The role of interleukin-10 in coronary artery disease

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MSc by Research

School of Science, Engineering and Environment

2023

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List of Abbreviations

BVH – Blackpool Victoria hospital

CABG - Coronary artery bypass graft surgery

CAD - Coronary artery disease

CTGF - Connective tissue growth factor

CV - Cardiovascular

DAILYS - Disability Adjusted Life YEARS

DCF - 2',7'-dchlorofluorescein

DCFDA - 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate

DCFH - dichlorodihydrofluorescein

DMEM - Dulbecco's Modified Eagle Medium

ECC - excitation-contraction coupling

ECHO - Echocardiogram

EDV - End diastolic volume

EF - ejection fraction

ELISA - Enzyme-linked immunosorbent assay

FBS - Foetal bovine serum

GRS - Genetic risk score

H_2O_2 - Hydrogen peroxide

HRP - Horseradish peroxidase

IL - Interleukin

IL-1β- Interleukin-1β

IL-6- Interleukin-6

IL-10 – Interleukin-10

IL-10R1- IL-10 receptor-1

IL-10R2 - IL-10 receptor-2

INF-γ - Interferon gamma

JAK - Janus Kinase

LDL – Low density lipoprotein

LV – Left ventricular

LVOT - Left ventricular outflow tract

NCX - Sodium-calcium exchanger

NF-κβ - Nuclear factor kappa-β

NLRP3 - NOD-like receptor protein 3

NOX - Nicotinamide adenine dinucleotide phosphate oxidase

OS - Oxidative stress

PARP-1 - Poly(ADP-ribose) polymerase 1

PASP - Pulmonary arteriole systolic pressure

ROS - reactive oxygen species

RV – Right ventricular

STAT - Signal transducer activator of transcription

TASPE - Tricuspid annular plane systolic excursion

TGF-β - Tissue growth factor-β

Th-1 – T-helper-1

TLR4 - Toll-like receptor 4

TMB - Tetramethyl-benzidine

TNF-a - Tumour necrosis factor alpha

WHO – World health organisation

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my principal supervisor Dr David Greensmith for the invaluable support and guidance provided during this research project, and mentorship towards my journey as an early career researcher.

Secondly, I would like to thank the Nidal Bittar and the whole team at Blackpool Victoria hospital, for their support recruiting patients and collating patient data. Along with a special thank you to all CADVCAD patients for without their participation this research would not exist.

I also would like to thank all members of the Greensmith lab, especially Dr Amy Foster, for without their constant motivation and academic support this thesis would not exist.

A special thank you to my parents, Karen Samphire, Graeme Noden, Darren Chester, and my sister Holly, as well as my partner Matthew Mills. For their constant love and support throughout my academic journey, thank you for encouraging me to pursue my dreams. For that I will forever be grateful, I dedicate this thesis to you.

Abstract

Coronary artery disease (CAD) is a leading cause of mortality and morbidity worldwide, accounting for 66,000 deaths annually in the UK. Atherosclerosis is the underlying pathophysiological mechanism which drives CAD progression, producing an inflammatory state within coronary arteries reflected by increased cytokine levels. Oxidative stress (OS) arises through imbalance of reactive oxygen species (ROS) and antioxidants and has been indicated as a key player in development and progression of atherosclerosis. We know increased levels of cytokines and OS are associated in cardiac dysfunction in other diseases, but it isn't clear in CAD.

This study sought to measure serum levels of IL-10 in a CAD patient cohort and correlate to indices of systolic and diastolic function. Alongside elucidating the role of pathological IL-10 levels on OS and cellular viability in challenged and non-challenged cells.

This study was conducted in accordance with IRAS ethical approval (ID: 247341). Patient blood samples were collected pre-operatively and IL-10 levels quantified using enzyme-linked immunosorbent assay. Those levels were then correlated to indices of cardiac dysfunction extracted from patient echocardiological (ECHO) records. Levels of oxidative stress and cellular viability were measured using DCFDA and MTT respectively on a microplate reader, in response to hydrogen peroxide challenge.

Average patient IL-10 concentrations were 4.20 pg/ml $\pm$ 0.01 pg/ml (n = 70). Peak E-wave velocity negatively correlated with plasma IL-10 concentration (n = 53, R^2 = 0.08, p = 0.04) however, did not correlate with further indices diastolic dysfunction – EDV. Indices of systolic function (EF, LVOT and PASP) did not correlate with IL-10. A significant increase in fluorescence was measured regarding oxidative stress (p = <0.001 and 0.004) in response to H_2O_2 treatment in addition to a significant decreased in relation to cellular viability (p = 0.02 and 0.03), which validated our method. Microplate reader data showed IL-10 played no role in oxidative stress nor a cardioprotective role in cells with a high baseline OS. These finds were also reflected in cellular viability, with IL-10 having no effect on cellular viability both in challenged and non-challenged cells.

Overall, these findings show IL-10 plays a lesser role regarding CAD, OS and cellular viability than expected. Limited correlations seen in this preliminary study suggest interleukin-10 to be of little use as a biomarker for assessing dysfunction in CAD in relation to both diastolic and systolic function. Further research is required to increase study power.

1.1 Introduction to coronary artery disease

Coronary artery disease (CAD) is one of the most prevalent diseases worldwide, estimated to affect 200 million globally and is a leading cause of mortality equating to 9.1 million deaths per annum (British Heart Foundation 2023). Disease aetiology and progression is strongly influenced by both genetic and lifestyle risk factors. Disease pathology is considered to be multifactorial, and the pathophysiological hallmark recognised as the development of atherosclerotic plaques. Plaque formation in the vessel lumen of the coronary arteries impede blood flow to the myocardium resulting in an inadequate supply of oxygen (ischemia). The disease manifests as stable or unstable angina, myocardial infarction (MI) or sudden cardiac death (Malakar *et al.* 2019).

1.2 Epidemiology of coronary artery disease

Cardiovascular diseases are the leading cause of mortality and morbidity with an estimated 620 million people affected worldwide, of which, CAD is the most prevalent equating to 9.1 million deaths, 34% of the total global death burden (figure 1) (British Heart Foundation 2023)**.** Disease progression is strongly linked to genetics, co-morbidities, controllable (obesity, inactivity, and smoking) and non-controllable (age, sex and race) risk factors.

Figure 1: Global prevalence of cardiovascular disease (British Heart Foundation 2023).

However, while the mortality rate in the UK has declined by 70% over the last 30 years (figure 2), prevalence has remained constant at 3% (Bhatnagar *et al.* 2016). Studies have contributed improvements in mortality to progression in modern medicine and reduction in cardiovascular risk factors rather than a decline in incidence of the disease (Unal *et al.* 2004, Bhatnagar *et al.* 2016).

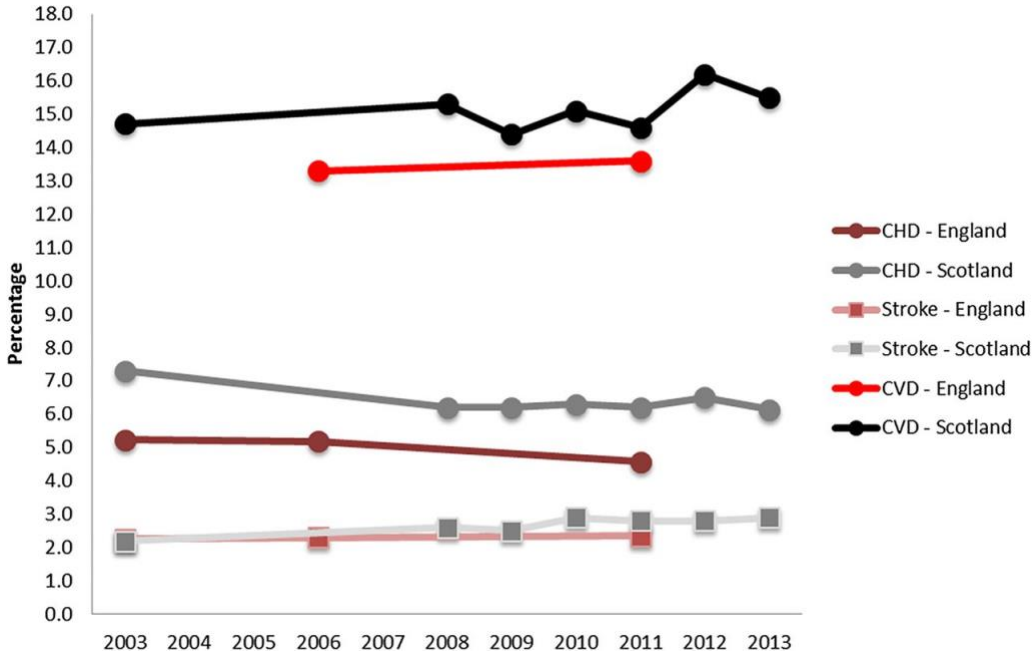

Figure 2: Trends in the prevalence of CHD, CVD and stroke in England and Scotland from 2003-2013 (Bhatnagar et al. 2016).

CAD is no longer solely associated with developed countries; epidemiological studies have shown an increase in the prevalence of CAD in developing countries and is a leading cause in disease burden (figure 3). Low- and middle-income countries account for 7 million deaths and 129 million Disability Adjusted Life Years (DALYs). Increase in disease burden has been linked to socio-economic and life-style changes as well as increased life span (Gaziano *et al.* 2010, Ralapanawa and Sivakanesan 2021). However, increase in mortality can also be accounted to lack of infrastructure in less industrialised countries leads to gross inequality's in cardiovascular health care (Kreatsoulas and Anand 2010).

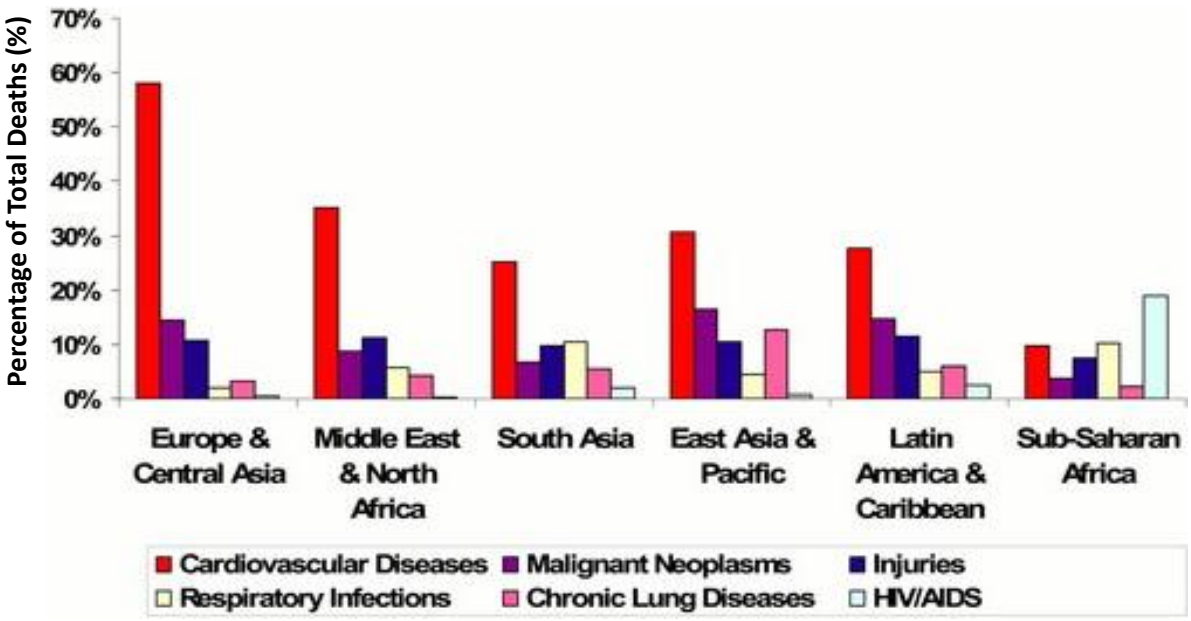

Figure 3: Major causes of death in low- and middle-income regions worldwide, cardiovascular diseases cause the highest percentage total of death in all continents except sub-Saharan Africa (Gaziano 2005).

Overall whist we observe a continuous fall in the incidence of CAD in the UK, we see no reduction in the prevalence. This may be equated to a decrease in mortality driven effective by pharmacological and surgical intervention. Global CAD burden is expected to increase in both developed and un-developed countries associated with a rise in risk factors and co-morbidities amongst the aging population. As global burden increases, we see a reflection in the economic burden of the disease, equating to £19 billion annually, as well as a £7.4 billion burden on the NHS (British Heart Foundation 2020 , National Institute for Heath and Care Excellence 2023)

1.3 Aetiology of coronary artery disease

The aetiology of CAD is considered to be extremely heterogeneous encompassing interplay between genetic and environmental risk factors. Various epidemiological studies have sought to elucidate the role of risk factors in CAD pathogenesis and progression. Risk factors identified fall into discreet categories; modifiable including obesity, hypertension, hyperlipidaemia and smoking and non-modifiable factors such as age and sex as well as genetic influence.

1.3.1 Modifiable risk factors

The Framingham study (1958) was the first to describe modifiable risk factors in CAD, the study highlighted hypertension, hyperlipidaemia, and obesity as factors of interest.

Hypertension is clinically defined by either a systolic blood pressure >140 mmHg or diastolic pressure >90 mmHg, with a reported prevalence of 31.1% worldwide (Fuchs and Whelton 2020). Hypertension is the leading preventable risk factor for CAD and is shown to directly attribute towards 57 % of CAD cases globally (Wu *et al.* 2015, Mills *et al.* 2020). Cardiovascular risk associated with hypertension is greatly reduced with effective anti-hypertensive therapy, therefore intensive statin therapy is recommended for patients diagnosed with clinical and pre-clinical CAD (Rosendorff *et al.* 2015).

Another considered risk factor in CAD is hyperlipidaemia, which refers to elevated cholesterol and triglyceride levels (>200 mg/dL) within blood (Nelson 2013). Excess levels of lipids have been associated with pro-atherosclerotic effects caused by enhanced platelet thrombus formation (Lacoste *et al.* 1995). Hyperlipaemia has been shown to not only play a direct role on mortality in CAD, but studies have also shown interplay between high cholesterol levels and hypertension (Rosendorff *et al.* 2015, Ariyanti and Besral 2019). However, findings from the Lipid Research Clinics Coronary Primary Prevention Trial found established therapeutic interventions, such as statins, correlated with a reduced risk of mortality and morbidity in CAD (Nelson 2013).

Finally, obesity has been indicated to play a key role in CVD development. The world health organisation (WHO) recognise obesity, defined as a body mass index of >30kg/m^2, as a global pandemic (World Health Organisation 2021). In the UK alone it is predicted 31% of adults and 15% of children to be classified as clinically obese, we also observe these trends globally (Powell-Wiley *et al.* 2021). Obesity is not only an independent risk factor for CAD associated noted to progress disease acceleration but also increases the probability of risk factors previously discussed. Therefore, weight loss through a combined decreased caloric intake and increased in physical activity is recommended in obesity and has been shown to be favourable in predicating lower incidences of CAD (Ades and Savage 2017).

1.3.2 Non-modifiable risk factors

Coronary artery disease aetiology has also been associated with non-modifiable risk factors such as age and gender.

Age plays a vital role in increased risk of cardiovascular disease through deterioration of functionality, leading to higher prevalence of CAD independent of gender in the aged population (Rodgers *et al.* 2019) . Increased prevalence, 70% in those aged 75, is directly related to CAD caused mortality and morbidity, resulting in major healthcare burdens worldwide (Yazdanyar and Newman 2009).

Gender has also been implicated as a risk factor in CAD, with an observed gender bias towards men. Compared to women there is an 17 % increased risk in CAD development in age matched males, development of CAD is also shown to be slower in women (Roeters van Lennep *et al.* 2002, Maas and Appelman 2010). The cardioprotective role observed in women (pre-menopausal) is believed to be related to the sex hormone oestrogen, specifically oestradiol. Oestradiol's antioxidant effects have been shown to limit cardiac remodelling and attenuate hypertrophy by mediating angiogenesis, oxidative stress, and fibrosis (Iorga *et al.* 2017).

Given non-modifiable risk factors cannot be controlled, decrease of CAD risk and treatment in is focused on associated lifestyle changes including weight management, increase in physical exercise and cessation of smoking (Rodgers *et al.* 2019).

1.3.3 Genetic risk factors

Genetic influence in CAD is considered to be equivalent to environmental risk factors. Familial clustering of CAD has been reported since the 1950s, early studies demonstrated heritability between first degree relatives a predicted onset of CAD (Dai *et al.* 2016). Early Swedish twin studies approximate the role of heritability in CAD is estimated to be 50% (McPherson and Tybjaerg-Hansen 2016). Numerous genes that underlie CAD as well as their associated loci have been identified through high-throughput genetic sequencing. Discovery of genetic factors has been vital in the development of the genetic risk score (GRS), allowing improved risk prediction and guidance for preventative treatment (Dai *et al.* 2016).

1.4 Echocardiology in coronary artery disease

Imagining techniques such as echocardiography (ECHO) are a key diagnostic and monitoring method in CAD. It is a non-invasive technique which uses ultrasound waves to provide comprehensive data regarding cardiac function and haemodynamic (Sadeghpour and Alizadehasl 2022). The basic principle of an ECHO regards the reflection of ultrasound waves in the range of 4 – 7 MHz from an external probe between different tissue densities (Mohamed *et al.* 2010). The waves reflect off the myocardial structures and are detected via a transducer, processed as information, and displayed as an image (Ashley and Niebauer 2004).

