UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Human Development and Health Academic Unit

Assessment of individual responses to antiplatelet therapy in cardiovascular disease: Insights with Short Thrombelastography

by

Vikram Khanna

Thesis for the degree of Doctor of Medicine

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ABSTRACT

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ASSESSMENT OF INDIVIDUAL RESPONSES TO ANTIPLATELET THERAPY IN CARDIOVASCULAR DISEASE: INSIGHTS WITH SHORT THROMBELASTOGRAPHY

Vikram Khanna

The pivotal role of platelets in atherogenesis and thrombosis is well established. Consequently, suppression of platelet function with antiplatelet therapy (APT) is an important therapeutic target for the prevention of cardiovascular events in high-risk populations, particularly in patients treated with percutaneous coronary intervention (PCI). Based on various *ex vivo* platelet function assays (PFAs), studies have consistently shown heterogeneity in responses to APT. This is especially important given that platelet reactivity has been associated with both atherothrombotic events and bleeding complications at opposite ends of the spectrum. Personalised APT based on assessing individual responses has therefore emerged as a logical solution to the dilemma of optimising clinical outcomes in PCI and CVD, however this has yet to be proven. Furthermore, a number of important questions regarding the delivery of this strategy remain outstanding before it can be implemented in clinical practice.

The studies presented in this thesis have universally employed Short Thrombelastography (s-TEG) to: (i) determine whether VerifyNow, a point of care PFA, overestimates the functional effects of clopidogrel compared to s-TEG, based on the inclusion of prostaglandin E1 as agonist; (ii) to evaluate whether responses to APT remain stable over time following hospital discharge thereby obviating the need for serial testing; and (iii) to compare the antiplatelet and anticoagulant effects of unfractionated heparin and bivalirudin, two adjunctive anticoagulant agents used commonly in the setting of primary PCI. Furthermore, there is anecdotal evidence suggesting that arachidonic acid (AA), a substrate of the cyclooxygenase-1 (COX-1) pathway blocked by aspirin, can stimulate clotting via alternative pathway(s). We therefore sought to investigate potential COX-1-independent mechanisms for AA-mediated clotting in a population of patients undergoing major vascular surgery on aspirin.

Studies in this thesis raise concerns about the stability of responses to APT over time and highlight the importance of an accurate and reliable assay of platelet function for the successful implementation of a personalised APT strategy. Furthermore, they offer important mechanistic insights into the contrasting pharmacodynamic effects of anticoagulant agents commonly used as adjuncts in PCI. Collectively, these studies could form the basis of larger clinical trials that may influence how these agents are prescribed in the future.

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DECLARATION OF AUTHORSHIP

I, Vikram Khanna, declare that this thesis entitled **'Assessment of individual responses to antiplatelet therapy in cardiovascular disease: Insights with Short Thrombelastography'** and the work presented in it are my own and have been generated by me as the result of my own original research. I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

7. Parts of this work have been published as listed in the next section.

Signed:.....

Date:.....

PUBLICATIONS AND AWARDS ARISING FROM THIS THESIS

Original papers

Khanna V, Hobson A, Mikael R, Sambu N, Englyst N, Curzen N. Does the VerifyNow P2Y12 assay overestimate "therapeutic response" to clopidogrel? Insights using short thrombelastography. Thromb Haemost 2014;**111**(6):1150-9.

Khanna V, Mikael R, Thayalasamy K, Sambu N, Dimitrov BD, Englyst N, Calver AL, Corbett S, Gray H, Simpson IA, Wilkinson JR, Curzen N. Does the response to aspirin and clopidogrel vary over 6 months in patients with ischemic heart disease? J Thromb Haemost 2015;**13**(6):920-30.

Khanna V, Armstrong, PJC, Warner TD, Curzen NC. Prostaglandin E1 potentiates the effects of P2Y12 blockade on ADP-mediated platelet aggregation in vitro: Insights using short thromboelastography. Platelets 2015; **27**(6):689-92.

Khanna V, Shahzad A, Thayalasamy K, Kemp I, Mars C, Cooper R, Roome C, Wilson K, Harris S, Stables R, Curzen N. Comparison of the antiplatelet and anticoagulant effects of bivalirudin versus unfractionated heparin with short thrombelastography in patients undergoing primary percutaneous coronary intervention: results from a HEAT PPCI Platelet Substudy. (Submitted for publication)

Presentations

Curzen N, **Khanna V**, Shahzad A, Stables R. Antiplatelet and Antithrombotic effects during contemporary primary angioplasty for ST-elevation MI: Observations from the HEAT PPCI Platelet Substudy. Presented by Professor Nick Curzen as a late-breaking trial at a Hot Line session on Antithrombotic Strategies at the EuroPCR meeting in Paris May 2015.

Awards

Khanna V, Curzen N. Does Arachidonic acid induced platelet aggregation vary according to the vascular inflammatory status? Winners of the 2014 **Josephine Lansdell Research Grant** awarded by the British Medical Association.

Book chapter

Khanna V, Gershlick A, Curzen N. (2016) Current Status of Oral antiplatelet therapy in percutaneous coronary intervention. In: Redwood S, Curzen N, Thomas M eds. Oxford Textbook of Interventional Cardiology (Second edition). Oxford University Press, Oxford. (Submitted)

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DEDICATION

This thesis is dedicated to the loving memory of my dear friend Keith Nicholas who sadly passed away in April 2015. You were a kind soul, a true gentleman and my best friend. I couldn't have done this and so much else in my life without you. I am very grateful for all that you have helped me accomplish and for having had you as 'my friend'. I miss you and will always cherish the wonderful memories we shared together.

ABBREVIATIONS

AA	Arachidonic acid
AAA	Abdominal aortic aneurysm
AC	Adenylyl cyclase
ACS	Acute coronary syndrome
ACT	Activated clotting time
Act F	Activator F
ADP	Adenosine diphosphate
AMI	Acute myocardial infarction
APT	Antiplatelet therapy
ARU	Aspirin reaction units
AST	Acute stent thrombosis
AT	Antithrombin
ATP	Adenosine triphosphate
AUC15	Area under the curve at 15 minutes
BMI	Body mass index
BMS	Bare metal stent
CABG	Coronary artery bypass grafting surgery
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
COX	Cyclo-oxygenase
CRP	C-reactive protein
CVA	Cerebrovascular accident
CVD	Cardiovascular disease

DAPT	Dual antiplatelet therapy
DES	Drug-eluting stent
DM	Diabetes mellitus
DTI	Direct thrombin inhibitor
ELISA	Enzyme-linked immunosorbent assay
ESC	European Society of Cardiology
FDA	Food and Drug Administration
GP	Glycoprotein
GPCR	G-protein coupled receptor
GPI	Glycoprotein IIb/IIIa inhibitor
HIT	Heparin-induced thrombocytopenia
HPR	High on-treatment platelet reactivity
IDR	Ischaemia-driven revascularisation
IL-6	Interleukin-6
LMWH	Low molecular weight heparin
LTA	Light transmittance aggregometry
MA	Maximum amplitude
MACE	Major adverse cardiovascular events
MEA	Multiple electrode aggregometry
MI	Myocardial infarction
NSTEMI	Non-ST-elevation myocardial infarction
OTPR	On-treatment platelet reactivity
OCPR	On-clopidogrel platelet reactivity
OR	Odds ratio
PAD	Peripheral arterial disease

PAR	Protease-activated receptor
PCI	Percutaneous coronary intervention
PFA	Platelet function assay
PF4	Platelet factor 4
PGE1	Prostaglandin E1
PKC	Protein kinase C
PLC	Phospholipase C
PPCI	Primary percutaneous coronary intervention
PRU	P2Y12 reaction units
RCT	Randomised-controlled trial
sCD40L	Soluble CD40 ligand
ST	Stent thrombosis
STEMI	ST-elevation myocardial infarction
s-TEG	Short Thrombelastography
TF	Tissue factor
TG	Thrombin generation
TIA	Transient ischaemic attack
TMC	Thrombin-mediated clotting
TNF	Tumour necrosis factor
TVR	Target vessel revascularisation
TXA_2	Thromboxane A_2
UFH	Unfractionated heparin
VASP	Vasodilator-stimulated phosphoprotein
VN-P2Y12	VerifyNow P2Y12 assay
vWF	von Willebrand factor

CHAPTER 1: INTRODUCTION

Cardiovascular disease (CVD) is the dominant cause of mortality worldwide. In Europe alone, it is responsible for around 4 million deaths annually, thus accounting for almost half of all deaths[1]. However, over the last three decades there has been a substantial decline in mortality from CVD, particularly in rates of coronary heart disease (CHD) which have more than halved in several European countries since 1980[2]. This era has witnessed dramatic advances in therapeutic interventions including drug-eluting intracoronary stents as well as improved primary and secondary prevention strategies.

Antiplatelet agents are an important component in a range of proven therapies that have contributed significantly to lowering morbidity and improving survival in CVD. Several landmark studies have established the clinical importance of antiplatelet therapy (APT) in improving outcomes across the spectrum of CVD including coronary artery disease (CAD), acute coronary syndrome (ACS), ischaemic stroke and peripheral arterial disease (PAD). The efficacy of APT emphasises the pivotal role of platelets in the pathogenesis of atherothrombotic cardiovascular events including ACS where occlusive platelet-rich thrombi aggregate at sites of plaque erosion or rupture.

Aspirin remains the antiplatelet of choice in a CVD. However, certain high-risk populations such as ACS and those undergoing percutaneous coronary intervention (PCI) with stents derive incremental benefit from the addition of a second antiplatelet agent, usually a $P2Y_{12}$ inhibitor. The latter group deserve special consideration as, despite dual antiplatelet therapy (DAPT), patients receiving intracoronary stents remain vulnerable to the potentially fatal sequelae of stent thrombosis (ST). Consequently, this has been a driver for the development of more potent and rapidly acting $P2Y_{12}$ inhibitors, some of which are now incorporated into evidence based clinical practice guidelines.

Conventional antiplatelet regimes employed in PCI target key platelet enzymes and receptors. However, despite effective inhibition of periprocedural platelet function with DAPT, the risk of recurrent ischaemic events remains substantial as platelets are reactive to a multitude of other mediators, most notably thrombin, which is a dominant effector of the coagulation cascade and a potent platelet activator. Plaque rupture whether *de novo* or iatrogenic in the context of PCI results in generation of thrombin and ultimately thrombus formation. Anticoagulant therapy therefore continues to be an important

adjunct for reducing periprocedural ischaemic complications, particularly in patients undergoing primary PCI (PPCI) for ST-elevation myocardial infarction (STEMI). Nevertheless, the optimal periprocedural antithrombotic regimen in this setting remains to be established.

Over the last two decades there has been a vast expansion in the antithrombotic armamentarium at the disposal of interventional cardiologists and physicians alike. There is however, greater awareness that whilst more aggressive combinations of antiplatelet and anticoagulant agents may achieve better protection against ischaemic events, this inevitably increases the risk of bleeding complications, which are now the most common complications following PCI. The latter is no longer considered a benign nuisance based on studies that have reported that major bleeding is associated with significant in-hospital and long-term morbidity and mortality. The balance between protection from ischaemic events and bleeding complications remains delicately poised, but how this challenge can be overcome remains enigmatic.

Furthermore, numerous observational studies have reported wide variability in individual responses to APT, determined using a variety of platelet function assays (PFAs). There is compelling evidence linking "poor response" to the risk of recurrent ischaemic events. At the other end of the therapeutic spectrum there is increasing evidence that "enhanced responders" are more susceptible to bleeding complications. Measuring individual patient responses may therefore provide reassurance of an adequate therapeutic response in some individuals or could help guide further modifications of APT based on platelet function testing in others. In theory, personalisation of APT in this manner could improve clinical outcomes by reducing the risk of recurrent atherothrombotic events whilst preventing an excess of associated bleeding complications. Nevertheless, this remains to be proven and there are yet several unanswered questions with respect to the prescribing of APT in CVD: -

i) What is the optimal APT regimen and duration of treatment, particularly in patients undergoing PCI who remain at risk of ST?
ii) Can personalising APT based on measuring responses with platelet function testing improve clinical outcomes?
iii) What is the ideal PFA for measuring responses to APT, and what cutoffs should be used to define an "inadequate response"?
iv) In whom should responses to APT be monitored routinely in clinical practice?

v) Is there an optimal interval for platelet function testing or do responses to APT remain stable over time?
vi) How should therapy be altered in patients with a sub-therapeutic pharmacodynamic response to standard therapy?
vii) In light of new and more potent antiplatelet agents, does the role of aspirin become redundant?

Some of these themes will be discussed and explored further in this thesis in a variety of subjects including: (i) healthy volunteers, (ii) patients with CAD on aspirin monotherapy or DAPT, (ii) patients undergoing PPCI for STEMI, and (iv) patients with PAD undergoing major vascular surgery.

1.1 The role of platelets

Platelets were first recognised as normal constituents of circulating blood almost incidentally by Max Schlutze as early as 1865[3]. However, it wasn't until 1882 that Giulio Bizzozero described their morphology and made the critical observation that they adhere to damaged blood vessels at the site of injury where they clump together forming aggregates. Through carefully designed experiments he described vividly, *"Blood platelets, swept along by the blood stream, are held up at the damaged spot as soon as they arrive at it. At first one sees only 2 to 4 to 6 (platelets); very soon the number climbs to hundreds. Usually some white blood cells are held up amongst them. Little by little the volume increases and soon the thrombus fills the lumen of the blood vessel, and impedes the blood stream more and more"[3].* Since the pioneering work of Bizzozero there has been considerable progress in our understanding of platelet morphology, biology and production as well as an appreciation of their role in:

a) Haemostasis – when platelets form a haemostatic plug at the site of vessel injury to arrest bleeding

b) Thrombosis – where platelet activation and aggregation following rupture or erosion of an atherosclerotic plaque leads to the clinical manifestations of thrombotic events such as ACS or ischaemic stroke

c) Inflammation – which underlies the formation and extension of atherosclerotic plaques

1.1.1 Platelet Production

Platelets mature from megakaryocytes as anucleate cells that lack genomic DNA but contain megakaryocyte-derived messenger RNA (mRNA) and the translational machinery requisite for protein synthesis. They also contain mitochondria and several types of secretory granules, the contents of which are released upon activation. They are considerably heterogeneous in size ranging from 2.0 to 5.0 microns in diameter[4]. Platelet production is regulated by thrombopoietin, a glycoprotein synthesised in liver and kidneys. They are released from bone marrow at a rate of approximately 10^{11} (i.e. one hundred billion) a day, and have a lifespan of around 7- 10 days in circulation[5]. With a normal range of between 150 to 400 X 10^9 platelets per litre of blood, there are approximately one trillion platelets circulating at any one time in an average adult.

1.1.2 Platelets in Thrombosis and Haemostasis

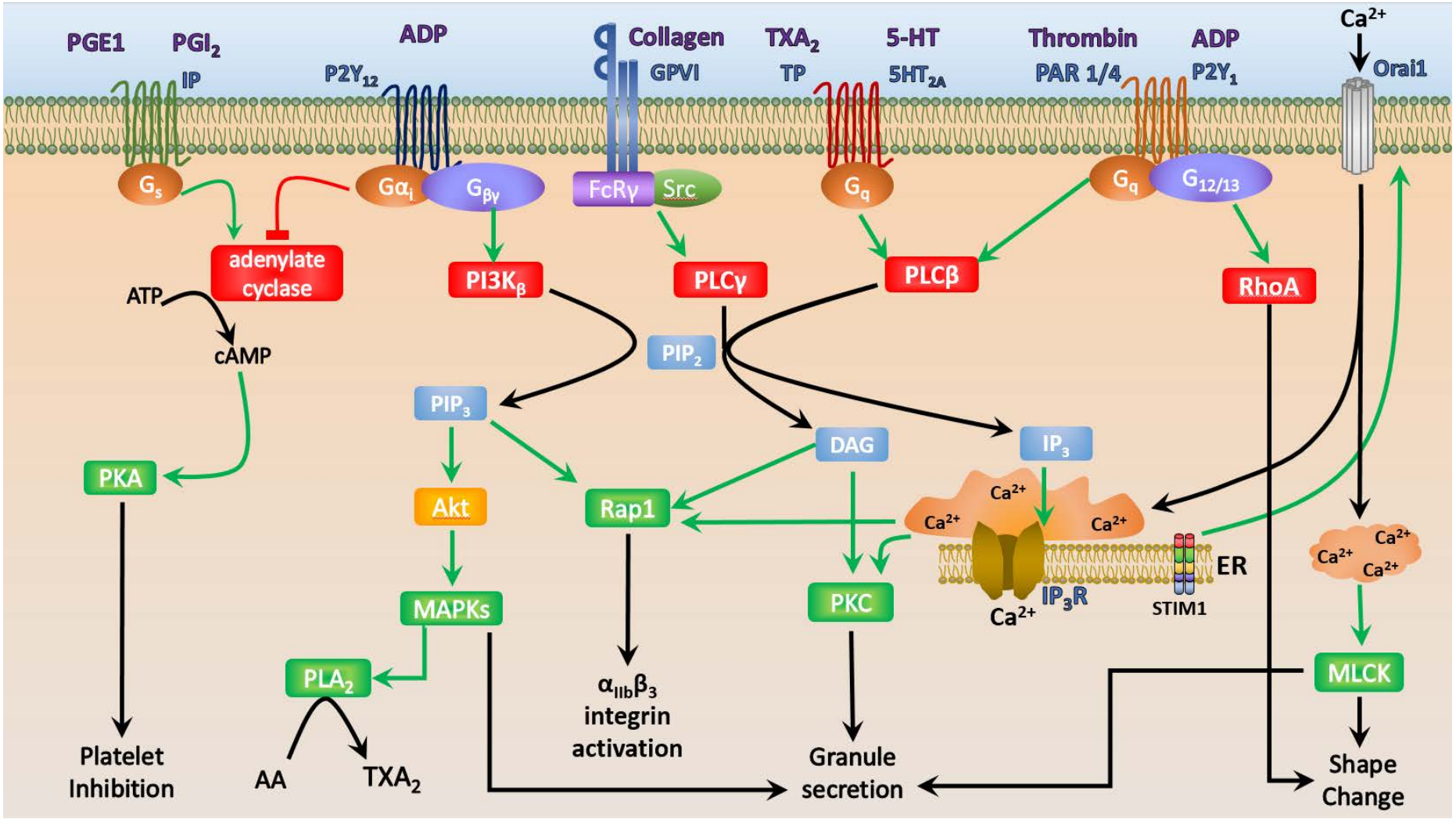
The pivotal role of platelets in haemostasis is emphasised by a bleeding diathesis observed in patients with thrombocytopenia and genetic thrombocytopathies. Under normal physiological conditions, circulating platelets remain quiescent and are discoid in shape owing to a highly specialised cytoskeletal structure. Within the circulation platelets are constantly exposed to endothelial-derived mediators including nitric oxide and prostacyclin that attenuate platelet reactivity at levels that prevent clotting. Nevertheless, upon detection of vascular injury, platelets anchor themselves on to the damaged vessel wall within seconds. Due to their small size, platelets typically concentrate adjacent to vessel wall during flow by a process known as "margination"[6]. The process through which rapidly circulating platelets slow down sufficiently to allow tethering to the damaged vessel wall under high shear forces is mediated by the highly thrombogenic constituents of the subendothelial matrix (von Willebrand factor (vWF), collagen, fibronectin, laminin) that become exposed when the endothelial layer is disrupted[7]. vWF is a large multimeric glycoprotein (GP) produced constitutively in endothelium (Weibel-Palade bodies) and megakaryocytes (α-granules). It is the major

ligand for one component (Ib) of the glycoprotein (GP) Ib/V/IX receptor complex. Based on this weak interaction collagen, the most reactive component of the extracellular matrix is also able to directly bind GPVI, an immunoglobulin receptor, on the platelet surface triggering a cascade of intracellular events mediated by calcium, resulting in platelet activation[8] (Figure 1.1).

Once adherent, a well characterised sequence of events leads to platelet activation and recruitment of further platelets. Platelet activation leads to a reorganisation of the actin cytoskeleton resulting in shape change from smooth biconcave discs to flat spiculated spheres with finger-like protrusions called filopodia extending from the plasma membrane[8]. These morphological changes permit firm anchoring of platelets to the vessel wall and adjacent platelets. Furthermore, translocation of negatively charged phospholipids to the outer platelet surface allows binding of clotting enzyme complexes catalyzing the conversion of inactive zymogens to serine proteases whose activity leads to a burst of thrombin generation via the coagulation cascade. Platelet shape change during activation results in significant growth in surface area available for this procoagulant activity[9].

Shape change is also accompanied by secretion of storage granules containing a vast array of vasoactive, pro-aggregatory, mitogenic and chemotactic substances. Platelets ordinarily have three major storage granules including lysosomes, dense granules and α-granules that play a pivotal role in the amplification of platelet activation. The latter are amongst the most abundant secretory organelles and contain a plethora of proteins including haemostatic factors (e.g. vWF, fibrinogen, Factor V), cytokines, proteases, growth factors and membrane markers of platelet activation (e.g. P-selectin, glycoprotein IIb/IIIa, platelet factor 4). By contrast, the morphologically distinct dense granules contain small molecules including, adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, histamine as well as ionised calcium. Besides degranulation, activation also results in *de novo* synthesis of thromboxane A_2 (TXA_2), a platelet activator and potent vasoconstrictor via the cyclooxygenase-1 (COX-1) pathway[10].

Activation of platelets results in the release of a variety of mediators including, TXA_2 and ADP which act in a paracrine or autocrine manner, creating feedback loops to amplify the initial platelet response and recruit further platelets to the site of injury. These diffusible mediators activate further platelets by binding G-protein–coupled receptors (GPCRs),



ADP *adenosine diphosphate;* ***FcRγ****, Fc receptor γ-chain;* ***5-HT****, 5-hydroxytryptamine or serotonin;* ***MLCK****, myosin light-chain kinase;* ***PAR-1/4****, protease-activated receptor 1 or 4;* ***PGE1****, prostaglandin E1;* ***PGI2****, prostacyclin;* ***TP****; thromboxane receptor.*

Figure 1.1 Summary of platelet receptor signalling pathways

Platelets are activated upon adhesion of extracellular matrix proteins like collagen to receptors GPIb-IX and GPVI, or by the action of soluble agonists on G-Protein coupled receptors (GPCRs) which activate an associated heterotrimeric G-protein through the exchange of a guanine diphosphate (GDP) for a guanine triphosphate (GTP). Agonist binding to GPCRs activates various effectors and their downstream signalling pathways, including: **(i)** the β isoform of phospholipase C (PLCβ) via Gqα, which generates inositol 1,4,5- triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2), causing an increase in cytosolic Ca^{2+} (initially through activation of inositol 1,4,5- triphosphate receptors on the endoplasmic reticulum (IP_3R) and secondarily through translocation of the calcium sensing stromal interaction molecule 1 (STIM1) to activate Orai1 calcium channels on the plasma membrane), and activation of protein kinase C (PKC); **(ii)** via G12α and G13α, reorganize the actin cytoskeleton and cellular machinery that drives platelet shape change through Ras homology gene family member A (**RhoA**), a small GTPase belonging to the Ras superfamily, that alternates between an inactive GDP bound state and an active GTP bound conformation through the action of specific guanine nucleotide exchange factors (GEFs); **(iii)** the activity of adenylate cyclase is either augmented via Gs or inhibited via Gαi, which otherwise causes platelet inhibition through the generation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP), resulting in the activation of protein kinase A (PKA); **(iv)** via Gβγ activation of the β-isoform of phosphoinositide 3-kinase (PI3K) results in the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). This results in $\alpha_{iib}\beta_3$ integrin activation through the generation of RAS-related protein 1 (Rap1), and granule secretion through generation of mitogen-activated protein kinase (MAPK) via the activation of Akt, a family of serine/threonine kinases. The latter MAPK pathway also results in the generation of thromboxane A_2 from the arachidonic acid (AA) pathway via the activity of phospholipase A_2 (PLA_2).

which triggers a cascade of inside-out signalling (Figure 1.1). Ultimately, this results in upregulation and conformational changes to the platelet integrin complex $\alpha_{IIb}\beta_3$, the final effector for platelet aggregation. These changes not only amplify the density of the $\alpha_{IIb}\beta_3$ integrin complex on the platelet surface, but also augment its affinity for adhesive substrates including the bivalent fibrinogen and multivalent vWF molecules that serve as bridges in binding to adjacent platelets.

The present schema of coagulation proposes three overlapping phases that lead to the generation of thrombin and subsequently fibrin: initiation, amplification, and propagation[11]. Coagulation is initiated by tissue factor (TF), a transmembrane glycoprotein constitutively expressed by vascular smooth muscle cells and fibroblasts. Exposure of TF to plasma following tissue injury allows it to form a complex with coagulation factor (F) VIIa, which causes activation of FX. The complex of FXa and FVa forms the "prothrombinase complex" that cleaves prothrombin to generate small quantities of thrombin[11]. In the "amplification phase", thrombin causes proteolysis of the GPCRs, protease-activated receptors (PAR) types 1 and 4 (Figure 1.1), resulting in platelet activation and secretion of FV and FXI from α-granules. Furthermore, thrombin activates components of the coagulation cascade, including FV, FVIII, FXI and IX, that set the stage for a burst of thrombin generation characteristic of the "propagation phase". In this final phase, FVIIIa/FIXa complex (termed "intrinsic tenase") and the prothrombinase complex assemble on the specialised negatively charged surface of activated platelets and accelerate the generation of FXa and thrombin, respectively[11]. Aside from causing platelet activation, an important function of thrombin includes the conversion of soluble fibrinogen to insoluble fibrin monomers, which polymerise to form fibrin strands. Moreover, it also activates FXIII that serves to stabilise the fibrin polymers to form a solid platelet fibrin meshwork that stabilises platelet aggregates[11].

In the event of vascular damage, concomitant activation of platelets and the coagulation cascade is therefore able to rapidly initiate formation of a haemostatic plug that arrests bleeding before tissue repair can commence. Haemostasis and thrombosis could be considered two sides of the same coin. Under normal physiological conditions, highly intricate regulatory mechanisms tightly control both thrombin generation and fibrin degradation (fibrinolysis) such that thrombus formation is contained both spatially and temporally. However, when these regulatory mechanisms are overwhelmed by uncontrolled pathological platelet aggregation and coagulation, thrombosis ensues,

leading to progressive luminal narrowing or thrombotic occlusion of the vessel. If sustained this can be potentially fatal or lead to ischaemia/infarction of the subtended vascular territory.

Atherosclerotic plaques with large lipid cores and thin fibrous caps are considered vulnerable to erosion or spontaneous rupture. Mechanical stress in the context of PCI is another important mechanism that can contribute to plaque instability. Once the endothelial layer is breached, the propensity for a developing clot to propagate is governed primarily by its relative composition of thrombogenic substrate. Plaques are well known to be heterogenous, and studies have shown that TF content appears to be a critical determinant of a plaque's thrombogenic potential[12].

1.1.3 Platelets in Atherogenesis and Inflammation

Endothelial denudation is no longer considered a necessary requisite for platelet adhesion and activation. Consequently, the prevailing dogma regards the role of platelets in the pathophysiology has evolved considerably. It has long been established that arterial thrombosis in the context of plaque rupture is orchestrated principally by platelet activation and aggregation. However, there is now greater appreciation that platelets are also important protagonists in the genesis of atherosclerotic plaques. For instance, in a murine model of atherosclerosis (apolipoprotein E knockout mice fed a western diet), platelet adhesion to regions of the carotid artery prone to atherosclerosis precedes both leukocyte adhesion and the formation of visible atherosclerotic lesions[13].

Normal healthy vascular endothelium prevents platelet activation and smooth muscle cell (SMC) proliferation through the production of prostacyclin and nitric oxide. Studies have shown that endothelial dysfunction represents a vascular phenotype that predisposes individuals to atherogenesis and future risk of cardiovascular events[14]. Inflammatory mediators and cytokines promote endothelial dysfunction. However, in the absence of overt atherosclerosis, baseline endothelial function is usually impaired in patients with cardiovascular risk factors. Inflammatory stimuli induce the translocation of Weibel-Palade bodies to the endothelial surface where vWF and P-selectin, the principle constituents of these storage granules, making them accessible to the circulation[15]. Consequently, the endothelial surface becomes more adhesive, thus allowing circulating

platelets to roll and tether themselves to activated endothelial cells, despite high shear stress prevalent in the arterial vasculature. Platelets firmly adhere to endothelial cells predominantly via the integrins GP $\alpha_{IIb}\beta_3$ and GP Ibα[16].

Adherent platelets undergo activation which results in conformational change as well as secretion of dense and alpha granules. Activated platelets themselves are a rich source of adhesive proteins, growth factors, mitogens, chemokines and cytokines. Amongst these is P-selectin (CD62P), a cell adhesion molecule, which is rapidly expressed on the surface following platelet activation. Binding to its ligand, P-selectin glycoprotein-1 (PSGL-1), which is constitutively expressed on leukocytes, facilitates the formation of platelet leukocyte aggregates, an important step in leukocyte transmigration into the vascular wall[16]. P-selectins are also pivotal in adhesion and migration of monocytes into the vessel intima where they differentiate into activated macrophages that take up lipoproteins to become lipid-laden foam cells, the hallmark of atherosclerotic lesions[16].

Another proinflammatory mediator released from activated platelets is CD40 ligand (CD40L), a trimeric transmembrane protein, which undergoes cleavage upon translocation to the platelet surface, generating a functional soluble fragment. Ligation of the CD40 receptor on endothelial cells by platelet-derived soluble CD40L results in production of reactive oxygen species and adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM-1) and E-selectin[17]. Moreover, it stimulates the release of chemokines and cytokines including interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) that attract neutrophils and monocytes[17]. The importance of CD40L in atherosclerosis is characterised by the finding that blockade of CD40L–CD40 signalling markedly inhibits atherosclerotic plaque formation and arterial lipid deposition in a murine model of hyperlipidaemia[18].

Platelet factor 4 (PF4; CXCL4) is the most abundant chemokine released from platelet α-granules. It inhibits proliferation, apoptosis and primes the differentiation of cell types both of the adaptive and innate immune system. There is a growing body of evidence which indicates that it plays a prominent role in atherosclerosis. In humans, PF4 was detected in early and late atherosclerotic lesions of the carotid artery, correlating with the histological and clinical severity of the disease[19]. Moreover, PF4 knockout mice (-/-), genetically engineered to be susceptible to atherosclerosis, had smaller atherosclerotic lesions compared to controls[20]. PF4 is thought to promote atherosclerosis by recruiting

and arresting monocytes at the lesion site, facilitating their differentiation into macrophages, inhibiting lipid catabolism and enhancing the uptake and esterification of oxidised lipoproteins into foam cells[21].

1.2 Purinergic Receptors

As described in Section 1.1.2, thrombosis is a dynamic processes mediated by the activity of various platelet agonists on membrane receptors either expressed constitutively or upregulated following platelet activation. Chief amongst these agonists is the purine, ADP, which in isolation is regarded as a weak agonist, inducing reversible aggregatory responses compared to more potent comparators including thrombin and collagen. However, ADP, when released at high concentrations from dense granules following platelet activation, constitutes an important secondary agonist, which plays a prominent role in the amplification of platelet responses induced by other agonists. Specifically, its spectrum of activity includes intraplatelet calcium elevation, TXA_2 synthesis, protein phosphorylation, shape change, granule secretion, activation of $\alpha_{IIb}\beta_3$, and aggregation. The ADP-induced signal is mediated by a family of purinergic receptors present in almost all mammalian tissues.

Presently, based on cloning, signal transduction and pharmacology, there are three distinct classes of purinergic receptors known as P1, P2X and P2Y receptors[22]. Four subtypes of the P1 receptor have been cloned all of which are coupled to G-proteins and preferentially bind adenosine. Conversely, the P2 receptor family preferentially binds adenosine triphosphate (ATP) amongst other purines (e.g. ADP) and pyrimidine nucleotides[23]. This family of receptors has been further subclassified into P2X ionotropic receptors of which seven subtypes have been isolated (i.e. $P2X_{1\text{-}7}$) and P2Y metabotropic G protein-coupled receptors (GPCRs) with eight subtypes cloned[23]. Human platelets are known to express three P2 receptors: $P2Y_1$, $P2Y_{12}$ and $P2X_1$ receptors on their surface.

1.2.1 $P2X_1$

$P2X_1$, is a ligand-gated cation channel found on platelets which causes a rapid influx of the divalent cation Ca^{2+}. Previously, $P2X_1$ was not believed to have a role in platelet

aggregation as it was wrongly considered an ADP receptor, though now it is established that it is activated by ATP only[24]. Moreover, its role in platelet function has been questioned because it rapidly desensitises and selective activation *in vitro* evokes a transient shape change without significant aggregation[25]. Studies have reported a synergy between ATP-driven $P2X_1$ and ADP-induced Ca^{2+} responses of P2Y receptors[26], whereby the latter is substantially attenuated following pharmacological inhibition of $P2X_1$ receptors[27]. $P2X_1$ has also been shown to participate in collagen[28] and shear-induced aggregation[29]. $P2X_1$-deficient mice show normal bleeding time but resistance to thromboembolism upon collagen-epinephrine injection. By contrast mice overexpressing this receptor display increased systemic thrombosis[30].

1.2.2 $P2Y_1$

The human $P2Y_1$ metabotropic receptor has the classic seven transmembrane domain structure of GPCRs. Radioligand binding studies have shown that human platelets express comparatively low (approximately 150) $P2Y_1$ receptor binding sites, which accounts for 20-30% of overall ADP binding sites on the platelet surface[31]. $P2Y_1$ is a $G\alpha_q$-coupled receptor linked to phospholipase C-β (PLCβ) which catalyzes the cleavage of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) to generate the second messengers inositol 1,4,5- triphosphate (IP_3) and diacylglycerol (DAG) (Figure 1.1)[32]. Whilst IP3 stimulates the mobilization of calcium (Ca^{2+}) from internal stores causing a rapid rise in intracellular Ca^{2+}, DAG causes direct protein kinase C (PKC) activation[32], which further regulates the function of other intracellular proteins through phosphorylation of their serine and threonine amino acid residues. Phosphorylation of myosin, a cytoskeleton regulatory protein, by myosin light chain kinase (MLCK) is an important mechanism for platelet shape change in response to $G\alpha_q$-mediated Ca^{2+}-mobilisation[33]. However, studies have shown that shape change in response to cellular agonists can also occur through activation of Rho kinase via $G_{12/13}$ mediated signalling, which can stimulate MLCK and myosin phosphorylation independently of $G\alpha_q$-mediated Ca^{2+}-mobilisation (Figure 1.1)[34].

$P2Y_1$ stimulation results in only weak and transient ADP-mediated aggregatory responses that are amplified and sustained by concomitant $P2Y_{12}$ activation[35]. Nevertheless, there is evidence that $P2Y_1$ activation is essential for complete expression of platelet responses to ADP stimulation. This is exemplified by the loss of intracellular Ca^{2+} mobilisation, absence

of platelet aggregation and shape change in response to ADP in $P2Y_1$ knockout mice. In addition to ADP, $P2Y_1$ also potentiates aggregation to other agonists such as collagen[36].

1.2.3 $P2Y_{12}$

A second ADP receptor, coupled to inhibition of adenylyl cyclase (AC) activity through activation of $G\alpha_{i2}$ was implicated from early studies[37], as $P2Y_1$ activation could not account for the aggregatory effects of ADP on its own. Nevertheless, the identity of this receptor known to be the molecular target of the efficacious antiplatelet agents, ticlopidine and clopidogrel, remained elusive until 2001 when the $P2Y_{12}$ receptor was cloned by Hollopeter *et al*[38]. Whilst pharmacologic inhibition and genetic deficiency of $P2Y_1$ can abrogate the calcium response to ADP, there is also evidence that the calcium responses evoked by $P2Y_1$ are potentiated and sustained by $P2Y_{12}$ dependent signalling pathways[39-41].

$P2Y_{12}$ is well known to be "negatively coupled" to adenylyl cyclase, which otherwise regulates platelet activity *in vivo* by the production of cyclic adenosine monophosphate (cAMP) (Figure 1.1). This pathway activates a cascade of protein kinase A-mediated phosphorylation events resulting in the inactivation of small G proteins of the Ras and Rho families, inhibition of Ca^{2+} mobilisation, and modulation of actin cytoskeleton dynamics (via phosphorylation of the vasodilator-stimulated phosphoprotein or VASP)[42]. As early as 1966, Kloeze *et* al had demonstrated that prostaglandin E1 (PGE1), an endogenous prostanoid, could inhibit ADP-mediated platelet aggregation[43]. This was later attributed to $G\alpha_s$ mediated stimulation of the AC-cAMP pathway[44]. Indeed, platelet cyclic nucleotides (e.g. cAMP and cGMP) interfere with all known platelet responses including release of Ca^{2+} from intracellular stores, degranulation, adhesion, cytoskeletal reorganisation, aggregation and apoptosis[45]. Inhibition of $P2Y_{12}$ would therefore be expected to potentiate the platelet inhibitory effects of endogenous prostanoids including nitric oxide, prostacyclin, and PGE1. Nevertheless, inhibition of adenylyl cyclase, with specific agents that do not stimulate $G\alpha_i$, is insufficient to cause platelet aggregation[46, 47]. A role for additional intracellular signalling pathways in ADP-mediated $\alpha_{IIb}\beta_3$ integrin activation and aggregation is therefore suggested.

Another intracellular pathway coupled to $G_{\beta\gamma}$ via $P2Y_{12}$, that plays an important role in

ADP-mediated platelet aggregation, involves the lipid kinases phosphoinositide 3-kinase (PI3K)[48, 49]. PI3K catalyses the phosphorylation of phosphoinositides on the inositol ring to produce the second messengers phosphoinositide (PI) (3,4,5)P3 and PI(3,4)P2, which regulate a broad range of platelet responses (Figure 1.1). Specifically, these include $P2Y_{12}$-induced TXA_2 generation[50] and activation of integrin $\alpha_{IIb}\beta_3$ via the small GTPase Rap1b, which is critical in platelet aggregation[51]. Moreover, Hardy *et al* demonstrated that $P2Y_{12}$-mediated activation of PI3K also contributes to sustaining cytosolic Ca^{2+} [40] via activation of phospholipase C[52], whereby Ca^{2+} signalling appears to represent a point of crosstalk between the two platelet ADP receptors (i.e. $P2Y_1$ and $P2Y_{12}$). $P2Y_{12}$ appears also to be important in regulation of DAG-mediated platelet signalling events[32], which is an important effector of the $G\alpha_q$ coupled $P2Y_1$ receptor.

Finally, the importance of $P2Y_{12}$ in sustaining aggregatory responses to ADP is exemplified by impaired secretion and aggregation in response to a range of platelet agonists including ADP, collagen, and thrombin in human platelets congenitally lacking $P2Y_{12}$ and knockout mice[53]. Bleeding time in these models was significantly prolonged, a finding also observed following pharmacological inhibition of the $P2Y_{12}$ receptor by specific antagonists[54].

1.3 Protease activated receptors

The serine protease thrombin is the most potent physiological agonist of human platelets and is critical not only in maintaining normal haemostasis but also in pathophysiology of atherothrombosis. Thrombin can evoke a wide array of platelet responses including shape change, granule secretion, synthesis and release of TXA_2, mobilisation of P-selectin, activation of GP $\alpha_{IIb}\beta_3$ and, ultimately, platelet aggregation. Most of the cellular effects of thrombin are initiated via activation of a family of GPCRs called protease-activated receptors (PARs).

The PAR1 receptor was the first member of this family to be identified[55] that all share a topology of seven transmembrane spanning alpha helices with four extracellular and intracellular domains. Thus far, four PARs including PAR1, PAR2, PAR3 and PAR4 have

been identified in the mammalian genome[56]. These receptors widely differ in their cellular expression, signalling, as well as their agonist specificity and affinity.

The specificity of thrombin interaction with PAR1 is markedly augmented by binding of the anion binding exosite 1 on thrombin to a domain on PAR1 that closely resembles the carboxyl terminus of the anticoagulant hirudin[57]. In contrast, studies have shown that PAR4 does not bind exosite 1 on thrombin[58]. This enzyme-substrate interaction between exosite 1 and PAR1 induces thrombin into a conformation that greatly enhances its proteolytic activity. Consequently, PAR1 is the dominant receptor for thrombin on human platelets that can cause platelet activation at subnanomolar concentrations, whereas, PAR4 is a lower affinity thrombin receptor that requires higher thrombin concentrations to achieve platelet activation[56]. Nevertheless, many favour the theory that PAR1 also serves as the cofactor for PAR4 by recruiting thrombin to the platelet surface and holding it in protease conformation[59].

PAR1 is activated when thrombin cleaves its N-terminal exodomain at a specific cleavage site between arginine residue at position 41 and serine residue at position 42[60]. This reveals a tethered ligand that forms an intramolecular complex with a ligand binging site on the receptor itself, thus activating heterotrimeric G-proteins coupled to it. The activation of $G_{12/13}$ by PAR1 and PAR4 activates Rho and Rho kinase, which phosphorylates and inhibits myosin light chain phosphatase, which in turn enhances myosin light chain phosphorylation and contraction of actin leading to rapid platelet shape change and granule release[56]. PAR1 and PAR4 also activate $G\alpha_q$ that is linked to phospholipase C-β (PLC-β), which hydrolyses the cleavage of membrane-associated PIP2 to produce IP3, which stimulates calcium mobilisation, and DAG, which activates PKC[56] (Figure 1.1). Neither PAR1 or PAR4 are coupled to $G\alpha_i$ in platelets but their activation does trigger the release of ADP from dense granules, an important secondary agonist in platelets, which activates the $G\alpha_i$ and $G_{\beta\gamma}$-coupled $P2Y_{12}$ receptor and downstream signalling pathways[56].

Several highly selective synthetic peptide agonists corresponding to the tethered ligand motif, generated by proteolytic cleavage, have been developed that can both bind and activate PARs independent of proteolysis or receptor cleavage. Thrombin receptor-activating peptide 6 (TRAP-6) is a potent PAR1 agonist that contains a six amino acid sequence corresponding to the new amino terminus of the cleaved thrombin PAR1

receptor[60]. These peptide agonists have been extremely useful experimentally not only in evaluating the effects of PAR activation *in vitro*, but also in the development of novel PAR1 antagonists that have been tested in phase III clinical trials[61, 62] and could represent an attractive therapeutic target for the prevention of atherothrombotic events.

1.4 Antiplatelet therapy

Advances in our understanding of thrombosis and haemostasis have provided an important foundation for identifying specific enzymes and receptors critical in pathways of platelet aggregation that can serve as important therapeutic targets for prevention of atherothrombotic events. In preceding decades, a range of novel antiplatelet agents have emerged and undergone extensive investigation. Large randomised controlled trials (RCTs) have confirmed the rational basis for APT in the prevention of adverse events in CVD. Appraisal of these landmark trials have informed the development of evidence-based guidelines, which recommend APT in a variety of clinical settings, including stable CAD, ACS, stroke, PAD and perhaps most importantly in PCI. Selection and duration of therapy with individual antiplatelet agents is governed by the clinical context and treatment strategy but broadly the therapeutic options include: -

i) Acetylsalicylic acid – aspirin

ii) Thienopyridines – ticlopidine, clopidogrel, prasugrel

iii) Reversible $P2Y_{12}$ inhibitors – ticagrelor, cangrelor, elinogrel

iv) Glycoprotein IIb/IIIa inhibitors – abciximab, tirofiban, eptifibatide

v) Phosphodiesterase inhibitors – dipyridamole, cilostazol

vi) PAR1 antagonists – vorapaxar, atopaxar

Below is a summary of the antiplatelet agents in widespread use, though importantly discussion of phosphodiesterase inhibitors and PAR1 antagonists has been omitted as these are outside the scope of this thesis.

1.4.1 Aspirin

Willow bark was used by Hippocrates to treat pain, fever and inflammation as far back as the fifth century BC. Salicin, the active ingredient of willow bark was isolated in the 1820's and acetylsalicylic acid (aspirin) was first synthesised in 1853. However, it wasn't until 1897 that scientists at Bayer Research Laboratories discovered that acetylation of salicylic acid made it more palatable and began marketing aspirin as an analgesic. Since discovery of its mechanism of action by Sir John Vane in 1971[63], aspirin has experienced a meteoric renaissance as an antiplatelet drug. Despite growing enthusiasm for emerging therapies, aspirin remains the most widely prescribed antiplatelet agent in CVD.

The platelet inhibitory effects of aspirin are attributable to irreversible acetylation of the serine residue at position 529 in cyclooxygenase-1 (COX-1)[64]. Consequently, the catalytic site on the enzyme (Tyrosine 385) cannot bind its substrate, arachidonic acid (AA), thereby preventing its conversion to prostaglandin H_2 (PGH_2)[64]. The latter is a precursor for prostanoid biosynthesis including thromboxane A_2 (TXA_2), a potent platelet activator and vasoconstrictor synthesised by the platelet-specific enzyme thromboxane synthase. Aspirin has a short half-life of only 15-20 minutes in systemic circulation. However, as aspirin irreversibly inhibits COX-1 and platelets lack the translational machinery to regenerate the enzyme, TXA_2-induced platelet aggregation is abolished for the lifetime of the platelet (usually 8 - 10 days)[65]. TXA_2 is highly unstable in aqueous solution and is rapidly converted to the more stable metabolite thromboxane B_2 (TXB_2).

There are two chemically distinct isoforms of the enzyme prostaglandin H_2 synthase (also known as COX-1 and -2) that are differentially expressed in humans. COX-1 is constitutively expressed in most tissues and serves important physiological functions in vascular haemostasis, gastric mucosal protection and maintenance of renal blood flow[64]. By contrast, the COX-2 isoform is barely detectable under physiological conditions though its expression can be rapidly induced by a variety of proinflammatory cytokines and growth factors[66]. Aspirin has ~166-fold greater activity against COX-1 than the COX-2[67]. By virtue of its differential selectivity for these isoenzymes, significantly higher doses of aspirin are required to suppress COX-2 activity sufficiently to achieve a therapeutic anti-inflammatory effect than those necessary for inhibiting platelet TXA_2 synthesis. Early studies demonstrated the inhibitory effects of aspirin on TXA_2 generation are almost

completely saturable in the dose range of 75 – 100 mg[68, 69].

RCTs support antiplatelet prophylaxis in secondary prevention of CVD. Specifically, the second instalment from the Antiplatelet Trialist's Collaboration, systematically reviewed 145 RCTs and reported that long-term aspirin reduced the cumulative incidence of serious vascular events such as vascular death, non-fatal MI and non-fatal stroke by about 25% across a broad category of patients with a history of arterial vascular disease[70]. Similarly, the landmark ISIS-2 study reported that daily aspirin given for a month reduced 5-week mortality by 23%, but given in addition to fibrinolytic therapy with streptokinase reduced mortality by 42% in patients presenting with acute myocardial infarction (AMI). A prospectively planned meta-analysis of two major trials [International Stroke Trial (IST) and Chinese Acute Stroke Trial (CAST)] evaluated in-hospital outcomes in 40,000 patients suspected of acute ischaemic stroke[71]. This showed a highly significant reduction of recurrent ischaemic stroke (1.6% Vs 2.3%, $p < 0.000001$) and modest reduction of death without further stroke (5.0% Vs 5.4%, $p = 0.05$) in favour of aspirin compared to control.

The benefits of aspirin in primary prevention of CVD are less convincing compared to CVD where the role of aspirin is well established. The Antithrombotic Trialists' Collaboration recently reviewed results from six primary prevention trials and showed aspirin significantly reduced non-fatal MI (0.18% Vs 0.23%, $p = 0.0001$) but had no significant effect on stroke (0.20% Vs 0.21%, $p = 0.4$) or vascular mortality (0.19% Vs 0.19%, $p = 0.7$)[72]. However, the incidence of major gastrointestinal and other extracranial bleeds was significantly higher in patients allocated to aspirin (0.10% Vs 0.07%, $p < 0.0001$). Presently, primary prevention trials do not support routine use of aspirin[73]. However, the benefits of aspirin may be greater than its hazards in certain populations at higher risk like patients with diabetes. Data in this cohort are conflicting thus far but this area of research will be informed by large ongoing studies[74, 75].

1.4.2 Thienopyridines

ADP released in large quantities from platelet dense granules upon platelet activation is an important secondary mediator crucial to the amplification of platelet responses to a variety of agonists. Ticlopidine, clopidogrel and prasugrel belong to the thienopyridine family of ADP-receptor antagonists, which act via irreversible inhibition of the platelet

$P2Y_{12}$ receptor. *In vitro,* these compounds have no antiplatelet activity, however, *in vivo* they undergo hepatic bioactivation by the cytochrome P450 enzyme system. The resulting active metabolites form disulphide bridges with the extracellular cysteine residues (Cys17 and Cys270) on the $P2Y_{12}$ receptor and thereby inactivate the receptor. The presence of a methoxycarbonyl group on clopidogrel and prasugrel molecules, affords them greater efficacy as well as a superior safety and tolerability profile compared with ticlopidine.

1.4.2.1 Ticlopidine

Historically, patients receiving intracoronary stents were co-administered anticoagulant regimens, including warfarin, of varying duration alongside aspirin to mitigate the risk of ST. Nonetheless, this hazard persisted despite intensive anticoagulation, invariably also resulting in excessive haemorrhagic complications. In the late 1990's several RCT's evaluated outcomes after stent implantation with a combination of aspirin and ticlopidine, a first generation thienopyridine. In the STARS study, the composite primary endpoint of death, MI, angiographically evident thrombosis, or target lesion revascularisation (TVR) at 30 days occurred in 3.6% randomised to aspirin monotherapy, 2.7% receiving combined aspirin-warfarin therapy, and 0.5% in patients assigned to combined aspirin-ticlopidine[76]. Indeed, DAPT with aspirin-ticlopidine was consistently superior to aspirin with anticoagulation in both medium-, mixed- (FANTASTIC and ISAR studies)[77, 78] and high-risk cohorts (MATTIS)[79] undergoing stent implantation. Furthermore, haemorrhagic complications were less common with DAPT in the ISAR, FANTASTIC and MATTIS studies and equivalent to aspirin-warfarin in STARS[76-79].

Unfortunately, compliance with ticlopidine was greatly limited by its poor tolerability due to side effects including rashes, diarrhoea, nausea, vomiting and abdominal pains. Further, haematological toxicity is the most important of its undesirable effects. Neutropenia occurred in 2.4%[80] and whilst marrow failure related to ticlopidine is mostly reversible, it can be complicated by life-threatening septicaemia. Rarely, ticlopidine causes aplastic anaemia which carries a high mortality[81]. In the CLASSICS study, 1,020 patients were randomised to either aspirin-ticlopidine or aspirin-clopidogrel for 28 days following successful stent implantation[82]. The primary endpoint, a composite of major bleeding, neutropenia, thrombocytopenia, or early discontinuation of study drug

occurred in 9.1% assigned to ticlopidine compared to 4.6% assigned to clopidogrel[82]. A meta-analysis of randomised and registry data from 13,955 patients demonstrated that compared to a reference of aspirin plus ticlopidine, a combination of aspirin and clopidogrel was associated with lower 30 day major adverse cardiac events (MACE) including all-cause mortality[80]. Owing to its improved efficacy and tolerability, clopidogrel has succeeded in entirely replacing ticlopidine as the default $P2Y_{12}$ inhibitor in clinical practice.

1.4.2.2 Clopidogrel

Clopidogrel, a second-generation thienopyridine, was granted approval from U.S. Food and Drug Administration (FDA) in 1997, based largely on the findings of the CAPRIE study which evaluated the long-term safety and efficacy of clopidogrel administration against aspirin monotherapy in a large cohort (19,185) of patients with a previous history of CVD[83]. This showed a reduction of 8.7% in the primary composite endpoint of ischaemic stroke, MI, or vascular death in favour of clopidogrel ($p = 0.043$), adding further impetus to the view that aspirin alone may not be sufficient for secondary prevention of ischaemic events in certain patient populations. A logical corollary was to evaluate the effect of concomitant aspirin with clopidogrel in populations at high risk of recurrent ischaemic events. This was first accomplished in the landmark CURE study, which randomised 12,562 patients presenting with NSTEMI within 24 hours to aspirin with either clopidogrel loading followed by maintenance or placebo[84]. The addition of clopidogrel reduced the composite primary endpoint of MI, stroke or cardiovascular death by 20% (absolute risk reduction of 2.1%) at 1 year compared to placebo ($p < 0.001$), though this was associated with more major but not fatal bleeding[84]. Analysis of data from a subgroup of patients treated with PCI similarly showed a 31% relative risk reduction in cardiovascular death and MI ($p = 0.002$) in the PCI-CURE Study[85].

The addition of clopidogrel (300 mg loading dose followed by 75 mg daily maintenance) to aspirin and fibrinolytic therapy in STEMI was subsequently evaluated in CLARITY-TIMI 28, a placebo-controlled study that enrolled 3,491 patients scheduled to undergo angiography during the index admission[86]. In addition to evidence of improved patency of the infarct-related artery (IRA) in the clopidogrel arm compared to placebo (Rate of occlusion in IRA: 15.0% Vs 21.7%, $p < 0.001$), this trial also showed a significant reduction

in cardiovascular death and ischaemic events at 30 days (11.6% Vs 14.1%, p = 0.03)[86]. The COMMIT study, another large trial of 45,852 STEMI patients demonstrated a 9% proportional reduction in death, reinfarction or stroke at 28 days (p = 0.002) in clopidogrel-treated patients compared to placebo. It is noteworthy that in both these trials there was no excess of major or minor bleeding in patients assigned to clopidogrel.

Perhaps the only study which failed to demonstrate overall benefit of concomitant aspirin and clopidogrel over aspirin monotherapy was the CHARISMA study which recruited 15,603 patients with known history of CVD or multiple risk factors[87]. Overall moderate bleeding was higher in the clopidogrel arm compared to placebo, but there was no increase in fatal bleeding according to the GUSTO criteria. Subgroup analysis showed a marginally significant reduction in the rates of MI, stroke and cardiovascular death in the symptomatic group. By contrast, primary efficacy event rates were numerically higher in the clopidogrel arm, though this did not reach levels of statistical significance[87].

These studies have informed clinical guideline committees who have given clopidogrel a class I recommendation for patients undergoing PCI for stable CAD, NSTEMI and STEMI.[88, 89]. In the latter group, however, the European Society of Cardiology (ESC) clinical guidelines recommend that clopidogrel is reserved for those where other potent therapies such as prasugrel or ticagrelor are either not available or contraindicated[88].

1.4.2.3 Prasugrel

Prasugrel is a novel third-generation thienopyridine, which provides more rapid, consistent and potent platelet inhibition compared to clopidogrel making it an attractive therapeutic alternative in coronary intervention[90]. These properties have been attributed to its rapid absorption and more efficient metabolism to a thiol-containing active metabolite, a process that is less reliant on the liver cytochrome P450 enzyme system compared to its predecessors[91]. Further to its superior pharmacological profile, a randomised, phase 2, dose-ranging study (JUMBO-TIMI 26) in 904 patients undergoing elective or urgent PCI showed there was no excess of haemorrhagic complications at 30 days compared to clopidogrel[92]. Subsequently, in the PRINCIPLE-TIMI 44 study patients undergoing cardiac catheterisation for planned PCI were randomised to prasugrel (60 mg loading dose followed by 10 mg maintenance) or high-dose clopidogrel (600 mg loading

dose followed by 150 mg daily maintenance)[93]. This showed that prasugrel achieved significantly greater inhibition of ADP-mediated platelet aggregation compared to high-dose clopidogrel at 6 hours and during the maintenance phase[93].

Inevitably, a large Phase III trial was conducted to adjudicate the remaining uncertainties about efficacy and safety of this novel thienopyridine in 13,608 moderate-high risk ACS patients scheduled for PCI[94]. The TRITON-TIMI 38 study demonstrated that prasugrel (60mg loading dose followed by 10mg maintenance dose) was associated with lower rates of cardiovascular death, non-fatal MI or stroke (9.9% Vs 12.1%, $p < 0.001$), as well as ST (1.1% Vs 2.4%, $p < 0.001$) at 15 months, compared to clopidogrel (300 mg loading dose and 5 mg maintenance dose). Correspondingly, prasugrel was also associated with higher rates of major bleeding (2.4% Vs 1.8%, $p = 0.03$) including fatal as well as non-fatal life-threatening bleeding. *Post hoc* analysis suggested that prasugrel is associated with no net clinical benefit or could even cause harm in subgroups of older patients (age ≥ 75 years), low body weight (< 60 kg) or prior history of stroke/transient ischaemic attack (TIA). Conversely, prasugrel conveyed greatest benefit in patients with diabetes and STEMI[94, 95], though importantly this benefit was not evident in a subset of patients undergoing primary PCI[95], now considered the optimal reperfusion strategy for STEMI.

