Characterisation of Inflammatory Mediators in Ischaemic Heart Disease

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Acknowledgments

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Abbreviations

ACE – angiotensin-converting enzyme

ACRP30 – adipocyte complement-related protein of 30kDa

ACS – acute coronary syndrome

ADAM10/17 – A disintegrin and Metalloproteinase 10/17

AMI – acute myocardial infarction

AMPK – AMP-activated protein kinase

APC – antigen presenting cell

Apo – apolipoprotein

ATP – Adenosine 5'-triphosphate

BMI – body mass index

BP – blood pressure

C/EBP – CCAAT-enhancer-binding protein

CABG - coronary artery bypass grafting

CAD – coronary artery disease

CCB – calcium channel blocker

CCR2 – C-C Motif Chemokine Receptor 2

COX – cyclooxygenase

CRP – C-reactive protein

CVD – cardiovascular disease

CXCL8 – C-X-C Motif Chemokine Ligand 8

DALY – disability-adjusted life year

DAMP – damage-associated molecular patterns

DASH – Dietary Approaches to Stop Hypertension

DC – dendritic cell

DD – death domain

EC – endothelial cell

EF – ejection fraction

ERK 1/2 – extracellular signal-regulated kinase 1/2

GRS – genetic risk score

GWAS – genome-wide association studies

HDAC-1 – histone deacetylase 1

HDL – high density lipoprotein

HMG-CoA – 3-hydroxy-3-methylglutary-coenzyme

ICAM-1, 2, 3 – intracellular adhesion molecule-1, 2, 3

IFN-γ – Interferon-γ

IHD – ischaemic heart disease

IKK – inhibitor of nuclear factor-κB kinase

IL-10 – Interleukin-10

IL-12 – Interleukin-12

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

IL-8 – Interleukin-8

iNOS – inducible nitric oxide synthase

IRF – interferon regulatory factor

IκB – inhibitor of nuclear factor-κB

JAK – Janus kinase

LDL – low density lipoprotein

LPS – lipopolysaccharide

LVEDVI – Left ventricle end diastolic volume index

LVESVI – Left ventricle systolic volume index

LVIDD – Left ventricular internal diameter end diastole

LVIDS – Left ventricular internal diameter end systole

LVOT – left ventricular outflow tract

MAPK – mitogen-activated protein kinase

MAPKKK – mitogen-activated protein kinase kinase kinase

M-CSF – macrophage colony-stimulating factor

MEKK1 – MEK kinase 1

MHC class II – major histocompatibility complex II

MI – myocardial infarction

MKK7-JNK – mitogen-activated protein kinase kinase 7- c-Jun N-terminal kinase

MLKL - mixed lineage kinase domain-like protein

MMP – matrix metalloprotease

MSK1/2 - mitogen- and stress-activated protein kinase ½

mTOR - mechanistic target of rapamycin

NF-κB – nuclear factor kappa B

NK cell – natural killer cell

NLRP3 – leucine-rich repeat pyrin domain containing 3

NO – nitric oxide

NPC1L1 – Niemann-Pick C1-like 1

NRF – NF-κB-repressing factor

NSTEMI – non-ST segment elevated myocardial infarction

OCT-1 – octamer-1

oxLDL – oxidised LDL

PAI-1 – plasminogen activator inhibitor-1

PAMP – pathogen associated molecular patterns

PASP – pulmonary arterial systolic pressure

PCI – percutaneous coronary intervention

PCSK9 – proprotein convertase subtilisin/kexin type 9

PECAM-1 – platelet endothelial cellular molecule 1

PGE1/2 – prostaglandin E1/E2

PIGF – placental growth factor

PRR – pattern recognition receptor

ROS – reactive oxygen species

ROS – reactive oxygen species

SAA – serum amyloid A

SHP-2/ERK – Src homology region 2-containing protein tyrosine phosphatase 2/ extracellular signal-regulated kinase

SMC – smooth muscle cells

SNP – single-nucleotide polymorphism

STAT – signal transducer and activator of transcription

STAT1/3 - signal transducer and activator of transcription 1/3

STEMI – ST-elevation myocardial infarction

SV – stroke volume

TAPSE – tricuspid annular plane systolic excursion

TCA – tricarboxylic acid

TCR - T-cell receptor triggering

TF – tissue factor

TGF-β – Transforming Growth Factor β

TGRL – triglyceride-rich protein

TH1/2 – T helper cells type 1/2

TIMP – tissue inhibitor of metalloproteinase

TIR – Toll-IL-1 receptor

TNFR - TNF-alpha receptor

TNF-α – Tumour Necrosis Factor α

Treg – regulatory T cell

UA – unstable angina

VCAM-1 – vascular cell adhesion molecule-1

VEGF – Vascular endothelial growth factors

VHDL – very low density lipoprotein

VSMC – vascular smooth muscle cell

WHO – World Health Organisation

Abstract

Coronary artery disease (CAD) is one of the most common causes of death in general population. In the UK it accounts for 64000 deaths annually and there are currently 2.3 million people living with CAD. Persistent inflammation of myocardium is believed to be the major driver of heart pathology. As such, inflammatory mediators such as cytokines have emerged as potential biomarkers for local and systemic inflammation. Therefore, this study aimed to determine if pre-operative cytokine levels are associated with adverse cardiac function in CAD patients. We also sought to optimise a protocol for the isolation of viable cardiac myocytes from human atrial tissue.

All experiments were conducted in accordance with IRAS ethical approval (REC reference: 18/LO/2219). Firstly, protocol for the isolation of cardiomyocytes from the atria of coronary artery bypass graft (CABG) surgery patients was adapted to obtain cells of optimal quality. In parallel, pre-operative levels of interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin10 (IL-10), tumour necrosis factor alpha (TNFα), and interleukin 12 (IL-12) were measured using a multiplexed cytometric bead array. Reference data from a healthy population was obtained using meta-analysis. Individuals were matched based on age, gender structure, BMI, hypertension status and history of tobacco use. Patient cytokine concentrations were then correlated with clinical data to identify correlations between indices of cardiac function and inflammatory mediators.

Levels of Interleukin-1β and IL-8 fell below the detection limit of the cytometric bead array assay. Detectable cytokine levels in patients were found to be at 3.15±0.44pg/ml for IL-6, 1.72±0.48pg/ml for IL-10, 1.63±0.19pg/ml for TNF-α, 1.72±0.07pg/ml for IL-12. Healthy population cytokine levels were as follow: 3.92±4.33pg/ml for IL-6, 4.40±6.11pg/ml for IL-10, 11.85±8.62pg/ml for TNF-α, and 16.15±12.97pg/ml for IL-12. Analysis showed that only TNF-α and IL-12 levels were significantly different between both groups ($p<0.0001$).

Correlation of cytokine levels with cardiac function showed significant relationships between ejection fraction and IL-6 ($p=0.0005$, $r=-0.5142$) and IL-12 ($p=0.0487$, $r=0.3061$), as well as stroke volume and IL-10 ($p=0.0070$, $r=-0.9539$), TAPSE and IL-6 ($p=0.0214$, $r=-0.4491$), and BMI and IL-10 ($p=0.0088$, $r=-0.4247$).

Overall, these findings show that preoperative levels of certain cytokines significantly correlate with certain indices cardiac function in our CAD patient cohort. These results may offer potential targets for therapeutic agents and treatment strategies for improving cardiac function in CAD patients.

Chapter 1 – Introduction

1.1 An introduction to coronary artery disease (CAD).

Coronary artery disease is a multifactorial phenomenon in which occlusion of coronary arteries leads to inadequate supply of oxygenated blood to myocardium and a demand-supply difference of oxygen. It manifests by stable or unstable angina, myocardial infarction (MI) or sudden cardiac death (Malakar et al., 2019). The primary cause of CAD is the atherosclerosis of coronary arteries shown in figure 1. The word "atherosclerosis" is the combination of two Greek words, "athero" and "sclerosis" which mean gruel and hardening respectively (Shao et al., 2020).

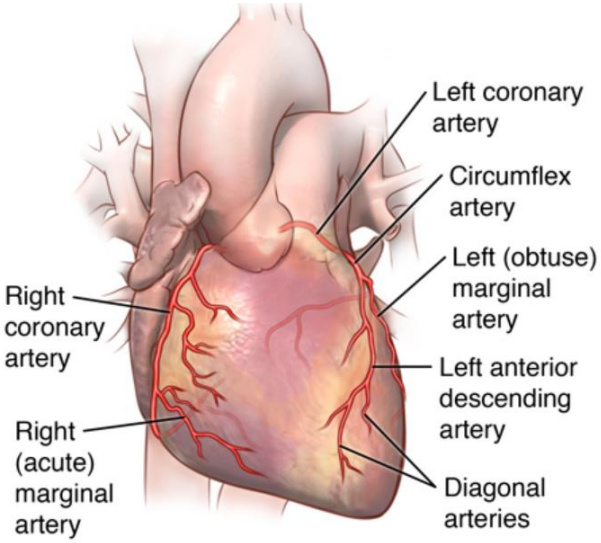

Figure 1. Coronary arteries of the heart. From: *Anatomy and function of coronary arteries*. (https://www.hopkinsmedicine.org/health/conditions-and-diseases/anatomy-and-function-of-the-coronary-arteries).

Coronary artery disease is one of the most common cause of death in the general population after dementia and Alzheimer's disease (McCullough, 2007). It accounts 64000 deaths in the UK and currently there is a 2.3 million of people living with coronary heart disease in the UK (British Heart Foundation, 2021). Despite these pessimistic facts, the statistics for CAD show that its burden declines in the UK: mortality rate between 2000 and 2019 dropped by nearly 60%. This decline however is not a result of declining incidence of the disease, but of the advances of modern medicine. That includes greater treatment focused on improving survival in the first four weeks after the cardiac event, investment in prevention measures and

population-wide primary prevention, such as reduction in tobacco and alcohol use, and improved diet with increased physical activity (Office for National Statistics, 2021)

It has been shown that not only lifestyle plays an important role in the development of CAD but also genetic and environmental factors interact together to determine phenotype of the disease (Girelli et al., 2009). These factors include hypertension, tobacco use, hyperlipidaemia, obesity diabetes mellitus and psychosocial stress that will be described in further chapters (Kelly & Fuster, 2010).

1.1.1 History of coronary artery disease.

The information on the emergence of CAD cannot be dated precisely. The first time the CAD was described in modern journals was in 1812 in the first issue of The New England Journal of Medicine and Surgery by John Warren M.D. (Warren, 1812). At the time, pathogenesis of coronary artery disease was unknown. It was often treated with a bed rest, opium, or bloodletting.

At the beginning of 20th century clinical medicine, physiology and pathology were advancing in separate yet parallel universes. Meaning that for decades observations by pathologists that described thrombotic occlusions were not related to myocardial ischaemia which was well known to physicians. Marriage of the symptoms of CAD with the clinical representation of MI led to formation of recommendations for patients, e.g., bed rest. Finally, the invention of electrocardiography in 1919 allowed to correctly diagnose patients with CAD. (Herrick, 1912, 1919).

It wasn't until 1960s when scientists and clinicians started to understand and manage myocardial infarction that struck down and killed many apparently healthy men in their 40s and 50s. The first study in 1948 tried to understand how heart disease develop by studying life styles of residents of Framingham, Massachusetts (Kannel et al., 1961). Researchers found that elevated blood pressure and cholesterol levels were associated with increased incidence of myocardial infarction. This discovery led to the formation of national programs that educated general public and clinicians about the importance of controlling these risk factors. That in turn resulted in dramatic decrease in age-adjusted cardiac death rates (Nabel & Braunwald, 2012). Identification of these risk factors led to unmasking the underlying mechanisms behind myocardial infarction. That in turn allowed to introduce the concept that

coronary heart disease and its complications can be prevented. Lowering blood pressure and cholesterol was further improved with new drugs that were forged as a result of successful collaboration between industry and academic medicine (Nabel & Braunwald, 2012).

Development of clinical studies in humans had shaped the field of vascular biology. It showed that thrombotic occlusion of an eroded or ruptured atherosclerotic plaque leads to myocardial infarction and that nitric oxide is a physiological dilator for blood vessels. This pioneering work by Furchgott, Ignarro and Murad has granted them a joint Nobel Prize in Physiology or Medicine in 1998 (Ignarro et al., 1987; Rapoport et al., 1983; Zawadzki et al., 1981).

These and other studies influenced a direction of following research. Investigators shifted their focus from preparations of heathy, intact vessels to cellular and molecular regulation and genes that encode proteins and RNAs responsible for formation of normal or diseased vessels (Nabel & Braunwald, 2012).

1.1.2 Incidence and epidemiology of coronary artery disease.

Cardiovascular diseases, that include heart disease, hypertension and stroke are number one cause of death globally and have major economic consequences that affect individuals, societies, and health systems across the world (figure 2). To meet economic challenges of CVD policymakers need to access reliable information about the national spend on healthcare as well as the economic burden of the disease. Across Europe financial burden of CVD accounts for around 10% of current health expenditures, however economic burden was estimated to cost EU economy €210 billion a year, with IHD contributing €51billion to the cost. This cost in UK was at €18.9 billion a year in 2014 and it represented 1.4% of Gross Domestic Product (GDP) (European Society of Cardiology, 2020).

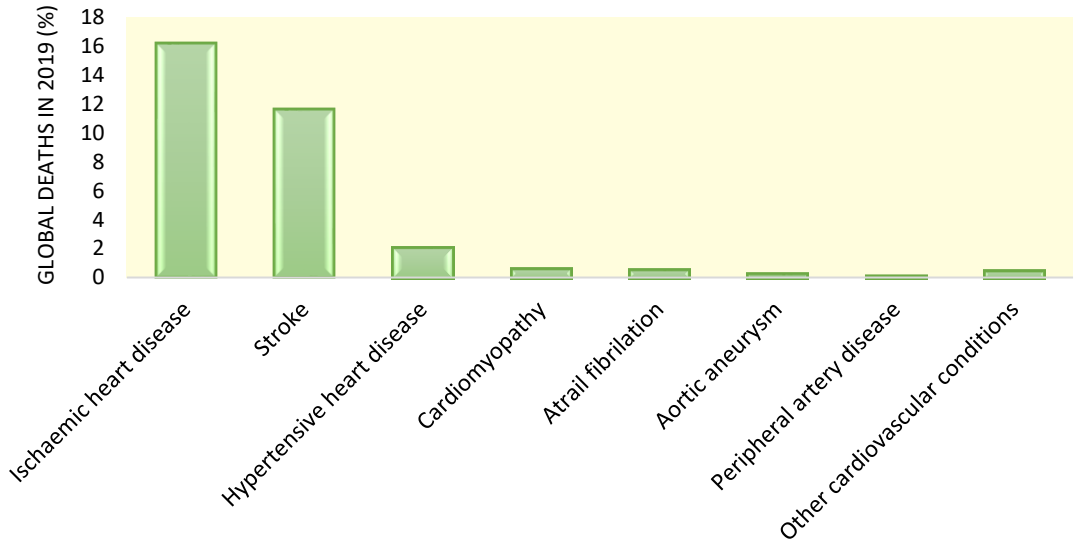

Figure 2. The contribution of cardiovascular disease to the global burden of death in 2019. These data show the importance of CAD worldwide. It is worth to mention that not all stroke deaths result from atherosclerosis and not all cases of cardiomyopathy are a result of ischaemic damage.

Burden of the disease is a concept developed by the WHO to describe death and loss of health caused by the risk factors and disease (World Health Organization, 2003). It is usually quantified by morbidity, mortality, and financial cost and is called disability-adjusted life years (DALYs). It provides a measure of health lost due to CVD. One DALY can be thought as one lost year of full health. The sum of DALYs is a measure of gap between current health status and ideal situation, where entire population lives to an advance age, free of disease or disability (World Health Organisation). In Europe IHD and stroke accounts for 82% of DALYs due to CVD. These declined from 7542 to 4530 per 100000 people between 1990 and 2017. Additionally, DALYs due to CVD in 2016 were almost twice as high in males as they were in females (Townsend et al., 2016).

CVD remains the most common cause of death in Europe, and more than three-fifths of all CVD occur in those aged >75 years. Deaths in those <70 years are often referred to as premature and are a particular concern with more than 60 million DALYs lost to CVD in Europe yearly and more that 4 million deaths accounting for 45% of all deaths (Townsend et al., 2016). Despite the fact that more women than men die from CVD , age standardised data shows that morbidity and deaths are higher in men than in women especially in individuals aged <70

years (Townsend et al., 2021). Despite sustained declines in CVD, it has remained the most common cause of death in Europe accounting for 47% of deaths in females and 39% deaths in males (figure 3). The decline of heart disease mortality has been seen in developed countries with advanced health care systems all around the world. In Netherlands the decline between 1980 and 2009 has been around 70%, while in UK and Ireland more that 60%. This decline has been paralleled by the decrease in the incidence of related mortality of acute myocardial infarction and stroke (Casolo et al., 2020).

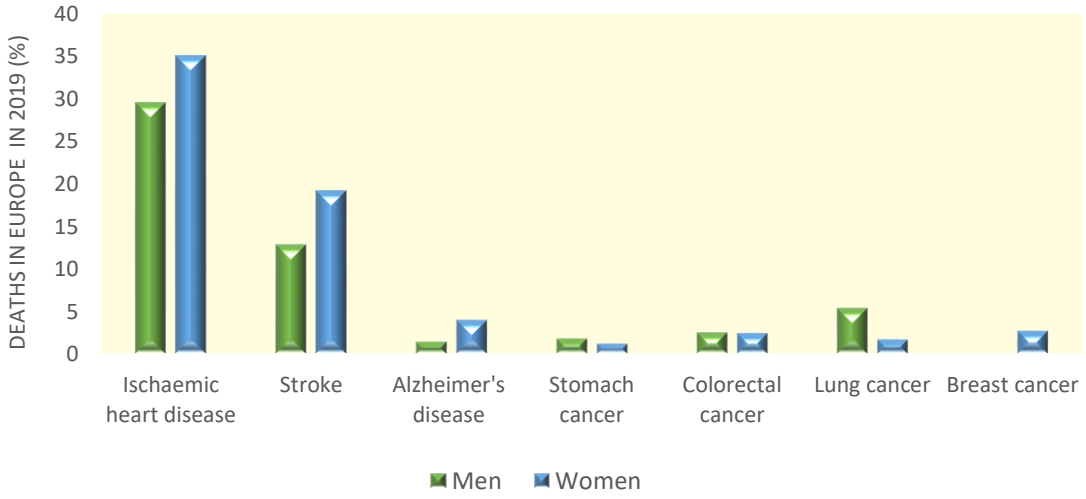

Figure 3. The most common death causes in Europe depending on sex.Interestingly, burden of coronary atherosclerosis also seems to ease. The study of 11223 patients in the Copenhagen City Heart Study showed that, in time, obstructive CAD became less common in men, but especially in women. Also, flow limiting stenosis in stable angina decreased in men by 20% and in women by 15% in year 2008 when compared to year 2000. In addition, the study showed that these changes were continuing and progressive (Jespersen et al., 2012). The same patterns are observed in the United Kingdom as shown in figure 4.

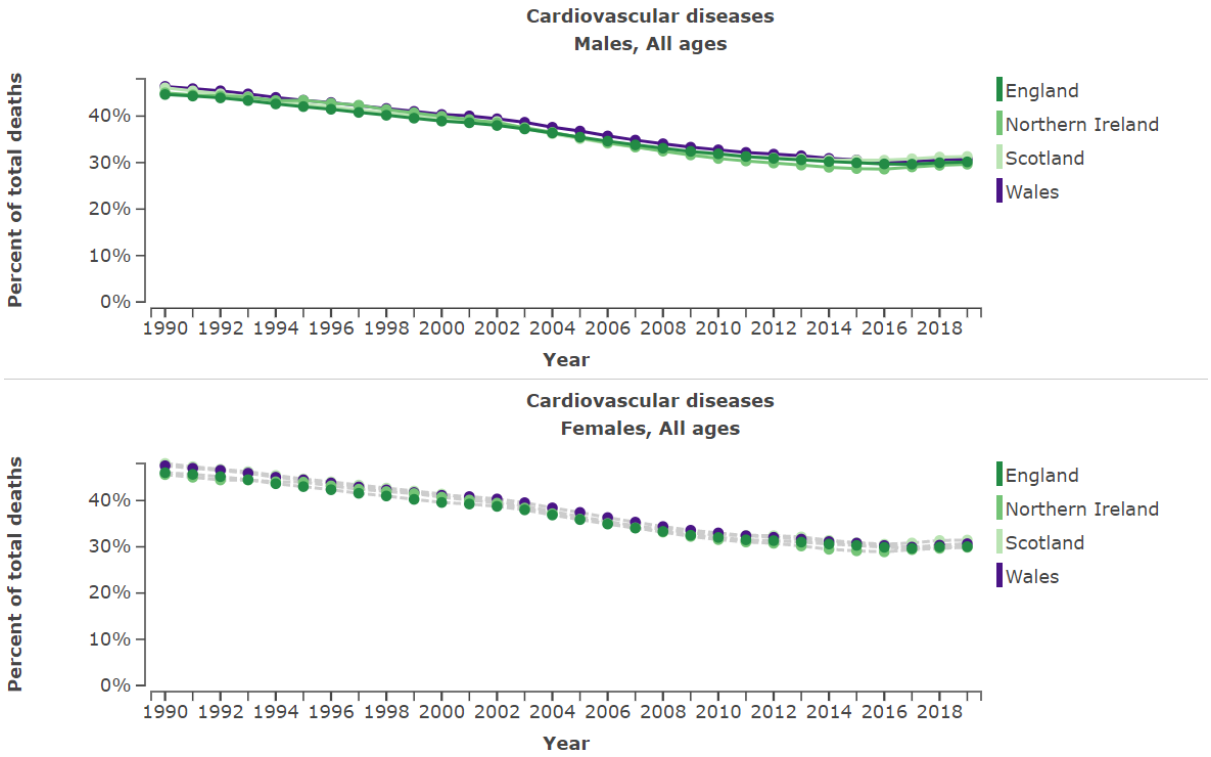

Figure 4. Difference in death rate from cardiovascular diseases between men and women in from 1990 to 2018 in England, Northern Ireland, Scotland, and Wales.

Decline in cardiovascular diseases events and mortality has been observed in all high-income countries around the world. It is generally accepted that it is due to improved control of changeable risk factors, like smoking or diet as well as pharmacological interventions, especially statins and anti-hypertensive drugs. However, government interventions in terms of unnecessary use of alimentary additives like trans-saturated fatty acids also have a significant impact on decrease of cardiovascular deaths (De Souza et al., 2015).

1.2 Aetiology and progression of coronary artery disease.

1.2.1 Lipids in atherosclerosis

Multiple population-based observational studies established that the highest incidence of atherosclerosis is most prevalent among older adults in societies that adopt Western lifestyle and diet (Board, 2014; Orekhov & Ivanova, 2017). There are multiple factors that are involved in the atherosclerosis initiation. Liver derived very-low density lipoprotein (VLDL) related to insulin resistance has an atherogenic effect due to its influence on the low-density lipoprotein

(LDL) species and on the level of high-density lipoprotein (HDL) by increasing the former and decreasing the latter. Noteworthily, HDL plays a protective role in atherosclerosis (Mehta & Shapiro, 2022). Despite long association of LDL with atherosclerosis, current studies show that rather that elevated LDL cholesterol, an elevation in triglyceride-rich lipoprotein (TGRL) and low HDL now comprise the major pattern of abnormality in many patients treated for cardiovascular disease (Nordestgaard & Varbo, 2014).

Atherosclerosis is a complex, chronic disease that originate in endothelial injury or accumulation of low-density lipoproteins (LDLs) within the arterial wall in the subendothelial layer of arterial vessels, which are generally prone to oxidation or modification (Moriya, 2019). These oxidised LDLs (oxLDLs) together with low-grade inflammation caused by the endothelial injury initiate innate and adaptive responses, which are responsible for development of atherosclerosis (Moriya, 2019). The formation of atherosclerotic lesions is not random, they take place at specific arterial regions, branching sites, where low and oscillatory endothelial stress occurs. Hence, they predominantly develop in walls of large and medium sized arteries (Summerhill et al., 2019).

1.2.2 Cell types involved in formation of early atherosclerosis.

It is widely recognised that monocyte adhesion is provoked by endothelial dysfunction of arterial wall. Endothelial injury is characterised by low-grade inflammation that triggers upregulation of cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), selectin and intracellular adhesion molecule-1 (ICAM-1) (Moriya, 2019). These molecules enable monocytes to adhere to the arterial wall where they will differentiate into macrophages under the stimulation by the macrophage colony-stimulating factor (M-CSF), that will engulf lipoprotein to become foam cells, the hallmark of atherosclerotic lesions. Macrophages involvement in atheroma formation are shown in figure 5. They do so by expressing scavenger receptors to take up oxidised LDLs. This accumulation of lipids induces the inflammation, which augments the atherosclerotic process, resulting in positive feedback loop and preclinical phase of the disease (Hulsmans et al., 2018; Lu et al., 2018). That accumulation of foam cells in the arterial intima leads to the formation of the initial lesion and fatty streaks that represent early phase of the atherogenic progression as well as increased synthesis of extracellular matrix components in subendothelial cells (Orekhov et al.,

1990). These macrophages together with smooth muscle cells undergo apoptosis and form a lipid-rich necrotic core of newly forming atheroma.

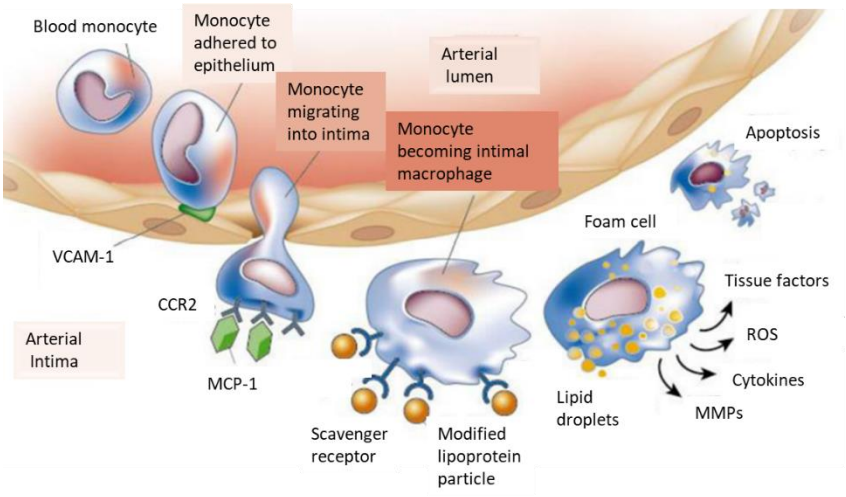

Figure 5. Macrophages in atherogenesis. Endothelial dysfunction starts inflammatory process and increase blood monocytes that activate adhesion molecules VCAM-1. Chemokines, including monocyte chemoattractant protein-1 (MCP-1) participate in this migration by interacting with monocyte receptor CCR2. These monocytes differentiate into macrophages in the intima and internalise lipoproteins to form foam cells. These cells secrete reactive oxygen species (ROS), local cytokines and matrix metalloproteases (MMPs). The latter may degrade extracellular matrix leading to plaque rupture that releases potent procoagulant protein tissue factor. Finally, macrophages undergo apoptosis and produce necrotic centre of the atherosclerotic centre. Adapted from: Geovanini & Libby, 2018Neutrophiles have been recently recognised as one of the most important contributors to the development of atherosclerosis. Like monocytes, they are recruited to the atherosclerotic lesion by chemokines produced by activated platelets and are also capable of recruiting monocytes by releasing granule proteins such as cathepsin-G or azurocidin. These granules have been reported to activate macrophages to form foam cells (Soehnlein, 2012).

T-lymphocytes (T-cells) are among the earliest cells present at the formation of the atherosclerotic plaque. Most chemokines and adhesion molecules that promote monocyte migration to intima are also responsible for recruitment of T-cells. Helper T-cells differentiate into T-helper cell type 1 (TH1) and T-helper cell type 2 (TH2) subtypes. Locally both types are controlled by cytokines or antigen presenting cells (APCs), but most cells in atherosclerotic lesions are TH1 cells. These cells secrete IL-2, tumour necrosis factor (TNF) and interferon-γ

(IFN-γ) which are known to promote inflammation via acting on vascular cells through macrophages (Ait-Oufella et al., 2006). On the contrary, TH2 cells produce anti-inflammatory IL-10 and regulatory T cells (Treg) secrete transforming growth factor β (TGF-β) that can limit inflammation and reduce smooth muscle cell proliferation as well as promote interstitial collagen synthesis (Nus & Mallat, 2016).

This cooperation between these cellular constituents of innate and adaptive immunity stimulates production of pro-inflammatory cytokines that sustain and amplify the local inflammatory response as shown in figure 6. Once established, atherosclerotic plaques progress to accumulation of lipid and lipid engorged cells. In addition, migration of smooth muscle cells from the media to the intima contribute to their accumulation in atherosclerotic plaque to comprise much of its bulk (Bennett et al., 2016).

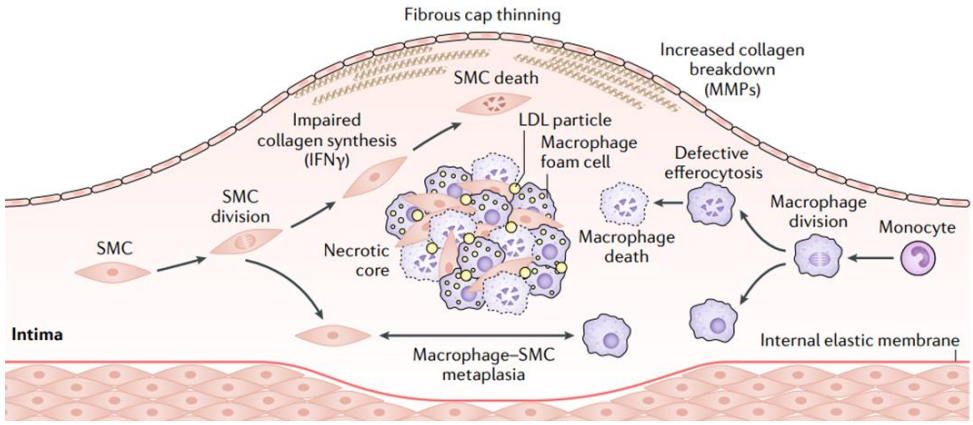

Figure 6. Progression of atherosclerotic lesions. During evolution of the atherosclerotic plaque, smooth muscle cells (SMC) produce extracellular matrix molecules that contribute to the formation of the plaque. Cellular mediators, such as IFN-γ dampen the ability of SMC to produce collagen and repair the fibrous cap on the necrotic core. Additionally, activated macrophages produce matric metalloproteases that degrade the collagen weakening the cap. This leads to cap susceptibility to rupture. SMC and macrophages proliferate within the cap and undergo apoptosis forming a necrotic core of the atheroma. Impaired clearance of death cells (efferocytosis) contributes to the formation of the necrotic core. Source: Libby et al., 2019.

1.2.3 Complications of atherosclerosis.

During much of the disease, the atherosclerotic plaque expands outward radially preserving the calibre of the arterial lumen. Eventually, however it begins encroaching the arterial lumen and lead to formation of flow-limiting lesions as shown in figure 7 (Libby et al., 2019). Consequent impairment of coronary artery perfusion can produce ischaemia and the symptoms of angina pectoris, especially when oxygen demand during physical effort is not met (Alexander et al., 2012).

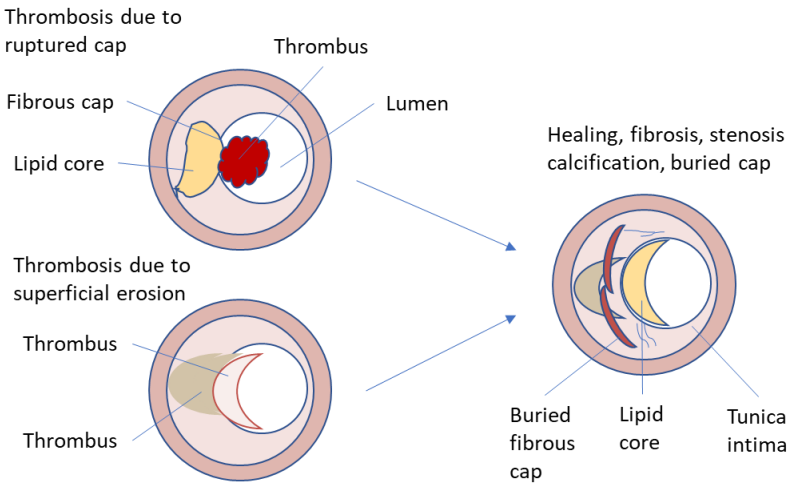

Figure 7. Atheroma complications including disruption and healing. The fracture of the fibrous cap on the atherosclerotic plaque permits pro-coagulants to access the core of the plaque occlude the vessels causing ischaemic event. Many thrombi do not occlude the vessel but undergo lysis. Resorbing thrombus can activate smooth muscle cells to migrate and platelets to produce extracellular matrix due to the production of platelet deriving growth factor and TGF-β. These processes lead to increased lesion volume and blocking of the arterial lumen. Plaques that lack well-defined lipid core have rich extracellular matrix and can provoke coronary thrombi by superficial erosion, where clots have a characteristic 'white' thrombus. By contrast the plaque rupture produce 'red thrombi' rich in erythrocytes and fibrin. Adapted from: Libby et al., 2019.

This obstruction leads to typical symptoms of CAD such as a substernal discomfort that radiates to the jaw, neck, back and arm, pressure-like feeling, heaviness. These symptoms last just few minutes and are usually a result of exertion,, cold, emotional stress or heavy meal, and can be alleviated by rest and/or nitro-glycerine (Malakar et al., 2019).

1.2.4 Plaque rupture and erosion

Rupture of the atherosclerotic plaque is the most common trigger of acute thrombosis of coronary arteries that cases myocardial infarction (Libby et al., 2019). There are two types of plaques. Ones termed 'vulnerable' have large lipid cores covered by a thin fibrous cap, whereas plaques with thicker fibrous caps and limited lipid accumulation are called stable plaques. Inflammatory process can not only hinder production of interstitial collagen impairing the ability of smooth muscle cells to maintain the rigidity of the fibrous cap, but also degrade key structural components of the fibrous cap of the lesion (Galis et al., 1995). Rupture of the plaque exposes content of the interior of the plaque to the blood compartment. Material in the plaque core can then trigger thrombosis – the most dreaded complication of atherosclerosis that can lead to acute coronary syndromes, peripheral artery disease, stroke and death (Martinod & Wagner, 2014). Despite these dangers, current therapies for atherosclerosis have shifted plaque characteristics to be more fibrous and less liable to rupture. Despite this, alternative thrombotic called plaque erosion seems to arise. Here, lesions have rich extracellular matrix without a thin fibrous cap, with few inflammatory leukocytes and little lipid (Quillard et al., 2017).

1.3 Risk factors of coronary artery disease.

One of most important achievements in cardiovascular medicine has been the identification of major risk factors for CAD and development of therapeutic strategies to prevent the disease and its progression (European Society of Cardiology, 2020). The most important study to do just that was called the Framingham Heart Study, which is a longitudinal, ongoing cardiovascular cohort study of residents of the city of Framingham, Massachusetts. Over the years it described and characterised most important risk factors that are responsible for more than 90% of the risk of acute myocardial infarction (Wawrzyniak, 2013). Coronary artery risks are shown in figure 8.