The first developed form was M-mode echocardiography, in which provides a 1D view with a high temporal and spatial resolution. However, the real anatomy of the heart cannot be well visualised but provides precise measurements of cardiac time intervals (figure 4A) (Fagiry *et al*. 2019). Major advancements have led to 2D and 3D echocardiography which today are the most commonly used method for assessment of cardiovascular diseases. Two-dimensional

ECHO's allows structures to be viewed in real time in a cross section of the heart, commonly used due to ease of use, price and repeatability (figure 4B/D) (Fagiry *et al*. 2019). The development of 3D echocardiograms in 1974 allows imagining of cardiac structures to be shown closely to their real forms (figure 4C). It has several advantages over 2D ECHO's including improvement of visualisation of complex structures, assessment of valvular dysfunction and calculation of cardiac volumes and mass however requires a higher degree of training (Maleki and Esmaeilzadeh 2012).

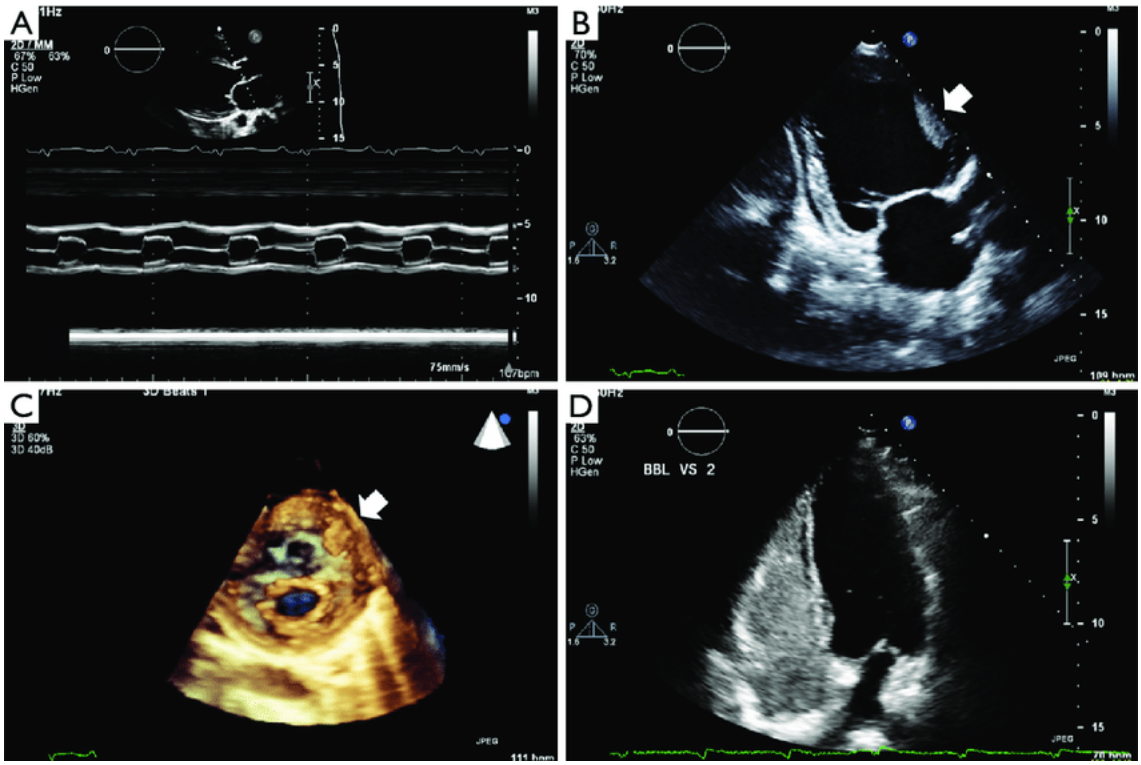

Figure 4: Comparative of different echocardiograph modalities. (A) M-mode imaging of the left atrium and aortic value. (B) 2D imaging of the left ventricle with mass adherent to anterior wall. (C) 3D view of the left ventricle with mass adherent to anterior wall. (D) 2D contrast echocardiography of the right ventricle (Di Franco *et al.* 2017).

Functional information pertained by analysis of echocardiography allows assessment of overall cardiac function this can be further explored in relation to both systolic and diastolic function using a variety of obtained ECHO parameters.

Systolic function describes normal contractility of the heart however, in pathology specifically cardiovascular diseases we observe systolic dysfunction represented by impaired ventricular contraction. The most validated method for measurement of systolic function is ejection fraction (EF), with a range between 55-70% considered normal (Klaeboe and Edvardsen 2019). Dysfunction is clinically identified by a reduction in EF measured at > 40% (Moon *et al.* 2009). Although most commonly used EF is not the only measure of systolic function that can be obtained from echocardiography, another commonly used parameter is left ventricular outflow tract obstruction (LVOT) which refers to the limitation of blood flow from the ventricle. Normal LVOT level is clinically defined as <30mmHg and is an useful index to determine hypertrophy or dilation within the heart (Slama *et al.* 2016). Systolic dysfunction in patients with CAD increases mortality and morbidity therefore effective diagnosis and monitoring through indices of cardiac function obtained by echocardiography is key towards disease progression and management.

As well as systolic, diastolic dysfunction can also be measured using indices of function obtained from ECHO's. Diastolic dysfunction refers to a disturbance in ventricular relaxation and filling (Leite-Moreira 2006). The most used ECHO parameter for assessment of diastolic function is E/a ratio which is defined as the ratio of peak early to late diastolic filling velocity (Chung *et al.* 2018). In dysfunction we observe a greater A velocity (late ventricular filling velocity) within the ratio and is characterised by a ratio <2.5 (Mottram and Marwick 2005). However other parameters such as peak E-wave velocity, velocity of blood flow during early diastole and end diastolic volume, defined as the volume of blood in the ventricle at the end of diastolic filling can also be used to measure and provide further information on diastolic dysfunction within the heart. In CAD diastolic function is the first cardiac function to be impaired therefore detection and monitoring through echocardiography is essential is disease diagnosis and management (Jamiel *et al.* 2016) .

1.5 Treatment

Treatment of CAD is dependent on disease severity and may encompass both secondary preventative and interventive action, individually specialised to each patient. Secondary preventative treatment focuses on prevention of recurrent coronary events through aggressive life-style changes. (Sigamani and Gupta 2022). Whilst interventive treatment focuses on pharmacotherapeutic and surgical intervention to aid in the reduction of further major cardiovascular events (Gaudel *et al.* 2022)

1.5.1 Life-style changes

Risk of developing CAD has been strongly linked to risk factors influenced by lifestyle habits, in turn emphasis on lifestyle changes to address risk factors is important in initial disease management (Mozaffarian *et al.* 2008). Risk factors include obesity, dyslipidaemia, hypertension, and diabetes therefore, treatment focuses on weight management through diet modification and increased physical activity, alongside smoking cessation.

1.5.2 Pharmacology

Pharmacological interventions are considered the cornerstone of CAD treatment usually comprising of combination of drug therapies, primarily anti-ischemic and anti-thrombotic agents, to regress atherosclerosis (Doenst *et al.* 2022).

Anti-ischemic therapy, typically beta blockers or calcium channel blockers are prescribed as first line cardioprotective agents, treatment is known to aid reduction in long term mortality, through improvement of ejection fraction, restriction of ventricular hypertrophy and lowering myocardial oxygen demand (Smith *et al.* 2006, Sigamani and Gupta 2022). Therapeutic mechanisms of action of beta blockers occur through restriction of β-adrenergic receptors

reduce heart rate and increase diastolic filling time to mitigate workload (Sigamani and Gupta 2022).

Anti-thrombotic therapy is associated with reduction of mortality and risk reduction of further cardiovascular (CV) events (MI and stroke) (Doenst *et al.* 2022). Anti-thrombotic action can be achieved various therapeutic approaches, one which is dual antiplatelet therapy using Acetylsalicylic acid (Aspirin) or Clopidogrel alongside a P2Y12 inhibitor (Floyd 2020). Aspirin mechanism of action is through reduction of thromboxane synthesis by inhibition of cyclo-oxygenase, required in plated aggregation and activation (Ornelas *et al.* 2017). Another method is through lipid-lowering agents, such as statins, that act through inhibition of platelet aggregation and coagulation cascade as well as exerting a cholesterol lowering effect which aids in plaque stabilisation (Violi *et al.* 2013).

1.5.3 Surgery

In severe CAD cases where lifestyle and pharmacological therapeutics are not adequate to stabilise or prevent symptoms and further CV events, surgical intervention is required. Revascularisation therapy consists of two surgical procedures percutaneous coronary intervention (PCI) in which a stent is placed to remove occlusion of the artery and restore bloody supply, or coronary artery bypass surgery (CABG) in which blood is bypassed from site of obstruction using healthy vessels usually from the arm or leg (figure 5) (Ahmad *et al.* 2023, Bachar and Manna 2023).

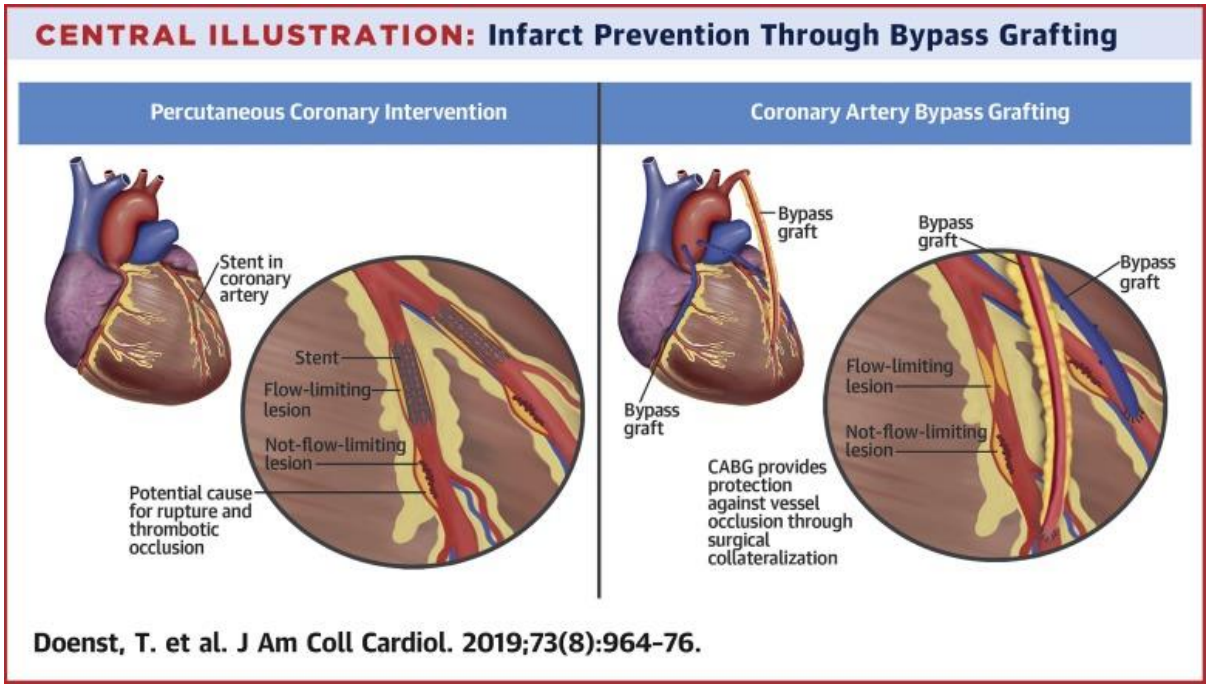

Figure 5: Comparative illustration of the mechanistic differences between PCI and CABG (Doenst *et al.* 2019).

PCI is recommended as treatment for acute coronary events due to minimally invasive procedure allowing speed of application whereas CABG is recommended as treatment of anatomically complex CAD and/or previous PCI intervention has failed (Doenst *et al.* 2022, Bachar and Manna 2023). Whist CABG is the gold standard in CAD treamtent, with 400,000 surgeries perfomed yearly, risk factors such as co-morbiidities and age may dictate eligability of surgical intervention (Alexander and Smith 2016).

1.6 Pathophysiology of coronary artery disease

The pathophysiology of CAD is complex however formation of atherosclerotic plaques is believed to be the hallmark. Inflammation has been documented as a key mechanism in which CAD pathogenesis is promoted as well as playing a major role in the development of atherosclerosis. Finally, myocardial dysfunction is believed to play a role in CAD however the

process which drive this are less understood one observed mechanism is through an increase in oxidative stress which is further discussed in section 1.7.2.

1.6.1 Atherosclerosis

The pathophysiological hallmark of CAD is believed to be the formation of atherosclerotic plaques within the coronary arteries, plaque instability extubated by the inflammatory response led to rupture and occlusion of coronary arteries by resulting thrombus. This occlusion in coronary arteries can cause myocardial infarction.

Atherosclerosis is characterised by calcification, accumulation of lipids and fibrosis elements within arteries. The process is initiated by endothelium activation resulting in a cascade of events that lead to activation if inflammatory pathways and vessel narrowing to promote atheroma formation (Jebari-Benslaiman *et al.* 2022).

The initial stage of atherosclerosis is formation of the fatty streak (fig 6). Formation is initiated by endothelial dysfunction caused by disturbed laminar flow, this allows low-density-lipoproteins (LDL) to cross the endothelial layer and accumulate in the intima (Kowara and Cudnoch-Jedrzejewska 2021). Oxidative modification of accumulated LDLs driven by inflammatory cells as well as atherogenic factors, lead to macrophage recruitment, via activation of macrophage receptor CD36 to transduce intracellular signalling to promote nuclear factor kappa B (NF-κB) activation and cytokine expression (Kowara and Cudnoch-Jedrzejewska 2021). This recruitment of immune cells to the intima by activated endothelial cells initiate to plaque development. Furthermore, phagocytosis of LDLs by macrophages promotes foam cell formation the prototypical cells to instigate formation of the fatty streak (Yu *et al.* 2013, Jebari-Benslaiman *et al.* 2022). Alongside activated inflammatory signalling pathways further promote plaque formation.

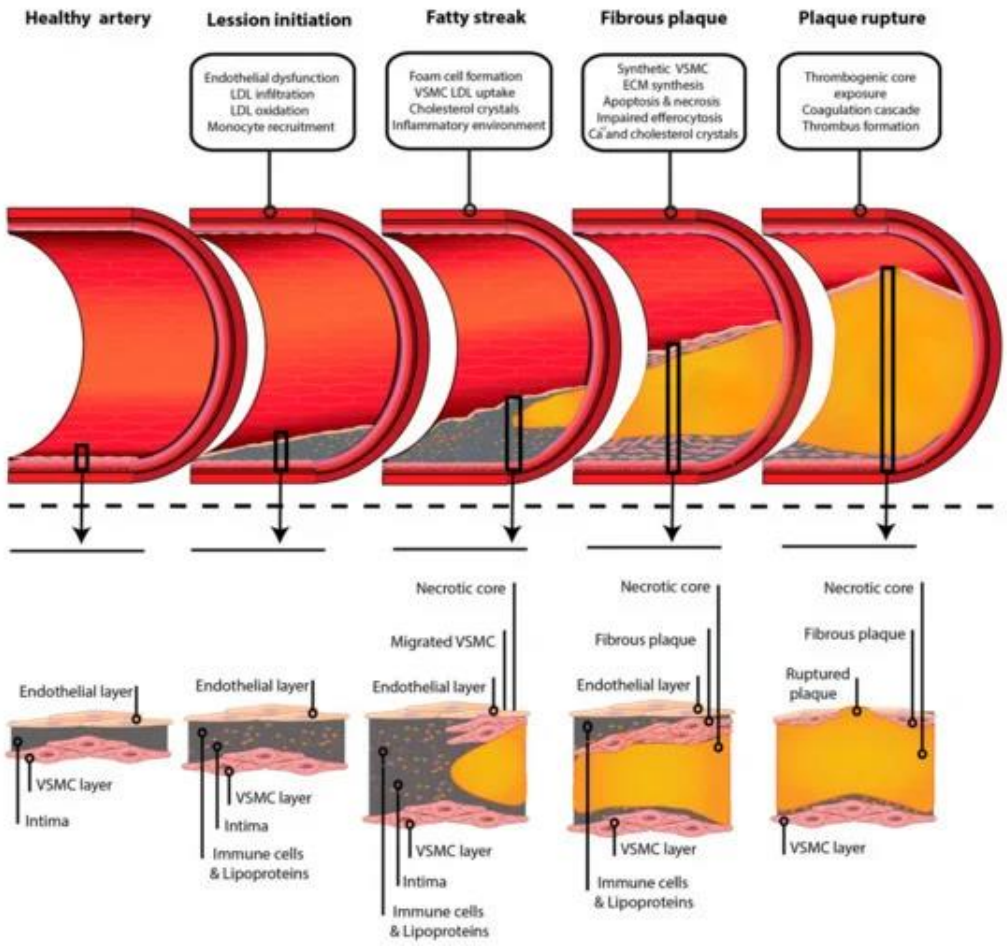

Figure 6: Outline of the events that contribute to atherosclerotic plaque formation, importantly fatty streak, and fibrotic plaque formation (Jebari-Benslaiman *et al.* 2022)

Final stage of atherosclerotic plaque formation is the development of the fibrotic plaque, commenced by transition of the fatty streak to intimal growing (figure 6) (Jebari-Benslaiman *et al.* 2022). The plaque consists of two key features; the necrotic core a cell free lipid rich area, and the fibrous cap to stabilise the core. The necrotic core is considered the nucleus of the plaque, within the core macrophage death and impaired apoptotic cell removal contribute to thrombogenicity, OS and inflammation which increases plaque vulnerability (Linton *et al.* 2016). The stability the fibrotic cap proves ensures the pro-thrombotic core cannot trigger thrombosis (Linton *et al.* 2016). Development of the necrotic core and fibrous cap, thus fibrotic plaque is indicative of advanced atherosclerosis and plaque regression is unlikely.