Whilst encouraging there are several critiques of the trial design and findings reported by the TRITON-TIMI 38 study worthy of consideration. Firstly, contrary to contemporary practice patients assigned to clopidogrel received a loading dose of 300 mg, instead of 600 mg recommended by current clinical guidelines[88, 89]. Secondly, randomisation and subsequent administration of the study drug were delayed until the coronary anatomy was known, a design that would be expected to favour prasugrel given its rapid onset of action. Finally, the benefit of prasugrel in preventing ischaemic events when broken down to its individual components was predominantly driven by a 24% risk reduction in non-fatal MI. Whilst, ST accounts for a proportion of these events, composition of the remainder is poorly defined, though it probably includes a sizeable component of asymptomatic periprocedural MI, the significance of which remains unknown. Prasugrel given as part of DAPT for the medical management of ACS was compared to a clopidogrel-based regimen in the TRILOGY-ACS study which randomised 9,326 patients to either prasugrel 10 mg daily or clopidogrel 75 mg daily. A lower dose of prasugrel 5 mg daily was selected arbitrarily in patients with body weight < 60 kg or age ≥ 75 years as these were the subgroups that exhibited no net benefit with prasugrel 10mg in TRITON-TIMI 38.

In the overall population there was no significant difference in the rate of cardiovascular death, non-fatal MI or stroke at 30 months between the two treatment arms (18.7% clopidogrel Vs 20.3% prasugrel, p = 0.45). Reassuringly, there was also no evidence of harm as no excess of bleeding in prasugrel-treated patients was reported, thus vindicating low-dose prasugrel in certain high-risk subgroups. Nevertheless, widespread use of prasugrel in ACS populations remains limited by its relatively slow offset of action. This was evident in TRITON TIMI-38 study where prasugrel was associated with significantly higher rates of coronary artery bypass grafting surgery (CABG)-related major bleeding[94]. Discontinuation of prasugrel for at least 7 days is therefore recommended prior to surgery to minimise major bleeding complications[88]. The active metabolite of prasugrel has a half-life of 3.7 hours, however, like all thienopyridines prasugrel binds $P2Y_{12}$ receptors irreversibly, thereby preventing recovery of platelet function for the lifespan of the platelet.

1.4.3 Reversible $P2Y_{12}$ Inhibitors

Unlike thienopyridines, novel $P2Y_{12}$ antagonists such as ticagrelor, cangrelor and elinogrel have shorter half-lives, bind reversibly to the $P2Y_{12}$ receptor and do not require metabolic activation via the hepatic cytochrome P450 system, thus overcoming important limitations of thienopyridine therapy.

1.4.3.1 Ticagrelor

Ticagrelor (previously AZD6140), an oral, direct-acting and reversible $P2Y_{12}$ receptor antagonist belongs to a new class of drugs called cyclopentyl-triazolo-pyrimidines. It binds directly to the $P2Y_{12}$ receptor to a site distinct from that of ADP which may prevent conformation changes that lead to activation of the GPCR[96]. Unlike thienopyridines, ticagrelor does not require metabolic activation, though AR-C124910, its major metabolite is also a potent $P2Y_{12}$ receptor antagonist. The ONSET-OFFSET study, a randomised comparison of ticagrelor, clopidogrel or placebo with aspirin in 123 patients with stable CAD showed a greater proportion of patients achieved inhibition of platelet aggregation (IPA) in excess of 50% at 2 hours with ticagrelor than clopidogrel (98% Vs 31%, $p < 0.0001$)[97]. Ticagrelor also had a more rapid offset, achieving similar levels of IPA

at 3 days to those achieved at 5 days following discontinuation of clopidogrel.

DISPERSE-2, was a phase II, dose-ranging trial designed to evaluate the safety and tolerability of ticagrelor (twice daily 90 mg or 180 mg) against standard dose clopidogrel in patients presenting with NSTEMI. This showed that ticagrelor produces greater and more consistent inhibition of platelet aggregation compared to clopidogrel without a significant difference in rates of major bleeding over weeks[98, 99]. In addition, a propensity for ticagrelor to induce dyspnoea and ventricular pauses was highlighted, which may be compatible with higher levels of circulating adenosine due to reduced cellular uptake which has been consistently reported with ticagrelor[99].

The PLATO study was a multicentre, randomized, double-blind, phase III trial that compared ticagrelor (loading dose of 180 mg, followed by a dose of 90 mg twice daily) with clopidogrel (300 mg loading dose followed by 75 mg daily) in 18,624 patients admitted with a spectrum of ACS[100]. At 12 months the primary efficacy endpoint, a composite of cardiovascular death, MI or stroke was met by 9.8% assigned to ticagrelor compared to 11.7% in the clopidogrel arm ($p < 0.001$). In contrast to prasugrel, there was a clear mortality benefit associated with ticagrelor therapy, as evidenced by lower rates of vascular death (4.0% Vs 5.1%, $p = 0.001$). Furthermore, the rate of angiographically proven definite ST was lower in the ticagrelor group (1.3% Vs 1.9%, $p = 0.0009$). Similar to DISPERSE-2, there was no overall excess of major bleeding. However, non-CABG-related major bleeding was higher in the ticagrelor arm (4.5% Vs 3.8%, $p = 0.03$) compared to clopidogrel. This study also confirmed a greater burden of dyspnoea amongst ticagrelor-treated patients (13.8% Vs 7.8%, $p < 0.001$) necessitating discontinuation of study drug in 0.9%. In contrast to prasugrel, ticagrelor was superior to clopidogrel in preventing ischaemic events in ACS patients managed medically regardless of whether there was an intention to treat without revascularisation or not[101, 102]. However, as with prasugrel in TRITON-TIMI 38, ticagrelor failed to demonstrate benefit in patients undergoing PPCI for STEMI[103], a population in which early platelet inhibition is imperative particularly as time to reperfusion is consistently improving. Subsequent pharmacodynamic studies have suggested that both prasugrel and ticagrelor have a delayed onset of action in STEMI patients, whereby only one third to half the patients have therapeutic levels of platelet inhibition at 2 hours[104, 105]. Moreover, early prehospital administration of ticagrelor in patients with STEMI did not appear to improve pre-PCI coronary reperfusion in the ATLANTIC study[106]. The delayed onset of action of ticagrelor and negative results of this

trial may well be related to delayed absorption of $P2Y_{12}$ resulting from co-administration of morphine in a large proportion of the study cohort.

Ticagrelor was first granted approval for marketing by the European Medicines Agency (EMA) in 2010 and by the FDA in 2011, and has since been incorporated into a number of evidence-based guidelines for the management of ACS[88, 107, 108]. The envelope for patients in whom ticagrelor is indicated for the prevention of ischaemic events could widen further following recent publication of the PEGASUS-TIMI 54 study[109]. This was a large, multinational trial of 21,162 high-risk patients with a prior history of MI (1-3 years before enrolment) which aimed to evaluate the efficacy of aspirin and either standard-dose ticagrelor (i.e. 90 mg twice daily) or low-dose ticagrelor (i.e. 60 mg twice daily), against placebo. Both doses of ticagrelor were associated with significantly lower rates of CV death, MI or stroke compared to aspirin alone (90 mg: 7.9% Vs 9.0%, $p = 0.008$; 60 mg: 7.8% Vs 9.0%, $p = 0.004$) over a median follow up of 33 months. This benefit when broken down to its individual components appeared to be driven largely by a reduction in non-fatal MI though mortality related to CHD was also significantly lower in the group receiving ticagrelor 90mg. There was however a higher incidence of major bleeding in both intervention groups compared to aspirin alone ($p < 0.001$) though the incidence of fatal bleeding or non-fatal intracranial haemorrhage were similar in all arms of the trial. Long-term tolerability, in this otherwise stable population, was a substantial dilemma with a significantly greater proportion discontinuing ticagrelor on account of dyspnoea (90 mg: 6.5% Vs 0.79%, $p < 0.001$; 60 mg: 4.55% Vs 0.79%, $p < 0.001$) than previously observed in PLATO.

Landmark studies evaluating prasugrel as well as ticagrelor have demonstrated superior efficacy coupled with an excess of major bleeding compared to clopidogrel in ACS populations[94, 100]. However, the most pertinent question regards ticagrelor therapy that as yet remains unresolved is whether it is more efficacious and safer in patients with ACS compared to prasugrel. ISAR-REACT 5 is an ongoing open-label, multicentre randomised trial designed specifically to answer this question[110].

1.4.3.2 Cangrelor

Cangrelor is a potent intravenous ATP analogue, which directly and competitively inhibits

ADP binding to the $P2Y_{12}$ receptor. It has a rapid onset of action achieving extensive inhibition of platelet aggregation within 2 minutes as it is administered parenterally, and does not require hepatic bioactivation[111]. Furthermore, it has a short plasma half life of 2-5 minutes as it is rapidly deactivated by plasmatic ectonucleotidases[112]. An infusion is therefore required to maintain steady state platelet inhibition, which recovers rapidly to baseline levels within 60-90 minutes following cessation[111]. There is evidence of a competitive pharmacodynamic interaction between cangrelor and clopidogrel, such that the sustained platelet inhibitory effects of clopidogrel are attenuated when administered concomitantly with cangrelor but not when given upon termination of its infusion[113]. Given its short half-life, it is conceivable that the unstable active metabolite of clopidogrel is mostly eliminated before it can irreversibly bind to $P2Y_{12}$ receptors in the presence of cangrelor which has a greater affinity for the receptor.

The efficacy and safety of cangrelor in patients undergoing PCI for stable angina or ACS was initially evaluated in two large Phase III trials. Both trials randomised patients in a double blind, double dummy, placebo-controlled design to either cangrelor (bolus of 30 μg/kg and infusion of 4 μg/kg/min) or clopidogrel 600 mg. Clopidogrel was administered to both arms at the end of PCI in CHAMPION-PLATFORM[114]. By contrast, the comparator group received clopidogrel within 30 minutes prior to PCI in CHAMPION-PCI[115]. Both trials launched almost concurrently but had to be terminated prematurely on account of futility. Specifically, neither CHAMPION-PCI (7.5% Vs 7.1%, p =0.59) nor CHAMPION-PLATFORM (7.0% Vs 8.0%, p = 0.17) demonstrated superiority of cangrelor over placebo in reducing the rate of death, MI or ischaemia-driven revascularisation (IDR) at 48 hours. There was no excess of major bleeding with cangrelor, with the exception of a single bleeding scale (i.e. ACUITY criteria) in the CHAMPION-PLATFORM trial[114]. Despite early termination there was a suggestion of benefit with cangrelor based on a reduction in ST and death in CHAMPION-PLATFORM, two key prespecified secondary endpoints[114]. Furthermore, it was considered that a suboptimal definition of procedure-related MI may have contributed to the overall negative result of the CHAMPION programme.

The CHAMPION-PHOENIX study was therefore designed to definitively assess whether cangrelor reduces ischaemic complications following PCI in patients with stable CAD or ACS[116]. There were several similarities in design to earlier studies in the programme, but also subtle differences including adoption of the universal definition of MI, use of either clopidogrel 300 mg/600 mg in the comparator arm based on operator discretion, and

inclusion of ST in the composite primary efficacy endpoint. The trial enrolled 11,145 patients and showed a significant reduction in death, MI, IDR or ST at 48 hours with cangrelor compared to placebo (4.7% Vs 5.9%, p = 0.005), which remained significant at 30 days. This result was predominantly driven by a reduction in MI (3.8% Vs 4.7%, p = 0.02) and ST (0.8% Vs 1.4%, p = 0.01). Furthermore, there was no excess of major or minor non-CABG related bleeding reported in the cangrelor arm.

Discontinuation of APT for sufficient duration, usually 5-7 days for thienopyridines, is necessary for adequate recovery of platelet function to minimise haemorrhagic complications following CABG surgery. Apart from causing inevitable delays to surgery, patients, particularly those with DES, are exposed to risks of further ischaemic events following premature discontinuation of APT. A Phase II trial evaluated whether cangrelor could be used safely for bridging thienopyridine-treated patients to CABG[117]. The BRIDGE study recruited 210 patients with ACS or treated with an intracoronary stent awaiting inpatient CABG. Patients were randomised to either cangrelor or placebo infusion for at least 48 hours but no less than 7 days, whereby the infusion was discontinued 1-6 hours prior to surgery. A greater proportion of patients treated with cangrelor had adequately low levels of platelet inhibition throughout the duration of treatment compared with placebo (98.8% Vs 19.0%, p < 0.01). Furthermore, there were no significant differences in major bleeding between cangrelor and placebo, whether related to CABG or not.

Presently, cangrelor has received approval from the FDA and EMA as an adjunct in the setting of PCI but not as a bridge to bypass surgery. Larger studies powered for clinical outcomes such as bleeding are necessary to evaluate the latter indication. Further trials are also required to compare cangrelor against contemporary $P2Y_{12}$ inhibitors in the context of PCI, particularly PPCI.

1.4.3.3 Elinogrel

Elinogrel is the latest direct-acting, reversible $P2Y_{12}$ antagonist to come under scrutiny in a series of Phase II clinical trials. Its availability in dual formulations is an important distinguishing attribute allowing it to be administered both intravenously and orally. This potentially eliminates the possibility of competitive interactions with its oral formulation during transition from intravenous therapy. As there is no requirement for hepatic

transformation to an active metabolite, platelet inhibition is achieved rapidly within 15 minutes. Conversely, it has a short plasma half-life of 8-12 hours and binds to the $P2Y_{12}$ receptor reversibly allowing for complete reversal of its pharmacodynamic effect within 24 hours[118].

The safety, efficacy and tolerability of elinogrel in both formulations were evaluated in a randomised, double-blind, dose-ranging, Phase 2b trial[119]. INNOVATE-PCI randomised 652 patients undergoing non-urgent PCI to either elinogrel (80 mg or 120 mg IV followed by oral elinogrel 50, 100 or 150 mg twice daily) or clopidogrel (300 mg or 600 mg followed by 75 mg daily). There was an excess of TIMI combined bleeding with elinogrel though this was driven almost exclusively by an increase in bleeding requiring medical attention (BRMA) rather than bleeding episodes meeting the TIMI major or minor criteria. In addition, there was also an increased incidence of combined clinical efficacy endpoints including death, MI, stroke, urgent target vessel revascularisation (TVR), ST and glycoprotein IIb/IIIa inhibitor (GPI) bailout therapy. Consequently, the development of elinogrel has now been terminated.

1.4.4 Glycoprotein IIb/IIIa Inhibitors

Glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) is a heterodimeric integrin complex found on platelet cell membranes, formed through non-covalent, Ca^{2+}-dependent association of α_{IIb} and β_3 subunits[120]. Platelet activation leads to conformational changes that augment the receptor's affinity for its ligands, chiefly fibrinogen, which facilitates interplatelet bridging between adjacent platelets to form stable aggregates. GPIs exert their antiplatelet effects by targeting this receptor, thus effectively interfering with the final common pathway in platelet aggregation. Presently, three different parenteral GPIs are available for clinical use including abciximab, eptifibatide, and tirofiban.

Abciximab is the Fab fragment of a murine/human chimeric monoclonal mouse antibody directed against $\alpha_{IIb}\beta_3$ to which it binds avidly with high affinity[121]. It can therefore remain bound to the receptor for the life span of the platelet, even though in plasma it has a short half life and is eliminated via the reticuloendothelial system within minutes. Nevertheless, platelet aggregation typically returns to baseline levels within 48 hours[120]. Unlike small molecule GPIs (e.g. tirofiban, eptifibatide), abciximab is not renally excreted

and therefore does not require dose adjustment in renal insufficiency. Small molecule GPIs are much more specific but have lower affinity for $\alpha_{IIb}\beta_3$ than abciximab. They have a plasma half life of 1.5-2.5 hours leading to rapid dissipation of their platelet inhibitory effects following cessation[120]. It is recommended that platelet counts are monitored routinely following administration of GPIs, given the risk of thrombocytopenia associated with their use, particularly abciximab.

A large number of trials have investigated the use of GPI in coronary intervention, however a large proportion of these were performed with older devices (e.g. balloon angioplasty) or before DAPT with a $P2Y_{12}$ inhibitor became the standard of care. Consequently, in the era of modern interventional cardiology, there is no substantial evidence that supports routine use of GPIs in elective PCI. Specifically, in the ISAR-REACT study, abciximab did not reduce the primary efficacy endpoint (composite of death, MI and TVR) compared to placebo in 2,159 patients pre-treated with high loading dose of clopidogrel 600 mg[122]. Similarly, there was no evidence of benefit of abciximab over placebo in a population with diabetes recruited to the ISAR-SWEET study[123].

The observation that high-dose clopidogrel obviated the benefit of GPIs in elective stenting prompted a similar line of investigation in ACS populations. The ISAR-REACT 2 study randomised 2,022 clopidogrel pretreated patients with NSTEMI or UA undergoing PCI to either abciximab (0.25 mg/kg of body weight bolus, followed by a 0.125 µg/kg/min) or placebo[124]. This showed that abciximab was associated with a reduction in rates of death, MI or TVR compared to placebo at 30 days (8.9% Vs 11.9%, p = 0.03), though a *post hoc* subgroup analysis showed these benefits appeared to be confined to those with elevated cardiac biomarkers. Furthermore, as demonstrated in the EARLY ACS study there is no evidence to support early routine GPI compared to delayed provisional use at the time of PCI, especially as upstream GPI carried a higher bleeding risk[125].

In patients with STEMI, PPCI has emerged as the optimal reperfusion strategy. Clinical evidence supporting use of GPIs in PPCI comes mostly from randomised trials such as ADMIRAL[126], CADILLAC[127] and ACE[128] which may no longer reflect contemporary management trends. Over the years, management of these patients has evolved substantially with numerous technical advances in stent technology, broader implementation of strategies to reduce bleeding (e.g. radial access) as well as expansion in the pharmacopeia of $P2Y_{12}$ inhibitors and anticoagulants used as adjuncts in PPCI.

BRAVE-3 was the most recent study to evaluate upstream abciximab against placebo (i.e. unfractionated heparin 70 U/kg followed by placebo infusion for 12 hours) in a PPCI population pretreated with loading doses of aspirin 500 mg and clopidogrel 600 mg[129]. This showed no significant reduction in infarct size determined by Technetium-99m (^{99m}Tc) sestamibi before hospital discharge between abciximab and placebo. Moreover, there was no difference in the combined secondary endpoint of death, MI, stroke or urgent TVR at 30 days (5.0% Vs 3.8%, p = 0.40), though the study was not powered to evaluate clinical outcomes.

Amongst patients treated with PCI, several studies have established the benefits of GPIs, though these come at modestly increased risks of bleeding complications. Moreover, the questionable benefit of GPIs in the setting of clopidogrel pretreatment have restricted use of GPIs to a bailout strategy for managing complications of PCI (intraprocedural thrombus formation, slow flow, threatened vessel closure), a recommendation supported by evidence-based guidelines[88].

1.5 Anticoagulant Therapy

A hypercoagulable state exists in patients undergoing coronary intervention as a consequence of the vasoocclusive process itself, but is also related to the direct trauma and distal embolisation associated with mechanical revascularisation. Adjuvant anticoagulant pharmacotherapies are therefore imperative during PCI to prevent thrombotic complications. Anticoagulant agents can be generally classified as follows: -

1) Parenteral
    a) Indirect
        i) Thrombin and Factor Xa – unfractionated heparin (UFH), enoxaparin
        ii) Factor Xa – fondaparinux

b) Direct

i) Thrombin inhibitor – hirudin, lepirudin, desirudin, bivalirudin, argatroban

ii) Factor Xa inhibitor - otamixaban

2) Oral

a) Indirect - Warfarin

b) Direct

i) Thrombin inhibitor – dabigatran, ximelagatran

ii) Factor Xa – rivaroxaban, apixaban, edoxaban

Below is an outline of the anticoagulant agents in widespread use in the cardiac catheterisation laboratory. I have consciously excluded a number of emerging and existing therapies (e.g. direct factor Xa inhibitors, warfarin) as discussion of all these is beyond the scope of the work in this thesis.

1.5.1 Structure and pharmacology of Heparin(s)

Heparin is a naturally occurring glycosaminoglycan polymer of varying molecular weight that has been used for its anticoagulant properties in a variety of clinical settings since 1935. These properties are derived from a unique pentasaccharide sequence, which mediates its binding to antithrombin III (AT-III). This results in conformational changes to the otherwise quiescent AT-III that allow it to form complexes with serine proteases, most notably thrombin (Factor IIa) and Factor Xa. Inactivation of the latter requires only the binding of AT-III to heparin polymers containing the pentasaccharide sequence. Conversely, inhibition of thrombin requires a longer sequence (≥ 18 saccharides ~ 5,400 daltons) that allows the formation of a ternary complex between heparin, AT-III and thrombin. Heparin-mediated bridging between the inhibitor and enzyme, which

augments their interaction, can only occur with thrombin in the fluid phase (i.e. not when bound to fibrin clot). Heparin loses its high affinity for AT-III following complex formation, allowing it to dissociate and align itself with free circulating antithrombin molecules to amplify its anticoagulant effect[131].

1.5.1.1 Unfractionated Heparin

UFH is a heterogenous preparation of heparin molecules of varying size ranging from 3,000 to 30,000 daltons[132]. Typically, only about one third of these molecules in an administered dose have anticoagulant activity. Furthermore, UFH exhibits a biphasic mode of clearance involving a rapid saturable phase through uptake and depolymerisation by macrophages and endothelial cells as well as a slower phase via a renal mechanism that follows first order kinetics. Larger species are cleared more rapidly than low molecular weight moieties, resulting in a variable half life which is dependent on dose and molecular weight of the constituent molecules[132]. Finally, heparin has a propensity for binding plasma proteins which can limit its responsiveness, potentially to a greater extent in ACS where higher concentrations of acute phase reactants are in circulation[130]. In concert, these factors confer a highly unpredictable anticoagulant profile with UFH which requires serial monitoring and dose titration to maintain clotting inhibition in a therapeutic range. Additionally, thrombocytopenia is a known adverse reaction of heparin therapy which can be further complicated by arterial or venous thrombosis. Heparin-induced thrombocytopenia (HIT) is a syndrome characterised by formation of antibodies to the antigenic complex of heparin and PF-4, a protein released by activated platelets.

1.5.1.2 Enoxaparin

Enzymatic depolymerisation of heparin yields shorter heparin molecules of low molecular weight in the range 1,000 – 10,000 daltons, with only 25-50% having sufficient span to inactivate thrombin. Compared to its precursor (i.e. UFH), LMWH exhibits less non-specific binding to plasma proteins and has a longer half-life (Enoxaparin: 2 - 4 hours after IV injection and 3 - 6 hours following a subcutaneous injection) resulting in a more predictable anticoagulation profile. Consequently, enoxaparin can be administered on a

once or twice daily basis, obviating the need for routine monitoring. In any case, conventional assays for assessing efficacy of UFH such as ACT are redundant for monitoring response to LMWH as these are more sensitive to the inhibitory effects of heparin on thrombin activity rather than factor Xa. Moreover, specific anti-Xa assays are available that can measure factor Xa activity in LMWH-treated patients where necessary. LMWH is cleared principally by the renal route; hence dosing needs to be adjusted in renal insufficiency. LMWH is also associated with a lower incidence of HIT, possibly because of limited non-specific binding in comparison to UFH.

1.5.1.3 Fondaparinux

Fondaparinux is a synthetic pentasaccharide sequence (1,728 daltons) that selectively inhibits factor Xa by interacting with AT-III, thereby preventing thrombin generation, but not inhibiting thrombin directly. Unlike heparin, it does not non-specifically bind plasma proteins therefore yielding a predictable pharmacokinetic profile, and precluding the need for routine monitoring. Furthermore, as it does not bind PF- 4, HIT is unlikely to be a complication of its use though there is some anecdotal evidence to the contrary[133]. Fondaparinux, has a renal mechanism of clearance resulting in a dose-dependent half life of approximately 17 hours which permits a once daily dosing regimen that has to be adjusted in renal insufficiency[133].

1.5.2 Evidence for heparin

1.5.2.1 Unfractionated Heparin

The use of UFH as an adjunct in PCI is founded largely on historical practice rather than substantiated by evidence from large randomised placebo-controlled trials. Nevertheless, it has a class I recommendation in the latest European guidelines[88]. Despite numerous limitations anticoagulation with UFH continues to be the standard in PCI as it is inexpensive, easily monitored and rapidly reversible. Indeed, it is the reference against which emerging anticoagulant agents are assessed. In the context of coronary intervention, UFH is administered as a fixed dose or weight-adjusted bolus, with additional doses adjusted according to the measured activated clotting time (ACT). The

optimal dosing regimen for UFH or level of ACT for prevention of ischaemic complications without an excess of bleeding remains unclear. It is however recommended that a lower dose of UFH is used if a GPI is being administered concomitantly[88].

1.5.2.2 Low-molecular weight heparin versus unfractionated heparin

In the era of PCI with stents, STEEPLE was the first large randomised study to evaluate the safety of enoxaparin (0.5 or 0.75 mg/kg) compared to UFH adjusted for ACT in 3,528 patients undergoing elective PCI. This showed that enoxaparin 0.5 mg/kg caused a significant reduction in rates of non-CABG-related bleeding at 48 hours (5.9% Vs 8.5%, p = 0.01), which was not observed with the higher dose (i.e. 0.75 mg/kg)[134]. The SYNERGY study showed that enoxaparin (1 mg/kg given every 12 hours) was non-inferior compared to UFH (bolus followed by infusion adjusted to activated partial thromboplastin time [aPTT]) in 10,027 high-risk NSTEMI patients managed with an early invasive strategy[135]. The recent ATOLL trial compared intravenous enoxaparin (0.5 mg/kg) to UFH (bolus 70-100 U/kg without concurrent GPI or 50-70 U/kg with GPI) adjusted against ACT in 910 patients undergoing PPCI for STEMI[136]. This showed a neutral outcome in terms of the primary endpoint which was a composite of numerous ischaemic endpoints and bleeding. However, a composite of death, recurrent MI or urgent revascularisation at 30 days was a more familiar key secondary endpoint in the trial which was met in 7.0% treated with enoxaparin compared with 11.0% treated with UFH (p = 0.015) with no reported excess in bleeding complications. Consequently, enoxaparin has been upgraded to a class IIa indication in PPCI for STEMI[88].

1.5.2.3 Indirect Factor Xa inhibitor versus unfractionated heparin

The OASIS-5 study was a double-blind, double-dummy trial of 20,078 high-risk NSTEMI patients designed to demonstrate non-inferiority of fondaparinux (2.5 mg) to enoxaparin (1 mg/kg twice daily) in terms of ischaemic outcomes and superiority for safety outcomes[137]. The primary outcome, which was a composite of death, MI and non-refractory ischaemia, was similar in both arms at 9 days (5.8% Vs 5.7%) meeting the criteria for non-inferiority (p < 0.007). Furthermore, there was evidence of superiority compared to enoxaparin with respect to major bleeding which was significantly reduced

at 9 days with fondaparinux (4.1% Vs 2.2%, p < 0.001). In patients undergoing an invasive procedure, there was an excess of catheter thrombus formation in the fondaparinux arm, although this was significantly attenuated following use of open label UFH during PCI.

OASIS-6 was a randomised double blind, double-dummy study in 12,092 STEMI patients comparing fondaparinux (2.5 mg once daily) to either UFH or placebo depending on whether UFH was mandated as per the reperfusion therapy received[138]. Whilst the trial overall showed reduced rates of death or reinfarction at 30 days in the fondaparinux arm (7.4% Vs 8.9%, p = 0.003), there was a trend towards harm in the subgroup treated with PPCI (6.0% Vs 4.0%, p = 0.12). Moreover, there was also an increased rate of guiding catheter thrombosis (22 vs 0, p < 0.001) in this subgroup. Based on these findings fondaparinux is not recommended in the context of PPCI[88]. Furthermore, in other ACS intended for invasive management, patients treated with fondaparinux should receive and additional anticoagulant with activity against thrombin (e.g. UFH or enoxaparin) at the time of PCI[88]. However, fondaparinux is recommended in non-urgent situations where an early invasive strategy isn't imminent, based on its favourable efficacy/safety profile.

1.5.3 Direct Thrombin Inhibitors

The observation that salivary secretions from *Hirudo medicinalis* (medicinal leeches) prevent blood clotting in 1884 led to discovery of hirudin, a highly specific and potent inhibitor of thrombin[139]. Though initially extracted laboriously from leeches, it became readily available in late 1980's through recombinant DNA technology in chiefly two isoforms: lepirudin and desirudin. Early clinical trials comparing recombinant hirudin to heparin in ACS[140] and PCI[141, 142] showed greater efficacy but raised safety concerns over the increased risk of bleeding associated with these agents. Presently, lepirudin is only approved for prevention of thrombosis in HIT and desirudin is indicated for thromboprophylaxis in patients undergoing elective hip replacement surgery[143]. Discussion of the small molecular oral direct thrombin inhibitors including dabigatran, argatroban and ximelagatran is beyond the scope of this thesis. Below is a detailed discussion of bivalirudin, the only DTI that has approval in patients undergoing PCI.

1.5.3.1 Pharmacology of Bivalirudin

Bivalirudin, a synthetic analogue of hirudin, is a bivalent DTI that binds to two distinct regions in the thrombin molecule (i.e. active catalytic site and the fibrinogen binding site or exosite 2) forming a 1:1 stoichiometric complex with thrombin. It is a polypeptide of 20 amino acids comprising a peptide (D-Phe-Pro-Arg-Pro) directed against the thrombin active site linked via a tetraglycine spacer to a synthetic dodecapeptide analogue of the C-terminus of hirudin[144]. Once bound, thrombin is rendered inactive only transiently. Circulating proteases including thrombin itself cleave bivalirudin close to the N-terminus liberating the amino-terminal moiety from the active site, thereby allowing resumption of thrombin's catalytic activities[145]. Thereafter, the C-terminus remnant can be displaced by fibrinogen allowing alignment to the thrombin active site requisite for its conversion to fibrin. Proteolytic cleavage of bivalirudin in this manner accounts for 80% of its elimination, the remaining occurring via renal excretion[145]. If renal function is normal, bivalirudin has a short plasma half-life of ~ 25 minutes, allowing rapid reversal of its anticoagulant effect within ~1 hour of terminating the infusion. Coagulation tests including aPTT, ACT, international normalised ratio (INR) and thrombin time (TT) all increase in a dose-dependent manner following administration of bivalirudin[145].

1.5.3.2 Bivalirudin versus unfractionated heparin in PCI

Unlike heparin, bivalirudin has the theoretical advantage that it does not bind plasma proteins, is not neutralised by PF4 antibodies, exerts its effector activity on thrombin directly without need for cofactors and can bind thrombin in the fluid phase as well as circulating clot-bound thrombin. Several randomised trials have examined whether this superior pharmacological profile translates to a favourable risk benefit ratio with bivalirudin in a variety of PCI settings.

Stable and Unstable Angina

In the era of DAPT and stents, bivalirudin (0.75 mg/kg bolus followed by 1.75 mg/kg/hr infusion for the duration of the procedure) was compared to UFH (140 U/kg) in 4,570 patients undergoing PCI for stable and unstable angina as part of the ISAR-REACT 3

study[146]. Importantly, all patients were loaded with both aspirin and clopidogrel (600 mg) at least 2 hours preprocedure. This showed no significant difference between the two arms for the primary efficacy endpoint, a composite of death, MI or urgent TVR at 30 days (8.3% Vs 8.7%, p = 0.57). However, the incidence of major bleeding was significantly higher in the control group receiving UFH (3.1% Vs 4.6%, p = 0.008)[146].

Non ST-elevation Myocardial Infarction

The combination of UFH plus GPI became standard adjunctive pharmacotherapy in PCI following ISAR-REACT 2 which showed that this combination was superior to UFH alone in biomarker positive patients pretreated with clopidogrel[124]. Inevitably, bivalirudin became increasingly compared to a combination of UFH and GPI. The ISAR-REACT 4 study randomised 1,721 NSTEMI patients undergoing PCI to either bivalirudin (0.75 mg/kg bolus followed by 1.75 mg/kg/hr infusion for the duration of the procedure) or UFH (70 U/kg) and abciximab 0.25 mg/kg followed by 0.125 µg/kg/min infusion for 12 hours)[147]. This showed that compared to bivalirudin, UFH and abciximab had a similar rate of the primary endpoint (composite of death, large recurrent MI, urgent TVR or major bleeding at 30 days) but were associated with an increased risk of major bleeding (4.6% Vs 2.2%, p = 0.02).

Similarly, the ACUITY study enrolled 13,819 NSTEMI patients and randomised them to bivalirudin alone, bivalirudin plus GPI or heparin (UFH or enoxaparin) plus GPI in equal proportion[148]. Both bivalirudin plus GPI and bivalirudin alone were non-inferior to heparin plus GPI with respect to the primary endpoint, a composite of death, MI or unplanned revascularisation for ischaemia at 30 days. The incidence of major bleeding was similar in both groups receiving GPI (5.7% with heparin Vs 5.3% with bivalirudin, p = 0.93), but bivalirudin alone was associated with significantly lower rates of bleeding compared to heparin plus GPI (3.0% Vs 5.7%, p < 0.001)[148]. Consequently, European guidelines recommend bivalirudin as an alternative to UFH plus GPI during PCI (Class I recommendation)[88].

ST-elevation Myocardial Infarction

Theoretically, the superior pharmacological profile of bivalirudin would be expected to most benefit patients with thrombotic coronary occlusion presenting as STEMI. Several large randomised studies have investigated this theory, but their results are confounded by differential use of GPI as well as varying durations of bivalirudin infusion. Table 1.1 provides a summary of the trials that have evaluated bivalirudin in a STEMI population thus far.

The landmark HORIZONS-AMI study (Table 1.1) showed that bivalirudin was associated with a significant reduction in net clinical outcome, which was chiefly driven by a reduction in rates of non-CABG-related major bleeding at 30 days (9.2% Vs 12.1%, p = 0.005)[149]. To date this remains the only study to demonstrate a survival benefit both in terms of all-cause and cardiac mortality, though this analysis lacked adequate power. The trial did report higher rates of acute ST (AST) with bivalirudin within 24 hours (1.3 % Vs 0.3 %, p< 0.001)[149]. A key limitation of this trial was that UFH was administered before angiography in approximately two thirds of the group assigned bivalirudin. Moreover, the differential use of GPI in this trial may explain the excess of major bleeding reported in the UFH arm where GPI use was mandated.

Evolving trends in interventional cardiology including potent $P2Y_{12}$ inhibitors, transradial access and selective 'bailout' use of GPIs mean that the outcome of the HORIZONS-AMI trial may no longer reflect modern practices. The EUROMAX trial (Table 1.1) therefore aimed to evaluate the relative efficacy and safety of bivalirudin in comparison to heparin (UFH or enoxaparin) in a more contemporary setting which included pre-hospital initiation of treatment, selective use of GPIs at operator discretion and greater use of radial access[150]. Bivalirudin was associated with a significant reduction in the incidence of death or non-CABG-related major bleeding at 30 days, regardless of whether GPI was used on a provisional or routine basis in the heparin arm. Similar to HORIZONS-AMI, this benefit was driven by lower rates of major bleeding though once again bivalirudin was associated with a significantly higher rate of AST. Nevertheless, GPI use was extremely disparate despite it no longer being mandated in the heparin arm, thereby not providing a direct head-to-head comparison of the two anticoagulant agents.

Given the higher incidence of AST reported with bivalirudin in STEMI trials, the synergistic effects of combining bivalirudin with prasugrel were postulated to be attractive in this group and therefore compared to standard therapy with heparin plus clopidogrel in the BRAVE-4 study[151] (Table 1.1). Notably, the use of GPI was similarly low in both groups and restricted to bailout only. The results can be regarded as exploratory only because the trial was terminated prematurely due to slow recruitment. Nevertheless, there was no significant difference in overall net clinical outcome or bleeding between the two treatments arms.

Prolonged bivalirudin infusion following the index procedure was explored as a potential strategy to reduce the risk of AST with bivalirudin in the BRIGHT study[152] (Table 1.1). Once again, bivalirudin demonstrated a net clinical outcome benefit over heparin, regardless of whether GPI use was routine or provisional. However, similar to previous studies this benefit was driven almost exclusively by a reduction in the rates of bleeding. Rates of ST were generally low but essentially similar in each arm. The results therefore suggest that a prolonged infusion could potentially mitigate the increased risk of AST reported with bivalirudin in earlier trials, a theory which has biological plausibility. The trial, however, had key limitations including a mixed population (88% STEMI), high-dose of UFH (100 U/kg) in the heparin only arm which would be expected to potentiate bleeding, and use of a locally manufactured formulation of bivalirudin.

The HEAT-PPCI study was a single centre, open-label randomised study comparing bivalirudin to UFH in a PPCI population[153] (Table 1.1). Selective bailout use of GPI in both arms provided an opportunity for direct comparison of these anticoagulant agents in a head-to-head fashion without differential use of GPI confounding results. Uniquely, a strategy of delayed consent allowed consecutive enrolment of an unselected population, thereby reflecting real world practice. Moreover, widespread use of radial access and potent $P2Y_{12}$ inhibitors such as prasugrel and ticagrelor ensured the trial was reflective of contemporary trends in PPCI. Compared with bivalirudin, UFH was associated with a significantly reduced incidence of the primary endpoint, a composite of death, reinfarction, stroke and unplanned TVR at 28 days (8.7% Vs 5.7%, $p = 0.01$). This was largely driven by an excess of AST (2.9% Vs 0.9%, $p = 0.007$) and associated reinfarction (2.7% Vs 0.9%, $p = 0.004$) in the bivalirudin arm[153]. Furthermore, this benefit in favour of UFH did not come at the expense of increased major bleeding which was similar in both arms (3.5% Vs 3.1%, $p = 0.59$). These findings are entirely discordant with the existing

evidence base and may be explained by differential use of GPI and higher doses of heparin administered in earlier studies. Indeed, a recent meta-analysis of 15 randomised trials reported that bivalirudin was associated with a reduction in major bleeding when compared to high-dose heparin (≥ 100 units/kg), but importantly not when the dose of heparin was < 75 units/kg (OR 1.04; 95% CI 0.94 - 1.14, p = 0.46)[154]. Important limitations of the HEAT-PPCI trial include an open-label, single centre design though concerns over under dosing of bivalirudin have also been raised. Results from a platelet function substudy of the HEAT-PPCI study will be presented in section 6 of this thesis.

Based on concerns over AST consistently reported with bivalirudin in randomised trials the European guidelines have downgraded bivalirudin to a Class IIa recommendation in PPCI for STEMI[88].

Table 1.1 Randomised clinical trials of bivalirudin in STEMI populations

	HORIZONS-AMI	EUROMAX	BRAVE-4	HEAT-PPCI	BRIGHT
Centres	123	65	3	1	82
Patients (N)	3,602	2,218	548	1,812	2,194
Treatment arms	**Bivalirudin:** 0.75 mg/kg bolus followed by 1.75 mg/kg/hr infusion for the duration of the procedure and optional 0.25 mg/kg/hr after the procedure **Heparin + GPI:** UFH 60 U/kg plus Abciximab (12 hr) or Eptifibatide (12 – 18 hrs)	**Bivalirudin:** 0.75 mg/kg bolus followed by 1.75 mg/kg/hr infusion for the duration of the procedure **Heparin:** UFH 100 U/kg without a GPI or 60 U/kg with a GPI; enoxaparin 0.5 mg/kg bolus GPI selection at operator discretion	**Prasugrel + Bivalirudin:** 0.75 mg/kg bolus followed by 1.75 mg/kg/hr infusion for the duration of the procedure **Clopidogrel + Heparin:** UFH 70–100 U/kg GPI selection at operator discretion	**Bivalirudin:** 0.75 mg/kg bolus followed by 1.75 mg/kg/hr infusion for the duration of the procedure **Heparin arm:** UFH 70 U/kg Abciximab (12 hours)	**Bivalirudin:** 0.75 mg/kg bolus followed by 1.75 mg/kg/hr infusion for the duration of the procedure and for up to 4 hours after. This could be followed by a reduced dose infusion for up to 20 hours 0.2mg/kg/hr **Heparin:** UFH 100 U/kg without GPI or 60 U/kg with a GPI **Heparin + GPI:** UFH 60 U/kg + tirofiban
Glycoprotein IIb/IIIa use	**Bivalirudin:** bailout (~ 7.5%) **Heparin arm:** routine (100%)	Optional in both arms. **Bivalirudin**: 3.9% upstream and 7.9% bailout **Heparin arm:** 58.5% routine and 25.4%	**Bivalirudin:** bailout GPI (3.0%) **Heparin arm:** bailout GPI (6.1%)	Bailout GPI in both arms **Bivalirudin:** bailout **(**13.5%) **Heparin:** bailout (15.5%)	**Bivalirudin arm:** bailout (4.4%) **Heparin arm:** bailout (5.6%) **Heparin + GPI arm**: routine tirofiban (18 - 36 hrs) (100%)
MACE at 30 days	5.5% Vs 5.4% (p = 0.95)	6.0% Vs 5.5% (p = 0.64)	4.8% Vs 5.5% (p = 0.894) *Includes ST*	8.7% Vs 5.7% (p = 0.01)	5.0% Vs 5.8% Vs 4.9% (p = 0.74)
Major bleeding	4.9% Vs 8.3% (p < 0.001)	2.6% Vs 6.0% (p < 0 .001)	14.1% Vs 12.0% (p = 0.54)	3.5% Vs 3.1% (p = 0.59)	4.1% Vs 7.5% Vs 12.3% (p < 0.001)
Stent thrombosis	**All:** 2.5% Vs 1.9% (p = 0.3) **AST:** 1.3% Vs 0.3% (p < 0.001)	**Definite:** 1.6% Vs 0.5% (p = 0.02) **AST:**1.1% Vs 0.2% (p = 0.007)	**Definite:** 1.1% Vs 1.5% (p = 0.98)	**All:** 3.4% Vs 0.9% (p = 0.001) **AST:** 2.9% Vs 0.9% (p = 0.007)	**All:** 0.6% Vs 0.9% Vs 0.7% (p = 0.77) **AST:** 0.3% Vs 0.3% Vs 0.3%

1.6. Antiplatelet therapy response variability

There is abundant evidence supporting a strategy of DAPT in patients suspected of ACS or those undergoing PCI with stents (as discussed in Section 1.4), though the optimal interval for switching to aspirin monotherapy remains unknown. Despite DAPT, a significant proportion of patients experience recurrent arterial thrombotic events which has given rise to the concept of "clinical antiplatelet resistance". Strictly, such events represent "treatment failure", which is not uncommon particularly in disorders of multi-factorial aetiology, unless there is absolute evidence of "true resistance" based on lack of pharmacodynamic inhibitory effect. Laboratory tests of platelet function that specifically explore platelet activation pathways targeted by APT can be employed for studying the pharmacodynamic effects of these drugs *ex vivo*. Indeed, PFAs have been pivotal in phase I and II trials during development of novel $P2Y_{12}$ inhibitors. However, as yet, the clinical utility of platelet function testing for confirming the adequacy of APT remains controversial.

Gurbel *et al* were amongst the first to demonstrate that individual responses to loading doses of clopidogrel (i.e. 300mg) administered following successful stent implantation are subject to considerable interindividual variability[155]. Importantly, they also showed that platelet aggregation responses to ADP (a $P2Y_{12}$ receptor agonist) in patients given loading doses of clopidogrel are critically dependent on baseline platelet reactivity prior to drug administration. Similarly, in a mixed population of patients and healthy volunteers, Serebruany *et al* showed that ADP-mediated platelet aggregation following clopidogrel treatment followed a normal distribution[156]. Numerous studies have confirmed these observations and as such it is widely accepted that individual patient responses to clopidogrel are considerably heterogeneous even when high loading doses are administered[157].

1.6.1 Mechanism of Variability

Assuming patient compliance and adequate dosing, a number of comorbid conditions as well as baseline clinical and demographic factors have been identified that can influence patient responses to APT. Specifically, these include age, gender, body mass index (BMI), smoking, diabetes mellitus (DM), ACS, poor left ventricular function and renal failure[158-

[161]. Moreover, by virtue of being an inactive prodrug requiring hepatic bioactivation, systemic availability of clopidogrel's active metabolite can potentially be attenuated by drug-drug interactions and genetic polymorphisms of key enzymes involved in its metabolism. Several pharmacodynamic studies have demonstrated that concomitant administration of lipophilic statins such as atorvastatin[162], proton pump inhibitors such as omeprazole[163, 164], or dihydropyridine calcium channel blockers such as amlodipine[165] can attenuate the platelet inhibitory effects of clopidogrel. Nevertheless, an adverse effect of clopidogrel-drug interactions on cardiovascular endpoints has never been demonstrated in clinical trials[166, 167].

Similarly, studies have shown that loss-of function genetic polymorphisms (e.g. *CYP2C19*2*) relating to enzymes key in clopidogrel metabolism are associated with a reduced pharmacodynamic effect of clopidogrel and increased risk of ischaemic events including ST[168-172] in patients treated with PCI. However, this specific polymorphism only accounts for a small proportion (between 5 to 12%) of the observed variability in clopidogrel response when included with established discriminators of clopidogrel response such as diabetes and BMI in a multivariable linear regression analyses[168, 173]. This implicates a multifactorial aetiology in clopidogrel response variability, and some factors may as yet be elusive or poorly understood. Other genetic polymorphisms leading to gain-of-function correspond to increased enzyme activity, however, their association with bleeding complications is contentious. Overall, the clinical utility of genetic testing remains to be established, but a substudy of the PEGASUS PCI study showed evidence that phenotyping of clopidogrel response based on platelet function testing was a better predictor of adverse events including ST compared to genotyping[174].

1.6.2 High platelet reactivity

As previously discussed (Section 1.1), platelet activation and aggregation play a key role in the pathophysiology of atherothrombosis and the merits of targeting pathways of platelet activation with agents such as aspirin and clopidogrel are well established (Section 1.2). However, variable responses to APT inevitably yield a proportion of individuals in whom despite APT, platelets remain persistently reactive. This phenomenon is termed "high residual platelet reactivity" (HRPR) and its clinical sequelae have been studied extensively in the past decade. HRPR could result from either insufficient inhibition of normal

baseline platelet reactivity or from adequate platelet inhibition of highly reactive platelets at baseline. An assessment of baseline platelet reactivity prior to initiation of treatment would therefore be necessary to differentiate between the two, which is often not logistically feasible in clinical scenarios such as ACS.

Earlier studies described response to APT in terms of the absolute difference between agonist-mediated platelet aggregation after treatment compared to a pretreatment baseline, whereby a <10% difference was considered representative of inadequate response[155]. However, using this schema Gurbel *et al* demonstrated that patients with low baseline platelet reactivity will have persistently low platelet reactivity regardless of whether their response to APT is adequate or not[175]. Furthermore, those with high baseline platelet reactivity are more likely to have persistently high platelet reactivity post treatment despite having an adequate response to APT thereby potentially underestimating risk of thrombotic events. It has therefore been proposed that an absolute measure of platelet reactivity during treatment (i.e. on-treatment platelet reactivity or OTPR) may be a more appropriate parameter for assessing response to APT.

The incidence of high on-treatment platelet reactivity (or HPR) in the literature varies widely between 0.5 to 65% in aspirin-treated patients[176, 177] and is around 25% in clopidogrel-treated patients though rates of up to 50% have been reported in some series[178, 179]. The variability in the reported incidence of HPR is likely the result of disparities in methodology between assays, cutoffs used to define HPR, study populations and the time interval at which platelet function testing is performed.

1.7 Platelet function testing

Historically, PFAs were reserved for the diagnosis of inherited or acquired haemorrhagic or clotting disorders. In recent times they have also found a role in the management of perioperative haemostasis. However, intercalated with the development of novel antiplatelet agents in recent decades, the role of platelet function testing has evolved to include assessment of response to APT in both clinical and research settings. The principles of each test and their potential advantages and limitations are discussed below:

1.7.1 Bleeding Time

Bleeding time, described by Duke in 1910, was the first test of *in vivo* platelet function[180]. It involves measurement of time to cessation of bleeding from a superficial forearm incision, a process requiring platelets for formation of a primary haemostatic plug. Despite modifications to standardise the test it remains of limited clinical utility as it lacks specificity for platelet function and can lead to scar formation.

1.7.2 Light Transmittance Aggregometry

The study of platelet function was revolutionised by the invention of light transmittance aggregometry (LTA) in the early 1960s[181], which is still considered the "gold standard" PFA. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) are obtained from citrated whole blood (WB) by a two-step centrifugation process. The optical density of PRP, a turbid suspension of cells, changes through formation of platelet aggregates following stimulation with a panel of agonists including ADP, AA, collagen and thrombin. A range of parameters including maximal platelet aggregation (MPA) can thus be measured normally at 5-10 minutes. Light transmittance through unstimulated PRP is used as a surrogate baseline (i.e. 0%) whereas PPP serves as an internal control that defines maximum aggregation (i.e. 100%). There are numerous observational studies that have reported an association between HPR with clopidogrel using LTA and adverse clinical outcomes post-PCI[182-186]. Nevertheless, the assay is not standardised and subject to considerable methodological variability which predictably results in poor reproducibility between laboratories. Moreover, there are other significant limitations that prohibit routine application of LTA in the assessment of responses to APT. Specifically, the assay is costly, labour intensive, time consuming and requires large sample volumes as well as a skilled operator adept at performing the assay and interpreting its results. Finally, and importantly, the assay dose not simulate normal physiology as it is performed under static conditions in isolated platelets rather than in whole blood.

1.7.3 Flow Cytometry

Flow cytometry is a technique that can be used to evaluate platelet activation. Prior to analysis, platelets are labelled with a fluorescently conjugated monoclonal antibodies. The suspension of cells in a blood sample are then aligned in single file and transported through a flow chamber by a process known as 'hydrodynamic focusing'[187]. A focused laser beam is directed at the sample stream, whereby the scattered fluorescence emitted by the particle-laser beam interaction is measured by photodetectors that amplify the output to generate a discernible electrical signal.

Platelet activation results in conformational changes to the $\alpha_{IIb}\beta_3$ integrin complex, and the monoclonal antibody PAC-1 selectively binds to an epitope on this receptor in its activated state[188]. Similarly, expression of P-selectin (CD62P) on the platelet surface increases following degranulation of α-granules as a result of platelet activation, thus allowing binding to leucocytes via P-selectin glycoprotein ligand-1 (PSGL-1). Platelet-leucocyte aggregates can be measured readily by flow cytometry and are a more sensitive marker of platelet activation than P-selectin expression[189]. Studies have shown that ADP-mediated platelet activation as measured by flow cytometry can be used to monitor response to $P2Y_{12}$ blockade[190-192].

The process of cell permeabilisation can be used to study intracellular antigens with flow cytometry[193]. VASP is an intracellular platelet protein which is not phosphorylated in its basal state. PGE1 stimulates phosphorylation of VASP via the adenylyl cyclase/cAMP pathway. By contrast, ADP binding to the $P2Y_{12}$ receptor inhibits this pathway, an effect which is blocked by $P2Y_{12}$ inhibitors. A platelet reactivity index (PRI), expressed as a percentage, can be derived by comparing VASP phosphorylation in whole blood with PGE1 alone and in combination with ADP. In this manner assessment of VASP phosphorylation can be used to monitor the efficacy of $P2Y_{12}$ inhibitor. A high PRI has been associated with adverse cardiovascular events in patients undergoing PCI[194, 195]. Moreover, there are several studies that have also demonstrated an association between low platelet reactivity (LPR) and bleeding complications in patients undergoing PCI[196-198].

The advantages of flow cytometry include low sample volumes and the use of whole blood making the assay more physiological. However, similar to LTA, the assay is expensive, poorly standardised and involves complex sample preparation therefore necessitating an experienced technician to perform and interpret results.

1.7.4 Impedance Aggregometry

Impedance aggregometry, or whole blood aggregometry (WBA) measures the shift in electrical impedance occurring in response to agonist-mediated platelet adhesion to the surface of two sensor electrodes immersed in a sample of diluted whole blood. The Multiplate analyser is a fully automated, computerised device that uses disposable cuvettes/electrodes in five test channels to evaluate platelet aggregation to a variety of agonists. Aside from the standard ADPtest which contains only ADP as reagent, a high-sensitivity test (ADPHS) is also available that includes PGE1 in addition to ADP based on the premise that this makes the assay more specific for evaluating $P2Y_{12}$ blockade by mitigating the contribution of the $P2Y_1$ receptor to ADP-mediated platelet aggregation. Whole blood anticoagulated with hirudin is diluted with saline pre-warmed to 37°C, before aggregation is initiated by a platelet agonist. The increase in impedance is graphically illustrated as arbitrary aggregation units (AU) plotted against time (AU*min) for six minutes. The most valuable parameter, area under the aggregation curve (AUC) is derived and recorded as units (U). The assay can be performed easily and rapidly without need for sample preparation making it a genuine POC assay.

Multiplate has been shown to be sensitive to the platelet inhibitory effects of APT and has moderate to good correlation with LTA[199-201]. Several large observational studies have reported an association between HPR with MEA and risk of recurrent ischaemic events following PCI including periprocedural MI and ST[202-205]. By contrast, there is also evidence that LPR as determined by the Multiplate analyser is associated with a greater risk of major bleeding in patients undergoing PCI[203, 204]. Furthermore, studies have shown that platelet function testing with the MEA ADPtest may have utility in identifying patients at risk of postoperative bleeding in cardiac surgery[206].

1.7.5 VerifyNow

VerifyNow by Accumetrics is a turbidimetric whole blood assay specifically developed to rapidly assess response to APT using a closed and fully automated system thereby obviating the need for sample handling. Blood is collected in a vacuette containing sodium citrate and incubated for the assay-specific recommended period. The vacuette is

then inserted in a single-use disposable cartridge containing a lyophilised preparation of human fibrinogen-coated beads, a platelet agonist, buffer and preservative. Activated platelets interact with the fibrinogen-coated beads in the mixing chamber causing agglutination, thereby increasing light absorbance which is measured in less than 5 minutes by an automated detector.

The Aspirin assay contains AA (1mM) as the agonist and reports results as aspirin reaction units (ARU). According to the manufacturer an ARU > 550 can be used to define aspirin resistance, as this threshold had a sensitivity of 91.4% and specificity of 100% for discriminating between patients who had or had not ingested aspirin (manufacturer packet insert). Moreover, this cutoff has been shown to be an independent predictor of adverse clinical outcomes post-PCI[207, 208].

In accordance with the VASP phosphorylation assay and Multiplate ADPHS test, the VerifyNow P2Y12 assay (VN-P2Y12) also employs PGE1 (PGE1, 22 nM) in addition to ADP (20 μM). In principle, PGE1 is purported to suppress the contribution of the $P2Y_1$ receptor to ADP–mediated platelet aggregation, thereby providing a pure assessment of the effect of $P2Y_{12}$ receptor inhibition. Results are expressed as P2Y12 reaction units (PRU) whereby higher numerical values represent platelet reactivity at the higher end of the spectrum. There are discrepant data in regard to the ideal "cutoff" value that optimally predicts adverse clinical outcomes post-PCI. Nevertheless, there is an abundance of observational data reporting a consistent association between HPR with clopidogrel and adverse clinical outcomes in populations undergoing PCI[182, 209-213]. Overall studies have shown moderate to good correlation between LTA and VN-P2Y12[214-217]. Nevertheless, several studies have reported a divergence from linearity at extremes of platelet inhibition whereby VN-P2Y12 has diminished sensitivity compared to LTA, particularly at high levels of platelet inhibition[218-221].

1.7.6 The Platelet Function Analyzer – PFA-100/Innovance PFA-200

PFA-100 is a whole blood assay developed in the 1990s as an *in vitro* surrogate for bleeding time, which has been updated in recent times to the Innovance PFA-200 system. A small volume of anticoagulated blood is aspirated into a capillary thus simulating arterial flow and through a microscopic aperture coated with either collagen and ADP

(CADP cartridge) or epinephrine (CEPI), thus simulating focal vascular injury. The time taken for platelet adhesion and clot formation under high shear stress to occlude the orifice is defined as the closure time (CT), which extends to a maximum of 300 seconds. A key limitation of the assay is that it remains sensitive to several variables including platelet count, haematocrit and levels of circulating vWF. Both cartridges are insensitive to the platelet inhibitory effects of $P2Y_{12}$ receptor blockers. A newly developed cartridge containing a smaller aperture coated with ADP, PGE1 and calcium (Innovance P2Y) has been shown to be sensitive to the platelet inhibitory effects of $P2Y_{12}$ inhibitors[222] though there is no evidence of prognostic utility for determining patients at risk of MACE in the clinical context of PCI[182]. There is however evidence that a short CT with CEPI assay in aspirin-treated patients is an independent predictor of MACE in patients with CVD[223] and those undergoing PCI for AMI[224]. However, the cutoff aperture CTs used to define aspirin low response vary significantly between individual studies signifying the need for better standardisation of this assay[225].

