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The Role of Interleukin-8 In Ischemic Heart Disease and Oxidative Stress

Anna Ioannidi

School of Science, Engineering, and Environment

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List of Abbreviations.

ANG II- angiotensin II

ASMR – Age-standardised mortality rates

CABG- Coronary artery bypass grafting

CAD- Coronary artery disease

CHF- congestive heart failure

CVD- Cardiovascular disease

CHD- Coronary heart disease

DCF- 2', 7'–dichlorofluorescein

DCFDA- 2',7'-Dichlorodihydrofluorescein diacetate assay

DMEM- Dulbecco's modified Eagle's medium

DNA- deoxyribonucleic acid

EC: endothelial cells

EDV- End diastolic volume

EF- Ejection fraction

ELISA- Enzyme-linked immunosorbent assay

eNOS- endothelial nitric oxide synthase

FBS- fetal bovine serum

GDMT- Guideline-directed medical therapy

H_2O_2-Hydrogen peroxide

HF -Heart failure

HFrEF- Heart failure with reduced ejection fraction

HR- Heart rate

IHD- Ischemic heart disease

IL-10- Interleukin-10

IL-1β- Interleukin-1β

IL-4- Interleukin-4

IL-6- Interleukin-6

IL-8- Interleukin-8

LDL- Low-density lipoprotein

LV- Left ventricle

LVOT-Left ventricular outflow tract.

MI- Myocardial infarction

NO- Nitric oxide

OS- oxidative stress

Ox-LDL- oxidized low-density lipoprotein

PASP- Pulmonary arterial systolic pressure

PCI- Percutaneous coronary intervention

PTCA- Percutaneous transluminal coronary angioplasty

RAAS: renin–angiotensin–aldosterone system

ROS- Reactive oxygen species

RV- Right ventricle

SNS: sympathetic nervous system

SV- Stroke volume

TAPSE- Tricuspid annular plane systolic excursion TMB-

Tetramethyl-benzidine

TNF -Tumor Necrosis Factor

VSMC-Vascular smooth muscle cells

Abstract.

Ischemic heart disease also known as coronary heart disease remains a primary cause of mortality and morbidity worldwide (Nowbar et al., 2019). Several mechanisms are involved in the development of the condition, such as chronic inflammation and the imbalance between the formation of reactive oxygen species (ROS) and antioxidant defences also called oxidative stress (Beteridge 2000). Chronic inflammation seems to be playing a key role in the progression of the disease as well as the long-term prognosis. Since the establishment of the link between inflammation and the prediction of cardiovascular events, several pro-inflammatory cytokines have been examined as potential biomarkers and therapeutic targets (Vilela & Fontes-Carvalho, 2021) .

To further elucidate potential biomarker , this study measured the levels of pro-inflammatory cytokine IL-8 in 52 CAD patients' serum with the goal to investigate the possible correlation between those levels to cardiac function. The average concentration of IL-8 was 5.77± 5.81 pg/mL (n=38), while 14 patients' IL-8 levels were undetectable.
Trends were observed between the correlation of IL-8 and Ejection fraction (EF) (p= 0.0684, r^2= -0.3163, n= 34) and the correlation between IL-8 and the Body Mass Index (BMI) (p=0.0784, r^2=0.2358. n=14). While other parameters did not correlate with IL-8. For example no correlation was observed between levels of IL-8 , Left ventricular outflow tract (LVOT) (p=0.4073, r^2=0.04330, n=18) or between Tricuspid annular plane systolic excursion (TAPSE) (n=21, r^2 = 0.009047, p= 0.6817). No correlations were observed

between the levels of IL-8 and the Age and Heart Rate (HR) (n=34, r^2 = 0.02481, p=0.3736) and (n=18, r^2 = 0.0970, p=0.2091) respectively.

In addition to that, no significant difference between the levels of IL-8 was observed between the male (n=16) and female (n=6) patients of the study cohort (p= 0.2851).

Finally, to further understand the involvement of IL-8 in IHD, different concentrations of the cytokine along with hydrogen peroxide, an established ROS inducer that was employed as a positive control, were introduced to rat cardiomyocytes to investigate the possible dose-dependent effects in oxidative stress production.

Data demonstrated that the two different concentrations of IL-8 (6 pg/mL and 50 ng/mL) alone did not cause a significant increase in oxidative stress levels (n= 8, p>0.05). However, an increase in OS was observed when 50 ng/mL were co-introduced to H9C2 cells along with 100μM of H_2O_2 (n= 8, p<0.01) compared to the H9C2 cells that were only exposed to the 100μM of H_2O_2. In addition to that both concentrations of 6pg/mL, 50 ng/mL IL-8 caused a significant increase in OS (n= 8, p<0.05).

These findings suggest that IL-8 could potentially be an important biomarker of ischemic heart disease and its manifestations in cardiac dysfunction. While its ability to increase levels of OS in an ischemic model supports IL-8 involvement in the interplay of inflammation and oxidative stress in CAD. Further research is important to increase the study's power and validate these results.

1. Introduction

1.1 Ischemic heart disease and coronary artery disease definition.

Ischemic heart disease (IHD) known as coronary artery disease (CAD) is a medical condition defined by reduced cardiac blood flow, which causes an imbalance in myocardial oxygen supply and demand (Shimokawa & Yasuda, 2008). It is caused primarily by atherosclerosis, a condition in which plaques progressively develop and constrict the coronary arteries that provide oxygen-rich blood to the heart, limiting blood flow (NHS, 2022). Toxic compounds like lactic acid and reactive oxygen species accumulate in the ischemic tissue resulting in pathogenesis and damage to the tissue, this is caused by insufficient clearance via the capillary and venous blood systems (Steenbergen & Frangogiannis, 2012).

IHD can result in symptoms such as short-term pain (angina), abnormal heartbeat (arrhythmia), irreversible heart muscle injury (myocardial infarction), and muscular atrophy (heart failure) (Choi et al., 2009). Most commonly, the underlying clinical cause of IHD is coronary artery disease (CAD), caused by an atherosclerotic blockage or spasm of the epicardial coronary arteries, or microvascular dysfunction through a multifactorial process that is set off by the injury to the endothelium called plaque formation (Crea et al., 2022). As a result, the terms ischemic heart disease and coronary artery disease (CAD) are frequently used interchangeably (Jensen et al., 2020).

However, IHD is a chronic, multi-stage condition that can present at any moment or even begin as an unstable condition, generally because of an acute atherothrombotic

event caused by plaque rupture or erosion (Jensen et al., 2020). Research has discovered that IHD can arise in the absence or presence of obstructive CAD and that obstructive CAD is only one aspect of the complex, multifactorial, pathophysiological process of IHD which includes inflammation, microvascular coronary dysfunction, endothelial dysfunction, thrombosis, and angiogenesis (Marzilli et al., 2012). To further support this theory studies conducted by Douglas et al., (2011) and Patel et al., (2010) concluded that some patients presenting with severe obstructive coronary atherosclerosis, neither complained of chest discomfort (angina) nor experienced any other myocardial ischemia symptoms. Other studies have also found that, a great portion of the patients that have undergone coronary revascularization to treat CHD presented with myocardial ischemia after a brief period, while the prognosis was not affected by the overall reduction or bypass of the stenosis (Alderman et al., 2004, Boden et al., 2007, Henderson et al., 2003).

Nevertheless, IHD continues to be the leading cause of myocardial infarction (MI), despite the advancements that have been made in the treatments of CHD and myocardial ischemia, with studies showing that in more than 70% of individuals, IHD was the primary cause of heart failure (HF) (Leancă et al., 2023). Further research on the progression, relation, and treatment strategies for the two diseases will help reduce the morbidity and mortality rates.

1.2 Epidemiology and the impact of ischemic heart disease.

Cardiovascular disease (CVD) describes every disease of the heart or the circulatory system and includes a wide range of disorders such as congenital heart disease, ischemic heart disease, cardiopulmonary arrest, angina, and myocardial infarction (Gaze, 2013). Heart disease remains the second leading cause of mortality after cancer resulting in around 17.9 million death per year worldwide and more than 160,000 deaths in the United Kingdom per year (Office for National Statistics, 2021).

In more detail, IHD is classified as the most prevalent among CVD and has resulted in more than 9 million deaths worldwide only in the year 2016 (Nowbar et al., 2019). In the UK alone around 66,000 people die each year from CAD which equates to about 1 death every 8 minutes (British Heart Foundation, 2023). In addition to that many individuals with non-fatal IHD live with chronic disabilities and severely affected quality of life (Moran et al., 2014). The prevalence of CAD is projected to continue to rise, owing not only to rising rates of obesity, diabetes, and metabolic syndrome as well as to the aging of the population. Researchers have shown that by the year 2030, 23 million people will have died from IHD (Khan et al., 2020).

Despite of this, deaths from IHD have been declining in England and Wales on an annual basis, with an overall decline of 48.1% from 2001 to 2019 (106,177 to 55,064 deaths respectively) (Office for National Statistics, 2021).

Age-standardized mortality rates (ASMRs) have also declined since 2001, from 246.5 to 96.1 fatalities per 100,000 patients in 2019 (Figure 1).

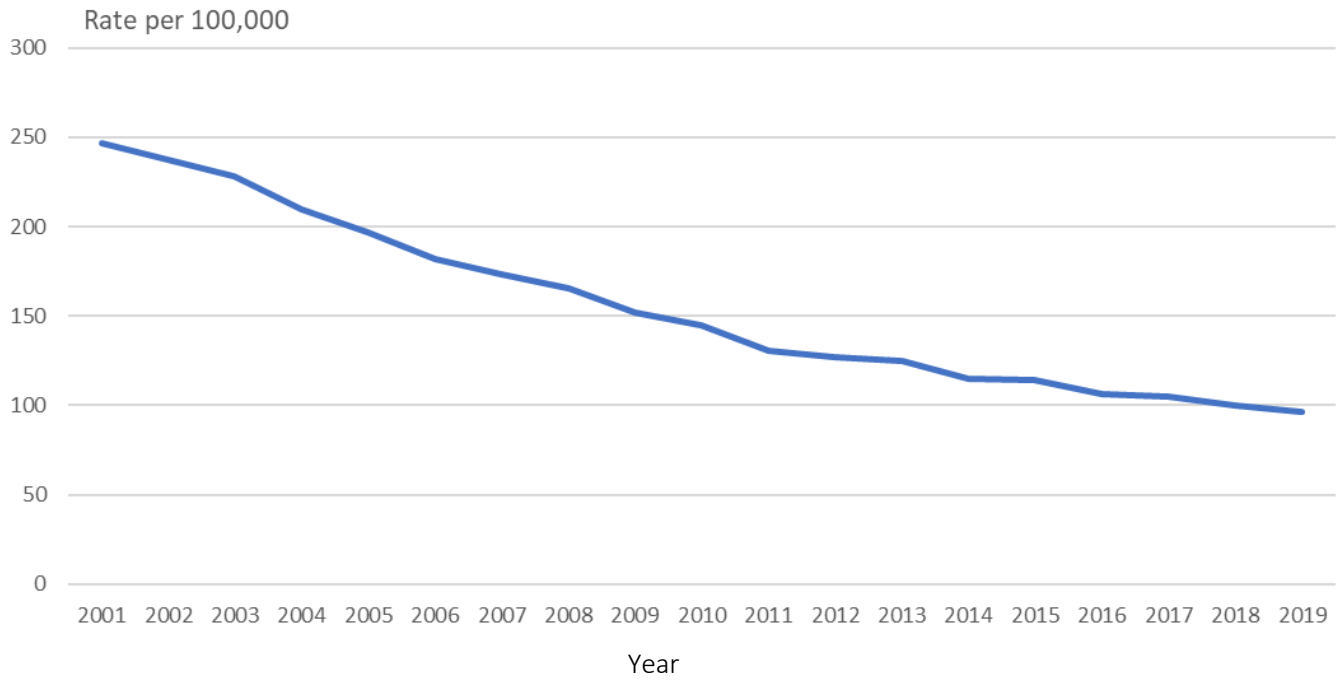

Figure 1. Age-standardized mortality rate (ASMR) per 100,000 people from ischemic heart disease in England and Wales from 2001 to 2019. Data for the graph adapted from Office for National Statistics 2021.

ASMRs have significantly decreased year after year, apart from 2014 to 2015 and 2016 to 2017 when the decline was not statistically significant (Office for National Statistics, 2021). In addition to that the prevalence of CVD is decreasing in the Western world, despite rising rates of lifestyle-related risk factors such as obesity, smoking, and type II diabetes mellitus (Amini et al., 2021). Advances in therapeutic and invasive intervention have contributed to that decrease (Kandaswamy & Zuo, 2018). Patients develop heart failure as a result of the improving outcomes for individuals with acute cardiac problems, which leads to the need for longer-term care and monitoring which consequently results in a higher health burden than the acute events alone (Gaze, 2013).

Finally, it should be mentioned that CVD remains a major economic burden of health and social care (Roth et al., 2020). According to Public Health England, CVD costs around £7.4 million

per year to the healthcare system and it was estimated that CAD cost the UK healthcare system around £1.73 billion in the year 1999 alone (Liu, 2002).

1.3 Aetiology and risk factors of Ischemic heart disease.

IHD is a progressive multifactorial disease therefore there are many genetic and environmental factors that can contribute to the progression of the disease (Gaze 2013, Gupta & Wood, 2019). According to studies conducted on patients with CAD and healthy controls of the general population 70% of patients with CAD can often present with several different risk factors while only 2%-7% of the healthy controls had none of the identified risk factors for CAD (Khan et al., 2020). The most common risk factors are age, smoking, obesity, hypertension, family history of IHD and CAD, diabetes, and chronic inflammation. Smoking and obesity have been proven to contribute to 36% and 20% of the cases of IHD respectively (Kivimäki et al., 2012).

Age is a strong irreversible risk factor for CVD and IHD as well. In both men and women, the likelihood of CAD increases after the age of 35. After the age of 40, men and women have a 49% and 32% risk of developing CAD respectively (Sanchis-Gomar et al., 2016, Roger et al., 2001). Furthermore, while both sexes share many of the same risk factors, it is important to consider the plethora of female-specific risk factors, given the underlying biological differences observed in cardiac structure, function, and pathophysiology. Women exhibit distinct cardiac characteristics, such as smaller left ventricles and coronary arteries, along with lower stroke volumes under physiological conditions compared to men (Beale et al., 2018, Taqueti, 2018). Moreover, women have been found to have higher pulse rates at rest, greater systolic function, and less diastolic compliance (Crea et al., 2015). On a cellular level, studies have shown that women

have lower glucose uptake and a preference for using fatty acids compared to men. However, women's mitochondria demonstrate higher resistance to ischemic reperfusion injury, contribute to decreased production of ROS and a higher capacity for antioxidants (Beale et al., 2018).

While these female-specific risk factors extend beyond physiological differences, encompassing factors such as exposure to menarche, pregnancy, anemia, hypertension, and menopause (Majidi et al., 2021). Even though obstructive CAD is the leading cause of mortality in women of all ages, the risk of CAD is considered to be relatively low until women reach menopause at around 51 years (Lerner & Kannel 1986, Shaw et al., 2002), with men and women only attaining similar prevalence rates in their seventh decade of life (Smith et al., 2001, Stangl, 2002). Overall, equivalent prevalence rates are attained for women 10 years older, so that the CAD prevalence rates of a 55-year-old male are comparable to those of a 65-year-old female (Lerner & Kannel 1986). Menopause is an indicator of cardiovascular biological changes in women. It significantly contributes to an increased risk of developing IHD after the age of 55 years (Aggarwal et al., 2018). During menopause, the levels of estrogen decrease causing an apparent impact on vascular function and cholesterol levels (Shaw et al., 2006). Furthermore, research has shown that menopause increases the risk of metabolic syndrome and truncal obesity (Wellons et al., 2012). For both sexes, however, age displays accumulating chronic exposure to all the other risk factors.

In more detail, arteriosclerosis is an age- related degenerative process that results in increased arterial stiffness (Wen et al., 2015). It is also considered to be a remodeling event of the medial layer caused by repeated hemodynamic stress. Despite being a unique disorder in terms of histopathology and pathophysiology, arterial aging can predispose, exacerbate, and speed up atherosclerotic disease via the enhancement of the proatherogenic blood flow conditions (Tsioufis et al., 2018).

Hypertension has been established as a risk factor for atherosclerosis, as a result of both the oxidative and mechanical stress it puts the arterial wall in, as well as the important role it plays in the rupture of the delicate thin-cap plaques (Brown et al., 2022). Studies have shown that three out of four patients have hypertension (Vongpatanasin, 2008). In a study conducted by Brown et al (2022), twelve modifiable risk factors were investigated, it was then proven that hypertension and smoking were the two major reasons for mortality. It should also be noted, that even though hypertension is an established risk factor for CAD only 54% of patients effectively manage their blood pressure (Vongpatanasin, 2008). Despite a large body of data connecting hypertension to atherosclerotic disease, the underlying pathophysiological pathways remain unknown. Arterial hypertension is a chronic systemic condition characterized by several functional and anatomical macrovascular and microvascular changes. Vascular changes represent an adaptive response to hemodynamic stress and form a cycle in which hypertension is frequently considered as both a source and a result. However, new data connects hypertension to oxidative stress and inflammation through a variety of molecular mechanisms (Yannoutsos et al., 2014).

Smoking has been established as a conventional modifiable risk factor for CAD. Tobacco use is generally acknowledged as the most complex risk factor for atherosclerosis (Barua & Ambrose, 2013). This intricacy can be explained by several reasons. To begin with, tobacco smoke consists of almost 4000 recognized and 100,000 unknown components (Smith, 2001). It is also known that smoking's detrimental effects are often dosage-dependent (Messner & Bernhard, 2014). Finally, there is evidence that supports that genetic background history might have an effect on susceptibility to smoking's harmful consequences (Hall, 2002). Smoking contributes significantly to CAD’s development and progression.

Cigarette smoke is thought to cause endothelial dysfunction by OS and inflammation , altering the lipid profile and producing a highly prothrombotic setting (Barua & Ambrose, 2013, Messner & Bernhard, 2014). The FDA has stated that the risk of CAD decreases to that of lifetime non-smokers after 4 years of quitting, but according to the CDC the risk of CAD decreases within 10 years (Brown et al., 2022).

Another important risk factor for CAD is a family history of CVD. Research has shown that patients under the age of 50 who have a family history of early cardiac disease have an elevated risk of dying from CAD (Bachmann et al., 2012). A different study conducted by Hajar (2017) has reported that a father or brother diagnosed with CAD before the age of 55, as well as a mother or sister diagnosed before the age of 65, could be identified as risk factors.

Last but not least another important risk factor for IHD is obesity. Obesity is the risk factor that increases the probability of developing additional CAD risk factors such as hypertension, hyperlipidemia, and diabetes mellitus (Ades & Savage, 2017). Overweight individuals were twice as prone to develop coronary heart disease after adjusting for demographics, smoking, physical activity, and alcohol consumption (Ndumele et al., 2016). In addition to that according to a study conducted by McGill & McMahan (1998), obesity was also linked to more complicated, elevated, and high-grade atherosclerotic coronary artery lesions.

