The Role of Serum Biomarkers and Oxidative Stress in Ischemic Heart Disease

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Master's By Research Thesis

2022

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Acknowledgements

I would like to begin with thanking everyone who has made this project possible and supported me throughout my academic studies.

In particular I would like to thank my principal supervisor Dr David Greensmith for all the guidance and support provided at both undergraduate and postgraduate level. I am grateful for all the opportunities you have provided me with to help me on my journey to become an independent research scientist.

Secondly, I would like to thank Vasanthi Vasudevan, Nidal Bittar and the whole team at Blackpool Victoria Hospital for recruiting the patients, collecting samples, and providing clinical data, an integral part of the project.

Thirdly I thank everyone in Dr David Greensmith's research team for providing your expertise, assistance, and encouragement throughout my research.

Finally, I would like to thank my family in particular my parents Wendy Riley and Carl Riley, and partner Tom Mannion for the love and support, and always believing in me and pushing me to achieve my goals throughout my academic studies.

List of Abbreviations

ADP - Adenosine diphosphate

AD(P)H - Nicotinamide adenine dinucleotide phosphate

ATP - Adenosine triphosphate

***BDM** - 2,3-butanedione monoxime*

BMI – Body-mass index

BP – Blood pressure

BSA - Bovine serum albumin

Ca^{2+} - Calcium ion

CABG - Coronary artery bypass graft

$CaCl_2$ – Calcium chloride

CAD – Coronary artery disease

CRP – C-Reactive protein

CTCF – Corrected total cell fluorescence

CVD – Cardiovascular disease

EC – Endothelial cell

ECC - Excitation-contraction coupling

ECHO - Echocardiology

ECG – Electrocardiogram

EDV – End-diastolic volume

EF – Ejection fraction

ELISA - Enzyme-linked immunosorbent assays

eNOS - Nitric Oxide Synthase

ER – Endoplasmic reticulum

ESV – End-systolic volume

H^+ - Hydrogen ion

HDI – Human development index

HDL – High density lipoprotein

HNE - 4-Hydroxynonenal

H_2O – Water

H_2O_2 – Hydrogen peroxide

HRA – Health research authority

HRP – Horseradish peroxidase

ICAM-1 – Intracellular adhesion molecule-1

ICD - Implantable cardioverter device

IHD – Ischemic heart disease

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

IL-18 – Interleukin-18

K^+ - Potassium ion

KCl – Potassium chloride

KH_2PO_4 - Monobasic potassium phosphate

LAA – Left atrial appendange

LCC – Left coronary cuspid

LDL – Low density lipoprotein

LMCA – Left main coronary artery

LV – Left ventricle

LVEF – Left ventricular ejection fraction

LVIDD – Left ventricular internal diameter, diastolic

LVIDS – Left ventricular internal diameter, systolic

LVOT – Left ventricular outflow tract obstruction

$MgSO_4$ – Magnesium sulphate

MIR - Mortality-to-Incidence ratio

MMP - Matrix metalloproteases

MOPS - 3-(N-morpholino) propanesulfonic acid

MRI - Magnetic resonance imaging

Na^+ - Sodium ion

NaCl – Sodium chloride

NC(C) – Non coronary cuspid

NCX - Na^+/Ca^{2+} exchanger

NF-kB - Nuclear factor-kB

NKA - Sodium potassium ATPase

NO – Nitric oxide

O_2 – di-oxygen

O2·- – Superoxide

$ONOO^-$ - Peroxynitrite

OS – Oxidative stress

Ox-LDL – Oxidised low-density lipoprotein

PASP – Pulmonary artery systolic pressure

Pi – Inorganic phosphate

PCI - Percutaneous coronary interventions

PT – Pulmonary tract

RCA – Right coronary artery

RCC – Right coronary cuspid

REC - Research and ethics committee

ROS – Reactive oxygen species

SarcKATP - Sarcolemma ATP sensitive K^+ channels

SERCA - Sarcoplasmic Endo-reticulum Calcium ATPase

SR - Sarcoplasmic reticulum

SV – Stroke volume

TAPSE – Tricuspid annular plane systolic excursion

TMB - Tetramethyl-benzidine

TNF- α - Tumour necrosis factor-α

TR Velocity – Tricuspid regurgitation velocity

VCAM-1 - Vascular cell adhesion molecule-1

VSMC – Vascular smooth muscle cell

Abstract

Ischemic heart disease also referred to as coronary artery disease (CAD) is a leading cause of death worldwide. The primary pathological mechanism underlying CAD is atherosclerosis, an inflammatory process associated with an accumulation of lipids and metabolic alterations within the coronary arteries, and increased cytokine levels and oxidative stress (OS) in the myocardium. Previous studies focused on the use of small animals to model the pathophysiology of CAD. However, CAD is a multifactorial disease and the extent to which inflammatory markers and oxidative stress are elevated in CAD patients is not fully understood. This limits the reliability of those previous studies.

To address this, this *preliminary* study sought to measure plasma levels of interleukin-1β (IL-1β; a pro-inflammatory cytokine), and oxidised-low density lipoprotein (Ox-LDL, a marker of OS) in a CAD patient cohort and correlate to indices of cardiac function. Furthermore, OS tolerance in isolated cardiac myocytes was examined to elucidate the role of reactive oxygen species (ROS), thus OS mechanisms in CAD pathology.

The study was conducted in accordance with IRAS ethical approval (ID: 247341). Preoperative plasma IL-1β and Ox-LDL levels were quantified using enzyme-linked immunosorbent assays. Indices of cardiac function were acquired from patient echocardiology records. In parallel right atrial appendage tissue was obtained for isolation of cardiac myocytes. Subsequently, Cytation fluorescence imaging was used to measure baseline OS and in response to hydrogen peroxide (H_2O_2) challenge.

Average serum IL-1β and Ox-LDL concentrations were 1.46 ± 0.26 pg/ml ($n = 31$) and 32.76 ± 6.15 ng/ml ($n = 37$) respectively. Peak E wave velocity negatively correlated with plasma IL-1β concentration ($n = 13$, $R^2 = 0.4506$, $p = 0.0120$). Tricuspid annular plane systolic excursion (TAPSE) positively correlated with plasma Ox-LDL levels ($n = 15$, $R^2 = 0.3067$, $p = 0.0322$). Other indices of systolic function, ejection fraction, did not correlate with either IL-1β or Ox-LDL. *Preliminary* Cytation fluorescence imaging analysis revealed a significant increase in fluorescence at both 100 µM *(27 ± 9 %, $n = 13$, $p < 0.05$)* and 200 µM *(62 ± 9 %, $n = 8$, $p < 0.001$)*, hydrogen peroxide concentrations. This was associated with a significant difference between patients *($n = 3$, $p < 0.001$)*.

These data show that IL-1β may be a valuable biomarker in assessing the severity and progression of myocardial dysfunction in CAD. Oxidative stress increased in response to H_2O_2 which validated our method. Importantly this OS was heterogeneous across patients, which may be a result of diminished antioxidant capacity in cardiac myocytes.

While these findings indicate that inflammation and OS play a crucial role in CAD pathology, further research is required to increase the study power and elucidate the underlying mechanisms.

1. Introduction

Cardiovascular disease (CVD) is an umbrella term for conditions that affect the heart or blood vessels. Generally, CVD can be broadly grouped into arrhythmias, myopathies, and vascular disease. Cardiovascular disease remains one of the greatest health challenges worldwide (McAloon *et al*., 2016) though different populations, regions, ethnicity, age, and gender can induce differences in patterns and severity of disease.

An important example of CVD is coronary artery disease (CAD) also referred to as ischemic heart disease (IHD) (Mendis *et al*., 2011). Typically, CVD is associated with the build-up of fatty deposits in the arteries termed atheroma's, linking to an increased risk of blood clots (World Health Organization, 2021).

1.1. Coronary Artery Disease

Coronary artery disease is caused by narrowing (stenosis) of the coronary arteries; the large blood vessels that supply the myocardium. This reduces oxygen supply to the heart (ischemia) manifesting as shortness of breath and chest pain (angina) during physical activity. In more severe cases whereby, the coronary artery is completely blocked, heart attacks can occur.

1.2. Incidence and epidemiology of Coronary Artery Disease

Cardiovascular diseases are a leading cause of death and disability worldwide, being strongly linked with unhealthy lifestyles and co-morbidities. In the UK alone, CAD contributes to 64,000 annual deaths resulting in a death every 8 minutes (British Heart Foundation, 2022c). Globally around 550 million people are living with CVD's, and as the survival rates and population age and size increase, the incidence is set to rise (Figure 1-1) (British Heart Foundation, 2022b). Of them, CAD is the most commonly diagnosed, with around 200 million people currently affected (British Heart Foundation, 2022b). With this disease comes clear symptoms including chest pain, nausea, excessive perspiration, fatigue, and palpitations. The pathology of CAD can be associated with "intermediate" risk factors, hereditary gene expression, and other morbidities including inflammation, diabetes, and hypertension (NHS, 2020).

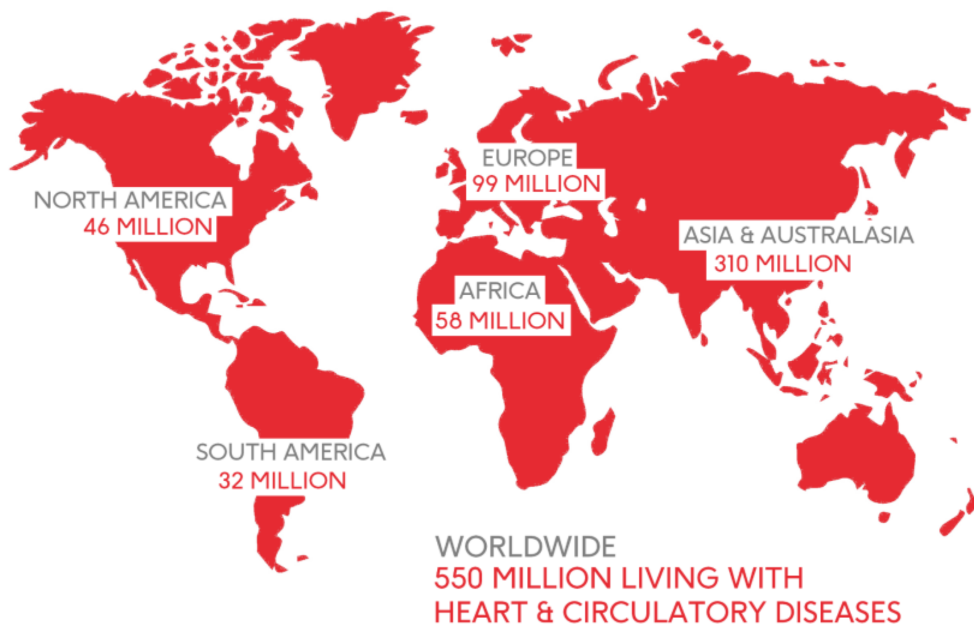


Figure 1-1. Prevalence of cardiovascular disease worldwide
Image showing Asia & Australasia having the highest prevalence of CVD and South America the lowest (British Heart Foundation, 2022e).

Generally, incidence and mortality rates of CAD vary between regions due to many factors including lifestyle, dietary habits, and access to health care (Amini *et al*., 2021). In relation to this, Mortality-to-Incidence ratio (MIR) is often measured to evaluate the burden of a disease. Interestingly incidence and mortality have shown to be affected by economic and social disparities, reflecting regional differences (Amini *et al*., 2021). As a result, the human development index (HDI) has become an important indicator for determining the progress, human development and living conditions of different countries, taking into consideration socio-economic factors that affect the health and national development of a population (Amini *et al*., 2021; United Nations Development Progress, 2022). Of benefit to the UK is its low ranking of 13, higher than the world average indicating this population is more knowledgeable, healthy, and has a higher standard of living in comparison to the 176 countries with higher ratings (Figure 1-2). Although CAD is still responsible for reduced quality of life and life expectancy imposing a significant burden on the economy in many countries, including the UK.

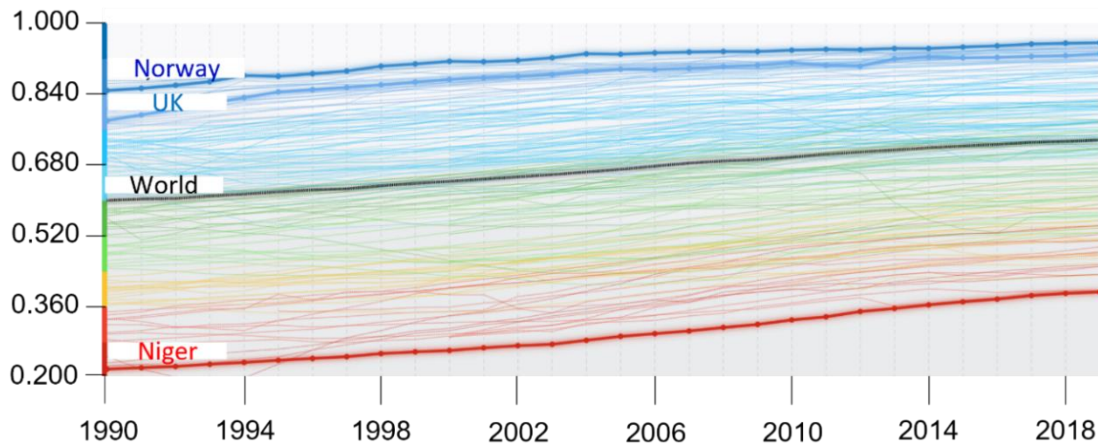


Figure 1-2. Yearly human development index.
HDI values are calculated using life expectancy at birth, expected years of schooling, mean years of schooling, and gross national income per capita. Graph showing a general increase in HDI across all countries with Norway ranked number 1, the UK ranked 13, and Niger the lowest at 189 by 2019 (United Nations Development Progress, 2022).

Coronary artery disease is the leading cause of mortality and loss of Disability Adjusted Life Years worldwide (Ralapanawa & Sivakanesan, 2021). With a significant burden falling on low- and middle-income countries (Forouzanfar *et al*., 2012; Moran *et al*., 2012). In line with the Global Burden of Disease estimates from 2001, 43 % of all CVD deaths are attributable to CAD, with a significant burden falling on low- and middle-income countries (Forouzanfar *et al*., 2012; Moran *et al*., 2012). Although as the average life expectancy increases and the global epidemic of rising systolic blood pressure (BP), the risk of dying from CAD continues to rise worldwide.

The incidence of CAD follows similarly with high prevalence found in low and middle-income countries. In particular, traditional risk factors including sedimentary lifestyle, smoking and obesity play crucial role for increased CAD prevalence in Latin America whilst in China the high CAD prevalence is largely at the fault of their high kilocalorie, meat and tropical oil dietary intake resulting in blood lipid alterations (Ferreira-González, 2014; Institute of Medicine (US) Committee, 2010). However, countries that are more industrialised increase the level of risk factors whilst also providing a higher level of medical facilities and generally better public health. Hence, the interplay of these two factors can greatly affect CAD incidence, prevalence, and mortality (Gaziano *et al*., 2010).

It is also important to acknowledge that ethnicity and genetic factors play a crucial role in risk and mortality. These differences are likely due to a combination of host susceptibility, genetic, and environmental factors which may provide important aetiological indications of CAD (Forouzanfar *et al*., 2012; Gaziano *et al*., 2010).

The incidence of CAD increases with age, independent of gender (Shahjehan. R & Bhutta. B, 2022) however, there are more men diagnosed with CAD both globally and, in the UK, than women (British Heart Foundation, 2022b, 2022c). Yet, there are sex-specific differences between men and women in relation to the prevention, investigation, and management of CAD. Generally, men develop CAD 8-10 years earlier than women, and this is largely due to menopause marking a significant cardiovascular-biological transition (Desai *et al*., 2021). Oestrogen loss after menopause appears to have a negative effect on arterial function, altering cholesterol profile, and increasing blood pressure and incidence of obesity, all of which are conventional risk factors of CAD (Desai *et al*., 2021). Globally there are more men physically active than women, this is an important preventative measure for the development of CAD risk factors such as depression, high blood pressure and cholesterol (National Clinical Guideline Centre (UK), 2014; World Health Organization, 2020).

Future cardiovascular burden is expected to be exacerbated by the aging population, diabetes epidemic in developed countries, and other associated cardiovascular risk factors which are continuing to rise. In the UK alone, CVD currently causes a significant strain on the economy and National Health Service costing around £7.4 billion a year (British Heart Foundation, 2022c; Public Health England, 2019). Hence, gaining a better understanding of CAD risk is necessary to better inform current and future actions, to address CAD worldwide reducing the far-reaching effects of associated morbidity and mortality.

1.3. Aetiology of Coronary Artery Disease

Epidemiological evidence indicates a number of risk factors contribute to CAD incidence and mortality. These include factors include smoking, stress, alcohol, high blood pressure (hypertension), high blood cholesterol (hyperlipidaemia), physical inactivity, overweight/obesity, diabetes, family history of heart disease, ethnic background, sex,

and age. Many of these risk factors can be controlled, treated, or modified, therefore reducing a person's likelihood of developing CAD. See Appendix A for more information on risk factors and their associated global mortality and burden.

In the UK alone around 28 % of adults are obese and a further 36 % have a body-mass index (BMI) classed as overweight (British Heart Foundation, 2022c). This increases the probability of other associated CAD risk factors such as diabetes, hypertension, and hyperlipidaemia. All of which are important for the pathology of CAD. One way to control an individual's weight is through healthier dietary patterns, with studies indicating a Mediterranean diet reduces CAD risk, obesity, blood pressure, cholesterol, and mortality (Chareonrungrueangchai *et al*., 2020; Gao *et al*., 2021).

High blood pressure medically referred to as hypertension is associated with the strongest evidence for the causation of CAD and leading cause of mortality in the UK (Fuchs & Whelton, 2020; Public Health England, 2019). Globally 54 % of myocardial infarctions and 47 % of CAD cases are attributable to hypertension (Wu *et al*., 2015). This is a consequence of the myocardium being overworked ultimately leading to blood vessel damage (Chhajer, 2014). As hypertension is associated with increased age, the burden and mortality on the older generation are set to rise (Kintscher, 2013).

There is an interplay between hypertension and hyperlipidaemia, both contributing to the damage of artery walls and further increasing the risk of CAD (Davis, 2021). Cholesterol is a fatty substance found in the blood made up of organic molecules termed lipids. It has an important role within the body to produce hormones, vitamin D, bile acids, and cell membranes. The majority of cholesterol is made within the liver and some intake from a person's diet. The two main types of cholesterol are low-density lipoproteins (LDL) responsible for increasing the risk of CAD, and high-density lipoproteins (HDL) which help protect against CAD (Australian Athroscelorsis Society, 2022; Soliman, 2018). Within the UK approximately 50 % of adults have cholesterol levels greater than the national average (> 5 mmol/L), so as no surprise a third of all CAD cases worldwide are attributable to hyperlipidaemia (Hill & Bordoni, 2022; Public Health England, 2019).

Moreover, diabetes, hypertension, and hyperlipidaemia have a substantial overlap in the aetiology and pathology of CAD (Cheung & Li, 2012; Chou *et al*., 2020; Leon & Maddox, 2015). All of these diseases are associated with elevations in pro-inflammatory mediators and oxidative stress (OS), important in CAD pathology (see sections 1.8 and 1.9). Diabetes is a common co-morbidity of CAD, as increased blood sugar levels result in damage to the inner lining of blood vessels, increasing the likelihood of stenosis. Consequently, adults with diabetes are 2-3 times more likely to develop CAD and almost twice as likely to die from heart disease or myocardial infarction due to reduced oxygenated blood flow (British Heart Foundation, 2022c). Diabetic patients have an increase in free-fatty acid release present in insulin-resistant fat cells, hence a rise in cholesterol, in addition to increased inflammation and oxidative stress, all of which correlate with hypertension and hyperlipidaemia (Cheung & Li, 2012; Leon & Maddox, 2015).

Smoking is a preventable risk factor that has negative effects on both the blood vessels and the myocardium. The unfavourable effects of nicotine include acute increases in BP and coronary vascular resistance, reduction in oxygen delivery increasing the risk of ischemia, enhancement of platelet aggregation, increased fibrinogen, and depression of HDL cholesterol (British Heart Foundation, 2021b; Rigotti & Pasternak, 1996). Smoking strongly correlates with CAD risk with cessation rapidly reducing cardiovascular morbidity and mortality (Rigotti & Clair, 2013).

As such determining the contributing factors of CAD is important for treatment and management programmes to develop personalised medicine. Furthermore, identifying these CAD risk factors and causes is essential for the development of prevention strategies set up to help contribute to a healthier population and environment. Examples of these include government salt, sugar, and kilocalorie reduction targets and the roadmap to smoke-free 2030 in the UK (Department of Health and Social Care, 2022; Public Health England, 2018). These initiatives subsequently contribute to a reduction in the risk and prevalence of CAD amongst other diseases.

1.4. Diagnosis and monitoring of Coronary Artery Disease

Initial assessment of CAD involves a thorough medical history check, including chest discomfort and related symptoms in addition to a risk factor assessment, to assess the likelihood of disease. This is followed by appropriate non-invasive testing, driven by the probability of disease outcome (BMJ Best Practice, 2021).

1.4.1. Medical history

The first step in CAD diagnosis includes a general health check at your GP surgery. The healthcare professional checks blood pressure, cholesterol levels and discusses your lifestyle in general covering factors such as exercise, smoking, and alcohol intake. Additionally, other factors will be explored including common co-morbidities of CAD including overweight/obesity, diabetes, and depression, along with any family history of CVD (British Heart Foundation, 2021a; NHS, 2020).

Blood pressure is recorded using two numbers with the first number (higher number) referring to systolic pressure and the second number (lower number) relating to diastolic pressure. Generally, in the UK, the ideal normal BP is considered to be between 90/60 mmHg and 120/80 mmHg. Although, it is important to note BP varies slightly between individuals, hence it is important to consult the healthcare professional for advice.

Cholesterol levels are checked using a small blood sample taken from the individual. A blood sample allows the measurements of a series of cholesterol parameters; total cholesterol, HDL (good cholesterol), LDL (bad cholesterol), fasting triglycerides (a fatty substance similar to LDL), non-fasting triglycerides, and total cholesterol to HDL ratio (NHS, 2022a). The average healthy levels in the UK are shown in Table 1-1, though it is important again to seek advice from a healthcare professional as these levels will vary slightly for each individual.

Table 1-1. Average healthy cholesterol parameters.
Highlighting average healthy levels of various parameters measured from blood samples in the UK, adapted from (NHS, 2022a)

Parameter	Average Healthy Level (mmol/L)
Total Cholesterol	≤ 5
HDL	≥ 1
LDL	≤ 4
Fasting Triglycerides	≤ 1.7
Non-fasting Triglycerides	≤ 2.3
Total Cholesterol to HDL Ratio	≥ 6

Secondly, the healthcare professional will identify the characteristics of any associated chest pain, termed angina. The Canadian Cardiovascular Society grading system helps characterise the severity of angina based on the level of activity that causes symptoms, this is widely adopted by many institutions worldwide and in the UK (Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria, 2010). This ranking ranges from class I where there is no limitation of ordinary activity to class IV where there is an inability to perform any physical activity without discomfort (See Table 1-2).

Table 1-2. Canadian Cardiovascular Society angina grading system.
Showing rankings of angina severity dependant on clinical observations Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria, 2010)

Class	Description of Angina Severity	
I	Angina with strenuous exertion only	Presence of angina during strenuous, rapid, or prolonged ordinary activity.
II	Angina with moderate exertion	Slight limitations of ordinary activities when they are performed rapidly, after meals, in cold, in wind, under emotional stress, during the first few hours after waking up, but also walking uphill, climbing more than one flight of ordinary stairs at a normal pace and in normal conditions.
III	Angina with mild exertion	Having difficulties walking one or two blocks or climbing one flight of stairs at normal pace and conditions, performing household chores.
IV	Angina at rest	No exertion is needed to trigger angina.

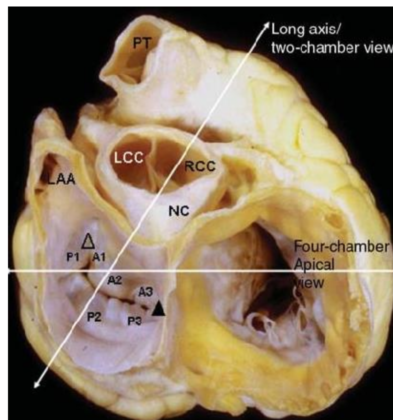
Moreover, the presence of typical angina indicates a clinical diagnosis of CAD. However further tests including electrocardiogram (ECG), X-rays, blood tests, magnetic resonance imaging (MRI), CT scans, and echocardiogram (ECHO) are used to confirm a diagnosis. Of particular importance for this study is ECHOs, as clinical data consisting of the patients' medical history and ECHO analysis is provided.

1.4.2. Echocardiogram

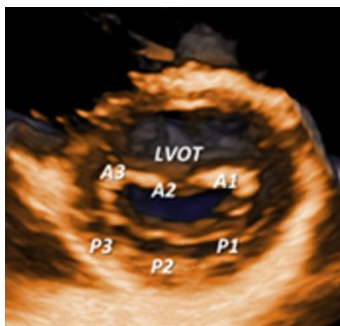
An echocardiogram is a non-invasive test that uses sound waves to look at the structure of the heart and surrounding vessels. The general procedure of a standard ECHO called a transthoracic ECHO uses a transducer on the outside of the chest, close to the position of the myocardium which sends pulses of high-frequency sound waves through the skin. The ultrasound waves reflect the "echo" of structures of your myocardium, creating an image of the moving heart and valves on a computer (NHS, 2022b).

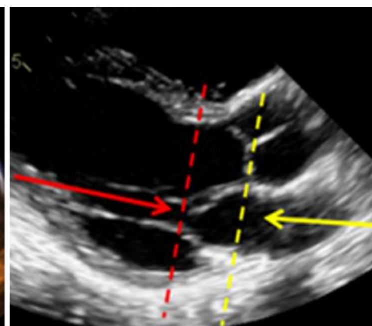
An echocardiogram can use echocardiography to assess multiple aspects of the functionality of the myocardium. M-mode echocardiology offers an ice-pick view of the myocardium in real-time, demonstrating tissue interfaces at varying distances (Geva & Van Der Velde, 2006). This spatially one-dimensional image of the myocardium is useful for measuring structures such as the size of the myocardium itself, the thickness of myocardium walls, and left ventricular dimensions and functions. Although this method provides exceptional resolution it lacks to provide much anatomic information, having been replaced with two-dimensional (2D) echo imaging (Figure 1-3) (Geva & Van Der Velde, 2006). The two-dimensional echo imaging technology allows a visual representation of myocardium structures in motion. Specifically, “flat” views of the myocardium chambers, valves, walls, and large blood vessels can be distinguished and examined using this technology (Houck *et al*., 2006). Although this technology allows quantitative analysis it is based on geometric assumptions about cardiac structure shape, being less reproducible and having limitations in relation to observing structures from multiple perspectives (European Society of Cardiology, 2022) (Figure 1-3). To overcome these limitations an emerging technology using three-dimensional (3D) echocardiology (Figure 1-3) is becoming more widely utilised having a diverse array of clinical applications. This technique takes into consideration volumetric imaging providing a more realistic anatomical display of the same myocardium structures in motion (European Society of Cardiology, 2022). Images from this technique allow easier recognition of structures for interpretation, providing more information on valvular, diastolic, and systolic dysfunction however, training of a higher level is required for this (Mayo Clinic, 2010).

