MEASURES OF VASCULAR DYSFUNCTION, MONOCYTE SUBSETS AND CIRCULATING MICROPARTICLES IN PATIENTS WITH DIFFUSE CORONARY ARTERY DISEASE

by

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ABSTRACT

Diffuse, multivessel coronary artery disease (CAD) affects about one third of patients with CAD and is associated with worse outcomes. Abnormal vascular stiffness and function (e.g., reflected by increased endothelial microparticles and diminished microvascular endothelial-mediated responses), cell mediated pro-inflammatory status (e.g., reflected by levels of specific monocyte subsets), and platelet function (e.g., increased monocyte-platelet aggregates (MPAs) and platelet microparticles) have established roles in CAD pathogenesis but their contribution to the unfavourable diffuse CAD form is unclear. The aim of this study was to compare measures of vascular function, monocyte subsets, MPAs, and endothelial and platelet microparticles in patients with diffuse and focal CAD and subjects without CAD. Additionally, prospective changes in these characteristics were analysed over one year. I found increased counts of aggregates of Mon2 monocyte subset with platelets and apoptotic endothelial microparticles in patients with diffuse CAD and I identified a negative correlation between Mon2 MPAs and microvascular endothelial function and increased diastolic elastance. My findings suggest that excessive levels of Mon2 aggregates with platelets and apoptotic endothelial microparticles could be important contributors to the diffuse type of CAD by a mechanism involving microvascular endothelial dysfunction and abnormal cardio-vascular interactions.

DEDICATIONS

I dedicate this thesis to my wife Natalie for her
love and patience throughout my medical and research training
and to my parents Ken and Vilma for their love and support.

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LIST OF CONTENTS

LIST OF FIGURES

SECTION I

SECTION II

SECTION IV

SECTION V

LIST OF TABLES

SECTION I

SECTION III

SECTION IV

SECTION V

ABBREVIATIONS

AC: Afro Caribbean

ACC: American College of Cardiology

ACE: angiotensin converting enzyme

ACh: acetylcholine

ACS: acute coronary syndrome

AHA: American Heart Association

AIx: Augmentation index

AMI: acute myocardial infarction

ANOVA: analysis of variance

AP: augmentation pressure

ARB: angiotensin receptor blocker

ArS: arterial stiffness

ASP: aortic systolic pressure

BCIS: British Cardiovascular Intervention Society

BD: Becton Dickinson

BMI: body mass index

BMP: bone morphogenic protein

BSA: body surface area

CABG: coronary artery bypass graft

CAC: coronary artery calcium

CAD: coronary artery disease

CCR: chemokine receptor

CD: cluster differentiation

CI: confidence interval

CKD: chronic kidney disease

CFR: coronary flow reserve

CSA: cross-sectional area

CT: computed tomography

CTCA: computed tomography coronary angiography

CV: cardiovascular

DNA: deoxyribonucleic acid

DTBT: door-to-balloon time

EaI: effective arterial elastance index

EC: endothelial cell

ECG: electrocardiogram

ED: endothelial dysfunction

EDP: end-diastolic pressure

EDPVR: end-diastolic pressure volume relationship

EDRF: endothelium derived relaxing factor

EDV: end-diastolic volume

Eed: end-diastolic elastance

Ees: end-systolic elastance

EF: ejection fraction

EMP: endothelial microparticles

EQ-5D-5L: euroqol-5 dimension-5 level

ESC: European Society of Cardiology

ESP: end-systolic pressure

ESPVR: end-systolic pressure volume relationship

ESS: endothelial shear stress

ESV: end-systolic volume

ET: endothelin

FBC: full blood count

FFR: fractional flow reserve

FSC: forward scatter

GP: glycoprotein

GTN: glyceryl trinitrate

GWAS: genome wide association study

HDL: high density lipoprotein

HF: heart failure

HIV: human immune deficiency virus

HR: heart rate

HTN: hypertension

ICAM: intercellular adhesion molecule

IFN: interferon

IL: interleukin

IQR: interquartile range

IRA: infarct related artery

IVCT: isovolumetric contraction time

IVRT: isovolumetric relaxation time

IVUS: intravascular ultrasound

LAD: left anterior descending (artery)

LDL: low density lipoprotein

LV: left ventricle/ventricular

LVEF: left ventricular ejection fraction

MACCE: major adverse cardio and cerebrovascular events

MAP: mean arterial pressure

MCP: monocyte chemoattractant protein

MI: myocardial infarction

MINAP: myocardial infarction national audit project

MMP: matrix metalloproteinase

Mon1: Monocyte subset 1 (CD14++CD16-CCR2+)

Mon2: Monocyte subset 2 (CD14++CD16+CCR2+)

Mon3: Monocyte subset 3 (CD14+CD16++CCR2-)

MOSS: Mediators of Social Support

MP: microparticle

MPA: monocyte-platelet aggregate

MRI: magnetic resonance imaging

MVD: multivessel disease

NADPH: nicotinamide adenine dinucleotide phosphate

NF-κB: nuclear factor-kappa beta

NHS: National Health Service

NICE: National Institute for Health and Care Excellence

NO: nitric oxide

OI: operator index

OMT: optimal medical therapy

OOH: out-of-hours

OR: odds ratio

OTHT: onset-to-hospital time

PAD: peripheral artery/arterial disease

PBS: phosphate buffered saline

PCI: percutaneous coronary intervention

PDGF: platelet-derived growth factor

PECAM: platelet and endothelial cell adhesion molecule

PET: positron emission tomography

PMP: platelet microparticle

PP: pulse pressure

PPCI: primary percutaneous coronary intervention

PWA: pulse wave analysis

PWV: pulse wave velocity

QOL: quality of life

RANTES: regulated on activation normal T cell expressed and secreted

RCT: randomised controlled trial

RNA: ribonucleic acid

ROS: reactive oxygen species

SA: South Asian

SD: standard deviation

SMC: smooth muscle cell

SNP: sodium nitroprusside

SPSS: statistical package for social sciences

SSC: side scatter

STCT: symptom-to-call time

STDT: symptom-to-door time

STEMI: ST-segment elevation myocardial infarction

SV: stroke volume

SWBH: Sandwell and West Birmingham NHS Hospitals

TDI: tissue Doppler imaging

TGF: transforming growth factor

TLR: toll-like receptor

TNF: tumour necrosis factor

t-PA: tissue plasminogen activator

TTE: transthoracic echocardiography

VAC: ventricular arterial coupling

VAS: visual analogue score

VCAM: vascular cell adhesion molecule

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

VSMC: vascular smooth muscle cell

VTI: velocity time integral

WBC: white blood cell

WE: White European

WHR: waist-to-hip ratio

PREFACE

This opening section of the thesis contains a brief introduction to the topic of advanced diffuse coronary artery disease (CAD) followed by a generalised overview of the pathological processes underlying atherogenesis. Subsequently I review the literature pertaining to diffuse CAD and explore the pathogenesis in more depth, before discussing features relevant to the diagnosis of normal coronary arteries and concluding with a section summary. I provide an overview of the experimental work undertaken in section two with methodology validation in section three. Section four details the results of retrospective analyses that I performed as part of my research training and to illustrate the clinical landscape of CV disease in the region, compare our data with previously published literature (with emphasis on the ethnic differences pertinent to this region of the UK) and introduce the experimental data detailed in section five. Within section five are the results of all the experiments I performed, followed by conclusions and suggestions for further work in section six.

SECTION I

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Despite falling rates of age-adjusted mortality, coronary artery disease (CAD) remains the leading cause of death worldwide[1, 2]. Advanced obstructive CAD (often diffuse in nature) is becoming an important entity of modern cardiology as the population ages. More patients with historical coronary revascularisation procedures no longer have suitable coronary anatomy for additional procedures once grafts have occluded and restenosis has occurred at angioplasty sites[3-5]. Advances in the treatment of diffuse obstructive CAD are hampered by a poor understanding of the factors implicated in its development.

Patients with diffuse CAD are at risk of developing refractory angina – resistant to conventional revascularisation techniques such as percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) – and not adequately controlled by optimal medical therapy (OMT). Studies have shown that up to 12% of symptomatic patients undergoing coronary angiography are ineligible for revascularisation[6]. Annual mortality rates in patients with refractory angina due to advanced CAD vary from 3-21% in randomised trials[7], and from 2-17% in registries[8]. One-year rates of myocardial infarction up to 25.5% have been reported with 71% of one cohort being re-hospitalised at least once during the year[6]. Patients with this condition often have restricted physical capacity and severely impaired quality of life[9]. This has stimulated interest in alternative strategies, but satisfactory treatments are still to be developed[10].

Although the likelihood of developing clinically significant CAD is closely linked to the presence of traditional risk factors (e.g. diabetes[4]) the extent of coronary disease among individuals possessing one or more conventional risk factor at any one point in time is

highly variable. Some patients develop diffuse multivessel involvement while others have a single focal stenosis. Indeed, rates of normal coronary arteries amongst people referred for elective (invasive) coronary angiography range from 5-50%[11].

CAD is associated with generalised vascular dysfunction. The purpose of this thesis is to test the hypothesis that stable patients with advanced diffuse CAD have increased measures of vascular dysfunction compared to stable patients with focal CAD and patients without CAD, and that the variation in these measures over one year will differ between patients with diffuse and focal CAD. I also propose that there will be a relationship between the measures of vascular dysfunction and biochemical markers of endothelial damage/dysfunction.

1.2 PRINCIPLES OF ATHEROGENESIS

1.2.1 Normal arterial structure

Healthy human arteries have a tri-laminar structure (Figure 1.1). The tunica intima is an inner layer of endothelial cells (ECs) lining the lumen called the endothelium. The endothelium is in direct contact with blood flow and arterial ECs possess highly regulated mechanisms of vital importance in maintaining vascular homeostasis. It is bound on the outside by internal elastic lamina, which separates the intima from the tunica media. The media contains numerous layers of SMCs. In large elastic arteries SMCs are arranged in a concentric manner within an elastin-rich extra cellular matrix which helps store kinetic energy required for the transmission of pulsatile flow. The media is bound on the outside by an external elastic lamina that separates the media from the adventitia. The adventitia consists mainly of fibroblasts, mast cells, and a matrix containing collagen and proteoglycans[12].

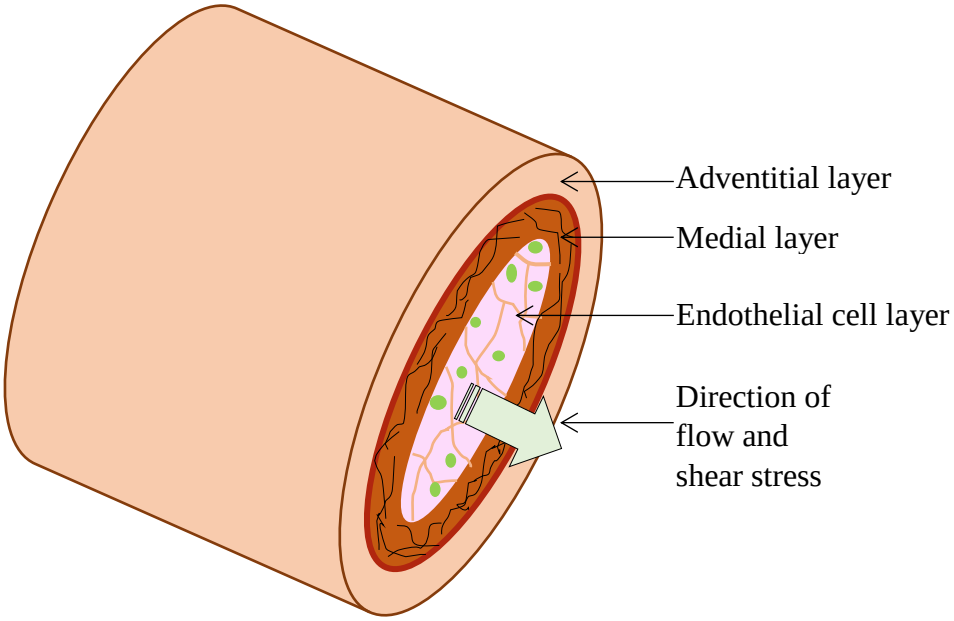


Figure 1.1 Schematic cross section of a human artery showing endothelial, medial and adventitial layers.

1.2.2 Normal endothelial function

The normal, healthy endothelium is the major regulator of vascular homeostasis – maintaining the balance between vasodilation and vasoconstriction, inhibition and stimulation of smooth muscle cell proliferation and migration, and thrombogenesis and fibrinolysis (Table 1.1)[13]. Initial insight as to the importance of the endothelium in vasomotor modulation came from animal experiments during the 1980s[14-19]. It subsequently became evident that the endothelium is an active paracrine, endocrine and autocrine organ capable of varying degrees of stimulation – a spectrum of endothelial disturbance exists that depends on the prevailing physiological, or pathophysiological, conditions[20-22]. Its main vasoregulatory functions include;

- Generation of nitric oxide (NO), a potent vasodilator originally identified as endothelium-derived relaxing factor (EDRF)[23]. Other endothelium-derived vasodilators include prostacyclin and bradykinin both of which also inhibit platelet aggregation[13].
- Generation of vasoconstrictor substances such as endothelin and angiotensin II. Angiotensin II is pro-oxidant[24] and both promote smooth muscle cell proliferation within the intimal layer[13].

In normal vascular physiology, NO is key to maintaining the vascular wall in a quiescent state by inhibiting inflammation, cellular proliferation, and thrombosis[25]. This is achieved through the expression of a set of protective genes (IκκB-a, A20, Bcl-2) that down-regulate the expression of transcription factor nuclear factor (NF)-κB since activation of NF-κB triggers endothelial activation and renders the endothelium more susceptible to apoptosis[21]. NO also limits oxidative phosphorylation in the mitochondria. Laminar shear

stress (Figure 1.1) is probably the major factor that maintains this quiescent, NO-dominated, endothelial phenotype with little or no expression of pro-inflammatory factors[26].

Table 1.1 Major function of mediators involved in the maintenance of normal vascular homeostasis

	Antithrombotic	**Prothrombotic**	**Vasodilator**	**Vasoconstrictor**
Nitric oxide	+	-	+	-
Bradykinin	+	-	+	-
Prostacyclin	+	-	+	-
Endothelin	-	-	+†	+‡
Thromboxane A_2	-	+	-	+
Prostaglandin H_2	-	-	-	+
Angiotensin II	-	-	-	+
Thrombomodulin	+	-	-	-
Tissue plasminogen activator	+	-	-	-
Tissue factor	-	+	-	-
Plasminogen activator inhibitor -1	-	+	-	-
Binding sites for factors IX and X	-	+	-	-

† acting on endothelial ET_B receptors, ‡ acting on vascular smooth muscle ET_B receptors.

1.2.3 Endothelial activation, injury and dysfunction

Endothelial disturbance is characterised by distinct, often overlapping, phases[21]. Endothelial activation occurs in two stages and represents a change from the quiescent phenotype described above toward one that involves the host defence response (characterised by the expression of pro-inflammatory substances e.g. cytokines), which may be physiological, for example in response to infection[27], or pathophysiological, in the presence of CAD risk factors. Essentially, a reduction of NO production leads to a lack of NO mediated cell signalling, thus creating an imbalance between endothelium-dependent vasodilators and the opposing effects of endothelium-derived vasoconstrictors such as angiotensin II. Under these circumstances endogenous nitric oxide synthase (eNOS) favours the production of reactive oxygen species (ROS) which can consume NO and 'activate' endothelial cell (EC) processes (Figure 1.2)[25]. Endothelial activation implies reversibility – the activated ECs may return to a quiescent state upon withdrawal of inflammatory stimuli[28]. Unchecked endothelial activation may either result in EC apoptosis leading to irreversible endothelial injury, EC fragmentation and separation from the intima (for example, in atherosclerosis)[21, 29], or EC dysfunction without evidence of injury (for example atherogenesis or increased permeability)[28, 29].

Endothelial dysfunction (ED) results from a persistent imbalance of vasoactive substances leading to an inability of the endothelium to regulate tissue perfusion. Irreversible EC injury may occur with chronic EC activation as critical levels of endothelial adhesion molecules, procoagulant molecules, cytokines and chemokines cause EC necrosis and other mural cell injury[21]. Circulating endothelial microparticles (EMPs), derived from activated or apoptotic cells, are markers of such EC damage.

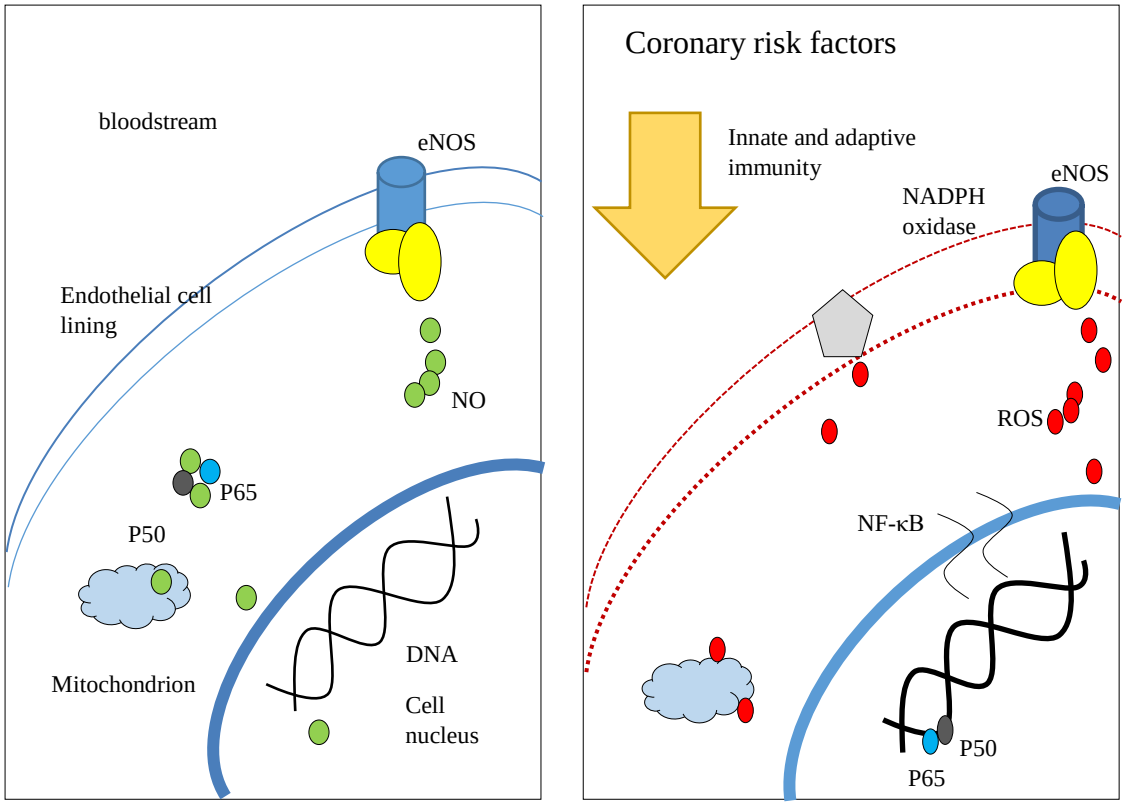


Figure 1.2 Endothelial activation in the presence of coronary risk factors (adapted from Deanfield et al[25]).

The left-hand panel shows normal, quiescent endothelial physiology induced by nitric oxide (NO, green circles). The right-hand panel reflects endothelial activation characterised by increased membrane permeability and reactive oxygen species (ROS, red circles) which now predominate, altering the function of cellular constituents leading to phosphorylation of transcription factors and mitochondria as well as protease activation (eNOS endothelial nitric oxide synthase, p50/p65 nuclear factor-κB transcription factor, NADPH nicotinamide adenine dinucleotide phosphate).

1.2.4 The impact of conventional modifiable and non-modifiable cardiac risk factors on endothelial function in patients with and without coronary artery disease

Numerous studies have examined the effect of conventional modifiable and non-modifiable risk factors on endothelial function since endothelial activation and dysfunction occur early in the evolution of CAD. Tables 1.2[30-65] and 1.3[42, 66-80] represent a summary of key data. According to epidemiological data, nine modifiable risk factors account for more than 90% of the proportion of risk of a first myocardial infarction[81]. Conventional CAD risk factors favour a reduction in L-arginine induced NO synthesis leading to an increase in ROS and subsequent oxidative stress (Figure 1.2). This alteration in endothelial function results in a chronic inflammatory process accompanied by a loss of antithrombotic factors and an increase in measures of endothelial activation and dysfunction which independently predict the risk of subsequent cardiovascular (CV) events in patients with and without CAD[82, 83]. While treatment or cessation of these risk factors may improve endothelial function[38] and, in some cases, reduce atherosclerotic progression[84], the presence of multiple CAD risk factors are synergistically detrimental to endothelial function and increase CV risk exponentially[81, 85, 86]. Hypertension (HTN) has a complex relationship with endothelial dysfunction, with alterations in regulatory pathways dependent upon the mechanism of the HTN. Regards the microvasculature (which is relatively spared from direct pressure effects), most studies agree that HTN is associated with impaired endothelial-dependent function but there has been divergence of opinion in relation to endothelial-independent function. Other factors (e.g. diabetes) strongly influence the response of the microvascular beds to HTN[43]. Ethnicity may be an important non-modifiable risk factor with South Asians tending to develop more severe

CAD at a younger age then other ethnic groups. However, there is conflicting evidence as to the impact of ethnicity per se on endothelial function (Table 1.3). Reduced capability of repair has been implicated[74]. Much of the excess CV risk in South Asians is explained by the increased prevalence of diabetes.

Table 1.2 Prospective studies of modifiable CAD risk factors on vascular endothelial and smooth muscle function in humans

Author/year	Design	Total participants	Indices of endothelial dysfunction and outcome
Diabetes			
Yaqoob 1993[30]	Cross-sectional Case control	78	53% of non-controls had elevated ED markers.
Makimattila 1996[31]	Cross-sectional Case control	27	Endothelium-dependent: independent blood flow ↓≈40% poor glycaemic control to normal subjects.
Johnstone 1993[32]	Cross-sectional Case control	31	Endothelium-dependent blood flow ↓ in diabetics ($p<0.01$).
Clarkson 1996[33]	Cross-sectional Case control	160	FMD significantly impaired in diabetics ($p<0.001$).
Caballero 1999[34]	Cross-sectional Case control	143	↓ Forearm iontophoresis of ACh ($p<0.001$) and ↑ ET-1, vWF, soluble ICAM and VCAM in diabetics and non-diabetics with diabetic first-degree relatives (all $p<0.05$).
Beer 2008[35]	Cross-sectional Case control	92	Iontophoretically applied ACh and SNP ↓ in diabetics compared with controls.
Williams 1996[36]	Cross-sectional Case control	44	Forearm iontophoresis with methacholine chloride and SNP ↓ in diabetics c/w controls ($p<0.005$).

Hypertension			
Panza 1990[37]	Cross-sectional Case control	36	Maximal forearm flow was higher in patient than controls ($p<0.0002$).
Spencer 2002[38]	Randomised substudy (ASCOT)	442	Patients with TOD had ↑ vWF ($P=0.002$). vWF independently predicted TOD ($p<0.05$).
Lip 1995[39]	Cross-sectional	151	Hypertensive patients had ↑ soluble P-selectin ($p=0.03$), vWF $p<0.01$) and fibrinogen ($p < 0.01$). DBP and vWF levels were significant predictors for fibrinogen levels ($p<0.05$).
Lip 2001[40]	Longitudinal Observational	102	Fibrinogen and vWF ↑in the malignant hypertension group>non-malignant hypertension group>normotensive group ($p < 0.001$). After mean BP reduction at 6-months both soluble P-selectin ($p<0.001$) and vWF ($p=0.0025$) were significantly reduced.
Felmeden 2003[41]	Longitudinal Observational	346	Plasma VEGF and vWF levels ↑ in hypertensive patients than controls (all $p<0.001$). VEGF and vWF levels correlated significantly with age, systolic and diastolic BP, 10-year CVD risk, and CVA risk scores (all $p<0.01$).
Taddei 2001[42]	Cross-sectional Case control	96	Vasodilation to ACh and SNP ↓ in essential hypertension compared with normotensive controls ($p<0.01$).
Shantsila 2011[43]	Cross-sectional Case control	95	Malignant hypertension patients ↓ endothelium dependent and independent micro- and macrovascular function than patients with hypertension and healthy controls.
Smoking			

Schmidt-Lucke 2005[44]	Cross-sectional	44	ACh mediated dilatation ↓ in smokers c/w controls (p<0.05)
Harmer 2014[45]	Randomised Substudy (FIELD)	193	Dilator responses to glyceryl trinitrate were ↓ in past and present cigarette smokers (p=0.005).
Newby 2001[46]	Cross-sectional	25	Current cigarette smoking was associated with ↓ coronary release of active t-PA (p<0.05).
Zeiher 1995[47]	Cross-sectional	96	Coronary FMD ↓ in smokers compared with non-smokers (p<.0001). Ratio of coronary FMD to nitroglycerine-induced dilation ↓ in smokers (p<0.001). FMD absent in smokers with CAD. Angiographic CAD (p<0.0001) and smoking (p<0.001) independently associated with reduced coronary dilation.
Johnson 2010[48]	Randomised	1504	1-year FMD increased by 1% in quitters (p=0.005), but did not change in those who continued smoking (p=0.643). Improved FMD among quitters remained significant on multivariable analysis (p=0.010).
Celermajer 1993[87]	Cross-sectional Case control	200	FMD was impaired or absent in smokers (p<0.0001). FMD in smokers inversely related to lifetime dose smoked (p<0.01).
Dyslipidaemia			
Vlahos 2014[50]	Cross-sectional Case control	60	HFH children exhibited ↓ FMD c/w controls (p<0.004).
Gilligan 1994[51]	Cross-sectional Case control	24	Maximal ACh induced flow ↓ in hypercholesterolaemics c/w controls (p=0.002).

Creager 1990[52]	Cross-sectional Case control	24	Maximal methacholine induced flow ↓ in hypercholesterolaemic subjects c/w controls ($p<0.05$).
Sorenson 1994[53]	Cross-sectional Case control	60	FMD impaired or absent in hypercholesterolaemic children c/w controls ($p<0.0001$). TC inversely correlated with FMD ($r= -0.61$, $p<0.0001$). In hypercholesterolaemic children, FMD inversely related to Lp(a) ($r= -0.61$, $P=0.027$). GTN-induced dilation present in all subjects but ↓ in hypercholesterolaemia ($p=0.023$).
Central obesity			
Al- Suwaidi 2001[54]	Cross-sectional Case control	397	% increase in coronary blood flow to ACh ↓ in obese patients c/w controls ($p=0.009$). Overweight (OR 1.55; 95% CI 1.2–2.0) and obesity (OR 2.41; 95% CI 1.5–4.0) status independently associated with impaired coronary endothelial function.
Francischetti 2011[55]	Cross-sectional	75	Structural and functional alterations in skin microcirculation are proportional to the increase in global and central obesity. Obese subjects with metabolic syndrome had limited functional capillary reserve.
De Ciuceis 2011[56]	Longitudinal Observational	53	Obesity independently predictive of ↑ media: lumen ratio and media cross-sectional area, together with impaired endothelial-dependent vasodilatation. After bariatric surgery and consistent weight loss microvascular structure and oxidative stress/inflammatory markers improved significantly ($p<0.05$).
Exercise			
Lee 2006[57]	Randomised	81	Patients completing cardiac rehabilitation ↓ levels of vWF, fibrinogen and D-dimer. FMD ↑ (all $p\leqslant0.001$). Levels unchanged in controls. Mean 24-hour systolic, diastolic and aortic BPs also reduced (all $p<0.05$).

Lewis 1999[58]	Longitudinal Observational	9	Endothelium-derived NO increased after 4 weeks cycle training.
Psychosocial factors			
Harris 2003[59]	Longitudinal	193	In women not using HRT, psychosocial factors (type A/anger) related to impaired FMD (p=0.03).
Do 2010[60]	Population survey	434	In age- and sex-adjusted models, hopelessness linearly correlated with soluble ICAM1 but not e-or p-selectin.
Fruit and vegetable consumption			
McCall 2009[61]	Randomised	112	A 1-portion increase F+V consumption, led to 6.2% improvement in forearm blood flow responses to intra-arterial ACh (p=0.03).
Plotnick 2003[62]	Randomised	38	4 weeks of powdered F+V and anti-oxidant supplement improved brachial artery FMD ($p<0.05$).
Ali 2011[63]	Randomised Cross-over	64	No significant difference in endothelial function with daily F+V supplement versus placebo for 8 weeks.
Regular alcohol intake			
Djousse 1999[64]	Longitudinal Cross-over	13	No benefit of red wine on endothelial function assessed by FMD.
Agewall 2000[65]	Randomised	12	FMD was significantly higher after drinking de-alcoholised red wine than after drinking red wine ($p < 0.05$)

ACE angiotensin converting enzyme, ACh acetylcholine, BP blood pressure, CAD coronary artery disease, CI confidence interval, CVA cerebrovascular atherosclerosis, CVD cardiovascular disease, c/w compared with, DBP diastolic blood pressure, ED endothelial dysfunction, ET-1 endothelin-1, FMD flow mediated dilation, F+V fruit and vegetable, GTN glyceryl trinitrate, HFH Heterozygous Familial Hypercholesterolaemia, HRT hormone replacement therapy, ICAM, intercellular adhesion molecule, Lp(a) lipoprotein (a), NO nitric oxide, OR odds ratio, SNP sodium nitroprusside, TC total cholesterol, TOD target organ damage, t-PA tissue plasminogen activator, VCAM vascular cell adhesion molecule, VEGF vascular endothelial growth factor, vWF von Willebrand Factor.

Table 1.3 Prospective studies of non-modifiable CAD risk factors on vascular endothelial and smooth muscle function in humans

Author/year	Design	Total participants	Indices of endothelial dysfunction and outcome
Gender			
Skaug 2013[66]	Cross-sectional	4739	Endothelial function assessed by FMD ↑ in females across all age groups. At age 40–49, prevalence of ED was significantly higher (p=0.03) in men.
Juonala 2008[67]	Cross-sectional	2265	FMD values ↑ in women (p<0.0001), present after risk factor correction but attenuated after adjustment for baseline brachial artery diameter.
Hashimoto 1995[68]	Longitudinal	34	FMD ↑ during the follicular and luteal phases of menstruation corresponding with higher levels of endogenous oestrogen (p<0.01 compared to age-matched males).
Celermajer 1994[69]	Cross-sectional	238	In men, FMD preserved until ≤40 years but declined thereafter at 0.21%/year. In women, FMD was stable until the early 50s, then declined at 0.49%/year (p=0.002 compared with men).
Family history			
Schächinger 1999[70]	Cross-sectional	150	Coronary endothelial-dependent dilation ↓ in patients with near normal coronary arteries and a positive family history (p=0.03). Remained significant after multivariable analysis (p=0.008).
Clarkson 1997[71]	Cross-sectional Case-control	100	FMD impaired in the family history group (p<0.005).
Age			
Skaug 2013[66]	Cross-sectional	4739	FMD ↓ with ↑ age in both genders up to 70 years for men and 80 for women (p<0.001).

Celermajer 1994[69]	Cross-sectional	238	↓ FMD in older age on multivariable analysis (r= -0.34, p < 0.0001).
Donato 2007[72]	Cross-sectional	95	Brachial artery FMD ↓≈50% in older versus young men (p<0.01). Nitrotyrosine ↑ in ECs obtained from the brachial artery and antecubital veins of older men (p=0.01 and p<0.05). NF-κB also elevated in older men and correlated with nitrotyrosine (r=0.51, p<0.05 [n=16]).
Taddei 2001[42]	Cross-sectional Case control	96	Endothelium-dependent vasodilation declined with aging (p<0.01).
Ethnicity			
Houghton 2002[73]	Cross-sectional	66	CBF response to ACh after L-arginine ↑ in African Americans compared to whites (p=0.0016).
Murphy 2007[74]	Cross-sectional Case control	49	Circulating endothelial progenitor cells ↓ in South Asian c/w Caucasian men. Ethnicity was the strongest predictor of EPC count (B=0.65, p=0.001)
Loehr 2004[75]	Cross-sectional Case control	1627	Adjusted absolute and % change in brachial artery diameter ↓ in African American c/w Caucasian (p<0.0001 and p=0.0002, respectively).
McCrohon 2000[76]	Cross-sectional Case control	40	On multivariate analysis, postmenopausal status was associated with impaired FMD in Caucasian, but not Chinese, females (p<0.002).
Jaumdally 2007[77]	Cross-sectional	87	No difference in the indices of angiogenesis, platelet activation, and ED between South Asians or White Europeans.

Makin 2003[78]	Cross-sectional	284	No difference in vWF, P-selectin, TF, or fibrinogen in South Asians with PAD compared to White Europeans.
Lip 1997[79]	Cross-sectional	225	No difference in vWF between black, white or South Asian hypertensive patients.
Gunarathne 2009[80]	Cross-sectional	293	In a multivariable analysis of arterial stiffness and endothelial function measurements in South Asian stroke survivors, South Asian ethnicity was an independent predictor.

ACh acetylcholine, ANOVA analysis of variance, CBF coronary blood flow, ECs endothelial cells, ED endothelial dysfunction, EPCs endothelial progenitor cells, FMD flow mediated dilation, GTN glyceryl trinitrate, NF-κB nuclear factor kappa B, PAD peripheral arterial disease, SD standard deviation, TF tissue factor, vWF von Willebrand Factor.

1.2.5 Non-conventional cardiac risk factors

A number of serological markers of arterial vulnerability are currently recognised[88]. Most reflect endothelial injury but do have a causal relationship with CAD. Possibly with the exception of hsCRP[89] and homocysteine[90], most current cardiovascular biomarkers add little primary predictive value to traditional risk factors[91].

1.2.6 Atherosclerotic plaque formation and morphology

1.2.6.1 A brief history

The first reports of 'hardening of the arteries' were recorded as early as the sixteenth century[92]. In the mid to late eighteenth century, William Heberden was among the first of a group of English physicians to describe 'a disorder of the breast' (anginal chest pain)[93]. He and colleagues Edward Jenner and Caleb Hillier Parry also described the process of calcification of the coronary arteries[94]. Sir Marc Ruffer was able to identify in 1911 degenerative arterial changes suggestive of atherosclerosis in the left subclavian artery from an Egyptian mummy[95]. His findings were later confirmed with more modern twentieth century tissue fixation methods[96]. Perhaps the most influential pathological insights were from Rudolph Virchow who, in 1856, described three processes he believed encouraged thrombogenesis[97] – abnormal blood constituents (haemostatic and fibrinolytic pathways), abnormal blood flow (viscosity and shear forces [rheology]) and abnormalities of the blood vessel wall (endothelial damage/dysfunction)[98]. This was later expanded by Druguid to include a role for platelets[99]. With the benefit of previous work, we now recognise the importance of the endothelium in regulating several physiological systems such that endothelial injury – leading to dysfunction – may disrupt sufficient cellular processes to facilitate fulfilment of the triad.

1.2.6.2 Response to injury

In the latter half of the twentieth century considerable advances in our understanding of atherogenesis have come from work by Russell Ross who published the 'response to (endothelial) injury' theory in a series of papers throughout the nineteen seventies[100, 101]. He outlined three main events preceding the development of atherosclerotic lesions; intimal proliferation of smooth muscle cells, formation by these cells of large amounts of connective tissue matrix including collagen, elastic fibre proteins and proteoglycans, and deposition of intra- and extracellular lipid which can contribute to a necrotic core in more advanced lesions[101]. The initial intimal event is caused by some form endothelial injury of which there are numerous possibilities ranging from traditional CAD risk factors (described previously) to less conventional mechanisms such as immunologic injury or infections. If the injuring stimulus persists, ECs undergo structural and functional changes which lead to exposure of the underlying collagen and platelet adherence and aggregation. Release of platelet factors and plasma constituents stimulates both medial and intimal smooth muscle cell proliferation[101]. Later versions of the theory emphasise endothelial dysfunction rather than denudation, implying potential reversibility and plaque regression under favourable circumstances. There is a balance of endothelial injury and repair – a cycle that may persist for many years – leading to progressive smooth muscle proliferation. In any case, the different forms injury increase the adhesiveness, coagulability and permeability of the endothelium[102].

Although a systemic disease, atherosclerosis is a characteristic response of particular arteries occurring most commonly in the aortic, coronary, cerebral, femoral and iliac arteries[100]. It affects different organ systems in different patients. Coronary atherosclerosis is the principal cause of coronary artery disease, in which atherosclerosis,

driven by underlying systemic inflammation, is present in the walls of the coronary arteries[103]. Amongst patients with comparable vascular risk factors, there is considerable lesion diversity.

1.2.6.3 Atherosclerotic plaque progression

Atherosclerosis is essentially a continuous process (Figure 1.3) however Stary et al[104], expanding on Ross' theory, outlined six distinct morphological stages of atheroma formation supported by the American Heart Association's committee on vascular lesions (Figure 1.4). They are classified according to their histological composition and structure. Within this process a pre-lesional sometimes reversible phase (types 1-3) occurring in the first 3 decades of life and a phase of progressive plaque growth (types 4-6) can be distinguished[105].

The principal morphologies and sequences can be considered to be a bi-directional continuum consisting of a multitude of pathogenetic developments, including macrophage foam cell formation and death, accumulation of extracellular lipid, displacement and reduction of structural intercellular matrix and smooth muscle cells, generation of mineral deposits, chronic inflammation, neovascularization, disruptions of the lesion surface, and formation and transformation of hematoma and thrombus to fibromuscular tissue[106]. These processes are facilitated by a complex network of cytokines, pro-inflammatory and anti-inflammatory mediators (Tables 1.4-1.6[107, 108]).

Any one of these processes may dominate (or be lacking) during lesion development. Some may continue throughout whilst others appear at various stages. Towards the latter stages of lesion progression, many of the processes run synchronously[106].

Figure 1.3

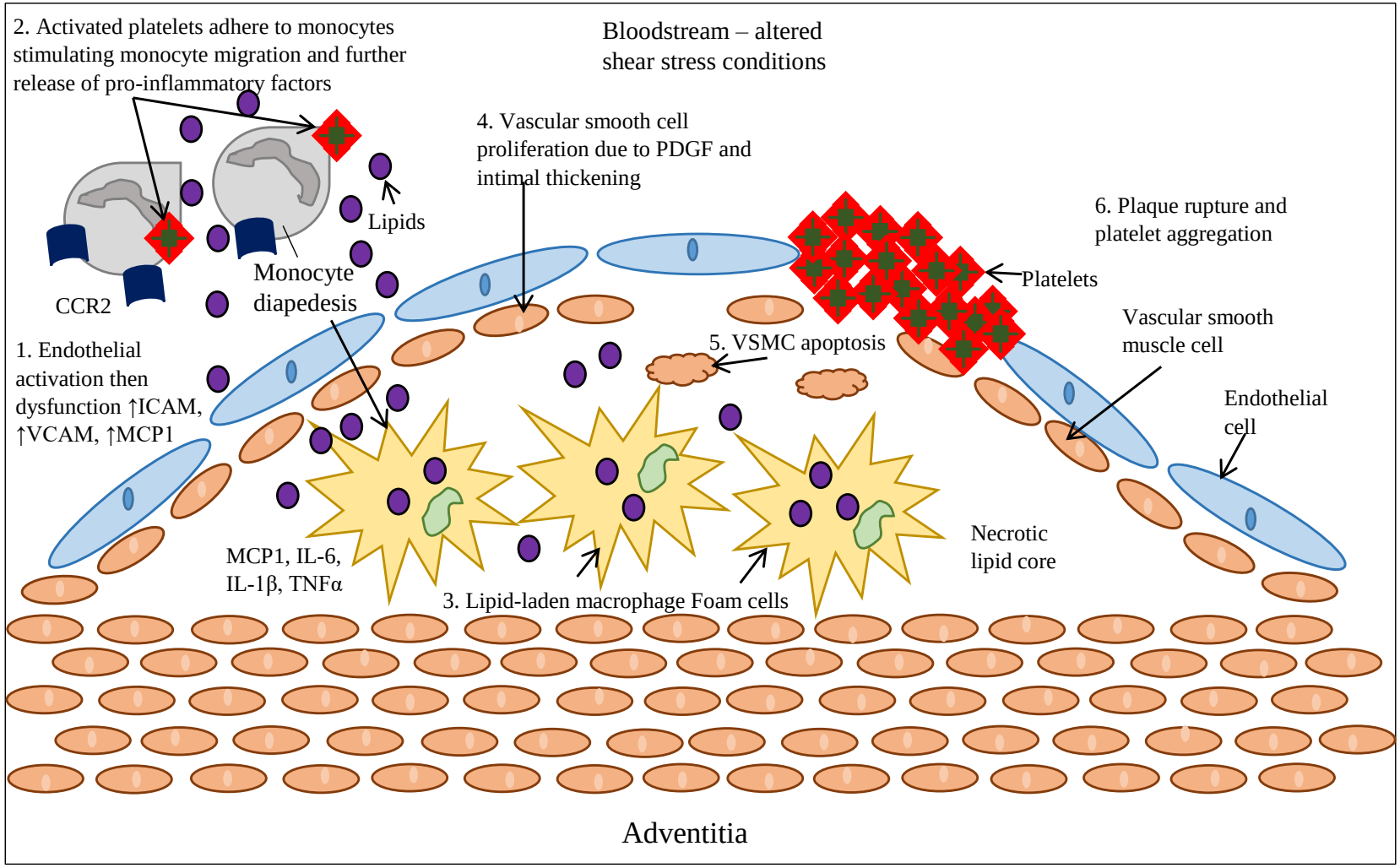

2. Activated platelets adhere to monocytes stimulating monocyte migration and further release of pro-inflammatory factors
Bloodstream – altered shear stress conditions
4. Vascular smooth cell proliferation due to PDGF and intimal thickening
6. Plaque rupture and platelet aggregation
Lipids
Monocyte diapedesis
CCR2
Platelets
Vascular smooth muscle cell
1. Endothelial activation then dysfunction ↑ICAM, ↑VCAM, ↑MCP1
5. VSMC apoptosis
Endothelial cell
MCP1, IL-6, IL-1β, TNFα
3. Lipid-laden macrophage Foam cells
Necrotic lipid core
Adventitia

Figure 1.3 Atherogenesis and the unstable atherosclerotic plaque

1. EC activation and dysfunction causes increased vascular permeability. Surface adhesion molecules (e.g. ICAM-1 and VCAM-1) adhere to ECs. 2. Attachment and infiltration of lipid and inflammatory cells into the subendothelial space. 3. Monocyte differentiation into macrophages, engulfing lipid to form foam cells and releasing pro-inflammatory cytokines (e.g. MCP1, IL-6, IL-1β) creating an inflammatory environment. 4. VSMCs proliferate in response to mitogens such as PDGF and synthesise collagen to form the fibrous cap that encloses the growing lipid core. 5. VSMC apoptosis, in part mediated by engagement of death receptors on VSMCs with death ligands on macrophages and T lymphocytes, and increased activity of matrix-degrading enzymes in the cap. 6. plaque rupture, with subsequent platelet attachment and thrombosis. (ICAM intercellular adhesion molecule, IL interleukin, MCP monocyte chemoattractant protein, PDGF platelet derived growth factor, VCAM- vascular cell adhesion molecule, VSMC vascular smooth muscle cell).

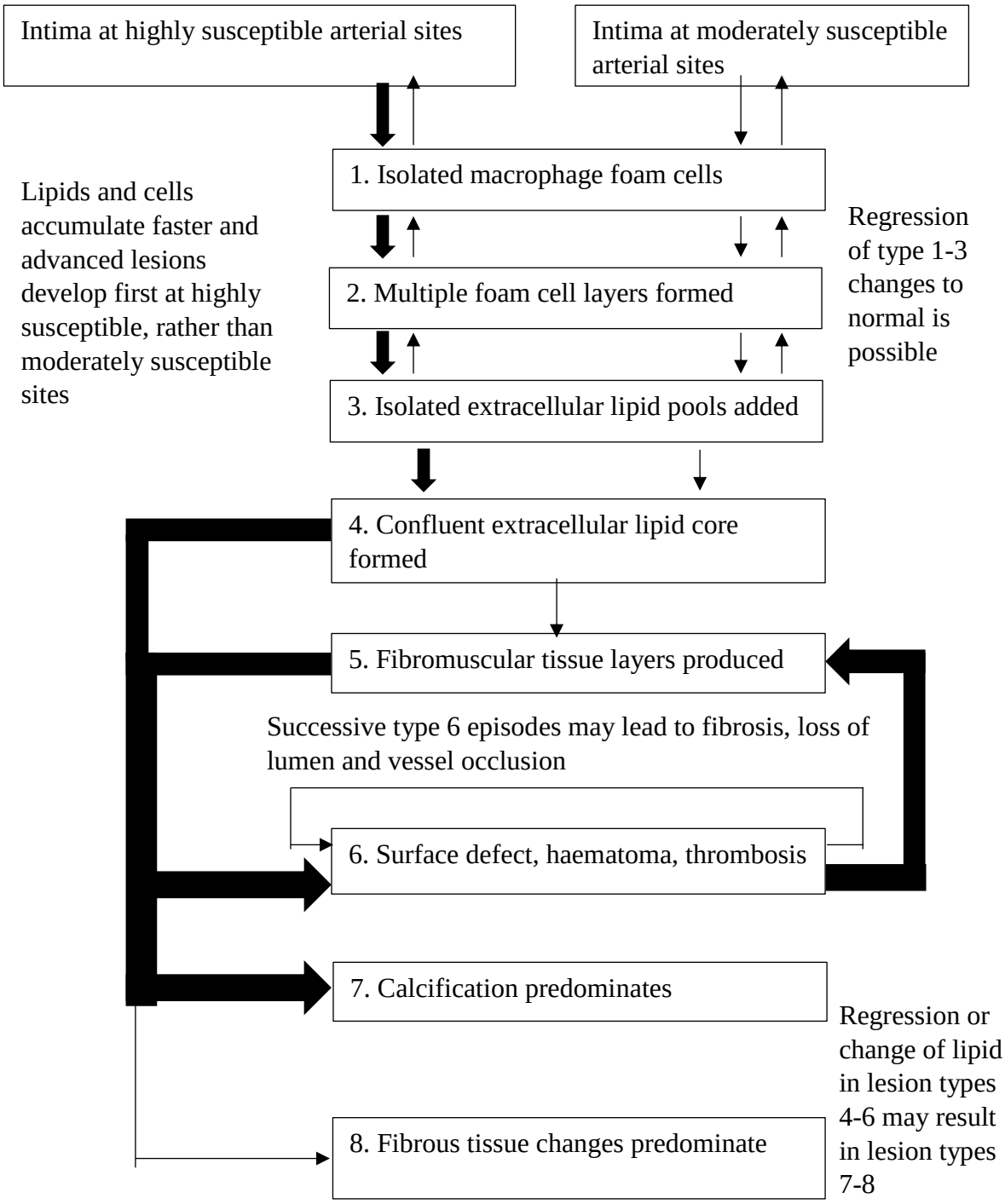


Figure 1.4 Progression of atherosclerosis. Adapted with permission, from Stary et al. Natural History and Histological Classification of Atherosclerotic Lesions. ATBV 2000;20:1177-1178.

The evolution of atherosclerotic lesions from type 1 to type 4 and of the possible subsequent pathways of progression to lesion types beyond type 4. The main histological characteristics of each step (lesion type) are listed.

Thick or thin arrows denote the relative ease with which lesions develop at specific sites, or they indicate the relative frequency and importance of a pathway.

Table 1.4 Inflammatory regulating enzymes and pro-inflammatory mediators in atherosclerosis

Enzyme	**Function**
5-lipoxygenase (5-LO)	-Produces leukotrienes and inflammatory lipid mediators.
12/15-lipoxygenase (12/15-LO)	-Facilitates lipid peroxidation and affects the adaptive immune response.
Heme Oxygenase -1 (HO-1)	-Reduces monocyte chemotaxis in response to LDL oxidation. -Facilitates antioxidant-dependent protection from atherosclerosis.
Paraoxonases	Hydrolyses oxidised lipids in LDL.
Pro-inflammatory mediators	
Oxidised low-density lipoprotein (OxLDL)	-Induces of foam cell formation. -Alters nitric oxide signalling. -Initiates endothelial activation. -Expresses adhesion molecules that accelerate leukocyte homing to the site of atherosclerosis.
C-Reactive Protein (CRP)	-Stimulates the production of IL-1α, IL-1β, IL-6, CXCL1, and CXCL8 by human monocytes in vitro. -Upregulation of liver X receptor-α. -Binds to minimally modified LDL preventing the formation of foam cells from macrophages.
Advanced Glycation End Products (AGE)	-Elicits oxidative stress. -Increases production of inflammatory cytokines and tissue factor. -Elevates expression of adhesion molecules.
Reactive Oxygen Species (ROS)	Activates SMC mitogenic signalling pathways.