1.4 Treatment of CAD.

Although CAD is not a completely curable condition, its progression can be effectively managed, the symptoms can be controlled and the risk of complications such as an MI can be lowered (Cassar et al., 2009).

Treatment options include risk factors minimization via changes in lifestyle, medications like guideline-directed medical therapy (GDMT), and revascularization/surgery. The aims of treatment are to reduce the progression of atherosclerosis, avoid or decrease complications such as mortality , and attempt to remove ischemic symptoms ,with the gaol of enchaning the quality of life and restoring functional capacity (Dababneh & Goldstein, 2023).

Individualized lifestyle modifications such as changes in diet, weight loss, stopping smoking, and frequent exercise can result in risk reduction. Most importantly, substantial focus should be given to treating hypertension, diabetes, and dyslipidemia since these conditions might have a direct effect on stable IHD outcomes and increase the risk of future events (Dababneh & Goldstein, 2023). There are two types of guideline-directed medical therapy (GDMT). The first category comprises medications that delay atherosclerotic lesions, reduce future myocardial infarction occurrences, and reduce mortality over time (Knuuti et al., 2019). Amongst these are antiplatelet medicines, beta-blockers, renin-angiotensin-aldosterone blockers, and lipid-lowering medications The second group targets symptoms directly, by attempting to eradicate angina (or angina-like symptoms) using nitrates, beta-blockers, calcium channel blockers, and new therapeutics such as anti-inflammatory drugs (Doenst et al., 2022).

If the blood vessels are narrowed due to atheroma build-up, or if symptoms cannot be managed with therapeutics, interventional treatments or surgery may be needed to open up or bypass blocked arteries (NHS UK, 2020). There are two basic procedures of revascularization in stable ischemic heart disease, percutaneous coronary intervention (PCI) or else percutaneous transluminal coronary angioplasty (PTCA), and balloon angioplasty (Schwartz, 2009). PCI is a non-surgical procedure during which a balloon is inflated into the blocked coronary artery to suppress the plaque into the arterial walls and restore normal blood flow to the heart muscle (Ahmad et al., 2022).

To maintain the width of the artery a metal stent (a wire mesh tube) is usually implanted. However, another alternative is drug-eluting stents, these stents trigger the release of medication that prevents the artery from closing up again (NHS UK, 2020). In addition to that CABG (coronary artery bypass grafting) is also known as bypass surgery a heart bypass, or coronary artery bypass surgery. Coronary artery bypass graft surgery is a general surgical technique during which arteries or veins originating from the patient's body are joined to the coronary arteries to bypass atherosclerotic narrowing and increase the blood supply to the coronary circulation delivering blood to the myocardium (Dababneh & Goldstein., 2022), which consequently causes the restoration of function, viability, and reduction of angina symptoms (Bachar & Manna, 2023). It should be noted that, while coronary artery intervention significantly decreased the chances of restenosis from occurring, the risk of MI and heart-related death does not significantly decrease (Holmes et al., 2004). This implies that it is critical to control the condition's development and inhibit recurrence with suitable medication management that will protect the myocardium at a cellular level.

1.5 Pathology of CAD.

Understanding the pathophysiology of IHD is necessary to investigate the complex interplay of factors, such as inflammation and OS that contribute to its development. This section aims to provide a comprehensive overview of the underlying mechanisms of IHD, starting with the physiological dynamics of the arterial wall and progressing to the pathological changes that occur in atherosclerotic plaque formation and subsequent MI.

1.5.1 Vascular pathology and the physiology of the arterial wall.

Before we can understand the pathology of IHD, we must understand the normal physiology of the vascular wall (Figure 2.)

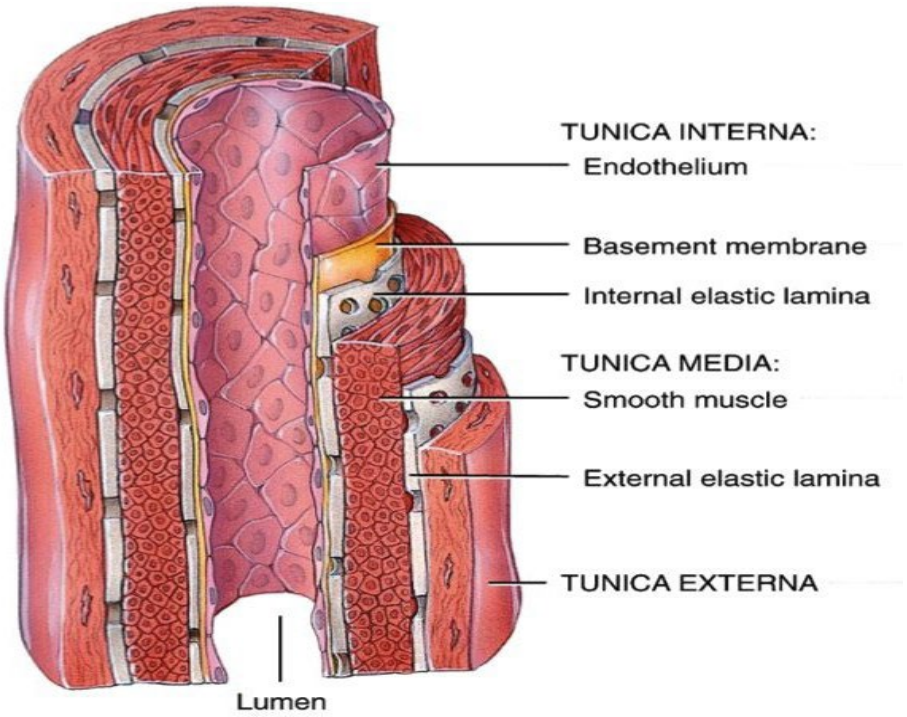

Figure 2. Illustration of the physiological structure of the layers of a medium-sized elastic artery. Image adapted from Almeida, 2013.

Arteries consist of three layers of tissue the *intima* or *interna, media*, and *adventitia* or *externa*. The *intima* layer consists of the endothelial surface, basement membrane, and internal elastic lamina, playing a crucial role in nutrient absorption and waste removal for cell homeostasis (Rahman & Siddik, 2023, Michel et al., 2022). The media layer, consisting of circular smooth muscle cells and elastin, allows for efficient blood flow and kinetic energy storage (Paul et al., 2021; Tousoulis et al., 2018). The outermost layer, the *adventitia*, is primarily made up of collagen fibrils and a small number of fibroblasts, inflammatory cells, adipocytes, and progenitor cells. *Tunica adventitia* also contains nerve terminals and the *vasa vasorum*. Under normal conditions, the *vasa vasorum* carries molecules from the blood to the *adventitia* by transport via the artery wall. (Galis et al., 2022, Gössl et al., 2003).

However, in response to chemical and paracrine cues, the *vasa vasorum* network might expand to boost the transport of oxygen and nutrients in atherosclerotic lesions. On the other hand, there is evidence supporting that *vasa vasorum* causes the formation of atherosclerotic plaques either as a result, or a direct cause of the atherosclerotic plaque's fragility, rupture, and as a product of the acute coronary syndrome (Herrmann, 2001, Ambrose & Singh, 2015, Kawabe & Hasebe, 2014).

1.5.2 Atherosclerotic Plaque Formation.

Atherosclerotic plaques are formed by the build-up of degenerative elements within the artery wall of the middle size and large arteries (Luk & Gotlieb, 2014). These accumulations are mostly made up of macrophages, lipid debris, connective tissue, and fibrous materials that are calcified to varying degrees depending on the regional wall and systematic features, as well as the chronicity of the lesions (Jebari-Benslaiman et al., 2022).

There are five main steps involved in the development of the plaque: LDL buildup in the *intima,* LDL oxidation, monocyte-macrophage recruitment, absorption of oxidized LDL by macrophage scavenger receptors, and transformation of macrophages into foam cells and the creation of a fibrous cap including smooth muscle cells, which allows plaque stability (Brown and Goldstein 1979, Fuster et al., 2005). Inflammatory cytokines (See section, 1.7.5) are involved in every stage of the process, establishing atherosclerosis as a chronic inflammatory disease (Tousoulis et al., 2018). In further detail, cholesterol-rich low-density lipoprotein (LDL) particles of minimum diameter travel through the endothelial layer and accumulate in the extracellular sub-endothelial space via the LDL receptor protein, a mosaic cell surface protein that identifies apolipoprotein B100 embedded in the LDL particle (Brown and Goldstein 1979, Fuster et al., 2005).

LDL lipoprotein also identifies apolipoprotein E, which is prevalent in chylomicrons and extremely low-density lipoprotein residues, as well as intermediate-density lipoprotein. Native LDL can be oxidized by artery endothelial cells, smooth muscle cells, and macrophages, through a scavenger receptor, and once oxidized the LDL may be taken up by monocytes and macrophages (Leitinger & Schulman, 2013).

Unlike the standard LDL receptor, this receptor absorption method is not inhibited by increasing cellular cholesterol ester concentration, resulting in the formation of lipid-laden foam cells (Betteridge, 2000). This causes the production of juvenile high fatty streaks within the endothelium. In addition to that the macrophages secrete lipids and cytokines into the *intima* (Linton & Fazio, 2003).

Cytokines promote intimal thickening by smooth muscle cell proliferation, which results in collagen secretion and fibrosis (Nilsson, 1993, Steen et al., 2020). As the lesion progresses, the medial layer of the arterial wall atrophies and the elastic lamina is disrupted (Linton & Fazio, 2003). In addition to that, collagen produces a fibrous cap over the site that appears hard and white called the fibrolipid or fibrous plaque. This plaque consists of macrophages as well as extracellular lipids (Gaze, 2013, Ross 1975). The exposed lipid core triggers platelet aggregation and gradually progresses to thrombosis. This lesion expands as a result of the persistent platelet aggregation, resulting in the narrowing of the artery lumen and causing endothelium vulnerability, causing localized ischemia (Gaze, 2013, Ross 1975).

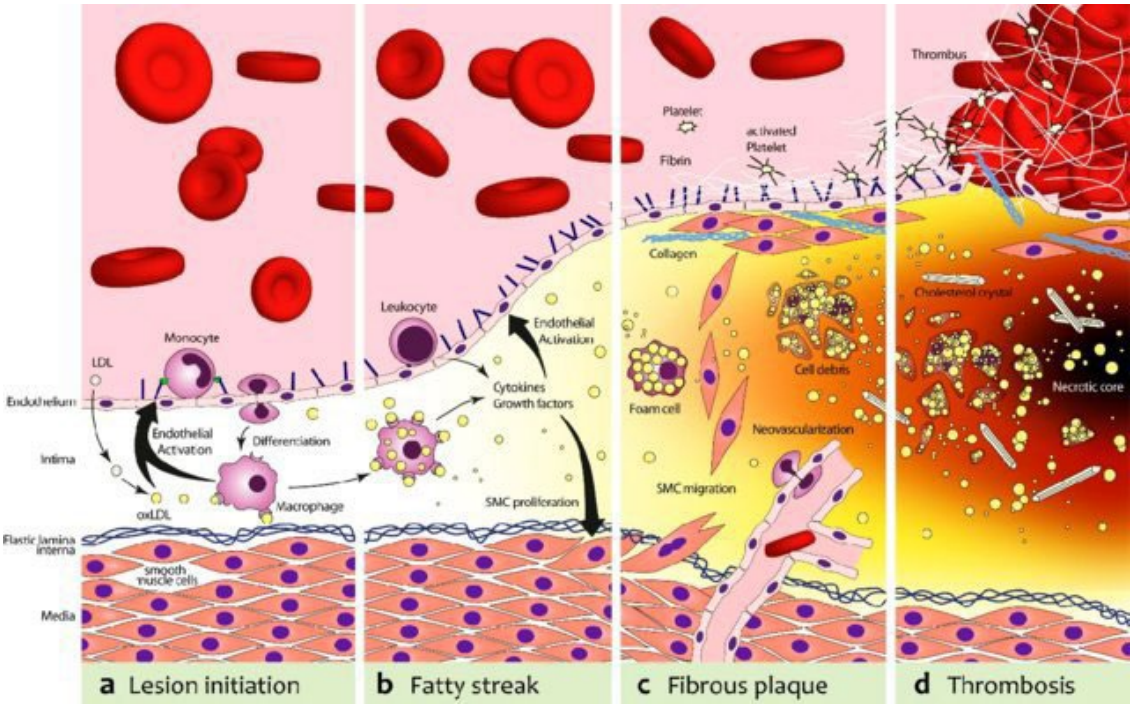

Figure 3. The figure shows the progression of the atherosclerotic pathogenesis. (a) In the first step, LDL is deposited in the endothelium and undergoes oxidative alteration, resulting in oxidized LDL (ox-LDL). OxLDL stimulates endothelial cells to produce adhesion molecules (VCAM-1, P-Selectin) and chemokines (for example, Monocyte Chemoattractant Protein-1 (MCP-1) and Interleukin 8 (IL-8). Monocytes are drawn to the intima and develop into pro-atherogenic macrophages. (b) Macrophages capture leftover oxLDL via their scavenger receptors, contributing to endothelial activation and, ultimately, leukocyte recruitment through the release of Tumor Necrosis Factor (TNF-) and IL-6. (b) Growing plaque volume encourages neovascularization. Smooth muscle cells (SMCs) that are proliferating help to maintain the nascent fibrous plaque. The deposition of fibrin and activated platelets on the defective endothelium that produces tissue factor (TF) and von Willebrand factor (vWF) creates a pro-thrombotic environment. (d) Apoptosis can lead to the release of cell debris and lipids from foam cells. Foam cells form when there is a dysregulation in lipid metabolism in macrophages. This process ultimately contributes to the formation of a necrotic core in atherosclerotic plaques. The plaque can potentially be destabilized by proteases produced

by foam cells. This can trigger plaque rupture, at which point extracellular matrix components (such as collagens, elastin, TF, and vWF) accelerate thrombotic events. Image adapted from (Steinl & Kaufmann, 2015).

A distal embolization of such a thrombus can migrate downstream and entirely obstruct smaller arteries. Finally, plaque rupture, fissure, or erosion can occur at any point of this development and may result in thrombotic occlusion and trigger acute coronary syndromes otherwise called myocardial infarction (MI) or else heart attack and can result in death (Institute of Medicine US, 2010, Marzilli et al., 2012).

1.5.3 Atherosclerosis to MI.

Myocardial ischemia is characterized by the imbalance between the myocardial oxygen supply and demand preventing the heart muscle to receive sufficient oxygen. This reduced blood flow is caused by the partial or total blockage of one or more of the coronary arteries (Hoffman 1987, Shimokawa & Yasuda, 2008).

The heart's structure and function are altered because of myocardial ischemia, which damages the myocardium and simultaneously starts compensatory processes in the damaged tissue, along with the production of new proteins, connective and fibrotic tissues (Cziraki et al., 2023). The damaging effects of cardiovascular diseases including MI and HF are attributed to these alterations observed in acute myocardial ischemia and reperfusion injury (Calvert, 2014). MI caused by myocardial ischemia causes a sudden blockage of the oxygen and nutrients supply myocardium (Sobel, 1974). Cardiac myocytes undergo substantial electrical, metabolic, and structural remodeling to survive under new conditions (Sobel, 1974).

Reperfusion injury is the term used to describe the damage to the myocytes via the myocardial stunning (myocardial hibernation), microvascular, endothelial injury, and necrosis following a myocardial ischemic event (Yellon, 2000). Chronic ischemia causes irreversible damage to heart cells (Lefer & Marbán, 2017). On the other hand, effective remodeling after a severe ischemia-reperfusion can significantly enhance patient outcomes via the rescuing of the myocardium (Jugdutt, 2010). In addition to that, myocardial ischemia is an important predictor of outcomes in individuals with IHD, studies have shown that the presence of myocardial ischemia in patients that presented with HF indicates a worse outcome and additionally, the outcome is defined by the severity and extension of atherosclerosis (Albakri, 2018).

1.6 Cardiac Remodelling in IHD.

Cardiac remodeling is the term used to describe the collection of molecular, cellular, and interstitial changes that occur following chronic ischemic conditions or MI, altering the structure and function of the heart (Pfeffer et al., 1979). Initially, cardiac remodeling can be an adaptive mechanism when the heart is challenged by effort, volume expansion, overload, or injury, helping to maintain cardiac output and compensate for increased demands (Maron & Pelliccia, 2006). However, this process may progress as a maladaptive response, impairing heart function and contributing to the progression of heart disease, resulting in complications like HF and arrhythmias (Brenner & Ertl, 2012).

Cardiac remodeling includes various structural and functional changes that respond to different cardiac insults. In response to an acute MI, cardiomyocyte necrosis triggers immuno-inflammatory pathways, complement activation, production of ROS, and inflammasome activation, leading to changes such as dilation, hypertrophy, and fibrosis (Cohn et al., 2000).

During the initial phase of remodeling after an MI, the infarcted wall dilates and thins, potentially leading to premature rupture, aneurysmal distortion, and intracavitary thrombus development (Sutton & Sharpe, 2000; Phan et al., 2019). Both the tissue surrounding the necrotic region and the distant myocardium are affected by acute MI, causing rapid increases in wall shear stress and chamber dilation (Calvieri et al.,2023).