(a)



(b) **(c)** **(d)**





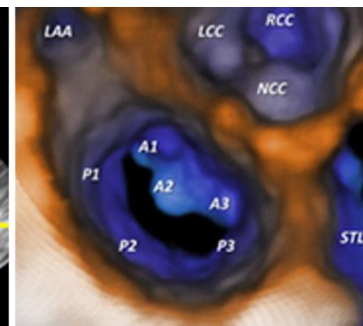


Figure 1-3. Comparison view of the mitral valve with 2D and 3D echocardiography. *(a) Mitral valve structure showing the base of the myocardium with the location of two and four chamber echocardiographic perspectives. (c) 2D echocardiology of the mitral valve can only be seen from the ventricular perspective, with arrows identifying mitral valve between left atrium (yellow) and left ventricle (red). 3D echocardiology allows the mitral valve to be visualised from the ventricular (b) and atrial (d) perspectives. Where the mitral valves anterior and posterior leaflet identified, with the latter having more visual indentations (scallops) which are numbered from anterolateral to posteromedial into P1, P2, and P3, and the anterior leaflet numbered corresponding to the posterior into A1, A2 and A3. Adapted from (European Society of Cardiology, 2022).*

Abbreviations: LVOT – Left ventricular outflow tract, LAA – Left atrial appendage, LCC – Left coronary cuspid, PT- Pulmonary tract, RCC – Right coronary cuspid, NCC – Non coronary cuspid.

Echocardiology analysis provides an array of information relating to the functionality and geometry of the heart and surrounding vessels allowing the assessment of systolic and diastolic function described below.

1.4.2.1. Systolic dysfunction

Systolic dysfunction refers to impaired ventricular contraction, clinically identified by the reduced left ventricular ejection fraction (LVEF) (Walls *et al*., 2018). This is usually due to damage or death of cardiac myocytes, or structural alterations as a result of ischemia,

reducing cardiac output and left ventricular stroke volume (Walls *et al*., 2018). As incomplete ventricular emptying occurs, an increase in ventricular end-diastolic volume and pressure are also often observed. Global systolic function is assessed using 3D ECHO, with an LVEF ≥ 50-55 % considered normal (Jander & Minners, 2017).

Patients with CAD and systolic dysfunction have higher mortality and morbidity rates, consuming more healthcare resources. Studies suggest that the clinical outcome of patients with CAD and left ventricular dysfunction who undergo revascularization surgery is highly dependent upon cardiac myocyte viability (Afridi *et al*., 1998).

It is also important to acknowledge the systemic effects of reduced LVEF, as the supply and demand of oxygen to vital organs are also negatively impacted, such as the kidneys and brain.

1.4.2.2. Diastolic dysfunction

Diastolic dysfunction is defined by impaired relaxation as a result of reduced ventricular compliance or increased resistance to ventricular filling during diastole (relaxation) (Myburgh, 2014). The term compliance refers to how easily a heart chamber expands when filled with a volume of blood. Typically, diastolic dysfunction accompanies systolic dysfunction, as both depend on one another. However, the incidence of patients with diastolic dysfunction and preserved systolic function is increasing (Walls *et al*., 2018).

Generally, this is observed due to one of three mechanisms; (1) impaired ventricular relaxation, (2) increased ventricular wall thickness, or (3) myocardial interstitial collagen accumulation (Walls *et al*., 2018). In CAD it is largely due to the impaired relaxation capacity which leads to an increase in ventricular filling pressure, clinically represented as congestive symptoms. Myocardial relaxation is an active process, requiring energy (Schwartz & Boheler, 1994). Therefore, the inability of cardiac myocytes to relax may be contributed to by the loss of mitochondrial function (1.7.1), hence low intracellular energy stores.

Diastolic dysfunction is determined by evaluating characteristics of diastolic relaxation, such as pressure and filling parameters; isovolumic relaxation or stiffness using echocardiology. It is important to note that the left ventricle (LV) size can be normal with diastolic dysfunction but ischemia, fibrosis, and myocardial hypertrophy can alter the

diastolic filling process (Seres, 2011). The non-invasive recognition of diastolic dysfunction suggests a poor prognosis in patients with CAD, acting as both an important prognostic and diagnostic tool (Ohara & Little, 2010).

1.5. Treatment of Coronary Artery Disease

There are a wide range of preventative and interventive treatments for CAD which are tailored to each patient's circumstance. The main emphasis is on therapeutic lifestyle changes to reduce an individual's preventable risk factors (Rippe, 2019; Wexler *et al.*, 2012). These changes focus on reducing blood pressure and cholesterol in particular through a reduced-sodium diet, dietary approaches to stop hypertension, weight loss, exercise, and reduced alcohol intake (Wexler *et al.*, 2012). Additionally, there are pharmacological treatments available to reduce hypertension and prevent atheroma build-up and rupture.

High cholesterol strongly correlates with an increased risk of atheroma build-up within the arteries therefore, statins such as atorvastatin, pravastatin, and simvastatin are often prescribed to reduce the production of cholesterol in the liver (British Heart Foundation, 2022f; NHS, 2021). Thrombotic events in CAD are a huge burden on healthcare budgets, thus anticoagulant use is recommended as a preventative measure (De Caterina, 2009). These include the following administration of aspirin, ticagrelor or coumadin (warfarin). For the reduction of BP, a number of treatments exist with beta blockers being the most favoured by cardiologists (British Heart Foundation, 2022g). These include atenolol, bisoprolol, and carvedilol which work to block adrenaline and noradrenaline, slowing down the heart rate and reducing the force of each beat (British Heart Foundation, 2022g; Bupa Healthcare, 2022). Other treatments for blood pressure include angiotensin receptor blockers reducing constriction of blood vessels, and calcium channel blockers reducing calcium influx into cells of the myocardium and blood vessels, again reducing contraction (British Heart Foundation, 2022a, 2022d). It is unknown what specific medication each patient in this study is taking prior to surgery.

Non-pharmacologic treatments are used in severe CAD cases whereby atherosclerotic plaque build-up is dangerous. These surgeries can either be coronary artery bypass graft (CABG) (Figure 1-4) or less invasive surgeries include percutaneous coronary

interventions (PCI) including implantable cardioverter defibrillator (ICD), angioplasty or stents (British Heart Foundation, 2021a).

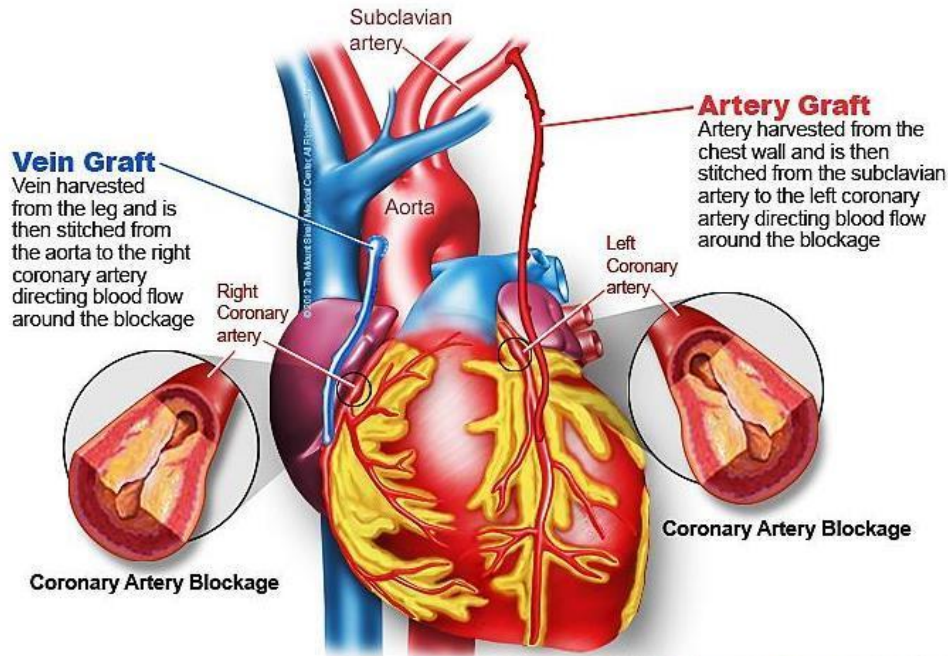


Figure 1-4. Coronary artery bypass graft.

Image showing vein and artery bypass grafts to the heart enabling blood to bypass the blocked area of the coronary artery. When a single bypass graft is required, the left internal thoracic artery is attached the left coronary artery. Typically, the great saphenous vein from the leg is used when more than one bypass graft is needed due to its size and ease of removing small segments. Once implanted the vein remodels to withstand higher blood pressure to mimic a more arterial function. (Icahn School of Medicine, 2012).

Around 400,000 CABG surgeries are performed each year ranking it as one of the most frequently performed major surgeries (Bachar & Manna, 2022). This is usually recommended when high-grade blockages are present in any major arteries and/or PCI has failed (Bachar & Manna, 2022). During this procedure a “healthy” blood vessel is used to form a new route for blood flow bypasses the blocked or narrowed artery (National Heart Lung and Blood Insitute, 2022).This provides patients with increased survival rates and improved quality of life by reducing associated morbidities, and having improved survival benefits compared to individuals receiving medical therapy or PCI alone (Serruys *et al*., 2009).

1.6. Vascular pathophysiology - Atherosclerosis

Coronary artery disease generally stems from structural and functional changes within the vascular structure of the coronary artery, an example of this is atherosclerosis. Atherosclerosis is an inflammatory pathological process characterised by the build-up of fibrous elements, lipids, and calcification within large arteries (Jebari-Benslaiman *et al*., 2022). This process is initiated by vascular endothelium activation which is followed by a cascade of events ultimately leading to vessel narrowing, activation of inflammatory pathways, and atheroma plaque formation (Jebari-Benslaiman *et al*., 2022) (Figure 1-5). The clinical manifestations of atherosclerosis affect 2 in 3 men and 1 in 2 women after the age of 40, with 50 % of all deaths associated with this pathology in westernised societies (Pahwa & Jialal, 2021; Robinson *et al*., 2009).

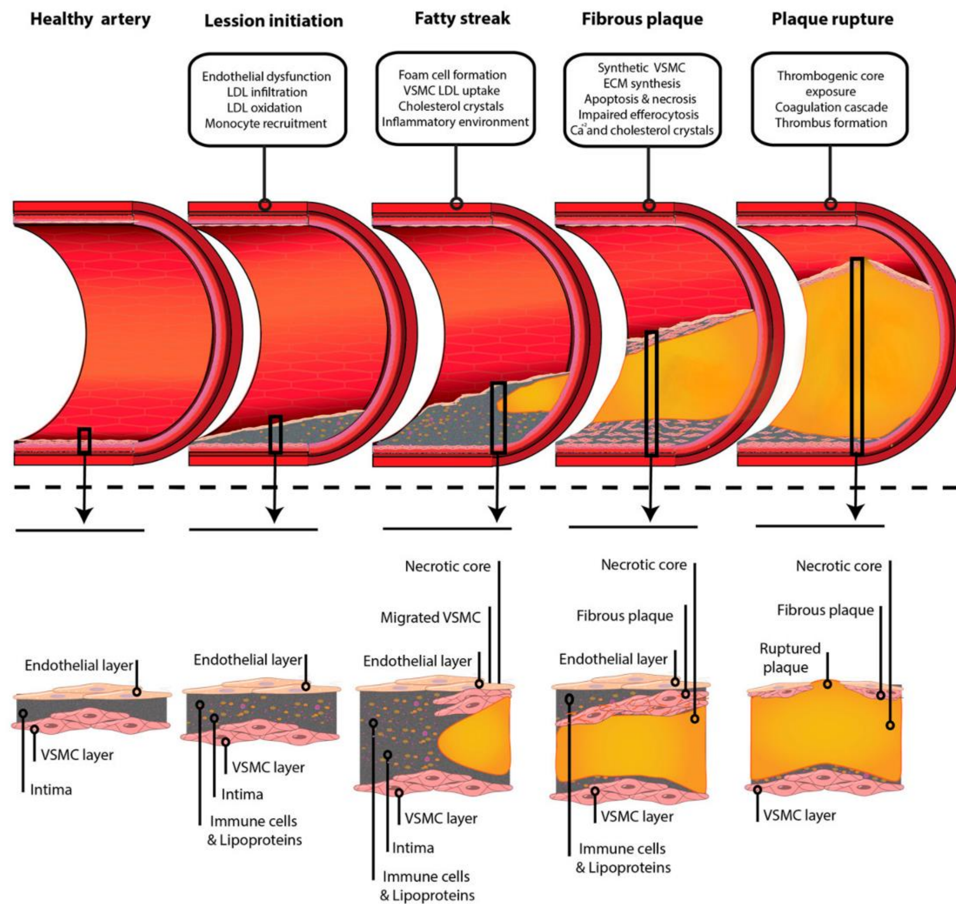


Figure 1-5. Schematic representation of atherosclerosis.

Highlighting key stages from lesion initiation to plaque rupture in atherosclerosis and important events that contribute to each stage (Jebari-Benslaiman et al., 2022).

1.6.1. Atherosclerosis initiation and fatty streak formation

Atherosclerosis is initiated upon endothelial dysfunction complemented by LDL retention and modification in the vascular intima. Modified LDLs in combination with other atherogenic factors are responsible for endothelial cell (EC) activation promoting inflammatory cell recruitment including monocytes (Lorey *et al*., 2022; Summerhill *et al*., 2019). As a result, the modified LDLs are trapped by differentiated monocytes (macrophages) and vascular smooth muscle cells (VSMC) promoting foam cell (lipid-laden macrophages) formation and fatty streak development in the vascular intima (Jebari-Benslaiman *et al*., 2022; Seidman *et al*., 2014) (Figure 1-5). Additionally, other inflammatory pathways are activated during this process which is responsible for exacerbating atherosclerotic plaque formation discussed in section 1.8.

1.6.2. Fibrous plaque development

When the fatty streak transitions to intimal growth the fibrous plaque (Figure 1-5) is developed. This is characterized by the existence of a cell-free and lipid-rich nucleus referred to as the necrotic core (Jebari-Benslaiman *et al*., 2022). This area continually increases in size due to macrophage death and impaired efferocytosis, a mechanism responsible for apoptotic cell removal (Gonzalez & Trigatti, 2017). This contributes to an inflammatory microenvironment, OS, and death of neighbouring cells all of which increase plaque vulnerability (Jebari-Benslaiman *et al*., 2022; Otsuka *et al*., 2016). The core is covered by fibres, developing the fibrous cap which aims to stabilise the plaque. This acts as a subendothelial barrier between the lumen of vessels and the necrotic core, providing structural support (Jebari-Benslaiman *et al*., 2022). These features are hallmarks of advanced atherosclerosis, hence plaque regression at this stage is unlikely. In fact, if exposure to proatherogenic factors continues, increasing the inflammatory response the fibrous cap can become weak and susceptible to rupture leading to blood clot formation (thrombus), and complete blockage of the artery resulting in myocardial infarction (Jebari-Benslaiman *et al*., 2022).

1.7. Myocardial Pathophysiology

Our understanding of vascular dysfunction observed in CAD is relatively advanced. However, this is not the case for our understanding of the cellular basis of myocardial dysfunction. We do know that CAD results in myocardial ischemia, it is likely that, the mechanical and electrical properties of single cardiac myocytes are altered. Cardiac myocytes are responsible for generating contractile force, thus the mechanical function of the myocardium.

1.7.1. Functional mitochondrial abnormalities

Cardiac myocytes contain high numbers of mitochondria in which the majority (60-70 %) of adenosine triphosphate (ATP) is required for excitation-contraction coupling (ECC) and the remaining 30 - 40 % is used for various ion pumps (Doenst *et al*., 2013).

In terms of ECC, the primary ATP-dependent utilising mechanisms are the myosin ATPase in the myofibril, the -Calcium ATPase Sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and, the sodium (Na^{+}), potassium (K^{+}) ATPase (NKA) in the sarcolemma (Figure

1-6) (Ingwall & Weiss, 2004). Thus, a high ATP supply is critically important to maintain normal heart function.

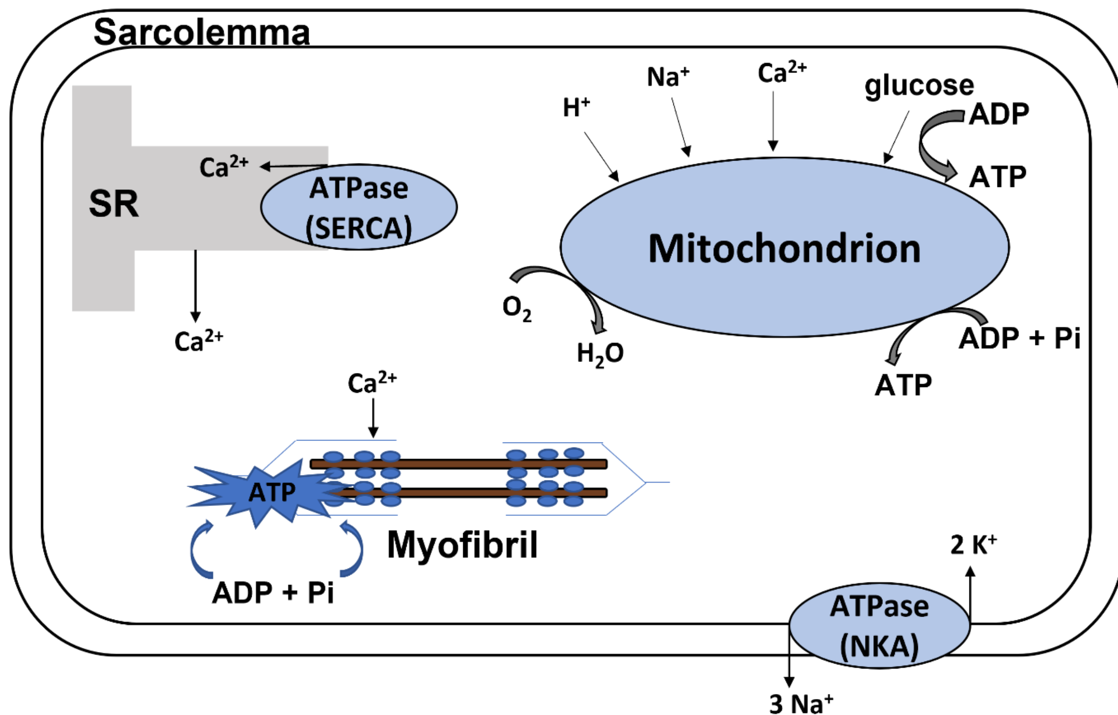


Figure 1-6. Diagram illustrating the integration of ATP synthesis and utilisation reactions in the myocardium.

The primary ATP-utilising reactions for cardiac myocytes are actomyosin ATPase in the myofibril, SERCA in the sarcoplasmic reticulum, and NKA in the sarcolemma. The main ATP synthesising pathways in the myocardium are via the oxidative phosphorylation and glycolytic pathway in the mitochondria.

Abbreviations: SR – Sarcoplasmic reticulum, ADP – Adenosine diphosphate, H^+- Hydrogen ion, H_2O – water.

Cardiac muscle contraction occurs by the myofilament sliding filament in much the same way as skeletal muscle. Cytoskeletal proteins form the basis of sarcomeres which are formed by contractile proteins such as myosin (thick filaments) and actin (thin filaments) along with regulatory troponin-tropomyosin complex (Kumar *et al*., 2020). Contraction is a cyclic, multistep process that involves the binding and hydrolysis of ATP requiring myosin ATPase for the movement of actin and myosin (Kumar *et al*., 2020; Zhou *et al*., 2020).

SERCA is an active transporter that hydrolyses ATP to provide energy for the active transport of Ca^{2+} into the Sarcoplasmic Reticulum (Bravo *et al*., 2013). Thus, SERCA is essential for the maintenance of ECC and the cardiac cycle (Periasamy & Huke, 2001).

SERCA promotes cardiac relaxation by lowering cytosolic Ca^{2+} and restoring intracellular Ca^{2+} required for diastole (Bhupathy *et al*., 2007; Periasamy & Huke, 2001). Important to note is that SERCA is susceptible and sensitive to oxidative modifications. Under pathological OS irreversible oxidation of cysteines such as sulfonylation, result in reduced SERCA activity (Takeshi Adachi *et al*., 2004). In response to reduced SERCA activity, elevated cytosolic Ca^{2+} levels affect the ECC compromising myocardial function for example decreased force generation, prolonged action potential, and damage to other ion channels key to ECC such as the ryanodine receptors (Boncompagni *et al*., 2006; Qaisar *et al*., 2018). In addition to affecting the ECC, Ca^{2+} homeostasis is important for mitochondrial function, thus elevated cytosolic Ca^{2+} can negatively impact cellular energy stores (T. Adachi *et al*., 2004).

Sodium (Na^+) regulation is also important for cardiac function as small alterations in Na^+ cytoplasmic concentration can have significant impacts on the heart, influencing Ca^{2+} and pH levels, and controlling heart contractility (Shattock *et al*., 2015; Swift *et al*., 2007). NKA is important for regulating the concentration gradient for Na^+ and K^+, whereby sodium ions are moved against their concentration gradient from the cytoplasm to the extracellular space utilising energy released via ATP hydrolysis (Shattock *et al*., 2015). This mechanism is necessary to generate and maintain the cardiac myocyte membrane potential allowing a steady-state resting potential to be reached, ready for the next cycle of depolarization (Figure 1-7) (Swift *et al*., 2007; Teissie & Yow Tsong, 1981). Reactive oxygen species (ROS)-mediated modifications to Na^+/Ca^{2+} exchanger (NCX) can also contribute to altered Ca^{2+} homeostasis and cardiac myocyte function. Though the effect of ROS on NCX activity is debated as ROS has been shown to both stimulate and decrease NCX activity (Zhang *et al*., 2016; Zhang *et al*., 2008). As intracellular Ca^{2+} is generally increased due to ROS-mediated reduced SERCA and ryanodine receptor activity, NCX favours its forward activity inducing a depolarization wave (Driessen *et al*., 2014).

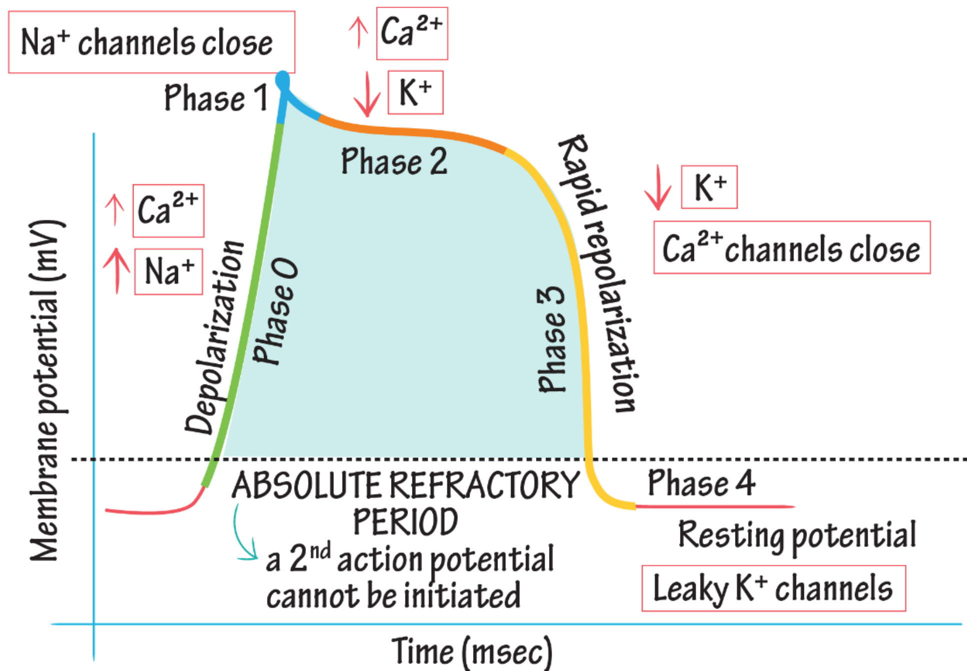


Figure 1-7. Cardiac muscle cell action potential.

The diagram highlights the role of key ions calcium, sodium, and potassium in cardiac conduction for the contraction-relaxation cycle. ("Physiology Glossary: Cardiac Muscle Action Potential," 2018)

Oxygen deprivation as a result of CAD prevents ATP formation by mitochondrial oxidative phosphorylation (Lemasters, 2010). Alternatively, glycolysis partially replaces ATP production after mitochondrial dysfunction, although in ischemia glycolytic substrates are rapidly diminished (Lemasters, 2010). Arrhythmogenesis is often favoured in response to mitochondrial dysfunction as the electrical stability of cardiac myocytes is altered (Gambardella *et al.,* 2017). The impact of mitochondria on cardiac myocyte excitability is mainly mediated via energy sensing, sarcolemma ATP sensitive K^+ channels (sarcKATP) (Gambardella *et al.*, 2017). Insufficient ATP supply to cardiac myocytes results in the activation of sarcKATP, as this ion channel is negatively regulated via intracellular ATP levels. In turn extrusion of K^+ is increased reducing the duration of the action potential and influx of Ca^{2+}, whilst the efflux of Ca^{2+} via NCX is favoured (Gambardella *et al.*, 2017; Nakaya, 2014). Consequently, intracellular Ca^{2+} is reduced and contractility is impaired.

Abnormalities in cardiac mitochondria can lead to reduced ATP production and energy supply, diminished autophagic mechanisms, increased ROS production (section 1.9), and cell death via apoptosis (Chistiakov *et al*., 2018).