1.6.2 Role of inflammation in atherosclerosis

Inflammation plays a crucial role in the pathophysiology of atherosclerosis, hence CAD. It is involved in all stages of atherosclerosis including plaque development, progression, and rupture (Mehu *et al.* 2022). Alongside elevated levels of inflammatory markers, such as cytokines, are predictive of cardiovascular events as well as correlate to CAD mortality.

During the early stages of atherosclerosis oxidised LDLs induce pro-inflammatory responses in endothelial cells and macrophages causing endothelial damage (Jebari-Benslaiman *et al.* 2022). LDL also activates signalling receptor toll-like receptor 4 (TLR4) dependent and independent via activation of (NF-κB) induces secretion of cytokines IL-1β, IL-6 and TNFα (figure 7) (Miller *et al.* 2005). Furthermore, binding of LDL and specific antibodies form immune complexes which induce inflammatory cytokine production and foam cell formation.

In the advanced stages after fatty streak formation, inflammation can influence the formation and destabilisation of collagen in the fibrinous cap. Whilst expression of inflammatory cytokine, IL-1β , released from T-cells and foam cells aid in degradation of collagen within the fibrous cap through promotion of matrix metalloproteases production leading to plaque instability and rupture which can result in CV events such as myocardial ischemia which promote progression of CAD (figure 6) (Alfaddagh *et al.* 2020).

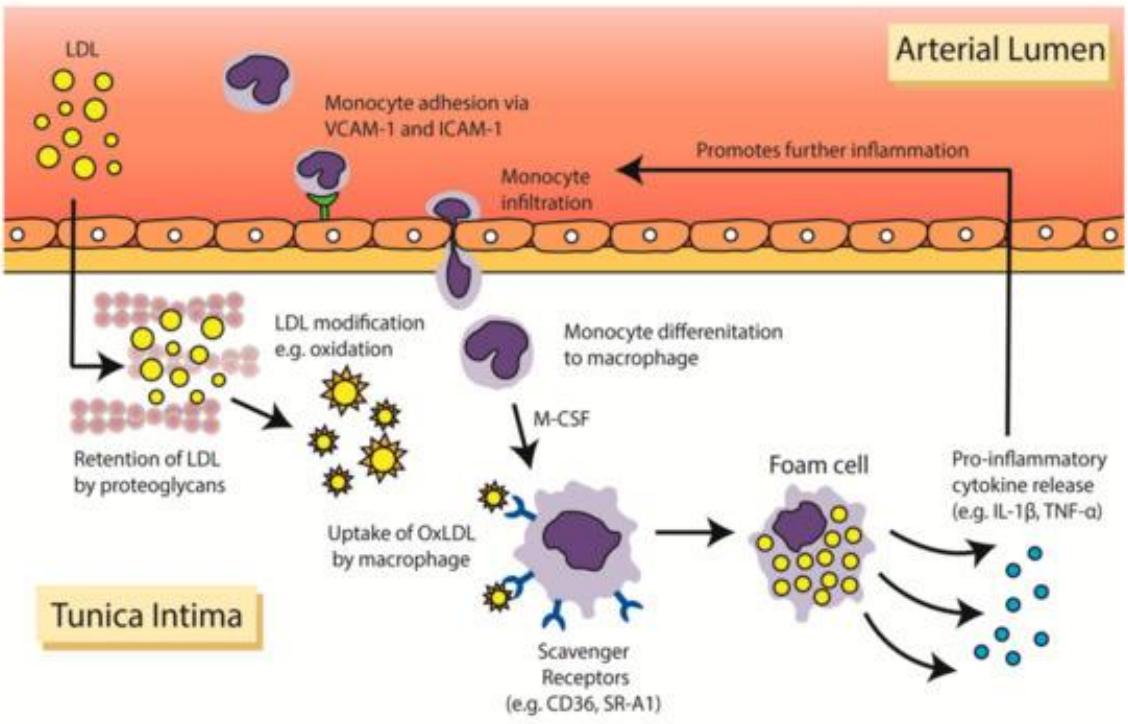

Figure 7: Diagram showing association between LDLs and monocytes in the activation of foam cells to induce pro-inflammatory cytokine release during inflammation (Nguyen *et al.* 2019).

Cells of the adaptive immune system, T and B lymphocytes, are equally important mediators of atherosclerosis. They are identified in plaques at the same time as macrophages and regulated macrophage elicited inflammatory response during atherogenesis. A diverse population of T cells are present and exhibit diverse functions within the atherosclerotic plaque. Secretion of pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-a) and interferon gamma (INF-y), by T-helper-1 (Th-1) lymphocytes accelerate atherosclerosis through activation of macrophages and endothelial cells (Wu *et al.* 2017, Nguyen *et al.* 2019). Whilst regulatory-T lymphocytes secret anti-inflammatory cytokine IL-10 and tissue growth factor-β (TGF-β) to downregulate the inflammatory process (Hansson 2005). B-lymphocytes act in similar manner, B2 cells secrete pro-inflammatory cytokines and activate Th-1 cells to exacerbate atherogenesis, whereas B1 cells produce antibodies and play a protective role in atherosclerosis (Nguyen *et al.* 2019).

1.7 Role of cytokines in coronary artery disease

Cytokines are small, secreted proteins of low molecular weight which exert pleiotropic effects to regulate intracellular communication between cells, through either autocrine, paracrine, or endocrine action. Cytokine is an umbrella term to describe; interleukins (produced by one leukocyte to exert its effect on another), lymphokines (produced by lymphocytes), monokines (produced by monocytes), chemokines (which exert chemotactic activity). The most predominant producer of cytokines are T-helper cells and macrophages however, they can be made by many other cell populations. Cytokines act in a network and are often produced in a cascade, within the network individual cytokines can secreted and act upon by multiple cell types in a synergist of antagonistic manner (figure 8) (Zhang and An 2007).

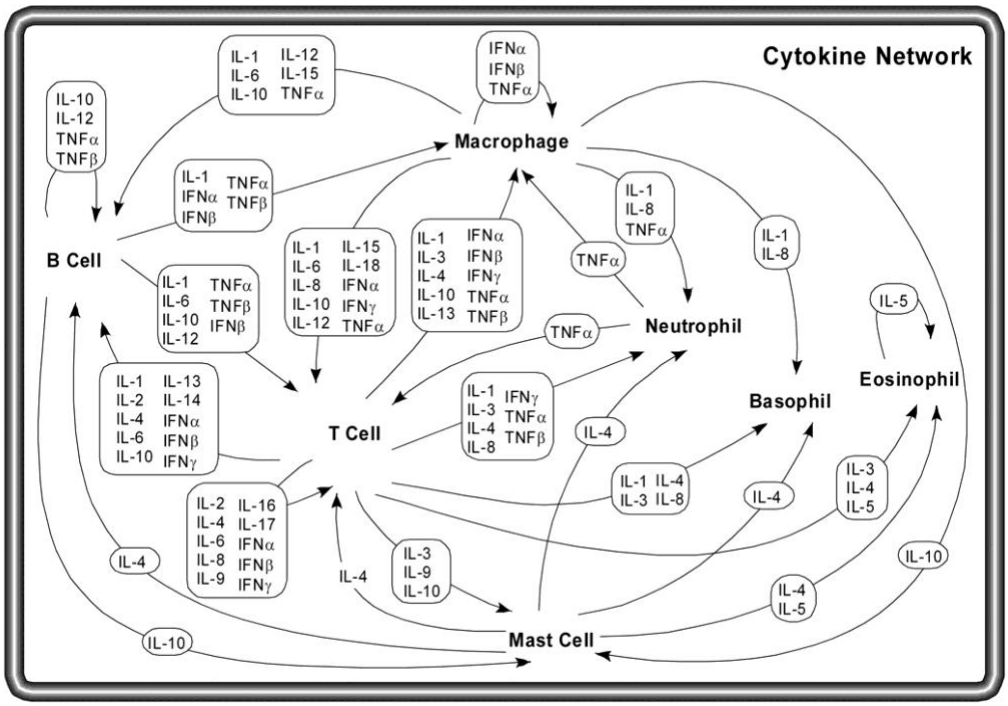

Figure 8: Diagram of the cytokine network, presenting the different immune cells which produce cytokines as well as the cell type each cytokine acts upon (Zhang and An 2007).

Cytokines, specifically interleukins, can be further categorised as pro- or anti-inflammatory. Pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α, upregulate inflammatory reactions via activation of immune cells as well as production and release of further cytokines (Kany *et*

al. 2019). Whilst anti-inflammatory cytokines, namely IL-10, exert immunoregulatory control on the pro-inflammatory response through suppression of antigens and immune mediators. Simultaneous release as well as balance between pro- and anti-inflammatory cytokines is essential in immune response and dysregulation is seen in the pathogenesis of many inflammatory diseases including atherosclerosis hence, CAD (Zhang and An 2007, Kany *et al.* 2019).

It is through cytokine mechanisms of inflammatory mediation in which dictates their importance in the pathogenesis of CVD, the mechanisms in which cytokines cause CAD are outlined in figure 9. CAD has strong association with pro-inflammatory cytokines IL-6, IL-1β, TNF-α in which they contribute to the formation of atherosclerotic plaque (Fatkhullina *et al.* 2016). Once the atheroma has been formed activated macrophages release various cytokines inducing tissue damage and inflammation, this cycle leads to further promotion of atherogenesis within the occluded vessels which induces endothelial dysfunction (Amin *et al.* 2020). Whilst expression of anti-inflammatory cytokines play an anti-atherogenic role and can attenuate plaque formation (Moss and Ramji 2016). As well as directly acting upon atherosclerotic lesions, IL-1, IL-6 and TNF-α can promote expression of pro-atherogenic genes (Yudkin *et al.* 2000).

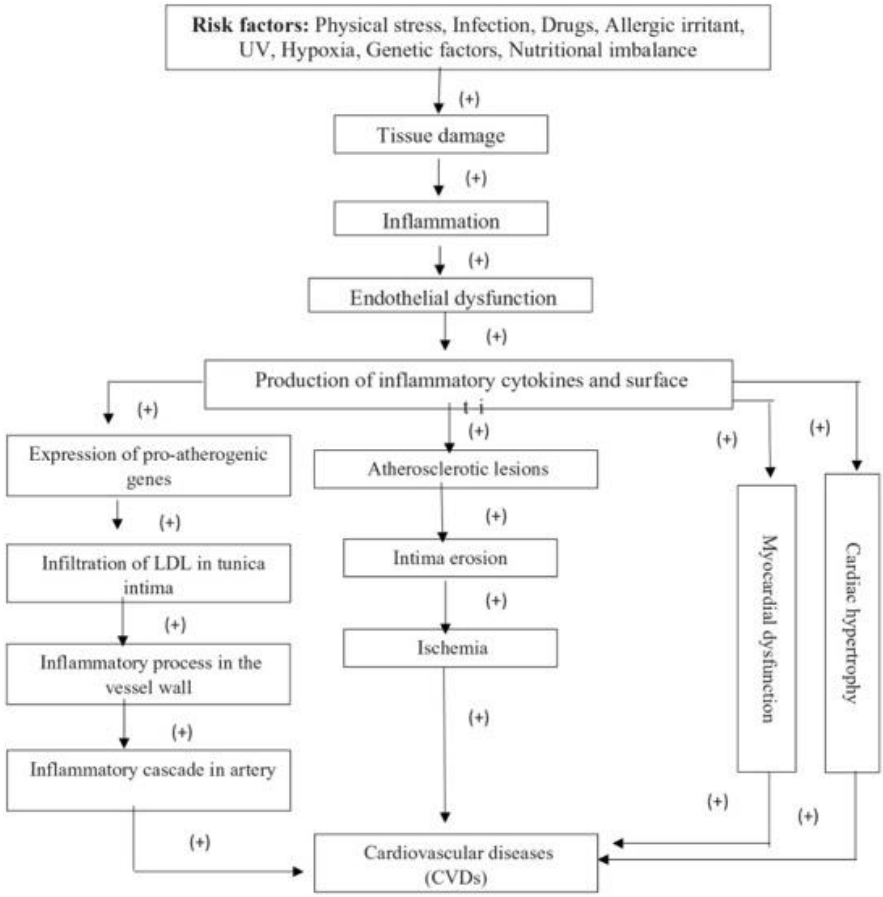

Figure 9: Mechanisms in which cytokine induced inflammation cause cardiovascular disease (Amin *et al.* 2020).

As well as the role cytokines play in the progression of atherosclerosis through inflammatory mediation, elevated levels of IL-1, IL-6 and IL-8 have been observed in atherosclerotic plaques in CAD patient cohort (Heinisch *et al.* 2005). Furthermore, elevated levels of important pro-inflammatory cytokines IL-6 and TNF-a have been associated with risk of CAD as well risk of mortality in CAD (Kaptoge *et al.* 2014, Clarke *et al.* 2018). Anti-inflammatory cytokines, such as IL-10, play a cardioprotective role in CAD through reduced atherogenic and improved plaque stability. However, human studies present conflicting evidence regarding association between IL-10 levels and risk of CVD events (Lakoski *et al.* 2008, Barcelos *et al.* 2019, Goldwater *et al.* 2019). These studies not only highlight the importance of inflammatory cytokines in progression of CAD but also showcase potential use as biomarkers for disease progression as well as predictors of mortality.

1.7.1 The role of interleukin-10

Interleukin-10 is a pleiotropic cytokine, with potent anti-inflammatory properties, known to play a role in inflammation and immune regulation (Iyer and Cheng 2012). In humans the IL-10 protein is encoded by the IL-10 gene and is a homodimer comprising of two 178-amino acid long subunits in humans. Major cellular sources of IL-10 include lymphocytes – specifically T-helper cells, monocytes, macrophages. However, B-cells, granulocytes – neutrophils and eosinophils, mast and natural killer cell are a possible important source of IL-10 (Mosser and Zhang 2008, Iyer and Cheng 2012).

The IL-10 receptor complex consists of two IL-10 receptor-1 (IL-10R1) and two IL-10 receptor-2 (IL-10R2) subunits. IL-10R1 binds with a high affinity to the IL-10 ligand, whereas IL-10R2 enables signal transduction following ligand binding (Verma *et al.* 2016). Binding between IL-10 and the IL-10 receptor complex activates Janus Kinase (JAK) / Signal Transducer and Activator of Transcription (STAT) signalling pathway. Activation of JAK1 through association with IL-10R1 and tyrosine kinase 2 through interaction with IL-10R2 induces activation of STAT family members STAT 1, 3 and 5 (Riley *et al.* 1999). Further phosphorylation allows dimerization of STAT molecules facilitating translocation to the nucleus, where it regulates various anti-apoptotic and cell cycle genes.

Furthermore, IL-10 controls inflammatory response through inhibition of pro-inflammatory cytokines from macrophages. Exposure to IL-10 is seen to repress inflammatory cytokines tumour necrosis factor alpha, IL-1, IL-6 and IL-12, as well as inflammatory chemokines (Mosser and Zhang 2008, Verma *et al.* 2016). Additionally, IL-10 can stimulate multiple cell types, specifically B-cells, as well as natural killer cells highlighting the complexity of IL-10 in immunoregulation and inflammation (Lakoski *et al.* 2008, Mosser and Zhang 2008).

The importance of IL-10 in CAD comes from its anti-inflammatory and anti-atherogenic properties, not only is IL-10 expression observed in early and advanced atherosclerotic plaques but associations between elevated levels and disease severity have been made in humans (Mallat *et al.* 1999, Lakoski *et al.* 2008, Barcelos *et al.* 2019). These studies highlight

the importance of IL-10 in CAD as a potential biomarker in disease diagnosis and progression, as well as highlight potential therapeutic benefits. Whist therapeutic potential of IL-10 in atherosclerosis, hence CAD has gained interest as of recent, there are limited clinical studies available. However, a recent study by Kim *et al* (2020) exhibited targeted nanocarrier delivery of IL-10 lead to significant regression in plaque size in a mouse model, whilst promising further studies are required to cooperate these findings (Kim *et al.* 2020).

1.7.2 Methods of detecting cytokines

Enzyme-linked immunosorbent assay (ELISA) was developed in 1971 pioneered by Swiss scientists Engvall and Perlmann (Aydin 2015). The technique detects and quantifies immunological reactions through the catalytic properties of enzymes (Alhajj *et al.* 2023), the specificity and sensitivity make ELISA the technique of choice for cytokine detection.

There are four main types of ELISA: direct, indirect, competitive and sandwich, however. The sandwich ELISA is mostly commonly used for cytokine detection and utilises a primary and secondary antibody to increase sensitivity and robustness. In this method, wells of the plate are coated with a primary 'capture' antibody, the sample is then added and incubated a specific time-period. After incubation, and following a washing step, a secondary 'detection' antibody is added and further incubated. Enzyme substrate is then added to produce a colorimetric change indicating detection of the antigen (figure 10) (Aydin 2015).

