ACUTE CORONARY SYNDROMES, PLATELETS AND THE ENDOTHELIUM

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ABSTRACT

Background: Acute coronary syndromes (ACS) are medical emergencies. Platelet and endothelial function are fundamental to the pathophysiology; and implicated in secondary conditions such as no reflow (NR). Guidelines support the administration of $P2Y_{12}$ antagonists in ACS treatment, but the broader effects are unclear.

Objectives: 1) Assess the impact of $P2Y_{12}$ inhibition (ticagrelor) on the platelet sensitivity to prostacyclin (PGI_2) and nitric oxide (NO) in coronary artery disease (CAD). 2) Review NR and identify at risk patients 3) Outline the optimal $P2Y_{12}$ antagonist in ACS patients with diabetes.

Methods: 1) Platelet and endothelial function assessed at baseline and after 3 days oral ticagrelor in CAD patients. Multiple aspects of platelet activation and sensitivity to PGI_2 and NO were examined by flow cytometry. 2) Prospective case-control study of STEMI patients with and without NR. Multiple regression identified independent predictors and a risk score established. 3) Meta-analysis of randomised trials with clinical outcomes for $P2Y_{12}$ inhibitors in ACS patients with diabetes.

Results: *Ex vivo* studies of CAD patients (n=63) demonstrated that oral ticagrelor induced only modest platelet inhibition in whole blood. However, it enhanced the inhibitory actions of PGI_2 and NO. Ticagrelor potently amplified PGI_2 inhibition of platelet-leukocyte aggregate formation (a measure of platelet inflammatory function). Ticagrelor improved endothelial reactive hyperaemic index (RHI), which correlated with platelet sensitivity. 24(13.9%) STEMI patients suffered NR, which significantly increased the risk of cardiovascular death. The independent predictors of NR were lesion complexity, systolic hypertension, weight<78kg, and history of hypertension. Systematic review of 7 studies, established newer $P2Y_{12}$ antagonists (ticagrelor and prasugrel) were optimal for ACS patients with diabetes; with a trend to prasugrel superiority in the reduction of major adverse cardiovascular events.

Conclusion: In patients with CAD, $P2Y_{12}$ antagonism by ticagrelor promotes inhibition of platelet haemostatic and inflammatory function by endogenous regulators; and improves endothelial function.

PUBLICATIONS

Articles

JA Rossington, J John, A Hoye. No reflow phenomenon: how, who and what to do. *Indian Journal of Cardiology.* 2019*;* in press

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Oral presentations

Ticagrelor has a beneficial synergistic relationship with endothelial vasodilators in patients with coronary disease. ACI Conference (YIA): London, January 2017 **Finalist**

Is efficacy of platelet aggregation inhibition by endothelial platelet antagonists enhanced by oral ticagrelor mediated $P2Y_{12}$ blockade in patients with coronary artery disease? HYMS Conference: York, June 2016

No reflow phenomenon and comparison to the normal flow population post primary percutaneous coronary intervention for ST elevation myocardial infarction. HYMS Conference: York, June 2016

Is efficacy of platelet aggregation inhibition by endothelial platelet antagonists enhanced by oral ticagrelor mediated $P2Y_{12}$ blockade in healthy subjects? Allam Lecture: Hull, April 2016

A systematic review and meta-analysis of optimal antiplatelet therapy for ACS patients with diabetes mellitus. Allam Lecture: Hull, April 2015

Poster presentations

No reflow phenomenon and comparison to the normal flow population post primary percutaneous coronary intervention for ST elevation myocardial infarction. ESC Acute Cardiovascular Care congress: Milan, March 2018

Is inhibition of platelet aggregation by endogenous endothelial platelet antagonists enhanced by oral ticagrelor mediated $P2Y_{12}$ blockade in patients with coronary artery disease? TCT conference: Washington DC, October 2016

The interaction between $P2Y_{12}$ receptor antagonism by ticagrelor and endothelial vasodilators in patients with coronary artery disease. Eurothrombosis: London, September 2016 **Prize winner**

Is efficacy of platelet aggregation inhibition by endogenous endothelial platelet antagonists enhanced by oral ticagrelor mediated $P2Y_{12}$ blockade? ESC congress: Rome, August 2016

No reflow phenomenon and comparison to the normal flow population post primary percutaneous coronary intervention for ST elevation myocardial infarction. HYMS Conference: York, June 2016

No reflow phenomenon: how, who and what to do. Allam Lecture: Hull, April 2016

A systematic review and meta-analysis of optimal antiplatelet therapy for diabetic patients with acute coronary syndromes. ESC congress: London, August 2015

A systematic review and meta-analysis of optimal antiplatelet therapy for diabetic patients with acute coronary syndromes. BCS conference: Manchester, June 2015

Is efficacy of platelet aggregation inhibition by endogenous endothelial platelet agonists enhanced by ticagrelor mediated $P2Y_{12}$ blockade. HYMS Conference: Hull, May 2015

ABBREVIATIONS

AA	Arachidonic acid
ABP	Actin-binding protein
AC	Adenylyl cyclase
ACEi	Angiotensin converting enzyme inhibitor
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
AHA	American Heart Association
AKAP	A kinase anchoring protein
APC	Anticoagulant-proteases activated protein C
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CAD	Coronary artery disease
CalDAG-GEFI	Diacylglycerol regulated guanine nucleotide exchange factor I
cAMP	Cyclic adenosine monophosphate
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcome Study
CCL	C-C motif cytokine
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CLEC	C-type lectin-like receptor
COX	Cyclooxygenase
CPTP	Cyclopentyl-triazolo-pyrimidine
CRACM	Calcium release activated calcium modulator
CRP	Collagen-related peptide
CVA	Cerebrovascular accident
CXCL	C-X-C motif cytokine
DAG	1,2- diacylglycerol
DAMP	Danger-associated molecular pattern
DM	Diabetes mellitus
DMC	Data monitoring committee
DNA	Deoxyribonucleic acid

DTS	Dense tubular system
EC_{50}	Half-maximal effective concentration
ECG	Electrocardiogram
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
eNOS	Endothelial nitric oxide synthase
ESAM	Endothelial cell specific adhesion molecules
ESC	European Society of Cardiology
FACS	Fluorescence-activated cell sorter
FCB	Fluorescent cell barcoding
GAP	GTPase activating protein
GP	Glycoprotein
GPCR	G protein-coupled receptor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GKIP	G kinase interacting protein
GRK	G-coupled receptor kinases
GSK-3	Glycogen synthase kinase-3
GSNO	S-nitrosoglutathione
GTP	Guanosine triphosphate
HR	Hazard ratio
HbA_{1c}	Glycated Haemoglobin
HDL-C	Cholesterol high density lipoprotein
HMG	3-hydroxy-3-methyl-glutaryl
IC_{50}	Half-maximal inhibitory concentration
IFI	Interferon inducible
IHD	Ischaemic heart disease
IL-1β	Interleukin -1β
IL1R1	Interleukin 1 receptor 1
IP3	Inositol 1,4,5 trisphosphate
IRAG	IP3 receptor-associated cGMP kinase substrate

ITAM	Immunoreceptor tyrosine-based activation motif
JAM	Junctional adhesion molecules
LAD	Left anterior descending artery
LAT	Linker for activation of T cells
LCx	Left circumflex artery
LDL-C	Cholesterol low density lipoprotein
LMS	Left main stem artery
MACE	Major adverse cardiovascular event
MARCKS	Myristoylated alanine-rich C kinase substrate
MBG	Myocardial blush grade
MCE	Myocardial contrast echocardiogram
MCP-1	Monocyte chemoattractant protein-1
MFI	Median fluorescence intensity
MINAP	Myocardial Infarction National Audit Project
MIP-1α	Macrophage inflammatory protein -1 α
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP-1	Matrix metalloprotease 1
MRI	Magnetic resonance imaging
NICE	National Institute of Clinical Excellence
NO	Nitric oxide
NOAC	Non-Vitamin K antagonist oral anticoagulants
NLR	NOD-like receptor
NLRC	NLR family CARD domain-containing protein
NR	No reflow phenomenon
NSTEMI	Non-ST elevation myocardial infarction
NT-ProBNP	N-terminal Pro-B Type Natriuretic Peptide
OCS	Open canalicular system
OR	Odds ratio
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern

PAR	Protease-activated receptor
PAT	Peripheral arterial tone
PBS	Phosphate buffered saline
PEAR	Platelet endothelial aggregation receptor
PECAM	Platelet endothelial cell adhesion molecule
PCI	Percutaneous coronary intervention
PDE	Phosphodiesterase
PFA	Paraformaldehyde
PG	Prostaglandin
PGI_2	Prostacyclin
PGSL-1	P-selectin glycoprotein ligand 1
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PLA	Platelet leukocyte aggregate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PPCI	Primary percutaneous coronary intervention
PSGL-1	P-selectin glycoprotein ligand-1
PRP	Platelet rich plasma
PTPRC	Protein tyrosine phosphatase receptor type C
PVDF	Polyvinylidene fluoride
RANTES	Regulated on activation, normal T cell expressed and secreted
RCA	Right coronary artery
RGS2	Regulator of G-protein signalling 2
RHI	Reactive hyperaemic index
RhoGEF	Rho-guanine nucleotide exchange factor
RIAM	Rap 1-B interacting adapter molecule

ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
sGC	Soluble guanylyl cyclase
SLAM	Signalling lymphocyte activation molecule
STEMI	ST elevation myocardial infarction
STIM	Stromal interaction molecule
TBI	Total binding index
TFPI	Tissue factor pathway inhibitor
TIMI	Thrombolysis in Myocardial Infarction
TRAP	Thrombin receptor activating peptide
TRPC	Transient receptor potential canonical
TXA2	Thromboxane A2
UK	United Kingdom
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

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AUTHOR'S DECLARATION

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1   CHAPTER ONE: GENERAL INTRODUCTION

1.1   INTRODUCTION

Acute coronary syndromes (ACS) is a spectrum of conditions that convey a huge clinical and financial burden, with potential devastating outcomes for affected patients. Well-established evidence demonstrates that presentation is in the majority of cases secondary to coronary atherosclerotic plaque rupture or erosion. With the acute syndromes and the antecedent stable plaque being closely linked with endothelial function and platelet interaction.

The interplay between the platelets and endothelium is constant, as haemodynamic marginalisation of platelets to the periphery of blood vessels ensures close cellular proximity. This is essential for healthy haemostasis but in diseased states promotes progression of a multicellular chronic inflammatory process that causes coronary atherosclerosis. (1,2) Sudden disruption of a plaque triggers platelet activation, by exposure of extracellular matrix proteins and release of secondary mediators, which act as paracrine regulators of platelet activation ensuring rapid thrombus formation and coronary artery occlusion.

The defining role platelets play in ACS has led to great interest in their potential to be a pharmacological target in the management of patients. A number of therapeutic agents are strongly recommended in guidelines by international experts, notably those targeting thromboxane A2 (aspirin) and the $P2Y_{12}$ receptor (ticagrelor, clopidogrel, prasugrel). (3,4)

This chapter will explore more extensively acute coronary syndromes, platelets and the endothelium; and their close relationship. Providing insight into the aims of this thesis.

1.2   ACUTE CORONARY SYNDROMES

1.2.1   Background

Coronary artery disease (CAD) remains the leading cause of death in industrialised countries, and this is expected to be mirrored in developing countries over the subsequent decades. (5) This cohort typically presents to medical attention with ACS, which accounts for 150,800 hospital admissions per annum in the United Kingdom (UK) and is a significant burden on resources with an overall loss to the UK economy of £3.6 billion. (6) Similar figures worldwide have led to a plethora of research into the field aiming to optimise patient management, improve clinical outcomes and reduce the financial implications.

1.2.2   Pathophysiology

CAD is diagnosed following identification of underlying coronary atherosclerosis. Atherosclerosis affects medium and large vessels and the term is derived from Latin, meaning gruel-like ("athero") hardening ("sclerosis"). (7) It is a process whereby lipid-rich plaque is laid within the vessel wall architecture. This encroaches on the luminal area (stenosis) and impedes blood flow to the myocardial tissue, resulting in ischaemia and potential necrosis (infarction).

Atherogenesis is still not fully understood but evidence suggests it is dependent on the relationship between the vessels endothelium, modified lipoproteins and chronic vascular inflammation/immunity promoted by modifiable and non-modifiable risk factors. (8) Well-described is the process in which monocytes, the largest of leukocytes, mobilise to the endothelial wall in response to expression of chemokines/cytokines acting as adhesion molecules (integrins, selectins). The permeability of the monolayer enables their transfer into the intimal layer. Here, they differentiate into macrophages and uncontrollably scavenge the oxidised LDL causing phenotypic transition into foam cells, forming fatty streaks. (8–10) Over subsequent decades the fatty streaks can progress into clinically significant and angiographically evident plaques. These form at certain sites driven by the association with shear stress and flow disturbance; most notably developing at areas of low shear stress e.g. the inside curvature of tortuous vessels and opposite the side branch in vessel bifurcations. (9) Plaque development occurs secondary to cytokine release by

foam cells, T-lymphocytes (TH-1) and platelet aggregates. This initiates migration of the medial smooth muscle cells into the intima, also triggering their proliferation. The relocated smooth muscle cells combined with secretion of abnormal amounts of collagen and other connective tissue proteins, results in development of a fibrous cap overlying a lipid rich pool. This pool is an accumulation of foam cells and cellular debris, with a necrotic core formed by death of the foam cells, while viable foam cells migrate to the edges. (9)

Plaque composition is not uniform with variability in cap to core ratio, which is independent of stenosis severity but important in determining stability. (11) A plaque with a thin cap (<65 microns thickness) and large necrotic core is vulnerable to rupture. A process primarily driven by T-cell release of interferon γ and CD40 ligand, decreasing collagen production by smooth muscle cells and increasing concentration of collagenase respectively. (8,9) In a third of cases (31%) plaque erosion, not rupture, is the pathophysiology underlying the ACS presentation. (12) This process is less dependent on inflammation, and described in plaques with thick caps rich in proteoglycans and the presence of raised systemic myeloperoxidase. (8,13) Following rupture/erosion the sudden exposure of the core constituents to the circulation triggers haemostasis and thrombus formation (see section 1.3.7).

Overall, ACS is a spectrum of conditions primarily characterised by the presence of an unstable atherosclerotic plaque within an epicardial coronary artery, which ruptures/erodes and induces overlying thrombus formation accompanied by localised vasoconstriction. The ACS umbrella term encompasses unstable angina, non-ST elevation myocardial infarction (NSTEMI) and ST-elevation myocardial infarction (STEMI). The latter two are defined by particular electrocardiographic changes which correlate pathologically to the extent of coronary obstruction, with STEMI a result of complete vascular occlusion. (Figure 1.1)
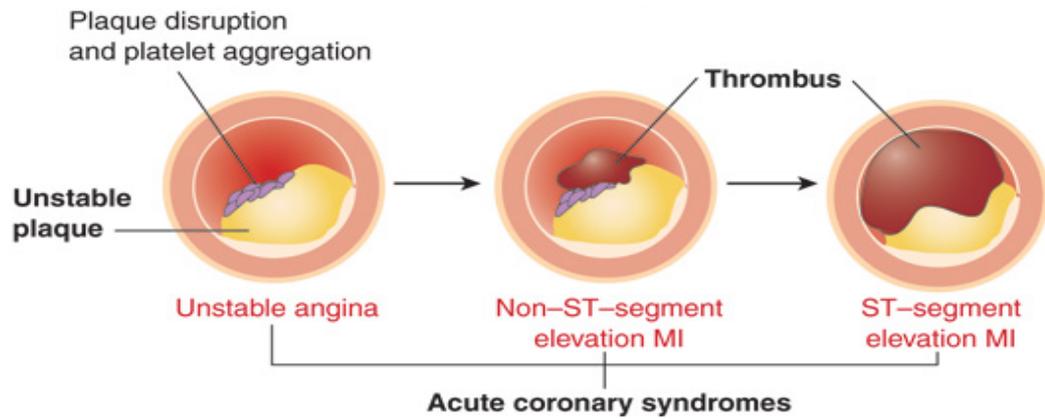
Figure 1. 1: Pathophysiological spectrum of Acute Coronary Syndromes

ACS is a spectrum of conditions classified by the disruption of an unstable atherosclerotic plaque with overlying thrombus formation. The degree to which this thrombus reduces distal coronary blood flow differentiates the diagnosis and management. Adapted from www.5minuteconsult.com. (14)
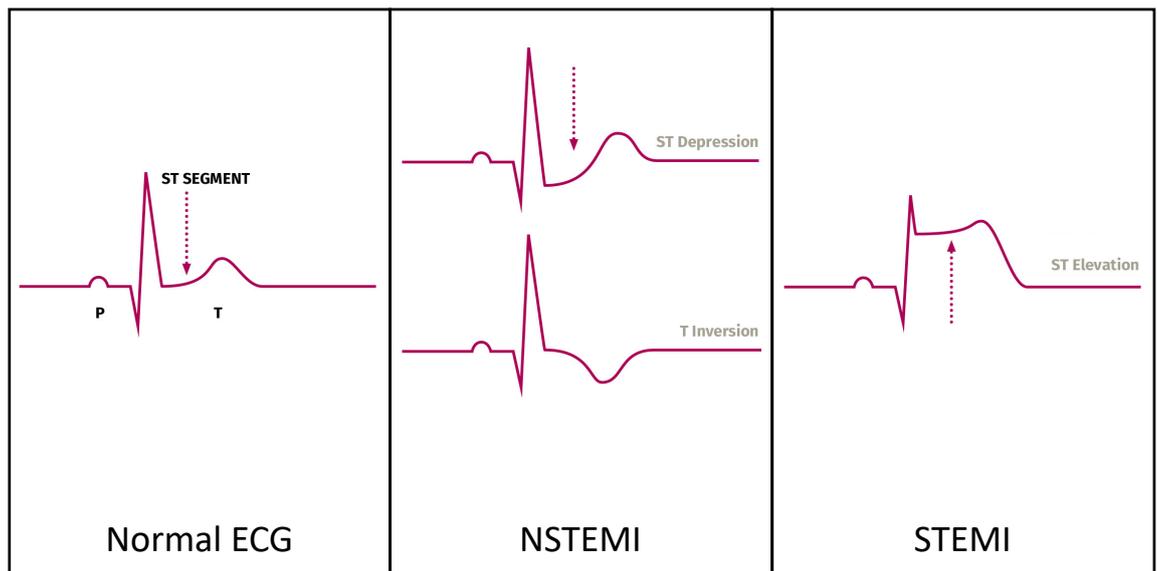
Figure 1. 2: Electrocardiographic changes in Acute Coronary Syndromes

Demonstration of ECG changes assisting diagnosis in ACS. Adapted from www.thrombosisadviser.com. (15)

1.2.3 Presentation

Cases of ACS, as the name would suggest, are sudden presentations classically with central chest pain radiating to the jaw and/or left arm associated with diaphoresis. Patients may also attend a medical centre with symptoms suggestive of the complications of myocardial infarction; such as heart failure, cardiac rupture, valvular regurgitation, dysrhythmia or cardiac arrest. In these cases, chest pain may not be a feature but rather breathlessness or collapse.

If any patient attends with these symptoms immediate acquisition of an electrocardiogram (ECG) is mandatory (3,4), which will depict certain classical features aiding diagnosis of the underlying aetiology. (Figure 1.2) The ECG is an instantaneous graphical representation of electrical signal generated by myocyte activity with voltage against time (seconds). Marked myocardial ischaemia and infarction cause a predictable disruption of this signal during ventricular repolarisation and depolarisation; making it an essential diagnostic tool and lends its results to the commonly used nomenclature in describing the nature of ACS, NSTEMI and STEMI which in turn defines the management strategy.

Unstable angina and NSTEMI may have no evidence of abnormalities on the ECG but may have changes suggestive of ischaemia particularly at times of symptoms, including ST-depression and T wave inversion. STEMI, as the name suggests must demonstrate elevation of the ST segments (≥1mm in the limb leads or ≥2mm in the precordial leads) in greater than two contiguous leads. Beyond simple diagnosis, the specific ECG leads that demonstrate ST-elevation may assist in identifying the anatomical location of the culprit lesion.

To complete the triad for the diagnosis of ACS, in addition to symptoms and ECG, the blood assay for troponin level is advocated. (3,4) Troponin C, I and T are found in skeletal and cardiac muscle. They have a role in regulation of muscle contraction via effecting the interaction between myosin and actin. Troponin C aids initiation of contraction with calcium binding, troponin I inhibits myosin's interaction with actin and troponin T forms a complex with tropomyosin on thin myofibril filaments. Troponin I and T have immunologically distinct isomers originating from cardiac myocytes compared to skeletal muscle. Therefore, a raised plasma concentration detected by the highly sensitive assay of

troponin I and T is highly specific (90-94%) and sensitive (≥90%) for myocardial damage. (16) The definition of acute myocardial infarction (MI) is dependent on both an elevated initial troponin result (>99% percentile the upper limit of normal range; assay specific) and detection of dynamic raise or fall (>20%) in serial troponin levels. (17)

1.2.4 Outcomes

The sudden reduction or absence of distal blood flow, thus starving myocytes of essential oxygen/energy, results in necrosis leading to cardiac dysfunction, dysrhythmia, rupture or death. Following a myocardial infarction (STEMI/NSTEMI) 30 day all-cause mortality is 9.7% in the UK. (18) For patients admitted with STEMI, prompt restoration of blood flow can reduce mortality. Over a 10 year period, the improved delivery of more effective reperfusion therapy to the UK STEMI population resulted in a fall in mortality from 12.4% to 8.1% as declared by the MINAP (Myocardial Infarction National Audit Project) dataset in 2014. (19)

Patients are also at increased risk of further vascular events well beyond 30 days following ACS presentation; with three-year cumulative rate of major adverse cardiovascular events (MACE) quoted as 20.4%. The subsequent event is equally likely to be related to the index culprit as a new untreated lesion. (20) The risks of these negative clinical outcomes are the focus of published and validated risk scores intended to identify high risk populations with the aim of modification of their management and potential outcomes. The GRACE (Global Registry for Acute Coronary Events) score (table 1.1 (21)) and TIMI (Thrombolysis in Myocardial Infarction group) risk score (22) are in routine clinical use; with the former preferred by guidance bodies as it provides the more accurate risk stratification due to its good discriminative power. (4)

Variable (on admission)	Unit	Risk category	Score
Age	years	**In-hospital mortality**	
Heart rate	Beats/min	**Low**	**≤108**
Systolic blood pressure	mmHg	**Intermediate**	**109-140**
Creatinine	μmol/L or mg/dL	**High**	**>140**
Signs of heart failure	Killip class	**6-month mortality**	
Cardiac arrest at admission	Yes/no	**Low**	**≤88**
ST deviation on ECG	Yes/no	**Intermediate**	**89-118**
Elevated cardiac enzymes	Yes/no	**High**	**>118**

Table 1. 1: GRACE risk score

Table of variables included in the GRACE in-hospital risk score calculated on admission. The subsequent score allows categorisation of patient into low, intermediate and high risk of death or death and MI. Ranges stated are for risk assessment of non ST-elevation ACS. (21)

1.2.5 Treatment

The fundamental principle of ACS treatment is to reduce ischaemia. In STEMI, reperfusion can be provided mechanically with percutaneous coronary intervention (PCI) or medically with thrombolysis. Throughout the ACS spectrum, antiplatelet therapy is an essential component of medical management.

Urgent reperfusion strategy has been the gold standard of care for STEMI since the 1980s, when thrombolysis with streptokinase (≤ three hours of presentation) was proven to significantly reduce all-cause mortality (risk ratio (RR): 0.74 p=0.0005). (23) In the 1990s the method of reperfusion was challenged and large trials showed that although thrombolysis markedly improved outcomes, switching to a PCI method derived an even greater one; (24,25) with evidence of both superior efficacy (death, re-infarction) and safety (major bleeding) endpoints. This resulted in the widespread adoption of primary PCI (PPCI) as the reperfusion strategy of choice in patients presenting with STEMI. (26,27)

Benefit was also found for invasive management of patients with non ST-elevation ACS (Unstable angina and NSTEMI), with reduction in mortality and re-infarction rates compared to those managed conservatively. (28–30) It is now routine for patients admitted to hospital with non ST-elevation ACS to undergo coronary angiography and revascularisation prior to discharge. (3,4) Although at present there is no mandate for this to be with the same urgency as STEMI, unless patients are high risk and unstable. (31)

Following coronary intervention, or in cases felt not appropriate for intervention, aggressive secondary prevention is initiated with a combination of therapy with a statin, angiotensin converting enzyme inhibitor, beta-blocker and dual antiplatelet therapy. The first major antiplatelet agent in common use was aspirin, a cyclooxygenase (COX) inhibitor, with evidence in patients with ACS having a significantly lower rate of MI and death following admission with acute unstable symptoms (RR: 0.49, p=0.005). (32) The initial studies into aspirin shaped the pharmacological management of ACS patients, with no future medication able to confer such a profound benefit in randomised trials over the comparator. Another class of antiplatelet agents, the $P2Y_{12}$ inhibitors, were initially investigated as an alternative monotherapy to aspirin but no statistical difference was determined in patients following MI. (33) However, the addition of a $P2Y_{12}$ inhibitor to

aspirin did demonstrate an additive benefit in multiple trials, when compared to therapy with aspirin alone. (34–36) There are different classes of P2Y$_{12}$ inhibitors with thienopyridines (clopidogrel and prasugrel) and triazolopyrimidine (ticagrelor). In contemporary practice, clinical preference is given to the newer agents in each group (prasugrel and ticagrelor) after significant reduction in the risk of clinical endpoints without detrimental effect on safety was established. (34,35)

Lipid lowering therapy has long since been integral to successful management of CAD predominately with HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase inhibitors, however its role beyond long-term benefits were not appreciated until studies examined ACS patients. HMG-CoA reductase is a rate-limiting enzyme in hepatic cholesterol synthesis. The result of its inhibition is to increase LDL uptake from the circulation into hepatocytes due to up-regulation of LDL receptor synthesis. Other effects, independent of LDL, are secondary to modification of several membrane-bound enzymes initiating improved endothelial function, diminished vascular inflammation, reduced platelet aggregation, and stabilisation of the atherosclerotic plaque. (37) Interestingly the "secondary" roles of statins are likely responsible for the reduction in recurrent symptomatic ischaemia rates in the short-term following admission with ACS found with intensive atorvastatin treatment. (38,39)

1.2.6   Ticagrelor

Ticagrelor [(1S, 2S, 3R, 5S)-3-[7-{[(1R, 2S)-2-(3,4-difluoro-phenyl) cyclopropyl] amino}-5-(propylthio)-3H-[1, 2, 3]-triazolo[4, 5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy) cyclopentane-1, 2-diol] is a cyclopentyl-triazolo-pyrimidine (CPTP) (figure 1.3), a non-thienopyridine non-ATP (adenosine triphosphate) analog drug, which is orally administered. This class is a non-competitive P2Y$_{12}$ receptor antagonist, inducing potent inhibition of adenosine diphosphate (ADP) mediated platelet activation. Discovery occurred during innovations into stable ATP analogs designed for pharmacological experiments. (40)

It differs from preceding P2Y$_{12}$ inhibitors (such as clopidogrel) as it is not a prodrug, and although metabolised in the liver into a single equally potent active metabolite (AR-C124910XX concentrations are maximally 40% of the parent drug), this step is not
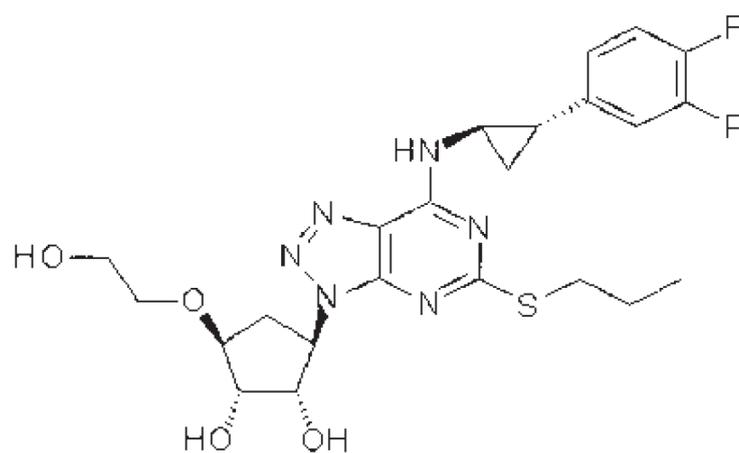
Figure 1. 3: Chemical structure of ticagrelor

From Van Giezen et al, 2005. (41)

obligatory for activity. (42,43) Therefore, unlike clopidogrel, ticagrelor's effects are not muted by inter-subject cytochrome P450 enzymic variability or the administration of CYP3A inhibitors. (43) In healthy subjects, ticagrelor is rapidly absorbed with platelet inhibitory effects evident after 30 minutes, reaching peak plasma concentration at approximately 90-120 minutes (42,43) and steady state at 2-3 days. (40) The half-life is approximately 8 hours (43) in those assuming normal metabolism and gastro-intestinal excretion (<1% renal excretion). (42–44)

Some studies have shown ticagrelor's benefits beyond those of inhibition of platelet activation. $P2Y_{12}$ receptors are also found in relative abundancy in vascular smooth muscle cells, where stimulation mediates vasoconstriction. (45) Thienopyridines have not been shown to inhibit this contraction, presumed to relate to the inability of the active metabolites to enter the vascular wall. However, this does not impede ticagrelor, and has been proven in murine and human models. (46) Canine models have shown significantly reduced infarct size and improved myocardial perfusion following coronary thrombotic occlusion; not seen with clopidogrel. (47) Canine coronary flow has also been shown to be potentiated by ticagrelor in response to reactive and adenosine induced hyperaemia. This may be connected to its inhibition of erythrocyte adenosine uptake confirmed in human samples. (48)

In addition to its $P2Y_{12}$ receptor antagonism, ticagrelor impedes adenosine reuptake by acting as a weak inhibitor of the equilibrative nucleoside transporter (ENT) 1. (49) Adenosine, a purine nucleoside, is intrinsic to multiple signalling pathways. Actions via the Gs coupled A_2 receptor subgroup are most pertinent to this chapter with roles in vasodilation, inflammation and platelet inhibition. (50,51) A_{2a} and A_{2b} receptors are both expressed in platelets (51) and coronary arterial endothelial cells (52), with increased expression of A_{2b} during periods of oxidative stress. (51) Adenosine has been shown in porcine coronary artery endothelial cells to increase cGMP production in a concentration (physiological) dependent manner through a nitric oxide (NO) pathway directly involving A_{2b} and A_{2a} receptors. (53,54)

A study in 2010 by Butler and Teng looked at the safety profile of ticagrelor in healthy volunteers (n=28). Multiple doses of ticagrelor were assessed, most significantly larger than the current recommended therapeutic dose. Adverse events recorded included

myalgia (11%) and gingival bleeding (4%). Two subjects required discontinuation of their medication; one for shortness of breath and one for a significant rise in liver transaminases. The latter was noted at day 4 following 300mg daily, and results had normalised 10 days following discontinuation. (42) A further two studies were reviewed by Teng et al looking at ticagrelor safety when given in combination with low dose aspirin in healthy volunteers. This showed it to be well tolerated and there were no serious adverse events or death. In both studies, there were no clinically meaningful changes in haematology, clinical biochemistry, urinalysis, vital signs, physical findings, or ECG data. (44)

DISPERSE-2, a multicentre international double blind randomised trial, explored safety and efficacy of ticagrelor in patients with ACS compared to clopidogrel, in addition to aspirin (n=990). (55) Although powered for safety endpoints a numerical trend was observed for lower MI rates with ticagrelor at 4 and 12 weeks. A small sub study (n=91) demonstrated significantly greater inhibition of ADP-induced platelet aggregation with ticagrelor in a dose dependent fashion at multiple time-points out to 12 weeks. (56)

PLATO (PLATelet inhibition and patient Outcomes) was the landmark multicentre double blind randomised trial investigating ticagrelor versus clopidogrel in the treatment of ACS in addition to low dose aspirin for 12 months. (34) Published in 2009, this study recruited 18624 patients internationally and demonstrated a significant reduction in the primary composite endpoint of cardiovascular death, MI and stroke at 12 months; with 9.8% of patients experiencing events on ticagrelor (90mg twice daily) compared to 11.7% on clopidogrel treatment (Hazard ratio (HR) 0.84 (95% confidence interval (CI) 0.77-0.92), p<0.001). (34) Evidence now goes onto support continuation of low dose ticagrelor (60mg twice daily) beyond the first 12 months in high risk patients post-MI, with reduction in the same hard composite endpoint (HR 0.84 (95% CI 0.74-0.95), p=0.004). (57)

Ticagrelor's superiority in cardiovascular disease led to further exploration of its potential role in other types of vascular disease; cerebrovascular (SOCRATES (58)) and peripheral vascular disease (EUCLID (59)) compared to aspirin and clopidogrel respectively. However, no statistical benefit was seen in the composite end point of cardiovascular death, MI and stroke in either of these large cohorts ((cerebrovascular disease: HR 0.89 (95% CI 0.78-1.01), p=0.07) or (peripheral vascular disease: HR 1.02 (95% CI 0.92-1.13), p=0.65)). (58,59)

One concern of antiplatelet therapy is that it may cause major bleeding. PLATO and DISPERSE-2 found that in patients treated with ticagrelor 90mg twice daily, major bleeding, as defined by TIMI and TIMI-defined criteria, occurred in 7.9% (n=9235, 12months) and 8.6% (n=334, 12 weeks) respectively. (34,55) As stated both trials compared patients on ticagrelor against clopidogrel with DISPERSE-2 finding no significant increase in the major or minor bleeding risk with ticagrelor (90mg twice daily). (55) The results of SOCRATES (compared to aspirin) and EUCLID (compared to clopidogrel) mirrored this outcome. (58,59) The landmark ACS study PLATO found statistical similarity in total major bleeding, but an increased rate of non-CABG and non-procedure-related major bleeding in the ticagrelor group, primarily after 30 days on study drug treatment. (34) Fatal bleeding was low and not significantly different in either group.

PLATO and DISPERSE-2 also recorded non-bleeding safety endpoints, highlighting that dyspnoea and ventricular pauses were reported more frequently in patients on ticagrelor than clopidogrel. (34,55) Both phenomena are felt likely to be secondary to the adenosine accumulation, and transient in most cases. In PLATO, dyspnoea occurred in 14.5% of patients who actually received ticagrelor medication versus 8.7% in the clopidogrel arm (n=18421). (60) 0.4% and 0.3% of these cases were reported as severe/incapacitating dyspnoea respectively. Interestingly, 15% of cases were ultimately attributed to ticagrelor (2.2% overall) against 6.9% of cases felt related to clopidogrel (0.6%) (p<0.0001). (60) In ACS ventricular pauses were more commonly found than clinical bradycardia, independent of the $P2Y_{12}$ antagonist. Detailed PLATO subgroup analysis (n=2,908) demonstrated an increased risk of ventricular pauses ($\geq$3 seconds) when ticagrelor (5.8%) was compared to clopidogrel (3.6%) after one week (RR 1.61 (95% CI 1.14-2.26), p=0.006). However, this variation was not significant at one month (2.1% vs 1.7%; RR 1.26 (95% CI 0.67-2.38), p>0.05). Further analysis suggested the pauses were asymptomatic and likely to originate from the sinus node. No detrimental clinical sequelae were recorded from these events. (61) In light of these publications, caution is recommended in the use of ticagrelor in patients with asthma, chronic obstructive airways disease and bradycardia in the absence of a permanent pacemaker. (62)

Consequently, given the favourable efficacy outcome data without major concerns regarding safety, ticagrelor is a mainstay of treatment of ACS, recommended in combination with aspirin by NICE for at least 12 months. (63)

This guidance may be amended in light of more recent trial data suggesting safety of a shorter duration of dual antiplatelet therapy in ACS patients undergoing invasive management with ongoing ticagrelor monotherapy. The GLOBAL LEADERS study (n=15968) showed that patients undergoing PCI had the same efficacy (all death and MI p=0.073) and safety outcomes (bleeding p=0.77) independent of antiplatelet strategy; standard 12 months dual antiplatelet therapy prior to aspirin monotherapy or 1-month ticagrelor and aspirin followed by a further 23 months of ticagrelor monotherapy. Importantly, this was also true for the ACS subgroup (n=7487), in which the standard dual therapy comparator was aspirin and ticagrelor (all death and MI p=0.19). (64) The TWILIGHT study (n=9006) specifically explored patients with clinical and angiographic high-risk features for bleeding and ischaemic events. The primary focus was safety and described reduction in bleeding (p<0.001) with termination of dual antiplatelet therapy at 3 months followed by ticagrelor monotherapy for 12 months compared to 15 months dual antiplatelet therapy prior to standard care in both arms. This reduction in bleeding was not at the expense of increased ischaemic events. (65) Subgroup analysis of patients presenting with non ST-elevation ACS (n=4614) compared the short dual antiplatelet strategy to aspirin and ticagrelor for 15 months and agreed bleeding rates were lower with the monotherapy group (HR 0.47 (95% CI 0.36-0.61), p<0.001) with no difference in ischaemic end points (HR 0.97 (95% CI 0.74-1.28), p=0.84). (66) The results of these studies demonstrate the safety of reduced duration of dual antiplatelet therapy in ACS managed with invasive strategy and supports a more tailored approach to individual patient care.

1.3   PLATELETS

1.3.1   Background

Platelets were first identified as discrete functional cellular components of whole blood in 1882 by Bizzozero, following evidence that platelets were the major constituent of "white clots" at site of vascular injury. (67) On average a human has $150x10^9$ to $400x10^9$ platelets per litre of circulatory volume, each with a life span of ten days and a third are located in the spleen. (68) While the discussion this far has only touched on platelets' involvement in the pathological process, their primary role is protective as a key player in haemostasis, preventing exsanguination from sites of vascular injury. They work synergistically with the local vascular response and the coagulation cascade to arrest haemorrhagic events.

1.3.2   Formation

In order to maintain a steady platelet count approximately $1x10^{11}$ platelets are produced daily, with scope for a ten-fold increase at times of high demand. (68) Megakaryocytes are large nucleated platelet precursor cells (50-100µm) and themselves are derived predominately from bone marrow localised haematopoietic stem cells; (69) both cell lines are regulated by the humoral glycoprotein thrombopoietin. Platelet formation occurs in two phases. During the first, each megakaryocyte matures over days expanding their platelet specific cytoplasmic content and growth factors inducing nuclear proliferation. The second "assembly" phase requires only hours, in which the megakaryocyte remodels developing 10-20 long processes called pro-platelets. The platelet then develops at the tip of the pro-platelet, with a pre-platelet intermediary step. This process routinely takes 5 days in humans. (69)
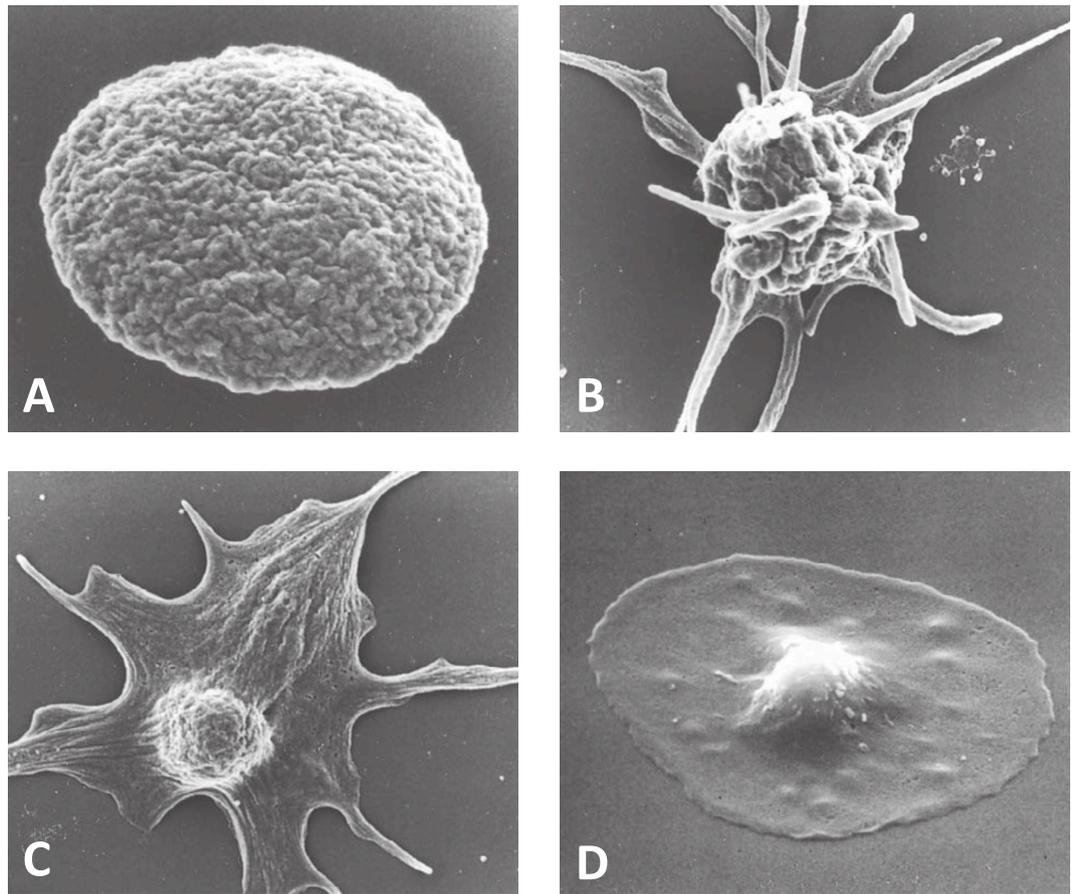
Figure 1. 4: Platelet structure from quiescent to activated

Low-voltage, high-resolution scanning electron microscope images of a dormant discoid platelet (A). During early activation the platelet becomes rounded and extends pseudopodia or filopodia (B). The cytoplasm begins to spread between these processes with formation of lamellipodia (C). Eventually the undulated surface is smoothed or "spread" as the platelet is fully activated (D). Adapted from White, 2007. (70)

1.3.3 Structure

Human platelets are small (~2–3 μm) anucleated cells, which circulate in a quiescent discoid state; a feature that enables vascular haemodynamics to drive them towards the endothelium and placing them in prime position for injury response. (71) Following activation, they undergo dramatic changes in shape and ultrastructure; the membranes become ruffled with cytoplasmic projections and the granules are centralised and discharged. (67) (Figure 1.4)

1.3.4 Platelet Membrane

The platelet membrane is multi layered with dynamic function beyond merely containing the cellular contents. The outermost stratum of the platelet is termed the glycocalyx and acts as a sensory layer. It expresses multiple glycoprotein receptors, which detect vascular signals, triggering adhesion and haemostatic activation. (70) The platelet unique glycocalyx covers a more typical lipid bilayer, predominately phospholipid, constituting the unit membrane. (72) Due to the increase in surface area of the platelet during activation (figure 1.4) the static nature of the unit membrane demands the presence of expandable undulations known as the open canalicular system (OCS) which, further to this acts as a region for granular fusion post activation. (71) The unit membrane is crucial in the potentiation of the clotting process by providing arachidonic acid implicated in the generation of prostaglandins including thromboxane A2. (72) Their roles will be explored later. Furthermore, platelet activation induces flipping of the anionic phospholipids from the cytoplasmic lipid layer to the outer surface providing a polar surface for factor induced conversion of prothrombin to thrombin. (72) Beneath the bilayer is a organelle-barren region known as the submembrane area, which acts as an important location for signalling involving cytoplasmic domains of the transmembrane receptors and the membrane contractile cytoskeleton. (70)

1.3.5 Platelet Cytoskeleton

The cytoskeleton is an intracellular matrix integral in determining the platelet shape and its main constituents are actin and tubulin, which provide a rigid backbone to the cell. (73) In the resting platelet polymerised tubulin forms microtubules circumnavigating the periphery in a ring structure, known as the marginal band, determining the discoid shape. (74) For structural support there are microfilaments predominately formed by the platelets most abundant protein actin and its association with membrane bound/cytoplasmic binding sites (spectrin and actin-binding protein; and myosin respectively). (71)

During activation the microtubules begin to coil, contributing to the rounding of the structure. At this stage there is still potential for relaxation back to quiescent state, however eventually the coil becomes increasingly compressed before newly polymerising microtubules form bridges generating new smaller rings and by which point the activation is irreversible. (74) The microfilamental structure is also altered, with initial shape rounding secondary to calcium dependent destruction of the existing actin filaments and membrane skeleton by processes including phosphorylation and proteolysis; and recoil of the core filaments into a central contractile ring. This enables the membrane to accommodate the developing protrusions created by new filament formation, which are permitted by the uncapping of barb-ended filaments and nucleation. As spreading occurs actin polymerises further and the filaments transition into more organised stress fibres. (75) Beyond platelet morphological change the cytoskeleton dynamics drives central granular association and mobilisation of signalling molecules to the plasma membrane; rendering it crucial in multiple aspects of platelet activation. (71)

1.3.6 Platelet Granules

The glycogen rich platelet cytoplasm contains small numbers of mitochondria (important for energy production), multivesicular bodies (with potential role in granular sorting) and secretory organelles. There are three main types of granules expressed in platelets: α granules; dense granules; and lysosomes. (70) Internalised in each platelet is 50-80 α granules (72) (diameter 0.2-0.4μm), which are enclosed stores of molecules critical for a multitude of platelet functions. (71) Contents are listed in table 1.2, and serve roles in

coagulation, inflammation, antimicrobial host defence, angiogenesis and wound healing. Release of the contents occurs on stimulation by fusing of the granular membrane with surface connected membranes of the OCS or plasma membrane. (76) The rapidly secreting dense granules are relatively sparse (2-7 per platelet (diameter 0.15μm)) and contain small soluble molecules that serve to potentiate platelet activation (table 1.2). (71,72) Although not fully understood, dense granular exocytosis also appears to occur by external membrane fusion. (77) Both the α and dense granules release molecules that interact with neighbouring platelets but also have an autocrine function, with positive feedback driving further activation. Lysosomes (diameter 300nm) are granules with enzymic contents (arylsulphatase, β-glucuronidase, acid phosphatase and catalase); and are proposed to have a role in digestion of components of the vascular wall matrix during aggregation. (72)

α Granules	Lysosomal Granules	Dense Granules
Albumin	Cathepsin D	Serotonin
Fibrinogen	Cathepsin E	ADP
Fibronectin	Carboxypeptidase A	ATP
Vitronectin	Carboxypeptidase B	Calcium
Osteonectin	Proline carboxypeptidase	Pyrophosphate
von Willebrand factor and antigen II	β-N-acetyl-D-hexosaminidase	
Thrombospondin	β-D-glucuronidase	
Platelet factor 4	β-D-galactosidase	
IgG, IgA, IgM	α-D-mannosidase	
C1 inhibitor	α-L-arabinofuranosidase	
Plasminogen	α-D-galactosidase	
Plasminogen activator inhibitor-1	α-L-fucosidase	
Platelet-derived collagenase inhibitor	β-D-fucosidase	
High molecular weight kininogen	β-D-glucosidase	
Protein S	α-D-glucosidase	
α$_2$-antitrypsin	Acid phosphatase	
α$_2$-macroglobulin	Arylsulphatase	
α$_2$-antiplasmin		
Multimerin		
Platelet basic protein		
β-thromboglobulin		
Histidine-rich glycoprotein		
Connective tissue activating protein III		
Neutrophil-activating protein II		
Platelet-derived growth factor		
Transforming growth factor β		
Endothelial cell growth factor		
Coagulation factor V and VIII		

Table 1. 2: Contents of platelet granules (77)

1.3.7 Platelets in haemostasis

The formation of a thrombus is a complex physiological process from platelet adhesion to an area of vascular endothelial damage through to activation and aggregation of platelets.

1.3.7.1 Adhesion

The first stage in haemostasis is adhesion of platelets to the sub-endothelial matrix due to damage or pathological process (oxidative stress). The process occurs via platelet integrins and glycoprotein (GP) receptors (VI predominately) following exposure to subendothelial extracellular matrix components such as collagen (integrin $\alpha 2\beta 1$), laminin ($\alpha 6\beta 1$) and fibronectin ($\alpha 5\beta 1$). (78,79) In situations of intravascular high shear stress the glycoprotein Ib-V-IX surface receptor is paramount, with collagen bound von Willebrand Factor (vWF) ligand slowing the circulating platelet velocity to "rolling" state (80) and enabling the more robust (via $\alpha 2\beta 1$ and GP VI) adhesions to form. (78)

1.3.7.2 Activation

The process of adhesion, beyond tethering the platelet, initiates the platelet activation cascade. The GP Ib-V-IX/vWF complex triggers the upregulation of integrin $\alpha_{IIb}\beta_3$ via inside-out signalling, altering cytoskeletal architecture and stimulating calcium (Ca^{2+}) mobilisation. (Figure 1.5) (81) GPVI binding results in non-covalent association with Fc receptor (FcR) associated immunoreceptor tyrosine-based activation motif (ITAM) and subsequent Src family kinases (Fyn/Lyn) induced tyrosine phosphorylation of the FcR γ-chain contained in the ITAM. (Figure 1.6) (82–84) This culminates in phosphoinositide 3-kinase (PI3K) dependent phospholipase C (PLC) $\gamma 2$ activation (85), Ca^{2+} mobilisation, integrin activation and granule secretion. (83)

C-type lectin-like receptor 2 (CLEC-2), a non-classical C-type lectin highly expressed platelet/megakaryocyte receptor, has more recently been implicated in platelet activation. Following ligand binding, with podoplanin or other extracellular matrix proteins, a pathway analogous to GP VI is initiated with the notable distinction of the single cytoplasmic motif
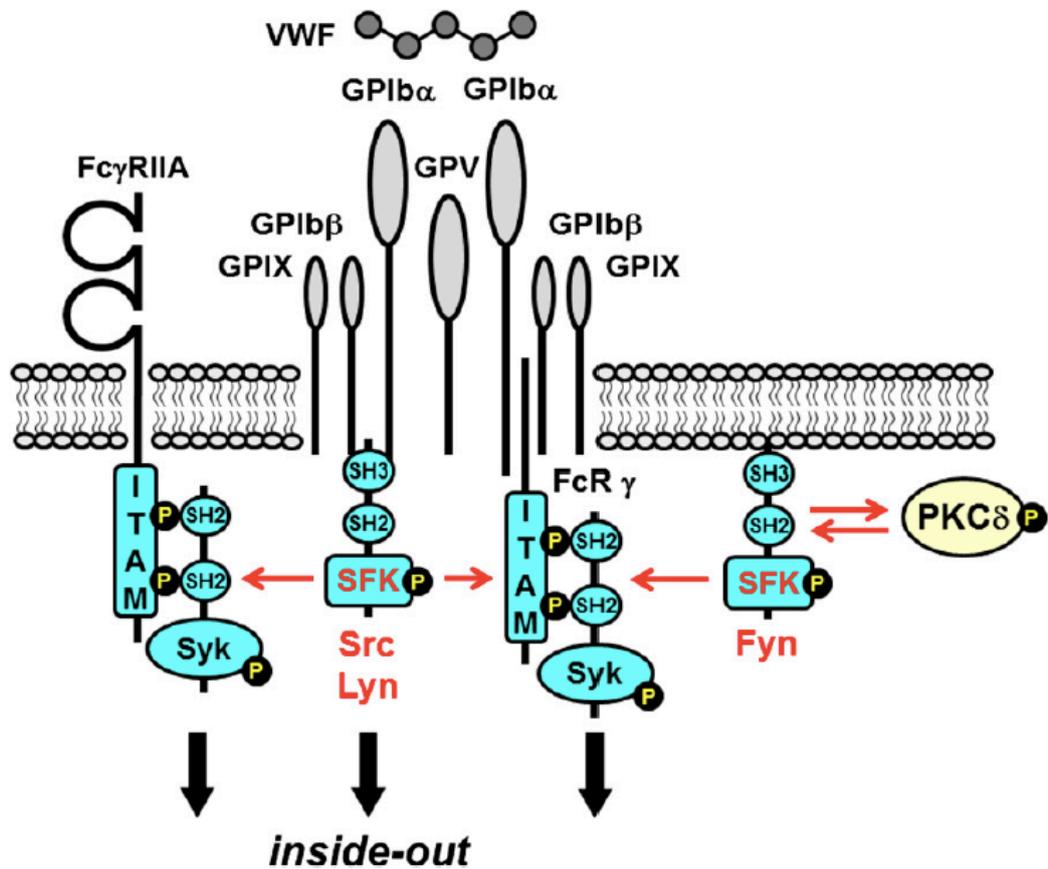
Figure 1. 5: GP Ib-V-IX signalling cascade

The receptor constitutes four subunits (Ibα, Ibβ, V and IX). Following vWF binding to the extracellular GP Ibα subunit, Src family kinase (SFK) Lyn become associated with the cytoplasmic tail of the GP Ibα and initiate inside-out signalling, with subsequent activations being enhanced by Src. Resultant phosphorylation of the ITAMs containing FcR γ chain and FcγRIIA promotes Syk activation. From Senis et al, 2014. (84)
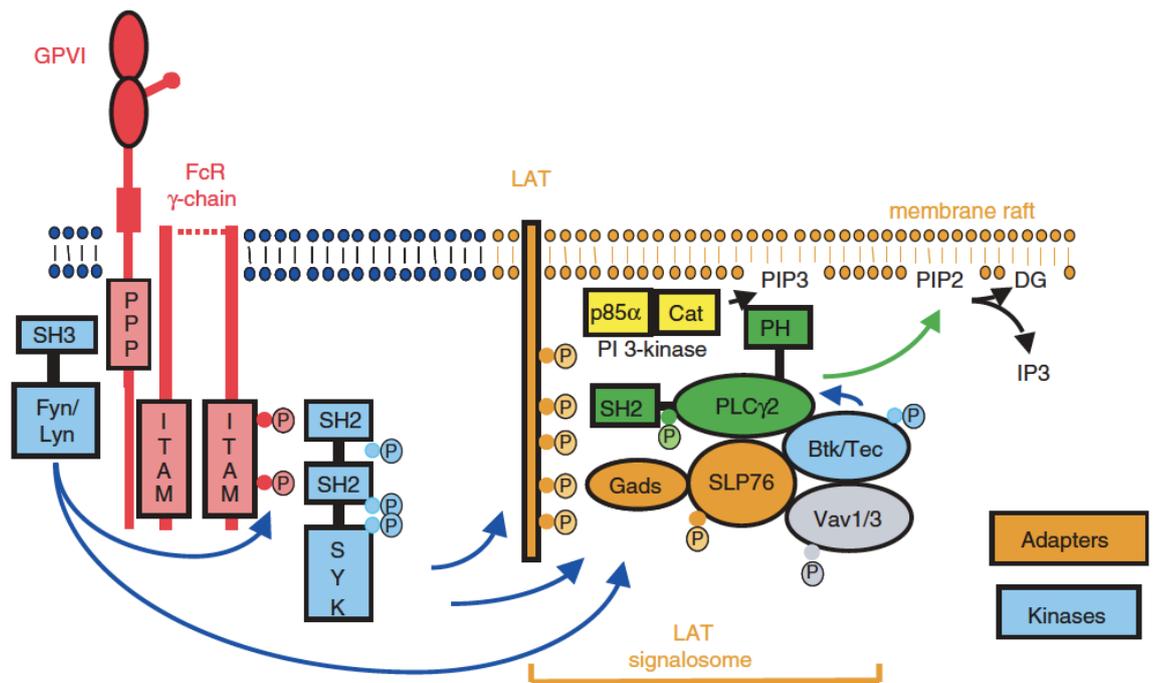
Figure 1. 6: GP VI signalling cascade

Collagen bound GP VI triggers tyrosine phosphorylation of the ITAM by Src family kinases (Fyn and Lyn), which are located on the proline rich region of the receptors cytosolic tail. This step induces Syk phosphorylation and subsequent formation of a LAT (linker for activation of T cells) (transmembrane adaptor) signalosome, and this in turn leads to PLCγ2 activation via direct and indirect stimuli. From Watson et al, 2005. (83)

(ITAM) rather than the GP VI associated tandem structure. (84,86) Interestingly, there is evidence of that both GP Ib-V-IX and GPVI receptors have crossover of roles, with GP Ib-V-IX also triggering the Syk dependent signalling cascade and GP VI inducing $\alpha_{IIb}\beta_3$ activation. (84,87)

PLCγ2 is the keystone to many of the subsequent activating processes within the platelet, including hydrolysation of phosphatidylinositol 4,5 bisphosphate to form the second messenger inositol 1,4,5 trisphosphate (IP3) and the membrane bound 1,2- diacylglycerol (DAG). (Figure 1.7) (80) IP3 binds to its named receptor, a Ca^{2+} selective ion channel, situated within the dense tubular system (DTS) driving an influx of calcium into the cytoplasm. (78) The now depleted DTS Ca^{2+} stores are detectable due to conformational change of the DTS's stromal interaction molecule (STIM) 1 membrane protein. The structural transformation allows binding to the Orai1 (also called calcium release activated calcium modulator (CRACM) 1) receptor on the platelet membrane inducing further Ca^{2+} influx. (88) DAG combines in the cytosol with phosphatidylserine bound calcium and the resulting receptor instigates the relocation of serine/threonine protein kinase C (PKC) to the membrane, where it is activated. (80) Multiple isoforms of PKC are known, with α, β, δ and θ isolated in human platelets and having varying roles including granular secretion (α, δ and θ), co-ordinated by PKC phosphorylation of many proteins including the SNARE complex, the cytoskeletal related pleckstrin and myristoylated alanine-rich C kinase substrate (MARCKS) and type II phosphatidylinositol-5-phosphate-4-kinase. Importantly, there is additional roles in thromboxane A2 (TXA2) synthesis (δ and θ) and platelet shape change (β (enhances spreading) and θ (sustain filopodia projections)). (89)
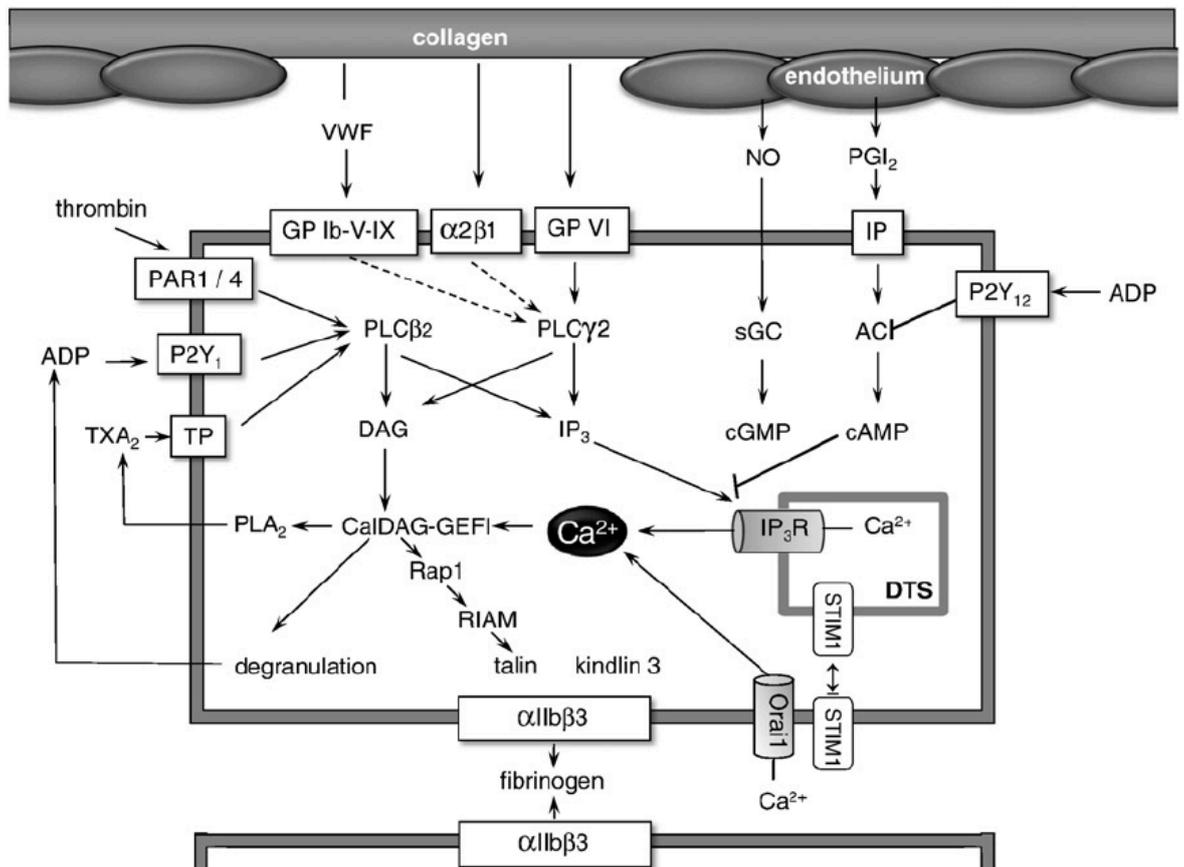
Figure 1. 7: Summary of receptor and signalling pathways involved in platelet activation

This overview is explored in depth over section 1.3. From Broos et al, 2011. (80)

The raise in intracellular Ca^{2+} supports these downstream effects of PKC driven phosphorylation, stimulating a multitude of critical processes for platelet activation, including morphological change, presentation of a pro-coagulant surface, the secretion of platelet granular (α and dense) content, the activation of glycoproteins, and the activation of Phospholipase A_2 (PLA_2). (78,90) PLA_2 that a prime enzyme in formation of arachidonic acid (AA), and subsequently TXA2. These pathways result in local accumulation of molecules like thrombin, TXA2, and adenosine diphosphate (ADP), which are important for the further recruitment of platelets and amplification of activation via their agonistic activity on respective G protein coupled receptors. (See section 1.3.9)

1.3.7.3 Aggregation

The final step in the cascade is platelet aggregation, which is facilitated through the irreversible transition of the platelet specific integrin $α_{IIb}β_3$ (or glycoprotein IIb/IIIa) receptor from a low affinity state to a high affinity state. PKC and/or Ca^{2+} via CalDAG-GEFI (diacylglycerol regulated guanine nucleotide exchange factor I) generates GTP-bound Rap1, which forms a complex with RIAM (Rap 1-B interacting adapter molecule) at the plasma membrane to recruit talin. In turn this complex binds to the receptors β subunit cytoplasmic tail transforming the integrin. (87) The conformational change allows outside-in signalling and the efficacious binding of fibrinogen, vWF and fibronectin. The attachment of these ligands results in multivalent linking between neighbouring platelets and formulating a firm platelet thrombus. (80) The stability of the aggregates is protected via further interactions by adhesion molecules (junctional adhesion molecules (JAM-A, JAM-C), endothelial cell specific adhesion molecules (ESAM), DNAM-1, platelet endothelial cell adhesion molecule (PECAM-1) and signalling lymphocyte activation molecule (SLAM)) and contact dependent signalling (Eph kinases, ephrins and platelet endothelial aggregation receptor (PEAR-1)). (91)

Tissue factor, contained within vessel medial and adventitial layers, mediates thrombin production by the extrinsic coagulation cascade. Thrombin is an important activator of platelets through PAR receptors and critical in the conversion of fibrinogen to fibrin. (Figure 1.8) (92)
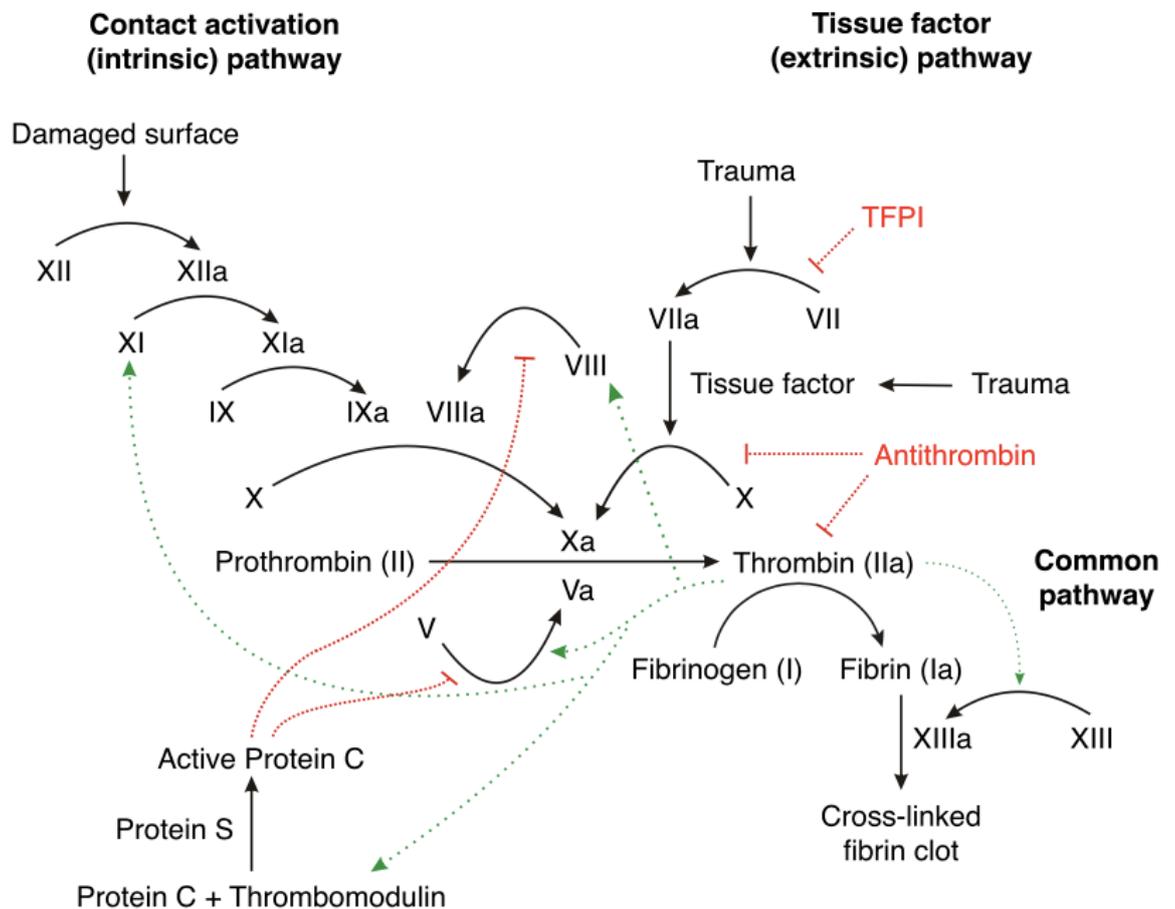
Figure 1. 8: The coagulation cascade

The coagulation cascade is fundamental in the production of fibrin and stabilisation of thrombus leading to formation of the haemostatic plug. There are two distinct pathways dependent on numerical coagulation factors - the contact activation (intrinsic) and the tissue factor (extrinsic) pathway - prior to the final common step. Green and red lines depict the positive and negative feedback loops respectively. From www.gnu.org. (93)

Finally, the polymerised fibrin mesh united with the activated platelets forms a secure haemostatic plug.

1.3.7.4 Adhesion Molecules

Adhesion molecules implicated in platelet haemostasis are numerous and integral to may stages of the process. Those most salient are discussed in more detail.