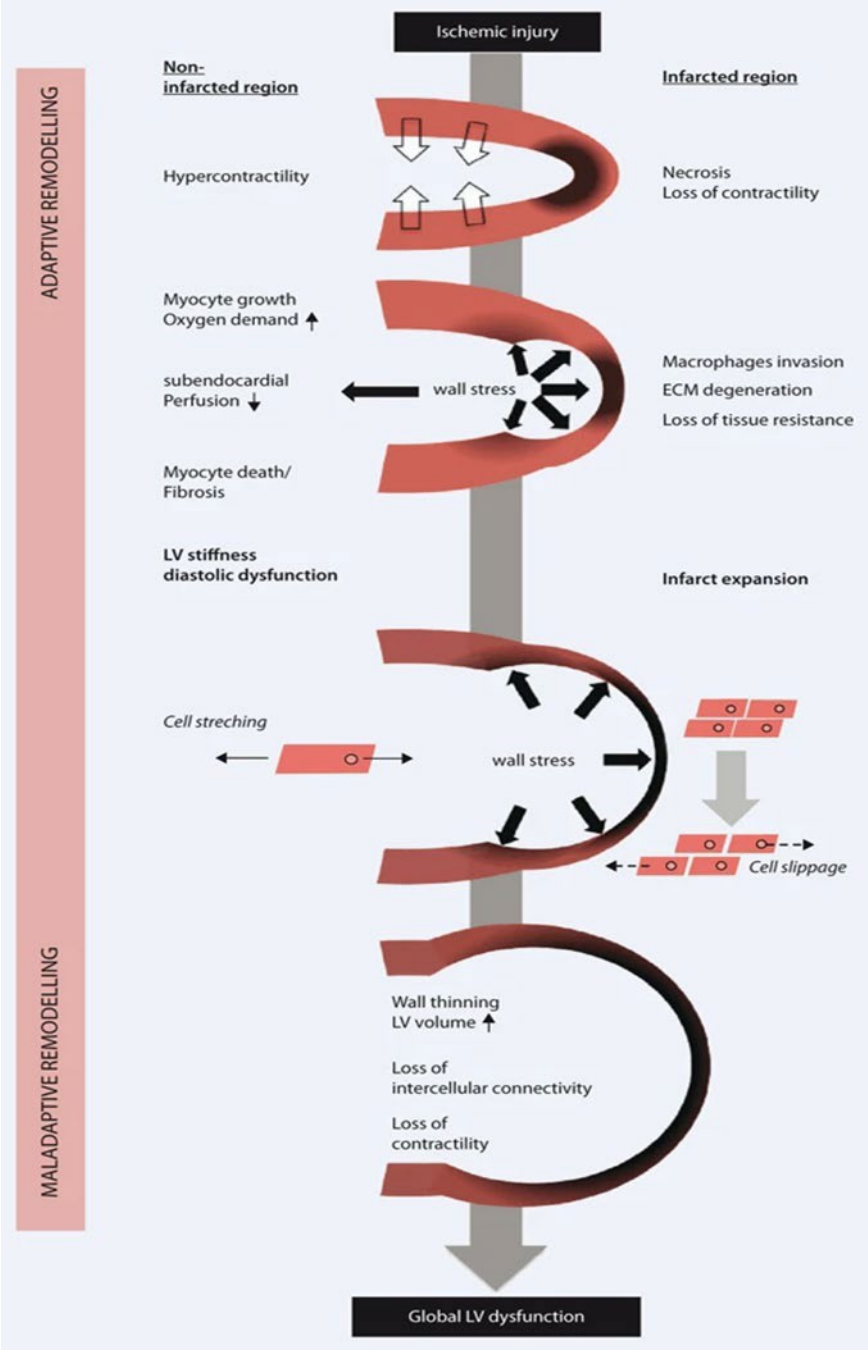

Figure 4. Schematic representation of the remodeling of the left ventricle following an MI. Image adapted from Brenner & Ertl, 2012

Regarding IHD, structural and functional changes predominantly affect the left ventricle, resulting in increased end-systolic and end-diastolic volumes and normal or decreased wall thickness (Ma et al., 2020). These alterations observed in IHD are the result of multiple ultrastructural, metabolic, and neutrally driven processes that occur in both infarcted and distant myocardium (Carrick et al., 2015, Del Buono et al., 2022). It should be noted that the heart is a naturally heterogeneous organ. The differences are found at a physiological level with the right and left side of the heart, as well as the atria and ventricles differ in cell constellations, hemodynamics, and electrical properties (Zimmer, 1994). While the heart naturally exhibits heterogeneity in its physiology, it undergoes further heterogenic modifications during passive ventricular remodeling in response to IHD (Kessler et al., 2014).

In further detail, the specific structural changes in cardiac remodeling include ventricular dilatation, particularly affecting the left ventricle in CAD. This enlargement attempts to increase the heart's filling capacity and maintain cardiac output despite reduced blood flow, but prolonged dilatation can weaken the heart's contractility (Roger, 2013). Moreover, the heart may undergo ventricular hypertrophy in response to the increased workload caused by narrowed arteries, initially enhancing contractility and blood pumping. However, sustained hypertrophy can lead to impaired diastolic filling and increased oxygen demand (Roger, 2013).

In terms of functional changes, remodeling can lead to impaired relaxation in CAD, compromising the heart's ability to relax and fill with blood during the diastole. This can further impact cardiac output and lead to heart failure with preserved ejection fraction (HFpEF) (Roger, 2013). Furthermore, remodeling changes, particularly myocardial fibrosis, can disrupt the normal electrical conduction system of the heart, leading to arrhythmias like atrial

fibrillation or ventricular tachycardia (Burchfield et al., 2013). Additionally, it should be noted that ischemic injury triggers significant electrical, metabolic, and structural remodeling modifications with myocytes, which play a critical role in the overall pathophysiology of IHD (Sobel, 1974).

At a cellular level, ischemic injury prompts an adaptive response to the limited oxygen supply. The cell stops the creation of ATP with the use of oxygen and alters from aerobic to anaerobic metabolism, producing ATP instead of glycogen and creatine phosphate (YI et al., 2003. Anaerobic metabolism causes the buildup of osmotic active products such as lactic acid and inorganic phosphates, lowering intracellular pH and affecting intracellular osmotic pressure (YI et al., 2003).

The availability of ATP is substantially decreased, and the energy-dependent Na^+/K^+ pump in the plasma membrane diminishes or loses its transport function. The cell can no longer maintain ionic gradients across the membrane. Failure of this active transport causes changes in the intracellular ionic contents (Berger & Garnier, 1999, de Haan & Hasaart, 1995, Hansen, 1984).

Na^+ concentration increases while K^+ concentration decreases, causing membrane depolarization. In the absence of membrane potential, Cl^- ions and substantial numbers of Ca^{2+} (de Haan & Hasaart, 1995, Hansen 1984) flow into the cell via the voltage-dependent ion channel. The combined impact of the aforementioned processes, the failure of active transport, the opening of voltage-dependent channels, and anaerobic metabolism, results in an unusually large concentration of catabolites and ions in the tricellular space. The net solute gain causes an influx of water down the osmotic gradient, with the goal of establishing an isosmotic pressure on both sides of the plasma membrane. As a result, the cell swells, which leads

to cytotoxic edema, also known as acute cell swelling (de Haan & Hasaart, 1995, Klatzo, 1994). The water influx causes the dilation and deterioration of vacuoles, mitochondria, rough endoplasmic reticulum, and Golgi bodies (Rajasekharan & Lee, 2020). If hypoxia persists, further changes occur, such as the creation of blebs on the cell's surface and mitochondrial enlargement (Lemasters et al., 1987).

1.7 Pathogenic mediators of IHD.

IHD is a complicated condition that is regulated by a wide range of pathogenic mediators. Cytokines and OS are recognized as primary contributors that have a significant influence on the development of the disease and its clinical manifestations. In this section. The mechanisms and importance of these two crucial factors are examined, as well as their significant impact on IHD.

1.7.1 Oxidative stress definition.

The imbalance between the production of reactive oxygen species (ROS) and antioxidant defense known as OS can lead to tissue damage (Kibel et al. 2020). ROS are the physiological by- products and, on some occasions, an on-purpose consequence of activated cells as well as normal cellular aerobic metabolism during oxygen reduction (Kibel et al. 2020). Apart from non-free radicals like hydrogen peroxide (H_2O_2), hypochlorous acid, or peroxynitrite, which all present oxidizing features and are involved in OS upregulation, ROS also includes unstable free radicals such as superoxide anion (O2-), hydroxyl radicals, or lipid radicals (Roberts & Porter, 2013).

At low concentrations, ROS is involved in various physiological functions like signaling, while high levels of ROS generation cause oxidation of DNA, proteins, carbohydrates, lipids,

and other biological macromolecules, resulting in OS, if antioxidant defenses are ineffective (Cai & Harrison 2000, Hinkle et al., 1991). The generation of ROS is necessary for vascular health, but it must be strictly regulated to avoid pro-atherogenic contributors including inflammation and endothelial dysfunction (Batty et al., 2022).

1.7.2 Endothelial Dysfunction and oxidative stress in IHD.

Endothelial dysfunction produced by OS is a characteristic of many cardiovascular disorders, including atherosclerosis, hypertension, and ischemia/reperfusion injury (Cai & Harrison, 2000, Panza et al., 1995). The endothelium consists of a layer of squamous cells that separates the flowing blood from the arterial wall. A healthy endothelium responds to vascular stress by providing endothelium-dependent vasorelaxation, controlling vascular permeability, and preventing platelet aggregation (Mensah, 2007). It responds quickly to mechanical stimuli, chemical stimuli, and humoral agents by generating a variety of mediators, including nitric oxide (NO) , to preserve the vasomotor tone and structure. Due to its powerful vasodilatory, anti-inflammatory, and antithrombotic properties, NO has a significant impact on endogenous antioxidant defense (Ignarro et al., 1987, Zhang & Gutterman, 2007). Vascular NO is generated from L-arginine with the help of a cytochrome p450 reductase-like enzyme which utilizes tetrahydrobiopterin, called endothelial nitric oxide synthase (eNOS) (Cai & Harrison, 2000).

Endothelial dysfunction is promoted when ROS generation exceeds antioxidant defence mechanisms and there is a disruption in the balance between NO bioavailability and ROS, generally known as oxidative stress (Cai & Harrison, 2000, Higashi et al., 2014). Endothelial dysfunction is defined as disrupted endothelial-mediated vasodilation, abnormal vascular reactivity, vasospasm, greater

production of chemotactic and adhesive molecules, elevated platelet activation, thrombus formation, elevated endothelial permeability , leucocyte adhesion, monocyte migration into the vascular wall, impaired regeneration of endothelial cells with smooth muscle cell proliferation and finally migration, which results in vascular injury (Fosterman ,2010 ,Montezano & Touyz,2011). Impaired endothelium-dependent vasodilation in the human coronary circulation is significant prognostic sign, as it predicts severe cardiovascular events and long-term prognosis (Kibel et al. 2020).

1.7.3 Oxidative stress in patients with CAD.

Numerous investigations have discovered an imbalance of prooxidants and antioxidants in CAD patients. OS has been established as a risk factor for the development of CAD, affecting patient onset, prognosis, and quality of life (Yang et al., 2019).

However apart from that, already established risk factors for cardiovascular disease such as hypertension, diabetes, smoking, genetic predisposition, and environmental factors all boost ROS production while decreasing endothelial NO production (Batty et al., 2022, Cai & Harrison 2000). OS generated by ischemia left ventricular (LV) dysfunction, and neuroendocrinological activation all play a role in the connection between HF and vascular disease (Münzel et al., 2017). ROS disrupts myocardial calcium handling, induces arrhythmias, and contributes to cardiac remodeling by promoting hypertrophic signaling, apoptosis, and necrosis (Münzel et al., 2017). Aside from being linked to atherosclerosis and CHD, OS can cause oxidative modification or damage to lipid peroxidation at a deoxyribonucleic acid (DNA)

and protein level, which can have a negative impact on the structure and function of the overall vascular system (Leopold & Loscalzo, 2008). A study conducted by Bhat et al. (2012), demonstrated that individuals with CAD have a considerable increase in lipid peroxidation. In addition to that the patients were found to have a substantial rise in total oxidant status and OS index, as well as a significant dicrease in total antioxidant status. This finding further supports the theory of the presence of an imbalance between oxidant and antioxidant molecules in CHD which increases the need for modification due to the potential to cause various co-morbidities.

1.7.4 The Link between Inflammation and oxidative stress in Atherosclerosis.

Chronic inflammation is the primary pathophysiologic process of atherosclerosis (Gregersen & Halvorsen, 2018. Atherosclerosis is a multisystemic, progressive, chronic inflammatory disease defined by the interaction of immunological and endothelial cells on the surface of the vascular endothelium, which results in the production of various proinflammatory biomarkers (Sima et al., 2018).

OS plays a vital role in regulating vascular homeostasis, which includes endothelial and smooth muscle cell growth, proliferation, migration, angiogenesis, apoptosis, vascular tone, host defenses, and genomic stability (Kibel et al., 2020). Studies have established the relationship between vascular endothelial inflammation and severe oxidative stress as an initiation of the atherosclerotic process (Higashi, 2022). The disruption of the balance between the synthesis of excess ROS and the antioxidant system results in the upregulation of OS, which leads to the development of atherogenic Ox-LDL, a primary indicator of atherogenesis (Kattoor et al., 2019).

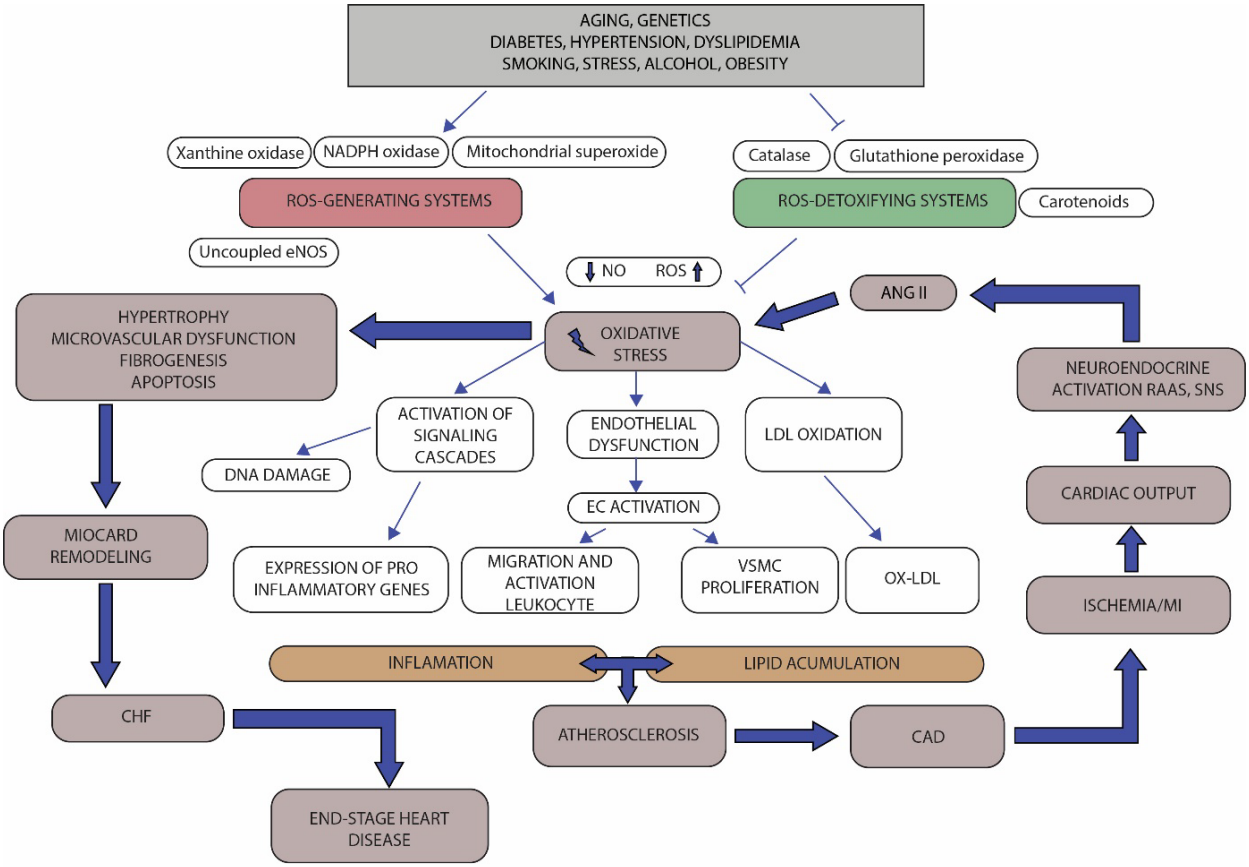

Figure 5. Schematic demonstration of the multi-stage involvement of oxidative stress in the development of atherosclerosis, as well as the pathophysiology of congestive HF. ANG II: angiotensin II, CHF: congestive heart failure, DNA: deoxyribonucleic acid, EC: endothelial cells, eNOS: endothelial nitric oxide synthase, LDL: low-density lipoprotein, RAAS: renin–angiotensin–aldosterone system, SNS: sympathetic nervous system, VSMC: vascular smooth muscle cells. Image adapted from Kibel, 2020

The generation of ROS from a variety of sources disrupts mitochondrial functionality and results in mitochondrial dysfunction (Yeh et al., 2018). Free fatty acids enter the tricarboxylic acid cycle in endothelial cells, where oxidation causes an excess formation of NADH, an important generator of ROS during oxidative phosphorylation (Tibaut & Petrovi|, 2016). Atherosclerotic lesions begin to form and further develop as a result of mitochondrial dysfunction, which further promotes ROS

production and OS (Tibaut & Petrovi|, 2016). In addition to that T- lymphocytes, mast cells, and foam cells that migrate into the intima during plaque formation (Section 1.5.2) produce several cytokines, that increase inflammation and ROS production (Mehta & Li, 1999 & Kattoor et al. 2017).

ROS and growth factors generated by these cells encourage smooth muscle cell migration and collagen deposition, resulting in further development of the atheromatous plaque (Libby, 2002). Smooth muscle cells' production of sarcoplasmic reticulum and their conversion to foam cells are both boosted by ROS In addition to that ROS causes the production of matrix metalloproteinases (MMPs), which damage the fibrous wall of the atheromatous plaque and the membrane of endothelial cells, resulting in the plaque's physical breakdown (Kattoor et al. 2017).

1.7.5 Cytokines what are they, and what is their role in inflammation?

Cytokines are a diverse group of soluble proteins, of molecular weight up to 20 kDa. They are produced and secreted by many types of cells but primarily by the immune cells and more specifically the helper T cells and macrophages, in response to pathogens and other antigens (Carpenter & Fitzgerald, 2017).

They play a vital role in both biological and physiological mechanisms at cellular, tissue, and organismal levels, such as the immune response to inflammation, blood pressure, control of cellular proliferation and differentiation, and the regulations of homeostasis (Gajdcs & Behzadi, 2020). The classification of cytokines is based on two principles: structure and function. For example, cytokines can be divided into different structural classes like chemokines, interleukins, interferons ,lymphokines and tumor necrosis factors , with each family playing a different role in mediating inflammation.

Cytokines have a variety of activities based on the receptors and cell types they bind to, including autocrine, paracrine, and endocrine functions (Masi et al., 2017). In autocrine action, a cytokine secreted by a specific kind of cell attaches to a secretory cell receptor. An example of autocrine action is the secretion of IL-1 by macrophages. The binding of IL-1 to receptors on macrophages stimulates these cells further and causes the release of other cytokines, including more IL-1 (King, 2007). During paracrine activity, the cytokine attaches to several cells in the surrounding region. For example, when TNF-a is secreted in low concentrations it acts on nearby endothelial cells to increase vasodilation and encourages these cells to release chemokines, a class of leukocyte-chemotaxis cytokines (Vitale & de Andrade Quintanilha Ribeiro, 2007).

Finally, in endocrine action, the cytokine reaches the circulation and attaches to a target cell that is furthest from the secretion cell. For instance, IL-6 exemplifies this mode of action by playing a crucial role in restoring normal hepatic function following liver damage (Michalopoulos, 2007). During a general acute phase response, IL-6 is released by immune cells at the site of damage. It is considered one of the key mediators that initiate the production of acute phase proteins by hepatocytes in the liver (Gauldie et al., 1992). The prevailing concept is that IL-6, upon release into the bloodstream, is taken up by the liver and serves as a trigger for local hepatocytes to commence the synthesis of acute-phase proteins (Norris et al., 2014).

They are frequently created in a cascade, where one cytokine encourages its target cells to release more cytokines (Gajdács & Behzadi, 2020). Cytokines can also work in a synergistic or antagonistic manner. Synergistic interactions occur when two or more cytokines act together, with antagonist interactions occur when two cytokines

generateopposing actions (Oppenheim ,2001). After binding to the target cell, they induce activation, proliferation, or migration of target cells (Zhang & An 2007).

Proinflammatory cytokines are primarily generated by activated macrophages and have a role in controlling inflammatory responses (Zhang & An, 2009). Chemotactic cytokines (or chemokines) are a broad family of small proteins that communicate via cell surface G protein-coupled heptahelical chemokine receptors. They are mostly known for their role in inducing cell migration, particularly that of white blood cells (leukocytes) (Hughes & Nibbs, 2018).

