The Role of Tumour Necrosis Factor-α in Coronary Artery Disease

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List of Abbreviations

ACD – Atherosclerotic coronary disease

ACE – Angiotensin-converting enzyme

ACS – Acute coronary syndrome

AGEs - Advanced glycation end products

AMI – Acute myocardial infarction

ASS - Arginosuccinate synthase

ATP – Adenosine triphosphate

BMI – Body mass index

BNP – Brain natriuretic peptide

BVH – Blackpool Victoria hospital

CABG - Coronary artery bypass graft surgery

CAD - Coronary artery disease

CICR - Calcium induced calcium release

CM-H_2DCFDA - chloromethyl derivative of H_2DCFDA

CN – Calcified nodule

CT - Computed tomography

CVD – Cardiovascular disease

DALYs – Disability adjusted life years

DAMPs – Damage associate molecular patterns

DCF - 2',7'-dichlorodihydrofluorescein

DCFDA - 2',7'-dichlorodihydrofluorescein diacetate

DMEM - Dulbecco's Modified Eagle Medium

EC – Endothelial cell

ECC - Excitation contraction coupling

echo – Echocardiogram

ECM – Extra cellular matrix

EDV - End diastolic volume

EF - Ejection fraction

ELISA - Enzyme-linked immunosorbent assay

FBS - Foetal bovine serum

ERK – Extracellular signal-regulated kinases

ETC - Electron transport chain

FADH - Reduced from of flavin adenine dinucleotide

GWAS – Genome-wide association studies

H_2O_2 - Hydrogen peroxide

HDL – High density lipoproteins

HIC – High income countries

IHD – Ischemic heart disease

IL - Interleukin

JNK – C -jun N – terminal kinases

RAGE - Receptor for advanced glycation end products

LDL – Low density lipoproteins

LMIC – Low to middle income countries

LV – Left ventricular

LVEF – Left ventricular ejection fraction

LVOT - Left ventricular outflow tract

MAPK – Mitogen activated protein kinase

MCP – 1 – Monocyte chemoattractant protein

MHCα – Alpha myosin heavy chain

MHCβ – Beta myosin heavy chain

mtDNA - Mitochondrial DNA

MI – Myocardial infarction

MMP – Matrix metalloproteinase

MRI –Magnetic resonance imaging

NADP – Nicotinamide adenine dinucleotide phosphate

NCD – Non communicable disease

NCX - Sodium-calcium exchanger

NET – Neutrophil extracellular traps

NFKB - Nuclear factor kB

NO- Nitric oxide

NOS- Nitric-oxide synthase

NOX – NADPH oxidase

OS - Oxidative stress

Ox LDL – Oxidised low-density lipoprotein

PASP - Pulmonary arteriole systolic pressure

PCSK9 – Proprotein convertase subtilisin/kexin type 9

PET – Position emission tomography

PPAR - Peroxisome proliferator-activated receptor

ROS - Reactive oxygen species

RYR – Ryanodine receptor

sdLDL – Small dense LDL

SERCA – Sarcoendoplasmic reticulum Ca^{2+} ATPase

SMC – Smooth muscle cell

SNP – Single nucleotide polymorphism

SR – Sarcoplasmic reticulum

SV - Stroke volume

TASPE - Tricuspid annular plane systolic excursion

TGFβ - Transforming growth factor beta

TLR - Toll-like receptor

TMB - Tetramethyl-benzidine

TNF-a - Tumour necrosis factor alpha

VCAM - Vascular cell adhesion molecule

VSMC – Vascular smooth muscle cell

WT - Wildtype

XO- Xanthine oxidase

XOR - Xanthine oxidoreductase

Abstract

Coronary artery disease (CAD) is a chronic inflammatory condition associated with high rates of mortality worldwide. Pro-inflammatory cytokines such as tumour necrosis factor (TNFα) play a key role in CAD pathogenesis, though the degree to which TNFα is elevated, or alters cardiac function in CAD remains unclear. CAD is also associated with elevated levels of reactive oxygen species (ROS) in the heart, though the extent to which this is dependent on cytokines isn't known. This preliminary study sought to (1) quantify plasma TNFα levels in a CAD patient cohort then correlate those levels to measurements of systolic and diastolic cardiac function, and (2) establish whether clinically relevant concentrations of (TNFα) elevate intracellular oxidative stress.

The study was conducted in accordance with local and IRAS ethical approval (IRAS ID: 247341). Blood samples were taken from consenting patients scheduled for revascularisation surgery. TNFα levels were measured using a high-sensitivity ELISA kit. Measurements of cardiac function were extracted from patient clinical records. Fluorescent imaging was used to quantify levels of oxidative stress and cytokine signalling in H9C2 rat myoblasts treated with supraphysiological and clinically relevant concentrations of TNFα.

Average plasma TNFα levels were 2.27 ± 0.35 pg/ml (n = 66). Preliminary data indicates that E/A ratio negatively correlates with TNFα concentration (p = 0.0489; r2 = n = 45). TNFα levels may correlate with LVIDD, though this did not reach significance (p = 0.06; r2 = 0.09; n = 38). We observed no significant correlations with other indices of systolic and diastolic function including EF (p = 0.6993; r2 = 0.0031; n = 51) and E/e ratio (p = 0.3297; n = 30). We observed no significant increase in oxidative stress following treatment with an acute or clinically relevant concentration of TNFa, which were 50ng/ml and 2pg/ml, respectively.

These preliminary data suggest that plasma TNFα levels may be of limited use as a diagnostic biomarker in CAD. Furthermore, clinically relevant concentrations of TNFα do not appear to elevate levels of oxidative stress in our H9C2 rat myoblast model. However, further work should aim to increase study power, stratify patients according to potential confounding variables upon complete acquisition of clinical data, and investigate cellular mechanisms that link TNFα levels with whole heart dysfunction

1. Introduction

Cardiovascular diseases (CVDs) refer to a group of disorders involving the blood vessels and the heart. These include diseases of the vessels supplying the brain, heart, or peripheral areas of the body (arms and legs), or conditions that cause abnormal heart function/damage. CVDs are the leading cause of death globally; approximately 32% of global deaths were attributed to these diseases in 2019 (WHO, 2024).

1.1 CAD

Coronary artery disease (CAD) is the most prevalent CVD; it is a leading cause of mortality in developed countries and accounts for approximately 17 million deaths globally each year (Nichols et al, 2014). It is also referred to as ischemic heart disease (IHD) or atherosclerotic cardiovascular disease (ACD) (Khan et al., 2020). Endothelial dysfunction and the accumulation of atherosclerotic plaques within the coronary vessels supplying the heart underpins the pathological basis of CAD. Myocardial and cardiovascular complications can arise when vessels supplying blood to the heart become narrow/blocked, or when plaque rupture results in thrombus formation (Papageorgiou, 2016). These processes can manifest clinically as shortness of breath, angina, ischemic cardiomyopathy, myocardial infarction (MI), and stroke as a result of CAD (Papageorgiou, 2016).

1.1.1 Incidence and epidemiology

Growing incidence and mortality rates of cardiovascular diseases like CAD are expected to continue – a trend that can be attributed to multiple factors including the sustained prevalence of obesity and diabetes, as well as an aging global population (Khan, 2020). Global burden of cardiovascular disease is currently estimated to be around 620 million: approximately 290 million and 260 million of which are women and men, respectively (British Heart Foundation, 2023).

Epidemiological patterns vary considerably throughout the global populace. In low to middle-income countries (LMIC's), CVD's are responsible for a significant portion of disease related deaths which would have previously been associated with infectious disease. This

epidemiological transition to non-communicable disease (NCD) burden in such regions has been analogous with developments in trade, infrastructure, and commercialization over the past several decades (Khan, 2020). Even as the effects of NCD's like CVD (and associated comorbidities) are mitigated, current trends in population aging, obesity and sedentary lifestyles are expected to further drive rates of incidence and mortality (Vedanthan et al., 2014; Gaziano, 2010).

In developed or high-income countries (HIC's), a progressive age-adjusted decline in mortality rate has been observed despite an overall increase in total burden (Vedanthan et al., 2014). This suggests that population ageing and growth are preeminent drivers of CVD burden in HIC's, in particular (Roth et al., 2020). Approximately 80% of CVD-related mortality occurs over the age of 60 in such regions – a stark contrast to that seen in LMIC's, where only 42% of CVD-related deaths occur within the same demographic (Gaziano et al., 2010). The perceived decline in mortality rates in HIC's is associated with numerous aspects aimed to prevent CVD development and improve patient outcomes following diagnosis. Firstly, prompt surgical and pharmacological intervention has significantly reduced the case-fatality rate of acute myocardial infarction (AMI). Procedures including coronary artery bypass surgery (CABG), revascularisation, and catheterisation are examples of methods that have been utilised to treat AMI and chronic disease (Ford & Capewell, 2011). Likewise, a more comprehensive and easily accessible range of medical treatments are available to patients in HIC's; treatment options previously limited to a small number of antiarrhythmic agents, beta blockers, and nitrates expanded to include thrombolytic agents, angiotensin converting enzyme (ACE) inhibitors, statins, and numerous others (Ford & Capewell, 2011; Malach & Imperato, 2006). The introduction of guidelines to limit the development of risk factors amongst the population also provided a pragmatic approach to preventing the development of CAD and other CVD's (Ford & Capewell, 2011).

Around 200 million people are affected by CAD globally - approximately 110 and 80 million are men and women, respectively (British Heart Foundation, 2023). Although CAD is the single leading cause of mortality among higher income regions (Gaziano et al, 2010), LMIC's contribute more than half of the global mortality burden and over two thirds of Disability Adjusted Life Years (DALY's) (Ralapanawa & Sivakanesan 2021; Vedanthan et al., 2014). Latin America and the Middle East have recently seen the highest increase in rates of incidence. For

these reasons, CAD prevalence in LMIC's somewhat reflect incidence patterns previously seen in developed countries (Vedanthan et al., 2014). The median age of mortality from CAD among men in LMIC's was ten years lower in 2010 than that observed in HIC's; early onset acute coronary syndrome (ACS) (thought to be provoked by early accretion of detrimental heath behaviours), along with suboptimal healthcare and treatment appear to be important contributing factors (Vedanthan et al., 2014; Karthikeyan et al., 2007). A genetic predisposition of some populations within these geographical locations can also make individuals more susceptible to CAD-associated risk factors. In South Asia for example, premature development of metabolic syndrome now appears to play a substantial role in the rising incidence of acute myocardial infarction and diabetes (Eapen et al., 2009).

The long-term decline in CAD mortality rates in some HIC's (including areas of the United States and the UK) has recently transposed; in some cases, incidence has once again started to rise. It is understood that this latest change in trend has been facilitated by enduring political, social, and economic impediments with regards to disease prevention (Gupta & Wood, 2019). For example, the advantages of improved treatment and prevention strategies are not shared equally between communities from different economic and racial groups (Havranek et al, 2015). This subsequently alters the state of social determinants – the conditions in which people spend their lives – and heavily influences an individual's risk of developing CAD or associated comorbidities. These might include factors such as: environment and infrastructure; access to well financed, quality universal healthcare; whether an individual is living in poverty; access to facilities that help to promote healthy lifestyle practices such as physical activity; education and social support etc. Furthermore, the implementation of health-conscious policies, or lack thereof, can also influence the control of such risk factors and CAD prevalence (Havranek, 2015).

CAD also poses a substantial economic burden which is projected to increase. For example, national expenditure in the US is expected to increase 2.5-fold over a period of 20 years (from 2015 – 2035) from $126 billion (Khera et al., 2020). Such economic burden can be incurred through the cost of healthcare, social care, and productivity costs due to mortality and morbidity (Luengo-fernandez et al., 2023). In 2021, CAD cost health and social care systems throughout the European Union €30 billion, with a major portion of the costs attributed to hospital care and pharmaceutical outlay. Furthermore, it has been estimated 500,000 working

years were lost as a result of CAD mortality. After also considering the financial burden of informal care and working days lost, a total cost of round €77 billion to the EU economy was estimated in the same year – this represented over a quarter of the burden calculated for all CVDs. Given this considerable impact, the importance of combative public health policy is further highlighted; policies should be developed to help to alleviate this burden, particularly when directing research expenditure towards areas that have the potential to aid prevention, diagnosis, and treatment (Luengo-fernandez et al., 2023).

1.1.2 Aetiology and risk factors

As acknowledged, risk factors often facilitate the formation of atherosclerotic plaques and subsequent thrombotic events. The identification of risk factors associated with CAD development has been a fundamental aspect of understanding the disease and reducing the far-reaching effects. This is mainly due to the fact that management of these factors play a significant role in prevention, and that they offer insight when it comes to predicting the onset of disease (Hajar, 2017).

1.1.2.1 Non-modifiable risk factors

Non-modifiable risk factors describe those which cannot be controlled, and include age, sex, ethnicity, and genetics. Age is one of the most influential risk factors for most vascular diseases, including CAD. According to the American Heart Association's 2019 update, 25.4% of female and 31% of male patients over the age of 80 years had some degree of CAD. Associated risk and prevalence increase with age for both men and women (Fadah et al., 2022). This is typically due to the accumulation of sufficient vascular changes/damage as individuals get older; arterial stiffness, vessel inflammation, and sustained endothelial dysfunction will eventually lead to vascular remodelling and escalate the risk of occlusion/thrombosis over time (Lakatta & Levy, 2003).

The general consensus is that CAD and related events of myocardial infarction is more frequent among men than women (Soler & Ruiz, 2010). In 2021, the BHF reported that the death of 42,434 men and 23,145 women were attributable to CAD – 15,921 and 9,153 of which were a result of MI, respectively (BHF, 2023). Pathophysiological disparities between the sexes also exist. Although pathology is multifactorial, interaction with biological sex

characteristics can influence aspects of the disease. For example, the features of coronary plaques in women tend to make them more susceptible to plaque erosion, whilst those in men have a propensity to rupture. Oestrogen is thought to have a protective effect against plaque rupture as this phenomenon is particularly rare in younger women (Davies, 2000). Men and women - despite sharing most of the same risk factors for CAD and other CVD's - also appear to have different levels of sensitivity to certain determinants (Mehta et al., 2016). Presentation of hypertension or diabetes increases risk of myocardial infarction more so in women, whilst the protective effect of exercise is less potent for men (Yusuf et al., 2004). Although, other disparities appear to contribute to an enduring problem in the management and treatment of atherosclerotic cardiovascular disease in women. For young women in particular, hospitalisation and mortality rates from acute myocardial infarction have not sufficiently improved. This has been attributed to poorer management of risk factors, longer delays seeking medical care and treatment, worsened state of comorbidities at time of presentation, reduced guidance-directed medical therapy/administration of therapeutics, and inadequate representation in medical trials (Holtzman et al., 2023).

Ethnicity is another non-modifiable determinant that can considerably alter the risk of developing CAD. Despite worldwide prevalence of the disease, certain ethnic groups are more inclined to develop CAD at higher rates, particularly due to the influence of westernised lifestyles and associated modifiable risk factors. Populations most at risk of subsequent metabolic and vascular dysfunction include South Asian, Mexican-American, and those of Afro-Caribbean decent. For instance, age-standardised mortality rates from CAD in the UK are around 50% higher in South Asian men and women when compared to white/Europeans (Forouhi & Sattar, 2006). Moreover, analyses of men within this demographic – all with a similar Body Mass Index (BMI) - revealed a predisposition to associated risk factors when compared to white/European men: South Asians had elevated central adiposity (Yajnik, 2001), increased systolic blood pressure, increased fasting and post glucose-loading insulin levels, and dysfunctional high-density lipoprotein (HDL) levels (Forouhi & Sattar, 2006; McKeigue et al., 1991). Hispanic and Japanese populations are disproportionately burdened with these risk factors in a similar way to South Asians, and the development of metabolic syndrome is a commonality amongst these groups (Llabre et al., 2015). For African Americans, the prevalence of hypertension is amongst the highest in the world and is perhaps the most

influential fisk factor for developing CAD in this population (Carnethon et al., 2017; Mills et al., 2016). Whilst African Americans (along with other populations of Afro-Carribean decent) are especially burdened with some of the risk factors described above, the prevalence of lipid dysfunction is lower than that observed in Caucasians (Carnethon et al., 2017). Fundamentally, genetic factors appear to have a prevailing effect on metabolic profile, insulin resistance, diabetes, and hypertension in particular. Differences in CAD susceptibility between ethnic populations encapsulate the importance of genetic elements, and how these influence the effects of common modifiable risk factors on CAD risk (Forouhi & Sattar, 2006).

Many studies and clinical observations have highlighted the heritable nature of CAD. Familial studies and genome wide association studies (GWAS) have both suggested that the heritability of the disease can be as high as 50%. Numerous genes have been implicated that are thought to be involved with causal pathways, including: metabolism of low-density lipoprotein (LDL) and triglyceride-rich lipoproteins; inflammatory mechanisms; cellular proliferation and vascular remodelling; and nitric oxide (NO) signalling. Indeed, up to 60 genetic variants have been detected, although the majority of these have been associated with only marginal increases in CAD risk. Nonetheless, some have significant associations with increased risk, and approximately 20% of these variants are located near genes involved in the pathways described above (Khera & Kathiresan, 2017).

Familial studies have revealed that having at least one parent with premature CVD independently increases risk (Lloyd-Jones et al., 2004), and even more so in individuals with sibling CVD (Murabito et al., 2005). More specifically, hereditary hypercholesterolaemia has been identified in familial studies. Mutations in genes encoding LDL receptors (resulting in impaired hepatic uptake of LDL), apolipoprotein B, and PCSK9 (where loss of function prevents LDL from binding to its receptor) lead to elevated circulating cholesterol, thus facilitating early CAD development (Khera & Kathiresan, 2017; Soria et al., 1989; Moulin et al., 2003). Alongside this, GWAS have since proven to be useful in recognising common genetic variants with higher frequency among CAD cohorts. Single nucleotide polymorphisms (SNPs) within the 9p21 gene locus have been repeatedly implicated in CAD, for example. These mutations have been shown to disrupt transcription factor binding sites that are involved with inflammatory response in vascular cell types, therefore emphasizing a connection between genetic susceptibility and dysfunctional inflammatory signalling (Harismendy et al., 2011). Such studies might expose

new pathological mechanisms leading to disease development as well as novel therapeutic targets.

1.1.2.2 Modifiable risk factors

There are also numerous individual and modifiable risk factors. The INTERHEART study concluded that a number of these modifiable risk factors accounted for over 90% of attributable risk of MI within a population (Joseph et al., 2017; Yusuf et al., 2004). Those most commonly associated with CAD pathogenesis are hypertension, diabetes (or diabetes mellitus), dyslipidaemia, smoking, and obesity.

Firstly, the association between CAD and hypertension is robust. The pathological effect of hypertension is understood to help facilitate and exacerbate atherosclerosis; arterial hypertension exerts additional mechanical stress on the vascular endothelium as a result of increased transmural pressure, thus reducing endothelial integrity (Malakar et al., 2019; Stauer, 1992). Studies show that moderate increases in blood pressure – even within the normal range – accompany a marked increase in CAD risk. The extent to which hypertension might contribute to modifiable disease burden may have been overestimated, however. Only in individuals with well-established hypertension has a reduction in CAD risk been observed following blood pressure reduction (Joseph et al., 2017; Pfeffer et al., 2016). In a similar way, decreasing blood pressure though the use of beta blockers and ACE inhibitors has only reduced CAD risk in those already highly susceptible, or with confirmed vascular disease (Joseph et al., 2017).

Type two Diabetes Mellitus (or simply known type two diabetes) is often considered a modifiable risk factor, and is another significant determinant of CAD risk and associated patient outcomes. The aetiological role of diabetes overlaps with a number of other risk factors including hypertension, dyslipidaemia, and obesity. It is characterised by a reduced uptake of glucose as a result of insulin resistance or deficiency, and subsequent hyperglycaemia and dyslipidaemia (D'Souza et al., 2009). Multiple pathological process involved in CAD pathogenesis can be accelerated or worsened in the presence of diabetes, which can be attributed to this metabolic dysregulation. Hyperglycaemia is known to induce vascular damage by means of multiple pathways: excess glucose (and its metabolic products) can cause osmotic stress; increased oxidative stress due to the accumulation of oxidising

molecules such as NADP+; and hypoxia in the vascular endothelium (Beckman et al., 2002). Increased transcription of pro-inflammatory cytokines (Du et al., 2000) and supressed nitric oxide (NO) production has also been observed as a result of elevated glucose flux (Du et al., 2001). Insulin resistance and subsequent hyperglycaemia can propagate further production of insulin, giving rise to hyperinsulinemia - which can independently impact CAD severity (D'Souza et al., 2009). In a similar way to hyperglycaemia, hyperinsulinemia provokes atherogenic effects such as increased production of inflammatory mediators and vascular smooth muscle cell (VSMC) proliferation (D'Souza et al., 2009; Hsueh & Law, 1999). The risk of mortality from CAD and other types of heart disease is 2-4 times higher in diabetic patients; a similarly increased risk was also seen following MI (Aronson & Edelman, 2014).

One of the preeminent features of atherosclerosis is lipid accumulation within the arterial wall, and so lipid dysfunction (or dyslipidaemia) is a potent risk factor for CAD. Current estimates suggest around 50% of the adult population has some form of dyslipidaemia (Hedayatnia et al., 2020; Joshi et al., 2014). The term encompasses various aetiological elements of lipid dysregulation such as hyperlipidaemia, hypercholesterolaemia, low HDL, and hypertriglyceridemia (Abd alamir et al., 2018). Two types of cholesterol – HDL and LDL – have paradoxical roles in CVD development. HDL's have a protective role in CAD and function to transport excess cholesterol to the liver (Rahmati-Ahmadabad et al., 2019). These lipids also have antioxidative and anti-inflammatory effects. Therefore, HDL deficiency can result in elevated levels of triglyceride-rich lipoproteins and LDLs which increase the likelihood of atherogenesis (Rosenson et al., 2015). Limited clearance of triglyceride-rich lipoproteins and LDL's is detrimental, particularly with regards to small dense low-density lipoproteins (sdLDL's), as these are more susceptible to oxidation and accumulate within the vessel wall (Yokota et al., 2016). Studies have demonstrated that various forms of dyslipidaemia independently increase the prevalence of multivessel CAD (whereby at least two coronary arteries are involved), even following multivariate adjustment for other risk factors (Abd alamir et al., 2018).

Over the past several decades, mean population BMI (and obesity) has steadily increased around the world. Around 11% of men and 15% of women are classified as obese (BMI >30) and in some places such as the United Kingdom, this figure exceeds 30% of the adult population (Di Cesare et al., 2019). As well as playing an integral aetiological role in the

development of risk factors described above (Flint et al., 2010), obesity/adiposity may independently contribute to CAD. Evidence suggests that adipocyte-derived inflammatory mediators contribute to a state of systemic inflammation that may perpetuate atherosclerosis. Reduced expression of cardio-protective adipokines has also been observed in obesity, which would usually exert beneficial effects via anti-inflammatory mechanisms (Berg & Scherer, 2005). Central or visceral adiposity is an important criterion where metabolic syndrome and cardiovascular disease are concerned. Prominent long-term studies have demonstrated that the degree of central obesity reflects the risk of cardiovascular disease in a linear fashion (Kannel et al., 1991), and BMI-determined obesity resulted in higher incidence of CVD (Wilson et al., 2002).

Whilst smoking is one of the most potent risk factors associated with CAD, it is also one of the most preventable. Despite a continuing decline in consumption over the past several decades, around 10% of CVD related deaths are still attributable to smoking (Messner & Bernhard, 2014; Machii & Saika, 2012). A dose-dependent relationship exists between the detrimental effects of smoking and CVD prevalence; the number of cigarettes smoked daily/smoking duration has a profound impact on atherogenesis and degree of vascular occlusion (Malakar et al, 2019). The chemical constituents of tobacco smoke induce various maladaptation's within vascular endothelial cells, including increased expression of adhesion molecules which encourages adherence of activated macrophages and platelets to the vessel wall; decreased NO availability, which is involved in the regulation of vascular tone; smooth muscle cell (SMC) proliferation and vascular remodelling; and activation of inflammatory processes. Smoking cessation is vital in preventing mortality from atherosclerotic disease and reversing existing damage (Messner & Bernhard, 2014).

1.1.3 Pathology

Structurally, most blood vessels (including larger vessels, venules, and arterioles) are comprised of several layers (Figure 1-1). The endothelium is the luminally-facing monolayer of endothelial cells (EC) and acts as the initial barrier between the blood and surrounding tissues. This, together with a subendothelial layer of collagen, forms the intima. Surrounding this is the media, which is a band of tissue consisting of SMCs, collagen, and elastin fibres. Finally, a dense matrix of connective tissue forms the adventitia, which is the outermost layer

of the vessel wall. The endothelium in particular plays a critical role in vascular homeostasis; it is sensitive to haemodynamic and chemical changes in the blood, and acts as a signal transducer to affect outer layers of the vessel wall. In vascular disease, excessive mechanical stress exerted on the vessel wall (usually a result of hypertension) and high concentrations of circulating metabolic factors are known to be significant effecters of atherogenesis and progression (Zhang et al., 2009; Jebari-Benslaiman et al., 2022).