	Amplifies platelet recruitment.
Toll-Like Receptors (TLRs)	-Induces production of pro-inflammatory cytokines and nitric oxide in macrophages. -Induces dendritic cell maturation. -Upregulates co-stimulatory molecules such as CD80 and CD86.

LDL low density lipoprotein, IL interleukin, CXCL chemokine (C-X-C motif) ligand, SMC smooth muscle cell, CD cluster differentiation.

Table 1.5 Cytokines in atherosclerosis

Cytokine	Cell Source	Function
IL-1α	Macrophages, lymphocytes, EC, SMC	-Pro-inflammatory, stimulates endothelial and SMC activation.
IL-2	Activated T cells	-T-cell growth factor, stimulates NK activity and Treg cells.
IL-4	Th2 cells, mast cells	-Proliferation and differentiation of B cells (Ig switching to IgG_1 and IgE) and Th2 cells (anti-inflammatory by inhibiting Th1 immune responses). -Stimulates VCAM-1.
IL-5	T cells, mast cells, EC	-Stimulates growth and differentiation of B cell antibodies.
IL-6	Macrophages, EC, SMC, T cells	-Differentiates myeloid cells. -Induces acute phase proteins and SMC proliferation.
IL-8	Monocytes, EC, T cells	-Pro-inflammatory, promotes leukocyte arrest.
IL-9	Th2 cells	-Promotes proliferation and differentiation of mast cells. -Stimulates IgE production. -Inhibits monocyte activation. -Stimulates TGF-β in monocytes.
IL-10	Macrophages, Th2, Treg and B cells, mast cells	-Anti-inflammatory, inhibits Th1 responses and promotes proliferation and differentiation of regulatory T cells.
IL-12	Th1 cells	-Pro-inflammatory, promotes NK and cytotoxic lymphocyte activity and induces IFN-γ.
IL-18	Macrophages	-Pro-inflammatory, induces IFN-γ and other Th1 cytokines. -Promotes Th1 development and NK activity.
IL-33	Th1 cells	-May neutralize harmful oxLDL.

		-Reduces IFN-γ production and other pro-inflammatory cytokines.
M-CSF	Macrophages, EC, lymphocytes	-Growth and differentiation of macrophages
TNF-α	Macrophages, T & B cells, NK cells, SMC	-Pro-inflammatory, fever, neutrophil activation, bone resorption, anticoagulant, tumour necrosis
TGF-β	Platelets, macrophages, Th3, Treg & B cells, SMC	-Anti-inflammatory and pro-fibrotic, promotes wound healing, angiogenesis and suppresses Th1 & Th2 immune responses.
IFN-γ	Th1 cells, NK cells, SMC	-Pro-inflammatory, promotes Th1 immune responses/secretion of Th1-associated cytokines. -Inhibits extracellular matrix synthesis by SMC.
CD40L	Platelets, T cells, NK cells, EC, SMC	-Pro-inflammatory, promotes Th1 immune responses/secretion of Th1-associated cytokines. -Stimulates MMP secretion.

EC endothelial cell, Ig immunoglobulin, IFN-ɣ interferon-gamma, IL interleukin, MMP matrix metalloproteinases, NK natural killer, OxLDL oxidised low-density lipoprotein, SMC smooth muscle cell, TGF transforming growth factors, Th T helper, VCAM vascular cell adhesion molecule.

Table 1.6 Chemokines in atherosclerosis

Chemokine	Function
CCL2	-Recruits monocytes from bone marrow into the arterial wall.
CCL5 and CXCL4	-Monocyte and T cell recruitment into the vessel wall. -Activates ECs by inducing the expression of E-selectin, NF-κB activation, and enhanced binding of oxLDL to ECs.
CXCL8	-Proliferation and migration of SMCs and ECs and affects neovascularisation.
Migration inhibitory factor (MIF)	-Inhibits monocyte migration. -Regulates lipid deposition, protease expression, and intimal thickening.

CCL2 chemokine (C-C motif) ligand 2, CXCL (C-X-C motif) ligand, EC endothelial cell, NF-κB nuclear factor kappa B, oxLDL oxidised low-density lipoprotein, SMC smooth muscle cell.

In stage 4 lesions, the cap constitutes pre-existing intima, which at susceptible sites undergoes adaptive thickening; however, the composition is like that of the normal intima. Most people with stage 4 atheroma will not have obstructive CAD because of the vessel's ability to remodel outwards[109]. Progression through these stages is not uniform and preferred sequences exist, for example, a lesion may become increasingly obstructive by repeatedly passing between stages five and six. Up to 61% of stable lesions without thrombi contain evidence of healed plaques (i.e. repeated plaque ruptures) at post-mortem[110]. Haematoma and/or thrombus formed during these repeated ruptures that do not produce clinical events are transformed into fibromuscular tissue, thus increasing cap size.

Monocyte-derived macrophages play a pivotal role in the progression of atherosclerosis. They are also implicated in the genesis of new vessels (angiogenesis) brought about by local hypoxia within the plaque as it grows[111, 112]. The microcirculation within the plaque increases transfer of leucocytes which may further facilitates plaque growth. This is supported by evidence from murine models where inhibition of angiogenesis has been shown to limit lesion expansion[113].

1.2.7 Platelets

Increasingly platelets are being recognised as important contributors to inflammation and both innate and adaptive immune responses. Activated platelets interact with all types of leucocytes, particularly monocytes, leading to up-regulation of a wide range of pro-inflammatory functions such as release of pro-inflammatory cytokines (Table 1.5), ROS production and endothelial adhesion (Figure 1.3). This is mediated through intracellular

compartments containing α-granules, lysosomes and dense core granules as well as a complex membranous system allowing storage and release of the various factors[114].

1.2.8 Genetic regulators

Patients in whom risk factors are present do not always develop CAD. Similarly, patients with relatively little background risk may develop severe CAD. Despite a significant inheritable component to CAD (~50%), technological limitations have meant the isolation of specific genes has not been forthcoming[115]. Following the advent of genome wide association studies (GWAS), our understanding of different patterns of gene expression has increased exponentially, leading to the identification of more than 50 CAD loci with genome wide significance. Approximately 70% of these genetic variants have no obvious mechanism (e.g. 9p21) whilst the remainder increase CAD risk via their influence on conventional risk factors (e.g. PCSK9). Trials of PCSK9 inhibitors have shown them to be effective in lowering LDL cholesterol[116] and reducing cardiovascular events[117].

Contemporary studies have revealed microRNAs (negative regulators of gene expression acting at the post-transcriptional level), that are involved in SMC regulation and further identified several target genes which are implicated in SMC pathophysiology. Paracrine microRNA regulated crosstalk between endothelial and SMCs has also been demonstrated, revealing a mechanism through which vascular cells communicate. Moreover, altering microRNA expression levels can prevent and even reverse the acquisition of SMC synthetic phenotype in vivo and reduce neointimal formation, thereby implicating miRNAs as therapeutic targets for vascular proliferative disease[118, 119].

1.2.9 Summary

Under normal homeostatic conditions the endothelium maintains vascular tone and blood fluidity with minimal expression of pro-inflammatory factors. CAD is a chronic multistage inflammatory disease beginning in the second and third decade of life. Progression depends on the balance of endothelial activation, injury and repair. The principal acute complication (and main cause of mortality) is myocardial infarction (MI) usually occurring because of plaque rupture or erosion followed by superimposed thrombosis. The influence of known risk factors and/or recently discovered genetic variants on EC biology in can increase lifetime CAD risk. Nonetheless, neither the severity nor the spatial heterogeneity of CAD are fully explained by conventional or novel risk factors and the mechanisms linking many of the newer genetic variants with CAD susceptibility are yet to be elucidated.

1.3 ADVANCED DIFFUSE CORONARY ARTERY DISEASE

Since the endothelium is equally exposed to injury stimulants such as lipoproteins, the distribution and morphology of CAD is challenging to explain in mechanistic terms. The complex patterns of disease and the fact that molecular genetic investigations have not yet revealed specific genetic variants for either CAD or AMI, suggest alternative mechanisms are involved. The following paragraphs review the epidemiology, pathogenesis and prognosis relating to diffuse, obstructive (advanced) CAD.

To gather as much relevant information as possible, on a subject that lacks a standard definition, I performed multiple searches of electronic bibliographic databases (i.e. MEDLINE, EMBASE and the Cochrane Database) hand searching references from included articles and abstracts from congress meetings. For the sections relating to definition, prevalence, pathogenesis and prognosis I used interchangeable phrases such as 'refractory angina', 'end stage coronary artery disease' and 'no option for revascularisation' – with the caveats that refractory angina does not always equate to advanced CAD and that people may be unsuitable for revascularisation for other reasons, therefore I undertook careful review of the angiographic criteria used in such studies.

Regards cardiac risk factors, pathogenesis, arterial stiffness, monocytes, monocyte-platelet aggregates and circulating microparticles I performed separate searches using the relevant headings in relation to coronary artery disease applying adjectives such as 'severe', 'diffuse', three-vessel' or multivessel'. Bibliographies of all selected articles and review articles were reviewed for other relevant articles.

1.3.1 Definition and prevalence

There is no single definition of diffuse CAD per se. Moreover, a number of phrases are used interchangeably to represent an expanding group of patients unsuitable for conventional revascularisation, or patients in whom therapeutic options have been exhausted[8]. The European Society of Cardiology (ESC) has defined refractory angina as "a chronic condition (>3 months) characterized by the presence of angina caused by coronary insufficiency in the presence of CAD which cannot be controlled by a combination of medical therapy, angioplasty, and coronary bypass surgery"[120]. A flow limiting lesion can be considered diffuse in the presence of; a significant (usually ≥70%) stenosis longer than 20 mm or multiple significant stenoses in the same artery or significant narrowing involving the whole length of the coronary artery[121, 122]. In some studies diffuse CAD has been defined when 'more than two-thirds of the left or right coronary artery is affected by irregularities or stenoses'[123]. The ACC/AHA classification system (Table 1.7) uses eleven variables to stratify lesions into one of four categories (A [simplest], B1, B2 and C [most complex]) predictive of PCI success[121]. Type A lesions are, by definition, discrete (<10mm). Type C lesions are diffuse (>20mm). Type B lesions display various complexities in between types A and C.

A Swedish survey in 1994–1995 found that 9.6% of patients referred for coronary angiography with stable angina were not candidates for revascularization due to advanced CAD[120]. In the Minneapolis Heart Institute series of CAD patients, 6.7% were on optimal medical management and not candidates for revascularization ('no option' patients) and a further 9.3% were not candidates for revascularization but received additional medical therapy[124]. Among 5767 CAD patients covered by the Euro Heart Survey 21% were medically managed following coronary angiography[125]. The same group also reported

that 14% of a subset of 4409 patients was ineligible for traditional revascularization[126]. In one catheterisation laboratory population, the prevalence of advanced CAD (defined as unsuitable for revascularisation) is >15%. In the Mediators of Social Support (MOSS) study, the same authors found that 41% of patients with three vessel CAD were unsuitable for revascularisation[4]. A more recent analysis of 16,215 patients undergoing coronary angiography revealed that 5.9% of people with advanced (three vessel) CAD (1.3% overall) had anatomy that precluded standard revascularisation techniques[127].

Table 1.7 ACC/AHA intracoronary lesion classification[121]

Type	Description
A	<10mm, discrete, concentric readily accessible, <45-degree angle smooth contour, little or no calcification, less than totally occluded, not ostial, no major side branch involvement, absence of thrombus.
B1	One of the following characteristics: 10-20mm, eccentric, moderate tortuosity of proximal segment, irregular contour, presence of any thrombus grade, moderate or heavy calcification, total occlusion <3 months old, ostial lesion or bifurcation lesion requiring two guide wires.
B2	Two or more of the following characteristics: 10-20mm, eccentric, moderate tortuosity or proximal segment, irregular contour, presence of any thrombus grade, moderate or heavy calcification, total occlusion <3 months old, ostial lesion or bifurcation lesion requiring two guide wires.
C	>20 mm diffuse, excessive tortuosity of proximal segment, total occlusion >3 months old and/or bridging collaterals inability to protect major side branches, degenerated vein graft with friable lesions.

1.3.2 Coronary artery disease severity indexes

A variety of scoring systems quantifying angiographic CAD burden exist (Table 1.8[128-134]), many of which have been validated against IVUS and correlate well with each other[135].

In 1993 Bogaty et al published a report detailing topographically distinct patterns of CAD in patients suffering an index MI compared to patients diagnosed with stable angina without prior MI[136]. They devised a system to describe CAD severity, extent and pattern (The Bogaty score). The authors noted a discrete pattern in 54.5% of patients with unheralded infarction compared to 8.5% of those with uncomplicated angina ($p<0.001$). On the other hand, multivessel disease was found in 74.5% of subjects with uncomplicated angina, and 29.1% in patients with MI. These findings were subsequently reproduced by Cianflone et al[137]. It was proposed that different pathogenic processes led to some people developing diffuse (but stable) disease and others more focal disease prone to instability rather than the same pathophysiological process randomly producing different clinical presentations.

Table 1.8 Coronary angiography severity scores validated against IVUS

Author (Year)	**Score**
Gensini GG (1983)[129]	Gensini
Ringqvist I (1983)[130]	CASS-50
Ringqvist I (1983)[130]	CASS-70
Dash H (1977)[131]	Duke Jeopardy
Mark DB (1994)[132]	Duke CAD Severity Index
Friesinger GC (1970)[133]	Friesinger
Sullivan DR (1990)[134]	Sullivan vessel
Sullivan DR (1990)[134]	Sullivan stenosis
Sullivan DR (1990)[134]	Sullivan extent
Jenkins PJ (1978)[128]	Jenkins

CASS coronary artery surgery study.

1.3.3 The role of coronary risk factors in determining disease topography

In general angiographic CAD burden increases with the number of documented CAD risk factors[138]. The nine modifiable risk factors listed in Table 1.2 explain more than 90% of the population attributable risk of acute MI in both men and women[81]. However, certain risk factors have been associated with particular CAD phenotypes (Table 1.9[123, 139-151]). The 'smokers paradox' for example refers to the observation in a number of pre-PPCI (primary percutaneous coronary intervention) studies[142], that smokers have improved survival after reperfusion following MI. Insights from large trials of the era suggested this was, in part, due to the fact that smokers presenting with acute MI had less extensive CAD than non-smokers[139, 141]. This was explained by the prothrombotic effects of smoking leading to index events at a younger age when there are less associated comorbidities[152]. Indeed, when these cofactors are adjusted for the difference is attenuated – completely in some studies[142] – partially in others[153].

South Asians (SAs) have the highest rate of CAD amongst all populations. Epidemiological studies suggest that the likelihood of developing CAD in SAs is twice that of Europeans and five times higher than Chinese people, irrespective of their country of residence[154]. Moreover, SA men and women are susceptible to premature (<40 years or pre-menopause), accelerated CAD with three vessel disease found in half of all SAs and one-third of premenopausal women[155]. Tillin et al[148] reported longer, more severe CAD in the proximal LAD segments of South Asian men compared to white European men although no difference in overall coronary artery calcification (CAC) scores. In this small underpowered study, South Asians were also noted to have significantly smaller proximal LAD, carotid and femoral lumens than Europeans even after adjustment for age (although South Asians were younger in this study anyway) and body surface area (BSA).

Interestingly, the difference in proximal LAD lumen diameter was eradicated in men with more advanced CAD, raising the possibility of either a tendency toward more outward remodelling in South Asians, or that inward remodelling occurs earlier in the atherogenic process and is then attenuated (hypothesised by the authors). Alternatively, comparable CAC scores between the two may indicate ongoing susceptibility to plaque rupture events. In contrast, Koulaouzidis et al[149] found significantly more aggressive and diffuse calcification in South Asians compared to white Europeans ($p = 0.003$ for CAC 101-400, $p = 0.004$ for CAC 401-1000) but interestingly this was not the case in patients under fifty, despite established evidence that CAD affects South Asian people earlier than other ethnicities.

Peripheral artery disease (PAD) evidenced by an ankle-brachial index <0.9 is associated with more complex lesions (B2 and C) at angiography but this could be a bi-directional association. However, the observation that South Asians have comparatively little PAD despite higher rates of diffuse or multivessel CAD would suggest that mechanisms behind PAD may relate to CAD but not vice versa[156].

Fischer et al[123] retrospectively examined the inheritable patterns of CAD in families where multiple members had been diagnosed. They did not identify any particular genetic component to the overall diffuseness of CAD and certainly no consistent genetic contribution regards 'focal versus diffuse' but did note an increased likelihood of diffuse CAD in the left coronary artery as compared to the right. This is the largest study of this kind so the significance of that finding is uncertain although the authors also report increased likelihood of siblings developing other high risk angiographic patterns such as ostial, proximal and left main disease, ectasia or calcification at presentation, which tend to be associated with more advanced CAD.

In the Mediators of Social Support (MOSS) study age and diabetes were the only univariate predictors of patients with advanced CAD unsuitable for revascularisation. Other recorded risk factors such as hypertension, smoking and gender were similar when compared to patients with advanced disease but suitable for revascularisation[4]. In another study[124], the presence of chronic kidney disease (CKD) was also more likely in patients not suitable for revascularisation however the presence of CKD may preclude revascularisation independently of CAD morphology and there was no multivariate analysis to delineate the true association.

It is also interesting to note that in a dedicated refractory angina registry 33% had never smoked, 31% had no history of hypertension, 34% had no family history of CAD and 63% had no history of diabetes[8].

Table 1.9 Influence of selected CAD risk factors on angiographic CAD phenotype

Author/year	Design	Total participants	Study outcome
Smoking			
Angeja/2002[139]	RCT subgroup analysis	2573	Single-vessel and non-LAD disease ↑ in smokers (both p≤0.01).
Mueller/1992[140]	RCT subgroup analysis	3339	Never smokers more LAD infarcts (p<0.001). Current smokers less MVD (p=0.05).
Barbash/1995[141]	RCT subgroup analysis	41021	Non-smokers ↑ TVD c/w smokers (p=0.001). Smoking associated with improved survival (OR 1.25, 95% CI 1.11 to 1.39)
Grines/1995[142]	Pooled RCT data	1619	Smokers larger MLD and less MVD.
Diabetes			
Dortimer/1978[143]	Retrospective case control	116	Diabetics had similar number of diffusely diseased vessels as controls (p>0.05). CAD more extensive but not more diffuse or inoperable than non-diabetics.
Gui/2009[144]	Cross-sectional Case control	546	Diabetics more severe and diffuse CAD c/w controls (Chinese population).
Melidonis/1999[145]	Cross-sectional	673	More severe, multifocal and diffuse forms of CAD in diabetics.
Wilson/1983[146]	Case control	116	Diabetics had more severe CAD in the mid segments of epicardial arteries but no difference in the proximal or distal segments.

Mosseri/1998[147]	Retrospective Case control	35	Coronary arteries and their branches significantly smaller in diabetics.
Family history			
Fischer/2005[123]	Retrospective Case control	882	Diffuse CAD more heritable in LCA than RCA (p=0.03). No. of diseased vessels and lesion length did not show significant heritable components but proximal, ostial and LMS disease did.
Ethnicity			
Tillin/2008[148]	Prospective substudy. Case control	83	South Asian men had more proximal LAD disease and longer lesions than European men. No difference in overall CAC.
Koulaouzidis/2013[149]	Retrospective cohort	202	South Asians over 50 years more diffuse CAD, MVD and CAC.
Burdoff/2002[150]	Prospective non-randomised	782	African Americans and Hispanic patients less CAD burden and CAC than White patients.
Gender			
Kyriakidis/1995[151]	Prospective	735	More MVD and higher Gensini scores in men.

CAC coronary artery calcification, CAD coronary artery disease, CI confidence interval, LAD left anterior descending, LCA left coronary artery, LMS left main stem, MLD minimal luminal diameter, MVD multivessel disease, OR odds ratio, RCA right coronary artery, RCT randomised controlled trial, TVD three-vessel disease.

1.3.4 Pathogenic mechanisms

The absence of an objective definition of diffuse CAD and the evolutionary nature of atherogenesis make the precise pathophysiological mechanisms underlying its development more challenging to pinpoint. Genetic variations alter the expression of immuno-modulatory receptors and vascular endothelial cells which may adapt the mechanism outlined in Figure 1.4, causing some people to cycle more frequently between stages five and six of the atherogenic process or accelerate the calcification process. The following paragraphs detail current understanding of the pathophysiological situation in patients with different expressions of CAD.

1.3.4.1 Endothelial shear stress

Despite the global effect of injury stimulants on arteries, the topography of atherosclerotic CAD is highly variable between individuals. Part of the difference can be explained by differences in blood flow induced shear stress to which the endothelium is exposed – endothelial shear stress (ESS) – brought about by curvatures or branches in the arterial tree which cause disturbed flow patterns[157]. Shear stress is tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall and is the product of blood viscosity and shear rate at the artery wall. Blood tends to flow quicker at the wall then it does at the centre of the lumen. Shear rate is the gradient of flow speed from the middle towards the wall[158].

Different forms of shear stress exist according to the force per unit area on the blood vessel wall. Laminar shear stress (>12dyn/cm^2 without oscillation) and pulsatile shear stress (>12dyn/cm^2 with oscillation) are atheroprotective and both result in up-regulated eNOS activity with subsequent quiescent EC phenotype[159]. In contrast, oscillatory shear

stress (0-0.5dyn/cm^2 with oscillation) cause up-regulation of monocyte chemoattractant protein (MCP)-1 and adhesion molecules through sustained release of atherogenic flow sensitive micro RNAs[160]. Turbulent blood flow, such as that caused by irregular wall geometry (e.g. bifurcations), leads to low (oscillatory) ESS which is detected by mechanoreceptors on the EC membrane. Signals are transmitted throughout the EC cytoskeleton initiating a cascade of inflammatory, proliferative and apoptotic pathways – thereby potentiating processes that favour the development and progression of atherosclerosis (Figure 1.5).

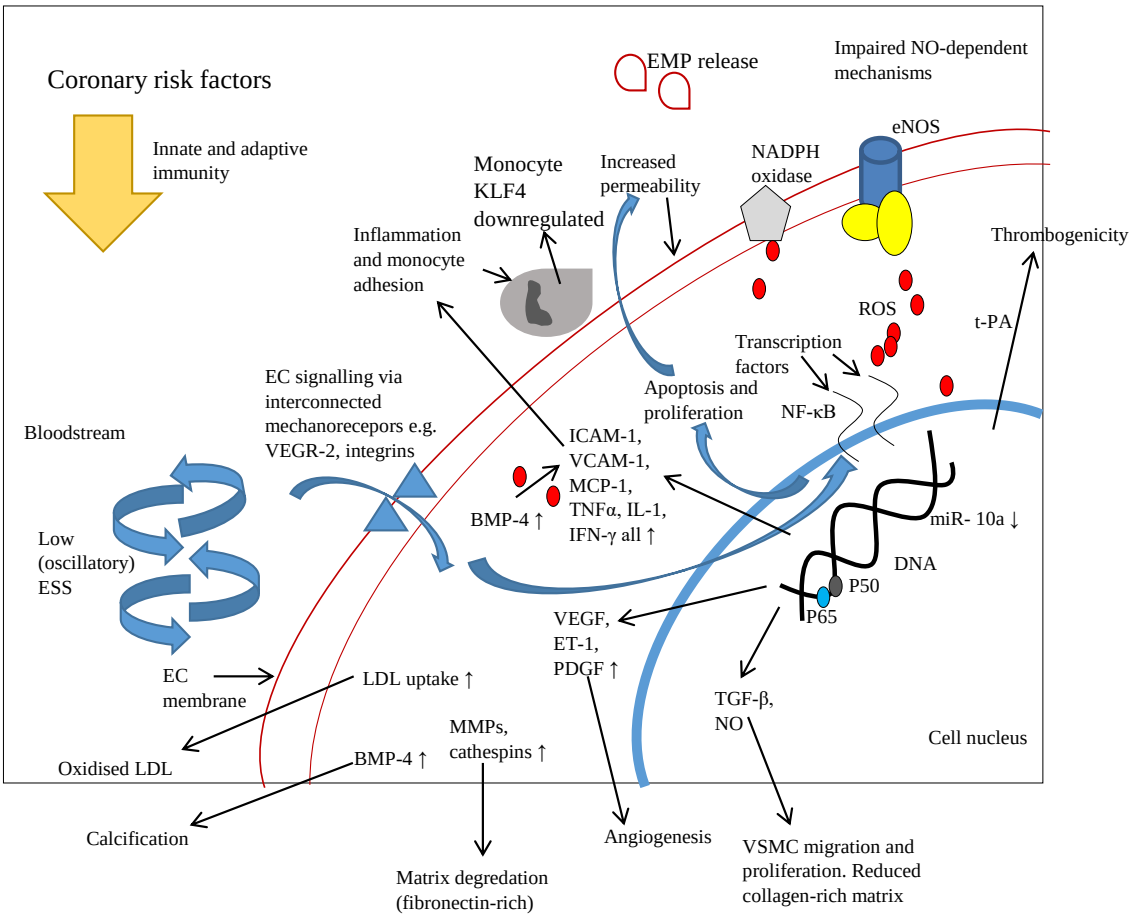


Figure 1.5 Pro-atherogenic pathways up-regulated by oscillatory shear stress.

Disturbed laminar flow causes low endothelial shear stress (ESS) which leads to endothelial activation, ultimately promoting atherogenesis, atherosclerotic plaque formation and progression, and vascular remodelling. BMP bone morphogenic protein; DNA deoxyribonucleic acid; EC endothelial cell; EMP endothelial microparticles, ET endothelin; ICAM intercellular adhesion molecule; IFN interferon; IL interleukin; KLF4 Krüppel-like factor 4, LDL low-density lipoprotein cholesterol; MCP monocyte chemoattractant protein; miR flow sensitive (mechano) micro RNA; MMP matrix metalloproteinase; NADPH nicotinamide adenine dinucleotide phosphate; NO nitric oxide; NOS nitric oxide synthase; NF nuclear factor; PDGF platelet-derived growth

factor; ROS reactive oxygen species (red circles); TGF transforming growth factor; TNF tumour necrosis factor; t-PA tissue plasminogen activator; VCAM vascular cell adhesion molecule; VEGF vascular endothelial growth factor; VSMC vascular smooth muscle cell.

The role of low ESS with a high oscillatory index in modifying EC function is fundamental to the development of atherosclerosis. On the other hand, very high ESS ($\geq$70dyn/cm^2) can produce endothelial damage, promote platelet deposition and may promote plaque rupture[161]. ESS undergoes continual modification in response to local surroundings and is influenced by the severity and duration of systemic risk factors, as well as arterial wall stiffness and remodelling[162]. Hence, plaque growth affects local ESS at the level of the plaque and immediately downstream which may cause a progressive cycle of atherosclerosis[163].

1.3.4.2 Vascular remodelling and ESS

Intracoronary imaging studies (Table 1.10[157, 163-169]) have improved our understanding of the different forms of vascular remodelling, their effect on ESS and their relationship to CAD progression and plaque stability. Vascular remodelling influences not only the degree of luminal encroachment at that site but also plaque composition. Large plaque burdens with low ESS independently predict progressive enlargement and luminal stenosis[163].

A lesion is more likely to become flow limiting (obscuring $\geq$70% of the lumen) if it undergoes constrictive remodelling (Figure 1.6) however in vivo intravascular studies have demonstrated considerable heterogeneity in the local haemodynamic environment within which each plaque is situated[158]. The majority of severe plaques exist in a physiological ESS, usually brought about by a combination of compensatory expansive and subsequent constrictive remodelling[164]. Once normal ESS is re-established, the pro-atherogenic molecular and cellular phenotype of ECs (shown in Figure 1.5) may be reversed. This could result in a severe but relatively focal stenosis – a Type A lesion.

Advanced lesions that were exposed to low ESS during their initial phases (influenced by the degree of inflammation) continue to experience low ESS, maintenance of pro-atherogenic EC phenotype and progression of CAD. Furthermore, Koskinas et al found that plaque-associated low ESS exacerbates atheromatous development in areas that already have a large plaque burden[164]. Thus, a severe (flow limiting) lesion associated with adjacent low ESS downstream could increase the diffuseness of that lesion.

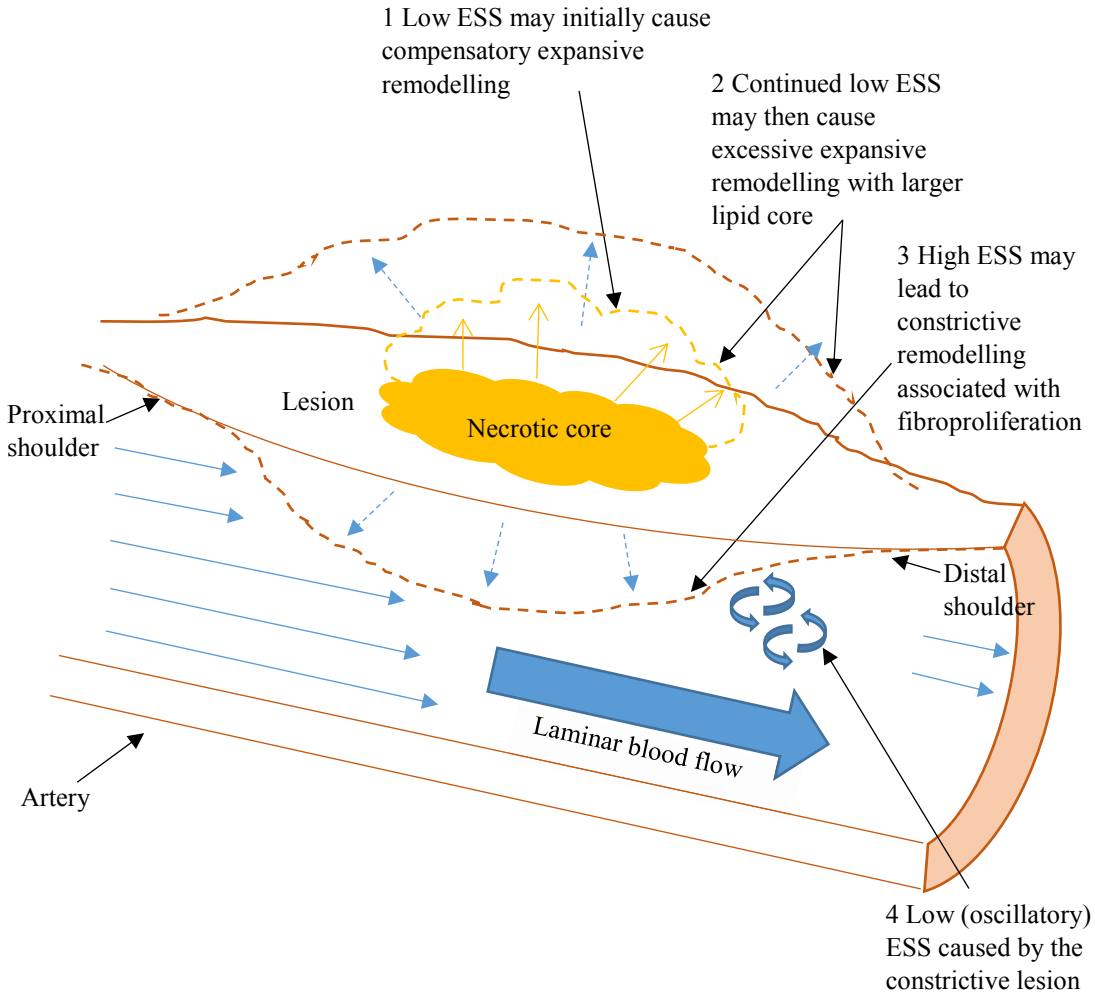


Figure 1.6 Types of vascular remodeling.

1. Low ESS may initially cause compensatory expansive remodelling. If ESS returns to physiological levels, then the lesion may remain quiescent. 2. If ESS remains low then excessive expansive remodelling may occur associated with larger plaque burden and thin capped fibroatheroma. 3. High ESS may lead to constrictive remodelling associated with fibroproliferation and luminal stenosis. 4. The lesion itself modifies ESS causing oscillatory flow patterns at the distal shoulder facilitating further lesion progression. Alternatively, if the constrictive lesion exists within physiological ESS, it may not progress.

Table 1.10 Natural history studies of endothelial shear stress and vascular remodelling in relation to coronary artery disease

Author/year	Design	Objective	Outcome/conclusions	Comment
Stone 2012[163]	Prospective longitudinal 506 patients PREDICTION study	Establish the vascular characteristics and local haemodynamic forces favouring coronary plaque progression and relate to clinical events.	-Baseline large plaque burden and low ESS predicted increase plaque area and decreased lumen area at FU.	-Significant change despite only 6-10 months gap. -ACS patients only, more inflammatory than stable patients.
Koskinas 2010[164]	Prospective longitudinal 15 pigs	Assess the role of ESS and VR in CAD.	-Most plaque caused compensatory expansive remodelling. -↓ ESS in areas of excessive expansile remodelling. -↓ ESS associated with plaque progression ($p<0.001$).	-Used IVUS. -Small numbers. -Diabetic and hyperlipidaemic state induced.
Asakura 1990[157]	Post-mortem Explanted human coronary arteries	Examination of mechanical factors involved in development of CAD	-Atherosclerosis more common at inner wall of curvatures and outer wall of bifurcations. -recirculated flow and ↓ ESS noted in these areas.	-Detailed exploration of flow patterns in all 3 coronaries. -Ex vivo.
Samady 2011[165]	Prospective longitudinal 20 patients	Assess the impact of ESS on CAD progression.	- ↓ ESS develop ↑ plaque progression and constrictive remodelling ($p<0.001$)	-Stable CAD patients. -Small numbers.

				-Non-obstructive CAD. -Used VH-IVUS.
Stone 2003[166]	Pilot longitudinal 6 patients.	Assess ESS in prediction of progression of CAD and ISR.	-↓ ESS showed ↑ in plaque thickness and outward remodelling at FU.	-IVUS used. -Non-obstructive (≤50%) disease in native vessel and IMT in stented segment. -short (6 months) FU.
Stone 2007[167]	Pilot longitudinal 13 patients	Investigate effects of different ESS environments on plaque and wall characteristics at 6 months.	-Remodelling responses to ↓ ESS are highly variable. -Plaque progresses in ↓ ESS areas.	-IVUS used. -Stable CAD. -Non-obstructive (≤50%) disease.
Vergallo 2014[168]	Retrospective cross section 21 patients	Assess relationship between local ESS and coronary plaque characteristics.	-↓ ESS had ↑ lipid-rich plaques (p=0.019), ↑ thinner fibrous cap (p=0.004), and ↑TCFA (p=0.037) -↓ ESS had ↑ cross-sections with lipid plaque (p=0.021) and ↑ macrophage density within the fibrous cap (p=0.017). -Coronary calcium not significantly different (p=0.24).	-Used OCT -ACS patients -96 patients excluded.

Papafaklis 2015[169]	Retrospective analysis of prospective data 374 patients from PREDICTION study	Assess role of local ESS in de novo lesions and progressive plaque eccentricity.	-Eccentric plaque formation at FU in normal segments predicted by baseline ↓ ESS ($p<0.001$), ↑ ESS circumferential heterogeneity ($p=0.001$), and their interaction ($p=0.026$). -↓ ESS ($p=0.003$) and ↑ plaque burden ($p=0.002$) at baseline predicted ↑↑ plaque eccentricity index with ↑ stenosis at FU in diseased areas.	-Significant change despite only 6-10 months gap. -ACS patients only, more inflammatory than stable patients.

ACS acute coronary syndrome, CAD coronary artery disease, ESS endothelial shear stress, FU follow up, ISR in-stent restenosis, IMT intima media thickness, IVUS intravascular ultrasound, OCT optical coherence tomography, TCFA thin capped fibroatheroma, VH-IVUS virtual histology-intravascular ultrasound, VR vascular remodelling.

1.3.4.3 Arterial stiffness

Arterial stiffness (ArS) refers to the ability of an artery to expand and contract in response to pressure changes[170]. Compliance (volume change [ΔV] in response to a change in blood pressure [ΔV/ΔP]) and distensibility (compliance relative to initial volume [ΔV/ΔP × V]) are measures (sometimes used interchangeably) that relate to wall stiffness. Distensibility can be measured directly using magnetic resonance or computed tomography[171]. Alternative non-invasive measures of ArS include pulse wave velocity (PWV), central and peripheral pulse wave analysis, aortic augmentation index (AIx) and arterial elastance (Ea)[172-174].

Increases in ArS favour a state of endothelial activation/injury – leading to subsequent damage/dysfunction – by altering the exposure of endothelial and vascular smooth muscle cells to ESS[175]. Animal models have shown that coronary perfusion falls with decreased aortic compliance[176, 177]. The alteration in shear forces could induce endothelial inflammation as well as causing subendocardial ischaemia with subsequent rise in ROS and pro-inflammatory cytokines. Furthermore, the outer wall of the ascending aorta receives blood supply from the vasa-vasorum which is linked to coronary circulation therefore reduced coronary perfusion could create a perpetuating cycle of abnormal aortic elasticity and worsening CAD[178]. Arterial stiffness is a strong predictor of future cardiovascular events and all-cause mortality and has been shown in numerous, mainly cross sectional, human studies to correlate with the presence, and in some cases, the severity of CAD (Table 1.11[171, 178-192]). Similar results were reported in a longitudinal analysis which, albeit retrospectively, paralleled worsening CAD with a decline in aortic distensibility[171].

Table 1.11 Studies of measures of arterial stiffness in humans with stable coronary artery disease

Author/year	Design	No. of participants	Objective	Outcome	Comment
Stefanadis 1987[178]	Cross sectional Retrospective	42	Determine differences in aortic distensibility between patients with and without CAD.	-CAD patients had similar aortic pressures but markedly lower distensibility.	-Normal coronary arteries defined as 0% stenosis – minor plaque excluded. -Small sample. -No differentiation of severity of CAD. -Aortic stiffness measured as distensibility.
Oberoi 2012[171]	Retrospective Longitudinal	164	Use CTCA to examine the relationship between changes in aortic stiffness and coronary atherosclerotic burden.	-CAD burden (assessed by segmental involvement score) was inversely linearly correlated with ADI ($p<0.001$).	-Aortic stiffness measured as distensibility. -Potential bias from interpretation of both outcome measures by the same investigator. -Severe disease (people with CABG or previous PCI) excluded. -Highly select group of people having two CTCAs in a year without having

					disease requiring intervention – hence only 164 eligible out of 3161 patients screened.
Nigam 2003[179]	Cross sectional	32	Evaluate the relationship between endothelial function and conduit vessel distensibility in normal subjects, patients with stable CAD, and patients with risk factors but no CAD.	-Flow mediated dilatation significantly correlated with central pulse pressure (r= −0.457, p=0.019), central systolic pressure (r= −0.442, p=0.024), peripheral pulse pressure (r= −0.393, p=0.039), peripheral systolic pressure (r= −0.398, p=0.036), and proximal aortic compliance (r= 0.390, p=0.049). -No correlation between FMD and AIx or PWV.	-Small sample size. -Flow mediated dilatation for endothelial function (macrovascular endothelial function). -Males only. -PWV, aortic compliance and augmentation index used for arterial stiffness. -Groups not age matched. -Correction required for too many variables relative to sample size – could lead to type 1 error.
Stefanadis 1990[180]	Cross sectional	46	Compared invasive measures of aortic distensibility with measures obtained non-invasively, using 2D and M mode echocardiography.	-Showed that estimates of aortic distensibility could be obtained non-invasively with a high degree of accuracy (r= 0.949, p=0.0001)	-Near perfect linear relationship between echo and catheter measurements. -Under estimation of pulse pressure using peripheral BP due to over estimation

					of diastolic BP relative to systolic BP.
Ahmadi 2011[181]	Cross sectional	229 (26 for the substudy)	Compare measures of aortic distensibility index with severity of CAD measured by CTCA. A substudy comparing CT with echo measurements was also performed.	-Excellent correlation between CT and echo (r^2= 0.94, p<0.001). -Strong inverse correlation between ADI and coronary artery calcium (CAC) (r= -0.69, p=0.0001). -Severity of CAC increased in each Framingham risk category as ADI decreased.	-Patients with established CAD excluded. -The presence of CAC as an outcome measure suggests CAD is established anyway. -ADI independently predicted significant CAD. -Luminal stenosis difficult to quantify with CT.
Bogren 1989[182]	Cross sectional	100	Compare regional aortic compliance in patients with and without CAD.	-Patients with CAD or syndrome X ↓ or no measurable aortic compliance.	-Very uneven groups (70 normal controls, 10 CAD patients, 7 syndrome X and 13 athletes).
Elbasan 2013[183]	Cross sectional	376	Assess the relationship between aortic distensibility and the extent and complexity of atherosclerotic lesions assessed with SYNTAX score (SS) in patients with stable CAD.	-SS ↑ in patients with low AD c/w the high AD group (separated according to median value – 18.5 ± 10.2 vs. 8.3 ± 5.9, p<0.001). -AD independently associated with age (β= –0.104, p=0.019), hypertension (β= –	-AD measured using echocardiography. -Large sample size relative to other studies of a similar nature. -Distribution assessed with Kolmogorov-Smirnov.

				0.202, p<0.001) and SS (β= –0.457, p<0.001).	Shapiro-Wilk better for this sample size. -No control group.
Yildiz 2008[184]	Cross sectional	96	Investigate the association between elasticity indexes of the aorta (aortic distensibility [AD] and aortic strain [AS]) and severity of CAD in patients with stable CAD.	-AD (beta= -0.577, p=0.003) and AS (beta= -0.494, p=0.021) independently associated with Gensini score on multiple linear regression analysis.	-Used echocardiography to measure aortic elasticity indexes. -Used Gensini score for CAD severity. -Patients in the control group had no CAD confirmed with angiography.
Siegel 2013[185]	Cross sectional substudy of the ROMICAT trial	293	Determine the relationship of AD to the presence and morphological features in coronary and thoracic atherosclerosis.	-The associations of AD with different morphologies of CAD were entirely explained by age and not independently related.	-Defined non-calcified plaque as well as calcified plaque. -Used Hounsfield units >130 but no Agatston classification used. -Simultaneous acquisition of the AD measurements and coronary plaque morphology.

					-Extent of CAD not assessed.
Fazlioğlu 2009[186]	Cross sectional	108	Evaluate the association of arterial elasticity indexes, serum uric acid and the presence and extent of CAD in patients with chronic stable angina.	-Small artery elasticity index (SAEI) independently reflected the extent of CAD. -Serum uric acid associated with extent of CAD Serum uric acid independently associated with systemic vascular resistance and SAEI.	-CAD severity assessed with the Gensini score. -Applanation tonometry used to assess arterial elasticity. -Stable angina diagnosed with symptoms, no document of ischaemia. -No control group. -SAEI only 22% of the variance of Gensini score.
Syeda 2003[187]	Cross sectional	151	Analyse to what degree SAEI and LAEI can reflect atherosclerotic burden as assessed by angiography.	-SAEI independently predicts diffuse disease but LAEI does not.	-Patients with valve disease included. -Classified angiographic disease as diffuse, focal or no CAD, no scoring system used – eyeball visual estimate >15mm for diffuse.
Takeuchi 2004[188]	Cross sectional	380	Examined the influence of CAD risk factors on the association between SAEI in	-SAEI significantly ↓ in CAD patients than no CAD patients whereas the large artery	-Large numbers.

			patients with and without CAD.	elasticity index (LAEI) was similar in patients with and without CAD. -The association between SAEI and CAD in male hypertensive patients independent of conventional risk factors for CAD.	-Severity of CAD not quantified. -Similar findings to Syeda et al. Japanese population.
Giannattasio 2007[189]	Cross sectional	101	Examine arterial stiffening in angina patients with CAD.	-Abdominal aortic and carotid distensibility lowest in the 3-vessel disease group c/w 2 and 1-vessel disease. -Suggests that large artery stiffening can be regarded as a marker of the coronary status.	-CAD grouped according to one, two or three-vessel disease. -Arterial distensibility measured at the radial, subdiaphragmatic aorta and carotid sites. -Medications continued. -All patients had angina (not defined). -Not fasted morning of appointment. -Contrast to Takeuchi and Syeda.

Dart 1991[190]	Cross sectional	93	Examine the effect of CAD and hypercholesterolaemia without CAD on age-related aortic stiffness.	-Presence of CAD associated with lower transverse arch aortic distensibility independent of age. -Paradoxical, unexplained finding that AD was higher in those with hypercholesterolaemia without CAD then in normal controls.	-Four groups investigated including post-transplant patients. -Multivessel CAD only in CAD group. Diffuse or focal not quantified. -Echocardiography of the transverse aortic arch. -All CAD subjects were men.
Kingwell 2002[191]	Cross sectional	96	Determine whether large artery stiffness independently predicts ischaemic threshold in patients with CAD.	-Ischaemic threshold related to a critical stenosis, not the extent of CAD. -Systemic arterial compliance, distensibility index and carotid augmentation index were independent predictors of shorter time to ischaemia.	-Measures of large artery stiffness included systemic arterial compliance, distensibility index, pulse wave velocity and carotid augmentation index. -CAD classified as 1, 2 or 3-vessel (1 or more ≥50% stenosis).
Wang 2014[192]	Cross sectional	501	Investigate the relationship between CAVI and NT-proBNP in hypertension and CHD subjects.	-CAVI and NT-proBNP ↑ in hypertension subjects with CHD ($p<0.001$). -NT-proBNP predicted CAVI ($\beta= 0.15$, $p=0.021$).	-Difference in medication not accounted for. -Weak correlation.

AD(I) aortic distensibility (index), AS aortic strain, BP blood pressure, CAC coronary artery calcium, CAD coronary artery disease, CAVI Cardio-ankle vascular index, CT computed tomography, CTCA computed tomography coronary angiogram, c/w compared with, FMD flow mediated dilatation, LAEI large artery elasticity index, NT-proBNP N terminal-pro brain natriuretic peptide, PWV pulsed wave velocity, SAEI small artery elasticity index, SS segmental score, SYNTAX SYNergy Between PCI With TAXUS and Cardiac Surgery.

1.3.4.4 Monocyte subsets and their platelet aggregates in stable CAD

1.3.4.4.1 Monocyte subsets

Atherosclerosis is a result of complex interaction between leucocytes (primarily monocytes), ECs, ESS, vascular remodelling and platelets, influenced by cardiac risk factors and mediated by various growth factors and cytokines. Monocytes are mononuclear phagocytes of myeloid origin that represent approximately six percent of the total leukocyte population in adults[193]. They are currently considered to exist as three distinct subsets, partly explaining their diverse functionality (Table 1.12); Mon1 CD14++CD16- (classical, about 85% of monocyte population), Mon2 CD14++CD16+ (intermediate, about 5%) and Mon3 CD14+CD16++ (Non-classical, about 10%)[194]. Monocytes play a key role in all stages of atherogenesis from vascular recruitment and diapedesis to and from injured ECs, to lipid-laden macrophage-like foam cell formation and repair following AMI. Moreover, specific subsets are differentially involved in a range of conditions including ACS, heart failure, stroke, kidney disease and inflammatory arthritis[195-199].

Knowledge from mouse models has informed our understanding of monocyte subset activity under homeostatic conditions in humans[200]. For example, comparisons of gene and protein expression in mice and humans have revealed several properties unique to the Mon3 (non-classical) population such as heightened metabolism and cytoskeletal features, consistent with their 'patrolling' phenotype[201]. Furthermore, the transcriptional range of Mon2 (intermediate) is in between that of Mon1 (classical) and Mon3[197]. Genes associated with maturity are more frequently found on Mon3. Indeed, studies have shown a significant shift from Mon1 to Mon3 with increasing age in healthy adults[202]. Whether

this is due to increased differentiation of CD16- monocytes or new production is not known.

Several clinical studies, summarised in Table 1.13, have evaluated circulating total monocytes and monocyte subsets in stable CAD and its associated CV risk factors[196, 203-213]. While many reports suggest increased 'CD16+' monocyte levels in more advanced CAD including in-stent restenosis, they are limited by the analysis of Mon2 and Mon3 as one CD16+ subset. Hristov et al[210] showed that Mon1 counts were highest ($p = 0.006$) and Mon3 counts lowest ($p = 0.001$) in patients with ≥5 CAD risk factors elucidating a link between monocyte mediated endothelial inflammation and traditional risk factors in CAD. The number of risk factors also predicted the percentage of Mon2 monocytes ($p=0.042$) but interestingly, the extent of CAD (one, two or three vessel) showed no association with individual subset counts. This could have been due to small numbers in each of the disease categories. Expanding this, Czepluch et al observed a relative increase in the percentage of Mon1 counts in CAD patients[214]. In contrast, Krychtiuk et al[211] found LDL particle size to be negatively correlated with Mon3 counts ($r=0.25$, $p=0.017$). Mon3 counts were also significantly increased in multivessel CAD whereas Mon1 and Mon2 were unchanged, although the lack of a control group in this study is a limitation. HDL cholesterol levels, on the other hand, have been negatively correlated with Mon2 counts in chronic kidney disease (CKD) patients[215]. Wrigley et al, in a study of acute ischaemic heart failure (HF), noted no difference in subset counts between their two control groups – one of CAD without HF, the other disease free[216]. Silent CAD in the latter is a possibility. Mon2 counts have been shown to predict CV events in different patient groups[208, 217, 218].