1.3.7.4.1 Von Willebrand Factor

Von Willebrand Factor (vWF) is a large (2050 amino acids) multimeric adhesive glycoprotein which binds to collagen, GP Ib-V-IX and GP IIb/IIIa, with an important role in platelet adhesion and aggregation as reviewed above, particularly at sites of high shear stress. (79) The endothelium stores vWF in Weibel-Palade bodies and platelets within the α granules. Platelet/megakaryocyte release accounts for approximately 20% of the plasma concentration (10µg/ml), resulting in an autocrine positive feedback pathway. (80,94) Inherited and acquired conditions leading to deficiency in vWF can have a notable clinical bleeding consequences. (95,96)

1.3.7.4.2 Fibrinogen

Fibrinogen is synthesised in the liver and exists as a soluble plasma glycoprotein (300 kDa) comprising of two peptides, each with three polypeptide chains (α, β and γ) (97) circulating in volumes of 2-4 mg/ml. (98) Platelets contain intracellular fibrinogen (cytosolic and within α granules) and express membrane bound molecules in resting states, with the GP IIb/IIIa receptor vital to fibrinogens endocytosis. (99) Fibrinogen has multiple functions, including proplatelet propagation in bone marrow (69), acute phase reactant (97), platelet activation from tyrosine kinase related outside in signalling (79) and platelet aggregation from GP IIb/IIIa–fibrinogen crosslinking and fibrin precursor role. (80)

1.3.7.4.3   Fibronectin

Fibronectin was first described in 1948 and named due to its relationship to fibrinogen and fibrin. This glycoprotein constitutes two subunits (250 kDa), each derived primarily of three repeating units (type I, II and III), bound together by two c-terminal disulphide bonds. (100) Hepatocytes produce soluble plasma fibronectin (230-650μg/ml), but cellular fibronectin is synthesised by numerous cells all generating a different isoform, the properties of which render them less soluble than their circulating relations. (100,101) Fibronectin binds to a number of integrins (notably $\alpha 5\beta 1$ and GP IIb/III) and contains major sites of binding for heparin, collagen and fibrin; designed to promote its role in embryological development, wound healing and importantly haemostasis. The molecule has an apparent dichotomous role in the latter process, with initial fibrin bound fibronectin contributing to aggregation and stabilisation of the thrombus, while non-fibrin bound fibronectin on the outer thrombus surface preventing further growth and uncontrolled propagation. (101)

1.3.7.4.4   P -selectin

P-selectin (CD62P) is the largest of the selectin family of adhesion molecules. It is contained predominately in platelet α-granules (102), though has also been described in dense granules and is also present in the Weibel-Palade bodies of endothelial cells. (103) These molecules constitute: a N-terminal lectin-like domain, an epidermal growth factor-like domain, a number of consensus repeats of a sequence found in complement regulatory proteins, a transmembrane domain, and a short cytoplasmic tail (103); and is approximately 140 kDa. (67) In quiescent state minimal P-selectin is expressed on the platelet surface, but following activation there is rapid (maximally 30 seconds to ten minutes) redistribution from the intracellular space to the external membrane. (67) This enables multiple ligands to bind to the platelet; including vWF, glycoprotein Ibα, sulphatides and P-selectin glycoprotein ligand -1 (PGSL-1). (67) The latter enabling adhesion to circulating leukocytes (notably neutrophils and monocytes) through a calcium dependent lectin-like mechanism (103) to form platelet-leukocyte aggregates. (104) The absence of the adhesion molecule on resting platelets surfaces and rapid externalisation following activation lends P-selectin to being a frequently used sensitive marker of platelet

activation. (105) It has been shown to be elevated in episodes of acute coronary syndromes (103,106), correlating well with established biomarkers. (106)

1.3.8 Secondary Platelet Activation

Platelet activation leads to degranulation and the release of numerous messenger molecules with autocrine and paracrine activity leading the amplification of the process of activation. These messengers include TXA2, adrenaline and ADP which exert their effect on specific platelet membrane receptors and share many common pathways are explored in this section. (Figure 1.7 and 1.9)

1.3.8.1 Thromboxane A2 Receptor

Thromboxane A2, produced in response to platelet activation, acts locally due to limitations of a very short half-life and binds to its specific G protein coupled receptor on the platelet (TPα receptor) and endothelial (TPβ receptor) surface to intensify the process. (107) The TP receptors are linked with $G\alpha_{12/13}$ and $G\alpha_q$ proteins, which trigger several signalling cascades. (78,108) $G\alpha_{12/13}$ activates intracellular Rho/Rho-kinase (ROCK) pathway causing phosphorylation of myosin light chain, and calcium independent shape change. (109) $G\alpha_q$ prompts PLC activity and downstream pathway has been discussed above. (108) Reduction in thromboxane A2 formation via cyclooxygenase inhibition is the primary antiplatelet function of aspirin. (110)

1.3.8.2 P2Y Receptors

The $P2Y_{12}$ receptor is a member of the P2 family within the eight metabotropic P2Y subtypes of G-protein coupled seven transmembrane domain receptors, first isolated in 2001 by Hollopeter et al. (67,111) It is predominately expressed on the surface of platelets but has also been localised to specific areas of the brain (glial cells) (111–113) and more recently $P2Y_{12}$ related mRNA has been identified in vascular smooth muscle cells. (45) The $P2Y_{12}$ receptor is preferentially controlled by adenine related nucleotides; with ADP acting
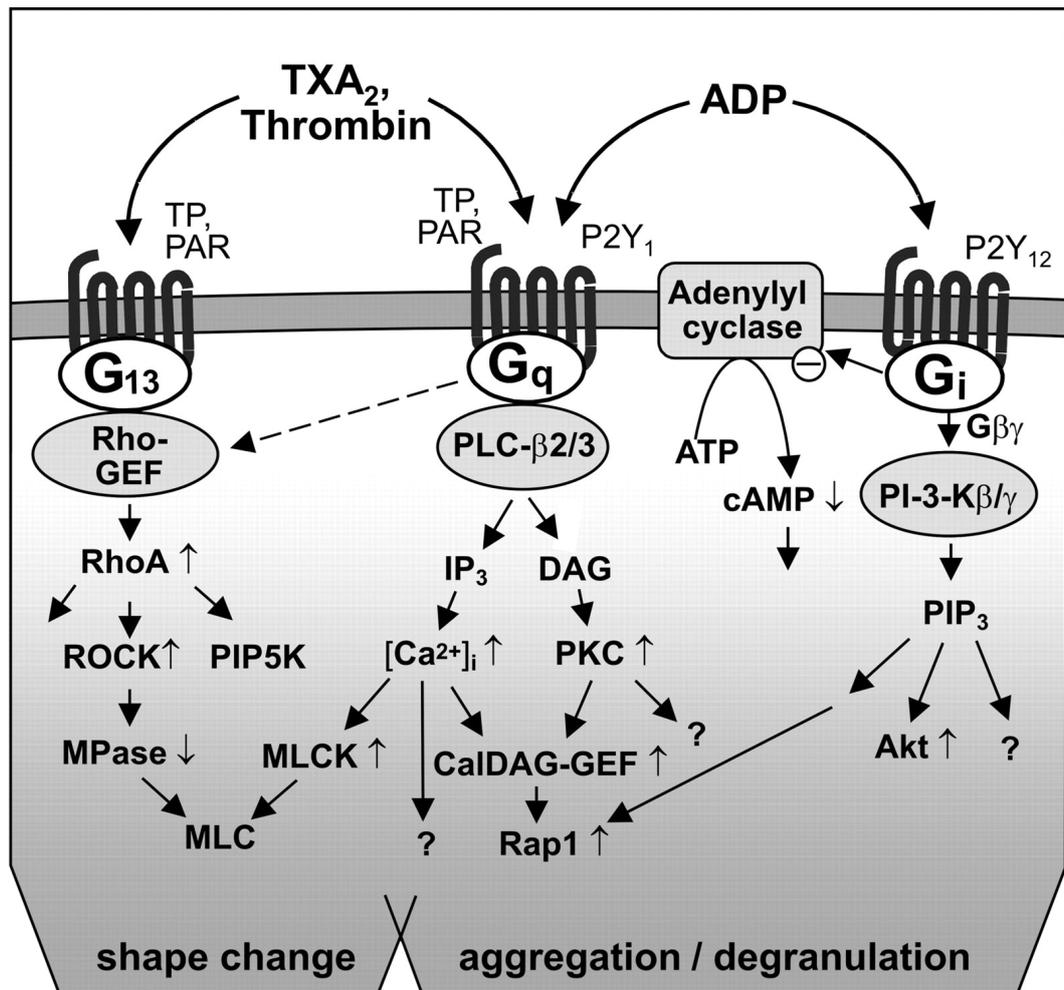
Figure 1. 9*: **G coupled receptors and associated signalling pathways in the control of platelet activation***

Detailed overview of signalling cascades involved with platelet activation following interaction with receptors and certain g proteins. RhoGEF relates to Rho-guanine nucleotide exchange factor; ROCK, Rho-kinase; phosphatidylinositol 4-phosphate 5-kinase (PIP5K); myosin phosphatase (MPase); MLC kinase (MLCK); DAG, diacyl glycerol; CalDAG-GEF, calcium and diacyl glycerol-regulated guanine nucleotide exchange factor and PIP₃. From Offermanns, 2006. (114)

as an agonist and in platelets an antagonistic response is seen with the related triphosphate and its analogues. (113)

It is well established that these receptors are coupled to the Gi protein (78,115), with activation in platelets inducing a fall in cAMP production due to the inhibition of adenylyl cyclase by Gi's $\alpha\iota$ subunit. Therefore, counteracting the endothelial prostacyclin's (PGI$_2$) attempt to maintain stasis in the inactive platelet. There is also evidence that P2Y$_{12}$ receptor agonism will interact with the PI3K pathway via the $\beta\gamma$ Gi subunit interacting with PI3Ks β and γ isoforms. (114) Resulting in phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$) mediated activation of the small GTPase Rap1B and the phosphorylation of serine–threonine protein kinase B/Akt , critical in the process of GP IIb/IIIa platelet aggregation. (115)

P2Y$_{12}$ receptor antagonists, ticagrelor, clopidogrel and prasugrel are used in routine clinical practice for reduction in adverse events following MI, cerebrovascular accidents, peripheral vascular atherothrombotic events and routine PCI.

The P2Y$_1$ receptor is the other member of the P2 family expressed on platelets and thus shares many common structural features with its P2Y$_{12}$ receptor relative. However, ADP stimulation of P2Y$_1$ receptors leads Gq receptor coupling (113), rather than Gi, and subsequent PLC activation. There is potentially G$_{12/13}$ activity also present, driving RhoGEF activation and shape change. (Figure 1.9) This is debated and is potentially mediated by Gq coupling rather than independent. (115)

1.3.8.4 Protease Activated Receptors

Thrombin in its active form is a protease with a plethora of actions across cell types. It is potent activator primarily drives aggregation and secretion in platelets, while endothelial stimulation initiates secretion of NO, PGI_2 and anticoagulants such as thrombomodulin and protein C; which raises confusion about its role as a driver of haemostasis. (Figure 1.10) (116) Its effects are mediated through interaction with G coupled cell surface receptors with 7 transmembrane domains called protease activated receptors (PARs), where cleavage of the extracellular N terminus allows the new terminus to form an irreversible ligand for auto-activation. (117) Four such receptors have been described, with PAR1 and PAR4 expressed on platelets, endothelial cells, leukocytes and only PAR1 in smooth muscle cells. (116) Their expression can be up and down regulated by different molecules and disease states. (117) Thrombin exhibits interactions with its specific binding site and exosite I on the PAR1 receptor, whereas it only binds to its specific site on PAR4. This conveys a delay in the receptor's activation when compared to PAR1, which acts as a co-factor in this process. (117) Other described proteases inducing PAR activation include anticoagulant-proteases activated protein C (APC), plasmin and zinc- dependent matrix metalloprotease 1 (MMP-1). (118)

PAR activation results in morphological receptor change and enables G protein coupling. PAR1 has affinity for $G\alpha_{12/13}$, $G\alpha_q$, $G\alpha_I$ and $G\beta_\gamma$ which triggers a multitude of pathways outlined in figure 1.10. PAR4 appears to also couple with $G\alpha_{12/13}$ and $G\alpha_q$. The cleavage process is permanent and therefore control of the signalling relates to the uncoupling of the G proteins via phosphorylation and arrestin binding of the cytoplasmic aspect of the receptor. Finally, the receptor is internalised and journeyed to lysosomes for degradation. (118)
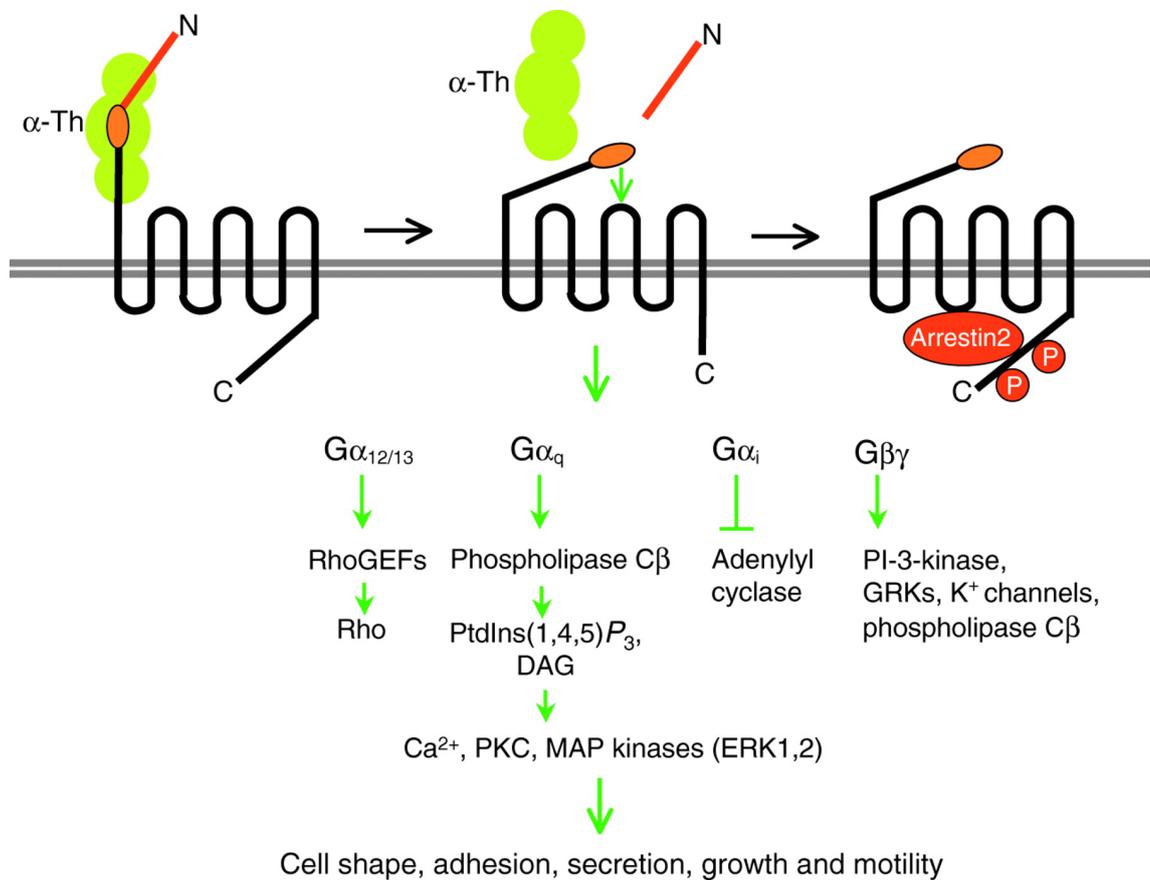
Figure 1. 10: PAR1 receptor activation and signalling pathway

Thrombin (α-Th) cleaves the N-terminus of PAR1 resulting in irreversible ligand binding and receptor activation. Subsequent coupling with an array of G proteins commence multiple signalling cascades. $G\alpha_{12/13}$ activates Rho; $G\alpha_q$ activates PLC and $G\alpha_I$ inhibits adenylyl cyclase. $G\beta_\gamma$ can activate PI3K, g-coupled receptor kinases (GRK) and PLC. PAR1 is uncoupled form G-protein signalling by rapid phosphorylation and arrestin binding. From Arora et al, 2007. (118)

1.3.9 Platelets in Acute Coronary Syndromes

Atherosclerotic plaque rupture is a complex process. Repeated plaque ruptures may be silent, and subsequently heal with no clinical manifestation. However, post-mortem studies have demonstrated that repeated plaque rupture will increase luminal narrowing, and healed ruptures are a frequent finding on post-mortem studies of men who have died because of sudden cardiac death. (1,119) Plaque disruption and restrictive/occlusive thrombus formation within the coronary artery is pathognomonic of ACS, thus demonstrating a clear pathophysiological distinction between this and a stable presentation due to a coronary stenosis. However, it is important to note the flow dynamics are increased in coronary stenoses and platelet activation is generally potentiated in areas of high shear stress. (108) Furthermore, in the post-stenotic region there is a fall in shear stress favouring and initiating stable discoid platelet aggregation. (120) In ACS, with progressive platelet propagation, eventually the flow will become stagnant, an environment preferential for fibrin and erythrocyte thrombi development. Unsurprisingly therefore, STEMI patients (complete vessel occlusion) have a higher degree of fibrin rich "red" thrombus compared to the unstable angina patient, where the platelet rich "white" thrombi predominate. (1) Beyond thrombus formation, platelets have a role in the development of atherosclerosis with endothelial cell adhesion aiding migration of leukocytes and their pro-inflammatory molecules to the vessel wall, which can accelerate plaque formation. (1) This can be exacerbated further by platelet/thrombus entry into the plaque following recurrent episodes of rupture and repair. (1)

Overall individuals with coronary artery disease have been shown to have increased circulating active platelets, microthrombi and platelet leukocyte aggregates, and this change has been demonstrated in stable disease as well as ACS. The extent of the increase in levels is a predictor of both short and long term major adverse cardiovascular events (MACE). (121)

1.4   ENDOTHELIUM

1.4.1   Background

The endothelium is a single layer of cells (0.2μm) existing as a semipermeable barrier between the lymphatic system or blood vessel wall and the lumen. In the context of this project the focus will be on the vascular endothelium acting as a homeostatic monitor, transporter and communicator between the smooth muscle and circulating cells with some organ specific functions. (122) So critical are these function that impairment of the endothelium has been implicated in early atherosclerosis. (Figure 1.11)

1.4.2   Haemostasis and Platelet Inhibition

A healthy intact endothelium is characterised by integrin binding to the extracellular matrix and its intercellular adhesions. (123) While the endothelium has integrity, it plays an important role in the prevention of thrombus formation by triggering anticoagulant, fibrinolytic and antiplatelet activity. In its anticoagulant function, endothelial cells release thrombomodulin, which activates protein C and prevents splicing of factor V-VIII into their respective active states (figure 1.8), and heparin sulphate, which via antithrombin III inhibits thrombin. (123) Tissue plasminogen activator, also released from the endothelium, induces fibrin dissolution (122), and is a substance so potent in its thrombolytic function that recombinant forms have extensive clinical use for treatment of pulmonary emboli (124), ischaemic cerebrovascular accidents (125) and MI. (26)

The synthesis and release of molecules implicated in control of platelet activation is one of the endothelium's most powerful roles. In a healthy endothelial cell it is this paracrine activity that predominates, although it also presents a negative charge to the circulating platelets acting as a repellent to guide them away from the vessel wall. (1) The molecules involved include PGI_2, NO (126) and CD39. CD39 is an ecto-ADPase, a cell surface enzyme with extracellular catalytic site resulting in hydrolysis of ADP to its monophosphate derivative and therefore inhibiting ADP-induced platelet activation. (123,127)
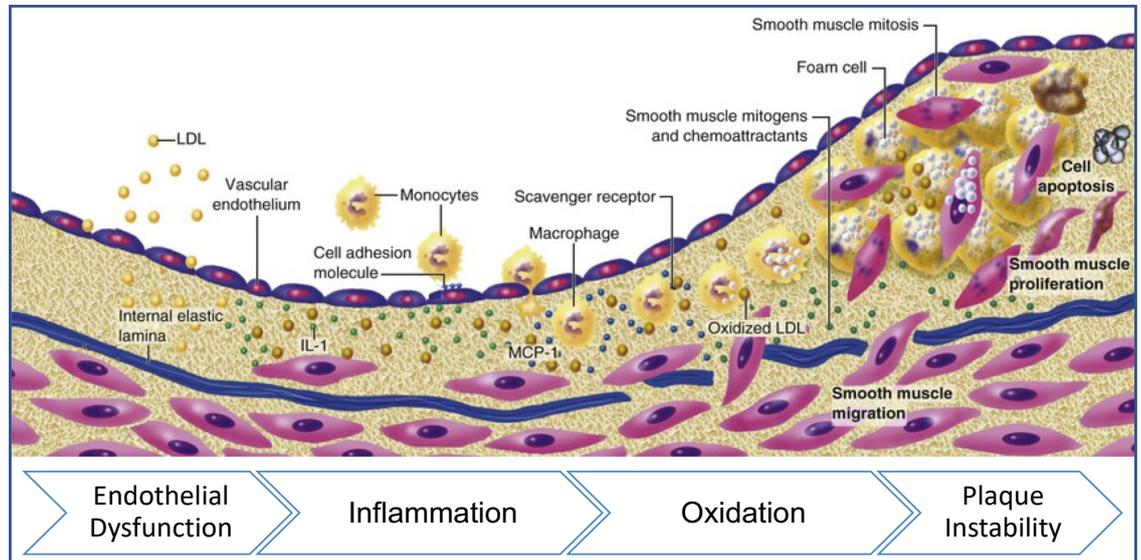
Figure 1. 11: Stages of an atherosclerotic plaque formation

Atherosclerosis develops over decades with endothelial dysfunction being the first stage before intimal accumulation of cytokines (Interleukin-1 (IL1), monocyte chemoattractant protein-1 (MCP-1)), LDL and macrophages. Adapted from Libby et al, 2008. (128)

1.4.2.1   Prostacyclin

Prostacyclin or prostaglandin I_2 (PGI$_2$) is a lipid messenger derived from arachidonic acid (AA) metabolism. First described in 1976 as an inhibitor of platelets and vasoconstriction. (129) Following AA release by phospholipase A_2, multiple enzymes including COX co-ordinates the transformation of AA to PGG$_2$ then PGH$_2$. It is PGH$_2$ that is the substrate for several different prostanoids (PGE$_2$, PGD$_2$, PGI$_2$, and TXA2) determined by the presence of specific synthases in variant tissues. For PGI$_2$ this is the role of the aptly named prostacyclin synthase (130), a membrane bound member of the P450 cytochrome family, whose activity is mainly located to endoplasmic reticulum in endothelial and smooth muscle cells. (131) Once produced PGI$_2$ has a rapid half-life of three minutes before degradation into the weak vasodilator 6-keto-PGF$_{1\alpha}$. (132) Albumin however can preserve PGI$_2$ and furthermore the protein can help regulate PGI$_2$ availability for its platelet receptor. (133)

PGI$_2$ acts locally and exerts its actions through the prostacyclin receptor (IP receptor), a plasma membrane G-coupled receptor expressed on platelets and arterial smooth muscle cells with seven transmembrane domains. (134) The paracrine nature of activity is supported by the negligible plasma concentrations of PGI$_2$ stable metabolite 6-keto-PGF$_{1\alpha}$ (3 nanograms per litre). (135) Binding to the IP receptor via the G protein $G_{\alpha s}$ activates adenylyl cyclase leading to the intracellular accumulation of cyclic adenosine 3',5'monophosphate (cAMP) and subsequent activation of protein kinase (PK) A. With high PGI$_2$ concentrations $G_{\alpha q}$ coupling stimulates PLC. (131)

1.4.2.2   Nitric Oxide

NO is a free radical gas first associated with endothelial cells in 1980 (136), and platelet interaction in 1987. (137) It is formed in mammals by a nitric oxide synthase (NOS) catalysed reaction between molecular oxygen and the abundant amino acid L-arginine, and has a half-life of only 10 seconds. (131) NOS has three isoforms, with the most relevant being constitutive eNOS (endothelial NOS) present in cardiac myocytes, renal mesangial cells, osteoblasts, osteoclasts, platelets and endothelium. (37)

Action	Effect
Inhibition of platelet activation and aggregation	Reduced Thrombosis Possible reduced vascular smooth muscle cell proliferation
Inhibition of platelet–leukocyte interaction	Reduced leukocyte recruitment
Inhibition of cell adhesion molecule expression	Reduced leukocyte adhesion and extravasation
Scavenging of lipid radicals	Reduced LDL oxidation
Inhibition of vascular smooth muscle cell proliferation	Reduced neointima formation Reduced production of extracellular matrix
Inhibition of tissue factor expression	Reduced thrombosis

Table 1. 3: The vasoprotective role of nitric oxide

The actions and the possible mechanism by which these actions could reduce atherosclerosis. (138)

NO is potent inhibitor of platelet aggregation and adhesion, and can therefore play a key role in controlling atherogenesis and thrombosis. (139) (Table 1.3) In platelets, NO diffuses through the platelet membrane and stimulates increased concentrations of the second messenger cyclic guanosine 3',5'monophosphate (cGMP) through activation of its intracellular receptor soluble guanylyl cyclase (sGC). (78,139,140) This accumulation leads to activation of cGMP-dependent PKG. Elevations in platelet cGMP are associated with reduced platelet aggregation in vitro. (141) Consistent with these observations, reducing cGMP/PKG signalling, as seen in PKG deficient mice, is associated with enhanced injury-induced vascular proliferation and platelet activation. (142)

1.4.2.3　　　　　Cyclic Nucleotide Signalling

Increasing concentrations of cAMP and cGMP results in the activation of their related protein kinase, PKA and PKG respectively to maintain platelet quiescence. The phosphorylation of a plethora of proteins by these kinases are thought to underpin their ability to control platelet activation.

PKA leads to phosphorylation of the myosin light chain kinase and IP3 receptor rendering them inert, this not only prevents activation but can assist in disaggregation. (131) PKG is an inhibitor of several pathways including IP3 stimulated Ca^{2+} release from the sarcoplasmic reticulum and TXA2 receptor function. It also promotes ATPase dependent refilling of intraplatelet tubules with Ca^{2+}. (78) There is evidence of a synergism between the two nucleotides (126) with one explanation being cGMP ability to limit cAMP breakdown. (143)

Both PKA and PKG phosphorylate vasodilator-stimulated phosphoprotein (VASP) (144), which when in its dephosphorylated form affects profilin-actin dynamics, with upregulation of polymerisation and filament bundle formation. (145) Further, it is accepted, following work with VASP knockout mice, that phosphorylation of the VASP molecule results in inhibition of GPIIb/IIIa. (146)

cAMP and cGMP are terminally degraded to related monophosphates (AMP and GMP) by intracellular phosphodiesterase (PDE) isoenzymes. PDE3a, and lesser extent PDE2, preferentially hydrolysing cAMP, whereas PDE5 will direct its action specifically to cGMP

inactivation. (141) This had led to development of PDE inhibitors as therapeutic agents to potentiate the effects on platelets and vascular tone by PGI_2 and NO. PDE3 inhibitors milrinone and PDE5 inhibitors sildenafil and dipyridamole are in common clinical use with roles in heart failure (147), pulmonary hypertension (148) and stroke (149).

While the role of the endothelium in regulation of platelet function is critical, following injury and activation it acquires a contrary function as a platelet activator, which can be both a homeostatic and a pathological process. Following stimulation, intracellular Weibel-Palade bodies migrate and fuse to the plasma membrane releasing their contents, which includes vWF, P-selectin, angiopoietin-2 (150) and endothelin-1 (151) mediating platelet adhesion, leukocyte recruitment, inflammation and vasoconstriction. The injury to the endothelium itself leads to exposure of the circulating constituents to the subendothelial matrix rich in proteins that trigger platelet activation. At sites of recurrent injury, the repeated confluence of platelet activation, inflammation and constriction leads to development of an atherosclerotic plaque, which is a self-perpetuating scenario as previously discussed.

Protease activated receptors (PAR) are also expressed on the endothelial surface, primarily mediated by thrombin activation of PAR1, although tryptase/coagulation factor binding with endothelial PAR2 is also evident. Activation instigates chemokine release (platelet activating factor and interleukin 6 and 8) and upregulation of adhesion molecules leading to neutrophil and monocyte recruitment and platelet adhesion. PAR1 also permeabilises the endothelium and causes localised oedema. (152) The interplay between endothelium, leukocytes and platelets generates a plethora of positive feedback loops and subsequent amplification of the cascade.

1.4.3   Vasomotor Tone

The endothelium has a close relationship with vascular smooth muscle cells coordinating tonal response to multiple stimuli culminating in vasoconstriction and/or vasorelaxation.

As the name suggests the "endothelial vasodilators", PGI_2 and NO, have well documented roles in vasodilation. Changes in shear stress or paracrine mediators, such as acetylcholine or bradykinin, lead to endothelial cell generation of the endogenous vasodilators, which

are released into the subendothelial space to interact with vascular smooth muscle cells. (153) NO diffuses into the cells, while PGI_2 interacts with its specific receptor on vascular smooth muscle cells, instigating the accumulation of cGMP and cAMP, respectively. (154) This culminates in relaxation/vasodilation by 4 distinct pathways: (i) the reduction in intracellular Ca^{2+} via increased uptake into sarcoplasmic reticulum and cellular efflux; (ii) hyperpolarisation of cells via activation of K^+ and inactivation of Na^+ channels; (iii) contractile inactivation via decreased MLCK and increased myosin light chain phosphatase (MLCP) activity; and (iv) direct effects on actin/actomyosin following cyclic nucleotide induced heat shock protein phosphorylation. (155)

The endothelium also promotes vasoconstriction in response to stimuli, such as adrenaline, inflammation and hypoxia, resulting in the release of Endothelin-1. (156) Endothelin-2 and -3 isoforms have limited/no endothelial synthesis. (151) Endothelin-1 (ET-1) is produced by three steps from Prepro-ET-1 mRNA to conversion of Big ET-1 to ET-1 by endothelin converting enzyme 1 or ECE 2 in alkalotic conditions. (151) ET-1 proceeds to interact with receptors on endothelial cells (ET_{B2}) and vascular smooth muscle cells (ET_A and ET_{B1}) with polar consequences; the former promoting NO and PGI_2 release (see above) and the latter opening Ca^{2+} channels increasing intracellular concentrations leading to vasoconstriction. Throughout the vasculature the proportional expression of receptor types vary with higher abundance of ET_A compared to ET_B in the arterial vessels. (154) Beyond ET-1, thromboxane A2 also triggers vasoconstriction by accumulating intracellular Ca^{2+} via signalling pathways mirroring its intra-platelet effects (section 1.3.8.1). (157)

1.4.4 Barrier Function

The endothelium interacts with its surroundings by acting as a barrier, while enabling communication between the lumen and the extracellular matrix/smooth muscle cells. The transport of signalling molecules can occur transcellular or paracellular. The former involves endocytosis, transcytosis and subsequent exocytosis via caveolae, cholesterol rich tubes, structurally dominated by the protein caveolin-1, connecting cellular surfaces or present as cytoplasmic vesicles. These allow the passage of macromolecules such as insulin, lipids, hormones and principally albumin to control oncotic pressure. Encapsulation

of eNOS by caveolae is an important step in the regulation of NO production. (158) Paracellular passage occurs between cells and is limited to molecules less than 3nm including urea, water, amino acids and glucose. Endothelial cells are held together by adherens junctions (vascular endothelial cadherin – catenin complexes), tight junctions (claudins, occludin and junctional adhesion molecules) and gap junctions (connexons); with the latter having only a limited role in the microvascular endothelium compared to the others. These junctions can be opened by mediators such as platelet activating factor and VEGF (vascular endothelial growth factor); increasing permeability. (159)

1.4.5   Endothelium in Disease

The term 'endothelial dysfunction' refers to the inability of the endothelium to regulate vascular homeostasis, which results in promotion of vasoconstriction, proinflammatory and prothrombotic effects, predominately through reduction in bioavailability of NO. (154,160) Endothelial dysfunction is frequently described as the precursor to atherosclerosis and unsurprisingly close links exist between documented risk factors for both conditions, such as smoking and diabetes mellitus.

1.4.5.1   Smoking

Cigarette smoking is a well described major risk factor for atherosclerosis, of which the potentiation of chronic arterial wall inflammation and endothelial dysfunction are key underlying mechanisms. (161) The cigarette smoke triggers a cascade driven by free radicals, both endogenous in origin and direct constituents of the smoke. Resulting in increased cytokines release, inflammation and oxidative stress to create a pro-atherosclerotic environment. (162) Another suspected pathway is related to smokers elevated levels of thiocyanate, leading to formation of hypothiocyanous acid which denatures eNOS and results in reduction of NO. (161)

Contributory to the atherosclerotic process and the increased incidence of ACS in smokers is the prothrombotic state induced by inhalation of cigarette smoke. Platelets are more readily activated compared to non-smokers, which may in part be linked to NO availability.

(163) Furthermore, smokers have increased inflammatory mediators such as fibrinogen (162) and lymphocytes (164) enhancing thrombus formation. While muted levels of basal and stimulated tissue plasminogen activator are seen, preventing thrombus degradation. (162) This prothrombotic milieu provides rationale behind the greater risk of stent thrombosis for smokers in cases of ACS managed with PCI compared to non-smokers (adjusted HR 1.44 (95% CI 1.07-1.94), p=0.02). (165)

Standard medical therapy is recommended in this cohort in guidelines yet their response to antiplatelet medication varies from non-smokers. Aspirin appears to have limited effect on platelet aggregation in this prothrombotic group. (166) Therefore, interestingly smokers appear to have greater clinical benefit and reduction in platelet reactivity related to clopidogrel administration compared to their non-smoking counterparts. (167) This finding extends to prasugrel, another thienopyridine (168), but not ticagrelor. (165)

1.4.5.2   Diabetes Mellitus

Diabetes mellitus (DM) is a chronic metabolic disease characterised by persistent hyperglycaemia. DM is a spectrum of conditions predominately categorised into two main subgroups (type 1 and type 2) differentiated by underlying aetiology. Type 1 DM is secondary to absence of insulin production due to autoimmune destruction of pancreatic β cells. Type 2 DM is secondary to a combination of metabolic disorders leading to increased glucose production, impaired glucose uptake and reduced insulin secretion. In the UK 3.5 million patients have been diagnosed with DM, of which 90% are type 2. (169)

In DM, the sustained hyperglycaemia results in a well-documented increased risk of atherosclerosis and ACS secondary to effects on the endothelium, inflammation and platelets.

Endothelial cells have been proven to be dysfunctional in altered glucose states. Oxidative stress in hyperglycaemia plays an important role in reducing NO bioavailability by direct degradation secondary to elevated reactive oxygen species (ROS) levels and changes to the functional capacity of eNOS. (160)

The low concentrations of NO also impacts on platelet function (160) and patients with DM are known to have increased platelet reactivity. (170–172) The hyperglycaemic state negatively impacts on a number of pathways involved in platelet activation beyond NO by generating a proinflammatory and prothrombotic environment. Patients with DM have increased levels of inflammatory cytokines, fibrinogen, endothelin, thrombin and vWF contributing to this milieu. (173)

Platelet targeted medication is again the mainstay of ACS treatment, with an important variation seen in response to therapy for patients with DM. The up regulation of $P2Y_{12}$ and GP IIb/IIIa receptors in DM is of particular interest as both pharmacological targets in ACS. (174,175) Furthermore, elevated serum esterases seen in DM increases the metabolism of both aspirin (176) and clopidogrel (174) reducing efficacy. Additional reduction in response to clopidogrel has been noted to be multifactorial (177,178), but not apparent with prasugrel or ticagrelor. (179)

The impact of DM on platelet function, endothelial function and $P2Y_{12}$ receptor antagonist therapy is expanded further in chapters four and seven.

1.4.6   Endothelial Function Testing

Given the knowledge that cardiovascular endothelial dysfunction is of such clinical importance, quantification of functional status has been sought keenly in human populations. It was first described in atherosclerotic patients in 1986 by Ludmer et al, with direct coronary angiographic assessment of left anterior descending artery luminal diameter response to infusions of acetylcholine. (180) Intravascular measurements are still performed by comparison of epicardial luminal diameter before and after infusions of vasoactive substances (e.g. acetylcholine); but microvascular function can also be judged using a flow wire technology. Placing a standard angioplasty wire containing ultrasound doppler technology with in the coronary vessel can enable direct measurement of phasic coronary flow. (181)

It is these invasive measures that remain gold standard (182), however, there has been increasing acceptance that non-invasive techniques have a role in research, using peripheral arteries as a surrogate marker. Their obvious advantage is the relative ease and low risk nature of testing. This led to advances in development and experimental validation has built up confidence. The most commonly used methods are flow mediated dilatation (FMD) (183) and EndoPAT (184), which measure macro and microvascular endothelial function respectively and have both been shown to correlate with their coronary vascular equivalents. (183,184) Both protocols utilise endothelial response to reactive hyperaemia secondary to arterial occlusion by a sphygmomanometer inflated above systolic blood pressure. The ischaemia should prompt release of multiple vasodilators including PGI_2 and NO, causing vessel dilation and reactive hyperaemia. FMD delivers a method of measuring the dilation response, but also has the ability to measure flow and has been robustly tested since its first use in 1992. EndoPAT primarily provides information on reactive hyperaemia and is a newer technology (2003) therefore does not have the same level of supporting literature. Although it has a notable benefit over FMD in its ease of use and independence of operator. (185) Both non-invasive measurements have been shown to independently predict cardiovascular events and all-cause mortality (RR 0.90 (95% CI 0.88-0.92) per 1% FMD increase and RR 0.85 (95% CI 0.78-0.93) per 0.1 increase in EndoPAT calculated reactive hyperaemic index). (186)

1.5   ACUTE CORONARY SYNDROMES, PLATELETS AND THE ENDOTHELIUM

Endothelial dysfunction is an independent predictor of adverse outcomes in ACS (187), and is established in the initiation and progression of atherosclerosis (156), a process which is the stable precursor to the majority of ACS presentations. *De novo* lesions are typically an angiographically mild stenosis on previous imaging; although associated with thin caps, high plaque burden and small luminal area (20); suggesting the "vulnerability" of the plaque is more important than the severity of the underlying lesion in causing ACS. Their instability is secondary to increased levels of inflammation and necrosis, and this is promoted in endothelial dysfunction due to the amplified levels of oxidative stress and down regulation of NO. (188)

Overall, endothelial disease prevents routine response to external triggers and leads to increased permeability, allowing lipid, inflammatory cell and cytokine passage into the vessel wall architecture. To magnify this prothrombotic state further, the dysfunctional endothelium is incapable of generating its endogenous antiplatelet agents (PGI_2/NO) (189) leading to platelet aggregation and vasoconstriction. The latter is exacerbated by the damaged endothelium having a reduced quantity of ET_{B2} receptors, enabling uncontrolled vasoconstriction generated from ET-1. (154) The increasing vascular shear stress associated with vasoconstriction is a stimulus for platelet activation. (108) This demonstrates the close interplay between the platelet and endothelium in this diseased state, perpetuating a constant positive feedback between vasoconstriction and aggregation creating a climate where disease begets disease. In keeping with this philosophy, patients with systemic conditions suffer a multitude of related pathologies to create a toxic milieu; such as patients with diabetes mellitus who have increased endothelial dysfunction (160), exaggerated platelet activation (170,171) and worse clinical outcomes in ACS. (190)

Cardiac endothelial dysfunction is present but not limited to the epicardial arteries and the microvasculature is equally at risk. Beyond chronic disease leading to symptomatic stable angina (191), acute endothelial damage has been demonstrated, and is instrumental in the development of a condition called "no reflow" phenomenon, which is seen almost exclusively in ACS. (Section 1.6) Interestingly, peripheral endothelial dysfunction does not appear to correlate with incidence. (192) It results in reperfusion injury of the myocardium in the absence of epicardial obstruction. This is due to damage of the endothelium and

subsequent relationship with inflammatory cells/mediators, free radicals and vasoconstrictors. (193) This conveys a significantly worse clinical outcome for this cohort. (194)

Current clinical guidelines formulate a standard pharmacological approach for patients presenting with ACS. (3,4) A number of these agents have been shown to not just improve clinical outcomes, but also endothelial function. It is known in high risk patients that angiotensin converting enzyme inhibitors (ACEi) reduce MACE (195), and also improve endothelial function. (196) Lipid lowering statin medication has been a well-established therapy in ACS, and reduce clinical events independent of cholesterol levels (197) with intensive dosing noted to be most beneficial. (38) Moreover, statins moreover positively enhance endothelium function. (198) Although it should be stated no data has shown this pharmaceutical optimisation of the endothelium independently impacts on clinical outcomes. Some medications not routinely used post ACS, but commonly used in the management of angina can also improve endothelial function including calcium channel antagonists (196). Yet, interestingly chronic use of non-organic nitrates, such as isosorbide mononitrate, has been shown to paradoxically worsen endothelial function (199), which contradicts the findings when dietary nitrates are administered. (200)

The close relationship between platelets and the endothelium is a key focus for research into potential therapeutics and understanding of established medications. In ACS, along with aspirin, $P2Y_{12}$ receptor inhibitors are a mainstay of antiplatelet treatment and interestingly *in vitro* studies suggest that restraining $P2Y_{12}$ signalling is associated with a paradoxical increase in platelet sensitivity to endothelial vasodilators (PGI_2 and NO) (139,201); which has translated to *ex vivo* data. (202) However, the clinical importance of these observations in patients is unclear.

1.6   PROJECT AIMS

ACS are a major cause of morbidity and mortality in the western world, supporting the need for research to advance understanding and improve population outcomes. As described, the endothelium and circulating platelets are integral to the pathophysiology of the condition, and this project intends to explore further these associations in patient cohorts. The focus will be on the interplay between platelet $P2Y_{12}$ antagonism and the endothelium in patients with CAD and ACS. (Figure 1.12) This will be achieved by:

1. Demonstrating synergism between $P2Y_{12}$ antagonism with ticagrelor and endogenous endothelial vasodilators on platelet inhibition using flow cytometry;

2. Investigating the effect of $P2Y_{12}$ inhibition with ticagrelor on endothelial function using EndoPAT;

3. Demonstrating that these effects persist in patients with CAD;

4. Assess the interaction between $P2Y_{12}$ inhibition, platelets and inflammation;

5. Review the endothelial condition of no reflow phenomenon seen in ACS, focusing on incidence, outcomes and management;

6. Explore independent predictors of no reflow phenomenon in patients with ST-elevation myocardial infarction;

7. Perform a systematic review and meta-analysis of the optimal $P2Y_{12}$ antagonist in patients with ACS and diabetes mellitus, a population at significant risk of endothelial dysfunction.
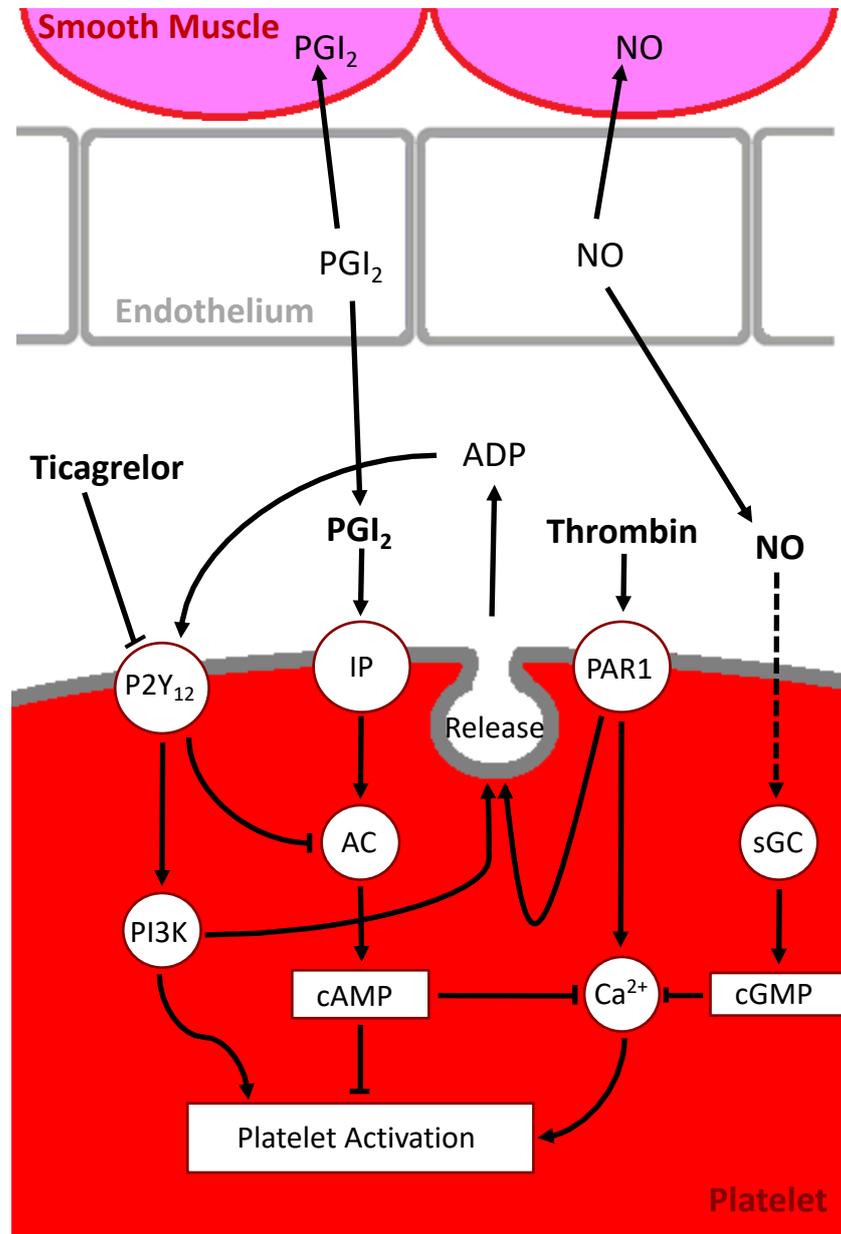
Figure 1. 12: Diagram of interaction and signalling pathways involving ticagrelor, platelets and endothelial vasodilators

2   CHAPTER TWO: PRINCIPLES OF THE METHODS

Below provides an overview of materials and methods, with specific protocols extensively detailed in the methods section of the relevant chapters.

2.1   MATERIALS: CHEMICALS, ANTIBODIES AND REAGENTS

S-nitrosoglutathione (GSNO) was purchased from Santa Cruz (Wembley, UK), while Cayman chemical (Michigan, USA) supplied Prostaglandin I_2 (PGI$_2$) and BD FACS Lysing Solution X10 was from BD Bioscience (Oxford, UK). Ethylenediaminetetraacetic acid (EDTA) was obtained from Calbiochem (Nottingham, UK). The platelet activator protease-activated receptor (PAR)1 agonist (SFLLRN) and PAR4 receptor agonist (AYPGKF) were from Cambridge Bioscience (Cambridge, UK). The P2Y receptor antagonists ticagrelor (P2Y$_{12}$) and adenosine-3'-phosphate-5'-phosphosulphate (A3P5P) (P2Y$_1$) were provided by AstraZeneca (Mölndal, Sweden) and Sigma-Aldrich (Poole, UK) respectively. Nigericin was also acquired from Sigma-Aldrich (Poole, UK).

Phycoerythrin (PE) conjugated mouse anti-human CD45 antibody, PE conjugated mouse anti-human CD14 antibody, PE mouse anti-human CD62P, PE mouse IgG1 (κ Isotype Control), fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD42a antibody, FITC mouse anti-human CD42b, FITC mouse IgG1 (κ Isotype Control) was acquired from BD Bioscience (Oxford, UK) and FITC rabbit anti-human fibrinogen antibody from Dako (Ely, UK).

Agents for phosphoflow included BD Phosphoflow Lyse/Fix Buffer (Oxford, UK). Pacific Blue NHS Ester, Alexa Fluor 488 NHS Ester and goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody (Alexa Fluor 647) were acquired from ThermoFisher Scientific (Cambridge, UK). PhosphoVASP (Ser157 and Ser239) antibodies were purchased from cell signalling technology (Danvers, USA). Paraformaldehyde (PFA) solution was from Electron Microscopy Sciences (Hatfield, USA).

R&D systems provided the Quantikine HS Human IL-1β Immunoassay (Minneapolis, USA) and the Fluorescent Labeled Inhibitors of Caspases (FLICA) assay was from ImmunoChemistry Technologies (Bloomington, USA). The different IL-1β antibodies were

acquired from ThermoFisher Scientific (Cambridge, UK) (unconjugated polyclonal antibody) and BioLegend (Cambridge, UK) (Alexa Fluor 647 conjugated anti-human IL-1B antibody).

2.2 METHODOLOGY OF BLOOD PREPARATION

Blood samples were taken from healthy donors and study participants, with independent protocols used to attain whole blood, platelet rich plasma (PRP) and isolated platelets.

2.2.1 Preparation of Whole Blood

The sample was drawn from the median cubital vein using a 21-gauge butterfly needle into a syringe. The initial 3mls aspirated was discarded to exclude venepuncture related platelet activation and then the required sample was collected into a second syringe containing the anticoagulant tri-sodium citrate (109mM) 9:1 (v/v), followed by gentle mixing. The blood was then ready for immediate whole blood testing.

2.2.2 Preparation of Platelet Rich Plasma

For this study PRP was obtained by centrifuging whole blood in a small volume falcon tube for 6 minutes ($200g$, 20°C). The resultant supernatant PRP layer was aspirated and placed in a PVC reagent reservoir for immediate processing.

2.2.3 Preparation of Isolated Platelets

Platelets were isolated from whole blood using acidic conditions to ensure platelets were quiescent during centrifugation. (203) Venous blood was collected as previously described and gently mixed with the anticoagulant acid-citrate dextrose (ACD; 29.9mM sodium citrate, 113.8mM glucose, 72.6mM NaCl, 2.9mM citric acid, pH 6.5) 4:1 (v/v). The sample was centrifuged in a large falcon tube for 15 minutes ($200g$, 20°C). The resulting PRP was transferred, using wide bore Pasteur pipettes, into small volume falcon tubes. To maintain

the acidic conditions 0.5mM citric acid is added prior to further centrifugation for 12 minutes (800g, 20°C). Following centrifugation, the resultant platelet-poor plasma was discarded. The platelet pellet was gently resuspended in 4ml of wash buffer (36mM citric acid, 10mM EDTA, 5mM glucose, 5mM KCl, and 9mM NaCl), and then centrifuged again for 12 minutes (800g, 20°C). The process was repeated before resuspending the remaining platelets in 1ml of modified Tyrodes (150mM NaCl, 5mM HEPES, 0.55mM NaH_2PO_4, 7mM $NaHCO_3$, 2.7mM KCl, 0.5mM $MgCl_2$, 5.6mM D-glucose, pH 7.4) in a single tube for counting and allowed to warm to 37°C prior to experimental use.

2.2.4   Platelet Counting

A Beckman Coulter Z1 Particle Counter (Beckman Coulter Life Sciences) was used to measure platelet numbers. The counter detects voltage pulses generated by the electrical resistance of a platelet as it passes through an aperture and records the number of events in a specific volume. (Figure 2.1) An automated reading was displayed equating to number of platelets per millilitre. Washed platelets (5µl) were suspended in a Coulter Accuvette containing Beckman Coulter Isotone II diluent (10ml), and an average of three readings was taken. Appropriate dilution was then performed for standardisation of platelet counts across experiments/donors.

2.3   METHODOLOGY USED FOR EXAMINATION OF PLATELET FUNCTION

2.3.1   Flow cytometry

Flow cytometry allows rapid identification and analysis of a single cells within complex mixtures such as whole blood samples. (204) The method uses unique validated patterns of light scatter generated by a cell as it passes through a laser beam enabling isolation of morphologically distinct populations and precise data collection. (Figure 2.2)
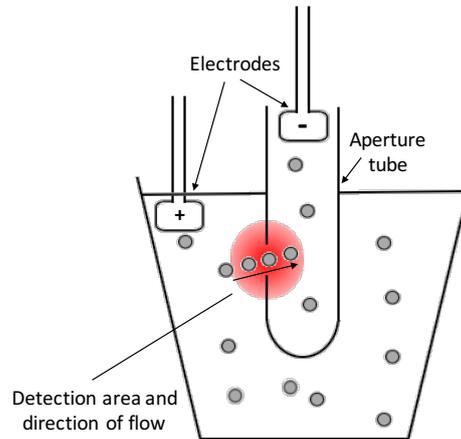
Figure 2. 1: Principle of the Coulter counter

Two electrodes generate a current in the conductive fluid which flows through the predetermined aperture. The impendence is measured in the detection area, which alters as non-conducting cells pass through, displacing fluid, thus allowing their number and volume to be measured.
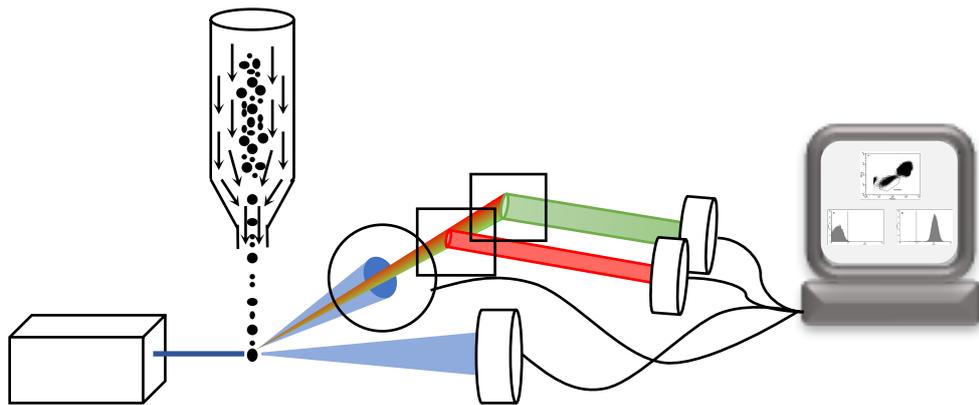
Figure 2. 2: Principles of flow cytometry

Fluidics involves the focusing of the fluid stream into a single cell file. Optics relates to the laser interaction with the cell. Blue light represents forward scatter and side scatter collected by scatter detectors. Fluorochrome emitted light (red and green) is recorded by collection optics. Electronics is the final stage where the information is converted into a digital output.

The prepared sample is injected into a fluid stream where hydrodynamic focusing creates a central sample core, secondary to the principles of laminar flow, presenting the resultant single file of cells at a fixed pressure to the cytometer's lasers. Divergence of the emitted beam on interaction with the cell is collected as forward scatter (FSC) and is directly proportional to the cellular size. Reflected or refracted light waves are detected as side scatter (SSC) and are proportional to the cell's internal complexity or granularity. The FSC and SSC pattern for each cell is akin to a finger print and allows specific populations to be isolated.

Fluorescence labelled antibodies are routinely incubated with the samples prior to analysis to confirm and quantify the presence of related antigens within the isolated cellular population. The cytometer's laser excitation energy is absorbed by the fluorochrome associated with the primary antibody and emitted, which is then detected by the collection optics. The intensity of the detected fluorescence is proportional to the number of fluorochromes, hence directly corresponds to the amount of antigen expressed.

There are numerous fluorochromes available which have variable maximal excitation wavelengths and modern flow cytometers have multiple lasers producing light across the spectrum. The fluorochrome absorbs the laser's light, taking its electrons from a resting to an excited state. This leads to conformational change into a stable form, losing energy as heat. The remaining energy is then emitted as fluorescence at a longer wavelength returning the fluorochrome to a resting/reusable state. The emitted wavelength is also specific and predictable for that fluorochrome. Multiple lasers and differing wavelength excitation/emission by fluorochromes allows the collection of distinct data and enables complex analysis of several antigens in different cell populations in a single sample, demonstrating the power of flow cytometry.

2.3.1.1   Materials

Experiments were performed on a FACS (Fluorescence Activated Cell Sorting) Calibur and a FACS LSRFortessa flow cytometers from BD Bioscience (Oxford, UK). Initial analysis was performed using the relevant software for each machine, BD Cellquest and BD FACSDiva, respectively. Further evaluation was done using FlowJo vX.0.7 software.

2.3.2   Light Transmission Aggregometry

Light transmission aggregometry (LTA) is the gold standard technique used to measure platelet aggregation. (205) The principles rely on quantification of transmission of light directed through a platelet suspension compared to a platelet-poor plasma sample, with the presumption that stirred resting platelets will be uniformly distributed throughout the sample obscuring the light (0% aggregation) and activated platelets will form large "clumped" aggregates enabling a significant increase in light transmission.

A classical recording following activation will show a sudden temporary reduction in light transmission secondary to platelet shape change, followed by a time-dependent progressive increase in transmission. (Figure 2.3) This occurs in two phases, firstly there is a primary phase which is reversible followed by a secondary irreversible phase. This provides a percentage of aggregation seen at a predetermined time course, in variable experimental conditions. Aggregation traces were recorded using the Chrono-Log platelet aggregometer and appropriate software (Aggro Link (Havertown, USA)).
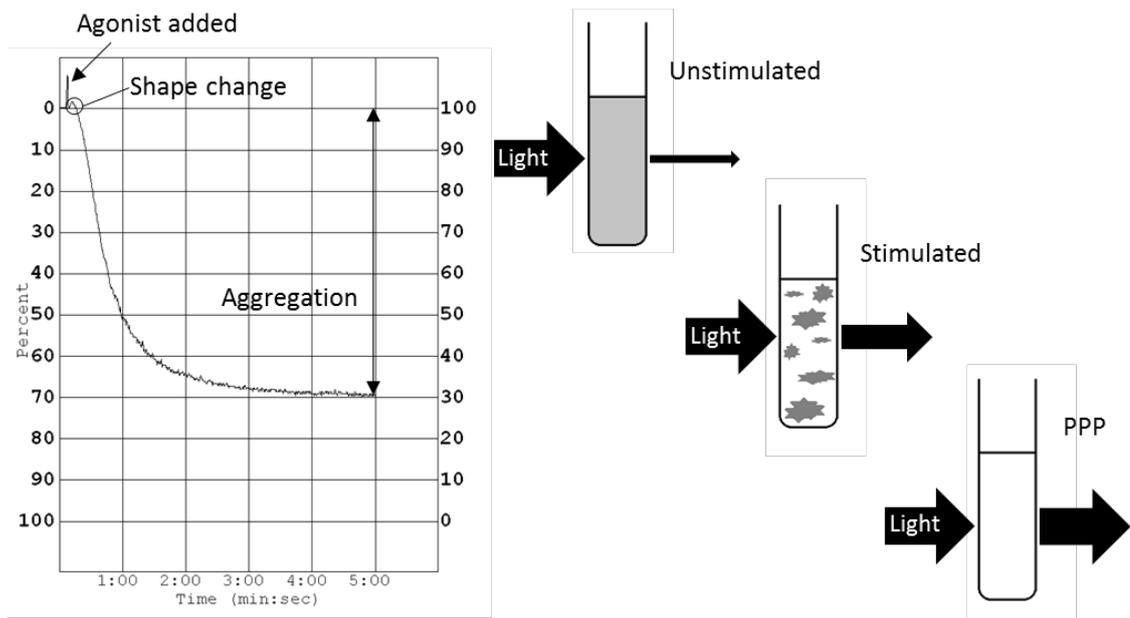
***Figure 2. 3: Principles of* Light Transmission Aggregometry**

Standard LTA curve demonstrating platelet aggregation representing the light detection when compared to platelet poor plasma sample.

2.4   ENDOTHELIAL ASSESSMENT

EndoPAT is a measure of microvascular endothelial function by recording the pulse wave amplitude, representing pulse volume, in the capillary beds of both index fingers of the participant. (Figure 2.4) This is measured by transducers in finger probes, with latex cuffs inflated to a pressure relative to baseline diastolic blood pressure to prevent venous pooling. The principle involves comparing the amplitudes before and after brachial artery occlusion of one (non-dominant) arm. In subjects with normal endothelial function release of the occlusion induces a hyperaemic response, and the software can analyse the magnitude of this against the control (non-occluded, dominant arm) to generate a result known as the reactive hyperaemic index (RHI). RHI is a known independent risk factor for cardiovascular events. (206)

2.4.1   Materials

Endothelial function was assessed with the EndoPAT 2000© using previously validated methodology (207,208), acquired from Itamar medical ltd (Caesarea, Israel). Itamar also supplied the disposable pneumatic endo probes and computer software.

Reactive Hyperaemic Index = (A/B) / (C/D)
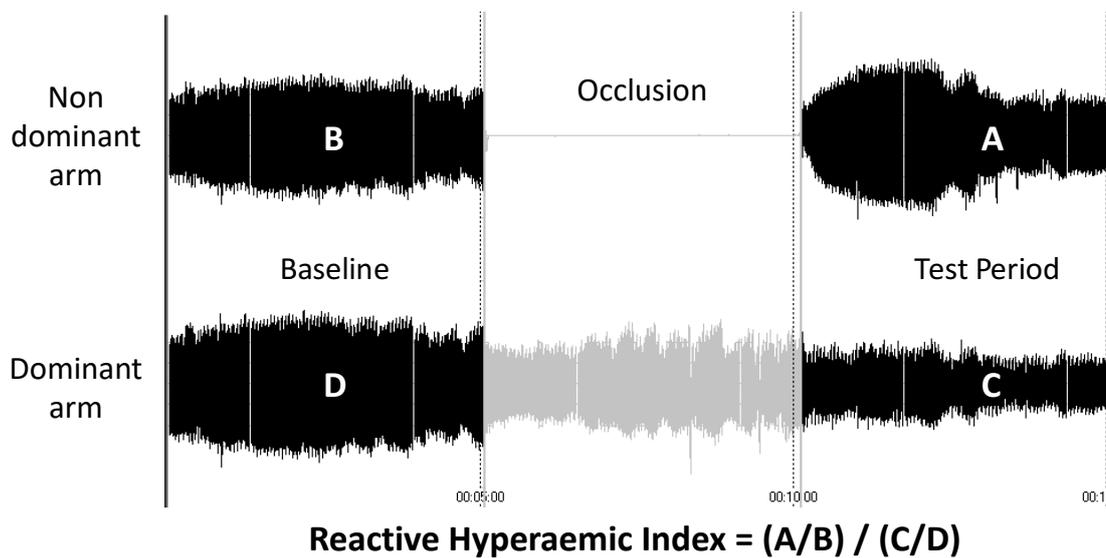
Figure 2. 4: Principles of EndoPAT

This is a normal recording from a non-dominant occlusive arm and a dominant control arm. This demonstrates a clean initial recording, satisfactory occlusion (grey segment) and evidence of increased wave amplitude in occluded arm post release (hyperaemia).

3   CHAPTER THREE: *IN VITRO* EXPERIMENTS

3.1   INTRODUCTION

Ticagrelor is an orally administered non-thienopyridine potent inhibitor of ADP-mediated platelet activation, acting as a non-competitive $P2Y_{12}$ receptor antagonist. In addition, ticagrelor impedes adenosine reuptake by ENT 1 inhibition [49], leading to further platelet function inhibition, inflammatory regulation and vasodilatation. [50] Clinically, it is the mainstay of treatment of ACS, recommended by National Institute for Health and Care Excellence (NICE) to be given in combination with aspirin for a minimum of 12 months. [63]

Intravascularly platelets are constantly modulated by endothelial derived vasodilators PGI_2 and NO. The haemodynamic marginalisation of platelets to the periphery of blood vessels ensures that they are "bathed" in these potent inhibitors during circulation. PGI_2 initiates a signalling cascade following binding to its G-coupled membrane receptor resulting in activation of PKA mediated by cAMP formation. [141] NO diffuses into the platelet and binds to intracellular sGC, resulting in the synthesis of the second messenger cGMP whose elevated concentrations in turn activate PKG. [37] PKG along with PKA co-ordinates the most potent endogenous platelet-inhibitory intracellular signalling pathways, including driving phosphorylation of VASP. These pathways cause broad inhibition of all aspects of platelet function and therefore form an interdependent and co-ordinated system for "global" modulation of platelet function. [141]

Ticagrelor and these endogenous platelet inhibitors share common roles in platelet inhibition and vasodilation. Observations that $P2Y_{12}$ inhibition can significantly enhance platelet sensitivity to NO and PGI_2, suggested additional mechanisms of action to those previously understood. [139,201] The aim of this chapter is to explore the association between ticagrelor and the endogenous endothelial vasodilators.

3.1.1 Objectives of this chapter

- Investigate the relationship between platelets and:
  - PAR receptor agonists;
  - NO;
  - PGI_2;
  - Ticagrelor;
  - Endothelial vasodilators and ticagrelor.

- To prepare for a clinical study:
  - Ensure evidence to proceed with a patient study;
  - Optimise experimental conditions.

3.2 METHODS

3.2.1 Ethics

Ethical approval has been granted by the Postgraduate Medical Institute (Hull York Medical School, Hull, UK).

3.2.2 Volunteers

Blood was donated following an informed consent by adult healthy volunteers. It was specified that no medication likely to interact with platelet function (aspirin/ibuprofen) was to have been taken in the preceding two weeks.

3.2.3 Methodology of blood preparation

Whole blood, platelet rich plasma (PRP) and isolated platelets were prepared as per previously outlined. (Section 2.2)

3.2.4 Methodology of platelet function testing

3.2.4.1 Flow cytometry

3.2.4.1.1 Sample preparation

The samples were prepared for flow cytometric analysis by well-established methods. (209,210) Each analysis had appropriate control samples with whole blood or PRP (five microlitres (5μl)) added to tubes containing concentration matched negative control fluorescence or platelet marker (5μl) antibodies and filtered modified Tyrode's buffer to allow a final sample volume of 50μl. In the absence of a reliable isotype control for fibrinogen, non-specific binding was measured in duplicate using standard fibrinogen antibody in the presence of EDTA (6mM). The role of ETDA was as an integrin inhibitor thus

preventing fibrinogen binding to $\alpha_{IIb}\beta_3$. Samples were then fixed with 0.2% formyl saline (450µl) and a minimum of ten minutes was mandated prior to analysis.

Experimental samples were prepared with single marker/fluorescence (5µl). The antibody was combined with adjusted volumes of modified Tyrode's buffer to ensure that the final sample volume was always 50µl. Whole blood or PRP (5µl) was added to the preparation and incubated with antagonists or agonists or combination as required per protocol. The fixed volume of the active substances was 5µl, so the concentration added was x10 the desired final concentration.

3.2.4.1.2   Analysis of samples

Samples were analysed using a BD FACS Calibur or BD FACS LSRFortessa (BD Bioscience). Prior to initial experiments; instrument settings for voltage, amplitude and mode were optimised using isotype controls. No compensation adjustment was required for single fluorescence measurements per sample.

The platelet population was initially identified using FSC and SSC; and confirmed using FITC-CD42b antibody. The gate was adjusted to attain ≥ 95% positive cells as compared to FITC-IgG1 control. (Figure 3.1) On each occasion background fluorescence was established with FITC and PE controls set at a threshold of 2% positive. All samples had 10,000 platelet events recorded with both percentage positive cells and median fluorescence intensity (MFI) quantified beyond the 2% threshold. The overall platelet mean fluorescence intensity was recorded. Analysis was performed using BD CellQuest Pro (FACS Calibur) or BD FACSDiva (FACS Fortessa) software. The percentage positive cells and the mean fluorescence intensity were used to calculate the total binding index (percentage positive cells x mean fluorescence intensity x 0.01), a validated quantification of the data. (211)
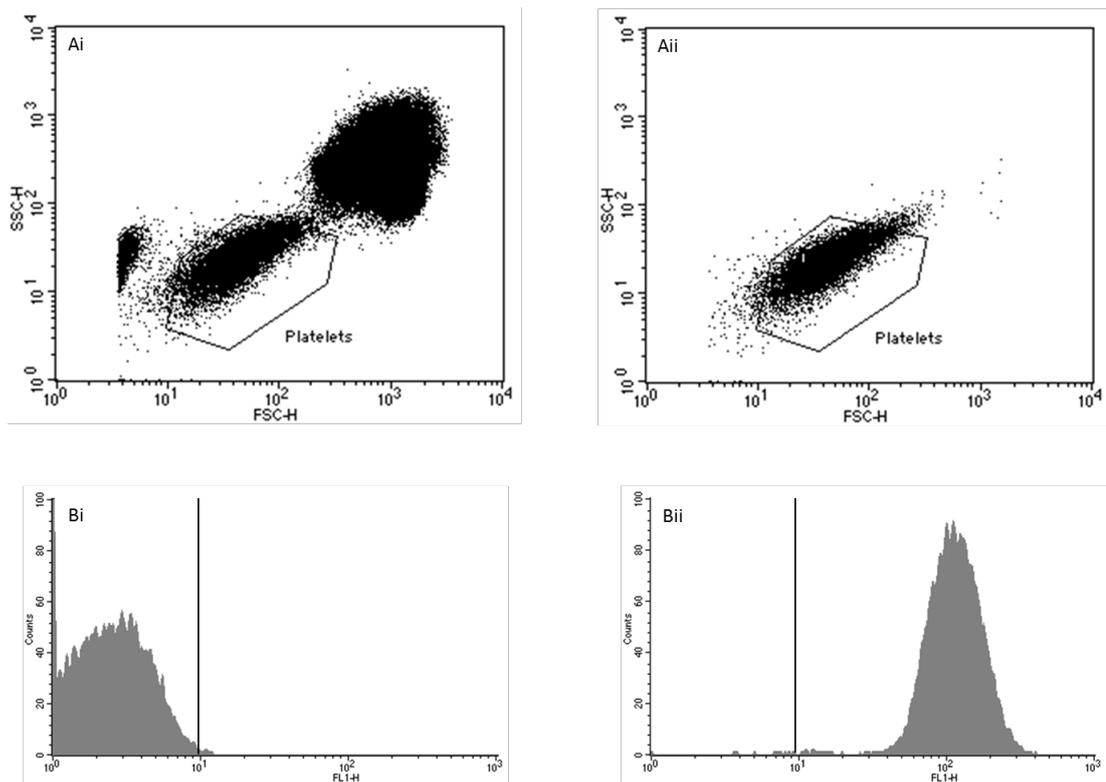
Figure 3. 1: Identification of the platelet population in whole blood and PRP

Platelet specific antibody CD42b bound to FITC (5µl) and its relevant isotype control (IgG-FITC, 5µl) was added to separate whole blood (Ai) and PRP (Aii) samples and analysed by flow cytometry. The platelet population was initially isolated based on the distinctive forward and side scatter (A). The histogram, representing data from 10,000 events within the outlined "platelet" population, demonstrates the background FITC fluorescence with isotype (Bi, IgG-FITC) and the rightward shift in the presence of a platelet unique marker (Bii, CD42b-FITC) confirming isolation of platelets (>95% cells positive).