Ischemia induces cardiac myocyte apoptotic cell death as it is responsible for initiating mitochondrial fragmentation, increasing mitochondrial permeability, and the release of cytochrome C, an initiator of a cascade of irreversible events which ultimately lead to apoptosis (Chistiakov *et al*., 2018). This plays an important role in ischemia- and induced cardiac remodelling, whereby fibroblasts replace apoptotic cardiomyocytes resulting in myocardium scarring and fibrosis.

Furthermore, autophagy is a survival mechanism used to remove damaged and old cell machinery (Glick *et al*., 2010). Severe ischemia can increase autography so that damaged mitochondria accumulate in cardiac myocytes, which can result in increased OS and apoptotic cell death accentuating scarring and fibrosis (Chistiakov *et al*., 2018). The loss of this mechanism has been demonstrated to be involved in the pathogenesis of various CVDs including CAD (Glick *et al*., 2010).

Cardiac remodelling in response to ischemia can have various detrimental effects on cardiac function, heightens inflammatory responses, and alters the size, shape, and function of the myocardium (Figure 1-8). The loss of contractility in infarcted regions results in residual volume and diastolic wall tension, additionally triggering hypercontractility in non-infarcted regions to maintain stroke volume (Brenner & Ertl, 2012; Liu *et al*., 2021). At sites of infarcted lesions, fibrosis and scarring are present, as the divergence between oxygen demand/perfusion as a result of the inflammatory increase in matrix-turnover and degradation with the loss of tissue layer connectivity (Brenner & Ertl, 2012; Sutton & Sharpe, 2000). Consequently, the wall thinning observed triggers infarct expansion. Since the non-ischemic region is constantly challenged to maintain cardiac performance, the cardiac myocytes grow in length leading to myocardium hypertrophy and cavity enlargement (Brenner & Ertl, 2012; Sutton & Sharpe, 2000). In particular, the left ventricle is a primary target for ischemic injury due to its heavy workload and increased muscularity (Madani & Golts, 2014). Often clinically represented as increased diameter, wall stress, and dilation correlating with ventricular stiffness and indices of global left ventricular dysfunction (Bonnema *et al*., 2008; Brenner & Ertl, 2012) (section 1.4.2.2 & 1.4.2.1).

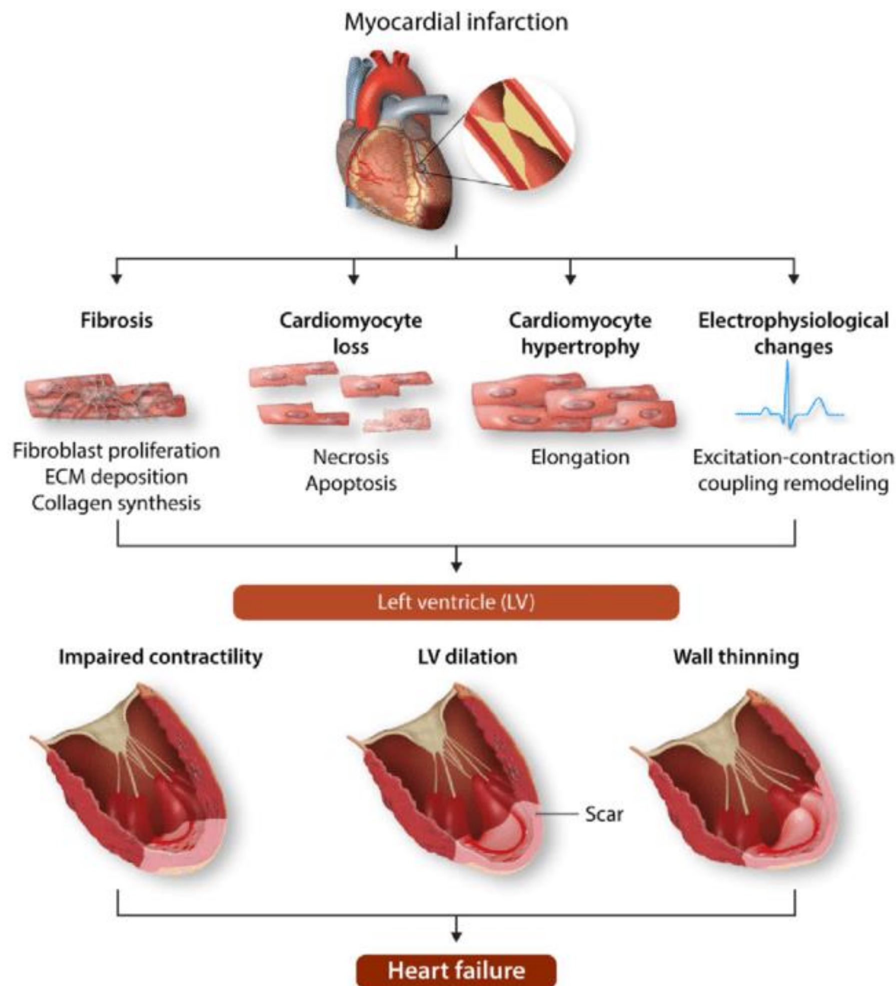


Figure 1-8. Pathological left ventricular remodelling post-myocardial infarction. *Following ischemia in the myocardium fibrous scar forms in infarcted tissue in addition to cardiac myocyte death. Adjacent to the infarcted region myocytes elongate and excitation-contraction is impaired. As a result, early remodelling is characterised by thinning, elongation, and ventricular dilation changing chamber shape from an elliptical to spherical (Riddell et al., 2020).*

Indeed, cardiac myocytes are extremely vulnerable to oxygen deprivation as hypoxic conditions induce mitochondrial dysfunction contributing to hypoxia-induced cardiac injury.

1.8. Inflammation and coronary artery disease

Inflammation consists of a complex, highly conserved cascade of molecular and cellular events. The cascade of events involves the increased permeability of micro-vessels, migration of cells, attachment of circulating cells in close proximity to the site of injury, cell apoptosis, and regeneration of tissues and micro-vessels (Schmid-Schönbein, 2006). The initiation of an inflammatory response increases circulating levels of pro-

inflammatory cytokines, proteins, and immune cell counts all of which can be measured within bodily fluids. Inflammation is vital to protect and heal the body against injury, infection, and harmful stimuli, however when it is chronically activated and sustained it often leads to injury and reduced survival, termed maladaptive (Alfaddagh *et al*., 2020). Inflammation is central to CAD pathogenesis and involved in all phases of atherosclerosis and vascular dysfunction. It also acts at a systemic level affecting the myocardium itself (Spagnoli *et al*., 2007).

1.8.1. Pathobiology of atherosclerosis and inflammation

Endothelial dysfunction, subintimal cholesterol accumulation, and atherosclerotic lesion formation are key drivers of the subintimal inflammatory response observed in CAD (Figure 1-9). However other factors such as smoking, diabetes, visceral adipose tissue, perivascular fat, ROS, and genetic traits can contribute to this increased inflammation. The binding, rolling and transmigration of inflammatory cells including monocytes is promoted at sites of early plaque initiation due to the upregulation of adhesion molecules including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and several selectins expressed on ECs (Alfaddagh *et al*., 2020; Jebari-Benslaiman *et al*., 2022). At a molecular level, when infiltrating macrophages engulf oxidised low-density lipoproteins (Ox-LDLs) the inflammasome, a complex cytosolic multiprotein is formed, this is an important step in propagating inflammation (Alfaddagh *et al*., 2020; Jebari-Benslaiman *et al*., 2022). As a result, IL-1β and Interleukin-18 (IL-18) are released from foam cells which further activate a variety of inflammatory cells including neutrophils, T- and B-Cells, increasing Interleukin-6 (IL-6), C-reactive protein and ROS production, all amplifying the inflammatory cascade within the vessel wall (Alfaddagh *et al*., 2020).

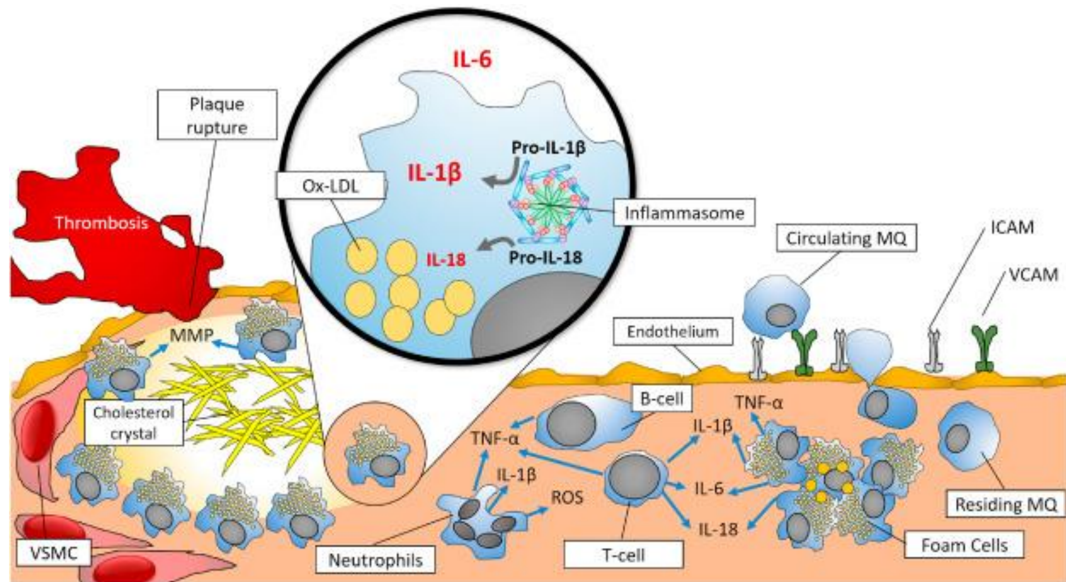


Figure 1-9. Inflammation and its role in atherosclerosis.

Image showing the role of infiltrating inflammatory cells to the area of initial endothelial dysfunction. With cytokines and reactive oxygen species involved in the process of initial fatty streak formation to plaque rupture (Alfaddagh et al., 2020).

Abbreviations: MMP – Matrix metalloproteases, MQ – Macrophage, Ox-LDL – Oxidised low-density lipoprotein, TNF-α - tumour necrosis factor-α.

1.8.2. Inflammation and plaque stability

Plaque architectural stability is affected by inflammation as this plays a role in the formation and destabilization of collagen in the fibrous cap (Figure 1-9). The migration and proliferation of VSMCs in the intima and the production of collagens important for fibrous cap formation are strongly influenced by cytokine, fibrogenic mediator, and growth factor release from foam cells, T-cells, and other inflammatory cells (Alfaddagh *et al*., 2020; Jebari-Benslaiman *et al*., 2022). However, IL-1β, a cytokine important for the production of matrix metalloproteases (MMP) released from the foam cells degrades this collagen within the fibrous cap, leading to plaque instability and increasing the risk of rupture (Alfaddagh *et al*., 2020). Hence, inflammatory mechanisms in this case can act as both protective and offensive.

1.8.3. The role of inflammatory cytokine, IL-1β, in myocardial dysfunction

Cytokines are a type of signalling molecule that are secreted from immune cells to control interactions and communications between cells (Zhang & An, 2007). An inflammatory response is tightly regulated via pro- and anti- inflammatory cytokine release. A number of cells secrete the same cytokine and each released can act on the

same cell (autocrine action) and a number of other cells (pleiotropy) shown in Figure 1-10 (Balkwill & Burke, 1989; Kishimoto *et al*., 1994; Zhang & An, 2007).

Figure 1-10. The cytokine network and its relationship to immune cells.
A number of cell types are involved in the immune system including, B cells, T cells, Macrophages, Neutrophils, Basophils, Eosinophils, and Mast cells, with their cytokine release acting synergistically or antagonistically. The main pathway that involves IL-1β in coronary artery disease is largely controlled via infiltrating macrophages secreting IL-1β at the initial vascular injury/dysfunction. This stimulates and sustains an inflammatory response through immune cell recruitment, leukocyte activation specifically neutrophils, and lymphocyte activation (B and T cells) which in turn stimulates further downstream pathways. (Zhang & An, 2007)

Proinflammatory cytokines are specifically involved in the up regulation of inflammatory mechanisms. Of particular interest to this study is the cytokine IL-1β as it is a key mediator in the inflammatory response during chronic disease and acute tissue injury, exhibited in CAD (Lopez-Castejon & Brough, 2011). This cytokine is secreted by a variety of cell types however, extensive research is primarily focussed on production within cells of the innate system such as monocytes and macrophages (Lopez-Castejon & Brough, 2011; Zhang & An, 2007).

Evidence suggests that IL-1β contributes to the pathology of CAD with the correlation of higher prevalence of CAD in higher IL-1β quartiles (Baldetti *et al*., 2019; Fairweather &

Rose, 2005). Studies investigating pro-inflammatory cytokines on whole heart function of rats found that IL-1β and tumour necrosis factor-α (TNF-α) in combination initially increases cardiac work and systolic pressure before a substantial reduction in cardiac work and coronary flow occurs with prolonged exposure (Schulz *et al*., 1995). Furthermore, this upregulation of IL-1β seen in animal heart failure models and human patients has confirmed its importance for the pathogenesis of cardiac dysfunction and adverse remodelling (Hanna & Frangogiannis, 2020; Xia *et al*., 2009).

Interleukin-1β is a typical pleiotropic cytokine, hence acting on various cells. At a cellular level, IL-1β driven cardiac remodelling and dysfunction is not completely understood however, several potential mechanisms have been proposed. One mechanism is IL-1β driven systolic dysfunction, thought to involve the disruption of calcium handling or suppression of B-adrenergic responses of cardiomyocytes, hence reduced contractility (Gulick *et al*., 1989; Kumar *et al*., 1996). Furthermore, as shown in section 1.8.1, IL-1β secretion stimulates adhesion and recruitment of other inflammatory cells, hence exacerbating inflammation in the myocardium resulting in further damage and dysfunction (Leszczynski *et al*., 1994; Saxena *et al*., 2013). Additionally, as IL-1β is involved in matrix-degradation (Section 1.8.2) this may contribute to the disruption of critical matrix-cardiomyocyte interactions which are important for cell survival, thus accentuating apoptosis (Saxena *et al*., 2013). This phenomenon may also play a role in increased fibrosis due to the activation of fibroblast-mediated matrix protein synthesis by increased fibrogenic growth factors, consequently leading to the thickening of chamber walls and scarring of the myocardium (Bageghni *et al*., 2019). Therefore, it is evident that IL-1β plays an important role in both atherosclerosis and myocardial dysfunction in CAD, thus a valuable marker to assess.

1.9. Reactive oxygen species and coronary artery disease

Oxidative stress in tissues occurs when the production of ROS overwhelms the antioxidant defence mechanisms, causing a redox imbalance. Reactive oxygen species are highly reactive and unstable oxygen-centred free radicals or oxidising agents. Common ROS include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($ONOO^-$).

Reactive oxygen species can be produced as a by-product of mitochondrial respiration, metabolism, or specific enzymes. At a cardiovascular level, the vast majority of cellular ROS comes from mitochondrial respiration via the electron transport chain. During oxygen consumption, in the electron transport chain, a small minority (1-2 %) of electrons are released to produce the superoxide radical (Chistiakov *et al*., 2018; Dubois-Deruy *et al*., 2020). In sustained ischemia uncoupling of the mitochondrial electron transport chain from ATP production occurs, resulting in an overproduction of ROS (Chen *et al*., 2008).

Increased OS is involved in the pathogenesis of various chronic diseases including atherosclerosis, CVD, cancer, and diabetes (Hayes *et al*., 2020). In particular, the association of OS and CAD is well documented, with elevated levels often observed in CAD patients (Lakshmi *et al*., 2013; Palazhy *et al*., 2015). The harmful effects of elevated OS levels in CAD include endothelial dysfunction, lipid peroxidation, the activation of inflammatory pathways, and myocardial dysfunction.

1.9.1. The role of nitric oxide in endothelial dysfunction

As discussed in section 1.6.1, endothelial dysfunction is important for the initiation of atherosclerotic plaque formation in CAD. This endothelial dysfunction can be explained through the reduction in NO bioavailability. L-arginine, an amino acid in ECs synthesises NO in a reaction catalysed by endothelial Nitric Oxide Synthase (eNOS). Nitric Oxide diffuses across cell membranes to the smooth muscle tissue of artery walls and generally is deemed an athero-protective molecule as it promotes smooth muscle fibre relaxation termed endothelial-dependant vasodilation (Figueroa *et al*., 2013). Nitric oxide is an important metabolite for reducing atherosclerosis progression by improving mitochondrial efficiency, endothelial function, angiogenesis, glucose clearance and insulin resistance whilst reducing inflammation, tissue oxidation and steatosis (Figure 1-11) (Litvinova *et al*., 2015).

Though, in the occurrence of cardiovascular risk factors such as diabetes, smoking, and hypertension, NO production is reduced. This is a consequence of pathologies which increase OS including CAD, as this promotes mediators that inhibit the activity of eNOS, hence reducing NO production (Schulz *et al*., 2011). Increased cellular ROS, thus OS, promotes the synthesis of pro-atherogenic cytokines (TNF-α, IL-1β, IL-6), adhesion

molecules (VCAM, ICAM), and chemokines via the activation of the transcription factor nuclear factor-kB (NF-kB) (Figure 1-11). These NF-kB activating stimuli are responsible for suppressing eNOS at an mRNA level and consequently at the protein level (Lee *et al*., 2014) . In hypertensive patients defects in the endothelium-derived NO system have been shown to link to abnormal endothelial-dependant vasodilation (Panza *et al*., 1993).

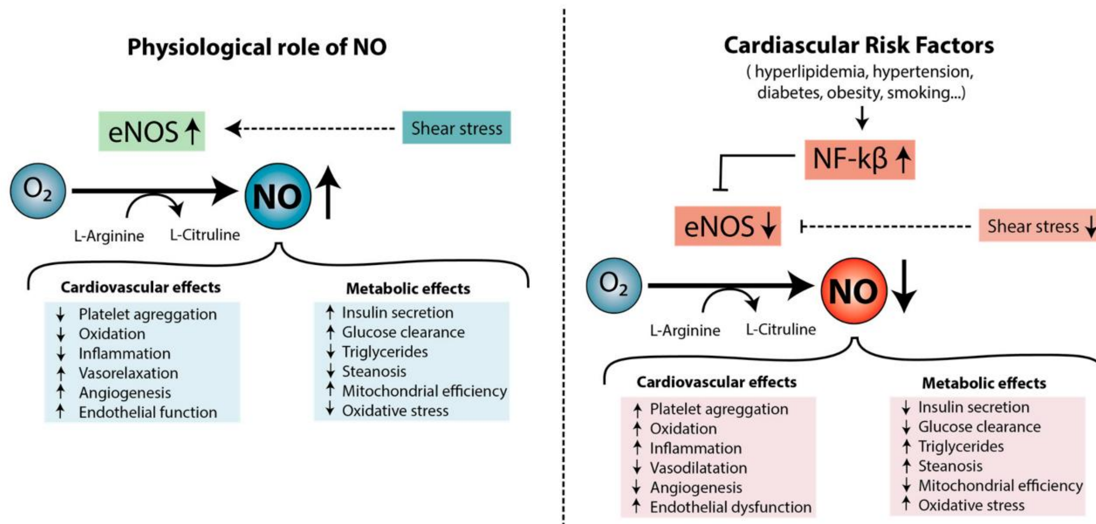


Figure 1-11. Nitric oxide and its cardiovascular and metabolic effects.
Cardiovascular metabolism is highly regulated by nitric oxide and is compromised by cardiovascular risk factors (Jebari-Benslaiman et al., 2022).

1.9.2. Role of oxidation in lipid modification

At sites of EC activation and inflammation, trapped LDLs are often chemically modified prior to being engulfed by macrophages. In particular, the oxidation of the LDLs (Ox-LDL) is facilitated due to the absence of protective plasma antioxidants. The LDLs can be oxidised by ROS in the extracellular media or directly by the enzymatic activity of phospholipases and lipoxygenase (Figure 1-12). These modified Ox-LDLs are important in atherosclerotic plaque formation being key inflammatory mediators.

Such modifications to LDLs can alter their interaction with the extracellular matrix of the vascular intima, hence assisting in their entrapment (Schneider *et al*., 2012). More importantly, Ox-LDLs possess a greater affinity for scavenger receptors on macrophages, therefore encouraging non-regulated uptake. However, the excess uptake of lipids by macrophages perpetuates the inflammatory response, and Ox-LDLs induce signalling cascades that activate NF-kB targets involved in inflammation development and progression (Jebari-Benslaiman *et al*., 2022). As a consequence, EC activation, monocyte

recruitment, and foam cell formation are maintained accelerating atherosclerotic plaque formation. Although LDL oxidation is the most common it is important to note other modifications such as glycosylation, acetylation and aggregation do occur contributing to atherosclerosis (Knott *et al*., 2003; Kruth *et al*., 1995).

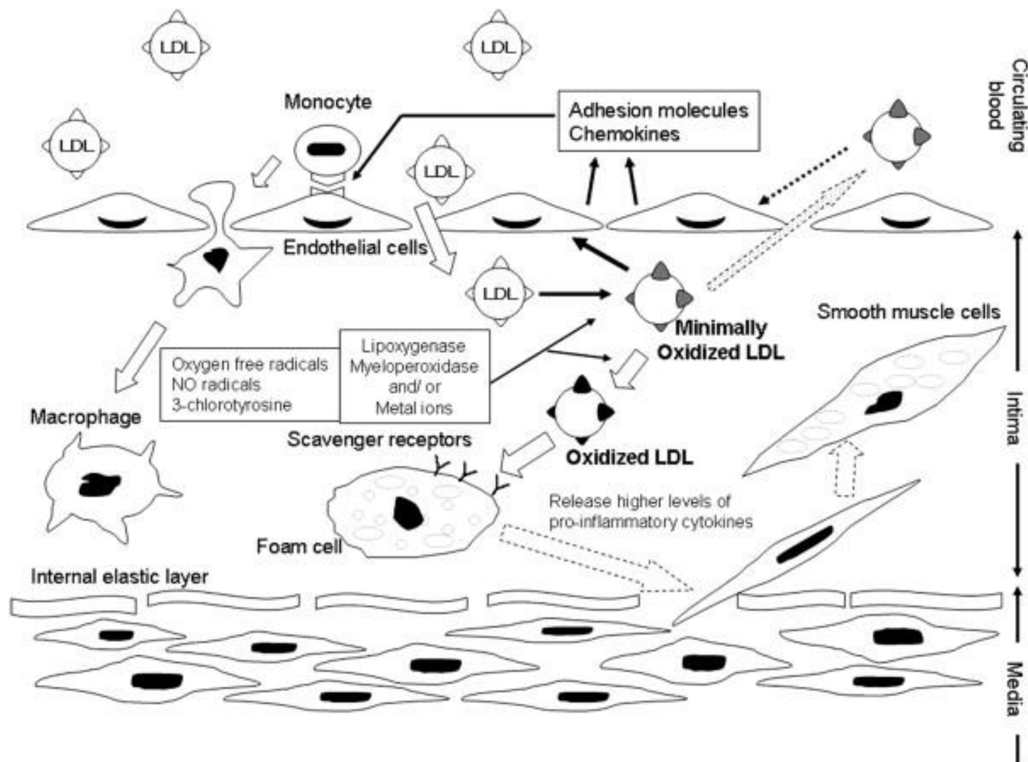


Figure 1-12. Mechanisms of LDL oxidation and foam cell formation.
A simple overview of the modification of LDLs in circulating blood which infiltrates into the intima as minimally oxidised LDLs. These can be subjected to further modification increasing their probability of engulfment by macrophages (Hiroshi Yoshidaab & Kisugia, 2010).

Those LDLs that are minimally oxidised can escape scavengers (macrophages) and re-enter the circulating blood system. Hence, these can be measured in serum from blood samples to indicate the extent of OS within a patient.

1.9.3. Role of reactive oxygen species in myocardial dysfunction

As previously discussed, mitochondria are important in cardiac myocyte function and physiological ROS production. Therefore, elevated levels of ROS, thus OS, can have detrimental effects on mitochondrial function and consequently heart function.

During ischemia, irreversible damage to the mitochondria occurs particular at a DNA level. This is due to mitochondrial DNA being highly susceptible to oxidative damage due to its low repair capacity and close proximity to the electron transport chain (Dubois-

Deruy *et al.*, 2020). This phenomenon has been demonstrated in murine models of myocardial infarction induced via 4 weeks of coronary ligation (Ide *et al.*, 2001). As a result of DNA damage, further mitochondrial dysfunction is observed, inducing, and exacerbating the disease.

Mitochondrial ROS is also able to directly oxidise proteins involved in the electron transport chain. Complexes I and III are often targets of the attack resulting in defective complexes increasing the reduction of di-oxygen (O_2) to superoxide ($O_2^{\cdot-}$) (Dubois-Deruy *et al.*, 2020). This increased flux of superoxide contributes to metabolic OS, genomic instability, cellular injury, hypertrophic signalling, and ultimately the shutdown of mitochondrial energy production (Dubois-Deruy *et al.*, 2020).

Calcium is an important player in the intracellular signalling system that transforms extracellular stimuli to the regulation of a number of key mechanisms including cardiac muscle contraction, cell proliferation, cell death, and gene expression (Castillo *et al.*, 2021). More recently increased levels of cardiac ROS have been shown to be associated with diastolic dysfunction in relation to disturbance of calcium homeostasis (Greensmith *et al.*, 2010). Reactive oxygen species can modify important calcium channels, particularly the ryanodine receptor in cardiomyocytes resulting in Ca^{2+} leak and relaxation stiffness of cardiomyocytes (Jeong & Dudley, 2015). Hence, contributes to the diastolic dysfunction discussed in section 1.4.2.2.

On the other hand, ROS also contribute to systolic dysfunction via disturbance of calcium homeostasis also. This occurs via the activation and oxidisation of stress kinases. (Wagner *et al.*, 2013). This particular activation results in sodium intracellular accumulation and prolonged action potentials. As a result, calcium entry is encouraged, and in combination with ROS-induced sarcoplasmic reticulum dysfunction, intracellular calcium drastically rises diminishing contractility (Wagner *et al.*, 2013).

Therefore, OS plays a key role in the cardiac dysfunction observed in CAD patients, some of which are measurable using ECHO or experimental methods on single cardiomyocytes.

1.10. Models of coronary artery disease

Disease models play an important role in the understanding of cardiovascular disease pathogenesis and the development of new therapeutics. There is a wide range of models used in cardiovascular research from transformed cell cultures to animal, and human models all providing important insights into CVD.