Table 1.12 Comparison of human monocyte subsets

Monocyte subset	**Antigen/receptor expression**	**Functions**
Mon1 (classical) CD14++CD16- Least mature	CD14, CD86+, CD45, HLA-DR, CCR2, CXCR 1, 2 and 4, CD62L, CD93, CD64, CD32, CD36, FCN1 (ficolin-1), SIRPA (signal-regulatory protein alpha), CCR1, CD11a/CD18, CD11b/CD18, CD36, class A and B scavenger receptor, AP-1 transcription factor genes, CLEC4D, IL-13Rα1, VEGFR1.	-Production of IL-10 and IL-6, IL-8, TNF-α, IL-1β and CCL2. -High myeloperoxidase and phagocytic activity - ingest native LDL. -Preferential differentiation into M1 macrophages. -CCR5 and CX3CR1 mediated recruitment to atherosclerotic plaques. -Angiogenesis -Recruitment during the early phase of MI. -Produce ROS.
Mon2 (intermediate) CD14++CD16+	CD14, CD16, CD86, CD45, MHC II, HLA-DR, CCR2, CX3CR1 CCR1, CCR2, CXCR2, CCR5, CD43 (ACE), Tie2 (angiopoietin receptor), CD64, CD36 scavenger receptor, CD11a/CD18, CD11b/CD18, CD11c/CD18 integrins, GFRα2, CLEC10A.	-Production of TNF-α or IL-1β upon LPS stimulation. -Stimulate CD4+ T-lymphocyte proliferation. -High affinity for VCAM-1 (particularly after MI). -May support endothelial regeneration through expression of Tie2 and other angiogenic cytokines.
Mon3 (non-classical) CD14+CD16++ Most mature	CD14, CD16, CD86, CX3CR1, CD45 HLA-DR, Nur77, LFA1 (lymphocyte function–associated antigen-1 [CD11a integrin]), CD11c/CD18, CD294, Siglec10, ILT4.	-Secretion of IL1-β and TNF-α (when triggered by viruses and nucleic acids). -Patrols the vascular endothelium -Lower phagocytic activity than classical.

		-Mediates removal of damaged ECs. -CCR5 mediated recruitment to atherosclerotic plaques. -Preferential differentiation into M2 macrophages and dendritic cells. -May emigrate from atherosclerotic plaque back to circulation. -Remove oxLDL from circulation. -Recruitment during reparative phase of MI. -Possibly arteriogenesis and vascular repair.

ACE angiotensin converting enzyme, CCL2 chemokine (C-C motif) ligand 2, CCR chemokine (C-C motif) receptor, CD cluster differentiation, CLEC C-type lectin domain family, CXCL (C-X-C motif) ligand, EC endothelial cell, GFRα2 GDNF family receptor alpha-2, HLA human leucocyte antigen, IL interleukin, ILT immunoglobulin-like transcript, LPS lipopolysaccharides, NF-κB nuclear factor kappa B, oxLDL oxidised low density lipoprotein, MHC major histocompatibility complex, MI myocardial infarction, ROS reactive oxygen species, SMC smooth muscle cell, TNF tumour necrosis factor, VCAM vascular cell adhesion molecule, VEGFR vascular endothelial growth factor receptor.

Table 1.13 Studies quantifying monocyte subsets in patients with stable coronary artery disease

Author/year	Design	No. of participants	Objective	Outcome	Comment
Ozaki 2012[203]	Cross sectional	125	Determine association of monocyte subsets with severity of CAD	-Significant association between CD14+CD16+ monocytes and severity of CAD.	-Monocytes analysed as two subsets. -Significant positive correlation between CD16+ monocytes and Gensini score. -Forty-four percent of 'no CAD' group had a history of coronary intervention.
Schlitt 2004[213]	Cross sectional	247	Examine levels of CD14+CD16+ monocytes in CAD versus control patients plus association with TNFα.	-Higher levels of CD14+CD16+ monocytes found in CAD patients. Significant association with TNFα found.	-Monocytes analysed as two subsets. -Control group might have had occult CAD. -Groups not matched for age and sex. -Study group included ACS and found no difference between ACS and stable.
Kashiwagi 2010[205]	Cross sectional	73	Evaluate the relationship between monocyte subsets and	-Association between CD14+CD16+ and presence of vulnerable plaques	-Monocytes analysed as two subsets.

			the presence, extent and vulnerability of non-calcified CAD assessed by CT.	(defined by positive remodelling and low attenuation plaque).	-Small patient numbers. -CT over estimates disease.
Liu 2010[206]	Cross sectional and longitudinal	71	Assessment of monocyte subsets as a marker of ISR after bare metal stent implantation after AMI.	-CD14+CD16+CX3CR1+ numbers ↑ in people with ISR and correlated with significantly with late lumen loss.	-Monocytes analysed as two subsets. -No control group. -Only BMS used. -Not clear proportion of pre-dilation versus direct stenting. -Unclear methodology (timing of blood samples, repeat angiogram driven by symptoms or pre-specified nine months).
Olivares 1993[207]	Prospective observational cohort	3779	Study different leucocytes as a risk factor for premature CAD in men	-Monocytes significantly higher in early CAD.	-Men only. -Mainly White Europeans. -Monocytes not classified as subsets. -Large numbers.

Rogacev 2012[208]	Prospective observational cohort	951	Analyse the relationship between human monocyte subsets and cardiovascular risk in patients referred for elective coronary angiography.	-Association found between CD14++CD16+ (intermediate) monocytes and cardiovascular events.	-Monocytes analysed as three subsets. -Median follow up 2.6 years. -Small (for epidemiological study) but largest epidemiological monocyte study so far.
Huang 2013[209]	Cross sectional	140	To compare levels of CD14+CD16+ monocytes in patients with coronary artery disease and blood stasis syndrome versus those with CAD and no blood stasis syndrome.	-Protein level of CD14+CD16+ monocyte levels increased in CAD patients with blood stasis syndrome c/w controls.	-Monocytes analysed as two subsets. -Inflammatory cytokines tumour necrosis factor-α and interleukin 1 also higher.
Shantsila 2014[196]	Cross sectional	103	Characterisation of monocyte subsets and their expression of receptors in patients with CAD.	-Total count of classical and intermediate monocytes and their MPAs were similar. However, non-classical MPAs were higher in CAD patients. Significant up-regulation of CXCR4 on non-classical monocytes and CD34 on all subsets.	-Monocytes analysed as three subsets. -No distinction of CAD severity.

Hristov 2010[210]	Cross sectional	80	Evaluation of associations of circulating monocyte subsets with cardiovascular risk and extent of CAD.	-Non-classical monocytes reduced in high risk (≥5 risk factors) patients with CAD. Classical monocytes highest in this group. Significant negative association between family history and non –classical.	-Monocytes analysed as three subsets. -No control group. -One, two, three vessel disease system used.
Krychtiuk 2015[211]	Cross sectional	90	Examine whether monocyte subsets are associated with sdLDL serum levels.	-Patients in the highest sdLDL tertile had highest levels of Mon3 which correlated ($R = 0.25$, $p = 0.017$) and lowest levels of Mon1. - MVD significantly increased Mon3 ($p = 0.015$). -Mon1 and Mon2 not associated with CAD severity.	-Used mean (SD) when absolute counts of individual subsets clearly skewed. -No control group.
Czepluch 2013[212]	Cross sectional	70	Investigate expression of atheroprotective KLF4 on monocyte subsets in patients with CAD	- Down regulation of KLF4 in all CAD monocyte subsets compared to control ($p < 0.01$ for all). -KLF4 lowest in Mon1 compared to Mon2 and	-Use of control group, albeit small. -Cannot exclude CAD in control group.

				Mon3 for both CAD and controls.	-Monocytes analysed as 3 subsets. -CAD severity/morphology not assessed.

ACS acute coronary syndrome, AMI acute myocardial infarction, BMS bare metal stent, CAD coronary artery disease, CD cluster differentiation, CT computed tomography, c/w compared with, ISR in-stent restenosis, KLF4 Krüppel-like factor 4, MPA monocyte-platelet aggregate, MVD multivessel disease, SD standard deviation, sdLDL small dense low-density lipoprotein, TNF-α tumour necrosis factor-alpha.

1.3.4.4.2 Monocyte-platelet aggregates

Activated platelets adhere to monocytes (as well as each other) and form monocyte-platelet aggregates (MPAs). This union alters the function of the monocyte. Experiments in healthy volunteers has revealed increased expression of CD16 on the surface of CD14++CD16- monocytes incubated with autologous platelets compared with CD14+CD16- monocytes in the medium alone ($p<0.001$) – an effect which correlated directly with degree of MPA formation ($r=0.88$, $p<0.0001$) and was associated with increased monocyte adhesion to ECs, and thus, a mechanism for atherosclerosis progression[219]. MPAs are a robust signal of platelet activation – increased levels are present in ACS and in patients undergoing PCI.

MPAs are early markers of diabetes, endothelial activation/dysfunction and subclinical atherosclerosis[220-222]. Studies generally show increased MPAs with the presence of CAD (Table 14[214, 222-225]) although they have tended to be small with homogenous populations[224, 225]. In patients undergoing elective single vessel PCI, Mickelson et al found increased MPA levels to be associated with target vessel restenosis and CAD progression requiring surgical revascularisation[225]. However, the study was limited by many confounders including recent AMI in two (out of eleven) subjects and heart failure in others plus four patients had MVD at baseline and would be more likely to progress independent of MPA levels. In contrast De Serafino et al recently found no relation between MPA levels and CAD extent ($r=0.005$, $p=0.45$)[222]. A major drawback is that MPAs were not analysed as separate subsets, so the relative contributions may have been overlooked.

Contemporary studies have the advantage that monocytes are correctly categorised as three subsets. Unfortunately, this makes them difficult to compare with studies performed previously. Whilst there is probably overall agreement that CD16 positivity seems to be more readily associated with more severe CAD phenotypes, considerable divergence of the available evidence exists such that the precise pathophysiological situation requires further clarification. Certainly there are significant changes in receptor expression across all monocyte subsets in CAD compared to controls[196, 212].

Table 1.14 Studies of monocyte-platelet aggregates in human coronary artery disease

Author	Design	No. of participants	Objective	Outcome	Comments
Furman 1998[223]	Cross sectional	38	To examine whether patients with stable CAD have increased platelet reactivity and an enhanced propensity to form MPAs.	-Stable CAD patients had ↑ circulating MPAs c/w controls.	-Small numbers. -Difference could be due to difference in risk factors between the groups. -Different subsets not analysed.
Di Serafino 2014[222]	Cross sectional	145	To see if MPA are associated with the presence of functionally significant coronary stenoses or with coronary arterial endothelial dysfunction.	-Neither MPA nor coronary endothelial function associated with functionally significant stenoses. -MPA negatively correlated with coronary endothelial function.	-Fractional flow reserve (FFR) used to quantify functional significance. -Uses the Bogaty score to quantify CAD pattern. -Even distribution of risk factors. -Four percent of people with a negative FFR are classified as having silent ischaemia.
Robinson 2006[224]	Cross sectional	40	Assess the relationship between platelet activation and endothelium-dependent	-Platelet-monocyte binding was higher in	-Male only study. -Small numbers.

			vasomotion in patients with CAD.	CAD patients than healthy men. -Significant inverse correlations were seen between substance P and acetylcholine induced vasodilatation and platelet-monocyte binding in CAD patients.	
Czepluch 2014[214]	Cross sectional	124	To examine MPAs and expression of CCR5 in individuals with coronary atherosclerosis.	-MPA formation ↑ in patients with CAD in all three monocyte subsets. -CCR5+ monocyte number ↓ in patients with CAD.	-Monocytes analysed as three subsets. -Multiple baseline differences not corrected for including age. -Attempted age-matched cohort in nine people. -No limitations mentioned.
Mickelson 1996[225]	Pilot cross-sectional and prospective longitudinal	11	To determine if leukocyte activation occurs, whether leukocyte-platelet complexes develop and whether there is any association between	-After angioplasty, LPAs ↑ (p=0.02). LPAs were also higher in the six patients that had clinical events during follow up.	-Small sample size. -All male population. -Heterogeneous population (some having had a recent AMI, some with LVSD).

			these findings and clinical outcome after elective single vessel coronary angioplasty.		-Pre-DES era.

CAD coronary artery disease, CCR5 chemokine receptor 5, c/w compared with, DES drug eluting stent, FFR fractional flow reserve, LPA leukocyte-platelet aggregate, LVSD left ventricular systolic dysfunction, MPA monocyte-platelet aggregate.

1.3.4.5 Circulating microparticles

Microparticles (MPs) are small, anucleoid phospholipid vesicles released from the plasma membranes of various source cells in response to stimuli such as apoptosis, platelet or endothelial activation, ESS, inflammatory cytokines or oxidative stress[226, 227]. MPs are primarily identified by their diameter (0.1-1μm), as opposed to exosomes (0.04-0.1μm), or apoptotic bodies (>1.5μm)[228]. Following appropriate stimuli, a cascade of events – beginning with calcium-dependent phosphatidylserine shift from the inner to the outer monolayer of the eukaryotic cell membrane – occurs, leading to MP formation[229]. Calcium-dependent proteolysis of the cytoskeleton causes intracellular changes that promote 'blebbing'[228]. Therefore, MPs contain the same cytoplasmic material and express the same surface proteins as the parent cell allowing them to be classified.

Factors precipitating an atherogenic EC phenotype, such as low ESS, favour MP formation in many cell types. Indeed, haemodynamic forces are a major influence driving EMP (endothelial microparticle) release from damaged ECs. EMPs in turn promote the expression of adhesion molecules on the EC surface which corroborates evidence from clinical studies that increased EMP levels predict the presence of CAD risk factors, increased arterial stiffness, coronary endothelial dysfunction and CAD in certain groups[230-233]. In some cases, MPs may be even more potent than their parent cells[234].

Clinical studies evaluating microparticles in CAD are summarised in Table 1.15[227, 231, 235-242]. Biasucci et al compared levels of EMPs, PMPs (platelet microparticles) and AMPs (apoptotic microparticles) in patients with stable angina, stable angina and PVD (peripheral vascular disease), non-ST-segment elevation myocardial infarction and ST-segment elevation myocardial infarction. The authors reported higher levels of all MPs in

subjects with ACS compared with stable patients, but no difference between the two stable groups[236]. However, prolonged endothelial activation may result in more severe CAD then PVD. Although Tan et al also found no correlation between PMPs and the degree of CAD[238]. Furthermore, Cui et al[235] reported no difference in EMPs or PMPs between stable CAD patients and controls (p=0.763 and p=0.853, respectively) but the numbers in both groups were small compared to the unstable groups. Also, asymptomatic CAD could have been present in the controls. In contrast, EMPs were predictive of CAD presence in a well powered Japanese diabetic cohort (OR 3.5, 95% CI 1.8 to 6.9, p<0.001). Post PCI MP measurement provides additional evidence that endothelial injury results in higher MP levels[227, 239]. Some of these studies are limited by small samples, male only populations and the lack of a control group[227, 236, 237, 239]. In any case the role of MPs in CAD severity remains inconclusive.

Whilst MPs are important mediators throughout many stages of atherogenesis some – neutrophil MPs for example – possess anti-inflammatory effects capable of down regulating macrophages, for example, murine models of MI have shown an excess of extracellular vesicles (size 118±4nm and 252±18nm, therefore in the MP range) from cardiomyocytes and endothelium within 24 hours of coronary artery ligation which facilitated monocyte transition from $Ly6C^{high}$ (proinflammatory) to $Ly6C^{low}$ (favouring repair)[243, 244]. Intra plaque MPs – derived mainly from leucocytes – have been shown to engulf immunoglobulins and have a proliferative effect on CD4 T lymphocytes, thus modulating tissue inflammation[245]. Moreover, low levels of PMPs and EMPs are present in healthy individuals, resulting from continuous low gradient turnover[228, 246].

Table 1.15 Studies of microparticles in human populations with stable coronary artery disease

Author	Design	No. of participants	Objective	Outcome	Comments
Sinning 2011[242]	Prospective longitudinal	200	Evaluate whether MPs are an independent predictor of cardiovascular outcomes in stable CAD patients.	Over median 6.1 (6-6.4) year follow up, CD31+/Annexin V+ EMPs were associated with increased cardiovascular mortality and need for revascularisation.	EMPs not associated with CAD extent 'number of diseased vessels'. No control group. No relation to all-cause mortality.
Cui 2013[235]	Cross sectional	110	Investigate endothelial, platelet, lymphocyte, monocyte and tissue factor positive microparticles in CAD patients and evaluate their correlation with Interleukin-6 and C-reactive protein.	-No difference in any microparticle population between stable angina patients and controls.	-Study examined ACS patients which did show significantly higher microparticle levels across all cell types.
Koga 2005[231]	Cross sectional	334	Examine whether CD144-EMP (endothelium-derived microparticles) is useful as a specific marker of endothelial cell dysfunction and to determine whether plasma levels of circulating	-CD144-EMP levels ↑ in DM patients with CAD c/w those without CAD. -CD144- EMP levels independently predicted CAD in patients with DM	-Japanese diabetic population undergoing coronary angiography. -Healthy controls angiographically proven absence of major CAD.

			CD144-EMP predict CAD in patients with type 2 DM.	without symptomatic episodes of angina.	-Controls age and sex matched.
Biasucci 2012[236]	Cross sectional and prospective longitudinal	76	To assess the release of microparticles and their association with inflammation and atherosclerotic burden.	-Atherosclerotic burden did not affect MP levels in stable patients (although levels of MP in ACS patients were higher than stable patients with or without PVD.	-Small sample size. -No control group. -Severity of CAD judged by ischaemic syndrome, not disease extent.
Werner 2006[237]	Cross sectional	50	To determine if endothelial function is influenced by the degree of endothelial cell apoptosis.	-On multivariate analysis, increased apoptotic microparticle counts predicted severe endothelial dysfunction (p=0.003).	-No control group. -Patients with no CAD or severe diffuse CAD were excluded. -Intracoronary acetylcholine used for endothelial function.
Horn 2015[227]	Cross sectional	28	To quantify the release of MPs during stent implantation into stable atherosclerotic lesions and compared the release between RCAs and SVG-RCAs.	-In patients with comparable plaque volume and composition in RCAs and SVG-RCAs, intracoronary PMPs and EMPs were increased after stent implantation into their RCAs and SVG-RCAs. No difference	-Male patients only. -Small sample size. -Symptomatic. -IVUS used to quantify plaque burden. -Limited to right coronary or graft to right coronary only.

				between RCA and SVG-RCA.	-MPs measured directly after and in the vicinity of, stent implantation.
Tan 2005[238]	Cross sectional	89	To determine if PMPs are related to severity of CAD.	-No evidence to suggest that PMPs are related to degree of CAD.	-Peripheral blood. -Age and sex matched controls.
Inoue 2006[239]	Cross sectional and longitudinal.	20	To compare circulating PMPs with that of P-selectin in patients with stable angina undergoing PCI.	-Circulating PMPs and P-selectin expression enhanced following stent implantation. -PMPs increased 24-48 h after coronary stenting in the coronary sinus whereas P-selectin increased 15 min after stenting and remained elevated for 48 h; the changes were less striking in peripheral blood.	-MPs measured in peripheral blood and coronary sinus. -Small sample. -Stent related changes. -First generation stents.
Craft 2003[240]	Cross sectional with paired sample analysis	113	To quantify PMPs in patients undergoing PTCA stratified according to treatment with abciximab.	-PMPs ↑ following angioplasty in the group without abciximab (p=0.019). -No change in PMP levels following angioplasty in	-Peripheral assessment of PMPs. -Not randomised. -Pre-DES.

				patients who received abciximab, despite requiring more complex angioplasty procedures.	
Craft 2004[241]	Cross sectional with paired sample analysis	19	To monitor PMP levels in the coronary circulation.	-Significant ↓ in arterial PMPs from heparinisation to contrast administration (p=0.001), followed by a significant ↑ to the end of angioplasty (p=0.004).	-Small sample. -Coronary sinus and coronary artery samples. -Pre-DES.

ACS acute coronary syndrome, CAD coronary artery disease, CD cluster differentiation, c/w compared with, DES drug eluting stent, DM diabetes mellitus, EMPs endothelial microparticles, IVUS intravascular ultrasound, MPs microparticles, PCI percutaneous coronary intervention, PMP platelet microparticles, PVD peripheral vascular disease, PTCA percutaneous transluminal coronary angioplasty, RCA right coronary artery, SVG-RCA saphenous vein graft- right coronary artery.

1.3.5 Prognosis of patients with CAD unsuitable for revascularisation

Despite improvements in age-adjusted mortality[1], diffuse obstructive CAD remains highly prevalent and confers a higher probability of adverse outcomes, particularly when conventional revascularisation is precluded[247]. Anatomic reasons prohibiting traditional revascularisation include severe diffuse CAD, collateral-dependent myocardium, multiple coronary restenoses, chronic total coronary occlusions, degenerated saphenous vein grafts, poor distal targets, or lack of conduits due to prior CABG – all of which equate to advanced CAD[124]. Despite much interest in alternative strategies including myocardial angiogenesis (protein, gene, or stem cell therapy),[248-252] novel pharmacological agents (for example ranolazine, which has recently shown no benefit in patients with incomplete revascularisation after PCI)[253], enhanced external counter pulsation[254], spinal cord stimulation,[255] and transmyocardial revascularisation, satisfactory treatments are still to be developed[7, 256-259].

Data on CAD patients not eligible for revascularisation – a highly subjective condition – is mainly limited to case series, extrapolation from patients undergoing coronary angiography and small randomised controlled trials (RCTs) of alternative therapies for refractory angina in which annual mortality is up to 21% (some of which are summarised in Table 1.16[4, 7, 8, 124, 127, 256, 259-266]). The wide range of RCT mortality outcomes is due, in part, to the low power of these studies for this outcome but a recent pooled analysis of randomised trials of transmyocardial laser revascularisation confirmed an overall mortality of around 12% in both the treatment and the control arms[5]. The Mediators of Social Support Study (MOSS) study of patients undergoing cardiac catheterization at

Duke University also suggested high mortality (38% at 2.2 year mean follow-up)[4]. In contrast, 1-year mortality from the Euro Heart Survey for patients with stable angina treated medically was 5%[125]. In patients ineligible for revascularization, 7% died at 1 year compared with 3.7% in the cohort eligible for revascularization[126]. Furthermore, event-free survival is low with 70-90% experiencing a MACE (major adverse cardiac event).

Table 1.16 Clinical endpoints in studies of patients with advanced CAD unsuitable for revascularisation

Author/year	Design/no. of patients unsuitable for revascularisation	Follow up (months)	Event rate (%)
Allen/1999[256]	RCT/275*	12	69 CV re-hospitalisation† 11 death†
Da Rocha/2005[260]	Prospective/51	12	43 death 39 NFMI 6 CHF 88 all
Frazier/1999[7]	RCT/192*	12	89 combined†‡ 21 death†§ 69 UA†
Aaberge/2000[259]	RCT/100*	12	8 death†
Aaberge/2002[261]	RCT/100*	43	24 death†
Allen/2004[262]	RCT/212*	60	48 death†
Burkhoff/1999[263]	RCT/182*	12	10 death† 77 UA†
Oesterle/2000[264]	RCT/221*	12	3 death† 56 combined‡
Schofield/1999[265]	RCT/188	12	4 death†
Kandzari/2001[4]	Prospective cohort/487	26††	38 death
Lozano/2014[127]	Retrospective/220	45††	62 death
Williams/2010[124]	Retrospective/493	36††	32 death§§
Henry/2013[8]	Prospective/1200	61††	17.5 death
Povsic/2015[266]	Retrospective/1908**	36††	13 death 17.1 death and NFMI 43.5 CV re-hospitalisation 52.2 MACCE

CHF congestive heart failure, CV cardiovascular, MACCE major adverse cardiovascular or cerebral event (defined as death, MI, stroke, cardiac rehospitalisation, and revascularisation), NFMI non-fatal myocardial infarction, RCT randomised controlled trial, UA unstable angina, * 1:1 transmyocardial revascularisation:medical therapy, † medical therapy arm only, ‡ acute myocardial infarction, death, unstable angina, and class IV angina § non cross-over, †† median, §§ in groups not receiving revascularisation, ** multivessel coronary artery disease present in 65%.

1.3.6 Conclusion

Substantial improvements in therapies for acute CAD coupled with higher general life expectancy and better prognoses from other comorbidities such as HIV and certain malignancies is likely to increase the number of patients experiencing complex CAD in future years. Despite some signs of efficacy in reducing symptoms and recurrent cardiac hospitalisations, novel therapies have shown no impact on mortality with some resulting in early mortality excess. The shortfall in evidenced based therapy drives an urgent requirement for a deeper understanding of the pathogenesis underlying this type of aggressive CAD.

1.4 NORMAL CORONARY ARTERIES – EXISTING AND CHANGING PATTERNS

1.4.1 Normal coronary arteries amongst patients referred to hospital

The prevalence of normal coronary arteries amongst the individuals referred for elective coronary angiography ranges from about 5% to 50% depending upon a number of factors[11]. There is significant inter-hospital variation, reflecting both the varying prevalence within the local population and the varying thresholds for undertaking invasive angiography. The definition used to represent 'normal' is an important consideration. In a Danish registry of 11223 patients receiving elective coronary angiography between 1998 and 2009, the rates of normal coronary angiography were 19% for men and 48% women (34% overall)[267]. This was using a definition of 0% luminal stenosis to represent normal. Interestingly, in a similar American registry study, 21% of elective coronary angiograms undertaken between 2007 and 2010 were considered normal – normal being defined as < 20% luminal stenosis. In a paper by Ashbury et al[268] examining the psychosocial impact of cardiac syndrome X in females, the definition of syndrome X was "patients aged <80 years with chest pain, positive exercise test for myocardial ischaemia and angiographically smooth coronary arteries." Alternatively Zachariae et al defined normal as <50% luminal stenosis[269]. Such a definition is likely to include a significant proportion of patients with structural and functional endothelial dysfunction and may have contributed to the negative findings in the study.

1.4.2 Prognosis

Registry data over the last thirty years generally convey an excellent prognosis for patients with normal or minimally diseased coronary arteries compared to the general population, often despite the persistence of a chest pain syndrome for many years

afterwards[270-273]. Kemp et al examined 21,487 patients' data from the Coronary Artery Surgery Study (CASS) between 1976 and 1979[270]. They identified 4,051 (19%) normal or near normal coronary angiograms (defined as "having no segment with a stenosis of 50% or greater and no left ventricular abnormality.") Of these, 3,136 (77%, 15% overall) were considered entirely normal whilst 915 had a stenosis >0% but <50%. Survival at seven years was 96% for those with completely normal arteries and 92% for those with <50% luminal stenosis. An ischaemic response to exercise (implying cardiac syndrome X) was not associated with increased mortality.

In contrast, recent evidence suggests that patients with suspected angina and normal coronaries may be at higher risk of adverse events than previously thought[274]. In a retrospective cohort taken from a Danish registry of 11,223 patients who had undergone coronary angiography for suspected CAD between 1998 and 2009, Jespersen et al identified 3,479 (31%) individuals with normal coronary arteries[274]. Normal was defined as 0% stenosis in all coronary arteries. If patients with diffuse, non-obstructive CAD (defined as 1-49% luminal narrowing) were included as normal (as they have been in some studies[269]) then a further 1704 patients would be analysed as normal increasing the proportion to 46% of the cohort. An asymptomatic control group was taken from the Copenhagen City Heart Study (n=5705). The overall 5-year MACCE (major adverse cardiovascular or cerebral event – defined in this case as cardiovascular mortality, hospitalization for MI, heart failure, or stroke) – free survival probabilities were 0.96 in the asymptomatic control group, 0.92 in patients with normal coronary arteries and 0.89 in people with mild diffuse disease. In the pooled analysis, multivariate-adjusted hazard ratios (HRs) for the risk of MACCE were 1.52 (95% confidence interval [CI] 1.27-1.83, $p<0.001$) for patients with normal coronary arteries and 1.85 (95% CI 1.51-2.28, $p<0.001$)

for patients with mild non-obstructive CAD. The increase in MACCE for both groups was driven by an increase in cardiovascular death and hospitalisation with heart failure or myocardial infarction. For all-cause mortality, normal coronary arteries and diffuse non-obstructive CAD were associated with HRs of 1.29 (95% CI 1.07-1.56, p=0.007) and 1.52 (95% CI 1.24-1.88, p<0.001) respectively, similar for men and women.

1.4.3 Further classification – when is normal normal?

A normal coronary angiogram does not signify the absence of atherosclerosis since only the lumen is visualised on an invasive angiogram (unless there is calcification, which may be seen as a silhouette without contrast). Therefore atherosclerotic disease associated with eccentric remodelling may not be appreciated[109]. Intracoronary imaging can be used to assess atherosclerotic burden. Experimental studies using IVUS (intravascular ultrasound) suggest that coronary plaques are present in approximately 30-50% of patients with angiographically normal coronary arteries[275, 276]. In light of the possibility of excess mortality in patients with chest pain and normal coronaries compared with the general population a sub-classification of people with normal coronary angiograms has been proposed to include measures of endothelial dysfunction and coronary flow reserve (Table 1.17)[277]. However, prospective longitudinal outcome data are lacking.

1.4.4 Non-invasive coronary angiography

Computed tomography coronary angiography (CTCA) provides a non-invasive structural assessment of coronary anatomy. The recently updated National Institute for Health and Care Excellence (NICE) guidelines (CG95) recommend CTCA as the first line investigation for symptomatic individuals with CAD[278]. An important advantage of CTCA compared to invasive angiography is the ability to visualise the arterial walls

thereby increasing its diagnostic sensitivity, although specificity remains an issue. The risk of adverse outcomes following normal CTCA are extremely low[279, 280].

1.4.5 Conclusion

The use of CT has grown significantly following increased demand brought about by the NICE guidelines for assessment of recent onset chest pain. Its higher sensitivity allows earlier detection of CAD with the chance for disease modifying therapy such statins.

Table 1.17 Subclassification of patients with angina and angiographically normal coronary arteries proposed by Bugiardini et al[277]

Subgroup	Description
1	Non-obstructive CAD, including patients with visible plaque by intravascular ultrasound (IVUS) and/or severe coronary endothelial dysfunction (vasoconstriction during acetylcholine infusion).
2	Non-atherosclerotic microvascular angina, referring to patients with normal IVUS and/or coronary endothelial function but reduced coronary flow reserve (CFR).
3	Normal coronary arteries, referring to people with normal IVUS, coronary endothelial function and CFR. Only then would patients be said to have 'non-cardiac chest pain'.

CAD coronary artery disease, CRF coronary flow reserve, IVUS intravascular ultrasound.

1.5 SECTION I SUMMARY

In this opening section, I have introduced the topic of my thesis, namely a study of the pathophysiology underlying advanced diffuse CAD. I began with a general overview of the fundamental concepts of atherogenesis, focusing on the importance of the vascular endothelium in maintaining homeostasis and traditional CAD risk factors in contributing to endothelial activation and an inflammatory phenotype, with sequential rise in novel risk markers, many of which interact to promote further inflammation. Recent genome wide association studies have resulted in the discovery of novel variants, although much work is needed to fully elucidate the mechanisms by which many of them exert their effects.

Following the general overview, I described the current literature pertaining to people with advanced diffuse forms of CAD (to whom this thesis relates) and people with normal coronary arteries. The subjectivity of diffuse CAD means that significant heterogeneity regarding prevalence and outcomes among published data exists. Intracoronary imaging has enhanced our understanding not only of disease morphology but also plaque composition and vascular remodelling. Vascular profiling has allowed us to apply computer-generated fluid based dynamics to estimate endothelial wall stress and flow patterns and correlate them to CAD progression. Relationships between large artery stiffness and progressive CAD have been established. Highly relevant markers of innate and adaptive immunity as well as platelet and endothelial activation have been correlated to the presence of CAD although studies diverge in the strength of the relationship between these markers and CAD severity, particularly since the recent change in monocyte nomenclature. The excess adverse outcomes and lack of treatment options

serves to highlight the importance of recognising this manifestation of CAD as challenge for the future.

SECTION II

OVERVIEW OF THESIS

In this section, I present my MD proposal, including a detailed description of the material and methods I used for patient selection and data collection.

2.1 AIMS AND HYPOTHESES

2.1.1 Objectives

The overall aim of this thesis was to provide a detailed comparison of the pathophysiological differences between patients with severe diffuse CAD and focal CAD, both also compared to subjects that maintain normal coronary arteries, despite similar traditional risk factors across all three groups. Emphasis was placed on measures of cardio-vascular interactions, arterial stiffness, systemic microvascular endothelial function, monocyte subsets, monocyte-platelet aggregates and circulating endothelial and platelet microparticles.

2.1.2 Hypotheses

I hypothesised;

- That patients with diffuse CAD would have abnormal cardio-vascular interactions, impaired systemic microvascular endothelial function and increased arterial stiffness compared to patients with focal coronary artery disease and subjects with normal coronary arteries and that these measures would deteriorate more prominently in subjects with diffuse CAD than in subjects with focal CAD.

- That patients with diffuse CAD would have increased levels of proatherogenic monocytes and endothelial and platelet microparticles compared to patients with focal CAD and individuals with normal coronary arteries and that longitudinal change in cardio-vascular interaction and microvascular function would be inversely proportional to longitudinal change in microparticles and proatherogenic monocyte subsets in diffuse CAD subjects.

2.2 MATERIALS AND METHODS

2.2.1 Study design

This was a prospective observational case-control study.

2.2.2 Study 1: Cross-sectional study

I compared fifty patients with diffuse CAD with forty age and sex matched 'disease' controls with focal CAD and fifty 'healthy' controls with normal coronary arteries (Figure 2.4).

2.2.2.1 Definitions

The term 'diffuse' is frequently used in literature to describe widespread involvement of an organ or anatomical structure by a pathological process. It is commonly used to subjectively describe advanced or extensive CAD however there is no standard definition. Throughout the published literature there are numerous examples of different models employed to quantify the severity and extent of CAD[136, 281-283]. Indeed, many semi-quantitative scoring systems have been validated against IVUS and shown to correlate with each other[135] (Section 1.3.2 and Table 1.8). 'Severity' tends to refer to the degree of luminal encroachment of a stenotic lesion whereas extent often refers to the number of main epicardial vessels with disease[123, 136]. The term 'diffuse' describes the morphology of a lesion, which may be mildly stenotic or limited to a single vessel.

In this thesis, diffuse CAD was defined if more than half the total length of at least two major epicardial coronary arteries contained stenoses ≥70% (assessed visually) (Figure 2.1). Patients with a single vessel chronic total occlusion were required to have diffuse CAD as defined above in at least one other major epicardial artery.

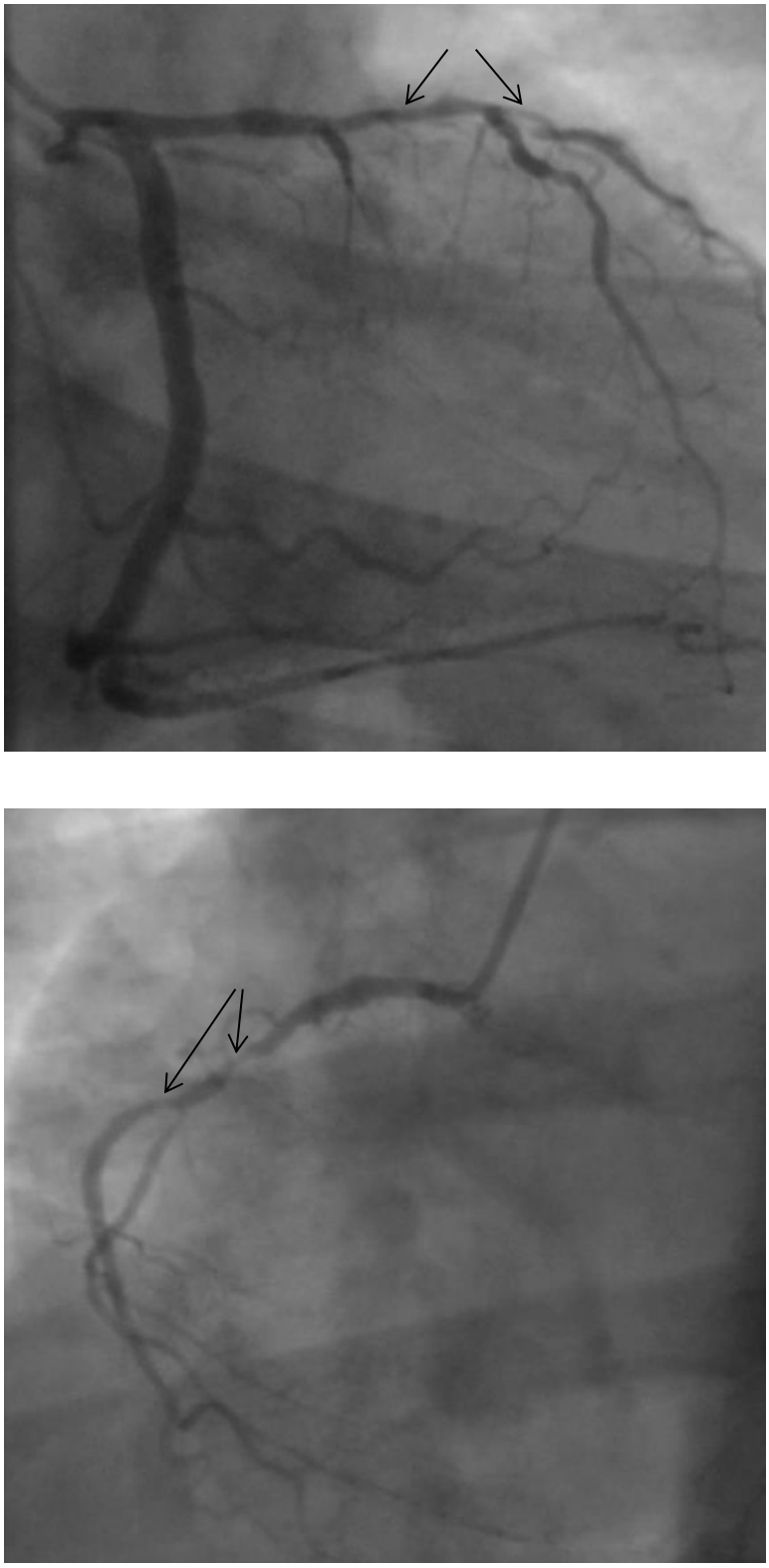


Figure 2.1 Example of diffuse two-vessel disease involving the left and right coronary arteries (arrows).

Focal CAD was defined as one significant (≥70%) type A or B1 lesion[284] (Section 1.3.1, Table 1.7) in one segment (i.e., proximal, mid or distal) of a single coronary artery with no more than mild (<50%) disease elsewhere in the coronary tree documented within the previous year.

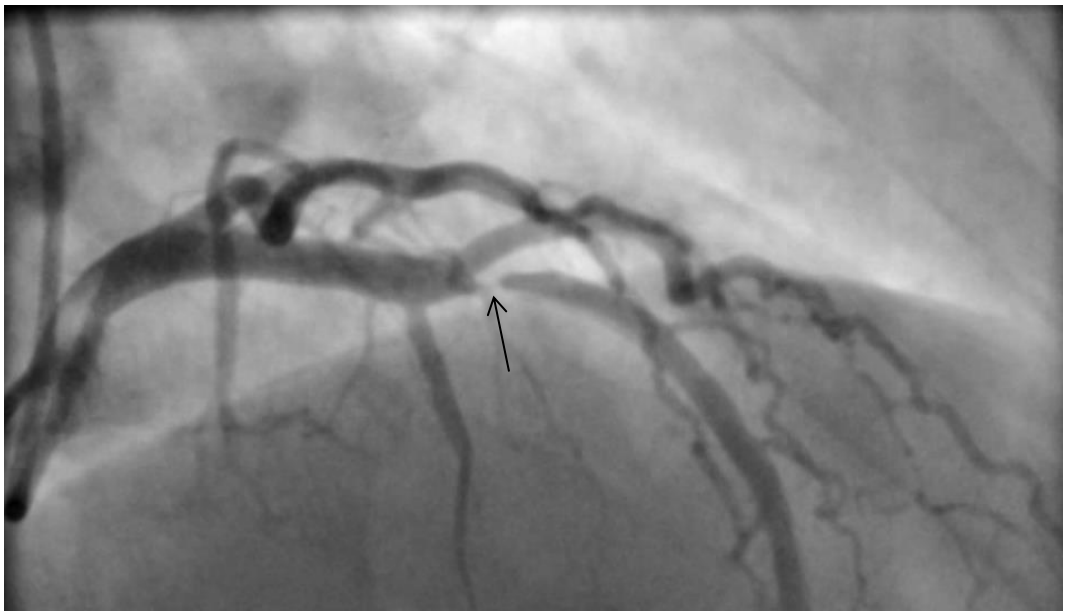


Figure 2.2 Example of a focal stenosis in the mid left anterior descending artery (arrow).

Normal coronary arteries were present if the invasive angiogram showed no visible lesions in any segments of any arteries; or if the computed tomography angiogram (both performed within the previous year) showed no evidence of calcified or non-calcified plaques (Agatston Score 0[285], Appendix 7).

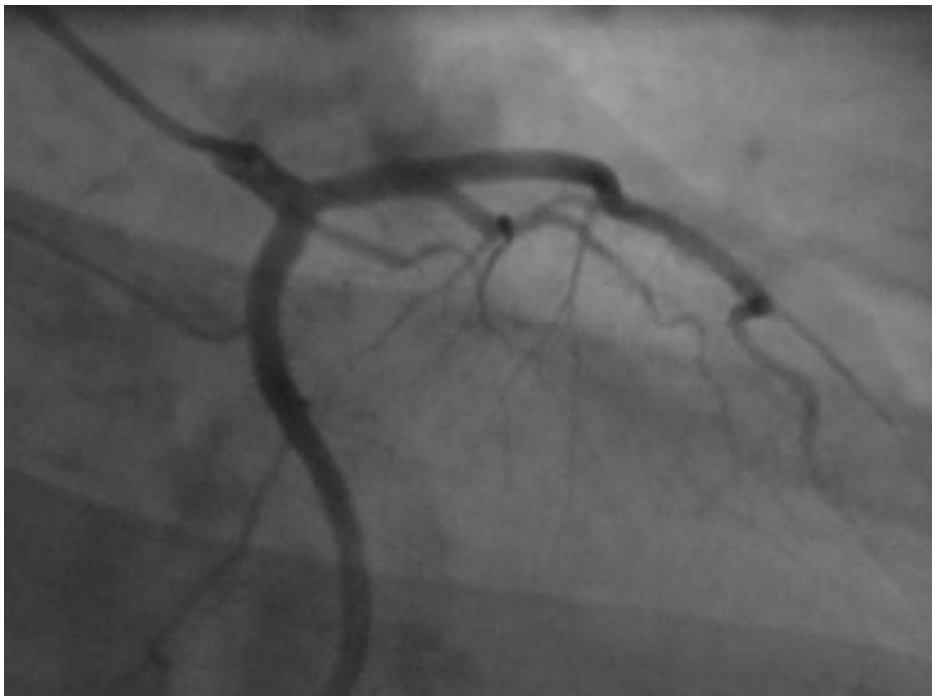


Figure 2.3 Example of normal coronary arteries.

2.2.2.2 Subject selection

I reviewed the angiograms of potentially eligible participants attending the Sandwell and West Birmingham Hospitals (SWBH) NHS Trust outpatient clinics, catheter laboratories, CT (computed tomography) coronary angiography suite, cardiac rehabilitation centres and cardiology diagnostic departments. I approached all patients without exclusion criteria who met the angiographic criteria described above either by telephone or in person. All participating subjects provided written informed consent which was verbally updated at each follow up appointment. The study was approved by the Black Country Research Ethics Committee (and the Research & Development department at SWBH in accordance with the Declaration of Helsinki. The study was insured and sponsored by the University of Birmingham

2.2.2.3 Study procedures

Subjects were invited to the research unit at City Hospital where I collected a detailed clinical history including cardiovascular risk factors, an EQ-5D-5L quality of life assessment and physical examination. Subjects were fasted and asked to abstain from smoking, caffeine or medication on the morning of their appointment except for insulin-dependent diabetics whose appointments I scheduled around their insulin regime. Sublingual GTN spray was permitted if necessary. Patients were asked to bring their medication with them so that they can take them straight after their appointment thereby minimising interruption to drug therapy. In all patients I performed laser Doppler flowmetry followed by transthoracic echocardiography (TTE), followed by analysis of the radial arterial pulse using applanation tonometry followed finally, and after 20

minutes of supine rest (since exercise causes mobilisation of Mon3[286]), by a 20ml blood sample 10mls of which was used for immediate flow cytometric analysis of monocyte subsets, monocyte-platelet aggregates, measurement of full blood count, biochemical and lipid profiles. The rest of the blood was centrifuged for 15 minutes at 3000g (~4000 revolutions per minute and the supernatant plasma removed and stored at minus 70^{o}C for batch analysis of circulating platelet and endothelial microparticles.

2.2.3 Study 2: Longitudinal study

I repeated all measures outlined in the cross-sectional study on participants with diffuse and focal CAD at six- and twelve months post baseline (Figure 2.4). Patients were studied at the same time of day for each follow up appointment to minimise the effect of diurnal variation on the study parameters.

2.2.4 Inclusion criteria

Participants were required to be over 18 years of age with angiographically proven CAD suitable for either one of the two study groups (definitions, section 2.2.2.1), or documented normal coronary arteries on either invasive or non-invasive (CT) angiography within the last year invasively or non-invasively.

2.2.5 Exclusion criteria

Subjects with the following conditions were excluded: left ventricular ejection fraction less than 35%, eGFR <30ml/min/1.73^{2}, more than mild valve disease, liver failure, any myocardial infarction as per the World Task Force classification[287] or coronary stenting within 3 months, stroke, non-cardiac or cardiac surgery within 12 months, active malignancy, autoimmune disease, haematological disorders, active infection, anaemia (<100g/l), age >80 years.

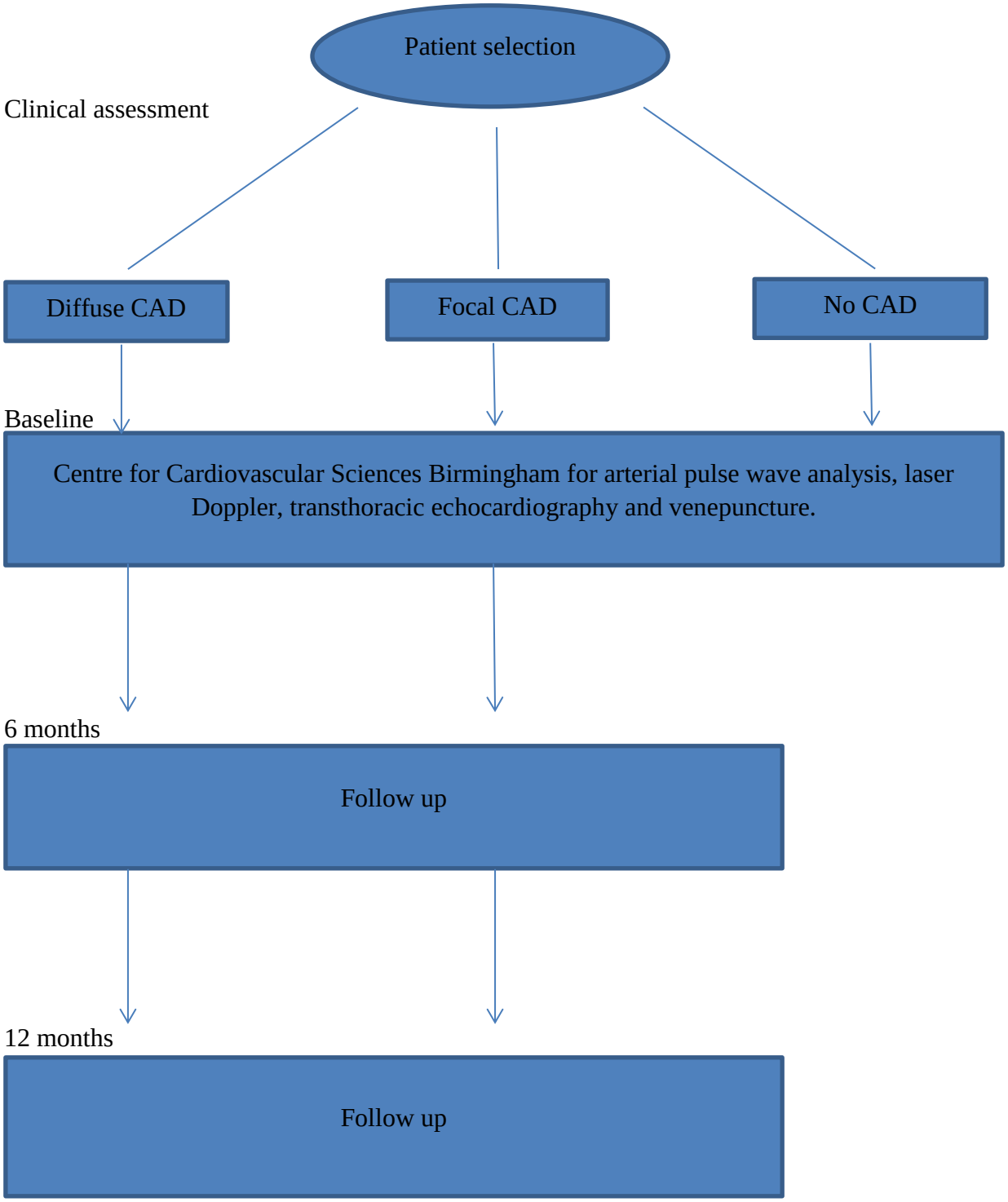


Figure 2.4 Research protocol.