3.2.4.2   Light Transmission Aggregometry

Washed platelets ($2.5x10^8$ platelets/ml) were stirred at 1000rpm for 1 minute at $37^{o}C$ for temperature equilibration. Samples were then monitored for 5 minutes with continuous stirring using a multichannel Chronolog aggregometer. In each case platelet poor plasma was used for calibration. The washed platelets were incubated for 5 minutes with ticagrelor ($5\mu M$) or vehicle control (dimethyl sulfoxide; DMSO, 0.5%). In some cases, PGI_2 or GSNO were added after 3 minutes. After 5 minutes the PAR1 peptide agonist ($10\mu M$) was added and aggregation was recorded for 5 minutes. Percentage aggregation was logged from this recording.

3.2.5   Methodology for Platelet-Leukocyte Aggregate Analysis

3.2.5.1   Sample preparation

For this assay platelets were identified using CD42a, and leukocytes identified using CD45. Whole blood ($30\mu l$) was stained with CD42a-FITC and/or CD45-PE ($5\mu l$) prior to the addition of platelet agonist or incubation with platelet inhibitors ($5\mu l$). Filtered modified Tyrode's was used to create the final working volume of $50\mu l$. Control tubes were stained with matched negative control fluorescently conjugated antibodies. Samples were fixed and lysed with diluted FACS lysis solution X10 (1:9 filtered dH_2O) ($450\mu l$) for 5 minutes. Lysis prevented erythrocyte contamination of the cytometric data, and a minimum period of 10 minutes was mandated prior to analysis to ensure effective lysis was complete.

3.2.5.2   Flow cytometric analysis of samples

As performed previously, the samples were analysed on BD FACS Calibur (BD Bioscience), and settings were optimised prior to the first experiments. As dual fluorescence was required for cell labelling, appropriate compensation values were manually calculated (figure 3.2) and fixed for all subsequent analyses.
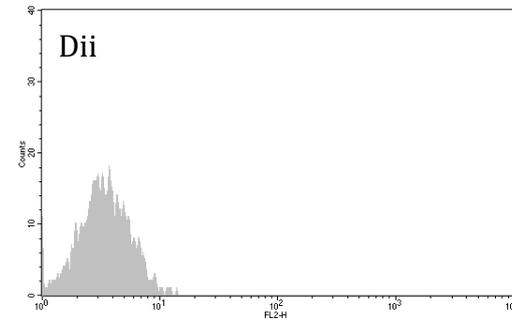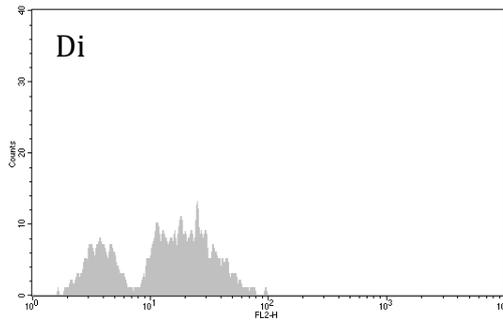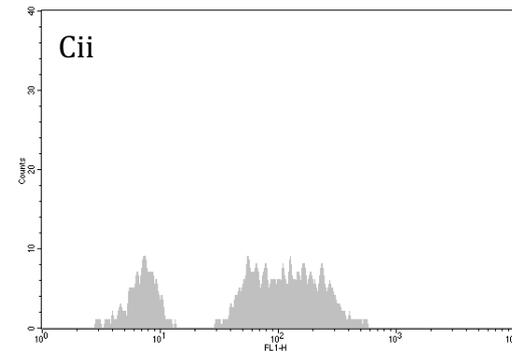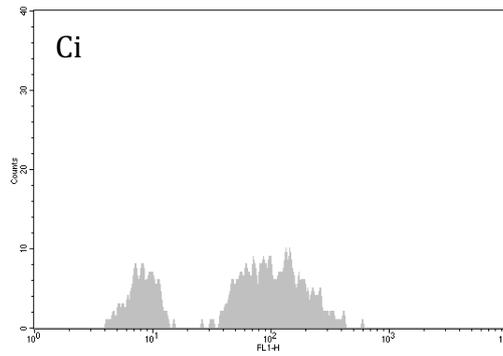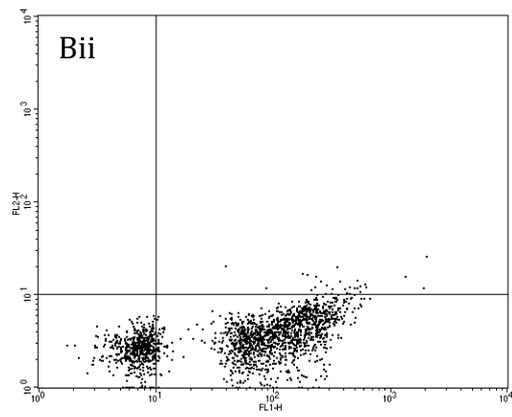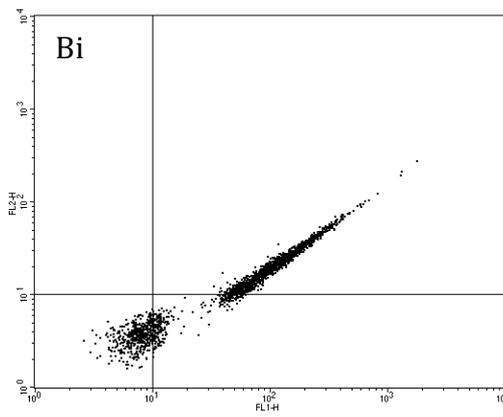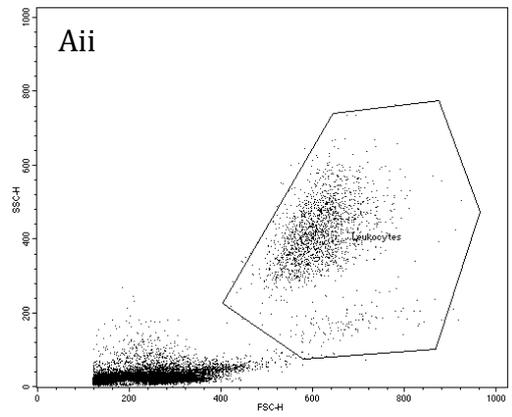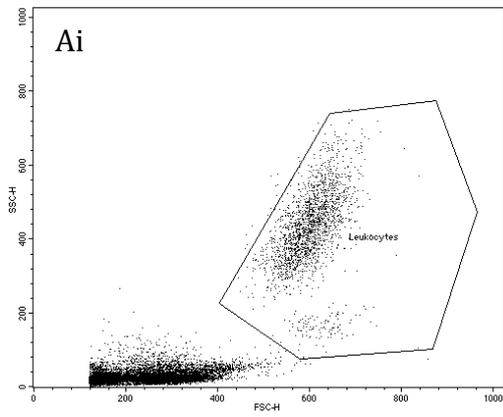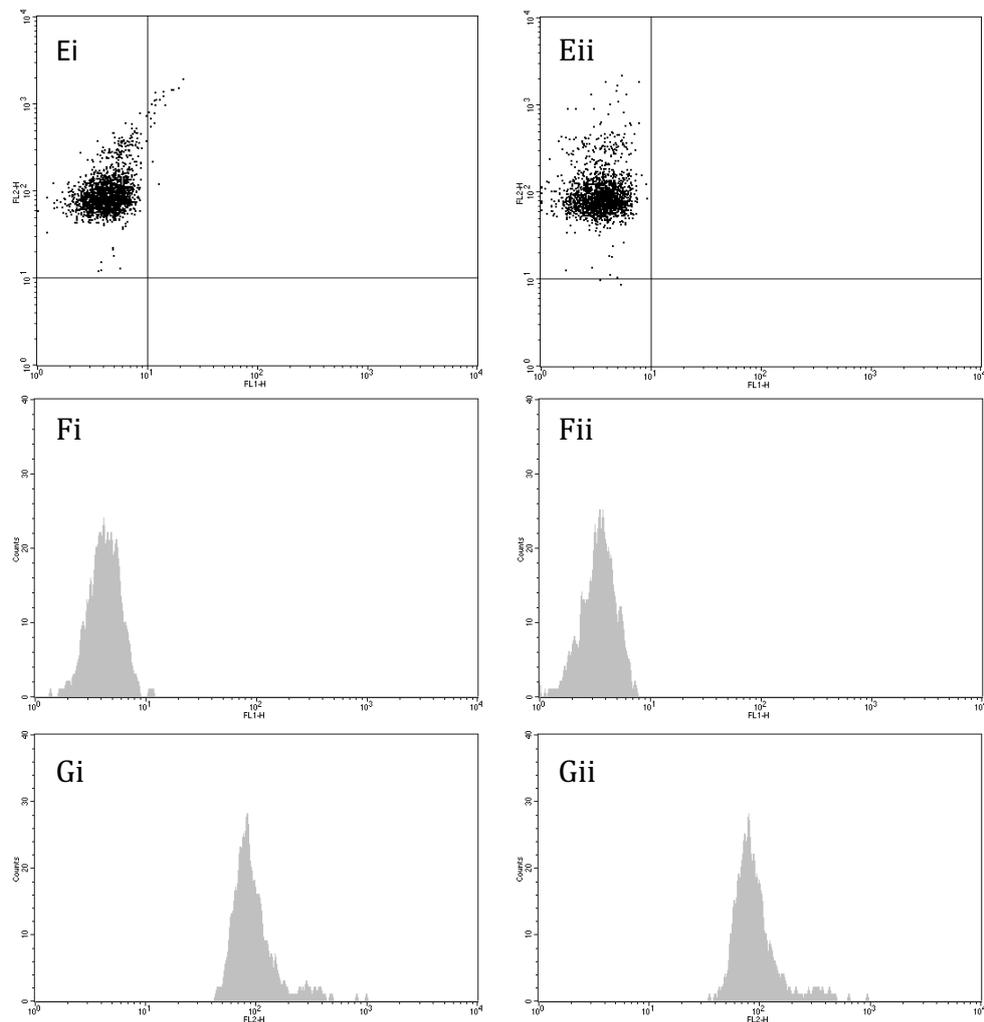
Figure 3. 2: Fluorescence compensation in flow cytometry

Whole blood (30μl) was combined with CD42a-FITC antibody and activated or CD45-PE. Samples were run on the flow cytometry multiple times with compensation adjustment performed before confirming optimisation. The results demonstrate isolation of the granulocyte and monocyte population basally (Ai) and following activation with PAR1 peptide agonist (Aii). Flow cytometry dot plots (B and E) and histograms (C, D, F and G) compare pre (i) and post (ii) application of compensation settings (FL1-0.8% FL2 and FL2-12% FL1). Prior to compensation there is marked evidence of FITC (FL1) contamination of FL2 demonstrated by fully activated (PAR1 10μM) whole blood sample labelled with CD42a-FITC (B-D) but following adjustment FITC no longer bleeds into the FL2 range (B-D). The crossover of PE into FL1 was less apparent, therefore minor modifications were required, with samples containing PE labelled CD45 used to confirm (E-G). Final images verify the absence of spectral overlap in FL2 for FITC and FL1 for PE.

Background fluorescence was established with FITC and PE controls set at a threshold of 2% positive. The leukocyte (granulocyte and monocyte) population was identified using SSC/FSC scatter plots and isolated using PE conjugated CD45 antibody specific for isoforms of the leukocyte common antigen (protein tyrosine phosphatase receptor type C (PTPRC)). The gate was adjusted to attain ≥ 98% positive cells as compared to PE conjugated IgG1. All samples had 2000 leukocyte events recorded; and the percentage of cells positive for both presence of the leukocyte label (CD45) and platelet label (CD42a) were recorded using FlowJo vX.0.7 software, as this was considered evidence of the presence of a platelet-leukocyte aggregate.

3.2.6   Statistical analysis

Data was analysed using GraphPad Prism Version 6.0c. and expressed as mean ± SEM, unless otherwise stated. Summary results (IC50, EC50) were obtained by fitting of data to a logistic equation. Direct statistical comparisons were carried out using Student's t-test (two groups) or one-way ANOVA (>2 groups). Overall effect was explored by two-way ANOVA, with additional post-test Bonferroni method multiple comparison. P values of <0.05 was considered to indicate statistical significance.

3.3   RESULTS

3.3.1   Platelet Activation

In the first instance whole blood assays were tested for the binding of fibrinogen and P-selectin associated expression to platelets. Initial experiments demonstrated that increasing concentrations of PAR1 and PAR4 peptide agonists resulted in a higher number of platelets expressing markers of activation ($p < 0.001$) (EC_{50} PAR1 1µM (1.06µM P-selectin expression and 1.02µM fibrinogen binding) and PAR4 30µM (30.1µM P-selectin expression and 30.3µM fibrinogen binding) (figure 3.3) and a greater quantity of the marker expressed by each of those activated platelets. (Figure 3.4)

3.3.1.1   Optimisation of flow cytometry protocol

Given that the final aim was to examine platelet function in patient samples, it was important to fully optimise the assay. In the first instance the concentration of activation marker antibody was examined. Two concentrations of antibody (2µl and 5µl) were used for each antigen. In both cases, increasing the concentration of antibody did not signficantly change the percentage of platelets expressing P-selectin or fibrinogen binding. However, with the higher concentration, the MFI was markedly greater (figure 3.5) and consequently 5µl was used for the rest of the experiments, and subsequent fluorescence controls were concentration adjusted.

The importance of incubation of the sample with fluorescence conjugated antibodies prior to addition of any agonist/antagonist was assessed due to practical requirement of pre-preparation of sample tubes in future experiments. Comparison of no incubation and a five minute incubation time prior to the addition of PAR1 peptide agonist demonstrated no difference between percentage positive cells or MFI. (Figure 3.6)
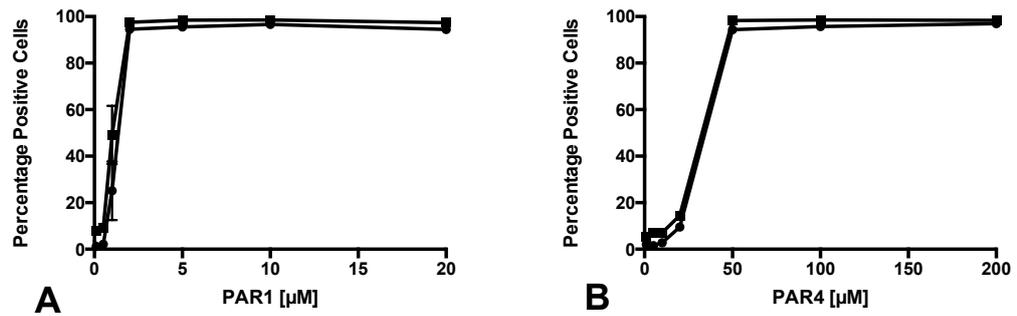
Figure 3. 3: Concentration response to PAR agonists

Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-20μM) (A, n=3) and PAR4 agonist (0-200μM) (B, n=2). Results demonstrating the percentage of platelets expressing markers of activation (P-selectin (circle ●) and fibrinogen binding (square ■) in response to incubation with the agonists. Error bars represent standard error of the mean.
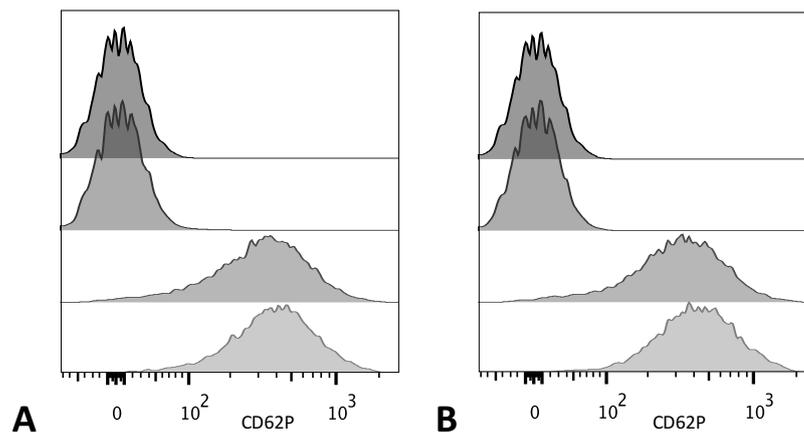
Figure 3. 4: Concentration response to PAR agonists

Whole blood was stimulated with variable doses of PAR1 peptide agonist (0 (top), 1μM, 5μM, 10μM (bottom)) (A) and PAR4 agonist (0 (top), 10μM, 50μM, 100μM (bottom)) (B). Flow cytometry histograms demonstrating the intensity of fluorescence exhibited by platelets expressing P-selectin, as a marker of activation, in response to incubation with the agonists.
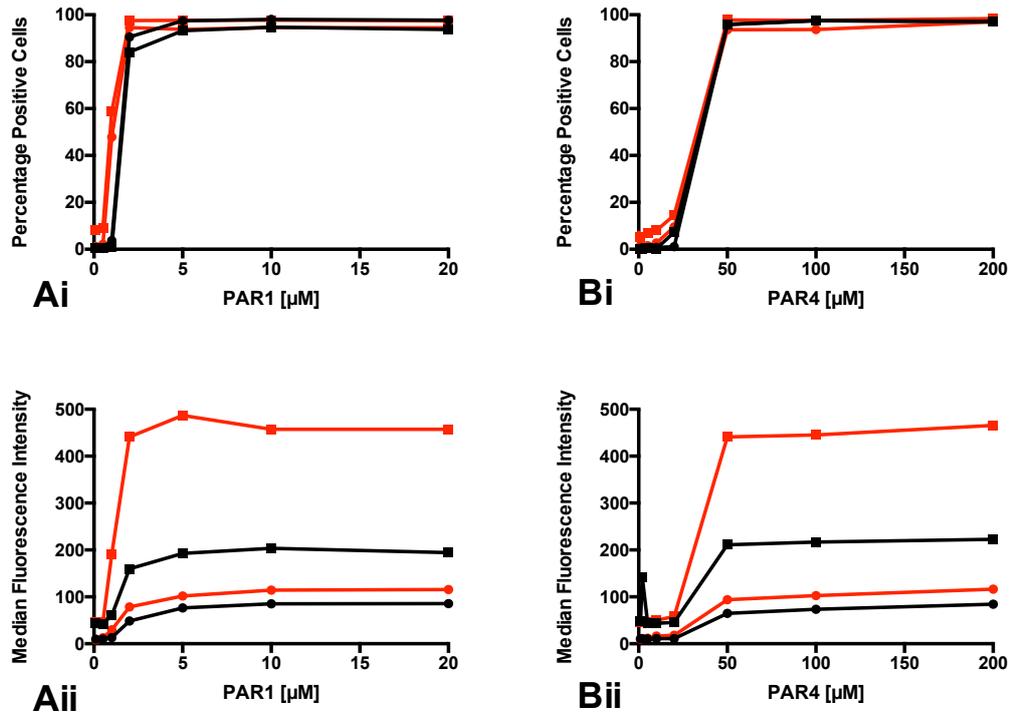
Figure 3. 5: Comparison of variable antibody concentrations

Whole blood sample dose response curves to PAR1 (i) and PAR4 (ii) agonist demonstrating percentage of platelets expressing P-selectin (circle ●) and fibrinogen binding (square ■) (A) and median fluorescence intensity (B) in samples with both 2µl (black) or 5µl (red) of respective antibody. (n=1)
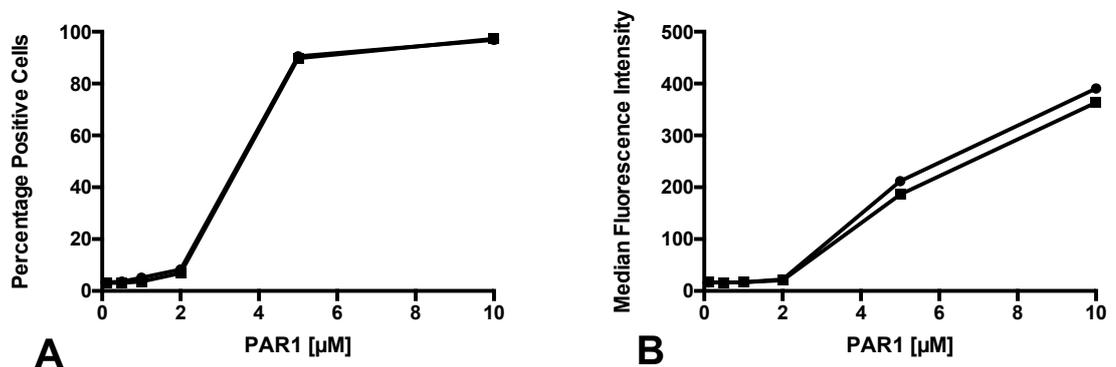
Figure 3. 6: Comparison of sample incubation time with antibodies.

Dose response curves to PAR1-activating peptide agonist showing percentage of positive platelets (A) and median fluorescence intensity (B) for P-selectin expression. Whole blood samples were immediately stimulated with PAR1 (0 minutes (circle ●)) or stimulated after 5 minutes (square ■) incubation with the antibody. (n=1)

Stability of the PAR peptide agonists (PAR1, PAR4) and timing of their addition to the sample was also investigated. The agonist was added to the room temperature experimental tube one hour before, one minute before or one minute after the whole blood (5μl). Results confirmed the robust stability of PAR agonists at room temperature, and no difference was seen in results when comparing timings. (Figure 3.7)

The results in section 3.3.1.1 confirmed that PAR1 and PAR4 peptide agonists induce platelet activation, which can be predictably quantified using flow cytometry and fluorescence associated with P-selectin expression and fibrinogen binding. Consistency in experimental protocol is of extreme importance and from these initial experiments a standard procedure was formulated. All samples would be prepared with 5μl whole blood/PRP, 5μl relevant antibody, and 5μl of appropriate agonist/antagonist concentration. Total sample volume would be made up to 50μl with modified Tyrode's buffer. PAR1 and PAR4 peptide agonists performed similarly in testing, but the decision was made to use the more potent activator (PAR1).
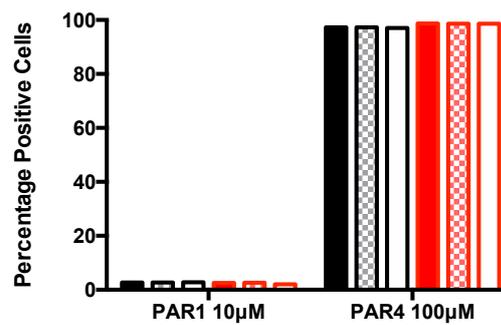
Figure 3. 7: Comparison of timing of agonist addition

Histogram of percentage of platelets positive for P-selectin expression (black) and fibrinogen binding (red) when PAR was added one hour before (closed bar), one minute before (patterned bar) and one minute (open bar) after addition of whole blood to the sample. (n=1)

3.3.2 Influence of nitric oxide on platelet activation

S-nitrosoglutathione (GSNO), a known nitric oxide donor, was explored as a potential potent inhibitor of platelet activation. It was preferable to utilise whole blood, as more representative of the *in vivo* state, and emphasises the power of population isolation in flow cytometry. Consequently, initial testing compared the ability of GSNO to inhibit platelet activation by PAR1 peptide agonist in whole blood compared to PRP. All samples were incubated with GSNO for two minutes prior to PAR1 peptide agonist ($10\mu M$) as recommended by previous validation. (212)

There was evidence of inhibition of activation with increasing doses of GSNO ($0-100\mu M$) in the PRP experiments (percentage positive cells for P-Selection expression $97.1\pm0.3\%$ to $92.0\pm1.8\%$ (GSNO $100\mu M$) and fibrinogen binding $84.9\pm2.7\%$ to $67.8.0\pm1.4\%$ (GSNO $100\mu M$)). However, there was no response with the whole blood sample from the same donor (P-Selection expression 98.05% to 98.7% (GSNO $100\mu M$) and fibrinogen binding 97.4% to 97.4% (GSNO $100\mu M$)). (Figure 3.8) This can be explained by the erythrocyte abundance within the whole blood sample; these red blood cells are accepted to be prolific scavengers of NO. (213)

NO has a well-established short half-life, and although GSNO's half-life is significantly longer at more than two minutes (214), careful consideration of appropriate timings was important. Results in PRP showed a standard dose response curve to GSNO when fixed at five minutes, however, at ten minutes the results suggested a less reliable response. (Figure 3.9)

In conclusion, the experiments show that GSNO produces a consistent response in PRP following incubation for two minutes prior and fixing five minutes after addition of the agonist.
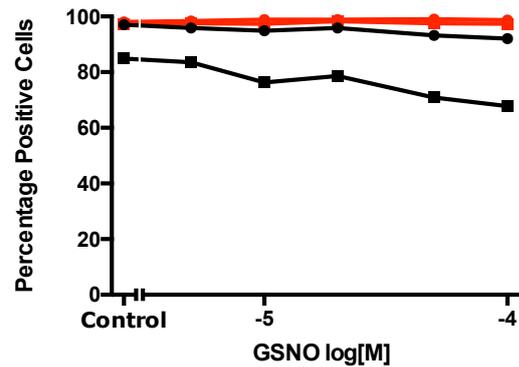
Figure 3. 8: Dose response curve to nitric oxide donor (GSNO)

Whole blood (red, n=1) and PRP (black, n=2) was incubated with increasing doses of GSNO (0-100μM) for two minutes prior to activation with PAR1 peptide agonist (10μM). Results demonstrate the percentage of platelets expressing markers of activation (P-selectin (circle ●) and fibrinogen binding (square ■).
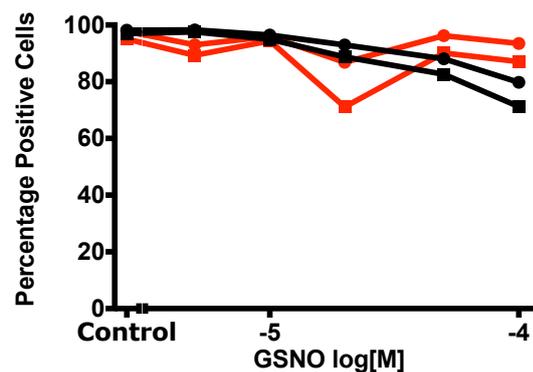
Figure 3. 9: Comparison of timing of sample fixing after GSNO incubation

PRP was incubated with increasing doses of GSNO (0-100μM) for two minutes prior to activation with PAR1 peptide agonist (10μM). Subsequently the samples were fixed at five (black) or ten minutes (red) with formyl saline. Results demonstrate the percentage of platelets expressing markers of activation (P-selectin (circle ●) and fibrinogen binding (square ■). (n=2).

3.3.3 Influence of prostacyclin on platelet activation

PGI_2 (0-10nM) induced inhibition of platelet activation was explored. In contrast to experiments with GSNO, PGI_2 induced a concentration dependent inhibition of P-selectin and fibrinogen binding in both whole blood and PRP (P-selectin p= 0.709 and fibrinogen p=0.285). (Figure 3.10) PGI_2 IC_{50} was 14nM in whole blood (P-selectin expression 14.0nM and fibrinogen binding 14.7nM), and 8nM in PRP (P-selectin expression 8.7nM and fibrinogen binding 7.9nM). To maximise consistency with *in vivo* conditions, future experiments were performed with PGI_2 in whole blood.
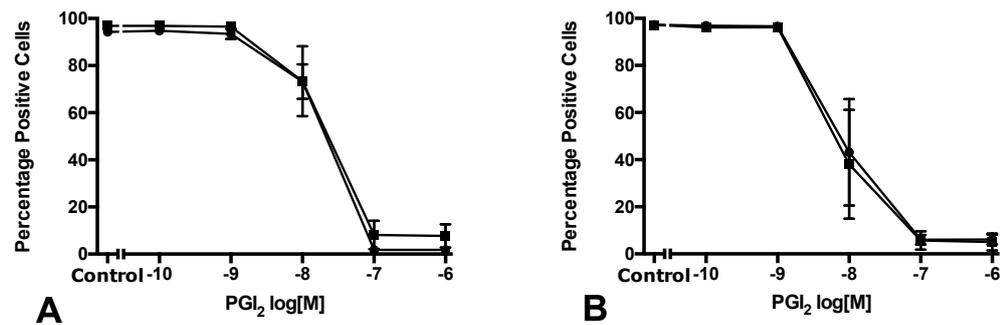
Figure 3. 10: Dose response curve to prostacyclin

Whole blood (A, n=3) and PRP (B, n=3) samples were incubated with increasing doses of PGI$_2$ (0-1μM) for two minutes prior to activation with PAR1 peptide agonist (10μM). Results demonstrate the percentage of platelets expressing markers of activation (P-selectin (circle ●) and fibrinogen binding (square ■). Error bars represent standard error of the mean.

3.3.4   Influence of ticagrelor on platelet activation

Published data on concentrations of ticagrelor *ex vivo* is sparse (139,215), with none available for experiments with PAR1 peptide agonists. Initial work demonstrated the importance of incubation of the whole blood sample with ticagrelor prior to activation with the PAR1 peptide agonist, therefore experiments comparing of incubation times (5-30mins) were performed with no benefit beyond the five minutes seen. (Figure 3.11)

To ensure that the inhibition was related to ticagrelor and not secondary to the vehicle, DMSO 0.5%, a direct comparison was made. (Figure 3.12). This was important as it is known that DMSO 0.5% may impact on platelet aggregation in concentrations as low as 1%. (216) Significantly lower levels of activation were seen in samples incubated with ticagrelor in response to PAR1 peptide agonist (p=0.046). Finally, with optimisation of the methods, the technique was confirmed in whole blood and PRP. (Figure 3.13) Ticagrelor inhibited P-selectin expression following PAR1 receptor agonism, with 10μM generating 13.1% inhibition in whole blood and 24.3% inhibition in PRP; IC$_{50}$ was calculated at 2.85μM and 4.01μM respectively.

Ticagrelor primarily targets the P2Y$_{12}$ adenosine diphosphate (ADP) receptor, as discussed in section 1.2.6. To investigate the antagonistic effect on ADP induced platelet activation whole blood was incubated with ticagrelor (0-10μM) or vehicle prior to ADP (0-100μM) activation and was found to induce a significant reduction in P-selectin expression and fibrinogen binding (p<0.001). (Figure 3.14) Results support previous studies findings that 5μM ticagrelor generated high levels of P2Y$_{12}$ receptor inhibition with only low levels of P-selectin expression and fibrinogen binding seen in response of ADP. ADP also exhibits activity on the P2Y$_1$ receptor, a transmembrane G-coupled receptor from the same family as P2Y$_{12}$. Within these experiments, a known selective and competitive P2Y$_1$ receptor antagonist adenosine-38-phosphate-58-phosphate (A3P5P, 100μM (maximal inhibition)) (217) was compared to vehicle, to ticagrelor, and also evaluated when given in combination with ticagrelor. This also significantly inhibited ADP induced platelet activation (p<0.001) but its activity was not as potent as ticagrelor (p<0.005). When the two antagonists were combined the negligible residual platelet activity seen with ticagrelor was abolished.
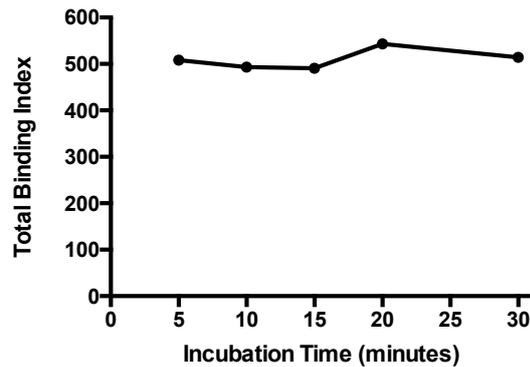
Figure 3. 11: Comparison of timing of ticagrelor incubation

Whole blood samples were incubated for varying time frame (5-30 minutes) with ticagrelor (5μM, DMSO 0.5%) prior to activation with PAR1 peptide agonist (5μM). Results demonstrate the total binding index for platelet P-selectin expression. (n=1)
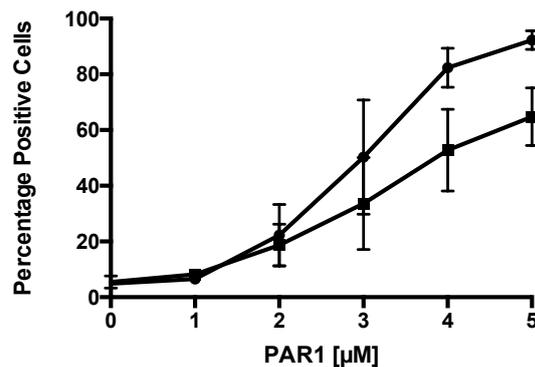
Figure 3. 12: Comparison of ticagrelor and vehicle response to PAR1 peptide agonist

Whole blood samples were incubated for 5 minutes with ticagrelor (5μM (square ■)) or vehicle (DMSO 0.5% (circle ●)) prior to activation with increasing doses of PAR1 peptide agonist (0-5μM). Results demonstrate the percentage of platelets positive for P-selectin expression. Error bars represent standard error of the mean. (n=3)
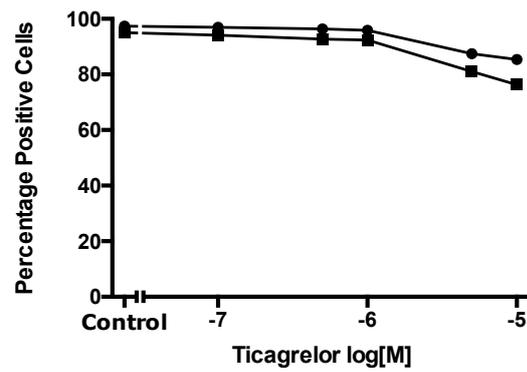
Figure 3. 13: Dose response curve to ticagrelor

Whole blood (circle ●) and PRP (square ■) samples were incubated for 5 minutes with increasing doses of ticagrelor (0-10μM) prior to activation with PAR1 peptide agonist (5μM). Results demonstrate the percentage of platelets positive for P-selectin expression. (n=2)
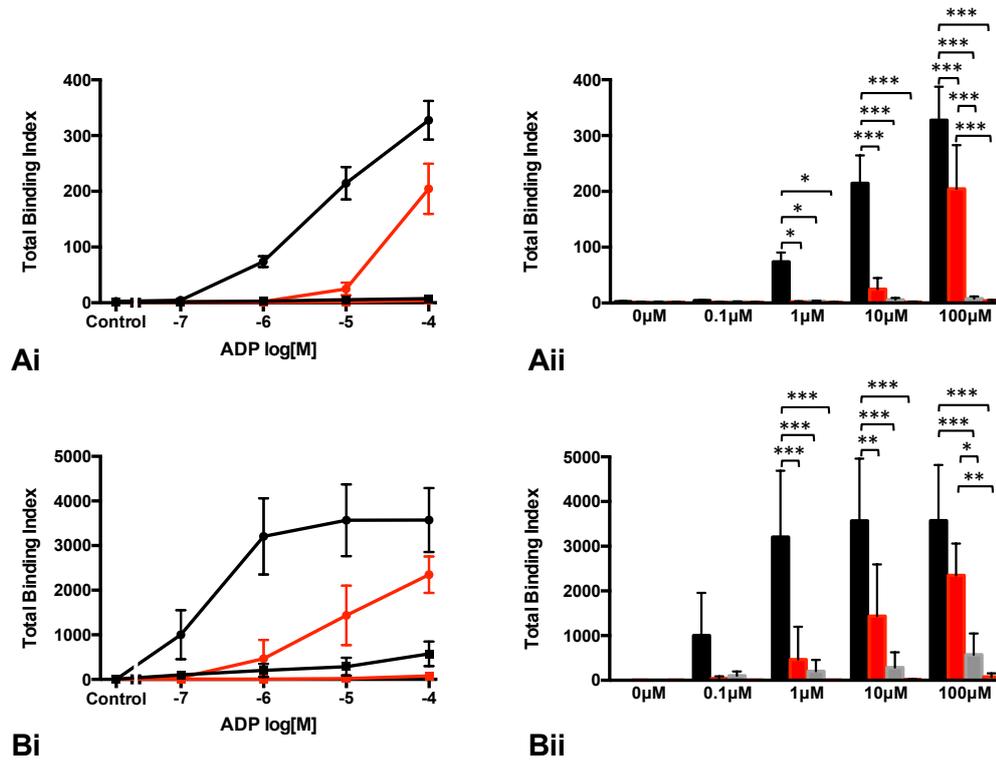
Figure 3. 14: Dose response curve to ADP in presence and absence of P2Y inhibitors

Whole blood samples were incubated for five minutes with vehicle, ticagrelor (5μM), A3P5P (100μM) or combined ticagrelor and A3P5P; prior to stimulation with variable doses of ADP (0-100μM). Results were analysed on the flow cytometer and charts depict total binding index for platelet P-selectin expression (A) and fibrinogen binding (B). Line chart represents data without (circle ●) and with ticagrelor (square ■); and in the presence (red) and absence (black) of A3P5P (i). Bar chart demonstrates vehicle (black bar), ticagrelor (grey bar), A3P5P (red bar) and combined ticagrelor/A3P5P (red outline/shaded grey bar) (ii). N=3 and error bar represents standard error of the mean. Conditions were compared using one-way ANOVA and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.

In conclusion ticagrelor, a potent P2Y$_{12}$ receptor antagonist, inhibits PAR1 peptide agonist induced platelet activation in both whole blood and PRP samples. Five minutes incubation with the inhibitor prior to activation, rather than longer periods, was sufficient for effect.

3.3.5   Ticagrelor and the endothelial vasodilators

The next step was to explore the potential synergism between P2Y$_{12}$ inhibition by ticagrelor and the endothelial vasodilators on platelets. Initial investigations explored the relationship between ticagrelor and GSNO in PRP, with results revealing both to be potent inhibitors of platelet activation (ticagrelor 5μM 81.5%, GSNO 1μM 47.5% and 10μM 98.2% reduction in TBI compared to activated control). (Figure 3.15)

Assessment of the nature of interaction between ticagrelor and NO was not possible from these experiments due to the inability of 5μM PAR1 peptide agonist to override the inhibition produced by incubation of PRP with high doses of the two antagonists. Therefore, to enable appropriate analysis the decision was made to increase the agonist concentration to saturate the sample. As anticipated there was a reduced level of inhibition generated by ticagrelor (figure 3.16) thus permitting required evaluation. After protocol change there was a statistical separation of the curves, showing a dose dependent response to GSNO in the presence and absence of ticagrelor (5μM) ($p < 0.001$), even at higher doses of the NO donor. This was replicated with fibrinogen associated fluorescence ($p = 0.002$). (Figure 3.17)

To explore the interaction between ticagrelor and NO, flow cytometric analysis of inhibition was performed demonstrating a significantly greater inhibition of P-selectin expression post ticagrelor ($p < 0.001$). (Figure 3.18) The pattern of this relationship was synergistic as the mean inhibition seen (GSNO 10μM in presence of ticagrelor = 44.7%) was beyond that of the additive inhibition (GSNO 10μM in absence of ticagrelor 11.7% plus ticagrelor in the absence of GSNO 8.0% = 19.7%). A similar pattern was seen in fibrinogen binding ($p = 0.001$) with the combined inhibition (ticagrelor 5μM and GSNO 10μM) observed to be greater than would be expected from an additive effect alone (66.2% versus 52.5%).
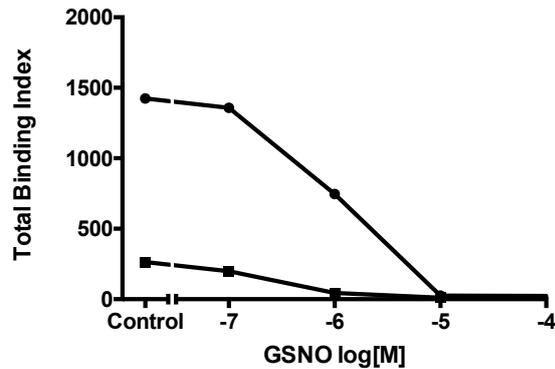
Figure 3. 15: Dose response curve to nitric oxide donor (GSNO) with and without ticagrelor

PRP was incubated with ticagrelor 5µM (square ■) or vehicle (DMSO 0.5% (circle ●)) for five minutes and then increasing doses of GSNO (0-100µM) added during the final two minutes prior to activation with PAR1 peptide agonist (5µM). Results demonstrate the total binding index (percentage positive cells x mean fluorescence intensity x 0.01) for P-selectin expression. (n = 2)
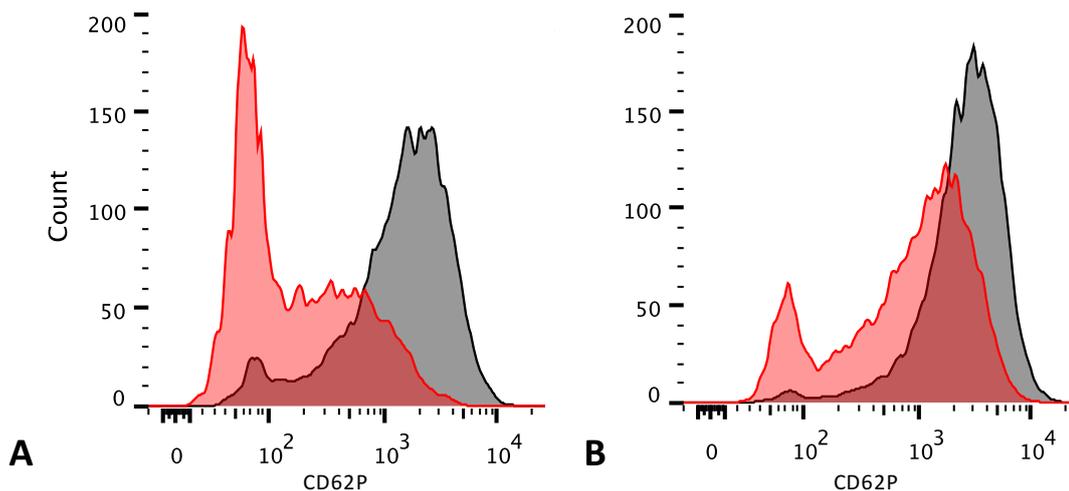
Figure 3. 16: Comparison of ticagrelor inhibition against 5µM and 10µM of PAR1 peptide agonist

Whole blood samples were incubated for 5 minutes with ticagrelor (5µM (red)) or vehicle (DMSO 0.5% (black)) prior to activation with PAR1 peptide agonist ((5µM (A) or 10µM (B))). Flow cytometry histograms demonstrate the intensity of fluorescence exhibited by platelets expressing P-selectin, as a markers of activation. (n=2)
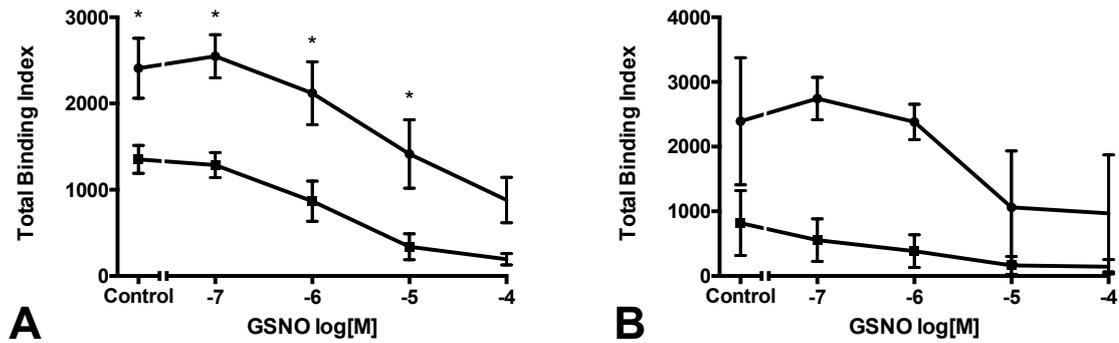
Figure 3. 17: Dose response curve to nitric oxide donor (GSNO) in the presence and absence of ticagrelor with 10μM PAR1 peptide agonist

*PRP was incubated with ticagrelor 5μM (square ■) or vehicle (DMSO 0.5% (circle ●)) for five minutes and then increasing doses of GSNO (0-100μM) added during the final two minutes prior to activation with PAR1 peptide agonist (10μM). Results demonstrate the total binding index for P-selectin expression (A, n=3) and fibrinogen (B, n=3). Error bars represent standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p < 0.05 comparing with and without ticagrelor.*
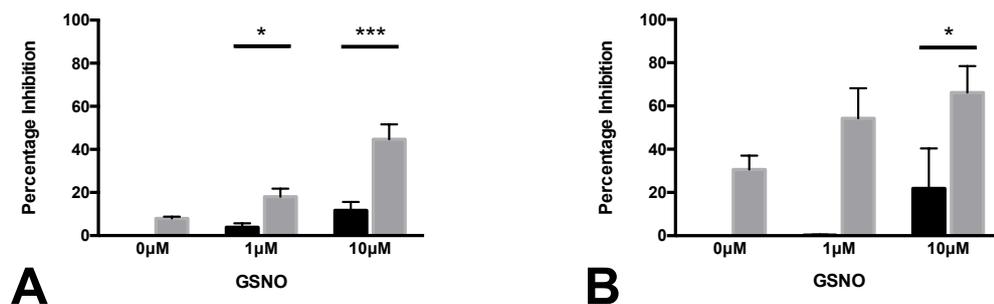
Figure 3. 18: Histogram of nitric oxide induced inhibition of platelet activation

*PRP was incubated with variable doses of GSNO (0-10μM) in the presence of vehicle (DMSO 0.5% (black)) or ticagrelor (5μM (grey)), prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry, with data recorded as percentage of platelets positive for P-selectin (A, n=4) and fibrinogen binding (B, n=3) associated fluorescence. Percentage inhibition was calculated as the percentage of positive platelets compared to the percentage in the maximally stimulated sample (100 − ((percentage of positive cells/percentage of cells positive in control) x 100). Error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05 and *** p<0.001.*

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Identical experimental conditions demonstrated parallel results for ticagrelor and PGI_2, with whole blood analysis revealing significantly lower numbers of platelets positive for P-selectin expression when treated with ticagrelor (p=0.032) and consequently a higher level of inhibition (p=0.019). (Figure 3.19 and 3.20) Ticagrelor was consistent, generating 8.2% inhibition of platelet activation; while 1nM of PGI_2 generated 1.1%. In combination, 36.9% inhibition was seen, which was four-fold greater than expected if the relationship was only additive (9.3%), supporting a synergistic interaction.

To further investigate the interaction, beyond surface markers, assessment was undertaken of the impact on platelet leukocyte aggregate (PLA) formation, a consequence of P-selectin expression on the platelet surface binding with the leukocyte P-selectin glycoprotein ligand-1. Evidence suggests this measure maybe a superior marker of platelet activation than P-selectin expression alone. (209) The results mirrored previous experiments with PAR1 peptide agonist (10μM) increasing PLA numbers and PGI_2 inducing inhibition recorded as the percentage of leukocytes expressing platelet specific fluorescence. (Figure 3.22) PAR1 peptide agonist induced high levels of PLA formation (control 7.9±0.2%, PAR1 peptide agonist 5μM 62.1±1.7% and 10μM 73.0±1.0%). Formation was overall impaired by the presence of ticagrelor (p=0.003), although could be overcome at maximal PAR1 peptide agonist concentrations (PAR1 peptide agonist 5μM 43.6±7.7% (p=0.004) and 10μM 61.0±4.6% (p=0.08)). PGI_2 prevented PAR1 peptide agonist induced PLA formation in a dose dependent fashion (PGI_2 0nM 72.5±1.4%, 0.1nM 68.8±2.2%, 1nM 69.3±1.5% and 10nM 16.7±4.4%) with higher concentrations being more potent than ticagrelor 5μM (PGI_2 76.9% and ticagrelor 13.8% inhibition). However, ticagrelor did statistically enhance the inhibition by PGI_2 (p<0.001) (PGI_2 0nM 62.4±2.7% (p=0.22), 1nM 53.7±7.2% (p=0.02) and 10nM 8.3±0.2% (p=0.40)). The methodology precluded GSNO assessment due to the requirement of whole blood.

Flow cytometric exploration of the effect on PAR1 peptide agonist induced platelet activation in the presence of ticagrelor and endothelial vasodilators provided evidence that there was a relationship which appeared synergistic in nature. To further investigate the platelet response, light transmission aggregometry was performed on isolated platelets in similar experimental conditions, to investigate if the results were independent of the methodology. Consistency was confirmed with evidence of a fall in aggregation following
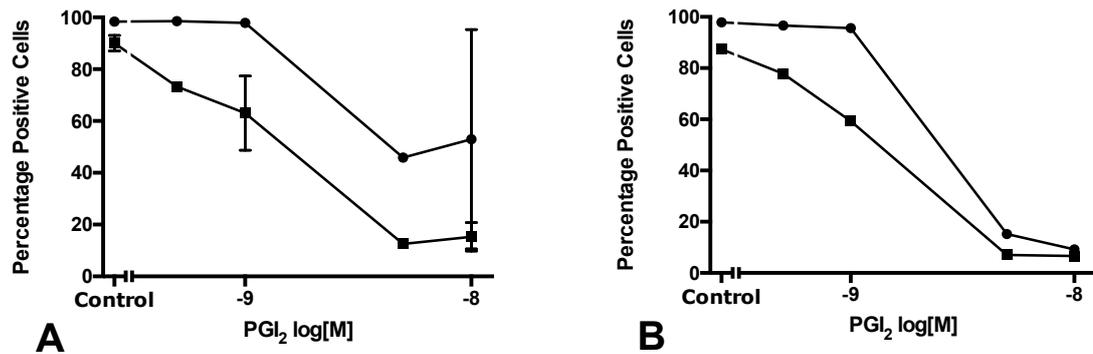
Figure 3. 19: Dose response curve to prostacyclin in the presence and absence of ticagrelor with 10μM PAR1 peptide agonist

Whole blood (A, n=3) and PRP (B, n=1) samples were incubated with ticagrelor 5μM (square ■) or vehicle (DMSO 0.5% (circle ●)) for five minutes and then increasing doses of PGI$_2$ (0-1nM) added during the final two minutes prior to activation with PAR1 peptide agonist (10μM). Results demonstrate the percentage of platelets positive for P-selectin expression. Error bars represent standard error of the mean.
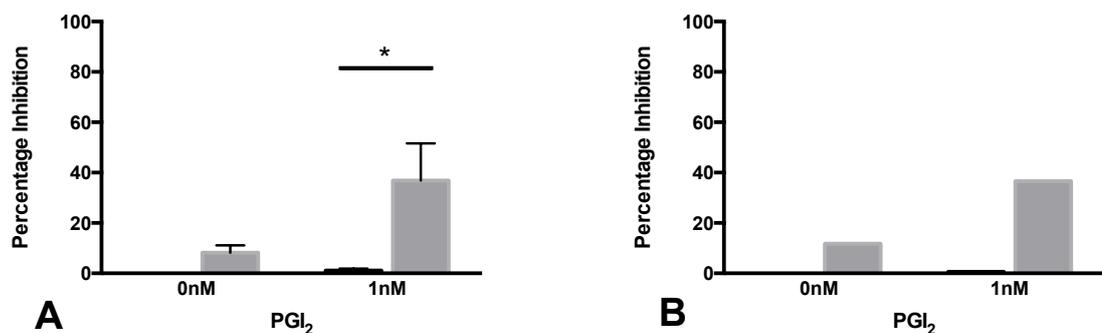
Figure 3. 20: Histogram of prostacyclin induced inhibition of platelet activation

Whole blood (A, n=3) and PRP (B, n=1) samples were incubated with PGI$_2$ (1nM) or buffer (0nM/control) in the presence of vehicle (DMSO 0.5% (black)) or ticagrelor (5μM (grey)), prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry, with data recorded as percentage of platelets positive for P-selectin associated fluorescence. Inhibition was calculated as percentage of positive platelets compared to the maximally stimulated sample (100 – ((percentage of positive cells/percentage of cells positive in control) x 100). Error bar represents standard error of the mean. The effect of was explored using Student's t-test and significance is shown as * p<0.05.
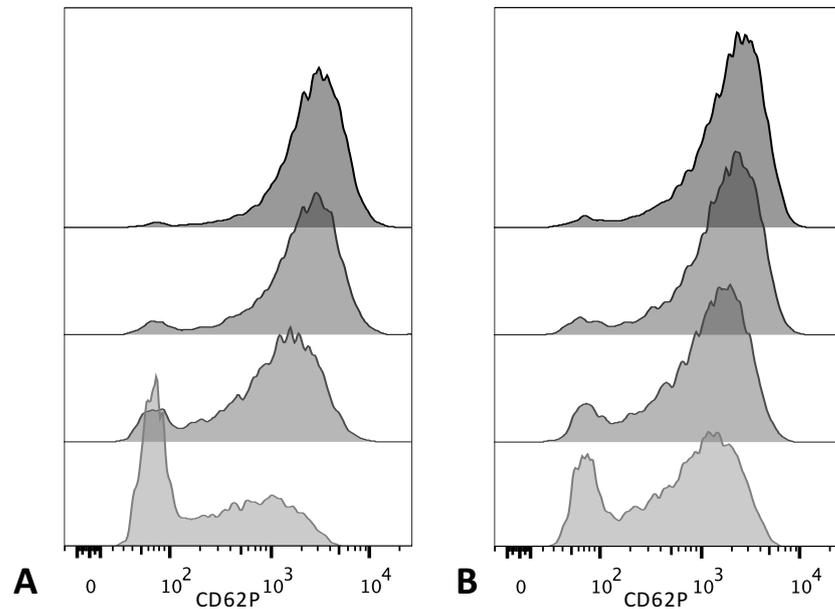
Figure 3. 21: Relationship between ticagrelor and endothelial vasodilators

Whole blood was incubated with PGI$_2$ (1nM) or buffer (0nM/control) in the presence of vehicle (DMSO 0.5%) or ticagrelor (5μM), prior to maximal stimulation with PAR1 peptide agonist (10μM) (A). PRP was incubated with GSNO (1μM) or buffer (control) in the presence of vehicle (DMSO 0.5%) or ticagrelor (5μM), prior to maximal stimulation with PAR1 peptide agonist (10μM) (B). Results show flow cytometry histograms demonstrating the intensity of fluorescence exhibited by platelets expressing P-selectin, as a marker of activation, in response to PAR1 peptide agonist (control (top)), PGI$_2$/GSNO, ticagrelor and ticagrelor/vasodilator combined (bottom).
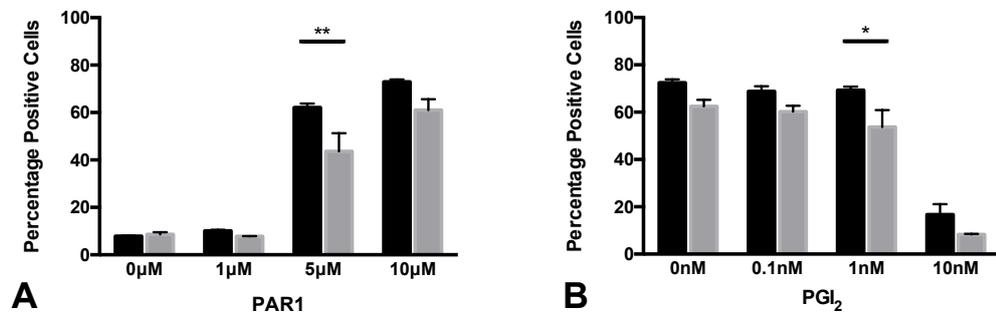
Figure 3. 22: Platelet leukocyte aggregate formation in response to PAR1 peptide agonist and prostacyclin

Whole blood was incubated with ticagrelor 5μM or vehicle (DMSO 0.5%) for five minutes and then increasing doses of PAR1 peptide agonist (0-10μM) (A) or PGI$_2$ (0-10nM) (B) added during the final two minutes prior to activation (PAR1 peptide agonist (10μM)). Samples were analysed using flow cytometry and quantified the number of cells positive for CD45, a leukocyte specific antibody, and CD42a, an antibody used to label platelets. These events represent presence of a PLA. Results show histograms representing the percentage of PLAs compared to the overall leukocyte population, comparing vehicle (black bars) to ticagrelor (grey bars). Error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05 and ** p<0.01. (n=3)

incubation with ticagrelor (5µM 51.7±8.3% aggregation) compared to control (71.0±6.7%), however low concentrations of PGI_2 (0.1nM 70.0±6.4%) and GSNO (0.1µM 71.7±4.4%) had no effect. Yet, when these low dose antagonists were investigated with ticagrelor (5µM) a synergistic response was seen (PGI_2 0.1nM 29.0±14.6% and GSNO 0.1µM 25.0±17.6%). Overall, ticagrelor significantly enhanced the inhibitory effect of both PGI_2 ($p < 0.001$) and NO ($p = 0012$). (Figure 3.23)

In conclusion, *in vitro* data by varying methodology and markers provides evidence to support a synergistic relationship between ticagrelor and the endothelial vasodilators, PGI_2 and NO.
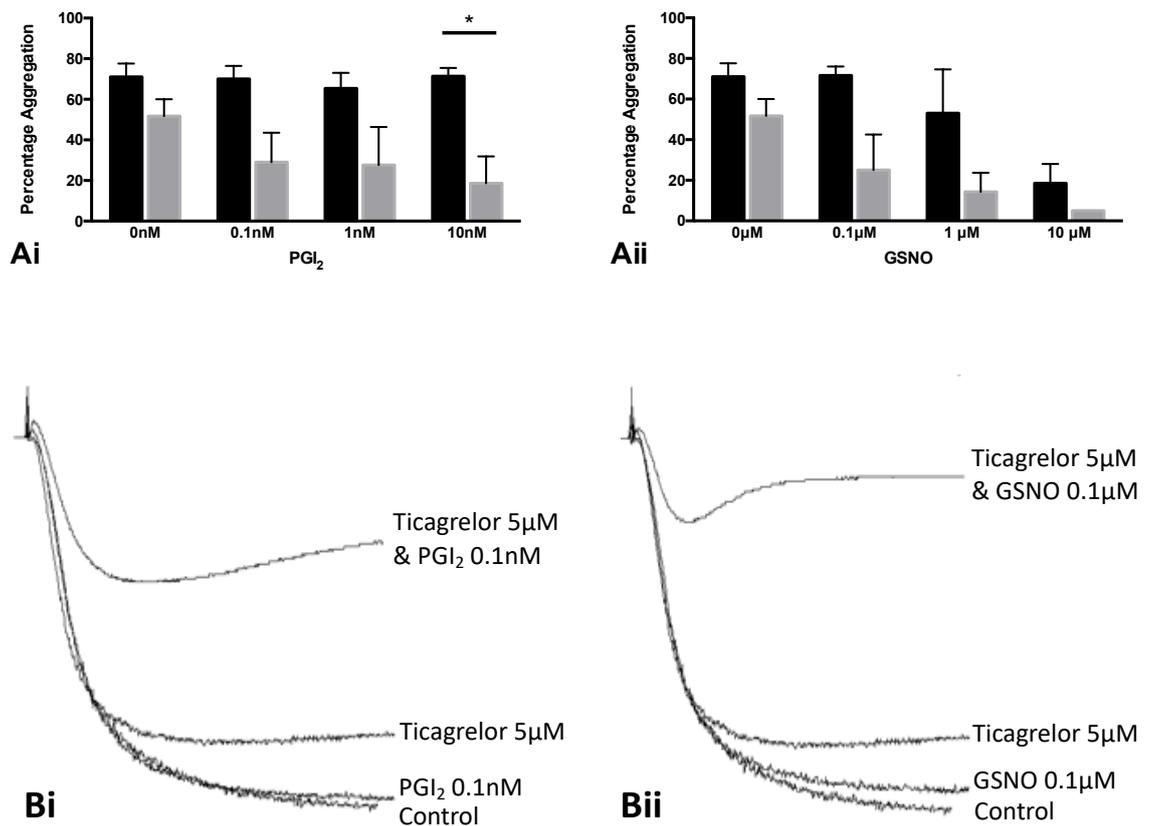
Figure 3. 23: Percentage of isolated platelet aggregation as measured with light transmission aggregometry

Isolated platelets were incubated with ticagrelor 5μM or vehicle (DMSO 0.5%) for five minutes and then increasing doses of PGI$_2$ (0-10nM) (i) or GSNO (0-10μM) (ii) added during the final two minutes prior to activation with PAR1 peptide agonist (10μM). Aggregation was tracked using light transmission aggregrometry. Results show histograms representing the percentage of measured aggregation following five minutes of recording for varying concentration of endothelial inhibitor in the absence (black bars) or presence of ticagrelor (grey bars) (A). The recorded traces for comparison are also present (B). Error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05. (n=3)

3.4   DISCUSSION

The primary aim of this chapter was to establish the primary methodology and use these to investigate the effect on platelets by known activators and inhibitors of function; and assess the interaction between them.

PAR1 and PAR4 agonists produced a predictable platelet response in whole blood, PRP and isolated platelets, with evidence of activation as measured by P-selectin expression, fibrinogen binding and PLA formation on flow cytometry; and aggregation by light transmission aggregometry. PAR1 peptide agonist was more potent compared to PAR4, as expected due to the higher abundance of the relevant receptor on platelets. (116) The inhibitory effects of GSNO could not be quantified in whole blood due to erythrocyte competition at the experimental concentrations, but in PRP it was found to inhibit PAR1 peptide agonist induced platelet activation. The whole blood effect can be only partially overcome by saturation of the sample with high concentrations of NO donor. (218) PGI_2 activity was evident in both whole blood and PRP with effective inhibition of activation induced by the PAR1 peptide agonist in nanomolar concentrations.

Ticagrelor's primary action is as a potent $P2Y_{12}$ antagonist. The $P2Y_{12}$ receptor, first isolated in 2001 by Hollopeter et al, is a member of the P2 family of G-protein coupled seven transmembrane domain receptors. (67,111) It is preferentially controlled by adenine related nucleotides with ADP acting as an agonist. (113) Ticagrelor in combination with a $P2Y_1$ inhibitor abolished ADP induced platelet activation and independently generated partial inhibition of PAR1 peptide agonism, evident even at concentrations beyond physiological activation. Incomplete inhibition of aggregation by thrombin receptor agonist with ticagrelor has been previously established (219) secondary to the availability of alternative signalling pathways (section 1.3.8.4). Interestingly, PGI_2 and NO inhibitory effect was amplified by ticagrelor beyond the additive interaction irrespective of quantifier or methodology, suggesting their relationship is synergistic.

3.5 CONCLUSIONS

This chapter has demonstrated that ticagrelor, PGI_2 and NO can all inhibit PAR1 peptide agonist induced platelet activation. Moreover, when used in combination there appears to be a synergistic relationship between *in vitro* ticagrelor and the endothelial vasodilators PGI_2 and NO. This finding was consistent across techniques and measures; supporting exploration of orally administered ticagrelor in patient groups using these optimised methodologies.

4   CHAPTER FOUR: IS EFFICACY OF PLATELET AGGREGATION INHIBITION BY TICAGRELOR MEDIATED P2Y$_{12}$ BLOCKADE DEPENDENT UPON ENDOGENOUS ENDOTHELIAL NITRIC OXIDE? (PLATENOX STUDY)

4.1   INTRODUCTION

The activation of blood platelets at sites of vascular injury is essential for haemostasis, but also makes a significant contribution to the development and progression of atherothrombosis, a multicellular, chronic inflammatory process that causes coronary artery disease (CAD). Triggering of platelet activation by extracellular matrix proteins results in the release of secondary mediators of aggregation including adenosine diphosphate (ADP) and thromboxane A2 (TXA2), which as paracrine regulators of platelet activation ensure rapid haemostasis at the site of injury. (78,90) This role has led to great interest in their potential to be a pharmacological target in the management of patients with CAD.

Changes in the pharmacological management of ACS over recent years (section 1.2.5) has dramatically improved outcomes but cardiovascular disease remains the biggest killer in the United Kingdom. (220) PLATO (PLATelet inhibition and patient Outcomes), was a landmark multicentre double blind randomised trial that demonstrated a reduction in all-cause mortality with ticagrelor compared to clopidogrel in patients with ACS. (34) Importantly, this benefit was evident without an increase in major bleeding risk, and consequently, ticagrelor has become a mainstay of treatment of ACS, recommended by NICE to be given in combination with aspirin for a minimum of 12 months. (63)

Complete understanding of the mechanism of action of the pharmacological agents is of profound importance to understand how to optimise benefit and minimise clinically significant side effects. The preceding chapter explored the potential relationship between ticagrelor and endogenous endothelial vasodilators in relation to platelet inhibition in *ex vivo* experiments. The aim of this chapter was to investigate the effect of oral ticagrelor on platelet sensitivity to PGI$_2$ and NO in not just the "healthy population" but also a CAD patient group.

4.1.1 Objectives of this chapter

The primary objective of the study was to demonstrate whether, in patients with CAD, blockade of the $P2Y_{12}$ receptor by ticagrelor will increase the platelet inhibitory actions of PGI_2 and NO. The secondary objectives of the study were to explore the effect of ticagrelor on endothelial function and evaluate if this correlated with the platelet inhibitory effect.

4.2   METHODS

This was a single centre clinical study based at Castle Hill Hospital, Cottingham, East Yorkshire, England. Subject participation is outlined in figure 4.1, with platelet and endothelial function testing performed while established on aspirin therapy and then repeated when additional oral ticagrelor reached steady state.

4.2.1   Study selection

64 patients and 10 healthy age-matched controls were recruited. All patients had confirmed CAD and were felt appropriate for percutaneous coronary intervention (PCI). The patient group was stratified into 4 sub-groups to allow detailed analysis:

1. Non-smoking patients and no history of diabetes mellitus
2. Non-smoking patients with diabetes mellitus
3. Smoking patients and no history of diabetes mellitus
4. Smoking patients with diabetes mellitus
5. Healthy controls

4.2.1.1   Subject recruitment

The patients were identified by their clinical cardiologists as requiring PCI, and consent was gained after adequate understanding of the participant information sheet. To aid recruitment, following major protocol amendment approval by the Ethics Committee, patients with known CAD requiring coronary angiography +/- PCI or requiring further lesion assessment with fractional flow reserve lesion/intravascular ultrasound were also considered. The control participants responded to local advertisement and provided informed consent.
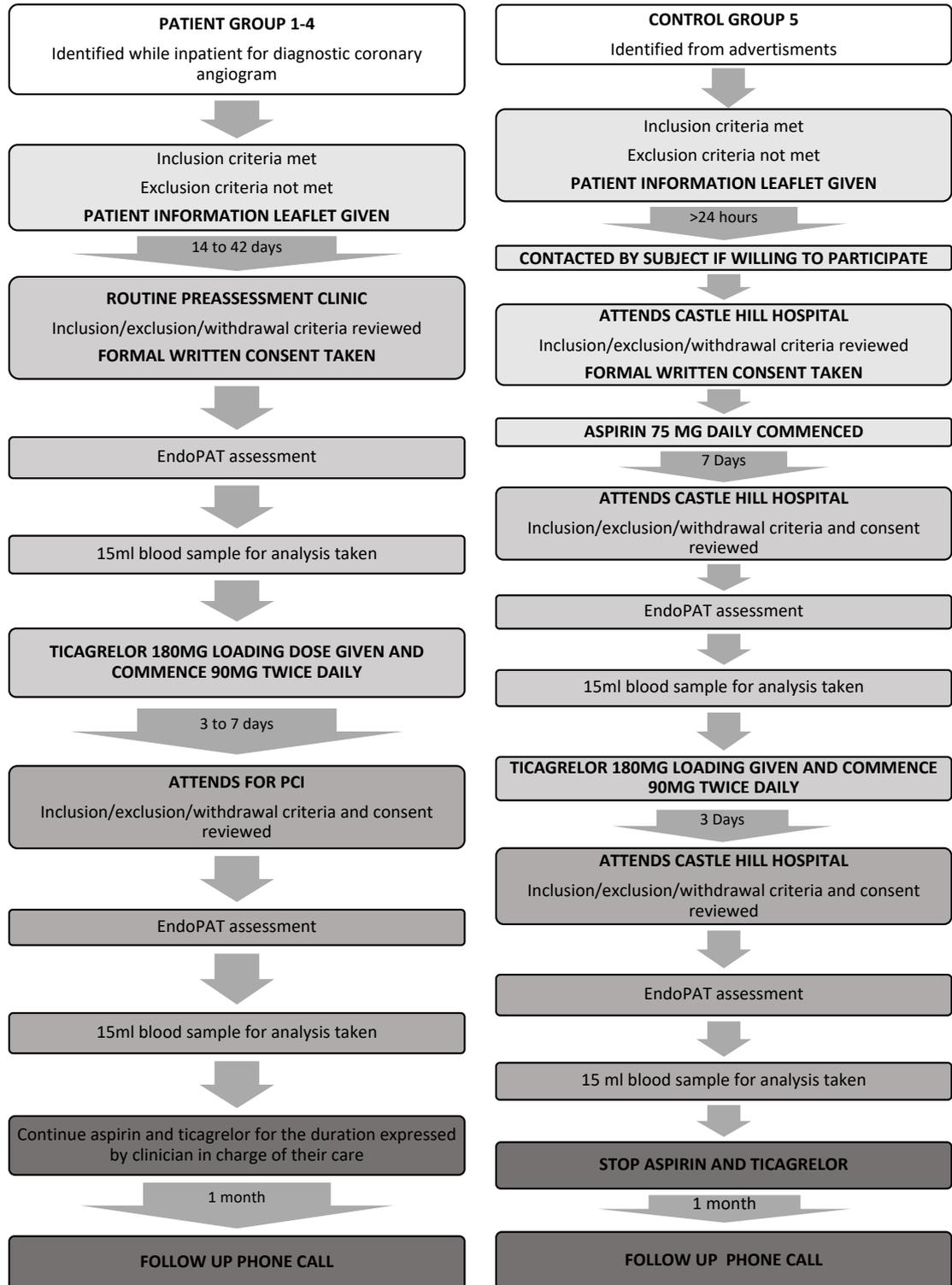
Figure 4. 1: Flow chart detailing participant involvement

All subjects had to meet comprehensive inclusion and exclusion criteria. During the period of the study if any of the withdrawal criteria were met, the subject was excluded from the study and none of the data was included in the final statistical analysis.

