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The Clinical Application Of Short Thrombelastography Platelet Function Testing In Cardiovascular Disease

by

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ABSTRACT

Platelets are key mediators in the pathogenesis of atherothrombosis. Antiplatelet therapy (APT) with aspirin and a P2Y12 receptor inhibitor is the cornerstone of treatment in patients with cardiovascular disease (CVD) and following percutaneous coronary intervention with stent implantation. Clinical studies have consistently demonstrated heterogeneity in responses to APT determined using *ex vivo* platelet function tests and reduced responsiveness is overwhelmingly associated with increased risk of adverse events including stent thrombosis (ST) and cardiovascular death. Nonetheless, assessment of responses to APT with a view to providing tailored treatment is currently not undertaken in routine clinical practice largely due to the lack consensus regarding the most appropriate platelet function test as well as controversy surrounding the optimal definition of APT hyporesponsiveness.

The objectives of the studies in this thesis are: Firstly, to determine the reproducibility and reliability of a well validated point-of-care platelet function test known as Short thrombelastography (TEG) in the assessment of responses to APT. Secondly, to employ Short TEG as a clinical tool to provide tailored APT in a consecutive series of patients admitted acutely with ST. Thirdly, to use Short TEG to conduct a series of clinically relevant experiments in patients with CVD as well as in healthy volunteers. Specifically, I investigated: (i) the effect of clopidogrel cessation one year after drug-eluting stent implantation on platelet reactivity and vascular inflammation, (ii) whether the pharmacological response to aspirin in patients with acute ischaemic stroke can be reliably determined from a functional test of AA-induced whole blood clotting, and (iii) whether there are any significant inter- or intra-individual differences in the antiplatelet effect of Plavix® (clopidogrel hydrogen sulphate) versus the cheaper generic clopidogrel salts that are in widespread clinical use in patients with CVD despite limited data to support their efficacy.

The results generated from these studies not only lend support to the concept of routine testing of responses to APT using a standardised and reliable test with a view to providing tailored therapy for *all*, but also provide insights into potential mechanisms by which P2Y12 receptor antagonists elicit their antiplatelet effects and, specifically, their interactions with aspirin. Our study findings could form the basis of large scale clinical trials that may have a significant impact on the future of APT prescribing in CVD.

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AUTHOR'S DECLARATION

I, Nalyaka Sambu, declare that the thesis entitled '**The clinical application of Short Thrombelastography platelet function testing in cardiovascular disease'** and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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

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

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

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

- I have acknowledged all main sources of help;

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

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

Signed: ……..

Date:…….

PUBLICATIONS IN PEER REVIEWED JOURNALS

Original papers

1. Sambu N, Radhakrishnan A, Englyst N, Weir N, Curzen N. "Aspirin resistance" in ischaemic stroke: Insights using short thrombelastography. *Journal of Stroke and Cerebrovascular Diseases*. 2013 Jul 16. In press

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ABBREVIATIONS

AA	Arachidonic acid
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
APT	Antiplatelet therapy
ATP	Adenosine triphosphate
AUC15	Area under the curve at 15 minutes
BMS	Bare metal stents
CABG	Coronary artery bypass graft surgery
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
COX	Cyclo-oxygenase
CT	Computed Tomography
CVD	Cardiovascular disease
CYP	Cytochrome
DES	Drug-eluting stents
ELISA	Enzyme-linked immunosorbent assay
ESC	European Society of Cardiology
FDA	Food and drug administration
GP	Glycoprotein
hsCRP	High sensitivity C-reactive protein
IL-6	Interleukin 6
LACS	Lacunar Stroke
LTA	Light transmittance aggregometry
MA	Maximum amplitude

MACE	Major adverse cardiovascular events
MI	Myocardial infarction
MRS	Modified Rankin Scale
NSTEMI	Non ST elevation myocardial infarction
PACS	Partial Anterior Circulation Stroke
PAR	Protease activated receptor
PCI	Percutaneous coronary intervention
PFA	Platelet function analyser
PKA	Protein kinase A
POCS	Posterior Circulation Stroke
PVD	Peripheral vascular disease
sCD40L	Soluble CD40 ligand
ST	Stent thrombosis
STEMI	ST elevation myocardial infarction
TACS	Total Anterior Circulation Stroke
TEG	Thrombelastography
TIA	Transient ischaemic attack
TNF	Tumour necrosis factor
TXA2	Thromboxane A2
VASP	Vasodilator-stimulated phosphoprotein
VWF	von Willebrand factor

CHAPTER 1: INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death and disability worldwide. The World Health Organisation estimates that by 2030 almost 24 million people will succumb to CVD. In Europe alone, it is responsible for over 4 million deaths per year accounting for almost 50 percent of all mortality. As such, research and development in modern medicine is largely focused on preventative and therapeutic strategies that will ultimately attenuate the scale of this mounting problem. The spectrum of CVD comprises coronary artery disease (CAD), which includes stable angina and acute coronary syndromes (ACS), ischaemic stroke and peripheral vascular disease (PVD). It is well established that platelets play an integral role in the onset, development and progression of these disease conditions and, thus, therapeutic targets for primary and secondary prevention of CVD are mainly focused on the multiple complex pathways involved in platelet activation and aggregation.

It is well documented that antiplatelet therapy (APT) in CVD improves clinical outcome, and this beneficial effect is particularly observed in those patients with CAD treated with percutaneous coronary intervention (PCI) and stent implantation. The latter are an important patient group because coronary artery stenting can be associated with the potentially fatal complication of stent thrombosis (ST). Although the aetiology of ST is multifactorial, it is well recognised that suboptimal or premature discontinuation of APT is one of the leading and potentially avoidable causes. Nonetheless, the choice of APT regime and optimal duration of treatment in patients undergoing PCI remains to be established and is the subject of ongoing debate.

Aspirin is undisputably the most widely prescribed antiplatelet agent across the entire spectrum of CVD. However, the previous decade has witnessed significant advances in the development of novel, more potent agents that have recently been incorporated into evidence-based clinical practice guidelines. Although these potent therapies have been shown to be more efficacious in clinical trials, they are associated with increased bleeding risks and, therefore, should not be used indiscriminately in all patient groups. As such, selection of the most appropriate antiplatelet agent for the individual patient should be based on a balance between safety and efficacy, but it remains to be determined how best to achieve this.

Clinical studies have consistently demonstrated significant heterogeneity in individual patient responses to APT, determined using various laboratory tests of platelet function and, furthermore, a clear link between inadequate response and increased risk of adverse cardiovascular events has been established. Nonetheless, clinical guidelines recommend standard doses of APT in all patients rather than optimising pharmacotherapy by providing individually tailored treatment guided by platelet function testing. This is mainly due to the lack of consensus regarding the most appropriate and reliable test that can be employed in frontline clinical practice to measure individual responses to APT, as well as controversy surrounding the optimal definition of inadequate response to APT. Further data are required from large scale clinical studies to provide some clarity on these issues.

Thus, there remain several unanswered clinical questions in the vast field of APT prescribing in CVD. Specifically:

a) What is the optimal APT regime and duration of treatment in patients with CVD, particularly those patients undergoing PCI who may be at risk of ST?

b) Given the advent of newer, more potent APTs, should aspirin remain the default antiplatelet agent or can its role as the default APT now be challenged?

c) How and in whom should responses to APT be measured in routine clinical practice and what cut-off levels of platelet inhibition could be considered adequate for a given clinical situation?

d) Is there a role for individually tailored APT based on platelet function testing and does this strategy result in improved long term clinical outcome in all patient groups?

The objectives of this thesis are to explore some of the above pertinent issues in a wide ranging group of subjects including: (i) patients with acute ST, (ii) stable patients on dual APT who have previously undergone PCI, (iii) patients with acute ischaemic stroke on aspirin, and (iv) healthy volunteers. Most of the work in this thesis has now been published in peer reviewed journals and, furthermore, has generated clinically relevant questions that may set the stage for future large scale clinical investigation.

1.1 The role of platelets

Platelets were first described in 1865 by Professor Max Schultze (1825-1874) and their specific role in haemostasis was subsequently established by Dr. Giulio Bizzozero (1846-1901) (1). Platelets are produced from bone marrow megakaryocytes at a rate of 10^{11} per day and have a normal lifespan of 7 to 10 days (2). They are anucleate cells that lack genomic DNA but contain megakaryocyte-derived messenger RNA required for protein synthesis. Platelets also contain mitochondria and several types of granules, the contents of which are released following platelet activation. Platelets play an integral role in:

a) Normal physiologic haemostasis, which occurs when platelets adhere to injured vessels walls, thereby restricting blood loss

b) Pathological atherothrombosis, which precipitates the acute clinical manifestations of myocardial infarction (MI), ischaemic stroke and PVD

c) Chronic inflammation, which underlies the formation and extension of atherosclerotic plaque

As first described vividly by Bizzozero in 1882 following a series of experiments: "*blood platelets, swept along by the blood stream, are held up at the damaged spot as soon as they arrive at it. At first, one sees only 2 to 4 to 6 (platelets); very soon the number climbs to hundreds…. little by little the volume increases and soon the thrombus fills the lumen of the blood vessel, and impedes the blood stream more and more…*"

In the undisturbed biological system, circulating platelets are quiescent. However, vascular injury secondary to insults such as spontaneous plaque rupture or PCI-induced endothelial damage allow exposure of the circulating platelets to the subendothelium thereby triggering platelet recruitment, activation and aggregation, a process that is mediated via multiple pathways described below.

1.1.1 Platelets in haemostasis

Circulating platelets recognise sites of vascular injury and adhere to the damaged vessel wall. The initial tethering of platelets is mediated by the interaction of the platelet membrane glycoprotein (GP) receptors with subendothelial matrix proteins

including von Willebrand factor (VWF) and collagen (3). The adherent platelets then become activated by a variety of agonists such as adenosine diphosphate (ADP), thromboxane A2 (TXA2) and thrombin, which bind to their distinct G-protein coupled receptors on the platelet surface (4). This results in the release of platelet granule constituents including ADP, adenosine triphosphate (ATP) and serotonin from dense granules, as well as fibrinogen, VWF, thrombospondin, growth factors and pro-coagulants from alpha granules. Furthermore, activated platelets also synthesize (*de novo*) and release the potent platelet activator TXA2. All the released products of platelet activation promote recruitment of additional platelets from the circulation to the site of vascular injury leading to potentiation and amplification of platelet aggregation and hence formation of a growing haemostatic plug. This platelet plug is anchored and stabilized by fibrinogen or VWF which build a network of inter-platelet bridges by binding to the platelet membrane receptors located in the GP IIb/IIIa integrin complex (5).

1.1.2 Platelets in plaque formation

Normal healthy vascular endothelium prevents platelet activation and adhesion through the production of inhibitory prostaglandins and nitric oxide. However, local disturbance in blood flow (for example, at the site of coronary artery bifurcations and/or in the setting of cardiovascular risk factors) can lead to minor injury of the vascular endothelium thereby exposing the subendothelium to circulating platelets. As described previously, the subendothelial matrix contains substances such as VWF and collagen that promote platelet activation and aggregation (3,4). Adherent platelets undergo conformational change that includes secretion of dense and alpha granules and this leads to an increase in vascular permeability, vascular smooth muscle proliferation and migration into the intimal layer. This is the process involved in initial plaque formation which, in itself, leads to further disturbance in local blood flow, further injury to the endothelium leading to further increase in platelet activity, inevitably resulting in a vicious cycle that leads to the progression of plaque formation.

1.1.3 Platelets in thrombosis

Atherosclerotic plaques are rich in collagen, fibronectin, thrombospondin and tissue factor. Erosion or rupture of an atherosclerotic plaque results in exposure of these thrombogenic substrates to circulating platelets, thereby promoting platelet adhesion and activation via specific receptors (described in section 1.1.1 above). Activated platelets promote the formation of thrombin from prothrombin. Thrombin is the most potent platelet activator and works via the protease activated receptors on the cell membrane of the platelet. Thrombin converts soluble fibrinogen into insoluble strands of fibrin. This leads to further platelet activation, aggregation and formation of a platelet and fibrin plug. Furthermore, activated platelets release substances (such as plasminogen activator inhibitor-1) which inhibit fibrinolysis and can lead to uncontrolled platelet activation and aggregation resulting in intraluminal thrombus, ischaemia and infarction.

1.1.4 Platelets in inflammation

Inflammation is characterised by a multitude of interactions between leucocytes, endothelial cells and platelets and, irrespective of its aetiology, inflammation results in endothelial activation. Inflammatory mediators trigger an imbalance between pro-coagulant and anticoagulant properties of the endothelium that can lead to local stimulation of the coagulation cascade, thereby affecting platelet function and thus thrombus formation. As previously described, circulating platelets are rapidly recruited to sites of vascular injury. During adhesion to damaged endothelium, activated platelets secrete pro-inflammatory cytokines, chemokines and other inflammatory mediators which promote chemotaxis, lead to leucocyte adhesion to the endothelium, and promote leukocyte activation and subsequent migration into subendothelial tissue (6,7). In addition, platelets in atherosclerotic lesions secrete a variety of substances including platelet activating factor and macrophage inflammatory protein-1α which promote further leukocyte chemotaxis, as well as growth factors and serotonin which stimulate the synthesis of smooth muscle cells, fibroblasts and collagen. Thus, platelets play an important role in inflammation by inducing a pro-inflammatory and pro-adhesive state in endothelial cells and leukocytes.

1.2 The evolution of antiplatelet therapy

APTs are the cornerstone for: (i) medical management of patients with ACS and stable CAD, (ii) protection against thrombotic complications of PCI, (iii) secondary prevention of ischaemic stroke, and (iv) treatment of acute and chronic PVD. The mechanisms by which antiplatelet drugs achieve their desired effect involves targeting the specific enzymes or receptors that are essential for the initiation of platelet activation and propagation of thrombus formation. The preceding decade has witnessed significant advances in the development and evolution of APT for the treatment of CVD and current clinical practice guidelines are based on robust data from landmark clinical trials. Antiplatelet agents can generally be categorised as follows:

a) Acetylsalicylic acid: aspirin

b) Thienopyridines: ticlopidine, clopidogrel and prasugrel

c) Reversible P2Y12 inhibitors: ticagrelor, cangrelor and elinogrel

d) GP IIb/IIIa inhibitors: abciximab, eptifibatide and tirofiban

e) Protease activated receptor (PAR)-1 antagonists: vorapaxar and atopaxar

f) Phosphodiesterase inhibitors: Cilostazol

g) Dipyridamole

Below is an outline of the antiplatelet agents in widespread clinical use, including novel therapies that have recently been approved for routine use and emerging therapies that are currently under investigation. We have excluded agents d) to g) from the discussion below as this is outside the remit of the work in this thesis.

1.2.1 Aspirin

Aspirin (acetylsalicylic acid) was first developed in the 1850's and subsequently patented and marketed by Bayer in 1889. For over 50 years, aspirin has been the foundation of APT and remains the most widely prescribed antiplatelet agent. Aspirin causes platelet inhibition by irreversible acetylation of a serine residue at position 529

on the cyclo-oxygenase (COX)-1 enzyme. This action prevents conversion of arachidonic acid (AA) to prostaglandin–H, thereby blocking the production of TXA2, a potent vasoconstrictor and stimulator of platelet aggregation (8). There are two distinct isoforms of the COX enzyme: COX-1 is constitutively expressed in most tissues and is the only functioning COX in platelets; whereas COX-2 is an inducible form of the COX enzyme that is undetectable in most tissues under normal physiological conditions but its expression is increased by stimuli that are implicated in the development of atherosclerosis and inflammation (9). Aspirin is an approximately 200-fold more potent inhibitor of the platelet enzyme COX-1 compared to the COX-2 isoform, hence the different dosage requirements of aspirin as an antithrombotic (COX-1) versus anti-inflammatory drug (COX-2) (10).

Aspirin has a rapid onset of action with a half-life in the human circulation of 20 minutes. However, due to its irreversible and, therefore sustained, antithrombotic effect it can be administered every 24 to 48 hours. Early human studies of platelet COX-1 inactivation have suggested that the antithrombotic effect of aspirin is saturable at doses in the range of 75 to 100mg (11,12).

Clinical trials have consistently demonstrated the beneficial effects of aspirin in a variety of clinical settings. Specifically, the Antithrombotic Trialist's Collaboration (13) which systematically reviewed 287 randomised trials in a heterogeneous cohort of patients reported an approximately 25% relative risk reduction in the cumulative incidence of death, MI or stroke with aspirin 75mg daily versus placebo. Of note, the majority of patients included in this meta-analysis had a previous history of CVD. Similarly, with regards to acute ST elevation MI (STEMI) patients, the landmark ISIS-2 study (14) showed that aspirin, in addition to fibrinolysis, was associated with significantly fewer deaths (8% vs. 13.2%), re-infarction (1.8% vs. 2.9%) and stroke (0.6% vs. 1.1%) compared with placebo.

Furthermore, aspirin remains unchallenged as the cornerstone of treatment following PCI, to attenuate the risk of the potentially catastrophic complication of ST. In the early days, patients routinely received either aspirin monotherapy or a combination of aspirin and complex anticoagulant regimes with disappointing results. Specifically, a high incidence of acute ST (with rates of up to 20%) were observed with aspirin monotherapy and this led to the advent of numerous clinical trials investigating the potential additive benefit of dual APT with aspirin and a thienopyridine (outlined in section 11.2.2 below). Data from these studies unequivocally demonstrated a

significant reduction in the incidence of ST and adverse ischaemic events with dual APT regimes (15-18) and this led to the development of clinical guidelines which recommended a combination of aspirin and a thienopyridine in all patients undergoing PCI.

Although the efficacy of aspirin in secondary prevention of CVD and following PCI is well established, its role in primary prevention is less certain (19-22). Recent data from primary prevention studies have failed to establish a clear benefit with aspirin compared to placebo and, furthermore, aspirin in this setting is associated with an unnecessary increased risk of gastrointestinal bleeding and haemorrhagic stroke (22-25). Thus, although aspirin is not routinely recommended for the primary prevention of CVD (26) this is a subject of ongoing debate, particularly in patients who are deemed to be at relatively higher risk of a primary cardiovascular event.

