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Good, Richard I.S. (2014) *Antiplatelet response to aspirin and clopidogrel in patients with coronary artery disease undergoing percutaneous coronary intervention*. MD thesis.

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**Antiplatelet Response to Aspirin and Clopidogrel in
Patients with Coronary Artery Disease Undergoing
Percutaneous Coronary Intervention**

by

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**A thesis submitted in fulfillment of the requirements
for the Degree of Doctor of Medicine
College of Medical, Veterinary and Life Sciences
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June 2013

Abstract

Aspirin and clopidogrel are cornerstone therapies in cardiovascular disease. In particular, they are almost universally prescribed in patients undergoing percutaneous coronary intervention (PCI). Evidence has emerged of a variation in the antiplatelet effects of aspirin and clopidogrel between individual patients with a suggestion of an increased risk of adverse cardiovascular events. However, the optimal method of measuring response to aspirin and clopidogrel remains uncertain. In light of this, the antiplatelet effects of both aspirin and clopidogrel were studied in patients with coronary artery disease, concentrating on patients undergoing PCI.

Initially, a pilot study of 40 patients investigated the use of thromboxane B2 (TxB2), VerifyNow Aspirin, VerifyNow P2Y12, platelet fibrinogen binding and intra-platelet vasodilator-stimulated phosphoprotein levels (VASP-PRI) to measure response to aspirin and clopidogrel. This was followed by a larger study assessing aspirin and clopidogrel response in 323 patients attending for coronary angiography with a view to PCI. These patients were tested by measuring TxB2, VerifyNow P2Y12, VASP and whole blood impedance platelet aggregation (WBPA). The primary objective was to investigate whether measures of aspirin or clopidogrel efficacy predicted peri-procedural myocardial necrosis following PCI. In addition, a small series of 10 patients had aspirin and clopidogrel response measured following stent thrombosis.

A wide variation in the antiplatelet effects of both aspirin and clopidogrel was found by all measures. Correlation between assays ranged from moderate to poor. Of particular interest, it was found that measurement of [TxB2] may facilitate the assessment of aspirin response in patients already taking clopidogrel. There was a high incidence of myocardial necrosis following coronary intervention assessed by elevation of troponin I. Only VerifyNow P2Y12 and VASP-PRI were associated with a significantly increased frequency of myocardial necrosis following PCI.

The data of this thesis confirm a wide variation in response to aspirin and clopidogrel. Good response to clopidogrel was associated with reduced myocardial necrosis during PCI. TxB2 may be the best measure of aspirin response for patients taking both therapies. How these measures may be incorporated into clinical practice remains uncertain.

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List of abbreviations

AA	arachidonic acid
ACEI	angiotensin converting enzyme inhibitor
ACS	acute coronary syndrome
ADP	adenosine diphosphate
AG	Professor Alison Goodall
AHA	American Heart Association
AM	Anne McGarrity
ARB	angiotensin receptor blocker
ARU	aspirin reaction units
ALBION	Assessment of the best Loading dose of clopidogrel to Blunt platelet activation, Inflammation and Ongoing Necrosis
ANCOVA	analysis of covariance
ARC	Academic Research Consortium
ARMYDA-2	Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty
ATTC	Antiplatelet Trialists' Collaboration
BD	Beckton Dickinson
BMI	body mass index
BMS	bare metal stent
BRAVE-3	Bavarian Reperfusion Alternatives Evaluation-3
CABG	Coronary Artery Bypass Grafting
CAPRIE	Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events
CATS	Canadian American Ticlopidine Study
CCB	calcium channel blocker
CHARISMA	Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance
CLARITY-TIMI 28	Clopidogrel as Adjunctive Reperfusion Therapy–Thrombolysis in Myocardial Infarction
CLASSICS	Clopidogrel Aspirin Stent International Cooperative Study
CK-MB	creatinine kinase - MB
COMMIT	Clopidogrel and Metoprolol in Myocardial Infarction

COX	cyclo-oxygenase
CPG	cationic propyl gallate
CREDO	Clopidogrel for the Reduction of Events During Observation
CURE	Clopidogrel in Unstable angina to prevent Recurrent Events
CVA	cerebrovascular accident
CV	coefficient of variation
Cx	circumflex
CYP	cytochrome P450
DAPT	dual antiplatelet therapy
DES	drug eluting stent
ECG	electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immune sorbent assay
EQC	Electronic Quality Control
ESC	European Society of Cardiology
FANTASTIC	Full anticoagulation versus ASpirin and TIClopidine after stent implantation
FITC	fluorescein isothiocyanate
FS	forward scatter
GPIIb/IIIa	glycoprotein IIb/IIIa
GRAVITAS	Gauging Responsiveness with A VerifyNow assay - Impact on Thrombosis And Safety
GUSTO	Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Arteries
HBS	HEPES buffered saline
HM	Helen Miller
HRP	horseradish peroxidase
HOPE	Heart Outcomes Prevention Evaluation
ISAR	Intracoronary Stenting and Antithrombotic Regimen
ISAR CHOICE	Intracoronary Stenting and Antithrombotic Regimen: Choose Between 3 High Oral Doses for Immediate Clopidogrel Effect

ISAR-REACT	Intracoronary Stenting and Antithrombotic Regimen- Rapid Early Action for Coronary Treatment
ISIS-2	Second International Study of Infarct Survival -2
ISR	in-stent restenosis
IVUS	intravascular ultrasound
LAD	left anterior descending
LIMA	left internal mammary artery
LMWT	low molecular weight
LMS	left main stem
LTA	light transmission aggregometry
MA	maximum clot strength (TEG)
MACE	major adverse cardiovascular event
MATTIS	Multicenter Aspirin and Ticlopidine Trial after Intracoronary Stenting
MFI	mean fluorescence intensity
MI	myocardial infarction
NHS	National Health Service
NSTEMI	non-ST elevation myocardial infarction
OM	obtuse marginal
PAR	protease-activated receptors
PCI	percutaneous coronary intervention
PFA	platelet function analyser
PGH2	prostaglandin H2
PLATO	PLatelet Inhibition and Patient Outcomes study
POBA	plain old balloon angioplasty
POPULAR	Do Platelet Function Assays Predict Clinical Outcomes in Clopidogrel-Pretreated Patients Undergoing Elective PCI?
PPACK	D-phenylalanyl-prolyl-arginine chloromethyl ketone
PPI	proton pump inhibitor
PRONTO	Plavix Reduction Of New Thrombus Occurrence
R	reaction time (TEG)
RCA	right coronary artery

RFPA	rapid platelet function analyser
RG	Richard Good
SAT	sub-acute stent thrombosis
SD	standard deviation
SS	side scatter
ST	stent thrombosis
STARS	STent Anticoagulation Restenosis Study
STEMI	ST-elevation myocardial infarction
SVG	saphenous vein graft
SYNTAX	SYNergy Between PCI With TAXus and cardiac surgery
TASS	Ticlopidine Aspirin Stroke Study
TDS	ter die sumendum
TEG	thromboelastography
TIA	transient ischaemic attack
TIMI	Thrombolysis In Myocardial Infarction
TJ	Tina James
TxA2	thromboxane A2
TxB2	thromboxane B2
ULN	upper limit of normal
VASP-PRI	Vasodilator-stimulated phosphoprotein – platelet reactivity index
WBPA	whole blood impedance platelet aggregometry
11DTxB2	11-dehydro thromboxane B2

Acknowledgements

I would like to thank my principal supervisors, Professor Keith Oldroyd and Professor Alison Goodall, for their advice, support, patience and encouragement throughout the design and execution of this research including the completion of this thesis. I would also like to thank Dr Alex McConnachie in the Robertson Centre for Biostatistics for his guidance with the statistical power calculations and data analysis.

I am extremely grateful to my friends and colleagues at the Western Infirmary and elsewhere for their help and assistance with this research. Support has come from many different sources to help with patient recruitment, blood sampling at the time of cardiac catheterisation and the challenges of collecting and analysing data from a large series of patients. There are too many people to thank individually but I would particularly like to thank Anne McGarrity and Helen Miller for their dedication in undertaking the majority of platelet function assays that form the core of this work.

I am very grateful to the British Heart Foundation, the North Glasgow University Hospitals NHS Trust West Research Endowment Fund, the Graham Wilson Travel Scholarship and Merck, Sharpe and Dohme US Medical School Grant Committee for their generosity in providing support for this project.

Most importantly, I would like to thank the patients who agreed to participate in this research project. I am humbled by the willingness they have shown to discuss and consent to participation in this research despite the considerable anxiety that exists at the time of invasive investigations and treatment.

Finally I would like to thank my family, and particularly my wife and boys, for their sacrifice in helping me to complete this work. This thesis is dedicated to them.

Author's declaration

I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Richard Good

June 2013

Chapter 1: Variation in response to aspirin

1.1 Aspirin in cardiovascular disease

1.1.1 Introduction

Aspirin is the most widely prescribed and regularly consumed medication in the world. Approximately one third of the adult population of North America is thought to be taking regular aspirin, amounting to billions of tablets consumed every year. However, despite such common acceptance, much controversy remains. The role in primary and long term secondary prevention has been questioned, aspirin continues to cause significant side effects, the optimal dose is uncertain and newer antithrombotic agents, that may prove superior to aspirin, continue to emerge.

In this chapter, I initially review the salient stages in aspirin's evolution, the evidence to support its use and recent controversies regarding widespread aspirin prescription for the prevention of cardiovascular disease. I also describe the evidence to support aspirin as a cornerstone therapy for patients undergoing coronary intervention. Leading on from this, I review the emergence of 'aspirin resistance' or poor response to aspirin, the assays used to measure aspirin response and whether these measures can predict clinical outcome.

1.1.2 The evolution of aspirin for the treatment of cardiovascular disease

The medicinal use of compounds containing salicylate derivatives has been documented for more than 3000 years (1). Notes from the mid-eighteenth century confirm the use of willow bark extract – known to be rich in salicylates – for its beneficial effects on pain, inflammation and fever. Towards the end of the 19th century, the chemical structure of acetylsalicylic acid was established and in 1897 it was marketed by the pharmaceutical company Bayer under the trade name Aspirin (2). Bayer lost the rights to the trademark aspirin during the First World War, but aspirin continued to grow in popularity particularly during the 1918 flu pandemic (1).

With such widespread usage, it became apparent that acetyl salicylic acid had additional blood thinning properties, initially thought to be an anticoagulant effect via interaction with the coagulation cascade. In 1948 a letter to *The Lancet* suggested that this may be of benefit in the treatment of thrombotic disease including coronary thrombosis and in 1949 two cases of unstable angina rapidly relieved by the administration of aspirin were reported (3,4). Perhaps the most persuasive early insights into the blood thinning properties of aspirin came from the little recognised physician L.L. Craven. He reported in 1950 the interesting observation that, since the introduction of aspirin gum for the relief of pain, he had experienced a significant number of post-procedural bleeds following tonsillectomy (5). Remarkably for that time, he then made the intellectual leap to suggest aspirin for the prevention of myocardial infarction (6).

In the late 1960s, the antithrombotic properties of aspirin were attributed to an inhibitory effect on platelet function (7). At the same time the interaction between coronary atherosclerosis, thrombosis and acute myocardial infarction was becoming increasingly apparent (8). In addition, there was also emerging evidence to suggest aspirin may have a beneficial effect in patients suffering from cerebrovascular thrombotic events (9). The natural extension of these advances was to re-examine the anecdotal evidence that aspirin may prevent cardiovascular events in at risk patients in the form of a clinical trial.

The first study to investigate the role of aspirin in cardiovascular disease was carried out between 1971 and 1973 (10). This landmark randomised, double blind, placebo controlled study investigated whether aspirin 300mg/day reduced mortality in 1239 patients following myocardial infarction. Although there was a reduction in mortality in the aspirin group, the primary endpoint did not reach statistical significance. Interestingly, the mean time from myocardial infarction to entry to the study was 9.8 weeks and on further subgroup analysis, differences in mortality were only seen in those patients recruited within 6 weeks of their index event. Subsequently, a raft of studies was published in patients with a history of stroke, TIA, myocardial infarction and unstable angina. Although individually these studies were often too small to yield statistically significant mortality and morbidity benefits, pooled analysis published in 1988 suggested a highly significant reduction in death, non-fatal MI and non-fatal stroke in those patients taking aspirin (11).

Following the somewhat mixed results of individual studies looking at the long term reduction of events in patients with documented history of previous myocardial infarction or unstable angina, and in light of prior suggestion of particular benefit to patients early in the post myocardial infarction period, the Second International Study of Infarct Survival (ISIS-2) was designed to investigate the benefits of aspirin in the setting of an evolving ST-elevation myocardial infarction (12). ISIS-2 randomised over 17000 patients, within 24 hours of the onset of symptoms of suspected MI, in a 2x2 factorial design, to receive placebo, aspirin, streptokinase or a combination of the two agents. Aspirin was prescribed as 160mg/day for 1 month and showed a significant reduction in death, reinfarction and stroke either alone or in combination with streptokinase. This early mega-study cemented the routine use of aspirin in patients experiencing a myocardial infarction.

The 2002 Antithrombotic Trialist's Collaboration (ATTC) meta-analysis illustrates the difficult task of assimilating the available data in this field into comprehensive recommendations (13). Although only considering trials published before 1998 and excluding studies smaller than 200 patients, 197 studies were included in the analysis. As a further complication, the indications for aspirin prescription include stable angina, coronary bypass grafting, coronary angioplasty, heart failure, atrial fibrillation, cardiac valve disease and surgery, peripheral vascular disease, haemodialysis, diabetes mellitus, and asymptomatic carotid disease. From this data, the group concluded that, in patients at high risk of cardiovascular events or those with known cardiovascular disease, regular aspirin reduces the risk of death, myocardial infarction and stroke by around 23%. For those patients without a history of thromboembolic disease the recommendations were less clear due to the trade-off between reduction in cardiovascular events and a higher incidence of bleeding.

The most recent guideline published in 2009 has attempted to address some of these difficulties by returning to the individual participant data from trials that had recruited at least 1000 participants randomised to aspirin or placebo and followed for at least 2 years (14). These more stringent criteria restricted the trials included to 6 primary prevention and 16 secondary prevention studies. The conclusion remained the same; a recommendation of aspirin for the secondary prevention of cardiovascular disease but a questionable role in primary prevention. In spite of this, there remains a degree of concern regarding the validity of longer term aspirin use and questions remain regarding the optimal dose (15).

1.1.3 Dosage of aspirin in cardiovascular disease

The first tablet form of aspirin marketed by Bayer in 1900 was approximately 325mg. Arbitrarily, a children's dose of $\frac{1}{4}$ this amount (approximately 81mg) was subsequently produced in 1922 (1). Early studies investigating the use of aspirin following myocardial infarction or cerebrovascular events used significantly higher doses of aspirin than would be employed in this setting today. This is reflected in the ATTC publication from 2002 where 34/65 studies used doses between 500mg and 1500mg per day. In this same analysis, 15/65 studies used aspirin doses of 150mg or less as would be commonly prescribed today (13). This is a reflection of the initial introduction of aspirin as an anti-inflammatory agent and even in today's practice doses as high as 4000mg/day may be used for this indication. However, over the last 30 years there has been a trend towards lower maintenance doses of aspirin for the prevention of cardiovascular disease. Considerable global variation remains. Centres in North America routinely prescribe 325mg/day. In contrast, European centres most commonly use doses between 75mg and 150mg per day.

Pharmacodynamic evidence suggests that a loading dose of 300mg aspirin achieves maximal early platelet inhibition with no incremental antiplatelet effect at higher doses (16). The celerity of this effect can be maximised by using an oral solution with peak plasma concentrations occurring 30 minutes from ingestion (17). The irreversible nature of aspirin's interaction with cyclooxygenase 1 (COX-1) means that chronic doses as low as 30mg/day have been shown to completely suppress thromboxane production in healthy volunteers (18). However, there is evidence that thromboxane production is persistently elevated in patients with cardiovascular disease and at least 50mg/day may be required (19).

The shift towards lower doses of aspirin for the long term treatment of cardiovascular disease has been driven by concerns regarding bleeding side effects. It has been shown that 30mg/day of aspirin reduces gastric prostaglandin levels by approximately 50% but complete inhibition is only achieved at doses higher than 1300mg/day (20). This finding is reflected in clinical data confirming a significantly higher incidence of bleeding in patients taking higher doses of aspirin (21). Thus, later studies of aspirin use in cardiovascular and cerebrovascular disease looked to evaluate whether an optimal dose of aspirin could be established to maximise the antithrombotic benefits whilst minimising any adverse events (22,23). From this data and

several post-hoc analyses of aspirin studies, the greatest risk reduction appears to be achieved using doses between 75 and 150mg/day, with no apparent further reduction in cardiovascular events with higher doses. Conversely, there appears to be a significant increase in adverse events in patients taking aspirin at a dose higher than 75mg/day (13,23). The post-hoc analysis of cardiovascular events and bleeding risk in the CURE study, concluded that higher doses of aspirin in combination with clopidogrel did not reduce cardiovascular events, but at doses >300mg did appear to be associated with increased bleeding (21,24). Interestingly, a more recent analysis of data from the PLATO study suggested that the mortality benefit of ticagrelor over clopidogrel was lost in patients recruited from North America who were more commonly prescribed aspirin 325mg although this did not appear to be related to increased bleeding (25).

1.1.4 Aspirin in percutaneous coronary intervention

Endothelial disruption is an integral part of coronary intervention. Balloon angioplasty and stent implantation cause plaque rupture and exposure of the thrombogenic subendothelial layers, as well as the release of numerous cytokines stimulating inflammation and platelet aggregation (26). Stent implantation in particular seems to amplify platelet activation with rapid deposition of platelets on the surface of stent struts (27). Early studies using aspirin and dipyridamole showed a dramatic (90%) relative reduction in the incidence of clinically significant coronary thrombosis following angioplasty including a reduction in abrupt vessel closure of up to 85% (28). Subsequent studies suggested that aspirin monotherapy, without dipyridamole, was equally effective (29) and thus preloading with aspirin prior to coronary intervention was established as a standard of care. Aspirin remains almost universally prescribed in patients undergoing coronary intervention, usually in combination with an additional platelet P2Y₁₂ receptor inhibitor (30). However, debate continues regarding the optimal dose of aspirin for coronary intervention with centres varying between 75mg/day and 325mg/day.

1.2 Variation in response to aspirin

1.2.1 Aspirin 'resistance'

The popular term aspirin 'resistance' describes both clinical resistance (having a thromboembolic event whilst taking aspirin) and biochemical resistance (failure to inhibit platelet function as measured by an appropriate assay). In most research, the end-goal of these two distinct concepts is common; the measurement of aspirin 'resistance' using a particular assay as a means of predicting patients at risk of future thromboembolic events, but they represent two different perspectives. A particular difficulty is the definition of biochemical resistance as it is assay dependent and requires an arbitrary cut-off. Historically, perhaps to facilitate statistical analysis, patients have been divided into those responding to aspirin and those who are 'resistant' but considering the response to aspirin as a continuous variable may be more appropriate.

The finding that the benefits of aspirin appeared more pronounced in males than females led early investigators to look into the variation in antiplatelet effects of aspirin (31). Further interest in measuring this variation in response in stroke patients led to a small pilot study investigating whether this impacted upon clinical outcome (32,33). The method of assessing aspirin response in this study was rather unusual. Platelet counts were compared between blood samples suspended in EDTA and those in an EDTA-formaldehyde buffer which prevents the aggregation of platelets. From these numbers 'Platelet Reactivity' was calculated as a marker of response to aspirin and according to studies of normal subjects a platelet reactivity of >1.25 was deemed abnormal. 180 subjects were followed-up for 2 years and all prescribed aspirin 500mg TDS. Of the 60 non-responder patients, 24 suffered death, MI or stroke at 60 months vs only 5/114 responders, a statistically significant difference ($p<0.001$). Although there are a number of limitations to this study and the method used to assess the antiplatelet effects of aspirin, it marks the first attempt to use a laboratory measure of aspirin response to predict cardiovascular events in at risk patients.

The second such study was published 4 years later. Patients with peripheral arterial disease undergoing balloon angioplasty underwent platelet function testing using whole blood

aggregometry. Residual activation of platelets in the presence of ADP and collagen was significantly associated with re-occlusion at the site of angioplasty (34).

Perhaps the most well regarded early work in this field was published by Gum *et al* in 2001 (35). This was the first data to appear using what many consider to be the ‘gold standard’ method of assessing the antiplatelet efficacy, optical light transmission platelet aggregometry (LTA). 325 patients with stable cardiovascular disease taking aspirin 325mg/day were tested using LTA in response to 10 μ mol ADP and 0.5mg/ml arachidonic acid. They were also tested using a novel platelet function analyser (PFA-100, Siemens Healthcare Diagnostics). Patients were deemed aspirin resistant by optical aggregation if they had a mean aggregation >70% to 10 μ mol ADP and >20% to 0.5mg/ml arachidonic acid (AA). ‘Semi-responders’ had one or the other markers of poor response. 18 patients (5.5%) were found to be aspirin ‘resistant’ and 78 patients (23.8%) semi-responders according to optical aggregation criteria. The PFA-100 criterion for aspirin ‘resistance’ was a closure time <193 seconds; 31 patients (9.5%) were aspirin resistant as defined by this binary cut-off value. Only 4 patients were aspirin resistant by both optical aggregation and PFA-100 testing.

In 2002, Eikelboom *et al* published data from the Heart Outcomes Prevention Evaluation (HOPE) study demonstrating significant variation in urinary thromboxane B₂ secretion between patients taking aspirin (36). The production of thromboxane A₂ (TxA₂), a powerful platelet agonist and vasoconstrictor, is the final step in the COX-1 enzyme cascade, which is the target of aspirin. TxA₂ is, however, unstable in serum and is rapidly metabolised to the more stable derivative 11-dehydro thromboxane B₂ and other metabolites, several of which are secreted by the kidneys. Urinary levels of 11-dehydro thromboxane B₂ were measured in 976 patients recruited to the HOPE study, who were deemed at high risk of cardiovascular events. Not only was a significant variation in levels found but there was a significant association between increased production of urinary thromboxane B₂ and risk of cardiovascular events (MI, stroke or cardiovascular death) with a hazard ratio of 1.8 between patients in the highest and lowest quartiles.

In the year following the publication of this data, Gum *et al* returned to their cohort of patients to publish their own follow-up. 315 of the 325 patients were followed-up at a mean time of 679 days following assessment of aspirin response. Using a composite of death, MI and

stroke, 4/17 aspirin resistant patients (24%) had experienced an event against 30/309 (10%) of responsive patients ($p=0.03$) (37).

Novel assays of 'aspirin resistance' have continued to emerge and in 2004 Chen *et al* published an observational study of 151 patients undergoing non-urgent percutaneous coronary intervention (38). Using a bedside point-of-care assay incorporating the agonist cationic propylgallate (VerifyNow Aspirin) patients were tested for aspirin resistance using the manufacturer's pre-determined cut-off of 550 ARU (Aspirin Reaction Units). Patients deemed aspirin resistant had a significantly increased incidence of myonecrosis determined by CK-MB or troponin elevation following PCI.

Since the publication of these early studies, there has been a surge in publications addressing variable response to aspirin therapy. However, there continues to be no consensus as to the best method of measuring aspirin response, the most appropriate criteria for defining biochemical aspirin 'resistance' and whether any therapeutic interventions can be guided by these assays in clinical practice. The salient publications will be discussed in the following section describing the proposed methods of measuring response to aspirin.

1.3 Assays for assessing response to aspirin

1.3.1 Challenges of assessing platelet function

Assessing platelet function by any method is notoriously difficult. Platelet function is modified by exercise and stress and there may also be a diurnal variation (39,40). In addition, the collection of blood samples may in itself affect platelet function and introduce significant variability. The calibre and quality of individual veins is heterogeneous, aspiration through needles or 'butterfly' devices causes platelet activation, as may drawing samples from arterial sheaths. Most centres collect samples in tubes containing 3.2% sodium citrate. The citrate chelates calcium ions thus disrupting the clotting process. However, there is some evidence that sodium citrate may promote platelet microaggregates through fibrinogen binding to the

GPIIb/IIIa receptor (41). Thus, some studies have advocated the use of the antithrombin combination hirudin and D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK) when testing for both aspirin and clopidogrel response (39,42).

Processing of samples following collection may also introduce a degree of variability. Delays to sample manipulation are important as endogenous nitric oxide and prostacyclin levels fall in the first 30 minutes after collection. Any method that involves manipulation of the sample such as fixation or washing can also bias the results.

1.3.2 (Optical) Light Transmission Aggregometry (LTA)

The 'gold standard', and most commonly referenced, method for assessing response to aspirin, and platelet inhibition in general, remains light transmission optical platelet aggregometry (LTA). It was first described by Born and O'Brien in 1962 (43). Whole blood is centrifuged at low speed to remove nucleated cells and erythrocytes. The resulting platelet rich plasma is collected and a portion further centrifuged at high speed to create a sample of platelet poor plasma to act as a reference. Light transmission through the platelet rich plasma following the addition of an agonist of choice is measured (Figure 1). As platelet aggregates form, transmission of light through the solution increases, usually reaching a maximum within 10 minutes. In most cases the agonist of choice is arachidonic acid, the substrate for the enzyme cascade inhibited by aspirin. However, in several studies collagen or an amalgamation of response to both arachidonic acid and adenosine diphosphate (ADP) have been used to assess aspirin response (35,44).

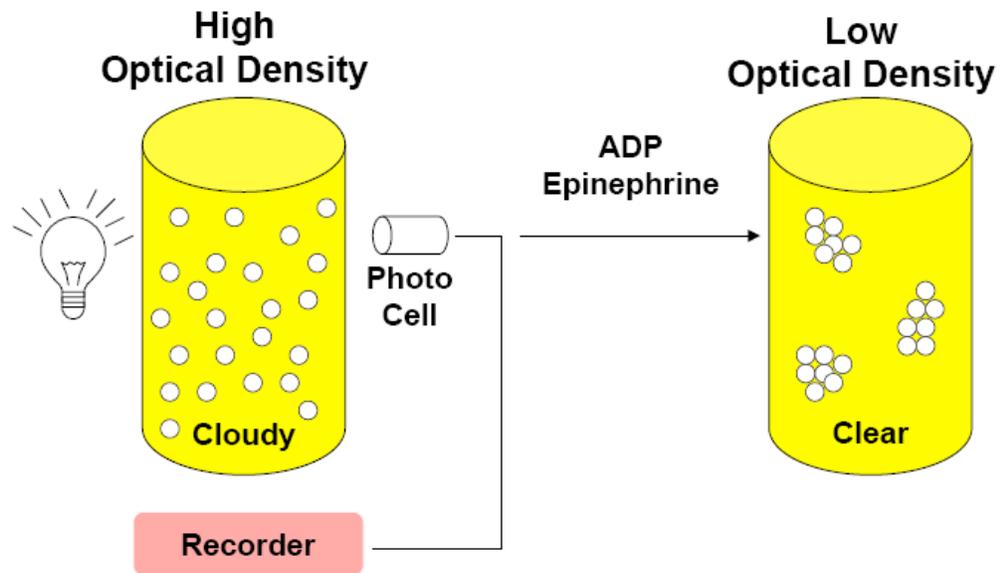


Figure 1: The principle of Born-type platelet aggregation

There are a number of inherent limitations to this method of assessing platelet function. It is well recognised that there is a degree of interoperator variability likely due to differences in pipetting and handling techniques which may have a profound effect on platelet activation. The method is both time consuming and labour intensive which has significant cost implications and makes the application of these assays on a routine basis and in the context of acute clinical scenarios difficult. In addition, the removal of platelets from the whole blood medium is non-physiological and may eliminate several important co-factors for aggregation. The centrifugation process may also preferentially remove the larger, more reactive platelets (45). Finally, the sensitivity of platelet aggregation in response to arachidonic acid and ADP, which are both relatively weak agonists, can be profoundly affected by other antithrombotic or anticoagulant agents.

As an illustration of some of these difficulties, a small study by Nicholson *et al* investigated LTA in response to 20mmol ADP in 2 subjects taking no antiplatelet therapy (46). LTA was performed at two different time points on six consecutive days by two different operators. They found standard deviations of 5.7 to 7.7% depending on the assay saline control concentrations. In addition they found that there was variation in results between operators and assays on the same day. However, the largest variation was between assays on different days

perhaps reflecting an additional physiological variation in platelet responsiveness from one day to the next. It is difficult to be certain how this type of result impacts on the results for patients on antiplatelet therapy, and there is little published evidence in this regard.

1.3.3 Whole Blood Impedance Platelet Aggregometry (WBPA)

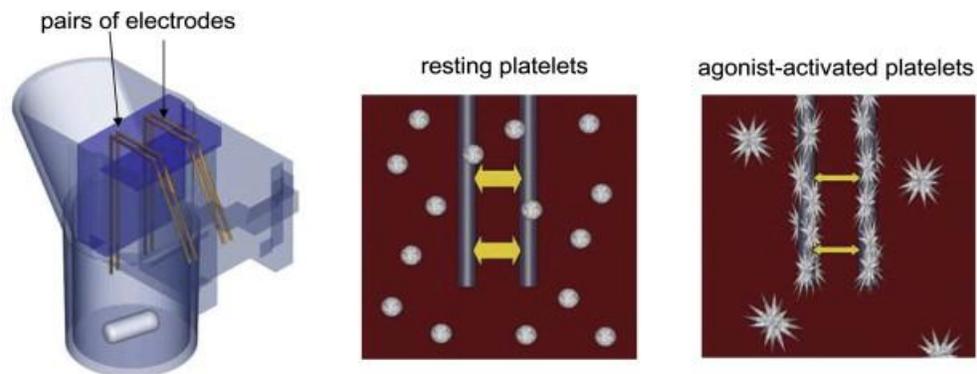


Figure 2: Principles of whole blood impedance platelet aggregometry

(With permission from Elsevier Ltd. Kozek-Langenecker, S. *Best Practice & Research Clinical Anaesthesiology*. Vol 24, March 2010 Pages 27-40)

Whole blood impedance platelet aggregometry avoids some of the limitations of optical platelet aggregation. First described by Cardinal *et al* in 1980, platelet aggregation is assessed by measuring the change in impedance between two fine electrodes immersed in a whole blood solution (47). After immersion of the electrodes, a platelet monolayer forms over the electrodes. Once this has occurred, a steady state of impedance is reached. The addition of a platelet agonist of choice stimulates further platelet aggregates to form between the electrodes increasing the impedance and reducing the flow of current (Figure 2). Maximal impedance is recorded after 6 minutes. Sample preparation is minimal, requiring simply the mixing of citrated whole blood with normal saline in a cuvette with an electrode and stir bar. The great appeal of this assay lies in its relatively straightforward protocol, with a likely reduction in interoperator variability and sample preparation time. Potentially, therefore, this assay could be incorporated more readily into the clinical environment with a minimal amount of operator training. However, there are a number of limitations. Reproducibility is notoriously poor and in this regard the quality of the electrodes is critical. Until recently, electrodes were reusable and care was required to remove all the aggregated platelet material from the surface of the

electrode between samples. This process could distort the electrodes thus affecting the subsequent impedance values. This problem has been eliminated by the introduction of disposable electrodes although reproducibility remains uncertain (48).

Stimulation with arachidonic acid (0.5-2 millimolar) has been used to assess aspirin response in WBPA assays. In addition, platelet response to stimulation with collagen is thought to be significantly affected by the production of TxA₂ and thus may also serve as a marker of response to aspirin (49). It is recommended that the assay be performed within 30 minutes of sample acquisition to minimise the effects of sample-aging on the result (50). As in LTA, platelet response to these agonists is sensitive to the effects of other antiplatelet or anticoagulant therapies and thus there is little evidence supporting the use of WBPA to assess aspirin response in patients taking dual antiplatelet therapy.

1.3.4 Measurement of thromboxane metabolites

Aspirin principally inhibits the enzyme COX-1, which catalyses the conversion of arachidonic acid to prostaglandin H₂ (PGH₂). PGH₂ is subsequently metabolised to several prostaglandins, prostacyclin and, by thromboxane synthase, to thromboxane A₂. Of these final products, it is the production of TxA₂ in platelets that contributes to the prothrombotic process (Figure 3). Thus, reduction in the production of TxA₂ is thought to be the primary anti-thrombotic effect of aspirin resulting in a beneficial clinical profile. Thromboxane A₂ is, however, extremely labile, being rapidly hydrolysed to thromboxane B₂. Thromboxane B₂ is more stable although, *in vivo*, is further metabolised to more than 20 derivatives including 11-dehydro-thromboxane B₂ and 2,3-dinor-thromboxane B₂ both of which are secreted by the kidneys.

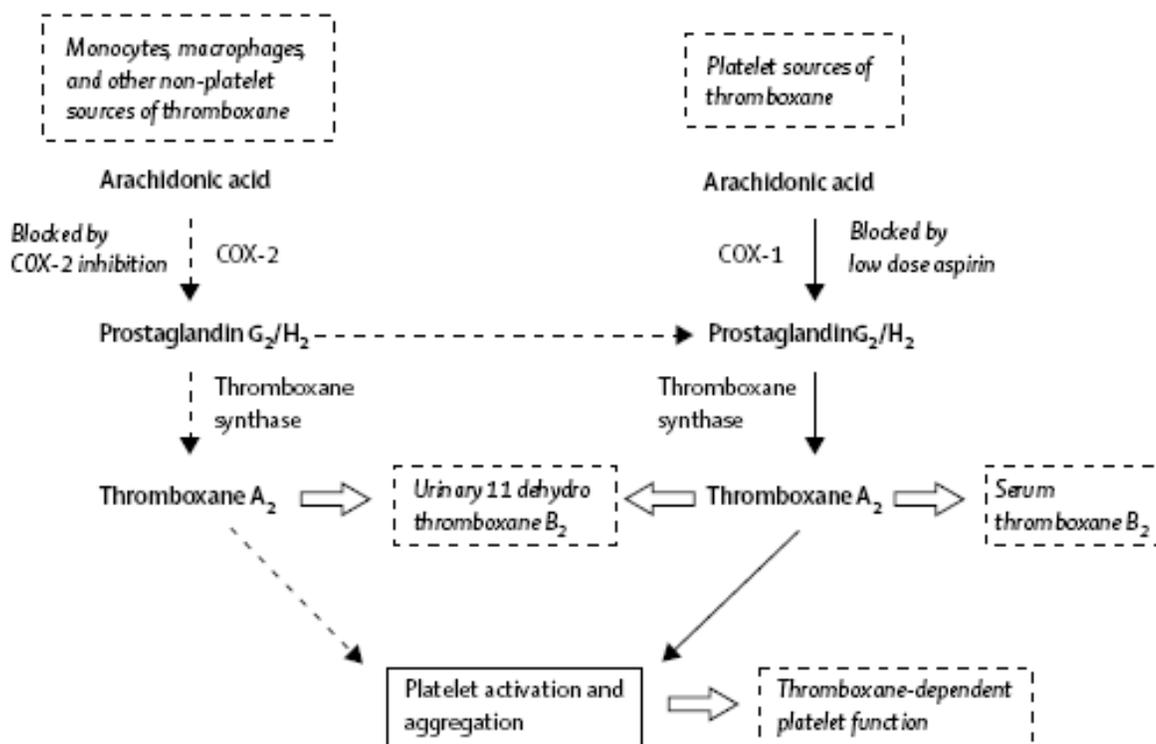


Figure 3: The secretion and metabolism of thromboxane in humans

(With permission from Elsevier Ltd. Hankey *et al* (51))

As a consequence, assays are available to measure serum or plasma levels of TxB_2 or the urinary metabolites 11-dehydro-thromboxane B_2 and 2,3-dinor-thromboxane B_2 . The commercially available assays to measure TxB_2 concentrations are competitive enzyme immunoassays. Once again this laboratory based assay presents a number of practical limitations to use in routine clinical practice. Principally, there is significant sample processing and sufficient samples need to be accumulated to run an assay plate. Both these factors have important cost and time implications.

Much controversy remains regarding the contribution of COX-1 dependent thromboxane generation to reduced response to aspirin therapy. Several studies have shown a consistent reduction in serum TxB_2 levels in patients taking aspirin (52-55). Failure to suppress TxB_2 levels to below those found in untreated individuals may relate to non or partial compliance with aspirin (55). Studies have also shown that residual TxA_2 production, albeit at low levels, does exist in aspirin treated patients and the level of TxB_2 has been shown to be higher in

patients with CAD (19). In addition, residual levels of serum TxB2 are significantly lower in patients taking higher doses of aspirin (52).

Collection of urine samples for measurement of TxB2 derivatives is a more straightforward, although less direct, measure of the antiplatelet effects of aspirin. However, some studies have failed to find a link between urinary TxB2 concentrations and platelet COX-1 activity (53). Certainly, although levels of urinary TxB2 concentration are reduced in patients treated with aspirin, the magnitude of this reduction is nothing like that seen in serum TxB2 concentrations. This suggests that urinary TxB2 may reflect alternative sources of thromboxane production such as endothelial cells or macrophages and may be a marker of risk but not a reliable marker of platelet inhibition by aspirin (36). In addition, urinary TxB2 is influenced by rates of clearance, the period during which urine is collected and renal function.

One potential advantage to TxB2 measurement as a means of monitoring response to aspirin therapy is the potential for this to be less affected by concomitant clopidogrel therapy. In theory, measuring the product of the enzyme pathway specifically inhibited by aspirin negates the effect of any other antiplatelet therapy not directly inhibiting this enzyme cascade. Clearly, the interaction is somewhat more complex as the platelet activation inherent to the production of a serum sample will inevitably involve multiple pathways of platelet activation (Figure 4). However, this may be less of an issue than in direct platelet function tests which simply measure the end-point of platelet activation and aggregation and which are profoundly affected by concurrent antiplatelet therapy. In addition, measuring directly the product of COX-1 is not dependent on the agonist that stimulates the platelets provided the stimulus is uniform throughout the study.

Figure has been removed due to Copyright restrictions

Figure 4: Complex interaction of agonists and pathways involved in platelet activation and aggregation

(Courtesy of Storey R.F. (56))

1.3.5 Platelet flow cytometry

The details of flow cytometric assessment of platelet function are described more fully in Chapter 2 when discussing the assessment of response to clopidogrel therapy. In principle, platelet antigens are targeted by labelled antibodies allowing quantification of platelet activation in response to an agonist of choice. However, no single or combination of internal or surface platelet ligand has been identified to reliably reflect platelet inhibition by aspirin (45,57). In addition, flow cytometry requires a significant degree of operator knowledge and skill, and is time-consuming and expensive, which further reduces any appeal for the use of this approach to assess aspirin response.

1.3.6 Rapid platelet function analyser (RPFA), VerifyNow Aspirin

Figure has been removed due to Copyright restrictions

Figure 5: Rapid platelet function analyser (Accumetrics, USA) and VerifyNow Aspirin cartridge

(From Accumetrics.com, San Diego, USA)

The Rapid Platelet Function Analyser (RPFA) (Accumetrics, San Diego, USA) was first developed to monitor inhibition of the GPIIb/IIIa receptor by abciximab (58). This cartridge based device uses a turbidimetric based optical detection system to measure platelet aggregation (59). Separate cartridges have been developed to assess platelet inhibition by aspirin (VerifyNow Aspirin) and by agents targeting the platelet P2Y₁₂ ADP receptor (VerifyNow P2Y12) (58). The test cartridge contains a lyophilised mixture of beads coated with human fibrinogen, platelet agonist, buffer and preservative. Whole blood is collected in a standard citrated tube, gently mixed and left for between 30 minutes and 4 hours. Following registration of a cartridge in the RPFA, the blood sample tube is impaled on to a needle embedded within the cartridge. Blood is aspirated into the chamber containing the lyophilised preparation of human fibrinogen-coated beads, platelet agonist and buffer. The original version of VerifyNow Aspirin used cationic propyl gallate (CPG), which has multiple effects on platelets and the coagulation cascade, as an agonist. However, the most recent version of the assay uses arachidonic acid as the agonist. Platelet activation leads to increased expression

of GPIIb/IIIa receptors which bind to the fibrinogen coated beads to form aggregates. In a similar fashion to light transmission aggregometry, as these aggregates form, light transmission through the medium increases and this change in optical signal is recorded (Figure 6).

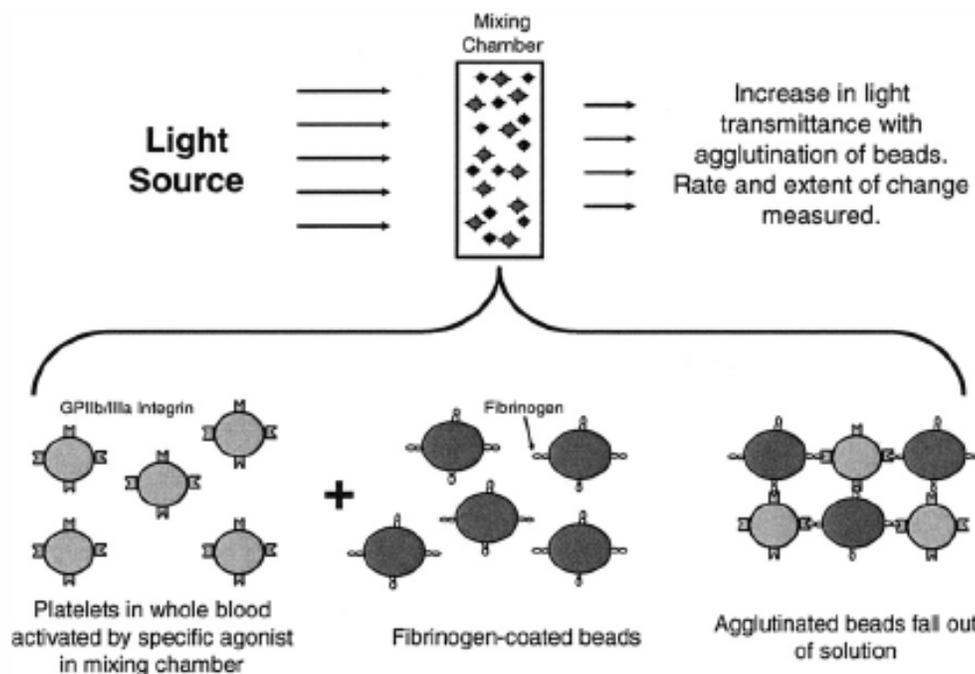


Figure 6: Principles underlying the VerifyNow cartridge to assess platelet aggregation in whole blood

(With permission from Elsevier Ltd. Michelson *et al* (60))

As with other aggregation based systems, it is sensitive to additional antiplatelet or anticoagulant treatments and sample acquisition and storage is important. Results are expressed in Aspirin Reaction Units (ARU). The assay is recommended to be used qualitatively with a dichotomous cut-off value of ≥ 550 ARU indicating a poor response to aspirin or 'aspirin resistance'. This cut-off was determined by a study of 24 subjects not taking aspirin or any other anticoagulant using the original CPG based cartridges (61). Validation of this original cartridge against optical light transmission platelet aggregation in response to epinephrine has suggested a strong correlation between these two assays ($r^2=0.9$) although as discussed in the following section further comparative studies have not shown such strong correlations (Figure 9).

1.3.7 Platelet function analyser (PFA-100)

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Figure 7: Platelet function analyser, PFA-100 (Siemens Healthcare Diagnostics)
(From www.medical.siemens.com, Siemens, USA)

The PFA-100® (Siemens Healthcare Diagnostics) cartridge based assay uses whole blood samples collected in sodium citrate (Figure 7). The cartridge contains a synthetic capillary through which blood is forced towards a membrane with apertures of 147µm. This membrane is coated with agonists of choice, most commonly collagen/ADP or collagen/epinephrine. As platelets aggregate the aperture is obstructed. The time to complete occlusion (closure time) is measured and, in patients taking aspirin, a time to occlusion within the normal reference range (<193 seconds) is regarded as aspirin ‘resistance’ (62). This assay has some appeal in that it is rapid, requires little sample preparation, and utilises only a small amount of whole blood. It also tries to mimic the physiologically relevant cessation of blood flow in a high shear stress environment. However, data suggests that the result is significantly affected by level of von Willebrand factor and haematocrit (63). As yet, no data has emerged to convincingly show that the aspirin response according to the PFA-100 assay influences clinical outcome. Indeed, two studies looking at periprocedural myocardial enzyme release and MACE rate found no-significant association with these clinical endpoints (64,65).

1.3.8 Thromboelastography (TEG)

This assay principally measures clot strength. A schematic is shown in (Figure 8). Clot formation between the rotating cup, which oscillates between 4 and 45 degrees, and the pin generates a signal in the torsion wire. The parameters of particular interest are the time to clot formation (Reaction Time, R) and the maximum clot strength (MA) which is thought to reflect platelet-fibrin interactions (66). It has been proposed that the antiplatelet efficacy of aspirin can be evaluated by using arachidonic acid as an agonist in the whole blood solution (67). In this case, whole blood samples are collected in tubes containing lithium and heparin, with heparin included to negate the effects of thrombin. This heparinised sample is transferred to a vial containing heparinase to neutralise the heparin. The rotating cup is also coated in heparinase.

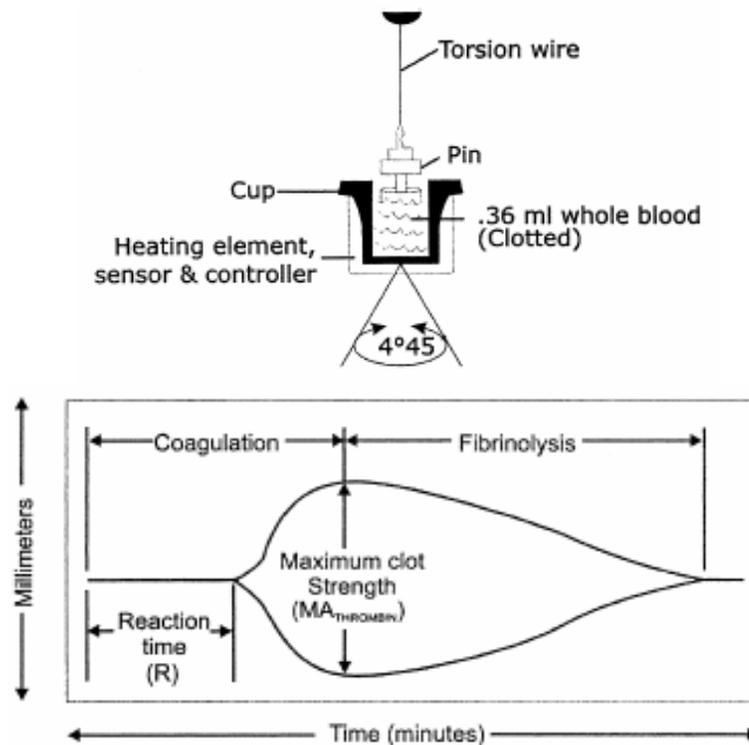


Figure 8: Principles of thromboelastography

(With permission from Elsevier Ltd. Gurbel *et al* (67))

1.3.9 Other assays to assess aspirin response

There are several other assays that have been proposed to study the antiplatelet effects of aspirin including the Plateletworks system using both arachidonic acid and collagen cartridges. This assay returns very much to Grottemeyers *et al*'s original study and determines platelet count in whole blood before and after the addition of an agonist of choice (32). The IMPACT cone and platelet analyser assess platelet adhesion and aggregation under flow conditions by measuring the percentage area of a membrane covered by platelet aggregates following the addition of an agonist of choice (68). Both arachidonic acid and collagen have been proposed for the assessment of aspirin response in this assay. Whilst there have been small studies using these assays to assess response to clopidogrel (69), there is very little clinical data published on the use of these assays for the assessment of aspirin response.

1.4 Comparison of methods to assess the response to aspirin

The introduction of new point-of-care assays to assess the response to aspirin has rekindled interest in variable response to aspirin therapy and the implications for the management of patients with cardiovascular and cerebrovascular disease. Several studies comparing the assays described above for the detection of aspirin response have been published.

In 2005, Harrison *et al* published an investigation of 100 patients with a history of TIA or stroke taking 75-150mg of aspirin per day for at least 4 weeks (70). Aspirin response was assessed by LTA using AA and 10 μ mol ADP as agonists. Using the criteria first proposed by Gum *et al*, aspirin 'resistance' was defined as >20% aggregation with AA or >70% aggregation following stimulation with ADP. They also used the PFA-100 and the Ultegra RPFA assays with cationic propyl gallate (CPG) as the agonist in the RPFA cartridges. They found levels of aspirin non-response of 12% and 14% with AA and ADP respectively. According to the RPFA, 17% of patients were aspirin resistant whilst the PFA-100 assay suggested 22% had a poor response to aspirin. However, of perhaps more significance than these rather arbitrary definitions of resistance, only 2/98 patients were poor responders by all the tests and agreement between the different assays was very poor (Figure 9).

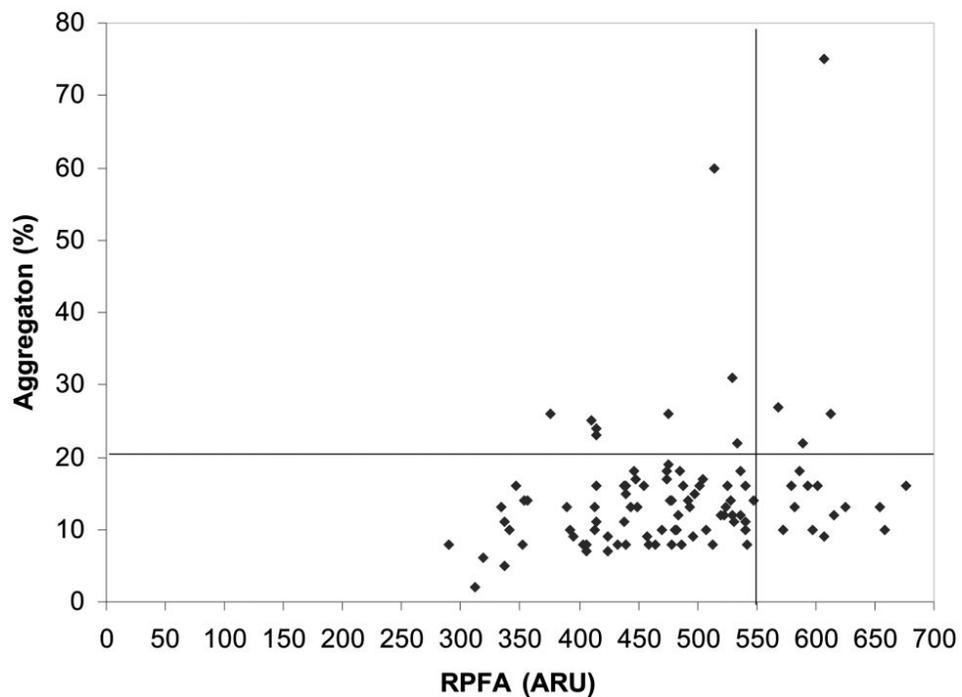


Figure 9: Comparison of light transmission aggregometry (LTA) to arachidonic acid and VerifyNow Aspirin (CPG) for the assessment of platelet inhibition by aspirin

(With permission from Wolters Kluwer Health. Harrison *et al* (70))

In 2007 Lordkipanidze *et al* published a similar study in patients with known CAD (44). The dose of aspirin in this 201 patient cohort ranged from 80mg/day to 1300mg/day (one patient only at the highest dose) and all patients had been on treatment for at least 1 month. Whole blood was collected for LTA using arachidonic acid and ADP as agonists, the RPFAs VerifyNow Aspirin (AA) assay, PFA-100 and finally WBPA using arachidonic acid. Urine samples were simultaneously collected for 11-dehydro-thromboxane B2 levels. Although not all patients had exactly the same assays, the results were similarly disappointing to those of Harrison *et al* with regard to correlation and agreement. The best correlation was between LTA and WBPA with arachidonic acid as the agonist ($r=0.24$, $p=0.001$). VerifyNow Aspirin, using the new arachidonic acid based cartridge for the RPFAs, had a very weak correlation with LTA ($r = 0.12$, $p = 0.06$). Overall, the prevalence of aspirin non-response as previously defined by LTA was only 4% although significantly higher with some assays (10-59%). Importantly, in keeping with data mentioned previously, correlation between urine levels of 11dTxB2 and LTA in response to arachidonic acid was also poor (53). Again this was suggested to be a reflection of the production of TxB2 by other blood components and from the kidneys.

