The ANOVAs revealed that there was no statistical significance between means (p=0.115).

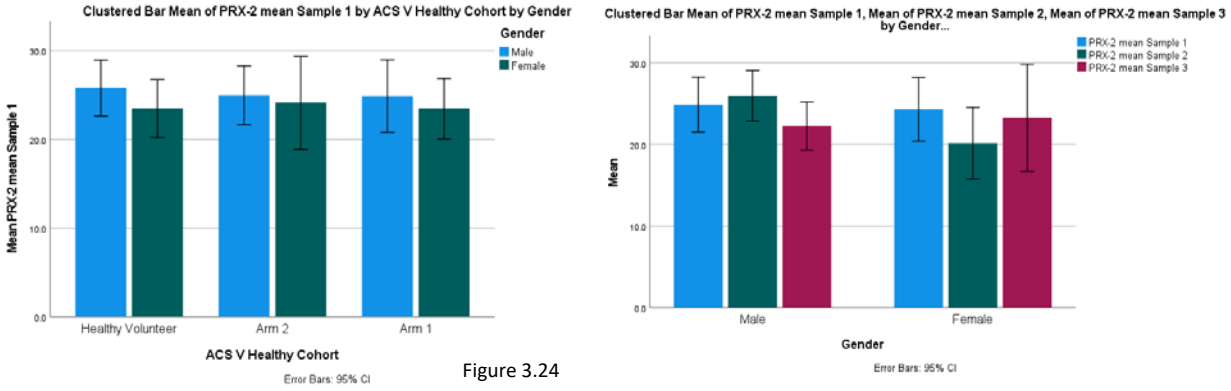


Figure 3.24

Figure 3.24 Gender stratified blood plasma PRDX-2 levels between healthy and ACS participant cohorts. The data presented illustrate mean plasma PRDX-2 levels between meals (light blue) and females (teal) for healthy volunteers and ACS Arm-1 and Arm-2 cohorts (left). The plot on the right shows the comparison between males and females at screening (sample 1), first follow-up (sample-2) and second follow-up (sample 3). Data presented are mean ± 95% CI, and analysed by two-way ANOVA Table 3.68 with Mauchly's test specificity for interaction p>0.05.

A Mauchly's Test of Sphericity was next performed to confirm the PRDX-2 ANOVA findings. This particular test evaluates sphericity in the data as appose to variance and is required to satisfy Assumption #8 (Section 2.24.2). The results are presented in Table 3.68.

Table 3.68. Mauchly's Test of sphericity between gender and healthy and ACS cohort.

Measure: Peroxiredoxin_2

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b] Greenhouse-Geisser	Epsilon[b] Huynh-Feldt	Epsilon[b] Lower-bound
Time	.900	4.330	2	.115	.909	1.000	.500

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

The Mauchly's test of sphericity indicated that the assumption of sphericity was met for the two-way interaction (p=0.115). This finding indicates that the relationship between the

different pairs of conditions is similar (i.e., males vs females, healthy vs ACS and sample time). Therefore, there is no evidence to suggest that plasma PRDX-2 concentration level differs between males and females, for healthy and ACS cohorts. The is also no difference in plasma PRDX-2 concentration between sample time and sex.

To evaluate the interaction between male vs female and healthy vs ACS, a Mauchly's multiple comparison 'within-subjects' test was performed, where individual participants are compared with themselves over time. Here the means were evaluated with respect to 'time' i.e., the point at which the blood sample was taken (sample-1, sample-2 and sample-3). The data presented in Table 3.69 illustrates that there was no statistically significant interaction between sample time and ACS Arm (p=>0.05. The data presented in Figure 3.24 shows that the mean data for males and females is parallel, indicating no change in plasma [PRDX-2] between groups or sex. Table 3.70 shows that there was no significant main effect on blood sample time vs gender or blood sample time vs ACS Arm vs gender (p>0.05).

Table 3.69. Multiple comparisons of blood plasma sample PRDX-2 levels between gender and ACS cohorts.

Measure: Peroxiredoxin_2
Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	58469.436	1	58469.436	623.122	<.001	.937
ARM	80.492	1	80.492	.858	.360	.020
Gender	82.214	1	82.214	.876	.355	.020
ARM * Gender	196.838	1	196.838	2.098	.155	.048
Error	3940.990	42	93.833			

Table 3.69

Next the interaction between male vs female and healthy vs ACS, was made by a Mauchly's multiple comparison 'between-subjects' test, where participant groups are compared over time. This type of test is more susceptible to individual participant variation. The results presented in Table 3.69 show that there was significance between ARM's (p=0.05) however, no significant interaction between ACS Arm and gender (p>0.05).

Table 3.70. Pairwise comparisons of blood plasma sample 1 PRDX-2 levels in healthy and ACS cohorts.

Dependent Variable: PRX-2 mean Sample 1

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Healthy Volunteer	Arm 2	.097	1.906	.960	-3.671	3.864
	Arm 1	.455	2.079	.827	-3.656	4.565
Arm 2	Healthy Volunteer	-.097	1.906	.960	-3.864	3.671
	Arm 1	.358	2.327	.878	-4.242	4.958
Arm 1	Healthy Volunteer	-.455	2.079	.827	-4.565	3.656
	Arm 2	-.358	2.327	.878	-4.958	4.242

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 3.70

As a final analytical step, a Tukey's Honestly Significant Difference (HSD) test was performed for plasma PRDX-2 concentration, which compares the ANOVA means based on the studentized data range. The data presented in Table 3.70 shows that there was no statistically significant interaction between the ACS and healthy cohort, with respect to plasma PRDX-2 concentration (p=0.05). The pairwise comparison illustrates that there is no significant difference between plasma PRDX-2 concentration for healthy volunteer's vs ACS participants (Table 3.71, p>0.05).

Table 3.71. Tukey multiple comparisons of blood plasma PRDX-2 levels between healthy and ACS cohorts.

Dependent Variable: PRDX-2 mean

Sample-1 Tukey HSD

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Healthy Volunteer	Arm-2	-.040	1.7722	1.000	-4.239	4.158
	Arm-1	.150	1.8860	.997	-4.318	4.618
Arm-2	Healthy Volunteer	.040	1.7722	1.000	-4.158	4.239
	Arm-1	.190	2.0401	.995	-4.643	5.024
Arm-1	Healthy Volunteer	-.150	1.8860	.997	-4.618	4.318
	Arm-2	-.190	2.0401	.995	-5.024	4.643

Based on observed means.

The error term is Mean Square (Error) = 2.406.

The Tukey's multiple comparison between-subjects test (Table 3.71) shows that there was no significant difference between plasma PRDX-2 concentration and cohorts ($p>0.05$).

Data filed – Thesis Two-way PRX-2.spv (Document1) Output & Thesis Two-way PRX-2.sav DataSet1) Data-IBM SPSS Statistics Data Editor.

3.50 Peroxiredoxin-2 (PRDX-2) Receiver Operator Curve (ROC).

Given the analysis presented above, which illustrates changes in the plasma biomarkers, Peroxiredoxin-2 (PRDX-2) for ACS participants a Receiver Operating Curve (ROC) analysis was next carried out. This was completed to ascertain the probability of event prediction, in this instance estimate whether an ACS event has occurred.

Sensitivity (true positives) for PRDX-2 for blood taken at screening (sample-1) was correctly predicted in 81%. Specificity, percentage of all observed as Healthy Cohort correctively predicted as 60.9 % See Table 3.72. The efficiency of PRDX-2 is calculated as (81%+60.9%) / (81%+60.9%+19%+39.1%) = 70.95%. In other words, blood plasma PRDX-2 predicts a correct diagnosis 70.95% of the time.

Table 3.72 Percentage accuracy in Classification [PRDX-2] biomarker

Peroxiredoxin 2	Presence of Heart Disease		Predicted ALL/ACS		
	Observed		Healthy Cohort	ACS Cohort	Percentage Correct
Step 1	Presence of Heart Disease ALL/ACS	Healthy Cohort	39	25	60.9
		ACS Cohort	15	64	81.0
	Overall Percentage				72.0

a. The cut value is .500

The positive predictive value (percentage correctly predicted) for plasma PRDX-2 concentration at screening, which relates to 'observed characteristics' compared to 'case predictive characteristics' is 100 x (64 ÷ (25 + 64)) = 71.9 % This means that 71.9 % of ACS cases are correctly predicted by evaluating plasma PRDX-2 concentration at screening. The negative predictive value, which relates to cases 'without the observed characteristics' compared to 'cases predicted not having the disease characteristic' is 100 x (39 ÷ (39 + 15)) = 72.2 %. This means that 72.2 %. of non-ACS cases are correctly predicted by evaluating plasma PRDX-2 concentration at screening.

Figure 3.25 illustrates the ROC curve was analysis for PRDX-2. Data included was plasma PRDX-2 concentrations at screening (sample-1) for the ACS cohort (n=80) and the healthy cohort (n=65). The area under the cure was determined as 0.820 (95% CI = 0.752 to 0.887).

Table 3.73. Area under the curve analysis for blood plasma PRDX-2.

Area under the curve

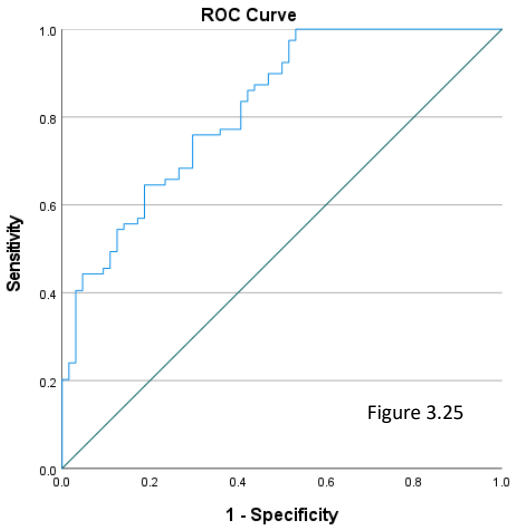


Figure 3.25

Test Result Variable(s): Predicted probability PRDX-2

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.820	.034	.000	.752	.887

Table 3.73

Figure 3.25 and Table 3.73 Receiver operator curve (ROC) analysis for blood plasma [PRDX-2]. The data presented illustrate the clinical utility of blood plasma PRDX-2 for the diagnosis of ACS. Blood plasma [PRDX - 2] concentrations for the healthy donor cohort i.e., 'true negatives' (specificity) was plotted with the ACS cohorts i.e., 'true positives' (sensitivity). The area under the curve was determined as 0.820 with a 95% CI or 0.752 to 0.888. This indicates that blood plasma PRDX-2 alone may predict a correct ACS diagnosis in 82% of cases.

Data filed – ROC PRDX – 2 Thesis.spv (Document1) Output and ROC All Biomarkers. Sav (DataSet1) Data-IBM SPSS Statistics Data

3.51 Kaplan-Meier

The ROC analysis presented above demonstrates clinical utility for each of the plasma biomarkers for ACS diagnosis, as determined by area under the curve. This was substantiated by specificity, sensitivity, and efficiency calculations, which showed that TRX biomarker was able to predict a correct result i.e., determine a 'true positive' and 'true negative' in >82% of cases. Therefore, to take this analysis further, it was next important to evaluate whether [PRDX-2], could reliably predict ACS participant outcomes, in this case the time to event endpoint was an ACS readmission.

3.52 Logistic Regression Predictions.

Using binomial logistic regression to predict if cases can be correctly predicted from the independent variables, it was then analysed which independent variable contributed and its statistical significance.

The variables in the equation below show each independent variable and statistical significance. The odds ratio ("Exp B" column) was used to predict the probability of an event occurring. Odds Ratio of each independent variable recorded below in tables A, B, C, D along with the confidence Intervals, showing the change in log odds occurring for one-unit change in independent variable, keeping the other independent variables constant.

Table 3.74. Logistic regression predicting likelihood of ACS event based on age, BMI, gender and peroxiredoxin-2

Table 3.74

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	Age	.088	.017	26.632	1	<.001	1.092	1.056	1.130
	BMI	.102	.055	3.485	1	.062	1.108	.995	1.233
	Gender (1)	.705	.436	2.617	1	.106	2.025	.861	4.760
	PRDX_2	-.022	.025	.792	1	.373	.978	.931	1.027
	Constant	-7.780	2.079	14.010	1	<.001	.000		

a. Variable(s) entered on step 1: Age, BMI, Gender, PRDX-2 mean Sample-1.

The statistical significance in respect to PRDX-2 found that age (p<0.001) added significantly to the predictions model. Whereas, BMI (p=0.062) and gender (p=0.107) did not. For [PRDX-2] males had 2.02 (95% CI, 0.861 to 4.760) times higher odds to exhibit ACS than females. [PRDX-2] had a value less than 1000, indicating a decreased odds for an increase of one of the other independent variables (Table 3.74).

Having satisfied the robustness of the plasma biomarkers [PRDX-2] a non-parametric Kaplan-Meier analysis was next performed to determine if these biomarkers impacted on participant prognosis and were able to predict the probability of an ACS readmission following stratification.

3.53 Kaplan-Meier all ACS participants peroxiredoxin-2 (PRDX-2)

The ACS cohort (n=79) displayed events and censoring at sample-1 (screening blood sample) as displayed in Table 3.75. The sample-1 [PRDX-2] quartile means were subsequently calculated.

Table 3.75. Readmission analysis of blood plasma PRDX-2 ng/ml at less than 25%, median and greater than 75% percentile as the cut-off values.

Table 3.75

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 19.50 25% percentile	18	2	16	88.9%
Median >19.60 ~ < 29.0 (23.60)	39	13	26	66.7%
> 30.60 75% percentile	22	8	14	63.6%
Overall	79	23	56	70.9%

For blood plasma analysis at sample-1 the ACS participants (n=79) were stratified according to PRDX-2 concentration. There were 18 participants in the <25% percentile (PRDX-2 <19.50 ng/ml), however 16 were censored (88,9%). For the 25%-75% inter-percentile range (PRDX-2 >19.60 ng/ml~<29.0 ng/ml), there were 39 participants, however 26 were censored (66.7%). Finally, there were 22 participants in the >75% percentile (PRDX-2 30.6 ng/ml), however 14 were censored (63.6%). Taken together, a total of 70% ACS participants were censored (Table 3.75).

For sample-1 statistical significance was not reached between <25% PRDX-2 concentration stratified ACS participants and >75% percentile concentrations, $\chi 2(1) = 3.287$, $p=0.070$ (Table 3.76) For sample-2 there were no statistically significant findings, however for sample-3 (6 months from index event in all participants) there was a statistically significant

difference in the readmission rate between the >75% PRDX-2 concentration stratified ACS participants, compared with the <25% PRDX-2 concentration, $\chi 2(1) = 6.429$, p=0.011 (Table 3.77).

Table 3.76. Multiple pairwise comparisons for blood plasma [PRDX-2] ng/ml cut off values in sample 1.

Table 3.76	Intervention Sample 1 ACS 25% ~ 75% percentile	< 19.50 25% percentile		Median >19.60 ~ < 29.0 (23.60)		> 30.60 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 19.50 25% percentile			2.752	.097	3.287	.070
	Median >19.60 ~ < 29.0 (23.60)	2.752	.097			.148	.701
	> 30.60 75% percentile	3.287	.070	.148	.701		

Table 3.77. Multiple pairwise comparisons for blood plasma [PRDX-2] ng/ml cut off values in sample 3.

Table 3.77	Intervention Sample 3 ACS 25% ~ 75% percentile	< 19.50 25% percentile		Median >19.60 ~ < 29.0 (23.60)		> 30.60 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 19.50 25% percentile			2.553	.110	6.429	.011
	Median >19.60 ~ < 29.0 (23.60)	2.553	.110			1.452	.228
	> 30.60 75% percentile	6.429	.011	1.452	.228		

Figure 3.26 illustrates a visual representation of the data presented in Table 3.76, there was no statistical significance in sample-1.

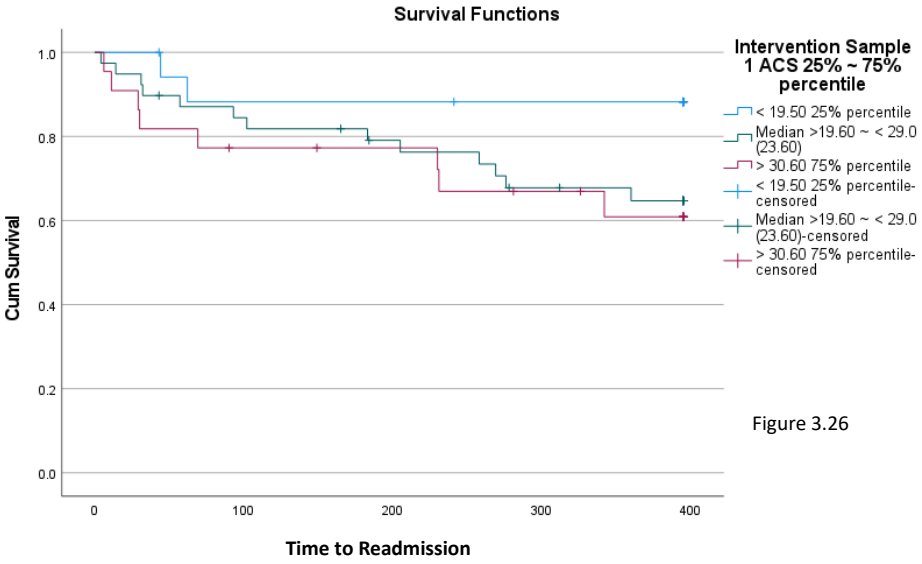


Figure 3.26

Figure 3.26. To determine whether blood plasma PRDX-2 (ng/ml) could predict participant outcome (overall survival without ACS readmission), Kaplan-Meier analysis was performed. Participant were stratified according to blood plasma [PRDX-2]. **1)** Blue line represents the 25% percentile, PRDX-2 <19. 50 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-2 >19.60 ~ <29.0 ng.ml. **3)** Red line represented the 75% percentile, PRDX-2 >30.60 ng/ml. χ^2 analysis, p=0.05.

Figure 3.27 however, illustrates a visual representation of the data presented in Table 3.69, highlighting that there was a significant reduction in readmissions due to a second ACS event for participants who had a plasma PRDX-2 concentration of <19.50 ng/ml at sample-3.

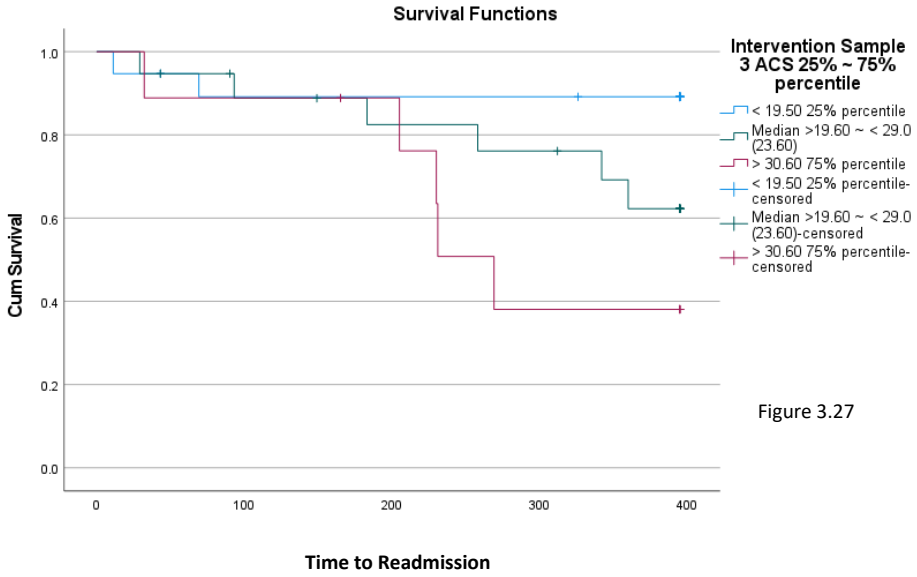


Figure 3.27

Figure 3.27. To determine whether blood plasma PRDX-2 (ng/ml) could predict participant outcome (overall survival without ACS readmission), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [PRDX-2]. **1)** Blue line represents the 25% percentile, PRDX-2 <19. 50 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-2 >19.60 ~ <29.0 ng.ml. **3)** Red line represented the 75% percentile, PRDX-2 >30.60 ng/ml. χ^2 analysis for 25% percentile vs 75% percentile = 6.49, p=0.011.

Data Filed - ALL KM Sample1-3 PRDX-2.SAV [DataSet1] and ALL KM Sample1-3 PRDX-2.SPV [Document5] IBM SPSS Statistics Output.

3.54 Kaplan-Meier for plasma peroxiredoxin-2 (PRDX-2) concentration percentiles.

To address the aim and objectives PRDX-2 plasma concentrations were then analysed to determine if indicative of an ACS event and/or predict a second event, the Kaplan-Meier analysis was systematically performed for all biomarkers. Initially, this was performed for the 'blood sample-1' PRDX-2 percentile concentrations. To recap, these percentile concentrations were calculated as <19.50 ng/ml for the <25% percentile (n=4), >19.60~<29.0ng/ml for the inter-percentile range (n=18) and 30.60ng/ml for the >75% percentile (n=12). Summarised data are presented in Table 3.78.

Table 3.78. Readmission analysis based on percentage of admissions and censored cases of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [PRDX-2] ng/ml as the cut-off values.

Table 3.78

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 19.50 25% percentile	4	2	2	50.0%
Median >19.60 ~ < 29.0 (23.60)	18	13	5	27.8%
> 30.60 75% percentile	12	8	4	33.3%
Overall	34	23	11	32.4%

The participants in the <25% range for plasma PRDX-2 concentration had the lowest time to readmission of 62.0 days (95% CI, 33.1 to 90.8) days. The time to readmission values for the inter-percentile and >75% plasma PRDX-2 concentration participants were much higher at 205 and 230 days respectively, see Table 3.79.

Table 3.79. Readmission analysis in days for baseline blood plasma PRDX-2 ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.79

Intervention Sample 1 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 19.50 25% percentile	115.667	51.343	15.035	216.298	62.000	14.697	33.194	90.806
Median >19.60 ~ < 29.0 (23.60)	202.388	35.310	133.179	271.596	205.000	96.783	15.305	394.695
> 30.60 75% percentile	199.912	54.007	94.058	305.765	230.000	226.997	.000	674.915
Overall	199.648	29.118	142.576	256.719	205.000	105.145	.000	411.083

a. Estimation is limited to the largest survival time if it is censored.

Assumption #4 (Section 2.24.4.1) states that for time to event statistics, there is similar censoring. The percentage of censored cases present in the <25 % percentile was 50.0%, compared to 27.8% and 33.3% for the inter-percentile and >75% percentile groups. Based on this, it is clear that the censoring of the healthy and ACS cohort groups was not similar. Figure 3.28 provides a visual representation of the data presented in Table 3.79. The plot shows a small interaction i.e., crossing of survival curves. However, in general those, participants with lower plasma PRDX-2 concentrations (<19.50 ng/ml) have a greater incidence of readmission than those with higher concentrations.

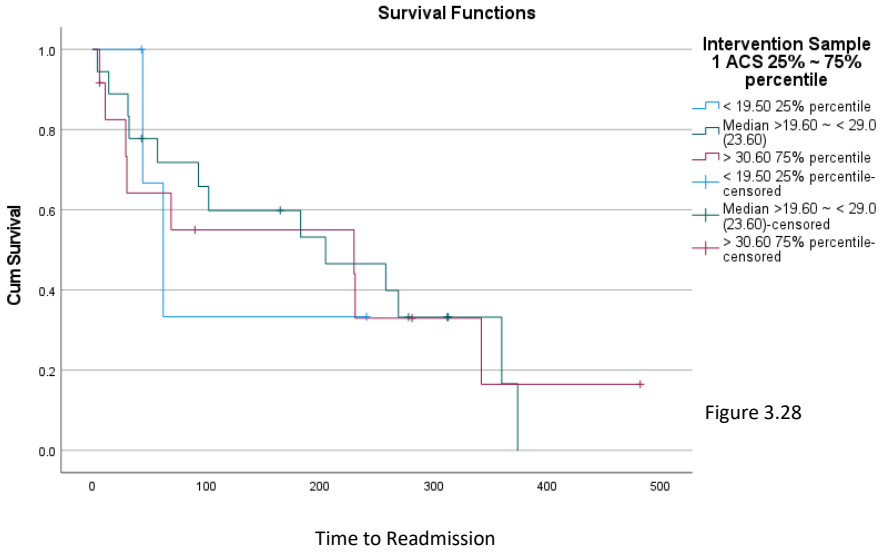


Figure 3.28

Figure 3.28. To determine whether blood plasma PRDX-2 (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [PRDX-2]. **1)** Blue line represents the 25% percentile, PRDX-2 <19. 50 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-2 >19.60 ~ <29.0 ng.ml. **3)** Red line represented the 75% percentile, PRDX -2 >30.60 ng/ml. χ^2 analysis p=>0.05.

To follow-on from this, a log rank test was conducted, which showed that there were no statistical differences in the admission rate for the three plasma PRDX-2 concentrations (>0.05)

Next the same analysis was conducted on plasma [PRDX-2] for blood sample-2 (first follow-up). The data presented in Table 3.80 shows the percentage of censored cases present in the <25% percentile (50.0% ACS participants), the inter-percentile range (22.2% ACS participants) and >75% percentile (16.7% ACS participants). These data illustrate that the proportion of censored groups was not similar.

Table 3.80. Readmission analysis at less than 25%, median and greater than 75% percentile of blood plasma PRDX-2 ng/ml as the cut-off values at sample 2.

Table 3.80

Intervention Sample 2 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 19.50 25% percentile	12	6	6	50.0%
Median >19.60 ~ < 29.0 (23.60)	9	7	2	22.2%
> 30.60 75% percentile	6	5	1	16.7%
Overall	27	18	9	33.3%

Participants that had low concentration of peroxiredoxin-2 concentrations had greater time to readmission of 342.0 days (95% CI, 79.7 to 604.2) days (Table 3.81). Median concentrations of PRDX-2, had readmission by 230.0 days (95% CI, 92.6 to 367.3) days compared to high concentrations admitted by 29 days (95% CI, 0.00 to 212.3) days.

Table 3.81. Readmission analysis in days for baseline blood plasma PRDX-2 ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.81

Intervention Sample 2 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 19.50 25% percentile	245.080	50.306	146.480	343.679	342.000	133.778	79.795	604.205
Median >19.60 ~ < 29.0 (23.60)	233.444	51.182	133.128	333.761	230.000	70.063	92.676	367.324
> 30.60 75% percentile	124.750	49.903	26.941	222.559	29.000	93.571	.000	212.398
Overall	215.428	31.364	153.954	276.901	230.000	34.637	162.111	297.889

a. Estimation is limited to the largest survival time if it is censored.

Figure 3.29 provides a visual representation of this data, highlighting that the >75% plasma PRDX-2 group display an increase in ACS readmission time, compared with those in the <25% and inter-percentile plasma range.

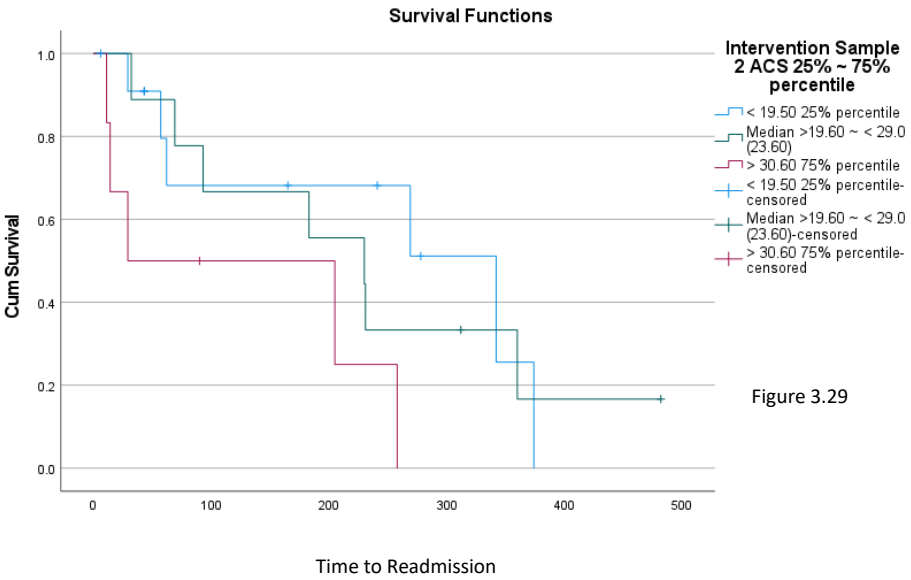


Figure 3.29

Figure 3.29. Readmission analysis using various blood plasma [PRDX-2] ng/ml cut-off values of baseline sample 2. To determine whether blood plasma PRDX-2 (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [PRDX-2]. **1)** Blue line represents the 25% percentile, PRDX-2 <19. 50 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-2 >19.60 ~ <29.0 ng.ml. **3)** Red line represented the 75% percentile, PRDX-2 >30.60 ng/ml. χ^2 analysis for 25% percentile vs 75% percentile = 4.76, p=0.009.

The survival readmission distributions for the three plasma PRDX-2 concentrations based on sample-2 was statistically significant, p=0.029 (Table 3.82), as determined by Log rank pairwise comparison.

Table 3.82 The survival readmission distributions for the healthy and ACS cohort, sample-2 blood plasma PRDX-2 ng/ml levels.

Table 3.82

	Intervention Sample 2 ACS 25% ~ 75% percentile	< 19.50 25% percentile		Median >19.60 ~ < 29.0 (23.60)		> 30.60 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 19.50 25% percentile			.038	.846	4.765	.029
	Median >19.60 ~ < 29.0 (23.60)	.038	.846			1.960	.162
	> 30.60 75% percentile	4.765	.029	1.960	.162		

Finally, analysis was performed for plasma [PRDX-2], based on blood collected at Sample-3 (2nd follow-up). The percent of censored ACS cases present in the <25 % plasma PRDX-2 concentration was 50%, compared with the inter-percentile range (40%) and >75 % (16.7%). Thus, the censored groups were not similar (Table 3.83).

Table 3.83. Readmission analysis of sample 3 percentage of admissions and censored cases at less than 25%, median and greater than 75% percentile of blood plasma [PRDX-2] ng/ml as the cut-off values at baseline.

Table 3.83

Intervention Sample 3 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 19.50 25% percentile	4	2	2	50.0%
Median >19.60 ~ < 29.0 (23.60)	10	6	4	40.0%
> 30.60 75% percentile	6	5	1	16.7%
Overall	20	13	7	35.0%

The participants in the <25% range for plasma PRDX-2 concentration had the lowest time to readmission of 69 days (95% CI, 0.00 to 155.8) days. The time to readmission values for the inter-percentile and >75% plasma PRDX-2 concentration participants were much higher at 258 and 230 days respectively, see Table 3.84.

Table 3.84. Readmission analysis in days for baseline blood plasma [PRDX-2] ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.84

Intervention Sample 3 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 19.50 25% percentile	209.375	123.582	.000	451.596	69.000	44.298	.000	155.825
Median >19.60 ~ < 29.0 (23.60)	238.333	47.768	144.707	331.960	258.000	95.607	70.611	445.389
> 30.60 75% percentile	200.125	35.933	129.696	270.554	230.000	26.615	177.836	282.164
Overall	225.788	34.338	158.485	293.092	231.000	23.349	185.237	276.763

a. Estimation is limited to the largest survival time if it is censored.

Figure 3.30 provides a visual representation of the data presented in Table 3.76. The plot shows a small interaction i.e., crossing of survival curves between inter-percentile and >75%. However, in general those, participants with lower plasma PRDX-2 concentrations (<19.50 ng/ml) have a greater incidence of readmission than those with higher concentrations.

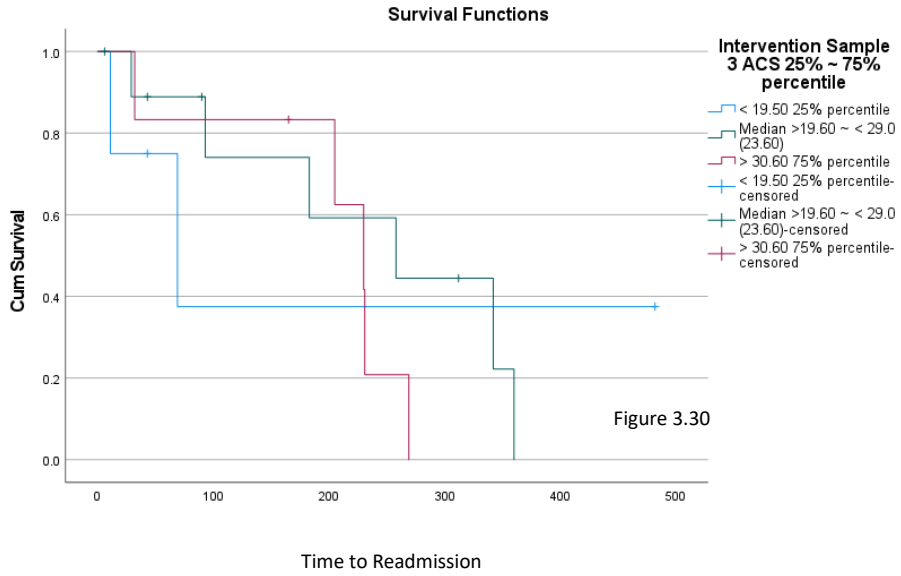


Figure 3.30

Figure 3.30. Readmission analysis using various blood plasma [PRDX-2] ng/ml cut-off values of baseline sample 3. To determine whether blood plasma PRDX -2 (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [PRDX-2]. **1)** Blue line represents the 25% percentile, PRDX-2 <19. 50 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-2 >19.60 ~ <29.0 ng.ml. **3)** Red line represented the 75% percentile, PRDX-2 >30.60 ng/ml. χ^2 analysis, p=>0.05.

A log rank (Mantel-Cox) test was run to determine if there were differences in the admission rates between the three concentrations there was no statistical significance between concentrations $\chi 2(2) = 0.789$, p=0.674.

Data filed- KM sample 1-3 PRDX-2 SC. spv [document11] output KM Sample 1-3 PRDX-2 SC.sav [DataSet1]data

3.55 Kaplan-Meier for lesions of Percutaneous Coronary Intervention.

The final set of analyses was to establish whether the PRDX-2 plasma biomarkers had any predictive value for ACS readmission with respect to ACS percutaneous coronary intervention (PCI) i.e., Right Coronary Artery (RCA), circumflex or Left Anterior Descending (LAD). ACS participants for blood sample-1 were subsequently stratified according to lesion of PCI as outlined in Table 3.85.

Table 3.85. Readmission analysis based on lesion of ACS event that resulted in baseline PCI

Table 3.85

Lesion of PCI	Total N	N of Events	Censored N	Censored Percent
RCA	39	13	26	66.7%
Circumflex	11	3	8	72.7%
LAD	30	8	22	73.3%
Overall	80	24	56	70.0%

Overall (n=80) ACS participants were included in this analysis, of these participants the PCI event is broken down as follows: RCA (n=39), Circumflex (n=11) and LAD artery (n=30), Table 3.85.

[PRDX-2] was reviewed with respect to the lesion of PCI event. As previously described, participants were further stratified according to plasma biomarker concentration i.e., <25%, inter-percentile range (>25% ~ <75%) and >75%. As previously stated, the design was non-event driven and all participant survival status was known at end of study. This limited the events of interest to (n=35) of all ACS admissions.

3.56 Peroxiredoxin-2 – Kaplan Meier readmission relating to AMI lesion.

Initially, the impact of PCI with respect to plasma PRDX-2 concentration was evaluated. This related to n=17 participants who received PCI to RCA, n=5 to circumflex and n=12 for LAD. The full breakdown is presented in Table 3.86.

Table 3.86. Readmission analysis based on lesion of PCI of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [PRDX-2] ng/ml cut- off values.

Table 3.86

Case Processing Summary

Lesion of PCI	PRXD -2 Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored N	Censored Percent
RCA	< 19.50 25% percentile	3	1	2	66.7%
	Median >19.60 ~ < 29.0 (23.60)	6	5	1	16.7%
	> 30.60 75% percentile	8	6	2	25.0%
	Overall	17	12	5	29.4%
Circumflex	Median >19.60 ~ < 29.0 (23.60)	3	2	1	33.3%
	> 30.60 75% percentile	2	1	1	50.0%
	Overall	5	3	2	40.0%
LAD	< 19.50 25% percentile	1	1	0	0.0%
	Median >19.60 ~ < 29.0 (23.60)	9	6	3	33.3%
	> 30.60 75% percentile	2	1	1	50.0%
	Overall	12	8	4	33.3%
Overall	Overall	34	23	11	32.4%

The data indicate that ACS participants who received PCI to LAD, had a greater readmissions rate for all concentrations of PRDX-2, the overall percentage was less when compared with circumflex and RCA. Table 3.87 shows that ACS participants who received PCI with LAD as culprit lesion had a median time of 93 days to readmission (95% CI, 36.9 to 149 days). For RCA participants, the time to readmission was longer at 230 days (95% CI, 42.9 to 417 days). Circumflex lesions had a median readmission time of 342 days; however, it was not possible to determine the 95% CI due to the low participant number (n=5).

Table 3.87. ACS readmission analysis based on lesion of ACS event that resulted in baseline PCI of PRDX-2 ng/ml levels as the cut-off values less than 25%, median and greater than 75% percentile.