As a result, chemokines play a critical role in immune system development and homeostasis, and they are involved in all defensive and damaging immunological and inflammatory responses (Yung & Farber, 2013). Immunoregulatory molecules also known as anti-inflammatory cytokines control the pro-inflammatory cytokine response. Cytokines act synergistically with soluble cytokine receptors, cytokine inhibitors, and other molecules to regulate the human immune response (Zhang & An, 2009). Their physiologic and pathologic roles in systemic inflammatory conditions are becoming more widely acknowledged. Interleukin IL-1 receptor antagonists, IL-4, IL-10, IL-11, and IL-13 are all valuable anti-inflammatory cytokines (Opal & DePalo, 2000).

1.7.6 Interleukin-8 in inflammation.

Interleukins are a type of cytokine that is secreted by many types of cells (Apostolakis et al., 2009). Their main role is to activate, differentiate, proliferate, and migrate cells of the immune system during inflammation as well as during an immune response (Vaillant & Qurie, 2023). IL-8 or (CXCLX8) is a pro-inflammatory cytokine produced

mainly by monocytes and macrophages in the external space as a result of different cellular stimuli (Waugh & Wilson, 2008). The primary way of action of IL-8 is to recruit monocytes and neutrophils through the development of a chemotactic gradient, that allows the inflammatory cells to migrate to the location of the increased concentration of the cytokine (Gimbrone et al., 1989). In vivo, this chemotactic gradient may be created by the binding of IL-8 to basement membrane proteins, which might cause cells to migrate and adhere to the site of inflammation, promoting the activation of monocytes and neutrophils (Remick, 2005). IL-8 has a unique list of characteristics, for example, it has been shown to be highly resistant to temperature, proteolysis, and acidic environments, therefore it can survive for long periods of time in a hostile environment around the site of the inflammation. Due to these unique characteristics of the cytokine, it is generated early in the immune response and may remain active for weeks (DeForge et al., 1992).

Finally, according to studies, IL-8 is sensitive to oxidants, while antioxidants have been found to reduce the production of it (DeForge et al., 1993). The relationship between IL-8 and the ischemic-induced OS in CVD, as a potential diagnostic and therapeutic target, makes this of particular interest.

1.7.7 The role of IL-8 in atherosclerosis and CAD.

Several studies have confirmed the presence of IL-8 in areas of arterial damage, while others have shown that IL-8 may play a role in various phases of atherosclerosis. In a study conducted by Rus et al. (1990), IL-8 was found in significant concentrations in the human artery atherosclerotic wall as cellular and extracellular deposits in the connective tissue matrix. The quantification of IL-8 using an enzyme-linked immunosorbent assay indicated that IL-8 levels were considerably higher in fibrous plaques than in normal intima (Rus et al., 1996).

Macrophages have been identified as the primary source of IL-8 in atherosclerotic plaques. They have been shown a have a higher capacity to create IL-8 than normal and patient blood monocytes, and they concluded that macrophages constitute a primary location of IL-8 mRNA synthesis in atherosclerotic plaques (Apostolopoulos et al., 1996). Similarly, Liu et al. (1997) discovered increased amounts of IL-8 in foam cells retrieved from human atherosclerotic tissue compared to monocytes or monocyte-derived macrophages in culture.

Once the association of IL-8 in atherosclerotic plaques was established, with macrophages being recognized as the primary source, several researchers tried to determine the mechanisms leading to IL-8 release in atherogenesis sites. Yue et al (1994), revealed that IL-8 is also a mitogenic and chemotactic factor for VSMCs, as it stimulated DNA synthesis and cell proliferation in both human and rat aorta SMCs in a concentration-dependent manner. Rydberg et al. (2003), demonstrated that hypoxia increased 25-hydroxycholesterol (25-OH-chol)-induced IL-8 secretion in human monocyte-derived macrophages and that both 25-OH-chol and hypoxia promote IL-8 secretion by raising the amount of the intracellular signaling molecule, H_2O_2.

An animal model developed by Boisvert et al. (1998), revealed more evidence that IL-8 contributes to atherogenesis. The researchers employed irradiated LDL receptor knockout mice that were repopulated with bone marrow cells missing the mouse equivalent of the IL-8 receptor, CXCR2. They concluded that double-knockout animals had fewer macrophages and less severe lesions than those who received bone marrow cells expressing the receptor. However, because CXCR2 has several ligands (CXCL1 and CXCL8), the latter research was not conclusive in establishing IL-8's function in atherosclerosis (Boisvert et al., 1998).

Weber et al. (2008), showed that macrophage migration inhibitory factor (MIF) might possibly be a

CXCR2 ligand alternative. The scientists went on to show that MIF has a pseudo-(E)LR motif that allows it to behave as a non-canonical CXCR2 ligand, and they concluded that this structural similarity may be the basis of pro-inflammatory MIF/CXCR2 interactions. As a result, CXCL1 deletion in LDLr/ mice decreases atherosclerosis less than bone marrow CXCR2 deficiency in LDLr/ mice, with effects on macrophage accumulation in established rather than early lesions. MIF, as a CXCR2 alternative ligand, may compensate for a shortage of CXCL1. (Weber et al. 2008, Zernecke et al. 2008).

However, the effect of direct IL-8 inhibition on the degree of myocardial injury experienced during reperfusion in New Zealand White rabbits was investigated by Boyle et al. (1998), demonstrating that IL-8 neutralization significantly reduced the degree of necrosis in a rabbit model of myocardial ischemia-reperfusion injury.

1.7.8 Elevated levels of IL-8 in the serum of CAD patients.

The literature is replete with data pointing to IL-8's possible connection in atherosclerosis, either as an indication or as a potential therapeutic target. However, the evaluation of whether IL-8 is a potential biomarker of short- or long-term prognosis in individuals with CAD is a recent popular area of research.

In a study conducted by Inoue et al. (2008), blood levels of eleven cytokines were investigated as prospective predictors of long-term prognosis in angiographically detected stable CAD. The researchers established that IL-8 was the only cytokine that could predict cardiovascular events autonomously of the other cytokines and C-reactive protein. In addition to that another study conducted by Rauchhaus et al. (2000) demonstrated that elevated levels of IL-8 in the plasma of individuals are linked to a raised risk of future CAD in otherwise healthy people.

In another study conducted by Zhou et al, (2001) levels of IL-8 were also found to be considerably elevated in the serum of patients with unstable angina or AMI compared to healthy controls, proposing that IL-8 is involved in the development of CAD. Finally, a study conducted by Romuk et al(2002), measured the levels of IL-8 levels in the serum of individuals with stable and unstable CAD. Elevated levels of IL-8 were observed in individuals with unstable CAD, this led the researchers to assume that IL-8 could potentially be a valuable clinical sign of unstable CAD.

1.8 The Study's Hypothesis and Aims.

Inflammation and OS play a key role in the development and progression of IHD. This project aims to investigate the role of inflammation in IHD by measuring IL-8 levels in 52 CAD patients' serum. Correlations between IL-8 concentrations and clinical parameters will be analyzed to assess relationships with cardiac function. Additionally, the study will explore IL-8's impact on OS regulation in rat cardiomyocyte cells (H9C2). Consequently, the aims of this study are to:

1. Measure serum levels of IL-8 in the CAD patient cohort.
2. Correlate the concentrations of IL-8 to indices of whole heart function.
3. Determine if clinically relevant levels of IL-8 increase levels of OS in H9C2 cells.

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2 Methodology

2.1 Ethical Considerations.

The project was conducted under the guidelines of the Research and Ethics Committee (REC), the Trust and research office, the Good Clinical Practice Guidelines, and the International Conference Harmonization. Ethical approval has been obtained from the Health Research Authority HRA and Health and Care Research Wales (HCRW), (IRAS project ID 247341), and the University of Salford's ethical committee, under ethics ID 5184. This study was a partnership between the cardiac surgery group at Blackpool Victoria Hospital and the University of Salford.

2.2 Patient Recruitment.

All the patients in this research had previously been diagnosed with CAD and were scheduled for regular coronary revascularisation surgery. Patients were recruited to the study by the Blackpool Victoria Hospital research team at their CABG preoperative assessment, and they provided written and informed permission for their involvement in the trial (Appendix D.) The patients that were recruited for this study met the following criteria mentioned in Table 1.

Table 1. Patients' recruitment criteria for the study.

Inclusion criteria	Exclusion criteria
Patients that have been diagnosed with CAD. Patients that have their CABG procedure scheduled at Blackpool Victoria Hospital. Age >18 Both biological sexes. Patients that have given both written and informed consent to be a part of the study.	Age <18 Patients who are non-English speaking

All volunteers had to be over the age of 18 and English-speaking, otherwise, they would be excluded from the research. It should be noted that there were no patients recruited solely for this study. All samples and data, that included the echocardiogram parameters such as age, weight, and biological sex were completely anonymized and were assigned to be collected by a unique research identifier.

2.3 Collection and preservation of blood sample.

Prior to the routine cannulation procedures in open-heart surgery for cardiopulmonary bypass grafting operation, a 10 mL blood sample was obtained at the site of anaesthesia and preserved under refrigeration (4 °C) in an EDTA tube. A courier service was used to transport the sample to the University of Salford with the travel time being estimated to be about an hour.

2.4 Preparation and storage of blood sample.

The blood was then transferred from the EDTA tube to a 15mL falcon tube and then centrifuged at 2000xg for 5 minutes. This process allowed for the separation of the serum (supernatant, top layer) from the cellular components, including platelets and red blood cells, which pelleted at the bottom of the tube. The serum was then carefully extracted, transferred to 1mL aliquots in cryovials, and stored in -80^{o}C freezers for future analysis.

2.5 Measurement of serum IL-8 levels by Enzyme-linked immunosorbent assay (ELISA) assay.

Enzyme-linked immunosorbent assay (ELISA) is a quantitative analytical method that depicts the antigen-antibody reactions through color change obtained by using an enzyme-linked conjugate and an enzyme substrate and is used to determine the presence and concentration of molecules in biological fluids (Hornbeck, 1992). The technique has found applications in a variety of sectors, and it is employed as a standard method in research and diagnostic laboratories all over the world. (Aydin, 2015 There are different types of ELISA's, and they are classified based on how the antigen is immobilized and detected. However, all categories follow the same basic principle. The antigen is either directly or indirectly immobilized in a microplate well by a particular antibody known as a capture antibody. The primary detection antibody is then introduced, resulting in an antigen- antibody complex (Shah & Maghsoudlou, 2016). The primary detection antibody can either be directly identified with an enzyme (direct ELISA) or coupled to a secondary antibody known as a 'secondary detection antibody' (indirect ELISA). It is important to note that the well is rinsed with a buffer solution between each stage. Finally, the presence of antigen in the sample manifests as a color indicator produced by the addition of a

substrate. The optical density measurement correlates positively to the amount of antigen in the sample (Shah & Maghsoudlou, 2016).

2.5.1 Standard curve preparation.

The Human IL-8 Simple Step Elisa kit was purchased (LOT number: 2101009021) from ABCAM, UK. The kit had a detection range of 3.91 pg/mL - 250 pg/mlL, with an analytical sensitivity of 1.8 pg/mL . All reagents were equilibrated at room temperature prior to use.

To ensure precision and accuracy in the quantification of IL-8 a set of standard curves was prepared (Figure 6.). Human IL-8 lyophilized recombinant protein was reconstituted to achieve the concentration of 4,000 pg/mL. Then was further diluted to a 250 pg/mL stock standard solution. The stock standard solution was then used to produce the following serial dilutions (Table 2.)

Table 2. Preparation of serial dilutions of standard concentrations of IL-8.

Dilution Step	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting concentration (pg/mL)	Final concentration (pg/mL)
Stock standard	25	375	4,000	250
1	150	150	250	125
2	150	150	125	62.50
3	150	150	62.50	31.25
4	150	150	31.25	15.63
5	150	150	15.63	7.81
6	150	150	7.81	3.91
Blank Control	0	150	N/A	N/A

The standard curves were constructed by plotting the absorbance at 450 nm against the corresponding concentrations of the standard solutions (Table 2.)

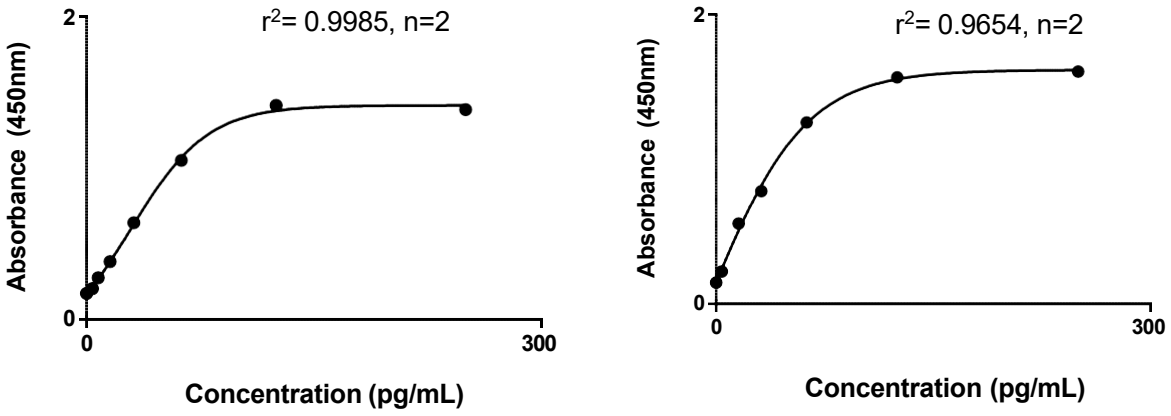

Figure 6. ELISA standard curves for IL-8. The concentration of the standard curve ranges between 0 - 250 pg/ml. All data are expressed as Mean ± SEM (n= 4). n demonstrates absorbance measurements

2.5.2 Reagents Preparation.

All reagents were equilibrated to room temperature before use. The wash buffer was prepared by diluting Wash Buffer PT 10X with deionized water in a 1:9 ratio, then it was mixed thoroughly and gently. The antibody cocktail was prepared by mixing the 10X Capture antibody, the 10X Detector antibody, and the antibody diluent 4BI in a 1:1:8 ratio.

2.5.3 ELISA experimental protocol for measurement of IL-8 concentrations.

The assay was used to quantify the concentrations of IL-8 in the patient's serum by forming the antibody-analyte sandwich complex. 52 patients' serums were removed from the storage -80^{o}C freezer and defrosted at room temperature of 20^{o}C. Once defrosted, 50 µL of each patient's serum was added in duplicates in two experimental wells along with 50µL of the antibody cocktail and was mixed gently before sealed and being incubated for an hour at room temperature in the plate shaker (FLUOstar Omega, BMG Labtech, Germany) at 400 rpm.

After one hour each well was washed three times with 350 µL of wash buffer and then 100 µL of tetramethyl-benzidine (TMB) development solution was added to each well. After incubating the plate for 15 minutes at the plate shaker at 400 rpm at room temperature 100 µL of stop solution was added to each well. The plate was shaken for 60 seconds before the endpoint reading at the plate reader (FLUOstar Omega, BMG Labtech, Germany) at 450 nm OD primary wavelength and 620 nm as the reference wavelength. As all readings were conducted twice and the difference between the wavelengths was averaged. Finally, to allow the quantification of the patient's serum concentrations of IL-8 all sample values were inter plotted against the standard curve. (Figure 6.)

2.6 H9C2 cell line as a cardiac model.

Cardiomyocytes derived from rat neonatal hearts are frequently utilized to study different cellular and molecular modifications that occur in the heart (Watkins et al., 2010). The choice of the H9C2 cell line for this set of experiments was based on several factors that make it a well-established model for cardiac research. More specifically, the H9C2 cell line is generated from embryonic rat ventricular tissue, (Kimes& Brandt, 1976), and even though they are unable to

beat they still present with many similar characteristics to primary cardiomyocytes, such as membrane shape, g-signaling protein production, and electrophysiological properties (Hescheler et al., 1991, Sipido & Marban, 1991). In addition to that when induced with hypertrophic drugs in vitro, they can exhibit hypertrophy-associated features (Huang et al., 2004, Watkins et al., 2010). Finally, several studies have used H9C2 cells to stimulate and investigate the conditions of ischemia in the heart, establishing the cell line as a standard for research (Buoncervello et al., 2019, Ekhterae et al., 1999, Liu et al., 2017).

2.6.1 Cell culture.

H9C2 rat cardiomyoblast cells were obtained from Public Health England ECACC and cultured in complete media containing Dulbecco's modified Eagle's medium (DMEM) obtained from Thermo Fisher Scientific UK, containing 10% (v/v) fetal bovine serum (FBS), 1% penicillin, streptomycin (100 µg/mL) at 37°C, and 5% (v/v) CO_2, fresh media was added to the cells every 2-3 days until 70% confluency.

2.7 Measurement of oxidative stress with the 2',7'-Dichlorodihydrofluorescein diacetate assay (DCFDA) .

ROS detection is frequently used as a marker for biological processes and cell cycle development, as well as to investigate the ways of action of a plethora of medications and substances (Lautraite et al., 2003). This is important because alterations in intracellular ROS levels can affect whether cells continue through the cell cycle, inhibit it, or go through apoptosis (Fan & Li, 2014). The quantity of ROS will eventually determine how quickly

the cell cycle is accelerated or inhibited, and this pace varies depending on the kind of cell, extracellular stimuli, and length of exposure (Fan & Li, 2014).

DCFDA is a non-fluorescent, lipophilic, and non-ionic compound that can diffuse via the cell membrane into the cytoplasm. DCFDA is deacetylated by intracellular esterases once it enters the cells, which activates the production of 2', 7'-dichlorofluorescein (DCFH), a non-fluorescent and impermeable to the cell membrane molecule that interacts with the intracellular ROS (Brandt & Keston, 1965, Rota et al., 1999). Depending on the cell type, initial incubation period, DCFDA concentration, esterase activity, and potential future leakage, intracellular levels of non-fluorescent DCFH may differ significantly (Chen et al., 2010). The experimental protocol of the DCFDA assay in each study can be altered depending on the design of the study and the hypothesis of each research group (Figueroa et al., 2018). In more detail, cells can be treated with the dye for a 30-minutes up to 1-hour period and the concentration of the DCFDA can range between 5 to 50 μM either before or after introducing the cells with a ROS equivalent compound (Chen et al., 2010, Valko et al., 2007). It is important to note that most reagents used for ROS detection are sensitive to light it is essential that the experiment is conducted with precautions while using the dye (Wu & Yotnda, 2011). DCFH oxidizes to fluorescent 2', 7'–dichlorofluorescein (DCF) after being introduced to ROS. This emitted fluorescence can be measured with either spectrofluorimetry or flow cytometry, with the intensity of the DCFH being correlated to ROS activity (Epling et al., 1992, Kim et al., 2005). Finally, the measurement of the DCFH is conducted once at a specific time point and the results generated are a measurable indicator of the levels of cellular OS (Chen et al., 2010, Figueroa et al., 2018).