Similar comparative data from other studies have suggested that correlation between these assays is poor and this is reflected in a wide variation in the reported incidence of aspirin ‘resistance’ (35,71-74). This may reflect variation in the assay properties, patient characteristics and the dose of aspirin used in the different populations. This uncertainty has been a major barrier to the incorporation of these novel point-of-care assays into clinical practice.

1.5 Aspirin response and clinical outcomes

Since the pivotal studies described above reporting variation in response to aspirin therapy, a number of additional studies have emerged investigating whether these measures of response influence clinical outcome in a variety of patient settings. Three recent meta-analyses have attempted the difficult task of amalgamating this data, which in the majority accrues from relatively small single centre studies (75-77).

Analysing these meta-analyses in detail exposes all of the difficulties with this important area of research. The studies included are uniformly small; the largest continues to be the series of 326 patients published by Gum *et al* in 2003 (37). Of the 12 studies pooled for the earliest publication, 7 different platelet function tests were reported including bleeding time, platelet count, PFA 100, urinary TxB2 concentration, plasma TxB2 concentration, Verifynow Aspirin (CPG) and LTA (using ADP, arachidonic acid, collagen and CPG or their combination as agonists) (75). Sofi *et al* in 2008, reported 11 studies of which 8 were based on the PFA-100 platelet function test (76). Indeed this assay was the most commonly used in all 3 meta-analyses and yet it is probably the most poorly correlated with the ‘gold standard’ of light transmission aggregometry (Figure 10). Krasopoulos *et al* reported 20 studies using 6 different platelet function tests including 2 studies based on TEG results (77). As with Snoep *et al*, the end point for two of the included studies was coronary artery bypass graft failure rather than cardiovascular events. Only 3 studies were included in all 3 meta-analyses (37,78,79).

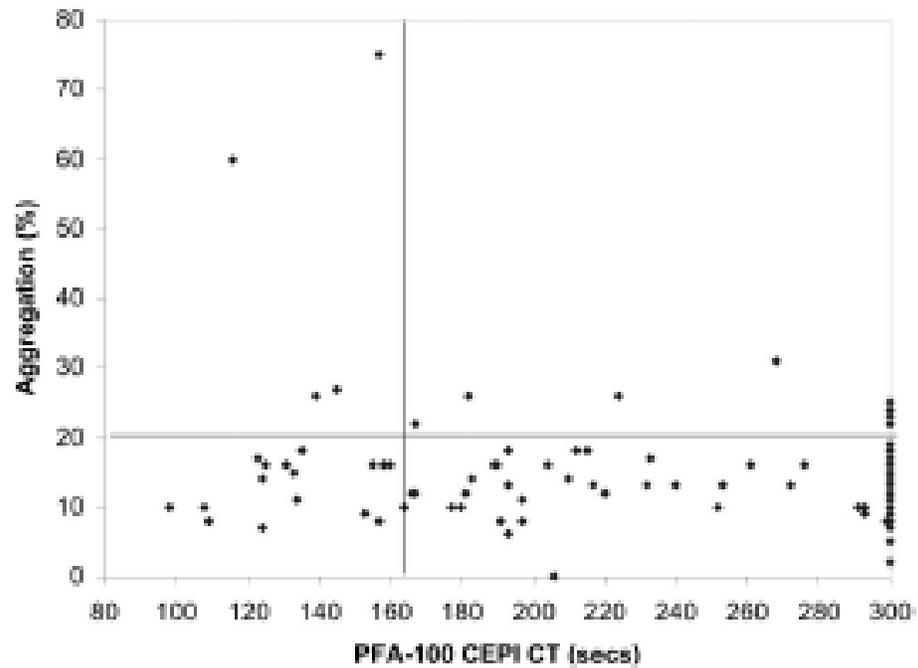


Figure 10: Poor correlation between LTA in response to arachidonic acid and PFA-100 CEPI for the assessment of response to aspirin

(With permission from Wolters Kluwer Health. Harrison *et al* (70))

Nonetheless, all 3 meta-analyses have reported a statistically significant clinical hazard associated with aspirin ‘resistance’ with an odd ratio of cardiovascular events between 3.11 (76) and 4.4 (75). Of particular note, Krasopoulis *et al* reported a hazard ratio of 5.99 for mortality (77). Such a striking effect on cardiovascular outcomes and mortality in patients with coronary disease is at odds with the results of randomised, placebo controlled trials of similar numbers of patients which have noticeably failed to show a clear benefit to aspirin over placebo for at risk patients (10). Although the funnel plots for these analyses were reassuring the authors of all 3 papers acknowledge that publication bias may exist. In addition, combining such measures that have separately been shown to correlate so poorly to produce a definitive opinion regarding the risks of ‘aspirin resistance’ seems unsound. It should be noted that although a proportion of patients in these studies did undergo coronary intervention, the main focus for recruitment was known coronary disease or an ACS event.

Returning to the original data supporting the use of aspirin in cardiovascular and cerebrovascular disease, it is worth recalling at this stage that the evidence to support the long term use of aspirin, beyond 6 weeks, in the prevention of thrombotic events remains an issue

of some debate. Large primary prevention studies in both females and diabetics have not supported the use of chronic low dose aspirin therapy in this setting (80,81). Long term randomised placebo controlled data for secondary prevention of cardiovascular events is lacking. It has, however, been clearly shown that for patients undergoing coronary angioplasty and stenting treatment with both aspirin and clopidogrel significantly reduces thrombotic events. Following drug eluting stent implantation, dual antiplatelet therapy (DAPT) is recommended for at least 1 year and in most cases aspirin lifelong thereafter (30). This population is therefore most likely to suffer from a reduced response to aspirin. Although several meta-analyses and clinical studies have emerged to investigate further the role of platelet function testing for clopidogrel response in patients undergoing PCI, the data for aspirin response in this context remains limited being restricted to retrospective data suggesting a role for poor response to aspirin in PCI patients suffering from stent thrombosis (82,83). Of course the picture in these circumstances is clouded by additional clopidogrel therapy which limits the use of assays to isolate the response to aspirin (67).

1.5.1 Measuring response to aspirin in patients taking dual antiplatelet therapy

In a study by Lev *et al*, aspirin response was measured prior to the introduction of clopidogrel using LTA to AA 1.6mmol, ADP 5 and 20 mcmol/l and VerifyNow Aspirin (CPG) (84). In contrast, Gori *et al* measured aspirin response using LTA to AA in patients taking DAPT and found a significantly higher aggregation response in patients that had suffered stent thrombosis (83). This has limited the data in this important patient group and contrasts with measurement of response to clopidogrel where there is a wealth of studies assessing clopidogrel response on DAPT. For example, Buch *et al* investigated aspirin and clopidogrel response and myocardial enzyme release following elective PCI and found that only 3.7% of patients were deemed aspirin resistant according to VerifyNow Aspirin when taking both aspirin and clopidogrel (85). An even more preclusive effect has been highlighted by the recent ARCTIC study which used VerifyNow Aspirin and VerifyNow P2Y12 to guide adjustments to antiplatelet therapy (86). VerifyNow Aspirin (AA) assays were run following the introduction of clopidogrel and only 5/1213 (0.004%) were found to be aspirin 'resistant' according to this assay.

1.6 Aetiology of variation in response to aspirin

Multiple mechanisms have been proposed to explain the variable antiplatelet effects of aspirin. As yet no single mechanism has been confirmed to cause clinically significant variation in aspirin response but it remains a field of much interest.

Non-compliance: It is recognised that compliance with long term cardiovascular therapies is limited (87). Most studies investigating aspirin resistance have sought to confirm compliance with aspirin therapy but in most cases this has been through verbal questioning only. Schwarz *et al* (55) found that 12/21 (57%) of patients on chronic aspirin therapy found to be ‘non-responders’ admitted to being non-compliant with aspirin therapy. In addition, following direct observation of ingestion of 325mg of aspirin, all bar one of these patients showed suppression of platelet aggregation. Weber *et al* also addressed this issue when investigating the effects of oral and in vitro administration of aspirin to help define aspirin ‘resistance’ (88).

Dose of aspirin: The dosing of aspirin highlights one of the main difficulties with monitoring aspirin response in clinical practice. Clinical studies have suggested that a maximal reduction of thromboembolic events can be achieved using aspirin doses from 75-150mg/day. This is also supported by laboratory evidence suggesting maximal suppression of thromboxane production with as little as 30mg/day. However, the measures of aspirin response used in the studies to date have shown that higher doses of aspirin will further reduce platelet activation (89). It is clinical efficacy that remains paramount when using these therapies, but it may be that tailoring the dose of aspirin to the individual depending on the response testing and cardiovascular risk may offer improved outcomes (90).

Intestinal absorption and pre-systemic portal metabolism: Aspirin is rapidly absorbed through the gastric mucosa and a proportion is hydrolysed during this process to salicylic acid, an inactive derivative. Variations in this absorption and hydrolysis process may play a role in aspirin resistance and it has been proposed that proton pump inhibitors may have an effect by promoting the mucosal esterases that are responsible for hydrolysis although there is evidence that this is not a significant effect (74,91). Once aspirin enters the portal circulation it interacts

with platelets but is also rapidly hydrolysed to salicylic acid, with a half life of 15 minutes (90).

COX-1 binding: The interaction between aspirin and the COX-1 enzyme is a potential source of variation. NSAIDS such as naproxen have been shown to reduce aspirin binding to COX-1 thus reducing the antiplatelet effect (92). There is also evidence from studies in patients undergoing coronary bypass surgery and carotid artery surgery of a transient increase in thromboxane production or platelet aggregation that is not prevented by the additional ex-vivo administration of aspirin (93-95). The mechanism by which this transient effect occurs has not been clarified although it has been proposed that the increased turnover of platelets that occur in the setting of major vascular surgery, the very short plasma half-life of aspirin and the use of heparin during surgery may play a role (94,96).

Alternative sources of thromboxane A₂: Endothelial cells, macrophages and monocytes produce TxA₂ from arachidonic acid and there is some suggestion that this non-platelet dependent production of thromboxane may be upregulated in patients with atherosclerotic disease and in inflammatory states (97-99). In particular, biomarkers of non-platelet related thromboxane synthesis are augmented by several cardiovascular risk factors such as smoking, diabetes, hyperlipidaemia and in the presence of acute coronary syndromes. Smokers have also been shown to have increased concentrations of urinary TxB₂ (100). These findings may explain the association between these risk factors for cardiovascular events and increased platelet activation (100-102).

Genetics: Multiple single nucleotide polymorphisms exist for both COX-1 and cyclooxygenase-2 (COX-2). There are also numerous polymorphisms of the genes involved in the pathways of thromboxane biosynthesis (103). In addition, there are suggestions that the P2Y₁ ADP receptor may modify platelet response to various agonists including thromboxane. It has been proposed that perhaps one third of the variation in response to aspirin may be attributable to these genetic polymorphisms but there is little certainty in this regard (104,105).

Duration of aspirin therapy: There is some suggestion that the antiplatelet effects of aspirin may diminish with prolonged treatment (106). This relatively small study of 150 patients used platelet function testing at baseline, 2 months and 24 months to evaluate optical platelet

aggregation in response to collagen. The reduced response to collagen was measured according to the delay between addition of collagen to the PRP and the time to onset of aggregation (lag-phase) and also maximal % aggregation. Both measures suggested a reduced response to aspirin over time. The other trigger for the notion that aspirin response is reduced with time comes from the observation from trials of GPIIb/IIIa inhibitors and low molecular weight heparin that patients established on aspirin therapy have a worse outcome following an ACS event (107,108). However, there are a number of risk factors such as hypertension, compliance and age itself that are likely to confound this association.

1.7 Conclusion

Chapter 1 has reviewed the evidence supporting the widespread use of regular, low dose aspirin for the prevention of cardiovascular events. It has also described the emerging evidence of a variation in response to aspirin between individual patients that was the driving force behind the inception of the research described in this thesis. Possible mechanisms to explain this variation have been outlined and will be revisited in Chapter 4 following the analysis of our own data. Evidence to support a link between measured aspirin response and clinical outcome has also been described, noting the limitations of the available data. In particular, this chapter has addressed the strengths and weaknesses of the available assays to measure aspirin response and I return to this important aspect in Chapter 3, 4 and 6 where I discuss the methods selected to assess aspirin response for this research. Chapter 2 now addresses similar issues related to clopidogrel and outlines the aims of this thesis.

Chapter 2: Variation in response to clopidogrel

2.1 Clopidogrel in cardiovascular disease

2.1.1 Introduction

Over the last 15 years, clopidogrel has established itself as a core therapy in patients with cardiovascular disease, most notably acute coronary syndrome and those undergoing percutaneous vascular intervention. Acting specifically on the platelet P2Y₁₂ ADP receptor, it has shown itself to be very well tolerated, with few side effects. It has tracked the expansion of interventional vascular procedures by providing reassurance that a potent combination of antiplatelet therapy will reduce short and long term risks associated with these procedures and has challenged the use of more powerful intravenous agents in these settings. However, despite the overwhelmingly positive benefits of clopidogrel, there remain some issues. In particular, it has become increasingly apparent that not all patients experience the same platelet inhibition on clopidogrel and this variation in response may have clinical significance. This is becoming increasingly important as newer, more potent and more expensive, ADP antagonists are emerging which may offer greater reassurance and clinical benefit in certain patient groups.

In this chapter, I review the data supporting the widespread use of clopidogrel in combination with aspirin for the reduction of cardiovascular events. I focus particularly on the evidence for patients with acute coronary syndromes and those undergoing percutaneous coronary intervention. Leading on from this, and building on the concepts of platelet function testing established in Chapter 1, I review the evidence of variable response to clopidogrel, including a review of the assays used to measure response to clopidogrel and the evidence that this may have important implications for clinical outcome.

2.1.2 The evolution of clopidogrel for the treatment cardiovascular disease

ADP was first proposed as an agent of platelet activation in 1960 (109). Although in isolation ADP is a relatively weak platelet agonist, it has important synergistic effects with other common platelet activators such as thrombin and collagen (49,110). ADP seems to hold a key role in the formation of platelet aggregates through conformational change and activation of the glycoprotein IIb/IIIa complex. In particular, stimulation by ADP promotes platelet shape change from discoid to spherical and spiculated (56). Both these steps are important in the formation of platelet aggregates although stabilisation of these aggregates is more dependent on other pathways leading to fibrin production. Whilst exogenous sources of ADP, for example from injured cells, play a role in platelet activation, the majority of ADP induced platelet activation is thought to result from endogenous ADP released from the platelet dense granules (56).

It has been demonstrated that two distinct platelet ADP receptors exist (111-113). P2Y₁ is largely responsible for instigating changes in platelet geometry whereas the conformational change and activation of the GPIIb/IIIa receptor complex is mediated through the P2Y₁₂ receptor. This purinergic receptor, which when activated inhibits adenylate cyclase, is coupled to a G protein triggering activation of the GPIIb/IIIa receptor complex through a number of subsequent pathways including phosphoinositide 3-kinases, Akt, Rap1B and potassium channels (114).

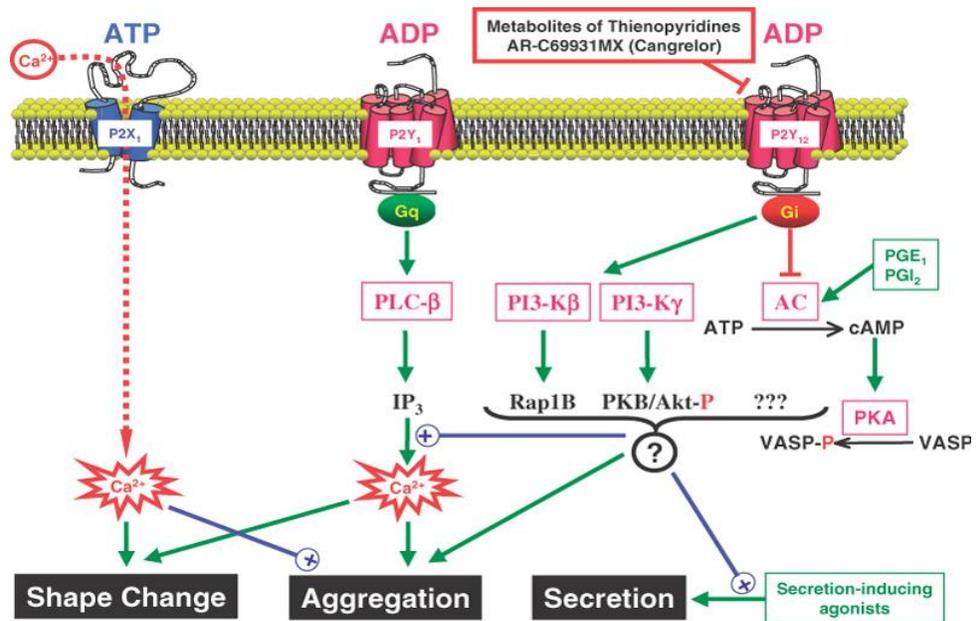


Figure 11: Mechanisms of action of the platelet P2Y receptors
 (With permission Michelson A.D., Platelets, Second Edition. Elsevier Inc/Academic Press. 2007)

In the 1970s, long before the ADP receptors had been identified, a group of compounds categorised as pyrimido-pyrimidines were being investigated for their ability to inhibit the effects of ADP on platelet activation (115). This work would lead to the development of the first thienopyridine derivative, ticlopidine, in the 1980s. Recognised for its antiplatelet properties, this was finally attributed to inhibition of the effects of ADP on platelet aggregation in 1991(116). Whilst investigation continued in to the molecular pharmacology of thienopyridines, trials had already begun into the clinical potential for this medication. As with the initial studies of aspirin use, much of the early focus was on patients suffering from cerebrovascular disease. The Canadian American Ticlopidine Study (CATS) of 1053 patients within 4 months of a thromboembolic stroke showed a 30% relative risk reduction in vascular death, myocardial infarction or recurrent stroke with ticlopidine over placebo (117). The Ticlopidine Aspirin Stroke Study (TASS) showed a 12% relative risk reduction of recurrent stroke or vascular death with ticlopidine over aspirin (118). A small, non-blinded study investigating the use of ticlopidine in unstable angina suggested a benefit similar to aspirin (119). However, at this point, interest turned to the use of ticlopidine in combination with aspirin for patients undergoing coronary intervention.

As discussed in Chapter 1, it is recognised that angioplasty and stenting cause significant disruption of the coronary vascular endothelium. Successful intervention to an atherosclerotic coronary lesion requires dilatation of the underlying medial and intimal layers. The relatively rigid luminal adventitia is usually fractured at its' weakest point exposing the undersurface of the plaque and the medial layers to flowing blood. These surfaces contain numerous procoagulant materials including platelet activators such as collagen and tissue factor that generates thrombin through the extrinsic coagulation pathway. Pathological and experimental studies have confirmed the formation of a platelet 'carpet' over the denuded surface and the role of thrombus formation in the complications associated with coronary intervention is clear (120,121). In view of this, aspirin and anticoagulants such as warfarin, heparin and hirudin were an integral part of the pharmacology associated with coronary intervention from the outset. However, the opportunity to add a second antiplatelet agent led to several studies investigating the addition of ticlopidine to aspirin in patients undergoing coronary intervention.

The Intracoronary Stenting and Antithrombotic Regimen (ISAR), Full anticoagulation versus ASpirin and TIClopidine after stent implantation (FANTASTIC), Multicenter Aspirin and Ticlopidine Trial after Intracoronary Stenting (MATTIS) and STent Anticoagulation Restenosis Study (STARS) studies randomised patients between aspirin-ticlopidine therapy and aspirin plus anticoagulant therapy (122-125). All showed a 30 day reduction in cardiac events using dual antiplatelet therapy over aspirin-anticoagulant therapy. ISAR, FANTASTIC and MATTIS also showed a lower incidence of haemorrhage and peripheral vascular complications. Interestingly, even in these early studies, investigators noted that the timing of the introduction of ticlopidine may play a further role in reducing cardiovascular events, particularly acute occlusion rates. In the FANTASTIC and ISAR studies, ticlopidine was administered at the time of stent insertion, but it is recognised that it may take 3-5 days of drug administration to reach maximal platelet inhibition. In keeping with this, both studies suggested very early complications were equivalent between the groups, with the benefit of ticlopidine only becoming apparent after 48-72 hours.

The use of ticlopidine was hampered by frequent side effects: gastrointestinal upset occurred in 30-50% of patients and skin rash was also a problem. However, of most concern was the development of neutropaenia in approximately 2% of patients on chronic therapy. In half of

these cases a severe reduction in neutrophils was seen with a number of fatalities reported and, as a consequence, interval full blood count testing was recommended for the first 3 months of treatment (117).

Clopidogrel, a second generation thienopyridine, is chemically very similar to ticlopidine (126,127). Both are prodrugs, requiring oxidation via the cytochrome P450-3A liver enzyme system to produce the active metabolite (128,129). Only about 15% of clopidogrel is metabolised to the active compound, with the remainder being hydrolysed by esterases to an inactive carboxylic acid derivative. The active metabolite binds irreversibly to the ADP P2Y₁₂ receptor leading to blockade of ADP binding at that site for the lifespan of the platelet. Initial dose finding studies suggested that inhibition of platelet aggregation with 75mg once daily of clopidogrel was equivalent to ticlopidine 250mg twice daily (126,130).

The first major clinical study using clopidogrel was the Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) trial (131). This randomised, double blind, placebo controlled study investigated the use of clopidogrel in patients with an established diagnosis of vascular disease in the form of ischaemic stroke, myocardial infarction or symptomatic peripheral arterial disease. Patients received either clopidogrel 75mg/day or aspirin 325mg/day with a mean follow-up of 1.91 years. There were some questions regarding the heterogeneity of the results for each of the subgroups of patients recruited but the study demonstrated a 0.51% absolute risk reduction (or 8.7% relative risk reduction) in the primary outcome of ischaemic stroke, myocardial infarction or vascular death in favour of clopidogrel. Importantly, however, there was no difference in the rate of neutropaenia between the groups and only very small differences in the rate of diarrhoea or rash were observed.

The Clopidogrel Aspirin Stent International Cooperative Study (CLASSICS) published in 2000 demonstrated that clopidogrel had a superior safety and tolerability profile compared to ticlopidine following coronary artery stenting (132). Although not powered to look for a reduction in cardiovascular events, there was some suggestion of benefit over ticlopidine when initiated with a 300mg loading dose. This landmark study led to the widespread adoption of clopidogrel over ticlopidine for patients undergoing coronary stent implantation.

Looking further at the use of clopidogrel in the setting of acute coronary syndromes, the Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) study investigated patients presenting with symptoms and investigations suggestive of unstable angina or non-ST-elevation MI to aspirin alone or aspirin and clopidogrel (133). This study showed a significant reduction in the combined primary endpoint of death, myocardial infarction or stroke in patients randomised to clopidogrel over placebo at a mean duration of 9 months. Of particular relevance was the pre-determined sub-study of patients undergoing percutaneous coronary intervention (PCI-CURE) where the 300mg loading of clopidogrel followed by 75mg/day for up to 1 year was compared to the standard 75mg/day regimen for 1 month started at the time of intervention (134). This reinforced the widespread practice of giving a 300mg loading dose of clopidogrel in patients introduced on to clopidogrel in the cardiac catheterisation laboratory at the time of stenting or presenting with an acute coronary syndrome.

To further evaluate the benefits of a 300mg loading dose of clopidogrel in patients undergoing PCI, the Clopidogrel for the Reduction of Events During Observation (CREDO) study randomised 2116 patients scheduled for elective PCI to either 300mg loading 3-24 hours prior to PCI followed by 75mg/day for 12 months or 75mg/day started immediate after completion of the intervention procedure (135). There was a significant 18.5% relative risk reduction in MACE in the pre-treatment group at 28 days with a 26.9% reduction seen at 1 year. In a pre-specified subgroup analysis, the benefit appeared to be confined to the 50% of patients receiving clopidogrel loading at least 6 hours prior to intervention and further analysis using linear splines to summarize the effects of time of pre-treatment suggest that the benefit was further confined to patients receiving clopidogrel loading at least 15 hours prior to coronary intervention.

Two years following publication of the results of the CREDO trial and following work demonstrating more rapid platelet inhibition with a 600mg loading dose of clopidogrel, the Intracoronary Stenting and Antithrombotic Regimen - Rapid Early Action for Coronary Treatment (ISAR-REACT) study was published (136,137). In this study, 2159 elective PCI patients were loaded with 600mg of clopidogrel at least 2 hours prior to coronary intervention. Patients were then randomised to receive either abciximab or placebo infusion during their intervention procedure started immediately following diagnostic angiography. All patients

then received clopidogrel 75mg twice daily following stent placement for a maximum of 3 days followed by 75mg/day thereafter. There was no difference between the abciximab or placebo groups in the incidence of MACE at 30 days, and there was no difference between patients loaded 2 hours before the procedure or those loaded >12hours from intervention. The Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty (ARMYDA-2) study randomised 255 patients to clopidogrel 300mg or 600mg 4-8 hours before elective PCI and found a significantly reduced incidence of peri-procedural cardiac enzyme elevation (CK-MB >3x ULN) in subjects receiving 600mg clopidogrel (138). This finding, however, was not supported by the subsequent PRAGUE-8 study which randomised patients attending for angiography with a view to PCI to receive either upfront clopidogrel 600mg >6 hours from the procedure or loading with clopidogrel following diagnostic angiography in the catheterisation laboratory (139). There was no significant difference in peri-procedural troponin release but there was an increase in minor bleeding according to a modified TIMI classification. It should be noted in this study that all procedures were undertaken via the femoral artery.

To complete the evaluation of clopidogrel therapy added to aspirin, in the spectrum of acute coronary syndromes, the CLARITY-TIMI 28 (Clopidogrel as Adjunctive Reperfusion Therapy–Thrombolysis in Myocardial Infarction) and COMMIT (Clopidogrel and Metoprolol in Myocardial Infarction) trials investigated the use of clopidogrel in patients with ST elevation myocardial infarction undergoing thrombolysis or PCI(140,141). Again a clear benefit in favour of clopidogrel was found. Interestingly, the recently published BRAVE-3 (Bavarian Reperfusion Alternatives Evaluation-3) study showed that for patients undergoing angiography with a view to PCI within 24hours of an acute STEMI, there was no additional benefit with the addition of abciximab if they had been pretreated with a 600mg loading dose of clopidogrel (142).

Finally, 15,000 patients at high risk of cardiovascular events were randomised to receive clopidogrel or placebo in addition to aspirin in the CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance) study (143). There was no additional benefit to DAPT in reducing cardiovascular events with an increased rate of GUSTO moderate bleeding. However, in patients who had previously experienced a

cardiovascular event, there did seem to be a small additional benefit. As a consequence, the use of DAPT beyond the treatment of ACS or outwith the context of PCI remains limited.

2.1.3 Dosage of clopidogrel in cardiovascular disease

As mentioned previously, much debate surrounds the dose and timing of clopidogrel loading in patients with acute coronary syndrome, especially those undergoing coronary intervention. In elective patients, the evidence from CREDO and ISAR REACT would support preloading with clopidogrel to ensure adequate platelet inhibition prior to stent placement (135,137). The counter argument is mainly based on concerns about bleeding risk. As PRAGUE-8 has suggested, when access is from the femoral route, there may be an increase in, predominantly minor, bleeding (139). There are also concerns that acute coronary syndrome patients loaded with clopidogrel may have an increased risk of bleeding during coronary artery bypass surgery (CABG) should that be the subsequent revascularisation option of choice. Both the the American Heart Association (AHA) and European Society of Cardiology (ESC) guidelines suggest that CABG be undertaken at least 5 days following cessation of clopidogrel therapy unless instability demands otherwise (144,145).

As interest grew in the issue surrounding the timing and dosing of clopidogrel in patients undergoing coronary stenting, research turned to the use of platelet function testing to further examine this important issue. As will be discussed subsequently, unlike aspirin, the antiplatelet effects of clopidogrel are more readily quantified. The mechanism of action is well defined and there is an appropriate agonist which directly stimulates the receptor targeted by clopidogrel. Various assays exist to determine the antiplatelet effects of clopidogrel but in a study of 54 patients published in 2002 and the subsequent PRONTO study (Plavix Reduction Of New Thrombus Occurrence), Gurbel *et al* investigated the onset and magnitude of platelet inhibition with a 300mg loading dose of clopidogrel followed by 75mg/day. A variety of platelet function assays were used to assess clopidogrel response including LTA, WBPA, the PFA-100 platelet function analyser and whole blood flow cytometry (146,147).

In the PRONTO study, 100 patients undergoing elective coronary intervention were divided in to 4 equal groups. All patients were taking regular aspirin. Group A to C received a 300mg

loading dose of clopidogrel between 3 and 24 hours prior to stent placement followed by 75mg at the time of intervention and daily thereafter. Group D received 75mg at the time of intervention and thereafter without preloading. There were no differences in 30 day clinical outcomes between the groups. However, the platelet studies confirmed that patients not loaded with clopidogrel had significantly reduced platelet inhibition to ADP for up to 2 days following coronary intervention. Interestingly, the level of inhibition in all four patient groups was reduced on day 2 following intervention but this was more pronounced in patients that had not been loaded (Figure 12). Data was consistent for aggregation and flow cytometry assays using ADP as the agonist.

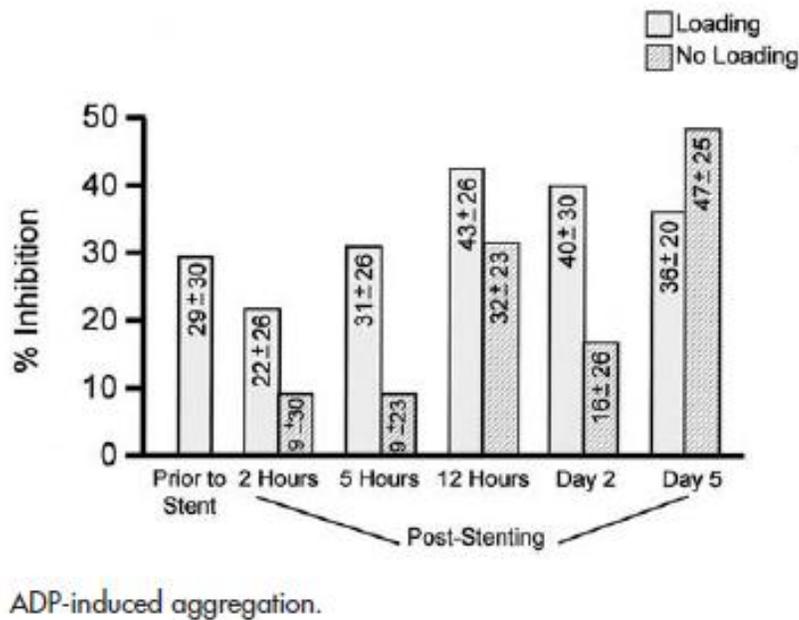


Figure 12: Inhibition of ADP induced platelet aggregation in patients included in the PRONTO study

(With permission from Elsevier Ltd. Gurbel *et al* (147))

Muller *et al* were the first group to publish a study looking at the antiplatelet effects of a 600mg loading dose of clopidogrel over 300mg (136). In 30 patients, they found that platelet inhibition was more rapid in patients receiving the higher loading dose. The equivalent platelet inhibition achieved after 48 hours following loading with 300mg was achieved within 4 hours. This rapid onset of platelet inhibition with 600mg was confirmed in a subsequent study by Angiolillo *et al* (148). To investigate the clinical implications of these studies, the ARMYDA-2 study investigated the occurrence of peri-procedural myocardial infarction, defined as CK-MB elevation >3x the upper limit of normal, following coronary intervention in patients

loaded with either 300mg or 600mg of clopidogrel 4-8 hours prior to coronary angiography (138). 4% of patients in the 600mg loading group experienced peri-procedural infarction against 12% in the 300mg loading group ($p=0.041$). These findings were also reflected in periprocedural troponin I elevation. Using an upper limit of normal of 0.08ng/ml, 26% experienced an elevation in the 600mg group against 44% in the 300mg group ($p=0.004$).

In 2005, Hochholzer *et al* published a larger study of 1001 patients undergoing coronary angiography who had platelet function testing for response to a 600mg loading dose of clopidogrel (149). Patients with recent MI were excluded. 428 patients subsequently underwent coronary intervention. They found a significantly increased aggregation response to 20 μ mol ADP in the cohort of patients undergoing testing less than 2 hours from loading. Beyond 2 hours there was no significant difference in platelet inhibition with time (ie 2-4 hours, 4-6hours or >6hours). It should be noted that at all time points there was a wide variation in platelet inhibition.

With the emerging evidence of incomplete inhibition of ADP aggregation even with 600mg loading doses of clopidogrel and some indication that there might be clinical benefit with increased loading doses of clopidogrel, two studies addressed whether using loading doses higher than 600mg would lead to further reduction in platelet activity. The ISAR CHOICE (Intracoronary Stenting and Antithrombotic Regimen: Choose Between 3 High Oral Doses for Immediate Clopidogrel Effect) study randomised 60 patients attending for diagnostic coronary angiography in a 1:1:1 ratio to 300mg, 600mg or 900mg of crushed clopidogrel tablets (150). Platelet function was assessed, using optical platelet aggregation to 5 and 20 μ mol ADP, at baseline and 4 hours following administration. Pharmacokinetic studies were also performed to detect clopidogrel and its active and inactive metabolites in plasma at baseline, 20, 40, 60, 120 and 240 minutes after administration. There were significantly higher plasma concentrations of the active metabolite in the 600mg and 900mg groups compared to the group receiving 300mg. However, there was no significant increase in active metabolite concentration between those in the 600mg and 900mg groups suggesting that intestinal absorption may peak at the 600mg oral dose. Peak metabolite concentrations occurred at 1 hour for all groups. In keeping with the pharmacokinetic data, ADP induced aggregation was significantly reduced in the 600mg and 900mg groups compared to the 300mg group but again

there was no significant difference in platelet aggregation to either 5 or 20 μ mol ADP between the 600mg and 900mg groups.

The ALBION trial (Assessment of the best Loading dose of clopidogrel to Blunt platelet activation, Inflammation and Ongoing Necrosis) did suggest some additional platelet inhibition with loading doses of clopidogrel greater than 600mg (151). In this study, 103 patients with non-ST-elevation ACS were randomised to 300mg, 600mg or 900mg loading dose of clopidogrel. Platelet function was assessed using optical aggregometry in response to 5 μ mol or 20 μ mol ADP or by flow cytometry using antibodies against PAC1, P-selectin or fibrinogen. Flow cytometry was also used to assess the level of vasodilator-stimulated phosphoprotein (VASP). Samples were taken at 30minutes, 1, 2, 3, 4, 5, 6 and 24 hours following administration of the loading dose. Although the small sample size limited the statistical power of this study, there was a significant increase in platelet inhibition with both 600mg and 900mg loading doses over 300mg. In addition, the data confirmed a significantly more rapid onset of platelet inhibition although all groups had reached a peak of inhibition by 6 hours. Even though the research did not include a direct comparison of 600mg and 900mg loading doses, there was a clear trend towards greater inhibition to 20 μ mol ADP and a reduction in the VASP platelet reactivity index (VASP-PRI) with the higher dose. There was no significant difference in clinical outcomes but again a trend was observed favouring a reduction in troponin release with the highest loading dose.

As part of the investigation into the optimal loading dose of clopidogrel, Kastrati *et al* also published a small study investigating whether a further loading dose of 600mg clopidogrel for patients already on 75mg/day of clopidogrel for >1 month produced any additional platelet inhibition (152). They found that there was a significant reduction in platelet aggregation in response to 5 μ mol or 20 μ mol ADP and also a further reduction in the expression of GPIIb/IIIa receptors and P-selectin. This small study was not powered to address clinical endpoints.

2.2 Variation in response to clopidogrel

As data was emerging concerning the clinical and antiplatelet effects of a loading dose of clopidogrel, and, in view of the concurrent data published regarding aspirin resistance, several

research groups noticed that there was a significant individual variation in the platelet inhibitory effects of clopidogrel. In 2002, Jaremo *et al* published a small flow cytometry study of 18 patients and demonstrated a wide variation in platelet p-selectin and fibrinogen expression following stimulation with ADP (153). All patients were sampled following a 300mg loading dose at the time of initiation and on day 2 of clopidogrel treatment. As an extension of the data generated by the PRONTO study, Gurbel *et al* further evaluated platelet inhibition to ADP at 30 days following coronary intervention in 96 patients taking clopidogrel 75mg/day and aspirin 325mg/day (147). This study demonstrated a wide variation in platelet aggregation induced by 5 μ mol/l ADP, 20 μ mol/l ADP and platelet P-selectin expression. They found a strong correlation between assay results at 5 and 30 days ($r = 0.8$ for ADP induced aggregation and $r = 0.7$ for p-selectin expression). They also noted that pre-treatment platelet reactivity influenced the magnitude of the antiplatelet response to clopidogrel in patients found to have a higher baseline platelet reactivity showing greater persistent activation in response to ADP on clopidogrel therapy. Serebruany *et al* published a much larger series looking retrospectively at platelet inhibition in 544 patients following the introduction of clopidogrel (154). The majority of patients were undergoing coronary intervention ($n=405$) but a proportion were also volunteers with risk factors for cardiovascular disease ($n=94$), had heart failure ($n=25$) or had suffered a cerebrovascular event ($n=20$). All subjects had platelet function testing by conventional optical platelet aggregation testing to 5 μ mol/l ADP both before and after the introduction of clopidogrel. All patients received either 5 days of 75mg/day or a loading dose of 300mg clopidogrel at least 3-4 hours prior to post-treatment platelet sampling. Both the absolute %aggregation in response to ADP and the change in %aggregation from baseline showed a wide variation which appeared to be normally distributed. The mean change in aggregation from baseline was 41.9% (SD 20.8%) and testing for normality revealed a trend towards hyporesponsiveness with a negative skewness of -0.1 using both Anderson-Darling and D'Agostino omnibus tests.

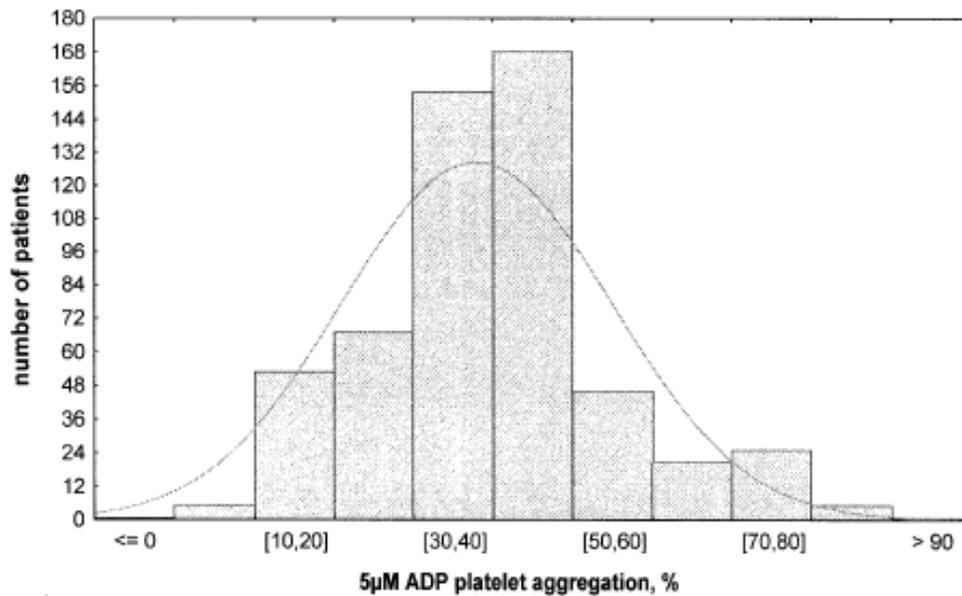


Figure 13: Frequency distribution of response to clopidogrel assessed by LTA to ADP
 (With permission from Elsevier Ltd. Serebruany *et al* (154))

2.2.1 Clinical outcomes associated with variable response to clopidogrel

Concurrent to the emergence of this data looking at platelet function testing in patients on clopidogrel therapy, data was emerging regarding the potential clinical implications of such variability. In 2003, Barragan *et al* published a small study investigating levels of VASP-phosphorylation in 16 patients who had suffered a sub-acute stent thrombosis (SAT) within 30 days of coronary intervention (155). 30 well-matched patients who had undergone coronary intervention without incident also had levels of VASP-phosphorylation measured for comparison. There was a significant increase in platelet reactivity in the SAT group (63.3% vs 39.8%, $p < 0.0001$). Muller *et al* also retrospectively assessed 3 patients who had experienced stent thrombosis and found high platelet aggregation in response to ADP in all 3 patients (156). These findings were replicated by Gurbel *et al* in 2005 (157). They studied platelet inhibition to ADP in 20 patients with confirmed SAT and compared the results to 100 well-matched patients who had previously undergone coronary intervention without incident. Both light transmission aggregometry in response to 5 and 20µmol ADP and GPIIb/IIIa expression in response to ADP were increased in patients who had experienced stent thrombosis. Of

interest, they did not find a significant difference in VASP-phosphorylation between the groups.

The first prospective study investigating the implications of clopidogrel response for clinical outcome was published by Matetzky *et al* in 2004 (69). 60 patient undergoing primary PCI for acute ST-elevation MI underwent platelet function testing at baseline and daily for 5 days after coronary intervention. Baseline samples were taking in the cardiac catheterisation laboratory prior to the introduction of eptifibatide. Clopidogrel was administered as a loading dose of 300mg immediately following PCI followed by 75mg/day thereafter for 3 months. Platelet inhibition was measured using optical aggregation in response to 5µmol ADP and a novel Cone-and-Platelet analyser. Patients were divided into quartiles according to the level of platelet inhibition on day 5 of treatment compared to baseline, with patients in the lowest quartile being deemed clopidogrel resistant. There were 8 major adverse cardiovascular events during the 6 months of follow-up. 7 of these events (88%) occurred in patients in the lowest quartile of platelet inhibition. One event occurred in a patient in the second lowest quartile of clopidogrel response. This exploratory prospective study has not been repeated in a larger cohort despite the dramatic results reported.

Hochholzer *et al* subsequently published follow-up data on the large series of patients tested for clopidogrel response to a 600mg loading dose, reported above, who had subsequently undergone PCI (149). 802 patients were tested by optical aggregometry to 5µmol ADP immediately prior to coronary intervention and assessed for major adverse cardiovascular events at 30 days, including target lesion revascularisation. Patients received 75mg/day of clopidogrel following the loading dose. 15/802 (2%) patients experienced MACE in this time period with a significant difference in events between those in the upper and lower halves of clopidogrel response. The hazard ratio for events between those patients with clopidogrel response below the median against those above the median was 6.71 (CI 1.52-29.41, p = 0.003). They found no association between aggregation response to ADP and troponin elevation following coronary intervention. It should be noted that in this observational study, 242/802 (30.2%) of the cohort underwent platelet function testing and PCI <2 hours from clopidogrel loading and these patients had a significantly reduced response to clopidogrel. As described above, testing of clopidogrel response <2 hours from a 600mg loading dose may not accurately reflect the steady state response (130,137).

A similar impact on clinical outcome was reported by Geisler *et al* in 2006 (158). 379 patients undergoing PCI were tested by optical platelet aggregation to 20µmol ADP and followed up for 3 months. The primary end-point was myocardial infarction, stroke or death. Using an arbitrary cut-off for low response to clopidogrel of >30% aggregation and after adjustment for other factors, this study again demonstrated a significant increase in events in the group with a low response to clopidogrel, HR 3.71, 95% CI 1.08-12.69; p = 0.037. All patients tested had received a loading dose of clopidogrel at least 6 hours prior to platelet function testing.

In the studies by Geisler *et al* and Hochholzer *et al*, blood samples were acquired within a few hours of clopidogrel loading (149,158). Bliden *et al* published a further study investigating 100 patients undergoing coronary intervention (75 elective, 25 urgent) all of whom had received clopidogrel 75mg/day for at least 1 month (159). Patients were tested for clopidogrel response using optical aggregation in response to 5µmol ADP and by TEG in response to 2µmol ADP. 23 patients (23%) experienced MACE within 1 year. These patients had a significantly higher residual platelet reactivity assessed by either LTA or TEG. Correlation between the two assays was good, r = 0.82.

Revisiting the use of VASP-PRI, Bonello *et al* investigated 144 consecutive patients undergoing coronary intervention in a single centre (160). Patients had been loaded with 300mg of clopidogrel at least 24 hours prior to platelet function testing and were divided into quintiles according to response to clopidogrel measured by VASP-PRI. Patients were followed-up for six months for MACE including repeat revascularization. 14% of the total cohort experienced an event during the period of the study. However, no events occurred in the patients in the highest quintile of clopidogrel response. Whilst suggesting a high negative predictive value with this degree of platelet inhibition, further discrimination of risk between the remaining quintiles was not seen.

As these studies have continued to emerge, the overwhelming majority of data suggests that response to clopidogrel measured after loading of 600mg >2 hours previously, 300mg >24 hours previously or following the administration of >5 days of 75mg/day is an independent risk factor for major adverse cardiovascular events and stent thrombosis in patients undergoing coronary intervention. The evidence described in detail to this point formed the basis and

support for the instigation and design of the research project that is the subject of this thesis investigating response to clopidogrel in patients undergoing coronary intervention.

Since this time, two meta-analyses have been published including the data described above and subsequent publications (161,162). Both of these meta-analyses have focused on patients undergoing coronary intervention rather than the wider population exposed to clopidogrel. Aradi *et al* included 20 studies with a total population of 9187 (162). As with similar analyses investigating aspirin response, several different assays were used including LTA (13 studies (5, 10 and 20 μmol ADP)), VASP-PRI (4 studies), VerifyNow P2Y12 (5 studies) and WBPA (2 studies). In addition, a wide range of criteria were used to define clopidogrel non-response and the prevalence of non-response varied from 80% to 6%. These authors have reported increased risks associated with an impaired response to clopidogrel including: non-fatal MI (OR 3.00), definite/probable stent thrombosis (OR 4.14), ischaemic events (OR 4.95) and, most significantly of all, cardiovascular mortality (OR 3.35) (162). However, there is extensive heterogeneity among the studies regarding the arbitrary cut-off for high platelet reactivity used to define response to clopidogrel and the data is not sufficient to clearly demonstrate which assay is optimal. This issue will be explored again in the following section describing in detail the assays used to measure response to clopidogrel.

2.2.2 Aetiology of variable response to clopidogrel

Pre-treatment platelet reactivity: Some studies have defined a poor response to clopidogrel on the basis of a comparison of pre and post-treatment platelet response to ADP (21,163). The majority of studies take an absolute value on clopidogrel treatment as the definition. Two studies (164,165) have shown that high pre-treatment platelet reactivity predicts a lower absolute inhibition to ADP on treatment. However, in the clinical environment it may be impractical to measure pre-treatment response. In addition, the factors that predict a high pre-treatment platelet reactivity have not been clearly determined save for the presence of diabetes mellitus.

Patient co-morbidities: Increased platelet reactivity on clopidogrel therapy in patients with Braunwald classification grade II or III angina has been reported. Data from several studies

have shown increased platelet reactivity and a concomitant reduced response to clopidogrel in patients with ACS (128). There is also evidence that patients with diabetes have higher baseline platelet reactivity and a reduced inhibition of platelet function following the introduction of clopidogrel (147,166,167).

Body mass index (BMI): Although earlier smaller studies have not shown a significant association between BMI and clopidogrel response assessed by LTA and VASP (159,160,168), the larger POPULAR (Do Platelet Function Assays Predict Clinical Outcomes in Clopidogrel-Pretreated Patients Undergoing Elective PCI?) study did show that patients with a higher BMI had a significantly increased residual platelet reactivity on clopidogrel assessed by LTA (5 and 20 μmol), VerifyNow P2Y12 and Plateletworks assays (167).

Concurrent medication: As described above, clopidogrel, as a prodrug, requires activation by cytochrome P450 isoenzymes in order to achieve a therapeutic effect. Several small ex-vivo studies have suggested an association with impaired inhibition of platelets when clopidogrel is administered concurrently with a lipophilic statin (169,170). Against this data, other studies have not found any interaction (154,171) and post hoc analyses of large clinical studies including CREDO and CHARISMA have not shown any association between the co-prescription of these medications and clinical outcome (27,172).

More recently, concern has been raised regarding a possible negative interaction between clopidogrel and proton pump inhibitors. A single study has demonstrated an increase VASP-PRI in patients taking both clopidogrel and omeprazole (173). Furthermore, evidence has emerged to suggest an increased risk of recurrent myocardial infarction in patients taking both therapies (174). Two subsequent meta-analyses have produced conflicting evidence and there remains uncertainty for clinicians trying to balance the risk of thromboembolic events against the risk of bleeding on DAPT (175,176).

Smoking: The early study by Matetsky *et al* suggested an association between smoking and increased antiplatelet effects of clopidogrel (69). Cigarette smoking is known to induce the liver enzyme CYP1A2 which is one of the enzymes that converts clopidogrel to its active metabolite (177). This association was confirmed in a study of 259 patients undergoing elective coronary stenting (178). Platelet aggregation and platelet expression of activated

GPIIb/IIIa receptor were both reduced in current smokers taking clopidogrel. Interestingly, a further analysis of data from the CHARISMA study showed a significant reduction in mortality for current smokers with established cardiovascular disease who were prescribed clopidogrel (179). There was also an associated increase in moderate or severe bleeding episodes in this group. This clinical data is in keeping with the suggestion from platelet studies of an increased antiplatelet effect of clopidogrel in smokers although it is subject to the acknowledged limitations of subgroup analysis.