1.2.2 Thienopyridines

Ticlopidine, clopidogrel and prasugrel represent three generations of thienopyridines that achieve their antiplatelet effect by selective and irreversible inhibition of the ADP P2Y12 receptor. They are inactive prodrugs that require oxidation to their active metabolite via the hepatic cytochrome P450 system. The active metabolite irreversibly binds to the platelet P2Y12 receptor, thereby inhibiting ADP-induced platelet aggregation.

1.2.2.1 Ticlopidine

Ticlopidine is a first generation thienopyridine approved for use in 1991. Early landmark studies showed that dual APT with ticlopidine and aspirin in patients undergoing PCI was superior to either aspirin alone or aspirin plus anticoagulation with warfarin, in reducing the risk of recurrent ischaemic events including ST (27). However, the main limitation with the use of ticlopidine was its safety profile. In particular, it is associated with an elevated risk of neutropenia and thrombocytopaenia, as well as poorly tolerated side effects including nausea, vomiting and rash. Meta-analysis of data (28) have shown that clopidogrel is at least as effective as ticlopidine in reducing thrombotic and ischaemic complications after PCI and is a safer and better tolerated agent. As such, ticlopidine has been entirely replaced in clinical practice by clopidogrel.

1.2.2.2 Clopidogrel

Clopidogrel is a second generation thienopyridine approved for use in 1997. It irreversibly binds to the P2Y12 receptor with a relatively slow onset of action. Following a 600mg loading dose, the time taken for clopidogrel to achieve its peak effect is approximately 4 hours, and a maintenance dose of 75mg requires at least 5 days to achieve approximately 50 percent steady state inhibition of ADP-induced platelet aggregation. In addition to its antiplatelet effect, clopidogrel also exhibits anti-inflammatory properties. Specifically, it has been shown to inhibit the expression of the inflammatory mediators CD40 ligand and CD62 P-selectin, both acutely and in patients on long term maintenance therapy (29-31). These inflammatory biomarkers are expressed on activated platelet membranes as well as on many cells of the immune and vascular systems, and are not only potent stimulators of vascular inflammation but also play an important role in the pathogenesis and progression of atherosclerosis (32-34).

The data in support of the clinical benefit of clopidogrel in the treatment of CVD is abundant. For example, the CAPRIE study (35), which was the first and only large randomised trial to evaluate the efficacy of clopidogrel versus aspirin monotherapy in the secondary prevention of atherothrombotic disease, observed a modest increase in efficacy with clopidogrel compared to aspirin. Specifically, there was a relative risk reduction of 8.7% in favour of clopidogrel for the composite endpoint of vascular death, MI or stroke ($p=0.043$). This paved the way for further studies investigating the clinical benefit of combining aspirin and clopidogrel as dual APT compared to aspirin alone in patients with CVD.

In the CURE study (36), the beneficial effect of aspirin plus clopidogrel loading followed by maintenance therapy in patients with non ST elevation MI (NSTEMI) was established. In this study, the composite primary endpoint of cardiovascular death, MI or stroke was significantly reduced by 20% with dual therapy compared to aspirin alone at up to 12 months follow up (9.3% vs. 11.4%, $p<0.001$). This benefit was maintained in a subgroup of patients who underwent PCI (16). The significant findings from this trial changed clinical practice virtually overnight.

Clopidogrel for the treatment of STEMI was investigated in the CLARITY study (37) that assessed the value of adding clopidogrel (300mg loading dose plus 75mg once daily) to aspirin plus fibrinolyic therapy for STEMI patients who were scheduled to

undergo coronary angiography during index hospitalization. They observed that the clopidogrel group had improved patency of the infarct-related artery as well as a significant reduction in cardiovascular death and ischaemic events at 30 days (7.5% vs. 12%, p=0.001), with no increase in major bleeding. This study excluded patients who were over 75 years of age and who presented more than 12 hours after the onset of symptoms. Another large trial in STEMI patients, the COMMIT study (38), also demonstrated benefit with clopidogrel compared to placebo in conjunction with aspirin. Specifically, they reported a 9% relative risk reduction in death, re-infarction or stroke with clopidogrel (p=0.002) and no significant difference between the two groups in major bleeding. However, in this study, a loading dose of clopidogrel was not administered and maintenance therapy was continued for just 4 weeks.

The optimal timing of clopidogrel pretreatment and duration of maintenance therapy when combined with aspirin in stable patients undergoing elective PCI was evaluated in the CREDO study (15). Pretreatment with clopidogrel 300mg at least six hours prior to PCI was associated with a reduction in major adverse cardiovascular events (MACE) including urgent target vessel revascularisation at 28 days (18.5% relative risk reduction, p=0.23). Furthermore, subsequent maintenance therapy for 12 months compared to treatment for only 4 weeks resulted in a significant reduction in the combined endpoint of death, MI and stroke (26.9% relative risk reduction, p=0.02). Similar benefit was reported in patients undergoing PCI in the acute setting, where maintenance dose clopidogrel, in conjunction with aspirin, was also continued for up to 12 months (16).

Although these studies clearly demonstrated the importance of long term clopidogrel, the optimal duration of therapy, particularly in patients undergoing PCI with drug-eluting stents (DES), remains uncertain. For example, smaller studies and observational data have shown that the mortality benefit of clopidogrel extends beyond 1 year in patients with DES (39-41). By contrast, the combined analysis of data from two randomised multicentre trials (42), found no significant difference in the combined risk of MI and cardiac death in patients with DES who received 12 versus 24 months of clopidogrel therapy (1.2% vs. 1.8%, p=0.17). Similar observations were reported in the more recent PRODIGY study that randomised 2013 patients undergoing PCI to either 6 or 24 months dual APT with aspirin and clopidogrel (43). They found no difference between the two groups in the composite endpoint of all cause mortality, non fatal MI or stroke at 2 years (10 vs. 10.1%,

p=0.93), but there was a significantly greater risk of bleeding in the 24 month clopidogrel group. Of note, this study included patients treated with both DES and bare metal stents (BMS) and no difference in ischaemic events between these subgroups was observed. Furthermore, the EXCELLENT study that randomised 1443 patients with DES to dual APT for 6 versus 12 months reported no significant difference between the two groups in the primary composite endpoint of cardiac death, MI and target vessel revascularisation (4.8% vs. 4.3%; p=0.001 for noninferiority) (44). However, the primary endpoint occurred more frequently at 6 months in a subgroup of diabetic patients.

Some clarity on this controversial issue may be provided from the large, prospective, randomised controlled DAPT study (45), which will assess the safety and efficacy of 12 versus 30 months of dual APT with aspirin and a thienopyridine in 20,000 patients undergoing PCI. The primary study endpoints include the incidence of MACE, ST and major bleeding. Study recruitment was recently completed and the results are expected to be disseminated in 2014.

With regards to the appropriate clopidogrel loading dose, several studies have shown that a higher loading dose of 600mg is more efficacious than the 300mg dose. For example, the ARMYDA-2 trial (46) observed that pretreatment with 600mg versus 300mg clopidogrel 4 to 8 hours prior to PCI in patients with stable angina or NSTEMI was associated with a significant reduction in the combined primary endpoint of death, MI or target vessel revascularisation at 30 days (4% versus 12%, p=0.041), largely driven by the reduction in peri-procedural MI. Similar findings were reported by Cuisett et al (47) who investigated ACS patients receiving a loading dose of clopidogrel 12 hours prior to PCI.

These data have led to the widespread implementation of clinical guidelines which recommend a loading dose of 600mg clopidogrel followed by maintenance therapy with 75mg for 12 months, in addition to aspirin, in all ACS patients treated medically or with PCI, as well as in stable patients undergoing elective PCI with DES. The optimal duration of dual APT remains controversial and unproven at the current time. However, the main limitations with clopidogrel include its irreversible platelet inhibition and relatively slow onset of action, and this has led to the development and testing of more potent agents, such as prasugrel and ticagrelor (outlined in sections 11.2.2.3 and 11.2.3) which have largely overcome these shortcomings. However,

these newer therapies are associated with increased bleeding risks and could, thus, be potentially dangerous if used indiscriminately in all patient groups.

1.2.2.3 Prasugrel

Prasugrel is a third generation thienopyridine approved for use in 2009. It achieves a higher, more consistent level of platelet inhibition with a more rapid onset of action compared to clopidogrel and this has been shown to be due to a more rapid production of the active metabolite (48). Prasugrel is administered orally at a dose of 10mg once daily.

The TRITON-TIMI-38 study (49) compared prasugrel with clopidogrel in 13608 ACS patients with confirmed evidence of CAD (established on coronary angiography prior to study inclusion) and who were scheduled to undergo PCI. At 15 month follow up, the prasugrel group had a significant reduction in the primary endpoint of cardiovascular death, MI or stroke (9.9% vs.12.1%, $p<0.001$) as well as in the incidence of ST (1.1% vs. 2.4%, $p<0.001$). However, prasugrel was associated with significantly higher rates of major and life-threatening bleeding, particularly in patients who were older (over 75 years of age), had a low body weight or prior history of stroke. Although the positive findings from TRITON are encouraging, there are important criticisms of this study which need to be taken into account: firstly, the driver for the difference in the combined clinical endpoint was non fatal MI (there were no differences between prasugrel and clopidogrel in terms of the other individual endpoints including death or stroke); secondly, a 300mg loading dose of clopidogrel was used, which is contrary to current clinical guidelines that recommend a higher 600mg loading dose in the routine treatment of ACS; thirdly, the study drug was given after coronary angiography and guidewire insertion in the majority of cases and this would be expected to favour the faster-onset prasugrel over the slower-onset clopidogrel. Furthermore, in subsequent published work dedicated solely to the STEMI subset in TRITON (50), there was no significant difference between prasugrel and clopidogrel in the combined primary endpoint in the subgroup of patients treated with primary PCI (6.6% vs. 8.2%, $p=0.144$), despite the fact that it is in this group that uptake of prasugrel is highest in the UK.

The recent TRILOGY-ACS study (51) investigated the potential benefit of prasugrel in ACS patients (NSTEMI or unstable angina) managed medically (i.e. without PCI).

9326 patients were randomised to receive either clopidogrel or prasugrel in addition to aspirin. The usual 10mg dose of prasugrel was reduced to 5mg in those patients who were over the age of 75yrs or had a body weight of less than 60kg, in view of previously reported elevated bleeding risks in these groups. At a median follow up of 17 months, there was no difference between the two groups in the combined primary endpoint of cardiovascular death, MI or stroke (20.3% vs. 18.7%, p=0.43). Furthermore, there was no significant difference in major bleeding between the two groups, suggesting that the lower 5mg dose of prasugrel is the safer option in patients who are at increased risk of bleeding.

Although prasugrel has a more rapid *onset* of action compared to clopidogrel, the major limitation it shares with clopidogrel is the slow *offset* of action due to irreversible inhibition of the P2Y12 receptor. This has important clinical implications in patients on thienopyridines who require coronary artery bypass graft surgery (CABG) (or any other urgent surgery), and who are, thus, faced with the inevitable delay of several days to allow recovery of platelet function in order to minimise the risk of major bleeding. This limitation of thienopyridine treatment led to the development of novel, potent but reversible P2Y12 receptor antagonists with shorter half-lives, such as ticagrelor (discussed in section 1.2.3.1 below).

1.2.3 Reversible P2Y12 inhibitors

Ticagrelor, cangrelor and elinogrel are direct-acting, reversible P2Y12 receptor antagonists. Unlike thienopyridines, they do not require metabolic activation via the hepatic cytochrome P450 system and, thus, have a more rapid onset of action.

1.2.3.1 Ticagrelor

Ticagrelor was approved for use in Europe in 2010. It belongs to the new chemical class cyclopentyl-triazolo-pyrimidines and is administered orally at a dose of 90mg twice daily. Ticagrelor has a rapid onset and offset of action; the time to peak platelet inhibition following a loading dose is approximately 2 hours and partial recovery of platelet aggregation occurs within 12 hours after discontinuation of treatment. Like prasugrel, ticagrelor has been shown to have greater potency and consistency of platelet inhibition compared with clopidogrel.

The PLATO study (52) evaluated the efficacy and safety of ticagrelor versus clopidogrel in 18624 patients with ACS treated either medically or with PCI. At 12 month follow up, there was a significant reduction in the primary composite endpoint of cardiovascular death, MI and stroke in the ticagrelor group (9.8% vs 11.7%, $p<0.001$). The rate of death from any cause was also significantly reduced with ticagrelor. However, ticagrelor was associated with a higher rate of non-CABG related major bleeding compared to clopidogrel (4.5% vs. 3.8%, $p=0.03$) and a significant increase in the incidence of dyspnoea (13.8% vs. 7.8%, $p<0.001$), which led to discontinuation of the drug in 0.9% of patients. A recent substudy of PLATO (53), which comprised the ACS group that were managed medically, showed consistent benefit with ticagrelor over clopidogrel in reducing the primary composite endpoint (12% vs. 14.3% $p=0.04$). However, as with prasugrel, the subgroup of patients in PLATO undergoing primary PCI for STEMI failed to show any significant benefit with ticagrelor versus clopidogrel (54).
Although ticagrelor is evidently an attractive alternative to thienopyridines, particularly when rapid *onset* and *offset* of platelet inhibition is required, the drug is administered twice daily, raising important issues about potential non-compliance. As such, the rapid *offset* could represent a limitation of this drug in real life when compliance may be suboptimal.

1.2.3.2 Cangrelor

Cangrelor is an intravenously administered direct P2Y12 receptor antagonist. Its rapid onset of action (achieves platelet inhibition within 15 minutes of initiation), short plasma half life (3 to 5 minutes) and reversibility within 60 minutes of cessation of treatment potentially make it an ideal candidate for use in specific clinical settings, including: (i) the acute peri-procedural setting during PCI, and (ii) as a bridge to mandatory surgery in those patients on dual APT following recent DES implantation. The clinical benefit of cangrelor in ACS patients undergoing PCI was evaluated in two large scale randomised trials, both of which were terminated early due to lack of efficacy. Specifically, CHAMPION-PCI (55) demonstrated that cangrelor, administered 30 minutes prior to PCI, was not superior to an oral loading dose of 600mg clopidogrel in reducing ischaemic events at 30 days, and CHAMPION-PLATFORM (56) showed that peri-procedural cangrelor during PCI was not superior

to placebo and was associated with a significant increase in major bleeding. As such, cangrelor is currently not recommended for routine use in this setting.
Current clinical guidelines recommend discontinuation of thienopyridines 5 to 7 days prior to major surgery to minimise bleeding risks. However, premature discontinuation of thienopyridines for any reason, particularly in patients with DES, is potentially dangerous. The BRIDGE study (57) investigated whether cangrelor was a safe and effective drug to bridge patients from irreversible P2Y12 inhibition to CABG surgery. Two hundred and ten patients on thienopyridines for ACS or following PCI and awaiting CABG surgery were included. They were randomised to either cangrelor (infusion of 0.75μg/kg/min) or placebo for at least 48 hours. CABG was undertaken within 7 days of randomization and the study drug was discontinued 1 to 6 hours prior to surgery. The primary efficacy endpoint was maintenance of platelet inhibition (as measured on platelet function testing) during drug infusion prior to surgery. The results suggested that cangrelor achieved and maintained adequate platelet inhibition compared with placebo with no increased risk of major bleeding. Although these results are encouraging, the study was relatively small in size with surrogate endpoints. Larger clinical outcome studies are needed prior to the adoption of cangrelor for routine use as a bridge to surgery.

1.2.3.3 Elinogrel

Elinogrel is the newest of the direct-acting, reversible P2Y12 receptor antagonists currently under investigation. It is unique in that it can be administered both intravenously and orally which would be of particular benefit for bridging acute peri-procedural intravenous APT with chronic oral maintenance therapy, thereby avoiding potential interactions between different agents.
Results from the phase 2 safety and efficacy INNOVATE-PCI study which randomised 652 patients undergoing elective PCI to clopidogrel or to intravenous elinogrel followed by oral elinogrel were presented at the European Society of Cardiology Congress 2010 (Stockholm, Sweden). They observed that, although elinogrel resulted in greater platelet inhibition compared with clopidogrel, there were no significant differences in ischaemic events at up to 120 days follow up between the two groups. Furthermore, elinogrel was associated with higher rates of vascular access site bleeding complications. Large scale phase 3 studies are needed.

1.3 Current antiplatelet therapy prescribing guidelines

APT prescribing guidelines in CVD are wide-ranging, complex, dynamic and, at times, inconsistent. They take into account data from historical landmark clinical studies as well as new and emerging data from large scale randomised trials. Below is an outline of the current European clinical practice guidelines on APT prescribing.

1.3.1 Acute coronary syndromes and percutaneous coronary intervention

In patients with STEMI, the European society of cardiology (ESC) recommend a loading dose of aspirin 150 to 300mg in all patients followed by maintenance therapy with 75 to150mg long term regardless of treatment strategy. In those patients receiving fibrinolysis, a loading dose of clopidogrel 300mg is recommended and, in those treated with primary PCI, a loading dose of either clopidogrel 600mg, prasugrel 60mg or ticagrelor 180mg is recommended. Dual APT with aspirin and a P2Y12 receptor inhibitor must be continued for up to 12 months with a strict minimum of 1 month in patients receiving BMS and 6 months in patients with DES. Maintenance treatment with either prasugrel or ticagrelor is "preferred" over clopidogrel in STEMI patients undergoing PCI (58).

In patients with NSTEMI, the ESC guidance for aspirin treatment is the same as for STEMI. Dual APT with aspirin and a P2Y12 receptor inhibitor should be maintained for 12 months in all patients with NSTEMI regardless of treatment strategy. Of the P2Y12 receptor inhibitors: (i) ticagrelor is recommended for all patients at "moderate to high risk" of ischaemic events, regardless of initial treatment strategy and including those patients pretreated with clopidogrel, (ii) prasugrel is recommended for P2Y12 inhibitor-naïve patients in whom coronary anatomy is known and who are proceeding to PCI, and (iii) clopidogrel is recommended in patients who cannot receive ticagrelor or prasugrel (i.e. if contraindicated or unavailable) (18).

In patients undergoing elective PCI for stable angina, a bolus dose of aspirin 150 to 300mg followed by maintenance therapy with 75 to 150mg long term is recommended in all patients. In addition, a loading dose of 300mg clopidogrel should be administered at least 6 hours prior to PCI, or a loading dose of 600mg administered at least 2 hours prior to PCI. Maintenance treatment with clopidogrel should continue for a minimum of 1 month in patients receiving BMS and for 6 to 12 months in patients treated with DES (59).