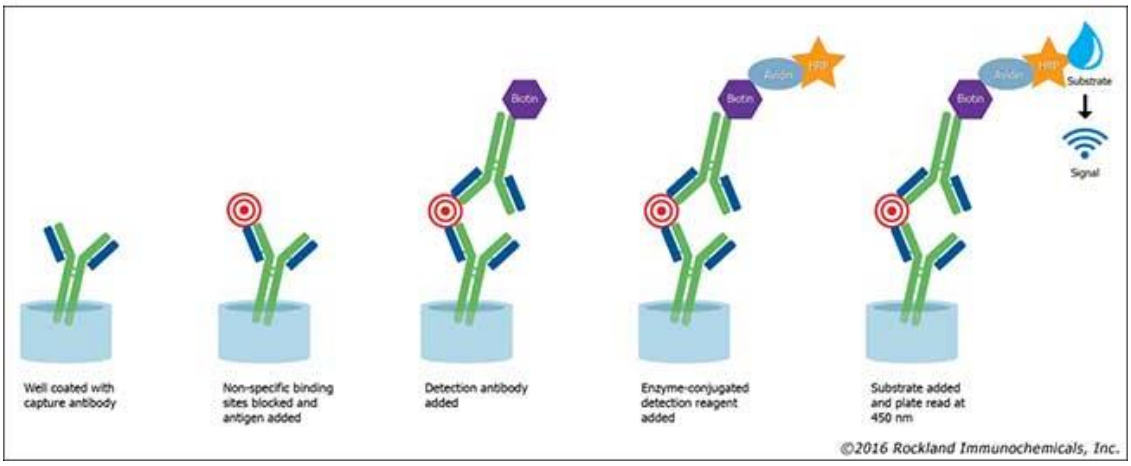

Figure 10: Principles of a sandwich ELISA (Optofluidic Bioassay 2021).

There are many advantages of using an ELISA specifically the reproducibility of the assay as well as the high sensitivity, specificity, and quantitative results. Another advantage is the wide commercial availability, as well as low-cost (Leng *et al.* 2008). However, there are disadvantages, kit performance is dependent on manufacture, antibody quality as well as operator skill (Zhou *et al.* 2010).

1.8 Role of oxidative stress in coronary artery disease

Oxidative stress arises due to dysregulations between ROS production and antioxidant defence. Reactive oxygen species (ROS) are oxygen containing free radicals that exhibit both beneficial and deleterious roles, examples include superoxide, hydroxyl radical and hydrogen peroxide (H_2O_2) (Dubois-Deruy *et al.* 2020). Reactive oxygen species can be produced by several pathways in cardiac myocytes, such as mitochondrial electron transport chain, nitric oxide synthases and NADP oxidases (Woo *et al.* 2021). In physiological conditions ROS are involved in cell differentiation, proliferation, and excitation-contraction coupling (ECC) in cardiomyocytes (D'Oria *et al.* 2020).

Dysregulation of ROS production, hence OS, has long been associated as a pathogenic mechanism in which drives CAD progression with elevated levels of oxidative stress observed in CAD patients (Lakshmi *et al.* 2013). Mechanisms in which OS induce damaged in the heart include myocardial dysfunction and activation of inflammatory pathways (Münzel *et al.* 2017, Aimo *et al.* 2020).

1.8.1 Role of oxidative stress in inflammation

As previously discussed, inflammation is a driving factor in the pathogenesis of CAD. One way in which inflammation is induced is through OS however, OS and inflammation have been found to have an interdependent relationship hence inflammation can further enhance OS leading to a cycle of damage (figure 11).

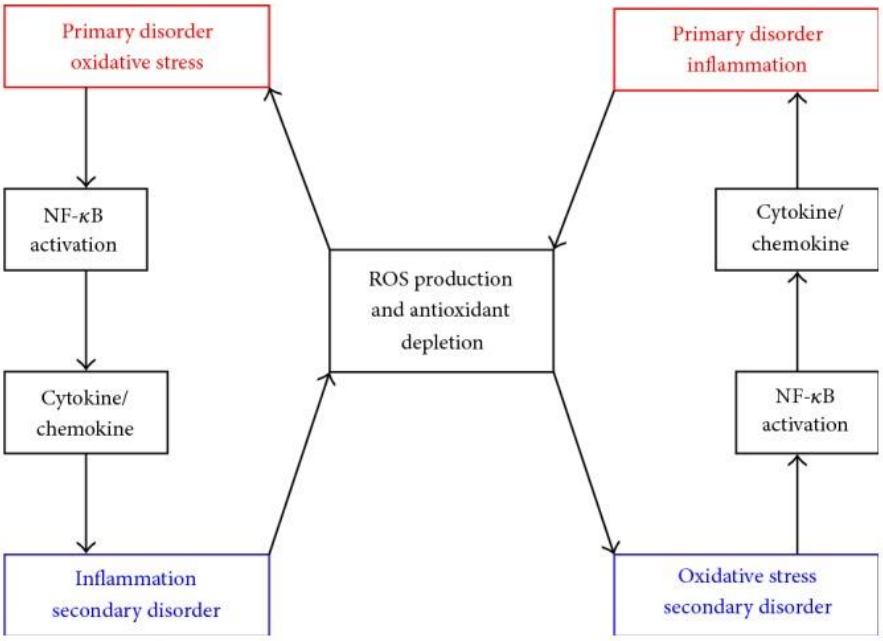

Figure 11: Interdependence between oxidative stress and inflammation. Both oxidative stress and inflammation can occur as a primary disorder, which is the first pathological process or event, and can both lead to further pathological events referred to as secondary disorders (Biswas 2016).

Oxidative stress induces inflammation through multiple key pathways. Firstly, ROS can induce inflammation through activation of NF-κβ, which is a transcription factor that regulates gene expression of pro-inflammatory cytokines (Biswas 2016). Oxidative stress can also act upon the innate immune system by activating NOD-like receptor protein 3 (NLRP3) inflammasome, which controls secretion and maturation of pro-inflammatory cytokines IL-1β and IL-18 (Shimada *et al.* 2012). There are multiple mechanisms in which ROS can activate the NLRP3 inflammasome, firstly by oxidised mitochondrial DNA during apoptosis and secondly by ROS released from damaged mitochondria causing IL-1β and localised inflammation (Shimada *et al.* 2012, Biswas 2016). Furthermore, increased levels of ROS can cause oxidative DNA damage which combined with upregulation in the expression of P53 leads to apoptotic cell death. ROS induced DNA damaged also causes impairment of cellular metabolic pathways and expression of inflammation meditator through stimulation of the nuclear enzyme poly(ADP-ribose) polymerase 1 (PARP-1) causing the development of an inflammatory state (Aimo *et al.* 2020).

As OS can induce inflammation, the inflammatory process can also induce OS. One pathway in which inflammation can induce oxidative stress is through production of ROS by activated phagocytes: neutrophils and macrophages. However, in pathological conditions exaggerated generation can lead to localised oxidative stress and tissue injury (Biswas 2016). Furthermore, pro-inflammatory cytokines play an important role in ROS generation. Overexpression of TNF- causes mitochondrial DNA damage as well as inhibiting antioxidant factors leading to an increase in ROS whereas, TGF-β increases mitochondria ROS generation through impairment of mitochondrial function and suppression of specific antioxidant – glutathione (Aimo *et al.* 2020). This cyclic process of Inflammation-OS induced damage further promotes progression and pathogenesis in CAD.

1.8.2 Role of oxidative stress in myocardial dysfunction

Oxidative stress is known to drive pathological conditions within cardiomyocytes that contribution to progression of myocardial dysfunction. One mechanism in which ROS contribute to myocardial dysfunction is through altered calcium homeostasis. Cardiac contraction is controlled through a process called excitation-contraction coupling (ECC), which is dependent on movement of intracellular calcium (Ca^{2+}) levels through Ca^{2+} channels. Entry of Ca^{2+} through L-type Ca^{2+} the sarcoplasmic reticulum triggers calcium-induced-calcium release from the ryanodine receptors, this influx of Ca^{2+} then binds to troponin C to induce contraction. Whilst removal of from the cytoplasm by SERCA and the sodium-calcium exchanger (NCX) induces cellular relaxation (Eisner *et al.* 2017, D'Oria *et al.* 2020). ROS can target several components of ECC. Firstly, ROS can interact with the ryanodine receptors, leading to increased open probability, irreversibly activation and increased Ca^{2+} leakage (Terentyev *et al.* 2008). Secondly, oxidative stress may induce modification in SERCA leading to irregular function (Balderas-Villalobos *et al.* 2013) . Abnormal function of key Ca^{2+} receptors and pumps lead to dysregulation in Ca handling therefore disturbed calcium homeostasis. Disturbed calcium homeostasis has been associated with both systolic and diastolic dysfunction in cardiomyocytes (Greensmith *et al.* 2010, Wagner *et al.* 2013).

Furthermore, ROS play a central role in the development of fibrosis which is seen to play a role in systolic and diastolic dysfunction in cardiomyocytes. ROS modulation of tissue growth factors, TGF-β and connective tissue growth factor (CTGF) play a key role in the development of fibrosis. ROS derived from nicotinamide adenine dinucleotide phosphate oxidase (Nox) – 2 can also activate T cell signalling pathways involved angiotensin-II induced differentiation and proliferation of cardiac fibroblasts (Fujii *et al.* 2005, Aimo *et al.* 2020). Chronic oxidative stress conditions can lead to prolonged fibrotic deposition results in hardening of the myocardium and poor tissue compliance leading to myocardial dysfunction (Jiang *et al.* 2021).

Finally, oxidative stress can induce cardiomyocyte apoptosis through modulation of signalling pathways. Firstly, ROS modulates the extrinsic signalling pathway which is stimulated by TNF-α, ROS can also activate an intrinsic pathway, regulated by B-cell lymphoma 2 (Bcl-2) family proteins through direct or calcium dependent opening of the mitochondrial permeability transition pore. Furthermore, activation of transcription factor p53 by ROS induced DNA damage further promotes the intrinsic pathway (Aimo *et al.* 2020). Oxidative stress induced apoptosis is an important mechanism which contributes to hypertrophic remodelling as well as cellular dysfunction reflected by ventricular remodelling in rodent and canine models. (Cesselli *et al.* 2001, Yamaguchi *et al.* 2003).

1.8.3 Methods of detection

As discussed in 1.8 OS arises due to excess generation of ROS, this overproduction is indicated in many pathological precursors that drive CAD namely atherosclerosis. However, as OS is a redox imbalance between oxidants and antioxidants it cannot be measured therefore oxidative stress conditions in are determined using direct measurements of cellular ROS levels (Katerji *et al.* 2019, Azzi 2022).

One method of directly measuring cellular levels of ROS is through the use of fluorogenic probes, one example being 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA). DCFDA assay is mostly used for measurement of cellular peroxides, specifically H_2O_2 in intact cells.

In the cytoplasm DCFDA is hydrolysed to form 2',7'-dichlorodihydrofluorescein (DCFH) by intracellular esterase's, then oxidised to the fluorescent molecule 2',7'-dchlorofluorescein (DCF) in the presence of ROS (H_2O_2). Fluorescent intensity can then be measured using a fluorescent plate reader, excitation, and emission wavelength of 498 and 522 nm respectively, and used as an estimation of cellular oxidative stress (Yang *et al.* 2014, Katerji *et al.* 2019)

There are several advantages of using the DCFDA assay to detect ROS, the dye is inexpensive, easy to use and extremely sensitive. However, the assay is not without limitations the fluorescent dye lacks specificity, shows sensitivity to photobleaching and is prone to dye leakage (Tetz *et al.* 2013, Wang and Roper 2014). Despite previously described limitations, DCFDA is suitable widely used assay to obtain general measurements of cytoplasmic oxidative stress and use in cardiomyocytes is strongly supported in the literature (Dai *et al.* 2011, Ludke *et al.* 2017, Rizvi *et al.* 2021).

1.9 Aims

This study aimed to quantify serum IL-10 levels in a CAD patient cohort whilst correlating to indices of cardiac function. With the main aim to determine the value of IL-10 as a biomarker for CAD. Additionally, it aimed to investigate the role of pathological IL-10 levels in a CAD cohort on oxidative stress and cellular viability.

The main aims of this study were:

1. Quantify serum levels of IL-10 in a CAD patient cohort.
2. Correlate quantified IL-10 levels to indices of cardiac function.
3. Investigate the role of pathological IL-10 obtained on oxidative stress in challenged and non-challenged cells.
4. Investigate the role of pathological IL-10 obtained on cellular viability in challenged and non-challenged cells.

2. Methods

2.1 Study design

The wider CAVCAD study is designed to characterise cardiac cellular function in coronary heart disease in a patient cohort.

This specific project sought to contribute to this by measuring serum cytokine levels of interleukin-10 in a cohort of 70 patients, whilst identifying correlates between serum cytokine levels and vascular function through ECHO parameters. To identify tissue biomarkers possibly associated with CAD. Alongside elucidating the role of the anti-inflammatory cytokine IL-10 on OS and cellular viability.

2.1.1 Ethical considerations

CAVCAD is approved by IRAS (ID: 247341). Local ethical approval was also obtained from the University of Salford (review code 3392).

2.1.2 Patient recruitment

Patients at Blackpool Victoria hospital (BVH) scheduled for coronary artery bypass graft surgery (CABG) were recruited during pre-operative assessment. A cohort of seventy patients were recruited that met the following criteria: diagnosis of CAD, CABAG surgery scheduled, age > 18 and either gender (Appendix A). Consent was obtained by participants recruited through completion of the patient consent form prior to surgery (Appendix B). All patient data and samples were fully anonymised. Once consent was obtained 10 ml of blood was taken pre-surgery and stored in an EDTA tube at 4^{o}C. Clinical ECHO data for each participant was also collected via routine pre-operative assessment.

2.2 Sample preparation

10ml blood samples were transferred from EDTA, an anticoagulant which inhibits clotting of the blood sample through chelation of blood calcium, coated blood containers into a 15 ml falcon tube. Then centrifuged at 2000xg for 5 minutes to separate whole blood into its constituents: serum, PMBC and red blood cells. After centrifugation the serum was aliquoted into cryovials for storage at -80^{o}C.

2.3 Quantification of interleukin-10 levels using ELISA

Sandwich ELISA (Thermofisher, USA) was chosen to quantify serum interleukin-10 levels. The high sensitivity kit had an analytic sensitivity of 0.05 pg/ml, with a detection range of 0.39 – 25 pg/ml. Prior to use all reagents were equilibrated to room temperature, then diluted according to manufacture instructions if required (appendix C).

2.3.1 Standard curve generation

A standard curve from known standard concentrations was used to quantitatively assess unknown IL-10 concentrations. The standards were prepared by reconstituting lyophilised human IL-10 for a concentration of 400 pg/ml and left to reconstitute at room temperature for 30 minutes. During the time taken to equilibrate, 225 µl of sample diluent was added to a further 7 Eppendorf tubes labelled with the standard concentrations. The reconstituted 400 pg/ml standard vial was further diluted 1:8 using sample dilution obtaining a concentration of 50 pg/ml. This was then used to prepare serial dilution shown in table 1 below. A negative control of only sample diluent was also prepared.

Table 1 : IL-10 standard concentrations used to create standard curve.

Tube Label	Standard Dilution	Concentration (pg/ml)
1	1:2	25.00
2	1:4	12.50
3	1:8	6.25
4	1:16	3.13
5	1:32	1.56
6	1:64	0.78
7	1:128	0.39
8 (negative control)	No dilution	0

2.3.2 Experimental protocol

Patient serum samples were removed from the -80°C freezer and thawed at room temperature. Before addition to the microwell plate each sample was vortexed. Prior to use each microstrip well was washed twice with 400 µl of 1X wash buffer. Once homogenized 100 µl of patient serum sample was added in duplicate to the microwell plate. Prior to addition the Biotin-conjugate was prepared, then 50 µl added to each well before being incubated on a microplate shaker (100 rpm) at room temperature for 2 hours.

After initial incubation microwells were washed 6 times using 1X wash buffer before addition of 100 µl of streptavidin-Horseradish peroxidase (HRP) and incubated at the same conditions for 1 hour.

Immediately prior to addition 1X amplification I solution was prepared; wells were then washed 6 times. 100 µl 1X amplification I solution was added and incubated a third time for a further 15 minutes. Wells were washed again 6 times and 1X amplification II solution prepared before addition of 100 µl to each well and was incubated for 30 minutes.

Cells were washed for a final time before 100 µl of tetramethyl-benzidine (TMB) was added and final incubation for 15 minutes in the dark. After 15 minutes 100 µl phosphoric acid (1M) (stop buffer) was added and a colorimetric change from blue to yellow was observed indicating the stop of the enzymatic reaction.

2.3.3 Quantification of IL-10 levels.

Absorbance levels of the microwell plate were measured immediately using FLUOstar Omega plate reader (BMG LabTech Germany) at 450 nm. Background wavelength was measured at 620 nm. Background absorbance levels were subtracted, and the average absorbance value of each standard and patient sample were calculated. A standard curve (figure 12) was plotted for IL-10 using the measured absorbance values. Patient serum concentrations were then extrapolated. IL-10 concentrations generated were then compared to clinical parameters on a patient-to-patient basis to determine whether IL-10 levels measured influenced cardiac function.