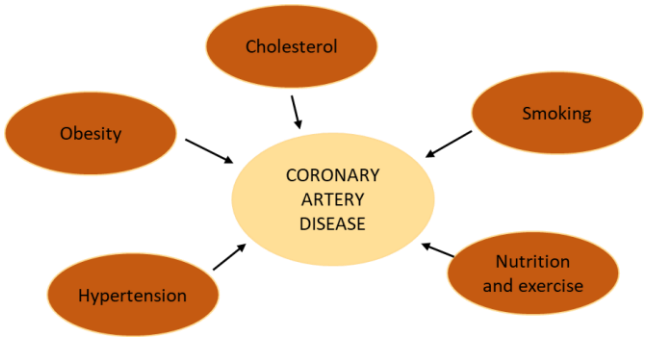

Figure 8. Various traditional risk factors associated with coronary artery disease.

Description of modifiable risk factors and health behaviours are presented below along with World Health Organisation reduction targets.

1.3.1 Hypertension as a risk factor in CAD.

The INTERHEART study estimated that 22% of MI in Europe are attributable to hypertension (Macleod et al., 2005). Current European Society of Cardiology guidelines define hypertension as a systolic blood pressure >140mmHg or a diastolic blood pressure >80mmHg (Whelton & Williams, 2018). Treatment to lower blood pressure provide significant protection against cardiovascular events. Lifestyle changes like weight loss and exercise are important however pharmacotherapy is usually necessary. Equally important is patient's adherence to the treatment of at least 80% for adequate reduction of blood pressure as lack of patient's discipline is the major cause of 'resistant hypertension' (European Society of Cardiology, 2020; Macleod et al., 2005). The WHO has set a target of a 25% relative reduction in the prevalence of elevated blood pressure to be achieved by 2025 (World Health Organisation, 2016b). Despite promising trends in declining the prevalence of elevated blood pressure, progress is to slow and it seems unlikely that the WHO target for 2025 will be achieved (European Society of Cardiology, 2020).

1.3.2 Total cholesterol as a risk factor for CAD.

Another risk factor described by the INTERHEART study refers to elevated total cholesterol. Prevalence in elevated total cholesterol exceeds 50% in Europe when compared with less than 30% in Africa and Southeast Asia (European Society of Cardiology, 2020). Low density cholesterol (LDL) is a key determinant of CVD risk and is a major target in risk reduction programmes. Current guidelines recommend statin therapy as a primary treatment for people at risk of heart attack or stroke as well as for patients with established CAD (Mach et al., 2019).

WHO data for 2009 showed that Europeans blood cholesterol concentration median was at 5.1mmol/L in both sexes. Hypercholesterolaemia (>6.2mmol/L) prevalence averaged at 15.6% in females and 14.3% in males (European Society of Cardiology, 2020).

1.3.3 Obesity as a risk factor for CAD.

Another risk factor for CAD is obesity. The worldwide prevalence of obesity (BMI>30kg/m^2) nearly tripled between 1975 and 2016. Now more people are obese than underweight globally (Malik et al., 2013). Additionally, the Global BMI Mortality Collaboration reported that for every 5kg/m^2 BMI increase, the risk of death increase by more than 33%, hence increased BMI is not only a risk for CAD but generally it is a risk of premature death for any reason (Flegal, 2017). Data showed that approximately one in five adults in Europe is obese. The most disturbing statistics show that obesity prevalence increased from 9.6% in 1980 to 22.6% in 2017 (European Society of Cardiology, 2020). WHO set a target to halt the increase in obesity prevalence by 2025, however statistics make it unlikely for this target to be achieved unless present trends are reversed (World Health Organisation, 2016a).

Obesity is an independent risk for CAD, but it is also a major contributor to the rampant epidemic of type 2 diabetes and now affects 60 million people in Europe. It doubles the risk of death and half of these deaths are the result of IHD or stroke (World Health Organisation). Diabetes is responsive to treatments with pharmacology however the knock-on effect for reducing the risk of CAD is minimal. The most important changes to successfully manage diabetes and to reduce the risk of CAD is through lifestyle changes, e.g., reducing body mass and moderate exercise (European Society of Cardiology, 2020). Who set a target to halt the increase in prevalence of obesity by 2025, however analysis of data for European countries clearly show that diabetes prevalence is not stopping. Quite the opposite, it starts to grow exponentially from 2000, where its prevalence was 1.6% in general population to 3.9% in 2015. This makes it highly unlikely the WHO diabetes target will be met unless these trends are reversed (Roglic, 2016).

1.3.4 Smoking as a risk factor of CAD.

Next important CAD risk factor is smoking. Tobacco use has been linked to many forms of cancer and cardiovascular disease and it causes approximately 6 million deaths per year that could have been avoided. Hence, it has been described as one of the single largest avoidable

health risks (European Commision: Eurostat, 2014; Hoffman & Tan, 2015). The WHO set the target to reduce prevalence of using tobacco by 30% to be achieved by 2025. Data is promising as smoking prevalence declined from 28% in 1995 to 21% in 2014 (European Society of Cardiology, 2020). This data makes the WHO target potentially achievable if the trend persists.

1.3.5 Nutrition and physical activity as a risk factor for CAD.

Finally, nutrition and physical activity have been recognised s risk factors for CAD. It has been shown that an intake of 800g/day of fruit and vegetables reduced the risk of CVD as well as all-cause mortality (Aune et al., 2017). The same is truth for the physical activity defined as minimum of 150 minutes of moderate activity per week or 75 minutes of vigorous activity per week. It has been shown this activity level reduces the risk of ischaemic heart disease or type 2 diabetes by nearly 10%. The cardiovascular benefit of physical activity is mediated by reduction in blood pressure, reduction of obesity and increase in HDL cholesterol as well as improved glucose metabolism (Lee et al., 2012).

CAD is a slow-developing disease that usually takes decades to manifest clinical features. This process is influenced by environmental and lifestyle choices, which, when decreased are extremely efficient for secondary prevention after clinical event, such as MI or angina. These risk factors usually manifest themselves in sixth and seventh decade of life which is less than optimal for primary prevention, thus modification of these risk factors early in life could markedly attenuate CAD with the currently available therapeutic repository (Roberts et al., 2021).

1.4 Genetic risk factors of coronary artery disease.

Epidemiologic studies suggest that all modifiable risk factors are under genetic control, however a positive family history is an independent predictor and genetic heritability accounts for 40-50% of risk associated with CAD (Roberts & Stewart, 2012). Common genetic variants including single-nucleotide polymorphism (SNPs) are believed to contribute to the genetic risk of the disease. Thanks to genome-wide association studies (GWASs) there is currently identified over 50 different loci with significant importance to the increased risk of CAD (Ozaki & Tanaka, 2016). Furthermore, application of genetic risk score (GRS) based on

individual's DNA has many advantages over conventional risk factors, because they are not determined based on age: can be defined at birth or any time thereafter (Roberts et al., 2021).

Predisposition to CAD is due to multiple DNA risk variants and is therefore called polygenic. List of all discovered genetic risk loci updated to March 2021 is attached as an appendix 3. The first genetic risk variant for CAD, 9p21, was discovered in 2007 and was shown to be present in approximately 75% of the world population, except Africans, for whom this variant is not a risk factor. This risk is mediated independently of conventional risk factors for CAD (Assimes & Roberts, 2016). Subsequently, more genetic loci were found that include 3q22, SLC22A3 and 12q24 (Erdmann et al., 2009; Gudbjartsson et al., 2009; Myocardial Infarction Genetics Consortium et al., 2009; Trégouët et al., 2009). All these discoveries led to formation of Coronary Artery Disease Genome-Wide Replication, and Meta-analysis (CARDIoGRAM) Consortium. International consortium and individual effort led to discovery of 171 genetic risk variants associated with CAD that are of genome-wide significance (Erdmann et al., 2018; Roberts et al., 2021).

1.5 Biomarkers of atherosclerosis in coronary artery disease.

Atherosclerosis is considered a low-grade inflammatory disorder associated with many pro- and anti-inflammatory biomarkers, which are shown in table 1.

Table 1. Classification of biomarkers associated with atherosclerosis in coronary artery disease. Adapted from: Martinez et al., 2020.

Classification	Biomarkers
Inflammatory	CRP, SAA, IL-6, IL-1β, TNF-α, MCP-1, suPAR
Endothelial and cell adhesion	VCAM-1, ICAM-1, L-selectin, E-selectin, endothelial MP
Matrix degrading or proteolysis	MMP-1, MMP-2, MMP-7, MMP-9, TIMP-1
Lipid	LDL, sdLDL, ox-LDL, HDL, TRL, Lp-PLA2, Lp(a), ApoA-I, ApoB, ApoE
Metabolic	Adipokines (resistin, adiponectin, FABP4), homocysteine, OPG
Hematologic	RDW, WBC count, neutrophil count, T lymphocytes, monocytes
Angiogenic and neovascularization	VEGF
Thrombosis-related	PAI-1
Other	miRNA

Process called trained immunity contributes to long-term inflammation. Here, innate immune cells adopt long term pro-inflammatory phenotype after a short exposure to pathogen and favour a macrophage phenotype expression, where it produces chemokines and cytokines associated with plaque formation, and increased foam cell formation at atherosclerotic plaques (Bekkering et al., 2016).

1.5.1 C-reactive protein as an inflammatory biomarker.

C-reactive protein (CRP), the first atherosclerosis biomarker to be identified, is a representation of an acute phase protein and is indicative of chronic inflammation levels. It is mainly produced in liver, however it is now reported to be also produced at other sites, such as in macrophages and in human aortic endothelial cells and is regulated by Interleukin-6 (IL-6) and tumour necrosis alpha (TNF-α) (Volanakis, 2001).

CRP induces its effect through several signalling pathways. It can activate extracellular signal-regulated kinase (ERK) 1 and 2, which is involved in the induction of expression of metal metalloproteinases (MMPs) in macrophages and endothelium and vascular smooth muscle cells (VSMC) proliferation. These signalling pathways are involved in many cellular processes, such as migration, proliferation, differentiation and apoptosis to name the few (Calabrò et al., 2009; Cirillo et al., 2005). Another pathway where CRP is involved, a Rho/Rho-kinase, is known to be involved in the inflammatory signalling that includes nuclear factor kappa B (NF-κB) activation (Kaibuchi et al., 1999). Nuclear factor kappa B plays pivotal role in inflammation and plaque instability promoting expression of multiple pro-inflammatory genes and most CRP-activated pathways culminate with NF-κB activation that amplifies inflammatory stimuli (Liuzzo et al., 2007).

C-reactive protein acts as a causal factor at each stage of atherosclerosis. The first stage of atherosclerosis consists of a low-density lipoprotein (LDL) intimal deposition and endothelial dysfunction resulting from nitric oxide (NO) imbalance leading to vasoconstriction and proinflammatory phenotype (Bisoendial et al., 2007). CRP decreases bioactivity of NO synthase (NOS) through CD32 crosslinking compromising NO-related vasorelaxation (Hattori et al., 2003). Furthermore, CRP decreases inducible nitric oxide synthase (iNOS) activity in VSMCs adding to endothelial dysfunction. It also interacts with LDL, which is oxidised by reactive oxygen species in endothelium to become oxidised LDL (ox-LDL) which stimulates

endothelial cells to express adhesion molecules. That leads to the recruitment of macrophages to intima to become “foam cells” (Zwaka et al., 2001).

1.5.2 Endothelial biomarkers.

Selectins (E, L and P) are cellular adhesion molecules from a family of cell surface glycoproteins involved in the rolling and anchoring of leukocytes to the vascular wall. They are expressed on all monocytes and granulocytes, and most lymphocytes.

All selectins participate in early steps of leukocyte recruitment at the endothelial surface at slightly different ways. They form weak bonds between activated endothelial cells and leukocytes allowing for their rolling and tethering to endothelium as shown in figure 9.

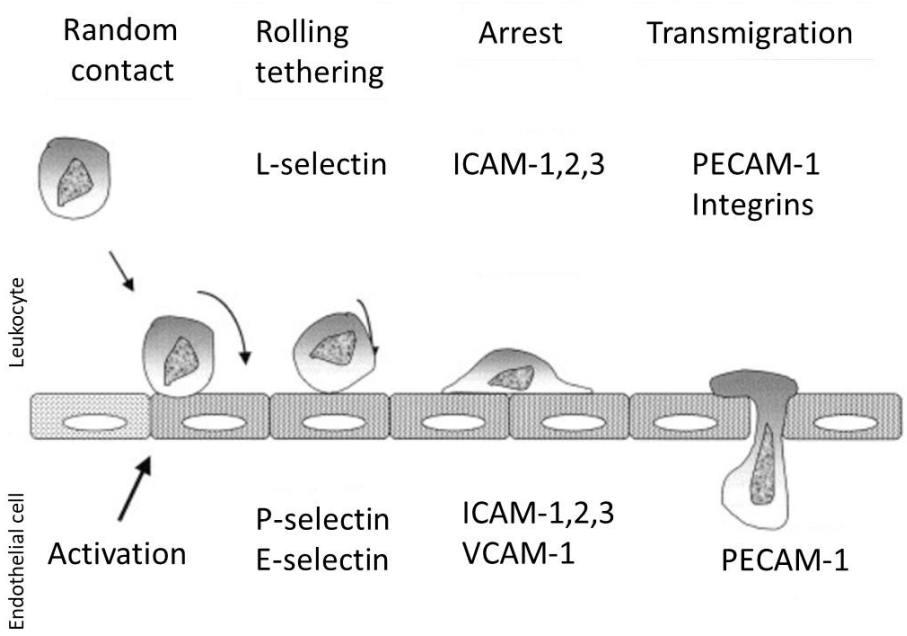

Figure 9. Representation of the leukocyte-endothelial cell-interactions during initial steps of atherosclerosis and role of different adhesion molecules in that process. Adapted from: Blankenberg et al., 2003.

Availability of selectins on cell surface is usually obtained in a few minutes and can last to few hours with a rapid decrease after a peak. The downregulation is important to limit or stop inflammatory processes that can have critical consequence on integrity of the vascular wall. Levels of rapidly removed from the cell surface selectins are measurable in blood (Hafezi-Moghadam et al., 2001).

Intercellular adhesion molecules (ICAM) are glycoproteins that are widely expressed at a basal level and can be upregulated by leukocytes, endothelial cells, pro-inflammatory cytokines and platelets. Out of all ICAMs, ICAM-1,2,3 mediate adhesion of leukocytes to activated endothelium by forming bonds with integrins and participate in leukocyte extravasation (del Pozo et al., 1994).

Platelet endothelial cellular molecule 1 (PECAM-1) is expressed by platelets, endothelial cells, and leukocytes. It is especially dense at the junctions between endothelial cells (Newton et al., 1997).

Finally vascular cell adhesion molecule-1 (VCAM-1) is transcriptionally induced on endothelial cell but is also present on myoblasts, macrophages, and dendritic cells. It is mainly involved in recruitment of blood cells by activated endothelium favouring their strong adhesion (Blankenberg et al., 2003).

1.5.3. Matrix degrading or proteolysis biomarkers

Matrix metalloproteinases (MMPs) belong to the family of metalloproteinases contain a zinc atom and a conserved methionine (Johnson, 2017). They play fundamental role in whole organism homeostasis, but also participate in the pathology of numerous major diseases such as cancer progression and atherosclerosis. In terms of vasculature, MMPs have the potential to degrade all the major components of the blood vessel wall. This is why their activity is tightly regulated by family of inhibitors called tissue inhibitors of metalloproteinases (TIMPs) to maintain balance in homeostasis (Khokha et al., 2013).

These proteases are regulated by multiple inflammatory cytokines, hormones, growth factors, and physical cell-cell interactions and are secreted by endothelial cells, VSMCs and macrophages. Due to MMPs role in homeostasis, the imbalance between MMPs and TIMPs have been shown to be evident in cardiovascular diseases (Brew & Nagase, 2010). The thrombogenic core of advanced atherosclerotic plaque is protected by a fibrous cap rich in collagen and MMP can break down fibrillar collagens within it and trigger plaque destabilisation (Libby, 2013). Furthermore, elevated plasma levels of MMP are predictive of cardiovascular events and resulting all-cause mortality (Cavusoglu et al., 2015).

1.5.4. Cholesterol and apolipoproteins as biomarkers.

The plasma lipoproteins are spherical particles consisting of a lipid core (triglycerides and/or cholesterol) surrounded by monolayer of so-called amphipathic lipids (phospholipids and unesterified cholesterol) as shown in figure 10.

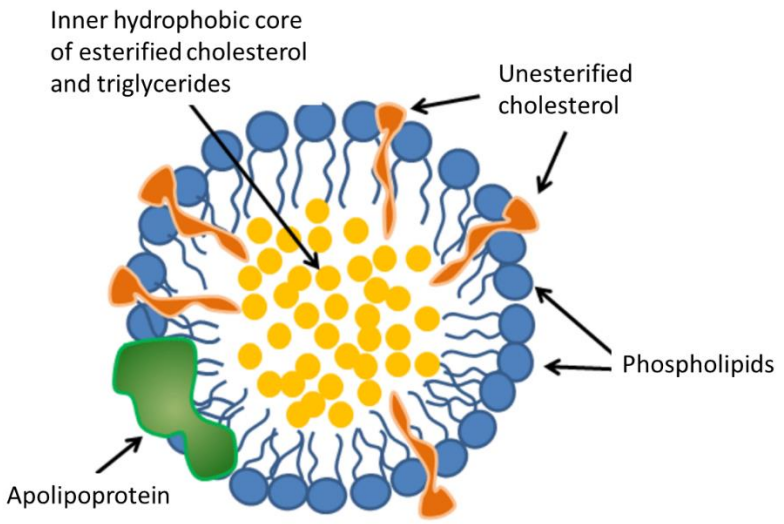

Figure 10. Schematic drawing of lipoprotein structure. Adapted from: Champe et al., 2005.

Lipoproteins are divided into low density cholesterol (LDL), high density cholesterol (HDL), very low-density cholesterol (VLDL) and chylomicrons which are rich in recently absorbed triglycerides. Currently it is considered that the "response-to-retention hypothesis" by Williams and Tabas (1995) explains the events that initiate atherogenesis (Williams & Tabas, 1998). It postulates that the initiating event is the retention and accumulation of LDL and other atherogenic proteins in artery wall. These proteins then directly or indirectly aggravate pathological features of initial lesions, such as cytokine production, smooth muscle proliferation, lipoprotein oxidation, monocyte migration into artery wall and macrophage foam cell formation (Olofsson & Boren, 2005).

The protein component of the lipoproteins – the apolipoprotein (apo) – is bound to the surface of the lipoprotein. Key role of apolipoproteins is in lipid transport in plasma and in central nervous system (Huang et al., 2004). There are currently six different apolipoproteins that have been identified to hold pro or anti-atherosclerotic properties:

1. Apolipoprotein A (apo A) is the major component of HDL and positively modify triglyceride levels in coronary artery disease patients (Patsch & Gotto Jr, 1996).

2. Apolipoprotein B (apo B) is associated with chylomicrons and LDLs. High levels are related to heart disease. Apo B/apo A ratio identified by the INTERHEART study have shown that it is better marker of vascular disease as well as better guide to the adequacy of statin treatment than any cholesterol index (McQueen et al., 2008).
3. Apolipoprotein C (apo C) activates lipoprotein lipase in capillaries, liberating fatty acids and monoglycerides from chylomicrons, where fatty acids are transported to muscle cells and adipocytes. Defects in production of apo C result in risk of early atherosclerosis (Sacks, 2006).
4. Apolipoprotein D (apo D) is a component of HDL in human plasma. There is evidence of its neuroprotective role due to the anti-inflammatory and anti-oxidant activity (Perdomo & Dong, 2009).
5. Apolipoprotein E (apo E) is involved in recognition of chylomicron remnants by the liver and is essential for normal catabolism of triglyceride-rich lipoprotein constituents (Marais, 2021).
6. Apolipoprotein H (apo H) also called glycoprotein I or beta-2 (B2gp1) is involved in physiological processes such as blood coagulation and homeostasis (Simó et al., 2008).

1.5.5 Adipokines as metabolic biomarkers.

Adipokines are molecules produced and secreted by the adipose tissue and act as paracrine or endocrine hormones. They have many functions including regulating fat distribution, inflammation, blood pressure and endothelial dysfunction as well as appetite and satiety (Blüher, 2014). The relations between metabolic status, adipokines and cardiovascular pathophysiology are shown in figure 11.

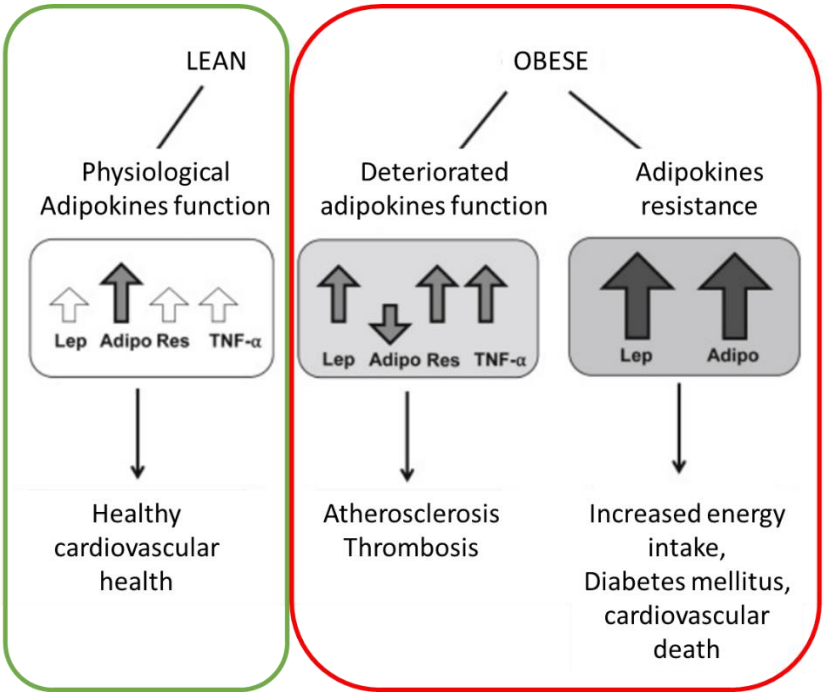

Figure 11. Relations between metabolic status, adipokines production and cardiovascular physiopathology. In the green panel: Lean subjects adipokine tissue has a physiological amount of adipokines (leptin, adiponectin and resistin) and cytokines (TNF-α); here prevalence of adiponectin is associated with a healthy coronary artery. Red panel left side: Obesity is associated with visceral fat accumulation and adipocyte hypertrophy with naturally accompanying infiltration of activated macrophages that lead to chronic low-grade inflammation. Higher amount of leptin, resistin and TNF-α as well as reduced adiponectin level led to higher risk of cardiovascular disease. Red panel right side: Lack of appropriate hormone response after engagement with the receptor characterises metabolic disturbance and obesity. It is often defined as resistance, where despite higher levels of leptin and adiponectin, their signalling cascades are defective. Adapted from: Ruscica et al., 2017.

Adiponectin is an example of adipokine. It is also named adipocyte complement-related protein of 30kDa (Acrp30) and is an adipocyte-specific secreted protein (Pajvani et al., 2003). Adiponectin has been characterised as a systemic insulin sanitiser that acts on many cells and decrease levels of inflammation. It does so by promoting macrophage polarisation towards anti-inflammatory M2 phenotype and activates AMPK and fatty acid oxidation in skeletal muscle. This anti-inflammatory function also translates into the atherogenesis and atherothrombosis (Ruscica et al., 2017).

Adiponectin has many protective functions in atherosclerosis. It decreases atheroma formation and improves plaque stability by increasing endothelial nitric oxide synthase (eNOS) activity in endothelial cells (Wang et al., 2014). It controls the expression of adhesion molecules, e.g., VCAM, ICAM and e-selectin involved in leukocyte recruitment resulting in dampening endothelial pro-inflammatory response. Finally it induces expression of tissue inhibitor of MMP-1 in macrophages and controls proliferation and migration of VSMC (Ruscica et al., 2017).

1.5.6 T lymphocytes as a hematologic biomarker.

Multiple studies have identified that atherosclerosis is initiated by the injury to the endothelial wall or accumulation and oxidation of LDLs within the arterial wall. These factors trigger innate and adaptive immune responses that are known to play key roles in the development of atherosclerosis. Monocytes, macrophages, T-lymphocytes and B lymphocytes are the main cell types that are involved in formation of the plaque (Bartekova et al., 2018).

T-lymphocytes are amongst the earliest cells to be recruited for the formation of the atherosclerotic plaque and have a crucial role in developing plaque. All subsets of T cells: CD4+, CD8+, NK and helper T cells have been recognised in human atherosclerotic plaque (Hansson & Hermansson, 2011). The variable expression of cytokines contributes to the polarisation of TH cells into different subtypes shown in figure 12. TH1 lymphocytes are more abundant than TH2 in the plaque and differentiate from the naïve T cells under stimulation with IL-12 and IL-18 cytokines produced by activated macrophages. Furthermore that stimulation elicit production of IFN-γ by TH1 cells promoting atherosclerosis progression. (Elhage et al., 2003).

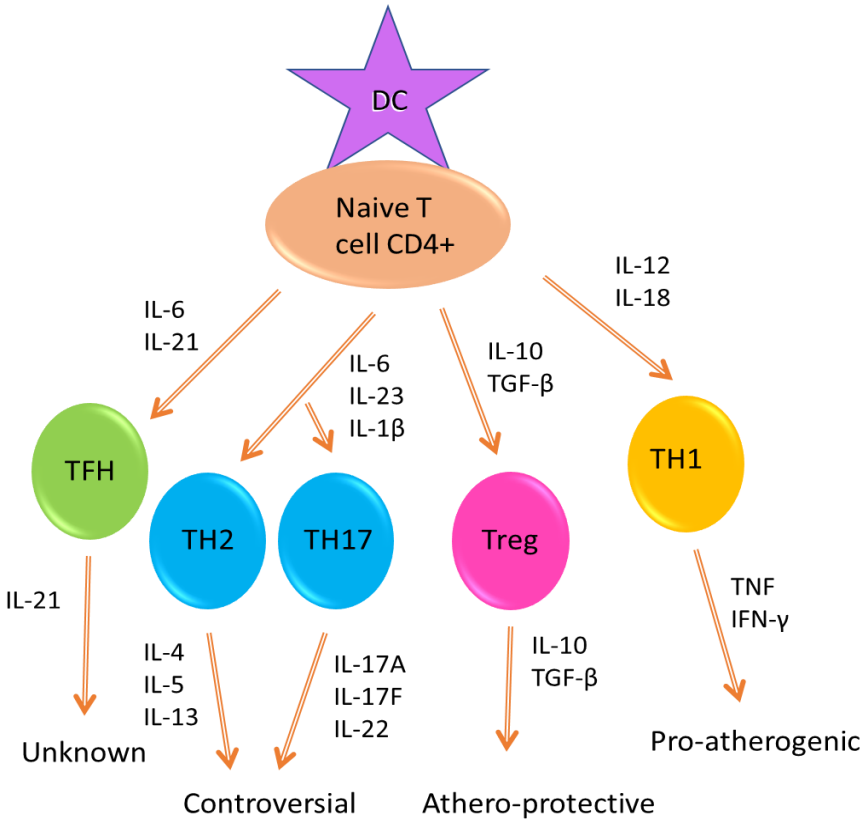

Figure 12. Different subtypes of T cells in atherosclerosis. Pro-atherogenic TH1: expression of IL-12 and IL-18 activates transcription factor T-bet that leads to production of pro-inflammatory cytokines such as TNF and IFN-γ. Atheroprotective Treg: Treg cells display transcription factor FOXP3 that is allows production of IL-10 and TGF- β in the presence of IL-10 and TGF-β. Controversial TH1 and TH2: Cytokines including IL-23 IL-6 IL-1β and TGF-β stimulate TH-17 to produce IL-22 and IL-17A/F via transcription factor RORγT. TH2 stimulated by the same cytokines produces IL-4, which, in a positive feedback loop, influences production of transcription factor GATA3. Excess production of IL-4 leads to inhibition IFN-γ. Unknown: Follicular helper T cell have the potential to release IL-21 and to express transcription factor BCL6. Adapted from: Roy et al., 2021.

TH1 differentiation occurs through IL-12 and IL-18 stimulation produced by activated macrophages in the atherosclerotic plaque. This differentiation leads to production of IFN-γ that promotes development and progression of atherosclerosis (Davenport & Tipping, 2003). Differentiation into TH17 lymphocytes is stimulated by many cytokines in the atherosclerotic plaque to produce IL-17 family of cytokines that activate various signalling pathways including ERK1/2 or NF-κB in various target cells. These include endothelial cells, smooth muscle cells,

TH1 and macrophages to produce pro-inflammatory cytokines such as TNF, IL-1β and granulocyte colony-stimulating factor (Abdolmaleki et al., 2019). Atheroprotective function of TH2 lymphocytes relies on production of IL-5, IL-13 and IL-4 which neutralise the effect of IFN-γ. Especially IL-4 showed antiatherogenic activity through downregulation of VCAM-1 MMP1 and A scavenger receptor on macrophages while IL-5 promote production of IgM antibodies against oxidated LDL by B lymphocytes (Roy et al., 2021). Finally, Treg cells have various critical roles in atherosclerosis. They act on other cells through the expression of IL-10 and TGF-β and can restrict autoimmunity by competing with other T cell subtypes for the set of antigens and MHC class II (MHCII) on antigen presenting cells by direct cytotoxic or inhibitory effect on effector cells and downregulating the expression of co-stimulatory molecules such as CD80/CD86 (Tse et al., 2013).

1.5.7 VEGF as an angiogenic and neovascularization biomarkers.

The VEGF is a family of heparin-binding proteins involved in angiogenesis, lymphopoiesis and lymph angiogenesis and regulation of inflammation and lipid metabolism. The VEGF family contains three proteins that are involved in regulation of blood vessel growth: VEGF-A, VEGF-B and placental growth factor – PIGF (Corlan et al., 2017). They are involved in inhibition of the inflammatory response, promote dilation and proliferation of lymphatic vessels and reduce oxidative stress, thus prevent atherosclerosis progress (Dabravolski et al., 2022). Circulating VEGF-A protein is the most studied in terms of atherosclerosis. Its levels in circulation are marker for revascularisation and identification of CAD severity (Mitrokhin et al., 2016). Interestingly VEGF-a has a dual purpose in atherosclerosis. It can protect endothelial cells by stimulating NO synthesis and stimulating the expression of anti-apoptotic proteins. However, it also prevents repair of the endothelial lesion hence promoting atherogenesis by promoting monocyte adhesion and endothelial permeability (Dabravolski et al., 2022).

1.5.8 Thrombosis – related biomarkers

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor (SERPIN) superfamily and is an important inhibitor of fibrinolytic pathways. Under physiological conditions PAI-1 prevents plasmin formation, however elevated levels of PAI-1 have been associated with pathological conditions such as MI and metabolic syndrome and greater coronary artery disease risk (Jung et al., 2017). Despite PAI-1 promising to be a good

indicator of CVD disease, current evidence support relationship between PAI-1 and atherosclerosis without inferring clear causality nor pathways involved (Song et al., 2017).

1.5.9. miRNA as CAD biomarker.

miRNA are defined as short, single stranded, noncoding RNA molecules, which influence the synthesis of proteins through their interactions with mRNAs (Thum & Mayr, 2012). They are able to inhibit certain protein expression by attaching to and silencing gene either via cleaving and degrading its target mRNA or by inhibiting the translation process and subsequently reduce protein synthesis and affect the function of cells. (Thum & Mayr, 2012). Thus, miRNA play a role in endothelial injury and consequent cell attachment, growth and inflammatory responses as well as regulate smooth muscle proliferation and macrophage activity (Lu et al., 2018).

One way that miRNA is involved in the formation of atherosclerotic plaque is via exacerbating endothelial cells (EC) senescence. Several miRNAs have been involved in this process. It has been shown to influence ECs proliferation and differentiation during cellular senescence by decreasing activity of genes responsible for longevity, proliferation and apoptosis (Lu et al., 2018). Another way miRNAs affect endothelium is through influencing the activation and infiltration of leukocytes via the vascular wall (Staszel et al., 2011). A key miRNA involved in this process is miR-126 which inhibits VCAM-1. Inhibition of this miRNA results in decrease expression of TNF-α resulting in the formation of lesions (Lu et al., 2018).

1.6 Clinical manifestations of coronary artery disease.

Coronary artery disease manifests itself by stable or unstable angina, myocardial infarction (MI) or sudden cardiac death which are the result of atherosclerosis, shown in figure 13. All these conditions that are a result of reduced oxygen supply to a heart are known as an acute coronary syndrome (Kloner & Chaitman, 2017).

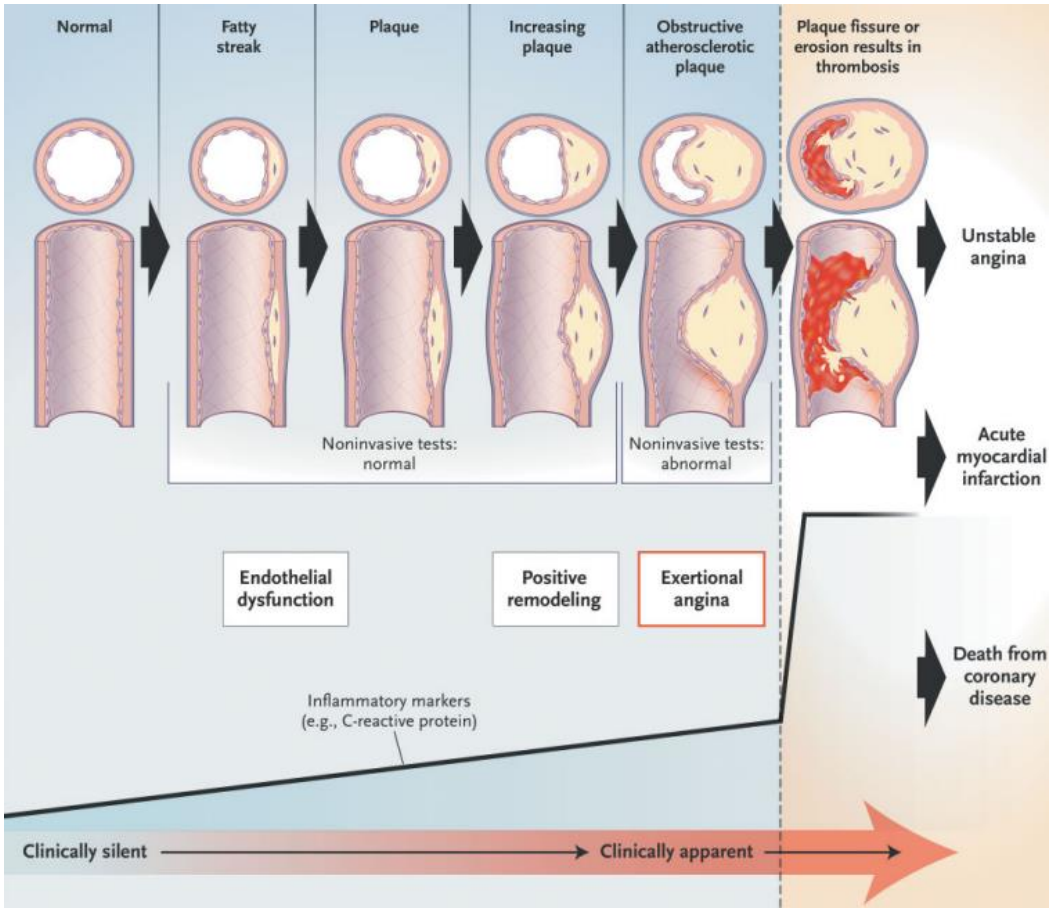

Figure 13. Typical progression of coronary atherosclerosis. Firstly, the plaque mass stays external to the lumen which allows lumen diameter to be maintained: this is known as a Glagov effect or positive modelling. As plaque starts growing into the lumen, the coronary artery diameter decreases. This pathology results in disturbed ratio of coronary supply and myocardial consumption. When this narrowing is of more than 65% to 75% it may result in transient ischaemia or angina. Source: Abrams (2005).