1.3.2 Ischaemic stroke

European guidelines on the management of acute ischaemic stroke recommend that aspirin at a dose of 160 to 325mg is administered within 48hrs of symptom onset (but not within 24hrs of receiving thrombolytic therapy) (60). Thereafter, aspirin should be continued for 2 weeks after the onset of stroke symptoms, at which time definitive long-term APT should be initiated

The guidance for long term APT in secondary stroke prevention is variable. For example, American guidelines (61) recommend either aspirin monotherapy at a dose of 50 to 325mg, clopidogrel monotherapy or a combination of aspirin 25mg and dipyridamole 200mg twice daily. They suggest that the selection of antiplatelet agent should be *"individualised on the basis of patient risk factor profiles, cost and tolerance"*. On the other hand, the recent UK NICE guidance (http://guidance.nice.org.uk/TA210) recommend clopidogrel monotherapy as first line treatment in all patients. Dual APT with aspirin and dipyridamole is only recommended in those patients in whom clopidogrel is contraindicated or not tolerated.

1.3.3 Peripheral vascular disease

The ESC recommend aspirin 75 to 100mg daily in patients with symptomatic PVD. In addition, Cilostazol is indicated in patients with intermittent claudication (62).

1.4 Antiplatelet therapy response variability

As discussed in section 11.2, APT is the cornerstone of treatment in the secondary prevention of CVD and following PCI to reduce the risk of adverse events, thereby improving long term clinical outcome. However, several clinical studies have shown that, despite apparently adequate antiplatelet treatment, a significant proportion of patients continue to experience recurrent ischaemic events. In some cases, this has been attributed to antiplatelet treatment failure, the mechanisms of which are not fully understood and likely to be multifactorial.

Clinical studies have consistently demonstrated heterogeneity in individual patient responses to APT measured using *ex vivo* platelet function tests (described in section

11.5) and it is well established that APT hyporesponsiveness (which is also referred to as APT "resistance" or "high on-treatment platelet reactivity") is clearly associated with increased risk of adverse events including ST and cardiovascular death (63-66). Nonetheless, current clinical guidelines recommend standard doses of treatment that do not take into account the well documented inter-individual variability in responses. Despite these data, monitoring responses to APT and tailoring of treatment according to individual response has not been widely implemented and is not recommended in routine clinical practice largely due to: (i) lack of a standardised definition for APT hyporesponsiveness, (ii) lack of a widely accepted platelet function test appropriate for use in frontline clinical practice, and (iii) lack of consistent data showing clinical outcome benefit from tailoring therapy.

1.4.1 Defining antiplatelet therapy hyporesponsiveness: what are we asking?

Defining true APT hyporesponsiveness (or "resistance") remains a challenging and contentious issue. The use of arbitrary and binary cut-off values to define high residual platelet reactivity and thus differentiate "responders" from "non-responders" in the clinical setting may be considered inappropriate for three main reasons. Firstly, therapeutic response is more likely to be a continuous variable and, thus, should not be considered in a dichotomous way. Secondly, the cut-off values that define APT hyporesponsiveness widely differ depending on the laboratory assay and methodology used and, thirdly, the aetiology and biological mechanisms by which antiplatelet agents fail to achieve their desired effect is likely to be multifactorial. A number of contributory factors have been identified including patient compliance, gender, drug-drug interactions, under-dosing, genetic variability in drug absorption and metabolism, accelerated platelet turnover and the presence of specific cardiovascular risk factors such as diabetes, smoking, hyperlipidaemia and obesity (67-73). All these factors should be taken into account when defining APT hyporesponsiveness and when identifying effective strategies to alleviate the clinical consequences of response variability. Unsurprisingly, therefore, the reported incidence of APT hyporesponsiveness varies significantly depending upon the assay and methodology used, the definition applied and the population group studied. Specifically, large systematic reviews and meta-analyses of data have shown that the incidence of APT

hyporesponsiveness in CVD varies between 0.4 to 65% with aspirin (66,74) and 4 to 30% with clopidogrel (64,75).

The optimal pharmacological definition of APT hyporesponsiveness should encompass failure of the antiplatelet agent to inhibit the specific target of its action. This should ideally be demonstrated by utilisation of a method that measures the activity of the target receptor before and after administration of the antiplatelet agent. However, in 'real-world' clinical practice pretreatment baseline platelet reactivity levels would be difficult to ascertain. Thus, an absolute measure of platelet reactivity during treatment (i.e. "on-treatment platelet reactivity") is generally used to define poor response instead (76).

As previously described, the antiplatelet effect of aspirin is mediated through irreversible inactivation of platelet COX-1, which is required for the conversion of AA into the precursors of TXA2. Thus, the definition of aspirin hyporesponsiveness should refer to its inability to inhibit platelet COX-1 dependent TXA2 generation despite evidence of aspirin intake. This can be determined either by directly measuring AA-induced platelet activation using platelet function assays or by measuring serum TXB2 levels. Low dose aspirin (40 to 60mg) has been shown to successfully inhibit over 95% of platelet COX-1 activity (77,78) and it has been suggested that the prevalence of aspirin hyporesponsiveness is rare when assessed by methods directly dependent on platelet COX-1 activity (79). Nonetheless, *clinical studies* investigating aspirin hyporesponsiveness typically utilise platelet function assays that are not specific to the COX-1 pathway and variably reflect the thromboxane-dependent component of platelet aggregation (80). This highlights one of the most important clinical limitations of testing responses to antiplatelet drugs: is the aim in the clinical setting to assess the response of the patient to the pharmacological target OR is the aim to assess the response of the patient's clotting to the drug? These may well be completely different questions with very different answers.

With regards to clopidogrel, its active metabolite irreversibly binds to platelet P2Y12 ADP receptors, thereby inhibiting ADP-induced platelet aggregation. Thus, the definition of clopidogrel hyporesponsiveness should strictly refer to evidence of increased P2Y12 ADP receptor activity despite clopidogrel treatment, and this can be determined using platelet function assays that specifically measure ADP-induced platelet aggregation (81). Again, the behaviour of, for example, isolated platelets to

ADP is potentially very different to the clotting of a patients whole blood in response to the same agonist. One of our challenges in the field of testing patient responses to APT is to decide which of these questions is the one we need to answer with a clinically relevant point-of-care test.

1.5 Methods of assessing responses to antiplatelet therapy

Platelet function testing was initially used as a screening tool to identify patients with clotting disorders and subsequently in the clinical management of bleeding to guide transfusion requirements. More recently, their role has expanded to include the assessment of the effectiveness of pro-haemostatic and APT in both clinical and research settings. Specifically, in ACS patients and following PCI, high residual platelet reactivity as determined using platelet function testing is a predictor of clinical risk and poor outcome. In this knowledge, we would therefore expect that if it was possible to identify patients with high residual platelet reactivity using a point-of-care test then it would be possible to modify the risk of such patients by tailoring their APT. So far, this aspiration has failed to translate into clinically meaningful outcomes. This may be, at least to some extent, because of a lack of suitable frontline tests. In this particular setting, the ideal platelet function test should be rapid, simple, reproducible and appropriate for use at the point of patient contact.

Historically, turbidometric light transmittance aggregometry (LTA) was considered the "gold standard" platelet function assay. LTA measures platelet aggregation in platelet rich plasma following *in vitro* stimulation with various agonists and is the most widely investigated method to predict clinical outcome (82-84). However, the assay is not standardised and is subject to many methodological variables (85). Furthermore, several limitations preclude its use in routine clinical practice including the need for an experienced technician, relatively large sample volumes and lengthy processing times. Electrical aggregometry, in contrast to LTA, measures increase in electrical impedance rather than light transmittance and utilises whole blood instead of platelet rich plasma (86,87).

Other platelet function assays employ flow cytometry either to assess activation-dependent changes on platelet surface membrane receptors such as P-selectin and GP IIb/IIIa or alternatively to measure intracellular signalling by vasodilator-stimulated

phosphoprotein (VASP) which is a specific biomarker for P2Y12 receptor activation (87). The advantages of flow cytometric techniques include small sample volumes and the use of whole blood but, like LTA, are limited by complex sample preparation and the requirement for skilled technicians. They cannot therefore offer point-of-care testing.

The desire for easy access to assessment of responses to APT combined with the technical limitations associated with aggregometry and flow cytometry have led to the development of point-of-care platelet function tests that can be utilised with relative ease outside of the clinical laboratory setting, require minimal sample handling and provide results within a relatively short period of time. This has facilitated an expansion in the role of platelet function testing to include the identification of patients in the acute clinical setting who are hyporesponsive to APT and are, as a result, at increased risk of adverse events. The currently available point-of-care tests include Accumetrics VerifyNow, modified Thrombelastography (TEG) Platelet Mapping, Multiple Platelet Function analyser (Multiplate), Platelet Function Analyser (PFA)-100 and PlateletWorks. The limitations of these point-of-care tests are that they are not standardised, they utilise different methodologies and cut-off values to define APT hyporesponsiveness, and, furthermore, the data on the correlation between the various tests is conflicting (88-90). Thus, defining true "resistance" or hyporesponsiveness to APT and determining the most appropriate assay that could be reliably used to measure individual responses to specific antiplatelet agents remains a challenging issue.

The principles and methodology of: (i) the individual point-of-care platelet function tests, and (ii) biomarkers of thromboxane metabolism that are used to measure response to aspirin, are described in detail overleaf.

1.5.1 Accumetrics VerifyNow

The VerifyNow (Accumetrics, CA, USA) system was formerly known as the Ultegra rapid platelet function analyser (91). It is a rapid automated whole blood assay that measures agglutination of fibrinogen coated beads in response to specific agonists for aspirin, thienopyridines and GP IIb/IIIa inhibitors. The agonists used include AA for the aspirin assay, a combination of ADP and prostaglandin E1 for the P2Y12 receptor assay (92) and a thrombin receptor activating peptide for the GP IIb/IIIa assay. The

principle of platelet function measurement is based on increase in light transmission that occurs following platelet aggregation and it measures platelet-to-platelet aggregation in a GP IIb/IIIa-dependent manner. The purpose of the fibrinogen coated beads is to augment this signal.

Its advantages are that it is easy to use and very portable, does not require sample preparation, only requires a 2ml volume of blood and the results are rapidly available. Although results are delivered within 5 minutes, it is recommended that the blood sample is incubated for a minimum of 30 minutes prior to aspirin testing and a minimum of 10 minutes prior to processing the P2Y12 assay. Data have a shown a good correlation between VerifyNow and LTA in assessment of responses to clopidogrel (93,94). Furthermore, several studies have reported that hyporesponsiveness to aspirin and clopidogrel, as defined by the VerifyNow assay, in patients undergoing PCI is associated with an increased risk of peri-procedural MI and adverse clinical outcome (63,90,95-97).

1.5.2 Multiple platelet function analyser (Multiplate)

The Multiplate analyser (Dynabyte, Munich, Germany) is a whole blood assay that utilises impedance aggregometry to measure responses to aspirin, clopidogrel and GPIIb/IIIa antagonists (98). It employs five test channels containing various agonists that stimulate platelet aggregation. The attachment of platelets to the Multiplate sensors generates an increase in impedance which is transformed into an aggregation tracing that is plotted against time and from which various parameters are measured. It requires only a small amount of blood (0.3ml per test), no sample preparation and has a rapid 10 minute test time. Multiplate has been shown to correlate well with LTA in measuring responses to APT (99-101) and large prospective studies have shown that aspirin and clopidogrel hyporesponsiveness, as determined using Multiplate in patients undergoing PCI, is an independent predictor for the occurrence of ischaemic events including peri-procedural MI and ST (102-104).

1.5.3 Platelet function analyser (PFA)-100

The PFA-100 system (Dade Behring, Marburg, Germany) is a whole blood assay that utilises cartridges containing collagen and epinephrine agonists to measure the

antiplatelet effects of aspirin. Platelet aggregation is determined by the time taken for the occlusion of an aperture in a membrane under high shear conditions. The assay does not require any sample preparation and the aperture closure time is up to 5 minutes. However, blood samples require manual pipetting and need to stand for at least 15 minutes prior to assaying. The disadvantages of this system are that the assay is affected by VWF levels (which are elevated following PCI) (105), platelet count and haematocrit levels. Furthermore, although the PFA-100 assay has a collagen/ADP cartridge, it has been shown to be insensitive to the effects of clopidogrel (106-108) and there are limited data to support its use in monitoring thienopyridines.
Several meta-analyses have shown that high residual platelet reactivity and aspirin hyporesponsiveness determined by the PFA-100 assay is associated with increased risk of cardiovascular events and may be useful in predicting adverse outcome in patients with CVD (109,110). However, the cut-off aperture closure times used to define aspirin hyporesponsiveness varied significantly between individual studies, signifying the need for better standardisation of this assay.

1.5.4 PlateletWorks

The PlateletWorks system (Helena Laboratories, TX, USA) utilises impedance aggregometry to measure the degree of platelet aggregation by way of single platelet counting (111). Platelet aggregation is stimulated by ADP, AA or collagen agonists. Only a small amount of whole blood is required and the results are available within 2 to 10 minutes depending on the agonist. However, blood samples must strictly be analysed within 10 minutes of collection, which can be difficult in a busy clinical setting leading to unreliable test results. The Plateletworks assay has been shown to correlate well with LTA (112) but there is very limited data on its use in the prediction of cardiovascular outcomes. Recent data have shown that high residual platelet reactivity determined by Plateletworks in patients undergoing PCI on clopidogrel therapy was associated with adverse clinical outcome (90).

1.5.5 Thrombelastography (TEG) Platelet Mapping

TEG (Haemonetics Corp, MA, USA) is a platelet function test that provides an overall assessment of *ex vivo* haemostatic function. It incorporates the interaction of

all the components of coagulation including platelets, fibrin, clotting factors and thrombin. The TEG method involves pipetting a small volume of blood into a cylindrical cup into which a stationary pin is suspended by a torsion wire. The cup oscillates and, as blood clots, changes in its viscoelasticity are transmitted to the pin which acts as a torque transducer converting the oscillations into an electrical signal to produce a TEG trace. Analysis of the TEG trace provides information on the speed and strength of clot formation as well as clot stability.

Unmodified TEG provides a non-specific assessment of global haemostasis and, thus, the effects of some abnormalities are obscured by other more dominant components of the coagulation system (such as thrombin). Modifications to the original TEG methodology including the use of specific platelet activators have allowed TEG to be used more specifically to assess the effects of APT (113) and, in this context, it has been shown to correlate closely with the historical "gold standard" method LTA (79,82,114,115).

A novel method of TEG Platelet Mapping, known as Short TEG, has recently been developed and validated by this group in Southampton (116). Short TEG allows a more rapid assessment of the effects of APT (within 15 minutes), thereby making it suitable for use as a point-of-care test in the acute clinical setting. However, the main limitation with the use of Short TEG in this setting is that the TEG analyser is a relatively bulky piece of equipment that is not particularly portable. Hence, although it offers point-of-care testing with regards to its rapidity of results acquisition, the equipment is relatively static and cannot be easily transported to the patient's "bedside" (unlike the VerifyNow analyser which is the most portable of all the point-of-care tests). Furthermore, the TEG analyser is sensitive to surrounding movement and temperature, factors that need to be taken into account when determining its ideal location and placement within the clinical environment.

The clinical studies that encompass this thesis utilise the Short TEG test. Section 1.9 covers in depth the development, validation and methodology of Short TEG.

1.5.6 Biomarkers of thromboxane metabolism

As previously described, aspirin achieves its antithrombotic effects through inactivation of COX-1 hence inhibiting TXA2 production. Serum TXB2 is a stable metabolite derived solely from platelet TXA2 and is pharmacologically the most

specific test to evaluate the effect of aspirin on platelets (117,118). By contrast, urinary TXA2 metabolites (11-dehydro-TXB2) are not specific because up to 30% of these metabolites are derived from extra-platelet sources. Serum TXB2 levels are measured by enzyme immunoassay, with less pre-analytical and technical variability, the potential for high throughput compared to standard platelet function tests and, therefore, low cost. However, this test requires skilled technicians and the results are not rapidly available, making it unsuitable for use in the acute clinical setting. Clinical studies have reported a moderate to poor correlation between serum TXB2 levels and various platelet function tests in the assessment of responses to aspirin (80,119-124).

1.6 Antiplatelet therapy hyporesponsiveness and risk of adverse events

Clinical studies have demonstrated a clear link between APT hyporesponsiveness, high on-treatment platelet reactivity, and adverse clinical outcome in patients with ischaemic stroke, PVD, stable CAD, ACS and following PCI. The evidence for this relationship is described in detail below.

1.6.1 Ischaemic stroke

Clinical studies have consistently reported an apparently high prevalence of aspirin hyporesponsiveness in the stroke population. Furthermore, data have shown an association between aspirin hyporesponsiveness and frequency of stroke recurrence, severity of neurological deficit and cardiovascular death. For example, Englyst et al (125) reported an incidence of aspirin hyporesponsiveness as high as 67% in 45 stroke patients measured using TEG. In this study, aspirin hyporesponsiveness was independently associated with stroke severity and occurred more frequently in lacunar than embolic strokes. Jeon et al (126) reported a positive association between aspirin hyporesponsiveness in 117 stroke patients determined using VerifyNow and early recurrent ischaemic lesions seen on brain imaging at 1 week. The reported incidence of aspirin resistance in this study was 13.7% and this was independently related to early recurrent ischaemic lesions occurring outside the vascular territories of index stroke within the first week of stroke (OR 6.01; 95% CI 1.29 - 28.09; p=0.023).

Grotemeyer et al (127) measured platelet reactivity prior to discharge in 180 stroke patients on aspirin. They demonstrated that 33% of patients exhibited high on-treatment platelet reactivity and, furthermore, there was a significantly higher incidence of recurrent stroke, MI and vascular death at 2 years in aspirin non-responders versus aspirin responders (40% vs. 4.4% $p<0.0001$).

1.6.2 Peripheral vascular disease

Mueller at al (128) investigated 100 patients with PVD on long term aspirin therapy undergoing peripheral limb balloon angioplasty. They observed a poor response to aspirin in 60% of patients as determined by whole blood aggregometry and this was a predictor of vessel re-occlusion at 18 months. The risk of re-occlusion was at least 87% higher ($p=0.0093$) in aspirin hyporesponders. Similarly, Ziegler at al (129) examined the incidence of vessel restenosis or re-occlusion at 12 months following angioplasty for PVD in 98 patients. They observed an increased risk of re-occlusion in clopidogrel hyporesponders compared with clopidogrel responders (55% vs. 13%) measured using the PFA-100 assay.