1.7.7 Biomarkers of thromboxane metabolism

As previously discussed (Section 1.4.1), TXA_2 is the principle product of AA metabolism via the COX-1 pathway resulting in platelet activation, recruitment of additional platelets to the site of vascular injury and potent vasoconstriction. It is, however, rapidly hydrolysed to TXB_2, a biologically inert and stable compound, of which only a small proportion is excreted unchanged in urine. Instead, it is predominantly metabolised to one of two major metabolites including 2,3-dinor-TXB_2 or 11-dehydro-TXB_2. It is possible to quantify the concentration of these metabolites in serum or urine by radioimmunoassay, enzyme-linked immunoassay (ELISA) or mass spectrometry.

Studies have shown that up to 30% of urinary TXB_2 metabolites are derived from extra-platelet sources[226], and this contribution may be further exacerbated in inflammatory disorders. Nevertheless, assays measuring TXB_2 remain the gold standard biochemical index for evaluation of aspirin response as they are highly sensitive and specific for the AA/COX-1 pathway targeted by aspirin. Moreover, there is evidence that an elevated TXB_2 levels are associated with adverse cardiovascular events in aspirin-treated patients at high risk of cardiovascular events[227] and those undergoing cardiac catheterisation[228]. However, there is poor correlation between assays measuring TXB_2 synthesis with those

evaluating the functional effects of aspirin on agonist-mediated clotting *ex vivo*[229-234]. Indeed, methodological variances in assays may explain the wide variability in the reported prevalence of aspirin resistance, which ranges from 0 - 67%[235-238]. Nevertheless, it is generally accepted that true aspirin resistance is uncommon with methods specifically evaluating the COX-1 pathway.

1.7.8 Thrombelastography

Thrombelastography (TEG) provides a global assessment of haemostatic function, including the contribution of all components of clotting including platelets, fibrin, clotting factors, and thrombin. The basic principle of TEG involves a stationary pin on a torsion wire being suspended into an oscillating cup in which whole blood is stimulated by agonists. As the cup rotates the torque generated is transmitted to the pin with progressively greater amplitude as the clot forms and stabilises. The resulting pin motion generates an electrical signal which is graphically illustrated as a TEG trace plotted against time which can be used to derive a complete profile of clot initiation, kinetics, strength and stability as well as fibrinolysis. Modifications to the standard TEG assay (called Modified TEG or TEG platelet mapping) eliminate thrombin activity, which obscures the effects of weaker agonists such as AA or ADP, by anticoagulation with heparin. With this and other modifications (discussed in Section 1.11.1), TEG has become more specific for assessing platelet function and sensitive to the platelet inhibitory effects of APT. In this regard TEG has shown strong correlation[239-242] and good agreement with LTA[242, 243], the historical "gold standard" methodology. Moreover, the assay has been shown to be reliable with good reproducibility[244, 245]. Recently, a new modification to TEG platelet mapping, known as Short TEG (s-TEG), has been developed and validated by this group in Southampton that allows a more rapid assessment of response to APT within 15 minutes[245, 246]. Nevertheless, the equipment necessary for TEG is bulky as a result of which it is not easily portable to the patient's bedside. Furthermore, the need for skilled operators and sample preparation has further precluded its wider application as a true POC device.

1.7.9 Summary

Despite better standardisation of assay methodology recently, there is generally moderate to poor correlation and agreement amongst assays of platelet function[201, 229, 230, 233, 247, 248], which means they cannot be used interchangeably. These discrepancies are thought to be a related to methodological variances amongst assays which commonly use different surrogates for aggregation (e.g. light transmittance, viscoelasticity, electrical impedance), different anticoagulants to preserve the sample, and different sample types (e.g. platelet rich plasma, whole blood etc). There is no overall consensus on which assay represents the optimal methodology for assessing patient responses to APT. The research studies in this thesis universally employ TEG for the assessment of platelet function. The original TEG methodology and its modifications that led to the development of s-TEG and its validation in CVD will be covered in depth in Section 1.11.

1.8 Platelet Reactivity Predicts Atherothrombotic Events

The consistent association between HPR and recurrent atherothrombotic events in patients with ACS, stable CAD, PCI, ischaemic stroke and PAD offer a persuasive argument for the "platelet function hypothesis". The evidence base supporting this adverse association is described in detail below.

1.8.1 Ischaemic Stroke

Studies have consistently reported high rates of aspirin resistance ranging from 14% to over 60% in patients presenting with ischaemic stroke[249-253]. Moreover, there is also evidence of an association between aspirin resistance and adverse clinical outcomes in this population. Grotemeyer *et al* assessed the prognostic utility of measuring predischarge platelet reactivity in a stroke population of 180 aspirin-treated patients. Over a follow up period of 24 months, the incidence of recurrent stroke, MI or vascular death was significantly higher in those with HPR on aspirin (33% of cohort) compared to those with normal platelet reactivity (40% Vs 4.4%, $p < 0.0001$)[252]. Jeon *et al* measured

platelet reactivity with the VerifyNow Aspirin assay in a stroke population of 117 aspirin-treated patients and evaluated early recurrent ischaemic lesions (ERILs) with diffusion-weighted imaging at one week. HPR on aspirin was reported in 13.7% of the cohort and this was independently associated with ERILs outside the vascular territories of index stroke (OR 6.01; 95% CI 1.29–28.09; p = 0.023)[249]. Finally, based on the TEG platelet mapping assay, Englyst *et al* reported an incidence of HPR on aspirin of 67% in a cohort of patients admitted with ischaemic stroke. This was independently associated with stroke severity and was more common in the context of lacunar strokes than embolic strokes[250].

The clinical sequelae of high on-clopidogrel platelet reactivity (OCPR) have not been extensively investigated previously in stroke populations. However, a recent study by Qiu *et al* evaluated markers of platelet activation including PAC-1, P-selectin and CD63 using flow cytometry in 198 clopidogrel-treated patients presenting with acute ischaemic stroke. The incidence of high OCPR varied considerably from 25.8% - 56.1% and it was independently associated with poor prognosis (i.e. Score > 2 on the modified Rankin Scale) and ischaemic events (a composite of non-fatal MI, non-fatal ischaemic stroke/TIA, and vascular death) at 12 months[254].

1.8.2 Peripheral Arterial Disease

Muller *et al* studied platelet function with WBA in 100 aspirin-treated patients undergoing elective peripheral balloon angioplasty for intermittent claudication. A low response to aspirin was noted in 60% of male patients and this was a predictor of vessel reocclusion at 18 months[255]. Similarly, Zeigler *et al* evaluated responses to APT with PFA-100 in 98 patients with undergoing percutaneous transluminal angioplasty (PTA) for PAD following treatment with aspirin and/or clopidogrel. The risk of complications during a 12 month follow up post-PTA was greater in nonresponders to clopidogrel compared to responders (55% Vs 13%).

Pastromas *et al* used VN-P2Y12 to assess platelet reactivity in 113 patients on long-term clopidogrel therapy for at least 3 months following infrainguinal PTA or stenting for intermittent claudication or critical limb ischaemia[256]. Clopidogrel non response was reported in almost 54% of patients in whom the cumulative TLR rate was significantly greater compared to clopidogrel responders (71.2% Vs 31.8%, p < 0.001). Furthermore,

TLR-free survival at ≤ 7 years was superior in responders than nonresponders (20.7% Vs 1.9%, p = 0.001). The same group measured preprocedure platelet reactivity in 100 clopidogrel-treated patients undergoing peripheral endovascular procedures for femoropopliteal PAD. Based on a receiver-operating characteristics curve analysis, a PRU > 234 was identified as the optimal cutoff to predict occurrence of the primary outcome measure, a composite of death, major stroke, major amputation, TVR or infrainguinal bypass surgery at 1 year (AUC: 0.88; 95% CI: 0.81 - 0.95; p < 0.0001). Patients with platelet reactivity above this threshold had an almost 17-fold greater risk of adverse events (HR: 16.9, 95% CI: 5 to 55, p < 0.0001)[257].

1.8.3 Stable Coronary Artery Disease

In a substudy of the HOPE trial, Eikelboom *et al* assessed aspirin response as the ability of aspirin to adequately supress urinary 11-dehydro TXB_2 levels in 976 aspirin-treated patients at high risk of cardiovascular events[227]. They observed a 1.8 times higher risk of the primary endpoint a composite of MI, stroke, or cardiovascular death, and a 3.5 times higher risk of cardiovascular death, in the highest quartile of urinary 11-dehydro TXB_2 compared to the lowest quartile. Gum *et al* studied functional aspirin resistance by measuring ADP- and AA-mediated platelet aggregation with LTA in 326 aspirin-treated patients[258]. The incidence of aspirin resistance was 5.2% in the study population and this was an independent predictor for the composite endpoint of death, MI or stroke (adjusted HR 4.14, 95% CI 1.42 – 12.06, p = 0.009) over a mean follow up of 679 +/- 185 days. Similarly, Chen *et al* evaluated aspirin response with the VerifyNow Aspirin assay in 468 aspirin-treated patients with stable CAD. They observed aspirin resistance in 27.4% of the study population and these patients were at significantly greater risk of the composite outcome of cardiovascular death, ACS, and stroke/TIA compared to the aspirin sensitive cohort (HR 3.12, 95% CI 1.65-5.91, p < 0 .001) over a mean follow up of 379 ± 200 days[207].

1.8.4 Percutaneous Coronary Intervention

A summary of selected studies evaluating the association between HPR and adverse clinical events in patients undergoing PCI following ACS, or for stable CAD is outlined in.

Table 1.2 Selected studies evaluating the association between high platelet reactivity and adverse ischaemic events in patients undergoing PCI

Source	Population	Assay (agonist)	Key clinical events (HPR *Vs* No HPR)
Geisler *et al* 2010[259]	**1,019** PCI patients (50.4% SA; 49.6% ACS)	**LTA** (20 µM ADP) at least 6 hours after LD	MACE at 3 months: 9.1% *Vs* 4.2 % (**adjusted OR 2.2**; 95% CI: 1.3 – 3.7; p = 0.002)
Breet *et al* 2010[182]	**1,069** elective PCI patients	**LTA** (20 µM ADP) at least 4 hours after LD	MACE at 1 year: 12.0% *Vs* 6.2% (**OR 2.05**; 95% CI: 1.3 – 3.2; p = 0.001)
Hochholzer *et al* 2006[260]	**802** elective PCI patients	**LTA** (5 µM ADP) at least 2 hours after LD	MACE at 30 days: 3.3% *Vs* 0.5% (**adjusted OR 9.6**; 95% CI 2.1 – 44.3; p = 0.004)
Parodi *et al* 2011[186]	**1,789** ACS patients undergoing PCI	**LTA** (10 µM ADP) 12-18 hours after LD	MACE at 2 years: 14.6% *Vs* 8.7% (**adjusted HR 1.49**; 95%CI 1.08 – 2.05; p = 0.02)
Buonamici *et al* 2007[261]	**804** patients (34% SA; 66% ACS) treated with DES	**LTA** (10 µM ADP) 12-18 hours after LD	ST at 6 months: 8.6% *Vs* 2.3% (**HR 3.08**; 95% CI 1.3 – 7.2; p = 0.009)
Price *et al* 2008[212]	**380** patients (93.7% SA or ischaemia) treated with DES	**VN $P2Y_{12}$ assay** (20 µM ADP + 22nM PGE1) at least 12 hours after LD	MACE at 6 months: 6.5% *Vs* 1.0%; (**OR 7.2**; 1.5 – 35.2; p = 0.008)
Marcucci *et al* 2009[209]	**683** ACS patients undergoing PCI	**VN $P2Y_{12}$ assay** (20 µM ADP + 22nM PGE1) within 24 hours of LD	MACE at 12 months: 13.2% *Vs* 4.7% (**HR 3.6**; 95% CI 1.5 – 9.1; p = 0.005)
Breet *et al* 2010[182]	**1,052** elective PCI patients	**VN $P2Y_{12}$ assay** (20 µM ADP + 22 nM PGE1) at least 4 hours after LD	MACE at 1 year: 13.3% *Vs* 5.7% (**OR 2.53**; 95% CI 1.6 – 3.9; p = 0.005)
Stone *et al* 2013[213]	**8,583** patients (48.3% SA; 51.7% ACS) treated with DES	**VN $P2Y_{12}$ assay** (20 µM ADP + 22 nM PGE1) at 6 / 12 hours after LD (600 mg / 300 mg)	ST at 1 year**:** 1.3% *Vs* 0.5% (**adjusted HR 2.49**; 95% CI 1.4 – 4.3; p = 0.001)
Sibbing *et al* 2009[262]	**1,608** patients (66.9% SA; 33.1% ACS) treated with DES	**MEA** (6.4 µM ADP) 4 [2 – 15] hours after LD	Definite ST at 30 days: 2.2% *Vs* 0.2% (**OR 9.40**; 95% CI 3.1 – 28.4; p < 0.0001)
Siller-Matula *et al* 2012[174]	**416** PCI patients (66% SA; 34% ACS)	**MEA** (6.4 µM ADP + 9.4 PGE1) at least 2 hours after LD	ST at 1 year**:** 12.5 *Vs* 0.3% (**adjusted OR 36.9**; 95% CI 4.3 – 319; p < 0.001)
Blindt *et al* 2007[263]	**99** ACS patients undergoing PCI	**VASP** (10 µM ADP + PGE1) 72-96 hours after LD	ST at 6 months: **adjusted OR 1.16**; 95% CI 1.01 – 1.33; p = 0 .04
El Ghannudi *et al* 2010[194]	**461** PCI patients (24.9% SA or ischaemia; 75.1% ACS)	**VASP** (10 µM ADP + PGE1) at least 6 hours after LD	Cardiac death at 9 ± 2 months: 7.9% *Vs* 2.2% (**adjusted HR 4.0**; 95% CI 1.1 – 14.8; p = 0.037)
Gurbel *et al* 2010[264]	**225** undergoing elective PCI	**TEG platelet mapping** (2 µM ADP) at 18-24 hours after LD	MACE at 3 years: **adjusted HR 10.9**; 95% CI 5.6 – 21.3; p < 0.001
Bliden *et al* 2007[240]	**100** elective PCI patients on chronic clopidogrel	**TEG platelet mapping** (2 µM ADP) pre-PCI	MACE at 1 year: **adjusted OR** 26.8; 95% CI 6.7 – 107.5; p < 0.001
Tang *et al* 2015[265]	**789** PCI patients (32.5% SA; 61.6%)	**TEG platelet mapping** (2 µM ADP) at least 6 hours after using clopidogrel	MACE at 1 year: 6.7% *Vs* 2.6% (**adjusted HR 2.6**; 95% CI 1.3 – 5.3; p = 0.005)

1.8.4.1 Elective PCI

Using the VerifyNow Aspirin assay, Chen *et al* reported aspirin resistance in almost one-fifth of their study population comprising 151 patients scheduled for elective PCI. The incidence of post-PCI myonecrosis was significantly greater in patients with aspirin resistance compared to the aspirin sensitive cohort[208]. Myonecrosis as defined by an elevation in cardiac biomarkers (e.g. troponin or creatinine kinase MB isoform) occurs commonly following PCI, likely from side branch occlusion or distal microembolisation. This has been associated with a higher risk of adverse clinical outcomes including mortality in some studies[266, 267] though the threshold predictive of adverse prognosis remains poorly defined.

Gurbel *et al* evaluated clopidogrel response with LTA preprocedure in 297 patients undergoing elective PCI and showed that HPR to ADP almost quadrupled the risk of post-PCI ischaemic events (OR 3.8; 95% CI 1.9 - 7.9, $p < 0.001$) over a 2 year follow up[183]. In the EXCELSIOR study, Hochholzer *et al* assessed platelet reactivity with LTA in 802 patients loaded with clopidogrel 600 mg immediately before elective PCI and observed higher rates of MACE in those in the highest quartiles of ADP-induced platelet aggregation compared to those in the lowest quartiles[260]. Whilst HPR on aspirin and clopidogrel individually have both been shown to have prognostic utility, there is growing acceptance that patients with a low response to both agents are at yet greater risk of post-PCI ischaemic events. Using the VerifyNow assay, Breet *et al* showed that dual non-response to APT with aspirin and clopidogrel was observed in 14.7% and carried the highest risk of MACE at 1 year in 422 patients undergoing elective PCI[268].

1.8.4.2 Acute Coronary Syndrome

Using the VN-P2Y12 assay, Marcucci *et al* evaluated OCPR in 683 patients loaded with clopidogrel undergoing PCI for ACS. Clopidogrel low response, defined in this study as a PRU ≥ 240, was an independent predictor of cardiovascular death (HR 2.55; 95% CI 1.08 to 6.07; $p = 0.03$) and non-fatal MI (HR 3.36; 95% CI 1.49 to 7.58; $p = 0.004$) at 1 year[209]. Similarly, Parodi *et al* assessed OCPR with LTA in 1,789 patients undergoing PCI for ACS and showed that HPR was associated with a higher risk of post-PCI thrombotic events including cardiac death, MI, urgent revascularisation and stroke (HR 1.49; 95% CI 1.08-

2.05; p = 0.02) at 2 year follow up[186].

There is an obvious paucity of clinical outcomes data specifically examining the impact of HPR in STEMI patients undergoing primary PCI, though this subgroup is represented in some observational studies recruiting ACS populations. In a small study of 60 STEMI patients, Matetzky *et al* stratified patients in four quartiles according to percentage reduction in ADP-induced platelet aggregation and found that all but 1 of the 7 recurrent cardiovascular events occurred in the lowest quartile[269]. Marcucci *et al* reported that residual platelet reactivity in 367 clopidogrel-treated STEMI patients was an independent predictor of MI severity based on peak cardiac biomarker values[270]. More recently, Jin *et al* used the VN-P2Y12 assay to measure OCPR in 181 STEMI patients undergoing PPCI and found that PRU ≥ 282 was the optimal cutoff for predicting post-PCI ischaemic events. Multivariate analysis showed high OCPR to be an independent predictor of the primary outcome measure, a composite of cardiovascular death, MI and stroke (HR: 8.13; 95% CI 1.79 – 37.03; p=0.007)[271].

1.8.4.3 Stent Thrombosis

ST is a life-threatening complication of PCI, which carries a mortality of up to 50%[272-274]. A large observational study of 3,021 patients by Airoldi *et* al showed that early discontinuation of thienopyridine therapy was the single most important determinant of ST (HR 13.74; 95% CI 4.04 - 46.68; $p < 0.001$) within the first six months following DES implantation[273]. It is therefore entirely conceivable that poor pharmacodynamic response in patients compliant with APT could be an important mechanism for ST. Buonamici *et al* prospectively evaluated this by measuring ADP-induced platelet aggregation with LTA in 804 patients receiving DES(s) and showed that clopidogrel nonresponse is an important predictor of definite ST (HR 3.08; 95% CI 1.32 - 7.16; p = 0.009) over a 6 month follow up[261]. Similarly, Sibbing *et* al measured ADP-induced platelet aggregation in whole blood with MEA in 1,608 patients undergoing PCI with DES(s) and showed that clopidogrel low responders had a significantly higher risk of definite ST at 30 days (2.2% vs. 0.2%; OR 9.4; 95% CI 3.1 - 28.4; p < 0.0001)[262]. The relationship between platelet reactivity and clinical outcomes including ST was examined in ADAPT-DES, a large multicentre registry of 8,583 patients treated with DAPT following DES implantation. This showed that high OCPR was strongly associated with ST (adjusted HR 2·49; 95% CI 1·43 – 4·31; p = 0·001) at 1 year[213],

though no adverse association with ischaemic events was evident in patients hyporesponsive to aspirin.

1.9. Platelet reactivity and bleeding

Since DAPT became the default approach in the DES era, ongoing research has focused on development of more potent adjunctive pharmacotherapies and evaluation of novel strategies that could yield further attrition of recurrent ischaemic events following PCI. Nevertheless, a logical and almost inevitable consequence of more aggressive prevention of thrombosis is a heightened risk of bleeding. Whilst previously this risk was considered an acceptable trade off for improved efficacy, there is now greater awareness that bleeding, the most common complication in PCI, is not entirely a benign entity. Firstly, studies showed that red blood cell transfusion in the context of PCI carries a higher risk of MACE, including mortality[275]. Furthermore, meta-analyses and registries from large patient populations have confirmed that bleeding in patients undergoing PCI is a strong predictor of not only MACE but also in hospital and long-term mortality[276-278].

Developing this insight into the scale and adverse sequelae of bleeding has led to a paradigm shift in both research as well as clinical practice in the field of coronary intervention. Firstly, there is broader implementation of strategies to reduce peri-procedural bleeding including the wider use of radial access, selective use of adjunctive therapies like GPIs, and greater deployment of vascular closure devices. Furthermore, a consensus definition of bleeding has now been proposed by the Bleeding Academic Research Consortium (BARC) to standardize comparisons of safety outcomes across trials which have been challenging to interpret due to heterogeneity amongst the prevailing classification systems[279]. Finally, the focus is now moving towards finding strategies that reduce ischaemic complications without causing excessive bleeding. The relationship between platelet reactivity and bleeding endpoints has therefore been evaluated to determine if there is an intermediate range where it is possible to attain an optimal equipoise between ischaemic and bleeding complications.

Sibbing *et al* were the first to provide evidence of such a therapeutic window when they evaluated this relationship in 2,533 clopidogrel-treated patients in whom platelet function testing was performed with MEA immediately before PCI. They observed that

the risk of major bleeding was significantly higher in patients with an enhanced response (low platelet reactivity of LPR) compared to the remaining patient population (2.2% Vs 0.8%; adjusted OR 3.5; 95% CI 1.6 – 7.3, p = 0.001)[203]. Similarly, based on a cutoff of PRI < 16 by the VASP assay, Bonello *et al* reported a significantly higher rate of bleeding events amongst patients with LPR compared to those without (15.6% Vs 3.3%; p < 0.001) in a population of 301 prasugrel-treated patients undergoing PCI for NSTEMI[196]. Finally, the large scale, multicentre ADAPT-DES registry measured platelet reactivity with the VerifyNow™ assay in over 8,665 patients successfully treated with a DES. In this study, Stone *et al* reported that platelet reactivity is inversely associated with bleeding (adjusted HR 0·73; 95% CI 0·61 – 0·89; p = 0·002)[213].

A recent collaborative analysis of 17 studies included over 20,000 thienopyridine-treated patients undergoing PCI, and stratified them into HPR, LPR and optimal platelet reactivity (OPR)[280]. This showed that patients with HPR have a 2.7-fold higher risk for ST and a 1.5-fold higher risk for mortality compared with those with OPR. By contrast, patients with LPR showed a 1.7-fold higher risk for major bleeding complications without any further reduction in the risk of ST compared to patients with OPR. These results suggest the existence of an optimal range of platelet inhibition that can be considered a "therapeutic window", within which the predicted risk of both ST and major bleeding after PCI is minimised.

1.10 Personalised antiplatelet therapy

It is well established that responses to APT, in particular clopidogrel, are variable. Moreover, HPR has been demonstrated to be a useful and independent predictor of post-PCI atherothrombotic events including ST (Section 1.8). This has been an important driver for the development and marketing of more potent and rapidly acting $P2Y_{12}$ inhibitors such as prasugrel and ticagrelor. Whilst these agents confer reduced risk of ischaemic events in some patient populations, this benefit needs to be considered cautiously in view of the increased risk of non-bypass surgery-related major bleeding events. Furthermore, there is growing evidence that a significant proportion of patients are hyporesponsive to prasugrel[281] and ticagrelor[282] as well. Extrapolating these limitations further it has been argued that a "one-size-fits-all" strategy may not therefore be optimal. Specifically, in

patients with HPR, it may be possible to reduce the risk of post-PCI adverse events and improve clinical outcomes by adopting a "personalised" approach that aims to achieve a pharmacodynamic response within an acceptable "therapeutic range".

Whilst promising, the concept of "personalised APT" has not permeated everyday clinical practice for several reasons. The requirement for a simple, accurate, reproducible, cost-effective and rapid POC assay of platelet function is essential if a strategy of personalised APT is to be delivered effectively. However, for any such test, establishing a scientifically and statistically well-validated cutoff value for the definition of therapeutically "adequate" or "inadequate" that relates to both the risk of thrombotic events at one end of the therapeutic spectrum, and bleeding at the other, is recognised to be highly challenging. In this context, it is notable that there is considerable controversy regards the optimal cutoff for HPR that identifies patients at risk of post-PCI thrombotic events with the VN-P2Y12 assay. A collaborative patient level meta-analysis of six major observational studies including 3,059 patients in patients undergoing PCI concluded that a PRU > 230 at the time of PCI had the highest predictive value for post-PCI ischaemic events[283]. However, studies in patients with stable CAD have favoured a much lower cutoff (i.e. PRU > 208)[211] whilst those enrolling a more high-risk cohort like STEMI patients have reported a much higher threshold to optimally define HPR (i.e. PRU > 282)[271].

Apart from the clinical context, it is plausible that the time interval at which platelet function testing is performed may also influence the response phenotype. Some studies have shown that levels of platelet reactivity decrease over time following an initial peak when baseline sampling is performed early, for example around the time of PCI. This may relate to early platelet activation in the context of the PCI procedure itself. Presently, there is considerable controversy regards the stability of platelet reactivity and response phenotype over time. The cutoff for platelet reactivity that defines HPR in the acute phase may not have predictive value when employed subsequently in the maintenance treatment phase. Moreover, the time interval that most reliably predicts future ischaemic events in patients undergoing PCI remains to be established. A study by Campo *et al* showed that OCPR was a stronger predictor of outcome when evaluated at 1 month (HR 28.5; 95% CI 8 – 104; $p < 0.01$) compared with a pre-PCI baseline (HR 3.1; 95% CI 1.3 – 7.3; $p < 0.02$)[284].

Finally, the most effective strategy to overcome HPR on clopidogrel remains enigmatic.

Potent $P2Y_{12}$ receptor such as prasugrel and ticagrelor represent evidence-based alternatives that have proven superior efficacy compared to clopidogrel in certain populations. However, a higher clopidogrel dosing regimen, if effective, could be a more cost-effective strategy to overcome HPR. In a prespecified subgroup analysis of patients undergoing PCI in the CURRENT-OASIS 7 trial, a seven-day double-dose clopidogrel regimen was associated with a reduction in 30 day cardiovascular events and ST compared with the standard dose clopidogrel[285]. Several small observational studies as well as large multicentre randomised studies have evaluated the strategy of personalised APT in PCI patients, as summarised below.

1.10.1 The evidence for personalised antiplatelet therapy

A study by Bonello *et al* was the first to demonstrate that adjusting the clopidogrel loading dose based on monitoring platelet reactivity can improve clinical outcomes after PCI in patients with HCPR. All eight adverse events occurred in the control group receiving placebo (10% Vs 0%; p = 0.007) compared to the VASP-guided group who received additional loading doses of clopidogrel[286]. Similarly, another multicentre study from the same group randomised 429 patients with high OCPR undergoing PCI to either placebo or up to three additional loading doses of clopidogrel 600 mg. The rate of ST (0.5% Vs 4.2%; p < 0.01) and MACE (0.5% Vs 8.9%; p < 0.001) was lower in the patients assigned to VASP-guided therapy compared to the control group[287]. Of note, 8% of patients in the VASP-guided group remained hyporesponsive to clopidogrel despite a 2,400 mg loading dose of clopidogrel.

Valgimigli *et al* enrolled 263 patients undergoing elective PCI with evidence of low response to either aspirin, clopidogrel or both with the VerifyNow assay and randomised them to receiving either tirofiban or placebo in a double-blinded manner. The incidence of peri-procedural MI (20.4% Vs 35.1%; RR 0.58; CI 0.39 – 0.88; p = 0.009) and MACE (3.8% Vs 10.7%; p = 0.031) were significantly lower in the tirofiban group compared with placebo[288]. Whilst these early trials support the platelet function hypothesis and the need for routine monitoring of response to APT, large randomised clinical trials were clearly needed to adjudicate the remaining uncertainties regards the optimal strategy for overcoming HPR.

In the GRAVITAS trial, 2,214 patients with high OCPR undergoing elective PCI with DES were randomised to either standard dose clopidogrel or a high-dose regimen which included an additional loading dose of 600 mg followed by a higher maintenance dose of 150 mg[289]. At 6 month follow up, the incidence of the combined primary endpoint of cardiovascular death, non-fatal MI or ST was identical in both treatment arms (2.3% Vs 2.3%; p = 0.97), with no observed excess of moderate or severe bleeding. The observed antiplatelet effect of higher-dose clopidogrel regimen was moderate given that persistent HPR was observed in 40% of the higher-dose cohort at 30 days. By comparison, HPR had resolved spontaneously without need for additional intervention in 38% of those receiving standard dose clopidogrel. Moreover, a *post hoc* analysis of the results suggested that a PRU > 208 was associated with a lower risk for cardiovascular events and might have been a more appropriate cutoff than the one used to define HPR in the main study (i.e. PRU > 230)[290].

However, the TRIGGER-PCI study used a lower cutoff of PRU > 208 to define HPR with VN-P2Y12 performed 2-7 hours after the first maintenance dose of clopidogrel on the day after PCI[291]. Accordingly, patients with HPR were randomly assigned to receive either standard dose clopidogrel or prasugrel 10 mg daily. Given a very low overall event rate the study was terminated early after randomisation of only 423 patients for reasons of futility. Nevertheless, at 3 months platelet reactivity in the prasugrel cohort had decreased significantly (245 [225 – 273] to 80 [42 – 124]; p < 0.001) compared to only a small albeit statistically significant decrease in the clopidogrel arm (249 [225 – 277] to 241 [194 – 275]; p = 0.001). An important critique of these two large multicentre randomised studies was that they recruited a low risk cohort predominantly comprising patients undergoing elective PCI for stable CAD. The actual rate of adverse events was therefore substantially lower than initially anticipated (~5%). It is likely that contemporary practices and technologies in PCI may have contributed to the improved outcomes observed in these studies. Consequently, the GRAVITAS trial in particular was underpowered to detect a rather optimistic relative risk reduction (50%) anticipated from treatment with high-dose clopidogrel.

In the ARCTIC study, 2,440 patients undergoing PCI with DES were randomised following coronary angiography to either a conventional strategy employing fixed dose regimens of aspirin and clopidogrel without platelet function testing or a monitoring arm where the treatment was adjusted based on measuring OTPR[292]. In the latter group, platelet

function testing with the VerifyNow™ assay was performed immediately before PCI and if HPR was identified then the protocol called for administration of a GPI and either an additional loading dose of clopidogrel 600 mg (80%) or a loading dose of prasugrel 60 mg (3%) followed by maintenance dose of clopidogrel 150 mg or prasugrel 10 mg respectively. The choice of thienopyridine and GPI was left to the discretion of the attending physician. By contrast, patients with a low response to aspirin during PCI were administered intravenous aspirin during the procedure. At 2 to 4 weeks post PCI, patients with a low response to clopidogrel either received an additional 75 mg increase in the maintenance dose of clopidogrel or were switched to prasugrel 10 mg. In the conventional arm, the use of aspirin, GPI and thienopyridine was left to the physician's discretion. ARCTIC showed no difference in the composite primary endpoint of death, MI, stroke, urgent revascularisation or ST at 1 year between the monitored and conventional therapy arms (34.6% Vs 31.1%; p = 0.10) and no difference in major bleeding 2.3% Vs 3.3% p = 0.15). Similar to GRAVITAS and TRIGGER-PCI, the majority of patients (two-thirds) were considered low-risk PCI for stable CAD, though the higher event rates were driven by peri-procedural MI defined by elevations in cardiac biomarkers. Moreover, the novel thienopyridine, prasugrel, only became available during the course of the trial and therefore was only administered infrequently (9% in the monitored group and 6% in the conventional therapy group). In keeping with other trials, HPR persisted on repeat testing at 2 to 4 weeks in a significant proportion of nonresponders (15.6%).

The MADONNA study was a relatively small study of 798 patients undergoing PCI (including 37% ACS), who were divided into two groups based on whether or not they received APT guided by platelet function testing with MEA[293]. All patients received a loading dose of clopidogrel 600 mg, however, those in the guided-therapy arm received either additional loading doses of clopidogrel (up to a maximum of 2400 mg over 4 days following repeat testing) or prasugrel 60 mg if HPR persisted. During the maintenance phase, all patients received the $P2Y_{12}$ inhibitor agent in accordance with that administered last during loading. Whilst not randomised and small, the MADONNA study did demonstrate a significant reduction in ST rates in the guided therapy group compared to the conventional therapy arm (0.2% Vs 1.9%, p = 0.027), though no differences in cardiovascular death or major bleeding were observed.

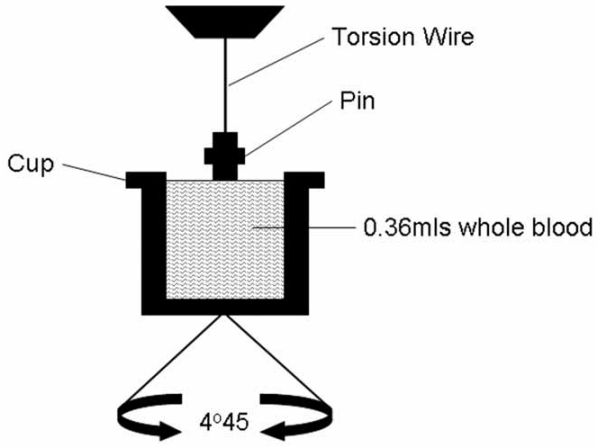
1.10.2 Summary

The results of these trials may have been limited by their design due to insufficient power, inadequacy of the personalised pharmacodynamic intervention and possible selection bias for low risk populations. Nevertheless, a recent meta-analysis comprising 4,213 patients from ten studies showed that in patients with HPR, intensified APT reduced the risk of thrombotic events without increasing the risk of major bleeding when compared to standard therapy with clopidogrel[294]. A meta-regression analysis suggested that personalised APT based on platelet function testing is only beneficial when the risk of ST is otherwise high, so this should be taken into consideration in future trials of tailored APT. Whilst there is no definitive evidence for a personalised approach, the consistency of association between platelet reactivity and post-PCI ischaemic as well as bleeding complications across several prospective observational studies including the large-scale multicentre ADAPT DES registry, offer a persuasive argument for a personalised approach to APT prescribing[213, 280].

1.11 The TEG Methodology

Whole blood activated by kaolin, which stimulates clotting via the intrinsic pathway, is manually pipetted into a cylindrical cup that is pre-warmed to 37°C. A stationary pin suspended on a torsion wire is immersed in this cup which oscillates through an angle of 4°45’ at a frequency of 0.1 Hz (Figure 1.2A). Platelet-fibrin linkage during clot formation allows the torque generated by the rotating cup to be transmitted to the immersed pin, which acts as a torque transducer converting pin motion into an electrical signal. The magnitude of the output is determined by clot strength, whereby the pin remains static as blood is in a liquid state, but as the clot develops pin motion occurs in phase with cup rotation. In this manner the viscoelastic properties of a clot are measured by the TEG analyser (Figure 1.2B) and graphically represented as a TEG trace plotted against time (Figure 1.3). Further quantitative analysis of this trace can be used to derive a complete profile of clot initiation, kinetics, strength and stability as well as fibrinolysis.

A



B

Figure 1.2 Thrombelastography: (A) a schematic representation of TEG methodology, and (B) a TEG analyser with two channels

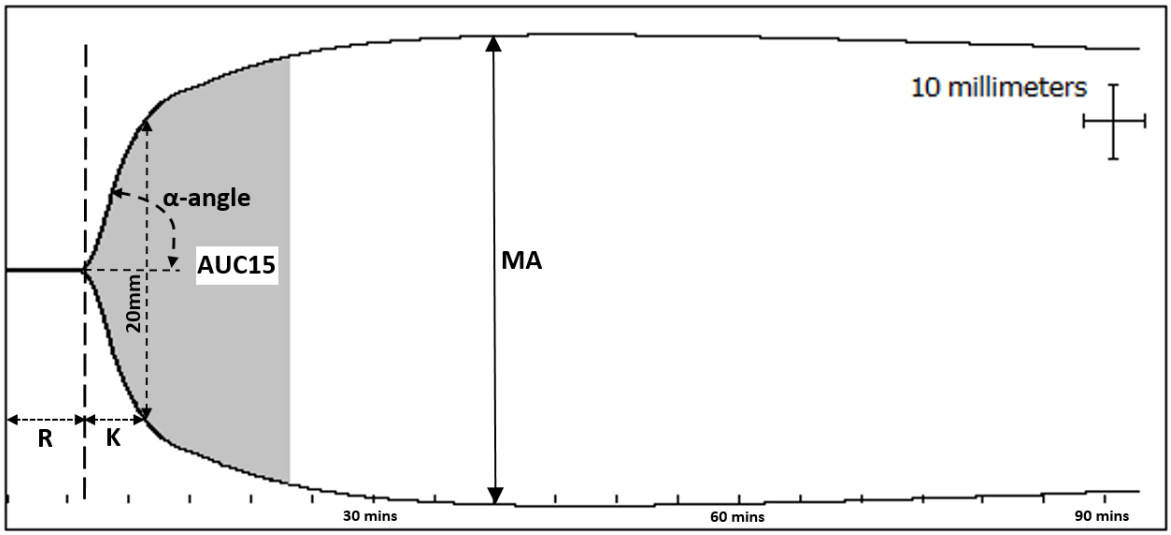


Figure 1.3 A representative TEG trace. *MA*, maximum amplitude; *R*, R time; *K*, K time; *AUC15*, Area under the curve at 15 minutes

Normal haemostasis involves the controlled activation of clot formation, spontaneously balanced by mechanisms of clot lysis. Therefore, a truly global analysis of haemostatic function requires assessment of both the fibrinolytic and coagulation systems. TEG incorporates all the components of clotting and measures the viscoelastic changes that occur following clot initiation, as the clot becomes stronger and is stabilised before it retracts and is broken down in the lytic phase. Analysis of the TEG trace can determine (a) the speed of clot generation (b) its strength and (c) its stability. Table 1.3 below summarises commonly assessed TEG variables[295].

Table 1.3 Commonly assessed TEG parameters

Parameter	Description and rationale for assessment
R time	Latent period between start of sample analysis to initial fibrin formation. Relates to plasma clotting and inhibitor activity
K time	The time taken for blood to attain a fixed level of viscoelasticity (i.e. 20 mm). It is a measure of clot kinetics
α-angle	The gradient formed by the angle of the initial trace. It represents the rapidity of fibrin build-up and cross-linking
MA	MA, or maximum amplitude, represents the ultimate clot strength of the clot. It represents the activity of fibrin and platelets.

As clotting is a dynamic and complex process, many conventional tests such as aPTT and platelet count assess only isolated components of the haemostatic system rather than being able to discriminate between the contributions of the individual components to haemostasis as a whole. The advantage of TEG is that it incorporates the interaction of all of the components of coagulation including platelets, fibrin, clotting factors, and thrombin as well as providing information about the quality of the clot.

1.11.1 Modifications of TEG

Conventional "unmodified" TEG provides a non-specific assessment of global haemostasis whereby the dominant effect of certain components (e.g. thrombin) can obscure certain clotting abnormalities. Modifications to TEG have broadened the scope of this technology by accelerating result acquisition, extending duration of sample viability for remote analysis and allowing more specific evaluation of the functional importance of individual components of haemostasis. These include: -

a) Sample activators like kaolin, celite or tissue factor to speed up result acquisition

b) Citrate for preserving blood for an extended duration allowing delayed sample

analysis, particularly in circumstances where the TEG apparatus is remote to the point of care

c) Specifically, designed cups coated with the enzyme heparinase can be used to neutralise heparin in patients anticoagulated with this agent in the context of cardiopulmonary bypass or PCI

d) Collecting samples in heparin eliminates the effect of thrombin, thereby allowing assessment of the platelet and fibrin contributions to clot formation

e) The addition of a GPI *in vitro* allows assessment of the relative contribution of fibrinogen to clot formation

f) Activator F™ (Act F), comprising a combination of reptilase and Factor XIIIa, generates a fibrin clot without platelet activation, thereby allowing isolation of the fibrin contribution to clot strength

g) Specific platelet agonists (e.g. ADP, AA) can be used to evaluate the functional effect of APT

1.11.2 TEG Platelet Mapping

In standard TEG, the final clot strength or maximum amplitude (MA) is predominantly a result of thrombin, which itself is a potent platelet activator. Indeed, when present, thrombin conceals the effect of other lesser agonists (e.g. AA and ADP) on clotting. Nevertheless, the use of heparin as anticoagulant in the sampling Vacutainer effectively annuls the effect of thrombin. Furthermore, the addition of Act F generates a fibrin network in which platelets can interact independently of thrombin. In the absence of alternate sources of platelet activation, the resultant clot is weak which is depicted on the TEG trace as a low amplitude trace. However, when other clotting pathways (e.g. AA or ADP) are stimulated unopposed by their respective inhibitors (e.g. aspirin or $P2Y_{12}$ antagonists) then clot strength is more analogous to that attained with thrombin. Maximal platelet activation generates a curve similar to unmodified TEG in the presence of thrombin. The effect of APT can therefore be established by comparing the unmodified TEG curve (representing maximal platelet activation) and the modified TEG

curve with either AA- or ADP-stimulation.

Aspirin achieves its antiplatelet effect by irreversible inactivation of COX-I, an enzyme involved in AA-metabolism. The pharmacodynamic response to aspirin can therefore be evaluated by comparing the unmodified curve in the presence of thrombin (maximal platelet activation), the heparinised sample with Act F alone (no platelet activation) and the modified TEG curve with AA-stimulation (residual platelet activation due to AA in the presence of aspirin). Similarly, the antiplatelet effect of a $P2Y_{12}$ receptor blocker or GPI can be assessed in the same way. This system is marketed by Haemoscope as the "Platelet Mapping Kit", and includes reagents to run four different channels including: (1) Kaolin (thrombin-mediated clotting); Activator F™; (3) Activator F™ and AA (1 mM); (4) Activator F™ and ADP (2 µM). With these modifications TEG Platelet mapping has a close correlation with LTA, the historical "gold standard" assay for assessing the pharmacodynamic effects of APT[239-242, 296].

1.11.3 Assessing response to antiplatelet therapy

Comparison of platelet reactivity following initiation of an antiplatelet agent to a pretreatment baseline can be used to determine an individual's response to APT. However, for obvious logistical reasons a baseline sample is not always feasible in routine clinical practice, particularly in acute settings such as STEMI. It would therefore be valuable to assess the response to APT as a one off "snapshot" during treatment without the need for a baseline sample. Based on TEG platelet mapping, studies have used the parameter percentage platelet inhibition (% PIn) to evaluate response to APT. This concept uses thrombin-mediated clotting as an internal reference as it represents maximum uninhibited platelet activation. The contribution of fibrin to clot formation is excluded to theoretically obtain a pure assessment of platelet contribution to whole blood clotting. The platelet inhibitory effects of aspirin can thus be determined based on the formula:

$$\textbf{\% Platelet Inhibition} = 100 - \left(\frac{MA_{AA} - MA_{Fibrin}}{MA_{Thrombin} - MA_{Fibrin}} \right) \times 100$$

Similarly, the platelet inhibitory effects of a $P2Y_{12}$ inhibitor such as clopidogrel can be determined using agonist-mediated clotting with ADP (i.e. MA_{ADP}). Whilst attractive, this index of platelet inhibition has several potential drawbacks. Firstly, there can be considerable delay in obtaining MA, sometimes in excess of 60 minutes. This is a significant impediment to utilising TEG as a genuine POC assay in acute clinical settings. Secondly, MA provides information solely on maximum clot strength and not speed of clot formation. Finally, the contribution of fibrin is excluded in the derivation of percentage platelet inhibition, which is difficult to justify given how integral it is in the adhesion of platelet aggregates via interaction with the $\alpha_{IIb}\beta_3$ complex[297]. Furthermore, it can be argued that this measure of platelet inhibition negates one of the primary advantages of TEG, namely its ability to evaluate overall whole blood clotting, including the effects of fibrin, rather than focusing in isolation on platelet function. The requirement for an assay that can detect the inhibitory effects of APT on overall whole blood coagulation rapidly and without the need for a pretreatment baseline as reference, led to the development of a novel method of analysis known as Short Thrombelastography (s-TEG) (Section 1.11.5).

1.11.4 TEG platelet mapping in PCI

Presently, TEG is principally employed in the management of haemostasis and guide blood component therapy in surgical, trauma, transplant and obstetric settings. However, TEG has also been investigated as a marker of thrombotic risk in patients undergoing PCI. Amongst the first to evaluate TEG for this purpose were Gurbel *et al* who measured thrombin-mediated clotting (TMC) following stimulation with kaolin, before and 1 day post-PCI, in 192 patients the majority of whom were on DAPT[296]. They showed that $MA_{Thrombin}$ was significantly greater (74 ± 5 Vs 65 ± 4, $p < 0.001$) and R time was significantly shorter (4.3 ± 1.3 Vs 5.9 ± 1.5, $p < 0.001$) after PCI in patients with ischaemic events over a 6 month follow up compared to those without. A combination of these two parameters in a linear regression model, was highly predictive of ischaemic events (OR 38.0; 95% CI 9.3 – 156.2; $p < 0.0001$).

A smaller study by the same group including 100 patients on chronic clopidogrel therapy

undergoing PCI for non-emergent indications evaluated platelet aggregation based on the TEG parameter MA_{ADP}[240]. This showed that HPR was significantly associated with ischaemic events at a 1 year follow up (OR 26.8; 95% CI 6.7 – 105; $p < 0.001$). The same group recruited 225 patients treated with DAPT undergoing non-emergent PCI, and correlated clinical outcomes over a 3 year follow up to TEG parameters measured 18-24 hours post PCI[264]. They reported that $MA_{ADP} > 47$ mm (HR 10.9; 95% CI 5.6 – 21.3; $p < 0.0001$) and $MA_{Thrombin} > 69$ mm (HR 3.5; 95% CI 1.9 – 6.4; $p < 0.0001$) were predictive of long-term ischaemic events after PCI. Finally, in the largest study evaluating the prognostic utility of TEG to date, Tang *et al* enrolled 789 consecutive patients on DAPT, undergoing coronary angiography as potential candidates for PCI. They observed a higher incidence of high OCPR in patients with ischaemic events over a one year follow up compared to those without (6.7% Vs 2.6%; HR 2.6; 95% CI 1.4 – 5.6; $p = 0.005$)[265].

1.11.5 Short Thrombelastography

The Southampton Coronary Research Group, in conjunction with the Department of Medical Physics and Bioengineering at Southampton, developed a purpose-built software programme able to calculate area under the TEG Curve (AUC) at a given time interval. Amongst the multitude of indices available routinely from analysis of a TEG trace, MA has been the one most extensively investigated though this has several limitations as discussed in section 1.11.3. By contrast, in addition to clot strength, AUC also incorporates the speed of clot formation (Figure 1.3). The importance of the latter is exemplified by TEG traces illustrated in Figure 1.4. Both traces achieve the same MA, however the relative difference in speed of clot formation is visibly appreciable, thus highlighting the importance of assessing both strength and speed of clot formation in combination.

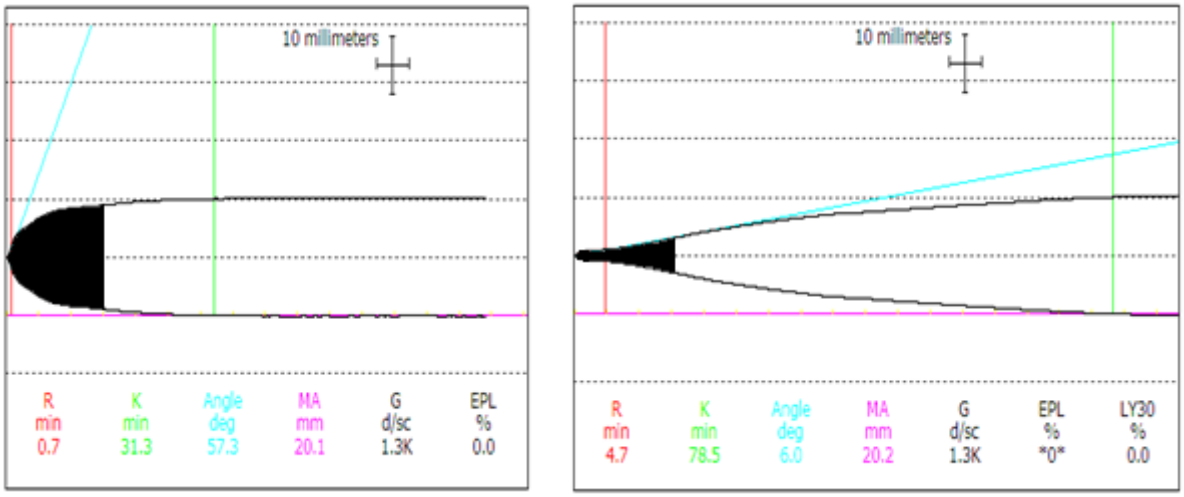


Figure 1.4 Area under the curve at 15 minutes in two separate traces with similar maximum amplitude (AUC15 shaded in black)

1.11.6 Validation of Short Thrombelastography

Based on measuring AUC at 60 minutes (AUC60), initial experiments in healthy volunteers demonstrated that TEG was sensitive to time-dependent responses to aspirin and clopidogrel when administered at loading and maintenance doses[298]. The concept of assessing clot strength and speed of clot formation with a single measure (i.e. AUC60) therefore proved interesting. Nevertheless, in an acute clinical setting rapid assessment of an individual's response to APT would be valuable to permit early implementation of more aggressive strategies to achieve a therapeutic antiplatelet effect. Consequently, under these circumstances an assay requiring over an hour to produce clinically meaningful information would have limited utility. The group therefore investigated whether the AUC principle could be reliably employed at a time before the MA was reached. A threshold of 15 minutes was chosen arbitrarily and the concept of AUC at 15 minutes (AUC15) was tested in healthy volunteers as well as patients being loaded with APT[246]. These experiments confirmed that with this modification, TEG can rapidly and reliably assess individual *ex vivo* clotting responses to aspirin and clopidogrel in a time sensitive manner. Most importantly for the AUC concept, they demonstrated an excellent correlation between AUC15 and MA ($r = 0.964$, $n = 560$, $p < 0.01$), thus dispelling concerns that 15 minutes might be too short to gather reliable and meaningful results (Figure 1.5).

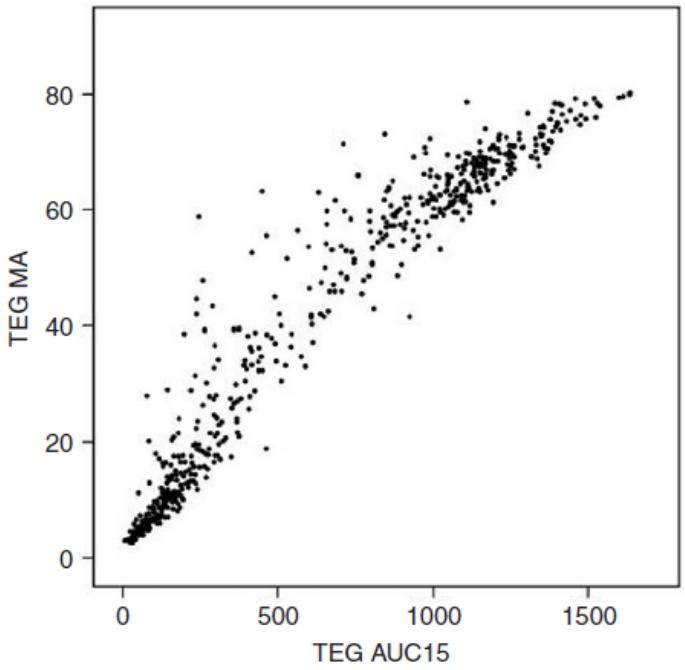


Figure 1.5 Scatter graph of correlation between MA and AUC15 (from Hobson *et al*[246])

Experiments in healthy volunteers and patients being loaded with aspirin and/or clopidogrel showed that s-TEG is a reproducible and reliable test of clotting responses to APT with minimal intra- (Figure 1.6A) and inter-individual variability (Figure 1.6B and 1.6C)[245].

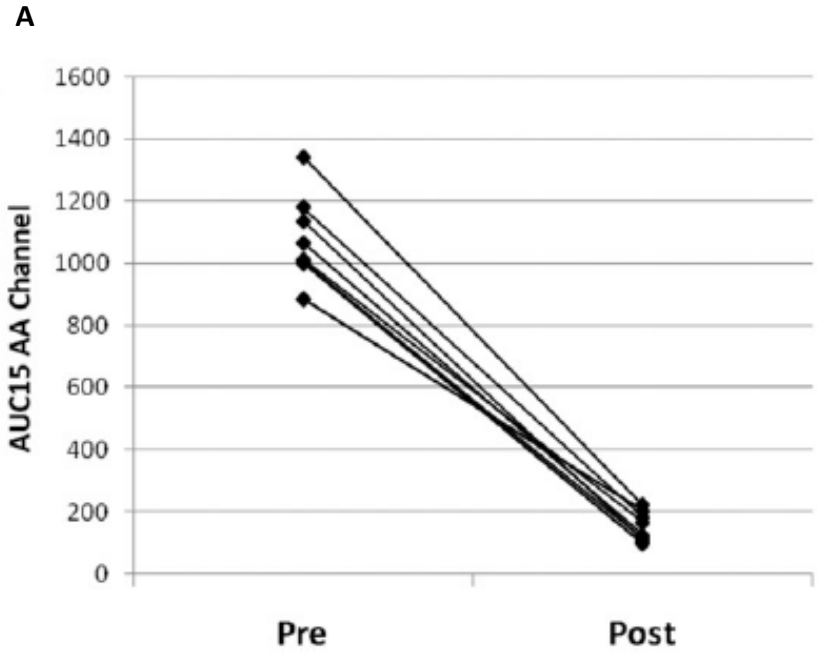


Figure 1.6A. Line graph demonstrating responses to APT with s-TEG in an individual before and 6 hours after loading with aspirin 300 mg on 10 occasions (Sambu *et al*[245])

B

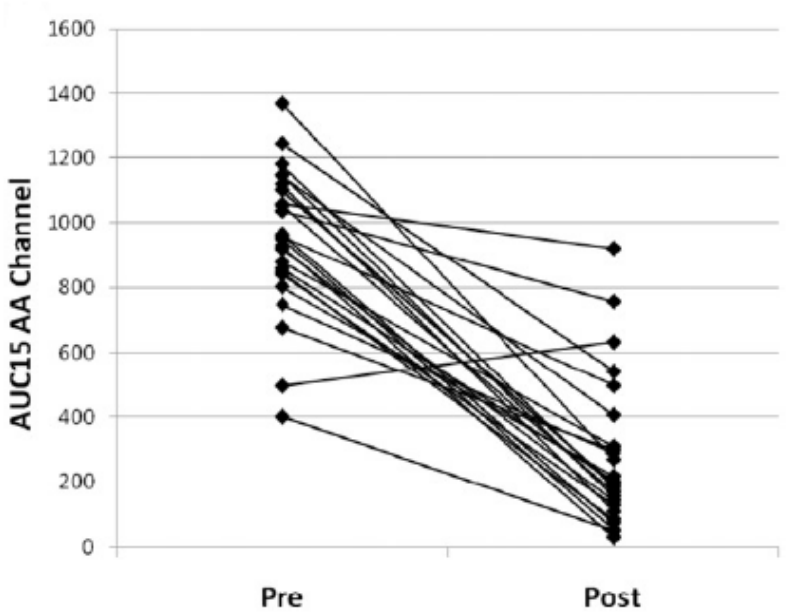


Figure 1.6B. Line graph demonstrating responses to APT with s-TEG in 25 volunteers before and 6 hours after loading with aspirin 300 mg (from Sambu *et al*[245])

C

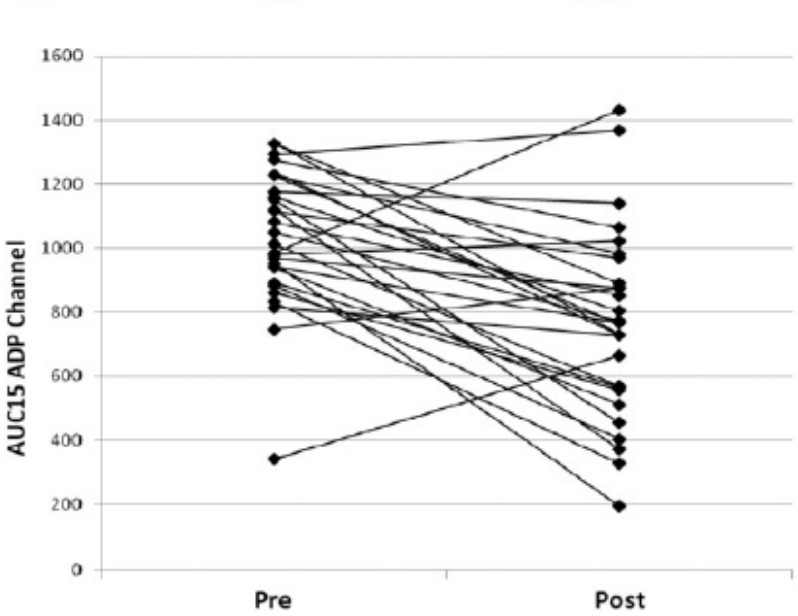


Figure 1.6C. Line graphs demonstrating responses to APT with s-TEG in 28 patients on low-dose aspirin undergoing PCI, before and 6 hours after loading with clopidogrel 600 mg (from Sambu *et al*[245])

1.11.7 Percentage Clotting Inhibition

The Southampton Group went on to evaluate whether AUC15 could be used to determine the inhibitory effect of APT on overall whole blood clotting *ex vivo* in a "snapshot" fashion, without the need for a pretreatment baseline sample. Similar to % platelet inhibition, they compared agonist-mediated clotting (e.g. ADP and AA) with those in response to thrombin which served as an internal control. By contrast however, contribution of the fibrin component to whole blood clotting was incorporated in this measure called percentage clotting inhibition (% CIn). Using AA as agonist, the inhibitory effect of aspirin on whole blood clotting can be determined using the following formula:

$$\text{Percentage clotting inhibition} = 100 - \left(\frac{AUC15_{AA}}{AUC15_{Thrombin}} \right) X \ 100$$

Similarly, the percentage clotting inhibition (% CIn) due to clopidogrel is calculated by substituting $AUC15_{ADP}$ as the nominator in the above formula. The Southampton group further established strong correlation between percentage clotting inhibition and the previously described percentage platelet inhibition ($r = 0.903$, $n = 320$, $p < 0.01$).