Susceptible plaque is the most important factor in acute coronary events, even more important than the degree of stenosis. It is because the acute coronary syndromes result from erosion or ulceration of the cap with subsequent intraluminal thrombosis. Additionally, atherosclerosis is considered to be a silent killer since the plaque within the vessel may not be obstructive hence remain clinically silent until it ruptures and causes associated consequences (Abrams, 2005).

1.6.1 Characteristics of angina pectoris.

Angina pectoris is caused by myocardial ischaemia and presents itself as a pain in the chest or adjourning area as a result of exertion related to myocardial function disorder. It is often described as a sense of "strangling and anxiety" or in the manner of squeezing, tight, suffocating and crushing. Discomfort is usually substernal with the left arm as a common radiant area. It is often exacerbated by exertion and/or anxiety, lasts 30 to 60 seconds and is relieved by rest and/or nitro-glycerine. (Shao et al., 2020). Angina pectoris is a common manifestation of IHD with an estimated prevalence of 3%-4% in UK adults. There are over 250000 invasive coronary angiograms performed annually with over 20000 new cases of angina. It uses significant portion of healthcare resources with over 110000 inpatient episodes each year that lead to substantial associated morbidity (Timmis et al., 2020).

1.6.1.1. Stable angina.

There are two major types of angina: stable and unstable. Features of chronic stable angina include complete reversibility of the symptoms and repetitiveness of attacks over time, typically months to years and there is usually a trigger such as stress or exercise (Kloner & Chaitman, 2017). Typical and atypical symptoms of angina are shown in table 2.

Table 2. Symptoms of stable angina: classical and atypical.

Classical symptoms of angina	Atypical symptoms of angina
Chest squeezing, heaviness, tightness, burning	Pain is sharp, knife-like, choking, pulsating.
Radiation to shoulder, neck jaw and inner arm	Pain in the chest, radiation non predictable
Relatively predictable	Cannot be predicted
Lasts 1-15 minutes	Can last between seconds to all day.
Subsides after nitro-glycerine is taken or when stressor is gone	Variable response to nitro-glycerine

Stable angina is the most prevalent manifestation of coronary artery disease, and its prevalence increases with age. Interestingly, despite a higher overall prevalence of CAD in men, stable angina is more common as an early presentation of CAD in women. The Women's

Ischaemia Syndrome Evaluation (WISE) study highlighted that over 2/3 of women has no obstructive CAD (absence of coronary vessel occlusion), but the disease symptoms were associated with significant impairments in health-related quality of life, such as anaemia or poorly controlled hypertension (Olson et al., 2003). In general, however as coronary atherosclerosis progresses, encroachment of the plaque into the lumen can result in haemodynamic obstruction and symptoms of stable angina.

1.6.1.2. Unstable angina.

Coronary atherosclerotic disease is the underlying cause of unstable angina (UA) in patients with acute myocardial ischaemia. The most common cause of unstable angina is the narrowing of the coronary artery due to a developing thrombus on the disrupted plaque that is nonocclusive (Goyal & Zeltser, 2022).

Unstable angina is heterogeneous syndrome with widely variable symptoms and prognosis characterised by prolonged or recent-onset symptoms where attacks are more unpredictable and can continue despite resting (Kloner & Chaitman, 2017). Unstable angina deals with reduced blood flow in coronary arteries causing a lack of perfusion to the heart. This block most commonly can be formed from the intraluminal plaque formation or thrombosis, vasospasm and elevated blood pressure or a combination of thereof (Goyal & Zeltser, 2022). Patients will often present with chest pain radiating to jaw or arms, and left and right sides can be affected, and shortness of breath. Statutory symptoms such as vomiting, nausea, dizziness and palpitations are also prevalent. One distinguishing factor of unstable angina is that the pain may not completely resolve with nitro-glycerine and/or aspirin (Goyal & Zeltser, 2022; Yeghiazarians et al., 2000). Another, patients have familiarity with the with the symptoms such as increase in episodes of chest pain that may take longer to resolve as well as severity of symptoms. It is important to understand these differences from the diagnostic point as these may indicate impending myocardial infarction and ST-elevation myocardial infarction (STEMI) hence should be evaluated promptly as the risks of morbidity and mortality are higher in UA that stable angina (Shah & Nathan, 2018). The key complications of unstable angina include myocardial infarction, stroke and death (Goyal & Zeltser, 2022).

In terms of unstable angina and its progression to MI a variety of biomarkers that are linked to inflammation can predict a reoccurrence of short term coronary events in patients after acute coronary events better than conventional risk factors (Morrow & Ridker, 2000). These

markers include pro- and anti-inflammatory cytokines, shed cell adhesion molecules, matrix metalloproteinases and acute-phase proteins. Interestingly these markers are common prognosticate of cardiovascular events in general population as well as in CAD patients and are likely to reflect fundamental mechanisms of the disease. (Ridker et al., 1997).

1.6.1.3 Myocardial infarction.

Myocardial infarction (MI), colloquially known as "heart attack" is caused by significant decrease or cessation of blood flow to a portion of myocardium. It might be silent and undetected, or it could be catastrophic leading to haemodynamic deterioration and sudden death. Most MIs are a result of underlying coronary artery disease (Ojha & Dhamoon, 2021). With coronary artery occlusion, myocardium is deprived of oxygen which, if prolonged, can lead to cell necrosis and death (Apple, 2012).

Myocardial ischaemia is responsible for the development of acute coronary syndrome (ACS) because of unstable angina plaque rupture. It can be divided into non-ST segment elevated myocardial infarction (NSTEMI) and ST segment elevated infarction (STEMI) (Landesberg et al., 2009). Differences between both types are shown in figure 14.

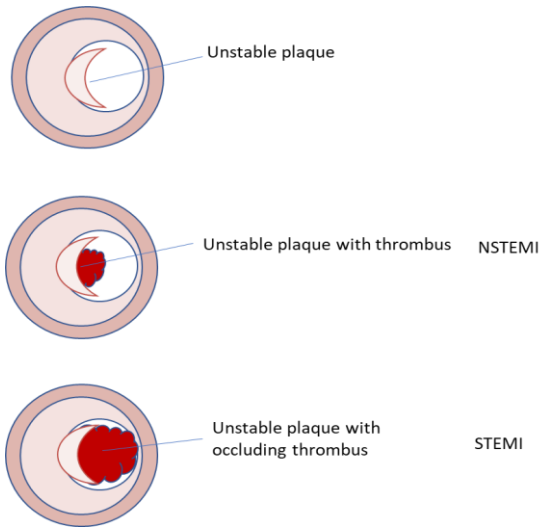

Figure 14. Pathological mechanisms responsible for cardiac ischaemia. STEMI – ST-segment elevation myocardial infarction, NSTEMI – non-ST segment elevation myocardial infarction.

STEMI is life threatening and time-sensitive emergency that results in complete thrombotic occlusion of the coronary artery. These patients have severe chest pain radiating to left arm, jaw and back and short-term mortality is high at around 30%. The remaining 70% of patients

have >5% mortality (Kingma, 2018). Patients with NSTEMI are presented with more heterogeneous symptoms, e.g. reduced blood flow without complete coronary occlusion, coronary embolism and myocarditis and have higher long-term mortality due to occurrence of comorbidities and CAD (Kingma, 2018).

Infarction produces necrosis by loss of myocardial structure and myocytes death followed by repair including formation of a scar. Coagulation necrosis is the most common type of myocardial necrosis caused mainly by reduction in coronary perfusion due to intracellular acidosis. In coagulative myocytolysis hydrolytic enzymes released by leukocytes and neutrophils lyse myocardial fibres. Formed lesions consist of loss of contractile proteins, oedema and nuclear changes such as fragmentation. Finally, coagulative myocytolysis is a result of activity of toxins such as carbon monoxide or nicotine (Daga et al., 2011).

1.7 Current treatments for coronary artery disease.

Current treatments include many options and include lifestyle changes to address risk factors associated with diet, levels of physical exercise, smoking cessation, and stress reduction. Pharmacology treatments can be focus to address hypertension, diabetes, lipid levels, and platelets levels. Finally they include interventional or surgical revascularisation (Wolf & Hunziker, 2020)

1.7.1 Lipid lowering pharmacotherapy.

It is indicated for primary and secondary prevention to include statins, proprotein convertase subtilisin/kexin type 9 (PCSK9)-inhibitors and ezetimibe to lower the LDL cholesterol levels.

Statins are the first line of treatments however they have a dose limiting side effect like myopathies, but this activity is not fully understood (Ward Natalie et al., 2019). Studies have shown that in patients with different levels of CAD risk, statins have reduced the risk of cardiovascular events and mortality, especially in secondary prevention patients (Musunuru, 2021). All statins are inhibitors of 3-hydroxy-3-methylglutary-coenzyme (HMG-CoA), a key enzyme in the synthesis of cholesterol. Reduction of endothelial dysfunction is mediated by induction of statin-dependant iNOS and thrombomodulin as well as reduction of secretion of IL-1β and IL-6 and reduction of ROS. Additionally athero-protection in endothelium is

enhanced through YAP/TAZ inactivation and reduced VCAM1 expression that leads to suppression of endothelial dysfunction (Li et al., 2019).

Ezetimibe is another type of drug used to reduce cholesterol uptake in the intestine and is used as a second line of augmentation of statin treatment. It inhibits the Niemann-Pick C1-like 1 (NPC1L1) protein in gastrointestinal track which modestly reduces levels of LDL cholesterol hence reducing CAD risk (Cannon et al., 2015; Grundy et al., 2019).

Activating mutations for PCSK9 were discovered in 2003 to be a cause of familial hypercholesterolemia; the protein is produced in the liver and secreted into bloodstream where it antagonises cellular LDL receptors. Inactivation of PCSK9 leads to reduced LDL cholesterol levels (Cohen et al., 2005). The same inactivating mutations were established to be highly protective against CAD, reducing its risk of up to 88% (Cohen et al., 2006). This observation led to formulation of PCSK9 inhibitors – monoclonal antibodies evolocumab and alirocumab that were to be administered via injections every few weeks. Randomised control trails FOURIER and ODYSSEY OUTCOMES have proved for this therapy to reduce cardiovascular events and for that reason, both are no included in guidelines as third-line agents (Fitzgerald et al., 2017; Ray et al., 2017).

1.7.2 Hypertension lowering pharmacology.

1.7.2.1 Beta blockers

Pathophysiological mechanisms of blood pressure as a risk factor for CAD are complex and include blood pressure as a physical force on the development of the arthroscopic plaque. In CAD treatment of arterial hypertension is beneficial, especially beta blockers (BBs) have shown to be superior to any other drug classes for use after a recent myocardial infarction (Weber et al., 2016). Primary blood pressure goal in patients with CAD is below 140/90 mmHg. It is especially important for patients after the MI where reduction of systolic blood pressure by 10mmHg and diastolic blood pressure by 5 mmHg resulted in CAD events reduction by 24% (Law et al., 2009).

The physical impact of high blood pressure can cause endothelial injury as a result of impairment in the synthesis and the release of vasodilator – nitric oxide and promotes accumulation of ROS and inflammatory mediators which facilitate thrombosis, vascular occlusion and, in result, atherosclerosis (Oparil et al., 2003). Additionally, renin-angiotensin-

aldosterone system (RAAS) can be dysregulated, as angiotensin II increases blood pressure and facilitates progression of atherosclerosis via vasoconstrictive and vascular remodelling effect. Taken together, the inflammation that is driven by these factors led to the idea that some antihypertensive agents such as angiotensin-converting enzyme (ACE) inhibitors can have beneficial anti-atherosclerotic effect in addition to their BP lowering effect (Dzau, 2005).

1.7.2.2 Nitro-glycerine

Nitro-glycerine has been a cornerstone therapy in treatments of angina and lowering blood pressure. The main effect includes veins and coronary arteries dilatation resulting in reducing end-diastolic volumes and improving coronary flow respectively. Nitrates are recommended for STEMI with mild heart failure and elevated blood pressure and for STEMI with moderate heart failure if there is no hypotension and no right ventricular infarction (Weber et al., 2010).

1.7.2.3 Calcium channel blockers

Long-lasting dihydropyridines calcium channel blockers (CCBs), amlodipine and nifedipine can be added if blood pressure remains elevated despite beta-blocker therapy. These agents act by causing vasodilation and decreasing peripheral resistance and wall tension thus reducing myocardial oxygen demand as well as increase myocardial oxygen demand by dilating coronary vessels (Black et al., 2003).

1.7.2.4 Angiotensin-Converting Enzyme

Angiotensin – Converting Enzyme (ACE) inhibitors are recommended for all patients after MI as it showed cardioprotective effect in hypertensive CAD patients (Fox, 2005). Despite ACE inhibitors are associated with only minimal blood pressure reduction, they are correlated with significant reduction in cardiovascular disease death, stroke and MI (Yusuf et al., 2000).

1.7.3 Anti-platelet agent - aspirin

The first successful anti-platelet agent was aspirin. Aspirin blocks the signalling pathways of cyclooxygenase (COX) so that it binds to the catalytic site and acetylate it. In result arachidonic acid, which is a substrate for COX, cannot gain access to catalytic site (Cox, 2020). Typically, it is prescribed for people who had MI to reduce the risk of blood clots forming.

1.7.4 Lifestyle changes

Smoking cessation is the most cost-effective secondary measure to prevent MI. It has pro-thrombotic effect, which has strong association with atherosclerosis and MI.

A diet low in saturated fat with focus on whole grains, vegetables, fruits, and the fish are considered cardioprotective. Target level for bodyweight is body mass index (BMI) of 20 to 25kg/m^2 and waist circumference of less that 80cm for female and 94cm for men (Ojha & Dhamoon, 2021). Summary of dietary guidelines is shown in table 3.

Table 3. Summary of dietary guidelines for patients with CAD. Source: (Shao et al., 2020)

	Population goals			
	Overall healthy eating pattern	Appropriate body weight	Desirable cholesterol profile	Desirable blood pressure
Major guidelines	Include a variety of fruits, grains, low-fat or non-fat dairy products, fish, legumes, poultry, lean meats	Match energy intake to energy needs, with appropriate changes to achieve weight loss when indicated	Limit foods high in saturated fat and cholesterol; and substitute unsaturated fat from vegetables, fish, legumes, nuts	Limit salt and alcohol; maintain a healthy body weight and a diet with emphasis on vegetables, fruits, and low-fat or non-fat dairy products

Dietary Approaches to Stop Hypertension (DASH), vegetarian and Mediterranean diets are currently the most recommended type of diet in terms of preventing cardiovascular disease. Studies have shown that DASH diet have reduced hypertension as coronary artery disease by 21% and Mediterranean diet decreased the risk of cardiovascular disease by 20-25% by having a positive effect on endothelium function, arterial stiffness and cardiac function (Aune et al., 2017; Mattioli et al., 2017; Sacks et al., 1999).

Healthy lifestyle, whether after the acute coronary syndrome or during the atherosclerosis, is not only limited to diet and exercise. It also includes the adherence to cardioprotective medicines. Shockingly, studies have shown that low adherence to pharmacotherapy were seen in 66% of individuals and high cardiovascular risk and 55% in patients with CAD two years after initiation of prescription (Naderi et al., 2012).

1.7.5 Procedures and surgery.

If blood vessels are narrow as the result of a build-up of atheroma or if the CAD cannot be controlled with pharmacology, interventional procedures or surgery may be needed. First coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI) were performed in 1964 and 1977 respectively for myocardial revascularisation (Riley et al., 2011).

Coronary artery bypass grafting (CABG) is the most performed cardiac surgery and most effective for the revascularisation for stenotic coronary arteries. This procedure is most commonly used to relieve myocardial ischaemia by constructing grafts to bypass atherosclerotic lesions and restore blood supply to distal coronary branches (Li et al., 2020). CABG is very effective in improving patient's prognosis, hence selecting patients is critical for good outcomes. This evaluation is based on coronary anatomy, extent of the disease and comorbidity. It is mainly performed in patients with stable CAD, with reduced EF (≤45%), diabetes and ischaemic mitral regurgitation as these patients can benefit from CABG (Bangalore et al., 2016).

Coronary angioplasty is also known as percutaneous coronary intervention (PCI) is a procedure during MI to quickly open blocked artery and reduce the amount of damage to the heart. Here, a tiny balloon is inserted temporarily into the damaged artery to enlarge it. To further decrease the chances of artery contraction ever again a stent (wire mesh tube) is inserted permanently to keep the artery opened. This procedure reduces the symptoms of blocked arteries such as pain and breath shortness (Malakar et al., 2019). This procedure is usually performed if there is less than 120 minutes from the diagnosis of ischaemic event (Ojha & Dhamoon, 2021).

1.8 The role of cytokines in inflammation.

Cytokines are small, non-structural proteins which are less than 40kDa in size. They are produced by nearly every cell and exert their pleiotropic effect to regulate immune response to inflammation, trauma and infection (Kany et al., 2019). Physiological role of the cytokines is tissue homeostasis and cellular activation, relocation, and differentiation (Dembic, 2015).

Cytokines are generally produced as a consequence of cell activation and serve as communicators of characteristic functions in specific tissues and have different modes of

action. In most cases they act locally on neighbouring cells. However, since cytokines are soluble molecules, they penetrate most tissues and are delivered by migration of white blood cells to distant locations. When they act systemically, they are often responsible for many general symptoms, e.g., in infection they are responsible for headache or fever (Dembic, 2015; Slifka & Whitton, 2000).

Dysregulation of the pro and anti-inflammatory cytokine balance is responsible for pathogenesis in many diseases. Furthermore cytokines can potentially trigger signalling cascades, where a small amount of protein can lead to devastating consequences and rapid mortality (Kany et al., 2019; Slifka & Whitton, 2000). Overview of various cells expressing different cytokines is shown in figure 15.

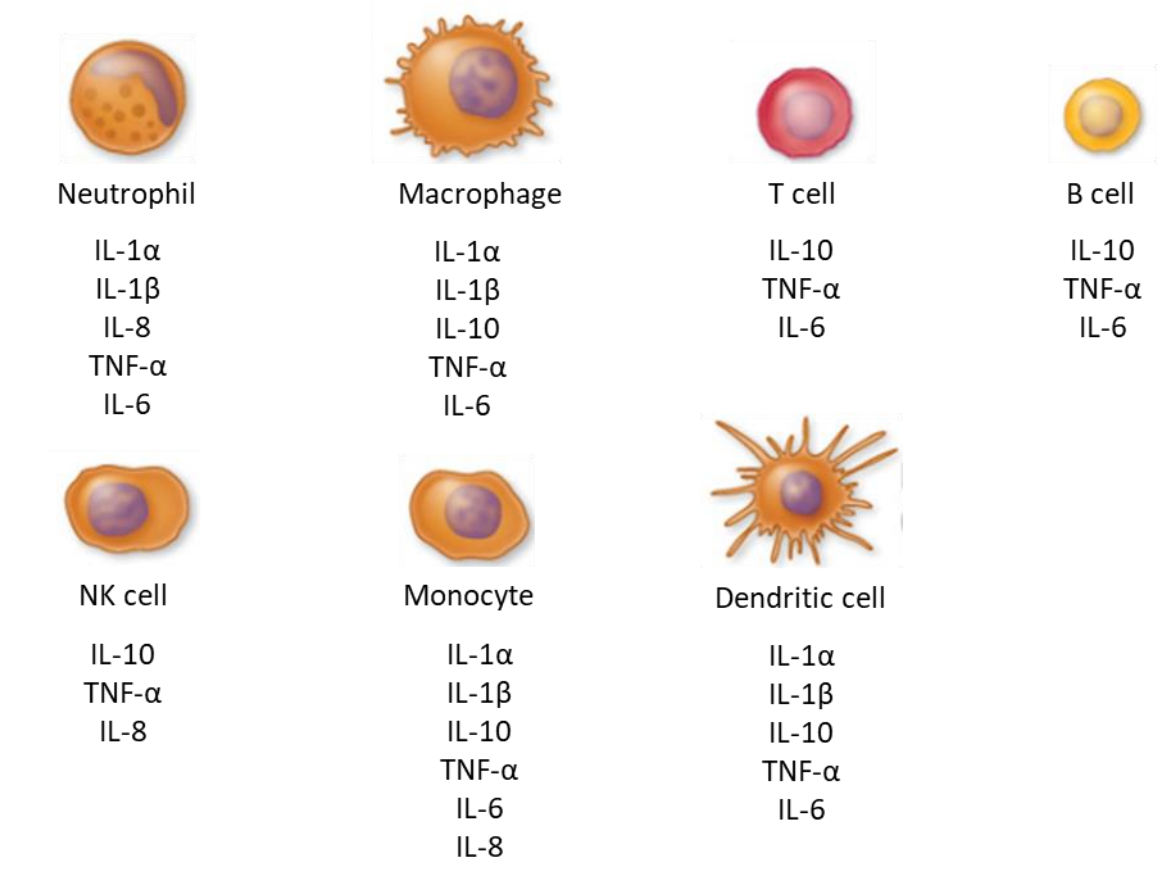

Figure 15. A diagram showing various cells expressing different cytokines. NK-natural killer cells, IL-interleukin, TNF-α-tumour necrosis factor-alpha. Adapted from Kany et al. (2019)

1.8.1 The role of cytokines in coronary artery disease.

Cytokines are recognised as important mediators of the progression from a normal autoimmunity to autoimmune disease. (Nichols et al., 2012). Among one of the conditions

under the cardiovascular disease umbrella is coronary artery disease caused by atherosclerosis – a chronic inflammatory condition that is characterised by reduced blood flow through the coronary artery (Narasimhulu et al., 2016). For this reason, cytokines emerged as an important biomarker for local and systemic inflammation.

In atherosclerosis immune reactions and inflammatory responses occur in the vessel wall. Lesions are filled with immune cells that coordinate and influence inflammatory responses. Initial lesions are filled with macrophages and T cells which both produce and secrete cytokines (Balkwill, 1993). Local markers of inflammation in atherosclerotic tissue include tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), where IL-6 production is stimulated by IL-1 and TNF-α. Furthermore, patients with coronary artery disease had increased plasma levels of IL-6, IL-1 and IL-8 in atherosclerotic plaque (Heinisch et al., 2005). Additionally, IL-12 stimulates T cells and natural killer cells (NK) to produce interferon gamma (IFN-γ) which induces proatherogenic processes in the atherosclerotic lesion. In contrast, IL-10 have an anti-inflammatory effect and inhibits cytokine secretion (Opstad et al., 2016).

1.8.1.1 Interleukin-1β (IL-1β).

In recent years there is an increasing evidence suggesting that inflammation plays key role in coronary artery disease and other atherosclerosis manifestations (Mai & Liao, 2020). The Interleukin-1 (IL-1β) is the main cytokine family associated with chronic and acute inflammation (Szekely & Arbel, 2018). Among the members of the family, IL-1β is predominantly produced by monocytes, macrophages and dendritic cells and its synthesis can be divided into several steps (figure 16). (Mai & Liao, 2020).

Firstly IL-1β binds to its receptor, type 1 (IL-1R1) and recruits the co-receptor chain termed the accessory protein (IL-1RAcP). This complex then recruits the adaptor protein MyD88 to the Toll-IL-1 receptor (TIR) domain of each receptor. Multiple kinases are phosphorylated and nuclear factor-κB (NF-κB) translocates to the nucleus where expression of vast number of inflammatory genes, including IL-1β, IL-6, IL-8 ensues (Weber et al., 2010). Another key player is the cytosolic molecular structure called leucine-rich repeat pyrin domain containing 3 (NLRP3) inflammasome that contains NLRP3 sensor molecule called nucleotide-binding domain and pro-caspase-I. Sensor molecule can be activated by an infectious stimulus (pathogen associated molecular patterns – PAMPs) or sterile stimuli (damage-associated molecular patterns – DAMPs) such as cholesterol crystals or oxidised low-density lipoproteins

(ox-LDL) accumulated under intimae. This activation is based on either formation of reactive oxygen species (ROS) or ATP binding to the P2X7 receptor and efflux of potassium to the extracellular space. As a result of activation of NLRP3 inflammasome, pro-caspase 1 is converted to an active enzyme and cleaves the IL-1 precursor in specialised secretory lysosomes or the cytosol, followed by secretion of mature IL-1β (Cremer et al., 2016).

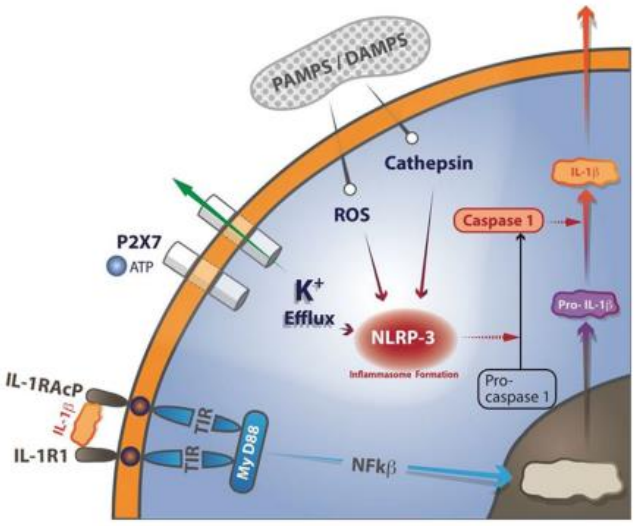

Figure 16. IL-1β synthesis and secretion. IL-1B binds to the IL-1R1 and recruits co-receptor IL-1RAcP. This complex recruits adaptor protein MyD88 to the TIR domain. Multiple kinases are phosphorylated, NF-κB translocates to the nucleus and transcription of pro-IL-1 begins. The NLRP3-inflammasome that consist of NLRP3 sensor molecule, adaptor protein and pro-caspase 1 can be activated by PAMPs or DAMPs. This activation is based on formation of ROS or ATP binding to P2X7 receptor and extracellular efflux of potassium. After activation of NLRP3-inflammasome, pro-caspase is converted to an active enzyme and cleaves IL-1 precursor in specialised lysosomes or cytosol to secrete mature IL-1β. Source: Szekely & Arbel, 2018.

1.8.1.2 Interleukin-8 (IL-8).

Chemokines are a type of chemotactic cytokines, and their function is to induce direct migration of cells to the site of inflammation. Structurally, they are small with a molecular mass of 8-10 kDa and are divided into four subfamilies: CXC, CC, CX3C and C. They differ by the number of amino acid bridges between the amino-terminal cysteine residues (Fernandez & Lolis, 2002). Chemokines exert their activity by binding to specific cell surface receptors with high affinity. Interleukin-8 also called C-X-C Motif Chemokine Ligand 8 (CXCL8) is a

chemokine dimer from the CXC family with cysteine residues with an intervening amino acid as shown in figure 17.

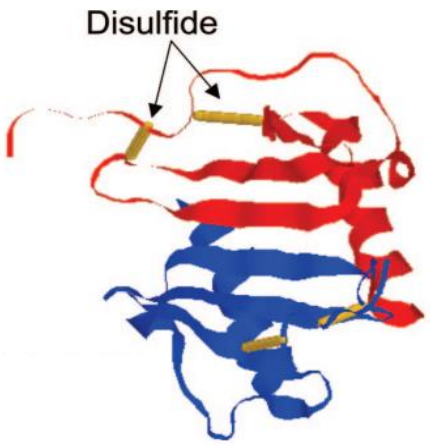

Figure 17. Interleukin-8 as dimer. Both chains are shown, one in red and one in blue. Disulfide bonds between cysteine residues are shown in yellow. Adapted from: Remick, D. G. (2005).

IL-8 is produced by many cell types including monocytes, lymphocytes, fibroblasts, endothelial cells and chondrocytes and is released only under inflammatory conditions (Matsushima et al., 2022). This intercellular signal of acute inflammation is hence used by many tissues and cell types to employ host defence and predominantly activate neutrophils. Multiple stimuli can induce secretion of IL-8 including bacterial particles, hypoxia, lipopolysaccharides as well IL-1, interleukin-6 (IL-6) and tumour necrosis factor- α (TNF-α) (Remick, 2005). There are three different mechanisms that lead to upregulation of IL-8 expression: de-repression of the IL-8 gene promoter, IL-8 mRNA stabilisation by the p38 mitogen-activated protein kinase (MAPK) pathway and trans-activation of IL-8 expression by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal Kinase (JNK) pathways (Brat et al., 2005). In unstimulated cells IL-8 gene promoter is repressed as a consequence of three events: (1) by the NF-κB-repressing factor (NRF) binding to negative regulatory element (NRE) blocking the NF-κB binding site, (2) histone deacetylase 1 (HDAC-1) induction of deacetylation of histone proteins and (3) octamer-1 (OCT-1) binding to the complementary strand of the promoter gene in the opposite direction of the CCAAT-enhancer-binding protein (C/EBP) binding site. In a presence of a stimulus, such as IL-1 or TNF-α, the activated p65 subunit of NF-κB translocates to the nucleus and binds to DNA. CCAAT-

enhancer-binding proteins (C/EBP) binds to promoter replacing octamer-1 (OCT-1) and recruits cyclic adenosine monophosphate response element binding protein (CREB) binding protein (CBP)/p300 resulting in histone hyperacetylation and chromatin remodelling. Therefore, IL-8 promoter is de-repressed, activator protein 1 (AP1) and NF-κB are phosphorylated and activate gene transcription of IL-8 as well as other anti-apoptotic genes (Ha et al., 2017; Hoffmann et al., 2002). Signal transduction is shown in figure 18.

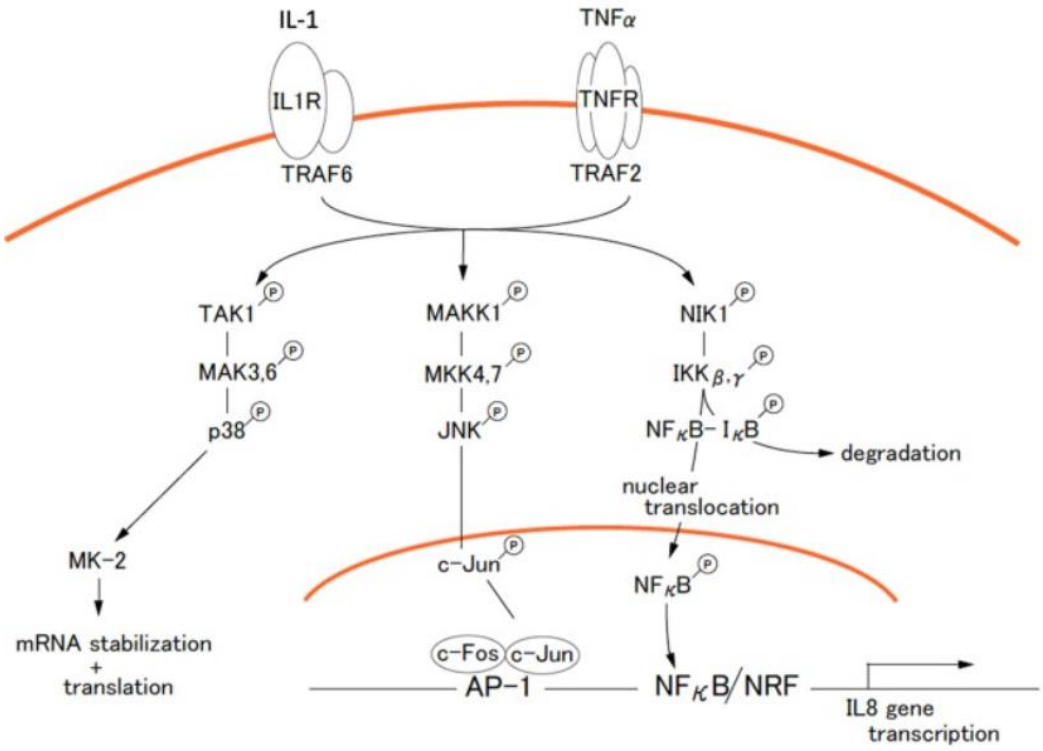

Figure 18. Transduction steps in cytokine-mediated IL-8 gene regulation. After IL-1 or TNF-αbinds to the cell surface it forms a multimeric receptor complex that recruits adaptor proteins TNF receptor associated factor 6 (TREF6) and TNF receptor associated factor 2 (TREF2) respectively. This oligomerisation triggers activation of various mitogen-activated protein kinase kinase kinase (MAPKKK). Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) and MEK kinase 1 (MEKK1) activates the mitogen-activated protein kinase kinase 7- c-Jun N-terminal kinase (MKK7-JNK) and the nuclear factor kappa-B/-γ pathways. The direct target of inhibitor of nuclear factor-κB (IκB) kinase (IKK) phosphorylates IκB allowing release of NF-κB, whereas JNK regulates phosphorylation of c-Jun and AP-1 transcription activity. The p65 subunit of NF-κB translocates to nucleus and binds to the NF-

κB site of the IL-8 promoter and interacts with nuclear respiratory factor 1 (NRF) and activator protein 1 (AP-1) transcription factors. Finally activated p38 phosphorylates mitogen-activated protein kinase (MK-2) to stabilise IL-8 mRNA contributing to upregulation of IL-8 production (Hoffmann et al., 2002; Matsushima et al., 2022; Umebashi et al., 2018). Source: Matsushima et al. (2022)

CXCL8 binds C-X-C Motif Chemokine Receptor 1 (CXCR1) and C-X-C Motif Chemokine Receptor 2 (CXCR2) receptors in humans. These receptors are found on many cells including neutrophils, monocytes, lymphocytes, and fibroblasts (Russo et al., 2014). Atherosclerotic plaque is characterised by the cellular infiltration with all these cells and monocyte-derived macrophages are key players. It is due to their ability to form foam cells and oxidation of lipoproteins accumulated to the blood vessels (Van Tits et al., 2011). There are multiple pathways that trigger production of IL-8 in the plaque. Monocytes increase CXCR2 expression on their surface for adhesion upon IL-8 stimulation. Oxidised derivative of cholesterol, 25-hydroxycholesterol, acts on retinoic-inducible gene I expressed on macrophages and induces production of IL-8 by activating interferon regulatory factor 1. Also endothelial cells contribute to inflammation by producing IL-8 as tissue factors and factor VIIa are also present in atherosclerotic plaques (Russo et al., 2014; Zernecke et al., 2008). Apart from the IL-8 pro-inflammatory activity, it can also be a contributor to a mechanism related to plaque rupture as it can inhibit Tissue Inhibitor of Metalloprotease-1 (TIMP-1) expression in macrophages by introducing an imbalance between matrix metalloproteinases and TIMPs at the atherosclerotic plaque sites (Moreau et al., 1999).