Table 3.87

		Mean[a]				Median			
				95% Confidence Interval				95% Confidence Interval	
Lesion of PCI	PRXD -2 Sample 1 ACS 25% ~ 75% percentile	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
RCA	< 19.50 25% percentile	151.500	63.286	27.459	275.541	62.000	.	.	.
	Median >19.60 ~ < 29.0 (23.60)	252.667	47.204	160.148	345.186	258.000	43.478	172.782	343.218
	> 30.60 75% percentile	124.750	41.061	44.270	205.230	30.000	94.752	.000	215.715
	Overall	189.968	33.797	123.726	256.209	230.000	21.564	187.734	272.266
Circumflex	Median >19.60 ~ < 29.0 (23.60)	132.333	58.505	17.664	247.003	183.000	.000	.	.
	> 30.60 75% percentile	342.000	.000	342.000	342.000	342.000	.	.	.
	Overall	237.400	70.660	98.906	375.894	342.000	.000	.	.
LAD	< 19.50 25% percentile	44.000	.000	44.000	44.000	44.000	.	.	.
	Median >19.60 ~ < 29.0 (23.60)	148.778	38.562	73.197	224.358	102.000	13.416	75.704	128.296
	> 30.60 75% percentile	175.000	74.953	28.091	321.909	69.000	.	.	.
	Overall	145.250	33.096	80.382	210.118	93.000	28.579	36.985	149.015
Overall	Overall	185.083	24.421	137.217	232.948	205.000	73.451	61.036	348.964

a. Estimation is limited to the largest survival time if it is censored.

The data presented in Figure 3.31 gives a visual representation of PCI lesions with respect to stratified plasma PRDX-2 concentrations (data also summarised in Table 3.87). As shown in Figure 3.45, whilst there were some interesting trends in the time to readmission based on stratified PRDX-2 concentration and lesion of PCI.

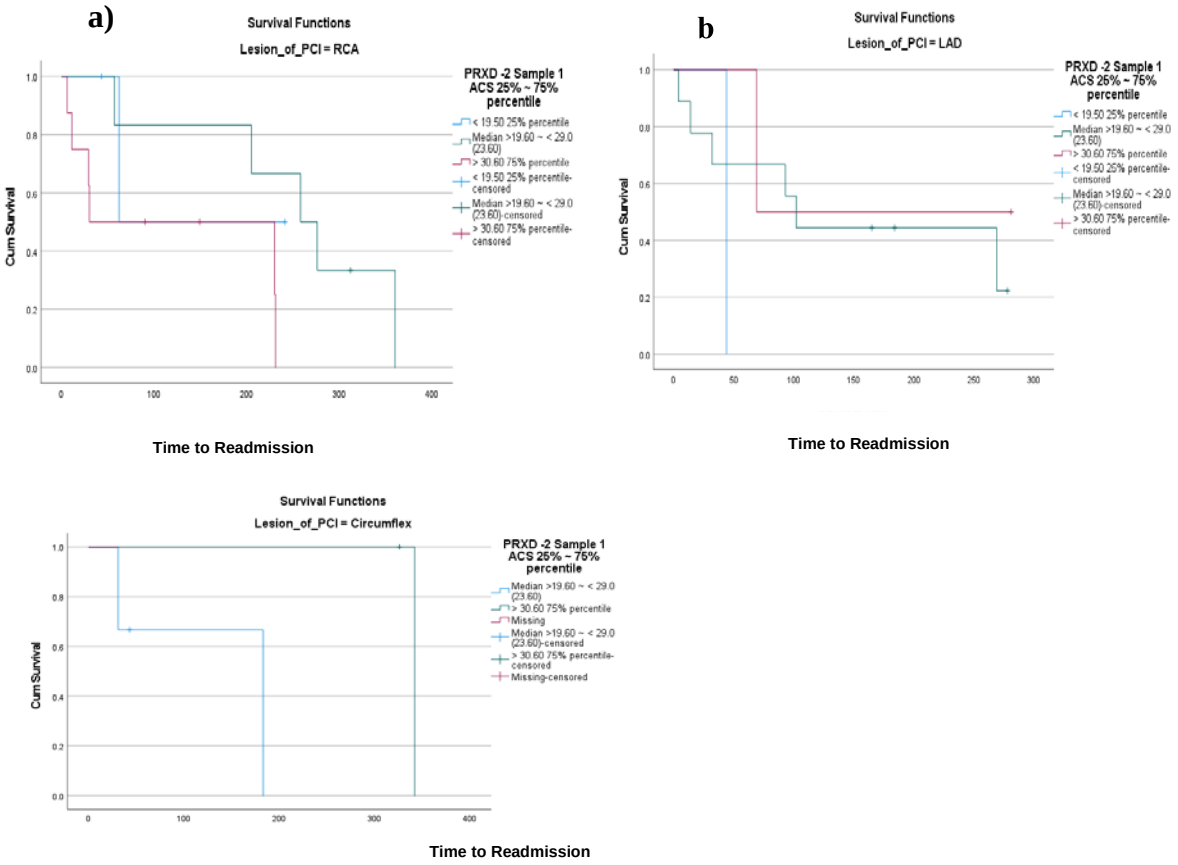


Figure 3.31 ACS Readmission analysis based on lesion of PCI of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [PRDX-2] ng/ml cut-off values. a) Represents PCI to RCA, **b)** represents PCI to LAD and **c)** represents circumflex PCI. [PRDX-2]. Blue line represents the 25% percentile, PRDX-2 <19. 50 ng/ml. Green line represents 25%-75% percentile, PRDX -2 >19.60 ~ <29.0 ng.ml. Red line represented the 75% percentile, PRDX-2 >30.60 ng/ml. χ^2 analysis for 25% percentile vs 75% percentile = 4.15, p=0.04.

Log rank pairwise between the three stratified PRDX-2 plasma concentrations and lesion PCI did show statistical significance between >75 ~ <25% plasma percentile concentrations, χ2(1) =4.157, p=0.041 (Table 3.88).

Table 3.88 The readmission distribution between lesion of PCI of baseline healthy and ACS cohort blood plasma PRDX-2 ng/ml levels.

Table 3.88		<19.50 25% percentile		Median>19.60~<29.0 (23.60)		>30.60 75% percentile	
	PRDX-2 Sample-1 ACS 25% ~ 75% percentile	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 19.50 25% percentile			2.216	.137	4.157	.041
	Median >19.60 ~<29.0	2.216	.137			.329	.566
	> 30.60 75% percentile	4.157	.041	.329	.566		

a. Adjusted for Lesion of PCI.

3.57 Brief discussion of peroxiredoxin-2 (PRDX-2) findings.

The data presented in this chapter illustrates that there is no overall differences in mean plasma PRDX-2 concentration for ACS participants (24.98 ng/ml) and healthy cohort (24.92 ng/ml). The plasma PRDX-2 reported in this chapter for healthy subject are lower than those reported previously by Wadley et al., 2019, which could be due to the analytical methods used. Interestingly, for the healthy cohort where inclusion numbers were relatively matched, the under 55's (n=36) had a mean plasma PRDX-2 concentration of 24.1 ng/ml compared to over 55's (n=29) who had a mean concentration of 26.0 ng/ml, an observation as previously highlighted (Chapter 3a) may be explained by an increase in age associated oxidative stress (Tan et al., 2018). For the ACS cohort, the numbers included were less equal, but a similar trend in PRDX-2 levels was observed, although these differences failed to reach significance (Figure 3.22).

The ROC analysis showed plasma PRDX-2 concentrations at baseline screening for the ACS cohort (n=80) and the healthy cohort (n=65) had an area under the curve determined as 82% (Figure 3.22). This finding illustrates potential clinical utility for PRDX-2 in predicting an ACS event. The most significant finding of the analysis presented in this chapter is the capacity of PRDX-2 plasma concentration to predict patient outcome, as illustrated at follow-up (Figure 3.29) and following stratification according to PCI intervention (Figure 3.31). Here Kaplan-Meier analysis was conducted to determine probability of 'time-to-readmission', based on biomarker stratification. The data presented in Figure 3.29 and Table 3.82 show that there is a significant risk of an ACS readmission at first follow-up, if plasma [PRDX-2) is >30.60 ng/ml, compared with [PRDX-2] <19.50 ng/ml, as indicated by a reduced time to readmission from 342 days to 29 days. This was also the case for blood samples analysed at second follow-up, i.e., 6-months following the primary event (Figure 3.26). These findings present a rationale to monitor ACS patient plasma PRDX-2 during recovery. During ACS recovery, the ischemia is treated through reperfusion (via PCI), a process which can cause further injury to the myocardium, by accelerating myocardial cell death through the generation of H_2O_2 (Simonis *et al*., 2012; Bae *et al.,* 2016). This type of damage is known as ischemia/reperfusion injury (IRI). Studies indicate that PRDX-2 may be released from damaged tissues during periods of inflammation (Mullen *et al.*, 2015). Since IRI is mediated by inflammation and H_2O_2 (Algoet *et al.,* 2022; He *et al.,* 2022), ACS patients with elevated PRDX-2 (>30.60 ng/ml) at follow-up may be recovering less well

from IRI, explaining the poorer outcome. Consistent with TRX (Chapter 3a), ACS patients in the >75% percentile for PRDX-2 at baseline had an increased risk of readmission for AMI patients presenting to LAD (Table 3.87). Interestingly, a study using a porcine pre-clinical model illustrated that IRI is most prominent in the LAD following reperfusion, compared with RCA (Rios-Navarro et al., 2021), which taken together with the previous discussion, could explain the poorer clinical outcome for the ACS patients evaluated herein.

In conclusion, the data presented in this chapter reveals that [PRDX-2] levels could reliably predict a correct diagnosis in ~82% cases and may indicate further cardiac muscle damage at follow-up, as mediated by potential IRI. Moreover, a high plasma PRDX-2 concentration at first and second follow-up is associated with an increased risk of ACS readmission, particularly for those patients presenting with AMI to LAD.

Chapter 3d – Peroxiredoxin-4 (PRDX-4)

3.58 Brief introduction to plasma peroxiredoxin-4 (PRDX-4) analysis.

Chapter 3a,b,c highlighted a role for TRX, TRXr and PRDX-2 in cardiovascular disease as potential representative biomarker candidates in predicting prognosis or outcomes. Here the analysis explores PRDX-4, which is known to be highly expressed in the liver and pancreas, with lowest expression in blood leukocytes and brain (Haridas *et al.,* 1998; Jin *et al.*, 1997). Interestingly, acute hyperglycemia is a common feature during the early phase AMI, regardless of diabetes status, causing a condition known as 'diabetic cardiomyopathy' which is a severe complication of AMI (Paolisso *et al.*, 2021; Ishihara 2012; Webster 2008; Zang et al.,2021). Increased ROS resulting in oxidated stress can overwhelm the availability of antioxidants or free radical scavengers in the system, leading to an oxidative stress (Das et al., 2014). As described in Section 1.6, PRDX-4 is small redox-regulating protein, which plays a crucial role in maintaining cellular redox homeostasis and cell survival. The function of PRDX-4 is to regulate cellular oxidative stress by reducing H_2O_2 to water in a thiol-dependent catalytic cycle (*Wood et al*., 2003; Elko *et al.*, 2021; Hoyle *et al.*, 2015). Previous studies have indicated that changes in blood concentrations of PRDX-4 (plasma or serum) may be indicative of an underlying oxidative stress and altered redox signalling (Schulte 2011a). This may exacerbate a pathological process, which can be detrimental to patient outcome in numerous conditions, including CVD (Schulte 2011a). In AMI, PRDX-4 may offer protection against the damaging effects of H_2O_2 and resulting oxidative stress in cardiomyocytes, as well as offer protection after an AMI aiding recovery (Jeong et al., 2021).

Thus, the following analysis as described in this chapter was conducted to determine if there are any changes in plasma [PRDX-4] between a healthy cohort of participants and those following an AMI / during an ACS event. Participant plasma samples were subsequently evaluated using an optimised ELISA for PRDX-4 (Section 2.23., Table 2.2 and Appendix Z). The data collected was subsequently analysed using various statistical methods as described in chapter 2. This chapter presents the results of this analysis is a logical order to ultimately

evaluate the clinical utility of PRDX-4 in the context of ACS and establish whether PRDX-4 could reliably predict ACS patient outcome. In this case the time to event endpoint was an ACS readmission.

Objectives:

a) To clarify the mean plasma concentrations for PRDX-4 for healthy volunteers, stratified based on sex and age., which will be used as baseline measurements for ACS comparison.

b) To evaluate the plasma concentrations levels of PRDX-4 for ACS patients stratified based on age and sex at initial diagnosis / screening and follow-up. Clinical utility may subsequently be evaluated.

c) Monitor the concentration level of PRDX-4 through ACS patient follow-up sampling, in order to evaluate whether this biomarker may be predictive of an ACS readmission.

d) Evaluate whether [PRDX-4] may predict readmission based on ACS patient stratified according to PCI.

3.59 Basic description statistics of PRDX-4.

To determine the mean plasma [PRDX-4] for the 'healthy cohort', any healthy volunteers with a medical history of diabetes mellitus (n=5), hypertension (n=10), family history of cardiovascular conditions (n=19), or inflammatory disorders (n=1) were removed from the analysis. This equated to a healthy volunteer population with no medical conditions (n=37).

This population had a similar plasma [PRDX-4] mean of 13.89 ng/ml ± 12.53 ng/ml, compared with the healthy cohort as a whole 13.94 ng/ml ± 11.28 ng/ml (n=64). Stratification of the ACS participant cohort into Arm-1 (n=34) and Arm-2 (n=44) revealed plasma PRDX-4 levels of 21.75 ng/ml ± 12.23 ng/ml and 16.94 ng/ml ± 11.96 ng/ml respectively. Next the 'smokers' were removed from the healthy cohort analyses, which resulted in a further drop in mean plasma PRDX-4 concentration to 11.98 ng/ml ± 12.43 ng/ml (n=26). The same

convention was applied to the ACS participants, which resulted in mean plasma PRDX-4 concentrations of 18.04 ng/ml ± 12.53 ng/ml for Arm-1 (n=5) and 19.55 ng/ml ± 12.53 ng/ml for Arm-2 (n=8). Statistical analysis showed that these [PRDX-4] values remained significantly higher for ACS participants, compared with the healthy ($p<0.05$), and smoking status has little impact on mean plasma PRDX-4 levels once an ACS has occurred.

Overall, males in the healthy cohort (n= 31) had a mean plasma PRDX-4 concentration of 15.91 ng/ml ± 12.07 ng/ml, compared with a female (n=32) value of 11.79 ng/ml ± 10.37 ng/ml, indicating that, for healthy males, the mean plasma [PRDX-4] is higher.

The mean [PRDX-4] for the healthy cohort was 13.93 ng/ml ± 11.38 ng/ml (n=64) compared with all ACS participants (n=78), which was higher at 19.04 ng/ml ± 12.24 ng/ml. Data was statistically significant demonstrated on a Mann-Whitney U test ($p<0.0077$), as shown in Figure 3.32 and Table 3.89.

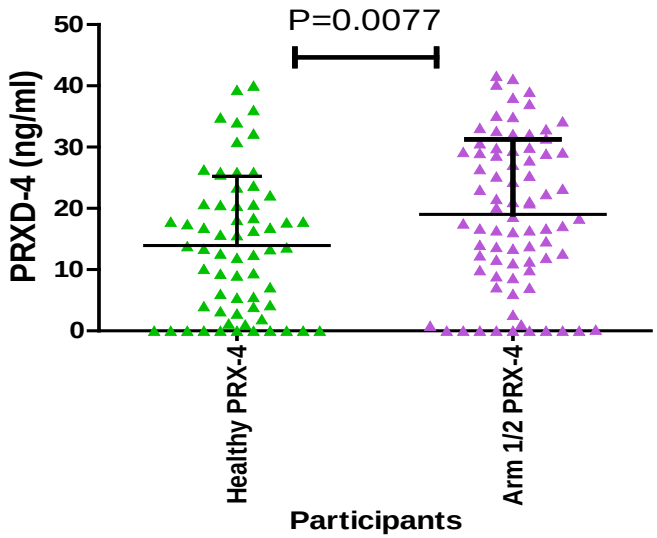


Figure 3.32 Overall blood plasma PRDX-4 levels between healthy and ACS participant cohorts. The data presented illustrate the spread of data between all healthy (n=65) and all ACS (n=80) plasma PRDX-4 levels (ng/ml). For healthy the mean PRDX-4 concentration was 13.9±11.4 ng/ml. For ACS the mean PRDX-4 concentration was 19.0±12.2 ng/ml. Data was statistically significant demonstrated on a Mann-Whitney U test. (p=<0.0077).

Table 3.89. Descriptive statistics for plasma PRDX-4 (ng/ml) concentration.

		PRDX-4		
ACS V Healthy Cohort	Sex	Mean	Std. Deviation	N
Healthy Volunteer	Male	15.913	12.0690	31

	Female	12.064	10.3239	33
	Total	13.928	11.2809	64
ACS Arm-1 and Arm-2	Male	19.586	12.7014	58
	Female	17.445	10.9307	20
	Total	19.037	12.2389	78
Total	Male	18.307	12.5399	89
	Female	14.094	10.7787	53
	Total	16.735	12.0486	142

3.60 Basic PRDX-4 analysis for ACS Arm-1.

Given the significant differences in plasma PRDX-4 levels observed between males and females for the healthy cohort, it was next important to evaluate this effect with respect to the ACS participants. For the Arm-1 ACS participants, males (n=26) had a mean plasma PRDX-4 concentration of 22.32 ng/ml mean ± 9.68 ng/ml, compared to females (n=9) 17.69 ng/ml ± 12.09 ng/ml. The effects of smoking status were next evaluated for Arm-1 ACS, where non-smokers or ex-smokes ≥12 months (n=25) had a mean plasma PRDX-4 concentration of 21.42 ng/ml ± 12.42 ng/ml compared to smokers/vapers (n=9) 22.64 ng/ml ± 12.38 ng/ml.

For Arm-1 ACS, the primary endpoint (readmission) occurred in (n=15) 41.6%, of which ACS admission (n= 10) 27.7% was the cause.

Comparing plasma PRDX-4 concentrations at follow-up appointments was challenging for those participants who were readmitted for causes other than ACS. However, mean PRDX-4 plasma concentrations for the readmission caused by a second ACS were 20.16 ng/ml ± 15.79 ng/ml (at baseline of those readmitted, n=10) compared to 24.07 ng/ml ± 11.77 ng/ml (at first follow-up, n=9, $p>0.05$).

3.61 Basic PRDX-4 analysis for ACS Arm-2.

The same analysis was carried out for ACS Arm-2 participants (n=44), who had their ACS event within 24 hours of their hs-cTn result. Here, the male cohort (n=32) had a mean plasma PRDX-4 concentration of 17.37 ng/ml ± 12.39 ng/ml, compared to females (n=12) which was 15.81 ng/ml ± 11.17 ng/ml. As with Arm-1, the Arm-2 participants were stratified according to smoking status. The non-smokers or ex-smokers ≥12 months (n=28) had a mean plasma PRDX-4 concentration of mean 18.37 ng/ml ± 12.58 ng/ml, compared to smokers/vapers (n=16) 14.45 ng/ml ± 10.72 ng/ml ($p>0.05$).

For the Arm-2 ACS participants, the primary endpoint (readmission) occurred in (n=20) 45.4%, of which readmission due to a second ACS accounted for (n= 14) 31.8% cases overall. The mean plasma PRDX-4 concentration at baseline for those with ACS readmission was 18.65 ng/ml ± 11.34 ng/ml, compared with the first and second follow-up samples, which were 23.26 ng/ml ± 12.93 ng/ml (n=8) and 23.03 ng/ml ± 13.32 ng/ml (n=6) respectively. In spite of this large increase in [PRDX-4] at second follow-up, data did not reach significance when compared to the mean baseline concentration.

Combining the Arm-1 and Arm-2 ACS data, the primary endpoint (readmissions) occurred in (n=34) 42.5% of participants. Where a second ACS was the cause, this was observed in (n=24) 30% of all readmissions. The mean plasma PRDX-4 concentration for all ACS participants readmitted with a second ACS event was 19.28 ng/ml ± 13.07 ng/ml, compared with 23.69 ng/ml ± 11.94 ng/ml and 22.96 ng/ml ± 13.76 ng/ml at first (n=17) and second (n=12) follow-up respectively. Taken together, the difference in mean compared with the ACS baseline plasma PRDX-4 concentration was significantly higher for first or second follow up or between other admissions, see Table 3.90.

Table 3.90 Descriptive statistics [PRDX-4] (ng/ml) biomarkers for readmission rates

ACS Readmissions		PRDX-4 (ng/ml)	SD	Non-ACS Readmission	PRDX-4 (ng/ml)	SD	Significance
Baseline	(n=24)	**19.28**	13.07	(n=10)	**21.48**	12.83	
Follow up 1	(n=17)	**23.69**	11.94	(n=11)	**17.17**	12.01	0.8769
Follow up 2	(n=12)	**22.96**	13.76	(n=7)	**18.91**	6.51	0.5804

3.62 Age comparisons at screening PRDX-4 for all participant groups.

For healthy volunteers at time of screening, the under 55's (n=35) had a mean plasma PRDX-4 concentration of 11.85 ng/ml ± 11.09 ng/ml compared, compared to over 55's (n=28) who had a mean concentration of 16.60 ng/ml ± 11.15 ng/ml. For the Arm-1 ACS participants aged under 55 (n=6) at time of screening had a mean plasma PRDX-4 concentration of 12.63 ng/ml ± 12.81 ng/ml compared, with over 55's (n=28) which was 23.70 ng/ml ± 11.41 ng/ml. For Arm-2 ACS participants, the under 55's (n=11)

had a mean plasma PRDX-4 concentration of 18.85 ng/ml ± 13.40 ng/ml compared with over 55's (n=33) which had a mean of 16.31 ng/ml ± 11.60 ng/ml (Figure 3.33).

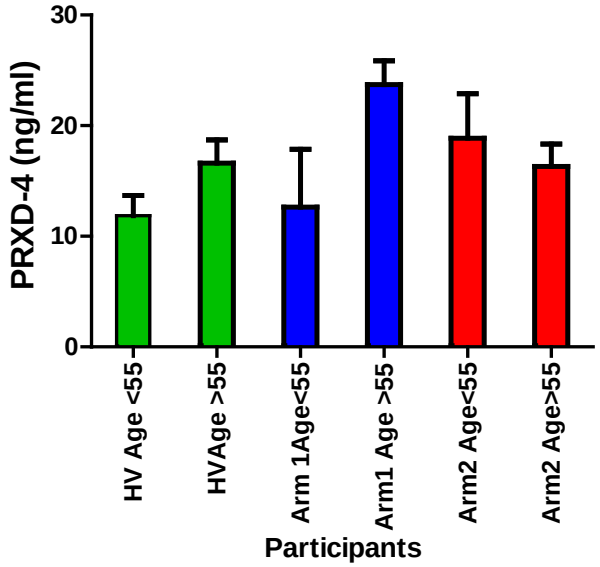


Figure 3.33 Age stratified blood plasma PRDX-4 levels between healthy and ACS participant cohorts. The data presented illustrate blood plasma PRDX-4 levels (ng/ml) following age stratification; <55 years or >55 years for a) healthy in green (<55 years, n=35, >55 years n=28), b) ACS Arm-1 in blue (<55 years n=6, >55 n=28) and c) ACS Arm-2 in red (>55 years n=11, >55 years n=33).

3.63 Two-way mixed ANOVA.

To follow on from the descriptive statistical analysis presented above, it was next important to evaluate the impact of sex (male / female) with respect to the plasma concentrations of PRDX-4. Since the sample population cohorts included a healthy population, along with ACS Arm-1 and Arm-2, a two-way mixed ANOVA was selected, as this would determine interaction between participant 'sex' and population cohort.

3.64 Assessment of outliers for peroxiredoxin-4 (PRDX-4).

For Sample-1 (baseline) for the healthy cohort (n=65), Arm-1 (n=36) and Arm-2 (n=44) the Boxplot data presented in Figure 3.34a indicated that there was no outlier.

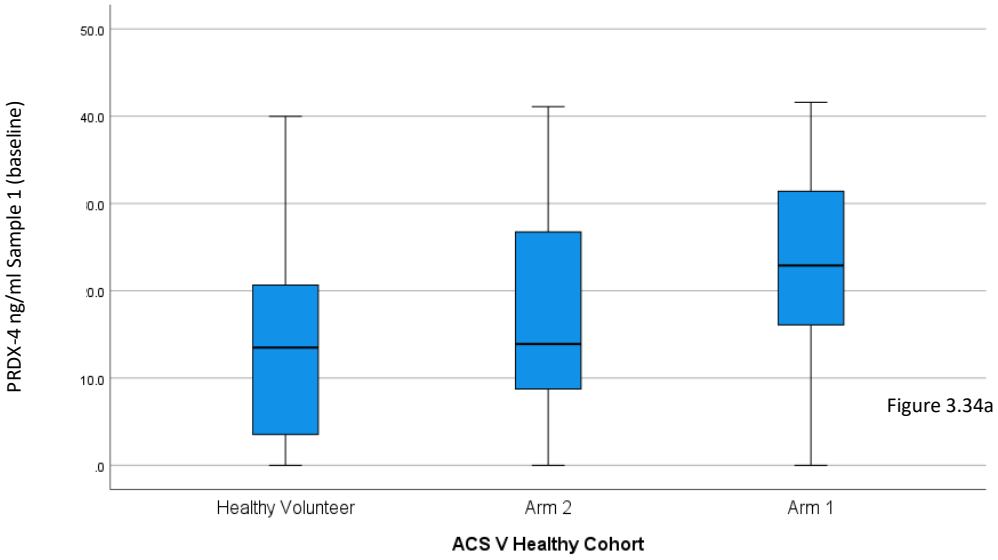


Figure 3.34a. Evaluation of 'outliers' for blood plasma [PRDX-4] sample 1, baseline bloods. The box and whisker plots are presented for blood plasma PRDX-4 (ng/ml), along with 'outliers' for healthy volunteers (n=80), ACS Arm-1 (n=36) and ACS Arm-2 (n=44).

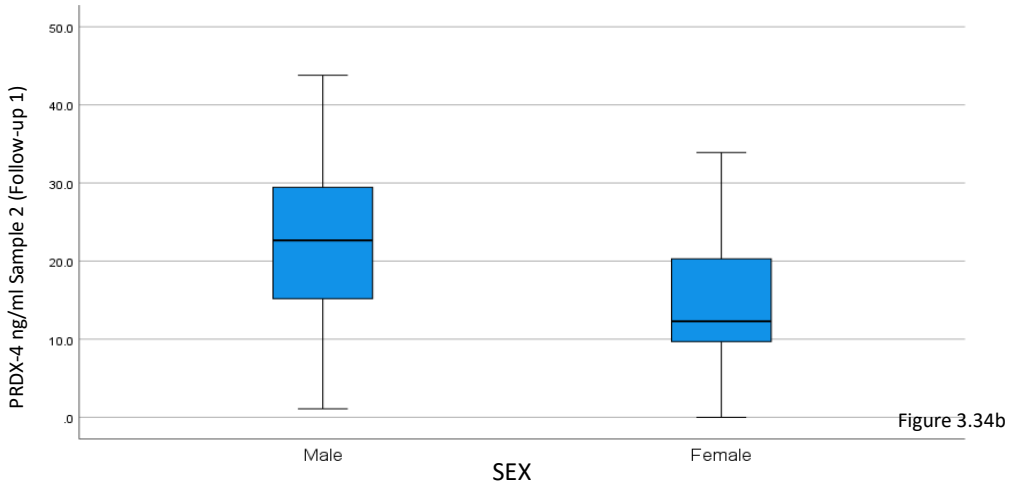


Figure 3.34b. Evaluation of 'outliers' for blood plasma [PRDX-4] Sample 2, Follow-up 1. The box and whisker plots are presented for blood plasma PRDX-4 (ng/ml), along with 'outliers' for healthy volunteers (n=80), ACS Arm-1 (n=36) and ACS Arm-2 (n=44) ACS Arm-1 Male (n=27) Female (n=9) and ACS Arm-2 Male (n=32) Female (n=12).

As illustrated in Figure 3.34b, for ACS Arm-1 (n=36) and Arm2 (n=44) for PRDX-4 there were no outliers.

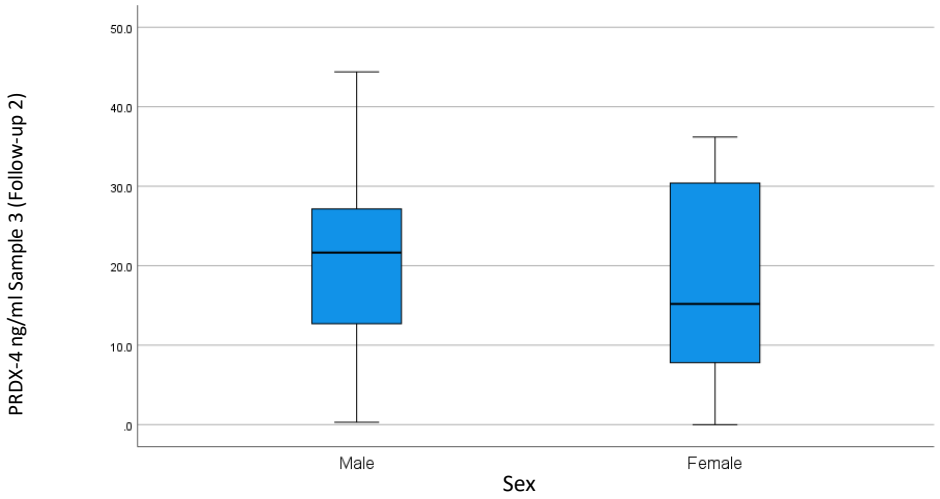


Figure 3.34c. Evaluation of 'outliers' for blood plasma [PRDX-4] Sample-3 (Follow-up 2). The box and whisker plots are presented for blood plasma PRDX-4 (ng/ml), demonstrating 'no outliers' for ACS Arm-1 Male (n=27) Female (n=9) and ACS Arm-2 Male (n=32) Female (n=12).

3.65 Test of normality with outliers [PRDX-4] sample 1, 2 and 3 including outliers.

Next a Shapiro Wilk's test was conducted to determine whether the data fitted a normal distribution for blood plasma [PRDX-4]. This test was initially conducted and found to have no 'outliers' included. The data are presented in Table 3.91 for sample-1 (a), sample-2 (b) and sample-3 (c). The data in Table 3.91 shows that, plasma PRDX-4 concentrations did not fit a normal distribution for all bloods taken at sample-1 ($p<0.001$). However, for females at blood sample-1 (Baseline), the plasma [PRDX-4] Arm-2 (n=44) concentration did fit a normal distribution ($p>0.05$).

Table 3.91a and b: Test of normality with no outliers peroxiredoxin-4 for blood sample-1 Healthy Cohort versus ACS (a), male and females (b).

(a) Tests of Normality

Table 3.91a

	ACS V Healthy Cohort	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
PRDX_4 mean Sample-1 (Baseline)	Healthy Volunteer	.108	64	.059	.933	64	.002
	Arm-2	.120	44	.120	.951	44	.058
	Arm-1	.132	34	.139	.933	34	.038

a. Lilliefors Significance Correction

(b) Tests of Normality

Table 3.91b

	Gender	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
PRDX_4 mean Sample-1 (Baseline)	Male	.083	91	.153	.949	91	.001
	Female	.102	51	.200*	.938	51	.010

*. This is a lower bound of the true significance. a. Lilliefors Significance Correction

3.66 Peroxiredoxin-4 (PRDX-4) Two-way mixed ANOVA.

A two-way mixed ANOVA was subsequently performed for plasma PRDX-4 concentration to establish whether there were interactions between the healthy cohort, the ACS cohort (Arm-1 and Arm-2) and sex (male and female). A summary of the data analysed in provided in Table 3.84.

Table 3.92. Case summary figures used for [PRDX-4] blood plasma between ACS verses healthy cohort participants and sex.

	ACS V Healthy (Cohort	Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
PRDX_4 mean Sample-1	Healthy Volunteer	64	98.5%	1	1.5%	65	100.0%
	Arm-2	44	100.0%	0	0.0%	44	100.0%
	Arm-1	34	94.4%	2	5.6%	36	100.0%

		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
PRDX_4 mean Sample-2	Male	32	34.4%	61	65.6%	93	100.0%
	Female	9	17.3%	43	82.7%	52	100.0%
PRDX_4 mean Sample-3	Male	32	34.4%	61	65.6%	93	100.0%
	Female	9	17.3%	43	82.7%	52	100.0%

Table 3.92

Initially, homogeneity of variance analysis was performed by the Levene's test for homogeneity of variance for [PRDX-4]. The data presented in Table 3.93 confirmed that for the [PRDX-4] data assessed for sample-1 (screening) and sample-3 (second follow-up) displayed equal variance across all analytical methods i.e., mean, median, median adjusted and trimmed mean (p>0.05).

Table 3.93. Levene's Test of equality of dependent variable for [PRDX-4].

		Levene Statistic	df1	df2	Sig.
PRXD_4 mean Sample 1	Based on Mean	.501	3	36	.684
	Based on Median	.461	3	36	.711
	Based on Median and with adjusted df	.461	3	32.366	.711
	Based on trimmed mean	.494	3	36	.689
PRXD_4 mean Sample 2	Based on Mean	.601	3	36	.619
	Based on Median	.580	3	36	.632
	Based on Median and with adjusted df	.580	3	34.107	.632
	Based on trimmed mean	.597	3	36	.621
PRXD_4 mean Sample 3	Based on Mean	1.024	3	36	.393
	Based on Median	.783	3	36	.512
	Based on Median and with adjusted df	.783	3	32.446	.512
	Based on trimmed mean	1.028	3	36	.392

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

Table 3.93

Since downstream statistical analysis involes multivariate analysis, the Box's test of 'equality of covariance matrices' was next conducted. This test indicates whether two or more covariance matrices are homogenous. For the [PRDX-4] homogeneity of covariance the null hypothesis was rejected, signifying that the covariances were not homogenous (p=0.503), see Table 3.94.

Table 3.94. The Box Test for homogeneity of equalities covariances.

Box's M	13.730
F	.943
df1	12
df2	1092.958
Sig.	.503

Tests the null hypothesis that the observed covariance matrices of the dependent variables are equal across groups.

Following this, the two-way mixed ANOVA was performed for [PRDX-4]. Which evaluated the difference between male and female subjects for the health and ACS (Arm-1 and Arm-2) cohorts (Figure 3.35). The ANOVAs revealed that there was no statistical significance between means (p=0.490).

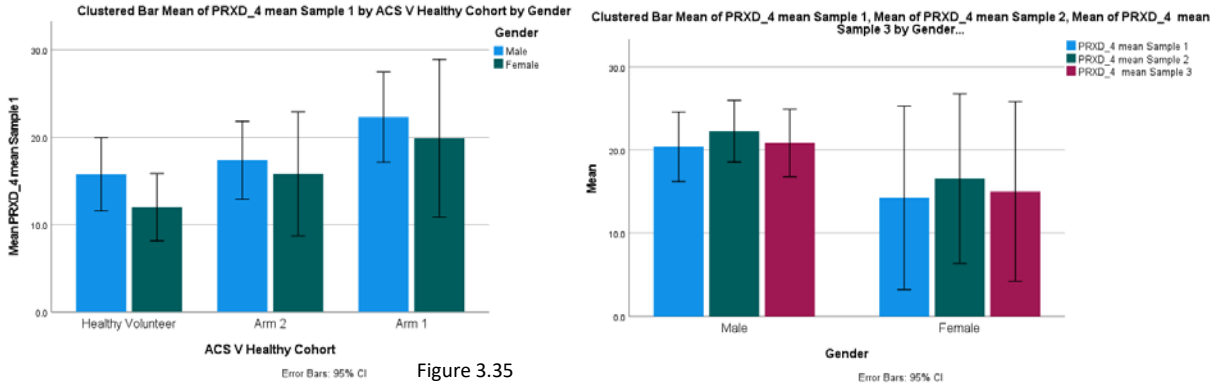


Figure 3.35

Figure 3.35 Gender stratified blood plasma PRDX-4 levels between healthy and ACS participant cohorts. The data presented illustrate mean plasma PRDX-4 levels between meals (light blue) and females (teal) for healthy volunteers and ACS Arm-1 and Arm-2 cohorts (left). The plot on the right shows the comparison between males and females at screening (sample 1), first follow-up (sample-2) and second follow-up (sample 3). Data presented are mean ± 95% CI, and analysed by two-way ANOVA Table 3.95 with Mauchly's test specificity for interaction p>0.05.

A Mauchly's Test of Sphericity was next performed to confirm the [PRDX-4] ANOVA findings. This particular test evaluates sphericity in the data as appose to variance and is required to satisfy Assumption #8 (Section 2.24.4.1). The results are presented in Table 3.95.

Table 3.95 Mauchly's Test of sphericity between gender and healthy and ACS cohort.

Measure: Peroxiredoxin_4

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b] Greenhouse-Geisser	Epsilon[b] Huynh-Feldt	Epsilon[b] Lower-bound
Time	.960	1.428	2	.490	.962	1.000	.500

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept + ARM + Gender + ARM * Gender
Within Subjects Design: Time

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Table 3.95

The Mauchly's test of sphericity indicated that the assumption of sphericity was met for the two-way interaction (p=0.490). This finding indicates that the relationship between the different pairs of conditions is similar (i.e., males vs females, healthy vs ACS and sample time).

Therefore, there is no evidence to suggest that plasma PRDX-4 concentration level differs between males and females, for healthy and ACS cohorts. The is also no difference in plasma PRDX-4 concentration between sample time and sex.

Table 3.96. Multiple comparisons of blood plasma sample PRDX-4 between healthy and ACS cohorts.

Dependent Variable: PRDX_4 mean Sample-1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1360.239[a]	2	680.119	4.947	.008	.066
Intercept	40857.843	1	40857.843	297.210	<.001	.681
ARM	1360.239	2	680.119	4.947	.008	.066
Error	19108.522	139	137.471			
Total	60234.970	142				
Corrected Total	20468.761	141				

a. R Squared = .066 (Adjusted R Squared = .053)

Table 3.96

To evaluate the interaction between male vs female and healthy vs ACS, a Mauchly's multiple comparison 'within-subjects' test was performed, where individual participants are compared with themselves over time. Here the means were evaluated with respect to 'time' i.e., the point at which the blood sample was taken (sample-1, sample-2 and sample-3). The data presented in Table 3.96 and 3.97 illustrates that there was statistically significant interaction between sample time and ACS Arm ($p<0.05$) but not gender ($p>0.05$). The data presented in Figure 3.35 shows that the mean data for males and females is parallel, indicating change in plasma [PRDX-4] between groups and sex. However, Table 3.89 shows that there was no significant main effect on concentrations ($p>0.05$).