Genetics: Polymorphisms in the hepatic enzymes involved in the oxidation of clopidogrel to an active metabolite have been described and are associated with clopidogrel response. Frere *et al*, in particular, studied over 600 patients with acute coronary syndrome and found that ADP induced platelet LTA and VASP-PRI were associated with polymorphisms in the hepatic enzyme CYP2C19*2 (180). Subsequent clinical studies of this polymorphism found a significant increase in the risk of death, MI or stroke in patients treated with clopidogrel (181,182). A recent meta-analysis, however, has not supported this association (183). It has also been proposed that variations in the genetic coding of platelet membrane receptors themselves may influence both baseline platelet reactivity and response to antiplatelet therapies that target these receptors. However, the data regarding common P2Y₁₂ polymorphisms is not consistent and a study by Angiolillo *et al* found no association between clopidogrel response and the P2Y₁₂ 34CT and 52GT polymorphisms (184). Studies continue to investigate genetic variation in the membrane glycoproteins that are integral to platelet activation and aggregation.

Compliance: As with aspirin, and indeed all cardiovascular therapies, there remains a significant issue regarding patient compliance. In most cases, assessing compliance relies on patient self-reporting which is notoriously unreliable. A meta-analysis of stroke patients on secondary prevention antiplatelet therapy has suggested non-compliance rates of between 12% and 52% (185). A retrospective study on clopidogrel metabolites in stored samples from patients with either CAD or previous stroke has supported non-compliance rates of 20% in the 6 months following introduction of clopidogrel therapy (186). Interestingly, non-compliance of patients participating in studies for more than 6 months was much less, suggesting that non-compliant patients may withdraw and discontinue study medication before then.

2.3 Assays to assess response to clopidogrel

In the case of aspirin, studies have used arachidonic acid alone, a combination of arachidonic acid and ADP, or other agonists such as epinephrine, collagen and CPG to assess the antiplatelet effects of aspirin. However, as clopidogrel is a specific ADP receptor antagonist, the use of ADP alone is almost universal to assess clopidogrel response.

It should be re-emphasised here that the predominant indication for clopidogrel therapy is in combination with aspirin to prevent complications following coronary intervention and for the treatment of acute coronary syndrome. Thus, in assessing the inhibition to platelet aggregation or activation in response to ADP all the studies are in fact assessing the effects of a combination of aspirin and clopidogrel. This may have important implications for differences between assay results. For example, an aggregation based assay may be influenced by variation in response to aspirin, whereas, an assay assessing the specific pathway targeted by the P2Y₁₂ receptor may not.

2.3.1 Optical light transmission aggregometry

The principle of light transmission aggregometry, to assess the response of platelets to an agonist of choice, has been described in Chapter 1. In the case of clopidogrel, the universal agonist employed is ADP although the concentration varies between studies. The majority of studies have used a final concentration of ADP between 5 and 20µmol with 10µmol being the most frequently reported (162). In a study by Gurbel *et al* and repeated by Lordkipanidze *et al* comparison was made between 5µmol and 20µmol ADP (147,187). Both concentrations demonstrated a variation in response to clopidogrel with higher aggregation in the 20µmol group. In the study by Gurbel *et al*, this variation was seen at different time points following loading and maintenance dosing of clopidogrel. However, the correlation or agreement between results with the two ADP concentrations was only moderate.

Although in most studies, peak aggregation induced by ADP has been used to measure clopidogrel response, different parts of the aggregation curve have been proposed to determine the effects of clopidogrel (42). The P2Y₁₂ receptor targeted by clopidogrel plays a particularly

prominent role in the stabilisation of platelet aggregates (56). In contrast, the P2Y₁ ADP receptor which is not affected by clopidogrel is thought to play a more prominent role in the initiation of platelet aggregation and platelet shape change (49). Thus, it has been proposed that evaluation of late aggregation or indeed disaggregation by optical platelet aggregometry may more accurately reflect changes associated with clopidogrel therapy (42).

In most cases a citrated whole blood sample using 3.8% sodium citrate is used for preparation of assay samples. Labarthe *et al* investigated the use of antithrombin as an alternative anticoagulant for the assays (42). They found that Hirudin/PPACK containing samples showed reduced late aggregation and disaggregation in patients on clopidogrel and thus produced significantly lower numbers of patients thought to be ‘resistant’ to clopidogrel. Interestingly, peak aggregation which is the parameter reported in the majority of the literature was not affected by the choice of anticoagulant.

In addition, it needs to be remembered that the environment created for platelet aggregation in this assay is non-physiological as platelets are separated from whole blood and subjected to artificial shear stress induced by a magnetic stir bar. Once again, samples must be prepared rapidly following acquisition, may take up to one hour to process and require a degree of technical expertise. Despite this and the widely accepted inconsistencies related to the operator, timing and the assay equipment discussed when considering aspirin, this assay continues to be regarded as the ‘gold standard’ method of assessing response to clopidogrel.

2.3.2 Platelet flow cytometry

Whole blood flow cytometry has been used to for a number of years to measure activation of platelets *in vitro* following the addition of an agonist of choice (188). As discussed in Chapter 1, no consistent combination of agonist and marker of platelet activation has been found to measure response to aspirin therapy. In contrast, there are several available antibodies covering surface platelet receptors or internal platelet antigens that are thought to accurately reflect clopidogrel response when used with ADP as an agonist (57).

A detailed description of the flow cytometry protocols used during this research is given in Chapter 3. In principle, two fluorophore labelled monoclonal antibodies are incubated with a diluted whole blood sample. Samples are usually collected in 3.2% sodium citrate as an anticoagulant. The first antibody binds to all platelets and allows for their identification within the other cellular material in the sample. Once identified, the second antibody is a marker of platelet activation and should reflect response of platelets to stimulation with ADP.

As mentioned previously, the final common pathway for platelet aggregation is activation of the surface GPIIb/IIIa receptor. This receptor binds fibrinogen leading to conformational change in the protein and further receptor binding. Antibodies to both the activated GPIIb/IIIa receptor (PAC-1) and to bound fibrinogen are commercially available. Studies have suggested CD62P (P-selectin) as a marker of response to clopidogrel as it reflects platelet degranulation but there are concerns that as P-selectin is lost from the surface of platelets in-vivo over time and this may not be an appropriate in-vitro surrogate marker (60). A comparative study has shown poor correlation between ADP induced LTA and platelet surface p-selectin expression (165).

Whilst these assays are essentially measuring general platelet activation, which is not specific for ADP, a relatively new flow cytometry assay does offer the potential to assess more specifically activation of the P2Y₁₂ ADP receptor (155). Vasodilator-stimulated phosphoprotein (VASP) is an intracellular actin regulatory protein and in platelets is a substrate for cAMP and cGMP dependent protein kinases (189). In response to ADP stimulation of the P2Y₁₂ receptor, VASP is dephosphorylated and hence inhibition of this receptor by clopidogrel leads to higher levels of intraplatelet phosphorylated VASP (Figure 14). It has been shown previously that phosphorylation of VASP plays an important role in the inhibition of fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ receptor and VASP knock-out mice have increased platelet aggregation, fibrinogen binding and P-selectin expression (190). It should be noted that both epinephrine and nitric oxide (NO) donor compounds may also lead to VASP dephosphorylation through cGMP. Conversely, prostaglandin E1 (PGE1)-activated adenylyl cyclase induces phosphorylation of VASP by cAMP-dependent protein kinases (Figure 15).

Figure has been removed due to Copyright restrictions

Figure 14: The interaction of PGE1, ADP and P2Y12 inhibitors (thienopyridines) on VASP phosphorylation

(From VASP assay package insert, Biocytex, France)

a good correlation to the 'gold standard' of optical aggregation and there is evidence of an influence on clinical outcome (155).

There are two features of this assay that make it particularly appealing as a laboratory based method of measuring response to clopidogrel. Firstly, the assay results are stable for 24 hours following sample acquisition (Figure 16) and this has significant implications for use in clinical or research applications (193). Of perhaps even greater significance as a method of assessing response to clopidogrel, or indeed any P2Y₁₂ ADP receptor antagonist, the specific relationship of VASP-phosphorylation to stimulation of this receptor makes the concurrent administration of other antiplatelet agents less likely to interfere with the assay results. As mentioned, when measuring response to clopidogrel using traditional ADP induced aggregation based assays, the result is usually an assessment of the antiplatelet effects of the combination of aspirin and clopidogrel. However, as outlined in Section 1.3, ADP has been used in combination with arachidonic acid as an agonist for the evaluation of aspirin 'resistance.' In addition, the use of GPIIbIIIa receptor antagonists, such as tirofiban or abciximab invalidates aggregation based assays due to their overwhelming inhibition of platelet aggregation at standard clinical doses (194,195). Work published since the research for this study was completed has confirmed the view that VASP may remain a valid reflection of clopidogrel response even after the administration of these agents (196). This has implications for the use of this assay to measure response to clopidogrel in acute situations, including STEMI patients undergoing primary PCI.

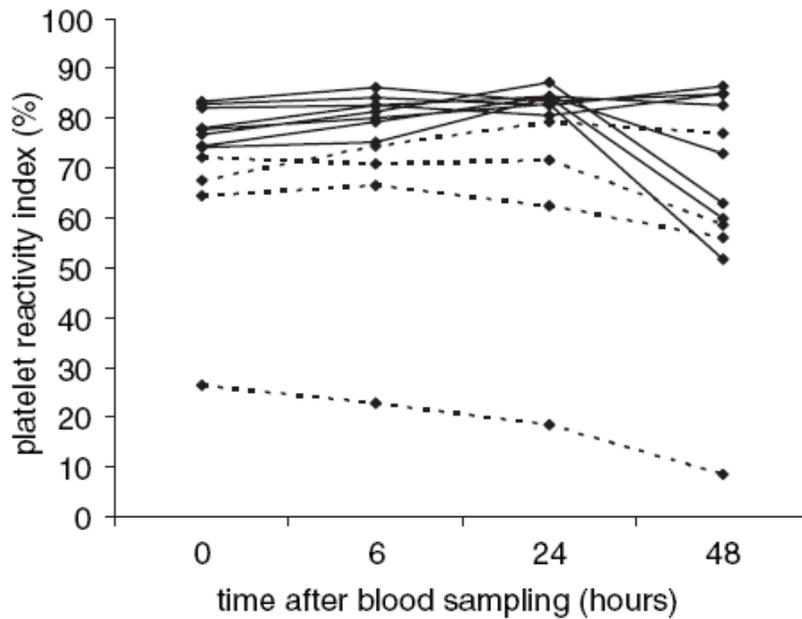


Figure 16: Stability of VASP-PRI for 24-48 hours following sample acquisition

(With permission from John Wiley and Sons. Aleil et al (193))

There are several additional advantages of flow cytometry over LTA. Platelets are stimulated in their physiological milieu including red and white blood cells which may affect platelet activation. There is minimal manipulation of the sample prior to stimulation with the agonist and only a small amount of sample is required. However, the assays are expensive, time consuming and require even greater technical expertise than optical aggregometry. Except for the VASP assay, samples must be processed rapidly following their acquisition.

2.3.3 Whole Blood Impedance Aggregometry

Again, the principles of this technique for measuring platelet inhibition have been described in the discussion of the assessment of aspirin response (Section 1.3.3). This assay measures the endpoint of fibrinogen dependent platelet aggregation. It is thought that the aggregate particles in impedance aggregometry are smaller than those created in optical aggregometry. It is worth repeating that this assay assesses platelet aggregation in the physiological milieu of whole blood where additional important co-factors will be present. Although there is significantly less preparation of samples than in optical platelet aggregation or flow cytometry, there

remains a degree of technical input and time. As with all other assays assessing response to clopidogrel, the principle agonist used is ADP with concentrations ranging from 5µmol to 20µmol (48,60,187). Subsequent to our study, Hochholzer *et al* have published a small study assessing response to clopidogrel in 27 patients using LTA, WBPA, platelet surface markers of aggregation by flow cytometry. The data has shown the limitation of WBPA in response to 20µmol ADP to differentiate response to clopidogrel with 88% of patients established on clopidogrel therapy showing little change in impedance following agonist administration (197).

2.3.4 Rapid Platelet Function Analyser, VerifyNow P2Y12

The principles of this cartridge based assay for the assessment of platelet inhibition have been discussed in Section 1.3.6. Whilst the agonist used in the aspirin cartridges has been modified, the cartridge VerifyNow P2Y12 (Accumetrics, San Diego, USA) has consistently used ADP (198). However, in addition to 20µmol ADP the cartridge assessing P2Y₁₂ inhibition also contains 22nmol prostaglandin E1 (PGE1). As with the VASP flow cytometry assay, the purpose of PGE1 is to suppress the increase in intraplatelet calcium levels caused by ADP stimulation of the P2Y₁ receptor. Thus, the combination of ADP and PGE1 provides a more accurate assessment of the specific effects of ADP on the P2Y₁₂ receptor. Unlike VerifyNow Aspirin, the cartridge includes a second channel containing Thrombin Receptor Activating Peptide (TRAP) to stimulate platelets maximally and act as a reference of baseline platelet activation. The assay cartridge uses 3.2% sodium citrate whole blood samples. Results are reported either as absolute values of aggregation from the ADP/PGE1 containing channel P2Y12 Reaction Units (PRU) or as a percentage inhibition relative to the aggregation achieved in the TRAP containing channel:

$$\% \text{inhibition} = ((\text{BASE-PRU}) / \text{BASE}) \times 100$$

Using VerifyNow P2Y12, Malinin *et al* investigated 147 patients with multiple risk factors for vascular disease all of whom were taking regular aspirin, for response to clopidogrel (199). The samples were acquired prior to clopidogrel loading, 24 hours after a 450mg loading dose and after subsequent administration of 75mg/day for 7 days. Samples were collected using a

venepuncture and vacutainer technique in to 3.2% sodium citrate and analysed in duplicate. There was no significant difference in the results between those taken at 24hours following a loading dose of clopidogrel and after 7 days of therapy. There was a highly significant reduction in platelet function following the introduction of clopidogrel but with a wide variation in the degree of inhibition. 30/147 (20%) of patients had less than 30% inhibition of PRU after clopidogrel therapy.

2.3.5 Platelet Function Analyser-100

Cartridges containing ADP and epinephrine are available for the PFA-100 system (Siemens Healthcare Diagnostics, Inc., Deerfield, IL) described in Section 1.3.7. However, data does not support this assay cartridge being sensitive for platelet inhibition by clopidogrel (60). A new PFA cartridge Innovance PFA P2Y is now available but published data remains limited.

2.3.6 Thromboelastography

As discussed in Section 1.3.8, this assay measures the degree of clot strength mediated by a platelet-fibrin interaction. Parameters include the time to first clot formation, reaction time (R) and maximum clot strength (MA). Using ADP as an agonist, this assay has been used to measure variation in response to clopidogrel. Again, blood is collected into heparinised tubes and subsequently the heparin is neutralised by the addition of heparinase and the presence of heparinase on the surface of the rotating cup.

A prospective study of 197 patients by Gurbel *et al* measured TEG parameters and LTA before and after the introduction of clopidogrel therapy. Both MA and R following ADP administration were found to be significantly associated with cardiovascular events following percutaneous coronary intervention (67,159). Indeed, MA was found to be significantly more sensitive and specific for the prediction of cardiovascular events than the more traditional light transmission aggregometry. Those patients deemed to have a high MA together with a short R were found to be at extremely high risk of further cardiovascular events following PCI (odds ratio 38.0, $p < 0.0001$) (67). Events in this study were cardiovascular death, MI, unstable angina or stroke requiring hospitalisation up to 6 months following intervention.

2.4 Comparison of assays to assess response to clopidogrel

Several of the studies described above, investigating variable clopidogrel response and the influence of this response on clinical outcomes, have used multiple assays to assess the degree of platelet inhibition to ADP. Even several years later, there remains little definitive opinion regarding the optimal method to apply in the clinical environment.

At the time of this research study design, comparative data was limited. The prominent studies in the literature were as outlined in Table 1. As the data summarised in this table illustrates, comparing the assays for assessing clopidogrel response was challenging. LTA in response to ADP was by far the most widely published assay, but concerns remained about utility in the clinical environment because of the demanding nature of the assay protocols. Activation of the GPIIb/IIIa complex appeared promising but several of the studies seemed to use no agonist when assessing this response. Only Hochholzer *et al* used ADP in this circumstance and did not formally report a correlation (149). The single study evaluating VASP-PRI seemed to suggest a more promising correlation but this was a small study of only 33 patients taking clopidogrel (193).

Date	Study	n	Assays	Comparative Data	Corr.	p value
2002	Gurbel <i>et al</i>	100	LTA 5µmol/L ADP LTA 1µg/mL collagen LTA 750µmol/L arachidonic acid WBPA 1 µg/mL collagen Flow cyt. (GpIIb/IIIa expression, PECAM-1, no agonist) PFA-100 ADP/Collagen	Not reported	-	-
2003	Gurbel <i>et al</i>	92	LTA 5, 20µmol/L ADP Flow cyt. (PAC-1 and P-selectin, no agonist)	LTA of 5 and 20 µmol/L ADP	0.6	Not reported
2005	Hochholzer <i>et al</i>	1001	LTA 5 and 20µmol/L ADP Flow cyt. 20µmol/L ADP (PAC-1 and P-selectin expression)	'Flow cytometry findings confirmed those of optical aggregation'	Not reported	
2005	Serebruany <i>et al</i>	544	LTA 5µmol/L ADP Flow cyt. PECAM-1	Change in activated GpIIbIIIa expression vs change in 5 µmol ADP aggregation	0.72	Not reported
2005	Angiolillo	48	LTA 6µmol/L ADP Flow cyt. (P-selectin, PAC-1)	LTA vs P-selectin LTA vs Activated GpIIb/IIIa expression	0.09 0.39	.59 .015
2005	Aleil	33	LTA 5µmol/L ADP Flow cyt. (VASP-PRI)	LTA vs VASP	0.72	<.0001
2005	Gurbel <i>et al</i>	160	LTA 20µmol/L ADP TEG no agonist	None reported	-	-
Corr. = correlation coefficient						

Table 1: Studies comparing assays to assess response to clopidogrel published before the initiation of this research.

Since the inception and recruitment of patients for this research, three large comparative datasets have now been published to address the variation in measurement of clopidogrel response between a number of these assays. These studies are summarised in Table 2 (167,187,200).

Date	Study	n	Patient group	Assays	Comparative data	
					Yes	No
2008	Lordkipanidze <i>et al</i>	116	Stable angina	LTA 5, 20 µmol ADP WBPA 5, 20 µmol ADP PFA-100 (Collagen-ADP) VerifyNow P2Y12	Yes	All between assay correlations (Figure 16)
2009	Cuisset <i>et al</i>	598	ACS	LTA 10 µmol ADP VASP-PRI	Yes	R=0.64, p<0.01
2010	Breet <i>et al</i>	1069	Elective PCI	LTA 5 and 20 µmol ADP VerifyNow P2Y12 Plateletworks ADP Impact-R PFA-100 collagen/ADP	No	Not reported

Table 2: Studies published since 2006 evaluating clopidogrel response using multiple assays

The study by Lordkipanidze *et al* provides the most detailed comparison of the majority of assays excluding flow cytometry (187). A summary of the correlation between the assays used is given in Figure 17.

Platelet function test	LTA ADP 20 μ M	WBA ADP 5 μ M	WBA ADP 20 μ M	PFA-100 [®]	VerifyNow [®] P2Y ₁₂
LTA ADP 5 μ M	0.902 ^a (0.862–0.931)	0.255 ^a (0.077–0.417)	0.307 ^a (0.133–0.463)	–0.270 ^a (–0.438 to –0.102)	0.370 ^a (0.152–0.554)
LTA ADP 20 μ M		0.291 ^a (0.112–0.449)	0.382 ^a (0.215–0.527)	–0.274 ^a (–0.434 to –0.097)	0.496 ^a (0.299–0.652)
WBA ADP 5 μ M			0.881 ^a (0.833–0.916)	–0.139 (–0.313 to 0.044)	0.187 (–0.046 to 0.401)
WBA ADP 20 μ M				–0.150 (–0.322 to 0.033)	0.293 ^a (0.066–0.491)
PFA-100 [®]					–0.334 ^a (–0.525 to –0.111)

^aCorrelation is significant at $P < 0.05$.

Figure 17: Correlation coefficient for 6 assays used to evaluate response to clopidogrel

(With permission from Oxford University Press. Lordkipanidze *et al* (187))

This data demonstrates a strong correlation between 5 and 20 μ mol ADP for LTA. It also shows a strong correlation between the results of 5 and 20 μ mol ADP in WBPA. However, correlation between LTA and WBPA is poor. VerifyNow P2Y12 has a significant correlation with both LTA (5 and 20 μ mol ADP) and WBPA (20 μ mol ADP) although the correlation is not strong. The correlation between different concentrations of agonist using the same assay does suggest that there is a degree of reproducibility within an assay method. This suggests that different assays may be measuring different aspects of platelet aggregation and clot formation and highlights the need for outcome data to support the use of these assays in clinical practice. It also confirms the findings of previous studies suggesting that the ADP/collagen PFA-100 cartridge system does not appear to reflect the antiplatelet effects of clopidogrel.

Which assay would best predict clinical outcome was addressed by Breet *et al* in their study of more than 1000 patients undergoing elective coronary stenting (167). These investigators used LTA to 5 and 20 μ mol ADP to assess clopidogrel response but also measured the antiplatelet effects of clopidogrel using 6 additional point-of-care assays that are commercially marketed to measure platelet function. These included VerifyNow P2Y12, Plateletworks, Impact R, IMPACT-R ADP, PFA 100 collagen/ADP and PFA 100 Innovance PFA P2Y. The primary outcome was a composite at 1 year of death, myocardial infarction, stent thrombosis and ischaemic stroke. Patients were also monitored for TIMI major and minor bleeding. A cut off threshold for high residual platelet reactivity was determined using ROC curves for each assay

against the primary outcome. Patients were then divided using these cut-off values and differences in outcome assessed using the Kaplan-Meier method and the log-rank test. LTA, VerifyNow P2Y12 and Plateletworks were all predictive of future cardiovascular events. The optimal cut off for the VerifyNow P2Y12 assay in order to discriminate between those patients at risk of events was 236 PRU which is in keeping with data from Gurbel *et al* (67). Interestingly, correlation coefficients between the predictive assays were not included among the results in the paper or in the additional appendix.

2.5 Conclusion

Leading on from Chapter 1 addressing the use of aspirin in cardiovascular disease, Chapter 2 has described the evidence to support the additional use of the antiplatelet agent, clopidogrel, in selected patients with cardiovascular disease. Recent publications reporting a variation in response to clopidogrel have been reviewed with the supporting meta-analyses of an association with cardiovascular outcomes. Chapter 2 contains a detailed review of the loading and maintenance regimens of clopidogrel, particularly prior to coronary intervention and this was critical in establishing the appropriate selection criteria for patients in this research. This chapter also includes a detailed analysis of the platelet function assays available to assess response to clopidogrel and this played a critical role in guiding the selection of platelet function tests described in Chapters 3, 5, 6 and 7.

2.6 Aims

At the inception of this research, there was an emerging body of evidence suggesting variable response to both aspirin and clopidogrel. Limited data suggested an association with adverse outcomes in patients with coronary artery disease and those undergoing PCI. However, the optimal method of measuring response to either aspirin or clopidogrel was not clear. In addition, novel assays to assess response to these therapies had recently emerged but without substantial clinical data to support their use in everyday practice. Finally, there was little published evidence regarding the measurement of both aspirin and clopidogrel response in the same cohort of patients.

The hypothesis tested in this thesis was that assays of aspirin or clopidogrel response would predict the incidence of myocardial necrosis following PCI measured by elevation in troponin I. This was accomplished by selecting a cohort of patients attending our cardiac catheterization laboratory for the investigation of coronary artery disease with a view to coronary intervention. In addition to this primary aim, this thesis presents the investigation of a number of other aspects of testing for aspirin and clopidogrel response;

- To assess the measurable variation in residual concentrations of TxB2 in serum ([TxB2]_S) and plasma ([TxB2]_P) in patients established on aspirin therapy. In particular, to investigate the difference between these measures, [TxB2]_{S-P}, as a novel marker of aspirin response that may more accurately reflect the residual ability of platelets to generate thromboxane despite aspirin therapy.
- To assess VerifyNow Aspirin for the measurement of aspirin response, including comparison with [TxB2]_{S-P}
- To compare the residual generation of thromboxane [TxB2]_{S-P} and WBPA in response to collagen and thrombin receptor activating peptide (TRAP) for the assessment of aspirin response in patients established on aspirin and clopidogrel.

- To investigate whether [TxB2]_{S-P} may be a valid measurement of aspirin response following the introduction of clopidogrel by comparing this measure before and after the introduction of this additional antiplatelet agent.
- To assess the assays VerifyNow P2Y12 and WBPA in response to ADP as potential point-of-care assays for the assessment of clopidogrel response in the cardiac catheterization laboratory and compare the results with the laboratory based flow cytometry assays VASP-PRI and platelet fibrinogen binding.
- To investigate response to clopidogrel measured by VerifyNow P2Y12, VASP-PRI and platelet fibrinogen binding before and after percutaneous coronary intervention.
- To compare the results of assays used to assess response to aspirin and those measuring response to clopidogrel in patients established on DAPT.
- To assess which patient characteristics predict a poorer response to aspirin or clopidogrel according to each assay including comorbidities, such as a recent acute coronary syndrome, and concurrent medications.
- To investigate whether platelet function testing using whole blood samples aspirated from an arterial sheath at the time of cardiac catheterization is equivalent to testing using samples collected by venepuncture.
- To investigate [TxB2]_{S-P}, VerifyNow P2Y12, VASP-PRI as measures of response to aspirin and clopidogrel in patients that have suffered a recent stent thrombosis and to assess how these measures compare to the larger population of patients.

Chapter 3: Materials and Methods

3.1 Patient selection and recruitment

All patients included in this research were recruited by RG in the Department of Cardiology, Western Infirmary Hospital, Glasgow between September 2005 and May 2007. They were aged between 18 and 85 years, and were able to provide informed consent. Ethical approval was granted by the West Research Ethics Committee in the form of three separate applications (see Appendix I-VI). Screening for all patients was performed by RG. Patients were recruited from the outpatient department, cardiology in-patient wards and the cardiac catheterisation day ward. Notes and medications were reviewed prior to approaching the patient to seek their informed consent. Due to the observational nature of this study it was decided not to keep a screening log of patients. Although every attempt was made to recruit all eligible patients, because of resource limitations and the nature of some of the assays being performed this proved impractical.

3.1.1 Exclusion criteria

Exclusion criteria that applied to all the studies were:

1. Age <18yrs
2. Administration of GPIIb/IIIa therapy within 14 days
3. Oral anticoagulation, ticlopidine, dipyridamole, or NSAID therapy
4. Pregnancy
5. Chest pain in the 12 hrs prior to PCI
6. Anaemia (Haemoglobin (Hb)<10g/dl)
7. Platelet count <100 or >500 x10⁹/l
8. Personal or family history of bleeding disorder
9. Non-compliance with aspirin therapy

Further inclusion and exclusion criteria will be detailed for each individual study in turn.

Following recruitment, patients were questioned regarding their demographics. Weight and height measurements were recorded and background medical history not covered in the screening process was sought including a history of hypertension, diabetes, dyslipidaemia, smoking, previous myocardial infarction, previous coronary intervention, or peripheral vascular disease. A full list of all current medications was recorded and through direct questioning or by review of case records, the duration of aspirin and clopidogrel therapy was established; in particular, if the patient was already taking clopidogrel, the timing and dose of clopidogrel loading was recorded.

3.2 Blood sample collection for platelet assays

All platelet assay venous blood samples were collected by RG. The utmost care was used to avoid unnecessary trauma and agitation to the sample both during and after collection. Where a suitable large calibre vein could be identified, platelet assay samples were obtained by venepuncture. Patients were asked to sit for 10 minutes prior to sampling and use of a tourniquet was kept to a minimum. Blood was drawn from a large calibre vein in the antecubital fossa using a 21G butterfly needle and vacutainer system. Sample tubes were filled to the indicated volume to ensure a consistent ratio of blood to additive. All samples were handled with appropriate precautions for hazardous material.

The number and type of sample tubes for each separate study is described with each protocol but in all sampling an initial tube of 3.2% sodium citrate was collected and discarded after which, further samples were collected sequentially into 3.2% sodium citrate, EDTA, thrombin or SST (polyester based polymer gel) as required.

In patients without a suitable calibre vein, arterial blood samples were taken directly from the arterial sheath by the cardiac catheterization laboratory medical staff. Immediately following insertion of a sheath into either the femoral or radial artery, 5mls of blood was aspirated and discarded following which blood was gently collected into a 20ml syringe and immediately transferred to the appropriate vacutainer tubes in the sequential order described above.

All tubes were mixed by gently inverting 5 times and immediately transferred to the laboratory for processing.

3.3 Platelet assays methods

3.3.1 Ultegra RFPA – VerifyNow Aspirin, VerifyNow P2Y12

Assay cartridges were purchased from Accumetrics (SanDiego, USA) and refrigerated at 4-6°C throughout transport and until use. Individual assay cartridges were placed in a room temperature environment at least 30 minutes prior to operation. The Electronic Quality Control (EQC) accompanying the RFPA system was run before testing on each day of sampling. As per the manufacturer's guidelines, samples were stored at room temperature for at least 30 minutes before assaying. All samples were processed by RG, AM and HM between 30 minutes and 2 hours of acquisition. When the sample was ready for processing, the assay cartridge (VerifyNow Aspirin or VerifyNow P2Y12) was removed from its packaging, and the protective sheath discarded from the needle. When prompted by the RFPA, the cartridge was inserted and the 2ml 3.2% sodium citrate vacutainer tube gently inverted 5 times to ensure uniform mixing. At the prompt from the instrument, the sample was impaled on to the needle following which aspiration and processing of the sample continued automatically. Within 5 minutes the result was displayed and recorded. For VerifyNow Aspirin, the result was displayed in aspirin reaction units (ARU) with a recommended cut-off for aspirin resistance of 550ARU. The VerifyNow P2Y12 assay results were recorded as an absolute value of platelet aggregation (PRU) from the ADP channel or the %inhibition of P2Y₁₂ calculated using the formula: %inhibition = (Base – PRU) / Base x 100. According to the manufacturers package insert, the intra-assay coefficient of variation (CV) for VerifyNow P2Y12 is 7.5% with an inter-assay CV between 10.6% and 15.5%.

3.3.2 Thromboxane B2 in serum and plasma

As described in Chapter 1, aspirin inhibits COX-1 which is integral to the platelet production of TxA₂. TxA₂ is unstable in vivo and rapidly converted to thromboxane B₂. Several

commercially available ELISA (Enzyme Linked Immuno-Sorbent Assay) assays can be used to measure the levels of TxB2. In addition to platelets, thromboxane is also produced by other cell lines including neutrophils, macrophages and endothelial cells. In most studies, assessment of thromboxane levels is made in serum samples following stimulation of platelets either through collection in non-anticoagulated tubes or by the addition of an agonist such as collagen (52,201). However, levels of TxB2 in serum may be influenced by background thromboxane generation from other sources and not solely representative of platelet production. This may be particularly important in the context of lower levels of thromboxane typically seen in aspirinated patients. Thus, in order to better reflect the residual ability of platelets to generate thromboxane, despite aspirin therapy, we sought to measure background thromboxane levels in anticoagulated samples of plasma with minimal platelet stimulation, [TxB2]_P, and simultaneously in maximally stimulated serum samples, [TxB2]_S. By subtracting the background concentration of TxB2 in plasma we sought to better reflect the residual ability of platelets to generate thromboxane despite aspirin therapy, [TxB2]_{S-P}.

Within 5 minutes of sample acquisition, a 3.2% sodium citrate whole blood tube was centrifuged for 30 minutes at 3000rpm and the plasma supernatant collected in two 0.5ml aliquots. Samples were immediately stored at -80°C. The thrombin containing tube was left at room temperature for 1 hour to encourage maximal platelet activation and aggregation. After 1 hour the tube was centrifuged at 3000rpm for 10 minutes and the serum supernatant collected in two 0.5ml aliquots and stored in -80°C for subsequent analysis. All the samples were processed by RG, AM and HM. All TxB2 assays were performed in the Department of Cardiovascular Sciences, Glenfield Hospital, Leicester by TJ under the supervision of AG. Following completion of patient recruitment, samples were transported on dry ice between the Western Infirmary and Glenfield Hospital following which they were again transferred to -80°C storage. Immediately prior to performing the ELISA assay, samples were gradually warmed from -80°C to room temperature

A commercially available assay for TxB2 was purchased from R&D Systems, Europe (cat. no. KGE011). This immunoassay is based on the principle of forward competitive sequential binding whereby TxB2 in the sample competes with horseradish peroxidase (HRP) labelled TxB2 for a limited number of binding sites on a mouse monoclonal antibody. 100µl of sample was incubated with the mouse monoclonal antibody to TxB2 for 2 hours on an orbital

microplate shaker. HRP-labelled TxB2 was then added and the incubation continued for another hour. The samples were washed to remove unbound material and a substrate solution added to determine the bound enzyme activity. After 30 minutes incubation at room temperature in a light protected environment, a stop solution was added and within 30 minutes the absorbance at 450nm recorded with a wavelength correction at 540nm. The intensity of colour was inversely proportional to the concentration of TxB2 in the sample. The samples were run in duplicate and all plates include a dilution series of standardised TxB2 to ensure an accurate calibration curve for each assay plate. According to the information supplied with the assay, the intra-assay CV is between 3.9% and 5.9% with an inter-assay CV of between 5.1 and 8.9%.

3.3.3 Whole Blood Platelet Aggregation

A Chronolog 592A whole blood impedance platelet aggregometer with disposable electrodes was purchased in October 2005 (Figure 18) This included the Aggo/Link Interface (Chronolog Corporation, USA) software used to record and analyse change in impedance over time. The aggregometer was switched on at least 30 minutes before sampling to allow the temperature in the wells to stabilize prior to performing assays.

Figure has been removed due to Copyright restrictions

Figure 18: Chronolog whole blood platelet aggregometer and disposable electrode

(From Chronolog.com)

A single 3.2% sodium citrate whole blood sample tube was collected for WBPA studies as described previously. All WBPA assays were run within 30 minutes of sampling. A cuvette with disposable electrode (Figure 18) was placed in each of the 2 wells. A magnetic stir bar was added to each cuvette. The sample tube was inverted gently three times to ensure adequate mixing. 0.5ml of citrated whole blood was pipetted to the base of the cuvette and diluted in a 1:1 ratio with normal saline. The sample was left for 5 minutes to warm to 37°C. The aggregometer was then activated and a trace of impedance was generated by the Aggro/Link software. Impedance recordings were observed to ensure a steady state value had been reached reflecting adequate warming of the sample and the formation of a platelet monolayer adhering to the exposed electrode in the solution. If steady state was not achieved, the calibration process was repeated for a further minute. Once steady state was achieved, recording began and the agonist of choice was pipetted into the base of each cuvette. Change in impedance was monitored for 6 minutes and the peak change recorded. Change in impedance was recorded to the nearest 0.5ohm. An example of the output from the Aggro/Link software for ADP and collagen 2µl is shown in Figure 19. The cuvette, electrode, stir bar and solution were then discarded and the process repeated for any further agonists required.

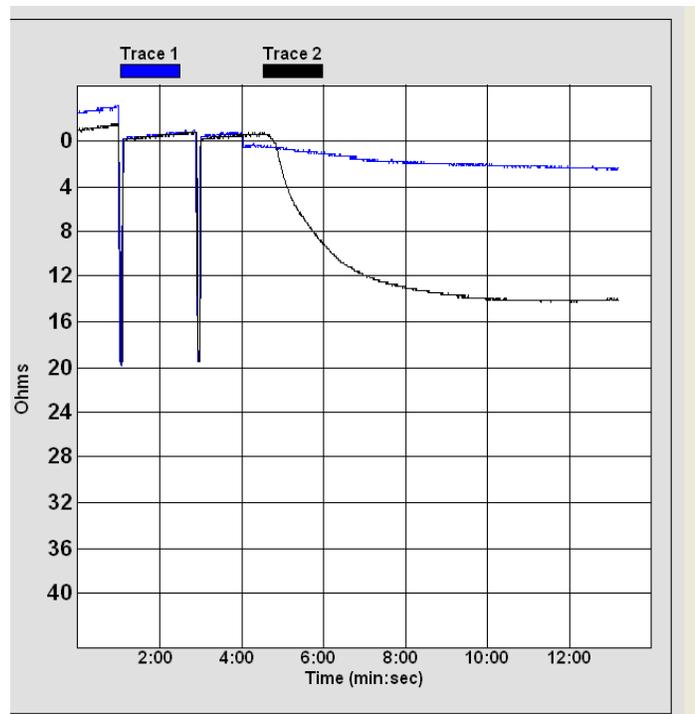


Figure 19: Output from the Aggro/Link Interface of WBPA in response to ADP 20mmol (Trace 1) and collagen 2µl (Trace 2)

3.3.4 Optical Light Transmission Aggregometry

Whilst we were able to produce appropriate aggregation curves using optical platelet aggregation, we found the demands of the technique to be cumbersome. Samples needed to be transferred rapidly to the laboratory and 20 minutes of centrifugation were required to produce platelet rich plasma followed by further centrifugation to produce platelet poor samples. After pipetting into aggregometry tubes and the addition of agonists an aggregation curve was complete within approximately 10 minutes. From the time of sampling we were unable to obtain a result consistently in less than 1 hour making LTA impractical as a point-of-care clinical test. In addition, we felt that this technique had been widely reported in the literature and other centres clearly had extensive experience with it in patients with cardiovascular disease. As such, we did not employ LTA in our studies.

3.3.5 Flow cytometric evaluation of platelet function

Flow cytometry allows the interrogation of individual cells or cellular structures by a focused beam of laser light. The reflection and refraction of this light is recorded by a series of photodiodes or photomultiplier tubes to produce an electronic signal proportional to the size and granularity of the cellular structure. In this way, individual components of whole blood, including platelets, can be separated and analysed. A simplified schematic is shown in Figure 20. Furthermore, once an individual particle has been identified, detailed emission characteristics of the particle can be quantified. In particular, if the sample has been previously incubated with appropriate fluorescently labelled antibodies the intensity of fluorescence emitted by the particle can be quantified and recorded using a photomultiplier tube sensitive to the wavelength of the emitted fluorescence. Multiple photomultiplier tubes permit the recording of multiple wavelength signals corresponding to multiple fluorescently labelled antibody markers. The measurement of fluorescence can be expressed as percentage positive particles above a threshold or as mean fluorescence intensity (MFI). As the markers used to monitor platelet function by flow cytometry are widely expressed in platelets, it is usually more appropriate to use MFI (188).

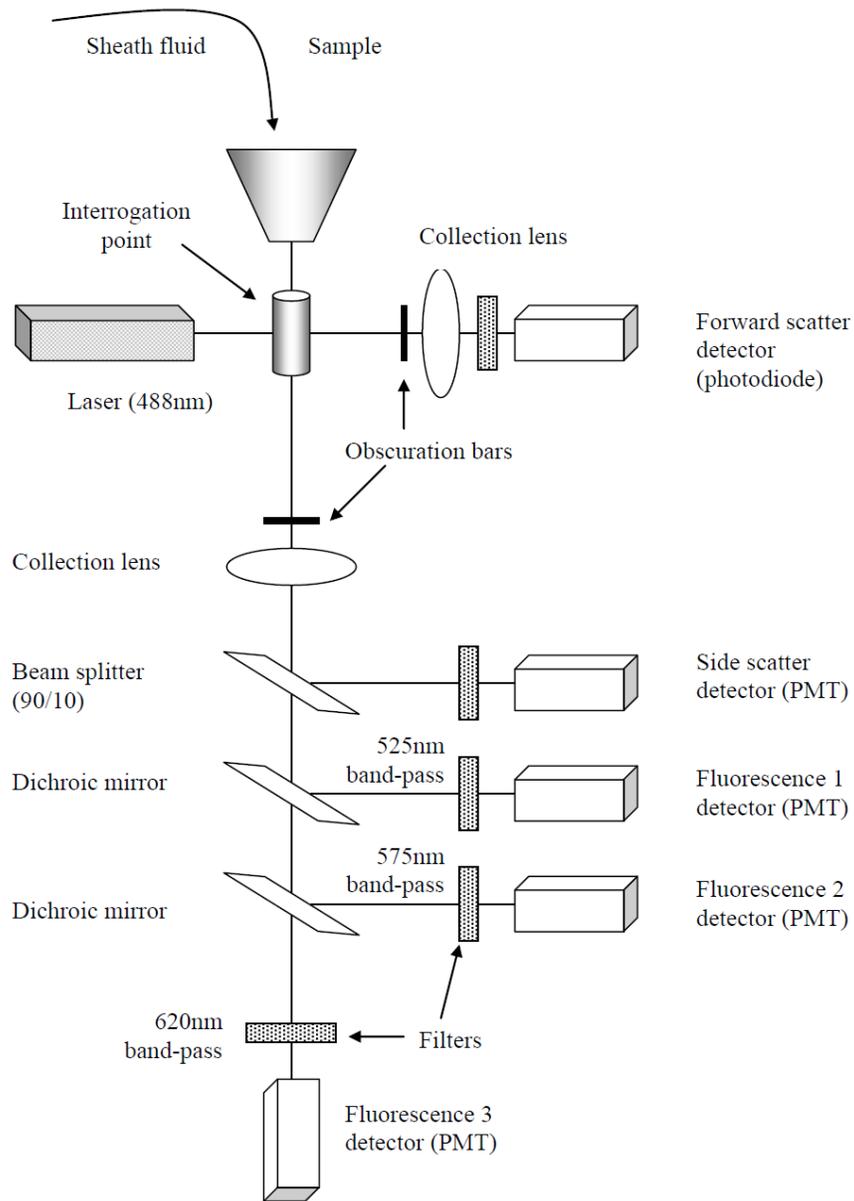


Figure 20: The principle components of a 3 channel flow cytometer. The sample is mixed with a large volume of sheath fluid to ensure separation of individual platelets. A beam splitter and subsequent dichroic mirrors progressively filter increasing wavelengths of the laser beam to isolate different fluoroscopic components.

(With permission from Springer. Goodall AH *et al* (188))

Preliminary assays were carried out to look at the effects of aspirin on the expression of platelet GPIIbIIIa and p-selectin which had been previously reported to show a wide range of variation in patients taking aspirin therapy (202). However, we found the results to be inconsistent and it was felt there was no reliable flow cytometric antibody marker to reflect inhibition of platelet function by aspirin.

Several whole blood flow cytometry assays have been shown to reflect response to clopidogrel. Two flow cytometry assays were selected to investigate the individual effects of clopidogrel on the activation of platelets by ADP: platelet fibrinogen binding and VASP-PRI.

3.3.6 Measurement of platelet fibrinogen binding by flow cytometry

The end point of platelet activation by ADP is the binding of fibrinogen to the activated GPIIbIIIa complex. In vivo, this fibrinogen binding leads to platelet aggregation, the release of thromboxane and subsequent platelet degranulation, introducing a number of additional markers to the platelet surface. However, during flow cytometry studies, assay preparation is designed to try and reduce platelet aggregation and preserve individual platelets that can be interrogated. Thus, little degranulation of platelets occurs following stimulation with ADP in these assays and therefore it was felt the best marker of platelet response to ADP would be the extent of platelet fibrinogen binding.

Immediately prior to patient sampling, assay tubes were prepared at room temperature by adding 50µl HEPES buffered saline (HBS), ADP (final concentration 10µmol) and FITC conjugated- antifibrinogen antibody (DAKO Cytomation, UK). Whole blood samples were collected in 3.2% sodium citrate as described previously. Samples were transferred to the laboratory at room temperature and processed within 10 minutes of acquisition. Using a Barkey pipette, 5µl whole blood was added to the base of the tube and the sample gently agitated to facilitate mixing. After 20 minutes incubation at room temperature, 0.5mls formal saline was added to fix and dilute the assay. A negative control was prepared with each sample containing 50µl HBS, antifibrinogen antibody and 2µl of 6mM EDTA to inhibit the binding of fibrinogen to GPIIbIIIa.

Flow cytometry was performed within 2 hours of assay fixation using a BD FACSCalibur 4-channel flow cytometer (BD Biosciences). Platelets were isolated using a forward scatter vs side scatter plot. The negative control was set at 2% to account for non-specific binding of this antibody to platelets. Results were recorded as change in % positive platelets against the negative control. All samples were run in duplicate. Using these duplicate samples an intra-assay coefficient of variation was calculated as 2.2%. A typical output from a patient tested following the introduction of clopidogrel is shown in Figure 21. All the assays were performed by RG, AM or HM.

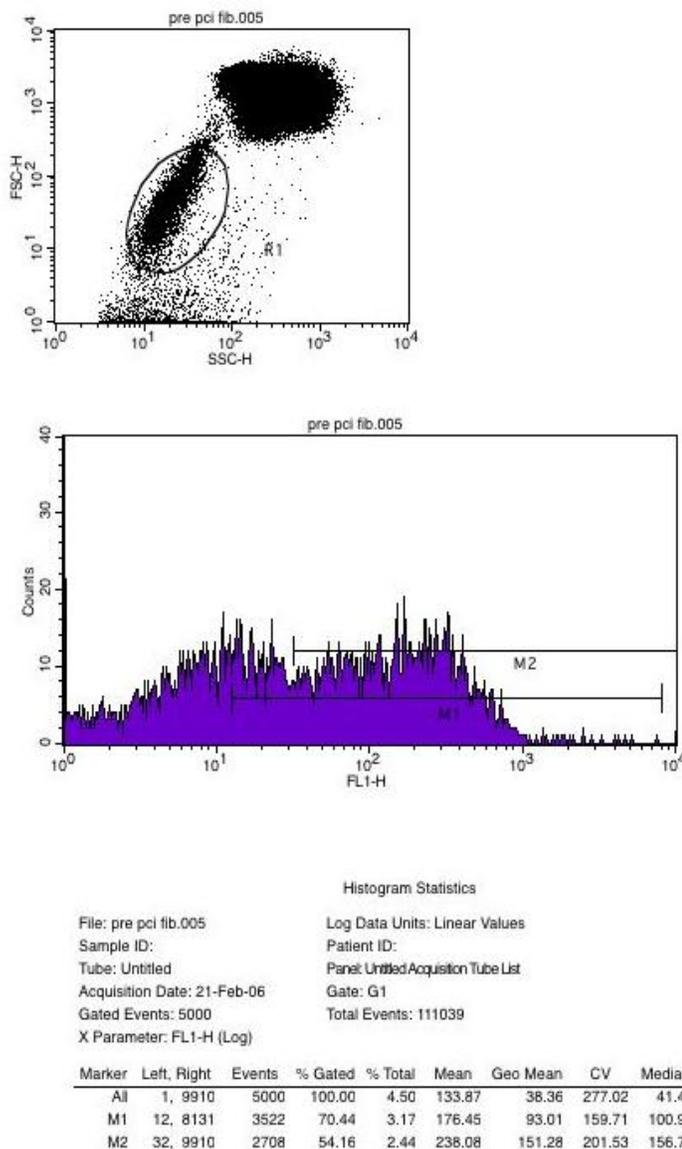


Figure 21: Typical output from the BD FACSCalibre flow cytometer for a patient immediately prior to coronary intervention established on clopidogrel therapy

(Upper) Forward scatter:side scatter plot showing the smaller particles within the region of interest (R1) that are isolated for further interrogation (Middle) Fluorescence histogram with M1 representing the margins set by the negative control of 2% to represent non-specific binding of the fluoroscene antibody to platelets. (Lower) Tabulated output with % positive platelets (%Gated).

3.3.7 Measurement of intraplatelet VASP-phosphorylation by flow cytometry

As previously described in Section 2.3.2, PGE1 stimulates VASP phosphorylation. The level of VASP phosphorylation in response to PGE1 is reduced by ADP stimulation of the P2Y12 receptor. Thus, inhibition of the ADP receptor by clopidogrel leads to persistently high levels of VASP-phosphorylation in response to PGE1. A commercially available assay (VASP/P2Y12, Biocytex France) uses flow cytometry to measure the extent of intraplatelet VASP-phosphorylation. Assays were purchased from Biocytex in pre-packaged boxes containing reagents for 10 assay samples. Unopened boxes were stored at between 2 and 8°C until use. Reagents were prepared as instructed by the manufacturer and each box was used within 1 month of opening. A whole blood sample was collected as described above in a vacutainer tube containing 3.2% sodium citrate. The sample was gently mixed at the time of collection and then stored at room temperature until processing. All assays were carried out within 24 hours of sampling.

In brief, as described in the manufacturers accompanying instructions, 3 test tubes were prepared, T1 containing PGE1 alone and T2 and T3 containing both PGE1 and ADP. 10µl whole blood was carefully pipetted in to each tube and the tubes gently homogenised for 1-2 seconds. Following 10 minutes of incubation paraformaldehyde fixative was added to prevent further platelet activation and a permeabilization agent (non-ionic detergent) was added to each tube. In addition, T1 and T2 received the primary monoclonal mouse antibody against serine 239-phosphorylated VASP (16C2) whilst T3 received a negative isotypic control mouse monoclonal antibody. After a further 5 minutes of incubation, a polyclonal antibody to anti mouse IgG conjugated to FITC was added together with a platelet counterstaining reagent (anti CD61-PE). In the final step all three samples were diluted to a final volume of 2050µl. Samples were run on a 4-channel BD FACSCalibur flow cytometer.

Samples were run twice to monitor consistency. Isolation of platelets was performed using T1. An FS and SS cytogram was used to separate leucocytes and then an FL2 threshold to exclude cellular debris. A selected region of interest was created to contain the particle demonstrating the appropriate platelet profile and the MFI recorded representing maximal VASP-phosphorylation in response to PGE1 alone (Figure 22). Following this, and without making any further changes to the flow cytometer settings, samples T2 and T3 were analysed and the

MFI of particles in the pre-specified region were recorded. Sample T2 represented the extent of VASP-Phosphorylation in response to ADP and PGE1 (Figure 23) and sample T3 acted as a negative control.