1.8.1.3 Interleukin-6 (IL-6).

Interleukin-6 (IL-6) is a soluble molecule with a pleiotropic effect on immune response, haematopoiesis, and inflammation. It is made of 212 amino acids and is 20-26kDa in size (Tanaka et al., 2014). IL-6 is produced by a variety of cells including T cells, monocytes/macrophages, endothelial cells, fibroblasts and hepatocytes (Choy & Rose-John, 2017). Interleukin-6 acts on many different tissues and cells. In hepatocytes it induces and mediates acute phase response by inducing acute phase proteins such as C-reactive protein (CRP) serum amyloid A (SAA), fibrinogen, thrombopoietin and complement C3. In haematopoiesis it promotes hematopoietic stem cell differentiation and megakaryocyte maturation that leads to release of platelets as well as activation of coagulation system by

inducing tissue factor (TF) on the surface of monocytes, which in turn leads to thrombin production and as a result hypercoagulable state and thrombosis (Tanaka et al., 2014, 2018).

Interleukin-6 promotes differentiation of activated B cells into Ig-producing cells as well as regulation of direction of differentiation of naïve $CD4^{+}$T cells. IL-6 in combination with transforming growth factor β (TGF-β) is essential for T helper 17 (Th17) differentiation at the same time inhibits TGF-β-induced regulatory T cell (Treg) differentiation. This Th17/Treg imbalance is pivotal in development of various chronic and autoimmune diseases (Kimura & Kishimoto, 2010).

Interleukin-6 can also induce excess production of vascular endothelial growth factor (VEGF) that leads to enhanced angiogenesis and increased vascular permeability. Vascular permeability by IL-6 itself or through VEGF can then lead to interstitial oedema resulting in tissue damage (Romano et al., 1997). These effects of IL-6 play a pathological role in tissue hypoxia, disseminated intravascular coagulation and multiple organ dysfunction named together as systemic inflammatory response syndrome (SIRS) (Tanaka et al., 2018).

Figure 19 provides detailed overview of IL-6 signalling. In the classical IL-6 signalling pathway IL-6 binds to the plasma membrane associated IL-6 receptor (IL-6R). To transduce signal this complex associate with the signal transducer glycoprotein 130 (gp130). This binding induces gp130 homodimerization and activation (Tanaka et al., 2018). Active complex recruits the JAK non receptor tyrosine kinase which phosphorylates the tyrosine residues of gp130. This phosphorylation generates recruitment sites for other proteins such as signal transducer and activator of transcription 1/3 (STAT1/3) and Src homology region 2-containing protein tyrosine phosphatase 2/ extracellular signal-regulated kinase (SHP-2/ERK), activating multiple signal cascades that lead to transcription of IL-6 target genes, such as c-myc, bcl2, cyclin D1 and several metalloproteinases (Choy & Rose-John, 2017). Additionally, STAT3 activation activates inhibitory adaptor proteins SOC1 and SOC3 which generate negative feedback loop by binding to JAK and gp130 (Villar-Fincheira et al., 2021). In trans-signalling model IL-6 signalling pathway a soluble, shedded IL-6R (sIL-6R) binds to IL-6 and exists as IL-6/sIL-6R complex in various body fluids including blood. Gp130 is expresses on all cells in the body while membrane bound IL-6R is expressed by many inflammatory cells including monocytes, macrophages, and granulocytes as well as hepatocytes. Thus, sIL-6R/IL-6 complex can bind to and stimulate cells that only express gp130. It has been assumed that classical IL-6 signalling

is associated with the immune response and trans-signalling with more systemic processes (Villar-Fincheira et al., 2021).

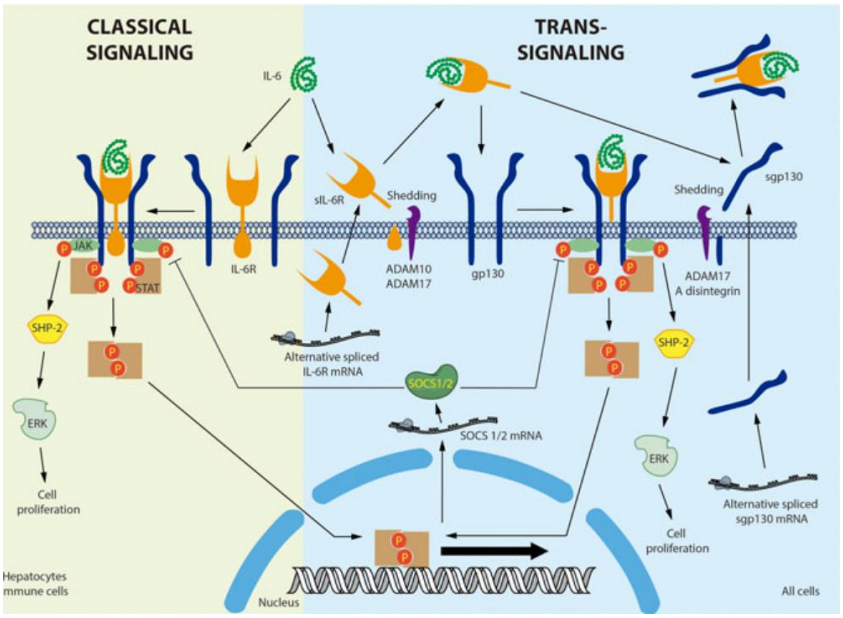

Figure 19. Interleukin classical and trans-signalling.In classical signalling IL-6 binds to a membrane bound IL-6R and associates with the gp130. It is followed by activation of JAK/STAT signalling. It is followed by activation of SHP2/ERK signalling pathway which is common with a trans-signalling IL-6 pathway. In the trans-signalling, sIL-6R is produced by shedding of the membrane bound IL-6R by A disintegrin and Metalloproteinase 10 or 17 (ADAM10 or ADAM17). Alternatively, IL-6/IL-6R complex binds membrane bound gp130 activating a signalling pathway described in classical signalling. Soluble gp130 (sgp130) is produced mainly by alternative splicing and shedding of the cell membrane by A disintegrin and ADAM17. Sgpd130 binds IL-6/sIL-6R complex and inhibits trans-signalling without affecting classical pathways. Finally in negative feedback loop JAK activity is inhibited by Stat-dependant expression of SOC1/2. Source: Villar-Fincheira et al. (2021).

Atherosclerosis, once thought to be just an accumulation of lipid in the arterial wall, is now recognised to have a prominent inflammatory component. It is not fully understood whether elevated IL-6 levels are a product of the cardiovascular disease or whether it is serving pathogenic function. It is clear however that IL-6, a pro-inflammatory cytokine plays a causal role in determining CVD risk (Fontes et al., 2015).

1.8.1.4 Interleukin-10 (IL-10).

Interleukin-10 (IL-10) is an anti-inflammatory cytokine with a crucial role in preventing inflammation and autoimmune diseases. It is a pleiotropic cytokine that inhibits cell-mediated immunity while enhancing humoral immunity (Kaur et al., 2009). It is a non-glycosylated protein of 18 kDa and exists as a non-covalently linked homodimer as shown in figure 20 (Dembic, 2015).

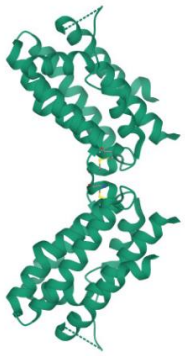

Figure 20. Crystal structure of human IL-10 homodimer. Source: Jones et al. (2002)

Interleukin-10 production is associated with many immune cells hence it has a crucial role as a feedback regulator of immune responses. It is expressed by cells of innate immune system such as dendritic cells (DC), macrophages, mast cells and neutrophils, as well as by the cells of adaptive immune system for example B cells, $CD8^+$ T cells, TH1, TH2, TH17 and Treg cells (O'Garra et al., 2008). Interestingly, recent studies showed that IL-10 is also produced by cardiac macrophages and epithelial cells (Hulsmans et al., 2018). IL-10 has an important effect on immune response. It acts on DC and macrophages and inhibits the development of Th1-type responses and supresses Th2 cells and allergic responses (Moore et al., 2001). In addition to autocrine inhibitory effect, IL-10 can be produced by TH1, TH2, TH17 and form an additional feedback loop that limits the innate effector function of macrophages and DC and their following activation of T-cells. On the other side, IL-10 boosts the differentiation of Treg cells that produce IL-10 providing a positive regulatory loop for its induction. Finally, IL-10 can activate mast cells and increase function of NK, B and $CD8^+$ T cells, however effects of this actions are not yet tested (Saraiva & O'garra, 2010).

The biological activities of IL-10 are dependent on two receptors, one with high affinity, IL-10R1 and the other with low affinity IL-10R2.

Expression of IL-10 is tightly regulated. In myeloid cells it is similar to that of pro-inflammatory cytokines, downstream of activation of multiple pattern recognition receptors (PRRs). MAPK are the key pathway regulating IL-10 expression. Activation of ERK1/2 downstream of MAP3 kinase tumour progression locus 2 (Tpl2) regulates its expression by toll-like receptor (TLR) - activated DCs and macrophages. The MAPK p38 also regulates production of IL-10 by these cells by as it is activated downstream of TLR signalling (Kaiser et al., 2009). Cooperation between p38 and ERK activates the mitogen- and stress-activated protein kinase 1 (MSK1) and MSK2 which, through the transcription factors cAMP response element binding protein (CREB) and cyclic AMP-dependent transcription factor 1 (ATFI1), stimulate production of IL-10 in TLR4-stimulated macrophages (Ananieva et al., 2008). Several NF-κB family members also regulate expression of IL-10 in macrophages by binding to the *IL10* locus. Finally, the phosphoinositide-3-kinase (PI3K)/serine/threonine/ protein kinase B (Act) cascade inhibits the expression of *IL10* in macrophages by blocking the kinase glycogen synthase kinase 3β and conversely potentiating production of IL-10 through ERK1/2 and mechanistic target of rapamycin (mTOR) (Ohtani et al., 2008).

In addition to direct PRR signalling, other cytokines and coreceptors regulate production of IL-10 in DCs and macrophages. Type 1 interferon (IFN) stimulates production of IL-10 by bone marrow-derived macrophages stimulated with lipopolysaccharides (LPS). It induces transcription of *IL10* gene and stabilises resulting mRNA through ERK and STAT1 activation (Howes et al., 2016).

In addition to macrophages, IL-10 is produced by all T cell subsets. IL-10 production occurs downstream of T-cell receptor triggering (TCR) which activates rat sarcoma virus (Ras) and consequently MAPK ERK1/2 and API1 as well as other transcription factors. Also, activation of STAT4, 4 and 6 and interferon regulatory factor (IRF) during Th cell differentiation contribute to *IL10* gene expression. Thus, mechanism required for differentiation of Th cells is also required for regulation of IL-10 production and forms a feedback regulatory loop to prevent pathology (Saraiva et al., 2020). The signalling pathways are shown in figure 21.

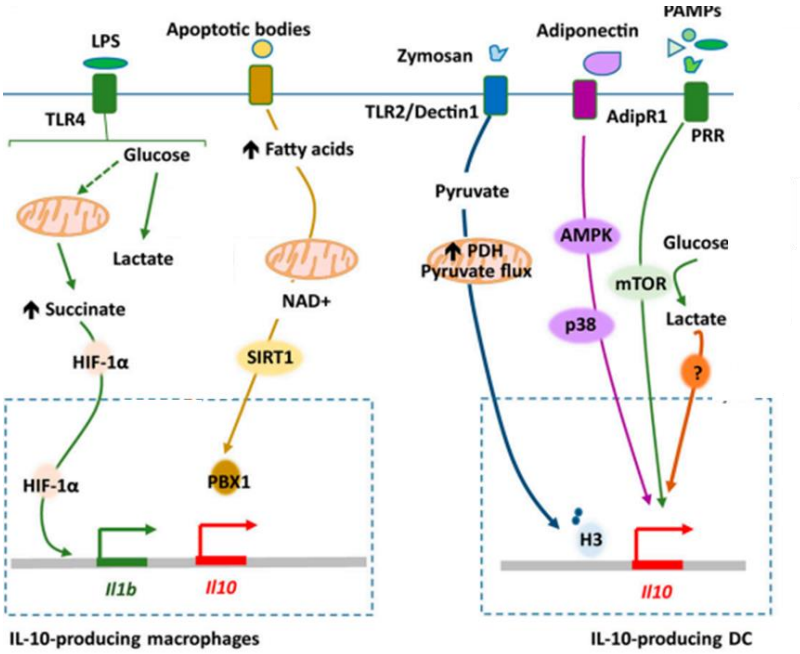

Figure 21. Signalling pathways of IL-10. Macrophages stimulated by TLR4 start producing of lactate and break the tricarboxylic acid (TCA) cycle. As a result, succinate accumulates and activates JHIF-1α which enhances transcription factor of *IL1β* gene. Increase in IL-10 production can also be increased by the enhanced fatty acid metabolism and is initiated by the PBX1 transcription factor. In DCs, IL-10 production is favoured by multiple pathways. The pyruvate flux downstream of the zymosan stimulation reinforce presence of acetylate histone 3 at the *IL10* gene promoter. Other metabolic regulators, such as AMPK and mTOR also contribute to the IL-10 production, however the exact pathway is unknown. Adapted from: Saraiva et al., 2020.

Myeloid cells play a critical role in innate immunity and are involved, as adherent macrophages, in the formation of early atherosclerotic lesions (Libby et al., 1996). In terms of atherosclerosis, the major function of IL-10 is to inhibit macrophage activation, MMPs, tissue factor and pro-inflammatory cytokines production, i.e., IL-2 and TNF-α, cyclooxygenase-2 expression in lipid loaded and activated macrophage foam cells, and anti-apoptotic activity (Hansson, 2001).

1.8.1.5 Tumour necrosis factor alpha (TNF-α).

Tumour necrosis factor alpha (TNF-α) is a glycoprotein with a transmembrane region. It is a homotrimer, as shown in figure 22, that by the action of proteases becomes soluble and in that form is present in the tissues (Dembic, 2015).

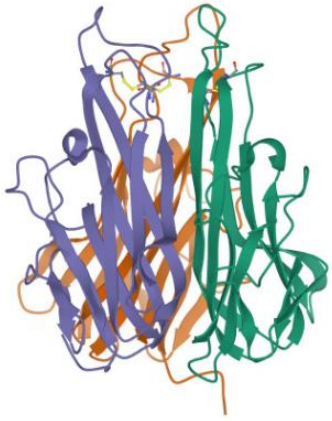

Figure 22. Crystal structure of human tumour necrosis factor alpha lymphokine homotrimer. Source: Maskos et al. (1998).

TNF-α is a multifunctional cytokine that has well established roles in innate and adaptive immunity as well as in physiological functions of immune cells. Abnormally increased and sustained levels of TNF are associated with pathogenic infection-like sepsis and autoimmune diseases. It has also become known as a adipokine since it is produced in adipose tissue in obesity, which lead to the appreciation obesity to be inflammatory in nature (Mathis & Shoelson, 2011).

Tumour necrosis factor alpha is produced by a range of immune and non-immune cells and is the first cytokine to appear within minutes of an injury or stress by a pro-inflammatory stimulus. It exists in two forms: 26kDa transmembrane trimer (mTNF) 17kDa soluble trimer (sTNF) (Sethi & Hotamisligil, 2021). Signal transduction by both trimers occurs following binding to one of two homotrimeric cell-surface receptors: TNF-alpha receptor I (TNFR1) which is a receptor for soluble ligand and TNF-alpha receptor II (TNFR2) mediates signalling of the membrane-bound ligand as shown in figure 23. While TNRF1 is expressed on all cell types in the body, TNRF2 is selectively expressed on endothelial cells, neurons and lymphocytes but it can also be induced in response to TNFR1 activation and signalling (Sethi et al., 2008). Tumour necrosis factor alpha induced TNFR1 leads to signal transduction in two

ways: stimulation of cell survival and expression of pro-inflammatory genes or apoptosis and cell death through the death domain (DD). In the first state (signalling complex I) transcription of pro-inflammatory genes and anti-apoptotic pathways is driven by many transcription factors, AP-1 and NF-κB being the most important. They regulate production of cytokines, growth factors, metalloproteinases, leukotrienes and adhesive molecules (Bradley, 2008). Unlike the signalling complex I which leads to survival, complex II directs towards apoptosis when NF-κB activation via complex one has failed (Ihnatko & Kubes, 2007). Signal pathway activated by TNFR2 supports cell activation, migration, and proliferation. It does so by induction of MAPK, Akt and NF-κB pathways (Zelová & Hošek, 2013).

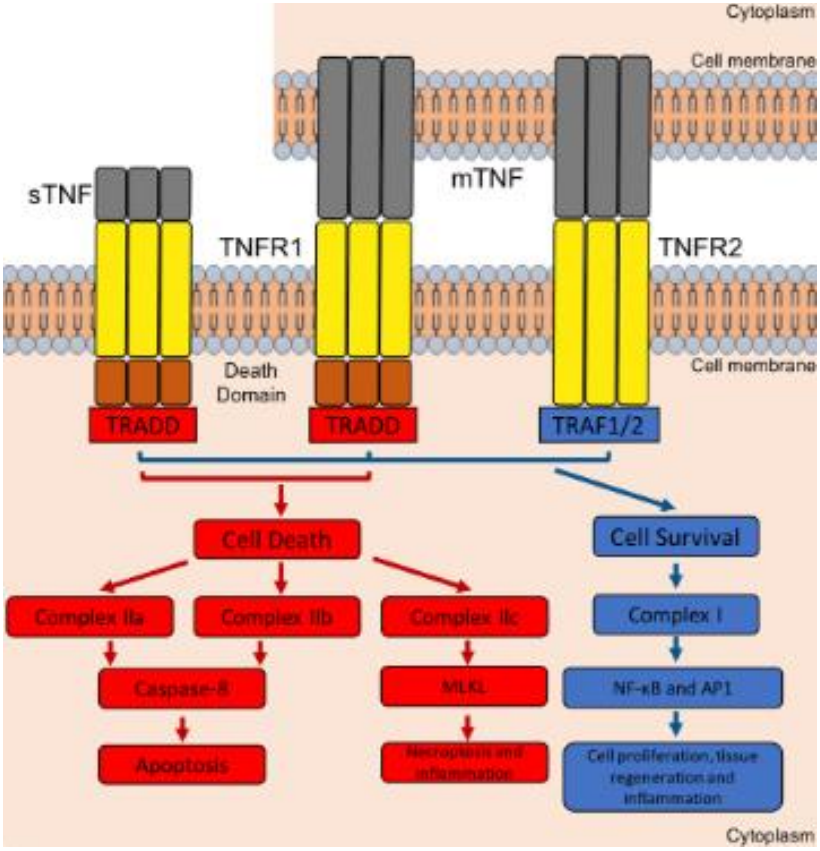

Figure 23. Tumour necrosis alpha receptor 1 and 2 (TNFR1/2) signalling pathways. TNFR1 can be activated by soluble TNF (sTNF) or membrane bound TNF (mTNF). TNFR1 contains a dead domain which interacts with TNFR1- associated death domain (TRADD). Depending on whether reception-interacting serine/threonine-protein kinase 1 (RIPK1) is ubiquitinated, the cell undergoes apoptosis via complexes IIa and IIb, necrosis via II c or cell survival, via complex I. Apoptosis via complexes IIa and I b is propagated through the cleavage of pro-caspase-8 to form caspase-8, while formation of complex II c leads to activation of mixed lineage kinase

domain-like protein (MLKL) and resulting necroptosis. In complex I cell survival is induced via activation of NF-κB and AP-1 transcription factors because of RIPK1 ubiquitination. TNRH2 is primarily by mTNF and interacts directly with TNFR-associated factor (TRAF) 1 and 2 to induce complex I with induction of homeostatic signals. It does not contain intracellular death domain. Source: Holbrook et al. (2019).

1.8.1.6 Interleukin-12 (IL-12).

Interleukin-12 (IL-12) is a heterodimeric protein with two disulfide-linked subunits: p35/p40 and IL-12p70, however a homodimer comprising only larger subunit also exists as an IL-12p40 as shown in figure 24. Interestingly, IL-12 component p40 can dimerise with IL-23p19 to form IL-23. The main source of IL-12 are macrophages, monocytes, DC and B cells and is produced in the infection and inflammation (Dembic, 2015).

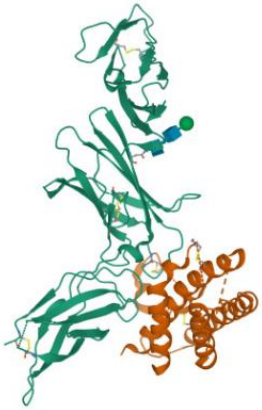

Figure 24. Crystal structure of human interleukin-12 homodimer. Larger subunit IL-12p70 shown in green, smaller subunit p35/40 shown in red. Source: *Human Interleukin-12* (https://www.rcsb.org/structure/1f45).

The interleukin-12 receptor is a heterodimeric complex of IL-12 Rβ1 and IL-12Rβ2 units. IL-12R is predominantly found on T cells and NK cells. Biological activity of IL-12 include stimulation of IFN-γ production by NK and activated Th1 cells, as well as inhibition of Th2 lines during infection and inflammation (Wojno et al., 2019). Signalling through the IL-12R depends on JAKs family members JAK2 and Tyrosine Kinase 2 (Tyk2) that are associated with IL-12 Rβ1 and IL-12Rβ2 respectively. Once IL-12 binds to IL-12R it phosphorylates IL-12βR. In turn, phospho-JAK2 phosphorylates the tyrosine residue Y800 which act as a binding site for SRC

homology domains (SH2) of STATs, especially STAT4. STATs are then phosphorylated by JAK2 and translocate to the nucleus where they serve as repressors or promoters of gene transcription, especially IFN-γ (Zundler & Neurath, 2015). Overview of signalling is described in figure 25.

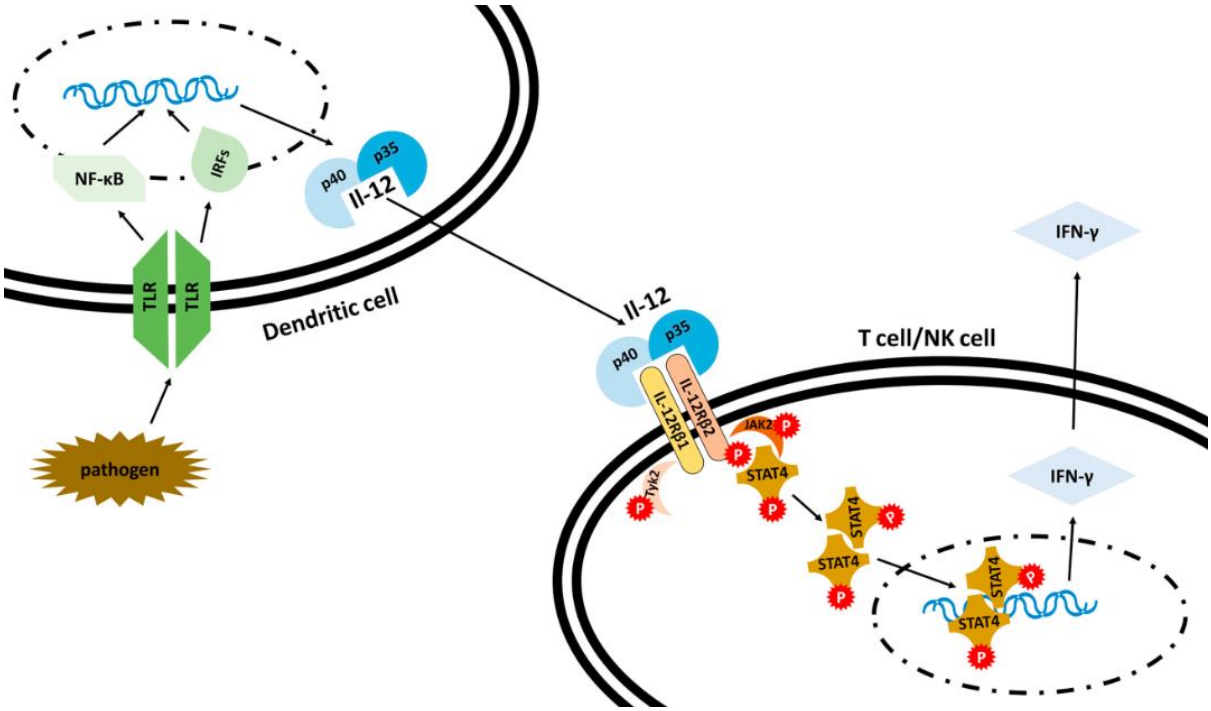

Figure 25. Secretion and signalling of IL-12. Phagocytic cells sense pathogen components via TLRs, that leads to activation of transcription factors such as NF-κB and interferon regulatory factors (IRFs) to induce transcription of IL-12p35 and Il-12 p40. Subsequently IL-12 p70 is released and recognised by IL-12R on NK and T cells. IL-12p40 binds to IL012Rβ1 and IL-12p35 binds to IL-12Rβ2. This triggers association of Tyk2 and JAK2 to the intracellular domains of the IL-12R receptor subunits. JAK2 then phosphorylates and enables binding of STAT4 to IL-12Rβ2 where it is phosphorylated by JAK2. Consequently, to homodimerization and translocation to the nucleus, STAT4 binds the promoters of different genes, most prominently IFN-γ. Source: Zundler & Neurath (2015).

The action of IL-12 on T cells and NK cells has multiple effects: macrophages are activated by IFN-γ to produce NO synthase and therefore release reactive oxygen species. This results in local inflammation and an enhanced phagocytic function (Becker et al., 2005). Interleukin-12 also drive the development of naïve T cells to Th1 cells and inhibits Th2 differentiation.

Additionally, $CD8^+$ T cells and NK cells are caused to proliferate and gain cytotoxic potential, and B cells are prompted to secrete antibody associated with Th1 responses.

1.9 Effects of the cytokines on the cardiac tissue.

The pleiotropic properties of cytokines allow them to work at the systemic, organ, or cellular levels. In terms of cardiac myocytes cytokines can have direct or indirect influence on their performance.

1.9.1 Effect of IL-1β on cardiac myocytes.

IL-1β is a pro-inflammatory cytokine that is produced by various cells, including immune cells and cardiac cells, in response to injury or infection. IL-1β can directly affect cardiac tissue by inducing a variety of cellular responses, including inflammation, hypertrophy, apoptosis, and fibrosis (Bujak & Frangogiannis, 2009).

Traditionally, IL-1 family has been associates with regulation of mitogenesis in smooth muscle and endothelial cells, lipoprotein metabolism, extracellular matrix production, vascular permeability and thrombogenic response to endothelial cells (Szekely & Arbel, 2018). It plays crucial role in the post-infarction inflammatory response and is involved in pathogenesis of cardiac remodelling where it impairs the contractility of cardiomyocytes and aggravate post-infarction reperfusion injury (Saxena et al., 2016).

IL-1β can activate the NF-kB signalling pathway, leading to the expression of various pro-inflammatory genes and the production of other cytokines, such as TNF-alpha and IL-6, which can also contribute to cardiac dysfunction (Weber et al., 2010). IL-1β can also induce cardiomyocyte hypertrophy and fibrosis by activating the TGF-β signalling pathway and promoting the production of extracellular matrix proteins leading to stiffening as well as thickening of walls of the heart muscle (Mariotti et al., 2006).

Current advances in cardiovascular research identified IL-1β to be involved in pathways that stimulate vascular smooth muscle via upregulation of growth factor-β (TGF-β), suppression of endothelial cells proliferation and their expression of adhesion molecules and modification of endothelium that favours thrombosis (Bujak & Frangogiannis, 2009; Mariotti et al., 2006). By inducing an inflammatory response in endothelial cells by increase expression of adhesion factors and chemokines, IL-1β promotes accumulation of inflammatory cells in blood vessels

and their invasion to local intima of blood vessels which often initiates atherosclerosis (Bevilacqua et al., 1985). These adhesion molecules include vascular cell molecule (VCAM-1), intercellular inflammatory cytokines (ICAM-1) and chemokines like monocyte chemoattractant protein (MCP-1). The latter is closely related to atherosclerosis as it employs mononuclear phagocytes (Mai & Liao, 2020). IL-1β also promotes proliferation and differentiation and activation of macrophages, monocytes as well as secretion of multiple inflammatory mediators including prostaglandins (Libby et al., 1988). It enhances expression of multiple inflammatory mediators: induces cyclooxygenase-2 (COX-2) which leads to production of prostaglandin E1 (PGE1) and E2 (PGE2), it enhances IL-1 expression and forms a positive feedback loop, and induces interleukin-6 (IL-6) and matrix metalloproteinase (MMP) generation (Dinarello, 2009).

In summary, IL-1β can directly affect cardiac tissue by inducing inflammation, hypertrophy, apoptosis, and fibrosis, and is involved in the pathogenesis of various cardiac diseases.

1.9.2 Effect of IL-8 on cardiac myocytes.

Interleukin-8 is a cytokine that is primarily produced by immune cells and plays a key role in inflammation and immune response. While IL-8 is not typically produced by cardiac tissue, it can indirectly affect the heart through its effects on the immune system (Russo et al., 2014).

In the context of cardiac tissue, IL-8 has been implicated in the development and progression of atherosclerosis, a chronic inflammatory disease that can lead to heart attacks and stroke. IL-8 can promote the migration and activation of immune cells within atherosclerotic plaques, which can lead to plaque destabilization and rupture (Van Tits et al., 2011).

IL-8 has also been shown to induce oxidative stress and promote in cardiac cells, which can contribute to cardiac dysfunction and injury. Elevated levels of IL-8 have been observed in patients with heart failure, and IL-8 has been proposed as a potential biomarker for heart failure prognosis (Dutka et al., 2020).

Overall, the effects of IL-8 on cardiac tissue are primarily mediated through its effects on the immune system and inflammation. Elevated levels of IL-8 can contribute to the development and progression of cardiovascular disease and may be indicative of poor prognosis in patients with heart failure.

1.9.3 Effect of IL-6 on cardiac myocytes.

Interleukin-6 is a cytokine that is produced by various cells in the body, including cardiac cells, in response to inflammation or infection. IL-6 has a wide range of effects on different cells and tissues in the body, including the heart. In the context of cardiac tissue, IL-6 has been shown to have both protective and detrimental effects.

On one hand, IL-6 can stimulate the production of other cytokines, such as IL-10, which can have anti-inflammatory and protective effects on the heart. IL-6 can also promote the growth and survival of cardiac cells, which can help to maintain cardiac function. IL-6 is upregulated during and after an acute myocardial infarction (AMI) and has a protective function as it prevents cardiomyocyte apoptosis, enhances tissue preservation, and triggers the preconditioning response.

On the other hand, IL-6 can also contribute to cardiac dysfunction and injury under certain conditions. For example, elevated levels of IL-6 have been observed in patients with heart failure, and IL-6 has been shown to induce apoptosis (programmed cell death) in cardiac cells, which can lead to cardiac dysfunction. When IL-6 signalling continues chronically these protective responses become pathogenic and depress myocyte function by decreasing contractility, enlarging left ventricle and turning on genes responsible for hypertrophy (Fontes et al., 2015). Not only the duration that IL-6 acts on the cardiac tissues is important but also the pathway that is activated during the response. Higher levels of sIL-6R, associated with trans-signalling, are particularly pathogenic as they are associated with worse disease outcomes, and cardiac tissue provides an example where duration of signalling from acute to chronic demonstrates transition from protective to pathogenic (Fontes et al., 2015; Terrell et al., 2006). Acutely, IL-6 protects myocytes from oxidative stress and induces anti-apoptotic program while chronically exposed myocytes IL-6 induces genes responsible for pathological hypertrophy as well as depression in the basal contractility leading to decreased function (Prabhu, 2004; Wollert et al., 1996).

Overall, the effects of IL-6 on cardiac tissue are complex and depend on various factors, such as the level and duration of IL-6 exposure, the presence of other cytokines or inflammatory mediators, and the overall health status of the individual.

1.9.4 Effect of IL-10 on cardiac myocytes.

Interleukin-10 is an anti-inflammatory cytokine that is produced by various cells in the body, including immune cells and cardiac cells. IL-10 has been shown to have beneficial effects on cardiac tissue, primarily through its anti-inflammatory and immunomodulatory properties.

IL-10 can modulate the immune response in cardiac tissue. IL-10 can inhibit the activation and migration of immune cells, such as macrophages, to sites of inflammation or injury in the heart. This can help to limit tissue damage and promote tissue repair. Following myocardial infarction (MI), the innate stress response to prevent heart against necrosis involves elevated levels of many pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α. In the early stages this promotes wound heling and scar formation, but excessive inflammation has an opposite effect: it results in damage of the normal structure and contractile function of the heart (Xu et al., 2021). Here, IL-10 can supress excessive inflammation and improve cardiac physiology by improving inflammation resolution and indirect stimulation of cardiac fibroblasts (Jung et al., 2017). Additionally, it suppresses neutrophil recruitment, inhibit TNF-αproduction, induce the expression of tissue inhibitor of metalloproteinases (TIMP)-1 and modulate the mononuclear cell phenotype (Frangogiannis et al., 2000).

Overall, IL-10 has beneficial effects on cardiac tissue through its anti-inflammatory and immunomodulatory properties. IL-10 may have therapeutic potential for the treatment of cardiovascular disease, but further research is needed to determine the optimal conditions and mechanisms of action for IL-10 treatment.

1.9.5 Effect of TNF-α on cardiac myocytes.

Tumour necrosis factor alpha is a pro-inflammatory cytokine that is primarily produced by immune cells, including macrophages and T cells. TNF-α has been implicated in the pathogenesis of various inflammatory and autoimmune diseases, including cardiovascular disease.

In the context of cardiac tissue, TNF-α has been shown to have both beneficial and detrimental effects, depending on the context and level of expression. It is one of the most common pro-inflammatory cytokines observed following cardiac stress (Kaur et al., 2009). TNF-α can induce apoptosis in cardiac cells, which can contribute to cardiac dysfunction and injury (Schumacher & Naga Prasad, 2018). Elevated levels of TNF-α have been observed in

patients with heart failure, therefore TNF-α has been proposed as a potential therapeutic target for the treatment of heart failure (Aimo et al., 2020).

However, TNF-α can also have beneficial effects on cardiac tissue. TNF-α can promote the growth and survival of cardiac cells, and can stimulate the production of other cytokines, such as interleukin-10, which can have anti-inflammatory and protective effects on the heart (Kambara et al., 2015; Urschel & Cicha, 2015). TNF-α can also promote angiogenesis in cardiac tissue, which can help to maintain cardiac function (Tachibana et al., 2017).

Overall, the effects of TNF-α on cardiac tissue are complex and depend on various factors, such as the level and duration of TNF-α exposure, the presence of other cytokines or inflammatory mediators, and the overall health status of the individual. Elevated levels of TNF-α can contribute to the development and progression of cardiovascular disease, but TNF-α can also have beneficial effects on cardiac tissue under certain conditions.

1.9.6 Effect of IL-12 on cardiac myocytes.

Interleukin-12 is a cytokine that is primarily produced by immune cells and plays a key role in the immune response to infection and cancer. While IL-12 is not typically produced by cardiac tissue, it can indirectly affect the heart through its effects on the immune system (Vignali & Kuchroo, 2012).