4.2.1.2   Inclusion criteria

- Age > 18 years

- CAD deemed to require PCI

- Patients with diabetes mellitus must have been established on oral or subcutaneous therapy

- Patients without diabetes mellitus must have had HbA1c levels between 20-41 mmol/mol

- Current smokers were those that have smoked >100 cigarettes and currently smoke on a daily basis

- Non-smokers must not have smoked for greater than 3 years (and not be on nicotine replacement)

- Healthy controls are non-smokers without medical history and not taking regular medication

4.2.1.3   Exclusion criteria

- Contra-indication to dual antiplatelet therapy
  - o Known bleeding disorders
  - o Known malignant disease
  - o Known myeloproliferative disease/malignant paraproteinaemia/heparin induced thrombocytopenia
  - o Previous intracranial bleed

- Already established on dual antiplatelet therapy

- Known moderate-severe liver or splenic failure

- Severe renal impairment

- Major surgery due within one month of enrolment or before completion of measurements

- Known allergy/intolerance to aspirin or ticagrelor

- Reaction or side effect of aspirin or ticagrelor resulting in discontinuation prior to completion

- Known allergy/intolerance to HMG CoA reductase inhibitor therapy (statins)

- Concurrent use of high dose simvastatin/lovastatin (>40mg daily)

- Currently taking medication that will interact with platelet function e.g. NSAIDS, antibiotics or herbal remedies

- Concurrent use of strong CYP3A4 inhibitors e.g. ketoconazole, clarithromycin, nefazodone, ritonavir, and atazanavir

- Concurrent use of strong CYP3A4 inducers e.g. rifampicin, dexamethasone, phenytoin, carbamazepine and phenobarbital

- Known sick sinus syndrome, second or third-degree AV block or bradycardia-related syncope without permanent pacemaker in situ

- Known severe asthma/COPD or worsening of dyspnoeic symptoms on ticagrelor

- Known severe gout

- Currently taking calcium channel antagonist

- Currently taking long acting nitrate

- Currently taking >15mg/week of methotrexate

- Women pregnant, breast feeding or of child bearing potential

- Require anticoagulation on warfarin or NOAC

- Platelet count <150 x10^9/L or >400 x10^9/L

- Known blood borne virus carrier

- Unable to give informed consent

- Involvement in a conflicting study

- Non-English speaker

4.2.1.4 Withdrawal of subjects

Subjects were withdrawn from the study in the following situations:

- Develop significant bleeding complications of medication requiring discontinuation of antiplatelet therapy prior to completion of the study

- Urgent surgery undertaken during the study resulting in discontinuation of antiplatelet therapy prior to completion of the study

- Reacted or developed side effects of aspirin or ticagrelor resulting in discontinuation prior to completion of the study

- Commenced medication that interacted with platelet function before completion of the study

- Commenced use of strong CYP3A4 inhibitors before completion of the study

- Commenced use of strong CYP3A4 inducers before completion of the study

- Worsening of dyspnoea in subjects with mild/moderate asthma/COPD resulting in discontinuation of ticagrelor prior to completion of the study

- Platelet count on initial sampling $< 150 \times 10^9$/L or $> 400 \times 10^9$/L

- Noncompliance with medication

- Subject wishes to no longer participate in the study (no reason required)

4.2.2   Experimental methods

4.2.2.1   Translation from *in vitro* to study

To enable the samples to be processed in a timely fashion individual FACS tube were not a viable option, so a multichannel pipette and 96 well plate was trialled in order to significantly reduce experimental time. This was particularly prudent as the aim was to analyse the fluorescence on separate samples and in duplicate. Although results were not identical there was a common proportional pattern seen with the well plate compared to FACS tubes if adequate mixing was performed, defined as eight controlled pipetting mix and five gentle agitations. (Figure 4.2)

Initial experiments had taken place using the FACS Fortessa machine and FACS Diva software, yet study site analysis required the FACS Calibur and BD CellQuest Pro to be used. Comparison was made using identical samples between the two machines with individually optimised settings. No difference was seen in the number of platelets positive for fluorescence, but the intensity of fluorescence was lower in the results from the FACS Calibur. (Figure 4.3) Interestingly, when we calculated the ratio of change in the MFI results, the data mirrored each other. (Table 4.1)

In conclusion, a methodology using the 96 well plate with adequate mixing and the FACS Calibur flow cytometer provided satisfactory results compared to previous protocols.
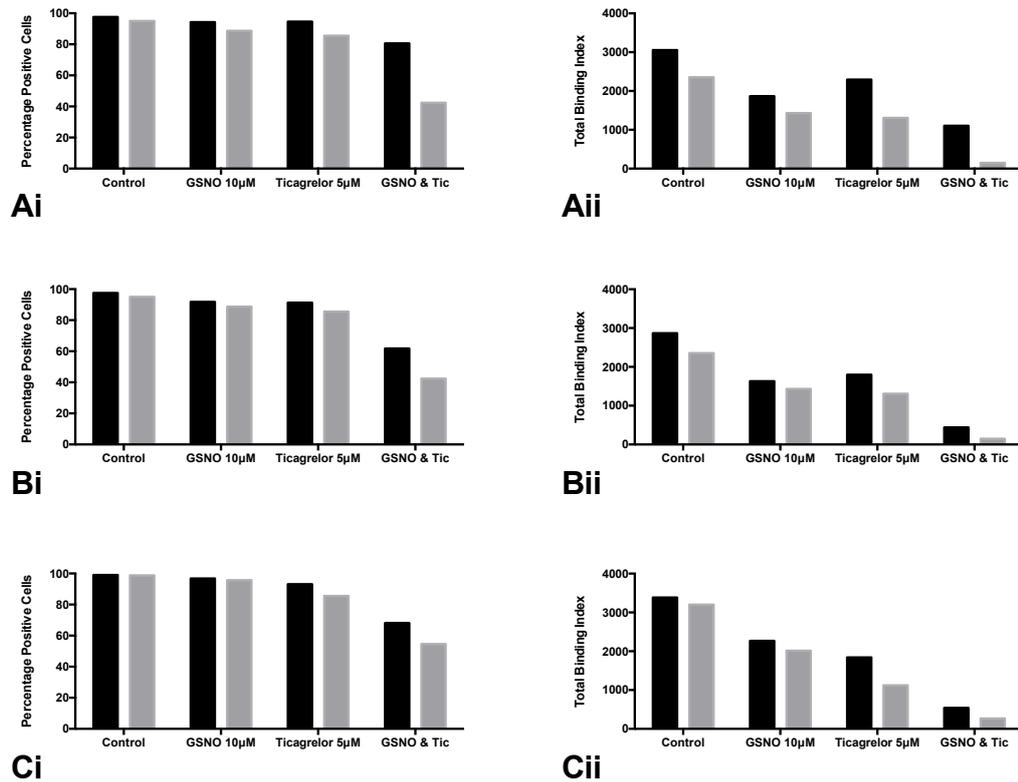
Figure 4. 2: Comparison of individual FACS tubes versus a 96 well plate

PRP was incubated with ticagrelor 5μM, GSNO 10μM or both inhibitors prior to activation with PAR1 peptide agonist (10μM). The samples were prepared either using a multichannel pipette and 96 well plate (black bars) or in individual FACS tubes (grey bars) as per previous methodology. Differing mixing techniques was compared in the newly adopted well plate technique (two controlled pipetting mix (A), five controlled pipetting mix (B) and eight controlled pipetting mix /five gentle agitations (C)). Histograms are presented demonstrating percentage of platelets positive for P-selectin expression (i) and the total binding index of those platelets (ii). (n=1)
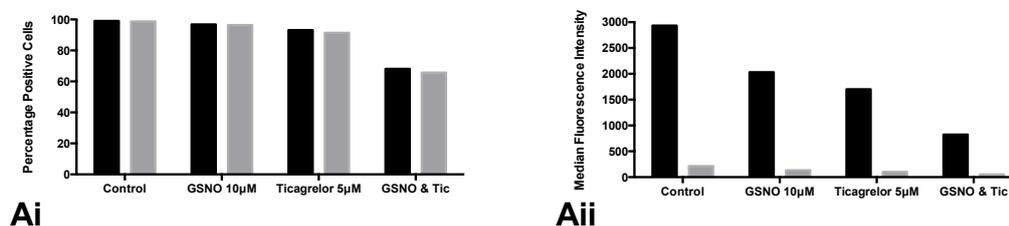
Figure 4. 3: Comparison of the flow cytometers

PRP samples were incubated with ticagrelor 5μM, GSNO 10μM or both inhibitors prior to activation with PAR1 peptide agonist (10μM). Results were analysed on the flow cytometer FACS Fortessa (black bars) and FACS Calibur (grey bars). Histograms are presented demonstrating percentage of platelets positive for P-selectin expression (i) and the median fluorescence intensity of those platelets (ii). (n=1)

	Median Fluorescence Intensity	
	FACS Fortessa	FACS Calibur
Control versus GSNO ratio	1.4	1.6
Ticagrelor versus GSNO & Ticagrelor ratio	2.1	2.0

Table 4. 1: Comparison of flow cytometer median fluorescence intensity ratio

4.2.2.2   Methodology of blood preparation

4.2.2.2.1   Preparation of whole blood

Blood was obtained from the consented study participants at two time points. First with steady state aspirin plasma concentration (day 0) and the second when established on dual antiplatelet therapy with aspirin and ticagrelor.

The sample was drawn from the median cubital vein of the dominant arm (non-occluded arm) post endothelial assessment using a 21-gauge butterfly needle. This was done in order to prevent phlebotomy induced changes in vascular tone from confounding the EndoPAT result, or localised platelet activation post occlusion interfering with activation results. At visit one the initial aspirated sample was collected into four vacuettes for routine analysis. One vacuette tube containing EDTA for full blood count and haemoglobin A1$_c$; one vacuette tube containing serum gel clot activator for biochemical and lipid profile; one vacuette tube containing 3.2% sodium citrate for coagulation screen; and one vacuette tube containing sodium fluoride with potassium oxalate for glucose levels. Then 9mls of blood, for functional assessment, was aspirated into a 10ml syringe containing the anticoagulant tri-sodium citrate (109mM) 1:9 (v/v), followed by gentle mixing. On repeat sampling during visit two, as blood was only required for functional analysis, the initial 3mls aspirated was discarded to exclude venepuncture related platelet activation and then 9mls collected into a syringe again with tri-sodium citrate (109mM).

The vacuette tubes were delivered to Castle Hill Hospital's pathology laboratory for processing as per standard sample protocols. 3mls of the sodium citrate combined sample was transferred to a 15ml falcon tube for platelet rich plasma preparation; and the remaining blood was kept for immediate whole blood testing.

4.2.2.2.2   Preparation of platelet rich plasma (PRP)

For this study PRP was obtained by centrifuging 3ml of whole blood in a 15ml falcon tube for 6 minutes (200g, 20°C). The resultant supernatant PRP layer was aspirated and placed in a PVC reagent reservoir for immediate processing.

4.2.2.3 Methodology for platelet function testing

A 96 well platelet was pre-prepared (figure 4.4) no longer than one hour prior to venepuncture and kept at 4°C in a light controlled environment to prevent fluorescence degradation.

4.2.2.3.1 Isotype controls and population markers

Each analysis had appropriate control samples with whole blood or PRP (5µl) added to wells containing concentration matched negative control fluorescence or platelet marker (5µl) and filtered modified Tyrode's buffer to allow a final volume of sample of 50µl. In the absence of a reliable isotype control for fibrinogen, non-specific binding was measured in duplicate using standard fibrinogen antibody in the presence of EDTA (6mM). Samples were then fixed with 0.2% formylsaline (450µl) and a minimum of ten minutes prior to analysis was mandated.

4.2.2.3.2 Response to PAR1 peptide agonist

At room temperature whole blood (5µl) was added to plate wells using an eight channel multi-tip pipette. The wells contained PE mouse anti-human CD62P or FITC rabbit anti-human fibrinogen antibody (5µl) and modified Tyrode's buffer to ensure total post blood volume of 45µl. 5µl of varying concentrations of PAR1 peptide agonist or buffer was added, and then the samples were fixed with formylsaline after 5 minutes. Gentle mixing following PAR1 addition was achieved with repeated controlled pipetting. All samples were performed in duplicate.

4.2.2.3.3 Response to endothelial derived platelet antagonists following activation

Whole blood (PGI_2 experiments) or PRP (GSNO experiments) (5µl) was added to the wells containing PE mouse anti-human CD62P or FITC rabbit anti-human fibrinogen antibody (5µl) and modified Tyrode's buffer to ensure total post blood/PRP volume of 40µl. 5µl of varying concentrations of the antagonist or buffer was added and left to incubate for two
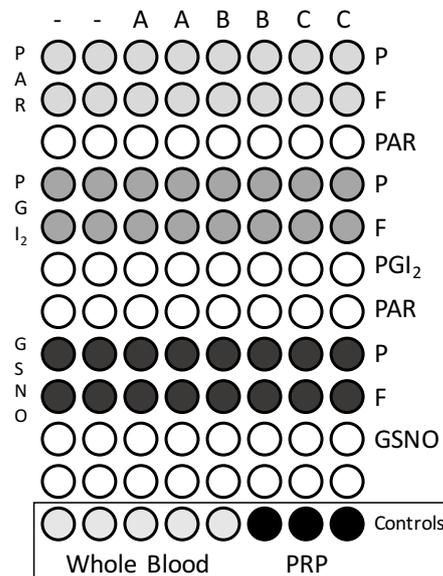
Figure 4. 4: Layout of the 96 well plate

PAR1 peptide agonist (light grey), PGI$_2$ (dark grey) and GSNO (black) dose response samples in duplicate for P-selectin (P) and fibrinogen antibodies (F). Aliquots of vehicle (-), PAR peptide agonist (A=1µM, B=5µM and C=10µM), PGI$_2$ (A=0.1nM, B=1nM and C=10nM), and GSNO (A=1µM, B=10µM and C=100µM) were placed in blank wells for rapid transition into sample wells at appropriate time points. Isotype controls for whole blood and PRP were also present for each subject.

minutes before addition of 10μM PAR1 peptide agonist (5μl). The samples were fixed with formylsaline after a further five minutes. Again, gentle mixing succeeded each 5μl addition. All samples were performed in duplicate.

4.2.2.3.4   Flow cytometric analysis of samples

Samples were transferred from the well into a FACS tube and analysed using a BD FACS Calibur. Prior to study recruitment; instrument settings for voltage, amplitude and mode were optimised using isotype controls. (Table 4.2) No compensation adjustment was required for this protocol as only a single fluorescence was measured per sample. On each occasion 10,000 events were recorded, and background fluorescence was established with FITC and PE controls set at a threshold of 2% positive. The platelet population was identified and isolated using FITC conjugated 42b antibody as previously described. (Section 3.2.4.1.2)

	Direct Functional Assessment			Platelet Leukocyte Aggregates		
	Voltage	Amplitude Gain	Mode	Voltage	Amplitude Gain	Mode
FSC	E00	1.00	Log	E00	1.32	Linear
SSC	350	1.00	Log	360	1.78	Linear
FL1	600	1.00	Log	555	2.47	Log
FL2	627	1.00	Log	525	5.08	Log

Table 4. 2: Voltage and Amplitude settings consistently used throughout study

4.2.2.4   Methodology for platelet-leukocyte aggregate analysis

4.2.2.4.1   Isotype controls and population markers

Each analysis had appropriate control samples with whole blood (30µl) added to tubes containing correctly concentration matched negative control fluorescence, platelet marker and/or leukocyte marker (5µl) and filtered modified Tyrode's to allow final volume of sample of 50µl. Samples were finally lysed with diluted FACS lysis solution X10 (1:9 filtered dH$_2$O) (450µl) and a minimum of ten minutes prior to analysis was mandated.

4.2.2.4.2   Response to PAR1 peptide agonist

At room temperature whole blood (30µl) was added to tubes pre-prepared with Tyrode's buffer (5µl), PE mouse anti-human CD45 (5µl), FITC mouse anti-human CD42a (5µl) and relevant PAR1 concentration/buffer (5µl). The samples were lysed with lysis solution after 5 minutes. Gentle mixing was achieved with finger tap induced gentle agitation. All samples were performed in duplicate.

4.2.2.4.3   Response to endothelial derived platelet antagonists following activation

At room temperature whole blood (30µl) was added to tubes pre-prepared with PE mouse anti-human CD45 (5µl), FITC mouse anti-human CD42a (5µl) and relevant PGI$_2$ concentration/buffer (5µl). Following 2 minutes incubation 10µM PAR1 peptide agonist (5µl) was added. The samples were lysed with lysis solution after a further 5 minutes. Gentle mixing was achieved with finger tap induced gentle agitation following each addition to tube. All samples were performed in duplicate.

4.2.2.4.4 Flow cytometric analysis of samples

As performed previously, the samples were analysed on BD FACS Calibur, and settings were optimised prior to participant recruitment. (Table 4.2) As dual fluorescence was required for cell labelling appropriate compensation values were manually calculated (figure 3.2) and fixed for all analyses. The analysis protocol has previously been described (section 3.2.5.2) and has been covered further in figure 4.5.
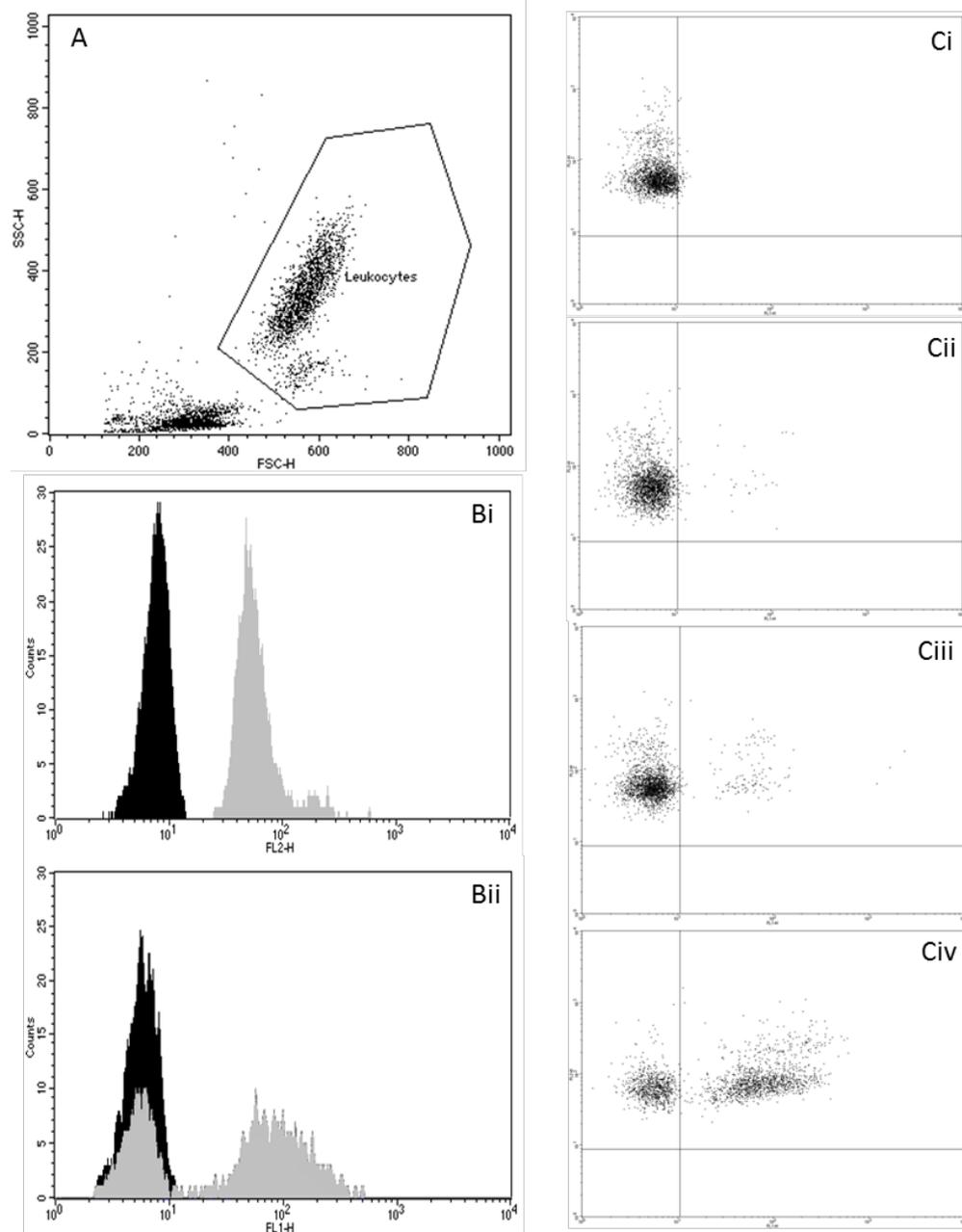
Figure 4. 5: Platelet leukocyte aggregate measurements

The leukocyte population was isolated based on FSC and SSC pattern (A) with background PE (fluorescence channel 2 (FL2) (Bi)) and FITC (fluorescence channel 1 (FL1) (Bii)) recorded (black histograms). Correct isolation was confirmed using PE conjugated CD45, a leukocyte specific marker (grey histogram Bi). A scatter plot of FL2 against FL1 for the CD45 labelled sample with IgG FITC control enabled FL1 background fluorescence grid to be set at 2% (Ci). Therefore, percentage of events in the right upper quadrant represent FITC CD42a positive / PE CD45 positive cells indicating a PLA. The percentage is seen to increase in response to PAR1 peptide agonist (0μM (Cii), 1μM (Ciii), 10μM (Civ, and grey histogram Bii)).

4.2.2.5   Methodology for phosphoflow and fluorescent cell barcoding

Phosphoflow and fluorescent cell barcoding (FCB) were performed as previously described. (221) Whole blood samples were again incubated with PGI$_2$ (0-100 nM) before addition of vehicle or PAR1 peptide agonist (10 μM) in a 96-well plate, before fixing with BD Phosphoflow Lyse/Fix Buffer for 10 minutes. The samples were then centrifuged for 10 minutes (*1000g*, 4°C) and the supernatant aspirated. The pelleted cells were resuspended in 200 μL of ice-cold 0.1% Triton X-100 in phosphate buffered saline (PBS) (1.8mM KH2PO4, 10mM Na2HPO4, 2.7mM KCl, 137mM NaCl, pH 7.4) to induce permeabilisation. Following a further 10 minutes (on ice) the plate was centrifuged as per previous settings, washed (removal of supernatant and resuspension in PBS (200μl)) and spun again. The next step after removal of the supernatant, was to resuspend in 100μl PBS and label with Pacific Blue NHS Ester (0, 0.02, 0.06, 0.18, 0.5 μg/mL) and Alexa Fluor 488 NHS Ester (0, 0.5 μg/mL) to create unique fluorescent barcodes for individual samples. (Figure 4.6) Barcoded samples were combined and stained with rabbit PhosphoVASP (Ser157, 2 μg/mL) for 30 minutes on ice. Between the fourth and fifth spin (10 minutes, 1000*g*, 4°C) was a further wash step, followed by the addition of the secondary fluorescence labelled antibody anti-rabbit Alexa Fluor 647 (1μg/ml) and mouse CD45-PE in 100μl of PBS incubated for 30 minutes in a darkened environment. A final wash step with associated centrifuge spins was mandated prior to resuspension in 150μl of PBS and transfer into tubes for FACS analysis. Processing of samples was assisted by Dr Benjamin Spurgeon PhD.

4.2.2.5.1   Flow cytometric analysis of samples

Combined and stained samples were acquired on an optimized BD FACS Fortessa. Platelets were isolated based on their unique forward and side scatter, while leukocytes were gated on side scatter versus CD45. Data was deconvoluted and analysed with Cytobank. (222)
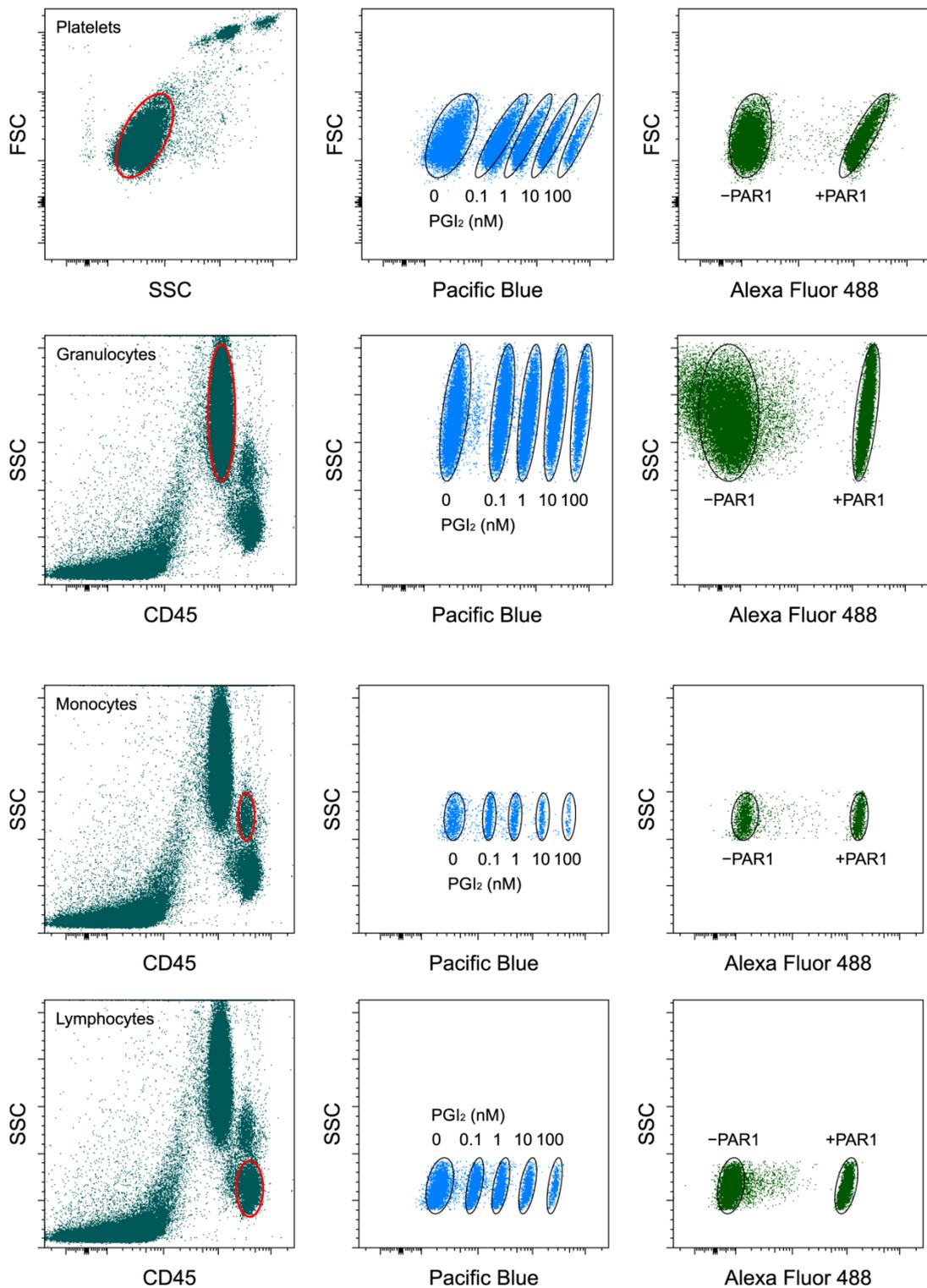
Figure 4. 6: Population isolation and sample bar coding

Scatter plots generated on flow cytometry demonstrating ability to isolate specific cell populations based on unique fluorescence and side scatter properties, allowing analysis of targeted cells representing different experimental conditions in a single tube.

4.2.2.6   Methodology for endothelial assessment

Study participants were assessed in a temperature controlled dimly lit quiet environment. While in a recumbent position a standard blood pressure reading was attained from the non-dominant arm at least five minutes prior to performing the recording. The pneumatic endo probes were placed in the index fingers of both hands and the manual sphygmomanometer cuff was positioned on the upper arm of the participant's non-dominant arm. Once the probes were inflated with an acceptable signal achieved, the participant was asked to relax in silence and the recording was commenced. A baseline, period of occlusion and post occlusion measurement were recorded, each for five minutes. The occlusion was obtained by rapid inflation of the cuff to 60mmHg greater than systolic blood pressure (minimum pressure 200mmHg and maximum pressure 300mmHg) confirmed with absence of pulsatile signal on the relevant probe reading. On completion of the test, satisfactory recording was confirmed, and automated analysis performed.

4.2.3   Data collection

Data was collected and stored on a standardised case report form and pseudo-anonymised on an encrypted uniform spreadsheet. Following analysis of the experiments described the variables below were collected.

4.2.3.1   Baseline data

Age	years		
Waist Circumference	cm		
Height	cm		
ECG	12 lead		
Smoking	Pack years (1 pack year = 20 cigarettes/day for 1 year)		
Medications	Allergies	Past Medical History	Bleeding Risk Assess

4.2.3.2   Baseline blood results

Full Blood Count		Renal Function		Liver Function	
Haemoglobin	g/L	Sodium	mmol/L	Alanine Transaminase	iU/L
White Cell Count	x10^9/L	Potassium	mmol/L	Alkaline Phosphatase	iU/L
Platelets	x10^9/L	Urea	mmol/L	Bilirubin	Umol/L
Mean Cell Volume	fL	Creatinine	µmol/L	Albumin	g/L
Mean Corpuscular Haemoglobin Concentration	g/L	estimated Glomerular Filtration Rate	ml/min		
Red Blood Cells	x10^{12}/L				
Mean Cell Haemoglobin	pg/cell				

Coagulation Screen		Other	
Prothrombin Time	seconds	HbA1c	mmol/mol
Activated Partial Thromboplastin Time	seconds	Chol (HDL/LDL)	mmol/L
Fibrinogen	g/L		

4.2.3.3 Platelet/leukocyte analysis

Results were collected when subject was on established aspirin therapy and following three days of oral ticagrelor to reach steady state.

Fibrinogen binding	Percentage positive cells (%)
	Median Fluorescence Intensity of positive cells (MFI)
	Mean Fluorescence Intensity
	Total Binding Index (TBI) (percentage positive cells x mean fluorescence intensity x 0.01)
	Percentage Inhibition (100 – ((percentage of positive cells/percentage of cells positive in control) x 100))
P-selectin expression	Percentage positive cells
	MFI
	Mean fluorescence intensity
	TBI
	Percentage Inhibition
Inhibition	Additive inhibition (percentage inhibition induced by ticagrelor alone added to the percentage inhibition induced by endothelial vasodilator alone)
	True inhibition (percentage inhibition induced by ticagrelor in combination with endothelial vasodilator)
Platelet Leukocyte Aggregates (PLA)	Percentage of cells positive for CD42a and CD45 associated fluorescence (%)
Vasodilator stimulated phosphoprotein phosphorylation (phosphoVASP)	MFI
	Fold change (MFI/MFI at baseline)

4.2.3.4   Endothelial function testing

Results were collected when the subject was established on aspirin therapy and following three days of oral ticagrelor.

- RHI (Reactive Hyperaemia Index)

    o The post-to-pre occlusion signal ratio in the occluded side, normalized to the control side and further corrected for baseline vascular tone

    o Normal: RHI > 1.67   Abnormal: RHI ≤ 1.67

- LnRHI (RHI after natural log transformation with a matched cut off)

    o Normal: LnRHI > 0.51   Abnormal: LnRHI ≤ 0.51

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4.2.3.5   Clinical follow up

At one month a final contact was made with the patient to ensure no adverse events or concerns.

4.2.4 Statistical analysis

Categorical variables are listed as numbers and percentage, while continuous variables stated mean and standard deviation. The primary analysis was using a paired t-test to look at the change in the variables from pre to post ticagrelor dosing. Area under the response curves (AUC) enabled assessment of overall effect. In measurements not suitable for AUC calculation, two-way ANOVA was used. The secondary focus of the analysis was to explore the effects of smoking, diabetes and endothelial function on these outcomes. One-way ANOVA analysis was undertaken by using various quantifications of fluorescence expression. Correlation was assessed using linear regression. P values of <0.05 were considered to indicate statistical significance. All analyses were undertaken on SPSS version 24. The final statistical methodology and study results were kindly verified by Hull York Medical School statistician Dr Victoria Allgar.

4.2.4.1   Sample size calculation

The sample size was based on Gremmel et al paper (105) findings, which quoted a percentage increase in P-selectin expression of 50.1% when comparing patients with and without diabetes, in the context of CAD. A sample size of 63 would have 95% power to detect a difference in means of 23.2 (e.g. a first condition mean of 46.3 and second mean of 69.5), based on a paired t-test with a 5% significance level. Due to the cohort being split into four sub groups, for ease we decided to recruit 64 patients.

4.2.5   Quality control and assurance

4.2.5.1   Peer review

This trial was peer reviewed and supported by University (Hull York Medical School) affiliated medical Professors/Consultants with specialist interests in platelet function, vascular pathology and endothelial function. The study protocol was also submitted to the Trans-Humber Consumer Research Panel for review and received their full endorsement of the study and associated subject related documents.

4.2.5.2   Monitoring

The study was monitored in accordance with Hull and East Yorkshire Hospitals NHS Trust Research and development (R&D) department's standard operating procedures to ensure compliance with International Conference for Harmonisation of Good Clinical Practice (ICH GCP) and the Research Governance Framework 2005. Data was collected and retained in accordance with the Data Protection Act 1998.

Interim analyses were performed after results obtained for the first 10 patients and following 50% recruitment by a data monitoring committee (DMC). No members of the committee were affiliated with the research team. This constituted two interventional cardiology consultants, a specialist registrar in cardiology and a member of the R&D team. They reviewed all the pseudo-anonymised data/results available and were made aware of any adverse events, adverse reactions or serious adverse events. No concerns were raised.

Within the protocol it was stipulated that if prior to the first DMC review there was a major bleeding event (Bleeding Academic Research Consortium (BARC) type 3 or 5), then an earlier meeting was to be conducted. Furthermore, if a greater than 8% major bleeding event rate occurred at any stage in the study, recruitment would be suspended and await formal investigation by the Sponsor in conjunction with the DMC. This percentage was guided by bleeding rates found in previous large cohort studies. (34,55)

4.2.5.3   Ethical considerations

4.2.5.3.1   Patient groups 1-4

Medication Safety

The patients were subject to minimal deviation from best/standard practice compared to their non-study counterparts. They were selected as clinically requiring PCI, and there was no delay to therapy. They received dual antiplatelet therapy, but for the study it was aspirin and ticagrelor rather than NICE recommendation of clopidogrel with aspirin. Although evidence does support superiority of ticagrelor compared to clopidogrel, in reducing adverse events without increasing bleeding rates, in the management of patients with stable angina in addition to ACS. (223) The ticagrelor was commenced three days prior to PCI which is contrary to routine, resulting in three days extra of therapy.

Patient Burden

The visits required were designed to coincide with routine attendance to Castle Hill Hospital to prevent unscheduled visitation requirements. However, the first pre-assessment visit involved a longer time frame than routine, by approximately 15-30 minutes. The visits included two relatively low risk procedures, venepuncture and EndoPAT. Venepuncture has a low risk of haematoma formation, bleeding, infection and vasovagal syncope. With appropriate preparation and aseptic technique these levels in the normal population are minimal. (Figure 4.1)

Confidentiality

Patients were informed that their personal information was to be stored externally to their notes, but reassurance was given to the safety of this information and no identifiable data was available electronically or on the final publication.

4.2.5.3.2 Control group

Medication Safety

This group were required to take 10 days of 75mg aspirin and also 3 days 90mg twice daily of ticagrelor which was not clinically indicated. In studies of "normal" subjects administered ticagrelor there is a low risk of serious adverse events. Butler and Teng looked at safety and tolerability in healthy volunteers, with many of the administered doses being significantly higher and for a longer duration than those used in this study, without concern. (42)

Subject Burden

Control subjects were required to commit to three visits over the course of ten days. Incorporated into these visits were two low risk procedures, venepuncture and non-invasive EndoPAT as above. (Figure 4.1)

Confidentiality

Subjects were informed that their information will be stored externally to any notes, but reassurance given to the security of this information. No identifiable data was available electronically or included in the final publication.

Incentive

In the absence of clinical benefit, with possible risk, there was financial incentive of transport payment and book token.

4.2.5.4 Ethics and R&D approval

This study was reviewed, and favourable outcome was granted by National Research Ethics Service Committee Yorkshire and the Humber – South Yorkshire (14/YH/0179). The study was conducted in accordance with the declaration of Helsinki.

The study was performed with Site Specific Assessment (SSA) approval and had Hull and East Yorkshire Trust R&D Department support. It was also registered with clinicaltrials.gov (NCT02169596).

4.3   RESULTS

Overall 74 subjects were recruited, with one participant withdrawal between first and second study visit due to ticagrelor intolerance secondary to dyspnoea. None of their data was included in the analyses.

4.3.1   Control characteristics and laboratory results

Ten healthy non-smokers (70% males) were recruited to the control arm of the study with a mean age of 56 ± 9 years. Baseline blood tests were unremarkable. (Table 4.3)

4.3.1.1   Platelet activation in response to PAR1 peptide agonist

The PAR1 peptide agonist (0-10μM) induced significant platelet activation (p<0.001), which was markedly inhibited following three days of oral ticagrelor (p<0.001). (Figure 4.7)

				Normal Range
Total number	10	Platelets (x10^9/L)	238 ± 35	(150-400)
Male (%)	7 (70)	Neutrophils (x10^9/L)	3.1 ± 0.9	(2.0-7.7)
Mean age (years)	56 ± 9	Monocytes (x10^9/L)	0.4 ± 0.1	(0.2-0.8)
Mean BMI	26.6 ± 2.0	Haemoglobin (g/L)	143 ± 11	(135-175)
Glucose (mmol/L)	5.1 ± 0.3	PT (secs)	11.5 ± 0.9	(10.0-13.5)
HbA1$_c$ (mmol/mol)	37 ± 4	APTT (secs)	33.8 ± 3	(28.0-38.0)
LDL Cholesterol (mmol/L)	3.3 ± 0.6	eGFR (ml/min)	95 ± 8	(>90)
HDL Cholesterol (mmol/L)	1.5 ± 0.3	Albumin (g/L)	38 ± 3	(35-55)

Table 4. 3: Basic demographic data of the healthy control participants

Results expressed as mean ± standard deviation, with normal ranges available for comparison.
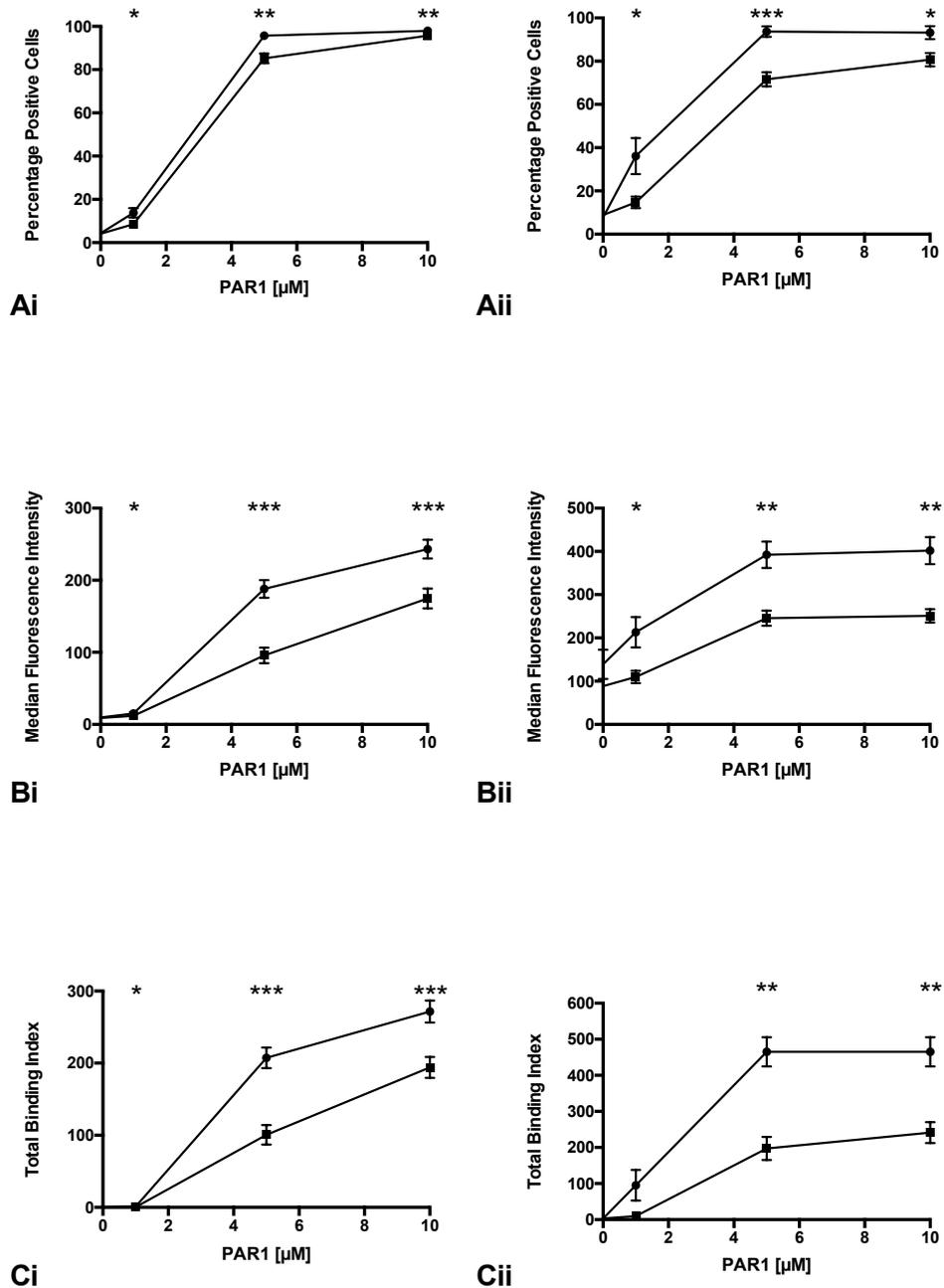
Figure 4. 7: Concentration response curve to PAR1 peptide agonist in healthy controls

*Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor. Samples were analysed by flow cytometry with data expressed as percentage of platelets positive for fluorescence (A), the median fluorescence expressed by the platelets (B) and the total binding index (C) for P-selectin expression (i) and fibrinogen binding (ii). N =10 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.*

4.3.1.2   Platelet response to prostacyclin

PGI_2 (0.1-10nM) inhibited platelet activation by a PAR1 peptide agonist in a dose dependent fashion, as measured by P-selectin expression and fibrinogen binding ($p<0.001$). (Figure 4.8) Post ticagrelor, PGI_2 induced a significantly greater level of inhibition and was greater than the expected if inhibition was additive. This difference reached statistical significance for P-selectin expression inhibition at 1 and 10nM PGI_2, where inhibition of $11.2\pm2.6\%$ and $57.1\pm9.1\%$ was seen, compared to the anticipated $3.6\pm0.6\%$ and $28.1\pm6.0\%$, respectively. (Figure 4.9)
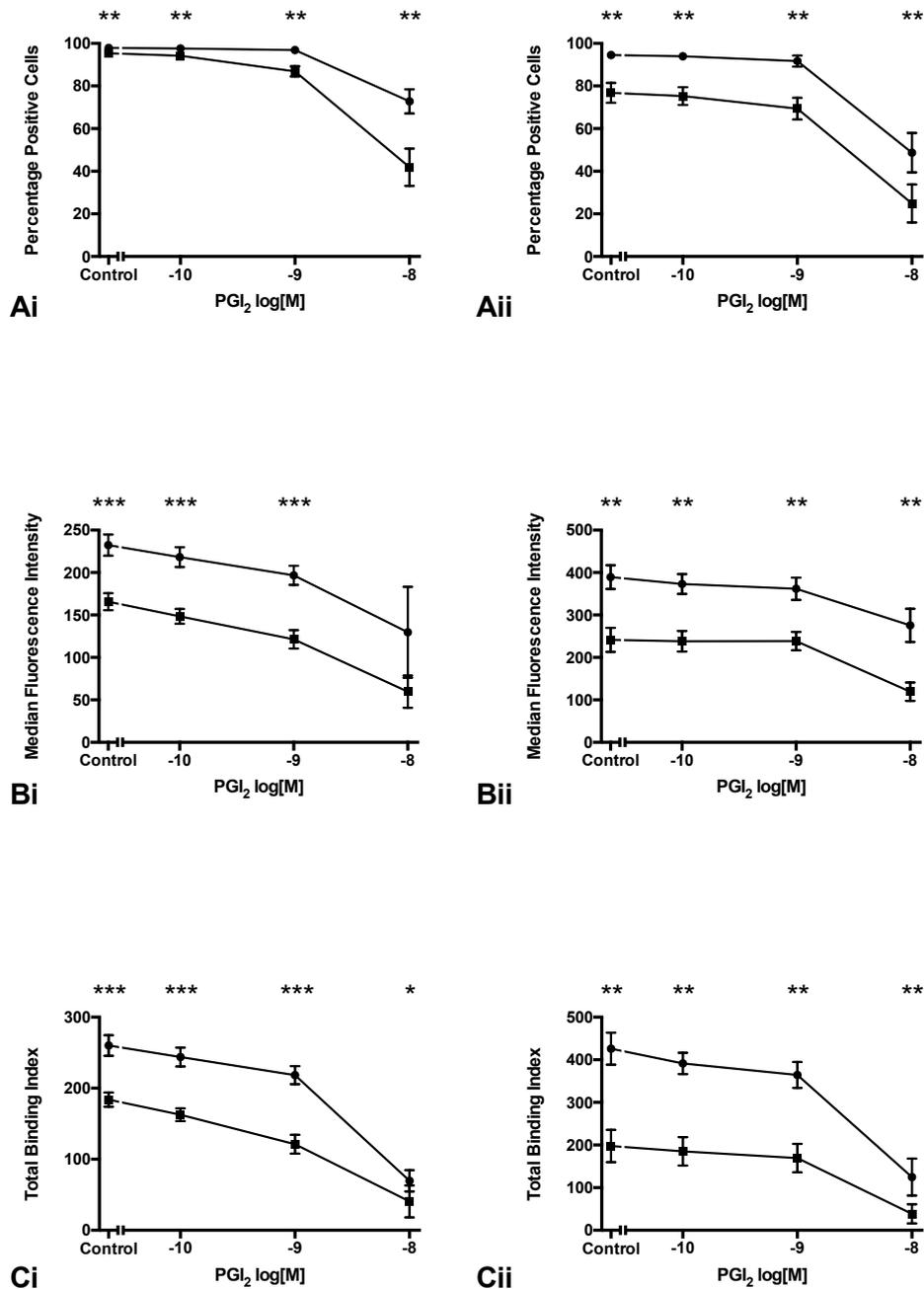
Figure 4. 8: Dose response curve to prostacyclin in healthy controls

*Whole blood was incubated with variable doses of PGI₂ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry with data expressed as percentage of platelets positive for fluorescence (A), the median fluorescence expressed by each platelets (B) and the total binding index (C) for P-selectin expression (i) and fibrinogen binding (ii). N=10 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.*
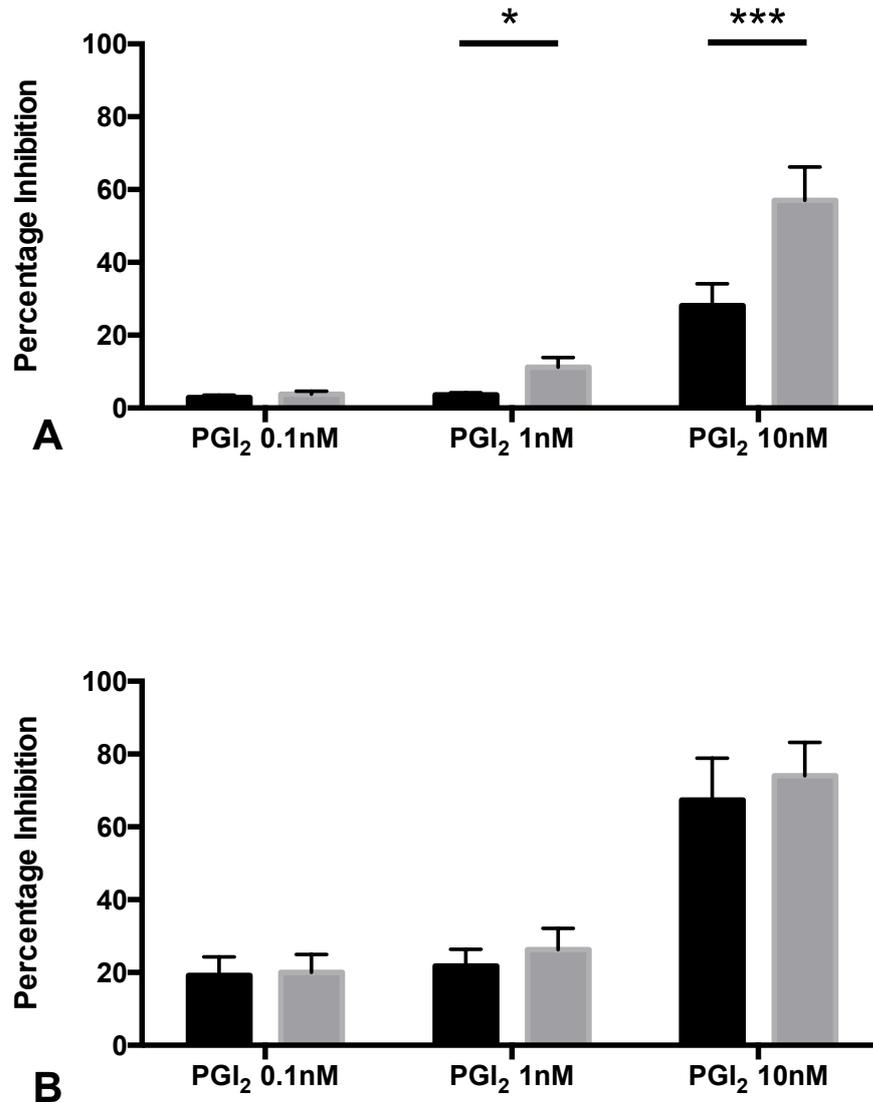
Figure 4. 9: Histogram of prostacyclin-induced inhibition of platelet activation in healthy controls

Whole blood was incubated with variable doses of PGI_2 (0-10nM) pre and post three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist ($10\mu M$). Samples were analysed by flow cytometry, with data recorded as percentage of platelets positive for P-selectin (A) and fibrinogen binding (B) associated fluorescence. Inhibition was calculated as percentage of positive platelets compared to the maximally stimulated sample ($100 - $ ((percentage of positive cells/percentage of cells positive in control) x 100). Bars represent additive inhibition (pre ticagrelor PGI_2 + ticagrelor alone inhibition) (black) compared to true post-ticagrelor inhibition seen in response to PGI_2 (grey). N=10 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4.3.1.3 Platelet response to nitric oxide

GSNO (1-100μM) caused a concentration dependent inhibition of fibrinogen binding and P-selectin expression (figure 4.10, $p<0.001$, except Bii). The results following ticagrelor were universally lower, all attaining a statistically significant reduction. Analysis of the expected additive inhibition versus actual post ticagrelor results revealed the same trend as PGI$_2$. Inhibition of P-selectin expression was significantly greater following ticagrelor with GSNO 1, 10, 100μM (observed levels 8.8$\pm$1.9%, 21.3$\pm$4.7%, and 29.9$\pm$4.6%, compared to the expected levels 5.0$\pm$1.2%, 9.0$\pm$2.9%, and 13.3$\pm$3.6% respectively). Similarly, fibrinogen binding was significantly increased with GSNO 10μM (observed level 64.8$\pm$9.4% compared to the expected level 50.1$\pm$9.3%). (Figure 4.11)
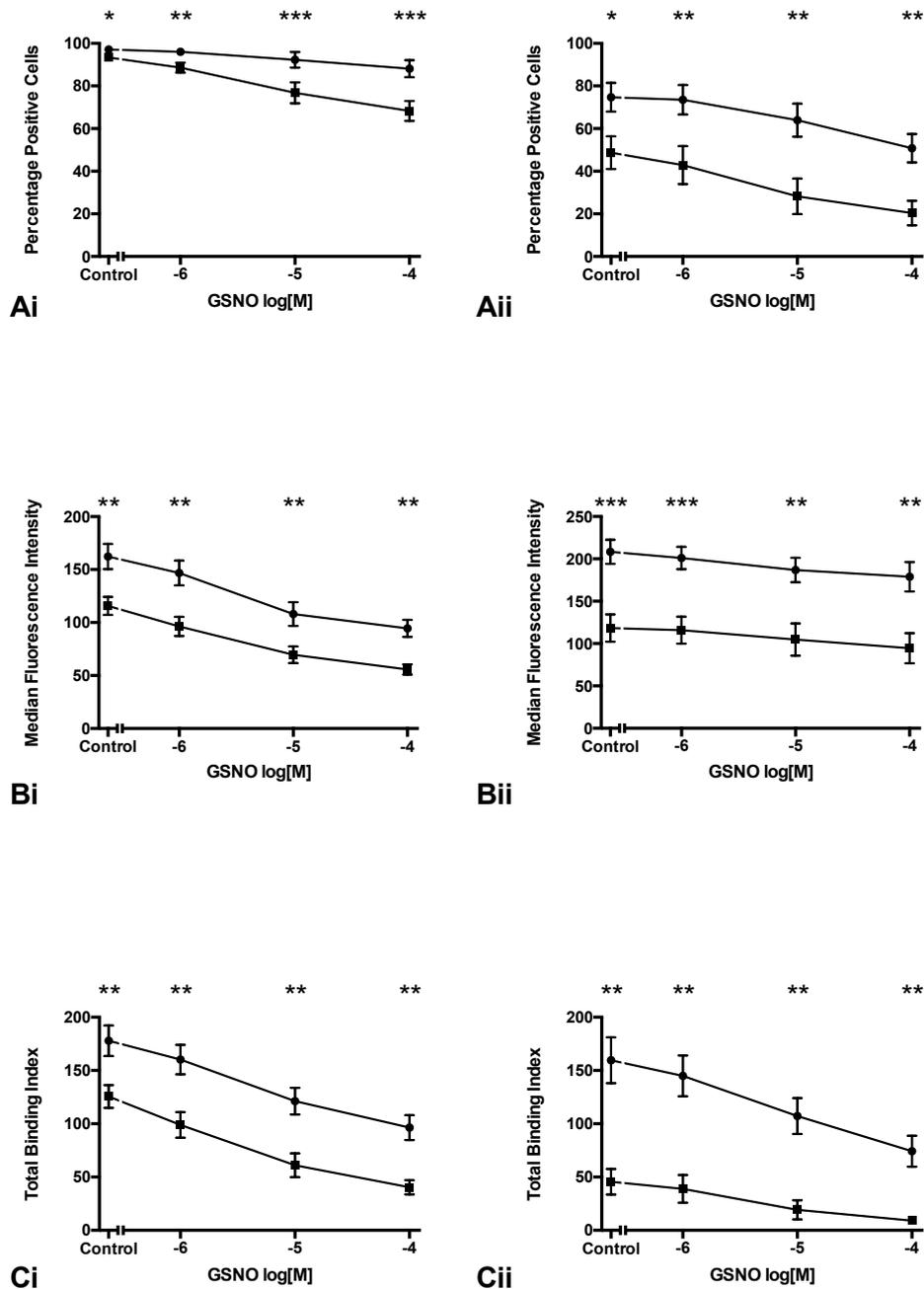
Figure 4. 10: Dose response curve to nitric oxide in healthy controls

PRP was incubated with variable doses of GSNO (0-100μM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry with data expressed as percentage of platelets positive for fluorescence (A), the median fluorescence expressed by each platelets (B) and the total binding index (C) for P-selectin expression (i) and fibrinogen binding (ii). N =10 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and ***p<0.001

Figure 4. 11: Histogram of nitric oxide induced inhibition of platelet activation in healthy controls

PRP was incubated with variable doses of GSNO (0-100μM) pre and post three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry, with data recorded as percentage of platelets positive for P-selectin (A) and fibrinogen binding (B) associated fluorescence. Inhibition was calculated as percentage of positive platelets compared to the maximally stimulated sample (100 − ((percentage of positive cells/percentage of cells positive in control) x 100). Bars represent additive inhibition (pre ticagrelor GSNO + ticagrelor alone inhibition) (black) compared to true post ticagrelor inhibition seen in response to GSNO (grey). N =10 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.

4.3.1.4 Platelet-leukocyte aggregate response

Incubation of whole blood with PAR1 peptide agonist (0-10μM) increased the percentage of platelet-leukocyte aggregates (PLA) formed, in a dose-dependent manner from 20.1$\pm$4.4% to a maximum of 86.7$\pm$2.8% (p<0.001). PLA formation correlated with the direct measures of platelet activation. Overall ticagrelor reduced PLA formation (p=0.001), most notably at the lower concentrations of PAR1 peptide agonist (1μM 27.7$\pm$4.6% to 16.9$\pm$3.2% post ticagrelor). However, at the highest concentration of the PAR1 peptide agonist (10μM) there was no statistical difference in the result (86.7$\pm$2.8% and 80.1$\pm$3.0% post ticagrelor, p=0.907). When the effect of PGI_2 was tested on aggregate numbers, the prostanoid was found to have little effect. However, PGI_2 post ticagrelor led to a substantial 59% reduction in PLA numbers (p=0.008) (activated baseline 84.3$\pm$2.9%, post PGI_2 10nM 81.9$\pm$2.8%, post ticagrelor 83.9$\pm$2.0%, post PGI_2 10nM and ticagrelor 48.8$\pm$8.9%). (Figure 4.12)

In conclusion, the control group data complemented the *in vitro* experimental conclusions with demonstration that ticagrelor and the endogenous endothelial dilators relationship was synergistic with regard to platelet inhibition and therefore supporting continuation of the study to enrolment of the patient cohort.
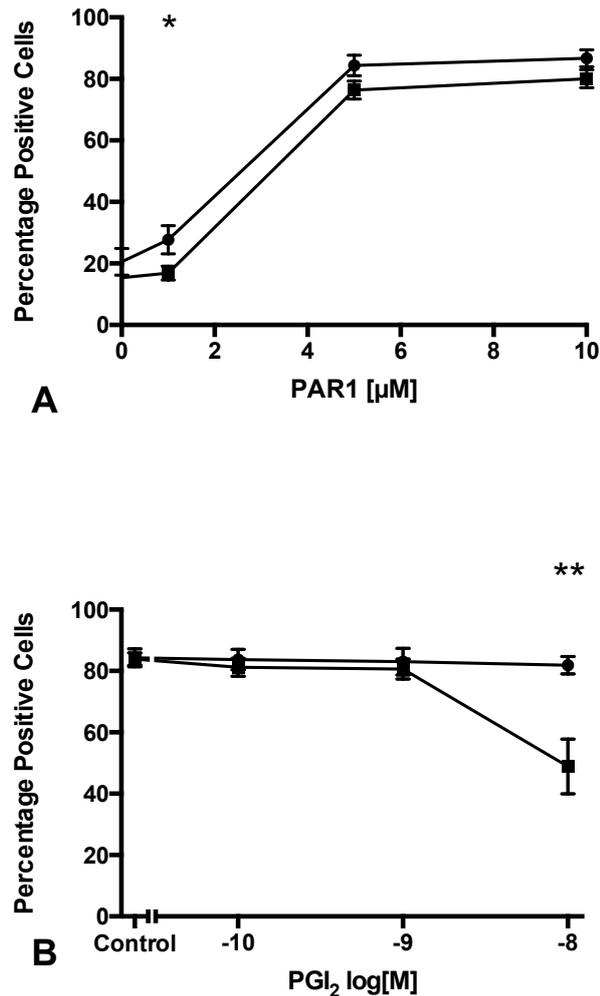
Figure 4. 12: Platelet leukocyte aggregate experiments in healthy controls

Concentration response curve to PAR1 peptide agonist (A) Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor.

Dose response curve to prostacyclin (B) Whole blood was incubated with variable doses of PGI$_2$ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM).

Samples were analysed by flow cytometry with data collected as percentage of cells expressing platelet (CD42a) associated fluorescence within the isolated leukocyte population, representing presence of a PLA. N=10 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.

4.3.2 Patient characteristics and laboratory results

There were 63 patients (47 male) with known CAD recruited to the study and their characteristics are outlined in table 4.4. The mean age of the population was 63 ± 9 years and included 25 patients (40%) with diabetes mellitus. This is a relatively high frequency as patients with diabetes were actively sought to enable subgroup analysis. This may also account for the increase in HbA1$_c$ levels (51mmol/mol), fasting glucose (7.3 mmol/L) and body mass index (29.7kg/m^2) seen. No marked abnormality was noted in the full blood count, liver function or renal function.

4.3.2.1 Platelet activation in response to PAR1 peptide agonist

The PAR1 peptide agonist induced a concentration dependent activation of the platelet population ($p<0.001$), with 98% of cells expressing P-selectin and fibrinogen binding at maximal activation (10μM). The EC$_{50}$ for the total binding index curve pre ticagrelor was 3.5μM and 2.1μM for P-selectin and fibrinogen binding respectively. Ticagrelor did not alter basal levels of activation but significantly inhibited the agonists effect even at low concentrations (1μM). (Figure 4.13)

	Patients	Normal Range	Controls	p value
Total number	63		10	
Male (%)	47 (75)		7 (70)	0.573
Mean age (years)	63 ± 9		56 ± 9	0.960
Mean BMI	29.7 ± 5.3		26.6 ± 2.0	**0.012**
Smokers (%)	25 (40)		0	
Diabetes Mellitus (%)	25 (40)		0	
Insulin therapy (%)	6 (24)			
Glucose (mmol/L)	7.3 ± 3.7		5.1 ± 0.3	**0.016**
HbA1$_c$ (mmol/mol)	51 ± 17		37 ± 4	**0.003**
LDL Cholesterol (mmol/L)	2.4 ± 1.1		3.3 ± 0.6	0.093
HDL Cholesterol (mmol/L)	1.2 ± 0.3		1.5 ± 0.3	0.624
Platelets (x10^9/L)	237 ± 50	(150-400)	238 ± 35	0.324
Neutrophils (x10^9/L)	4.8 ± 1.6	(2.0-7.7)	3.1 ± 0.9	0.096
Monocytes (x10^9/L)	0.7 ± 0.2	(0.2-0.8)	0.4 ± 0.1	0.05
Haemoglobin (g/L)	140 ± 12	(135-175)	143 ± 11	0.797
PT (secs)	11.4 ± 0.8	(10.0-13.5)	11.5 ± 0.9	0.328
APTT (secs)	32.7 ± 3.1	(28.0-38.0)	33.8 ± 2.9	0.982
eGFR (ml/min)	86 ± 19	(>90)	95 ± 8	0.077
Albumin (g/L)	38 ± 3.0	(35-55)	38 ± 3.2	0.607

Table 4. 4: Basic demographic data of the patient group

Continuous data expressed as mean ± standard deviation and categorical characteristics expressed as number (percentage). Control data included and compared using one-way ANOVA and chi-squared test for continuous and categorical data respectively. p<0.05 was deemed significant.
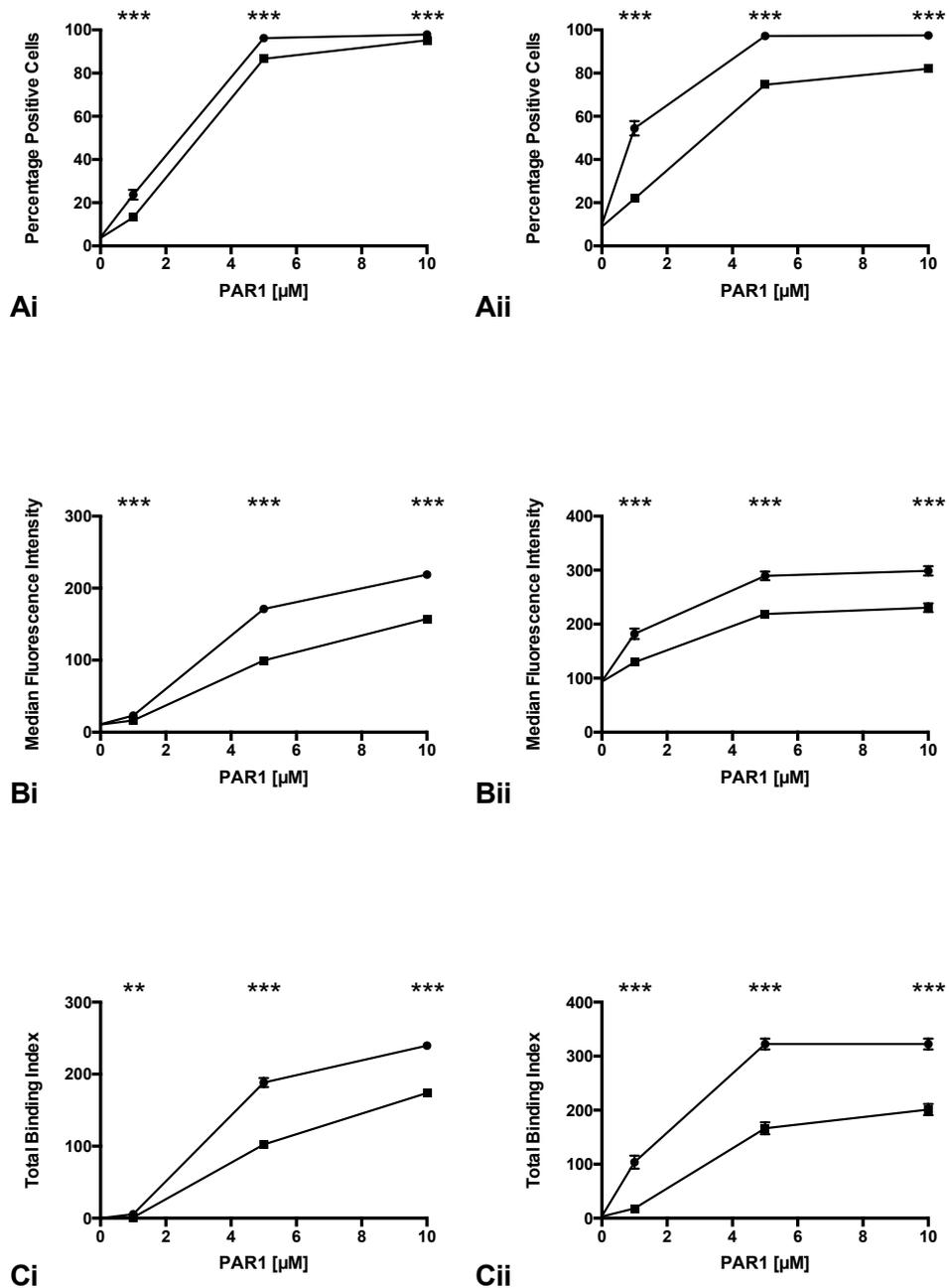
Figure 4. 13: Concentration response curve to PAR1 peptide agonist in patients

Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor. Samples were analysed by flow cytometry with data expressed as percentage of platelets positive for fluorescence (A), the median fluorescence expressed by each platelets (B) and the total binding index (C) for P-selectin expression (i) and fibrinogen binding (ii). N=63 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.