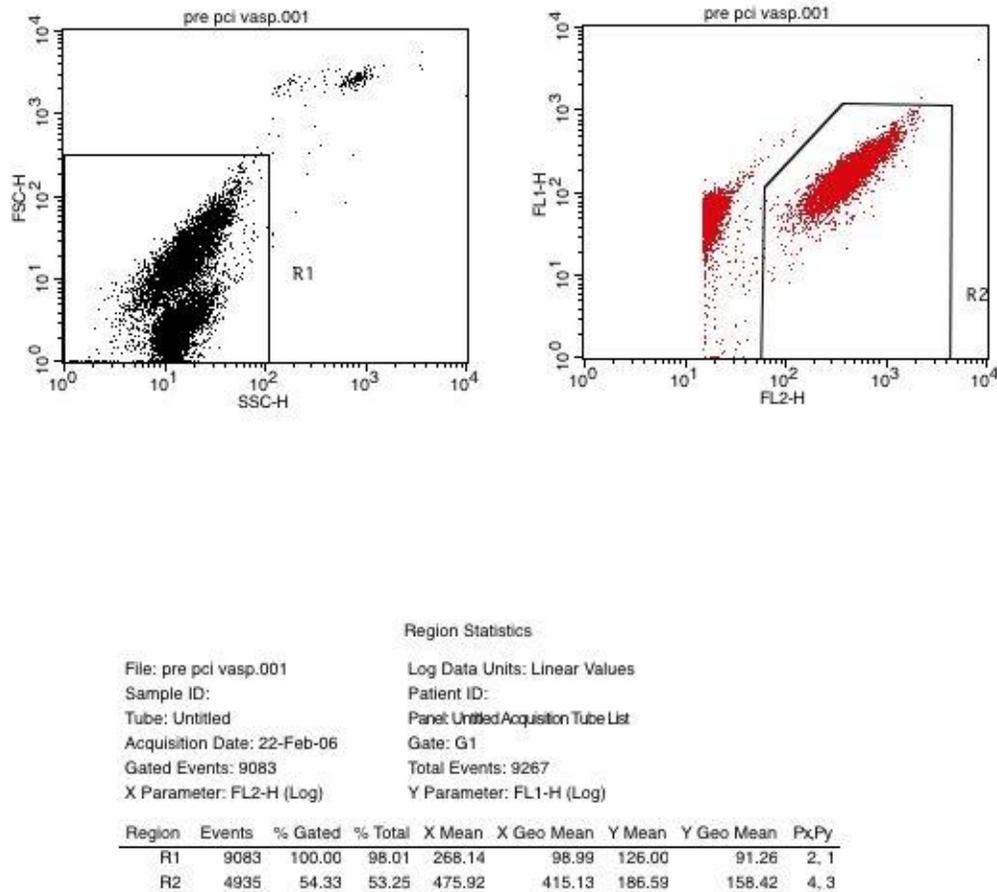
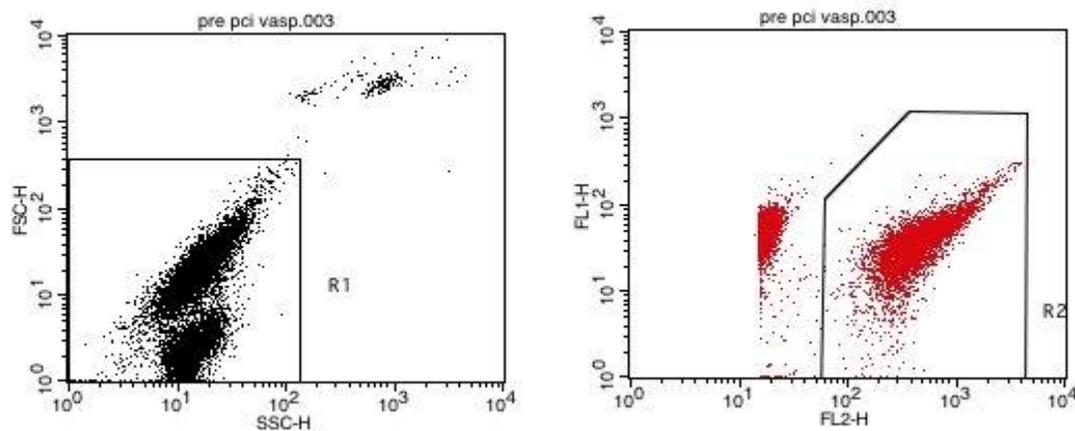


Figure 22: Typical output of VASP assay tube T1 containing PGE1 alone.

(Upper left) Forward scatter:side scatter plot to isolate platelets, (upper right) FL2 threshold was included to eliminate cellular debris following which particles with the appropriate FL1:FL2 profile are selected using a region of interest (R2). (Lower) Tabulated output showing the mean fluorescence intensity (MFI(T1)) for platelets isolated from R2.



Region Statistics								
File: pre pci vasp.003	Log Data Units: Linear Values							
Sample ID:	Patient ID:							
Tube: Untitled	Panel: Untitled Acquisition Tube List							
Acquisition Date: 22-Feb-06	Gate: G1							
Gated Events: 8665	Total Events: 8853							
X Parameter: FL2-H (Log)	Y Parameter: FL1-H (Log)							
Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	PxPy
R1	8665	100.00	97.88	300.84	108.44	44.31	37.39	2, 1
R2	4924	56.83	55.62	515.88	432.36	40.42	32.45	4, 3

Figure 23: Typical output of VASP assay tube T2 containing PGE1 and ADP.

Mean fluorescence intensity (MFI(T2)) of platelets isolated from the region of interest (R2) is used to calculate the platelet reactivity index

Subsequently, the corrected MFI (MFIC) for tubes T1 and T2 was calculated by incorporating the negative control result MFI(T3) according to the following calculations;

$$MFIC_{(PGE1)} = MFIC(T1) = MFI(T1) - MFI(T3)$$

$$MFIC_{(PGE1 + ADP)} = MFIC(T2) = MFI(T2) - MFI(T3)$$

A platelet reactivity index was then calculated using the formula;

$$\text{PRI}(\%) = \{(\text{MFIC}(\text{T1}) - \text{MFIC}(\text{T2})) / \text{MFIC}(\text{T1})\} \times 100$$

All blood samples were collected by RG. All assays were performed by RG, AM or HM. Intra and inter-lot assay variation are provided graphically as mean \pm SD in the package insert supplied by the manufacturer.

3.4 Additional blood sample collection

All patients had haemoglobin and platelet counts performed prior to recruitment in to the study to ensure they met the appropriate criteria. Samples had also been collected to assess renal function. These samples were performed by medical, nursing or ancillary staff at the Western Infirmary as part of the patient's routine clinical care. All samples were processed in the clinical laboratories or the Western Infirmary and Gartnavel General Hospital.

3.5 Coronary angiography and percutaneous coronary intervention

Coronary angiography and PCI were carried out according to clinical indication and the primary operator's discretion. Procedural information was collected retrospectively by RG. All data was taken from the cardiac catheterization laboratory database report (Minerva™) or from the clinical case record. Data collected included the extent of atherosclerotic CAD, whether PCI was undertaken and the number of lesions treated. For each lesion the reference vessel diameter, lesion morphology (de novo, in-stent restenosis, calcification, occlusion), type of stent used and total length of stent deployed were recorded. The use of GPIIb/IIIa antagonists was entirely at the discretion of the operator but their use was also recorded. Any additional features of the intervention documented by the operator including dissection, side branch occlusion or patient symptoms during the procedure were also noted.

3.6 Troponin measurement pre and post percutaneous coronary intervention

All patients had a sample collected for the assessment of troponin I at the time of platelet assay sample collection immediately prior to coronary angiography. Patients undergoing

intervention were reviewed the day following the procedure. Further blood samples were collected either by RG or ancillary medical staff for the assessment of post-procedural troponin elevation. Post-procedural troponin samples were collected between 12 and 24 hours post-procedure.

3.7 Funding of the research

Several sources of funding supported this research. The main research project was funded by a British Heart Foundation Junior Research Fellowship (FS/06/031). However, for the pilot study work, funding was also obtained from the North Glasgow University Hospitals NHS Trust West Research Endowment Fund and an unrestricted educational grant from Merck, Sharpe and Dohme US Medical School Grant Committee. Collaboration with Leicester University was also facilitated by support from the Graham Wilson Travel Scholarship Award, West Glasgow University Hospitals NHS Trust.

3.8 Conduct and monitoring of the research

All studies were monitored by the West Glasgow University Hospitals NHS Trust Research and Development Department and were approved by the West Ethics Committee, Western Infirmary, Glasgow (06/S0709/11).

3.9 Statistical methods

Statistical methods are described with each study.

Chapter 4: Variation in the antiplatelet effects of aspirin assessed by measurements of thromboxane, whole blood platelet aggregation and VerifyNow Aspirin

Summary

Following review and discussion of the published data available at the inception of this research as outlined in Chapter 1, I wished to investigate the measurement of aspirin response in a cohort of patients with CAD undergoing PCI. I specifically planned to investigate assays that may offer a rapid, point-of-care option for the clinical environment. However, I was also keen to investigate whether any assay may be valid in the presence of additional clopidogrel therapy. In light of these factors and the features and limitations described in Chapters 1, 2 and 3, I investigated TxB2 concentrations in serum and plasma, WBPA in response to collagen and TRAP and VerifyNow Aspirin. This chapter describes these investigations in the form of 3 separate studies.

Study 4.1 investigates variation in TxB2 concentration in 123 patients taking aspirin alone. In a subset of 83 patients I also assess this measurement following the addition of clopidogrel therapy to investigate what effect this has and whether this assay may be a valid reflection of aspirin response in patients on dual antiplatelet therapy. In the remaining 40 patients I also tested for aspirin response using the VerifyNow Aspirin (AA) assay for comparison with TxB2 levels.

Study 4.2 follows on from the data of Study 4.1 and investigates the measurement of TxB2 concentrations in plasma and serum in a larger cohort of 323 patients attending for coronary angiography who were established on dual antiplatelet therapy with aspirin and clopidogrel. This patient cohort comprised 83 patients recruited as part of Study 4.1 and an additional 240 patients already established on DAPT at the time of recruitment (Figure 47). In addition, I also measured WBPA in response to collagen and TRAP in these patients to investigate whether this relatively simple and inexpensive assay may offer a practical solution to measuring aspirin response in the cardiac catheterisation laboratory.

Finally, Study 4.3 presents a series of small studies that were done as part of the pilot work of this project investigating the potential utility of VerifyNow Aspirin for measuring response to aspirin.

Study 4.1: Variation in thromboxane B2 in serum and plasma in patients taking regular low dose aspirin with and without clopidogrel therapy

4.1.1 Introduction

The antiplatelet effects of aspirin are mediated largely through inhibition of the production of TxA_2 . As discussed in chapter 1, previous work has suggested that thromboxane production may remain elevated despite aspirin therapy. This may reflect a 'resistance' to the therapeutic effects of this medication and may have significant clinical sequelae. As highlighted previously, no test has been shown to reflect consistently aspirin response, and all currently available tests are influenced by the addition of a second antiplatelet agent such as clopidogrel.

Using the methodology outlined in Chapter 3, I sought to investigate whether measurement of the residual ability of platelet to generate thromboxane, $[\text{TxB}_2]_{\text{S-P}}$ would confirm variation in response to aspirin therapy in a cohort of routine patients attending our centre. I investigated whether any patient characteristics might affect the residual level of thromboxane production. Crucially, I examined whether the introduction of clopidogrel therapy would influence the level of $[\text{TxB}_2]_{\text{P}}$, $[\text{TxB}_2]_{\text{S}}$ or $[\text{TxB}_2]_{\text{S-P}}$ and, if so, whether the measurable response to clopidogrel influenced the magnitude of this effect.

4.1.2 Patient selection

Patients with suspected CAD attending for angiography with a view to PCI were sampled to assess $[\text{TxB}_2]_{\text{S}}$ and $[\text{TxB}_2]_{\text{P}}$ prior to the introduction of clopidogrel. Previously described exclusion criteria were applied. All patients had been taking aspirin 75mg/day for at least 5 days and were naive to clopidogrel at the time of recruitment. The remaining selection criteria are those described in section 3.1.

Following recruitment, patients were started on clopidogrel. In a subgroup of patients a second sample was taken following the introduction of clopidogrel. As a reflection of the available data outlined in Chapter 2 and in keeping with the local practice in our centre, a patient was

deemed to have been adequately treated with clopidogrel if they had received a loading dose of 600mg > 2 hours previously, a 300mg loading dose >24hrs previously or had received 75mg/day of clopidogrel for at least 5 days.

In a separate subgroup of patients samples were also collected for the assessment of aspirin response using VerifyNow Aspirin. Figure 24 summarises the design of study 4.1.

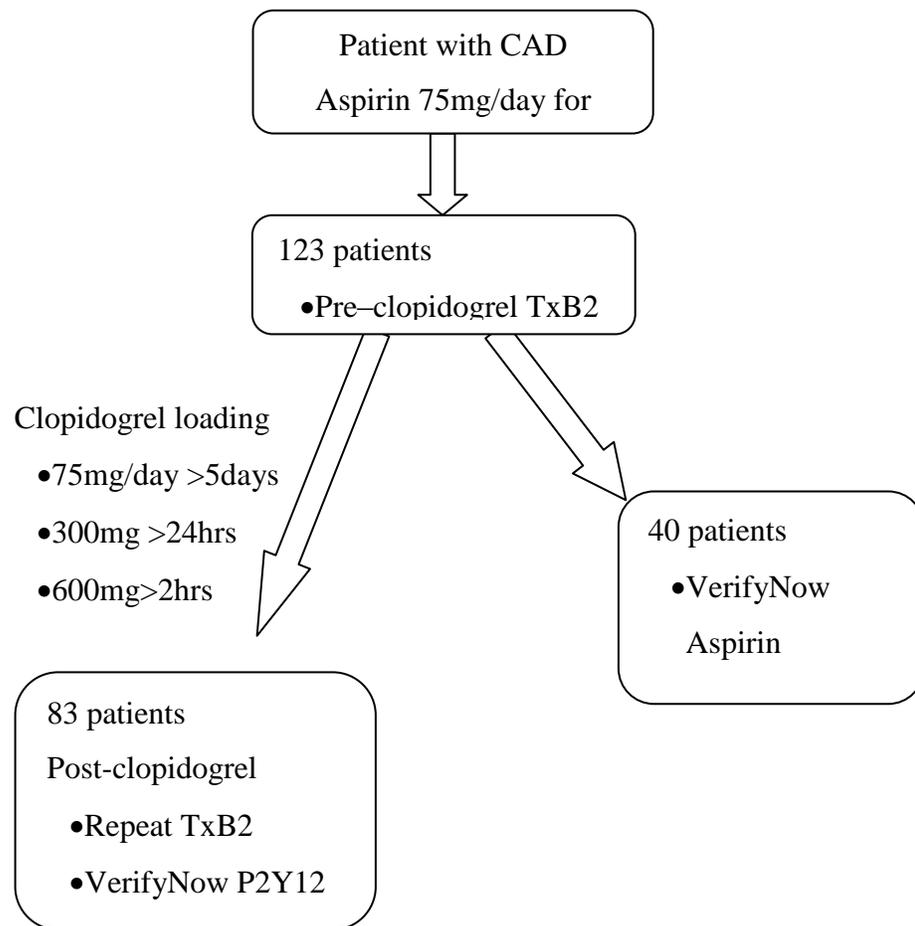


Figure 24: Flow diagram illustrating the design of study 4.1

4.1.3 Sample acquisition and processing

Whole blood samples were taken from a large calibre vein by RG. An initial 3.5ml 3.2% sodium citrate tube was filled and discarded followed by a further 3.5ml 3.2% sodium citrate tube and lastly a 3ml glass vacutainer tube containing thrombin in strict sequential order. The samples were immediately mixed by gently inverting 5 times. The 3.5ml citrated tube and thrombin samples were processed to generate frozen samples of plasma and serum (see Section 3.3).

Following the introduction of clopidogrel, a second series of samples was collected either by venepuncture or following the insertion of an arterial sheath at the time of coronary angiography (see Section 3.3). In addition to the series of samples described above, further 3.8ml and 2ml (supplied by Accumetrics) 3.2% sodium citrate tubes were collected for the assessment of response to clopidogrel. The 3.8ml tube was set aside for the assessment of VASP-phosphorylation within 24 hours. After 30 minutes, the 2ml citrated tube was used to undertake clopidogrel response testing using VerifyNow P2Y12. All TxB2 samples and assays were processed according to the protocol described in Section 3.3.

4.1.4 Statistical methods

This was an exploratory study to assess the variation in thromboxane concentrations in this group of patients both before and after the introduction of clopidogrel. The distribution of results are summarised as the median, interquartile range and range of values for each assay. Negative values of $[TxB2]_{S-P}$ were excluded on the basis of biological implausibility. TxB2 concentrations showed a strongly positively skewed distribution. Logarithmic transformations produced a distribution of concentrations approaching normality. This transformed data was used in simple linear regression analysis to investigate whether any baseline characteristic was associated with $[TxB2]_{S-P}$. Patients characteristics with a significant correlation were subsequently included in a stepwise, multiple regression model. Correlations between log transformed $[TxB2]_P$, $[TxB2]_S$ and $[TxB2]_{S-P}$ before and after clopidogrel, and between $\log_{10}[TxB2]_{S-P}$ and VerifyNow Aspirin data were calculated using Pearson's correlation coefficient. Differences between log transformed $[TxB2]_P$, $[TxB2]_S$ and $[TxB2]_{S-P}$ before and

after clopidogrel were investigated using paired sample t-tests. Significance was taken at the $p < 0.05$ level for all statistical tests. Assuming a normal distribution of log transformed concentrations of TxB2 with a standard deviation of 0.34, a sample size of 70 patients should have an 80% power to detect a difference between thromboxane concentration before and after clopidogrel of 0.115. Thus to allow for a margin of error in collection and sampling, I sought to recruit 80 patients with samples before and after clopidogrel.

4.1.5 Results

One hundred and twenty-three patients were recruited to the study. The demographics of these patients are shown in Table 3. A summary of thromboxane concentrations before the introduction of clopidogrel is given in Table 4. Four paired thromboxane samples prior to the introduction of clopidogrel were erroneously processed. A further 4 samples produced plasma levels of TxB2 that were higher than those of serum. This was deemed to be biologically implausible and hence these were excluded leaving 115 paired serum and plasma samples prior to the introduction of clopidogrel for analysis. In 36/115 patients (31%), $[TxB2]_{S-P}$ was greater than 1000pg/ml with 5 patients (4%) having $[TxB2]_{S-P} > 10000$ pg/ml. Seven out of 115 patients (6%) had $[TxB2]_P > 1000$ pg/ml. Of these 7 patients 6 also had $[TxB2]_{S-P} > 1000$ pg/ml.

	N=123	Linear Regression (N=115)*	
		Coefficient (95% CI)	p
Patient characteristic			
Age(yrs)			
Mean±SD	64.4 ± 10.2	-0.015 (-0.025 – -0.006)	0.002
Male(%)	81(66)	0.003 (-0.211 – 0.216)	0.980
BMI(kg/m²)			
Mean±SD	27.7 ± 4.4	0.020 (-0.003 – 0.043)	0.084
Ethnicity			
Caucasian(%)	121(98.4)	-	-
SE Asian(%)	2(1.6)	-	-
Medical History			
Hypertension(%)	69(56.1)	-0.161 (-0.362 – 0.041)	0.117
Diabetes(%)	17(13.8)	-0.084 (-0.377 – 0.209)	0.570
Family History(%)	61(49.6)	0.028 (-0.173 – 0.230)	0.781
Dyslipidaemia(%)	81(65.9)	-0.051 (-0.265 – 0.162)	0.635
Previous TIA/CVA(%)	13(10.6)	-0.071 (-0.397 – 0.255)	0.666
Previous MI(%)	33(26.8)	0.098 (-0.130 – 0.326)	0.397
Previous CABG(%)	12(9.8)	-0.153 (-0.479 – 0.173)	0.354
Smoking Status			
Current	25(20.3)	0.281 (0.035 – 0.527)	0.026
Medication			
Statin	107(87)	0.300 (0.005 – 0.596)	0.046
Beta Blocker	89(72.4)	0.003 (-0.224 – 0.229)	0.982
ACE Inhibitor	47(38.2)	-0.063 (-0.271 – 0.144)	0.545
Calcium Channel Blocker	49(40)	0.064 (-0.159 – 0.287)	0.570
Blood parameters			
Haemoglobin (Mean±SD)	13.8 ± 1.4	-0.011 (-0.122 – 0.100)	0.838
Platelets(Mean±SD)	253 ± 69	0.002 (<0.001 – 0.004)	0.188
Creatinine(Mean±SD)	92 ± 20	-0.007 (-0.013 – 0.001)	0.030

Table 3: Patient characteristics for study 4.1.

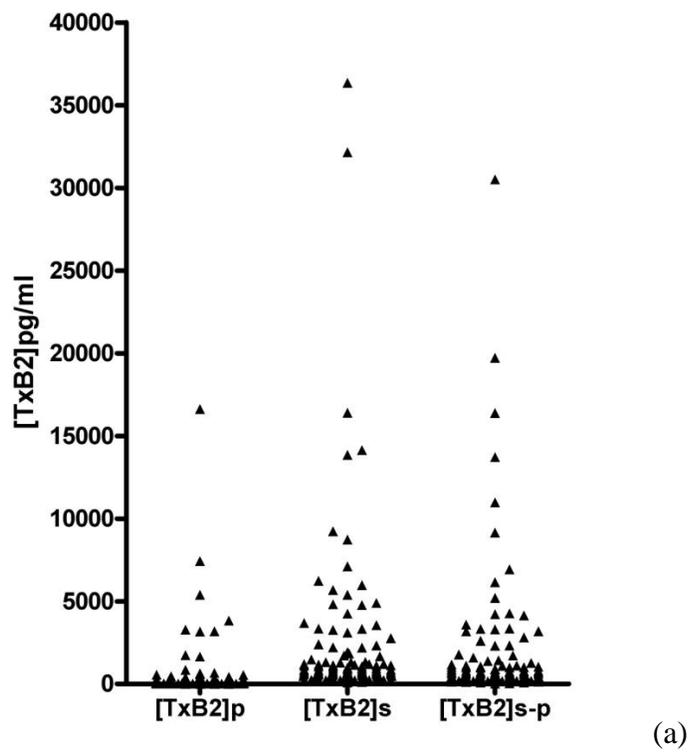
*Dependent variable [TxB2]_{S-P}

Of the original 123 patients, we were able to obtain a second series of samples for the estimation of Tx_{B2} following the introduction of clopidogrel in 83 patients. Of these, 4 samples were erroneously processed and were not able to be analysed. In addition, 8 of these samples produced plasma concentrations of Tx_{B2} greater than that in the serum sample and were again excluded on the basis of biological implausibility. This left a total of 71 patients with paired Tx_{B2} concentrations after clopidogrel therapy. Of these, 2 patients did not have a measurable pre-clopidogrel sample and were thus excluded from this analysis leaving a total of 69 patients with matched samples before and after clopidogrel. The summary statistics of the data are shown in Table 4. Forty-two of the 69 patients (61%) had received clopidogrel 75mg/day for >5days, 18/69 (26%) 300mg >24hrs previously and 9/69 (13%) 600mg >2hours but < 24hrs prior to sampling. Fifteen of the 69 samples (22%) were collected from the arterial sheath at the time of angiography.

	Pre-Clopidogrel (n=115)			Post-Clopidogrel (n=69)		
	Median	IQR	Range	Median	IQR	Range
[Tx _{B2}] _P (pg/ml)	110	148	9 – 16620	136	138	16 - 5301
[Tx _{B2}] _S (pg/ml)	773	1018	85 - 36344	673	701	200 - 36344
[Tx _{B2}] _{S-P} (pg/ml)	646	842	19 - 30511	491	612	119 - 31043
VerifyNow P2Y12 (%inhibition)	-	-	-	45	41	6 - 97
VASP- PRI (%activation)	-	-	-	58	31	1 - 88
	(n=40)					
VerifyNow Aspirin (ARU)	428	94	366 - 653	-	-	-

Table 4: Summary statistics of [Tx_{B2}] in plasma and serum before and after clopidogrel therapy and VerifyNow Aspirin.

All concentration frequency distributions showed a strong positive skew and kurtosis (Figure 25 (a)). To address this, several transformations were attempted. A logarithmic transformation produced a data set for all concentrations which approached a normal distribution (Figure 25 (b)) although not according to the strict Kolmogorov-Smirnov or Shapiro-Wilk criteria. Further analysis of the distribution of [TxB2] in patients taking both aspirin and clopidogrel is given in as part of Study 4.2 using a larger dataset of 323 patients taking both aspirin and clopidogrel.



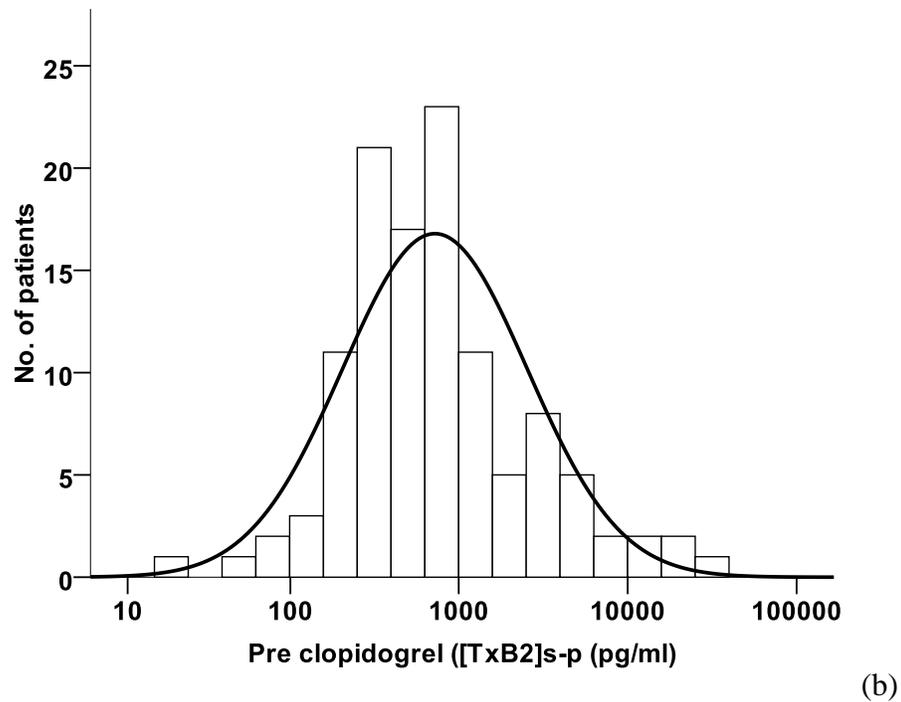


Figure 25: Pre clopidogrel [TxB₂]

(a) Distribution of [TxB₂]_P, [TxB₂]_S and [TxB₂]_{S-P} (n=115) (b) [TxB₂]_{S-P} frequency distribution using a log scale

The log₁₀ transformed data of pre-clopidogrel [TxB₂]_{S-P}, were used to investigate whether any baseline characteristics would predict the residual ability of platelets to generate thromboxane despite aspirin therapy. Univariate linear regression showed significant associations between log₁₀[TxB₂]_{S-P} and age, statin therapy, current smoking and creatinine level (Table 3). When these variables were subsequently included in a forward stepwise multiple regression analysis, age alone was the only independent negative predictor of [TxB₂]_{S-P} prior to the introduction of clopidogrel therapy (Table 5).

Model		Unstandardized Coefficients ^a		Standardized Coefficients ^a	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.827	0.309		12.374	0.000
	Age	-0.015	0.005	-0.286	-3.173	0.002

a. Dependent Variable: $\text{Log}_{10}([\text{TxB2}]_{\text{S-P}})$

Table 5: Multiple regression model of Pre Clopidogrel $[\text{TxB2}]_{\text{S-P}}$ and patient characteristics

A subgroup of 40 patients was tested for response to aspirin using the VerifyNow Aspirin assay prior to the introduction of clopidogrel. Only 2 patients were deemed aspirin resistant ($\text{ARU} > 550$) according to this assay. Correlation between VerifyNow Aspirin ARU and $[\text{TxB2}]_{\text{S-P}}$ was poor (Figure 26). However, the patient with the highest residual generation of TxB2 (36,000pg/ml) also had the highest VerifyNow Aspirin ARU (653 ARU). The remaining patient deemed aspirin resistant by VerifyNow had a modest residual $[\text{TxB2}]_{\text{S}}$ of 1040pg/ml with a $[\text{TxB2}]_{\text{S-P}}$ of 980pg/ml.

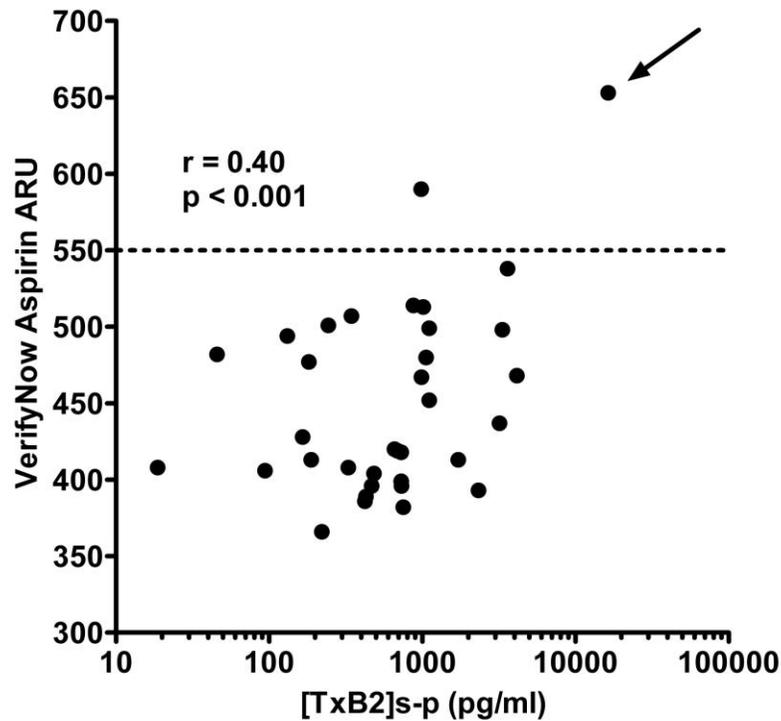


Figure 26: Correlation of $\log_{10}[\text{TxB2}]_{\text{S-P}}$ and VerifyNow Aspirin assays.

The highest $[\text{TxB2}]_{\text{S-P}}$ was seen in one of 2 patients deemed aspirin resistant by VerifyNow Aspirin (arrowhead).

There were 69 patients with paired samples of plasma and serum before and after the introduction of clopidogrel. Spearman's correlation coefficients are shown for each of the paired data sets (Figure 28). Similar results for Pearson's correlation coefficients were found using the \log_{10} transformed data; $\log_{10}[\text{TxB2}]_{\text{P}}$ ($r=0.863$, $p<0.001$), $\log_{10}[\text{TxB2}]_{\text{S}}$ ($r=0.827$, $p<0.001$), $\log_{10}[\text{TxB2}]_{\text{S-P}}$ ($r=0.778$, $p<0.001$) $[\text{TxB2}]_{\text{P}}$, $[\text{TxB2}]_{\text{P}}$, $[\text{TxB2}]_{\text{S}}$ and $[\text{TxB2}]_{\text{S-P}}$ all tended to be lower following the introduction of clopidogrel but using the \log_{10} transformed concentrations of TxB2, paired t-tests showed that only $[\text{TxB2}]_{\text{P}}$ was significantly reduced following the introduction of clopidogrel (Figure 27).

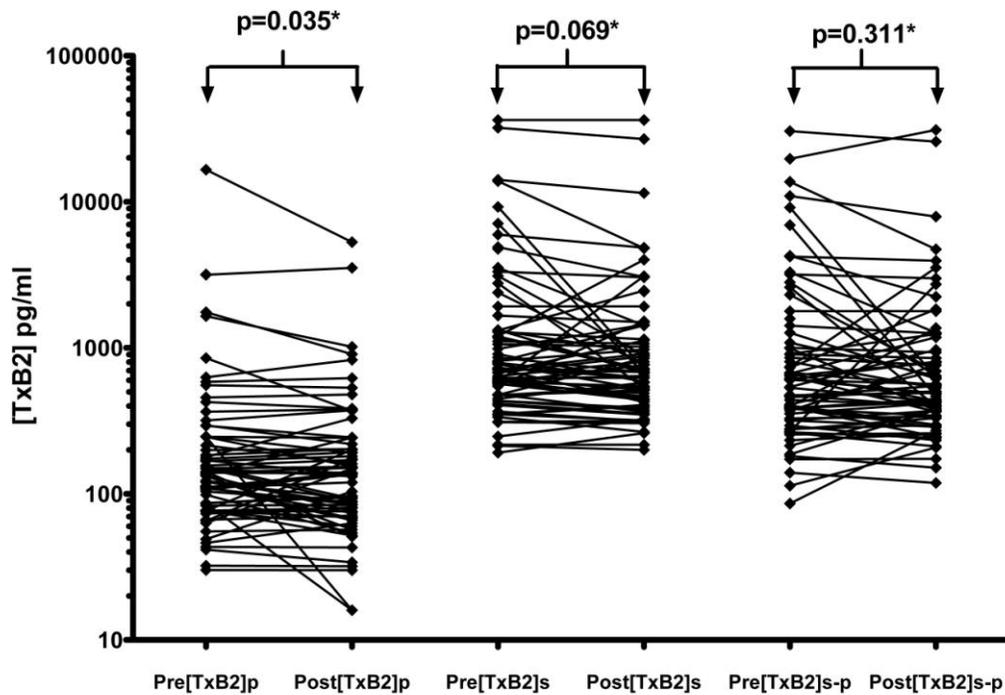
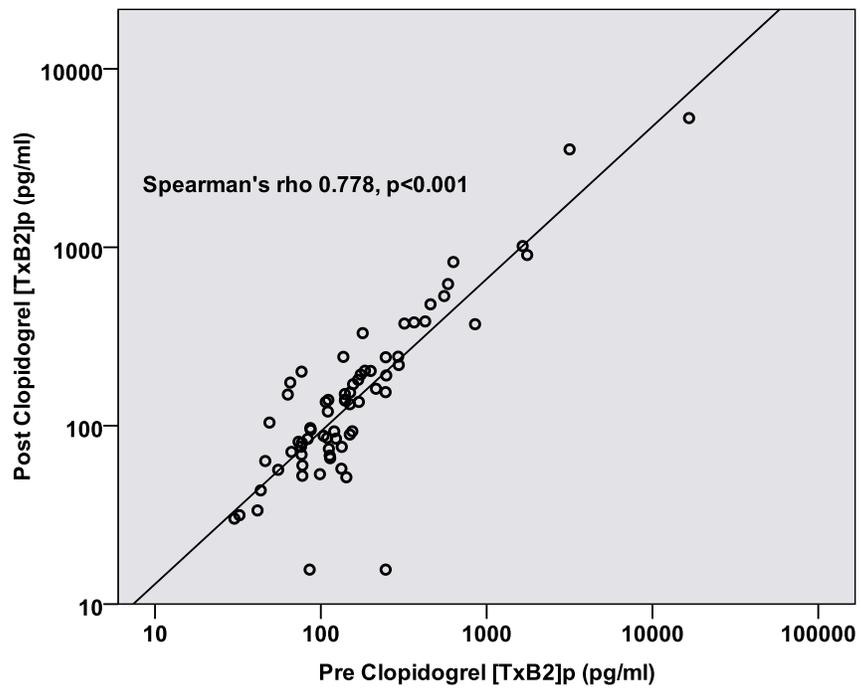


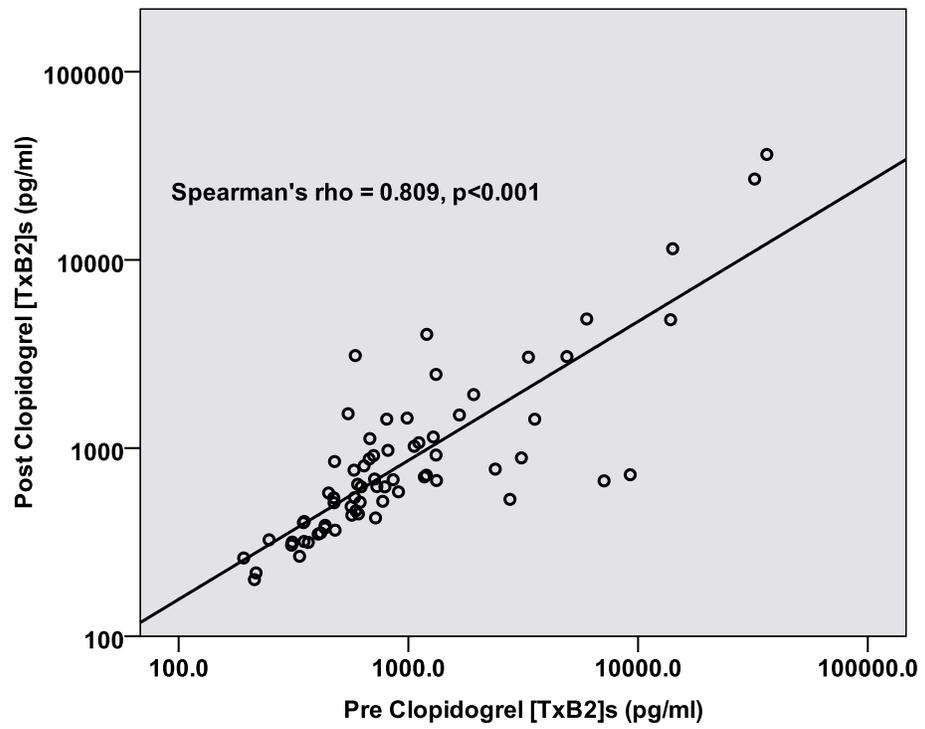
Figure 27: Paired samples of thromboxane B2 before and after the introduction of clopidogrel

*p-values are for paired sample t-test using \log_{10} transformed [TxB2]

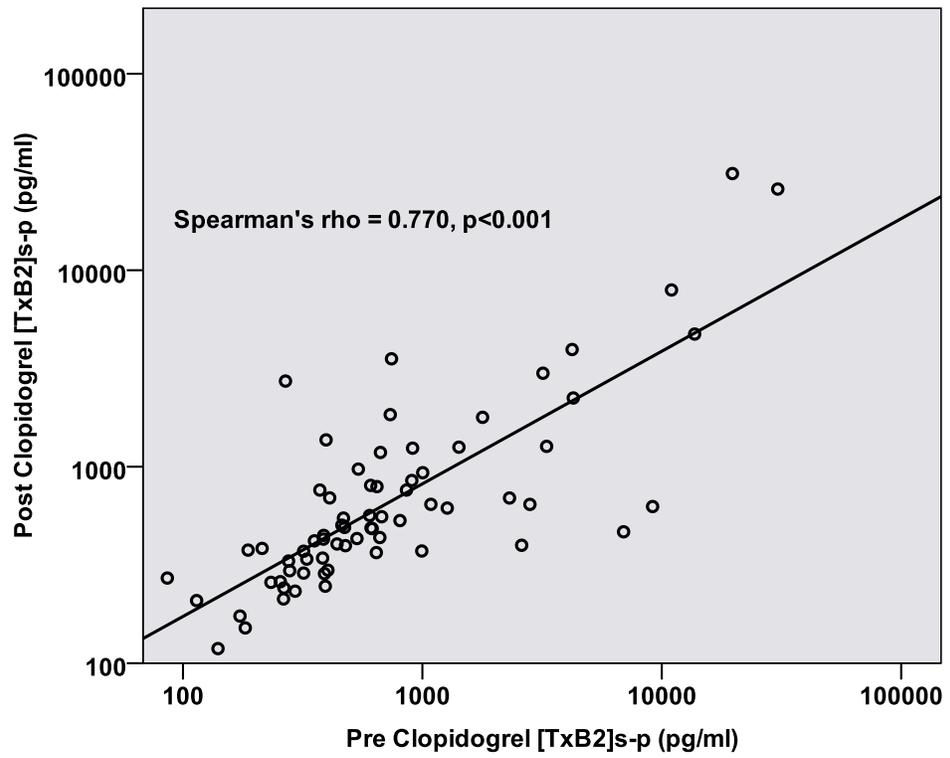
At the time of acquiring TxB2 samples following the introduction of clopidogrel, blood was also collected to allow the assessment of response to clopidogrel using both VASP-phosphorylation and VerifyNow P2Y12. The variation in clopidogrel response is summarised in Table 4. A linear regression model suggested that the magnitude of response to clopidogrel measured using either the VerifyNow P2Y12 assay or VASP-PRI assay does not effect the change in thromboxane B2 concentration (Δ TxB2) in either plasma, serum or their difference observed following the introduction this additional antiplatelet agent (Figure 29)



(a)



(b)



(c)

Figure 28: Spearman's rho correlation coefficients between [TxB2] before and after the introduction of clopidogrel

(a) [TxB2]_P, (b) [TxB2]_S, (c) [TxB2]_{S-P}

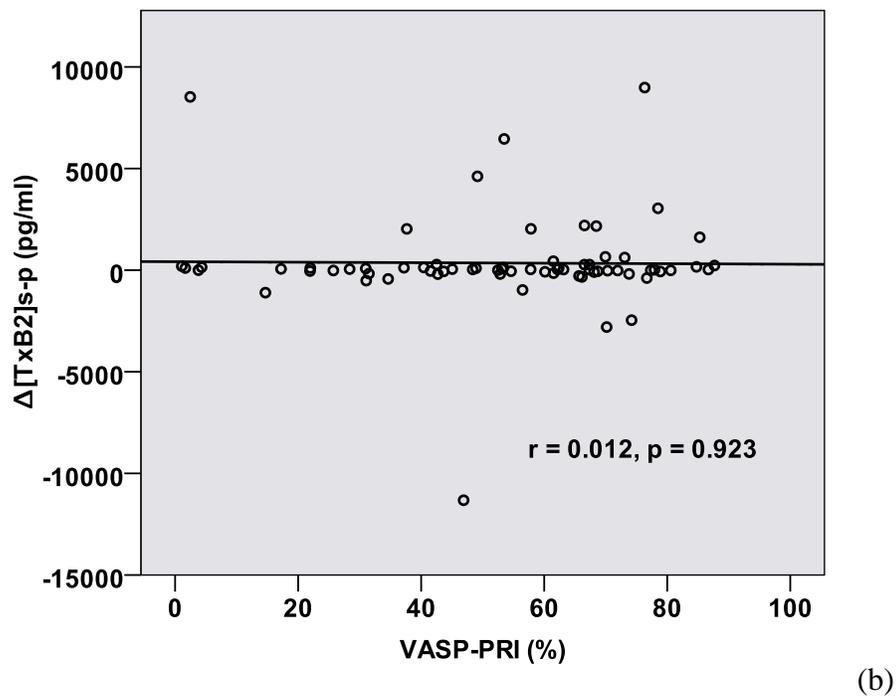
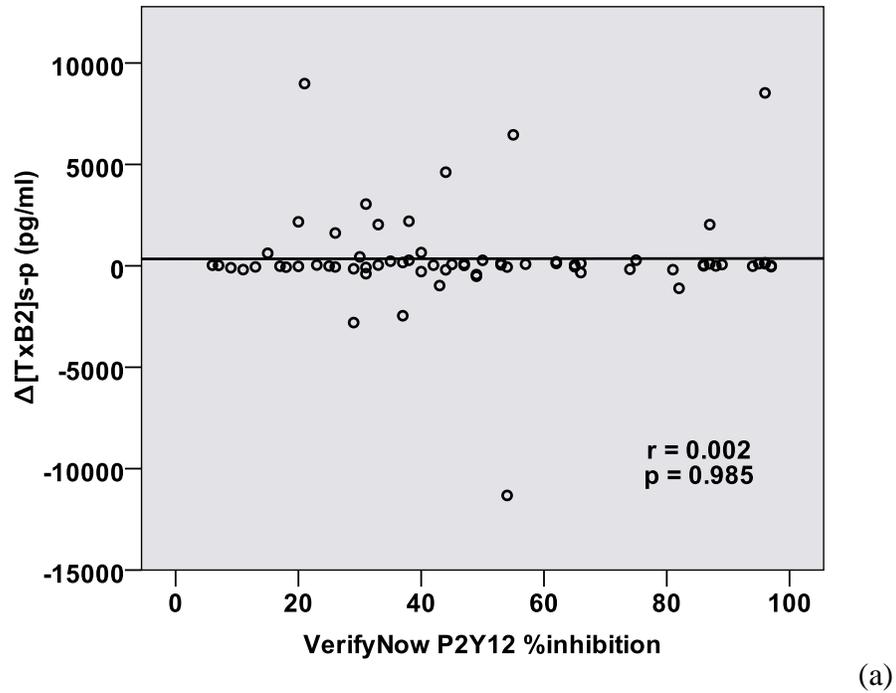


Figure 29: Change in [TxB2]_{s-p} following the introduction of clopidogrel vs clopidogrel response
The response to clopidogrel measured by (a)VerifyNow PY12 or (b)VASP-PRI does not influence change in concentration of TxB2 observed following the introduction of this additional antiplatelet agent

4.1.6 Discussion

This study investigated the variation in TxB2 concentration in patients with suspected CAD taking regular low dose aspirin therapy as a potential marker of reduced response to this therapy. In addition, it investigated whether this marker of antiplatelet effect may have some validity in this patient population following the introduction of clopidogrel, an additional antiplatelet agent.

One hundred and twenty-three patients were recruited prior to the introduction of clopidogrel therapy. The demographics of this population fit well with those seen in contemporary studies of CAD (Table 4) there being a large male preponderance, mean age of 64 years and an anticipated distribution of comorbidities (25). In addition, this patient group was well treated with contemporary cardiovascular therapies, including statins, ACEI and anti-anginal agents. Of these 123 patients, final concentrations of TxB2 in plasma and serum were available for analysis in 115 patients (93.5%).

The principle finding of this study was a measurable variation in concentration of TxB2 in both plasma and serum. These measured concentrations can be combined to produce a novel marker of the residual ability of platelets to generate thromboxane, namely [TxB2]_{S-P}. As has been previously demonstrated using [TxB2]_S, these concentrations are low compared with normal controls and, in keeping with previously published data, highly positively skewed (52,72,89).

It was found that 36 patients (31%) had [TxB2]_{S-P} >1000pg/ml suggesting residual ability to generate thromboxane despite aspirin therapy. In 5 of these patients (4%) [TxB2]_{S-P} exceeded 10,000pg/ml. These levels of TxB2 are approaching those seen in stimulated serum samples from normal, non-aspirinated individuals and would suggest either non-compliance or non-response to aspirin therapy. Although an order of magnitude less, levels of [TxB2]_{S-P} >1000pg/ml may have important clinical implications due to the amplification of platelet activation that occurs through several other pathways including ADP receptor stimulation. In this regard, the near normalisation of [TxB2]_{S-P} concentrations following logarithmic

transformation is interesting and may be helpful in determining a threshold of clinically relevant residual thromboxane generation.

An additional finding of importance was a poor correlation between [TxB2]_{S-P} and VerifyNow Aspirin. This highlights the difficulty of measuring response to aspirin in clinical practice and is in keeping with data from other groups showing poor correlation between tests of aspirin response (44,70). As yet, we remain uncertain of the best test to use and the interpretation of meta-analyses suggesting association with increased cardiovascular risk are potentially confounded by the different tests used and poor correlation between tests.

Although not meeting strict criteria for normality, a logarithmic transformation approached normality and facilitated investigation of patient features that may influence this variation. Of the demographic characteristics recorded in our population, only age had an independent weak negative correlation with [TxB2]_{S-P}. This finding is in contrast to previously published data, using urinary 11-dehydro TxB2, suggesting an increased incidence of aspirin ‘resistance’ with increasing age (203) and a significant association between increased urinary 11-dehydro TxB2 concentration and female gender, hypertension, diabetes and current smoking. We did not replicate these findings in our dataset. No concurrent medication appeared to influence the distribution of the residual TxB2 concentration. Haemoglobin concentration and platelet count also had no effect.

I was able to repeat these assays in 83/123 patients following the introduction of clopidogrel therapy. Of these samples 69 (83.1%) were subsequently available for analysis. This data confirmed variation in TxB2 in both serum and plasma. Most importantly it offered some insight into the reproducibility of these assay results with strong and significant correlations between concentrations before and after clopidogrel (Figure 28). There appeared to be a trend towards a lower concentration of TxB2 following the introduction of clopidogrel therapy but only the change in [TxB2]_p was statistically significant.

This is the first data to demonstrate a test of the antiplatelet effects of aspirin that may be applicable in patients taking concomitant platelet P2Y₁₂ ADP receptor antagonists. To further support this, I investigated whether the magnitude of response to clopidogrel assessed using both a bedside assay (VerifyNow P2Y₁₂) and laboratory based assay (VASP-PRI) would have

any influence on the small change in concentration seen. This data was strongly against any such influence with no discernible effect detected (Figure 29). As newer, more potent and uniform ADP receptor antagonists have been introduced into clinical practice since this research was undertaken, it is encouraging that these assay results may remain relevant for patients taking aspirin and either prasugrel or ticagrelor.

We were unable to obtain paired concentrations in 8/123 patients (6.5%) prior to the introduction of clopidogrel and 12/83 (14.5%) following the introduction of clopidogrel. This suggests a degree of unreliability using this assay which would not be acceptable for routine use in clinical practice. It should be emphasised, however, that this was a new technique to our department and samples were transferred for processing from Glasgow to Leicester. These factors may have influenced the number of sample errors that occurred. To counteract act this problem and improve the reliability of our data a repeat set of samples could have been taken.

Despite the encouraging results, we have a relatively small dataset, particularly of samples before and after clopidogrel therapy, and further studies would be warranted to confirm the findings. In addition, although patients were questioned at the time of recruitment regarding compliance with their aspirin and clopidogrel therapies, no formal testing was undertaken to confirm this. Various methods exist to examine whether these findings can be influenced by the addition of ex-vivo aspirin (52,55). On reflection, preparing plasma samples with indomethacin, a non-selective COX enzyme inhibitor, added to the sample tube might have helped to reduce any further production of thromboxane during processing of the plasma sample.

Ultimately, the question remains whether these small, albeit reproducible, residual concentrations of TxB₂ that are generated following stimulation of platelets in patients taking both aspirin and clopidogrel therapy are of clinical significance. This was the basis for continuing to recruit a larger series of patients undergoing coronary angiography with a view to PCI. This dataset is reported in Study 4.2.

Study 4.2: Variation in thromboxane concentration and whole blood platelet aggregation in patients taking aspirin and clopidogrel

4.2.1 Introduction

If variation in aspirin response is an important clinical entity then the greatest impact is likely to be in those patients with known cardiovascular disease. In particular, we recognise the critical importance of dual antiplatelet therapy in preventing thrombotic complications in patients undergoing PCI. At the time of PCI, most patients in European centres have already been commenced on dual antiplatelet therapy. In addition, data from the CURE study supports the early use of clopidogrel following an ACS presentation in order to reduce the risk of cardiovascular events (204). This makes the recruitment of patients in these settings naive to clopidogrel very difficult as has been demonstrated by recent studies investigating alternative ADP receptor antagonists that have permitted randomisation following the introduction of clopidogrel (25). Thus a test of aspirin response that remains valid despite additional clopidogrel therapy would potentially be of great value. In Study 4.1, I demonstrated that the residual concentrations of TxB₂ following the introduction of clopidogrel correlated strongly with those on aspirin alone. In light of this, I sought to assess the residual concentrations of TxB₂ in plasma and serum in a larger cohort of patients with suspected CAD established on dual antiplatelet therapy.

The assessment of [TxB₂] is laboratory based and the currently available assays would not allow this to be done as a point-of-care test. No assay has previously been reported as accurately assessing aspirin response in patients on dual antiplatelet therapy. Whole blood platelet aggregometry could be applied as a point of care assay, providing a rapid assessment of platelet aggregation. Thus, in the same cohort of patients I sought to investigate impedance aggregometry in response to collagen and TRAP to assess potential utility for assessing response to aspirin and clopidogrel. This also provided an opportunity to compare these results with the generation of TxB₂.

4.2.2 Patient selection

Patients with suspected CAD attending for angiography with a view to PCI were sampled to assess the levels of TxB₂ in serum and plasma. Previously described inclusion and exclusion criteria were applied (Chapter 3.1). All patients had been taking aspirin 75mg/day for at least 5 days and had been introduced onto clopidogrel. As described previously, a patient was deemed to have been adequately treated with clopidogrel if they had received a loading dose of 600mg > 2 hours previously, a 300mg loading dose >24hrs previously or had received 75mg/day of clopidogrel for at least 5 days.