Interleukin-12 as a pleiotropic cytokine leads to collagen synthesis, preserved cardiac function, positive inotropy and inhibition of pro-inflammatory cytokine production (Shirazi et al., 2017). It has been shown that in myocarditis, IFN-γ produced by the activity of IL-12 increased macrophage and neutrophils population in the heart facilitating viral clearance protecting it from dilated cardiomyopathy and congestive heart failure (Fairweather & Rose, 2005). However, it is still not clear whether IL-12 has a pro or anti-inflammatory function.

It has been shown that IL-12 have significantly higher level of expression in patients with CAD and atherosclerosis and are closely correlated with the progression of these diseases that can lead to heart attack and stroke. It is also one of the first cytokines expressed in atherosclerotic lesion (Rahman & Fisher, 2018). IL-12 can promote the activation and proliferation of immune cells within atherosclerotic plaques, which can lead to plaque destabilization and rupture, hence IL-12 has been proposed as a potential biomarker for cardiovascular disease.(Poizeau et al., 2020).

Overall, the effects of IL-12 on cardiac tissue are primarily mediated through its effects on the immune system and inflammation. Elevated levels of IL-12 can contribute to the development and progression of cardiovascular disease and may be indicative of poor prognosis in patients with heart failure.

1.10 Aims of the study.

This MSc by research project acted as a 57-patient pilot study to determine if pre-operative cytokine levels of interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor alpha (TNFα), and interleukin-12 (IL-12) are associated with adverse cardiac function in CAD patients. This formed the main aim of the study.

From this main overall aim, several sub aims have been generated:

- To optimise myocyte isolation from the cardiac tissue obtained during the routine Coronary Artery Bypass Graft (CABG) from the patients whose serum was used to quantify cytokines.
- To measure IL-8, IL-1β, IL-6, IL-10, TNFα, IL-12 in patient cohort and correlate it with indices of coronary artery disease.
- To provide reference values from a healthy population using meta-analysis.
- To correlate plasma cytokine levels with indices of cardiac function.

2. Methods

2.1 Study design

This study was designed to observe the correlations that may exist between cytokine levels and indices of cardiac dysfunction in the coronary artery disease in a large cohort of patients.

2.1.1 Ethical considerations

Ethical approval has been obtained in accordance with the standards of the International Conference on Harmonization, Good Clinical Practice Guideline, Research Ethics Committee and applicable government, Trust and Research Office policies, regulations, and guidelines. The University of Salford has approved this project (STR1920-07) and it has also received IRAS approval (REC reference: 18/LO/2219).

Ethical considerations include human and animal tissue procurement and handling, informed patient consent to research study, patient information confidentiality and patient data randomisation and anonymisation.

2.1.2 Patient recruitment

Patients were recruited from Blackpool Victoria Hospital. Suitable patients were undergoing planned elective coronary artery bypass graft surgery (CABG). Patients gave informed consent before taking and storage of heart tissue and blood samples. Once recruited, 10ml of blood was taken from patients before the CABG. In this procedure blood supply to the heart was rerouted from the coronary arteries to the graft. Diseased coronary arteries were not removed, but the blockage was bypassed as showed in the figure 26 (Kuhn & Lynch, 2016).

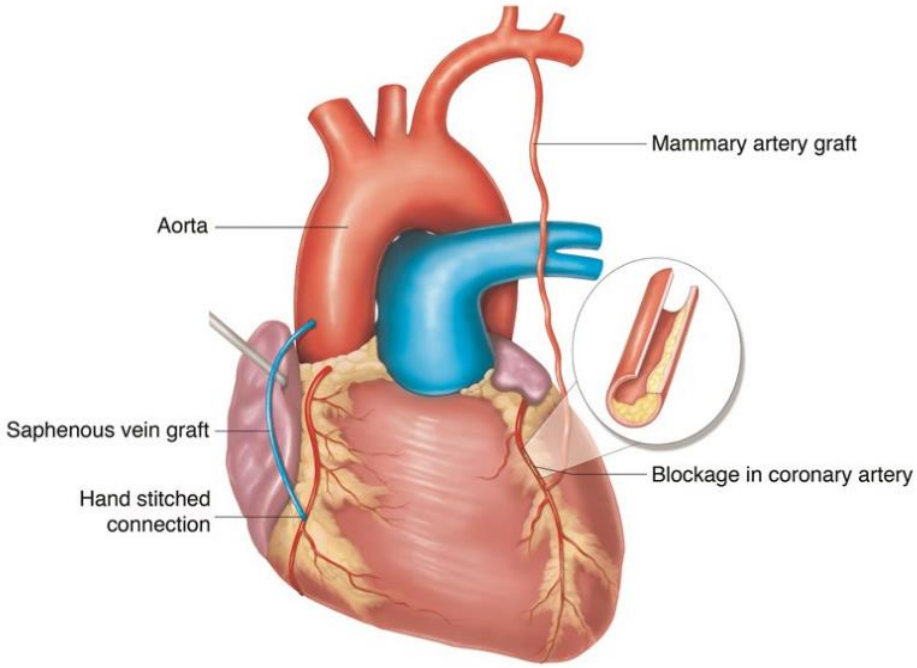

Figure 26. Diagram showing a double coronary artery bypass surgery where blood supply is restored to both coronary arteries. Source: *Coronary artery bypass grafting*. (https://www.lhch.nhs.uk/our-services/adult-heart-surgery/coronary-artery-bypass-grafting/)

During the operation a small portion of right atrial appendage tissue was removed. This was to accommodate the cardiopulmonary bypass machine as the heart was stopped for the duration of the procedure. This tissue is usually discarded as clinical waste, however for the purpose of this study it was saved.

Alongside the blood and atrial tissue samplings, a series of clinical and demographic information was collected.

2.2 Patient serum sample isolation.

Venous blood samples were collected from the patients preoperatively and stored on ice until they arrived at the laboratory. To separate serum from red and white blood cells samples were immediately centrifuged at 2000xg for 10 minutes in swing out bucket rotor centrifudge. Figure 2 shows the blood sample before and after centrifugation.

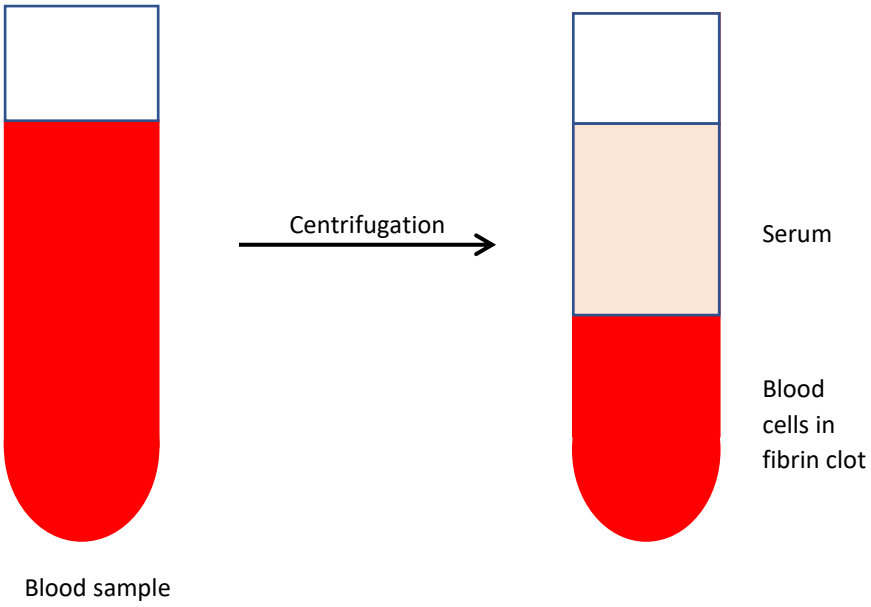

Figure 27. Obtaining serum from whole blood sample.

After centrifugation, serum was carefully aspirated and aliquoted into cryovials with volumes ranging between 200µl and 1000µl before storing at -80°C for later analysis.

2.3 Myocyte isolation

During the routine open-heart surgery for cardiopulmonary bypass grafting, the tip of the right atrial appendage can be excised upon insertion of the right atrial cannula of the heart-lung machine (Nummi et al., 2017). Hence it can be used for the isolation of atrial cardiomyocytes. After excision, tissue sample was transferred to a 50ml tube with a sterile Ca^{2+}-free transport solution and stored on ice until arrival at the laboratory. Samples were used for the isolation protocol immediately and no tissue or cells were stored. Approach presented in this study used a modified protocol based on Voigt et al. (2013) methodology.

Before the atrial myocyte isolation protocol was performed, it required a list of prearrangements and a n equipment set up showed in figure 28. Firstly, solutions required for the protocol were made. Reagents used for these solutions are shown in table 4, 5 and 6.

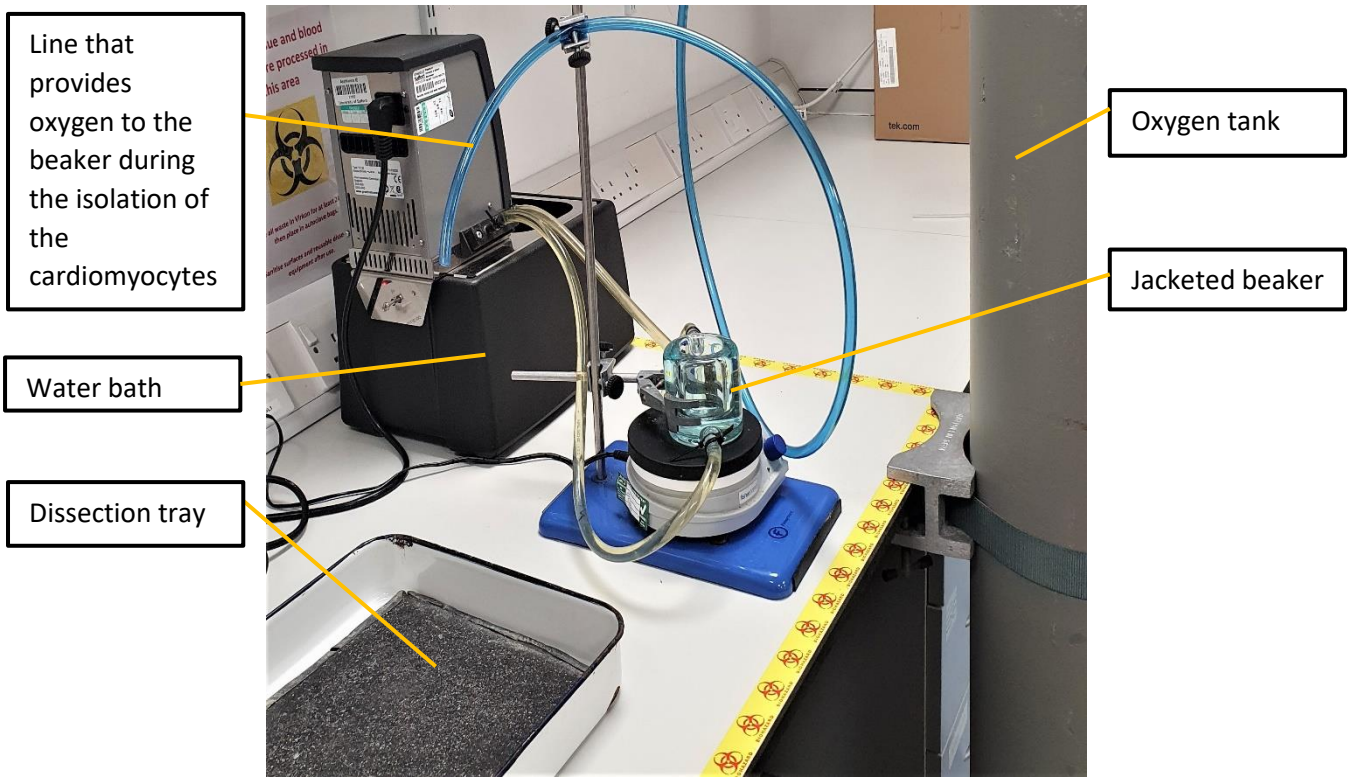

Figure 28. Equipment set up for the isolation of the cardiomyocytes.

Firstly, the of Ca^{2+}-free solution was prepared and comprised of glucose (Sigma-Aldrich, G8270, ≥99.5%), 3-(N-Morpholino) propane sulfonic acid (MOPS) (Sigma-Aldrich, M1254, ≥99.5%), sodium chloride (NACL) (Sigma-Aldrich, S9888, ≥99.0%), taurine (Sigma-Aldrich, T0625, ≥99%), monopotassium phosphate (KH_2PO_4) (Sigma-Aldrich, P0662, ≥99.0%), magnesium sulphate ($MgSO_4$) (Sigma-Aldrich, M7506, ≥99.5%), and potassium chloride (KCl) (Sigma-Aldrich, P9541, ≥99.0%).

Table 4. Reagents and their concentrations used to make 100ml of Ca^{2+}-free solution.

Ca^{2+}-free solution was pH adjusted with 1M NaOH to obtain pH 7.00.

Ca^{2+}-free solution (pH 7.00)		
Constituent	**mM**	**100ml**
Glucose	20	0.36032g
MOPS	5	0.1048g
NaCl	100	0.5844g
Taurine	50	0.6256g
KH_2PO_4 (1M)	1.2	0.12ml
$MgSO_4$ (1M)	5	0.5ml
KCl (1M)	10	1ml

Storage solution contained glucose, taurine, DL-β-Hydroxybutyric acid (Sigma-Aldrich, 166898, 95%), L-glutamic acid (Sigma-Aldrich, G1251, ≥99%), albumin (Sigma-Aldrich, A3059, ≥98%), KH2PO4 and KCl.

Table 5. Reagents and their concentrations used to make 100ml of storage solution. Storage solution was pH adjusted with 1M KOH to obtain pH 7.40.

Storage solution (pH 7.40)		
Constituent	**mM**	**100ml**
Glucose	10	0.18g
Taurine	10	0.12516g
DL-β-Hydroxybutyric acid	10	0.126g
L-glutamic acid	70	1.03g
Albumin	1%	0.1g
KH_2PO_4 (1M)	10	1ml
KCl (1M)	20	2ml

Transport solution included 2,3-butanedione monoxime (BDM) (Sigma-Aldrich, B0753, ≥98%), glucose, MOPS, NaCl, taurine, KCl, KH_2PO_4 and $MgSO_4$.

Table 6. Reagents and their concentrations used to make 100ml of transport solution. Transport solution was pH adjusted with 1M NaOH to obtain pH 7.00.

Transport solution (pH 7.00)		
Constituent	**mM**	**100ml**
BDM	30	0.299g
Glucose	20	0.54g
MOPS	5	0.104g
NaCl	100	0.579g
Taurine	50	0.620g
KCl (1M)	10	1.ml
KH_2PO_4 (1M)	1.2	0.12ml
$MgSO_4$ (1M)	5	0.5ml

After solutions were prepared, thermocirculator was switched on to maintain the temperature of the jacketed beaker at 37°C. Subsequently, 10ml of Ca^{2+}-free solution was kept in a plastic beaker at 4°C and 50ml of Ca^{2+}-free solution was stored in a 100ml glass cylinder in a water bath at 37°C.

Patient's cardiac tissue was delivered at 4°C in a clinical sample box. Tissue sample was transferred together with the transport solution into a large weighing boat and any fatty tissue was dissected off using scissors. Sample was weighed, with samples of <50mg discarded as unsuitable for cell isolation. Appropriate samples were transferred to a beaker containing 10ml of Ca^{2+}-free solution at 4°C. The sample was then cut into smaller chunks of approximately $1mm^3$ in size.

The following steps were performed at 37°C under continuous gassing with 100% O_2. Tissue chunks together with Ca^{2+}-free solution were transferred into a jacketed beaker and stirred carefully for 3 minutes with a magnetic bar. Tissue chunks were allowed to settle down and supernatant was carefully strained through the nylon mesh (200µm). Restrained tissue chunks were returned to the beaker with forceps and beaker was refilled with 10 ml of Ca^{2+}-free solution to repeat the washing steps two more times.

Selection of the enzymes has been performed by the previous study and recombinant collagenase type I in combination with protease type XXIV were used to optimise this protocol. This was aimed to obtain the maximum number of functional cells.

Next step involved preparation of the enzymatic solutions E1 and E2 needed for the tissue digestion. Collagenase I (Lot: 49HI9435, Worthington-Biochem, USA) and Protease XXIV (7.0-14.0 units/mg, Sigma-Aldrich, USA) were weighted into the plastic beakers and stored at room temperature. The enzymes were diluted in 10 ml of Ca^{2+}-free solution just before use. Enzyme solutions were prepared as follows:

- E1: 9.079mg of collagenase and 4.76mg of protease were diluted in 10 ml of Ca^{2+}-free solution.
- E2: 9.079mg of collagenase only was diluted in 10 ml of Ca^{2+}-free solution.

After the washing steps, tissue chunks were re-suspended with 10ml of enzyme solution E1 and stirred carefully for 5 minutes. It was followed by addition of 20µl of 10mM $CaCl_2$ solution to obtain a final concentration of 20µM Ca^{2+}. After a further 10 minutes of stirring the supernatant was carefully strained through the nylon mesh (200µm) and tissue chunks were returned from the mesh into the beaker.

Tissue chunks were resuspended in 10ml of enzymatic solution E2 with the immediate addition of 20µl of 10mM $CaCl_2$ solution to obtain a final concentration of 20µM Ca^{2+}. Solution was carefully stirred for 5 minutes. Three tubes were filled with 10 ml of storage solution with added 20µl of 10mM $CaCl_2$ solution to obtain a final concentration of 20µM Ca^{2+}. After 5 minutes, the stirring was stopped, and the supernatant was carefully strained through a nylon mesh (200µm) into a separate 15 ml tube. At this point the O_2 was halted.

Tissue chunks were resuspended in the beaker with 10ml of storage solution containing 20µM Ca^{2+} and cells were further dissociated by the gentle mechanical trituration using a 3ml Pasteur pipette for 2 minutes. After this time, supernatant was carefully strained through the nylon mesh (200µm) into a 15 ml tube. This step was repeated two more times, strained supernatant was collected into two further 15 ml tubes. Following the mechanical trituration, tubes were centrifuged at 95xg for 10 minutes. After centrifugation the supernatant was discarded from all the tubes and cells in each tube were resuspended in 1.5ml of storage solution at room temperature.

To adjust each tube to the final concentration of Ca^{2+} 7.5µl of 10mM $CaCl_2$ was added to each tube and incubated for 10 minutes followed by subsequent addition of 7.5µl of 10mM $CaCl_2$ and further 10 minutes incubation. Final addition of 15µl of 10mM $CaCl_2$ led to a final Ca^{2+} concentration of 0.2mM.

2.4 Meta-analysis

PubMed was searched for English literature published from January 1990 to October 2022 which stated levels of interleukin-1β (IL-1β), Interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12p70 (IL-12) and tumour necrosis factor α (TNF- α) in healthy individuals. Searching included search terms and combination of words related to cytokines, e.g., Interleukin-6, IL-6, and healthy subjects, e.g., control, healthy individuals. Studies were eligible for inclusion if they recruited participants from a general population and had a control group without any pre-existing condition. Only studies conducted on human populations were evaluated. Study level characteristics were extracted based on information that included: study date, number of control subjects, sampling method (i.e., serum samples), subject age, and sex.

The inclusion criteria that were considered in this meta-analysis were: (1) age of the control subjects, (2) data for cytokine levels was extractable from the text of the study, (3) cytokine levels were measured in the serum samples.

The exclusion criteria were: (1) duplicate studies, (2) studies other than original research or review works, e.g., Comments or Letters, (3) single digit control group.

Data for this meta-analysis was extracted based on inclusion and exclusion criteria. Extracted information included, authors names, year of publishing, number of controls and their sex, and levels of all cytokines that were studied. Eligible studies were recognised by the student according to PRISMA guidelines and checklists. Finally, out of 38 studies, five were identified as suitable for this meta-analysis.

2.5 Cytometric bead array.

Cytometric bead array (CBA) is a powerful tool for measuring cytokines and other soluble proteins in biological samples. Compared to other techniques of cytokine measurement, CBA has several advantages (Castillo & MacCallum, 2012). It is high throughput method where in a single sample multiple cytokines are measured simultaneously allowing for efficient analysis of cytokine profiles(Castillo & MacCallum, 2012). Another advantage of CBA is its sensitivity; it can detect cytokines at concentration as low as 1pg/ml in a small sample. Therefore it is especially useful for analysing cytokine profiles in samples with limited volume such as serum or plasma (Tarnok et al., 2003). Furthermore, CBA assays are highly accurate and reproducible, with low inter- and intra-assay variability that can be used to measure cytokines in a variety of sample types, including serum, plasma and cell culture supernatant (Moncunill et al., 2014). Therefore, CBA is a widely used technology for detecting and quantifying cytokines in physiological and pathophysiological conditions.

Physiological levels of cytokines refer to the normal, healthy levels of these proteins in the body. Here, CBA can be used to detect and quantify physiological levels of cytokines in various bodily fluids, such as blood, plasma, and serum. Research show that multiple cytokines are detectible at physiological levels in healthy subjects regardless of age and gender (Kleiner et al., 2013; Tarnok et al., 2003; Yamaguchi et al., 2019).

On the other hand, pathophysiological levels of cytokines refer to abnormal or excessive levels of these proteins that are associated with various diseases and conditions. For example, pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-1β are detectable by the cytometric bead array in inflammatory conditions such as rheumatoid arthritis and sepsis (Fukue et al., 2022; Mera et al., 2011). Anti-inflammatory cytokines, such as IL-10 are detected

in conditions such as allergies and autoimmune diseases (Reynolds et al., 2018; Zhang et al., 2021).

In summary, CBA is a powerful tool for detecting and quantifying cytokines in both physiological and pathophysiological conditions in clinical and research settings.

2.5.1 Optimisation experiment.

Before the cytometric bead array was performed, the protocol required an optimisation for known concentrations.

Before patient cytokines could be analysed a standard curve needed to be created to determine the concentration of interleukin- 1β, Interleukin- 8, interleukin-6, interleukin-10, interleukin-12p70 and tumour necrosis factor α based on known cytokine concentrations.

The standards were prepared by pooling lyophilised human inflammatory cytokine standards from the BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines kit. Standards were reconstituted with 2ml of the assay diluent in a 15 ml centrifuge tube and left to equilibrate for 15 minutes at room temperature.

After the equilibration period, reconstituted proteins were gently mixed with a pipette and a serial dilution was conducted in the patters shown in table 7. Negative control contained the assay diluent only.

Table 7. Standard concentrations and dilution factors following the standard dilution of standard spheres from the BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines kit sets.

Tube label	Concentration (pg/ml)	Standard dilution
1 (Top standard)	5000	1:1
2	2500	1:2
3	1250	1:4
4	625	1:8
5	312.5	1:16
6	156	1:32
7	80	1:64
8	40	1:128
9	20	1:256
10	10	1:512
11	7.5	1:1024
12	5	1:2048
13	4	1:4096
14	3	1:8192
15	2	1:16384
16	1	1:32768
17 (negative control)	0	No dilution

For the purpose of this project standard curve was created with the serial dilutions at the lower end of the theoretical detection limit (<40pg/ml). These limits are presented in table 8.

Table 8. Limit of detection for BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines kit (*BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit Instructional Manual*, 2019).

Cytokine	Limit of detection (pg/ml)
IL-8	3.6
IL-1β	7.2
IL-6	2.5
IL-10	3.3
TNFα	3.7
IL-12p70	1.9

Following the dilutions of standards, capture beads were prepared. This was done by calculating the number of tests to be conducted and determining the correct volume of capture beads, as 1 test required 10µl of capture beads for each cytokine, e.g., 3 standard dilutions = 3 assay tubes that require 10µl of each capture bead. There was six different cytokines standard beads, so 6 x 10µl = 60µl, then 60µl x 3 standard dilutions = 180 µl of total volume of the capture beads.

Once calculated, each capture bead vial was vortexed for 3 to 5 seconds and correct volume was pipetted into a tube and vortexed. The mixed capture beads were then incubated for 30 minutes at room temperature, protected from the light. After incubation, capture beads were vortexed and 50µl was added to individual 1.5 ml Eppendorf tubes followed by addition of 50µl of the human inflammatory cytokine standards prepared earlier. It was followed by 90 minutes incubation at room temperature, protected from light.

Following incubation, 1ml of wash buffer was added to each assay tube and centrifuged at 200xg for 5 minutes. Supernatant was aspirated and discarded, while approximately 100µl of liquid was left in each tube. Each sample was resuspended in 50µl of human inflammatory cytokine PE detection reagent and tubes were gently agitated to break down the pellet. Samples were incubated for 90 minutes at room temperature, protected from light.

After incubation, 1ml of wash buffer was added to individual tubes and tubes were centrifuged at 200xg for 5 minutes. Supernatant was carefully aspirated and discarded. Pellet was resuspended in 300µl of wash buffer before analysis by flow cytometry.

2.5.2 Cytometric bead array for the analysis of the unknown cytokine concentrations in patient's serum samples.

Patient samples were defrosted at room temperature immediately before the analysis. Remaining serum was refrozen and kept at -80°C.

Once defrosted, 50µl of serum was aliquoted in triplicate in Eppendorf tubes. After this, 50µl of prepared mixed capture beads (see 2.5.1) was added to the sample followed by gentle pipetting. Samples were incubated at room temperature in the dark for 90 minutes.

After incubation, 1ml of wash buffer was added to the samples and tubes were centrifuged at 200xg for five minutes. Supernatant was aspirated and removed and approximately 100µl of liquid was left in each tube. Pellet was resuspended in 50µl of human inflammatory cytokine PE detection agent, gently agitated with pipette and incubated in darkness at room temperature for 90 minutes. Subsequently, 1ml of washing buffer was added to each tube and centrifuged at 200xg for five minutes. The supernatant was discarded, samples were resuspended in 300µl of wash buffer, and analysed using flow cytometry.

2.6 Flow cytometry analysis of the serum samples and standards for the cytometric bead array.

After samples were prepared, they were analysed by flow cytometry. During this analysis, six different capture beads for six different cytokines (IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70) were recognised from a general bead population. Each cytokine detection bead was confirmed from the general population by analysing a single bead population to ensure accurate measurements and minimise errors from analysing bead population in bulk. Once the detection beads were analysed, they were compared to the fluorescent signatures of other beads in the sample to determine their identity (figure 29 B).

Cytokine-positive events were distinguished from the main bead population based on their fluorescence intensity by gating strategy, where phycoerythrin (PE) fluorescence intensity of

the cytokine staining identified cytokine-positive events. Beads had a distinct fluorescence intensity in a allophycocyanin (APC-A) channel which allowed to distinguish main bead population from all cytokine-positive events. Then a gate was drawn based on the intensity of the APC-A channel to include most of the beads in the sample, excluding debris and non-specific events (figure 29 A). Additionally, gate included population with high cytokine staining intensity in PE channel that excludes background and non-specific staining (figure 29C).

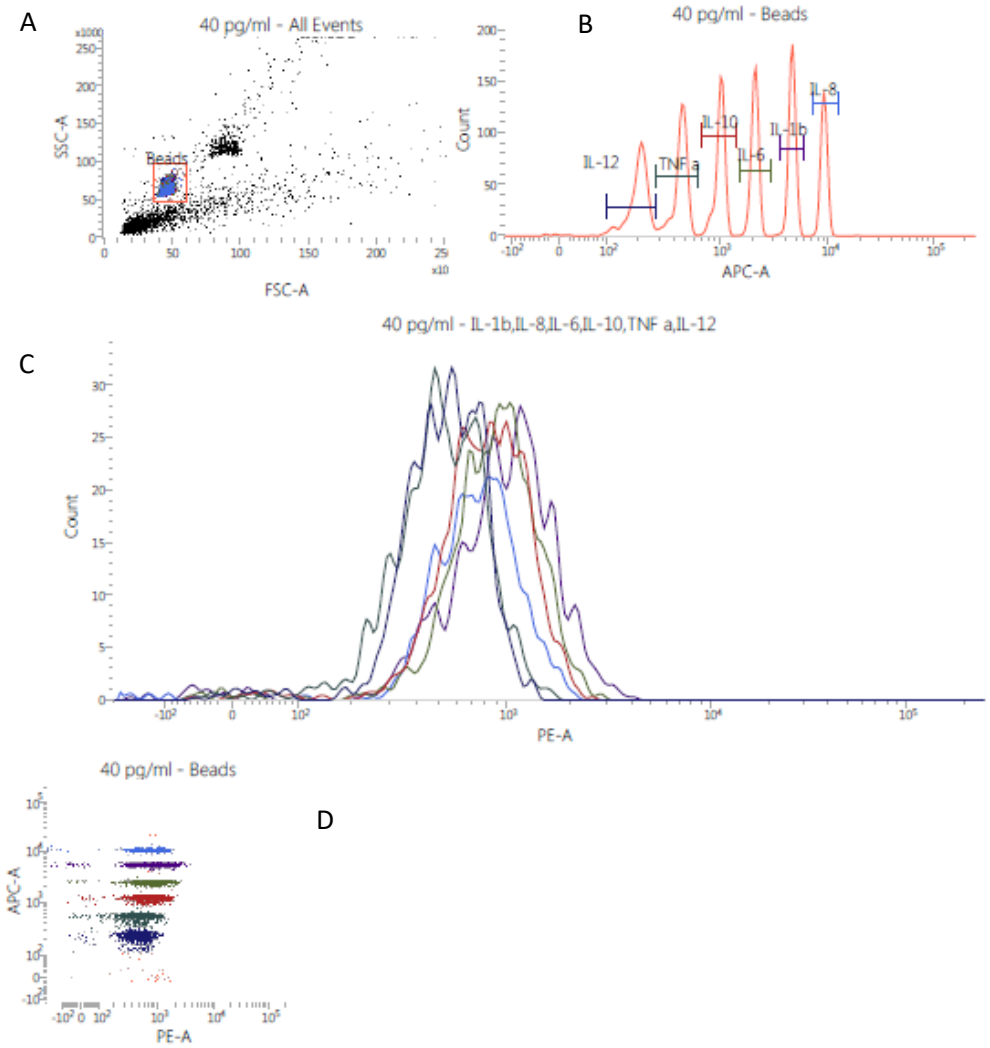

Figure 29. Example of gating for and differentiation of IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 cytokine. Forward scatter (FSC) and side scatter (SSC) were used to identify the bead population and gates were set around the bead population to exclude any debris (A). Beads with different fluorescent intensities were coated with antibodies specific to different cytokines and produced distinguished peaks seen on the diagram B. The cytokine specific phycoerythrin (PE) fluorescence intensity was used to determine the concentration of the

cytokines in the sample (C). Final diagram (D) represents allophycocyanin (APC-A) fluorescence intensity which is proportional to the complexity of measured cytokines, and PE fluorescence intensity which is proportional to the expression level of the cytokines. Gating template is a courtesy of Dr Matthew Jones (Jones, 2018).

The fluorescence intensity measured with flow cytometry was proportional to the cytokine concentration in the sample and was quantified from a calibration curve generated during optimisation of the cytometric bead array method (see 2.5.1). Concentrations of the cytokines were then correlated with the patient's clinical data to determine any correlations.

2.7. Statistical analysis

All statistical analysis was performed on GraphPad Prism software version 5.03 (GraphPad Software Inc., San Diego, CA, USA). Assessment of the normality was performed using Shapiro–Wilk test. Statistical analysis was performed with two tail student t-test and the linear regression analysis. Correlations were performed using Pearson product-moment correlation coefficient. All results were expressed as mean ± SE of the differences of means and were considered significant for $p<0.05$.

3. Results.

3.1 Optimisation of cardiomyocyte isolation protocol.

Isolation of viable cardiomyocytes from the heart tissue is an important precursor for many areas of cardiac pathophysiological research. The isolation protocol used here was adapted from Voigt et al. (2013). The adaptations were required because study was performed under different circumstances. For example, it took between 90-120 minutes for the sample to arrive to the laboratory from the time of extraction and following his protocol resulted in no viable cardiomyocytes in every isolation.

Qualitative visual examination of atrial cardiomyocytes was used to assess the success of the optimisation of the isolation protocol.

3.1.1. Enzymatic concentration.

Isolating cardiomyocytes is a delicate process involving the use of enzymes to disrupt the extracellular matrix that holds cells together in heart tissue. For this, enzyme concentration is an important consideration. Figure 30 shows the results of concentration optimisation.

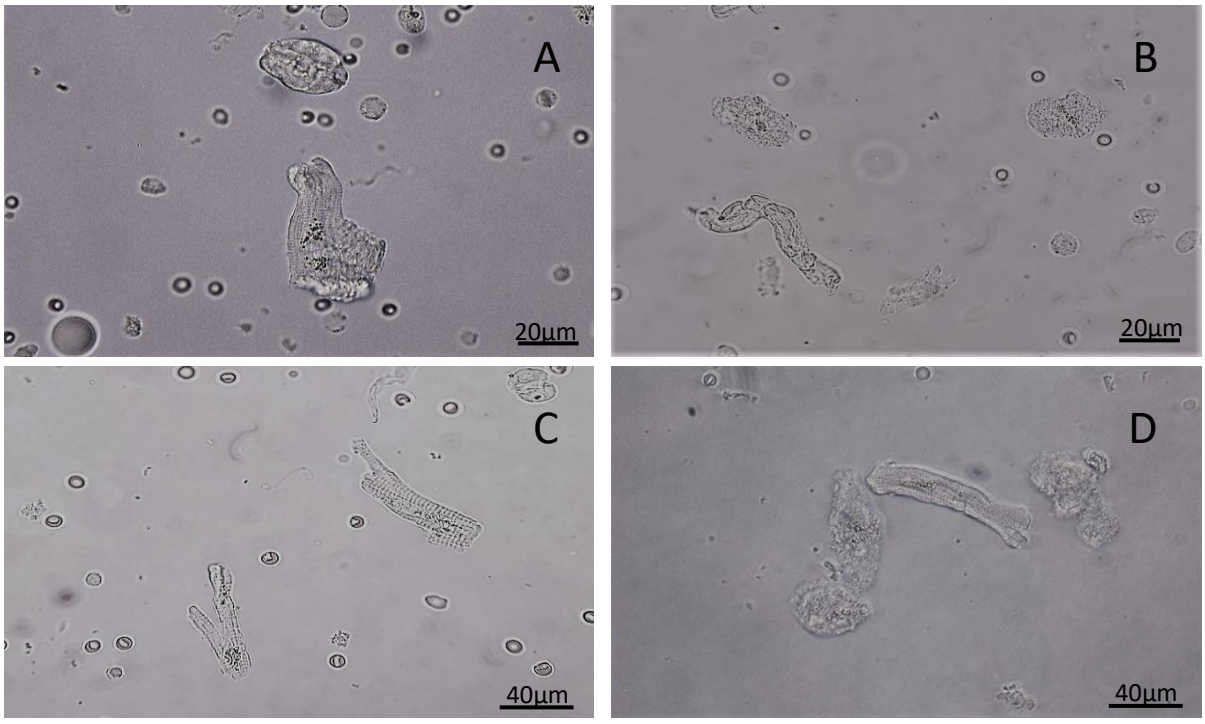

Figure 30. Isolated cardiomyocytes after two step digestion with collagenase I and protease type XXIV. A and B – cells that were dead and showed spherical morphology or were in the process of dying where they lost typical cardiomyocyte elongated shape for a twisted or

shortened architecture with minimal visible striation (collagenase I at 1.059mg/ml, protease at 0.714mg/ml). C and D – Isolated cardiomyocytes after two step digestion with lowered collagenase I concentration. Around 5% cells were viable and kept the typical elongated shape with distinguishable sarcomeres. Granularity within cells as well as irregular borders were also a common feature (collagenase I at 0.9079mg/ml, protease at 0.476mg/ml).