Table 3.97. Post Hoc multiple comparisons of blood plasma sample [PRDX-4] between healthy and ACS cohorts and gender.

Dependent Variable: PRXD_4 mean Sample 1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1645.200[a]	5	329.040	2.377	.042	.080
Intercept	31226.520	1	31226.520	225.611	<.001	.624
ARM	940.114	2	470.057	3.396	.036	.048
Gender	176.464	1	176.464	1.275	.261	.009
ARM * Gender	29.377	2	14.688	.106	.899	.002
Error	18823.561	136	138.409			
Total	60234.970	142				
Corrected Total	20468.761	141				

a. R Squared = .080 (Adjusted R Squared = .047)

Table 3.97

Next the interaction between male vs female and healthy vs ACS, was made by a Mauchly's multiple comparison 'between-subjects' test, where participant groups are compared over time. This type of test is more susceptible to individual participant variation. The results presented in Table 3.98 show that there was significance between ACS Arm-1 and healthy cohort ($p<0.05$) however, no significant interaction between ACS Arm-2 and healthy cohort ($p>0.05$). The was also a statistical significance between ACS Arm-1 and 2 at sample 3 (Follow-up three) Table 3.100. Taken together, these multiple comparison tests indicate that, the time at which the blood sample was taken has an impact on plasma PRDX-4 level.

As a final analytical step, a Tukey's Honestly Significant Difference (HSD) test was performed for plasma PRDX-4 concentration, which compares the ANOVA means based on the studentized data range. The data presented in Table 3.98 shows that there was statistically significant interaction between the ACS and healthy cohort, with respect to plasma PRDX-4 concentration ($p<0.05$). The pairwise comparison illustrates that there is significant difference between plasma PRDX-4 concentration for healthy volunteer's vs ACS Arm-1 (Table 3.98, $p=0.05$) but no statistical significance between healthy volunteer's vs ACS Arm-2 ($p>0.05$).

Table 3.98. Pairwise comparisons of blood plasma PRDX-4 levels in Healthy and ACS cohorts.

Dependent Variable: PRDX_4 mean Sample-1

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-	Std. Error	Sig.[b]	95% Confidence Interval for Lower Boun	95% Confidence Interval for Upper Boun
Healthy Volunteer	Arm-2	-2.719	2.476	.274	-7.615	2.177
	Arm-1	-7.239*	2.797	.011	-12.769	-1.708
Arm-2	Healthy Volunteer	2.719	2.476	.274	-2.177	7.615
	Arm-1	-4.519	3.102	.147	-10.653	1.615
Arm-1	Healthy Volunteer	7.239*	2.797	.011	1.708	12.769
	Arm-2	4.519	3.102	.147	-1.615	10.653

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 3.99. Tukey multiple comparisons of blood plasma sample 1 PRDX-4 levels between healthy and ACS cohorts

Dependent Variable: PRXD_4 mean Sample 1

Tukey HSD

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Healthy Volunteer	Arm 2	-3.015	2.3040	.393	-8.475	2.445
	Arm 1	-7.819*	2.4967	.006	-13.735	-1.903
Arm 2	Healthy Volunteer	3.015	2.3040	.393	-2.445	8.475
	Arm 1	-4.804	2.6864	.177	-11.170	1.562
Arm 1	Healthy Volunteer	7.819*	2.4967	.006	1.903	13.735
	Arm 2	4.804	2.6864	.177	-1.562	11.170

Based on observed means.
The error term is Mean Square(Error) = 138.409.

Table 3.99

*. The mean difference is significant at the .05 level.

Table 3.100. Pairwise comparisons of blood plasma sample 3 PRDX-4 levels in Healthy and ACS cohorts.

Dependent Variable: PRXD_4 mean Sample 3

(I) ACS V Healthy Cohort	(J) ACS V Healthy Cohort	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Arm 2	Arm 1	-8.121*	3.996	.049	-16.190	-.051
Arm 1	Arm 2	8.121*	3.996	.049	.051	16.190

Based on estimated marginal means

Table 3.100

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 3.100 show that there were significant differences in plasma PRDX-4 concentrations between ACS Arms ($p<0.05$). Taken together, the PRDX-4 two-way mixed ANOVA highlighted an interaction between ACS Arm and blood sample time (Muchly test), which was driven by the female cohort. This is highlighted by differences in plasma PRDX-4 concentration between males and females (Tukey test).

Data filed – Thesis Two-way PRX-4.spv (Document6) Output and Thesis Two-way PRX-4.sav DataSet1) Data-IBM SPSS.

3.67 Peroxiredoxin-4 (PRDX-4) receiver operator curve (ROC).

Following the analysis presented above, which illustrates changes in the plasma biomarkers, peroxiredoxin-4 (PRDX-4) for ACS participants a Receiver Operating Curve (ROC) analysis was next carried out. This was completed to ascertain the estimate the probability of event prediction, in this instance to ascertain the probability of whether an ACS event has occurred.

Sensitivity (true positives) for [PRDX-4] for blood taken at screening (sample-1) was correctly predicted in 78.2%. Specificity, percentage of all observed as Healthy Cohort correctively predicted as 64.1 %. See Table 3.101. The efficiency of [PRDX-4] is calculated as (78.2%+64.1%) / (78.2%+64.1%+21.8%+35.9%) = 71.15%. In other words, blood plasma [PRDX-4] predicts a correct diagnosis 71.15% of the time.

Table 3.101 Percentage accuracy in classification for [PRDX-4] biomarker.

Table 3.101

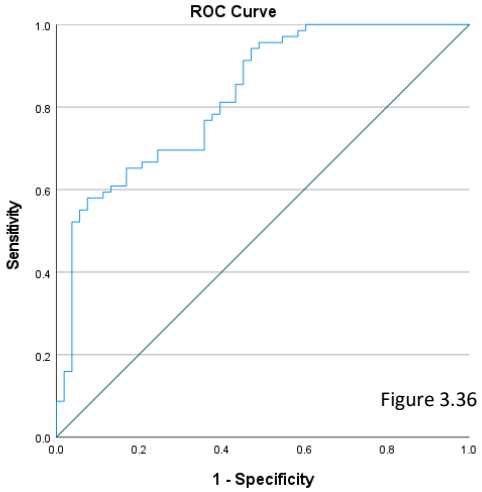
			Predicted		
			Presence of Heart Disease ALL/ACS		
	Observed		Healthy Cohort	ACS Cohort	Percentage Correct
Step 1	Presence of Heart Disease ALL/ACS	Healthy Cohort	41	23	64.1
		ACS Cohort	17	61	78.2
	Overall Percentage				71.8

a. The cut value is .500

The positive predictive value (percentage correctly predicted) for plasma PRDX-4 concentration at screening, which relates to 'observed characteristics' compared to 'case predictive characteristics' is 100 x (61 ÷ (23 + 61)) = 72.6 %. This means that 72.6 % of ACS cases are correctly predicted by evaluating plasma PRDX-4 concentration at screening. The negative predictive value, which relates to cases 'without the observed characteristics' compared to 'cases predicted not having the disease characteristic' is 100 x (41 ÷ (41 + 17)) = 70.6 %. This means that 70.6 % of non-ACS cases are correctly predicted by evaluating plasma PRDX-4 concentration at screening.

Figure 3.36 illustrates the ROC curve was analysis for [PRDX-4]. Data included was plasma PRDX-4 concentrations at screening (sample-1) for the ACS cohort (n=80) and the healthy cohort (n=65). The area under the curve was determined as 0.826 (95% CI = 0.826 to 0.899).

Table 3.102. Area Under the Curve analysis for blood plasma PRDX-4



Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Boun	Upper Bound
.826	.036	.000	.826	.899

Figure 3.36 and Table 3.102 Receiver operator curve (ROC) analysis for blood plasma [PRDX-4]. The data presented illustrate the clinical utility of blood plasma PRDX-4 for the diagnosis of ACS. Blood plasma PRDX-4 concentrations for the healthy donor cohort i.e., 'true negatives' (specificity) was plotted with the ACS cohorts i.e., 'true positives' (sensitivity). The area under the curve was determined as 0.826 with a 95% CI or 0.753 to 0.889. This indicates that blood plasma PRDX-4 alone may predict a correct ACS diagnosis in 82.6% of cases.

Data filed – ROC PRDX – 4 Thesis.spv (Document1) Output and ROC All Biomarkers. Sav (DataSet1) Data-IBM SPSS Statistics Data

3.68 Kaplan-Meier.

The ROC analysis presented above demonstrates clinical utility for each of the plasma biomarkers for ACS diagnosis, as determined by area under the curve. This was substantiated by specificity, sensitivity, and efficiency calculations, which showed that [PRDX-4] biomarker was able to predict a correct result i.e., determine a 'true positive' and 'true negative' in >82.6% of cases. Therefore, to take this analysis further, it was next important to evaluate whether [PRDX-4], could reliably predict ACS participant outcomes, in this case the time to event endpoint was an ACS readmission.

3.69 Logistic regression predictions.

Using binomial logistic regression to predict if cases can be correctly predicted from the independent variables, it was then analysed which independent variable contributed and its statistical significance.

The variables in the equation below show each independent variable and statistical significance. The odds ratio ("Exp B" column) was used to predict the probability of an event occurring. Odds Ratio of each independent variable recorded below in tables A, B, C, D along with the confidence Intervals, showing the change in log odds occurring for one-unit change in independent variable, keeping the other independent variables constant.

Table 3.103. Logistic regression predicting likelihood of ACS event based on age, BMI, gender and peroxiredoxin-4.

Table 3.103		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	Age	.083	.016	25.985	1	<.001	1.087	1.053	1.122
	BMI	.093	.054	2.908	1	.088	1.097	.986	1.220
	Gender (1)	.771	.436	3.132	1	.077	2.162	.920	5.076
	PRDX_4	.012	.018	.404	1	.525	1.012	.976	1.048
	Constant	-7.977	1.992	16.033	1	<.001	.000		

a. Variable(s) entered on step 1: Age, BMI, Gender, PRDX-4 mean Sample-1.

The statistical significance with respect to [PRDX-4] found that age (p<0.001) added significantly to the predictions model. However, BMI (p=0.088) and gender (p=0.77) did not add. For [PRDX-4] males had 2.16 (95% CI, 0.920 to 5.076) times higher odds to exhibit ACS than females (see Table 3.103).

Having satisfied the robustness of the plasma biomarkers [PRDX-4] a non-parametric Kaplan-Meier analysis was next performed to determine if these biomarkers impacted on participant prognosis and were able to predict the probability of an ACS readmission following stratification.

3.70 Kaplan-Meier All ACS participants peroxiredoxin-4 (PRDX-4).

The ACS cohort (n=78) displayed events and censoring at sample-1 (screening blood sample) as displayed in Table 3.104. The sample-1 PDRX-4 quartile means were subsequently calculated.

Table 3.104. Readmission analysis based on blood plasma [PRDX-4] ng/ml at less than 25%, median and greater than 75% percentile as cut-off values.

Table 3.104

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 9.9 25% percentile	20	6	14	70.0%
Median >10.00 ~ < 29.00 (18.45)	37	11	26	70.3%
> 29.2 75% percentile	21	7	14	66.7%
Overall	78	24	54	69.2%

For blood plasma analysis at sample-1 the ACS participants (n=78) were stratified according to PRDX-4 concentration. There were 20 participants in the <25% percentile (PRDX-4 <9.9 ng/ml), however 14 were censored (70.0%). For the 25%-inter-percentile range (PRDX-4 >10.00 ng/ml~<29.00 ng/ml), there were 37 participants, however 26 were censored (70.3%). Finally, there were 21 participants in the >75% percentile (PRDX-4 >29.2 ng/ml), however 14 were censored (66.7%). Taken together, a total of 69.2% ACS participants were censored (Table 3.104). For sample-1, sample-2 and sample-3 there were no statistically significant findings. Sample-3 (6 months from index event in all participants) there was no statistically significant difference in the readmission rate between the >75% PRDX-4 concentration stratified ACS participants, compared with the median PRDX-4 concentration, $\chi 2(1) = 3.078$, $p=0.079$ (Table 3.105).

Table 3.105. Multiple pairwise comparisons for blood plasma [PRDX-4] ng/ml cut off values in sample 3.

Table 3.105

	Intervention Sample 3 ACS 25% ~ 75% percentile	< 9.90 25% percentile		Median >10.00 ~ < 29.00 (18.45)		> 29.25 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 9.90 25% percentile			.340	.560	1.084	.298
	Median >10.00 ~ < 29.00 (18.45)	.340	.560			3.078	.079
	> 29.25 75% percentile	1.084	.298	3.078	.079		

Figure 3.37 illustrates a visual representation of the data presented in Table 3.105, there was no significant reduction in readmissions due to a second ACS event at sample-3.

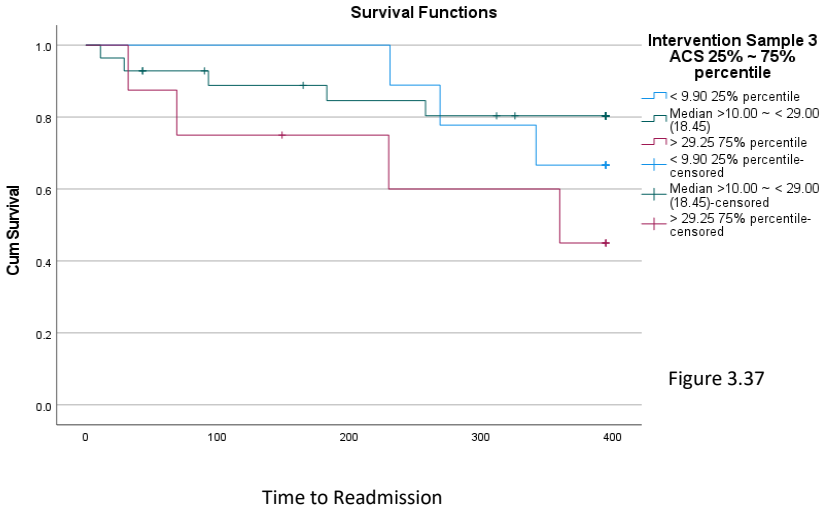


Figure 3.37

Figure 3.37. Readmission analysis using various blood plasma [PRDX-4] ng/ml cut-off values. To determine whether blood plasma PRDX -4 (ng/ml) could predict participant outcome (overall survival without ACS readmission), Kaplan-Meier analysis was performed. Participants were stratified according to blood plasma [PRDX-4]. **1)** Blue line represents the 25% percentile, PRDX-4 <9.99 ng/ml. **2)** Green line represents 25%-75%. percentile, PRDX-4 > 10.00 ~ <29.00 ng.ml. **3)** Red line represented the 75% percentile, PRDX-4 >29.25 ng/ml. χ^2 analysis p=>0.05.

Data Filed - ALL KM Sample1-3 [PRDX-4].SAV [DataSet1] and ALL KM Sample 1-3 PRDX-4.SPV [Document5] IBM SPSS Statistics Output.

3.71 Kaplan-Meier for plasma peroxiredoxin-4 concentration percentiles

To address the aim and objectives the final PRDX-4 plasma concentrations were then analysed to determine if indicative of an ACS event and/or predict a second event, the Kaplan-Meier analysis was systematically performed for PRDX-4. Initially, this was performed for the 'blood sample-1' PRDX-4 percentile concentrations. To recap, these percentile concentrations were calculated as <9.9 ng/ml for the <25% percentile (n=7), >10.00~<29.0 ng/ml for the inter-percentile range (n=17) and 29.2ng/ml for the >75% percentile (n=10). Summarised data are presented in Table 3.106.

Table 3.106. Readmission analysis based on percentage of admissions and censored cases of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma PRDX-4 ng/ml as the cut-off values.

Table 3.106

Intervention Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 9.9 25% percentile	7	6	1	14.3%
Median >10.00 ~ < 29.00 (18.45)	17	11	6	35.3%
> 29.2 75% percentile	10	7	3	30.0%
Overall	34	24	10	29.4%

The participants in the <25% range for plasma PRDX-4 concentration had the lowest time to readmission of 93.0 days (95% CI, 0.00 to 222.2) days. The time to readmission values for the inter-percentile and >75% plasma PRDX-4 concentration participants were higher at 102 and 231 days respectively, see Table 3.107.

Table 3.107. Readmission analysis in days for baseline blood plasma [PRDX-4] ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.107

Intervention Sample 1 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 9.9 25% percentile	131.190	53.903	25.541	236.840	93.000	65.932	.000	222.226
Median >10.00 ~ < 29.00 (18.45)	196.045	48.662	100.668	291.422	102.000	42.185	19.318	184.682
> 29.2 75% percentile	216.856	38.362	141.667	292.045	231.000	31.846	168.582	293.418
Overall	187.889	28.606	131.821	243.958	183.000	84.329	17.715	348.285

a. Estimation is limited to the largest survival time if it is censored.

Assumption #4 (Section 2.24.4.1) states that for time to event statistics, there is similar censoring. The percentage of censored cases present in the <25 % percentile was 14.3%, compared to 35.3% and 30.0% for the inter-percentile and >75% percentile groups. Based on this, it is clear that the censoring of the healthy and ACS cohort groups was not similar.

Figure 3.38 provides a visual representation of the data presented in Table 3.99. The plot shows a small interaction i.e., crossing of survival curves between <25 and inter-percentile. However, in general those, participants with <25% plasma PRDX-4 concentrations (<9.9 ng/ml) have a greater incidence of readmission than those with inter-percentile concentrations, those with >75% (>29.2ng/ml) show an initial reduction readmission.

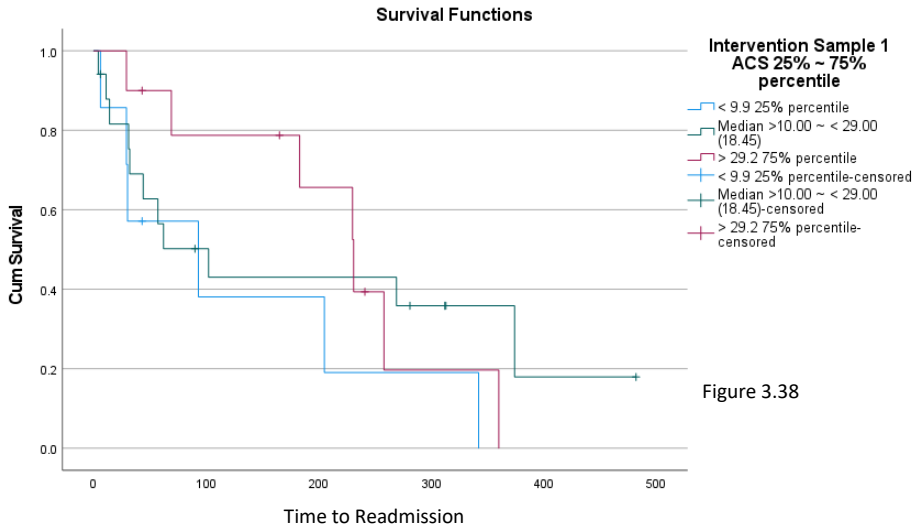


Figure 3.38

Figure 3.38. Readmission analysis using various blood plasma [PRDX-4] ng/ml cut-off values of baseline sample 1. To determine whether blood plasma PRDX-4 (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood [PRDX-4]. **1)** Blue line represents the 25% percentile, PRDX-4 <9.99 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-4 > 10.00 ~ <29.00 ng.ml. **3)** Red line represented the 75% percentile, PRDX-4 >29.25 ng/ml. χ^2 analysis p=>0.05.

To follow-on from this, a log rank test was conducted, which showed that there were no statistical differences in the admission rate for the three plasma concentrations, $\chi2(2) = 2.009$, p=0.366.

Next the same analysis was conducted on plasma [PRDX-4] for blood sample-2 (first follow-up). The data presented in Table 3.108 shows the percentage of censored cases present in the <25% percentile (60.0% ACS participants), the inter-percentile range (28.6% ACS participants) and >75% percentile (28.6% ACS participants). These data illustrate that the proportion of censored groups was not similar.

Table 3.108. Readmission analysis at less than 25%, Median and greater than 75% percentile of blood plasma PRDX-4 ng/ml as the cut-off values at sample 2.

Table 3.108

Intervention Sample 2 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 9.9 25% percentile	5	2	3	60.0%
Median >10.0 ~ < 28.0 (18.45)	14	10	4	28.6%
> 29.2 75% percentile	7	5	2	28.6%
Overall	26	17	9	34.6%

Participants with the >75% plasma PRDX-4 concentration had a median time to readmission of 183.0 (95% CI, 40.8 to 325.1) days. The inter-percentile PRDX-4 plasma concentration group which was 258.0 days (95% CI, 216.2 to 299.7) days. The confidence interval was not able for the <25% group due to n=5, where participants censored was n=3.

Table 3.109. Readmission analysis in days for baseline blood plasma PRDX-4 ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.109

Intervention Sample 2 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 9.9 25% percentile	160.500	58.958	44.942	276.058	57.000	.	.	.
Median >10.0 ~ < 28.0 (18.45)	244.193	41.431	162.988	325.397	258.000	21.277	216.298	299.702
> 29.2 75% percentile	163.911	63.914	38.639	289.182	183.000	72.507	40.886	325.114
Overall	216.821	32.813	152.507	281.134	231.000	50.372	132.271	329.729

a. Estimation is limited to the largest survival time if it is censored.

Figure 3.39 provides a visual representation of this data in Table 3.110, highlighting that the intra-percentage plasma [PRDX-4] group display a reduction in ACS readmission time, compared with those in the inter-percentile range.

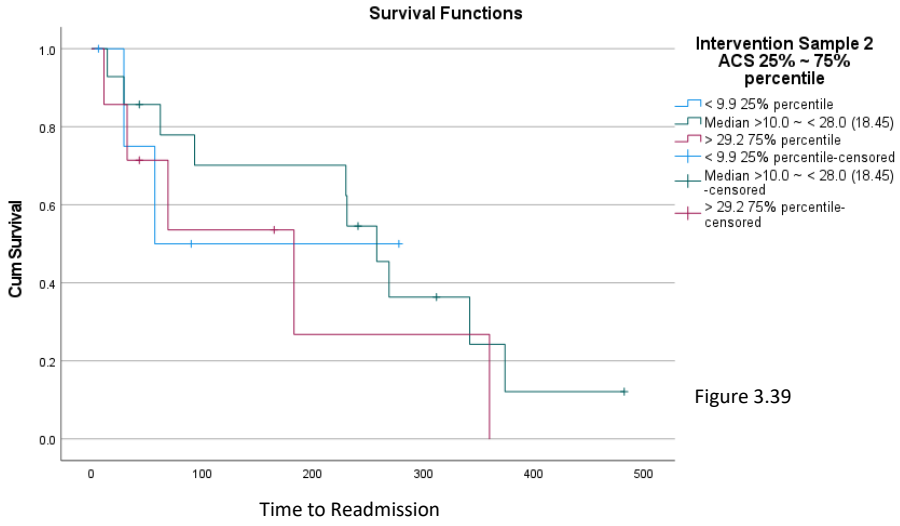


Figure 3.39

Figure 3.39. Readmission analysis using various blood plasma [PRDX-4] ng/ml cut-off values of baseline sample 2. To determine whether blood plasma PRDX -2 (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood [PRDX-4]. **1)** Blue line represents the 25% percentile, PRDX -4 <9.99 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-4 > 10.00 ~ <29.00 ng.ml. **3)** Red line represented the 75% percentile, PRDX -4 >29.25 ng/ml. χ^2 analysis p=>0.05.

The survival readmission distributions for the three plasma PRDX-4 concentrations based on sample-2 were not statistically significant, p>0.05 (Table 3.110), as determined by Log rank pairwise comparison.

Table 3.110 The survival readmission distributions for the three healthy and ACS cohort sample 2 blood plasma PRDX-4 ng/ml levels.

Table 3.110

	Intervention Sample 2 ACS 25% ~ 75% percentile	< 9.9 25% percentile		Median >10.0 ~ < 28.0 (18.45)		> 29.2 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 9.9 25% percentile			.080	.777	.027	.870
	Median >10.0 ~ < 28.0 (18.45)	.080	.777			1.107	.293
	> 29.2 75% percentile	.027	.870	1.107	.293		

Finally, analysis was performed for plasma PRDX-4, based on blood collected at Sample-3 (2nd follow-up). The percent of censored ACS cases present in the <25 % plasma PRDX-4 concentration was 0%, compared with the inter-percentile range (54.0%) and >75 % (20.0%). Thus, the censored groups were not similar (Table 3.111).

Table 3.111. Readmission analysis in days for baseline blood plasma PDRX-4 ng/ml levels as the cut-off values at various cut offs for participants readmitted with ACS.

Table 3.111

Intervention Sample 3 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
			N	Percent
< 9.90 25% percentile	3	3	0	0.0%
Median >10.00 ~ < 29.00 (18.45)	11	5	6	54.5%
> 29.25 75% percentile	5	4	1	20.0%
Overall	19	12	7	36.8%

Participants with the <25% plasma PRDX-4 concentration had a median time to readmission of 269 days (95% CI, 2.08 to 436.1) days and inter-percentile plasma PRDX-4 concentration group had a median readmission time of 93.0 days (95% CI, 40.0 to 146.0) days compared to >75% PRDX-4 plasma concentration group which was 69 days. See Table 3.112 for full summary.

Table 3.112. Readmission analysis of baseline blood plasma [PRDX-4] ng/ml levels as the cut-off values less than 25%, median and greater than 75% percentile for participants readmitted with ACS.

Table 3.112

Intervention Sample 3 ACS 25% ~ 75% percentile	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
< 9.90 25% percentile	280.667	32.570	216.830	344.503	269.000	31.027	208.187	329.813
Median >10.00 ~ < 29.00 (18.45)	255.807	65.013	128.381	383.232	258.000	90.905	79.826	436.174
> 29.25 75% percentile	172.750	75.782	24.217	321.283	69.000	99.000	.000	263.040
Overall	228.576	36.637	156.768	300.384	231.000	42.997	146.725	315.275

a. Estimation is limited to the largest survival time if it is censored.

Figure 3.40 provides a visual representation of this data, highlighting that the >75% plasma [PRDX-4] group display an increase in ACS readmission time, compared with those in the <25% and inter-percentile range.

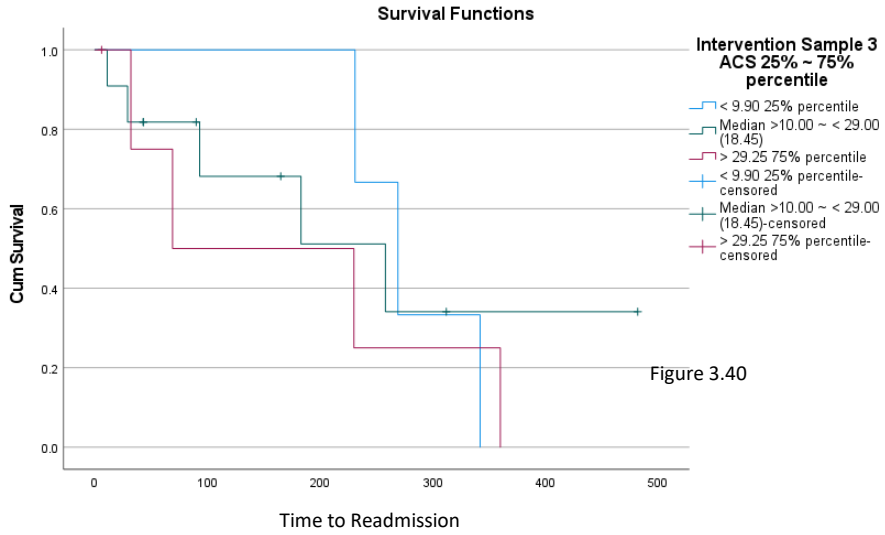


Figure 3.40

Figure 3.40. Readmission analysis using various blood plasma [PRDX-4] ng/ml cut-off values of baseline sample 3. To determine whether blood plasma PRDX -4 (ng/ml) could predict participant readmission rate (overall survival), Kaplan-Meier analysis was performed. Participants were stratified according to blood [PRDX-4]. **1)** Blue line represents the 25% percentile, PRDX-4 <9.99 ng/ml. **2)** Green line represents 25%-75% percentile, PRDX-4 > 10.00 ~ <29.00 ng.ml. **3)** Red line represented the 75% percentile, PRDX-4 >29.25 ng/ml. χ^2 analysis p=>0.05

The survival readmission distributions for the three plasma PRDX-4 concentrations based on sample-3 were not statistically significant, p>0.05.

Data filed- KM sample 1-3 PRDX -4 SC. spv [document 13] output KM Sample 1-3 PRDX -4 SC. sav{dataset1]

3.72 Kaplan-Meier for lesions of Percutaneous Coronary Intervention (PCI).

The final set of analyses was to establish whether the [PRDX-4] plasma biomarkers had any predictive value for ACS readmission with respect to ACS percutaneous coronary intervention (PCI) i.e., Right Coronary Artery (RCA), circumflex or Left Anterior Descending (LAD). ACS participants for blood sample-1 were subsequently stratified according to lesion of PCI as outlined in Table 3.113.

Table 3.113. Readmission analysis based on lesion of ACS event that resulted in baseline PCI

3.113

Lesion of PCI	Total N	N of Events	Censored	
			N	Percent
RCA	39	13	26	66.7%
Circumflex	11	3	8	72.7%
LAD	30	8	22	73.3%
Overall	80	24	56	70.0%

Overall (n=80) ACS participants were included in this analysis, of these participants the PCI event is broken down as follows: RCA (n=39), Circumflex (n=11) and LAD artery (n=30), see Table 3.113.

[PRDX-4] was reviewed with respect to the lesion of PCI event. As previously described, participants were further stratified according to plasma biomarker concentration i.e., <25%, inter-percentile range (>25% ~ <75%) and >75%. As previously stated, the design was non-event driven and all participant survival status was known at end of study. This limited the events of interest to (n=35) of all ACS admissions.

3.73 Peroxiredoxin-4 – Kaplan-Meier readmission relating to Acute Myocardial Infarction lesion.

Initially, the impact of PCI with respect to plasma [PRDX-4] concentration was evaluated. This related to n=1 participants who received PCI to RCA, n=5 to circumflex and n=11 for LAD. The full breakdown is presented in Table 3.114.

Table 3.114. Readmission analysis based on lesion of PCI of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma PRDX-4 ng/ml cut- off values.

Table 3.114

Lesion of PCI	PRXD-4 Sample 1 ACS 25% ~ 75% percentile	Total N	N of Events	Censored	
				N	Percent
RCA	< 9.9 25% percentile	5	4	1	20.0%
	Median >10.00 ~ < 29.00 (18.45)	7	4	3	42.9%
	> 29.2 75% percentile	6	5	1	16.7%
	Overall	18	13	5	27.8%
Circumflex	< 9.9 25% percentile	1	1	0	0.0%
	Median >10.00 ~ < 29.00 (18.45)	2	1	1	50.0%
	> 29.2 75% percentile	2	1	1	50.0%
	Overall	5	3	2	40.0%
LAD	< 9.9 25% percentile	1	1	0	0.0%
	Median >10.00 ~ < 29.00 (18.45)	8	6	2	25.0%
	> 29.2 75% percentile	2	1	1	50.0%
	Overall	11	8	3	27.3%
Overall	Overall	34	24	10	29.4%

Patients presenting with AMI to in LAD where [PRDX-4] was <25%, had a much higher time to readmission of 93.0 days compared with LAD AMI for [PRDX-4] in the medium range of 44 days (95% CI, 36.9 to 149 days), see Table 3.115.

Readmission times were higher for all PRDX-4 concentrations for RCA participants at 230 days to readmission (95% CI, 42.9 to 417 days), with circumflex PCI showing the highest time to readmission at 345 days, but CI unable to compute.

Table 3.115. ACS readmission analysis based on lesion of ACS event that resulted in baseline PCI of [PRDX-4] ng/ml levels as the cut-off values less than 25%, median and greater than 75% percentile.

Table 3.115

		Mean[a]				Median			
				95% Confidence Interval				95% Confidence Interval	
Lesion of PCI	PRXD-4 Sample 1 ACS 25% ~ 75% percentile	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
RCA	< 9.9 25% percentile	95.000	46.592	3.681	186.319	30.000	1.095	27.853	32.147
	Median >10.00 ~ < 29.00 (18.45)	186.571	47.785	92.912	280.230	276.000	166.747	.000	602.825
	> 29.2 75% percentile	236.167	49.512	139.123	333.210	231.000	13.717	204.114	257.886
	Overall	181.025	33.069	116.209	245.841	230.000	95.422	42.973	417.027
Circumflex	< 9.9 25% percentile	342.000	.000	342.000	342.000	342.000	.	.	.
	Median >10.00 ~ < 29.00 (18.45)	178.500	104.298	.000	382.925	31.000	.	.	.
	> 29.2 75% percentile	183.000	.000	183.000	183.000	183.000	.	.	.
	Overall	237.400	70.660	98.906	375.894	342.000	.000	.	.
LAD	< 9.9 25% percentile	93.000	.000	93.000	93.000	93.000	.	.	.
	Median >10.00 ~ < 29.00 (18.45)	127.625	41.516	46.254	208.996	44.000	49.497	.000	141.015
	> 29.2 75% percentile	117.000	33.941	50.475	183.525	69.000	.	.	.
	Overall	132.545	33.590	66.709	198.382	93.000	31.928	30.421	155.579
Overall	Overall	174.583	24.142	127.265	221.902	205.000	80.541	47.139	362.861

a. Estimation is limited to the largest survival time if it is censored.

The data indicate that ACS participants who received PCI to LAD, had a greater readmissions rate for all plasma concentrations of PRDX-4, the overall percentage was less when compared with circumflex and RCA. Table 3.115 shows that ACS participants with culprit lesion at PCI was LAD, had a median time of 93 days to readmission (95% CI, 30.4 to 155.6) days. For RCA participants, the time to readmission was longer at 230 days (95% CI, 42.9 to 417) days. Circumflex lesions had a median readmission time of 342 days; however, it was not possible to determine the 95% CI due to the low participant number (n=5). Visualised data in Figure 3.41.

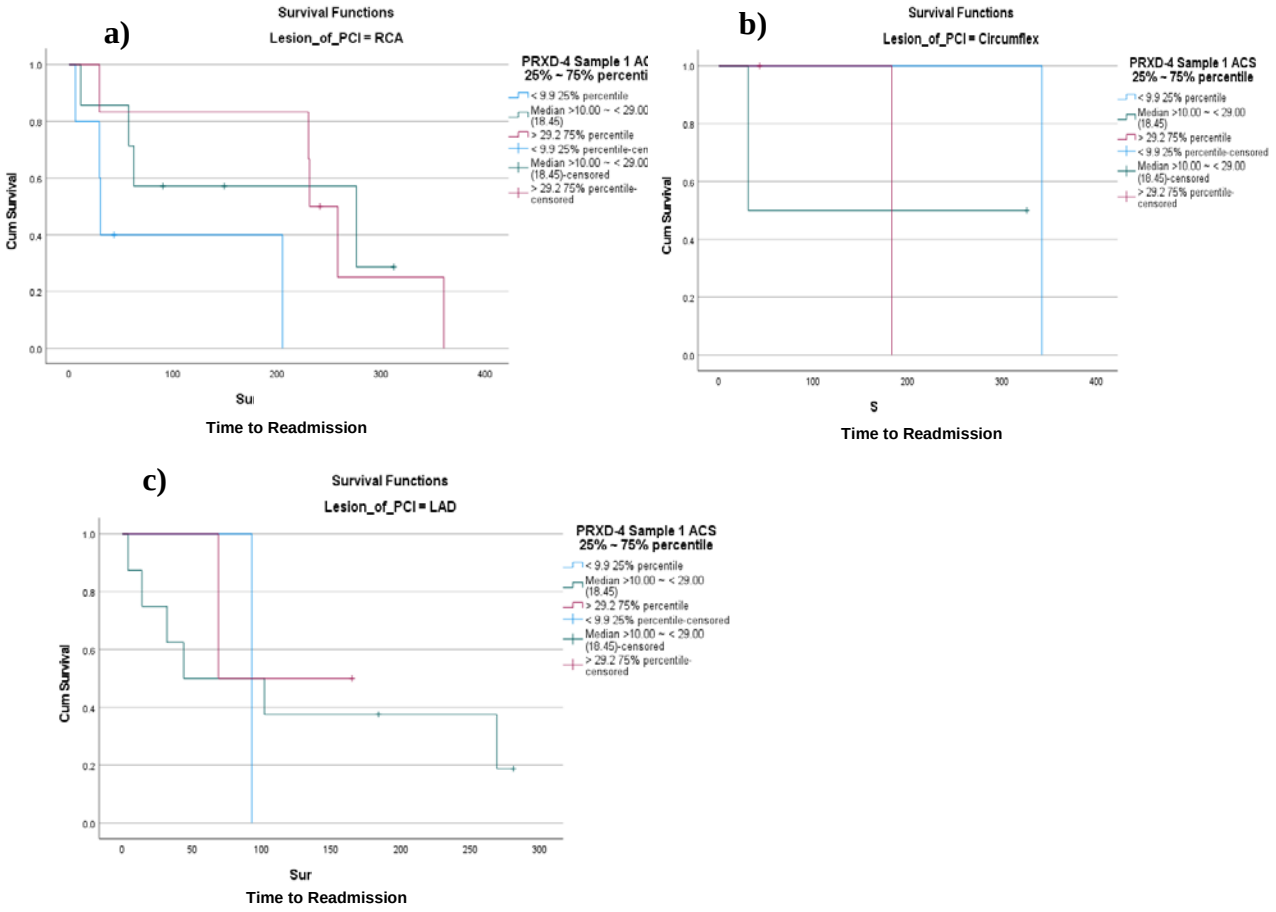


Figure 3.41. ACS Readmission analysis based on lesion of PCI of baseline blood levels at less than 25%, median and greater than 75% percentile of blood plasma [PRDX-4] ng/ml cut-off values. a) Represents PCI to RCA, **b)** represents circumflex PCI and **c)** represents PCI LAD. [PRDX-4]. Blue line represents the 25% percentile, PRDX-4 <9.99 ng/ml. Green line represents 25%-75% percentile, PRDX-4 > 10.00 ~ <29.00 ng.ml. Red line represented the 75% percentile, PRDX-4 >29.25 ng/ml. χ^2 analysis p>0.05

Log rank pairwise between the three stratified PRDX-4 plasma concentrations and lesion PCI did not show statistical significance (P>0.05) (Table 3.108).