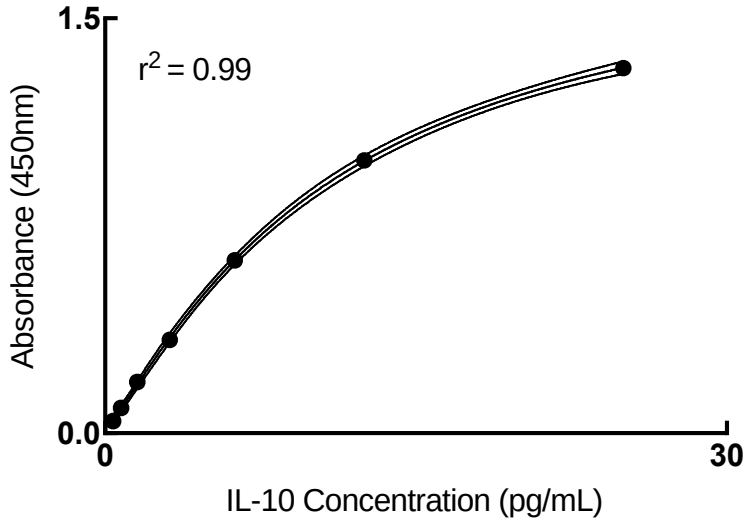

Figure 12: IL-10 ELISA standard curve ranging from 0.39 – 25 pg/ml, data points are displayed as mean with 95% confidence intervals.

2.4 Cell Culture of H9C2 cell line

The rat cardiac myoblast cells line, H9C2 (CRL-1446), were purchased from Public Health England (UK). Culturing and experimentation on the H9C2 cell line were performed aseptically in a class II biological safety cabinet (NuAire, USA), maintained in an incubator (NuAire, USA) at 37^{o}C with 5 % CO_2.

H9C2 cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK) supplemented with 10 % heat inactivated foetal bovine serum (FBS) (Gibco, UK) and 1% penicillin-streptomycin (10,000 units/ml and 10,000 μg/ml) (Gibco, UK) in T_{175}, surface area 175 cm^3, cell culture flask until a confluency of 80% was achieved. DMEM media was changed twice weekly. Cells were then either passaged at a ratio of 1:3 for continuation of culture or seeded at a density of 25,000 cells per well for experimental purposes.

2.4.1 Disassociation of H9C2 cell line

Once cells reached the desired confluency of 80%, they were washed using 10ml of phosphate buffered saline (PBS) (Sigma, USA) and treated with 8 ml of Trypsin-EDTA solution (Gibco, UK) for 5 minutes at 37^{o}C until the cell layer was detached. 10ml of complete media was added to neutralise the Trypsin. The cell suspension was then centrifuged at 200 xg for 5 minutes, the supernatant was discarded, and cell pellet resuspended in complete DMEM media.

2.4.2 Cell Count for seeding

To calculate the volume of cells required to seed experiments a cell count of our suspension was performed using a haemocytometer. The trypan blue exclusion method was used (Strober 2001). 10 μl of the cell suspension was mixed in a 1:1 ratio with 0.4% trypan blue solution (Gibco, UK). 10 μl of this mixture was added to our haemocytometer and a cell count was

performed using a Leica DM500 microscope (Leica, Germany) with a total magnification of x40.

Cell count performed included cells that fell into the large grids only; cells on the bottom or right edge of the grid were excluded and any cells which up took the dye (non-viable) were excluded from the cell count. Total cell count was calculated from the addition of the 4 large grids**.** The concentration of cells per ml of solution was then calculated by multiplying the average cell count by 2 (the trypan blue dilution factor), then multiplied by $x10^4$. This gave the total cell count per ml. The volume of the cell suspension required to seed experimentation was then calculated using the total cell concentration. Once calculated, the volume of cell suspension required was diluted using desired volume of media for experiments.

2.5 Measurement of cellular viability

Cells were seeded in a 96 well plate (Fisher Scientific, USA) at a density of 3.5 $x10^4$ cells per well. They were incubated for 24hr at 37°C, 5 % CO_2 to attach. After 24 hours the media was removed and replaced with concentrations of IL-10, in wells not treated with the cytokine the media was replaced (figure 13).

Plates were then incubated for a further 24 hours, after treatment supernatant was removed from each well. Further treatment of H_2O_2 was applied to wells indicated in figure 13 and incubated for 1 hour**.**

After treatment the supernatant was removed from all wells and replaced with 100 µl of 0.5 mg/ml MTT reagent (Thermoscientific, UK). Then incubated at 37°C for a further 3 hours until a yellow colour change occurred indicating production of yellow tetrazolium salts. The supernatant was aspirated and 100 µl of DMSO was added. Each well was then thoroughly mixed ensuring dissolution of the purple formazan crystals. Absorbance was measured at 540 nm, with background absorbance measured at 690 nm using a FLUOstar Omega plate reader (BMG LabTech Germany).

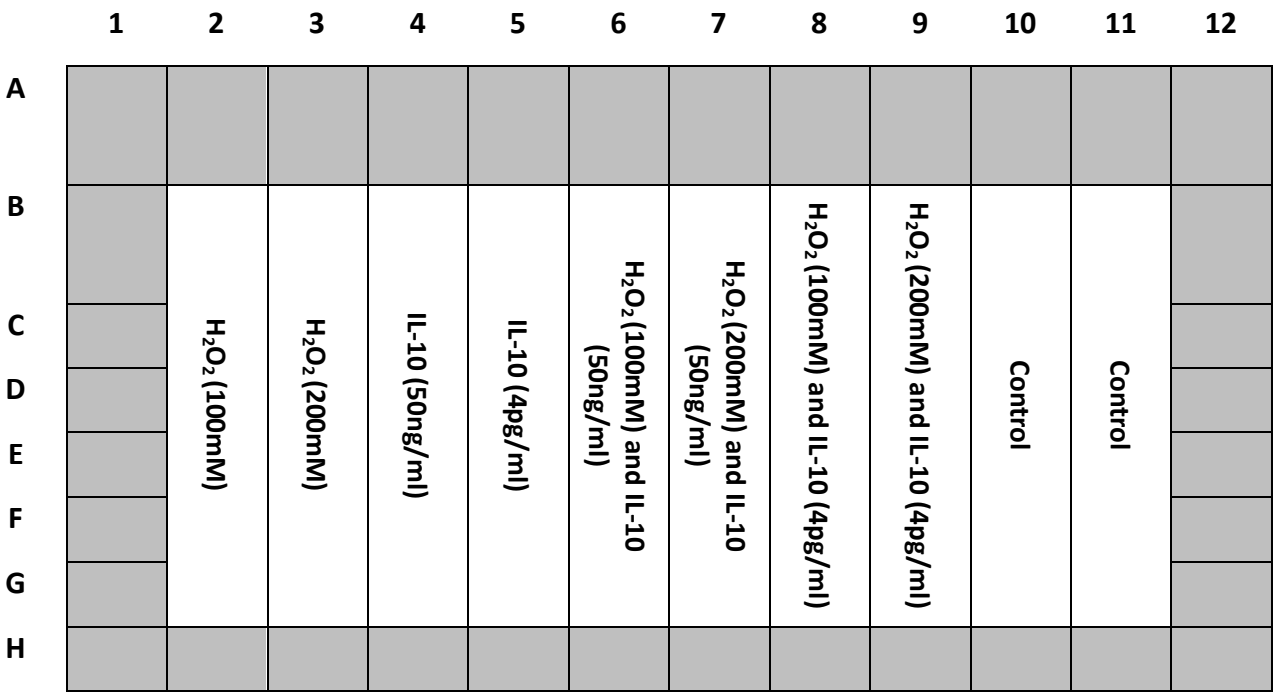

Figure 13: 96 well plate layout for MTT assay

2.6 Measurement of intracellular oxidative stress

Cells were seeded in a 96 well black clear bottom plate (Fisher Scientific, USA) at a density of 25 x10^4 cells per well. They were incubated for 24hr at 37°C, 5 % CO_2 to attach. After 24 hours the media was removed and pre-treated with two concentrations of IL-10, in wells not treated with the cytokine the media was replaced.

Plates were then incubated for a period of 1 or 24 hours, after treatment supernatant was removed from each well. Half the plate (figure 14) was loaded with 100 µl of the fluorescence dye DCFDA at a concentration of 5 µM for 30 minutes. In wells not loaded with the fluorescent dye media was replaced.

Supernatant was aspirated from each well and a secondary treatment with hydrogen peroxide (H_2O_2) was applied to wells indicated in figure 14 and incubated for 1 hour. Fluorescence

intensity was detected at excitation wavelength of 485 nm and emission wavelength of 520 nm using FLUOstar Omega plate reader (BMG LabTech Germany).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		H_2O_2 (100mM)	H_2O_2 (200mM)	IL-10 (50ng/ml)	IL-10 (4pg/ml)	H_2O_2 (100mM) and IL-10 (50ng/ml)	H_2O_2 (200mM) and IL-10 (50ng/ml)	H_2O_2 (100mM) and IL-10 (4pg/ml)	H_2O_2 (200mM) and IL-10 (4pg/ml)	Control	Control	
C												
D												
E												
F												
G												
H												

Figure 14: 96 well plate layout for DCFDA assay. Row B-C were loaded with fluorescent dye DCFDA, whilst row E-F were not loaded and remained the unstained controls.

2.7 Statistical analysis

Statistical analysis for all data were performed using GraphPad Prism 9 (GraphPad Software, Inc, USA). Normality of the data were assessed using Shapiro-Wilk normality test; all data were found to be normally distributed, therefore was analysed using parametric statistical tests. Statistical significance was accepted when $p < 0.05$.

Correlations between IL-10 serum levels and clinical parameters, measurements of cardiac function gathered from patient ECHO data, were conducted using Pearson product-moment correlation coefficient (Pearson R) measuring the linear correlation between the two variables: concentration and clinical parameter.

Cellular viability was analysed using a one-way ANOVA with Tukey post hoc test to allow comparison of treatment, IL-10 or H_2O_2, against control. Intracellular oxidative stress was analysed using a one-way ANOVA with a Tukey post hoc test to allow comparison of treatment, IL-10 or H_2O_2, against control.

3. Results

3.1 Quantification of mean serum IL-10 levels

As per 2.3, serum interleukin-10 concentrations were quantified using sandwich enzyme linked immunosorbent assay conducted in duplicate from a cohort of 70 patients. IL-10 levels were measured to (1) determine a mean level thus a clinically relevant concentration for further experiments (cell viability, oxidative stress) and (2) to provided patient-by-patient data for clinical correlations.

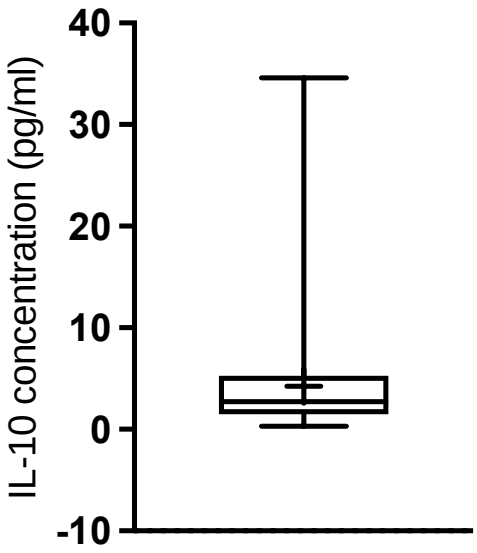

Figure 15: Box and whisker plot of patient IL-10 concentrations. Mean IL-10 concentration 4.20 pg/ml ± 0.01 pg/ml. Max and minimum range between 0.3 pg/ml and 34.6 pg/ml.

Patient interleukin-10 concentrations range from 0.3 pg/ml to 34.6 pg/ml with a mean value 4.20 pg/ml ± 0.01 pg/ml (n = 70) (figure 15). This mean value determined the clinically relevant concentration used throughout future experiments.

3.2 Patient demographic

To date, pre-operative serum and clinical data were collected from 70 CAVCAD patients, represented in appendix D and E. Systolic function was further divided into left and right ventricular function. Indices of interest for left ventricular function, table 2, were ejection fraction (EF) and left ventricular outflow tract (LVOT). Those of interest for right ventricular function, table 3, were pulmonary arteriole systolic pressure (PASP) and tricuspid annular plane systolic excursion (TASPE). Diastolic function could not be further divided into left and right ventricular function due to lack of clinically used ECHO measurements. Therefore, diastolic function was analysed using E/a ratio, peak E-wave velocity and end diastolic volume (EDV) (table 4). Comparative normal values for all indices of cardiac function measured are seen in appendix F.

Table 2: Indices of left ventricular systolic function. Shown are the mean values, standard error of the mean and number of patients analysed.

Measurement	Mean	SEM	n
EF (%)	49.51	1.40	63
LVOT (m/s)	0.95	0.03	45

Table 3: Indices of right ventricular systolic function. Shown are the mean values, standard error of the mean and number of patients analysed.

Measurement	Mean	SEM	n
PASP (mmHg)	26.53	2.19	17
TASPE (cm)	2.14	0.06	41

Table 4: Indices of diastolic function. Shown are the mean values, standard error of the mean and number of patients analysed.

Measurement	Mean	SEM	n
E/a ratio	0.91	0.04	55
Peak E-wave velocity (m/s)	0.72	0.03	56
EDV (ml)	103.67	8.20	18

Alongside indices of cardiac function further clinical parameters such a gender, age and body mass index (BMI) were collated for each patient (appendix G), average patient data for each parameter can been seen in table 5 below.

Table 5: Patient clinical parameters; age, BMI and heart rate. Shown are the mean values, standard error of the mean and number of patients analysed.

Measurement	Mean	SEM	n
Age	67	1.1	65
BMI	28	1.5	48
Heart rate	69	1.4	33

3.3 Correlations of IL-10 levels to systolic function

Serum IL-10 levels were correlated to indices of systolic function. Parameters chosen to explore correlates with left ventricular function were ejection fraction (EF) and left ventricular outflow tract (LVOT). Right ventricular function was investigated using pulmonary arteriole systolic pressure (PASP) and tricuspid annular plane systolic excursion (TAPSE).

3.3.1 Left ventricular function

Patient IL-10 concentrations were correlated with indices of left ventricular function, EF and LVOT.

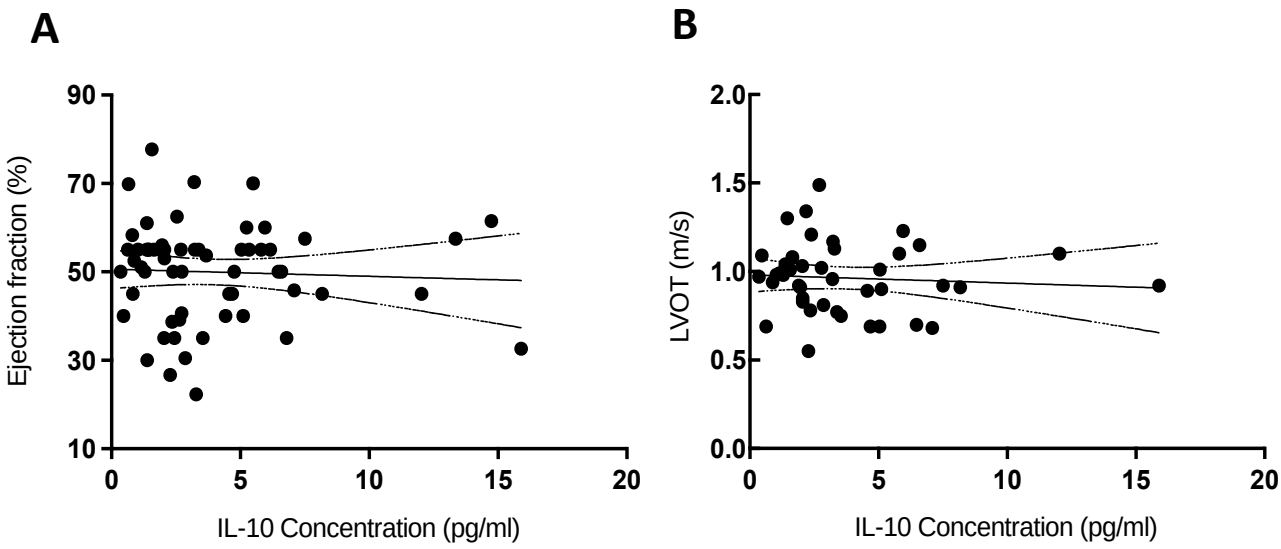

Figure 16: Left ventricular systolic function Vs IL-10 concentration. **(A)** Comparison of ejection fraction and IL-10 concentration. **(B)** Comparison of left ventricular outflow tract (LVOT) and IL-10 concentration. Data points expressed as mean ± SEM, with 95% confidence intervals.

Neither ejection fraction (n = 60, r^2= 0.002, p =0.71) nor LVOT (n = 42, r^2= 0.005, p =0.65) correlated with IL-10 concentration.

3.3.2 Right ventricular function

Patient IL-10 concentrations were correlated with indices of right ventricular function, PASP and TASPE.