1.10.1. Animal models

Animal models used within cardiovascular research have provided important insights into the pathophysiology of CVDs. In particular explanted animal myocardium and corresponding isolated primary cells have been utilised for centuries to monitor cardiac cell physiology and electrophysiology improving our knowledge of the functionality of the myocardium (Zaragoza *et al*., 2011). Small animal models such as mice and rats and larger models including rabbits, pigs, and sheep are often used in cardiovascular research. However coronary lesions are difficult to induce and are naturally rare, so CAD induction in these models often involves pharmaceutical or surgical interventions that result in no or limited coronary lesions (Liao *et al*., 2015). Thus, the CAD pathophysiology and progression in animal models are not fully representative of that observed in humans. In particular significant differences are observed between small animal models and human cardiomyocytes in terms of heart rate, Ca^{2+} cycling, expression of key ion channels, myofilament composition, and cellular electrophysiology (Zuppinger, 2019). These physiological differences are reduced between larger models and humans providing more relevant information on the anatomical structure and pathophysiological changes in response to pressure overload (Zuppinger, 2019). Whilst the differences between the human and animal myocardium becoming more exacerbated in pathological conditions (Liao *et al*., 2015; Zuppinger, 2019). Furthermore, animal models are relatively expensive, require long-term housing, strict quality control, and have ethical concerns.

1.10.2. Cell cultures

The *in vitro* modelling of the mammalian myocardium remains a challenge due to its complexity and highly differentiated nature. The use of 2D cell cultures has been used for many years uncovering many cellular mechanisms of the myocardium (Zuppinger,

2016). These cell lines include mouse atrial cardio myoblasts (HL-1,) and rat cardio myoblasts (H9c2, ATC® CRL-1446™). Cell cultures allow long term experiments as a cell line can be kept in culture over many months and single experiments to run over the course of weeks. However, the traditional 2D cultures do not provide information on the electrical and paracrine crosstalk between different cell types in the 3D network that form the myocardium (Zuppinger, 2016). Moreover, techniques now allow cardiac cells to be examined in a tissue-like, 3D environment resembling a more *in vivo* response. Historically 3D cultures have been used for drug testing in cancer biology and more recently branched into the cardiovascular field for drug and toxicology testing. Regenerative medicine has been the driving force for 3D cultures with the prospective to replace lost cells of the heart after cardiac infarction (Zuppinger, 2016). Innovations in the field of 3D cell culture techniques including the technology of reprogramming somatic cells into induced pluripotent stem cells (iPSC) allow the production of human cardiomyocytes as an alternative to primary rodent cells (Zuppinger, 2016). With the addition of numerous strategies, the selection and maturation of these human-iPSCs have improved which offers a better option that represents human cardiomyocyte biology compared to other alternatives. Their contribution to cardiovascular medicine has become widely recognised for example describing cardiac channelopathies such as arrhythmogenic right ventricular dysplasia, familial hypertrophic cardiomyopathy, and long-QT syndromes (Karakikes *et al*., 2015). This is important as it encapsulates a patient's individual disease at a molecular and cellular level for the use in disease modelling, and personalised drug medicine contributing to advances in precision medicine (Karakikes *et al*., 2015). Nevertheless, 3D culture techniques do have drawbacks including the availability of differentiated cells, inconsistent efficacy of the differentiation process, batch-to-batch variation, time consumption, and expense of equipment (Zuppinger, 2019).

Therefore, it would be advantageous to use *in vitro* screening models of the myocardium that allow the study of environmental factors, gene mutations, and drugs preferentially on a human genetic background. The use of human models is the most clinically relevant way to understand cardiac physiology and regarding this study, the mechanisms underlying oxidative stress in CAD.

1.11. Aims of the study

The primary pathological mechanism leading to CAD is atherosclerosis, an inflammatory process associated with an accumulation of lipids and metabolic alterations within the arteries, noticeable by elevated cytokine levels and oxidative stress. Previous studies focus on the use of small animal models to mimic the metabolic and pathophysiology of CAD. However, CAD is a complex multifactorial disease hence, this *preliminary* study aimed to address this by investigating patient levels of IL-1β, a marker of inflammation, and Ox-LDL, a marker of OS, to correlate to indices of cardiac function.

By quantifying the levels of these biomarkers in a CAD patient cohort, this study seeks to determine whether IL-1β and Ox-LDL are valuable biomarkers of the underlying pathophysiology of CAD. Additionally, determining patient oxidative stress tolerance will provide the pathway for future investigation into the underlying mechanisms of oxidative stress. In particular, the study aimed to:

1) Optimise the isolation of human cardiac myocytes
2) Quantify the levels of IL-1β and Ox-LDL in patient serum samples
3) Investigate the isolated cardiac myocyte oxidative stress tolerance in response to varying concentrations of hydrogen peroxide
4) Correlate the findings of aims (2) and (3) to indices of cardiac function

It is important to acknowledge the technical difficulty of isolating primary cardiac myocytes from human tissue. Thus, a key focus on optimising the isolation procedure was initially required, (aim (1)), to allow any further experiments on the myocytes and continuation of the study.

2. Methodology

2.1. Study design

The main aim of the study is to investigate the effects of CAD on cardiac function, and to assist in the development of better treatment options and surgical outcomes. With the study designed to identify the link between the known vascular dysfunction and myocardium dysfunction through ECHO parameters and cardiac cellular dysfunction, determine the role of oxidative stress in CAD, and correlate findings with specific biomarkers in patient serum. Data collected summarised in Table 2-1. The study was set up via a collaboration with academics at The University of Salford and cardiothoracic surgeons at the Lancashire Cardiac Centre, Blackpool Victoria Hospital.

Table 2-1. Table summarising data collected during the complete study since Nov 2019 (complete patient data) and the data collected/used for the contents of this thesis (data used).

Note: Complete patient serum data represents ALL patients collected with differences in complete tissue due to not being processed for experimentation and ECHO's not processed by BVH as yet.

Complete Patient Data	n	Data Used	n
Tissue	67	Tissue	18
Serum	83	Serum	37
ECHO	52	ECHO	37

An important component of the initial study design was the sample size ruling, taking into consideration the experimental optimisation that was required. This decision was based solely on similar experimental designs conducted on animal models which typically advise that optimisation along with associated experiments requires an n of 10 per group. As a result, an n of 90 patient samples was initially deemed sufficient to complete the objectives given for this study, but since has been extended.

2.1.1. Ethical considerations

The study was carried out in accordance with Research and Ethics Committee (REC) regulations, Trust and Research Office policies and procedures, Good Clinical Practice Guidelines, and standards of the International Conference Harmonization. Before research commenced approval from the Health Research Authority (HRA) and Health

and Care Research Wales (Appendix B) (**IRAS 247341**) was required in addition to a favourable opinion from London - Camberwell St Giles Research Ethics Committee (Appendix C). Furthermore, approval from the ethical committee at the University of Salford was approved under the ethics code **3391**.

2.1.2. Patient recruitment

Patients were recruited during their pre-operative assessment who were scheduled for a coronary artery bypass graft surgery (CABG) at Blackpool Victoria Hospital (BVH). Those recruited met the following criteria shown in Table 2-2.

Table 2-2. Recruitment criteria for the study.

Inclusion Criteria	Exclusion Criteria
Patient with CAD	Non-Adults < 18
Revascularisation surgery scheduled	Non-English-speaking patient
Age > 18	
Male or Female	
Written, informed patient consent	

Those recruited were required to give consent, completing the patient information sheet and consent form prior to surgery (Appendix D). Once recruited a blood sample was taken pre-surgery and the right atrial appendage tissue was taken during surgery (Figure 2-1). The blood sample was taken at the point of anaesthesia and stored in an EDTA tube at 4 °C. After surgical resection the tissue sample was transferred into a sample tube containing sterile 0.02 mM Ca^{2+} -transport solution at 4 °C (Table 2-3). Additionally, clinical data for each patient was collected via routine pre, during, and post-operative assessment, consisting of the following information: Echo data, BMI, Weight, and Sex. All the samples and data collected were fully anonymised, using a unique study identifier.

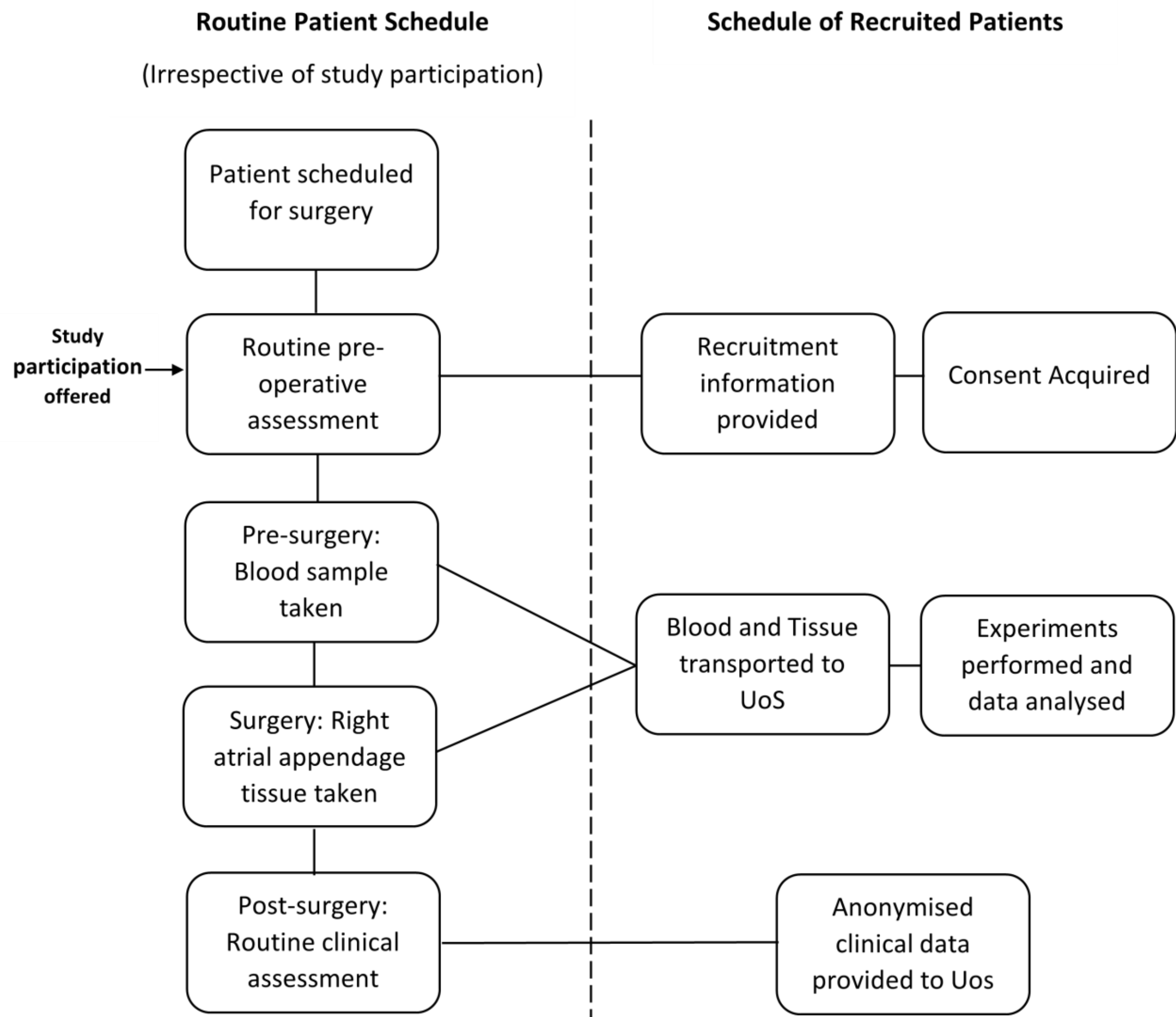


Figure 2-1. Patient schedule.
Flowchart showing routine patient schedule and additional steps involved with patients recruited to the study.

The clinical data was used as a patient CAD prognostic tool. A comparative analysis was performed between the clinical data, relative levels of OS, and concentrations of serum IL-1β and Ox-LDLs. This comparison identified any correlations between vascular dysfunction relating to OS and cardiac dysfunction on a cellular level.

2.2. Human cardiomyocyte isolation by chunk digestion

The study required the isolation of human cardiomyocytes from right atrial appendage tissue using an adapted version of the protocol described in Niels *et al*. (2013) study. The development of the protocol used involved extensive optimisation and the preparation of three solutions: transport, isolation, and experimental (2.2.1). An overview of the apparatus used in the isolation procedure is shown in Figure 2-2.

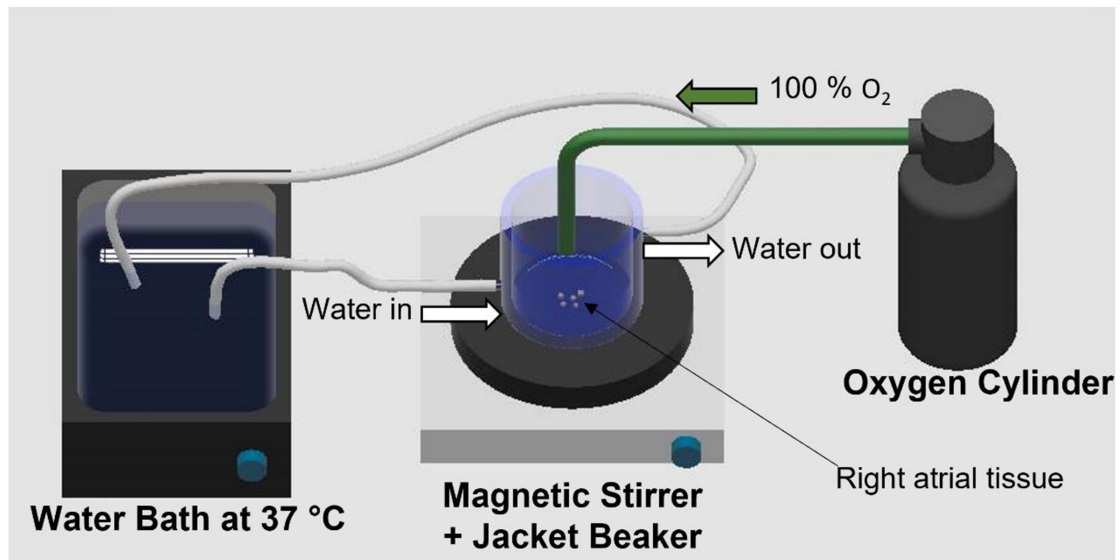


Figure 2-2. A visual summary of isolation apparatus.
The jacketed beaker incorporates an integral jacket in which heated water (37 °C) circulates through from the water bath keeping the sample at a controlled temperature. The jacket beaker is placed on a magnetic stirrer for mixing the sample and solution(s) to aid in complete dissociation. Additionally, an oxygen cylinder supplies 100 % oxygen to the sample.

2.2.1. Solution preparation

The basic composition of the solutions used for cardiomyocyte isolation is generally formulated based on established physiological saline solutions used within cardiac research (Voigt *et al*., 2015). The following section will discuss the importance of the different constituents used in the transport, isolation, and experimental solutions.

Table 2-3. Constituent of the transport solution.

Constituent	Company	CAT No.	Concentration (mM)
BDM	Sigma-Aldrich	B0753	30
$CaCl_2$	Fluka Analytics	21114	0.02
Glucose	Sigma-Aldrich	G8270	20
KCl (1M)	EMSURE	104936	10
KH_2PO_4 (1M)	Sigma-Aldrich	P5655	1.2
$MgSO_4$ (1M)	Honeywell	M7506	5
MOPS	Sigma-Aldrich	M1254	5
NaCl	Sigma-Aldrich	S3014	100
Taurine	Sigma-Aldrich	86329	50
pH	-	-	7.00
Adjusted with	-	-	NaOH

Abbreviations: BDM – 2,3, Butanedione monoxime, KH_2PO_4 - Monobasic potassium phosphate, $MgSO_4$ – Magnesium sulphate, MOPS – 3-Morpholino)-1-sulfonic acid.

Table 2-4. Constituent of the isolation solution

Constituent	Company	CAT No.	Concentration (mM)
$CaCl_2$	Fluka Analytics	21114	0.02
Glucose	Sigma-Aldrich	G8270	20
KCl (1M)	EMSURE	104936	10
KH_2PO_4 (1M)	Sigma-Aldrich	P5655	1.2
$MgSO_4$ (1M)	Honeywell	M7506	5
MOPS	Sigma-Aldrich	M1254	5
NaCl	Sigma-Aldrich	S3014	100
Taurine	Sigma-Aldrich	86329	50
pH	-	-	7.40
Adjusted with	-	-	NaOH

Table 2-5. Constituents of the enzyme buffer 1 and 2 solution

Constituent	Company	CAT No.	Concentration (mM)
Isolation Solution	-	-	-
Collagenase I	Sigma-Aldrich	LS004196	0.9079 mg/ml
Proteinase XXIV	Sigma-Aldrich	P8038	0.357 mg/ml

Table 2-6. Constituents in the experimental solution

Constituent	Company	CAT No.	Concentration (mM)
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A3059	1
$CaCl_2$	Fluka Analytics	21114	0.02
DL-B-Hydroxybutyric acid	Sigma-Aldrich	H6501	10
Glucose	Sigma-Aldrich	G8270	10
KCl (1M)	EMSURE	104936	20
KH_2PO_4 (1M)	Sigma-Aldrich	P5655	10
L-glutamic Acid	Sigma-Aldrich	G1251	70
$MgSO_4$ (1M)	Honeywell	M7506	5
Taurine	Sigma-Aldrich	86329	10
pH			7.40
Adjusted with			KOH

2.2.1.1. Importance of solution components to cell survival

Bovine Serum Albumin

Increased sarcolemma permeability often results in excessive osmotic swelling. Therefore, the addition of BSA helps maintain colloid osmotic pressure, hence preventing cell damage during isolation and the reintroduction of Ca^{2+} (Voigt *et al.*, 2015).

2,3-butanedione monoxime

The extensive influx of Ca^{2+} into the cytosol of cardiomyocytes is a detrimental effect of the calcium paradox discussed further in 4.2.2, resulting in hypercontraction of a cell. 2,3-butanedione monoxime is an important constituent of the transport solution being a reversible inhibitor of cardiomyocyte contracture (Hall & Hausenloy, 2016). As it has the ability to uncouple excitation-contraction producing a cardioprotective effect during transport (Voigt *et al*., 2015). However, BDM does possess some detrimental side effects including the depletion of ATP stores and the alteration of calcium handling and action potential of a cell hence, it is not used within the isolation or experimental solution.

Ion Concentrations

Extracellular concentrations of Na^{+}, K^{+}, and Ca^{2+} ions are important during the isolation process to maintain the physiological functions of the cardiomyocytes. Hence, there is a higher concentration of NaCl used relative to KCl within all the solutions, as it is required for the functionality of Na, K -ATPase (Yasaman *et al*., 2022). However, low levels of Ca^{2+} are used throughout the isolation procedure to assist in diastolic arrest, decrease in myocardial metabolic demand, and cell separation at intercalated discs all of which are important for increasing the quality and quantity of isolated cells (Voigt *et al*., 2015).

Taurine

During the isolation of cardiomyocytes, important cellular compounds are often lost therefore, higher levels of taurine supplemented in the transport and isolation solution are essential. Taurine contributes to the reduced loss of Ca^{2+} from cardiomyocytes and limits intracellular Na^{+} rise when in low Ca^{2+} -containing solutions (Hansen *et al*., 2006). Hence, promotes contractile recovery by having a positive effect on the Ca^{2+} tolerance of isolated cardiomyocytes (Voigt *et al*., 2015).

Glucose

Glucose is a known energy source required by cardiomyocytes for the generation of ATP to maintain contractile function, so is a constituent for enhanced cell survival (Locke, 1895; Shao & Tian, 2015).

Enzyme Digestion

Isolation of single cells requires extracellular matrix (ECM) breakdown, this is attenuated by mechanical (trituration) and chemical methods. However, the use of tissue-dissociating enzymes is the most important factor for cell dissociation. The use of collagenase and protease in combination is generally used to isolate cells from mammalian atria, as suggested by Kono *et al*., in 1969. It is thought that collagenase is necessary to instigate digestion by initial cleavage of the collagen in the ECM, so that collagen fragments are then accessible for hydrolytic digestion by less specific proteases (Barrett *et al*., 2004; Kono, 1969; Voigt *et al*., 2015).

2.2.2. Human tissue source

Patients with CAD that are undergoing revascularisation surgery usually have the tip of the right atrial appendage removed, this tissue is normally subjected to clinical waste. However, Dr David Greensmith's laboratory used this tissue to isolate atrial cardiomyocytes. The blood and tissue samples were placed in a UN3373 bag on frozen gel packs within a polystyrene-lined UN3373 box and transported to the University of Salford with transport times ranging between 50-120 minutes.

2.2.3. Cleaning of the tissue

Any visible fat on the tissue sample was removed using scissors before being weighed and transferred to 0.02 mM Ca2+ -isolation solution

Table 2-4), previously stored at 4 °C. The tissue sample was then cut into smaller chunks approximately 1 mm^3 in size. The tissue chunks along with the 0.02 mM Ca^{2+} -isolation solution was then transferred to the jacketed beaker, under continuous gassing under high purity O_2 (99.99 %, BOC, Manchester, UK) at room temperature and stirred for 3 minutes using a magnetic flee. The resultant supernatant was strained through a 200 µm nylon mesh (BioDesign Inc, USA) and the tissue chunks were returned to 10 ml of 0.02 mM Ca^{2+} -isolation solution for washing a further two times.

2.2.4. First enzymatic digestion

All the following steps were then performed in the jacketed beaker under continuous gassing with 100 % O_2 at 37 °C. The chunks were resuspended in 10 ml of Enzyme Buffer 1, containing 0.9079 mg/ml Collagenase I (Worthington Biochemical Corporation, USA)

and Proteinase XXIV (Sigma Aldrich, UK) see Table 2-5, and stirred for 5 minutes. The resultant supernatant was then filtered through a 200 µm nylon mesh into a 15 ml falcon tube (Tube A) for further analysis, returning any tissue chunks to the jacketed beaker.

2.2.5. Second enzymatic digestion

The tissue chunks were then transferred into 10 ml of Enzyme Buffer 2, containing Collagenase I only (Table 2-5), and stirred for 5 minutes. The resultant supernatant was then filtered through a 200 µm nylon mesh into a new 15 ml falcon tube (Tube B) for further analysis, returning any tissue chunks to the jacketed beaker. The O_2 gas was then switched off to prevent any bubble formation in the following steps. The tissue chunks were resuspended in 5 ml of 0.02 mM Ca^{2+} -experimental solution (Table 2-6). Further dissociation was then performed by mechanical trituration using a Pasteur Pipette with the tip removed to prevent cell damage. This was performed a further two times, each for a 3-minute period with the supernatant being filtered through a 200 µm nylon mesh into one 15 ml falcon tube (Tube C).

2.2.6. Final preparations for experiments

All tubes A, B, and C were centrifuged for 10 minutes at 95 x g and the supernatant was carefully aspirated, ensuring the cell pellet was not disturbed. The cell pellets were then resuspended in an appropriate volume of 0.02 mM Ca^{2+} -experimental solution and pooled together. Calcium concentration was then gradually increased to 0.2 mM Ca^{2+} in a stepwise manner from 0.02 mM to 0.05, 0.1, and finally 0.2 mM with 10-minute incubation periods between each calcium addition. It was important that cells were examined at each calcium concentration, to monitor cell viability and quality.

2.3. Cell quality and viability assessment

Cell quality and viability assessment was carried out after isolation. This was carried out by viewing cells at 40 x magnification on a confocal microscope (Zeiss). Viable, good-quality isolated cardiomyocytes are rod-shaped with clear striations along the whole length of the cell and do not contract or wave without stimulation. Poor quality cardiomyocytes may be of an irregular shape, lack defined striation, are “grainy” in appearance, and often wave uncontrollably. Dead cardiomyocytes undergo contracture appearing as round shapes, (Figure 2-3). Using this knowledge, distinguishing between

live and dead cells, a simple observation of the quality of cells and cell viability was able to be conducted.

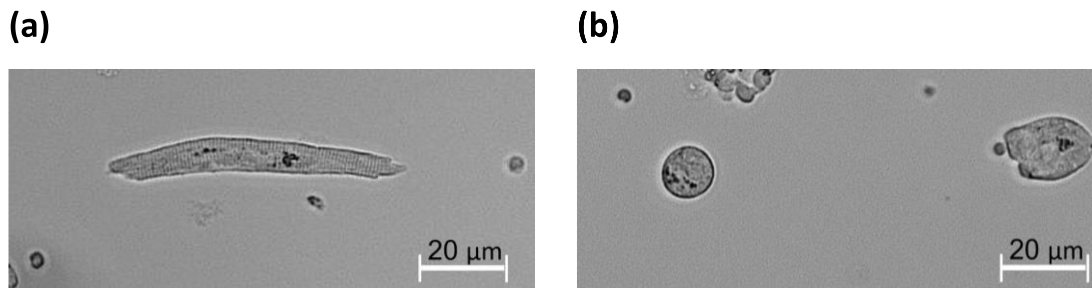


Figure 2-3. Human atrial cardiomyocyte morphology.
(a*) Showing viable atrial cardiomyocyte with clear striations along the whole length of the cell. (b) Showing dead atrial myocyte with circular morphology.*

A simple ranking system was developed (Figure 2-4) to distinguish between good and poor-quality cells based on morphological characteristics. Whereby cardiac myocytes were ranked from +1, being poor quality, to +3 being the best quality cells. Those cells ranked +3 possessed typical atrial cardiac myocyte morphology being short, narrow and rectangular, whilst also having uniform striations along the whole length of the cell demonstrating intact myofilaments and associated proteins within the myofibril. As the ranking number decreased this was associated with the loss of morphological characteristics. This was important in assisting in the assessment of optimisation.

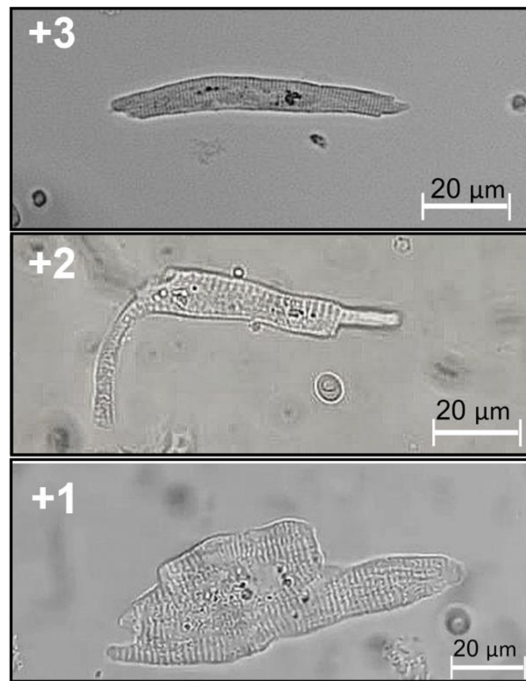


Figure 2-4. Cardiac myocyte quality ranking system.