1.11.8 Short TEG as a research tool

Following the validation of s-TEG, the Southampton Research Group used this modified assay in a number of investigator-led observational and mechanistic studies.

a) Assessment of responses to aspirin and clopidogrel in PCI patients who have experienced stent thrombosis whilst on APT[299]

b) The evaluation of gender specific differences in response to aspirin and clopidogrel[159]

c) The assessment of responses to clopidogrel in patients presenting with ACS[300]

d) The effect of higher loading doses of clopidogrel 900 mg in healthy volunteers and

patients hyporesponsive to clopidogrel 600 mg[301]

e) The effects of clopidogrel on "aspirin-specific" pathways of platelet inhibition and its potential clinical implications[302]

f) The effects of clopidogrel cessation after one year of DAPT following DES implantation[303]

g) The prevalence of non-response in patients presenting with ST and the feasibility of using s-TEG to personalise APT in this population[304]

h) A cross-over study designed to evaluate equivalent inhibition of ADP-induced platelet aggregation with branded Plavix compared to a generic preparation containing clopidogrel hydrochloride[305]

i) Whether s-TEG, a functional test of AA-induced whole-blood clotting, can reliably determine the true biochemical response of patients with ischemic stroke to aspirin[251]

1.12 Inflammation platelet reactivity and atherothrombosis

The role of platelets in atherogenesis and inflammation has previously been discussed in section 1.1.3. Recently, advances in our understanding of CVD support the belief that inflammation plays a key role not only in the development and progression of atherosclerosis, but also in plaque destabilisation and subsequent atherothrombosis. This is further supported by the finding of accelerated atherosclerosis and increased cardiovascular mortality in chronic inflammatory disorders[306] including systemic lupus erythematosus (SLE)[307], rheumatoid arthritis (RA)[308], and inflammatory bowel disease (IBD)[309].

1.12.1 Inflammation and Acute Infection

There is increasing evidence from epidemiological studies suggesting a link between

cardiovascular events including AMI and stroke in the immediate aftermath of an acute infection. In a case-control study of 1,922 AMI patients and 7,649 matched controls, Meier *et al* found an increased risk of AMI associated with acute respiratory tract infection (OR 3.0, 95% CI [2.1-4.4]; $p < 0.001$)[310]. In a retrospective cohort study, Smeeth *et al* evaluated 20,486 persons with a first myocardial infarction and 19,063 persons with a first stroke who received influenza vaccine. The risks of both events were substantially higher after a diagnosis of respiratory tract infection with a peak incidence in the first three days (incidence ratio: MI 4.6, 95 % CI [4.4-5.5]; stroke 3.3; 95 % CI [2.8-3.6])[311]. Finally, Clayton *et al* performed a case-control study including 11,155 MI cases and 9,208 stroke cases, in which they showed a high risk of both events in the seven days following respiratory infection (adjusted OR; MI 2.1, 95% CI [1.4 – 3.2]; stroke 1.9, 95% CI [1.2-3.0])[312].

1.12.2 Prognostic utility of inflammatory biomarkers

Several inflammatory biomarkers have shown promising predictive power for the assessment of atherothrombotic risk. Amongst these, high sensitivity C-reactive protein (hsCRP) has been the most extensively studied. Elevated CRP levels have been found in patients with stable CAD as well as in ACS populations, and has been shown to be an independent predictor of future ischaemic events. A large prospective study followed up nearly 28,000 postmenopausal women for over 8 years and showed that CRP was a better predictor of cardiovascular risk than LDL cholesterol[313]. Moreover, elevated concentrations of CRP at baseline in patients undergoing PCI[314, 315], and high-risk noncardiac surgery (especially vascular surgery)[316] are predictive of adverse outcomes.

In an early study, aspirin was found to reduce cytokine and CRP levels in patients with chronic stable angina [317]. This effect of aspirin correlates with the finding that, in apparently healthy men enrolled to the Physicians' Health Study, the largest benefit of aspirin in reducing the risk of a first MI was observed in those with the highest levels of CRP[318]. Similarly, a substudy of the CREDO trial evaluated 1 year outcomes in 1,468 patients with baseline CRP data available and found the composite incidence of death, MI or stroke to be higher in the highest two tertiles compared to the lowest tertile (11.4% vs. 6.4%, $p = 0.003$)[319]. Moreover, clopidogrel therapy was associated with a lower incidence of the combined endpoint at one year compared to placebo in the highest two tertiles of

CRP (9.1% vs. 13.5%, p = 0.04) but not in the lowest.

Similarly, increased levels of interleukin-6 (IL-6) have been reported in patients with stable and unstable angina[320]. Furthermore, healthy subjects who have higher IL-6 levels have been shown to be more likely to develop cardiovascular disease (RR 2.3, 95% CI [1.3-4.3]; p = 0.005)[321]. In patients with ACS, it increases the likelihood of reinfarction and adverse outcomes[322, 323]. CD40 ligand (CD40L) is another marker of inflammation with potential prognostic utility. Raised levels of soluble and membrane bound CD40L are detectable in patients with stable as well as unstable angina and MI[324, 325]. Moreover, elevated levels of preprocedural CD40L have been shown to correlate with late restenosis in patients undergoing angioplasty[326]. Even in a large population of apparently healthy women, soluble CD40L was an independent predictor of future cardiovascular risk (RR 3.3; 95% CI [1.2 – 8.6]; p = 0.01)[327].

1.12.3 Inflammation and platelet reactivity

A growing body of evidence now suggests that long-term APT may exert benefit not only by reducing platelet activation, but also by reducing inflammation through its anti-inflammatory properties. Conversely, it has also been proposed that inflammation may augment platelet reactivity thereby potentially reducing antiplatelet drug efficacy[328]. Indeed, a study by Gori et al showed that ACS patients on DAPT with a dominant proinflammatory cytokine profile had a significantly higher risk of having HPR in comparison to those with a balanced or anti-inflammatory profile[329]. This suggests that a patient's inflammatory state may have a modulating role on the effect of APT on platelet reactivity.

A study by Ge *et al* showed that at 6 months follow up after elective PCI, soluble CD40L (sCD40L) was an independent predictor of clopidogrel non-response (HR 3.0; 95% CI 1.3 – 3.3; p = 0.04)[330]. In a cohort of 903 patients undergoing PCI for symptomatic CAD, Müller *et al* showed that levels of IL-6 and CRP were significantly higher in nonresponders to aspirin and clopidogrel compared to responders, based on MEA[331]. Furthermore, those in the highest stratum of both CRP and residual platelet aggregation had the highest cumulative incidence of post-PCI MACE compared to those in the lowest stratum (p < 0.001). Similarly, in 500 patients undergoing PCI in the ARMYDA-CRP study, Patti *et al*

demonstrated that the incidence of periprocedural MI was greatest in individuals with both elevated baseline CRP levels and HPR to ADP compared to those without either (16.6% vs. 3.6%; OR 4.3; 95% CI 1.5 – 12.6;p = 0.008)[332]. Finally, Bernlochner *et al* demonstrated that an elevated CRP (i.e. > 5 mg/L) was associated with significantly greater ADP-induced aggregation in 1,223 stable patients on long-term DAPT following stent implantation[333]. Furthermore, they noted that elevated CRP was an independent predictor of HPR to ADP in this population (OR 1.6; 95% CI 1.1-2.4; p = 0.009).

1.12.4 Summary

The influence of inflammation on overall clotting is likely orchestrated by a complex interplay between all components of the immune and haemostatic system that ultimately results in endothelial dysfunction, activation of the coagulation cascade, impaired function of physiologic anticoagulants and suppressed fibrinolytic activity. Nevertheless, the precise mechanisms implicated in the advent of an atherothrombotic event in the context of an acute inflammatory episode, particularly in patients established on long-term APT, warrants further investigation.

1.13 Cyclooxygenase-independent pathway of AA-mediated clotting

As previously discussed in section 1.4.1, aspirin targets the platelet AA/COX-1 pathway, though it does have weaker activity against COX-2 at higher doses. Assays measuring TXB_2, the dominant stable metabolite of this pathway, remain the gold standard biochemical index for evaluation of aspirin response as they are highly sensitive and specific for the AA/COX-1 pathway targeted by aspirin. Nevertheless, a myriad of tests have been used to describe aspirin response based on the ability of aspirin to inhibit platelet aggregation/activation to a variety of agonists including AA, ADP, collagen, and epinephrine, some of which are not specific for the AA/COX-1 pathway. Unfortunately, there is poor to moderate correlation amongst these different tests of platelet function. Furthermore the results of these functional tests do not correlate well with serum TXB_2 levels, the biochemical gold standard assay[229, 230, 232-234, 247].

1.13.1 Functional versus biochemical aspirin resistance

Previous work from this group using s-TEG has questioned the validity of employing AA-induced clotting as a test for aspirin efficacy. Firstly, clopidogrel can potentiate the effects of aspirin on AA-mediated clotting such that 8 out 10 aspirin nonresponders were converted to responders by the addition of clopidogrel[302]. Secondly, in the CESSATION study, as well as the expected increase in ADP-induced clotting, a significant and unexpected increase in AA-induced clotting was observed following the discontinuation of clopidogrel (but continuation of aspirin monotherapy) after one year of DAPT[303]. This rebound phenomenon following cessation of clopidogrel has been subsequently confirmed in other studies[334]. Importantly, serum TXB_2 levels remained consistently suppressed in the CESSATION study, thus confirming effective and persistent COX-1 inhibition due to ongoing aspirin therapy[303]. Similarly, in patients presenting with acute ischaemic stroke treated with aspirin, this group demonstrated high clotting responses in up to 67% to AA-stimulation despite negligible TXB_2 levels[251].

These data, for which as yet there is no mechanistic explanation, have important clinical implications. Firstly, they show that the functional effects of aspirin are only variably detected by assays that employ AA as agonist, a test which may therefore be flawed for the detection of response to aspirin therapy. This may be clinically relevant as it could lead to inappropriate misclassification of subjects as aspirin nonresponders despite adequate inhibition of platelet COX-1 activity. Secondly, these and other data indicate that there may be a recruitable aspirin-independent pathway (i.e. COX-1 independent pathway) for AA-induced clotting. Finally, an understanding of this phenomenon may provide insight into the increasingly observed incidence of very late ST (i.e. ST more than one year after stent implantation) in patients on aspirin monotherapy[304]. Is it possible that the mechanism for ST in this setting is not a transient failure of aspirin to suppress COX-1-mediated TXA_2 synthesis but instead represents a diversion of AA-mediated clotting down a recruitable COX-1-independent pathway?

1.13.2 Lipoxygenase pathway

A COX-1 independent pathway has also been implicated by a study from Frelinger *et al*, who showed that persistent AA-mediated aggregation despite aspirin could not be further suppressed by *in vitro* aspirin or indomethacin, suggesting a COX-1 and COX-2 independent mechanism[335]. Indeed, a COX-1 independent pathway has also been implicated in increased ADP- and AA-mediated aggregation following administration of UFH in aspirin treated patients undergoing carotid endarterectomy. In this study by McMahon *et al*, paradoxical potentiation of ADP-and AA-mediated aggregation appeared to correlate with a concomitant increase in 12-hydroxyeicosatretraenoic acid (12-HETE), a stable metabolite of the 12-lipoxygenase (12-LO) pathway, whilst plasma TXB_2 levels remained unchanged[336]. This potential mechanism for AA-mediated but COX-1-independent clotting is plausible for several reasons. Firstly, it is well known that both the COX-1 and 12-LO pathways are enzymatically coupled to the same cellular substrate, AA. Furthermore, studies have shown that thrombin stimulation results not only in the generation of TXB_2 but also similar increases in 12-HETE production[337]. This was further shown to be temporally related to increased expression of P-selectin, and both were inhibited in a dose-dependent manner by inhibitors of 12-LO, but not aspirin. Similarly, Nyby *et al* have previously demonstrated that a highly specific inhibitor of 12-LO attenuated thrombin-mediated Ca^{2+} mobilisation and platelet aggregation[338].

The metabolism of AA via 12-LO represents an attractive alternate pathway not susceptible to the platelet inhibitory effects of aspirin, which could conceivably account for persistent AA-mediated clotting despite effective COX-1 inhibition. Further studies to systematically investigate the role of the 12-LO pathway on platelet function and the major determinants of these effects are warranted. In Chapter 7 of this thesis, the association between vascular inflammation and its effects on AA-mediated aggregation as well as AA-metabolism via the COX-1 and 12-LO pathways will be investigated further.

1.14 Summary of study objectives

The focus of my thesis is to explore pertinent clinical themes relevant to optimising

antiplatelet and anticoagulant therapy in cardiovascular disease based on platelet function testing, predominantly with s-TEG.

1.14.1 Pooled analysis comparing s-TEG to VerifyNow P2Y12 assay

This group had previously consistently observed HPR on various $P2Y_{12}$ inhibitors with s-TEG despite VerifyNow reporting a therapeutic response in a proportion of individuals presenting with ST. Based on these observations we hypothesised that VerifyNow may overestimate the functional response to clopidogrel compared to s-TEG. I therefore conducted a detailed pooled analysis of all patients and volunteers in whom simultaneous assay with s-TEG and VerifyNow had been performed. This comparison revealed striking results that raised concerns about the validity of using VN-P2Y12 as a test for assessing response to clopidogrel. Furthermore, in an additional subset of patients on DAPT with aspirin and clopidogrel, I explored whether the inclusion of PGE1 (i.e. simulating the reagents used in VN-P2Y12) could account for the observed discrepancies between s-TEG and VN-P2Y12.

1.14.2 Effect of PGE1 on $P2Y_{12}$ blockade *in vitro*

Based on the results of the above pooled analysis we designed *in vitro* experiments in healthy volunteers with the aim of systematically evaluating the dose-response relationship effect of PGE1 on $P2Y_{12}$ inhibition of ADP-mediated aggregation with s-TEG. As personalised APT is critically dependent on accurate selection of patients with a suboptimal response, evaluating the effect of PGE1 on $P2Y_{12}$ blockade is very relevant as inaccurate selection individuals at risk of recurrent adverse events could conceivably compromise the clinical benefit of a personalised strategy in randomised trials employing assays that utilise PGE1 as agonist.

1.14.3 Response variability to antiplatelet therapy over time

Few studies have investigated the variability of response to APT over time and results so far are conflicting. It is therefore unknown whether individual responses remain stable

over time. A single predischarge assessment of response to APT may be sufficient if responses remain stable over time in patients established on DAPT. Conversely, if this is not the case then the optimal interval for performing platelet function testing remains to be established. We therefore hypothesised that responses to aspirin and clopidogrel remain stable over time and prospectively performed a longitudinal analysis of response to APT over a six-month period in patients established on APT.

1.14.4 HEAT-PPCI platelet substudy

The optimal antithrombotic regimen in PPCI has recently been a subject of intense debate. Whilst bivalirudin is considered to have a more favourable pharmacological profile compared to UFH, this has not translated into better protection against atherothrombotic events in RCTs. Conversely, concerns have been raised with respect to an early hazard of AST associated with bivalirudin. Furthermore, controversy exists with regards to the comparative platelet inhibitory effects of these anticoagulant agents. We therefore performed a predefined platelet function substudy of the HEAT-PPCI trial which randomised patients undergoing PPCI to either UFH or bivalirudin. The aim of this substudy was to compare the antiplatelet and anticoagulant effects of these anticoagulants based on PFAs including MEA and s-TEG.

1.14.5 Does AA-mediated clotting vary with inflammation

Serum TXB_2 is the gold standard biochemical test for evaluating the pharmacodynamic effect of aspirin on its pharmacological target, COX-1. However, several *ex vivo* PFAs assess response to aspirin in terms of AA-mediated clotting, which may be flawed. This group has previously described a dynamic ability of AA to induce clotting even when TXB_2 has effectively been suppressed by aspirin. This observation can be wrongly interpreted as "aspirin resistance" based simply on an AA-mediated test in isolation. We sought to investigate: (a) COX-1-independent mechanisms for AA-mediated clotting; (b) whether the dynamic, recruitable nature of this response to AA could be associated with the inflammatory status. We used a model of "on/off" vascular inflammation offered by studying patients undergoing elective major vascular surgery.

CHAPTER 2: METHODOLOGY

2.1 Study participants

The study participants included in our (i) pooled analysis comparing s-TEG to VerifyNow P2Y12 assay, (ii) *in vitro* evaluation of PGE1 with s-TEG, (iii) HEAT-PPCI platelet substudy, and (iv) prospective clinical studies evaluating platelet function performed at University Hospital Southampton (UHS) are outlined below. The inclusion and exclusion criteria for each study are described in their individual sections in the results chapters.

2.1.1 Pooled analysis comparing s-TEG to VerifyNow

Previous studies were undertaken by Dr Alex Hobson in 43 healthy volunteers and 143 patients with either stable CAD or those undergoing angiography as potential candidates for PCI. In addition to s-TEG, all participants in this cohort underwent simultaneous assay with VerifyNow. Furthermore, we also included 27 patients from a prospective study evaluating variability in platelet reactivity over time in patients treated with DAPT (Section 2.1.4 A) who also underwent simultaneous testing with VerifyNow and s-TEG. I performed a pooled analysis of data obtained from these experiments.

2.1.2 Effects of PGE1 and $P2Y_{12}$ blockade on ADP-mediated aggregation *in vitro*

I prospectively recruited 7 healthy volunteers on no medical therapy at the William Harvey Research Institute, Barts and the London School of Medicine and Dentistry.

2.1.3 HEAT-PPCI Platelet Substudy

Of the 1,812 patients enrolled to the HEAT-PPCI study in the Liverpool Heart and Chest Hospital, 457 patients had evaluable platelet function data from either MEA, s-TEG or both and comprised the substudy cohort. I performed an analysis of results obtained from this platelet substudy.

2.1.4 Prospective clinical studies

I prospectively recruited patients admitted to the Wessex Cardiothoracic Centre and the Vascular Surgical Unit at UHS, and enrolled the following subjects: -

(A) Response variability to APT over time - 40 patients scheduled to continue DAPT for at least 6 months following hospital discharge.

(B) AA-mediated clotting in vascular surgery - 26 patients established on aspirin therapy, undergoing major elective vascular surgery have completed the study so far. For the study to be adequately powered, a minimum sample size of 40 patients had been estimated (see section 7.2.4). Further recruitment of the remaining 14 subjects therefore remains outstanding, however, results for the patients who have completed the study so far have been presented in Section 7.

2.2 Research ethics and approval from other local and national bodies

Studies relating to the pooled analysis in section 2.1.1 and the prospective clinical study detailed in Section 2.1.4 (A) were approved by the Southampton and Southwest Hampshire Research Ethics Committees. *In vitro* experiments described in section 2.1.2 were covered by St. Thomas's Hospital Research Ethics Committee 07/Q0702/24, which provides blanket approval for obtaining blood samples from healthy volunteers on no medical therapy for *in vitro* experiments. Study (B) in section 2.1.4 was approved by the Essex, East of England Research Ethics Committee.

The HEAT-PPCI study uniquely employed "delayed informed consent" in which patients were randomly allocated treatment and underwent angiography in an emergency setting whereby no attempt was made to discuss the trial or to seek consent during this phase. Surviving patients or their appropriate representatives were subsequently approached for formal consent to continue as trial participants, to use their data and to allow contact for the 28 day follow up. This strategy was approved by the National Research Ethics Service (North West, Liverpool East) and the UK Medicines and Healthcare Products Regulatory Agency. Moreover, given the high early hazard associated with STEMI a number of patients died after randomisation but before consent could be obtained. The study had

approval from the National Confidentiality Advisory Group to include clinical data and outcome measures from these patients. A substantial amendment to the study protocol relating to obtaining a second blood sample on the day after PPCI was approved by the Liverpool East, North West Research Ethics Committee.

In addition, all studies were approved and sponsored by the UHS Research and Development department and registered on the National Institute for Health Research (NIHR) Clinical Research Network Portfolio database. The only exception to this was the *in vitro* studies described in section 2.1.2, which were performed entirely at the William Harvey Research Institute.

2.3 Consent

Written informed consent was obtained prior to enrolment for all studies listed in section 2.1, with the exception of the HEAT-PPCI platelet substudy, where a strategy of delayed consent was employed (Section 2.2), and the *in vitro* studies described in section 2.1.2 where verbal consent was obtained before blood sampling by venepuncture.

2.4 Study Methods

2.4.1 Blood sampling

I personally performed blood sampling as described below, in approximately 75% of the participants in the prospective clinical studies described in Section 2.1.4. I am grateful for the help of three medical students Rand Mikael (BMedSci student), Kala Thayalasamy (BMedSci student), and Alexander Ashby (MMedSci student) who performed blood sampling in 25% of the participants sampled at the UHS site. I also performed venesection in all participants recruited to the *in vitro* studies described in section 2.1.2 which were performed at the William Harvey Research Institute. In the historical cohorts (Groups A –E) included in the pooled analysis of studies comparing s-TEG to VerifyNow (section 2.1.1), venesection was performed by Dr Alex Hobson. Finally, in the HEAT-PPCI platelet substudy (section 2.1.3) blood sampling was performed entirely by the research team at the Liverpool Heart and Chest Hospital.

Venesection was performed from the antecubital fossa in the majority of cases. In some patients undergoing major vascular surgery (i.e. section 2.1.4 B), blood sampling was performed either via an arterial line in the radial artery or a jugular central venous catheter *in situ* during the early post-operative phase. Similarly, blood sampling at the end of the PCI procedure was undertaken via the arterial sheath in patients recruited to the HEAT-PPCI study (section 2.1.3). During venesection blood was taken with an 21-gauge needle with the assistance of a disposable tourniquet. Using a three-way tap, the first 2ml of blood were drawn into a 5 ml syringe and discarded as per the manufacturer's instructions. Similarly, if blood sampling was performed via an arterial line or a central venous catheter, then the first 10 ml of blood aspirated with a 10ml syringe was discarded. Thereafter, blood was collected for performance of the experiments as follows: -

1) In the majority of instances blood sampling was performed in the vicinity of the TEG installation. Consequently, there was no need for the use of citrate to arrest clotting prior to sample analysis for assessing thrombin-mediated clotting with TEG. In these instances, 2 ml of blood was collected in a 5 ml syringe without use of anticoagulant.
2) Where blood sampling was performed at the point of care, remote from the TEG installation, blood samples for assessing thrombin-mediated clotting were collected in 2 ml 3.2% sodium citrate vacutainers (citrate which chelates calcium and therefore arrests clotting). Sample analysis was deferred for at least 15 minutes following collection as per the manufacturer's instructions. This method of preserving samples prior to analysis was employed in the HEAT-PPCI substudy (section 2.1.3) and vascular surgery study (section 2.1.4 B)
3) Blood was also collected in a 6 ml lithium heparin (102 units) vacutainer and gently inverted 5 times before sample analysis for TEG platelet mapping was performed.
4) For VerifyNow, blood was collected in two 2 ml 3.2% sodium citrate vacutainers and allowed to incubate for a period (30 minutes for the Aspirin assay and 10 minutes for the P2Y12 assay) prior to sample analysis as per the manufacturer's instructions.
5) For studies (A) and (B) in section 2.1.4, which required the measurement of serum TXB_2 and inflammatory biomarkers additional blood was collected into two serum

separating tubes (SSTs), using the BD vacutainer collection system. These samples were centrifuged after 30 minutes of collection at 1000 X g for 15 minutes following which aliquots of 250 µl were immediately frozen at -80°C for future batch analysis. Details are described in section 2.4.5.

6) For MEA in the HEAT-PPCI substudy, blood was collected in 3ml vacutainers containing recombinant hirudin at a specified concentration of > 15 µg/ml.

2.4.2 TEG platelet mapping

Samples were analysed using a computerised TEG Analyser (Haemonetics Corp., Braintree, MA, USA) (Figure 1.2B). Electronic quality controls (eTest) were performed daily for all channels. The manufacturer also provides two biological controls (i.e. Level I and Level II) that provide a standard of reference for normal and abnormal anticoagulation patterns, which were performed monthly in each channel. The controls contain animal citrated whole blood, including platelets and plasma, stabilisers, and buffer. These are lyophilised and are reconstituted with distilled water before using them as samples in the presence of calcium chloride. Level I is specifically formulated to simulate normal blood, whilst Level II simulates an abnormal blood sample of a bleeding patient. The results of blood samples obtained from patients are considered unreliable if the controls produce tracings with parameters outside the normal reference range provided by the manufacturer for each control. In the event of a failure to obtain the expected values, both the biological controls were repeated with a fresh batch to exclude deterioration of biological controls as a potential reason for the failure. Technical support from the manufacturer could potentially be sought if biological controls failed repeatedly despite using separate batches.

All reagents were allowed to reach room temperature before being reconstituted and were then utilised within 120 minutes. The four standard TEG platelet mapping channels are: (a) Kaolin (the “thrombin” channel): kaolin stimulates clotting via the intrinsic coagulation pathway leading to thrombin generation and maximal platelet activation as thrombin is a potent platelet agonist, (b) Activator F™ (Act F) (the “fibrin channel): a mixture of reptilase and factor XIIIa, which generates fibrin clot without causing platelet activation (c) ADP: a combination of Activator F™ and ADP (2µM), (d) AA: a combination of Activator F™ and AA (1mM).

In the HEAT-PPCI platelet substudy (section 2.1.3), and the AA in vascular surgery study section 2.1.4 [B]), we collected blood samples for assessing thrombin-mediated clotting in citrate as the TEG installation was remote to the point of care. Thrombin-mediated clotting was assessed with kaolin in the presence of 20 µl 0.2 M calcium chloride (CK channel). Furthermore, as a proportion of patients in these studies received heparin we used an additional channel in which we analysed kaolin-activated citrated blood in the presence of heparinase (i.e. blue cup and pin coated with heparinase), to neutralise the effects of heparin (CKH channel).

For the assessment of thrombin-mediated clotting, 1ml of whole blood (either plain blood in the absence of anticoagulant or citrated) was mixed by gentle inversion in a vial containing 1% kaolin solution (Haemonetics Corp., Braintree, MA, USA). Thereafter kaolin-activated blood was analysed in one or more of the following ways:

i) *Kaolin (K)* - 360 µl of kaolin-activated blood is manually pipetted into a plain cup (i.e. not coated with heparinase)
ii) *Citrated kaolin (CK)* – 340 µl of kaolin-activated citrated blood was manually pipetted into a plain cup (i.e. without heparinase) and mixed with 20 µl of 0.2 M calcium chloride.
iii) *Citrated kaolin with heparinase (CKH)* – 340 µl of kaolin-activated citrated blood is manually pipetted into a blue cup (coated with heparinase) and mixed with 20 µl of 0.2 M calcium chloride.

For the remaining three TEG platelet mapping channels (i.e. fibrin, ADP and AA), 10 µl of Activator F™ was manually pipetted into each plain cup. 10µl of AA (1 mM) was placed in the AA channel cup and 10 µl of ADP (2 µM) was placed in the ADP channel cup. 360 µl of heparinised blood was then added to the fibrin, AA and ADP channel cups and mixed with the reagents. Samples were run at least until the MA had been reached but in the majority of instances until 60 minutes had elapsed.

In studies evaluating the effects of PGE1 on ADP mediated aggregation (i.e. section 2.1.1 – Group F or Section 2.1.2), 10 µl of PGE1 (Sigma, Poole, UK) was used in addition to standard concentrations of Activator F™ and ADP to achieve the desired concentration of PGE1 in whole blood (i.e. 11 nM or 22 nM).

2.4.3 VerifyNow

Testing with VerifyNow in the retrospective pooled analysis from section 2.1.2 (Groups A-E) was performed by Dr Alex Hobson. In the prospective studies looking at response variability to APT over time (section 2.1.4 [A]) VerifyNow analysis was performed predominantly by myself with the assistance of medical students as detailed in section 2.4.1. Electronic quality control was carried out on a daily basis. Level 1 and Level 2 Quality Controls were carried out on every new batch of both Aspirin and P2Y12 assays (i.e. every 25 assays). Vacutainers were mixed gently by inversion 5 times immediately after filling and incubated at room temperature prior to analysis as outlined in section 2.4.1. The vacuette was inserted in a single-use disposable cartridge (assay-specific) containing a lyophilised preparation of human fibrinogen-coated beads, a platelet agonist, buffer and preservative. The aspirin assay contains AA (1 mM) as the agonist and reports results as aspirin reaction units (ARU). The VerifyNow P2Y12 assay employs PGE1 (22 nM) in addition to ADP (20 µM) and results are expressed as P2Y12 reaction units (PRU).

2.4.4 Multiple Electrode Aggregometry

Testing with MEA for the HEAT-PPCI substudy was performed exclusively by the research team at the Liverpool Heart and Chest Hospital. Electronic quality control was performed on a daily basis. Whole blood (300 µl) anticoagulated with hirudin was diluted 1:1 with 0.9% saline pre-warmed to 37°C before stirring for 3 minutes. Thereafter, platelet aggregation was initiated by addition of ADP (6.5 µM) or AA (0.5 mM) to the test cells. The change in impedance resulting from the adhesion and aggregation of platelets on the surface of two silver-coated highly conductive copper electrodes was analysed in each sensor unit for 6 minutes. This is plotted against time, which is used to derive area under the aggregation curve (AUC) expressed in arbitrary aggregation units (U). All materials including reagents were obtained from the manufacturer (Roche diagnostics, Mannheim, Germany).

2.4.5 Serum Thromboxane B_2

Serum TXB_2 levels were measured in duplicate by competitive enzyme immunoassay using commercially available kits from R&D Systems (Abingdon, UK) according to the manufacturer's instructions. Measurement of serum TXB_2 levels for the variability in response to APT study (section 2.1.4 [A]) was performed by Dr Nicola Englyst at the Institute of Developmental Sciences, Faculty of Medicine, University of Southampton. Measurement of serum TXB_2 levels is part of the protocol for the AA in vascular surgery study (section 2.1.4 [B]), however as enrolment to this study is incomplete at present, batch analysis of frozen serum samples remains outstanding.

2.4.6 Inflammatory biomarkers

Measurement of inflammatory biomarkers including CD40 ligand (CD40L), high sensitivity C-reactive protein (hS-CRP), Interleukin-6 (IL-6), and tumour necrosis factor (TNF)-α is a prespecified component of the AA in vascular surgery study (section 2.1.4 [B]). Moreover, we have collected and stored serum samples at -80°C for future batch analysis as outlined in section 2.4.1. However, as enrolment to this study is incomplete at present, batch analysis of frozen serum samples remains outstanding.

2.5 Study definitions

Previous studies employing TEG platelet mapping measured percentage platelet inhibition (%PIn) to define response to APT, as described in section 1.11.3. The cutoff values of <50% for aspirin and <30% for clopidogrel were associated with subsequent ischaemic events[239, 240]. This group has previously shown that percentage clotting inhibition (%CIn) described in section 1.11.7 correlates well with %PIn and, as such, the above thresholds have been used to define HPR in these studies.

In VN-P2Y12 assay, a subject was defined as having HPR for a measured PRU of ≥ 230. This cutoff was reported as the optimal cutoff to define HPR by VN-P2Y12 in a collaborative meta-analysis[283]. This cutoff was subsequently used in the GRAVITAS trial

for defining HPR[212, 289]. In pooled analysis comparing s-TEG to VerifyNow, an additional alternative cutoff of PRU ≥ 208 was also evaluated in our study because this lower level more recently has been reported as being predictive of future adverse cardiovascular events (ACE) in patients with stable CAD[211, 291, 339]. For the VerifyNow™ Aspirin assay, a subject was defined as having HPR for a measured ARU ≥ 550[340].

For the MEA ADPtest a patient was defined as having HPR for an AUC > 46 U based on previous studies[203, 262] and the latest consensus document of the Working Group for On-Treatment Platelet Reactivity[341].

Previous studies have shown that administration of 100 mg aspirin daily leads to more than 98% steady-state inhibition of platelet COX-1 activity and has been associated with residual serum TXB_2 concentrations of less than 10 ng/ml[342]. This cutoff is therefore consistent with an adequate response to aspirin[335] and has been used in these studies.

2.6 Statistical analysis

Statistical advice was sought from medical statisticians Dr Borislav Dimitrov and Scott Harris at the Public Health Sciences & Medical Statistics department, University of Southampton. Continuous variables are presented as the mean ± 95% confidence interval (CI) of the mean or mean ± standard error of the mean[52] for normally distributed data. Normality of data was confirmed using the Shapiro-Wilk test. If not normally distributed, continuous variables are presented as median and interquartile range (IQR). Categorical variables are presented as frequencies (percentages). At all times a p value of <0.05 was considered to represent significance. Statistical analyses were performed using SPSS versions 19, 20 and 21 (IBM Corp., Armonk, NY, USA).

The statistical methods employed for each study are described in their individual sections in the results chapters.

CHAPTER 3: RESULTS

Does the VerifyNow P2Y12 assay overestimate "therapeutic response" to Clopidogrel?

Insights using short thrombelastography

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Summary

In contrast to short thrombelastography (s-TEG) which utilises adenosine diphosphate (ADP) alone, the VerifyNow P2Y12 assay (VN-P2Y12) additionally uses prostaglandin E1 (PGE1) as agonist to assess response to P2Y12 inhibitors. Based upon previous observations, we hypothesised that VN-P2Y12 overestimates the therapeutic effects of clopidogrel. Simultaneous assay with s-TEG and VN-P2Y12 was performed in 43 healthy volunteers and 170 patients either on or off clopidogrel. Furthermore, in 27 patients on clopidogrel 75 mg we compared the effects of adding 22 nM PGE1 to ADP on platelet aggregation in s-TEG to ADP alone. A higher proportion of individuals had a result indicating high platelet reactivity (HPR) with s-TEG than VN-P2Y12 in (i) 43 clopidogrel naïve volunteers (95.3% vs 81.4%, p = NS); (ii) 28 volunteers loaded with clopidogrel 600 mg (39.3% vs 10.7 %, p = < 0.01); (iii) 123 clopidogrel naïve patients (93.5% vs 78%, p = < 0.0001); (iv) 47 patients on clopidogrel 75 mg (42.6% vs 4.3%, p = < 0.0001). In 59 patients loaded with clopidogrel 600 mg/900 mg, a greater proportion had a "therapeutic response" with VN-P2Y12 compared to s-TEG, regardless of the threshold for defining HPR with VN-PY12 (P2Y12 reaction units ≥ 230 or 208). Furthermore, adding PGE1 to ADP in s-TEG potentiated the anti-aggregatory effects of clopidogrel compared with ADP alone. In conclusion, VN-P2Y12 overestimates the functional effects of clopidogrel in some individuals, possibly because it utilises PGE1 in addition to ADP. This could have implications for the ability of VN-P2Y12 to stratify patients as "responders" or "non-responders" to clopidogrel.

Keywords

Clopidogrel, high platelet reactivity, thrombelastography, VerifyNow P2Y12 assay, prostaglandin E1

3.1 Introduction

Platelet activation and aggregation play a key role in the pathophysiological development of MI and ST. In patients undergoing PCI with stents, a combination of aspirin and a $P2Y_{12}$ receptor inhibitor, most commonly clopidogrel, represents essential therapy. However, response to clopidogrel between individuals is heterogeneous[155, 156], and studies have consistently shown an association between HPR and subsequent adverse ischaemic events[209, 210, 212, 296, 341]. These factors have been a driver for the introduction of stronger, faster acting $P2Y_{12}$ inhibitors such as prasugrel and ticagrelor[94, 100]. Although these agents confer reduced risk of ischaemic events in some patients, they are also associated with increased rates of bleeding[343].

One management strategy in PCI patients would therefore be to give all patients clopidogrel, but assess individual platelet reactivity with a view to tailoring therapy with stronger agents in those patients with HPR. This strategy is dependent upon the availability of a simple, accurate point of care test of platelet function. Recent large randomised trials including GRAVITAS[289], ARCTIC[292] and TRIGGER-PCI[291] have all employed the VN-P2Y12 assay to detect HPR in order to modify therapy. VN-P2Y12 is a turbidimetric whole blood assay that measures agglutination of fibrinogen-coated beads in response to a combination of prostaglandin E1 (PGE1) and the platelet agonist adenosine diphosphate (ADP)[344]. All the randomised trials of tailoring therapy using VN-$P2Y_{12}$ to detect and stratify patients with HPR have failed to demonstrate a positive outcome for this strategy.

This group has previously observed in a population who have had DES ST that some patients exhibited HPR on $P2Y_{12}$ inhibitors using s-TEG[304], yet had a response in the "therapeutic range" for VN-P2Y12. As a result of this observation we therefore speculated that VN-P2Y12 may be overestimating the degree of inhibition of ADP-induced blood clotting in some individuals, possibly as a result of the presence of PGE1 (in addition to ADP) in the VN-P2Y12 assay. The aims of the experiments reported here were as follows: (i) to compare simultaneous responses to ADP in s-TEG and VN-P2Y12 in healthy volunteers on and off clopidogrel; (ii) to compare simultaneous responses to ADP in s-TEG and VN-P2Y12 in patients on and off clopidogrel; and (iii) to test whether adding PGE1 to the ADP in s-TEG would lead to an increase in the apparent "therapeutic response" to clopidogrel in patients compared to ADP stimulation alone.

3.2 Methods

3.2.1 Study population

Exclusion criteria for healthy volunteers

a) an antiplatelet agent or non-steroidal anti-inflammatory (NSAID) drug within 14 days

b) a history of peptic ulceration, asthma, or bleeding

Exclusion criteria for patients

i. an NSAID or antiplatelet agent other than those specified in the study

ii. anticoagulation medication in the preceding 28 days

iii. a history of clopidogrel intolerance, bleeding diathesis or major haematological disturbance precluding the use of DAPT

iv. planned use of GP IIb/IIIa inhibitors during PCI

v. STEMI

3.2.2 Study protocols

Simultaneous assay with s-TEG and VN-P2Y12 was performed in 43 healthy volunteers and 170 patients either on or off clopidogrel. A breakdown of the participants in each group, their antiplatelet regimen and the time points at which sampling was undertaken is provided in Table 3.1.

Table 3.1 Summary of the number of subjects in each population, their health status, antiplatelet regimen and schedule of sampling

Group	N	Population	Clopidogrel	Aspirin	Sampling	Study
A	15	Healthy Volunteers	None	No		Sambu et al[245]
B	28	Healthy Volunteers	600 mg LD	No	Pre-LD and 6 hours after LD	Hobson et al[301]
C	64	Patients with stable CAD	None	75 mg OD		Sambu et al[245]
D	59	Patients being considered for elective PCI	LD 600 mg (n=30); or LD 900 mg (n=29)	75 mg OD	Pre-LD and 1, 2, 6 and 24 hours after LD	Hobson et al[301]
E	20	Patients post-PCI	75 mg OD	75 - 150 mg OD		Sambu et al[245]
F	27	Post angiography +/- PCI	75 mg OD	75 - 150 mg OD	24 hours post-angiography	Khanna et al[345]

CAD, Coronary artery disease; PCI, Percutaneous coronary intervention; OD, once daily; LD, Loading dose

3.2.3 Blood sampling and analysis

Venesection and sample analysis were performed as specified in the Methods Section 2.4.1. In addition, in Group F an extra channel was used, which contained not only standard concentrations of Activator F and ADP, but also 22 nM PGE1, the same concentration of PGE1 used in the VN-P2Y12 assay.

3.2.4 Definition of high platelet reactivity

HPR was defined with s-TEG as percentage clotting inhibition (% Cln) $AUC15_{ADP} \leq 30\%$[241, 246]. For VN-P2Y12, a subject was defined as having HPR for a measured PRU of ≥ 230. This cutoff was reported as the optimal cutoff to define HPR by VN-P2Y12 in a collaborative meta-analysis[283]. This cutoff was also used in the GRAVITAS trial for defining HPR[212, 289]. In addition, an alternative cutoff of PRU ≥ 208 was also evaluated in our study because this lower level more recently has been reported as being predictive of future MACE in patients with stable CAD[211, 291, 339].

The derivation and validation of percentage % CIn is described in detail in section 1.11.7. Briefly, %CIn $AUC15_{ADP}$ is calculated using the formula:

$$\% \text{ CIn } AUC15_{ADP} = 100 - [(AUC15_{ADP} / AUC15_{Thrombin}) \text{ X } 100]$$

Similarly, % CIn $AUC15_{ADP/PGE1}$ is calculated using the following formula:

$$\% \text{ CIn } AUC15_{ADP/PGE1} = 100 - [(AUC15_{ADP/PGE1} / AUC15_{Thrombin}) \text{ X } 100]$$

3.2.5 Statistical analysis

Continuous variables are presented as the mean +/- 95% confidence interval (CI) of the mean, unless stated otherwise. Correlation between continuous variables was assessed using Pearson's correlation. Categorical variables are presented as percentages. Agreement between the two assays was assessed by Cohen's kappa coefficient (κ). Values of $\kappa < 0.4$ are considered to denote poor agreement, and > 0.7 good agreement. McNemar's test was used to determine if the proportions of patients exhibiting a response in the "therapeutic range" were significantly different with VN-P2Y12 compared to s-TEG. Comparison of paired means of any parameter over time was done using the paired two-tailed t-test. A p value < 0.05 was considered to represent statistical significance. Statistical analyses were performed using SPSS version 19 (IBM Corp., Armonk, NY, USA).

3.3 Results

Simultaneous assay by s-TEG and VN-P2Y12 was undertaken in 43 healthy volunteers and 170 patients either on or off clopidogrel and the key parameters measured by both assays are summarised in Table 3.2. A larger proportion of individuals had a result indicating HPR with s-TEG than VN-P2Y12 and this was significant in all populations except in clopidogrel naïve volunteers (Table 3.2).

Table 3.2 Summary of key results with s-TEG and VN-P2Y12 and rates of HPR

Population	N	Mean +/- 95% confidence interval of the mean			High Platelet Reactivity (HPR)		
		MA_{ADP}	$AUC15_{ADP}$	PRU	VN-P2Y12	s-TEG	P value
Clopidogrel naïve volunteers (Groups A and B)	43	61.5 ± 2.4	1000.3 ± 65	255.9 ± 13.3	81.4 % (35)	95.3 % (41)	NS
Volunteers after taking clopidogrel (Group B)	28	34.8 ± 6.9	511.9 ± 118.3	104.1 ± 28.8	10.7 % (3)	39.3 % (11)	< 0.01
Clopidogrel naïve patients (Groups C and D)	123	60.9 ± 1.7	1045.1 ± 39.7	257.5 ± 7.7	78 % (96)	93.5 % (115)	< 0.0001
Patients taking clopidogrel (Groups E and F)	47	46.4 ± 3.9	687.5 ± 79.5	123.3 ± 17.4	4.3 % (2)	42.6 % (20)	< 0.0001

3.3.1 *Correlation and agreement*

The correlation between PRU and $AUC15_{ADP}$ was poor to strong (correlation coefficient between 0.22 and 0.74) depending on the population analysed (Table 3.3). The correlation between PRU and % CIn $AUC15_{ADP}$ also varied between weak and strong (correlation coefficient between 0.26 to – 0.77) (Figure 3.1, Figure 3.2 and Table 3.3).

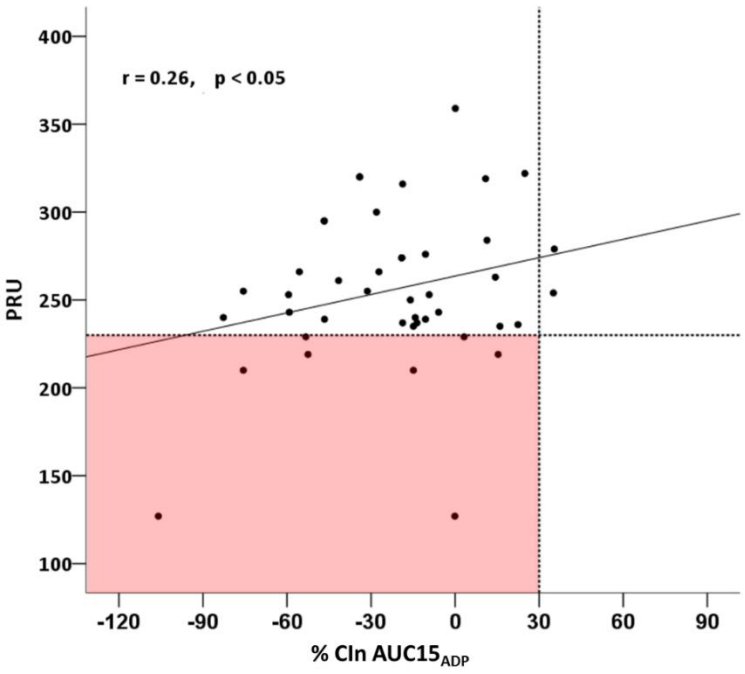
Table 3.3 Correlation, agreement, concordance and discordance between results from s-TEG and VN-P2Y12 in different populations of healthy volunteers and patients either on or off clopidogrel. The underlined results represent the primary source of discordance between VN-P2Y12 and s-TEG.

Population	N	Correlation coefficient (r)		Agreement (κ)	Concordant Results		Discordant Results	
		PRU and AUC15	PRU and % CIn $AUC15_{ADP}$	PRU and % CIn $AUC15_{ADP}$	HPR with VN-P2Y12 and s-TEG	NPR with VN-P2Y12 and s-TEG	NPR with VN-P2Y12 but not s-TEG	NPR with s-TEG but not VN-P2Y12
Clopidogrel naïve volunteers (Groups A and B)	43	0.22	0.26 †	-0.08	76.7 % (33)	0 (0)	**18.6 % (8)**	4.7 % (2)
Volunteers after taking clopidogrel (Group B)	28	0.74 ††	-0.77 ††	0.31 †	10.7 % (3)	60.7 % (17)	**28.6 % (7)**	0 (0)
Clopidogrel naïve patients (Groups C and D)	123	0.42 ††	-0.22 †	0.20 ††	75.6 % (93)	4.1 % (5)	**17.9 % (22)**	2.4 % (3)
Patients taking clopidogrel (Groups E and F)	47	0.37 ††	-0.21	0.07	4.3 % (2)	55.3 % (26)	**38.3 % (18)**	2.1 % (1)

HPR, high platelet reactivity; NPR, normal platelet reactivity (therapeutic response); †† P < 0.005; † P < 0.05

Agreement between VN-P2Y12 and s-TEG as assessed by Cohen's kappa coefficient was poor (κ between –0.08 to 0.313) depending on the population analysed (Table 3.3). Overall, the concordance between VN-P2Y12 and s-TEG results was in the range of 59.6% – 79.7% (Table 3.3). Discordance in results reported by these assays was primarily driven by participants who had HPR with s-TEG, but in whom VN-P2Y12 reported a response in the "therapeutic range" (i.e. PRU ≥ 230) (Figure 3.1, Figure 3.2 and Table 3.3). Whilst this trend was observed in all populations, it included those subsets who were clopidogrel naïve at the time of testing, in some of whom VN-P2Y12 suggested that they were "responding" to the drug that they had never taken. Specifically, of the 43 volunteers naïve to clopidogrel at the time of testing, a response in the "therapeutic range" was reported by VN-P2Y12 in 8 (18.6%) compared to 2 (4.7%) with s-TEG. Furthermore, of the 123 patients who hadn't taken any clopidogrel at the time of testing, a response in the "therapeutic range" was reported by VN-P2Y12 in 27 (22%) compared to 8 (6.5%) with s-TEG.

A



B

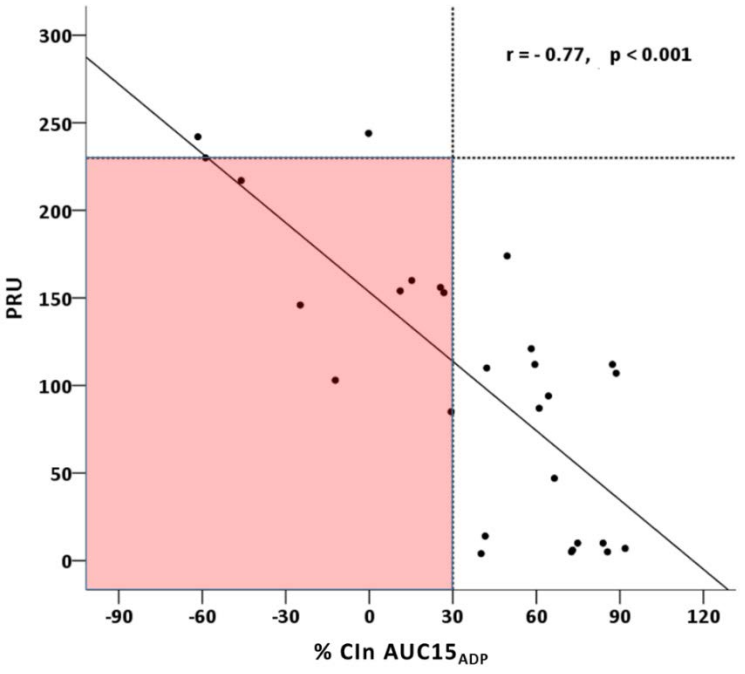


Figure 3.1 Scatter graphs illustrating correlation and agreement between PRU and % CIn $AUC15_{ADP}$ in healthy volunteers: (A) who have not received any clopidogrel, (B) who have been loaded with clopidogrel 600 mg. The shaded area represents a region in which VN-P2Y12 reports "therapeutic response" (NPR) to clopidogrel whilst s-TEG shows HPR.

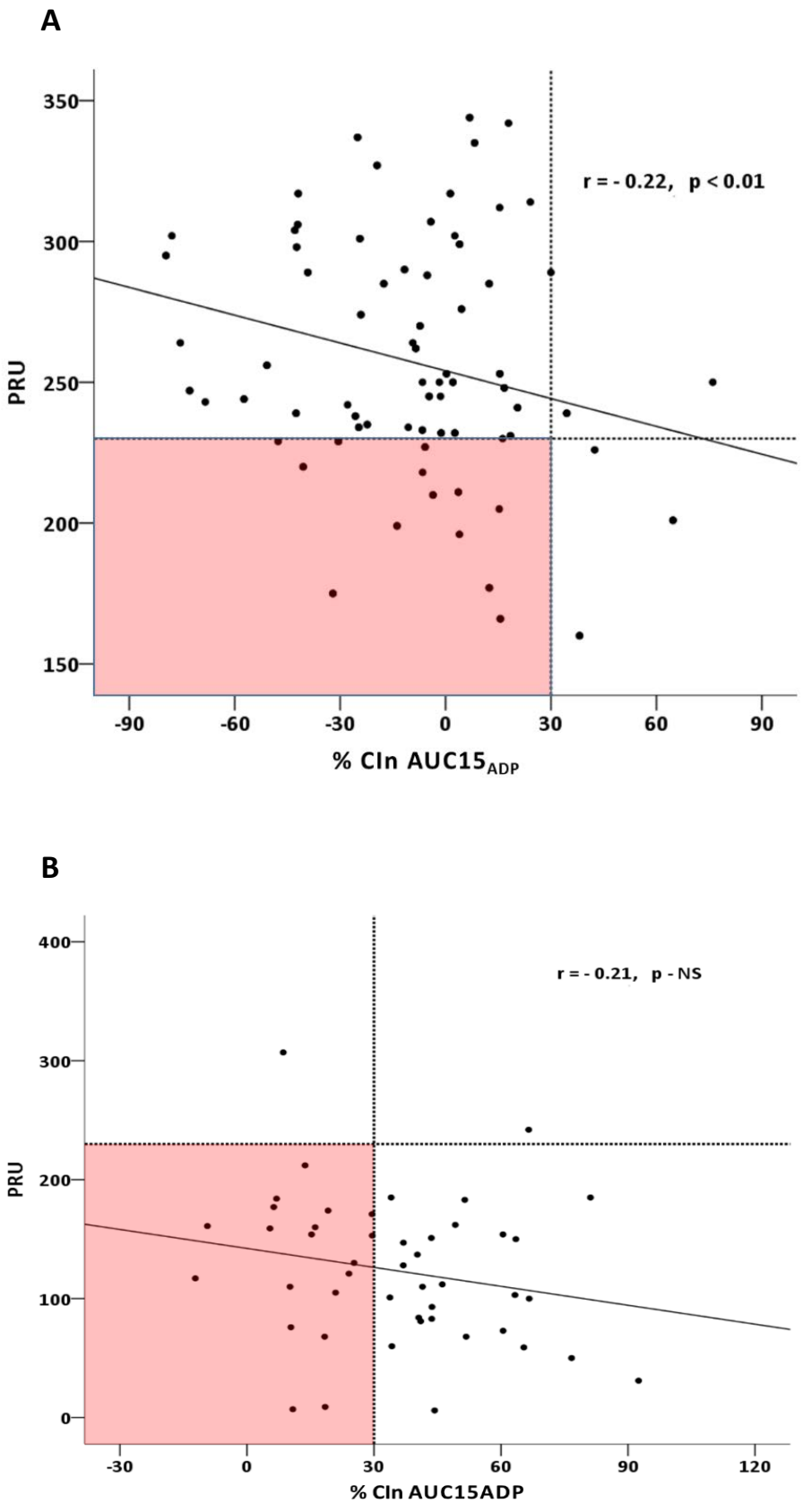


Figure 3.2 Scatter-graphs illustrating correlation and agreement between PRU and % CIn $AUC15_{ADP}$ in patient populations: (A) who have not received any clopidogrel, (B) who have been loaded with clopidogrel 600 mg. The shaded area represents a region in which VN-P2Y12 reports "therapeutic response" (NPR) to clopidogrel whilst s-TEG shows HPR.