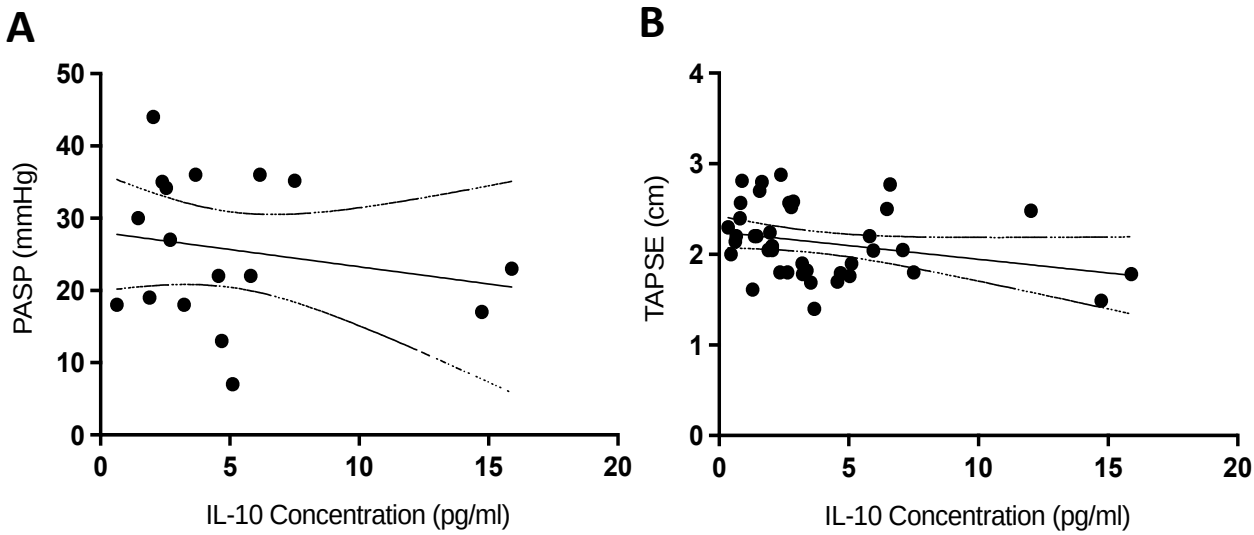

Figure 17: Right ventricular systolic function Vs IL-10 concentration. (A) Comparison of pulmonary arteriole systolic pressure (PASP) and IL-10 concentration. **(B)** Comparison of tricuspid annular plane systolic excursion (TAPSE) and IL-10 concentration. Data points expressed as mean ± SEM, with 95% confidence intervals.

PASP did not correlate with IL-10 levels (n = 17, R^2 = 0.04, p = 0.43) however, TASPE (n = 41, R^2 = 0.08, p = 0.08) correlated negatively but insignificantly with IL-10.

3.4 Correlations of IL-10 Levels to diastolic function

Correlations were made between indices of diastolic function, E/a ratio, Peak E-wave velocity and end diastolic volume (EDV) and patient serum IL-10 levels.

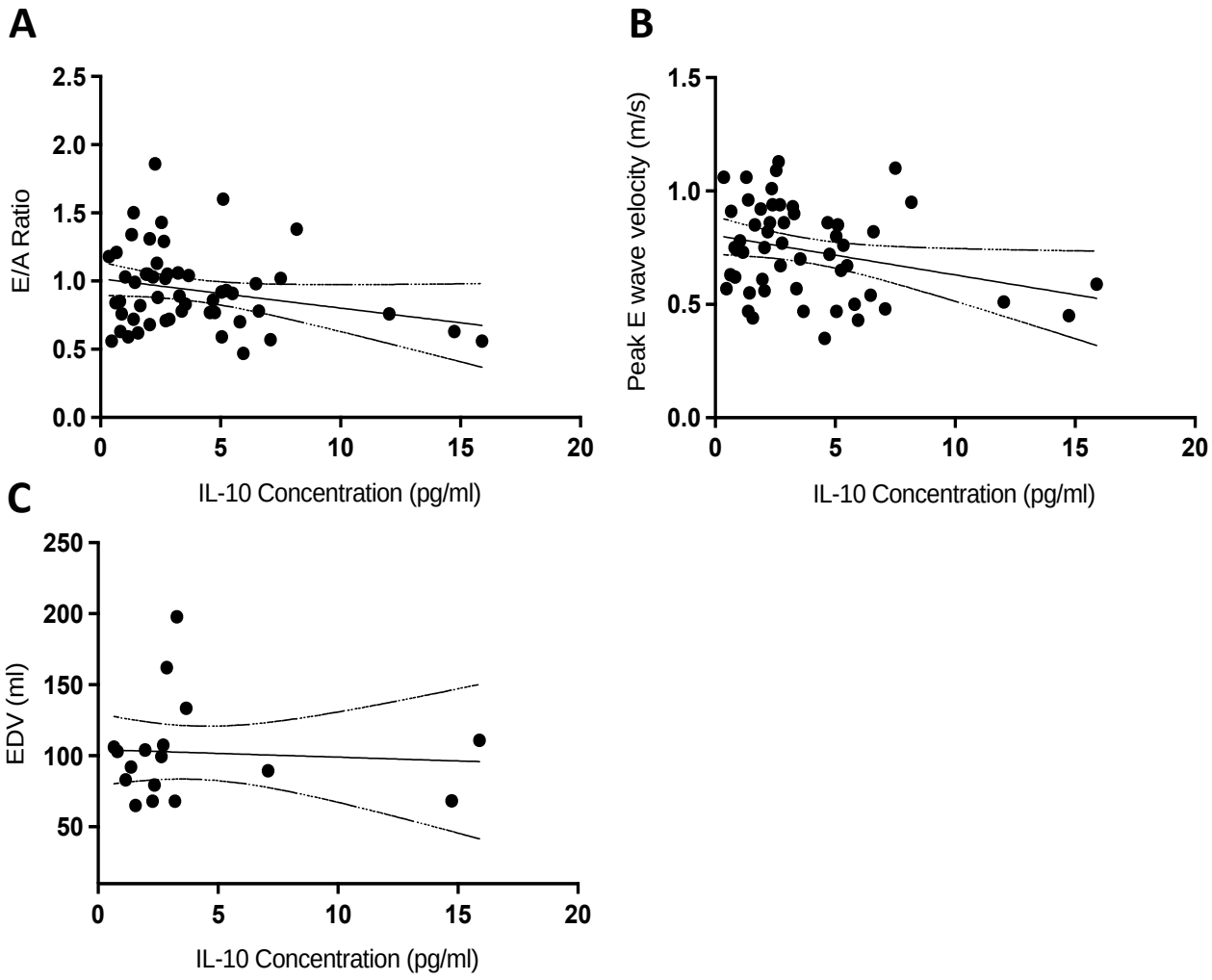

Figure 18: Diastolic function Vs IL-10 concentration. (A) Comparison of E/A ratio and IL-10 concentration. **(B)** Comparison of peak E wave velocity and IL-10 concentration. **(C)** Comparison of end diastolic volume and IL-10 concentration. Data points expressed as mean ± SEM, with 95% confidence intervals.

Peak E-wave velocity showed a significant negative correlation with IL-10 concentration (n = 53, R^2 = 0.08, p = 0.04). E/A ratio correlated negatively but insignificantly with IL-10 (n = 52, R^2 = 0.05, p = 0.08). EDV did not correlate with IL-10 concentration (n = 17, R^2 = 0.005, p = 0.80).

3.5 The effect of IL-10 on cytoplasmic oxidative stress

Initial experiments sought to measure the increase in cytoplasmic oxidative stress produced by hydrogen peroxide (a reactive oxygen species) in a H9C2 cell line. This manoeuvre was performed as a positive control to test the reliability of the method described in section 2.6. To determine if IL-10 plays a protective role against cytoplasmic oxidative stress cells were pre-treated at two different time points – 1 hour and 24 hours before secondary treatment of hydrogen peroxide for 30 minutes.

3.5.1 Does 1-hour treatment of IL-10 attenuate H_2O_2 induced cytoplasmic oxidative stress and are these effects concentration dependent?

Initial hydrogen peroxide challenge was performed to determine sensitivity of our method to measure increase in oxidative stress. The pathological effects of IL-10 at a concertation of 4 pg/ml were measured in our CAD patient cohort and determined our clinically relevant concentration throughout. In addition, a supraphysiological concentration was chosen based on previous studies (Kaur *et al.* 2006, Dhingra *et al.* 2009, Krishnamurthy *et al.* 2009). These concentrations of IL-10 (4 pg/ml and 50 ng/ml) were investigated over an hour period.

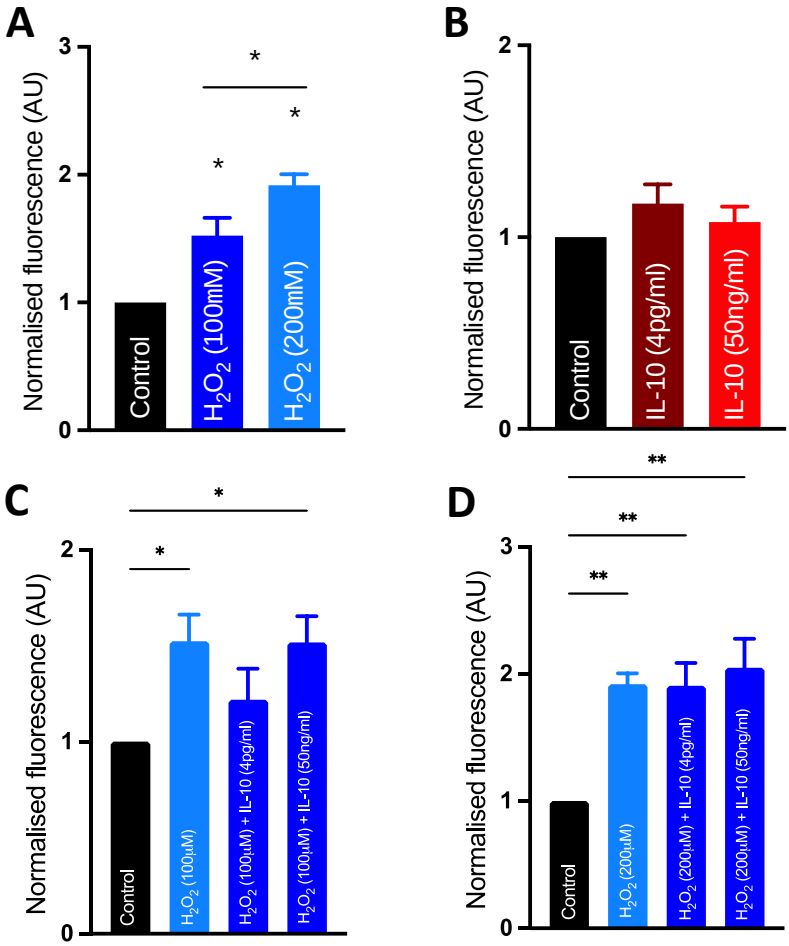

Figure 19: Effect of 1 hour treatment of interleukin-10 and hydrogen peroxide on H9C2 cell line. (A) Mean normalised fluorescence of cardiac myocyte cell line (H9C2) in control, 100 µM and 200 µM H_2O_2. **(B)** Mean normalised fluorescence of cardiac myocyte cell line (H9C2) in control, 4 pg/ml and 10 pg/ml IL-10. **(C)** Mean normalised fluorescence of cardiac myocyte cell line (H9C2) treated with 100 µM H_2O_2, combination treatment of 100 µM H_2O_2 + 4 pg/ml IL-10 and combination treatment of 100 µM H_2O_2 + 50ng/ml IL-10. **(D)** Mean normalised fluorescence of cardiac myocyte cell line (H9C2) in 200 µM H_2O_2, combination treatment of 200 µM H_2O_2 + 4pg/ml IL-10 and combination treatment of 200 µM H_2O_2 + 50 ng/ml IL-10. Significance was determined using a one-way ANVOA (n per treatment = 18 wells, 6 plates).

Treatment with hydrogen peroxide induced a significant increase in fluorescence at both 100 µM and 200 µM H_2O_2 (n = 18 wells p = 0.004. n = 18 wells p = <0.001 respectively) (figure 19A). Neither our physiological concentration, 4 pg/ml of IL-10 nor our pathological control,

50 ng/ml induced a significant increase or decrease in fluorescence (n = 18 wells, p = 0.244. n = 18 wells, p = 0.732) (figure 19B). Pre-treatment with IL-10 did not produce a significant decrease in fluorescence at both 100 μM and 200μM H_2O_2 as seen in figure 19C and D.

3.5.2 Does 24-hour treatment of IL-10 attenuate H_2O_2 induced cytoplasmic oxidative stress and are these effects concentration dependent?

Further to previous experiments (section 3.5.1) the role of IL-10 on oxidative stress was then elucidated over a 24hr period.

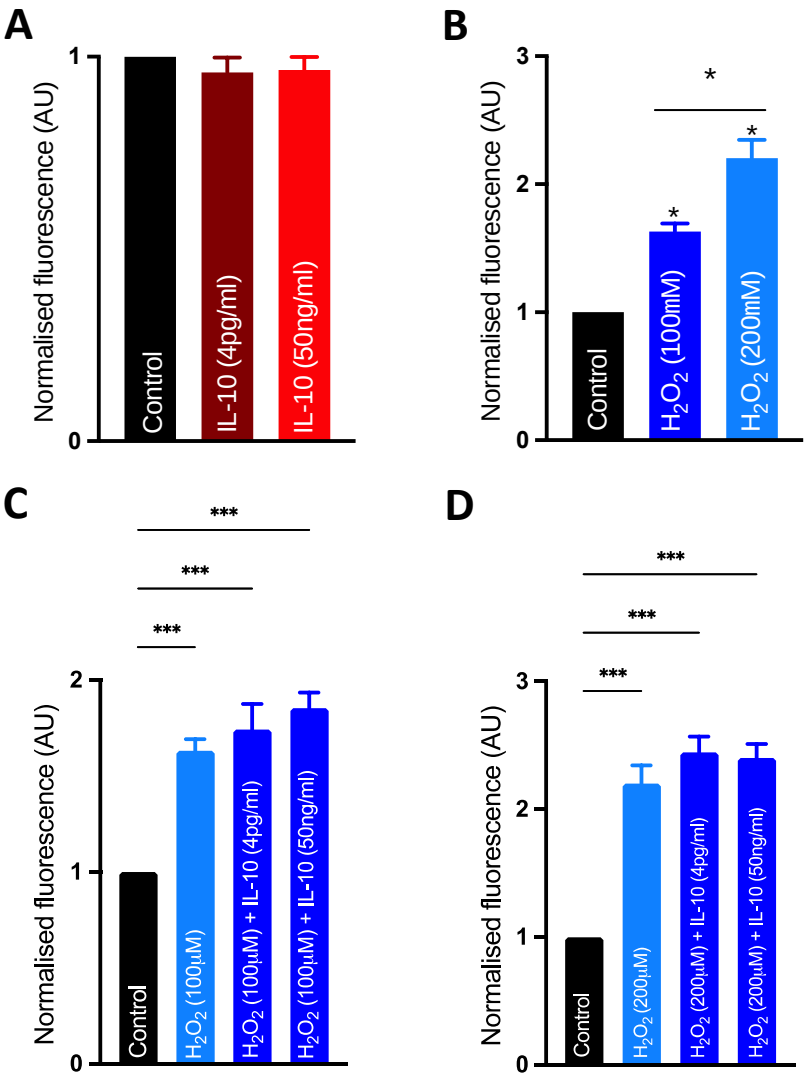

Figure 20: Effect of 24-hour treatment of interleukin-10 and hydrogen peroxide on H9C2 cell line. **(A)** Mean normalised fluorescence of cardiac myocyte cell line (H9C2) in control, 100 μM and 200 μM H_2O_2. **(B)** Mean normalised fluorescence of cardiac myocyte cell line (H9C2) in control, 4 pg/ml and 50 ng/ml IL-10. **(C)** Mean normalised fluorescence of cardiac myocyte cell line (H9C2) treated with 100 μMH_2O_2, combination treatment of 100 μM H_2O_2+ 4pg/ml IL-10 and combination treatment of 100 μM H_2O_2+ 50 ng/ml IL-10. **(D)** Mean normalised fluorescence of cardiac myocyte cell line (H9C2) in 200 μM H_2O_2, combination treatment of 200 μM H_2O_2+ 4 pg/ml IL-10 and combination treatment of 200 μM H_2O_2+ 50 ng/ml IL-10. Significance was determined using a one-way ANVOA (n per treatment = 15 wells, 5 plates).

Treatment with hydrogen peroxide induced a significant increase in fluorescence at both 100 μM and 200 μM H_2O_2 (n = 15 wells p = <0.001. n = 18 wells p = <0.001 respectively) (figure 20A). Treatment with both 4 pg/ml and 50 ng/ml did not produce significant increase or decrease in fluorescence (n = 18 wells, p = 0.614. n = 15 wells, p = 0.696) (figure 20B). No significant decrease in fluorescence was observed after combination treatment of both IL-10 and H_2O_2 at both concentrations as seen in figure 20C and D.

3.6 The effects of IL-10 and H_2O_2 on cellular viability

Further experiments sought to establish the effect of experimental concentrations of IL-10 on cellular viability, assessed over a 24-hour period. Initial experiments sought to measure the effect of hydrogen peroxide on cellular viability, this was used as our positive control. Cells were then subsequently exposed to 4 pg/ml (the clinically relevant concentration derived in 3.1) and 50 ng/ml (a relatively supraphysiological concentration) of IL-10, alongside treatment with a sublethal concentration of hydrogen peroxide, 100 μM and 200 μM.