Ranking +1 striations are not present in whole of cell, membrane not fully defined/intact, and not rectangular in shape/stacked cells. Ranking +2 striations present but not uniform across the cell, membrane intact but not completely rectangular in shape/torn edges. Ranking +3 uniform striations across the whole length of the cell, membrane intact and long rectangular shape

Using this knowledge on cell morphology gave way for simple cell counts to be conducted distinguishing between live/good quality cells and dead/poor quality cells. From this percentage cell viability was able to be calculated using Equation 2-1. This gave us a sense of cell yield after isolation preparations for further downstream analysis.

$$\%\ cell\ viability = \frac{Total\ number\ of\ viable\ cells\ across\ three\ fields\ of\ view}{Total\ number\ of\ cells\ counted\ across\ three\ fields\ of\ view}\ x\ 100$$

Equation 2-1. Percentage cell viability.

2.4. Measurement of intracellular oxidative stress and tolerance in response to hydrogen peroxide (H_2O_2)

2.4.1. The fluorophore

CellROX deep red reagent (Invitrogen, UK) is a cell-permeant dye with an excitation/emission maximum of ~644/665 nm (Figure 2-5) (Technologies, 2012). When excited, fluorescence is proportional to the level of oxidation thus, CellROX reports intracellular oxidative stress.

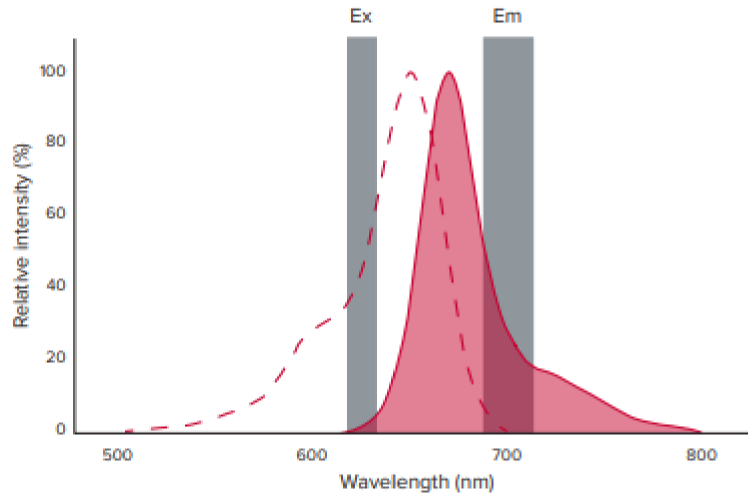


Figure 2-5. The fluorescence response curve of oxidised CellRox Deep Red Reagent. *Excitation (dotted line) and emission (solid line) spectra of the oxidised CellRox (Ha, 2018).*

2.4.2. Hydrogen peroxide

Oxidative stress is implicated in the pathogenesis of various diseases. To model this, ROS hydrogen peroxide (H_2O_2), is frequently added to cells (Greensmith *et al*., 2010). This molecule readily diffuses through cells and tissues initiating immediate cellular effects such as altering cell morphology, proliferation, and cell recruitment (Sies, 2017). The metabolic and regulatory role of H_2O_2 has become increasingly recognised within research. So, the availability and application of this compound have drastically increased within many research laboratories.

2.4.3. Assessment of oxidative stress using the cytation cell imaging platform

Isolated atrial myocytes were separated in equal volumes (300 µl) into three 15 ml centrifuge tubes. The cells were then treated with an equal volume (300 µl) of specific concentrations of H_2O_2 (Honeywell, USA), shown in Figure 2-6. Following treatment, the myocytes were incubated for 30 minutes at room temperature.

After incubation, the cells were hand centrifuged for 3 minutes to allow pellet formation. The cell pellets were then resuspended in 300 µl of 5 µM CellROX deep red dye before incubating for a further 30 minutes at room temperature, in the dark.

Following incubation, the cells were hand centrifuged again for 3 minutes and then resuspended to prevent further CellROX loading. The cell pellets were then resuspended in an appropriate volume of experimental solution (Table 2-6), before plating 100 µl into

the corresponding wells of a 96 well-plate (Figure 2-6). The brightfield and fluorescence of the contents of each well were then immediately measured using the Cytation cell imaging system with the excitation recorded at 644 nm and emission at 665 nm (Biotek, USA). Images under brightfield and the corresponding fluorescence were saved as TIF files for further evaluation. For each patient 10-14 cells were analysed over the range of H_2O_2 used, with a minimum of two cells required for each concentration.

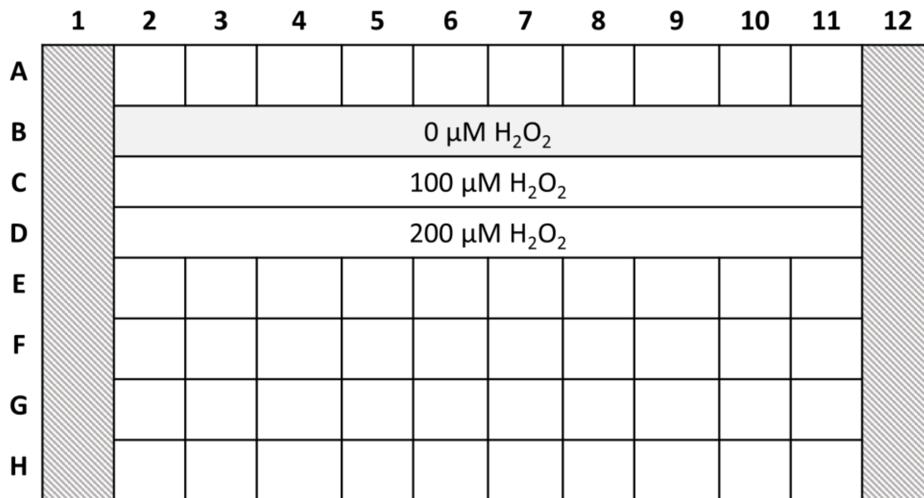


Figure 2-6. 96-Well plate layout for imaging on Cytation.

2.4.4. Calculation of fluorescence as an indicator of oxidative stress and tolerance

The relative fluorescence of each cell analysed was quantified using ImageJ (Schneider *et al*., 2012). The fluorescent image was first overlayed with the brightfield image of the same field of view. From this, the area, mean grey value, and integrated density were taken for each cell and its background by manually drawing around the cell itself and then 3 points around the cell. Background fluorescence is an unwanted and unobserved fluorescent signal often termed noise; thus, it is important to measure this to obtain the true fluorescence of the cell reducing bias. The corrected total cell fluorescence (CTCF) was then able to be calculated using the following formula (Equation 2-2).

$$integrated\ density - (area\ of\ selected\ cell \times mean\ fluorescence\ of\ background\ readings)$$

Equation 2-2. Corrected total cell fluorescence equation.

2.5. Serum isolation

Blood was transferred from EDTA tubes into a 15 ml falcon tube and centrifuged at 2000 x g for 5 minutes to split whole blood into serum (top), PBMC (buffy coat) and red blood

cells (RBC – bottom). The serum was then removed and aliquoted into cryovial tubes labelled with the corresponding patient number and stored at -80 °C. All volumes of whole blood, serum, RBCs, and aliquots were recorded appropriately for each patient.

2.6. Quantification of cytokine levels using Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA immunoassay has been at the forefront of protein research for over 50 years producing high sensitivity, qualitative and quantitative results (Tighe *et al*., 2015). Generally, ELISA immunoassays are based on the principle of specific antigen-antibody interactions which are measured via colorimetric or fluorescent imaging.

Multiple variations of the ELISA technique have been established since it's development in 1971 (Bidwell *et al*., 1976). These differences are usually associated with the type of reporter molecule, or when the antibody is added to the sample before or after primary antibody addition (Jones, 2017).

All ELISA share fundamental principles. The first step involves the immobilisation of the antigen of interest in the sample to the surface of a coated microtiter plate (BioRad). This is accomplished by direct absorption to the plate's surface or using a "capture antibody" that is specific to the target antigen. The latter is typically used for sandwich ELISA's. Following immobilization, a detection antibody is added that binds to the absorbed antigen to form the antigen-antibody complex. This detection antibody is either conjugated directly with an enzyme such as horseradish peroxidase (HRP) or provides a binding site for a labelled secondary antibody. The enzyme labels produce a visible colour change when provoked by a chromogenic substrate addition or the appropriate wavelength of light to stimulate fluorescence. The amount of colour change correlates to the amount of target antigen in the sample, allowing qualitative and quantitative measurements to be conducted (Gan & Patel, 2013).

2.7. Serum quantification of Interleukin-1β using ELISA

This interleukin-1β high sensitivity ELISA kit (ThermoFisher Scientific, UK) had a detection range of 0.16-10 pg/ml, with an analytical sensitivity of 0.05 pg/ml. All

reagents were brought to room temperature and diluted correctly before use (Appendix E).

2.7.1. Standard curve preparation

To allow quantitative assessment of IL-1β, a standard curve was prepared in advance. The standards were prepared by reconstituting a vial of lyophilised human interleukin-1β to obtain a concentration of 500 pg/ml. After equilibration, this was further diluted to a 20 pg/ml stock which was then used for serial dilution to standard concentrations as per (Table 2-7).

Table 2-7. IL-1B standard concentration serial dilutions.
Showing a 1:2 dilution conducted for each standard from 20 pg/ml stock to 0.16 pg/ml.

Tube Label	Standard Dilution	Resultant Concentration (pg/ml)
Stock Vial	1:25	20.00
1 (Top Standard)	1:2	10.00
2	1:4	5.00
3	1:8	2.50
4	1:16	1.25
5	1:32	0.63
6	1:64	0.31
7	1:128	0.16
8 (Negative Control)	No Dilution	0

Each well was washed twice with 400 µl of 1X wash buffer prior to the experiment.

2.7.2. Serum sample preparation

Patient serum samples were taken from the -80 °C freezer and allowed to defrost at room temperature. They were then vortexed and 100 µl added in duplicate to the microwell plate.

2.7.3. Interleukin-1β ELISA experimental protocol

A flow diagram summarising the key steps in the ELISA protocol is shown in Figure 2-7. Following the addition of the standard, sample, and control, 50 µl of 1X biotin-conjugate

monoclonal antibody was added to each well and *first incubated* at room temperature in the dark overnight (~15 hours). The subsequent incubation steps were performed in the dark on a microplate shaker. Each well was washed 6 times before the addition of 100 µl 1x streptavidin-Horseradish peroxidase (HRP) for the *second incubation* of 1 hour. The wells were then washed again a further 6 times, before the *third incubation* with 100 µl 1X amplification solution I for 15 minutes. The wells were washed again 6 times before 100 µl 1X amplification solution II was added to each well and the *fourth incubation* occurred for 30 minutes. The wells were then washed 6 times before the addition of 100 µl of tetramethyl-benzidine (TMB) for the *fifth incubation* at room temperature in the dark. The enzyme reaction was then stopped after 15 minutes by adding 100 µl 1M Phosphoric acid (*stop reaction*) to each well.

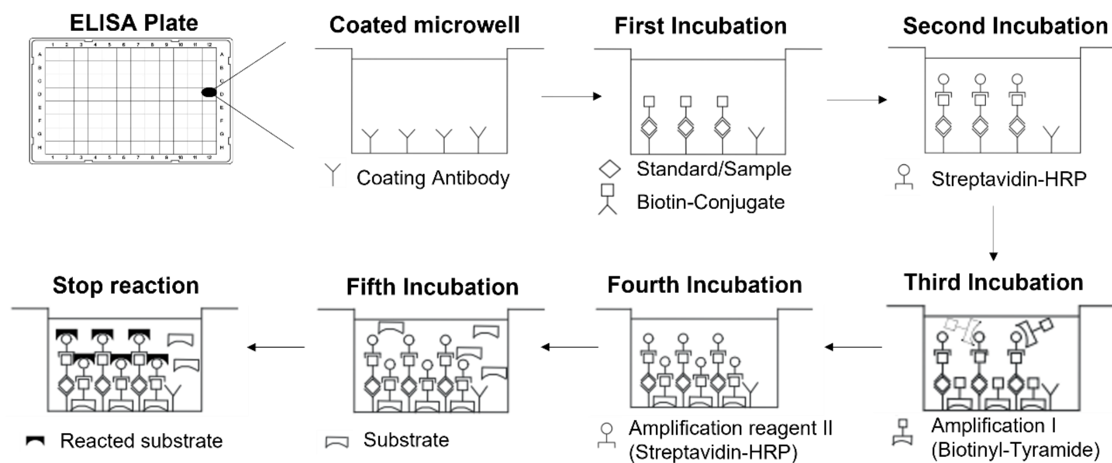


Figure 2-7. ELISA flow diagram.

2.8. Serum quantification of Ox-LDL using ELISA

The Ox-LDL ELISA kit (abcam, UK) has a detection range of 3.125-200 ng/ml, with an analytical sensitivity of 1.875 ng/ml. All reagents were brought to room temperature and diluted correctly before use (Appendix F).

2.8.1. Standard curve preparation

To allow quantitative assessment of Ox-LDL, a standard curve was prepared in advance. The standards were prepared by reconstituting a vial of lyophilised human Ox-LDL to obtain a concentration of 200 ng/ml, the top standard. After equilibration, this was used for serial dilution to standard concentrations as per Table 2-8.

Table 2-8. Ox-LDL standard concentration serial dilutions.
Showing a 1:2 dilution conducted for each standard from 200 to 3.125 ng/ml.

Tube Label	Standard Dilution	Resultant Concentration (pg/ml)
1 (Top Standard)	1 ml	200.00
2	1:2	100.00
3	1:4	50.00
4	1:8	25.00
5	1:16	12.50
6	1:32	6.25
7	1:64	3.125
8 (Negative Control)	No Dilution	0

Each well was washed twice with 350 µl of 1x wash buffer prior to the experiment.

2.8.2. Ox-LDL ELISA experimental protocol

Patient samples were prepared according to section 2.7.2 and followed a similar protocol to Figure 2-7 excluding third and fourth incubation with amplification reagents. All preceding incubation steps were at 37 °C in the dark. After standard, sample, and control addition the plate was incubated for 90 minutes. The contents of the wells were removed without washing before 100 µl of 1x biotin-detection antibody was added. The plate was then incubated for a further 60 minutes. The plate was washed 3 times before 100 µl 1x HRP-Streptavidin conjugate was added and incubated for 30 minutes. The plate was washed a further 5 times and 90 µl of TMB was added to each well. After 15 minutes of incubation, 50 µl of the stop solution was added to each well to stop the enzyme reaction.

2.9. Cytation Imaging and quantification of IL-1β and Ox-LDL

Each microwell was immediately measured on a FLUOSTAR omega plate reader at 450 nm as the primary wavelength, and 620 nm as the reference wavelength. The average absorbance values for each set of duplicate standards and samples were calculated and normalised to the negative control. Using the standards corrected absorbance values a standard curve was plotted for both interleukin-1β (Figure 2-8a) and Ox-LDLs (Figure

2-8b) allowing the extrapolation of biomarker concentrations in the unknown patient serum samples.

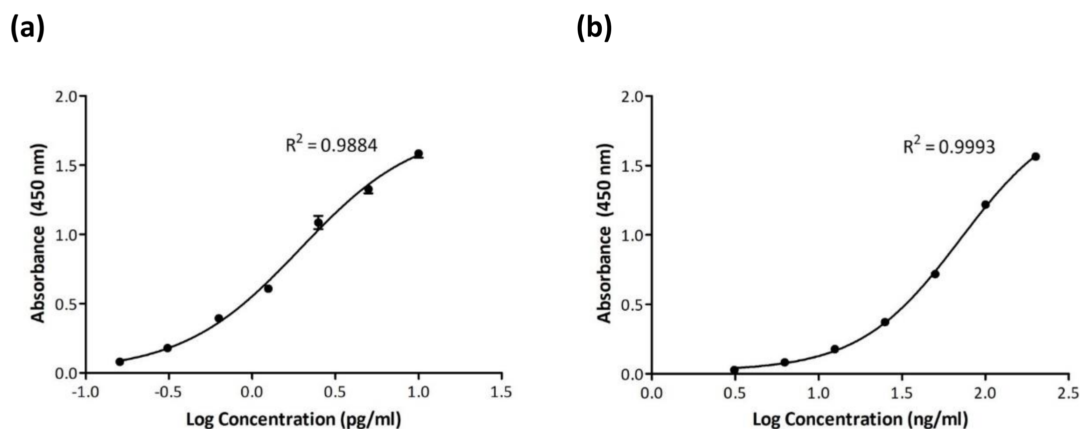


Figure 2-8. ELISA standard curves.
(a*) IL-1β standard curve ranging from 0.16-10 pg/ml. (b) Ox-LDL standard curve ranging from 3.125-200 ng/ml. Data expressed as mean ± SEM (n = 4).*

Before analysis of ROS fluorescence in cardiac myocytes, cells were analysed on ImageJ (2.4.4) and normalised to the control, untreated cell, from the same patient.

2.10. Statistical analysis and data management

Data were analysed using GraphPad Prism 5 (GraphPad Software, Inc, USA.) Statistical significance was accepted when p value < 0.05. Normal distribution was not assumed, hence non-parametric tests were conducted on data.

Electronic copies of clinical data were sent to the University of Salford where analysis was conducted on Microsoft excel. This entailed highlighting appropriate data, organising, and converting information into an accessible format for further analysis.

Analyses of cell viability was performed using two-tailed unpaired T-tests to determine the effects of isolation parameters (temperature and calcium).

Comparisons of average fluorescence of cardiac myocytes when treated with 100 µM H_2O_2 and 200µM H_2O_2 were evaluated using two-tailed unpaired T-tests to compare response (fluorescence) to the compound (H_2O_2). In order to compare directly between patients' responses to H_2O_2 a two-way analysis of variance (ANOVA) was conducted. This allowed the estimation of how the quantitative variable, fluorescence, changes in response to two independent variables, patient, and H_2O_2 concentration.

Correlations between serum biomarker levels and clinical data were conducted using the Pearson R statistical test to measure the linear correlation between the two variables.

3. Results

3.1. Optimisation of cardiac myocyte isolation

Voigt's *et al*. (2013) protocol described in section 2.2 was optimised by adjusting several variables. The outcome of this procedure was evaluated using cell quantity and quality assessment defined in section 2.3.

3.1.1. Digestion time

Initial preparations used the digest times reported by Voigt *et al.* (2013) but resulted in considerable over digestion as demonstrated by a reduced quality ranking (Figure 2-4). Consequently, digestion time was reduced from 10 minutes to 5 minutes. This change was visually assessed using the protocol described in section 2.3.

When digestion time was reduced to 5 minutes a higher proportion of isolated cells were visually ranked as +3 (Figure 2-4), having a uniform striated appearance across the whole length of cell.

3.1.2. Mechanical trituration

Niels Voigt *et al*. (2015) states that trituration is crucial for ECM breakdown for cell separation, for acceptable cell yields. Despite this, trituration can have adverse impacts on cell morphology. Thus, cardiac myocyte morphology and yield were visually analysed (Figure 3-1) before trituration which normally occurs after the second digest with enzyme buffer II (2.2.5).

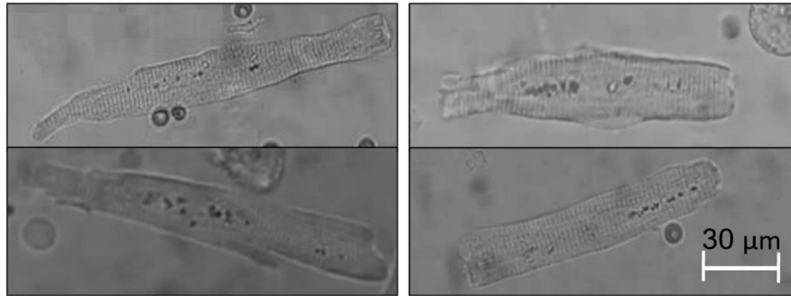


Figure 3-1. Before trituration Vs after trituration cell morphology.
Before trituration (left) cells are longer and thinner whilst after trituration (right) morphology is shorter and wider.

The pre-trituration yield was reduced highlighting the importance of the step. However, post-trituration cardiac myocytes tended to be shorter in length, wider in diameter and in some cases having torn edges (Figure 3-1). This was visually confirmed by randomly selecting live cells from before trituration and after trituration. The following observations were made, before trituration 70 % of the live cells were longer in length whilst after trituration 85 % of the live cell population were shorter in length. As a result, cells were also collected and pooled from the 1st and 2nd digest, to increase cell yield and have a more varied cell pool.

3.1.3. Temperature

The narrow optimum temperature range of the enzymes collagenase and protease limited adjustments to temperature during the digestion phase of the isolation procedure. However, the initial washing step described in section 2.2.3 does not use solutions that contain such enzymes. Thus, the effect of reducing the temperature of this step was examined.

Following temperature reduction cell viability increased from 19.1 ± 3.21 % *(n = 19)* to 21.7 ± 3.71 % *(n = 3)* (Figure 3-2). Although this is not significant Niels Voigt *et al*. (2015) indicate the reduced temperature is important for cardiac myocyte protection from excessive calcium influx during reintroduction. Thus, the initial washing step was continued at room temperature.

3.1.4. Calcium concentration

Calcium presents a paradox for cell isolation. While normal cell function requires relatively high extracellular Ca^{2+} levels, such levels are not suitable for digestion then long-term transport, and storage. As such, the effect of altering the Ca^{2+} concentration in the storage solution was tested (Figure 3-2).

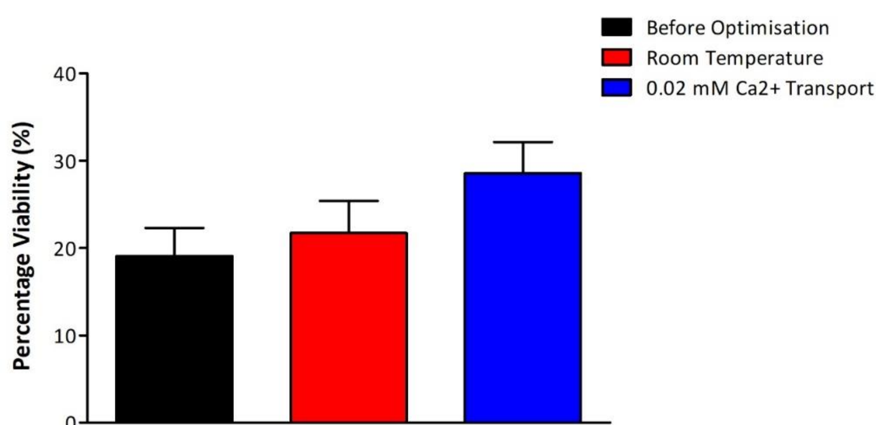


Figure 3-2. Percentage viability in response to changing variables.
Percentage increase in cell viability was visually observed when the washing step temperature was reduced to room temperature (red) and this further increased when 0.02 mM Ca^{2+} was added to transport solution (blue) which previously contained no Ca^{2+}. Each change was conducted for a minimum of three isolations before the next parameter was changed in in a stepwise manner. Data presented as mean ± SEM.

As the transport solution was modified from no calcium content to containing a low content of 0.02 mM, a further increase in average cell viability to 28.6 ± 3.57 % *(n = 3)* was observed.

By monitoring cell quality and quantity in response to specific variables, modifications to the original Niels *et al*. (2013) protocol were made to enhance further downstream analysis and continuation of the project.

3.2. Validation of technique for measuring intracellular oxidative stress

3.2.1. Validation using sheep ventricular myocytes

Sheep cells were selected for initial validation in order to reserve human cells for other experiments. The fundamental protocol for measuring oxidative stress by cytation (as described in 2.4.3) was established by a previous research student. Here, the sensitivity

of the technique was tested by exposing sheep ventricular myocytes to increasing hydrogen peroxide while measuring the resulting fluorescence (Figure 3-3).

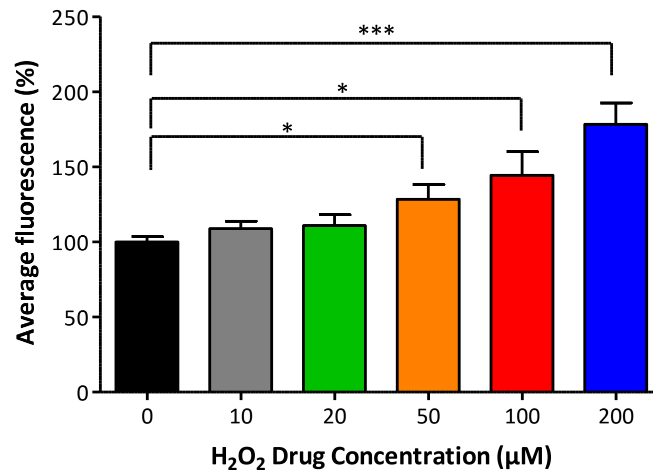


Figure 3-3. Response to hydrogen peroxide challenge in sheep ventricular cardiomyocytes.

Sheep ventricular cells were treated with varying H_2O_2 concentrations. The concentrations of H_2O_2 of 50 µM+ resulted in a significant increase in fluorescence in comparison to the control (0 µM). Data expressed as mean ± SEM.

Hydrogen peroxide produced a concentration-dependant increase in fluorescence that reached significance at 50 µM *(an increase of 28 ± 9 %, n = 10, $p < 0.05$)*, 100 µM *(44 ± 16 %, n = 10, $p < 0.05$)* and 200 µM *(78 ± 14 %, n = 11, $p < 0.001$)*.

3.2.2. Validation using human atrial myocytes

Following successful validation in sheep, we confirmed similar sensitivity to increased levels of oxidative stress using human cells (Figure 3-4).

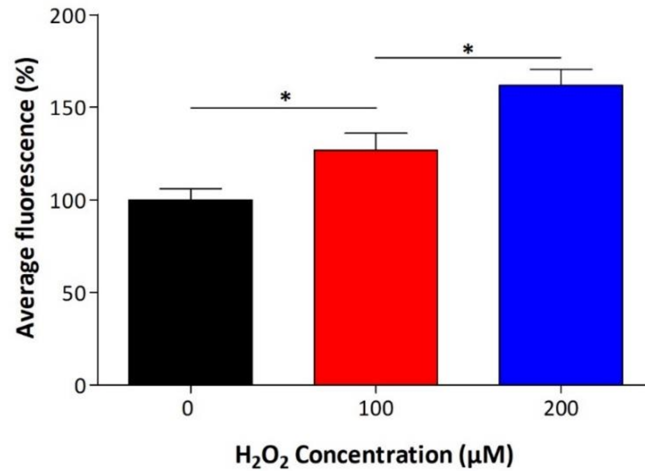


Figure 3-4. Reactive oxygen species fluorescence in response to hydrogen peroxide for all patients grouped.