Table 3.116 The readmission distribution between lesion of PCI of baseline of healthy and ACS cohort blood plasma PRDX-4 ng/ml levels.

Table 3.116

	PRXD-4 Sample 1 ACS 25% ~ 75% percentile	< 9.9 25% percentile		Median >10.00 ~ < 29.00 (18.45)		> 29.2 75% percentile	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	< 9.9 25% percentile			1.370	.242	1.977	.160
	Median >10.00 ~ < 29.00 (18.45)	1.370	.242			.110	.740
	> 29.2 75% percentile	1.977	.160	.110	.740		

a. Adjusted for Lesion of PCI .

3.74 Brief discussion of peroxiredoxin-4 findings.

The data presented in this chapter showed an overall statistically significant increase in mean plasma PRDX-4 concentration for ACS participants (19.0 ng/ml) compared to the healthy cohort (13.9 ng/ml) ($p<0.0077$). Following censoring, the healthy cohort population had a mean plasma [PRDX-4] as 11.9 ng/ml, giving a potential predictor for an upper limit of normal (ULN). There was an identified difference between sexes, where healthy females displayed the higher concentrations between the in ACS cohort (17.4 ng/ml) and healthy population (12.0 ng/ml). Thus, this finding may be important in the future for determining female AMI patient prognosis.

To the best of knowledge at the time of writing, this study is unique in its evaluation of plasma PRDX-4 in a healthy cohort in this way. However, other studies have evaluated PRDX-4 in healthy participants to understand how plasma values change during stress exercise. The values reported in this chapter are higher than those reported by Wadley et al., (2019) which evaluated younger individuals (median 29 years) and showed baseline the concentration of ~5.5 ng/ml for PRDX-4, which almost doubled 60 minutes post exercise (Wadley *et al*., 2019). For the healthy cohort, inclusion numbers were relatively matched in numbers, the under 55's (n=34) had a mean plasma PRDX-4 concentration of 11.8 ng/ml compared to over 55's (n=29) who had a mean concentration of 16.6 ng/ml.

The results presented in Table 3.96 and 3.97 illustrate significance differences between ACS Arm-1 and the healthy cohort ($p<0.05$) with no significant difference between ACS Arm-2 and healthy cohort ($p>0.05$). Furthermore, there was a statistical significance between ACS Arm-1 and 2 at sample 3 (Follow-up 2), which was six months post AMI.

Hyperglycemia is encountered in up to 50% of all STEMI AMI patients, whereas previously diagnosed Diabetes Mellitus is present in only 20% of patients, suggesting pancreatic involvement in STEMI AMI (Wahab 2002; Zarich et al., 2007; Kosiborod et al., 2010). Mouse models have indicated that PRDX-4 can protect pancreatic islet β-cells against oxidative injury (Ding *et al*., 2010). Therefore, there may be a link between PRDX-4 expression and metabolic disturbances occurring during a STEMI AMI that mediate hyperglycemia, which can burden patient outcome. However, this is speculative and the mechanism driving this notion needs to be elucidated. Other studies have indicated a protective role for PRDX-4 in heart failure and highlight a decrease in 'dilated

cardiomyopathy' in patients with elevated PRDX-4 levels (Ahmed *et al.*, 2020; Jeong et al., 2021). Interestingly, the mean plasma PRDX-4 concentration presented here at baseline for ACS readmission was 18.65 ng/ml, compared with the first and second follow-up samples, which were 23.26 ng/ml and 23.03 ng/ml respectively. PRDX-4 levels increase through the course of AMI recovery. This finding could thus potentially explain the poorer outcomes for participants with lower concentrations (Table 3.107), where protection from adverse cardiovascular events due to hyperglycemia are reduced (Eter and Masri 2015), as well as a reduction in cardiomyopathy (Ahmed *et al.*, 2020; Jeong et al., 2021). These findings represent a rational for monitoring the ACS patient's plasma PRDX-4, particularly for previously diagnosed diabetic and non-diabetic STEMI AMI patients (Savu et al., 2012; Eter and Masri 2015).

The ROC showed plasma PRDX-4 concentrations at baseline screening for the ACS cohort (n=80) and the healthy cohort (n=65) had an area under the curve determined as 82.6% thus, providing confidence in the biomarkers analysed would positively predict 4 in 5 ACS events. To investigate the impact of plasma [PRDX-4] on ACS readmission rates, Kaplan-Meier analysis was conducted to determine probability of 'time-to-readmission', based on biomarker stratification. Initial admissions were recorded in days from PCI (Appendix AA) and any admission that was not due to an ACS admission was censored. The ACS participants (n=80) were thus stratified according to PRDX-4 concentration at this point. There were 20 participants in the <25% percentile (PRDX-4 <9.9 ng/ml), however 14 were censored (70.0%) for non-ACS readmissions. For the 25%-75% median range (PRDX-2 >10.00 ng/ml~<29.0 ng/ml), there were 37 participants, however 26 were censored (70.3%). Finally, there were 21 participants in the >75% percentile (PRDX-4 >29.00 ng/ml), however 14 were censored (66.7%). The data show that ACS patients within the >75% percentile at second follow up, (after six months) reduced of readmission, potentially offering a possible cardiac protection.

Participants who had AMI in LAD, had a much greater readmission rate with low concentrations (<9.9 ng/ml) of PRDX-4 concentrations, with readmission within 93.0 days thus supporting the notion that higher levels offer not only cardiovascular protection, but reduce readmissions. Evaluating plasma [PRDX-4] at ACS screening may therefore be clinically relevant for risk stratifying patients at point of diagnosis, which may have some prognostic implications.

In conclusion, the data presented in this chapter highlight differences between plasma [PRDX-4] for healthy individuals and ACS patients, which is particularly pronounced in females. Plasma PRDX-4 levels reliably predicted correct diagnosis in ~82% cases and decreased levels could be a significate deprivation and predicative of patients requiring closer monitoring post AMI.

Several limitations with hs-cTn as a diagnostic biomarker were identified in Chapter 1, which included the notion that hs-cTn levels only increase after the AMI has occurred, therefore likely significant damage to the myocardium has already occurred (Thokala *et al.*, 2012; Raskovalova *et al.*, 2014). Also, determination of the ULN for hs-cTn can be challenging, which may pose problems for diagnosing certain demographics i.e., younger females (Ungerer *et al*., 2016; Apple *et al*., 2017; Humphries., 2020). Furthermore, serum concentration levels of hs-cTn does not accurately predict whether a patient is likely to be readmitted with a secondary ACS event. Finally, whilst hs-cTn is indicative of myocardial damage / necrosis, and possibly extent, it does not predict the coronary artery lesion that has caused the AMI in the first place, or indeed how the lesion of PCI relates to prognosis (Iftikhar *et al.,* 2022). These limitations provided a rationale for the overall aim of this study, which was to evaluate plasma levels of TRX, TRXr, PRDX-2 and PRDX-4 in ACS from the onset of chest pain, and at various time-points following the event i.e., 1-3 month and 6 months after the AMI. It was hoped that this information may help predict readmissions and overall outcome, but also assist with the diagnosis of younger females, as well as determining the prognostic impact following medical intervention i.e., PCI. To investigate this overall aim, the following objectives were identified:

a) Clarify the mean plasma concentrations for TRX, TRXr, PRDX-2 and PRDX-4 for healthy volunteers, stratified based on sex and age., which will be used as baseline measurements for ACS patient comparisons, as well as clinical utility evaluation, since the ‘healthy population’ are identified as ‘true negatives’ (specificity).

b) Evaluate plasma concentrations levels of TRX, TRXr, PRDX-2 and PRDX-4 for ACS patients stratified based on age and sex at initial diagnosis / screening and follow-up. Clinical utility may subsequently be evaluated, as the ‘ACS patients’ represent the ‘true positives’ (sensitivity).

c) Monitor the plasma concentration levels of TRX, TRXr, PRDX-2 and PRDX-4 through ACS patient follow-up sampling, in order to assess whether these biomarkers may be predictive of an ACS readmission.

d) Evaluate whether TRX, TRXr, PRDX-2 and PRDX-4 may predict readmission based on ACS patient stratified according to PCI.

4.1. General consideration of the plasma changes in TRX, TRXr, PRDX-2 and PRDX-4.

This study is unique in various aspects and is the first to collectively assess changes in these specific plasma biomarker concentrations in healthy populations, compared to ACS patients at diagnosis and through the disease course (follow-up). It was important that baseline levels of each biomarker were established for the identified 'healthy population' (n=65), so that comparison with the ACS patient cohort (n=80) could be made. The data presented in chapter 3 shows the average baseline measurements for the healthy population cohort, which related to 10.80 ng/ml for TRX, 0.63 ng/ml for TRXr, 24.98 ng/ml for PRDX-2 and 13.93 ng/ml for PRDX-4 (Figures 3.1, 3.11, 3.21 and 3.32). A recent study involving healthy individuals sought to establish changes in plasma concentration of TRX, TRXr, PRDX-2 and PRDX-4 in response to exercise, a process which is linked to the transient generation of ROS (Wadley *et al*., 2019). The authors showed that, at baseline the concentration of each biomarker 'pre-exercise' was ~2.6 ng/ml for TRX, ~17 ng/ml for TRXr, ~3.5 ng/ml for PRDX-2 and ~5.5 ng/ml for PRDX-4 (Wadley *et al*., 2019). The participants were then subjected to 2 different exercise protocols [1 muscle damaging and 1 non-damaging] and monitored for changes in the biomarkers at 3 time points 1) immediately post-exercise, 2) 30 mins post and 3) 60 mins post (Wadley *et al*., 2019). There are clear defenses in the baseline measurement between Wadley *et al*., (2019) and the data presented in chapter 3. It must be noted that in the Wadley *et al*., 2019 study, the participants were grouped according to exercise type, each of which had a mean participant age of 29 ± 5 for the energy matched trials group (n=9), compared with a mean age of 25 ± 9 for the eccentric based resistance exercise group (n=16). It is not reported what proportion of the participants were male or female, however the study reported plasma concentration increases in certain biomarkers in response to exercise, including TRX and PRDX-4 which almost doubled 60 minutes post exercise (Wadley *et al*., 2019). By

comparison, the data presented in chapter 3 for healthy volunteers relates to a higher aged population 48.9 ± 17.9 years which is more closely representative of the ACS patient cohort. This enabled stratification of the healthy cohort into <55 and >55 age groups, with 49% male and 51% female (Table 3.1). An explanation for the comparable increase in plasma biomarkers in the healthy cohort as presented in chapter 3, may be due to the observations that oxidative stress increases with age and is associated with the development of many age-related diseases, including CVD (Luo *et al.,* 2019; Izzo *et al.,* 2021). Therefore, the increases observed in the baseline plasma biomarker as reported in Figures 3.1, 3.11, 3.21 and 3.32 relative to Wadley *et al*., (2019) could reflect indolent underlying diseases of the aged, in which chronic oxidative stress is a pathological feature, although this is speculative (Ling Tan *et al.,* 2018).

It is challenging to draw more meaningful and direct comparisons with the literature, due to the lack of studies examining plasma concentrations of TRX, TRXr, PRDX-2 and PRDX-4 in health with respect to a young vs an aged population. Stratification of the healthy population by age revealed that the <55-year-old cohort had a significantly lower average plasma concentrations for TRX, TRXr and PRDX-4 compared with the >55-year-old cohort (Figures 3.2, 3.12 and 3.33). Thus, these findings provide the first evidence to suggest that an increase in the plasma concentration of these biomarkers maybe associated with age, supporting the notion that 'oxidative stress' increases with age, which may be due to low grade underlying undiagnosed chronic disease(s) (Luo *et al.,* 2019). In fact, the plasma concentrations for the <55 aged healthy cohort was 8.82 ng/ml for TRX and 13.93 ng/ml for PRDX-4 respectively which are more closely matched to the Wadley study (Wadley *et al.,* 2019). Chapter 3 shows that PRDX-2 changes little across the stratified age groups. This was also observed by Wadley *et al.,* (2019), where PRDX-2 remained constant through following exercise, whereas other biomarkers e.g., TRX and PRDX-4 increased (Luo *et al.,* 2019). Taken together, the data indicates that 3 out of the 4 biomarkers for the healthy cohort (TRX, TRXr and PRDX-4) show significant increases between age groups. To the best of knowledge, this study is the first to demonstrate this trend with respect to age in health. Since age impacts the plasma concentrations, these data are thus important for interpreting the ACS patient data, as discussed below.

Previous to this study, other researchers had evaluated blood plasma levels of TRX (Mongardon *et al.,* 2013) and PRDX-4 (Abbasi *et al.,* 2012) in cardiac syndromes. For the TRX study by Mongardon and colleagues, a total of 176 cardiac arrest syndrome patients were evaluated, and comparisons made between stratified 'survivor' and 'non-survivor' populations. The plasma TRX for both patient populations were evaluated on admission, as well at day 2 and day 3 post admission. The authors used a commercially available TRX ELISA kit (Randox Biosciences) for plasma TRX concentration estimation. Their findings indicated that the survivor group had average plasma TRX concentrations of 22 ng/ml on admission, compared with non-survivors who were significantly higher at 74 ng/ml, concluding the higher concentration level of plasma TRX was associated with high-risk patients (Mongardon *et al.,* 2013). The data presented in chapter 3 agrees with the general trend in plasma TRX concentration as reported by Mongardon *et al*., showing that high-risk ACS patients i.e., those patients readmitted with a secondary ACS had average plasma TRX concentrations of >30.60 ng/ml at first diagnosis, compared with low-risk patients, i.e., those who were not readmitted with a secondary ACS, where the average plasma TRX levels <13.40 ng/ml ($p<0.05$, Figure 3.1). Unlike the Mongardon *et al*., study, a commercially available ELISA kit was not used for the data presented in chapter 3, rather optimisation for each biomarker was conducted in a way where the primary antibody could be switched out, leaving the secondary antibody and detection steps identical. This method extended the linear part of the curve (see appendix W, X, Y, and Z). In this way, the detection limit for each test antigen (TRX, TRXr, PRDX-2 and PRDX-4) was reduced to 3 ng/ml, thus increasing assay sensitivity. This may in part explain that, whilst the overall trend in the data presented herein for TRX is relatable to the Mongardon study, the average plasma concentrations reported are slightly lower. Interestingly, Mongardon *et al*., (2013) also evaluated TRX clinical utility by ROC analysis and demonstrated that, plasma TRX concentrations for cardiac arrest syndrome on admission was able to predict death within 24h, from a median area under the curve of 0.84 (Mongardon *et al*., 2013). In other words, plasma TRX concentration could predict cardiac arrest syndrome death in 84% of cases within 24 hours. This finding is fascinating and further supports the data presented herein, where it is demonstrated from the ROC analysis that plasma TRX level was able to predict an AMI in 81.9% of cases at screening (Figure 3.5). Although this study is not about finding an alternative for hs-cTn, this ROC analyses does demonstrate a clinical utility for TRX

nonetheless, substantiating it's use as a potential predictive biomarker.

PRDX-4 has also gained attention in the literature, linking in the concept of 'oxidative stress' and CVD (Abbasi *et al.,* 2012). In a study by Abbasi *et al*., the authors identify PRDX-4 as a risk factor for CVD, concluding that elevated serum PRDX-4 was linked to a higher risk of a CVD event, but more importantly a poor risk outcome (Abbasi *et al.,* 2012). The method used for detection of PRDX-4 by Abbasi and colleagues was a novel 'sandwich Immunoluminometric assay (ILMA)', which was previously developed and optimised by the co-authors (Schulte *et al.,* 2010). This assay presents the data in 'U/L arbitrary units' and is therefore difficult to draw direct comparisons with the data presented herein, which used an afore mentioned optimised in house ELISA (ng/ml). However, observation regarding general trends in data can be made, as shown in Figure 3.32 and Table 3.3, which illustrate an increase in the average plasma PRDX-4 for the healthy cohort 13.9 ng/ml relative to 19.0 ng/ml for the ACS patients at screening, although data did not reach significance. However, ROC analysis reveals that PRDX-4 has the capacity to correctly predict an AMI in 82.6% of cases, as determined by the median area under the curve of 0.826 (Figure 3.36), demonstrating for the first time a clinical utility for PRDX-4 in ACS diagnosis. Interestingly, when ACS participants baseline, measurements were stratified according to plasma PRDX-4 percentile, it is noted that for the middle and upper quartile (PRDX-4 >10 ng / ml), PRDX-4 predicted readmission in 75% of ACS patients who received a PCI to LAD (Table 3.113), however these data did not reach significance. Whilst the data presented demonstrate a general trend with regards to increased PRDX -4 at screening and ACS readmissions, which agrees with the data presented by Abbasi *et al*., no overall statistical significance was reached. This could be a reflection of the differences in assay detection sensitivity between ILMA technique used by Abbasi *et al*., and the in-house ELISA Schulte et al. (Abbasi *et al*., 2012; Schulte *et al*; 2010). Moreover, the sample size for the Abbasi *et al*., study was significantly larger at 8,141 participants, compared with 151 herein, which is important for downstream statistical analysis for which the denominator is involves 'n' (Holmes *et al*., 2016). Taken together with respect to the literature discussed, the data presented in chapter 3 supports TRX as a predictive biomarker in the context of ACS and substantiates the previous findings relating to cardiac arrest syndrome. For PRDX-4, clinical utility is defined in chapter 3, with general trends in the data that suggest an increased plasma PRDX-4 level is associated with an AMI, which may have some prognostic implications for

predicting readmissions where patients received a PCI to LAD.

There are few studies which have examined plasma PRDX-2 in the context of CVD. However, an interesting study by Eter and Al-Masri (2015) investigated four PRDX isoforms (1,2,4 and 6) in the context of type-2 diabetes mellitus and CVD development risk (Eter and Al-Masri, 2015). Using commercially available ELISA kits (Wuhan EIAab Science Co) to detect serum PRDX, the authors demonstrated that non-type-2 diabetic CVD patients (n=25) had an average serum PRDX-2 concentration of 20.37 ng/ml, compared with the diabetic CVD patients (n=53) 36.61 ng/ml ($p<0.05$), however the authors did not include information on the 'healthy population'. In chapter 3, it is demonstrated that overall, ACS patients had an average plasma PRDX-2 concentration of 24.92 ng/ml (Table 3.61), a value which agrees with the non-diabetic CVD patients reported by Eter and Al-Masri (2015). Although the data presented in chapter 3 does not consider the impact of type-2 diabetes on ACS, this was factored into the initial screening questionnaire for the patients recruited into this study. Stratification of ACS patients based on this revealed that, type-2 diabetic ACS patients (n=22) had an average plasma PRDX-2 concentration of 23.91 ng/ml, compared with non-diabetic ACS patients (n=57) 25.30 ng/ml ($p>0.05$), data not shown. The deference in these findings compared with Eter and Al-Masri could be explained by the differences in study design. For example, Eter and Al-Masri excluded all CVD patients who had experienced ACS event within 6 months at point of screening, therefore establishing the impact of type-2 diabetes on baseline levels of PRDX-2 in CVD high risk patients generally (Eter *and* Al-Masri 2015). Eter and Al-Masri thus pose questions regarding to a more 'chronic' state of oxidative stress in CVD. However, for the study presented herein, the focus is on ACS patients at point of AMI diagnosis and how changes in plasma concentrations of PRDX-2 may predict outcome. Thus, the focus here is on an 'acute' event. It has been recently shown in a pre-clinical model that, PRDX-2 expression is decreased in myocardial tissue directly after an AMI, which may explain why plasma PRDX-2 concentrations normalise between type-2 diabetic and non-diabetic patients (Li *et al*., 2020). Interestingly, Li *et al*., formally demonstrated that administration of recombinant PRDX-2 protected the myocardium from further damage post AMI, by lowering ROS and inhibiting the THR4/Nf-κB pathway (Li *et al*., 2020) This raises the possible application of PRDX-2 as an anti-oxidant therapy for ACS patients, and warrants further investigation. To

the best of knowledge, PRDX-2 clinical utility is yet to be evaluated in the context of ACS. The ROC analysis presented in Figure 3.25 shows for the first time that PRDX-2 was able to predict a primary AMI in 82% of cases, as determined by the area under the curve. Thus supports a role for PRDX-2 in ACS. Taken together, the data presented in chapter 3 reveals similar plasma PRDX-2 concentrations to previous studies, however stratification of ACS patients based on type-2 diabetes had little impact, which may be explained by the observation that myocardial PRDX-2 expression is reduced following an AMI.

There appears to be no literature with respect to changes in blood / plasma concentrations of TRXr, that focusses on cardiac conditions e.g., CVD / ACS. Therefore, the data presented in chapter 3 is particularly novel. The data shown in Figure 3.11. demonstrates significant increases in plasma TRXr in ACS patients compared to the healthy donor controls ($p<0.001$). This increase was most apparent for the <55-year-old ACS patients, which increased from 0.41 ng/ml for the aged matched healthy donor cohort to >1.20 ng/ml for the ACS patients, representing a ~3-fold increase (Figure 3.12). Another important finding is the TRXr ROC analysis (Figure 3.15), which shows that plasma TRXr concentration on first admission was able to predict an AMI in 85% of cases (median area under the curve 0.85). This finding substantiates the clinical utility of plasma TRXr in ACS which warrants further investigation. Further discussion around TRXr is presented in the sections below.

Taken together, the data presented illustrate significant changes in some of the biomarkers with respect to ACS vs the healthy donor control, in particular TRX, TRXr with trends for PRDX-4. However, the ROC analysis for ACS patients and healthy donor controls at screening (sample 1), for TRX (Figure 3.5), TRXr (Figure 3.15), PRDX-2 (Figure 3.25) and PRDX-4 (Figure 3.36) in which true positives (sensitivity) were plotted against false positives (1-specificity) across various cut-offs, demonstrates area under the curve values of >0.80 for each biomarker (summarised in Figure 4.1).

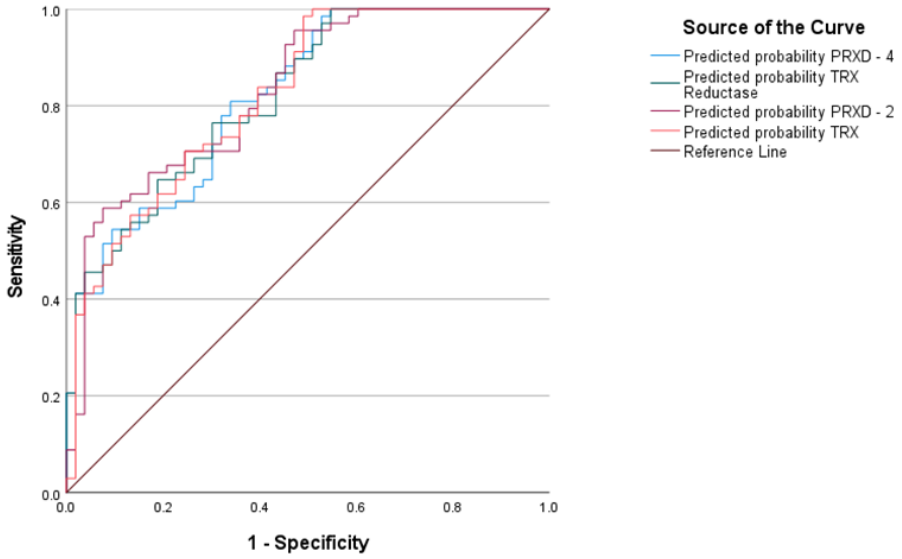


Figure 4.1. Combined ROC Analysis for TRX, TRXr, PRDX-2 and PRDX-4. Area under the curve for TRX 0.819 (95% CI, 0.752 to 0.888), TRXr 0.850 (95% CI, 0.761 to 0.940), PRDX-2, 0.820 (95% CI, 0.752 to 0.887), PRDX-4, 0.826 (95% CI, 0.753 to 0.899).

These data validate for the first time that each biomarker has a discriminant capacity for AMI diagnosis (Hajian-Tilaki 2013). Whist these will not replace the gold standard 'hs-cTn' (Su *et al*., 2015) and (Reichlin *et al.,* 2011) they do nonetheless support clinical utility.

4.2. Establishing an upper limit of normal for TRX, TRXr, PRDX-2 and PRDX-4.

Atherosclerosis is most common inflammatory pathological process known to be associated with development of cardiovascular disease (Mangge *et al.,* 2014; Jeong *et al.,* 2021; Hadri *et al*., 2021) and recent focus on oxidative stress is thought to be linked to certain cardiovascular diseases. The existing research exists on oxidation of low density-lipoproteins (LDL) and is evident in plaque formation leading to potential atherosclerosis and cardiovascular disease (Poznyak et al 2021). To this end, total cholesterol (TC) levels were established for ACS participants at screening (Table 3.0) however, this information is not available in the healthy cohort. Moreover, the oxidation status of the 'LDL' (OxLDL) was not determined for the ACS patients. Therefore, considering there were no secular changes within the ACS cohort, TC levels were not considered in this study.

However, known oxidative stress contributing factors were considered, which included

diabetes (previously discuss with respect to PRDX-2 in section 4.1), cigarette smoking, pre-existing health conditions e.g., family history of heart disease and hypertension, all of which are reported to increase the risk of developing CVD (Bonomini *et al*., 2008; Lubrano, *et al*., 2019). In order to ascertain an average concentration for each biomarker within participant groups, screening for these known risk factors was performed (Table 3.0). Other known risk factors for cardiovascular diseases such as sedentary and unhealthy lifestyle (Dubois-Deruy *et al*., 2020) were difficult to ascertain, although BMI was noted (Table 3.0). It was also challenging to accurately determine smoking status in both the ACS and healthy cohort, which is a known cause of oxidative stress, and associated with increased risk of developing CVD (Kamceva et al., 2012; Dikalov *et al*., 2018; Messner *et al.,* 2014). Thus, for the ACS cohort ~60% of participants reported as current smokers compared with 40% for the healthy group (Table 3.0).

To establish an upper limit of normal (ULN) for each biomarker, which may be important for the consideration of plasma TRX, TRXr, PRDX-2 and PRDX-4 a clinical setting, healthy participants who reported as having diabetes (insulin and non-insulin dependent), any of the afore mentioned pre-existing health conditions, a family history of heart disease / hypertension, and smokers, were excluded from the calculation. Taking everything together, the remaining healthy participants formed a recommended mean for use as a baseline percentile figure of a 'healthy population', which may be used to recommend as potential ULN's in the clinical setting. For each biomarker, these values were determined as; 10.34 ng/ml ± 10.45ng/ml for TRX, 0.78 ng/ml ± 1.65 ng/ml for TRXr, 27.20 ng/ml ± 9.92 ng/ml for PRDX-2, and 11.98 ng/ml ± 12.43 ng/ml for PRDX-4. Interestingly, by removing the 'smokers' the base line mean calculations for TRX, TRXr and PRDX-4 concentration levels reduced slightly, however the PRDX-2 concentration increased from PRDX-2 25.77 ng/ml ± 9.29 ($p<0.02$).

4.3. Plasma concentrations of TRX, TRXr, PRDX-2 and PRDX-4 following 'male/female' stratification.

For this analysis, a two-way mixed ANOVA method was used. This method was selected in order to establish whether there was an interaction between gender, within the healthy and ACS cohort for each of the plasma biomarkers (Maxwell & Delaney, 2004). In this way it could be determined whether each biomarker was significantly different between the

Arm-1 and Arm-2 ACS groups and the healthy cohort at each sample time point. The healthy cohort only had one sample taken at screening. For the ACS cohort, in addition to sample-1, subsequent samples were taken at 1-to-3-month follow-up (sample-2), and 6-month follow-up (sample-3). Depending on whether the interaction was statistically significant or not determined how and which results were reported with full explanation.

Previous studies have indicated differential sensitivity of oxidative stress between males and females, with studies generally agreeing that females are less susceptible to oxidative stress (Kender *et al*., 2017; Vina *et al.,* 2011). This can therefore make interpretation of the data presented here challenging, since average plasma concentrations of TRX, TRXr, PRDX-2 and PRDX-4 may differ between males and females. Therefore, direct comparisons were performed using a two-way mixed ANOVA to evaluate mean pair changes of each biomarker between the healthy cohort, and the ACS participants, with respect to sex.

For the ACS patients stratified to Arm-1 and Arm-2, the data presented in chapter 3 (Figure 3.11) showed a statistically significant difference in mean plasma TRXr concentration at screening (sample-1) for ACS Arm-2 patients, compared with healthy donor controls (p=0.014), but not for ACS Arm-1 patients, although the mean was higher. This difference could be explained by fact that, the Arm-2 patients were those where the blood sample was taken at time of ACS diagnosis, therefore during the AMI, whereas samples were taken at a later time point for the Arm-1 patients. Thus, the overall reduction in plasma TRXr in Arm-1 patients could reflect the half-life of TRXr, a concept which is an important factor in cardiac biomarker evaluation (Jacob *et al*., 2018). There is no information regarding TRXr plasma half-life in the context of ACS, however Wadley *et al*., (2019) demonstrated a return to normal plasma TRXr concentrations 60 minutes post exercise, which may indicate a short plasma half-life for TRXr in general. However, further research is required to establish this. For ACS participants, TRXr plasma concentrations incrementally decreases for both Arm-1 and Arm-2 participants when comparing the samples taken at screening baseline (sample-1), first (sample-2) and second follow-up (sample-2). Although these differences in means did not reach statistical significance, the effects were far more pronounced for females, which displayed a ~2-fold higher plasma TRXr concentration at screening compared to males, reducing to equivalent concentrations at second follow-up (Figure 3.14). These finding suggest that plasma TRXr concentration

may be sex discriminant in the context of ACS warranting further investigation.

There was no significant difference between ACS patients in general and the healthy volunteer cohort for plasma TRX concentration, but there was a significant increase between sampling time for Arm-1 and Arm-2, for both males and females (Figure 3.4). However, no significance was found with TRX between cohort or gender. Prior to this, only two previous studies have been conducted that assess TRX levels over time (Soejima *et al.,* 2003) and more recently (Vichova *et al.,* 2021). Soejima *et al*., (2003) concluded that, plasma TRX levels were increased in AMI patients, which differentiated AMI from stable angina and chest pain syndrome. The AMI patients were monitored over a 4-week period, during which the mean plasma TRX concentration incrementally decreased by ~15% (Soejima *et al.,* 2003). Whereas the data presented here show a slight upward trend at follow-up sample 2 (~4weeks), although these data did not reach significance (Figure 3.4). The Soejima *et al*., study did include males and females, however no further stratification of the data was made. The study by Vichova *et al.,* focused on plasma TRX concentration over a shorter timeframe (hours), and demonstrated a link between the time taken between reperfusion by PCI and increased levels of TRX, however in agreement with the data presented in Figure 3.4 these increases did not reach statistical significance (Vichova *et al.,* 2021). Taken together, it is difficult to draw direct comparison with the literature and explain the findings herein, due to lack of research. However, increased oxidative stress levels have been associated with elevated plasma TRX concentration in ACS, whereby attenuation of the oxidative stress was associated with decreasing TRX levels (Whayne *et al*., 2015). Therefore, the role of TRX in maintaining the wider redox homeostasis may be more important in ACS, compared with its potential role as a prognostic biomarker.

For PRDX-4, there was a general increase in plasma concentration for both males and females at screening for Arm-1 and Arm-2 ACS patients, compared with the healthy donor cohort, with males displaying higher levels across the board (Figure 3.35). These increases were significant when comparing the healthy cohorts with Arm-1 and Arm-2 in general ($p<0.05$), however the differences between males and females for each cohort did not reach significance. These findings support a previous study by Abbasi and colleagues, who investigated the role of plasma PRDX-4 as a risk of CVD events mortality (Abbasi *et al*., 2012). As previously discussed, this study recruited 8,141 participants (aged 28 to 75 years), 52.6% of which were women. The authors demonstrate that, PRDX-4 was inversely

associated with the female sex (p=0.03), a finding which is supported by the trend data presented in Figure 3.35. Taken together, these findings support the notion that, ACS females have lower plasma PRDX-4 levels relative to male counterparts, warranting further investigation.

Unlike PRDX-4, the PRDX-2 data (Figure 3.24) is interesting in the sense that the plasma concentrations were generally equal between ACS participants and healthy cohort, with no statistical significance between ACS patient 'Arms' or sex, although there were slightly lower levels for females compared to males in each cohort (p=0.155). Interestingly, deficiency of PRDX-2 was found to accelerate atherosclerotic plaque formation in mice (Park *et al*., 2011). However, it is difficult to relate how this may impact the ACS patients included in this study, or how males or females may be differentially affected by this in general, given the little observable changes plasma PRDX-2 (Figure 3.24). More recently, Jeong *et al*., (2020) found increased expression of PRDX-2 in abdominal aortic aneurysms, with lower levels in healthy humans and mice, but there was no further stratification according to sex. Taken together, with the previously discussed data relating to PRDX-2, it appears that plasma concentrations for this particular biomarker bear little importance with respect to ACS or male vs female stratification.

4.4. Plasma TRX, TRXr and PRDX-2 as ACS predictive biomarkers.

Up to this point, the data discussed has related to overall change in the biomarkers in ACS relative to healthy volunteers. Age and sex stratification was also considered along with respective ROC analysis. Given the changes plasma concentration observed, along with the ROC analysis findings, it was next important to evaluate the predictive capacity of each biomarker, the endpoint of which was an AMI readmission.

The data presented in Figure 3.6 highlights the importance of monitoring ACS patient plasma TRX. These data show that, at second follow-up (blood sample-3), patients who were stratified in the bottom percentile (<25%) for plasma TRX concentration (<8.42 ng/ml) were low risk of ACS readmission compared with patients in the top percentile (>13.40 ng/ml, p=0.009). This study is the first of its kind to follow-up ACS patients in this way and report this finding, however prognostic links to the impact of TRX for other ischemic diseases are reported. For example, high TRX (>20.0 ng/ml) is associated with a negative outcome in ischemic stroke patients, p<0.0001 (Qi *et al*., 2015). The Kaplan Meier log rank

data also demonstrated a significant statistical significance with respect to TRXr for blood sample-1 (Figure 3.17, p=0.010), illustrating for the first time that blood plasma TRXr concentration at screening may predict an ACS readmission. Here, ACS patients were stratified according to one of three plasma TRXr concentrations i.e., bottom percentile (<25%), inter-percentile range (>25% ~ <75%) and upper percentile (>75%). When comparing these patient groups, the data show that ACS patients who fall within the upper percentile for plasma TRXr concentration (>2.00 ng / ml) had a significantly lower overall risk of ACS readmission compared with those patients in the inter-percentile (p=0.022) and lower percentile range (p=0.002). This related to a median of 57 days before ACS readmission for the lower percentile group and 69 days for the inter-percentile range group, compared with a median of 230 days before readmission for the upper percentile patients. This study is the first to demonstrate a prognostic significance for plasma TRXr in the context of ACS. However, a recent study demonstrated that serum TRXr is predictive of 28-day survival in sepsis (Li *et at.,* 2021). This study examined 187 patients diagnosed with sepsis, whereby the authors demonstrate that patients who had high levels of serum TRXr (>38.27 ng / ml) had a better 28-day prognoses compared with patients who had low levels of serum TRXr (<38.27 ng / ml). The authors noted the essential role of oxidative stress in sepsis and highlighted a potential protective role for TRXr overexpression in sepsis, which may modulate the balance between pro- and anti-inflammatory processes (Li *et at.,* 2021). Thus, a similar interpretation may be drawn for the data presented herein, whereby high levels of plasma TRXr could indicate overexpression in the myocardium, which may in turn balance the oxidative stress. Therefore, the ACS patients in the >75% (>2.00 ng/ml) group benefit from higher levels of TRXr, which may protect the myocardium from lethal amounts of oxidative stress during and AMI, as well as promote recovery. Support for this notion comes from a recent study, which demonstrated in a pre-clinical model that overexpression of TRXr was able to protect vascular smooth muscle cells from cell death by lowering intracellular ROS (Park., 2019a). This finding was corroborated in a more recent study showing that overexpression of TRXr protects pulmonary smooth muscle cells against oxidative stress mediated by arsenic trioxide (Park.,2019b). Taken together it is therefore conservable that, raised plasma TRXr in ACS may indicate overexpression in the myocardium, which enhances ACS patient prognosis by lowering the risk of ACS readmission.

PRDX-2 was also interesting and demonstrated prognostic significance, most notably for plasma concentrations from blood drawn at first follow-up (sample-2), see figure 3.38. Here, ACS patients in the >75% for plasma PRDX-2 concentration (>30.60 ng/ml) had a significantly higher risk (p=0.009) of ACS readmission compared with ACS patients in the <25% for plasma PRDX-2 concentration (<19.50 ng/ml). PRDX-2 is previously reported as a risk factor for CVD, particularly when type-2 diabetes is a comorbidity (Eter and Al-Masri., 2015) however, there are no studies which have examined PRDX-2 in ACS patients who have been 'followed-up'. Taken together, plasma concentrations of TRXr as diagnosis have implications in predicting ACS patient's outcome, whereas PRDX-2 at follow-up also indicate prognosis, at which point plasma TRXr has little predictive capacity. Thus, monitoring each of these biomarkers through the disease and recovery course may provide important risk factors for determining ACS patient outcome.