2.7.1 The use of Hydrogen peroxide to model oxidative stress.

Under healthy conditions, heart development, cardiac calcium management, excitation-contraction coupling, and vascular tone are all regulated by cardiac ROS signaling (Burgoyne et al., 2012). Pathological conditions of unregulated ROS production result in elevated levels of ROS, which can cause OS via oxidative damage to DNA, proteins, lipids, and cell death (Richardson & Schadt, 2014). Numerous cardiac disorders including IHD have been linked, to some extent, to an increase in the formation of ROS (Kattoor et al., 2017, Tsutsui et al. 2011, Samman Tahhan et al., 2017). ROS can manifest as oxygen-free radicals such as superoxide, hydroxyl radicals, and peroxyl radicals, as well as non-radicals such as H_2O_2 (Fink, 2002). To investigate the mechanisms under which hydrogen peroxide increases the production of ROS in CVD several studies have introduced it to cells (Ali et al., 2013, Ogawa et al., 2004, Wu et al., 1996).

2.7.2 Experimental protocol of DCFDA assay.

The H9C2 cells were plated at 20,000 cells per well into dark clear bottom 96-well microplates (deep Well Polypropylene Microplates) , purchased from Thermo Fisher Scientific. The cells were then incubated for 24 hours at 37 °C in 5% CO_2 to allow adherence. All reagents used in the DCFDA assay were equilibrated to room temperature before use (Table 3.)

Table 3. Reagents used in DCFDA assay.

Compound	Company purchased	Product no.	Concentration
Interleukin-8	SIGMA- ALDRICH	I1645	6 pg/mL, 50 ng/mL
H_2O_2	Honeywell, USA	K1460	100 mM, 200 mM
DCFDA	Thermo Fisher Scientific	C6827	5µM

Firstly, the cells were washed with 100 µL of phosphate-buffered saline (PBS) and the relevant wells were exposed to 100 µL of set concentrations of interleukin-8 (Figure 7.), 100 µL of serum-free media was then added to the remaining wells. The plate was then incubated for 1 hour at 37 °C in 5% CO_2. After the incubation, all media from the plate was extracted and discarded, then the relevant wells were exposed to 100 µL of 5 µM of DCFDA while 100 µL of serum-free media was then added to the remaining wells under low light conditions to avoid degradation of the DCFDA. The plate was then covered with aluminum foil to avoid exposure to light and then incubated for 30 minutes at 37 °C in 5% CO_2. After the incubation, all reagents were extracted and discarded. Finally, the relevant wells were treated with set concentrations of hydrogen peroxide, (Figure 7.), and serum-free media was added to the remaining wells. The plate was finally incubated for 30 minutes at 37 °C in 5% CO_2. After the incubation and while in the H_2O_2, the fluorescence of each

well was measured using the multi-mode fluorescence plate reader (FLUOstar Omega, BMG Labtech, Germany) at excitation 485 nm/emission 590 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A		H_2O_2 100µM	H_2O_2 200µM	IL-8 50 ng/mL	IL-8 6pg/mL	H_2O_2 100µM & IL-8 50 ng/mL	H_2O_2 100µM & IL-8 6pg/mL	H_2O_2 200µM & IL-8 50 ng/mL	H_2O_2 200µM & IL-8 6pg/mL	Ctrl	Ctrl	
B		DCFDA 5 µM										
C		DCFDA 5 µM										
D		DCFDA 5 µM										
E												
F												
G												
H												

Figure 7. DCFDA assay 96-well plate layout for reading under the fluorescence microplate reader. Rows B, C, and D of the plate were exposed to 5 µM of DCFDA. Columns 10 and 11 acted as the negative controls containing only serum-free media.

2.7.3 Calculation of ROS production using the fluorescence microplate reader.

In the presence of ROS, the DCFDA assay analyses the conversion of non-fluorescent DCFDA to the fluorescent molecule 2',7'-dichlorofluorescein (DCF). The fluorescence signal's strength is proportional to the quantity of DCF produced, which represents the levels of ROS in the sample.

In this study, in order to quantify the fluorescence signal of each treatment group, the difference in mean fluorescence intensity between the stained and unstained wells was calculated. The mean fluorescence intensity measurements of the stained wells were subtracted from the fluorescence intensity measurements of the unstained wells to obtain the difference.

The fluorescence intensity measurements were further normalized to allow comparison and interpretation of data across the different treatment groups. The control group served as a standard and was normalized to the mean fluorescence intensity of 1. Finally, to calculate the normalized results of the mean fluorescence intensity measurements of each treatment group were dived by the mean fluorescence value of the control group. This enabled the expression of fluorescence values from each treatment group relative to the control group, allowing further analysis.

2.8 Statistical Analysis.

Statistical analysis was conducted using GraphPad Prism 5 (GraphPad Software, Inc, USA. All data are expressed as mean±SEM, and the presence of statistical significance was assessed at a significance level of $p<0.05$. The Shapiro-Wilk test was utilized to evaluate normality.

Correlation analysis was performed to determine the potential relationship between the IL-8 concentrations as determined by the ELISA assay and the patient's clinical parameters. Correlations that passed the normality test were analyzed using the Pearson correlation and the statistical significance of the relationship was estimated using the Pearson coefficient along with their associated p-values. The correlation that did not pass the normality test was examined using the Spearman rank correlation coefficient along with the associated p-values.

In addition to that the Mann-Whitney U test was used to investigate a potential statistically significant difference between the concentrations of IL-8 in the male and female patients of the study's cohort, as the IL-8 concentration data were found to be non-normally distributed via the Shapiro-Wilk test. (Appendix C.)

Finally, to investigate the potential significance of OS production between different treatment conditions of IL-8 and H_2O_2 one-way ANOVA with post-hoc Tukey's Multiple Comparison Test was utilized.

3 Results.

3.1 Quantification of serum IL-8 concentrations.

The serum levels of IL-8 in 52 patients of the study cohort were measured via sandwich ELISA assay. Amongst the patients, it was observed that in 14 patients (26.92 % of the patient cohort) the IL-8 levels were undetectable, therefore excluded from any further analysis. The mean value of IL-8 based on the n=38 measurable values is 5.77± 5.81 pg/mL.

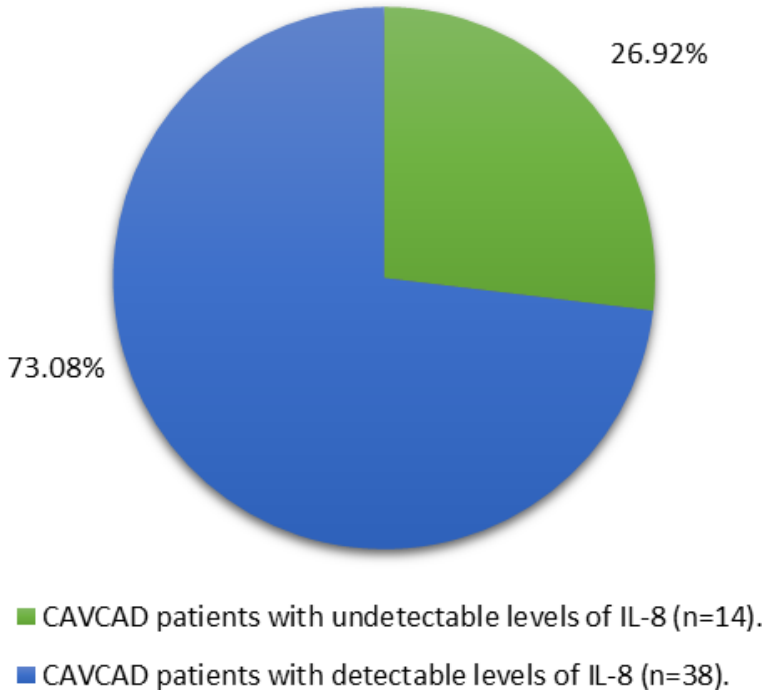

Figure 8. Schematic representation of the percentage of patients with detectable and undetectable levels of IL-8.

3.2 Patient cohort demographics.

Clinical data along with echocardiograms in (Appendix B.) for individual patients of the study cohort were supplied. The parameters of clinical relevance for ischemic heart disease are presented below in Table 4.

Table 4. Clinical parameters of patient cohort (n=38) .

Clinical Parameter	Mean	SEM	N
Ejection fraction (%)	50.21	1.81	34
Left ventricular outflow tract (LVOT) peak velocity (m/s)	0.94	0.04	18
Tricuspid annular plane systolic excursion (cm)	2.1	0.07	21
Body mass index (BMI) (kg/m^2)	31.1	1.54	14
Heart rate (bpm)	67.72	2.77	18
Age (Y)	64.76	1.89	34

3.3 Correlation of IL-8 with indices of cardiac function.

The individual values of each patient's IL-8 serum concentrations in (Appendix A.) were then correlated with indices of cardiac function provided by the patient's echocardiography data in (Appendix B.)

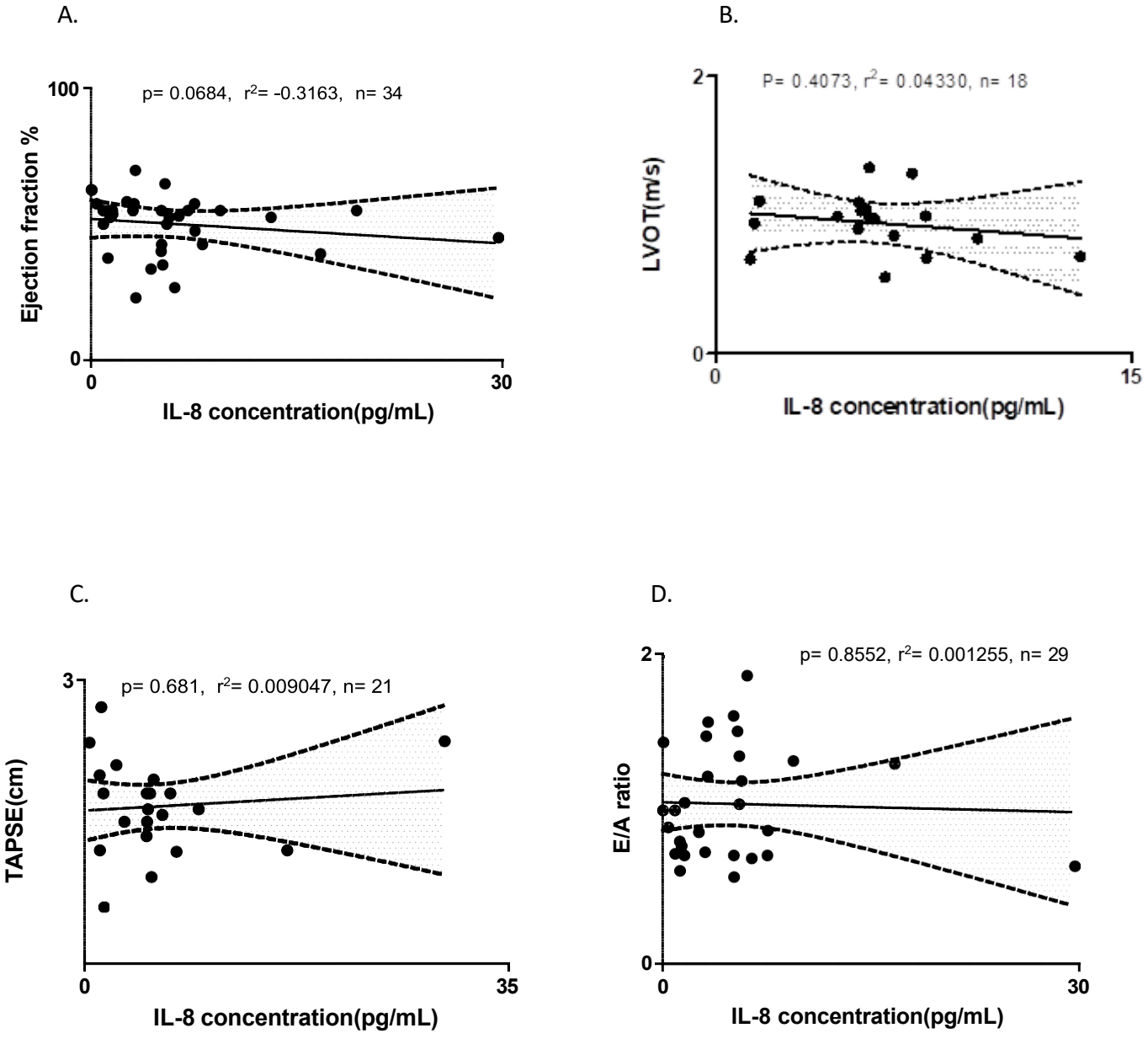

Figure 9. Correlations of patients' IL-8 levels with cardiac indices. A. Correlation of patients' IL-8 serum levels and ejection fraction. (n=34), B. Correlation of patients' IL-8 serum levels and left ventricular outflow tract peak velocity (n=18) C. Correlation of patients'

IL-8 serum levels and Tricuspid annular plane systolic excursion (n= 21). D. Correlation of patient's interleukin-8 serum levels and peak velocity blood flow from left ventricular relaxation in early diastole (the E wave) (E/A ratio) (n=29).

According to preliminary findings, there is no significant correlation between IL-8 levels and ejection fraction (n=34, r^2 = 0.03043, p=0.3238) (Figure 9.A.).

Furthermore, data revealed that there was no correlation between Left ventricular outflow tract (LVOT) peak velocity and Tricuspid annular plane systolic excursion (TAPSE) either n=18, r^2 = 0.04330, p=0.4073 (Figure 9.B.) and respectively (Figure 9.C.).

Finally, no significant correlation was found between IL-8 serum levels and peak velocity blood flow from left ventricular relaxation in early diastole (E/A ratio) (n=29,

r^2 = 0.001255, p= 0.8552) (Figure 9.D.).

3.3.1 Correlation of IL-8 with demographic factors and physiological measurements.

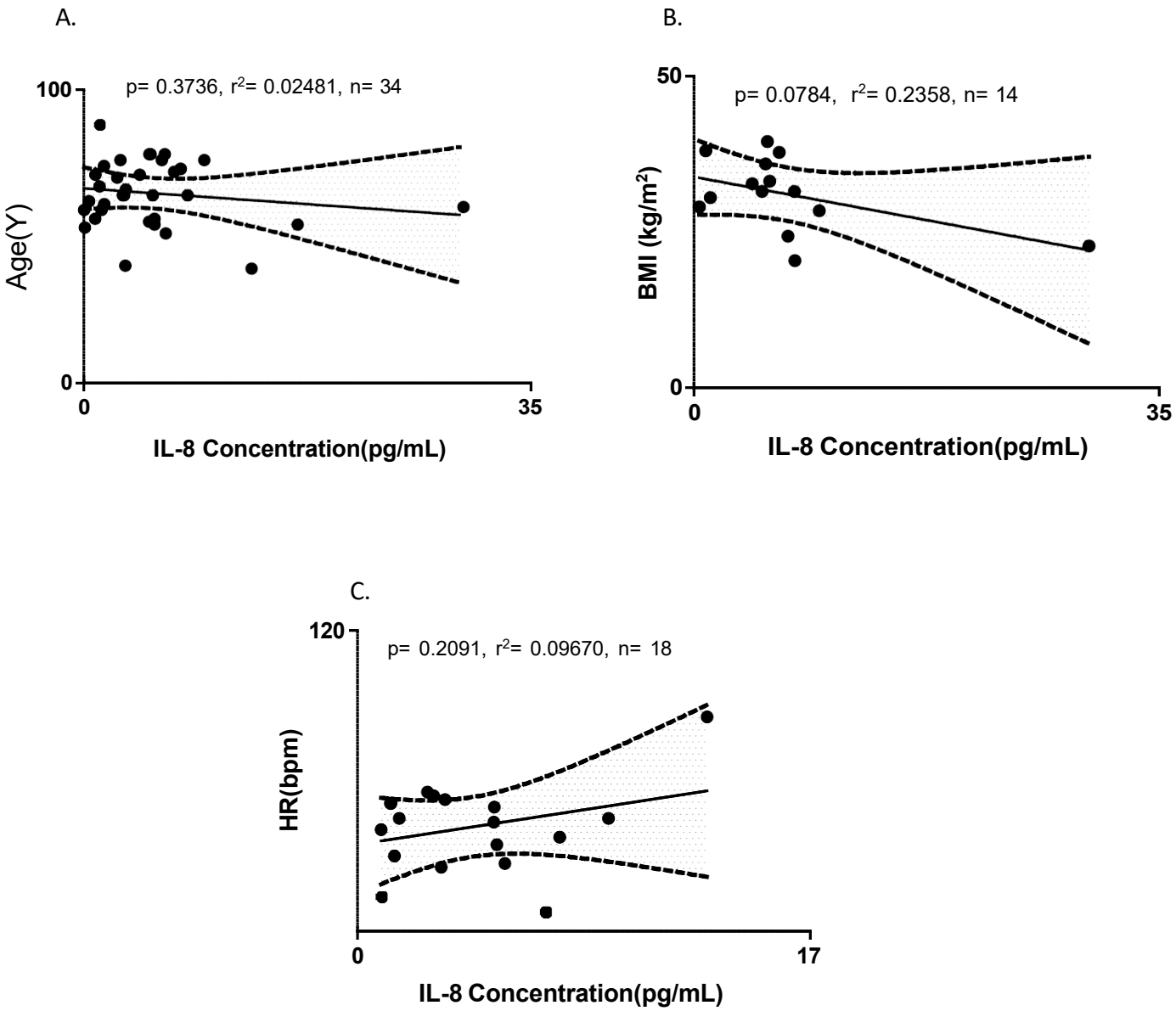

Figure 10. Correlation of patients' IL-8 levels with demographic factors and physiological measurements. A. Correlation of patients' IL-8 serum levels and age (n= 34). B. Correlation of patients' IL-8 serum levels and BMI (n=14), C. Correlation of patients' IL-8 serum levels and heart rate (n=18)

According to preliminary findings, there is no significant correlation between IL-8 levels and age (n=34, r^2 = 0.02481, p=0.3736) (Figure 10.A.) Furthermore, data revealed that there was no correlation between levels of IL-8 and body mass index (n=14, r^2 = 0.2358, p=0.0784) (Figure 10.B.). It should be noted that these data are likely underpowered (n=14), therefore caution should be exercised when drawing conclusions. Finally, statistical analysis that there is no correlation between the concentrations of IL-8 and heart rate, (n=18, r^2 = 0.0970, p=0.2091) (Figure 10.C.).

3.4 Comparison of levels of IL-8 in female and male CAD patient cohort.

As mentioned in Section 1.3 the manifestations of IHD differ between the two sexes. The levels of IL-8 in this study's female and male patients were evaluated to further investigate a potential variation in inflammatory biomarker levels.