*The effects of H_2O_2 on isolated all human cardiac myocytes on ROS fluorescence when treated for 30 minutes. * Indicates statistically significant differences between control and 100 µM H_2O_2, in addition to 100 µM and 200 µM H_2O_2 at $p < 0.05$. Data expressed as mean ± SEM.*

Hydrogen peroxide produced a concentration-dependant increase in fluorescence. 100 µM H_2O_2 increased fluorescence by 27 ± 9 % *($n = 13$, $p < 0.05$)*, 200 µM H_2O_2 increased fluorescence by 62 ± 9 % *($n = 8$, $p < 0.05$)*.

3.3. Patient tolerance to oxidative stress

A hydrogen peroxide challenge was used to determine the relative sensitivity to oxidative stress from three patients (Figure 3-5).

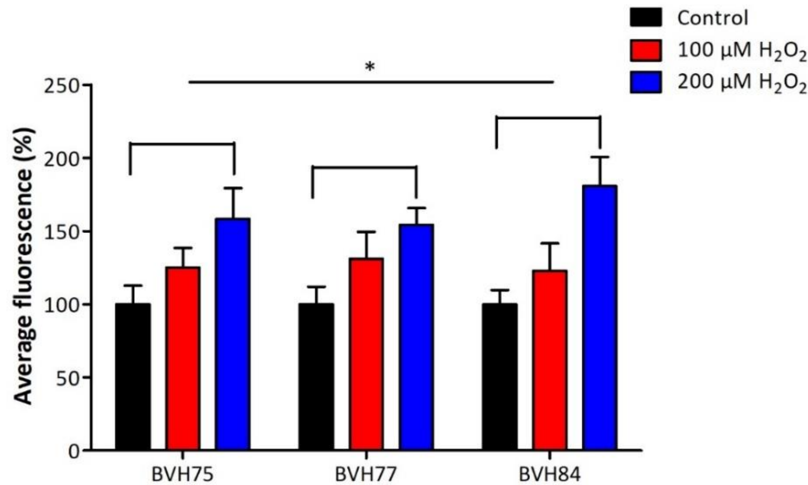


Figure 3-5. Patient-to-Patient average fluorescence in response to hydrogen peroxide.

*The effects of H_2O_2 on ROS fluorescence in isolated human cardiac myocytes from three patients when treated for 30 minutes. BVH(x) indicates the patient ID number. * indicates a statistically significant difference in fluorescence between patients at p <0.05. Data expressed as mean ± SEM.*

This *preliminary* study showed a significant difference in fluorescence displayed between the patients when treated with 200 µM H_2O_2 *(n = 3, p <0.001).* Patient BVH75 displayed a 25 ± 13 % *(n = 4, p >0.05)* increase in fluorescence when treated with 100 µM H_2O_2 and a 58 ± 21 % *(n = 2)* at 200 µM H_2O_2 when compared to control independently. Patient BVH76 exhibited a 31 ± 18 % *(n = 5, , p >0.05)* increase in fluorescence at 100 µM H_2O_2 and 54 ± 11 % *(n = 4, , p >0.05)* increase at 200 µM H_2O_2 compared to control. At 100 µM H_2O_2 patient BVH84 displayed a 23 ± 19 % *(n = 4, p >0.05)* and an 80 ± 20 % *(n = 2)* increase at 200 µM H_2O_2 relative to control.

3.4. Quantification of mean serum IL-1β and Ox-LDL levels

3.4.1. Absolute levels

Using sandwich enzyme-linked immunosorbent assays (2.6) standard curves were developed (Figure 2-8) to calculate the concentration of IL-1β and Ox-LDL levels in patient serum samples.

Individual patient data for IL-1β displayed in (Appendix G) and Ox-LDL in (Appendix H) were then used for the correlations presented below in sections 3.6 and 3.7.

The mean IL-1β at 1.46 ± 0.26 pg/ml *(n = 31)* while that of Ox-LDL was 32.76 ± 6.15 ng/ml *(n = 37)*.

3.4.2. *Correlation of IL-1β and Ox-LDL*

The concentration of IL-1β was correlated to Ox-LDL levels in patient serum samples. This was conducted to determine if there was a correlation between inflammatory pathways involving IL-1β and oxidative stress relating to vascular dysfunction in CAD (Figure 3-6).

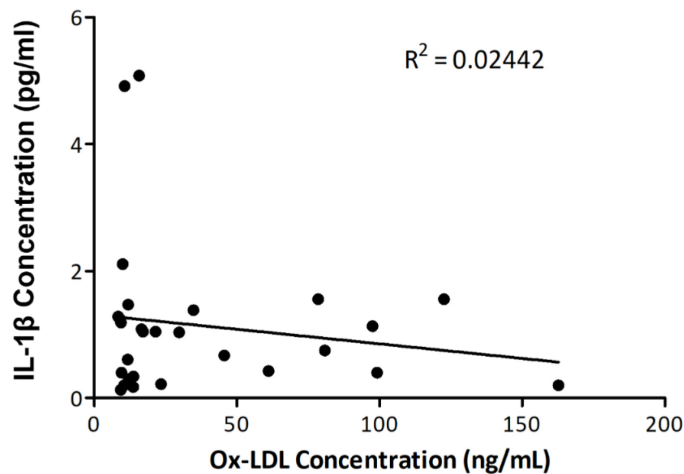


Figure 3-6. IL-1β Vs Ox-LDL concentration in patient serum samples.

As shown in Figure 3-6 there was no correlation between IL-1β and Ox-LDL concentration in patient serum samples tested *($n = 28$, $R^2 = 0.02442$, $p > 0.05$)*.

3.5. Patient demographics

Patient's clinical data from preoperative assessment and ECHO was provided for each patient demonstrated in Appendix I, with normal reference values in Appendix J. Indices of cardiac function that were of particular interest related to either systolic or diastolic function are displayed in Table 3-1.

Systolic function was analysed using ejection fraction (EF), referring to the percentage of blood ejected from the myocardium during systole and tricuspid annular plane systolic excursion (TAPSE) measuring the longitudinal contraction of the tricuspid base toward the apex during systole. Diastolic function was monitored using mitral valve

peak E wave velocity representing early diastolic filling and the E/A ratio, a ratio of early (E) to late (A) ventricular filling.

Table 3-1. Indices of cardiac function in patient cohort analysed.
The table displays mean values, standard error of the mean, and the number of patients analysed.

Measurement	Mean	SEM	n
EF (%)	46.04	2.82	21
TAPSE (cm)	2.13	0.079	15
Peak E-wave velocity (m/s)	0.71	0.048	20
E/A Ratio	0.908	0.087	20

3.6. Correlation of Interleukin-1β with indices of cardiac function

Patient IL-1β levels (see Appendix G) were correlated with indices of cardiac function. The indices of systolic function (Figure 3-7) and diastolic function (Figure 3-8) were correlated to inflammatory mechanisms using serum IL-1β on a patient-to-patient basis.

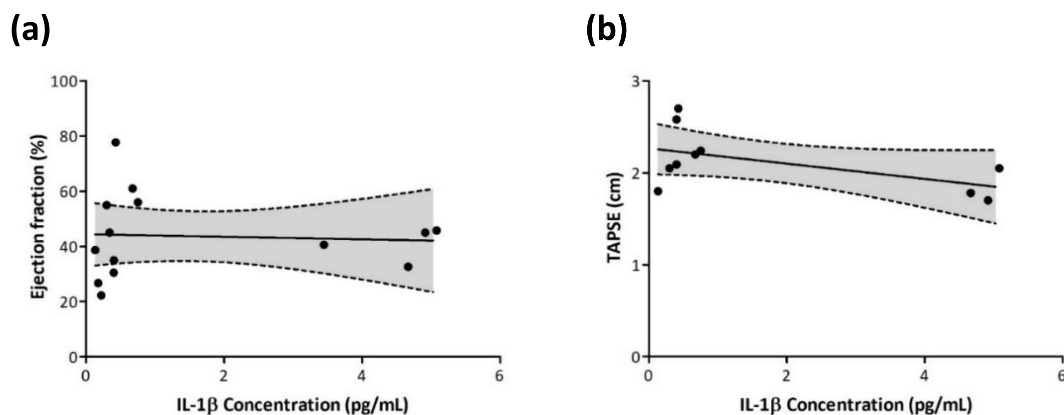


Figure 3-7. Systolic function Vs IL-1β concentration.
(a) Comparison of patient ejection fraction and IL-1β concentration. (b) Comparison of patient tricuspid annular plane systolic excursion and IL-1β concentration.

Early indications from this data suggests that EF does not correlate with IL-1β levels *(n = 14,* $R^2 = 0.003736$*,* $p > 0.05$*)*. *Preliminary* results suggest that TAPSE does negatively correlate with IL-1β, though this did not reach significance $R^2 = 0.2983$, $p > 0.05$. These data are likely underpowered (*n = 10*)

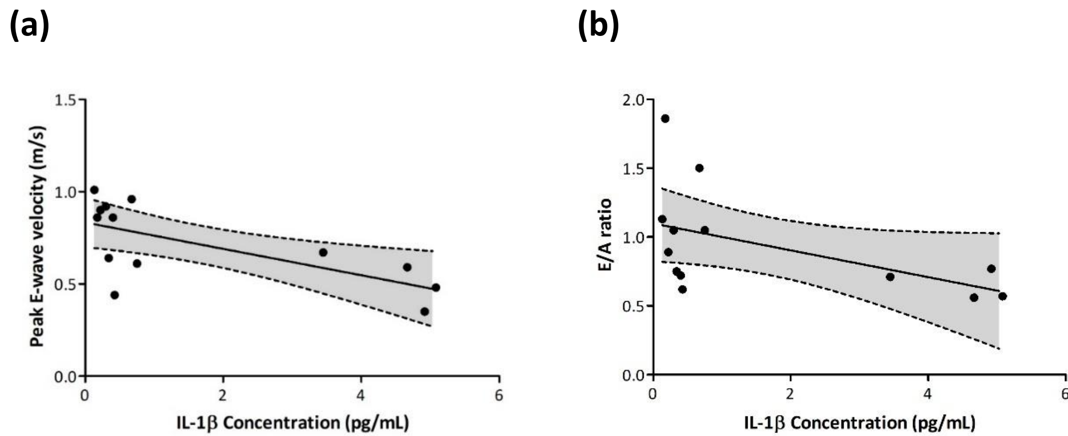


Figure 3-8. Diastolic function Vs IL-1β concentration.
(a) Comparison of patient peak E wave velocity and IL-1β concentration. (b) Comparison of patient E/A and IL-1β concentration.

Peak E wave velocity negatively correlated with IL-1β concentration *(n = 13, R^2 = 0.4506, p = 0.0120)*. *Early indications* also revealed a negative correlation with E/A ratio *(n = 13, R^2 = 0.2658)*, though *p >0.05*.

3.7. Correlation of Ox-LDL with indices of cardiac function

Patient Ox-LDL levels (Appendix H) were correlated with indices of cardiac function. The indices of systolic function (Figure 3-9) and diastolic function (Figure 3-10) were correlated to oxidative pathways using serum Ox-LDL on a patient-to-patient basis.

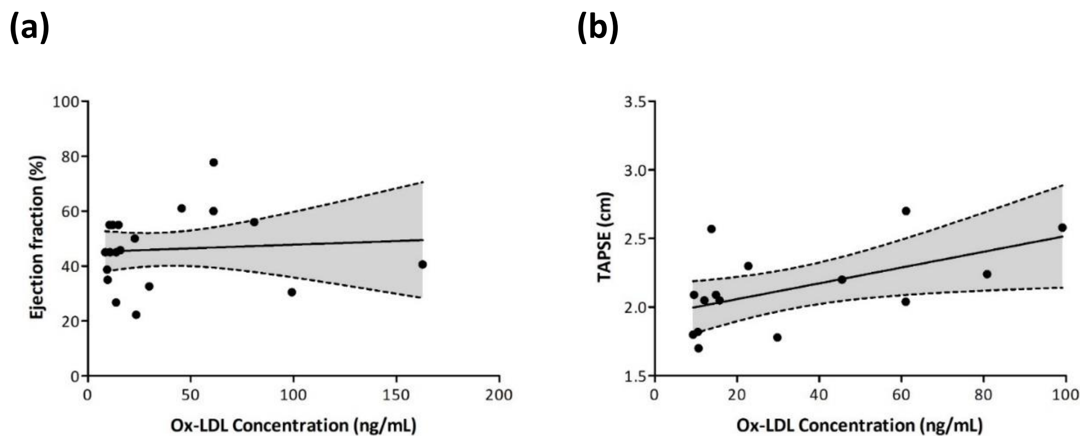


Figure 3-9. Systolic function Vs Ox-LDL concentration.
(a) Comparison of patient ejection fraction and Ox-LDL concentration. (b) Comparison of patient tricuspid annular plane systolic excursion and Ox-LDL concentration.

Ejection Fraction did not correlate with Ox-LDL concentration *(n = 21, R^2 = 0.006407, p >0.05)*. Interestingly, TAPSE showed a positive correlation with Ox-LDL levels *(n = 15, R^2 = 0.3067, p = 0.0322).*

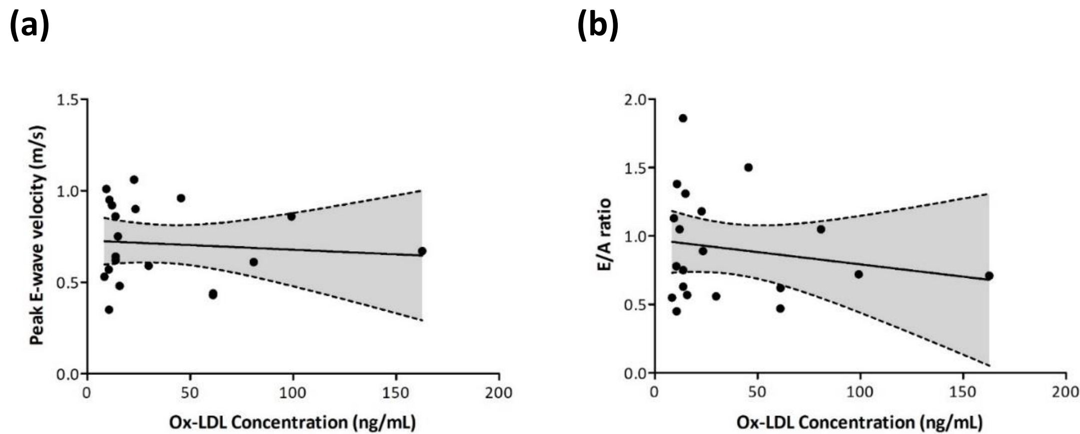


Figure 3-10. Diastolic function Vs Ox-LDL concentration.

(a) Comparison of patient peak E wave velocity and Ox-LDL concentration. (b) Comparison of patient E/A ratio and Ox-LDL concentration.

Neither peak E wave velocity *(n = 20, R^2 = 0.008640, p >0.05)* nor E/A ratio *(n = 20, R^2 = 0.03407, p >0.05)* correlated with Ox-LDL levels.

3.8. Oxidative stress response as a predictor of CAD severity

Preliminary oxidative stress response in patient samples was compared to biomarker levels to assess any links to CAD pathology.

3.8.1. Does tolerance of oxidative stress correlate to serum biomarkers

Correlations between average ROS fluorescence and corresponding IL-1β levels (Figure 3-11) or Ox-LDL (Figure 3-12) in the three patients discussed in section 3.3 was conducted.

The patient serum IL-1β were as follows BVH75 1.28 ± 0.003 *(n = 4)*, BVH77 1.24 ± 0.008 *(n = 4)* and BVH84 1.56 ± 0.003 *(n = 4)*. The average fluorescence for each patient when treated with 100 µM H_2O_2 and 200 µM H_2O_2 is shown in Figure 3-5.

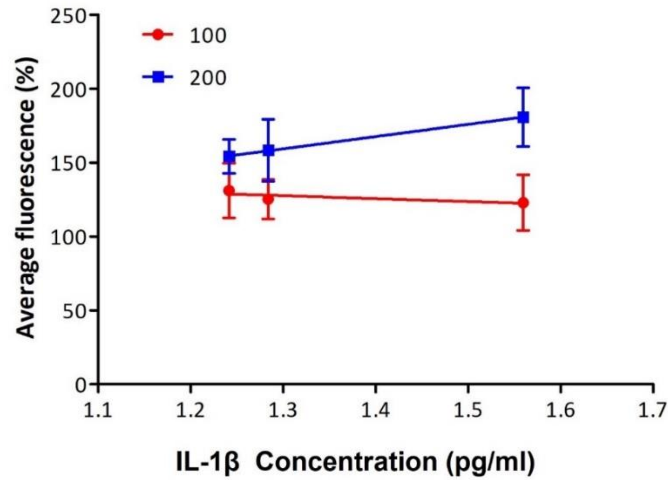


Figure 3-11. Comparison of average ROS fluorescence in response to H_2O_2 and IL-1β serum concentration.

Average fluorescence increases relative to control for patient BVH75, 77, and 84 when treated with 100 µM H_2O_2 (red) and 200 µM H_2O_2 (blue) compared to IL-1β serum concentration. Data expressed as mean ± SEM (n =3).

This *preliminary* study showed there was no correlation between tolerance of OS when treated with 100 µM H_2O_2 and IL-1β concentration *(n = 3, R^2 = 0.6927, p >0.05)*. Although when treated with 200 µM H_2O_2 levels of IL-1β levels positively correlated *(n = 3, R^2 = 0.9995, p = 0.0139)*.

The average patient Ox-LDL levels were 8.41 ± 0.005 *(n = 4)* for BVH75, 9.05 ± 0.003 *(n = 4)* for BVH77 and 78.49 ± 0.095 *(n = 4)* for BVH84.

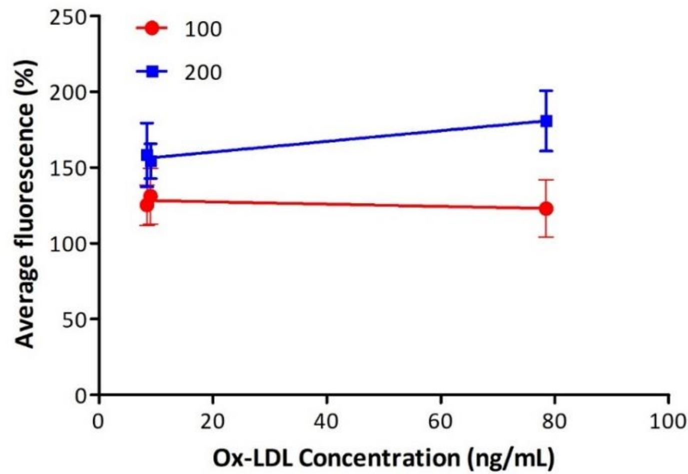


Figure 3-12. Comparison of average ROS fluorescence in response to H_2O_2 and Ox-LDL serum concentration.

Average fluorescence increases relative to control for patient BVH75, 77, and 84 when treated with 100 µM H_2O_2 (red) and 200 µM H_2O_2 (blue) compared to Ox-LDL serum concentration. Data expressed as mean ± SEM (n =3).

No correlation observed between Ox-LDL levels and OS tolerance when treated with 100 µM H_2O_2 *($n = 3$, $R^2 = 0.5007$, $p > 0.05$)* or 200 µM H_2O_2 *($n = 3$, $R^2 = 0.9770$, $p > 0.05$)*.

4. Discussion

4.1. The need for human cardiac tissue within cardiovascular research

Cardiovascular disease remains a major contributor to global morbidity and mortality. Although the link between risk factors of CAD is well established our understanding of the exact cellular and molecular mechanisms underpinning cardiovascular pathology is minimum (Sobey *et al*., 2022). Therefore, current treatment focuses on the management of symptoms rather than the disease itself (Sobey *et al*., 2022). Although much of our understanding of CVD pathology is attributable to research conducted in animal models, they may not be able to fully recapitulate human disease states (Huang & Janssen, 2022). As species differences have long been assumed to be a leading cause for the failure of translation between animals and humans. Thus, the need for human-based models has become increasingly important in cardiovascular research to bridge the knowledge gap.

Cardiovascular research aims to improve the health and understanding of human disease. However important mechanisms, physiology, and physiological changes of the myocardium differ between human and animal models in both disease and non-disease states including the contraction-relaxation cycle, a distinct difference in ion channels, and the presence of transverse tubules in the atria of some large animals (D'Souza *et al*., 2014; Gussak *et al*., 2000; Richards *et al*., 2011). As the immense emphasis on translational research raises, the use of human tissue is becoming more common. As such the Heart Tissue Bank set up in the Netherlands collects and stores biomaterial and clinical data from individuals with and without cardiac diseases in order to boost a wide range of cardiac disease-related fundamental and translational studies (Henkens *et al*., 2022).

Although the use of such models comes with difficulties including acquisition time, tissue protocol optimisation, and the struggle to acquire and define a “control” sample (Huang & Janssen, 2022). Furthermore, it is important to understand that research is the secondary goal in human studies as the individuals’ health and treatment for the disease are paramount, thus there are many confounding variables that researchers cannot control for (Huang & Janssen, 2022). Limitations for human models are discussed further

in sections 4.2 and 4.8.3. As the human-model component of this study includes patients with variations in disease state, BMI, age, and treatment all these variables have to be taken into consideration when drawing direct conclusions from results. These issues highlight the crucial need for both animal and human models to fully understand CVD pathology.

4.2. What variables determine the quantity and quality of isolated cardiac myocytes?

The isolation of viable human atrial cardiac myocytes from right atrial specimens routinely obtained during surgery allows the direct investigation of CAD on atrial myocytes. Although atrial myocytes can vary considerably between patients and in response to external variables, therefore it is important that the isolation procedure is optimised and remains perpetual (Voigt *et al*., 2015). Since the first cardiac myocyte isolation protocol was described in 1955, a number of protocols have since been established to isolate atrial and ventricular cardiac myocytes from a variety of species including mice, rats, rabbits, sheep, dogs, and humans (Voigt *et al*., 2013). Utilising the literature available several factors affecting cardiac myocyte yield and quality have been identified, influencing the optimisation steps in this study. A thorough analysis of available literature revealed several variables that may affect cell yield and quality including enzyme digest, temperature, pH, and ion content.

Important to note is the effect of sample size on the number of isolated cardiac myocytes measured. The average size of right atrial appendage tissue (after removing the fat) used for this project was 0.24 ± 0.022 g *(n = 18)*. In Voigt *et al*., (2013) study samples between 0.2 – 0.6 g were used for isolation, thus the samples received at the UoS were at the lower end of the scale. Furthermore, the resultant cell pellet was split in two for separate students and experimentation, reducing the quantity of isolated cells for oxidative stress cytation. This typically yielded a cell count of ≈ 10 good quality isolated cells per half a pellet. Thus, any samples < 0.1 g were not processed, as the the cell yield would have been too small. In combination with the factors discussed in section 4.8.3 the number of isolated cardiac myocytes measured were expected to be fewer than other studies.

4.2.1. Variables to consider for enzymatic digestion

The goal of the optimisation steps was initially to isolate an increased yield of rod-shaped, striated, and non-over-digested cardiac myocytes. The most important step for cell dissociation, hence the resultant single cardiac myocytes, was enzyme digestion. The time, pH, and temperature of the conduction of this step were important for complete digestion.

It is well established that 37 °C is the optimum temperature for enzyme activity without denaturation (Voigt *et al*., 2015). Although a method isolating human atrial myocytes at 30 °C has been reported, the majority of laboratories perform enzyme digestion between 35-37 °C (Van Wagoner *et al*., 1997; Voigt *et al*., 2015). Similarly, every enzyme has an optimum pH, and outside the optimum changes in the bonding pattern occur altering the shape of the active site (Bisswanger, 2014). Collagen I is a highly purified enzyme having an optimum pH range between 6.3-8.8 and has limited use when used alone, instead in conjunction with proteases (XXIV) which has an optimum pH of 7.5 (Bosch *et al*., 1999; Kono, 1969). Thus, it was important that temperature and pH were tightly monitored to maintain a steady rate of reaction for maximum cell yield.

Collagen is important to initiate digestion by cracking the collagen structure allowing the breakdown of collagen fragments by protease (Voigt *et al*., 2015). The protease enzyme was less specific so the breakdown of other ECM macromolecules such as glycoproteins and proteoglycans can be targeted in addition to important proteins such as myocyte ion channels (McCarthy *et al*., 2011; Rajamani *et al*., 2006). This non-specificity can be detrimental to myocyte quality; thus, the decision to reduce the 1st enzyme digestion time to five minutes was determined.

4.2.2. Variables to consider for reducing the calcium paradox

The second goal for the optimisation process was to increase cell viability, thus reducing the number of “dead” and “waving” cells. The term waving refers to myocytes that are in a state of hyper-contracture. This was first observed by Zimmerman *et al.* in 1960 describing significant lysis of cardiac myocytes after the reintroduction of physiological levels of calcium in isolated rat myocardium, terming the event “calcium paradox” (Zimmerman *et al*., 1967; Zimmerman & Hülsmann, 1966). Unexpectedly the cell

membranes of the cardiac myocytes were also altered, resulting in cell necrosis which clarifies the term “paradox” (Zimmerman & Hülsmann, 1966).

Many successful protocols take advantage of the Ca^{2+} - free buffers for perfusion and/or washing of the sample. It is suggested that this period of no calcium is important to allow the separation of myocytes at intercalated discs (Yates & Dhalla, 1975). It is assumed it disrupts Ca^{2+} - dependant cadherins that are responsible for mediating adherents and desmosomal junctions between cells (Leckband & Sivasankar, 2012). On the contrary, it can have deleterious effects that are thought to occur from the excessive rise in intracellular Ca^{2+} which results in excess enzyme release, exhaustion of high-energy phosphates, mitochondrial swelling, and structural damage (Piper, 2000). Protecting the cells from these deleterious effects of the calcium paradox is important to increase cell yield and quality.

The calcium paradox is strongly influenced by temperature. Calcium perfusion at 4 °C followed by increasing temperature has been shown to allow recovery of cardiac function (Holland & Olson, 1975). Whilst in a constant temperature of 37 °C led to significant myocyte damage, termed myocytolysis (Holland & Olson, 1975). This is speculated to be a result of the reduced temperature preventing the separation of the glycocalyx, glycoproteins that cover the cell membrane (Rich & Langer, 1982). This restricts Ca^{2+} re-uptake during reintroduction. Thus, Niels *et al*. (2015) suggest initial washing at room temperature to benefit from the protective effects of reduced temperatures. This was confirmed in our study where an increase in cell viability was observed, though not significant when washing at room temperature (Figure 3-2).