1.6.3 Stable coronary artery disease

Gum et al (130) investigated aspirin hyporesponsiveness using LTA in a prospective study of 326 patients with stable CAD. They observed a 5.2% incidence of aspirin hyporesponsiveness and a greater than three-fold increase in the composite endpoint of death, MI and stroke in this group at a mean follow up of 679+/-185 days. Chen et al (131) reported a higher incidence of aspirin hyporesponsiveness of 27.4% in 422 stable CAD patients using VerifyNow, and a significant increase in the primary composite outcome of cardiovascular death, ACS and stroke was reported in the aspirin-resistant versus aspirin-sensitive group (15.6% vs. 5.3% $p<0.001$). In the HOPE study, Eikelboom et al (132) assessed aspirin hyporesponsiveness using urinary 11-dehydro TXB2 levels. They observed a 1.8 times higher risk of the composite endpoint of MI, stroke or death from vascular disease, and a 3.5 times higher risk of cardiovascular death, in the quartile with the highest levels (representing least effect of aspirin) compared with the lowest quartile.

1.6.4 Percutaneous coronary intervention

The clinical implications of APT hyporesponsiveness in patients undergoing PCI are well described. Geisler et al (83) showed that clopidogrel hyporesponsiveness, measured using optical aggregometry, is associated with increased risk of cardiovascular death, MI and stroke in patients undergoing elective PCI. Similar findings were reported by Bliden et al (115) who observed a significant increase in adverse ischaemic events in patients undergoing PCI on chronic clopidogrel therapy who exhibited high on-treatment platelet reactivity measured using optical aggregometry and TEG. Chen et al (95) investigated the effect of aspirin hyporesponsiveness following elective PCI in patients pretreated with clopidogrel and found that aspirin hyporesponders (determined using VerifyNow) were at increased risk of myonecrosis (51.7% vs. 24.6%, p=0.006). Myonecrosis is defined by a rise in cardiac enzymes and previous studies have consistently shown that elevated cardiac enzymes following PCI is associated with higher risk of death, MI and repeat revascularisation. Similar findings have been observed by Marcucci at al (133) who have shown that high post treatment platelet reactivity in patients undergoing PCI for STEMI is also associated with an increased incidence of myonecrosis and is an independent predictor of MI severity, irrespective of other clinical, laboratory and/or procedural parameters.

With regards to dual APT, there is increasing evidence to suggest that dual hyporesponders represent a unique patient group who are at significantly high risk of ischaemic complications following PCI. Specifically, Lev et al (63) compared the response to clopidogrel in aspirin responsive versus aspirin hyporesponsive patients undergoing PCI using optical aggregometry, flow cytometry and VerifyNow. They found that 50% of aspirin hyporesponders were also hyporesponsive to clopidogrel and, furthermore, dual hyporesponsiveness was associated with a greater than two-fold increase in the rate of myonecrosis compared to dual drug-sensitive patients. These findings are supported by Gori et al (134) who reported a significant increase in the risk of ST during a 6 month follow up period in patients undergoing PCI who exhibited dual APT hyporesponsiveness compared to isolated clopidogrel or aspirin hyporesponsiveness.

More recent studies using point-of-care assays including Multiplate, TEG and VerifyNow in patients undergoing PCI in both the acute and elective setting have also

reported a clear link between APT hyporesponsiveness and recurrent ischaemic events, cardiovascular death and ST at short and long term follow up (up to 3 years). A large prospective study by Sibbing et al (102) investigated clopidogrel response status using Multiplate in patients receiving DES (n=1608) and demonstrated that clopidogrel hyporesponsiveness was a strong and independent predictor of ST. The cumulative incidence of definite ST within six months was significantly higher in the hyporesponder group compared to the responder group (2.5% vs. 0.4%; $p<0.001$).

A summary of clinical studies investigating the relationship between APT hyporesponsiveness and clinical outcome in patients undergoing PCI following an ACS, or for the elective treatment of stable CAD, is outlined in Table I. These studies collectively demonstrate a strong and consistent association between APT hyporesponsiveness in patients undergoing PCI and adverse clinical events. Specifically, out of the 22 studies summarised in Table I, 14 (i.e. two-thirds) investigated clopidogrel alone, 5 investigated both aspirin and clopidogrel and just 3 investigated aspirin alone. Thus, the link between *clopidogrel* hyporesponsiveness and clinical events is more commonly described in the literature than with any other antiplatelet agent.

In summary, therefore, the data undoubtedly suggest that APT response variability is a clinically relevant entity and that hyporesponsiveness determined using various laboratory assays of platelet function is a credible predictor of adverse clinical events. However, there are notable differences in the reported prevalence of APT hyporesponsiveness between the laboratory assays used to measure response to therapy. What is clearly needed is a widely available, standardised and reproducible test with well-defined, validated cut-off values representative of clinical risk that can be used to reliably measure responses to APT in routine clinical practice.

Study	N	Clinical setting	Test	Agent studied	Clinical outcome in hyporesponders
Sibbing et al (135)	1608	PCI with DES	MEA	Clopidogrel	↑ST at 6months
Breet et al (90)	1069	Elective PCI	VerifyNow PlateletWorks LTA	Clopidogrel	↑CV death, ST and ischaemic events at 12months
Gurbel et al (136)	225	Nonemergent PCI	TEG LTA	Clopidogrel	↑Ischaemic events at 3yrs
Eshtehardi et al (103)	219	PCI	MEA	Aspirin and clopidogrel	↑CV death, ST and ischaemic events at 30days
Marcucci et al (96)	683	PCI in ACS	VerifyNow	Clopidogrel	↑CV death and MI at 12months
Price et al (97)	380	PCI with DES	VerifyNow	Clopidogrel	↑ CV death, non-fatal MI and ST at 6months
Patti et al (137)	160	PCI (not primary PCI)	VerifyNow	Clopidogrel	↑MACE at 30days
Cuisset et al (138)	120	Elective PCI	VerifyNow	Clopidogrel	↑ peri-procedural myonecrosis
Gurbel et al (139)	297	Elective PCI	LTA	Clopidogrel	↑Ischaemic events at 3years
Bliden et al (115)	100	Elective PCI	TEG LTA	Clopidogrel	↑CV death, ST and ischaemic events at 12months
Foussas et al (140)	612	Nonemergent PCI	PFA-100	Aspirin	↑CV death and rehospitalisation for non-fatal MI at 12months
Cuisset et al (141)	190	PCI for ACS	LTA	Aspirin and clopidogrel	↑Post PCI myonecrosis
Marcucci et al (133)	367	PCI for ACS	PFA-100 LTA	Aspirin and clopidogrel	↑Post PCI myonecrosis
Buonamici et al (142)	804	PCI with DES	LTA	Clopidogrel	↑ST at 6months
Lev et al (63)	150	Elective PCI	VerifyNow LTA	Aspirin and clopidogrel	↑Post PCI myonecrosis
Marcucci et al (143)	146	PCI for ACS	PFA-100	Aspirin	↑MACE at 12months
Geissler et al (83)	379	PCI for ACS and stable angina	LTA	Clopidogrel	↑CV death and ischaemic events at 3months
Hochholzer et al (84)	802	Elective PCI	LTA	Clopidogrel	↑MACE at 30days
Gurbel et al (82)	192	Elective PCI	TEG LTA	Aspirin and clopidogrel	↑CV death and ischaemic events at 6months
Gurbel et al (144)	120	Elective PCI	LTA	Clopidogrel	↑Post PCI myonecrosis
Chen et al (95)	151	Elective PCI	VerifyNow	Aspirin	↑post PCI myonecrosis
Matetzky et al (145)	60	Primary PCI for STEMI	LTA	Clopidogrel	↑Ischaemic events and ST at 6months

Table I. Antiplatelet therapy hyporesponsiveness and clinical outcome in patients undergoing PCI

1.7 Tailoring antiplatelet therapy

The growing body of evidence demonstrating a link between APT hyporesponsiveness and risk of adverse events, has raised the question as to whether APT should be routinely adjusted according to individual level of response and whether tailored therapy could lead to improved clinical outcome. At present, the only guideline recommendation supporting tailored therapy based on platelet function testing is in patients undergoing PCI in whom ST may be a *"catastrophic or lethal"* event (such as unprotected left main stem or last patent coronary vessel). In these patients, the American College of Cardiology/American Heart Association have recommended that *"platelet aggregation studies may be considered and the dose of clopidogrel increased to 150 mg per day if less than 50% inhibition of platelet aggregation is demonstrated...."* (Class IIb, level of evidence C recommendation) (146). However, the recommended method with which to assess platelet inhibition is not described and there are no specific recommendations for tailoring aspirin therapy. Furthermore, as yet, there is a paucity of data to support the attractive theory that identifying patients with high on-treatment platelet reactivity and then modifying their therapy to achieve greater levels of antiplatelet response actually translates into clinical benefit.

1.7.1 The evidence for personalised antiplatelet therapy

Two small randomised multicenter studies evaluating the clinical benefit of VASP-guided incremental loading doses of clopidogrel in patients undergoing PCI (for stable angina and ACS) who were hyporesponsive to clopidogrel, have demonstrated that dose adjustment compared with placebo resulted in a significant reduction in MACE (0% vs. 10%, $p=0.007$ and 0.5% vs. 8.9%, $p<0.001$) and ST (0.5% vs. 4.2%, $p<0.01$) at 1 month without any increase in major bleeding events (147,148). Of note, 14% of patients remained hyporesponsive to clopidogrel despite additional doses of up to 2400mg. Although the findings from these early studies potentially supported the need for routine testing for APT hyporesponsiveness in patients undergoing PCI, large scale randomised studies were clearly required to determine the most appropriate alternative therapeutic option(s) in those patients who were hyporesponsive to standard doses of clopidogrel. Furthermore, these studies utilised

the VASP assay, which is technically demanding and not widely available, hence limiting its applicability to routine clinical practice.

Other more recent large, randomised trials have failed to support the concept that personalised therapy yields a lower clinical event rate. For example, the large randomised prospective GRAVITAS study (149) investigated the outcome of tailored clopidogrel therapy in 2214 patients undergoing PCI who exhibited high on-treatment platelet reactivity measured using VerifyNow. Patients were randomised to either continuing with the usual 75mg dose of clopidogrel or to receiving an additional loading dose of 600mg followed by a higher maintenance dose of 150mg daily. At 6 month follow up, the combined primary endpoint of cardiovascular death, MI and ST was identical in both groups (2.3%) with no significant difference in bleeding events. Of note, the majority of patients included in this study had stable CAD and low risk clinical presentations which raised the question as to whether tailored APT may only benefit specific higher risk patient groups such as those presenting with ST, high risk ACS patients or diabetics. One of the main criticisms of the design of the GRAVITAS study is that it was significantly underpowered to show an effect, because the 50% relative risk reduction aimed for with just doubling the maintenance dose clopidogrel was overoptimistic. Furthermore, the investigators predictions of an event rate of around 5% turned out to be unrealistic, given that the actual event rate was half that. These limitations need to be taken into account when interpreting the GRAVITAS study findings.

Furthermore, the randomised TRIGGER-PCI study (150) investigated the efficacy, safety, and antiplatelet effect of prasugrel compared with clopidogrel in patients with high on-treatment platelet reactivity on clopidogrel, measured using the VerifyNow assay. Like GRAVITAS, this study only included the low risk, stable CAD population undergoing PCI. The findings demonstrated that, although prasugrel resulted in effective platelet inhibition compared to clopidogrel, there was no difference between the two groups in the primary endpoint of cardiovascular death and MI at 6 months. In fact, of the 423 patients studied, the primary endpoint did not occur in any of the patients assigned to prasugrel versus only one patient in the clopidogrel group. Thus, the trial was prematurely halted when it became apparent that they would not see enough clinical endpoints to deliver a meaningful result.

More recently, the multicenter ARCTIC study randomly assigned 2440 patients (70% were stable electives) scheduled for PCI with DES to either platelet function

monitoring using VerifyNow and adjustment of APT in patients who had an inadequate response, or to conventional treatment without platelet function testing (151). In the monitored group, platelet function testing was performed immediately prior to PCI and repeated at 2 to 4 weeks following stent implantation. Patients who were hyporesponsive to clopidogrel at the time of PCI received either an additional loading dose of clopidogrel (80%) or a loading dose of prasugrel (3%) and this was followed by maintenance therapy with either clopidogrel 150mg or prasugrel 10mg. The choice of thienopyridine and the additional use of GPIIb/IIIa inhibitors during PCI was left to the Physician's discretion. At 2 to 4 weeks post PCI, patients who were hyporesponsive to clopidogrel were either switched to prasugrel 10mg or received a 75-mg increase in their maintenance dose of clopidogrel. Aspirin hyporesponders were treated with intravenous aspirin at the time of PCI followed by higher maintenance therapy at discharge and/or at subsequent testing 2 to 4 weeks later. In the non-monitored conventional treatment group, the use of both aspirin and clopidogrel or prasugrel as well as GPIIb/IIIa inhibitors was left to the Physician's discretion and/or was in accordance with local and international guidelines. Of note, prasugrel was rarely used in this study (9% of patients in the monitored group and 6% of patients in the conventional treatment group at discharge) owing to its late availability in the trial and its off-label use in stable elective patients. The study findings demonstrated that there was no difference in the composite primary endpoint of death, MI, ST, stroke or urgent revascularisation at 1 year in the monitored group versus the conventional treatment group (34.6% vs. 31.1%, p=0.10), and no difference in major bleeding (2.3% vs. 3.3%, P=0.15). Furthermore, it was observed that approximately one third of patients (35%) were hyporesponsive to clopidogrel prior to PCI and just 8% of patients were hyporesponsive to aspirin. At repeat testing up to 4 weeks later following drug adjustment, only 15% of patients were hyporesponsive to P2Y12 inhibitor. Although these data do not support the routine use of platelet function testing with a view to providing tailored APT in patients undergoing PCI, the majority of patients in this study (over two thirds) were stable, low risk cases (similar to the GRAVITAS and TRIGGER-PCI groups) and, therefore the findings may not be applicable to the higher risk ACS patients. Furthermore, no further platelet function testing was undertaken in the 15% of patients who were hyporesponsive to P2Y12 inhibitor at the second visit and, therefore, we do not know whether drug adjustment ultimately had any impact on platelet reactivity in this group. The use of more potent

P2Y12 inhibitors, rather than increasing the maintenance dose of clopidogrel, may have been the more appropriate intervention in all patients demonstrating high on-treatment platelet reactivity. Further large scale investigation is needed in the higher risk patient groups and including the more potent P2Y12 inhibitors.

The MADONNA study was a relatively small study that included patients with ACS undergoing PCI (n=798) and investigated the clinical benefit of individualised APT in clopidogrel hyporesponders, determined using the Multiplate assay (152). Following a 600mg loading dose of clopidogrel, patients were allocated in a non-blinded manner to either the "tailored treatment" (n=403) or "non-tailored treatment" (n=395) group. In the tailored group, clopidogrel hyporesponders (26%) received either repeated loading doses of clopidogrel 600mg (up to a maximum of 2400mg) or a single dose of prasugrel 60mg until adequate platelet inhibition had been achieved, followed by maintenance treatment with whatever agent was administered during reloading. In the non-tailored group, clopidogrel hyporesponders (25%) did not undergo any additional reloading and received maintenance treatment in the usual way with the standard 75mg dose of clopidogrel. At 30 day follow up, there was a significant reduction in the primary endpoint of ST in the tailored therapy group (0.2% vs. 1.9%, p=0.027) but no difference in cardiovascular death or major bleeding was observed. Although these results are encouraging, the study has limitations including its non-blinded, non-randomised design and the fact that the APT regimes differed in the tailored treatment group such that the numbers actually treated in each group were relatively small.

In summary, therefore, robust data demonstrating clinical benefit with tailored APT in patients with high on-treatment platelet reactivity are lacking. What remains to be established is whether tailored therapy may only be beneficial in a specific subset of patients who are perceived to be at comparatively higher risk of adverse events and whether this strategy leads to improved long term clinical outcome. Adequately powered clinical trials are required to address these clinically relevant questions.

1.7.2 Stent thrombosis

PCI with stent insertion is now the commonest method of coronary revascularisation in the UK. ST is an important, potentially life-threatening, complication of coronary stent placement with a cumulative incidence of 0.5 to 1% per year in DES (153-155), and a reported mortality of up to 45% (156). Definite ST is defined as clinical

presentation with an ACS associated with angiographic or pathologic evidence of stent occlusion or thrombus (157). Based on the elapsed time since stent implantation, ST is classified as early (0-30 days post stent implantation), late (greater than 30 days and less than 12 months post stent implantation) and very late (greater than 12 months post stent implantation).

Whilst the aetiology of ST is likely to be multifactorial, it is now well described that premature discontinuation of or inadequate response to APT are important risk factors. Specifically, hyporesponsiveness to either aspirin or clopidogrel that results in high residual platelet reactivity has been consistently shown in multiple studies to be associated with an increased risk of ST (82,115,135,142,156,158-162) and dual APT hyporesponsiveness has been shown to be an independent predictor of ST and cardiac death (134). Furthermore, data have shown that there is a significantly higher incidence of aspirin and/or clopidogrel hyporesponsiveness in patients with ST compared to controls (163-167). For example, Rajendran et al (167) demonstrated in a recent small study (n=56) that aspirin and/or clopidogrel hyporesponsivess occurred in 75% of patients with ST vs. 2.5% of controls ($p<0.001$) measured using the VerifyNow assay. Repeat platelet function testing in this group, following 2 weeks of double-dose APT, showed a significant improvement in platelet reactivity in over 80% of patients.

These data indicate that ST patients may possess a prothrombotic tendency (consistent with the significant proportion found to have high on-treatment platelet reactivity) and, thus, represent a unique group who may specifically benefit from tailored APT and in whom the more potent antiplatelet agents could play a crucial role.

1.8 Clopidogrel withdrawal and the "rebound phenomenon"

As described previously in section 1.3, current clinical guidelines recommend 12 months of clopidogrel (in addition to aspirin) in ACS patients managed medically or with PCI, as well as in stable elective patients undergoing PCI with DES. However, the data in relation to the optimal duration of clopidogrel, particularly in the context of PCI with DES, are discrepant and controversial (168).