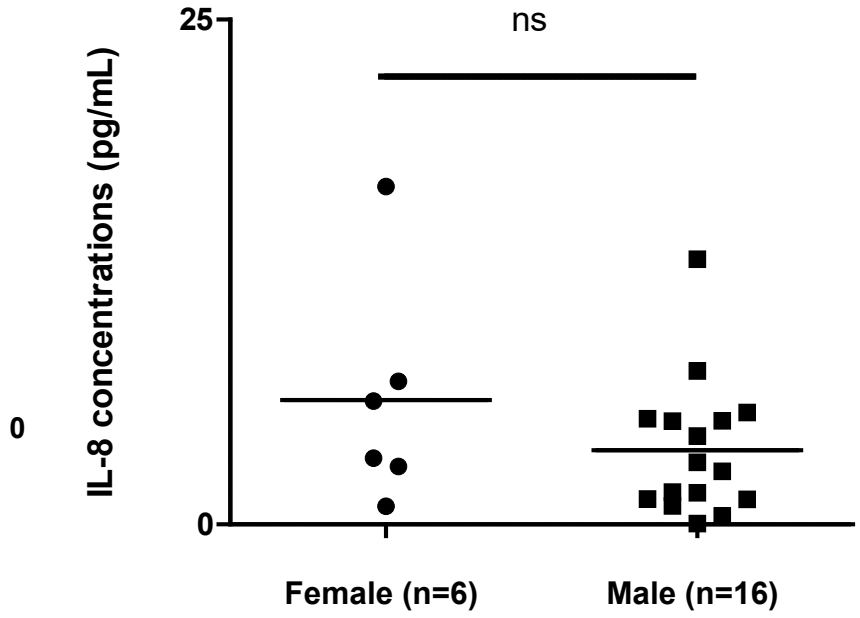

Figure 11. Comparison of interleukin-8 Levels in Female and Male CAD patients, n=6 and 16 respectively. The graph demonstrates individual data points for each patient's IL-8

concentrations along with the mean value represented by the horizontal line. Ns demonstrated the absence of a significant difference between the levels of interleukin-8 in the male patients and the levels of IL-8in the female patients.

After establishing that the data do not follow a normal distribution (See Section 2.14), to investigate a potential difference between the serum levels of interleukin-8 in the female and male patients of the study cohort a Mann-Whitney U test was employed.

A p-value of 0.2851 was established, demonstrating no significant difference in IL-8 levels between female n=6 and male n=16 patients. It should be noted that the 14 patients whose IL-8 levels were undetectable have been excluded from this analysis. Furthermore, it is important to acknowledge that the data presented in Figure 11. Could potentially be underpowered, and therefore limit the detection of significant differences between the two groups (p=0.2851).

3.5 DCFDA assay optimisation.

As mentioned before DCFDA is a commonly used assay to measure the production of ROS. In this study, the DCFDA assay was employed to evaluate OS levels in the different treatment groups of the study. To investigate the sensitivity of the assay, positive control was established, and the fluorescence intensity in response to ROS production was measured after a 30-minute incubation period with H_2O_2 of 100µM and 200µM (Figure 12.)

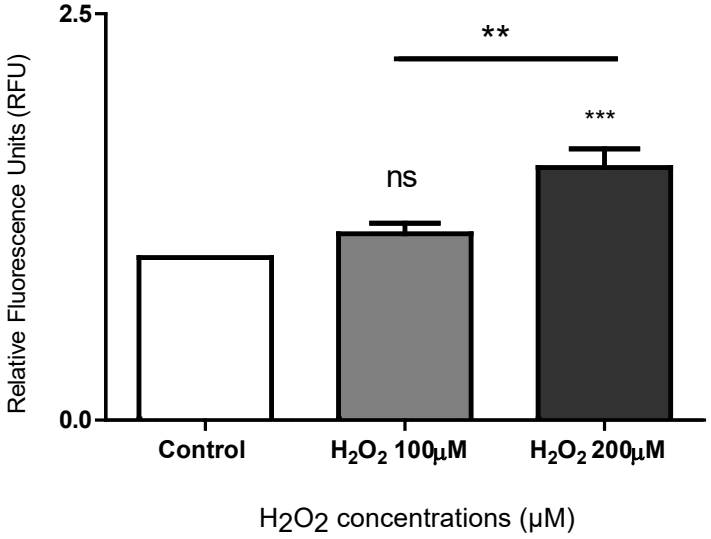

Figure 12. The effects of 100µM and 200µM of hydrogen peroxide in ROS fluorescence(n=8).

*** symbolizes the statistical difference between the control group and the 200µM H_2O_2, ($p < 0.001$), ns indicates the absence of statistical significance between the control group and 100µM H_2O_2, ($p > 0.05$).** indicates a statistical difference between the 100µM H_2O_2 and the 200µM H_2O_2, ($p < 0.01$). All data are presented as mean ± SEM (n=8). The data presented have been normalized to the control (untreated wells).

Figure 12. shows that 100µM H_2O_2 did not produce a significant increase in fluorescence (n=8, $p > 0.05$). However, 200µM H_2O_2 increased fluorescence by 0.408 5±0.372 Relative Fluorescence Units (n=8, $p < 0.001$). In addition to that, a significant increase was observed between the two concentrations of H_2O_2 (n=8, $p < 0.01$).

3.5.1 The effects of IL-8 in ROS fluorescence.

H9C2 cells were treated with different concentrations of IL-8 to investigate the potential dose-dependent increase in OS regulation compared to the untreated/ control group. (Figure 13.).

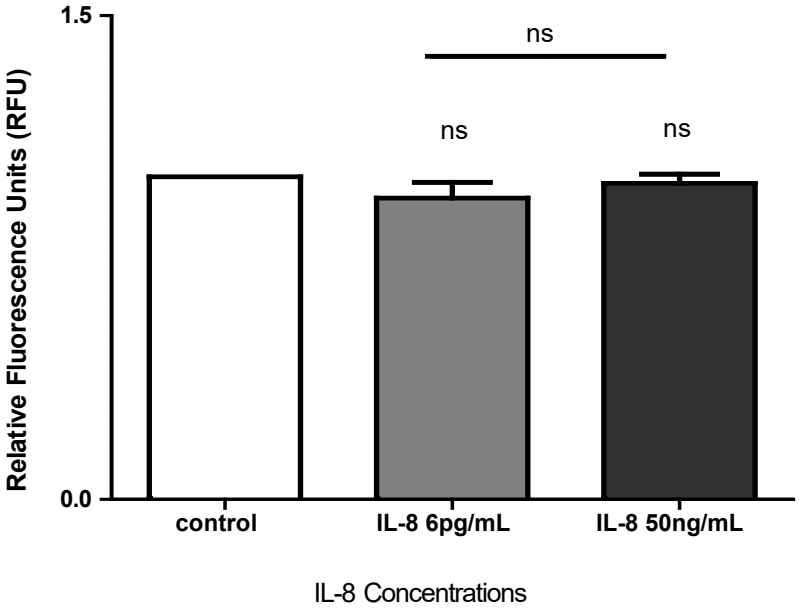

Figure 13. The effects of different concentrations of IL-8 in ROS fluorescence (n=8).

Ns indicates the absence of a significant difference ($p > 0.05$). All data are presented as mean ± SEM (n=8). The data presented have been normalized to the control (untreated wells).

Over the time course of the experiment, 6 pg/mL and 50 pg/mL IL-8 produced no significant increase in fluorescence ($p>0.05$). In addition to that, no significant difference in fluoresces between the two concentrations was observed. ($p>0.05$) (Figure 13.).

3.5.2 The effects of IL-8 in ROS fluorescence in the presence of 100µM of H_2O_2.

In section 3.5, 100µM H_2O_2 did not cause a significant increase in the fluorescence compared to the untreated group. In addition to that in section 3.5.1 the two concentrations of IL-8 did not cause a significant increase in fluorescence compared to the untreated group. To further investigate the potential interplay between H_2O_2 and IL-8 the two concentrations of IL-8 were added to cells along with 100µM H_2O_2 (Figure 14.).

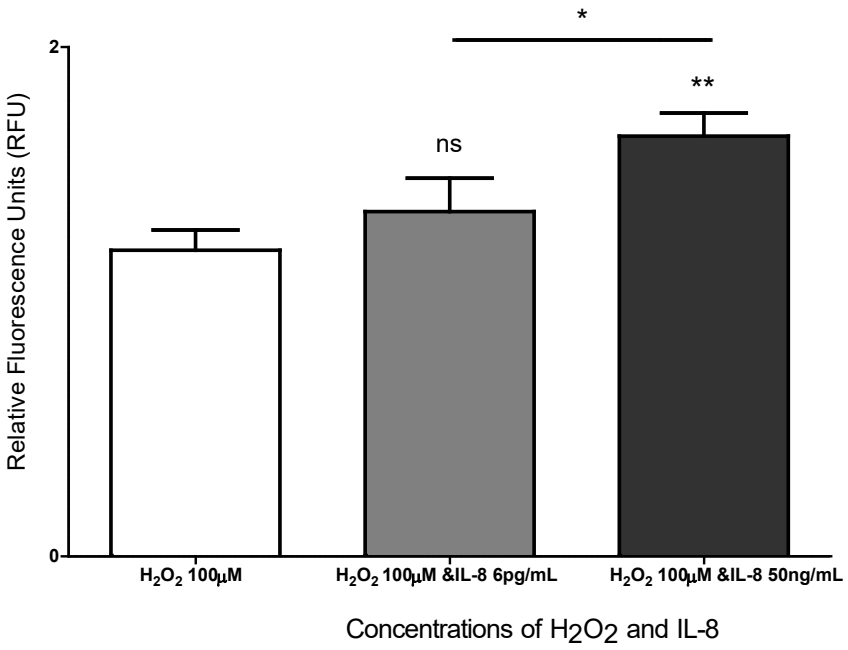

Figure 14. ROS fluorescence measurements in response to treatment with different concentrations of IL-8 in the presence of 100µM of H_2O_2 (n=8). Ns is used to demonstrate the absence of a significant difference. ** represents the significant difference in fluorescence ($p<0.01$). Finally, * represents the significant difference ($p< 0.05$). All data are presented as mean ± SEM (n=8). The data presented have been normalized to the control (untreated wells).

In the presence of 100µM H_2O_2, 6pg/mL IL-8 produced a small (0.158±0.088) but insignificant increase in Relative Fluorescence Units (n= 8, $p>0.05$). However, 50 ng/mL IL-8 increased fluorescence by 0.329±0.147 (n=8, $p<0.01$). Finally, a significant increase in fluorescence (0.170 ±0.05) between the two concentrations was observed (n=8, $p<0.05$) (FIgure14.).

3.5.3 The effects of IL-8 in ROS fluorescence in the presence of 200μM of H_2O_2.

After successfully establishing that 200μM of H_2O_2 caused a significant increase in the fluorescence measurements of the H9C2 cell line compared to the untreated/control group, the study explored the potential dose-dependent increase in OS production when the H9C2 cells were also treated with different concentrations of IL-8 (Figure 15.).

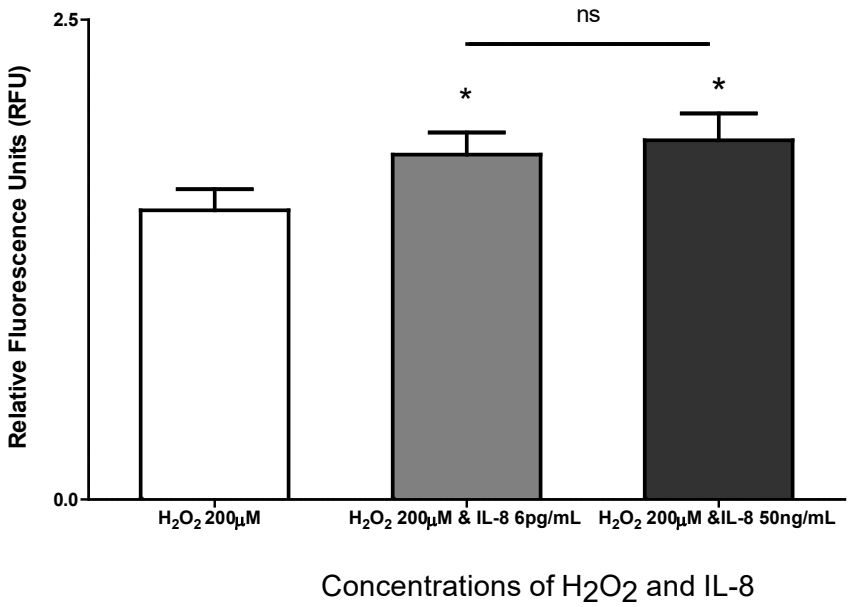

Figure 15. ROS fluorescence measurements in response to treatment with different concentrations of interleukin-8 when H9C2 cells have been pre-sensitized with 200μM of H_2O_2. * is used to depict the significant ($p<0.05$) NS is used to demonstrate that there is no significant difference in the fluorescence ($p>0.05$) All data are presented as mean ± SEM (n=8). The data presented have been normalized to the control (untreated wells).

In the presence of 200μM H_2O_2, 6pg/mL IL-8 produced a significant increase in Relative Fluorescence Units (0.283 ± 0.015) (n= 8, $p<0.05$). 50 ng/mL IL-8 increased fluorescence by 0.329±0.147 (n=8, $p<0.05$). Finally, there was not a significant increase in fluorescence between the two IL-8 concentrations (n=8, $p>0.05$) (Figure 15.).

4 Discussion.

IHD remains one of the primary causes of mortality and morbidity worldwide (Nowbar et al., 2019). Chronic inflammation and the imbalance between the production of ROS and antioxidant defense, also called OS play a major role in the development and progression of the condition, along with the patient's prognosis (Beteridge 2000). Since the establishment of both these factors as manifestations of iIHD, several pro-inflammatory cytokines as well as the involvement of ROS have been investigated.

IL-8 is a proinflammatory cytokine that is produced by various types of cells and plays a major role in atherosclerosis via the activation, proliferation, and migration of monocytes and neutrophils to the site of its increased concentration (Gimbrone et al., 1989). In addition to that the unique characteristics of the IL-8, such as the high resistance to changes in temperature, proteolysis, and acidic environments allow the cytokine to survive and remain active at the site of inflammation for several weeks (DeForge et al., 1992). Since studies have shown that IL-8 plays a role in the formation and establishment of atheromatic plaque on the arterial walls, there has been a great deal of interest in determining whether the cytokine can potentially be a biomarker to predict cardiovascular events and consequently a therapeutic target. Studies have shown that higher levels of IL-8 in patients' serum have been associated with an increased risk of developing CAD (Romuk et al., 2002). While other studies have suggested that IL-8 is an indicator of overall mortality rather than an independent indicator of CAD development (Cavusoglu et al., 2015, Panichi et al., 2005) . It is known that CHD affects both the systolic and diastolic function of the heart due to the remodeling that occurs after prolonged periods of ischemia or an MI.

Systolic dysfunction is defined as impaired left ventricular contraction, which results in a lower EF and decreased cardiac output (Litle & Applegate, 1993). Important cardiac measures such as left ventricular ejection fraction (LVEF) (John et al., 2022), the LV outflow tract (LVOT velocity) (De Azevedo Filho et al., 2021), and the tricuspid annular plane systolic excursion (TAPSE) (Berman et al., 1979) are generally reduced in CAD patients with systolic dysfunction, suggesting decreased contractile performance (Nagueh et al., 2009).

Diastolic dysfunction, on the other hand, is characterized by abnormal relaxation and poor filling of the LV during diastole (Zile & Brutsaert, 2002). One of the essential parameters for assessing diastolic function is the E/A ratio, which represents the ratio of early (E to late) A transmitral flow velocities during diastole (Motram, 2005). An abnormal E/A ratio can indicate impaired LV relaxation or increased LV filling pressure, both of which are associated with diastolic dysfunction (Miter et al., 2017). In spite of the fact that CAD has an established effect on cardiac function, not many studies have investigated the potential relationship between the levels of IL-8 with cardiac dysfunction in IHD.

This study measured the levels of pro-inflammatory cytokine IL-8 in 52 patients diagnosed with CHD and correlated the concentrations with indices of cardiac function provided by the patients' echocardiography data.

4.1 Are levels of Interleukin-8 elevated in CAD patients?

The serum levels of IL-8 in 52 patients of the study cohort were measured via sandwich ELISA assay. The development of standard curves (Figure 6.) allowed the precise quantification of the concentrations of IL-8. The concentrations of IL-8 in the patient cohort ranged from 0.03 - 29.71 pg/ mL, (n=38) while amongst the patients, it was observed that in 14 patients (26.92 % of the

patient cohort) the IL-8 levels were undetectable. These undetectable IL-8 levels were further investigated in the context of available literature. In a meta-analysis conducted by Borun (2023), the levels of IL-8 in 369 healthy individuals were found to be 10.99 ± 9.14 pg/mL. This suggested that the levels measured in these 14 patients can be considered clinically irrelevant to patients with IHD. Therefore, they were excluded from any further analysis. The inclusion of results below the assay's limit of detection is a crucial factor in our research. While undetectable IL-8 levels have been excluded from our analysis due to their probable clinical insignificance in patients with IHD, it is important to note that this exclusion may have an effect on the overall interpretation of IL-8 dynamics in our study. Excluding undetectable values results in a dataset with lower variability, making it simpler to identify potential relationships or trends associated with IL-8 in the setting of IHD. Inclusion of these undetectable values and the establishment of reference ranges could help future research. The mean value of IL-8 based on the n=38 measurable values is 5.77± 5.81 pg/mL. These levels cannot be insinuated to be elevated as there were no healthy patients recruited solely for the study, therefore no baseline of normal values of IL-8 However, it should be noted that several studies have found levels of IL-8 in healthy individuals to range from 2.7 up to 34.31pg/ml (Kuloglu et al., 2011, Serban et al., 2021).

4.1.1 Do levels of Interleukin-8 correlate with indices of cardiac function?

The involvement of IL-8 in atherogenesis along with the correlation of high levels of the cytokine with increased risk of acute cardiovascular events such as an MI or HF have been thoroughly investigated (Velsquez et al., 2014). However, only a few studies have examined the potential relationship between the levels of IL-8 and overall heart function. Reports have shown that high levels of IL-8 have correlated with an increased risk of left ventricular dysfunction (Mocan et al., 2019).

In this study, the levels of IL-8 of patients diagnosed with CHD were correlated to cardiac parameters. Firstly, we assessed the relationship between the levels of IL-8 and parameters determining systolic function. Primary data indicated that there was no correlation between the levels of IL-8 and EF (p=0.0684). While the p-value between EF and IL-8 measurements was found to be 0.0684 in our study, it is important to note that the study is underpowered and despite the limitations imposed by the sample size (n=34), a noticeable trend was observed that suggests a potential relationship between EF and IL-8. With a larger sample size, this trend could potentially be substantiated and provide more robust evidence. A study conducted by John et al, (2022) showed that CHD increases the risk of impaired EF (EF <55 %). To further support this finding in this study, 52.9 % (n=18) of the patients had an EF <55 %. A low EF is established as an indicator of HF (Vedin et al., 2017), with IHD remaining the primary etiology (Cleland & McGowan, 1999). However, preserved EF >55%, in CAD patients was found to cause greater deterioration in cardiac function and higher mortality rates during the follow-ups in comparison with patients presented without substantial coronary lesions (Greenberg, 2014).

Another important parameter to examine systolic function in CAD is the left ventricular outflow tract (LVOT) peak velocity. LVOT refers to the measurement of the maximum rate of blood flow calculated in the left ventricular outflow tract during systole. Low values of LVOT peak velocity (<1 m/s) have been associated with aortic valve stenosis (Stewart et al., 1997). Atherosclerosis shares underlying pathways and mechanisms with aortic valve stenosis, therefore both conditions frequently co-exist (Stewart et al., 1997). Studies have also shown that about 15 to 80% of patients with severe aortic stenosis also have CAD (De Azevedo Filho et al., 2021).