Demographic details were recorded for each patient together with details of all additional pharmacological therapies. Patients were questioned directly regarding compliance with both aspirin and clopidogrel and were excluded if they had been non-compliant over the previous 7 days.

4.2.3 Sample acquisition and processing

Blood samples were collected either by venepuncture of a large calibre vein by RG or following the insertion of an arterial sheath at the time of coronary angiography, as described in Section 3.2. An initial 3.5ml 3.2% sodium citrate tube was collected and then discarded followed by a further 3.5ml 3.2% sodium citrate tube and a 3ml glass vacutainer tube containing thrombin in strict sequential order. All samples were immediately mixed by gently inverting 5 times. The 3.5ml citrated tube and thrombin samples were processed to release frozen samples of plasma and serum. Thromboxane B₂ assays were carried out according to the protocol described in Section 3.3.2. WBPA was performed according to the protocol described in Section 3.3.3. For this study, collagen 2µl (WBPA-Col 2), 5µl (WBPA-Col 5) and TRAP 5µmol (WBPA-TRAP) were used as agonists.

4.2.4 Statistical methods

This study was part of a larger project to assess the variation in both aspirin and clopidogrel response in patients on dual antiplatelet therapy undergoing coronary intervention. Our power calculations were based on contemporary data published at the time suggesting that there may be up to a 4-fold difference in periprocedural myocardial necrosis assessed by troponin elevation between those patients in the highest and lowest quartiles of clopidogrel response. The details of this power calculation are given in Chapter 6. The aim was to recruit 240 patients undergoing coronary intervention.

As in Study 4.1, the distribution of all assays are summarised as the median, interquartile range and range of values for each assay. Negative values of $[TxB2]_{S-P}$ were excluded on the basis of biological implausibility. Again, logarithmic transformation of thromboxane data approached normality and thus was used in a stepwise, multiple regression analysis to investigate any significant associations between $[TxB2]$ the demographic characteristics of the patients, concurrent medications and blood parameters. Similar regression analysis was used to investigate any association with WBPA results. Significance was taken at the $p < 0.05$ level.

4.2.5 Results

A total of 323 patients were recruited to the study. The demographic details of these patients are given in Table 6.

Patient characteristic	N=323	*log ₁₀ ([TxB2] _{S-P}) (N=304)		*WBPA-Col 2 (N=320)		*WBPA-Col 5(N=320)		*WBPA-TRAP 5 (N=320)		
		Std. coefficient	p	Std. coefficient	p	Std. coefficient	p	Std. coefficient	p	
Age (mean±SD)	64.3 ± 10.2	-0.137	0.016	0.174	0.012	-	-	-	-	
Male gender(%)	223 (69)	-0.142	0.011	-	-	-	-	-	-	
BMI(kg/m ² ; mean±SD)	28.1 ± 5.0	0.205	<0.001	0.112	0.048	-	-	0.121	0.045	
Hypertension(%)	170 (52.6)	-0.118	0.039	-	-	-	-	-	-	
Diabetes(%)	54 (16.7)	-	-	-	-	-	-	-	-	
Current smoker	70 (21.7)	-	-	-	-	-	-	-	-	
Medication										
Statin	292 (90.4)	-	-	-	-	-0.222	0.001	-	-	
Beta blocker	250 (77.4)	-	-	-	-	-	-	-	-	
ACE inhibitor	170 (52.6)	-0.125	0.029	-	-	-	-	-	-	
PPI(%)	86 (26.6)	-	-	-	-	-	-	0.129	0.040	
Blood parameters (Mean±SD)										
Haemoglobin (g/dl)	13.7 ± 1.5	-	-	-	-	-	-	-0.175	0.005	
Platelet count (x10 ⁹ /l)	251 ± 68	-	-	0.230	0.001	0.173	0.010	0.378	<0.001	

Table 6: Patient characteristics for Study 4.2 including significant predictors of each platelet function assay according to a multiple regression analysis

*Dependent variable for stepwise multiple regression analysis

A summary of the distribution of [TxB2] in plasma, serum and their difference is given in Table 7 and graphically depicted in Figure 30. Nine samples were erroneously processed and were excluded. In addition a further 10 samples produced plasma concentrations of TxB2 greater than those in the corresponding serum sample and were excluded on the grounds of biological implausibility. This left a total of 304 cases for subsequent analysis. Ninety-one of the 323 patients (30%) had a [TxB2]_{S-P} >1000pg/ml with 7/323 patients having [TxB2]_{S-P} >10000 pg/ml. Ten of the 323 patients had a [TxB2]_P >1000 pg/ml. All of these patients had [TxB2]_{S-P} >4500 with 5 patients having [TxB2]_{S-P} >10000pg/ml.

	Median	IQR	Range
[TxB₂]_P (pg/ml)	125	134	15 – 11400
[TxB₂]_S (pg/ml)	766	980	110 – 36344
[TxB₂]_{S-P} (pg/ml)	628	881	52 – 31043
WBPA Collagen 2µl (Ohms)	8.5	5.0	0.5 – 18.5
WBPA Collagen 5µl (Ohms)	13.5	3.0	4.5 – 23
WBPA TRAP 5µmol (Ohms)	12.5	5.0	4.0 – 28.5

Table 7: Summary statistics of TxB2 concentrations and WBPA in response to collagen and TRAP.

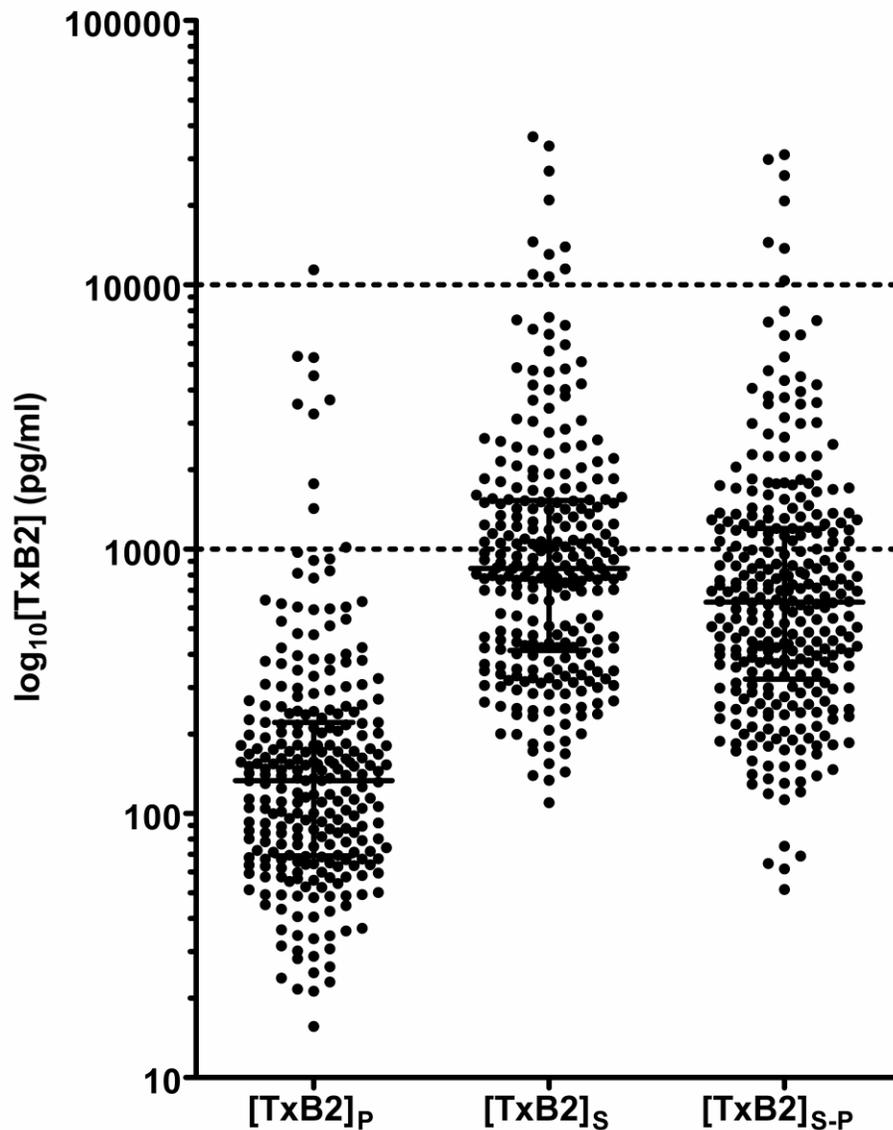


Figure 30: Illustrative distribution column plot for concentration of TxB2 including median and IQR.

Upper dotted lines represents [TxB2]_{S-P} >10000pg/ml which would suggest non-compliance or non-response to aspirin. Lower dotted line represents [TxB2]_{S-P} >1000pg/ml suggesting significant residual thromboxane generation.

In keeping with previously published data and the findings of study 4.1, the concentrations of thromboxane show a marked positive skew. Once again several transformations were applied in an attempt to normalise the data for subsequent analysis. As Figure 31 demonstrates, and in keeping with the findings of Study 4.1, logarithmic transformation produced a frequency distribution curve approaching normality. This transformed data was used in a backward

stepwise multiple regression model to investigate whether any patient characteristics influenced the extent of residual [TxB2] generation.

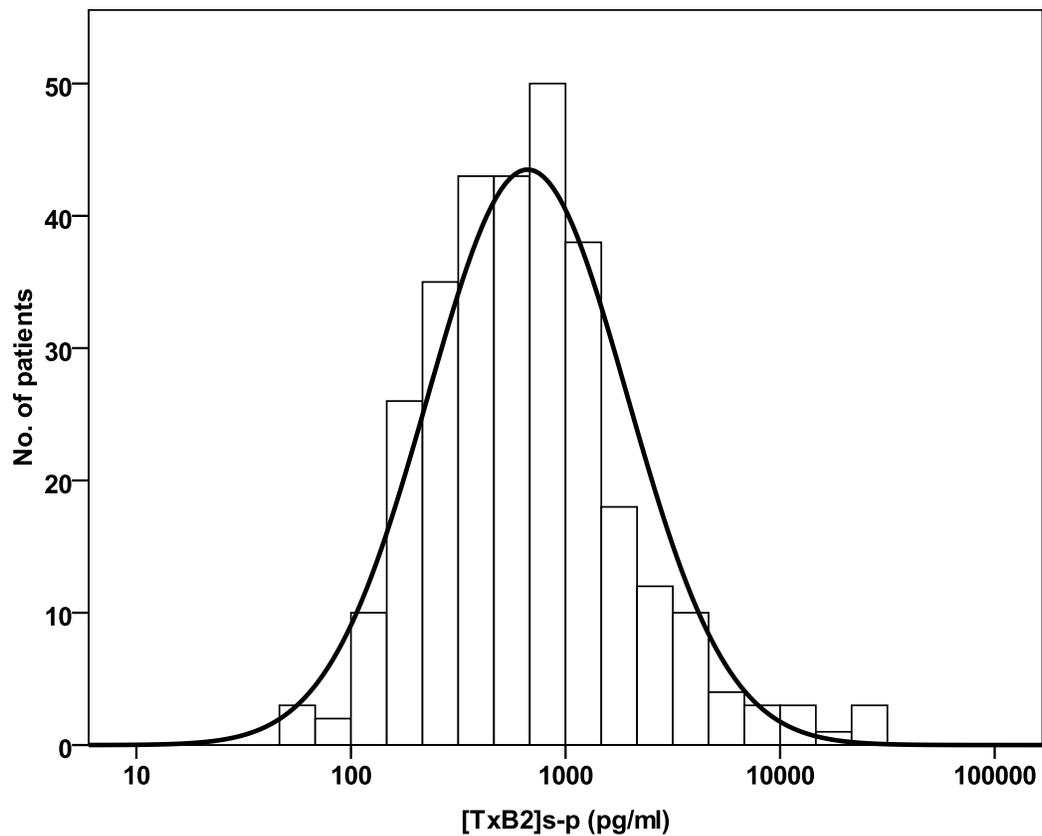


Figure 31: Frequency distribution of [TxB2]_{S-P} on a logarithmic scale.

Table 6 shows the output from the backward stepwise multiple regression model using $\log_{10}[\text{TxB2}]_{S-P}$ as the dependent variable and demographic details, medications and blood parameters as predictors. This shows weak but significant effects with a positive association with BMI and negative association with age (as was shown in Study 4.1), male gender and hypertension.

Similar analysis using $\log_{10}[\text{TxB2}]_P$ as a dependent variable found a statistically significant negative correlation with male gender (std. coefficient = - 0.161, p 0.005) but no other demographic predictor. $\text{Log}_{10}[\text{TxB2}]_S$ showed a positive correlation with BMI (std coeff. = 0.180, p=.001) and negative correlations with male gender (std. coeff. = -0.136, p= .016) and age (std. coeff. = -0.125, p=.027) similar to the findings of $[\text{TxB2}]_{S-P}$.

I assessed whether there were any significant differences between the samples according the method of sample acquisition. Neither the mean nor variance between the two methods of acquisition was significantly different for $\log_{10}[\text{TxB2}]_p$, $\log_{10} [\text{TxB2}]_s$ or $\log_{10} [\text{TxB2}]_{s-p}$ (Table 8).

	Levene's Test for Equality of Variances		t-test for Equality of Means			
	F	p	t	p (2-tailed)	Mean Diff.	SE Diff.
$\text{Log}_{10}[\text{TxB2}]_p$	0.166	0.684	-0.594	0.553	-0.0323	0.054
$\text{Log}_{10}[\text{TxB2}]_s$	0.177	0.674	-0.257	0.798	-0.0143	0.055
$\text{Log}_{10}[\text{TxB2}]_{s-p}$	0.895	0.345	-0.199	0.842	-0.0119	0.059

Table 8: A comparison of [TxB2] in plasma and serum in samples acquired by venepuncture or by aspiration from an arterial sheath prior to coronary angiography.

A summary of WBPA results following the addition of collagen 2 μ l, collagen 5 μ l and TRAP 5 μ mol is given in Table 7. Three hundred and twenty of the 323 samples (99.1%) were successfully processed. The distribution of all three assay results approached normality although did not meet the strict Kolmogorov-Smirnov or Shapiro-Wilk criteria. Figure 32 shows the frequency distribution histogram of change in impedance following the addition of 2 μ l collagen (WBPA-Col 2) to whole blood.

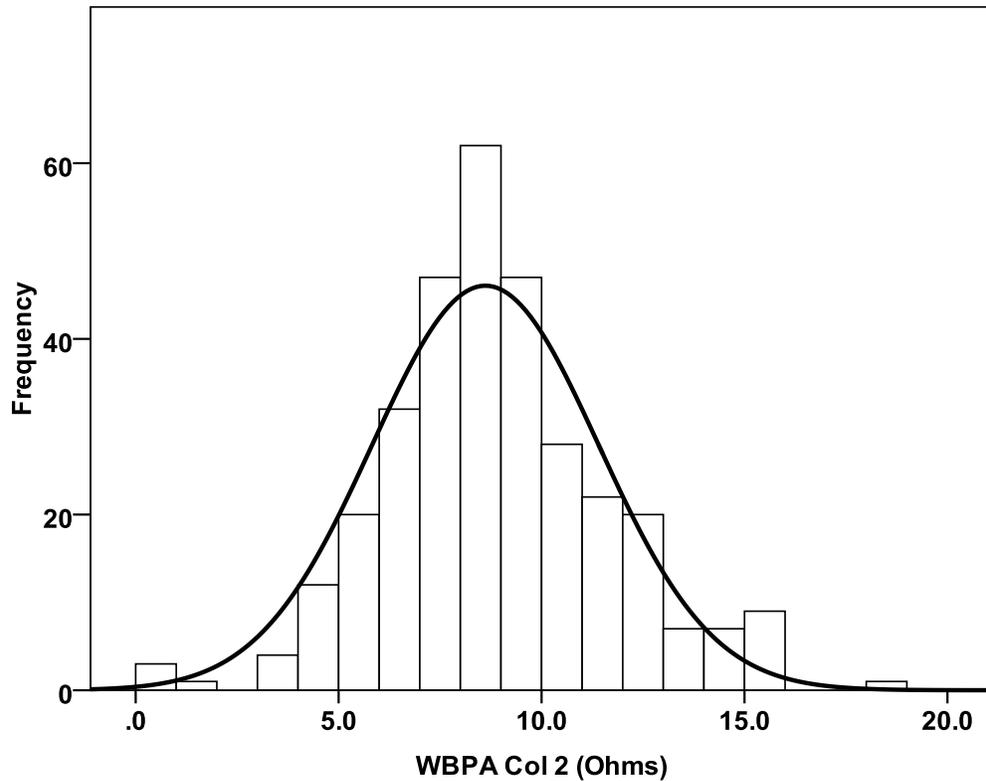


Figure 32: Frequency distribution of change in impedance induced by Collagen 2µl.

		WBPA-Col 5	WBPA-TRAP	Log ₁₀ [TxB2] _{S-P}
WBPA-Col 2	Pearson Correlation	0.497**	0.392**	0.139*
	Sig. (2-tailed)	<0.001	<0.001	0.015
WBPA-Col 5	Pearson Correlation	-	0.284**	0.059
	Sig. (2-tailed)	-	<0.001	0.306
WBPA-TRAP	Pearson Correlation	-	-	0.068
	Sig. (2-tailed)	-	-	0.242
* Correlation is significant at the 0.05 level (2-tailed)				
** Correlation is significant at the 0.01 level (2-tailed)				

Table 9: Correlation coefficients between WBPA assays and [TxB2]

Correlation coefficients between the WBPA assays and [TxB2]_{S-P} are shown in Table 9. They are generally poor, with the strongest correlation between WBPA-Col 2 and WBPA-Col 5. Only WBPA-Col 2 showed any correlation with [TxB2]_{S-P} (Figure 33). Using a stepwise multiple regression analysis, I investigated whether any patient characteristic influenced the result of WBPA assays. These results are summarized in Table 6. Age was found to have a significant positive correlation with the effects of collagen 2µl on whole blood but not collagen 5µl or TRAP. Increasing BMI was associated with WBPA-Col 2 and WBPA-TRAP. The only association that was consistent across all 3 WBPA assays was with increasing platelet count.

Again, I was keen to confirm that these assays were not affected by the method of sample acquisition. As Table 10 confirms, there were no significant differences in the mean or variance of samples collected by venepuncture or from the arterial sheath at the start of the angiography procedure.

	Levene's Test for Equality of Variances		t-test for Equality of Means			
	F	p	t	p (2-tailed)	Mean Diff.	SE Diff.
WBPA-Col 2	0.031	0.860	-0.77	0.441	0.261	0.338
WBPA-Col 5	3.384	0.067	-1.22	0.223	-0.499	0.409
WBPA-TRAP	2.547	0.112	-1.52	0.128	-0.734	0.481

Table 10: Independent sample t-test comparing WBPA assay results between samples acquired from the arterial sheath and by venepuncture.

Although the principle aim of this part of the research was to investigate the utility of WBPA for the assessment of aspirin response, neither TRAP nor collagen are agonists that specifically target the platelet aggregation pathway inhibited by aspirin. Thus their effects on WBPA are

likely to be influenced by the presence of clopidogrel. This is discussed more fully in Chapter 6 where the relationship between the assays used to assess response to clopidogrel, TxB2 concentrations and WBPA in response to collagen and TRAP is presented.

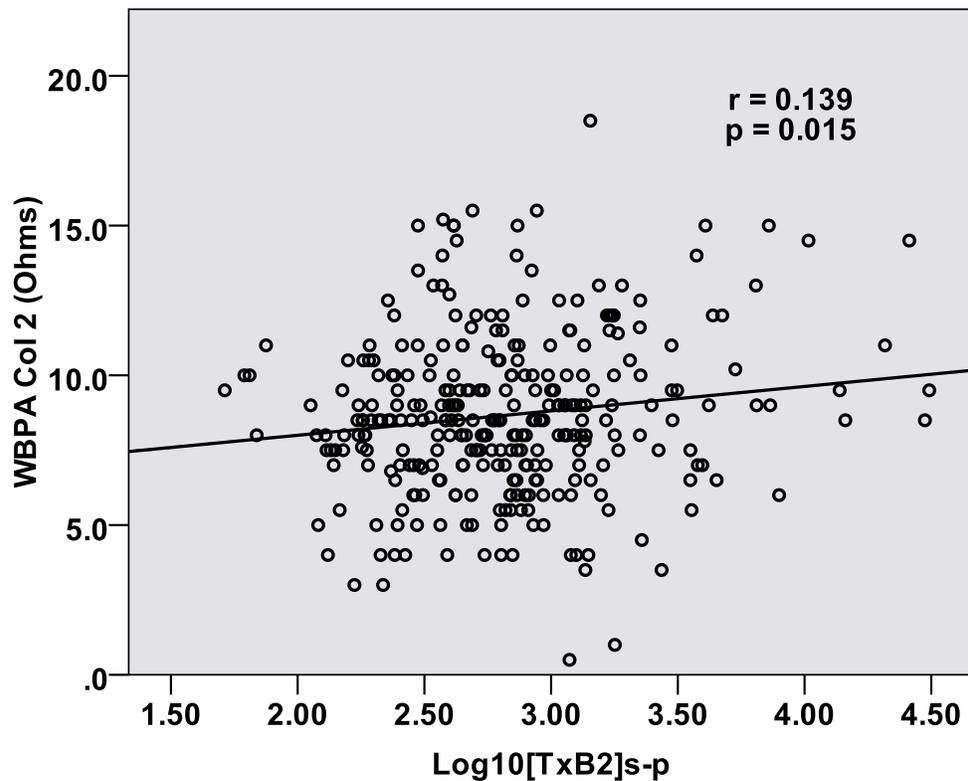


Figure 33: Correlation between WBPA in response to 2 μ l collagen and \log_{10} [TxB2]_{S-P}

4.2.6 Discussion

The principle aim of Study 4.2 was to investigate the residual ability of platelets to generate thromboxane ([TxB2]_{S-P}) in a large series of patients taking DAPT. I also investigated whole blood impedance aggregometry in response to collagen or TRAP in these patients to find out whether this might serve as a rapid, point-of-care test of the antiplatelet effects of these therapies which could potentially be used in the cardiac catheterisation laboratory for the assessment of antiplatelet response. All the assays showed a wide variation in platelet response.

Three hundred and twenty- three patients established on both aspirin and clopidogrel were studied. Once again, the demographics of these patients are in keeping with contemporary studies of patients undergoing investigation for CAD with a predominantly male population, and a high prevalence of hypertension, dyslipidaemia and a history of previous cardiovascular disease. Concomitant therapy was also appropriate with >90% of patients receiving statin therapy, >75% beta-blocker therapy and a high prevalence of additional anti-anginal agents. 17% of patients had a history of diabetes. Ninety-nine percent of the patients recruited were Caucasian, reflecting population demographics in the West of Scotland. Two hundred and thirty-three of the 323 patients (72%) had blood samples taken by venepuncture. The remaining samples (90/323, 28%) were acquired from the arterial sheath prior to coronary angiography. There was no discernible difference in the platelet function assay distributions between the different methods of blood sample collection.

In keeping with the findings of Study 4.1, this study showed a wide variation in the measurement of [TxB2]_P, [TxB2]_S and [TxB2]_{S-P}. All concentrations showed positively skewed distributions that approached normality following logarithmic transformation. The data were used in a stepwise multiple regression model to investigate whether any patient characteristics significantly predicted the residual generation of thromboxane on aspirin therapy. This multiple regression model demonstrated a significant positive correlation with BMI, suggesting that obese patients may have a reduced response to 75mg/day of aspirin. There was a negative correlation with male gender, suggesting that female patients may also have a reduced response to aspirin therapy at this dose. This gender difference is in keeping with previous studies that have suggested higher levels of urinary [TxB2] (203) or platelet aggregation in females taking aspirin (37,205). There also appeared to be a negative correlation with age, suggesting that younger patients may have a reduced response to aspirin therapy. This result is at odds with the findings of Faraday *et al* and Gum *et al* who both saw an increased incidence of aspirin ‘resistance’ in older patients but are consistent with the results of Study 4.1 (35,203). There was a negative correlation with a history of hypertension which has not been reported previously. Studies have suggested higher serum concentrations of thromboxane in diabetics but this was not suggested by the data for [TxB2]_P, [TxB2]_S or [TxB2]_{S-P} (206). Cigarette smoking which has also previously been found to predict aspirin resistance was not found to have any significant impact in this study (35). Studies of

aggregation based assays for the assessment of aspirin response have suggested an association with higher platelet count and haemoglobin concentration (35,205). No association with [TxB2]_{S-P} was found in this study.

Whilst [TxB2]_{S-P} was generally lower than that expected in normal individuals, 30% had a concentration >1000pg/ml. Five of the 304 samples (2%) yielded a [TxB2]_{S-P} >10000pg/ml suggesting that this small number of patients are either non-compliant or truly 'resistance' to aspirin. The normalization of [TxB2] following logarithmic transformation is interesting. It is known that many biological systems operate using feedback pathways leading to marked signal amplification. Platelets are no exception, and hence, even relatively small concentrations of [TxB2] may play a significant role in platelet aggregation.

Using WBPA in response to collagen (2µl and 5µl) and TRAP, a wide variation in change in impedance in response to all 3 agonists was found with a distribution that approached normality. Correlation between the results of different agonists was generally poor. This perhaps highlights the limitations in terms of reproducibility of this assay although, due to resource constraints, we did not test this with repeated measurements (60). WBPA-Col 2 showed a significant correlation with [TxB2]_{S-P} suggesting that thromboxane generation may be contributing to general platelet aggregation in response to collagen, but the association was weak and not reproduced by the other WBPA assays.

Regression analysis using baseline patient demographics and blood parameters as predictors showed inconsistent associations between WBPA and patient characteristics. Both WBPA-Col 2 and WBPA-TRAP suggested a significant positive effect of increasing age, which is at odds with the effect of age on [TxB2] but in keeping with previously published data. I found a consistent association between all WBPA assays and platelet count which is in keeping with previously published data (50). Once again, the method of sample collection did not appear to influence the assay results.

It must be emphasised that these patients were taking both aspirin and clopidogrel. As an aggregation based assay, using agonists that are not specific for the pathway inhibited by aspirin. It was therefore highly likely that the results may be significantly influenced by the extent of response to clopidogrel. This exploratory data is presented in Chapter 6, but it is

worth mentioning here that a very clear and significant effect of clopidogrel response assessed by either the VerifyNow P2Y₁₂ assay or the VASP-PRI assay and the results of WBPA was found. This is not surprising as it is known that ADP plays a central role in the amplification of the aggregation response to several platelet agonists. It does, however, once again highlight the difficulty of using aggregation based assays for the assessment of aspirin response in patients taking additional P2Y₁₂ antagonists.

It may be that the clinical utility of the more general platelet function assays, such as WBPA in response to collagen, is not for their ability to measure the individual response to aspirin therapy but as a means of risk stratifying patients who may benefit from more intensive platelet inhibition therapy. I further discuss this view and investigate whether clinical outcome is influenced by these assay results in Chapter 6.

Study 4.3: VerifyNow Aspirin for the assessment of the antiplatelet effects of aspirin

4.3.1 Introduction

The study by Chen *et al* (38) demonstrated a significant association between aspirin ‘resistance’ measured by the VerifyNow Aspirin system and the incidence of periprocedural myocardial infarction following elective coronary intervention. The Department of Cardiology, Western Infirmary, Glasgow purchased an Ultegra RPFA in 2005 and during the recruitment of patients for the studies described above, and those in Chapter 5, I investigated the utility of this assay for the assessment of patients with CAD. The appeal lay particularly in its ease of use, portability and the rapidity with which results were available. All of these features would facilitate the incorporation of VerifyNow Aspirin into routine practice in the cardiac catheterisation laboratory.

Before committing to the use of this assay to measure aspirin response in a large cohort of patients, I sought to gain initial experience to evaluate whether the results in our hands were comparable to the previously published data and would offer a reliable way of measuring response to aspirin therapy. This initial pilot work was complicated by the manufacturer changing the agonist used in the assay in July 2005 from cationic propyl gallate to arachidonic acid. The findings of three additional preliminary studies are described in this section.

Study 4.3.1: Investigation of the use of VerifyNow Aspirin to differentiate aspirinated vs non-aspirinated patients

Patient selection

A series of 46 patients attending the cardiology outpatient clinic in our hospital taking aspirin 75mg/day and no other antiplatelet or anticoagulant agent were recruited as part of the studies described in Section 4.1 and 5.1. In addition, I tested a series of 17 normal controls taking no

antiplatelet or anticoagulant therapy. The exclusion criteria described in Chapter 3.1 were also applied.

Sample acquisition and processing

All samples were obtained by venepuncture as described in Section 3.2 by RG. The VerifyNow Aspirin assays (using cationic propyl gallate based cartridges) were run between 30 minutes and 2 hours of sampling as described in Section 3.3.1. Results were recorded as ARU units with aspirin ‘resistance’ defined as >550ARU.

Results

Using the original cationic propyl gallate based cartridge, I demonstrated a wide range of values with one patient meeting the criteria for aspirin resistance (>550 ARU), (Figure 34). Of some concern, we found 15/17 (88%) of control subjects had results suggesting an adequate response to aspirin therapy.

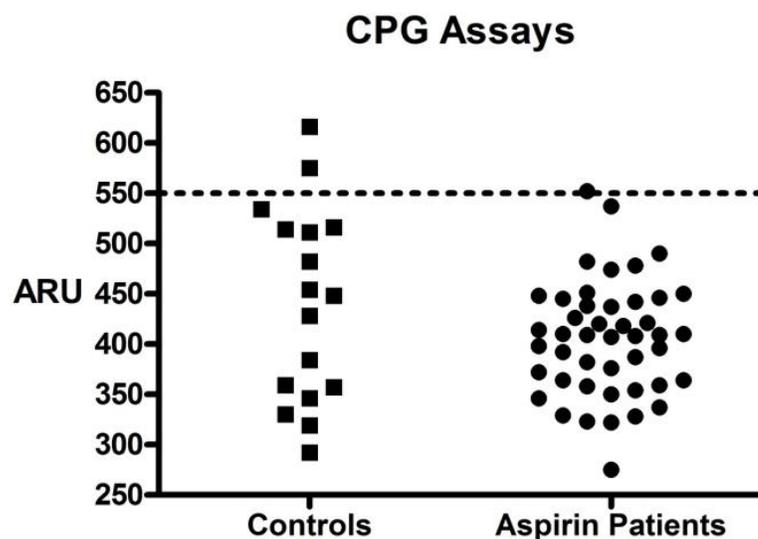


Figure 34: Assessment of aspirinated vs non-aspirinated subjects using VerifyNow Aspirin cationic propyl gallate assay

Study 4.3.2: Comparison of VerifyNow Aspirin using cationic propyl gallate and arachidonic acid as agonists

In July 2005, as I was accumulating these results, Accumetrics changed the agonist within the cartridge to arachidonic acid, the more commonly used agent for assessing response to aspirin. Accordingly, I repeated this assessment on another series of patients with known CAD taking regular low dose aspirin (75mg/day). The cut-off for aspirin resistance recommended by the manufacturer remained the same (550ARUs).

Forty patients underwent testing with the new arachidonic acid based assay (Study 4.1). These results were compared to the initial 46 patients tested using the CPG based assay. It was found that levels of ARU were significantly higher than those seen with the original assay (mean \pm SD ARU 405 ± 8 vs 458 ± 10 , $p= 0.001$) (Figure 35). Three of the 40 patients (7.5%) were resistant using the new assay compared to 1/46 (2%) of patients tested with the CPG based assay.

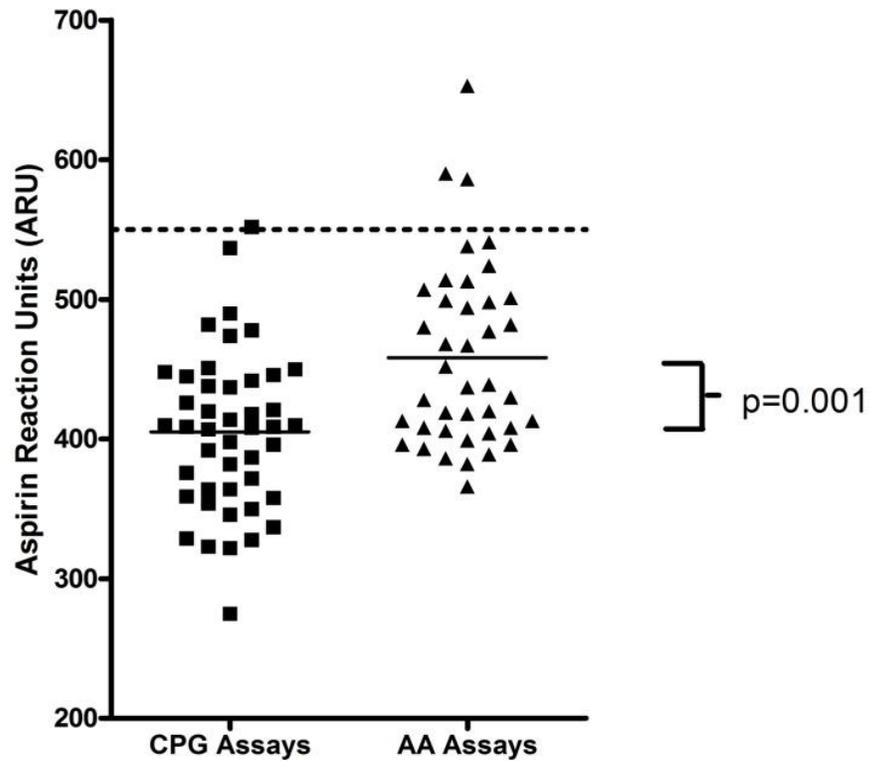


Figure 35: Ultegra RPFA cationic propyl gallate(CPG) vs arachidonic acid(AA) assay cartridges

*p-value represents an unpaired t-test. Dotted line represents the recommended threshold for defining aspirin resistance. Solid lines represent mean ARU for each assay.

Study 4.3.3: The effects of clopidogrel on aspirin response measured by VerifyNow Aspirin

I explored whether the AA based VerifyNow Aspirin assay might have any applicable use for patients taking clopidogrel. With the results from the CURE study (204) showing benefit to patients treated early following admission with suspected acute coronary syndrome (ACS) and, in light of the popular practice in Europe of pre-treating elective patients with clopidogrel prior to coronary intervention, an assay capable of assessing the antiplatelet effects of aspirin in the presence of clopidogrel would be particularly appealing. I tested a small series of patients attending for coronary intervention (n=9) before and after the planned introduction of clopidogrel. Clopidogrel had been administered as a loading dose of 300mg or 600mg and sampling occurred at least 24 hours from the introduction of clopidogrel. The results showed

significantly reduced ARU post clopidogrel therapy, 379 ± 22 vs 440 ± 55 , $p=0.007$ (Figure 36).

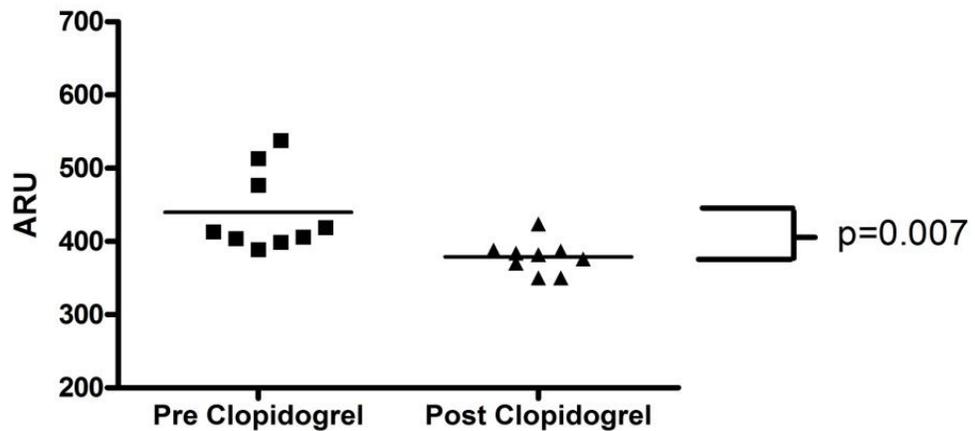


Figure 36: Ultegra RPFAs, VerifyNow Aspirin before and after clopidogrel treatment
*p value represents a paired t-test between samples before and after clopidogrel.

Discussion

These 3 small studies carried out as part of the pilot work of this research, demonstrated very concerning variation in the results of VerifyNow Aspirin. Study 4.3.1 was of particular concern as 87% of normal control patients taking no antiplatelet therapy were found to have assay results in keeping with aspirin response. Study 4.3.2 also raised concern. Although not randomised, there was a significant difference in the distribution of assay results. I found it somewhat surprising that an assay that had just been found to predict peri-procedural myocardial damage and which was receiving considerable media and academic attention was being changed to a different assay with no associated validation data. Finally, the results of study 4.3 confirmed our own concern that this assay was unlikely to accurately reflect aspirin response in patients taking both aspirin and clopidogrel.

As a result of these investigations, it was decided that the data was not supportive enough to warrant further investigation of this assay in a larger population of patients, particularly those taking DAPT. This decision has been recently justified by the study by Collet *et al* (86) which

found only 46/1213(3.9%) of patients to be aspirin resistant according to VerifyNow Aspirin when taking both aspirin and a P2Y₁₂ inhibitor.

4.4 Conclusion

Studies 4.1 and 4.2 have investigated the residual ability of platelets to generate thromboxane despite aspirin therapy. The results have shown a variation in this assessment of aspirin response that approaches normality following logarithmic transformation. They have shown that this measure of aspirin response may be valid even after the introduction of a P2Y₁₂ antagonist, clopidogrel. It was also shown that there is measurable variation of WBPA in response to collagen and TRAP in patients taking both aspirin and clopidogrel, although correlation between these assays was weak. Finally, these studies have demonstrated that VerifyNow Aspirin does not appear to produce a reliable assessment of aspirin response, particularly in patients on dual antiplatelet therapy.

Chapter 5: Assessing response to clopidogrel in patients with coronary artery disease

Introduction

Chapter 2 described the evolution of the use of clopidogrel to the current position where it is almost universally prescribed for patients suffering from acute coronary syndrome or undergoing percutaneous coronary intervention. Although newer agents have emerged over the last few years to challenge this dominant position, pricing pressures and the wider familiarity with clopidogrel across the medical specialities and ancillary staff managing cardiology patients is likely to mean that clopidogrel remains a widely prescribed medication for these patients for some time to come.

As I outlined, over the last 10 years, evidence has emerged to support a variation in individual response to clopidogrel and the rationale for the alternative agents is largely based on their more potent and uniform antiplatelet effect. However, in some patient groups, this more potent antiplatelet activity may lead to an increased bleeding rate. Thus, the optimal platelet inhibition for the individual to maximise protection against thrombotic events whilst minimising the risks of bleeding remains uncertain across the spectrum of cardiology patients.

At the time of patient recruitment for this research, variation in response to clopidogrel was still a novel phenomenon. In addition, it was, and remains the case, that the optimal assay to assess clopidogrel response is uncertain. This chapter describes my investigation of measurable response to clopidogrel. I sought to compare several selected assays and investigate whether any patient characteristics or concurrent pharmacological therapies might predict the variation in response.

Study 5.1 VerifyNow P2Y12, fibrinogen activation and VASP-phosphorylation before and after the introduction of clopidogrel

5.1.1 Introduction

From 2003-2005, several research groups published data supporting a variation in response to clopidogrel (69,154,156,207). The majority of this data used optical platelet aggregation in response to ADP as the principle means of assessing the variability. Flow cytometric measurement of fibrinogen activation in response to ADP was also recognised as a marker of response to clopidogrel (147). However, 2 novel commercial assays emerged during this period; VerifyNow P2Y12 and flow cytometric measurement of VASP-phosphorylation (193,208). As discussed in Chapter 2, these novel assays presented some advantages over more traditional methods. VerifyNow P2Y12 is a rapid, easy to use cartridge-based assay that has the potential to be used as a bedside test in the clinical environment by staff with minimal training. VASP-PRI is a more time consuming, laboratory-based assay which measures the concentration of an intraplatelet phosphoprotein that is specifically affected by stimulation of the platelet P2Y₁₂ receptor, the target of clopidogrel. It also has the advantage of producing stable results for at least 24 hours after sampling. Thus, VerifyNow P2Y12 appealed on the basis of potential clinical utility and VASP-PRI on the basis of specificity and flexibility in the management of laboratory resources. I therefore sought to investigate the change in these 2 assay results following the introduction of clopidogrel in a pilot study of patient attending for coronary angiography and intervention.

It has been suggested that coronary intervention itself may stimulate platelet activation and there is data demonstrating that the assessed response to clopidogrel may vary over the days following PCI (67,147). However, most patients undergoing intervention either electively or following an ACS (excluding ST elevation myocardial infarction) are discharged the day following this procedure. In light of this, I also investigated how the assessed response to clopidogrel using these assays changes on the day following intervention. As a reference assay, I measured platelet fibrinogen binding following stimulation with ADP using flow cytometry.

5.1.2 Patient selection

Patients with suspected CAD attending for angiography with a view to PCI were recruited prior to the introduction of clopidogrel. Exclusion criteria previously described were applied. All patients had been taking aspirin 75mg/day for at least 5 days and were naive to clopidogrel at the time of recruitment. The remaining selection criteria are those detailed in Section 3.1. Following recruitment, patients had a baseline sample collected for the assessment of platelet activation in response to ADP according to the three assays; VerifyNow P2Y12, VASP-PRI and platelet fibrinogen binding. Patients were then introduced on to clopidogrel prior to their PCI procedure. A second sample was taken following the introduction of clopidogrel immediately prior to coronary angiography. As a reflection of the available data outlined in Chapters 2 and 3, and in keeping with the local practice in our centre, a patient was deemed to have been fully introduced onto clopidogrel if they had received a loading dose of 600mg > 2 hours previously, a 300mg loading dose >24hrs previously or had received 75mg/day of clopidogrel for at least 5 days. In those patients that underwent PCI, a third sample was collected to reassess the response to clopidogrel by all three assays 12-24 hours following the procedure.

5.1.3 Sample collection and processing

All samples were collected from a large calibre vein by RG as previously described (Section 3.2). An initial 3.5ml 3.2% sodium citrate tube was collected and then discarded followed by 2 further 3.5ml 3.2% sodium citrate tubes and a 2ml 3.2% sodium citrate tube supplied by Accumetrics. The retained sample tubes were mixed by gently inverting 5 times.

Details of sample processing are given in Chapter 3. In summary, prior to sample acquisition, flow cytometry tubes were prepared for the assessment of fibrinogen binding to platelets. A 3.8ml citrated whole blood tube was utilised within 10 minutes of sampling for these assays. Samples were run in duplicate to assess reproducibility. After 30 minutes, the 2ml citrated

tube was used to test clopidogrel response using the VerifyNow P2Y12 system. A single citrated tube was set aside for the assessment of VASP-PRI within 24 hours.

5.1.4 Statistical Methods

This was an exploratory study to investigate the utility of these assays for the assessment of clopidogrel response. The distribution of assay results was summarized using the median, interquartile range and range for each assay. Assay results from the 3 sample time points were compared using paired t-tests. The 3 different assay results at each time point were also compared using Pearson correlation coefficients with an assumption of normality.

	n=41
Demographic	
Age (yrs)	
Mean±SD	64.7 ± 10.6
Male (%)	25 (61)
BMI (kg/m ²)	
Mean±SD	26.7 ± 6.0
Ethnicity	
Caucasian (%)	41 (100)
Medical History	
Hypertension (%)	24 (59)
Diabetes (%)	6 (15)
Family History (%)	16 (39)
Dyslipidaemia (%)	24 (59)
Previous TIA/CVA (%)	5 (12)
Previous MI (%)	12 (29)
Previous PCI (%)	7 (17)
Previous CABG (%)	4 (10)
Smoking Status	
Current (%)	8 (20)
Medication	
Statin (%)	31 (76)
Beta Blocker (%)	17 (66)
ACE Inhibitor (%)	12 (29)
ARB (%)	1 (2)
Calcium Channel Blocker (%)	16 (39)
Nitrates (%)	8 (20)
K⁺ Channel Activators (%)	11 (27)
Proton Pump inhibitor (%)	7 (17)

Table 11: The demographics of patients included in Study 5.1

5.1.5 Results

41 patients were recruited to the study. The demographics of these patients are shown in Table 11. A summary of results for the three assays used to assess platelet activation in response to ADP are provided in Table 12. In 2 patients, I was unable to obtain a sample prior to coronary angiography. Of the 39 patients sampled prior to coronary angiography, 28 went on to have coronary intervention and thus had a further sample taken 12-24hours following this procedure for the assessment of clopidogrel response. Of these patients, 2 received intravenous GPIIb/IIIa inhibitor (1 abciximab, 1 tirofiban) during the procedure and hence their post-PCI results were excluded. Duplicate assays were undertaken for the measurement of fibrinogen platelet binding. The correlation between these duplicate samples was excellent ($r^2 = 0.94$) thus the average value was calculated.

	Pre Clopidogrel			Pre-PCI			Post-PCI		
	(n=41)			(n=39)			(n=26)		
	Med.	IQR	Range	Med.	IQR	Range	Med.	IQR	Range
Fibrinogen binding (% +ve)	94	6	82-99	78	18	51-99	76	26	30-97
VerifyNow P2Y12 %inhibition	10	12	0-15	40	44	1-96	52	43	1-95
VASP-PRI	84	10	63-99	62	31	9-93	61	26	13-88

Table 12: Variation in clopidogrel response before clopidogrel, before PCI and after PCI assessed using 3 different assays.

		VASP-PRI	VerifyNow P2Y12 (%inhibition)
Fibrinogen binding (%+ve)	Pearson Correlation	0.65	-0.76
	p (2-tailed)	<0.001	<0.001
VASP-PRI	Pearson Correlation	-	-0.76
	p (2-tailed)	-	<0.001

Table 13: Correlation coefficients between the assays used to measure clopidogrel response

Significant correlations were seen between all 3 assays at all three time points, with the strongest between VerifyNow P2Y12 and VASP-PRI ($r = 0.768$, Table 13). The weakest correlation was between VASP-PRI and fibrinogen binding ($r = 0.65$, Figure 37) It should be noted that the correlation coefficients between VerifyNow P2Y12 and the other assays are negative given that this assay presents results as %inhibition of ADP response.

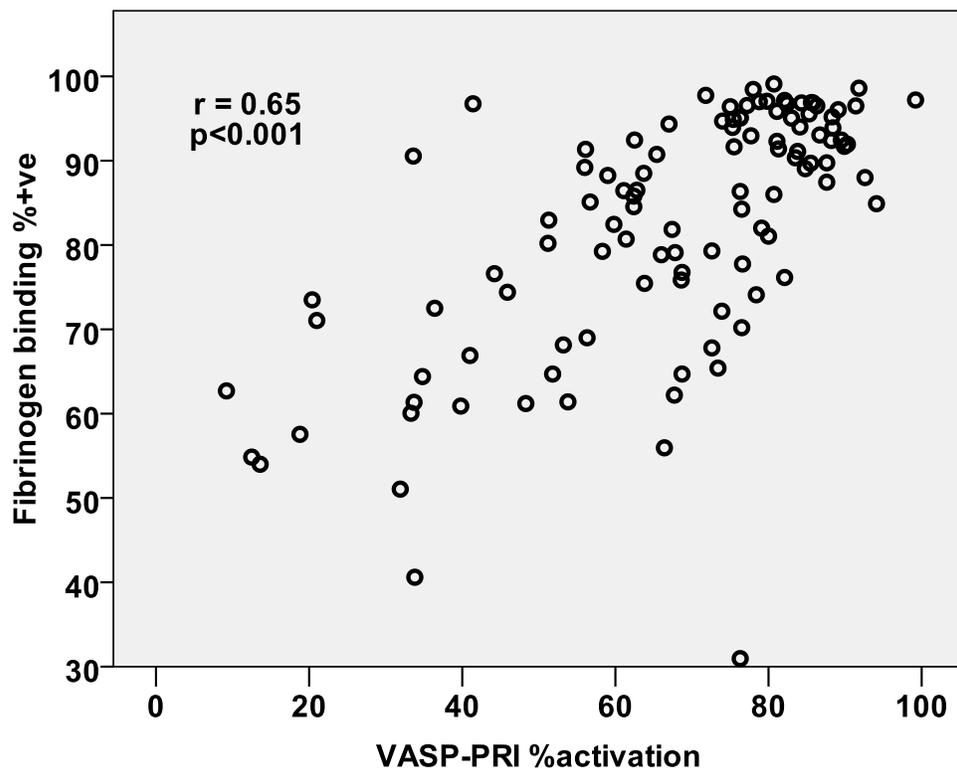


Figure 37: Correlation between fibrinogen binding and VASP-PRI for all assays (baseline, pre-PCI and post-PCI)

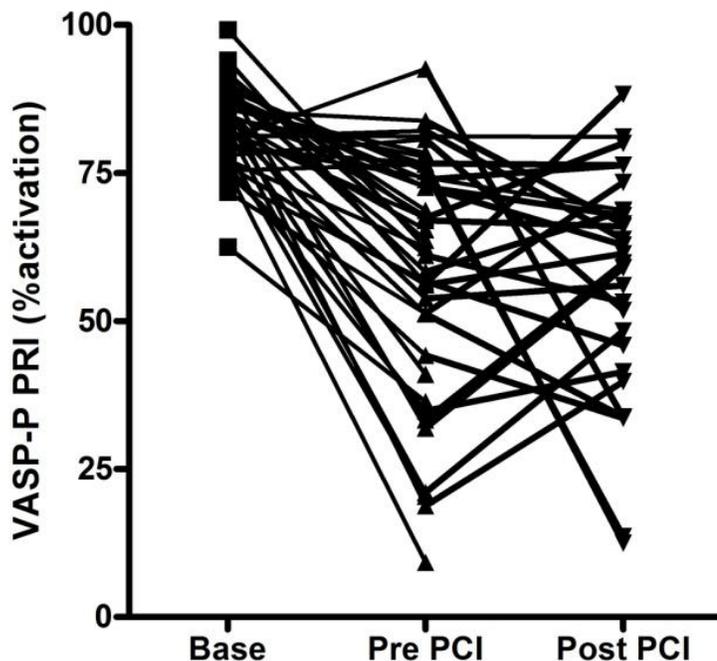


Figure 38: VASP-PRI results for patients from study 5.2

Base = before clopidogrel; Pre PCI = after clopidogrel, before coronary intervention; Post PCI = after coronary intervention

There was clearly a significant change in all three assays following the introduction of clopidogrel. There was a trend towards greater platelet inhibition to ADP following PCI. Comparing the mean values between these data sets using paired sample t-tests suggested, for this relatively small cohort of 26 patients, the differences did not reach statistical significance (Verify Now P2Y12, mean dif. +4.4%, $p = 0.13$; VASP-PRI, mean dif -3.8%, $p = 0.10$; fibrinogen binding, mean dif. -3.0%, $p = 0.181$).