3.3.2 Patients loaded with Clopidogrel 600 mg/900mg

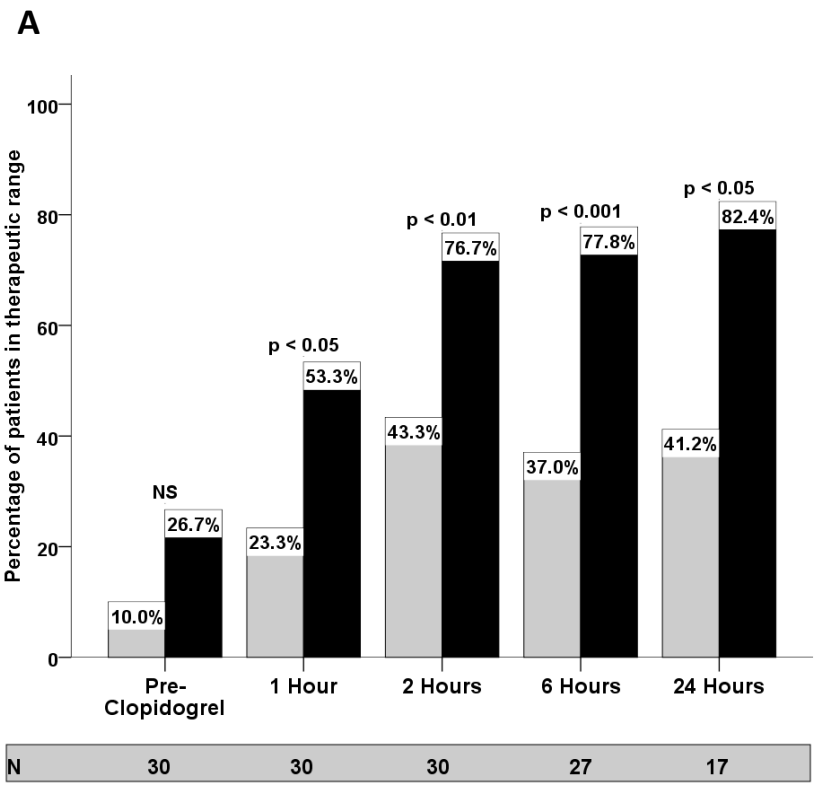
Simultaneous assay by s-TEG and VN-P2Y12 was undertaken in 59 patients being loaded with clopidogrel 600 mg/900 mg (Group D) as summarised in Table 3.4. Data was available for analysis in 89.3% (clopidogrel 600 mg) and 90.3% (clopidogrel 900 mg) of the planned data points. Missing data were due either to early hospital discharge or to having received abciximab/bivalirudin during PCI. Following the loading dose of clopidogrel 600 mg/900 mg, both $AUC15_{ADP}$ and PRU reduced significantly in a time-dependent manner (Table 3.4, Figure 3.3 and Figure 3.4).

Table 3.4 $AUC15_{ADP}$ and PRU at baseline and four time points after preloading 59 patients with clopidogrel 600 mg/900 mg. Results are represented as mean ± 95% CI of the mean. The p value given is for comparison with the baseline time point before administration of clopidogrel.

	Loading with Clopidogrel 600mg			**Loading with Clopidogrel 900mg**		
	N	**$AUC15_{ADP}$**	**PRU**	**N**	**$AUC15_{ADP}$**	**PRU**
		Mean ± 95% CI	**Mean ± 95% CI**		**Mean ± 95% CI**	**Mean ± 95% CI**
Baseline	30	1010.9 ± 80.4	252.6 ± 16.8	29	1079 ± 72.9	270.9 ± 15.2
1 hour	30	904.3 ± 77.8 †	220.9 ± 21.6 †	29	879.1 ± 94.2 ††	213.2 ± 27.9 ††
2 hours	30	738.9 ± 119 ††	158.7 ± 30.4 ††	29	745.6 ± 101.2 ††	152.3 ± 27.3
6 hours	27	738.7 ± 101.4 ††	153.3 ± 32.4 ††	25	632.3 ± 101.8 ††	121.1 ± 27.4 ††
24 hours	17	822.5 ± 128.2 †	146.7 ± 39 ††	19	728.7 ± 112.8 ††	103.2 ± 30.5 ††

† *P < 0.05;* †† P < 0.005; CI, *confidence interval*

In patients being loaded with clopidogrel 600 mg a response in the "therapeutic range" was seen at baseline (i.e. pre-loading) in 8 patients (26.7%) by VN-P2Y12 compared to 3 patients (10%) with s-TEG (p = NS). In the clopidogrel 900mg subgroup, 3 patients (10%) had a response in the "therapeutic range" with VN-P2Y12 at baseline in contrast to none with s-TEG.



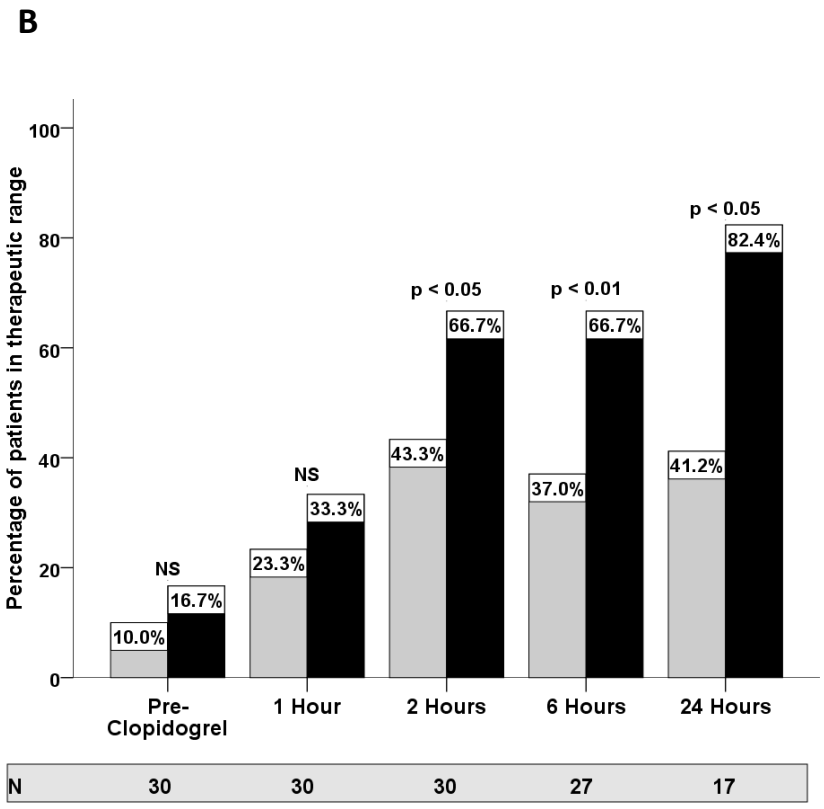


Figure 3.3 Comparison of s-TEG and the VN-P2Y12 assay before and at four time points after loading 30 patients with clopidogrel 600 mg. (A) Percentage of patients in therapeutic range (% CIn $AUC15_{ADP} \geq 30\%$ - shaded grey, and PRU ≤ 230 – shaded black), (B) Percentage of patients in therapeutic range (% CIn $AUC15_{ADP} \geq 30\%$ - shaded grey, and PRU ≤ 208 – shaded black).

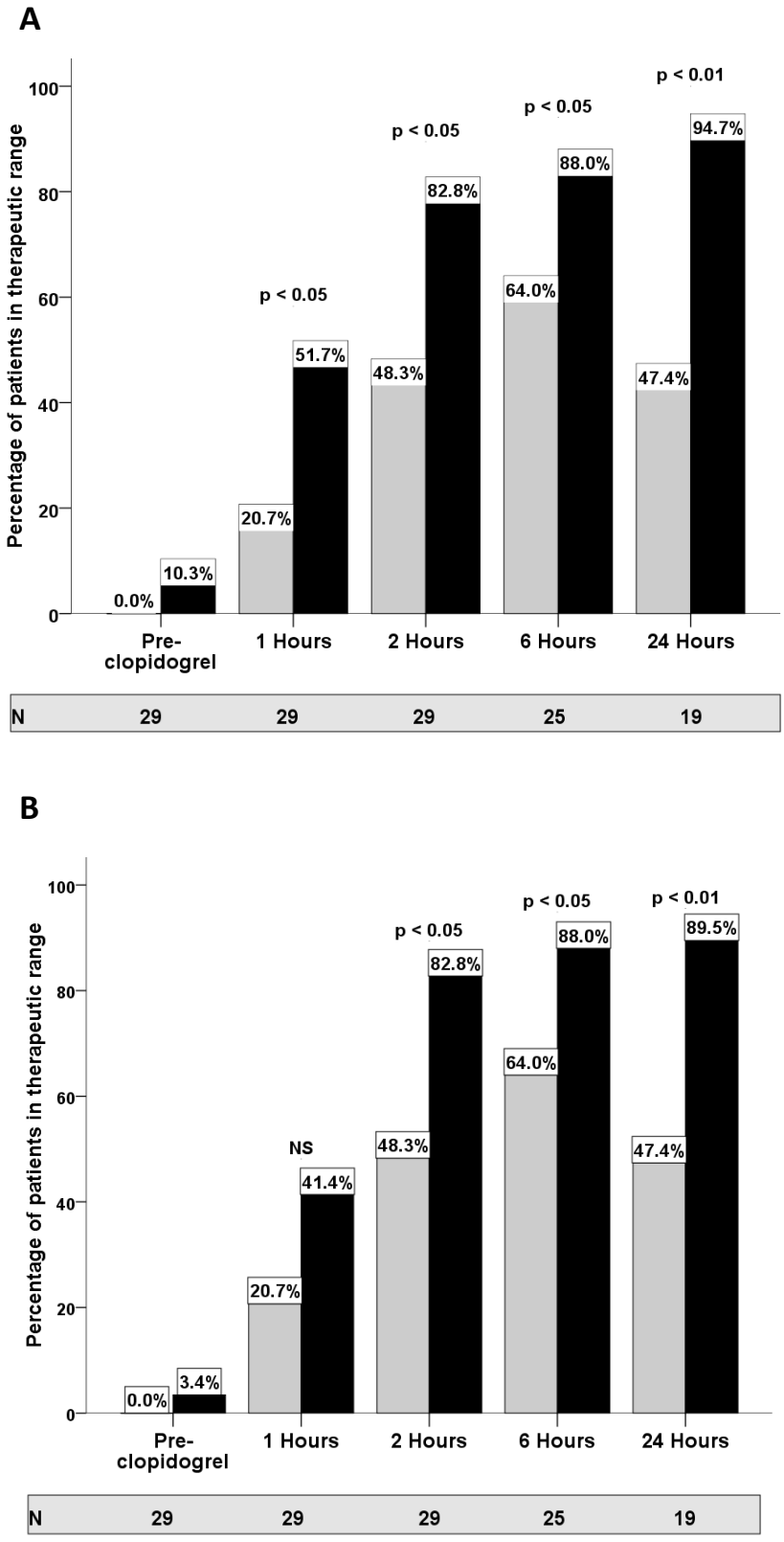


Figure 3.4 Comparison of s-TEG and VN-P2Y12 before and at four time points after loading 29 patients with clopidogrel 900 mg. (a) Percentage of patients in therapeutic range (% CIn $AUC15_{ADP}$ ≥ 30% - shaded grey, and PRU ≤ 230 – shaded black), (b) Percentage of patients in therapeutic range (% CIn $AUC15_{ADP}$ ≥ 30% - shaded grey, and PRU ≤ 208 – shaded black).

Using the therapeutic threshold of % CIn $AUC15_{ADP}$ > 30% and PRU < 230, there was a statistically significant difference in the proportion of patients exhibiting a "therapeutic response" with VN-P2Y12 and s-TEG at all time points subsequent to loading with clopidogrel 600 mg/900 mg (Figure 3.3A and 3.4A). Even when using the PRU threshold of 208, this discrepancy persisted at the 2, 6 and 24-hour time points in all patients being loaded with clopidogrel (Figure 3.3B and 3.4B).

3.3.3 Effect of PGE1 on ADP-mediated aggregation

In 27 patients on DAPT the *ex vivo* clotting response to ADP alone was compared in the s-TEG assay with responses to a combination of ADP and PGE1 (Group F). The mean $AUC15_{ADP}$ (Figure 3.5A) was significantly lower and correspondingly the mean % CIn $AUC15_{ADP}$ (Figure 3.5B) was significantly higher in the PGE1/ADP channel compared to the ADP-only channel. Furthermore, the response of 6 patients lies in the "therapeutic range" of s-TEG when using % CIn AUC15 in the ADP/PGE1 channel, but was in a range indicating HPR (i.e. hyporesponsive) when evaluated in the ADP-only channel. The degree of concordance between VN-P2Y12 and s-TEG improves from 70.4% to 92.6% when using AUC15 from the ADP/PGE1 channel as opposed to the ADP-only channel.

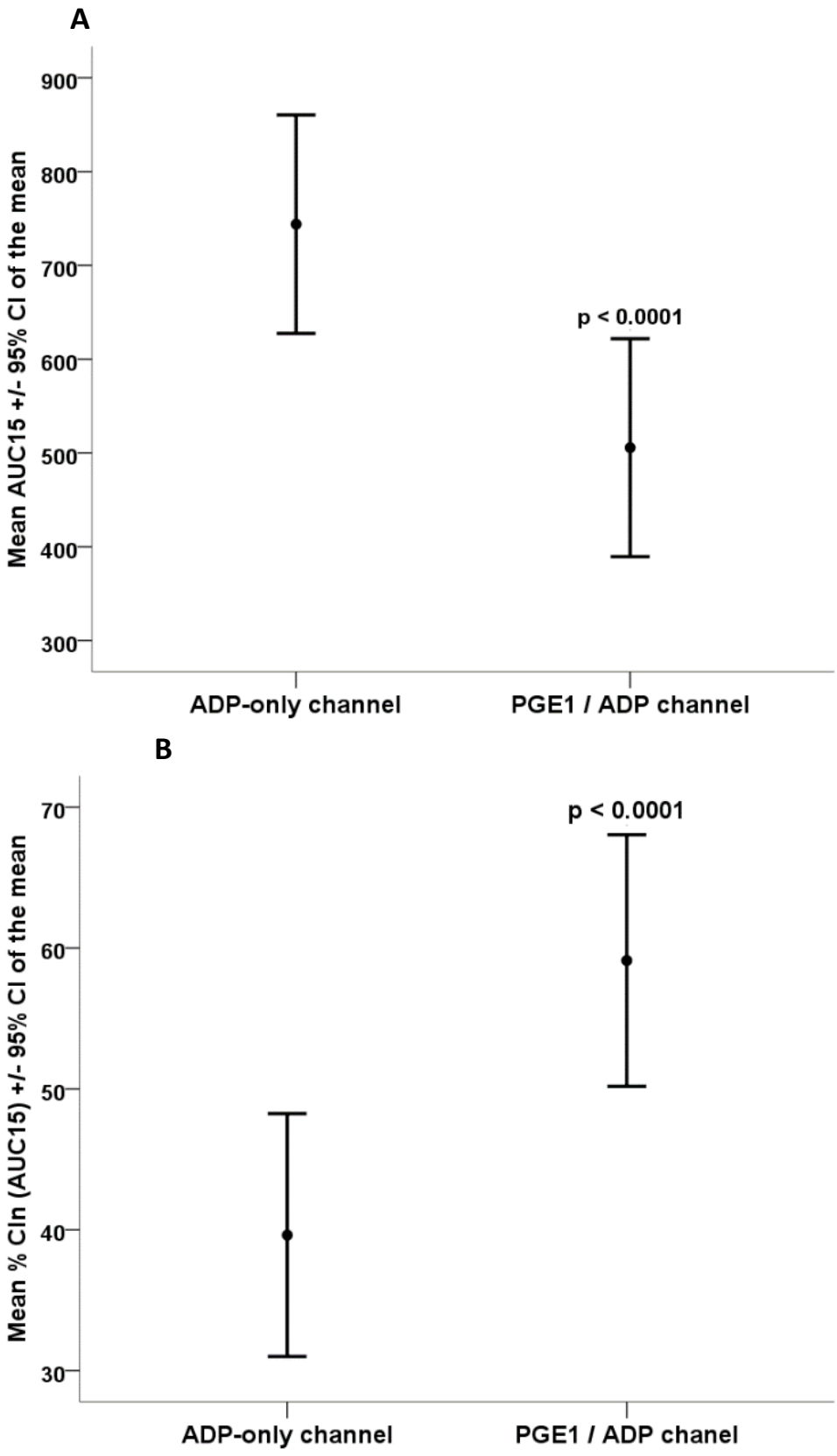


Figure 3.5 Error bar graphs comparing (A) AUC15, and (B) % CIn AUC15 in ADP/PGE1 channel to ADP-only channel in patients on DAPT

3.4 Discussion

This study describes the following key results. Firstly, that VN-P2Y12 finds a larger proportion of individuals taking clopidogrel are within its "therapeutic range" than s-TEG. Secondly, that this excess in reported clopidogrel "responders" with VN-P2Y12 compared to s-TEG is consistent in healthy volunteers as well as patients and is seen regardless of whether these populations were taking clopidogrel or not. Thirdly, that even when the PRU threshold denoting HPR is arbitrarily decreased from PRU ≥ 230 to PRU ≥ 208, VN-P2Y12 reported a larger proportion of subjects within its "therapeutic range" than s-TEG. Finally, one possible mechanism for the observed excess of apparent responders to clopidogrel with VN-P2Y12 is that when PGE1 is added to ADP in s-TEG we observed a consistent reduction in whole blood clotting, thus simulating an apparently greater "therapeutic response" compared to ADP alone.

Given that there is a very well described association between HPR and thrombotic events in patients requiring $P2Y_{12}$ inhibitors[346], the options for reducing the risk for those individuals who do not achieve a therapeutic response are limited, especially since robust definitions of HPR remain contentious[347]. The requirement for a simple, rapid point-of-care test of platelet function is clear cut if a strategy of personalised $P2Y_{12}$ inhibitor therapy is to be delivered accurately. Amongst a range of candidates, VN-P2Y12 has emerged as the only truly POC test that accurately describes, in a snapshot fashion, the platelet reactivity response of an individual to $P2Y_{12}$ therapy. Nevertheless, it is notable that recent randomised trials employing VN-P2Y12 have not shown any benefit for this "tailoring of therapy" approach[289, 291, 292]. However, these trials have been critically dependent for the intervention at randomisation upon a binary allocation of each patient into two arms of 'adequate responder' or 'inadequate responder' to clopidogrel. It is clear that if the employed assay does not, in fact, accurately reflect the true clotting response of some patients to ADP-stimulation (even if it does reflect the degree of $P2Y_{12}$ inhibition), then a proportion of the trial population would be allocated incorrectly at randomisation.

The VN-P2Y12 assay has been compared with several platelet function assays including vasodilator-stimulated phosphoprotein (VASP) phosphorylation [348], Whole-blood aggregometry [248], Multiple electrode platelet aggregometry (MEA) [349], as well as the Platelet function analyzer (PFA-100). The outcome of these studies indicates modest

correlation at best. Although there are some studies showing a good correlation (correlation coefficients ranging from 0.62 to 0.86) between LTA and VN-P2Y12 [214-217], this has not been consistent in patients with stable CAD [248].

The consistency of our data present a persuasive case that VN-P2Y12 overestimates response to clopidogrel in some subjects. Inevitably this leads us to speculate about a possible mechanistic explanation for these observations. The main difference between the assays is that s-TEG uses ADP alone, whereas VN-P2Y12 uses a combination of ADP and PGE1 as agonists. Given their opposing effects on platelet aggregation, PGE1 is a 'physiological antagonist' of ADP. Nevertheless, it is important to highlight that in the context of this platelet function assay, PGE1 is considered an agonist as it neither binds to nor activates the P2Y receptors responsible for expressing the proaggregatory effects of ADP.

The inclusion of PGE1 is based on the 'theoretical' justification that this makes the test more specific for $P2Y_{12}$ blockade by reducing the contribution of the $P2Y_1$ pathway on ADP-mediated platelet aggregation. ADP-mediated activation of the $P2Y_1$ receptor causes platelet aggregation by increasing phospholipase C-mediated mobilisation of Ca^{2+} from intracellular stores. PGE1 inhibits this pathway, and thus platelet aggregation, by stimulating adenylate cyclase-mediated production of cAMP. As $P2Y_{12}$ antagonists block ADP-mediated inhibition of adenylate cyclase, PGE1 would be expected to potentiate the antiaggregatory effects of a $P2Y_{12}$ inhibitor. Indeed, Fox *et al* have described the synergistic effects of combining PGE1 with a $P2Y_{12}$ blocker on inhibiting ADP-mediated increases in intracellular Ca^{2+} levels[350]. More pertinently however, they demonstrated that combining PGE1 with a $P2Y_{12}$ inhibitor (clopidogrel or cangrelor) produces a greater inhibitory effect on platelet aggregation than that seen with either agent on its own. Thus, the addition of PGE1 to ADP may inadvertently exaggerate the apparent therapeutic effects of clopidogrel observed by VN-P2Y12.

Based upon this potential mechanism, we assessed the effects of running simultaneous s-TEG channels with ADP alone or ADP plus PGE1 at a concentration (22 nM) replicating that in the VN-P2Y12 assay. The results confirm that adding PGE1 to ADP leads to reduced clotting and therefore to an apparently greater therapeutic effect of clopidogrel than is observed in the ADP-only channel. These results do suggest that VN-P2Y12, whilst indeed providing a pure assessment of the effect of $P2Y_{12}$ receptor inhibition, may not be

providing a true indication of the actual blood clotting consequence of taking clopidogrel. The suitability of this assay as the sole arbiter at the point of randomisation in an interventional trial of tailored $P2Y_{12}$ inhibitor therapy is therefore uncertain.

These experiments have several limitations. Firstly, the number of subjects is small. Secondly, the endpoints are assay-dependent rather than based on clinical outcomes. Thirdly, the studies were all conducted by a research team in a single centre. Finally, the investigators were unblinded. As such the data can be seen as hypothesis-generating only.

3.5 Conclusion

VN-P2Y12 produces an overestimate of the functional effects of clopidogrel in some individuals, possibly because it utilises PGE1 as an agonist in addition to ADP. This observation could have implications for the ability of the VN-P2Y12 assay to detect and stratify patients as “responders” or “nonresponders” to clopidogrel. Further mechanistic and clinical outcomes data are required.

CHAPTER 4: RESULTS

Prostaglandin E1 potentiates the effects of P2Y12 blockade on ADP-mediated platelet aggregation *in vitro*: Insights using short thromboelastography

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Abstract

In addition to adenosine diphosphate (ADP), a number of platelet function tests including the VerifyNow P2Y12 assay (VN-P2Y12) employ prostaglandin E1 (PGE1) to improve specificity for P2Y12 blockade by mitigating the contribution of the P2Y1 pathway on ADP-mediated platelet aggregation. Using short thromboelastography (s-TEG), we have previously shown that VN-P2Y12 overestimates the functional effect of clopidogrel in some individuals. We investigated whether PGE1 systematically increases the inhibitory effects of P2Y12 blockade on ADP-mediated platelet aggregation in an *in vitro* model. Using s-TEG, we measured ADP-induced platelet aggregation either in the presence or absence of PGE1 (11 or 22 nM) in blood samples taken from healthy volunteers pre-incubated with prasugrel active metabolite (PAM; 0, 1, 3 or 10 μM). Individually, both PGE1 ($p < 0.02$) and PAM ($p < 0.0001$) inhibited ADP-mediated platelet aggregation in a dose-dependent manner, as expected. Furthermore, inclusion of PGE1 augmented inhibition of ADP-mediated platelet aggregation in response to PAM ($p < 0.02$) in a dose-dependent manner such that a 10-fold higher dose of PAM was required to attain equivalent inhibition of ADP-mediated platelet aggregation to that achieved by 1 μM PAM in the presence of 11 nM PGE1. In conclusion, PGE1 potentiates the anti-aggregatory effects of P2Y12 blockade on ADP-mediated platelet aggregation. Assays that employ PGE1 with ADP may therefore overestimate therapeutic response to prasugrel in a proportion of individuals, potentially making them unsuitable candidates for guiding delivery of personalized antiplatelet therapy.

4.1 Introduction

A significant proportion of clopidogrel-treated patients exhibit HPR[155, 156], which is associated with recurrent ischaemic complications[209, 210, 212, 341]. Several strategies to personalise $P2Y_{12}$ inhibitor therapy in patients with HPR on clopidogrel have been investigated. However, with the exception of two relatively small studies by Bonello *et al* which used the VASP phosphorylation assay[286, 287], all large randomised trials of tailored APT have failed to demonstrate a clinical benefit[290-292]. It is notable that these trials all employed the VN-P2Y12 assay. VN-P2Y12 is a turbidimetric whole blood assay that measures agglutination of fibrinogen-coated beads in response to 22 nM prostaglandin E1 (PGE1) and 20 µM ADP.

This group has extensively validated s-TEG, an *ex vivo* whole blood PFA, modified from TEG platelet mapping based on the novel parameter AUC15[245, 246, 298]. Using s-TEG, we have previously shown that VN-P2Y12 overestimates the functional effect of clopidogrel in some patients, possibly because it utilises PGE1 in addition to ADP[351] (Chapter 3). PGE1 is purported to make PFAs more specific for detection of $P2Y_{12}$ blockade by mitigating the contribution of the $P2Y_1$ pathway on ADP-mediated platelet aggregation. PGE1 acts by stimulating adenylyl cyclase activity resulting in raised intracellular cAMP levels[352]. As $P2Y_{12}$ antagonists block ADP-mediated inhibition of adenylyl cyclase, PGE1 would thus be expected to potentiate the antiaggregatory effects of $P2Y_{12}$ inhibitors. In our previous experiments in clopidogrel-treated patients, the addition of PGE1 to ADP potentiated the antiaggregatory effects of clopidogrel compared to ADP alone[351].

The aim of the current experiments was to investigate whether PGE1 systematically increases the platelet inhibitory effects of $P2Y_{12}$ blockade in an *in vitro* model.

4.2 Methods

4.2.1 Study population

Blood was collected from 7 healthy volunteers on no regular medications. The first 2 ml were discarded as normal, then blood was drawn into four 4 ml lithium heparin vacutainers. Aliquots of 2 ml heparinised blood were pre-incubated for 30 minutes at

37°C with either vehicle (0.1% dimethylsulfoxide [DMSO]), 1, 3 or 10 μM prasugrel active metabolite [353].

4.2.2 Blood sampling and analysis

Venesection and sample analysis were performed as specified in the Study Methods in Section 2.4.1. However, in this study the standard s-TEG methodology was adapted so that effects of PGE1 on ADP-induced platelet aggregation could be investigated. In addition to standard concentrations of Activator F and ADP (2 μM), we used either vehicle (0.1 M phosphate buffer), 11 or 22 nM PGE1.

4.2.3 Statistical analysis

Data are presented as mean ± standard error of the mean[52]. The interaction between PGE1 and PAM on ADP-mediated platelet aggregation (i.e. $AUC15_{ADP}$) was assessed by two-way repeated measures ANOVA followed by post-hoc t-tests using the Bonferroni adjustment. One-way repeated measures ANOVA was used for analysing the effect of a single factor (either PGE1 or PAM alone). A p value < 0.05 was taken to represent statistical significance. Statistical analyses were performed using SPSS Version 20 (IBM Corp., Armonk, NY, USA).

4.3 Results

Following pre-incubation with PAM or vehicle (0.1% DMSO), blood samples taken from 7 healthy volunteers were assayed with s-TEG using standard agonist concentrations (i.e. Activator F and ADP 2 μM) and either vehicle (i.e. 0.1 M phosphate buffer) or PGE1 at 11 or 22 nM.

4.3.1 PAM alone

In the absence of PGE1, PAM caused concentration-dependent inhibition of ADP-induced platelet aggregation ($p < 0.0001$), as expected (Figure 4.1). All concentrations of PAM

significantly inhibited ADP-induced platelet aggregation compared to vehicle. No significant differences were detected in ADP-mediated platelet aggregation between 1 μM and 3 μM PAM ($p = 0.06$) or 3 μM and 10 μM PAM ($p = 0.25$).

4.3.2 PGE1 alone

Overall, PGE1 also significantly inhibited ADP-induced platelet aggregation ($p < 0.02$) in the absence of PAM, although 11 nM PGE1 only had a small insignificant effect (Figure 4.1). Furthermore, there was no significant difference between the two PGE1 concentrations on ADP-mediated platelet aggregation in the absence of PAM ($p = 0.06$).

PGE1 with PAM

In combination, 11 nM PGE1 and 1 μM PAM reduced $AUC15_{ADP}$ from 768.1 ± 78.5 (no PAM or PGE1) to 185.4 ± 30.9. A 10-fold higher concentration of PAM (i.e. 10 μM) was required to achieve equivalent inhibition of ADP-mediated platelet aggregation in the absence of PGE1 (Figure 4.1).

Using PGE1 and PAM as factors in a two-way repeated measures ANOVA, both PAM ($p < 0.0001$) and PGE1 ($p < 0.00001$) significantly inhibited ADP-mediated platelet aggregation individually. Post-hoc analysis showed no significant differences between 3 and 10 μM PAM ($p = 0.51$). Whilst PGE1 significantly inhibited ADP-mediated platelet aggregation compared to vehicle (11 nM $p = 0.003$; 22 nM $p = 0.002$), there were also important differences between 11 nM and 22 nM PGE1 ($p = 0.008$). There were no significant differences in $AUC15_{ADP}$ at different doses of PAM (i.e. 1, 3 and 10 μM) in the presence of PGE1 (11 nM or 22 nM).

There was significant interaction between PAM and PGE1 as co-factors in a 2-way ANOVA ($p = 0.018$). Specifically, the null hypothesis that the effect of PAM on ADP-mediated platelet aggregation is the same at all doses of PGE1 can be rejected.

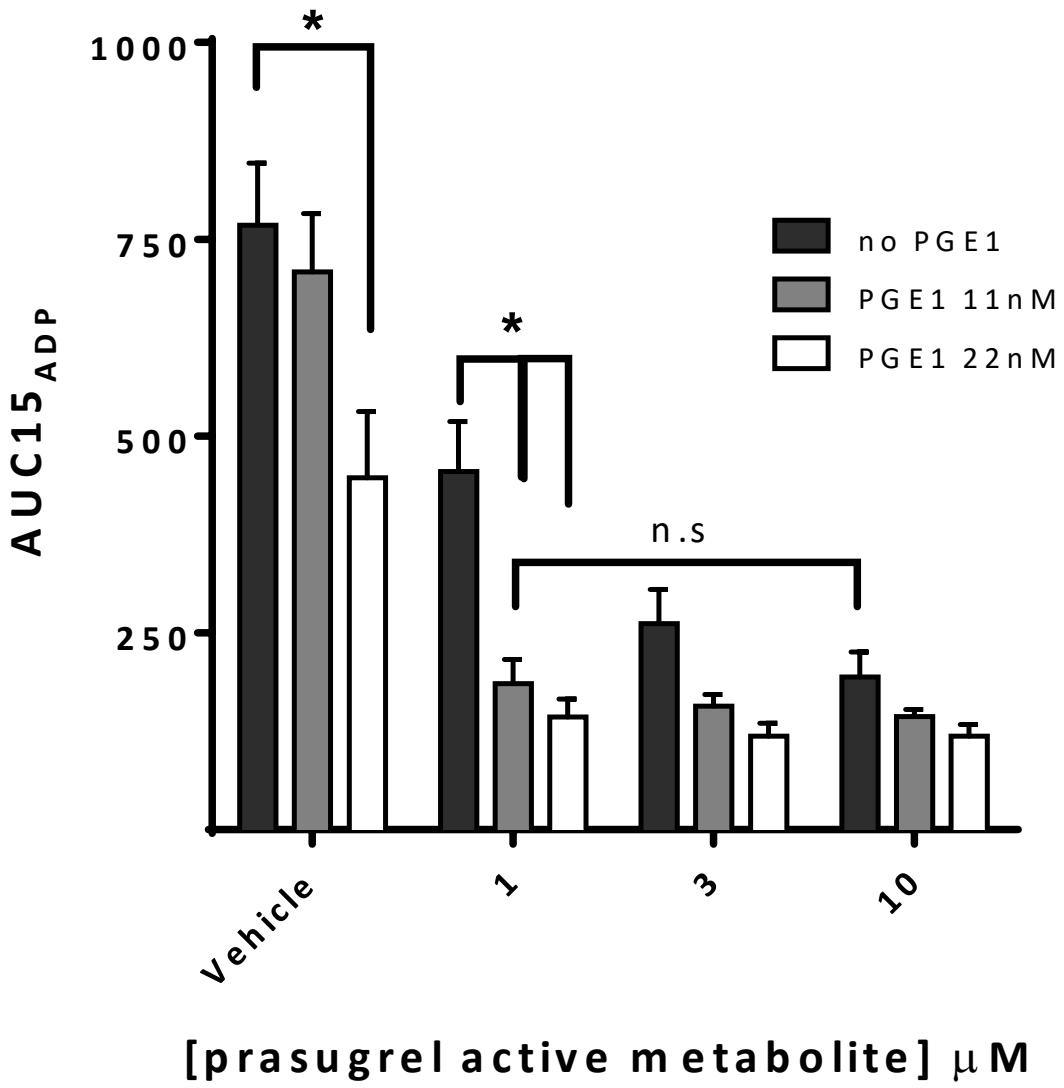

Figure 4.1 Bar graph showing ADP-mediated platelet aggregation (i.e. $AUC15_{ADP}$) in the absence (vehicle) or presence of PGE1 (11 nM or 22 nM after whole blood from 7 healthy volunteers was pre-incubated with prasugrel active metabolite (PAM; vehicle, 1, 3, or 10 μM). Data reported as mean ± SEM. (* $p < 0.05$)

4.4 Discussion

The results of this study support the hypothesis that PGE1 potentiates the antiaggregatory effects of PAM *in vitro* in a dose-dependent manner. This finding is consistent with both our previous data in clopidogrel-treated patients[351] and those of Fox *et al* who also demonstrated the synergistic effects of combining PGE1 with $P2Y_{12}$

blockers on ADP-mediated rises in $[Ca2+]_i$ and platelet aggregation[350]. Furthermore, studies have similarly shown that $P2Y_{12}$ antagonists increase the sensitivity of platelets to the inhibitory effects of other endogenous autacoids including prostacyclin[354] and nitric oxide[355].

$P2Y_1$ is a G_q-coupled receptor linked to phospholipase C-β (PLCβ) which catalyzes the cleavage of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) to generate the second messengers inositol 1,4,5- triphosphate (IP_3) and diacylglycerol (DAG)[32]. Whilst IP3 stimulates the mobilization of calcium (Ca^{2+}) from internal stores causing a rapid rise in intracellular Ca^{2+} levels, DAG causes direct protein kinase C (PKC) activation[32]. The P2Y12 receptor is coupled to $G\alpha_i$ which causes inhibition of adenylyl cyclase activity through activation of the $G\alpha_i$ G-protein subunit and phosphoinositide 3-kinase (PI3K) activity via activation of the γ,β-subunits [23].

Concomitant activation of both $P2Y_1$ and $P2Y_{12}$ receptors is considered essential to elicit a normal aggregatory response to ADP. However, $P2Y_1$ is reported to produce comparatively weak and transient aggregation whilst $P2Y_{12}$ is believed to play a more dominant role in ADP-mediated platelet aggregation[54]. In this study, we demonstrated that the dose of PAM required to achieve equivalent platelet inhibition to that attained with 10 µM PAM was reduced by a factor of ten in the presence of 11 nM PGE1. Given the modest effects of 11 nM PGE1, it is difficult to explain the profound effect PGE1 and PAM in combination have on ADP-mediated aggregation, if PGE1 is putatively only suppressing the $P2Y_1$ pathway in isolation. Moreover, there is increasing evidence of complex reciprocal cross-talk in the downstream signalling pathways of these two G-protein-coupled receptors (i.e. $P2Y_1$ and $P2Y_{12}$)[32, 40, 41]. The theory that inclusion of PGE1 suppresses the contribution of the $P2Y_1$ pathway on ADP-mediated platelet aggregation in isolation therefore seems implausible, particularly as PGE1 acts on the AC-cAMP pathway[350], a substrate coupled to the $P2Y_{12}$ receptor.

Overall, individual platelet function assays employ a unique design and methodology to assess a specific aspect of platelet function and measure this end point as a marker of response to APT in order to predict clinical outcomes. Each assay reports values on an arbitrary scale and within this dynamic range some assays may perform better or worse at different levels of $P2Y_{12}$ blockade. The VN-P2Y12 assay appears to have a limited dynamic range compared to LTA, the historical gold standard PFA, and lacks sensitivity at

extremes of platelet inhibition. Jakubowski *et al* described a limited dynamic range of the VN-P2Y12 assay where correlation with 20 µM ADP-induced platelet aggregation was best at intermediate levels of platelet inhibition, with divergence from linearity reported at either high or low levels of platelet inhibition[214]. Varenhorst *et al* also found low sensitivity of the VN-P2Y12 assay at high levels of inhibition of ADP-induced platelet aggregation with LTA[218]. Indeed, there are several reports confirming that VN-$P2Y_{12}$ and other devices that include PGE1 like the VASP phosphorylation assay are unable to differentiate between high levels of $P2Y_{12}$ inhibition detected by LTA, an assay that employs only ADP as agonist[219-221]. This is likely explained by the inclusion of PGE1 as agonist in the VN-P2Y12 assay and the demonstrated synergy between PGE1 and $P2Y_{12}$ blockade. In the present study, there were no significant differences in the platelet inhibitory effects of PAM at 1, 3 or 10 µM in the presence of PGE1 (11 nM or 22 nM).

In a recent study, Kreutz *et al* demonstrated that platelet aggregation in response to 20 µM ADP with LTA was significantly greater in patients with diabetes compared to those without diabetes on DAPT, but only in the presence of PGE1 (22 nM and 88 nM)[356]. Furthermore, platelet reactivity was similarly greater in patients with diabetes when response to clopidogrel was assessed with VN-P2Y12. Similarly, Chirkov *et al* noted that higher concentrations of PGE1 were required to reverse ADP-mediated platelet aggregation in patients with stable CAD compared to healthy volunteers[357]. This suggests that responses to PGE1 may be blunted in certain patient populations in whom PFAs that employ PGE1 could potentially classify them inappropriately as nonresponders.

Limitations of this study include a small sample size and the fact cAMP levels were not measured in parallel to elucidate the molecular basis for the observed synergy between PGE1 and $P2Y_{12}$ blockade.

4.5 Conclusion

PGE1 potentiates the antiaggregatory effects of PAM *in vitro*. PFAs that employ PGE1 with ADP are likely to overestimate the functional response to $P2Y_{12}$ inhibitors. The suitability of such assays for determining the adequacy of $P2Y_{12}$ inhibitors in trials of tailored APT is therefore uncertain. More clinical trial evidence is now required.

CHAPTER 5 – RESULTS

ORIGINAL ARTICLE

Does the response to aspirin and clopidogrel vary over 6 months in patients with ischemic heart disease?

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Summary. *Background:* Dual-antiplatelet therapy (DAPT) with aspirin and a P2Y12 inhibitor, mostly clopidogrel, is the default therapy in both acute coronary syndrome (ACS) and after intracoronary stents. It is well established that responses to antiplatelet therapy (APT), particularly clopidogrel, are subject to considerable interindividual variability. *Objectives:* We investigated whether responses to APT in individuals vary significantly over time. *Methods:* Simultaneous assay with VerifyNow™ and short thrombelastography (s-TEG) was performed before and at four time points over 6 months after hospital discharge in 40 patients receiving DAPT. Serum thromboxane B_2 levels were also measured. *Results:* While aspirin response units (ARU) by VerifyNow™ and serum thromboxane B_2 levels remained stable over time, arachidonic acid (AA)–mediated platelet aggregation with s-TEG (i.e. area under the curve at 15 min in AA channel, $AUC15_{AA}$) increased at 1 week compared with predischarge ($P < 0.008$). In addition, platelet reactivity units (PRU) by VerifyNow™ ($P = 0.046$) and adenosine diphosphate (ADP)–mediated platelet aggregation with s-TEG (i.e. $AUC15_{ADP}$) also increased at 1 week compared with predischarge ($P = 0.026$). There were no significant changes in either future response may therefore be flawed. The design of future strategies to assess individual responses for tailored therapy needs to take this into account.

Keywords: aspirin; clopidogrel; platelet aggregation; platelet function tests; thrombelastography.

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5.1 Introduction

In patients undergoing PCI with stents, the default APT strategy constitutes a combination of aspirin with a $P2Y_{12}$ inhibitor to reduce platelet reactivity (PR) in order to prevent adverse cardiovascular events, particularly ST. However, studies employing a variety of PFAs have reported wide variability in individual responses to APT[155, 156] and an association between HPR and increased risk of recurrent atherothrombotic events[176, 177, 212, 283, 296, 358]. Despite this, responses to APT are not assessed routinely in clinical practice for several reasons. Firstly, there is no universally accepted method for assessing a patient's response to APT. Secondly, there is no standardised definition for what constitutes "hyporesponsiveness" (i.e. HPR) as the cutoffs used in clinical studies are highly assay-specific, and poorly validated to ischaemic or bleeding events. Finally, there is insufficient evidence to support modifying APT in patients shown to have HPR since all large randomised trials investigating this tailored approach have failed to show benefit for a strategy of "personalised APT"[289, 291, 292].

Studies investigating the ability of PFAs to predict ischaemic events or to personalise APT have largely relied on a single isolated measurement of OTPR immediately before, during or soon after PCI. A small number of studies have documented the evolution of OTPR and efficacy of APT during follow up beyond one month. It therefore remains to be established whether individual responses to aspirin and clopidogrel vary significantly over time. This question has important clinical implications, particularly in relation to the stability of individual patient risk of ST.

In this study we performed a longitudinal analysis of response to APT over 6 months in patients with ACS[356] and stable CAD on DAPT[213]. The study employed both VerifyNow™ (Accumetrics Inc., CA USA) and s-TEG, two point-of-care assays that use whole blood for the assessment of *ex vivo* platelet function. The study hypothesis was that responses to aspirin and clopidogrel do not vary significantly over time, and therefore an isolated snapshot assessment of OTPR prior to hospital discharge can reliably identify patients with HPR who may benefit from individually tailored APT.

5.2 Methods

5.2.1 Study population

Patients listed for cardiac catheterisation as potential candidates for PCI (i.e. admitted electively with stable CAD or acutely with ACS) at University Hospital Southampton were eligible to participate in this study if they were scheduled to continue DAPT with aspirin and clopidogrel for at least 6 months following hospital discharge. Specific exclusion criteria were presentation with STEMI; use of glycoprotein IIb/IIIa inhibitors during hospital admission, severe bleeding diathesis, thrombocytopenia (< 100 x 10^9 cells/L); anaemia (< 10 d/dL); regular therapy with NSAIDs, steroids, anticoagulants or antiplatelet agents other than aspirin and clopidogrel. All patients were loaded with and maintained on aspirin and clopidogrel prior to cardiac catheterisation in accordance with international guidelines and local practice.

5.2.2 Study procedures

Simultaneous assay with VerifyNow and s-TEG was performed in 40 patients on the day after cardiac catheterisation but prior to hospital discharge (t0) and subsequently at 1 week (t1), 1 month (t2), 3 months (t3) and 6 months (t4). The time interval between loading with APT and first sampling (i.e. t0) was not standardised as this depended on the timing of cardiac catheterisation which was governed by the schedule of the cardiac catheterisation laboratory. Compliance with APT was encouraged through comprehensive patient education and standardised patient interviews at each follow up visit.

5.2.3 Blood sampling and analysis

Venesection and blood sampling were performed as detailed in the Study Methods (section 2.4.1). Agonist-mediated platelet aggregation was measured using s-TEG and VerifyNow. Furthermore, quantitaive determination of serum TXB_2 concentrations was performed, using competitive enzyme immunoassay.

5.2.4 Definitions

As discussed in the section 2.5, for s-TEG, the cutoffs % CIn $AUC15_{AA} \leq 50$ and %CIn $AUC15_{ADP} \leq 30\%$ have been used to define HPR on aspirin and clopidogrel respectively. Similarly, for VerifyNow, the cutoffs ARU ≥ 550 and PRU of ≥ 230 were used to define HPR on aspirin and clopidogrel respectively. Finally, a serum TXB_2 concentrations of less than 10 ng/ml on aspirin was used to define an adequate response to aspirin in this study.

5.2.5 Statistical analysis

Continuous variables are presented as the mean ± 95% confidence interval of the mean (CI) for normally distributed data or median and interquartile range (IQR) if not. Normality of data was confirmed using the Shapiro-Wilk test. Correlation between continuous variables was assessed using Pearson's correlation coefficient (r) for parametric data or Spearman's rank correlation coefficient (r_s) for non-parametric data. Statistical analysis of differences in continuous variables over time was performed using the repeated-measures analysis of variance (ANOVA) test followed by t-tests corrected for multiple comparisons using the Bonferroni adjustment. Categorical variables are presented as frequencies (percentages). Agreement within assays at different time points was assessed by the coefficient of agreement, Kappa (κ), where values of $\kappa \leq 0.4$ are considered to denote poor agreement, and > 0.7 good agreement. Heterogeneity in the proportion of patients exhibiting HTPR over the course of the study was analysed using the Cochran-Q test, and for comparison of two individual time points was analysed with McNemar's test. A p value < 0.05 was considered to represent statistical significance. Statistical analyses were performed using SPSS version 21 (IBM Corp., Armonk, NY, USA).

5.3 Results

Simultaneous assay with VerifyNow and s-TEG was undertaken in 40 patients at the predischarge baseline 23.7 ± 0.9 hours after cardiac catheterisation ± PCI (t0), 7.1 ± 0.7 days (t1), 31.4 ± 5.4 days (t2), 91.4 ± 4.0 days (t3) and 182 ± 6.2 days (t4). One patient

withdrew having completed four of the five study time points and another patient missed a single follow up visit. For s-TEG there was additional missing data due to errors related to sample processing. In total, of the 800 AUC15 measurements (4 channels, in 40 patients over 5 time points) there were 18 missing values (1.9 %). To avoid loss of power for longitudinal analysis, and based upon expert statistical advice, missing values were imputed using expectation maximisation based upon the appropriate reference group values. Similarly, for VerifyNow, 5 of the 400 measurements (including ARU and PRU from 40 patients at 5 time points) were missing (1.3 %) which were imputed as above. Finally, for serum TXB_2 levels there were 4 missing values out of a possible 200 measurements (2%) which were imputed as above.

5.3.1. Baseline characteristics

Baseline characteristics are summarised in Table 5.1. Most patients (80%) were maintained on aspirin 75 mg once daily and clopidogrel 75 mg once daily as their DAPT regimen, whilst in eight patients (20%) a higher maintenance dose of aspirin (i.e. 150 mg once daily) was used.

Table 5.1 Summary of baseline characteristics.

Variable	Study cohort (N=40)
Age (years)	59.2 +/- 12.4
Sex	
Male (%)	31 (77.5)
Female (%)	9 (22.5)
Risk Factors	
Body Mass Index (kg/m^2)	29.9 +/- 6.23
Diabetes Mellitus (%)	5 (12.5)
Hypertension (%)	20 (50)
Hyperlipidaemia (%)	34 (85)
History of cerebrovascular disease (%)	2 (5)
Previous myocardial infarction (%)	6 (15)
Previous PCI (%)	6 (15)
Previous bypass surgery (%)	6 (15)
Current smoker (%)	16 (40)
Ex-smoker (%)	14 (35)
Family history of premature CAD (%)	19 (47.5)
Diagnosis	
Acute coronary syndrome (%)	27 (67.5)
Stable CAD (%)	13 (32.5)
Management	
Medical management (%)	6 (15)
PCI (%)	34 (85)
Drug therapy on discharge	
Beta-blockers (%)	37 (92.5)
ACE-inhibitors (%)	28 (70)
Angiotensin receptor blockers (%)	6 (15)
Statins (%)	40 (100)
Calcium channel blockers (%)	9 (22.5)
Proton-pump inhibitors (%)	13 (32.5)
Antiplatelet therapy maintenance dose	
Aspirin 75 mg (%)	32 (80)
Aspirin 150mg (%)	8 (20)
Clopidogrel 75mg (%)	40 (100)
Laboratory investigations	
Haemoglobin (g/L)	145.1 +/- 12.9
White cell count x 10^9 /L	8.3 +/- 2.0
Platelet count x 10^9 /L	236 +/- 58.4
Creatinine clearance	78.2 +/- 11.2

ACE, angiotensin-converting enzyme; PCI, percutaneous coronary intervention; CAD, coronary artery disease

5.3.2 Variability within whole study population

5.3.2.1 Short TEG

The key parameters reported by s-TEG are summarised in Table 5.2 and Figure 5.1. Repeated-measures ANOVA test showed no statistically significant differences in $AUC15_{Thrombin}$ over time (p = 0.06). However, there were significant differences in $AUC15_{AA}$ (p = 0.05), $AUC15_{ADP}$ (p = 0.04), $AUC15_{Fibrin}$ (p = 0.001) over time. Pairwise evaluation (using Bonferroni's adjustment for multiple comparisons) showed that this effect was driven by a significant increase in values at 1 week compared to baseline for $AUC15_{AA}$ (p < 0.008) and $AUC15_{ADP}$ (p = 0.026). $AUC15_{Fibrin}$ was also significantly different between 1 week and 6 months (p = 0.002) (Table 5.3). Beyond the 1-week time point there were no further significant changes in $AUC15_{AA}$ or $AUC15_{ADP}$ (Table 5.3).

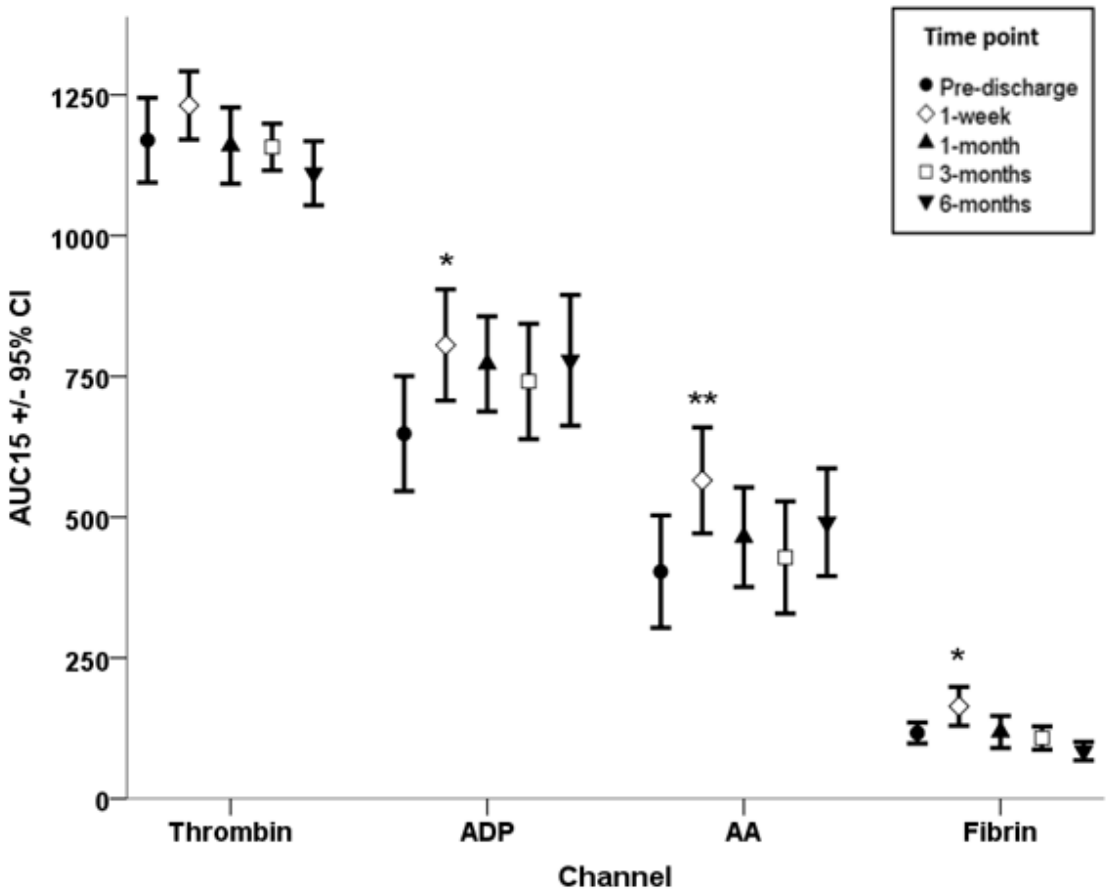


Figure 5.1 Error bar graph of changes in thrombin, ADP, AA and fibrin mediated clotting responses with s-TEG in 40 patients on DAPT over 6 months (* p < 0.05; ** p < 0.01).

5.2 Summary of key parameter measured over 6 months in patient on DAPT

	Short TEG								VerifyNow				TXB_2	
	$AUC15_{Thrombin}$		$AUC15_{ADP}$		$AUC15_{AA}$		$AUC15_{Fibrin}$		ARU		PRU		ng/ml	
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Pre-discharge	1169.5	72.9	648.3	99	403.3	96.6	116.7	18	446.3	25.0	120.6	19.2	1.75	0.47
1 week	1231.3	58.6	805.8	95.8	565.2	91.2	164	33.4	424.3	21.6	141	22.7	2.14	0.69
1 month	1159.9	65.7	772.1	81.8	464.4	85.7	118.5	27.6	443.6	22.3	148.7	19.4	2.12	0.74
3 months	1157.3	40.3	741.1	99.3	428.4	96.5	107.6	19.9	452.5	23.2	153	21.5	1.51	0.50
6 months	1110.8	55.2	778.6	112.5	490.9	92.7	84.4	15.8	457.5	25.9	140.2	21	1.71	0.85

Table 5.3 Pairwise evaluation of key parameters between individual time points corrected for multiple comparisons. († $p < 0.05$; †† $p < 0.01$)

Parameter	Time points	1 week	1 month	3 months	6 months
$AUC15_{Thrombin}$	**Pre-discharge**	0.87	1.0	1.0	1.0
	1 week		0.53	0.11	**0.035 †**
	1 month			1.0	1.0
	3 months				1.0
$AUC15_{ADP}$	**Pre-discharge**	**0.026 †**	0.11	0.21	0.26
	1 week		1.0	1.0	1.0
	1 month			1.0	1.0
	3 months				1.0
$AUC15_{AA}$	**Pre-discharge**	**0.008 ††**	1.0	1.0	1.0
	1 week		0.74	0.38	1
	1 month			1	1
	3 months				1
$AUC15_{Fibrin}$	**Pre-discharge**	**0.014 †**	1.0	1.0	0.07
	1 week		0.35	0.09	**0.002 ††**
	1 month			1.0	0.28
	3 months				0.60
ARU	**Pre-discharge**	0.47	1.0	1.0	1.0
	1 week		1.0	0.56	0.72
	1 month			1.0	1.0
	3 months				1.0
PRU	**Pre-discharge**	**0.046 †**	**0.002 ††**	**0.004 ††**	0.39
	1 week		1.0	1.0	1.0
	1 month			1.0	1.0
	3 months				0.38
Serum TXB_2	**Pre-discharge**	1.0	1.0	1.0	1.0
	1 week		1.0	0.6	1.0
	1 month			0.61	1.0
	3 months				1.0

The rates of HPR with s-TEG before hospital discharge were 30% on aspirin (Figure 5.2A) using % CIn $AUC15_{AA} < 50\%$ as the cutoff and 27.5% on clopidogrel (Figure 5.2B) using CIn $AUC15_{ADP} < 30\%$ as the cutoff. Using the Cochran Q test there was no significant

heterogeneity in the rates of HPR on aspirin (p = 0.53) overall across the study time points. By contrast, the proportion of patients exhibiting HPR on clopidogrel were significantly heterogeneous (p = 0.03). Specifically, the rates of HPR on clopidogrel at 1 week and 6 months (50%) were significantly higher (p = 0.01) than observed at baseline (27.5%) (Figure 5.2B). Furthermore, rates of HPR on clopidogrel at 1 month and 3 months (52.5%) were also significantly higher (p = 0.02) than reported at baseline (27.5%) (Figure 5.2B).