The results of our research are consistent with those of earlier studies, with 61.11% (n=11) of the patient group exhibiting reduced LVOT peak velocity (1 m/s). However, the concentrations of IL-8 did not significantly correlate with the LVOT peak velocity measurements of the patient's cohort (p= 0.4073) . Finally, to assess the right ventricular systolic function in relation to the levels of IL-8, the concentrations of the cytokine were inter ploted with the tricuspid annual plane systolic excursion (TAPSE) measurements. Over the years the role of the right ventricle's function to the overall heart function has been a topic of controversy. Due to the restoral location and complicated structure, the RV was often overlooked during echocardiography (Friedberg & Redington, 2014).

Nonetheless, later studies supported that the function of the RV is an important aspect of the overall heart function and that it can predict symptomatic severity and prognosis in many CVD (Aloia et al., 2016). Both acute MI and chronic IHD have been linked to changes in RV function (Berman et al., 1979). During ischemia, the RV has been reported to be less vulnerable than the LV (Rallidis et al., 2014). This can be atributed to the fact that at rest the LV extracts roughly 75% of the oxygen carried by coronary blood flow compared to the RV which extracts about 50% (Rallidis et al., 2014). This allows the right chamber to increase the extraction of oxygen during situations of elevated oxygen demand caused by stress or ischemia (Zong et al., 2005).

In this study, 9.52% (n=20) of the patients had a TAPSE <1.7 cm, which is an indicator of impaired RV function. However, levels of IL-8 did not correlate with the measurements of the TAPSE (p= 0.6817).

Diastolic function during IHD is also altered to various extents. In CHD, diastolic dysfunction is an

early sign in the chronological sequence of ischemic abnormalities, the ischemic cascade distinguishes diastolic problems of the LV as an early sign in the chronological sequence of ischemic events (Strk et al., 1995). Beginning with an oxygen demand-supply imbalance and metabolic phenomenon, sometimes occurring before systolic dysfuncfton, electrocardiographic changes, or angina (Strk et al., 1995).

Diastolic dysfuncfton is a prediction of mortality even in patients presenting with normal LV systolic function (AlJaroudi et al., 2012). In addition to that diastolic dysfunction has been linked to endothelial dysfunction, increased levels of OS and coronary microvascular dysfunction (Paulus & Tschpe, 2013, Sorop et al., 2018).

The echocardiographic evaluation of diastolic function is usually assessed by measuring the transmitral flow parameters such as early (E) and late (A) diastolic filling velocities, as well as the E/A ratio (Motram, 2005). E wave refers to the passive blood flow from the left atrium to the left ventricle, or else the peak velocity of the early rapid filling wave, and A wave refers to the late diastolic peak velocity of the atrial filling. An E/A ratio <1 is associated with abnormal left ventricular relaxation, while the average of the E/A ratio is considered normal when between 1 to 1.5 (Garca-Fernndez, et al., 1999, Galderisi, 2005). Early diastolic relaxation is considered an active event that requires more energy than passive late diastolic motion. This has been found to be the physiological explanation behind the low e-wave and a reverse E/A ratio as the most precise indicators of hypoperfusion (Garca-Fernndez, et al., 1999).

The data in this study further support previous research, while 55.17% of the patients (n=16) had an E/A ratio < 1. In addition to that, several studies have proven the involvement of proinflammatory cytokines in diastolic dysfunction. A study conducted by Lee et al, (2012) demonstrated that the levels of proinflammatory cytokines correlated significantly with echocardiography parameters for LV diastolic dysfunction.

Therefore, to investigate the relationship between IL-8 and diastolic dysfunction that there is no significant correlation ($p=0.8552$, $n= 29$). Nevertheless, due to the complex multifactorial nature of CHD, which includes several different mechanisms such as endothelial dysfunction, plaque formation, and vascular inflammation, patients may experience a variety of different symptoms, alterations in cardiac function, and changes in levels of inflammatory biomarkers depending on the severity of their condition. As a result, the interpretation of the findings of this preliminary study should be exercised with caution. Further research will strengthen the credibility of these findings as well as increase the study's statistical power.

4.1.2 Do levels of IL-8 correlate with the patient's demographics and physiological measurements?

Aging is known to cause several physiological functions to gradually deteriorate as well as increase the prevalence of diseases such as atherosclerosis, hypertension, MI, and stroke (Lakata & Levy, 2003). Pathological changes that occur in the cardiovascular tissues during aging include hypertrophy, altered diastolic function, decreased LV systolic reverse capacity, increased arterial stiffness, and reduced endothelial function (Lakata & Levy, 2003, North & Sinclair, 2012). Furthermore, elevated levels of circulatory cytokines and pro-inflammatory markers have also been linked to aging (Michaud et al., 2013). This is due to the changes to the immune system also called immunosenescence a term used to describe the elevated secretion of cytokine by the adipose tissue (Michaud et al., 2013).

However, a study investigating the levels of IL-8 in accordance with aging revealed that the cytokine was decreased in healthy individuals between 65-79 (39.4 ± 8.3 ng/mL) compared to the 20-27 age group (66.4 ± 5.0 ng/mL) (Clark & Peterson, 1994). Finally, in a study conducted by Zhou et al. (2001), the serum levels of IL-8 were found to be significantly higher in patients with

unstable angina or AMI compared to the healthy control subjects, suggesting the involvement of IL-8 in the process of IHD (Apostolakis et al., 2009). In this study, the mean age of the patient cohort was 64.76 ± 1.89 (n=34), however, the levels of IL-8 did not correlate with the age of the patients (p=0.3736).

Nonetheless, due to the complexity of age-related immune system changes, other factors may have influenced the levels of IL-8 production in the patient's cohort.

One of the most important risk factors in the development of CHD is obesity (Atique et al., 2016). According to studies, regardless of the presence of other risk factors, obesity can lead to the development of CVD autonomously (Cercato & Fonseca, 2019, Powell-Wiley et al., 2021). Obesity is the term used to describe an increase in body fat accumulation with a variety of different factors contributing to its pathogenesis. A body mass index of ≥30 kg/m^2 is used as a baseline to assess obesity (Yusuf et al., 2005). Adipose tissue, previously thought to be an energy storage organ, plays an important role in the production of secretory factors called adipocytokines or adipokines that directly affect close or remote tissues (Ouchi et al., 2011). Adipose tissue operates as an endocrine organ that regulates the progression of several pathophysiological processes in diseases like CAD (Ouchi, 2016). In addition to that another study have further supported the relationship between inflammation and obesity, including elevated levels of different inflammatory biomarkers (Khanna et al., 2022). To further contribute to that, the BMI index of the patients was correlated with the levels of IL-8 to investigate a potential relationship.

In the study, 64.28% of patients (n=9) were found to have a BMI > 30. Regardless of the fact that the p-value between BMI and IL-8 concentrations in our studies was shown to be 0.0784, it is crucial to consider that the study is underpowered. Nevertheless, despite the restrictions imposed by the sample size (n=14), a definite pattern was observed that may point to a connection

between BMI and IL-8. This patern may be confirmed, with the increase of the study's statistical power.

In addition to that it should be noted that the BMI should be taken into account with caution due to the limitations, as it does not take into consideration various body compositions (Azab et al., 2018). For example the same BMI value can be shared by the same fit individual with a greater muscle mass and unfit individuals with a larger fat mass (Azab et al., 2018). Moreover, BMI is overlooks differences in fat distribution amongst individuals (Gonzalez et al., 2017) Finally, BMI fails to take into account differences amongst individuals such as biological sex and ethnicity (Jeong et al., 2023). It has been reported that different ethnicities can exhibit different ratios of muscle, bone and fat even when the BMI value of an individual is the same. This can result in the misclassification of individuals from specific ethnic groups, rendering BMI an unreliable indicator of health risks (Jeong et al., 2023). Therefore, other measurements may be more beneficial in providing insights into a individual's muscle mass, bone density and body fat distribution which would offer a more comprehensive insight of health risks and guidance on personalized interventions (Bazzocchi et al., 2016 ,Tinsley et al., 2021). Other measurements may include waist circumference, Waist-to-Hip Ratio (WHR) and Body Composition Analysis (Muscogiuri et al., 2023).

The risk of cardiovascular morbidity and death is increased for CAD patients presenting with elevated resting heart rates. The mismatch in supply and demand that results in ischemia and angina has been reported to be caused by an increase in cardiac activity and a decrease in diastolic filling time (Axsom & Bangalore, 2012). Although a lower HR is linked to a beter prognosis, it is currently under examination whether pharmacological reduction of the HR has any real benefits or is just a sign of increased risk and worse outcomes in patients (Axsom & Bangalore, 2012). A study of 25,000 patients with known or suspected CHD surveyed for 14.7 years revealed that

a resting HR greater than or equal to 83 bpm was associated with a higher risk of all-cause

and cardiovascular mortality, independently of age, hypertension, diabetes, impaired ejection fraction, and medication (Diaz et al., 2005). Another study, conducted on 4162 patients by Bangalore et al. (2010), demonstrated that HR does not follow a linear relationship with CAD patients' outcomes, but rather a J-shape one. Patients with a resting HR lower than 59 bpm had an increased risk of cardiovascular events, as did the patients presenting with a HR higher than 70bpm. In addition to that, a HR of 60-69 beats per minute was shown to have the lowest risk of a cardiovascular event (Bangalore et al., 2010). Furthermore, accumulating data from clinical research suggests a relationship between inflammatory activation and arrhythmias (Lazzerini et al., 2023). This is due to the specific role that pro-inflammatory cytokines play in arrhythmogenesis, which includes direct cardiac effects and indirect actions brought on by systemic cytokine alterations (Lazzerini et al., 2023).

In this study, 0% of the patients presented with tachycardia (HR >100 bpm), however, 22.22% of the patients (n= 4) presented with bradycardia (HR< 60 bpm). Furthermore, levels of IL-8 were ploted against the HR measurements of the patients. Primary data demonstrated that there is no correlation between IL-8 levels and HR in CAD patients ($p = 0.2091$, $n=18$). However, the correlation's statistical power is underpowered and therefore raises the possibility for inclusive findings. As a result, caution should be exercised before drawing conclusions from these data.

4.1.3 Do the interleukin-8 levels in the research cohort's male and female participants differ?

Inflammatory responses have been thoroughly investigated however the role of sex steroid hormones in the regulation of cytokines is yet to be determined.

Studies have investigated the potential difference in the immune response between the sexes, with men producing higher levels of cytokines than women (Bernardi et al., 2020, Kaptoge et al., 2013). It has been proven that men that have been diagnosed with CHD have lower levels of androgens compared to healthy men, (Pugh et al., 2003) and that testosterone hormone therapy has been successful in downregula ng the levels of cytokines in inflammation (Mohamad et al., 2018).On the other hand, elevated levels of IL-8 in women have been associated with an increased risk of all-cause mortality (Moreno Velsquez et al., 2014, Ridker et al., 2017). In this study, it was found that sex is not associated with levels of IL-8 It should be noted however that the female participants' data (n= 6) were underpowered compared to the male participants (n=16). Several studies have reported on the underrepresentation of women in cardiovascular research for HR, CHD, and acute coronary syndrome (Jin et al., 2020). This observation was also confirmed by this study, increasing the need for larger-scale studies focusing on female patient cohorts. This is essential because, in contrast to the extensive research on the male population, the differences in the processes of IHD in the female population are still undetermined. A beter understanding of CHD in women will allow the establishment of better treatment strategies and therefore better patient outcomes.

4.2 Do inflammatory biomarkers increase levels of oxidative stress?

As mentioned before inflammation and OS play a key role in the pathogenesis of CAD. Understanding the complex interactions between inflammation and OS has garnered more ate ntion in recent years.

The aim of this study was to investigate the interplay between the inflammatory biomarker IL-8 and OS in IHD.

The data, therefore, suggest that H_2O_2 can modulate OS levels in a concentration on-dependent manner. To achieve that embryonic rat cardiomyocytes were exposed to various concentrations of IL-8 alone or in combination with H_2O_2 to assess the rise in OS levels.

4.2.1 Does the DCFDA assay work?

H_2O_2 is a known OS inducer, as mentioned before in Section 2.7.1, and IHD is one of the many conditions in which the upregulation of ROS occurs. (Katoor et al., 2017) Several studies have induced OS with the use of different concentrations of H_2O_2 and have concluded that concentrations higher than 1 μM can cause growth arrest and cell death (Antunes & Cadenas, 2001, Stone & Yang, 2006). In more detail, it has been demonstrated that in concentrations higher than 120 μM up to 150 μM a temporary growth arrest was observed, while concentrations between 250 to 400 μM caused a permanent growth arrest (Gülden et al., 2010).

Therefore, to validate the reliability and sensitivity of the DCFDA assay as well as to mimic the ischemic conditions in the cells, H_2O_2 was employed as a positive control.

Interestingly, when the H9C2 cells were treated with the lower concentration (100μM of H_2O_2) for 30 minutes the levels of OS did not significantly increase in comparison with the cells that were untreated ($p>0.05$). On the contrary, when the cells were treated with 200μM of H_2O_2 for 30 minutes the levels of OS significantly increased with a difference in Relative Fluorescence Units of 0.3482±0.115 ($p<0.001$). Subsequently, a significant increase between the cells treated with the lower concentration (100μM of H_2O_2) compared to the cells that were treated with the higher concentration (200μM of H_2O_2) was observed (Figure 12.).

The significant increase in ROS production detected a er the treatment with the higher concentration of H_2O_2 highlights its potential to mimic the conditions of IHD. It should be noted however that the concentrations and exposure periods employed in this study were specific to the study's experimental design. Additional studies will allow a further understanding of the dose and time-dependent relationship between H_2O_2-induced OS.

4.2.2 Does Interleukin-8 increase oxidative stress in H9C2 cells?

Inflammation is a pathological state characterized by the entry of immune cells into the vascular wall, migration of the immune cells into the tissue, and the generation of ROS by these cells, resulting in tissue damage (Chaterjee, 2016). In addition to that, OS triggers inflammation via the activation of the nuclear transcription factor kappa B (kNF-B), responsible for the control of critical genes that encode proinflammatory cytokines, chemokines, and leukocyte adhesion molecules (Kibel, A. et al. 2020), and this in return can cause further production of ROS (Chaterjee, 2016).

IL-8 is a chemokine thoroughly investigated for its involvement in atherogenesis due to its chemoatractant and mitogenic effects on vascular smooth muscle cells along with the migration of monocytes into the subendothelial space of the atheromatic lessons (Boekholdt et al., 2004, Gerszten et al., 1999). In addition to that several studies have investigated the relationship between IL-8 and OS but have mostly focused on the upregulation of IL-8 production due to OS.

In this study, the effects of different concentrations of IL-8 on the levels of OS were investigated. The preliminary data demonstrated that both concentrations (50 ng/mL and 6pg/mL) when introduced to the H9C2 cell line for 1 hour had an insignificant difference in OS levels compared to the levels of OS in the untreated cells. ($p > 0.05$)

The data reveal that the specific concentrations and treatment period of IL-8 did not have a significant change in OS in the H9C2 cell line. Since CHD is a chronic multifactorial condition IL-8's involvement may result from time and concentration-related factors, as well as from the contribution of other more complex mechanisms that this study's model did not fully capture. Therefore, to further comprehend these mechanisms more research is required to investigate the effects of IL-8-mediated OS while taking into consideration the chronic nature of CHD.

4.2.3 Do different concentrations of interleukin-8 increase oxidative stress in presensitized H9C2 cells with 100µM of H_2O_2?

To investigate the interplay between inflammation and OS in the ischemic model H9C2 cells were then co-treated with 100µM of H_2O_2 and different concentrations of IL-8. Interestingly, when the cells were co-treated with 100µM of H_2O_2 and 50 ng/mL of IL-8 a significant increase in OS was observed ($p<0.01$). This result appears in contrast with our previous observations in Sections 4.2.1 and 4.2.2 which supported that 100µM of H_2O_2 and IL-8 separately did not significantly increase OS. However the concentration equivalent to the average levels of IL-8 measured in the study's patient cohort (6 pg/mL) was found to cause an insignificant increase in OS when introduced to H9C2 cells along with 100µM of H_2O_2 compared to the cells that were solely treated with 100µM of H_2O_2. These findings support the statement that IL-8 could potentially have a role in enhancing OS in CHD when ROS production is increased due to other mechanisms implicated in the conditions pathogenesis.

4.2.4 Do different concentrations of interleukin-8 increase oxidative stress in presensitized H9C2 cells with 200μM of H_2O_2?

To further support the previous findings of the study, 200μM of H_2O_2 and different concentrations of IL-8 (50 ng/mL and 6 pg/mL) were introduced to H9C2 cells. It was observed that both concentrations of 50 ng/mL and 6 pg/mL along with 200μM of H_2O_2 significantly increased levels of OS compared to the cells that were solely treated with 200μM of H_2O_2 ($p<0.05$).

This indicates that the presence of IL-8 even at low concentrations can promote the OS-inducing effects of H_2O_2 at this concentration.

The results emphasize the possible synergistic relationship of IL-8 and H_2O_2 in inducing OS in the cardiac model. Further research can potentially explore the mechanisms under which IL-8 increases OS when in the presence of H_2O_2 as well as further support the findings of this research by increasing the study's power.

4.3 Impact of therapeutics in inflammation in coronary heart disease.

Each patient's treatment plan depends heavily on the severity of the condition, the symptoms, and the lifestyle of each individual. Since inflammation is a proven manifestation of CHD the majority of medication prescribed to patients has anti-inflammatory effects (Pello Lázaro et al., 2021). In addition to that and as mentioned before the medication history of this study's cohort is unknown. Therefore, the findings should be interpreted with extra caution as it is uncertain whether the parameters investigated in this study have been affected by medication. The most effective CAD therapeutics, as described in Section 1.4, include beta-blockers, statins, and anti-inflammatory drugs.

4.3.1 Beta-blockers.

Beta-blockers were first introduced in the 1960s for the treatment of angina, and ever since they have been widely used for a variety of conditions such as hypertension, arrhythmias, and HF (Joseph et al., 2019). The way they work is by binding to beta-adrenoceptors, preventing the circulatingsympathetic hormones adrenaline and norepinephrine from binding. Beta-blockers have various effects on the cardiovascular system depending on reception affinity (Andreasen & Andersson, 2018). The first set of beta-blockers created was non-selective with equal affinity for β1 and β2 receptors. While the second group was more cardio-selective andhad a higher affinity for β1 receptors, which are primarily found in cardiomyocytes and the conduction system (Andreasen & Andersson, 2018). The positive effect of sympathetic inhibition in IHD has been shown to be due to the decreased HR and contractility of myocytes, which consequently decreases myocyte oxygen consumption.This lowering of oxygen demand and contractility have been suggested to be the main ways of actionn against angina pain (Andreasen & Andersson, 2018).