5.1.6 Discussion

Although a relatively small number of patients, this pilot study produced important results. In a population representative of the West of Scotland patient attending for coronary angiography for suspected coronary disease, taking regular aspirin therapy, I was able to demonstrate using

three different assays that there was a measurable reduction in platelet response to ADP following the introduction of clopidogrel. I also showed that, in keeping with work from others, the extent of platelet inhibition in response to clopidogrel varied considerably. In addition, testing platelet response the day following coronary intervention suggested a trend towards increased platelet inhibition by clopidogrel at this time point. This is at odds with work from Gurbel *et al* (147).

The optimal time point to measure clopidogrel response remains uncertain. However, the introduction of GPIIb/IIIa inhibitors at the time of PCI may have significant effects on the assay results, particularly VerifyNow P2Y12 and flow cytometric measurement of fibrinogen platelet binding, both of which are affected by GPIIb/IIIa receptor inhibitors. There is some evidence that VASP-PRI is less affected by GPIIb/IIIa inhibitors (209). In view of this, the most practical time point to measure clopidogrel response is in preloaded patients prior to coronary angiography/intervention.

It also remains uncertain which assay is superior for measuring clopidogrel response. The results of this study demonstrated that although all three methods confirm a wide variation in clopidogrel response, correlations between the assays were only moderate.

I found the measurement of fibrinogen binding to platelets to be particularly cumbersome. The assay tubes required preparation immediately before blood sampling and patients could only be tested when there were the available staff and resources. In addition, this assay had already been reported elsewhere and in our cohort it showed the weakest correlation with the other clopidogrel response assays. The strongest correlation was between VerifyNow P2Y12 and VASP-PRI. VerifyNow P2Y12 offered a simple and rapid assessment of clopidogrel response requiring minimal training. Although time consuming, VASP-PRI appealed due to the direct relationship with stimulation of the P2Y12 receptor by ADP. In addition, the stability of this assay for 24-48 offered considerable logistical advantages. As a consequence, these assays became the focus of my further investigation of clopidogrel response.

Ultimately, the primary concern was whether these assay results have any bearing on clinical outcome. Again, a much larger dataset was required. Study 5.2 describes the assessment of response to clopidogrel in the cohort of patients recruited for Study 4.2.

Study 5.2: Response to clopidogrel assessed by VASP-Phosphorylation , VerifyNow P2Y12 and Whole Blood Platelet Aggregation in patients with coronary artery disease

5.2.1 Introduction

Following the successful demonstration of measurable response to clopidogrel shown in the pilot work of Study 5.1, I sought to explore this variation in a larger cohort of patients attending for coronary angiography with a view to PCI. As discussed in the Chapter 4, in European centres such as ours, preloading with clopidogrel prior to elective coronary intervention is widespread and patients with acute coronary syndromes are also introduced onto clopidogrel at the earliest opportunity. Thus, recruiting patients prior to clopidogrel therapy is challenging. In principle, I was interested in the variation of the antiplatelet effects of clopidogrel in patients established on this therapy. However, in order to better validate the assays, where patients could be recruited prior to the introduction of clopidogrel, a baseline assessment of platelet response to ADP was also performed.

As highlighted in study 5.1, VerifyNow P2Y12 and VASP-PRI offered the most attractive combination of features for measuring clopidogrel response in clinical practice. I also felt that WBPA could offer a relatively straightforward and inexpensive method of assessing antiplatelet response with little published data. Therefore, I also measured WBPA in response to ADP.

5.2.2 Patient selection

Patients with suspected CAD attending for angiography with a view to PCI were recruited. Previously described exclusion criteria (Section 3.1) were applied. All patients had been taking aspirin 75mg/day for at least 5 days. If the patient was naive to clopidogrel, an initial sample was taken prior to the introduction of this therapy for the assessment of VASP-PRI, VerifyNow P2Y12 and WBPA in response to ADP.

Immediately prior to PCI all patients had a sample taken for the measurement of response to clopidogrel. As described above, a patient was deemed to have been fully introduced onto clopidogrel if they had received a loading dose of 600mg > 2 hours previously, a 300mg loading dose >24hrs previously or had received 75mg/day of clopidogrel for at least 5 days. If the patient had not received one of these loading regimens they were excluded from the study. Demographic details were recorded for each patient together with details of all additional medications. Patients were questioned directly regarding compliance with both aspirin and clopidogrel and were excluded if appropriate.

5.2.3 Sample acquisition and processing

All samples prior to the introduction of clopidogrel were collected from a large calibre vein by RG as described in Section 3.2. An initial 3.5ml 3.2% sodium citrate tube was collected and then discarded followed by 2 further 3.5ml 3.2% sodium citrate tubes and a 2ml 3.2% sodium citrate tube supplied by Accumetrics. Samples collected prior to coronary angiography were collected by RG either by venepuncture or following the insertion of an arterial sheath at the time of coronary angiography. The retained sample tubes were mixed by gently inverting 5 times.

All samples were processed by AM, HM or RG as described in Section 3.3. Within 30 minutes a 3.8ml citrated tube was used to undertake whole blood platelet aggregometry using 20mmol ADP as the agonist. After 30 minutes, the 2ml citrated tube was used to undertake clopidogrel response testing using the VerifyNow P2Y12 assay. A single citrated tube was set aside for the assessment of VASP-Phosphorylation within 24hours.

5.2.4 Statistical methods

This study was part of a project to assess the variation in both aspirin and clopidogrel response in patients on dual antiplatelet therapy undergoing coronary intervention. The power calculations were based on contemporary data published at the time suggesting that there may be up to a 4-fold difference in outcome between those patients in the highest and lowest

quartiles of clopidogrel response(149). The details of this power calculation are given in Chapter 6. The aim was to recruit 240 patients undergoing coronary intervention.

The distributions of all assay results were summarized as the median, interquartile range and range of values. Standard descriptive statistics were used to assess the demographics and clinical characteristics of the patients. Distribution of clopidogrel response was assessed for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. A backward stepwise multiple regression analysis was used to investigate whether any patient characteristics concurrent medication or blood parameters influenced absolute clopidogrel response measured by the three different assays. Assay results were compared using Pearson correlation coefficients. An ANCOVA test was used to investigate whether either the method of blood sampling or the loading regimen of clopidogrel had any significant impact of assay results after correcting for patient characteristics.

Using the cohort of patient for whom samples were available before the introduction of clopidogrel I investigated the relationship between post-clopidogrel assay results and the change in assay result from baseline using Pearson correlation coefficients.

5.2.5 Results

A total of 323 patients were recruited for the study. The demographics and clinical characteristics of these patients are identical to those of Study 4.2, Table 14.

	n=323	*VerifyNow P2Y12 (PRU) (n=321)		*VerifyNow P2Y12 (%in) (n=321)		*VASP-PRI (n=320)		*WBPA-ADP (n=322)	
		Std. coefficient	p	Std. coefficient	p	Std. coefficient	p	Std. coefficient	p
Age (mean±SD)	64.3 ± 10.2	0.233	<0.001	-0.142	0.009	-	-	0.184	0.001
Male (%)	223(69)	-0.109	0.041	-	-	-	-	-0.169	0.002
BMI(kg/m², mean±SD)	28.1 ± 5.0	0.179	0.001	-0.170	0.002	-	-	-	-
Hypertension (%)	170(52.6)	-	-	-	-	-	-	-	-
Diabetes (%)	54(16.7)	0.151	0.049	-0.172	0.002	0.149	0.008	0.160	0.003
Smoker (%)	70(21.7)	-	-	-	-	-	-	0.119	0.035
Statin (%)	292(90.4)	-	-	-	-	-	-	-	-
PPI (%)	86(26.6)	0.193	0.001	-0.170	0.003	-	-	0.130	0.023
Hb. (g/dl, mean±SD)	13.7 ± 1.5	-0.350	<0.001	0.145	0.029	-	-	-0.230	0.001
Plt. count (x10⁹/l, mean±SD)	251 ± 68	-0.222	<0.001	0.229	0.001	-	-	0.132	0.045
Creatinine (µmol/l, mean±SD)	95 ± 20	-	-	-	-	-	-	-	-

Table 14: Characteristics of patients recruited to Study 5.2 with the results of the multiple regression model investigating the influence on platelet function testing

Table 15 summarises the variation in clopidogrel response assessed by the three assays. All 322/323 patients had WBPA assays performed successfully. VerifyNow P2Y12 assays produced an error message in 2 patients and 3 VASP-PRI assays did not produce a meaningful result due to errors in 3 patients' samples. Table 15 also includes the baseline assay results for the 83 patients who were recruited prior to the introduction of clopidogrel. There were no errors in sampling or processing for these 83 patients.

	Pre-Clopidogrel			Post-Clopidogrel		
	Median	IQR	Range	Median	IQR	Range
VerifyNow P2Y12 %inhibition	11	11	1 - 40	40	33	0 - 99
VerifyNow P2Y12 PRU	259	52	168 - 365	141	152	6 - 308
VASP-PRI	82	8	29 – 100	57	24	1 - 91
WBPA-ADP (Ohms)	8.0	4.0	0 – 23.0	4.0	5.0	0 – 14.0

Table 15: ADP based platelet function assays before and after clopidogrel therapy

Individual changes in the results of the three different assays are shown in Figure 39. Six patients had less inhibition of platelets following the introduction of clopidogrel according to the VerifyNow P2Y12 assays, with 11 patients and 5 patients respectively showing similar results with VASP-PRI and WBPA to ADP.

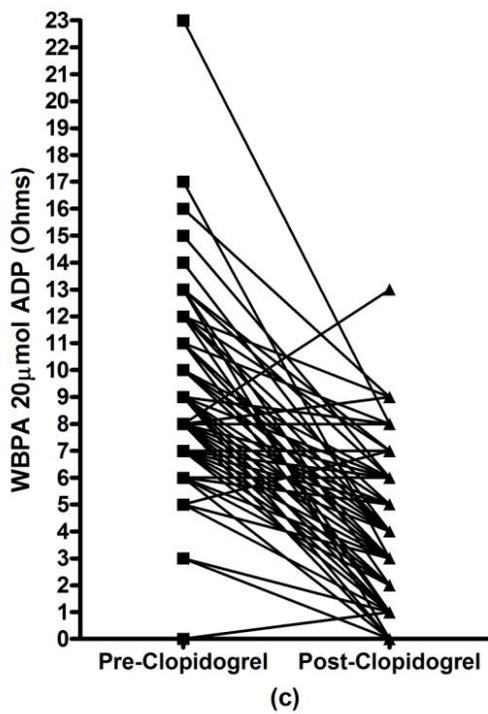
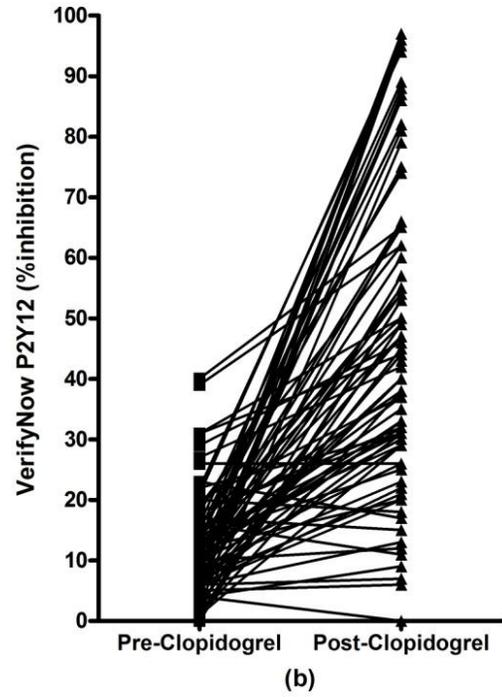
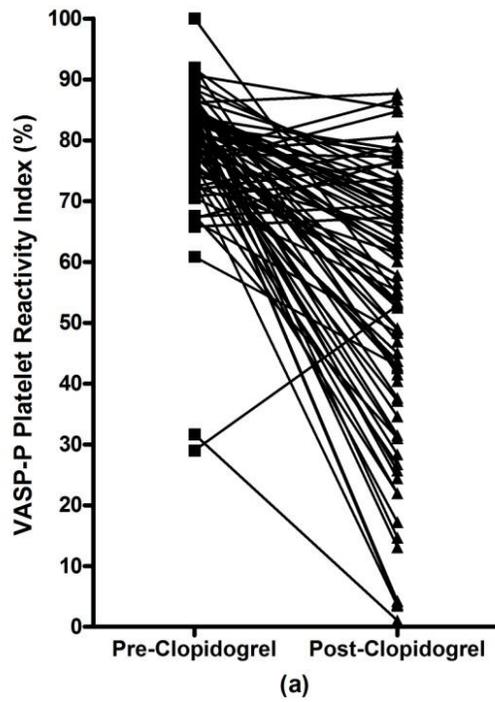
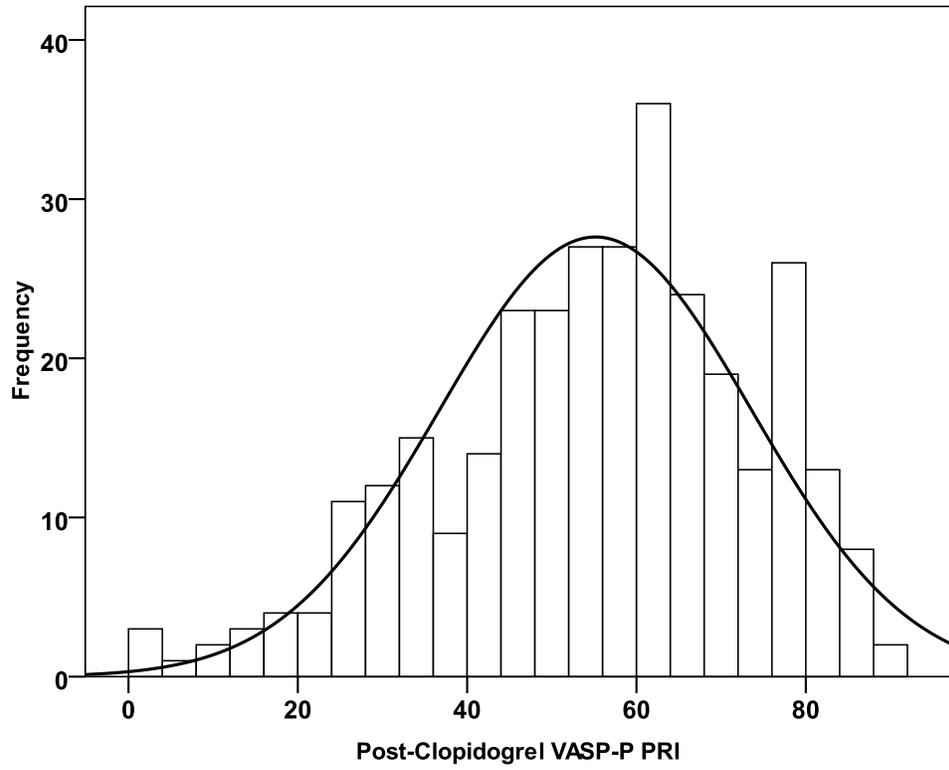
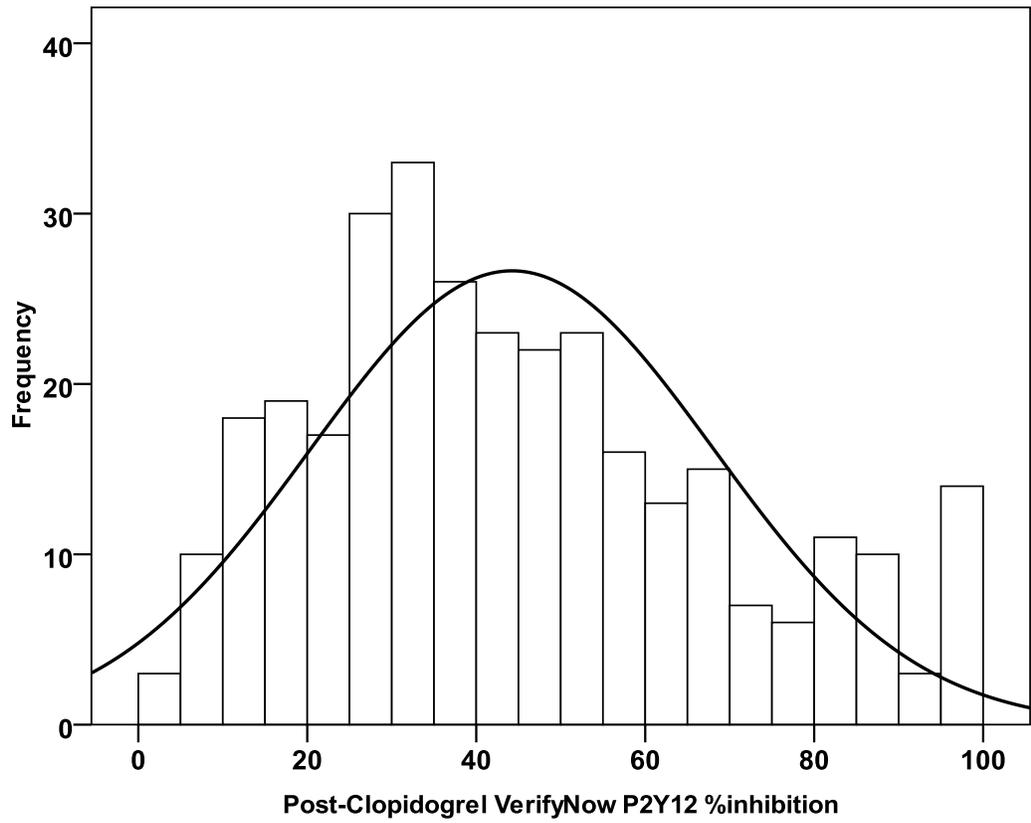


Figure 39: Change in platelet function assays following the introduction of clopidogrel (a) VASP-PRI (b) VerifyNow P2Y12 (c) WBPA-ADP

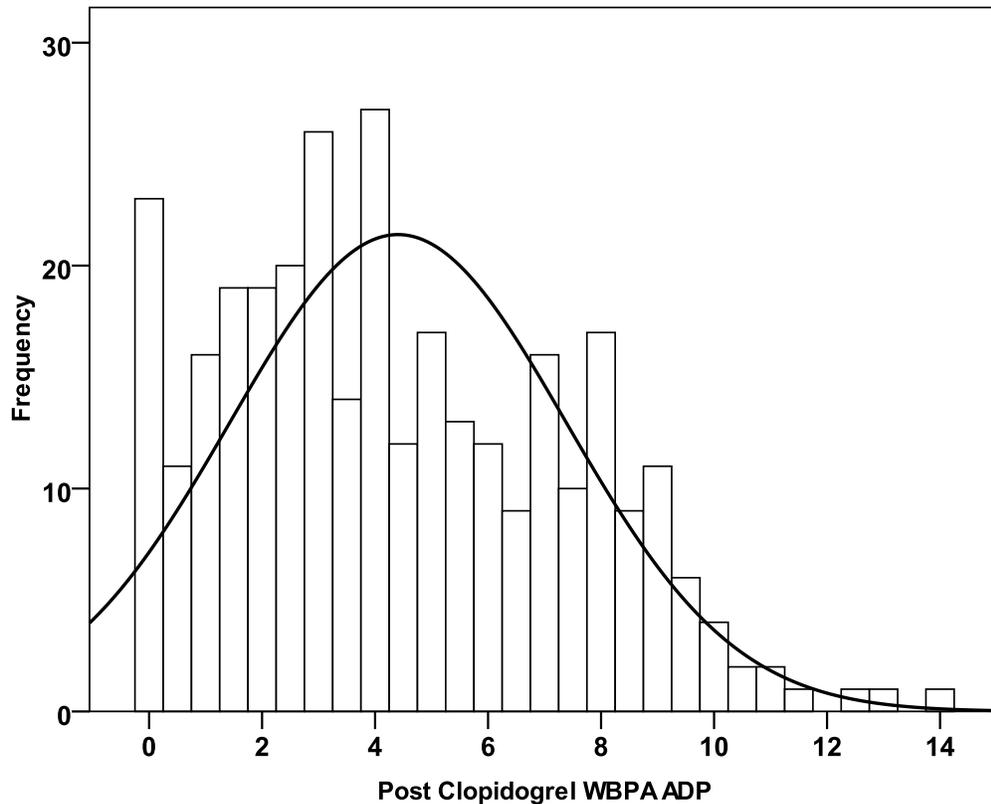
The distributions of assay results post-clopidogrel approached normality but did not meet the criteria according to either Shapiro-Wilk or Kolmogorov-Smirnov tests. All assay distributions were skewed towards a poorer response to clopidogrel. The frequency distribution histogram for VASP-PRI, VerifyNow P2Y12 and WBPA to ADP are shown in Figure 40. From our own data prior to the introduction of clopidogrel and the available literature, a cut off of <30% platelet inhibition or >70% PRI despite clopidogrel therapy has been labelled as ‘clopidogrel resistant’. Using these values, 105/321 (32.7%) of patients demonstrated clopidogrel resistance according to the VerifyNow P2Y12 assay and 73/320 (22.8%) of patients according to the VASP-PRI assay. Only 51/320 (15.9%) of patients were deemed clopidogrel ‘resistant’ by both these assay methods (Figure 43a). The study by Breet *et al* demonstrated a cut-off of absolute aggregation response to ADP in the VerifyNow P2Y12 assay (PRU) >236 was most predictive of MACE in at risk patients. According to this cut-off, only 61/321 (19%) of patients were poor responders to clopidogrel.



(a)



(b)



(c)

Figure 40: Frequency distribution of clopidogrel response assays (a) VASP-P PRI (b) VerifyNow P2Y12 (c) WBPA-ADP.

It has been suggested that change in platelet reactivity following the introduction of clopidogrel is more predictive of risk than the absolute platelet reactivity once established on therapy (163). This change in platelet reactivity to ADP was calculated for each of the three assays for the 83 patients for whom there were assay results before and after the introduction of clopidogrel. Again, a wide variation in distribution was found. The Pearson's correlation between absolute platelet reactivity on clopidogrel and change in platelet reactivity was very strong for both VASP-P PRI ($r^2 = 0.80$, $p < 0.001$) and VerifyNow P2Y12 ($r^2 = 0.91$, $p < 0.001$), Figure 41. Correlation for WBPA was much weaker ($r^2 = 0.54$, $p < 0.001$).

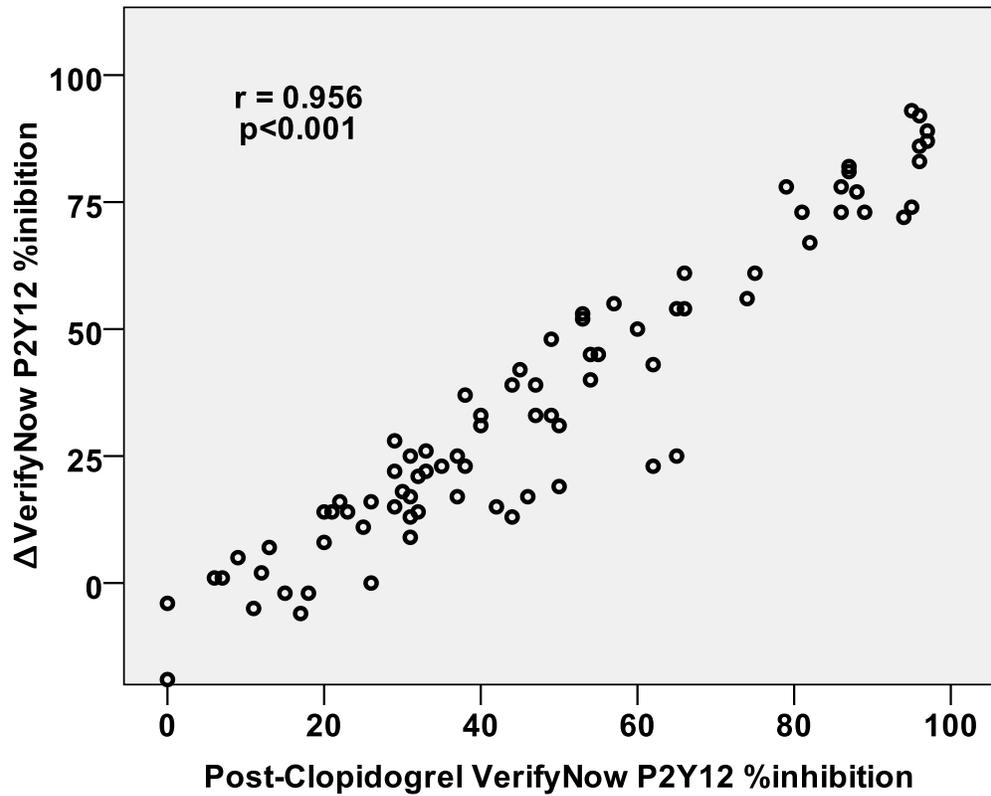


Figure 41: Correlation between change in VerifyNow P2Y12 %inhibition following the introduction of clopidogrel and absolute VerifyNow P2Y12 %inhibition.

The impression that assay results at baseline were not predictive of clopidogrel response is confirmed in Figure 42 which clearly demonstrates a negligible influence of baseline VerifyNow P2Y12 result on the final clopidogrel response assessed by the same assay. Similar findings were seen for VASP-PRI ($r = -0.048$, $p = 0.672$). There was a weak but significant correlation between pre and post clopidogrel results for WBPA-ADP ($r = 0.273$, $p = 0.013$).

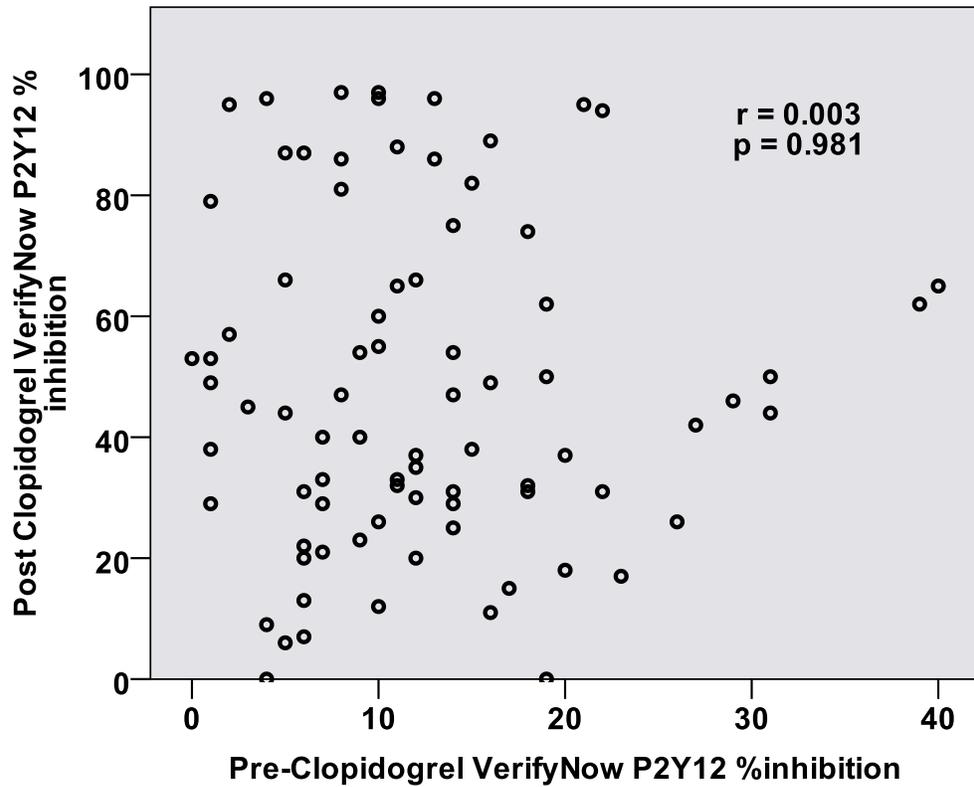
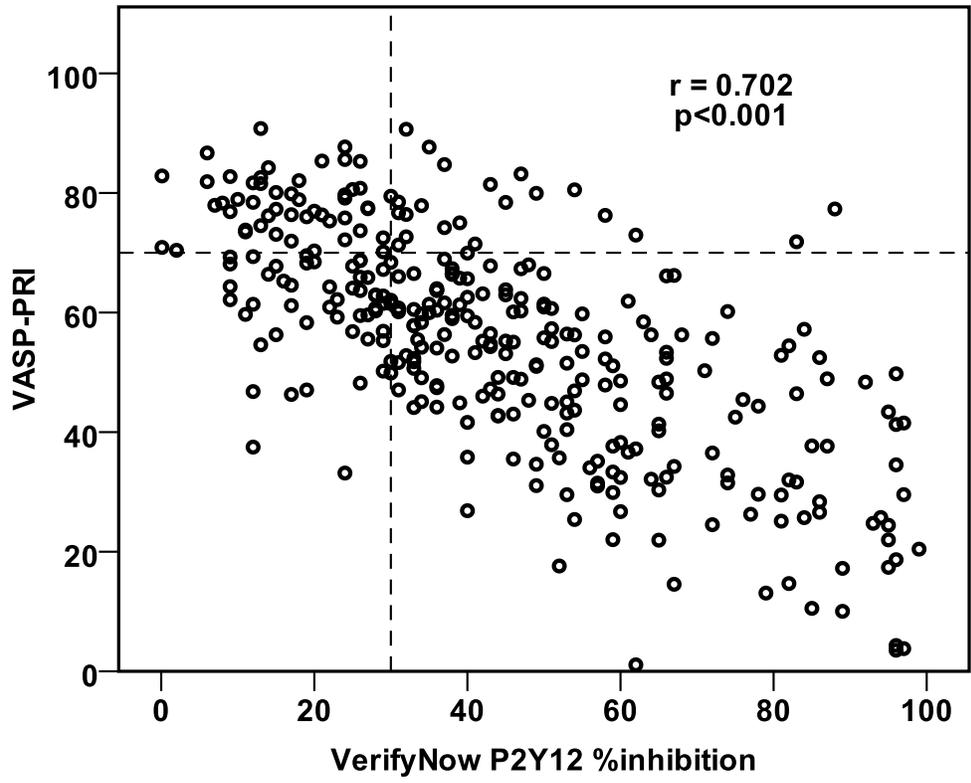
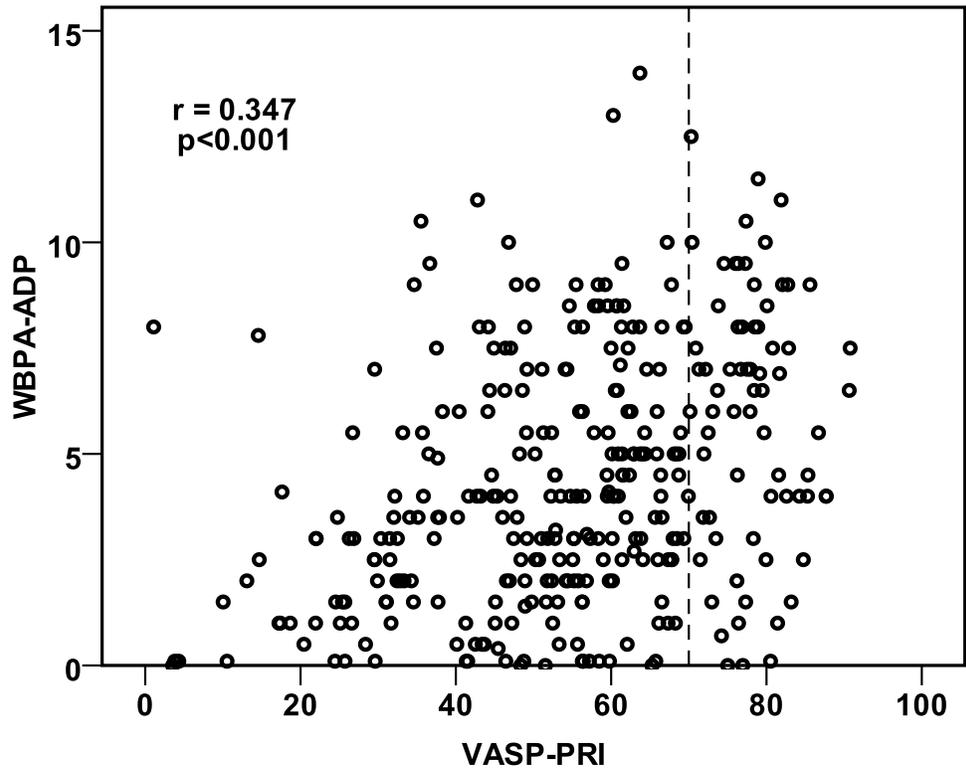


Figure 42: Pre vs Post-clopidogrel response measured by VerifyNow P2Y12.

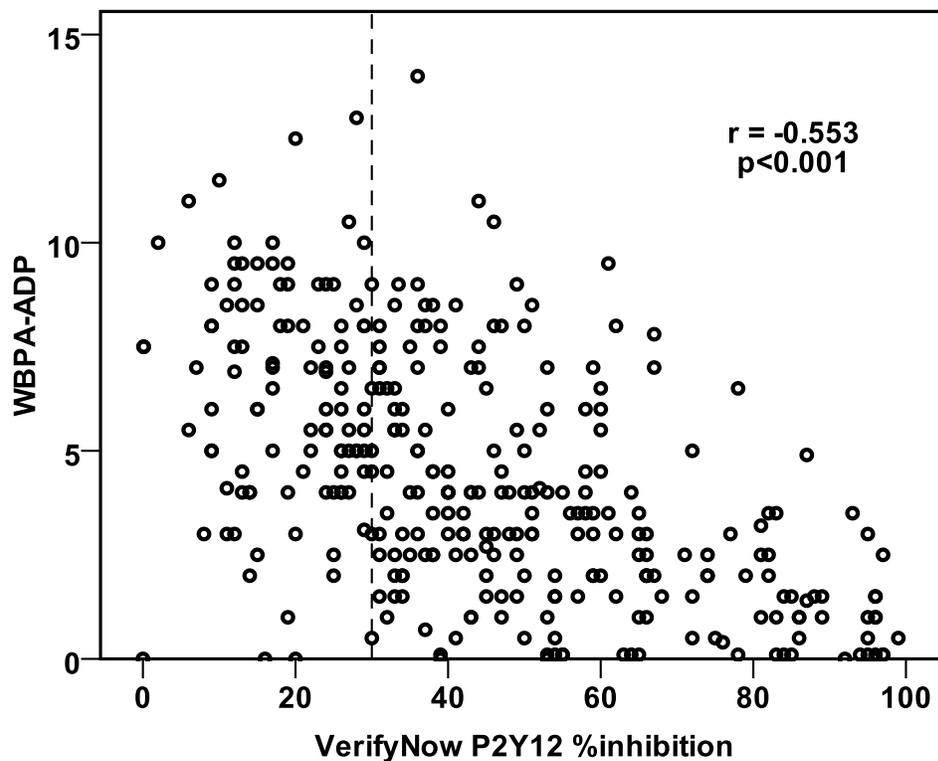
Overall correlation was strongest between VASP-PRI and VerifyNow P2Y12 with only weak correlations between WBPA and these assays (Figure 43).



(a)



(b)



(c)

Figure 43: Correlations between the three assays used to measure clopidogrel response.

(a) VerifyNow P2Y12 %inhibition vs VASP-PRI, (b) VASP-PRI vs WBPA-ADP (c) VerifyNow P2Y12 %inhibition vs WBPA-ADP. Dotted lines represent thresholds of clopidogrel ‘resistance’ suggested by previous publications.

To confirm the validity of these analyses between assays, I also generated Bland-Altman plots. Figure 44 shows this data for the comparison of VerifyNow P2Y12 %inhibition and VASP-PRI using (100-VASP-PRI (%activation)) as the comparator to VerifyNow %inhibition. It demonstrated only a very small bias but a large systematic error with limits of agreement ($\pm 2SD$) from -34 to +33.

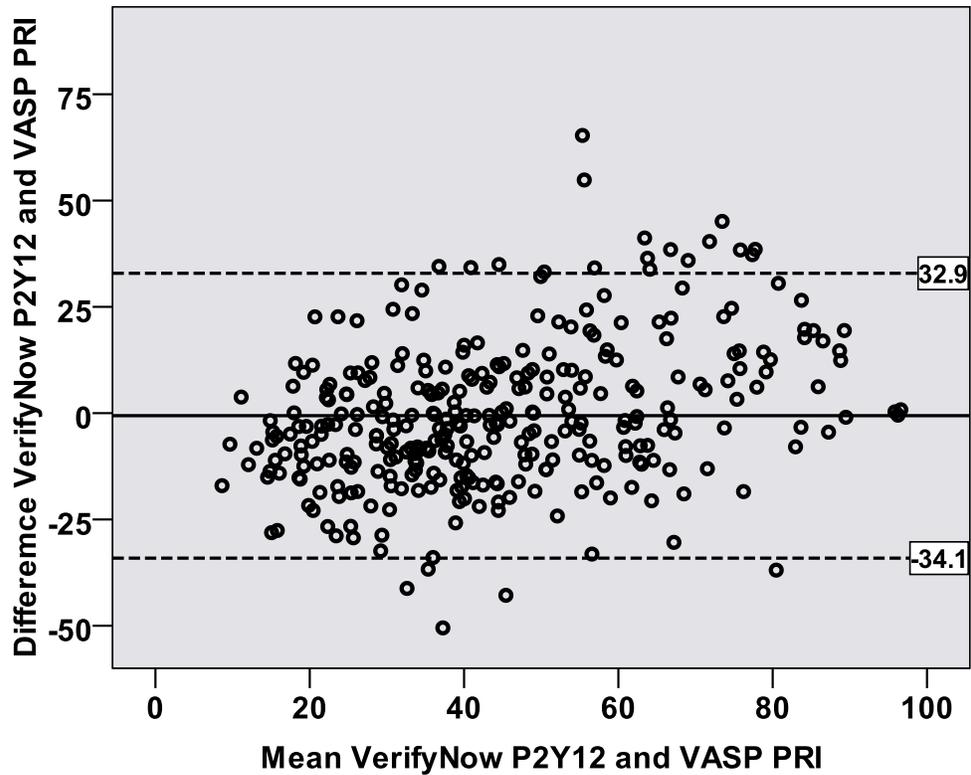


Figure 44: Bland-Altman plot of VerifyNow P2Y12 %inhibition and (100-VASP-PRI).

As in Study 4.2, I confirmed there was no significant effect on assay results of the method of sample collection using an independent sample t-test for each assay (Table 16). 233/323 blood samples (72%) were collected by venepuncture and 90/323 (28%) directly from the arterial sheath at the time of coronary angiography.

	Levene's Test for Equality of Variances		t-test for Equality of Means	
	F	Sig.	Mean Difference	Sig. (2-tailed)
VerifyNow P2Y12 %inhibition	.001	.974	-5.115	.087
VASP-PRI	.576	.449	.24235	.916
WBPA-ADP	1.649	.200	.312	.401

Table 16: Independent t-test comparing distribution of clopidogrel response between samples collected by venepuncture and those from the arterial sheath.

In order to investigate whether any of the patient characteristics described in Table 14 influenced the extent of platelet inhibition by clopidogrel, these factors were entered into a backward stepwise multiple regression model for each assay in turn. Table 14 indicates the standardised coefficient and level of significance for those variables retained by the multiple regression model for each assay. These results are discussed more fully below. However, it is worth noting at this stage that all three assays suggested that diabetic patients had a greater activation in response to ADP (Figure 45).

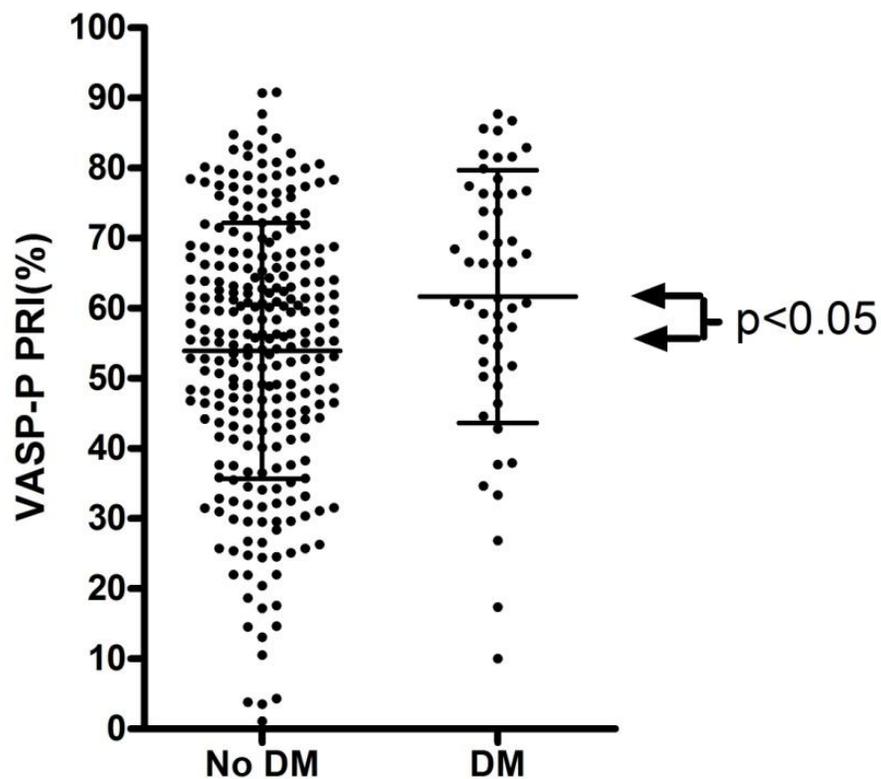


Figure 45: VASP-PRI in diabetic and non-diabetic patients, demonstrating a significant difference in response to clopidogrel between these two groups

The regression analysis also suggested an influence in both aggregation based ADP assays from the concurrent administration of a PPI with a reduced response to clopidogrel. Again, this is discussed more fully below but graphically this effect is shown in Figure 46 for

VerifyNow P2Y12 %inhibition. Such an effect was not seen for VASP-PRI although there was a trend towards a lower response.

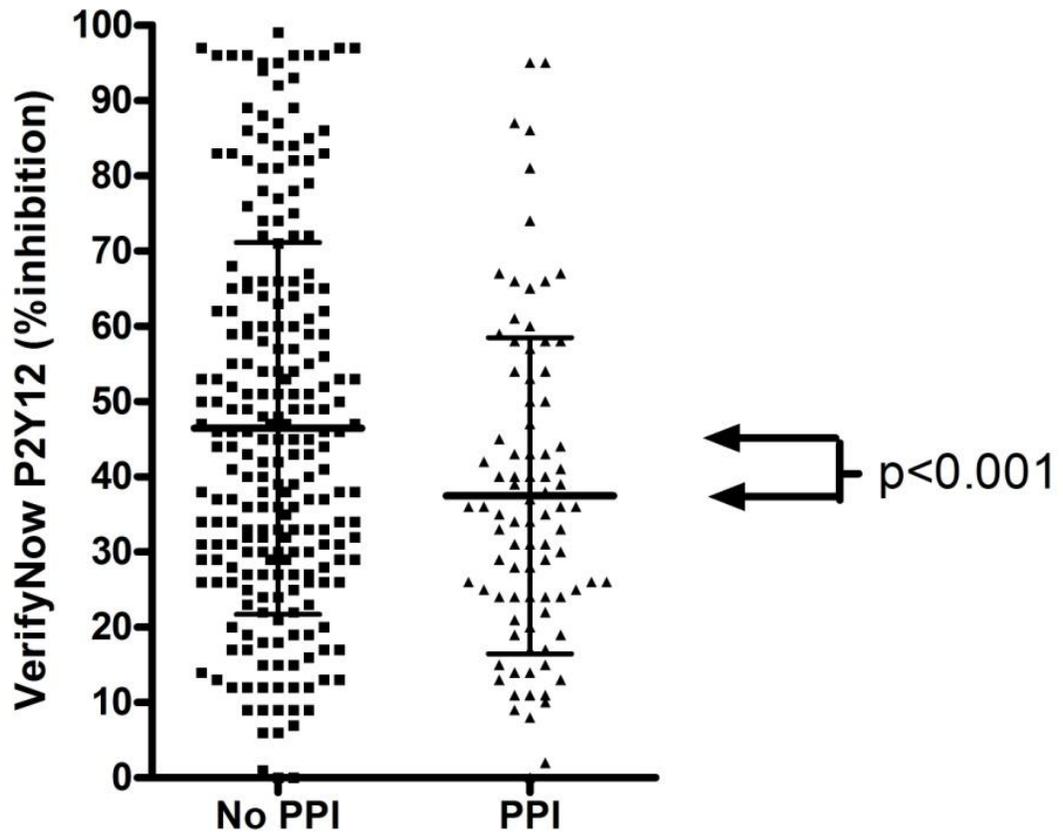


Figure 46: VerifyNow P2Y12 %inhibition according to whether the patient was taking a proton pump inhibitor in conjunction with dual antiplatelet therapy.

I found that haemoglobin level and platelet count appeared to have no association with the extent of platelet inhibition measured by VASP-PRI. There was an association between haemoglobin level and platelet count for both the aggregation based assays, VerifyNow P2Y12 %inhibition and WBPA-ADP. Although higher haemoglobin levels appeared to be associated with greater inhibition of platelets by clopidogrel according to both assays, higher platelet count seemed to increase the extent of platelet inhibition by clopidogrel measured by VerifyNow P2Y12 whilst being associated with greater platelet aggregation according to WBPA-ADP. The finding for WBPA-ADP is in keeping with the similar analysis performed for WBPA assays in Study 4.2.

Finally, I carried out a retrospective analysis to compare the three different loading regimens of clopidogrel; those within 24 hours of receiving a 600mg loading dose (Group 1, n=85), those loaded with either 300mg or 600mg between 24hrs and 5 days prior to sampling (Group 2, n=82) and those taking clopidogrel 75mg for more than 5 days (Group 3, n=153). Using an ANCOVA test incorporating diabetes, BMI and PPI use as covariates, there was no difference in assay results between the three groups. The result for VASP-PRI is shown in Table 17.

Tests of Between-Subjects Effects

Dependent Variable: Post-Clopidogrel VASP-PRI

	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	5700.130 ^a	5	1140.026	3.499	.004
Intercept	15946.333	1	15946.333	48.945	.000
DiabetesYN	1787.485	1	1787.485	5.486	.020
BMI	1596.358	1	1596.358	4.900	.028
PPI	1008.240	1	1008.240	3.095	.080
Clopidogrel Loading Group	232.207	2	116.103	.356	.701
Error	102302.079	314	325.803		
Total	1081436.215	320			
Corrected Total	108002.209	319			

a. R Squared = .053 (Adjusted R Squared = .038)

Table 17: ANCOVA test to investigate variation in response according to the three different loading regimens of clopidogrel.

5.2.6 Discussion

Following the work of Study 5.1, in Study 5.2, I sought to examine the measurement and variation in response to clopidogrel in a larger cohort of patients attending for coronary angiography with a view to PCI. The principle finding of this study was to confirm this variation in response to clopidogrel using 3 different assays. Of additional importance, moderate correlation between the assays VerifyNow P2Y12 and VASP-PRI was found. Correlation with WBPA-ADP was poor and suggests this assay may not offer a reliable method of measuring clopidogrel response in clinical practice.

Platelet function testing is notoriously difficult due to the sensitivity of platelets to exposure to any pro-coagulant environment. In an ideal situation all patients would have platelet function assays performed on samples collected in exactly the same way, preferably the method of venepuncture described in Chapter 3. However, the busy clinical environment makes this requirement for testing very demanding and time consuming. Thus, if platelet function testing is to be routinely performed in the setting of patients attending for angiography, it would be significantly more convenient if samples could be collected directly from the arterial sheath. This data, in combination with the results of Study 4.2, support the use of whole blood samples collected from the arterial sheath prior to coronary angiography.

Earlier studies have suggested that individual variation in clopidogrel response is normally distributed but other data suggests the distribution is skewed towards a poor response to clopidogrel (21,187). Surprisingly, given the importance in interpreting the statistical tests employed, this receives little attention in the published reports. The data presented here confirms skewed distributions for all three assays towards a poorer response to clopidogrel. Bearing in mind the limitations of applying these models to this distribution of data, a multiple regression model demonstrated that several patient characteristics seemed to predict a poorer response to clopidogrel. It has been shown previously that diabetic patients have heightened platelet reactivity and a poorer response to clopidogrel (210,211). Numerous pathways are thought to be involved in this difference including platelet receptor expression, intra-platelet metabolic pathways and alterations in the metabolism of clopidogrel in diabetics (212). All three of our assays demonstrated a significantly reduced inhibition to ADP in diabetic patients

taking clopidogrel (Table 14). In keeping with this finding, it has been shown more recently that diabetic patients may derive less clinical benefit from clopidogrel and improved outcomes with more potent and uniform inhibitors of the P2Y₁₂ receptor (213,214).

No other patient characteristic showed a consistent effect on clopidogrel response across all three assays. However, both aggregation based assays, VerifyNow P2Y₁₂ and WBPA-ADP demonstrated significantly increasing platelet reactivity in older patients. Recently, this finding has been confirmed using LTA and VerifyNow P2Y₁₂ in a large study of patients with CAD receiving three different regimen of P2Y₁₂ ADP receptor blockade (215). The mechanism of this difference remains uncertain. It may relate to heightened baseline platelet reactivity in these patients, in part due to increased co-morbidities. An alternative explanation may be differences in the metabolism of clopidogrel in this population. Against this, the effect was also seen in patients taking prasugrel and it is noteworthy that the effect is not seen in the VASP-PRI assay which is thought to be more specific for inhibition of the P2Y₁₂ ADP receptor rather than platelet aggregation per se.

Both VerifyNow P2Y₁₂ and VASP-PRI assays suggested that increasing BMI may reduce platelet inhibition in response to clopidogrel. This is again in keeping with subsequently published data (216). It is likely to be multi-factorial in origin but putative mechanisms include altered platelet cytosolic calcium concentration and differences in cytochrome P450 metabolism in obese patients.

Male gender appeared to be significantly associated with a greater response to clopidogrel assessed by WBPA-ADP and VerifyNow P2Y₁₂ PRU. Lev *et al* have previously reported a higher incidence of antiplatelet non-response in female patients (84). In a similar fashion, WBPA-ADP also suggested significantly increased platelet activation in response to ADP in current smokers. This is at odds with the data described in Chapter 2 suggesting upregulation of clopidogrel response in smokers and must therefore be treated with caution (178,179).

It has been widely reported in the literature that there may be a reduced response to clopidogrel in patients taking concurrent proton pump inhibitors (174). This is a finding that was echoed by our data from both aggregation based ADP assays. In our series, patients treated with a PPI were taking either omeprazole (n=49), lansoprazole (n=36) or esomeprazole (n=5). These agents are metabolised by the liver enzyme CYP2C19 which also plays a role in

the metabolism of clopidogrel. The mechanism by which this difference may arise has not been firmly established. Although retrospective data suggests that the concomitant use of PPIs and dual antiplatelet therapy may have a detrimental effect on clinical risk (175,176), the single randomised control study to assess this was not supportive (217). This observation may be due to confounding factors associated with the co-prescription of these agents. It is interesting that a similar finding was not seen with VASP-PRI which is thought to be more specific for the P2Y₁₂ receptor.