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4.3.2.2 Platelet response to prostacyclin

PGI_2 and ticagrelor both independently inhibited platelet activation (TBI compared to stimulated baseline $p \leq 0.002$. P-selectin expression activated baseline TBI 236±6, post PGI_2 10nM 88±6, post ticagrelor 177±5. Fibrinogen binding baseline TBI 318±10, post PGI_2 10nM 130±11, post ticagrelor 181±10). (Figure 4.14) PGI_2 inhibited platelet P-selectin expression and fibrinogen binding in a concentration dependent exponential fashion (P-selectin 1.6±0.2% (1nM) and 21.8±2.7% (10nM); and fibrinogen 1.6±0.4% (1nM) and 34.1±3% (10nM)). While in whole blood ticagrelor induced 2.3±0.3% inhibition of P-selectin expression and 17.0±1.4% inhibition of fibrinogen binding in the activated platelets.

PGI_2 and ticagrelor were shown to act synergistically, with the combined antagonism of platelet activation being significantly greater than that expected by additive effect alone. The anticipated inhibition of PGI_2 10nM and ticagrelor on markers of platelet activation was 22.5±2.9% and 51.0±3.8% (P-selectin and fibrinogen binding, respectively), calculated from the addition of the independent percentages of inhibition. However, the observed inhibition was 59.3±2.0% and 88.0±11% (P-selectin and fibrinogen binding, respectively), representing a 2.6 and 1.7-fold increase. Thus, providing evidence for the enhanced antiplatelet activity of PGI_2 in the presence of ticagrelor. (Figure 4.15)
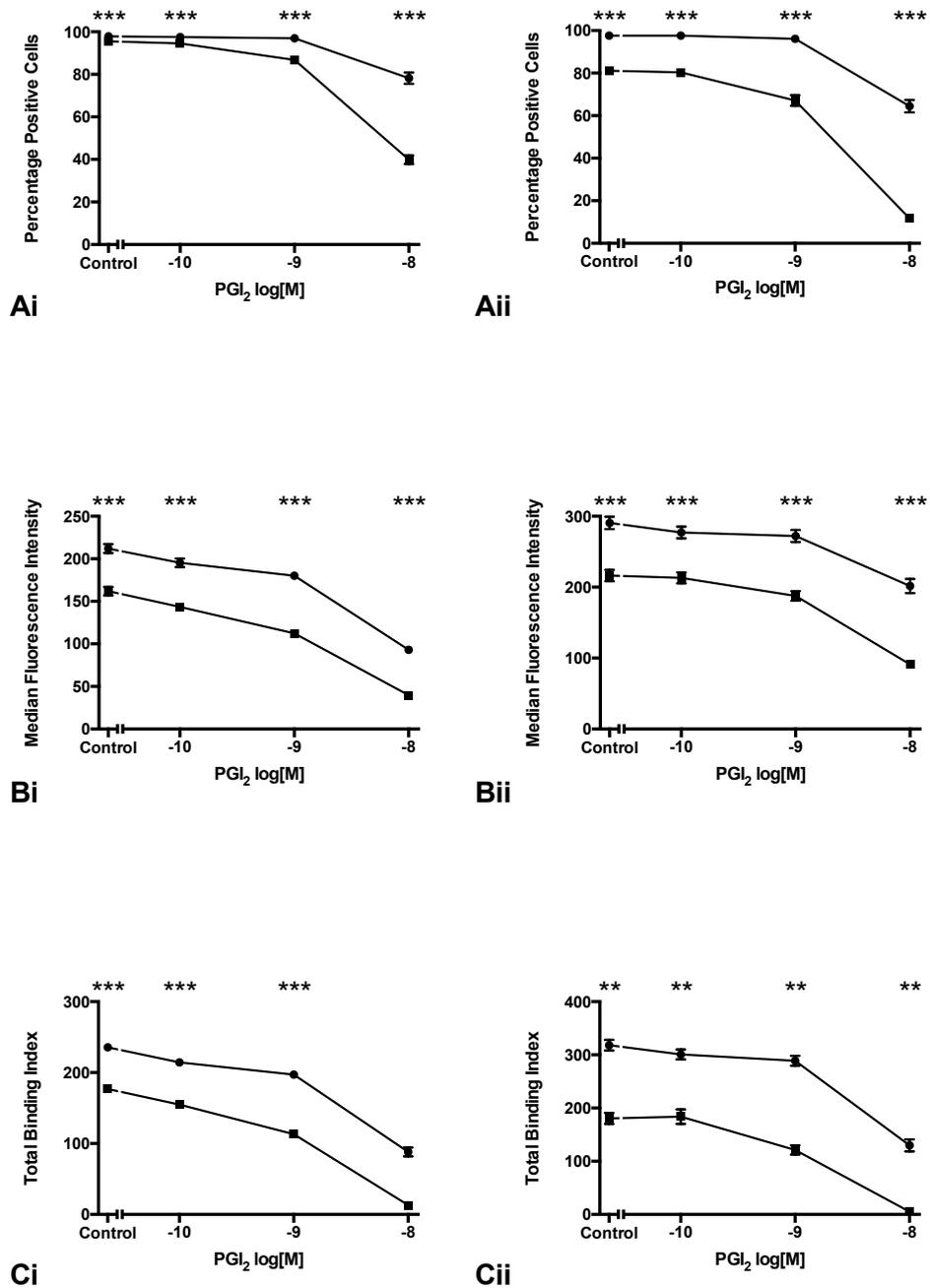
Figure 4. 14: Dose response curve to prostacyclin in patients

*Whole blood was incubated with variable doses of PGI₂ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry with data expressed as percentage of platelets positive for fluorescence (A), the median fluorescence expressed by each platelets (B) and the total binding index (C) for P-selectin expression (i) and fibrinogen binding (ii). N=63 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.*

Figure 4. 15: Histogram of prostacyclin induced inhibition of platelet activation in patients
Whole blood was incubated with variable doses of PGI₂ (0-10nM) pre and post three days
of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10µM). Samples
were analysed by flow cytometry, with data recorded as percentage of platelets positive for
p-selectin (i) and fibrinogen binding (ii) associated fluorescence. Inhibition was calculated
as percentage of positive platelets compared to the maximally stimulated sample (100 −
((percentage of positive cells/percentage of cells positive in control) x 100). Data displayed
for pre (black) versus post (grey) ticagrelor (A) and additive inhibition (inhibition by
ticagrelor alone plus inhibition by relevant PGI₂ concentration alone) (red) versus actual
inhibition seen post ticagrelor (grey) (B). Flow cytometry histograms are shown for PGI₂
10nM (C) (control (grey), PGI₂ 10nM alone (black outline), ticagrelor alone (red outline) and
PGI₂ post ticagrelor (red)) and are representative of the mean. N=63 and error bar
represents standard error of the mean. Results were compared using Student's t-test and
significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.

4.3.2.3   Response to nitric oxide

NO and ticagrelor were significant inhibitors of platelet activation in response to PAR1 peptide agonism. (TBI compared to stimulated baseline $p < 0.001$. P-selectin expression activated baseline TBI 215 ± 5, post GSNO 100µM 139 ± 5, post ticagrelor 161 ± 5. Fibrinogen binding baseline TBI 211 ± 9, post GSNO 100µM 133 ± 8, post ticagrelor 71 ± 6). (Figure 4.16) GSNO reduced the percentage of platelets exhibiting markers of activation relative to concentration (P-selectin $1.5 \pm 0.3\%$ (10µM) and $3.8 \pm 0.6\%$ (100µM); and fibrinogen $8.3 \pm 0.9\%$ (10µM) and $16.5 \pm 1.4\%$ (100µM)). In platelet rich plasma, ticagrelor generated $2.2 \pm 0.4\%$ inhibition of P-selectin expression and $35.4 \pm 2.6\%$ inhibition of fibrinogen binding.

The findings for GSNO post ticagrelor were consistent with PGI_2 and demonstrated synergism of the inhibitors with a 2.8 and a 1.3-fold increase on anticipated inhibition of P-selectin expression and fibrinogen binding, respectively. The calculated additive inhibition for P-selectin and fibrinogen was $6.0 \pm 0.8\%$ and $52.7 \pm 3.5\%$ respectively, compared to the actual inhibition seen of $16.5 \pm 1.4\%$ and $68.8 \pm 2.4\%$. This indicates that ticagrelor enhances the platelet inhibitory action of NO beyond an additive effect. (Figure 4.17)
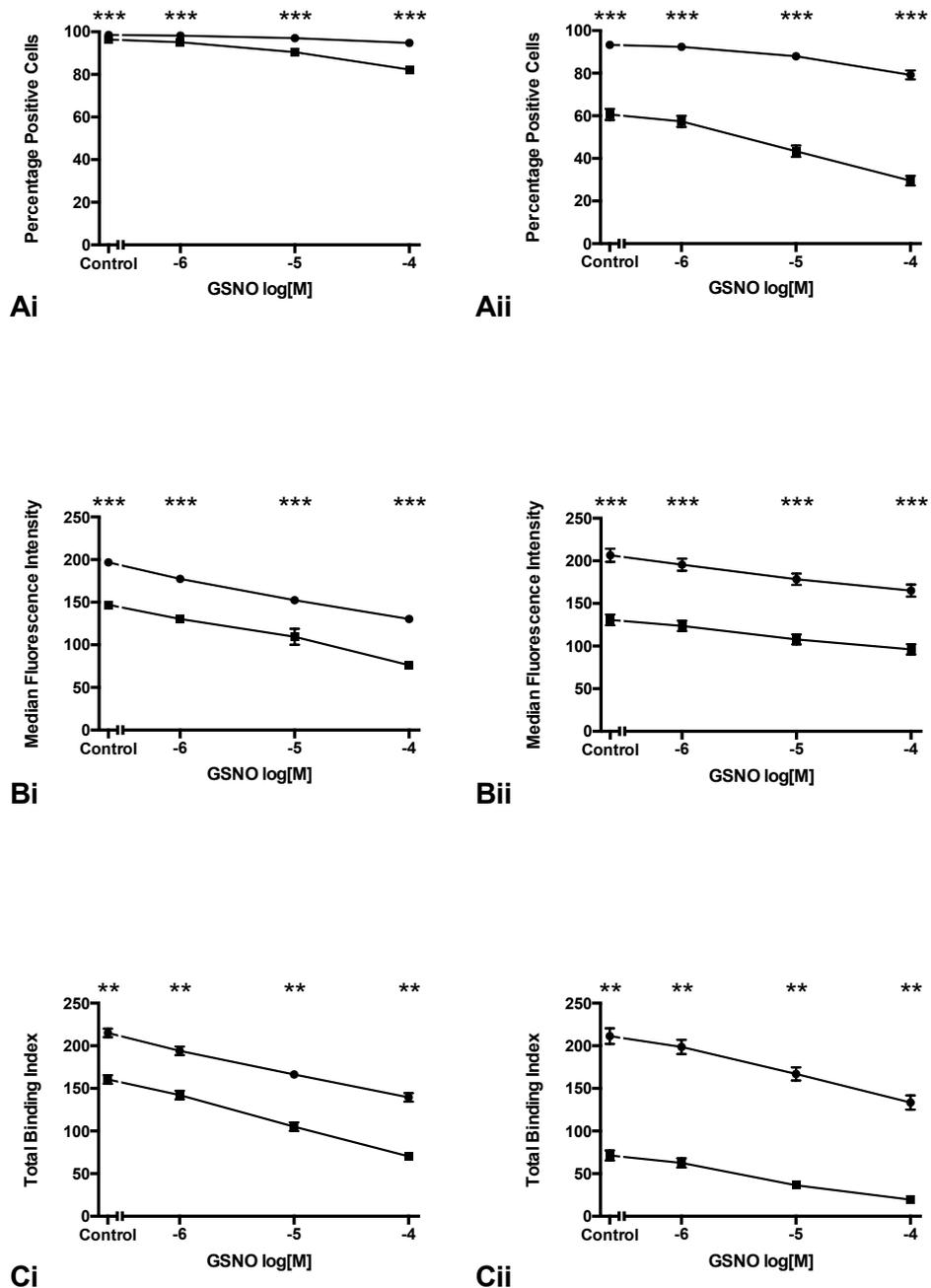
Figure 4. 16: Dose response curve to nitric oxide in patients

*Platelet rich plasma was incubated with variable doses of GSNO (0-100μM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry with data expressed as percentage of platelets positive for fluorescence (A), the median fluorescence expressed by each platelets (B) and the total binding index (C) for P-selectin expression (i) and fibrinogen binding (ii). N=63 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.*

Figure 4. 17: Histogram of nitric oxide induced inhibition of platelet activation in patients

Platelet rich plasma was incubated with variable doses of GSNO (0-100μM) pre (black) and post (grey) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry, with data recorded as percentage of platelets positive for p-selectin (i) and fibrinogen binding (ii) associated fluorescence. Inhibition was calculated as percentage of positive platelets compared to the maximally stimulated sample (100 − ((percentage of positive cells/percentage of cells positive in control) x 100). Data displayed for pre (black) versus post (grey) ticagrelor (A) and additive inhibition (inhibition by ticagrelor plus inhibition by relevant GSNO concentration alone) (red) versus actual inhibition seen post ticagrelor (grey) (B). Flow cytometry histograms are shown for GSNO 100μM (C) (control (grey), GSNO 100μM alone (black outline), ticagrelor (red outline) and GSNO post ticagrelor (red)) and are representative of the mean. N=63 and error bar represents standard error of the mean. Results were compared using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.

4.3.2.4   Platelet-leukocyte aggregate response

Activation of platelets by PAR1 peptide agonist resulted in the formation of PLA (p<0.001), with increase in percentage of PLAs from 5.4±0.4% to 72.5±1.5% (PAR1 peptide agonist 10μM). Overall ticagrelor limited aggregate numbers compared to the uninhibited samples (p<0.001). However, the ticagrelor effect was overcome when maximally stimulated (72.5±1.5% and 72.8±1.4% post ticagrelor (p=0.859), figure 4.18 A). PGI$_2$ had minimal impact on PLA numbers with notable percentage reduction only seen after 10nM (17%, p<0.001).

In support of the direct measures of platelet activation, there was again a synergistic relationship between ticagrelor and PGI$_2$ (activated baseline 70.5±1.7%, post PGI$_2$ 10nM 53.9±2.4%, post ticagrelor 69.0±1.6%, post PGI$_2$ 10nM and ticagrelor 21.2±1.5%); with a three-fold greater level of inhibition seen with 10nM PGI$_2$ following ticagrelor (23.5% inhibition compared to 69.9% post ticagrelor). (Figure 4.18 B & C)
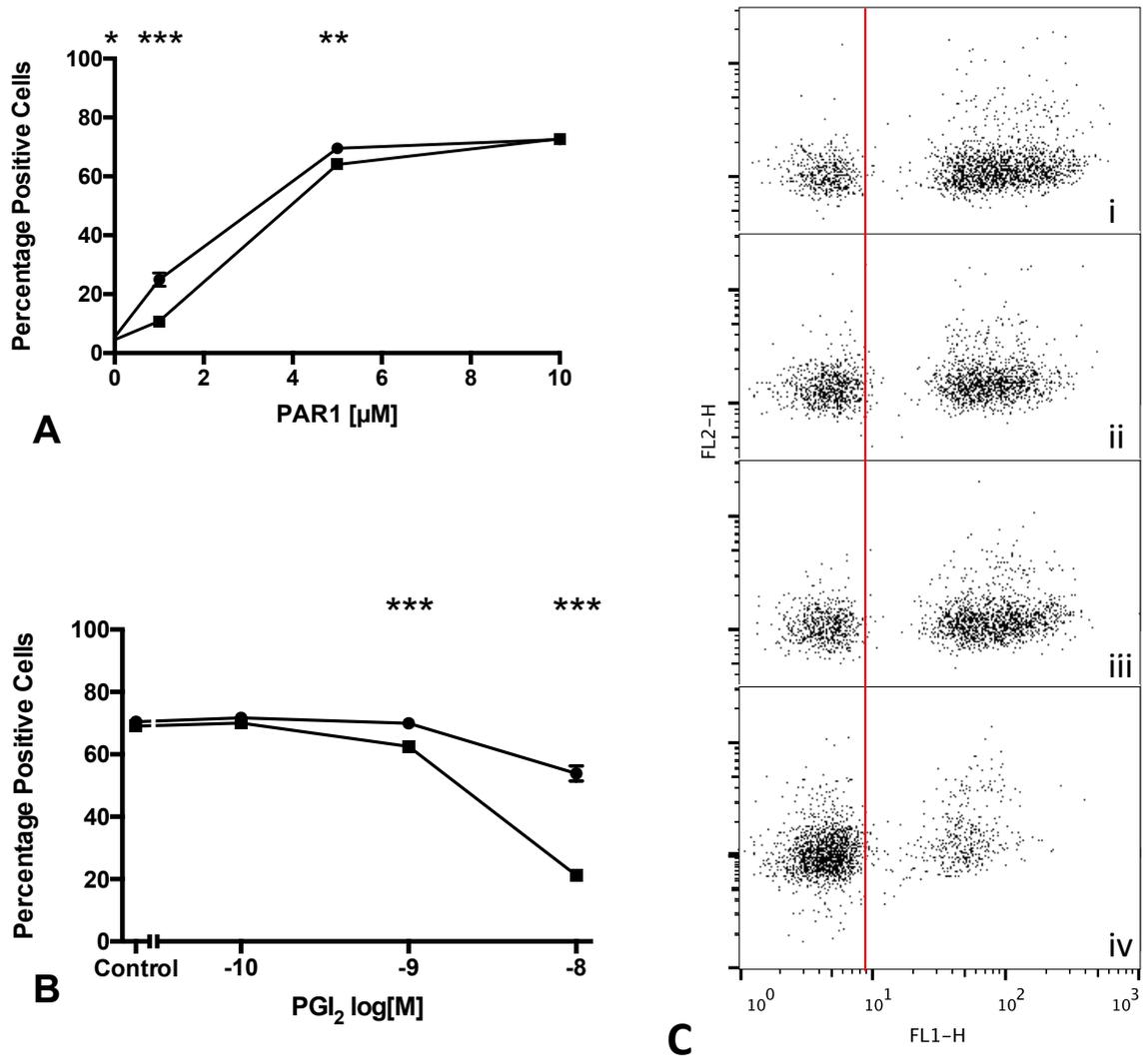
Figure 4. 18: Platelet leukocyte aggregate experiments in patients

Concentration response curve to PAR1 peptide agonist (A). *Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor.*

Dose response curve to prostacyclin (B & C). *Whole blood was incubated with variable doses of PGI_2 (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM) (B). Samples were analysed by flow cytometry (C) with data collected as percentage of cells expressing platelet (CD42a) associated fluorescence within the isolated leukocyte population (events (dots) beyond red line), representing presence of a PLA. (control (i), PGI_2 10nM alone (ii), ticagrelor (iii) and PGI_2 post ticagrelor (iv)). N=63 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.*

4.3.2.5   VASP phosphorylation response

Given that PGI_2 inhibits platelets through the effects of PKA, a common pathway with $P2Y_{12}$ receptor, it was important to examine this signalling pathway through measurement of phosphoVASP. PAR1 peptide agonist did not have any effect on phosphoVASP ($p=0.128$). Increased levels of phosphorylation are driven by PKA/G and so associated with quiescent cells. Consistent with this, increase in levels of phosphoVASP was seen in response to PGI_2 (control MFI 121 ± 6 to 604 ± 43 (PGI_2 10nM) and control MFI 144 ± 9 to 514 ± 68 (PGI_2 10nM), with and without PAR1 receptor agonism, $p<0.001$). Again, the pattern was suggestive of an enhanced response to PGI_2 in the presence of ticagrelor ($p<0.001$), which alone did not impact on phosphoVASP (baseline ticagrelor MFI 119 ± 5 to PGI_2 10nM post ticagrelor 708 ± 41 and ticagrelor 127 ± 5.6 to PGI_2 10nM post ticagrelor 735 ± 56, with and without PAR1 receptor agonism).

In conclusion, patients with CAD demonstrate increasing levels of platelet activation in a concentration response to the PAR1 peptide agonist, which was inhibited by the endothelial vasodilators and ticagrelor individually. In combination with ticagrelor, PGI_2 and NO work synergistically in patients with CAD as demonstrated using multiple measures of platelet activation.
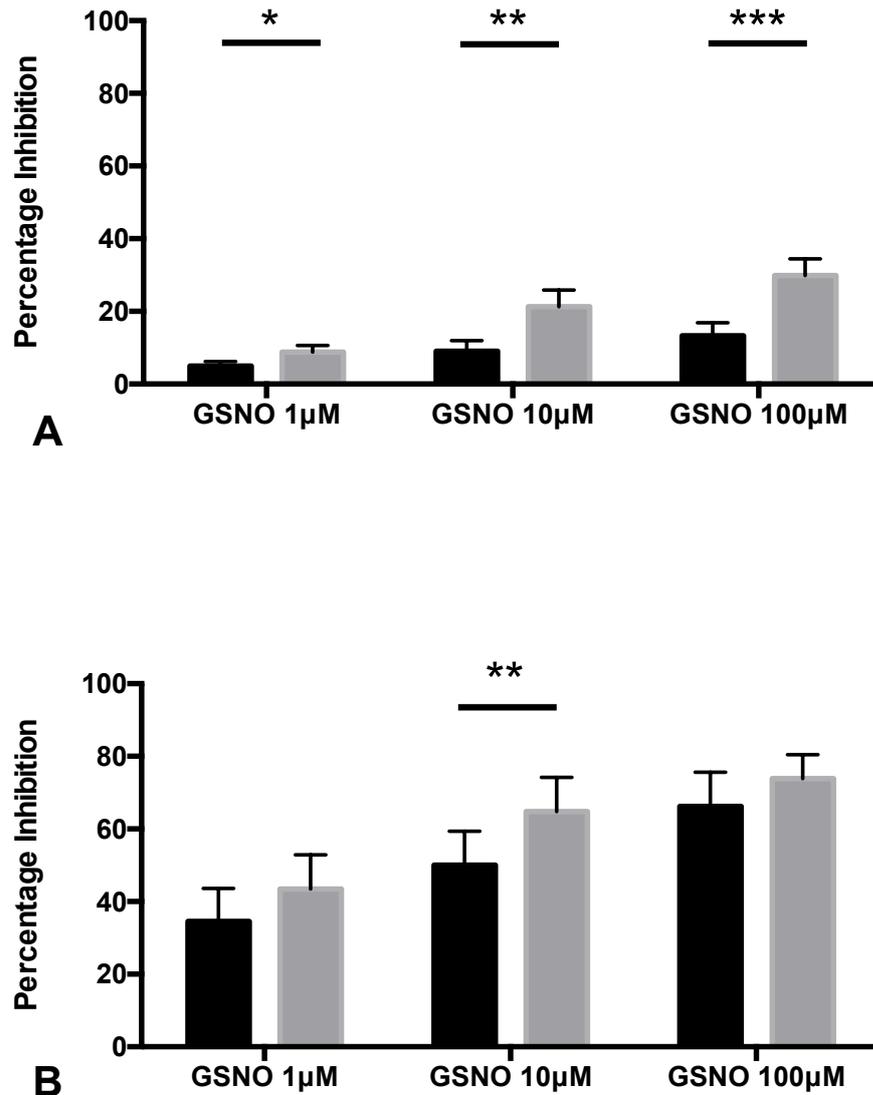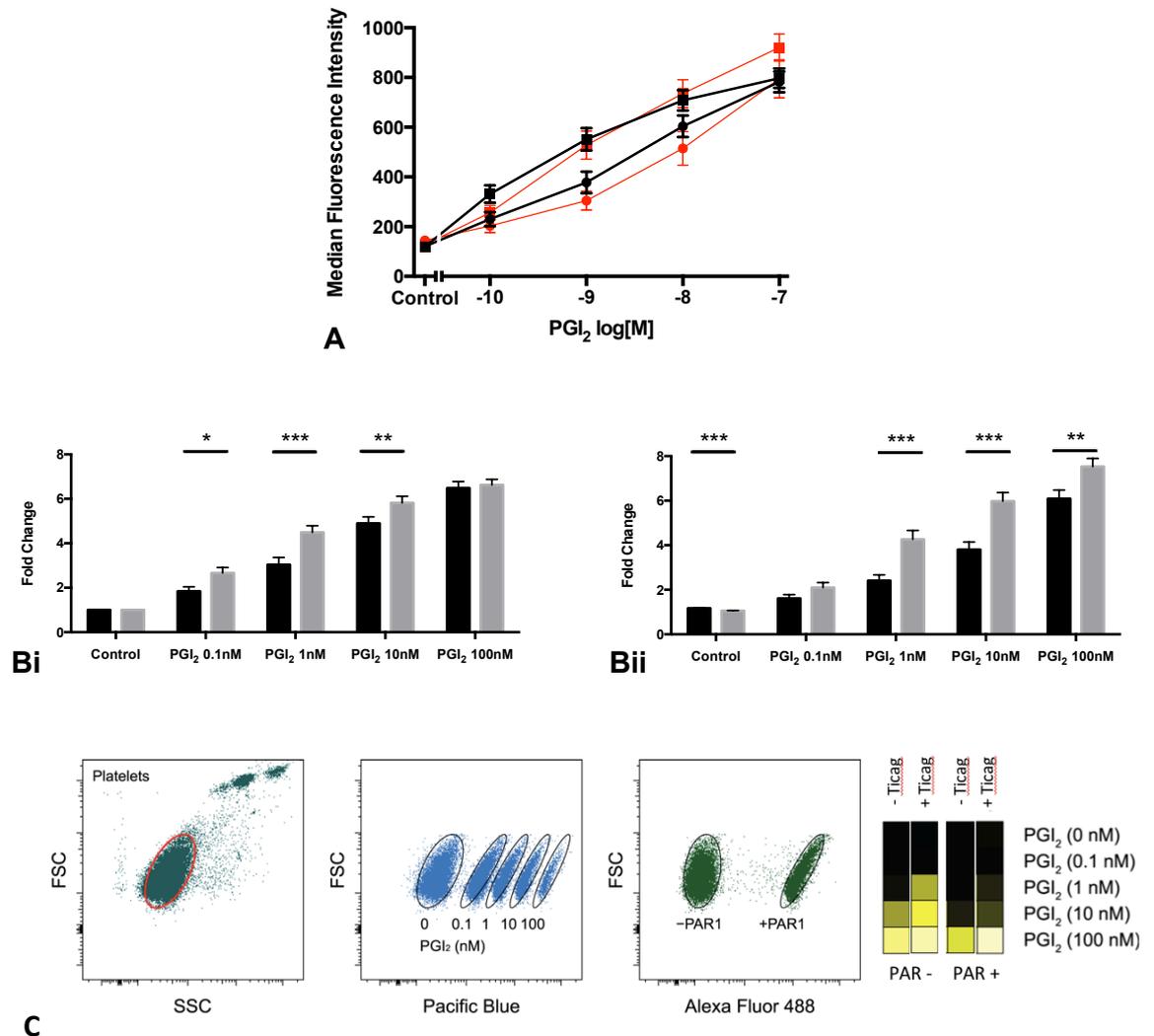
Figure 4. 19: VASP phosphorylation in response to prostacyclin in patients

*Whole blood was incubated with variable doses of PGI₂ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, without presence of agonist (black) or prior to maximal stimulation with PAR1 peptide agonist (10μM) (red) (A). Histograms depict fold change in median fluorescence intensity pre (black) and post (grey) ticagrelor with PAR1 peptide agonist (ii) and without PAR1 peptide agonist (i) (B). Samples were studied with flow cytometry and deconvoluted/analysed with Cytobank, with each experimental condition being isolated using fluorescent bar coding, and unique cell scatter (C). Heat maps quantify phosphoVASP expression with low levels (dark) through to high levels (light). N=58 and error bar represents standard error of the mean. The effect of ticagrelor was explored using Student's t-test and significance is shown as * p<0.05, ** p<0.01 and *** p<0.001.*

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4.3.3   Comparison of characteristics and laboratory results

The data was further reviewed to assess for variation in response to ticagrelor and the endothelial vasodilators between patients in predetermined sub-groups: diabetes mellitus; smoking; and endothelial dysfunction.

4.3.3.1   Influence of diabetes mellitus

Patients were classified as suffering with DM if they were managed with oral hypoglycaemic medications or insulin. To be included in the group without diabetes mellitus the patient needed to have no established diagnosis of diabetes and HbA1c level of less than 42mmol/mol on initial testing. All patients with diabetes mellitus in this study had a diagnosis of type II diabetes mellitus.

As anticipated the cohort with diabetes had significantly higher levels of glucose and recorded HbA1c ($p<0.001$). In addition, the group had statistically greater BMI ($p=0.002$) and lower HDL-C ($p=0.014$) and haemoglobin ($p=0.035$). (Table 4.5)

In common with the overall results, increasing platelet activation was seen with increasing concentrations of the PAR1 peptide agonist, with inhibition induced following the oral ticagrelor. The presence of diabetes mellitus did not vary this response (pre vs post ticagrelor area under TBI dose response curve P-selectin expression $p=0.121$ and fibrinogen binding $p=0.541$). (Figure 4.20)

Ticagrelor enhanced the inhibition induced by PGI_2 and NO alone. In both studies this was statistically more than the expected values if the relationship was additive ($p\leq0.001$ except for the non-significant value for PGI_2 0.1nM and GSNO 1μM related fibrinogen binding). This finding was statistically similar in the cohort with diabetes mellitus compared to those without (pre vs post ticagrelor area under TBI dose response curve for PGI_2 $p=0.371$ & $p=0.661$, and GSNO $p=0.355$ & $p=0.487$ with values for P-selectin and fibrinogen binding respectively). (Figure 4.20)

	No Diabetes Mellitus	Diabetes Mellitus	p value	Normal Range
Total (n)	23	25		
On Insulin	0	6 (24)		
Male (%)	17 (74)	19 (76)	0.871	
Smoker	9 (39)	7 (28)	0.425	
Mean age (years)	61 ± 10	65 ± 8	0.201	
Mean BMI	26.8 ± 4.4	31.6 ± 5.4	**0.002**	
Glucose (mmol/L)	5.1 ± 0.5	10.4 ± 4.4	**<0.001**	
HbA1$_c$ (mmol/mol)	38.3 ± 2.7	66.8 ± 17.7	**<0.001**	
LDL Cholesterol (mmol/L)	2.3 ± 1.1	2.1 ± 1.0	0.533	
HDL Cholesterol (mmol/L)	1.3 ± 0.4	1.1 ± 0.2	**0.014**	
Platelets (x10^9/L)	234 ± 41	236 ± 52	0.895	(150-400)
Neutrophils (x10^9/L)	4.8 ± 1.7	4.8 ± 1.9	0.978	(2.0-7.7)
Monocytes (x10^9/L)	0.7 ± 0.2	0.7 ± 0.2	0.912	(0.2-0.8)
Haemoglobin (g/L)	142.6 ± 10.4	134.9 ± 13.8	**0.035**	(135-175)
PT (secs)	11.4 ± 0.7	11.4 ± 0.9	0.909	(10.0-13.5)
APTT (secs)	32.9 ± 2.8	32.3 ± 2.4	0.455	(28.0-38.0)
eGFR (ml/min)	87.4 ± 15.9	85.4 ± 25.6	0.75	(>90)
Albumin (g/L)	38.9 ± 2.9	37.6 ± 3.2	0.128	(35-55)

Table 4. 5: Basic demographic data of the patients comparing those with diabetes mellitus and those without

Continuous data expressed as mean ± standard deviation and categorical characteristics expressed as number (percentage). Comparison using one-way ANOVA and chi-squared test for continuous and categorical data respectively. p<0.05 was deemed significant.
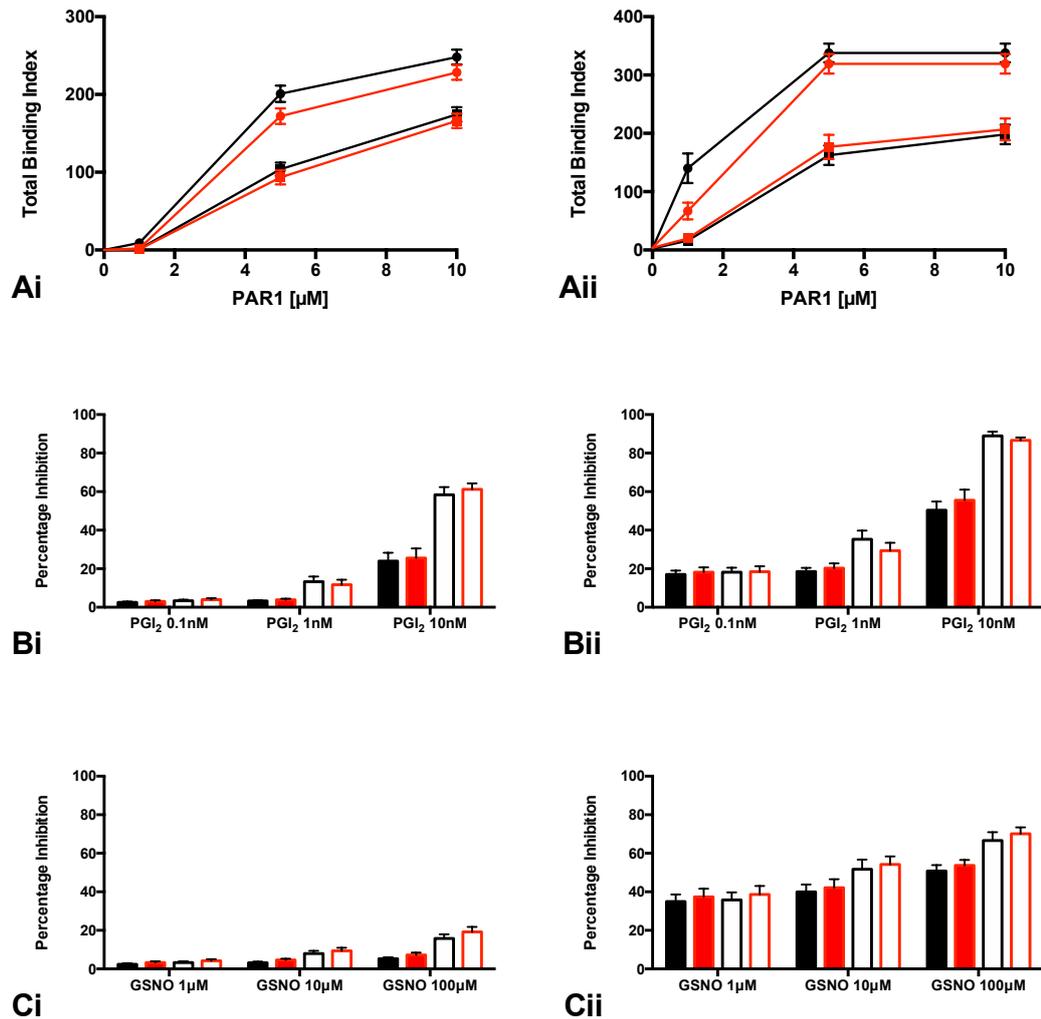
**Figure 4. 20: Comparison of platelet function between patients with and without diabetes mellitus**

Whole blood was stimulated with variable concentrations of PAR1 peptide agonist (0-10µM) pre (circle ●) and post (square ■) three days of oral ticagrelor. Samples were analysed by flow cytometry with data expressed as the total binding index for P-selectin expression (i) and fibrinogen binding (ii) in patients with diabetes mellitus (red) and those without diabetes mellitus (black) (A). Samples were incubated with variable doses of PGI_2 (0-10nM) (B) or GSNO (0-100µM) (C) for two minutes pre and post three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10µM). Results demonstrate percentage of inhibition seen post ticagrelor (open bars) and the relationship to the expected additive inhibition (inhibition generated by PGI_2 or GSNO alone plus ticagrelor induced inhibition, closed bars). N=48 and error bar represents standard error of the mean.

The suggestion of lower activation in the cohort with diabetes mellitus failed to reach significance with levels of P-selectin expression and fibrinogen binding. However, there was a statistically lower level of PLA formation in response to PAR (p=0.002) and a muted reduction was seen in response to ticagrelor with and without PGI_2 in the group with a history of diabetes mellitus (p<0.001). (Figure 4.21)

There was no difference in quantified VASP phosphorylation in either the platelet (p=0.371 and p=0.344 with stimulation) or monocyte population (p=0.399 and p=0.832 with stimulation) comparing groups with and without diabetes mellitus. An important change was seen post ticagrelor in both groups when the samples had been activated with PAR1 peptide agonist and presence of PGI_2 1nM (platelets) and 10nM (platelets and monocytes) (p<0.05). (Figure 4.22)

Overall no conclusive difference was demonstrated in the results for patients with diabetes mellitus when compared to their counter-parts without diabetes mellitus.
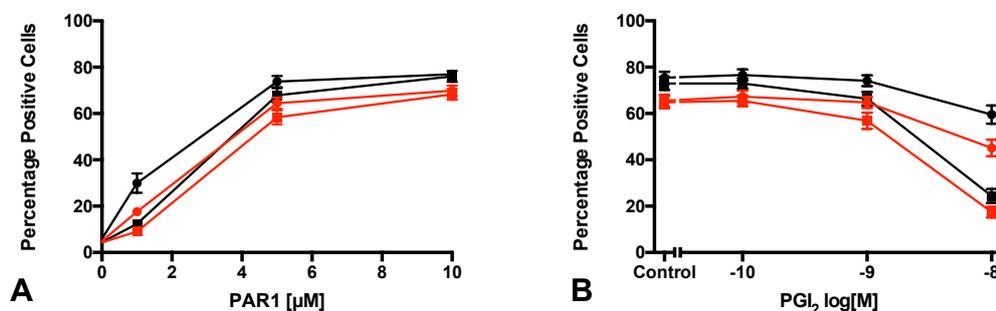
Figure 4. 21: Comparison of platelet leukocyte aggregate formation between patients with and without diabetes mellitus

Concentration response curve to PAR1 peptide agonist (A). Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor, in patients with diabetes mellitus (red) and those without diabetes mellitus (black). Dose response curve to PGI$_2$ (B). Whole blood was incubated with variable doses of PGI$_2$ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry with data collected as percentage of cells expressing platelet (CD42a) associated fluorescence within the isolated leukocyte population, representing presence of a PLA. N=48 and error bar represents standard error of the mean.
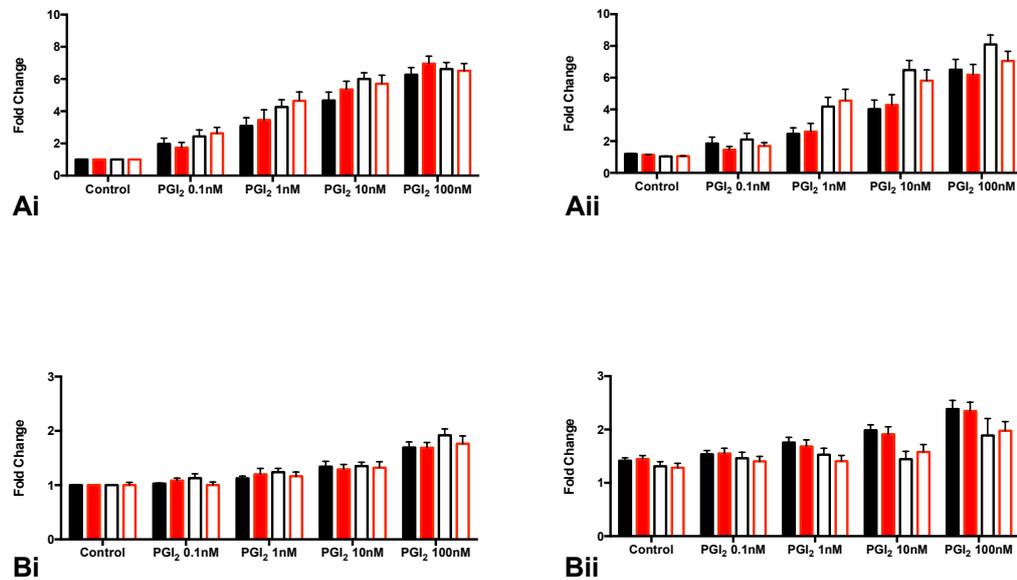
Figure 4. 22: Comparison of VASP phosphorylation between patients with and without diabetes mellitus

Whole blood was incubated with variable doses of PGI_2 (0-10nM) pre (closed bars) and post (open bars) three days of oral ticagrelor, without presence of agonist (i) or prior to maximal stimulation with PAR1 peptide agonist (10μM) (ii). Samples were studied with flow cytometry and deconvoluted/analysed with Cytobank. Histograms depict fold change in phosphoVASP related median fluorescence intensity in patients with (red) and without (black) diabetes mellitus in the platelet (A, n=44) and monocyte (B, n=35) populations. Error bar represents standard error of the mean.

4.3.3.2 **Influence of smoking**

Patients were categorised as smokers if they had smoked more than 100 cigarettes and remained an active daily smoker, while non-smokers were required to have not smoked for over 3 years.

The smoking cohort were found to be statistically younger (p=0.003), with higher levels of LDL-C (p=0.002), neutrophils (p=0.001) and haemoglobin (p=0.002) on routine blood tests. (Table 4.6) Consistent with previous results, both populations demonstrated increasing platelet activation with increasing concentrations of the PAR1 peptide agonist, with ticagrelor inhibiting this activation significantly (pre vs post ticagrelor area under TBI dose response curve for fibrinogen and P-selectin p<0.001). Smoking did not influence this result (P-selectin p=0.322 and fibrinogen p=0.325). (Figure 4.23 A)

The synergism was again demonstrated between ticagrelor and the endothelial platelet antagonists with statistical greater inhibition seen post ticagrelor compared to the calculated additive inhibition. (p≤0.004 for all concentrations except for PGI_2 0.1nM P-selectin expression in smokers (p=0.049) and non-significant values for PGI_2 0.1nM and GSNO 1μM (non-smokers only) fibrinogen binding). Numerically there appears to be lower inhibition seen across all concentrations and measures in the smoking group, with significant lower inhibition present in a number of post ticagrelor conditions (PGI_2 P-selectin 62±2.4% vs 54.9±3.5%, fibrinogen 88.9±1.4 vs 86.7±1.6%, GSNO P-selectin 18.4±2.0% vs 13.7±1.4%, fibrinogen 69.0±3.2% vs 68.4±3.5%; non-smokers vs smokers, figure 4.23 B&C). However, this failed to reach overall statistical difference (pre vs post ticagrelor area under TBI dose response curve for PGI_2 p=0.167 & p=0.096, and GSNO p=0.051 & p=0.129 with values for P-selectin and fibrinogen binding respectively).

	Non-Smoker	Smoker	p value	Normal Range
Total (n)	38	25		
Male (%)	27 (71)	20 (80)	0.433	
Diabetes Mellitus	18 (47)	7 (28)	0.128	
Mean age (years)	65 ± 9	59 ± 7	**0.003**	
Mean BMI	29.9 ± 5.2	39.5 ± 5.6	0.751	
Glucose (mmol/L)	7.7 ± 4.1	6.8 ± 3.1	0.393	
HbA1$_c$ (mmol/mol)	51 ± 16	50 ± 20	0.745	
LDL Cholesterol (mmol/L)	2.0 ± 0.8	2.9 ± 1.2	**0.002**	
HDL Cholesterol (mmol/L)	1.2 ± 0.3	1.2 ± 0.3	0.781	
Platelets (x10^9/L)	231 ± 47	245 ± 55	0.311	(150-400)
Neutrophils (x10^9/L)	4.3 ± 1.3	5.6 ± 1.8	**0.001**	(2.0-7.7)
Monocytes (x10^9/L)	0.6 ± 0.2	0.7 ± 0.2	0.088	(0.2-0.8)
Haemoglobin (g/L)	137 ± 12	146 ± 10	**0.002**	(135-175)
PT (secs)	11.6 ± 0.7	11.1 ± 0.7	**0.017**	(10.0-13.5)
APTT (secs)	32.5 ± 3.8	32.9 ± 1.6	0.629	(28.0-38.0)
eGFR (ml/min)	81 ± 18	92 ± 20	**0.022**	(>90)
Albumin (g/L)	38 ± 3	37.9 ± 2.9	0.916	(35-55)

Table 4. 6: Basic demographic data of the patients comparing non-smokers versus smokers

Continuous data expressed as mean ± standard deviation and categorical characteristics expressed as number (percentage). Comparison using one-way ANOVA and chi-squared test for continuous and categorical data respectively. p<0.05 was deemed significant.
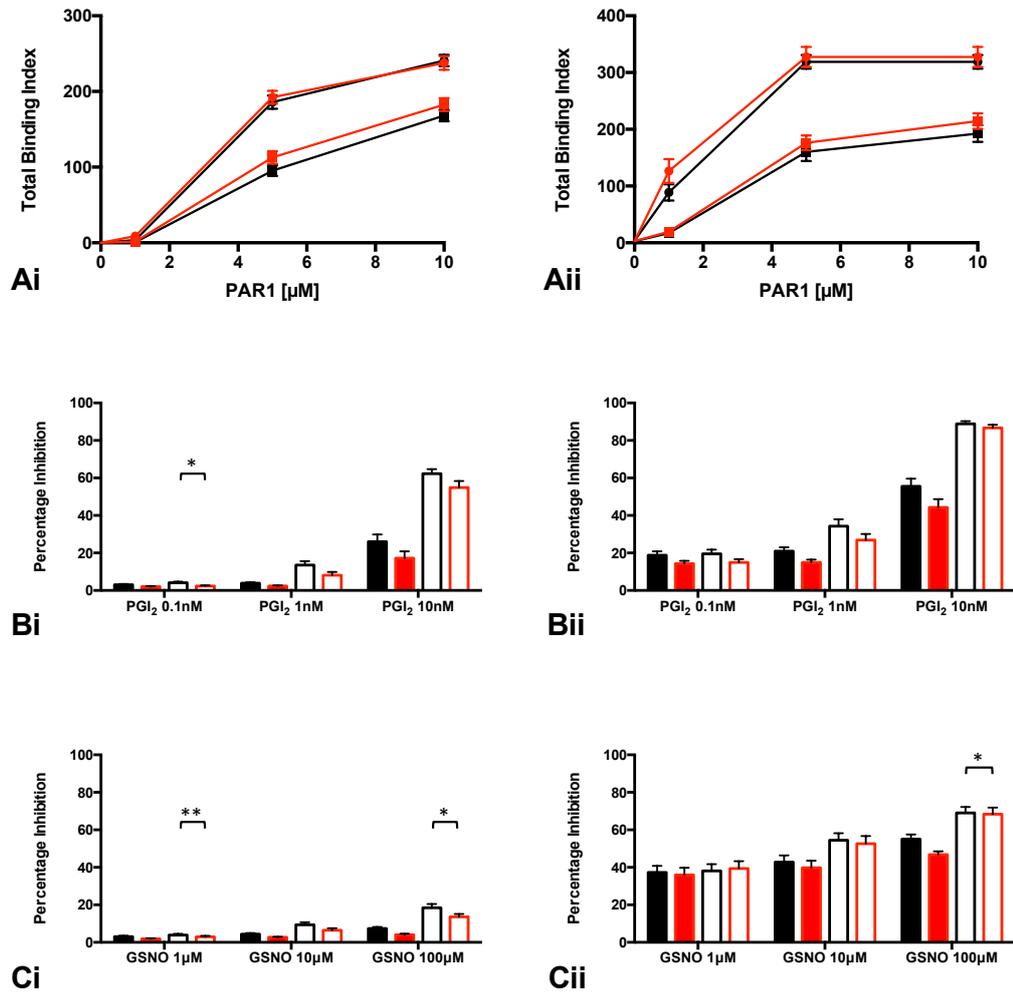
Figure 4. 23: Comparison of platelet function between non-smoking versus smoking patients

*Whole blood was stimulated with variable concentrations of PAR1 peptide agonist (0-10µM) pre (circle ●) and post (square ■) three days of oral ticagrelor. Samples were analysed by flow cytometry with data expressed as the total binding index for P-selectin expression (i) and fibrinogen binding (ii) in patients who smoke (red) and those who do not smoke (black) (A). Samples were incubated with variable doses of PGI$_2$ (0-10nM) (B) or GSNO (0-100µM) (C) for two minutes pre and post three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10µM). Results demonstrate percentage of inhibition seen post ticagrelor (open bars) and the relationship to the expected additive inhibition (inhibition generated by PGI$_2$ or GSNO alone plus ticagrelor induced inhibition, closed bars). N=63 and error bar represents standard error of the mean. The impact of smoking was explored using one-way ANOVA and significance is shown as * p<0.05 and ** p<0.01.*

The platelet-leukocyte aggregate data followed an identical pattern to the previous results with a significant difference pre and post ticagrelor, independent of smoking status, with PAR1 $1\mu M$ during PAR1 concentration response and PGI_2 10nM during PGI_2 dose response ($p<0.001$). Non-smokers also demonstrated a fall in post ticagrelor PLA formation at doses of PAR1 $5\mu M$ ($61.6\pm2.6\%$ vs $67.7\pm2.6\%$, non-smokers vs smokers, $p=0.003$) and PGI_2 1nM ($60.4\pm2.6\%$ vs $65.7\pm2.7\%$, non-smokers vs smokers, $p=0.001$). However, this did not represent an overall difference in ticagrelor effect between the smokers versus non-smokers (pre vs post ticagrelor comparison for PAR dose response curve $p=0.181$ and PGI_2 dose response curve $p=0.256$). (Figure 4.24)

Smoking status did not alter the direction of influence of ticagrelor on VASP phosphorylation in either cell type, with an increase in levels compared to PGI_2 alone in platelets and a fall in monocytes when stimulated with PAR1 peptide agonist. When analysing the impact of smoking on phosphoVASP there was a statistical reduction seen in the smoking cohort present in both the platelet ($p<0.002$) and monocyte ($p<0.001$) populations (platelet fold change 6.9 ± 0.4 vs 5.8 ± 0.4 and 6.7 ± 0.5 vs 5.1 ± 0.6 with PAR1 peptide agonist; monocytes fold change 1.8 ± 0.1 vs 1.5 ± 0.1 and 2.5 ± 0.1 vs 2.2 ± 0.1 with PAR1, non-smokers vs smokers, figure 4.25). Smokers showed an impact generated by ticagrelor with PGI_2 1 & 10nM, and 1-100nM with PAR1 receptor agonism in platelets. Non-smokers demonstrated a significant ticagrelor effect in the presence of PAR1 peptide agonist in monocytes (PGI_2 10 & 100nM) and platelets (PGI_2 0, 1 & 10nM) ($p<0.05$).

In conclusion, there was no difference in levels of activation or overall inhibition in smokers compared to non-smokers. In the presence of ticagrelor, a higher concentration of NO donor generated lower levels of inhibition in the smoking group. They also had statistically lower levels of VASP phosphorylation. Future research would be interesting to explore this potential interaction further by analysing a larger cohort size.
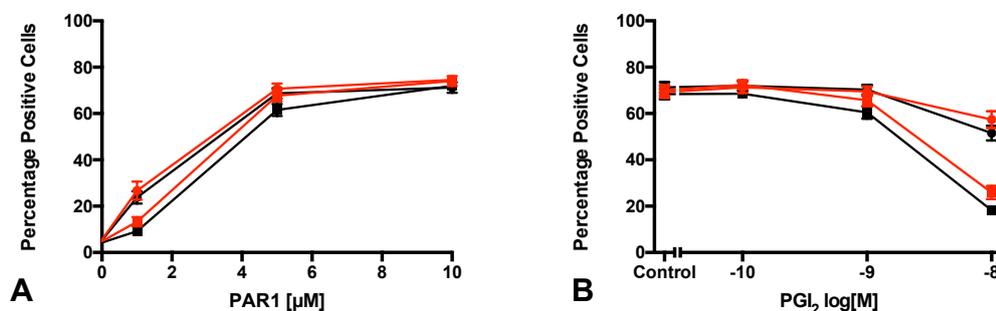
Figure 4. 24: Comparison of platelet leukocyte aggregate formation between non-smoking versus smoking patients

Concentration response curve to PAR1 peptide agonist (A). Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor, in smoking patients (red) and non-smokers (black). Dose response curve to PGI$_2$ (B). Whole blood was incubated with variable doses of PGI$_2$ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry with data collected as percentage of cells expressing platelet (CD42a) associated fluorescence with in the isolated leukocyte population, representing presence of a PLA. N=63 and error bar represents standard error of the mean.
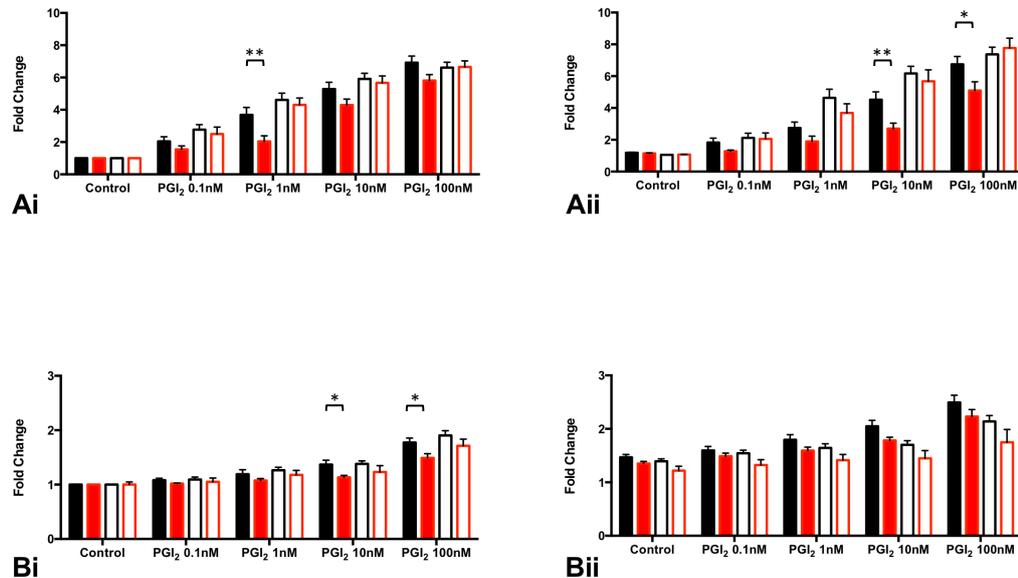
Figure 4. 25: Comparison of VASP phosphorylation between non-smoking versus smoking patients

Whole blood was incubated with variable doses of PGI$_2$ (0-10nM) pre (closed bars) and post (open bars) three days of oral ticagrelor, without presence of agonist (i) or prior to maximal stimulation with PAR1 peptide agonist (10μM) (ii). Samples were studied with flow cytometry and deconvoluted/analysed with Cytobank. Histograms depict fold change in phosphoVASP related median fluorescence intensity in patients who smoker (red) and non-smokers (black) in the platelet (A, n=58) and monocyte (B, n=48) populations. Error bar represents standard error of the mean. The impact of smoking was explored using one-way ANOVA and significance is shown as * p<0.05 and ** p<0.01.

4.3.3.3 Influence of endothelial dysfunction

The impact of abnormal endothelial function was explored by comparing patients with normal against those with abnormal function. Impaired endothelial function was defined as those patients with an EndoPAT result at baseline (reactive hyperaemic index ≤ 1.67).

Twenty-five patients had impaired baseline endothelial function. This cohort had a statistically higher BMI (p=0.041) and circulating monocyte level (p=0.015). (Table 4.7) No difference was seen in the baseline haemodynamics between normal and impaired endothelial function (heart rate 65 ± 13 and 66 ± 11 (p=0.812), blood pressure $138\pm24/77\pm12$ and $134\pm18/74\pm8$ (p=0.403/0.225) respectively).

Ticagrelor significantly inhibited PAR1 peptide agonist induced platelet activation (pre vs post ticagrelor area under TBI dose response curve for fibrinogen and P-selectin p<0.001). Endothelial dysfunction did not impact this result (P-selectin p=0.305 and fibrinogen p=0.574). (Figure 4.26 A) Again, the beyond additive relationship was supported by results in both groups. (figure 4.26 B&C) Similarities between groups were matched with overall analysis stating no variances seen (pre vs post ticagrelor area under TBI dose response curve for PGI$_2$ p=0.353 & p=0.852, and GSNO p=0.703 & p=0.352 with values for P-selectin and fibrinogen binding respectively).

	Normal Function	Impaired Function	p value	Normal Range
Total (n)	38	25		
Male (%)	28 (74)	19 (76)	0.577	
Diabetes Mellitus	14 (37)	11 (44)	0.840	
Smoker	17 (45)	8 (32)	0.320	
Mean age (years)	63 ± 10	63 ± 8	0.951	
Mean BMI	28.6 ± 5.2	31.4 ± 5.1	**0.041**	
Glucose (mmol/L)	7.2 ± 3.9	7.5 ± 3.6	0.804	
HbA1$_c$ (mmol/mol)	50 ± 18	53 ± 16	0.44	
LDL Cholesterol (mmol/L)	2.3 ± 1.1	2.5 ± 0.9	0.485	
HDL Cholesterol (mmol/L)	1.2 ± 0.3	1.1 ± 0.3	0.056	
Platelets (x10^9/L)	234 ± 53	240 ± 47	0.679	(150-400)
Neutrophils (x10^9/L)	4.6 ± 1.7	5.2 ± 1.5	0.197	(2.0-7.7)
Monocytes (x10^9/L)	0.6 ± 0.2	0.8 ± 0.2	**0.015**	(0.2-0.8)
Haemoglobin (g/L)	139 ± 12	143 ± 13	0.229	(135-175)
PT (secs)	11.3 ± 0.8	11.5 ± 0.7	0.463	(10.0-13.5)
APTT (secs)	32.5 ± 2.4	32.9 ± 4.1	0.635	(28.0-38.0)
eGFR (ml/min)	86 ± 18	85 ± 17	0.883	(>90)
Albumin (g/L)	38 ± 3	38 ± 3	0.714	(35-55)

Table 4. 7: Basic demographic data of the patients comparing those with normal and impaired endothelial function

Continuous data expressed as mean ± standard deviation and categorical characteristics expressed as number (percentage). Comparison using one-way ANOVA and chi-squared test for continuous and categorical data respectively. p<0.05 was deemed significant.
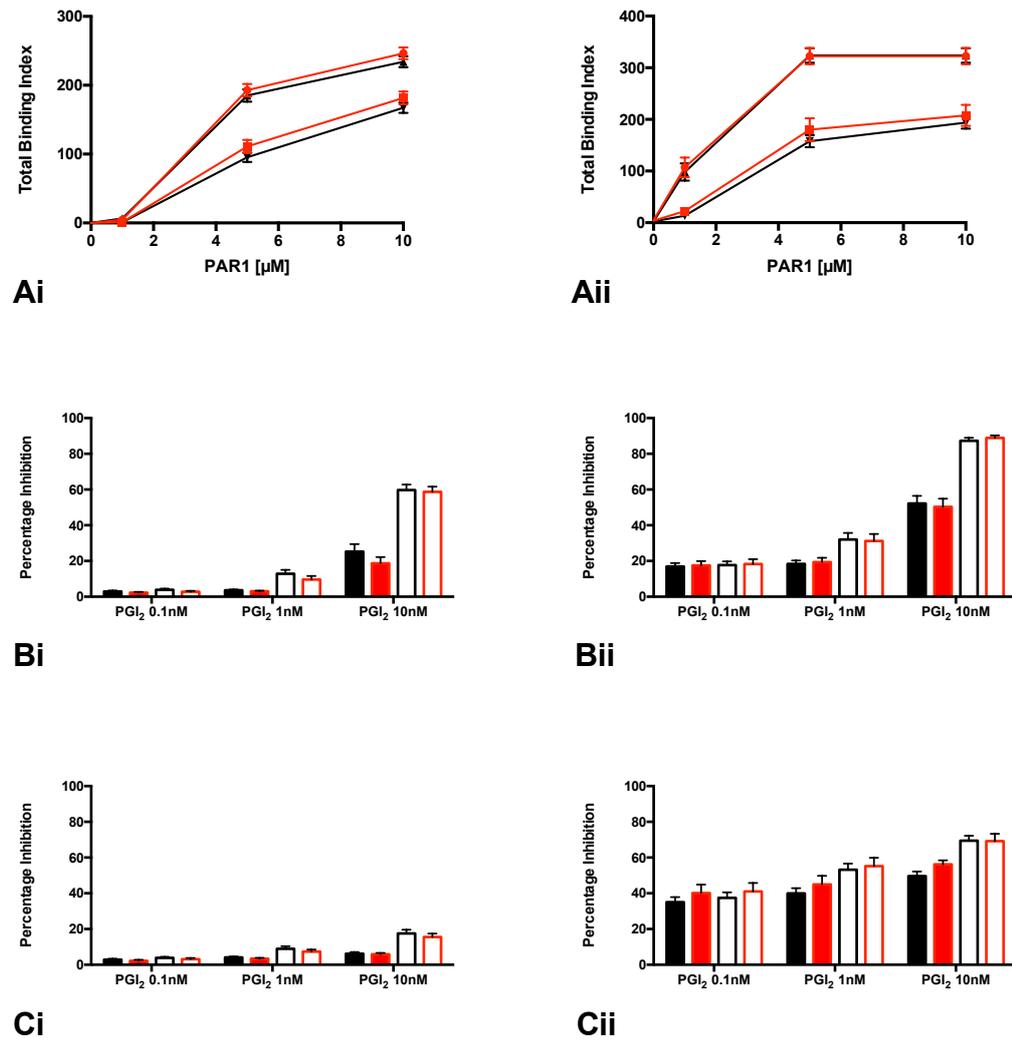
Figure 4. 26: Comparison of platelet function between patients with normal and impaired endothelial function

Whole blood was stimulated with variable concentrations of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor. Samples were analysed by flow cytometry with data expressed as the total binding index for P-selectin expression (i) and fibrinogen binding (ii) in patients with impaired (red) and those with normal endothelial function (black) (A). Samples were incubated with variable doses of PGI$_2$ (0-10nM) (B) or GSNO (0-100μM) (C) for two minutes pre and post three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Results demonstrate percentage of inhibition seen post ticagrelor (open bars) and the relationship to the expected additive inhibition (inhibition generated by PGI$_2$ or GSNO alone plus ticagrelor induced inhibition, closed bars). N=63 and error bar represents standard error of the mean.

The pattern of response to PAR1 peptide agonist and PGI_2, in regard to effect on PLA formation, was identical to previous. Ticagrelor significantly reduced percentage aggregates at PAR1 $1\mu M$ during PAR1 *concentration* response and PGI_2 10nM during PGI_2 dose response ($p<0.001$) in patients with normal and impaired endothelial function. Those with normal function demonstrated further significant results with PAR1 $5\mu M$ ($p=0.001$) and PGI_2 1nM ($p<0.001$). Overall there was no statistical difference in response relative to baseline endothelial function (pre vs post ticagrelor comparison for PAR dose response curve $p=0.810$ and PGI dose response curve $p=0.991$). (Figure 4.27)

Patients with impaired endothelial function had a significant phosphoVASP response to ticagrelor in several conditions in platelets (PGI_2 1nM and PGI_2 0, 1 & 10nM following stimulation ($p\leq0.01$)) and monocytes at PGI_2 100nM ($p=0.007$). Those patients with normal function showed a ticagrelor effect in a greater number of conditions, particularly when PAR1 peptide agonist was added (PGI_2 0, 10 & 100nM in platelets PGI_2 0-100nM in monocytes ($p<0.05$)). PGI_2 1 & 10nM also revealed a statistical increase in phosphoVASP in the absence of PAR1 in platelets ($p\leq0.01$). Overall review of phosphoVASP found that there was a statistical difference in results determined by baseline endothelial function in platelets in the absence of PAR1 peptide agonist ($p=0.032$), and monocytes independent of PAR1 peptide agonist ($p<0.001$). However, the nature of this effect does not display a clear pattern and only a single analysis was noted to be significantly different. These results should therefore be taken with caution. (Figure 4.28)

In conclusion, the patient's baseline endothelial function did not appear to affect the platelet response to ticagrelor.
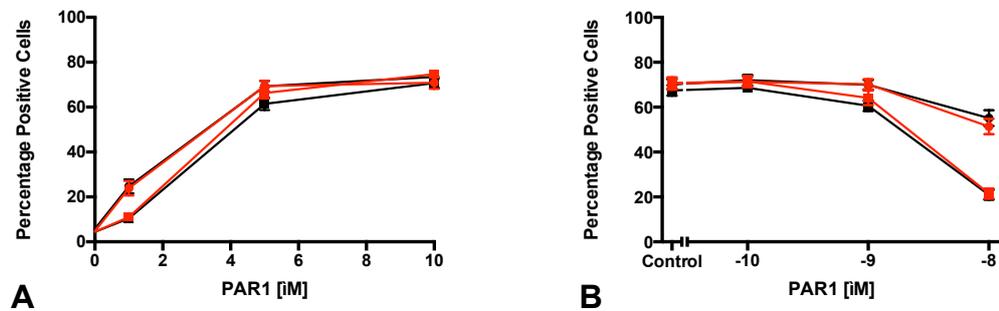
Figure 4. 27: Comparison of platelet leukocyte aggregate formation between patients with normal and impaired endothelial function

Concentration response curve to PAR1 peptide agonist (A). Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor, in patients with impaired (red) and those with normal endothelial function (black). Dose response curve to PGI$_2$ (B). Whole blood was incubated with variable doses of PGI$_2$ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Samples were analysed by flow cytometry with data collected as percentage of cells expressing platelet (CD42a) associated fluorescence within the isolated leukocyte population, representing presence of a PLA. N=63 and error bar represents standard error of the mean.

Figure 4. 28: Comparison of VASP phosphorylation between patients with normal and impaired endothelial function

Whole blood was incubated with variable doses of PGI$_2$ (0-10nM) pre (closed bars) and post (open bars) three days of oral ticagrelor, without presence of agonist (i) or prior to maximal stimulation with PAR1 peptide agonist (10μM) (ii). Samples were studied with flow cytometry and deconvoluted/analysed with Cytobank. Histograms depict fold change in phosphoVASP related median fluorescence intensity in patients with impaired (red) and those with normal endothelial function (black) in the platelet (A, n=55) and monocyte (B, n=46) populations. Error bar represents standard error of the mean. The impact of endothelial dysfunction was explored using one-way ANOVA and significance is shown as * p<0.05 and ** p<0.01.

4.3.3.4   Influence of cardiovascular status

To assess if co-morbidity influenced the outcomes a comparison between the healthy control group against the "cardiovascularly sickest" patient cohort, defined as smokers with CAD and a history of diabetes mellitus, was performed. There were seven patients and ten healthy aged matched (p=0.224) controls included in this analysis, noting the small numbers will affect the statistical relevance of results.

The baseline investigations revealed the patient group to be heavier (p=0.028) with higher HbA1$_c$ and glucose levels (p≤0.002). HDL-C was in lower circulatory concentrations and the leukocyte numbers were significantly higher in patients (p≤0.002). The differences back the concept of well versus sick. (Table 4.8)

The PAR1 peptide agonist produced a standard concentration response curve in both groups with lower levels of fibrinogen binding pre-ticagrelor seen in the patient group (p=0.003). Yet this was not apparent when only assessing percentage of activated platelets (p=0.265), suggesting this was driven by reduced fibrinogen binding by each activated platelet. Ticagrelor successfully inhibited this activation independent of co-morbidity (patients p≤0.02 and controls p≤0.001). The ticagrelor effect on aggregation could be further assessed by calculating the mean difference of area under the curves (AUC) pre versus post ticagrelor. (Figure 4.29A) The P-selectin expression percentage difference of total binding index AUC was 32.0% for patients and 41.8% in the controls (p=0.278), with fibrinogen differences 33.1% for patients and 56.6% for control reaching statistical significance (p=0.008). The percentage of activated platelets in basal conditions post ticagrelor was not statistically different between groups (P-selectin expression (7.0% in patients and 4.1% in controls (p=0.425)), and fibrinogen binding (11.9% in patients and 8.9% in controls (p=0.373))).