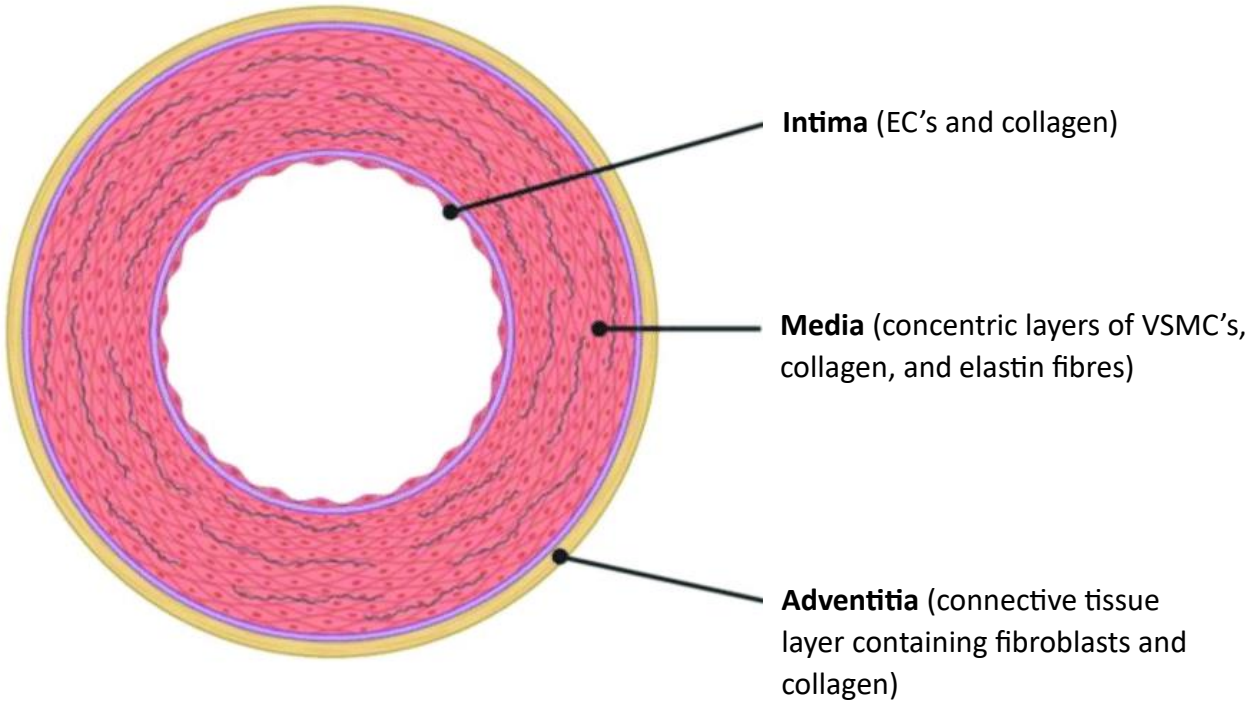

Figure 1-1. Cross-sectional view of the arterial wall. Schematic showing the three layers of the arterial wall: the Intima, Media, and Adventitia. Atherosclerosis typically initiates and develops within the Intima. Adapted from (González-Clemente et al., 2021).

Vascular lesion development is multifactorial, with a wide range of factors impacting the biochemical and pathological mechanisms involved in atherosclerosis (Jinnouchi et al., 2019). The process begins with endothelial activation/dysfunction, then continues with fatty streak formation, fibrous plaque formation, and ultimately plaque rupture (Jebari-Benslaiman et al., 2022).

1.1.3.1 Atherosclerosis

Areas of the vessel wall that are exposed to turbulent blood flow are more susceptible to endothelial dysfunction and lesion development. This is due to the fact that mechanical stress in these locations will more likely compromise endothelial integrity, and circulating LDL will remain in these areas for longer periods of time (Jebari-Benslaiman et al., 2022). Subsequent endothelial damage and LDL infiltration are known to promote EC activation, along with increased expression of adhesion molecules. LDLs then begin to accumulate within the intima. Here, LDLs are susceptible to oxidation by free radicals (such as superoxide), and the formation of oxidised LDLs (oxLDL's) promote monocyte migration into the vessel wall. The monocytes then differentiate into macrophages that engulf the oxLDLs. These lipid-filled cells are known as foam cells from which increased cytokine expression initiates further recruitment and accumulation of inflammatory cells within the intima (Fatkhullina et al., 2016). This process forms the basis for fatty streak formation, and later fibrous plaque development. It is also important to note the role of the inflammatory response involved in atherogenesis; exposure to inflammatory agents such as TNFα and IL-1 (among others) contribute to sustained phenotypic modulation of the vessel wall, which further enhances conditions for plaque development. Thus, a positive feedback loop between unresolved inflammation and foam cell accumulation exists to exacerbate the state of disease (Jebari-Benslaiman et al., 2022).

Advanced stage atherosclerosis is characterised by the formation of a necrotic core within the plaque, followed by the development of a fibrous cap. The growing number of foam cells remain internalised within the vessel wall and eventually undergo necrotic cell death *in situ* (Jebari-Benslaiman et al., 2022). The resulting lipid-rich region is known as the necrotic core – this tends to destabilise the relatively secure structure of the plaque, where it becomes susceptible to rupture and thrombus formation. To reduce the potential exposure of necrotic material, a subendothelial layer of migrated VSMCs and extra cellular matrix forms a 'fibrous cap' on the luminal side of the necrotic core. These derivative cells, however, no longer express the contractile phenotype of the SMCs located in the media. Instead, they exhibit a phenotype that elicits cellular proliferation and migration in response to vascular injury and the secretion of various growth factors from adjacent foam cells retained in the intima or media. Although this may restore plaque stability – depending on fibrous cap/extra cellular

matrix (ECM) composition – this process contributes to the development and occlusive nature of the lesion (Jebari-Benslaiman et al., 2022). Along with sustained exposure to a pro-atherogenic environment, plaques with a large necrotic core and a thin fibrous cap are most vulnerable to rupture (Jebari-Benslaiman et al., 2022; Stefanadis et al., 2017).

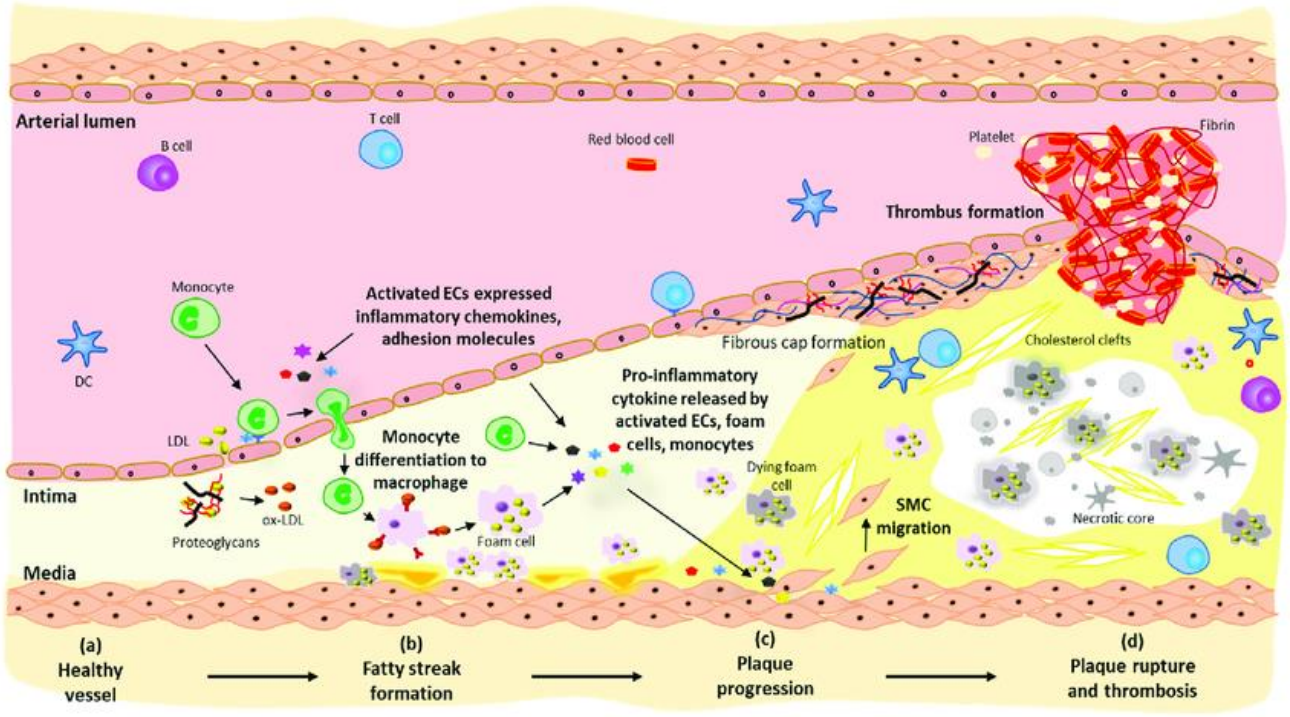

Figure 1-2. Endothelial pathology of atherosclerosis. (a) Normal arterial structure with uncompromised endothelium. (b) Endothelial activation and dysfunction in response to stress and other associated risk factors allowing the infiltration of lipids and monocytes, which is facilitated by adhesion molecules, chemokines, and pro-inflammatory cytokines. Phagocytosis of oxidised lipids leads to the formation of foam cells – the basis of fatty streak formation. (c) Subsequent further release of cytokines and growth factors promote proliferation and migration of vascular SMCs, which then secrete large amounts of collagen and other ECM components. This forms the 'fibrous cap' which lies between the endothelium and necrotic core. (d) A continuation of these processes and the release of metalloproteinases (MMP's) leads to a thinning fibrous cap, and eventually plaque rupture. Exposure of pro-thrombotic material to the circulation results in thrombus formation (Nguyen et al., 2022).

1.1.3.2 Consequences of plaque formation

Plaque rupture, erosion, and calcified nodule are three typical mechanisms of coronary thrombus. Plaque rupture occurs when pro-thrombotic material within the necrotic core is exposed to blood flow through the fibrous cap, leading to luminal thrombus formation at the site of the plaque. Although the cause of rupture has not been fully elucidated, it has been shown that macrophage infiltration – and their secretion of various collagenases – may disrupt the continuity of the of the fibrous cap (Jinnouchi et al., 2019; Dollery et al., 2003). Where the necrotic core is exposed, platelets, fibrin, and red blood cells aggregate and can lead to partial or total occlusion of the vessel (Jinnouchi et al., 2019).

In contrast to the mechanisms and plaque characteristics involved in rupture, plaques that are prone to erosion have different phenotypic qualities. For instance, these tend to have a small or absent necrotic core, instead consisting mainly of proliferative VSMC's with extensive ECM beneath the endothelium. As the plaque develops, cytokines and other pro-inflammatory factors induce endothelial desquamation; the endothelium is eroded, leaving ECM components (such as proteoglycans and collagen) and VSMC's exposed to the circulation (Baaten et al., 2023). In addition, the blood is also exposed to neutrophil extracellular traps (NETs). NETs are comprised of cellular material (DNA, cytokines, pro-oxidant material) left from neutrophil cell death to which platelets adhere and aggregate. This facilitates thrombus formation which may settle and develop distal to the atheroma. According to Fahed & Jang (2021), acute coronary syndrome that occurs as a result of plaque erosion is most prevalent in younger (average 53 years) female patients. These patients also tend to have low-to-moderate risk lesions and less complex disease (Fahed & Jang, 2021). These findings, however, may vary in different patient cohorts.

Least common are calcified nodules (CN). Patients that seem to be particularly susceptible to these lesions are older, diabetic, or present with chronic kidney disease. This type of thrombus also only occurs in the context of extensive calcified CAD (Jinnouchi et al., 2019). The mechanisms involved in CN development remain unclear, however CN thrombi are characterised by an erupted calcific nodule through the endothelium or a thin fibrous cap. From here, a similar pattern of platelet aggregation/accumulation of thrombotic material occurs (Torii et al., 2019).

Sufficient coronary perfusion is absolutely necessary for physiological functioning, and this should be maintained when oxygen demand is increased. A significant pathological mechanism in CAD that induces myocardial injury is ischemia, which occurs when blood flow is interrupted or stopped completely (ischemic) as a result of occlusive plaques or thrombi, leaving areas of the myocardium without oxygen (hypoxic). This causes an imbalance between myocardial oxygen supply and demand. Myocardial ischemia (and subsequent hypoxia) can occur both in the circumstance of stable CAD and ACS (Smit et al., 2020). With the former, ischemia occurs as a consequence of reduced coronary flow reserve. This refers to the capacity for coronary perfusion and is defined as the difference between basal coronary flow and maximal coronary flow. When atherosclerosis causes luminal narrowing, increased hydrodynamic resistance will abate blood flow and therefore flow reserve. Coupled with endothelial dysfunction and inadequate vasodilation, this decrease in flow reserve will increase the potential for myocardial injury (Smit et al, 2020; Crossman, 2004).

Acute coronary syndrome describes the following complications of advanced atherosclerosis: unstable angina, myocardial infarction, and sudden coronary death (Jinnouchi et al., 2019). Unstable angina is experienced as a result of highly occluded vessels; here, the oxygen demand of the myocardium is not sufficiently met by the supply of blood, even at rest. Myocardial infarction most commonly occurs as a result of superimposed luminal thrombus – a result of plaque rupture -, but can rarely arise through coronary embolism, thrombosis within non-diseased vessels, or coronary spasm (Burke & Virmani, 2007). With regards to sudden coronary death, around 40-50% of cases are a result of significant narrowing of the lumen (at least 75% reduction in cross-sectional area) with stable coronary plaques. The rest, however, are usually a consequence of luminal/coronary thrombus (Jinnouchi et al., 2019).

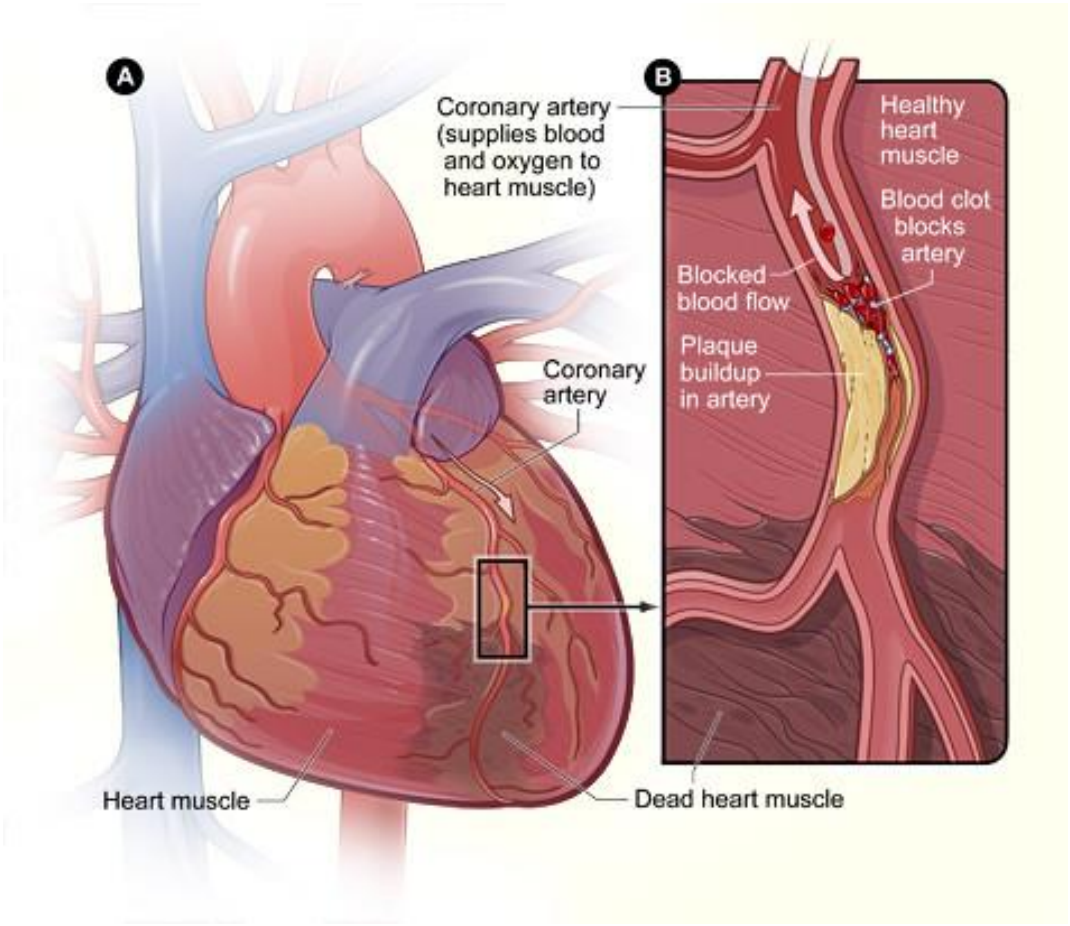

Figure 1-3. Acute myocardial infarction. (a) Myocardial ischemia and hypoxia will result in injury or death of the tissue supplied by the occluded vessel. The extent of infarct zone will depend on the location of the blockage: blockages that occur near the apex may result in minimal injury, whereas blockages closer to the aortic arch will result in a more extensive infarct zone, and potential fatality. (b) Mature atherosclerotic plaque and thrombus formation. Image (NHS, 2022).

1.1.3.3 Cardiac remodelling

In response to ischemic injury and other CAD-associated factors (haemodynamic and metabolic changes, inflammation) the myocardium undergoes structural and functional modification. To begin with, these regional and global alterations may serve as compensatory mechanisms. However, sustained exposure to the aforementioned factors will perpetuate these changes until they become pathogenic and induce myocardial dysfunction. This process is known as cardiac remodelling (Heusch et al., 2014). Vascular stenosis and hypertension can facilitate concentric remodelling of the myocardium; this usually presents as heart failure with preserved ejection fraction (HFpEF) (Heusch et al., 2014). Despite the fact that EF is maintained, ultrastructural remodelling in this condition is characterised by an increased ventricular mass to volume ratio – the outcome being increased ventricular stiffness and

impaired diastolic relaxation (Ohtani et al., 2012). Heart failure with reduced ejection fraction (HFrEF), on the other hand, is often characterised by increased end diastolic volume to wall thickness ratio (chamber dilation). Ventricular dilation is indicative of impaired contractility. HFrEF can arise in response to diastolic dysfunction, recurrent infarcts, and neurohumoral activation (ie sympathetic nervous system and renin-angiotensin-aldosterone system) – all of which cause or exacerbate cardiac injury (McMurray et al., 2012). Furthermore, ventricular failure, pulmonary hypertension, and valvular malfunction can lead onto atrial remodelling and associated arrhythmias like atrial fibrillation. In each of these conditions where either myocardial stiffness or impaired contractility is the source of failure, pathological reorganisation of the vessels and individual cardiomyocytes is the underlying mechanism (Heusch et al., 2014). This is inevitably based on intrinsic changes taking place on a cellular level that undermine the global function of the heart.

Cardiomyocytes undergo various significant changes in disease that might impair the heart's capacity for relaxation and contraction. Geometric changes within the myocardium are, in part, due to a divergence from physiological morphology of cardiomyocytes. For instance, cellular hypertrophy occurs in response to haemodynamic overload via the activation of specific signalling pathways. In brief, volume overload may induce 'eccentric' hypertrophy; longitudinal addition of sarcomeres gives the cell an elongated appearance, which translates to the ventricular dilation seen in HFrEF. Pressure overload can likewise lead to systolic wall stress and produce the concentric hypertrophy typical of HFpEF. Here, lateral sarcomere extension will result in ventricular wall thickening and impaired relaxation (Figure 1-4) (Hartupee & Mann, 2016; van Berlo et al., 2013).

Altered gene expression can be observed in hypertrophy that could upset the balance of excitation and contraction within the heart. An example of this is a reversion to a foetal or neonatal phenotype: increased expression of beta-myosin heavy chain (β-MHC) along with decreased alpha-myosin (α-MHC) heavy chain; and decreased SERCA2 expression (an ATPase associated with the sarcoplasmic reticulum, which is involved in the regulation of intracellular calcium) (Bers, 2001). Changes in expression of regulatory myofilament proteins has also been highlighted. It is likely that each of these mechanisms contribute to sarcomere disarray and a subsequent reduction in contractile function (Hartupee & Mann, 2016).

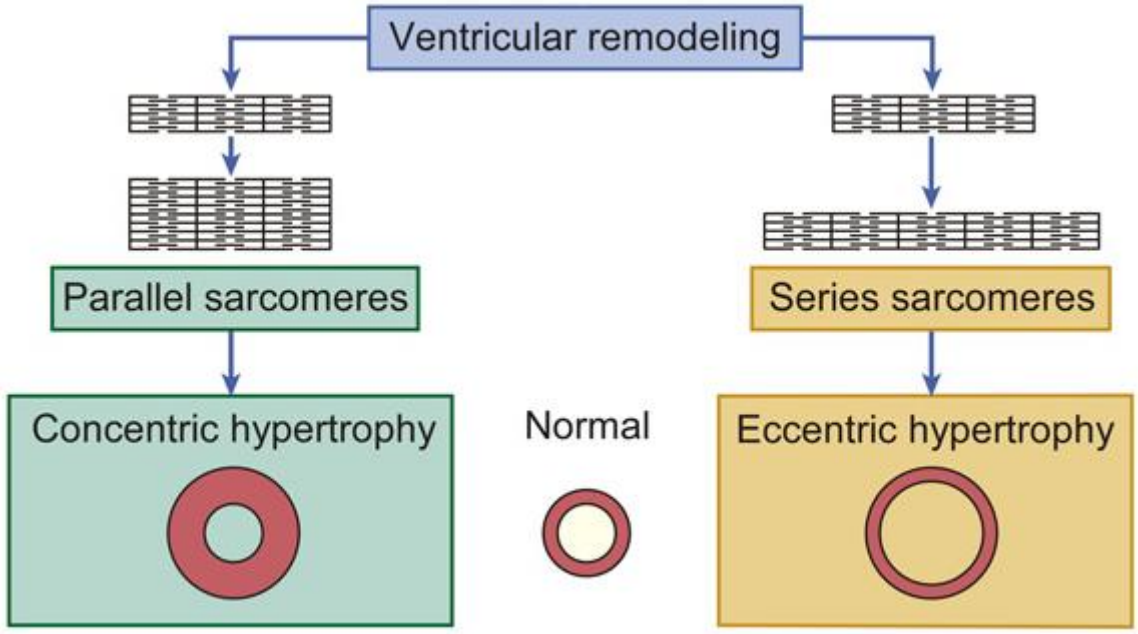

Figure 1-4. Ventricular remodelling. Pressure overload – a response to stenosis or hypertension – causes an increase in systolic wall stress, leading to widening of ventricular cardiomyocytes through the parallel addition of sarcomeres. This concentric remodelling is characteristic of HFpEF. Likewise, volume overload leads to an increase in diastolic wall stress, series addition of sarcomeres and cell lengthening; this ventricular dilation is characteristic of the eccentric remodelling seen in HFrEF. (Hartupee & Mann, 2016).

Signalling pathways involved in this type of remodelling are extensive and complex, but a particular set of pathways that should be highlighted are the mitogen-activated protein kinase (MAPK) pathways. More specifically, the ERK (extracellular regulated protein kinase) pathways, JNKs (C-Jun N-terminal kinases), and the P38 MAPK. These act to phosphorylate various substrates within the cascade, some of which are nuclear transcription factors; activation of some of these genes are early indicators of hypertrophy such as C-Jun and BNP (expressed in response to stretching caused by volume overload) (Bers, 2001). The clinical manifestations of the cardiac remodelling discussed here will be outlined in sections 1.1.4 and 1.1.5.

1.1.3.4 Changes to cardiac contractility

The collective process, from the generation of an action potential to contraction and relaxation within the cardiomyocyte, is known as excitation contraction coupling (ECC). Calcium homeostasis plays an integral role in this process, where calcium release, recapture, and storage facilitate physiological cardiac function. Disruption in calcium handling, therefor, will often result in cardiac dysfunction (Wang et al., 2020). Here, a concise overview of the events involved in this process will be provided.

ECC begins with a wave of excitation along the sarcolemmal membrane, thus depolarisation of the transverse tubules (T-tubules). This is detected by L-type calcium channels which then open. The resulting influx of calcium into the cytoplasm activates proximal calcium-gated calcium channels called ryanodine receptors (RyRs) (Bers. 2001). These are associated with the membrane of the sarcoplasmic reticulum (SR), which is an intracellular calcium store. More calcium is then released from the SR into the cytosol - a process that is termed calcium-induced calcium release (CICR). Thus, cytoplasmic Ca^{2+} levels increase and bind troponin, activating cross-bridge cycling thence myocyte shortening (Bers, 2001). In order for relaxation to take place, the diastolic Ca^{2+} concentrations must be restored. This is facilitated via four calcium transport systems: SR Ca-ATPase (SERCA), which mediates calcium reuptake back into the SR; Na/Ca exchanger (NCX) and sarcolemmal-ATPase, both of which are responsible for calcium efflux into the extracellular space; and the mitochondrial import system (Figure 1-5) (Bers, 2001).