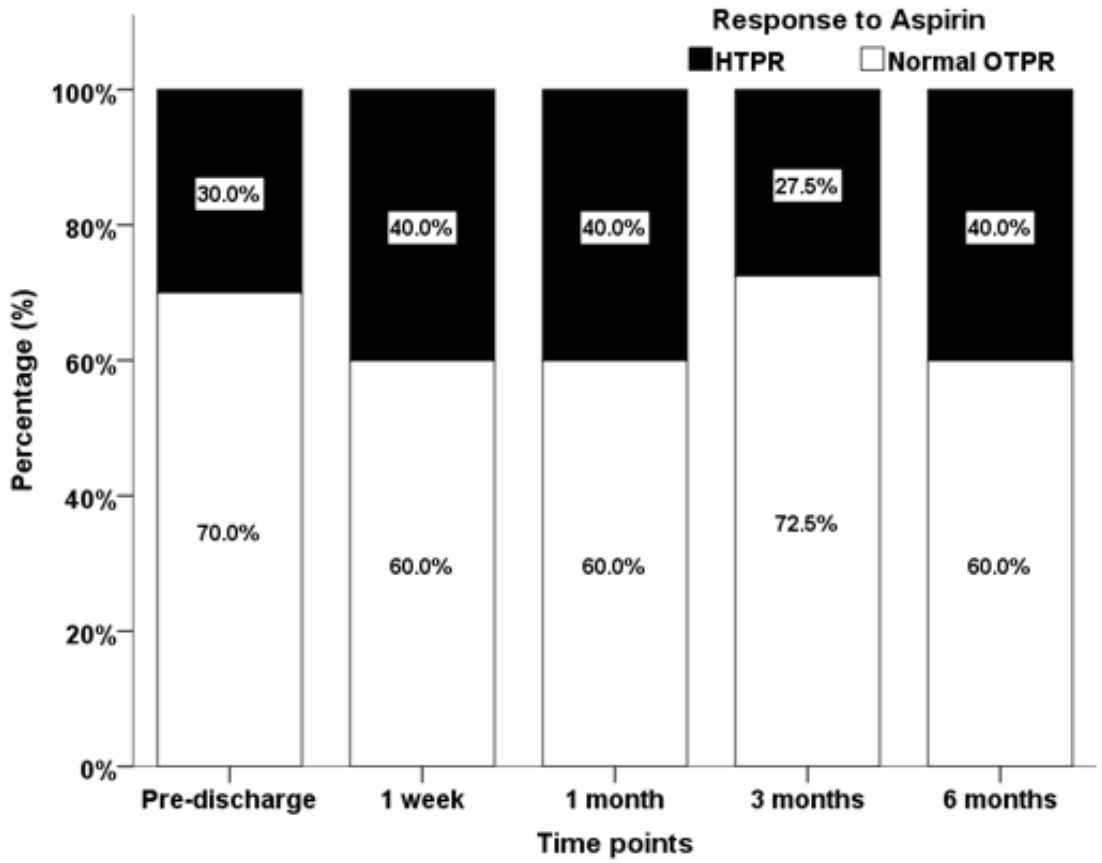


Figure 5.2A Bar chart displaying rates of normal OTPR (shaded white) and HTPR (shaded black) with s-TEG in 40 patients on aspirin

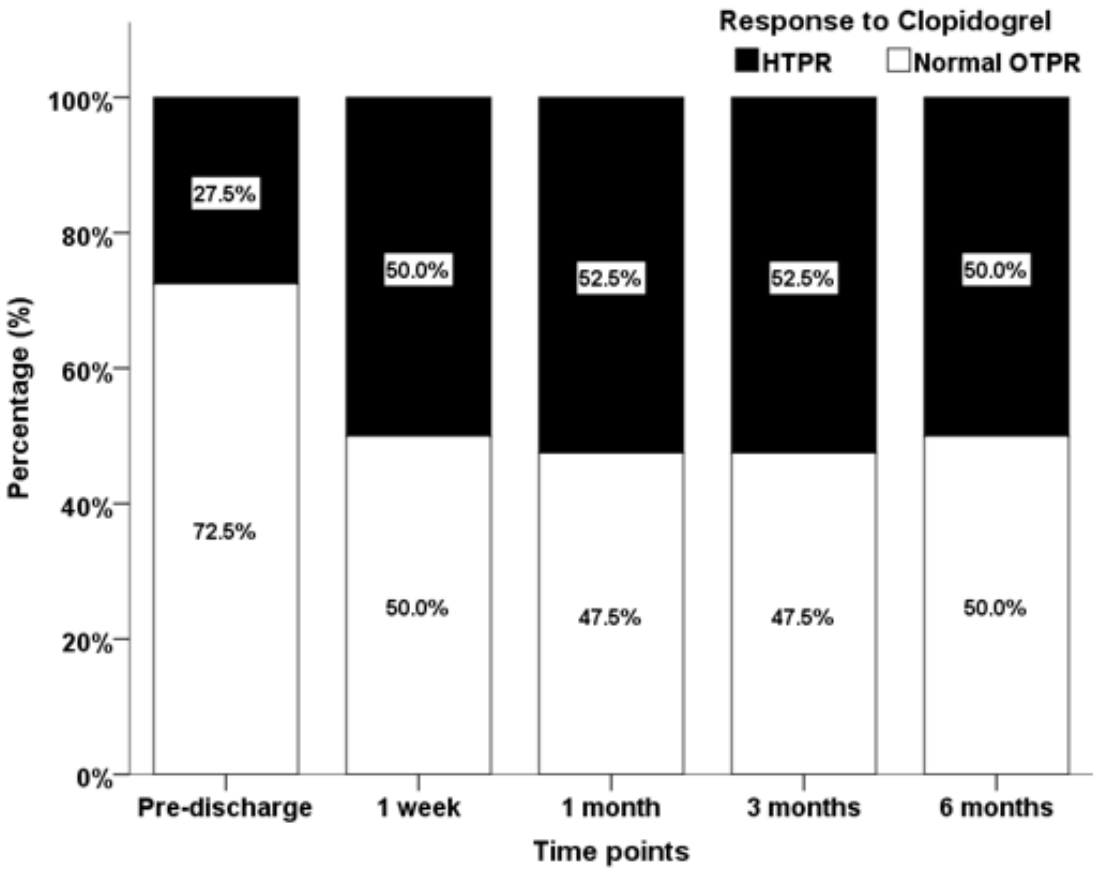


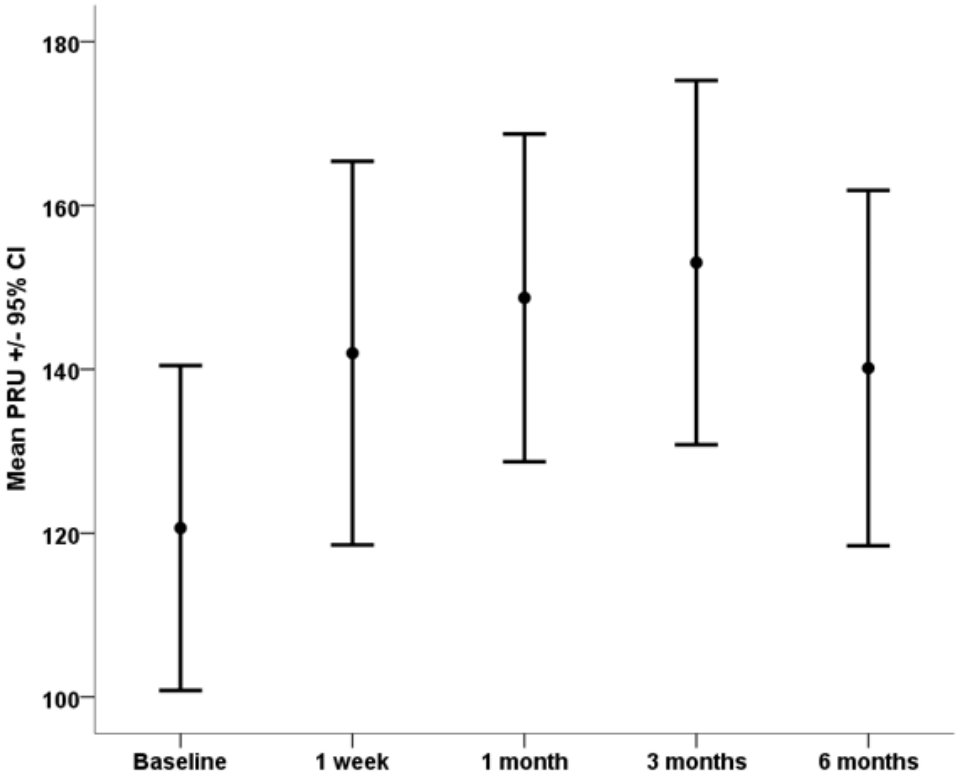
Figure 5.2B. Bar chart displaying rates of normal OTPR (shaded white) and HTPR (shaded black) with s-TEG in 40 patients on clopidogrel

However, based on the Cochran Q test, there were no statistically significant differences in the proportion of patients exhibiting HPR on aspirin ($p = 0.53$) or clopidogrel ($p = 0.99$) beyond 1 week (i.e. excluding t0 in an analysis across the remaining study time points).

5.3.2.2 VerifyNow

The key parameters measured by VerifyNow are summarised in Table 5.2 (Figure 5.3A and 14B). Repeated-measures ANOVA showed no statistically significant differences in ARU values over time. However, PRU values did vary significantly over time ($p = 0.002$). This effect was largely driven by significant increases in PRU values at 1 week ($p = 0.046$), 1 month ($p = 0.002$) and 3 months ($p = 0.004$), compared to baseline (Table 5.3). There were no significant changes in PRU values beyond 1 week (Table 5.3).

A



B

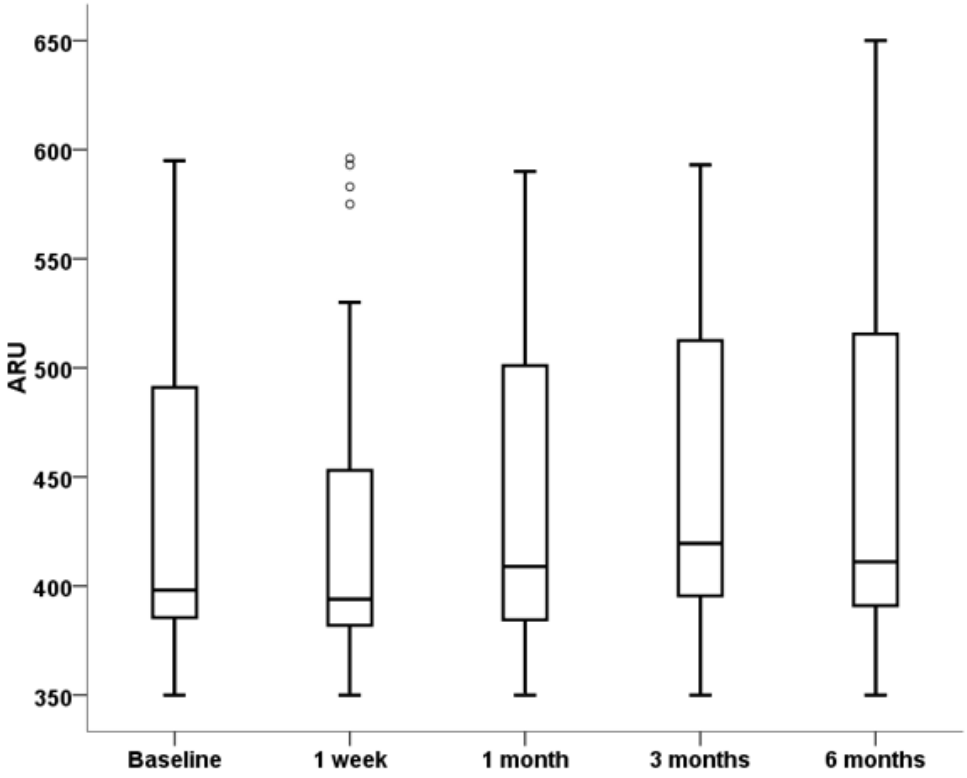


Figure 5.3 Error bar graph showing mean +/- 95% CI of the mean for (A) ARU measured by the VerifyNow Aspirin assay, and (B) PRU measured by the VN-P2Y12 assay in 40 patients on DAPT over 6 months

The prevalence of HPR with the VerifyNow assay predischarge was 20% (Figure 5.4A) using 550 as the ARU cutoff to define HPR on aspirin, and 2.5% (Figure 5.4B) using 230 as the PRU cutoff to define HPR on clopidogrel. Using the Cochran Q test there was no significant heterogeneity in the rates of HPR on aspirin (p = 0.69) overall across the study time points (Figure 2c). However, the variability in rates of HPR on clopidogrel were statistically significant (p = 0.02) (Figure 2d). Specifically, the proportion of patients exhibiting HPR on clopidogrel at 3 months (17.5%) was significantly higher (p = 0.03) than observed at baseline (2.5%). However, based on the Cochran Q test, there were no statistically significant differences in the proportion of patients exhibiting HPR on aspirin (p = 0.71) or clopidogrel (p = 0.11) beyond 1 week (i.e. excluding t0 in an analysis across the remaining study time points).

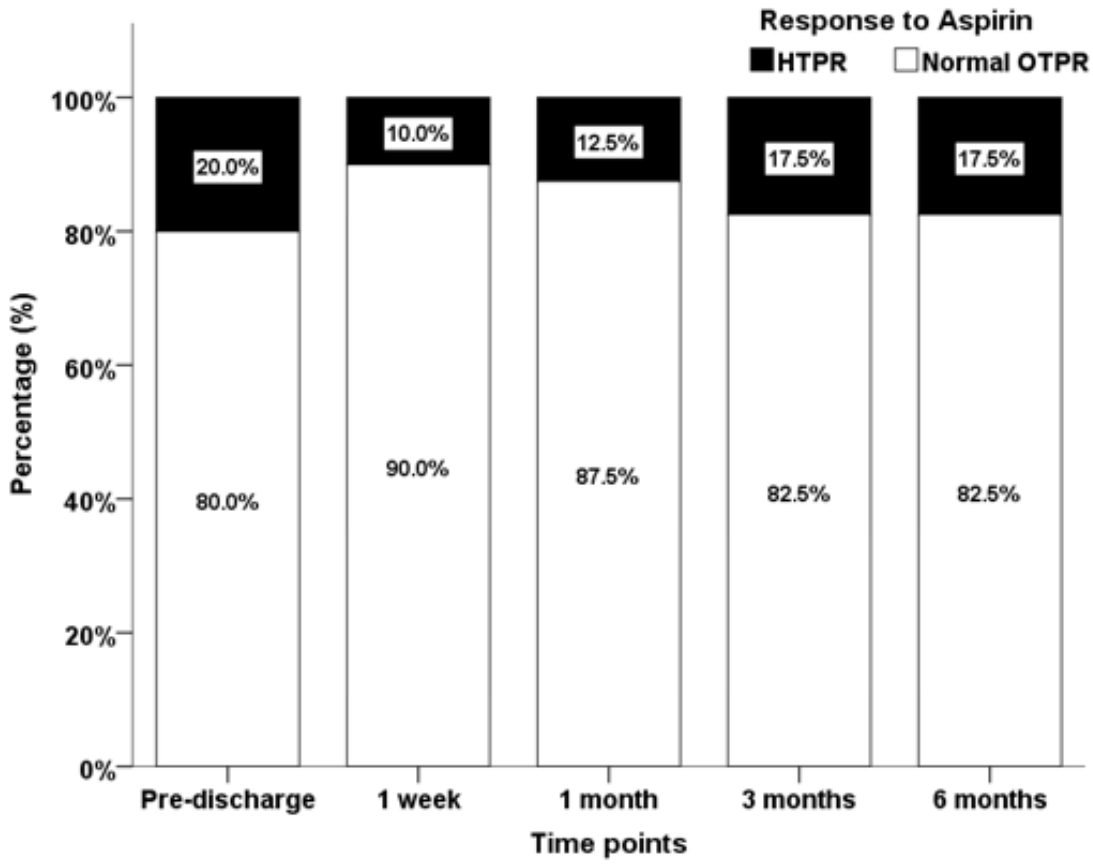


Figure 5.4A. Bar chart displaying rates of normal OTPR (shaded white) and HPR (shaded black) with VerifyNow aspirin assay in 40 patients on aspirin

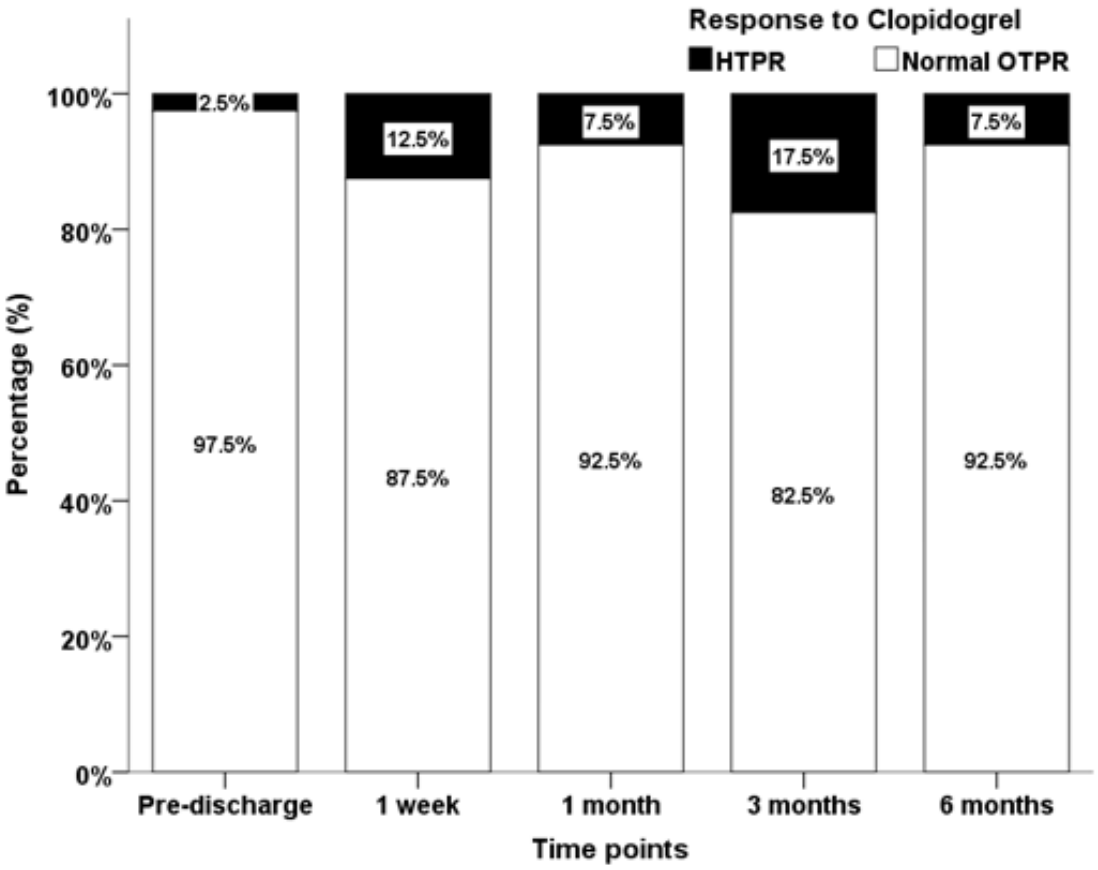


Figure 5.4B. Bar chart displaying rates of normal OTPR (shaded white) and HPR (shaded black) with the VN-P2Y12 assay in 40 patients on clopidogrel

5.3.2.3 Serum Thromboxane B_2 Levels

Median serum TXB_2 values were 1.36 ng/ml (IQR 0.56 to 2.52) at baseline, 1.17 ng/ml (IQR 0.45 to 3.24) at 1 week, 1.29 (IQR, 0.51 to 2.87) at 1 month, 1.02 (IQR 0.34 to 2.26) at 3 months and 1.05 (IQR 0.35 to 2.16) at 6 months (Figure 5.5). Repeated-measures ANOVA showed no statistically significant differences in TXB_2 levels over time. With the exception of one patient who had an isolated value above the 10 ng/ml threshold at 6 months, serum TXB_2 levels remained consistently suppressed over six months confirming effective inhibition of platelet cyclooxygenase-1 (COX-1) activity by aspirin.

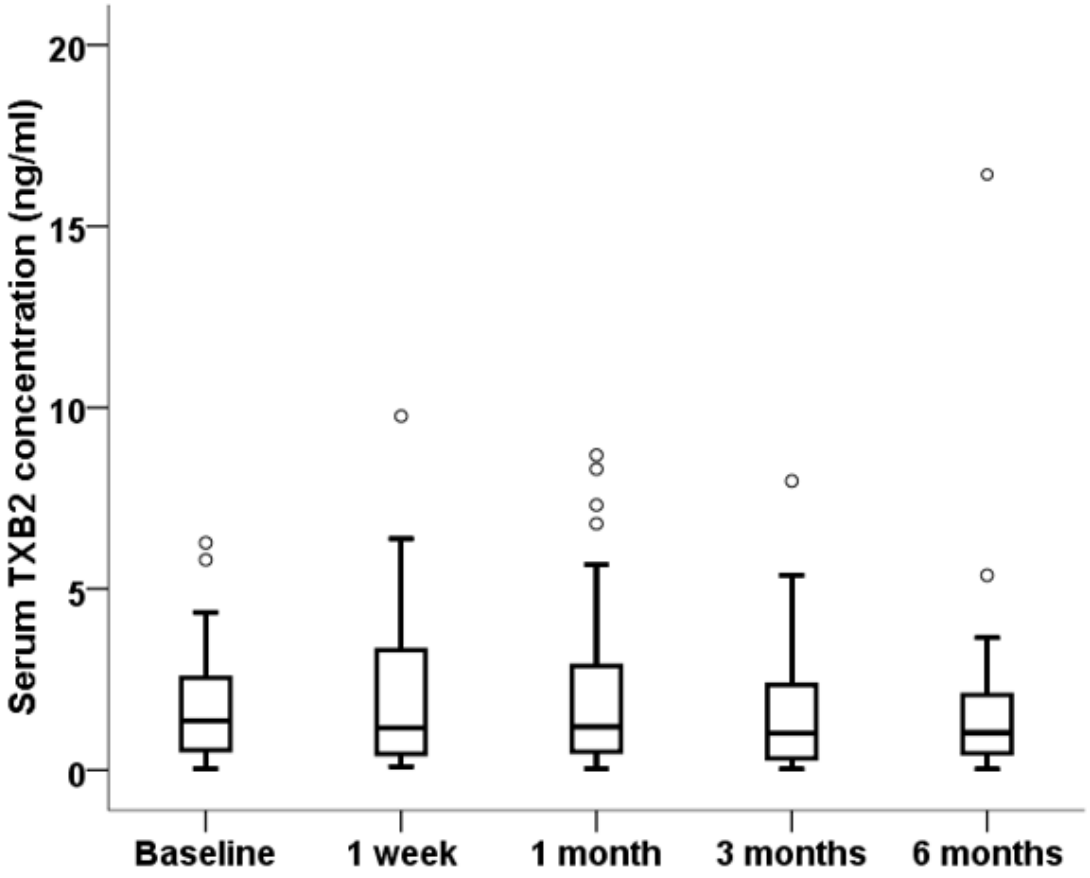


Figure 5.5 Box plot diagram showing median, interquartile range and outliers for serum thromboxane B_2 (TXB_2) over 6 months

5.3.3 Intra-individual variability

Based on an analysis of the entire study population we identified a number of key parameters including $AUC15_{AA}$, $AUC15_{ADP}$, and PRU that varied significantly over the study time points. Further pairwise comparison of these results suggests that the main driver for variability in these parameters was an increase in platelet reactivity between baseline and 1 week beyond which there were no significant changes as shown above. Figure 5.6 illustrates individual changes in platelet reactivity for all patients between baseline and 1 week in key parameters that showed significant variability over time.

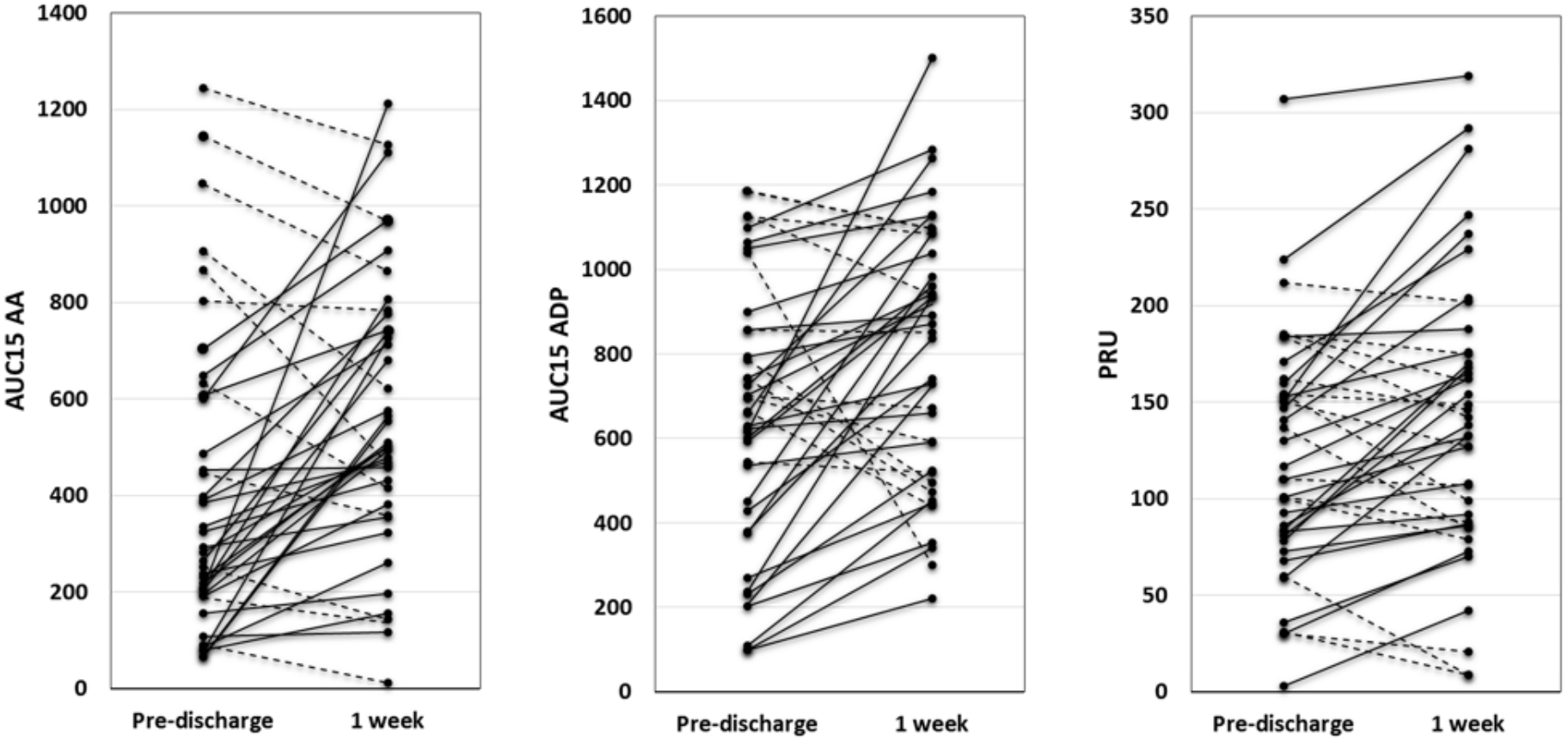


Figure 5.6 Line graphs showing individual changes in (a) $AUC15_{AA}$; (b) $AUC15_{ADP}$; (c) PRU between predischarge and 1 week in 40 patients on DAPT. Solid black lines represent patients in whom platelet reactivity increased and dotted black lines represent patients in whom platelet

5.4 Discussion

The dominant finding described by this study is that AA- and ADP-mediated clotting responses in patients on DAPT vary significantly between predischarge and 1-week time points, but are stable thereafter. Specifically, there was a consistent, and significant, increase in platelet reactivity in response to both AA and ADP at 1 week after discharge, thus disproving the study hypothesis. This raises a question mark over a strategy that employs a single predischarge assessment of platelet reactivity as the sole arbiter for individual patient response to DAPT as part of a personalised medicine strategy.

A number of previous studies have performed a longitudinal analysis of PR in patients on aspirin and the results thus far have been conflicting[359, 360]. Potential limitations of these studies include that they employed a multitude of agonists (e.g. collagen, ADP and epinephrine) which are not considered specific to the COX-1 pathway targeted by aspirin and used different patient populations that were not taking concomitant clopidogrel. Saw *et al* showed no significant differences in AA-induced platelet aggregation with LTA and VerifyNow in 26 patients undergoing PCI during follow up over 12 months[361]. By contrast, we have shown that AA-mediated clotting did vary over time when assessed with s-TEG, an effect that was driven by highly significant differences between $AUC15_{AA}$ at 1 week compared to baseline ($p = 0.008$). Helgason *et al* used LTA to measure AA-induced platelet aggregation in 306 aspirin-treated patients following ischaemic stroke and reported that aspirin response status fluctuated considerably from complete inhibition to partial inhibition and vice-versa in patients who underwent repeat testing at intervals of 6 months[362].

Previous work from this group using s-TEG has raised questions about the validity of employing AA-induced clotting as a test for aspirin efficacy. Firstly, it was reported that clopidogrel can potentiate the effects of aspirin on AA-mediated clotting[302]. Secondly, in the CESSATION study[303], which included patients who were due to stop clopidogrel at one year after receiving a DES, we observed that as well as the expected increase in ADP-induced clotting there was also a significant and simultaneous increase in AA-induced clotting. Importantly, serum TXB_2 levels remained consistently suppressed, thus confirming effective and persistent COX-1 inhibition due to ongoing aspirin therapy. Finally, we also demonstrated supratherapeutic clotting responses to AA-stimulation despite negligible TXB_2 levels in aspirin-treated patients with ischaemic stroke[251].

Consistent with other reports[335, 363], our previous data and results from this study suggest that clotting responses to AA can be mediated via aspirin/COX-1 independent pathways and *ex vivo* PFA's that employ AA as agonist to test aspirin response may be flawed. It is therefore important to exercise caution when interpreting the findings of the present and previous studies using AA-stimulation to assess aspirin response over time, particularly as "aspirin resistance" is rare when assessed using the biochemical gold standard, serum TXB_2. In this context it is, however, reassuring to observe stability of AA-induced clotting from 1 week through six months.

A number of studies have performed longitudinal analyses of platelet reactivity in clopidogrel-treated patients over time and the results have again been discrepant[155, 284, 361, 364-369]. However, these studies investigated heterogeneous patient populations managed with different antiplatelet regimens and in whom platelet function testing was performed at varying intervals in relation to both clopidogrel loading and timing of PCI. A key finding of the present study is that there was a significant increase both in mean platelet reactivity (i.e. PRU and $AUC15_{ADP}$), and in rates of HPR on clopidogrel from baseline.

Increasingly, it is becoming recognised that prevalence of HPR can vary significantly over time amongst clopidogrel-treated patients undergoing PCI[284, 365, 367, 369]. For example, a study performed in 102 patients with stable CAD or ACS (including STEMI) showed that rates of HPR as measured by the VASP phosphorylation assay reduced significantly from 67% at baseline (i.e. 6-24 hours post-PCI) compared to 1-month (50%)[369]. Furthermore, there were also considerable changes in the OTPR phenotype in the first month (31-39%) during which there was a significant reduction in the platelet reactivity index (PRI), but also after 1 month (25-45%) during which PRI was steady state. Similarly, a study by Amoah *et al* assessed OTPR with VerifyNow in 151 ACS patients pretreated with clopidogrel before PCI and found significantly higher rates of HPR during angiography (49%) compared to 1 month (28%)[367]. Finally, the largest study to date assessing OCPR over time recruited 300 patients undergoing PCI for stable CAD or ACS (excluding STEMI patients) in whom a loading dose of clopidogrel 600 mg was given at least 12 hours previously. The prevalence of HPR was significantly higher at baseline immediately before PCI (36%) compared to 1 month and these remained stable at 6 months. So, whist these studies demonstrate variability in the prevalence of HPR on clopidogrel over time, they are discrepant with our current data.

Our findings suggest that in addition to inter-individual variability in responses to clopidogrel, intra-individual variability over time may also exist. In contrast, Codner *et al* reported no change in mean PR measured by VerifyNow and MEA over 30 days in 57 patients admitted with acute MI[368]. A potential limitation of that study, however, is that the patient cohort comprised predominantly STEMI patients, and in-hospital baseline samples were taken at 3-4 days post-PCI, by which time PR may have achieved steady-state. Siller-Matula *et al* showed that PR, as measured by three different PFA's, was significantly higher immediately post-PCI compared to one day thereafter in 30 patients following elective PCI. As patients were on DAPT including clopidogrel 75mg/day, for 3 months on average, they concluded that it was procedure-related platelet activation that explained the reduced platelet inhibition noted immediately after PCI. Furthermore, in five additional patients who also underwent platelet function testing pre-PCI and 2 days post-PCI, maximal platelet inhibition was observed at day 1 post-PCI after which PR increased on day 2. A number of other studies have also demonstrated that the maximal platelet inhibitory response of clopidogrel on ADP-induced platelet aggregation occurs at around 24 hours, after which it diminishes somewhat before achieving steady state[269, 361]. These observations are consistent with our current data. These early changes in PR could be related to levels of vascular inflammation which may be transiently elevated in the context of ACS or PCI in some cases. The evolution of OTPR in clopidogrel-treated patients both before and in the initial hours as well as days after PCI therefore warrants further investigation. If platelet inhibitory responses to clopidogrel peak around 24 hours post-PCI but are not subsequently maintained, then this raises fundamental questions about the validity of performing early platelet function testing before steady state is achieved. Furthermore, early sampling in relation to PCI may be detrimental to the ability of a PFA to identify patients at greatest risk of post-PCI ischaemic events. This is supported by the findings of Campo *et al*, who showed that OCPR was a stronger predictor of outcome when evaluated at 1 month (HR: 28.5, 95% CI: 8 to 104, $p < 0.01$) compared with a pre-PCI baseline (HR: 3.1, 95% CI: 1.3 to 7.3, $p < 0.02$)[284]. It remains to be established if there is a specific time point that reliably predicts future ischaemic events in patients undergoing PCI. This would be of critical importance for the design and implementation of a strategy for tailored DAPT therapy in PCI patients.

It is, however, reassuring that there were no significant changes in the PR or rates of HPR on clopidogrel after 1 week with either assay, particularly in the light of previous evidence. A study by Jaitner *et al*[364] assessed ADP-induced platelet aggregation at weekly intervals over three weeks of monitored therapy in 31 patients on long-term DAPT. Despite being on clopidogrel for more than 12 months, the OTPR phenotype changed in more than 40% of patients when PR was assessed with LTA (5 µM ADP) and in 6% when PR was assessed with MEA. Studies have shown only limited correlation and agreement between various PFA's. Intra-individual variability over time may also be an assay-specific phenomenon. In particular, previous studies by this group demonstrated poor correlation and agreement between s-TEG and VerifyNow, which could be related to the inclusion of PGE1 in the VN-P2Y12 assay as this appears to potentiate the antiaggregatory effects of clopidogrel[351].

There are several limitations to this work. Firstly, the sample size was small. Secondly, the study was performed in a single centre. Thirdly, whilst compliance was encouraged through patient education before hospital discharge and assessed thoroughly at follow up visits, non-compliance to the APT protocol cannot be completely excluded. Fourthly, the duration of antiplatelet therapy prior to sampling was not standardised for logistical reasons, which could conceivably impact our results. Finally, the study population was heterogeneous in terms of presenting diagnosis (i.e. ACS or stable CAD) and smoking status[161], which could also be a potentially confound our results.

5.5 Conclusion

In conclusion, this study demonstrates important variability in responses to DAPT not only between individuals but also within individuals over time. In this regard the platelet inhibitory responses to aspirin and clopidogrel peak within 24 hours after angiography, but these reduce significantly at 1 week and remain stable thereafter out to 6 months. The use of a single early (predischarge) PFA as an indicator of future response to DAPT is therefore flawed. The design of future strategies to assess individual responses for tailored therapy need to take this into account.

CHAPTER 6 – RESULTS

Comparison of the antiplatelet and anticoagulant effects of bivalirudin versus unfractionated heparin in patients undergoing primary PCI: results from the HEAT PPCI Platelet Substudy

Presented as a Late Breaking Trial in a Hotline session on Antithrombotic strategies - EuroPCR meeting in Paris on May 21st 2015

Abstract

Background: Higher rates of AST have been reported with bivalirudin in randomised trials evaluating bivalirudin versus unfractionated heparin, without mechanistic explanation. Furthermore, data are discrepant regards the effects of bivalirudin on platelet function. The aim of this prespecified HEAT-PPCI substudy was to compare antiplatelet and anticoagulant effects of bivalirudin and unfractionated heparin (UFH).

Methods: In the HEAT-PPCI trial, patients undergoing PPCI for STEMI were randomised to receive either UFH or bivalirudin before angiography. Of 1,812 participants, 457 (25.2%) had evaluable platelet function data. Platelet function testing with MEA and s-TEG was performed (i) immediately at the end of procedure (EOP) and (ii) at 24 hours. In addition to ADP and AA-mediated platelet aggregation we also assessed thrombin-mediated clotting (TMC) using kaolin with (CKH) and without heparinase (CK).

Results: There were no significant differences between UFH and bivalirudin in AA-, ADP- and TRAP- (MEA only) mediated platelet aggregation with either assay at EOP or 24 hours. However, whilst UFH obliterated TMC, bivalirudin prolonged R time (19.7 min [15.9 – 25.4] vs. 8.4 min [7.5 - 10]; $P < 0.0001$), K time (2.4 min [1.9 - 3.4] vs. 2.2 min [1.8 - 2.7]; $P = 0.007$) and significantly increased maximum clot strength (MA 62.7 mm [58.7 - 67.4] vs. 58.6 [55 - 63]; $P = 0.0005$) compared to control in response to kaolin.

Conclusions: There were no differences between UFH and bivalirudin with respect to AA-, ADP- and TRAP-mediated platelet aggregation. However, whilst UFH obliterated TMC, bivalirudin prolonged clot initiation and propagation delays clot initiation but also potentiated maximum clot strength. These data may offer an insight into the mechanism of AST in bivalirudin-treated patients.

6.1 Introduction

In patients undergoing PPCI for STEMI, the risk of recurrent ischaemic events remains substantial. Despite pharmacological inhibition of periprocedural platelet function with conventional DAPT, platelets remain reactive to a multitude of other mediators, most notably thrombin, a potent platelet agonist and the final effector of the coagulation cascade. Furthermore, recently concerns have been raised in STEMI patients with respect to the delayed onset of action of new oral $P2Y_{12}$ inhibitors[104, 105] previously reported to have a more reliable and superior antiplatelet effect compared to clopidogrel[94, 100]. Adjunctive anticoagulant therapy therefore still remains critical for improving outcomes in patients undergoing percutaneous coronary intervention PPCI.

There is uncertainty about the optimal adjunctive antithrombotic regimen in PPCI. Historically, UFH has been the default anticoagulant administered during PCI. Unlike heparin, bivalirudin has the advantage that it does not bind plasma proteins, is not neutralised by platelet factor 4 antibodies, exerts its effector activity on thrombin directly without need for co-factors and can bind thrombin in the fluid phase as well as circulating clot-bound thrombin[370]. However, despite its favourable profile, net outcome benefits demonstrated in RCTs have been largely driven by reductions in bleeding complications rather than incremental benefits in reducing ischaemic events[146-149, 371], and this generally when compared to heparin plus GPI, rather than heparin alone.

Evolving trends in PPCI including use of potent $P2Y_{12}$ inhibitors, transradial access and selective 'bailout' use of GPI means that these trials no longer reflect contemporary practices. The HEAT-PPCI study was a randomised comparison of UFH and bivalirudin (with selective 'bailout' use of GPI in both arms) in patients undergoing PPCI and demonstrated a significant reduction in MACE in favour of UFH without excessive bleeding complications[153]. This benefit was largely driven by a higher incidence of AST in the bivalirudin arm, an observation consistent with previous trials[149, 371]. However, the mechanism of AST in bivalirudin-treated patients remains poorly understood. Furthermore, data on the antiplatelet effects of bivalirudin are discrepant.

The primary objective of this prespecified HEAT-PPCI platelet substudy was to compare the antiplatelet and anticoagulant effects of bivalirudin and UFH in patients undergoing PPCI for STEMI.

6.2 Methods

6.2.1 Study population and design

All patients in this platelet substudy were part of the HEAT-PPCI (How Effective are Antithrombotic Therapies in Primary Percutaneous Coronary Intervention Study) trial[153], a single centre, open label, randomised study comparing bivalirudin to UFH in a PPCI population. A strategy of delayed consent was employed in this study as discussed in section 2.2.

The selection inclusion and exclusion criteria were identical to those for the HEAT-PPCI study[153]. Specifically, adults (≥18 years age) were enrolled unless they had known intolerance, hypersensitivity, or contraindication to any trial drug; active bleeding at presentation; artificial ventilation, reduced conscious level or other factors precluding the administration of oral APT; their physician refused to administer antiplatelet loading (uncertain diagnosis or risk of bleeding); or if they had previously been enrolled in this trial. Additionally, patients who received a GPI were excluded from the present platelet substudy.

Platelet function testing with MEA at the end of the index procedure (EOP) was planned from inception of the trial to compare the differential effects of $P2Y_{12}$ inhibitors on platelet reactivity and clinical events, which is not presented in this thesis. However, in a second phase of the platelet substudy, we sought to compare the antiplatelet and anticoagulant effects of bivalirudin and heparin. In this phase we (i) performed platelet function testing with both MEA as well as s-TEG, and (ii) began collecting an additional blood sample at 24 hours. Platelet function data at the 24-hour time point where possible was therefore only performed in a limited proportion of the substudy population. A substantial amendment to the study protocol relating to obtaining a second blood sample at 24 hours was approved by the Liverpool East, North West Research Ethics Committee.

Due to logistic considerations patients were enrolled to this substudy only during the core operational hours of the research investigation laboratory at the Liverpool Heart and Chest Hospital.

6.2.2 Study procedures

All patients received DAPT before PPCI as per routine practice at the host institution and its referring emergency departments. After entry to the catheter laboratory, and before the angiographic findings were known, patients received the assigned drug[153]. UFH was given as a bolus dose of 70 U/kg bodyweight before the procedure. Additional doses were administered if ACT values 5–15 min after the bolus dose or at the EOP were less than 200s. Bivalirudin was given as a bolus of 0.75 mg/kg followed by infusion of 1.75 mg/kg/hr for the duration of the procedure. A rebolus of 0.3 mg/kg was administered if ACT values 5–15 min after the bolus dose or at the EOP were less than 225 s. ACT was monitored with Actalyke XL MAX-ACT system (Helena Laboratories, Beaumont, TX, USA). Angiography and PCI were performed without imposing any study-related restrictions in accordance with prevailing best local practice as determined by the attending interventional cardiologist.

6.2.3 Blood sampling and analysis

Venesection and blood sampling were performed as detailed in the section 2.4.1. Platelet function was assessed with s-TEG and/or MEA as described in section 2.4.2 and 2.4.4 respectively. TMC was assessed in citrated blood both in the absence (CK) and presence of heparinase (CKH), which neutralises the effects of heparin (section 2.4.1). In patients treated with UFH, the latter provides a pure assessment of TMC. It therefore served as a control channel for comparison of the anticoagulant effects of bivalirudin and UFH.

6.2.4 Study endpoints and definitions

The primary aim of this platelet substudy was to compare the pharmacodynamic effects of UFH and bivalirudin on agonist-induced aggregation. Additionally, we evaluated the prespecified combined clinical efficacy endpoints of the HEAT-PPCI trial[153], which was a composite of all-cause mortality, CVA, reinfarction or additional unplanned TLR. We further evaluated all the individual components of this primary efficacy endpoint as well as AST per study group based on the presence or absence of HPR to ADP. The latter was defined as MEA ADPtest > 46 U based on previous studies[203, 262] and the latest consensus document of the Working Group for On-Treatment Platelet Reactivity[341].

6.2.5 Statistical analysis

As continuous variables were not normally distributed, data are presented as median with interquartile range (IQR), and compared using the Mann-Whitney U test. Normality of data was tested using the Shapiro-Wilk test. Categorical variables are presented as frequencies (%). The interaction between the study arm and HPR on the hazard of the primary clinical efficacy outcome measure was tested in a Cox proportional hazard model. Survival curves were constructed using the Kaplan-Meier method. For individual components of the primary efficacy outcome measure and AST, event-free survival amongst patients with HPR compared to those with no HPR were assessed per study group using the log-rank test. A p value < 0.05 was considered to represent statistical significance. Statistical analyses were performed using SPSS version 21 (IBM Corp., Armonk, NY, USA).

6.3 Results

As outlined in the study flow chart (Figure 6.1), of the 1,812 patients randomised in the HEAT-PPCI trial 457 (25.2 %) had evaluable platelet function data. A significant proportion of patients enrolled to the trial did not have blood samples taken for platelet function testing (67.8%) as they presented outside the core operating times of the research analysis laboratory. Furthermore, 86 patients who received GPI were also excluded from the analysis. Finally, 15 bivalirudin-treated patients were excluded from this analysis as they exhibited a pattern of TMC consistent with heparin contamination. Specifically, TMC was obliterated in the CK channel in these patients, consistent with the effects of heparin, but restored in the presence of heparinase (CKH channel), which neutralises heparin. On careful review of source data there was evidence of only one patient having received fondaparinux and one patient receiving UFH prior to randomisation. Platelet function data at the 24-hour time point for 21 patients (14 in the UFH arm and 7 in the bivalirudin arm) bearing this characteristic pattern were excluded from the analysis.

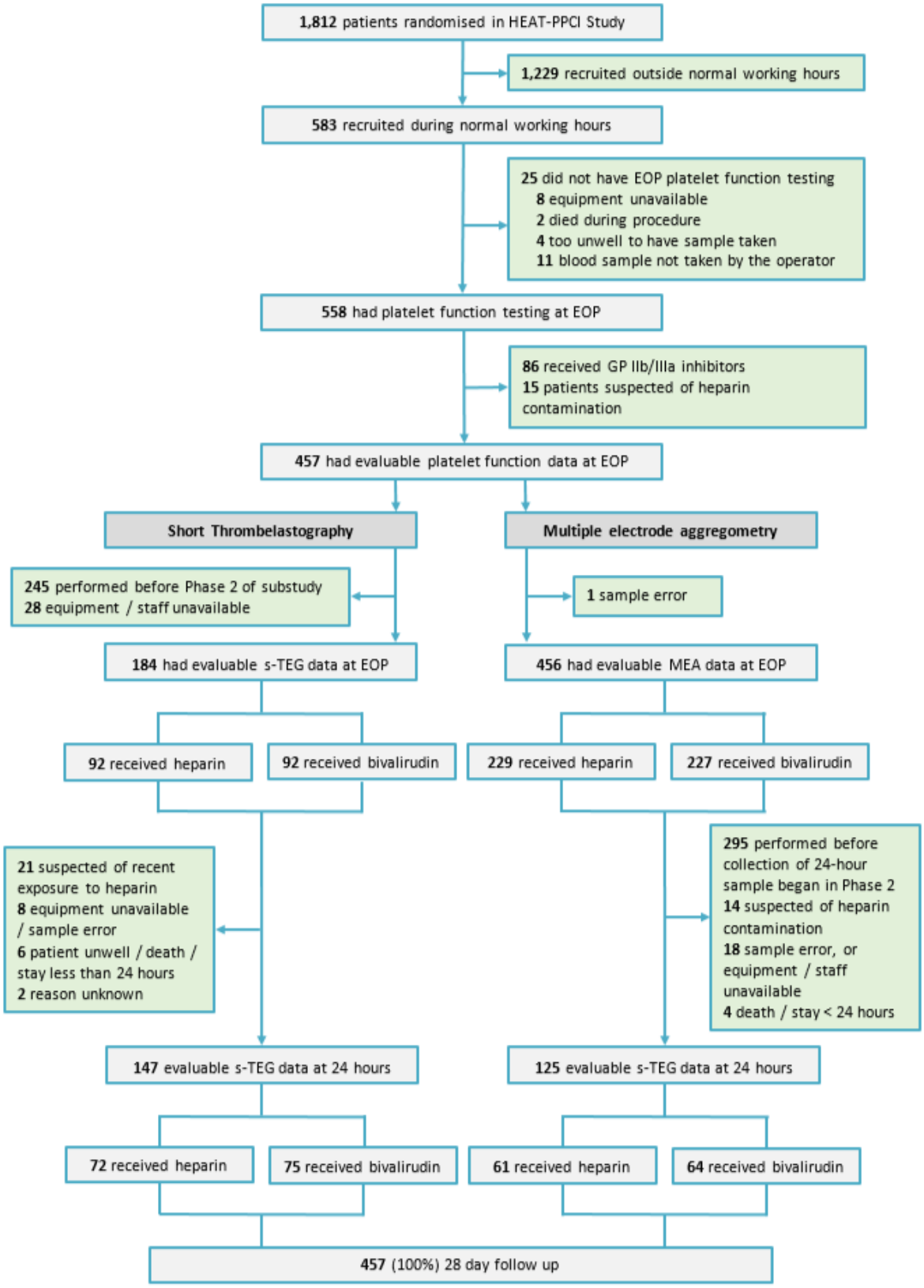


Figure 6.1 Study flow chart

Baseline characteristics in the overall substudy population (Table 6.1) were well matched between patients treated with bivalirudin (n = 227) and UFH (n = 230). Clinical follow up at 30 days was completed (100%) for all 457 patients.

Table 6.1 Summary of baseline characteristics, laboratory investigations and APT

	Bivalirudin (n = 227)	Heparin (n = 230)
Age, years	66 [55 – 74.5]	65 [54 - 74]
Sex, female	67/227 (29.5%)	67/230 (29.1%)
Bodyweight, kg		
Data available	196 (86.0%)	203 (88.3%)
Median [IQR]	77.8 [66.8 - 87.4]	78.5 [68.7 - 89]
Ethnicity, White	216/223 (96.9%)	218/226 (96.5%)
Comorbidities		
Hypertension	96/227 (42.3%)	99/229 (43.2%)
Hyperlipidaemia	86/223 (38.6%)	88/228 (38.6%)
Diabetes Mellitus	26/227 (11.5%)	30/229 (13.1%)
Family history of CVD	100/225 (44.4%)	101/226 (44.7%)
Current Smoker	83/226 (36.7%)	86/226 (38.1%)
Previous myocardial infarction	31/227 (13.7%)	25/230 (10.9%)
Previous PCI	16/227 (7.0%)	14/229 (6.1%)
Previous CABG	8/227 (3.5%)	5/230 (2.2%)
Platelet count		
Data available	223 (98.2%)	227 (98.7%)
Median [IQR]	235 [190 – 288.5]	227 [190 - 264]
Estimated GFR		
Data available	224 (98.7%)	228 (99.1%)
Median [IQR]	81 [63 - 90]	79 [64.5 - 90]
Haemoglobin		
Data available	225 (99.1%)	228 (99.1%)
Median [IQR]	13.6 [12.3 - 14.6]	13.8 [12.7 -14.8]
$P2Y_{12}$ inhibitor		
Clopidogrel	25/227 (11.0%)	15/230 (6.5%)
Prasugrel	54/227 (23.8%)	71/230 (30.9%)
Ticagrelor	148/227 (65.2%)	141/230 (61.3%)
None	0	3/230 (1.3%)
Patients on other oral anticoagulants		
Warfarin	6/227 (2.6%)	4/230 (1.7%)
Fondaparinux	10/227(4.4%)	15/230 (6.5%)
Low-molecular-weight heparin	1/227 (0.4%)	0
Other	1/227 (0.4%)	0
Time between LD and EOP sampling (minutes)		
Aspirin		
Data available	218 (96.0%)	213 (92.6%)
Median [IQR]	95 [77 - 114]	94 [78 - 110]
$P2Y_{12}$ inhibitor		
Data available	218 (96.0%)	213 (92.6%)
Median [IQR]	68 [42 - 100]	60 [42 - 95]

6.3.1 Antiplatelet effects of heparin versus bivalirudin

There were no significant differences in ADP-, AA- or TRAP-mediated platelet aggregation between the two groups at EOP or the 24-hour time (Table 6.2). Similarly, there were no

Table 6.2 Comparison of agonist-mediated platelet aggregation in patients treated with unfractionated heparin to those treated with bivalirudin at the end of the PCI procedure and 24-hour time point

		End of Procedure					24 hours				
		UFH		Bivalirudin			UFH		Bivalirudin		
		N	Median [IQR]	N	Median [IQR]	P value	N	Median [IQR]	N	Median [IQR]	P value
MEA	ASPItest	229	24 [17 - 32]	227	22 [15- 30]	0.08	60	10.5 [5.5 - 18]	62	9.5 [4 - 15]	0.21
	ADPtest	229	71 [33 - 98]	227	64 [32.5 - 93]	0.33	61	22 [15 – 29]	63	17 [14.5 – 25.5]	0.17
	TRAPtest	224	119.5 [93.5 - 140.5]	222	120.5 [96 - 148]	0.35	60	84.5 [61 – 103.5]	64	74 [54 - 98]	0.17
s-TEG	$AUC15_{AA}$	91	457.3 [225.9 - 648.5]	92	379.4 [251.6 – 573.3]	0.60	68	150.1 [104.7 - 318.7]	73	227.6 [142.7 – 298.8]	0.06
		91	460.8 [195.2 - 732.9]	92	537.4 [303.1 – 890.2]	0.08	70	140 [77.1 - 309.9]	74	186.5 [119.2 – 301.5]	0.09

s-TEG, Short Thrombelastography; MEA, Multiple electrode aggregometry; IQR, Interquartile range; UFH, unfractionated heparin

significant differences in $AUC15_{ADP}$ or $AUC15_{AA}$ between UFH and bivalirudin at EOP or the 24-hour time point (Table 6.2).

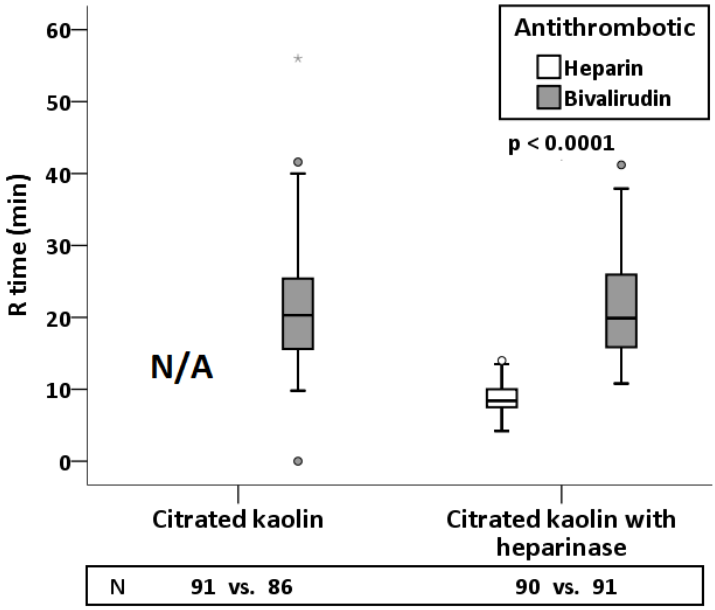
6.3.2 Anticoagulant effects of heparin versus bivalirudin

In the control channel (i.e. CKH channel in patients treated with UFH), the median and IQR for R time was 8.4 min [7.5 - 10], K time was 2.2 min [1.8 - 2.7], α-angle was 59.8° [54.5 - 65.4], MA was 58.6 mm [55 - 63], and $AUC15_{CKH}$ was 965.7 [859.8 - 1090.4]. By contrast, in patients treated with UFH in the absence of heparinase (i.e. CK only), clotting in response to kaolin (i.e. TMC) at EOP was obliterated, at least for the duration of the observation period. Consequently, no further TEG parameters including R time, MA, K time, α-angle or AUC15 could be derived at this time point.

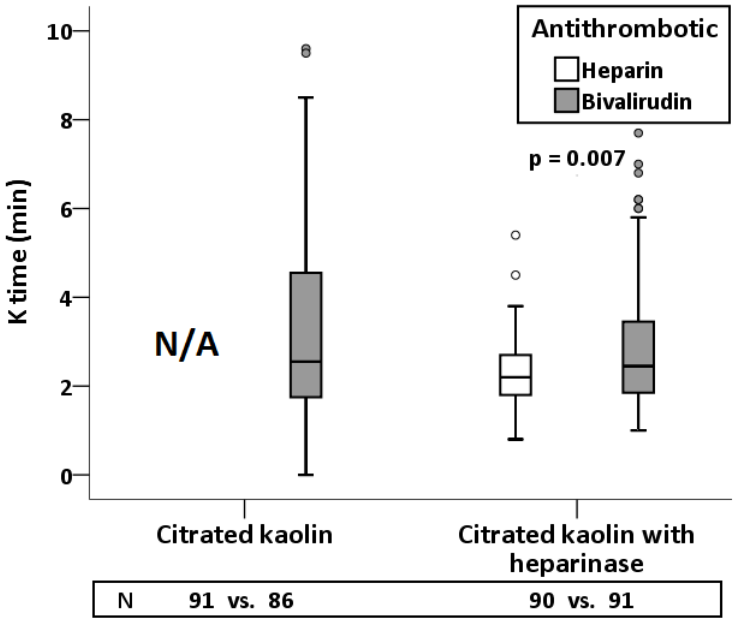
Compared to control (i.e. CKH channel in patients treated with UFH) (n=90), bivalirudin (n = 91) significantly prolonged R time (19.7 min [15.9 – 25.4] vs. 8.4 min [7.5 - 10]; $P < 0.0001$), prolonged K-time (2.4 min [1.9 - 3.4] vs. 2.2 min [1.8 - 2.7]; $P = 0.007$) and increased MA (62.7 [58.6 - 67.4] vs. 58.6 [55 - 63]; $P = 0.0005$). There were no significant differences in the α-angle (58.6° [47.3 - 64.1] vs. 59.8° [54.5 - 65.4]; $P = 0.06$) and $AUC15_{CKH}$ at EOP (1011.2 [808.6 - 1161] vs. 965.7 [859.8 - 1090.4], $P = 0.72$) between patients treated with bivalirudin compared to control (Figure 6.2).