There are some limitations with the study design of this research project that require reflection at this point. This study was limited by not being an 'event driven study', whereby some censoring observations between ACS recruitment Arms was noted. For example, the data analysis was performed following the removal of 'non-events of interest' such as asthma, and surgical / trauma admissions, the patients of which were still 'at risk' of having an ACS re-admission following the censored event. An assumption when performing the Kaplan-Meier analysis was that, patients who were censored had the same survival prospects as those who were followed in study (Stel *et al.*, 2011). Thus, when comparing the Arm-1 with Arm-2 patients who were censored at time of admission (Figure 3.0), it is possible that those in Arm-1 are in general healthier that those in Arm-2, and thus may explain why Arm-1 has a better prognosis than Arm-2, who were recruited into the study at the time of the AMI. Or it might be that case that those participants recruited at point of AMI, and thus receiving a PCI, were potentially healthier to fulfil the duration of the follow up period. As there is no 'recovery from an AMI' endpoint, only 'readmission rate' this implies a limited bias in the time to event probability. The predictive biomarker analysis discussed in this section combined Arm-1 and Arm-2 patient data. Unfortunately, it was not possible to expand this analysis out to evaluate Arm-1 and Arm-2 individually, due to lack of sample size in each Arm following censoring. This is unfortunate, since this information would be important for establishing whether time of sampling at point of recruitment (Sample-1) could bias the data. Thus, to help reduce any admission censoring bias (Arm-1

vs Arm-2), a final set of analysis was performed which evaluated the impact of the differences within each lesion of PCI (LAD, circumflex or RCA), since time to event was based on PCI date for both Arms. The rationale for this analysis was outlined in the introduction, and informed the aim and objectives of this study. Taking everything together, this final analysis sought to establish whether plasma biomarker concentration with respect to lesion of PCI had any significance to ACS readmission rates.

All plasma biomarkers for each ACS patients at baseline (sample-1) were systematically analysed following stratification in accordance with PCI i.e., LAD, circumflex or RCA. The data presented show that for the >75% plasma TRXr concentration (>2.00 ng/ml), patients who had an AMI due to blockage of the LAD or RCA, had in general a statistically significant longer time to ACS readmission, compared with the <25% plasma TRXr ACS patients (Figure 3.20). Interestingly, there were no ACS readmissions for LAD patients that were placed in the >75% plasma TRXr group (p=0.02). This is the first study of its kind to highlight this observation for TRXr. However, recently a study noted that TRX levels tended to be elevated in patients 1-hour post-AMI but decreased again 6-hours following the PCI (Vichova *et al.,* 2021). Moreover, TRX levels were found to be higher when participants were subject to longer times to reperfusion (>6 hours). Although not statistically significant, there was an over trend in the >75% plasma TRX concentration for circumflex ACS patients, who had a shorter time to readmission compared with the <25% plasma. In contrast to the data discussed previously, when plasma PRDX-2 was examined with respect to PCI, it was noted that patients in the >75 plasma concentration had a better prognosis i.e., time to readmission who received a PCI to the LAD (Figure 3.31, p=0.04). The observation may be explained by a recent publication by Jeong and colleagues, who reviewed the potential of the PRDX family of proteins in CVD, and highlighted that low expression of PRDX-2 is specifically in linked to CVD disease progression, since the protective antioxidant capacity of PRDX-2 which benefits vascular cells is lost (Jeong *et al*., 2021). Thus, in the context LAD cardiovascular plaques, those patients with higher expression of PRDX-2 may have an advantage. This notion is supported by Li and colleagues who demonstrate through *in vitro* modelling that, upregulation of PRDX-2 in carotid arteries protects the cells from ROS and slows the development of atherosclerosis (Li *et al*., 2021). However, it is not known how intracellular expression of PRDX-2 in carotid arteries or myocardial cells correlates with plasma concentration level. In a large international

study, that examined treatment outcomes of ACS patients following PCI in 6 wealthy countries, it was reported that England had the highest 30-day readmission rate at 23.1% patients (Cram *et al*., 2022). Whilst this study did not focus on the clinical impact of plasma biomarkers of oxidative stress in this context, it did highlight shortcomings with regards to ACS readmissions. The data presented herein may therefore identify high risk patients based on lesion of PCI and plasma biomarker concentration, so that intervention may be taken to minimise ACS readmission. One such intervention may be to treat patients who have low plasma PRDX-2 and have received a PCI to the LAD with intravenous recombinant PRDX-2. In a recent preclinical study, intravenous administration of recombinant PRDX-2 was shown to reduce ROS in myocardial cells, and protect the myocardium from cell death (Li *et al*., 2020).

To summarise, the key findings discussed here address the objectives of this research study and demonstrate for the first time that:

a) Healthy participants have lower plasma levels of three of the four biomarkers, TRX, TRXr, PRDX-4 compared to ACS participants. This observation was more apparent when participants were stratified according to age, i.e., <55 years old vs >55 years old and sex i.e., male vs female.

b) Each biomarker demonstrated clinical utility as determined by the ROC analysis, area under the curve >0.80 discriminative for ACS. This means that, irrespective of the standard diagnostic test i.e., hs-cTn, each of the biomarkers evaluated can predict an ACS in 4/5 cases.

c) The biomarkers were significant with respect to predicting readmission rates. Specifically**, i)** TRX baseline sample at diagnosis predicts readmission for ACS patients whose plasma TRX concentration falls within the upper percentile, **ii)** TRXr baseline sample at diagnosis predicts readmission for ACS patients whose plasma TRXr concentration falls within the lower percentile and **iii)** PRDX-2 first follow-up sample predicts readmission for ACS patients whose plasma PRDX-2 concentration falls within the upper percentile.

d) Plasma concentrations for some biomarkers was linked to the initial index event cardiac lesion of the AMI, which taken together allowed for subsequent risk of readmission prediction. Specifically, **i)** TRXr bottom percentile plasma concentration sample at diagnosis / screening predicts readmission for ACS patients who received PCI to LAD and **ii)** PRDX-2 bottom percentile plasma concentration sample at diagnosis / screening predicts readmission for ACS patients who received PCI to LAD.

4.5. Concluding remarks.

Whilst hs-cTn remains the gold-standard for diagnosing AMI, it cannot predict further events. Exploring all 4 oxidative stress markers and whether they bear any prediction on event rates or readmission in ACS was novel, adding to the current knowledge and highlighting potential clinical utility. Given the previous literature, it could be reasonably hypothesised that these biomarkers may change during the disease course, and therefore may be used as a diagnostic tool to predict ACS patient readmission. The data presented in this study demonstrates that TRX, TRXr and PRDX-4 are significantly raised compared to healthy cohort, which enables for the first time an upper limit of normal to be established, guiding clinical practice. Moreover, all biomarkers demonstrated clinical utility as informed by the ROC analysis.

TRXr and PRDX-4 represent exciting clinical benefits for monitoring plasma levels at baseline to risk stratify ACS patients at point of diagnosis. Since patients with low baseline levels of TRXr perform worst clinically, establishing ways to increase this may improve the antioxidant defence protection during ACS, enabling tissues / cells to repair, which may have a positive impact on ACS readmissions. Moreover, for PCI of the RCA and LAD, low levels of TRXr and PRDX-2 biomarkers predicted ACS readmission. This information may inform clinical outcome which in turn may highlight strategies to improve ACS readmission rates in England, e.g., recombinant PRDX-2 therapy when culprit lesion during PCI is the LAD. The significance of these findings in ACS alone is novel, but to demonstrate clear detectable differences in ACS and in readmission rates convincingly adds to the body of evidence to support oxidative stress in ACS. Given the rich data highlighting the role of oxidative stress imbalances occurring during the course of an ACS event gives prudence for further research into establishing ways of increasing the plasma concentrations of TRXr and PRDX-2 at index event. Furthermore, monitoring concentrations specifically in patients

presenting with AMI to RCA or LAD could further benefit from increased concentrations, monitoring at baseline could indicate outcomes and predict a readmission.

Given the limitations with regards to establishing sufficient numbers for patient stratification analysis following censoring, further research with a larger population of similar study design, including interventions of antioxidant is warranted to evaluate the impact on patient outcome. This will also substantiate the data presented but demonstrate the prognostic implication of these biomarkers for predicting a secondary event / reducing the risk of hospitalisation. The development of therapies to restore rapid homeostatic equilibrium of oxidative stress in the physiological process of CVD as a prevention of damage and/or a predictor of event is thus valid. Thus, with further research the impact could potentially be significant to clinical practice. With larger sample sizes, in parallel with development of commercially available assays or Point of Care devices to measure the biomarkers could be a rapid revolutionary cost-effective diagnostic tool in clinical practice, as well as inform new therapeutic approaches. Taken together, this could significantly benefit heart disease patient outcomes related to oxidative stress and reduce readmissions.

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Appendix A

National Institute for Health Research

Clinical Research Network: West Midlands
Worcestershire Clinical Research Unit
Newtown Road
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WR5 1LF

Tel: 01905 760256
Web: www.crn.nihr.ac.uk/wmidlands
Email: crnwestmidlands@nihr.ac.uk

9 March 2017

Angela Doughty
Worcester Royal Hospital
Charles Hastings Way
Worcester
WR5 1DD

Dear Angela

Project Title: Monitoring Intracellular and Extracellular Markers of Oxidative Stress in Acute Coronary Syndrome

I confirm that Worcestershire Acute Hospitals NHS Trust will act as Sponsor for the above study provided the conditions below are met. The decision was made on the basis of the information provided in the protocol and correspondence that is recorded and logged on the Trust's research management systems.

Conditions

- *The Researcher obtains Research Ethics Committee and Health Research Authority approval for the study*
- *The Researcher does not begin any research activities without obtaining NHS confirmation of capacity and capability to do so.*

As Sponsor, Worcestershire Acute Hospitals Trust will provide insurance as per HSG (96) 48 for the study and ensure the study is conducted in accordance with the Research Governance Framework and all applicable regulatory requirements.

Appendices 1 and 2 set out the allocation of responsibilities between Chief Investigator and Sponsor, including clear delegation of duties and expectations in relation to trial management, monitoring and conduct.

As Chief Investigator you must ensure that the above trial does not commence at any site until all applicable approvals have been obtained and agreements finalised (where applicable). This letter does not constitute confirmation of NHS Permission at the participating organisation.

Yours Sincerely

Charlotte Passingham
R&D Manager (Acting), Worcestershire Acute Hospitals NHS Trust

Appendix A

Appendix 1:

Allocation of roles & responsibilities under the Research Governance Framework:

	Yes	No	N/A	Responsibility of	Comments
Is the study encompassed by the EU Clinical Trials Directive/UK Clinical Trials Regulations 2004?		X		The Researcher	Study classes as a basic science study with human participants
Is the appointed sponsor also the funder?		X		The Researcher	Study being undertaken as part of educational course – no external funding applied for
Is/are there a financial contract(s) in place for the study?			X	n/a	Not required
Are honorary contracts in place for all staff dealing with NHS patients, their samples, tissues or data?			X	n/a	All staff are employed by WAH NHS Trust
Is there a clear written agreement between the key stakeholders in this research outlining the divisions of responsibilities?		X		The Researcher	Researcher to undertake all responsibilities
Are there adequate arrangements in place for the provision of compensation for the study?	X			The Sponsor	Not a clinical trial, no compensation anticipated
Has confirmation of capacity and capability been obtained?		X		The Researcher	This process will commence in line with HRA Approval
Has the study undergone an internal peer review?			X	n/a	Not required for educational studies
Has the study undergone an external peer review			X	n/a	Not required for educational studies
Has a statistician been involved with the study?		X		The Researcher	
Are there adequate resources in place to allow for the collection of and analysis of quality data?	X			The Researcher	

Are there adequate measures to ensure all data is protected?	X			The Researcher	In line with Trust policy and Data Protection Act
Has study been registered with the data protection officer at the local institution?		X		The Researcher	
Have adequate health & safety measures been taken for the conduct of the study?	X			The Researcher	
Have consumers been involved in the development & execution of the study?			X	n/a	

Allocation of roles & responsibilities under the RGF	**Yes**	**No**	**N/A**	**Responsibility of**	**Comments**
Has main Research Ethics Committee Approval been granted?		X		The Researcher	To be applied for
Has identification of any intellectual property taken place?			X	n/a	
Are there adequate arrangements for parties to be alerted to any developments as the study progresses?	X			The Researcher	The researcher is responsible for keeping the Sponsor up to date with regards to any new developments
Are there adequate arrangements to deal with misconduct & fraud?	X			The Sponsor	As per Trust policy

Appendix A

Appendix 2: Additional Delegation of Sponsor Duties:

The following duties are delegated to the Chief Investigator:

1. Prepare documents for REC/HRA and NHS Confirmation of Capacity and Capability including Information Sheets and Consent Forms and completion of relevant forms in the Integrated Research Application System (IRAS)

2. Prepare an appropriate data collection tool for study data according to protocol and ensure patient confidentiality is maintained

3. Maintain a Trial Master File encompassing all documentation in relation to the study from application, conduct and study close-out

4. Conduct study in compliance with the Research Governance Framework and Good Clinical Practice

5. To notify the REC and R&D Office of any amendments to trial or protocol

6. Submit annual reports to the NHS Trust and Research Ethics Committee

7. Notify the Trust and REC of end of study or early termination

8. Disseminate research findings in accordance with Protocol

9. To ensure all study documentation is archived at the end of the study

Appendix B

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

CONSENT FORM

Title of Project: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Participant Identification Number for this study:

Name of Researcher: Angela Doughty

Please initial boxes

1	I confirm that I have read the Patient Information Sheet dated 25th April 2017 for the above study and I have had the opportunity to consider the information, ask questions and have had these answered satisfactory.	☐
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3	If I withdraw from the study, I agree that any data already collected may be used as part of the results of the study.	☐
4	I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from University of Worcester, Worcestershire Acute NHS Trust or regulatory authorities where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
5	I agree to blood samples being collected for the purpose of this study.	☐
6	I agree that my participation involves completing a questionnaire and blood samples at the start of study and at 1-3 and 6 months after hospital discharge. Healthy volunteers will just be one visit at time of consent. At 12 months after admission my study medical notes will be reviewed.	☐
7	I give permission for my contact details (including my name, address, phone number, date of birth) to be stored on the trial database and used by the research team only for administration purposes of the study.	☐
8	I agree to my General Practitioner being informed of my participation in the study.	☐
9	I agree to take part in the above study.	☐

Name of Participant_____Signature_____Date_____

Name of Person Taking Consent_____Signature_____Date_____

Original to be filed with patient source notes, copy 1 for patient and scan to patient's medical records.

Appendix C

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

Title of Project: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

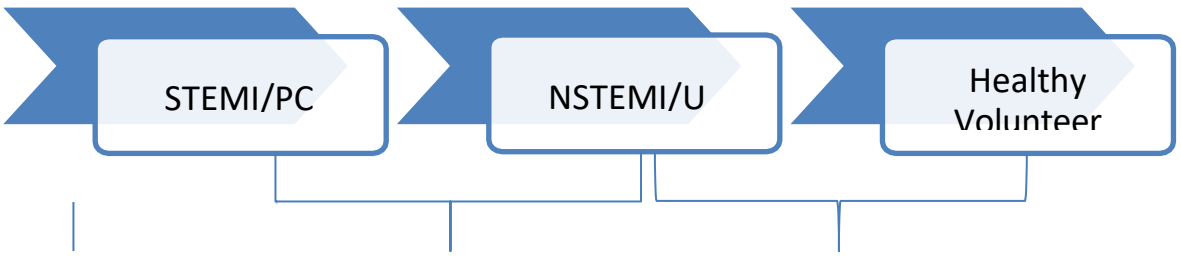
Participant Identification Number for this study:

_____.

Inclusion Criteria	>18 years Consenting Healthy Volunteer	Exclusion Criteria	< 18 years Admission for second stagged procedure
	Hospitalised with STEMI > PCI/NSTEMI or Unstable Angina		UA, STEMI, NSTEMI complicated by trauma, GI Bleeding
	Hospitalised with Stable Angina or Chest Pain no ACS Diagnosis.		Serious/severe co-morbidities in the opinion of the researcher which may is life limiting (i.e., < 6mths)
	Hospitalised Chest Pain/ Hx ACS > 12 months		Inability to consent

Diagnosis Criteria for Suspected ACS – confirm with at least one of below from Standard Care.

- Troponin-T level on Admission and/or 3 hours after admission. MI: Change in serial Troponins >10ng/L with one result > 20ng/L. Negative: both <20ng/L
- ECG abnormalities - e.g., ST depression >0.5mm documented from standard care.



Inclusion/Exclusion

Consent *
Inclusion/ Exclusion *
Demographics *
Medication History *
Local/Study Bloods *
CV Hospital Admissions *
Physical Examinations *
Study Bloods 1
Medical notes review 2
AE report 2

Appendix C

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

Enrolment Date___/___/_____ **Participants Number**_____

Informed consent obtained ☐ Any Exclusion fulfilled ☐

All Inclusion Criteria fulfilled ☐ Healthy Volunteer ☐

Participant Demographics

Age ☐ Years Height ☐ cm Weight ☐ kg

Gender Male ☐ Female ☐

Race Caucasian ☐ Black ☐ Oriental ☐ Other ☐ Unknown ☐

Final Diagnosis

STEMI ☐ NSTEMI ☐ U/Angina ☐ S/ Angina ☐ Non-ACS ☐

Circle as Appropriate

Risk Factors

Smoking Never Smoked Current Former Unknown

Hypertension YES ☐ No ☐

Previous Cardiovascular Disease **(Include year of diagnosis)**

MI Prior PCI Prior CABG Angina Heart Failure TIA/Stroke PVD

Previous Non-CVD **(Include year of diagnosis)**

Cancer YES ☐ No ☐

Appendix C

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

COPD /Other Chronic lung conditions YES ☐ No ☐

Medication List as appropriate and state if active on discharge.

	Concomitant	**Discharge**
Antiplatelet	YES ☐ No ☐	YES ☐ No ☐
Acetylsalicylic Acid /Clopidogrel /Ticlopidine/Prasugrel /other (specify) (Circle as appropriate)		
Anticoagulants	YES ☐ No ☐	YES ☐ No ☐
Detail		
Beta blockers	YES ☐ No ☐	YES ☐ No
☐ Detail		
ACE Inhibitors/ARBS	YES ☐ No ☐	YES ☐ No
☐ Detail		
Statins	YES ☐ No ☐	YES ☐ No
☐ Detail		

Appendix C

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

Index event

- Time of admission ……
- Onset time of chest pain…….
- Time of Troponin T……
- Time of trial bloods……
- Cardiac Catheterisation……Primary…….Lesion……RCA/Cx/LAD
- ECG
- Local Labs

  - Troponin 1 ☐ 2 ☐
    Time drawn ……/…… drawn ……/……
  - CKMB
  - Total Cholesterol ☐ mmol/L
    HDL ☐
    LDL ☐
  - Study Blood 1 ☐ 2 ☐
    Time drawn ……/…… drawn ……/……

Appendix D

University of Worcester

Worcestershire NHS
Acute Hospitals NHS Trust

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

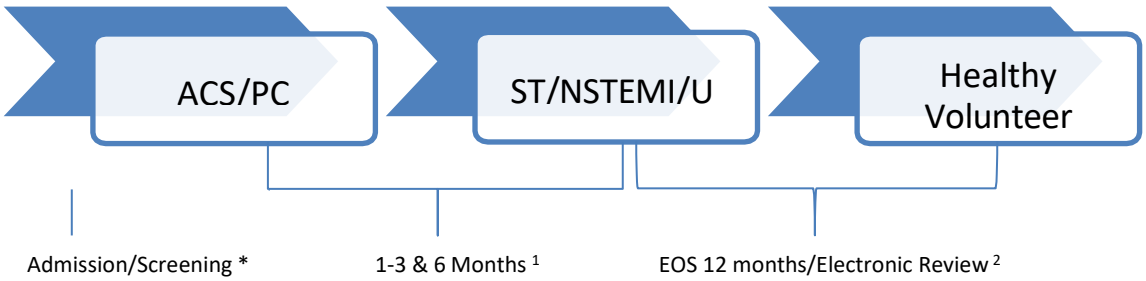
Title of Project: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Participant Identification Number for this study: _____.

Inclusion Criteria	>18 years Consenting Healthy Volunteer	Exclusion Criteria	< 18 years Admission for second stagged procedure
	Hospitalised with STEMI > PCI/NSTEMI or Unstable Angina		UA, STEMI, NSTEMI complicated by trauma, GI Bleeding
	Hospitalised with Stable Angina or Chest Pain no ACS Diagnosis.		Serious/severe co-morbidities in the opinion of the researcher which may is life limiting (i.e., < 6mths)
	Hospitalised Chest Pain/ Hx ACS > 12 months		Inability to consent

Diagnosis Criteria for Suspected ACS – confirm with at least one of below from Standard Care.

- Troponin-T level on Admission and/or 3 hours after admission. MI: Change in serial Troponins >10ng/L with one result > 20ng/L. Negative: both <20ng/L
- ECG abnormalities - e.g., ST depression >0.5mm documented from standard care.



Inclusion/Exclusion

Consent *	CV Hospital Admissions *
Inclusion/ Exclusion *	Physical Examinations *
Demographics *	Study Bloods 1
Medication History *	Medical notes review 2
Local/Study Bloods *	AE report 2

Appendix D

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

Enrolment Date___/___/_____ **Participants Number**_____

Informed consent obtained ☐ Any Exclusion fulfilled ☐

All Inclusion Criteria fulfilled ☐ Healthy Volunteer ☐

Participant Demographics

Age ☐ Years Height ☐ cm Weight ☐ kg

Gender Male ☐ Female ☐

Race Caucasian ☐ Black ☐ Oriental ☐ Asian ☐ Other ☐

Circle as Appropriate

Risk Factors

Smoking Never Smoked Current Former Unknown

Hypertension YES ☐ No ☐

Family History of heart disease YES ☐ No ☐

Medical History

……

……

……

Medication List as appropriate

……

……

……

Appendix E

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

Title of Project: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Patient Information Sheet: 25th April 2017

Patient Information

You are invited to take part in the above titled research study, your decision to take part is entirely voluntary. Please take time to read this information sheet and ask further questions if required. The study involves your medical data being collected from routine hospital practice and an extra blood sample analysed.

The following information will detail the study to you and help you to decide whether you wish to take part or not. Before you do decide I would like you to understand why the research is being done and what it would involve for you.

The study researcher will go through this information sheet with you and answer any questions you may have.

Background

Cardiovascular (heart) disease accounts to approximately 5% - 10% of hospital admissions with symptoms suggestive of acute heart problems known as Acute Coronary Syndrome, so timely diagnostic tests to rule a heart attack in or out is of paramount importance.

What is Acute Coronary Syndrome?

Heart disease occurs when the blood vessels that supply the heart progressively become narrowed (atherosclerosis) and blocked.

Acute Coronary Syndrome (ACS) refers to a sudden/rapid condition to the heart and is divided into heart attacks or unstable angina for health professionals to treat the conditions.

Appendix E

A tracing of your heart by an electrocardiogram (ECG) clinical symptoms and blood test are used to diagnose as part of standard care when admitted into hospital and heart problems are suspected.

Currently to diagnose a heart attack we measure a compound released from the heart muscle into the blood following heart attack (cardiac markers). However, cardiac markers can take several hours following a heart attack to be detectable.

It is known that during a heart attack a reaction in the blood happens that we call oxidative stress. This reaction causes a different compound to be released called allantoin.

What is the purpose of the study?

The purpose of this study is to develop an assay (a blood test) to measure this allantoin and then measure blood in patients admitted with angina or a heart attack, to compare against participants with no heart problems (healthy volunteers).

All your medical data collected will remain anonymous, so that your identity is protected.

Do I have to take part?

No, it is up to you to decide whether or not to take part, taking part is entirely voluntary and your standard care will not be changed if you do or do not take part in the study. If you decide to stop after bloods have been tested, we will continue to use the data unless you advise us to withdraw you completely from the study in which case the information collected will not be used in the final results.

Why have I been asked to take part?

You have been invited to take part in this study because you have been admitted to hospital with chest pain, diagnosed with angina or a heart attack or as a healthy volunteer (See table 1).

If you choose to take part in this study you will be asked to sign a consent form, you will receive a copy of the signed document and a copy will go in your medical notes, we will let your GP know that you have agreed participation.

Even if you agree to take part you may withdraw from the study at any time without giving reasons why and without any consequence to any planned treatment. If you do not participate in the study your medical care will not be affected.

What will happen to me if I take part?

After informed consent has been gained:

•Brief questionnaire about your medical history, medicines you are currently taking, tobacco and alcohol use will be collected.

Appendix E

•Blood tests: Approximately four-eight teaspoons of blood will be taken for testing blood at time of consent and future visits.

• Other diagnostic test and blood results to confirm your heart diagnosis, plus liver and kidney function test results will be documented from your standard care hospital bloods.

The questionnaire and hospital bloods are recorded because liver and kidney function, smoking and alcohol use may cause different reactions in the blood. Documenting at the beginning of study will help with accurate analysis of results when all data is analysed at the end of the study.

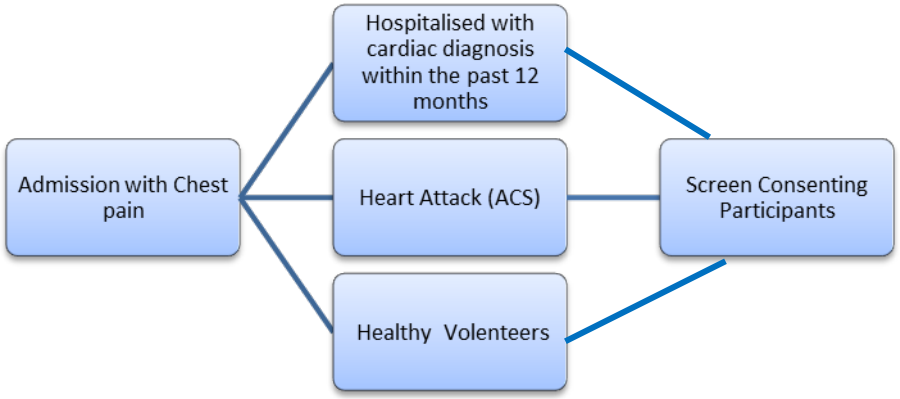


Table 1 – Study Design

What will happen to me if I take part - continued?

If you have confirmed heart conditions, we would like to repeat the blood sample and questionnaire one to three months later and six months after your first visit. (Approximately four-eight teaspoons of blood will be taken for testing blood at each visit) Your hospital record will be reviewed at 12 months after discharge to check your wellbeing; this will be classed as your end of study visit.

If you enter as a healthy volunteer or non-confirmed heart conditions, we will only collect the single sample of blood (See Tables 1 and 2).

Assessment Schedule (Table 2)

Visit	Screening	2-3 & 6 Months	12 Months
Informed consent	X		
Inclusion/Exclusion	X		
Study bloods (4-8 mls)	X	X	
Demographics i.e., Vitals Height/Weight/ECG	X		
Medication	X		
Smoking & Alcohol	X		

Appendix E

Questionnaire	X		
Hospital Admissions review	X	X	X

What are the possible side effects or risks of taking part?

The study will collect data on your health and treatment; therefore, it does not present any additional risk for you other than the ones related to taking blood samples, it will not alter the standard procedure of care.

Taking blood may cause you to feel faint, bruising, pain or bleeding from the puncture site.

What are the possible benefits of taking part?

There may not be any benefit for you from taking part in this study, although previous participants in similar studies often find it useful having extra time to ask questions with the experienced members of the hospital staff.

The information obtained from this study may help improve treatment for people with similar disease.

What if there is a problem?

If you have a problem, you can contact the study researcher on 01905 733844 or the complaints department can be contacted via the Patients Advisory Liaison Service (PALS) on 0300 123 1732.

Will my taking part in this study be kept confidential?

The study will follow good clinical practice in accordance with ethical reviews from the University of Worcester and Worcestershire Acute Trust's Research and Development approval.

Your research data will be collected confidentially and anonymised. You will be assigned a unique individual number; you will not be identified by name externally.

Who has reviewed the study?

Research in the NHS is reviewed by an independent group of people called a Research Ethics Committee. The study is approved from both Worcestershire Acute NHS Trust and University of Worcester.

Who is organising and funding the research?

The study is being conducted for a post graduate degree programme. The study coordinator is funded by the participating trust to complete the post graduate degree; the equipment is supplied from the researcher, research department and Worcester University. As the study progresses some funding may be sought for post graduate Students.

Appendix E

What will happen to the results of the Study?

The results of this study will form part of a thesis for the author's degree. Results may be published in part and/or shown at medical meetings but participants will becompletely anonymous.

Participants wishing to find out the results of the study may contact the researcher on the below contact details at the end of the published thesis.

What if I have further questions?

If you have any question about the study you can contact: Angela Doughty Cardiology Research Co-Ordinator, Worcestershire Acute Hospitals NHS Trust, Charles Hasting Way, Worcester. 01905 733844.

Thank you very much for taking time to consider participation in this study.

Appendix F

Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD

Worcestershire NHS
Acute Hospitals NHS Trust
Cardiology Research Dept. 01905 733844

VERBAL CONSENT SHEET TO INVITE PARTICIPATION TO THE OXY ACS STUDY

Title of Project: Development and application of a high throughput assay for monitoring oxidative stress in Acute Coronary Syndrome

This should be read to the patients and the result of the discussion documented in the clinical notes.

- Your doctors have diagnosed that you are having a heart attack, which means that it is likely that one of your heart arteries is blocked reducing blood supply to your heart muscle.
- We are undertaking a research study to learn more about reactions in the blood during/after a heart attack, so that we can try to predict and/or reduce occurrence in the future.
- In this hospital the usual procedure is to perform blood test on admission to help diagnosis of a heart attack.
- If you agree, I would like to take an extra blood sample.
- This will be taken when usual bloods are routinely taken in this situation.
- If you agree, but bloods have already been taken we will take some additional bloods when you are stable and comfortable. These will not be standard but we will reduce amount of times bloods are taken where possible.
- After you have had your early treatment and are recovering, we will provide you with further information about the study and you will have another opportunity to discuss this and decide if you wish to carry on in the study or not.
- You will be asked to fill in a questionnaire and we would like to follow you up in the outpatient department.
- If you decide not to take part, we will discard the blood sample.
- Whether you decide to take part or not is entirely up to you, and in any case, you will receive the best care we can provide for your condition.

Appendix G

Worcestershire NHS
Acute Hospitals NHS Trust
Worcestershire Royal Hospital
Charles Hasting way
Worcester

WR5 1DD
Cardiology Research Dept. 01905 733844

Re: Study Title: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Dear

I am undertaking a postgraduate degree in collaboration with Worcester University conducting a study entitled as above.

Following your recent hospital admission, you may be eligible to take part in the above study if you would like to.

I work at Worcestershire Acute NHS Trust as the Cardiology Research Nurse and I am conducting the study as part of my post-graduation degree in collaboration with University of Worcester.

Enclosed with this letter is a detailed patient information sheet detailing the study involvement. Please could you read this very carefully? The information explains about the study and why it is being done.

I am contacting you now so you have time to consider the study and raise any queries or questions.

It is important to know that you are under no obligation to take part in this study. If you decide not to take part in this study it will not affect the care that you are receiving from the hospital.

If you do decide to take part you are free to withdraw at any time without giving a reason.

If you would like to participate or discuss further, please contact me Angela Doughty on 01905733844.

Many thanks for taking the time to read the information and consider taking part.

Yours sincerely

Sr. Angela Doughty
Cardiology Research Co-Ordinator Enc. Patient Information leaflet

University of Worcester

Worcestershire NHS
Acute Hospitals NHS Trust

XY - ACS

Temperature Log- Fridge/Freezer

Study: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Date	*Patient Enrolment number*	*Temperature Fridge/Freezer*	*Date shipped to University of Worcester Comments, temperature excursions etc..*

Worcestershire Acute Hospitals NHS Trust
Worcestershire Royal Hospital
Charles Hasting way
Worcester
WR5 1DD
Cardiology Research Dept. 01905 733844

Re: Study Title: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome.

Dear

I work at Worcestershire Acute NHS Trust as the Cardiology Research Nurse and I am undertaking a postgraduate degree in collaboration with Worcester University conducting a study entitled as above.

Enclosed with this letter is a detailed participant information sheet detailing the study involvement. Please could you read this very carefully? The information explains about the study and why it is being done.

I am writing to you to invite you to take part as a healthy volunteer?

It is important for you to know that you are under no obligation to take part in this study. If you decide not to take part in this study it will not affect future care from the hospital.

If you do decide to take part you are free to withdraw at any time without giving a reason.

I am contacting you now so you have time to consider the study and raise any queries or questions. If you would like to participate or have any further questions, please contact me on 01905 733844.

Many thanks for taking the time to read the information and consider taking part.

Yours sincerely

Sr. Angela Doughty

Cardiology Research Co-ordinator

Enc. Patient Information leaflet

Worcestershire NHS
Acute Hospitals NHS Trust
Worcestershire Royal Hospital
Charles Hasting way
Worcester

WR5 1DD
Cardiology Research Dept. 01905 733844

Appendix I

Re: Study Title: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Dear Doctor,

Re: Patient name:
DOB:
Address:

Your patient as named above has kindly agreed to participate in the above study, this letter is for your information only.

Patients who present with or have previously had a chest pain admission are being recruited along with healthy controlled volunteers.

The main aim of this study is to learn more about oxidative stress in cardiovascular disease at time of admission.

I'm taking detailed participant history and extra blood tests for analysis against acute coronary episodes.

No additional treatment apart from the standard care for this condition is applied. Follow-up is carried out at 1-3, 6 months post admission with an additional blood test, and then an electronic admission review at 12 months post admission.

Please contact a member of the research team below should you have any questions regarding the study or your patient's participation.

Yours sincerely,

Angela Doughty

Appendix K

Material Transfer Agreement

This Agreement is made by and between:

a) Worcestershire Acute Hospitals NHS Trust, Aconbury East. Charles Hasting way, Worcester, WR51DD ("the Donor Institution") and

b) Institute of Science & The Environment. University of Worcester. St Johns Campus. Henwick Grove. Worcester. WR2 6AJ {"the Recipient Institution")

This Agreement records the terms under which the Donor Institution will make available consenting participants blood samples (the "Material"). The term "Material" includes all unmodified progeny generated from the material supplied and that part of all derivatives and the derivative's progeny which contains any of the material supplied or its progeny. The Recipient Institution will hold the Material on the terms of this Agreement and solely for the purpose of **Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome** ("the Research Project") within the research group of ("the Dr [redacted]. (Recipient Scientist").

1. The Material may only be used by those under the Recipient Scientist's direct supervision in the Recipient Institution's laboratories under suitable containment conditions, and in compliance with all applicable statutes and regulations. **THE MATERIAL MAY NOT BE USED IN HUMAN SUBJECTS OR FOR CLINICAL OR DIAGNOSTIC P U R P O S E S .**

2. The Recipient Institution will not transfer the Material to any other body, or permit its use within the Recipient Institution other than by the Recipient Scientist's research group, without (in each case) prior written consent from the Donor Institution. The Material may not be used by the Recipient Scientist in research which is subject to the provision of any rights to a commercial third party without prior written consent.

3. The Recipient Institution understands that the Material is experimental in nature, and may have hazardous properties. The Donor Institution makes no representations and gives no warranties either express or implied in relation to it: for example, no warranties are given about quality or fitness for a particular purpose; or that the use of the Material will not infringe any intellectual property or other rights of third parties. The Donor Institution will not be liable for any use made of the Material.

4. Except to the extent prohibited by law, the Recipient Institution assumes all liability for damages which may arise from its receipt, use, storage or disposal of the Material. The Donor Institution will not be liable to the Recipient Institution for any loss, claim or demand made by the Recipient Institution, or made against the Recipient Institution by any other party, due to or arising from the use of the Material by the Recipient Institution, except to the extent the law otherwise requires.

5. The liability of either party for any breach of this Agreement, or arising in any other way out of the subject matter of this Agreement, will not extend to loss of

business or profit, or to any indirect or consequential damages or losses.

6. The Recipient Scientist will acknowledge the source of the Material in any publication reporting on its use. If the Recipient Scientist wishes to include in a publication any information which has been provided by the Donor Institution with the Material and which was clearly marked as "confidential" and "proprietary" at the point of disclosure ("Confidential Information"), the Recipient Scientist will request permission from the Donor Institution, providing a copy of the text before publication takes place.

7. **Nothing in this Agreement grants the Recipient Institution any rights over the Material (other than as specifically granted by this Agreement} or under any patents, rights over to use, or permit the use of, any products or processes containing, using, or directly derived from the Material for profit-making or commercial purposes ("Commercial Use").** If the Recipient Institution wishes to make Commercial Use of the Material or a product directly derived from the Material it agrees to negotiate in good faith with the Donor Institution or its representative for the grant of an appropriate license or the conclusion of a revenue sharing agreement, if justified. The Donor Institution will have no obligation to grant a license.

8. Nothing included in this Agreement shall prevent the Donor Institution from being able to distribute the Material to other commercial or non-commercial entities, including any intellectual property protection being undertaken by the Recipient Institution on any new use made with the Material.

9. This Agreement shall commence on the date of last signature below and will (subject to earlier termination pursuant to clause 10) continue for the duration of the Research Project.

10. The Donor Institution may terminate this Agreement if the Recipient Institution is in material breach of any of the terms of this Agreement and, where the breach is capable of remedy, the Recipient Institution has failed to remedy the same within one month of service of a written notice from the Donor Institution specifying the breach and requiring it to be remedied.

11. Upon completion of the Research Project or earlier termination under clause 1O the Recipient Institution will discontinue all use of the Material, and upon the Donor Institution's direction, return or destroy the Material, unless permission to retain the Material is specifically provided in writing by the Donor Institution to the Recipient Institution.

12. This Agreement shall be governed by English Law, and the English Courts shall have exclusive jurisdiction to deal with any dispute which may arise out of or in connection with this Letter Agreement.