Collagenase I was used at a 1.059mg/ml and protease at 0.714mg/ml and that resulted in cells being over digested and in terms of their functionality and granularity with many cells not viable as shown in figure 30 A and B. Cells were deformed, twisted and without their typical cylindrical rod-shape. Some cells became round with visible membrane blebs (irregular membrane) and sarcomere was displaced centrally.

Firstly, collagenase I concentration was considered. Lowering its concentration from 1.059mg.ml to 0.9079mg/ml, resulted in improved cell shape. However, after changing the protease concentration and lowering it considerably, from 0.714 mg/ml to 0.476mg/ml, myocytes morphology improved significantly (figure 30 C and D). Higher proportion of cells displayed a rod-shaped characteristic with well organised perpendicular striation. Despite higher number of successfully isolated, functional cardiomyocytes, overall viability remained at 5%. Therefore, next optimisation step looked at adjustment of time needed for digestion.

3.1.2 Enzymatic digestion time.

Figure 31 shows the effects of digestion time on the preparation.

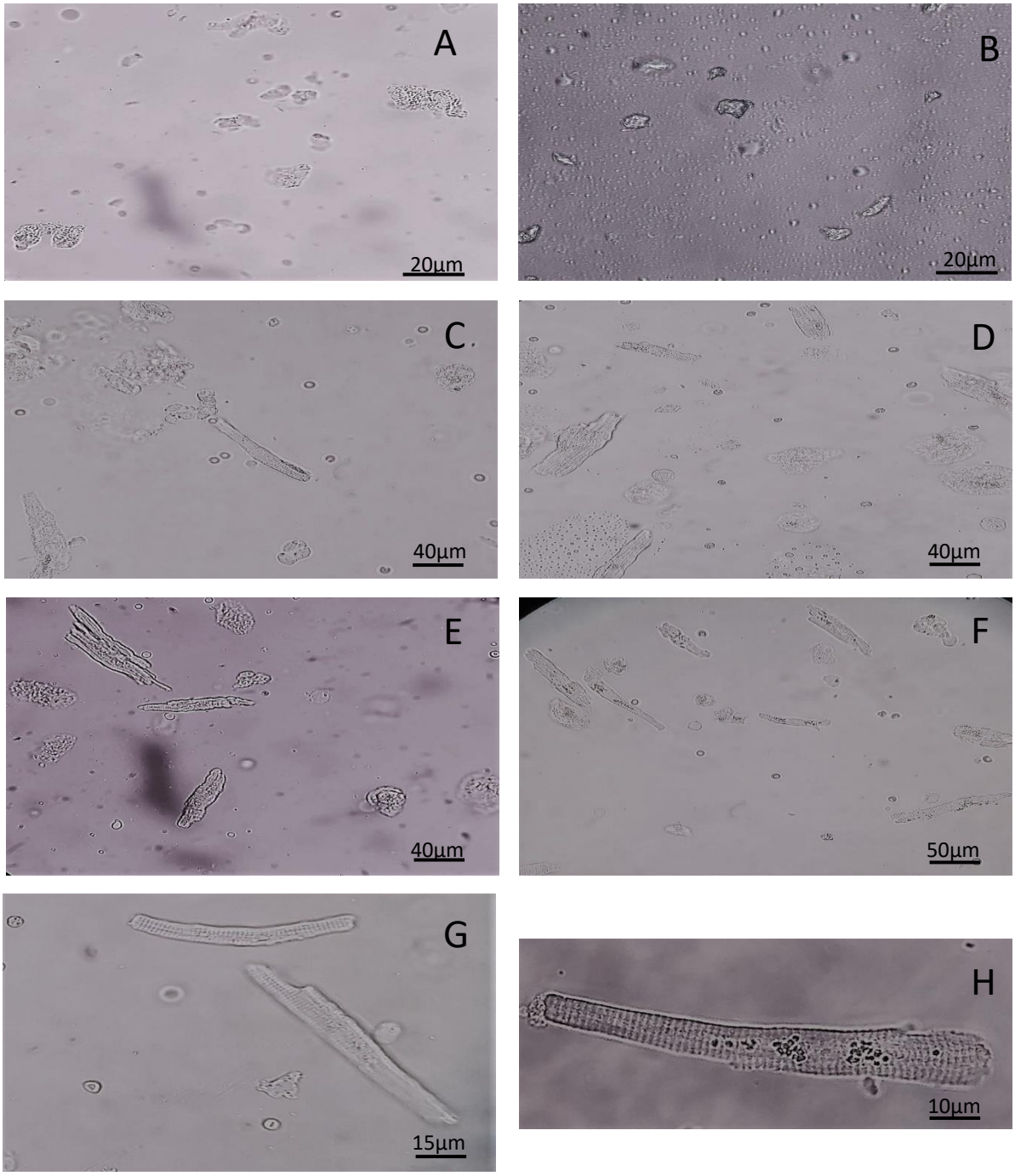

Figure 31. The effect of digestion time on myocyte viability. A+B; 35-minute primary and 10-minute secondary digestion. C+D; Isolated 20/10 minutes for first/second digestion. E+F; 15/10 minutes for first/second digestion. G+H; 15/5 minutes for first/second digestion (G, H).

Initial digestion times were selected according to Voight et al. (2013) i.e. 35 minutes for the first and 10 minutes for the second digestion (Voigt et al., 2013). This resulted in all isolated cells rounded and dead as indicated by figures 31 A and B.

Reducing the primary digesting time to 20 minutes improved yield and morphology as shown by figures 31 C and D. Immediately after isolation, alive cells maintained mostly rod-shape appearance with intact sarcomeres. At this stage only ~5% of cells were viable after isolation and would form club ends or die within a few hours in storage solution. Cellular deterioration was marked by shape distortion, e.g., shrinkage that would compromise cell size.

Further reduction of first digestion time to 15 minutes resulted in better quality cells with distinguished border and visible striation as well as improved yield as shown by fig 31 E and F. At this optimisation phases up to 40% of cells were viable with intact border, however some would still display blebs or round up after isolation.

The final optimisation step involved shortening of second digestion time to 5 minutes. It resulted in clearly separated cardiomyocytes with intact membrane and typical lengths of 110-140 µm (figure 31 G, H). Cells presented distinct striations with some granulation across the cell. There was no membrane 'blebbing' and no apparent morphological differences between rod shaped cells. Further reduction of digestion time for first and second digest resulted in cardiomyocytes that were not fully dissociated (not shown).

To conclude, optimal times were 15 minutes and 5 minutes for first and second enzymatic digestion respectively.

Optimisation protocol have altered enzyme concentration and digestion time to obtain cells that would have typical cardiomyocyte morphology. Additionally, this protocol was repeatable as well as cellular yield was comparable between isolations and ranged from 0% to 45% averaging at 19.74%.

3.1.3 Effect of tissue mass on cell viability.

To determine whether tissue mass influenced the preparations success, tissue mass was corelated with viability as shown by figure 32.

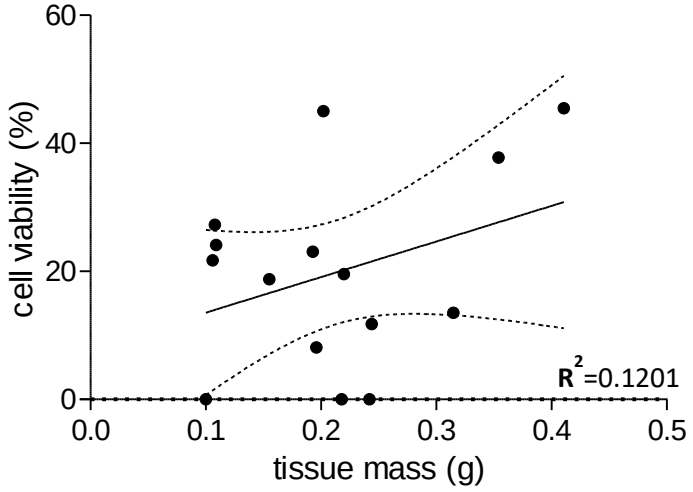

Figure 32. Correlation between the cardiac tissue mass and isolated cardiomyocyte viability (p=0.2, r=0.3465, n=15). Dashed lines represent 95% confidence interval.

There was no significant correlation between tissue mass and viability (p=0.2) (Figure 32). However, these data are likely under powered and an important trend - which suggests cell viability is dependent on starting mass - is apparent.

3.2 Optimisation of the cytometric bead array for measurement of cytokine concentration in patient samples.

To measure serum concentrations of interleukin-1β (IL-1β), Interleukin- 8 (IL-8), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12p70 (IL-12) and tumour necrosis factor α (TNF- α) using cytometric bead array required generation of standard curves. Flow cytometry gating for the cytometric bead array had already been optimised for similar studies in the lab. Standard curves for all cytokines are showed in Figure 33.

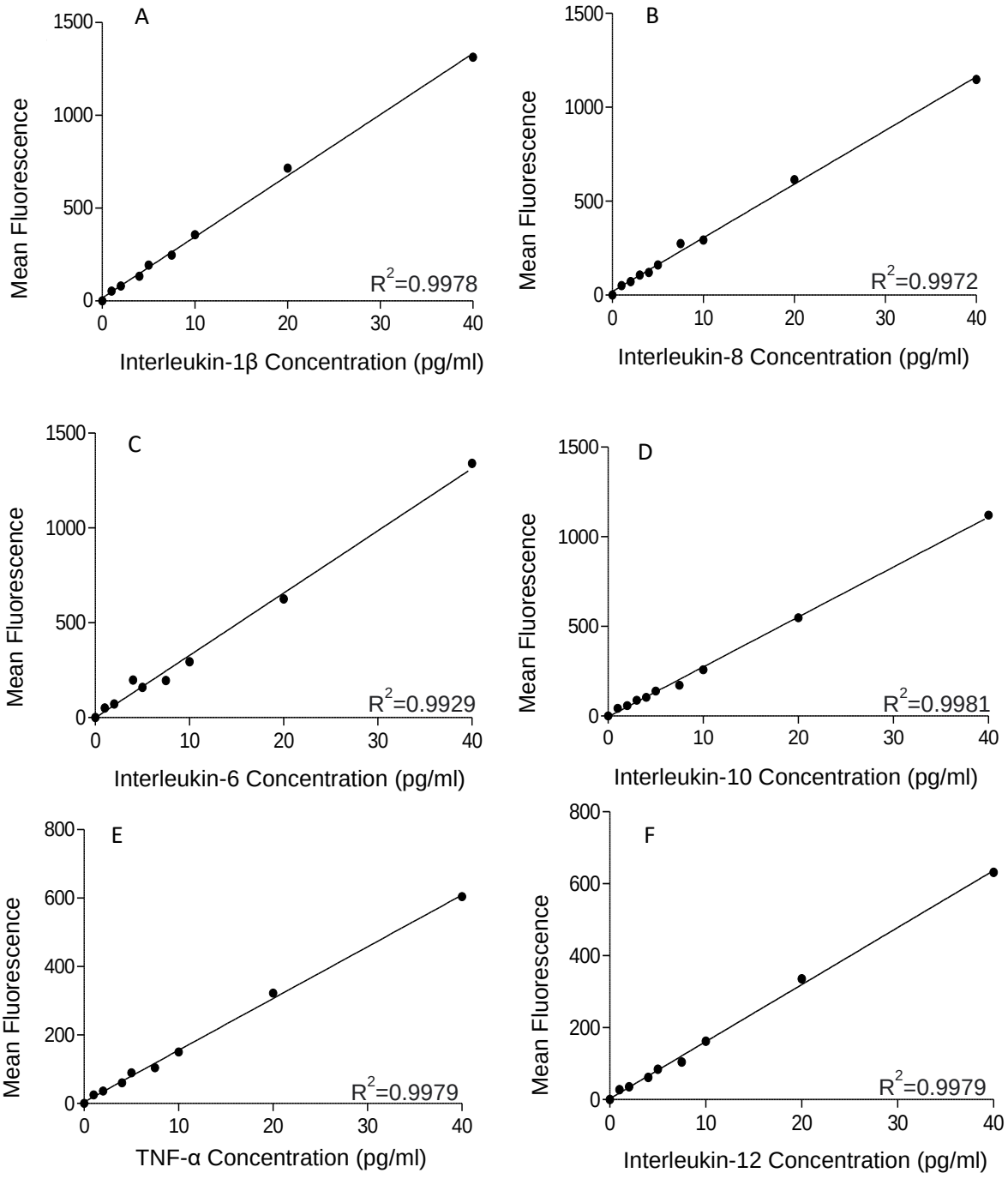

Figure 33. Standard curve generated for interleukin-1β (A), Interleukin-8 (B), interleukin-6 (C), interleukin-10 (D), interleukin-12p70 (E), and tumour necrosis factor α (F).

These standard curves were used to determine the concentration of interleukin- 1β, Interleukin- 8, interleukin-6, interleukin-10, interleukin-12, and tumour necrosis factor α in CAVCAD study patients.

3.3 Patient cohort demographics.

As per section 2.1.2, 57 right atrial appendage, and serum samples from patients undergoing artery bypass graft surgery were received.

3.3.1 Patient demographics

Clinical data and samples were collected from 57 CAVCAD study patients who satisfied the inclusion criteria stated in Appendix 2. Demographic and clinical characteristics of the study population are stated in table 9. Control data was obtained via meta-analysis as described in section 2.1.2. Patients were matched based on age, gender structure, BMI, hypertension status, and history of tobacco use.

Table 9. Characteristics of CAD study patients participating in the study.

Characteristics	CAD patients	Control
Age (y)	68.2±1.4 (n=43)	64.8±7.86 (n=369)
Male (%)	70% (n=37)	41% (n=369)
BMI (kg/m^2)	29.16±1.45 (n=37)	24.76±2.85 (n=369)
History of tobacco use	22 (38.6%) (n=44)	61 (21.6%) (n=283)

3.4 Quantification of serum cytokine concentrations.

Serum of 57 patients was quantified based on their cytokine concentration. It was performed with cytometric bead array as described in section 2.5.2.

3.4.1 Patient cohort cytokine analysis with cytometric bead array.

Cytometric bead array was used to measure interleukin-1β (IL-1β), Interleukin- 8 (IL-8), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12p70 (IL-12) and tumour necrosis factor α (TNF- α) in patient serum calculations. This was conducted in triplicate. Levels of these cytokines are shown in figure 34.

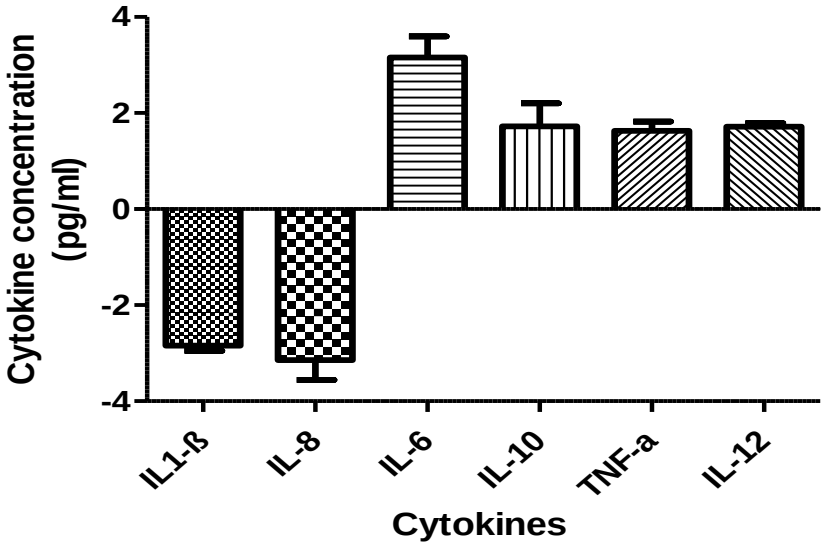

Figure 34. Mean cytokines levels in CAVCAD study patients' samples (n=57). Data shown as mean±SE.

Due to the multiplex aspect of the cytometric bead array, patient cytokines levels for IL-1β and IL-8 showed negative values, because they were below the detection limit of the assay (figure 34). The remaining cytokines i.e., IL-6, IL-10, TNF-α and IL-12 showed positive values and were used for further analysis.

Table 10. Mean cytokines levels in CAVCAD study patients' samples (n=57). Data shown as mean±SE.

Cytokine	Mean cytokine concentration (pg/ml) patients' samples
Interleukin- 1β	-2.84±0.10
Interleukin- 8	-3.14±0.41
Interleukin- 6	3.15±0.44
Interleukin-10	1.72±0.48
Interleukin- 12p70	1.72±0.07
Tumour Necrosis Factor α	1.63±0.19

Serum levels of IL-6, IL-10, IL-12 and TNF-α were detectable at single digit values. Levels of these cytokines ranged between 1.72±0.07 pg/ml for IL-12 and 3.15±0.44 pg/ml for IL-6 as shown in table 10. Levels of IL-1β and IL-8 in serum samples were detected at -2.84±0.10

pg/ml and -3.14±0.41 pg/ml respectively. Serum IL-6, IL-10, IL-12 and TNF-α were 3.15±0.44pg/ml, 1.72±0.48 pg/ml, 1.72±0.07 pg/ml and 1.63±0.19 pg/ml respectively. Patient raw data is supplied in appendix 4.

3.4.2 Healthy controls cytokine analysis from meta-analysis.

It was not possible to acquire serum from healthy volunteers, so a meta-analysis was performed to provide a comparative population. Studies were chosen based on criteria specified in section 2.4. Average cytokine levels for healthy population are shown in figure 35.

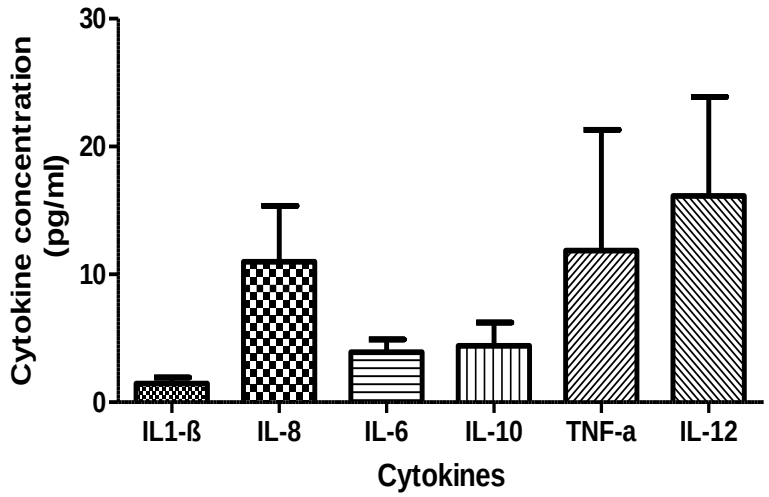

Figure 35. Mean cytokines levels in healthy volunteers from a meta-analysis. Data shown as mean ± SE.

Levels of Interleukin-1β, Interleukin-8, interleukin-6, interleukin-10, interleukin-12, and tumour necrosis factor-α were detectable in control samples from meta-analysis. Number of samples differed for each cytokine: for interleukin-1β n=231, for interleukin-8 n=369, for interleukin-6 n=317, for interleukin-10 n=194, for interleukin-12p70 n=173, and for tumour necrosis factor α n=283.

Table 11. Mean cytokines levels in healthy volunteers from a meta-analysis (n=369). Data shown as mean±SE

Cytokine	Mean cytokine concentration (pg/ml) in control group
Interleukin- 1β	1.47±2.35
Interleukin- 8	10.99±9.14
Interleukin- 6	3.92±4.33
Interleukin-10	4.40±6.11
Interleukin- 12p70	16.15±12.97
Tumour Necrosis Factor α	11.85±8.62

Numerical data after analysis is shown in table 11. Among the healthy cohort determined by the meta-analysis, cytokine levels ranged from the lowest of 1.47±2.35 pg/ml for IL-1β to the highest of 16.15±12.97 pg/ml for IL-12. Levels of IL-8 were at 10.99±9.14 pg/ml and IL-6 were at 3.92±4.33 pg/ml, followed by the second highest cytokine levels of TNF-α at 11.85±8.62 pg/ml.

3.4.3 Comparison of patient and controls cytokine levels.

Here, only valid levels of serum cytokine levels from CAVCAD patients and healthy subject from meta-analysis were compared to assess any level of significance between both populations. Evaluation of these relationships are showed in figure 36.

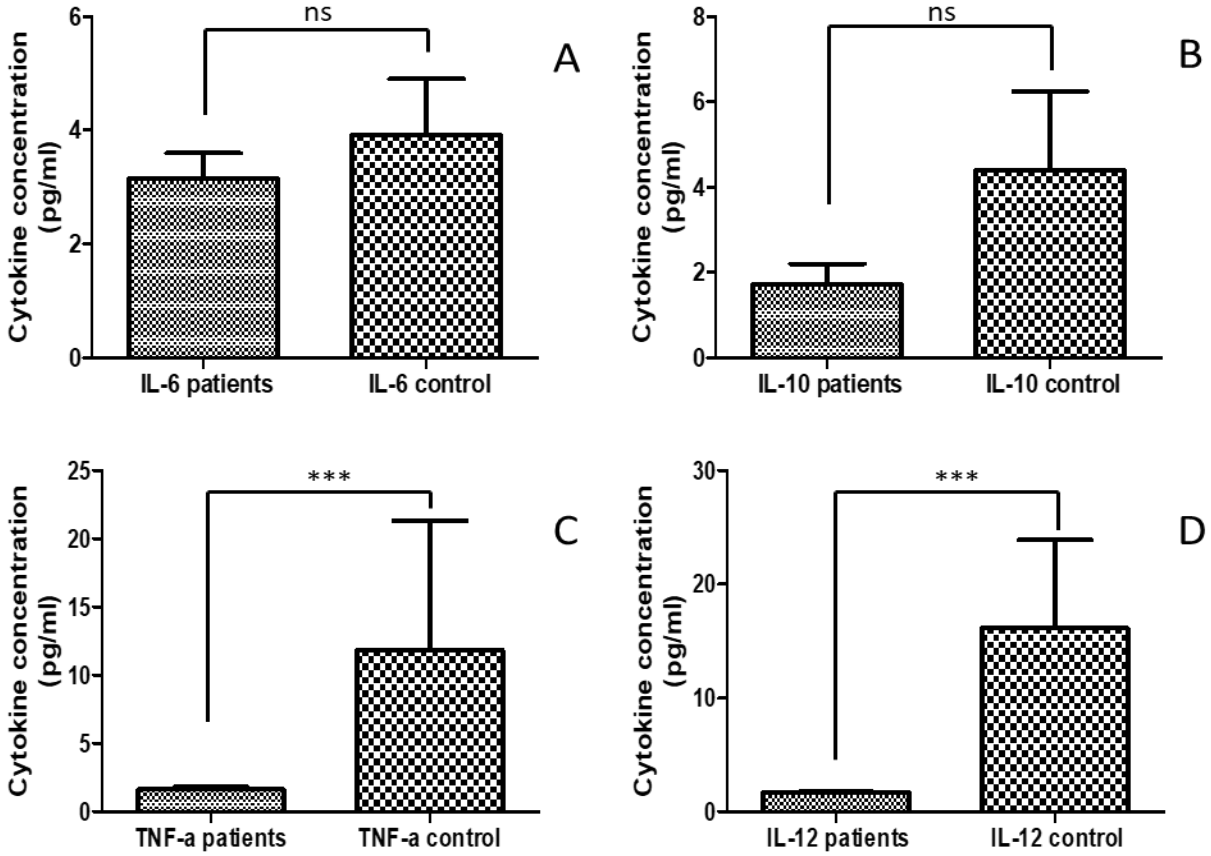

Figure 36. Comparison of cytokine levels in 57 CAVCAD study patients and 369 healthy individuals from the meta-analysis. Panel A shows comparison between IL-6 levels in both populations, panel B, C and D show comparison between IL-10, TNF-α and IL-12 levels in both populations respectively. ***=p<0.0001, ns=not significant.

All cytokines in this study were detected in control serum samples at higher concentrations than in patient cohort. Despite that, results for IL-1β and IL-8 were removed as they cannot be compared to patient levels. That allowed to compare levels of IL-6, IL-10, TNF-α and IL-12.

Data presented in section 3.4.1. and 3.4.2 was compared. Cytokine concentration in control samples were higher for every cytokine when compared to patient data. The levels of serum IL-12, and TNF-α were significantly higher in the control group (16.15±12.97 and 11.85±8.62 respectively) than in CAVCAD patients (1.72±0.07 and 1.63±0.19 respectively) (p<0.0001). Levels of serum IL-6 and IL-10 in patient samples (3.15±0.44 and 1.72±0.48 respectively) and control samples (3.92±4.33 and 4.40±6.11 respectively) did not differ significantly.

3.5 Patient clinical data

A range of clinical data was chosen to correlate to the serum cytokine levels, as shown in table 12. Clinical data was obtained from patient hospital reports as well as from performed echo cardiography. Collating this data provided a robust record of cardiac function and hemodynamic.

Table 12. *Clinical parameters of patients undergoing CAD for n=57. Data shown as average±SE*

Clinical parameter	Average value for n=57
Ejection fraction	47.42±1.72 %
Stroke volume	45.21±3.78 ml
End systolic volume	65.67±10.46 ml
End diastolic volume	110.49±10.88 ml
Left ventricular outflow tract (LVOT) peak velocity	0.97±0.04 m/s
Pulmonary arterial systolic pressure (PASP)	26.23±2.82 mmHg
Creatinine at the time of the surgery	86.04±4.46 µmol/L
Tricuspid annular plane systolic excursion (TAPSE)	20.11±1.13 mm
Heart rate	68.23±1.77 bpm
Left ventricle end diastolic volume index (LVEDVI)	64.12±11.88 ml/m^2
Left ventricle systolic volume index (LVESVI)	41.15±13.06 ml/m^2
Left ventricular internal diameter end systole (LVIDS)	3.24±0.19 cm
Left ventricular internal diameter end diastole (LVIDD)	4.71±0.13 cm
BMI	29.16±1.45 kg/m^2

Following the collating step (not shown), correlations between the cytokines and clinical data were performed.

3.6 Correlation of cytokine concentration to indices of cardiac function.

Levels of serum cytokine concentrations of CAVCAD study patients (section 3.4.1) were correlated to the indices of cardiac function (section 3.4)

3.6.1. Ejection fraction (EF).

Ejection fraction was the first parameter that was correlated with levels of patient serum cytokines that included IL-1β, IL-8, IL-6, IL-10, TNF-α and IL-12. Analysis showed in Figure 37.

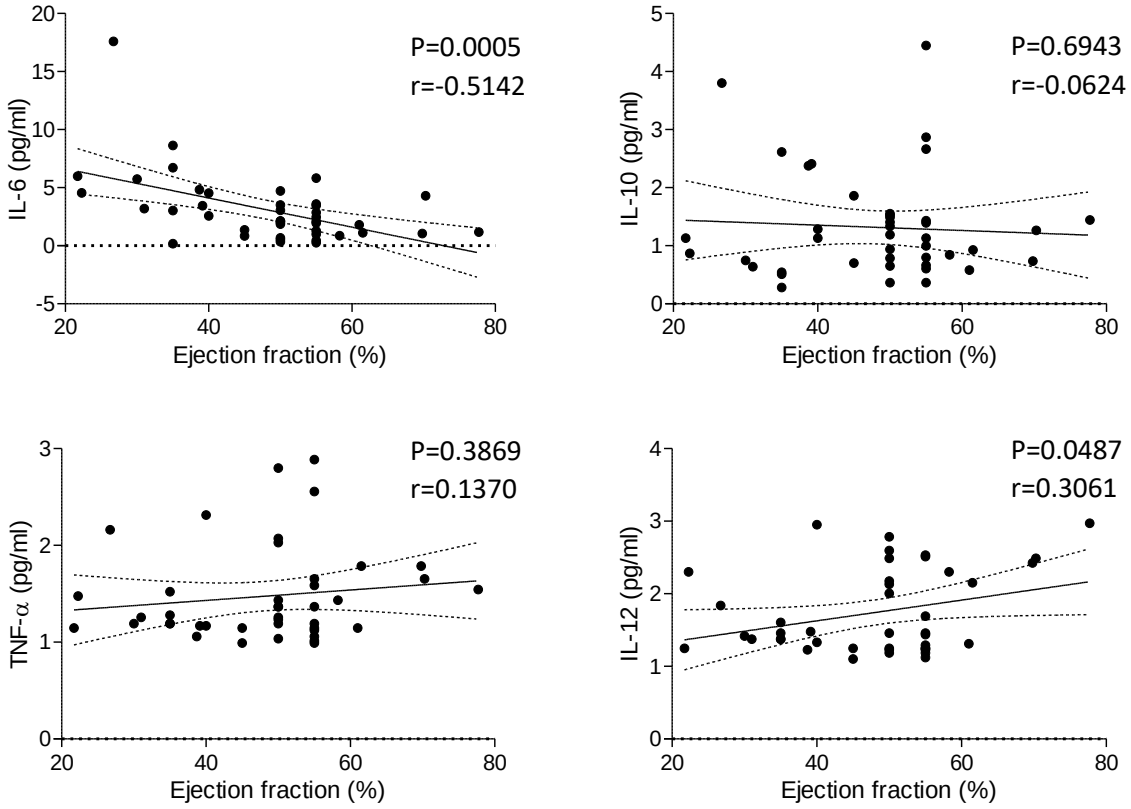

Figure 37. Correlation between serum cytokine (IL-6, TNF-α and IL-12, n=49) levels and ejection fraction in CAD patients, where r is a correlation coefficient and dashed lines represent 95% confidence interval.

Among 57 patients that had their serum analysed IL-6 (p=0.0005, r=-0.5142) significantly negatively correlated with the ejection fraction while IL-12 significantly positively correlated with EF (p=0.0487, r=0.3061). Interleukin-6 showed the highest negative correlation and IL-12 highest positive correlation. Additionally, TNF-α (p=0.3869, r=0.1370) correlated positively. For IL-6 this correlation was moderate (0.36>r>0.67) whilst remaining correlations were weak (0.1<r<0.35) (Akoglu, 2018). IL-10 (p=0.6943, r=-0.0624) did not correlate with EF.

3.6.2. Stroke volume (SV).

Second parameter chosen for correlations was stroke volume and analysis of this association is shown in figure 38.

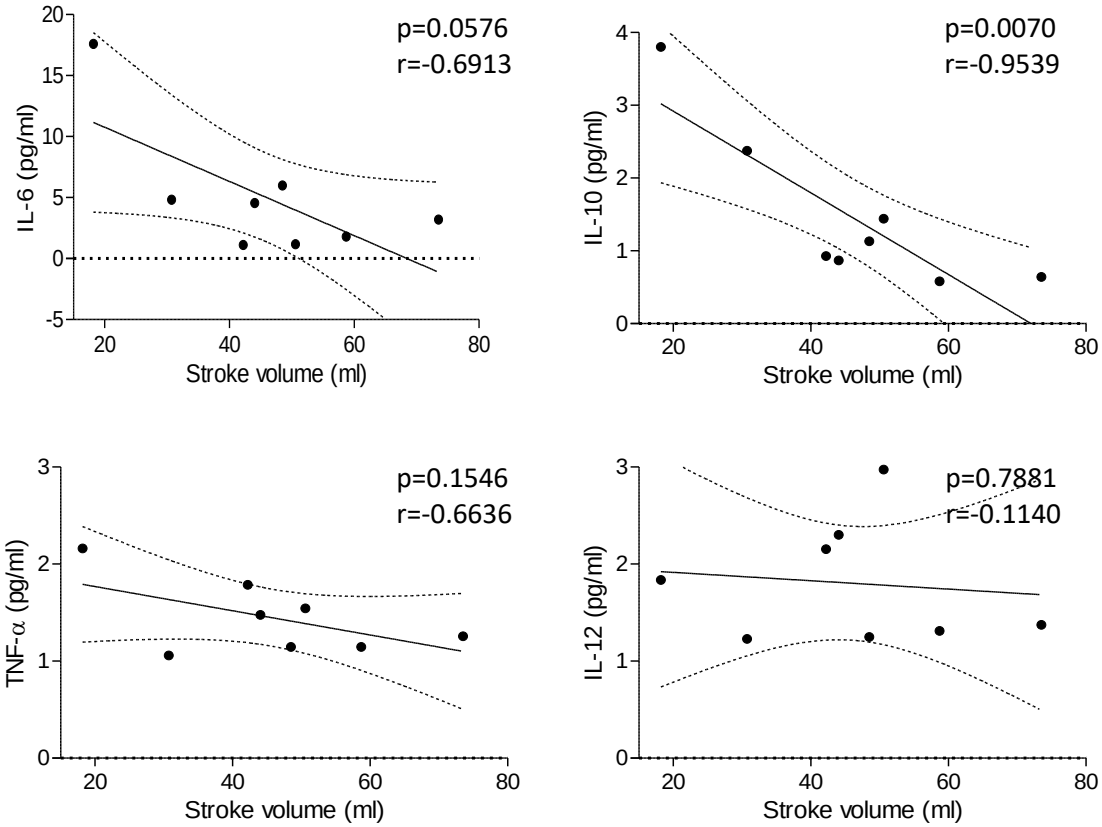

Figure 38. Correlation between serum cytokine (IL-6, TNF-α and IL-12, n=13) levels and stroke volume in CAD patients, where r is a correlation coefficient and dashed lines represent 95% confidence interval.

After determining the cytokines levels in the serum, correlation of these six cytokines and stroke volume was examined. Analysis of patient serum cytokines levels and EF showed that all correlations were negative. It was significant and strong for IL-10 (p=0070, r=-0.9539). Interleukin-10 showed strongest negative correlation. Furthermore, IL-6 (p=0.0576, r=-0.6913) and TNF-α (p=0.1546, r=-0.6636) correlations were not significant but moderate. Finally, IL-12 (p=0.7881, r=-0.1140) and stroke volume correlation was weak and not significant.

3.6.3 Tricuspid annular plane systolic excursion (TAPSE).

Fourth parameter chosen for correlations with patient serum cytokines levels was tricuspid annular plane systolic excursion, data is presented in figure 39.