At 24 hours there were no significant differences in any TEG parameters (R time, K time, α angle, MA or AUC15) between patients treated with UFH and bivalirudin, regardless of whether heparinase was present (i.e. CKH) or not (i.e. CK only) (Figure 6.3).

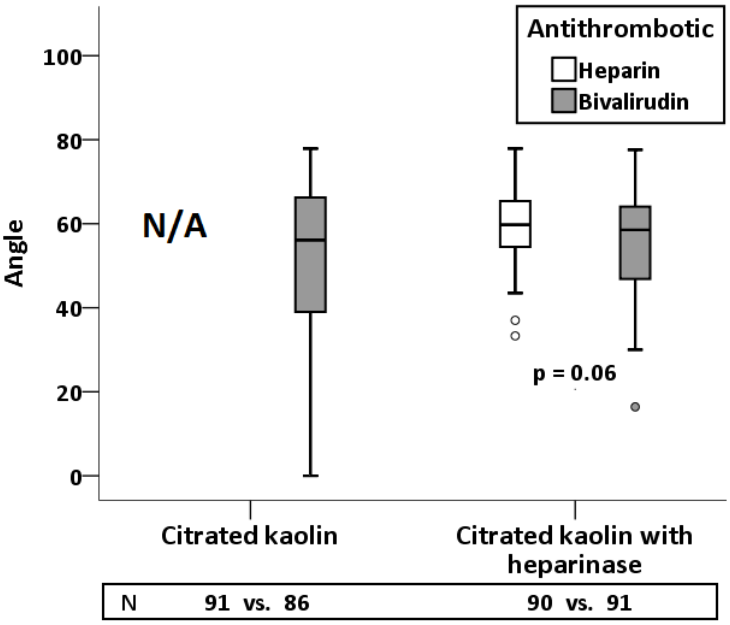
A


Antithrombotic
Heparin
Bivalirudin
p < 0.0001
R time (min)
60
50
40
30
20
10
0
N/A
Citrated kaolin
Citrated kaolin with heparinase
N
91 vs. 86
90 vs. 91

B


Antithrombotic
Heparin
Bivalirudin
p = 0.007
K time (min)
10
8
6
4
2
0
N/A
Citrated kaolin
Citrated kaolin with heparinase
N
91 vs. 86
90 vs. 91

C


Antithrombotic
Heparin
Bivalirudin
p = 0.06
Angle
100
80
60
40
20
0
N/A
Citrated kaolin
Citrated kaolin with heparinase
N
91 vs. 86
90 vs. 91

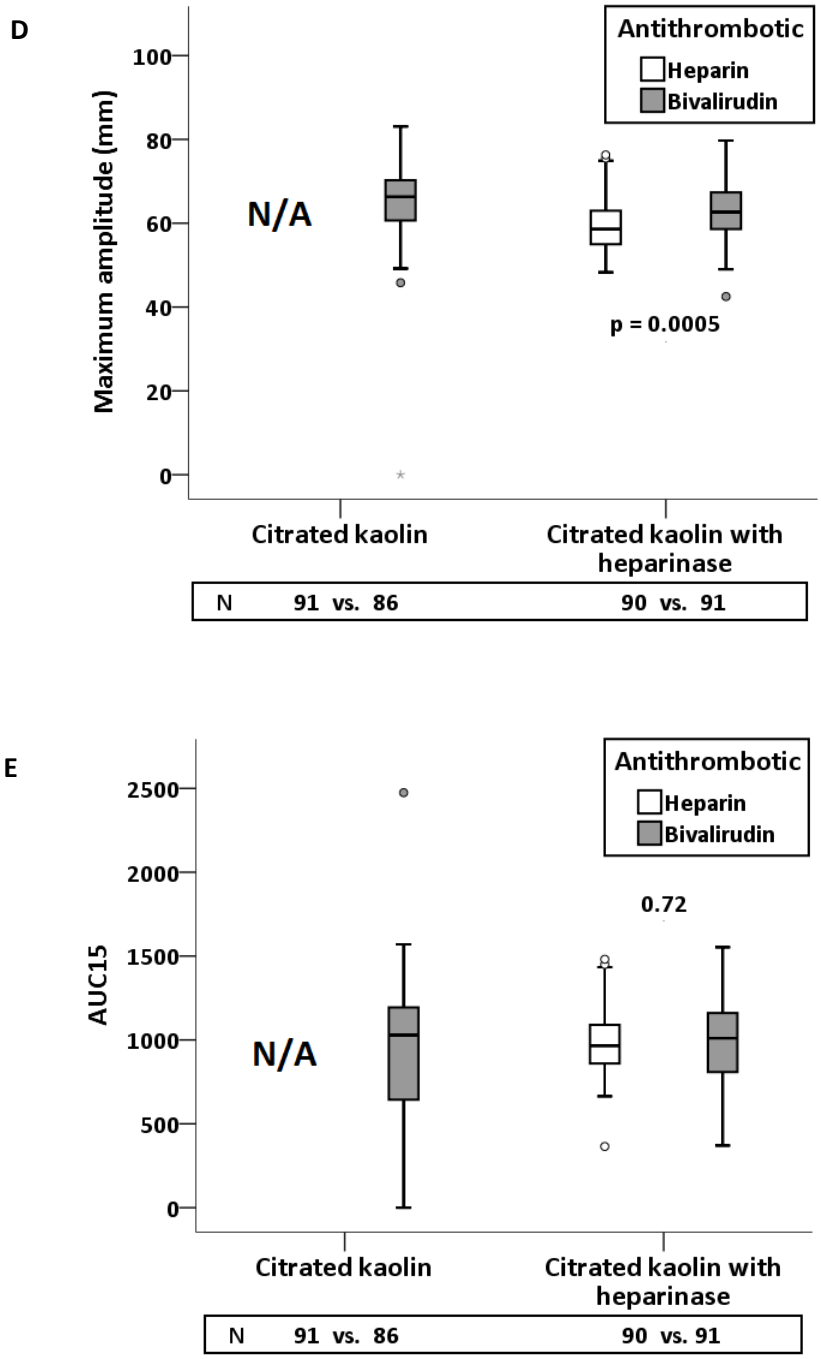
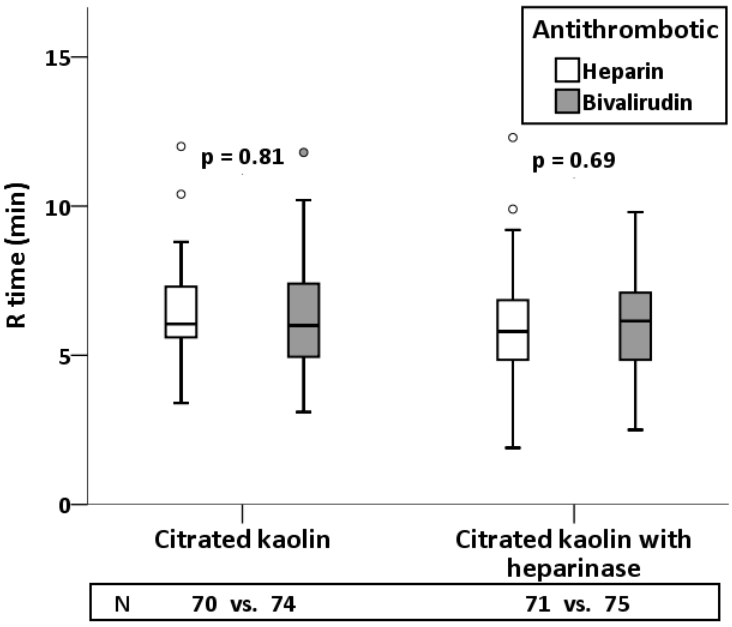
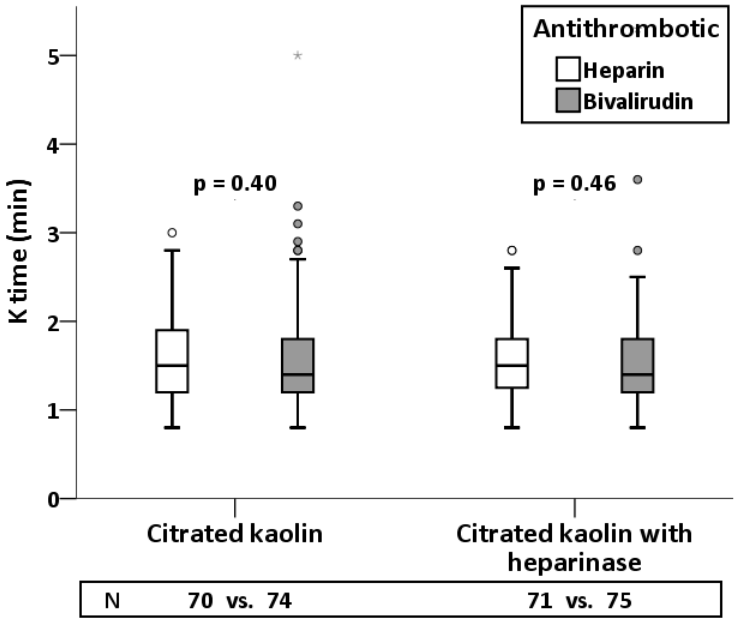


Figure 6.2 Comparison of TEG indices between patients treated with UFH and bivalirudin at EOP when TMC was assessed in the presence (CKH channel) and absence of heparinase (CK channel): (A) R time; (B) K time; (C) α-angle; (D) MA; and (E) AUC15.

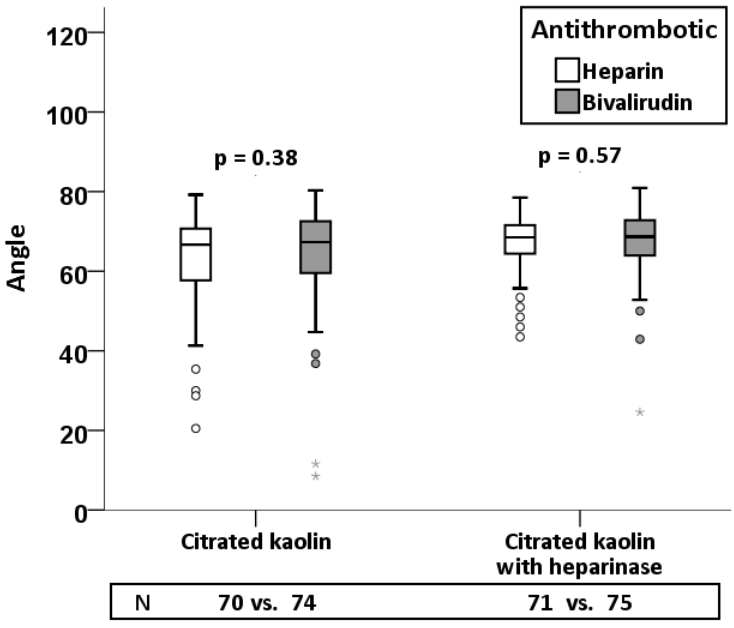
A


Antithrombotic
Heparin
Bivalirudin
R time (min)
15
10
5
0
p = 0.81
p = 0.69
Citrated kaolin
Citrated kaolin with heparinase
N
70 vs. 74
71 vs. 75

B


Antithrombotic
Heparin
Bivalirudin
K time (min)
5
4
3
2
1
0
p = 0.40
p = 0.46
Citrated kaolin
Citrated kaolin with heparinase
N
70 vs. 74
71 vs. 75

C


Antithrombotic
Heparin
Bivalirudin
Angle
120
100
80
60
40
20
0
p = 0.38
p = 0.57
Citrated kaolin
Citrated kaolin with heparinase
N
70 vs. 74
71 vs. 75

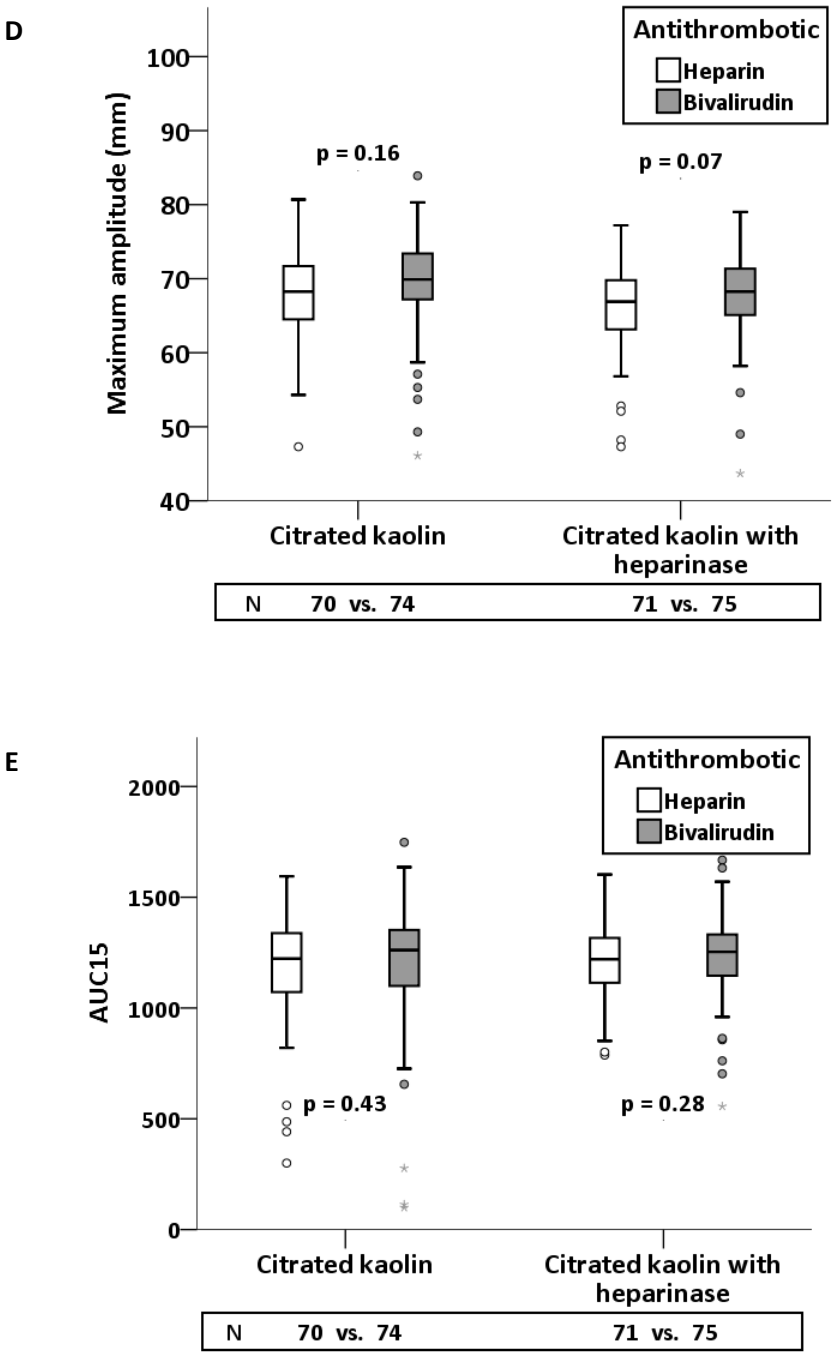


Figure 6.3 Comparison of TEG indices between patients treated with UFH and bivalirudin at 24 hours when TMC was assessed in the presence (CKH channel) and absence of heparinase (CK channel): (A) R time; (B) K time; (C) α-angle; (D) MA; and (E) AUC15.

6.3.3 Clinical outcomes per group and HPR

Of the 456 patients who had evaluable platelet function data with MEA at EOP, 299 (65.6%) exhibited HPR and 157 had no HPR (34.4%). The cumulative incidence of the primary efficacy endpoint was numerically higher in patients with HPR (22/299 = 7.4%) compared to those with no HPR (6/157 = 3.8%) though this did not reach conventional levels of statistical significance (OR 2.0; 95% CI: 0.8 to 4.8; *P* = 0.14). In patients treated with UFH, the incidence of the combined primary efficacy endpoint occurred in 3 of the 152 patients with HPR (2%) and 1 of the 77 patients with no HPR (1.3%) (OR 1.5; 95% CI: 0.2 to 14.5; p = 0.72) (Figure 6.4A). In patients treated with bivalirudin, the incidence of the combined primary efficacy endpoint occurred in 19 of the 147 patients with HPR (12.9%) and 5 of the 80 patients with no HPR (6.3%) (OR 2.1; 95% CI: 0.8 to 5.8; *P* = 0.13) (Figure 6.4B). There was no evidence of an interaction between HPR and the study intervention (p = 0.783) with respect to the primary efficacy endpoint (Figure 6.4) or its individual components (Table 6.3).

As a significant proportion of primary efficacy events occurred within the first 24 hours (i.e. 9 AST), and MEA data at 24 hours was only available in a limited proportion of individuals it was not possible to perform meaningful analysis of clinical outcomes at this time point. Furthermore, as the TEG assay was not performed in the early course of the trial there was no value in evaluating clinical outcomes with this assay, particularly given the low overall event rate (i.e. 28/456 = 6.1%).

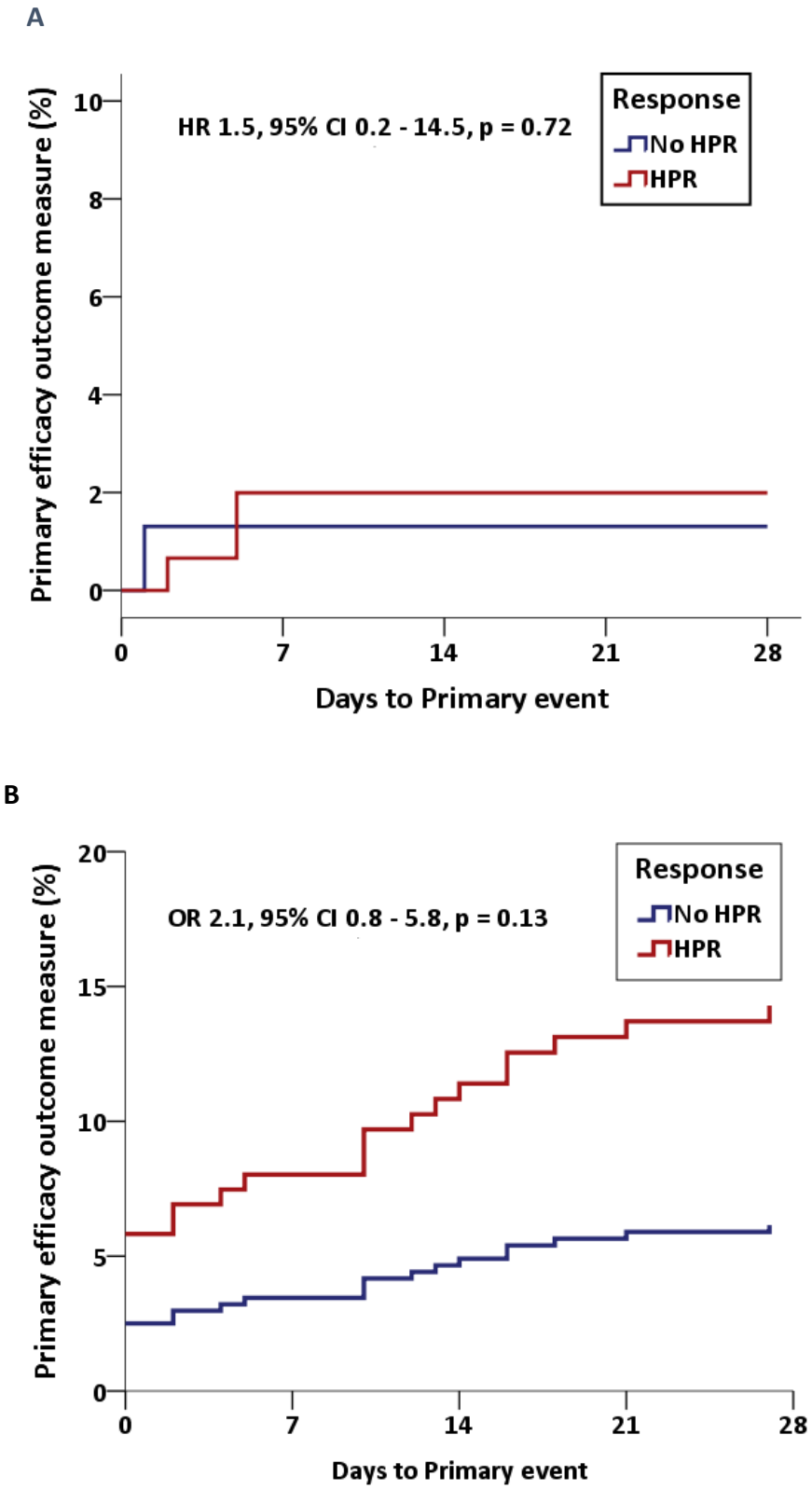


Figure 6.4 Kaplan-Meier curves illustrating the cumulative incidence of the combined primary efficacy outcome measure (a composite of death, cerebrovascular accident, reinfarction, urgent target lesion revascularisation) 28 days following PPCI based on the presence of HPR to ADP at the EOP using MEA: (A) patients treated with unfractionated heparin (n = 229); (B) patients treated with bivalirudin (n = 227).

Table 6.3 Clinical outcomes per study group and HPR

Outcome	Total	UFH (n = 229)			Bivalirudin (n = 227)		
		HPR (n = 152)	no HPR (n = 77)	p Value	HPR (n = 147)	no HPR (n = 80)	p Value
Primary efficacy outcome measures	28	3 (2%)	1 (1.3%)	0.72	19 (12.9%)	5 (6.3%)	0.13
Death	15	1 (0.7%)	1 (1.3%)	0.63	9 (6.1%)	4 (5.0%)	0.72
Cerebrovascular accident	4	1 (0.7%)	1 (1.3%)	0.62	2 (1.4%)	0	0.29
New MI or reinfarction	11	1 (0.7%)	0	0.48	9 (6.1%)	1 (1.2%)	0.09
Unplanned TLR	10	0	0	N/A	9 (6.1%)	1 (1.2%)	0.09
Definite AST	9	0	0	N/A	8 (5.4%)	1 (1.2%)	0.12

AST, acute stent thrombosis; TLR, target lesion revascularisation; HPR, high platelet reactivity; MI, myocardial infarction; UFH, unfractionated heparin

6.4 Discussion

There are two principal findings reported in this platelet substudy of the HEAT-PPCI trial. Firstly, based on MEA there were no significant differences in ADP-, AA- and TRAP-mediated platelet aggregation between UFH and bivalirudin at either time point (i.e. EOP or 24 hours), a result that was confirmed with s-TEG (ADP and AA only). Secondly, based on viscoelastic measures of clotting with s-TEG, UFH obliterated TMC. By contrast, bivalirudin prolonged time to clot initiation (R time) and propagation (K time), although recovery of thrombin activity upon decay of bivalirudin was associated with significantly greater maximum clot strength (i.e. MA) compared to control. This may provide an alternative mechanistic explanation for the higher incidence of AST using bivalirudin reported in HEAT-PPCI[153] and other studies evaluating bivalirudin as an adjunctive anticoagulant in PPCI[149, 150].

Using flow cytometry, previous studies have compared the effects of bivalirudin and UFH on agonist-mediated platelet activation, both *ex vivo*[372, 373] and *in vitro*[374-376] with mixed results. Bivalirudin has been shown to have modest inhibitory effects on platelet activation though some authors have reported that it potentiates P-Selectin surface expression[377]. By contrast, UFH has mostly been shown to potentiate platelet activation[378-380], possibly by initiating outside-in signalling via direct binding to $\alpha_{IIb}\beta_3$ integrin[381], though again there are some conflicting reports[382]. However, the overall significance of differential surface expression of platelet activation markers with these anticoagulant agents on agonist-mediated clotting and clinical outcome measures remains poorly understood.

A study by Gallandat Huet *et al* in cardiac surgical patients demonstrated that both bivalirudin and UFH significantly attenuated ADP-mediated platelet aggregation when used to preserve blood, although the effects of bivalirudin were more pronounced[383]. By contrast, using MEA and LTA, Sibbing *et al* demonstrated that in PCI patients preloaded with clopidogrel, whilst UFH had no significant effects, bivalirudin resulted in significant incremental inhibition of ADP-mediated aggregation[384]. Such antiaggregatory effects, albeit modest in magnitude, are significant and therefore, at least in theory, might be expected to mitigate the early hazard of periprocedural ischaemic events including AST. However, thus far there is no consistent randomised data supporting a superiority for bivalirudin over UFH with respect to ischaemic endpoints. On the contrary, bivalirudin

has been consistently associated with an elevated risk of AST[149, 150, 153], at least in the absence of a prolonged post-PCI infusion[152], a finding which has led to downgrading of recommendations for bivalirudin to class IIa in the recent European guidelines[88].

In the present platelet substudy there were no significant differences in ADP-, AA-, and TRAP-mediated platelet aggregation between UFH and bivalirudin with MEA at either time point (i.e. EOP or 24 hours). Furthermore, s-TEG also found no significant differences between groups with respect to ADP- and AA-mediated platelet aggregation. Our findings are consistent with the DEACON study, which found no significant differences between UFH and bivalirudin in ADP-induced aggregation with LTA in patients undergoing PCI, regardless of whether they had been treated with GPI[385].

Whilst conventional assays for monitoring heparin-related blood clotting (e.g. ACT and activated partial thromboplastin time) terminate early after fibrin gel formation occurs in response to trace amounts of thrombin (< 5%), TEG continues profiling several aspects of clot dynamics on a temporal basis beyond the initiation phase (i.e. clot propagation) which is typically characterised by a burst of thrombin generation (> 95%). TEG is therefore ideally suited for comprehensive characterisation of the effects of thrombin inhibitors on the viscoelastic properties of clot formation. Indeed, several studies have systematically evaluated a range of thrombin inhibitors with TEG, based on *in vitro* testing in healthy volunteers[386-390]. In accordance with our findings, these studies have shown that all thrombin inhibitors, including UFH and bivalirudin, prolong time to clot initiation (i.e. R time) and clot propagation (i.e. K time and α-angle) in a dose-dependent manner. However, the effects of direct thrombin inhibitors (DTIs) in prolonging clot initiation and propagation are modest in comparison to those of UFH, which has the steepest concentration-response curve, and this is consistent with our findings. In the present study, UFH obliterated TMC at EOP, at least for the duration of the observation period. These findings are also consistent with previous reports demonstrating that UFH not only inhibits clot initiation and propagation, but also significantly attenuates clot strength and rigidity in a concentration-dependent manner[386, 389, 390].

To our knowledge, the present study is the first to demonstrate that bivalirudin augments maximum clot strength, a finding that could have important implications in the context of AST. By contrast, the existing evidence is discrepant with respect to the reported effects of bivalirudin on the tensile strength of a clot (i.e. MA). Specifically, Young *et al* showed

that clot rigidity and elasticity were relatively preserved even at high doses of bivalirudin, once kaolin-activated clotting finally commenced following a prolonged lag period[386]. By contrast, Taketomi *et al* showed that at therapeutic concentrations (similar to those employed in PCI) bivalirudin resulted in modest increases in MA in response to direct stimulation with thrombin, when compared to control, though this was not statistically significant in their study[388]. It is noteworthy that we evaluated the pharmacodynamic effects of these anticoagulants *ex vivo* in a large real world population of STEMI patients undergoing PPCI which likely represents a more prothrombotic state than that prevalent in blood from healthy volunteers tested *in vitro*.

The differences between UFH and bivalirudin reported in this study are consistent with their known pharmacological properties. Specifically, heparin-mediated binding of antithrombin leads to the formation of a ternary complex and irreversible conformational changes to the active site of thrombin. By contrast, bivalirudin, comprises a peptide directed against the thrombin active site linked via a tetraglycine spacer to a synthetic dodecapeptide analogue of the C-terminus of hirudin[144] that binds to exosite 1 on thrombin (i.e. the binding site for fibrinogen and thrombomodulin). Once bound in a bivalent fashion (i.e. to the active site and exosite 1), thrombin is rendered inactive only transiently. Circulating proteases including thrombin itself cleave bivalirudin close to the N-terminus liberating the amino-terminal moiety from the active site, thereby allowing resumption of thrombin's catalytic activities[145]. Its relatively short plasma half life (~ 25 minutes) has been cited as a plausible explanation for the higher incidence of AST associated with bivalirudin. Undoubtedly, this mechanistic explanation has greater biological relevance given that there is now also convincing evidence for a delayed onset of action of $P2Y_{12}$ inhibitors in STEMI[104, 391]. Consequently, this has prompted analyses of prolonged post-PCI infusions of bivalirudin as a strategy to mitigate the higher incidence of AST, which has shown encouraging results[152, 392].

Nevertheless, numerous studies have also reported that at low concentrations, bivalirudin[389, 390, 393] and other DTIs[394, 395], produce a paradoxical increase in tissue factor (TF)-triggered thrombin generation (TG) *in vitro*. Furthermore, studies have also shown that TF-stimulation results in a paradoxical rise in markers of TG (e.g. prothrombin fragment 1+2, thrombin-antithrombin complex) in blood samples treated with bivalirudin[383, 390] or other DTIs[395]. However, no increases in TG have been reported in patients treated with Factor (F) Xa inhibitors or UFH which also has intrinsic antithrombin-

mediated activity against various clotting factors including FXa[389, 390]. This exaggerated TG at low concentrations of DTIs has been attributed to the suppression of the thrombin-thrombomodulin (TM)-induced negative feedback inhibition of protein C activation. Activated protein C (APC) naturally exerts a potent anticoagulant effect via proteolysis of factors (F) Va and VIIIa, thereby attenuating TG. Linder *et al* have demonstrated that a variety of direct (e.g. hirudin and melagatran) and indirect thrombin inhibitors (e.g. UFH) inhibit APC generation to a similar extent[396]. A critical role for APC and TM in exaggerated TG associated with DTIs at low concentrations is suggested by the following observations from previous studies: (i) enhanced TG was not observed in the absence of protein C in both rat models[397] and human plasma[394, 395]; (ii) the extent of enhancement of TG in human plasma was greater in the presence of TM compared to in its absence[394, 395]; and (iii) APC reversed enhancement of TG in a dose-dependent manner[394].

Following proteolytic cleavage, the carboxyl-terminal remnant of bivalirudin transforms into a competitive inhibitor of exosite 1 substrates including fibrinogen and TM[145], thus potentially exerting an ongoing inhibitory effect on thrombin-TM mediated activation of protein C. We can therefore speculate that a mechanism of rebound clotting activation could potentially explain the increase in clot strength at EOP and excess of AST observed in patients treated with bivalirudin. Nevertheless, further studies to elucidate the precise mechanism and clinical significance of this phenomenon are necessary.

Agonist-induced platelet aggregation was significantly higher at EOP compared to 24 hours with both assays. Consequently, despite early administration of DAPT and widespread use of potent $P2Y_{12}$ inhibitors in the substudy (> 90%) a significantly higher proportion of the cohort exhibited HPR to ADP with MEA at EOP compared to the 24-hour time point (65.4% vs. 6.0%). These results are consistent with previous reports which showed a delayed onset of the pharmacodynamic effects of prasugrel and ticagrelor in the setting of PPCI for STEMI, thereby suggesting a possible role for a rapidly acting intravenous $P2Y_{12}$ inhibitor such as cangrelor in this population. Moreover, these results also confirm that neither anticoagulant agent (i.e. UFH or bivalirudin) has potent inhibitory effects on agonist-mediated platelet aggregation. The incidence of the primary efficacy endpoint in this substudy was numerically higher in HPR versus no-HPR patients (12.9% vs. 6.3%) treated with bivalirudin, though not statistically significant (p = 0.13). However, a platelet substudy of ISAR-REACT 4 trial by Sibbing *et al* did report significantly higher incidence of death, MI and urgent TLR in patients with HPR treated with bivalirudin

in the context of PCI for non-ST elevation MI[398]. These apparent discrepancies with our data are likely explained by a disproportionately higher incidence of HPR at EOP combined with a low overall event rate in this substudy population. It is also noteworthy that all 9 AST events in this substudy occurred in patients treated with bivalirudin. This is unlikely to represent a chance finding as an excess of AST in STEMI patients treated with bivalirudin has been previously reported.[149, 150, 153]. An important limitation of this substudy was that the pharmacodynamic effects of $P2Y_{12}$ blockade had not reached steady state by the time platelet function testing was performed at the EOP. It is therefore difficult to confidently exclude an interaction between the study intervention received and clinical impact of HPR to ADP though trends from our data and results from previous studies suggest such an interaction may exist.

This substudy has several limitations that merit consideration. Firstly, we have assumed that baseline platelet reactivity prior to drug administration was similar in both treatment groups, though this was not formally confirmed. Secondly, we were unable to compare the effects of these anticoagulant regimens at therapeutic doses on TG as this was neither measured directly nor assayed via surrogate markers. Finally, we have not examined the validity of using the CKH channel in patients treated with UFH as a control group for comparison against patients treated with bivalirudin.

6.5 Conclusion

In conclusion, whilst there were no significant differences between UFH and bivalirudin with respect to their effects on platelet aggregation in response to ADP, AA and TRAP, we did observe important differences in the viscoelastic properties of clot formation in response to thrombin stimulation. Whilst heparin obliterated TMC bivalirudin prolonged clot initiation and propagation, but was also associated with a significant increase in maximum clot strength. These findings may provide some insight into the mechanism of the elevated risk of AST observed in bivalirudin patients observed in HEAT-PPCI and other trials, which may be related to loss of negative feedback inhibition of TG via APC. Further mechanistic and clinical studies are required to determine the optimal antithrombotic strategy in the setting of PPCI for STEMI.

CHAPTER 7 – RESULTS

Does arachidonic acid-induced clotting vary with the vascular inflammatory status: Insights with short thrombelastography

Abstract

Background: This group has previously observed high clotting responses to AA with s-TEG despite negligible serum TXB_2 levels in patients with CVD, suggesting the existence of a COX-1 independent pathway(s) of AA-mediated clotting. Furthermore, there is some evidence of an association between inflammation and the risk of platelet-mediated atherothrombotic events.

Objectives: We therefore sought to test the hypothesis that *ex vivo* whole blood responses to AA vary with levels of vascular inflammation, independent of the AA/COX-1 pathway. We selected patients undergoing elective major vascular surgery to study this hypothesis as this offers a model of intense but transient inflammation with a rapid onset/offset in a population already established on aspirin.

Methods and Results: Thus far 30 patients have been enrolled to this ongoing study aiming to recruit a sample size of n = 40. Of these, 26 have completed follow up and been included in this interim analysis. 15 (57.7%) had open abdominal aortic aneurysm (AAA) repair and 11 (42.3%) had bypass surgery. Platelet reactivity was measured with s-TEG before as well as 2 hours, 1 day, 2 days, 3-5 days and 3 months after surgery. Serum samples for measurement of inflammatory biomarkers and products of AA metabolism (i.e. TXB_2 and 12-HETE) by ELISA have been stored for future batch analysis upon completion of study. We observed significant differences in $AUC15_{AA}$ ($p < 0.0001$), $AUC15_{ADP}$ ($p = 0.003$) and $AUC15_{Thrombin}$ ($p < 0.0001$) in the perioperative period. This was driven by an early attenuation in agonist-mediated aggregation at 2 hours postprocedure, subsequently increasing significantly by days 3 to 5. Nevertheless, only $AUC15_{Thrombin}$ was significantly greater in the postoperative period compared to a preoperative baseline ($p = 0.001$).

Conclusion: Whilst there were significant changes in AA-mediated clotting in the perioperative period it remains to be seen whether these are associated with levels of vascular inflammation and moreover if these occur in a COX-1-independent manner. The

postoperative hypercoagulable state described here may further explain the link between vascular inflammation and atherothrombotic events which warrants further investigation.

7.1 Introduction

Whilst there is robust evidence for aspirin in secondary prevention of CVD[399], approximately 10 to 20% patients experience recurrent vascular events despite long-term aspirin therapy[400]. This has given rise to the concept of "aspirin resistance", though strictly "treatment failure" would represent a more accurate definition of this phenomenon, which is not uncommon amongst drug therapies. A more objective definition of aspirin response should encompass aspirin's effect on its principle target, i.e. COX-1 dependent TXA_2 production. This can be assayed by measuring serum concentrations of TXB_2, the dominant stable metabolite of this pathway, which remains the "gold standard" biochemical index for aspirin response.

However, conventional PFAs employ AA, a COX-1-specific agonist, to assess the functional effects of aspirin. Previous work from this group has demonstrated high clotting responses to AA with s-TEG despite negligible TXB_2 levels in patients: - (i) stopping clopidogrel after 12 months of DAPT[303]; (ii) patients presenting with acute ischaemic stroke treated with aspirin[251]; and (iii) patients with CAD on long-term DAPT[345]. These data suggest that employing functional tests, even those based on the COX-1 specific agonists (i.e. AA), may be flawed for the assessment of aspirin response. Furthermore, these data advocate the existence of a potential aspirin-independent pathway (i.e. COX-1 independent pathway) for AA-induced clotting, which has been eluded to in previous studies[335].

In the present study, we postulated that vascular inflammation may be an important determinant of AA-mediated clotting via a COX-1-independent pathway. There is accumulating evidence that inflammation plays a key role not only in the development and progression of atherosclerosis, but also in plaque destabilisation and subsequent atherothrombosis. This is further supported by the finding of accelerated atherosclerosis and increased cardiovascular mortality in chronic inflammatory disorders[306] including SLE[307], RA[308], and IBD[309]. Epidemiological studies have also demonstrated an early hazard of atherothrombotic events in the context of acute infection, which typically heralds a systemic inflammatory response[310-312]. Several inflammatory biomarkers including IL-6[321], CD40L[327] and CRP[314-318, 401] have also shown predictive power for the assessment of atherothrombotic risk. Moreover, a growing body of evidence now suggests that inflammation augments platelet reactivity[328-330, 332, 333], thereby potentially reducing APT

efficacy.

We therefore hypothesised that *ex vivo* whole blood responses to AA (and other agonists including ADP or thrombin) vary with the vascular inflammatory status in an aspirin- (i.e. COX-1) independent manner. We specifically chose to study this hypothesis in patients undergoing elective vascular surgery for the following reasons: (i) surgery is well documented to induce an acute, and transient, vascular inflammatory response which includes activation of complement, recruitment of white blood cells (predominantly neutrophils) and release of various proinflammatory cytokines including tumour necrosis factor (TNF-α) and IL-6[402]; (ii) aspirin is typically continued in the perioperative period[403] to mitigate the risk of myocardial injury associated with surgery in this population[404, 405]; (iii) the rapid onset/offset of inflammation associated with vascular surgery represents an attractive model in which to test our hypothesis.

In addition to measuring serum TXB_2 levels to assess COX-1 inhibition by aspirin, we also simultaneously measured serum 12-HETE, a stable metabolite of the platelet 12-lipoxygenase (12-LOX) pathway. This relatively poorly understood enzymatic pathway which shares a common substrate with the COX-1 pathway (i.e. AA) has been reported to have both pro- as well as antiaggregatory effects[406-409], though no unanimous effect has been determined. A secondary objective of this study was to determine whether 12-LOX activity as determined by serum 12-HETE levels correlate with AA-mediated clotting when aspirin is effectively suppressing TXB_2 production.

7.2 Methods

7.2.1 Study population

This is an ongoing single centre study aiming to recruit 40 patients undergoing major vascular surgery (e.g. open aortic abdominal repair [AAA], bypass surgery) on an elective basis at the University Hospital Southampton. All patients were established on maintenance dose of aspirin (i.e. 75mg daily) for at least five days. Patients were excluded if they had an acute intercurrent infection, acute limb ischaemia or were admitted as an emergency for ruptured AAA. Other specific exclusion criteria included liver failure, renal failure requiring dialysis, thrombocytopenia, and regular concomitant use of NSAIDs, steroids, oral anticoagulants, COX-2 inhibitors or APT other than aspirin.

Furthermore, patients were withdrawn from the study for the following reasons: (i) stopping aspirin in the postoperative period, (ii) transfusion of platelets, or (iii) transfusion of more than two units of packed red cells. In the event of a withdrawal, an additional participant meeting the study criteria will be recruited to achieve the targeted sample size.

7.2.2 Study procedures

All patients routinely received periprocedural UFH (5,000 units). Furthermore, aspirin was continued throughout the perioperative period and administered intravenously where there were concerns regards intestinal absorption or where oral drug administration was contraindicated on clinical grounds. Compliance with aspirin following hospital discharge was encouraged through comprehensive patient education and enquired about at the 3-month study visit.

7.2.3 Study design

Blood sampling was performed as specified in the Study Methods (section 2.4.1). Blood samples were taken at the following prespecified time points: (i) *t1* - within 48 hours of the surgical procedure, (ii) *t2* - 2 hours postprocedure, (iii) *t3* - 1-day postprocedure, (iv) t*4* - 2 days postprocedure, (v) *t5* - 3 to 5 days postprocedure, and (vi) *t6* – at least 3 months. At each time point, platelet reactivity was measured using s-TEG, as described in the methods section 2.4.2.

In addition, duplicate blood samples, allowed to clot for 30 minutes in SSTs, were centrifuged at 1000 X g for 15 minutes and aliquots of the resultant supernatant were stored at -80°C for later batch analysis. Upon completion of enrolment these serum samples will be assayed by ELISA for inflammatory biomarkers (IL-6, CRP, CD40L and TNF-α) and products of AA metabolism (i.e. TXB_2 and 12-HETE).

7.2.4 Statistical analysis

The sample size calculation for this study was derived by a medical statistician and based upon the CESSATION study[303]. Based on an observed difference of 171.6 between mean $AUC15_{AA}$ at 48 hours and baseline with a standard deviation of 283.4, where N = 33 in the CESSATION study, the effect size was assumed at 0.603. Using the G-Power software package (Version 3.1.3, Universitat Kiel, Germany, 2010), a sample size of n = 38 was assumed to be sufficient for a two-tailed, matched pairs t-test to detect with p = 0.05 (i.e. α or type 1 error) and 95% power such a significant difference in AA-mediated platelet aggregation following vascular surgery, as a primary outcome. However, this calculation has assumed a normal distribution of the outcome data. Allowing for a lack of normal distribution, for instance, the non-parametric Wilcoxon signed-rank matched-pairs test, yielded a minimum necessary sample of 40 (effect size = 0.6, α = 0.05, power = 95%).

Continuous variables are presented as mean and 95% CI of the mean if normally distributed or median and interquartile range (IQR) if not. Statistical analysis of differences in continuous variables over time was performed using the repeated-measures analysis of variance (ANOVA) test followed by correction for multiple comparisons using the Bonferroni adjustment. Categorical variables are presented as frequencies (percentages). A p value < 0.05 was considered to represent statistical significance. Statistical analyses were performed using SPSS version 21.

7.3 Results

A total of 30 patients have been enrolled into this study so far. Of these, four have been excluded from the analysis for the following reasons: (i) one patient died early in the postoperative period, (ii) one patient had their surgical procedure cancelled due to hospital bed crisis, (iii) one patient received a transfusion of more than two units of packed red cells (a prespecified exclusion criteria), and (iv) one patient withdrew their consent to participate after completing two study time points. Thus, study data for the remaining 26 patients is presented here. In addition, one patient refused to undergo blood sampling at a single in-hospital time point. Furthermore, 5 patients refused to

attend for the 3 month follow up visit and one person changed APT to clopidogrel by this time point.

All patients received aspirin on the day of sampling though the time interval between administration and blood sampling varied as illustrated in Table 7.1. This interval was prolonged at the preprocedure time point as generally patients being admitted electively for vascular surgery were instructed to arrive late afternoon to facilitate patient flow in the hospital. Furthermore, the time interval between aspirin administration and sampling at the 2 hours postprocedure time point (*t2*) was governed by the schedule of the surgical theatres and the procedure length.

Table 7.1 Time interval (minutes) between administration of aspirin and blood sampling at each study time point

	Time		
	N	Mean	95% CI
Preprocedure	26	483.4	89.9
2 hours	26	582.0	67.0
Day 1	26	323.6	83.0
Day 2	25	297.8	69.3
Days 3 to5	25	277.6	67.7
3 months	20	179.7	56.5

7.3.1 Baseline characteristics

Baseline characteristics for this cohort have been summarised in Table 7.2. Of the 26 patients analysed here, 22 (84.6%) were male with an average age of 67.7 ± 4 years (mean ± 95% CI). Fifteen patients (57.7%) underwent vascular surgery for an AAA repair, eleven (42.3%) had bypass surgery for either subcritical limb ischaemia (SLI) (n = 8) or repair of a peripheral aneurysm (n = 3). The cardiovascular risk factor profile included hypertension (73.1%), hyperlipidaemia (80.8%), diabetes mellitus (11.5%), active smoking (42.3%), and ischaemic heart disease (42.3%).

Table 7.2 Baseline characteristics, laboratory investigations, procedural data and drug use

Variable	Study cohort (N = 26)
Demographics	
Age, years	67.7 ± 4
Gender, Male	22 (84.6%)
Body mass index (kg/m^2)	25.7 ± 1.5
Ethnicity, White	26 (100%)
Risk factors	
Hypertension	19 (73.1%)
Hyperlipidaemia	21 (80.8%)
Diabetes mellitus	3 (11.5%)
Current smoker	11 (42.3%)
Ischaemic heart disease	11 (42.3%)
Previous myocardial infarction	6 (23.1%)
Previous PCI	6 (23.1%)
Previous CABG	3 (11.5%)
Baseline laboratory investigations	
Platelet count X 10^9 / litre	247 ± 54.3
Estimated GFR (ml/min)	70.7 ± 6
Haemoglobin (g/litre)	142.2 ± 6.1
Vascular surgical procedures	
Abdominal aortic aneurysm repair	15 (57.7%)
Bypass surgery for SLI	8 (30.8%)
Peripheral aneurysm repair with surgical bypass	3 (11.5%)
Concomitant medical therapy	
β-blocker	7 (26.9%)
ACE inhibitor or ARB	15 (57.7%)
Statin	19 (73.1%)
Calcium channel blocker	14 (53.8%)
Proton pump inhibitor	8 (30.8%)
Insulin	(7.7%)

7.3.2 Platelet reactivity with s-TEG

The key parameters reported by s-TEG are summarised in Table 7.3 and illustrated graphically in Figure 7.1.

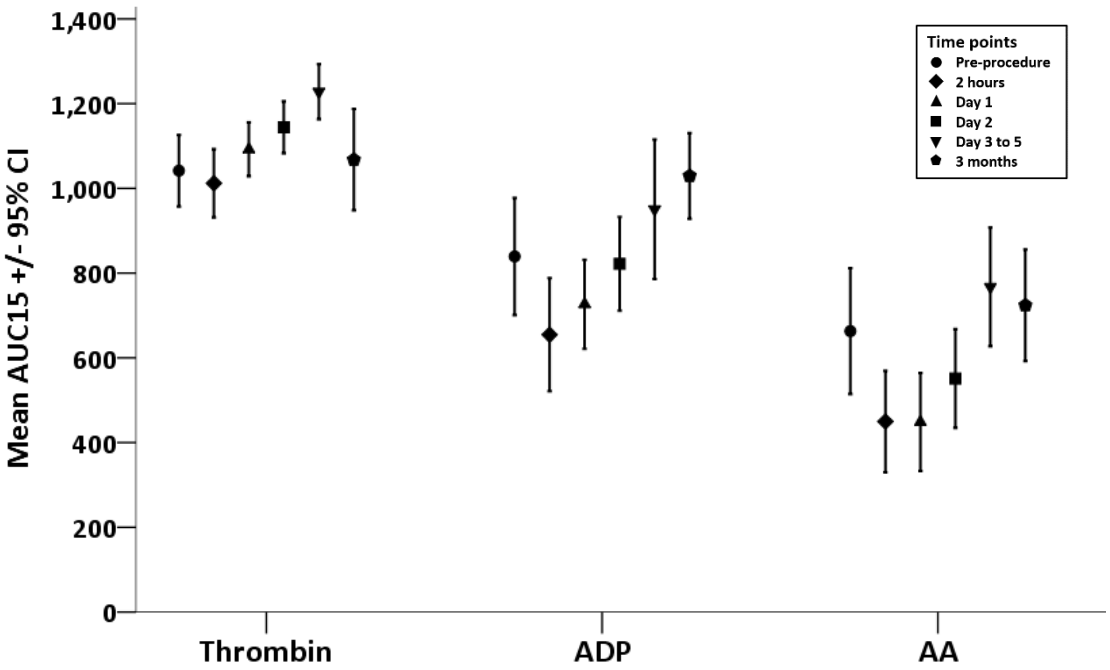


Figure 7.1 Error bar graph showing changes in thrombin, ADP, AA, and thrombin-mediated clotting responses measured with s-TEG in 26patients on aspirin undergoing elective major vascular surgery

7.3.2.1 Arachidonic acid

Repeated-measures ANOVA showed significant differences in $AUC15_{AA}$ ($p < 0.0001$) in the perioperative period (i.e. *t1* to *t5*) as illustrated by Figure 7.2. This was driven firstly by an initial attenuation of $AUC15_{AA}$ from baseline to 2 hours ($p = 0.007$), reaching a nadir at day 1 postprocedure ($p = 0.04$) (Table 7.3 and 7.4). Subsequently, $AUC15_{AA}$ increases significantly at *t5* (i.e. 3 to 5 day time point) compared to 2 hours ($p = 0.001$) and day 1 ($p = 0.003$) postprocedure (Table 7.3 and 7.4). Whilst $AUC15_{AA}$ increases at *t5* to values above baseline, this was not statistically significant (Table 7.4). At 3 months, mean $AUC15_{AA}$ is comparable to baseline values (Table 7.3).

Table 7.3 Summary of ADP-, AA-, and thrombin-mediated clotting before and over 3 months following major vascular surgery

	$AUC15_{Thrombin}$			$AUC15_{ADP}$			$AUC15_{AA}$		
	N	Mean	95% CI	N	Mean	95% CI	N	Mean	95% CI
Preprocedure	26	1041.7	84.6	26	839.2	138.5	25	663.1	148.9
2 hours	26	1012.1	80.6	26	654.8	133.7	26	449.7	119.9
Day 1	26	1092.1	63.7	26	726.3	105.0	26	448.6	115.7
Day 2	25	1144.3	61.6	25	821.9	110.7	25	551.1	116.8
Days 3 to5	25	1228.3	65.1	25	950.5	165.0	25	767.3	140.4
3 months	20	1067.8	121.7	20	1029.4	103.0	20	724.1	133.9

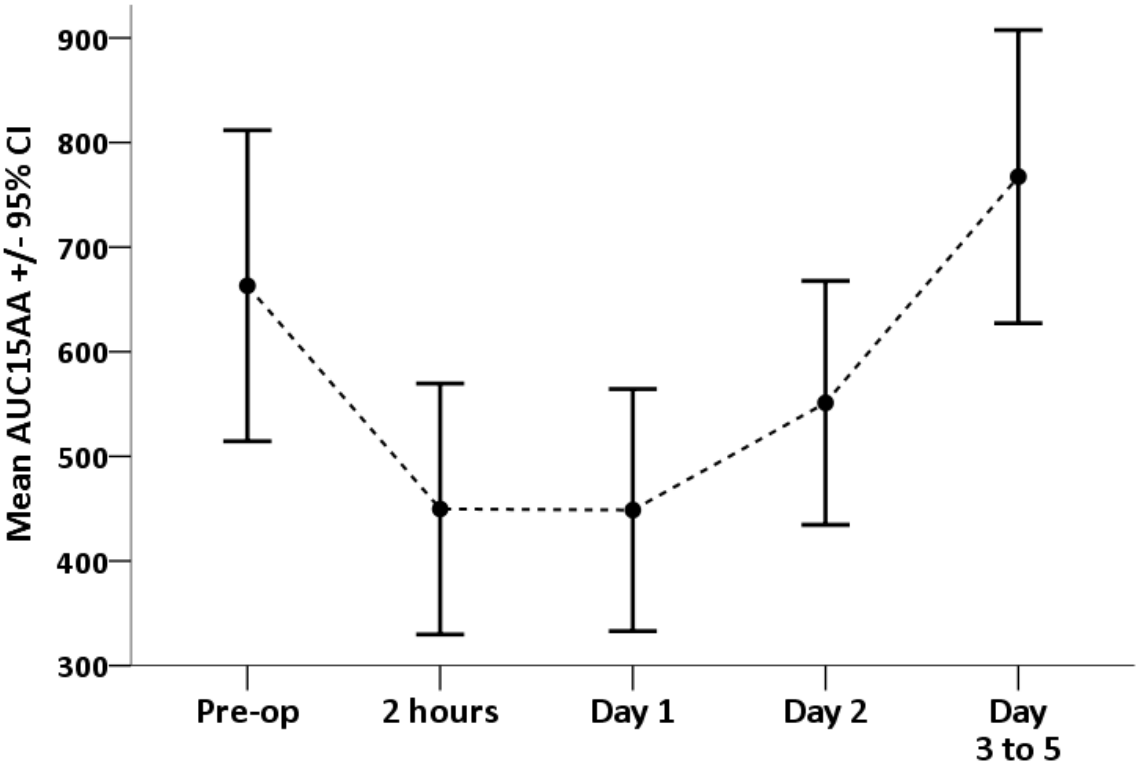


Figure 7.2 Error bar graph showing mean ± 95% confidence interval of $AUC15_{AA}$ in the perioperative period (i.e. *t1* to *t5*) in patients undergoing major vascular surgery

Table 7.4 Pairwise evaluation of ADP-, AA- and thrombin-mediated aggregation between individual time points corrected for multiple comparisons

Parameter	Time points	2 hours	1 day	2 days	3 to 5 days
$AUC15_{Thrombin}$	**Pre-op**	1.0	1.0	0.07	**0.001**
	2 hours		0.07	**0.004**	**0.0001**
	1 day			0.75	**0.007**
	2 days				0.11
$AUC15_{ADP}$	**Pre-op**	**0.044**	0.36	1.0	1.0
	2 hours		1.0	0.15	**0.021**
	1 day			0.77	0.08
	2 days				1.0
$AUC15_{AA}$	**Pre-op**	**0.007**	**0.04**	1.0	1.0
	2 hours		1.0	1.0	**0.001**
	1 day			0.94	**0.003**
	2 days				0.053

7.3.2.2 Adenosine diphosphate

Repeated-measures ANOVA showed significant differences in $AUC15_{ADP}$ ($p = 0.003$) in the postoperative period (i.e. *t1* to *t5*) as illustrated by Figure 7.3. This was driven by an initial attenuation of ADP-mediated aggregation (i.e. $AUC15_{ADP}$) which is evident as early as 2 hours postprocedure compared to baseline ($p = 0.044$) (Table 7.3 and 7.4). Subsequently, $AUC15_{ADP}$ increases significantly at *t5* (i.e. day 3 to 5) compared to at 2 hours ($p = 0.021$) postprocedure (Table 7.3 and 7.4). Mean $AUC15_{ADP}$ at *t5* is numerically higher compared to baseline though this is not statistically significant on pairwise comparisons (Table 7.4). Paradoxically, mean $AUC15_{ADP}$ at 3 months is numerically higher compared to any in-hospital time point (Table 7.3).