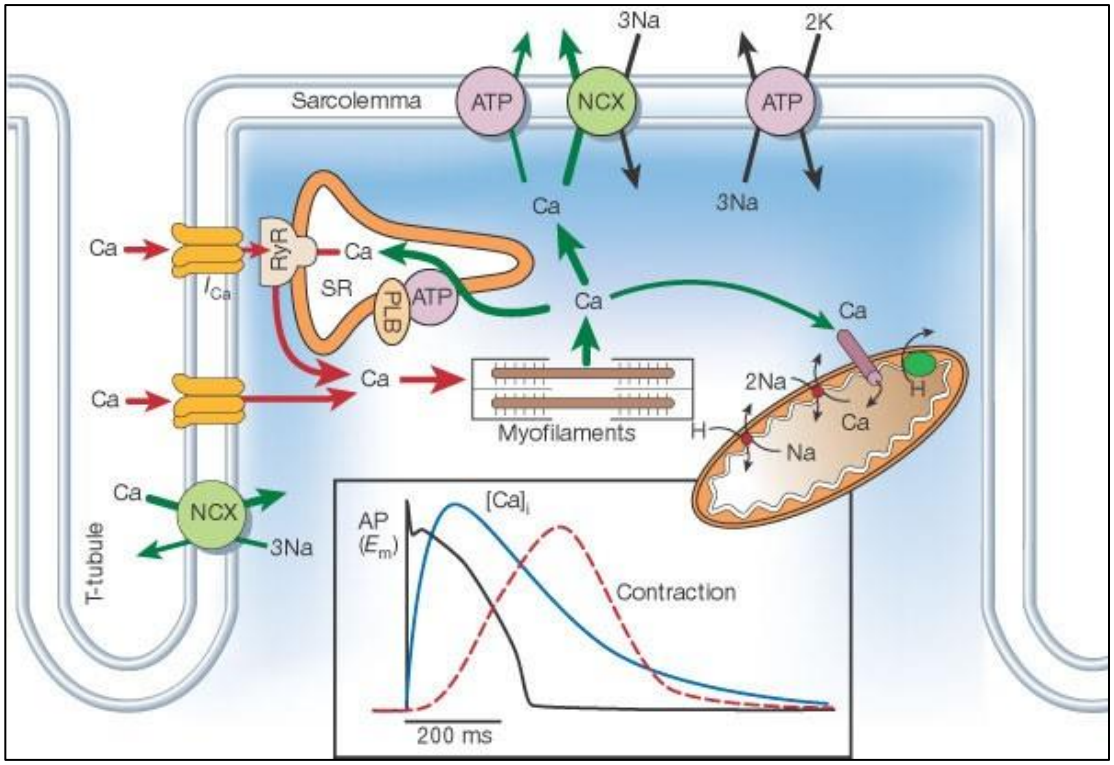

Figure 1-5. Cardiomyocyte ECC and Ca handling. Also illustrated is the relative timeframe of action potential generation, changes in intracellular Ca^{2+} (via Ca^{2+} transient amplitude), and myocyte contraction. Adapted from (Bers, 2002). Abbreviations: NCX – Na/Ca exchanger; ATP – ATPase; SR – sarcoplasmic reticulum; RyR – ryanodine receptor; PLB – phospholamban.

There are a number of ways that dysfunctional calcium cycling might lead to reduced ventricular function, such as that seen following ischemic injury. However, a focal point of this dysregulation seems to be the SR. Reduced SR calcium load has been considered the predominant cause of reduced Ca^{2+} transient amplitude, prolonged Ca^{2+} transient duration and transient decay. Firstly, diastolic SR Ca^{2+} leak has been recognised in HF (Marks, 2013). This leakage is mediated by ryanodine receptor 2 (RyR2) under chronic hyperadrenergic stimulation, which prompts stress induced RyR2 oxidation and depletion of a channel complex protein called calstabin2. Normally, said channel complex helps to stabilise the RyR2 channel whilst in a closed state. When the complex is deficient in calstabin2, the channels become sensitive to diastolic calcium levels; erroneous CICR from the SR during diastole depletes SR Ca^{2+} stores and activates further Ca^{2+} influx (Marks, 2013). The effects of "leaky" RyR2 may be amplified with reduced expression of the SR CaATPase (SERCA2), or persistent inhibition of SERCA2 by phospholamban, which is a protein that modulates SERCA2 Ca^{2+} sequestration. In

both cases, SR Ca^{2+} reuptake will be reduced, and SR calcium load will be diminished (Marks, 2013; Lompré et al., 2010).

Reduced ATP production that is observed in the failing myocardium can be attributed to alterations in cardiac energy metabolism. The decrease in ATP output is synonymous with a decrease in the mitochondria's capacity to oxidise carbohydrates and fatty acids. Instead, a compensative rise in glucose uptake – and glycolysis as a means of metabolising this energy substrate – often results in increased production of protons and lactate, which harm cardiac efficiency (Heusch et al, 2014; Lopaschuk et al., 2010). This dependence on glycolysis when oxidative metabolism is reduced is also accompanied by adoption of a foetal phenotype. This is characterised, for example, by reduced expression of metabolic enzymes such as peroxisome proliferator-activated receptor (PPAR) (α) and PPAR (ϒ), which participate in the regulation of mitochondrial biogenesis and expression of enzymes involved in fatty acid oxidation (Heusch et al., 2014; Finck & Kelly, 2007).

1.1.4 Clinical presentation and diagnosis of CAD

Diagnosis of CAD usually begins when a patient presents with one or more associated symptoms. The most common initial symptom – which typically presents in approximately 50% of patients (Kannel, & Feinleib 1972) – is angina. This usually manifests as sternal discomfort or pressure lasting several minutes, and may be accompanied with pain in the shoulder, jaw, or arm. For stable angina, this pain typically occurs following physical effort or psychological stress when at least one major coronary vessel is obstructed. Symptom severity will depend on the degree of occlusion within the vessel and how may major vessels are affected. Unstable angina will occur with mild exertion or at rest (Cassar et al., 2009). Other symptoms may be experienced instead or alongside angina including epigastric pain, fatigue, and dyspnoea (breathlessness), or the patient may even be asymptomatic (Cassar et al., 2009).

Initial examination by a physician will not likely lead to an immediate diagnosis, particularly in patients where symptoms are not severe such as cases with stable angina. However, patients will usually be assessed to determine the presence of risk factors like those previously discussed, comorbidities such as chronic lung disease, evidence of peripheral vascular disease, and a family history of CAD (Cassar et al., 2009). Based on this risk factor assessment, further

investigation may be prudent before a comprehensive treatment strategy is put in place. A series of non-invasive tests can be carried out to confirm angina, and therefore a clinical diagnosis of CAD (Cassar et al., 2009). Electrocardiography (ECG) performed at rest is perhaps the most common procedure to use during initial investigations, and a diagnosis is usually made if there is evidence of ST-T wave abnormality or LV dysfunction. Although findings may appear normal for many patients with stable angina, the test has significant prognostic value and predictability for patient outcomes, nonetheless (Cassar et al., 2009). Exercise testing with ECG can likewise be used for such individuals. Non-invasive imaging may otherwise be suitable for individuals that are unable to exercise, or previous testing has not provided sufficient information to make a diagnosis; adenosine perfusion imaging, positron emission tomography (PET), computed tomography (CT), and magnetic resonance imaging (MRI) are used depending on the clinical circumstances and laboratory expertise. Additional - albeit more invasive - techniques can be used to give definitive answers as to whether atherosclerotic disease is present, the magnitude, and location of plaques. Coronary angiography is an example that might be applied for investigation in high-risk patients (Cassar et al., 2009).

1.1.5 Echocardiological diagnosis and treatment of CAD

1.1.5.1 Echocardiography

Of particular interest in this study is the use of echocardiography (or echo) to assess potential vascular and myocardial abnormalities. Echocardiography can provide a comprehensive, anatomical picture of the heart's structure and function. It has remained an elemental part cardiovascular evaluation since its inception in the late 1970's (Anavekar & Oh, 2009; Holen & Simonsen, 1979; Hatle et al., 1978). 2D echocardiography in conjunction with Doppler imaging can be used for the appraisal of systolic and diastolic function and can provide insight into the haemodynamic workings of the heart (Anavekar & Oh, 2009). In essence, cross-sectional images of the myocardium are virtually produced from flow data that is generated based on the backscattering and compression of soundwaves (ultrasound) (Otto, 2024). A transducer is used to emit these high-frequency sound waves that will refract from structural components of the heart or vessels (NHS, 2022).

2D imaging produces a real-time view of structural components in cross-sectional tomographic slices (Faletra et al., 2018). Parameters are typically based on measurements of

contraction, and volumes; Left ventricular wall thickness, potential wall motion abnormalities, and LV ejection fraction can be identified with 2D imaging (Klaeboe & Edvardsen, 2018).

Doppler was originally developed to identify the velocity, direction, and flow characteristics of blood within the vessels and heart. Subsequent evolution of the technique to Doppler imaging is now an effective way to determine the velocity and direction of myocardial tissue as well as blood, based on the frequency shift (Δf) of these moving targets (Anavekar & Oh, 2009).

1.1.5.2 Assessment of systolic function

Analysis of systolic function has a vital impact on diagnosis, prognosis, and treatment strategy; left ventricular systolic function is usually the primary focus here, when evaluated with echocardiography (Cameli et al., 2015). There are numerous parameters that are relied upon to provide a thorough evaluation of LV systolic function. One of the most popular is the left ventricular ejection fraction (LVEF). LVEF is defined as the ratio of stroke volume (SV) – which is the difference between end-systolic and end-diastolic volumes – and the end-diastolic volume. This allows a basic assessment of the hearts 'pump function' and is expressed as a percentage. As per the American Society of Echocardiography: LVEF>55% is considered 'normal' (Appendix E); measurements ranging from 45 - 54% represent mild systolic dysfunction; 30 – 44% represent moderate systolic dysfunction; and LVEF < 30% is categorised as severe (Cameli et al., 2016; Lang et al., 2006).

Despite improvements in long term prognosis for patients with left ventricular systolic dysfunction, hospitalisation for this type of heart failure remains an important predictor of mortality. In a report based on the Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure (OPTIMIZE-HF), the risk of death during initial hospitalisation was higher in patients with LV systolic dysfunction when compared to those with preserved LV systolic function (Flaherty et al., 2009; Fonarow et al., 2007). Also, in patients with CAD and systolic dysfunction, the extent of angiographic burden has been shown to reflect prognosis and long-term survival (Gheorghiade et al., 2006; Felker et al., 2002).

1.1.5.3 Assessment of diastolic function

Diastolic dysfunction, on the other hand, is characterised by abnormal/impaired myocardial relaxation or increased filling pressures. Normally, the elastic recoil action of the myocardium allows ventricular relaxation and creates a positive trans mitral pressure gradient; around 80% of ventricular filling occurs during this early phase of diastole. As the ventricle fills with blood, ventricular pressure momentarily exceeds atrial pressure, and blood flow through the mitral valve decreases. The positive pressure gradient is then restored following atrial contraction, and the final 15-20% of ventricular filling occurs during late phase diastole (Anavekar & Oh, 2009). In the same way that ventricular contraction can be impaired, pathological events and mechanisms can lead to diastolic abnormalities such as: ventricular fibrosis and pericardial constriction; increased filling pressures and slower rate of ventricular filling. Further complications such as valvular malfunction, pulmonary venous congestion (Tschöpe & Lam, 2012), and ultimately low cardiac output will likely occur.

Due to the complexity involved in the evaluation of diastolic function, no single parameter holds particular significance when making a diagnosis; instead, a case for grading diastolic function is built depending on multiple parameters (Morrissey, 2016). For example, Mitral valve inflow patterns can be determined based on parameters like peak E wave (early phase) and peak A wave (late phase) velocity, E/A ratio, and also mitral valve deceleration time. Likewise, filling pressures might be alluded to by measuring parameters such as TR velocity and E/e ratio (Morrissey, 2016). Grading of diastolic dysfunction is based on conclusions drawn from the observation of such parameters (as per Table 1-1) (Kossaify & Nasr, 2019; Nagueh et al., 2016). Guidelines for appropriate grading state that patients must first be evaluated to ascertain whether LVEF is preserved or affected. Different algorithms for the interpretation of parameters need to be followed in each case to establish pressure status and grade (Morrisey, 2016; Nagueh et al., 2016).

Table 1-1. Grading of diastolic dysfunction.

Grade	Echocardiographic observation
Grade I	Impaired relaxation and subsequent reduced suction of the ventricle during early phase filling
Grade II	Increased stiffness of the ventricle, and pseudonormalisation of transmitral flow patterns can be seen as a result of elevated filling pressures
Grade III	Restrictive filling and significantly reduced compliance of the ventricle, along with elevated filling pressures

1.1.6 Pathological mediators of CAD

In reality, the processes involved in both vascular and myocardial remodelling which ultimately leads to dysfunction are more extensive and complex than is described here. However, a key mediator of CAD that drives many of these pathobiological aspects is inflammation. Inflammation plays a central role in the development of CAD and other cardiovascular diseases, on both local and systemic levels. So much so that inflammatory biomarkers have been shown to be predictive of CVD's. Conditions including previously described risk factors, pathogenic infection, autoimmune disease, and phycological stress can promote endothelial damage (Henein et al., 2022; Grover-Páez & Zavalza-Gómez, 2009); A maladaptive response can often result in chronic inflammatory activation that leads to atherogenesis/plaque development.

All phases of CAD development involve an elaborate network including innate and adaptive immune response. The activity – whether that be pro- or anti-inflammatory – of protein mediators and immune cells is modulated in a way that can either propagate or mitigate disease development (Jebari-Benslaiman et al., 2022). For example, phenotypic differentiation of macrophages - which may exert opposing effects - is dependent on the presence of other immune cells or inflammatory mediators: T helper (Th) cells generally influence differentiation into the M1 phenotype, which is pro-inflammatory/pro-atherogenic;

T regulatory (Treg) cells, however, induce the M2 phenotype, which is 'resolving' or anti-inflammatory (Tabas & Lichtman, 2017). In the former context, the occurrence of various other pro-atherogenic processes may also contribute to the inflammatory environment (Henein et al., 2022; Wesseling et al., 2018) to influence the progression of disease.

Cytokines are an integral part of inflammatory signalling in CAD. Epithelial cells undergo a functional reprogramming when exposed to cytokines, particularly during chronic inflammation. These proteins are generated by components of the inflammatory network such as T-lymphocytes, dendritic cells, macrophages, as well as SMCs and ECs. Pro-inflammatory cytokines in high concentrations can downregulate nitric oxide synthase, induce oxidative stress (OS), and provoke EC apoptosis (Kofler et al., 2005). One of the many ways that pro-inflammatory cytokines (such as TNFα and IL-1b) might promote atherogenesis is through induced expression of adhesion molecules ((VCAM)-1 – which is a cell adhesion molecule-, and intracellular adhesion molecule (ICAM)-1) within the activated endothelium. These cytokines can also induce hepatic production of C-reactive protein, and the activation of LOX-1 (an endothelial transmembrane receptor for oxLDL) (Zhang, 2008). Each of these contribute to further lipid accumulation, recruitment/infiltration of circulating leukocytes, and therefore foam cell formation (Henein et al., 2022; Raggi et al., 2017). Additionally, cytokines influence fibrous cap composition and stability; TNFα and IL-1b can affect the regulation of mRNA's which, in turn, induce the secretion of particular metalloproteinases (MMPs) from macrophages. These are a family of endopeptidases where, in this context, catalyse the degradation of collagen within the fibrous cap and increase the risk of thrombosis (Henein et al., 2022; Kowara et al., 2017).

Reactive oxygen species (ROS) are also important mediators in physiological and pathological cell signalling, and they are heavily involved in the chronic state of inflammation seen in CAD. Their interaction with cytokines, certain signalling pathways (such as MAPKs), activation of transcription factors, and their role in mitochondrial dysfunction often has pathological consequences in the context of CAD. The interplay between ROS and these processes described above help to facilitate endothelial dysfunction, atherosclerosis, myocardial dysfunction, and myocardial remodelling (Elahi et al., 2000). Examples of such will be discussed in more detail in section 1.3.

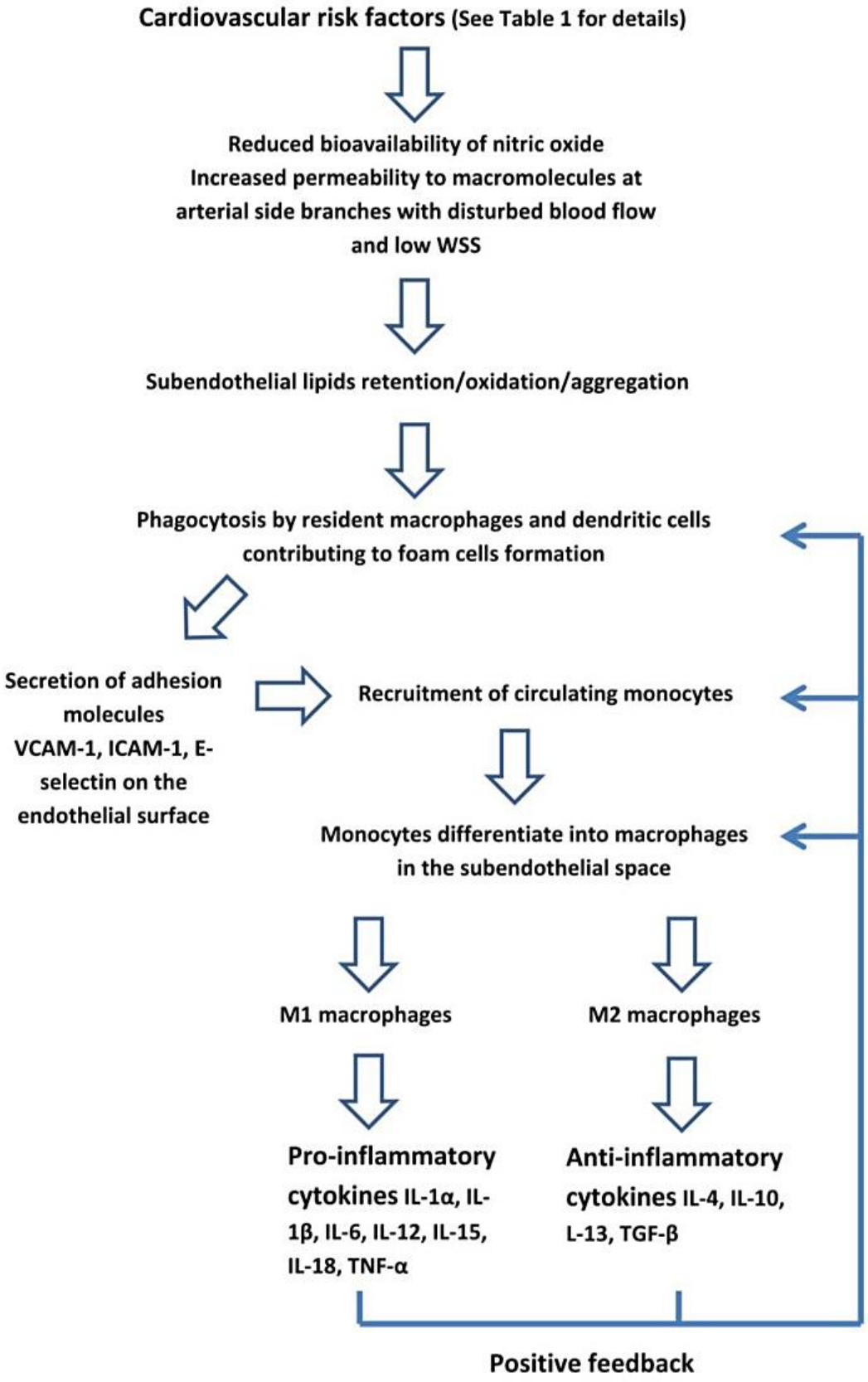

Figure 1-6. Inflammatory mediators involved during the early development of atherosclerosis. WSS: wall shear stress. VCAM-1: vascular cell adhesion molecule. ICAM-1: intercellular adhesion molecule. (Henein et al., 2022).

To summarise, the pro-atherogenic relationship between lipid acquisition/retention within the plaque and inflammation is a fundamental element of CAD. Modified lipids activate various cells of the immune system and begin an inflammatory cascade that is modulated by many different cytokines, chemokines, and other soluble factors (Henein et al., 2022). In turn, these affect almost all aspects of plaque composition, development, ECM activity, and ROS generation that continuously propagate this unresolved cycle of inflammation (Figure 1-6). The significance of this inflammatory involvement in CAD can also be corroborated by the fact that the anti-inflammatory effects of some drugs used to treat and manage the disease can provide the greatest benefit to the patient. For example, the reduction in vascular inflammation seen following treatment with statins may be more conducive than their action in lowering levels of LDLs (Henein et al., 2022; Diamantis et al., 2017). Gaining a thorough understanding of these inflammatory mechanisms may help to uncover therapeutic opportunities to lower the burden of CAD (Henein et al., 2022).

1.2 TNFα

1.2.1 What is TNFα?

Cytokines are pleiotropic proteins that are heavily implicated in the inflammatory response and atherosclerosis. TNFα is a proinflammatory cytokine often produced by macrophages and various cell types during acute inflammation, and is responsible for a diverse range of signalling cascades - frequently leading to necrotic injury and apoptosis (Medina-leyte et al., 2021). TNFα typically binds two receptors: TNFR-1 and TNFR-2 (Idriss & Naismith, 2000). Many of the TNFα signalling pathways are initiated through the binding of TNFR-1 and are mediated through an intracellular 'death domain'. TNFR-2 may indirectly mediate TNFR-1 signalling through the activation of endogenous TNFα, leading to subsequent autotropic or paratropic TNFR-1 activation (Idriss & Naismith, 2000; Grell et al., 1999). However, binding of each receptor can activate signalling pathways that confer contradictory roles: beneficial functions include tumour resistance, protection against infectious agents, and immune stimulation (via activation of monocytes, neutrophils, platelets, and enhanced NK cell killing ability); on the other hand, excessive TNFα circulation can amplify infection pathology, which may prove fatal (Idriss & Naismith, 2000; Fiers, 1991).

1.2.1.1 TNFα signalling

One of the major pathways activated in TNFα signalling is the NF-KB pathway (Morgan & Liu, 2010; Vallabhapurapu & Karin, 2009). It is often associated with cell survival and involves a process whereby TNFα facilitates the translocation of the NF-KB transcription factor into the nucleus (Morgan & Liu, 2010; Vallabhapurapu & Karin, 2009). This involves the recruitment of a signalling complex called IKK. This complex is subsequently activated by a number of enzymes, resulting in IKK induced phosphorylation/dissociation of a protein (IKBa) associated with the P105/NK-KB gene product. With this, the IKBa nuclear export signal is lost, and the P105/NF-KB complex is free to move into the nucleus and activate transcription (Morgan & Liu, 2010; Devin et al., 2000). Ensuing transcription of associated genes lead to physiological immune responses such as cell proliferation and survival. In circumstances where NF-KB activity is depleted or inhibited, TNFR-1 has been shown to initiate cell death via apoptosis or necrosis (Dondelinger et al., 2015).

The MAPK pathways are also significant in TNFR-1 signalling (Morgan & Liu, 2010). These include ERK, JNK, and P38 MAPKs, and their initiation is largely dependent on a key TNFR-1 signalling molecule known as TRAF-2 (Morgan & Liu, 2010; Devin et al., 2003). All three are known to act on multiple cellular targets that exert pro-death and pro-survival effects. However, ERK is most often associated with pro-survival action (Morgan & Liu, 2010).

Downstream activation of JNK is facilitated through the overexpression of various dominant negative molecules (such as ASK1) which interact with TRAF2 in a stimulation dependent manner (Morgan & Liu, 2010; Tobiume et al., 2001). JNK signalling is believed to have both pro-apoptotic and anti-apoptotic effects; these might be carried out by inducing the upregulation of pro-apoptotic genes or by influencing pro- and anti-apoptotic mitochondrial proteins (Morgan & Liu, 2010).

The P38 pathway, on the other hand, is initiated through the activity of RIP (receptor-interacting protein) – a death domain kinase associated with TNFR-1 (Devin et al., 2003). P38 plays a significant role in pro-inflammatory cytokine production, induction of enzymes involved in connective tissue remodelling, and expression regulation of inducible nitric oxide synthase (iNOS) (Rolski & Błyszczuk, 2020).

Crosstalk between the pathways mentioned can modulate their effects. An example of this is between NF-KB and JNK, where NF-KB helps to prevent sustained activation of JNK, thus mitigating its potentially pathological effects (Rolski & Błyszczuk, 2020). In a similar way, increased ROS production can also result in NF-KB-induced expression of antioxidant proteins, but in other cases can lead to further ROS production and cellular damage (Blaser et al., 2016).