2.3 STUDY METHODS

2.3.1 Assessment of arterial and ventricular elastance

2.3.1.1 Echocardiography

I performed M-mode, two and three-dimensional transthoracic echocardiography (TTE) and tissue Doppler imaging (TDI) on all participants using a Phillips iE33 ultrasound machine (Bothel, WA, USA). Modern off-line QLAB software (Xcelera, Phillip [iE33] Ultrasound Quantification Module, USA) was used for quantification of left ventricular (LV) function. I took an average of three measurements for all parameters. I calculated the effective arterial elastance index (EaI) by dividing central aortic pressure[288] (derived during pulse wave analysis – section 2.3.1.2) by 3D stroke volume (SV) followed by adjustment for body surface area (BSA). End-systolic elastance (Ees) was calculated as central aortic pressure (a.k.a end-systolic pressure [ESP]) divided by end-systolic volume (ESV). End-diastolic elastance (Eed) was calculated by first estimating the end-diastolic LV pressure using the Nagueh formula[289] – the ratio of early mitral inflow velocity (E) to early mitral annular diastolic TDI velocity (E prime [E'] – E/E'x1.24)+1.9, divided by SV[290, 291]. Arterial-ventricular interaction (Ea/Ees) is thus derived and indexed to BSA. The interassay coefficients of variation (CVs) for Ea, Ees and Eed were 4.4%, 5.1% and 9.8% respectively (Section III).

2.3.1.2 Pulse wave analysis (PWA)

I measured the carotid-radial pulse wave and aortic augmentation index (AIx) using a SphygmoCor device (SphygmoCor, AtCor medical, Sydney, Australia). Radial artery waveforms were recorded over 10 seconds using a high-fidelity hand-held applanation tonometer to perform pulse wave analysis and calculate (AIx) using an inbuilt modern

SphygmoCor CVMS software system (Version 8). The reason for using PWA was to provide an accurate measure of central aortic blood pressure (ESP) as this is also estimated by the software. I measured the average of three brachial artery pressure measurements following twenty minutes supine rest. This technique was previously validated in our laboratory. My average inter- and intra-assay CV for AIx were 7.8% and 4.1% respectively (Section III).

2.3.2 Assessment of microvascular function

2.3.2.1 Cutaneous iontophoresis with Laser Doppler assessment of blood flow

Iontophoresis is the application of an electrical current across the skin which allows the delivery of ionic compounds to the cutaneous microcirculation[292]. The technique has been used in various studies to evaluate microvascular endothelial function[43, 51, 293, 294]. Following twenty minutes supine rest, I positioned two small drug delivery chambers on the right forearm of each subject approximately 5cm apart (Figure 2.5) and placed a few drops of 1% acetylcholine [ACh] (Sigma-Aldrich, Dorset, UK) into the positively charged proximal chamber and 1% sodium nitroprusside [SNP] into the negatively charged distal chamber. I used a twelve minute protocol as previously published by our department[43, 295]. This consisted of one minute of baseline recording, followed by one minute of iontophoresis at 100mA, followed by ten minutes of post-iontophoretic recording since maximal smooth muscle vasodilation typically occurs several minutes later than endothelial-dependent vasodilation. ACh – a positively charged compound – is repelled through the skin during the application of electrical current (MIC1-e iontophoresis controller, Moor Instruments, Axminster, UK) whilst SNP (negatively charged) is repelled from the chamber containing the negative electrode. ACh acts on the endothelium to induce (endothelial-dependent) cutaneous vasodilation whereas SNP, a nitrate donor, acts directly on vascular smooth muscle (endothelial-independent vasodilation), thereby acting as a control for the experiment. The cutaneous blood flow (or flux) was observed using two optical lasers (one for each chamber) attached to a Laser Doppler perfusion monitor (DRT, Moor Instruments, Axminster, UK). Results were expressed as maximum relative percentage change in perfusion. The average inter- and intraassay CVs were 32% and 33% for Ach; and 21% and 31% for SNP (Section III).

Patients were studied in quiet, darkened, temperature-controlled room (between 19-23ºC according to manufacturer guidelines).

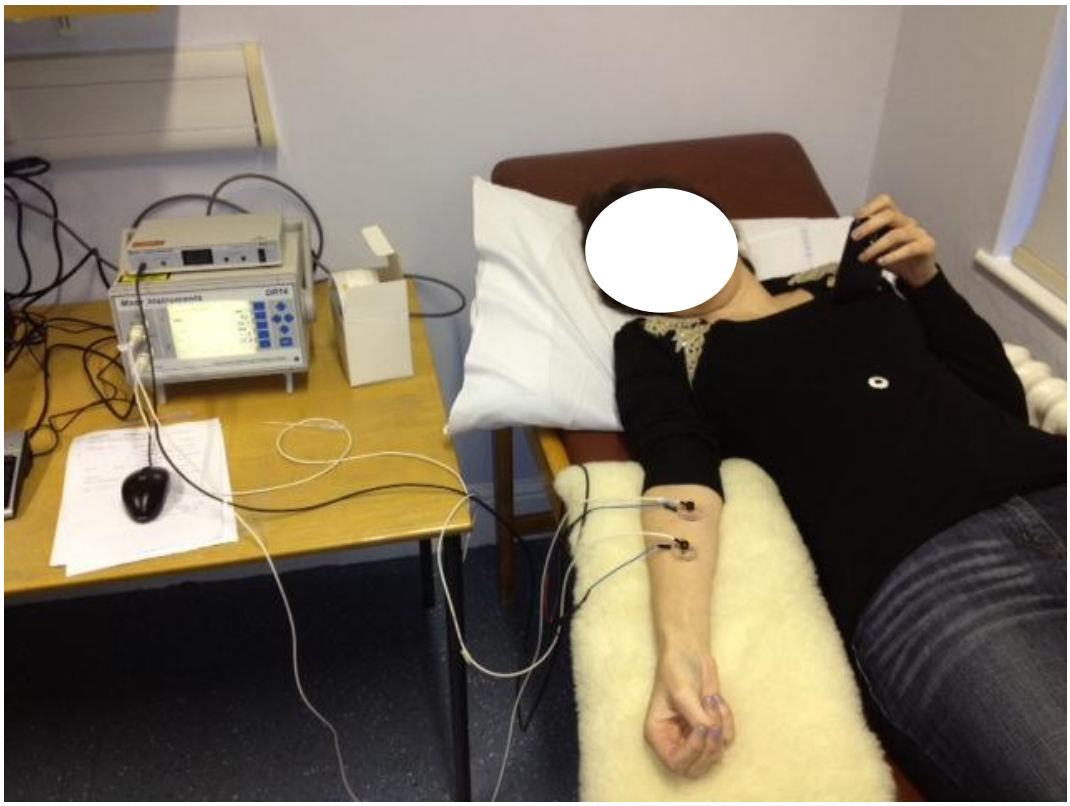


Figure 2.5 Example measurement of cutaneous microvascular function.

2.3.3 Assessment of monocyte subsets and monocyte-platelet aggregates

2.3.3.1 Monocyte subsets

Flow cytometric analysis of monocyte subsets was performed by me using the Becton Dickinson (BD) FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) as previously described by our group[296] (Figure 2.6). Mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK), anti-CD14-PE (clone MϕP9, BD), anti-CD42a-PerCP (clone Beb1, BD) and anti-CCR2-APC (clone 48607, R&D) is mixed with 50 microlitres (μl) of fresh EDTA anticoagulated whole blood in TruCount tubes (BD) containing a strictly defined number of fluorescent count beads. After incubation for 15 minutes, red blood cells are lysed by 450μl of lysing solution® (BD) for 15 minutes, followed by dilution in 1.5 ml of phosphate buffer solution (PBS) and immediate flow cytometric analysis. Monocyte subsets are defined as CD14++CD16-CCR2+ (Mon1, classical), CD14++CD16+CCR2+ (Mon2, intermediate) and (Mon3, non-classical) in accordance with contemporary nomenclature[194].

2.3.3.2 Monocyte-platelet aggregates

Monocyte-platelet aggregates (MPAs) were defined as events positive to both the monocyte markers described above and CD42a (glycoprotein IX). The total MPA count and the MPA count associated with each monocyte subset highlighted above were similarly expressed as a proportion of the number of count beads. Therefore, MPAs associated with Mon1 (classical) are CD14++CD16-CCR2+CD42a+, MPAs associated with Mon2 (intermediate) are CD14++CD16+CCR2+CD42a+ and MPAs belonging to Mon3 (non-classical) subset are CD14+CD16++CCR2-CD42a++. From this, I calculated

the percentage of each monocyte subset aggregated to platelets. I prepared all samples for monocyte and MPA analysis within two hours of venesection as previous work from our department has shown that significant change in absolute count can occur after two hours left standing[297]. The average inter- and intra-assay CV is 13% and 7% for total monocytes, and 12.5% and 14% for total MPAs (Section III).

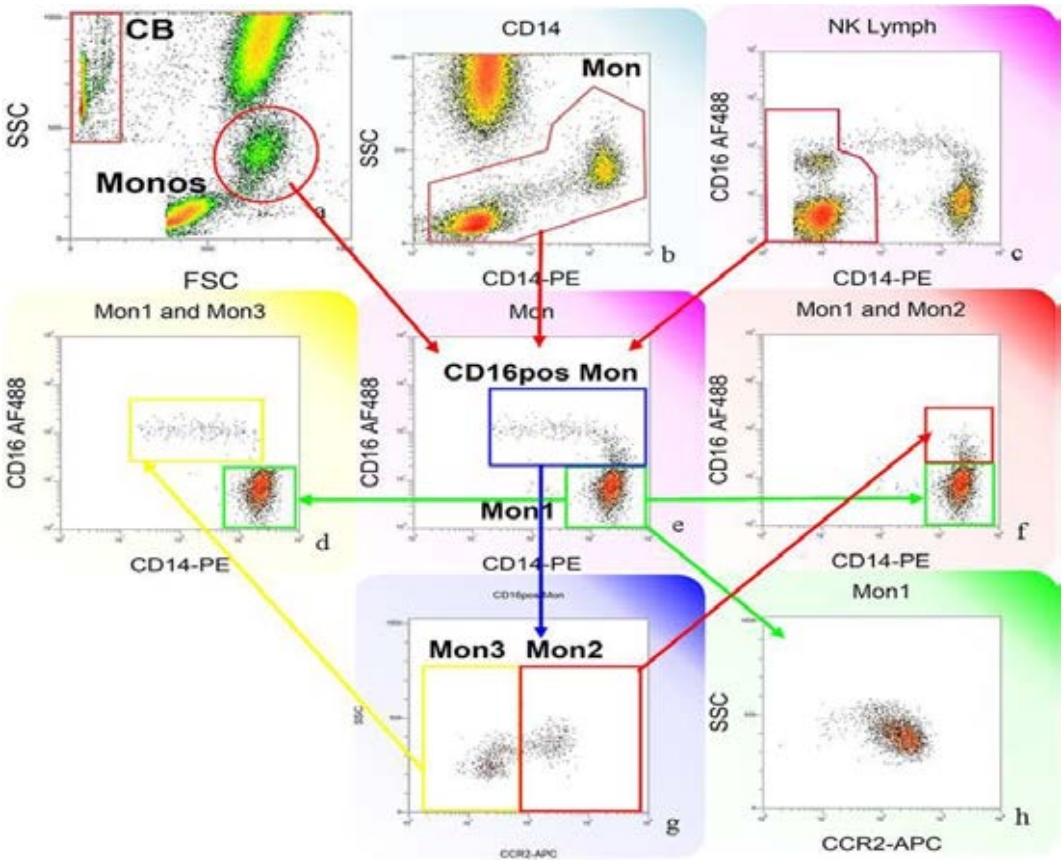


Figure 2.6 Gating strategies and presentation of monocyte subsets.

Monocytes are initially defined according to their forward scatter (FSC) and side scatter (SSC) properties (a). Granulocytes (neutrophils) are then excluded by their higher degree of SSC and lack of CD14 expression (b). CD14- natural killer cells are distinguished from CD14+ monocytes on the CD16 versus CD14 panel (c). Mon1 (green box) are CD14++CD16- cells (d, e and f). CD16+ cells (Mon2 and Mon3) are then distinguished according to their expression of CCR2 (g). Mon2 are CD14++CD16+CCR2+ (red box, g and f). Mon3 are CD14+CD16++CCR2- (yellow box, d and g) (reproduced with permission[298]).

2.3.4 Assessment of circulating platelet and endothelial microparticles

Plasma was separated by centrifugation and frozen at -70°c for batched analysis at the end of recruitment and follow up. I quantified plasma microparticles using the Apogee A50 flow cytometer (Apogee Flow Systems, Hertfordshire, United Kingdom). The Apogee machine has the advantage of being able to measure very small microparticles below 500 nanometres. The protocol was previously established in our department using polystyrene beads of 110, 200, 500 and 1000 nanometre diameter (Apogee Flow Systems, Hertfordshire, United Kingdom) to set up the microparticle-size gate in the two small angle light scatter detectors (LS1 and LS2)[299]. Microparticles were defined as events with sized between 110 and 1000 nm and gated to test their binding to anti-CD42b (platelets), and anti-CD31 (endothelial) antibodies. Prior to each batch, I calibrated the Apogee cytometer using Apogee Mix beads (Apogee Flow Systems, Hertfordshire, UK) to confirm that the flow rate and bead location within the gates remained stable. Logarithmic scales were used for all channels. Intra-assay CVs for EMPs and PMPs were 5.3% and 14.1% respectively (Section III).

Stored platelet-rich plasma was subject to further centrifugation at 13,000g for 5 minutes to make platelet-free plasma. Platelet microparticles were stained with 5µl of 10% biotinylated anti-human CD42b (glycoprotein [GP] Ib) antibody (Abcam, Cambridge, United Kingdom) and incubated for 30 minutes, at which point 2.5µl of 10% streptavidin-Alexa Fluor 647 nm-R-phycoerythrin (PE) tandem conjugate (Molecular Probes, Invitrogen, Carlsbad, CA) was added, followed by a second incubation of 30 minutes. I then diluted the sample with 950µl of filtered phosphate buffered saline (PBS) (final dilution 1:20). I followed the same procedure with endothelial microparticles instead using biotinylated anti-human CD31 antibody (Abcam, Cambridge, United Kingdom).

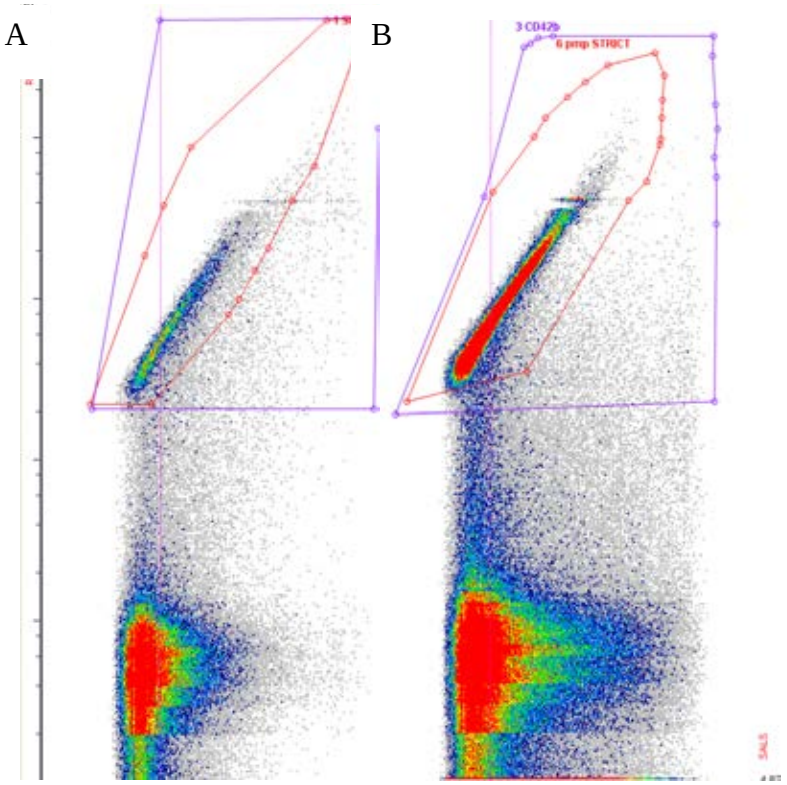


Figure 2.7 Example dot plots showing enumeration of microparticles.

Strict CD31 (endothelial, A) and CD42b (platelets, B) positive events are recorded within the red gates according to previously determined bead sizes 110-1000nm (picture taken during my data collection).

2.3.5 Laboratory measurements

Routine clinical haematological (full blood count [FBC]) and biochemical (urea and electrolytes, cholesterol [total, HDL and non-HDL] and triglycerides) measures were performed by SWBH Trust clinical pathology laboratories (XN10 analyser, Sysmex, Milton Keynes, UK for haematology and Abbott Architect C4000, Abbott laboratories, Illinois, USA for biochemistry).

2.3.6 Health-related quality of life

Of the available measures, the EQ-5D (EuroQuol 5 dimension) has become one of the most commonly used generic preference-based measures in economic evaluation. The EQ-5D comprises five dimensions of health (mobility, self-care, usual activities, pain/discomfort, and anxiety/depression). The EQ-5D-5L has five levels of severity on each dimension of health (superseding the previous version EQ-5D-3L, with three levels). There is also a visual analogue score (VAS), for people to rate their overall health from 0-100. The EQ-5D-5L and VAS are simple, standardised, generic measures that are applicable to a wide range of conditions, treatments and populations[300-304]. Subjects can self-classify their overall health status as a single index value. The tool has been assessed and validated in a large European stable CAD cohort, including UK patients[305]. Broadly, instruments for measuring HrQOL are generic or disease-specific. I felt a generic tool was more appropriate since disease-specific tools tend either to focus on a single recent event or diagnosis, or are only applicable to people with symptoms, whereas many of my cohort with established CAD have been event free for months or years. Permission for use in my study was granted by EuroQuol Research Foundation.

2.4 STATISTICAL CONSIDERATIONS

2.4.1 Power Calculation

From previous work, I propose that arterial elastance index (EaI) will be increased by 2/3rd of a standard deviation in patients with diffuse CAD compared to focal CAD and a similar magnitude of difference between subjects with focal CAD and normal controls (i.e. about 1.3±0.5 mm Hg/ml/m^2 in focal patients, 1.7±0.5 mm Hg/ml/m^2 in patients with diffuse disease, and 1.0±0.3 in normal controls). Thus, to achieve this difference in variance at 1-β = 0.8 and $p<0.05$ (ANOVA *F* statistic approximately 10), 45 subjects per group are required. For the longitudinal study, I expect an increase in EaI by 0.5 SD over a 6-month period with significant change in the disease control group, paired data on n=35 patients are required. This calculation is also sufficient for the measures of endothelial function and biomarkers[43, 306, 307].

2.4.2 Statistical Analysis

Continuous data were subject to Shapiro-Wilks test for normality of distribution. Normally distributed continuous data are expressed as mean and standard deviation (SD). Non-normally distributed continuous data are expressed as median and interquartile range (IQR). Comparisons between the three groups were performed with one-way analysis of variance (ANOVA) with Tukey's post hoc test of pairwise comparisons (for normally distributed data) or Kruskal-Wallis test with Dunn-Bonferroni's post hoc method (for non-normally distributed data). Categorical data are expressed as number (%) and compared using the chi-squared test with appropriate degrees of freedom or Fishers Exact test for comparisons between two groups. Correlations between continuous study measures were performed using Spearman rank method. Independently predictive

associations were assessed using multivariate linear or logistic regression models as appropriate. Longitudinal data were analysed using Friedman test. A two-sided $p < 0.05$ is considered significant. Data obtained were summarised in spreadsheets (Microsoft Excel) and analysed using SPSS version 23.

As part of my doctorate training, I attended tutorials in statistical methodology from Dr Andrew Blann and Dr Eduard Shantsila at the University of Birmingham Institute of Cardiovascular Sciences.

SECTION III

VALIDATION AND REPRODUCIBILITY

This section shows a record of all validation experiments I performed prior to recruitment to ensure competence with the methodology and accurate recording of data.

3.1 LASER DOPPLER FLOWMETRY

Coefficients of variation (CVs) were assessed on four subjects over three days and recorded as percentage increase from mean baseline to maximum flow. The average interassay CVs were 32% for ACh and 21% for SNP (Table 3.1).

Table 3.1 Interassay coefficients of variation for acetylcholine and sodium nitroprusside

Subject	Agonist	1st day	2nd day	3rd day	Mean	SD	CV (%)
1	ACh	816	1478	1161	1152	321	29
	SNP	1153	1627	848	1209	393	33
2	ACh	647	1929	1436	1337	647	48
	SNP	2148	1780	1872	1933	192	10
3	ACh	1113	1341	665	1040	344	33
	SNP	1262	928	851	1014	218	22
4	ACh	355	503	502	453	85	19
	SNP	876	670	626	724	133	18

ACh acetylcholine, SNP sodium nitroprusside.

Intra-assay CVs were assessed three times in the same day, on three subjects and recorded as percentage increase to the nearest whole number from mean baseline to maximum flow (flux). Average intra-assay CVs were 33% for ACh and 31% for SNP.

Table 3.2 Intra-assay coefficients of variation for iontophoresis of acetylcholine and sodium nitroprusside

Subject	Agonist	1st time	2nd time	3rd time	Mean	SD	CV (%)
1	ACh	202	123	109	145	50	35
	SNP	264	437	727	476	234	49
2	ACh	1432	989	710	1044	364	35
	SNP	846	655	499	667	174	26
3	ACh	847	677	460	661	194	29
	SNP	1337	1150	910	1132	214	19

ACh acetylcholine, SNP sodium nitroprusside.

3.2 PULSE WAVE ANALYSIS

Measures recorded using the SphygmoCor device (Section II) are shown below. I studied four healthy volunteers measured on three consecutive days to ascertain interassay CVs. Data are recorded to a maximum of 3 significant figures.

Table 3.3 Interassay coefficients of variation for pulse wave analysis, aortic augmentation index, heart rate and operator index

Subject	Measurement	Day 1	Day 2	Day 3	Mean	SD	CV
1	Ao S	117	116	117	117	0.58	0.5%
	Ao D	93	93	93	93	0.00	0.0%
	MAP	104	104	103	104	0.58	0.6%
	AIx	2	-1	1	1.5	0.7	47.1%
	HR	50	54	49	51.00	2.65	5.2%
	OI	100	100	89	96.3	6.35	6.6%
2	Ao S	118	119	120	119	1	0.8%
	Ao D	82	82	82	82	0	0.0%
	MAP	99	99	100	99.3	0.58	0.6%
	AIx	4	6	8	6	2	33.3%
	HR	63	67	65	65	2	3.1%
	OI	97	90	90	92.3	4.04	4.4%
3	Ao S	125	121	122	123	2.08	1.7%
	Ao D	83	83	84	83.3	0.58	0.7%
	MAP	102	101	101	101	0.58	0.6%
	AIx	9	2	5	5.33	3.51	65.8%

	HR	61	69	67	65.7	4.16	6.3%
	OI	98	91	99	96	4.36	4.5%
4	Ao S	126	123	128	126	2.52	2.0%
	Ao D	89	89	90	89.3	0.58	0.6%
	MAP	104	102	105	104	1.53	1.5%
	AIx	31	26	48	35	11.5	33.0%
	HR	51	63	52	55.3	6.66	12.0%
	OI	100	92	96	96	4	4.2%

AIx Aortic augmentation index, AoD Aortic diastolic pressure (central), AoS Aortic systolic pressure (central), CV Coefficient of variation, HR Heart rate, MAP Mean arterial pressure (central), OI Operator index, SD standard deviation.

The average interassay CV for each measure is shown in Table 3.4.

Table 3.4 Average interassay coefficient of variation for pulse wave measures

Measurement	**Mean interassay CV (%)**
Ao S (mmHg)	1.25
Ao D (mmHg)	0.33
MAP (mmHg)	0.83
AIx (%)	44.8
HR (bpm)	6.65
OI (arbitrary)	4.93

AIx Aortic augmentation index, AoD Aortic diastolic pressure (central), AoS Aortic systolic pressure (central), CV Coefficient of variation, HR Heart rate, MAP Mean arterial pressure (central), OI Operator index.

For intra-assay CVs of pulse wave measures three healthy volunteers were studied three times in succession on the same day (Table 3.5).

Table 3.5 Intra-assay coefficient of variation for pulse wave measures

Subject	Parameter	1st	2nd	3rd	Mean	SD	CV (%)
1	Ao S	114	115	115	115	0.58	0.5
	Ao D	74	74	74	74	0	0.0
	MAP	92	94	93	93	1	1.1
	AIx	11	12	12	11.7	0.58	4.9
	HR	52	53	53	52.7	0.58	1.1
	OI	100	81	100	93.7	11	11.7
2	Ao S	106	98	98	100	4.62	4.6
	Ao D	69	69	69	69	0	0.0
	MAP	83	81	82	82	1	1.2
	AIx	26	19	20	21.7	3.79	17.5
	HR	44	44	43	43.7	0.58	1.3
	OI	90	98	95	94.3	4.04	4.3
3	Ao S	125	120	119	121	3.22	2.6
	Ao D	83	83	83	83	0	0.0
	MAP	102	100	97	99.7	2.52	2.5
	AIx	9	1	-6	5.0	5.66	113
	HR	61	63	65	63	2	3.2
	OI	98	93	95	95.3	2.52	2.6

AIx Aortic augmentation index, AoD Aortic diastolic pressure (central), AoS Aortic systolic pressure (central), CV Coefficient of variation, HR Heart rate, MAP Mean arterial pressure (central), OI Operator index, SD standard deviation.

Average intra-assay CVs for the measures in Table 3.5 are shown in Table 3.6.

Table 3.6 Average intra-assay coefficients of variation for pulse wave analysis, aortic augmentation index, heart rate and operator index

Parameter	Mean intra-assay CV (%)
Ao S (mmHg)	2.57
Ao D (mmHg)	0
MAP (mmHg)	1.60
AIx (%)	45.2
HR (bpm)	1.87
OI (arbitrary)	6.20

AIx Aortic augmentation index, AoD Aortic diastolic pressure (central), AoS Aortic systolic pressure (central), CV Coefficient of variation, HR Heart rate, MAP Mean arterial pressure (central), OI Operator index.

Inter- and intra-assay CVs for AIx were high due to low numbers including some minus figures recorded in younger healthy volunteers. I repeated them in older healthy volunteers (mean age 58±2.6) more representative of the age of my study population. The results are shown in Tables 3.7 and 3.8.

Table 3.7 Repeat AIx interassay coefficients of variation

Subject	Day 1	Day 2	Day 3	Day 4	Mean	SD	CV (%)
1	23	27	24	22	24	2.16	9
2	36	35	34	32	34.3	1.71	5
3	32	26	27	29	28.5	2.65	9.3

CV Coefficient of variation.

Average interassay AIx CV (%) = 7.8

Table 3.8 Repeat AIx Intra-assay coefficients of variation

Subject	1	2	3	4	5	Mean	SD	CV (%)
1	39	37	36	39	38	37.8	1.30	3.4
2	27	27	27	25	27	26.6	0.89	3.4
3	32	36	36	36	37	35.4	1.95	5.5

CV Coefficient of variation.

Average intra-assay CV for AIx (%) = 4.1

3.3 MONOCYTES

Table 3.9 shows interassay CVs for total monocytes and total MPAs performed using samples from the same three individuals for three consecutive days.

Table 3.9 Interassay coefficients of variation for total monocytes and monocyte-platelet aggregates

Subject	Day 1		Day 2		Day 3		Mean		SD		CV (%)	
	Mon	MPA	Mon	MPA	Mon	MPA	Mon	MPA	Mon	MPA	Mon	MPA
1	312	31.9	241	26.9	277	30.5	277	29.8	35.5	2.6	12.8	8.7
2	318	31.8	466	54.5	409	50.4	398	58.2	74.4	10.1	18.7	17.4
3	362	43.5	426	54.7	414	49.7	400	49.3	34.1	5.63	8.5	11.4

CV coefficient variation, Mon monocyte, MPA monocyte-platelet aggregate, SD standard deviation.

Average interassay CV is 13% for total monocytes and 12.5% for total MPAs.

To determine intra-assay variability, I took a blood sample from each subject and then, using that single sample, prepared three further samples for consecutive analysis.

Table 3.10 Intra-assay coefficients of variation for monocyte subsets and monocyte-platelet aggregates

Subject	Parameter	Sample 1	Sample 2	Sample 3	Mean	SD	CV (%)
1	Mon 1	270	226	246	248	22.1	8.9
	Mon 2	37.3	26.0	27.1	30.2	6.22	20.6
	Mon 3	15.8	14.9	14.5	15.1	0.66	4.4
	Total Mon	323	267	288	293	28.4	9.7
	MPA 1	28.6	17.0	24.9	23.5	5.93	25.2
	MPA 2	9.6	4.98	7.90	7.50	2.35	31.3
	MPA 3	1.45	2.28	3.18	2.30	0.87	37.6
	Total MPA	39.7	22.2	36.0	33.3	8.09	24.3
2	Mon 1	508	488	490	495	10.9	2.2
	Mon 2	75.3	66.6	76.0	72.6	5.24	7.2
	Mon 3	33.9	34.3	36.2	34.8	1.24	3.6
	Total Mon	617	589	602	603	14.0	2.3
	MPA 1	38.9	42.8	42.1	41.3	2.06	5.0
	MPA 2	10.7	6.85	10.4	9.33	2.15	23.1
	MPA 3	5.61	4.9	7.07	5.86	1.11	18.9
	Total MPA	55.3	54.6	59.6	56.5	2.71	4.8
3	Mon 1	140	169	157	156	14.7	9.4
	Mon 2	18.7	23.0	20.4	20.7	2.16	10.4
	Mon 3	14.4	15.3	15.0	14.9	0.43	2.9
	Total Mon	173	207	193	191	17.2	9.0
	MPA 1	10.8	12.5	10.7	11.3	1.03	9.0
	MPA 2	2.84	4.62	3.26	3.57	0.93	26.0
	MPA 3	2.25	2.29	1.91	2.15	0.21	9.7
	Total MPA	15.9	19.4	15.9	17.1	2.05	12.0

Mon monocyte, MPA monocyte-platelet aggregate.

The average intra-assay CVs from the monocyte measures are shown in Table 3.11.

Table 3.11 Average intra-assay coefficients of variation for monocyte measures

Monocyte and MPA subset measures	**CV (%)**
Mon1	7
Mon2	13
Mon3	4
Total Mon	7
MPA 1	13
MPA 2	27
MPA 3	22
Overall MPA	14

Mon monocyte, MPA monocyte-platelet aggregate.

3.4 MICROPARTICLES

I prepared ten samples each from the frozen plasma of four healthy volunteers. Five samples were for elucidation of endothelial microparticles and five for platelet microparticles (Tables 3.12 and 3.13). After experimenting with different dilutions, I found that a 1:20 dilution of sample to phosphate buffered saline (PBS) produced the most reliable results.

Table 3.12 Intra-assay coefficients of variation for EMPs

Sample (1,000,000/µl)	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**	**Mean**	**SD**	**CV (%)**
1	4.4	3.8	3.6	4.3	4.4	4.1	0.37	9.0
2	10	10	11	10	9.9	10	0.26	2.6
3	12	13	13	13	13	13	0.45	3.5
4	1.1	11	11	11	12	11	0.35	3.1
5	9.7	9.3	11	11	9.0	9.9	0.82	8.3

Average EMP intraassay CV 5.3%

Table 3.13 Intra-assay coefficients of variation for PMPs

Sample (10,000/µl)	Test 1	Test 2	Test 3	Test 4	Test 5	Mean	SD	CV (%)
1	303	387	394	379	202	333	81.9	24.6
2	244	252	258	213	191	232	28.5	12.3
3	1.05	1.13	0.95	0.80	1.03	0.99	0.13	12.8
4	1.76	1.65	1.60	2.19	1.71	1.78	0.23	13.1
5	637	697	700	626	579	647	51.1	7.8

Average PMP intra-assay CV 14.1%

3.5 ECHOCARDIOGRAPHY

I performed all the measures required to derive the elastance values on three healthy volunteers on three consecutive days (Tables 3.14 and 3.15).

Table 3.14 Interassay coefficients of variation for echocardiographic measures

Subject	Measure	Day 1	Day 2	Day 3	Mean	SD	CV (%)
1	Ea	2.66	2.73	2.71	2.70	0.04	1.3
	Ees	3.32	3.81	3.76	3.63	0.27	7.4
	Ea/Ees	0.83	0.67	0.69	0.73	0.09	11.9
	Eed	0.08	0.09	0.09	0.09	0.01	6.7
	EF	55	60	62	59	3.61	6.1
2	Ea	1.65	1.69	1.72	1.69	0.04	2.1
	Ees	2.20	2.30	2.3	2.27	0.06	2.5
	Ea/Ees	0.75	0.69	0.72	0.72	0.03	4.2
	Eed	0.04	0.04	0.03	0.04	0.01	15.7
	EF	57	59	60	59	1.53	2.6
3	Ea	1.4	1.7	1.6	1.57	0.15	9.8
	Ees	3.7	4.1	4	3.93	0.21	5.3
	Ea/Ees	0.37	0.4	0.33	0.38	0.04	9.6
	Eed	0.08	0.09	0.08	0.08	0.01	6.9
	EF	72	69	67	69	2.52	3.6

CV coefficient of variation, Ea arterial elastance, Eed end-diastolic elastance, EF ejection fraction, Ees end-systolic elastance, SD standard deviation.

Table 3.15 Average coefficient of variation for each measure of elastance and left ventricular ejection fraction

Parameter	Mean CV (%)
Ea	4.4
Ees	5.1
Ea/Ees	8.6
Eed	9.8
EF	4.1

CV coefficient of variation, Ea arterial elastance, Eed end-diastolic elastance, EF ejection fraction, Ees end-systolic elastance.

3.6 SECTION SUMMARY

In this section, the CVs for the methods used in this thesis are presented. In general, they are low and in keeping with previous literature. Agarwal et al found that iontophoresis of ACh measured as relative percentage change had an average CV of 25.5% with ranges from 23% to 39%[308]. The sphygmoCor technique is well established with good repeatability and reproducibility demonstrated in a variety of populations[309-311]. If anything, AIx tends to be the most variable as in young highly compliant vessels, the AIx can be negative. There is little data on monocyte and microparticle CVs as they will depend upon individual laboratory methods. Previous data from our lab has shown absolute monocyte count CV to be around 5%, EMPs <13% and PMPs 18.2% (unpublished data). Echocardiographic measures of LV ejection fraction and volumes (used to estimate elastances) are well known to have intra- and interobserver variabilities of 5-10% depending upon the method used. Three-dimensional, non-contrast measures (as used in my study) have been shown to have the lowest temporal variability[312]. I did not assess interobserver variation as I was to be the sole operator throughout the study.

SECTION IV

OBSERVATIONAL ANALYSES OF CLINICAL DATA – THE LOCAL BURDEN OF CORONARY ARTERY DISEASE

4.1 SECTION INTRODUCTION

The United Kingdom (UK) comprises an ethnically diverse society with approximately 13% (8.7 million) of the population belonging to black and minority ethnic groups (BMEGs) amongst which there is considerable heterogeneity. Although resident throughout the UK, large clusters of BMEGs appear in metropolitan areas of major conurbations[313]. Birmingham, the second most populous city in the UK, has approximately three times the national average of BMEGs[314].

The major burden of cardiovascular (CV) disease within these groups is partially explained by the higher prevalence of traditional CV risk factors such as hypertension and diabetes. Although extensive, there is considerable inter- and intra-ethnic variability of CV disease. For example, African Americans have higher rates of CAD than Afro-Caribbeans living in Britain or natively, who are more likely to develop cerebrovascular or renal disease than CAD. On the other hand, the South Asian diaspora have similar epidemic proportions of CAD as do those who remain on the subcontinent[313]– defined as India, Pakistan, Sri Lanka, Nepal, and Bangladesh – and within which, there is also significant heterogeneity[315, 316]. Almost 30% of the Birmingham population are South Asian.

Epidemiological evidence shows that many South Asian groups – male and female – living in the western world have a higher burden of cardiovascular disease than other ethnicities[315, 317]. Furthermore, CAD develops at a younger age (often <40 years in men) and is more aggressive in South Asians[318]. While major risk factors like diabetes and abdominal obesity are more prevalent in South Asians, other important risk factors such smoking are much less, creating a paradox[319]. Some authors have suggested that South

Asians have smaller coronary artery lumens thus theorising that even the same burden of CAD becomes severe more quickly than it otherwise would[320]. Others have reported a difference in high and low-density lipoprotein (HDL and LDL) particle size as a potential contributor. The smaller LDL particles found in Asian Indians are more susceptible to oxidative stress and hence more atherogenic, whilst the smaller HDL particles identified in the same group are less protective than larger ones[321].

Whilst the precise underlying mechanisms are unclear, many of these observations are reflected in the following analyses of our own clinical cardiology databases, which delineate the wider CAD population from which my prospective study patients were selected.

4.2 ETHNIC DIFFERENCES IN THE DIURNAL VARIATION OF SYMPTOM ONSET TIME FOR ACUTE ST-ELEVATION MYOCARDIAL INFARCTION – AN OBSERVATIONAL COHORT STUDY

4.2.1 Introduction

A morning peak of ST-segment elevation myocardial infarction (STEMI) has traditionally been described however variations of this common perception exist[322-324]. Studies regarding South Asian populations are conflicting with some showing complete absence of circadian rhythm[323] and others reporting an excess between midnight and noon[324]. There is little data examining circadian symptom onset of acute myocardial infarction (AMI) in Afro Caribbeans. This study compares the circadian rhythm of STEMI onset in White European (WE), South Asian (SA) and Afro Caribbean (AC) patients.

4.2.2 Hypothesis

There will be differences in peak time of presentation of AMI between the ethnicities.

4.2.3 Methods

The methods described below have been published. I did all the data collection, analysis and wrote the final paper.

4.2.3.1 Patients and setting

In a retrospective survey of our British Cardiovascular Intervention Society (BCIS) database, I identified consecutive patients undergoing primary percutaneous coronary intervention (PPCI) for acute STEMI at our institution (Sandwell and West Birmingham Hospitals [SWBH] NHS Trust) between January 2008 and June 2014. The BCIS database

collects data from all hospitals in the UK that perform PCI and records information about every procedure performed[325].

PCI is defined as the use of any coronary device to approach, probe, or cross one coronary lesion with the intention of performing a coronary intervention. The database is compliant with UK data protection legislation. Data are collected prospectively at each hospital, electronically encrypted, and transferred online to a central database. Each patient entry offers details of the patient journey, including the method and timing of admission, inpatient investigations, results, treatment, and outcomes[325]. Acute STEMI was diagnosed in the setting of chest pain accompanied by an ECG demonstrating ST-segment elevation. Afro Caribbean, WE and SA patients were categorised into groups. Symptom onset time was based on patient recollection during the history and the ambulance record. Hospital arrival time was based on the recorded arrival time at the emergency department (ED). For patients already in hospital it was taken as the symptom onset time. Both timings were categorised into six 4-hour intervals (00:00-03:59, very early morning; 04:00-07:59, early morning; 08:00-11:59, morning; 12:00-15:59, afternoon; 16:00-19:59, evening; and 20:00-23:59, night). Definitions of the clinical and procedural characteristics recorded for each group are listed in Appendix 7.

4.2.3.2 Outcomes

We recorded and compared the proportion of each ethnic group with symptom onset and hospital arrival in a particular time category. We also analysed the association between symptom onset time and door-to-balloon time (DTBT). DTBT was defined as the time in minutes from the arrival at the PCI capable centre to inflation of the first device restoring patency to the infarct related artery (IRA). To remove ‘nonsense’ results from the analysis

brought about by incorrect recording of dates associated with the times (leading to several minus figures) we excluded patients with a recorded door-to-balloon time of less than 10 minutes. We also excluded patients with a DTBT of greater than 300 minutes, partly to include the patients who had the most to gain from myocardial salvage and partly to exclude recording errors.

4.2.3.3 Statistical analysis

Continuous, normally distributed variables are presented as mean and standard deviation (SD) – non-normal data as median plus interquartile range (IQR). Continuous data were analysed using one-way analysis of variance (ANOVA) if normally distributed or Kruskal-Wallis H Test if not normally distributed. Categorical variables are presented as numbers and percentages and were analysed using Chi Square. Standard and hierarchical multiple regression were used to determine the predictive capabilities of variables where necessary. A two-sided p value <0.05 was considered statistically significant. SPSS version 21 was used for analysis.

4.2.4 Results

4.2.4.1 Patients

A total of 1505 patients underwent PPCI between the specified time points and are included in the analysis. The cohort consisted of 68 ACs (4.5%), 1021 (68%) WEs, 407 (27%) SAs, 7 (0.5%) 'other' and 2 (0.1%) unspecified cases. After exclusion of the 7 'other' and 2 non-specified cases 1496 patients remained in the analysis.

Baseline and procedural data with unadjusted p values are shown in Table 4.1. WEs were significantly older at symptom onset ($p<0.001$), more likely to be current smokers ($p<0.001$) and less likely to be diabetic ($p<0.001$) than SA and AC patients. South Asians

were less often female (p=0.007), and had significantly more hypercholesterolaemia (p=0.007) whilst both SAs and ACs were more likely to have hypertension (p=0.013). White Europeans had fewer radial procedures (p=0.011) whilst SAs were more likely to have multivessel disease (p=0.046). South Asians and ACs tended to have left anterior descending (LAD) artery involvement more often (p=0.058).

Table 4.1 Clinical characteristics by ethnicity

Characteristic	Afro Caribbean (n=68)	White European (n=1021)	South Asian (n=407)	p value
Age (SD)	57 (14)	63 (13)	58 (14)	<0.001
Females n (%)	22 (32)	277 (27)	81 (20)	0.007
Elderly n (%)	10 (15)	235 (23)	60 (15)	0.001
Family history of CAD n (%)*	15 (22)	319 (32)	142 (36)	0.051
Previous MI n (%)	8 (12)	163 (16)	80 (20)	0.125
Previous CABG n (%)	1 (1.5)	26 (2.5)	19 (4.7)	0.082
Previous PCI n (%)	7 (10)	131 (13)	59 (14)	0.545
Diabetes n (%)	24 (35)	153 (15)	156 (38)	<0.001
Hypertension n (%)	39 (57)	475 (47)	219 (54)	0.013
Hypercholesterolaemia n (%)*	21 (31)	425 (43)	195 (50)	0.007
Ventilation pre-admission n (%)	2 (2.9)	59 (5.8)	24 (5.9)	0.603
Cardiogenic shock n (%)	9 (13)	124 (12)	61 (15)	0.352
Current smoking n (%)*	26 (38)	450 (46)	108 (28)	<0.001
PVD n (%)*	3 (4.4)	86 (8.5)	23 (5.7)	0.122
Previous stroke n (%)	3 (4.4)	52 (5.1)	15 (3.7)	0.545
Renal failure n (%)*	1 (1.5)	24 (2.4)	15 (3.8)	0.313
Radial n (%)	46 (68)	531 (54)	235 (61)	0.011
Circulatory support n (%)	5 (7.6)	110 (11)	53 (13)	0.279
Thrombus aspiration n (%)	27 (40)	445 (44)	181 (44)	0.762
LAD culprit n (%)	33 (49)	429 (43)	197 (50)	0.058
Longest stent (mm)	23 (18-38)	23 (16-31)	23 (18-32)	0.339
Largest balloon median (IQR)	3.25 (3.0-3.5)	3.25 (3.0-3.5)	3.25 (3.0-3.5)	0.439

GP 2b/3a inhibitors n (%)	20 (29)	317 (31)	131 (32)	0.844
No. of vessels attempted (mean+SD)	1.07 (0.26)	1.07 (0.35)	1.12 (0.46)	0.046
In hospital death n (%)	6 (8.8)	64 (6.3)	30 (7.4)	0.580

* One-way ANOVA, ** denominator reflects cases reported, § Kruskal-Wallis, all other tests Chi square, SD standard deviation, IQR interquartile range, CAD coronary artery disease, MI myocardial infarction, PCI percutaneous coronary intervention, CABG coronary artery bypass graft, PVD peripheral vascular disease, LAD left anterior descending (artery), GP glycoprotein.

4.2.4.2 Outcomes

4.2.4.2.1 Symptom onset

The proportions of each group that developed symptoms during each categorised time are shown in Table 4.2. Overall the highest incidence of AMI symptom onset was between 08:00 and noon (n=352) with WE and SA ethnicities mirroring this trend (both $p < 0.001$). A significantly higher proportion of the SA population, compared to the AC and WE population in this sample, developed symptoms between 20:00 and 23:59 (p=0.026) (Figure 4.1.) This remained significant in a multivariate logistic regression model after adjustment for age and gender (odds ratio [OR] 1.54, confidence interval [CI] 1.12-2.11, p=0.008) and after further adjustment for other significantly different baseline variables (diabetes, hypertension, hypercholesterolaemia and current smoking) (OR 1.60, CI 1.12-2.28, p=0.009.) Forty four percent of WEs developed symptoms between 08:00 and 15:59, with a significantly higher proportion experiencing symptoms between 12:00 and 15:59 when compared with SAs alone (p=0.011 data not shown.) After adjustment for the same variables this remained significant at p=0.017 (OR 1.52 CI 1.08-2.14.) Afro Caribbean ethnicity was not associated with diurnal variation in AMI symptom onset (symptom onset between 08:00-12:00 versus 20:00-23:59 p=0.19).