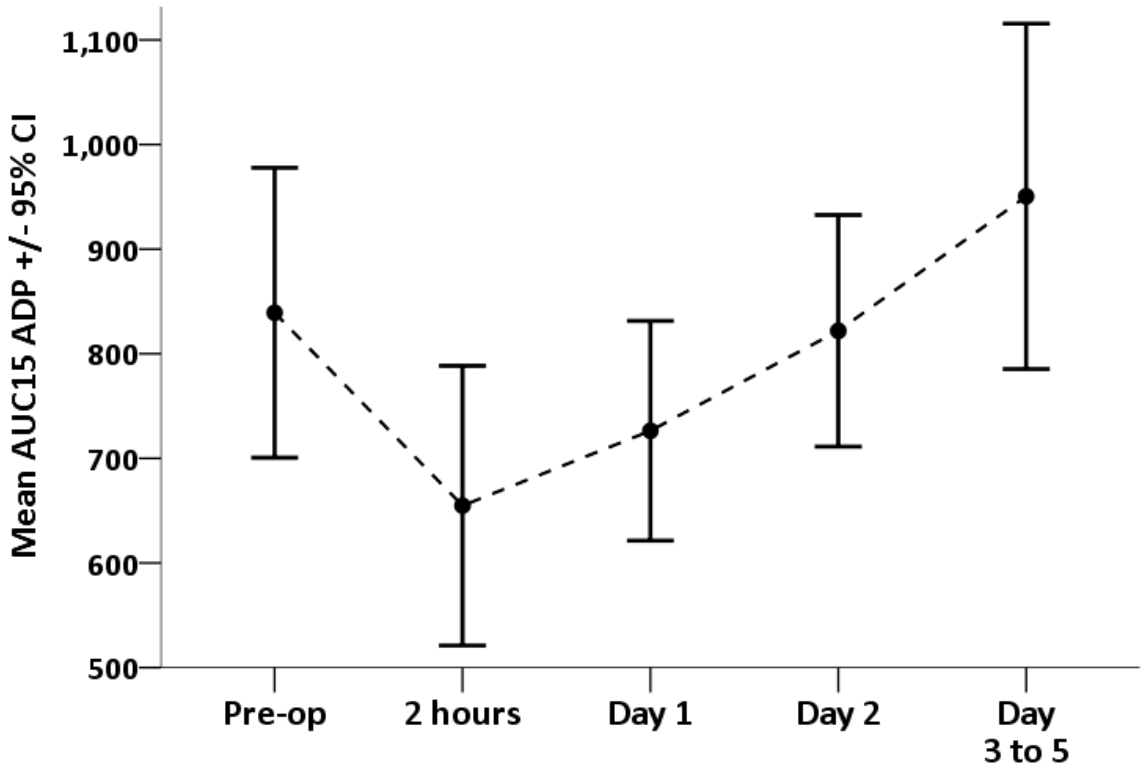


Figure 7.3 Error bar graph showing mean ± 95% confidence interval of $AUC15_{ADP}$ over the perioperative period (i.e. *t1* to *t5*) in patients undergoing major vascular surgery

7.3.2.3 Thrombin-mediated clotting

Repeated measures ANOVA (N = 24) showed significant differences in $AUC15_{Thrombin}$ ($p < 0.0001$) in the perioperative period (i.e. *t1* to *t5*) as illustrated by Figure 7.4. This effect of

time on $AUC15_{Thrombin}$ was largely driven by significant increases at *t5* compared to baseline (p = 0.001), 2 hours (p = 0.0001) and day 1 postprocedure (p = 0.007) (Table 7.3 and 7.4). $AUC15_{Thrombin}$ was significantly greater at the final in-hospital time point (i.e. day 3 to 5 or *t5*) compared to the preprocedure baseline (p = 0.017). At 3 months $AUC15_{Thrombin}$ was comparable to the preprocedure baseline (Table 7.3).

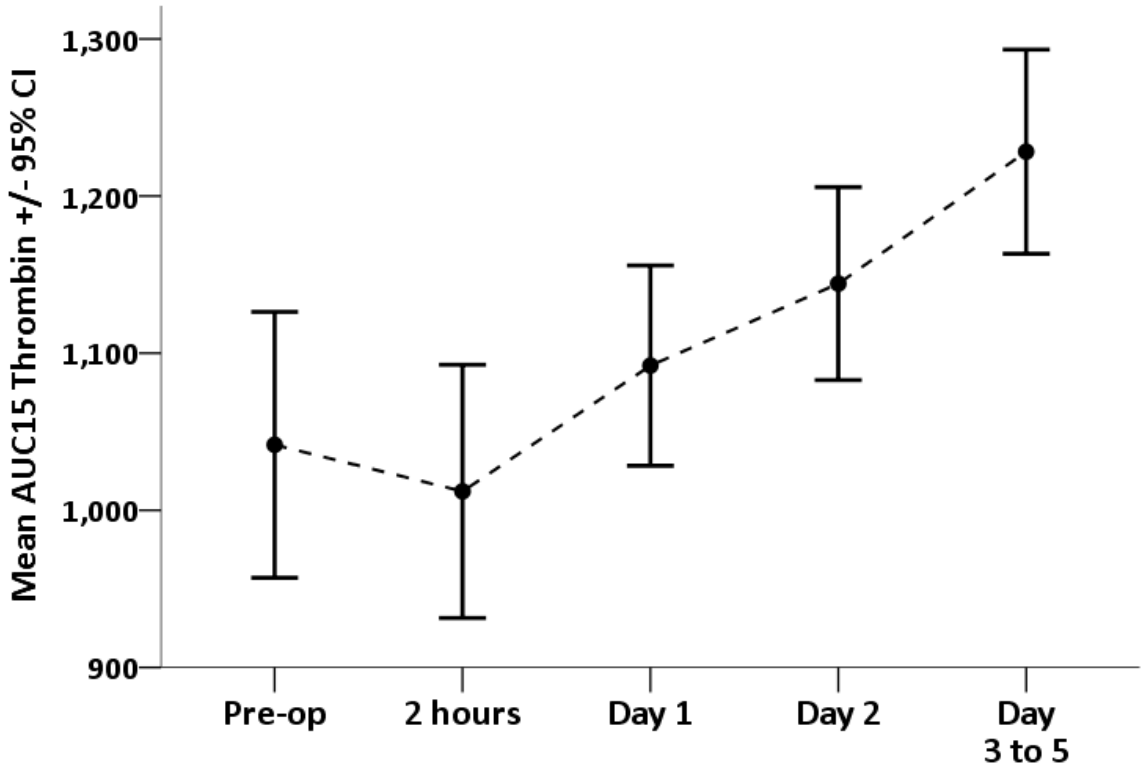


Figure 7.4 Error bar graph showing mean ± 95% confidence interval of $AUC15_{Thrombin}$ over the perioperative period (i.e. *t1* to *t5*) in patients undergoing major vascular surgery

7.4 Discussion

At present, enrolment to this study is incomplete and assay of inflammatory biomarkers as well as products of AA metabolism (i.e. TXB_2 and 12-HETE) remains outstanding. Nevertheless, platelet function data obtained with s-TEG in 26 patients enrolled to the study thus far has been evaluated in this interim analysis yielding the following key results. Firstly, whilst there was a significant and transient attenuation of AA- and ADP-induced platelet aggregation evident within 2 hours following surgery, recovery of platelet function was not associated with significant potentiation of agonist-mediated

clotting above preoperative baseline values. By contrast, this study demonstrated that major vascular surgery is associated with a hypercoagulable state as evidenced by a linear increase in thrombin-mediated clotting in the perioperative period resulting in significantly higher $AUC15_{Thrombin}$ at *t5* compared to baseline.

A number of studies have previously evaluated the functional and biochemical effects of aspirin in patients undergoing cardiac surgery, and these have largely concluded an impaired response to aspirin in the early postoperative period. Based on aggregometry[410] and MEA[411], Bednar *et al* reported significant inhibition of AA-induced aggregation and TXB_2 only occurred on postoperative day 5 in patients undergoing CABG. Similarly, Zimmerman *et al* demonstrated impaired inhibition of AA-induced aggregation and TXB_2 production in aspirin-treated patients undergoing cardiac surgery in the early post-operative period despite addition of aspirin *in vitro*[412]. It is, however, important to note that these studies recruited patients in whom aspirin and other antiplatelet therapy had been withdrawn several days previously, only to be recommenced after surgery. By contrast, all patients recruited to the present study were already established on aspirin for at least 5 days prior to surgery and continued daily aspirin in the perioperative period. Accordingly, we did not observe a potentiation of AA-mediated platelet aggregation in this cohort of aspirin-treated patients.

In patients undergoing MVS, the data with respect to evolution of AA-induced clotting in the post-operative period are conflicting. In contrast to our findings, Rajagopalan *et al* showed that AA-induced clotting increased significantly at postoperative day 1 to 5 in aspirin-treated patients predominantly undergoing MVS for SLI[404]. Similarly, Collins *et al* demonstrated that AA-induced aggregation was potentiated from the time of reperfusion to 24 hours postprocedure[413]. Both studies employed the VerifyNow assay to study AA-mediated platelet aggregation. By contrast, Schneider *et al* did not observe a significant increase in AA-induced aggregation with LTA in the perioperative period in patients undergoing non-emergent vascular surgery, though this study recruited a mixed population mainly comprising patients undergoing carotid endarterectomy, which would be expected to generate a much smaller systemic inflammatory response[414]. Samama *et al* noted a significant increase in ADP-mediated aggregation in the perioperative period in patients undergoing open AAA repair, but no significant potentiation of AA-induced clotting was observed[415]. In the present study there was no potentiation of AA-induced aggregation observed with s-TEG, up to day 5 post-surgery.

In agreement with our findings, previous studies have demonstrated an early attenuation of AA- and ADP-mediated aggregation post-surgery. Based on TEG platelet mapping, Weitzel *et al* demonstrated a significant decrease in MA_{ADP}, MA_{AA} and $MA_{Collagen}$ immediately post-CPB, compared to a pre-CPB baseline in patients undergoing cardiac surgery[416]. Similarly, based on TEG platelet mapping and MEA, Agarwal *et al* demonstrated a significant reduction in AA- and ADP- mediated platelet aggregation post-CPB compared to baseline in patients undergoing cardiac surgery on aspirin therapy[417]. Finally, Zimmerman *et al* also demonstrated a significant decrease in AA-induced platelet aggregation on post-operative day 1 compared to baseline, in patients undergoing aortic valve replacement off-aspirin[418]. Importantly, this does not appear to be a cardiac surgery- or CPB-specific phenomenon as Oberweis *et al* have also demonstrated similar decreases in AA and collagen-induced platelet reactivity early in the postoperative phase following non-emergent orthopaedic surgery[419].

The mechanism of these early changes in AA- and ADP- mediated clotting remains poorly understood. At present, it remains to be seen whether the perioperative changes in AA-mediated platelet reactivity reported in the present study are associated with changes in the vascular inflammatory status that inevitably follow MVS. Furthermore, it will be interesting to note whether these changes occur in an aspirin-independent manner (i.e. irrespective of aspirin's effect on TXB_2 generation) and if so whether these are paralleled by changes in the activity of the 12-LOX pathway as determined by assay of 12-HETE.

Another key finding of this study was that despite intraoperative and postoperative anticoagulation with heparin, MVS was associated with a hypercoagulable state as evidenced by a significant increase in thrombin-mediated clotting compared to baseline. This in itself is not a novel finding as studies have previously demonstrated that surgery, particularly major surgery, results in a prothrombotic state[420, 421]. Diamantis *et al* showed that markers of thrombin generation, including thrombin-antithrombin complex (TAT) and prothrombin fragment (F1+2) are elevated immediately after surgery and remain elevated for at least 72 hours in patients undergoing open or laparoscopic cholecystectomy. Similarly, Parolari *et al* demonstrated higher levels of F1+2 and TAT complex at least 30 days following both conventional as well as off-pump CABG. Finally, Collins *et al* have also demonstrated increased levels of TAT complex in the early post-operative period following lower limb bypass surgery for SLI. Furthermore, studies have also shown a decrease in natural anticoagulants including protein C and antithrombin III

that normally limit thrombin generation, in the context of abdominal aortic surgery. Finally, surgery is commonly associated with an increase in levels of fibrinogen, an acute phase protein synthesised in the liver, which plays a key role in clotting and is commonly associated with activation of coagulation.

There are important considerations and limitations to this data. Firstly, this is an interim analysis from an ongoing study and the sample size is small. Secondly, whilst serum samples for measurement of inflammatory biomarkers and products of AA-metabolism have been collected, these will only be analysed upon completion of the study. Thirdly, the study population includes a mixed cohort of individuals undergoing open abdominal surgery as well as those undergoing lower limb bypass surgery, however both procedures are associated with a significant systemic inflammatory response. Finally, several studies have shown that haematocrit can affect results obtained with platelet function testing and certainly this has been shown with TEG[422, 423] and VerifyNow[424, 425]. It is therefore conceivable that our results could have been confounded by changes in haematocrit post-surgery. Nevertheless, it has been suggested that this association may represent an assay-specific *in vitro* phenomenon that is independent of intrinsic changes in platelet reactivity as no association between haematocrit and ADP-induced platelet aggregation were shown with LTA which is performed in PRP in the absence of red blood cells[425-427], or MEA [427].

7.5 Conclusion

In conclusion, this study demonstrates that whilst major vascular surgery results in a hypercoagulable state, this is not associated with a potentiation of AA- and ADP-mediated platelet aggregation compared to baseline in aspirin-treated patients. Nevertheless, we did observe significant changes in AA-mediated clotting in the perioperative period. It will be interesting to observe whether these are associated with changes in levels of vascular inflammation and if they occur in an COX-1-independent manner, once this data becomes available. Vascular inflammation is likely to be an important factor in the development of the postoperative hypercoagulable state described here. If subsequent analysis of a completed cohort confirms these findings, then it will raise interesting questions about

the link between vascular inflammation and thrombotic events which will demand further investigation.

CHAPTER 8 – DISCUSSION

8.1 Summary of findings

The role of platelets in inflammation, atherogenesis, haemostasis and thrombosis is well established. Furthermore, there has been considerable progress in our understanding of the complex downstream signalling pathways that cascade from the activation G-protein coupled receptors which are pivotal in the expression and amplification of platelet responses to a multitude of agonists. These advances in platelet biology have helped inform the development of novel APT presently under investigation in preclinical studies and randomised clinical trials. Nevertheless, the spectrum of APT presently licensed for primary and secondary prevention of CVD remains dominated by aspirin and $P2Y_{12}$ inhibitors. Indeed, concomitant inhibition of the ADP-$P2Y_{12}$ pathway with clopidogrel and the AA-COX-1 pathway with aspirin has been a major strategy for the prevention of ischaemic complications in patients undergoing PCI.

However, studies employing a variety of *ex vivo* PFAs have consistently reported wide variability in individual responses to APT and unequivocally demonstrated that HPR, in particular whilst on clopidogrel, is an important determinant for recurrent atherothrombotic events post-PCI. Whilst potent $P2Y_{12}$ inhibitors afford a substantial absolute risk reduction in MACE of around 2% compared to clopidogrel, a "ceiling effect" on net clinical benefit is suggested by the significantly higher rates of non-procedure-related major bleeding associated with these agents. A universal strategy of potent $P2Y_{12}$ blockade may therefore be detrimental for those at high risk of bleeding complications. However, several characteristics such as older age, anaemia, chronic renal failure, female sex, and diabetes, that are independent predictors of bleeding, are also associated with ischaemic events post-PCI. Several observational studies have suggested that platelet reactivity represents a common metric that can provide incremental predictive value for discriminating between those at greater risk of bleeding at one end of the therapeutic spectrum versus those at greatest risk of post-PCI ischaemic events at the other. Moreover, these studies have also suggested the existence of a "therapeutic window" associated with the lowest risk of both thrombotic and bleeding complications.

These prospective observational studies demonstrating an association between PR and ischemic events as well as bleeding complications, offer a persuasive argument for

personalised APT. Nevertheless, despite this body of evidence, routine platelet function testing has not yet permeated everyday clinical practice for several reasons. Firstly, there is no universally accepted method for assessing a patient's response to APT. Secondly, there is no standardised definition for what constitutes "hyporesponsiveness" (i.e. HPR), because the cutoffs used in clinical studies are highly assay specific and poorly validated to ischaemic or bleeding events. Finally, there is insufficient evidence to support modifying APT in patients shown to have HPR, because all large randomised trials investigating this tailored approach have failed to show benefit for a strategy of personalised APT. However, the results of these trials may have been limited by their design due to insufficient power, inadequacy of the pharmacodynamic intervention, and possible selection bias for low-risk populations.

It is noteworthy that all large randomised trials of personalised APT including GRAVITAS, TRIGGER-PCI and ARCTIC employed the VN-P2Y12 assay to detect HPR. Based on previous discrepancies between VN-P2Y12 and s-TEG in patients presenting with DES ST, we hypothesised that VN-P2Y12 may overestimate the functional effects of clopidogrel in some individuals. In Chapter 3, I demonstrated that VN-P2Y12 finds a larger proportion of healthy volunteers and patients who had taken clopidogrel were within its "therapeutic range" compared to s-TEG. Paradoxically, VN-P2Y12 also reported a higher proportion of clopidogrel "responders" compared to s-TEG in individuals who were "clopidogrel-naïve" at the time of testing. An assay that overestimates the functional effects of clopidogrel may fail to identify a proportion of individuals with an "inadequate response" that otherwise might have derived benefit from intensified APT. Moreover, such a test could commit patients responding adequately to clopidogrel to more intensive therapy. We speculated that the inclusion of PGE1 in VN-P2Y12 may provide a mechanistic explanation for the discrepancy between results reported by these two *ex vivo* whole blood PFAs. In chapter 3 we also demonstrated that the addition of PGE1 in the same concentration as that employed by the VN-P2Y12 assay resulted in potentiation of the antiaggregatory effects of clopidogrel with s-TEG. Furthermore, inclusion of PGE1 in s-TEG also resulted in improved concordance (> 90%) between the two assays.

We systematically evaluated the effects of PGE1 on $P2Y_{12}$ blockade in an *in vitro* model using PAM. In Chapter 4, I showed that PGE1 potentiates the antiaggregatory effects of PAM in a dose-dependent manner. Furthermore, inclusion of PGE1 resulted in a ten-fold reduction in the dose of PAM required to achieve the degree of platelet inhibition

attained by PAM alone in the absence of PGE1. These results are readily understandable given that PGE1 stimulates adenylyl cyclase activity resulting in potentiation of intracellular cAMP. The latter, like cGMP, is a cyclic nucleotide known to supress broad categories of platelet responses including release of Ca^{2+} from intracellular stores, granule secretion, activation, adhesion and aggregation. Furthermore, cyclic nucleotides are capable of inhibiting a multitude of activation pathways including those stimulated by ADP, thrombin, TXA_2 and collagen. Furthermore, studies have shown considerable cross-talk between various GPCRs, and in the case of the purinergic receptors $P2Y_1$ and $P2Y_{12}$, this is thought to occur at the level of Ca^{2+} signalling. The argument that inclusion of PGE1 makes PFAs more specific for $P2Y_{12}$ blockade by "suppressing the contribution of the $P2Y_1$ receptor on ADP-mediated platelet aggregation" in isolation therefore seems increasingly facile.

Nevertheless, numerous observational studies have consistently demonstrated that HPR based on VN-P2Y12, is indeed an independent predictor of MACE in patients undergoing PCI. It may be possible to reconcile these observations given that inclusion of PGE1 to some extent likely simulates *in vitro* the platelet inhibitory function of vascular endothelium *in vivo*. When intact, the vascular endothelium releases endogenous autacoids including nitric oxide and prostacyclin that maintain platelets in a quiescent state by augmenting intracellular concentrations of cyclic nucleotides including cAMP and cGMP. However, endothelial dysfunction is a hallmark of CVD and various risk factors for atherosclerosis including, hypercholesterolaemia, hypertension, diabetes and smoking are known to be important determinants of endothelial function. It is therefore unlikely that a standard concentration of PGE1 *in vitro* can accurately simulate the physiological state *in vivo* on an individual basis.

Studies have also suggested that responses to PGE1 may be blunted in certain populations including diabetes[356] and stable angina[357]. Inclusion of PGE1 may therefore confound response to $P2Y_{12}$ blockade by underestimating inhibition of ADP-mediated aggregation in some individuals. This may explain why the threshold that optimally defines HPR with VN-P2Y12 appears to vary in different clinical syndromes and disease states. For instance, a Korean study reported a higher threshold of PRU > 272 was highly predictive for adverse cardiovascular events in AMI but not in a non-AMI setting (i.e. stable angina or UA)[428]. Indeed, studies including GRAVITAS have suggested that a lower threshold of PRU > 208 had greatest prognostic utility in a population of patients with

stable angina[211, 290]. Similarly, another study reported vastly different thresholds for HPR to predict clinical events after PCI in patients with diabetes compared to those without[429]. These discrepancies may represent differential responses to PGE1 rather than reflecting genuine differences in the platelet inhibitory effects of $P2Y_{12}$ inhibition. Thus, whilst assays employing PGE1 may retain some predictive value for post-PCI ischaemic events, this may be underestimated in certain populations. Conceivably, this may well impact the ability of an assay employing PGE1 to accurately stratify patients into "responders" and "nonresponders" in a trial of personalised therapy.

Though inter-individual variability of response to APT is well documented, uncertainty remains over the stability of OTPR over time. In chapter 5, I demonstrated important variability of platelet reactivity over 6 months in patients on long-term DAPT. This was driven by significant increases in AA- and ADP-mediated aggregation at one week compared to a predischarge baseline (usually within 24 hours of angiography). We did not measure platelet reactivity before cardiac catheterisation or even immediately postprocedure, though studies have suggested that platelet reactivity and activation do increase transiently immediately postprocedure. In our study the platelet inhibitory responses to APT were greatest (i.e. platelet reactivity was lowest) prior to hospital discharge at baseline (i.e. around 24 hours postprocedure). Similar trends have been shown in some longitudinal studies previously. As patients had been established on DAPT for at least 24 hours prior to testing at baseline in our study, it was speculated that these early changes in PR observed in our study may be related to levels of vascular inflammation, which may be transiently elevated in the context of ACS or the procedure itself (i.e. angiography or PCI), which, in the vast majority of cases, included PCI with stents. This study was however underpowered to confidently examine the interaction of these factors on variability of PR over time. Moreover, levels of inflammatory biomarkers were not specifically measured in this study. Further studies are therefore required to better understand potential mechanisms of the early but transient attenuation of PR reported in this study.

If trends reported in our study are confirmed, then this would suggest that "early" testing of platelet function prior to reaching steady state may yield inaccurate results in a proportion of individuals. Incidentally, platelet function testing in both GRAVITAS and TRIGGER PCI was performed within 24 hours of PCI. Our findings raise concerns about using a single predischarge assessment of PR as the sole arbiter for individual patient

response to DAPT as part of a personalised medicine strategy. It may be that serial measurements of platelet reactivity may be required for adequately monitoring the efficacy of APT. Alternatively, a long-term strategy of APT may be better guided by PFT performed preprocedure or when platelet reactivity is steady state postprocedure. The optimal time point with greatest prognostic utility with respect to the hazard of future MACE also remains to be established and would be of critical importance for the design and implementation of a strategy for tailored DAPT therapy in PCI patients.

Whilst the role for platelet function testing in guiding personalised APT has been under investigation recently, it has been extensively employed in clinical research for pharmacodynamic evaluation and dose selection of novel antiplatelet agents for some time. In chapter 6, we used two different PFAs (i.e. MEA and s-TEG) to evaluate differences between the antiplatelet and anticoagulant effects of UFH and bivalirudin in patients undergoing PPCI. In this substudy of the HEAT-PPCI trial we demonstrated no significant differences between these antithrombotic agents with respect to ADP-, AA- and TRAP-mediated platelet aggregation. These results from a comparatively larger cohort are in contrast to those from several smaller observational series that have predominantly shown that heparin causes platelet activation in comparison to bivalirudin which was shown to inhibit platelet function in some series. However, platelets exhibit a variety of different biological responses due to activation of distinct but often intercalated pathways. Furthermore, other than endothelial denudation, a variety of physiological and pathological stimuli such as proinflammatory cytokines, infectious agents and shear stress can cause platelet activation. Outside-in signalling via the platelet $\alpha_{IIb}\beta_3$ integrin receptors has been implicated in heparin-induced platelet activation. However, the fact that we found no significant differences between heparin and bivalirudin in agonist-mediated aggregation (i.e. ADP, AA and TRAP) raises questions about the clinical significance of differential expression of platelet activation markers reported in some studies. Regardless, these results suggest that neither anticoagulant has potent antiplatelet effects.

There were however, significant differences between UFH and bivalirudin with respect to thrombin-mediated clotting measured by s-TEG, which was obliterated in patients treated with UFH. By comparison, whilst bivalirudin caused prolongation of clot initiation and propagation, it was also associated with a significantly greater clot strength. The latter finding may be related to loss of negative feedback inhibition of thrombin generation via

APC and may provide some insight into the mechanism of the elevated risk of AST observed in patients treated with bivalirudin. Nevertheless, the higher incidence of AST associated with bivalirudin may be explained by its relatively short plasma half-life, particularly in light of delayed action of $P2Y_{12}$ inhibitors in STEMI. This argument is supported by encouraging results from studies employing a prolonged post-PCI bivalirudin infusion. However, further prospective randomised studies specifically evaluating the relative efficacy, optimal dose and safety of a prolonged infusion of bivalirudin are required. In addition, further *in vitro* and *ex vivo* studies measuring TG, TM and APC are required to understand the mechanism of increased clot strength associated with bivalirudin reported in our study.

In Chapter 7, I have described interim results from an ongoing study where we are evaluating whether AA-mediated platelet aggregation, as measured with s-TEG, varies with the vascular inflammatory status in an aspirin-independent manner. The basic premise for this investigation follows from previous findings of high clotting responses to AA despite adequate suppression of TXB_2 reported previously by our group in different patient populations (i.e. stroke and those on long-term DAPT following PCI). Similarly, we have observed aspirin-insensitive AA-mediated clotting in the variability study reported in Chapter 5. In the vascular surgery study, we observed an early attenuation in ADP- and AA-mediated clotting, a finding that has been previously observed in various settings including cardiac and orthopaedic surgery. Interestingly, in the variability study described in chapter 5, we also reported that the platelet inhibitory response to aspirin and clopidogrel on AA- and ADP- mediated aggregation peaked early after angiography at baseline (i.e. 24 hours postprocedure). The mechanism behind attenuation of platelet reactivity in either setting (i.e. surgical and non-surgical) remains poorly understood and warrants further investigation. Measurement of inflammatory biomarkers in the vascular surgical study presently remains outstanding, however, this may offer some insight into the early attenuation of ADP- and AA mediated platelet reactivity in the first 24 hours after surgery. The study does, however, clearly demonstrate a significant increase in $AUC15_{Thrombin}$ following major vascular surgery despite intra- and post-operative anticoagulation with UFH and LMWH respectively. This hypercoagulable state in the early postoperative phase may explain the relatively high incidence of perioperative MI (~ 5%) in patients undergoing non-cardiac surgery previously reported in some studies such as

the randomised POISE trial[430]. Therapeutic strategies targeting this prothrombotic state to improve clinical outcomes following major surgery warrant further investigation.

8.2 Relevance of findings

In studies described here, insights gained with s-TEG have raised concerns about the stability of platelet reactivity over time in patients on DAPT and moreover the appropriateness of using assays such as VN-P2Y12 that employ PGE1 to measure response to $P2Y_{12}$ blockade. Our observations with s-TEG have also provided potential mechanistic explanations for the higher incidence of AST reported in PPCI patients treated with bivalirudin as well as for the higher incidence of perioperative MI in patients undergoing surgery. Further studies are required to confirm and better understand our findings.

The research presented in this thesis raises important questions pertinent to the clinically relevant dilemma of improving clinical outcomes in PCI, and CVD at large. Optimisation of current approaches and therapies are as important to that endeavour as innovation. In this regard, personalised APT seems a logical corollary to the therapeutic counterpoise between superior efficacy and inferior safety of potent $P2Y_{12}$ inhibitors compared to clopidogrel. Similarly, whilst bivalirudin has the theoretical advantage of a better pharmacological profile (i.e. direct, predictable and reversible) compared to UFH, this has not translated into superior clinical outcomes (i.e. MACE or bleeding complications) compared to UFH alone in the HEAT-PPCI trial. Moreover, concerns regarding a higher rate of AST in patients treated with bivalirudin have been reaffirmed. Whilst these concerns may be potentially overcome by a prolonged infusion, the safety and efficacy of this approach compared to a reference of UFH alone are unproven. Furthermore, the optimal duration and dosing of such a post-PCI bivalirudin infusion are yet to be determined.

A personalised strategy of intensifying APT in patients with a poor pharmacodynamic response to clopidogrel has the advantage of not subjecting all patients to a high bleeding risk, unless there is objective evidence for an inadequate therapeutic response based on

platelet function testing. Though logical, such a strategy cannot be implemented in everyday clinical practice whilst some important questions remain unanswered. Firstly, what is the ideal platelet function assay for determining individual responses to APT? A suitable assay would be rapid, reproducible, have a standardised methodology, be easy to perform, and reliable at differentiating patients with an adequate versus inadequate therapeutic response. Whilst VerifyNow has dominated clinical research over the last decade as a true POC assay, our group has raised legitimate concerns over the validity of employing PGE1 as agonist for assessing response to $P2Y_{12}$ inhibitors. The optimal threshold for defining HPR also remains a subject of debate. Furthermore, the population that would benefit most from intensified APT is poorly defined. A recent meta-analysis suggested that the net clinical benefit of intensified APT depended upon the baseline risk of ST on clopidogrel. The optimal pharmacodynamic intervention to overcome HPR in those with a poor response to clopidogrel also remains to be established. Whilst potent $P2Y_{12}$ inhibitors such as prasugrel and ticagrelor have been compared to clopidogrel in randomised studies, there is a relative dearth of observational and randomised data comparing these agents to each other. A head-to-head comparison of prasugrel and ticagrelor in patients admitted with ACS being managed with a planned invasive strategy is anticipated in the ongoing ISAR-REACT 5 study[431]. Finally, the optimal interval for performing platelet function testing to determine response to APT warrants further investigation, in light of the early variability of platelet reactivity reported in chapter 5.

The well established role of aspirin as part of DAPT in patients undergoing PCI has recently been challenged in the era of potent $P2Y_{12}$ inhibitors[432]. This has partly been driven by observations that apart from the entirely predictable effects on the ADP-$P2Y_{12}$ pathway, $P2Y_{12}$ inhibitors also have significant inhibitory activity against the AA-COX-1 pathway. Indeed, studies have shown that aspirin has no additive platelet inhibitory effects on agonist-mediated aggregation (i.e. including in response to AA) when combined with PAM or ticagrelor *in vitro*[433-435]. In the CAPRIE trial, clopidogrel (75mg once daily) was superior to aspirin monotherapy in a broad group of patients with CVD[83]. In the MATCH study, the addition of aspirin to clopidogrel had no significant effect on major vascular events compared to clopidogrel alone in patients with recent ischaemic stroke or TIA[436]. In PCI patients on oral anticoagulation in the WOEST trial, clopidogrel was safer with respect to bleeding complications compared to DAPT (i.e. aspirin plus clopidogrel), without an excess of thrombotic events over a 1-year follow up[437]. This growing body of

evidence raises important questions about the relative contribution of aspirin to the balance of risk versus benefit in patients undergoing PCI on potent $P2Y_{12}$ inhibitors. This precise question is the subject of investigation in the GLOBAL LEADERS trial (ClinicalTrials.gov Identifier: NCT01813435), which is presently randomising an all comer's PCI population (i.e. ACS and stable CAD) to either aspirin and ticagrelor for 1 month followed by ticagrelor monotherapy for 23 months or standard therapy (i.e. DAPT for 12 months followed by aspirin monotherapy for 12 months).

8.3 Future perspectives

The research described in this thesis is predominantly derived from investigator-led observational or mechanistic studies performed in a single centre. The sample size was therefore inevitably restricted which was an important limitation of this work. As such these studies can be regarded as hypothesis-generating only. Nevertheless, based on s-TEG and other PFAs, we have provided some important insights that warrant further investigation. I have already highlighted a number different ways in which my research could be elaborated upon.

The future of personalised antiplatelet therapy remains uncertain amid dampened enthusiasm for this strategy in light of the serially negative large randomised trials. The association between platelet reactivity and adverse ischaemic or bleeding events highlights the limitations of a "one-size-fits-all" approach, but does not in itself justify widespread implementation of personalised APT. Moreover, several uncertainties regard the optimal methodology for such an approach remain outstanding. Chief amongst these is the careful and accurate selection of patients who based on the demonstration of HPR merit intensified APT. To that end, the requirement for a simple, easy, rapid and reliable POC test of platelet function is clear cut if a strategy of personalised APT is to be delivered accurately. Amongst a range of candidates, thus far, VerifyNow has emerged as the only true POC assay that could potentially meet these requirements. However, our work has raised important concerns about the validity and accuracy of using such an assay which employs PGE1 as agonist.

Several attributes of the TEG technology make it a viable and attractive alternative for the delivery of personalised APT. Recent studies in a PCI population have further expanded the evidence base for the prognostic utility of TEG[265], where previously there was a relative paucity of data. Whilst s-TEG is capable of assessing individual time-dependent responses to APT in just 15 minutes, an important limitation of the TEG 5000 analyser has always been the need for skilled operators and sample preparation which has precluded its wider application as a true POC device. This has recently been addressed by the manufacturers who have developed a new device called TEG 6s. This novel technology is based on measuring the harmonic motion of a pendant drop of blood in response to external vibration. As the sample transitions from a liquid state to a gel-like state during clotting, the modulus of elasticity and resonant frequency increases. The instrument measures these variations in resonant frequency during clotting and lysis and displays the results on a touchscreen display. Like VerifyNow the assay makes use of disposable cartridges pre-filled with the reagents requisite for platelet mapping.

In contrast to its predecessor, the new TEG 6s device is simple, compact, and does not require any complex sample preparation. I have helped design, and sought ethical approval for a series of experiments that will evaluate the reproducibility and ability of TEG 6s to detect time-dependent changes in clotting responses to standard agonists in healthy volunteers and patients being loaded with aspirin and/or clopidogrel. Furthermore, the study which has already commenced recruitment will also evaluate the correlation and consistency of results against the well-established TEG 5000 series. A genuine POC device which produces timely and equivalent results, if validated, could potentially improve the standard of care in a variety of clinical settings including PCI.

Strategies for improving clinical outcomes following PCI have evolved tremendously over time and are gradually approaching a point of “diminishing returns”. In this regard, major bleeding is proving to be the Achilles heel of adjunctive pharmacotherapy in PCI. Further progress in this discipline will therefore be critically dependent upon an exhaustive re-appraisal of existing practices including the relative importance of aspirin in DAPT, the role of long-term anticoagulation in ACS/PCI, the ideal duration of DAPT, and the optimal adjunctive antithrombotic regimen in PPCI. Moreover, a strategy of personalised APT is logical given the established association between platelet reactivity and adverse events post-PCI, though this remains to be demonstrated in carefully designed and adequately powered randomised studies.

CHAPTER 9 – APPENDICES

9.1 Variability of Antiplatelet therapy over time

9.1.1 Research participant information sheet

Cardiology Trials Office
Cardiovascular and Thoracic Unit
University Hospital Southampton NHS Foundation Trust
Tremona Road
Southampton
SO16 6YD

Tel 02380798538

Research Participant Information Sheet and Consent Form

1. Study Title – Antiplatelet therapy response variability

Intra-individual variability in responses to aspirin and clopidogrel in patients with ischaemic heart disease: is there evidence of reduced responsiveness or "tolerance" to antiplatelet therapy over time?

2. Invitation Paragraph

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish.

Ask us if there is anything that is not clear or if you would like more information.

3. What is the purpose of the study?

Aspirin and clopidogrel are essential antiplatelet agents (drugs used to prevent platelets from binding together and forming blood clots) that are widely used in the treatment of coronary heart disease. They reduce the risk of adverse events including recurrent heart attacks and admissions to hospital with unstable angina. In addition, they are particularly important in patients who have undergone coronary stent implantation as they reduce the risk of stent thrombosis (blood clots forming within stents) which often leads to a heart attack
Current clinical guidelines recommend aspirin for life and clopidogrel for up to 12 months in all patients who have had a heart attack and/or who have received a drug-eluting coronary stent However, despite aspirin and clopidogrel treatment a significant proportion of patients continue to experience recurrent adverse clinical events. It has been suggested from previous clinical studies that this may be due to the fact that some patients respond poorly to aspirin and/or clopidogrel and are, thus, considered "resistant" to these drugs.

This study will seek to determine, through a series of blood tests: (i) the proportion of patients with coronary heart disease who are resistant to aspirin and/or clopidogrel treatment measured using a simple, rapid, bedside platelet function test, (ii) whether their responses remain the same or vary significantly over a period of time, and (iii) whether their responses are related to blood marker levels of inflammation The findings from this study may support the need for routine measurement of responses to aspirin and clopidogrel in all patients with coronary heart disease in the future in order to identify those patients who are "resistant" to these drugs and who would, therefore, benefit from individually tailored treatment (i.e. increasing their aspirin dose or use of an alternative antiplatelet agent).

4. Why have I been chosen?

You have been chosen because you are receiving regular aspirin and clopidogrel treatment for one of the following reasons:

(i) you have been admitted to hospital with a heart attack or unstable angina and may or may not have undergone coronary stent implantation
(ii) you have been admitted to hospital for an elective coronary angiogram to investigate your angina symptoms and have undergone drug-eluting coronary stent implantation

5. Do I have to take part?

No. Your participation in this study is entirely voluntary. It is up to you to decide whether to take part or not. If you do decide to take part, you are free to leave the study at any time and without giving a reason. This will not affect your future medical care in any way. Furthermore, your study doctor may withdraw you from the study if they feel this is in your best interest or if the study is stopped early.

If you do participate, you will be given this information sheet to keep and be asked to sign a consent form.

6. What will happen to me if I take part and what do I have to do?

After discussing the study and having read the information sheet the study doctor will address any questions you may have regarding the study. You will be given sufficient time to decide whether you would like to take part. If you decide to participate, the study doctor will sign a consent form with you at the time of your admission to hospital.
Your participation will involve a total of 5 blood tests over a 6 month period. The first blood sample will be taken during your inpatient hospital stay and the remaining 4 samples will be taken at subsequent visits to the hospital. At each visit approximately 3 tablespoons (30mls) of blood will be taken. Details regarding the timing of blood tests and follow up are outlined below.

Summary of study procedures

Time period	Intervention
At hospital admission	Consent to study
Prior to hospital discharge	First blood sample taken
1 week after hospital discharge	Second blood sample taken
1 month after hospital discharge	Third blood sample taken
3 months after hospital discharge	Fourth blood sample taken
6 months after hospital discharge	Fifth blood sample taken **This marks the end of study participation**

For the time that you are in the study, the study doctor will enquire how you are feeling at every blood test appointment and will need to carefully monitor all the other medications that you are taking. Please speak with your study doctor before taking any non-prescription drugs or drugs prescribed by another doctor. There are no changes required to your routine care other than a requirement for the research staff to carefully monitor all your medication.
If you decide to withdraw from the study, you will be asked to return to your study doctor for a final visit to ensure that there are no outstanding safety concerns that need to be addressed prior to your discontinuation in the study.
If you feel unwell at any time during the trial, please tell the study doctor. If you seek emergency care, or if hospitalisation is required, please inform the treating doctor that you are participating in a research study.

7. Expenses and payments

Once you have attended for the final study visit, please contact the research office to arrange reimbursement of travel expenses. You will be paid for travel expenses related to your participation in the study such as public transport, petrol and car park charges. This will then be forwarded to you in the form of a personal cheque

8. What are the possible disadvantages and risks of taking part?

When a needle is inserted into your vein to draw blood, you may experience pain, bruising, swelling, bleeding, irritation or infection at the site of the puncture, or you may experience dizziness or faintness**.** Approximately 3 tablespoons (30mls) of blood will be collected at each appointment. In the unlikely event of an injury caused by taking part in the study, appropriate medical treatment will be provided.

9. Risks for Women of Childbearing Potential

The risk to women of childbearing potential participating in this study is nil, since no study drug will be administered during this study. Nevertheless, if you become pregnant during this study, please notify your study doctor.

10. What are the alternatives for diagnosis or treatment?

All standard treatment and care will remain available.

11. What are the other possible disadvantages and risks of taking part?

Occasionally during the course of a study you may be found to have a previously undiagnosed medical condition. In this situation your study doctor will take the necessary steps to ensure you receive appropriate treatment.

12. What are the possible benefits of taking part?

There may be no direct benefit to you from taking part in the study. However, if the results of your blood tests appear clinically significant we will inform the Consultant Cardiologist directly responsible for your care. You may feel that you benefit from the additional close follow up during the course of the study. The results of the study may add to the understanding of your condition and could be helpful for future patients.

13. What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed.

14. What if relevant new information becomes available?

Sometimes during the course of a research study, new information becomes available about the treatment that is being studied. If this happens, your study doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide to continue in the study you may be asked to sign an updated consent form. In addition, new information may lead your study doctor to consider withdrawing you from the study if they feel it is in your best interest. If for any reason the study is stopped prematurely, the study doctor will explain the reasons why and arrange for your care to continue.

15. What will happen if I don't want to carry on with the study?

Your participation in this study is entirely voluntary. It is up to you to decide whether to take part or not. Even if you do decide to take part, you are free to leave the study at any time without

giving a reason. If you decide to withdraw your consent, your study doctor will ask your agreement to perform the final evaluation and to collect the data. The study doctor will need to use the data collected up to the point of your withdrawal (including stored samples) as this data will be relevant and useful in the final data analysis. If you do not agree, your stored samples will be destroyed and none of the data collected will be included in our data analysis.

16. What if there is a problem?

If you have any problems, concerns, complaints or other questions about any aspect of the way you have been approached or treated during the course of this study, the normal complaints mechanisms of the NHS are available to you. You should preferably contact the investigator first, Professor N Curzen on telephone 023 8079 4972. Alternatively you may contact the hospital complaints department on telephone number 023 8079 6325.

17. Will my taking part in this study be kept confidential?

Your study doctor and the research staff will collect information about you. This information, called data, will be entered without your name, into a local database. In the database a number will replace your name. All the data collected will be kept confidential.
Only authorised personnel will enter the data into the database. The researchers organising this study will take all necessary steps to protect your privacy.
Your identity, including your name, will not be revealed in any compilation, study report or publication at any time. Your study doctor will maintain a confidential list linking your name to the number and only authorised persons will have access to this list.
You have access rights to your data and the possibility to rectify the data according to local law and procedures.
In order to make sure that the data collected from you is correct, it is necessary for a local representative from Research & Development to directly compare data with your medical records. Such checks will only be done by qualified and authorised personnel. All such persons are required to keep the data confidential.

18. Involvement of the General Practitioner/Family Doctor

Your family doctor (GP) will be notified of your involvement in this study if you give permission to do so.

19. What happens to any samples I give?

Your study doctor will collect the blood samples. Two blood samples will be analysed on-site immediately after they have been taken and one sample will be frozen and stored securely in the hospital Pathology Department. Access to this department is restricted by an entry key-pad. This allows several samples to be tested at once making efficient use of testing equipment. The frozen samples will be analysed on-site and will be destroyed following analysis. They will not be used for any additional testing.

20. What will happen with the results of the research study?

The data collected will be used for study evaluation only. Members of health authorities, such as the Medicines and Healthcare products Regulatory Agency (MHRA) and Research Ethics Committees or other organisations required by law may review the study data. Your data will be used in publications but your identity will not be revealed in any compilations or study reports**.**

21. Who is organising and funding the research?

The study has been designed by Professor Nicholas Curzen who is a Consultant Cardiologist and Professor of Interventional Cardiology within The Wessex Cardiac Unit, Southampton General Hospital. Contact details are as follows:

Professor Nicholas Curzen
Professor of Interventional Cardiology and Consultant Cardiologist
Wessex Cardiac Unit
Southampton General Hospital
Hants
Tel: 023 8079 8538
Fax: 023 8079 5174

Neither Professor Curzen or the hospital is paid for your participation in this study. However, financial support for a study doctor has been provided by a company called Haemonetics Ltd.

22. Who has reviewed the study?

An Independent Ethics Committee, South Central Southampton A Research Ethics Committee has reviewed the objectives and the proposed conduct of the study and has given a favourable opinion of it. It has also been reviewed by the hospital's Research & Development Department.

23. Contact details:

If you have any questions regarding the study or in case of study related injury you should contact the doctor running the study:

Professor Nicholas Curzen
Professor of Interventional Cardiology and Consultant Cardiologist
Southampton General Hospital
Tel: 023 8079 4972

or

The Cardiology Trials Office
Southampton General Hospital
Tel: 023 8079 8538.

If you have any questions regarding your patient rights as they relate to the study, you should contact INVOLVE (Promoting public involvement in NHS, public health and social care research) at Wessex House, Upper Market Street, Eastleigh, Hants, SO50 9FD or tel 023 8065 1088.

9.1.2 Consent form

Cardiology Trials Office
Southampton General Hospital
Tremona Road, Southampton
SO16 6YD

Patient Identification Number for this trial: _____

PATIENT CONSENT FORM

Title of Study: Intra-individual variability in responses to aspirin and clopidogrel in patients with ischaemic heart disease: is there evidence of reduced responsiveness or "tolerance" to antiplatelet therapy over time?

Name of Researcher: Professor Nick Curzen

Please initial each box

1. I can confirm that I have read and understood the information sheet (version 2 dated 27th January 2012) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I have been provided with the details of the known or foreseeable side effects and risks of the study procedures.	☐
3. I understand my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
4. I understand that, if I withdraw from the study, the data collected up until the point of study withdrawal will be used in the final data analysis unless I choose to decline.	☐
5. I understand that sections of my medical notes and data collected during the study may be looked at by responsible individuals from the sponsor (NHS Trust) or from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
6. I agree that the data collected for the study may be used for the purpose described, and to its processing and archiving in a coded form to protect the confidentiality of my personal data. This will not waive any rights that I have under local law.	☐
7. I agree to my GP being informed of my participation in the study	☐
8. I agree to take part in the above study	☐

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Name of Patient	Date and Time	Signature
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Name of person taking consent	Date and Time	Signature

9.2 Vascular Surgical Study

9.2.1 Research participant information sheet

Cardiology Trials Office
Cardiovascular and Thoracic Unit
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Tel 02380798538

Research Participant Information Sheet and Consent Form

PART 1

1. Study Title

Assessment of Arachidonic acid induced platelet aggregation in patients undergoing elective vascular surgery using Short TEG: Does the response to Arachidonic Acid vary according to the vascular inflammatory status?

2. Invitation Paragraph

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information.

3. What is the purpose of the study?

Aspirin (a drug that prevents cells called platelets sticking together to form a clot) has been shown to significantly reduce future major adverse cardiovascular and cerebrovascular events (MACCE) (e.g. heart attack, stroke,

death) in a broad category of patients. A number of international guidelines therefore recommend continuing taking aspirin around the time of an operation unless the associated risk of bleeding from continuing aspirin is greater than the potential risks of stopping aspirin.

However, in some patients aspirin is unable to prevent clot formation, a phenomenon known as "aspirin resistance." The frequency of aspirin resistance varies from 2 to 67 %, depending on the population studied. Nevertheless aspirin resistance has been reported in patients having open heart surgery (Coronary artery bypass surgery or CABG) or stents to treat narrowing's in heart arteries. Despite taking aspirin around 10-20% of patients experience recurrent MACCE in the general population and this could partly be due to aspirin resistance. This raises concerns about the effectiveness of aspirin not only in patients undergoing CABG or stents, but also in other procedures like vascular surgery, where aspirin is also commonly continued. Furthermore, the cause of aspirin resistance remains poorly understood.

This study aims to determine the frequency of aspirin resistance in patients undergoing vascular surgery and to investigate whether aspirin resistance is associated with future MACCE in this population. Furthermore, we will investigate whether aspirin resistance is related to inflammation which is caused by vascular surgery. The findings from this study may support the need for routine assessment of a patient's response to aspirin. Furthermore, if inflammation is related to aspirin resistance then the study may provide greater understanding about the cause of aspirin resistance.

4. Why have I been chosen?

You have been chosen because you have a definite indication for vascular surgery and are on regular treatment with aspirin. Furthermore you meet all the inclusion and exclusion criteria prespecified in the study.

5. Do I have to take part?

No. Your participation in this study is entirely voluntary. It is up to you to decide whether to take part or not. If you do decide to take part, you are free to leave the study at any time and without giving a reason. This will not affect your future medical care in any way. Furthermore, your study doctor may withdraw you from the study if they feel this is in your best interest, if the study is stopped early or if your clinical situation changes such that you no longer meet the eligibility criteria to continue in the study (e.g. if aspirin needs to be stopped for clinical reasons).

If you do participate, you will be given this information sheet to keep and be asked to sign a consent form.

6. What will happen to me if I take part and what do I have to do?

After discussing the study and having read the information sheet, a member of the research team will address any questions you may have regarding the study. If you are willing to participate in the study you will need to provide your consent formally by signing a consent form.

Blood tests will then commence according to the Following schedule:-

(i) T0 - Pre-operative (up to 48 hours before surgery)

(ii) T1 - Post-operative (2 hours post)

(iii) T2 – Post-op day 1

(iv) T3 – Post-op day 2

(v) T4 – Post-op day 3-5

(vi) T5 – 3 months post-operation

Approximately 1-2 tablespoons (20mls) of blood will be taken on every occasion.

For the time that you are in the study, we will need to know about any medication that you are taking and will enquire how you are feeling at every blood test appointment. If you have been discharged by your hospital doctor prior to completion of your blood tests, we will arrange for the outstanding blood tests to be undertaken on an outpatient basis.

Three months after your surgical procedure you will be invited back to the hospital for a visit during which you will have a final blood sample taken and will be asked about any problems encountered since your discharge. The investigator may also contact your general practitioner (GP) to obtain more specific information with regards to any adverse events since your operation for the purposes of follow up if you give your permission to do so.

If you decide to stop participating in the study prior to the end of the study, you will be asked to return to the hospital to have the final procedures performed. This final visit will ensure there are no outstanding safety concerns that need to be addressed prior to your discontinuation in the study.

If you seek emergency care or if hospitalisation is required at any time during the study, please inform the treating doctor that you are participating in a research study.

7. Expenses and payments

If you are discharged from hospital prior to completion of your blood tests, we will arrange for the outstanding blood tests to be undertaken on an outpatient basis. You will be paid for travel expenses related to your participation in the study such as public transport, mileage and car park charges.

8. What are the possible disadvantages and risks of taking part?

When a needle is inserted into your vein to draw blood, you may experience pain, bruising, swelling, bleeding, irritation or infection at the site of the puncture, or you may experience dizziness or faintness**.** Approximately 1-2 tablespoons (20mls) of blood will be collected at each appointment. In the unlikely event of an injury caused by taking part in the study, appropriate medical treatment will be provided.

9. Risks for Women of Childbearing Potential

The risk to women of childbearing potential participating in this study is nil, since no study drug will be administered during this study. Nevertheless, if you are a woman and become pregnant during this study, please notify your study doctor.

10. What are the alternatives for diagnosis or treatment?

All standard treatment and care will remain available.

11. What are the other possible disadvantages and risks of taking part?

Occasionally during the course of a study you may be found to have a previously undiagnosed medical condition. In this situation the investigator will take the necessary steps to ensure you receive appropriate advice.

12. What are the possible benefits of taking part?

There may be no direct benefit to you from taking part in the study. You may however feel that you benefit from the additional close follow up during the course of the study. The results of the study may add to the understanding of your condition and could be helpful for future patients.

13. What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

14. Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

15. Contact details:

If you have any questions regarding the study or in case of study related injury you should contact the doctor running the study:

Professor Nick Curzen
Professor of Interventional Cardiology and Consultant Cardiologist
University Hospital Southampton NHS Foundation Trust
Tel: 02380 794972

or

The Coronary Research Group
University Hospital Southampton NHS Foundation Trust
Tel: 02380 798538.

If you have any questions regarding your patient rights as they relate to the study, you should contact INVOLVE (Promoting public involvement in NHS, public health and social care research) at Wessex House, Upper Market Street, Eastleigh, Hants, SO50 9FD or Tel 023 8065 1088.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering taking part, please continue to read the additional information in Part 2 before making any decision.

PART 2

16. What if relevant new information becomes available?

Sometimes during the course of a research study, new information becomes available about the treatment that is being studied. If this happens, your study

doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide to continue in the study you may be asked to sign an updated consent form. In addition, new information may lead your study doctor to consider withdrawing you from the study if they feel it is in your best interest. If for any reason the study is stopped prematurely, the study doctor will explain why.

17. What will happen if I don't want to carry on with the study?

Your participation in this study is entirely voluntary. It is up to you to decide whether to take part or not. Even if you do decide to take part, you are free to leave the study at any time without giving a reason. If you decide to withdraw your consent, your study doctor will ask your agreement to perform the final evaluation and to collect the data. If you do not agree, no new data on you will be collected. If you decide to withdraw from the study we will need to use the data collected up to the point of your withdrawal.

18. What if there is a problem?

If you have any problems, concerns, complaints or other questions about any aspect of the way you have been approached or treated during the course of this study, the normal complaints mechanisms of the NHS are available to you. You should preferably contact the Chief Investigator first, Professor Nick Curzen on telephone 02380 794972. It may be helpful to contact the Patient Support Services for confidential advice, information or support on 02380796325 or patientsupportservices@uhs.nhs.uk or Patient Support Services, Mailpoint 81, C Level, Centre Block, University Hospital Southampton NHS Foundation Trust, Tremona Road, Southampton, SO16 6YD.

19. Will my taking part in this study be kept confidential?

Your study doctor and the research staff will collect information about you. This information, called data, will be entered without your name, into a local database. In the database a number will replace your name. All the data collected will be kept confidential.

Only authorised personnel will enter the data into the database. The researchers organising this study will take all necessary steps to protect your privacy.

Your identity, including your name, will not be revealed in any compilation, study report or publication at any time. Your study doctor will maintain a confidential list linking your name to the number and only authorised persons will have access to this list.

You have access rights to your data and the possibility to rectify the data according to local law and procedures.

In order to make sure that the data collected from you is correct, it is necessary for a local representative from Research & Development to directly compare data with your medical records. Such checks will only be done by qualified and authorised personnel. All such persons are required to keep the data confidential.

20. Involvement of the General Practitioner/Family Doctor

Your GP will be notified of your involvement in this study if you give permission to do so. Your GP may be contacted to get specific information relating to any adverse events since your operation, if you give permission to do so.

21. What happens to any samples I give?

The study doctor will collect the blood samples. A small quantity of blood will be analysed immediately after it has been taken (to test for aspirin resistance) and the rest will be frozen and stored securely in the Institute of Developmental Sciences. Access to this department is restricted by an entry key-pad. Blood samples may be stored for up to 24 months. This allows several samples to be tested at once making efficient use of testing equipment. The blood samples will be destroyed following analysis.

23. What will happen with the results of the research study?

The data collected will be used for study evaluation only. Members of health authorities, such as the Medicines and Healthcare products Regulatory Agency (MHRA) and Research Ethics Committees or other organisations required by law may review the study data. Your data will be used in publications but your identity will not be revealed in any compilations or study reports.

24. Who is organising and funding the research?

The study is sponsored by the University Hospital Southampton NHS Foundation Trust. The study has been designed by Professor Nick Curzen who is a Professor of Interventional Cardiology within the Cardiovascular & Thoracic Unit, University Hospital Southampton NHS Trust. Contact details are as follows:

Professor Nicholas Curzen
Professor of Interventional Cardiology and Consultant Cardiologist

Wessex Cardiac Unit
University Hospital Southampton NHS Foundation Trust
Tremona Road
Southampton SO16 6YD
Tel: 023 8079 8538
Fax: 023 8079 5174

Professor Curzen is not paid for your participation in this study. However, the study is supported by Haemonetics Corporation who provide study equipment, supplies and financial support to pay for the salary of a study doctor. Haemonetics Corporation have no commercial interests in the study and are not involved in any aspect of sample collection or its management. Furthermore they are not involved in data interpretation and analysis of results obtained from the study or generation of manuscripts prepared for publication in peer-reviewed medical journal.

25. Who has reviewed the study?

An independent ethics committee (NRES committee East of England – Essex) has reviewed the objectives and proposed conduct of the study and has given a favourable opinion of it. The study has also been reviewed by the Hospital's Research & Development Department.

Thank you for taking time to read this sheet.

You will receive a copy of this information sheet and the signed consent form should you wish to participate in this study.

9.2.2 Consent form

Cardiology Trials Office
Cardiovascular and Thoracic Unit
Southampton General Hospital
Tremona Road, Southampton
SO16 6YD

Research Participant Identification Number for this trial: _____

PARTICIPANT CONSENT FORM

Title of Study: Assessment of Arachidonic acid induced platelet aggregation in patients undergoing elective vascular surgery using Short TEG: Does the response to Arachidonic Acid vary according to vascular inflammatory status?

Name of Researcher: Professor Nicholas Curzen

	Please initial boxes
1. I confirm that I have read and understand the information sheet version 2.0 dated 20/09/2013, for the above study and have had the chance to ask questions.	☐
2. I have been provided with the details of the known or foreseeable side effects and risks of the study procedures.	☐
3. I understand that sections of my medical records may be looked at by responsible people from the sponsor (NHS Trust) or from regulatory authorities, where it is relevant to my taking part in research study. I give permission for these people to look at my medical records.	☐
4. I agree that the data collected for the study may be used for the purpose described, and to its processing and archiving in a coded form to protect the confidentiality of personal data.	☐
5. I agree to my general practitioner being informed of my participation in the study	☐
6. I agree to my general practitioner being contacted for getting specific follow up information relating to adverse events during the 3 month follow up period of the study.	☐
7. I agree to take part in the above study.	☐
8. I understand that my decision to take part in this study is voluntary and that I am free to withdraw from the study at any time, without giving any reason, without my medical care or legal rights being affected.	☐

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Name of participant	Date and Time	Signature
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Name of person receiving consent	Date and Time	Signature

10 – REFERENCES

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