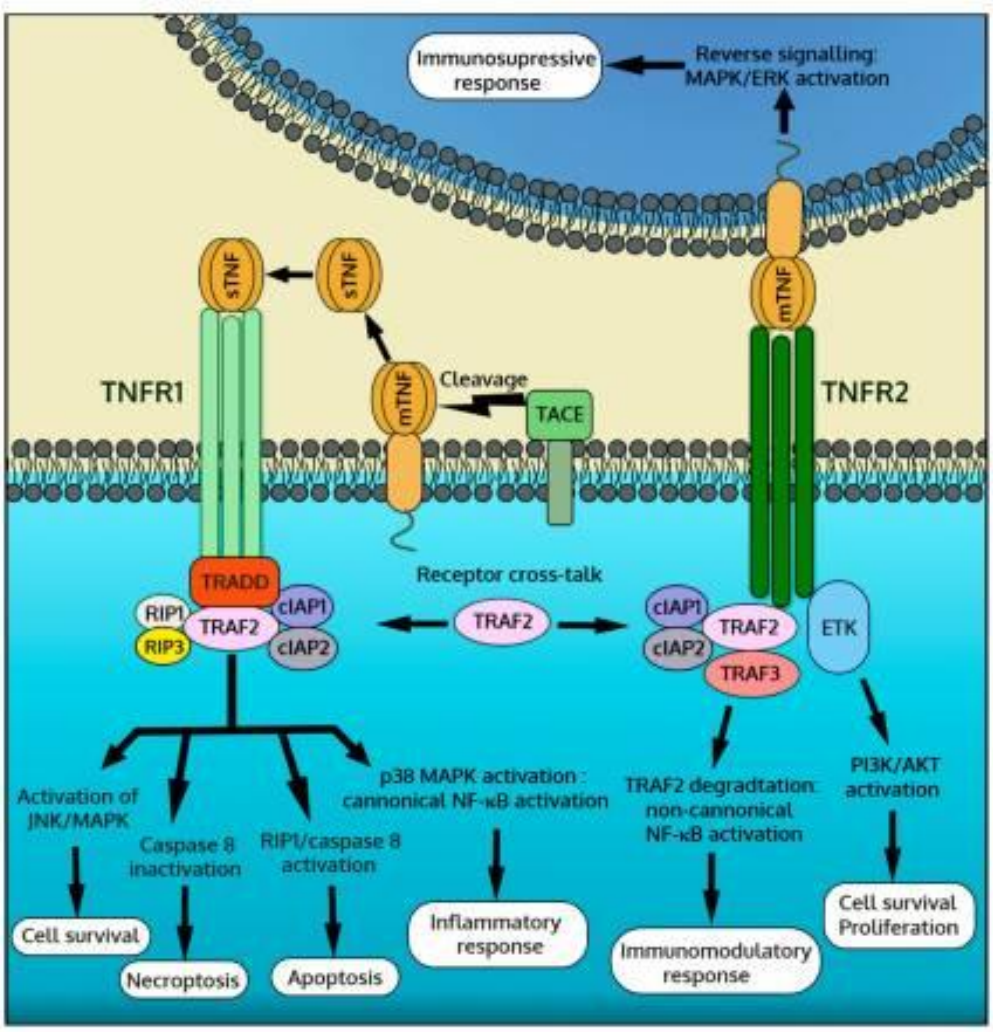

Figure 1-7. TNFα signalling pathways. Outline of the downstream pathways involved in TNFα signalling, including those mediated by TNFR1 and TNFR2 (Rolski & Błyszczuk, 2020).

Low levels of ROS also have a regulatory function in TNFR-1 signalling. However, excessive ROS accumulation will typically lead to dysfunction, DMA damage, and cell death. TNFα can directly influence the activity of three primary sources of cellular ROS (Blaser et al., 2016). For instance, mitochondrial ROS are produced physiologically

during oxidative metabolism and ATP production. However, TNFα induced ROS can promote further mitochondrial ROS production in response, thus creating a positive feedback loop between the two (Blaser et al., 2016; Morgan & Liu, 2010). TNFα can also facilitate the cellular capacity for ROS production via certain TNFα signalling events (such as those involved in P38 MAPK activation), where NADPH oxidase constituents may be upregulated, or NADPH oxidase activity is modulated through the phosphorylation of component proteins such as P47phox (Morgan & Liu, 2010). A multitude of other enzymes involved in ROS production, albeit to a lesser extent, can also contribute to TNFα-induced ROS accumulation or oxidative stress. These include lipoxidases, xanthine oxidase, and cyclooxygenases, among others (Morgan & Liu, 2010),

1.2.2 Role of TNFα in CAD

1.2.2.1 TNFα and Endothelial dysfunction

The signalling events described above are known to support atherogenesis, lesion development, vascular inflammation and remodelling, ROS production, EC apoptosis, and thrombosis (Zhang et al., 2009). Aside from those highlighted in sections 1.1.6 and 1.2.1.1, a significant way in which TNFα can exert potentially deleterious effects on the endothelium is through its regulation of nitric oxide synthase (NOS) expression, and thus subsequent bioavailability of nitric oxide (NO). Of particular importance is TNFα-induced downregulation of the isoform eNOS (expressed by EC's) and the subsequent decrease in NO availability (Zhang et al., 2009). To reiterate, this can reduce vascular capacity for vasodilation and increase endothelial vulnerability to mechanical stress. One of the several mechanisms that have been proposed to account for this apparent downregulation is that TNFα can increase the rate of associated mRNA degradation (Yoshizumi et al., 1993). In addition, other studies have demonstrated that TNFα's involvement in NO regulation may be actioned via the citrulline/NO cycle; decreased mRNA and protein expression of argininosuccinate synthase (ASS) (a key protein mediator of the cycle that produces NO) directly resulted in reduced NO availability (Zhang et al., 2009; Gao et al., 2007). With this being said, it should also be mentioned that TNFα may have a paradoxical role in eNOS modulation. Some data suggests that TNFα can in fact activate eNOS to further increase NO production and protect against TNFα-induced apoptosis (Bulotta et al., 2001). De Palmer et al (2006) demonstrated a

potential mechanism where neutral-sphingomyelinase-2 (N-SMase2) was activated by TNFα, followed by sphingosine-kinase-1 (SK1) activation and production of sphingosine-1-phosphate (Sph1P). Inhibition of these pathway messengers prevented eNOS activation. This TNFα-mediated activation of eNOS increased NO production and decreased E-selectin expression (thereby reducing dendritic cell adhesion to the endothelium) in human endothelial cells. However, the evidence to suggest that TNFα reduces NO availability seems to be more robust. and enhances leukocyte extravasation through increased expression of adhesion molecules. This discrepancy in vascular NO can be exacerbated by the production of ROS (Zhang et al., 2009).

There is evidence that multiple CAD risk factors such as obesity, smoking, and physical inactivity play a part in modulating TNFα signalling, which may contribute to their effect on endothelial dysfunction and CAD development. Moreover, some risk factor management strategies (regular exercise, dietary supplementation) can mitigate the potential for endothelial dysfunction by inhibiting TNFα signalling or production (Zhang et al., 2009). TNFα appears to play a particularly significant role in the relationship between inflammation and lipid metabolism, and therefore intravascular lipid accumulation and vascular dysfunction. Specifically, a relationship between TNFα and hyperlipidaemia has been observed (Popa et al., 2007); TNFα serum concentrations have been seen to fluctuate in accordance with dietary cholesterol. Correlations have been shown to exist between TNFα and very low-density lipoprotein (VLDL), LDL cholesterol, and triglycerides, whilst a negative relationship is observed with HDL cholesterol levels (Popa et al., 2007). Likewise, the anti-inflammatory benefits of lipid-lowering statins have been demonstrated when targeting TNFα. TNFα induced activation of NF-KB, generation of ROS, and adhesion molecule upregulation was abated in human aortic endothelial cells following treatment with atorvastatin (Wu et al., 2013). Statins and fibrates have also been shown to ameliorate elevated TNFα levels in patients with type IIa and IIb dyslipidaemia patients (Jovinge et al., 1998).

1.2.2.2 TNFα and Myocardial dysfunction

Under physiological conditions, cardiomyocytes do not express TNFα. However, these cells can elaborate TNFα in response to stress (Levine et al., 1990), and this cytokine has been implicated in cardiac remodelling on both a global and cellular level (Sun et al., 2007). As

mentioned in previous sections, pressure overload (occurring as a result of vascular stenosis and hypertension) for example can lead to concentric remodelling of the myocardium – in this situation, TNFα expression is upregulated (Sun et al., 2007). This could suggest a potentially significant role in the processes that characterise cardiac remodelling such as inflammation, fibrosis, hypertrophy, and apoptosis (Sun et al., 2007; Kassiri et al., 2005). Sun et al (2007) used aortic banding on C57/BL wild-type (WT) and TNF-knockout ($TNF^{-/-}$) mice to model pressure overload and subsequent remodelling. In WT mice, rates of apoptosis in cardiomyocytes were significantly increased, whilst this affect was attenuated in ($TNF^{-/-}$) mice. This coincided with elevated levels of caspase 8 levels in the WT group, suggesting caspase activation during TNFα signalling within the myocardium in response to pressure overload. Similar results were seen in these WT mice when examining fibrosis and hypertrophy: increased levels of focal fibrosis were seen, along with increased expression of transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF) in comparison to ($TNF^{-/-}$) mice; pump function was further reduced in WT mice compared with ($TNF^{-/-}$) mice, indicating that TNFα signalling is involved in LV hypertrophy and the resultant loss of contractile function. In addition, the upregulation of MMPs and corresponding matrix dysregulation/degradation could also be seen in the WT mice.

Similar findings have been shown in post-infarcted models. Infarcted ($TNF^{-/-}$) phenotype models also demonstrated less extensive infarct/peri-infarct zones, reduced expression of intracellular adhesion molecules, and decreased infiltration of inflammatory cells into the myocardium (Sato et al., 2006). Particular knockout of TNFR-1 ($Tnfrsf1a^{-/-}$) reduced NF-KB activation, preserved LV function (Rolski & Blyszczuk, 2020; Hamid et al., 2009), and increased survival (Rolski & Blyszczuk, 2020; Monden et al., 2006). Thus, it is plausible that local production of TNFα might significantly modulate the processes leading to cardiac remodelling, and ultimately the functionality of the heart in CAD.

1.2.2.3 TNFα and Contractility

The impairment of cardiomyocyte contractility following ischemic injury can be attributed to the significant generation of oxygen radicals (oxidative stress) and increased cytoplasmic Ca^{2+} concentrations – both of which have been found to be mediated by TNFα (Tian et al., 2015).

Inotropic effects have been observed when investigating TNFα induced alterations in Ca^{2+} handling, and this has been demonstrated in multiple studies using animal models and human primary cardiomyocytes (Duncan et al., 2007; Yokoyama et al., 1993; Oral et al., 1997). In human iPSC-derived cardiomyocytes for example, TNFα treatment provoked an increase in cellular ROS and caspase dependent apoptosis. Alongside, incidence of abnormal calcium transients and calcium sparks were detected – the latter of which are Ca^{2+} release events from the SR, resulting in SR calcium leakage, reduced SR content, and increased baseline cytoplasmic Ca^{2+} (Saraf et al., 2020). Not only can this reduce contractility as discussed in section 1.1.3.4, but can propagate the activation of arrhythmogenic contraction (Saraf et al., 2020). In addition to this, TNFα treatment can also been shown to induce differential expression of multiple ion channels, such as downregulated SERCA-associated ATP2A2 and ATP2A3, NCX, and RYR2 (Saraf et al., 2021), further contributing to the potential for dysregulated Ca^{2+} cycling. Rabit models have demonstrated decreased contractility in response to prolonged TNFα exposure (Goldhaber et al., 1996). Here, TNFα induces production of myocardial NO, resulting in reduced Ca^{2+} influx through the inhibition of voltage gated Ca^{2+} channels and decreased myofilament sensitivity (Saini et al., 2005; Finkel et al., 1992; Goldhaber et al., 1996). In a similar way, TNFα treatment has been shown to depress cardiac contractile function in rat cardiomyocytes – again, where a reduction in SR Ca^{2}+ content produced a reduction in intracellular calcium and therefore myocyte shortening (Greensmith & Nirmalan, 2023). Cailleret et al (2004) observed similar effects through a reduction in the rate of calcium induced calcium release – a process which appears to be mediated by the sphingosine phospholipid. These effects could be attenuated with N-acetylcysteine (NAC), which suggests that TNFα's effects on contractility can be exerted through its pro-oxidant activities (Cailleret at al., 2004).

1.3 ROS

The term reactive oxygen species is used to describe oxygen radicals containing one or more unpaired electrons (such as superoxide, Hydroperoxyl, pyroxyl, and hydroxyl radicals), along with other non-radical oxidising agents such as H_2O_2. Due to the highly reactive nature of ROS, they interact with most molecules; this can lead to dysfunction or damage to cellular

constituents such as DNA, proteins, and membrane lipids (Bayir, 2005). Under physiological conditions, cellular ROS are produced and involved in various cellular processes, including mitochondrial ATP production and electron transport, signal transduction, enzymatic reactions, gene expression, activation of transcription factors, and antimicrobial defence. ROS mediated damage is mitigated by a number of antioxidant proteins and their relative substrates (Hong et al., 2024). Maintaining this cellular redox state is crucial in avoiding ROS accumulation and the pathological state of oxidative stress (Bayir, 2005).

1.3.1 Cellular sources of Reactive Oxygen Species

There are three main sources of cellular ROS: NADPH oxidase; the mitochondria; and enzymatic reactions involving lipoxidases, xanthine oxidase, and cyclooxygenases, amongst others. NADPH oxidase is a membrane bound protein complex that uses NADPH to reduce molecular oxygen, thus generating superoxide radicals (Blaser et al., 2016). NADPH oxidase is typically expressed by leukocytes to aid in the defence against pathogenic agents by exerting oxidative damage (Frey et al., 2009). However, leukocyte activation can often occur in response to pro-inflammatory mediators such as TNFα, leading to the recruitment and activation of associated NADPH oxidases. These protein complexes are also expressed in vascular ECs, fibroblasts, and SMCs (Bayir, 2006; Frey et al., 2009).

The mitochondria are another prominent source of potential ROS production. This is where the vast majority of cellular oxygen is reduced via electron carriers during mitochondrial metabolism and ATP synthesis; the metabolic products of the Krebs cycle (NADH and $FADH_2$) transfer electrons through the electron transport chain (ETC) – this, along with the subsequent efflux of protons, drives the production of ATP Via ATP synthase activation (Chen & Butow, 2005). The ETC comprises four complexes located on the inner mitochondrial membrane. Complex I and III are the main sources of O_2^- production, particularly in the context of ischemia/reperfusion injury within the heart. Damage to either of these complexes have shown to result in enhanced production of O_2^-, leading to mitochondrial dysfunction, oxidative stress, and cell death (Kang et al., 2018). However, mitochondria-derived accumulation of ROS does not usually occur unless the function of the mitochondrial inner membrane is altered (Bayir, 2006; Nohl et al., 2005). Under physiological circumstances, the action of superoxide

dismutase and catalases can remove subsequently formed superoxide and H_2O_2 (Boveris et al., 1972).

Finally, certain enzymatic reactions might contribute towards the production of cellular ROS. One example is Xanthine oxidase (XO), which is an oxidised form of Xanthine oxidoreductase (XOR). XOR – in either of its forms, including XO – catalyses several steps of the purine degradation pathway (Seyshell et al., 2024), whereby H_2O_2 and superoxide is produced as a byproduct. Expression of XOR/XO is typically localised in endothelial and epithelial cells, however physiological expression levels are low but can be upregulated in response to certain pro-inflammatory mediators or hypoxia (Bayir, 2006; Berry & Hare, 2004).

The classical pathway for the removal of superoxide and H_2O_2 is outlined below (Fig 1-8).

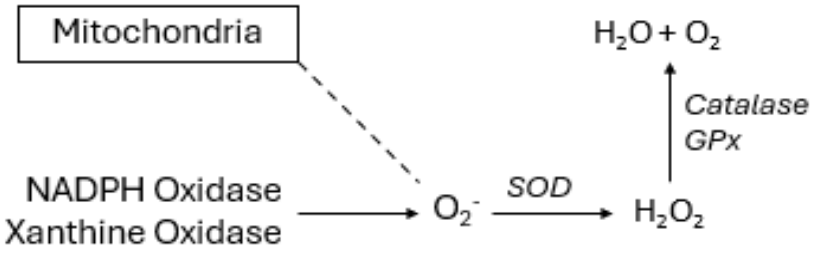

Figure 1-8. Key enzymes involved in the production and removal of cellular ROS. NADPH Oxidase and Xanthine oxidase produce radicals such as superoxide, removal of which is facilitated by the activity of antioxidant proteins (Bayir, 2006).

Superoxide dismutase (SOD) initially facilitates this removal by converting superoxide into H_2O_2 and O_2 (Figure 1-9).

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Figure 1-9. SOD-facilitated removal of superoxide. (Bayir, 2006).

H_2O_2 is then removed by the action of either catalases or peroxidases to form O_2 and $2H_20$, or $2H_2O$, respectively:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O+O_2$$

$$H_2O_2 \xrightarrow{\text{Peroxidase}} H_2O$$

Figure 1-10. catalase and peroxidase facilitated removal of H_2O_2. (Bayir, 2006).

The latter reaction depicted in Figure 1-10 also uses various reductants to reduce H_20_2 to H_2O, such as glutathione, thioredoxin, and ascorbate (Halliwell & Gutteridge, 1985; Bayir, 2006).

1.3.2 Oxidative Stress

The accumulation of oxidants, or ROS, or a depletion of antioxidants can alter the cellular redox state and leave molecular targets susceptible to oxidative damage. Failure to remove O_2^- or H_2O_2 results in further the generation of highly reactive radicals, such as the particularly reactive hydroxyl radical ($OH^{\cdot}$). Reactions between superoxide (O_2^{-}) or H_2O_2 and transition metals such as Fe(II) and Cu(I) facilitate this ROS production which often leads to oxidative stress (Figure 1-11) (Bayir, 2006).

Resultant DNA damage can manifest as DNA single strand breaks, DNA base damage, cross link formation between DNA and proteins, and guanine hydroxylation (Bayir, 2006; Cadet et al., 1994). Lipid cell membranes are also a frequent target of oxidative attack. Peroxidation of polyunsaturated fatty acid chains ultimately leads to further formation of peroxyl radicals, which can then propagate these damaging effects to adjacent membrane lipids as well as target nearby membrane proteins. As a consequence, membrane fluidity and integrity may be compromised. Likewise, various types of oxidative protein modification may lead to loss of function and enzyme inhibition (Bayir, 2006).

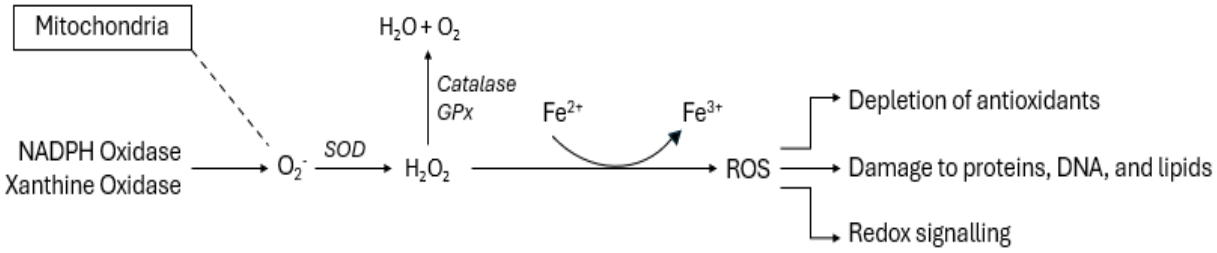

Figure 1-11. Oxidative stress. When the cellular redox state is altered, ROS production may exceed antioxidant capacity. In this context, reactions between various transition metals and O_2^- or H_2O_2 facilitate the production of highly reactive species such as the hydroxyl radical. ROS accumulation will then exert oxidative damage on molecular targets (Bayir, 2006).

1.3.3 The role of ROS in CAD

It has been well established that ROS play a fundamental role in the progression of cardiovascular disease, and CAD in particular; ROS and their involvement in various intracellular signalling cascades can be implicated in multiple stages of CAD progression or complications associated with the disease, including endothelial dysfunction, atherosclerosis, and ischemic/reperfusion injury (Panth et al., 2016).

1.3.3.1 ROS and endothelial dysfunction

In terms of endothelial dysfunction, accumulation of ROS can directly lead to oxidative damage, or mediate signalling cascades that have deleterious effects on the functionality of the endothelium. An example of this is the activation of NF-KB to initiate a signalling cascade that upregulates the production of pro-inflammatory cytokines like TNFα, thus propagating further ROS production (Forrester et al., 2018). Here, inflammatory mediators can induce EC and leukocyte production of O_2^- via the activation of Xanthine oxidase, mitochondrial oxidase, NOS, and NADPH oxidase (Panth et al., 2016).

The latter appears to be the primary source in various models of vascular disease and are prevalent in human vessels and atherosclerotic coronary arteries. In the context of diabetes, elevated basal levels of O_2^- correspond with increased expression of NADPH oxidase subunits including the previously mentioned p47Phox, which suggests that higher levels of oxidative stress might be a fundamental factor responsible for heightened CAD severity in these

patients (Brown et al., 2021; Zhang, 2008). Furthermore, oxidative stress in the setting of hyperglycaemia can induce the production of advanced glycation end (AGE) products, which also serve as ligands to activate the receptor for AGEs (RAGE). By themselves, AGEs can directly alter the framework of the vessel wall; reduced elasticity can arise due to the formation of AGE-induced intermolecular crosslinks and subsequent capture of plasma components (Bachschmid et al., 2012). However, signalling events following RAGE binding exert pro-coagulant and pro-inflammatory effects. Such signalling events include NADPH oxidase activation; NF-KB and MAPK signalling; and increased expression of TNFα, adhesion molecules, and tissue factor (TF) (Zhang, 2008) – many of which exacerbate the production ROS and perpetuate the cycle between inflammation and oxidative stress in the dysfunctional endothelium. It has also been demonstrated that NADPH oxidase-dependant pathways/generation of ROS is required for the upregulation MCP-1 and VCAM-1 in ECs, particularly in relation to TNF-α-induced upregulation of these proteins (Chen et al., 2004).

ROS can also facilitate the production of reactive nitrogen species (RNS), which have similar physiological and pathological roles as ROS. As previously discussed, NO generally has protective, vasodilatory effects. In the presence of O_2^-, however, reactions between the two can produce highly reactive intermediates such as peroxynitrite ($ONOO^-$). In high concentrations, RNS such as ONOO- exert nitrosative damage on various biomolecules and proteins resulting in subsequent loss of function (Alhayaza et al., 2020; Drew & Leeuwenburgh, 2002). Even excessive NO production has been linked to further production of ROS/RNS, particularly through the suppression of cytochrome c oxidase (which function to reduce molecular oxygen to water) (Alhayaza et al., 2020; Bolisetty & Jaimes, 2013).

1.3.3.2 ROS and myocardial dysfunction

Many of the hypertrophy-inducing pathways that may be activated by genetic and extracellular stimuli – some of which have already been highlighted –are directly and indirectly mediated by ROS, via their action to activate signal transduction factors within these respective pathways. Examples include NF-KB, MAPKs, and tyrosine kinase pathways, which are stimulated by the likes of TNFα and angiotensin II (Ang II) (Moris et al., 2017). The latter, for instance, induces the production of ROS following G-coupled receptor binding. The resultant hypertrophic effects of angiotensin II are affected largely by ROS-mediated kinases;

Ang II-induced upregulation of β-MHC is contingent upon ROS-dependant activation of ERK (Moris et al., 2017; Shih et al., 2001).

Additionally, interstitial fibrosis that occurs following cardiac fibroblast activation has been found to be closely associated activation of NADPH oxidase - particularly NOX 2 (Moris et al., 2017; Liu et al., 2009). As well the pro-fibrotic effects NOX2, NADPH oxidase inhibition (and thus inhibition of associated production of ROS) has been shown to have positive inotropic effects to improve contractility, which was facilitated by enhanced phospholamban phosphorylation (Moris et al., 2017; Kubin et al., 2011). This demonstrates that ROS could potentially directly affect cardiac contractile function.

The pathogenic interplay between mitochondrial dysfunction and ROS production is particularly damaging within the ischemic or failing myocardium. Not only are mitochondria abundant in cardiomyocytes to meet the high demand for ATP production, but these organelles are also heavily involved in Ca^{2+} handling and cellular viability within the heart. Since mitochondrial DNA (mtDNA) is in close proximity to the respiratory chain, it is especially susceptible to ROS-induced damage or mutation. Eventually, the accumulation of such mutations may result in decreased production of proteins encoded by mtDNA and insufficient production of ATP. This process, in turn, promotes further generation of ROS and perpetuates this destructive cycle (Bou-Teen et al., 2021; Tsutsui et al., 2006). In addition, mtDNA mutations present as damage associated molecular patterns (DAMPs) and activate various pattern recognition receptors (PRRs) (including toll-like receptors [TLRs]). This triggers a state of chronic-sterile inflammation within the heart; production of pro-inflammatory cytokines and recruitment of immune cells contribute to myocardial damage in response to ischemic injury and is implicated in the development of heart failure (Nakayama & Otsu, 2018).

The relationship between acute I/R injury and the mitochondrial permeability transition pore (mPTP) is well studied. During increased mitochondrial Ca^{2+} uptake/Ca^{2+} overload, oxidative stress signalling, and ATP depletion, the mPTP tends to open. In such conditions which often characterise myocardial cellular dysfunction, opening of the mTP allows the influx of water and various solutes to enter the mitochondrial matrix. Mitochondrial swelling, along with the release of cytochrome c and other mediators of apoptosis, then induce cell death following ischemic injury (Dridi et al., 2023; Li et al., 2010). mtDNA release that occurs in this way can

also sustain the state of sterile inflammation described above. Although mPTP opening is not dependent on the production of ROS, the adverse effects of both on mitochondrial dysfunction appear to be concurrent (Mendoza et al., 2024; Haworth & Hunter, 1979). It is not surprising, therefore, that the action of antioxidants and mPTP inhibitors are being explored as therapeutic targets against mitochondrial damage (Su et al., 2022). These, along with enhanced mitochondrial production of ROS, erroneous activation of MAPKs, and Ca^{2+} dysregulation are perhaps one of the most significant pathobiological factors affecting cardiomyocyte viability and myocardial function following ischemic injury (Sucher et al., 2009).

1.4 Aims and Objectives

The evidence for the involvement of TNFα in myocardial function is compelling. It is clear that the role of this pro-inflammatory cytokine is multifactorial, complex, and even sometimes paradoxical. However, oxidative stress appears to be an elemental consequence of TNFα signalling that leads to injury and dysfunction. This has been well established with supra-pathological and -physiological treatments in various models, however more research is needed to investigate these potential effects on both cellular and global levels in a clinically relevant context.