	Control	Smokers with Diabetes	p value	Normal Range
Total (n)	10	7		
Male (%)	7 (70)	6 (86)	0.484	
Mean age (years)	56 ± 9	61 ± 7	0.224	
Mean BMI	26.7 ± 2.0	31.2 ± 5.4	**0.028**	
Glucose (mmol/L)	5.1 ± 0.3	10.8 ± 3.4	**0.002**	
HbA1$_c$ (mmol/mol)	37 ± 4	75 ± 21	**<0.001**	
LDL Cholesterol (mmol/L)	3.2 ± 0.7	2.4 ± 1.4	0.113	
HDL Cholesterol (mmol/L)	1.5 ± 0.3	0.9 ± 0.1	**0.001**	
Platelets (x10^9/L)	238 ± 34	255 ± 43	0.359	(150-400)
Neutrophils (x10^9/L)	3.1 ± 0.9	6.3 ± 2.6	**0.002**	(2.0-7.7)
Monocytes (x10^9/L)	0.4 ± 0.1	0.8 ± 0.2	**<0.001**	(0.2-0.8)
Haemoglobin (g/L)	143 ± 11	143 ± 17	0.978	(135-175)
PT (secs)	11.5 ± 0.9	11.4 ± 1.0	0.988	(10.0-13.5)
APTT (secs)	33.8 ± 2.9	32.7 ± 1.5	0.341	(28.0-38.0)
eGFR (ml/min)	95 ± 8	103 ± 30	0.413	(>90)
Albumin (g/L)	38 ± 3	37 ± 4	0.701	(35-55)

Table 4. 8: Basic demographic data of the participants comparing healthy controls versus coronary artery disease patients with history of diabetes mellitus and active smoking

Continuous data expressed as mean ± standard deviation and categorical characteristics expressed as number (percentage). Comparison using one-way ANOVA and chi-squared test for continuous and categorical data, respectively. p<0.05 was deemed significant.
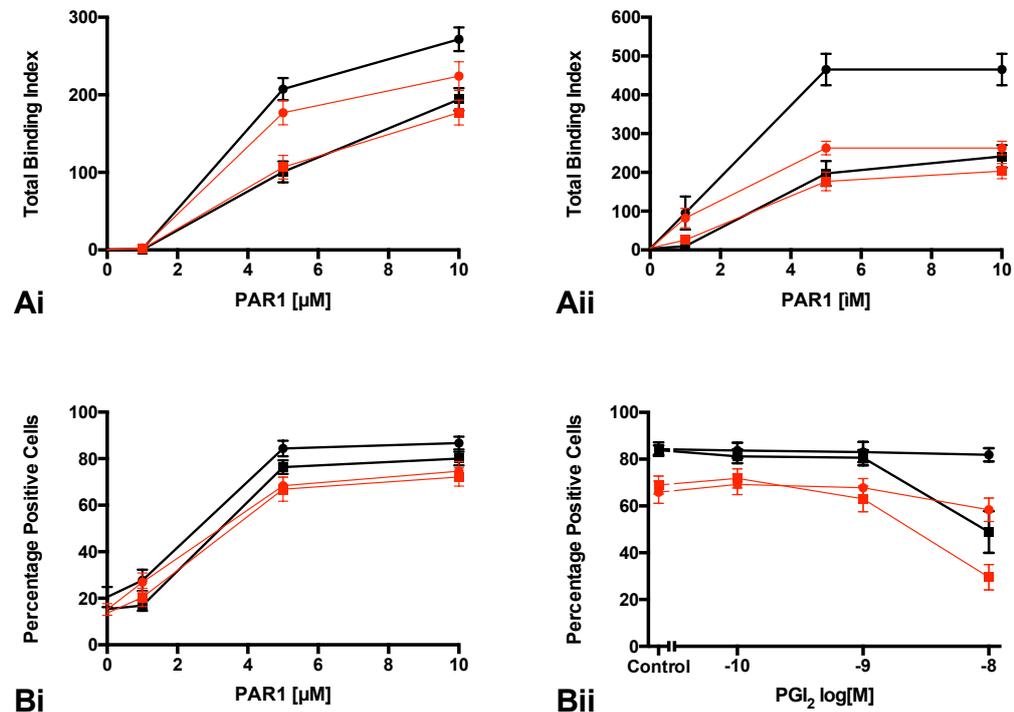
Figure 4. 29: Comparison of platelet function between healthy controls and sick patients.

Concentration response to PAR1 peptide agonist (A) were whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre (circle ●) and post (square ■) three days of oral ticagrelor. Samples were analysed by flow cytometry with data expressed as the total binding index for P-selectin expression (i) and fibrinogen binding (ii) in patients (red) and controls (black). PLA formation (B) Whole blood was stimulated with variable doses of PAR1 peptide agonist (0-10μM) pre and post three days of oral ticagrelor, in patients (red) and controls (black) (ii). Or whole blood was incubated with variable doses of PGI$_2$ (0-10nM) pre and post ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). (ii) Samples were analysed by flow cytometry with data collected as percentage of cells expressing platelet (CD42a) associated fluorescence within the isolated leukocyte population, representing presence of a PLA. N=17 and error bar represents standard error of the mean.

Platelet-leukocyte aggregates were formed during sample stimulation with PAR1 peptide agonist, to a lesser extent in those with cardiovascular disease (PAR response p=0.042 and PGI$_2$ response p=0.006). (Figure 4.29B) Interestingly in the patient cohort there was no effect by ticagrelor on this activation (p=0.417), whereas the control subjects saw a reduction in PLA numbers post three days of oral ticagrelor (p=0.012). This resulted in a statistical difference between the groups in response to ticagrelor (p=0.001). PGI$_2$ alone failed to inhibit PLA formation in either group, but consistent with previous results, when PGI$_2$ 10nM was added post ticagrelor there was a significant drop in percentages; 29% in patients (p=0.006) and 33% in controls (p=0.008). This outcome just failed to reach significance for a greater response in the control group (p=0.06).

PGI$_2$ and NO both led to a concentration dependent inhibition of P-selectin expression and fibrinogen binding without significant differences between populations. At the highest concentration of PGI$_2$ (10nM) inhibition of P-selectin in patients was 10.9±1.5% vs 25.6±6.0% in controls; while fibrinogen binding was 30.2±5.1% vs 48.8±9.5%. Observed sensitivity to GSNO demonstrated inhibition of P-selectin was 1.8±0.1% vs 9.5±3.6% for patients compared to controls while fibrinogen binding was 9.6±1.6% vs 33.8±5.6%. Following three days of oral ticagrelor the level of inhibition was universally increased. (PGI$_2$ P-selectin 58.7±6.7% vs 57.1±9.1%, fibrinogen 86.3±3.4% vs 74.0±9.2%; GSNO P-selectin 13.3±3.2% vs 29.9±4.6%; GSNO fibrinogen 74.4±4.9% vs 73.9±6.6%).

Overall in this small population of subjects ticagrelor again appeared to synergistically interact with both PGI$_2$ and NO. (Figure 4.30) Within the patient group this difference was noted to be significantly greater than the additive effect for P-selectin expression (PGI$_2$ 1-10nM and GSNO 1-100μM) and fibrinogen binding (GSNO 10μM) (p<0.05). The control cohort results also noted this in P-selectin (PGI$_2$ 0.1-10nM and GSNO 10-100μM) and fibrinogen binding (PGI$_2$ 1-10nM and GSNO 100μM) (p<0.05).
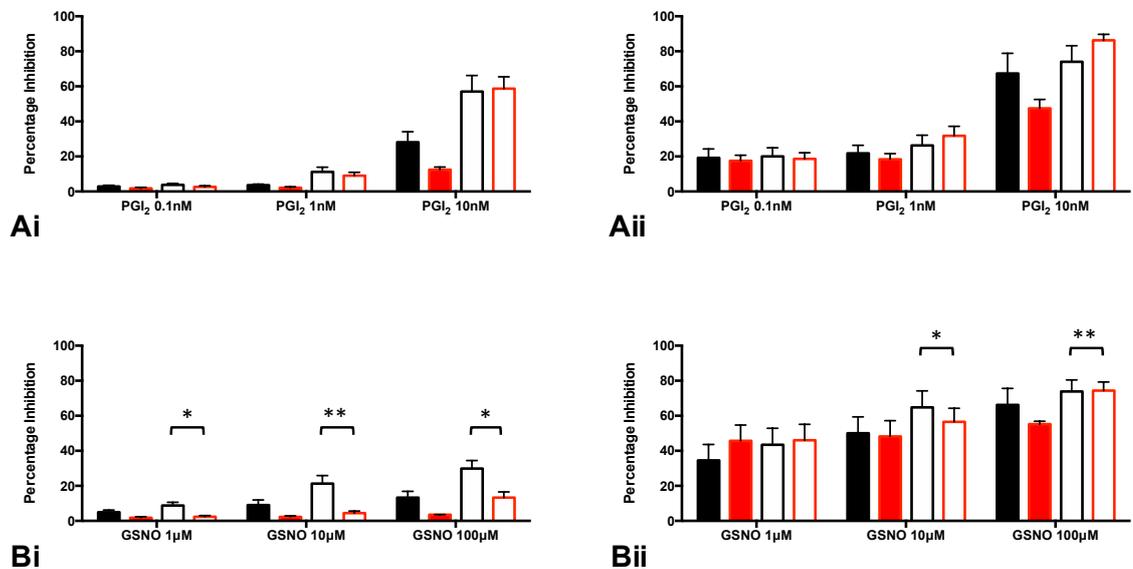
Figure 4. 30: Comparison of platelet function between healthy controls and sick patients with response to prostacyclin and nitric oxide donor

Samples were incubated with variable doses of PGI₂ (0-10nM) (A) or GSNO (0-100μM) (B) for two minutes pre and post three days of oral ticagrelor, prior to maximal stimulation with PAR1 peptide agonist (10μM). Results demonstrate percentage of inhibition seen post ticagrelor (open bars) and the relationship to the expected additive inhibition (inhibition generated by PGI₂ or GSNO alone plus ticagrelor induced inhibition) for P-selectin expression (i) and fibrinogen binding (ii) in patients (red) and controls (black). N=17 and error bar represents standard error of the mean. The differences between healthy controls and sick patients were explored using one-way ANOVA and significance is shown as * p<0.05 and ** p<0.01.

In conclusion, there was a muted response to ticagrelor in the inhibition of fibrinogen binding and PLA formation in cardiovascularly sick patients compared to healthy individuals. Furthermore, patients appeared to be desensitised to endothelial vasodilatory antiplatelet action.

4.3.4   Haemodynamic and endothelial function results

All subjects in the study also underwent endothelial function testing pre and post three days of oral ticagrelor. The overall result and subgroup analyses are discussed below.

4.3.4.1   Control results

Ten control subjects showed normal haemodynamic measures which showed no significant difference following ticagrelor. (Table 4.9) Their reactive hyperaemic index was 2.19 ± 0.5 (mean $\pm$ standard deviation) at baseline and 2.42 ± 0.6 following the ticagrelor regime (p=0.225). (Figure 4.31)

	Pre ticagrelor	Post ticagrelor	p value
Heart rate	60±10	63±9	0.278
Systolic blood pressure	141±20	139±15	0.67
Diastolic blood pressure	85±6	84±6	0.429

Table 4. 9: Haemodynamic measures pre and post ticagrelor in control subjects

N=10 and results are quoted in mean $\pm$ standard deviation. The effect of ticagrelor was explored using Student's t-test with a p value<0.05 deemed significant.
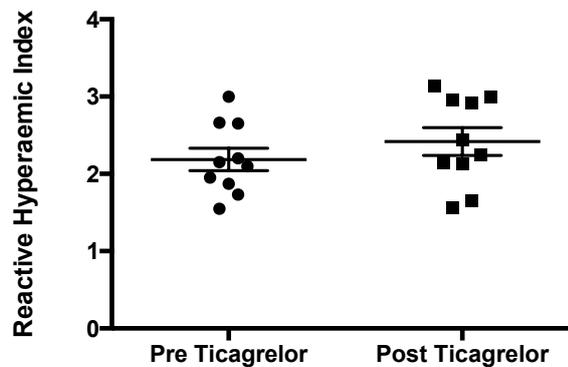
Figure 4. 31: Endothelial response to ticagrelor in control subjects

Reactive hyperaemic index was recorded using the validated EndoPAT method pre (circle ●) and post (square ■) three days of oral ticagrelor. N=10 and error bar represents standard error of the mean.

4.3.4.2 Patient results

Sixty-three patients had an unremarkable haemodynamic profile at baseline with no significant change after ticagrelor in their blood pressure. There was a statistical increase in heart rate, which is surprising given reports of bradycardia associated with ticagrelor during initiation. (26) (Table 4.10) Overall, the patient group demonstrated an increase in the mean reactive hyperaemic index following therapeutic ticagrelor (1.91±0.6 to 2.04±0.7, p=0.049). (Figure 4.32) Although numerically lower there was no significant difference between these and the control results for baseline RHI (p=0.15), post ticagrelor RHI (p=0.12) or the change in RHI (p=0.102).

	Pre ticagrelor	Post ticagrelor	p value
Heart rate	65±12	69±14	**0.003**
Systolic blood pressure	136±22	137±19	0.806
Diastolic blood pressure	76±11	74±13	0.328

Table 4. 10: Haemodynamic measures pre and post ticagrelor in patients

N=63 and results are quoted in mean ± standard deviation. The effect of ticagrelor was explored using Student's t-test with a p value<0.05 deemed significant.
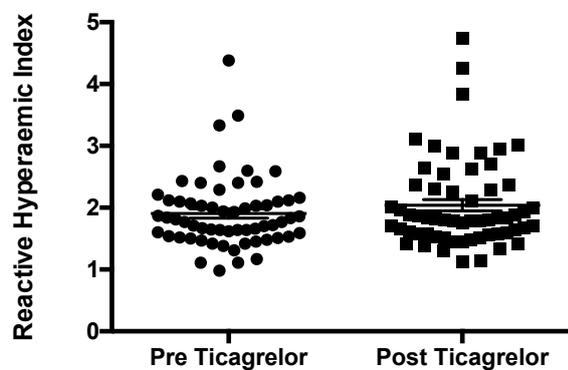
Figure 4. 32: Endothelial response to ticagrelor in patients

Reactive hyperaemic index was recorded using the validated EndoPAT method pre (circle ●) and post (square ■) three days of oral ticagrelor. N=60 and error bar represents standard error of the mean.

4.3.4.3   Comparison

As with platelet function testing prespecified subgroups were reviewed to look for differences in baseline endothelial function and reaction to ticagrelor.

The presence of diabetes mellitus had no influence on the blood pressure of patients throughout the study. Those with diabetes mellitus did have a lower heart rate pre-intervention, although this change did not persist post ticagrelor. (Table 4.11) Patients with known diabetes mellitus had a lower reactive hyperaemic index before (1.75±0.4 vs 2.09±0.7, diabetes mellitus vs non, p=0.054) and after ticagrelor (1.83±0.5 vs 2.30±0.9, diabetes mellitus vs non, p=0.036) as compared to the normoglycaemic patients. There was demonstration of a clear dampening response to ticagrelor (Δ pre/post ticagrelor 0.07 vs 0.2, diabetes mellitus vs non, p=0.028). (Figure 4.33)

The haemodynamics of patients who smoked against the non-smokers showed comparable results throughout the study. (Table 4.12) Smoking status failed to show any impact on RHI. There was no difference in measurements before or after ticagrelor, and the change seen was unwavered (p=0.444). (Figure 4.34)

Patients with endothelial dysfunction exhibited a significantly lower RHI compared to those patients with a normal baseline function (1.46±0.2 vs 2.22±0.6, p<0.001). This difference persisted post ticagrelor (1.66±0.3 vs 2.31±0.8, p<0.001). Interestingly however the ticagrelor effect on RHI was far larger in those with impaired baseline endothelial function (Δ pre/post ticagrelor 0.19±0.3 vs 0.09±0.6, p<0.001). (Figure 4.35) No variation was seen in the haemodynamics of patients with impaired endothelial function at baseline (defined as RHI ≤ 1.67) against the patients with normal function. (Table 4.13)

	No diabetes mellitus		Diabetes mellitus		Pre	Post
	Pre ticagrelor	Post ticagrelor	Pre ticagrelor	Post ticagrelor	p value	p value
Heart rate	69±11	71±16	62±12	68±12	**0.048**	0.516
Systolic blood pressure	139±19	138±19	133±24	136±21	0.396	0.684
Diastolic blood pressure	73±9	75±7	76±9	71±18	0.273	0.381

Table 4. 11: Haemodynamic measures pre and post ticagrelor in patients with and without diabetes mellitus

N=48 and results are quoted in mean $\pm$ standard deviation. The impact of diabetes mellitus was explored using one-way ANOVA with a p value<0.05 deemed significant.
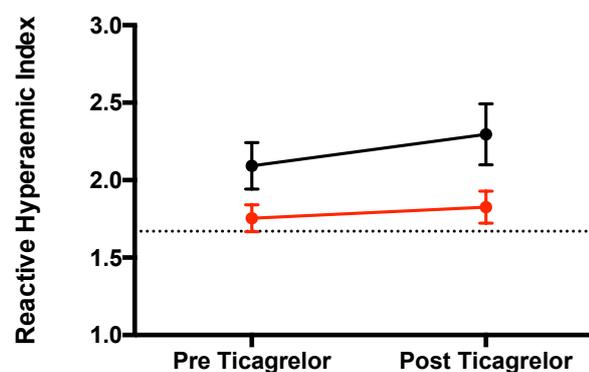
Figure 4. 33: Effect of diabetes mellitus on the endothelial response to ticagrelor

Reactive hyperaemic index was recorded using the validated EndoPAT method pre and post three days of oral ticagrelor. Comparison of patients with (red, n=24) and without diabetes mellitus (black, n=22). Error bar represents standard error of the mean.

	Smokers		Non-smokers		Pre	Post
	Pre ticagrelor	Post ticagrelor	Pre ticagrelor	Post ticagrelor	p value	p value
Heart rate	65±12	68±13	66±12	69±15	0.838	0.952
Systolic blood pressure	137±23	135±17	136±21	138±20	0.85	0.549
Diastolic blood pressure	78±13	75±7	74±9	73±15	0.174	0.549

Table 4. 12: Haemodynamic measures pre and post ticagrelor in smokers and non-smoking patients

N=63 and results are quoted in mean $\pm$ standard deviation. The impact of smoking was explored using one-way ANOVA with a p value<0.05 deemed significant.
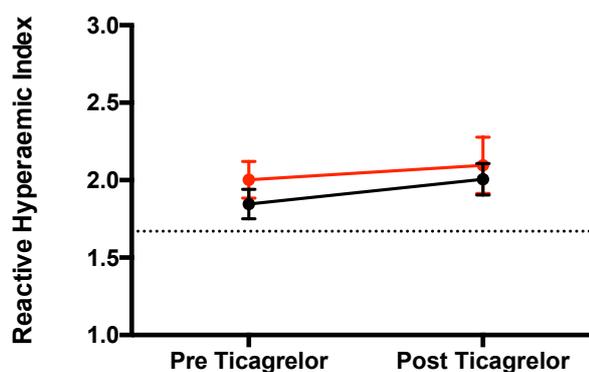
Figure 4. 34: Effect of smoking on the endothelial response to ticagrelor

Reactive hyperaemic index was recorded using the validated EndoPAT method pre and post three days of oral ticagrelor. Comparison of non-smokers (black, n=37) and smokers (red, n=23). Error bar represents standard error of the mean.

	Impaired endothelial function		Normal endothelial function		Pre	Post
	Pre ticagrelor	Post ticagrelor	Pre ticagrelor	Post ticagrelor	p value	p value
Heart rate	66±11	68±14	65±13	69±14	0.812	0.837
Systolic pressure	134±18	132±16	138±24	140±20	0.403	0.099
Diastolic pressure	74±8	73±9	77±12	74±14	0.225	0.771

Table 4. 13: Haemodynamic measures pre and post ticagrelor in patients with endothelial dysfunction and those with normal endothelial function

N=63 and results are quoted in mean ± standard deviation. The impact of endothelial dysfunction was explored using one-way ANOVA with a p value<0.05 deemed significant.
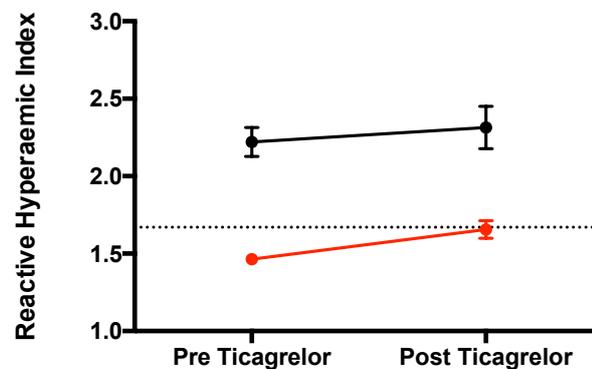
Figure 4. 35: Effect of impaired baseline endothelial function on the endothelial response to ticagrelor

Reactive hyperaemic index was recorded using the validated EndoPAT method pre and post three days of oral ticagrelor. Comparison of patients with impaired baseline endothelial function (red, n=25) and patients with normal baseline function (black, n=35). Error bar represents standard error of the mean.

In comparison of "sick" versus well no difference was seen between their heart rate or systolic blood pressures during the study. Interestingly the diastolic blood pressure was persistently higher in the control group, likely a result of the medical therapy used in the patient group. (Table 4.14)

Neither group attained a significant reactive hyperaemic index response to ticagrelor as the numbers were under-powered for this secondary outcome (p=0.581 for patients and controls as above). However, as with their platelet testing, the cardiovascular comorbid patients had a muted response to ticagrelor (1.8±0.2 to 1.86±0.3 post ticagrelor (Δ 0.06) in the patients versus 2.19±0.5 to 2.42±0.6 post ticagrelor (Δ 0.23) in controls). The difference post ticagrelor RHI as well as the overall change in RHI (pre versus post ticagrelor) was statistically significant in the patients as compared to controls (p=0.043 and p=0.026, respectively). The baseline results appeared to be lower in the patient population, but this was not significant (p=0.073). (Figure 4.36)

	Control		Patients		Pre	Post
	Pre ticagrelor	Post ticagrelor	Pre ticagrelor	Post ticagrelor	p value	p value
Heart rate	60±10	63±9	63±10	66±15	0.559	0.646
Systolic blood pressure	141±20	139±15	137±22	138±17	0.727	0.857
Diastolic blood pressure	85±6	84±6	73±13	74±3	**0.022**	**0.002**

Table 4. 14: Haemodynamic measures pre and post ticagrelor in healthy controls and smoking patients with cardiovascular disease and a history of diabetes mellitus

N=63 and results are quoted in mean ±standard deviation. Data was compared using one-way ANOVA with a p value<0.05 deemed significant.
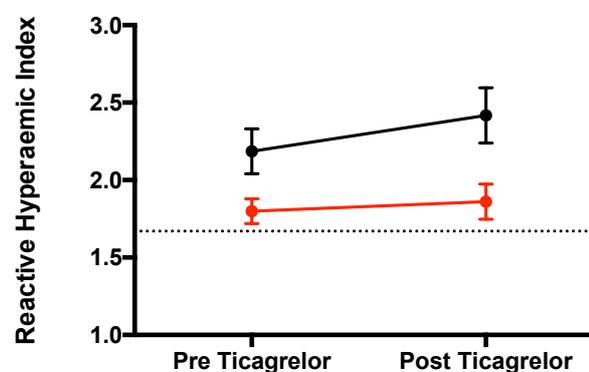
Figure 4. 36: Effect of high cardiovascular risk compared to controls on the endothelial response to ticagrelor

Reactive hyperaemic index was recorded using the validated EndoPAT method pre and post three days of oral ticagrelor. Comparison of smoking patients with history of diabetes mellitus (red, n=7) and aged matched controls (black, n=10). Error bar represents standard error of the mean.

4.3.5 Correlation between platelet and endothelial response to ticagrelor

To explore if the platelet functional response to ticagrelor in patients with CAD could be predicted by endothelial response a linear regression model was used. The results showed that the greater the increase in RHI induced by ticagrelor, the greater the effect of ticagrelor on reducing the total binding index for P-selectin (p=0.025) and fibrinogen binding (p=0.02). Therefore, suggesting correlation in the systemic response to ticagrelor. (Figure 4.37)
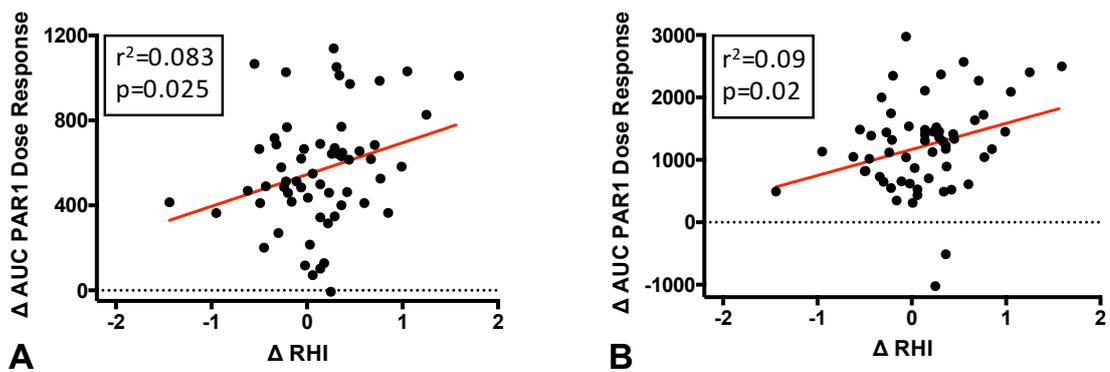
Figure 4. 37: Scatter plot of platelet and endothelial function correlation

The graphs show the relationship between change in area under the PAR1 peptide agonist dose response curve for P-selectin (A) or fibrinogen binding (B) total binding index and the change in reactive hyperaemic index from baseline to following three days of oral ticagrelor in patients with CAD. (n=63) Correlation was assessed using linear regression.

4.4   DISCUSSION

We have demonstrated a clear synergistic relationship between the $P2Y_{12}$ inhibitor ticagrelor and the endothelial vasodilators (PGI_2 and NO) in their role as potent inhibitors of platelet activation. The results establish, for the first time, a significant reduction in platelet activation (fibrinogen binding) and granular secretion (P-selectin expression) in response to PGI_2 and NO in the presence of oral ticagrelor in patients with CAD. Furthermore, we revealed the novel concept that PGI_2 induces a reduction in PLA formation and that this effect is significantly amplified in the presence of ticagrelor. Beyond the relationship with endothelial vasodilators we have shown a statistical improvement in the reactive hyperaemic index, as a marker of endothelial function, after 3 days of ticagrelor. Furthermore, this endothelial response correlated with the extent of inhibition of platelet activation achieved by ticagrelor.

The inhibition of platelet activation by ticagrelor, PGI_2 and NO has long been established (40,129,139), and was confirmed in the results for both "healthy" controls and patients in the experimental conditions used.

Endothelial derived PGI_2 controls platelets aggregation through the activation of cAMP signalling cascades, causing global inhibition of platelet function (131) via phosphorylation of a number of substrates, including VASP. (144) ADP ligation of $P2Y_{12}$ blocks activation of adenylyl cyclase and the tonic inhibition of platelet activation by cAMP. (115) Thus $P2Y_{12}$ inhibitors not only prevent ADP-induced activator signalling, but also release the inhibitory effects of adenylyl cyclase. Therefore, PGI_2 and ticagrelor derived platelet inhibition share a common pathway. In patients following a standard regime of ticagrelor, the current study has demonstrated an improved ability for PGI_2 to inhibit agonist-induced activation of integrin $\alpha_{IIb}\beta_3$, α-granule secretion of P-selectin and PLA formation. In fact, platelet cAMP levels have been discovered to be significantly elevated with PGI_2 in combination with $P2Y_{12}$ inhibition. (201,202) Several elegant *in vitro* studies have established that $P2Y_{12}$ blockade improved PGI_2 and cAMP derived platelet inhibition. (139,201)

However, it must be stated that experiments quantifying cyclic nucleotides are plagued by technical difficulties involved in isolating platelets and preventing the potential basal activity of phosphodiesterase. To avoid this, the study used multiplexed whole blood flow cytometry quantification of VASP phosphorylation to enable examination of the status of

platelet cAMP signalling, thereby preventing the requirement for cell isolation. This methodology discovered that PGI$_2$ was associated with increased phosphoVASP in platelets and was further amplified in the presence of ticagrelor. This supports the concept that ticagrelor improves platelet sensitivity to cAMP signalling and underlies its ability to promote inhibition of both platelet activation and P-Selectin expression. Ticagrelor alone failed to generate an increase in phosphoVASP and this is potentially related to the acknowledgement that cAMP and phosphoVASP are not the important messengers of ADP-induced platelet aggregation. (224,225) Similarly, PAR1 peptide agonists had no impact on platelet phosphoVASP, which has not previously been demonstrated. However, as the protease receptors have multiple targets and are not solely dependent on cAMP signalling (118) this is not unexpected.

Further research in healthy subjects also supports our results with P2Y$_{12}$ antagonism increasing the antiplatelet effect of NO, as well as PGI$_2$. (202) Research has shown that the NO sensitisation does not appear secondary to accumulation of its associated cyclic nucleotide, with cGMP (226) failing to increase beyond levels driven by NO donors alone. (139,202) However, there is suggested crosstalk between cGMP and cAMP with increasing baseline concentrations of the former mopping up PDE 3, a nucleotide degradation enzyme (141), enabling accumulation of cAMP. (143,227) There is also evidence that NO interaction may be further downstream still and is completely dependent of the PI3K pathway. (139) This less direct mechanism may explain the relatively muted findings with NO compared to PGI$_2$ and potentially supporting multiple levels of interaction for both endothelial vasodilators and ticagrelor.

P-selectin acts as a ligand for the formation of pro-inflammatory PLA (103) and their presence in whole blood was also measured. Whilst ticagrelor did not influence PLA formation in the context of PAR1 activation, it did significantly increase the ability of PGI$_2$ to prevent these pro-inflammatory cell associations. It is unclear if this response is entirely driven by altered platelet activation with reduction of P-selectin expression, or an independent process. P2Y$_{12}$ inhibitors have been shown to suppress inflammatory pathways, with ticagrelor having greatest potency. (228) The exact mechanism by which this occurs remains uncertain, but hypotheses include directly through platelet–leukocyte interaction, or via other P2Y$_{12}$ expressing cells such as smooth muscle, where stimulation

upregulates monocyte chemoattractant protein-1 (MCP-1) (229) and induces Interleukin-6 production. (230) However, the described synergism may not be isolated to inhibition of the $P2Y_{12}$ receptor. Ticagrelor has a weak role in adenosine accumulation due to prevention of reuptake through the ENT1 receptor. (49) Adenosine, a purine nucleoside, is intrinsic to multiple signalling pathways with roles in endothelial vasodilation, inflammation and platelet inhibition. (50,51) Relevantly, adenosine has been shown in porcine coronary artery endothelial cells to increase cGMP production in a concentration (physiological) dependent manner through NO pathway (53,54) and may also reduce monocyte migration into tissue. (50) This adenosine effect could be potentiated further by degradation of adenosine triphosphate (ATP) to adenosine, following ticagrelor-induced ATP release via anion channels from erythrocytes. (231)

The synergism between the endothelial vasodilators and ticagrelor was consistent across populations and independent of technique, although each method quantified different markers. P-selectin expression and fibrinogen binding are well understood markers of overall platelet activation. PLA formation is also an indication of activation assessing the platelets interaction with leukocytes rather than inter-platelet activity. However, it is thought to be a superior marker of *in vivo* platelet activation compared to surface expression alone. (209) PhosphoVASP is specific for cyclic nucleotide signalling and not universally affected during activation. Measurement of phosphoVASP is of increasing use in research specifically for the assessment of $P2Y_{12}$ inhibition (232), noting that correlation with P-selectin expression has been demonstrated. (233)

The results highlight the close relationship between the endothelium and vascular homeostasis, with endothelial dysfunction causing promotion of vasoconstriction, pro-inflammatory and pro-thrombotic effects, creating an environment which encourages atherosclerosis. (160) Given the systemic nature of the condition it is unsurprising that peripheral dysfunction correlates well with coronary results. (184) It is established that endothelial function is a predictor of cardiovascular risk and has prognostic implications in those with established CAD. (37, 36) In those with dysfunctional endothelium, failure to improve function despite optimisation of medical therapy results in a significantly higher cardiovascular event rate. (234) The present study has shown that not only does ticagrelor improve reactive hyperaemic index, a measure of endothelial function, but that this also

correlates with the platelet inhibitory response to ticagrelor. Given that endothelial dysfunction is characterised by reduced bioavailability of NO/PGI$_2$ and the observations of improved platelet sensitivity to the vasodilators by ticagrelor, it appears that beyond its ability to inhibit ADP-mediated platelet activation, one of the key benefits of this drug is to improve platelet and endothelial sensitivity to PGI$_2$ and NO. In accordance with a previous study (235), this response was most evident under conditions of endothelial dysfunction where bioavailability is low. Interestingly, ticagrelor is associated with increased eNOS and COX-2 expression *in vitro* (236), enzymes which are fundamental in the production of NO and PGI$_2$ respectively, and improved endothelial function in ACS patients. (237) With further links to adenosine involvement. (236,238) This suggests that there could be a two-pronged action both improving the availability of endothelial-derived platelet inhibitors, as well as increasing platelets' sensitivity to those inhibitors. The findings are not isolated to ticagrelor with evidence of "endothelial improvement" also previously seen with the thienopyridine P2Y$_{12}$ inhibitor clopidogrel (238,239). Studies have shown a significantly greater improvement seen with ticagrelor compared to clopidogrel (237,238) and prasugrel (237), perhaps reflecting the potential additional benefit of the ENT1 action of ticagrelor. Certainly correlation has been demonstrated between adenosine levels and increase in RHI. (238)

The overall results highlight an important mechanism by which ticagrelor exacts its clinical effect in CAD patients with additional functions beyond the antiplatelet action, supporting its superiority in landmark trials. (34) It also raises the possible role of combination pharmacotherapy with nitrates or agents that improve endothelial function. This concept is supported by a study that found enhanced platelet inhibition in patients treated with clopidogrel when combined with long acting nitrates. (240)

4.4.1   Comparison

Within clinical practice it is important to ensure that findings are consistent across the patient population, with particular interest in those at greatest risk. Following prespecified subgroup analysis, the interaction between ticagrelor and the endothelial vasodilators was persistent, with clear synergism demonstrated. However, variance of response was seen in different populations.

Patients with CAD and diabetes mellitus demonstrated increased BMI, lower HDL-C and lower haemoglobin levels compared to patients without diabetes. The presence of high BMI and low HDL-C are expected as part of the metabolic syndrome displayed in type II diabetes mellitus. Reduced HDL-C is impactful at multiple levels; it has antithrombotic properties (241), and there is evidence that increasing HDL-C can stabilise atherosclerotic plaque. (242) The explanation of anaemia seen in diabetes is primarily related to chronic kidney disease, indeed a numerical but not statistical fall in eGFR was seen in this cohort. It is also linked to the chronic inflammatory state secondary to hyperglycaemia and anaemia of chronic disease. (243)

The primary findings in the cohort with diabetes mellitus were identical to those of the main study. However, contrary to published data, there was a trend to lower levels of activation of platelets (170,171) and PLA formation. (244,245) It is unclear why our findings are as such, although it must be acknowledged this did not meet statistical significance. One possible explanation is the methodology. Most studies demonstrating higher activation used light transmission aggregometry or similar validated techniques such as VerifyNow $P2Y_{12}$ assay (170,171,246), and there is not universal correlation between these techniques and flow cytometry assessment of P-selectin or fibrinogen quantification. (247) Another explanation is the data presented as total binding index accounts for both the number of platelets activated and the extent to which each platelet is activated. Here, when only the percentage of platelets activated was considered, the differential is non-existent, suggesting that the difference is primarily related to the level of activation. A publication with similar methodology to this study found no difference in platelet activation in peripheral vascular disease patients between those with and without diabetes mellitus. However elevated activation was seen in patients with concomitant CAD and

diabetes mellitus, suggesting that the interaction is beyond just the presence of diabetes mellitus. (105)

In essence, there was no impact by the patient's DM status and their response to ticagrelor. This is in agreement with clinical data suggesting that both groups benefit from ticagrelor therapy (reduction in cardiovascular death, MI, or stroke: HR 0.88 (0.76-1.03) $p_{interaction}$ =0.49). (190) Interestingly, in patients with diabetes mellitus there was a muted response to ticagrelor in PLA formation, a technique felt to be a better assessment of function, and a less dynamic RHI change. Diabetes mellitus is known to result in endothelial dysfunction in keeping with the findings of the study. (160,248) Previous studies have found a trend to a reduced anti-platelet response to $P2Y_{12}$ antagonists in diabetes mellitus. (249,250) Metabolism is important in this negative response, with lower levels of circulating active clopidogrel metabolite found in patients with diabetes. (251) A recent report also demonstrated a reduction in ticagrelor metabolism, as a surrogate for absorption (metabolism not required for ticagrelor activation), in patients with diabetes compared to patients without. (252) However, ticagrelor remains the most potent $P2Y_{12}$ antagonist in this cohort. (249,253) The hyperglycaemic state in diabetes may cause increased expression of $P2Y_{12}$ receptors through nuclear factor (NF)-κB signalling pathway upregulating the relevant mRNA. (254) This would explain a reduced response to antagonists compared to normoglycaemic patients.

The patients who were smokers, were younger and had increased circulating levels of LDL-C, neutrophils and haemoglobin; results which could be anticipated. Smoking is a recognised risk factor for CAD, so smokers tend to present at an earlier age. LDL-C has been well established as increased in smokers (255), and furthermore smokers oxidise more LDL-C than their non-smoking counterparts contributing to their cardiovascular risk. (256) Neutrophilia in smokers has also previously been reported along with an elevated neutrophil to lymphocyte ratio. This is a marker of systemic inflammation, associated with inflammatory cytokine driven endothelial dysfunction, platelet activation and atherosclerotic plaque formation. (257) Elevation of the neutrophil/lymphocyte ratio also plays a role in predicting endothelial/platelet driven conditions such as no reflow phenomenon in ACS. (258) Hypoxaemia and chronically elevated levels of

carboxyhaemoglobin in smokers, stimulates red cell production (and increased levels of haemoglobin) through increased levels of erythropoietin. (164)

There was no statistical difference between results for smokers versus non-smokers. This correlates with clinical findings. In subgroup analysis of PLATO, there was no impact of smoking on the primary outcome (cardiovascular death, MI, stroke: HR 0.83 (0.68-1.00) $p_{interaction}$=0.5). (259) Early clinical and platelet aggregation studies investigating $P2Y_{12}$ antagonism paradoxically suggested smokers benefitted the greatest from clopidogrel therapy. (167) This finding has also been evident with prasugrel (168), hypothesising that enhanced P450 cytochrome metabolism in smokers was important. (260) The study results did suggest a numerically lower level of inhibition in response to PGI_2 with a number of concentrations reaching significance post ticagrelor. This could be related to the reduction in response to PGI_2 in smokers (261), or potentially the heightened reaction to activatory triggers compared to non-smokers. (163) The findings of lower phosphoVASP in smokers is not a new phenomenon with lower levels in basal and activated samples (262), supporting the diminished response to PGI_2.

Smoking has been implicated in decreasing insulin sensitivity driving hyperglycaemia, and in patients with diabetes mellitus this negatively influences their metabolic control. (263) This means that beyond both risk factors contributing to CAD, the risk is exponentially worse in combination. In the current study therefore, a comparison was made between this cohort (smokers with diabetes mellitus) deemed to have "impaired cardiovascular status", and healthy aged-matched controls. The main observational and biochemical variations between the two groups have been explained above. Diabetes mellitus and smoking generate a pro-inflammatory environment (264,265) and so in keeping with this, an elevation in neutrophils and monocytes compared to the healthy population was observed.

In accordance with the trend seen in the population with diabetes mellitus, when compared to a healthy population, this high-risk group had a muted platelet and endothelial response to ticagrelor. Despite the low cohort numbers this difference reached statistical significance in multiple total effect measurements. Although not statistically demonstrated in this small cohort reduced sensitivity to both PGI_2 and NO has been hinted

at in several high-risk groups (266,267) and CAD (268); and implies that hyposensitivity to endogenous antiplatelet mediators may play a role in heightened thrombotic risk. There appears to be a plethora of effects related to the relevant risk factors both for and against the pharmacodynamics/kinetics of ticagrelor discussed above, that are most apparent when compared to the "well". Exploration of this effect with larger populations to encompass measures of platelet function as well as clinical outcomes would be very interesting and potentially important.

4.4.2   Study Limitations

The study is a single centre trial that enrolled predominantly men and with limited ethnic diversity. This limits the generalisability of the findings to all populations. *In vivo* experiments are superior in design for extrapolation of results to real world practice, however due to the short half-life and localised action of PGI_2 and NO this was not feasible. Ticagrelor is licenced for use in ACS or history of recent MI but these populations were not investigated, primarily for prevention of treatment delay and practical purposes, limiting the impact of the results on clinical application. The study was powered to investigate the overall effect and therefore the numbers are too small for a comprehensive subgroup analysis.

4.5 CONCLUSIONS

This study has demonstrated, for the first time in patients with CAD, that $P2Y_{12}$ inhibition by oral ticagrelor, enhances the platelet inhibitory effect of the endothelial vasodilators PGI_2 and NO. Furthermore, ticagrelor was shown to increase the reactive hyperaemic index, measured as a marker of endothelial function; and this correlated with the anti-platelet response to ticagrelor.

It will be important to explore further the pathways involved in this synergism and investigate the implications of the findings in an ACS population.

5 CHAPTER FIVE: IMPACT OF TICAGREOR MEDIATED P2Y₁₂ INHIBITION AND PROSTACYCLIN ON INFLAMMATORY CELLS

5.1 INTRODUCTION

P-selectin facilitates the heterotypic interaction of platelets with both endothelial cells and leukocytes through P-selectin glycoprotein ligand-1 (PGSL-1). (103) The formation of platelet-leukocyte aggregates is a key marker of platelet activation *in vivo,* and such aggregates are known to be raised in a number of pathologies associated with CAD. (245,269) The previous chapter concluded that a ticagrelor regime alone did not influence PLA formation in context of PAR1 receptor activation in patients with CAD. However, it did significantly increase the ability of PGI_2 to prevent these pro-inflammatory cell associations. Although previously described in healthy individuals (228), this is the first demonstration in patients. It is unclear if this response is entirely driven by altered platelet activation, with reduction of P-selectin expression, or an independent inflammatory process generated in these experimental conditions.

Inflammation is integral to not just ACS but also the formation of the underlying chronic atherosclerosis, with well-established roles of the platelet within this pathophysiology. The activated platelets close relationship with the leukocyte, most notably the monocyte, primarily drives/attracts the immune cell to the vascular wall and promotes migration through the endothelial wall. Following subsequent transition into a macrophage the cell combines with oxidised LDL to form a foam cell complex. (8–10) This is a major component of the atherosclerotic plaque. As mentioned the inflammation cascade and platelets are critical in the ACS events following plaque rupture. (Section 1.3.9)

An inflammatory response is dependent on well characterised understood interactions between leukocytes and the endothelium, with stimulated endothelial expression of adhesion molecules attracting leukocytes which facilitates local targeted extravasation of the cells. (270) Beyond their haemostatic role platelets have also been implicated in immune modulation and promotion of inflammation. (271) The important clinical consequences of this as a pathological process has resulted in this being an area of great scientific and clinical interest.

Following activation, externalisation of P-selectin from the α-granule results in binding to circulating monocytes and neutrophils via its counter receptor PSGL-1, an established interaction. (272) However, the α-granules also contain a cacophony of both precursor and mature inflammation-mediating factors including C-C motif cytokine (CCL) 3 (macrophage inflammatory protein -1 α (MIP-1α)), CCL5 (regulated on activation, normal T cell expressed and secreted (RANTES)), C-X-C motif cytokine (CXCL) 4 (Platelet factor 4), CXCL14, CD40L and Interleukin-1β (IL-1β). (273,274)

Chemokines like MIP-1α and RANTES stimulate endothelial activation, inducing adhesion and further activation of the monocyte population. Further inflammatory targets include eosinophils, basophils, natural killer cells and dendritic cells; increasing antigen presentation to T cells. (274) CXCL 4, despite its alternative nomenclature (platelet factor 4), is not isolated to platelets/megakaryocytes, with signalling responses demonstrated in nearly all nucleated blood/vascular cells, promoting inflammation and inhibiting angiogenesis. (275) CXCL 14 has only recently been proven to be released from platelets resulting in potent monocyte attraction and inhibition of endothelial cell migration. (276) CD40L acts as a ligand for its associated transmembrane protein CD40, a member of the tumour necrosis factor receptor family. CD40 is predominately expressed in B cells, dendritic cells, monocytes, platelets, macrophages and non-hematopoietic endothelial cells. While CD40L is routinely seen following activation of T cells, B cells, platelets and, in times of inflammation, it is also detected in monocytes, basophils and mast cells. (277) CD40L is rapidly expressed by activated platelets, interacting directly with endothelial cells to stimulate leukocyte attraction and subsequent passage into the inflamed/injured tissue. (278)

Activated platelets can induce endothelial release of IL-1β as an important step in the inflammatory process (279), but also generate platelet derived IL-1β contributing to systemic and vascular inflammation. Within the quiescent platelet no IL-1β is detectable, only its precursor pro-IL-1β stored in polysomes. IL-1β production is dependent on cleaving of pro-IL-1β into the active form by the pro-inflammatory non-apoptotic cysteine protease Caspase-1. Activation of this is triggered by the formation of a multiprotein inflammasome complex (280,281), components of which are present in the platelet. (273) Multiple NOD-like receptor (NLR) family members have been implicated in the activation of Capsase-1.

NLRP3 is the most understood with three key pathways of activation characterised. (Figure 5.1) However NLRP1, NLRP6, NLRC4 (NLR family CARD domain-containing protein 4), AIM2 (absent in melanoma 2) and IFI16 (Interferon inducible) are also implicated in Caspase-1 activation, a pivotal process for innate immunity. These molecules are more directly driven by the diseased state, with triggers such as bacterial constituents (NLRP1, NLRC4), bacterial toxins (NLRP1), intestinal microbes (NLRP6) or pathogen DNA (AIM2, IFI16). (281) Upon platelet activation IL-1β is released to the haemostatic plug and to the endothelial cells stimulating neutrophil adhesion. (282) There is also a platelet autocrine function via the Interleukin 1 receptor 1 (IL1R1), magnifying the activatory response (283) and priming with stimulation of further pro-IL-1β generation. (280) These observations were the basis for a large scale clinical trial (CANTOS) targeting IL-1β with a human monoclonal antibody, demonstrating a significant reduction in major adverse cardiovascular events in patients at risk post myocardial infarction. (284) This study highlights inflammation and IL-1β activation as an area of keen academic interest.
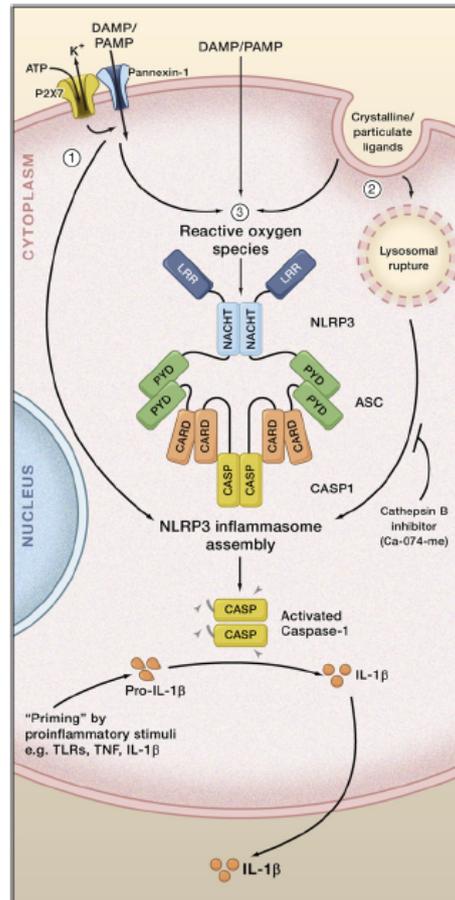
Figure 5. 1: ***Outline of NLRP3 inflammasome activation and triggering of mature IL-1β production*** (280)

Diagrammatic representation of three models of NLRP3 activation: 1) ATP influx triggers P2X$_7$ receptor-dependent pore formation by the Pannexin-1 channel, enabling danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) to enter the cytosol which act as direct NLRP3 agonists; 2) Crystalline or particulate NLRP3 agonists are engulfed, with subsequent lysosome rupture due to the nature of the contents. Discharge of these contents into the cytoplasm enables processes such as cathepsin-B dependent NLRP3 ligand formation; 3) Amplification occurs with ATP, DAMPs, PAMPs and crystalline/particulate agonists generating reactive oxygen species (ROS) leading to NLRP3 inflammasome complex formation. The final common step is NLRP3 driven Caspase-1 activation and maturation of pro-IL-1β into IL-1β. From Schroder et al, 2010.

5.1.1   Objectives of this chapter

- Expand previous exploration of the effects of PAR1 peptide agonist, PGI_2 and ticagrelor on VASP phosphorylation in patients with CAD to include inflammatory cell lines:

  - Monocytes;

  - Granulocytes;

  - Lymphocytes.

- Preliminary platelet work to investigate the role of:

  - Caspase-1.

5.2 METHODS

The details related to ethics, participant recruitment, blood sampling and preparation have been covered in prior chapters. Therefore, the focus of this section will be restricted to new methods.

5.2.1 Methodology of phosphoflow and leukocyte barcoding

The key principles of phosphoflow and fluorescence cell barcoding (section 4.2.2.5) have been covered. For this chapter, leukocytes were gated based on side scatter versus CD45, with median fluorescence intensity (MFI) recorded and subsequent fold change calculated. Data was deconvoluted and analysed with Cytobank. This work was carried out with the assistance of Dr Benjamin Spurgeon.

5.2.2 Methodology of Caspase-1 measurement

Fluorescent Labeled Inhibitors of Caspases (FLICA) assay from ImmunoChemistry Technologies (Bloomington, USA) was used with amendment of the validated protocol (285) to quantify caspase activity. The principle of the assay is covalent binding to active caspase enzymes of the fluorescence labelled inhibitor (FITC), which exhibits the ability to diffuse in and out of cells until bound. Therefore, residual fluorescence will be extracellular and removed during wash steps; however, the bound intracellular fluorescence can be measured effectively with single fluorescence flow cytometry.

5.2.2.1 Sample preparation

Isolated platelets (5×10^8) (20μl) or whole blood (30μl) was stimulated with concentration adjusted agonist (5μl), ensuring final sample volume of 50μl (modified Tyrode's buffer supplemented with Ca^{2+}(2μM)). Dependent on the experiment some required incubation with ticagrelor (5μM) or vehicle (DMSO, 0.5%) for five minutes prior to stimulation. The samples were then stored at 37ºC for prespecified period (15-60minutes) before addition of the FLICA reagent (150x stock diluted 1:14 prior to use,5μl). The samples were then

placed in a light controlled 37°C environment for a further one hour to enable fluorescence binding, prior to fixing with BD Phosphoflow Lyse/Fix Buffer for 10 minutes. The samples were then centrifuged for 10minutes (1000g, 4°C) and the supernatant aspirated. The pelleted cells were resuspended in 300µl of Tris buffered saline (TBS) (150mM NaCl, 20mM Tris base, pH7.4). Subsequently the samples were spun (10minutes, 1000g, 4°C), aspirated, resuspended (300µl TBS) and spun again. Finally, all remaining supernatant was removed and the cells resuspended in 200µl of TBS before analysis with flow cytometry.

5.2.2.2 Flow cytometric analysis of samples

Analysis was performed on FACS Fortessa using previously optimised settings. Platelet, granulocyte and monocyte populations were isolated based on their individual scatter, and confirmed using cell specific labelling (CD42b, CD45 and CD14 respectively) in the whole blood experiments. The number of recorded events in the isolated platelet preparations was 10000, while in whole blood it was guided by monocyte events (lowest cellular concentration) at 1000 events. FITC fluorescence associated with the FLICA assay was stored for each population, recording median fluorescence intensity.

5.2.3 Statistical analysis

Data was analysed using GraphPad Prism Version 6.0c. and expressed as mean ± SEM, unless otherwise stated. Direct statistical comparisons were carried out using Student's t-test (two groups) or one-way ANOVA (>2 groups). Overall effect was explored by two-way ANOVA, with additional post-test Bonferroni method for multiple comparisons. P value of <0.05 was considered to indicate statistical significance.

5.3   RESULTS

5.3.1   VASP phosphorylation

During quantification of platelet signalling, with measurement of vasodilator stimulated phosphoprotein phosphorylation (phosphoVASP), evaluation of the effects on granulocytes, monocytes and lymphocytes signalling in the same whole blood sample were undertaken. Analysing multiple experiments in a single tube, isolating each condition using fluorescent bar-coding; thus, demonstrating further advantages of whole blood experimentation and flow cytometry.

The monocyte population expressed increased levels of phosphoVASP (Ser[157]) in the presence of PAR1 peptide agonism ($p<0.001$). Also, there was a raise in phosphoVASP expression with a dose dependent response to PGI_2 ($p<0.001$) (MFI 1779$\pm$157 to 3039$\pm$305 (PGI_2 100nM)), and this was independent of the presence of PAR1 peptide agonist ($p<0.001$) (MFI 2486$\pm$210 to 4346$\pm$512 (PGI_2 100nM) with PAR1 peptide agonist). The presence of ticagrelor significantly altered this response at higher concentrations of PGI_2, showing a 17.3% reduction in fold change when the whole blood sample was stimulated with PAR1 peptide agonist (PGI_2 100nM) ($p<0.001$). (Figure 5.2)

The granulocyte population demonstrated a significant increase in expression of phosphoVASP following PAR1 agonist stimulation ($p<0.001$). PGI_2 displayed no effect on phosphoVASP levels in these cells ($p=0.85$), unless in the presence of PAR1 activation which conveyed a small but significant reduction in phosphoVASP ($p=0.001$) (MFI: 15132$\pm$1896 and 19487$\pm$2186 (PAR1 agonist) to 17946$\pm$2323 (PGI_2 100nM & PAR1 agonist)). However, there was no impact on phosphoVASP by ticagrelor in granulocytes ($p=0.85$ and 0.64 with PAR1 agonist). (Figure 5.3)

The lymphocyte population analysis found overall PAR1 peptide agonist ($p<0.001$) to increase phosphorylation of VASP. A dose dependent response was seen with PGI_2 ($p<0.001$) (MFI: 537$\pm$50 to 703$\pm$64 (PGI_2 100nM)) and this finding was consistent in the presence of PAR1 peptide agonist ($p<0.001$) (800$\pm$77 to 1041$\pm$119 (PGI_2 100nM) with PAR1 peptide agonist). Furthermore, ticagrelor overall significantly lowered levels of phosphoVASP ($p=0.0064$) demonstrating a 12% fall in levels independent of presence of PAR1 activation. (Figure 5.4)
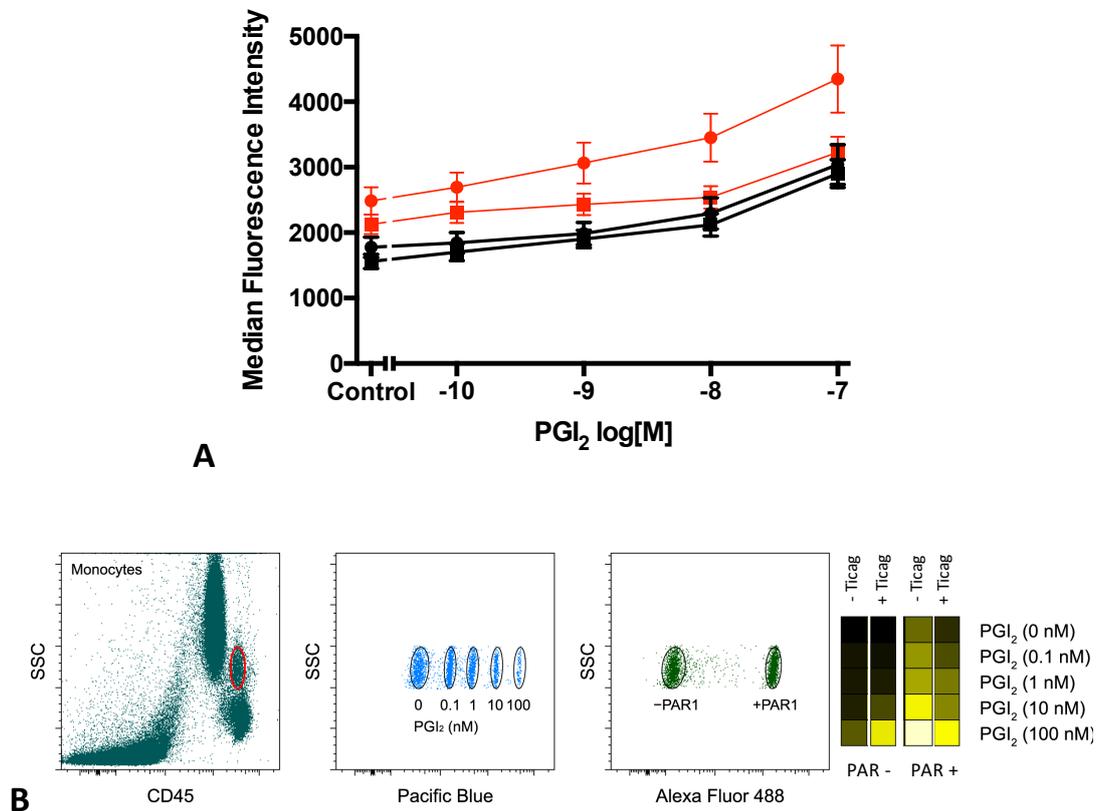
Figure 5. 2: VASP phosphorylation in response to prostacyclin in monocytes

Whole blood was incubated with increasing doses of PGI₂ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor. The experiments were performed in presence of agonist (red) or absence of PAR1 peptide (10μM) (black) (A). Samples were studied with flow cytometry and deconvoluted/analysed with Cytobank, with each experimental condition being isolated using fluorescent bar coding, and unique cell scatter (B). Heat maps quantify phosphoVASP *expression with low levels (dark) through to high levels (light). N=51 and error bars represents standard error of the mean.*

A

B
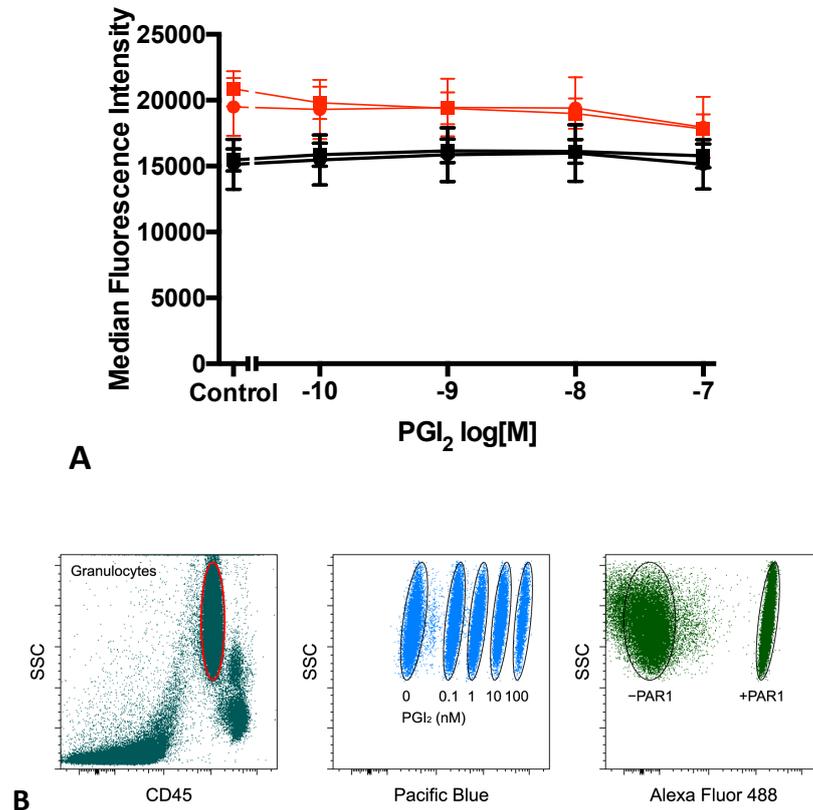
Figure 5. 3: VASP phosphorylation in response to prostacyclin in granulocytes

Whole blood was incubated with increasing doses of PGI$_2$ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor. The experiments were performed in presence of agonist (red) or absence of PAR1 peptide (10μM) (black) (A). Samples were studied with flow cytometry and deconvoluted/analysed with Cytobank, with each experimental condition being isolated using fluorescent bar coding, and unique cell scatter (B) N=61 and error bars represent standard error of the mean.
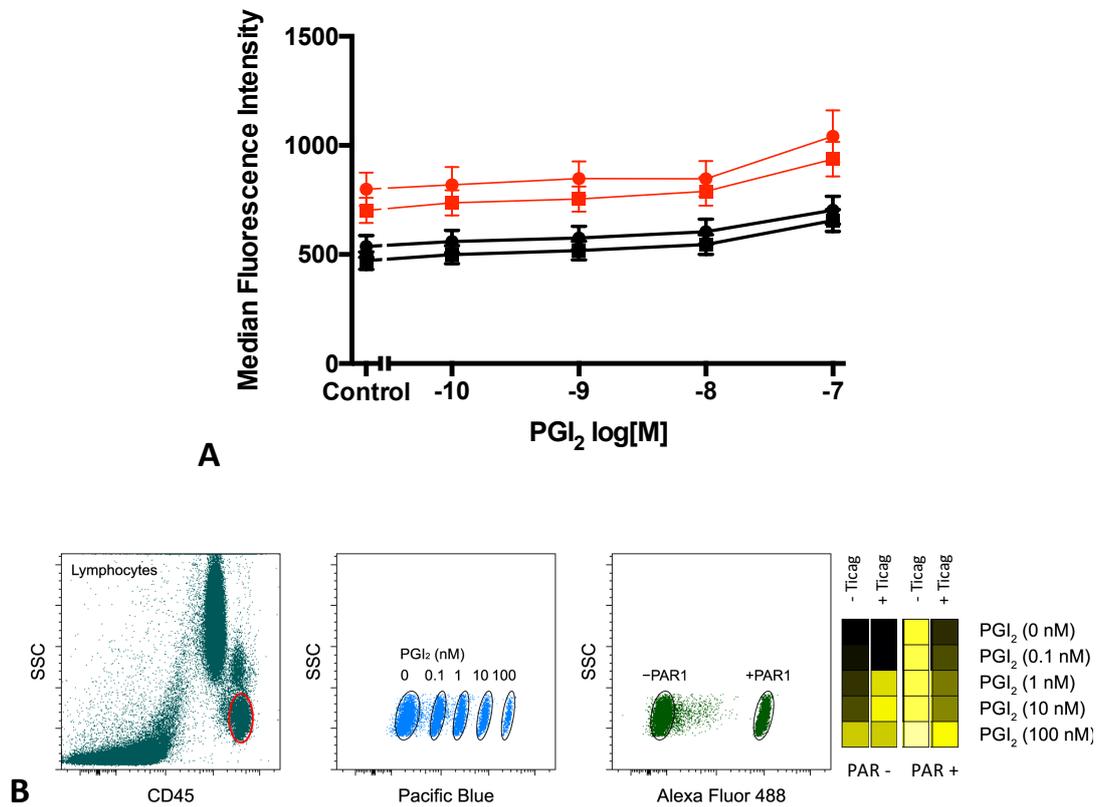
Figure 5. 4: VASP phosphorylation in response to prostacyclin in lymphocytes

Whole blood was incubated with increasing doses of PGI₂ (0-10nM) pre (circle ●) and post (square ■) three days of oral ticagrelor. The experiments were performed in presence of agonist (red) or absence of PAR1 peptide (10μM) (black) (A). Samples were studied with flow cytometry and deconvoluted/analysed with Cytobank, with each experimental condition being isolated using fluorescent bar coding, and unique cell scatter (B). Heat maps quantify phosphoVASP *expression with low levels (dark) through to high levels (light). N=61 and error bars represent standard error of the mean.*

Considering the research covered in the introduction and the significant effect on leukocyte signalling induced by PGI_2 and ticagrelor, further experiments were undertaken to investigate the interaction between platelets, leukocytes, inflammation and potentially $P2Y_{12}$ inhibition.

5.3.2 Caspase activity

Caspase activity has previously been recorded in platelets (286) and this was confirmed with low basal levels detected in isolated platelets and a dose dependent response seen to nigericin and PAR1 peptide agonist. Incubation with the agonists for thirty minutes before addition of the FLICA (60minutes before lysing/fixing) ensured a predictable response suggesting a capacity for NLRP3 inflammasome activation within the platelet. (Figure 5.5)

Repeated experiments were consistent, and results demonstrated that both nigericin and PAR1 peptide agonist induce platelet Caspase-1 activation, with nigericin being the more potent activator (MFI: 42 ± 6, 131 ± 63, 429 ± 309, 650 ± 323 (nigericin 0-20μM) and 38 ± 6, 76 ± 23, 96 ± 18 (PAR1 agonist 0-10μM)). The increase from basal levels however failed to reach statistical significance ($p=0.306$ nigericin, $p=0.11$ PAR1 agonist). (Figure 5.6)

The same conditions were further used to explore if ticagrelor exhibited any effect on the ability of agonist to increase platelet Caspase-1 levels. (Figure 5.7) No predictable impact was demonstrated with ticagrelor but in these results nigericin did produce a significant overall increase in Caspase-1 (MFI: basal 43 ± 4 and 20μM nigericin 1080 ± 290) ($p=0.001$).
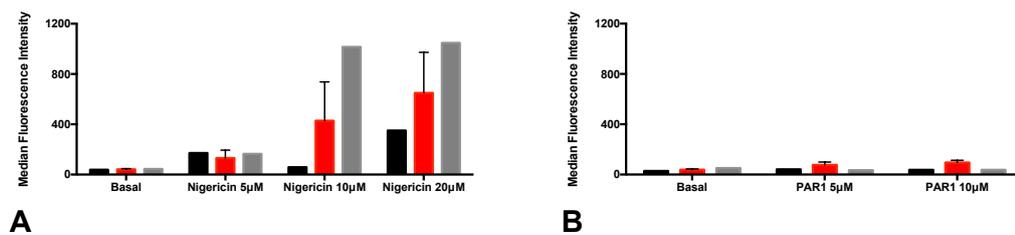
Figure 5. 5: Incubation response of Caspase-1 to nigericin and PAR1 peptide agonists in platelets

Isolated platelets were stimulated with variable concentrations of nigericin (0-20μM) (A) or PAR1 peptide agonist (0-10μM) (B) for 15 (black bars, n=1), 30 (red bars, n=4) or 60 minutes (grey bars, n=2 (A) and 1 (B)). The samples were then incubated for 60 minutes with FLICA before lysing, washing and analysing on FACS Fortessa flow cytometer. Results are median fluorescence intensity in histogram format comparing timing and concentration. Error bars represent standard error of the mean.
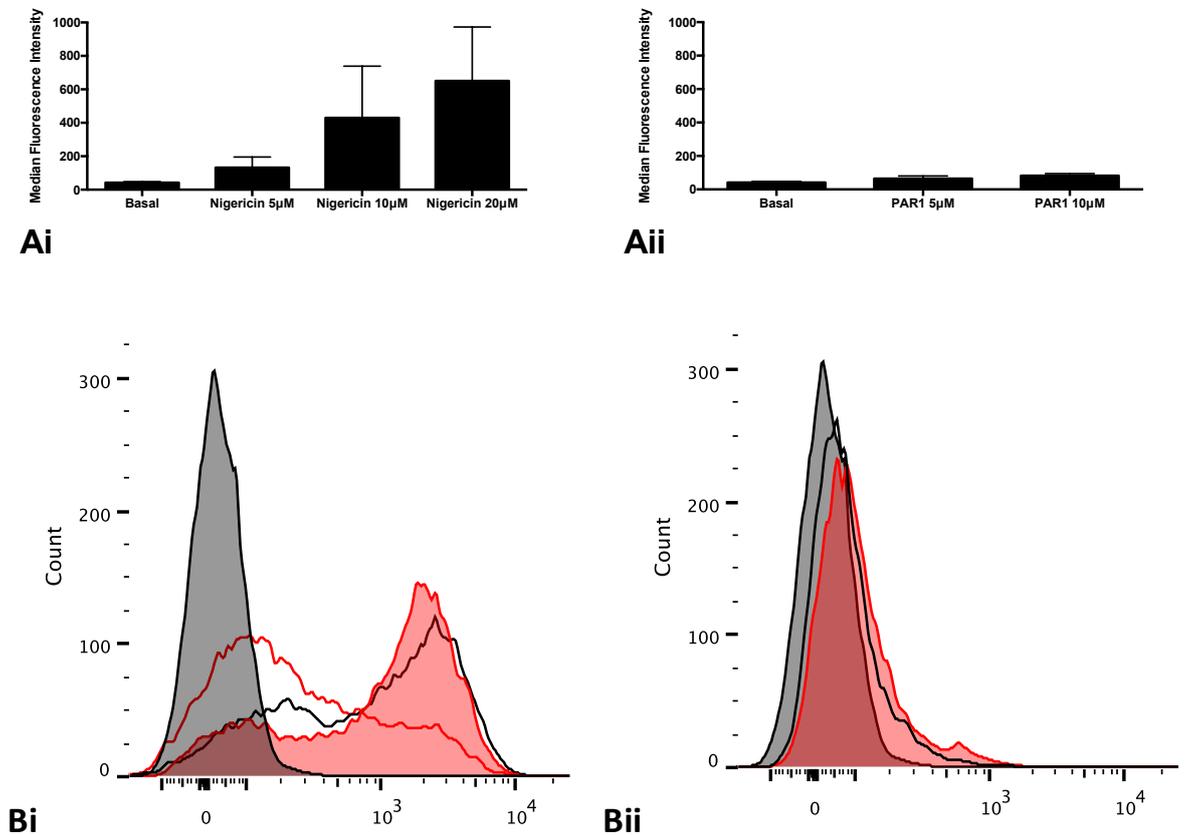
Figure 5. 6: Caspase-1 levels in isolated platelets in response to nigericin and PAR1 peptide agonist

Isolated platelets were stimulated with variable concentrations of nigericin (0μM (grey), 5μM (red line), 10μM (black line) and 20μM (red)) (i, n=4) or PAR1 peptide agonist (0μM (grey), 5μM (black line) and 20μM (red)) (ii, n=5) for 30 minutes. The samples were then incubated for 60 minutes with FLICA before lysing, washing and analysing on FACS Fortessa flow cytometer. Results shown as median fluorescent intensity (A) and flow cytometry histograms (B). Error bars represent standard error of the mean.
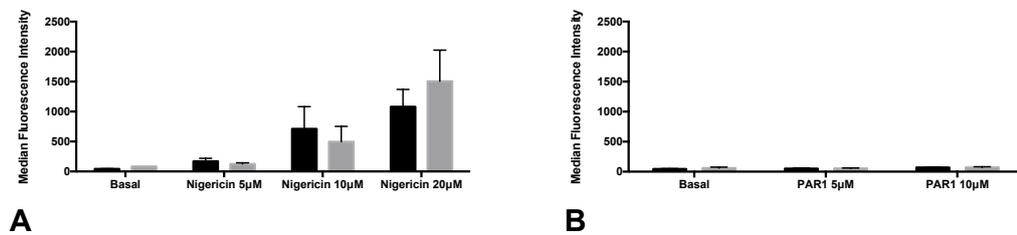
Figure 5. 7: Effect of ticagrelor on Caspase-1 in platelets

Isolated platelets were incubated with vehicle (black bars) or ticagrelor (5μM) (grey bars) for 5 minutes before stimulation with variable concentrations of nigericin (0-20μM) (A) or PAR1 peptide agonist (0-10μM) (B) for 30 minutes. The samples were then incubated for 60 minutes with FLICA before lysing, washing and analysing on FACS Fortessa flow cytometer. Results are median fluorescence intensity in histogram format comparing concentrations. Error bars represent standard error of the mean (n=3).