There have also been numerous contradictory reports in the literature regarding possible interactions with co-administered statin therapy. The proposed mechanism is thought to involve the CYP3A4 pathway and was thought to be particularly prominent with atorvastatin. However, neither of the three methods I used to assess the response to clopidogrel suggested a significant interaction between statin use and clopidogrel response. This finding was true when patients taking atorvastatin were considered separately (data not shown). Subsequent to the initial suggestion of an interaction, several further publications have also found no significant interaction, thus supporting our own findings (172).

With regard to blood parameters, I found that VASP-P PRI was not affected by haemoglobin or platelet count. However, both aggregation based assays did appear to show significant interaction with both haemoglobin levels and platelet count. It should be recalled that the criteria for patient recruitment (Chapter 3) excluded those patients with values outside the normal range. The VerifyNow P2Y₁₂ assay showed increasing inhibition of platelet aggregation by clopidogrel as both platelet count and haemoglobin increased. WBPA-ADP suggested greater inhibition by clopidogrel with rising haemoglobin but a reduced inhibition with rising platelet count. There is no clear rationale for this disparity. Previous studies have also suggested an interaction with haemoglobin and platelet count. In the POPULAR study (167) the findings for VerifyNow P2Y₁₂ %inhibition were similar to our own with patients in the high platelet reactivity group having a significantly lower haemoglobin and platelet count. Interestingly, when assessed by LTA, the finding for haemoglobin was consistent with this, but for platelet count there was a trend towards the opposite effect, much as was found with WBPA-ADP.

It remains uncertain which assay is most appropriate for the assessment of response to clopidogrel. Whilst there now seems little doubt that these assays are measuring an important variation in the antiplatelet efficacy of clopidogrel, and that this variation has significant clinical sequelae, no assay has clearly emerged as the definitive test upon which to base clinical decisions and alterations in patient treatment. As I have discussed at length in Chapters 2 and 3, each of the assays has practical and cost implications that must be considered. In addition, the consistency of results between the assays is limited. My research results support this difficulty. The correlation coefficient was strongest between VerifyNow P2Y12 %inhibition and VASP-P PRI, perhaps the two leading contenders for more widespread clinical use, but the correlation was not strong. Using the criteria of a 70% cut-off as outlined above, 75/320 patients (23%) would be deemed clopidogrel 'resistant' according to one or other assay, but not both (Figure 43(a)). As shown in Figure 44, the Bland-Altman plot suggests that there is a systemic error in the measurements provided by these two assays rather than a proportional error. Whilst the bias between the assay results is small (0.59%), the 95% limit of agreement lies between +32.9% and -34.1% representing 67% of the assay range. The results between WBPA-ADP were even less discriminating with very poor correlations. My feeling is that WBPA-ADP, although appealing from a pragmatic point-of-view, does not offer a robust assessment of clopidogrel response that could be used in clinical practice.

As part of this study, we were able to recruit a proportion of the patients prior to the introduction of clopidogrel in order to measure their platelet response to ADP at baseline. Some authors have suggested that baseline platelet reactivity may be important in predicting response but we did not confirm this hypothesis (163). Graphically this is demonstrated for VerifyNow P2Y12 in Figure 42 showing little correlation between the baseline measurement of platelet response to ADP and the subsequent inhibition once established on clopidogrel. In addition, I examined whether the population groups that appeared to have a reduced response to clopidogrel according to all 3 assays, namely diabetic patients and those taking a regular proton pump inhibitor, had higher pre treatment platelet reactivity. Although the numbers were small (10/83 patients with DM, 16/83 patients taking a proton pump inhibitor) there appeared to be no significant difference in the baseline response to ADP according to any of the three assays. Importantly, from a resource point of view, this makes the measurement of platelet activation in response to ADP prior to the introduction of clopidogrel redundant. As well as significant cost savings, this makes the recruitment of patients with acute coronary syndromes

more feasible. Despite every effort, I was only able to obtain samples prior to the introduction of clopidogrel in 83/323 patients (26%) highlighting the difficulty that any clinician or future researcher would encounter.

With regard to the loading regimen of clopidogrel, I have demonstrated that there is no significant difference between the three different loading regimens described in Chapter 3 and above. This again has important implications for the potential clinical use of these assays and for further research. Whilst by no means a definitive study of this issue, the data does add to the already published research described in Chapter 2 suggesting that these three loading regimens do offer a degree of equivalence.

5.3 Conclusion

Primarily, this part of my research demonstrated convincing support for the concept of a variable response to clopidogrel. This variation in response, although approaching a normal distribution, tends towards a poorer response to clopidogrel. I have shown that the presence of diabetes and the use of PPIs appears to significantly reduce the individual response to clopidogrel and for 2 of the three assays, increasing BMI also seems to reduce clopidogrel response. The effects of platelet count and haemoglobin seem to depend on the characteristics of the assay employed to measure clopidogrel response and the manufacturers' guidance regarding the limitations of these assays for patients with blood counts outwith the normal range must be borne in mind in both clinical and research use.

These data have negated any requirement to measure response to ADP prior to the introduction of clopidogrel in this patient group as this does not seem to predict clopidogrel response. Whether ADP response in patients taking aspirin alone influences clinical outcome has been discussed in Chapters 1 and 4 but is not a focus for this research. Measuring ADP response at a time-point following coronary intervention may be appropriate but there are associated pragmatic issues. It is likely that immediately prior to coronary intervention/angiography in patients already introduced onto clopidogrel remains the most appropriate time to test patients. Our data supporting the use of arterial blood samples taken at the time of sheath insertion instead of venous sampling, adds to the practicality of this approach.

With regard to the individual assays, the technical and time demands of flow cytometric analysis of fibrinogen binding render it impractical as a routine clinical test. It also does not appear to offer any great advantage in terms of reproducibility compared to the other assays although this was not extensively investigated in this project. Flow cytometry to measure VASP-PRI continues to appeal and I feel is now becoming the 'gold standard' method to directly measure inhibition of the platelet P2Y₁₂ receptor by clopidogrel. The stability of this assay for at least 24 hours following sampling is a great advantage when coordinating the recruitment and sampling of patients as well as the availability of staff and equipment to carry out the assays. In addition, this assay, together with VerifyNow P2Y₁₂, produced the strongest correlations. A further advantage of VASP-PRI in acute patients may be that it is minimally

affected by the concomitant use of GPIIb/IIIa antagonists in sharp contrast to the other aggregation based assays (196).

My experience with VerifyNow P2Y12 has reinforced the undoubted potential of the assay to act as a bedside point-of-care assay that could be relatively easily incorporated into clinical practice with minimal staff training. Although there is significant cost associated with purchasing the assay cartridges, these are relatively small in comparison with the cost involved in running a flow cytometer and associated trained staff. However, the correlation with VASP-PRI was only moderate and this remains a significant concern when considering the incorporation of this assay into clinical practice. It is critical, if clinical decisions are to be made on the basis of these assay results, that clinicians can be confident that the assays are producing accurate and reliable results which reflect response to treatment. I do not think that the correlations between the assays we have used, nor indeed those reported elsewhere with light transmission aggregometry are strong enough to be sure of this. As we have shown, a significant proportion of patients have a relatively poor response to clopidogrel by either VerifyNow P2Y12 or VASP-PRI but not both and this is troubling.

The hope had been that WBPA in response to ADP would offer a relatively inexpensive and rapid assay for the assessment of response to clopidogrel. Unfortunately, my experience with this assay was not positive. Correlation with the more widely recognised assays was poor and I was not confident that these results were an accurate representation of antiplatelet efficacy. I certainly could not envisage this form of impedance aggregometry being utilised in clinical practice.

These difficulties of reliability and agreement between assays have been highlighted elsewhere, particularly in studies looking at aspirin response as mentioned previously. Despite 40 years of platelet studies, the definitive methods to measure the antiplatelet effects of aspirin or clopidogrel has not been established. However, interest and enthusiasm remains, and continues to be fuelled by the ultimate question of whether the assay results can predict clinical outcome, enable risk stratification of patients or guide treatment decisions. This was the subject of the next part of my research.

5.3.1 Limitations of Studies 5.1 and 5.2

Due to the additional costs and complexities involved in undertaking a clinical trial this research was designed as an observational study. It was therefore difficult to control for many of the variables that are encountered in clinical practice. For example the acceptance of different loading regimens of clopidogrel was necessary in order to be able to recruit patients in our routine clinical environment without interfering with the clinical decision making process. As such, measurements of clopidogrel response have been undertaken in some patients 2 hours after a 600mg loading dose and in some 24hours after a similar dose. This may have had an impact on the results, although the available data suggest that this is not significant. Similarly, the introduction of clopidogrel could not be delayed in order to gain baseline samples in all patients. Again, data from the limited number of patients that were recruited before the introduction of clopidogrel suggested that baseline pre-treatment measurements do not predict the response to clopidogrel.

Ideally, to assess the reliability and reproducibility of the assay results, we would have repeated the assay at the same time-point and also considered sampling again at a different time point, perhaps when all patients had received clopidogrel 75mg/day for more than 1 week. Unfortunately the limited financial and personnel resources available to us precluded this. As with aspirin, I was most concerned to measure clopidogrel response prior to coronary intervention, as this is the point where changes in clinical management are most likely to be made.

As I have discussed, recent data has suggested that genetic polymorphisms may play a role in the aetiology of clopidogrel response. We were not able to incorporate this interesting aspect into our study and no samples were stored for future genetic testing.

Finally, although patients were questioned verbally at the time of sampling regarding compliance with clopidogrel therapy, no formal measurement of plasma clopidogrel metabolites was undertaken. Clearly, this may play an important role in explaining clopidogrel

response and some of the rather unusual variations seen particularly in our pilot study data before and after PCI.

Chapter 6: Aspirin and clopidogrel response and the incidence of myocardial necrosis following percutaneous coronary intervention

Summary

Chapters 4 and 5 have explored the assessment of aspirin and clopidogrel response in a cohort of patients attending our hospital with CAD. I have demonstrated a measurable variation in response to both these agents. The next stage of my research was to investigate whether there was an interaction between the measured response to aspirin and that of clopidogrel and to investigate whether any of these assays predict clinical outcome for the subset of patients undergoing percutaneous coronary intervention. The primary outcome measure was elevation in troponin following coronary intervention.

Study 6.1: Correlation between measures of aspirin and clopidogrel response in patients attending for coronary angiography with a view to PCI

6.1.1 Introduction

As described in Chapters 4 and 5, 323 patients attending for coronary angiography prior to possible coronary intervention were recruited to the main study for the assessment of aspirin and clopidogrel response. Eighty-three patients were sampled before and after clopidogrel therapy and 240 patients were recruited after being established on DAPT. Measurable variation in the antiplatelet effect of both of these agents was confirmed. However, there is a complex interaction between the pathways that promote platelet activation and aggregation. For example, ADP is secreted by platelets following degranulation in response to a number of stimuli and acts a co-factor promoting sustained platelet aggregation. Thus, response to ADP is likely to play a role in any aggregation based assay for the assessment of aspirin response. In Study 4.1, I demonstrated that there was no significant reduction in the generation of thromboxane ($[TxB2]_{S-P}$) by platelets stimulated by thrombin following the introduction of

clopidogrel. In addition, the concentration of thromboxane in serum and plasma did not appear to be influenced by the magnitude of clopidogrel response assessed by VASP-PRI and VerifyNow P2Y12. Using the enlarged dataset of patients I now investigated further whether there was any association between response to clopidogrel and response to aspirin in patients established on both these therapies.

As discussed in Chapter 2 there has been some suggestion that patients suffering from acute coronary syndromes have increased platelet activation and therefore may be more prone to have an impaired response to either aspirin or clopidogrel in the context of an acute event. This enlarged dataset provided an opportunity to further investigate whether patients presenting acutely with unstable angina or non-STEMI demonstrated any significant difference their response compared to those with a stable presentation.

6.1.2 Patient selection

As described in Chapter 4 and 5, patients attending for coronary angiography with a view to PCI were recruited for the assessment of aspirin and clopidogrel response. Inclusion and exclusion criteria are detailed in these chapters. A summary flow chart of the main research study to illustrate the distribution of patients that were recruited prior to clopidogrel treatment, once established on DAPT and those that underwent coronary intervention is shown in Figure 47.

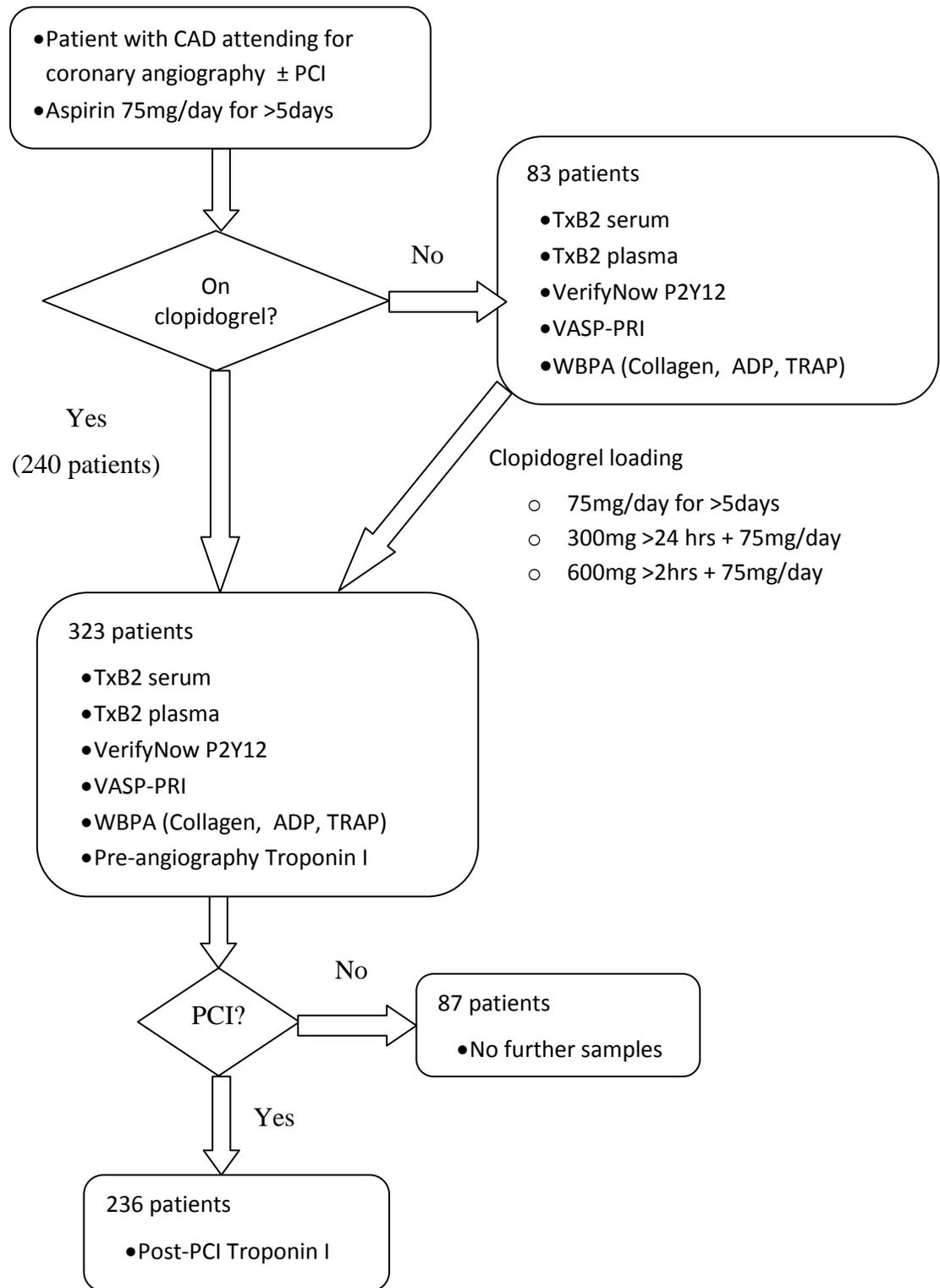


Figure 47: Flow chart summarising patient recruitment and sampling for the main research project.

6.1.3 Sample acquisition and processing

Details of the sample collection and processing for the assessment of platelet function have been extensively described in Chapters 3, 4 and 5.

6.1.4 Statistical methods

Log transformed [TxB2] data was used for all analyses. Pearson's correlation coefficients were used to compare the results of all assays. Comparison between acute and stable patients was made for both pre-clopidogrel and post-clopidogrel assay results.

6.1.5 Results

Table 18 shows the correlation coefficients between the assays used to measure aspirin and clopidogrel response. Reassuringly, further analysis using non-parametric testing (Spearman's rho) produced correlation coefficients and levels of significance that were almost identical to these results (data not shown).

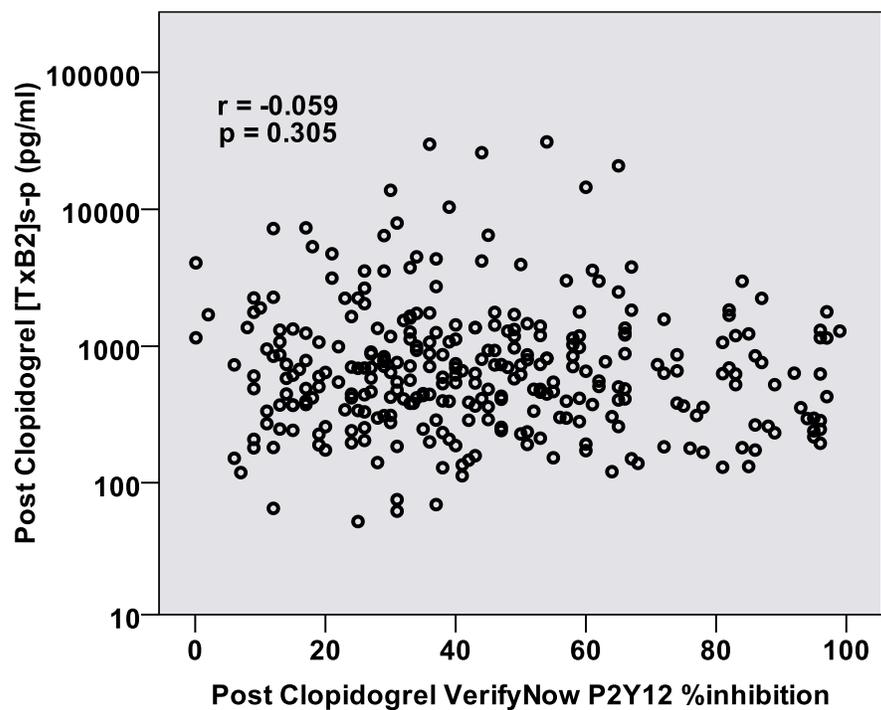
Platelet Function Assay		VerifyNow P2Y12 %inhibition	VASP-PRI	WBPA-ADP
Log₁₀[TxB2]_{S-P}	Pearson Correlation	-0.059	0.067	0.115 [*]
	Sig. (2-tailed)	0.305	0.243	0.046
WBPA-Col 2	Pearson Correlation	-0.291 ^{**}	0.095	0.569 ^{**}
	Sig. (2-tailed)	<0.001	0.099	<0.001
WBPA-Col 5	Pearson Correlation	-0.201 ^{**}	0.060	0.321 ^{**}
	Sig. (2-tailed)	<0.001	0.302	<0.001
WBPA-TRAP	Pearson Correlation	-0.167 ^{**}	0.078	0.432 ^{**}
	Sig. (2-tailed)	0.004	0.177	<0.001

Table 18: Pearson's correlation coefficients between assays assessing response to aspirin and clopidogrel.

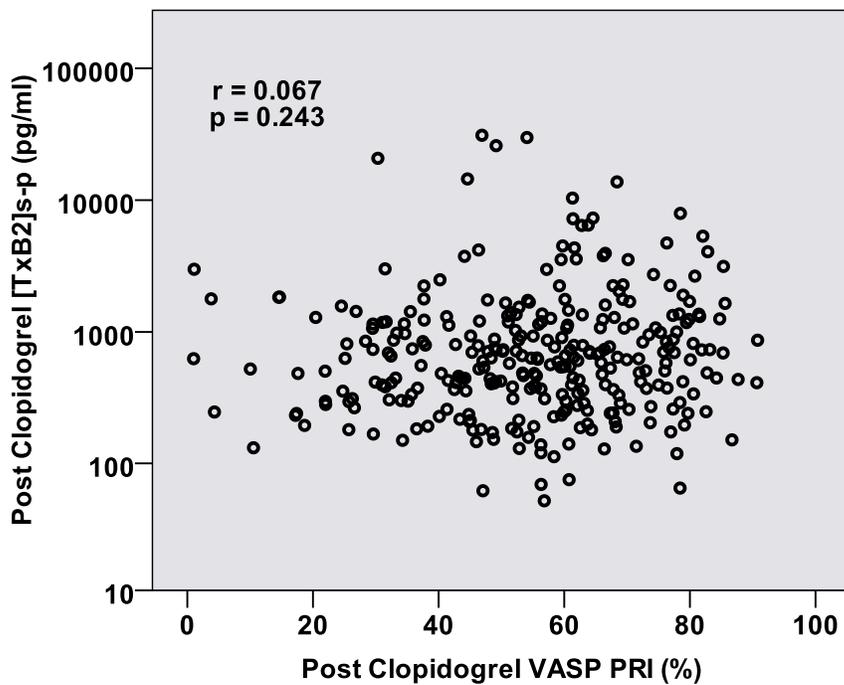
**** significant at the p<0.001 level, *significant at the p<0.05 level.**

As previously reported in Chapter 4 from a smaller dataset of 69 patients, there was no correlation between $\log_{10}[\text{TxB2}]_{\text{S-P}}$ and either VerifyNow P2Y12 %inhibition or VASP-PRI. There did appear to be a weak but significant positive correlation between $[\text{TxB2}]_{\text{S-P}}$ and WBPA-ADP. There were significant correlations between both aggregation based assays used for the assessment of response to clopidogrel (VerifyNow P2Y12, WBPA-ADP) and WBPA in response to collagen and TRAP. WBPA in response to either collagen or TRAP was not significantly associated with VASP-PRI.

Graphically, the absence of correlation between $[\text{TxB2}]_{\text{S-P}}$ and VASP-PRI or VerifyNow P2Y12 is shown in Figure 48 and the weak correlation between VerifyNow P2Y12 %inhibition and WBPA-Col 2 in Figure 49.



(a)



(b)

Figure 48: Correlation of [TxB2]_{s-p} and clopidogrel response assessed by; (a) VerifyNow P2Y12 and (b) VASP-PRI.

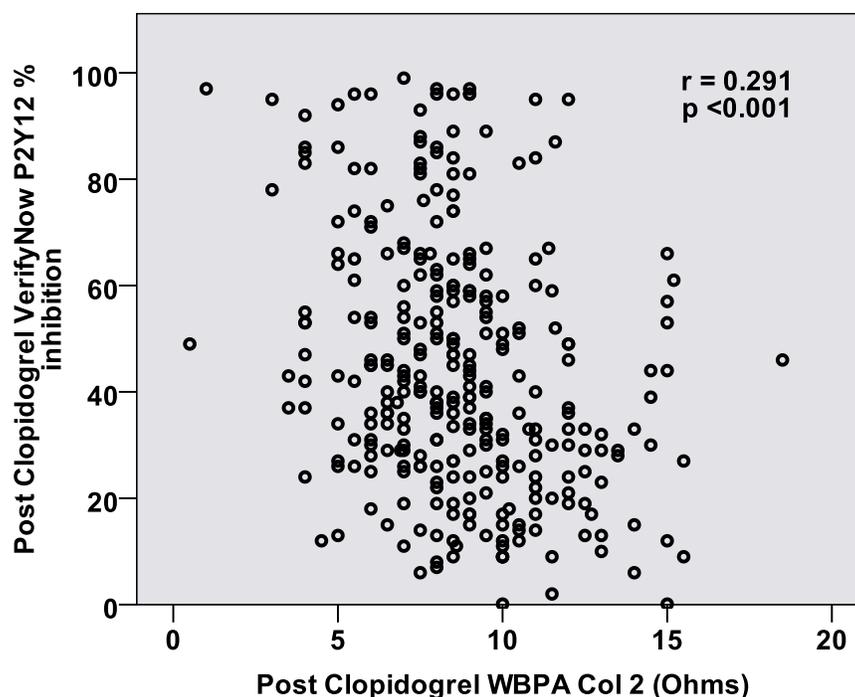


Figure 49: Correlation between WBPA-Col 2 and VerifyNow P2Y12 %inhibition.

Presentation		Number of patients (%)	Total no. (%)
Pre-clopidogrel (n=83)	Stable angina	67(80)	Stable 69 (83)
	Convalescent MI	2(2)	
	Unstable angina	6(7)	Acute 14 (16)
	Non-STEMI	8(9)	
Post-Clopidogrel (n=323)	Stable angina	199(62)	Stable 218 (67)
	Convalescent MI	19(6)	
	Unstable angina	39(12)	Acute 105 (33)
	Non-STEMI	66(20)	

Table 19: Mode of presentation of patients recruited to Studies 4.2, 5.2, 6.1 and 6.2

Of the 323 patients recruited to this study, we were able to obtain baseline platelet function in 83 patients. The mode of presentation of these patients is shown in Table 19. As expected, recruiting patients with ACS prior the initiation of clopidogrel was extremely difficult and

able to be done in only 13 patients. Even in patients established on clopidogrel therapy, due to the other inclusion and exclusion criteria, only approximately one third of patients recruited to this study presented with an ACS.

Despite these limitations, I investigated whether there was any significant difference in the assay results between patients with stable CAD and those with ACS. These results are summarised in Table 20 for samples collected prior to the initiation of clopidogrel and in Table 21 for samples taken from patients established on DAPT.

Pre-Clopidogrel platelet function assay	Acute mean±SD	Stable mean±SD	Levene's Test for Equality of Variances p	t-test for Equality of Means p
VerifyNow P2Y12 (%inhibition)	11 ± 6	12 ± 9	0.203	0.745
VASP-PRI	81 ± 6	79 ± 11	0.286	0.493
WBPA-ADP	9.3 ± 2.8	8.7 ± 3.3	0.579	0.582
WBPA-Col 2	9.5 ± 2.1	9.9 ± 3.2	0.390	0.685
WBPA-Col 5	15.5 ± 3.7	14.5 ± 3.8	0.493	0.413
WBPA-TRAP	17.5 ± 4.1	14.2 ± 4.5	0.806	0.015
Log₁₀[TxB2]_{S-P}	2.92 ± 0.42	2.89 ± 0.55	0.426	0.864

Table 20: Comparison of pre-clopidogrel platelet function assay results between patients presenting acutely and those with a stable presentation.

Post Clopidogrel platelet function assays	Acute Mean±SD	Stable Mean±SD	Levene's Test	
			for Equality of Variances p	t-test for Equality of Means p
VerifyNow P2Y12 %inhibition	43±25	45±23	0.124	0.476
VASP-PRI	55±20	55±18	0.113	0.952
WBPA-ADP	4.6±3.1	4.3±2.9	0.532	0.312
WBPA-Col 2	9.2±2.7	8.3±2.8	0.910	0.011
WBPA-Col 5	14.3±3.5	13.4±3.5	0.656	0.036
WBPA-TRAP	13.3±4.1	12.3±4.2	0.448	0.039
Log₁₀[TxB2]_{S-P}	2.83±0.41	2.81±0.52	0.094	0.701

Table 21: Comparison of platelet function assay results on DAPT between patients presenting acutely and those with a stable presentation.

There was a significant difference between the ACS and stable patients for WBPA-TRAP prior to the introduction of clopidogrel (Table 20). Once established on DAPT, this difference was maintained for WBPA-TRAP but in addition, WBPA-Col 2 and WBPA-Col 5 also showed a significantly increased aggregation in ACS patients (Table 21). VerifyNow P2Y12, VASP-PRI, WBPA-ADP and the levels of TxB2 all suggested slightly increased platelet activation both before and after the introduction of clopidogrel in ACS patients but the differences were small and did not reach statistical significance.

6.1.6 Discussion

The principle finding of study 6.1 was an absence of any significant correlation between measurement of [TxB2]_{S-P} and the two principle assays for the assessment of clopidogrel response, VerifyNow P2Y12 and VASP-PRI. This confirms the findings from Study 4.1 for a much larger dataset of patients established on DAPT and provides further support for the

validity of this assay to assess response to aspirin in patients taking an additional ADP receptor antagonist. I did find a weak correlation between [TxB2]_{S-P} and WBPA-ADP, a result which may reflect a lack of specificity for any single pathway when using WBPA to assess clopidogrel response. This is in keeping with the data presented in Chapter 4 showing weak correlation between [TxB2]_{S-P} and WBPA in response to collagen.

In addition, the data shows significant correlations between all of the whole blood platelet aggregation assays. In particular, VerifyNow P2Y₁₂ is significantly correlated with the WBPA response to both collagen and TRAP. It is interesting to reflect that this is not the case for VASP-PRI. It would therefore appear that the VASP assay is measuring a marker of platelet function that is more specific for inhibition of the P2Y₁₂ receptor rather than platelet aggregation in general. In that respect, it may be the stronger candidate when considering assays to guide clinical decision making such as alterations to dose or the selection of more powerful agents to inhibit the P2Y₁₂ receptor.

As a reflection of the inclusion and exclusion criteria, the majority of patients we recruited had stable CAD. I found no significant difference in the distribution of assay results between acute and stable presentation patients for VerifyNow P2Y₁₂, VASP-PRI, WBPA-ADP or [TxB2]_{S-P}. There were significant differences between WBPA-TRAP both before and after the introduction of clopidogrel with heightened platelet reactivity in acute patients. This data would support the studies investigating inhibition of the protease-activated receptors (PAR) to thrombin, in ACS patients (218). WBPA in response to collagen was also significantly higher in patients established on DAPT following an ACS presentation. To some extent, these findings confirm the suggestion from previous studies that patients suffering from an ACS have generally increased platelet activation (99,219). Changes in platelet activation as a result of acute coronary syndrome, in a similar fashion to those observed during exercise, are small and may, therefore, be largely masked by those changes associated with the introduction of antiplatelet agents. However, it may be that acute patients attending for coronary intervention should be a target for enhanced antiplatelet therapy because of heightened residual platelet activation on standard doses of aspirin and clopidogrel.

This data also counters concerns regarding compliance as a determinant of variation in response (186). Patients recruited following an ACS presentation had been hospitalised prior

to coronary angiography. Medications had been administered by nursing staff and hence non-compliance should not be an issue. The finding that any minor differences between acute and stable patients were weighted towards a lower response to antiplatelet therapy in acute patients suggests that compliance amongst the elective patients attending for coronary intervention was good. In this regard, I would reiterate that patients were questioned regarding compliance with both aspirin and clopidogrel. Throughout the department the importance of compliance with antiplatelet therapy due to the risks of stent thrombosis and significant morbidity and mortality was repeatedly emphasised to patients. Although not conclusive, it does indicate that non-compliance may not have played a major part in the observed variation in response that these results have shown.

Study 6.2: Aspirin and clopidogrel response and peri-procedural myocardial necrosis following PCI

6.2.1 Introduction

The primary objective of this research was to investigate whether variation in response to aspirin and clopidogrel had a significant impact on clinical outcome. Antiplatelet therapy has played a pivotal role in improving the safety and outcome for patients undergoing coronary intervention and this patient population is most likely to demonstrate any clinically important consequences of poor response to antiplatelet treatment. I investigated whether there was any difference in myocardial necrosis following PCI, measured by troponin I elevation, among patients according to response to either aspirin or clopidogrel.

6.2.2 Patient selection

The exclusion and inclusion criteria for the patients recruited to the main study have been extensively discussed. Patients were included in this part of the research if they went on to have coronary intervention by either balloon angioplasty or coronary stent implantation (Figure 47). Patients on whom PCI had been attempted but the lesion could not be crossed by a balloon were excluded.

6.2.3 Platelet function assays and measurement of cardiac enzymes

The platelet function assay methods have been previously described. As reported in Chapters 4 and 5, I undertook measurement of [TxB2]_{S-P}, WBPA-ADP, WBPA-Col 2, WBPA-Col 5, WBPA- TRAP, VerifyNow P2Y12 and VASP –PRI. Patients recruited prior to the introduction of clopidogrel also had these assays performed on aspirin alone.

At the time of platelet function assay sample acquisition, all patients had a sample collected for measurement of serum troponin I (pre-PCI). The threshold of this assay was 0.04µmol/l. In patients who subsequently underwent coronary intervention by either balloon angioplasty or coronary artery stent implantation, a further sample was collected between 12 and 24 hours

following the coronary intervention (post-PCI). The difference between the pre and post-PCI troponin assays was calculated and any rise attributed to the intervention procedure.

6.2.4 Statistical methods

Patient characteristics and the details of the intervention procedures performed were analysed using standard descriptive statistics. Platelet assay results for this group of patients were analysed using appropriate summary statistics. Troponin assay results were normalised for regression analysis using a logarithmic transformation. Stepwise regression was used to investigate whether baseline patient characteristics or procedural features were significantly associated with a rise in troponin following PCI. Subsequently, for the primary analysis, patients were divided into quartiles according to each platelet function assay and the frequency of troponin elevation (either any elevation or >10x ULN) between the upper and lower quartile was compared using a Chi square test. Significance for all analysis was taken at the 0.05 level.

Power calculation: The principle aim of our study was to investigate whether there was a significant difference in the incidence of troponin elevation following coronary intervention between the upper and lower quartiles of aspirin or clopidogrel response. We estimated the incidence of the primary end-point as 20%. The intention was to detect a 4-fold difference in the primary end-point between the upper and lower quartiles of clopidogrel response with a power of 90% and a 2 sided p-value <0.05. Statistical software (nQuery v4.0) suggested a sample size of 240 patients undergoing PCI.

6.2.5 Results

Of the 323 patients recruited to the study, 236 underwent PCI immediately following platelet function testing with the remainder being managed conservatively. The demographics of these patients are shown in (Table 22). I found no significant differences in any of the demographic characteristics or blood parameters, including baseline troponin, between these two groups.

	PCI (n=236)	No PCI (n=87)	p value
Age (mean ± SD)	64.2 ± 10.4	64.8 ± 9.5	0.58
Male (%)	163 (69)	60 (69)	0.96
BMI (kg/m², mean ± SD)	28 ± 5.0	28.2 ± 5.1	0.68
Caucasian (%)	233 (99.5)	86 (99)	0.87
Hypertension (%)	125 (53)	44 (52)	0.84
Diabetes (%)	38 (16)	16 (18)	0.63
Family History (%)	125 (53)	38 (44)	0.14
Dyslipidaemia (%)	144 (61)	59 (68)	0.26
Previous TIA/CVA (%)	20 (8.5)	14 (16)	0.08
Previous MI (%)	81 (34)	33 (38)	0.55
Previous PCI (%)	25 (11)	6 (7)	0.28
Previous CABG (%)	25 (11)	6 (7)	0.28
PVD (%)	33 (14)	7 (8)	0.11
Current smoker (%)	52 (22)	18 (21)	0.79
Statin (%)	217 (92)	75 (86)	0.16
Beta-blocker (%)	187 (79)	63 (72)	0.22
ACE Inhibitor (%)	123 (52)	47 (54)	0.76
ARB (%)	37 (16)	9 (10)	0.19
CCB (%)	88 (37)	30 (34)	0.64
Nitrates (%)	63 (27)	20 (23)	0.49
K⁺ channel activators (%)	65 (28)	34 (39)	0.06
Proton pump inhibitor (%)	68 (29)	23 (26)	0.67
ACS/NSTEMI (%)	70 (30)	34 (39)	0.13
Haemoglobin (g/dl, mean ± SD)	13.6 ± 1.4	13.9 ± 1.6	0.21
Platelet count (x10⁹/l, mean ± SD)	251 ± 68	254 ± 69	0.82
Creatinine (µmol/l, mean ± SD)	94 ± 18	97 ± 23	0.34
Trop I pre-PCI (µmol/l; median, range)	<0.04, <0.04–10.70	<0.04, <0.04-9.39	0.63
Trop I post-PCI (µmol/l; median, range)	0.14, <0.04 – 20.30	-	-
Change in Troponin (µmol/l; median, range)	0.10, <0.04 - 20.30	-	-

Table 22: Patient characteristics for participants that underwent coronary intervention and those managed medically

Number of vessels treated (%)	n=236 (%)
1	171 (72.5)
2	68 (26.5)
3	2 (1)
Length of procedure(mins)	
Mean±SD	73 ± 29
Total stent length	
Mean±SD	26.7 ± 6.0
Lesion location	n=313 (%)
LMS	5 (2)
LAD/Diagonal	118 (38)
Cx/OM	86 (27)
RCA	100 (32)
SVG	4 (1)
Lesion morphology	
ISR	6 (2)
Bifurcation	29 (9)
Chronic occlusion	13 (4)
Lesion treatment	
Stenting	299 (96)
POBA	14 (4)
Reference vessel diameter	
Mean±SD	3.0 ± 0.4
Number of stents per lesion	
Mean±SD	1.6 ± 0.9
Stent type	n=344 (%)
DES	167 (49)
Taxus	126 (37)
Endeavour	31 (9)
Cypher	10 (3)
BMS	163 (47)
Driver	74 (22)
Liberte	87 (25)
Costar	2 (1)

Table 23: Procedural details for patients that underwent coronary intervention.

In total, 303 vessels were treated with 70/236 patients receiving multivessel PCI (Table 23). Three hundred and thirteen lesions were treated using a total of 344 stents split almost equally between drug eluting (49%) and bare metal (47%). Fourteen patients (4%) received balloon angioplasty without stenting. The distribution of lesions between the three main coronary artery territories is shown in the table, with only a small number of patients receiving PCI to a LMS or a saphenous vein graft lesion, 9/313(3%). There were no LIMA artery interventions in this cohort. The average number of stents per patient was 1.6 with a mean total stent length of 27mm. The average procedural time was 73 minutes (range 25 – 180 minutes). The vast majority of lesions were de novo, with only 5 patients (2% of lesions) receiving coronary intervention for in-stent restenosis.

	Pre-Clopidogrel (n=58)			Post-Clopidogrel (n=236)		
	Median	IQR	Range	Median	IQR	Range
[TxB2] _P (pg/ml)	152	239	32 – 16621	130	149	16 - 11400
[TxB2] _S (pg/ml)	810	1908	217 - 36344	760	856	110 - 36344
[TxB2] _{S-P} (pg/ml)	617	1232	86 - 30511	623	841	52 - 31043
VerifyNow P2Y12 PRU	260	64	168 -365	154	113	0 - 384
VerifyNow P2Y12 % inhibition	12	10	1 - 31	43	31	0 - 97
VASP-PRI (%activation)	81	9	29 - 91	57	25	1 - 91
WBPA-ADP (Ω)	8.5	4.0	5 - 17	3.5	5.0	0 - 14
WBPA-Col 2 (Ω)	10.0	3.0	4 - 18	8.5	3.0	1 - 19
WBPA-Col 5 (Ω)	14.0	6.0	7 - 23	13.5	4.0	5 - 23
WBPA-TRAP (Ω)	13.5	6.0	7 – 25	11.5	5.0	5.5 - 28.6

Table 24: Platelet function assay results for patients undergoing PCI

Platelet function testing for the 236 patients who underwent PCI is shown in Table 24. There were no significant differences for any of the platelet function tests between patients who did or did not undergo PCI (data not shown). Troponin results before and after coronary intervention are summarised in Table 22. A somewhat unexpected finding was 13/165 (8%) of stable patients had a measurable troponin prior to PCI (range 0.05 – 5.28 μ mol/l). One hundred and sixty three of the 236 patients (69%) demonstrated a rise in troponin following coronary intervention, with the magnitude of this rise showing, as expected, a positively skewed distribution. Logarithmic transformation of these assay results approached normality although did not meet the strict Kolmogorov-Smirnov or Shapiro-Wilk criteria, Figure 50. Sixty six of the 236 patients (28%) showed a change in troponin of greater than 10x the upper limit of normal (0.4 μ mol/l) and 36/236 (13%) patients had a rise in troponin >1 μ mol/l.

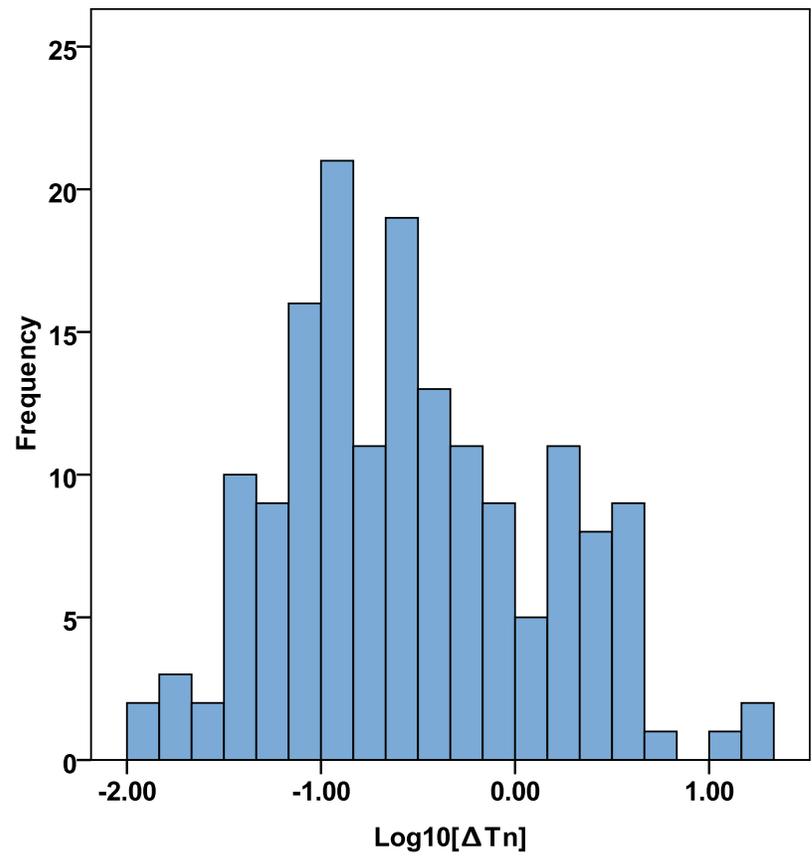


Figure 50: Frequency distribution of log10 transformed Troponin elevation following PCI

Using a stepwise multiple regression analysis, I investigated whether any of the baseline characteristics of age, diabetes, acute presentation, a detectable troponin prior to PCI, stent length or total procedure time had any significant association with Δ Tn. The only factor that was significantly associated with Δ Tn was the length of the procedure (standardised coefficient 0.407, $p = 0.001$). This result was replicated for a threshold of troponin elevation $>10\times$ ULN ($0.4\mu\text{mol/l}$), (standardized coefficient 0.366, $p = 0.001$).

Our predefined primary endpoint was to compare the number of positive troponin events in patients in the upper quartile of each platelet function test with those in the lower quartile. Table 25 summarises the results of these chi-square tests using two thresholds of troponin elevation, either any detectable elevation from prior to PCI or an elevation $>10\times$ the lower limit of detection (ie $0.40\mu\text{mol/l}$). This table shows that only VerifyNow P2Y12 PRU and %inhibition were predictive of troponin elevation at both these thresholds. There were significant differences between the upper and lower quartiles of VASP-PRI response for any detectable troponin elevation post PCI, but this significant difference was lost at the higher threshold of Troponin I. None of the other platelet function assays appeared to predict troponin elevation following PCI. The frequency of troponin elevation for each quartile is shown in Figure 51.

		Chi-square (χ^2) test	
		Trop $>0.04\text{ng/l}$	Trop $>0.4\text{ng/l}$
Log₁₀[TxB₂]_{s-p}	(n = 110)	2.72, p=0.10	0.176, p=0.68
VerifyNow P2Y12 PRU	(n = 117)	6.47, p=0.01	6.83, p=0.01
VerifyNow P2Y12 % inhibition	(n = 116)	6.89, p=0.01	5.93, p=0.02
VASP-PRI	(n = 117)	4.84, p=0.03	0.76, p=0.39
WBPA-ADP	(n = 108)	0.71, p=0.40	0.51, p=0.47
WBPA-Col 2	(n = 116)	0.02, p=0.90	1.61, p=0.30
WBPA-Col 5	(n = 116)	1.38, p=0.24	2.23, p=0.14
WBPA-TRAP	(n = 119)	0.12, p=0.74	0.12, p=0.73

Table 25: Chi-square test comparing troponin elevation between the upper and lower quartiles of each platelet function test.

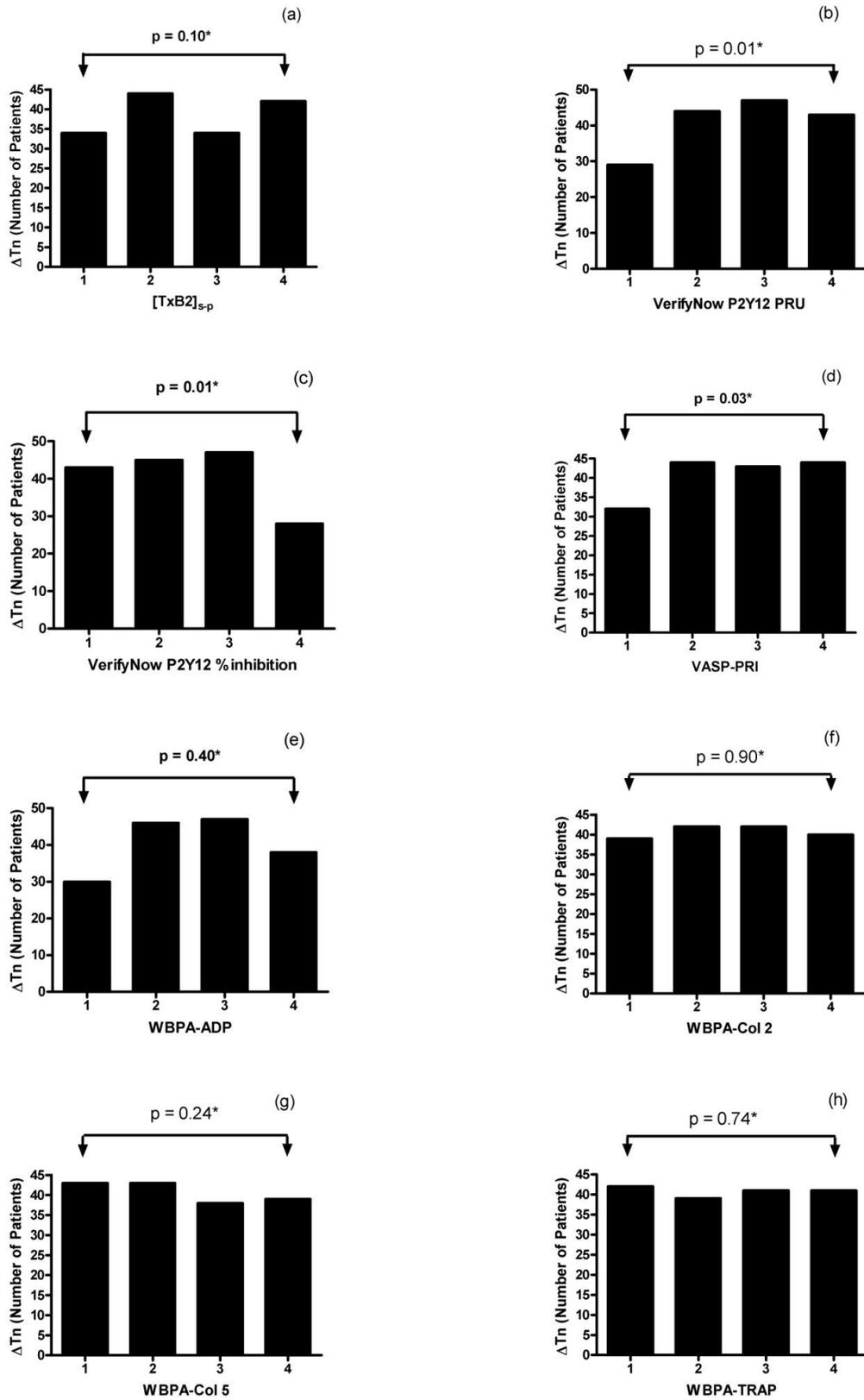


Figure 51: Frequency of troponin I elevation following PCI for each quartile of platelet function assay results.

6.2.6 Discussion

The objective of this research project was to investigate whether response to antiplatelet therapy could predict the incidence of post-procedural myocardial necrosis in patients undergoing coronary intervention. This study has found that a low clopidogrel response assessed by VerifyNow P2Y12 and VASP-PRI is associated with an increased frequency of troponin elevation following PCI.

Of the 323 patients recruited to this project, 236 patients (73%) underwent PCI and were thus eligible for this part of the study. Some elective patients with positive or equivocal non-invasive testing prior to diagnostic angiography were recruited on the basis that they may undergo coronary intervention directly after coronary angiography if the anatomy was suitable. In addition 104/323 (32%) of patients were recruited on the background of an acute presentation and 70/104 (67%) of these patients went on to have coronary intervention. This is a higher percentage than would usually proceed to follow-on coronary intervention following an ACS presentation in our centre but that reflects a selection bias toward ACS patients that I felt would be more likely to undergo intervention given all the available details of their presentation.

Comparison between the cohort of patients undergoing PCI and those initially managed medically showed no statistically significant difference in the baseline demographic or medications (Table 22). A comparison of platelet function assays also showed no significant difference between the two groups.

The interventions performed in the 236 patients are summarised in Table 23. The majority of patients (73%) underwent single vessel PCI and only 2 patients underwent three vessel PCI. There were 5 patients (2%) undergoing PCI to the LMS which reflects recruitment of some patients that were concurrently in the SYNTAX study of PCI vs CABG (220). The majority of patients received coronary intervention with stent implantation. Patients receiving POBA only either had treatment for a lesion that was deemed too small to merit stenting or had additional comorbidities that might preclude the prolonged antiplatelet therapy required following the placement of a drug eluting stent.

The only procedural characteristic that was predictive of troponin elevation was length of procedure. This is certainly an understandable result as these patients are likely to have undergone intervention for complex and challenging anatomy with increased pre and post-stent ballooning. They are also more likely to have suffered a complication such as side branch occlusion or no-reflow demanding additional procedure time. Unfortunately, due to the need for rapid processing of the majority of platelet assays the procedural data was collected retrospectively and operators varied considerably in the extent of the details recorded for each case making more detailed analysis difficult.

Troponin elevation following PCI occurred in 163/236 (69%) of patients. This is a higher figure than that quoted in the literature and is likely to reflect the change in threshold of our troponin assay from 0.2ng/l to 0.04ng/l shortly before recruitment to this study began (221). It is reported that using a high sensitivity troponin assay may significantly increase the number of positive troponin results in this patient group (222,223). It is interesting that 13/165 (8%) of elective patients had a positive troponin prior to coronary intervention; this prompts the question as to whether this group of patients should have a high sensitivity troponin checked routinely prior to angiography. We know a raised troponin is a major predictor of outcome in patients with coronary disease and it's identification may lead to alterations in management.