Accepted and Agreed *by an authorised signatory* on behalf of

Accepted and Agreed on behalf of

Appendix K

Institute of Science & The Environment. University of Worcester, St Johns Campus. Renwick Grove. Worcester.
WR26AJ

Research andDevelopment
Worcestershire Acute Hospitals NHS Trust.
Aconbury East. Charles Hasting way. Worcester, WR51DD

Name: Dr [redacted]

Position: Senior Lecturer in Biochemistry

Signature: - -=C.:--- - '='((..-

Date: 23rd June 2017

Name: [redacted]

Position. R&D Manager

Signature: {LG '(v'.... •

Date: 12/07/17

Appendix M

University of Worcester

Worcestershire Acute Hospitals NHS Trust

1. **Site Contact Information**
2. **Protocol**

2.1 IRAS
2.2 Registration for Research degree - Approved
2.3 Research Proposal - Research Degree Board
2.4 Standard Operating – Blood transfusion session

3. **Standard Operation Procedures**

3.1 The Process of obtaining informed consent

4. **Ethical Requirements**

4.1 Site NHS Sponsorship 09th March 2017
4.2 Research Ethics Committee (REC) Approvals 26th May 2017
4.3 Health Research Approvals (HRA) Approval 30th May 2017
4.4 R&D Local Approvals
4.5 University of Worcester Ethical Submission and Correspondence
4.6 R&D Local/REC, HRA approvals correspondence

5. **Investigator/Site Documentation**

5.1 CVs of investigators
5.2 Delegation of duties

6. **Signed Consent Forms**

Note to file – Consents stored confidentially

7. **Subject Information**

7.1 Patient information Leaflet
7.2 Consent forms
7.3 Verbal Assent
7.4 Source Documentation/Questionnaires
7.5 Invitation Letter Hospital Discharge Patient
7.6 Invitation Letter Healthy Volunteers
7.7 GP Letter
7.8 Monitoring reports

8. **Screening Logs**

8.1 Screening Log
8.2 Patient Enrolment and appointment log Healthy Patients
8.3 Patient Enrolment and appointment log ACS Patients
8.4 Completed consent visit tracker V_25_04_2017 with SAE's

9. **Laboratory**

9.1 ISE Research Laboratory information and code of practice
9.2 IOSH Responsible research
9.3 Local Laboratory Accreditation and Ranges
9.4 Blood preparation and transport guidelines
9.5 Calibration records WAHT – 40 freezer/Fridge/Centrifuge

10. **Sample transfers**

10.1 Material Transport Agreement

10.2 UN3373 transfer label
10.3 Training of Transporting Dangerous Goods

11. Site Sample Logs

12. 11.1 Temperature Log – Fridge/Freezer log

13. Authorised Signature Log

12.1 CV's and GCP of Site Personnel

14. Adverse Events

15. Training Material and Logs

15.1 Presentation for site set up
15.2 Training material and training log

16. Correspondence

Worcestershire NHS
Acute Hospitals NHS Trust

Appendix N

XY - ACS

Blood processing and transfer guidelines transfer

Please review recommendations below on how to prepare 4/8ml EDTA Purple Top vacutainer tubes for analysis.

1. Invert the tubes 5 times immediately after collection (as soon as removed from the needle). Label with patient number.
2. Keep the sample at Ambient/room temperature between draw and centrifugation (keep this time under 30 minutes)
3. Centrifuge blood at 491* x g for 5 min, and mark levels of haematocrit (red, lower layer) and plasma (straw, upper layer) on the tube, as demonstrated in picture.
4. Aspirate the plasma and add to fresh centrifuge tube (keep this on dry ice for later – see step 9)
5. Fill the haematocrit tube to marked level of original plasma with Hanks Balanced Salt (HBS) solution. Cap and invert a few times to gently mix. Centrifuge at 491* x g for 5 min.
6. Aspirate HBS solution and discard in virkon.
7. Replace HBS to the original haematocrit level with sterile PBS adjusted to contain 40% v/v glycerol (prepare this solution separately and pass through 0.22-micron filter).
8. Immediately aliquot plasma into transfer vial and place it straight into -20° C freezer.
9. Centrifuge plasma (from step 4) at 2,000 x g for 15 min (depletes the platelets). The resulting supernatant is the plasma.
10. Aliquot plasma evenly in 0.5 ml aliquots cryovials (3-4) and freeze at -20 o C. Place ambient sample in plastic blood UN3373 in freezer ready to transfer.

Samples to be stored in -20 ^{o}C Freezer and shipped to UoW on dry ice every 2 weeks.

X 4mls EDTA	UoW	Consent/ 2-3 months and 6months *
Trop T	Local Labs	Record for arm stratification
CK-MB	Local Labs	Record for arm stratification
LFT's	Local Labs	For eligibility criteria
eGFR/U&E	Local Labs	For eligibility criteria

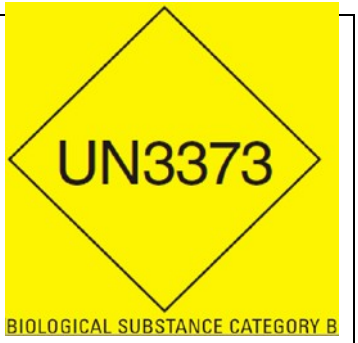
Key = *Centrifuge at 491 RC = 2300 S and 2000 RC = 4600S

Appendix N

- Place frozen individual blood samples in plastic UN3373 transfer pouches and fully submerge fully in dry ice. Immediately transfer to University of Worcester and store in –80 °C freezer. Update freezer log in Section 11.1 in site file.

URGENT DELIVERY

To
University of Worcester
Biomedical Research Group
Institute of Science & Environment
Henwick Grove
Worcester
WR2 6AJ



From
Cardiology Research,
Worcestershire Acute
Hospital, Aconbury East,
Worcester, WR5 1DD
Telephone: 01905 733844

Appendix O

Database Key

A Patient Number
B Consent Date
C AGE
D BMI
E Gender Male 1 Female 2
F Race
- 1 = Caucasian
- 2= Black
- 3 = Oriental
- 4 = Asian
- 5 = Other

G Date of INDEX PCI
H Smoking Status
- 1 = Current Smoker
- 2 = Never Smoked
- 3 = Former Smoker
- 4 = Unknown
- 5 = Vape smoker

I Hyper BP
- Y = 1 N = 2

J Diabetes
- 1 = No History
- 2 = Diet Controlled
- 3 = Medication
- 4 = Insulin

K Family History
- Y = 1 N = 2

L ARM
- 1 = Healthy Volunteers
- 2 = ACS/PCI
- 3 = ACS/12 Month History
- 4 = Heart Failure

M ACS
- 1 = UA
- 2 = NSTEMI
- 3 = STEMI

N Cancer
- Y = 1 N = 2

O **COPD**
- Y = 1 N = 2

P DAPT/SAPT
- Y = 1 N = 2

Q **Anti Coag**
- Y = 1 N = 2

R **Beta Blockers**
- Y = 1 N = 2

S **ACE/ARBS**
- Y = 1 N = 2

T **Statin**
- Y = 1 N = 2

U **Onset of Chest pain**

V **Lesion of PCI**
- 1 = RCA
- 2 = Circumflex
- 3 = LAD

W NT-Pro BNP ng/L
X Trop 1 Date
Y Trop 1 Time
Z Trop 1
AA Trop 2 Study Blood 1 Date
AB Peak Trop
AC Study Blood 1 Date
AD Study Blood 1 result
AE Study Blood 2 Date
AF Study Blood 2 result
AG Study Blood 3 Date
AH Study Blood 3 result
AI Study Blood 2 Days from Trop 1 (OR 2 If closer)
AJ Peak TCK
F = 25-200 M = 40-320 u/L
AK TC mmol/L
AL HDL
AM LDL
AN Trig (0 - 1.8)
AO Creatinine 62-106
AP eGFR mL/min Ex <30
AQ ALT (0 – 40)
AR End of Study (EOS)
AS End Points/SAE's
AT Patient Number

Appendix P

University of Worcester

Worcestershire NHS
Acute Hospitals NHS Trust

Patient Enrolment and appointment Log

Study: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Record of all patients who have provided written consent* using the ethics approved Informed Consent form for this study. Record follow up due date and tick when completed.

*patient consent is required prior to any study specific procedures and prior to use of a patient's data or biological samples for this clinical trial.

Patient initials	Date of Consent	Date Screened	Enrolment Number 001,002…	Follow up 1-3,6 mths Due dd/mm/yy	Tick	Follow up 12 mths Due dd/mm/yy	Tick	If NOT ENTERED give reason, eg: not eligible & reason, unwilling, etc..

Appendix R

NHS
Health Research Authority

West Midlands - Solihull Research Ethics Committee

Please note: This is the Favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

26 May 2017

Mrs Angela Doughty
[redacted]

Dear Mrs Doughty

Study title:	**Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome**
REC reference:	**17/WM/0132**
IRAS project ID:	**189016**

Thank you for your letter, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Management permission must be obtained from each host organisation prior to the start of the study at the

site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Costing template (commercial projects) [Permission to use retained equipment]	N/A	13 March 2017
Covering letter on headed paper [Approval Submission]	V1	10 March 2017
GP/consultant information sheets or letters [GP Letter]	V1	01 November 2015
IRAS Application Form [IRAS_Form_16032017]		16 March 2017
IRAS Checklist XML [Checklist_18052017]		18 May 2017
Letter from funder [Approval Letter]	N/A	07 December 2016
Letter from sponsor [Sponsorship Confirmation]	N/A	09 March 2017

Letters of invitation to participant [Invite letter medically discharged patients]	V1	01 July 2016
Letters of invitation to participant [Invite Healthy volunteers]	v1	08 August 2016
Letters of invitation to participant [Invite Healthy volunteers]	V1	08 August 2016
Non-validated questionnaire [Patient Questionnaire]	V1	01 July 2016
Other [WAT01 Informed Consent SOP]	1.0	10 October 2016
Other [CV - Amy Cherry]		27 March 2017
Other [CV - Professor ian maddock]		
Participant consent form [Assent]	V1	22 October 2016
Participant consent form [Consent Form]	V2	25 April 2017
Participant consent form [Verbal Consent]	V2	25 April 2017
Participant information sheet (PIS) [Patient Information Leaflet]	V1	12 July 2016
Participant information sheet (PIS) [Patient Information Leaflet]	V2	25 April 2017
Referee's report or other scientific critique report [Expert Reveiw 1]	V1	29 September 2015
Research protocol or project proposal [Final RDB1 Proposal]	V1	16 November 2016
Summary CV for Chief Investigator (CI) [CV & GCP]	NA	29 September 2016
Summary CV for student [CV]	NA	29 September 2016
Summary CV for supervisor (student research) [CV Supervisor]	N/A	09 March 2017
Summary, synopsis or diagram (flowchart) of protocol in non technical language [UoW Lay Term Application for Ethical Approval]	V1	09 March 2017

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form

available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

17/WM/0132	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project. Yours

sincerely

Signature removed

Dr Rex [redacted] Chair

Email: NRESCommittee.WestMidlands-Solihull@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mrs [redacted], NIHR Clinical Research Network: West Midlands*

Full Set of Project Data IRAS Version 5.3.1

Appendix Q

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please complete the questions in order. If you change the response to a question, please select 'Save' and review all the questions as your change may have affected subsequent questions.

Please enter a short title for this project (maximum 70 characters) Oxidative Stress in Acute Coronary Syndrome

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product

( ) Clinical investigation or other study of a medical device

( ) Combined trial of an investigational medicinal product and an investigational medical device

( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice

( ) Basic science study involving procedures with human participants

( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology

( ) Study involving qualitative methods only

(●) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)

( ) Study limited to working with data (specific project only)

( ) Research tissue bank

( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

a)	(●)	( )
b)	(●) Yes	( ) No
c)	( )	(●)
d)	(●)	( )

2a. Please answer the following question(s):

	Yes	No
Will you be taking new samples primarily for research purposes (i.e., not surplus or existing stored samples), including any removal of organs or tissue from the deceased?		
Will you be using surplus tissue or existing stored samples identifiable to the researcher?		
Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?		
Will you be processing identifiable data at any stage of the research (including in the identification of participants)?		

***3.* In which countries of the UK will the research sites be located?** *(Tick all that apply)*

- [x] England
- [ ] Scotland
- [ ] Wales
- [ ] Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

- (•) England
- ( ) Scotland
- ( ) Wales
- ( ) Northern Ireland
- ( ) This study does not involve the NHS

4. Which applications do you require?

IMPORTANT: If your project is taking place in the NHS and is led from England select 'IRAS Form'. If your project is led from Northern Ireland, Scotland or Wales select 'NHS/HSC Research and Development Offices' and/or relevant Research Ethics Committee applications, as appropriate.

- [ ] IRAS Form
- [x] NHS/HSC Research and Development offices
- [ ] Social Care Research Ethics Committee
- [x] Research Ethics Committee
- [ ] Confidentiality Advisory Group (CAG)
- [ ] National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D Offices in Northern Ireland, Scotland and Wales the CI must create NHS/HSC Site Specific Information forms, for each site, in addition to the study wide forms, and transfer them to the PIs or local collaborators.

For participating NHS organisations in England different arrangements apply for the provision of site specific information. Refer to IRAS Help for more information.

5. Will any research sites in this study be NHS organisations?

(•) Yes ( ) No

Yes No

Yes No

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5a. Are all the research costs and infrastructure costs (funding for the support and facilities needed to carry out research e.g. NHS Support costs) for this study provided by a NIHR Biomedical Research Centre, NIHR Biomedical Research Unit, NIHR Collaboration for Leadership in Health Research and Care (CLAHRC), NIHR Patient Safety Translational Research Centre or a Diagnostic Evidence Co-operative in all study sites?

Please see information button for further details.

Yes No

Please see information button for further details.

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5b. Do you wish to make an application for the study to be considered for NIHR Clinical Research Network (CRN) Support and inclusion in the NIHR Clinical Research Network Portfolio?

Please see information button for further details.

○ Yes ◉ No

The NIHR Clinical Research Network provides researchers with the practical support they need to make clinical studies happen in the NHS e.g., by providing access to the people and facilities needed to carry out research "on the ground".

If you select yes to this question, you must complete a NIHR Clinical Research Network (CRN) Portfolio Application Form (PAF) immediately after completing this project filter question and before submitting other applications. Failing to complete the PAF ahead of other applications e.g., HRA Approval, may mean that you will be unable to access NIHR CRN Support for your study.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the Confidentiality Advisory Group to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

◉ Yes ○ No

Please describe briefly the involvement of the student(s):

Student is fully coordinating the study as part of Mphil/PhD research degree, research works in Cardiology Research Department and is now conducting this as part of degree.

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

Yes No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

Yes No

Appendix Q Full Set of Project Data IRAS Version 5.3.1

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (e.g., participants)?

◯ Yes ◉ No

Integrated Research Application System
Application Form for Research limited to working with human tissue samples and/or data

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familiar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Oxidative Stress in Acute Coronary Syndrome

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:

Monitoring Intracellular and Extracellular Oxidative Stress in Acute Coronary Syndrome

A2-1. Educational projects

Name and contact details of student(s):

Student 1

	Title Forename/Initials	Surname
	Mrs Angela	Doughty
Address	[redacted]	

	Worcestershire Acute NHS Trust
	[redacted]
Post Code	[redacted]
E-mail	a[redacted]y@worcsacute.nhs.uk
Telephone	[redacted]
Fax	[redacted]

Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/ degree:
PhD in Biomedical Sciences

Name of educational establishment:
University of Worcester

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Name and contact details of academic supervisor(s):

Academic supervisor 1

	Title	Forename/Initials	Surname
	Dr	Steven	Coles

	University of Worcester,
	St Johns Campus, Henwick Grove,
Post Code	WR2 6AJ
E-mail	[redacted]
Telephone	[redacted]
Fax	[redacted]

Academic supervisor 2

	Title Forename/Initials Surname
	Dr [redacted]
Address	[redacted]
	University of Worcester,
	[redacted]
Post Code	[redacted]
E-mail	[redacted]
Telephone	[redacted]
Fax	

Academic supervisor 3

	Title Forename/Initials Surname
	Dr [redacted] [redacted]
Address	Institute of Science & The Environment,
	University of Worcester,
	[redacted]
Post Code	[redacted]
E-mail	[redacted]
Telephone	[redacted]
Fax	

Please state which academic supervisor(s) has responsibility for which student(s):
Please click "Save now" before completing this table. This will ensure that all of the student and academic supervisor details are shown correctly.

Student(s)	Academic supervisor(s)
Student 1 Mrs Angela Doughty	Dr Steven Coles Dr [redacted] Dr [redacted]

A copy of a current CV for the student and the academic supervisor (maximum 2 pages of A4) must be submitted with the application.

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A2-2. Who will act as Chief Investigator for this study?

- (●) Student
- ( ) Academic supervisor
- ( ) Other

Full Set of Project Data IRAS Version 5.3.1

A3-1. Chief Investigator:

	Title Forename/Initials Surname
	Mrs Angela Doughty
Post	Cardiology Research Coordinator
Qualifications	RGN BSc Honors Independent Nurse Prescriber
Employer	NHS
Work Address	Worcestershire Acute NHS Trust [redacted]
Post Code	[redacted]
Work E-mail	[redacted]
* Personal E-mail	[redacted]
Work Telephone	[redacted]

* Personal Telephone/Mobile [redacted]

Fax [redacted]

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*
A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application.

A4. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and HRA/R&D reviewers that is sent to the CI.[redacted]

[redacted]

Registration of research studies is encouraged wherever possible. You may be able to register **Appendix Q**

A5-1. Research reference numbers. *Please give any relevant references for your study:*

Applicant's/organisation's own reference number, e.g., R & D (if available):

access publisher. If you have registered your study, please give details in the "Additional reference number(s)" section.

A5-2. Is this application linked to a previous study or another current application?

○ Yes ◉ No

Please give brief details and reference numbers.

2. OVERVIEW OF THE RESEARCH

To provide all the information required by review bodies and research information systems, we ask a number of specific questions. This section invites you to give an overview using language comprehensible to lay reviewers and members of the public. Please read the guidance notes for advice on this section.

A6-1. Summary of the study. *Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments' Research Ethics Service, this summary will be published on the Health Research Authority (HRA) website following the ethical review. Please refer to the question specific guidance for this question.*

The aim of this study is to monitor markers of oxidative stress in patients with acute heart problems, compared to healthy volunteers with no diagnosed heart conditions.
Oxidative stress refers to the excessive production of free radicals in the body. In normal conditions the production of oxygen free radicals is balanced by efficient systems of antioxidants which are molecules capable of 'scavenging' oxygen free radicals or Reactive Oxygen Species (ROS).
If there's an inadequate antioxidant defense, ROS may lead to tissue damage. Markers of oxidative stress are not fully understood in patients with heart conditions. We will also look at the omega 3 and 6 levels to see if any emerging traits or benefits.
This study will attempt to clarify by gaining blood samples from patients following heart attacks, chest pain admissions to hospital and compare to 'healthy' volunteers, plus gain data on other factors that impact oxidative stress such as smoking, diet and medication.
Analysis will concentrate on range levels of oxidative stress, there prevalence as a diagnostic marker for

heart conditions, and if any correlations with readmission rates to see if there are detectable levels useful for diagnosis or predictions of potential hospital readmissions.
Blood measurement will also provide an opportunity to trial a new flow cytometric assay which has been developed at the University of Worcester.

A6-2. Summary of main issues. *Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.*

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, R&D office or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

Written informed consent from patients will be obtained before participation in the study. Patients invited to participate will be given a Patient Information Sheet to read and take away with them and patients can discuss it with others if they wish. The information sheet will tell the patients the purpose of the study, what will happen if they take part and detailed information about the conduct of the study. Patients will be given the

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opportunity to ask any member of the research team if there is anything that is not clear of if they would like more information. Patients will be allowed to take time to decide whether or not they wish to take part.
The patients' medical records and the data collected for the study will be looked at by authorised employees of Worcestershire's Acute Hospital only. All information that is collected during the study will be kept strictly confidential in the Cardiology Research Department. Each patient will be given a
Unique number and their name and other information that could potentially identify them will not be entered onto any documentation or samples that leave the site. Only the local research team will have access to their name. The recorded data will be kept in a secure location.
A Material Transfer Agreement between Worcestershire Acute Hospitals NHS Trust and Institute of Science & the Environment, University of Worcester will be adhered to at all times.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. *Please tick all that apply*:

- [ ] Case series/ case note review Case control
- [ ] Cohort observation
- [ ] Controlled trial without randomisation Cross-sectional study
- [ ] Database analysis
- [ ] Epidemiology Feasibility/ pilot study
- [ ] Laboratory study
- [ ] Metanalysis
- [ ] Qualitative

research

Questionnaire, interview or observation

study Randomised controlled trial

Other (please specify)

A10. What is the principal research question/objective? *Please put this in language comprehensible to a lay person.*

• Identify through questionnaire factors which may impact normal aerobic metabolic differences including participant's age, gender, lifestyle/past and present recreational such as smoking, diet, previous medical experiences/ generational attitudes of which may alter external stress indications.
• Explore specific plasma biomarkers of oxidative stress, including but not limited to; allantoin, GSH, TrX, and PRDX in healthy volunteers compared to those during and after an ACS event. Measurements will include 'total levels' as well as specific oxidation state.

Evaluate the intracellular redox status in peripheral blood mononuclear cells using a novel flow cytometry assay (developed at the University of Worcester) in healthy volunteers compared to those during and after an ACS event. Intracellular redox status of GSH, TrX and PRDX will also be evaluated (depending on cell

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• number).
• Evaluate erythrocyte lipid markers of oxidative stress as well as general membrane lipid composition and omega 3 and 6 levels. Recent literature indicates that certain fatty acids provide a protective role against cardiovascular disease
and that erythrocyte cell membranes proved an excellent representation of general lipid composition and redox status.
• Perform data analysis on all parameters measured and compare with standard clinical test data e.g. troponin T and total cholesterol. ROC analysis will be performed on subsequent ROS biomarker information in order to evaluate clinical utility.

A11. What are the secondary research questions/objectives if applicable? *Please put this in language comprehensible to a lay person.*

A12. What is the scientific justification for the research? *Please put this in language comprehensible to a lay person.*

The research of allantoin levels in humans as a biomarker for oxidative stress is limited, further research is warranted a) to investigate/clarify upper or lower limits as a mean for healthy volunteers b) for levels of serum allantoin in patients with acute ACS to investigate its prevalence to Troponin T levels
b) monitor if levels bear any prevalence to readmission rates.
Other novel markers of oxidative stress that are poorly understood in the context of CVD will also be investigated, including serum TRX, PRDX and GSH. The research will also provide opportunity to robustly trial a new flow cytometric assay for the high-throughput detection of intracellular oxidative stress in leukocytes, which may reveal a novel biomarker.

This study intends to compare the blood test troponin T levels, with markers of oxidative stress.
Investigating levels allantoin as a specific biomarker of oxidative stress in patients with heart disease and it's prevalence in monitoring for heart disease.

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Full Set of Project Data IRAS Version 5.3.1

A13. Please summarise your design and methodology. *It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.*

The project aims to explore quantitative data from blood sampled in participants with Acute ACS and healthy volunteers/siblings from the ACS arms.

Recruitment will run over twelve months; based on an average of two - four patients per week, potentially provide approximately over 200 participants, at least 50 % will be from ACS arm.

It is intended to collect data from consenting participants using structured questionnaires plus samples of blood following the acute ACS phase. This will be validated by collecting documented standard care data and Troponin T results.

Participants will be asked if they would be willing to return 2-6 months later for repeat sampling and/or follow up if readmitted. If participant has siblings an extra patient information leaflet will be given at discharge with researchers contact details for them to pass to siblings for considering potential participation as a controlled healthy volunteer.

Data will be collected identically in the non-ACS patients/healthy participants but only approached for a single appointment.

A database check for any serious adverse events within a year of hospital admissions will be conducted twelve months following recruitment as an end of study visit.

Consented participants will have a sample of blood taken (EDTA blood test - approximately 8 mls venous blood) the peripheral venous blood samples will be collected as soon as possible after stabilisation of admission along with a urine sample.
Interview questionnaires will be completed by the participants, information will include demographical and lifestyle indicators for comparatives to serum allantoin as certain elements such as cigarette smoking is known to increase allantoin

A14-1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

Design of the research

- [ ] Management of the research
- [ ] Undertaking the research
- [ ] Analysis of results
- [ ] Dissemination of findings
- [x] None of the above

Give details of involvement, or if none please justify the absence of involvement.

4. RISKS AND ETHICAL ISSUES

RESEARCH PARTICIPANTS

A15. What is the sample group or cohort to be studied in this research?

Select all that apply:

Blood

Cancer

- [x] Cardiovascular

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Full Set of Project Data IRAS Version 5.3.1

Congenital Disorders

Dementias and Neurodegenerative Diseases

Diabetes

Ear

Eye

Generic Health Relevance

Infection

Inflammatory and Immune System

Injuries and Accidents

Mental Health

Metabolic and Endocrine

Musculoskeletal

Neurological

Oral and Gastrointestinal

Paediatrics

Renal and Urogenital

Reproductive Health and Childbirth

Respiratory

Skin

Stroke

Gender:	Male and female participants
Lower age limit: 18	Years
Upper age limit:	No upper age limit

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A17-1. Please list the principal inclusion criteria (list the most important, max 5000 characters).

-Aged 18 years or older
Diagnosed with confirmed Acute Coronary Syndrome (ACS)STEMI, NSTEMI, Unstable Angina
- Troponin-T level on Admission and 3 hours after admission. Negative: both <20ng/L MI: Change in serial Troponins >10ng/L with one result > 20ng/L
ECG abnormalities - e.g. ST depression >0.5mm documented from standard care.
-Hospitalised with Stable Angina or Chest Pain no Diagnosis.
-Consenting Siblings Over 18
-Consenting Healthy Volunteers over 18.

A17-2. Please list the principal exclusion criteria (list the most important, max 5000 characters).

-STEMI, NSTEMI, Unstable Angina complicated by trauma, GI Bleeding, Admission for Staged PCI.
-Presence of any circumstance which in researchers' opinion could significantly limit the complete follow up of patient's, e.g. Tourist, Psychiatric disturbances
- Previous Recruitment to this study.
- Inability to consent

RESEARCH PROCEDURES, RISKS AND BENEFITS

A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *These include seeking consent, interviews, non-clinical observations and use of questionnaires.*

A21. How long do you expect each participant to be in the study in total?

12 Months

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

The study collects data on health and treatment; therefore, it does not present any additional risk other than the ones related to taking blood samples, it will not alter the standard procedure of care.
Taking blood may cause patient to feel faint, bruising, pain or bleeding from the puncture site. This will be minimised as per standard trust policies. Researcher is experienced phlebotomist.

Additional study visits: free parking will be made available to participants who make extra visits for purposes of the study to minimise the burden resulting from this.

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Informed Consent and Process and discussion	1	0	1 hour	Angela Doughty

Intervention or procedure	1	2	3	4
Blood Sample for Biochemistry testing	2	0	10 mins	Research Nurse
Weight Measurement	1	0	2 mins	Research Nurse
Height Measurement	1	0	2 Mins	Research Nurse

A24. What is the potential for benefit to research participants?

There may not be any benefit from taking part in this study, although previous participants in similar studies often find it useful having extra time to ask questions with the experienced members of the hospital staff.
The information obtained from this study may help improve treatment for people with similar disease.

RECRUITMENT AND INFORMED CONSENT

Full Set of Project Data IRAS Version 5.3.1

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27-1. How will potential participants, records or samples be identified? Who will carry this out and what resources will be used? *For example, identification may involve a disease register, computerised search of social care or GP records, or review of medical records. Indicate whether this will be done by the direct care team or by researchers acting under arrangements with the responsible care organisation(s).*

All patients presenting with chest pain will be invited to participate in the study.
Potential patients will be approached by the researcher or referred by the health care staff and owing to the urgent nature of the admission information will be provide as soon as clinically stable to discuss.
Patients will be approached for eligibility at the time of their presentation to hospital or as an out-patient's clinics.
The researcher will explain the study, give them the Patient Information Sheet (PIL) to read and answer any questions the patient may have in accordance ICH- GCP.
If the patient is willing to participate in the study, then they will be asked to complete the consent form.

Once written informed consent is obtained a copy will be given to the patient and a copy filed in medical notes.

During the research consultation information will be sought regarding siblings, if they do have siblings they will be asked if they would approach them and give a copy of the PIL and contact card and attend a single identical appointment as a controlled participant.

If Participants would like more time to consider their decision, then the research team will arrange to call them at a prearranged time to determine if they would like to take part and to get them to complete the consent form. For inpatients, if the patient would like more time to consider taking part, then a member of the researcher will return in person at a prearranged convenient time to see if they would like to take part or not.

The participant will be followed up once in clinic at 2-6 months from consent. Follow up will be by visit and with a review of the medical records at 12mths End of Study (EOS). Participants of the controlled group/healthy volunteers will have one visit only.

At each follow up point information on pre-existing and new medical conditions, hospital admissions, changes to medication and other therapies will be recorded by the research team. If the patient is deceased this will be recorded along with the cause(s) of death.

Appendix Q

A27-2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

No

Please give details below:
Patients will be identified at Worcestershire Acute Hospital as part of the researcher's professional role.
Professional standards and confidentiality will be adhered to at all times.

A27-3. Describe what measures will be taken to ensure there is no breach of any duty of confidentiality owed to patients, service users or any other person in the process of identifying potential participants. *Indicate what steps have been or will be taken to inform patients and service users of the potential use of their records for this purpose. Describe the arrangements to ensure that the wishes of patients and service users regarding access to their records are respected. Please consult the guidance notes on this topic.*

Only the researcher and members of the Cardiology Research Department will have access to patient information.

All identifiable data will be removed and allocation of a unique number. this will be used on all external documents and samples shipped.

A27-4. Will researchers or individuals other than the direct care team have access to identifiable personal information of any potential participants?

○ Yes ◉ No

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

○ Yes ◉ No

A29. How and by whom will potential participants first be approached?

Potential patients will be approached by the researcher.
Patients will be approached for eligibility at the time of their presentation to hospital or as an out patient's clinics.

Patient's relatives will provide information sheets to siblings for potential inclusion.

Standard care will not be disrupted.

A30-1. Will you obtain informed consent from or on behalf of research participants?

◉ Yes ○ No

If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

If you are not obtaining consent, please explain why not.

Please enclose a copy of the information sheet(s) and consent form(s).

Appendix Q

A30-2. Will you record informed consent (or advice from consultees) in writing?

Yes No

A31. How long will you allow potential participants to decide whether or not to take part?

Participants can consent from admission to discharge. If discharged then they can still participate but will be stratified to a different group.

Participants form control groups can have as long as required while screening open.

A33-1. What arrangements have been made for persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs?*(e.g. translation, use of interpreters)*

Although no arrangements have been made for participants for whom English is not a first language or participants who may not adequately understand verbal explanation, assistance will be provided on an individual basis as and when required. Only participants who can fully understand the implications of participating in this study will be recruited

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

◯ The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.

◉ The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.

◯ The participant would continue to be included in the study.

◯ Not applicable – informed consent will not be sought from any participants in this research.

◯ Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

If you plan to retain and make further use of identifiable data/tissue following loss of capacity, you should inform participants about this when seeking their consent initially.

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)?*(Tick as appropriate)*

Access to medical records by those outside the direct healthcare team

Access to social care records by those outside the direct social care team

Electronic transfer by magnetic or optical media, email or computer networks

Sharing of personal data with other organisations

Appendix Q

Export of personal data outside the EEA

Use of personal addresses, postcodes, faxes, emails or telephone numbers

Publication of direct quotations from respondents

Publication of data that might allow identification of individuals

Use of audio/visual recording devices

Storage of personal data on any of the following:

Manual files (includes paper or film)

NHS computers

Social Care Service computers

Home or other personal computers

University computers

Private company computers

Laptop computers

Further details:

A37. Please describe the physical security arrangements for storage of personal data during the study?

All NHS sites adhere to the data protection act and Caldicott principles.
Documents containing personal data are stored in locked rooms with limited access.

The researcher has Clinical Governance training yearly.

A38. How will you ensure the confidentiality of personal data? *Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g., anonymisation or pseudonymisation of data.*

All patient data will be anonymised by the research team. Each patient entering the study will be given a case specific number. This number replaces the use of any personal identifiable data.

This should ensure that there is no risk to patient confidentiality.
The research will keep a site file and retain a list of case specific study numbers for patients, this list will not leave the site.

A40. Who will have access to participants' personal data during the study? *Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.*

The researcher will store the site files and patient files in the cardiology research office which is locked at all times.

The Cardiology Research team will have access and are governed by the same data protection and Caldicott

Storage and use of data after the end of the study

A41. Where will the data generated by the study be analysed and by whom?

Dr Steven J Coles, Director of Studies, Angela Doughty PhD student

Award Leader in Biochemistry,

Appendix Q

Institute of Science & The Environment,
University of Worcester,
St Johns Campus, Henwick Grove,
Worcester, UK.
WR2 6AJ

Tel: ++44 (0[redacted]

A42. Who will have control of and act as the custodian for the data generated by the study?

Post Qualifications Work Address
Title Forename/Initials Surname Mrs AngelaDoughty
Cardiology Research Coordinator
RGN BSc Honours Independent Nurse Prescriber Worcestershire
Acute NHS Trust
Charles hasting Way
Post Code [redacted]
Work Email [redacted]
Work Telephone [redacted]
Fax [redacted]

A43. How long will personal data be stored or accessed after the study has ended?

- ○ Less than 3 months
- ○ 3 – 6 months
- ○ 6 – 12 months
- ○ 12 months – 3 years
- ◉ Over 3 years

If longer than 12 months, please justify:
Store for 15 years in our archive room.

A44. For how long will you store research data generated by the study?

Years: 15
Months: 0

A45. Please give details of the long term arrangements for storage of research data after the study has ended.
Say where data will be stored, who will have access and the arrangements to ensure security.

It will be archived with the hospitals Cardiology Research Departments Clinical trials.

INCENTIVES AND PAYMENTS

A46. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives

○ ◉

for taking part in this research?

Yes No

Appendix Q

A47. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?

Yes No

A48. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

Yes No

NOTIFICATION OF OTHER PROFESSIONALS

A49-1. Will you inform the participants' General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?

◉ Yes ○ No

If yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A49-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?

◉ Yes ○ No

It should be made clear in the participant's information sheet if the GP/health professional will be informed.

PUBLICATION AND DISSEMINATION

A50-1. Will the research be registered on a public database?

○ Yes ◉ No

Please give details, or justify if not registering the research.
Part of a PhD/MphiL research programme. If able to register as part of the NHS Organisation then this will be completed.

Registration of research studies is encouraged wherever possible.
You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. If you are aware of a suitable register or other method of publication, please give details. If not, you may indicate that no suitable register exists. Please ensure that you have entered registry reference number(s) in question A5-1.

A51. How do you intend to report and disseminate the results of the study?*Tick as appropriate:*

☑ Peer reviewed scientific journals

☐

☑

☑

Internal report

Conference presentation

Publication on website

Other publication

Appendix Q

Submission to regulatory authorities

Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators

No plans to report or disseminate the results

Other (please specify)

A52. If you will be using identifiable personal data, how will you ensure that anonymity will be maintained when publishing the results?

Not applicable

A53. Will you inform participants of the results?

No

Please give details of how you will inform participants or justify if not doing so.
Patients have contact numbers for the Cardiology Department and after degree completion they will be advised they can ring for results but this will not be routinely conducted at the end of the study.

5. Scientific and Statistical Review

A54-1. How has the scientific quality of the research been assessed? *Tick as appropriate:*

Independent external review

within a company

Review within a multi−centre research group

Review within the Chief Investigator's institution or host organisation

Review within the research team

☑ Review by educational supervisor

Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
Pear reveiw reports enclosed and changes addressed.

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/ institution.

A56. How have the statistical aspects of the research been reviewed? *Tick as appropriate:*

Review by independent statistician commissioned by funder or sponsor

Other review by independent statistician

Review by company statistician

Review by a statistician within the Chief Investigator's institution

Review by a statistician within the research team or multi−centre group

Review by educational supervisor

Other review by individual with relevant statistical expertise

Appendix Q

No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

Title Forename/Initials Surname
Dr Steven Coles
PhD

Department

Institution Work AddressInstitute of Science & The Environment, University of Worcester,
St Johns Campus, Henwick Grove, [redacted]
++44 (0)[redacted]

[redacted]
[redacted]uk

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

Relationship of Oxidative Stress in those suffering Acute Coronary Syndrome and healthy humans.

A58. What are the secondary outcome measures?*(if any)*

A59. What is the sample size for the research? *How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.*

Total UK sample size: 200
Total international sample size (including UK):
Total in European Economic Area:

Further details:

A60. How was the sample size decided upon? *If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.*

The sample size was based on other previously published studies and practical considerations such as the number of potential participants within the local area, and the maximum number of participants who can be accommodated within the eligibility criteria for the project to run effectively.
A sample size calculation on assuming equal sized groups was performed, relating to the normalised mean fluorescence intensity of the flow cytometric assay for evaluating intracellular redox potential (a principal technique of this study). Our preliminary preclinical data for the assay has a standardised difference of 0.59, based on a target difference of 0.49 and standard deviation of 0.83. Therefore, a sample size of ~120 is required for a p value of 0.05 with a power of 0.9 (in accordance with Whitley and Ball, 2002). The sample size selected will account for statistical calculations in the case of unequal sized groups, should this be the case at the end of the study.
It must be noted that the study is constrained by the time available to the Chief Investigator in carrying out a part time

PhD and within the maximum period of registration for doctoral study within the University of Worcester regulations. The sample size is a reflection of the feasibility of the study within the timeframe.

A61-1. Will participants be allocated to groups at random?

Yes No

Appendix Q

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

The qualitative data will be obtained from evaluative questionnaires, field notes and by carrying out semi-structured interviews. The supervisory team will check the analysis to ensure that all aspects of the data are fairly represented.