5.4   DISCUSSION

Endothelial vasodilators trigger the cyclic nucleotide cascade, culminating in the protein kinase driven phosphorylation of VASP. PGI_2 and NO prompt their cyclic nucleotides (cAMP/cGMP) to activate PKA and PKG respectively. These phosphorylate a variety of substrates including VASP. (144) VASP is a 46/50kDa (VASP/PhosphoVASP) phosphoprotein isolated in platelets and other cell types including granulocytes and lymphocytes. (287) The role of VASP in platelet activation has been previously covered (section 1.4.2.3). Broadly, levels of phosphorylated VASP (phosphoVASP) are inversely proportional to platelet activation. PhosphoVASP formation has a potent effect on actin dynamics and subsequent platelet cytoskeletal change important in activation, preventing the polymerisation of actin despite it still binding with VASP. (145) This means that phosphoVASP, by well-established methodologies, is a measure of platelet activation and marker of cyclic nucleotide driven cell signalling. (288,289) In platelets, there are three sites of cyclic nucleotide dependent phosphorylation - Ser^{157}, Ser^{239} and Thr^{278} - which have variable kinetics and affinity dependent on the active enzyme. Ser^{157} is the preferred site of PKA related phosphorylation both *in vitro and in vivo*. Ser^{239} is the PKG related preferential site *in vitro*, however, there is no Ser predilection *in vivo*. Thr^{278} is phosphorylated by both enzymes but only following Ser interaction. (290)

Platelet derived VASP has been demonstrated to be critical to the relationship of platelets with circulating neutrophils, as phosphorylation reduces the formation of platelet-neutrophil aggregates. This reduction correlates with reduced ischaemia/reperfusion myocardial injury. (291) However, the role of VASP within leukocytes is currently incompletely defined. Data suggests that VASP is expressed in leukocytes, and in ischaemia VASP deficiency leads to increased monocyte and neutrophil infiltration. (292) In neutrophils PKA induced transient VASP Ser^{157} phosphorylation is triggered, following stimulation by chemoattractant molecules effecting cellular actin polymerisation and migration. (293) The relationship between monocytes and phosphoVASP is less well described, but experiments using THP-1 monocytes differentiated into macrophage-like cells do contain VASP with phosphorylation shown to increase following introduction of infection. (294) Therefore, contrary to platelet-derived phosphoVASP reducing

activation/PLA formation, data suggests accumulation of leukocyte phosphoVASP is indicative of activation of the inflammatory cascade.

Our results in patients with CAD demonstrate detectable levels of phosphoVASP in monocytes, granulocytes and lymphocytes. Following addition of PAR1 peptide agonist VASP phosphorylation was increased across all leukocytes. The role of the protease activated receptor in platelets is relatively well understood (section 1.3.8.4); and crosstalk between PAR1 receptor activation in platelets and leukocytes may be influential in this finding. However, they are also expressed in monocytes (295,296), macrophages (295), granulocytes (297) and lymphocytes (296) with PAR1 receptor the most abundant; so potentially this finding could be secondary to a direct action. (296) PAR1 receptor activation have been shown to increase monocyte chemoattractant protein-1 (MCP-1) suggesting it drives inflammation. Anti-inflammatory messengers such as IL-4 down-regulate PAR1 expression. (295) The results demonstrating increased levels of leukocyte phosphoVASP in response to PAR1 receptor activation are consistent, as both actions have been linked with increased inflammation.

PGI_2 analogues (iloprost, cisaprost) and medications targeting prostanoids (ibuprofen, indomethacin) are in common clinical use for a plethora of conditions; including targeting the inflammatory processes. Like other prostaglandins, PGI_2 is known to have a role as a mediator in pain and inflammation as prostacyclin receptor deficient mice have a reduced response to stimuli. (298) Nonetheless, counter evidence has suggested an anti-inflammatory role for PGI_2 in immunoglobulin mediated allergic inflammation (299); implying an integral but complex role of PGI_2 in inflammation dependent on pathophysiology. This in part may explain the variable phosphoVASP response to PGI_2 by the different leukocytes; as monocytes and lymphocytes had a dose dependent increase in keeping with increased inflammation, while granulocytes had a reduction following PAR1 receptor activation. The granulocyte response maybe related to the neutrophil increase in VASP phosphorylation being transient (293), or possibly a consequence of the granulocyte basophil population, cells implicated in allergic reactions, having a converse response.

$P2Y_{12}$ inhibitors have been shown to suppress inflammatory pathways, with ticagrelor having greatest potency, potentially related to the increase circulating levels of adenosine. (228) The exact mechanism by which this occurs remains uncertain, but hypotheses include

directly through platelet–leukocyte interaction or via other $P2Y_{12}$ expressing cells such as smooth muscle where stimulation upregulates monocyte chemoattractant protein-1 (MCP-1) (229) and induces IL-6 production. (230) Lymphocytes and monocytes do express $P2Y_{12}$ receptors (300), with greatest abundance in lymphocytes. We observed a small but significant reduction in phosphoVASP in lymphocytes but a more evident reduction in monocyte phosphoVASP, especially in the presence of PAR1 peptide agonist. This supports the involvement of PKA induced VASP phosphorylation in the anti-inflammatory effect of ticagrelor. Neutrophils do not express $P2Y_{12}$ receptor (301), possibly explaining why we failed to elicit any response in the granulocyte population. Yet it has been shown that the presence of the thienopyridine $P2Y_{12}$ antagonists still prevents neutrophil activation independent of absence of neutrophil located receptors (301), suggesting the interaction is complex and beyond solely a direct cyclic nucleotide effect on leukocytes.

The patient results triggered further preliminary work to assess inflammation in platelets and the effect of replicated experimental conditions. Focusing on Caspase-1 as a chief mediator of IL-1β driven inflammatory response, in keeping with current understanding Caspase-1 was isolated in platelets (286) and levels increased in response to known potent NLRP3 activator nigericin. (302,303) To a lesser extent, PAR1 peptide agonist also induced a dose dependent increase in intra-platelet Caspase-1. PAR1 receptor activation has been shown to have a role in a number of inflammatory processes and systems, and apoptosis following interaction with other caspases (304,305); but to the best of our knowledge no data has been published regarding PAR1 receptor agonism and Caspase-1 production.

No direct interplay between ticagrelor and Caspase-1 levels were seen but, interestingly, the combination of $P2Y_{12}$ antagonism and pharmacological Caspase-1 inhibition has been shown to reduce infarct size in rat hearts when administered at reperfusion. (306) This suggests the potential of a common downstream pathway.

5.5   CONCLUSIONS

Leukocytes contain detectable levels of phosphoVASP, and phosphorylation is increased in response to PAR1 peptide agonism. PGI_2 also impacts VASP conversion to phosphoVASP but this is variable in keeping with anti-inflammatory and inflammatory data dependent on process/tissue involved. Ticagrelor affected VASP signalling in monocytes and lymphocytes, cells known to have $P2Y_{12}$ receptors. Careful review of the literature suggests that this complex signalling cascade potentially plays an important role, therefore it remains an area of interest.

6 CHAPTER SIX: NO REFLOW PHENOMENON AND COMPARISON TO THE NORMAL FLOW POPULATION POST PRIMARY PERCUTANEOUS CORONARY INTERVENTION FOR ST ELEVATION MYOCARDIAL INFARCTION: A CASE-CONTROL STUDY (NORM PPCI STUDY)

6.1 INTRODUCTION

The results from previous chapters in this thesis have provided further insight into the crosstalk between platelets and the endothelium. Furthermore, the role of inflammatory cells in this interaction. The academic interest in this area is substantial, from bench, through translational to clinical studies, as there is potentially significant benefit to be seen on outcomes in patients with ACS. Following the translational earlier work, the next step was a more clinical exploration of interaction between ACS, platelets and the endothelium. A condition intrinsically linked with inflammation, platelet activation and endothelial dysfunction but incompletely understood is "no reflow" phenomenon (NR) (see section 1.5), a cohort that have poor outcomes in ACS and which is the focus of the next chapter.

6.1.1 Background

Urgent reperfusion strategy has been the gold standard of care for STEMI since the 1980s when thrombolysis was proven to significantly reduce all-cause mortality. (23,307) In the 1990s this method of reperfusion was challenged with superior efficacy (reduction of death, re-infarction) and safety endpoints (major bleeding) demonstrated in those managed with PCI. (24,25,308) These findings resulted in the wide spread adoption of primary PCI (PPCI) as the reperfusion strategy of choice in patients presenting with STEMI, where feasible, to deliver in a timely fashion. (26,27) Following successful epicardial arterial patency, if myocardial perfusion is not re-established, then this is known as NR, and was first described in humans with acute myocardial infarction in 1992 by Ito et al. (309) Emphasising the importance of the work in the previous chapters NR is heavily associated with an inflammatory process involving leukocytes, platelets and the endothelium; and the crosstalk between them.

Multiple modalities can diagnose failure of myocardial perfusion, including electrocardiographic (ST-resolution), angiographic (Thrombolysis in Myocardial Infarction (TIMI) flow, myocardial blush grade (MBG) (table 6.1)), echocardiographic (Myocardial Contrast Echocardiogram (MCE)) and magnetic resonance imaging (MRI).

To be formally classified as NR there must also be no evidence of mechanical obstruction, flow limiting dissection, vessel spasm or heavy thrombus burden. (310) Although acknowledged in the thrombolysis era the phenomenon has been increasingly recognised since the adoption of PPCI. (26,27) Importantly, impaired perfusion may still be present despite evidence of TIMI III flow and is frequently under reported/recognised. NR has a negative impact on prognosis and is associated with a larger size of infarction. (194,311) In this chapter we will give an overview of NR before exploring those at risk with published models, and the challenges of managing these patients.

TIMI score		Blush Grade	
0	No flow	0	No blush
I	Flow at a standstill	I	Blush but no clearance
II	Flow but sluggish	II	Blush but sluggish clearance
III	Normal flow	III	Blush and cleared within 3 cardiac cycles

Table 6. 1: Thrombolysis in Myocardial Infarction Flow and Blush Grade definitions

(312,313)

6.1.2   Pathophysiology

The pathophysiology of NR is multifactorial and broadly can be defined by 4 distinct groups: 1) Distal atherothrombotic embolisation; 2) ischaemic injury; 3) reperfusion injury; and 4) susceptibility of coronary microcirculation to injury. (193) Distal embolisation is a result of debris consisting of thrombi, endothelial cells and lipid matrix migrating downstream from the primary lesion leading to microvascular obstruction and further injury. (314) Ischaemic injury is proportional to the duration of obstruction secondary to the described thrombi, endothelial protrusions and extrinsic compression caused by oedematous change in the myocytes. Reperfusion injury is seen following sudden exposure of the endothelium in the distal vessel and surrounding tissue to neutrophils. These initiate an inflammatory cascade, which results in direct endothelial and tissue damage. Furthermore, neutrophils aggregate with platelets to form micro-thrombi, which can also contribute to the mechanical occlusion. This is compounded by the release of endogenous vasoconstrictors from platelets, neutrophils and damaged endothelial cells. (193,310)

6.1.3   Incidence and outcomes

The incidence of NR quoted in the literature is highly variable ranging from 5-65%. (193,194,315) This inconsistency is in part dependent on the definition of NR and the sensitivity of the defining methodology. In particular, angiographic evaluation is relatively insensitive as compared to imaging with cardiac MRI or MCE.

In one study of STEMI patients, the rate of angiographic NR was 29% and was associated with a significantly worse outcome at 5 years (Hazard Ratio (HR) 1.66 (95% confidence interval (CI) 1.17-2.36), p=0.004); interestingly the Kaplan Meier graph lines continue to diverge. This difference was more significant when focused on cardiovascular death (12.4% vs 6.3%; Odds Ratio (OR) 2.18 (95% CI 1.46-3.27), p=<0.001). (194) This negative association is consistent across all registries (311,315) and reflects the adverse morbidity of larger infarct size, increased frequency of LV dysfunction, cardiogenic shock, stroke and predisposition to arrhythmia. (311,316,317) Those with persistent NR evident beyond 6 months (20%) confer a worse outcome still. (316)

6.1.4 Predictors

Given the importance of the condition a number of studies and registries have aimed to identify those at greatest risk of NR, with the hope that this would help influence management and improve outcomes. The largest registry from America analysed a population of 291,380 ACS patients (STEMI in 63%), NR was reported in 6553 (2.2%) with the independent predictors of NR outlined in table 6.2. (311)

Early NR (occurring between pre-dilation and prior to stent insertion) was associated with delayed time to reperfusion, systolic hypotension and increased thrombus burden. This early NR appears to be particularly important clinically. In a study of 420 patients with STEMI, early NR was associated with a significantly higher rate of all-cause mortality (p=0.034) and total major adverse cardiovascular events (MACE) compared to NR that occurred after stent insertion. (318)

Iwakura et al evaluated a STEMI population and identified hyperglycaemia, initial TIMI 0 flow, anterior infarct territory, absence of pre-infarct angina, male sex and increasing age as independent predictors that could be identified pre-intervention. (319) Hyperglycaemia was stated as the strongest independent predictor whether NR was identified on MCE (319) or angiography; with 44.4% of hyperglycaemic patients experiencing NR as compared with 23.5% in normoglycaemic patients (p=0.02). (320) Hyperglycaemia (defined as glucose level >8.9mmol/L (>160mg/dL)) had a risk ratio (RR) for NR of 12.1 (95% CI 2.7-61.2). (319) Interestingly, the association of NR appears to relate to the presence of an acutely elevated glucose and not the presence of pre-existing diabetes mellitus or elevation of HbA1c. (194,319,321) Therefore its plausible hyperglycaemia is responsive rather than causal; secondary to a glucagon stress response and not predisposition to DM. Studies have shown that the hyperglycaemic state does lead to increased platelet aggregation driven by a multitude of pathways including increasing p-selectin expression, impaired function of endogenous antiplatelet agents/endothelial vasodilators (160),

	OR	95% CI	Chi-square	p value
Cardiogenic shock at presentation	1.83	1.69-1.98	213	<0.001
Lesion length (per 10mm increase)	1.17	1.14-1.20	143	<0.001
Age (per 10-year increase)	1.14	1.12-1.17	134	<0.001
ACC/AHA class C lesion	1.47	1.36-1.59	102	<0.001
STEMI versus none	1.39	1.30-1.48	100	<0.001
Current smoker	0.78	0.74-0.83	72	<0.001
Pre-procedure TIMI flow			64	<0.001
0 (vs 3)	2.12	1.83-2.45		<0.001
1 or 2 (vs 3)	1.84	1.60-2.12		<0.001
Bifurcation lesion	1.29	1.19-1.40	36	<0.001
Symptom onset to admission >12h	1.18	1.10-1.26	23	<0.001
Previous PCI	0.82	0.75-0.90	18	<0.001
Signs of heart failure at presentation	1.17	1.07-1.28	12	<0.001

Table 6. 2: Factors independently associated with no-reflow in patients presenting with acute myocardial infarction undergoing PCI

Multivariate analysis (311)

amplified platelet adhesion (175), a pro-inflammatory environment (173) and increased platelet turnover. (322) These effects are all potential contributors to the increased risk of NR.

There is evidence to suggest that NR may be more common in men, although the causal factors are unclear. Animal models have postulated that this may reflect the presence of circulating endogenous oestrogen (a vasodilator) as well as an increased response to the vasodilatory effects of nitric oxide seen in females. (323,324) Conversely, male animals have increased levels of the vasoconstrictor thromboxane. There is also evidence that there may be gender-specific differences in microcirulatory responses including the reaction to platelet-induced vasodilation. (323)

A recently identified pre-procedural predictor of NR is epicardial adipose tissue (EAT) thickness. EAT is true visceral fat deposit localised beneath the visceral pericardium, which can be visualised and measured non-invasively by echocardiography. EAT thickness correlated closely with metabolic risk factors and was observed to be increased in patients with NR (3.9mm±1.7 vs. 5.4±2, p=0.001), where NR was diagnosed by failure of ST resolution. Further statistical analysis demonstrated EAT to have good accuracy as a predictor of NR. (325)

Procedural findings of an initial TIMI flow ≤2 confers a twofold risk of NR. (316) Indeed, if there is TIMI 0 flow following guidewire insertion then the risk of final TIMI flow ≤2 is 32%, as compared to 10% in those with evidence of any flow after guidewire insertion. Importantly, this difference translates into mortality benefit (HR 0.51 (95% CI0.28-0.94), p=0.029). (326,327)

NR has also been linked with lesion severity and the degree of disease burden. (311,328) This is supported by recent data from a study of cardiac computerised tomography in patients within 1 week following admission with a STEMI, which declared an increased calcium scoring (>100) was independently associated with increased risk of NR (OR 4.4 (95% CI 1.17-16.3)). (329) Aortic valve calcification has been shown to reflect the presence of coronary atherosclerosis and evidence of non-obstructive aortic sclerosis is associated with NR independent of other characteristics (OR 1.05 (p=0.039)). (330)

A multivariate analysis by Abdi et al concluded that although lesion severity was suggestive of an increased risk of NR, lymphocyte count and thrombus burden were stronger predictors. (331) A number of studies have linked lymphocyte count to NR risk, however, not all studies show that lymphocyte/neutrophil count are directly important but more weight has been given to the resulting ratio (332), with a higher Neutrophil-lymphocyte ratio (NLR) associated with increased risk of no reflow (NLR >4.34 had a 72% sensitivity and 70% specificity in predicting NR). (333–336) In addition to NLR, platelet lymphocyte ratio (PLR) has also been shown to be a strong marker of NR phenomenon (PLR ≥126 had a sensitivity of 73% and specificity of 71%). (258,335) Another biochemical result declared as an independent risk factor for NR is the presence of hypoalbuminaemia (<37.5g/l). (337)

6.1.5 Risk scores

Two publications have attempted to produce a risk scoring system based on the predictors of NR previously identified. The score from Dogan et al is illustrated in table 6.3 and figure 6.1. A value of ≥4 has a sensitivity and specificity for NR of 47% and 88% respectively. (338)

The score by Wang et al has better sensitivity and specificity (76% and 71% respectively for a score ≥14), but is a much more complex system that involves a number of variables (table 6.4 and figure 6.2). (339)

These scoring systems were published in 2015 and are yet to be externally validated. In addition, both incorporate the results of blood investigations (lymphocyte/neutrophil count) which are not commonly available at the time of PPCI, meaning that they have some limitation in terms of impacting on upfront patient treatment to reduce the incidence of NR.

Symptom-balloon time		Lymphocyte count		Blood sugar level	
≥3.5h	3	≥ 1830 uL	2	≥225mg/dl (12.5 mmol/L)	1
<3.5h	0	<1830 uL	0	<225 mg/dl (12.5 mmol/L)	0

Table 6. 3: Dogan et al clinical risk scores for no-reflow (338)

Variable		OR (95% CI)	p value	β-Coefficient	Risk Score
Age ≥ 55 years	Yes	3.15(2.23-4.44)	<0.001	1.147	5
	No				0
Pain to PCI ≥ 4h	Yes	1.69(1.25-2.28)	0.001	0.522	2
	No				0
Admission glucose ≥ 12mmol/L	Yes	2.03(1.38-3.02)	<0.001	0.712	4
	No				0
Neutrophil count ≥ 8.81x10^9/L	Yes	6.36(4.66-8.69)	<0.001	1.851	8
	No				0
Killip Class 4	Yes	1.87(1.30-2.69)	0.001	0.626	3
	No				0
Pre-PCI thrombus score ≥ 2	Yes	3.23(2.06-5.06)	<0.001	1.173	5
	No				0
Collateral circulation grade ≤ 1	Yes	1.55(1.03-2.34)	0.037	0.439	2
	No				0

Table 6. 4: Wang et al model for a clinical risk score for no-reflow (340)
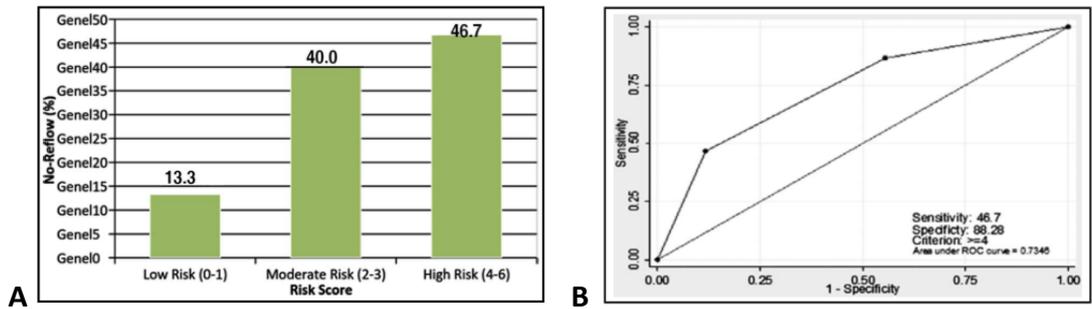
Figure 6. 1: *Rates of no-reflow in patients with low, moderate and high-risk scores (A) ROC analysis of the no-reflow risk model in the study group (B)* (338)
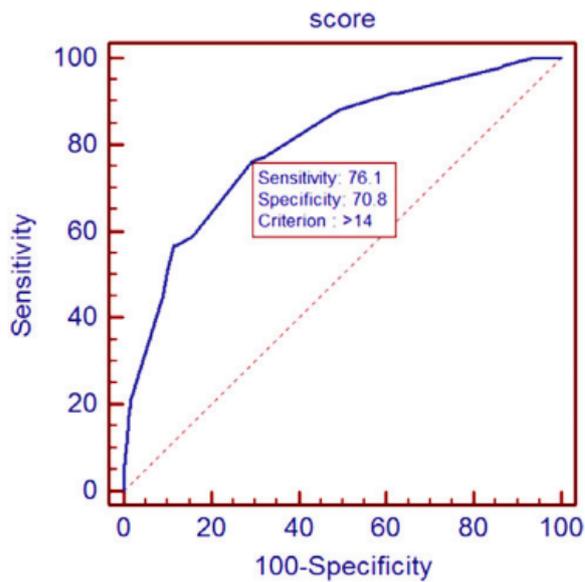
Figure 6. 2: *ROC curve for the NR risk model based on the Wang et al test dataset* (340)

6.1.6   Intervention/treatment

Given the poor outcomes related to NR there has been a plethora of research into potential therapeutic options both in the prevention of the condition and treatment following occurrence, exploring medication and procedural interventions.

6.1.6.1   Pharmacological

A clear causative factor in no reflow development is platelet activation and thrombus formation, leading consideration of these pathways as therapeutic targets. Glycoproteins (GP) IIb and IIIa are found on platelet membranes and upon calcium-dependent activation form an integrin complex capable of binding fibrinogen, a process which is essential to platelet aggregation. GP IIb/IIIa inhibitors (abciximab, eptifibatide, tirofiban) have therefore frequently been postulated as agents for the prevention and management of NR, though this has to weighed against the associated increased risk of bleeding. A study by Jia et al evaluated the role of routine upfront tirofiban administered in the emergency room as compared to more selected use only in patients shown to have high thrombus burden on angiography. The results showed a reduction in the use of tirofiban in the selected group (57%) but no difference was demonstrated in terms of the final TIMI flow or rate of bleeding. (341) Similarly, other antithrombotics have not shown favourable results as potential preventative agents. Early animal studies looking at the novel oral anticoagulant dabigatran found no evidence of reduction in NR. (342) HEAT PPCI, a study investigating the outcomes of STEMI patients managed with heparin versus bivalirudin, also found no difference in the end procedure TIMI flow. (343)

Tirofiban has also been evaluated as a potential treatment for established NR. A total of 162 patients with STEMI and NR were randomised to either a bolus of intracoronary tirofiban (25µg/kg) or placebo (saline). The study found a significant improvement in end procedure TIMI flow in the tirofiban arm (p=0.001). This promisingly translated in to a statistical reduction in 24hour cardiac enzymes and in-hospital MACE rates (p=0.013). (344) Currently there appears no importance attributable to the route of administration of GP IIb/IIIa (345), but novel techniques have begun the exploration of intra-lesion delivery.

Considered in collaboration with antithrombotic agents a potential pharmacology target for medication may be the endothelium and/or vascular resistance. Adenosine is an endogenous nucleoside, which acts as a vasodilator and also inhibits activation of mechanisms critical in the process of NR. Adenosine's therapeutic role in prevention of NR was conclusively reviewed in 2015 by Cochrane, assessing its use in patients presenting with acute myocardial infarction. The review showed that adenosine administration resulted in statistically fewer episodes of reduced end procedure TIMI flow (9 studies; n=907; 97% delivered intracoronary) with RR of 0.62 (95% CI 0.42-0.91). All-cause mortality both short term (RR 0.61 (95% CI 0.25-1.48)) and long term (RR 0.78 (95% CI 0.22-2.74)) followed this with a trend but failed to reach significance. (346)

Nicorandil combines nitrate properties and adenosine triphosphate (ATP) potassium channel agonism, with some speculation to its anti-free radical and neutrophil modulating properties. (347) A recent meta-analysis by Wu et al (2013) reviewed RCTs where nicorandil was administered prior to reperfusion therapy (7 studies, n=1284). This showed that those treated with nicorandil had significantly fewer end-procedure TIMI≤2 flow (RR 0.57 (CI 95% CI 0.42-0.79)) compared to placebo, conferring a reduction in episodes of heart failure (RR 0.41 (95% CI 0.22-0.75)) but not death (RR 0.39 (95% CI 0.09-1.67)). (348) The clinical application of these positive findings is muted due to the limited worldwide availability of the intravenous preparation. Indeed, the majority of the studies in the review were limited to Japan, where it's accessible.

There is limited evidence supporting adenosine's or nicorandil's role in the treatment of established NR. Other therapeutic options investigated include calcium channel antagonists and epinephrine. The RECOVER trial (n=102) showed that in patients with established NR, verapamil and diltiazem delivered directly through a microcatheter were superior to nitroglycerin, significantly improving corrected TIMI frame count; however, there was no benefit to clinical secondary outcome rates. (349) Another small study, retrospectively observing patients with resistant NR despite initial pharmacological management, investigated the effects of bolus doses of intracoronary epinephrine (100-400mcg) via an over-the-wire balloon. This showed an improvement in angiographic markers of NR without detrimental haemodynamic changes. (350)

Statin treatment is known to have anti-inflammatory and anti-platelet activation effects, as well as convincing evidence of limiting plaque progression. (351,352) Therefore, perhaps unsurprisingly, data collected from patients chronically pre-treated with standard dose statin therapy has shown a statistically lower rate of NR, compared to those statin naïve, independent of underlying lipid profiles. (353,354)

6.1.6.2   Non-pharmacological

Whether thrombectomy has a role in PPCI for STEMI has been considered at length following the positive results from the TAPAS trial (n=1071), which showed that thrombus aspiration was associated with a significant reduction in MBG 0-1. (355) The Bavry et al meta-analysis supported the TAPAS results with MGB III evident more commonly in patients treated with aspiration catheters (12 studies) (RR 1.69 (95% CI 1.26-2.28)) and embolic protection devices (8 studies) (RR 1.18 (95% CI 1.02-1.38)). (356) However, two subsequent, adequately powered large multicentre trials TASTE (n=7244) (357) and TOTAL (n=10732) (358) designed to evaluate routine thrombectomy during PPCI were disappointingly neutral in terms of a difference in clinical endpoints compared to standard therapy without thrombectomy. However, NR or end procedure TIMI flow were not recorded. (359) Registry data suggests this absence of benefit persists to 5 years. (360)

Another procedural technique to try to reduce NR is direct stenting. In recent meta-analyses, this was shown to significantly reduce the incidence of NR (OR 0.48 (95% CI 0.31-0.75)) (361) and OR 0.4(95% CI 0.24-0.65)) (362)). However, direct stenting is not always feasible and therefore this result is invariably skewed by the inherent risk of bias this generates; consolidated by the less convincing odds when analysis is limited to randomised data only (OR 0.65 (95% CI 0.28-1.48) (362) and OR 0.65 (95% CI 0.29-1.46) (363)). Historically, stent post-dilation in STEMI has not been recommended, yet retrospective observational data (n=160), although at high risk of bias, found no increased risk of NR in the post-dilation group, measuring TIMI flow and MBG. (364) Subsequently, perhaps more emphasis should be placed on stent optimisation with treatment of under expansion, while preventing over expansion (dilation ≥20% beyond the vessels true diameter) which significantly increases the risk of NR. (365)

In fact, considering not stenting at all during PPCI may be a shrewd therapeutic decision. Although a relatively small cohort (n=101) the results were striking in the DEFER STEMI trial that included patients undergoing PPCI with at least one risk factor for NR. Risks included clinical history >6hours, older than 65 years, culprit artery abnormalities (TIMI 0/1 initially, heavy thrombus burden), or signs of microvascular injury with persistent ST-elevation despite initial reperfusion. Patients were randomised to either standard therapy or a strategy of restoring perfusion (with a balloon or thrombus aspiration catheter), administering pharmacotherapy (abciximab), followed by stent implantation deferred for 4-16 hours. Overall, the use of IIb/IIIa inhibitors and thrombectomy was similar in both groups. The primary endpoint of evidence of no or slow reflow, was highly statistically significant in favour of deferred stenting (OR 0.16 (95% CI 0.03-0.63, p=0.005). (366) This strategy was also found to be safe as although two patients in the deferred group needed urgent re-intervention, overall the level of myocardial salvage was much greater in the deferred group. We are now awaiting a large multi-centre trial to look at this as a feasible strategy within the modern healthcare system.

Work has also explored advancements in stent design. The MGuard stent has a stainless-steel strut (MGuard Prime has a cobalt chromium strut) covered by a 20μm fibre micronet intended to prevent distal embolization. Initial publications gave conflicting reports in the prevention of NR. (367,368) Improvement in TIMI flow and ST resolution has been demonstrated; however, this did not translate to benefit in MBG, TIMI count rate, cardiac MRI evidence of microvascular obstruction or clinical endpoints, arguably the more important outcomes. (369) The GUARDIAN trial is awaited comparing MGuard to thrombectomy and stent. (370)

Another non-pharmacological strategy evaluated is early therapeutic hypothermia (within 20minutes) (target reduction to 32°C). In animal models this has been shown to markedly reduce NR and infarct size, however this has not translated to human trials. Although safe, early cooling of conscious patients did not reduce NR. (371,372)

6.1.6.3 Therapy in combination

As outlined above there is limited evidence to support the adoption of any individual intervention and this has led to consideration of combination therapy in NR prevention. In 2013, REOPEN AMI (n=240) looked at adenosine vs nitroprusside vs saline in patients with STEMI combined with thrombus aspiration and abciximab. This showed additional benefit with high dose adenosine in ST resolution but no clinical benefit derived. (373) A study by Zhou et al in 2015 randomised 216 patients at high risk of no reflow, as established by the Wang score (table 4) (339), to either combination treatment with atorvastatin 80mg pre-treatment, intracoronary adenosine (140µg/min/kg), tirofiban (10µg/kg bolus then infusion 0.15µg/kg/min) and intra-procedural thrombus aspiration, versus conventional care at the discretion of the treating physician. The low risk cohort (n=405) outcomes were also observed for comparison. The incidence of NR (diagnosed by MCE 72hours post PCI) and MACE rates were reported, comparing between randomised high-risk groups and against the low risk population. Incidence of NR in the combination therapy group was 2.8%, which was similar to that of the low risk group 2.7% and was significantly lower than that in control group (35.2%, p< 0.01). (374)

6.1.7 Summary of overview

No reflow phenomenon is an under-recognised condition determined by the endothelial, platelet and inflammatory responses of patients during ACS, that has a negative impact on major adverse cardiovascular events (MACE). Careful angiographic assessment should be made to ensure cases of NR are appreciated, and therapeutic options considered.

Prevention of NR is superior to the cure, considering the limited therapeutic options with significant evidence base. Therefore, understanding the clinical characteristics and predictors of NR is of importance to help identify, in a timely fashion, those individuals at greatest risk. A cohort who would benefit from upfront mono or combination therapy and consideration of deferring definitive culprit lesion stent implantation. This chapter reports the findings of a case-control study designed to provide further insight into NR and the

patients who suffer from the condition in the setting of ST-elevation myocardial infarction (STEMI).

6.1.8   Objectives of this chapter

The primary objective of the chapter was to evaluate the contemporary incidence of NR in consecutive patients presenting with STEMI to a single tertiary centre, and who were treated with primary percutaneous coronary intervention (PPCI).

Secondary outcomes included detailed evaluation of the baseline clinical, procedural and angiographic findings used to identify the independent predictors of NR; both pre and peri procedurally.

An additional pre-specified analysis evaluated the clinical adverse event rate at 30 days defined as a composite of cardiovascular death, MI or cerebrovascular accident.

6.2   METHODS

This was an open single centre prospective case-control study, which recruited consecutive patients from December 2015 to June 2016. The overall cohort was defined as all patients attending with STEMI undergoing PPCI. Cases were subjects who suffered NR and the control comparators were those that did not have evidence of NR as defined below. Clinical outcomes were documented in-hospital and at 30 days post-STEMI.

The definition of NR required: (194)

- Angiographic evidence of reopening of the occluded coronary artery and no evidence of flow-limiting residual stenosis (<50%), dissection, vessel spasm, or thrombus burden;
  And, either

- Angiographic documentation of a TIMI flow grade ≤II; (312)
  or

- A TIMI flow grade III with a myocardial perfusion grade 0 or I, at least 10 min after the end of the PPCI procedure. (313)

6.2.1   Subject selection

6.2.1.1   Subject recruitment

- Identification
  1. Patients were identified at point of presentation with STEMI, following acceptance for PPCI.

- Consent
  1. Written consent was taken in accordance with the declaration of Helsinki for contact at 30 days (telephone or email (subject preference)) and prior to any contact beyond routine care.
  2. Patients who failed to survive to discharge were included in the study without consent, as per approval from the research ethics committee.

6.2.1.2   Inclusion criteria

- Age ≥ 18 years

- Presenting with STEMI

    o   Defined as the presence of chest pain lasting >20 min associated with electrocardiographic changes meeting criteria for PPCI (ST-segment elevation ≥1 mm in at least 2 limb ECG leads or ≥2 mm in at least 2 contiguous precordial leads or left bundle branch block of new onset or ST-segment depression ≥2 mm in at least 2 contiguous precordial leads (V1-3/4) suggestive of true posterior STEMI). Diagnostic confirmation on coronary angiography was mandated in all patients

- Appropriate for PPCI

6.2.1.3   Exclusion criteria

- PPCI not performed e.g. due to non-flow limiting coronary artery disease

- Failure to cross/wire culprit lesion

- Prior or adjunctive thrombolysis

- Patients who do not survive the procedure to leave the catheterisation laboratory

6.2.1.4   Withdrawal of subjects

During the recruitment period no subjects were withdrawn from the study. When a subject could not be contacted follow up (n=3) outcome data was available from collected MINAP (Myocardial Ischaemia National Audit Project) data, a compulsory audit including STEMI outcomes.

6.2.2   Data collection

Data, procedure and results were recorded during the inpatient stay and careful monitoring for adherence to protocol timescale was mandated for all subjects. Protocols were provided to all appropriate clinical areas, with educational sessions for staff provided prior to commencement of the study. A standardised case record file was utilised for recording all variables outlined below.

6.2.2.1   Baseline characteristics

- Age (years)

- Sex (male/female)

- Weight (kilograms)

- Height (metres)

- Smoker (current/ex/never) and pack years

- Performance status (WHO score 0-5) (Table 6.5)

- Past medical history

    o   Diabetes mellitus (including length of diagnosis and treatment received)

        ▪   Known complications of DM

    o   Hypertension

    o   Hypercholesterolaemia

    o   Coronary artery disease (CAD)

        ▪   Previous PCI/CABG

    o   Heart Failure

    o   Cerebrovascular Disease

    o   Peripheral Vascular Disease

    o   Chronic Kidney Disease (KDOQI (Kidney Disease Outcomes Quality Initiative) stage IV or V)

- Family history of CAD

- Past medication history prior to admission

    o Betablockers

    o Angiotensin converting enzyme inhibitor (ACEi)/angiotensin receptor
      blocker

    o Statin

    o Aspirin

    o Other antiplatelet agents e.g. clopidogrel

    o Warfarin or non-vitamin K antagonist oral anticoagulants (DOAC)

    o Glycaemic control

        ▪ Metformin/Sulphonylureas/Gliptins/Insulin

Grade	Explanation of activity
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house-work, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair
5	Dead

Table 6. 5: WHO Performance status

(adapted from Eastern Cooperative Oncology Group (ECOG) score) (375)

6.2.2.2 **Admission and procedural characteristics**

Data related to the initial event and procedure were documented by the attending PPCI team as per routine care. Acquired from procedural notes/nursing records during admission.

- Admission blood pressure (mmHg)

- Admission heart rate (beats per minute)

- Admission oxygen saturations (percentage)

- Inspired oxygen (FiO$_2$ percentage)

- Symptom to door time (minutes)

- Door to balloon time (minutes)

- Premedication (and timing)

    - Aspirin/Clopidogrel/Ticagrelor/Prasugrel (dose)/Heparin (dose)

- Blood glucose (mmol/L) from ambulance sheet or procedural report

- Glycoprotein IIb/IIIa inhibitors (dose and timing)

- Intracoronary nitrate (dose and timing)

- Intracoronary nitroprusside (dose and timing)

- Adenosine (dose and timing)

- Atropine (dose and timing)

- Intravenous fluids (volume and timing)

- Activated clotting time(s) (seconds) (including time of result(s))

- Intra-Aortic Balloon Pump use (pre/post PCI)

- Temporary pacing wire insertion

- Use of adjunctive thrombectomy

- Pre-dilation with balloon (details of size and inflation pressure)

- Stent (details of size(s), type, and inflation pressure)

- Post-dilation with balloon (details of size and inflation pressure)

- Screening time (minutes)

- Radiation exposure (dose area product)

6.2.2.3   In-hospital investigation results

Information related to the relevant hospital admission was acquired from patient notes and computerised hospital care records. Standard data and results felt pertinent to NR risk as highlighted previously were collected. (Section 6.1.4)

- Fasting glucose levels (mmol/L) – in subjects with elevated admission blood glucose or known diabetes

- HbA1c (mmol/mol) – in subjects with elevated blood glucose or known diabetes

- Time from admission until initiation of hyperglycaemia treatment (as applicable)

- Troponin (ng/ml) (12-24 hours after onset of chest pain)

- N-terminal Pro-B Type Natriuretic Peptide (NT-proBNP) (pg/ml) (within 24 hours of admission)

- C reactive protein (mg/L) (initial result)

- Renal function (eGFR/Cr) (initial result)

- Haemoglobin (g/L) (admission result)

- Neutrophil count (x10^9/L) (initial result)

- Platelet count (x10^9/L) (initial result)

- Cholesterol (mmol/L)

- 60 minutes post PPCI ECG (determine presence of ≥70% reduction in ST-elevation)

- Left ventricular impairment on echocardiography within 24 hours of admission (non/mild/moderate/severe)

- Aortic valvular sclerosis on echocardiography. This required all of the following:

  1. Irregular, non-uniform thickening of portions of the aortic valve leaflets or commissures, or both

  2. Thickened portions of the valve with an appearance suggesting calcification

  3. Non-restricted or minimally restricted opening of the aortic cusps and peak continuous wave velocity across the valve of

- Epicardial adipose tissue (EAT) on echocardiography (cm)

    - EAT is described as the echo-free space between the outer myocardial wall and the visceral pericardial layer in the PLAX view. This was measured during end-systole at the midline point (perpendicular to the aortic annulus) on the free wall of the right ventricle and an average of three cycles recorded

6.2.2.4   Definitions: clinical outcomes

Clinical outcomes were retrieved from medical notes for in-hospital events and following patient contact at 30 days.

Cardiovascular Death

Cardiovascular death was defined as unexplained sudden death, or death secondary to acute myocardial infarction (MI), heart failure, or arrhythmia.

Myocardial infarction

MI was defined as ischaemic chest pain lasting longer than 20 minutes, with additional abnormalities of either cardiac enzymes and/or the ECG.

- o Cardiac enzymes:
    - Within 24 hours of index event: cardiac enzyme levels not diagnostic;
    - Prior to normalisation of cardiac enzymes: 50% increase in troponin T level compared to previous level;
    - Following normalisation of cardiac enzymes: troponin T levels above upper limit of normal.
- o ECG:
    - Within 24 hours: new ST-elevation;
    - Otherwise a significant ST-segment change, development of new Q waves in ≥2 contiguous ECG leads, or new left branch bundle block pattern.

Cerebrovascular accident

Cerebrovascular accident (CVA) was defined by the sudden onset of loss of neurologic function caused by an ischemic or haemorrhagic event, which persisted for greater than 24 hours.

Repeat revascularisation

Revascularisation was defined as any unplanned coronary intervention (either percutaneous or surgical).

Major Adverse Cardiovascular Event

MACE was defined as the composite of cardiovascular death, MI, CVA.

Heart failure

Heart failure was quantified by clinical signs and categorised by the Killip class system. (Table 6.6)

In hospital clinical outcomes recorded:

- Worse Killip class (defined table 6.6)
- Red cell transfusion required (number of units)
- Cardiovascular death
- Death
- Myocardial infarction
- Cerebrovascular accident (infarct/haemorrhage)
- Repeat unplanned angiography
- Repeat unplanned revascularisation

30-day clinical outcomes:

- Death
- Heart failure (Killip class)
- Myocardial infarction
- Cerebrovascular accident (infarct/haemorrhage)
- Repeat unplanned angiography
- Repeat unplanned revascularisation

Killip Class I	No clinical signs of heart failure
Killip Class II	Rales or crackles in the lungs, a third heart sound and elevated jugular venous pressure
Killip Class III	Frank pulmonary oedema
Killip Class IV	Cardiogenic shock (systolic blood pressure <90mmHg) and evidence of peripheral vasoconstriction (oliguria, cyanosis or sweating)

Table 6. 6: Killip class definitions (376)

6.2.2.5   Coronary angiography characteristics

The PPCI angiography images were analysed in detail. Operators were instructed to acquire images of the non-culprit vessel first and perform initial/final runs of long enough duration for venous filling to be evident. Following re-establishment of flow, a single lesion defining image was required. Final images were mandated at 30 frames/second in prespecified views (left coronary system: left lateral view; right coronary artery: right anterior oblique (377)) for assessment of myocardial blush grade. If, at the end of the procedure TIMI flow was less than III, clinicians waited for a minimum of at least ten minutes before acquiring the final images. During this time, additional therapy could be administered at their discretion.

Angiography review:

- Initial TIMI flow (0-III) (see table 6.1 for definitions)

- Evidence of intra/post-procedural no reflow phenomenon (see earlier definition)

- End procedure TIMI flow (0-III)

- End procedure myocardial blush grade (0-III) (see table 6.1 for definitions)

- Thrombus classification (0-5) (table 6.8)

- Culprit vessel (left anterior descending (LAD)/right coronary artery (RCA)/left circumflex artery (LCx)/other)

- Multi-vessel flow limiting disease (number of vessels with >50% lesion (LAD, RCA, LCx, left main stem (LMS) or side branch diameter >2mm))

- Lesion characteristics

    o Classification (American College of Cardiology/American Heart Association type A/B1/B2/C) (table 6.7)

    o Ostial

    o Bifurcation

    o Collateralisation (Rentrop Score 0-III) (table 6.9)

    o Reference vessel diameter (mm) (Quantitative Coronary Angiographic System (QCA))

- o Lesion severity: minimum luminal area diameter stenosis (%) (QCA system if patency or classified as 100% if occluded)

- o Lesion length following re-established flow (mm) (QCA system)

- o Post-PCI minimal luminal diameter (mm) (QCA system)

Angiographic assessment was analysed retrospectively by two investigators independently. QCA guidance/scale definitions was provided to maximise consistency. QCA calibration was performed using the guiding catheter, with measurement of the reference diameter performed close to the lesion, using the proximal vessel wherever possible. Lesion length was recorded in millimetres following re-establishment of flow. The post-PCI minimal luminal diameter (mm) was recorded as the mean of two orthogonal measurements. Any discrepancies on the analysis were further reviewed and if no consensus reached a third investigator was involved.

Type A Lesions (requires all characteristics)	Type B Lesions B1 has 1 characteristic B2 has ≥2 characteristics	Type C Lesions (requires any characteristic)
Discreteness (<10 mm) Concentricity Readily accessible Location in a non-angulated segment (<45°) Smoothness of contour Little or no calcification Absence of total occlusion Non-ostial location Absence of major branch involvement Absence of thrombus	Tubular shape (10-20 mm) Eccentricity Accessibility influenced by moderate tortuosity of the proximal segment Location in a moderately angulated segment (>45°, <90°) Irregularity of contour Moderate/severe calcification Presence of thrombus Ostial location Bifurcation lesion requiring double guide wires Total occlusion <3 months	Diffuseness (>20 mm) Excessive tortuosity of the proximal segments Location in an extremely angulated segment (>90°) Total occlusion >3 months Inability to protect major side branches Degeneration of older vein grafts with friable lesions

Table 6. 7: American College of Cardiology/American Heart Association Lesion Classification. (378,379)

Grade	Definition
0	No cine angiographic characteristics of thrombus present
1	Possible thrombus is present, with such angiography characteristics as reduced contrast density, haziness, irregular lesion contour, or a smooth convex "meniscus" at the site of total occlusion suggestive but not diagnostic of thrombus
2	There is definite thrombus (small size), with greatest dimensions ≤1/2 the vessel diameter
3	There is definite thrombus (moderate size), but with greatest linear dimension >1/2 but <2 vessel diameters
4	There is definite thrombus (large size), as grade 3, but with the largest diameter ≥2 vessel diameters
5	Total occlusion

Table 6. 8: Thrombus classification (380)

Grade	Definition
0	None
1	Filling of side branches of the artery to be dilated via collateral channels without visualisation of the epicardial segment
2	Partial filling of the epicardial segment via collateral channels
3	Complete filling of the epicardial segment of the artery being dilated via collateral channels

Table 6. 9: Rentrop classification of collateral circulation (381)

6.2.3   Statistical analysis

Sample size calculation was based on 21% of patients developing angiographic NR. (339) The aim in this observational study was to have data on 30 patients with NR, thus requiring at least 143 subjects in the recruited cohort.

Continuous characteristics were expressed as mean and standard deviation, and categorical data expressed as numbers/percentages. Continuous data was analysed using one-way ANOVA and categorical characteristics were analysed using chi-squared to assess for correlation with NR. Simple binary logistic regression models were used to observe any association between the correlated baseline clinical and angiographic factors with NR (reported as odds ratio with 95% confidence interval). Multiple backward stepwise logistic regression methodology enabled identification of independent variables in the prediction of NR. ROC curve analysis allowed conversion of continuous variables into dichotomous data with optimised cut off identified. The final categorical model was assessed with multiple logistic regression and a risk score calculated based on the β co-efficient. Two-sided p values of <0.05 were considered to indicate statistical significance, yet p<0.1 was felt to require further assessment. All analyses were undertaken on SPSS version 24. The final statistical methodology and results were kindly verified by HYMS statistician Dr Victoria Allgar.

6.2.4   Quality control and assurance

6.2.4.1   Peer review

This study was peer reviewed by HYMS Professors with research interest in cardiovascular disease and independent Interventional Cardiologists.

6.2.4.2   Monitoring

The study was monitored in accordance with Hull and East Yorkshire Hospitals NHS Trust Research and development department's standard operating procedures to ensure compliance with International Conference for Harmonisation of Good Clinical Practice (ICH

GCP) and the Research Governance Framework 2005. Data was collected and retained in accordance with the Data Protection Act 1998.

6.2.4.3   Ethical considerations

Treatment

There was no change to treatment compared to those not involved in the study.

Subject Burden

As part of normal care participants answered routine clinical questions during their inpatient stay and were subject to 30-day contact either by phone call or email (patient preference).

Confidentiality

Subjects were informed that their information would be stored externally to case notes, but reassurance given to the security of this information. No identifiable data was available electronically or included in the final publication.

6.2.4.4   Ethics and R&D approval

The study was performed following a favourable opinion from the Research Ethics Committee (Yorkshire and the Humber – Bradford Leeds (15/YH/0360)), Site Specific Assessment (SSA) approval and Hull and East Yorkshire Hospitals NHS Trust R&D approval.

6.3 RESULTS

A total of 173 patients (68% male) were recruited over the six-month period, with a mean age of 63 years (range 34-91years). NR occurred at some point during the PPCI in 24 cases (13.9%). The timing of NR was following stent implantation in 11 (45.8%); post-thrombectomy in 1 (4.2%), after pre-dilation in 6 (25%) and following post-dilation in 6 (25%).

6.3.1 Case-control characteristics

The characteristics of those developing NR were compared to the control group (those who did not develop NR) (table 6.10 and 6.11). The former group were older, more likely to have a history of hypertension, and presented with higher systolic blood pressures. Beyond this no significant difference was found in the history or initial investigations between the two groups.

Interestingly, the population with NR did not have a greater troponin elevation (p=0.805) compared to the control group. However, the admission NT-proBNP level was significantly greater (p=0.047). (Table 6.12) In keeping with this a numerical trend was evident for reduced left ventricular function in the NR cohort, however this did not reach statistical importance (p=0.502). Admission pathology results demonstrated a higher creatinine level (p=0.038). The haemoglobin level was numerically lower, though this was not statistically significant (p=0.066).

Overall, the mean maximal ST-elevation on the ECG was 3mm, which did not differ between groups. The frequency of resolution of the ECG changes was also comparable (64% control group and 57% NR group (p=0.530)).

		No Reflow		
		No n=149	Yes n=24	p value
Age (years)		62±13	70±13	**0.004**
Male		104(70)	14(58)	0.263
Smoker		62(42)	5(22)	**0.062**
Ex-smoker		46(31)	8(36)	0.619
Pack year history (years)		30±22	20±19	0.133
Functional class (WHO)	0	125(85)	17(71)	0.806
	1	14(10)	5(21)	
	2	8(5)	1(4)	
	3	0	1(4)	
Weight (kg)		85±20	77±14	**0.087**
Height (m)		1.7±0.1	2±0	0.535
Systolic blood pressure (mmHg)		138±28	151±30	**0.028**
Diastolic blood pressure (mmHg)		80±16	80±18	0.930
Heart rate (beats/min)		75±19	78±20	0.544
Oxygen saturation (%)		96±3	97±2	0.798
Inspired oxygen concentration (%)		26±17	24±13	0.666
Admission blood sugar (mmol/L)		8±3.3	8.6±3.6	0.419
Maximal ST elevation (mm)		3±2	3±1	0.215
Epicardial adipose tissue (cm)		0.5±0.2	0.5±0.3	0.507
Aortic valvular sclerosis		13(13)	2(14)	0.883

Table 6. 10: Baseline characteristics of patients presenting with STEMI comparing those with no reflow to controls

Continuous variables expressed as mean ± standard deviation with correlation investigated with one-way ANOVA. Categorical variables expressed as number (percentage) and correlation investigated with chi-squared test. P<0.05 was deemed significant, however, p<0.1 (bold) suggested further analysis required.

	No Reflow		
	No n=149	Yes n=24	p value
Diabetes mellitus	21(14)	3(13)	0.834
Hypertension	44(30)	12(50)	**0.047**
Hypercholesterolaemia	35(23)	6(25)	0.872
Coronary artery disease (CAD)	26(17)	4(17)	0.925
Congestive cardiac failure	3(2)	1(4)	0.519
Cerebrovascular disease	2(1)	1(4)	0.325
Peripheral vascular disease	3(2)	1(4)	0.515
Chronic kidney disease	1(1)	0(0)	0.687
Family history of CAD	52(35)	4(18)	0.110
Betablocker	21(14)	2(8)	0.440
ACEi	19(13)	5(21)	0.288
Angiotensin receptor blockers	10(7)	3(13)	0.329
Statin	42(28)	8(33)	0.606
Aspirin	25(17)	4(17)	0.989
Dual antiplatelet therapy	2(1)	0(0)	0.568
Warfarin	7(5)	1(4)	0.908
DOAC	1(1)	1(4)	0.137
Metformin	14(9)	3(13)	0.635
Sulphonylurea	1(1)	0(0)	0.687
Gliptins	4(3)	1(4)	0.688
Insulin	4(3)	0(0)	0.417

Table 6. 11: Past medical and drug history of patients presenting with STEMI comparing those with no reflow to controls

Continuous variables expressed as mean ± standard deviation with correlation investigated with one-way ANOVA. Categorical variables expressed as number (percentage) and correlation investigated with chi-squared test. P<0.05 was deemed significant, however, p<0.1 (bold) suggested further analysis required.

		No Reflow		
	Normal Range	No	Yes	p value
Troponin (ng/L)	(<14)	3720±3291	3898±2703	0.805
NT ProBNP (pg/ml)	(<400)	1556±2499	3305±7708	**0.047**
Creatinine (µmol/L)	(<120)	79±22	91±32	**0.038**
eGFR (ml/min)	(>90)	89±26	78±32	**0.059**
Haemoglobin (g/L)	(135-175)	142±18	135±16	**0.066**
Neutrophil (x10^9/L)	(2.0-7.7)	11.1±11.5	11.5±4.1	0.844
C reactive protein	(<10)	19±36	16±30	0.759
Platelet (x10^9/L)	(150-400)	248±95	216±73	0.122
Total cholesterol (mmol/L)		5.1±1.2	5.5±1.8	0.187
Albumin (g/L)	(35-55)	36±5	34±4	**0.096**
Resolution of ECG ST change		88(64)	12(57)	0.530
Left ventricular dysfunction	Non	62(43)	7(29)	0.502
	Mild	46(32)	8(33)	
	Moderate	29(20)	6(25)	
	Severe	9(6)	3(12.5)	

Table 6. 12: Inpatient investigation results of patients presenting with STEMI comparing those with no reflow to controls

Continuous variables expressed as mean ± standard deviation with correlation investigated with one-way ANOVA. Categorical variables expressed as number (percentage) and correlation investigated with chi-squared test. P<0.05 was deemed significant, however, p<0.1 (bold) suggested further analysis required.

Ticagrelor was the predominant P2Y$_{12}$ antagonist used, consistent across cases and controls (22 (91.7%) NR group and 142 (97.3%) control group (p=0.169)). No patients received prasugrel and nine patients received clopidogrel. In seven cases a dose of ticagrelor was given in addition to the clopidogrel, of the remaining two patients one had NR. Four patients (2.4%) did not receive any P2Y$_{12}$ inhibition, likely secondary to clinical condition, but all therefore received intravenous IIbIIIa inhibitor. The patients who suffered NR had a longer delay to balloon inflation compared to those who did not have NR, although this did not reach statistical significance. (Table 6.13)

Analysis of the angiographic images concluded that the culprit vessel, location, length of the lesion or thrombus class did not influence the incidence of NR. (Table 6.14) However the classification of the lesion overall, a composite like endpoint variable, did convey an important correlation with development of NR and increased lesion complexity (p=0.001).

Initial TIMI flow did not significantly differ between the cases and control patients, although as anticipated there was a highly significant reduction in final TIMI flow and myocardial blush grade (p<0.001).

Relating to the coronary interventional procedure a lower dose of heparin was used in the NR group, however there was no reduction in the ACT, and this most likely reflects the lower weight of the NR patient cohort. Numerically, but not significantly, higher levels of IIb/IIIa inhibitor use was seen in NR, with statistically more given intracoronary in this cohort (p=0.036). No difference in the use of nitrates, adenosine or nitroprusside. (Table 6.15)

No variance was seen in levels of stent implantation, type, size or inflation pressure. However, patients in the NR group had significantly more predilation at higher pressure, perhaps reflecting the higher complexity of the lesion. Stent post-dilation was performed in a larger proportion of patients in the NR group, though this did not reach statistical significance.

	No Reflow		
	No n=149	Yes n=24	p value
Symptoms to door time (mins)	511±915	681±1185	0.420
Symptoms to balloon time (mins)	543±918	720±1195	0.403
Door to balloon time (mins)	32±14	39±41	0.106
Symptoms to aspirin time (mins)	435±941	628±1268	0.406
Symptoms to P2Y$_{12}$ antagonist (mins)	510±923	656±1205	0.504

Table 6. 13: Timings of important clinical intervention in patients presenting with STEMI comparing those with no reflow to controls

Continuous variables (minutes (mins)) expressed as mean ± standard deviation with correlation investigated with one-way ANOVA. P<0.05 was deemed significant, but p<0.1 (bold) suggested further analysis required.

Table 6.14		No Reflow		
		No (n=149)	Yes (n=24)	p value
Culprit Vessel	LAD	52(35)	7(29)	0.828
	RCA	72(48)	14(58)	
	LCx	15(10)	2(8)	
	Other	10(7)	1(4)	
Initial TIMI flow	0	105(71)	20(83)	0.400
	1	16(11)	1(4)	
	2	18(12)	1(4)	
	3	10(7)	2(8)	
Final TIMI flow	0	0	2(8)	**<0.001**
	1	4(3)	5(21)	
	2	11(7)	15(63)	
	3	134(90)	2(8)	
Final MBG	0	3(2)	8(33)	**<0.001**
	1	5(3)	7(29)	
	2	9(6)	8(33)	
	3	128(88)	1(4)	
Lesion severity (% occlusion)		98±5.5	97.8±8.3	0.869
Thrombus class	0	3(2)	0	0.150
	1	7(5)	0	
	2	2(1)	1(4)	
	3	13(9)	0	
	4	17(11)	3(13)	
	5	107(72)	20(83)	
Presence of multivessel disease		95(64)	20(83)	**0.059**
Collaterals class	0	82(55)	9(38)	0.284
	1	27(18)	7(29)	
	2	30(20)	7(29)	
	3	9(6)	1(4)	
Reference vessel diameter (mm)		3±0.6	3±0.5	0.963
Ostial lesion location		10(7)	1(4)	0.635
Bifurcation lesion		23(15)	4(17)	0.877
Lesion length (mm)		15.2±10.3	18.3±7.5	0.165
Classification	A	0	0	**0.001**
	B1	48(32)	1(4)	
	B2	85(57)	15(63)	
	C	16(11)	8(33)	
Post intervention minimal luminal area (mm)		2.9±0.6	3±0.6	0.425

Table 6. 14: Angiographic characteristics of patients presenting with STEMI comparing those with no reflow to controls

Continuous variables expressed as mean ± standard deviation with correlation investigated with one-way ANOVA. Categorical variables expressed as number (percentage) and correlation investigated with chi-squared test. P<0.05 was deemed significant, but p<0.1 (bold) suggested further analysis required.

Table 6.15	No Reflow		
	No n=149	Yes n=24	p value
Heparin dose (units)	8054±2347	6818±2260	**0.022**
Activated clotting time (secs)	290±82	304±79	0.549
IIbIIIa inhibitor use	34(23)	8(33)	0.265
Intracoronary nitrate use	87(60)	15(65)	0.661
Intracoronary nitrate dose (mcg)	267±154	317±182	0.250
Nitroprusside use	0	0	
Adenosine use	4(3)	2(8)	0.169
Adenosine dose (mg)	1.8±2.7	0.5±0.2	0.540
Atropine use	28(19)	3(13)	0.440
Atropine dose (mcg)	621±181	900±794	0.103
Intravenous fluid use	42(29)	11(46)	**0.090**
Fluids volume (ml)	646±256	636±234	0.908
Predilation performed	103(70)	24(100)	**0.002**
Predilation balloon diameter (mm)	2.5±0.5	2.5±0.4	0.848
Predilation balloon inflation pressure (atm)	12±3	14±4	**0.047**
Stent inserted	140(96)	24(100)	0.312
Stent diameter (mm)	3.2±0.6	3.3±0.7	0.465
Stent inflation pressure (atm)	15±2	15±3	0.988
Post dilation performed	55(38)	13(54)	0.126
Post dilation balloon diameter (mm)	3.6±0.5	3.7±0.6	0.559
Post dilation balloon inflation pressure (atm)	16±4	17±4	0.334
Thrombectomy use	60(41)	8(33)	0.472
IABP use	1(1)	1(4)	0.141
Temporary pacing wire use	3(2)	0(0)	0.480
Screening time (mins)	12.31±7.4	14.45±11.7	0.233
Radiation exposure (DAP)	5553±3427	5618±2940	0.930

Table 6. 15: Procedural characteristics of patients presenting with STEMI comparing those with no reflow to controls

Continuous variables expressed as mean ± standard deviation with correlation investigated with one-way ANOVA. Categorical variables expressed as number (percentage) and correlation investigated with chi-squared test. P<0.05 was deemed significant, but p<0.1 (bold) suggested further analysis required.

6.3.2 Clinical Outcomes

The clinical outcomes are documented in table 6.16. Patients with NR were seven times more likely to die in-hospital compared to those without NR (OR 7.0 (95% CI 1.3-36.7), p=0.022). There was a numerical increase in evidence of clinical heart failure during the patient's hospital admission in those with NR compared to the control group, but this failed to reach statistical significance. (Figure 6.3) For those patients who did survive to 30 days, there was no difference in symptoms of heart failure between the groups. (Figure 6.4)

Post discharge at 30 days, 17 (11.6%) and 3 (14.3%) of the control and NR of patients had an unplanned readmission to hospital, respectively (p=0.754). 33% of those admissions related to cardiovascular diagnoses in the NR cohort which was not markedly different to 24% in the control group. There was a single cardiovascular death post discharge and this patient did not have NR. However, NR did confer a greater risk of cardiovascular death from index event to 30 days (p=0.024). (Table 6.17)

The 30-day rate of MACE was 6.5% and was significantly higher in the NR group (16.7% versus 4.8%), conveying a fourfold increase (OR 4.0 (95% CI 1.1-14.8), p = 0.04).

	No Reflow		
	No (n=149)	Yes (n=24)	p value
Transfusion	1(0.7)	0	0.687
Cardiovascular death	3(2)	3(12.5)	**0.009**
Non-cardiovascular death	0	0	
Myocardial infarction	2(1.3)	0	0.568
Cerebrovascular accident (infarct)	1(0.7)	0	0.687
Cerebrovascular accident (haemorrhagic)	1(0.7)	0	0.687
Repeat Revascularisation	4(2.7)	0	0.220

Table 6. 16: In-hospital clinical outcomes

Results quoted as number (percentage of relevant population) and correlation investigated with chi-squared test. P<0.05 deemed significant.

	No Reflow		
	No (n=149)	Yes (n=24)	p value
Cardiovascular death	4(2.7)	3(12.5)	0.024
Non-cardiovascular death	1(0.7)	0	0.703
Myocardial infarction	2(1.4)	1(4.8)	0.701
Cerebrovascular accident (infarct)	1(0.7)	0	0.687
Cerebrovascular accident (haemorrhagic)	1(0.7)	0	0.687
Repeat Revascularisation	4(2.7)	0	0.220

Table 6. 17: Day 30 clinical outcomes

Results quoted as number (percentage of relevant population) and correlation investigated with chi-squared test. p<0.05 deemed significant.
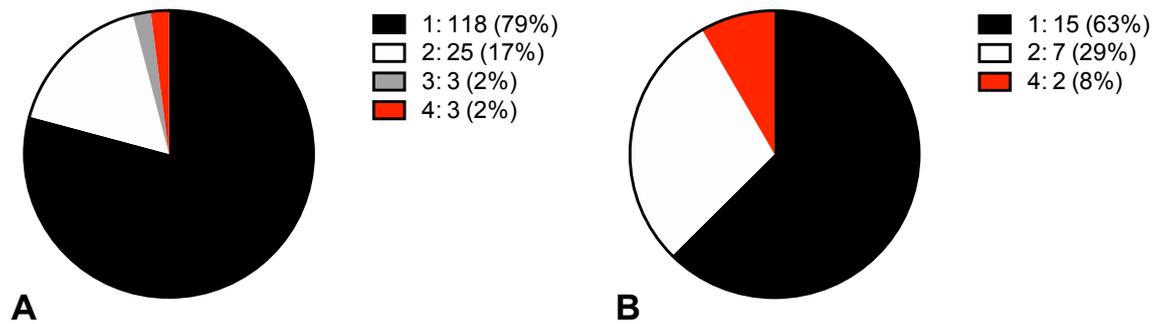
Figure 6. 3: In-hospital clinical heart failure (Killip class)

Chart depicting heart failure symptoms, as quantified by Killip class, in patients who did not experience NR (A) and those patients that did (B). Key states Killip class: number (percentage). Chi-squared analysis p = 0.056.
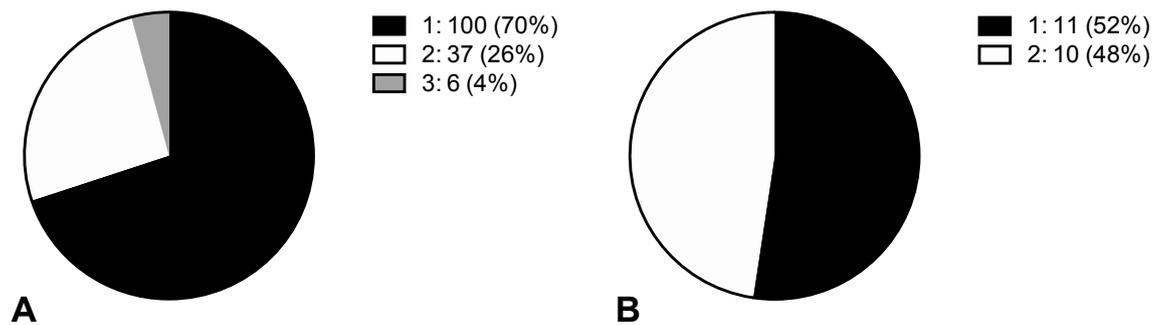
Figure 6. 4: 30-day clinical heart failure (Killip class)

Chart depicting of heart failure symptoms, as quantified by Killip class, in patients who did not experience NR (A) and those patients that did (B). Key states Killip class: number (percentage). Chi-squared analysis p=0.286.