In addition to that, clinical trials have shown that the administration of beta-blockers reduces mortality and morbidity in patients following an MI and in patients with HF with reduced EF (HFrEF) (Joseph et al., 2019). This seems to be due to the beta-blockers ability to reduce OS (Nakamura et al., 2011). A study conducted by Kukin et al. (1999) concluded that both metoprolol, a β1-selective blocker, and carvedilol, an α, and β blocker with antioxidant action, decreased plasma lipid peroxidation in patients with HF while improving cardiac function.

Another study conducted by Chin et al (2003) further supported that bisoprolol and carvedilol decreased the levels of lipid hydroperoxides in HF patients. Finally, a study conducted by Nakamura et al., 2002 discovered that carvedilol decreased the quantities of 4-hydroxy-2-nonenal HNE-modified protein by 40% in patients with HF, the most reliable marker of lipid peroxidation as a result of ROS damage (Nakamura et al., 2011, Toyokuni et al., 1995).

By considering the already-established connection between inflammation and OS in CHD , this evidence demonstrates that the levels of OS and the parameters investigated in this study will be impacted in the patients who have received beta-blocker treatment.

4.3.2 Statins.

Statins are known to lower the chances of cardiovascular events and mortality rates in patients with CHD . This is achieved by the blockage of 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, which is important for lowering LDL cholesterol levels in the blood (Lim, 2013). Statins were first introduced in 1987, and various studies have subsequently been conducted to evaluate their advantages in the outcomes of CHD patients (Jukema et al., 1995, Nissen et al., 2004). Nowadays, a wide range of patients are prescribed statins as both a primary and secondary prevention of CVD. Statin treatment has been proven to have pleiotropic effects that are independent of lowering the LDL-cholesterol levels of the blood. For example, improved endothelial function, (Dupuis et al., 1999) decreased vascular inflammation, (Ridker et al., 1998), and decreased platelet adhesion and thrombosis are a few of the benefits of the medication (Lacoste et al., 1995).

In further detail, statins have been shown to increase the amount of eNOS generated by endothelial cells, which upregulates the bioavailability of NO. This is a crucial component of the statins' anti-inflammatory activity given the anti-inflammatory and vasodilatory effects of NO (Laufs et al., 1998, Wassmann et al., 2002). In a clinical setting, statins have been shown to reduce the levels of pro-inflammatory cytokine levels in patients presented with metabolic syndrome, diabetes mellitus, or hypercholesterolemia (Golia et al., 2014). While other studies have reported on the downregulation of C-reactive peptides, chemokines, cytokines, and adhesion molecules, as well as the modulation of T-cell action due to statins administration (Corsonello et al., 2010).

All these studies support the potential involvement of the statins in lowering the cytokine levels of this patient's cohort if used in their treatment plan.

4.3.3 Anti-inflammatory Therapeutics.

Since the establishment of the role of inflammation in atherogenesis and the progression of CHD, several studies have attempted to assess the effectiveness of different anti-inflammatory medications in CHD patients (Wang et al., 2021, Wudexi et al., 2021). A study conducted by Ridker et al (2017), demonstrated that the administration of canakinumab, a therapeutic monoclonal antibody targeting IL-1β and C-reactive protein led to a significantly lower rate of recurrent cardiovascular events in 10,061 patients with a previous MI event. This is important since several studies have documented the time and concentration-depended production of IL-8 by IL-1β (Hwang et al, 2004). Another trial of 5522 individuals with a recent MI event reported that receiving 0.5mg of colchinine once a day reduced the incidence of cardiovascular events when compared to those who received the placebo (Nidorf et al.,2020).

In addition to that, nonsteroid anti-inflammatory drugs (NSAIDs) such as aspirin (acetylsalicylic acid) have been widely prescribed to patients with CHD due to their antithrombotic and anti-inflammatory activity (Rothwell et al., 2010).

A study conducted by Gao et al., (2009), demonstrated that the treatment of patients with cardiovascular metabolic syndrome with a range of doses of aspirin 100 to 300 mg/day decreased the levels of inflammatory biomarkers such as C-reactive protein, IL-6, and TNF-a.

To further support that finding, a study conducted by Berk et al, (2013) demonstrated that aspirin has the ability to lower the levels of inflammatory cytokines like TNF-a and IL-8 but was unable to lower negative immunoregulatory cytokines like IL-4 and IL-10.

4.3.4 IL-8 as a therapeutic target of IHD.

However, targeting the production of IL-8 as a potential therapy for CHD should be investigated thoroughly for numerous reasons. This is because IL-8 plays a role in several physiological processes. In addition to that, the upregulation of IL-8 is not only observed in CHD but in other conditions as well. Blocking the production of IL-8 may affect the immune system, disrupt homeostasis as well have other systemic effects beyond the cardiovascular system. Therefore, before establishing IL-8 as a therapeutic target, it seems essential to investigate the possible side effects and long-term consequences.

4.4 Limitations.

In spite of the significant results obtained by this research, it is important to highlight certain drawbacks that could potentially have affected the findings and therefore should be considered when interpreting the data. Certain limitations of the project were inevitable, while others might potentially be exploited to improve future work.

4.4.1 Human Models.

IHD, as previously established, is a complicated medical condition that can develop due to a variety of reasons and can present with a unique set of symptoms and degrees of severity in each patient**.** Due to this, the effects of the disease and therefore its manifestation on each participant of this study's cohort may differ and should be taken under consideration when interpreting the results of this research. The existence of other disorders including diabetes, autoimmune diseases, and other comorbidities, as well as the medications for these conditions, may have an impact on the levels of biomarkers in the patient cohort.

In addition to that, the patient cohort of the study was recruited after arranging a bypass procedure. It is therefore possible that some of the patients might have been on long-term medication plans designed to lower any inflammatory levels as well as decrease the heart rate and treat other potential symptoms of IHD before the procedure. Additionally, it's worth mentioning that there was no available information regarding the medications used by the patients in our cohort. Therefore, due to these specific circumstances mentioned above, further clinical data for each patient would be beneficial in order to be able to investigate the impact of these factors on CHD.

4.4.2 Data , sample collection and storage of the sample .

Another limiting factor of the study was the data collection as well as the travel time. Despite the diligent efforts of the Blackpool Victoria Hospital surgeons and the University of Salford research team, certain parameters could not be fully captured. As a result, the sample size and available clinical parameters for the analysis were limited, which may have impacted the statistical power of the study. These limitations are valuable to be acknowledged since they may affect the robustness of our findings.

We recognize that the travel time of the blood sample between Blackpool Hospital and the Salford University research laboratory might also play a role in the consistency of the finding. Travel times are an inevitable limitation in research, and during this study the travel time was estimated to be around 1 hour. While all necessary precautions were taken, including the use of appropriate storage containers and cooling equipment to minimize any potential degradation of the sample, the limitation is important to be noted as it may introduce a degree of variability in the results.

Finally, the freeze-thaw effect was not specifically examined in this study. This is important to be noted as the freeze-thaw cycles might have an impact on the stability of the cytokine and therefore the robustness of the results. While every precaution was taken to minimize a possible impact of the freeze-thaw cycles on the patient ‘s samples, future research could potentially investigate this aspect.

4.4.3 Sample size.

Another important limitation of the study is the small sample size (n=52) which may have limited the generalizability of the results. As this is a preliminary study the low number of participants may have impacted the statistical power and ability to detect significance in the relationship between clinical parameters and the concentrations of IL-8. In addition to that, it is noteworthy that for the specific correlations, the sample size was even more limited (n=38). This reduced sample size was due to the fact that, in a portion of the patients, the levels of IL-8 were undetectable compared to the original sample size that was tested with the ELISA assay (n=52).

4.4.4 Cell lines.

Animal cell lines are frequently utilized in research to mimic many aspects of human pathology. In further detail, rats have been a preferred animal model in cardiovascular research due to the similarities they share in anatomy and physiology with humans (Bryda, 2013). Even though animal models provide valuable insights into coronary heart disease mechanisms, they cannot fully capture the complexity of the human disease and the manifestations of it. As previously noted, CHD is a complex, multifactorial and chronic condition making it challenging to exactly mimic in animals. Therefore caution should be exercised when extrapolating results form animal models in vitro to clinical setting in vivo.

4.5 Future work.

Future research in coronary heart disease including the exploration of IL-8 as a biomarker holds the potential of enhancing our understanding of the condition's pathophysiology and improving diagnostics and treatment options.

Larger scale studies employing healthy control participants would allow the establishment of IL-8 reference ranges in individuals without CHD. This will provide a comparative foundation for determining the significance of the cytokine detected in CAD patients, and whether they deviate from the normal values.

In addition to that, it is essential to consider alternative models for a more comprehensive understanding of the condition. For example, genetic mouse models can provide valuable insights into the genetic foundations of coronary heart disease and cytokine responses. Furthermore, further investigations of the impact of IL-8 inhibitors on ROS production in the context of coronary heart disease would be beneficial. This approach could reveal valuable information regarding the possible therapeutic relevance of targeting IL-8 to mitigate OS-induced damage in the cardiovascular system.

To investigate sex-based differences in levels of cytokines involved in CHD , future research must take into account the underrepresentation of women in cardiovascular research and conduct studies with an equal number of participants from both biological sexes. Additional clinical parameters could potentially provide further understating of CHD mechanisms, for example, each patient’s level of severity, symptoms, history of previous cardiovascular events, and treatment record could be interpolated to investigate the relationship between levels of specific inflammatory biomarkers involved in CAD and these parameters.

Lifestyle also plays a significant role in CVD and inflammation, therefore, the levels of inflammatory biomarkers may fluctuate over time depending on the stimuli. Measuring the level of cytokines at different time points will allow the capture of temporal variability and will enable the determination of the underlying mechanisms and progression of the disease.

Future research will also focus on the interplay of inflammation and upregulation of OS. Inflammatory cytokines involved in CAD pathogenesis and progression will be feasible to being introduced to different cell lines in vitro, to investigate the underlying mechanisms by which they are involved in promoting the OS-inducing effects of the known ROS inducers.

4.6 Conclusion.

Preliminary data from this study demonstrated that IL-8 did not correlate with most patients' cardiac indices as well as demographics and physiological measurements. However, a clear trend was observed between the levels of IL-8 and BMI. These findings suggest IL-8 could potentially be a valuable biomarker for CAD.

In addition to that our data suggested that the were no significant differences between the levels of IL-8 in the female and male patients of the study cohort.

Furthermore, the DCFDA assay revealed that IL-8 solely was unable to increase the levels of OS in healthy cells, while it did contribute to an increase in H9C2 cells with a pre-existing elevated baseline of OS.

These findings provide credence to the hypothesis that the interplay between inflammation and OS plays a significant role in the pathophysiology and development of CAD. Despite this, the results from this study are preliminary, thus future research should try to strengthen the power of the study in order to validate these findings.

Appendix A. Individual Interleukin-8 serum concentrations.

Patient no.	IL-8 concentration (pg/ml)	SD	SEM
BVH 1	5.11	0.013	0.006
BVH 3	1.57	0.004	0.023
BVH 4	5.13	0.001	0.002
BVH 6	2.62	0.013	0.006
BVH 7	3.24	0.047	0.023
BVH 8	2.86	0.004	0.002
BVH 9	3.14	0.001	0.0006
BVH 10	0.03	0.0008	0.0004
BVH 11	7.41	0.011	0.0056
BVH 12	3.28	0.008	0.0042
BVH 13	1.39	0.001	0.0006
BVH 14	5.53	0.005	0.0028
BVH 15	7.08	0.006	0.0031
BVH 16	5.40	0.009	0.0047
BVH 17	6.10	0.016	0.008
BVH 18	16.73	0.077	0.038
BVH 19	3.06	0.002	0.0012
BVH 20	4.38	0.004	0.0023
BVH 21	7.56	0.065	0.032
BVH 22	5.23	0.004	0.0024

BVH 23	13.12	0.051	0.025
BVH 24	0.89	0.023	0.011
BVH 25	0.91	0.003	0.0015
BVH 26	1.59	0.02	0.012
BVH 27	0.93	0.01	0.01
BVH 42	8.12	0.05	0.028
BVH 46	6.33	0.05	0.025
BVH 51	5.15	0.23	0.11
BVH 52	29.71	0.6	0.3
BVH 53	5.71	0.24	0.12
BVH 54	9.43	0.29	0.14
BVH 70	5.53	0.01	0.007
BVH 71	0.07	0.003	0.001
BVH 72	7.59	0.08	0.041
BVH 73	6.42	0.006	0.003
BVH 75	1.24	0.021	0.003
BVH 76	19.37	0.003	0.01
BVH 77	0.41	0.008	0.004

Appendix B. Individual patients' clinical data and echocardiograms.

Patient no.	SV (ml)	EF %	EDV (ml)	LVOT (m/s)	PASP (mmHg)	TAPSE (cm)	Weight (kg)	HR (bpm)	Age (y)	Gender (F/M)
BVH 1	-	40	-	0.9	7	1.9	88.9	69	55	M
BVH 2	-	55	-	-	24	1.8	-	74	88	M
BVH 3		55	-	1.1	22	2.2	-	70	74	M
BVH 4		55	-	-	22	2.2	-	73	78	M
BVH 5	-		-	-	-	-	-	-	-	-
BVH 6	-	58.3	-	-	-	2.4	-	77	70	M
BVH 7	-	69.81	-	-	-	-	-	-	40	-
BVH 8	-		-	-	-	-	-	76	76	F
BVH 9	-	57.5	-	-	-	-	-	57	64	-
BVH 10	-	62.71	-	-	-	-	-	-	59	-
BVH11	-	-	-	-	-	-	-	-	-	-
BVH 12	-	22.95	-	-	-	2	-	75	66	F
BVH 13	-	52.5	-	0.94	-	2.81	-	60	59	-
BVH14	-	50	-	0.98	-	1.6	-	58	54	-
BVH15	-	55	-	1.3	30	2.2	63.8	45	72	F
BVH16	57.8	64.9	89.15	1.04	-	2.2	110	-	64	-
BVH17	18.2	26.7	68.1	0.55	-	-	-	-	76	F
BVH18	-	39.16	-	-	-	1.8	-	-	54	F
BVH 19	-	55	-	-	-	-	-	-	64	M
BVH20	56	33.5	167	0.99	42	-	-	-	71	M

BVH21	-	57.5	-	0.99	-	-	-	-	73	-
BVH22	-	35	-	1.03	44	2.09	-	63	78	M
BVH 23	-	52.5	-	0.7	11	-	-	97	39	M
BVH 24	-	55	-	-	-	-	89	67	56	F
BVH 25	-	50	-	-	-	-	-	49	71	M
BVH 26	73.5	53.7	137	-	36	-	-	-	61	M
BVH 27	-	-	-	-	-	-	-	-	-	-
BVH42	-	42.5	-	-	-	-	-	-	64	-
BVH46	-	-	-	-	-	-	-	-	78	-
BVH51	-	42.5	-	1.09	-	2	-	-	-	-
BVH52	-	45	-	-	-	2.57	54	-	60	-
BVH53	-	52	-	0.97	-	2.3	105	-	-	-
BVH54	-	55	-	0.83	-	2.09	92	70	76	-
BVH70	-	-	-	1.34	-	-	107.5	-	56	M
BVH 71	-	62.5	-	-	34.2	-	-		53	-
BVH72	-	47.5	-	0.69	13	1.79	55.5	65	73	M
BVH73	-	53	-	0.85	-	2.05	121	-	51	-
BVH 75	-	37.5	-	0.68	-	2.33	98.8	74	67	M
BVH76	-	-	-	-	-	-	-	-	-	-
BVH 77	-	57.5	-	-	-	2.56	94	-	62	M

Appendix C. HRA and HCRW approval letter

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

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

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

21 January 2019

Dear Dr Greensmith

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

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

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

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

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

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

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

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

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

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

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

What are my notification responsibilities during the study?
The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

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

The sponsor contact for this application is as follows:

Name: Professor Sheila Pankhurst

Tel: 0161 295 5171

Email: s.pankhurst@salford.ac.uk

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

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

IRAS project ID	247341

Yours sincerely

Joanna Strickland
Assessor

Email: hra.approval@nhs.net

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

Appendix C. HRA and HCRW approval letter

NHS
Health Research Authority

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

Telephone: 0207104 8204

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

19 January 2019

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

Dear Dr Greensmith

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

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

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact hra.studyregistration@nhs.net outlining the reasons for your request. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

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

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

Conditions of the favourable opinion

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

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

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

Extract of the meeting minutes

Approved documents

The documents reviewed and approved were:

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

Membership of the Proportionate Review Sub-Committee

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

There were no declarations of interest.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators

- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

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

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

Yours sincerely

Pp
REC Manager

Mr John Richardson
Chair

Email: nrescommittee.london-camberwellstgiles@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

"After ethical review – guidance for researchers"

Copy to: *Mrs Helen Spickett, Blackpool Teaching Hospitals NHS Foundation Trust*

Lead Nation

England: HRA.Approval@nhs.net

London - Camberwell St Giles Research Ethics Committee

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

Committee Members:

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

Also in attendance:

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

Appendix D. Patient information and consent form.

Blackpool Teaching Hospitals NHS Foundation Trust

Clinical Research Centre

2nd floor, Area 5 Blackpool Victoria Hospital Whinney Heys Road

Blackpool FY3 8NR

Tel: 01253 (9)53559

Email: helen.spickett@nhs.net

PATIENT INFORMATION SHEET

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

An Invitation to participate

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

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

What is the purpose of the study?

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

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

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

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

the bypass operation. We want to know if leaving these fat cells around the blood vessel will improve surgical outcome.

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

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

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

Do I have to take part in the study?

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

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

If you decide to participate in this study, you will be asked to sign a consent form then, before or after you are put to sleep for your surgery a 5mL blood sample will be taken from you so that we can look at circulating markers in your blood. When you are undergoing your surgery, some of the heart tissue

(approx. 0.5-1cm) and some of the leftover mammary artery / saphenous leg vein which is used as for the bypass, both of which are normally removed and discarded during the operation, will be kept in an experimental solution to be transported to the University of Salford so we can look at how the cells in these tissues work. We will use scientific equipment to keep the tissue alive so we can explore how they work and the signals from them, small pieces of tissue will be preserved so we can look at the structure of the tissue and the levels of specific proteins within it. A computer program will be used to find out whether any changes we see are linked to your recovery.

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

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

What will happen to my blood sample and tissue?

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

What are the possible benefits of taking part?

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

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

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

Indemnity and Compensation?

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

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

Will my taking part in the study be kept confidential?

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

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

If you decide to withdraw from the study at any point, we will continue to use any data collected up to your withdrawal. We will not contact you about the study from this point forward. A decision to

withdraw at any time or a decision not to take part will not affect the standard of care you receive or the treatment that you are receiving.

What if there is a problem?

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

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

What will happen to the results of the study?

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

Who can I contact for further information?

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

Thank you for taking the time to read this information.

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 27th November 2019 (version 1.3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	
I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
I give permission for my cardiac tissue, internal mammary artery and saphenous leg vein, which would be normally discarded to be supplied to The University of Salford. I also give my permission for an extra blood sample to be taken for identification of biomarkers.	
I give permission to The University of Salford to store and distribute my samples to any researchers whose work has appropriate ethical approval and who are conducting high quality medical research on the prevention, diagnosis and / or the treatment of ischemic heart disease or other associated diseases.	

I agree to take part in the above study

_____

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

_____

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

taking consent

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

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