Quantitative data (epidemiologic and anthropometric data, blood markers, clinical data, etc.) will be presented as range, mean and standard deviation of the mean. Tests of normality will be performed (using IBM's SPSS software or similar) to determine whether the different datasets are normally distributed or not.
Where results are normally distributed, a suitable t-test or ANOVA (one or two way) will be carried out to identify any statistically significant differences between groups. When significant interactions are detected, Tukey's Honestly Significant Difference post hoc test will be employed to identify the different individual groups. Where data collected after the first admission are being compared to data collected after the second admission, a single tailed paired t-test will be used to test for statistical significance. If results are not normally distributed (non-parametric), Mann-Whitney U tests will likely to be performed for this purpose. Differences will be considered statistically significant if $p \leq 0.05$.
Missing data will be defined either as MAR (missing at random) or NMAR (not missing at random). Where data is missing at random either the participants with the missing data will be excluded from the analysis, or a LOCF technique will be used (Last Observation Carry Forward) (Dancey et al. 2012).

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. *Please include all grant co−applicants, protocol co−authors and other key members of the Chief Investigator's team, including non-doctoral student researchers.*

Under the Research Governance Framework for Health and Social Care, a sponsor outside the UK must appoint a legal representative established in the UK. Please consult the guidance notes.

A65. Has external funding for the research been secured?

Funding secured from one or more funders

External funding application to one or more funders in progress

No application for external funding will be made

What type of research project is this?

Standalone project

Project that is part of a programme grant

Project that is part of a Centre grant

Project that is part of a fellowship/ personal award/ research training award

Other

Other – please state:

A66. Has responsibility for any specific research activities or procedures been delegated to a subcontractor (other than a co-sponsor listed in A64-1) ? *Please give details of subcontractors if applicable.*

No

Appendix Q

A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another country? No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A6-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68-1. Give details of the lead NHS R&D contact for this research:

Title Forename/Initials Surname Mrs [redacted]
[redacted]
NIHR Clinical Research Network: West Midlands Worcestershire
Acute Hospitals NHS Trust R&D Management Offices
Worcestershire Clinical Research Unit, Newtown Road WR5 1HN
[redacted]

[redacted]

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

A69-1. How long do you expect the study to last in the UK?

Planned start date: 02/02/2016
Planned end date: 02/02/2020
Total duration:
Years: 4 Months: 0 Days: 1

A70.

Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial

Voce Viva for Research Degree

A71-1. Is this study?

Single centre

Multicentre

A71-2. Where will the research take place? *(Tick as appropriate)*

England

Total UK sites in study 1

Does this trial involve countries outside the EU?

No

A72. Which organisations in the UK will host the research? *Please indicate the type of organisation by ticking the box and give approximate numbers if known:*

NHS organisations in England

Appendix Q

Independent (private or voluntary sector) organisations

☑ Educational establishments

Independent research units

Other (give details)

Total UK sites in study: 0

A73-1. Will potential participants be identified through any organisations other than the research sites listed above?

○ Yes ◉ No

A74. What arrangements are in place for monitoring and auditing the conduct of the research?

Research Degree Board University of Worcester

A76. Insurance/ indemnity to meet potential legal liabilities

Note: in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland

A76-1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? *Please tick box(es) as applicable.*

Note: Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

☑ NHS indemnity scheme will apply (NHS sponsors only)

Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-2. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? *Please tick box(es) as applicable.*

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

☑

NHS indemnity scheme will apply (protocol authors with NHS contracts only)

Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-3. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

Note: Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at these sites and provide evidence.

☑ NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)

Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

Please enclose a copy of relevant documents.

Appendix Q

A78. Could the research lead to the development of a new product/process or the generation of intellectual property?

○ Yes ◉ No ○ Not sure

Part B: Section 4 – Use of residual or existing stored human tissue (or other human biological materials)

1. What types of human tissue or other biological material will be included in the study?

Standard bloods for inclusion, EDTA 8mls for research

2. Will the samples be released to the researcher:

In fully anonymised form? *(Link to stored tissue and data is broken)*

○ Yes ◉ No

In linked anonymised form? *(Linked to stored tissue but donor not identifiable to researchers)*

◉ Yes ○ No

In a form in which the donor could be identifiable to researchers?

○ Yes ◉ No

3. Has consent been obtained previously to use the samples for research

◉ Consent has been given for all samples

○ Consent has been given for some of the samples

○ No consent has been given

4. Please outline what consents are already in place, distinguishing between different groups of samples where appropriate.

Local labs will be taken as part of standard care, where possible if able to assent at same time as local bloods to reduce procedures for patients. Full consent will be taken prior to any other action.

Assent/Consent to obtain blood for the purpose of the study and to transfer to the university will be obtained.
The donor site will keep ownership at all times.

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

○ Yes ◉ No

8. What types of test or analysis will be carried out on the samples?

Troponium T, liver function, CKMB, Total Cholesterol, LDL, HDL, will be obtained from Standard Care bloods to confirm Eligibility.

lipid and metabolite targets of ROS, plasminogen and uric acid
protein/peptide targets of ROS,
glutathione (GSH),
thioredoxin (TrX)
Peroxiredoxin (PRDX).

○ ◉ Omega 3 and 6 levels

9. Will the research involve the analysis or use of human DNA in the samples?

○ Yes ◉ No

10. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

11. If so, will arrangements be made to notify the individuals concerned?

○ Yes
○ No
◉ Not applicable

12. Who is the holder of the samples?

Please tick either/both boxes as applicable.

NHS pathology department(s) / diagnostic archive(s)
Specific details of each department/archive are not required

☑ Other research tissue bank(s) or sample collection(s)
Please provide further details of each bank/collection below

Appendix Q

Name of the research tissue bank (or other collection):

Does the bank/collection hold a license from the Human Tissue Authority to store tissue for research?
○ Yes ○ No

REC reference no. (if the bank/collection is ethically approved):
☑

	Title Forename/Initials Surname
	Dr Steven Coles
Address	Institute of Science & The Environment,
	University of Worcester,
	St Johns Campus, Henwick Grove,
Post Code	[redacted]
Telephone	[redacted]
Fax Mobile	
Email	[redacted]
Contact point	[redacted]
	Institute of Science and the Environment

13. Will any of the samples be imported from outside the UK?

No

14. Please give details of where the samples will be stored, who will have access and the custodial arrangements.

Blood samples will be shipped on the day of collection to University of Worcester (UoW) at least fortnightly in accordance with biomedical transfer regulations.
Material Transfer Agreement
This Agreement is made by and between:
a) Worcestershire Acute Hospitals NHS Trust, Aconbury East, Charles Hasting way, Worcester, WR5 1 DD ("the Donor Institution") and
b) Institute of Science & The Environment, University of Worcester, St Johns Campus, Henwick Grove, Worcester, WR2 6AJ ("the Recipient Institution")
Signed Agreement enclosed.
The Material may only be used by those under the Recipient Scientist's direct supervision in the Recipient Institution's laboratories under suitable containment conditions, and in compliance with all applicable statutes and regulations. THE MATERIAL MAY NOT BE USED IN HUMAN SUBJECTS OR FOR CLINICAL OR DIAGNOSTIC PURPOSES.

15. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

Return to current holder of the samples

Transfer to another tissue bank

(If the bank is in England, Wales or Northern Ireland a licence from the Human Tissue Authority will be required to store relevant material for possible further research.)

Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

Storage by research team as part of a new research tissue bank

(The institution will require a storage license for research from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

Part B: Section 5 – Use of newly obtained human tissue(or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?

Whole Blood EDTA 8mls

2. Who will collect the samples?

Chief Investigator will collect or be present during routine collection

Appendix Q

The donor site will keep ownership at all times.

10. Will the research involve the analysis or use of human DNA in the samples?

☐ Yes ◉ No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

In future research?

○ Yes ○ No ◉ Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

○ Yes ◉ No

8. Will the samples be stored: *[Tick as appropriate]*

In fully anonymised form? *(link to donor broken)*

◉ Yes ○ No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*

○ Yes ◉ No

In a form in which the donor could be identifiable to researchers?

○ Yes ◉ No

12. If so, will arrangements be made to notify the individuals concerned?

○ Yes ○ No ◉ Not applicable

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Blood samples will be shipped on the day of collection to University of Worcester (UoW) at least fortnightly in accordance with biomedical transfer regulations.
Material Transfer Agreement
This Agreement is made by and between:
a) Worcestershire Acute Hospitals NHS Trust, Aconbury East, Charles Hasting way, Worcester, WR5 1 DD ("the Donor Institution") and
b) Institute of Science & The Environment, University of Worcester, St Johns Campus, Henwick Grove, Worcester, WR2 6AJ ("the Recipient Institution")

Appendix Q

14. **What will happen to the samples at the end of the research?** *Please tick all that apply and give further details.*

Transfer to research tissue bank

(If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue Authority to store relevant material for possible further research.)

Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

Storage by research team as part of a new research tissue bank

(The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

Disposal in accordance with the Human Tissue Authority's Code of Practice

Other

Not yet known

Please give further details of the proposed arrangements:

PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. *For NHS sites, the host organisation is the Trust or Health Board. Where the research site is a primary care site, e.g. GP practice, please insert the host organisation (PCT or Health Board) in the Institution row and insert the research site (e.g. GP practice) in the Department row.*

Research site	Investigator/ Collaborator/ Contact

Appendix S

NHS
Health Research Authority

Mrs Angela Doughty
Cardiology Research Coordinator
Worcestershire Acute NHS Trust
Charles Hasting Way
[redacted]
[redacted]@nhs.net

Email: hra.approval@nhs.net

30 May 2017

Dear Mrs Doughty,

Letter of HRA Approval

Study title:	**Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome**
IRAS project ID:	**189016**
REC reference:	**17/WM/0132**
Sponsor	**Worcestershire Acute Hospitals NHS Trust**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

Appendix S

IRAS project ID	189016

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators",* issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants

and sponsors. You are invited to give your view of the service you have received and the application

Appendix S

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procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **189016**. Please quote this on all correspondence.

Yours sincerely

[redacted]

Assessor

Email: hra.approval@nhs.net

Copy to: *Dr Steven Coles, Worcester University [Academic Supervisor]* [redacted];
Dr [redacted]*, Worcester University [Academic Supervisor]*
[redacted]
Dr [redacted]*, Worcester University [Academic Supervisor]*
[redacted]
Mrs [redacted]*, NIHR Clinical Research Network: West Midlands [Sponsor Contact & Lead NHS R&D Contact]*
[redacted]nihr.ac.uk

Appendix S

IRAS project ID	189016

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
Contract/Study Agreement [Material Transfer Agreement (University of Worcester & Worcester Acute Hospitals NHS Trust)]		24 November 2015
Costing template (commercial projects) [Permission to use retained equipment]	N/A	13 March 2017
Covering letter on headed paper [Approval Submission]	V1	10 March 2017
GP/consultant information sheets or letters [GP Letter]	V1	01 November 2015
IRAS Application Form [IRAS_Form_16032017]		16 March 2017
IRAS Application Form XML file [IRAS_Form_16032017]		16 March 2017
IRAS Checklist XML [Checklist_16032017]		16 March 2017
IRAS Checklist XML [Checklist_18052017]		18 May 2017
Letter from funder [Approval Letter]	N/A	07 December 2016
Letter from sponsor [Sponsorship Confirmation]	N/A	09 March 2017
Letters of invitation to participant [Invite Healthy volunteers]	V1	08 August 2016
Letters of invitation to participant [Invite letter medically discharged patients]	V1	01 July 2016
Letters of invitation to participant [Invite Healthy volunteers]	v1	08 August 2016
Non-validated questionnaire [Patient Questionnaire]	V1	01 July 2016
Other [WAT01 Informed Consent SOP]	1.0	10 October 2016
Other [CV - [redacted]]		27 March 2017
Other [CV - Professor [redacted]]		
Participant consent form [Assent]	V1	22 October 2016
Participant consent form [Consent Form]	V2	25 April 2017
Participant consent form [Verbal Consent]	V2	25 April 2017
Participant information sheet (PIS) [Patient Information Leaflet]	V1	12 July 2016
Participant information sheet (PIS) [Patient Information Leaflet]	V2	25 April 2017
Referee's report or other scientific critique report [Expert Review 1]	V1	29 September 2015
Research protocol or project proposal [Final RDB1 Proposal]	V1	16 November 2016
Summary CV for Chief Investigator (CI) [CV & GCP]	NA	29 September 2016
Summary CV for student [CV]	NA	29 September 2016
Summary CV for supervisor (student research) [CV Supervisor]	N/A	09 March 2017
Summary, synopsis or diagram (flowchart) of protocol in non-technical language [UoW Lay Term Application for Ethical Approval]	V1	09 March 2017

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Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.

For information on how the sponsor should be working with participating NHS organisations in England, please refer to the *participating NHS organisations*, *capacity and capability* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections in this appendix.

The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Name: Mrs [redacted]
[redacted] Tel: [redacted]
Email: [redacted]nihr.ac.uk

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	This is a non-commercial single site study taking place in the NHS where that single NHS organisation is also the study sponsor. Therefore, no study agreements are required.

4.2	Insurance/indemnity arrangements assessed	Yes	Where applicable, independent contractors (e.g., General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the activities expected of them

IRAS project ID	**189016**

Section	HRA Assessment Criteria	Compliant with Standards	Comments
			research study.
4.3	Financial arrangements assessed	Yes	External funding has not been obtained for the study.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	Human Tissue Act A Material Transfer Agreement is to be used between Worcester Acute Hospitals NHS Trust and the Institute of Science & the Environment, University of Worcester.
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	REC Favourable Opinion was obtained from West Midlands – Solihull Research Ethics Committee on 26 May 2017.
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Appendix S

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
This is a non-commercial single site study taking place in the NHS where that single NHS organisation is also the study sponsor. Therefore there is only one site type involved in the research. If this study is subsequently extended to other NHS organisation(s) in England, an amendment should be submitted to the HRA, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England.

IRAS project ID	189016

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.
This is a single site study sponsored by the site. The R&D office will confirm to the CI when the study can start.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A Principal Investigator should be appointed at study sites. GCP training is not a generic training expectation, in line with the HRA statement on training expectations.

Appendix S

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
The sponsor has confirmed that all study activities will be undertaken by local staff who have a contractual relationship with the relevant organisation. Therefore no honorary research contracts or letters of access are expected for this study.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
The applicant has indicated that they do not intend to apply for inclusion on the NIHR CRN Portfolio.

Appendix T

IRAS PROJECT ID 189016, REC Reference 17/WM/0132: Amendment acknowledgement and implementation information

You forwarded this message on Mon 20/08/2018 12:10
You forwarded this message on Mon 20/08/2018 12:10
hra.amendments@nhs.net <noreply@harp.org.uk>

To:
- DOUGHTY, Angela (WORCESTERSHIRE ACUTE HOSPITALS NHSTRUST);
- [redacted]@nihr.ac.uk

Cc:
- [redacted]@nihr.ac.uk

Thu 10/05/2018 14:49

New Site Amendment, Implementation Information

Dear Mrs Doughty

IRAS Project ID:	189016
Short Study Title:	Monitoring Oxidative Stress in Acute Coronary Syndrome
Date complete amendment submission received:	10 April 2018
Sponsor Amendment Reference Number:	
Sponsor Amendment Date:	25 April 2018
Amendment Type:	Non-substantial
For new sites in Northern Ireland and/or Scotland:	Please start to set up your new sites. Sites may not open until NHS management permission is in place.
For new sites in England and/or Wales:	For studies which already have HRA and HCRW Approval: **This email also constitutes HRA and HCRW Approval for the amendment, and you should not expect anything further**. Please start to set up your new sites. Sites may not open until the site has confirmed capacity and capability (where applicable). For studies which do not yet have HRA and HCRW Approval: **HRA and HCRW Approval for the initial application is pending**. You can start the process of setting up the new site but cannot open the study at the site until HRA and HCRW Approval is in place and the site has confirmed capacity and capability (where applicable). For studies with HRA Approval adding Welsh NHS organisations for the first time. Please take this email to confirm your **original HRA Approval letter is now extended to cover NHS organisations in Wales. You now have HRA and HCRW Approval.** Please start to set up your new sites. Sites may not open until the site has confirmed capacity and capability (where applicable).

Thank you for submitting an amendment to add one or more new sites to your project. This amendment relates solely to the addition of **new sites**.

Appendix T

What should I do next?

Please set up the new site(s) as per the guidance found within IRAS. **Please note** that processes change from time to time so please use the most up to date guidance about site set up.

If your study is supported by a research network, please contact the network as early as possible to help support set up of the new site(s).

If you have listed new sites in any other UK nations **we will** forward the information to the national coordinating function(s) for nations where the new site(s) are being added. In Northern Ireland and Scotland, NHS/HSC R&D offices will be informed by the national coordinating function.

Note: you may only implement changes described in the amendment notice.

Who should I contact if I have further questions about this amendment?

If you have any questions about this amendment please contact the relevant national coordinating centre for advice:

- England – hra.amendments@nhs.net
- Northern Ireland – research.gateway@hscni.net
- Scotland – nhsg.NRSPCC@nhs.net
- Wales – research-permissions@wales.nhs.uk

Additional information on the management of amendments can be found in the IRAS guidance.

User Feedback

We are continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the amendment procedure. If you wish to make your views known please use the feedback form available at: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

Please do not hesitate to contact me if you require further information.

Kind regards

[redacted]

Health Research Authority
Ground Floor | Skipton House | 80 London Road | London | SE1 6LH
E.hra.amendments@nhs.net
W. www.hra.nhs.uk

Sign up to receive our newsletter HRA Latest.

Partner Organisations:
Health Research Authority, England NIHR Clinical Research Network, England
NHS Research Scotland NISCHR Permissions Co-ordinating Unit, Wales
HSC Research & Development, Public Health Agency, Northern Ireland

Appendix U

Notification of Non-Substantial/Minor Amendments(s) for NHS Studies

This template **must only** be used to notify NHS/HSC R&D office(s) of amendments, which are **NOT** categorised as Substantial Amendments.
If you need to notify a Substantial Amendment to your study then you MUST use the appropriate Substantial Amendment form in IRAS.

Instructions for using this template

- For guidance on amendments refer to http://www.hra.nhs.uk/research-community/during-your-research-project/amendments/
- This template should be completed by the CI and optionally authorised by Sponsor, if required by sponsor guidelines.
- This form should be submitted according to the instructions provided for NHS/HSC R&D at http://www.hra.nhs.uk/research-community/during-your-research-project/amendments/which-review-bodies-need-to-approve-or-be-notified-of-which-types-of-amendments/ . If you do not submit your notification in accordance with these instructions then processing of your submission may be significantly delayed.

1. Study Information

Full title of study:	Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome
IRAS Project ID:	189016
Sponsor Amendment Notification number:	
Sponsor Amendment Notification date:	
Details of Chief Investigator:	
Name [first name and surname]	Angela Doughty
Address:	Worcestershire Acute Hospitals NHS Trust Aconbury East, Charles Hasting way Worcester,
Postcode:	WR5 1 DD
Contact telephone number:	[redacted]
Email address:	[redacted]@nhs.net
Details of Lead Sponsor:	

Appendix U

Partner Organisations:
Health Research Authority, England NIHR Clinical Research Network, England
NHS Research Scotland NISCHR Permissions Co-ordinating Unit, Wales
HSC Research & Development, Public Health Agency, Northern Ireland

Name:	[redacted]
Contact email address:	[redacted]@nhs.net
Details of Lead Nation:	
Name of lead nation *delete as appropriate*	England
If England led is the study going through CSP? *delete as appropriate*	No
Name of lead R&D office:	Worcestershire Acute NHS Trust [redacted]

Appendix U

Partner Organisations:
Health Research Authority, England NIHR Clinical Research Network, England
NHS Research Scotland NISCHR Permissions Co-ordinating Unit, Wales
HSC Research & Development, Public Health Agency, Northern Ireland

2. Summary of amendment(s)

This template **must only** be used to notify NHS/HSC R&D office(s) of amendments, which are **NOT** categorised as Substantial Amendments.
If you need to notify a Substantial Amendment to your study then you MUST use the appropriate Substantial Amendment form in IRAS.

No.	Brief description of amendment *(please enter each separate amendment in a new row)*	Amendment applies to *(delete/ list as appropriate)*		List relevant supporting document(s), including version numbers *(please ensure all referenced supporting documents are submitted with this form)*		R&D category of amendment *(category A, B, C) For office use only*
		Nation	**Sites**	**Document**	**Version**	
1	Approach lay people attending the local blood donor service if they would like to participate as a healthy volunteer. The same PIL and Consent will be used. The blood transfusion service will take bloods so no	England	All sites or list affected sites			
		Northern Ireland	All sites or list affected sites			
		Scotland	All sites or list affected sites			

	additional indemnity insurance required. Blood Donor service have approved my attendance following full scrutiny. I will discuss with volunteers and gained informed consent as with all 'healthy volunteer' participants. No amended documents are required it is just a different location. I will ship to the single site WAHT and process bloods in the same way as transporting from WHAT to UoW. Location to recruit more healthy volunteers is just to increase a wider demographic gender and age population as only hospital staff invited to participate have been predominantly female. Many may just be given PIL and attend WAHT at a later date.	Wales	All sites or list affected sites			

Appendix U

Partner Organisations:

Health Research Authority, England
NIHR Clinical Research Network, England
NHS Research Scotland
NISCHR Permissions Co-ordinating Unit, Wales
HSC Research & Development, Public Health Agency, Northern Ireland

2						
3						
4						
5						

[Add further rows as required]

Appendix U

Partner Organisations:
Health Research Authority, England NIHR Clinical Research Network, England
NHS Research Scotland NISCHR Permissions Co-ordinating Unit, Wales
HSC Research & Development, Public Health Agency, Northern Ireland

3. Declaration(s)

Declaration by Chief Investigator

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment(s) to be implemented.

Signature of Chief Investigator: C#@d”t}]8sQt ……

Print name: …….Angela Doughty……

Date: …26/04/2018……

Optional Declaration by the Sponsor’s Representative (as per Sponsor Guidelines)

The sponsor of an approved study is responsible for all amendments made during its conduct.

The person authorising the declaration should be authorised to do so. There is no requirement for a particular level of seniority; the sponsor’s rules on delegated authority should be adhered to.

- I confirm the sponsor’s support for the amendment(s) in this notification.

Signature of sponsor’s representative: ……

Print name:……

Post: ……

Organisation:……

Date:……

Appendix V

Patient Screening Log - Confidential

Study: Development and application of a high throughput assay for monitoring oxidative stress in acute coronary syndrome

Group _ACS

Record of patients who have provided written consent* using the ethics approved Informed Consent documents for this study

*patient consent is required prior to any study specific procedures and prior to use of a patient's data or biological samples for this clinical trial.

Consent Date	Screen Number	Name/hospital Number	Telephone Number	Address

Appendix W

Procedure outline for TRX antibodies

1. The night prior to ELISA, prepare plate(s) using various dilutions of antigen and incubate in fridge. Number the strips and use marker to format layout if possible.

Samples 100ul of 40 samples on each plate in duplicate (as labeled below)
Standards: TRX stock 1mg/ml
Add 100ul /well for all dilutions (detailed below in yellow)
Add 1ul stock/1000ul PBS (1:1000)
Add 200ul of above+1800ul PBS (further 1:10 = 100ngs/ml = top concentration)
Doubling dilutions (500ul above+500PBS for 6 more dilutions (50ngs/ml down to 1.5625ngs/ml).
Add 100ul buffer only to H1 and H2 (in brown)
Leave antigen on wells o/n

ngs/ml

100	100	1	rep	9	rep	17	rep	25	rep	33	rep
50	50	2	rep	10	rep	18	rep	26	rep	34	rep
25	25	3	rep	11	rep	19	rep	27	rep	35	rep
12.5	12.5	4	rep	12	rep	20	rep	28	rep	36	rep
6.25	6.25	5	rep	13	rep	21	rep	29	rep	37	rep
3.125	3.125	6	rep	14	rep	22	rep	30	rep	38	rep
1.562	1.562	7	rep	15	rep	23	rep	31	rep	39	rep
0	0	8	rep	16	rep	24	rep	32	rep	40	rep

2. Pre wash wells with 200µl of PBS/Tw/Cs. Empty well contents over sink and tap dry on absorbent material.

3. Block all wells with 1% Casein – 200µl per well for 30 mins at room temperature.

4. Wash wells with PBSTwC – 200µl per well, empty over sink and tap dry as previous.

5. Add 100µl/well of TRX primary mouse antibody (in bag in freezer). Use 1ul/10 mls (3 plates here so 3ul/30 mls) across whole of 3 plates. Allow to incubate on bench for 1 hour at RT (on bench).

6. Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

7 Add 100ul/well 1:500 (ie 60ul/30 mls here)) Anti-mouse- Biotin (from my fridge R10 second shelf down RHS 2 bottles on blue rack). Allow to incubate on bench for 1 hour at RT (on bench).

8 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

9 Add 100ul/well Streptavidin Peroxidase (HRP) solution (regardless of mouse or rabbit, from my fridge R10 same place) (1:8000 ie 3.6ul/30 mls) in PBSTwC. Incubate at room temperature for 1 hour.

Appendix W

10 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

11 Make up TMB in Phosphate citrate immediately prior to adding.

12 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

13 Add TMB 100µl per well and keep microtitre plate on plate shaker at 300rpm in dark and allow color development. TMB is light sensitive.

14 Allow TMB to develop blue color for up to 20-30 minutes but this will depend on background.

15 To stop the reaction, add 50µl stop solution of 20% (or 1.5M) Sulphuric Acid (stored in safety cabinet in R10 outer room).

16 Read at wavelength of 450nm using ELISA plate reader in technician's lab.

Other assays:

For other assays (PRDX-2, PRDX-4 & TRX-Reductase) replace the antigen and primary antibody with appropriate concentrations from the excel document (also attached).

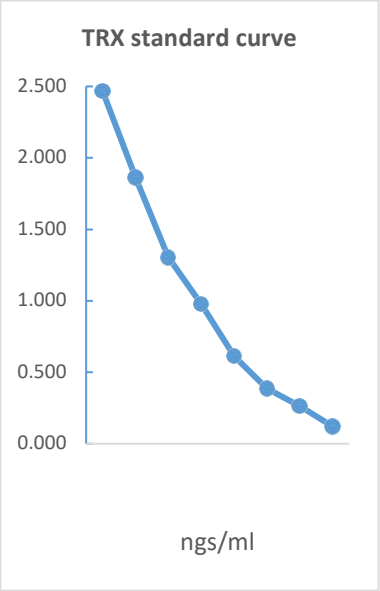
If the primary antibody is rabbit rather than mouse be aware to replace the anti-mouse- biotin step with anti-rabbit-Biotin (this in frozen aliquots in the fridge) (check other concentrations as well as sometimes Strep-HRP conc sometimes different (all in excel sheet).

At the back of the risk assessment ELISA you will find out how to make up the various buffers.

Appendix W

Optimisation Thioredoxin (TRX)
TRX standard curve was optimised as part of training so optimisation tables not all available.

				Average
100	2.487	2.503	2.415	2.468
50	1.957	1.868	1.762	1.862
25	1.397	1.292	1.224	1.304
12.5	1.019	0.973	0.943	0.978
6.25	0.637	0.494	0.704	0.612
3.125	0.410	0.390	0.361	0.387
1.5625	0.246	0.273	0.274	0.264
0	0.116	0.118	0.127	0.120



1:10000 dilution of the primary antibody (1 µl in 10 ml) with a 1:500 dilution of the Anti-mouse- Biotin (60 µl in 30 ml) provided the optimal ratio and used for procedure (Appendix Y)

Primary antibody	TRX	1 in 10,000	anti mouse		
Secondary antibody		1 in 500	anti mouse biotinylated		
Streptavidin-HRP		1 in 8000			

Primary Antibody - used from in house stock (ab51064).
Anti- Mouse (Biotin) from in house stock (SLS B7264) used
HRP - in house stock (SLS S5512).

Secondary antibody
1:8000 streptavidin

Appendix X

Procedure outline for TRX reductase antibodies

1. The night prior to ELISA, prepare plate(s) using various dilutions of antigen and incubate in fridge. Number the strips and use marker to format layout if possible.

Samples 100ul of 40 samples on each plate in duplicate (as labeled below)
Standards: TRXr stock 0.5mg/ml (500 ng/ml)
Add 100 µl /well for all dilutions (detailed below in yellow)
Add 1 µl stock to 1ml PBS (1:499)/add to 9mls PBS (10mls per plate)
(further 1:5 = 100ngs/ml = top concentration)
Doubling dilutions (200ul above + 500PBS for 6 more dilutions (50ngs/ml down to 1.5625ngs/ml).
Add 100ul buffer only to H1 and H2 (in brown)
Leave antigen on wells o/n

ngs/ml

100	100	1	rep	9	rep						
50	50	2	rep	10	rep						
25	25	3	rep	11	rep						
12.5	12.5	4	rep	12	rep						
6.25	6.25	5	rep	13	rep						
3.125	3.125	6	rep	14	rep						
1.562	1.562	7	rep	15	rep						
0	0	8	rep	16	rep						

2. Pre wash wells with 200µl of PBS/Tw/Cs – 3 times. Empty well contents over sink and tap dry on absorbent material.

3. Block all wells with 1% Casein – 200µl per well for 30 mins at room temperature.
1% Casein x3 10mls

4. Wash wells with PBS/Tw/C 3 times – 200µl per well, empty over sink and tap dry as previous.

5. Add 100µl PBS/Tw/ and TRXr primary Rabbit antibody to each well
Allow to incubate on bench for 1 hour at RT (on bench).
Use 10 µl /10 mls (1:1000 Primary)
10 µl – 10 mls – 1 plate
30 µl – 30 mls – 3 plates

6. Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

7 Add 100ul/well 1:5000 (i.e., 60 µl /30 mls here) Anti-Rabbit- Biotin (from fridge bottles on blue rack). Allow to incubate on bench for 1 hour at RT (on bench).
2 µl – 10 mls – 1 plate 1 µl: 5000 (optimization)
6 µl – 30 mls – 3 plates

Appendix X

8 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

9 Add 100ul/well Streptavidin Peroxidase (HRP) solution (regardless of mouse or rabbit from blue tray) (1:8000 i.e., 3.6ul/30 mls) in PBSTwC. Incubate at room temperature for 1 hour.
1.2 µl – 10 mls – 1 plate
3.6 µl – 30 mls – 3 plates

10 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

11 Make up TMB in Phosphate citrate immediately prior to adding.

12 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

13 Add TMB 100µl per well and keep microtitre plate on plate shaker at 300rpm in dark and allow color development. TMB is light sensitive.

14 Allow TMB to develop blue color for up to 30 minutes.

15 To stop the reaction, add 50µl stop solution of 20% (or 1.5M) Sulphuric Acid (stored in safety cabinet in R10 outer room).

16 Read at wavelength of 450nm using ELISA plate reader in technician's lab.

Standards: 0.5 Micrograms (µl) = 500 Nanograms (ng)
Serial Dilution

- Took 0.5 µl/ml of TRXr Antigen and diluted

1:1000 to give 0.5 µl/ml (500ng/ml)
This was 1 µl of Antigen and 999 µl of PBS/Tw/C

- Took 500 µl/ ml diluted antigen and diluted further 1:5 to give

100 µl /ml

- Here we did 300 µl of the 500 µl and added 1200 µl (2x 600 µl) of PBS/Tw/C

Next prepared a serial dilution of the 100 µl to 1:2 antigen (750 µl Antigen/ 750 µl of PBS)

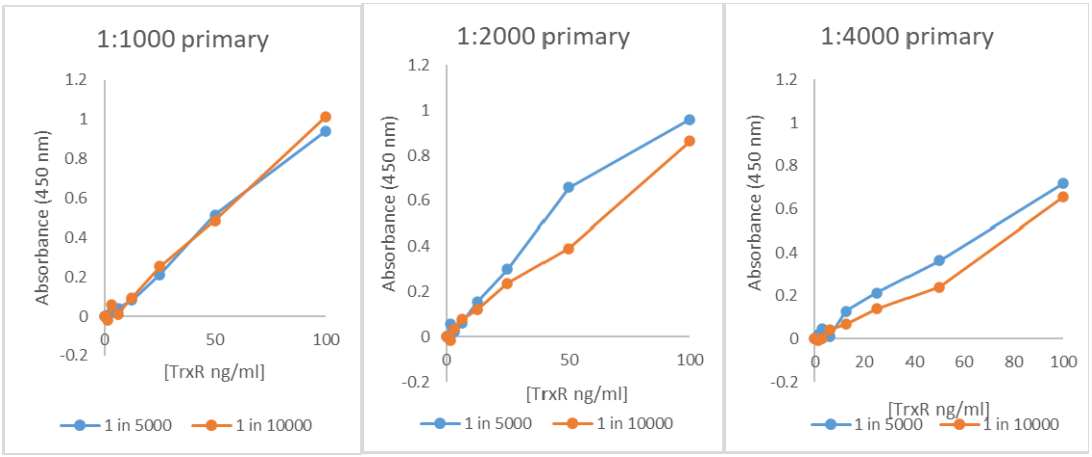
This gave 50, 25, 12.5. 6.25, 3,125, 1.562

Appendix X

Optimisation Thioredoxin-reductase (TRXr)

[Antigen] ng/ml	Absorbance (450 nm)					
secondary	1 in 5000	1 in 5000	1 in 5000	1 in 10000	1 in 10000	1 in 10000
primary	1 in 1000	1 in 2000	1 in 4000	1 in 1000	1 in 2000	1 in 4000
	A	B	C	D	E	F
100	1.249	1.195	0.965	1.275	1.122	0.938
50	0.822	0.893	0.611	0.746	0.651	0.523
25	0.52	0.535	0.461	0.513	0.498	0.422
12.5	0.39	0.389	0.377	0.353	0.379	0.35
6.25	0.348	0.294	0.259	0.27	0.337	0.326
3.13	0.327	0.256	0.293	0.319	0.297	0.285
1.56	0.314	0.29	0.266	0.24	0.245	0.274
0	0.308	0.235	0.248	0.26	0.26	0.283

	Normalised					
	1 in 1000	1 in 1000	1 in 2000	1 in 2000	1 in 4000	1 in 4000
	1 in 5000	1 in 10000	1 in 5000	1 in 10000	1 in 5000	1 in 10000
100	0.941	1.015	0.96	0.862	0.717	0.655
50	0.514	0.486	0.658	0.391	0.363	0.24
25	0.212	0.253	0.3	0.238	0.213	0.139
12.5	0.082	0.093	0.154	0.119	0.129	0.067
6.25	0.04	0.01	0.059	0.077	0.011	0.043
3.125	0.019	0.059	0.021	0.037	0.045	0.002
1.5625	0.006	-0.02	0.055	-0.015	0.018	-0.009
0	0	0	0	0	0	0



TRXr - 1:1000 dilution of the primary antibody (5 µl in 10 ml) with a 1:5000 dilution of the anti-rabbit biotin (2 µl in 10 ml) provided the optimal ratio and used for procedure. (See Appendix Z)

Primary Antibody - ab124954 Rabbit monoclonal TRX reductase (EPNCIR129) to TXNRD Lot no: GR241418-14. Secondary antibody Goat Anti-Rabbit IgG H&L (Biotin) Lot No: GR3356030 - Used 1:8000 streptavidin HRP - in house stock (SLS S5512).

Appendix Y

Procedure outline for PRDX-2 antibodies

1. The night prior to ELISA, prepare plate(s) using various dilutions of antigen and incubate in fridge. Number the strips and use marker to format layout if possible.

Samples 100ul of 40 samples on each plate in duplicate (as labeled below)
Standards: PRXr stock 0.5mg/ml
Add 100ul /well for all dilutions (detailed below in yellow)
Add 1ul stock/1000ul PBS (1:1000)
Add 100ul of above+1800ul PBS (further 1:5 = 100ngs/ml = top concentration)
Doubling dilutions (300ul above+500PBS for 6 more dilutions (50ngs/ml down to 1.5625ngs/ml).
Add 100ul buffer only to H1 and H2 (in brown)
Leave antigen on wells o/n

ngs/ml

100	100	1	rep	9	rep	17	rep	25	rep	33	rep
50	50	2	rep	10	rep	18	rep	26	rep	34	rep
25	25	3	rep	11	rep	19	rep	27	rep	35	rep
12.5	12.5	4	rep	12	rep	20	rep	28	rep	36	rep
6.25	6.25	5	rep	13	rep	21	rep	29	rep	37	rep
3.125	3.125	6	rep	14	rep	22	rep	30	rep	38	rep
1.562	1.562	7	rep	15	rep	23	rep	31	rep	39	rep
0	0	8	rep	16	rep	24	rep	32	rep	40	rep

2. Pre wash wells with 200µl of PBS/Tw/Cs. Empty well contents over sink and tap dry on absorbent material.

3. Block all wells with 1% Casein – 200µl per well for 30 mins at room temperature.

4. Wash wells with PBSTwC – 200µl per well, empty over sink and tap dry as previous.

5. Add 100µl/well of TRXr primary Rabbit antibody. Use 5ul/10 mls (1:2000 dilution) - Allow to incubate on bench for 1 hour at RT (on bench).
Use 5ul/10 mls (1:2000 Primary)
5 µl – 10 mls – 1 plate
15 µl – 30 mls – 3 plates

6. Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

7 Add 100ul/well 1:5000 Anti-Rabbit- Biotin (from my fridge R10 second shelf down RHS 2 bottles on blue rack). Allow to incubate on bench for 1 hour at RT (on bench).
Use 2µl/10 mls (1:5000 Primary)
2 µl – 10 mls – 1 plate
6 µl – 30 mls – 3 plates

Appendix Y

8 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

9 Add 100ul/well Streptavidin Peroxidase (HRP) solution (regardless of mouse or rabbit, from my fridge R10 same place) (1:8000 i.e., 3.6ul/30 mls) in PBSTwC. Incubate at room temperature for 1 hour.

10 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

11 Make up TMB in Phosphate citrate immediately prior to adding.

12 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

13 Add TMB 100µl per well and keep microlitre plate on plate shaker at 300rpm in dark and allow color development. TMB is light sensitive.

14 Allow TMB to develop blue color for up to 20-30 minutes but this will depend on background.

15 To stop the reaction, add 50µl stop solution of 20% (or 1.5M) Sulphuric Acid (stored in safety cabinet in R10 outer room).