This study aimed to quantify plasma TNFα levels in a CAD patient cohort whilst correlating to indices of cardiac function. With the main aim to determine the value of TNFα as a biomarker for CAD. Additionally, it aimed to investigate the role of clinically relevant TNFα levels in a CAD cohort on oxidative stress.

The main aims of this study were:

1. Quantify plasma levels of TNFα in a CAD patient cohort.
2. Correlate quantified TNFα levels to indices of cardiac function.
3. Determine whether clinically relevant TNFα concentrations can elevate intracellular oxidative stress.

2. Methods

2.1 Study Design

This project was carried out as part of the wider clinical study, CAVCAD, which aims to describe cardiac cellular function in a CAD patient cohort.

Specifically, this project quantified plasma levels of TNFα in a cohort of 66 patients and sought to identify potential correlates with indices of cardiac function obtained through echocardiographic assessment. Alongside this, the effects of TNFα on cellular oxidative stress within a H9C2 rat myoblast cell line model was also investigated.

2.1.1 Ethical considerations

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

2.1.2 Patient recruitment

Patients that were scheduled for coronary artery bypass graft (CABG) surgery were recruited to take part in the study during pre-operative evaluation at Blackpool Victoria Hospital (BVH). Inclusion criteria were as follows: patients previously diagnosed with CAD, scheduled for revascularisation (CABG) surgery, >18 years of age, and either gender was recruited. Consent was obtained from participants prior to surgery upon completion of the patient consent form (appendix A). Subsequent samples and data were completely anonymised. Pre-surgical samples were then collected; 10ml of blood was collected from each patient and stored in an EDTA tube at 4°C. Echo data was collected during the pre-operative assessment.

2.2 Sample preparation

Blood samples were then transferred from the EDTA coated tube into a 15 ml falcon tube. These were subsequently centrifuged at 2000xg for 5 minutes to isolate blood constituents; plasma was appropriately aliquoted into cryovials and stored at -80°C.

2.3 Quantification of TNFα levels using ELISA

High sensitivity sandwich ELISA kits (Thermofisher, USA; product code: BMS223HS) were used to quantify plasma TNFα levels, with a detectable range between 0.31 - 20 pg/ml. All reagents were allowed to come to room temperature prior to use and then diluted according to manufacturer instructions when required.

2.3.1 Standard curve generation

A standard curve was generated using the absorbance from a series of known, serially diluted concentrations. From this, the unknown TNFα levels of patients were extrapolated. Known standards were prepared by first reconstituting the lyophilised control with distilled water for 10-30 minutes, with continued agitation to ensure homogenous solubilisation. The solubilised control was pre-diluted 1:20 in sample diluent. Then, 225µl of the pre-diluted standard (40pg/ml) was added to the first tube and mixed to obtain the first standard at a concentration of 20pg/ml. The serial dilution was repeated a further 6 times in this way to create the points of the standard curve (Table 2-1). The final Eppendorf contained only sample diluent to serve as a blank negative control (0pg/ml). Absorbance levels were measured at 450 nm and background wavelength was measured at 620 nm using FLUOstar Omega plate reader (BMG LabTech Germany). Background absorbance levels were subtracted. Average absorbance values for each standard were calculated, and a standard curve (Figure 2-1) was plotted for TNFα.

Table 2-1: TNFα ELISA standard concentrations.

Standard	Standard Dilution	Concentration (pg/ml)
1	1:2	20
2	1:4	10
3	1:8	5
4	1:16	2.5
5	1:32	1.25
6	1:64	0.63
7	1:128	0.31
8 (negative control)	No dilution	Blank

2.3.2 Experimental protocol

Patient plasma samples were removed from the freezer and allowed to thaw at room temperature. Microwell strip(s) were then washed twice with 400µl of prepared (1X) wash buffer per well. Samples were vortexed and then added to the microwell plate in duplicate (100µl per experimental well). Biotin conjugate was prepared before adding 50µl to each sample well. Microwell strips were covered with adhesive film and incubated for 2 hours at room temperature on a microplate shaker.

Following this initial incubation period, Streptavidin-HRP was prepared (Appendix B) immediately prior to use. Sample wells were aspirated and washed 6 times. 100µl of diluted streptavidin-HRP was added to all wells and covered with adhesive film to incubate for 1 hour on a microplate shaker at room temperature.

Amplification diluent (1X) and Amplification Solution 1 were prepared immediately prior to use. Adhesive film was removed, and wells were again aspirated before being washed 6 times. 100µl of amplification solution 1 was then added to sample wells, before being incubated in previously described conditions for 15 minutes.

Amplification solution 2 was prepared immediately prior to use, and wells were aspirated and washed a further 6 times. 100µl of amplification solution 2 was then added to sample wells and incubated for a further 30 minutes.

Following this, sample wells were again washed 6 times and 100µl of tetramethyl-benzidine (TMB) substrate solution was added to wells. These were incubated for 10-20 minutes without exposure to direct light, before addition of 100µl of Stop solution was added to each well to stop the enzymatic reaction (indicated by a colour change from blue to yellow). Wells were read immediately on the microplate reader.

2.3.3 Quantification of TNFα levels

Using the average absorbance values measured from each patient sample, plasma concentrations were then extrapolated from the standard curve (Appendix C). TNFα concentrations generated were subsequently compared to clinical parameters (Appendices D and E) on a patient-to-patient basis to determine whether measured TNFα levels correlated with cardiac function.

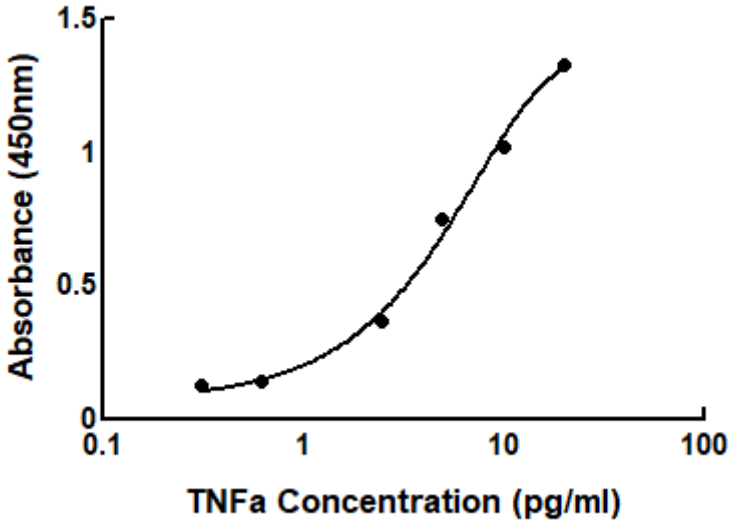

Figure 2-1: **TNFα ELISA standard curve**. Data are displayed as the mean with 95% CI.

2.4 Cell culture (H9C2 rat cardiac myoblast cell line)

The H9C2 rat cardiac myoblast cell line was purchased from Public Health England (UK) (CRL-1446). Cell culturing protocols and experimentation were performed in a class II biological safety cabinet (NuAire, USA) under aseptic conditions, and the cell line was maintained in an incubator (NuAire, USA) at 37°C with 95% air/5 % CO_2.

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK), which was supplemented with 10 % foetal bovine serum (FBS) (Gibco, UK) and 1% penicillin-streptomycin (10,000 units/ml and 10,000 μg/ml) (Gibco, UK). Cells were sub-cultured in T175 (175 cm3 surface area) cell culture flasks until confluency reached 70-80%. Cells were sub-cultured/passaged at a ratio of 1:3, typically every three days with the addition of fresh media. Cells were seeded at a density of 25,000 cells per well for experimentation during the subculturing procedure

2.4.1 Dissociation of H9C2 cell line

Cells were washed using 10ml of phosphate buffered saline (PBS) (Sigma, USA) once confluency was reached before treatment with 7 ml of Trypsin-EDTA solution (Gibco, UK) at 37°C. After approximately 5 minutes, cells were observed to confirm detachment of the monolayer. Once cells had sufficiently detached, completed media was added to neutralise the Trypsin. This suspension was then centrifuged at 125 xg for 5 minutes. The cell pellet was resuspended in 5ml of complete media for appropriate sub-culturing.

2.4.2 Cell counting

Cell counts were performed using a haemocytometer to determine the volume of suspension needed to seed plates at the desired density. 10 μl of cell suspension was resuspended with 0.4% trypan blue solution (Gibco, UK) at a 1:1 ratio. 10 μl of this mixture was added to a haemocytometer and the cell count was performed using a Leica DM500 microscope (Leica, Germany) (magnification X40). Non-viable cells (those which took up the blue dye) were excluded. Average cell count of the grid squares included were calculated and multiplied by the dilution factor of trypan blue (factor of 2), before then multiplying by $x10^4$ to give the total

cell count per ml of suspension. The volume of suspension needed to seed wells at the required density could then be calculated.

2.5 Measurement of cellular ROS

Cells were seeded in a 96 well black clear bottom plate (Fisher Scientific, USA) at a density of 25,000 cells per well before a 24hr incubation period to allow cell attachment. Afterwards, media was removed, and experimental wells were pre-treated with a clinically relevant (2.6 pg/ml) or supraphysiological (50 ng/ml) concentration of rat TNFα (PeproTech). Media was replaced in cells not treated with the cytokine. Following an incubation period of 1 or 24 hours, relevant wells (Figure 2-2) were loaded with 100 µl of CM-H_2DCFDA (5 µM) for 30 minutes. Media was replaced accordingly in unstained wells. Supernatant was subsequently removed from each well following incubation, and treatment with hydrogen peroxide (H_2O_2) was applied to the wells treated in synergy before further incubation for 30 minutes. Media only was used throughout on stained and unstained controls. Fluorescence intensity was detected at an excitation wavelength of 485 nm and emission wavelength of 520 nm using FLUOstar Omega plate reader (BMG LabTech Germany).

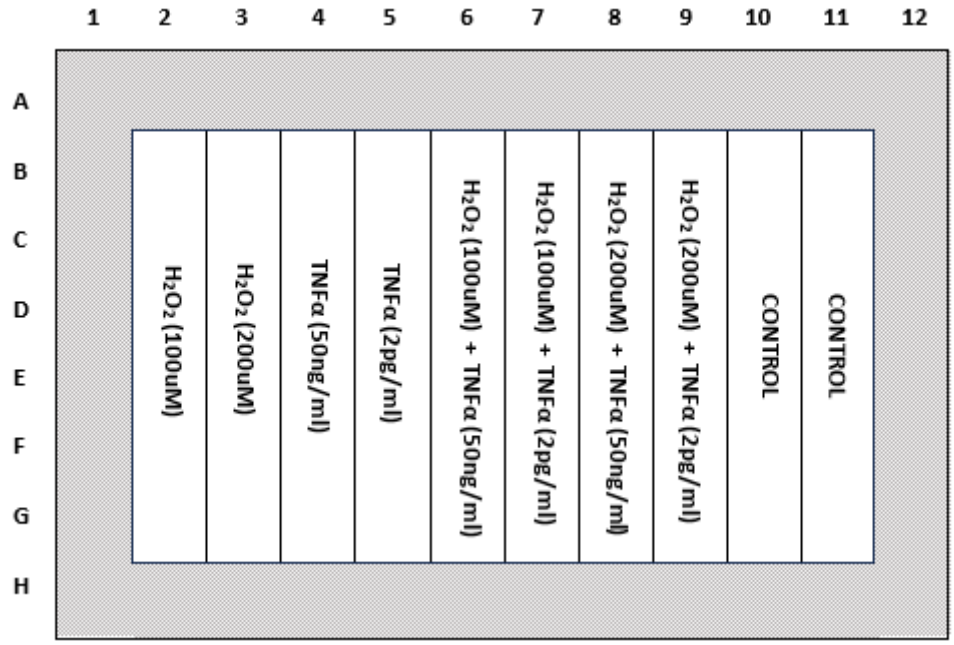

Figure 2-2: DCFDA assay plate layout. This assay was performed in a 96-well plate. Rows B-D were stained with the fluorescent dye, CM-H_2DCFDA, and E-G remained unstained.

2.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc, USA). Shapiro-Wilk normality testing was performed to assess data distribution. Normally distributed data was analysed using the parametric Pearson product-moment correlation coefficient (Pearsons) to measure the linear correlation between indices of cardiac function and plasma TNFα concentrations. EDV, E/A ratio, and E/e ratio were not normally distributed; instead, Spearman's Rank correlation coefficient was used to measure linear correlation with these parameters. Statistical significance was accepted when $p < 0.05$.

Intracellular levels of ROS were analysed using the non-parametric Kruskal-Wallis test with a Dunn's multiple comparison post hoc test to allow comparison of treatment (TNFα or H_2O_2) against control. Two-way ANOVA with Bonferroni post-tests were used to assess the effects of time and treatment on intracellular ROS.

3. Results

3.1 Quantification of plasma levels of TNFα

High sensitivity sandwich enzyme-linked immunosorbent assays were used to develop standard curves from which individual patient plasma TNFα levels could be extrapolated. These were later correlated against multiple indicators of cardiac function obtained from patient clinical records. Average concentration of TNFα within this cohort was 2.27 ± 0.35 pg/ml (n = 66).

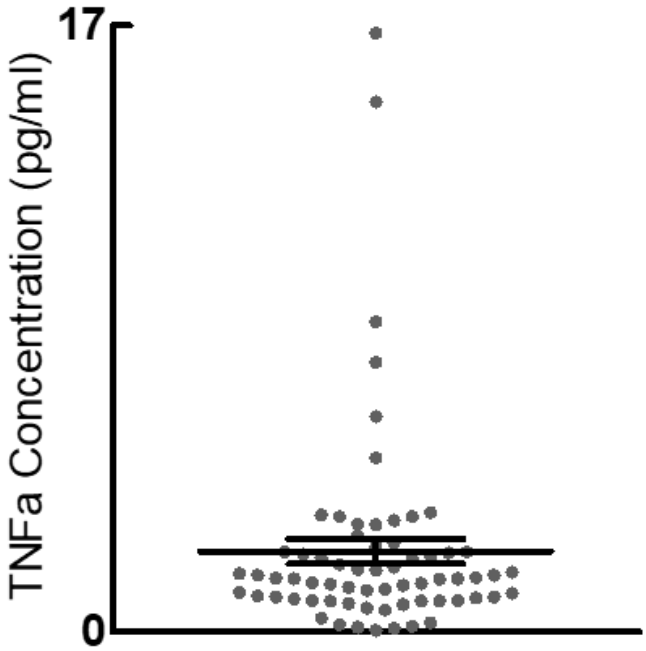

Figure 3-1. Patient serum TNFα concentrations. Average displayed as mean ± SEM.

3.2 Patient clinical parameters

Various clinical and echo parameters were recorded during preoperative evaluations for each patient. Parameters associated with systolic and diastolic function were extracted; those prioritised are presented below.

Table 3-1: ECHO parameters used to measure cardiac function within CAD patient cohort. Table displays each measurement with corresponding mean and standard error values along with the number of patients analysed.

Parameter	Mean	SEM	n
TAPSE (cm)	2.13	0.06	37
Stroke volume (ml/m^2)	49.12	4.18	13
LVOT (m/s)	0.95	0.04	36
ESV (ml)	49.14	7.34	13
Ejection Fraction (EF)	49.72	1.56	51
LVIDs (cm)	3.30	0.10	36
LVIDd (cm)	4.69	0.11	38
E/A ratio	0.94	0.05	45
Peak E wave (m/s)	0.72	0.03	46
EDV (ml)	97.99	7.83	13
TR Velocity (m/s)	2.47	0.11	22
E/e ratio	9.41	0.47	30

3.3 Correlation of plasma TNFα levels with indicators of systolic function

The following indicators of systolic function were selected to correlate against levels of TNFα on a patient-to-patient basis: stroke volume, ejection fraction, TAPSE, LVOT, LVIDs, and ESV. These indices are measured during or at the end of systole/contraction. Stroke volume is an indicator of left ventricular function and measures the volume of blood leaving the left ventricle. Ejection fraction is the percentage of blood in the heart that is ejected during systole. TAPSE (tricuspid annular plane systolic excursion) is commonly used to assess right ventricular systolic function; this measures dislocation of the tricuspid ring towards the right ventricle. LVOT (left ventricular outflow tract) peak velocity shows the speed at which blood flows from the left ventricle into the aorta. LVIDs (left ventricular internal diameter end-systole) is the distance measured between the wall of the left ventricle and the interventricular septum at the end of contraction. ESV (end-systolic volume) refers to the volume of blood remaining in the ventricle following contraction and immediately prior to ventricular refilling.

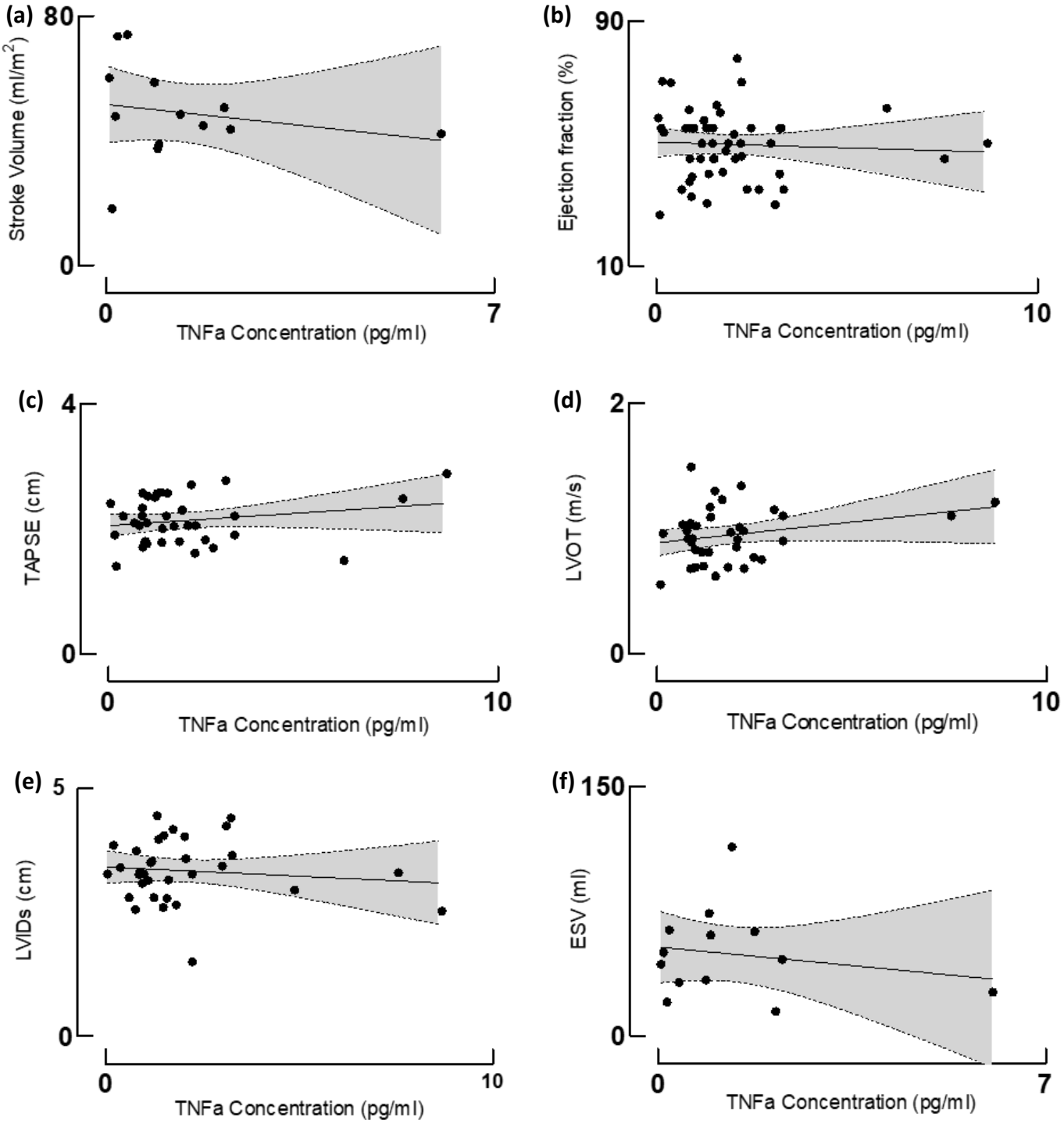

Figure 3-2. Indices of systolic function correlated against serum TNFα levels.

(a) Correlation of stroke volume against TNFα concentration. (b) Correlation of ejection fraction against TNFα concentration. (c) Correlation of TAPSE against TNFα concentration. (d) Correlation of LVOT against TNFα concentration. (e) Correlation of LVIDs against TNFα concentration. (f) Correlation of ESV against TNFα concentration. Pearson test was used to measure linear correlation. The grey area on each scatter plot represents the 95% CI band.

Figure 3-2 shows that TNFα levels do not correlate with stroke volume ($p = 0.5035$; $r^2 = 0.04168$; $n = 13$), ejection fraction ($p = 0.6993$; $r^2 = 0.0031$; $n = 51$), TAPSE ($p = 0.2251$; $r^2 = 0.0418$; $n = 37$), LVOT ($p = 0.1213$; $r^2 = 0.0691$; $n = 36$), LVIDs ($p = 0.5393$; $r^2 = 0.01228$; $n = 36$), or ESV ($p = 0.5256$; $r^2 = 0.0376$; $n = 13$).

3.4 Correlation of plasma TNFα levels with indicators of diastolic function

Furthermore, indices of diastolic function were chosen to compare against patient TNFα levels and were as follows: LVIDd, E/A ratio, TR velocity, E/e ratio, peak E wave, and EDV. These are measured at during or at the end of diastole/relaxation. LVIDd (left ventricular internal diameter end-diastole) is the measurement of the internal diameter at the end of ventricular relaxation. E/A ratio analyses the biphasic filling of the left ventricle, examining blood flow during the first phase (E wave) of diastole in relation to the second phase (A wave) brought on by atrial contraction. Peak E wave velocity, as described above, refers to the velocity of passive flow into the left ventricle during early diastole. Lastly, EDV (end-diastolic volume) evaluates the volume of blood left within the ventricle at the end of diastole.

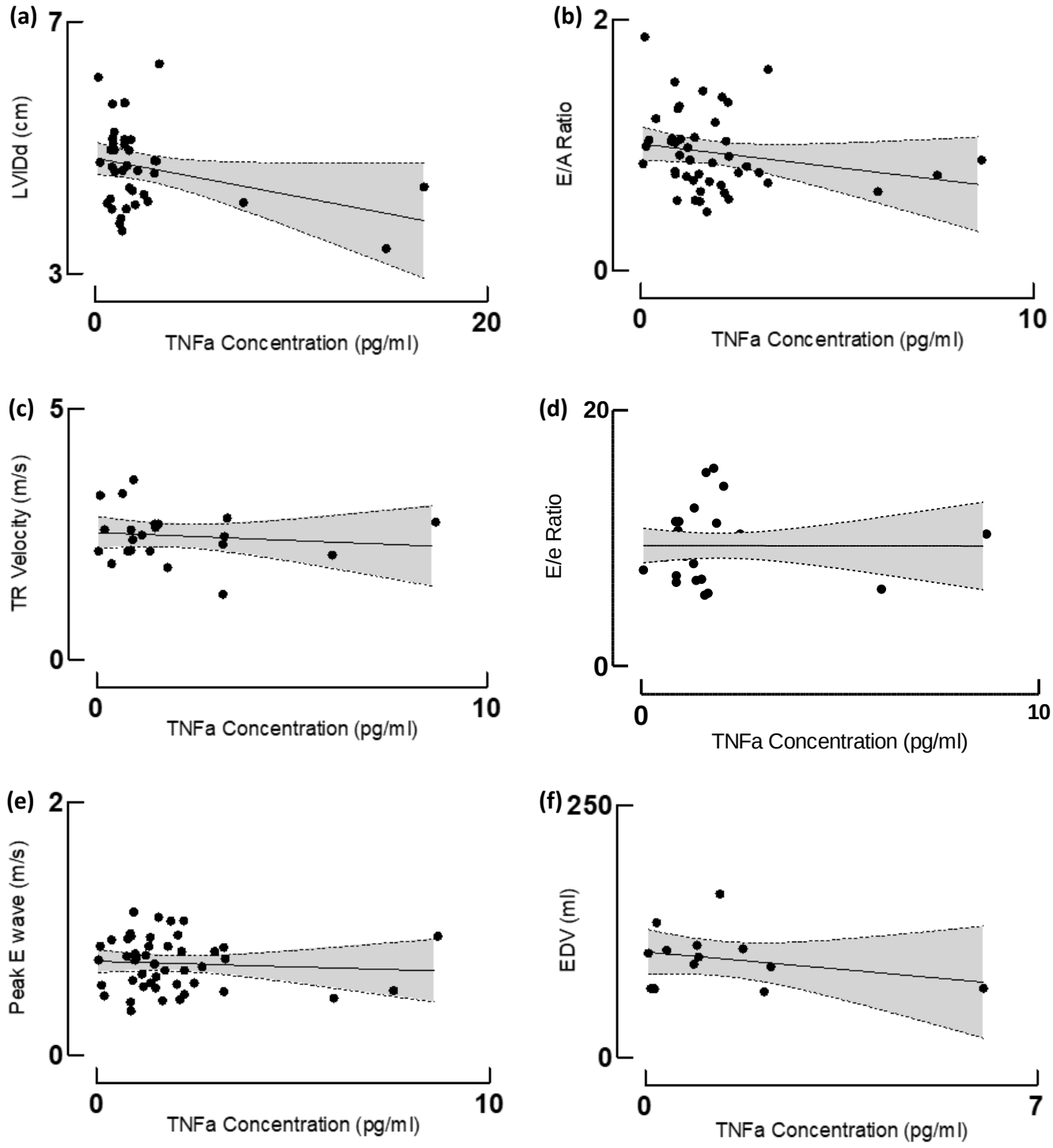

Figure 3-3. Indices of diastolic function correlated against serum TNFα levels.
(a) Correlation of LVIDd against TNFα concentration. (b) Correlation of E/A ratio against TNFα concentration. (c) Correlation of TR velocity against TNFα concentration. (d) Correlation of E/e ratio against TNFα concentration. (e) Correlation of peak E wave velocity against TNFα concentration. (f) Correlation of EDV against TNFα concentration. Pearson test was used to determine linear correlation. Spearmans test was used to determine linear correlation with EDV, E/A ratio, and E/e ratio. . The grey area on each scatter plot represents the 95% CI band.