Table 4.2 Timing of symptom onset throughout the day

Time	Afro Caribbean (n=68)	White European (n=1020)	South Asian (n=405)	p value
00:00-03:59	9 (13)	127 (12)	59 (14)	0.580
04:00-07:59	12 (18)	159 (16)	74 (18)	0.465
08:00-11:59	12 (18)	250 (24)	90 (22)	0.320
12:00-15:59	12 (18)	202 (20)	57 (14)	0.038
16:00-19:59	13 (19)	157 (15)	53 (13)	0.322
20:00-23:59	10 (15)	125 (12)	72 (18)	0.026

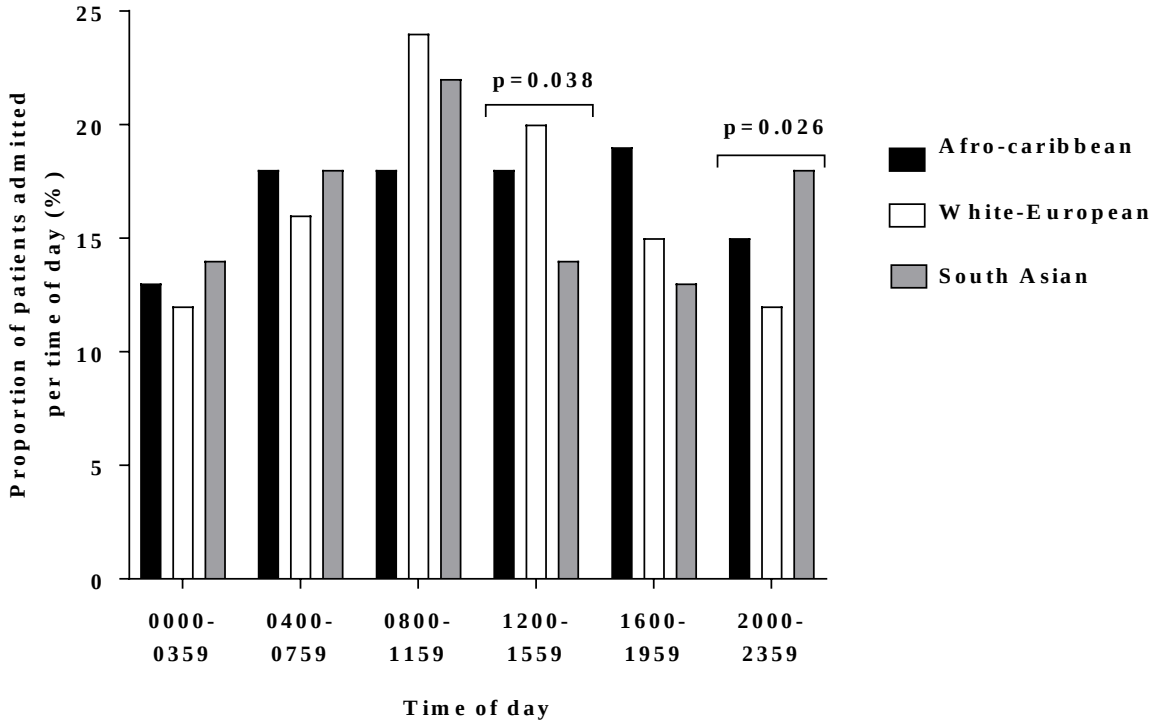


Figure 4.1 Diurnal variation of symptom onset times by ethnicity (p values unadjusted).

4.2.4.2.2 Hospital presentation

Despite the differences between ethnicities in the timing of symptom onset throughout the day there did not appear to be any differences in the timing of presentation to hospital (Table 4.3) other than an increased likelihood for Afro Caribbeans to arrive in the evening (16:00-19:59).

Table 4.3 Time of arrival at PCI centre (door time)

Time	Afro Caribbean (n=68)	White European (n=1019)	South Asian (n=407)	p value
00:00-03:59	8 (12)	123 (12)	50 (12)	0.989
04:00-07:59	5 (7.4)	112 (11)	57 (14)	0.144
08:00-11:59	16 (24)	260 (25)	107 (26)	0.876
12:00-15:59	13 (19)	234 (23)	76 (19)	0.187
16:00-19:59	17 (25)	169 (17)	54 (13)	0.038
20:00-23:59	9 (13)	121 (12)	63 (15)	0.181

4.2.4.2.3 Quality measures

The application of criteria to exclude spurious measures enabled us to analyse DTBT in 1370 (92%) patients. Symptom onset between 12:00 and 15:59 was found to be predictive of reduced DTBT at multivariate analysis after correcting for age, gender, ethnicity, diabetes, cardiogenic shock and pre-procedural ventilation (p=0.034). Symptom onset between 12:00 and 15:59 conferred a higher likelihood of achieving the internationally recognised DTBT target of less than 90 minutes (OR 0.66, 95% CI 0.47-0.92; p=0.016). DTBT was marginally longer in those whose symptoms developed at night (20:00-23:59), although this did not reach statistical significance (p=0.09).

4.2.5 Discussion

Notable findings include a morning excess of AMI onset in WE and SA patients but not for ACs, a second night time (20:00-23:59) peak for SAs and an association between afternoon symptom onset and shorter DTBT.

To my knowledge this is the first report of a possible bimodal distribution of AMI symptom onset in SAs who appeared to have a significantly higher night time (20:00-23:59) onset than other ethnicities but this would need to be confirmed with larger numbers (with specific tests for bimodality incorporated into the study design) as would the apparent absence of diurnal variation of AMI symptom onset in ACs. This could be related to ethnic differences in cortisol production[326]. Heart rate and blood pressure also vary less throughout the day in ACs[327], which may be related to the reduced cortisol variation.

Symptom onset time is an important predictor of increased pre-hospital delay, reperfusion times and subsequent infarct size[328-330]. The fact that white patients appear to develop symptoms more frequently during the afternoon than other ethnicities may partly explain the association found in some studies between increased reperfusion time and non-white ethnicity[331].

Limitations include low numbers of Afro Caribbean patients in the cohort, the geographical diversity of the South Asian and Afro Caribbean populations leading to considerable heterogeneity within those groups, and the fact that symptom onset time can be difficult to determine precisely due to inconsistencies in patient recollection.

4.2.6 Conclusion

South Asian ethnicity was associated with an increased likelihood of night time (20:00-23:59) symptom onset compared with WE and AC ethnicities, whilst WEs were more likely than other ethnicities to develop symptoms in the afternoon (12:00-15:59). In contrast to SAs and WEs, ACs did not show a morning excess of AMI symptom onset.

4.3 SYMPTOM-TO-DOOR TIMES IN PATIENTS PRESENTING WITH ST-ELEVATION MYOCARDIAL INFARCTION – DO ETHNIC OR GENDER DIFFERENCES EXIST?

4.3.1 Introduction

The relative contribution of ethnicity itself as an independent predictor of poorer hospital performance markers, such as symptom-to-door time (STDT) or door-to-balloon time (DTBT), leading to poorer outcomes in AMI due to CAD has been evaluated in several studies with conflicting conclusions[325, 332, 333]. Recent analyses have shown no difference in in-hospital mortality between SAs and patients of other ethnicities after adjustment for risk factors[325, 334]. Some investigators report an increased likelihood that SAs and female patients develop atypical chest pain, which may delay the diagnosis of AMI[335]. We recently reported an increased tendency for SA patients to develop symptoms of AMI at night[336], which may affect the immediacy by which people seek help. Despite the perception that these groups have uncharacteristic pain, there is evidence that they seek immediate help just as frequently (if not more so) than WEs and males[337]. This paper examines the association between pre-hospital delay, female gender and ethnic minority patients (AC and SA).

4.3.2 Hypothesis

Women and ethnic minority (AC and SA) patients present to hospital later after the onset of symptoms than their WE and male counterparts.

4.3.3 Methods

4.3.3.1 Study population and setting

At the time of my analysis twenty-four-hour PPCI was delivered to half a million people across the two acute hospital sites at SWBH NHS Trust. The same body of cardiologists covered both sites when on call and the patients' location at the onset of symptoms determined where they were taken. Now, following re-configuration in September 2015, all patients are brought to a single site for coronary intervention (see appendix 7 for procedural definitions).

Using the SWBH version of the BCIS database (described in 4.2.3.1) consecutive patients receiving PPCI for STEMI between January 2008 and January 2013 were identified. STEMI was defined using standard ECG criteria in the presence of ischaemic chest pain. Analyses were performed with the cohort grouped according to gender and then according to ethnicity (SA, AC or WE.)

4.3.3.2 Outcomes

The main outcome measure was STDT which is defined as the time in minutes between a patient arriving at a PCI capable hospital (not first medical contact) and the time of symptom onset according to the patient's recollection. Other outcome measures include the symptom-to-call time (STCT) which is defined as the difference between the onset of symptoms and the call for medical assistance, and in hospital mortality which is defined as death from any cause prior to discharge or at 30 days if the latter was shorter. We excluded those patients with a documented STDT of less than 10 minutes and those patients who were already in hospital at symptom onset.

4.3.3.3 Statistical Analysis

Baseline characteristics were compared across gender types using the Chi squared test for categorical variables and Student's T test or one-way analysis of variance (ANOVA) for continuous normally distributed variables. For non-normally distributed variables the Mann-Whitney U test or the Kruskal-Wallis test was used. Discrete variables are expressed as numbers and percentages, continuous variables as mean and standard deviation or median and interquartile range. Differences in STDT were analysed with non-parametric tests and subject to multivariate analysis to adjust for age, ethnicity (in the female population), gender (between the ethnic groups) and significant baseline variables. In hospital mortality was examined using chi square for between group differences and logistic regression to account for age, gender and differences in baseline characteristics. A p value <0.05 was considered significant. Data were analysed using SPSS 21.

4.3.4 Results

4.3.4.1 Patients

Altogether 1157 patients underwent PPCI during the study period. Eighty patients who were already in the cardiac centre at symptom onset, seventeen inter-hospital transfers and 40 patients with a recorded STDT less than 10 minutes were excluded. One thousand and twenty patients remained in the analysis of which 768 (75%) were male (Table 4.4). South Asian (n=263) and AC (n=38) patients were more likely to be diabetic and younger than WE patients (n=719) and there was significantly less smoking amongst SAs (Table 4.5). Women were more likely to be diabetic, hypertensive and older than males (p=0.019, <0.0001 and <0.001 respectively). They were less likely to smoke, have peripheral

vascular disease or have a family history of CAD, (p=<0.001, 0.009 and 0.039 respectively) and women were more likely to require intra-aortic balloon support than men. There were no differences in the requirement for any form of circulatory support between the ethnic groups.

Table 4.4 Gender related clinical and procedural characteristics

Variable	**Gender (n=1020)**		**p value**
	Male (n=768)	**Female (n=252)**	
Clinical			
Age (mean±SD)	59 (13)	67 (14)	<0.001**
Family history of CAD n (%)	255 (34)	65 (27)	0.04
Previous MI, n (%)	134 (17)	46 (18)	0.77
Previous CABG, n (%)	24 (3)	7 (3)	0.78
Previous PCI, n (%)	109 (14)	33 (13)	0.66
Diabetes n (%)	136 (18)	62 (25)	0.02
Hypertension n (%)	353 (45)	152 (61)	<0.001
Hypercholesterolaemia n (%)	321 (42)	118 (47)	0.18
Ethnicity n (%)			
W-E	530 (69)	189 (75)	
A-C	27 (4)	11 (4)	0.092
SA	211 (27)	52 (21)	
Ventilation pre-admission n (%)	29 (4)	4 (2)	0.09
IABP n (%)	60 (8)	30 (12)	0.04
Current smoking n (%)	327 (44)	77 (31)	<0.001
PVD n (%)	48 (6)	28 (11)	0.009
Previous stroke n (%)	29 (4)	16 (6)	0.08

Renal failure n (%)	16 (2)	8 (3)	0.27
Procedural			
Glycoprotein inhibitors n (%)	290 (38)	85 (35)	0.40
Radial access n (%)	400 (52)	118 (48)	0.32
Thrombus removal n (%)	348 (45)	105 (43)	0.49
Mean no. of DES implanted (SD)	1.14 (1.06)	1.01 (1.06)	0.09**
Median stent diameter mm (IQR)	3.25 (3.0-3.5)	3.0 (2.75-3.5)	0.002*
Median stent length mm (IQR)	23 (15-30)	20 (15-32)	0.78*
Outcomes			
Median STDT (IQR)	113 (68-247)	132 (72-312)	0.07*
Median STCT (IQR)	100 (45-253)	125 (55-316)	0.03*
Median DTBT (IQR)	68 (55-85)	72 (59-89)	0.09*
In hospital death n (%)	33 (4)	27 (11)	<0.001

SD standard deviation, CAD coronary artery disease, MI myocardial infarction, CABG coronary artery bypass grafting, PCI percutaneous coronary intervention, W-E White European, A-C Afro-Caribbean, SA South Asian, IABP intra-aortic balloon pump, PVD peripheral vascular disease, DES drug eluting stent, mm millimetres, STDT Symptom-to-door-time, STCT symptom-to-call-time, DTBT door-to-balloon-time, IQR interquartile range, *Mann-Whitney U, **Student's T test, all other p values derived from chi-squared test, † export catheter.

Table 4.5 Clinical and procedural characteristics by ethnic group

Variable	Ethnicity (n=1020)			p value
	White-European (n=719)	Afro-Caribbean (n=38)	South Asian (n=263)	
Clinical				
Age (mean±SD)	63	55	58	<0.001*
Male n (%)	530 (74)	27 (71)	211 (80)	0.09
Family history of CAD n (%)	217 (31)	12 (32)	91 (36)	0.34
Previous MI, n (%)	120 (17)	6 (16)	54 (21)	0.35
Previous CABG, n (%)	21 (3)	0 (0)	10 (4)	0.42
Previous PCI, n (%)	98 (14)	4 (11)	40 (15)	0.68
Diabetes n (%)	91 (13)	13 (34)	94 (36)	<0.001
Hypertension n (%)	337 (47)	23 (61)	145 (55)	0.05
Hypercholesterolaemia n (%)	294 (41)	13 (34)	128 (49)	0.02
Ventilation pre-admission n (%)	22 (3)	1 (3)	10 (4)	0.82
Current smoking n (%)	314 (45)	16 (43)	74 (29)	<0.001
PVD n (%)	58 (8)	1 (3)	7 (3)	0.64
Previous stroke n (%)	34 (5)	0 (0)	11	0.69
Renal failure n (%)	16 (2)	0 (0)	8 (3)	0.81
IABP n (%)	62 (9)	2 (5)	24 (9)	0.90
Procedural				
Glycoprotein inhibitors n (%)	259 (36)	14 (37)	100 (38)	0.35
Radial access n (%)	342 (48)	23 (61)	150 (57)	0.004
Thrombus removal† n (%)	312 (44)	17 (45)	122 (46)	0.74
Mean no. of DES implanted	1.05 (1.03)	1.13 (1.22)	1.26 (1.08)	0.06*

Median stent diameter mm	3.25	3.5	3.25	0.66***
Median stent length mm	23	20	23	0.51***
Outcomes				
Median STDT (IQR)	115 (69-250)	147 (74-572)	124 (68-267)	0.37***
Median STCT (IQR)	108 (48-261)	147 (72-690)	106 (45-299)	0.32***
Median DTBT (IQR)	69 (55-84)	85 (69-107)	71 (57-90)	0.012
In hospital death n (%)	44 (6.1)	3 (7.9)	19 (7.2)	0.96

SD standard deviation, CAD coronary artery disease, MI myocardial infarction, CABG coronary artery bypass grafting, PCI percutaneous coronary intervention, PVD peripheral vascular disease, IABP intra-aortic balloon pump, DES drug eluting stent, mm millimetres, STDT symptom-to-door-time, STCT symptom-to-call-time, DTBT door-to-balloon-time, IQR interquartile range, *Analysis of variance (ANOVA), ***Kruskal-Wallis test, all other p values Chi-squared test, † export catheter.

4.3.4.2 Outcomes

Females trended towards longer unadjusted STDT (p=0.07) and had significantly longer unadjusted STCT (p=0.03). After correction for age and ethnicity there was no association between female sex and longer STDT or STCT (p=0.15 and p=0.1 respectively). Unadjusted in-hospital mortality was significantly higher in females (p<0.001) but this association was not present on logistic regression after adjustment for age (odds ratio [OR] 0.69 95% confidence interval [CI] 0.40-1.18, p=0.17)

The crude STDT was not different between the ethnic groups (p=0.37). On linear regression analysis after adjustment for age, gender and diabetes SAs showed a trend towards longer STDT than other ethnic groups (p=0.08). Afro Caribbeans did not (p=0.58). Overall SAs showed a trend towards increased hospital mortality which was present on stepwise multivariate logistic regression after adjustment for age and gender (odds ratio [OR] 1.7 95% CI 0.96-3.10, p=0.07), but after adjustment for diabetes there was no association with mortality (OR 1.5, 95% CI 0.80-2.86, p=0.21). There was no univariate association between AC ethnicity and mortality (OR 1.6 95% CI 0.46-5.76, p=0.45).

4.3.5 Discussion

This study was designed to assess the impact of ethnicity and gender on STDT in patients who develop STEMI whilst in the community. We showed a trend towards longer STDT for both females and SAs that disappeared after adjustment for differences in baseline variables between men and women and between SAs and other ethnic groups. Afro Caribbean ethnicity did not confer a higher likelihood of pre-hospital delay.

Symptom-to-door time accounts for the largest proportion of total ischaemic time[338] and, in many cases, is broadly divided into time of symptom onset to call for medical assistance; and call for medical assistance to arrival at the emergency department. The latter usually consists of the time taken for emergency medical services to arrive at the patient's location and then transfer them to the nearest PPCI centre. Other variations of this process exist – patients may initially call their own doctor or transport themselves to a non-PCI capable facility. Previous studies have suggested that SAs are less likely to call emergency medical services following the onset of chest pain[338, 339] and use of such services is associated with shorter STDT. This may contribute to the trend observed in this study. The reasons behind this are not clear but could relate to the increased prevalence of atypical coronary ischaemic symptoms such as abdominal pain, nausea and fatigue without chest pain although as far as I am aware this has only been observed in patients presenting with stable symptoms[319]. In any case the data presented here do not support prehospital delay as a contributor to mortality in SAs which seems to be explained by the excess of cardiovascular risk factors particularly diabetes. Indeed, a recent large registry analysis has shown no difference in mortality between SAs and WEs after adjustment for diabetes[325].

Contrary to previous studies[340] we found no significant gender differences in STDT, suggesting that pre-hospital delay does not contribute to higher inpatient mortality seen in females post STEMI. In accordance with several other studies the excess early female mortality in this cohort was entirely explained by advancing age and its associated comorbidities[341-343]. In contrast to this and other studies, de Boer et al[344] found that the higher female mortality rate did not disappear after adjustment for baselines characteristics. The authors speculate that factors leading to longer symptom-to-balloon

times may have been responsible for this observation in their cohort but were not able to support this owing to a lack of data.

Over the last thirty years, community interventions to reduce patient associated delays have had mixed success. Some studies reported no change in patient behaviour despite an increase in knowledge because of the intervention[338, 345, 346]. Others observed significant reductions in pre-hospital time delay associated with an increased proportion of patients diagnosed with AMI or unstable angina[347, 348]. Some reported significant increases in non-cardiac chest pain attendances whilst others did not. No studies, including one randomised controlled trial, have shown any effect on mortality.

4.3.6 Limitations

Similarly to the previous analysis, there were small numbers of AC patients and time of symptom onset was determined from patient recall and may be inaccurate especially if asked during pain. Significant language barriers might exist between hospital staff and a proportion of the patients also leading to inaccurate recording and inputting of data; however, English-speaking family members are often present or staff members on duty may share the same language as the patient. As an observational study, there is always the possibility of inherent biases and confounders brought about by data that is missing or incorrectly entered although every effort was made to validate our findings by referring to the case notes when necessary. Although, a single centre study, the findings are highly relevant to our everyday clinical practice.

4.3.7 Conclusion

In this retrospective, observational cohort study neither female gender nor ethnicity could be shown to be associated with significant pre-hospital delay. Any differences in, or trends towards increased mortality are explained by differences cardiac risk factors.

4.4 SIMULTANEOUS COMPUTERISED ACTIVATION OF THE PRIMARY PERCUTANEOUS CORONARY INTERVENTION PATHWAY IMPROVES DOOR-TO-BALLOON TIME BUT NOT MORTALITY

4.4.1 Introduction

The major morbidity and mortality caused by the presence of CAD is AMI brought about by the sudden occlusion of one or more coronary arteries, the sequelae of which includes sudden cardiac death and heart failure. The latter half of the previous century has seen vast improvements in the treatment of this potentially devastating complication of CAD culminating in the latest generation of stent technology based on our current understanding of endothelial vascular biology. This has been combined with innovation within hospitals throughout the UK to deliver models of care aimed at providing prompt revascularisation in the setting of AMI. Where ST-segment elevation is present on an ECG the preferred strategy is PPCI[349]. Since our PPCI service began in 2005 considerable efforts have been made to implement evidence-based strategies to reduce DTBT for acute STEMI patients both inside and outside of normal working hours but, mainly due to logistical reasons, DTBTs are more difficult to achieve out-of-hours ([OOH] – 17:00-0900 weekdays, weekends and public holidays)[350].

This paper details the impact of a new protocol introduced in mid-July 2009 to alert the out-of-hours PPCI team. Prior to then, team members were telephoned separately following discussion with the interventionist. Since then, the team has been activated directly by the emergency department or paramedics independently of the cardiologist using a computerised paging system which transmits simultaneously to dedicated mobile telephones carried by each member of the team. Essentially the protocol change was

independently associated with reduced DTBT but mortality throughout the study periods was unchanged.

4.4.2 Hypothesis

The introduction of simultaneous computerised activation of the PPCI pathway would result in shorter DTBT and an increase in the overall proportion of patients achieving DTBT under 90 minutes as recommended in international guidelines and that this would reduce overall mortality.

4.4.3 Methods

4.4.3.1 Patient population

I analysed consecutive patients undergoing OOH PPCI for acute STEMI at our institution between 2007 and 2012 using SWBH NHS Trust Myocardial Ischaemia National Audit Project (MINAP) data. The study cohort was divided into two groups, patients presenting before the introduction of the computerised alert protocol (Group 1) and patients presenting afterwards (Group 2). The MINAP is a national registry of patients admitted to hospital with acute coronary syndrome. It was established in 1999, in response to the national service framework for coronary heart disease, to examine the quality of management of myocardial infarction in hospitals in England and Wales[351]. We excluded 106 patients who were either transferred from other hospitals or developed AMI whilst already in hospital or had a DTBT greater than 300 minutes.

4.4.3.2 Outcome measures

The main outcome measures were DTBT and all-cause pre-discharge and one-year mortality. DTBT is defined in section 4.2.3.2. To evaluate the proportion of patients

meeting target DTBT we divided patients into two groups: <90 minutes and ≥90 minutes and analysed them as categorical variables. We also recorded clinical characteristics and STDT (defined in section 4.3.3.2) since any mortality difference would need to account for this. STDT was categorised as less than 2 hours or greater than 2 hours.

4.4.3.3 Statistical analysis

Based upon strategies already known to improve DTBT[350] we expected to see a reduction of approximately 15 minutes in group 2 as compared with group 1. Baseline characteristics were compared using the Fisher's Exact test or the Chi squared test for categorical variables and analysis of variance for continuous variables. Discrete variables are expressed as numbers and percentages, continuous variables as mean and standard deviation or median and interquartile range. Differences in DTBT were tested in univariate and multivariate logistic analyses and a p value <0.05 was considered significant. The data were analysed using SPSS 21.

4.4.4 Results

Out of a total of 1340 patients, 1234 patients (mean age 61, 75% male) were included in the analysis of which 793 (64%) patients received OOH PPCI – 295 patients in Group 1 and 498 patients in Group 2. The clinical characteristics and outcomes of each group are shown in Table 4.6.

Table 4.6 Clinical characteristics, symptom-to-door time, door-to-balloon time, in-hospital and one-year mortality of patients receiving out-of-hours primary percutaneous coronary intervention before and after implementation of the computerised alert protocol

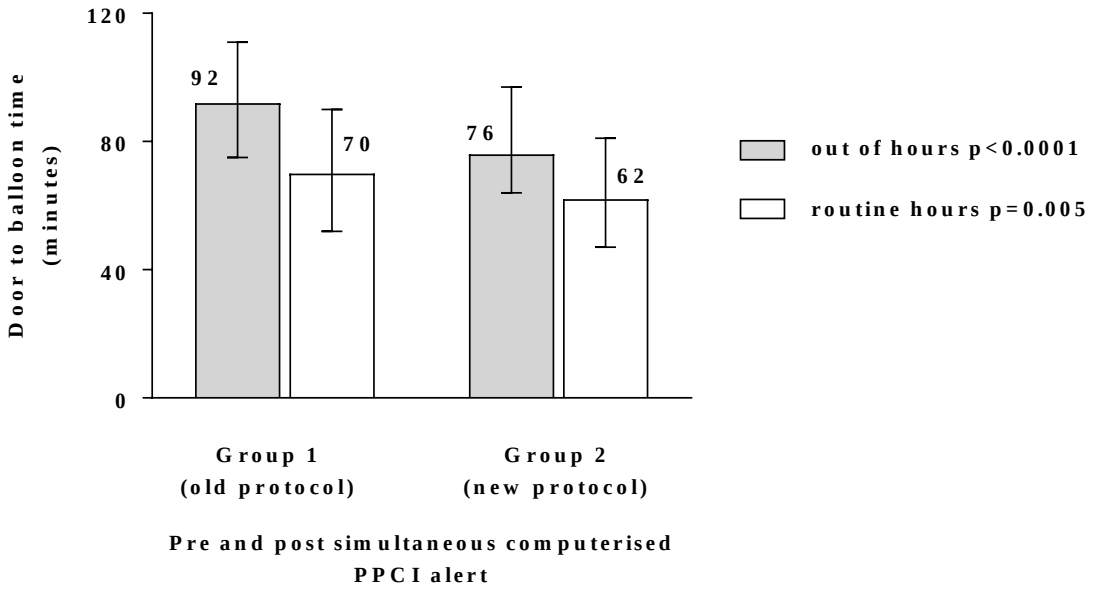
Characteristics	Pre-computerised PPCI activation OOH n = 295	Post computerised PPCI activation OOH n = 498	p value
Mean Age (SD)	61 (13.3)	60 (13.9)	0.60§
Male, n (%)	213 (72)	369 (74)	0.56
WE, n (%) AC, n (%) SA, n (%)	204 (69) 7 (2) 72 (24)	334 (67) 16 (3) 139 (28)	0.69†
Previous MI, n (%)	56 (19)	73 (15)	0.11
Angina, n (%)	60 (20%)	86 (17)	0.09†
Hypertension, n (%)	147 (50)	247 (50)	0.43†
Hypercholesterolaemia, n (%)	110 (37)	206 (41)	0.33
PVD, n (%)	6 (2)	21 (4)	0.11
CVA, n (%)	9 (3)	30 (6)	0.08
CKD, n (%)	9 (3)	15 (3)	1.00
Diabetes, n (%)	48 (16)	100 (20)	0.18
Previous PCI, n (%)	36 (12)	58 (12)	0.82
Previous CABG, n (%)	8 (3)	13 (3)	1.00
Smoking, n (%)	195 (66)	339 (68)	0.69
Cardiac arrest at presentation, n (%)	25 (8)	54 (11)	0.33
Anterior infarction, n (%)	108 (37)	209 (42)	0.49
Thienopyridine platelet inhibitor, n (%)	493 (98.9)	294 (99.6)	0.42

IV glycoprotein inhibitor, n (%)	183 (62)	199 (40)	<0.0001
OTHT minutes, median (IQR)	123 (63-296)	113 (67-269)	0.96
DTBT minutes, median (IQR)	92 (75-11)	76 (64-97)	<0.0001
DTBT < 90 minutes, n (%)	141 (48)	349 (70)	<0.0001
In-hospital mortality, n (%)	12 (4)	25 (5)	0.60
1-year mortality, n (%)	18 (6)	49 (10)	0.09

† Chi square test, § student's T test, all others Fisher's exact test, WE White-European, AC Afro-Caribbean, SA South Asian, MI myocardial infarction, PVD peripheral vascular disease, CVA cerebrovascular accident, CKD chronic kidney disease, PCI percutaneous coronary intervention, CABG coronary artery bypass grafting, IV intravenous, OOH out-of-hours, OTHT (symptom) onset to hospital time, IQR Interquartile range, DTBT door-to-balloon time.

4.4.4.1 Door-to-balloon time

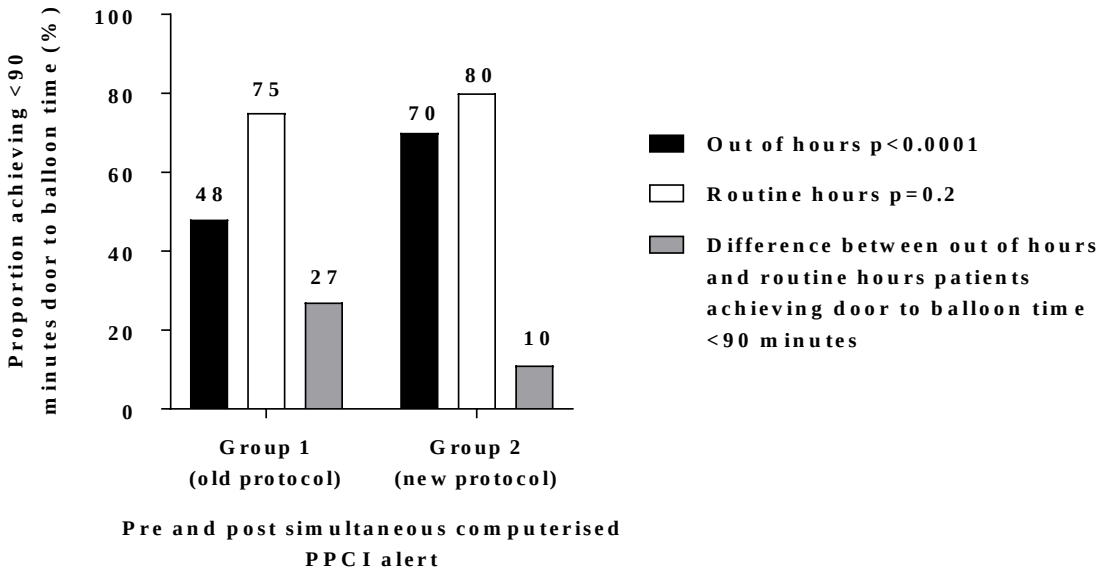
The unadjusted median DTBT were 92 minutes (interquartile range [IQR] 75-111) for Group 1 and 76 minutes (IQR 64-97) for Group 2 (odds ratio [OR] 2.6, 95% confidence interval [CI] 1.9-3.4, p<0.0001). This also remained significant on multivariate logistic regression analysis after adjustment for age, gender, ethnicity, admitting hospital, direct 999 admission (defined as an emergency ambulance from the community, as opposed to self-presentation), cardiac failure at presentation, cardiac arrest on admission, and previous coronary artery bypass grafting (OR 2.8, 95% CI 1.6-5.0, p<0.0001). Figure 4.2 shows the median DTBT for Groups 1 and 2 compared with patients presenting during routine hours over the same period.



PPCI primary percutaneous coronary intervention

Figure 4.2 Door-to-balloon times of patients admitted to hospital out-of-hours and in routine hours before and after the computerised alert protocol.

The proportion of patients admitted to hospital OOH achieving a DTBT <90 minutes increased from 48% (n=141) in Group 1 to 70% (n=349) in Group 2 (p<0.0001). The difference in the proportion of OOH patients achieving a DTBT <90 minutes as compared with patients admitted during routine hours are shown in Figure 4.3.



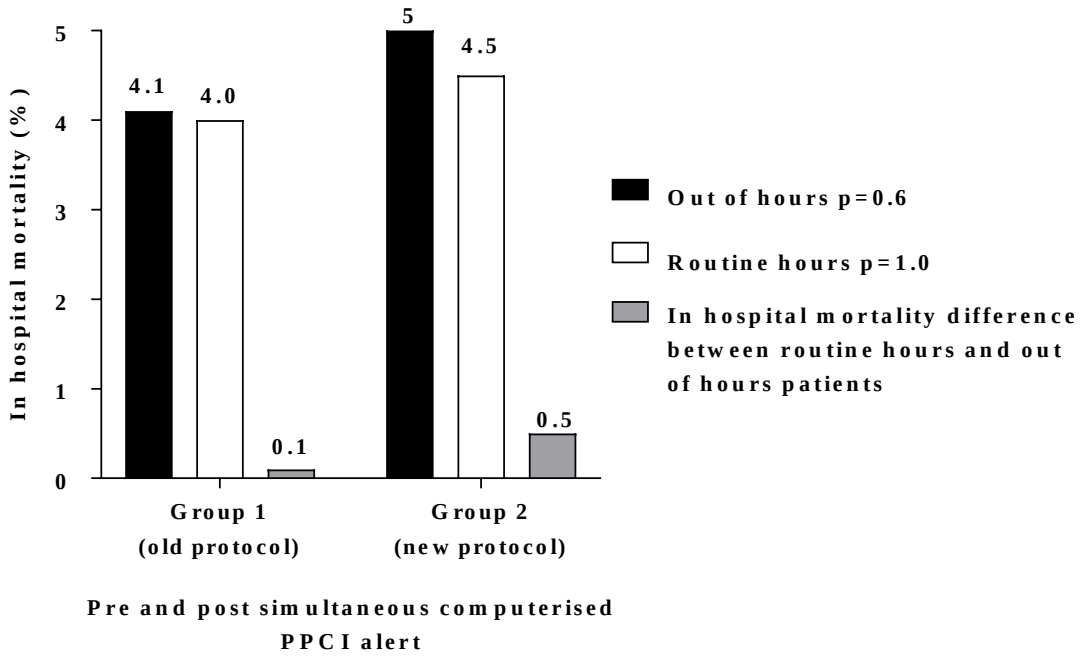
PPCI primary percutaneous coronary intervention

Figure 4.3 The proportion of patients out-of-hours achieving a door-to-balloon time of less than 90 minutes as compared with patients presenting in routine hours before and after the introduction of the simultaneous primary percutaneous coronary intervention alert.

4.4.4.2 All-cause mortality

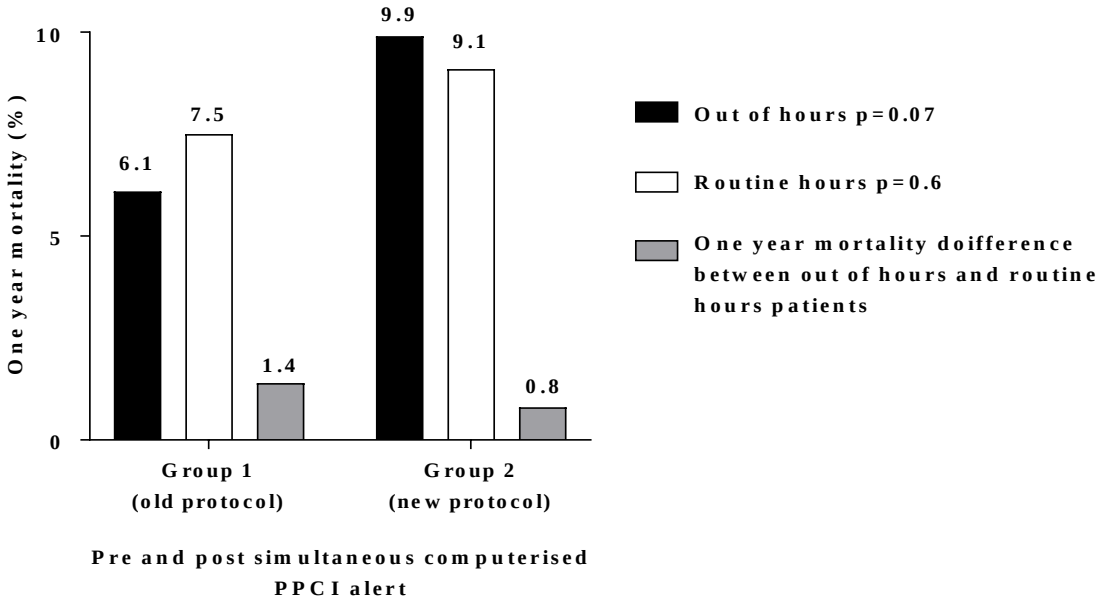
In hospital mortality was 4.1% (n= 12) in Group 1 and 5% (n=25) in Group 2 (p=0.60). All-cause mortality at 1 year was 6.1% (n=18) in Group 1 and 9.9% (n=49) in Group 2 (p=0.09). To exclude possible biases due to modifications in the standard of care of patients (such as pharmacological therapy, interventional procedure and physician expertise), we analysed the mortality rates for PPCI performed in the same two periods but during working hours (Figures 4.4 and 4.5). On regression analysis introduction of the new system was not related to any change in in-hospital or one-year mortality (OR 0.8, 95% CI 0.4-1.6, p=0.54 and OR 0.59, 95% CI 0.34-1.04, p=0.07 respectively).

Analysis of between group mortality rates after exclusion the of 120 patients with cardiac arrest lead to no difference in in-hospital [1.9% (Group1) vs 1.2% (Goup2), p= 0.44] or 1-year [4.4% (Group1) vs 5.6% (Group2), p=0.41] mortality, either overall or out-of-hours [in hospital 1.5% (Group 1) vs 1.4% (Group 2) p=1.0, and 1 year 3.4% (Group 1) vs 5.9% (Group 2) p =0.16].



PPCI primary percutaneous coronary intervention

Figure 4.4 In-hospital mortaliy in patients admitted out-of-hours and during routine hours before and after the computerised alert protocol.



PPCI primary percutaneous coronary intervention

Figure 4.5 One-year mortality in patients admitted out-of-hours and during routine hours before and after the introduction of the protocol.

4.4.4.3 Symptom-to-door time

The proportion of patients presenting OOH with a STDT longer than 2 hours prior to the protocol introduction was 51% (134/262) and after the protocol was 46% (227/489), p=0.22. When we analysed overall mortality over the five-year study period according to STDT (less than 2 hours versus longer than 2 hours) we found no difference in in-hospital or 1-year death rates (3.8% [15/390] vs 5.0% [18/361] p=0.48 and 7.7% [30/390] vs 8.9% [32/360] p=0.60 respectively).

4.4.5 Discussion

This study shows a significant improvement in DTBT following the introduction of the new alert protocol for patients admitted to hospital OOH – with a significantly higher proportion of patients achieving the internationally recognised target of less than 90 minutes. Despite the reduced DTBT, mortality remained unaltered – whereby (although not the primary purpose of this paper) the mortality rates of patients treated OOH compared with routine hours are similar in group 1 despite a difference in DTBT of 22 minutes.

Strategies known to reduce DTBT include: having emergency department physicians activate the catheterisation laboratory, having a single call to a central page operator activate the laboratory, activating the laboratory whilst the patient is en route to the hospital, expecting staff to arrive in the catheterisation laboratory within 20 minutes after being paged, having a cardiologist on site and having staff in the emergency department and the catheterisation laboratory use real time data feedback[350, 352]. The protocol we

introduced incorporated three of these strategies so it is not surprising that DTBTs improved significantly.

Studies to date evaluating the association of both short and long-term mortality with DTBT have been conflicting[353-356]. Previous work from our department has suggested that only the highest risk patients benefit from the shortest DTBT possible[357]. As this population accounts for the minority of PPCI cases the benefit in this group may be lost in the overall mortality rates. There is also the possibility that once a lower threshold has been reached (for example 2 hours) then no further overall mortality benefit is seen until the next lowest threshold (for example 30 minutes) is reached, with smaller reductions not producing the effect required to see mortality reduction.

Our data are in keeping with recent evidence which suggests that despite an improvement of DTBT in many hospitals, short and longer-term mortality remain unaltered[358]. In one study the median DTBT decreased from 83 minutes in 2005/6 to 67 minutes in 2008/9, with 83% of patients achieving a DTBT of less than 90 minutes in 2008/9 compared with 59% in 2005/6 and no corresponding improvement in mortality[358]. Parikh et al[359] also found that implementing similar strategies as part of a 'Code STEMI' brought about significant reductions in DTBT but had little impact on in-hospital mortality. The main limitation of that study was the sample size. The present study supports those findings in a larger cohort and adds longer term mortality data. Brodie et al[354] found that total time to reperfusion had little effect on one and six-month mortality in those undergoing PPCI. Interestingly we also found no difference in overall early or late mortality rates between patients with a STDT less than 2 hours compared with longer than 2 hours, even after

correcting for baseline variables and DTBT (data not shown). Strategies focusing on reducing this component of total ischaemic time, specifically symptom-to-call time, have had limited success in the past[346, 360] which might account for why 50% of patients still take longer than 2 hours to arrive at hospital after the onset of myocardial ischaemia with some taking considerably longer. In contrast, Rathore et al believe that DTBT should be as short as possible, advocating the implementation of targets below the current 90-minute threshold to achieve an in-hospital mortality reduction of 0.8% below 60 minutes and 1.3% below 30minutes. This is important for myocardial salvage and infarct size but many observational studies, including the present study, do not support this strategy, rather suggesting the current target of 90 minutes is appropriate, certainly in terms of mortality.

4.4.6 Limitations

These data are from a single, medium volume centre so results may not be generalisable to other centres however they raise important questions regarding the appropriateness of further strategies to reduce door to balloon time with its associated cost. Presently, there is no robust method in place to record data for patients that activate the primary PCI pathway but do not require intervention so we were unable to ascertain the proportion of 'false alarm' patients in each of the two groups. We excluded 106 patients from the analysis. Whilst these patients may have had different clinical characteristics to those studied which could affect the outcome, the level of confounding that would be brought about by their inclusion justifies their exclusion. The MINAP data focuses on the clinical aspects of myocardial infarction management rather than the technical aspects of the intervention therefore we have not included procedural information e.g. stent type or

access site. However, audit data from our department suggests that our use of techniques known to improve mortality such as drug eluting stents and radial access has increased since the beginning of the study period. This would be expected, if anything, to have a positive effect on mortality. Also, we did not have data on infarct size. Finally, as symptom time is self-reported by the patient and therefore subject to recall bias, ambulance records rather than notes from the emergency department were used to record the symptom onset time.

4.4.7 Conclusion

The change in protocol was independently associated with a reduction in OOH DTBT and a significant increase in the number of OOH patients achieving a DTBT of less than 90 minutes. Despite this, there was no significant change in in-hospital or one-year mortality.

4.5 SECTION SUMMARY

The proportion of WE and SA patients with symptomatic CAD approximately represents that of the wider regional populace. Afro-Caribbean patients are underrepresented compared to their proportion of the wider community which is consistent with other reports of low CAD rates in this group[313]. Other demographical features of CAD patients, such as the proportion of females and older patients, the younger age of onset of symptomatic and more aggressive CAD in SAs and the distribution of traditional risk factors across ethnicities, are in keeping with the literature. As far as possible I applied this information to my recruitment to ensure evenly matched groups for the pathophysiological studies and thus minimise confounding. At our institution, it is relatively unusual to find no visible CAD in patients referred for invasive coronary angiography. This could reflect a high referral threshold. However, the fact that emergency admission rates for CAD and stroke in Birmingham are above the national average[361] despite having a younger population (almost 50% of Birmingham's population are under thirty[314]) would suggest that the burden of CAD is still a major problem in this area.

SECTION V

PATHOPHYSIOLOGICAL INSIGHTS FROM MY PROSPECTIVE DATA

5.1 SECTION OUTLINE

This section contains the results of my experiments. Firstly, I present the cross-sectional and longitudinal pathophysiological data consisting of the vascular studies (arterial and ventricular elastance indices, aortic augmentation pressure and index and cutaneous iontophoresis) followed by biomarkers (monocyte subsets, monocyte-platelet aggregates and circulating microparticles), and secondly, the health-related quality of life data.

5.1.1 Hypotheses to be tested

The aims and hypotheses are discussed in detail in Section II. Briefly, the hypotheses are that;

- Patients with diffuse CAD will have increased arterial and ventricular elastance indices with abnormal ventricular-arterial interaction, increased aortic augmentation index and worse endothelial-dependent microvascular function than patients with focal CAD or no CAD.
- Diffuse CAD patients will have higher levels of Mon2 and Mon3, MPAs associated with Mon2 and Mon3, and larger numbers of circulating endothelial and platelet microparticles compared to patients with focal CAD or no CAD.
- Subjects with diffuse CAD have poorer quality of life than subjects with focal CAD or normal coronary arteries.
- At one-year follow up patients with diffuse CAD will exhibit a worsening trend of these measures than patients with focal CAD or no CAD.

5.2 RESULTS

5.2.1 Clinical Characteristics

A total of 140 patients (age 57 ± 8.5, 61% male, 64% white-European) were included in the cross-sectional study (Table 5.1).

The groups were matched for age (p=0.28), gender (p=0.56) and conventional risk factors. South Asians (24% of the study population) tended to have more diffuse CAD (p=0.07). There was no significant difference in smoking rates, although current smoking was observed less in focal CAD patients (many of whom were ex-smokers) than in diffuse or no CAD patients (p = 0.09).

Diffuse CAD patients had significantly more previous coronary bypass surgery (p<0.001) whereas patients with focal CAD had more previous PCI (p=0.07). Subjects without CAD had significantly less secondary preventive medication (aspirin, beta blocker, angiotensin-converting enzyme/angiotensin receptor blocker or statin) than either of the two study groups (p<0.001 for all). Subjects without CAD had significantly higher blood pressure (p<0.01) and pulse rates (p=0.04). There were no significant differences in either the use of secondary prevention medication or blood pressure and pulse recordings between the two CAD groups.

Table 5.1 Demographic and baseline clinical characteristics

Characteristic	**Diffuse CAD (n = 50)**	**Focal CAD (n = 40)**	**Normal (n = 50)**	**p value**
Age, mean (SD)	59 (7)	57 (10)	56 (8)	0.28*
Males	33 (66)	22 (56)	31 (58)	0.56
Afro-Caribbean White European South Asian	3 (6) 29 (58) 18 (36)	3 (7.5) 31 (78) 6 (15)	8 (16) 29 (58) 10 (20)	0.07
Diabetes Insulin dependent	13 (26) 5 (10)	7 (18) 2 (5)	10 (20) 2 (4)	0.59 0.43
Hypertension	28 (56)	15 (38)	27 (54)	0.17
Hypercholesterolaemia	34 (68)	23 (58)	31 (62)	0.59
Previous MI	29 (58)	17 (43)	-	0.14
Previous PCI	35 (70)	35 (88)	-	0.05
Previous CABG	18 (36)	0	-	<0.001
Previous stroke	2	2	1	0.73
Angina	12 (24)	13 (33)	-	0.37
Current smoker Ex-smoker Never smoked	7 (14) 17 (34) 26 (52)	3 (8) 24 (60) 13 (33)	7 (14) 17 (34) 26 (52)	0.09
Premature CAD in family	18 (36)	12 (30)	16 (32)	0.65
Aspirin	46 (92)	38 (95)	12 (24)	<0.001
Beta blocker	39 (78)	31 (78)	8 (16)	<0.001
ACE/ARB	43 (86)	28 (70)	17 (34)	<0.001
Statin	47 (94)	37 (93)	27 (52)	<0.001
Height cm, mean (SD)	168 (9.7)	169 (10)	169 (10)	0.79*
Weight kg, mean (SD)	80 (16)	83 (15)	88 (21)	0.08*
BMI kg/m^2, mean (SD)	28 (5)	29 (4)	31 (7)	0.11*

WHR, mean (SD)	0.94 (0.05)	0.92 (0.09)	0.95 (0.09)	0.17*
BSA m^2, mean (SD)	1.89 (0.20)	1.94 (0.21)	1.97 (0.25)	0.17*
Systolic BP mmHg, mean (SD)	126 (18)	126 (17)	137 (16)	0.001*
Diastolic BP mmHg, mean (SD)	71 (9.7)	75 (10)	84 (10)	<0.001*
Pulse rate bpm, mean (SD)	64 (11)	67 (12)	70 (13)	0.04*

Data are expressed as number and percentage unless otherwise stated, *One-way analysis of variance, all others Chi-square test, ACE angiotensin converting enzyme, ARB angiotensin receptor blocker, BMI body mass index, BSA body surface area, BP blood pressure, bpm beats per minute, CABG coronary artery bypass graft, MI myocardial infarction, PCI percutaneous coronary intervention, WHR waist-to-hip ratio.

5.2.2 Baseline laboratory data

Total and non-HDL cholesterol were higher in patients with no CAD compared to both CAD groups (both p<0.0001), but similar between the two CAD groups (Table 5.2). Other measures were similar across the groups.

Table 5.2 Baseline laboratory blood tests

Laboratory measure	**Diffuse CAD (n = 50)**	**Focal CAD (n = 40)**	**Normal (n = 50)**	**p value**
Haemoglobin, g/L	140 (131-149)	140 (127-137)	146 (129-153)	0.26
WBC, 10^9cells/L	6.7 (5.9-8.1)	7.3 (6.2-8.3)	6.9 (5.5-8.7)	0.66
Neutrophils, 10^9cells/L	3.9 (3.4-4.9)	4.4 (3.6-5.1)	4.0 (2.9-5.2)	0.75
Lymphocytes, 10^9cells/L	2.0 (1.5-2.4)	2.1 (1.4-2.6)	1.9 (1.5-2.5)	0.97
Monocytes, 10^9cells/L	0.5 (0.4-0.6)	0.6 (0.5-0.7)	0.6 (0.4-0.7)	0.37
Eosinophils, 10^9cells/L	0.16 (0.1-0.29)	0.21 (0.13-0.36)	0.18 (0.1-0.27)	0.17
Basophils, 10^9cells/L	0.04 (0.03-0.06)	0.05 (0.04-0.07)	0.05 (0.03-0.07)	0.65
Platelets, 10^9cells/L	233 (183-272)	244 (199-287)	251 (200-301)	0.35
eGFR, ml/min/1.73m^2 *	83 (71-90)	82 (71-90)	81 (71-90)	0.96
Cholesterol, mmol/L	3.8 (3.4-4.5)	4.0 (3.0-4.7)	4.9 (3.9-5.5)	<0.0001
HDL, mmol/L	1.2 (1.0-1.3)	1.2 (1.0-1.5)	1.3 (1.1-1.5)	0.18
Non-HDL, mmol/L	2.7 (2.0-3.4)	2.5 (2.0-3.5)	3.5 (2.8-4.4)	<0.0001

Triglycerides, mmol/L	1.3 (0.8-1.7)	1.3 (0.9-1.8)	1.4 (1.1-2.1)	0.25

Data are expressed as median and interquartile range and analysed using Kruskal-Wallis Test with Dunn's post-hoc multiple comparisons test unless otherwise stated, WBC white blood cells, eGFR estimated glomerular filtration rate, g/l grams per litre, * values above 90 are not individually measured so the true medians are likely to be underestimated, HDL high density lipoprotein, mmol/L millimoles per litre.