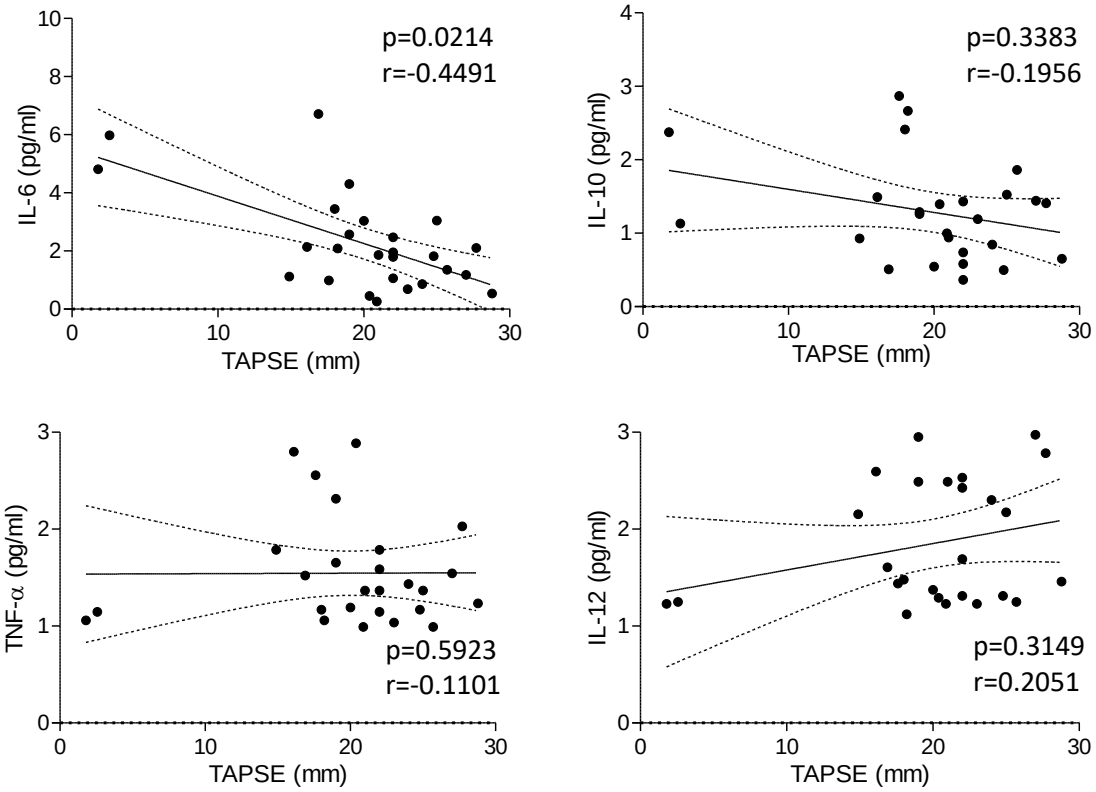

Figure 39. Correlations between serum cytokines (IL-6, TNF-α and IL-12) levels and pulmonary artery systolic pressure in CAD patients, where r is a correlation coefficient and dashed lines represent 95% confidence interval.

Analysis of these correlations showed that IL-6 (p=0.0214, r=-0.4491) was moderately, negatively, and significantly correlated to TAPSE. This was also the highest correlation for this clinical parameter. For IL-10 (p=0.3383, r=-0.1956) and TNF-α (p=0.5923, r=-0.1101) correlations were weak, negative, and not significant. For IL-12 (p=0.3149, r=0.2051) this correlation was weak and not significant but positive. Also, IL-12 showed highest positive correlation.

3.5.3. Body mass index (BMI).

Finally, figure 40 shows correlations of body mass index with the patients' cytokines levels.

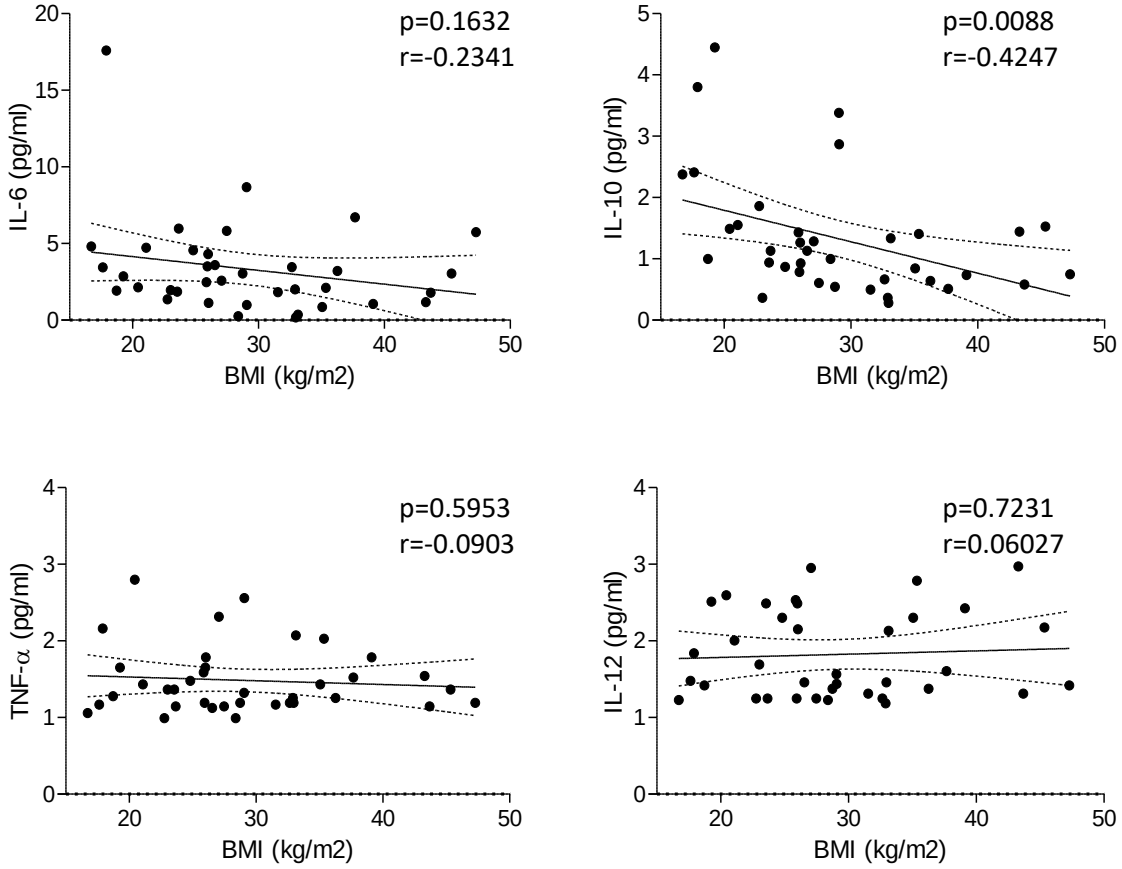

Figure 40. Correlation between serum cytokine (IL-6, TNF-α and IL-12, n-=37) levels and BMI in CAD patients, where r is a correlation coefficient and dashed lines represent 95% confidence interval.

Next parameter that was taken into the consideration for running the correlations with was a body mass index. Analysis showed that correlations were negative, significant, and moderate for IL-10 (p=0.0088, r=-0.4247). Additionally, IL-6 (p=0.1632, r=-0.2341) correlated with BMI negatively. weakly and not significantly. Furthermore, TNF-α (p=0.5953, r=-0.0902) and IL-12 (p=0.7231, r=0.0602) did not correlate with BMI.

3.6.4 Correlation summary

Other parameters were correlated include end systolic volume, end diastolic volume, pulmonary arterial systolic pressure, creatine at the time of the surgery, heart rate, left ventricle end diastolic volume index, left ventricle systolic volume index, left ventricle internal diameter end diastole but none of them significantly correlated with the cytokines levels or

were not relevant for this study (data not shown). Pearson r values and significance of the parameters is shown in Table 13.

Table 13. Correlation matrix of the cytokines expressed in serum of CAD patients and clinical parameters. All Pearson correlations are shown. Grey denotes significant correlations. *=p<0.05, **=p<0.005, ***=p<0.001, n=57.

Clinical parameter	IL-6	IL-10	TNF-α	IL-12
Ejection fraction	-0.5142***	-0.06246	0.1370	0.3061*
Stroke volume	-0.6913	-0.8539**	-0.5536	-0.1140
End systolic volume	0.1506	-0.1859	-0.1959	-0.1449
End diastolic volume	-0.08514	-0.4526	-0.3643	-0.1679
Left ventricular outflow tract (LVOT) peak velocity	-0.4118*	-0.4771**	-0.0271	0.03742
Pulmonary arterial systolic pressure (PASP)	0.009497	0.09133	-0.5847*	-0.5238
Creatinine at the time of the surgery	0.08003	-0.02362	-0.1621	-0.1177
Tricuspid annular plane systolic excursion (TAPSE)	-0.6225***	-0.2915	0.005729	0.2741
Heart rate	-0.1110	0.2529	-0.1076	-0.2408
Left ventricle end diastolic volume index (LVEDVI)	0.8976*	-0.2536	0.1166	0.3721
Left ventricle systolic volume index (LVESVI)	0.8726	-0.1037	0.04705	0.3494
Left ventricular internal diameter end systole (LVIDS)	-0.2385	-0.5683*	-0.5193*	-0.1908
Left ventricular internal diameter end diastole (LVIDD)	0.02587	-0.3083	-0.1758	0.08670
BMI	-0.2341	-0.4247**	-0.09025	0.06027

Cytokines that have a significant correlation with the clinical parameters include IL-6 that correlated with ejection fraction (p=0.0005), LVOT (p=0.0265), TAPSE (p=0.0007) and LVEDVI (p=0.0387). Also, significant correlation was that of IL-10 and stroke volume (p=0.0070), LVOT (p=0.0089) and BMI (p=0.0088)). Tumour necrosis alpha correlated significantly with PASP

(p=0.0458) and LVIDS (p=0.0393). Finally, IL-12 significantly correlated with EF (p=0.0487). All significant correlations were moderate to strong. No significant correlations were found with multiple clinical parameters including end systolic volume, end diastolic volume, creatinine level at the time of surgery, heart rate and LVESVI.

4.Discussion

Coronary artery disease has been found to be the leading cause of death in developed and developing countries. It arises from atherosclerosis and is inflammatory in nature (Shao et al., 2020). The underlying pathogenesis involves a disturbed equilibrium of lipid accumulation, immune responses and their clearance that is governed by multiple pro- and anti-inflammatory mediators including cytokines (Kany et al., 2019). Multiple cytokines are secreted at the site of the atherosclerotic lesion by the various cells present at the site, including smooth muscle cells as well as multiple innate and adaptive immune cells. These cytokines exert their effect via surface receptors and their effect is mediated by intracellular secreting molecules (Fairweather & Rose, 2005).

It has been shown that Interleukin-8, Interleukin-6, Interleukin-10, tumour necrosis factor-α and Interleukin-12 play major role in chronic inflammation in atherosclerosis progression, therefore, it is of crucial importance to understand how and why these molecules affect development of atherosclerosis and whether their activity can be translated to the heart function.

Cardiomyocytes from diseased hearts are under complex remodelling that involve changes in cell structure, membrane ion currents and excitation contraction coupling. These changes are responsible for the alterations leading to diastolic and systolic dysfunction in cardiac patients. Most information about cardiac diseases come from animal models which revealed physiological and biophysical properties of cardiomyocytes, but they do not necessary provide relevant models of human cardiac diseases (Coppini et al., 2014; Pimpalwar et al., 2020). Therefore, it is of paramount importance to successfully isolate viable myocytes from human myocardium to understand pathophysiology of heart myocytes and justify therapeutic methodologies.

Taken together, isolation of viable cardiomyocytes is crucial to understand pathology in the heart that is evoked by molecules, such as cytokines, secreted in response to inflammation. This understanding is furthered by correlating levels of circulating cytokines to indices of heart function where relationships can be discovered that can lead to finding the cause of the changes seen in the myocytes in the first place.

4.1. Optimisation of myocyte isolation.

Direct isolation of primary cells from tissues and organs preserves important cell characteristics for *in vitro* studies so are used in a plethora of biomedical applications. Disassociation of cells from the organ of interest is possible with the use of collagenases and proteases. However, the choice and concentration of these enzymes is critical for optimal yield and viability (Campora et al., 2018). Initially, this study followed the protocol of Voigt et al. (2013) to isolate cardiac myocytes from the atrial appendage removed during planned CABG operation (Voigt et al., 2013). However, the prolonged delivery time of the atrium samples necessitated further optimisation.

4.1.1 Does enzyme concentration and digestion time influence yield and viability?

Concentration of enzymes and digestion time play a fundamental role in obtaining single cells that maintain their properties for suitable period after extraction. Collagenase I that was obtained from *Clostridium histolyticum* is a crude preparations including more than 30 enzymes and are routinely used on its own or in combination with another enzyme in cardiomyocyte isolation. Protease XXIV greatly improves digestion efficiency when compared to collagenase I alone (Voigt et al., 2015). Hence, for the isolation of cardiac myocytes in this study Collagenase type I and Protease XXIV were used in combination as in Voigt et al. (2013) protocol but their concentrations were fine tuned to achieve best results.

To do so, cell suspension was observed after each isolation and firstly concentration of enzymes used for the digestion were balanced. Collagenases are widely used in all methods of cardiomyocyte isolation whether it is human or animal model (Campora et al., 2018; Voigt et al., 2015). Here, collagenase I was used, and its concentration was adjusted to 0.9079mg/ml, which was showed in other studies to work efficiently in isolation of cardiomyocytes (Ohler et al., 2009; Peeters et al., 1995). Additionally, protease XXIV was used to improve digestion efficiency and to reduce inconsistency due to collagenase batch variability (Wolska & Solaro, 1996). Firstly, protease was used at concentration of 0.714mg/ml which resulted in over digestion and cell death possibly due to cleavage of essential surface or extracellular matrix proteins (Judd et al., 2016). This study optimised protease concentration to 0.476mg/ml which allowed to obtain myocytes of typical morphology and size.

Second part of optimisation process involved modification of length of digestion. First isolations resulted in cells that were round or blebbing indicating cellular death after 35 minutes for primary digestion and 10 minutes for secondary digestion. Times that myocytes were in the first and second digest were gradually reduced what resulted in better quality of the cardiomyocytes as well as improved viability. Finally, digestion time was reduced to 15 minutes for first digestion with protease and collagenase, and 5 minutes for the second digestion with collagenase only. It is not uncommon for the cardiomyocyte isolation protocols to have different times for the enzymatic digestion of the cardiac tissue. It is a consequence of using different heart models as well as different heart disease models to investigate remodelling differences in healthy and diseases hearts (Peeters et al., 1995; Voigt et al., 2015).

In conclusion, obtaining cardiomyocytes of optimal quantity and quality from human atrium varies not only between laboratories but also between protocols used to isolate these cells. Hence optimising existing protocol to specific needs of this study was essential and resulted in an average yield of viable cardiomyocytes at 19.74%, which is less than reported in other studies (Engel et al., 1994; Voigt et al., 2013). This is possibly the result of the prolonged delivery time, as studies showed that successful isolation with good yield depends on a rapid initiation of the procedure after sample collection from the patient (Pimpalwar et al., 2020).

Finally, it is not currently known if cells isolated with this method are feasible for intracellular calcium cycling and contractile function investigations. This will have to be elucidated by further studies.

4.2 Are cytokines elevated in coronary artery disease?

Atherosclerosis remains an important global health burden and despite preventative measured there is still no cure. Pathogenesis results from a complex interplay between oxidative stress, inflammation and lipid metabolism (Poznyak et al., 2021). A key role in inflammation is played by cytokines, especially interleukins, which are produced in high quantities at the inflammation site (Bester & Pretorius, 2016).

It is well known that cytokines are modulators of immune responses and lack of balance between pro- and anti-inflammatory cytokines has a critical role in development of atherosclerosis which is the foundation of coronary artery disease (Wolf & Hunziker, 2020).

This study evaluated serum levels of Interleukin-1β, Interleukin-8, Interleukin-6, Interleukin-10, Tumour Necrosis Factor α and Interleukin-12 in a cohort of 57 patients that underwent planned CABG procedure.

In this project, analysis of serum cytokine levels in patients showed that IL-1β and IL-8 concentrations were below the detection limit of the cytometric bead array, hence they showed negative values after analysis. Consequently, both cytokines have been removed from further analysis. Previous studies showed that chronic inflammation produces low, almost physiological levels of both cytokines (Bester & Pretorius, 2016). Therefore, it is not uncommon for cytokines to be present in serum below the limit of the detection for the conventional immunoassays including cytometric bead array. This is because most cytokines act locally at specific sites of immune activity. Thus, it is reasonable to assume that only in abnormal cases would they be measured in high amounts in systemic fluids, like serum (Dabitao et al., 2011; Tarnok et al., 2003).

4.2.1 Is IL-6 elevated in coronary artery disease?

Interleukin-6 is a pleiotropic cytokine that exhibit a broad range of biological activities in multiple systems. Depending on the injury, it displays both -pro and anti-inflammatory properties (Tanaka et al., 2018). It has been shown that it plays an important role as a central regulator of inflammation found in the atherosclerotic plaque, however its contribution is controversial. It has a cardioprotective function during and after the acute heart event, but also have a destructive function when is expressed chronically at the site of plaque (Fontes et al., 2015).

This study revealed that concentration of circulating IL-6 was low at 3.15±0.44pg/ml, which was lower than in healthy population determined by the meta-analysis. It was surprising as it was expected to be elevated due to the inflammatory character of the disease and IL-6 causal contribution to atherothrombosis (Daniels, 2017). Nonetheless, studies showed that low lL-6 serum concentration is not uncommon in chronic diseases like obesity, type 2 diabetes mellitus, or rheumatoid arthritis (Maggio et al., 2006; Mirza et al., 2012). On the contrary, levels of circulating IL-6 in acute response like trauma or sepsis may increase 1000 fold (Marecaux et al., 1996; Stensballe et al., 2009). Therefore, low levels of IL-6 in patient cohort may suggest the low levels of inflammation and chronic disease.

Despite multiple factors influencing the IL-6 levels in CAD patients, the most important is the outcome – low levels of the circulating IL-6. Multiple studies have shown causal relationship between the coronary artery disease and increased levels of Interleukin-6, therefore its low levels decrease the risk of not only cardiovascular but of all-cause mortality (Su et al., 2013).

The Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) have shown that lowering circulating IL-6 levels with the canakinumab, a human therapeutic monoclonal antibody targeting IL-1β, significantly decreased major adverse cardiovascular events rates by 15% to 17% at doses of 150 and 300mg respectively (Ridker et al., 2018). This pivotal study showed that targeting pathways leading to generation of IL-1β or its downstream mediators, such as IL-6 might provide comparable benefits especially in people with low plasma IL-6 levels (Ridker et al., 2018). Similar effects were observed with different agents that target IL-6 signalling. Ziltivekimab neutralises IL-6 ligand, while tocilizumab and sarilumab block the IL-6 receptor via classical and trans-signalling pathways (Libby, 2021). Therefore, lower IL-6 levels can also be the indirect result of suppression of IL-1β by one of these agents. Despite promising results with the use of monoclonal antibodies they are not currently in the National Institute for Health and Care Excellence (NICE) recommendation for treatment of the atherosclerosis. (Duerden et al., 2015).

The recommendation however strongly advice lifestyle changes such as diet and exercise. Therefore, low serum IL-6 levels in patients' samples could be less the result of the pharmacotherapy and more because of following current guidelines in treatment of atherosclerosis, that include permanent lifestyle changes such as Mediterranean diet, regular exercise, stress management and intervention groups. Studies have shown that adherence to lifestyle changes by the CAD patients decreased circulating levels of IL-6 to physiological level (Dod et al., 2010; Palmefors et al., 2014; Schumacher et al., 2006).

4.2.2 Is IL-10 elevated in coronary artery disease?

IL-10 is a prototypic anti-inflammatory cytokine made in macrophages and TH2 lymphocytes. Its main role in atherosclerosis includes inhibition of macrophage activation, MMPs, pro-inflammatory cytokines production and cyclooxygenase-2 expression in lipid-loaded and activated macrophage foam cells (Han & Boisvert, 2015). These actions of IL-10 counterbalance the inflammation in the plaque.

Analysis of patient's samples in this study revealed that IL-10 levels are low, at 1.72±0.48pg/ml, however levels of proinflammatory cytokines are also low. It is not uncommon to find low levels of IL-10 in patients with advanced CAD. Study by Caligiuri et al. (2003) showed that IL-10 levels in atherosclerotic mice were associated with the disease progression and high levels were observed in early phases followed by decrease in IL-10 at the advanced phases (Caligiuri et al., 2003). Additionally, another study showed that lower circulating IL-10 were associated with higher plaque burden (Battes et al., 2014). These findings support the statement that low levels of IL-10 are associated with higher plaque burden in atherosclerosis and can indicate that patient cohort was more susceptible to plaque rupture, since high levels of IL-10 in atherosclerosis and after MI have a protective function as it augments the MMP activity and promotes plaque stability and protection from MI (Holven et al., 2006).

Given the anti-inflammatory properties of IL-10, higher levels would be expected to protect against atherosclerosis. Undeniably, data suggests that higher levels of IL-10 at the time of acute coronary syndrome event is protective against future complications (Heeschen et al., 2003; Zhang et al., 2016). Epidemiologic data, however, is less conclusive. Some studies showed that in clinically stable populations higher levels of IL-10 are related to increased risk for cardiovascular events (Lakoski et al., 2008; Welsh et al., 2011). A possible explanation is that low levels of IL-10 in patient samples are produced in response to low levels of circulating cytokines, such as IL-6 and TNF-α (Goldwater et al., 2019). This further explains the lower levels of circulating IL-10 in patient samples when compared to control.

Furthermore, study by George et al. (2012) found that IL-10 levels are reduced in patients with vulnerable coronary plaques compared to patients with stable angina (George et al., 2012). Szodoray et al. (2006) found higher levels of IL-10 in patients with acute coronary syndrome than either patients with stable CAD (Szodoray et al., 2006). Therefore, it can be concluded that patients underwent planned CABG surgery rather than being operated on due to the acute incident. Hence, IL-10 concentration is low due to reduced number of Treg cells in response to low grade inflammation.

Altogether low-grade inflammation produces low levels of circulating IL-10 in atherosclerotic patients.

4.2.3 Is TNF-α elevated in coronary artery disease?

TNF-α is a proinflammatory cytokine. Its elevated levels are associated with development of atherosclerosis (Dopheide et al., 2015). Increased levels of TNF-α were found in older adults and were associated with the risk of clinical diagnosis of atherosclerosis with or without comorbidities. Additionally, patients that are survivors of myocardial infarction also showed increased levels of TNF-α (Keul et al., 2019)

Analysis of patient circulating TNF-α concentration showed that they are at low level of 1.63±0.19pg/ml. Generally, high levels of TNF-α are associated with infection, elevated levels are often recognised in autoimmune diseases with chronic inflammation such as rheumatoid arthritis or inflammatory bowel disease as well as in atherosclerosis (Steyers & Miller, 2014). Low level of circulating TNF-α, however is associated with improved cardiac function in humans, and to lesser degree in animal models (Rolski & Błyszczuk, 2020). It appears that low levels of TNF-α in patient cohort might have protective function.

Additionally, patient cohort was compared to controls matched for age. Since age is a causative factor in elevated TNF-α it might be that both groups have elevated TNF-α levels regardless of accompanying diseases. Furthermore, higher TNF-α levels in a healthy population might be actually indication of inflammation somewhere in the body promoting atherosclerosis that control subjects were not aware at the time of the study.

As discussed previously, lower levels of IL-6 may be due to the life-style changes that are recommended for patients with atherosclerosis. Lifestyle changes are often associated with improvements in diet which can result in improved lipid profile. Studies have shown that HDL-associated apolipoprotein A-1 decreases TNF-α production through inhibiting contact-mediated activation of monocytes by binding to stimulated T cells (Hyka et al., 2001; Keul et al., 2019).

Taken together, low TNF-α levels in patient samples might be a result of inhibition due to improved diet as well as low inflammation levels that match the healthy cohort determined by meta-analysis.

4.2.4 Is IL-12 elevated in coronary artery disease?

Interleukin-12 is a pro-inflammatory cytokine which is associated with cardiovascular disease.

Analysis of circulating IL-12 levels in patient cohort showed that its level is low at 1.72±0.07pg/ml. Patients in this study had significantly lower levels of circulating IL-12 when compared to healthy cohort ascertained by the meta-analysis. Given the role of IL-12 in infectious and autoimmune disease it was expected to be found at a higher level, especially since recent studies recognised atherosclerosis as a potential autoimmune disease (Hedar et al., 2021; Sima et al., 2018). Additionally, clinical data indicates that IL-12 expression is altered in atherosclerosis and CAD, however there are multiple factors like overweight, smoking or type 2 diabetes, that are associated with the expression of IL-12 suggesting that it might be involved in in more ways than one in the same inflammatory environment (Ye, Wang, Wang, Liu, Yang, Wang, Xu, Ye, Zhang, & Lin, 2020; Ye, Wang, Wang, Liu, Yang, Wang, Xu, Ye, Zhang, & Zhou, 2020). Additionally, studies have shown that expression of IL-12 is closely related to levels of oxLDL in the serum. The higher the level of oxLDL the higher the IL-12 production in foam cells in arthroscopic plaque. (Bolayır, 2016). Furthermore, interleukin-12 accumulates in human atherosclerotic plaques, however it can be also detected in circulation, where it can serve as a biomarker for events such as MI, unstable angina, and death (Zhou et al., 2001).

Taken together, low levels of IL-12 in patient cohort might be due to fact that this is a statistically underpowered pilot study. Increasing number of samples and obtaining further clinical data might result in its explanation.

4.3 Do cytokine levels correlate with indices of heart dysfunction?

Cytokines have a diverse and pleiotropic effect on multiple cells in response to environmental stress including all resident cell types in myocardium. They can impact myocardial function and affect both, myocyte contractility and the extracellular matrix (Prabhu, 2004). Therefore, understanding the correlations between levels of circulating cytokines in CAD patients and heart dysfunction may help to understand causal relationship between both.

4.3.1 Does ejection fraction correlate with cytokine levels?

Ejection fraction (EF) is a measurement, expressed as a percentage of how much blood the left ventricle pumps out with each contraction. Current guidelines for general clinical practice describe normal ventricular ejection fraction (LVEF) as ≥50%, 41-49% midrange and <40% is considered reduced (Butler et al., 2014). Measuring the EF is crucial to assess whether patient

had previous acute cardiac events and/or whether it is related to a heart failure (Gerber et al., 2016). In this project ejection fraction correlated with IL-6 and IL-12.

4.3.1.1 IL-6

Patients' ejection fraction was measured together with other heart parameters by echocardiogram before the routine CABG procedure. The average EF of 57 patients was within the midrange category at 47.42±1.72% and significantly negatively correlated with IL-6 ($p=0.0005$, $r=-0.5142$). This indicates that decrease in circulating IL-6 lead to increase in ejection fraction. It is common to see reduced ejection fraction inversely correlated with IL-6, especially in MI patients (Ritschel et al., 2014). The correlation is also similar in heart failure patients where IL-6 levels are higher in patients with HFrEF and lower in HFpEF (Niethammer et al., 2008). Therefore, midrange category of EF in this study, which is just below the 50%, can be explained by the low levels of the IL-6, however causal effect is not confirmed.

This slightly reduced EF might be indicating some damage from possible previous acute cardiac event. However, it is not known if any of the patients underwent myocardial infarction before the echocardiogram was performed or is developing heart failure (HF) (Gerber et al., 2016). Multiple studies have shown that levels of IL-6 are associated with progression to heart failure whether it was for patients with CAD or otherwise healthy individuals (Chia et al., 2021; Fedacko et al., 2014; Povar-Echeverría et al., 2021). Therefore, patients low IL-6 levels may indicate that progression of patients to HF with or without preserved ejection fraction is unlikely despite some damage to the heart.

4.3.1.2 IL-12

In this project, patient ejection fraction significantly correlated with the levels of circulating IL-12 ($p=0.0487$, $r=0.3061$). This correlation was positive and moderate. This indicate that the increase in IL-12 results in increase in EF. Elevated IL-12 has been linked with arterial stiffness in healthy subjects, atherosclerosis and cardiac remodelling as it increases oxidative stress that affects vascular and myocardial function (Yong et al., 2013; Zhang et al., 2006). Studies have shown that reduction of IL-12 activity, especially IL-35, which is an IL-12 family member, improves left ventricle function and remodelling in heart failure and acute MI. Additionally, study by Ikonomidis et al. (2017) showed that inhibition of IL-12 in psoriatic patients was associated with improvement to coronary flow reserve and arterial elasticity which can

potentially lead to improved ejection fraction and as a result inverse correlation between both parameters (Ikonomidis et al., 2017). This might suggest that due to the lack of statistical power in this study, the correlation between IL-12 and EF shows to be positive, however the literature review indicates that negative correlation is more probable. Increasing patient cohort should resolve this issue.

Studies have also shown that coronary arteries without focal stenosis are generally considered non-flow limiting. However, atherosclerosis as a diffuse process without focal stenosis, causes a continuous, graded pressure fall along arterial length which contributes to myocardial ischaemia (De Bruyne et al., 2001). Patient cohort had a reduced left ventricular ejection fraction. Obstructed vessels in atherosclerosis can lead to increased resting coronary flow leading to lower coronary flow reserve values, which can be used as a marker of coronary dysfunction (Joh et al., 2022).

However, blood pressure is also affected by the IL-12 and its inhibition has been showed to reduce systolic blood pressure (Ye, Wang, Wang, Liu, Yang, Wang, Xu, Ye, Zhang, & Lin, 2020).

Taken together, despite low IL-12 levels in patient cohort, suggesting preserved ejection fraction according to previous studies, their EF is low. It might be due to previous ischaemic incident and increased fibrosis resulting in stiffness of left ventricle hence diminished coronary flow.

4.3.2 Does stroke volume correlate with cytokine levels?

Stroke volume (SV) represents a volume of blood ejected from the ventricle with each heartbeat and is the difference between end-diastolic and end-systolic volumes. The normal range is 50 to 100ml (Sidebotham & Le Grice, 2007). Stroke volume is a haemodynamic variable of left ventricular function that is important in assessing patients with coronary artery disease. In CAD patients it is common to find normal cardiac output and stroke volume at rest, however with the progress of atherosclerosis or in the presence of previous infarction that left a scar on the left ventricle, stroke volume and cardiac output drop with exercise (Stelfox et al., 2006). In this study stroke volume of patient cohort measured with echocardiogram is below the lower limit of the normal range and correlated with IL-10.

4.3.2.1 IL-10

The average stroke volume for the patient cohort was 45.21±3.78 ml which is just below the lower end of the range for the normal value. Interleukin-10 correlated significantly with stroke volume and this correlation was strong and negative (p=0.0070, r=-0.9539). This implies that increase in IL-10 is followed by the decrease in stroke volume. Previous studies showed that IL-10 correlates inversely with atherosclerosis progression (Dopheide et al., 2015; Sikka et al., 2013). Despite that it seems that there is no direct correlation between IL-10 and stroke volume in atherosclerotic patients. Therefore, IL-10 probably acts on stroke volume indirectly via another mechanism.

Lower circulating levels of IL-10 are associated with higher plaque burden (Battes et al., 2014). Animal studies have shown that low levels of circulating IL-10 are associated with vascular stiffness and increased blood pressure as well as hypertrophy and systolic and diastolic dysfunction (Sikka et al., 2013). Reduced stroke volume is a part of systolic dysfunction that might be assigned to old age in otherwise healthy individuals (Dean, 2007). In coronary artery disease however it is associated with heart failure, especially in heart failure with preserved ejection fraction (HFpEF) where ventricular hypertrophy results from myocardial fibrosis (Farris et al., 2017). Additionally, myocardial ischaemia and coronary artery disease are associated with left ventricular diastolic dysfunction which is the result of decrease in compliance of coronary arteries bypassing the surface of the heart and travelling between the myocardium that would cause a constricting effect on the ventricular wall like that caused by myocardial fibrosis (Lv et al., 2022). This mechanism might be an underlying cause of reduced stroke volume in patients, as studies showed that ventricular filling is decreased in fibrotic heart reducing the preload on the heart (Guccione et al., 2011).

4.3.3 Does tricuspid annular plane systolic excursion correlate with cytokine levels?

Right ventricular (RV) function impacts the outcomes in cardiac diseases, such as myocardial infarction and heart failure. Tricuspid annular plane systolic excursion (TAPSE) is a simple feasible marker of RV dysfunction. A rise in pulmonary hypertension, secondary to aortic or mitral valve disease leads to dilatation of RV leading to enlargement of the tricuspid annulus and tricuspid regurgitation (Khan et al., 2019). Normal TAPSE size is >19mm, borderline 15-19mm and abnormal <15mm (Duanmu et al., 2020). Additionally pulmonary hypertension

(PAH) was previously shown to be associated with atherosclerotic disease. Here TAPSE correlated with IL-6.

4.3.3.1 IL-6

Patients in this study had a low levels of circulating IL-6 and normal size TAPSE of 20.11±1.13mm. Interleukin-6 moderately, significantly, and negatively correlated with the TAPSE ($p=0.0005$, $r=-0.5142$). This indicates a moderate association between both parameters and decrease in IL-6 is followed by the increase in the the size of TAPSE. This association was confirmed by other studies where it was also inversed and significant in patients with or without pulmonary arterial hypertension (PAH) where elevated serum IL-6 levels correlated with worse right ventricle function and associated mortality (Bening et al., 2019; Prins et al., 2018). Therefore, increased IL-6 levels associated with atherosclerosis are involved in pathogenesis of RV. Patients' cohort had an average TAPSE within the normal range which was negatively correlated with the IL-6 levels. This correlation might indicate that right ventricle function in these patients is preserved and hence they are protected from the pulmonary atrial hypertension as well as from the RV dysfunction.

Elevated levels of IL-6 in PAH patients and in animal models were associated with greater symptomatic burden, however there is no clear understanding between elevated IL-6 and RV dysfunction (Giovanardi et al., 2012). Despite that, study by Prins et al, (2018) have shown that patients with higher levels of serum IL-6 had worse RV function indicating its role in pulmonary vascular remodelling resulting in increased mortality (Prins et al., 2018).

4.3.4 Does BMI and correlate with cytokine levels?

Chronic inflammation is known to promote obesity, which is a major risk factor for coronary artery disease (Furukawa et al., 2017). It is strongly associated with increased systemic oxidative stress which correlates with the extend of fat accumulation and has been linked to atherosclerosis susceptibility (Furukawa et al., 2017). In this project BMI correlated with IL-10.

4.3.4.1 IL-10

Patients IL-10 levels are lower than those of the healthy population as determined by meta-analysis, and they are negatively correlated with the patients BMI. This corelation is significant and moderate ($p=0.0088$, $r=-0.4247$). Additionally, BMI is elevated, and patients can be

classed as overweight (29.16±1.45 kg/m^2). Studies have shown that circulating IL-10 in CAD patients are lower than controls regardless of any other comorbidity (Gupta et al., 2012; Kumari et al., 2018). Additionally, circulating levels of IL-10 are low in individuals with metabolic syndrome that increase the risk of heart disease, especially in severe CAD further confirming the IL-10-BMI correlation found in this study. (Barcelos et al., 2019; Esposito et al., 2003)

Therefore, it is not unreasonable to conclude that patients' IL-10 concentration might be correlated with BMI due to the increased weight but there also might be another factor that have not yet been associated with low IL-10 and BMI levels in CAD patients.

4.4 Could levels of circulating cytokines influence cardiac muscle function in patient cohort?

Cytokines are signalling molecules that play important roles in regulating immune responses, inflammation, and tissue repair. Some cytokines have been shown to directly affect cardiac muscle function, while others can indirectly affect cardiac function by causing systemic inflammation (Kany et al., 2019).