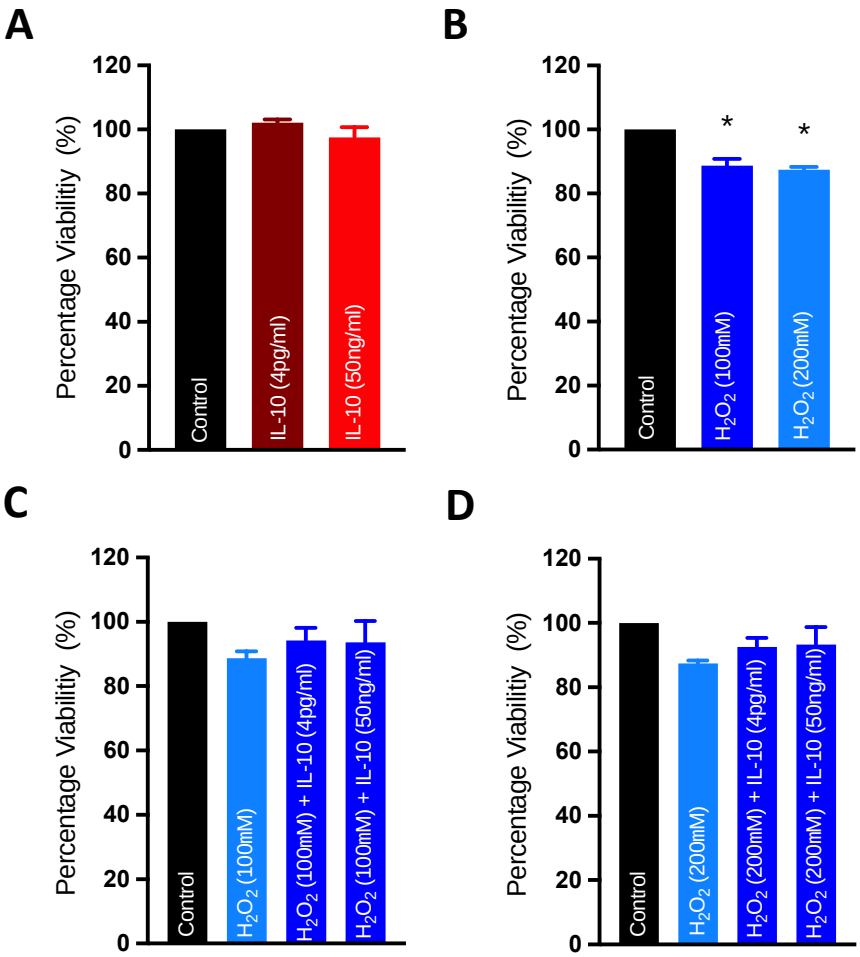

Figure 21: The effect of IL-10 and hydrogen peroxide on cell viability over 24 hours. (A) Percentage viability of cardiac myocytes in control, 100 μM and 200μM H_2O_2 treatment. **(B)** Percentage viability of cardiac myocytes in control, 4 pg/ml and 50 ng/ml IL-10 treatment. **(C)** Percentage viability of cardiac myocytes 100 μM H_2O_2, combination treatment of 100 μM H_2O_2 + 4 pg/ml IL-10 and combination treatment of 100 μM H_2O_2 + 50 ng/ml IL-10. **(D)** Percentage viability of cardiac myocytes in 200 μM H_2O_2, combination treatment of 200 μM H_2O_2 + 4 pg/ml IL-10 and combination treatment of 200 μM H_2O_2 + 50 ng/ml IL-10. Significant results were determined using a one-way ANOVA (n per treatment = 18 wells, 3 plates).

Treatment of both 100 μM and 200 μM of H_2O_2 (figure 21A) saw a significant reduction in cell viability by 11.3 % and 11.6 % (n= 16 wells, 3 plates. p = 0.03 and p = 0.02 respectively). Subsequent exposure to IL-10 4 pg/ml did not significantly reduce cell viability (n = 18 wells, 3 plates, p = 0.73) nor did IL-10 50 ng/ml (n = 15 wells, 3 plates, p = 0.67) (figure 21B). On

average combination treatment of 100 μM and IL-10 4 pg/ml saw no significant reduction in cell viability (n = 18 wells, 3 plate. p = 0.65) nor did combination of 100 μM and IL-10 50 ng/ml (n = 16 wells, 3 plates. p = 0.60) (figure 21C). Finally, combination treatment of 200 μM and IL-10 4 pg/ml saw no significant reduction in cell viability (n = 18 wells, 3 plates. p = 0.36) nor did combination of 200 μM and IL-10 50 ng/ml (n = 17 wells, 3 plate. p = 0.42) (figure 21D).

4. Discussion

4.1 Average IL-10 concentration in CAD patient cohort

The role of inflammatory biomarkers such as cytokines in CAD have been extensively researched (Zhang *et al.* 2016). Balance between pro- and anti-inflammatory cytokines plays a critical role in developing CAD whilst also possibly correlating with its outcome (Wolf and Hunziker 2020). Expression of IL-10 is known to be associated with the development of atherosclerotic plaques in humans; the key pathogenic phenomenon in CAD) (Mallat *et al.* 1999). Furthermore, elevated levels have been detected in CAD patients when compared to healthy controls (Szodoray et al., 2006). Alongside use of atherosclerotic mouse models, Eefting *et al* (2007) demonstrated the anti-inflammatory and anti-atherogenic properties of IL-10. Highlighting IL-10 as an important biomarker in the progression of CAD (Lakoski *et al.* 2008). Given this combined evidence for a role of IL-10 in the pathogenesis of CAD, it stands to reason IL-10 may be a useful prognostic indicator.

Analysis of our patient cohort revealed average IL-10 levels to be 4.2 pg/ml. Due to a lack of control patients in our study we cannot conclude whether the serum levels detected are elevated. However previous study by Heeschen *et al* (2003) looking at serum levels of IL-10 in a cohort of 547 patients with an acute coronary syndrome, a subset of CAD, determined elevated levels of interleukin-10 to be > 3.5 pg/ml. A further study by Mirhafez *et al* (2015) in which the relationship between several cytokines and CAD were studied, measured the serum levels of 98 control subjects and reported IL-10 levels to be 0.78 pg/ml. Therefore, levels measured in the present study in comparison to previous studies implies IL-10 are elevated in our cohort.

Previous studies have shown higher IL-10 levels are associated with a lower incidence of CAD; therefore, it is possible elevated IL-10 may have a protective role in our patient cohort (Barcelos *et al.* 2019). Other evidence suggests no relation between baseline levels of IL-10 and risk if cardiovascular events in patients free from overt cardiovascular disease (Goldwater

et al. 2019), suggesting IL-10 to only be a prognostic marker of coronary artery disease in a pathological model and not a marker of subclinical CAD.

One caveat surrounding reports of absolute concentrations is differences in detection methods / sensitivity. While we are confident (based on methods and comparison to previous work outlined above) our patient IL-10 levels are clinically representative, it is the correlation of *relative* levels to those of cardiac function that is of interest in this study.

4.2 Do levels of interleukin-10 correlate with indices of cardiac function?

Cardiac remodelling can be defined as changes in ventricular function, size and shape that occur due to myocardial injury. Pathological ventricular remodelling due to myocardial infarction (MI), appears initially compensatory but is later linked to heart failure progression (Sharpe 2004). Cardiac remodelling in CAD has been associated with activation of pro-inflammatory cytokines such as IL-6 and TNF-a (Ono *et al.* 1998).

IL-10 has been shown to limit infiltration of inflammatory cells in vascular injury (Rajasingh *et al.* 2006). A mouse study by Krishnamurthy *et al* (2010) found treatment with IL-10 inhibits TNF-α, as well as other pro-inflammatory cytokines, known to have adverse cardiac remodelling effects. In addition, IL-10 may attenuate remodelling without cytokine involvement through direct effects on protease activity (Krishnamurthy *et al.* 2010).

Therefore, we hypothesised that IL-10 levels in our CAD patient cohort would correlate with indices of cardiac function and be a possible marker for CAD severity and future cardiovascular events.

4.2.1 Do IL-10 levels correlate with indices of systolic cardiac function?

In health, systolic function describes normal contractility of the heart and is quantitatively assessed by ejection fraction. Normal values range between 55% and 75%. Systolic function in CAD can be determined either by left or right ventricular dysfunction with both having different aetiologies, usually with left having proceeded right dysfunction (Voelkel *et al.* 2006). Left ventricular (LV) dysfunction is a commonly used prognostic predictor in CAD and is associated with higher morbidity and mortality. It is associated with risk of further cardiovascular events such as sudden death, arrhythmia, or heart failure (Singh *et al.* 2015). Right ventricular (RV) systolic dysfunction is due to myocardial stress with the most common cause being left-sided heart failure however pulmonary hypertension also plays an important role (Haddad *et al.* 2008).

4.2.1.1 Do levels correlate with indices of left ventricular systolic function?

Left ventricular systolic dysfunction is characterised by reduced ventricular function, typically defined using echocardiography with an ejection fraction less than 40% (Moon *et al.* 2009). We see higher incidence of cardiovascular events in patients with left ventricular hypertrophy reflected by larger LV internal dimension and lower ejection fraction (58 ± 10%) compared to non-CAD groups (Zabalgoitia *et al.* 2001). Given studies have shown IL-10 treatment to attenuate hypertrophic remodelling and improve cardiac function in multiple animal models (Stumpf *et al.* 2008, Verma *et al.* 2012). We proposed IL-10 levels would correlate to indices of LV systolic function, specifically ejection fraction.

Two parameters were chosen to evaluate the relationship between IL-10 and LV systolic function in our CAD cohort: ejection fraction and left ventricular outflow tract obstruction. We saw no significance between IL-10 concentration and either measurement of systolic function – EF and LVOT (figure 16) - therefore concluding that LV systolic function did not correlate with IL-10 levels in our patient cohort. Our data contradicts that known about the

cardio-protective role of IL-10 in pressure overload. Study by Verma *et al* (2012) displayed improved LV function in mouse models of pressure overload hypertrophy and heart, evidenced by increased ejection fraction and fractional shortening. This is corroborated by the findings of Stumpf *et al* (2008) in which IL-10 treatment correlated with improvement of left ventricular function in a heart failure rat model after experimental myocardial infarction and a significant decrease in impairment after MI indicated by increase in fractional shortening was observed.

We speculate that our contradicting results are due to the lower circulating levels of IL-10 detected in our CAD patient cohort compared to the supraphysiological levels used in animal models in the above stated studies when elucidating correlations between IL-10 and systolic function – EF and LVOT.

4.2.1.2 Do levels correlate with indices of right ventricular systolic function?

Right ventricular systolic dysfunction is an established prognostic factor in CAD and is associated with increased mortality and morbidity after CABG. A study by Sumin *et al* (2022) demonstrated an increase in right systolic dysfunction associated with TASPE after CAD patients underwent routine CABG surgery (Sumin *et al.* 2022). Therefore, we aimed to discover whether RV systolic function would correlate with IL-10 levels in our CAD cohort. Two clinically relevant measurements of RV function were chosen to investigate: PASP and TASPE.

Interleukin-10 levels did not correlate with PASP, but early indications may be negative in relation to TASPE (fig 17). Therefore, early negative correlations may reflect lower TASPE values in patients with higher IL-10 levels. Previous studies have elucidated lower TASPE values to be associated with poor outcomes in CVD (Nakagawa *et al.* 2020, Tomasoni *et al.* 2023). From our correlates we speculate there is no cardioprotective role of IL-10 regarding RV systolic function reflected by TASPE. These findings compare with Rondelet *et al* (2012), to which no change in the expression of IL-10 was observed in RV failing in systemic-pulmonary

shunting in pigs (Rondelet *et al.* 2012). However, there are limited studies into the specific role IL-10 plays on right ventricular systolic function in CAD to compare our findings.

4.2.2 Do IL-10 levels correlate with indices of diastolic function?

Several studies have shown diastolic insufficiency in CAD proceeded by reduced left ventricular diastolic and myocardial stiffness due to cardiac remodelling, with right ventricular diastolic dysfunction being more common than systolic in stable CAD populations (Pagliaro *et al.* 2020, Sumin *et al.* 2022). However, the prognostic value of diastolic dysfunction, ventricular filling, is less studied.

In our study, we observe a negative correlation with E/a ratio but at this preliminary stage of the study not significant (figure 18A). However, we do observe correlates between IL-10 and diastolic dysfunction further supported by negative correlation with peak E-wave velocity (figure 18B). Correlates between E/a ratio and peak E-wave velocity indicate impaired ventricular relaxation and reduction in passive filling of the heart. This suggests impairment of cardio myocyte relaxation through actions of IL-10. These findings compare with human studies which display significant correlations between serum IL-10 levels after MI and diastolic impairment measured via E/A ratio (Karpinski *et al.* 2008). Although, contradicts findings from Kosmala *et al* (2008) in which diastolic dysfunction, measured via E/a ratio and peak E-wave velocity, did not significantly correlate with IL-10 levels in patients with stable angina.

However, we observed no correlation between IL-10 concentration and EDV (figure 18C), our chosen measurement of cardiac preload determined by venous filling pressure, in our stable CAD cohort. Our data contradicts that seen in myocardial infarction, where IL-10 treatment (50 ng/ml) is seen to significantly reduce EDV as well as improve left ventricular function post-MI (Jung *et al.* 2017). This study shows contradicting data in small rodent models to what is seen in ours. However, the levels of IL-10 used were super-pathological compared to our endogenous concentration. This suggest IL-10 may not play as significant a role on cardio protection against LV remodelling as previously believed, however more investigation is required.

The effects of IL-10 on systolic and diastolic function are less researched than its pro-inflammatory counter parts and there is also a lack in other literary evidence to support our findings. Therefore, further investigation on the effects of IL-10 on cardiac function is required.

4.3 Is our method to measure oxidative stress robust?

Oxidative stress plays a key role in the pathophysiology of CAD through the excessive accumulation of reactive oxygen species and/or imbalance of antioxidant defence.

Oxidation of DNA and proteins by ROS leads to tissue damage and subsequently DNA damage and protein denaturation resulting in loss of membrane pump function. ROS induced damaged to sarcoplasmic reticulum membrane calcium pumps impairs reuptake of sodium and calcium thus leading to impairment of cardiac calcium handling (Görlach *et al.* 2015). ROS can also act by recruitment of pro-inflammatory cytokines, IL-6 and TNF-α. These not only contribute to intracellular calcium dysregulation but also induce massive inflammatory response and increase free radical sources via NADPH-oxidase in response to neutrophil recruitment (Vichova and Motovska 2013).

Hydrogen peroxide, a non-radical ROS, is produced via two main mechanisms. Firstly, by oxidase driven enzymatic reduction of oxygen or via catalysed enzymatic by superoxide dismutase and/or spontaneous reactions (Liochev and Fridovich 2007, Handy *et al.* 2009). As the mechanisms in which H_2O_2 induces oxidative stress is well studied, the expected increase in oxidative stress saturation validates our method of detection. This allowed us to test the capability and sensitivity of the method in detection of OS in cells where OS already exists.

Cells were exposed to two sub-lethal hydrogen peroxide concentrations (100 μM and 200 μM) as a positive control. These concentrations were chosen based on previous studies in which 100 μM and 200 μM H_2O_2 induced significant but sublethal levels of oxidative stress (Qiu *et al.* 2019, Zhu *et al.* 2019). A significant concentration-dependent increase in cytoplasmic

oxidative stress was observed after exposure, validating the sensitivity of this method to detect increases in fluorescence indicative of increase in oxidative stress (figure 19 & 20A).

4.4 Does IL-10 increase cytoplasmic oxidative stress at different time points?

Inflammatory cytokines play a central role in not only induction of oxidative stress but also in the pathogenesis of CAD. Anti-inflammatory IL-10 has been shown to inhibit production of pro-inflammatory cytokines including those with known roles in oxidative stress (Bolger *et al.* 2002). It has also been shown to have a direct effect on the inhibition of ROS in macrophages (Dokka *et al.* 2001). Therefore, we sought to investigate whether IL-10 levels lead to an independent increase or decrease in oxidative stress in cardiac myocytes.

We observed no increase in IL-10 (4 pg/ml and 50 ng/ml) induced OS at both 1 and 24 hours (figure 19 & 20B). To our knowledge there are limited studies that explore the direct effect of IL-10 on OS however, our data supports that of Kaur *et al* (2006) in which IL-10 caused no change in levels of intracellular ROS dictated by DFCDA in non-challenged cardiomyocytes. These suggest the protective role of IL-10 through its antioxidant like properties may play less of an important role than thought in OS in. Therefore, we can deduce IL-10 is not modifying baseline oxidative stress in our cell line; further investigation is required to tease the mechanism of action.

4.5 What is the effect of IL-10 on cells with high baseline oxidative stress?

Balance between pro- and anti-inflammatory cytokines is vital to maintain health; oxidative stress has been shown to increase production of inflammatory cytokines. This cycle leads to further increased OS levels and diminished antioxidant capacity (Elmarakby and Sullivan

2012). Recent studies have shown IL-10 to exhibit antioxidant activity therefore, treatment of IL-10 may protective role against ROS (H_2O_2) evoked OS (Nematollahi *et al.* 2019).

We found IL-10 caused no attenuation in the levels of oxidative stress after treatment with hydrogen peroxide (100 μM and 200 μM) inferring a lack of a cardio-protective H_2O_2 evoked oxidative stress (figure 19C/D & 20C/D). However, to our knowledge limited studies have been conducted on IL-10 in cardiomyocytes with increased baseline oxidative stress induced by H_2O_2. A study by Kaur *et al* (2006) demonstrated IL-10 treatment alone had no effect on oxidative stress in adult rat cardiac myocytes. However, prevented TNF-α generated oxidative stress suggesting balance between pro- and anti-inflammatory cytokines to be of more importance in mediating OS than action alone (Kaur *et al.* 2006). These findings are supported by Dhingra *et al* (2007, 2009) who demonstrated IL-10 to have shown beneficial effects in towards inhibiting TNF-α induced oxidative stress in through inhibition of ERK pathways (Dhingra *et al.* 2007, Dhingra *et al.* 2009) .