5.2.3 Arterial and ventricular elastance indices, ventricular-arterial coupling and radial applanation tonometry

5.2.3.1 Cross-sectional analysis

EaI was lower in diffuse CAD patients compared to the control group (p=0.03), but no different to the focal CAD group (p=0.45) (Table 5.3). Central ASP (ESP) was significantly lower in both the diffuse and focal CAD groups compared to the normal group (p=0.004 and p=0.009 respectively), as was central mean arterial pressure (p<0.001) (Table 5.4). Compared with controls and patients with focal CAD, subjects with diffuse CAD had higher early mitral inflow and tissue Doppler velocities (p=0.008 and p=0.01) and higher EDPs and EDVs (p=0.01 and p=0.04). LV-Ees and LV-Eed were not significantly different between the groups (p=0.19 and p=0.70 respectively). AIx and AP were similar between the groups (p=0.87 and p=0.79 respectively). The median OI was >95% for each group with no significant differences.

5.2.3.2 Longitudinal analysis

During follow up patients with diffuse CAD showed an increase in LV Eed (p=0.02) with no change in Ees (p=0.79), EaI (p=0.11) or VAC (p=0.67) (Table 5.5). Patients with focal CAD showed no longitudinal change in elastance (p=1.00 for Ees, p=0.31 for Eed, p=0.22 for EaI and p=0.89 for VAC). Aortic augmentation index was unchanged

throughout follow up in both groups (p=0.59 and p=0.49 for diffuse and focal CAD respectively).

Table 5.3 Baseline echocardiographic parameters and measures of elastance

	Diffuse (N = 50)	**Focal (N = 40)**	**Normal (N = 50)**	**p value**
LVOTd, cm	1.9 (1.80-2.00)	2.0 (1.83-2.10)	2.0 (1.90-2.10)	0.07
CSA, cm^2	2.83 (2.54-3.14)	3.14 (2.61-3.46)	3.14 (2.83-3.46)	0.05
Mitral E, cm/s	94 (20)	83 (18)	85 (15)	0.008*
IVRT, ms	90 (70-110)	80 (70-100)	90 (80-100)	0.33
IVCT, ms	60 (50-70)	55 (40-70)	50 (40-70)	0.66
LVOT VTI, cm	22.4 (20.0-25.4)	20.5 (18.6-22.8)	21.6 (18.8-24.0)	0.13
EDP, mmHg	15 (13-18)	13 (12-15)	13 (12-16)	0.01
ESP (ASP), mmHg	115 (104-130)	119 (104-129)	131 (116-140)	<0.001
EDV index, ml/m2	44 (38-50)	39 (33-45)	42 (36-48)	0.04
ESV index, ml/m2	14 (12-17)	12 (10-17)	14 (12-16)	0.27
SV index, ml/m2	28 (25-33)	26 (22-31)	28 (23-31)	0.20
LVEF, (%)	65 (7)	67 (7)	65 (7)	0.38*
Diastolic TDI, cm/s	11 (9-13)	9 (8-11)	9 (8-11)	0.01
Ees, $mmHg/ml/m^2$	7.2 (6.5-10.3)	9.2 (6.8-11.5)	9.2 (7.5-10.7)	0.12
EaI, $mmHg/ml/m^2$	4.2 (3.4-5.0)	4.3 (3.6-5.3)	4.7 (4.1-6.0)	0.05
VAC	1.9 (1.4-2.5)	2.0 (1.7-2.5)	1.8 (1.5-2.5)	0.64
Eed, $mmHg/ml/m^2$	0.34 (0.29-0.47)	0.38 (0.28-0.44)	0.34 (0.26-0.41)	0.61

Data are expressed as median and interquartile range and analysed using Kruskal-Wallis Test unless otherwise stated, *one way analysis of variance, ASP aortic systolic pressure, CSA cross-sectional area, EaI arterial elastance index, EDP end-diastolic pressure, EDV end-diastolic volume, Eed end-diastolic elastance, Ees left ventricular end systolic elastance, ESP end-systolic pressure, ESV end-systolic volume, IVCT

isovolumetric contraction, IVRT isovolumetric relaxation, LVEF left ventricular ejection fraction, LVOTd left ventricular outflow tract diameter, LVOT VTI left ventricular outflow tract velocity time integral, SV stroke volume, TDI tissue Doppler imaging, VAC ventricular-arterial coupling.

Table 5.4 Estimation of central blood pressures and aortic augmentation index

Derived variable	**Diffuse (n = 50)**	**Focal (n = 40)**	**Normal (n = 50)**	**p value**
Central MAP, mmHg	87 (77-95)	89 (79-100)	100 (92-106)	<0.001
Central PP, mmHg	46 (11)	42 (11)	44 (11)	0.43*
AIx@75bpm, (%)	23 (8)	24 (10)	25 (10)	0.59
AP, mmHg	13 (9.3-19)	14 (8-19)	13 (8.3-19)	0.79
OI	97 (92-98)	96 (87-99)	96 (90-98)	0.79

Data are expressed as median and interquartile range and analysed using Kruskal-Wallis Test unless otherwise stated, *one-way analysis of variance, AIx@75bpm aortic augmentation index adjusted for heart rate, AP augmentation pressure, ASP aortic systolic pressure, MAP mean arterial pressure, mmHg millimetres of mercury, OI operator index, PP pulse pressure.

Table 5.5 Longitudinal analysis of arterial and ventricular elastance and aortic augmentation index

Stiffness parameter	CAD morphology	Baseline	6 months	12 months	p value
Ees, mmHg/ml/m^2	Diffuse	7.2	8.4	8.5	0.79
	Focal	9.0	8.7	8.5	1.00
Eed, mmHg/ml/m^2	Diffuse	0.35	0.34	0.41	0.02
	Focal	0.36	0.38	0.35	0.31
EaI, mmHg/ml/m^2	Diffuse	4.3	4.1	4.7	0.11
	Focal	4.2	4.7	5.0	0.22
VAC	Diffuse	1.9	2.0	1.9	0.67
	Focal	2.0	1.9	1.8	0.89
AIx@75bpm (%)	Diffuse	23	24	25	0.59
	Focal	27	26	25	0.49

AIx@75bpm aortic augmentation index adjusted for heart rate, CAD coronary artery disease, EaI arterial elastance index, Eed end diastolic elastance, Ees End systolic elastance, VAC ventricular-arterial coupling.

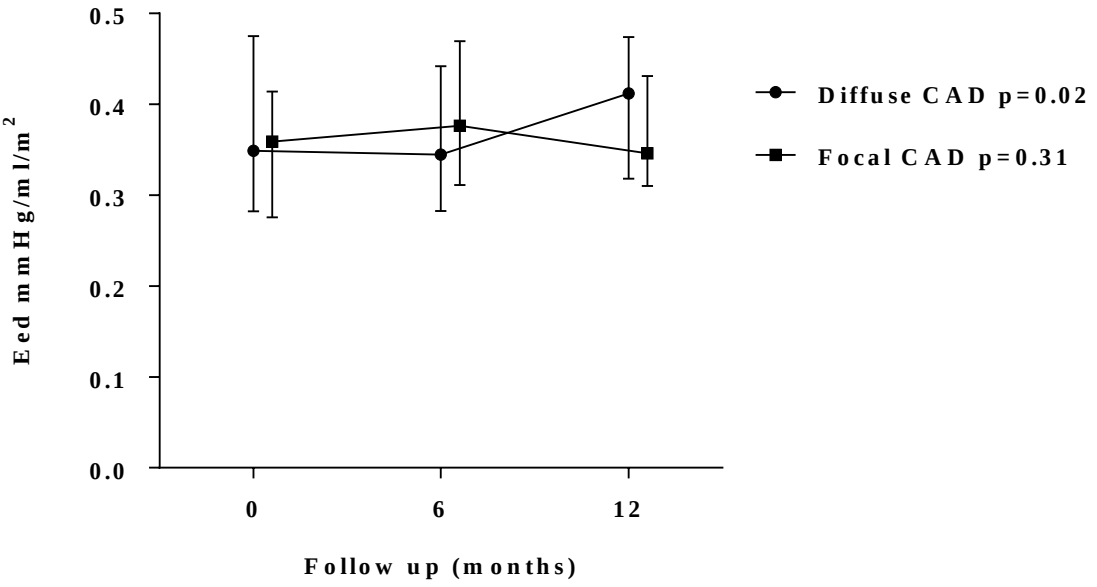


Figure 5.1 Longitudinal left ventricular end-diastolic elastance index.

5.2.4 Cutaneous microvascular function

5.2.4.1 Cross-sectional analysis

There was a strong trend towards worse microvascular response to ACh (endothelial dependent function) in patients with diffuse CAD than in subjects with focal CAD or normal coronaries (p=0.06). There was no difference between the groups in endothelial-independent function (p=0.74) (Table 5.6 and Figure 5.2).

5.2.4.2 Longitudinal analysis

Endothelial-dependent microvascular function improved throughout follow up in patients with diffuse CAD (p=0.036). Endothelial independent function was unchanged throughout the follow up period in both groups (Table 5.7).

Table 5.6 Cross-sectional analysis of microvascular endothelial function

Flow	Diffuse CAD	Focal CAD	No CAD	p value
Endothelial-dependent function (response to acetylcholine)				
Baseline, U	26 (15-55)	23 (14-39)	22 (14-33)	0.31
Peak, U	181 (139-225)	185 (150-221)	193 (153-228)	0.55
Increase, %	568 (275-973)	627 (357-1164)	847 (465-1246)	0.06
Endothelial-independent function (response to sodium nitroprusside)				
Baseline, U	14 (12-25)	14 (11-21)	13 (10-21)	0.28
Peak, U	173 (124-222)	159 (128-210)	187 (110-229)	0.87
Increase, %	870 (558-1483)	1047 (602-1340)	1026 (607-1574)	0.62

Data expressed as median and interquartile range and analysed with Kruskal-Wallis test, units are arbitrary flux units unless otherwise stated, CAD coronary artery disease.

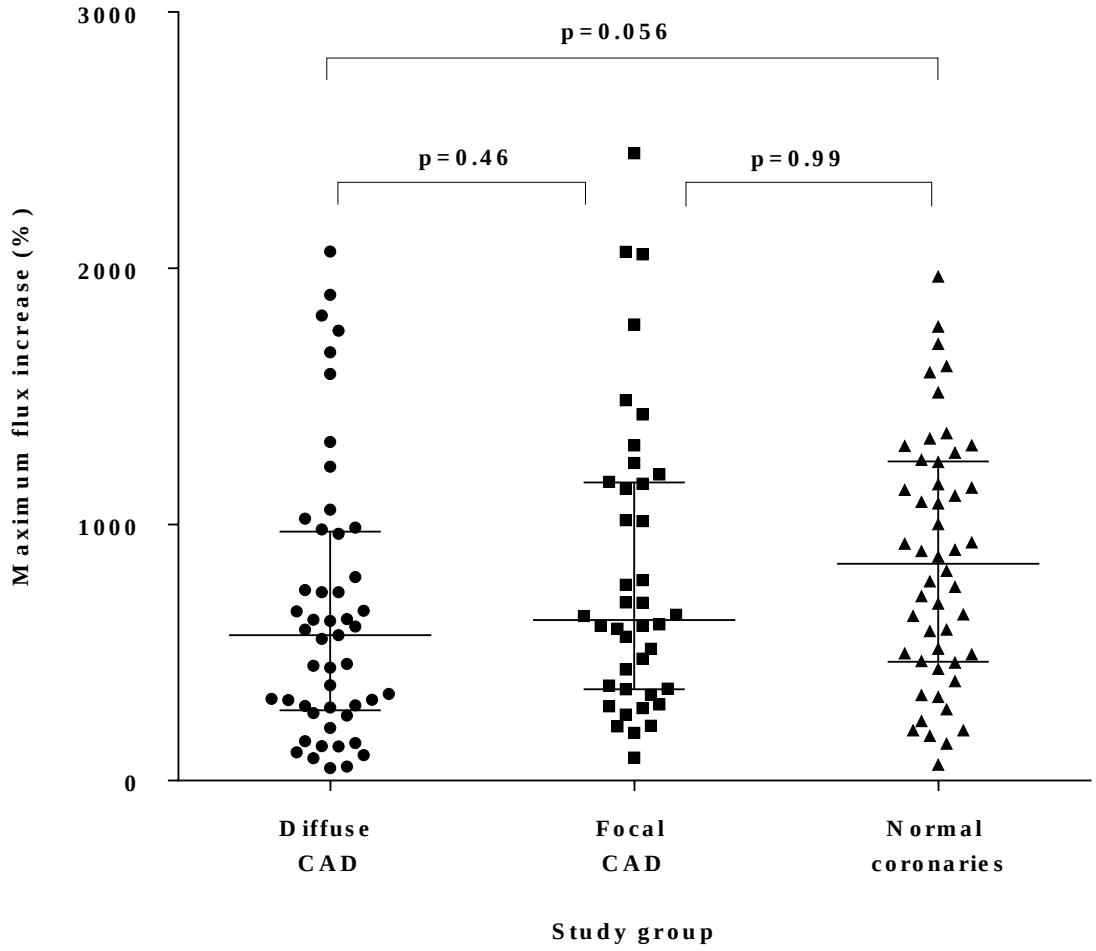


Figure 5.2 Maximum endothelial-dependent vasodilation for each group.

Table 5.7 Longitudinal analysis of microvascular endothelial function

CAD morphology	Baseline	6 months	12 months	p value
Endothelial-dependent function (response to acetylcholine)				
Diffuse CAD	445 (218-742)	651 (398-941)	691 (498-1211)	0.036
Focal CAD	605 (301-1123)	861 (658-1258)	882 (511-1340)	0.47
Endothelial-independent function (response to sodium nitroprusside)				
Diffuse CAD	854 (584-1514)	782 (481-1511)	800 (531-1152)	0.72
Focal CAD	1071 (548-1340)	1171 (601-1589)	1039 (685-1355)	0.97

Data expressed as median and interquartile range and analysed with Kruskal-Wallis test, units are arbitrary flux units unless otherwise stated, CAD coronary artery disease.

5.2.5 Monocyte subsets and monocyte-platelet aggregates

5.2.5.1 Cross-sectional analysis

There were no between group differences in monocyte subsets (p=0.65 for Mon1, p=0.14 for Mon2, p=0.83 for Mon3, p=0.62 for total monocytes) (Table 5.8).

MPAs associated with Mon2 were significantly higher in the diffuse CAD group compared to the focal CAD or control groups (p=0.003 and p=0.004, respectively) (Table 5.8 and Figure 5.3).

The proportion of Mon2 aggregated to platelets was significantly higher in the diffuse group compared to the focal group (p=0.007). MPAs associated with Mon1, Mon3 and total MPAs were similar between the groups (p=0.48, p=0.71, p=0.21, respectively).

5.2.5.2 Longitudinal analysis

There was a significant reduction in total MPA counts in patients with diffuse (p<0.001) and focal CAD (p=0.006) over 12 months follow up (Table 3). MPAs with Mon1 declined significantly in both groups (p<0.048 for diffuse CAD and p=0.02 for focal CAD); MPAs with Mon2 declined only in patients with diffuse CAD (p<0.001). Total monocytes and monocyte subsets did not change significantly during 12-month follow up (Table 5.9).

Table 5.8 Cross-sectional analysis of monocyte subsets and monocyte-platelet aggregates

Counts, per µl	**Diffuse CAD (n = 50)**	**Focal CAD (n = 40)**	**No CAD (n = 50)**	**p value**
Total monocytes*	480 (138)	511 (157)	487 (161)	0.62
Mon1*	397 (124)	427 (143)	413 (143)	0.59
Mon2	34 (24-55)	32 (14-54)	24 (17-40)	0.09
Mon3	39 (25-49)	44 (26-59)	37 (25-58)	0.93
Total MPA	53 (41-65)	52 (35-68)	42 (28-74)	0.21
MPA with Mon1	34 (29-48)	36 (26-54)	32 (18-55)	0.48
MPA with Mon2	10 (4-14)	5 (2-8)	5 (2-7)	<0.001
MPA with Mon3	5 (4-9)	6 (4-9)	5 (3-8)	0.71

Data are expressed as median and interquartile range unless otherwise stated, * mean and standard deviation, CAD, coronary artery disease; MPA, monocyte-platelet aggregate.

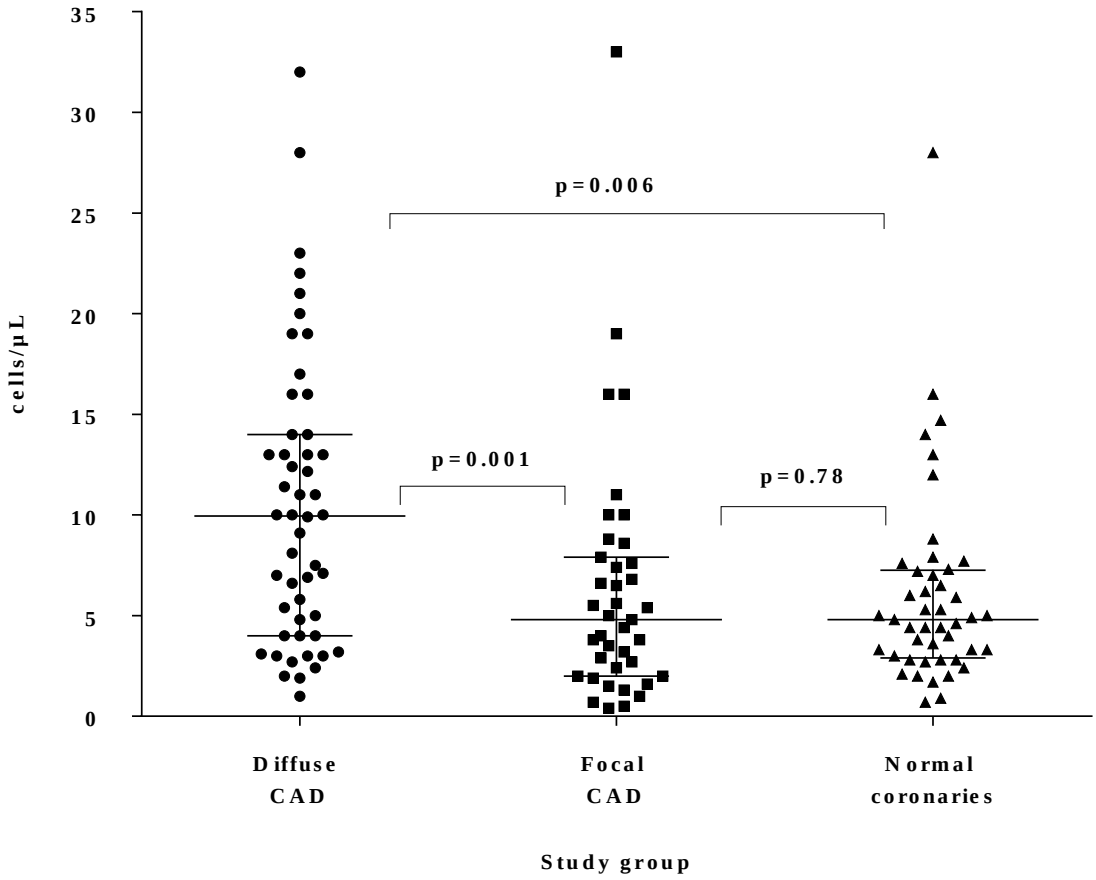


Figure 5.3 Monocyte-platelet aggregates associated with Mon2 (CD14++CD16+CCR2+) for each study group.

Table 5.9 Longitudinal analysis of monocyte subsets and monocyte-platelet aggregates

Counts, per µl	CAD morphology	Baseline	6 months	12 months	p value
Total monocytes	Diffuse	479 (151)	471 (139)	469 (142)	0.70
	Focal	530 (151)	528 (156)	469 (191)	0.43
Mon1	Diffuse	395 (136)	391 (110)	394 (125)	0.57
	Focal	440 (145)	444 (141)	392 (172)	0.33
Mon2	Diffuse	34 (23-54)	27 (19-55)	23 (13-40)	0.06
	Focal	32 (14-53)	24 (11-46)	22 (7.9-46)	0.52
Mon3	Diffuse	40 (26-51)	32 (24-59)	40 (32-62)	0.24
	Focal	46 (30-63)	44 (36-53)	40 (28-60)	0.33
Total MPA	Diffuse	53 (41-69)	48 (32-59)	41 (28-54)	<0.001
	Focal	50 (33-70)	50 (35-69)	34 (28-47)	0.006
MPA with Mon1	Diffuse	34 (28-48)	33 (25-44)	31 (20-43)	0.048
	Focal	36 (26-54)	36 (24-54)	25 (22-40)	0.02
MPA with Mon2	Diffuse	10 (5.2-16)	5.3 (3.1-9.9)	2.7 (1.7-4.2)	<0.001
	Focal	5 (2.2-7.8)	3.9 (1.9-7.8)	3 (1.5-4.1)	0.14
MPA with Mon3	Diffuse	5.4 (3.9-8.9)	4.9 (3.2-8.6)	5.5 (3.9-6.8)	0.30
	Focal	6.4 (4.4-8.6)	5.6 (4.0-8.4)	4.2 (3.7-6.4)	0.11

Data are expressed as median and interquartile range unless otherwise stated, * mean and standard deviation, CAD coronary artery disease, MPA monocyte-platelet aggregate.

5.2.6 Platelet and endothelial microparticles

5.2.6.1 Cross-sectional analysis

Baseline counts of EMPs were significantly higher in patients with diffuse CAD than in patients with focal CAD (p=0.004) or normal coronaries (p=0.016) (Figure 5.4). There were no differences in PMP counts (p=0.62) (Table 5.10).

5.2.6.2 Longitudinal analysis

EMPs showed a significant decline over 12 months in subjects with diffuse CAD (p<0.001) and in subjects with focal CAD (p=0.007). PMP levels increased at 6 months and declined below baseline at 12 months in diffuse CAD patients (p=0.007) and were unchanged in subjects with focal CAD (p=0.18) (Table 5.11).

Table 5.10 Cross sectional analysis of endothelial and platelet microparticles

Microparticle type	Diffuse CAD	Focal CAD	No CAD	p value
EMP (1,000,000/μl)	10 (7.9-11)	7.4 (4.8-9.4)	8.4 (5.4-9.8)	0.002
PMP (10,000/μl)	9.1 (1.9-250)	14 (2.0-210)	4.9 (1.4-137)	0.62

Data are expressed as median and interquartile range, CAD coronary artery disease; EMPs endothelial microparticles, PMPs platelet microparticles.

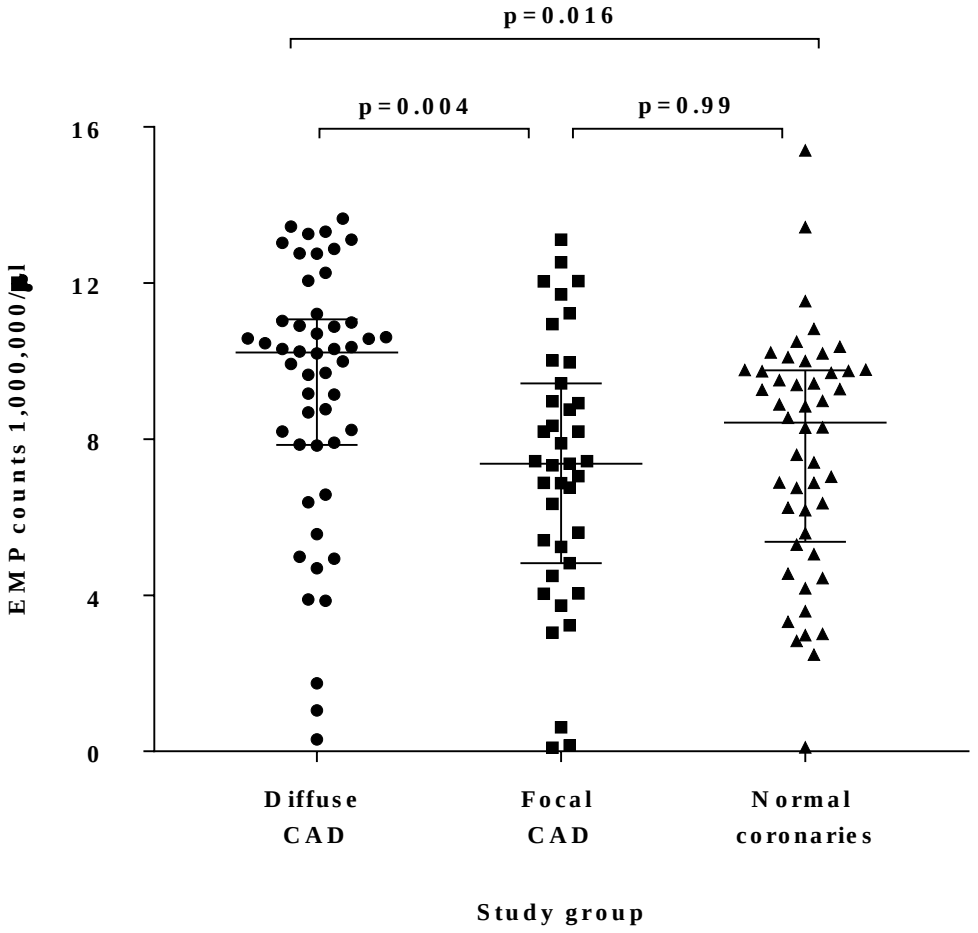


Figure 5.4 Endothelial microparticles by study group.

Table 5.11 Longitudinal analysis of endothelial and platelet microparticles

Counts	CAD morphology	Baseline	6 months	12 months	p value
EMP (1,000,000/µl)	Diffuse	10 (8.2-11)	9.1 (5.7-11)	4.4 (1.4-9.8)	<0.001
	Focal	7.7 (5.8-10)	4.1 (1.1-6.7)	5.6 (1.5-11)	0.007
PMP (10,000/µl)	Diffuse	7.2 (1.7-224)	16 (1.8-287)	2.3 (1.0-61)	0.007
	Focal	14 (1.6-278)	6.4 (1.1-109)	3.1 (1.1-144)	0.18

Data are expressed as median and interquartile range, CAD, coronary artery disease; EMPs endothelial microparticles, PMPs platelet microparticles.

5.2.7 Correlation and regression analyses of pathophysiological data

There was a significant negative correlation between counts of MPAs with Mon2 and endothelium-dependent microvascular function in patients with diffuse CAD (r= -0.37, p=0.008) – a correlation not present in patients with focal CAD or normal coronary arteries. Longitudinal analysis showed a persistent inverse relationship between the two variables in patients with diffuse CAD (r= -0.32, p=0.03) (Figure 5.5). There was no correlation between EMPs and microvascular endothelial function in diffuse CAD patients.

Whilst MPAs associated with Mon1 also declined significantly during follow up in diffuse CAD patients, they did not correlate with microvascular function (r= -0.08, p=0.61).

In focal CAD patients, there were also significant declines in total MPA and Mon1 related MPAs during follow up, but these did not correlate with microvascular function (both r=0.13, p=0.44).

On multivariable logistic regression levels of MPAs with Mon2 and EMPs were independent predictors of diffuse CAD after adjustment for age, gender, ethnicity, body mass index, previous myocardial infarction and use of beta blocker, ACE/ARB, statin, aspirin or clopidogrel (odds ratio [OR] 1.11, 95% confidence interval [CI] 1.02-1.19, p=0.012 for MPAs with Mon2 and OR 1.46, 95% CI 1.09-1.96, p=0.01 for EMPs) (Tables 5.12 and 5.13). The predictive capability persisted after further adjustment for previous revascularisation (PCI and/or CABG) (OR 1.11, CI 1.02-1.20, p=0.014 for MPAs with Mon2 and OR 1.40, CI 1.05-1.86, p=0.023 for EMPs).

The difference in EaI between the study groups was not present after correction for blood pressure (OR 1.28, 95% CI 0.86-1.91, p=0.22).

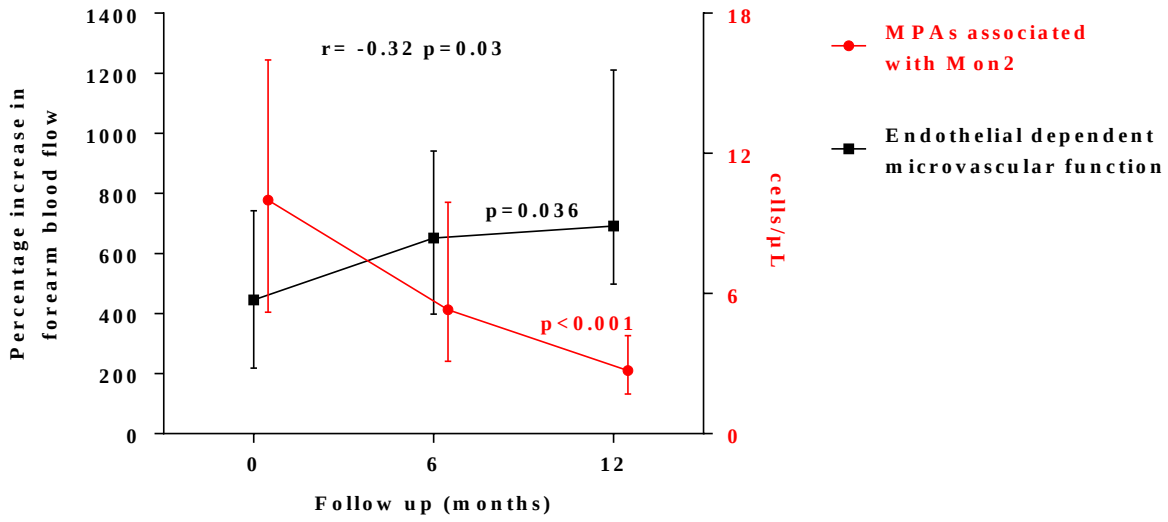


Figure 5.5 Longitudinal decline in monocyte-platelet aggregates associated with Mon2 parallels improvement in microvascular endothelial function in patients with diffuse coronary artery disease. r= -0.32, correlation coefficient of the change in measures over 12 months.

Table 5.12 Multivariable logistic regression of predictors of diffuse coronary artery disease – Mon2 related monocyte-platelet aggregates

Variable	**Odds ratio (95% confidence interval)**	**p value**
Age	1.05 (0.99-1.11)	0.12
Gender	0.66 (0.24-1.80)	0.41
White ethnicity	0.40 (0.15-1.11)	0.08
Mon2 monocyte-platelet aggregates	**1.10 (1.02-1.19)**	**0.01**
Body mass index	0.96 (0.86-1.07)	0.45
Previous myocardial infarction	3.17 (1.14-8.83)	0.03
Beta blocker	1.90 (0.63-5.78)	0.26
ACE inhibitor ARB	2.34 (0.78-7.05)	0.13
Statin	2.67 (0.55-13.1)	0.23
Aspirin	4.87 (1.11-21.4)	0.04
Clopidogrel	1.51 (0.56-4.09)	0.42

ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker.

Table 5.13. Multivariable logistic regression of predictors of diffuse coronary artery disease – endothelial microparticles

Variable	Odds ratio (95% confidence interval)	p value
Age	1.04 (0.99-1.11)	0.15
Gender	0.68 (0.25-1.80)	0.43
White ethnicity	0.60 (0.21-1.66)	0.32
Endothelial microparticles	**1.46 (1.09-1.96)**	**0.01**
Platelet microparticles	1.22 (0.81-1.83)	0.35
Body mass index	0.98 (0.89-1.09)	0.72
Previous myocardial infarction	4.19 (1.47-12.0)	0.007
Beta blocker	1.63 (0.55-4.86)	0.38
ACE inhibitor ARB	2.19 (0.72-6.71)	0.17
Statin	3.54 (0.65-19.2)	0.14
Aspirin	5.31 (1.22-23.1)	0.03
Clopidogrel	2.25 (0.83-6.07)	0.11

ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker.

5.2.8 Health related quality of life

All patients completed the both parts of the EQ-5D-5L questionnaire at each visit (Figures 5.6-5.10). Mobility, self-care, usual activities and anxiety/depression were similar between the study groups (p=0.32, p=85, p=0.81 and p=0.32, respectively) and there was no difference in visual analogue score at baseline (Figure 5.11) (p=0.97). Differences were present in the level of pain and discomfort reported between patients with focal CAD and subjects with normal coronaries. Participants with normal coronaries more often reported severe pain, while focal CAD patients reported more moderate and extreme pain (p=0.02) (Figure 5.9). There were no significant changes in quality of life measures during follow up (Figure 5.12).

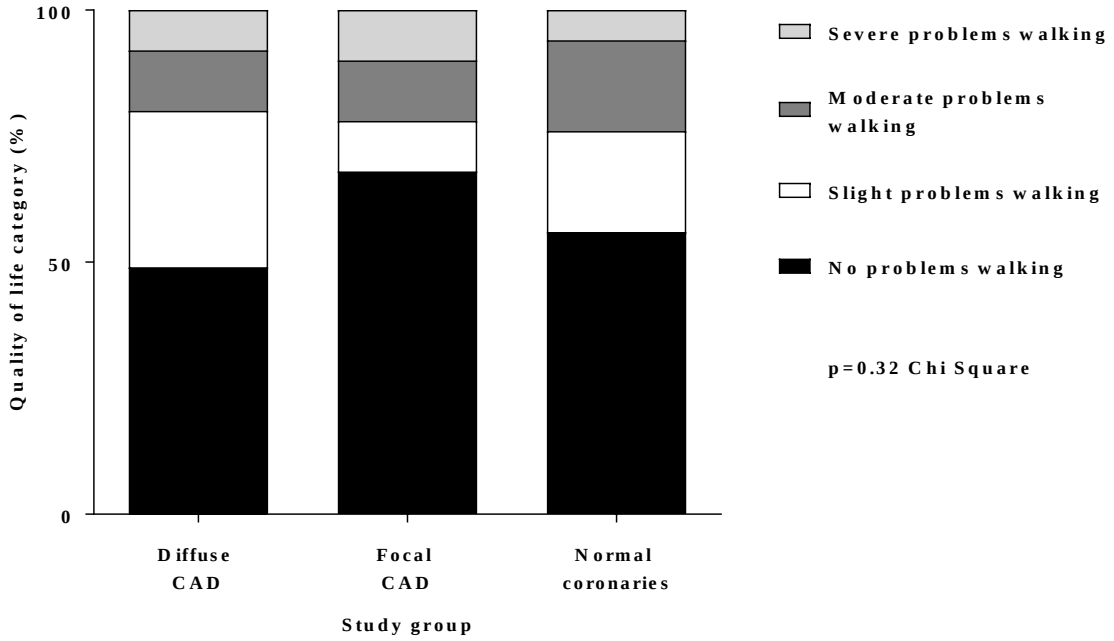


Figure 5.6 EQ-5D-5L: mobility. Shows the proportion of patients in each group reporting each degree of difficulty with mobility.

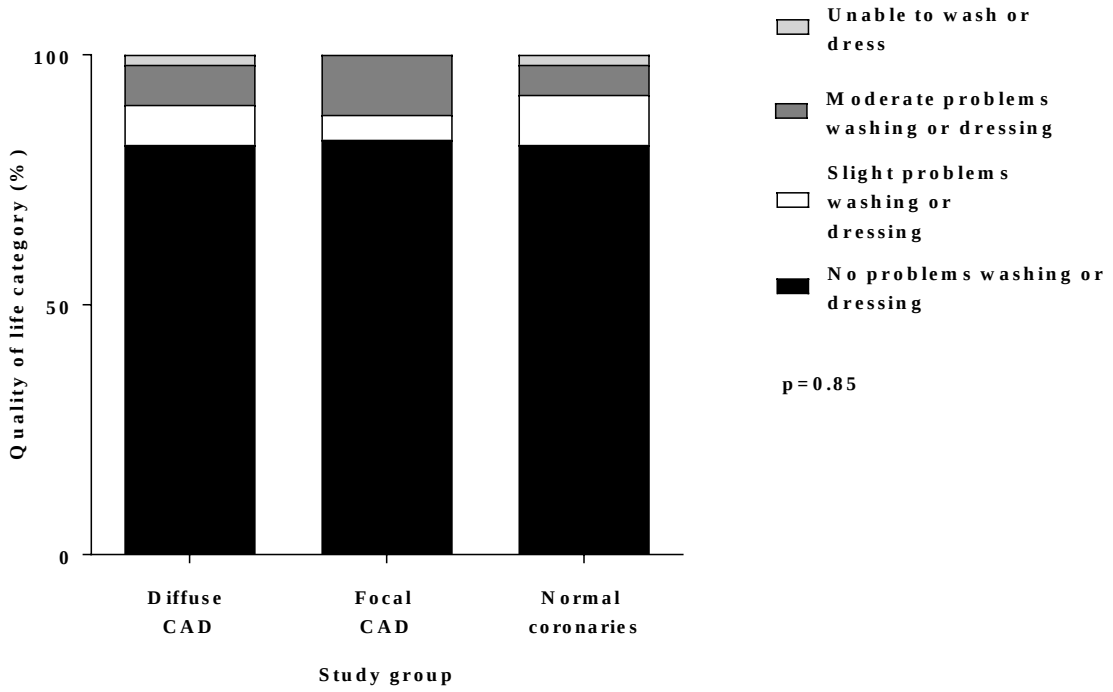


Figure 5.7 EQ-5D-5L: self-care. Shows the proportion of patients in each group reporting each degree of difficulty with self-care.

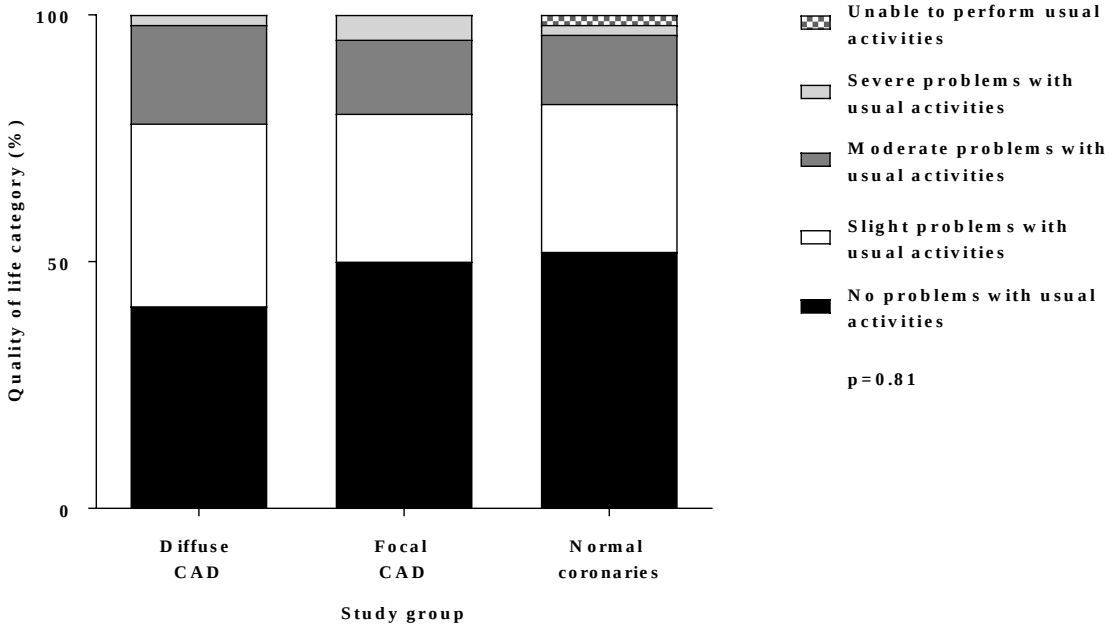


Figure 5.8 EQ-5D-5L: usual activities. Shows the proportion of patients in each group reporting each degree of difficulty with their usual activities.

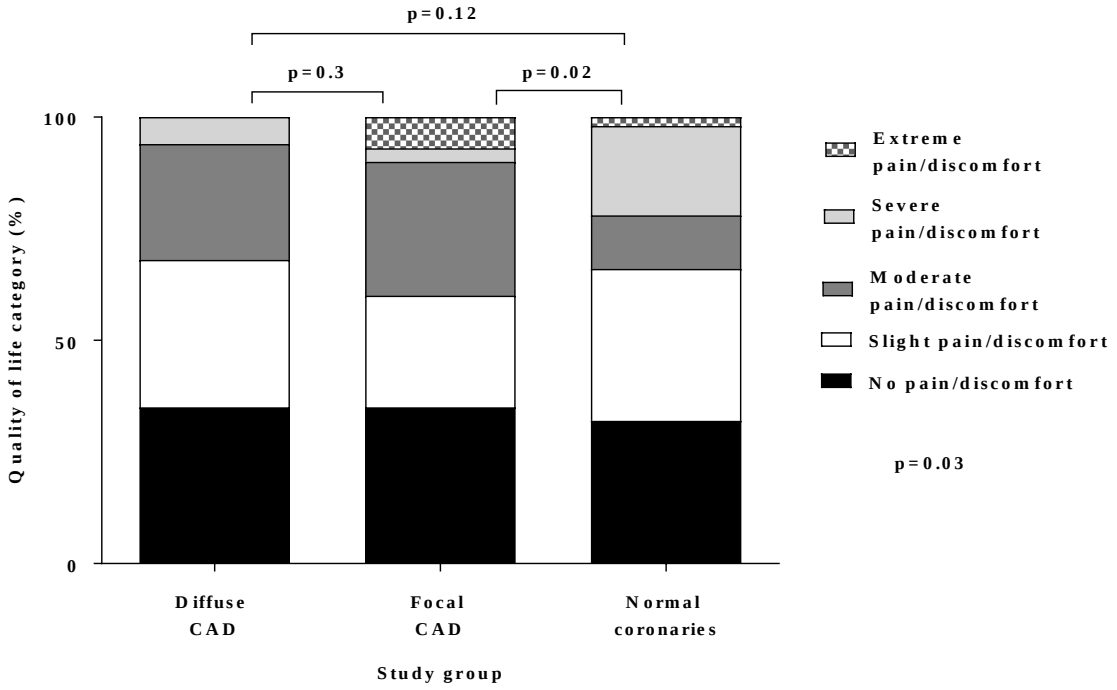


Figure 5.9 EQ-5D-5L: pain/discomfort. Shows the proportion of patients in each group reporting each degree of pain and/or discomfort.

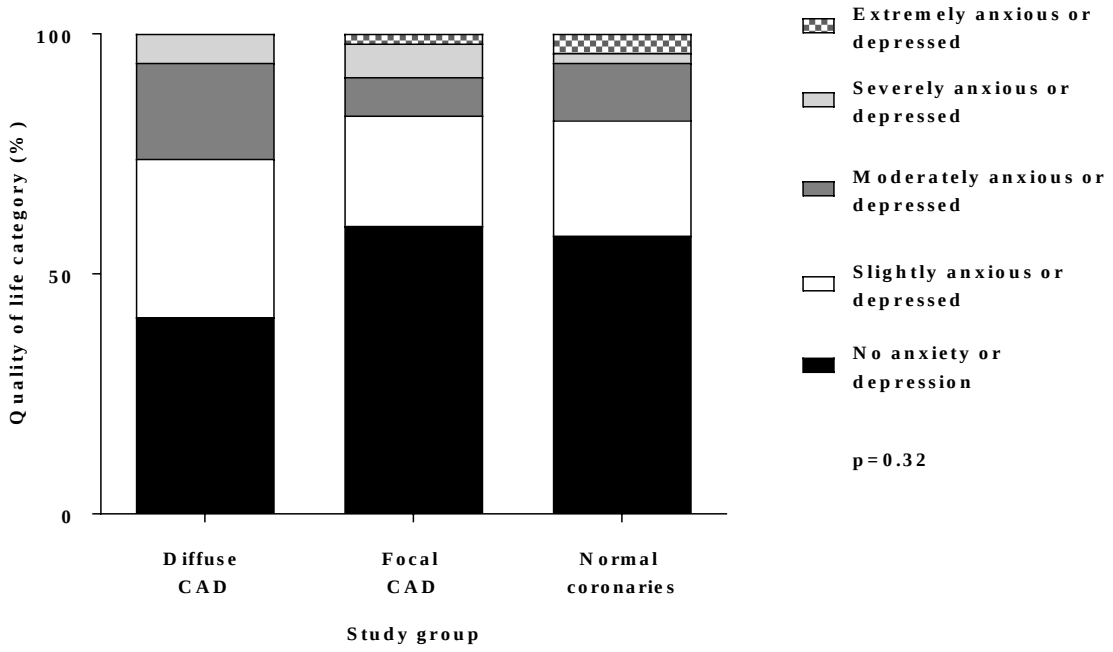


Figure 5.10 EQ-5D-5L: anxiety/depression. Shows the proportion of patients in each group reporting each degree of anxiety and/or depression.

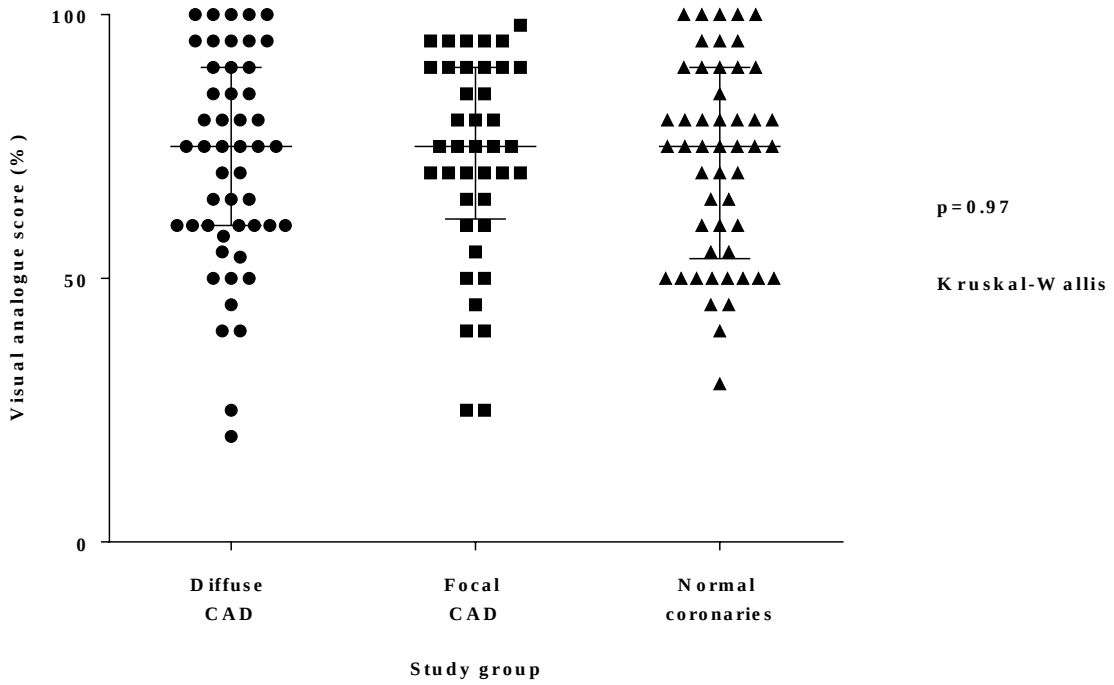
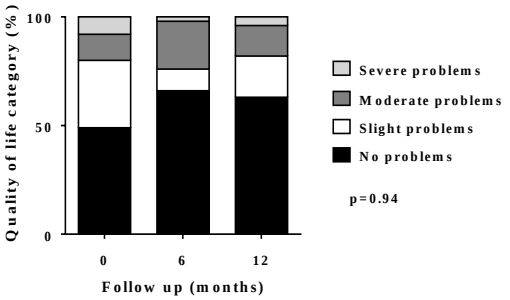
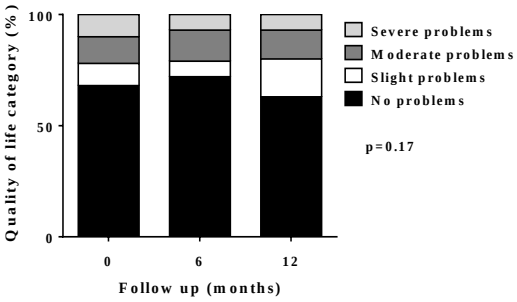


Figure 5.11 EQ-5D-5L: visual analogue score – subjects were asked to rate their overall health on a scale marked 0-100. The graph above shows median response with interquartile range for each group.