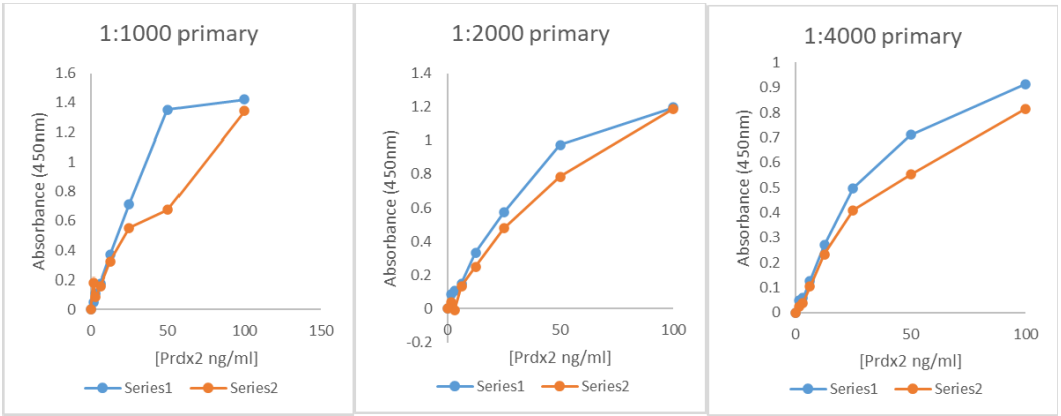
16 Read at wavelength of 450nm using ELISA plate reader in technician's lab.

Appendix Y

Optimisation Peroxiredoxin-2 (PRDX-2)

secondary	1 in 5000	1 in 5000	1 in 5000	1 in 10000	1 in 10000	1 in 10000
primary	**1 in 1000**	**1 in 2000**	**1 in 4000**	**1 in 1000**	**1 in 2000**	**1 in 4000**
	A	B	C	D	E	F
100	1.547	1.342	1.023	1.458	1.308	0.927
50	1.48	1.119	0.819	0.789	0.904	0.664
25	0.84	0.718	0.606	0.667	0.596	0.522
12.5	0.496	0.479	0.378	0.438	0.369	0.344
6.25	0.307	0.294	0.235	0.271	0.253	0.216
3.13	0.24	0.249	0.165	0.198	0.108	0.151
1.56	0.179	0.232	0.157	0.296	0.157	0.136
0	0.127	0.144	0.108	0.114	0.118	0.112

	Normalised					
	1 in 1000	1 in 1000	1 in 2000	1 in 2000	1 in 4000	1 in 4000
	1 in 5000	1 in 10000	1 in 5000	1 in 10000	1 in 5000	1 in 10000
100	1.42	1.344	1.198	1.19	0.915	0.815
50	1.353	0.675	0.975	0.786	0.711	0.552
25	0.713	0.553	0.574	0.478	0.498	0.41
12.5	0.369	0.324	0.335	0.251	0.27	0.232
6.25	0.18	0.157	0.15	0.135	0.127	0.104
3.125	0.113	0.084	0.105	-0.01	0.057	0.039
1.5625	0.052	0.182	0.088	0.039	0.049	0.024
0	0	0	0	0	0	0



PRDX-2 - 1:2000 dilution of the primary antibody (5 µl in 10 ml) with a 1:5000 dilution of the anti-rabbit biotin (2 µl in 10 ml) provided the optimal ratio and used for procedure.

Primary Antibody - ab133481 Rabbit mononclonal (EPR5155) to Peroxiredoxin 2/PRP. Lot no: GR92033-13. Secondary antibody Goat Anti-Rabbit IgG H&L (Biotin) Lot No: GR3356030. Used 1:8000 streptavidin HRP - in house stock (SLS S5512).

Appendix Z

Procedure outline for PRDX-4 antibodies

1. The night prior to ELISA, prepare plate(s) using various dilutions of antigen and incubate in fridge. Number the strips and use marker to format layout if possible.

Samples 100ul of 40 samples on each plate in duplicate (as labeled below)
Standards: TRXr stock 0.5mg/ml
Add 100ul /well for all dilutions (detailed below in yellow)
Add 1ul stock/1000ul PBS (1:1000)
Add 100ul of above+1800ul PBS (further 1:5 = 100ngs/ml = top concentration)
Doubling dilutions (125ul above+500PBS for 6 more dilutions (50ngs/ml down to 1.5625ngs/ml).
Add 100ul buffer only to H1 and H2 (in brown)
Leave antigen on wells o/n

ngs/ml

100	100	1	rep	9	rep	17	rep	25	rep	33	rep
50	50	2	rep	10	rep	18	rep	26	rep	34	rep
25	25	3	rep	11	rep	19	rep	27	rep	35	rep
12.5	12.5	4	rep	12	rep	20	rep	28	rep	36	rep
6.25	6.25	5	rep	13	rep	21	rep	29	rep	37	rep
3.125	3.125	6	rep	14	rep	22	rep	30	rep	38	rep
1.562	1.562	7	rep	15	rep	23	rep	31	rep	39	rep
0	0	8	rep	16	rep	24	rep	32	rep	40	rep

2. Pre wash wells with 200µl of PBS/Tw/Cs. Empty well contents over sink and tap dry on absorbent material.

3. Block all wells with 1% Casein – 200µl per well for 30 mins at room temperature.

4. Wash wells with PBSTwC – 200µl per well, empty over sink and tap dry as previous.

5. Add 100µl/well of 1:2500 PRX4 primary Rabbit antibody
Use 27.5 µL /70 mls (7 plates)
Allow to incubate on bench for 1 hour at RT (on bench).

6. Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

7 Add 100 µL /well 1:4000 (i.e., 60ul/30 mls here) Anti-Rabbit- Biotin (In fridge on blue rack). Use 20µL /80 mls (7 Plates)
Allow to incubate on bench for 1 hour at RT (on bench).

8 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

9 Add 100ul/well Streptavidin Peroxidase (HRP) solution (regardless of mouse or rabbit) (1:8000 i.e., 3.6ul/30 mls) in PBSTwC. Incubate at room temperature for 1 hour.

Appendix Z

10 Make up TMB in Phosphate citrate immediately prior to adding.

11 Following incubation above, flick out the well contents, tap dry and wash all wells 3 x 200µl with PBSTwC. Tap dry on absorbent pad between each wash.

12 Add TMB 100µl per well and keep microtitre plate on plate shaker at 300rpm in dark and allow color development. TMB is light sensitive.

13 Allow TMB to develop blue color for up to 20-30 minutes but this will depend on background.

14 To stop the reaction, add 50µl stop solution of 20% (or 1.5M) Sulphuric Acid (stored in safety cabinet in R10 outer room).

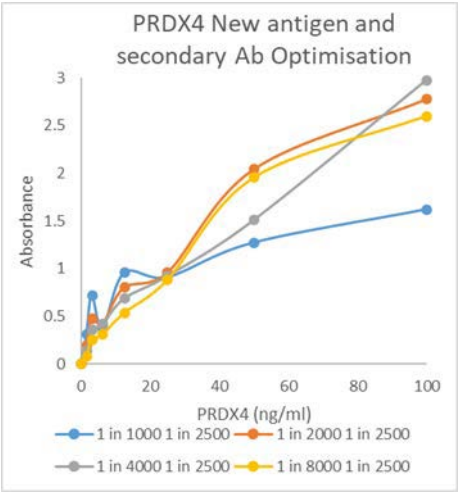
15 Read at wavelength of 450nm using ELISA plate reader in technician's lab.

Appendix Z

Optimisation Peroxiredoxin-4 (PRDX-4)

[Antigen] ng/ml	Absorbance (450 nm)			
secondary	1 in 1000	1 in 2000	1 in 4000	1 in 8000
primary	1 in 2500	1 in 2500	1 in 2500	1 in 2500
	A	B	C	D
100	2.5836	3.5628	3.5013	2.9992
50	2.2365	2.8293	2.0372	2.3549
25	1.8756	1.7458	1.4529	1.2841
12.5	1.9201	1.5947	1.2175	0.9408
6.25	1.3423	1.2091	0.9517	0.7167
3.13	1.6852	1.2632	0.8861	0.6578
1.56	1.2781	0.9832	0.6721	0.4844
0	0.9639	0.7886	0.5298	0.4030

	Normalised			
	1 in 1000	1 in 2000	1 in 4000	1 in 8000
	1 in 2500	1 in 2500	1 in 2500	1 in 2500
100	1.6197	2.7742	2.9715	2.5962
50	1.2726	2.0407	1.5074	1.9519
25	0.9117	0.9572	0.9231	0.8811
12.5	0.9562	0.8061	0.6877	0.5378
6.25	0.3784	0.4205	0.4219	0.3137
3.125	0.7213	0.4746	0.3563	0.2548
1.5625	0.3142	0.1946	0.1423	0.0814
0	0	0	0	0



PRDX-4 - 1:2500 dilution of the primary antibody (27.5 µL /70 mls for 7 plates) with a 1:4000 dilution of the anti-rabbit biotin (20µL /80 mls (7 Plates) provided the optimal ratio as optimal linear curve (grey) and used for procedure.

Primary Antibody - ab85331 Recombinant human Peroxiredoxin 4 protein Lot no: GR3377600-1 Secondary antibody Goat Anti-Rabbit IgG H&L (Biotin) Lot No: GR3356030. Used 1:8000 streptavidin HRP - in house stock (SLS S5512).

Appendix AA

Kaplan-Meier 'time to re-admission' censoring data to satisfy Assumption#4., Section 2.24.4.1

Key:

Participant Numbers: **Blue Arm 1 and Red Arm 2**

Time to Readmission: **Censored and ACS Admission**

NUMBER	CONSENT	PROCEDURE	Admission
09 03/08/17 – 28/12/17 **28+30+31+30+28=147** **Time to event 147 days** 03/08/17 – 07/08/18 **365 + 4 = 369** **Time to Event 369 days**	03/08/17	01/08/17	01/08/17- 04/08/17 Lateral STEMI **28/12/17** **Admission for bleeding on antiplatelets** **07/08/18 – 08/08/18** **admission Crescendo TIA**
11 04/08/17 – 15/02/18 27+30+31+30+ +31+31+15 = 195 Time to Event 195 days	04/08/17	30/06/17	15/02/18 ICD 12/09/18 Ophthalmology outpatient
13 29/08/17 – 27/09/17 2 + 27 = 29 Time to Event 29 days 29/08/17 – 05/10/17 2 + 30 + 5 = 37 Time to Event 37 days 29/08/17 – 12/10/17 2+30+12 = 44 Time to Event 44 days	29/08/17 RIP 12/10/17		27/09/17 – 08/10/17 Admission Dilated Cardiomyopathy 05/10/17 ICD De Activated
17 27/09/17 – 2/10/17 4+2 = 6 Time to Event 6 days	27/09/17 RIP 2/10/17	26/09/17	26/09/17 – 29/09/17 STEMI Trop 4616 ng/L
19 19/09/17 – 10/12/17 11+31+30+10 = 82 Time to Event 82 days **Censored**	19/09/17 10/12/17 C/P Admission A/E Trop 7.7ng/L	17/12/16 19/12/16 staged	17/12/16 – 20/12/16 Admission STEMI

19/09/17 - 24/04/18 13+31+30+31+31+ 28+31+24= 219 Time to Event 219 days Censored 19/09/17 - 26/09/18 365 +7 = 372 Time to Event 372 days			24/04/18 – 25/04/18 Admission Urology 26/09/18 – 01/10/18 Admission Community Acquired Pneumonia (CAP)
20 02/10/17- 31/10/2017 Time to event 28 days 02/10/17 - 16/06/18 28+30+31+31+28+31 +30+31+16 = 256 Time to event 256 days	02/10/17	11/06/17 1.Trop 5053 ng/L 21/06/17 2.Trop 240 ng/L 16/06/18 1.Trop <5 ng/L 2.Trop<5 ng/L	10/6/17 - 13/6/17 PPCI 31/10/17 ED chest pain unusual heartbeat 16/06/18 ED Chest pain
23 Reported a re-PCI Censored No Evidence DW Pt No chest pain	05/10/18	07/06/17 09/10/18 most vessels appeared normal	05/06/17 – 08/06/17 NSTEMI Admission Referred by RT to Leicester 21/11/17 re Spontaneous Coronary Artery dissection SCAD
32 13/10/17 – 08/12/17 18+30+8 = 56 Time to Event 56 days Censored 28/02/2018 18 +30+31+31+28 = 138 Time to Event 138 days RIP 03/06/2018 18+30+31+31+28+31+30 31+3= 233	13/10/17 A/E Chest Pain 08/12/17 Trop 16.2 ng/L 09/12/17 Trop 16.5 ng/L Ca diagnosis RIP 03/06/18	12/10/17	12/10/17 – 16/10/17 STEMI Anterior 12 /10/17 Trop 763 ng/L @ 10:26 29/12/17 out patient voice changes following cardiac event and septoplasty 20/02/18 Outpatient Mild emphysema 28/02/18 urgent referral lung cancer not cancer referred urgently to Respiratory
35 24/10/17 – 23/12/17 7+30+23 = 60 Time to Event 60 days 24/10/17 – 25/03/18 7+30+31+31+28+	24/10/17	23/06/17PCI prox LAD 13/07/17 staged to RCA with rotoblation	NSTEMI POCI LAD PREV CCS Class 11 Angina IHD 22/12/17 – 24/12/17 Admission

25 =152 **Time to Event 152 days** 24/10/17 – 04/05/18 **7+30+31+31+28+31+** Appendix AA **92 days** 24/10/17 – 26/06/2018 **7+30+31+31+28+31+** **30+31 +26 = 245** **Time to Event 245 days**		23/12/17 PCI RCA with DCB and Cx with 1 x DES	25/03/18 seen Dermatology BCC left temple **04/05/18 seen Colorectal Clinic Colonoscopy and OGD MDT 22/05/18** Transverse Colon lesion in keeping with moderately differentiated adenocarcinoma Suspicion liver mets pulmonary nodules **Admitted 19/06/18 - 26/06/2018** **Laparoscopic R hemicolectomy converted to open** **Defunctioning ileostomy in ICU tfr to 20/06/18 - 26/06/18** **oncology METS**
38 02/11/2017 - 24/09/18 **28+31+31+28+31+30** **+31+30+31+31+24=326** **Time to Event 326 days**	02/11/17	15/06/17 1.Trops 86 ng/L 2.Trops 73 ng/L	15/6/17 - 16/07/17 **24/9/18** **Surgery admission**
39 10/11/2017 - 25/10/18 20+31+31+28+31+30+ 31+30+31+31+30 =324 Time to Event 324 days	10/11/17	08/11/17 1.Trops 4079 ng/L 2.Trop 16078 ng/L 25/10/18 1.Trops 55 ng/L 2.Trops 60 ng/L ~~19/5/20~~ > 395 days ~~1.Trops 55 ng/L~~ ~~2.Trops 54 ng/L~~	08/11/17 – 11/11/17 Trop confirmed 25/10/18 Admission
40 10/11/17 - 08/02/18 **19 + 31+31+8 = 89** **Time to Event 326 days**	10/11/17	10/11/17 1.Trop 78 ng/L 2.Trop 315 ng/L	10/11/17 - 12/11/17

			08/02/18 gastroenterology Admission
41 10/11/2017 - 05/01/18 20 + 31+5 = 56 Time to Event 56 days	10/11/2017 10/11/17 - 5/01/18	10/11/17 PPCI LAD 1 x DES noted to have focal aneurysmal segment on proximal LAD just distal to critical pLAD lesion 05/01/18 elective re angio for assessment post PCI	10/11/17 – 13/11/17 NSTEMI - developed Anterior STEMI on table Angina IHD ~~01/10/19 – 03/10/19 UTI~~
42 27/11/17 - 06/01/18 **Censored** **3+31+6 = 40** **Time to Event 40 days** 27/11/17 - 08/01/18 **Censored** **3 + 31 +8 = 42** **Time to Event 42 days**	27/11/17	24/11/17 PCI to RCA 4 x DES	NSTEM 23/11/17 – 27/11/17 NTEMI **06/01/18 Admission NIDDM Hypertension** **08/01/18 Eye surgery left re do ptosis surgery** 22/02/18 seen falls clinic
43 24/11/17 - 23/7/18 **Censored** 6+31+31+28+31+30+ 31+30+30+23 = 241 Time to event 241 days	24/11/17	22/11/17 Trop 43.9 ng/L 24/11/17 Trop 31.2 ng/L	22/11/17 - 25/11/17 **23/7/18** **ED T/O admission**
48 09/01/18 - 16/11/18 **Censored** 22+28+31+30+ 31+30+31+31+30+31+16 = 311 Time to event 311 days	09/01/18	06/01/18 1.Trops 177 ng/L 2.Trops 559 ng/L	06/01/18 - 09/01/18 27/06/2018 Gastroenterology **16/11/18** **MRI Bx Prostate** 25/11/18 General Surgery 08/01/2019 TURP ~~MDT 01/02/19 Adenocarcarcinoma of prostate – radiotherapy 19/06/19~~

55 09/02/18 – 12/04/18 10 + 31 + 12 = 53 Time to event 53 days	09/02/18 A/E Hereford CP Admission 12/04/2018 Crea 121 K+ 4.3 RO Cardiac.	03/07/17 PCI 25/01/18 PCI ACS – UAP Severe stenosis mid LAD Pressure Wire + DES x 1 09/02/18	Elective PCI LMS into LAD Indication Unstable angina see prev PCI Mild disease mid LAD Continuing angina now admitted to Hereford with Unstable Angina TnT negative Indication chest pain admitted 05/02/18 to Hereford with hx of central chest pain radiating to jaw Clinical diagnosis IHD Excellent stent result Minor pinching of Diagonal2 & lesion in very distal PDA
56 20/02/18 – 14/03/18 08+14 = 22 Time to Event 22 days **Censored** 20/02/18 - 03/10/18 **26 + 31 + 30 + 31 + 31 + 30 + 31 + 30 + 3 = 243** **Time to event 243 days** **Censored** 20/02/18 - 11/11/18 **26 + 31 + 30 + 31 + 31 + 30 + 31 + 30 + 31+ 11 = 282** **Time to event 282 days**	20/02/18 Admission 14/03/18 – 16/03/18 1.Trop 23.0 ng/L 2.Trop 22.3 ng/L	12/02/18 Inferior STEMI late presentation Small Radial – 5 F PPCI to Occluded RCA 2 x overlapping DES	12/02/18 – 19/02/18 STEMI PCI Admission 14/03/18 – 16/03/18 H Failure patient **Admission 03/10/18 – 03/10/18** **Anaemia** **11/11/18** **Elective Gen Surgery**
60 **Censored** **Reported TIA/CVA – require date not confirmed**	04/04/18	26/02/18 Severe RCA lesion 2 x overlapping DES AF since initial ECG 01/03/18 1.Trops 699 ng/L	18/02/18 Inferior STEMI - PEA with thrombolysis

		07/03/18 2.Trops 63 ng/L	
61 27/02/18 – 07/05/18 2 + 31 + 30 + 7 = 69 Time to event 69 day	27/02/18	06/05/17 1.Trops 76 ng/L 2.Trops 145 ng/L 7/5/18 1.Trops 11.3 ng/L 2.Trops 11.1 ng/L	6/5/17 - 20/5/17
73 **Censored** 15/03/18 - 23/01/19 **16+30+31+30+31+31 +30+31+30+31+23 = 314** **Time to event 314 days**	19/03/18	16/03/18 1.Trops 281 ng/L 19/03/18 2.Trops 1362 ng/L	16/3/18 – 20/3/18 **23/1/19 – 01/02/19** **T/O admission**
71 **Censored** 15/03/18 - 08/05/2018 **16 + 30 + 8 = 54** **Time to event 54 days** **Censored** 15/03/18 - 20/06/2018 **16 + 30 + 31 + 20 = 97** **Time to event 278 days**	15/03/2018	24/11/17 1.Trops 111 ng/L 25/11/17 2.Trops 136 ng/L	24/11/17 - 27/11/17 PPCI 08/05/2018 20/06/2018 Gynae admission
78 **Censored** 20/04/18 - 23/01/18 **10 + 31 + 30 + 31 + 31 + 30 + 31 + 30 + 31 + 23 = 278** **Time to event 278 days**	20/04/18	01/11/17 1.Trops 17.8 ng/L 2.Trops 36.7 ng/L	01/11/17 - 03/11/17 23/01/18 Admission Asthma
79 **Censored** **Reported Event of Ca but found one prior to consent and one >395 days.**	26/04/18	1.Trops 40 ng/L 2. Trops 100 ng/L	26/04/18 - NSTEMI Hastings 02/01/18 1 x DES to Cx ~~08/09/17 Likely BCC left nasal labial region~~ ~~Possible SCC left arm~~ ~~09/10/19 non healing plaque left upper ear~~

80 **Censored** **Reported of re PCI** **No Evidence.**	01/05/18	This was one from Cardiac Rehab no procedure in our lab 1.Trops 289 ng/L 2.Trop 290 ng/L	NSTEMI Des to LAD 06/12/17 Sandwell Stent to LAD ~~03/09/19 Urology~~
86 05/06/18 – 21/01/19 25+31+31+30+31+ +30+31+21 = 230 Time to Event = 230 days 05/06/18 – 15/03/19 25+31+31+30+31+ +30+31+31+28+15 = 283 Time to Event = 283 days	05/06/18	04/05/18 staged PCI to RCA IHD offered PCI to RCA +/- - Om1 21/01/19 PCI 15/03/19Angina Successful PCI OM1 with 1 x DES	13/04/18 diag IHD Elective PCI to RCA with 2 overlapping DES 27/09/18 physio For MRI lumbar spine 21/01/19 Indication ANGINA Admission 20/02/19 – 23/02/19 Urology Listed for PCI to OM
89 22/06/18 – 28/07/18 8 + 28 = 36 Time to Event 36 days	22/06/18	22/6/18 Trop 76 ng/L 22/06/18 Trop 94 ng/L Trop AE 28/7/18 Trop 21.2 ng/L	22/06/18 ED DC 25/06/18 28/07/18 ED visit chest pain
93 **Censored Over 395 days** 29/06/2018 -12/09/19 **365+2+31+31+12** **Time to event** **441 days**	29/06/18 Admission 12/09/2019 Trop 12 ng/ml	09/05/16 Single discrete lesion in SVG to RCA treated PCI 1 x DES 29/06/18 PCI to SVG to Distal RCA 12/09/19 Successful PCI to SVG to OM with DES	Indication Unstable angina CABG following ACS and PCI to RCA in 2012 NSTEMI As above plus SVG – OM/D1/rPDA and LIMA to LAD Conclusion Trop neg ACS **Admitted** **12/09/19** NSTEMI
94 06/07/18 - 01/04/19 25 + 31 +30 + 31+30 +31 +31 +29+31+1 = 270 Time to Event = 270 days	06/07/18	06/07/18 PCI to LAD 06/07/18 ACS NSTEMI 05/07/18 – 07/07/18	Indication ACS Diabetic Successful PCI to LAD with long DES Clinical Diagnosis ACS NSTEMI Lesion in small OM2

Censored Over 395 days 06/07/18 – 24/10/19 365 +24 + 31 +30 + 24 = 474 Time to Event = 474 days **Beyond 365 + 30 days**		BRISTOL 01/04/2019 Chest pain/ angina admission. Attempting to locate trops. 24/10/2019 – 25/10/2019 BRI re PCI x1 stent to LAD.	Long lesion in proximal LAD subtending diagonal Successful PCI to LAD with long DES with provisional approach to diagonal, final kiss and POT Admission 05/07/18 – 07 /07/18
97 09/07/18 – 19/10/18 21+31+30+19=101 Time to Event 101 days	09/07/18	09/07/18 ACS NSTEM 19/10/18 – 22/10/18 Trop 476 ng/L	09/07/2018- Tfr from Hereford chest pain Known COPD PAF hypertension and PVD PREVIOUS PCI to LAD 2015 GGH chronic occluded RCA longstanding LMS and ostial disease turned down surgery at BHI so tight LAD treated alone Clinical diagnosis ACS NSTEMI If ongoing or difficult symptoms re MDT for CABG
98 12/07/18 – 19/09/18 19+31+19 = 69 Time to Event 69 days 12/07/18 – 25/10/18 **Censored** 19 + 31 + 30 + 25 = 105 Time to event 105 days	12/07/18	11/07/18 PPCI (DES) VF arrest during procedure treated with DC Cardioversion no CPR	Admitted 11/07/18 – 14/07/18 Anterior STEMI HFailure team 07/09/18 19/09/2018 CP admission to A/E Trops 1 = 43.2 ng/L 2 = 39.9 ng/L 25/10/18 Dermatology BCC left tragus – biopsy proven 05/02/19 BCC excised
100	24/08/18	24/08/18	23/08/18 – 25/08/18

24/08/18 – 10/07/18 **7+31+31+30+31+31** **28+31+30+31+28+31** **30+31+30+10 = 321** **Censored** **Time to Event 321 days**		PCI to Cx and RCA Mid Cx DES x 2	PREV PCI to RCA CTO 2003 Transient inferior ST elevation on admission ECG now normal Clinical Diagnosis IHD Unobstructed LAD and patent RCA BMS Severe lesion in mid RCA and Mid Cx 2 vessel PCI 2 x ultimaster stents **Colorectal appt 10/07/19 for colonoscopy** Endoscopy 14/9/19
102 **Censored Over 395 days** 30/08/18 - 25/12/19 365+30+31+30+25 = 481 **Time to Event beyond 365 + 30 days**	30/08/18	28/08/18 PPCI to LAD (diagonal) 1 x DES Diffuse disease in proximal LAD/CX and proximal RCA Distant history of surgery for Ca throat ~~27/12/19 diagnostic~~	28/08/18 – 31/08/18 STEMI Anterior with bradycardia and inferior changes **25/12/19 ACS admitted chest pain** ~~Echo 31/12/19 moderate LVSD EF44%~~
105 05/09/2018 - 09/09/18 = 4 days Time to Event 4 days 05/09/18 – 14/09/18 = 9 days Time to Event 9 days	05/09/18 09/09/18 Admission C/P +ve trops 1 – 44.9 ng/L 10/09/18 2 – 44.7 ng/L 10/09/18 14/09/2018 Admission with /CP Angina -ve Trops 1 – 14.8 ng/L 2 – 13.4 ng/L	05/09/18x smoker HTN Hx of stroke PCI to LAD and Circumflex 2 x DES 05/09/18 NSTEMI Denies angina x smoker Hx of stroke BCC left cheek 09/09/17 Bowens disease or superficial BCC frontal scalp 09/09/17	Admitted 02/09/18 – 06/09/18 NSTEMI Clinical Diagnosis IHD NSTEMI presentation Likely LAD 30/12/19 likely oral planus 09/01/20 trigger finger 10/03/20 physio cervical spine pain

		Letter 07/03/19 h hernia mentioned ? old 30/12/19 Likely oral planus	
107 10/09/2018 - 21/09/18 = 11 days Time to Event 11 days	10/09/18	09/09/18 1.Trop 97 ng/L 20/09/18 2.Trop 162 ng/L	09/09/18 – 01/09/18 CCU 21/09/18 – 22/09/18 Elective PCI
112 28/09/2018 – 17/05/19 2+31+30+31+31+28+ 31+30+17=231 Time to event 231 days 28/09/2018 – 30/10/19 2+31+30+31+31+28+ 31+30+31+30+31+31+ 30+ 28=354 365 + 30 = 395 Time to Event 395 days	28/09/18	28/9/18 ED chest pain 17/05/19 Chest pain 1.Trop <5 ng/L 08/11/19 A/E Trop 5.8 ng/L	28/09/18 – 01/10/ 18 18/06/19 Gastro inv 07/08/19 Endoscopy 09/11/2019 ~~CP – Re Angio~~ ~~-Ve trops Mild In-stent~~ ~~Restenosis' RCA Mild~~
114 19/10/18 - 02/11/18 12+ 2 = 14 days Time to event 14 days 19/10/18 - 28/03/19 12 + 30 + 31 +31 +28 + 20 = 152 Time to Event 152 days 19/10/18 - 19/08/19 **12+30+31+31+28+31** **+30+31+30+31+19 =304** **Time to event 304**	19/10/18 02/11/18 Trop 11.0 ng/L 28/03/19 – A/E admission -ve 1. Trop 9.6 ng/L 2.Trop 8.0 ng/L	19/10/18 Normal Coronary arteries MI NOCA treat as ACS for MRI	17/10/18 NSTEMI 02/11/18 – 03/11/18 overnight chest pain **19/08/19 Referred to endocrinology** **Fine needle aspiration for thyroid surgery.** **MRI picked up enlarged thyroid gland**
115 19/10/18 - 19/11/2018 365 + 30 = 395 Time to Event 395 days	19/10/18 Re PCI 19/11/2018	19/10/18 - 22/10/18 ACS NSTEM 30/10/2018 Trop 1284 ng/L PCI to LMS/Cx with rotational atherectomy 4 xDES	HGH chest pain PCI to LAD 2015 GGH Admitted HGH with Angina

		Dissection of OM1/OM2 bifurcation with loss of flow in OM2 with Timi 1 flow 19/10/19 PCI to LMS /Cx 19/10/18 – 22/10/18 Trop 476 ng/L 19/11/19 No stents	
119 23/11/18 – 18/02/19 7+ 31+ 31+ 18 = 87 Time to Event 87 days	23/11/2018 18/02/2019 C/P admission +ve trops 1 – 78 ng/L 2 – 80 ng/L 3 – 88 ng/L	23/11/2018 PCI ACS NSTEMI large vessels Single vessel disease IFR positive Successful PCI to mid LAD 1 x DES	21/11/18 – 24/11/18 Indication ACS 1st presentation Chest pain RBBB with intermittent anterior T wave inversion 18/02/19 – 22/02/19 Chest Pain
123 **Censored** **04/01/19 - 10/01/19** **Time to event 6 days** 04/01/19 - 13/02/19 27 + 13 = 40 Time to event 40 days 04/01/19 – 02/06/19 27+ 28+ 31 +30 + 31 + 2 = 149 Time to event 149 days **Censored** **04/01/19 – 06/06/19** **27 + 28 + 31 + 30 + 6 = 153** **Time to event 153 days** **Censored Epilepsy** **04/01/19 – 16/11/19** **27+ 28+ 31+ 30+ 31+30 +31+ 31+ 30+ 31+ 16 = 316** **Time to event 316 days**	04/01/19 13/02/19 Trop 1. 40.6 ng/L Trop 2. 34.6 ng/L CP admission 02/06/19 1. Trops 36.9 ng/L 2. Trops 39.6 ng/L	31/12/18 PPCI STEMI Inferior DES x 2 to pRCA occlusion Severe bystander mLAD disease 04/01/19 CAD Staged procedure to Bystander LAD disease	31/12/18 – 05/01/19 STEMI Inferior Successful staged PCI to LAD 3 stents **10/01/19 – 11/01/19** **SOB** 13/02/19 - 15/02/19 Trop1. 40.6 ng/L Trop 2. 34.6 ng/L 02/06/19 CP **6/06/2019 CVA** **Letter 06/06/19 states left ischaemic stroke (occipital and temporal lobe)** **Censored Epilepsy** **16/11/19 – 18/11/19** **Epilepsy** 27/11/19- 28/11/19 Jacksonian Epilepsy 12/12/19 – 18/12/19 Epileptic Seizure?

			09/01/2020 says Tremor not seizure
124 **Censored** 04/01/19 - 11/10/19 **27+ 28+ 31+ 30+ 31+30 +31+ 31+ 30+ 31+ 11 = 311** **Time to event 311 days**	04/01/19	04/01/19 Successful PCI to re stenosis of proximal LAD stent (IFR positive) -POBa and drug eluting balloon Occluded inferior wall branch Cx – OM2	03/01/19 - 07/01/19 NSTEMI Prev stent x 2 2007 at UHCW Clinic 06/03/19 CAD Discharged from clinic 20/08/19 Dr S Lipid clinic. TCK 66 U/L -VE **11/10/19 – 17/10/19** **13/10/19 fracture of ankle repair**

Key:
Participant Numbers: **Blue Arm 1** **and** **Red Arm 2**
Time to Readmission: **Censored** **and** **ACS Admission**

Appendix BB

Statistician feedback from Laerd and in-house, University of Worcester statistical review for appropriateness of statistical method used, interpretation of analysis and execution.

Berenice Mahoney <[redacted]>

To:

- DOUGHTY, Angela (WORCESTERSHIRE ACUTE HOSPITALS NHS TRUST)

Thu 15/06/2023 14:59

Dear Angela

Thank you for sending me the assumption checking summary document, and the relevant thesis chapters. I have reviewed these, focusing on the appropriateness of the assumption checking and analyses, and their execution. I am satisfied with the technical appropriateness of these and congratulate you on doing such a thorough job with a valuable and interesting data set.

Good luck and, albeit a little ahead of things, congratulations!

Kind regards

Dr Bere Mahoney

You will receive a response to your email within three working days. E.g., if you email within office hours on a Friday you should typically receive a response by the end of the following Wednesday. Working days/ typical email response times are Monday - Friday between 9am and 5pm excluding University closed days. If I am on leave/out of office when you email, I will respond within three working days of the date that I return (return date in the out of office response).

Dr Béré Mahoney CPsychol CSci AFBPsS FHEA
Principal Lecturer in Psychology | MPhil/PhD Course Leader School of Psychology
Social Science Lead, Three Counties Medical School
ONS Approved Safe Researcher
British Psychological Society Register of Chartered Psychologists & Register of Expert Witnesses https://www.bps.org.uk/lists/DIR/view/psychologist/16900?page
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Online tutorials via Collaborate are bookable using this link https://calendly.com/dr-mahoney-tutorials/dr-mahoney-tutorials
Please note: in line with University policy, I can only respond to student emails sent from official University of Worcester email accounts
University of Worcester Follow us on Facebook, Instagram, Twitter and YouTube

Dr Adam Lund <[redacted]>

To:

- DOUGHTY, Angela (WORCESTERSHIRE ACUTE HOSPITALS NHS TRUST);

Tue 10/05/2022 21:34

Hi Angela,

In terms of your message about the two-way mixed ANOVA, I understand that you had a statistically significant two-way interaction effect and ran simple main effects. In this

regard, what type of design do you have (e.g,. 2 x 2, 2 x 3, 3 x 3, etc.). Also, were the simple main effects also statistically significant, and if so, for the within-subjects factor or between-subjects factor? As for the difference in descriptive statistics, please could you provide an example stating from which tables (e.g., the Descriptive Statistics table and Estimated Marginal Means table) the results were different. Whilst I'm afraid that I cannot provide statistical consulting, we may be able to add a Note to the guide to help.

With kind regards,

Adam

On Thu, 13 Jan 2022 at 16:10, Dr Adam Lund <[redacted]> wrote:
Hi Angela,

You are very welcome. I'm glad that the Kaplan-Meier guide helped!

In terms of more professional looking graphics, SPSS Statistics is currently a little weak on this front, although it is making improvements. You can find SPSS Statistics guides to help via the main menu under "SPSS Statistics" and then the left-hand menu, "Charts & graphs". However, I should mention that we are working on a completely new section to help with data visualisation, including a Graph Selector (much like our Statistical Test Selector). We will also be adding guides to help with graphing using R, since so many of the professional looking graphs you see in journal publications are created using R (N.B., it is very flexible when it comes to data visualisation).

As for the two-way ANOVA, this can handle unbalanced designs. Also, whilst I'm afraid that I cannot provide statistical consulting, the residuals for each cell of the design, based on your Shapiro-Wilk tests and Normal Q-Q plots are clearly non-normal, and there are a lot of outliers. There appears to be a positive skew in the Normal Q-Q plots (e.g., compare them to the diagram the bottom of the following page to see if you feel that this is correct: https://statistics.laerd.com/premium/spss/tfn/testing-for-normality-in-spss.php). Therefore, you may want to consider a transformation of the dependent variable such as a $\log_{10}$ transformation (for strongly, positively skewed data), inverse transformation (for extremely, positively skewed data), or square root transformation (for moderately, positively skewed data): https://statistics.laerd.com/premium/spss/t/transformations-in-spss.php. Transformations are often a process of trial-and-error where you may need to try more than one transformation to see if any work, and if so, which works best. After applying the transformation, you need to re-run all the tests of assumptions using the transformed dependent variable (instead of the original dependent variable) to see if your data (residuals) now fits with the two-way ANOVA model. If it does, you need to continue with the transformed dependent variable throughout your analysis. Please note that this does come with some drawbacks in terms of interpretation, since the metrics of transformed variables can have relatively little intuitive meaning. However, some transformations can be back-transformed (e.g., a $\log_{10}$ transformation). Also, we plan to make some significant upgrades to our two-way ANOVA guide in the next 7-10 months

(possibly sooner) to include more help with violations of assumptions and transformations. I appreciate that this may be too late for your current analysis, but I can certainly let you know when this upgraded guide becomes available if this is of interest.

In terms of your other guide request, we hope to upgrade our SPSS Statistics guide on multiple regression to show how to deal with polytomous independent variables (i.e., independent variables with three or more categories) in 5-8 months, so I can certainly let you know when this upgraded guide becomes available. In the meantime, although we do not go through the interpretation for polytomous independent variables, we do have an SPSS Statistics guide on "Creating dummy variables", which you can access via the main menu under "SPSS Statistics" and then the side menu under "Getting started". Alternatively, if you are logged into the site, you can access the guide here: https://statistics.laerd.com/premium/spss/dv/dummy-variables-in-spss.php.

I hope that helps.

With best wishes,

Adam

From: Dr Adam Lund <[redacted]>
Sent: 10 January 2022 15:53
To: DOUGHTY, Angela (WORCESTERSHIRE ACUTE HOSPITALS NHS TRUST)
Subject: Re: Laerd Statistics Feedback

Hi Angela,

In terms of your message, whilst I'm afraid that I cannot provide statistical consulting, if you can let me know a little more about the problem you are having when running your two-way ANOVA, hopefully I can then let you know if we have an SPSS Statistics guide in the site to help or when one can be added.

As for the presentation of charts, again, if you could let me know a little more about the type of chart you are trying to create, hopefully I can then help.

With best wishes,

Adam