4.6 Does H_2O_2 decrease cellular viability in H9C2 cell line?

Cellular death plays a critical role in the pathogenesis of many cardiovascular diseases, with primary methods through apoptosis and necrosis. In myocardial infarction, a subset of CAD, we see a short-lived burst of cell death within the ischemic zone over 24 hours. However, we also see cell death occurring for longer time periods due to residual ischemia and cardiac remodelling of the infarct zone but at a lesser magnitude (Konstantinidis *et al.* 2012).

It is well known that cellular death via apoptotic pathways can be induced by ROS. A study by Xie *et al* (2014) demonstrated 100 μM to 500 μM hydrogen peroxide invokes a significant loss of cellular viability while promoting apoptosis in cardiomyocytes in a dose dependent manner. Based on these findings 100 μM and 200 μM of H_2O_2 were selected to study cellular viability in our study.

Exposure to 100 μM and 200 μM of hydrogen peroxide significantly reduced cellular viability to 88.7 % and 88.4 % respectively. This significant, albeit not concentration-dependent, reduction in cellular viability may be due to differences in treatment period compared to previous studies (Xie *et al.* 2014, Qiu *et al.* 2019). As mentioned above previous studies have shown H_2O_2 invoked reduction in cellular viability. In this study the use of H_2O_2 as a positive control allowed validation of the method of detection through observed reduction in viability (figure 21A).

4.6.1 Does IL-10 alter baseline cellular viability?

IL-10 plays a central role maintaining physiological balance within the body, with pleiotropic effects on immunoregulation and inflammation. Focusing on the interactions between IL-10 and cardiomyocytes, the cytokine is known to play a cardioprotective role through deactivation of macrophages in pathological conditions (Mallat *et al.* 1999). In this study we sought to answer whether IL-10 alters baseline cellular viability in non-challenged cells (control).

To our knowledge this is one of few studies to *directly* measure the effects of IL-10 on cell viability in cardiomyocytes. We demonstrate that IL-10, 4 pg/ml and 50 ng/ml, has no effect on cellular viability in non-challenged cells (figure 21B). These findings compare with rodent primary cardiomyocytes in which exposure to IL-10 (10 ng/ml) showed no significant change in number of apoptotic cells (Dhingra *et al.* 2009). However, these findings were contradicted in a recent study by Malik *et al* (2022), in which IL-10 pre-treatment (10 ng/ml) caused a significant increase in cellular viability (100%) after 48hrs .

These findings suggest further investigation into the influence of IL-10 on cellular viability is required alongside suggesting possible heterogeneity between in-vitro models.

4.6.2 What is the effect of IL-10 on cells with baseline reduction in cellular viability?

Maintenance and protection of cardiac myocytes is important after a major cardiovascular event; anti-inflammatory properties of IL-10 have been demonstrated to aid in the maintenance of tissue repair as well as contribute to the stability of atherosclerotic plaques (Kamaly *et al.* 2016, Jung *et al.* 2017). However, little is known on the interactions between IL-10 a cardiomyocyte cell death in CAD (Yang *et al.* 2020). Previously we demonstrated both physiology and supraphysiological levels of IL-10 have no effect on cellular viability in non-challenged cells (fig 21B). To further explore the relationship between IL-10 and cellular viability we repeated those experiments on cardiomyocytes with a baseline reduction in cellular viability though the use of a hydrogen peroxide challenge.

This study observed no attenuation in cellular viability in cells pre-treated with IL-10 compared to the H_2O_2 group (figure 21C & D). This suggests IL-10 exerts no cardioprotective effect in response to H_2O_2-induced cellular death in the H9C2 cell line. Our finding contradicts that seen in MI in which upregulation of IL-10 expression after insult might regulate macrophage activation and promote transformation towards a repair-type phenotype preventing further cellular death. Regulation of apoptosis through IL-10 regulation of anti- and apoptotic gene balance offers a further protective role through myocardial tissue remodelling (Yang *et al.* 2020). Further supported by Malik *et al* (2022), in which pre-treatment of primary cardiomyocytes with IL-10 significantly increased cellular viability in response to treatment of doxorubicin, a cytotoxic agent that induces apoptotic cell death through a H_2O_2 medicated mechanism (Wang *et al.* 2004).

4.7 Study limitations

There were several factors that impacted the effectiveness of this study. The first limitation was the issue of power; this study only included a cohort of 70 patients. Due to sample size the correlations found in our cohort may not be representative of all patients in this study and therefore we could deduce they imply a trend rather than absolute correlation. An increased cohort would also allow patients to be grouped based on co-morbidities or gender, provided by clinical records, allowing deduction of further correlations.

The second limitation regards the collection of patient data (ECHO and clinical) from Blackpool Victoria hospital. ECHO and clinical data were not available for all patients in this study, with further gaps in key clinical parameters on a patient-by-patient basis. Currently a placement student has overtook the collection of data from research nurses at BVH, this should ensure complete collection of clinical data for future patients and will also allow collection of missing patient data which will help strengthen our correlations by increasing study power.

The final limiting factor involved the cell line used to investigate cytokine role in oxidative stress and viability. At the time of this study this was the only in-vitro model available in the lab. Also, as experiments were performed over a period of 24 hours this ruled out the use of primary cardiomyocytes. H9C2 is a cardio myoblast cell line, an embryonic precursor, which presents a similar phenotype to cardiomyocytes. It can be differentiated into a more cardiomyocyte subtype however, both forms are widely used in the literature to investigate cellular and molecular cardiac events. Going forward, optimisation of the differentiation protocol would be continued, and data collected repeated in differentiated cells to ensure reproducibility.

4.8 Future work

This study identified serum IL-10 concentrations in limited CAD patient cohort, whilst making correlations to indices of cardiac function. Future work would focus on increased study power through continuous patient recruitment alongside collation of robust ECHO data. This would statistically validate correlations observed in this initial patient cohort.

Furthermore, as mentioned in section 4.7, H9C2 are a cardiac myoblast cell line and therefore not completely representative of cardiac myocytes, unless differentiated. Going forward, optimisation of the differential process would be performed and further data regarding interplayed between IL-10, OS and viability would be obtained to validate data collected in this study. If not possible other cardiac myocyte cell lines, such as HL-1, could be explored. An advantage of HL-1 is that it would also allow electrophysiological studies on the effects of clinically relevant IL-10 and OS on calcium handling (White *et al.* 2004).

Clearly IL-10 plays a lesser role regarding CAD, OS and cellular viability than expected. Future work would shift focus to other cytokines that play a key role in CAD. This would further expand our cytokine panel, as well as allowing exploration of interplay between key pro- and anti-inflammatory cytokines. The role of a cytokine network in CAD has been previously addressed therefore, studying the cytokine network as a dynamic system more useful to elucidating the cellular effects of inflammation and oxidative stress in CAD.

4.9 Conclusion

In conclusion this study identified pathophysiological concentrations of IL-10 in a CAD patient cohort, whilst correlating with indices of cardiac function. Measured levels of IL-10 showed early indication of correlations with TASPE, indices of systolic function. As well as correlations with E/a ratio and peak E-wave velocity, indices of diastolic function. Limited correlations seen in this preliminary study suggest interleukin-10 to be of little use as a biomarker for assessing dysfunction in CAD in relation to both diastolic and systolic function. Further research is required to increase study power.

To our knowledge it is one of the only studies that has investigated the cellular effect of pathophysiological relevant cytokine concentrations on oxidative stress and cellular viability. Microplate reader data showed IL-10 played no role in oxidative stress nor a cardioprotective role in cells with a high baseline OS. These finds were also reflected in cellular viability, with IL-10 having no effect on cellular viability both in challenged and non-challenged cells. These findings clearly show IL-10 plays a lesser role in OS and cellular viability than expected in CAD.

To summarise this study concludes IL-10 may be of limited use as a biomarker for whole heart dysfunction, oxidative stress and cellular viability in CAD but demonstrates the importance of accounting the entire cytokine network in disease progression.

Appendices

Appendix A: Study recruitment criteria

Inclusion criteria	Exclusion criteria
Diagnosis of CAD CABG surgery scheduled Male of female Age > 18	Non-English-speaking patients Age < 18

Appendix B: Patient recruitment consent form.

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NHS Foundation Trust

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PATIENT INFORMATION SHEET

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 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 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 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.

Blackpool Teaching Hospitals NHS
NHS Foundation Trust

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 6th December (version 1.1) 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 give permission for my cardiac tissue and internal mammary artery which would be normally discarded and blood samples to be supplied to The University of Salford, and for The University of Salford to store and distribute them 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 taking consent	**Date**	**Signature**

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

Appendix C: ELISA reagent preparation

Wash buffer (x1)

Number of strips	Wash buffer concentrate (ml)	Distilled water (ml)
1-6	25	475
1-12	50	950

Assay buffer (x1)

Number of strips	Assay buffer concentrate (ml)	Distilled water (ml)
1-6	2.5	47.5
1-12	5.0	95.0

Biotin detection antibody
make a 1:100 dilution prior to use

Number of strips	Biotin-conjugate (ml)	Sample diluent (ml)
1-6	0.03	2.97
1-12	0.06	5.94

Streptavidin-HRP
make a 1:200 dilution prior to use

Number of strips	Streptavidin-HRP (ml)	Assay buffer (ml)
1-6	0.03	5.97
1-12	0.06	11.94

Amplification diluent
make a 1:2 dilution prior to use

Number of strips	Amplification diluent (ml)	Distilled water (ml)
1-6	3	3
1-12	6	6

Amplification reagent I
Make a 1:100 dilution prior to use

Number of strips	Amplification reagent I (ml)	Amplification diluent (ml)
1-6	0.03	2.97
1-12	0.06	5.94

Amplification reagent II
Make a 1:250 dilution prior to use

Number of strips	Amplification reagent II (ml)	Assay buffer (ml)
1-6	0.012	2.988
1-12	0.024	2.979

Appendix D: Mean IL-10 serum concentrations (pg/ml)

Patient ID	IL-10 concentration (pg/ml)	SEM
1	5.11	0.0048
2	14.74	0.0171
3	5.80	0.0110
4	6.17	0.0380
5	1.56	0.0009
6	0.81	0.0015
7	0.66	0.0032
9	2.00	0.0025
8	6.60	0.0013
10	6.48	0.0188
11	3.21	0.0111
12	3.28	0.0088
13	0.88	0.0032
14	1.29	0.0052
15	1.45	0.0165
16	1.37	0.0032
17	2.28	0.0028
18	2.64	0.0035
19	1.38	0.0022
20	1.38	0.0025
21	5.33	0.0443
22	2.03	0.0103
23	3.54	0.0280
24	1.42	0.0024
25	2.73	0.0095
26	3.67	0.0015
27	1.49	0.0031
28	2.35	0.0139
29	1.44	0.0055
30	1.89	0.0105
31	3.23	0.0119
32	5.04	0.0904
33	2.86	0.0384
34	34.62	0.0061
35	5.50	0.0434
36	4.76	0.0101
37	5.23	0.0126
38	3.32	0.0096
39	6.80	0.0283
40	13.35	0.0247
41	1.65	0.0600
42	4.43	0.0846

43	12.02	0.1155
44	0.63	0.0016
45	5.05	0.3298
46	2.43	0.1446
47	1.15	0.0046
48	2.39	0.0120
49	0.30	0.0011
50	1.03	0.0110
51	0.46	0.0025
52	0.83	0.0018
53	0.35	0.0014
54	2.05	0.0140
57	3.38	0.0049
58	5.95	0.0098
59	7.51	0.0271
60	8.17	0.0031
61	6.37	0.0130
62	4.56	0.0021
63	1.97	0.0143
66	7.08	0.0143
67	15.90	0.0321
68	2.72	0.0114
69	2.78	0.0013
70	2.18	0.0268
71	2.54	0.0030
72	4.68	0.1330
73	2.05	0.0889
74	2.70	0.0481

Appendix E: Individual patient echocardiogram data

Patient ID	EF	LVOT	PASP	TASPE	E/a ratio	Peak E-wave	EDV
1	40	0.9	7	1.9	1.6	0.85	
2	61.5		17	1.49	0.63	0.45	68.38
3	55	1.1	22	2.2	0.7	0.5	
4	55		36				
5	77.73	1.01		2.7	0.62	0.44	65.1
6	58.3			2.4	0.85	0.75	103.17
7	69.81			2.2	1.21	0.91	106
9							
8	50	1.15		2.77	0.78	0.82	
10	50	0.7		2.5	0.98	0.54	
11	70.29	0.958		1.9			68
12	22.3	1.13			0.89	0.9	197.84
13	52.5	0.94		2.81	0.76	0.74	
14	50	0.98		1.61	1.34	1.06	
15	55	1.3	30	2.2			
16	61	1.04		2.2	1.5	0.96	92.135
17	26.7	0.55			1.86	0.86	68.1
18	39.14			1.8	1.29	1.13	99.41
19	55				0.72	0.47	
20	30						
21	55					0.76	
22	35	1.03	44	2.09			
23	35	0.75		1.69	0.83	0.7	
24	55				0.99	0.55	
25	50						
26	53.7		36	1.4	1.04	0.47	133.5
27							
28	38.7	0.78		1.8	1.13	1.01	79.45
29							
30	55	0.92	19	2.05	1.05	0.92	
31	55	1.17	18	1.78	1.06	0.93	
32	55	0.69		1.76	0.92	0.8	
33	30.5	0.81		2.58	0.72	0.86	162
34							
35	70				0.91	0.67	
36	50				0.77	0.72	
37	60				0.93	0.65	
38							
39	35						
40	57.5						
41	55	1.08		2.8	0.82	0.85	

42	40						
43	45	1.1		2.48	0.76	0.51	
44	55	0.69	18	2.14	0.84	0.63	
45		1.01			0.59	0.47	
46	35						
47	51	0.99			0.59	0.73	83.12
48	50	1.21	35	2.88	0.88	0.94	
49							
50	55	0.98			1.03	0.78	
51	40	1.09		2	0.56	0.57	
52	45			2.57	0.63	0.62	
53	50	0.97		2.3	1.18	1.06	
54	55	0.83		2.09	1.31	0.75	
55	45	0.62	32.7		0.55	0.53	
56	30.2	1.12		2.27	0.74	0.48	127.89
57	55	0.77		1.82	0.78	0.57	
58	60	1.23		2.04	0.47	0.43	
59	57.5	0.92	35.2	1.8	1.02	1.1	
60	45	0.91			1.38	0.95	
61							
62	45	0.89	22	1.7	0.77	0.35	
63	56	0.91		2.24	1.05	0.61	104
64	45	0.81	29.6		0.75	0.64	
65							
66	45.8	0.68		2.05	0.57	0.48	89.595
67	32.6	0.92	23	1.78	0.56	0.59	110.985
68	40.6				0.71	0.67	107.45
69		1.02		2.52	1.05	0.77	
70		1.34			1.03	0.82	
71	62.5		34.2		1.43	1.09	
72	45	0.69	13	1.79	0.86	0.86	
73	53	0.85		2.05	0.68	0.56	
74	55	1.49	27	2.57	1.02	0.94	

Appendix F: Normal echocardiogram values

Measurement	Normal Range
Ejection Fraction (%)	**55 - 70**
LVOT (m/s)	**0.8 – 1.0**
PASP (mmHg)	**< 35**
TASPE (cm)	**1.5 – 2.5**
E/a ratio	**1 – 2**
Peak E-wave velocity (m/s)	**0.6 – 0.8**
EDV (ml)	**56 – 104**

Appendix G: Individual patient demographics

Patient ID	Gender	Age	BMI	Heart rate
1	Male	55	27.06	69
2	Male	89	22.44	52
3	Male	74	25.86	70
4	Male	78	19.27	75
5	Male	70	43.28	
6	Male	71	35.06	77
7	Male	69	39.10	
8				
9	Male	62	38.82	68
10	Male	60	36.14	70
11	Male	66	25.99	
12	Female	68	29.21	
13		59		60
14	Female	54	20.43	58
15	Female	73	23.03	
16	Male	65	35.92	
17	Female	76	17.91	
18	Female	53	17.63	
19	male	64		
20	Male	72	32.88	67
21	Male	73	32.65	
22	Male	78	28.73	63
23	Male	40	37.65	71
24	Female	56	38.02	67
25	Male	72	25.95	
26	Male	61	36.26	
27				

28	Female	81	16.73	
29				
30	Female	72	32.89	
31	Male	57	26.53	
32	Male	69	28.41	82
33	Female	69	24.02	73
34				
35	Male	70	29.05	
36	Male	76	21.08	
37				61
38				
39	Male	74	32.95	78
40		67		70
41	Male	58	32.33	80
42	Male	64		
43	Male	71	31.53	
44	Male	69	23.55	60
45	Female	71	18.72	65
46	Male	78		
47	Male	60		
48		66		80
49				
50		61		
51		78		
52		60	22.77	
53		70	33.14	
54		76	28.39	70

55			78		68
56			60		58
57	Male		74		
58	Male		68		
59			75	24.26	69
60			66	34.41	60
61					
62			72	31.25	66
63			57	35.49	
64			46	22.41	88
65					
66		60	28.72737719	75	
67		74	0	69	
68	Male	79			
69	male	58		76	
70	male	56	39.48576676		
71		53			
72	male	73	20.38567493	65	
73		51	37.76411473		
74	female	59			

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