In the absence of Ca^{2+} contraction is halted in myocytes and upon Ca^{2+} reintroduction, the regeneration of contractile force is a marker of health. A study in isolated rat myocardium demonstrated that exposure of more than five minutes eliminated contractile regeneration (Alto & Dhalla, 1981). This indicates that restricting Ca^{2+} - free exposure may be advantageous in isolated cardiac myocytes. Although other studies have indicated that too short exposure to Ca^{2+} solution can affect myocyte separation at intracellular junctions, whilst too long exposure can yield Ca^{2+} intolerant myocytes (Lalevée *et al*., 2003; Varró *et al*., 1993). Due to the long transport times of the tissue

used in the study, a nominally low Ca^{2+} transport solution was favourable, increasing cell viability and Ca^{2+} tolerance (Figure 3-2).

As a result of the factors discussed above the protocol was altered to include 0.02 mM Ca^{2+} from the point of surgical resection and throughout the isolation procedure, to prevent Ca^{2+} overload. Secondly the initial washing step was reduced to room temperature to aid in the protective effects of reduced temperature on cell quality. Furthermore the 1st digestion time was reduced to five minutes to reduce over-digestion probability, which may have been aided by the small sample size.

4.3. Patient recruitment and clinical analysis

The recruitment process for this study ensured all patients were adults (> 18), English-speaking, and clinically diagnosed with CAD. Initially, a patient cohort of 90 individuals was agreed, upon which was later extended to allow the continuation of the study.

Analysis of the ECHO data provided allowed the identification of indices of cardiac function. This analysis revealed that 67 % of the patient cohort analysed (n = 21) had an ejection fraction < 55 %, widely recognised as reduced LVEF (Sweitzer *et al*., 2008). According to various studies lower EFs are often observed in severe CAD patients, strongly correlating with heightened heart failure risk (John *et al*.; Squeri *et al*., 2012). Although others indicate that preserved EF, observed in 40 % of our patient cohort, can be explained through CAD pathophysiology as myocardial ischemia causes both diastolic and systolic dysfunction (Hwang *et al*., 2014). Increased mortality and increased deterioration in ventricular function are often observed making the management and treatment of CAD with preserved EF difficult and often ineffective (Hwang *et al*., 2014).

The right ventricular (RV) function is also important in the functionality of the heart; however, it is often forgotten because of its retrosternal position, complex structure, and shape (Aloia *et al*., 2016). Although, RV function is thought to closely correlate with symptoms and exercise capacity playing a crucial role in the morbidity and mortality of CVD (Aloia *et al*., 2016). A useful and old method for the monitoring of systolic RV function is the measurement of TAPSE, whereby the patients analysed in this study ranged between 1.4 – 2.7 cm. There is no specific cut-off value for TAPSE however, lower values are associated with worse cardiovascular outcomes (Tamborini *et al*., 2007).

Various studies have suggested cut-off values between the range of 1.5 – 2.0 cm with those outside these values associated with reduced prognosis in numerous patient subgroups (Forfia *et al*., 2006; Ghio *et al*., 2001; Kjaergaard *et al*., 2007).

In relation to diastolic dysfunction, LV diastolic filling is an important indicator. Though LV filling can be influenced by other factors including HR, BP, age, and systolic function (Alexander, 1994). Various studies have demonstrated that abnormal filling patterns are associated with CAD, especially during acute ischemia (Carroll & Carroll, 1991). The measurement of peak E wave velocity and E/A velocity along with other parameters are valuable in the assessment of diastolic dysfunction. As CAD and associated ischemia result in a shift in diastolic pressure-volume contributed to by abnormal myocardial relaxation, acute chamber dilation, and increased LV stiffness (Carroll & Carroll, 1991; Chetrit *et al*., 2021). In particular, the increase in left atrial pressure are often attributable to CAD pathology including reduced LV compliance, fibrosis, and increased ECM (Chetrit *et al*., 2021). In this patient cohort an average peak E velocity of 0.71 ± 0.048 m/s *(n = 20)* and a E/A ratio of 0.908 ± 0.087 *(n = 20)* were noted. In accordance with other studies, this is indicative of impaired myocardial relaxation as less suction is observed at lower E velocities and E/A ratios less than 1.0 (Nair. R & Lamaa, 2022; Quinones, 2021). Those patients with an E velocity < 0.5 cm indicate a low pulmonary capillary wedge pressure reducing oxygenated blood flow around the body which can lead to cardioplegic shock (Nair. R & Lamaa, 2022; Quinones, 2021).

Analysis of ECHO data in the patient cohort was crucial to highlight the indirect effects of a primary vasculopathy on the myocardium. The results of this study show that alternative indices of systolic and diastolic function may be important to acknowledge when assessing CAD pathology.

As previously mentioned, there are differences in disease state associated with gender. In women CVD, including CAD, is often overlooked with current disease management programs primarily conducted in men (Eastwood & Doering, 2005). Although breast cancer is often considered their biggest threat, women are twice as likely to die from CVD in the UK (British Heart Foundation, 2022h; Mosca *et al*., 2004). The mechanisms underlying atherosclerosis are thought to display some gender-mediated differences. The anatomy of vascular structure differs amongst men and women, with many studies

observing smaller diameter vessels in women than men, independent of body size. This suggests a plausible explanation for women to be more prone to vascular occlusion than men (Eastwood & Doering, 2005; Sheifer *et al*., 2000). Evidence also suggests this smaller vascular diameter to increase adverse cardiac events, with a CABG study showing the operative mortality for women (4.5 %) to be higher than men (1.9 %) (Eastwood & Doering, 2005; Fisher *et al*., 1982). Although smaller vessel diameter results in women being less likely to experience acute events associated with total occlusion, they are more likely to have angina related partial occlusion (Hochman *et al*., 1999). Men typically display acute coronary syndrome, with ST elevation myocardial infarction aiding in CAD diagnosis (Eastwood & Doering, 2005). As a result of complicated signs and symptoms women are less likely to receive appropriate treatment, increasing myocardial damage and increasing mortality in many scenarios (Elsaesser & Hamm, 2004; McSweeney *et al*., 2001; McSweeney *et al*., 2003). This is backed up from the gender data that was supplied for all of the patients recruited during the whole study as 26 were male and 10 women.

Furthermore, the initial clinical data shows promising results associated with coronary artery disease. As more clinical data is processed it is important to explore more potential links.

4.3.1. Differences in heart chambers

Right atrial appendage tissue was used in this study as it was surgical waste, however many myocardial dysfunctions tend to focus on alterations within the ventricles. This is due to the ventricles containing greater muscle mass to generate the power required for the circulation of blood, hence, often thought to be the power horse for cardiac function.

Not only do the atria and ventricles differ from each other in terms of size, muscle mass, and wall thickness but also their functionality relating to intracavity pressure, contractile properties, ion channel composition and electrophysiological characteristics (Burashnikov *et al*., 2007; Caballero *et al*., 2010). As clinical and experimental scenarios can vary this adds an additional complexity to the structural and functional differences observed between the atria and ventricles (Li *et al*., 2000; Pogwizd *et al*., 2001). Thus, we would expect the morphology of the atrial cardiac myocytes to be slightly less

complex, smaller in size and have less contractility than ventricular cells. Furthermore heterogeneity amongst atrial cardiac myocytes between patient is contributed to by the in vivo effects of drugs and devices in each patient (Voigt *et al*., 2015).

Also, important to note is the differences in the left and right side of the myocardium. The primary two coronary arteries in the myocardium are the right coronary artery (RCA) and the left main coronary artery (LMCA). THE LMCA is the larger of the two being the primary source of blood for the left atrium and ventricle, whilst the RCA supplies the right atrium and ventricle in addition to the left ventricular posterior and inferior walls (Ogobuiro *et al*., 2022). Thus, the section of myocardium that is subject to reduced blood flow and associated pathologies is dependent upon the location of the coronary artery blockage. This is crucial to highlight when drawing conclusions on any results obtained from the isolated cardiac myocytes, as blockage location for each patient is unknown.

Hence, an investigation into the tissue from the LV would be a more representative study as this would highlight the direct effects of coronary artery disease on the myocardium. Although there are complications to obtaining tissue from the left ventricle during the CABG surgery as this section of the heart remains untouched, therefore would breach ethics.

4.4. Do levels of IL-1β correlate with indices of cardiac function?

It first emerged in the late 1990s that CAD could be considered an inflammatory pathology (Alexander, 1994; Ross, 1999). This is understood as inflammation plays a pivotal role in atherosclerosis, with the exacerbation of the inflammatory response facilitating plaque formation and rupture (Fioranelli. M *et al*., 2018). The pro-inflammatory cytokine IL-1β is known to mediate both systemic and local inflammatory responses to infection and injury, contributing to chronic disease. Various *in vitro* studies have highlighted the importance of IL-1β as a regulatory protein in atherosclerosis as elevated levels are often observed in CAD patients (Fioranelli. M *et al*., 2018; Galea *et al*., 1996). However, few studies have investigated the effects of IL-1β alone on myocardial function and structure. Therefore, this study aims to determine if concentrations of IL-1β can be used as a marker of CAD severity and pathology.

In the patient cohort analysed the IL-1β concentrations ranged between 0.13 – 5.08 pg/ml (n = 31). We cannot deduce whether these levels are elevated or not due to the lack of control subjects. Although a study conducted on 1292 human participants in the surrounding areas of Florence found that 44 individuals suffered from acute myocardial infarction and 348 had dyslipidaemia. In those participants their average serum IL-1β levels were 0.76 ± 0.61 and 0.52 ± 0.25 pg/ml (Di Iorio *et al*., 2003). Myocardial infarction and dyslipidaemia are associated with coronary artery disease, therefore may contribute to the higher levels of IL-1β observed in some of our patients serum. Important to note is that serum IL-1β concentration may not correlate to cytokine production capacity only representing a fraction of the real production of activated immune cells, thus an indirect marker of inflammation (Di Iorio *et al*., 2003). However studies conducted in coronary artery specimens have concluded that levels of IL-1β do correlate with CAD severity (Galea *et al*., 1996). Other previous evidence suggests that IL-1β plays a vital role in atherosclerosis, thus CAD pathology via; (1) stimulation and proliferation of VSMC, (2) expression of adhesion molecules on endothelial cells, (3) endothelium modification promoting coagulation and thrombosis and (4) acceleration of atherosclerosis in murine models (Chen *et al*., 2018; Offner *et al*., 1996). This left us to believe that the IL-1β concentrations in the CAD patient cohort would correlate with indices of cardiac function.

It is known that resident and recruited immune cells are present during early cardiac disease. These cells modulate both cardiomyocyte function and cardiac injury, thus impacting cardiac function. However, the systolic function did not correlate with patient IL-1β concentrations relating to EF, whilst there are *early* indications of a negative correlation with TAPSE (Figure 3-7). A similar phenomenon is found in sepsis patients, whereby elevated levels of IL-1β are often observed and contractility is impaired but no alterations in EF are seen (Busch *et al*., 2021). On the contrary, these findings do not compare with animal models which display elevated IL-1β along with reduced LV systolic function in response to myocardial infarction (Chen *et al*., 2018).

On the other hand, diastolic function (Peak E wave) negatively correlated with IL-1β concentration (Figure 3-8), with patients that have higher concentrations of IL-1β tending to have lower peak E velocities. This suggests IL-1β impairs the relaxation of

cardiac myocytes. Other studies investigate the cellular effects of IL-1β whereby Radin *et al*. (2008) confirmed a reduced calcium removal which induced prolonged relaxation represented as impaired LV relaxation. Additionally, *early* indications of the E/A ratio negatively correlating with IL-1β patient concentration are seen (Figure 3-8). This may be due to a number of factors that can affect the E/A ratio including the presence of mitral valve abnormalities, heart rate and rhythm, and aortic insufficiencies. All of these factors in the patient cohort are not provided, thus this must be acknowledged when drawing conclusions. There are also differences in findings from different models whereby IL-1β does not alter cardiac myocyte relaxation, thus diastolic function, in young sheep (Hadgraft & Greensmith, 2018). This contradicts findings from Duncan *et al*. (2010) and Radin *et al*. (2008) studies whereby diastolic dysfunction and impaired relaxation are observed in rat ventricular cardiac myocytes.

These findings indicate that IL-1β may be a useful biomarker in CAD pathology. However, inflammation is a complex biological response involving a variety of cells, cytokines, chemokines, and acute-phase proteins so the effect of IL-1β alone does not truly represent the underlying pathology of CAD. Hence, the effects on myocardial structure and function may be negligible at a whole heart level. As such, more investigation on the effects of IL-1β on cardiac function at a cellular level is necessary.

4.5. Do levels of Ox-LDL correlate with indices of cardiac function?

Mitochondrial and cytosolic sources of oxidative stress are known to contribute to the development and progression of CAD, with it becoming a target of therapeutic intervention. The measurement of circulating oxidative stress levels offers a non-invasive, valid tool with studies confirming correlations between circulating OS and CVD severity (Rubattu *et al*., 2019).

In the patient cohort analysed the serum Ox-LDL levels ranged between 8.37 – 162.68 pg/ml (*n = 37)*. Previous studies have highlighted elevated circulating Ox-LDL in several disease states including atherosclerosis and sepsis, associated with increased oxidative stress and inflammation (Behnes *et al*., 2008; Jin *et al*., 2012). Additionally various *in vitro* and animal studies, and correlational and epidemiological studies in humans have suggested that Ox-LDL may promote atherosclerosis (Shen *et al*., 2013). Hence, those

patients with higher serum Ox-LDL levels may indicate heightened CAD severity, oxidative stress, and inflammation. Furthermore, sufficient evidence in multiple population subgroups demonstrates that reduced Ox-LDL levels are associated with the presence of scavenger receptors on endothelial cells and specialized macrophages, these are capable of removing modified lipids, Ox-LDLs, from circulation (Holvoet *et al*., 2007; Van Berkel *et al*., 1991). Thus, patients with increased Ox-LDL levels may be associated with an inability to remove these from circulation.

Surprisingly there is little data available that links oxidative stress, specifically Ox-LDL, to myocardium (dys)function in the general population. This is remarkable as there is sufficient evidence of the following, (1) vasculopathy to which ROS and Ox-LDL link, (2) the ability of cardiac myocytes, endothelial cells, and blood cells to produce ROS, and (3) mechanisms of OS-related cardiac damage (Lefer & Granger, 2000; Sawyer *et al*., 2002). Therefore, this study aims to determine whether Ox-LDL is a valuable biomarker in assessing cardiac dysfunction in relation to CAD.

Our data implies elevated Ox-LDL affects myocardium function, reflecting systolic dysfunction but not diastolic dysfunction. Interestingly a positive correlation between systolic dysfunction and Ox-LDL concentration was observed when using precocious indices, TAPSE (Figure 3-9), rather than the typical measure of EF. Ernst *et al*. (2008) study conducted in a Belgian population with previous cardiovascular disease, demonstrated that the traditional measures of both systolic and diastolic function did not show any correlation to Ox-LDL, unlike advanced indices. Concluding that Ox-LDL does impact cardiac structure and function in a gender-specific manner, irrespective of traditional risk factors, lifestyle, inflammation, and vascular damage (Rietzschel *et al*., 2008). On the contrary heart failure studies have demonstrated Ox-LDL plasma levels to correlate with lower EF, the severity of clinical symptoms, and mortality (George *et al*., 2006; Tsutsui *et al*., 2002). However, OS in heart failure is secondary to the disease, whilst in CAD it has a primary role. This may explain the differences observed in our patient cohort. We know that OS, and consequently Ox-LDL is associated with plaque build-up within arteries leading to stenosis, arterial stiffness, and in turn increasing systolic blood pressure (SBP) (Brinkley *et al*., 2009). Additionally, an increase in pulse wave velocity (PWV) is often associated which causes reflected waves to return during

systole, amplifying SBP and further imposing a greater workload on the myocardium (Dao *et al*., 2005). As a result, subclinical changes in the myocardium are often observed in response to hypertension including ventricular strain and diastolic dysfunction often being observed before any reduction in EF is seen (Tran *et al*., 2020). This phenomenon may explain the increase in systolic function, relating to TAPSE, as the excursion of blood is increased as a result of elevated SBP.

A few mechanistic explanations for the alterations in cardiac functions can be put forward. Firstly Ox-LDL is a marker of OS which consequently leads to radical-mediated myocardial damage. As previously mentioned, there are a number of sources of ROS which can cause inhibitory effects on systolic dysfunction. This may occur as a result of membrane protein oxidation and lipid peroxidation, disturbances of calcium homeostasis on the mitochondria, SR, and sarcolemma leading to contractile abnormalities (Zorn-Pauly *et al*., 2005). Secondly, Ox-LDL is a by-product of ROS exposure with experimental data highlighting the importance of NO/redox signalling in contractility and hypertrophy (Rietzschel *et al*., 2008). Whereby ROS has the potential to be adaptive and maladaptive, as at low ROS levels fibrosis, thus hypertrophy is favoured and at high levels, myocyte death is a consequence (Pimentel *et al*., 2001). Finally, limited data implies that Ox-LDL can cause direct damage to the myocardium (Fearon, 2006). In Zorn *et al*. (2005) study Ox-LDL induced myocyte cell damage and alterations in electrophysical activity in guinea pig ventricular cardiac myocytes were observed. Important to note that these changes were dependent upon the lipid hydroperoxide content of the Ox-LDL. As a result of these proposed mechanisms, the circulating levels of Ox-LDL may be useful in determining both the systemic and metabolic state in CAD patients.

The hypothesis that Ox-LDL form an integral part in the development of atherosclerotic lesions was formulated over 30 years ago (Tsimikas & Witztum, 2001). Although it is thought that fully Ox-LDL do not exist within the blood due to the multitude of circulating antioxidants. In contrast minimally Ox-LDLs were initially described by Avogaro *et al*., (1988) and Sevanian *et al*., (1996) where these modifications are not recognised by scavenger receptors. Various studies have measured plasma levels of Ox-LDL using sandwich ELISA and found that elevated levels of Ox-LDL were found in CAD

patients relative to controls, also showing some correlation with disease severity (Ehara *et al*., 2001; Holvoet *et al*., 1999; Holvoet *et al*., 1998; Itabe & Ueda, 2007). With this being said in Ehara *et al*., (2001) study only 7 out of the 45 acute myocardial infarctions were distinguished in the unstable angina subset (*n = 45*). This suggests that it may not be robust enough to be used alone but in combination with other Ox-LDL epitopes or inflammatory markers it may provide useful insights into risk, diagnosis, plaque progression and pathophysiology of atherosclerosis . Although this data along with other studies does confirm Ox-LDL can be measured within the bloodstream, the etiology of specific oxidation epitopes remains unknown. The source of these epitopes could be associated with the direct release from plaques, ischemic injury, or remote inflammatory sources (Tsimikas & Witztum, 2001). Thus, to confirm our findings it would be necessary to explore specific epitopes of Ox-LDLs, other inflammatory markers in both the blood and more importantly within the vascular structure and associated plaque removed during surgery.

4.6. Preliminary study- The measurement of oxidative stress tolerance using cytation analysis

Oxidative stress in cardiac myocytes illustrates the myocyte injury as a result of increased ROS production and/or decreased antioxidant reserve. The production of ROS within cardiac myocytes occurs through the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (AD(P)H) oxidase, and xanthine dehydrogenase/xanthine oxidase (Tsutsui *et al*., 2008). In order to keep up with the demand for ATP synthesis, and producing energy, cardiac myocytes have the highest volume density of mitochondria (Freshney *et al*., 2007). Under physiological conditions, small amounts of ROS are produced by mitochondrial respiration, although these free radicals can be detoxified by the scavenging mechanisms of the cardiac myocytes (Tsutsui *et al*., 2008). Therefore, monitoring OS tolerance in isolated cardiac myocytes may provide important insights into the relationship between OS and CAD pathology.

The *preliminary* findings indicate that there is heterogeneity in OS tolerance among patients observed. The antioxidant capacity of the cardiac myocytes may contribute to this difference between patients, as H_2O_2 concentrations remained consistent, and the

tolerance of each patient was measured based on their base line OS levels. The depression of antioxidant reserves in cardiac myocytes generally stems from exhaustion and/or changes in gene expression (Dhalla *et al*., 2000). DNA damage and total antioxidant capacity have been reported in CAD patients independently, however little is understood linking to OS. Although it is well known that DNA damage occurs in response to ROS, previous reports in atherosclerotic lesions reveal increased mutation rates and widespread microsatellite instability (Casalone *et al*., 1991; McCaffrey *et al*., 1997). Nevertheless, there are several CAD risk factors known to contribute to DNA damage and consequently reduced antioxidant capacity. Numerous studies highlight smoking to increase DNA damage and decrease total antioxidant capacity (Andreassi, 2003; Nair. R & Lamaa, 2022; Petruzzelli *et al*., 1997). Additionally, it has been shown in hyperlipidaemia patients there is an increase in lymphocyte DNA damage as a result of OS and reduced total antioxidant capacity (Harangi *et al*., 2002; Marnett, 2000). Furthermore, evidence suggests that DNA damage increases with age as a result of an age-related decrease in antioxidant capacity (Andreassi, 2003; Goukassian *et al*., 2000).

In order to gain a better understanding of the possible mechanism(s) at fault for the differences in OS tolerance between patients, clinical and ECHO data is required along with a measurement of total antioxidant capacity.

4.6.1. Do levels of serum biomarkers correlate with oxidative stress tolerance?

Increased levels of inflammatory markers typically parallel OS, contributing to the structural and functional damage underlying CAD. Therefore, the relationship between OS tolerance and serum biomarkers was examined initially in a small patient cohort. This analysis revealed that IL-1β serum concentrations do correlate with cardiac myocyte OS tolerance when treated with 200 μM H_2O_2 (Figure 3-11). Firstly, it is important to understand that concentrations of 200 μM H_2O_2 significantly increased fluorescence, hence oxidative stress saturation, which we would expect to diminish the antioxidant capacity of the cell. Whilst it is also known that inflammation induces OS also reducing the cellular antioxidant capacity (Khansari *et al*., 2009). As inflammation and OS come hand in hand in contributing to the pathogenesis of CAD, the correlation between IL-1β and OS tolerance was no surprise (Lee *et al*., 2013). However, no correlation between Ox-LDL and OS tolerance was observed in the same patient cohort. This may be due to

Ox-LDL having a primary effect on the vascular dysfunction seen in CAD. Although oxidative modifications of LDL are a consequence of elevated ROS, numerous studies have shown negative links to antioxidant status (Nour Eldin *et al*., 2014).

As these findings are based upon a small sample size, the study power is needed to be increased to confirm these findings. Additionally, the independent effects of both IL-1β and Ox-LDL may be tested on isolated cardiac myocytes to monitor their response to OS tolerance.

4.7. The effects of therapeutics on Coronary Artery Disease pathophysiology

The therapeutics that CAD patients receive is dependent upon the clinical manifestations and lifestyle of the patient. Therefore, dependant on the medication given different elements of CAD pathophysiology are targeted. This could alter some of the parameters investigated within the study. As we do not know currently know the medication history of the patients in the study extra consideration when drawing conclusions needs to be undertaken, as drug administration could contribute to some of the findings observed. The treatments described in section 1.5 are of importance as these are commonly used to manage CAD symptoms.

4.7.1. Statins

The management of high cholesterol with statin therapy is often used for CAD to lower LDL levels. In other CVDs, such a stroke were plasma Ox-LDL levels are elevated, significant reductions have been observed in such plasma Ox-LDL levels after only 7 days of statin therapy (Tsai *et al*., 2014). This is due to Ox-LDL levels positively correlating with total serum cholesterol and LDL levels (Andican *et al*., 2008; Tsai *et al*., 2014). Hence, lower levels of serum Ox-LDL found in some of the patients recruited in this study may be contributed to by the patient being on statin therapy, dosage, and length of treatment. In turn this may consequently affect oxidative stress in the myocardium as LDL and Ox-LDL is lowered stopping further plaque build-up.

Inflammatory mediators are demonstrated to be effective targets of secondary preventions in CAD, including IL-1β. However, the effects of statins on IL-1β are a topic of conflict. Evidence suggests statins also have a significant immunomodulatory

property independent of lipid lowering, being able to alter protein prenylation (Greenwood *et al.*, 2006). This is associated with reduced inflammation decreasing circulating IL-6 and TNF-α, but paradoxically increase IL-1β and IL-18 production in monocytes (Mandey *et al.*, 2006; Rosenson *et al.*, 1999). Thus, this is important to consider when measuring serum IL-1β levels and assessing the inflammatory environment in the patient serum.

4.7.2. Beta-Blockers

Beta-blockers are often used to control the heart rhythm, treat angina, and reduce blood pressure. Many clinical trials have supported their use in treating CVD, with both ß1 adrenoreceptor blockers (metoprolol and bisoprolol) and a nonselective ß1 and ß2 adrenoreceptor blocker (carvedilol) demonstrating to increase mortality and improve cardiac function in patients with heart failure (Dargie, 1999; Fowler, 2004; Wikstrand, 2000). A mechanism by which this is thought to work is by the reduction in oxidative stress at systemic level in the plasma and myocardium itself (Nakamura *et al.*, 2002). In Kukin *et al.*, (1999) study beta blockers, metoprolol and carvedilol, reduced plasma lipid peroxidation in heart failure patients, further confirmed in Chin *et al.*, (2003) study by bisoprolol and carvedilol in congestive heart failure patients. Furthermore, endomyocardial biopsy samples obtained from patients with dilated cardiomyopathy displayed a 40 % reduction in 4-Hydroxynonenal (HNE)-modified myocardial proteins when treated with carvedilol (Nakamura *et al.*, 2002). This is important as HNE is cytotoxic product formed as a result of ischemia thus, beta-blockers show potential anti-ischemic properties (Nakamura *et al.*, 2011). Hence, any patients who were prescribed with beta-blockers in this study may have reduced oxidative stress within the isolated cardiac myocytes and Ox-LDL levels within the serum samples.

4.7.3. Other therapies

In addition to the traditional therapies used to treat CAD, there are other targeted treatments for associated disorders. This can include therapies for angina such as trimetazidine, which increases the cell tolerance to ischemia, thus reducing oxidative stress (Bobescu *et al.*, 2021). Furthermore, patients who are diabetic receiving oral drugs such as metformin, exenatide, vildagliptin have shown to have reduced markers of oxidative stress and inflammation (Wronka *et al.*, 2022). This highlights the importance

of considering medicine records of the patients recruited during this study as this may alter the conclusions drawn from results.