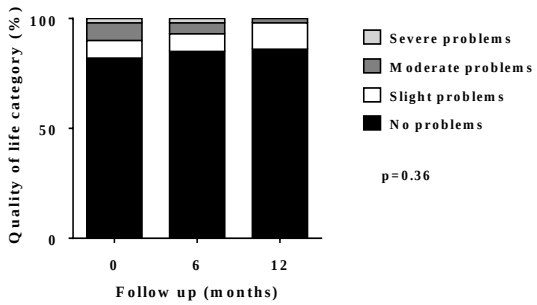
Diffuse CAD mobility over time



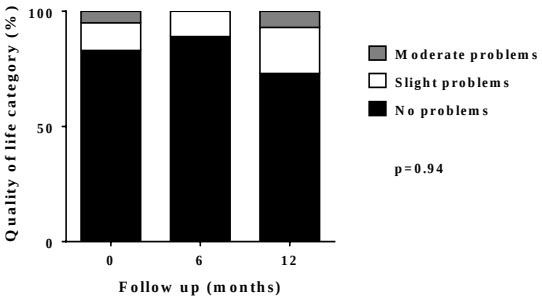
Focal CAD mobility over time



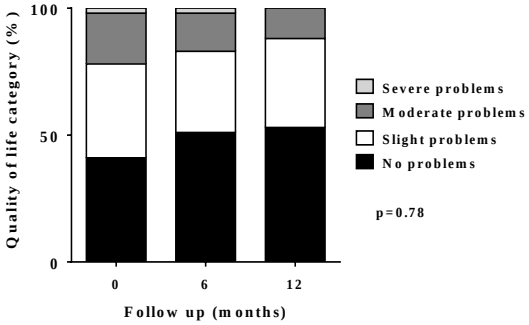
Self-care over time in patients with diffuse CAD



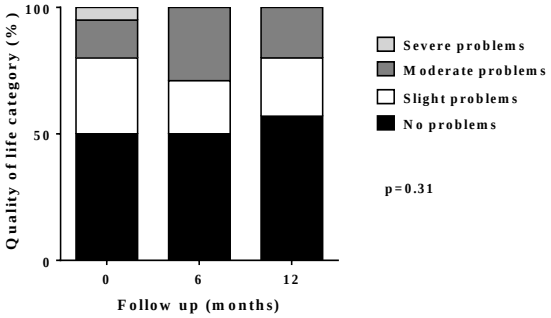
Self-care over time in patients with focal CAD



Usual activities over time in patients with diffuse CAD



Usual activities over time in patients with focal CAD



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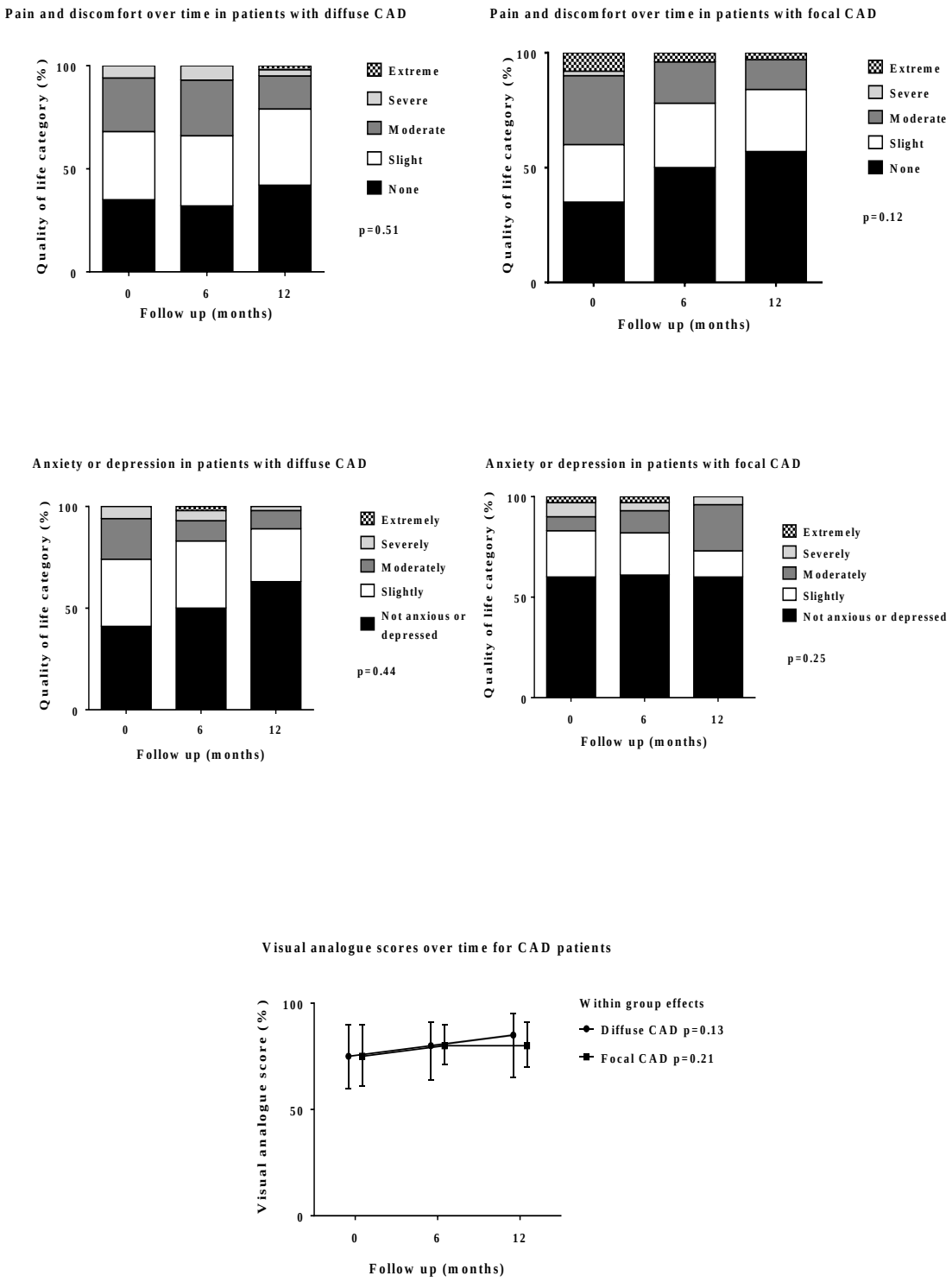

Figure 5.12 Changes in quality of life measures in the diffuse and focal CAD groups over 12 months follow up.

The bar graphs show the changes in the proportion of patients reporting each level of the 5 quality of life measures (mobility, self-care, usual activities, pain and /or discomfort and anxiety and /or depression). Diffuse CAD patients are on the left and focal CAD patients are on the right. The visual analogue scale is shown as a line graph of each group with median values plus interquartile ranges at each follow up visit.

5.3 DISCUSSION

5.3.1 Ventricular and arterial elastance, ventricular-arterial coupling and aortic augmentation index

The cross-sectional analysis showed no difference between the study groups in measures of EaI, LV Ees or VAC. Over twelve months there was a significant increase in LV Eed from baseline in patients with diffuse CAD compared to patients with focal CAD despite increased ACE inhibitor use in the former.

Although we found no difference in any of the stated measures of arterial or ventricular stiffness between the groups at baseline, we did note that patients with diffuse CAD had higher EDP, EDV, early mitral inflow and tissue Doppler velocities than patients with focal CAD, or normal coronary arteries. These measures indicate that diastolic stiffness is increased in patients with diffuse CAD despite similar LV Eed, (due to concomitant increases in EDP and EDV). Furthermore, longitudinal analysis showed an increase in LV Eed from baseline in patients with diffuse CAD, despite increased ACE inhibitor use in that group. Thus, these data support a temporal relationship between CAD severity and diastolic ventricular stiffness.

Only a few studies have examined measures of elastance and VAC in CAD[362, 363] for example Kadoi et al[362] evaluated Ees and VAC in 16 patients with, and four patients without CAD in anaesthetised patients undergoing CABG using transoesophageal echocardiography. The 16 CAD patients were stratified by LVEF. The study found that Ees, EaI and VAC in patients with CAD and normal ventricular function was similar to patients without CAD (undergoing elective abdominal surgery). Only patients with reduced LVEF showed a deterioration in elastance. In general, other studies have also

found LV Ees, VAC and EaI to be similar in patients with CAD compared to subjects without CAD[362-364].

Elastance measures have been more widely studied in the setting of heart failure and hypertension since its component variables (pressure and volume) are more readily affected by these conditions then they are by CAD. Indeed, long term use of secondary prevention drugs in the CAD groups may account for why, if anything, EaI was lower in those groups.

I observed a longitudinal increase in Eed in diffuse CAD patients. While this could be due to chance, diastole is more sensitive to ischaemia than systole and studies have shown that diastolic function is impaired more readily than systolic function in the context of ischaemia, meaning that the local changes in coronary blood flow that occur with more extensive CAD preferentially affect diastolic mechanisms[365, 366]. Indeed, several biomarkers associated with coronary endothelial dysfunction correlate with diastolic dysfunction in patients with CAD[367] and a recent study has shown that coronary pulse-wave velocity is high relative to aortic pulse-wave velocity in people with stable CAD, implying that differential anatomical increases in stiffness parameters are important, and supporting the fact that changes in ventricular stiffness measures may occur in the absence of aortic stiffness measures[368]. Oberoi et al[171] examined the relationship between CAD burden and aortic distensibility in a retrospective, longitudinal study using CTCA and found that CAD progression at 12 months follow up CTCA correlated with decline in aortic distensibility. Although limited by its retrospective design, it is one of the only studies (aside from the present one) to assess the relationship between CAD and arterial-ventricular stiffness longitudinally. The present study is also strengthened by the fact that the differences observed cannot be accounted for by age or disproportionate cardiac risk

factors. I saw a trend towards increased EaI and a significant increase in Eed during follow up in diffuse CAD patients that was not present in focal CAD patients. AIx remained static in both groups. Increased EaI (decreased compliance) promotes early wave reflection leading to increased systolic and pulse pressure and reduced diastolic pressure and coronary perfusion. While I did not see evidence of this within the year, these findings raise the possibility that a longer follow up period might reveal a temporal relationship between CAD severity and EaI, as well as with diastolic stiffening, especially since no change in Eed was seen in patients with focal CAD. Whether LV Eed can predict those patients with severe CAD at risk of developing ischaemia-associated cardiomyopathy would need to be evaluated in larger prospective studies.

I found no difference in AIx between the groups at baseline or longitudinally, suggesting it is not associated with the severity or progression of CAD. There have been inconsistencies in the literature regarding the usefulness of AIx as a marker of CAD[369-371]. These results are in keeping with several previous studies. Hope et al found no association between central AIx and the extent or severity of CAD assessed using the modified Gensini's and Sullivan's extent scores[372]. Patvardhan et al found no association with AIx and the presence of CAD[373]. Differences in the populations studied could be a factor in the variance, as could differences in the degree of CAD assessed for example Mcleod et al excluded cases with flow limiting lesions[371]. The impact of arterial wave reflection may just be related to age and other comorbidities, explaining why those with normal coronaries (but similar risk factor prevalence) have similar reflected wave contribution to central aortic pressures.

5.3.2. Cutaneous microvascular function

Patients with diffuse CAD had a strong trend towards worse microvascular endothelial function than patients with focal CAD or normal coronaries. Impaired cutaneous microvascular endothelial function has been shown to correlate with CAD risk factors such as diabetes[374], obesity[375], hypertension[376] and dyslipidaemia[377] as well as CAD itself, and has been used as a surrogate for coronary microvascular function in previous studies[378-381].

The attenuated overall group difference could be due to the presence of traditional CAD risk factors in my normal coronary artery control group. Indeed, my normal group was relatively hypertensive compared to the study groups. While studies using completely healthy individuals as controls have the capability to demonstrate larger differences[382] the impact of other risk factors combined with CAD can confound interpretation. My study has the advantage that coronary anatomy was known for each group and demonstrates a clear trend that supports the presence of worse microvascular endothelial dysfunction in patients with diffuse CAD compared to patients with focal CAD or patent coronary vessels[382-386].

Impaired coronary vasoregulation may involve several pathways. In the present study sodium nitroprusside was used to assess endothelium-independent vasodilation in the microvasculature and showed that maximal perfusion achieved was similar across all three study groups. Firstly, this proves that the drug delivery method was efficacious and secondly, suggests that CAD severity/diffuseness is linked predominantly to endothelium-dependent pathways as reported in other studies[387, 388].

The longitudinal analysis showed a significant improvement in endothelium-dependent microvascular function in patients with diffuse CAD that was not present in subjects with focal CAD which was associated with a decline in MPAs associated with Mon2.

5.3.3 Monocyte subsets and monocyte-platelet aggregates

Perhaps the most notable finding of my study is that levels of MPA associated with Mon2 were twice as high in patients with diffuse CAD as compared to the other groups. They were also found to be independently predictive of the presence of diffuse CAD. This is the first study that assesses the impact of MPAs related to specific monocyte subsets in patients with diffuse CAD.

In previous studies MPA levels were increased in humans with stable CAD[214, 223] as well as those with acute MI[389] and those undergoing PCI[390]. In a small study, Mickelson et al noted that in patients undergoing elective single vessel PCI, higher MPA levels were associated with subsequent target vessel restenosis and progression of CAD requiring surgical revascularisation[225]. My study supports a role for Mon2 related MPAs in the development of accelerated CAD. Indeed, chemokine-chemokine receptor (CCR) 5 (RANTES - regulated on activation normal T-cell expressed and secreted), a potent chemoattractant factor for monocytes and T cells, is highly expressed on CD16+ monocytes, especially Mon2[391]. CCR5 has a prominent role in human atherosclerotic lesion progression.

This study also showed that MPAs with Mon2 were inversely related to microvascular endothelial function (Figure 5.5). Di Serafino et al[222] prospectively assessed the contribution of coronary endothelial dysfunction and functionally significant obstructive CAD – measured by fractional flow reserve (FFR) – to circulating MPA levels. The

authors reported higher MPA levels in subjects with coronary endothelium dysfunction as well as an inverse correlation between the two.

Platelet activation has a key role in monocyte activation and recruitment to activated endothelium. MPAs are not only recruited to Von Willebrand Factor expressed on activated endothelial cells, but also induce the expression of additional platelet-borne receptors on the monocyte surface which can support the adhesion of unaggregated monocytes[392]. Thus, interaction between monocytes and platelets is an important link between vascular inflammation and thrombosis and may be more relevant than monocytes alone for the development of more aggressive CAD, with the present study providing further delineation and implicating MPAs associated with Mon2. These observations may reflect the fact that CCR5 (RANTES) and CCR2, potent chemoattractant factors for monocytes and T cells, are highly expressed on Mon2 – approximately 8-fold higher than on Mon3[391]. Both chemokine receptors have prominent roles in human atherosclerotic progression. In fact, several surface markers relevant to atherosclerosis are preferentially expressed on Mon2 monocytes in healthy donors and, therefore, may be upregulated in CAD. For example, CD11b regulates leucocyte adhesion to activated endothelium, VEGFR (vascular endothelial growth factor receptor) 1 and Tie, which have major roles in angiogenesis – important for the progression of inflammation and the development of collateral circulation in advanced CAD,[194] or TLR (toll-like receptor) 4. TLR4 signalling promotes CAD progression in a variety of ways, not least by mediating monocyte infiltration and subsequent macrophage FOAM cell formation within activated endothelium. It also mediates the release of several inflammatory cytokines (such as IL6, MCP1, and TNFα) from activated monocytes via receptors that are more prevalent on the Mon2 subset[194, 393]. Indeed, a previous study found that Mon3

($CD14^{dim}$) monocytes did not express CCR2 at all, and, in comparison to Mon2, produced no reactive oxygen species and expressed little mRNA for myeloperoxidase and lysozyme in response to TLR4, indicating that Mon2 produce most of the proinflammatory cytokines in that setting. Mon3 monocytes were found to possess an anti-inflammatory profile in the context of TLR4[394]. Thus, the findings from the present study likely reflect the imbalance of the immune system in favour of proinflammation – and increased monocyte-platelet cross talk in the Mon2 monocyte subpopulation – in people with advanced CAD, that is not present in people with focal CAD.

The excess of Mon2 related MPAs could also be a marker of plaque vulnerability, as well as the extent of stable plaque disease, as alluded to in some studies limited by the lack of individual subset analysis[389, 395]. Many previous studies have reported Mon2 and Mon3 together (as CD16+ monocytes). Nonetheless CD16+ monocytes have been associated with severity of CAD[203], late lumen loss due to in-stent restenosis of bare metal stents[206] and cardiac events[396]. Contemporary data suggest that Mon2 is likely to have been driving these associations and the present study emphasises the importance of platelet interaction with the Mon2 subset in the context of diffuse CAD.

There is a paucity of longitudinal data evaluating monocyte or MPAs in patients with diffuse coronary artery disease. In my study, patients with diffuse CAD showed a significant decline in Mon2 related MPAs during follow up. Importantly, this decline was paralleled by a significant improvement in endothelial-dependent microvascular function in that group. This was not observed in subjects with focal CAD. These findings imply that microvascular dysfunction could be an important part of the mechanism linking a Mon2 MPA driven, proinflammatory state, to progression of diffuse CAD.

As this was a non-interventional study however, the longitudinal changes observed in both measures could be due to many reasons e.g. variations in platelet and/or monocyte regulation[223, 397] or increased ACE inhibitor use in diffuse CAD patients[398]. Down regulation of MPA formation following monocyte recruitment into atherosclerotic lesions could theoretically allow for concomitant lesion progression and Mon2 related MPA decline. In addition to its chemoattractant effect,[399] animal studies have shown RANTES to be particularly prominent during the chronic phase of inflammation, resulting in increased CCR 1 and CCR5 expression, as well as increased numbers of monocytes and macrophages within areas of inflammation[400]. Furthermore, CCR5 receptor blockade results in experimental atherosclerotic lesion reduction, reduction in the recruitment of monocytes, mast cells, and neutrophils and, in some cases, near-complete resolution of inflammation[400-403]. Activated platelets promote CCL5-mediated monocyte arrest on injured endothelium, thus enhancing monocyte recruitment into developing atherosclerotic lesions. Increased CCR5+ cell recruitment into atherosclerotic lesions may explain the reduced levels of Mon2 related MPAs over time in patients with diffuse CAD, a phenomenon noted in previous cross-sectional studies[214] but expanded upon here with longitudinal data.

Nevertheless, whilst it is beyond the scope of this study to pinpoint the precise reason behind the longitudinal changes, the presence of a negative correlation between microvascular endothelial function and Mon2 related MPAs could be an important finding in patients with diffuse CAD and requires further study. A recent investigation by Taqueti et al[247] found that reduced coronary flow reserve (CFR) – an index of microvascular function – is associated with adverse cardiovascular outcomes

independently of luminal stenosis and extent of CAD. There was also an association between CAD extent and microvascular function. One might hypothesise therefore, that Mon2 related MPAs could underlie the association between reduced CFR and poor clinical outcomes – a notion implied by De Serafino's work[222] – and supported by a population study in patients undergoing elective coronary angiography[208].

5.3.4 Platelet and endothelial microparticles

My study also shows for the first time that CD31+/CD42b- EMPs are significantly higher in patients with severe, diffuse CAD compared to subjects with focal CAD or normal coronary arteries.

CD31 (platelet endothelial cell adhesion molecule [PECAM]-1) is a constitutive antigen expressed in high levels on mature endothelial cells. Its expression on EMPs is thought to reflect those EMPs derived mainly through endothelial cell apoptosis[404]. Persistent inflammatory stimulation of vascular endothelium leads to apoptosis and promotes progression of atherosclerosis so it is likely that endothelial cell apoptosis is a key pathophysiological mechanism differentiating focal CAD from more aggressive forms of the disease where reparative capability is reduced[405]. Indeed, we found no difference in EMP levels between subjects with focal CAD versus subjects with normal coronary arteries implying that distinct pathways – favouring endothelial cell death – may be involved in the development of diffuse coronary disease in middle age.

Reports regarding the impact of severity of stable CAD on MPs are mainly limited to descriptions of the stable control groups in experiments involving patients with acute

coronary syndrome[235, 236], many of which include either control subjects with undocumented coronary anatomy, or no control group[235-237]. This study demonstrates an association between apoptotic EMPs and severity of stable CAD in subjects with multiple, equivalent risk factors[231, 406]. It shows a pattern of changes consistent with some related studies in the field. Koga et al showed that CD144 EMPs independently predict the presence of CAD in patients with diabetes mellitus without symptomatic episodes of angina[231]. CD144 – an inducible antigen – is specific for EMPs derived from endothelial activation[407]. In a prospective study of 200 patients with stable CAD, Sinning et al found that circulating CD31+/annexin V+ MP levels above the median were associated with a higher risk of death from cardiovascular causes and need for revascularisation during 6-year follow up[242]. This would support CD31 EMPs as a marker of more aggressive CAD phenotypes. Interestingly, Sinning's group reported only a non-significant trend (p=0.08) in the association between EMPs and CAD extent. This most likely reflects the contribution of coronary endothelial dysfunction to subsequent plaque rupture events in people with single vessel CAD, since multivessel CAD is known to carry a worse prognosis than single vessel CAD[237, 408].

In vitro experiments demonstrate that CD31 antigen is also expressed on ~5% of activated and 10% of non-activated platelets[409], however, we saw no difference in PMP (CD42b+) levels between the groups, implying that the MPs were predominantly endothelial in origin.

Incorporating data from numerous in vitro experiments, MPs are now considered important vectors of biological information in many pathological (and physiological)

processes though their role in healthy individuals is less well understood. Thus, more than simply being a marker of severe endothelial dysfunction and possibly advanced CAD, EMPs also act as intercellular messengers, promoting the synthesis and release of proinflammatory cytokines, stimulating the expression of adhesion molecules (particularly ICAM-1) and augmenting the expression of adhesion molecule counter-receptors – such as CD11b – on Mon2 monocytes, potentially enhancing monocyte-platelet aggregation[194, 228]. Rautou et al confirmed some of these in vitro findings when they observed that exposure to (mainly leucocyte) MPs isolated from advanced atherosclerotic plaques in humans rapidly increased intercellular adhesion molecule-1 at the endothelial cell surface, suggesting that the proinflammatory effects of MPs occur throughout the atherosclerotic process[410]. Indeed, in some cases, MPs may be even more potent than their parent cells which could explain the relative abundance of EMPs in patients with accelerated CAD found in the present study [234].

Potential benefits of EMP formation on endothelial integrity include increased endothelial progenitor cell release[411], control of cell death mechanisms and induction of adaptive immunity[412]. Hence, EMPs may reflect the severity of the endothelial injury, but also the need for reparative angiogenesis due to atherosclerotic lesions of coronary arteries[413]. These favourable regulatory functions have mostly been studied in vitro so their relevance in vivo remains undetermined.

Throughout follow up, I observed a decline in EMPs in both CAD groups (although the effect was stronger in patients with diffuse CAD). As mentioned in section 5.3.4, this could be due many cofounding reasons. The extent to which this decline reflects improved

cutaneous microvascular function in the diffuse CAD group is unclear. Previous studies have correlated apoptotic EMPs with coronary endothelial function although in their analysis, Werner et al exclude subjects with severe CAD (those with surgical disease) and normal coronaries[237]. One reason for the lack of correlation in the present study could be the range of MP data acquired, such that despite clear differences in median values there was considerable overlap of interquartile ranges. This remains an important limitation in the enumeration of MPs and restricts their use in clinical practice.

5.3.5 Health related quality of life

There was no difference in HrQOL between the groups. There are several possible reasons for this. HrQOL is closely related to symptoms and many studies have shown an improvement in HrQOL following coronary revascularisation (during which symptoms are often resolved) particularly where pre-operative/procedural health status was poor[414-418]. Patients with normal coronaries on the other hand may have persistent unexplained symptoms which can adversely affect QOL by a number of mechanisms such reduced physical activity[419] or higher levels of anxiety. Even in the absence of symptoms, the groups were age, gender and risk factor matched which meant that subjects with normal coronaries had other conditions that could contribute to worse QOL such as diabetes[420] or hypertension[421]. Several patients in my diffuse CAD group had had previous CABG. Randomised data comparing HrQOL after CABG versus PCI has been inconsistent with some showing more improvement after CABG[422] and others no difference[423, 424].

Longitudinal analysis revealed no significant change in either CAD group. VAS scores also remained similar in both groups which could represent small group numbers or be consequential of a relatively short follow up period. However, the follow up time is

consistent with comparable studies some of which have shown change as early as three months[425, 426] albeit in response to the treatment of CAD, whereas the objective here was simply to provide an evaluation of general HrQOL between the groups.

Male sex and white ethnicity showed a trend towards higher VAS scores whereas Afro Caribbean ethnicity, diabetes and physical inactivity showed a trend toward lower VAS scores. Despite not meeting statistical significance, these are valid signals as similar observations have been made consistently throughout the literature[427-430]. Many studies have also described increasing age as a negative predictor of VAS scores which was not apparent in my results as my cohort was relatively young.

5.4 LIMITATIONS

Despite the careful design of this study, there are several limitations to consider.

5.4.1 Limitations of study design

This is single centre, observational data and is therefore susceptible to inherent bias. There is a selection bias since all patients were, at some point, symptomatic. However, the experimental data are prospective. Also, to strengthen the results by reducing unconscious bias, data analysis would have ideally been performed by an investigator who was unaware of the study participants' group allocation.

The degree of luminal stenosis in CAD patients was assessed visually which could have resulted in misclassification of some cases. I made every effort to minimise this potential by selecting patients in whom the severity and morphology of disease was obvious.

It is routine practice at our centre to provide PCI at the time of coronary angiography to stable patients who require it. Therefore, due to ethical considerations, patients were most often studied post angioplasty. Although no patients were studied within two months of stenting, I cannot rule out the possibility of long-term modification of peripheral MPA levels by intracoronary stents.

AIx was not obtained at the time of cardiac catheterisation. Rather, they were determined weeks, months or sometimes years later. However, to make sure that the measures taken represented the intended disease morphology, patients with normal coronaries or focal CAD were studied a maximum of one year post coronary angiography on the basis that significant progression of flow limiting CAD is unlikely under one year. Patients with

diffuse CAD are already at an advanced stage of the continuum of CAD and cannot go 'backwards'.

One-year follow up may be considered short for detecting changes in vascular function (especially those measured using ultrasound) although previous similar studies have shown differences in measures of aortic distension over one year[171]. Most studies assessing longitudinal monocyte and microparticle dynamics have been following an intervention (pharmacological or otherwise)[390, 431, 432], exercise[433] or acute illness[216, 434, 435]. However, this study was designed to investigate the natural history of these biomarkers and their behaviour in patients with stable CAD and was the most pragmatic timeframe in which to complete the study. Furthermore, CAD is a continuous process. As such, both the cross sectional and longitudinal measures in this study represent a snapshot in the natural cardiovascular history of the subjects involved and it is impossible to accurately determine the pattern of measures outside of the study timeframe. However, due to legitimate time constraints, only one-year follow up data could realistically be achieved.

5.4.2 Methodological limitations

The non-invasive measurement of Ees index from ESP/ESV index ratio assumes that the theoretical volume when no pressure is generated (V_o – which has not been well defined in humans) is negligible compared with end-systolic volume. Several single beat methods have been proposed to estimate the end systolic pressure volume relationship (ESPRV)[290, 436, 437], with the method employed by Bombardini et al[364] being perhaps the most easily applicable. The linearity of ESPVR simplifies its prediction using this approach however it should be viewed as an approximation, particularly for end-diastolic pressure-volume

relationship (EDPVR), where the curve is less linear. However, the same approach was used for all patients and previous studies have shown that the LV EDPVR can be reasonably estimated from a single pressure-volume point, and the predicted relationships are generally well correlated with directly measured data[438]. Also, the use of radial applanation tonometry in my study allowed more accurate central systolic blood pressure estimation than previous studies applying a correction factor to the brachial systolic pressure[290, 364].

The methodology used to detect and exclude CAD should be considered. Invasive coronary angiography provides information about luminal pathology only. Subjects with eccentric vascular remodelling may have CAD but demonstrate apparently normal coronaries. CTCA is a sensitive tool for the detection of vascular wall calcification and abnormal remodelling in CAD[439]. To identify subjects with no CAD at all (i.e. no extra-luminal CAD), I tried to use CTCA as much as possible. Unfortunately, due problems with missed or cancelled appointments and the high proportion of females referred for CTCA, I still had to recruit subjects from the invasive lists but since focus of my study was *flow limiting* CAD, I would not consider this a major limitation.

I quantified microvascular function in the peripheral circulation which is an indirect assessment of central processes, although studies have validated this approach[440] and moreover, invasive measurement such as coronary flow reserve has its own limitations[441]. Iontophoresis of acetylcholine (laser Doppler) has been shown to have good reproducibility when performed in a controlled environment[308].

Laser Doppler flowmetry (used to assess microvascular function) is sensitive to various factors which can affect the reliability of the measures. To mitigate these, I strived to

achieve highly standardised conditions, for example each person was studied in the same, temperature-controlled room (specified according to manufacturer guidelines). Follow up measures were performed at the same time of day (where possible) by the same operator (myself), using the same technique each time. Despite this approach, heterogeneity in the microvascular responses can be brought about by uncontrollable factors such as variation in capillary density or the number of hair follicles between patients[442].

Nonspecific, current induced vasodilatation (the so called galvanic response caused by the process of iontophoresis itself rather than the vasoactive drugs) is an important confounding factor[443]. Current induced release of neuropeptides is a popular theory but the mechanisms are incompletely understood[293]. Various strategies have been applied to avoid this effect such as the limitation of current strength or total iontophoretic charge[444], or special vehicle solutions[445] but the lack of a standardised protocol makes between-study comparisons difficult[293].

There is also the possibility of under estimation of MPA counts due to EDTA causing the platelets to clump together. EDTA-dependent chelation of calcium, at the appropriate temperature, can change the expression of the platelet glycoprotein 2b receptor, allowing autoantibodies to bind and induce platelet aggregation. However, this effect should be negligible, and the same methodology was used for all subjects.

5.4.3 External confounders

Interpretation of the longitudinal analyses are hampered by uncontrollable confounders such as changes in diet or exercise or unspecified changes to a patient's circumstances leading to other subtle lifestyle changes. Operator variation was kept to a minimum by performing batch analysis of plasma microparticles at the close of the study, minimising

the impact of factors such as antibody denaturation or shifts in calibration parameters over time.

EQ-5D does not contain any disease-specific measures however it has been validated in patients with ischaemic heart disease[303, 304]. I did not correct for psychosocial factors such as income, education or marital state but these have mainly been shown to be of importance in studies relating to ethnicity which was matched across my study groups.

5.5 SUMMARY OF KEY RESULTS

- MPAs associated with Mon2 and EMPs were associated with diffuse CAD. Levels of both markers were significantly higher than in patients with focal CAD or subjects with normal coronary arteries.
- There was a significant inverse correlation between Mon2 related MPAs and endothelial-dependent microvascular function in patients with diffuse CAD. The correlation was evident at baseline and on longitudinal analysis.
- There was a strong trend towards worse endothelial-dependent microvascular function in patients with diffuse CAD compared with focal CAD or subjects without CAD.
- MPAs associated with Mon1, MPAs associated with Mon3 and total MPAs were similar between the groups at baseline.
- Total monocytes and monocyte subsets were similar between the groups at baseline (although patients with diffuse CAD showed a non-significant trend towards higher Mon2 levels).
- Arterial and ventricular elastance indices (EaI, Ees and Eed), VAC and AIx were similar between the groups at baseline.
- During follow up there was a significant increase in Eed index in diffuse CAD patients.
- Quality of life measures were similar between the groups and essentially unchanged throughout follow up.

SECTION VI

SUMMARY OF FINDINGS, CONCLUSIONS AND FUTURE DIRECTIONS

6.1 SUMMARY OF FINDINGS

Section IV contains retrospective analyses of data collected routinely in patients with acute CAD and served to highlight the clinical burden of CAD locally in terms of acute myocardial infarction. I found that South Asian ethnicity is associated with late evening presentation to hospital with STEMI, that there was no difference in symptom-to-door time between men and women or different ethnic groups and that despite the successful reduction of door-to-balloon time in our centre, mortality from STEMI had remained constant throughout the 5-year study period.

Section V contains the results of my experiments. The main finding is a significant excess of MPAs associated with Mon2 and circulating EMPs in patients with established, diffuse flow limiting CAD compared to patients who developed flow limiting CAD more focally (affecting a single segment of one coronary artery) or patients who, despite similar age and traditional risk factors, had normal coronary arteries (Section V, Tables 5.8 and 5.10).

During 12 months follow up, I observed a decline in Mon2 related MPAs that was inversely correlated with improvement in endothelial-dependent microvascular function in patients with diffuse CAD (Section V, Figure 5.5). EMPs also declined significantly during follow up.

After adjusting for the effect of blood pressure medications I found no baseline differences in elastance measures (LV Ees, LV Eed, EaI, VAC or AIx) between the groups (Section V Tables 5.3 and 5.4). However, EDP, EDV, early mitral inflow and tissue Doppler velocities were all higher in patients with diffuse CAD compared to participants with focal, or without CAD. Furthermore, Eed increased during follow up in patients with diffuse CAD and remained static in patients with focal CAD.

I did not detect a significant difference in monocyte levels between the groups at baseline or during follow up.

There was no difference in self-reported HrQOL between the groups at baseline or on follow up (Section V, Figures 5.6-5.12).

6.2 CONCLUSION

The present thesis represents a detailed assessment of cardio-vascular interactions, arterial stiffness, systemic microvascular endothelial function, monocyte subsets, monocyte-platelet aggregates and circulating microparticles in patients with severe, diffuse CAD, patients with focal CAD and subjects with no CAD.

Patients with diffuse CAD had similar cardio-vascular interactions and aortic augmentation indices to patients with focal CAD and people with normal coronary arteries but they also had higher LV filling pressures and volumes and my findings indicate that patients with diffuse CAD may be more susceptible to changes in diastolic elastance.

Levels of Mon2 related MPAs and apoptotic EMPs were higher in patients with diffuse CAD than in subjects with focal CAD or normal coronaries. Mon2 related MPAs correlated inversely with endothelial-dependent microvascular function in subjects with diffuse CAD and suggest that microvascular endothelial apoptosis could be an important determinant of this unfavourable type of CAD. Levels of PMPs between the groups were similar which would be consistent with overall low-level platelet activation in stable CAD compared to acute thrombo-occlusive states.

Reported HrQOL was similar across all three groups which likely reflects the impact of undiagnosed chest pain in subjects with normal coronary arteries versus those who are diagnosed with CAD and subsequently treated.

6.3 FUTURE DIRECTIONS

Improvements in the treatment of acute CAD mean more people reach a point where CAD is advanced and diffuse, thus making it less amenable to conventional therapies thus increasing the mortality and morbidity associated with the condition. Future directions should perhaps be focused on a) identifying people at risk of developing the condition, and b) cell-specific targeting of the immune system.

In this thesis, I show that improvement of microvascular endothelial-dependent function in patients with diffuse CAD over one year parallels a decline in Mon2 associated MPAs. Future prospective longitudinal studies could examine whether these changes relate temporally to CAD burden, with intravascular imaging or CTCA.

Mon2 monocytes have been associated with poorer cardiovascular outcomes. Follow up time was a limitation of my study, but further research could focus on whether MPAs with Mon2 are better predictors of adverse outcome than Mon2 alone. Prospective long-term follow-up of people with focal CAD, with serial measurements of MPAs associated with Mon2, to see who develops diffuse CAD and whether Mon2 related MPAs are truly predictive of this type of CAD would be beneficial and would strengthen the case for their use as pharmaceutical targets.

Monocytes as delivery vehicles for therapeutic agents is an area that has seen recent attention, predominantly in mice[446]. Feasibility in humans for CAD treatment is not known.

The underlying mechanism by which platelets regulate the activation, polarisation and differentiation of monocytes is thought to involve activation of the nuclear factor ‘kappa-

light-chain-enhancer' of activated B cells (NF-κB) pathway and signal transduction via phosphorylation of Lyn kinase[447]. This requires clarification since the precise role of Mon2 is still being defined. It is possible that MPAs associated with Mon2 facilitate a functional transition from Mon1 and Mon2. On the other hand, Mon2 related MPAs may enhance the production of anti-inflammatory cytokines or angiogenic factors. IL10, produced preferentially by Mon2, is known to inhibit the NF-κB pathway so evidently a complex interplay exists. The recent CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) trial randomised over 10,000 patients to 50mg, 150mg or 300mg of Canakinumab respectively against placebo and found a statistically significant reduction in the primary endpoint (non-fatal myocardial infarction, any non-fatal stroke, or cardiovascular death) with the 150mg dose compared to placebo[448]. Canakinumab, an IL1ß inhibitor, effectively reduced high sensitivity C reactive protein and whilst it may benefit stable CAD who have ongoing inflammatory risk after appropriate lipid-lowering therapy, it did so at the expense of increased risk of fatal infection. Herein lies the real challenge of the future – targeting the immune system with enough specificity to slow, or even halt the progression of CAD whilst maintaining adequate defence against infection.

Molecular imaging techniques such as the tracer principle (labelling a biomolecule with a radioisotope and following its distribution throughout the body) have gained substantial interest over the past decade. Their diverse range of applications includes imaging of inflammatory cells such as macrophages as well as platelets and angiogenesis[449]. More recently multi-modal hybrid imaging (PET/MRI) has been reported to quantify both monocyte-associated myocardial inflammation and remodelling[450]. Ultimately these techniques may be routinely used to study human monocyte subsets and MPAs and could

be used in conjunction with measures of systemic microvascular function to identify patients at risk of developing diffuse CAD.

APPENDICES

Appendix 1: Published and submitted papers from this thesis

Brown RA, Lip GYH, Varma C, Shantsila E Apoptotic endothelial microparticles are increased in patients with diffuse coronary artery disease irrespective of microvascular function. Under submission.

Brown RA, Lip GYH, Varma C, Shantsila E. Impact of Mon2 monocyte-platelet aggregates on human coronary artery disease. Eur J Clin Invest. 2018;48:e12911.

Brown RA, Shantsila E, Varma C, Lip GYH. Current understanding of atherogenesis. Am J Med. 2017;130:268-282.

Brown RA, Shantsila E, Varma C, Lip GY. Epidemiology and pathogenesis of diffuse obstructive coronary artery disease: the role of arterial stiffness, shear stress, monocyte subsets and circulating microparticles. Ann Med. 2016;48:444-455.

Brown RA, Shantsila E, Varma C, Lip GY. Symptom-to-door times in patients presenting with ST elevation myocardial infarction-do ethnic or gender differences exist? QJM. 2016;103:175-80.

Brown RA, Lip GY, Varma C, Shantsila E. Ethnic differences in the diurnal variation of symptom onset time for acute ST elevation myocardial infarction - An observational cohort study. Int J Cardiol. 2015;187:414-6.

Brown RA, Varma C, Connolly DL, Ahmad R, Shantsila E, Lip GY. Simultaneous computerised activation of the primary percutaneous coronary intervention pathway reduces out-of-hours door-to-balloon time but not mortality. Int J Cardiol. 2015;186:226-30.

Appendix 2: Published abstracts from this thesis

Brown RA, Lip GYH, Varma C, Shantsila E. Monocyte-platelet aggregates associated with CD14++CD16+ monocytes predict diffuse coronary artery disease: Relationship to microvascular function. Eur Heart J (2016) 37 (Suppl 1): 599-983 DOI: http://dx.doi.org/10.1093/eurheartj/ehw433.

Brown RA, Lip GYH, Varma C, Shantsila E. CD14++CD16+CCR2+ monocyte-platelet aggregates are associated with severity of coronary artery disease. Hämostaseologie (2016) Suppl 1;A63.

Brown RA, Lip GYH, Varma C, Shantsila E. CD14++CD16+CCR2+ monocytes are increased in diffuse coronary artery disease. Heart 2015;101:Suppl 4 A90 doi:10.1136/heartjnl-2015

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Appendix 3: Standard Operating Procedure; pulse wave analysis

SOP 106

Pulse wave analysis (PWA)

Sern Lim: November 2008

updated by Richard Brown: January 2014

Background

Left ventricular (LV) contraction generates a forward travelling pressure wave. Some of this pressure wave will be reflected at sites of impedance mismatch (e.g. bifurcations and lesion sites). Increased arterial stiffness results in earlier return of the reflected pressure wave in systole rather than diastole. The reflected pressure wave will increase systolic blood pressure, termed pressure augmentation and may be expressed as the augmentation index. Increased arterial tone, such as that associated with endothelial dysfunction also increases the reflected wave and the augmentation index. The analysis of the arterial pressure wave, termed pulse wave analysis (PWA) may therefore provide information on the functional properties of the arterial system[451, 452].

1. Equipment

You will need:

- Patient.
- SphygmoCor with pressure transducer.
- Laptop computer with SphygmoCor software.
- Power cables and USB connection cables.
- ECG monitoring dots.
- BP recorder.
- Salbutamol inhalers and GTN spray.

2. Preparation

    a. Allow the subject to rest in the room for about 10 minutes before scanning. The test should be done in a supine position.
    b. While the patient is resting, you should connect the power cables to the SphygmoCor machine (at the back) and the laptop computer. The computer should then be connected to the SphygmoCor machine (use the USB connection at the back). The ECG cables should be connected to the SphygmoCor machine (in front where it says 'ECG').
    c. Switch on the SphygmoCor machine first. The lights in front will blink. When the lights stop blinking and green light stays on 'ready', turn on the computer. This sequence will allow the software in the computer to recognise the SphygmoCor.

d. The SphygmoCor icon should be on the computer desktop. Double-click to activate the software. A warning will come on the screen, just click ‘allow’.

e. You will see PWA and PWV on the top left of the screen. Click on PWA for pulse wave analysis.

f. Click ‘create new’ on the top right of the screen. You will need to enter the patient details in the relevant boxes (e.g.: date of birth) and click ‘update’.

g. Click on PWA on the top left for pulse wave analysis and then ‘study’ on the top left of the screen. Choose radial or carotid by ticking the box. Measure the patient’s brachial blood pressure (Omron). Take 3 measurements and use the average of the last two readings.

h. Enter BP measurements. Height and weight if you have the data.

i. Place the ECG leads on the patient and click ‘Capture data’ on the top right of the screen. You are now ready to go!

3. In the drawer of the tonometer machine, you will find the Millar tonometer with a plastic cap to protect the high-fidelity tip (this cap is important, DO NOT lose it!). The tonometry should be connected already. DO NOT disconnect the tonometer. Remove the plastic cap and it is ready for use.

4. Feel for the radial or carotid pulse (depending on which you want to measure) and place the tonometer over the pulsation. You will see a pressure trace on screen. The screen will automatically adjust the scale to accommodate the pressure trace. You need to make sure the trace is consistent for at least 12 seconds. To obtain

the measurement, press the spacebar on the laptop. The machine will not take the last 2 seconds of recording to give you time to let go and press the spacebar.

5. The computer screen will then change to the data screen with the ensemble-averaged pressure trace (radial or carotid depending on the one you have chosen) shown and the derived central arterial pressure trace. First thing to do: check the quality control box on the top left of the screen. If this number is red, then the data is of insufficient quality and the measurement will need to be repeated.
6. The data will be available at the bottom of the screen. At the top right of the screen, click 'Export' to save the data in your folder. You can store the data as a text file or jpeg (the screen shot). I would suggest you store both, then your study is done!

Potential problems

1. The high-fidelity tonometer is very sensitive to pressure (that is what it is meant to do!). You will need practise to achieve a consistent level of pressure to obtain reproducible readings. It is generally more difficult for carotid artery compared to the radial artery.
2. To avoid any problems with the computer, it should NOT be used for any other reasons (e.g.: surfing the internet). The computer and the tonometer (plus ALL the cables and ECG leads) should ALWAYS be kept together.
3. Every operator should create a folder for him/herself and store ALL the data in that folder. This should avoid confusion and the relevant data may be easily retrieved.

Appendix 4: Standard Operating Procedure; assessment of peripheral microvascular endothelial function by laser Doppler iontophoresis

Standard Operating Procedure 107

Measurement of cutaneous microvascular endothelial function

Written by Richard Brown January 2014

Health and Safety / COSHH

Some subjects may be allergic to Ach & SNP[453]. Some may respond adversely to the electrical current. Obtain consent.

The function of large arteries such as the aorta, brachial and femoral can be assessed by their dilation response to changes in blood flow (i.e. flow mediated dilatation, FMD). This assessment is not possible in the study of small arteries and arterioles, often within vascular beds such as the skin. However, the recent development of perfusion imaging can assess these small arteries[454]. The present SOP is to enable this assessment.

Introduction

Laser Doppler perfusion imaging is based on four separate technologies

- (a) the Doppler shift phenomenon (change of the wavelength of the moving object proportionally the speed of the object, i.e., blood cells),
- (b) the ability of the Laser imaging system to detect this phenomenon,

- (c) perfusion of skin with pharmacologically active drugs (acetylcholine [Ach] and sodium nitroprusside [SNP]) that will alter small blood vessels close to the surface of the skin, and
- (d) the electrical delivery of these agents into the skin (iontophoresis).

Thus, the method allows an evaluation of the perfusion of surface tissues (e.g. skin) in real time scale. Changes in skin blood flow can be determined in response to various stimuli. Vasoactive substances can be used to alter the flow of blood in the skin vessels if delivered by an appropriate means.

Iontophoresis is a delivery method that uses the charge of a vasoactive substance to allow it to be driven through the skin using an electric current. Sodium nitroprusside is a vasoactive substance with a negative charge that can be delivered through the skin to the microcirculation; once it has passed through the skin it acts as a nitrate donor and therefore acts directly on the vessel's smooth muscle to cause vasodilatation. Thus, it is independent of the endothelium in its vasodilatory action. Acetylcholine on the other hand, is a positively charged molecule that can similarly be delivered to the subcutaneous tissues by iontophoresis, but it acts on the endothelium rather than on the smooth muscle wall, causing endothelial release of nitric oxide. Thus, acetylcholine acts in an endothelium-dependent way on the vessel wall to produce vasodilatation. The vasoactive substances are provided close to the skin in a small perfusion chamber that also acts as an electrode. A second electrode is required to complete the circuit, and this is attached to a nearby section of skin.

1. Test solutions

Quantities of 1% acetylcholine chloride (Ach) and 1% sodium nitroprusside (SNP) (both from Sigma-Aldrich) must be prepared in sterile filtered distilled water (Fistreem International) and appropriate volumes (approximately 1ml aliquots) stored in plastic tubes in a fridge at 4°C for up to 1 month. These solutions are transferred from the plastic tubes to the largest of the three holes in the centre of the iontophoresis chamber with a plastic disposable transfer pipette (stored in the preparation room and the laboratory). Ensure the reagents reach room temperature. SNP solution should be protected from the light, so you will need something to wrap the test tube in, such as some tinfoil or inside a glasses case.

2. Preparing the Laser Doppler Flow meter (MoorSoft DRT4 Axminster Devon, UK)[455].

The laser requires at least 10 minutes to warm up so do this well before the subject is assessed.

1. To switch on the LD, press the black switch at the back of the Laser Doppler block. (grey box)

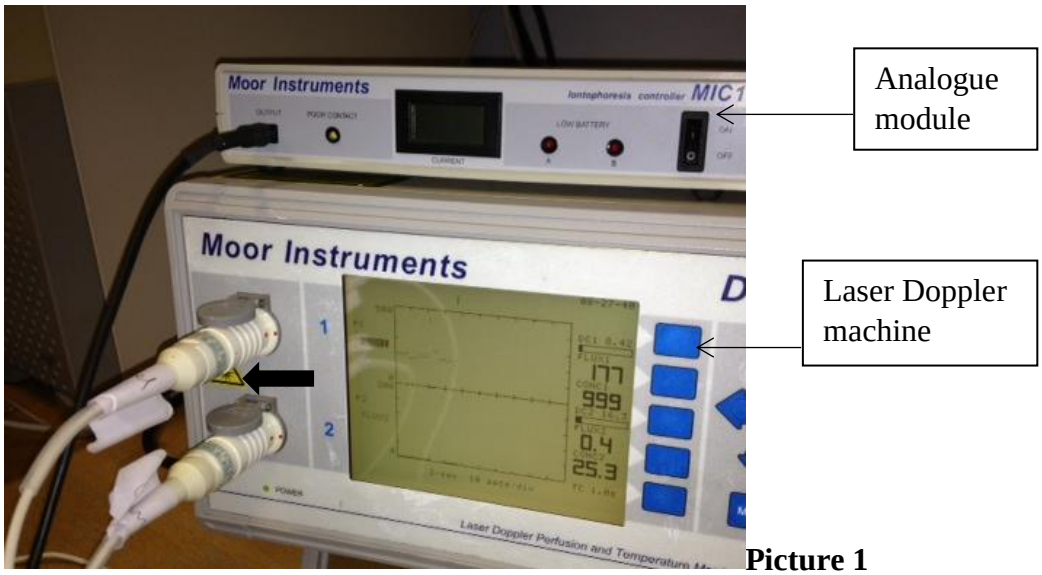
2. Turn on the computer (button front panel).