4.4.1 Could levels of IL-6 influence cardiac muscle function in patient cohort?

In general, high levels of IL-6 are associated with increased risk and severity of atherosclerosis. This is because IL-6 can activate various pro-inflammatory signalling pathways that promote the recruitment of immune cells to the arterial wall, exacerbating the inflammatory response and contributing to plaque formation and progression (Tanaka et al., 2018).

On the other hand, low levels of IL-6 may have a different effect on cardiomyocyte function in atherosclerosis. Cardiomyocytes are the muscle cells that make up the heart and are responsible for its contractile function (Reiss et al., 2017). Recent studies have suggested that IL-6 may play a protective role in cardiomyocyte function and that low levels of IL-6 may impair cardiac function in atherosclerosis through imbalance between IL-6 and IL-10 (Feng et al., 2022; Reiss et al., 2017). Animal studies found that IL-6-deficient mice had impaired cardiac function compared to wild-type mice, suggesting that IL-6 is necessary for normal cardiomyocyte function (Chen et al., 2017).

Overall, while high levels of IL-6 are generally considered detrimental in atherosclerosis, low levels of IL-6 seen in the patient group may also have negative effects on cardiomyocyte function. Therefore, it is not unreasonable to conclude that patient cohort has low levels of circulating IL-6, however it may inflict a negative effect on cardiomyocyte function.

4.4.2 Could levels of IL-10 influence cardiac muscle function in patient cohort?

Several studies have suggested that low levels of IL-10 may contribute to the pathogenesis of atherosclerosis and cardiovascular disease. In particular, low levels of IL-10 have been shown to impair cardiomyocyte function in atherosclerosis (Ortega-Rodríguez et al., 2020; Shukor et al., 2023).

Cardiomyocytes are the cells that make up the heart muscle, and their function is essential for maintaining proper heart function. In atherosclerosis, chronic inflammation can lead to the accumulation of immune cells and lipid deposits within the arterial walls, which can cause damage to the heart muscle and impair cardiomyocyte function (Hansson, 2001).

Low levels of IL-10 can exacerbate this inflammatory response, leading to increased oxidative stress, apoptosis, and impaired cardiac function (Aimo et al., 2020; Bu et al., 2022). Additionally, it has been shown that low IL-10 levels can lead to increased expression of pro-inflammatory cytokines, such as TNF-α and IL-6, which can further contribute to cardiomyocyte dysfunction (Chen et al., 2020).

Furthermore, low levels of IL-10 have been linked to the development of cardiac fibrosis, a condition characterized by the accumulation of collagen fibres within the heart muscle (Stafford et al., 2020). Cardiac fibrosis can impair cardiac function and increase the risk of heart failure (Xu et al., 2021).

In summary, low levels of IL-10 seen in the patient cohort can contribute to the development of cardiomyocyte dysfunction in atherosclerosis by promoting inflammation, oxidative stress, and cardiac fibrosis. Therefore, strategies aimed at increasing IL-10 levels may have potential therapeutic benefits in the treatment of atherosclerosis and cardiovascular disease.

4.4.3 Could levels of IL-12 influence cardiac muscle function in patient cohort?

IL-12 has been shown to play a role in the pathogenesis of atherosclerosis by promoting the differentiation and activation of Th1 cells, which produce pro-inflammatory cytokines such as IFN-γ (Vignali & Kuchroo, 2012).

In terms of cardiomyocyte function, low levels of IL-12 may have an indirect effect on cardiomyocytes by altering the immune response within the arterial wall. In atherosclerosis, activated macrophages and T cells secrete pro-inflammatory cytokines, including IL-1β, TNF-α, and IFN-γ, which can lead to the development of atherosclerotic plaques and contribute to plaque instability.

Studies have shown that IL-12 deficiency in mice leads to reduced Th1 responses and atherosclerosis development, but also increases the prevalence of Treg cells, which produce anti-inflammatory cytokines such as IL-10 (Mushenkova et al., 2021). This shift in the immune response from pro-inflammatory Th1 to anti-inflammatory Treg cells can decrease the production of pro-inflammatory cytokines within the arterial wall, which can ultimately lead to reduced plaque formation and stabilization of existing plaques (Davenport & Tipping, 2003; Mushenkova et al., 2021).

Overall, while low levels of IL-12 seen in the patient group, may have a beneficial effect on the development and progression of atherosclerosis, their direct effect on cardiomyocyte function is unclear. The primary effect of IL-12 in atherosclerosis appears to be on the immune response and inflammation within the arterial wall, which can indirectly affect cardiomyocyte function.

4.4.4 Could levels of TNF-α influence cardiac muscle function in patient cohort?

TNF-a is produced by various cell types, including macrophages and smooth muscle cells within atherosclerotic plaques, and promotes the recruitment and activation of immune cells, leading to the progression of the disease (Zelová & Hošek, 2013).

In cardiomyocytes, TNF-a can induce apoptosis and disrupt calcium homeostasis, leading to impaired contractile function (Ing et al., 1999). Additionally, TNF-a can activate signalling

pathways that promote oxidative stress and inflammation, which can further contribute to cardiomyocyte dysfunction (Bajaj & Sharma, 2006; Ing et al., 1999).

Studies have shown that reducing TNF-a levels can improve cardiomyocyte function in atherosclerosis. For example, in animal models of atherosclerosis, treatment with anti-TNF-a antibodies improved left ventricular function and reduced myocardial inflammation and fibrosis. In human studies, treatment with anti-TNF-a agents has been associated with improved cardiac function in patients with rheumatoid arthritis, a condition characterized by chronic inflammation and increased risk of atherosclerosis (Kaur et al., 2009; Saraf et al., 2021).

Overall, lower TNF-a levels in patient cohort my suggest improved cardiomyocyte function by reducing inflammation and oxidative stress, and by preventing apoptosis and calcium dysregulation.

4.5 Limitations of the study.

This study had various factors that had introduced limitations. One of the limitations of the study was the fact that patient's cohort was heterogeneous and came from the diverse population of Blackpool region. The only common factors for these patients were the fact that they were consented adults that were to undertake planned CABG procedure. This produced a range of cytokine concentration that did not take into consideration comorbidity status, genetic polymorphism, ethnicity or lifestyle.

Correlation can be interpreted as the association between two variables however it cannot be used to indicate causal relationship. Therefore, understanding the nature of correlational analysis is crucial in correct interpreting the result. This study looked not only at levels of cytokines in serum of patients undergoing CABG procedure, but also correlated these cytokines with indices of heart function. Despite that, causality cannot be ascertained.

Another limiting factor was the cytometric bead array method itself. It was optimised and used to detect levels of cytokines below the detection limit of the kit. That could have resulted in mathematical variation to results, due to extrapolation, and cause increased coefficient variation. Especially if the cytokines have very low signal above the background noise, should

be excluded from the final sample analysis and this is what happened with IL-1β and IL-8 concentrations in patient samples. This very low signal of both cytokines is most probably due to their concentration not being pathologically elevated. Studies have shown that cytometric bead array assays are particularly convenient in the presence of infection or trauma where cytokine concentrations are high (Tarnok et al., 2003). To resolve this issue immunoassay with higher sensitivity, like ELISA can be used to quantify cytokines levels more accurately in patients with chronic diseases, where circulating cytokine concentrations are low.

Next limiting factor is the clinical data retrieval for patient cohort. Echocardiogram and clinical data were not available for every patient in this study, furthermore not all the details were consistent between all the patients, despite research nurses and clinicians' best efforts. Clinical data also did not provide a description of any treatments that patient was adhered to before the CABG that could lower the serum cytokines levels which could be the explanation for atypically low cytokine concentrations that were identified in this study, but further clinical data is required to confirm this statement.

Another limiting factor is the patient cohort size used in this study; only 57 patient samples were analysed so far. Since it only represents small patient cohort, the statistical analysis may have not identified significant differences between variables. Therefore, this study might only recognise trends rather than be representative for all the patients in this study.

4.6 Future work.

Further research must be conducted to firstly recruit the study participants and process obtained samples until 100 patients' goal is reached. Then the samples will be analysed for the IL-1β, IL-8, IL-6, IL-10, TNF-α and IL-12. That analysis will reach the statistical power to validate the trends from the pilot study.

Additionally, validation of acquired data is essential. Serum samples showed low concentration of cytokines. Use of more sensitive immunoassay like ELISA will be necessary to show that optimisation of cytometric bead array to the lower end of detection range can still produce reliable and reproducible data.

During this study, samples were taken only at the time of surgery. Future studies might investigate into serum cytokine changes and add more time points after the surgery to assess how the cytokine concentration change and whether it influences long time survival.

Further to this investigations, concentrations of other molecules could be measured to provide more complex picture of how the CAD affects the patients. It can include molecules that are important in low grade inflammation linked to atherosclerosis, such as IFNγ, TGFβ and IL-4. This profile can be further expanded by other molecules that affect atherosclerosis progression and have diagnostic and prognostic value, such as apolipoprotein A and B, oxLDL, high sensitivity CRP, homocysteine, and creatinine.

Once serum cytokine concentrations and clinical data are obtained from the full patient cohort they can be correlated and might provide significance where it was not seen in the pilot study.

4.7 Conclusion

In conclusion, this research showed that levels of Interleukin-1β, Interleukin-8, Interleukin-6, Interleukin-10, Tumour Necrosis Factor α and Interleukin-12 in atherosclerotic patients correlate with multiple indices of heart function and can serve as potential biomarkers of the severity of disease and its control by pharmacotherapy and lifestyle changes. Results from this pilot study show trends in the data, however an increased study power is required to statistically validate the results.

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6. Appendices

Appendix 1. Patient recruitment consent form.

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PATIENT INFORMATION SHEET

<u>An Invitation to participate</u>

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

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

What is the purpose of the study?

The study is looking at how coronary artery disease (CAD), also known as ischemic heart disease (IHD) can affect your heart tissue and blood vessels so that we can work to develop better treatments and improve the surgical outcome for all patients in the future. There are two questions we want to ask: 1. Do changes in how heart cells work contribute to the progression of your disease? 2. Do the blood vessels which are used for your bypass work better if they have their normal fat tissue around them?

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

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

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

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

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

Do I have to take part in the study?

It is up to you to decide whether to take part or not. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to

take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive or the treatment that you are receiving.

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

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

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

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

What are the possible benefits of taking part?

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

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

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

Indemnity and Compensation?

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

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

Will my taking part in the study be kept confidential?

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

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

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

What if there is a problem?

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

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

What will happen to the results of the study?

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

Who can I contact for further information?

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

Thank you for taking the time to read this information.

Blackpool Teaching Hospitals NHS
NHS Foundation Trust

INFORMED CONSENT FORM

Patient Research Identification Number:

Name of Researcher: David Greensmith and Sarah Withers

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

Please Initial box

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

I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

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

I agree to take part in the above study ☐

_____

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

_____

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

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

Appendix 2. Patient inclusion and exclusion criteria.

Inclusion Criteria:

1 Patients will have IHD and will be scheduled for routine coronary revascularisation surgery, on pump or off pump

2 Age > 18

3 Patients will have given written, informed consent.

Exclusion Criteria:

1 Non-adults (under the age of 18).

2 Non-English speaking patients.

Appendix 3. Summary of genome-wide significant CAD risk loci.

Source: (Erdmann et al., 2018; Roberts et al., 2021)

OD's – odds ratio

EAF – effect allele frequency

SNP – single nucleotide polymorphism

Table A.

No	Position	Lead SNP	EAF	OR	Gene(s) at locus
1	chr1: 2252205	rs36096196	T (0.15)	1.05	MORN1,SKI
2	chr1: 3325912	rs2493298	A (0.14)	1.06	PRDM16, PEX10, PLCH2, RER1
3	chr1: 38461319	rs61776719	A (0.53)	1.04	FHL3, UTP11, SF3A3, MANEAL, INPP5B
4	chr1: 55496039	rs11206510	T (0.82)	1.08	PCSK9
5	chr1: 56962821	rs17114036	A (0.91)	1.17	PPAP2B
6	chr1: 109822166	rs599839	A (0.78)	1.11	SORT1, PSCR1, CELSR2
7	chr1: 115753482	rs11806316	G (0.66)	1.04	NGF,CASQ2
8	chr1: 151762308	rs11810571	G (0.79)	1.07	TDRKH, RP11-98D18.9
9	chr1: 154422067	rs4845625	T (0.47)	1.06	IL6R, AQP10, ATP8B2, CHTOP, UBAP2L
10	chr1: 169094459	rs1892094	C (0.50)	1.04	ATP1B1, BLZF1, CCDC181, F5,NME7, SELP, SLC19A2

Table B.

11	chr1: 200646073	rs6700559	C (0.53)	1.04	DDX59, CAMSAP2, KIF14
12	chr1: 201872264	rs2820315	T (0.30)	1.05	LMOD1, IPO9, NAV1, SHISA4, TIMM17A
13	chr1: 210468999	rs60154123	T (0.15)	1.05	HHAT, SERTAD4, DIEXF
14	chr1: 222823529	rs17465637	C (0.74)	1.14	MIA3, AIDA, C1orf58
15	chr1: 230845794	rs699	G (0.42)	1.04	AGT, CAPN9, GNPAT
16	chr2: 21286057	rs515135	C (0.83)	1.07	APOB
17	chr2: 44073881	rs6544713	T (0.30)	1.06	ABCG5, ABCG8
18	chr2: 45896437	rs582384	A (0.53)	1.03	PRKCE, TMEM247
19	chr2: 85809989	rs1561198	T (0.45)	1.06	VAMP5,VAMP8, GGCX
20	chr2: 145801461	rs2252641	C (0.46)	1.06	ZEB2, TEX41
21	chr2: 164957251	rs12999907	A (0.82)	1.06	FIGN
22	chr2: 188196469	rs840616	C (0.65)	1.04	CALCRL,TFPI
23	chr2: 203745885	rs6725887	C (0.15)	1.14	WDR12, CARF, FAM117B, ICA1L,NBEAL1
24	chr2: 216304384	rs1250229	T (0.26)	1.07	FN1, ATIC, LOC102724849, ABCA12, LINC00607
25	chr2: 218683154	rs2571445	A (0.39)	1.04	TNS1, CXCR2, RUFY4
26	chr2: 227100698	rs2972146	T (0.65)	1.07	LOC646736, IRS1, MIR5702

Table C.

27	chr2: 233633460	rs1801251	A (0.35)	1.05	KCNJ13, GIGYF2
28	chr2: 238223955	rs11677932	G (0.68)	1.03	COL6A3
29	chr3: 14928077	rs748431	G (0.36)	1.04	FGD5
30	chr3: 46688562	rs7633770	A (0.41)	1.03	ALS2CL, RTP3
31	chr3: 48193515	rs7617773	T (0.67)	1.04	CDC25A, SPINK8, MAP4, ZNF589
32	chr3: 49448566	rs7623687	A (0.86)	1.07	RHOA, AMT, TCTA, CDHRA, KLHDC8B, and others
33	chr3: 124475201	rs142695226	G (0.14)	1.08	UMPS, ITGB5
34	chr3: 132257961	rs10512861	G (0.86)	1.04	DNAJC13, NPHP3, ACAD11, UBA5
35	chr3: 136069472	rs667920	T (0.78)	1.05	STAG1, MSL2, NCK1, PPP2R3A
36	chr3: 138119952	rs2306374	C (0.18)	1.12	MRAS, CEP70
37	chr3: 153839866	rs12493885	C (0.85)	1.07	ARHGEF26
38	chr3: 156852592	rs4266144	G (0.32)	1.03	CCNL1, TIPARP
39	chr3: 172115902	rs12897	G (0.41)	1.04	FNDC3B
40	chr4: 3449652	rs16844401	A (0.07)	1.07	HGFAC,RGS12, MSANTD1
41	chr4: 57838583	rs17087335	T (0.21)	1.06	REST, NOA1
42	chr4: 77416627	rs12500824	A (0.36)	1.04	SHROOM3, SEPT11, FAM47E, STBD1

Table D.

43	chr4: 81181072	rs10857147	T (0.27)	1.06	PRDM8, FGF5
44	chr4: 82587050	rs11099493	A (0.69)	1.04	HNRNPD, RASGEF1B
45	chr4: 96117371	rs3775058	A (0.23)	1.04	UNC5C
46	chr4: 120901336	rs11723436	G (0.31)	1.05	MAD2L1, PDE5A
47	chr4: 146782837	rs35879803	C (0.70)	1.05	ZNF827
48	chr4: 147472512	rs1878406	T (0.15)	1.10	EDNRA
49	chr4: 156635309	rs7692387	G (0.81)	1.08	GUCY1A1a
50	chr4: 169687725	rs7696431	T (0.51)	1.04	PALLD, DDX60L
51	chr5: 9556694	rs1508798	T (0.81)	1.05	SEMA5A, TAS2R1
52	chr5: 55860781	rs3936511	G (0.18)	1.04	MAP3K1, MIER3
53	chr5: 121413208	rs1800449	T (0.17)	1.09	LOX
54	chr5: 131667353	rs273909	G (0.14)	1.07	SLC22A4
55	chr5: 131867702	rs2706399	G (0.51)	1.07	IL5, RAD50
56	chr5: 142516897	rs246600	T (0.48)	1.05	ARHGAP26
57	chr6: 1617143	rs9501744	C (0.87)	1.05	FOXC1
58	chr6: 12927544	rs12526453	C (0.67)	1.10	PHACTR1, EDN1

Table E.

59	chr6: 22583878	rs35541991	C (0.31)	1.05	HDGFL1
60	chr6: 31888367	rs3130683	T (0.86)	1.09	C2, C4A, and others
61	chr6: 35034800	rs17609940	G (0.75)	1.07	ANKS1A, UHRF1BP1
62	chr6: 36638636	rs1321309	A (0.49)	1.03	CDKN1A,PI16
63	chr6: 39174922	rs10947789	T (0.76)	1.07	KCNK5
64	chr6: 43758873	rs6905288	A (0.57)	1.05	VEGFA, MRPL14, TMEM63B
65	chr6: 57160572	rs9367716	G (0.68)	1.04	PRIM2,RAB23, DST, BEND6
66	chr6: 82612271	rs4613862	A (0.53)	1.03	FAM46A
67	chr6: 126717064	rs1591805	A (0.49)	1.04	CENPW
68	chr6: 134214525	rs12190287	C (0.62)	1.08	TCF21, TARID (EYA4−AS1)
69	chr6: 150997401	rs17080091	C (0.92)	1.05	PLEKHG1, IYD
70	chr6: 160961137	rs3798220	C (0.02)	1.51	LPA, SLC22A3, LPAL2
71	chr6: 161143608	rs4252120	T (0.73)	1.07	PLG, LPAL2
72	chr7: 1937261	rs10267593	G (0.8)	1.04	MAD1L1
73	chr7: 6486067	rs7797644	C (0.77)	1.04	DAGLB, RAC1, FAM220A, KDELR2
74	chr7: 12261911	rs11509880	A (0.36)	1.04	TMEM106B, THSD7A

Table F.

75	chr7: 19036775	rs2023938	C (0.10)	1.08	HDAC9
76	chr7: 45077978	rs2107732	G (0.91)	1.06	CCM2, MYO1G
77	chr7: 107244545	rs10953541	C (0.80)	1.08	BCAP29, GPR22
78	chr7: 117332914	rs975722	G (0.4)	1.03	CTTNBP2, CFTR, ASZ1
79	chr7: 129663496	rs11556924	C (0.62)	1.09	ZC3HC1, KLHDC10
80	chr7: 139757136	rs10237377	G (0.65)	1.05	PARP12,TBXAS1
81	chr7: 150690176	rs3918226	T (0.06)	1.14	NOS3
82	chr8: 18286997	rs6997340	T (0.31)	1.04	NAT2
83	chr8: 19813180	rs264	G (0.86)	1.11	LPL
84	chr8: 22033615	rs6984210	G (0.06)	1.08	BMP1, SFTPC, DMTN, PHYHIP, DOK2, XPO7
85	chr8: 106565414	rs10093110	G (0.58)	1.03	ZFPM2
86	chr8: 126490972	rs2954029	A (0.55)	1.06	TRIB1
87	chr9: 22125503	rs1333049	C (0.46)	1.29	ANRIL, CDKN2B-AS
88	chr9: 110517794	rs944172	C (0.28)	1.04	KLF4
89	chr9: 113169775	rs111245230	C (0.04)	1.14	SVEP1
90	chr9: 124420173	rs885150	C (0.27)	1.03	DAB2IP

Table G.

91	chr9: 136154168	rs579459	C (0.21)	1.10	ABO, SURF6, GBGT1
92	chr10: 12303813	rs61848342	C (0.36)	1.04	CDC123, NUDT5, OPTN
93	chr10: 30335122	rs2505083	C (0.38)	1.07	KIAA1462
94	chr10: 44775824	rs1746048	C (0.87)	1.09	CXCL12
95	chr10: 82251514	rs17680741	T (0.72)	1.05	TSPAN14, MAT1A,FAM213A
96	chr10: 91002927	rs1412444	T (0.42)	1.09	LIPA
97	chr10: 104719096	rs12413409	G (0.89)	1.12	CYP17A1, CNNM2, NT5C2
98	chr10: 105693644	rs4918072	A (0.27)	1.04	STN1,SH3PXD2A
99	chr10: 124237612	rs4752700	G (0.45)	1.03	HTRA1, PLEKHA1
100	chr11: 5701074	rs11601507	A (0.07)	1.09	TRIM5, TRIM22, TRIM6, OR52N1, OR52B6
101	chr11: 9751196	rs10840293	A (0.55)	1.06	SWAP70
102	chr11: 10745394	rs11042937	T (0.49)	1.03	MRVl1, CTR9
103	chr11: 13301548	rs1351525	T (0.67)	1.05	ARNTL
104	chr11: 43696917	rs7116641	G (0.31)	1.03	HSD17B12
105	chr11: 65391317	rs12801636	G (0.77)	1.05	PCNX3, POLA2, RELA, SIPA1, and others
106	chr11: 75274150	rs590121	T (0.30)	1.05	SERPINH1

Table H.

107	chr11: 100624599	rs7947761	G (0.28)	1.04	ARHGAP42
108	chr11: 103660567	rs974819	T (0.32)	1.07	PDGFD
109	chr11: 116648917	rs964184	G (0.13)	1.13	APOA1-C3-A4-A5
110	chr12: 7175872	rs11838267	T (0.87)	1.05	C1S
111	chr12: 20220033	rs10841443	G (0.67)	1.06	RP11-664H17.1
112	chr12: 54513915	rs11170820	G (0.08)	1.10	HOXC4
113	chr12: 57527283	rs11172113	C (0.41)	1.06	LRP1, STAT6
114	chr12: 95355541	rs7306455	G (0.9)	1.05	NDUFA12,FGD6
115	chr12: 111884608	rs3184504	T (0.44)	1.07	SH2B3, FLJ21127, ATXN2, and others
116	chr12: 118265441	rs11830157	G (0.36)	1.12	KSR2
117	chr12: 121416988	rs2244608	G (0.35)	1.06	HNF1A, OASL, C12orf43, and others
118	chr12: 124427306	rs11057401	T (0.69)	1.08	CCDC92
119	chr12: 125307053	rs11057830	A (0.15)	1.07	SCARB1
120	chr13: 28973621	rs9319428	A (0.32)	1.06	FLT1
121	chr13: 33058333	rs9591012	G (0.66)	1.04	N4BP2L2, PDS5B
122	chr13: 110960712	rs4773144	G (0.44)	1.07	COL4A1, COL4A2

Table I.

123	chr13: 113631780	rs1317507	A (0.26)	1.04	MCF2L, PCID2,CUL4A
124	chr14: 58794001	rs2145598	G (0.42)	1.03	ARID4A, PSMA3
125	chr14: 75147552	rs3832966	I (0.46)	1.05	TMED10, ZC2HC1C, RPS6KL1, NEK9, EIF2B2e, ACYP1
126	chr14: 94838142	rs112635299	G (0.92)	1.13	SERPINA2,SERPINA1
127	chr14: 100133942	rs2895811	C (0.43)	1.07	HHIPL1, YY1
128	chr15: 65024204	rs6494488	A (0.82)	1.05	OAZ2, RBPMS2, TRIP4, and others
129	chr15: 67455630	rs56062135	C (0.79)	1.07	SMAD3
130	chr15: 79089111	rs3825807	A (0.57)	1.08	ADAMTS7
131	chr15: 89574218	rs8042271	G (0.9)	1.10	MFGE8, RP11-326A19.4, ABHD2
132	chr15: 91416550	rs17514846	A (0.44)	1.07	FURIN, FES
133	chr15: 96146414	rs17581137	A (0.75)	1.04	gene desert
134	chr16: 56961074	rs1800775	C (0.51)	1.03	CETP
135	chr16: 72096666	rs1050362	A (0.38)	1.04	DHX38, HP, DHODH
136	chr16: 75387533	rs3851738	C (0.60)	1.07	CFDP1, BCAR1
137	chr16: 81906423	rs7199941	A (0.4)	1.04	PLCG2, CENPN

Table J.

138	chr16: 83045790	rs7500448	A (0.77)	1.07	CDH13
139	chr17: 2126504	rs216172	C (0.37)	1.07	SMG6, SRR
140	chr17: 17543722	rs12936587	G (0.56)	1.07	Ral1, PEMT, RASD1, SMCR3, TOM1L2
141	chr17: 27941886	rs13723	G (0.49)	1.04	CORO6, BLMH, ANKRD13B, GIT1, SSH2, EFCAB5
142	chr17: 30033514	rs76954792	T (0.22)	1.04	COPRS, RAB11FIP4
143	chr17: 40257163	rs2074158	C (0.18)	1.05	DHX58, KAT2A, RAB5, NKIRAS2, DNAJC7, KCNH4, HCRT, GHDC
144	chr17: 45013271	rs17608766	C (0.14)	1.07	GOSR2, MYL4, ARL17A, and others
145	chr17: 46988597	rs46522	T (0.53)	1.06	UBE2Z, GIP, ATP5G1
146	chr17: 59013488	rs7212798	C (0.15)	1.08	BCAS3
147	chr17: 62387091	rs1867624	T (0.61)	1.04	PECAM1, DDX5, TEX2
148	chr18: 47229717	rs9964304	C (0.38)	1.04	ACAA2, RPL17
149	chr18: 57838401	rs663129	A (0.26)	1.06	PMAIP1, MC4R
150	chr19: 8429323	rs116843064	G (0.98)	1.14	ANGTPL4
151	chr19: 11163601	rs1122608	G (0.77)	1.14	LDLR, SMARCA4
152	chr19: 17855763	rs73015714	G (0.2)	1.06	FCHO1, COLGALT1

Table K.

153	chr19: 32882020	rs12976411	A (0.91)	1.33	ZNF507, LOC400684
154	chr19: 41854534	rs8108632a	T (0.48)	1.05	HNRNPUL1, CCDC97, TGFB1, B9D2
155	chr19: 45395619	rs2075650	G (0.14)	1.14	APOE, APOC1, TOMM40, PVRL2, COTL1
156	chr19: 46190268	rs1964272	G (0.51)	1.04	SNRPD2, GIPR
157	chr20: 33764554	rs867186	A (0.89)	1.07	PROCR,ASIP, NCOA6, ITGB4BP/EIF6and others
158	chr20: 39924279	rs6102343	A (0.25)	1.04	ZHX3, PLCG1, TOP1
159	chr20: 44586023	rs3827066	T (0.14)	1.04	PCIF1, ZNF335, NEURL2,PLTP, MMP9
160	chr20: 57714025	rs260020	T (0.13)	1.04	ZNF831
161	chr21: 30533076	rs2832227	G (0.18)	1.04	MAP3K7CL,BACH1
162	chr21: 35599128	rs9982601	T (0.15)	1.18	MRPS6, SLC5A3, KCNE2
163	chr22: 24262640	rs180803	G (0.97)	1.20	ADORA2A
164	6p21.3	rs3869109	G (0.55)	1.16	HLC-C,HLA-B, HCG27
165	chr2: 19809058	rs2123536	T (0.39)	1.25	TTC32-WDR35
166	chr4: 156730909	rs1842896	T (0.76)	1.23	GUCY1A3
167	chr6: 32449331	rs9268402	G (0.59)	1.17	C6orf10
168	chr12: 88605319	rs7136259	T (0.39)	1.21	ATP2B1

Table L.

169	6p24.1	rs6903956	A (0.08)	1.51	C6orf105
170	chr3: 17099388	rs4618210	A (0.45)	0.91	PLCL2
171	chr19: 2111529	rs3803915	A (0.21)	0.89	AP3D1-DOT1L-SF3A2

Appendix 4. Cytokine levels measured in all 57 patients.

IL1-β – Interleukin-1β

IL-8 – Interleukin-8

IL-6 – Interleukin 6

IL-10 – Interleukin-10

TNF-α – Tumour Necrosis Factor – α

IL-12 – Interleukin-12

Table A.

Cytokine	IL1-β	IL-8	IL-6	IL-10	TNF-α	IL-12
Average	**-2.844239266**	**-3.143888472**	**3.155294935**	**1.725189282**	**1.629527562**	**1.717950276**
SD	**0.775420724**	**3.10838905**	**3.355644442**	**3.612004104**	**1.472174308**	**0.565350556**
n	**57**	**57**	**57**	**57**	**57**	**57**
SE	**0.102706984**	**0.411716188**	**0.444465964**	**0.478421631**	**0.194994251**	**0.07488251**
BVH 1	-3.180579596	-0.591358327	2.572973412	1.287217415	2.314314996	2.952030366
BVH 2	-3.889131821	0.657168528	1.122330767	0.928189662	1.785354404	2.152922003
BVH 3	-3.383023089	-1.489830924	2.47152987	1.430828516	1.586994181	2.531447017
BVH 4	-3.453878311	4.332738241	2.857015328	4.44666164	1.653114255	2.51041785

Table B.

BVH 5	-4.506584475	-6.005530857	1.183196892	1.442796108	1.542914132	2.973059534
BVH 6	-4.577439697	-4.138574812	0.858577559	0.84441652	1.432714009	2.300126175
BVH 7	-3.605710931	-5.655476599	1.061464642	0.736708194	1.785354404	2.42630118
BVH 8	-3.656321804	-2.434977422	2.725138724	1.418860925	1.498834083	2.804826194
BVH 9	-2.887036531	-2.656678452	2.10633312	1.406893333	2.027794675	2.783797026
BVH 10	-3.767665725	-2.878379482	3.049758057	1.52656925	1.366593935	2.17395117
BVH 11	-3.737299201	-3.531814098	4.307657973	1.263282232	1.653114255	2.489388682
BVH 12	-4.769761015	-0.684706129	4.561266827	0.868351704	1.476794058	2.300126175
BVH 13	-3.787910075	-6.378922066	1.639692829	1.059833172	1.763314379	2.468359515
BVH 14	-3.21094612	-3.321781543	2.136766183	1.490666475	2.799195539	2.594534519
BVH 15	-3.251434818	-2.831705581	1.954167808	0.36571285	1.366593935	1.690280319
BVH 16	-3.433633962	-5.993862382	1.791858141	0.581129501	1.146193688	1.311755305
BVH 17	-1.49017643	2.384102869	17.59676192	3.800411685	2.160034823	1.837484491
BVH 18	-3.474122661	6.573085495	3.445387869	2.412171041	1.168233712	1.479988644
BVH 19	-3.180579596	-6.600623097	16.76492488	0.353745258	1.146193688	1.374842807
BVH 20	-1.277610762	-3.333450018	5.738011909	0.748675786	1.190273737	1.416901142

Table C.

BVH 21	-3.291923517	-3.438466296	3.465676578	0.664902644	1.190273737	1.248667802
BVH 22	-3.281801342	-0.614695278	3.039613703	0.545226726	1.190273737	1.374842807
BVH 23	-2.553004768	-0.90640716	6.711869909	0.509323951	1.520874107	1.606163649
BVH 24	-2.259461703	-2.213276391	5.819166743	0.605064685	1.146193688	1.248667802
BVH 25	-2.127873432	-2.294955718	3.516398348	0.784578561	1.190273737	1.248667802
BVH 26	-3.403267438	-5.678813549	3.20192337	0.64096746	1.256393811	1.374842807
BVH 27	-2.998380452	-5.970525431	0.635401767	0.605064685	0.925793441	1.248667802
BVH 28	-2.978136103	-1.011423437	4.814875681	2.376268266	1.058033589	1.227638635
BVH 29	-3.069235675	-4.080232436	1.538249287	0.401615625	1.035993564	1.332784472
BVH 30	-3.048991325	-5.538791846	2.015033933	0.36571285	1.256393811	1.1855803
BVH 31	-2.998380452	-6.495606819	3.587408828	1.131638722	1.124153663	1.458959477
BVH 32	-1.257366413	-5.28208539	0.990454163	2.866939528	2.556755268	1.437930309
BVH 33	-2.49227172	-3.391792394	5.981476409	1.131638722	1.146193688	1.248667802
BVH 34	-1.945674289	-3.835194455	5.240938556	27.69969243	11.87968571	2.699680357
BVH 35	-1.044800745	4.881156579	8.669730262	3.381545974	1.322513885	1.564105314
BVH 36	-2.725081737	-0.451336624	4.723576494	1.550504434	1.432714009	2.005717831

Table D.

BVH 37	-2.259461703	-5.258748439	1.020887225	1.813791453	3.262036057	1.564105314
BVH 38	-2.765570435	-5.30542234	1.4469501	0.521291543	1.190273737	1.374842807
BVH 39	-2.806059134	-5.550460321	0.17890583	0.281939707	1.190273737	1.458959477
BVH 40	-2.77569261	-5.293753865	0.889010621	0.413583217	0.925793441	1.206609467
BVH 41	-2.289828227	-3.20509679	0.818000142	0.473421175	1.124153663	1.479988644
BVH 42	-2.502393894	-1.559841775	4.540978119	1.131638722	1.168233712	1.332784472
BVH 43	-2.431538672	-0.894738684	1.822291204	0.497356359	1.168233712	1.311755305
BVH 44	-2.502393894	-4.255259565	1.86286862	0.940157254	1.366593935	2.489388682
BVH 45	-2.563126942	-6.017199333	1.923734745	0.999995213	1.278433836	1.416901142
BVH 46	-2.47202737	-1.384814646	8.639297199	2.615620101	1.278433836	1.374842807
BVH 47	-1.520542953	-6.425595967	1.355650912	2.077078471	1.476794058	1.564105314
BVH 48	-2.046896035	-6.273905789	0.533958226	0.652935052	1.234353786	1.458959477
BVH 49	-2.785814784	-3.076743562	3.61784189	1.299185007	0.925793441	1.26969697
BVH 50	-2.411294322	-6.402259017	1.274496079	0.796546153	1.01395354	1.1855803
BVH 51	-2.90728088	-4.698661626	0.686123538	1.191476681	1.035993564	1.227638635
BVH 52	-2.765570435	3.947678557	1.355650912	1.86166182	0.991913515	1.248667802

Table F.

BVH 53	-2.451783021	-5.655476599	0.351359851	1.335087782	2.071874724	2.131892835
BVH 54	-2.482149545	-7.650785872	0.260060663	0.999995213	0.991913515	1.227638635
BVH 55	-2.502393894	-5.317090816	0.83828885	0.700805419	1.146193688	1.10146363
BVH 57	-2.441660846	-3.695172752	2.086044412	2.663490468	1.058033589	1.122492798
BVH 58	-2.441660846	-4.325270417	0.452803392	1.394925741	2.887355638	1.290726137