Studies have shown a clear link between cessation of long term clopidogrel therapy and adverse events including ST (169-173). Specifically, a significant proportion of

these events occur within days or weeks after clopidogrel is discontinued and this effect has not only been observed in patients with coronary stents, but also in ACS patients managed medically. For example, Ho et al (169) reported a clustering of adverse clinical events within the initial 90 days after stopping clopidogrel in a large retrospective study of 3137 ACS patients on dual APT treated either medically or with PCI. In the medically treated group, the mean duration of clopidogrel therapy was 278 days; death or MI occurred in 17% of patients with 61% of the events occurring 0 to 90 days after cessation of clopidogrel. Similarly, in PCI treated patients, mean duration of clopidogrel therapy was 302 days; death or MI occurred in 7.9%, with 58.9% of these events taking place within the initial 90 days after clopidogrel cessation. The relative increase in adverse events in the early 90 day period after stopping clopidogrel was nearly two-fold higher than at later time periods (i.e. 91 to 360 days). This group went on to confirm and expand their findings in a retrospective cohort study in 1656 ACS patients receiving clopidogrel therapy (170). They observed a similar two-fold increase in the risk of death or MI in the 0 to 90 day interval after clopidogrel cessation compared with later time intervals. These findings were consistent across all patient subgroups evaluated, i.e. medically managed versus PCI treated patients, DES versus BMS and with clopidogrel therapy duration of greater than versus less than 6 months.

The findings from these studies led to speculation that cessation of clopidogel may be associated with a “rebound” effect that is prothrombotic, pro-inflammatory or both and is directly responsible for adverse clinical events. Although the term “rebound phenomenon” is now relatively widely used in the context of clopidogrel therapy, its definition is unclear and is often subject to misinterpretation. Specifically, the two most widely accepted interpretations of “rebound” are: (i) simply that an adverse clinical event occurs shortly after, and because of, clopidogrel cessation, and (ii) that, as a result of clopidogrel cessation, one or more parameters of either platelet reactivity or vascular inflammation reaches a level that is higher than it was at baseline before clopidogrel therapy was ever initiated. The latter is the stricter and more scientifically accurate definition of “rebound” but is more difficult to ascertain in clinical practice (i.e. outside the realms of carefully designed research) due to the lack of availability of a comparative, baseline pre-clopidogrel assessment.

The postulated mechanisms and pathophysiology of the observed phenomenon of a clustering of clinical events (that are currently matters for speculation) include:

a) increased platelet activation resulting in a prothrombotic tendency due to the direct loss of the effect of clopidogrel on the inhibition of ADP-induced platelet aggregation

b) increase in biomarkers of inflammation resulting in a pro-inflammatory state and increased local vascular inflammation which could be prothrombotic

c) loss of the synergistic effect of clopidogrel alternative pathways of platelet aggregation, such as the AA-induced pathway which is predominantly affected by aspirin

The supportive data with regards to the hypotheses (a) and (b) are inconsistent. For example, Angiolillo et al (174) investigated the effect of clopidogrel withdrawal on platelet reactivity (measured using LTA) and inflammatory biomarkers in 54 diabetic patients on long term dual APT following PCI. Blood samples were taken at 12 months post PCI (on dual APT) and 1 month after clopidogrel cessation. They observed a significant increase in ADP-induced platelet aggregation as well as an increase in inflammatory biomarkers hsCRP and surface P-selectin at 1 month. However, this was a small study confined to the diabetic group so the results may not be directly applicable to the heterogeneous population. Furthermore, the determination of a possible "rebound" effect would necessitate pre-clopidogrel baseline data which were lacking. The DECADES study (175) examined the effect of clopidogrel cessation on markers of inflammation 12 months after implantation of DES in the non-diabetic population (n=96). A significant increase in the inflammatory biomarkers sCD40L and P-selectin were observed between 2 and 4 weeks after clopidogrel withdrawal, further raising the question as to whether this was due to a genuine rebound pro-inflammatory state associated with clopidogrel withdrawal. Unfortunately, no data were obtained regarding platelet reactivity or, indeed, baseline pretreatment inflammatory biomarker levels.

By contrast, a small randomised study in 64 patients undergoing PCI with DES did not demonstrate a rise in platelet reactivity following clopidogrel withdrawal (176). Specifically, ADP-induced platelet aggregation was measured using both LTA and Multiplate over various time points during treatment with clopidogrel and following cessation of therapy. They examined the effect of abrupt cessation of clopidogrel therapy versus tapered withdrawal and found no significant difference between the

two groups and no significant increase in platelet reactivity from 2 to 8 weeks following clopidogrel withdrawal.

More recently, the PACT study (177) measured platelet reactivity using LTA, Multiplate and flow cytometry, as well as inflammatory biomarker levels (soluble CD40L) at six time points (from 1 to 45 days) after clopidogrel withdrawal in 15 healthy volunteers who were also taking aspirin. Baseline pretreatment platelet reactivity and inflammation were also measured in this study. They observed that discontinuation of clopidogrel resulted in a recovery of platelet reactivity to baseline but did not result in rebound platelet hyper-reactivity or in an increase in inflammatory biomarker levels. However, this was a very small study conducted in healthy volunteers. Further large scale randomised patient studies are clearly warranted.

With regards to hypothesis (c) above, this is based upon the simple, but still controversial, notion that clopidogrel exerts some of its antiplatelet activity via the AA-TXA2 pathway, as well as via the more established P2Y12 ADP mechanism. The obvious implication of this would be that when clopidogrel is discontinued, then both pathways would be affected and this would result in an increase in both ADP- and AA-induced platelet aggregation. This would manifest as an apparent reduction in the response of an individual patient to aspirin when clopidogrel is stopped as assessed by AA-induced platelet reactivity. There is accumulating evidence to suggest that clopidogrel may also influence the AA-TXA2 pathway, thereby potentiating the effect of aspirin (178-181). For example, a recent small study has shown that standard doses of clopidogrel suppress the production of *in vivo* TXA2 urinary metabolites (11-dehydro-TXB2) to the same extent as aspirin in healthy volunteers (181). Further data have also shown that patients who were initially labelled as ‘non-responders’ to aspirin, as determined by as determined by LTA and TEG, were converted to ‘responders’ as a result of increased inhibition of AA-induced platelet aggregation following the addition of clopidogrel therapy (178,182). Further support of the “potentiation theory” is demonstrated by Angiolillo et al (174) who reported that, following clopidogrel withdrawal, 44% of patients exhibited a ‘poor response’ to aspirin determined using the PFA-100 system, but yet when these patients were taking aspirin and clopidogrel concomitantly their AA-induced platelet aggregation profiles were consistent with an apparent ‘normal’ response to aspirin.

Thus, these data sponsor the hypothesis that clopidogrel may influence AA-mediated platelet aggregation and its cessation could therefore lead to loss of the aspirin-synergistic effect resulting in "rebound" attenuation of the antiplatelet effect of aspirin. If this mechanistic theory were examined and proven in larger studies, it would mean that patients who are relatively hyporesponsive to aspirin would be at particular risk of adverse events when clopidogrel is discontinued. It would follow that all patients requiring dual APT, either in the context of PCI or following an acute coronary event, would need to undergo an individualised assessment of their response to aspirin and clopidogrel with a view to tailoring treatment according to their level of response.

Further robust data are needed from large scale randomised studies to: (i) further elicit the mechanisms and pathophysiology behind the "rebound" effect (ii) specifically assess platelet reactivity and vascular inflammation both pretreatment as well as post clopidogrel cessation (iii) determine precisely how clopidogrel achieves its effects including the extent to which it influences aspirin-specific pathways of platelet aggregation, and (iv) determine whether the "rebound phenomenon" is also exhibited in the newer, more potent third generation thienopyridines.

If the "rebound phenomenon" was proven, then results from these studies would help identify strategies to attenuate the its effect and therefore reduce the incidence of adverse clinical events. Specifically, this would potentially involve important changes in APT prescribing guidelines such as extending the duration of clopidogrel therapy, tapered rather than abrupt interruption of chronic clopidogrel therapy or, indeed, tailoring APT regimes to the individual patient, particularly those who are found to be hyporesponsive to aspirin.

1.9 Thrombelastography

The Thrombelastograph Haemostasis System (TEG, Haemonetics Corp, MA, USA) provides an overall assessment of *ex vivo* haemostatic function, thus incorporating the interaction of all the components of coagulation including thrombin, platelets, fibrin and clotting factors. TEG utilises whole blood to provide a graphic representation of speed of clot formation and lysis (183,184). First developed in 1948, it was used initially as a research tool (185). Over the last two decades, the development and

modernisation of TEG has facilitated its use in the setting of cardiac, hepatic and obstetric surgery to differentiate between haemostatic and surgical causes for bleeding and thus guide clotting factor replacement, platelet transfusion and fibrinolysis treatment (186-191). Modifications to the original TEG methodology have improved its ease of use and allowed it to be used to more specifically assess platelet function *ex vivo* in the context of dynamic clot formation. This has led to an expansion in the potential applications of TEG to include the detection of the effects of APT on an individuals clotting response.

1.9.1 TEG methodology

Kaolin activated blood at 37°C is placed in a cylindrical cup that oscillates by 4 degrees 45‟ at a frequency of 0.1 Hertz. Suspended within the cup by a torsion wire is a stationary pin. As the cup oscillates there is a 1mm gap between it and the pin. The wire acts as a torque transducer (Figure 1).

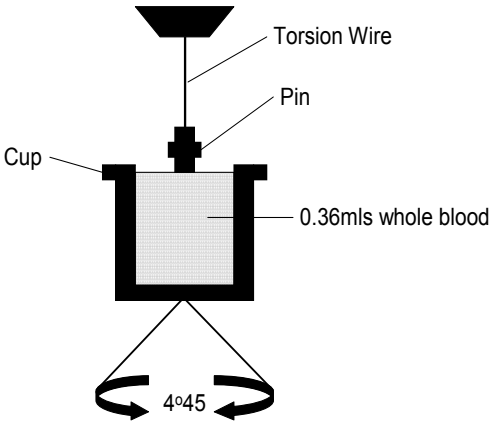

Figure 1. Schematic representation of the TEG methodology

When whole blood is in its liquid form, cup oscillation has no impact on the pin. As blood clots, fibrin strands link the pin and the cup and changes in the viscoelasticity of the blood are therefore transmitted to the pin. The resulting torque generates an electrical signal whose magnitude can be plotted as a function of time to produce a

TEG trace (192,193) (Figure 2). Thus, as blood clots there is a progressive increase in the signal amplitude to a maximum.

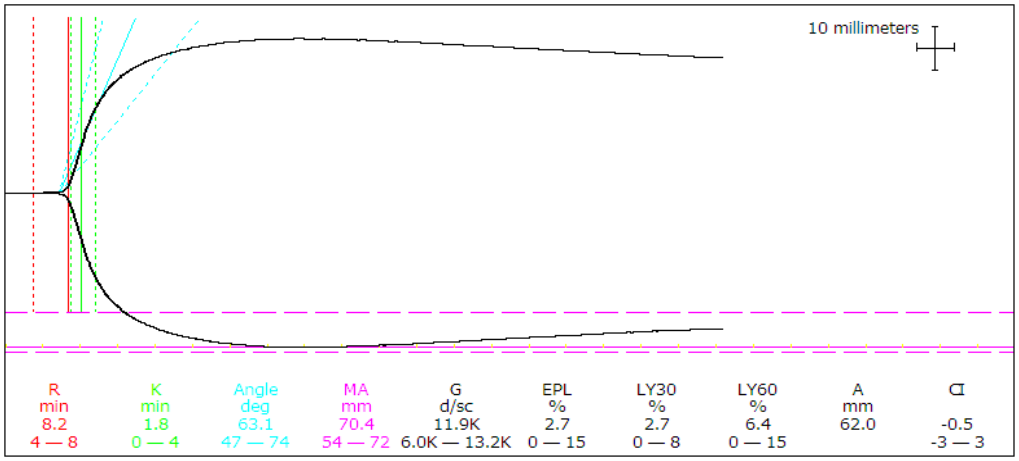

Figure 2. A standard TEG trace

Normal haemostasis involves the controlled activation of clot formation, spontaneously balanced by mechanisms of clot lysis; therefore a truly global analysis of haemostatic function requires assessment of both the fibrinolytic and coagulation systems. TEG measurements incorporate both of these components by providing continuous real time information on the viscoelastic properties of the evolving clot from the time of initial fibrin formation, through to platelet aggregation, fibrin cross linkage and clot strengthening to clot lysis (194). Analysis of the TEG trace can determine: (i) the speed of clot generation (ii) its strength, and (iii) its stability (191). Table II summarises the commonly assessed TEG parameters.

Although clotting is a dynamic process, some conventional tests, such as activated partial thromboplastin time and platelet count, only assess isolated components of the haemostatic system and are unable to predict the role of these components in the context of haemostasis as a whole. The advantage of TEG is that it incorporates the interaction of all of the components of coagulation including platelets, fibrin, clotting factors, and thrombin as well as providing information about the quality of the clot (195).

Parameter	Description and rationale for assessment
R time	Time from the start of a sample run until initial fibrin formation. It relates to plasma clotting factor and inhibitor activity.
K time	Time taken for the blood to achieve a fixed level of viscoelasticity. It represents clot kinetics.
α angle	The angle formed by the gradient of the initial trace. It represents the speed of clot formation and measures the rapidity of fibrin build-up and cross-linking.
MA	MA, or maximum amplitude, indicates the ultimate strength of the fibrin clot. It represents platelet aggregation and is a direct function of the maximum dynamic properties of fibrin and platelet bonding.
LY30	Measures the rate of amplitude reduction 30 minutes after MA. This represents clot lysis.

Table II. Commonly assessed TEG parameters

1.9.2 Recent TEG modifications

Conventional "unmodified" TEG provides a non-specific assessment of global haemostasis and, as such, the effects of some clotting abnormalities are obscured by other more dominant components of the coagulation system (such as thrombin). Modifications to the original TEG methodology (187,196,197) have improved its ease of use and reproducibility and allowed it to be used to more specifically assess the functional importance of the different components of the haemostatic system. These modifications include the use of:

a) Sample activators such as celite, kaolin and tissue factor to speed up result acquisition

b) Citrated samples to allow a longer delay before sample testing

c) Heparinised samples to eliminate the effect of thrombin, thereby allowing assessment of the contribution of platelets and fibrin to clot formation

d) Platelet GP IIb/IIIa inhibitors to allow the assessment of the relative contribution of fibrinogen to clot formation

e) Activator F™, which comprises a mixture of reptilase and Factor XIIIa, to activate fibrin formation without affecting platelets thereby allowing the assessment of the contribution of fibrin to clot formation

f) Platelet activators AA and ADP to allow the antiplatelet effects of aspirin and P2Y12 receptor inhibitors such as clopidogrel to be assessed

1.9.3 The principle of TEG Platelet Mapping

In standard TEG, the maximum amplitude (MA) is representative of clot strength and is largely dependent on thrombin. Thrombin is a powerful platelet activator and overwhelms the effect of other less potent platelet activators such as AA and ADP. By taking blood into a tube that contains heparin, thrombin is inhibited. The subsequent addition of Activator F™ generates a fibrin network in which platelets can interact independent of thrombin. Without alternative sources of platelet activation there is minimal platelet activation and therefore minimal response on the TEG curve, which is depicted by a low amplitude MA. However, other platelet activators such as AA or ADP can be added to the TEG sample and, in the absence of inhibition of their specific pathways of action (e.g. with aspirin or clopidogrel respectively), this results in an increase in the amplitude of the MA. Maximal platelet activation generates a curve similar to unmodified TEG in the presence of thrombin. The effect of antiplatelet agents can therefore be established by comparing the unmodified TEG curve (representing maximal platelet activation) and the modified TEG curve with either AA- or ADP-stimulation.

Aspirin achieves platelet inhibition by permanent inactivation of COX-1, an enzyme involved in platelet AA metabolism. The effect of aspirin can therefore be calculated by comparing the unmodified curve in the presence of thrombin (maximal platelet activation), the heparinised sample with Activator F™ alone (no platelet activation) and the modified TEG curve with AA-stimulation (residual platelet activation due to AA in the presence of aspirin). The effect of P2Y12 and GP IIb/IIIa receptor antagonists on platelet inhibition can be assessed in a similar fashion, utilising ADP-stimulation. This system is marketed by Haemoscope as the TEG “Platelet Mapping

Kit". It utilises four different channels on the TEG machine to detect the effects of APT acting via the AA and ADP pathways (113,115). The four channels contain: (1) Thrombin (2) Activator F™ (3) Activator F™ and AA (1mM) (4) Activator F™ and ADP (2μM).

With these modifications, TEG Platelet Mapping correlates closely with the historical "gold standard" platelet function assay LTA in the assessment of responses to aspirin and clopidogrel (79,82,114,115,198). The specific advantages of TEG Platelet Mapping over other platelet function tests are that it has the ability to detect responses to both aspirin and clopidogrel, it can determine the summative effect of multiple medications and it is a whole blood assay (unlike LTA) that also provides information on clot formation and clot lysis.

1.9.4 TEG Platelet Mapping in the assessment of responses to antiplatelet therapy

The effects of antiplatelet agents can either be calculated by comparing a sample containing APT with a baseline sample (113) or by calculating the parameter known as "percentage platelet inhibition" (79,114). The percentage platelet inhibition is derived from the MA of the TEG trace. Previous clinical studies have suggested that MA is a predictive tool for ischaemic events after non-cardiac surgery (199) and following PCI (82), making it an attractive and clinically relevant parameter. The percentage platelet inhibition in response to AA- or ADP- stimulation is calculated by comparing the clot with fibrin alone with maximal platelet activation due to thrombin and platelet activation due to AA or ADP.