4.8. Limitations

The effectiveness of the study was impacted by several factors, some of which were uncontrollable and others that may be rectified using additional tests and procedures.

4.8.1. Global Covid-19 pandemic

The global pandemic, coronavirus 2019, resulted in a country-wide lockdown in the UK with effect from March 2020. This study originally commenced in November 2019, thus laboratory operations during the pandemic were suspended.

The collaboration with Blackpool Victoria Hospital and their ability to perform CABG surgery during the pandemic had a knock-on effect on the number of samples obtained. Thus, it has taken three years to obtain the originally confirmed 90 samples. As such the human tissue sampling has only recently been able to progress past the optimisation stage.

4.8.2. Patient recruitment and data collection

Up until September 2022, the surgeons at Blackpool Victoria Hospital have been responsible for all aspects of patient recruitment, data collection, and analysis. However, this is a time-consuming process for the study and is secondary to the objectives of the surgeons whose responsibility is to primarily conduct the surgical procedure. As a placement student is due to start who will help process the patients' data. This will be beneficial to fill in any gaps in already obtained samples and any future samples, increasing the study power which is one of the main limitations currently.

4.8.3. Human models

Human model-based studies are the most direct and appreciated source in the understanding of human physiology in both disease and "healthy" states. However, the use and acquisition of human tissue provide clear limitations to the study. Generally, human tissue is less abundant in comparison to other models, specifically within the study as this depended upon the rate of CABG surgeries and the patient recruitment and approval, limiting the sample number. Furthermore, time delays between tissue

excursion and acquisition by the research team are inevitable in human studies. In relation to this study, the transport time was deemed to be a significant limitation with long travel times, increasing the cold ischemic time of the tissue which negatively impacts myocyte viability. The influence of a patient's lifestyle, comorbidities, and prior treatment alters a tissue's phenotype causing variable results. These variables make it particularly difficult to come to definite conclusions, as the need for more clinical data on the patients is required to an allow analysis of these factors on the disease outcome.

Taking these limitations into account, there is a strong emphasis on the need for identifying a human "control", again difficult to obtain, or the use of animal models to compare disease and non-disease states and to clarify our findings. As such the use of animal models, including mice, offers a close equivalent that can be tested quickly and over a long period of time. In combination with human models, this would give an even better understanding of the underlying mechanisms involved in CAD pathogenesis and its effects on the myocardium both on a whole heart level and cellular level.

4.9. Future work

This study has identified that IL-1β and Ox-LDL can be measured in patient serum samples representing systemic inflammation and oxidative stress. Further to this study has focused on the downstream effects of inflammation and oxidative stress on a whole heart level. Therefore, additional research will focus on the cellular effects of inflammation and ROS and the underlying mechanisms which occur.

Experimental data will be continuously collected investigating OS tolerance in response to H_2O_2, in the hope to increase the study power and continue to find patterns in patient variability. Alongside this future work will explore whether H_2O_2 and IL-1β have any effects on Ca^{2+} handling and contractility. As previous studies primarily conducted in animal models show that systolic Ca^{2+} is reduced in response to IL-1β, although a decrease in contractility is not observed (Hadgraft & Greensmith, 2018). Thus, the mechanisms of IL-1β may be complex requiring further investigation into ion channel activity via patch clamping. Furthermore, previous work in rat ventricular myocytes has demonstrated that H_2O_2 causes a concentration-dependant decrease in SERCA activity, reducing SR Ca^{2+} content thus, diminishing systolic function

(Greensmith *et al*., 2010). In order to determine specific mechanisms, caffeine can be used to inhibit SR Ca^{2+} reuptake, inhibiting SERCA activity and allowing the investigation of NCX activity (Greensmith & Nirmalan, 2013; Reggiani, 2021). Furthermore, OS and antioxidant inhibitors can be used to determine the underlying mechanisms of OS within cardiac myocytes.

Inflammatory cytokines also act as OS mediators, influencing ROS production. The balance between ROS production by pro-inflammatory cytokines, specifically IL-1β, can alter myocyte function. For example, the activation of the L-arginine-NO pathway in response to IL-1β is known to modulate contractility (Evans *et al*., 1993). Redox signalling can promote phospholamban phosphorylation, a key regulator in cardiac contractility, increasing Ca^{2+} removal and consequently relaxation (Ho *et al*., 2014). Additionally, impaired relaxation in acute inflammation of the myocardium has been associated with elevations in NO and ROS (Li *et al*., 2013). Therefore, it would be beneficial to the study if the future investigation of IL-1β on OS and consequently myocyte function was examined.

All of the above would be compared to ECHO data for each patient to determine whether cellular (dys)function in the isolated cardiac myocytes is translational to the whole heart function.

4.10. Summary

This *preliminary* study is one of the very few to retrieve experimental data on isolated cardiac myocytes from human tissue. This provides promising data for future continuation of the study further investigating the cellular effects of inflammation and oxidative stress.

Enzyme-linked immunosorbent assays have proven to be valuable in assessing systemic inflammatory cytokines and oxidative stress markers. With IL-1β serum concentrations showing links with TAPSE, index of systolic function as well as peak E wave and E/A ratio, indices of diastolic function. Whilst Ox-LDL positively correlating with TAPSE, an index of systolic function. This suggests that IL-1β may be useful biomarker in the assessment of impaired cardiac function in CAD.

Whereas Ox-LDL may be useful in the assessment in OS mechanisms, it does not currently show promise for assessing cardiac dysfunction.

Cytation analysis confirmed to some extent that ROS are present in the isolated cardiac myocytes. With initial oxidative stress tolerance testing confirming heterogeneity between patients. This may be due to diminished antioxidant mechanisms, through ROS induced DNA damage, although the exact reasoning behind this cannot yet be confirmed without additional testing to confirm mechanisms of underlying OS.

These findings confirm that inflammation and OS do play a role in CAD pathology however, these are *preliminary* results so future research aims to increase the study power to confirm our findings.

1. Appendix

Appendix A. Global Risk Factors for Heart and Circulatory Diseases

Modifiable Risk Factor & Attributable Burden	2019 CVD Deaths (million)	% Of Burden
High systolic blood pressure (hypertension)	10.0	54
Dietary risks (poor diet)	6.9	37
High LDL cholesterol	4.4	24
High fasting glucose (diabetes)	3.8	20
Air pollution (particulate matter)	3.5	19
High body-mass index (overweight/obesity)	3.2	17
Tobacco (smoking and second-hand smoke)	3.2	17
Kidney function (renal failure)	1.7	9

Appendix B. HRA and HCRW approval letter

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

Dr David Greensmith
Lecturer
University Of Salford
University Of Salford
Peel, G35
Greater Manchester
M5 4WT

21 January 2019

Dear Dr Greensmith

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Characterisation of cardiac cellular and vascular function in coronary artery disease**
IRAS project ID:	**247341**
Protocol number:	**TBC**
REC reference:	**18/LO/2219**
Sponsor	**University Of Salford**

I am pleased to confirm that HRA and Health and Care Research Wales (HCRW) Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and Wales, as well as any documentation that has been updated as a result of the assessment.

Following the arranging of capacity and capability, participating NHS organisations should **formally confirm** their capacity and capability to undertake the study. How this will be confirmed is detailed in the "*summary of assessment*" section towards the end of this letter.

You should provide, if you have not already done so, detailed instructions to each organisation as to how you will notify them that research activities may commence at site following their confirmation of capacity and capability (e.g. provision by you of a 'green light' email, formal notification following a site initiation visit, activities may commence immediately following confirmation by participating organisation, etc.).

It is important 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 of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?
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.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Professor Sheila Pankhurst

Tel: 0161 295 5171

Email: s.pankhurst@salford.ac.uk

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **247341**. Please quote this on all correspondence.

IRAS project ID	247341

Yours sincerely

Joanna Strickland
Assessor

Email: hra.approval@nhs.net

Copy to: *Professor Sheila Pankhurst [sponsor contact]* s.pankhurst@salford.ac.uk
Mrs Helen Spickett, Blackpool Teaching Hospitals NHS Foundation Trust [Lead R&D contact] helen.spickett@nhs.net

Appendix C. HRA and Camberwell St Giles REC

Health Research Authority

London - Camberwell St Giles Research Ethics Committee
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 0207104 8204

<u>Please note</u>: 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

19 January 2019

Dr David Greensmith
Lecturer
University Of Salford
Peel, G35
Greater Manchester
M5 4WT

Dear Dr Greensmith

Study title:	**Characterisation of cardiac cellular and vascular function in coronary artery disease**
REC reference:	**18/LO/2219**
Protocol number:	**TBC**
IRAS project ID:	**247341**

The Proportionate Review Sub-committee of the London - Camberwell St Giles Research Ethics Committee reviewed the above application on 25 December 2018.

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 favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact hra.studyregistration@nhs.net outlining the reasons for your request. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above

research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

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 HRA and HCRW Approval (England and Wales)/ 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. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

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).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Extract of the meeting minutes

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Covering letter on headed paper [Covering Letter]		05 December 2018
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance]	1	16 July 2018
IRAS Application Form [IRAS_Form_14122018]		14 December 2018
IRAS Checklist XML [Checklist_18012019]		18 January 2019
Participant consent form [PCF V2]	2	18 January 2019
Participant information sheet (PIS) [PIS]	2	18 January 2019
Participant information sheet (PIS) [PIS V2 Clean]	2	18 January 2019
Participant information sheet (PIS) [PIS V2 With Track Changes]	2	18 January 2019
Research protocol or project proposal [Proposal]	0002a	06 December 2018
Response to Request for Further Information		18 January 2019
Summary CV for Chief Investigator (CI) [DG CV]		06 December 2018
Summary CV for supervisor (student research) [SW CV]		06 December 2018

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

There were no declarations of interest.

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

<u>Reporting requirements</u>

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/

With the Committee's best wishes for the success of this project.

18/LO/2219	**Please quote this number on all correspondence**

Yours sincerely

Pp
REC Manager

Mr John Richardson
Chair

Email: nrescommittee.london-camberwellstgiles@nhs.net

Enclosures:	*List of names and professions of members who took part in the review*
	"After ethical review – guidance for researchers"
Copy to:	*Mrs Helen Spickett, Blackpool Teaching Hospitals NHS Foundation Trust*
	Lead Nation
	England: HRA.Approval@nhs.net

London - Camberwell St Giles Research Ethics Committee

Attendance at PRS Sub-Committee of the REC meeting on 25 December 2018

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Jennifer Bostock	Philosopher of Psychiatry	Yes	
Dr Cynthia Ruth Butlin	Retired Medical Practitioner	Yes	
Mr John Richardson	Retired Director of COREC: former Ecumenical Officer for Churches Together in South London	Yes	Chair of PRSC meeting

Also in attendance:

Name	*Position (or reason for attending)*
Mr Paolo Buscemi	REC Assistant

Appendix D. Patient information and consent form

Blackpool Teaching Hospitals NHS
NHS Foundation Trust

Blackpool Teaching Hospitals NHS Foundation Trust
Clinical Research Centre
2nd floor, Area 5
Blackpool Victoria Hospital
Whinney Heys Road
Blackpool
FY3 8NR
Tel: 01253 (9)53559
Email: helen.spickett@nhs.net

PATIENT INFORMATION SHEET

Study Title: Characterisation of cardiac cellular and vascular function in coronary artery disease (CAVCAD)

An Invitation to participate

We would like to invite you to participate in the CAVCAD study.

You are being invited to take part in a research study. Before you decide whether or not to take part it is important for you to understand why the research is being done and what is involved. Please take time to read the following information carefully and discuss it with others if you wish. Please ask if there is anything that you are unclear about or if you would like more information.

What is the purpose of the study?

The study is looking at how coronary artery disease (CAD), also known as ischemic heart disease (IHD) can affect your heart tissue and blood vessels so that we can work to develop better treatments and improve the surgical outcome for all patients in the future. There are two questions we want to ask: 1. Do changes in how heart cells work

contribute to the progression of your disease? 2. Do the blood vessels which are used for your bypass work better if they have their normal fat tissue around them?

We know that for the heart to beat properly, there is a cyclical rise and fall of calcium in the cells. This needs a coordination of channels and pumps for it to happen properly. We think that when these processes go wrong, this can affect how well the heart pumps, we would like to try and understand the mechanisms involved so that we can consider new therapeutic strategies in the future.

When you have your surgery, a small section of blood vessel will be used to help bypass the ischemic (without oxygen supply) part of your heart; this vessel is known as the internal mammary artery. Most of your blood vessels in your body have a layer of fat cells around them, these cells release signals which help the blood vessels to work properly. Normally, your surgeon will take these cells off when using it in the bypass operation. We want to know if leaving these fat cells around the blood vessel will improve surgical outcome.

Both of these questions can be asked without any change to your surgery, as we will look at the cells and blood vessels back in our laboratory at the University of Salford. No tissue will be taken that would not be taken anyway as a routine part of your surgery.

Why have I been asked to take part in the study?

You have been invited to take part in this study because you have ischemic heart disease and are about to undergo coronary revascularisation to help improve how your heart works.

Do I have to take part in the study?

It is up to you to decide whether to take part or not. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive or the treatment that you are receiving.

What do I have to do as part of this study?

If you decide to participate in this study, you will be asked to sign a consent form then, before or after you are put to sleep for your surgery a 5mL blood sample will be taken from you so that we can look at circulating markers in your blood. When you are undergoing your surgery, some of the heart tissue (approx. 0.5-1cm) and some of the leftover mammary artery / saphenous leg vein which is used as for the bypass, both of which are normally removed and discarded during the operation, will be kept in an experimental solution to be transported to the University of Salford so we can look at how the cells in these tissues work. We will use scientific equipment to keep the tissue alive so we can explore how they work and the signals from them, small pieces of tissue will be preserved so we can look at the structure of the tissue and the levels of specific proteins within it. A computer program will be used to find out whether any changes we see are linked to your recovery.

Your personnel data such as name address and telephone number will not be stored, this information will be kept by your doctor at the treating hospital.

By signing the consent form, you are also agreeing that the clinical team can access some information from your patient records, including age, how well you recover and medication.

What will happen to my blood sample and tissue?

Your tissue and blood sample will be sent to the University of Salford for their experiments. This may involve the storage of your tissue at University of Salford. When your tissue is no longer required for the study, it will be destroyed.

What are the possible benefits of taking part?

It is unlikely that you will experience any direct benefit from taking part in this study. However, the information gained from the study will help to develop further research which may help improve the treatment of future people who suffer from similar types of ischemic heart disease.

What are the potential risks of taking part in the study?

There are no additional risks through participating in this study, as the tissue we want to take is tissue which your surgeon would normally discard during surgery.

Indemnity and Compensation?

The Sponsor has an additional insurance policy in place for the completion of this study. This insurance will cover any additional unforeseen problems that may occur as a result of carrying out the study.

You will not be paid for your participation in the study.

Will my taking part in the study be kept confidential?

Yes, any information about you that is shared with the Sponsor as part of this study will be anonymous. Your name and address will be removed from all information so that you cannot be recognized from the information. All information about you will be handled in confidence. The study will also be carried out in accordance to Ethical and Research Governance Guidelines that are followed when completing any type of research within the NHS. If you decide to take part in the study your medical records and the data collected for the study will be looked at by authorised persons from within the research team. In addition, your records may also be viewed by employees of the regulatory authorities to ensure that the study is being carried out correctly.

What will happen if I want to withdraw from the study?

If you decide to withdraw from the study at any point, we will continue to use any data collected up to your withdrawal. We will not contact you about the study from this point forward. A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive or the treatment that you are receiving.

What if there is a problem?

If you are concerned at any point about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. You can contact the **Research Team using the details in the letter head**.

If you remain unhappy and wish to complain formally through the NHS complaints procedure, you can contact the **Patient Advice and Liaison Service (PALS) at the hospital on (01253) 955588/89**

What will happen to the results of the study?

The results of the research will be the property of the Sponsor. They may choose to present the results at a medical conference or publish the research results in a medical journal. We will be happy to send you an end of study report if you are interested in what we find.

Who can I contact for further information?

For further information regarding the study, you can contact the **Research Team using the details in the letter head**.

Thank you for taking the time to read this information.

INFORMED CONSENT FORM

Patient Research Identification Number:

Name of Researcher: David Greensmith and Sarah Withers

Title of Research: Characterisation of cardiac cellular and vascular function in coronary artery disease

Please Initial box

I confirm that I have read and understand the information sheet dated the 27th November 2019 (version 1.3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
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.	
I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
I give permission for my cardiac tissue, internal mammary artery and saphenous leg vein, which would be normally discarded to be supplied to The University of Salford. I also give my permission for an extra blood sample to be taken for identification of biomarkers.	
I give permission to The University of Salford to store and distribute my samples to any researchers whose work has appropriate ethical approval and who are conducting high quality medical research on the prevention, diagnosis and / or the treatment of ischemic heart disease or other associated diseases.	

I agree to take part in the above study

____

Name of Patient **Date** **Signature**

____

Name of Person **Date** **Signature**

taking consent

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix E. Interleukin-1β reagent preparation

Wash buffer (1X)

- Mix gently to avoid foaming and remains stable for 30 days at 2-25°C

Number of Strips	Wash Buffer Conc (20x) mL	Distilled Water (mL)
1-6	25	475
1-12	50	950

Assay buffer (1x)

- Mix gently to avoid foaming and remains stable for 30 days at 2-8 °C

Number of Strips	Assay Buffer Conc (20x) (mL)	Distilled Water (mL)
1-6	2.5	47.5
1-12	5.0	95.0

Biotin-detection antibody (1x)

- Make a 1:100 dilution, mix gently and use within 30 minutes of dilution

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1-6	0.03	2.97
1-12	0.06	5.94

Streptavidin-HRP (1x)

- Make a 1:200 dilution, mix gently and use within 30 minutes of dilution

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (mL)
1-6	0.03	5.97
1-12	0.06	11.94

Amplification diluent (1x)

- Make a 1:2 dilution immediately prior to use

Number of Strips	Amplification Diluent (2x) (mL)	Distilled Water (mL)
1-6	3	3
1-12	6	6

Amplification solution I (1x)

- Make a 1:100 dilution immediately prior to use

Number of Strips	Amplification Regent I (mL)	Amplification Diluent (1x) (mL)
1-6	0.03	2.97
1-12	0.06	5.94

Amplification solution II (1x)

- Make a 1:2300 dilution immediately prior to use

Number of Strips	Amplification Reagent II (mL)	Assay Buffer 1x (mL)
1-6	2.61×10^{-3}	5.99739
1-12	5.22×10^{-3}	11.99488

Appendix F. Ox-LDL reagent preparation

Wash buffer (1x)

- Mix gently to avoid foaming and remains stable for 30 days at 2-25°C

Number of Strips	Wash Buffer Conc (25x) (mL)	Distilled Water (mL)
1-6	15	375
1-12	30	750

Biotin-detection antibody (1x)

- Make a 1:100 dilution and mix gently

Number of Strips	Biotin Concentrate (mL)	Antibody Dilution Buffer (mL)
1-6	0.048	4.752
1-12	0.096	9.504

Streptavidin-HRP conjugate (1x) (SABC)

- Make a 1:100 dilution avoiding direct light

Number of Strips	Streptavidin-HRP (mL)	Strep Dilution Buffer (mL)
1-6	0.048	4.752
1-12	0.096	9.504

Appendix G. Mean IL-1β serum concentrations (pg/ml)

Patient ID	IL-1β concentration	SEM
BVH5	0.43	0.013
BVH12	0.22	0.005
BVH16	0.67	0.016
BVH17	0.17	0.002
BVH22	0.40	0.006
BVH28	0.13	0.003
BVH30	0.30	0.003
BVH33	0.40	0.016
BVH62	4.92	0.027
BVH63	0.75	0.001
BVH64	0.34	0.012
BVH65	0.49	0.023
BVH66	5.08	0.015
BVH67	4.67	0.008
BVH68	3.45	0.076
BVH69	4.05	0.002
BVH70	2.56	0.029
BVH71	1.19	0.006
BVH72	2.11	0.064
BVH73	1.47	0.015
BVH74	1.39	0.004
BVH75	1.28	0.003
BVH76	1.13	0.031
BVH77	1.24	0.008
BVH78	1.09	0.027
BVH79	1.03	0.031
BVH80	0.20	0.003
BVH81	0.61	0.014
BVH82	0.85	0.006
BVH83	1.05	0.007
BVH84	1.56	0.003

Appendix H. Mean Ox-LDL serum concentration (ng/ml)

Patient ID	Ox-LDL concentration	SEM
BVH5	61.12	0.025
BVH12	23.43	0.013
BVH16	45.53	0.030
BVH17	13.70	0.020
BVH22	9.54	0.005
BVH28	9.29	0.002
BVH30	12.04	0.002
BVH33	99.16	0.012
BVH52	13.75	0.006
BVH53	22.74	0.012
BVH54	14.84	0.004
BVH55	8.37	0.004
BVH57	10.46	0.002
BVH58	61.03	0.036
BVH59	11.81	0.006
BVH60	10.75	0.006
BVH61	11.56	0.005
BVH62	10.63	0.003
BVH63	80.85	0.115
BVH64	13.78	0.005
BVH66	15.73	0.005
BVH67	29.79	0.186
BVH68	162.68	0.111
BVH69	17.10	0.009
BVH70	122.59	0.076
BVH71	9.36	0.004
BVH72	9.95	0.002
BVH73	11.89	0.002
BVH74	34.81	0.027
BVH75	8.41	0.005
BVH76	97.56	0.011
BVH77	9.05	0.003
BVH78	16.55	0.023
BVH80	10.46	0.014
BVH81	11.76	0.014

BVH83	21.49	0.005
BVH84	78.49	0.095

Appendix I. Patient echocardiogram and clinical data

	EF (%)	SV (ml)	ESV (ml)	EDV (ml)	TAPSE (cm)	LVOT (m/s)	PASP (mm/Hg)	LVIDS (cm)	LVIDD (cm)
BVH5	77.7	50.60	14.50	65.10	2.70	1.01	-	-	-
BVH12	22.3	44.05	153.80	197.80	-	1.13	-	4.99	6.12
BVH16	61.0	58.70	33.44	92.14	2.20	1.04	-	3.26	5.05
BVH17	26.7	18.20	49.90	68.10	-	0.55	-	-	4.70
BVH22	35.0	-	-	-	2.09	1.03	44.0	-	6.33
BVH28	38.7	30.71	48.74	79.45	1.80	0.78	-	2.83	4.52
BVH30	55.0	-	-	-	2.05	0.92	-	3.74	4.03
BVH33	30.5	48.50	113.50	162.00	2.60	0.81	-	4.44	4.97
BVH52	45.0	-	-	-	2.57	-	-	-	4.64
BVH53	50.0	-	-	-	2.30	0.97	-	-	5.71
BVH54	55.0	-	-	-	2.09	0.83	-	-	4.32
BVH55	45.0	-	-	-	-	0.62	32.7	4.05	5.25
BVH57	55.0	-	-	-	1.82	0.77	-	-	-
BVH58	60.0	-	-	-	2.04	1.23	-	-	4.26
BVH60	45.0	-	-	-	-	0.91	-	3.58	4.72
BVH62	45.0	-	-	-	1.70	0.89	22.0	3.31	4.96
BVH63	56.0	58.20	45.80	104.00	2.24	0.91	-	4.10	5.70
BVH64	45.0	-	-	-	-	0.81	29.6	3.50	4.56
BVH66	45.8	43.70	45.90	89.60	2.05	0.68	-	3.27	6.13
BVH67	32.6	37.23	73.56	110.99	1.78	0.92	23.0	-	-
BVH68	40.6	44.85	62.60	107.45	-	-	-	4.17	5.07

Abbreviations: EF - Ejection fraction, SV – Stroke volume, ESV – End systolic volume (Left Ventricle), EDV – End diastolic volume (Left Ventricle), TAPSE – Tricuspid annular plane systolic excursion, LVOT – Left ventricular outflow tract (Peak Velocity), PASP - Pulmonary arterial systolic pressure, LVIDS – Left ventricular internal diameter end systole, LVIDD - Left ventricular internal diameter end diastole.

	Peak E wave (m/s)	EA Ratio	E'E Ratio	TR Velocity (m/s)	Weight (kg)	HR (bpm)	Age	BMI (kg/m^3)	M/F
BVH5	0.44	0.62	9.60	-	115	-	70	43.28	M
BVH12	0.90	0.89	-	2.61	72	-	68	29.21	F
BVH16	0.96	1.50	11.30	-	110	-	65	39.92	M
BVH17	0.86	1.86	17.09	3.27	47	-	76	17.91	F
BVH22	-	-	-	3.31	86	63	78	28.73	M
BVH28	1.01	1.13	22.54	2.58	53	-	81	16.73	F
BVH30	0.92	1.05	9.69	2.16	76	-	72	32.89	F
BVH33	0.86	0.72	12.36	-	67	73	69	24.02	F
BVH52	0.62	0.63	6.79	1.54	54	-	60	22.77	-
BVH53	1.06	1.18	11.18	-	105	-	70	33.14	-
BVH54	0.75	1.31	9.05	-	92	70	76	28.40	-
BVH55	0.53	0.55	-	2.63	-	68	78	-	-
BVH57	0.57	0.78	10.33	-	-	-	74	-	M
BVH58	0.43	0.47	5.69	-	-	-	68	-	M
BVH60	0.95	1.38	14.07	-	103	60	66	34.41	-
BVH62	0.35	0.45	6.55	2.18	80	66	72	31.25	-
BVH63	0.61	1.05	7.85	-	105	-	57	35.49	-
BVH64	0.64	0.75	-	2.48	64	88	46	22.41	-
BVH66	0.48	0.57	9.80	-	90	75	60	28.72	-
BVH67	0.59	0.56	10.61	2.39	-	69	74	-	-
BVH68	0.67	0.71	-	-	-	-	79	-	M

Abbreviations: TR Velocity – Tricuspid regurgitation velocity, HR – Heart rate, BMI – Body mass index, M/F – Male/Female.

Appendix J. “Normal” reference values for ECHO data

ECHO Parameter	“Normal” Myocardium
EF (%)	55-70
SV (ml)	60-120
ESV (ml)	19-50
EDV (ml)	56-104
TAPSE (cm)	1.5-2.5
LVOT (m/s)	0.8-1.0
PASP (mm/Hg)	≤35
LVIDS (cm)	2.0-4.0
LVIDD (cm)	3.5-5.6
Peak E wave (m/s)	0.6-0.8
EA Ratio	1-2
E’E Ratio	<8
TR Velocity (m/s)	<2.55

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