E/A ratio negatively correlated with TNFα concentration ($p = 0.0489$; $n = 45$). The data suggests that patient TNFα levels negatively correlate with LVIDd, however this did not reach significance ($p = 0.0624$; $r^2 = 0.0932$; $n = 38$). No significant correlations were observed with TNFα levels and TR velocity ($p = 0.5767$; $r^2 = 0.01584$; $n = 22$), E/e ratio ($p = 0.3297$; $n = 30$), peak E wave ($p = 0.6279$; $r^2 = 0.0054$; $n = 46$), or EDV ($p = 0.7890$; $n = 13$).

3.5 The effects of TNFα on cellular oxidative stress

Hydrogen peroxide (H_2O_2) was primarily used as a positive control during introductory experiments; these sought to ascertain that levels of cellular oxidative stress would increase in a rat myoblast cell line (H9C2) in the presence of an oxidising agent. Cells were then treated with TNFα to determine whether inflammatory mechanisms produced a similar rise in oxidative stress after 1 hour and 24 hours. Cells were also treated in synergy with TNFα and H_2O_2 to establish whether TNFα induced further increase in oxidative stress in the presence of another oxidising agent. During which, cells were incubated with H_2O_2 for 30 minutes following treatment with the cytokine. Throughout these experiments, n refers to the number of wells for each experimental condition.

3.5.1 Validation of fluorometric oxidative stress experiment

To determine whether the sensitivity of the technique described in section 2.5 was sufficient to detect a rise in oxidative stress in our model (should one exist), hydrogen peroxide was applied at the following concentrations: 100µM and 200µM.

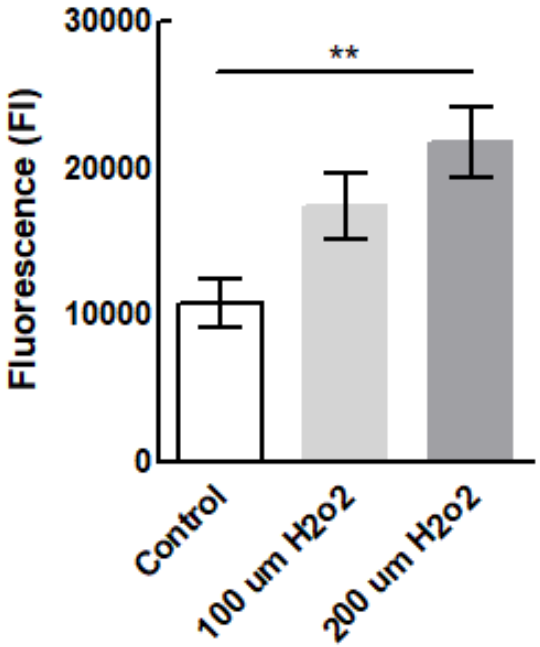

Figure 3-4. The effects of hydrogen peroxide on cellular oxidative stress in H9C2 rat myoblast cell line. Mean fluorescence of H9C2 in control (n = 102), 100µM H_2O_2 (n = 51), and 200µM H_2O_2 (n = 51). Error bars indicate ± SEM. Statistical significance was reached when $p < 0.05$. n relates to the number of experimental wells for each condition.

As demonstrated in Figure 3-4, H_2O_2 produced a concentration-dependant increase in oxidative stress. Treatment with 100µm H_2O_2 produced a moderate increase in fluorescence when compared to the untreated control, though this did not reach significance (p = >0.05, n = 51); treatment with 200µM induced a significant increase in fluorescence, and thus oxidative stress, when compared with the control (p = <0.01, n = 51).

3.5.2 The effects of TNFα on oxidative stress

A clinically relevant TNFα concentration of 2pg/ml was determined based upon on mean plasma levels obtained from our CAD patient cohort. The effect of TNFα at this concentration was examined after both 1 hour and 24-hour treatments.

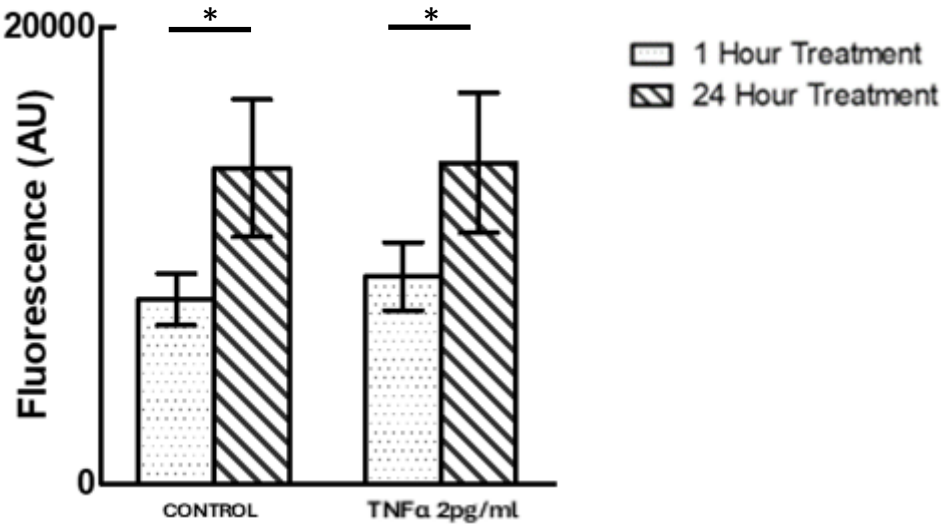

Figure 3-5. The effects of 2pg/ml TNFα on cellular oxidative stress in H9C2 rat myoblast cell line. Mean fluorescence of H9C2 in untreated control (1 hour n = 54; 24 hours n = 48) and with TNFα at 2pg/ml (1 hour n = 27; 24 hours n = 24) after 1 hour and 24-hour treatments. Two-way AVOVA and Bonferroni post hoc statistical tests were used to determine significance.

Fluorescence was significantly increased in experiments with 24-hour treatment when compared with 1 hour treatment, including the untreated controls ($p = <0.05$). However, no significant increase in fluorescence was observed following treatment with TNFα at 2pg/ml after 1 hour ($n = 27$, $p = >0.05$) or 24 hours ($n = 24$, $p = >0.05$) when compared to corresponding controls. To determine whether TNFα fundamentally produced an increase in oxidative stress, these experiments were repeated with a supraphysiological concentration of 50ng/ml.

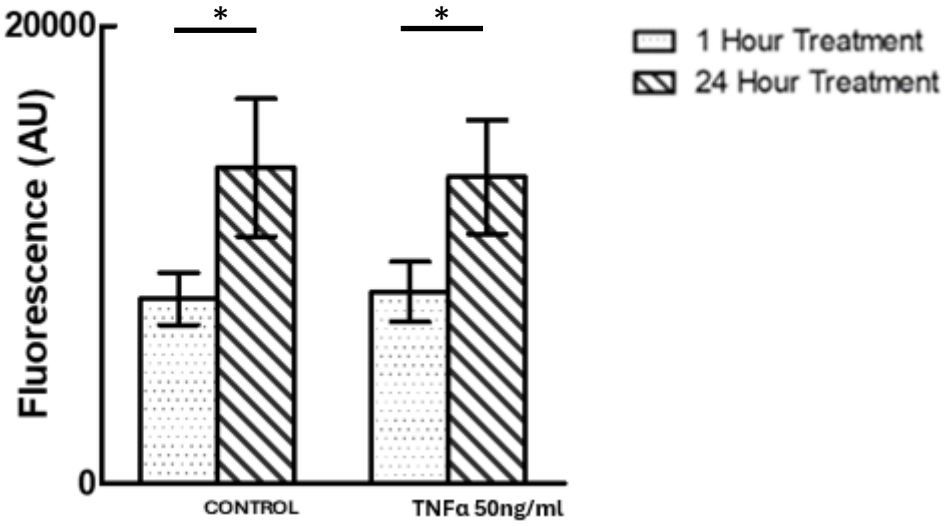

Figure 3-6. The effects of 50ng/ml TNFα on cellular oxidative stress in H9C2 rat myoblast cell line. Mean fluorescence of H9C2 in untreated control (1 hour n = 54; 24 hours n = 48) and with TNfα at 50ng/ml after 1-hour (n = 27) and 24-hour (n = 24) treatments. Two-way AVOVA and bonferroni post hoc statistical tests were used to determine significance.

Likewise, Fluorescence was significantly increased in experiments with 24-hour treatment when compared with 1 hour treatment, including the untreated controls ($p = <0.05$). No significant increase in fluorescence was observed following treatment with TNFα at 50ng/ml after 1 hour ($n = 27$, $p = >0.05$) or 24 hours ($n = 24$, $p = >0.05$) when compared with corresponding controls.

3.5.3 The effects of TNFα on oxidative stress in cells with elevated baseline levels of OS

H9C2 cells were also treated in synergy with TNFα and H_2O_2 to establish whether TNFα induced further increase in oxidative stress in the presence of another oxidising agent. During which, cells were incubated with H_2O_2 for 30 minutes following 1 hour or 24- hour treatment with the cytokine.

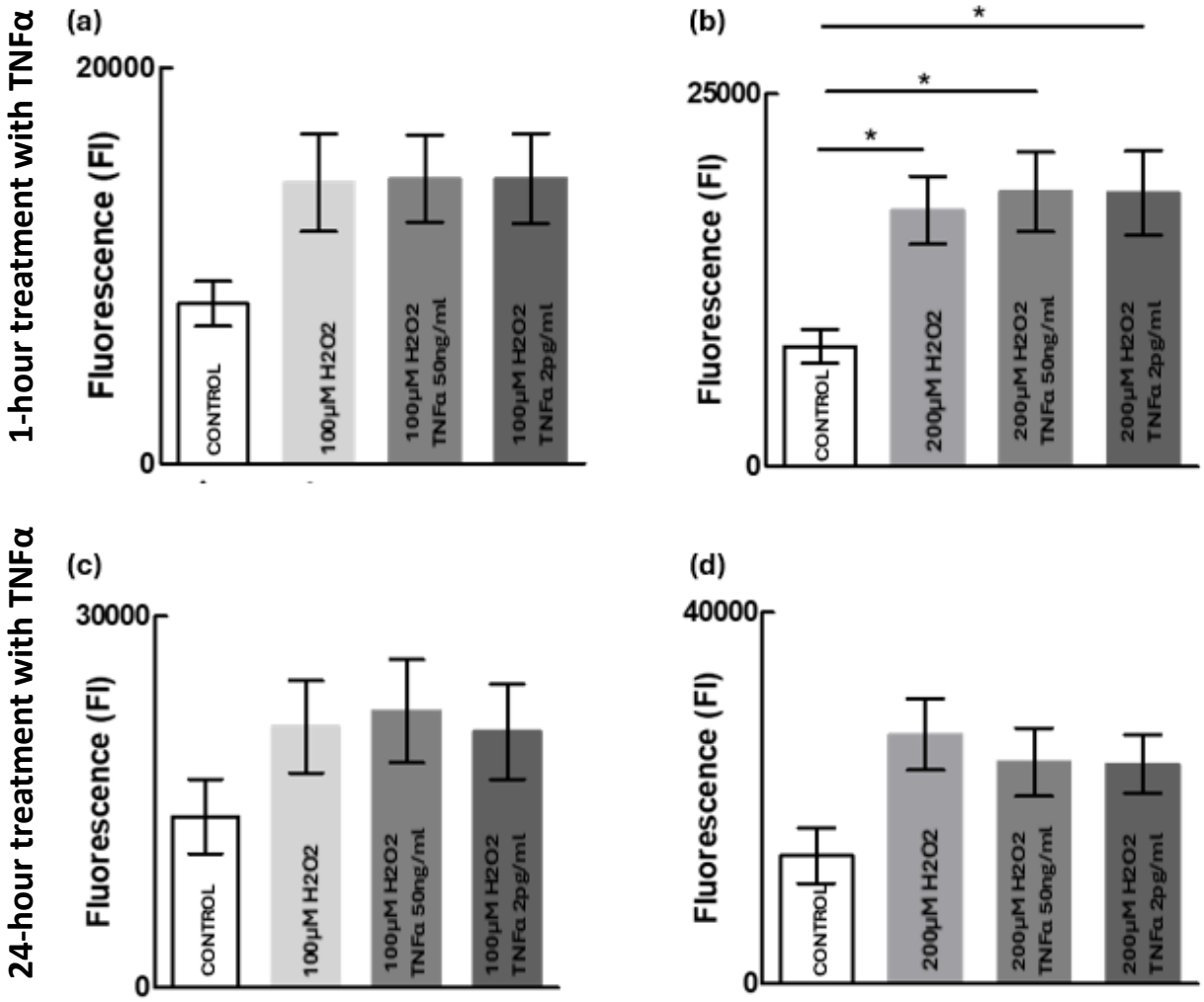

Figure 3-7. The effects of TNFα and hydrogen peroxide on cellular oxidative stress in H9C2 rat myoblast cell line. (a) 1 hour treatment with TNFα: mean fluorescence of H9C2 in control (n=54), 100μm H_2O_2 (n = 51), 100μm H_2O_2 + TNFα 50pg/ml (n = 27), and 100μm H_2O_2 + TNFα 2pg/ml (n = 27). (b) 1 hour treatment with TNFα: mean fluorescence of H9C2 in control (n = 54), 200μm H_2O_2 (n = 51), 200μm H_2O_2 + TNFα 50pg/ml (n = 27), and 200μm H_2O_2 + TNFα 2pg/ml (n = 27). (c) 24-hour treatment with TNFα: mean fluorescence of H9C2 in control (n = 48), 100μm H_2O_2 (n = 51), 100μm H_2O_2 + TNFα 50pg/ml (n = 24), and 100μm H_2O_2 + TNFα 2pg/ml (n = 24). (d) 24-hour treatment with TNFα: mean fluorescence of H9C2 in control (n = 48) , 200μm H_2O_2 (n = 51), 200μm H_2O_2 + TNFα 50pg/ml (n = 24), and 200μm H_2O_2 + TNFα 2pg/ml (n = 24). One-way AVOVA and Tukey post hoc statistical tests were used to determine significance.

No further increase was seen in cells treated with 100µm H_2O_2 in combination with either concentration of TNFα after 1 hour (each with n = 27, p = >0.05) (Figure 3-7 a). Cells treated with a combination of 200µm H_2O_2 and TNFα at both 50ng/ml and 2pg/ml for 1 hour displayed a significant increase in fluorescence when compared to the control (each with n = 27, p = <0.05). However no further significant increase in fluorescence was observed when compared to treatment with 200µM H_2O_2 alone (p = >0.05) (Figure 3-7 b). No further increase was seen in cells treated with 100µm H_2O_2 in combination with either concentration of TNFα after 24 hours (each with n = 24, p = >0.05) (Figure 3-7 c). Interestingly, Cells treated with a combination of 200µm H_2O_2 and TNFα at both 50ng/ml and 2pg/ml for 24 hours displayed no significant increase in fluorescence when compared to the control or when compared to treatment with 200µm H_2O_2 alone (each with n = 24, p = >0.05) (Figure 3-7 d).

4. Discussion

4.1 What are the cytokine concentrations in CAD?

Atherosclerosis in CAD has been associated with inflammation on both a local and systemic level. This is not surprising considering the inflammatory nature of CAD, and the significant role that the inflammatory cascade plays in disease progression. Therefore, serum and plasma cytokine levels have been a key target in the development of a method for non-invasive disease stratification. With this being said, correlations between circulating cytokine levels and CAD severity have yet to be clearly defined; the same is true when assessing the predictive capacity of cytokines in patients suspected to have the disease (Iside et al., 2023).

Prospective studies have reported associations between proinflammatory cytokines (such as IL-6, IL-1β, INF-ϒ and TNFα, amongst others) and adverse cardiac events or cardiovascular diseases – including CAD (Amin et al., 2020). Particular emphasis has so far been placed on C-reactive protein (CRP) and IL-6 as strong predictors (Bucova er al., 2008). Given that TNFα is involved in the synthesis and regulation of both (Paffen & Demaat, 2006), and is independently involved in most aspects of CAD pathology, it could be expected that elevated TNFα levels could have similar power in this context.

Numerous studies have found that serum TNFα levels are, in fact, elevated in patients with confirmed CAD when compared to controls, and also correlate to disease severity. Fadhil Jarfaar et al (2023) quantified the serum levels of a varied cytokine panel in both CAD and non-CAD cohorts using ELISA kits. Here, a significant increase in TNFα levels was observed in the CAD cohort when compared to the control group, which were 26.92 ± 7.05 pg/ml and 22.94 ± 7.37 pg/ml, respectively (P = 0.001). Similarly, Almassabi et al (2023) reported a significant increase in TNFα serum concentrations within patients with confirmed CAD (38.25 ± 10.93pg/ml) when compared with healthy controls (20.60 ± 2.99 pg/ml) (P = 0.001). Moreover, Gotsman et al (2008) found increasing levels of serum TNFα were also associated with the severity of obstructive disease; the lowest TNFα concentration tertile was associated with the lowest extent of vessel involvement (number of vessels with >70% stenosis) within the cohort, and the highest tertile was accordingly associated with the higher numbers of severely obstructed vessels. The patients analysed in this study included those with stable CAD, unstable angina, and myocardial infarction. Angiographic assessment revealed varied

levels of coronary atherosclerosis, ranging from non-obstructive to triple vessel disease with >70% stenosis. Median serum TNFα concentration within this group was 6.7 pg/ml.

Conversely, the elevated TNFα concentrations observed in patient groups when compared to control groups is not a ubiquitous finding. There are also multiple studies that report no significant elevation in CAD subjects. Iside et al (2023) grouped subjects into three categories - controls, non-obstuctive CAD, obstructive CAD – and determined serum cytokine concentrations using flow cytometry, with a multiplex bead-based immunoassay panel. Whilst significantly increased levels of multiple cytokines (including IL-6) were determined in the two CAD groups when compared to healthy subjects, there was no statistical variation in TNFα concentrations between either of the three groups (control: 12.9 pg/ml; non-obCAD: 13.5 pg/ml; obCAD: 12.1 pg/ml). Other studies supported this data: using similar methods of cytokine quantification to assess similar groups, Liu et al (2022) found that no significant difference was observed between these cohorts (non-CAD: 1.83 pg/ml; mild CAD: 1.73 pg/ml; and severe CAD: 1.76 pg/ml) (P = 0.491); likewise, Mirhafez et al (2015) demonstrated no elevation in serum TNFα concentration when comparing controls to a number of CAD-confirmed groups (control: 1.80 pg/ml; non-obstructive CAD: 1.80 pg/ml; obstructive CAD: 1.83 pg/ml; CABG: 1.86 pg/ml).

The average TNFα levels reported in the CAD cohorts cited above demonstrate the highly variable nature of quantified cytokine profiles - even when comparing analogous groups from separate studies that have used similar methods of detection. For this reason, it is difficult to define a 'range' for TNFα concentration that may characterise the plasma of CAD patients, as well as the plasma of healthy controls.

This poses challenges when trying to ascertain whether average TNFα concentrations in our own study (2.27 ± 0.35 pg/ml) are elevated (or not) without a control group to directly compare. A meta-analysis revealed that serum TNFα concentrations in healthy controls have been reported to range from nondetectable (Li et al, 2018) to 232.33 pg/ml (Yamaguchi et al., 2019). Whilst it can be speculated that the average TNFα concentration for the CAD cohort recruited to this study is not elevated, it is important to emphasise that levels vary depending on the sample collective. Controls should also be appropriately matched/stratified for age, gender, and ideally excluded if present factors were likely to affect inflammatory profile (Kumari et al., 2018). Besides this, variability will likely exist when quantifying cytokine levels

when using different methods of detection; lack of standardisation in this aspect can also be problematic when comparing TNFα levels determined in this study to CAD cohorts or control groups measured elsewhere.

4.2 Does plasma TNFα concentration correlate with cardiac function?

Given that TNFα has been shown to mediate fibrosis, hypertrophy, leukocyte infiltration, EMC alteration, and ROS generation in the myocardium in response to stressors such as overload or ischemic injury, then it is reasonable suspect that levels of circulating TNFα may correlate to indices of cardiac function. However, the myocardial response to inflammation or injury is complex and often variable. The initial depressive or stimulatory contractile response is largely dependent on the existing physiological (or pathological) milieu, which will influence the signalling pathways activated (outlined in sections 1.2.2) in such a response (Prabhu, 2004). This is usually followed by a 'late' (after 3 hours), typically cardio-depressive response that is more sustained. Again, this is also dependant on a multitude of secondary mediators and the effects of the initial response to injury. The interplay between these pathways and the timing of their activation plays an important role in apparent cytokine-induced cardiac dysfunction caused by both acute and chronic pathology (Prabhu, 2004).

4.2.1 Historical context

TNFα has received considerable attention whilst evaluating cytokine-induced cardiac dysfunction as it is often considered a prototypic proinflammatory cytokine. The apparent delayed, negative inotropic effects of which have been observed experimentally both in vivo and in vitro. Multiple studies have demonstrated profound systolic and diastolic dysfunction during the 'late' response following intravenous TNFα infusion in canine and rat models; these effects have been observed through parameters such as LVEF, end-systolic elastance (Ees), derivative of pressure over time (dP/dt) which represents the ratio of pressure change within the ventricle during isovolumetric contraction, and preload-recruitable stroke work (PRSW) (Prabhu, 2004; Herbertson et al., 1995; Eichenholz et al., 1992; Natanson et al.,1989; Pagani et al., 1992). Whilst the concentrations of TNFα used in these studies ranged from 30 – 120 µg/kg – which may be considered suprapathological -, similar observations were made in rats

treated with a pathophysiologically relevant concentration of 2.5 µg/kg via osmotic infusion over a period of 15 days. Here, Bozkurt et al (1998) reported a time-dependant reduction in myocyte shortening, LV function, and LV dilation at the end of the infusion period. These effects were fully or partially reversible when infusion ended or following administration of a soluble TNFα agonist. This also demonstrates that circulating pathological concentrations of TNFα have the potential to produce some of the clinical manifestations of cardiac dysfunction seen in CAD and related HF. Several studies have also demonstrated that TNFα can initially have a positive inotropic effect on cardiac contractility (0-3 hours) before the late, cardiodepressive response is initiated (Pagani et al., 1992; Walley et al., 1994; Murray & Freeman, 1996). This increase in contractility happened independently of changes to heart rate or cardiovascular reflex responses (Murray & Freeman, 1996). Thus, TNFα may have a direct intrinsic effect on myocardial contraction, which is then followed by the depressive response which is dependent on the activation of secondary mediators and altered gene expression (Prabhu, 2004).

The biphasic response often observed in vivo has been mirrored in vitro, albeit with less consistent findings. Although the latter phase of this cytokine-induced response was still found to be cardiodepressive, some studies reported immediate suppressive – rather than stimulatory – effects. For example, Hadgraft & Greensmith (2018) found that 50 ng/ml treatment with TNFα reduced contractility in isolated field stimulated sheep ventricular myocytes. The onset of this effect was almost immediate (<10s); a 17% reduction in systolic Ca^{2+} amplitude and 20% reduction in sarcomeric shortening could be accounted for by a 27% reduction in SR Ca^{2+} content. With this being said, a brief increase in systolic Ca was observed prior to the apparent suppression of contractility. Kumar et al (1996) presented similar findings using electrically paced rat cardiac myocytes. Here, TNFα induced depression in maximal extent and peak velocity of myocyte shortening in a dose-dependent manner. Despite the differences in the immediate response on contractility between in vitro and in vivo experiments which can be attributed to multiple factors, such studies indicate that TNFα can directly and indirectly alter cardiac function. It has also been shown that elevated levels of TNFα in some CAD patient groups can be sustained for up to 8 months post MI, rather than a transient increase in levels immediately following coronary occlusion (Ridker et al., 2000). These levels may be maintained as a result of increased infiltration of inflammatory cells into

the peri-infarct zone (Marx et al., 1997), or persistent myocardial expression of TNFα in areas outside of the infarct zone (Irwin et al., 1999). If this is the case, then TNFα could be consistently exerting pathological effects to induce maladaptive cardiac remodelling – offering predictive insight for patients at higher risk of recurrent events. Moreover, this gives reason to anticipate that TNFα levels in CAD might directly correlate to indicators of cardiac function. Correlations have already been observed between serum TNFα levels and diastolic dysfunction in HF. As well as elevated TNFα levels being associated with patient cohorts exhibiting more extensive LV dysfunction, levels have also been correlated with increased left atrial volume (LAVI), which was not seen in controls (El Missiri et al., 2020).