Thus, the percentage platelet inhibition (%PI) due to aspirin can be calculated using the formula:

$$\%\,PI = 100 - \frac{(MA\ AA\ channel - MA\ Fibrin\ channel)}{(MA\ Thrombin\ channel - MA\ fibrin\ channel)} \times 100$$

And, similarly, the percentage platelet inhibition (%PI) due to clopidogrel (or other P2Y12 receptor antagonist) can be calculate using the formula:

$$\% \text{ PI} = 100 - \frac{(\text{MA ADP channel} - \text{MA Fibrin channel})}{(\text{MA Thrombin channel} - \text{MA fibrin channel})} \times 100$$

Whilst this method has the advantage that the calculated TEG results are easily interpretable, there are potential shortcomings. Firstly, there can be a considerable delay (often over 1 hour) before the MA value is obtained making it a relatively slow tool for point-of-care testing in the acute clinical setting. Secondly, the MA provides information solely on clot strength and not the speed of clot formation. Thirdly, the percentage platelet inhibition subtracts the response seen in the fibrin channel in an attempt to describe the effect of platelets on whole blood coagulation. However, fibrin is itself involved in platelet aggregation via its action on the integrin alpha (IIb) beta receptors and is integral to thrombus formation (200). Thus, this means of calculation negates one of the fundamental benefits of TEG (namely its ability to detect clinically relevant changes in overall blood clotting, including the effects of fibrin, rather than isolated platelet function). The requirement for a more rapid assessment of the effects of antiplatelet agents on whole blood coagulation, incorporating the effects of fibrin and thrombin and without the need for a baseline reference sample, led to the development of a novel method known as Short TEG.

1.9.5 Development and validation of Short TEG

Over the last 5 years, the Coronary Research Group at University Hospital Southampton NHS Foundation Trust has pursued a research theme dedicated to investigating and validating the use of modified TEG for assessment of individual, time-dependent responses to APT (aspirin and clopidogrel) with the aim of further refining the technique in order to produce a more rapid point-of-care test. This is based upon the following assumptions:

a) TEG is ideally placed to assess platelet-induced whole blood clotting but will only be clinically useful if it can be performed as a rapid, point-of-care test

b) Given the well-documented heterogeneity of responses to aspirin and clopidogrel, as well as the link between reduced responsiveness and clinical events, there would be important clinical benefits in several fields of cardiovascular medicine if it were possible to assess individual response to APT

1.9.6 Area under the TEG response curve

The Southampton Coronary Research Group, in conjunction with the Department of Medical Physics and Bioengineering at Southampton, developed a purpose specific software programme to calculate the area under the TEG response curve (AUC) at any time point. AUC incorporates both the rate of rise (speed of clot formation) as well as the MA (strength of clot) of the TEG trace. An example of AUC at a specific time point on two TEG traces is highlighted in black in Figure 3. As illustrated, the TEG traces have an identical MA (representing clot strength) but the speed of clot formation significantly differs. Hence, there is a dramatic difference in AUC at this specific fixed time point due to the difference in speed of clot formation, despite an almost identical MA. This clearly demonstrates why an assessment of MA (clot strength) in isolation can be deceptive and it is important to take into account *both* the strength *as well as* speed of clot formation.

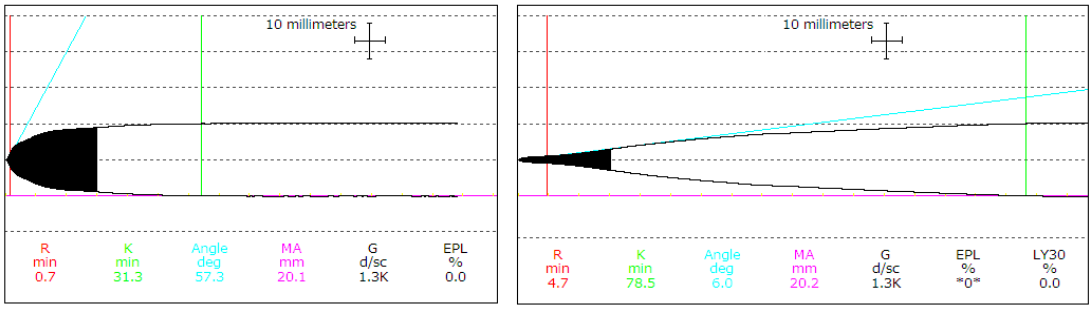

Figure 3. Area under the TEG response curve (AUC) at an identical time point on two TEG traces (AUC is highlighted in black).

1.9.7 The principle of Short TEG

The early experiments conducted by the Southampton Group (113) validated the ability of TEG to determine individual time-dependent responses to aspirin and clopidogrel and specifically employed the novel parameter AUC. Using groups of healthy volunteers, they demonstrated that AUC at 60 minutes provided a reliable and reproducible measurement of individual responses to loading and maintenance doses of aspirin and clopidogrel. The concept of AUC therefore proved interesting but the measurement, like the more conventional TEG parameter MA, required 60 minutes from the time of clot formation. In the acute clinical setting, where point-of-care testing could potentially be utilised to tailor doses of APT, the test result would need to be available more rapidly. Therefore, they investigated whether AUC could be reliably employed even before the MA was reached. The time point of 15 minutes was chosen and the concept of AUC at 15 minutes (AUC15) was subsequently successfully developed and tested (116). Most importantly for the concept, they established that correlation between MA and AUC15 was excellent, thus removing the concern that 15 minutes was too short a time to provide reliable and meaningful results (Figure 4). The AUC15 TEG analysis method is known as Short TEG.

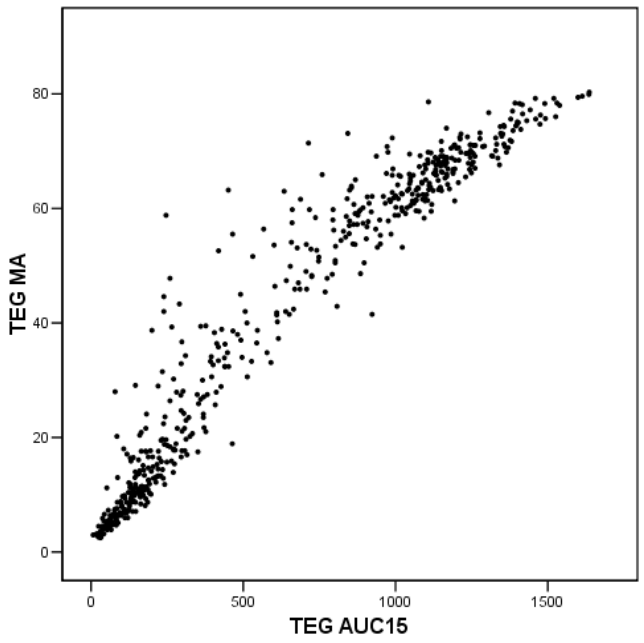

Figure 4. Scatterplot showing the correlation between TEG MA and AUC15 (R=0.964, n=560, p<0.01)

1.9.8 Percentage clotting inhibition

As outlined above, AUC15 provided a rapid and reliable method of assessing a TEG trace. The Southampton Group went on to determine the optimal method of calculating responses to APT employing the AUC15 parameter, and without the need for any baseline pretreatment values that would be impractical in the acute clinical setting. Based on AUC15, they derived and validated the percentage clotting inhibition which describes, as closely as possible, the absolute effect of antiplatelet medication on the overall *ex-vivo* clotting response of an individual as a snapshot result (116). The percentage clotting inhibition due to aspirin or P2Y12 receptor inhibitors is calculated by comparing AA- or ADP- induced clotting responses respectively with response to thrombin, which represents an invariable internal control.

Thus, the percentage clotting inhibition (%CI) due to aspirin is calculated using the formula:

$$\%\ CI = 100 - \frac{(AUC15\ AA\ channel)}{(AUC15\ Thrombin\ channel)} \times 100$$

And, similarly, the percentage clotting inhibition (%CI) due to clopidogrel (or other P2Y12 receptor antagonist) is calculated using the formula:

$$\%\ CI = 100 - \frac{(AUC15\ ADP\ channel)}{(AUC15\ Thrombin\ channel)} \times 100$$

The Southampton Group subsequently established that percentage clotting inhibition correlated well to the previously described method, percentage platelet inhibition (116) as illustrated in Figure 5.

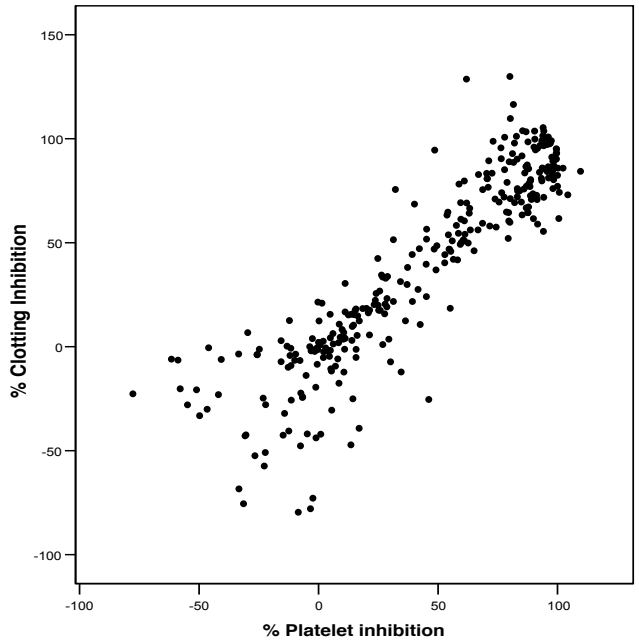

Figure 5. A scatterplot showing the correlation between percentage clotting inhibition and percentage platelet inhibition (R=0.903, n=320, p<0.01)

1.9.9 Cut-off values used to define antiplatelet therapy hyporesponsiveness

Previous studies that employed modified TEG to measure response to aspirin and clopidogrel have used a percentage platelet inhibition cut-off value of less than 50 and less than 30 to define hyporesponsiveness to aspirin and clopidogrel respectively and have been able to demonstrate a correlation with subsequent clinical events using these thresholds (79,115). As described previously, the Southampton Group have demonstrated that Short TEG percentage clotting inhibition correlates well with percentage platelet inhibition (116) and, as such, the above cut-off values are used to differentiate responders from non-responders on Short TEG.

1.9.10 The application of Short TEG

Following the validation of the Short TEG method, the Southampton Group used this test to conduct the following series of experiments:

a) Assessment of responses to aspirin and clopidogrel in PCI patients who have experienced ST whilst on APT (165)

b) The evaluation of gender-specific differences in responses to aspirin and clopidogrel (69)

c) The assessment of responses to clopidogrel in patients presenting with ACS (201)

d) The effect of higher clopidogrel loading doses (900mg) in clopidogrel hyporesponders (202)

e) The effects of clopidogrel on "aspirin-specific" pathways of platelet inhibition and its potential clinical implications (178)

1.9.11 Summary of previously published work on the validation Short TEG

1. Swallow R, Agarwala R, Dawkins K, Curzen N. Thromboelastography: potential bedside tool to assess the effects of antiplatelet therapy? *Platelets* 2006;17:385-92.

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3. Hobson A, Petley G, Dawkins K, Curzen N. A novel fifteen minute test for assessment of individual time-dependent clotting responses to aspirin and clopidogrel using modified Thrombelastography. *Platelets* 2007;18:497-505.

4. Hobson A, Dawkins K, Curzen N. Antiplatelet effects of licking an aspirin tablet can be detected by thrombelastography. *Acute Cardiac Care* 2008;10:62-3.

5. Hobson A, Petley G, Morton G, Dawkins K, Curzen N. Point-of-care platelet function assays demonstrate reduced responsiveness to clopidogrel, but not aspirin, in patients with drug-eluting stent thrombosis whilst on dual antiplatelet therapy. *Thrombosis Journal* 2008;21:1-6.

6. Hobson A, McKenzie D, Kunadian V, Purcell I, Zaman A, Dawkins D, Curzen N. Malignancy: an unrecognised risk factor for coronary stent thrombosis? *Journal of Invasive Cardiology* 2008;20:E120-3.

7. Hobson A, Qureshi Z, Banks P, Curzen N. Effects of clopidogrel on "aspirin-specific" pathways of platelet inhibition. *Platelets* 2009;20:386-90.
8. Hobson A, Curzen N. Improving outcomes with antiplatelet therapies in percutaneous coronary intervention and stenting. *Thrombosis Haemostasis* 2009;101:23-30
9. Hobson A, Qureshi Z, Banks P, Petley G, Curzen N. Gender and responses to aspirin and clopidogrel: Insights using short thrombelastography. *Cardiovascular Therapeutics* 2009;27:246-52
10. Cotton JM, Worrall AM, Hobson AR, Smallwood A, Amoah V, Dunmore S, Nevill AM, Rajendra R, Vickers J, Curzen N. Individualised assessment of response to clopidogrel in patients presenting with acute coronary syndromes: a role for short thromboelastography. *Cardiovascular Therapeutics* 2010;28:139-46
11. Hobson A, Qureshi Z, Banks P, Curzen N. The potential value of near patient platelet function testing in PCI: randomised comparison of 600mg versus 900mg clopidogrel loading doses. *Thrombosis* 2010;908272

1.10 Summary of our study objectives and their clinical relevance

The focus of my thesis is the clinical application of Short TEG in CVD. Below is an outline of my study objectives and their clinical relevance.

1.10.1 Short TEG reproducibility and correlation

The development and validation of Short TEG provided a novel method of assessing individual responses to aspirin and clopidogrel in a reliable and time-dependent manner (15 minutes), thus, making it appropriate for use as a point-of-care assay in the acute clinical setting. As previously described, the advantages of Short TEG over other point-of-care tests are that it is a whole blood assay that provides information on overall blood clotting tendency and, furthermore, it can be used to assess responses to both aspirin and P2Y12 receptor antagonists. However, the VerifyNow system is currently the most commonly used point-of-care test in the assessment of responses to APT mainly because of its relative ease of use, no requirement for sample preparation

and widespread availability. Thus, our first project entailed an investigation into the reproducibility of Short TEG and its correlation with VerifyNow in the assessment of responses to APT. I was able to effectively demonstrate that Short TEG is not only easily reproducible but also correlates well with VerifyNow in the assessment of responses to both aspirin and clopidogrel.

1.10.2 The clinical application of Short TEG: observations from a 'real world' patient registry of stent thrombosis

After having demonstrated the reproducibility and reliability of Short TEG as a rapid point-of-care test (and in view of the previous extensive work conducted by the Southampton Group on the validation of this method), our next step was to employ Short TEG as a clinical tool in 'real-world' frontline clinical practice. Specifically, I utilised Short TEG to assess responses to aspirin and clopidogrel in patients presenting acutely with ST and tailored treatment according to individual level of response. Our rationale for providing this clinical service was based on: (i) the well-described heterogeneity in individual responses to aspirin and clopidogrel (ii) the significantly higher reported incidence of APT hyporesponsiveness in patients with ST, and (iii) the clear link between reduced responsiveness and risk of adverse clinical events. As previously discussed, ST is a potentially life-threatening complication of PCI and, therefore, these patients represent a unique high risk group that would especially benefit from personalised APT. I conducted a comprehensive analysis of the prospective data I collected from this 'real-world' patient registry and made some fascinating observations that may form the basis of future large scale clinical studies in this field.

1.10.3 The prospective clinical studies

Finally, I employed Short TEG to conduct three prospective clinical studies that explored pertinent and clinically relevant issues all based on the theme of platelet function testing in the field of cardiovascular medicine. The rationale and objectives of these studies are outlined below.

1.10.3.1 An investigation into the effect of clopidogrel withdrawal in a cohort of patients one year after DES implantation.

I investigated a potential mechanism for the observed clustering of adverse events that have been reported following the cessation of long term clopidogrel. I measured ADP- and AA-induced platelet aggregation using Short TEG, as well as serum TXB2 and inflammatory biomarker levels at multiple time points. I observed an unexpected and significant time-dependent, aspirin-independent increase in AA-induced platelet reactivity following clopidogrel withdrawal, indicating that clopidogrel may exert some of its antiplatelet activity via the AA-pathway thereby potentiating the effect of aspirin. The findings from this study not only provided new insights into a potential mechanism for the clustering of adverse events observed after clopidogrel withdrawal, but also suggested that AA-induced clotting may not be a reliable test for measuring clinical response to aspirin. The latter formed the basis of the hypothesis of our next prospective study.

1.10.3.2 AA-induced clotting response in patients with ischaemic stroke: does this reflect the activity of aspirin at its pharmacological target?

My next study investigated whether responses to aspirin can be reliably determined from a functional test of AA-induced clotting and whether there is a relationship between aspirin responsiveness and vascular inflammation. Response to aspirin was determined by measuring AA-induced platelet aggregation on Short TEG as well as from the biochemical test, serum TXB2. We specifically chose to study this hypothesis in the ischaemic stroke population because the literature describes an apparently high prevalence of aspirin hyporesponsiveness in this particular patient group. I observed a significant discrepancy between AA-induced platelet reactivity and serum TXB2 in the assessment of responses to aspirin. These findings could pave the way for large scale clinical studies which may potentially have important implications on the way in which apparent aspirin hyporesponsiveness or "resistance" is diagnosed and managed in the future.

1.10.3.3 Is there a difference in pharmacological efficacy between Plavix® and generic clopidogrel?

My final study was a randomised prospective study which investigated a controversial issue that was the focus of ongoing debate among interventional cardiologists at the time of study planning: Clopidogrel exists in various different salt formulations but all published data that have demonstrated its beneficial effect are based entirely on the hydrogen sulphate salt that is contained in the branded product Plavix®. In view of the relatively high cost of Plavix®, a number of cheaper generic versions of the drug were increasingly being used in Europe as an alternative to Plavix® in all patients in whom clopidogrel was indicated. This widescale switch to generic clopidogrel was mainly driven by cost reasons, despite the lack of evidence to show that the pharmacodynamic effect of generic clopidogrel was equivalent to Plavix®. Thus, the objective of this study was to address the unease amongst clinicians, particularly interventional cardiologists, who required some reassurance that the cheaper generic clopidogrel salts shared the same degree of antiplatelet efficacy as Plavix® and was potentially safe to use in patients undergoing PCI, in whom the complication of ST could be fatal.

CHAPTER 2: METHODOLOGY

2.1 Study participants

The study participants included in our (i) reproducibility and correlation studies, (ii) ST patient registry, and (iii) prospective clinical studies are outlined below. The inclusion and exclusion criteria for each study are described in their individual sections in the results chapter.