6.3.3 Hyperglycaemic effect

As described in section 6.1.4 hyperglycaemia has been proposed as an important predictor of NR. To further investigation of the impact of glycaemic control on the incidence of the NR, subgroup exploration was completed. This group included those with diagnosed diabetes mellitus or elevated admission blood glucose (≥10mmol/L). The "hyperglycaemic" population had a mean admission blood sugar of 12.3±5.8mmol/L, with a fasting glucose of 9.4±2.9 mmol/L and HBA1c 73±28 mmol/mol.

The rate of recorded NR was 15.6% (5/32), demonstrating no increased risk compared to those with normoglycaemia 13.1% (17/130) (OR 1.2 (95% CI 0.4-3.6), p = 0.707). The level of admission blood glucose (p=0.770), fasting blood glucose (p=0.247) and HbA1c (p=0.652) had no correlation with diagnosis of no reflow.

6.3.4 Prediction

Comprehensive review of the collated data revealed several characteristics that were statistically correlated to NR. The data was investigated further for consideration of whether a prediction model could be generated.

6.3.4.1   Logistic regression

All characteristics with a correlation p value of <0.1 underwent individual logistic regression for prediction of NR. (Table 6.18) Caution was used however with those results that could be considered secondary to NR, rather than contributory.

This determined increasing age, higher creatinine levels, higher systolic blood pressure, lower heparin dose and angiographic classification of the lesion, to be significant predictors of NR.

		Odd Ratio	95% Confidence Interval	p value
Age		1.05	1.02-1.09	**0.005**
Weight		0.97	0.95-1	**0.081**
NT-ProBNP		1.00	1-1	0.103
Creatinine		1.02	1-1.03	**0.045**
Haemoglobin		0.98	0.95-1	**0.068**
Albumin		0.93	0.85-1.01	0.1
eGFR		0.98	0.97-1	**0.061**
Heart failure (Killip Class)		1.66	0.97-2.84	**0.066**
Systolic blood pressure		1.02	1-1.03	**0.03**
Heparin dose		1.00	1-1	**0.021**
Predilation balloon inflation pressure		1.17	1-1.62	**0.05**
Aspirin to balloon time		1.00	1-1.01	0.13
Smoker		0.38	0.13-1.08	**0.07**
Hypertension		2.39	1-5.72	**0.051**
Intracoronary IIb/IIIa use		6.59	0.88-49.22	**0.066**
Pre-dilation performed			Not recordable	0.997
Intravenous fluid use		2.12	0.88-5.1	**0.095**
Presence of multivessel disease		1.73	0.64-4.63	0.28
Classification	B2 vs B1	8.47	1.09-66.13	**0.042**
	C vs B1	24.0	2.78-206.96	**0.004**
	C vs B2	2.83	1.03-7.81	**0.043**

Table 6. 18: Odds ratios for characteristics demonstrating correlation to NR

Figures show OR, 95% confidence interval and subsequent p value. ORs associated with continuous data (Killip class included) above one confers an increase in odds for every increase in units versus ratio below one representing an increase in odds for every unit decrease. ORs associated with categorical data above one is the increase in odds related to presence of the characteristic. P<0.05 was deemed significant, however if <0.1 was considered for the prediction model.

6.3.4.2 Multiple logistic regression

A prediction model was considered containing all those variables deemed statistically important (age, classification, systolic blood pressure, hypertension, non-smoker) and could be quantified pre-intervention. (Table 6.19) In addition, symptoms to PCI time and admission glucose were included in the model as both values had been judged important in established risk models. (Section 6.1.5 (338,339)) History of hypertension, lesion classification, systolic blood pressure on admission and patient weight were independent predictors of NR. (Table 6.20)

Pre-Coronary Intervention	Intra/post Intervention	Secondary to NR?
1. Age	1. Heparin dose	
2. Lesion classification	2. Creatinine ←——————→	1. Creatinine
3. Systolic blood pressure	3. Predilation balloon inflation pressure	
4. Hypertension		
5. Non-smoker	4. eGFR ←——————→	2. eGFR
6. Weight	5. Intracoronary IIb/IIIa	3. Heart failure
	6. Haemoglobin ←——————→	4. Haemoglobin
	7. Intravenous fluids use	

Table 6. 19: Important prediction variables divided into subcategories

All variables found to have a p value <0.1 on logistic regression are divided into categories on whether information available pre-coronary intervention or intra/post intervention, or if data may be influenced by presence of NR rather than contributing to risk of NR. The order represents the statistical weighting with 1 associated with the most significant p value.

	Odd Ratio	95% Confidence Interval	p value
Hypertension	3.7	1.1-12.1	0.033
Lesion classification			0.009
B2 vs B1	16.8	1.8-156.7	0.013
C vs B1	48.4	4.1-575.8	0.002
Systolic blood pressure	1.0	1.0-1.1	0.005
Weight	0.95	0.91-0.98	0.006

Table 6. 20: Pre-interventional predictors of no reflow phenomenon

Results of a multiple logistic regression model with backward stepwise analysis showing OR, 95% confidence interval and p value, where p<0.05 was deemed significant.

6.3.4.3　　　Risk score

To enable risk score analysis, the continuous variables were transformed into binary outcomes. Systolic blood pressure greater than or equal to 138mmHg (area under the curve (AUC) 0.626 (95% CI 0.507-0.744), p=0.049)) had a 66.7% sensitivity and 49.7% specificity for NR prediction. Weight of less than 78kg (AUC 0.618 (95% CI 0.490-0.746), p= 0.088) conferred 55% sensitivity and 67.9% specificity for NR. (Figure 6.5)

Further assessment following re-interpretation of the measurements found the final model to be of good fit (Hosmer-Lemeshow 2.274, p=0.943) and a significant predictor of NR (chi-square 28.6, p<0.001). Approximately 31.3% variation in NR outcome can be explained by the grouping of the characteristics using Nagelkerke pseudo R^2 value. (Table 6.21)

There was a significant difference between the risk score values of the patients who suffered NR (4.1±1) compared to those who did not (2.6±1) (p<0.001), and the risk score was considered a good test analysis (AUC 0.823 (95% CI 0.723-0.923), p<0.001). (Figure 6.6)

In depth review of the ROC curve stated a score of 3 or above had 90% sensitivity and 49.8% specificity (3/67 (7.1%) (number of NR/total population (%)). A score of 4 or above had 75% sensitivity and 76.5% specificity (5/24 (17.2%)). A score of 5 or above had 50% sensitivity and 94.1% specificity (10/18 (55%)).

Therefore, patients with a risk score of 4 and above have a moderate risk, and those with 5 and above a high risk of proceeding to develop NR.
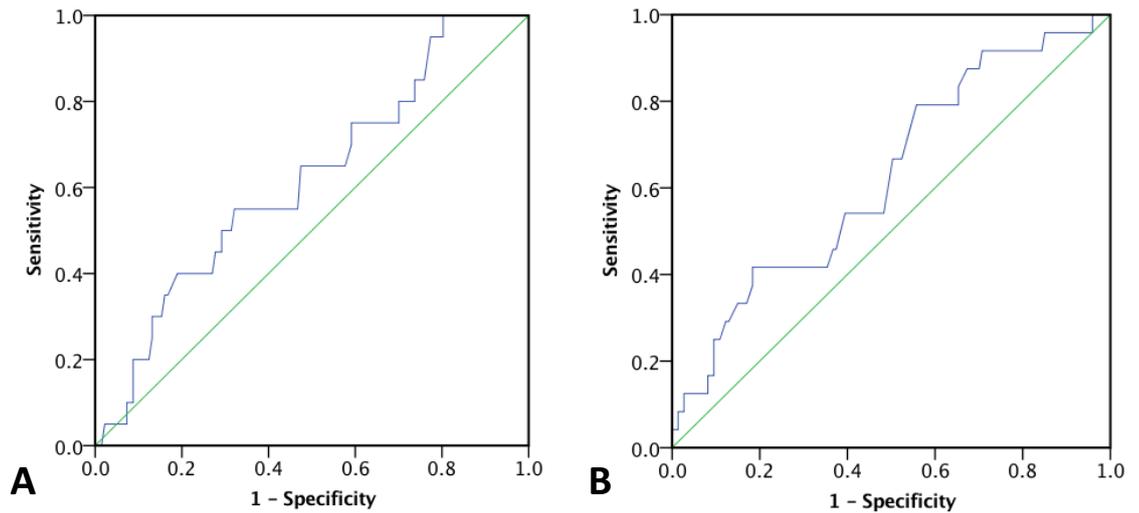
Figure 6. 5: ***Receiver operating characteristic (ROC) curve analysis of continuous characteristics***

Weight (A) and systolic blood pressure (B) were explored with a ROC curve analysis.

	Odd Ratio	95% Confidence Interval	β Coefficient	p value	Risk Score
Lesion classification				**0.005**	
B2	10.5	1.3-88.8	2.35	**0.03**	**2**
C	37.7	3.9-369.2	3.63	**0.002**	**3**
SBP ≥138mmHg	4.2	1.3-13.8	1.43	**0.019**	**1**
Weight <78kg	3.3	1.1-9.8	1.20	**0.03**	**1**
Known hypertension	3.2	1.1-9.1	1.15	**0.034**	**1**

Table 6. 21: Pre-interventional risk score

Exploration of categorical characteristics in a multiple logistic regression, entered in order or statistical importance. Results demonstrate odd ratio, 95% confidence interval, β coefficient and p value. Risk score was calculated using the β coefficient ratio compared to the lowest value. (hypertension 1.15)
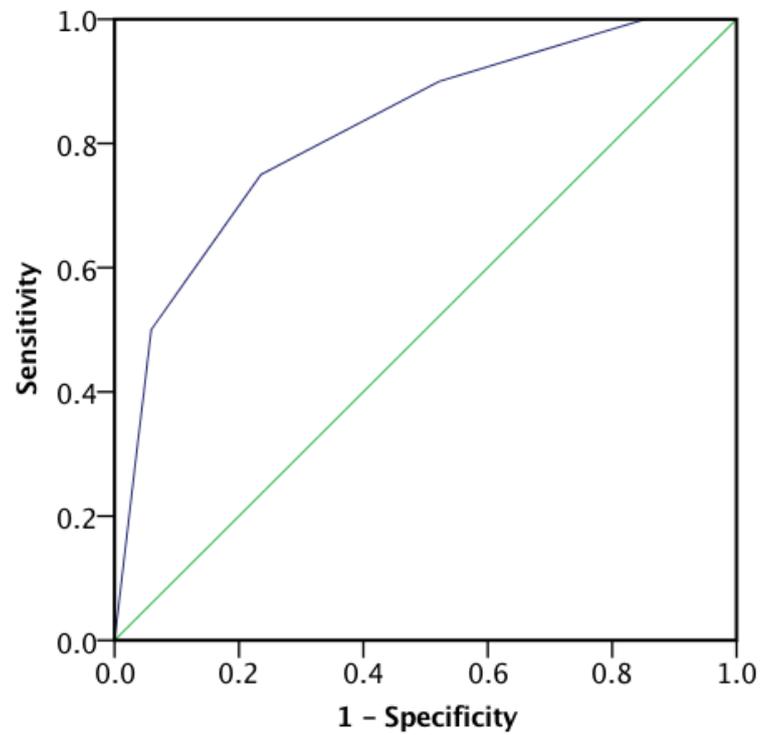
Figure 6. 6: ROC curve analysis for the no reflow prediction model

ROC curve of the multiple logistic regression model in predicting no reflow phenomenon, plotting true positives against false positives. Area under the curve 0.823 (95% CI 0.723-0.923) (p<0.001).

6.4   DISCUSSION

No reflow phenomenon occurred in 13.9% of a contemporary population of patients presenting with STEMI, which was lower than anticipated. This inconsistency is partly dependent on the definition of NR and the sensitivity of the defining methodology. Imaging with cardiac MRI or MCE is considered gold standard compared to the relatively less sensitive angiographic classification. (382,383) Angiographic diagnosis of NR is commonly used in research (318,328,384) and was selected in this study not just due to the impact on patients and cost implications of other modalities, but also the increased relatability to every day clinical practice.

Understanding the pathophysiology may give further explanations for this reduction in events. NR, as covered in section 6.1.2, has a multifactorial aetiology and broadly can be defined by 4 distinct groups. 1) Distal atherothrombotic embolisation; 2) ischaemic injury; 3) reperfusion injury; and 4) susceptibility of the coronary microcirculation to injury. (193) Distal embolisation is a result of debris (thrombi, endothelial cells and lipid matrix) (314) and ischaemic injury is relative to the duration of obstruction, secondary to the debris, protrusions and compression. Subsequently, thrombi/platelets play a critical role in development of the condition (252), and therapeutic practice switch in favour of newer $P2Y_{12}$ inhibitors (26) could be contributory to the lower incidence. Ticagrelor was the near universal $P2Y_{12}$ antagonist used in this STEMI population and is a more potent antiplatelet agent compared to clopidogrel. (385) However, demonstration of NR reduction in randomised groups has not been seen. (386)

This data did suggest a longer time elapse from symptoms to antiplatelet therapy in the NR group although non-significant. This is consistent with the findings of the present study, where there was a delay in all timed variables for patients who had NR. In particular, longer symptom to balloon time implies prolonged obstruction / ischaemic injury which may be a mechanism of increased risk of NR. Although the findings in this cohort failed to reach statistical significance, larger studies support its importance. (311) Another plausible contributing factor is UK geography, with relative short distances to medical contact and final PPCI therapy compared to some of our international counterparts.

In accordance with previously published data, NR was associated with a fourfold increase in major cardiovascular adverse events at 30 days. (311,315) Studies have demonstrated that this impact persists out to 5 years. (194)

It is therefore very important to be able to predict individuals at risk of NR to potentially help guide interventional strategy. The data model found lesion severity quantified by B2/C classification had the highest predictive value and its contribution to the risk score is a logical predictor. As described, pathophysiology of NR is in part triggered by reperfusion injury secondary to inflammation and vasoconstriction induced by platelets, neutrophils and damaged endothelial cells (193,310); a response directly proportional to lesion severity/length. Indeed NR has been previously linked with lesion severity and the degree of disease burden. (311,328)

NR is primarily a disease of the endothelium and microvasculature, both of which are negatively impacted by the presence of long term systemic hypertension. (387–389) Hence hypertension results in a myocardial vasculature more susceptible to NR. It is conceivable that admission systolic hypertension is driven by either uncontrolled/undiagnosed hypertension or systemic adrenergic stress response induced by the STEMI presentation. The former is important to acknowledge as hypertensive control can improve coronary microvascular function. (390) Myocardial ischaemia is known to stimulate catecholamine release and the renin-angiotensin system resulting in systemic vasoconstriction proportional to the degree of ischaemia. (391) Mirroring this is the vasoconstriction seen in the coronary vessels during infarction (392), and so systemic hypertension represents greater ischaemia and increased vasoconstriction resulting in higher levels of ischaemic and perfusion injury contributing to NR.

Weight was also predictive with lower values representing an increase in risk. Patients with reduced habitus may convey a potentially systemically unwell / frailer population. Inversely, it may be more appropriate to consider increased weight being protective against NR. Although morbid obesity has been shown to convey a worse outcome for cardiovascular disease, overweight or obese states have been found to be protective. (393,394) Adipocytes are linked with anti-inflammatory cascades and reduction in oxidative stress (395), which leads to consideration of "healthy" overweight/obesity.

Epicardial adipose tissue measurements, which has been shown to directly correlate to increased metabolic cardiovascular risk (396), were similar in both our cohorts suggesting a "healthier" obese state in the control population.

Independent predictors of NR identified in other previous risk models (338,339) were evaluated. Interestingly marked hyperglycaemia was infrequently seen in our group (blood glucose ≥12mmol/l was 6.9%), which may relate to its failure to contribute to the model. Age, and time from onset of symptoms to therapy, and high creatinine levels were significantly different between the groups, however were not found to be independently predictive. Differences found may relate to the population/ethnicity or clinical practice variation. Previously published data has found increasing age and creatinine levels in populations with more complex/severe coronary disease (397), a variable found to be the most powerful independent predictor of NR.

6.4.1   Limitations

The study was performed in a single centre in the United Kingdom with a cohort almost solely Caucasian, which may limit its relevance to all populations. Furthermore, NR was diagnosed on angiography, rather than more sensitive methods such as cardiac MRI or MCE. However, this methodology was purposeful as felt closer to real world practice.

6.5   CONCLUSIONS

NR is an important condition seen during PPCI for STEMI; it conveys an increased risk of MACE. Independent predictors of NR, identified prior to coronary intervention, were increased lesion complexity, systolic hypertension on admission, weight <78kg, and previous history of hypertension. Further international multicenter validation of this risk model is required. Furthermore, research is needed to explore whether subsequent modifications to the interventional strategy in patients evaluated to be at high risk of NR will have any impact on its occurrence.

7   CHAPTER SEVEN: SYSTEMATIC REVIEW AND META-ANALYSIS OF OPTIMAL P2Y$_{12}$ BLOCKADE IN DUAL ANTIPLATELET THERAPY FOR PATIENTS WITH DIABETES MELLITUS AND ACUTE CORONARY SYNDROMES (398)

7.1   INTRODUCTION

Globally, the prevalence of Diabetes mellitus (DM), a group of conditions categorised by sustained hyperglycaemia, is increasing. (173,174) As previously discussed, (section 1.4.5.2 & 4.4.1) this population is well described as having increased platelet reactivity (170–172) and therefore it is unsurprising that in large landmark antiplatelet trials as many as 15-39% of all patients presenting with ACS have a background of DM. (35,399) This figure correlates well with registry data percentages (GRACE registry 26%, Swedeheart registry 24%, PACIFIC registry 35%). (400–402) Furthermore this population is known to have worse mortality and morbidity outcomes compared to patients without diabetes; independent of other co-morbidities. (403) Hence the importance of research focused on this cohort.

Published data suggests increased aggregation of platelets in DM is driven primarily by hyperglycaemia affecting a multitude of pathways including increasing p-selectin expression via activation of protein kinase C, impaired function of endogenous antiplatelet agents such as NO and PGI$_2$ (160), amplified platelet adhesion (175), a pro-inflammatory environment (173) and increased platelet turnover. (322) Importantly up regulation of P2Y$_{12}$ signalling and GPIIb/IIIa surface receptors are also implicated. (175,174) Therefore, with the focus of pharmacological management of ACS being the reduction of thrombus burden and platelet reactivity (4,404) targeting P2Y$_{12}$ receptors is of great importance particularly in this population, who may stand to receive the most benefit.

Until recently clopidogrel was the most widely used P2Y$_{12}$ receptor inhibitor in addition to aspirin, following randomised control trial data showing a reduction in cardiovascular death, MI and stroke. (36,405,399) However, in patients with DM there has been a suggestion of a muted response to clopidogrel, which has been cited as multifactorial, including genetic, metabolic, cellular and clinical. (177,178) This has increased the interest in more novel P2Y$_{12}$ receptor antagonists, such as prasugrel and ticagrelor. Published data has led to preferential use of these agents in the general population (34,35,406) and possible better outcomes with prasugrel in the cohort with diabetes. (173,322) This is

interesting given *ex vivo* results in chapter four which demonstrated a muted response to ticagrelor inhibition of PLA formation and endothelial function in stable CAD patients with DM. No specific data has been systematically reviewed with both direct and indirect comparison of $P2Y_{12}$ antagonists for the management of ACS patients with DM.

7.1.1 Objectives of this chapter

To establish, through the available literature, the optimum antiplatelet therapy practice for management of patients with DM who present with ACS.

The specific review questions:

(1) In combination with aspirin, which is the superior agent for $P2Y_{12}$ blockade to improve the primary outcome of CV death, non-fatal MI and non-fatal stroke (CVA)?

(2) Is this benefit outweighed by increased risk of major bleeding (secondary outcome)?

7.2 METHODS

This review was reported in accordance with the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) guidelines (407), and a protocol prior to embarking on the search was written.

7.2.1 Protocol and question development (PICOS)

7.2.1.1 Population

The review population required diagnosis of DM with no differentiation made between type. All articles relevant to ACS and $P2Y_{12}$ blockade were careful reviewed for eligibility to ensure no important data was excluded. If the publication was not restricted to patients with DM, only the relevant sub group data was reviewed. If no isolated results for patients with DM were present, or available after contacting the author, the study was excluded.

7.2.1.2 Interventions and comparators

The intervention explored in the review is $P2Y_{12}$ blockade in the management of ACS in patients with DM, including oral and intravenous administration. The studies may compare $P2Y_{12}$ inhibitors against each other or against placebo.

7.2.1.3 Outcomes

Primary outcomes measured included, but not restricted to, mortality, MACE or revascularisation. Secondary outcomes included safety profile focusing on bleeding rates. Studies were not required to report all outcomes of interest to meet inclusion criterion.

7.2.1.4   Study design

Due to the number of large studies focused on the pharmacological management of ACS, to reduce the risk of bias while still maintaining adequate data, the data search was restricted to randomised control trials to optimise quality. The review considered all randomised control trials with no language or date restriction. The methodology of all papers meeting inclusion criteria was reviewed and carefully scrutinised for possible bias with the Cochrane risk of bias assessment tool. (24) To reduce the possibility of publication bias, we searched through conference abstracts to identify any more studies eligible for inclusion.

7.2.1.5   Inclusion criteria

- Human

- Adult (≥ 18-year-old)

- Randomised Control Trial

- Acute Coronary Syndromes

    o   Unstable Angina

    o   Non-ST Elevation MI

    o   ST Elevation MI

- Patients with DM – analysed independently of total population

    o   Type 1

    o   Type 2

- Comparing $P2Y_{12}$ inhibitor vs placebo or two differing $P2Y_{12}$ receptor antagonists

    o   Clopidogrel

    o   Prasugrel

- o Ticagrelor

- o Cangrelor

- o Elinogrel

- Outcomes to be clinical

  - o Death

  - o MI

  - o Revascularisation

  - o Bleeding

  - o Any other clinical outcome

- No outcome data on platelet function (e.g. VerifyNow)

- No data on ticlopidine due to limited clinical use of medication in UK

- No language restriction

- No date restriction

7.2.2 Search strategy and study selection

Medline and Embase databases were systematically searched up to 18th June 2014 with no date or language restrictions. The search threads were limited to human, adult (≥18 years) and randomised controlled trials evaluating; clopidogrel, cangrelor, ticagrelor, prasugrel, elinogrel, P2Y12 receptor antagonist, P2Y12 receptor inhibitor, ADP receptor antagonist or ADP receptor inhibitor. The results were combined with the Boolean operator "OR" and linked to Medical Subject Headings (MESH).

Following exclusion of duplicates the retrieved titles and abstracts were independently screened by two authors for relevant studies with focus on CAD. The full texts for remaining studies were obtained.

The authors reviewed these residual articles by electronically searching the studies for the word stems "diab", "mellitus" or "DM". Studies were excluded if they did not contain these terms, or only included in baseline patient characteristics. Each figure was individually searched to ensure no data relating to a DM subset. Also explored was the documentation of supplementary data using stem "supplement", "append" and "online". This data was then evaluated for results relevant to the review. Medically trained peers fluent in required language translated the foreign language papers discovered.

Finally, the authors again independently assessed for the presence of clinical outcome data for ACS (ACS, ST elevation MI, non-ST elevation MI and unstable angina) distinct from stable disease. The remaining studies were analysed to confirm there was clinical outcome data of $P2Y_{12}$ receptor inhibition in patients with DM presenting with ACS. Any discrepancies in results were resolved by group consensus.

In parallel to the systematic search process, we searched for studies in major cardiology conference abstract databases (American Heart Association (AHA), American College of Cardiology and European Society of Cardiology (ESC)). Further we retrieved other meta-analyses of antiplatelet agents reviewing the studies included and manually searched the references to ensure no relevant studies missed.

7.2.3 Data extraction

Two investigators scrutinised the resulting studies and relevant data was extracted, as outlined in the protocol. This included: title, author(s), country, publication year, study period, patient population, treatment arms, outcome definition, follow up duration, overall incidence, subgroups of patients with DM, number of patients and relative risks with 95% confidence interval.

The outcome measures included both primary and secondary outcome measures. Primary outcome measures mentioned: CV mortality; non-fatal MI; non-fatal CVA; and any other relevant clinical endpoint. Secondary outcomes incorporated, but were not limited to, major bleeding. Methodological quality was assessed via the "The Cochrane Collaboration's tool for assessing risk of bias" utilising the protocol or design and rationale

papers to aid. (408) Any discrepancies were resolved by group meeting and discussion with a third investigator if agreement not met.

7.2.4   Data synthesis and statistical analysis

The eligible studies were entered into RevMan 5 software package, and the statistical methods were programmed into RevMan 5.3 analysis software.

The number in each comparator group and the number of events were extracted. For the dichotomous data the risk ratios (RR) along with 95% confidence intervals were calculated. In cases of common comparators and outcomes the results were pooled using the fixed effects and random effects models dependent on heterogeneity. Heterogeneity was explored with the Cochrane Q statistic, which was considered to be significant if $p<0.10$ and if significant a random effect model was used to allow generalisation of the results. Z tests were used to test for the overall effect.

Indirect comparison analysis of relative risk was performed to evaluate prasugrel versus ticagrelor, and both newer P2Y12 inhibitors against control. This was completed using Bucher's method. (409)

7.3   RESULTS

7.3.1   Literature search

1162 articles were identified on the search of Medline and Embase following restriction to human adult randomised control trials. Review of the title and abstract excluded 896 papers, with elimination of 184 articles due to failure to mention diabetes beyond baseline characteristics. Absence of clinical outcomes or separate acute coronary syndromes cohort resulted in exclusion of further 67 articles. Therefore 15 papers, consisting of 6 study populations were included in the analysis. (Figure 7.1) (35,36,60,399,190,406,410–418) Study populations, design and outcomes are documented in table 7.1.

Five papers evaluated clopidogrel as the intervention of interest in three study populations. Two studies compared clopidogrel to placebo in addition to aspirin therapy (36,399,414) and one population examined double dose clopidogrel versus standard dose therapy in a 2 by 2 factorial design with aspirin low and high dose. (415,416)

Seven papers were written based on data from two study cohorts comparing prasugrel to clopidogrel (35,406,410–413,418) and three relevant papers generated from single study examining ticagrelor versus clopidogrel. (60,190,417) The initial trials were all found to be high quality and low risk of bias, as demonstrated in table 7.2; using Cochrane risk of bias tool. (408)

IDENTIFICATION

Results Identified from Database and other Searches
n=1162

SCREENING

Title and Abstract Review

Excluded n=896

Not related to coronary artery disease or $P2Y_{12}$ inhibitors

ELIGIBILITY

Text, Figure and Supplement Review

Excluded n=184

No comment on diabetes mellitus beyond baseline characteristics

Excluded n=67

No clinical outcomes or not restricted to ACS patients

INCLUDED

n=15

**15 articles from
6 study populations**
Clopidogrel vs Placebo: 3 studies
Prasugrel vs Clopidogrel: 2 studies
Ticagrelor vs Clopidogrel: 1 study

Figure 7. 1: Flow chart of study identification and inclusion

STUDY	Patient Population	Treatment 1	Treatment 2	Primary Outcome	Secondary Outcome	Follow Up	No of DM Patients (% of pop)	Risk Ratio	CI 95%
CURE	NSTEMI/UA patients within 24hours	Clopidogrel 75mg OD	Placebo	Composite CV death/MI /CVA	Composite CV death/MI/CVA/ ischaemia Bleeding Heart failure Revascularisation	12months	2840 (23%)	0.85	(0.72-1.01)
PCI-CLARITY	STEMI patients receiving fibrinolysis due to undergo PCI at day 2-8	Clopidogrel 300mg at time of fibrinolysis then 75mg OD	Placebo at time of fibrinolysis	Composite CV death/MI /CVA from time of PCI	Before PCI MI/CVA Bleeding	30days	282 (15%)	0.60	(0.26-1.36)
CURRENT-OASIS	ACS referred for PCI	Clopidogrel loading 600mg Day2-7 150mg OD then 75mg OD either low or high dose aspirin	Clopidogrel loading 300mg then 75mg OD with either low or high dose aspirin	Composite CV death/MI /CVA	CV death Any death MI,CVA Recurrent ischaemia Stent thrombosis Bleeding	30days	5880 (23%)	0.85	(0.69-1.05)
TRITON – TIMI 38	ACS with scheduled PCI	Prasugrel 10mg OD	Clopidogrel 75mg OD	Composite CV death/MI /CVA	Bleeding Ischaemia Revascularisation Stent thrombosis	15 months	3146 (23%)	0.72	(0.60-0.85)
TRILOGY	NSTEMI/UA patients <75 years old without revascularisation	Prasugrel 10mg OD	Clopidogrel 75mg OD	Composite CV death/MI /CVA	Bleeding Ischaemia Stent thrombosis	30 months (median 17.1)	2811 (39%)	0.87	(0.76-1.00)
PLATO	ACS patients (both PCI and medically managed)	Ticagrelor 90mg BD	Clopidogrel 75mg OD	Composite CV death/MI /CVA	Bleeding Any death Stent thrombosis	12 months	4662 (25%)	0.89	(0.77-1.02)

22: **Table of study methodology and key primary result in the population with diabetes mellitus**

	Sequence Allocation	Conceal ment	Blinding	Blinding of Outcome	Incomplete data	Selective Reporting
CLARITY	L	L	L	L	L	L
CURE	L	L	L	L	L	L
CURRENT -OASIS	L	L	L	L	L	L
PLATO	U	L	L	L	L	L
TRITON- TIMI 38	U	U	L	L	L	L
TRILOGY	U	L	L	L	L	L

L: Low risk of bias, U: Unclear risk of bias, H: High risk of bias

Table 7. 1: Risk of Bias Assessment (408)

7.3.2   Diabetes mellitus versus no diabetes

The importance of assessing the DM population in the ACS patient group was highlighted by the universal agreement that patients with diabetes have a worse outcome, notably with the predominant primary composite endpoint (CV mortality, non-fatal MI and non-fatal CVA) (399,190,411) and MI. (417) Management of DM patients with insulin conferred a worse primary outcome still. (411) (Figure 7.2) Insulin use is likely to be a surrogate marker for diabetes severity as the population with type 1 diabetes will comprise only a minority of these patients. Data from PLATO shows that type 1 diabetes accounted for only 4% of their total population with diabetes. (190)

In the studies that statistically assessed for interaction between patients with and without diabetes, the majority found that there was no significant difference in the overall results. (190,406,416) The exception to this was TRITON-TIMI 38 which found that the population with diabetes had superior benefit from prasugrel compared to clopidogrel in the reduction in fatal/non-fatal myocardial infarction (HR 0.60 (95% CI 0.48-0.76)(DM patient) versus HR 0.82 (95% CI 0.72-0.95) (patient without DM ($P_{interaction}$ = 0.02)) and composite of all cause death, MI, CVA and major bleed unrelated to CABG (HR 0.74 (95% CI 0.62-0.89) versus HR 0.92 (95% CI 0.82-1.03 ($P_{interaction}$ = 0.05)). (35,411)
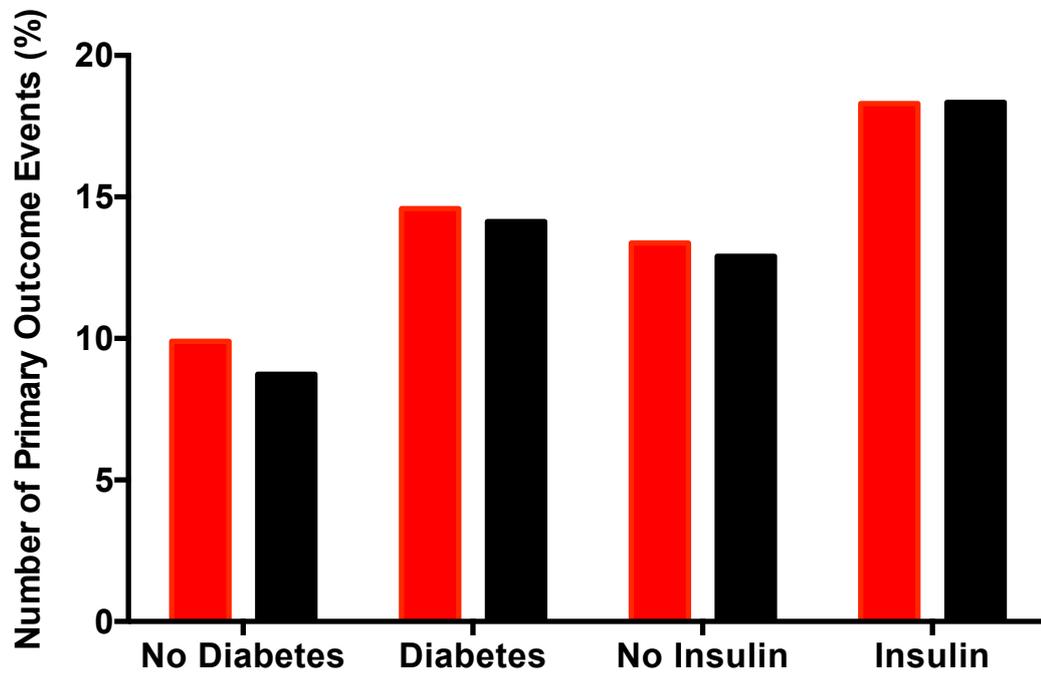
Figure 7. 2: Comparison of outcomes in patient with diabetes mellitus versus patients without diabetes mellitus

Primary outcome event rate (CV death, non-fatal MI and non-fatal CVA) percentage of total population in TRITON-TIMI 38 (prasugrel, red bars) and PLATO (ticagrelor, black bars) studies, exploring effects of presence of diabetes and nature of management. (190,411)

7.3.3   Clopidogrel versus placebo

In 3122 patients from two randomised studies, treatment with clopidogrel was associated with a significant reduction in CV death, non-fatal MI and non-fatal stroke at 30-360 days (RR 0.84 (95% CI 0.71-0.99)). The larger study looked at patients independent of management strategy (414), were as the second studied only those undergoing an invasive strategy. (399) There was no statistical heterogeneity (Q= 0.68, P= 0.41, I^2= 0%) so fixed effects were calculated. (Figure 7.3)

7.3.4   High versus low dose clopidogrel

In light of the favourable results of clopidogrel versus placebo CURRENT-OASIS 7 looked at 25,086 patients referred for PCI following presentation with ACS. Patients were assigned either double dose or standard therapy clopidogrel and high dose or low dose aspirin. In the pre-specified sub group of DM there was a non-significant benefit of higher dose clopidogrel in primary composite end point rate (CV death, non-fatal MI and CVA) with RR 0.85 (95% CI 0.69-1.05), p= 0.13. (416)

	CLOPIDOGREL		PLACEBO		WEIGHT
	Events	Total	Events	Total	(%)
CURE	200	1405	240	1435	94.4
PCI-CLARITY	8	133	15	149	5.6
TOTAL	208	1538	255	1584	100

CURE: 0.72 0.85 1.01

PCI-CLARITY: 0.26 0.60 1.36

TOTAL: 0.71 0.84 0.99

0.00 0.20 0.40 0.60 0.80 1.00 1.20 1.40

⟵ Favours Clopidogrel   Favours Placebo ⟶
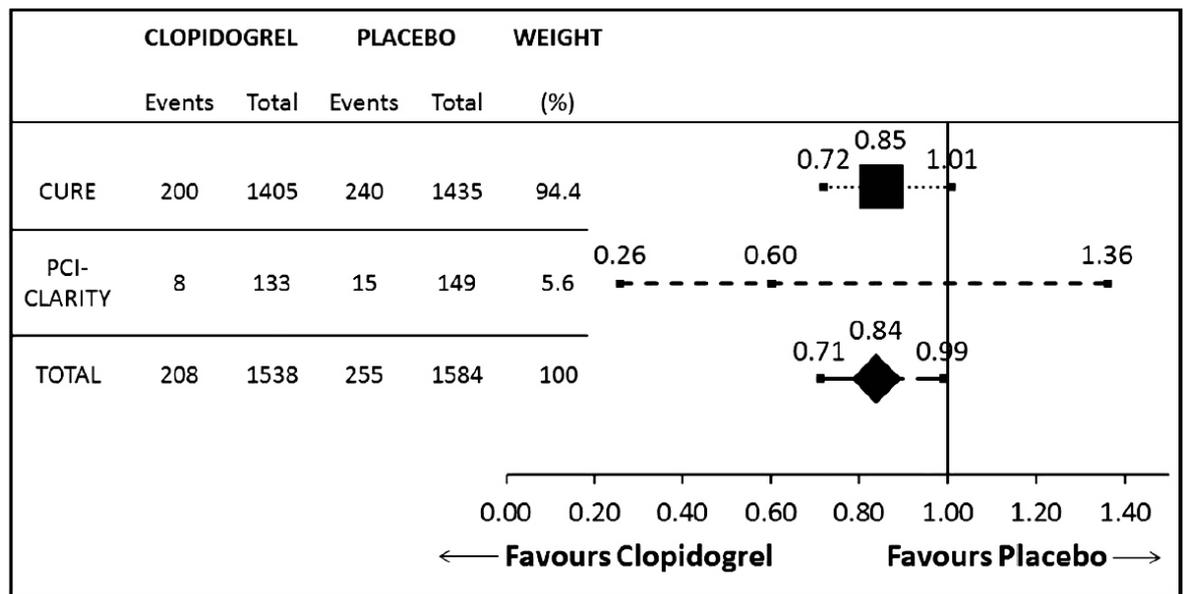
Figure 7. 3: Clopidogrel versus placebo

Risk ratio with 95% confidence intervals for the primary composite endpoint of CV death, non-fatal MI and non-fatal CVA in the population with diabetes mellitus comparing clopidogrel versus placebo in addition to aspirin.

7.3.5 Ticagrelor versus clopidogrel

The PLATO study compared ticagrelor and clopidogrel as P2Y$_{12}$ inhibitor in addition to aspirin. The DM patient population was a pre-specified subgroup that was subsequently analysed showing additional benefit with ticagrelor reducing composite of CV death, non-fatal MI and non-fatal CVA (RR 0.89 (95% CI 0.77-1.02), p= 0.10) over a 12month follow up period. (190) Interestingly, compared to the whole study population (RR 0.85 (95% CI 0.78-0.92), p= 0.0002) the DM patient cohort results were not statistically significant. (34) Reassuringly, in the patients with DM there was no increased risk of major bleeding as defined by PLATO (190) with RR 0.95 (95% CI 0.81-1.10), p= 0.47.

7.3.6 Prasugrel versus clopidogrel

The two studies with prasugrel and clopidogrel as comparators were TRITON-TIMI 38 and TRILOGY (35,406), which evaluated the ACS population managed with scheduled PCI and no revascularisation respectively. A total population of 6690 with diabetes were monitored for events predetermined with the composite primary end point of CV death, non-fatal MI and CVA, for the duration of follow up (14.5-30months). Heterogeneity was observed (Q= 3.15, P= 0.08, I^2= 68%), so the risk ratio was calculated using the random effects model. The overall effect was in favour of prasugrel in the population with DM RR 0.80 (95% CI 0.66-0.97) (figure 6.4).

From the TRITON-TIMI 38 data we can also elicit benefits of prasugrel over clopidogrel in the population with diabetes, with reduction in MI (RR 0.62 (95% CI 0.50-0.76), p= <0.01) and definite/probable stent thrombosis (RR 0.56 (95% CI 0.36-0.86), p= 0.01) events. This advantage is consolidated in the knowledge there is no increase in major bleeding risk associated (RR 0.95 (95% CI 0.61-1.46), p= 0.81). (411) Interestingly TRITON-TIMI 38's outcomes appear better in the DM patient cohort compared to the total enrolled population whose RR 0.82 (95% CI 0.74-0.91), p= <0.01. (35)
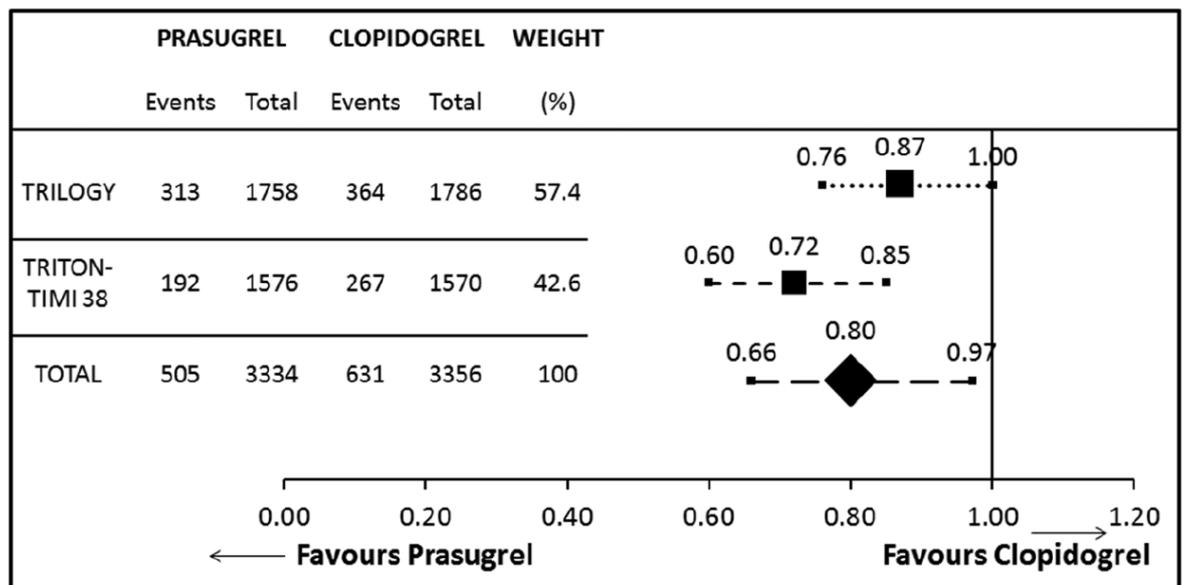
Figure 7. 4: Prasugrel versus clopidogrel

Risk ratio with 95% confidence intervals for the primary composite endpoint of cardiovascular death, non-fatal myocardial infarction and non-fatal stroke in the population with diabetes mellitus comparing prasugrel versus clopidogrel in addition to aspirin.

7.3.7 Insulin dependent patients with diabetes mellitus versus non-insulin dependent patients with diabetes mellitus

Two studies further divided the population with DM, categorising by insulin use. (190,411) Analysis showed that the benefit of the newer P2Y$_{12}$ inhibitor was more pronounced in the group treated on insulin with a reduction in risk of 36% with prasugrel and 22% with ticagrelor, compared to 24% and 8% respectively in the non-insulin treatment arm. However these were not adequately powered and failed to reach statistical significance (P$_{interaction}$= 0.2 for prasugrel and P$_{interaction}$= 0.13 for ticagrelor). (190,411) Reassuringly there was no increased risk of major bleeding risk in any group, independent of antiplatelet or DM management strategy (figure 7.5).
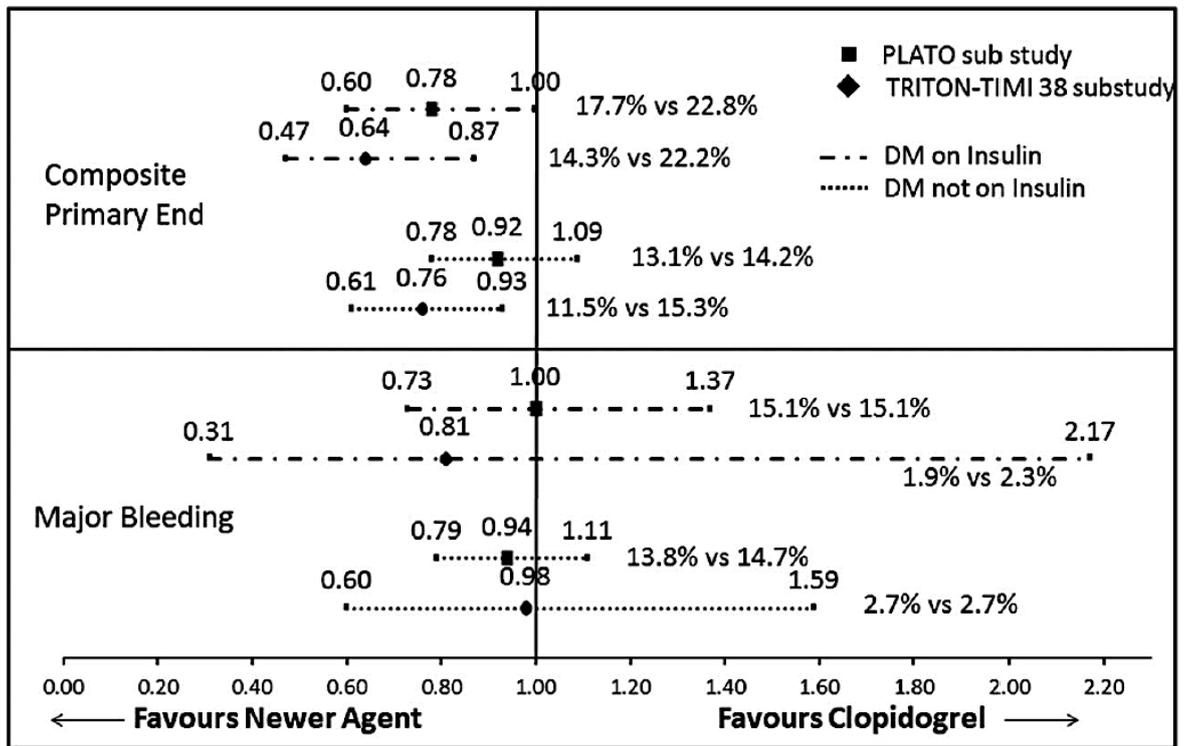
Figure 7. 5: Prasugrel and ticagrelor versus clopidogrel

Risk ratio with 95% confidence intervals for the primary composite endpoint of cardiovascular death, non-fatal myocardial infarction and non-fatal stroke. Also, major bleeding in the populations with diabetes mellitus managed with and without insulin; comparing prasugrel and ticagrelor versus clopidogrel in addition to aspirin. Event rate percentage of newer agent versus clopidogrel.

7.3.8   Indirect comparison - ticagrelor versus prasugrel

In the patient population with DM we were able to indirectly compare a number of outcomes, which showed no statistical difference, however, a trend to prasugrel superiority with regards to the composite primary endpoint of CV death, non-fatal MI and non-fatal CVA, particularly in the cohort undergoing PCI. (190,411,417) A significant 33% risk reduction in MI events favouring prasugrel was noted. Regarding safety parameters there was no difference in definite/probable stent thrombosis (RR 1.19 (95% CI 0.58-2.45)) or major TIMI bleeding events (RR 0.94 (95% CI 0.59-1.51)) (figure 7.6).
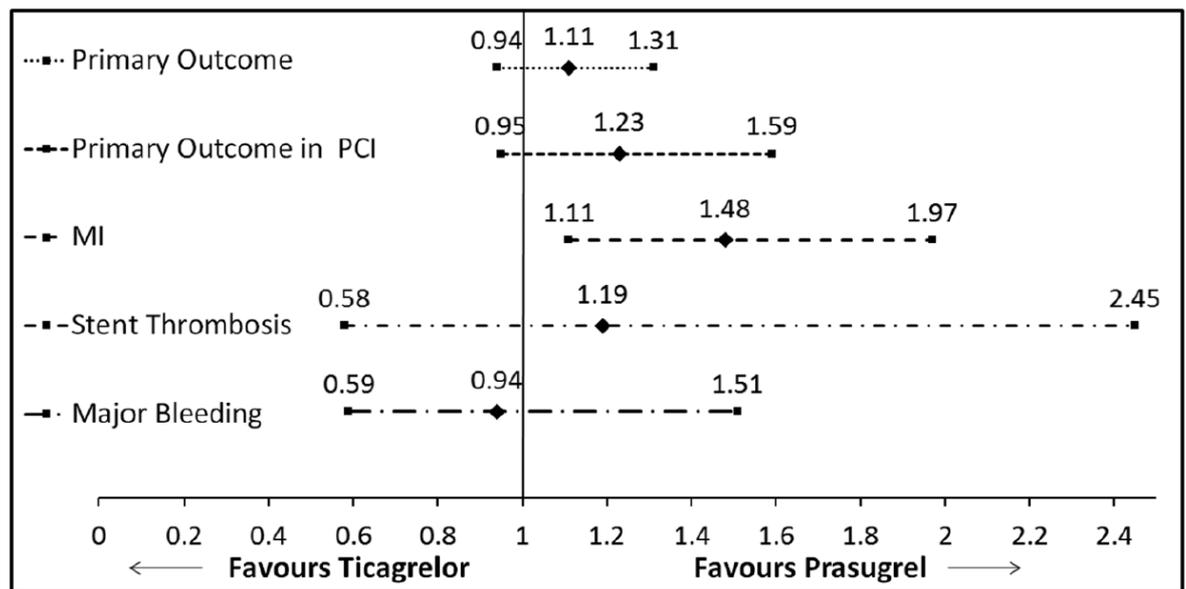
Figure 7. 6: Ticagrelor versus prasugrel

Risk ratio with 95% confidence intervals for the primary composite endpoint, primary composite endpoint in those undergoing PCI, myocardial infarction, definite/probable stent thrombosis and major bleeding (TIMI classification) in the population with diabetes mellitus indirectly comparing ticagrelor versus prasugrel in addition to aspirin.

7.3.9 Newer P2Y$_{12}$ inhibitor versus placebo

Using the same method of indirect comparison, we confirmed the superiority of the newer agents against placebo. This showed a marked reduction in composite end point of cardiovascular death, non-fatal MI and CVA (figure 7.7). There was further suggestion of prasugrel's clinical superiority in this population.
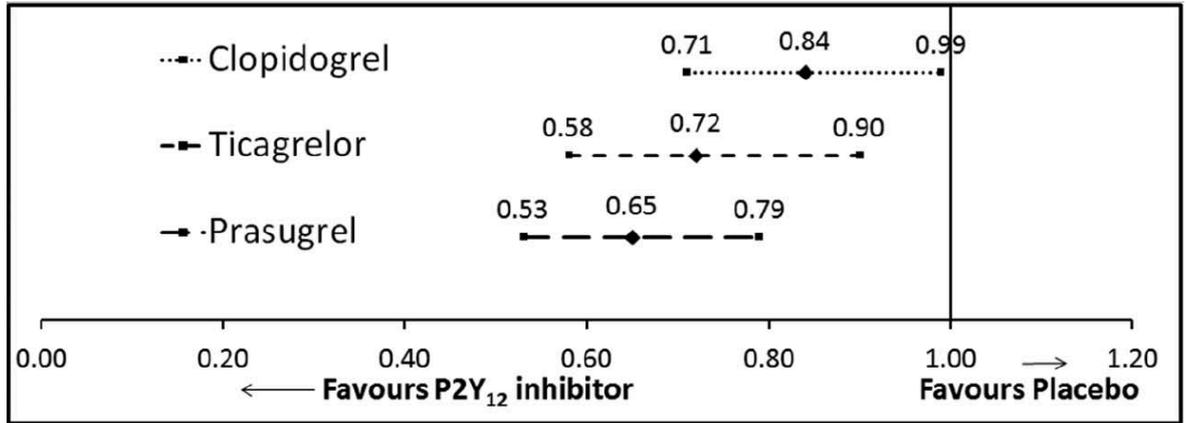
Figure 7. 7: P2Y₁₂ blockade versus placebo

Risk ratio with 95% confidence intervals for the primary composite endpoint of cardiovascular death, non-fatal myocardial infarction and non-fatal stroke in the population with diabetes mellitus comparing P2Y₁₂ blockade versus placebo in addition to aspirin.
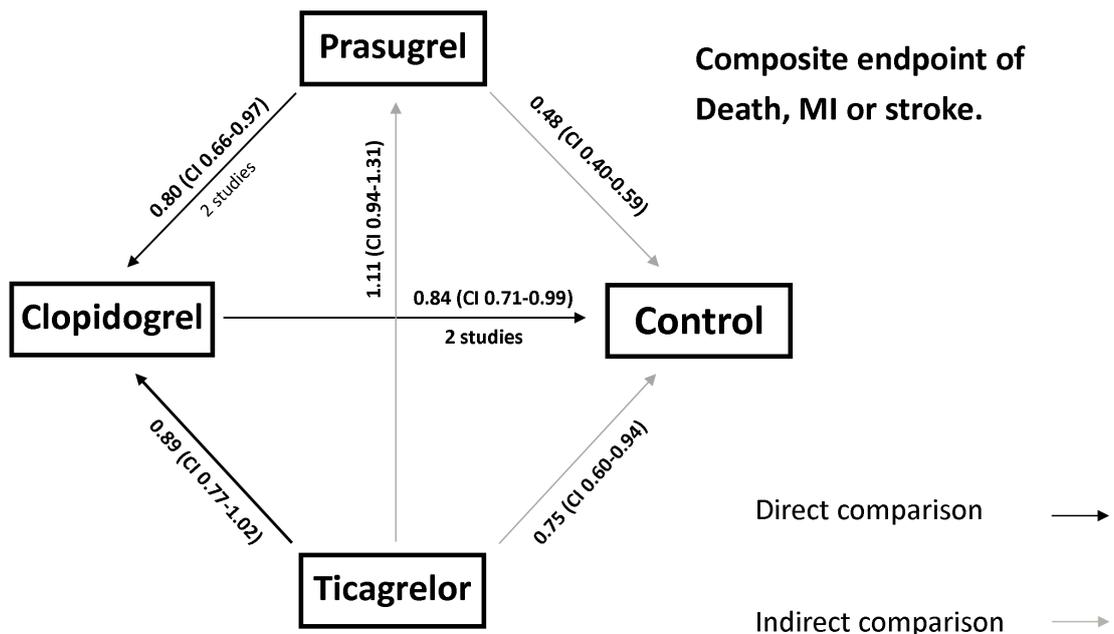
Figure 7. 8: Risk ratios for clopidogrel, prasugrel, ticagrelor and placebo for composite endpoint

Black lines represent direct comparison and grey lines represent indirect comparison

7.4   DISCUSSION

This review shows that all evaluated P2Y$_{12}$ inhibitors are superior in preventing ischaemic events compared to placebo in ACS patients with DM. The newer agents such as ticagrelor and prasugrel have a more favourable outcome when directly compared to clopidogrel, although ticagrelor failed to reach statistical significance. This was without an increased risk of bleeding.

There may be added value in the cohort managed on insulin, but these results were not powered to reach statistical importance. When indirectly analysed there is a significant benefit in reduction of events of MI with prasugrel compared to ticagrelor in the patient with DM group; with a trend to superiority relating to primary clinical outcomes especially in those undergoing PCI.

7.4.1   Possible Mechanisms

The clinical outcomes of ACS patients with DM when clopidogrel is combined with aspirin are favourable, in reduction of ischaemic events, versus placebo with aspirin. Patients with diabetes are more likely to be resistant to aspirin (176) and in addition, have increased P2Y$_{12}$ receptor signalling. (175) Therefore, this cohort may have amplified benefit from the addition of a P2Y$_{12}$ receptor antagonist to the COX inhibitor aspirin compared to their counterparts without DM.

Patients with DM have a higher level of platelet reactivity compared to the population without DM. (170) So the search for more potent inhibitors is especially important in this group. As demonstrated in the analysis, prasugrel and ticagrelor are superior to both placebo and clopidogrel. Regarding the newer agents versus clopidogrel these findings are supported by trials of platelet function testing showing increased inhibition of aggregation with less variable response. (179) This is likely to be multifactorial, but a large contributory is non-response to clopidogrel. Research shows that depending of method of assessment 38-44% of patients with DM are non-responders to clopidogrel compared to 8-17% of without DM at 24 hours. (172) The level of poor response is still apparent at 7 days (24-

53%) (178,179) and is combined with lower levels of circulating active clopidogrel metabolite. (178,251)

Non-response to clopidogrel is a well-documented phenomenon attributed to a number of plausible factors such as increased clearance, reduced absorption altered metabolism to the active clopidogrel metabolite, and higher platelet turnover. (174) Also potential genetic factors have been linked possibly related to the abnormalities in CYP expression in the DM population. (419) However the predominant mechanism appears to be the abnormal metabolism of clopidogrel within the DM population rather than dysfunction of the $P2Y_{12}$ receptor or its signalling pathway, as ex-vivo addition of active metabolite results in almost complete normalisation of response compared to controls. (178,251)

In non-responders increased on-medication platelet reactivity is significantly correlated to MACE (CV death, ACS or stroke), with an increase in event rate from 13.2% to 37.7% at 2 years in patients with DM whose level of platelet reactivity is in the upper 4th quartile. (246) Concern over none/sub responders led to exploration of high dose clopidogrel (150mg) in the patients with DM. Although this did significantly reduce platelet aggregation, nonetheless the majority remained categorised as a sub-responder (420), which may explain the lack of statistically important benefit to double dose clopidogrel in clinical outcomes. (416)

This has solidified the potential importance of the newer agents, prasugrel and ticagrelor. In the general population, they have been shown to be superior to clopidogrel in suppression of aggregation (177,385,421), even in those where patients are subjected to double dose clopidogrel. (179) Contributory to this is the difference in metabolism of prasugrel and ticagrelor, which decreases the time of onset of action and also limits the potential for drug interactions. Ticagrelor itself is a directly acting reversible agent (34), whereas prasugrel, a thienopyridine, still requires two activation steps akin to clopidogrel. However the first step is rapid, mediated by plasma esterases, and the next by CYP liver enzymes; which does not appear to be under the same CYP inhibitor influences as clopidogrel. (422) Interestingly higher circulating esterases are found in the patients with DM (176), which in theory would ameliorate this activation step, while inversely would enhance clopidogrel's conversion to inactive metabolite. (174)

The population of patients with DM requiring insulin therapy is trending towards increased benefit from the newer medications. As mentioned previously this represents a marker of severity and duration of diabetes, and functional studies have shown a muted response to clopidogrel in patients treated with insulin compared to oral medication. (423) This would suggest that the insulin-dependent group would have added benefit from a more potent $P2Y_{12}$ inhibitor. Further to this insulin has been noted *in vitro* to reduce ADP induced platelet aggregation by inhibiting the $P2Y_{12}$ pathway, which paradoxically is in agreement with the findings of the review, as patients requiring insulin therapy are those who are most highly resistant to insulin. (423)

Against the results of this clinical outcome analysis favouring prasugrel are small studies of platelet reactivity testing. These studies of have suggested superiority of ticagrelor over prasugrel at 5-30 days (424–426) even in the DM specific population. (253) Thirty patients with DM undergoing PCI following an admission with ACS were evaluated in a prospective single blinded, single centre crossover study comparing ticagrelor with prasugrel. All patients were pre-loaded with clopidogrel and platelet reactivity was tested at day 0, 15 and 30 using VerifyNow $P2Y_{12}$ functional assay. On day 15 patients were crossed over to the alternative comparator. The authors concluded that ticagrelor achieved a significantly higher level of platelet inhibition compared to prasugrel at 30days. Possible explanations are the pre-treatment with alternative thienopyridine to prasugrel (clopidogrel), although as the authors point out this is more consistent with clinical practice, and the lack of wash out period. Notably, prior to the crossover of patient groups from one comparator to the other, the difference in level of platelet inhibition was not statistically important. (253) Does this mean reversible versus non-reversible agents would skew the results? Agreement in the discussion is reached that this is only functional testing and may not relate to clinical outcomes, particularly as small population functional tests do not per se correlate with large study clinical outcomes. (427) It does however raise the question of possibly alternative pathways unrelated to platelet reactivity being involved, in keeping with chapter four results. Furthermore, theoretically, ticagrelor's functional superiority might be at the expense of a higher bleeding risk (424), however no clinical outcome evidence was found in the analysis to support this.

The variation between the direct functional and indirect clinical findings solidifies the need for a direct comparison study between ticagrelor and prasugrel with robust clinical and safety endpoints. We therefore await with great anticipation the results of the ISAR REACT 5 randomised control study (428), which will directly compare ticagrelor and prasugrel in those presenting with ACS undergoing an invasive strategy. The DM population is a clear pre-specified subgroup although patients with DM will be likely underrepresented in this invasive study. (34)

7.4.2   Guidance

European Society of Cardiology (ESC) and American Heart Association (AHA) both have clear guidance about antiplatelet management in ACS with published guidelines for patients presenting with both non–ST-elevation-ACS (NSTE-ACS) and STEMI, all supporting the use of $P2Y_{12}$ blockade in addition to aspirin (Class IA). There is already a move based on results of the landmark trials reviewed towards isolated guidance for patients with DM. (3,4,26,27)

The 2011 ESC guidelines support the preferential use of prasugrel or ticagrelor, over clopidogrel, in the DM population. Prasugrel should be only considered $P2Y_{12}$ antagonist naïve patients who due to undergo PCI for NSTE-ACS, assuming they are not at high risk of life threatening bleeding. (4,429) No specific preference is suggested in the STEMI group, where again prasugrel and ticagrelor are favoured. (26)

There are differing views in the 2014 ACCF/AHA (American College of Cardiology Foundation) NSTE-ACS guidelines, which state that with regards to antiplatelet therapy, patients with DM should be managed identically to the cohort without DM. They support the use of clopidogrel or ticagrelor (IB) with preference to ticagrelor where the patient is undergoing early invasive or ischaemia guided strategy. Prasugrel is not recommended for upfront therapy in any patient with ACS, unless undergoing PCI and not at high risk of bleeding. (3) In the 2013 ACCF/AHA STEMI guidelines all $P2Y_{12}$ inhibitors are given the same level of evidence without preference. But a minor comment is made to consider prasugrel

in younger patients with DM with low bleeding risk, ability to continue dual antiplatelet therapy and no planned surgery in the next year. (27)

In 2014 the UK NICE have followed suit with support for prasugrel in the DM cohort that present with ACS (STEMI and NSTE-ACS included) and who have a planned invasive strategy. (430) They determined that in comparison to clopidogrel, prasugrel is judged to be the more cost-effective treatment of NSTE-ACS patients with DM managed within the UK National Health Service framework.

This chapter's findings support the advice from NICE and we await future guidelines from AHA/ESC with regards to a more tailored approach to ACS management.

7.4.3   Strengths and Limitations

The original studies included in this analysis were large multicentre randomised controlled trials with careful pre-specification of the DM subgroup in the majority. (34,35,406,416) All the studies were found to have low risk of bias using the Cochrane risk of bias tool detailed in table 2. However, the analysis is restricted to published literature, all in peer-reviewed journals, but an element of publication bias cannot be excluded. The remaining papers where ad hoc subgroup analysis cannot be excluded are at obvious risk of statistical error. (399,414)

The definition of DM is not clearly explored in every study population, which may be open to differing interpretation. Four clearly state previous diagnosis of DM was required. However, this was only inferred in others. Only PLATO analysed data of all subjects on the basis of HbA1$_c$ which would enable pick up of patients with previously undiagnosed DM, arguably those at highest risk.

Indirect comparison analysis does require homogeneity of those compared, which is supported by the findings of the Cochrane Q statistics observing absence of heterogeneity in the clopidogrel versus control group. Random effects relative risk was required for the combination risk of prasugrel compared to clopidogrel in light of heterogeneity, which needs to be taken into account when considering the primary outcome indirect comparison. However, the remaining outcomes indirectly compared are single study

comparisons with similar design. In the analysis of major bleeding risk results generated from identical scoring schemes (TIMI) were utilised to maximise homogeneity.

7.5 CONCLUSIONS

In patients with DM who present with ACS the addition of a $P2Y_{12}$ receptor inhibitor is superior to placebo in reducing CV mortality, non-fatal MI and non-fatal CVA without significantly increasing major bleeding events. There is a trend to superiority of prasugrel in this cohort particularly in those undergoing PCI, without amplified risk of major bleeding. There is a need for the most effective anti-platelet strategy for patients with DM to be further explored with dedicated randomised controlled studies.

8   CHAPTER EIGHT: CLOSING REMARKS

8.1   CONCLUSIONS

The results of this thesis have concluded that $P2Y_{12}$ inhibition with ticagrelor enhances the antiplatelet effect of the endogenous vasodilators PGI_2 and NO, as demonstrated *in vitro* and *ex vivo* conditions by flow cytometry measurement of multiple markers of platelet activation. This effect was similarly observed for the first time in patients with established CAD, who overall trended to a reduced sensitivity to PGI_2, NO and ticagrelor compared to healthy controls.

Furthermore, the reactive hyperaemic index (RHI), as a measure of endothelial function, increased in response to oral ticagrelor. This was evident in both healthy aged matched controls and to a statistical level in the CAD patient cohort; with a tendency to reduced sensitivity to ticagrelor in the CAD group who also have diabetes mellitus and are smokers. Interestingly, the increase in endothelial RHI measurements by ticagrelor correlated with the degree of inhibition of platelet activation by ticagrelor, as measured by P-selectin expression and fibrinogen binding.

Platelets and leukocytes interactions are associated with platelet activation and the foundation of platelet facilitated inflammatory response. The results established that PGI_2 inhibited platelet-leukocyte aggregate formation in CAD patients and healthy controls, which has not previously been shown in CAD. Ticagrelor augmented the ability of PGI_2 to prevent PLA formation in both populations. In addition to this, ticagrelor had an effect on intracellular cyclic nucleotide signalling in monocytes and lymphocytes, suggesting that there may be a potential non-platelet mediated influence on inflammation by $P2Y_{12}$ inhibition.

Platelets, leukocytes and inflammation are heavily implicated in the no reflow phenomenon (NR), an important though often under-appreciated condition seen in ACS. As observed, NR negatively impacts patient clinical outcomes, increasing MACE primarily driven by cardiovascular death. Limited evidence exists for effective treatment of NR. A review of current published data indicates that prevention is the best strategy. Identification those at highest risk is therefore of importance. In the STEMI population, the

independent predictors of NR were increased lesion complexity, systolic hypertension on admission, weight <78kg and previous history of hypertension. This new information has the potential to enable operators to initiate therapy/modify the interventional strategy.

ACS patients are a heterogeneous population, with certain sub groups at greater risk, both in terms of occurrence as well as poorer outcome. This includes ACS patients with diabetes mellitus, who following systematic review of the available data, benefit from addition of a $P2Y_{12}$ receptor inhibitor in reducing CV mortality, non-fatal MI and non-fatal CVA compared to placebo. The newer $P2Y_{12}$ antagonists conveyed a greater benefit still compared to clopidogrel. This is in the absence of significantly increasing major bleeding events. Using direct and indirect analysis a trend to superiority of prasugrel was seen, compared to ticagrelor, particularly in those undergoing PCI without amplified risk of major bleeding.

In summary, work in this thesis has demonstrated important effects of $P2Y_{12}$ blockade with ticagrelor in patients with CAD on platelets, the endothelium and the inflammatory cross-talk. In light of the burden of CAD and ACS on the health of the population, this remains a key area of academic interest. These findings open new avenues for research with the aim of impacting of patient outcomes and potentially reducing serious complications of platelet aggregation in the coronary vasculature, such as NR.

8.2   FUTURE RESEARCH

The translational work has highlighted important findings related to P2Y$_{12}$ inhibition, and platelet/endothelial function. From this initial work several research questions have been raised, requiring further exploration:

1. Current research does not adequately explain the mode of interaction between P2Y$_{12}$ inhibition and endothelial vasodilators. Further breakdown of the pathways involved are required to provide greater insight;

2. Additional evaluation of the clinical importance of this interaction related to ticagrelor is necessary, were potential further increase in the antiplatelet effect of ticagrelor to reduce ischaemic events must be carefully balanced with the risk of bleeding;

3. The correlation of RHI response to ticagrelor and its antiplatelet effect could be considered further, potentially lending itself to a simple non-invasive bedside assessment as a surrogate marker in future research;

4. The significance of the varying response in the most high-risk patients (smokers who also have DM) should be validated and investigated. This work should go on to evaluate the clinical implications and target therapy accordingly;

5. Finally, reproducing this study in patients with ACS rather than stable CAD, would provide further understanding and increase relevance given the current licence for ticagrelor only in ACS or post recent MI.

Inflammation has a critical role in CAD and ACS. Ticagrelor may have an indirect and direct effect on the inflammatory cascade from preliminary work. Further research is required to improve understanding.

A score for stratifying the risk of NR in a STEMI patient quantifiable at point of urgent admission has the potential to be of key clinical importance and prospective validation studies are required. If of good predictive value, it would have a role in future therapeutic research and everyday clinical practice guiding current preventative therapies.

In the systematic review it was clear that optimal antiplatelet therapy in patients with DM is still unclear, and much of the data is provided by underpowered subgroup analysis. Dedicated randomised trials are needed to explore this at-risk population in further detail.

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