Although the significance of troponin elevation following coronary intervention remains contentious, a large proportion of patients experience a rise in this specific marker of myocardial damage following coronary intervention. These rises are likely to be multifactorial and will include mechanical complications during PCI and distal embolisation. Despite this, antiplatelet therapy is likely to play an important role in reducing thrombus burden associated with these complications and thus we felt this was an outcome measure that could be explored in a manageable number of patients for a single centre study.

Guidelines have recently suggested a high sensitivity troponin $>5xULN$ in the context of symptoms, ECG changes or a procedural complication as being the definition of periprocedural myocardial infarction, although there remains little evidence that this carries prognostic significance (224). On this basis, I also analysed the data to test for any significant association between troponin elevation $>10xULN$ and platelet function testing.

As Table 25 demonstrates, only VerifyNow PRU and %inhibition were consistently predictive of troponin elevation following PCI. VASP-PRI was predictive of any troponin elevation but this significant result was lost at the higher threshold of troponin. None of the assays used to assess response to aspirin predicted troponin elevation.

As discussed in Chapter 5, more recent larger studies looking at outcome following PCI have found VerifyNow P2Y₁₂ to be the most strongly associated with clinical outcome, in particular using the absolute value of PRU (225). This is in keeping with the data collected and suggests this assay may have a role to play in the clinical management of these patients. The results using the VASP-PRI assay are inconsistent and this again is largely in keeping with other published data (162). It highlights the challenges of using these assays in clinical practice when agreement between them is moderate. As discussed previously, WBPA in response to ADP does not appear to be discriminating enough to use in clinical practice. It is interesting that the difference between the ADP assays VASP-PRI and VerifyNow P2Y₁₂ remains when looking at clinical outcome. The basis for utilising VASP-phosphorylation as a measure of inhibition of the P2Y₁₂ receptor to ADP is sound. The inconsistency with VerifyNow P2Y₁₂ both in terms of response to clopidogrel and in clinical outcome suggests that these two assays may be measuring different things. Indeed it may be that the aggregation based assays are measuring a more complex pathway of response to ADP rather than simply inhibition of the P2Y₁₂ receptor and thus are more indicative of clinical risk. This raises the question as to whether it is clopidogrel response that is important in determining clinical outcome or an overall marker of platelet reactivity. This would be in keeping with subsequently published data. The GRAVITAS study of 2700 patients undergoing PCI for stable angina or ACS showed no clinical benefit to increasing the dose of clopidogrel in those patients determined to have a poor response to clopidogrel determined by VerifyNow P2Y₁₂ PRU >230 (226). In addition, in both elective patients (227) and ACS patients (86,219), alterations in antiplatelet therapy on the basis of clopidogrel response testing did not appear to show any clinical benefit.

The results also show that the measurement of variation in response to aspirin remains an uncertain clinical tool despite promising early data. Neither the WBPA assays nor measurement of TxB₂ concentrations in serum and plasma appeared to predict troponin elevation following PCI. As reported in Chapter 1, a meta-analysis by Krasopoulos *et al*

looking at 20 studies of between 30 and 326 patients has suggested that there is a significantly increased risk of cardiovascular morbidity in patients deemed to be poor responders to aspirin(77). However, this meta-analysis highlights all of the limitations of the available data in this field. Multiple different assays were used in the 20 studies including bleeding time, platelet aggregation, TEG, WBPA, platelet adhesion, VerifyNow Aspirin (using cationic propyl gallate as the agonist) and the PFA-100 system. As has been highlighted in Chapters 1 and 4, agreement between these assays remains poor and thus it draws into question the validity of this analysis. As the author acknowledges, these studies were performed in a variety of patient groups including elective PCI, ACS, stroke patients, those with peripheral vascular disease and in patients undergoing CABG. Finally, it should be noted that 6/20 studies measured aspirin response in patients taking both aspirin and clopidogrel and, in the remainder, patients were taking aspirin monotherapy. However, in only 3/20 studies were patients taking both aspirin and clopidogrel at the time of platelet function testing.

Thus, it is reasonable to suggest that within the cardiovascular community, we remain uncertain regarding the best method to measure aspirin response, the patient population at highest risk from impaired response to aspirin, and whether measurements of aspirin response can predict clinical outcome. In addition, for patients with CAD, and especially those undergoing coronary intervention, the influence of clopidogrel therapy on any test to measure aspirin response remains a significant challenge.

6.3 Conclusion

Chapter 6 has brought together the results of Chapters 4 and 5 measuring variation in response to aspirin and clopidogrel. In Study 6.1 I sought to investigate the interaction between measures of aspirin and clopidogrel response. Most importantly, leading on from the findings of Chapter 4, it was shown that [TxB2]_{S-P} is not related to measurement of clopidogrel response assessed by VASP-PRI and VerifyNow P2Y12 in this much larger cohort of patients on DAPT. This confirms the proposal that [TxB2] may be the best method of assessing response to aspirin in patients taking additional clopidogrel therapy. In Study 6.2, I investigated the subgroup of patients that underwent coronary intervention following recruitment to this research and showed a small but significant increase in the incidence of myocardial necrosis following PCI in those patients with a lower response to clopidogrel assessed by VASP-PRI or VerifyNow P2Y12. Neither [TxB2]_{S-P} nor WBPA were predictive of myocardial necrosis following PCI.

Chapter 7: Response to aspirin and clopidogrel in patients suffering stent thrombosis

7.1 Introduction

As outlined in Chapters 1 and 2, aspirin and clopidogrel have played a major role in the reduction of thrombotic complications associated with coronary intervention. One of the principle concerns of the interventional cardiology community is stent thrombosis. Although current bare metal and third generation drug eluting stent construction together with minimal stenting strategies (including single stent bifurcation techniques) appear to have significantly reduced the risk of stent thrombosis, there remains concern that patients are vulnerable to this complication despite dual antiplatelet therapy (228,229). In addition, the clinical consequences of such an event remain severe (230).

At the time of this research, major concerns were being raised regarding the risk of late stent thrombosis in patients receiving drug eluting stents (231). This led to the publication of more formal definitions of stent thrombosis (ST) for incorporation into clinical trials and publications by the Academic Research Consortium (ARC) (232). In brief, stent thrombosis was classified as definite or probable based on the presentation and whether there was angiographic evidence of thrombosis in or adjacent to the stent. In addition, ST was defined according to time from stent implantation; acute (<24hrs), sub-acute (24hrs-30days), late (30 – 365 days) and very late (>365days).

Evidence has emerged to suggest that this complication may be more common in patients with a poor response to aspirin and clopidogrel but the large numbers of patients required for prospective studies given the small number of events has made it difficult to confirm this with any degree of certainty. Other groups have, however, reported that aspirin and clopidogrel response in those patients that have suffered stent thrombosis is reduced (82,157)

Clearly, poor compliance with therapy is likely to place patients at a higher risk than poor response in itself and this has been addressed by the interventional community through emphatic communication to patients and the associated healthcare team. Nonetheless, recent

data continues to suggest that up to 2% of patients may be non-compliant with DAPT at 30 days following stent implantation (233). It is interesting that rates of stent thrombosis are lower than this suggesting that although antiplatelet therapy is important, multiple other factors, both mechanical and patho-physiological are in play.

As part of this research, I sought to measure response to aspirin and clopidogrel in patients presenting to our institution with definite stent thrombosis at any time point following stent implantation and compare these results to those found in our larger population of patients. Although the numbers were small, this provided a further opportunity to assess the validity of the assays being employed for the potential risk stratification of patients undergoing coronary intervention.

7.2 Patient selection

Attempts were made to recruit all patients attending our institution who had presented with stent thrombosis between March 2006 and May 2007. As the centre was a tertiary referral centre for the West of Scotland, some patients returned to their parent hospital before they could be recruited and have blood samples taken thus reducing the number of possible patients. Exclusion criteria were inability to take aspirin or clopidogrel due to bleeding or hypersensitivity and inability to provide informed consent.

Only patients with angiographically proven stent thrombosis (ARC definite) were included. Patients were recruited following intervention to relieve their acute complication. All patients gave informed consent and the study was given ethical approval by the West Research Ethics Committee.

7.3 Sample collection and processing

Venous blood samples were taken from a large calibre vein in the antecubital fossa using the approach outlined in Chapter 3. All patients received a GPIIb/IIIa antagonist (either abciximab or tirofiban) at the time of acute intervention for stent thrombosis. In view of the profound effects of these agents on aggregation based assays for clopidogrel response, we elected to

wait at least 72 hours before platelet function testing for those patients receiving tirofiban and at least 10 days for those patients receiving abciximab given the respective half-lives of the two agents. Additional exclusion criteria were dipyridamole or NSAID therapy, anaemia (Hb<10g/dl), platelet count <100 or >500 x10⁹/l.

An initial 3.5ml 3.2% sodium citrate tube was collected and then discarded followed by 2 further 3.5ml 3.2% sodium citrate tubes, a 2ml 3.2% sodium citrate tube (supplied by Accumetrics) and by a 3ml glass vacutainer tube containing thrombin in strict sequential order. All samples were immediately mixed by gently inverting 5 times. A 3.5ml citrated tube and the thrombin containing tube were processed to release samples of plasma and serum. (Chapter 3). Between 30 minutes and 2 hours of sampling, the 2ml citrated tube was used to undertake clopidogrel response testing using the VerifyNow P2Y12 system (Chapter 3). A single citrated tube was set aside for the assessment of VASP-PRI within 24hours (Chapter 3).

7.4 Statistical methods

This was an observational study of patients with confirmed stent thrombosis. No formal power calculation was undertaken. Patient demographics, procedural information and assay results were assessed using standard descriptive statistics. Assay results were compared to those seen in a larger population of patients taking aspirin and clopidogrel using an unpaired t-test for normally distributed data or a Mann-Whitney test for non-parametric data.

7.5 Results

10 patients presented to our institution over the course of the recruitment period with definite stent thrombosis and were recruited. The demographics of these patients are given in Table 26. Patients were divided equally between males and females. Only one patient had a history of non-insulin dependent diabetes mellitus. Seven out of 10 patients (70%) were receiving concomitant proton pump inhibitor therapy at the time of stent thrombosis a significantly higher proportion than seen in our general population.

	Stent Thrombosis n=10	Study patients n=323	p value
Age (yrs)			
Mean ± SD	57.2±12.3	64.3±10.2	0.03
Male (%)	5(50)	223(69)	NS
BMI (kg/m²)			
Mean ± SD	25.6 ± 2.3	28.1±5.0	NS
Medical History			
Hypertension (%)	4(40)	170(52.6)	NS
Diabetes (%)	1(10)	54(16.7)	NS
Family history (%)	5(50)	163(50.5)	NS
Dyslipidaemia (%)	6(60)	203(62.8)	NS
Previous TIA/CVA (%)	2(20)	34(10.5)	NS
Previous MI (%)	7(70)	114(35.3)	NS
Previous CABG (%)	0(0)	31(9.6)	NS
Smoking Status			
Current	3(30)	70(21.7)	NS
Medication			
Statin	10(100)	292(90.4)	NS
Beta-blocker	7(70)	250(77.4)	NS
ACE Inhibitor	6(60)	170(52.6)	NS
Proton pump inhibitor	7(70)	86(26.6)	<0.01

Table 26: Patient characteristics of stent thrombosis patients compared to our larger data set of patients tested for aspirin and clopidogrel response.

All patients presented with ST-elevation myocardial infarction and were treated with emergency percutaneous intervention. Two patients underwent unsuccessful thrombolysis with reteplase prior to coronary intervention. All patients received intravenous GPIIb/IIIa antagonist therapy at the time of acute intervention and for 12 hours following the procedure.

The GPIIbIIIa antagonist use was split equally between abciximab and tirofiban. Six patients had received a drug eluting stent in the original procedure with the remaining 4 receiving bare metal stents. Time from the original intervention until stent thrombosis varied considerably Figure 52. In 5 patients, ST occurred <10 days from the date of intervention (3 BMS, 2 DES). The remaining 5 patients had stent thrombosis >220 days from intervention (4 DES, 1 BMS).

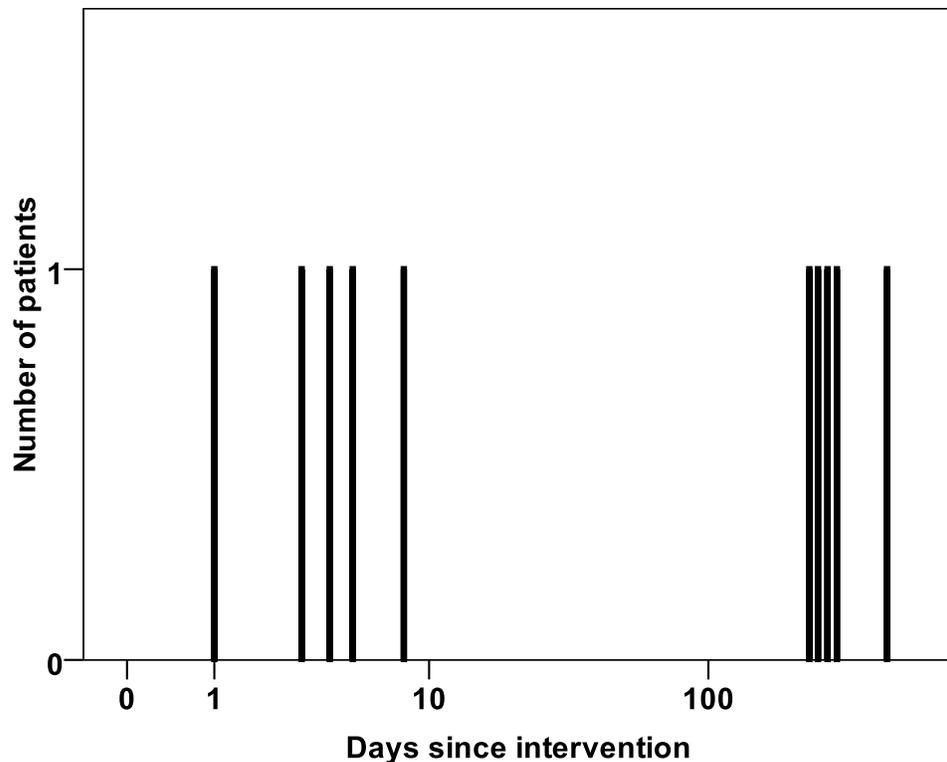


Figure 52: Number of days from stent implantation to stent thrombosis.

IVUS investigation was performed in 4 patients at the time of emergency intervention. Three of these studies revealed stents that were significantly undersized compared to the reference vessel.

No patient admitted non-compliance with aspirin therapy. Four patients had stopped clopidogrel prior to stent thrombosis. One patient had been inadvertently been discharged without clopidogrel following their PCI procedure. One patient admitted to non-compliance with clopidogrel therapy and the other 2 patients had stopped clopidogrel appropriately following their original intervention.

Platelet function assays were carried out between 4 and 18 days from presentation according to the GPIIb/IIIa antagonist that had been used. The results of platelet function testing are given in Table 2. Using non-parametric testing for [TxB₂]_P, [TxB₂]_S and [TxB₂]_{S-P}, significantly higher concentrations of thromboxane were found in all three of these parameters in patients that had suffered from stent thrombosis compared with the larger populations studied in Chapters 4, 5 and 6.

	Stent Thrombosis (n=10)		No Stent Thrombosis (n=323)		p value
	Median	Range	Median	Range	
[TxB ₂] _P (pg/ml)	204	73-1871	125	15-11400	0.04*
[TxB ₂] _S (pg/ml)	2580	525-16282	766	110-36344	<0.01*
[TxB ₂] _{S-P} (pg/ml)	2207	452-15923	628	52-31043	<0.01*
	Mean	±SD	Mean	±SD	
VerifyNow P2Y₁₂ %inhibition	39	25	50	27	0.55 [†]
VerifyNow P2Y₁₂ PRU	193	102	159	80	0.33 [†]
VASP-P PRI %activation	59.5	20.4	53.0	22.8	0.57 [†]
* Mann-Whitney test; [†] Independent sample t-test					

Table 27: Comparison of platelet function assays for patients suffering stent thrombosis.

Neither VerifyNow P2Y₁₂, nor VASP-PRI showed significantly increase platelet activation in response to ADP for all patients suffering a stent thrombosis. However, when just considering the 4 patients suffering sub-acute stent thrombosis (<30 days) on dual antiplatelet therapy there was a poorer response to clopidogrel (VASP-PRI mean 63%, VerifyNow P2Y₁₂ %inhibition mean 26%) but on account of the small number of patients this did not reach statistical significance.

7.6 Discussion and conclusion

I have shown that patients suffering from stent thrombosis who are compliant with aspirin therapy, have significantly increased residual [TxB2]_P, [TxB2]_S and [TxB2]_{S-P}. In the small number of patients suffering sub-acute stent thrombosis (<30 days from the index procedure) who are compliant with clopidogrel there appears to be reduced response to clopidogrel. The data is in keeping with previous findings of a reduced response to clopidogrel in patients that have suffered acute stent thrombosis (82,155-157,200). Clearly, non-compliance with clopidogrel is a factor in the occurrence of stent thrombosis. Since the time of recruitment to this study, both national and international guidelines have been modified to recommend 12 months of dual antiplatelet therapy for patients receiving a drug eluting stent to try and combat the incidence of late stent thrombosis in this patient group. In one of these patients this would not have altered the presentation as their event happened >1 year from original stent implantation.

Although previous studies have sought to measure response to aspirin in patients suffering stent thrombosis, this has been done using aggregation based assays with the agonist arachidonic acid either alone or in combination with ADP (82,84,157). Aggregation based assays have not been validated for the assessment of aspirin response in the presence of additional clopidogrel therapy, and indeed in these studies aspirin response as measured by these assays was not found to be significantly increased in patients with stent thrombosis. However, as outlined in Chapters 4 and 6, we have shown that there is a minimal interaction between measures of [TxB2]_{S-P} and clopidogrel response and it is our contention that this assay provides the best method to assess response to aspirin in patients taking DAPT. The results from this group of patients suffering stent thrombosis while taking regular aspirin therapy would support this. Indeed, it is possible that very late stent thrombosis after DES implantation or late stent thrombosis after BMS implantation are related to failure of aspirin therapy, unmasked by the 'appropriate' withdrawal of clopidogrel treatment.

The finding that the patients suffering stent thrombosis were significantly younger than the larger patient population studied previously supports the finding of higher concentrations of thromboxane in younger patients reported in Chapter 4. In addition, the high prevalence of PPI

use is also concerning given that the interaction between PPIs and clopidogrel has been proposed as a mechanism of stent thrombosis (174).

Chapter 8: Thesis conclusion

The principle aim of the research described in this thesis was to investigate the ability of a variety of platelet function assays to characterise individual variation in the antiplatelet effects of aspirin and clopidogrel. The influence of this variation on outcome following percutaneous coronary intervention was then examined.

As a reflection of platelet inhibition by aspirin, the residual ability of platelets to generate thromboxane B₂, [TxB₂]_{S-P}, was assessed. To do this, I used the novel technique of measuring background concentration of TxB₂ in plasma to try and eliminate the effects of thromboxane generation by other cells and tissues. A wide variation in the level of [TxB₂]_{S-P} was found although absolute concentrations were, in general, an order of magnitude below those seen in normal individuals. Importantly, these results were reproduced following the introduction of clopidogrel. As well as confirming the validity of these measurements, the results also served to support the use of this method of assessing response to aspirin for patients taking DAPT. This is a unique feature of this research as no assay has been shown to represent the efficacy of aspirin in patients taking an additional P₂Y₁₂ receptor inhibitor. Point-of-care assays in the form of WBPA and VerifyNow Aspirin did not correlate well with [TxB₂] and this thesis does not support their use as assays in clinical practice. We did not find a significant association between [TxB₂]_{S-P} and peri-procedural myocardial necrosis following PCI.

Four different assays were used to investigate variation in response to clopidogrel. Correlation between assays was strongest between VASP-PRI and VerifyNow P₂Y₁₂, although it was only moderate; a finding that raises concerns about the potential use of these assays to guide clinical decisions. However, a good response to clopidogrel assessed by both these assays was associated with a significantly lower incidence of peri-procedural myocardial necrosis. From these findings and other reported research, VerifyNow P₂Y₁₂ appears to be the strongest candidate for incorporation into clinical practice although recent clinical trial evidence using the results of VerifyNow P₂Y₁₂ to alter platelet ADP receptor inhibitors has been disappointing.

This raises an interesting dilemma. If one is to believe the data supporting an increased level of cardiovascular risk associated with low clopidogrel response measured by VerifyNow P2Y₁₂, why is the alteration of treatment to more potent P2Y₁₂ receptor inhibition not having a clinical benefit. As well as limitations imposed by the study protocols and the size of the studies, a clue may lie in the lack of significant association with long term clinical outcome seen using VASP-PRI. It is possible that aggregation based assays are not specifically measuring P2Y₁₂ receptor inhibition but rather a more general marker of platelet activation/inhibition in response to ADP. This may point to increased clinical risk, as has again been suggested by recent abstract data presented from the ADAPT-DES registry (234), but making adjustments to P2Y₁₂ receptor inhibition may not significantly lower this risk, as was found in both the GRAVITAS and ARCTIC studies (86,226). It is worth recalling that the pathways involved in platelet activation are very complex (Figure 4).

Nonetheless, aspirin and clopidogrel remain critical therapies in the treatment of cardiovascular disease, particularly for patients undergoing coronary intervention. Thus large scale clinical trial data assessing response to aspirin and clopidogrel are important and it is encouraging that these studies continue to emerge.

This thesis has highlighted a number of other important points. Much of the research in this area has come from relatively small single centre studies. These studies have tended to define an arbitrary cut-off for 'resistance' and have also used several platelet function tests that do not correlate well. Thus meta-analyses based on these small studies supporting their ability to predict clinical outcome may be flawed. It is known that both prasugrel (235) and ticagrelor (236) almost universally inhibit ADP induced platelet aggregation by more than 70%. It is noteworthy, therefore, that >13000 patients in the TRITON-TIMI 38 trial (214) derived only a small cardiovascular benefit from prasugrel in reducing cardiovascular events and >18000 patients with ACS were needed to show a significant benefit of ticagrelor over clopidogrel in the PLATO study (25). A small, single centre study, such as this research, with <350 PCI patients is unlikely to demonstrate a significant clinical impact in these circumstances. This raises the issue of how this type of research is planned and coordinated in order to ensure the data are robust and clinically relevant without relying on subsequent meta-analyses with all their inherent limitations.

The introduction of the newer P2Y₁₂ inhibitors, prasugrel and ticagrelor, into more widespread clinical use will clearly further complicate the situation, and may obviate any concern regarding variable inhibition of platelets to ADP. However, these agents are more expensive and data supporting their use comes from single, large, randomised clinical trials. Further clinical studies are ongoing but TRITON-TIMI 38 has already highlighted that, using these more potent platelet inhibitors, there may be a danger of increased bleeding in certain patient groups such as the elderly or those with a history of previous stroke (214). Thus, clopidogrel is likely to continue to play a major role in the treatment of cardiovascular patients and those undergoing PCI while further clinical trial and registry data accumulates.

Finally, the remarkable contrast between the evolution of aspirin and clopidogrel described in Chapters 1 and 2 shows the great strides evidence based treatment in cardiovascular medicine has made in the last 40 years. Aspirin use has evolved from an anti-inflammatory treatment to a role in cardiovascular disease based initially on observational data from small groups of patients with a multitude of presentations. Meta-analysis of these data supports the widespread use of this therapy. The role of aspirin for the long term primary prevention of cardiovascular disease continues to be challenged but the role of aspirin in coronary intervention is far more secure. In contrast, over the last 15 years, a robust body of clinical trial evidence has emerged to support or refute the use of clopidogrel in virtually all aspects of cardiovascular disease.

8.1 Future directions of research

I believe the further investigation of [TxB2]_{S-P} in large scale clinical studies is warranted. The normalisation of data following logarithmic transformation and the amplification inherent in the pathways of platelet activation suggest that these concentrations may have a significant impact. The data from our small series of stent thrombosis patients adds additional support to this.

The future management of patients with cardiovascular disease will undoubtedly involve more complex models of risk than are currently employed. Antiplatelet therapy may become more individualised based on patient characteristics such as age and BMI as well as comorbidities such as diabetes and pharmaco-genetic profiling. Platelet function testing may play a role in

the assessment of this risk and in guiding therapeutic decisions. Of course, as highlighted above, this risk will include bleeding as well as thromboembolic events and it may be that greater inhibition of platelet aggregation may be harmful in certain groups. This is now also a focus for further research.

Finally, I would suggest that the next obvious target for the cardiovascular community in the field of antiplatelet therapy is to challenge the role of aspirin. Whether an agent can be envisaged that can inhibit thromboxane production by platelets without reducing gastric prostaglandin secretion remains to be seen. However, the use of aspirin instead of clopidogrel for the long term treatment of patients following stent implantation also needs addressed. In treating an aging population of patients with previous coronary intervention who are at increased risk of significant bleeding events it may be that clopidogrel or one of the newer P2Y₁₂ receptor antagonists is the better choice of agent.

Appendix I: Patient information sheet for pilot study

Informed Consent Form

Version number 1.0

September 2005

THIS SHEET HAS BEEN APPROVED BY THE WEST ETHICS COMMITTEE

INFORMATION SHEET FOR PATIENTS/VOLUNTEERS IN CLINICAL RESEARCH PROJECT

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 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. Take time to decide whether or not you wish to take part.

Thank you for reading this.

Brief Title of Project

Resistance to antiplatelet therapy in patients undergoing PCI. A pilot study.

What does this title mean?

We know that blood clots are responsible for heart attacks and strokes. They also occur when patients have stents placed in narrowed arteries (Percutaneous Coronary Intervention , PCI) and may cause damage to the heart during these procedures. We give aspirin and another similar medication called clopidogrel (antiplatelet therapy) to help prevent clots forming and thus the risk of heart attacks, strokes and damage to the heart during and after PCI. However, some patients still experience events. This may be due to resistance to the effects of these therapies.

What is the purpose of the study?

The purpose of this study is to establish that we can measure variations in an individual's response to aspirin and clopidogrel using simple blood tests. This would be done before you undergo PCI and again following the procedure. We would also like to record the amount of damage to the heart muscle after stents have been placed in the narrowed heart arteries. We can measure the amount of damage using a blood test. Importantly none of these tests will affect the drug or stent treatment you receive

The follow-up will be for 12 months at which point you will be telephoned to establish whether you have had any further problems since the procedure was done.

Our hope is that if we can detect patients who are resistance to antiplatelet therapy and, therefore, at higher risk of complications from PCI, we can alter their medication to help reduce this risk.

Why have I been chosen?

You have been chosen to take part in this study because you have had symptoms (such as chest pain), an exercise test or blood tests that suggest you have narrowings in the heart arteries. You may have already had an special x-ray test called an angiogram to confirm this. Your consultant has suggested that you have balloons and stents placed in you arteries to relieve the narrowings.

You are already taking aspirin regularly to try and prevent clots forming in the arteries of the heart. You will also be given another tablet called clopidogrel before you undergo this procedure again to help prevent the formation of blood clots. We want to test your blood to see how well these medications are working before and after you have the procedure.

We intend to recruit 40 patients all of whom will undergo the same blood tests.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

If you are interested in taking part, we will assess your suitability by means of a review of your case notes and previous investigations at the time of your attendance at clinic or whilst on the ward. You will then be asked to read this information leaflet and be given time to decide whether you wish to take part in the study.

All patients who agree to take part in the study, after providing their written consent, will have a full medical history taken, including all medications. They will also undergo a brief physical examination. This should take no more than 15 minutes. We will then carry out a blood test (approx. 2 tablespoons) while you are taking aspirin alone, to check for response to this therapy. (Blood samples will be drawn from the arm and should cause no more than a small jaggging sensation. You would have a blood test at this stage as part of your normal care.) You will then be given clopidogrel as part of the standard preparation prior to PCI.

Immediately before you undergo the procedure we will perform a further blood test to check the response to clopidogrel therapy and the amount of heart damage markers in your blood. You will then undergo the procedure with placement of heart artery balloons and stents. None of the tests we have done will affect the procedure you have done.

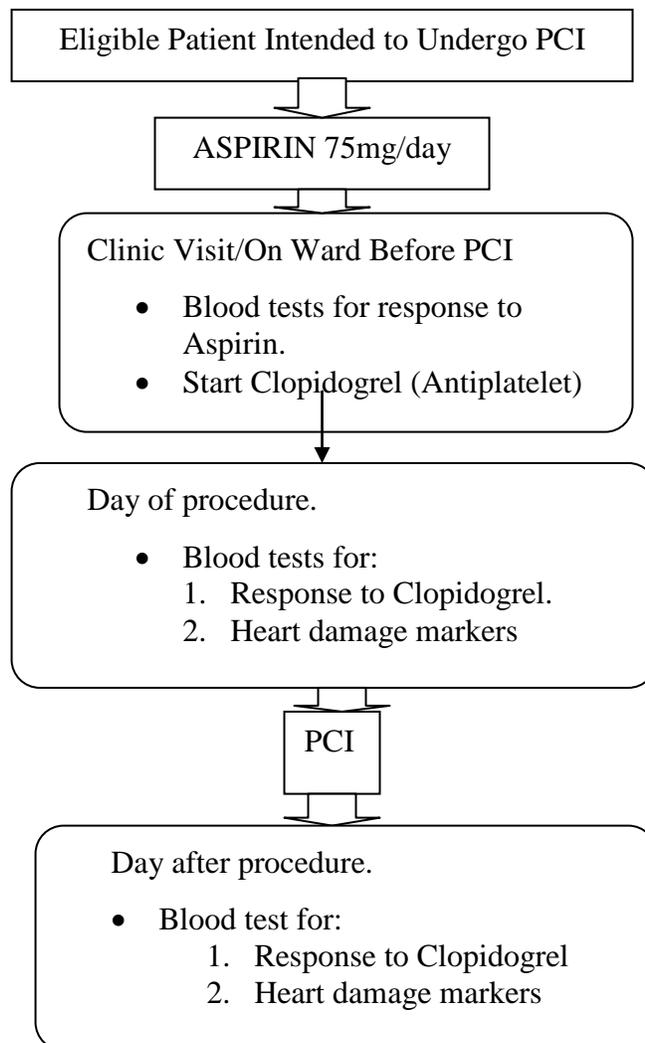
If you do not have any balloons or stents placed in the arteries of the heart then we shall thank you for your participation in this study and nothing else shall be required to be done.

If you have balloons or stents then the day after the procedure we will do a further blood test to check for damage to heart muscle and again measure the response to clopidogrel treatment. You would have a blood test at this stage as part of your normal care.

We will telephone you at home a year after your PCI and ask a few questions regarding your general health, medications and any health problems you have had in this period of time. This should take no more than 10 minutes.

This study does not involve any experimental treatment, there are no restrictions to your lifestyle, and you should continue to live as you normally would.

Flow Chart of Study Design



What are the side effects of any treatment received when taking part?

There are no additional treatments given as part of this study.

What are the potential risks or disadvantages to me if I have these tests performed?

Having your blood taken is occasionally uncomfortable and some people may feel faint. There is a small risk of bleeding, bruising or infection at the puncture site following the blood test.

What are the possible benefits of taking part in this study?

You may not benefit directly from taking part in the study, however the information that we get from this study may help us to improve the treatment of future patients undergoing PCI and may help prevent heart attacks and strokes.
You will not be paid for taking part in this study.

What happens if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the condition being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study, or whether it might be in your best interest to withdraw, or be withdrawn from the study.

What if something goes wrong?

If you are harmed as a result of taking part in this study, there are no special compensation arrangements. If you are harmed as a result of someone's negligence, then you may have grounds for legal action, but you may have to pay for it.
If you wish to complain, or have any concerns about the way you have been treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect your medical care in any way, either now, or in the future.

If you are, or are likely to become, pregnant you should not participate in the study.

Will my taking part in this study be kept confidential?

All information which is collected during the course of the research will be kept strictly confidential. Any information which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

If you decide to take part in this study, your General Practitioner will be advised of your participation and the tests that you will undergo.

What will happen to the results of the research study?

When the final results become available, they will be submitted to medical journals (magazines) where they will be considered for publication. The final results will also be submitted to national and international medical conferences, where they will be considered for presentation.

If you would like a copy of the results, please ask your study doctor.

Who is organising and funding the research?

This research has been sponsored by the West Research Endowments Fund. The medical staff receive no payment for including you in the study.

Who has reviewed the study?

This study has been reviewed by the local Research Ethics Committee. More details can be provided, on request, by your study doctor.

Who can I contact to get more information about this study?

You are encouraged to ask questions at any time during the study.
Please contact:-

Study Doctor: Dr Richard IS Good
Cardiology Research Unit 0141-211 6390

Supervisor: Dr Keith G Oldroyd 0141 211 2337

Independent Doctor: Dr Stephen D Robb 0141 211 1903

Thank you for taking the time to read this patient information leaflet.
You should be given a copy of this and the signed consent form to keep.

Appendix II: Consent form for pilot study

WEST ETHICS COMMITTEE

FORM OF CONSENT FOR PATIENTS/ VOLUNTEERS IN CLINICAL RESEARCH PROJECT

Title of Project:

Resistance to antiplatelet therapy in patients undergoing PCI. A Pilot Study

Please Circle

I confirm that I have read and understand the information sheet dated September 2005 for the above study and have had the opportunity to ask questions. Yes No

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reasons, without my medical care or legal rights being affected. Yes No

I understand that sections of any of my medical notes may be looked at by medical staff co-ordinating and running the study, and I give permission for these individuals to have access to my records. Yes No

I agree to take part in the above study. Yes No

I agree to give permission to store my samples until the tests have been carried out. Yes No

I agree to give permission to inform my GP about my participation in the above study. Yes No

By signing this form you give consent to your participation in the project whose title is at the top of this page. You should have been given a complete explanation of the project to your satisfaction and have been given the opportunity to ask questions. You should have been given a copy of the patient information sheet approved by the West Ethics Committee to read and to keep. Even though you have agreed to take part in the research procedures you may withdraw this consent at any time without the need to explain why and without any prejudice to your care.

Consent:

I,.....(PRINT)

of.....

give my consent to the research procedures above, the nature, purpose and possible consequences of which have been described to me

by....DR RICHARD INNES SHIELDS GOOD.(Study Doctor)

Patient's signature.....Date.....

Doctor's signature.....Date.....

Appendix III: Patient information sheet for main research project

Informed Consent Form

Version number 1.0

January 2006

THIS SHEET HAS BEEN APPROVED BY THE WEST ETHICS COMMITTEE

INFORMATION SHEET FOR PATIENTS/VOLUNTEERS IN CLINICAL RESEARCH PROJECT

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 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. Take time to decide whether or not you wish to take part.

Thank you for reading this.

Brief Title of Project

Does variation in response to oral antiplatelet therapy influence outcome in patients undergoing PCI?

What does this title mean?

We know that blood clots are responsible for heart attacks and strokes. They also occur when patients have stents placed in narrowed arteries (Percutaneous Coronary Intervention , PCI) and may cause damage to the heart during these procedures. We give aspirin and another similar medication called clopidogrel (antiplatelet therapy) to help prevent clots forming and thus the risk of heart attacks, strokes and damage to the heart during and after PCI. However, some patients still experience events. This may be due to resistance to the effects of these therapies.

What is the purpose of the study?

The purpose of this study is to establish that we can measure variations in an individual's response to aspirin and clopidogrel using simple blood tests. This would be done before you undergo PCI and again following the procedure. We would also like to record the amount of damage to the heart muscle after stents have been placed in the narrowed heart arteries. We can measure the amount of damage using a blood test. Importantly none of these tests will affect the drug or stent treatment you receive

The follow-up will be for 6 months at which point you will be telephoned to establish whether you have had any further problems since the procedure was done.

Our hope is that if we can detect patients who are resistance to antiplatelet therapy and, therefore, at higher risk of complications from PCI, we can alter their medication to help reduce this risk.

Why have I been chosen?

You have been chosen to take part in this study because you have had symptoms (such as chest pain), an exercise test or blood tests that suggest you have narrowings in the heart arteries. You may have already had a special x-ray test called an angiogram to confirm this. Your consultant has suggested that you have balloons and stents placed in you arteries to relieve the narrowings.

You are already taking aspirin regularly to try and prevent clots forming in the arteries of the heart. You may have also be given another tablet called clopidogrel before you undergo this procedure again to help prevent the formation of blood clots. We want to test your blood to see how well these medications are working before you have the procedure.

We intend to recruit 310 patients. All 310 patients will have a blood sample immediately before the stenting procedure. If you have not yet started taking clopidogrel we would like to do an additional blood test before you start taking this medication to assess the effects of aspirin alone.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

If you are interested in taking part, we will assess your suitability by means of a review of your case notes and previous investigations at the time of your attendance at clinic or whilst on the ward. You will then be asked to read this information leaflet and be given time to decide whether you wish to take part in the study.

All patients who agree to take part in the study, after providing their written consent, will have a full medical history taken, including all medications. They will also undergo a brief physical examination. This should take no more than 15 minutes. If you have not yet started taking clopidogrel then we will carry out an initial blood test (approx 2 tablespoons) to check the response to aspirin therapy alone. (Blood samples will be drawn from the arm and should cause no more than a small jaggging sensation. You would have a blood test at this stage as part of your normal care.) You will then be given clopidogrel as part of the standard preparation prior to PCI.

Immediately before you undergo the procedure we will perform a further blood test to check the response to clopidogrel and aspirin therapy together and the amount of heart damage markers in your blood. You will then undergo the procedure with placement of heart artery balloons and stents. None of the tests we have done will affect the procedure you have done.

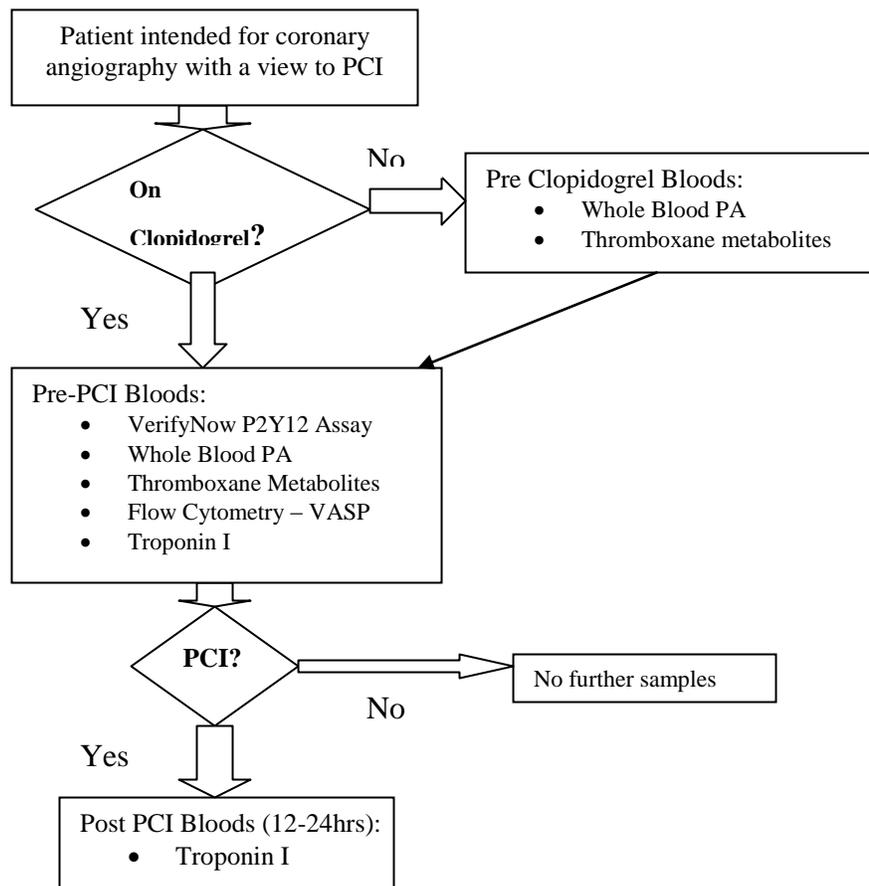
If you do not have any balloons or stents placed in the arteries of the heart then we shall thank you for your participation in this study and nothing else shall be required to be done.

If you have balloons or stents then, the day after the procedure, we will do a further blood test to check for damage to heart muscle. You would have a blood test at this stage as part of your normal care.

We will telephone you at home 6 months after your PCI and ask a few questions regarding your general health, medications and any health problems you have had in this period of time. This should take no more than 10 minutes.

This study does not involve any experimental treatment, there are no restrictions to your lifestyle, and you should continue to live as you normally would.

Flow Chart of Study Design



What are the side effects of any treatment received when taking part?

There are no additional treatments given as part of this study.

What are the potential risks or disadvantages to me if I have these tests performed?

Having your blood taken is occasionally uncomfortable and some people may feel faint. There is a small risk of bleeding, bruising or infection at the puncture site following the blood test.

What are the possible benefits of taking part in this study?

You may not benefit directly from taking part in the study, however the information that we get from this study may help us to improve the treatment of future patients undergoing PCI and may help prevent heart attacks and strokes.
You will not be paid for taking part in this study.

What happens if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the condition being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study, or whether it might be in your best interest to withdraw, or be withdrawn from the study.

What if something goes wrong?

If you are harmed as a result of taking part in this study, there are no special compensation arrangements. If you are harmed as a result of someone's negligence, then you may have grounds for legal action, but you may have to pay for it.
If you wish to complain, or have any concerns about the way you have been treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect your medical care in any way, either now, or in the future.

If you are, or are likely to become, pregnant you should not participate in the study.

Will my taking part in this study be kept confidential?

All information which is collected during the course of the research will be kept strictly confidential. Any information which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

When the final results become available, they will be submitted to medical journals (magazines) where they will be considered for publication. The final results will also be submitted to national and international medical conferences, where they will be considered for presentation.

If you would like a copy of the results, please ask your study doctor.

Who is organising and funding the research?

This research has been sponsored by the West Research Endowments Fund. The medical staff receive no payment for including you in the study.

Who has reviewed the study?

This study has been reviewed by the local Research Ethics Committee. More details can be provided, on request, by your study doctor.

Who can I contact to get more information about this study?

You are encouraged to ask questions at any time during the study.
Please contact:-

Study Doctor: Dr Richard IS Good
 Cardiology Research Unit 0141-211 6390

Supervisor: Dr Keith G Oldroyd 0141 211 2337

Independent Doctor: Dr Stephen D Robb 0141 211 1903

Thank you for taking the time to read this patient information leaflet.
You should be given a copy of this and the signed consent form to keep.

Appendix IV: Consent form for main research project

WEST ETHICS COMMITTEE

FORM OF CONSENT FOR PATIENTS/ VOLUNTEERS IN CLINICAL RESEARCH PROJECT

Title of Project:

Does variation in response to oral antiplatelet therapy influence outcome in patients undergoing PCI?

Please Circle

I confirm that I have read and understand the information sheet dated January 2006 for the above study and have had the opportunity to ask questions. Yes No

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reasons, without my medical care or legal rights being affected. Yes No

I understand that sections of any of my medical notes may be looked at by medical staff co-ordinating and running the study, and I give permission for these individuals to have access to my records. Yes No

I agree to take part in the above study. Yes No

I agree to give permission to store my samples until the tests have been carried out. Yes No

By signing this form you give consent to your participation in the project whose title is at the top of this page. You should have been given a complete explanation of the project to your satisfaction and have been given the opportunity to ask questions. You should have been given a copy of the patient information sheet approved by the West Ethics Committee to read and to keep. Even though you have agreed to take part in the research procedures you may withdraw this consent at any time without the need to explain why and without any prejudice to your care.

Consent:

I,.....(PRINT)

of.....

give my consent to the research procedures above, the nature, purpose and possible consequences of which have been described to me

by....DR RICHARD INNES SHIELDS GOOD.(Study Doctor)

Patient's signature.....Date.....

Doctor's signature.....Date.....

Appendix V: Patient information sheet for stent thrombosis study

Informed Consent Form

Version number 1.0

June 2006

THIS SHEET HAS BEEN APPROVED BY THE WEST ETHICS COMMITTEE

INFORMATION SHEET FOR PATIENTS/VOLUNTEERS IN CLINICAL RESEARCH PROJECT

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 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. Take time to decide whether or not you wish to take part.

Thank you for reading this.

Brief Title of Project

Platelet inhibition in patients following stent thrombosis.

What does this title mean?

When patients have stents placed in coronary arteries (Percutaneous Coronary Intervention, PCI) we give aspirin and another similar medication called clopidogrel (antiplatelet therapy) to help prevent clots forming and the stents suddenly blocking. Despite this, a small number of stents still block off (stent thrombosis.) This may be due to resistance to the effects of these therapies.

What is the purpose of the study?

We now have special tests that can measure how well aspirin and clopidogrel are preventing the formation of blood clots. The purpose of this study is to establish whether, in patients who have suddenly blocked a stent, there is a reduced effect of aspirin and clopidogrel.

Our hope is that if we can demonstrate that patients who suffer stent thrombosis have a reduced response to antiplatelet therapy, we may be able to alter their medication to help prevent this complication in the future.

Why have I been chosen?

You have been chosen to take part in this study because you have previously had a stent placed in your heart arteries and this stent suddenly blocked. We intend to recruit all local patients who have had this complication.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

If you are interested in taking part, we will assess your suitability by means of a review of your case notes and previous investigations at the time of your attendance at clinic or whilst on the ward. You will then be asked to read this information leaflet and be given time to decide whether you wish to take part in the study.

All patients who agree to take part in the study, after providing their written consent, will have a full medical history taken, including all medications. They will also undergo a brief physical examination. This should take no more than 15 minutes.

Patients who agree to take part in the study will have a simple blood test carried out. Blood samples (approximately 2 tablespoons) will be drawn from the arm and should cause no more than a small jaggging sensation.

Following this we will thank you for your participation in the study and nothing else shall be required to be done.

This study does not involve any experimental treatment, there are no restrictions to your lifestyle, and you should continue to live as you normally would.

What are the side effects of any treatment received when taking part?

There are no additional treatments given as part of this study.

What are the potential risks or disadvantages to me if I have these tests performed?

Having your blood taken is occasionally uncomfortable and some people may feel faint. There is a small risk of bleeding, bruising or infection at the puncture site following the blood test.

What are the possible benefits of taking part in this study?

You may not benefit directly from taking part in the study, however the information that we get from this study may help us to improve the treatment of future patients undergoing PCI and may help prevent heart attacks and strokes.
You will not be paid for taking part in this study.

What happens if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the condition being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study, or whether it might be in your best interest to withdraw, or be withdrawn from the study.

What if something goes wrong?

If you are harmed as a result of taking part in this study, there are no special compensation arrangements. If you are harmed as a result of someone's negligence, then you may have grounds for legal action, but you may have to pay for it.

If you wish to complain, or have any concerns about the way you have been treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect your medical care in any way, either now, or in the future.

If you are, or are likely to become, pregnant you should not participate in the study.

Will my taking part in this study be kept confidential?

All information which is collected during the course of the research will be kept strictly confidential. Any information which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

When the final results become available, they will be submitted to medical journals (magazines) where they will be considered for publication. The final results will also be submitted to national and international medical conferences, where they will be considered for presentation.

If you would like a copy of the results, please ask your study doctor.

Who is organising and funding the research?

This research has been sponsored by the West Research Endowments Fund. The medical staff receive no payment for including you in the study.

Who has reviewed the study?

This study has been reviewed by the local Research Ethics Committee. More details can be provided, on request, by your study doctor.

Who can I contact to get more information about this study?

You are encouraged to ask questions at any time during the study.
Please contact:-

Study Doctor: Dr Richard IS Good
 Cardiology Research Unit 0141-211 6390

Supervisor: Dr Keith G Oldroyd 0141 211 2337

Independent Doctor: Dr Stephen D Robb 0141 211 1903

Thank you for taking the time to read this patient information leaflet.
You should be given a copy of this and the signed consent form to keep.

Appendix VI: Consent form for stent thrombosis study

WEST ETHICS COMMITTEE

FORM OF CONSENT FOR PATIENTS/ VOLUNTEERS IN CLINICAL RESEARCH PROJECT

Title of Project:

Platelet inhibition in patients following stent thrombosis.

Please Circle

- | | | |
|--|-----|----|
| I confirm that I have read and understand the information sheet dated June 2006 for the above study and have had the opportunity to ask questions. | Yes | No |
| I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reasons, without my medical care or legal rights being affected. | Yes | No |
| I understand that sections of any of my medical notes may be looked at by medical staff co-ordinating and running the study, and I give permission for these individuals to have access to my records. | Yes | No |
| I agree to take part in the above study. | Yes | No |
| I agree to give permission to store my samples until the tests have been carried out. | Yes | No |

By signing this form you give consent to your participation in the project whose title is at the top of this page. You should have been given a complete explanation of the project to your satisfaction and have been given the opportunity to ask questions. You should have been given a copy of the patient information sheet approved by the West Ethics Committee to read and to keep. Even though you have agreed to take part in the research procedures you may withdraw this consent at any time without the need to explain why and without any prejudice to your care.

Consent:

I.....(PRINT)

of.....

give my consent to the research procedures above, the nature, purpose and possible consequences of which have been described to me

by....DR RICHARD INNES SHIELDS GOOD.(Study Doctor)

Patient's signature.....Date.....

Doctor's signature.....Date.....

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Publications containing work undertaken for this thesis

Good RIS, McGarrity A, Sheehan R, James TE, Miller H, Stephens J, Watkins S, McConnachie A, Goodall AH, Oldroyd KG. Variation in thromboxane B2 concentrations in serum and plasma in patients taking regular aspirin before and after clopidogrel therapy. *Accepted, Platelets, November 2013*

Presentations to Learned Societies of work undertaken for this thesis

Good RIS, Goodall AH, Oldroyd KG. The effect of clopidogrel on the generation of thromboxane B2 in patients taking regular, low dose aspirin therapy. *Poster presentation, ESC World Congress of Cardiology, Paris, August 2011*

Good RIS, McGarrity A, Miller H, Hillis WS, Goodall AH, Oldroyd KG. Assessing individual response to clopidogrel in patients undergoing coronary intervention. *Poster presentation, American College of Cardiology Congress, New Orleans, March 2007*

Good RIS, McGarrity A, Miller H, Hillis WS, Goodall AH, Oldroyd KG. Platelet inhibition by clopidogrel is not affected by concurrent statin or ACE inhibitor therapy. *Poster presentation to the Medical Research Society, February 2007*

Good RIS, McGarrity A, Miller H, Hillis WS, Goodall AH, Oldroyd KG. Assessing the response to clopidogrel in patients undergoing coronary intervention. *Poster presentation at the 8th UK Platelet Meeting, September 2006 (Abstract, Platelets)*

Good RIS, McGarrity A, Miller H, Hillis WS, Goodall AH, Oldroyd KG. Aspirin resistance in cardiovascular patients; comparison between a novel point-of-care assay and generation of thromboxane B2. *Poster presentation at the 8th UK Platelet Meeting, September 2006 (Abstract, Platelets)*

Good RIS, McGarrity A, Miller H, McConnachie A, Hillis WS, Goodall AH, Oldroyd KG. Measuring response to clopidogrel in patients undergoing coronary intervention; a comparison of two methods. *Oral presentation, British Cardiac Society, June 2007*

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