2.1.1 Short TEG reproducibility and correlation studies

Previous studies were undertaken by Dr. Alex Hobson in healthy volunteers as well as in patients undergoing PCI to evaluate the reproducibility and reliability of Short TEG as well as its correlation with VerifyNow. I analysed the pool of data obtained from these experiments. The following subjects were included:

A. *Assessment of intra-individual baseline variability:* 1 volunteer at 20 time points on no medication

B. *Assessment of intra-individual variability in response to aspirin:* 1 volunteer at 10 time points pre- and 6 hours post a loading dose of aspirin 300mg

C. *Assessment of inter-individual baseline variability:* 56 volunteers on no medication

D. *Assessment of inter-individual variability in response to aspirin:* 25 volunteers pre- and 6 hours post a loading dose of aspirin 300mg

E. *Assessment of inter-individual variability in response to clopidogrel:* 28 patients pre- and 6 hours post a loading dose of clopidogrel 600mg

F. *Comparison of Short TEG and VerifyNow:* (i) 25 volunteers pre- and 6 hours post a loading dose of aspirin 300mg; (ii) 30 patients pre-, 1, 2, 6 and 24 hours post a loading dose of clopidogrel 600mg; (iii) 29 patients pre-, 1, 2, 6 and 24 hours post a loading dose of clopidogrel 900mg; and (iv) 20 patients on maintenance therapy with aspirin 75mg and clopidogrel 75mg for at least 14 days

2.1.2 A prospective registry of patients with ST receiving tailored APT

I prospectively recruited 43 consecutive patients admitted acutely with ST, measured their response to APT using Short TEG and tailored treatment according to their level of response. I then analysed the data obtained from this 'real-world' patient registry.

2.1.3 Prospective clinical studies: (i) withdrawal of clopidogrel in patients with DES, (ii) response to aspirin in patients with ischaemic stroke, and (iii) randomised study of Plavix® vs. generic clopidogrel in healthy volunteers

I prospectively recruited healthy volunteers as well as patients admitted to the Wessex Cardiothoracic Centre and the Acute Stroke Unit at University Hospital Southampton. I enrolled the following subjects:

A. 38 patients receiving maintenance therapy with aspirin and clopidogrel following PCI with DES
B. 36 patients with acute ischaemic stroke receiving maintenance therapy with aspirin 300mg
C. 17 healthy volunteers receiving loading (300mg) and maintenance (75mg) doses of clopidogrel (Plavix®) and its generic salt formulation

2.2 Research Ethics Committee and Research and Development department approvals

The reproducibility studies in section 2.1.1 and the clinical studies (A) and (C) in section 2.1.3 above were approved by the Southampton and South West Hampshire Research Ethics Committee. Study (B) in section 2.1.3 was approved by the North Wales Research Ethics Committee. Furthermore, study (C) in section 2.1.3 was categorised as a Clinical Trial of an Investigational Medical Product and, therefore, required additional approval from the Medicines and Healthcare products Regulatory Agency (MHRA).

In addition, the studies were approved and sponsored by the University Hospital Southampton NHS Foundation Trust local Research and Development department

and were registered on the National Institute for Health Research (NIHR) Clinical Research Network Portfolio database.

2.3 Consent

All subjects provided written informed consent prior to study inclusion. Study (B) in section 2.1.3 was approved by the Research Ethics Committee to include participants who lacked mental capacity (as result of their stroke) and were, therefore, unable to provide informed consent. In this situation, study recruitment was undertaken in accordance with the Mental Capacity Act 2005 (outlined in our study protocol) and consent was obtained via the participant's legal representative.

2.4 Study methods

2.4.1 Blood sampling

I personally performed venesection in approximately 90% of the participants in the ST patient registry in section 2.1.2 as well as in the clinical studies in section 2.1.3. I am grateful for the help of my two medical students Ashwin Radhakrishnan (BMedSci student) and Hazel Dent (Intercalated BSc student) who performed venesection in the remaining 10% of these cases under my supervision. In the reproducibility studies in section 2.1.1, venesection was performed by Dr. Alex Hobson.

Venesection was undertaken from the antecubital fossa in the majority of cases (some patients on the Acute Stroke ward had intravenous cannulae or previous multiple venesection attempts in the antecubital fossa as part of their routine inpatient care. In these situations, venesection was performed from the largest alternative peripheral vein we could locate). In all cases, blood was taken using a tourniquet with an 18gauge needle. Using a three-way tap, the first 2mls of blood was drawn into a 5ml syringe and later discarded. Thereafter, 8mls of blood was drawn into a 10 ml syringe and utilised as follows: 1ml was directly placed into a kaolin vial for immediate TEG analysis; 6mls was then gently trickled into a lithium heparin vacutainer® tube, mixed by gentle inversion 5 times and put aside for subsequent TEG analysis which was

undertaken 30 minutes after blood collection. Details of the TEG analysis method are described in section 2.4.2 below.
For studies (A) and (B) in section 2.1.3, which entailed serum TXB2 and inflammatory biomarker analyses, additional blood was collected into a 5ml serum SST and 4ml EDTA vacutainer® tube using the BD vacutainer® collection system. These samples were centrifuged within 30 minutes of collection at 1000 x g for 15 minutes and immediately frozen and stored at minus 80 degrees centigrade for future batch analysis. Details are described in sections 2.4.4 and 2.4.6 below.

2.4.2 TEG Platelet Mapping Method

Samples were run in the four TEG channels and analysed using a computerised TEG Analyser (Haemoscope Corp, MA). Electronic quality controls were performed on a daily basis for all channels and both Level I and Level II wet quality controls were performed on a monthly basis. All reagents were allowed to reach room temperature before being reconstituted and were then utilised within 60 minutes.
The four channels used were: (1) Kaolin (the "thrombin channel"); (2) Activator F™ alone (the "fibrin channel"); (3) Activator F™ + AA (the "AA channel"); and (4) Activator F™ + ADP (the "ADP channel"). For the thrombin channel, 1ml of blood was mixed by gentle inversion in a vial containing 1% kaolin solution (Haemoscope Corp, MA) and 360µl of this mixed sample was then pipetted into the thrombin channel cup. For the other three channels (i.e. the fibrin, AA and ADP channels), 10µl Activator F™, a mixture of reptilase and factor XIII (Haemoscope Corp, MA), was pipetted into each cup. 10µl (1mM) of AA was then pipetted into the AA channel cup and 10µl (2µM) of ADP was pipetted into the ADP channel cup. 360µl of heparinised blood was then added to the fibrin, AA and ADP channel cups and mixed with the reagents. Samples were run until the MA was reached.

2.4.3 Presentation of TEG results

MA results (in millimetres) are derived directly from the TEG software and presented to 1 decimal place. AUC results (millimetre-minutes) calculated from the Areafinder 2.1 software are rounded to the nearest whole number. Results are presented as the mean ± 95% confidence interval of the mean.

The percentage clotting inhibition derived from AUC15 (described in section 1.9.8) is used to calculate responses to APT. A percentage clotting inhibition of less than 50 in the AA channel represents an inadequate response to aspirin, and a percentage clotting inhibition of less than 30 in the ADP channel represents an inadequate response to clopidogrel or other P2Y12 receptor inhibitor.

2.4.4 Serum Thromboxane B2 methods

Serum TXB2 analysis was performed in duplicate using commercially available competitive enzyme-linked immunosorbent assay (ELISA) kits from R&D systems (Abingdon UK) and Cayman Chemicals (Michigan, U.S.A) according to manufacturer's instructions. TXB2 analysis in study (A) in section 2.1.3 was performed by Professor Timothy Warner and his medical student Philip Leadbeater at the William Harvey Research Institute, Barts and the London School of Medicine and Dentistry. TXB2 analysis in study (B) in section 2.1.3 was performed by myself under the direct supervision of Dr. Nicola Englyst at the Institute of Developmental Sciences, Faculty of Medicine, University of Southampton.

2.4.5 Presentation of thromboxane B2 results

Previous studies have shown that administration of 100mg aspirin daily has led to more than 98% steady state inhibition of platelet COX-1 activity and has been associated with residual serum TXB2 concentrations of less than 10ng/ml (203). Thus, a cut-off value of serum TXB2 of <10ng/ml is consistent with an adequate response to aspirin (179) and this is the specific cut-off value used in our study. Results are presented as the mean ± 95% confidence interval of the mean.

2.4.6 Inflammatory biomarker methods

The inflammatory biomarkers soluble CD40 ligand (sCD40L), high sensitivity C-reactive protein (hsCRP), Interleukin-6 (IL-6) and tumour necrosis factor (TNF)-alpha were measured in duplicate using commercially available ELISA kits from R&D Systems (Abingdon, UK) according to manufacturer's instructions. The analysis was performed by myself under the direct supervision of Dr. Nicola Englyst at the Institute of Developmental Sciences, Faculty of Medicine, University of Southampton.

2.4.7 Presentation of inflammatory biomarker results

The reference ranges used for the individual inflammatory biomarkers were in accordance with the manufacturer's instructions. Results are presented as the mean ± 95% confidence intervals of the mean.

2.4.8 VerifyNow methods

VerifyNow testing in the correlation studies in section 2.1.1 was performed by Dr. Alex Hobson. Electronic quality control was carried out on a daily basis. Level 1 and Level 2 Quality Controls were carried out on every new batch of both Aspirin and P2Y12 assays. Vacutainers were mixed gently by inversion 5 times immediately after filling. Analysis was performed between 20 and 60 minutes of venesection for P2Y12 assays and 30 and 60 minutes for Aspirin assays. The VerifyNow results were recorded as presented on the machine.

2.4.9 Statistical methods

Statistical advice was sought from Professor Paul Roderick and Medical statistician Scott Harris at the Southampton Statistical Sciences Research Institute, University of Southampton. The statistical methods employed for each study are described in their individual sections in the results chapter.

CHAPTER 3: RESULTS

ORIGINAL ARTICLE

"Short" thrombelastography as a test of platelet reactivity in response to antiplatelet therapy: Validation and reproducibility

NALYAKA SAMBU[1,2], ALEX HOBSON[1], & NICK CURZEN[1,2]

Platelets, May 2011; 22(3): 210–216

Abstract

Background: A significant proportion of patients receiving dual antiplatelet therapy following percutaneous coronary intervention experience recurrent ischaemic events despite standard doses of treatment. Although clinical studies show significant heterogeneity in antiplatelet therapy responses, routine testing is not undertaken due to (i) lack of a standardized test, and (ii) poor clarity with regards to definition of abnormal responses. Short Thrombelastography (s-TEG) is a validated test that allows rapid measurement of clotting responses to antiplatelet therapy.

Objectives: This study sought to determine the reproducibility of s-TEG and to compare s-TEG with VerifyNow in assessment of responses to antiplatelet therapy.

Methods: (i) intra-individual variability was assessed using s-TEG Area Under the Curve (AUC15) and maximum amplitude (MA) in one volunteer at 20 time-points on no medication and at 10 time-points pre and post 300 mg aspirin treatment (ii) inter-individual variability was determined from a retrospective analysis of data on MA and AUC15 obtained from 56 volunteers on no medication, 25 volunteers pre and post 300 mg aspirin treatment and 28 patients pre and post 600 mg clopidogrel treatment (iii) a comparison between AUC15 arachidonic-acid (AA) channel and VerifyNow aspirin response units (VN ARU) and between AUC15 adenosine diphosphate (ADP) channel and VerifyNow P2Y12 reactivity units (VN PRU) was obtained from retrospective analysis of data at 370 and 296 time-points respectively.

Results: There was minimal intra-and inter-individual variability in MA and AUC15 in the AA, ADP and thrombin channels. There was a good correlation between AA AUC15 and VN ARU ($r = 0.701$, $p < 0.001$) and between ADP AUC15 and VN PRU ($r = 0.609$, $p < 0.001$).

Conclusions: s-TEG is a reproducible and reliable near-patient test that correlates well with VerifyNow. Large scale studies are needed to determine its potential role in individually tailored antiplatelet therapy.

Study objectives: To investigate the reproducibility and reliability of Short TEG as a rapid, point-of-care platelet function test and to determine whether Short TEG correlates well with the more widely used VerifyNow assay in the assessment of responses to APT.

3.1 Introduction

Dual APT with aspirin and clopidogrel is the default therapy for both ACS and following PCI. However, a significant proportion of these patients experience recurrent ischaemic and thrombotic events on standard doses of treatment and clinical studies have demonstrated significant heterogeneity in patient response to APT. Specifically, relative hyporesponsiveness and high residual platelet reactivity is associated with increased risk of adverse events including ST and cardiovascular death (63,84,115,134,142,166). Despite this evidence, routine testing for APT hyporesponsiveness is currently not undertaken largely due to the lack of a standardised and widely accepted platelet function assay appropriate for routine clinical use

TEG is a platelet function test that provides an overall assessment of *ex vivo* haemostatic function, thus incorporating the interaction of all the components of coagulation including thrombin, platelets, fibrin and clotting factors. TEG utilises whole blood to provide a graphical representation of the speed of clot formation, clot strength and stability. Modifications to the original TEG methodology have improved its ease of use and allowed it to be used to more specifically assess the effects of APT on an individual's clotting response. Conventionally, APT response has been determined from the MA of the TEG trace which can take over 1 hour to be reached making it a relatively slow tool for point-of-care testing. As a result, the novel TEG-derived parameter AUC15, which provides an assessment of the effects of APT in 15 minutes, has been developed and extensively validated. The TEG AUC15 method, known as Short TEG, has been previously described in section 1.9.7 (116).

The aims of this study are: (i) to assess the reproducibility of Short TEG by an assessment of both intra- and inter-subject variability, and (ii) to compare Short TEG with the widely used VerifyNow system in assessment of responses to APT.

3.2 Methods

3.2.1 Study population

Exclusion criteria for volunteers

All individuals were excluded if they had taken any antiplatelet or non-steroidal anti-inflammatory medication within 14 days. Those individuals receiving APT were excluded if they had a history of peptic ulceration, bronchial asthma or bleeding

Exclusion criteria for patients

Individuals were excluded if they had taken antiplatelet or anticoagulant medication other than aspirin in the preceding 28 days or if they had a history of clopidogrel intolerance, recent bleeding, major haematological disturbance, malignancy or planned use of GP IIb/IIIa inhibitors.

3.2.2 Study protocols

For the assessment of intra- and inter-individual variability the TEG MA value was measured and AUC15 calculated using Short TEG.

1. Assessment of intra-individual baseline variability

Venesection was performed on one volunteer at 20 different time points, each at least two weeks apart and at least 2 weeks from the administration of any antiplatelet or non-steroidal anti-inflammatory medication.

2. Assessment of intra-individual variability in response to aspirin

One volunteer received high dose aspirin 300mg on ten separate occasions. Aspirin was administered at least 2 weeks from any other antiplatelet or non-steroidal anti-inflammatory medication. Venesection was performed immediately before and 6 hours after drug administration.

3. Assessment of inter-individual baseline variability

Venesection was performed in 56 healthy volunteers at least 2 weeks from the administration of any antiplatelet or non-steroidal anti-inflammatory medication.

4. Assessment of inter-individual variability in response to aspirin and clopidogrel

(i) 25 healthy volunteers received high dose aspirin 300mg. Venesection was performed immediately before and 6 hours after drug administration
(ii) 28 patients on aspirin 75mg and undergoing planned PCI, received a loading dose of clopidogrel 600mg. Venesection was performed immediately before and 6 hours after drug administration.

5. Comparison of Short TEG and VerifyNow

Venesection was performed in the following groups of healthy volunteers as well as patients undergoing PCI and samples were analysed using Short TEG and VerifyNow: (i) 25 volunteers pre and 6hrs post 300mg aspirin (ii) 30 patients pre, 1, 2, 6 and 24hrs post 600mg clopidogrel (iii) 29 patients pre, 1, 2, 6 and 24hrs post 900mg clopidogrel (iv) 20 patients on dual APT with aspirin and clopidogrel

3.2.3 Blood sampling and analysis

Venesection and sample analysis were performed as specified in the Study Methods in section 2.4.

3.2.4 Statistical analysis

The coefficient of variation was calculated for the MA and AUC15 in all four TEG channels. Data are presented as the mean ± 95% confidence intervals of the mean. Correlations between AUC15 of the AA channel and VN ARU and between AUC15 of the ADP channel and VN PRU were calculated using Pearson's correlation. Significance was determined using paired two-tailed t-tests with a p value of <0.05 considered to represent significance.
Agreement between the cut-off points for Short TEG and VerifyNow in defining APT hyporesponsiveness was assessed by the Kappa *(κ)* statistic. Values of $\kappa < 0.2$ indicate poor agreement; $\kappa = 0.2$ to 0.4 indicate fair agreement; $\kappa = 0.41$ to 0.6 indicate moderate agreement; and $\kappa > 0.61$ indicate good agreement.

3.3 Results

3.3.1 Intra-individual baseline variability

The MA results obtained from one volunteer at 20 different time points were expressed as mean ± 95% confidence intervals of the mean. The coefficient of variation is 5.8 to 7.8% for the thrombin, ADP and AA channels, with a higher value obtained from the fibrin channel (45.8%). The higher coefficient of variability in the fibrin channel is largely due to the lower recorded MA in this channel. As can be seen in Figures 6A and B, the confidence intervals remain narrow. Figures 6A and 6B illustrate MA and AUC15 data with confidence intervals in all four TEG channels.