3. Start MoorSoft DRT4 icon (Laser Doppler software) on the desktop. If this is not already on the desktop it will require installing which will in turn require administrator privileges. The software for V2.0 can be downloaded from this link; https://dl.dropboxusercontent.com/u/107876031/DRT4/Setup.exe

The registration code for the DRT4 was given to me by the engineers as WB4JG PSLDW ZH PSLDW which will need to be entered into the software once installed and run. If you have any problems with this you can email Dave Bridges on dbridges@moor.co.uk or Brian Lock on lock@moor.co.uk.

It is important to run the DRT4 machine via the software on the computer and not through the LD machine itself (by simply pressing 'run' – the top blue button). This is because trying to download data from the DRT 4 to the PC/laptop can be unreliable. Once the software has been installed, you will need to make sure your PC/laptop is communicating with the DRT4 (LD machine). This is done via the grey 9 pin cable provided which plugs into the back of the hospital PC in research room 4. If, however, you wish to use a personal laptop you may require a USB converter as this 9-pin connection is not available on some laptops. The DRT4 uses a non-standard RS232 pinout so the USB converter will have to be bought from moor instruments if required. The software should already be configured to the manufacturer's specifications. Once connected, you should be able to instruct the DRT4 using your computer.

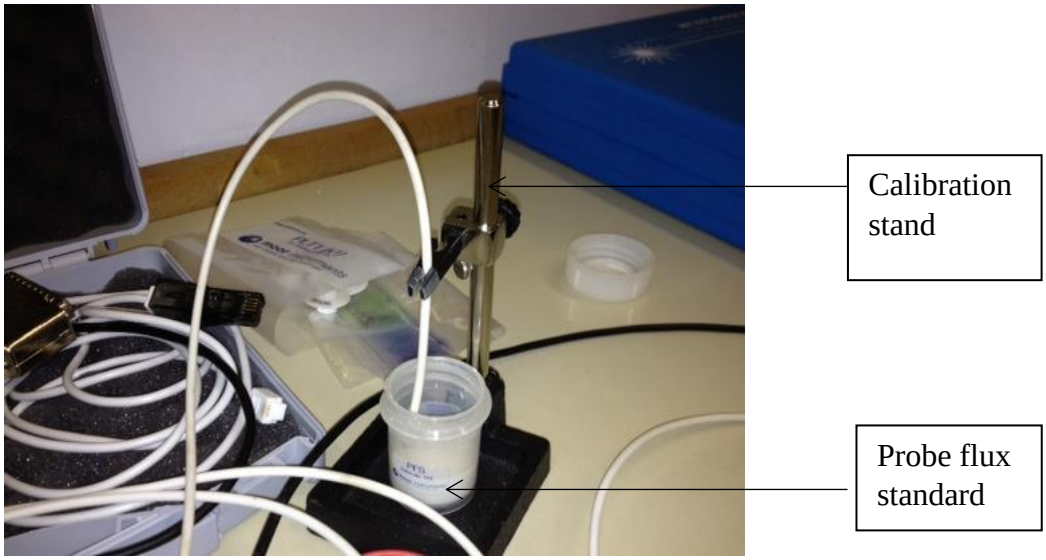
4. If the machine has not been used for a while between research fellows or in any case 1/month it will require calibrating. First take the white optical probe labelled 'channel 1' and plug it into the top space on the left-hand side of the LD/DRT4 machine. Then take the other white optical probe labelled 'channel 2' and plug that into the bottom of the two holes on the left-hand side of the machine (picture 1). You then need to press the yellow triangular shaped button (which looks more like a hazard warning then a button) located in between the two ports for the lasers to come on (black arrow, picture 1).



Picture 1

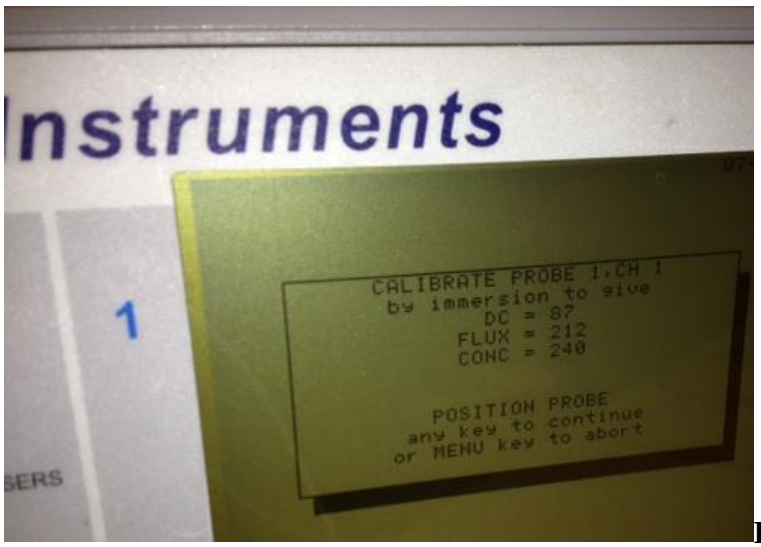
Next take the calibration stand which should fit together as shown in picture 2. This is for holding each optical wire in position during calibration. The small bottle of

milky fluid is the probe flux standard (PFS) used for calibration. Place this in the base of the calibration stand as shown.



Picture 2 Calibration stand with PFS

On the DRT 4 opening screen there should be an option saying, ‘probe calibration’. Press the blue button corresponding to this then select ‘recalibrate probe’, press ‘menu’ (which is synonymous with ‘enter’). For channel 1 select probe 1 followed by ‘enter/menu’ and immerse channel 1 (probe 1) into the PFS. Make sure the detector part of the probe is in the centre of the fluid (i.e. not up against the wall of the bottle). Do the same for channel 2. Once calibration is complete (picture 3) press the button to exit. The machine is now ready to use on a patient.



Picture 3

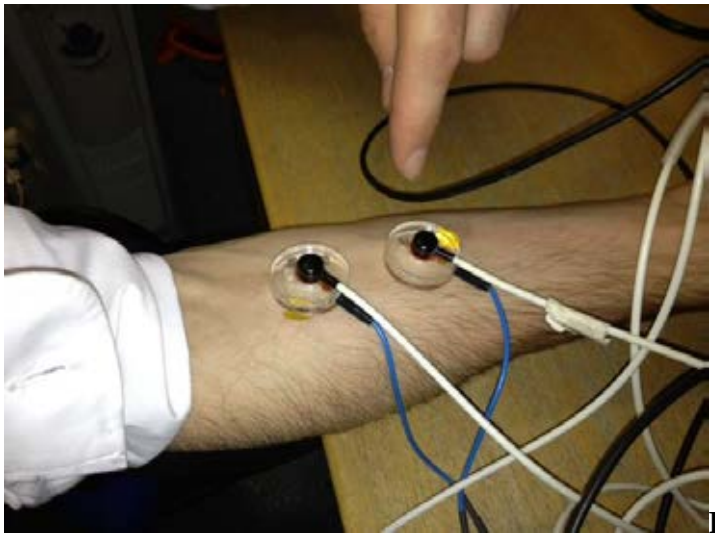
3. Patient preparation

3.1. Use right forearm, it should be clean (picture 4). Technically one could use either forearm but there are high degrees of variability between them (see below) so consistency is essential.

3.2. Ask the patient to lie down on a couch and place their arm on a comfortable cloth pad next to them on the couch. The room temperature should be constant (ideally 19-23°C). Make sure the participant is comfortable and the hand is stable.

3.3. Remove any hair from the forearm gently with a single-use razor.

3.4. Use a Skin Prep (e.g., Skintact) gently to prepare the area of the scans, leave the patient to rest for 20 minutes. Sleeping should be discouraged as this will affect microvascular flow.



Picture 4

4. Iontophoresis and Data acquisition

4.1. During assessment, the patient is laying on the couch comfortably in a constant room temperature room for 20 minutes before the start of measurements (picture 5). All measurements should be performed in dim light. The forearm is placed on a supportive cloth/pillow.

4.2. A hairless area of forearm skin in the chosen arm is cleaned with a 40% ethanol swab and the iontophoresis chambers (glass chambers with a larger hole in the middle and two smaller ones towards the edge (MoorSoft UK) placed over the skin about 5-10cm apart. They are attached using the double-sided adhesive discs supplied.

4.3. The iontophoresis chamber attached to the red wire (positive charge) should be proximal to the one attached to the black wire.

4.4. The proximal chamber is then filled with about 0.5 ml Ach (using transfer pipette) whilst ensuring that no air bubbles are visible below the glass cover. SNP (0.5ml) goes into the distal chamber. The optical probes (channels 1 and 2) then need to be placed into

the central hole in the glass chambers (on top of where the fluid has been placed). Channel 1 goes into the proximal chamber with Ach in (red wire) and channel 2 to the distal chamber with the SNP in. Turn on the analogue input/output module for the DRT4 using the black switch at the back. The green screen will then illuminate with mA which should read 0.01 or 0.02. No electrical current will be delivered until the appropriate phase of the protocol (which is controlled from the computer), so it is best to switch the machine on at this point lest you forget and run the entire protocol without the analogue module turned on.

Picture 5

4.5. The protocol should now be ready to run. Go to 'File, new' on the software. Provided the optical lasers are turned on and the DRT4 is connected to the computer you should see flux readings on the computer screen (as well as on the LD screen – which should be the same). The standard protocol is 60 seconds baseline recording (analogue module turned on but no current) followed by 60 seconds iontophoresis of both drugs (analogue module delivers a current to facilitate iontophoresis – patient may feel a tingling sensation) followed by 600 seconds (10 minutes) of drug response monitoring making a total of 12 minutes. The software controls all the timings, so you do not have to press anything except the 'ion' icon under the green play sign in the middle of the toolbar when you and the patient are ready (picture 6 arrow). You will hear a beep denoting the start of baseline and then another beep after a minute has passed denoting the start of iontophoresis (at which point the current on the analogue machine should read 101mA providing there is good skin contact) and a third and final beep denoting the end of iontophoresis and start of drug response monitoring; the machine will automatically stop at the end of 12 minutes. You will see flux readings on your screen for each of the two channels simultaneously (F1 and F2 where F stands for flux). The flux is an arbitrary figure derived from the velocity of the red blood cells multiplied by their concentration multiplied by constant.

Picture 6

4.6 At the end of 12 minutes the two glass chambers can be removed from the patient's forearm and cleaned.

4.7 Complete the procedure by wiping excess fluids from the subject, disposing of waste material etc. Power down the LD and switch it off.

5 Analysis of results

After the readings have been taken, the images are analysed using the dedicated software package provided by the manufacturer. Before selecting a region of interest (ROI) it is worth saving the recording as a DRT file in case you wish to re-analyse it later. To do this go to 'File and 'Save as' and click 'OK' on the DRT window that opens subsequently.

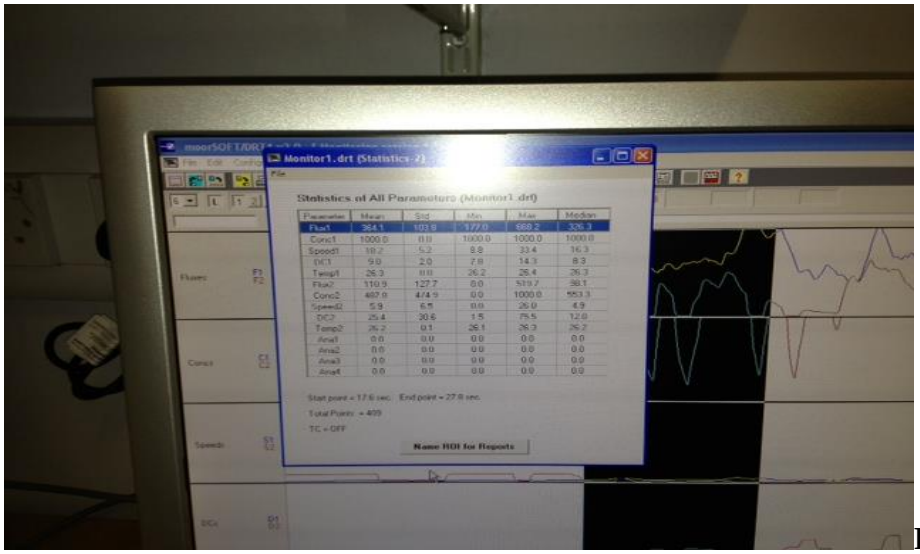
1. To select an ROI (the period of data collection you wish to analyse). Left-click the mouse pointer just under the first triangular marker in the white bar at the top of the screen underneath the toolbar and drag it across to the second marker. You have now selected the baseline 60 seconds.

2. Let go with the mouse and click 'Analysis' 'New statistics'. You will then have a choice. Choose 'Show statistics of all selected parameters' click OK.

3. The software will now display some basic descriptive statistics of the area that you have selected. These are your baseline flux readings (picture 7).

4. Click 'File' (in the top of the new window with the stats in, not the one on the main screen) and 'Save' to save the information in a folder for the patient. If you want to add a study to an existing patient click 'File' 'Append'.

5. Repeat the process this time with the ROI between the second and third triangular markers denoting the maximal flux during drug iontophoresis and again for the final 10 minutes of recording.



Picture 7

6. Save changes in the same way for each ROI as in step 4. Files can be saved to 'My Documents' or to the Moor Soft Machine however when opening up the data in excel files are more easily found from My Documents.

7. Select **Export** and **Images and calculations to Excel** to export results to Microsoft Excel; alternatively, you can open excel and import the text files which will be converted by excel into a compatible format (Microsoft 2010) but then when you save the file it needs to be as an xls file. The values for each time point are used to determine the peak response (in terms of absolute perfusion units, and also % change from baseline), and area under the curve for the perfusion over the 10-minute experiments.

8. Save results obtained and print them if necessary.

9. Close software.

10.Turn off the computer and switch off DRT4.

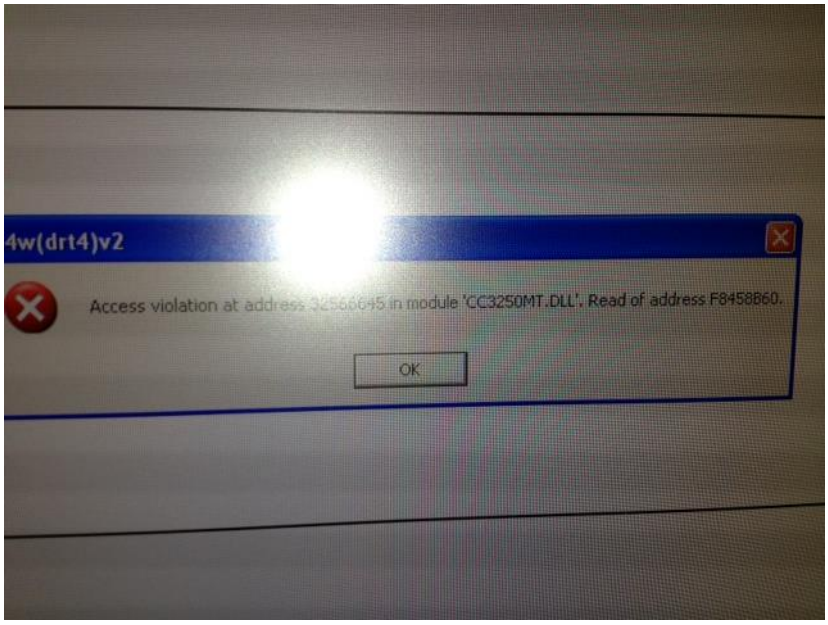
11. Write up the result in a legible form.

Troubleshooting

These are one or two difficulties that I had;

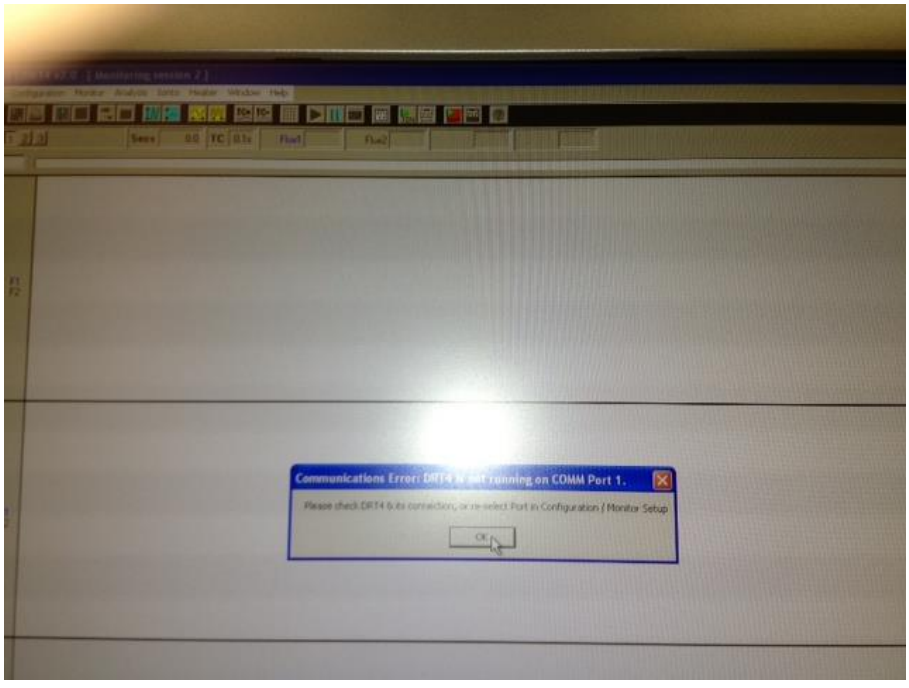
If you see either of these two messages below (which you shouldn't now the software has been properly installed and set up and all relevant installation files received from the manufacturer), it will require a combination of trust IT and MoorSoft engineers to resolve. The MoorSoft engineers were very helpful and able to deal queries via telephone and email.

This error message means that one of the files required for installation is either missing or cannot be seen by the software.



Picture 8

This error message means that the computer cannot ‘see’ the DRT4 owing to a COMM port issue.



Picture 9

Appendix 5: Standard Operating Procedure; enumeration of monocyte subsets and monocyte-platelet aggregates

STANDARD OPERATING PROCEDURE

Absolute count of monocyte subsets and assessment of surface marker expression on them by Flow Cytometry
(extraction of the absolute count protocol)

N.B. Use of the flow cytometry is forbidden
Without having been officially trained

Written by: E Shantsila, L Tapp, B Wrigley 2011

Required pre-training

1. SOPs on venepuncture, good clinical practice
2. SOP 195 – general operation of the flow cytometer

Contents

1. Introduction

Monocytes are circulating blood cells participating in innate immunity, inflammatory response as well as other processes such as angiogenesis, formation of tissue macrophages and dendritic cells etc. Monocyte include several subsets that can be discriminated based on surface expression of CD14 (lipopolysaccharide receptor) and CD16 (Fc gamma receptor III).

This method describes enumeration and characterisation of:
- CD14+CD16- monocytes (about 85%),
- CD14+CD16+ monocytes (also CCR2+, about 5%) and
- CD14lowCD16+ monocytes (also CCR2-, about 10%),

2. Materials and Supplier contact details:

1) BD “FACS Flow” Running solution [Becton Dickinson, Catalogue No. 342003] 10L containers.

BD “FACS Clean” Cleaning Solution [Becton Dickinson, Catalogue No. 340345].

2) 3 ml BD Falcon tubes [Becton Dickinson, Catalogue No. 352054].
3) BD Lysing solution [Becton Dickinson Catalogue No. 349202].
4) Sterile Phosphate Buffered Saline solution, 0.5L bottles [Invitrogen Ltd, Catalogue No 20012-068].
5) BD TruCount tubes [Becton Dickinson Catalogue No. 340334].
6) CD16-Alexa Fluor 488-conjugated monoclonal antibody [AbD Serotec, Oxford, UK, Cat No. MCA2537A488].
7) CD14 -PE conjugated monoclonal antibody [R&D Systems Europe Ltd, Cat No. FAB3832P].
8) CCR2-APC conjugated monoclonal antibody [R&D Systems Europe Ltd, Cat No. FAB151A].
9) CD42a- conjugated monoclonal antibody [Becton Dickinson, Catalogue No. 340537].
10) Clear pipette tips [Alpha Laboratories Limited Catalogue No FR1250 1250µl Fastrak Refill NS].
11) Yellow pipette tips [[Alpha Laboratories Limited Catalogue No FR1200 200µl Fastrak Refill NS].
12) Pipettes required – 2-20µl (grey)
    5-40µl (red)
    40-200 µl (yellow)
    200-1000µl (purple)
    10-10µl and 100-1000µl digital

3. Detailed method

3.1 General Preparation

3.1.1 Lysing solution.

Make from 50ml concentrate 10x FACS Lysing Solution (kept at room temperature). Dilute with 450ml distilled water in ½ litre bottle. This solution should not be used if it is older than a month (kept at room temperature).

3.1.2. Master mixes
Master mixes are made by responsible person in clearly labelled tubes from dark glass. Estimated number of samples to be done from one master mix should not exceed monthly number of samples done

3.1.3. Time from blood sample collection to beginning of sample preparation should be less 30 min, and must not be more than 60 min.

3.2 Blood sample preparation

3.2.1. Place EDTA blood sample on rotator.
3.2.2. Label tube for absolute count (AC).

3.2.3. Place Antibodies from the ‘master mix’: 12.5µl (grey) - place on opposite side to count beads. Do not touch the pellet! If this happened – change the tube.

3.2.4. Add blood to the tube.

- Take 10-100µl digital pipette.
- Set to 2 x 50µl.
- Withdraw blood then expel by pressing ‘RESET’, take up blood once again.
- Wipe pipette tip to remove excess blood.
- Eject 50µl into AC tube – keep the tip well above the metal greed. Do not touch count beads.
- Replace tube top.
- Replace rest of blood into EDTA blood tube (keep it in case you need to repeat the procedure).

3.2.5. Vortex all samples (level 3).

3.2.6. Place samples in dark for 15 minutes (taken from when last sample prepared).

3.3 Start-up procedure [See SOP 195 on General Operation]

- Turn on FC and after few seconds computer.
- Empty waste (right container) and refill with 360ml distilled water and 40ml bleach (ensure no bleach on gloves).
- Fill machine with left container by FacsFlow to appropriate level (level of indentation).
- Pressurize.
- Press LOW/PRIME with the arm closed (distilled water in place)- once PRIME light goes off press once more.
- Remove distilled water and place ‘top right’ on rack.
- Insert FacsClean tube (2ml of FacsClean) – opened arm 1 minute, closed 5 minutes - then remove and place ‘top left’ on rack- set FC on ‘high/run’.
- Insert a tube with sterile PBS – 1 minute open arm, 5 minutes closed arm.

3.4. Further sample preparation

3.4.1. Red blood cell lysing.

- Use purple pipette(200-1000µl).
- Put 450µl in AC tube.
- Place tops on tubes and vortex.
- Place AC tube in dark for 15 minutes- after which add 1.5mls of PBS, vortex - sample ready to run.

3.5. Sample acquisition

3.5.1. Absolute count

- Run FacsComp if you are first user of the flow cytometer during the day
- Press CellQuest icon
- ‘FILE’ – ‘Open document’ – ‘Data 1’ – ‘Mon protocols’ – ‘Mon no wash’
- ACQUIRE – ‘Connect to cytometer’
- CYTOMETER – ‘Choose instrument settings’ – ‘Mon no wash’ – ‘Set’ – ‘Done’
- Change appropriate directory to save flow data
- Change file name

- Place AC into cytometer and press ‘Acquire’ (press ‘Acquire’ on top bar, press ‘Counters’) - once finished, print

3.5.2. Expression of surface markers

- ‘FILE’ – ‘Open document’ - choose – ‘Mon wash Luke/Ben’
- CYTOMETER – ‘Instrument settings’ – ‘Open’ - choose ‘Mon wash Luke/Ben’ – ‘Set’ – ‘Done’
- ‘WINDOWS’ – ‘Show browser’
- Change directory and file name as before
- Vortex and run tubes 1-5, changing file on each sample (1, 2, 3 etc)- print after each

3.4 Shut down procedure [See SOP 195 on General Operation]

1. In this section, we re-use tubes 1 and 2 with distilled water and FACS clean respectively.

2. Install FACS Clean tube 2 over the SIP needle. Press button ‘**High’** and ‘**Run’** on the panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn’t empty completely.

3. Now replace the side arm under the Falcon tube and allow to run for approximately 5 minutes. This cleans the inner portion of the aspiration sheath and the FACS machine itself.

4. Repeat steps 2 and 3 with the distilled water tube 1. Once step 3 is complete, Leave the sheath in falcon tube 1 containing distilled water and press ‘**STANDBY’.**

5. Open the reservoir draw and depressurize the machine by moving the “Vent Valve” toggle switch to the up/rear position. The machine will hiss as it depressurizes.

6. Leave the machine on for a further 5 minutes to allow the laser lamp to cool. Turning the machine off prematurely will result in the lamp cracking.

7. Finally power down the FACSCalibur (green button) and Apple Mac.

8. Clean up!

9. Note: * IF THE SYSTEM IS TO BE USED AGAIN ON THE SAME DAY…

   LEAVE THE SYSTEM ON STANDBY and then

   DEPRESSURISE THE SYSTEM.

Appendix 6: Standard Operating Procedure; enumeration of platelet and endothelial microparticles

STANDARD OPERATING PROCEDURE 209

Enumeration of Platelet Microparticles using Apogee A50 Flow Cytometry

Silvia Montoro Garcia and Eduard Shantsila
September 2011

Updated by Dr Jackson Lau and Dr Xiong Qinmei: April 2015

Note: Use of the flow cytometry is forbidden without official training

Required pre-training

3. SOP on venepuncture, good clinical practice

Contents

1. Introduction

Cellular microparticles (MP) are submicron biological structures released from different types of cells (e.g., from platelets, leucocytes, endothelial cells, red blood cells) via remodelling of plasma membrane in response to numerous conditions, including activation and apoptosis[456]. MPs are generally defined as small (0.1 to 1μm) membrane fragments that often expose the anionic phospholipids phosphatidylserine and membrane antigens representative of their cellular origin. These characteristics discriminate MP from exosomes, which are smaller (<0.1 nm), originate from intracellular multivesicular bodies, and differ in antigenic composition.

Some MPs have prothrombotic properties irrespectively from their origin. However, presence of certain markers (e.g., tissue factor) on MP surface substantially enhances thrombogenic properties. Flow cytometry allows the identification and enumeration of different types of MP based on their size, surface antigens and, usually, utilisation of count beads[457].

The A50-Micro flow cytometer was shown to be superior to most conventional flow cytometer for MP analysis due to its design specifically focused for analysis of small events. Conventional flow cytometers are limited to measuring particles larger than about 500nm (y small angle light scatter [forward scatter, FS]). For example, the lower limit of resolution of BD FACSCalibur is 5.0μm. Apogee A50 discriminates 20nm particles (latex beads), and it also benefits from a volumetric sampling system that accurately counts MP per microliter without using count beads. An additional advantage of Apogee A50 is a fluidics system that can be refilled with distilled water, saving money on expensive reagents.

This SOP describes enumeration of CD42b (GPIb)+ platelet-derived microparticles (PMP). N.B. the SOP may be amended for more detailed analysis of MP subpopulations, for example, Annexin V positive events or other MPs types such as monocyte or endothelial MPs.

2. Materials and Supplier contact details:

1) Distilled water from the still in the tissue culture room

2) Eppendorfs tubes of 1.5mL, Copolymer PP 'ultra-clear, snap-shut' [Appleton Woods, #KC 135]

3) Filtered and sterile 1% Phosphate Buffered Saline solution, 0.5L bottles [Life Technology, #10010-023]
(0.22μM filters and syringes are available on the shelves in the tissue culture room)

4) CD42b-Biotin conjugated monoclonal antibody, 50μg vial. [Abcam, #AB 30400]
Antibody can be separated into multiple aliquots and stored in -70°C freezer.
Once thawed, to be kept in fridge and use within 7 days.
AVOID repeat freeze-thaw action, heat sensitive.

5) Streptavidin- Alexa Fluor -647-R-PE conjugate, 100μL vial. [Life Technology, S20992]
DO NOT FREEZE, heat sensitive.
Light sensitive keep wrapped in foil.

6) Red pipette tips, Fastrak Refill Pack NS, 10μL extended microtips, 14 x 96 per pack [Alpha Laboratories, #FR 1010].

7) Yellow pipette tips, Fastrak Refill NS, 200μL extended microtips, 14 x 96 per pack [Alpha Laboratories, #FR1200].

8) Sodium Azide, 99.9%, 25gram bottle. [Sigma Aldridge, # 438456-25G] or ProCLin™ 300 50mL bottle [Sigma Aldridge, # 48912-U].

9) ApogeeMix beads for Flow Cytometer performance assessment, 25mL bottle [Apogee Flow Systems, Hertfordshire, United Kingdom # 1493].

10) Apogee A50-Micro Flow Cytometer [Apogee Flow Systems, Hertfordshire, United Kingdom].

3. Detailed method

3.1 General Preparation

1. Peripheral vein blood sample is to be collected with a 21-gauge needle without applying haemostasis into sodium citrate Vacutainer tubes (BD Diagnostics). Discard the first vacutainer as this may have cell that are activated by the trauma (especially platelets). Use only the second vacutainer to generate clinical data

2. Platelet-poor plasma (PPP) must be prepared by centrifugation for 15 min at 2860g (circa 4000 rpm).

3. Aliquots to be frozen should be centrifuged additionally 2 min at 13000g (rendering platelet free plasma, PFP) and frozen in 1.5mL tubes (0.5mL per tube) at -70°C until use.

3.2 Sample Preparation (to be performed in dark)

1. PPP must be slowly thawed before sample staining at room temperature (RT) and not under hot water because it could damage MPs.

2. Avoid repeat freeze-thaw action of plasma as it will degrade MP numbers.

3. Prepare dilutions 1/10 of the CD42b-biotin conjugate and the streptavidin on the morning of analysis, do not store the rest for other days. This recipe is for the analysis of ten samples of plasma. Increase or reduce the volumes of reagents in accordance.

    a. Mix 12.5µL of CD42b-biotin antibody and 112.5µL of sterile and filtered PBS in a 1.5mL Eppendorf.

    b. Mix 12µL of Streptavidin-PR conjugate and 108µL of sterile and filtered PBS in a 1.5mL Eppendorf.

4. Put 5µL of the 1/10 diluted CD42b-biotin in a 1.5mL Eppendorf.

5. Take 50µL of thoroughly vortexed plasma sample with ‘wet tip’ reverse pipetting technique and add to the tube above without touching antibodies.

6. Gently vortex the sample. Incubate in the dark at room temperature for 30 minutes

7. Put 2.5µL of the 1/10 diluted Streptavidin-PE conjugate into the 1.5mL Eppendorf.

8. Gently vortex the sample again. Incubate in the dark at room temperature for 30 minutes (polystyrene cube 'cool box').

9. Add 950µL of filtered PBS, to achieve 1:20 dilution. Rate of dilution of samples may change depending on concentration of MP in different antibody used.

10. The sample is now ready to be analysed.

3.3 Start-up procedure for Apogee A50 Flow Cytometry

Part 1 – Restoring reagents and general preparation

1. Switch on Flow Cytometer by pressing the only big, black bottom on the machine. The attached computer will thus be automatically switched on.

2. The sheath (distilled water) fluid reservoir is located at the top of the flow cytometer and the waste reservoir is located on the floor under the flow cytometer.

3. Carefully lift the top of the sheath fluid reservoir (half way along the top of the machine) and fill it with distilled water to the level indicated by a red float (little plastic float in the clear vertical plastic tube).

   The cytometer requires up to 2-3 refills over a continuous 8 hours running period. Ensure that distilled water still is kept running.

4. Add small amount (1 plastic spatula full) of sodium azide or ProCLin™ 300 into the sheath reservoir upon every refill of distilled water, this will retard bacterial or fungal growth in sheath and flow cell.
   Never allow the distilled water in the sheath reservoir to run out.
   Ensure gloves are worn and good ventilation, as pungent odour may be released upon dissolving sodium azide.
   Avoid direct contact with sodium azide.

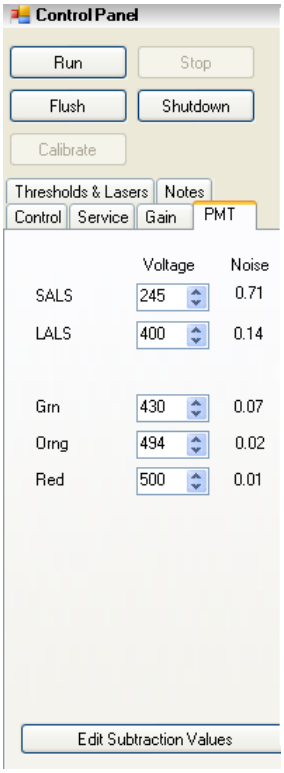
5. Carefully disconnect/unscrew the waste container and empty contents down sink with plenty of water.
   Ensure adequate ventilation as pungent fumes may be released.
   Never run flow cytometer with full waste container, as built up of back pressure will "aspirate" waste back into flow cell.

Part 2 – Apogee Histogram and software preparation

6. The computer will automatically open the Apogee Histogram Software. Go to SERVICE at the right hand top corner of the screen, open it, and press 'Flow Cell Clean', this will automatically clean the system and needle before starting. When it is complete it will say so (< 5 minutes).

7. Then press ‘Remove air in syringe’. Status will then move to ‘Idle clean’.

8. Before running any samples, ensure the following instruments checks are completed:



(i) Settings for PMT Voltage:

PMT Gates	Voltage	Noise
SALS	245	0.71
LALS	400	0.14
Grn	430	0.07
Orng	494	0.02
Red	500	0.01

- Ensure that Voltage settings are optimised to ensure most optimal detection of MP.

- Alteration in voltage will lead to increase excitation and emission, and lead to significant alteration of results (upwards shift of all results).

(i) PMT: Noise level

PMT Gates	Voltage	Noise
SALS	245	0.21
LALS	400	0.14
Grn	430	0.08
Orng	494	0.04
Red	500	0.03

Ensure that noise levels are <1.0 in all gates prior to running any actual samples.

Noise is due to background dust in flow cell and decreases accuracy of reading, usually increase after leaving Apogee A50 standing idle for > 48 hours.

Reduction in noise can be achieved by multiple times of “FLUSH”, running multiple times of filtered distilled water [see part (iii)below] and ‘Flow Cell Clean’.

Persistent, elevation of noise level may require servicing to replace parts.

(ii) Settings for Control:

	µL/min (running rate)	µL (aspirate volume)
SAMPLE	10.5	150

This setting allows for 150µL sample to be aspirated and analysed at 10.5µL/min.

Reduction in running rate will increase precision, but lengthen running time of each sample.

In specific circumstances of flushing flow cell to remove dirt and to reduce noise level, one may run sample at a faster rate (101µL/min) and after aspirating a maximum volume (400µL).

3.3 Running Samples.

Note: This must be learned from an experienced operator and please seek scientific staff support to clarify any queries.

1. Click on File (top left) → "Open settings and data" to open instrument settings.

2. Click on the appropriate settings located in 'Documents'. This will open the instruments settings for PMPs quantification settings.

3. The settings are predefined to enumerate CD42b-positive events with size less 1µm but more ~100 nm. This has been done during optimization of the protocol with different size beads (100, 200, 400, 500nm, 1µm and 2µm).

4. Open swing arm at bottom right of the flow cytometer, place the sample and put the arm in the 'running' position (from "clean position") to acquire the sample (in a 1.5 mL Eppendorf). The system will automatically aspirate from the sample without pressing any button, once the arm is switched to the "running" position. **ENSURE that there is adequate volume to prevent aspiration of air.**

5. The machine will say 'Aspirate sample', and then 'Running sample'.

6. The sample will now run until the event collection is complete; the time of the acquisition will depend on the volume aspirated and running speed. If the software buffer is full (1,000,000 events) a message will appear asking whether the sample should be acquired further (with erasing the last data) or the acquisition should be stopped.

7. Press "NO" and the sample will be stopped. If you want to stop the sample before reaching the limit of buffer size, press "STOP" during acquisition (at the right-hand corner of the software).

8. After the acquisition stops, move arm to the ‘clean’ position and discard the Eppendorf with the sample. The system will automatically clean the needle and tubes with pre-determined 2 flush cycles.

9. Save data by clicking in “Save data file as” under “File”, the system will also autosave the data file in “My documents”.

10. Absolute number of MP of interest will be expressed as events/µL at the bottom of the CD42b+ window gate. This number must be multiplied by the plasma dilution (such as x20).

11. Obtain all the data and apply in into the appropriate spreadsheet of your project.

3.4 Shut-down procedure

10. After moving the arm to the clean position and discarding the Eppendorf, the system can be cleaned and shutdown. Press button “Shutdown” at the right corner of the software panel and leave the system for cleaning (takes 2 - 4 minutes). This will be done automatically.

11. After the system has been automatically cleaned, a window will appear “Shutdown completed”, close the software and the control panel.

12. Finally, shut down the computer, this will automatically shut down the cytometer too.

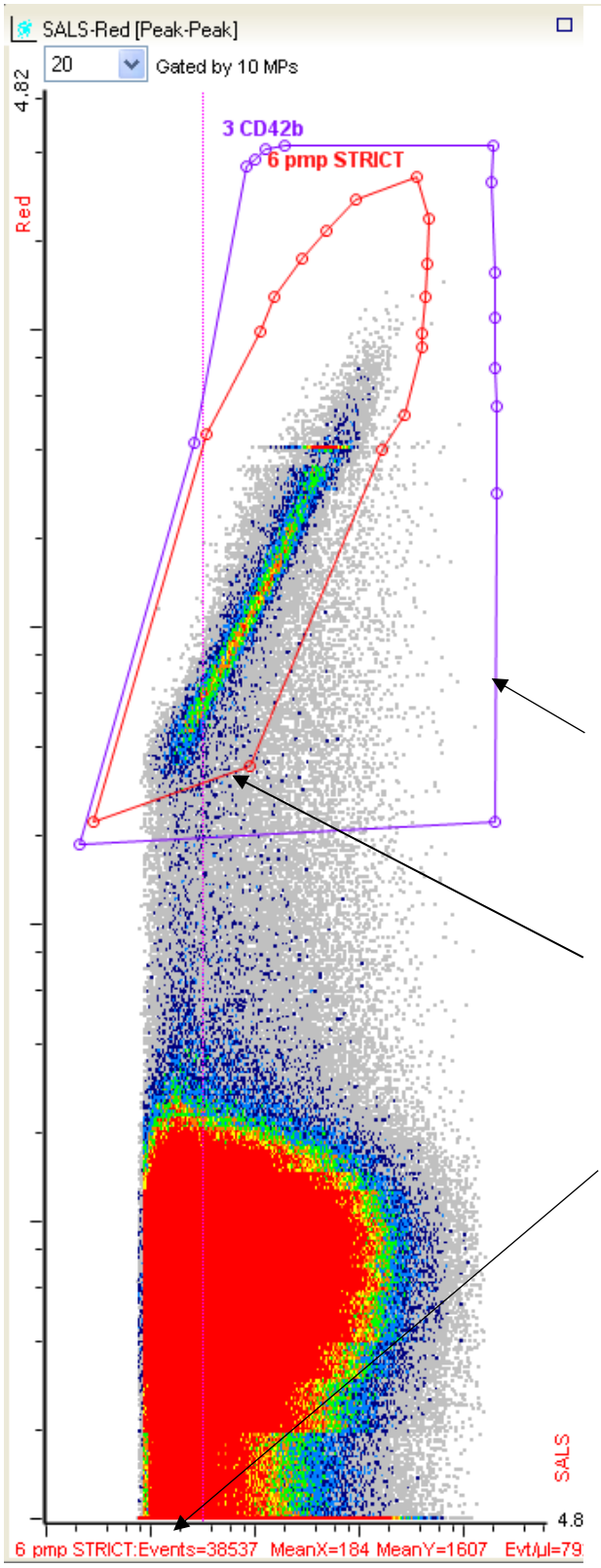
13. Clean up the work station.

14. Note: Leave the cytometer on if to be used again later that day.

4. Interpretation of the results

Platelets microparticles (PMPs) determined by CD42b positive events.

Plot 1: CD42b event rate detection by Apogee A50 in prepared sample.



The vertical (Y) axis (RED) is the staining with CD42b-Streptavidin red fluorochrome for platelets. The horizontal (x) axis (SALS) is shows small angle scatter.

Note the gate which defines the number of CD42b positive events.

These have been set by the results of the previous isotype experiments.

Purple Region-of-Interest (ROI), signifies potential CD42b events due to detection of excited fluorochrome.

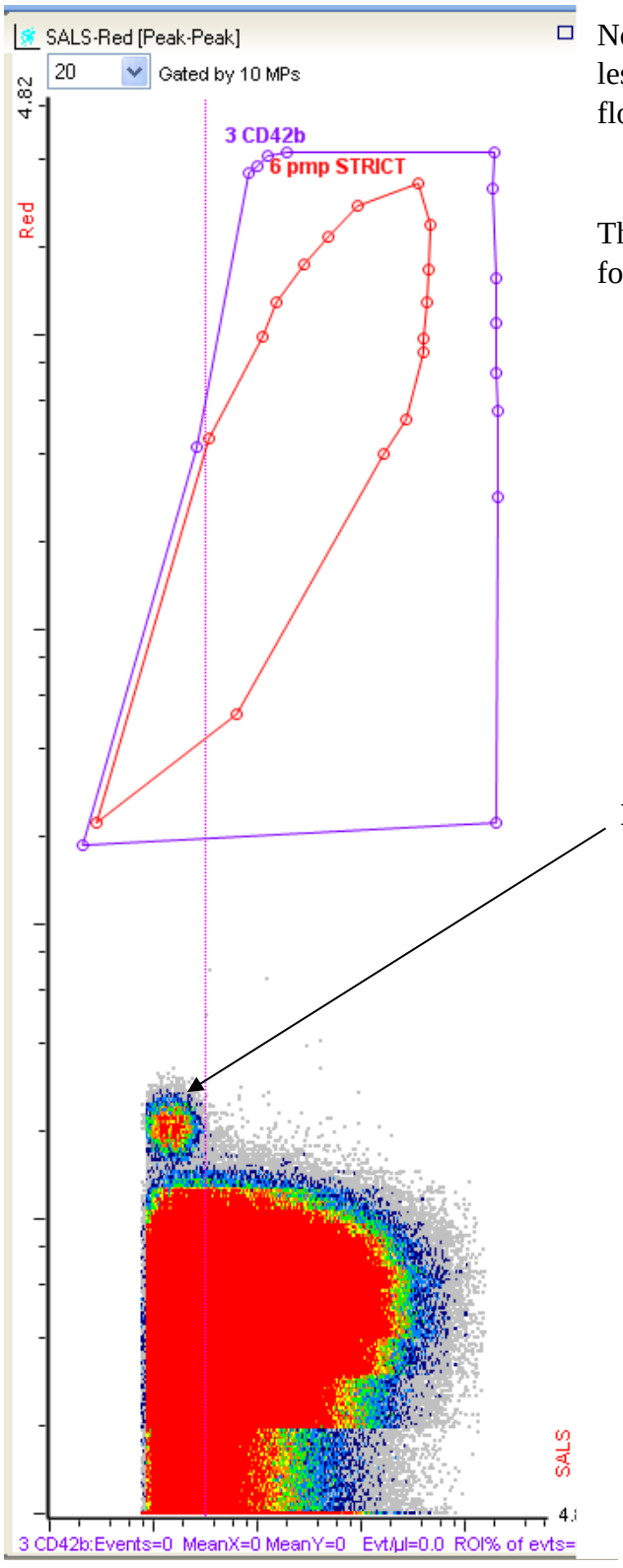
Red ROI suggests area of strict CD42b/PMP events.

At the bottom of the PMPs plot, the number of positive events per µL in selected ROI is demonstrated = 38537.

Once dilution factor (x100) is factored into the calculation = 3,853,700/µL in undiluted plasma.

Isotype control

Plot 2: CD42b event rate detection by Apogee A50 in isotype control.



Note identical layout but much less emission is detected by the flow cytometer.

Thus, event rates in ROI was found to be zero.

Lack of excitation of PMP

Validation (CD42b Antibody for PMP detection)

Duplicate agreement was obtained for thoroughly vortexed plasma from the same tube.

Intra-assay coefficients of variation were determined on 5 aliquots of plasma from 6 subjects (i.e. n=5 per assessment).

Results as below:

	CD 42b				
	Sample-A	Sample-B	Sample-C	Sample-D	Sample-E
Test-1	128039	58239	79105	80513	76148
Test-2	118054	53321	78123	79159	74879
Test-3	102355	60523	81970	76315	68953
Test-4	94560	43648	60218	57320	92747
Test-5	87561	39758	42370	67731	53072
SD	16696.62	9067.309	16865.46	9693.464	14289.51
Mean	106113.8	51097.8	68357.2	72207.6	73159.8
CV	**15.70%**	**17.80%**	**24.70%**	**13.40%**	**19.50%**

Mean intra-assay CV: 18.2%.

***As the steps described above are identical for other antibody (such as CD31 antibody), this protocol is transferrable for detection of endothelial antibody.**

Validation (CD31 Antibody for EMP detection)

	CD 31				
	Sample-A	Sample-B	Sample-C	Sample-D	Sample-E
Test-1	220070	235734	222953	212050	142516
Test-2	221210	209686	208422	200096	189074
Test-3	216376	291001	259805	204056	162329
Test-4	183635	201572	261570	193917	162607
Test-5	179830	247365	209547	183571	136927
SD	20653.5	35448.3	26402.4	10731	20536.5

Mean	204224.2	237071.6	232459.4	198738	158690.6
CV	**10.10%**	**14.90%**	**11.40%**	**5.40%**	**12.90%**

Mean intra-assay CV: 12.9%.

STANDARD OPERATING PROCEDURE 209

Enumeration of Platelet Microparticles by Apogee A50

Signed off……Andrew Blann…… …… 18th October 2011.

Appendix 7: Definitions of the clinical and procedural characteristics relating to sections 2.2.2.1, 4.2.3.1 and 4.3.3.1

The following are defined according to BCIS (British Cardiovascular Intervention Society) criteria. Current smoking: regularly smoking one or more cigarette per day or having stopped within the previous month. Family history: any first degree relative with premature coronary heart disease (males under 55; females under 65). Diabetes: patients currently using oral hypoglyaemics and/or insulin. Hypercholesterolaemia: total cholesterol above 5.2 mmol or use of hypocholesterolaemic drugs. Hypertension: current use of antihypertensives. Cerebrovascular event: loss of neurological function caused by an ischaemic insult with residual symptoms at least 72 hours after the episode. Renal failure: creatinine > 200 mmol/L or a functioning transplant irrespective of creatinine or current dialysis. Cardiogenic shock: systolic blood pressure < 100mmHg with pulse rate > 100 beats per minute or symptoms and signs consistent with hypoperfusion (e.g. clamminess or coolness) or requiring inotropes.

Coronary angiography and percutaneous coronary intervention were undertaken using standard techniques from the radial or femoral artery as appropriate. Patients undergoing percutaneous coronary intervention received guideline recommended dual antiplatelet therapy and anticoagulation with intravenous heparin at 70-100U/Kg or intravenous bivalirudin at 0.75 mg/kg followed by 1.75 mg/kg/hr for the duration of the procedure.

CT imaging was performed using a dual source 256 slice, 128 row CT scanner with 0.5mm Stellar detector elements, 280ms of gantry rotation time and a total z-axis coverage of 38.4 mm per rotation (SOMATOM® Definition Flash, Siemens Healthcare

Limited, Surrey, UK). For heart rates over 70 beats per minute, 5–20 mg of intravenous metoprolol was administered immediately before the scan. GTN spray was given sublingually if systolic blood pressure was ≥ 110 mm Hg. Lesions with an attenuation greater than 130 Hounsfield Units were identified as calcified. Total coronary artery calcium scoring was performed with inbuilt software according to Agatston criteria (Agatston Score 0 – no coronary calcium, 1-100 – mild coronary calcium, 101-400 – moderate coronary calcium and >400 – severe coronary calcium[285]).

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