4.2.2 Does TNFα correlate with diastolic dysfunction?

In our CAD patient cohort, the inverse correlation between plasma TNFα levels and E/A ratio reached significance ($p = 0.0489$; $n = 45$). This suggests that higher circulating levels of TNFα could be associated with lower E/A ratio: a potential early indicator of diastolic dysfunction. This association has been demonstrated elsewhere in patients with HFpEF (Abernethy et al., 2018). In such cases, a proposed reduction in NO bioavailability and protein kinase G signalling results in a reduction in the phosphorylation of titin-associated protein kinase A sites, which is accompanied by increased phosphorylation of protein kinase C sites. These events increase the stiffness of titin, and thus impair passive diastolic filling (Abertheny et al., 2018). Given that CAD is one of the main etiological sources of HF, and that inflammation initiated within the vascular endothelium plays a key role in the pathogenesis of HFpEF, the pathological mechanisms that link TNFα to diastolic impairment could be analogous between the two diseases.

Prolonged or slowed relaxation is indicative of such diastolic impairment, and this can become apparent during the early stages. E/A ratio is one of the key parameters used in the evaluation of diastolic function. This parameter essentially evaluates the proportionality of diastolic filling during the early (E) and late (A) phase of diastole via measurement of transmitral flow. Ventricular filling during the E wave is significantly influenced by left atrial compliance and the rate at which the ventricle relaxes; these determine the pressure gradient between the left atrium and left ventricle through the LV inflow tract. Impaired relaxation reduces the

transmitral pressure gradient, thus reducing E velocity and E/A ratio (<1 in younger patients; < 0.5 in elderly patients) (Mottram & Marwick, 2005; European Study Group on Diastolic Heart Failure, 1998). As the reduction in rate of relaxation is sustained into mid-diastole, this is reflected by prolongation of the E deceleration slope. At this early stage of dysfunction, these patients may only experience symptoms with exertion. However, even mild diastolic impairment is associated with an increased risk of cardiovascular events (Mottram & Marwick, 2005; Redfield et al., 2003).

In the specific context of CAD, delayed relaxation may be observed following myocardial ischemia, where resultant regional LV dysfunction may cause dyssynchronous relaxation; in this way, it has been suggested that ischemic areas of the myocardium that are supplied by stenotic vessels will lead to these regional abnormalities (Bonow et al., 1985). This type of pathology particularly affects the dynamics of early ventricular filling (Mottram & Marwick, 2005; Gibson, 2003). The etiological source of a lower E/A ratio for some of the patients within our study cohort might be attributable to chronic ischemia, myocardial infarction, or HF (Espersen et al., 2022). Nonetheless, it could be speculated that plasma TNFα levels could be predictive of wall motion abnormalities and therefore early diastolic dysfunction in patients with coronary heart disease.

It must be emphasised, however, that this assumption is highly speculative in the framework of this longitudinal study. Firstly, patients within our cohort were not stratified according to clinical or demographic risk factors that are known to be associated with regional wall motion score index (WMSI) or TNFα levels. These include age, diabetes, BMI, and systolic BP (hypertensive) among others (Espersen et al., 2022). Therefore, it is possible that TNFα levels within our cohort are reflective of the presence of certain cardiovascular risk factors as opposed to the diastolic dysfunction that is characterised by a decrease in E/A ratio. Furthermore, studies have demonstrated that diastolic dysfunction is associated with age, diabetes, and hypertension in the absence of ischemic heart disease; Sundqvist et al (2020) demonstrated that these factors were associated with reduced tissue doppler velocities, higher E/e ratio, lower E/A ratio, and higher LAVI in an outpatient population with either mild or no valvular disease. Whilst it is likely that increased levels of inflammation (and potentially TNFα) associated with these factors might play a role in diastolic dysfunction, this could lessen the predictive capacity of TNFα alone in the context of CAD. In the same way, it is important

to consider that clinical groups are often highly heterogeneous; often, a lack of biological symmetry exists between individuals within such cohorts. This presents challenges with regards to precarious interpretation results or data, and a need to stratify patients in a meaningful way (Marquand et al., 2016). Such analyses in our own study can be appropriately rectified following further acquisition of patient clinical data and increased study power.

Another consideration is whether a correlation between plasma TNFα levels and E/A ratio alone is sufficient to suggest an association between TNFα concentrations and diastolic dysfunction; without associations between this cytokine and other indices of diastolic dysfunction alongside, it may be impertinent to suggest a definitive relationship between TNFα and diastolic impairment. Currently, diastolic dysfunction is difficult to define without invasive techniques. When using echocardiography, a diagnosis of diastolic impairment is often based on the exclusion of systolic dysfunction – or HFpEF. However, there have been reports that echocardiographic indices do not always accurately portray the true diastolic workings of the left ventricle, which casts doubt on the assumption that diastolic impairment is the cause of HFpEF (Bench et al., 2009). This is also relevant when considering the cause of apparent cardiac dysfunction that manifests in CAD.

This is particularly true for parameters such as E/A ratio, peak E and A wave velocities, and E deceleration time – all of which are based on the transmitral flow profile. Firstly, patient age should be adjusted for prior to classification (as normal, impaired, pseudonormal, or restrictive diastolic function) and results typically require careful interpretation. This is due to the fact that they are variable depending on loading conditions and can therefore offer conflicting information (Mottram & Marwick, 2005). The extent of diastolic dysfunction is usually synonymous with LV filling pressure, which ultimately impacts the transmitral flow pattern. For instance, slowed relaxation will cause a decrease in E velocity whilst the elevated atrial pressure acts to increase this parameter. These two factors also have opposing influence on E deceleration time. Thus, these effects that accompany diastolic dysfunction tend to become compensated and then over-compensated in terms of the E/A ratio and E deceleration time; the relationship between these indicators are non-linear as a result of these loading effects, and different filling patterns in individual patients may reflect worsening diastolic pathology or altered loading conditions (Mottram & Marwick, 2005; Garcia et al.,

1998). For this reason, parameters that are less dependent on loading should be incorporated into the interpretation of diastolic function (Mottram & Marwick, 2005).

Another parameter that also appeared to have a negative association with plasma TNFα levels in this study was the left ventricular internal diameter end diastole (LVIDd), although this did not reach significance ($p = 0.0624$). This indicator is used as part of the geometric assessment of the left ventricle. Although geometric indices like LVIDd are not ubiquitously used for clinical diagnosis of diastolic dysfunction, they may prove to useful when supplementing other parameters like E/A ratio (Hamza et al., 2020). Typically, this parameter is used in the context of systolic HF; here, internal ventricular diameters can be used to determine whether ventricular dilation is contributing to a patient's cardiac dysfunction (Matthew et al., 2017). But more often, is used alongside other geometric indices such as interventricular septum thickness in diastole (IVSTd) and posterior wall thickness in diastole (PWTd) – all of which should be indexed according to body surface area (Matthew et al., 2017). From these, key measurements that are perhaps more relevant for possible diagnoses can be calculated, such as left ventricular mass (LVM) and relative wall thickness (RWT) (Ojji et al., 2015). LVIDd has also been shown have conflicting association with diastolic dysfunction; both positive correlations (Germing et al., 2011; El Aouar et al., 2013) and negative correlations have been shown to exist (Hamza et al., 2020; ul Haq et al., 2017).

In the present study, it is important to stress that the suggestion of a definitive association between TNFα and LVIDd is an extrapolation; again, perhaps a more significant correlation would emerge where study power is increased, and complete clinical data is ascertained for all patients included. This would allow a more thorough geometric evaluation for each patient and appropriate indexing (ie for body surface area) of this parameter. Likewise, it would have been beneficial to carry out multivariate analysis to adjust for concomitant factors such as gender, age, hypertension, and diabetes (Gottdiener at al., 2003).

Finally, and fundamentally, it should also be emphasised that correlation does not necessarily indicate causation between one variable and another. Correlational analysis is a useful tool to assess the strength of association between two variables and is able to accommodate data that is not normally distributed (Hung et al., 2017). Furthermore, certain tests such as the Peason correlation coefficient have been found to reliably predict the relationship between variables in underpowered data sets (Hung et al., 2017; Havlicek & Peterson, 1976).

Nonetheless, these types of analyses can only provide a measure of association – not causation. One of the most important reasons has already been discussed: an apparent relationship between variables could be impacted by variables that have not been observed (Hung et al., 2017). In the present study, this could relate to confounding variables such as the presence of risk factors within the patient cohort. An additional reason that observed correlations should be interpreted with caution is the fact that significance testing can overused on the same data set – a phenomenon that occurs often in clinical studies (Hung et al., 2017). In this case, comparing quantified TNFα levels with twelve indicators of cardiac function would have more probability of producing a Type 1 error, where there is a 5% chance (if $p < 0.05$ determines significance) that significant results are in fact a random occurrence (Hung et al., 2017). In a similar way where the importance of a significant finding might be exaggerated, small sample sizes can also produce such results. However, these correlations are often unstable; for this reason, it has been recommended that sample sizes are ideally between 150 – 200 and that fundings should be carefully interpreted until verified by other similar studies (Hung et al., 2017; Guadagnoli & Velicer, 1988).

4.2.3 Does TNFα correlate with Systolic function?

In this study, no correlation was observed between plasma TNFα levels within our CAD patient cohort and any measure of systolic function; this is despite the fact that evidence supporting the role of inflammation in reduced systolic function is more robust than that presented for diastolic function (Gullestad et al., 2012). Historically, it has been well established that the cardiac inflammation that might occur as a result (or in the absence of) ischemic injury can induce pathogenic remodelling and systolic impairment (Rolski & Błyszczuk, 2020; Van Linthout & Tschöpe, 2017). Specific mechanisms by which TNFα might negatively affect systolic function have been previously described (section 1.2.2.2 and 1.2.2.3), and multiple studies using transgenic mouse and rat models have further demonstrated how cardiac-restricted overexpression of TNFα might adversely affect cardiac remodelling and tissue viability following MI (Sun et al., 2004; Berthonneche et al., 2004; Bryant et al., 1998). Furthermore, systemic administration of this cytokine at levels comparable to those observed in patients with HF has induced dilated cardiomyopathy consistent with the HFrEF phenotype in such models (Gullestad et al., 2012; Bozkurt et al., 1998).

Patients with HFrEF and atherosclerotic disease have previously been characterised by elevated circulating levels of TNFα and other pro-inflammatory cytokines (Xing et al., 2017; Li et al., 2020), and in some cases, subsequent improvement in systolic function were associated with a reduction in these inflammatory mediators (Shao et al., 2018).

However, the causative association between TNFα and reduced systolic function is not straightforward. This has been made apparent by several studies highlighting the dual action of TNFα on the myocardium. For example, transgenic deletion of TNFR-2 has been shown to lead to an exacerbated state of cardiomyopathy (Higuchi et al., 2004), and pharmaceutical inhibition of TNFα can worsen cardiac function by enhancing cardiomyocyte apoptosis, post infarction (Wang et al., 2018). Whilst anti-TNFα therapeutics might reduce the risk of developing cardiovascular disease, such therapies have yielded disappointing results with regards to improving systolic function in clinical studies and may even prove to be harmful (Rolski & Błyszczuk, 2020; Baniaamam et al., 2020; Mann et al., 2004; Chung et al., 2003; Padfield et al., 2013). Nonetheless, the literature suggests that TNFα levels do generally correlate with – or associate – to systolic function. Perhaps full multivariate analysis would have provided clearer insight with regards to assessing correlations between circulating TNFα levels and indicators of systolic function in our own study, or such associations will emerge upon continued patient recruitment and analysis.

4.3 Validation of methodology for the measurement of intracellular oxidative stress

In the present study, we used a well-established and reliable fluorescent probe, CM-H_2DCFDA ((5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) at a concentration of 5 µm, to quantify levels of general cellular ROS in H9C2 rat myoblasts. This dye is applicable to most cell types, and although it is considered H_2O_2-specific, the range of detectable ROS include thiol, hydroxyl, and carbonate radicals (Oparka et al., 2016). CM-H_2DCFDA passively diffuses through the cell membrane; in the cytosol, it then hydrolysed via cellular esterases to form CM-H_2DCF. This is subsequently oxidised by molecular oxygen or other radical species thus producing the fluorescent product, CM-DCF (Oparka et al., 2016).

During the introductory experiments, cells were treated with sublethal concentrations of H_2O_2 (100 µM and 200 µM) that are known to increase cellular OS, as per previous studies (Suematsu et al., 2003; ref). A dose-dependent increase in fluorescence was observed, thus demonstrating the validity and sensitivity of the technique to detect a pathological increase in OS; a moderate increase was observed in cells treated with 100 µM H_2O_2 ($p = >0.05$) and a significant increase was detected in those treated with 200 µM H_2O_2 ($p = <0.01$).

4.4 Does TNFα induce oxidative stress?

Subsequent experiments challenged cells with TNFα at both our clinically relevant concentration (2 pg/ml) and at a supraphysiological concentration (50 pg/ml) (Das et al., 2010), treated for 1 hour and 24 hours. Surprisingly, no significant increase in ROS was observed at either concentration or timepoint when compared to corresponding controls.

Cells were then treated with both concentrations of TNFα for 1 hour and 24 hours, but in combination with 100 µM or 200 µM H_2O_2 to ascertain whether TNFα further increased levels of ROS in cells with elevated baseline levels. In this set of experiments, cells treated with TNFα for 1 hour in synergy with 200 µM H_2O_2 produced a significant increase in ROS when compared to controls. However, no significant difference was observed between these and cells treated with TNFα alone for 1 hour. Instead, this increase corresponded to the significant increase observed in cells treated with 200 µM H_2O_2 during the introductory experiment. Interestingly, when this was repeated with 24-hour treatments with TNFα, no increase was observed in cells

treated in combination with 200µM H_2O_2. This could suggest that treatment with TNFα over a longer timeframe actually attenuated the effects of 200µM H_2O_2, to a degree. There is evidence to suggest that cells previously exposed to some level of oxidative stress can exhibit adaptive changes in their response upon repeated exposure to oxidative agents (Goffart et al., 2021). Perhaps 24-hour exposure to TNFα (despite no detectable increase in ROS) elicited a protective cellular outcome following subsequent exposure to H_2O_2 in a similar way.

Nonetheless, these results are surprising given the fact that pathological initiation and progression of CAD is facilitated by the chronic state of vascular inflammation, where oxidative stress has been found to be a key mediator in the regulation of vascular homeostasis (Kibel et al., 2020). Oxidative stress is a pathological commonality shared between the vascular and cardiac dysfunction seen in CAD, often occurring in the myocardium following chronic, transient, and acute myocardial ischemia. Furthermore, many of the risk factors associated with CAD play a considerable part to exacerbate this inflammatory and oxidative state (Kibel et al., 2020).

Mechanisms by which TNFα has previously been shown alter the vascular and myocardial redox state has been demonstrated in-vitro and in animal models. This is alongside clinical evidence that these effects are applicable to the pathology seen in CAD patient cohorts. Such examples of these mechanisms have been outlined in more detail in previous sections (1.2.2.1, 1.3.3.1, and 1.3.3.2). In brief, TNFα-induced OS in the vascular endothelium has been shown to occur as a result of its effects on the regulation of eNOS expression, eNOS uncoupling, increased extravasation of immune cells into the vascular wall, adverse NFKB activation/signalling, fibrogenic and osteogenic activation, and recruitment of NADPH oxidases (Senoner & Dichtl, 2019; Farrar et al., 2015). Similar pathways are concurrent in the myocardium. Enhanced NADPH, NF-KB, and mitochondrial enzyme signalling can increase myocardial ROS production, and in turn result in cardiac hypertrophy; increased expression of MMP's and TGF-β which reduces cardiac compliance; and ROS-mediated caspase activation which initiates cardiomyocyte apoptosis (Senoner & Dichtl, 2019; Kibel et al., 2020). Furthermore, the effects of TNFα on calcium induced calcium release and reduced contractility also appears to be somewhat mediated by its pro-oxidant activity (Cailleret et al., 2004).

In vitro experiments have demonstrated that TNFα elicits the production of ROS in various cell types. Rahman et al (2002) treated a human alveolar epithelial cell line with TNFα (10 ng/ml), and 100mM H_2O_2 as a positive control. The subsequent rise in oxidative stress elicited by both treatments was established from a concurrent decrease and increase in the antioxidant reduced glutathione (GSH) and oxidised glutathione (GSSH), respectively. The pro-oxidant action of TNFα in this case was effected via histone acetylation, which was associated with activation of NF-KB and AP-1 transcription factor DNA binding. These mechanisms also led to a significant increase in IL-8 production in treated cells. In human umbilical vein endothelial cells (HUVECs), two-hour treatment with TNFα (5 ng/ml) produced a significant increase in cellular ROS as detected with CM-H_2DCFDA staining. TNFα-induced ROS production was then depreciated following pre-treatment with GLP-1 receptor agonist, Liraglutide. This reversal coincided with repressed induction of NADPH subunits ($p22^{phox}$ and $gp91^{phox}$), inhibited protein kinase C alpha (PKCα) translocation, increased expression of catalayse and SOD, and a reduction in NF-BK activation and apoptosis (Shiraki et al., 2012). In primary cultured neonatal rat ventricular myocytes, Suematsu et al (2003) demonstrated that one-hour treatment with TNFα (10 ng/ml) produced a significant increase in cellular ROS; again, H_2DCFDA staining detected an initial increase in fluorescence in as early as 30 minutes. Southern blot analysis in this study also revealed a simultaneous dose-dependent decrease in mitochondrial DNA (mtDNA) copy number. These effects were transposed with the antioxidant α-tocopherol, and separately, with sphingomyelinase inhibitor D609. Taken together, these suggest that TNFα induced OS in these cells - along with resultant damage to mtDNA - is mediated by the sphingomyelin-ceramide pathway. Such findings corroborate those of other studies and suggest that this pathway could be implicated as a mechanism by which TNFα contributes to myocardial remodelling and dysfunction.

One potential reason that TNFα did not have elicit an independent increase in cellular ROS during these experiments may have been due to the relatively short treatment timeframe of 1 hour or 24 hours. Whilst studies cited above have shown an acute increase in ROS in response to various concentrations of the cytokine, more prolonged exposure has been required elsewhere to evoke a significant response. Saraf et al (2021) used cardiomyocytes derived from human induced pluripotent stem cells (hiPSC's) and treated with a range of concentrations of TNFα over a period of four days. Treatments ranged from 1 – 100 ng/ml.

Similar to our own study, DCFDA was used as a fluorescent probe to quantify cytoplasmic ROS. Here, TNFα-treated cells only elicited a significant increase after 2 days of treatment when compared to controls. At the end of this treatment period, however, cells treated with each concentration all demonstrated increased levels of ROS that was dose dependent. RNA-Seq analysis carried out on cells treated with 20 ng/ml for 4 days revealed the differential expression of 6565 genes; many of these were related to the upregulation of TNFR-1 and its associated signalling pathways, which is consistent with the cardiotoxic and inflammatory response induced by TNFα (Saraf et al., 2021; Al-Lamki et al., 2009; Defer et al., 2007). Perhaps a longer treatment – particularly with our clinically relevant concentration – would have better represented the chronic inflammatory environment present within the patient cohort in our own study.

Alternatively, another reason that TNFα-induced increase in ROS was not observed in our own study may be due to the multifaceted nature of TNFα signalling, thus producing paradoxical effects (Blaser et al., 2016). Even in clinical settings, high TNFα levels have characterised patients with well-compensated heart failure and increased protection against ischemic injury (Defer et al., 2007; Sack, 2000; Sack, 2002).

Studies conducted by Tada et al (2001) and Sakon et al (2003) demonstrated how the interplay between TNFα-induced activation of NFKB and MAPK pathways can modulate cellular outcome to mitigate oxidative stress and promote cellular survival. TNFα receptor associated factors (TRAFs) are signalling intermediates implicated in NFKB and JNK activation (Sakon et al., 2003; Mak & Yeh, 2002). Here, murine embryonic fibroblasts (MEFs) were harvested from TRAF2 and TRAF5 double knockout (DKO) mice and wild type mice. TNFα-induced NFKB activation in the DKO MEFs was abated; these cells were highly susceptible to injury and cell death (Tada et al., 2001). Additionally, DKO MEFs exhibited prolonged JNK activation when exposed to TNFα, which was not observed in WT MEFs. The notion that this manner of prolonged or delayed activation of JNK occurring in the absence of NFKB (as opposed to immediate or transient activation) induces cellular damage/death has also been established elsewhere (Tang et al., 2002; Maeda et al., 2003). NFKB is thought to prevent cytotoxicity in such circumstances by facilitating the upregulation of antiapoptotic genes (such as XIAP, encoding X-linked inhibitor of apoptosis) (Tang et al., 2001), and proteins (such as GADD45β) that directly target/inhibit JNK activators (Papa et al., 2004).

More importantly, TNFα induced NFKB activation can positively regulate TNFα induced ROS production. Sakon et al (2003) demonstrated this in the aforementioned DKO MEF's; an increase in ROS was observed in these NFKB-defective cells when stimulated with TNFα, which was not observed in WT cells. The cytotoxic effects/ROS accumulation exhibited by the DKO cells was attenuated in the presence of an antioxidant, suggesting that the pro-survival mechanisms in WT cells function to mitigate the pro-oxidant effects of sustained TNFα-induced JNK/MAPK activation (Sakon et al., 2003).

As outlined in section 1.2, the contradictory effects of TNFα are often exerted through downstream signalling events associated with the two primary receptors for the cytokine – TNFR-1 and TNFR-2. Both receptors can each induce differential outcomes, depending on the context of the biological environment. ROS generation induced by TNFα plays an intimate role in mediating these signalling pathways downstream, which ultimately affect the outcome of these signalling events (Blaser et al., 2016). TNFR-1 is ubiquitously expressed by most cell types and can interact with both soluble and membrane-bound TNFa. TNFR-2 is expressed by endothelial cells, immune cells, and cardiomyocytes, and is only sensitive to membrane bound TNFα (Blaser et al., 2016; Grell et al., 1995; Torre-amione et al., 1995). Signalling pathways activated by the latter are more often implicated in immune modulation, cell survival, and tissue regeneration (Blaser et al., 2016; Faustman & Davies, 2010). This is largely due to the fact that TNFR-2 lacks the intracellular death domain (TRADD) that is associated with TNFR-1, which prevents the initiation of caspase-mediated apoptosis during downstream signalling events facilitated by TRADD activation (Rolski & Błyszczuk, 2020; Wajant & Siegmund, 2019).

Although the exact functional role of the TNFR's is not completely understood, there is evidence that TNFα can also exert these protective effects on the myocardium through TNFR-2 signalling (Defer et al., 2007). Inhibition of TNFR-1 in vivo have demonstrated that preferential activation of TNFR-2 has beneficial effects on post-ischemic remodelling, cardiac function, and cell survival (Ramani et al., 2004; Higuchi et al., 2004). Defer et al (2007) attempted to delineate the respective roles of TNFR-1 and TNFR-2 signalling in isolated rat cardiomyoctyes. This study found that the use of neutralising monoclonal antibodies (Mabs) for each receptor conferred opposing, dose dependant effects on cellular ROS production. AntiTNFR-1 Mabs inhibited ROS generation and decreased the inotropic effects (measured via

Ca^{2+} transient amplitude and fractional shortening) of the early (positive) and late (negative) response to TNFα infusion (25 ng/ml). In addition, TNFR-1 inhibition had a suppressive effect on TNFα-induced cell death. Similar results relating to these parameters were seen in NAC-treated rats. Conversely, cardiomyocytes incubated with antiTNFR-2 Mabs prior to TNFα exposure exhibited increased ROS production, rapid loss of contractile function, and exacerbated TNFα induced cell death following TNFα treatment. These highlight the fact that pro-oxidant and damaging effects of TNFα are largely exerted through TNFR-1 signalling, whilst also drawing attention to the significant interplay between ROS and TNFR-1 signalling. Moreover, these results also demonstrate that TNFα can act in a cardioprotective matter to mitigate these harmful effects through TNFR-2 activation/signalling. Although it appears that TNFR-1 is the predominant effector on cellular outcome, TNFR-2 can act as a significant limiting factor to positively self-regulate the effects of this cytokine (Defer et al., 2007). Such mechanisms facilitating protection against ROS accumulation could have been at play in our rat myoblast cell line when exposed to TNFα.

4.5 Does time alone induce oxidative stress in vitro?

An observation that was made during our experiments, that should be noted, was that time alone appeared to induce the production of ROS in our cultured cells; control cells exhibited increased levels of cellular ROS during 24-hour post treatment experiments when compared to those conducted 1 hour post treatment. This could be attributable to multiple factors, some of which should be considered in future experiments to better replicate physiological or pathophysiological conditions.

Certain culturing procedures may have an impact on cellular redox status. Cell trypsinisation, for example, has been shown to decrease levels of reduced glutathione (GSH) (Halliwell, 2003; Reiners et al., 2000). Exposure to trypsin-containing solutions when detaching adherent cell lines in preparation for sub culturing procedures caused a 95% loss in cellular GSH in some cases without impacting cellular viability. This will likely predispose cultured cells to a state of oxidative stress, particularly those being used at a higher passage number. With this being said, loss of glutathione in this way is typically transient; GSH levels return normal levels after a period of 24 hours. Nonetheless, this draws attention to the fact that cytotoxic evaluation

may be influenced by the time elapsed between trypsinisation and exposure to a toxic agent (Reiners et al., 2000).

Conditions in which cells are cultured can often promote ROS accumulation in the absence of treatment with oxidising or cytotoxic agents. One way in which these conditions can impose a state of increased oxidative stress is due to the fact that cultured cells are often exposed to relatively hyperoxic conditions in comparison to analogous cell types in vivo (Halliwell, 2003; Alva et al., 2022). Whilst cell culture is often performed at 95% air/5% CO2, with approximately 150 mm/Hg oxygen tension, in vivo O2 concentrations typically range from 1 to 10 mm/Hg (with the exception of some cell types) (Halliwell, 2003). Therefore, under culturing conditions, ROS generation facilitated by electron leakage and cellular enzymes may be increased. Another potential factor that may influence the cellular redox state of cultured cells is the culturing media; media is often deficient in antioxidants, whilst providing a pro-oxidant environment at the same time. Poor solubility or stability of antioxidants like vitamin E and C may prevent ideal supplementation of culturing media (Halliwell, 2003; Leist et al., 1996). However, supplementation with inorganic metal salts to aid cell growth can simultaneously help to catalyse reactions involved in free radical production (Halliwell, 2003; Aruoma & Halliwell, 1987). These, along with increased cell density in experimental wells incurred over the additional 24-hour period, may explain the time dependant increase in ROS in control cells in our own experiments. Conditions such as these, as well as characteristics of cells that are maintained in culture, should be contemplated when interpreting data – particularly when investigating ROS-induced signalling pathways assumed to be taking place in vivo (Halliwell, 2003).

4.6 The effect of therapeutics on CAD pathophysiology

Effective management of CAD, associated risk factors, and symptoms of angina have been established through the use of various therapeutic agents (Malakar et al., 2019). Pharmacological management has assumed a significantly important role in recent times and is also used as a robust form of secondary prevention (Kovacic & Fuster, 2011). It should be acknowledged that these therapeutics may have impacted the patient variables within this study; medication prescribed to patients scheduled for routine revascularisation surgery

within this cohort were unknown and may have affected absolute cytokine measurements or echo parameters reflecting cardiac function. Below, commonly used drug therapeutics and their potential effects on these parameters are described.

β-Blockers are a mainstay for the treatment of hypertensive patients because of the negative inotropic and chronotropic action of these drugs. Cardioselective β-Blockers are most commonly used in these circumstances, but non-selective β-Blockers such as Propranolol are still prescribed for the treatment of angina. The subsequent reduction in heart rate and contractile force allows more time for diastolic filling, decreases myocardial oxygen demand, thereby improving coronary perfusion and reducing symptoms of angina (Rosendorff et al., 2007). Studies have reported conflicting results regarding the effects of β-blockade on echocardiographic assessment. Findings presented by Eichhorn et al (2003) suggested that β-blocker treatment was associated with increased contractile reserve and improved LVEF in patients with CHF. Contrarily, Li et al (2014) found that this treatment had no significant modulatory effect of LVEF, nor any electrocardiographic parameters used during assessment, although a reduction in LVM was observed. Thus, treatment with β-blockers may also affect geometric dimensions used to characterise cardiac remodelling.

In cases where the administration of beta blockers does not sufficiently reduce blood pressure (or contraindications are present), then calcium channel blockers (CCB's) may be used in place of or in combination (BHF, 2023). CCB's encourage vasodilation and decrease vascular resistance, and nondihydropyridine agents (one of two major categories of CCB's) can also be used to slow sinus discharge rate and is faster acting. The anti-hypertensive effects of CCB's may also improve LV diastolic function. Takami & Shigematsu (2003) demonstrated a significant decrease in E deceleration time which occurred in conjunction with a significant increase in E/A ratio in patients treated with certain agonists (cilnidipine and amlodipine, sensitive to N-type Ca^{2+} channels) after a three-month period.

Angiotensin Converting Enzyme (ACE) inhibitors have similar antihypertensive effects as CCB's by decreasing sympathetic activity and inducing vasodilation. Inhibition of ACE reduces the bioavailability of angiotensin II, which is an important effector molecule in the Renin-Angiotensin System (RAS), and thus reduces the molecule's hypertensive effects (Cutrell et al., 2023; Atlas, 2007). These are particularly useful when hypertension persists in patients with

heart failure or left ventricular dysfunction (Rosendorff et al., 2007). These therapeutics have the capacity to increase stroke volume and thus improve cardiac output in patients with CHF and prevent disease development in patients with asymptomatic LV dysfunction (Khalil et al., 2001).

To bring levels of LDL cholesterol within recommended limits (<70 mg/dL) in patients with dyslipidaemia, statins remain the primary drug of choice (Koskinas et al., 2017). Statins are considered most efficient at reducing circulating plasma cholesterol; they work my inhibiting HMG-CoA, a key enzyme involved in endogenous cholesterol synthesis. Inhibition of this enzyme also has pleiotropic effects that enhance the drugs antiatherogenic mechanisms (Stancu & Sima, 2001); Statins have been shown to also exert anti-inflammatory effects, albeit with inconsistent results in various clinical trials (Abbasifard et al., 2022). Abbasifard et al (2022) conducted a meta-analysis to evaluate the effects of statins on plasma/serum levels of a number of inflammatory markers in patients with CVDs. Here, analyses revealed that TNFα was significantly reduced in response to statin administration. There are additional non-statin alternatives that may be used for patients with atherosclerotic cardiovascular disease, such as proprotein convertase subtilisin-kexin type 9 (PCSK9) inhibitors and cholesterol absorption inhibitors. Intensive treatment with all of these drugs have demonstrated significant clinical benefits – each 1mmol/L reduction in LDL cholesterol has been associated with a 19% decrease in major vascular events (Koskinas et al., 2017). Additionally, this class of lipid-lowering drugs have also been shown to affect plasma concentrations of TNFα in patients presenting with unstable plaques and dyslipidaemia; after a three-month treatment period, TNFα levels (along with IL-18 and IL-6) were found to be significantly reduced (Basiak et al., 2022).

A number of other key drug therapies are available to aid the management of CAD. Aspirin is often administered to patients with occlusive vascular disease to reduce the risk of thrombus formation and is usually prescribed indefinitely for patients following revascularisation procedures. These drugs have anti-inflammatory and cardioprotective properties. The former effects are exerted through the inhibition of pro-inflammatory signalling pathways facilitated by NFKB, and cyclooxygenase (COX-1 and COX-2) (Morris et al., 2009). In a similar way to statins, Gao et al (2009) demonstrated that treatment with an intermediate dose (300mg) over a three-week period significantly decreased serum levels of inflammatory markers in

patients with metabolic syndrome, including TNFα. Similar suppressive effects exerted by aspirin on these inflammatory markers have been observed elsewhere, whilst levels of immunoregulatory markers such as IL-10 remained unaffected (Goldstein et al., 2006). Thus, such studies provide evidence that aspirin administration might preferentially supress or attenuate elevated levels of proinflammatory cytokines, like TNFα, in patients with chronic inflammatory conditions (Berk et al., 2013) such as CAD.

4.7 Clinical Implications

Biomarkers that are developed for CAD should ideally be accurate, reproducible when using standardised techniques, minimally invasive, highly specific or sensitive for the intended identified outcome, and independently explain a certain portion of the outcome irrespective of established predictors for it to have potential clinical use (Vasan, 2006). For screening potential, it is beneficial that proposed biomarkers have known reference limits. For diagnostic use – particularly when identifying ischemic injury – biomarkers with myocardial specificity is favourable, and release should correspond with the degree of ischemic burden. Finally, prognostic/treatment capacity is based on the biomarker's ability to provide additional information alongside a known alternative prognostic index, be useful when determining management strategy or appropriate drug treatment, and managing the progression of disease (Vasan, 2006).

It is well established that TNFα - along with other inflammatory, oxidative, and metabolic mediators – is intimately associated with the signalling cascades involved with immune cell recruitment, atherogenesis, plaque progression, rupture, and myocardial dysfunction in CAD (Libby, 2002). However, the predictive capacity of potential circulating biomarkers such as this has still not been fully elucidated. Nonetheless, there is compelling evidence of strong individual and mutually adjusted associations between these and CAD development (Kaptoge et al., 2013). In a clinical context, these could potentially aid disease stratification and offer predictive insight (Subirana et al., 2018). This evidence is also relevant with specific regards to TNFα; as previously highlighted here, the literature supports that serum TNFα levels are often elevated in CAD patient cohorts when compared to controls, and that this cytokine has the power to independently predict CAD severity or the extent of vessel involvement (Subirana et

al., 2018). TNFα levels have also been linked with recurrent coronary events following MI when comparing these individuals with controls (Ridker et al., 2000). Given the fact that TNFα is suspected to play a role in cardiac dysfunction and remodelling (Nian et al., 2004), further investigation of the correlates in our own study could suggest that TNFα has both diagnostic and predictive capabilities in CAD patient cohorts at risk of cardiac dysfunction or ACS.

In spite of this, these findings are not consistent according to numerous studies. For example, Inoue et al (2008) demonstrated that – following multivariate analysis - plasma levels of several inflammatory mediators (including TNFα) did not have any independent predictive capacity for cardiovascular events in a stable CAD patient group over a 7-year period. Liu et al (2022) likewise demonstrated that TNFa serum levels did not predict severity of disease in patients suspected to have CAD. Studies such as these highlight the reason why the potential use for cytokine biomarkers in CAD is still heavily contested (Zakynthinos & Pappa, 2009). One of the main challenges that limit the use of cytokines as biomarkers for CAD is this apparent lack of definable parameters and the need for highly sensitive tools. Moreover, such tools – such as ELISA kits – will inevitably capture significant fluctuations in levels which reflect other biological factors (Kim et al., 2023; Ueland et al., 2015).

4.8 Limitations

The work carried out here was completed over a period of 12 months, but was part of a wider, ongoing longitudinal study. This can present challenges with regards to consistent data collection and acquisition over a prolonged period of time (Caruana et al., 2015). This is applicable to areas of this study conducted at both of the contributing institutions. With regards to the collection of echo data, consistent and standardised measurement of parameters can be difficult to enforce. Measurement of these parameters may differ depending on the clinical personnel carrying out the examinations, and also depending on the anatomy of the patient. Examination of echocardiographs will also rely on subjective interpretation (Bella et al., 2002). Consideration should also be given to the fact that the quantification of cytokine levels from patient samples recruited earlier on in the study may not be representative of plasma levels at the time the samples were collected (Leng et al., 2008). Despite appropriate storage of clinical samples, it is possible that some degree of

protein degradation during prolonged storage could influence individual and absolute cytokine levels within the cohort.

Although ELISAs are still used conventionally and regarded as the gold standard technique for the quantification of proteins or cytokines in biological samples, there are limitations associated with this assay (Leng et al., 2008). Perhaps the most significant is the fact that ELISAs provide a cross-sectional analysis of the inflammatory profile, which is in itself a highly variable and dynamic phenomenon, particularly when considering chronic inflammatory diseases like CAD (Leng et al., 2008; Nathan, 2002). Thus, the cellular and molecular events associated with the inflammatory cascade in vivo are not discernible in this context – a reality that is exacerbated by the fact that this technique only has the capacity to evaluate levels of a single cytokine at a time. Furthermore, the quality of the antibody used, skill of the individual performing the assay, experimental conditions, and kit manufacture will likely impact performance (Leng et al., 2008; Aziz et al., 1999).

Whilst DCFDA is a widely used and relatively uncomplicated by means of measuring cellular ROS levels, there may be certain limitations of the technique to consider. One of these is the fact that cellular peroxides are only detectable by the probe when they are decomposed to form radicals, which depends on the presence of certain catalysts like transition metals within the culture medium (Eruslanov & Kusmartsev, 2009; Tampo et al., 2003). Considering that H_2O_2 induced a detectable increase in fluorescence during our initial experiments, it was unlikely that a lack of such catalysts within our culture medium affected the validity of the technique. However, it should be considered that this might have been a limiting factor. Cellular esterase activity should also be considered when using this probe; the activity of which can differ between cell types/cell lines. Cells with lower levels of activity will not hydrolyse the probe as efficiently, thus reducing the potential for ROS detection (Eruslanov & Kusmartsev, 2009).

4.9 Future work

Potential future work within this study could first seek to ascertain whether correlations between plasma TNFα levels and indicators of diastolic function are robust. Focus on continued patient recruitment and acquisition of complete clinical data would serve to

validate existing findings, and potentially identify other associations. This increased study power would also allow for effective stratification of patients according to concomitant risk factors and diseases severity/complexity. Conducting these analyses within a control data set, if possible, would also help to validate findings and increase the power of this study.

Given extensive evidence linking TNFα to the development of CAD, its complex role in vascular and myocardial pathophysiology, and oxidative stress signalling, it could be beneficial to characterise the cellular mechanisms in action based on our clinically relevant concentration – with particular attention to those influencing cellular redox state at this concentration.

Nonetheless, the findings of this and other studies provide conflicting results with regards to the biomarker potential of TNFα in CAD. Therefore, future focus could be shifted to alternative inflammatory markers in this respect in order to further develop our cytokine profile and investigate the effects of inflammatory signalling and oxidative stress in CAD.

4.10 Summary

To summarise, this preliminary study quantified levels of plasma TNFα within a CAD patient cohort to determine potential correlations with indices of systolic and diastolic function. Whilst no correlations were evident between TNFα levels and indicators of systolic function, significant and moderate correlations were observed with E/A ratio and LVIDd, respectively. These might suggest the potential value of TNFα as a biomarker for diastolic dysfunction in CAD, although further analysis is needed to further validate and strengthen these associations.

Studies assessing the cellular effects of clinically relevant cytokine levels in CAD on oxidative stress are thus far limited; in this study, data obtained using the fluorescent probe CM-H2DCFDA showed that TNFα did not independently increase cellular ROS levels at a clinically relevant or supraphysiological concentration in H9C2 cells. furthermore, neither concentration of TNFα provoked further increase in cellular ROS in cells with elevated baseline levels of ROS. This might suggest that TNFα plays a less significant role in ROS-mediated cardiac dysfunction in CAD than was initially expected.

In conclusion, TNFα may be of limited use as a biomarker for whole heart dysfunction and oxidative stress in CAD, but may help to gain further understanding of the inflammatory profile that characterise CAD patients.

Appendix

Appendix A. Patient information and consent form

Blackpool Teaching Hospitals NHS
NHS Foundation Trust

Blackpool Teaching Hospitals NHS Foundation Trust
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Whinney Heys Road
Blackpool
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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 you are put to sleep for your surgery a 5mL blood sample will be taken from you so that we can look at circulating markers in your blood. When you are undergoing your surgery, some of the heart tissue (approx. 0.5-1cm) and some of the leftover mammary artery which is used as for the bypass, both of which are normally removed and discarded during the operation, will be kept in an experimental solution to be transported to the University of Salford so we can look at how the cells in these tissues work. We will use scientific equipment to keep the tissue alive so we can explore how they work and the signals from them, small pieces of tissue will be preserved so we can look at the structure of the tissue and the levels of specific proteins within it. A computer program will be used to find out whether any changes we see are linked to your recovery.

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

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

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

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 bo

I confirm that I have read and understand the information sheet dated the 6th Decembe (version 1.1) for the above study. I have had the opportunity to consider the information, a questions and have had these answered satisfactorily. ☐

I understand that my participation is voluntary and that I am free to withdraw at any time witho 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 stu may be looked at by individuals from the Sponsor, from regulatory authorities or from the NH Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

I give permission for my cardiac tissue and internal mammary artery which would be norma discarded and blood samples to be supplied to The University of Salford, and for T University of Salford to store and distribute them to any researchers whose work has appropriate ethical approval and who are conducting high quality medical research on the prevention, diagnosis and / or the treatment of ischemic heart disease or other associated diseases. ☐

I agree to take part in the above study ☐

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

Name of Person taking consent	**Date**	**Signature**

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

Appendix B. TNFα ELISA reagent preparation

Wash Buffer (1x)

- Mix gently to avoid foaming. Store at 2° to 25°C. Remains stable for 30 days.

Number of strips	Wash Buffer Concentrate (20x) (mL)	Distilled water (mL)
1 – 6	25	475
1 - 12	50	950

Assay Buffer (1x)

- Mix gently to avoid foaming. Store at 2° to 8°C. Remains stable for 30 days.

Number of strips	Assay Buffer Concentrate (20x) (mL)	Distilled water (mL)
1 – 6	2.5	47.5
1 - 12	5.0	95.0

Biotin-Conjugate

- Make a 1:100 dilution of concentrated Biotin-Conjugate solution with Assay Buffer (1x). Use within 30 minutes of dilution.

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

Streptavidin-HRP

- Make a 1:400 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x). Use within 30 minutes of dilution.

Number of strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 – 6	0.015	5.985
1 - 12	0.030	11.970

Amplification diluent (1x)

- Make a 1:2 dilution immediately prior to use

Number of strips	Amplification diluent (2x) (mL)	Distilled water (mL)
1 – 6	3	3
1 - 12	6	6

Amplification Solution I

- Make a 1:300 dilution of Amplification Reagent I with Amplification diluent (1x) immediately prior to use

Number of strips	Amplification Reagent I (mL)	Amplification Diluent (1x) (mL)
1 – 6	0.018	5.382
1 - 12	0.036	10.764

Amplification Solution II

- Make a 1:250 dilution of Amplification reagent II with Amplification diluent (1x) immediately prior to use

Number of strips	Amplification Reagent II (mL)	Amplification Diluent (1x) (mL)
1 – 6	0.022	5.378
1 - 12	0.044	10.456

Appendix C. Mean TNFα plasma concentrations (pg/ml)

Patient ID (BVH)	TNFα Concentration (pg/ml)	SEM
1	3.2403	0.0015
2	6.0428	0.1465
3	3.2403	0.0020
4	1.3157	0.1515
5	2.1258	0.0028
6	0.0559	0.0006
7	0.3828	0.0045
8	1.0855	0.0176
9	3.0135	0.0216
10	1.1985	0.0036
11	0.1663	0.0017
13	0.6096	0.0198
14	2.2213	0.0133
15	1.4921	0.0062
16	0.8667	0.0201
17	0.1007	0.0030
18	0.9500	0.0110
19	1.6003	0.0125
20	3.1243	0.0196
21	3.2778	0.0025
22	0.6693	0.0163
23	2.6933	0.0366
24	0.1318	0.0083
26	0.2078	0.0082
29	0.8670	0.0061
30	0.8017	0.0165
31	1.3692	0.0023
32	0.9914	0.0043
33	1.3367	0.0032
34	14.8470	0.0169
35	2.2435	0.0103
36	1.4795	0.0033
37	1.1054	0.0116
39	2.3813	0.0252
40	4.8832	0.0085
43	7.5618	0.0134
46	3.3491	0.0182
47	0.2597	0.0009
48	8.6888	0.0118
49	16.7740	0.0994
50	0.7762	0.0046
51	1.3841	0.0062
52	1.5200	0.0044

Patient ID (BVH)	TNFα Concentration (pg/ml)	SEM
53	1.8967	0.0188
54	0.9871	0.0040
55	1.5065	0.0079
57	2.4938	0.0106
58	1.6806	0.0009
59	1.6334	0.0067
60	2.0733	0.0057
61	1.7311	0.0059
62	0.8855	0.0022
64	1.1664	0.0083
65	1.4990	0.0057
66	2.2419	0.0017
67	0.9255	0.0036
68	1.7480	0.0060
69	1.0130	0.0091
70	2.1676	0.0112
71	1.5879	0.0025
72	1.8237	0.0210
73	2.0462	0.0197
74	0.8841	0.0046
75	0.8699	0.0036
76	3.0298	0.0166
77	1.2542	0.0030

Appendix D. Patient Echocardiogram data (Systolic)

Patient ID	SV	EF	TAPSE	LVOT	LVIDs	ESV
1		40	1.9	0.9		
2	42.22	61.5	1.49			26.16
3		55	2.2	1.1		
4		55				
5	50.6	77.73	2.7	1.01	2.11	14.5
6	60.15	58.3	2.4			43.02
7	74	69.81	2.2			32
8						
9		50	2.77	1.15		
10		50	2.5	0.7	3.49	
11	47.8	70.29	1.9	0.958		20.2
12	44.05	22.3		1.13	4.89	153.79
13						
14		50	1.61	0.98		
15		55	2.2	1.3		
16	58.695	61	2.2	1.04	3.26	33.44
17	18.2	26.7		0.55		49.9
18	38.91	39.14	1.8			60.5
19						
20		30				
21		55			3.07	
22		35	2.09	1.03		
23		35	1.69	0.75		
24		55				
25		50				
26	73.5	53.7	1.4		3.85	63.5
27						
28	30.71	38.7	1.8	0.78	2.83	48.74
29						
30		55	2.05	0.92	3.74	
31		55	1.78	1.17	3.97	
32		55	1.76	0.69		
33	48.5	30.5	2.58	0.81	4.44	113.5
34						
35		70			1.5	
36		50				
37						
38						
39		35				
40						
41						
42		40				
43		45	2.48	1.1	3.3	

Patient ID	SV	EF	TAPSE	LVOT	LVIDs	ESV
44		55	2.14	0.69	3.04	
45				1.01	2.72	
46		35				
47						
48		50	2.88	1.21	2.52	
49						
50		55		0.98	2.56	
51		40	2	1.09		
52		45	2.57			
53		50	2.3	0.97		
54		55	2.09	0.83		
55		45		0.62	4.05	
56	40.39	30.2	2.27	1.12		87.5
57		55	1.82	0.77		
58		60	2.04	1.23		
59						
60		45		0.91	3.58	
61						
62		45	1.7	0.89	3.31	
63	58.2	56	2.24	0.91	4.1	45.8
64		45		0.81	3.5	
65						
66	43.695	45.8	2.05	0.68	3.27	45.9
67	37.425	32.6	1.78	0.92		73.56
68	44.85	40.6			4.17	62.6
69			2.52	1.02		
70				1.34		
71		62.5			2.78	
72		47.5	1.79	0.69	2.65	
73		53	2.05	0.85	4.02	
74		55	2.57	1.49		
75		37.5	2.33	0.68		
76						
77		57.5	2.56		2.8	

Appendix E. Patient Echocardiogram data (Diastolic)

Patient ID	LVIDd	E/A ratio	TR velocity	E/e ratio	Peak E wave	EDV
1		1.6	1.3		0.85	
2		0.63	2.08	6	0.45	68.38
3		0.7	2.3	10	0.5	
4				8		
5	3.88	0.62			0.44	65.1
6		0.85	2.16	7.5	0.75	103.17
7		1.21	1.91		0.91	106
8						
9		0.78			0.82	
10	4.6	0.98			0.54	
11						68
12	6.12	0.89	2.61		0.9	197.84
13				8.51		
14	4.12	1.34		9.46	1.06	
15			2.7			
16	5.05	1.5		11.3	0.96	92.135
17	4.7	1.86	3.27		0.86	68.1
18		1.29	3.58	11.3	1.13	99.41
19				5.55		
20						
21	4.79		2.45		0.76	
22	6.33		3.31			
23		0.83			0.7	
24	4.15	0.99			0.55	
25						
26	4.97	1.04	2.59	8.55	0.47	133.5
27						
28	4.52	1.13	2.58	22.54	1.01	79.45
29						
30	4.03	1.05	2.16	9.69	0.92	
31	4.97	1.06	2.16	9.8	0.93	
32	3.68	0.92			0.8	
33	4.97	0.72		12.36	0.86	162
34						
35	3.4	0.91			0.67	
36		0.77			0.72	
37				8.59		
38						
39						
40						
41						
42						
43	5	0.76		8.55	0.51	

Patient ID	LVIDd	E/A ratio	TR velocity	E/e ratio	Peak E wave	EDV
44	4.13	0.84	2.09	7.06	0.63	
45	3.79	0.59	2.43	8.56	0.47	
46	5.29		2.82			
47				8.45		
48	4.77	0.88	2.74	10.32	0.94	
49						
50	4.38	1.03			0.78	
51	4.19	0.56		6.69	0.57	
52	4.64	0.63		6.79	0.62	
53	5.71	1.18		11.18	1.06	
54	4.32	1.31		9.05	0.75	
55	5.25	0.55	2.63		0.53	
56	5.72	0.74		7.47	0.48	127.89
57		0.78		10.33	0.57	
58	4.26	0.47		5.69	0.43	
59				15.13		
60	4.72	1.38		14.07	0.95	
61						
62	4.96	0.77	2.18	6.55	0.35	
63	5.7	1.05		7.85	0.61	104
64	4.56	0.75	2.48		0.64	
65						
66	5.13	0.57		9.8	0.48	89.595
67		0.56	2.39	10.61	0.59	110.985
68	5.07	0.71			0.67	107.45
69	4.37	1.05			0.77	
70	4.62	1.03			0.82	
71	4.64	1.43	2.7		1.09	
72	4.03	0.86	1.83	15.47	0.86	
73	5.13	0.68			0.56	
74	4.1	1.02	2.58	7.07	0.94	
75	5.15	0.79			0.42	
76						
77	4.8	0.88			0.79	

Appendix F. Normal reference values for ECHO parameters

Echocardiogram Parameter	"Normal" range
SV (ml/m3)	60 - 120
EF (%)	55 - 70
TAPSE (cm)	1.5 - 2.5
LVOT (m/s)	0.8 - 1.0
LVIDs (cm)	2.0 - 4.0
ESV (ml)	19 - 50
LVIDd (cm)	3.5 - 5.6
E/A ratio	1.0 - 2.0
TR Velocity (m/s)	< 2.55
E/e Ratio	< 8
Peak E Wave (m/s)	0.6 - 0.8
EDV (ml)	56 - 104

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