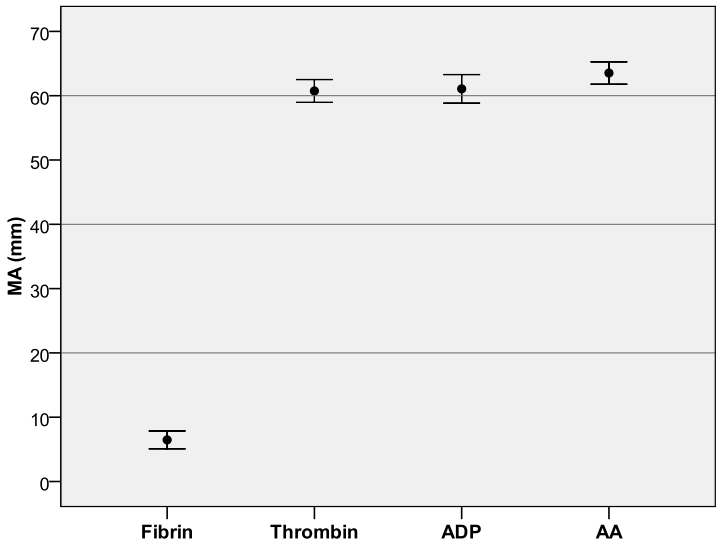

Figure 6A. Intra-individual baseline variability in all four channels of modified TEG illustrated using maximum amplitude (MA) (ADP – adenosine diphosphate; AA – arachidonic acid)

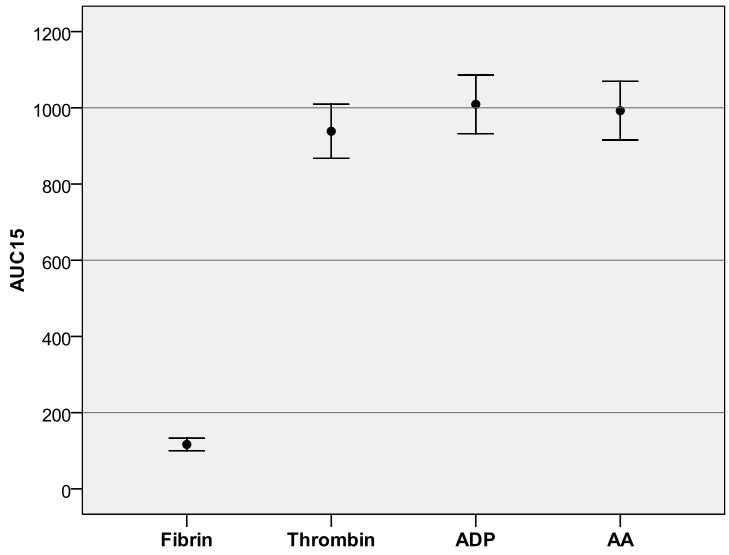

Figure 6B. Intra-individual baseline variability in all four channels of modified TEG illustrated using Area Under the curve at 15 minutes (AUC15) (ADP – adenosine diphosphate; AA – arachidonic acid)

3.3.2 Intra-individual variability in response to aspirin

The MA and AUC15 results obtained immediately before and 6 hours after administration of high dose aspirin 300mg in one volunteer on ten separate occasions are shown in figures 7A and 7B. There was a reliable and reproducible decrease in the MA from 62.8±1.8 to 9.2±2.3 ($p<0.001$) and in the AUC15 from 1052±85.5 to 152±30 ($p<0.001$) pre and post aspirin therapy (values are expressed as mean ± 95% confidence intervals of the mean).

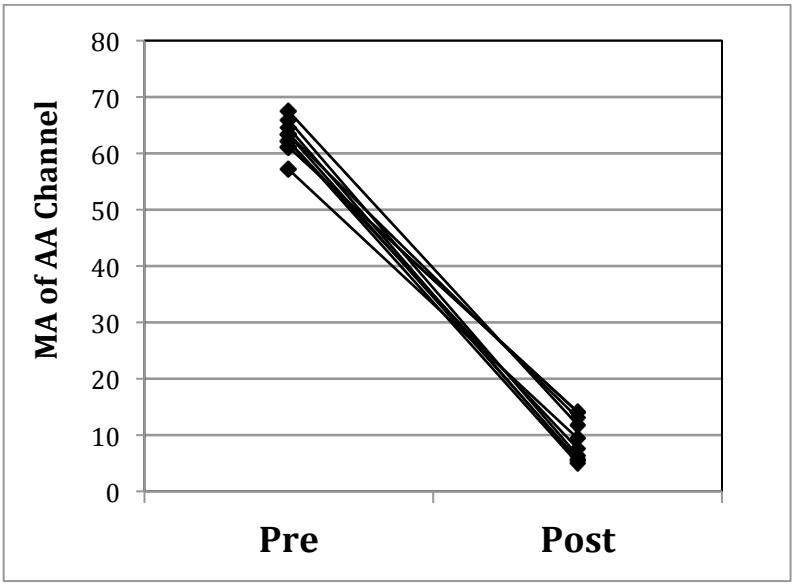

Figure 7A. Intra-individual response to aspirin therapy: the maximum amplitude (MA) of the arachidonic acid (AA) channel immediately prior to and 6 hours after administration of a 300mg dose of aspirin in one volunteer on 10 separate occasions

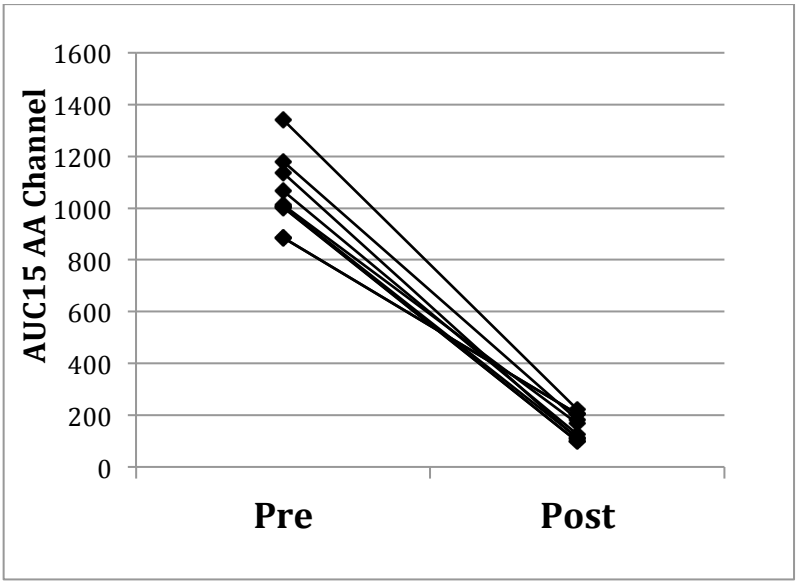

Figure 7B. Intra-individual response to aspirin therapy: the Area Under the Curve at 15 minutes (AUC15) of the arachidonic acid (AA) channel immediately prior to and 6 hours after administration of a 300mg dose of aspirin in one volunteer on 10 separate occasions

3.3.3 Inter-individual baseline variability

The MA results obtained from 56 volunteers were expressed as mean ± 95% confidence intervals of the mean. The coefficient of variation is between 11.9 to 19.2% for the thrombin, ADP and AA channels, with a higher value obtained from the fibrin channel (86.3%). Figures 8A and 8B illustrate MA and AUC15 data with confidence intervals obtained in all four channels.

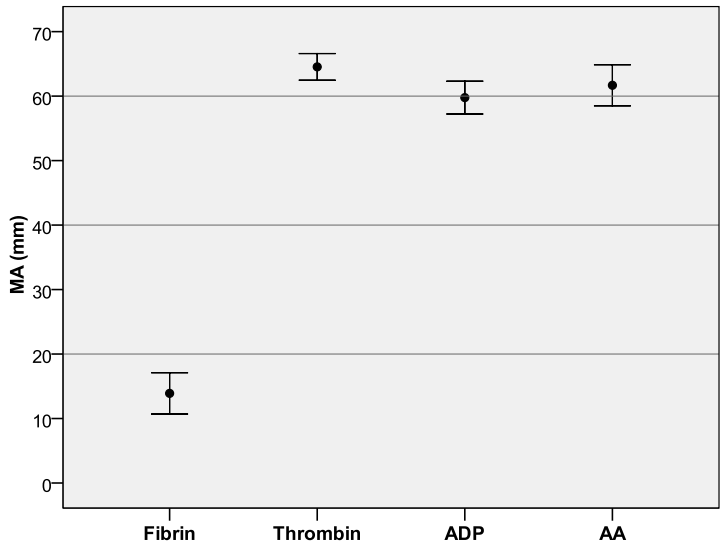

Figure 8A. Inter-individual baseline variability in all four channels of modified TEG illustrated using maximum amplitude (MA) (ADP – adenosine diphosphate; AA – arachidonic acid)

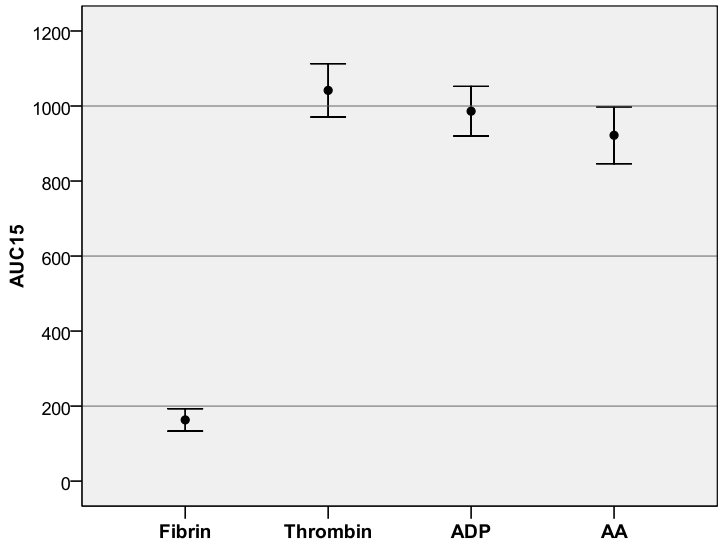

Figure 8B. Inter-individual baseline variability in all four channels of modified TEG illustrated using Area Under the Curve at 15 minutes (AUC15) (ADP – adenosine diphosphate; AA - arachidonic acid)

3.3.4 Inter-individual variability in response to aspirin and clopidogrel

(i) Aspirin

The MA and AUC15 results in the AA channel obtained immediately before and 6 hours after administration of high dose aspirin in 25 healthy volunteers are shown in figures 9A and 9B. There was a decrease in the MA from 63.9±2.7 to 21.1±7.8 ($P<0.001$) and in the AUC15 from 953.4±86.8 to 277±90.4 ($p<0.001$) pre- and post aspirin therapy (values are expressed as mean ± 95% confidence intervals of the mean).

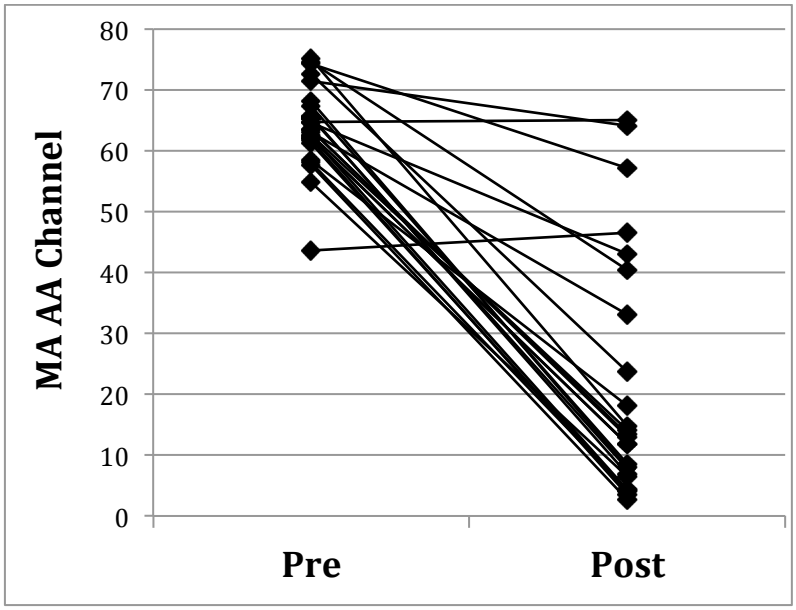

Figure 9A. Inter-individual response to aspirin therapy: the maximum amplitude (MA) of the arachidonic acid (AA) channel immediately prior to and 6 hours after administration of a 300mg dose of aspirin in 25 healthy volunteers

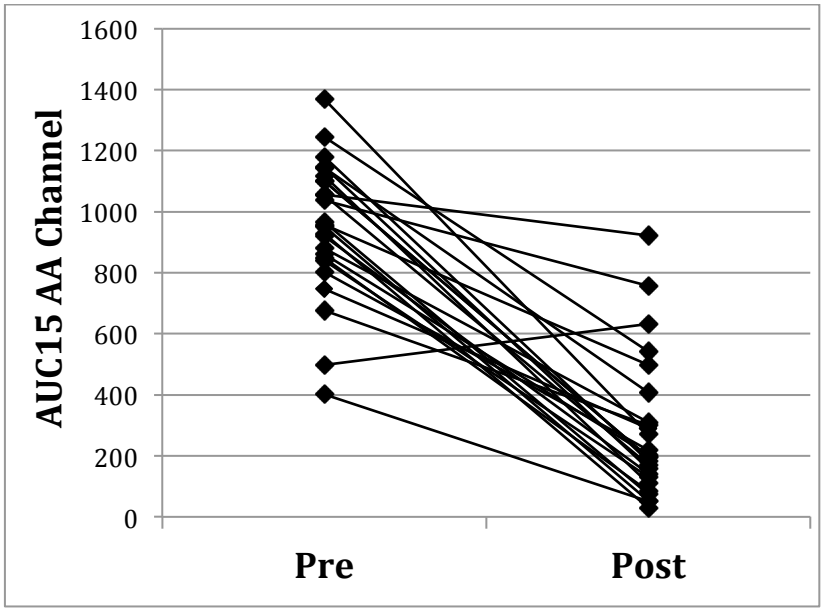

Figure 9B. Inter-individual response to aspirin therapy: the Area Under the Curve at 15 minutes (AUC15) of the arachidonic acid (AA) channel immediately prior to and 6 hours after administration of a 300mg dose of aspirin in 25 healthy volunteers

(ii) Clopidogrel

The MA and AUC15 results in the ADP channel obtained immediately before and 6 hours after administration of clopidogrel 600mg in 28 patients undergoing PCI are shown in figures 10A and 10B. There was a decrease in MA from 60.7±3.3 to 45.1±5.5 ($p<0.001$) and in the AUC15 from 1032.8±79.4 to 763.5±109.1 ($p<0.001$) pre and post clopidogrel therapy (values are expressed as mean ± 95% confidence intervals of the mean).

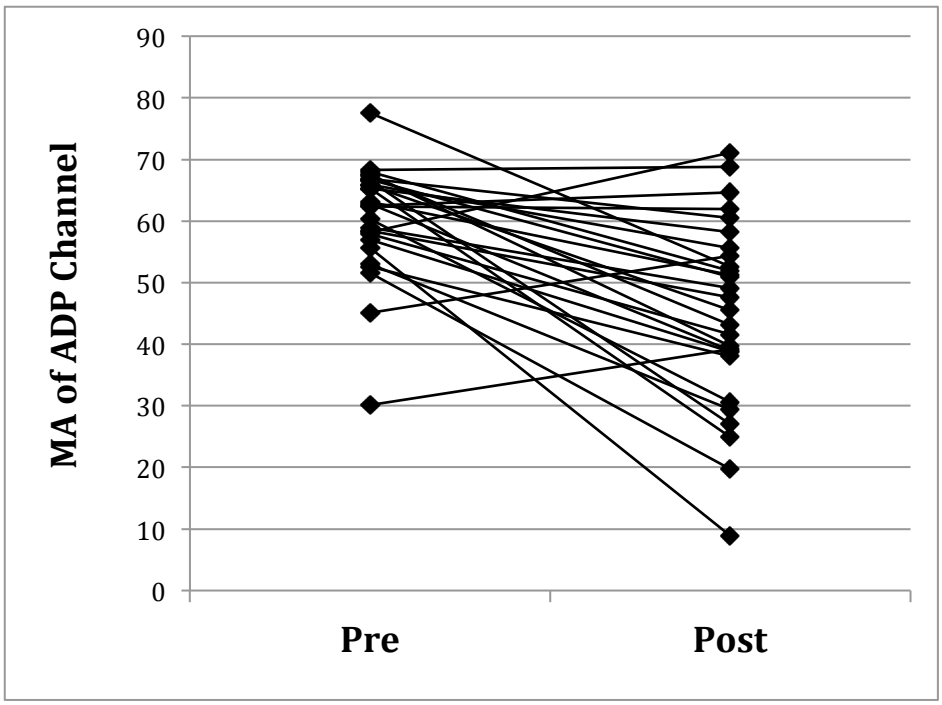

Figure 10A. Inter-individual response to clopidogrel therapy: the maximum amplitude (MA) of the adenosine diphosphate (ADP) channel immediately prior to and 6 hours after administration of a 600mg dose of clopidogrel in 28 patients on low dose aspirin undergoing PCI

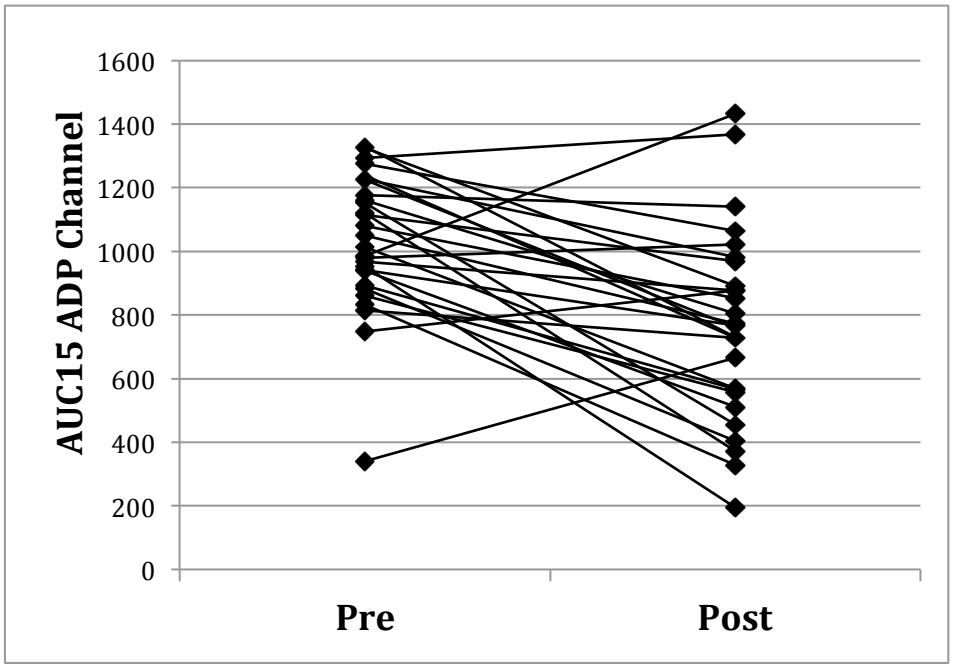

Figure 10B. Inter-individual response to clopidogrel therapy: the Area Under the Curve at 15 minutes (AUC15) of the adenosine diphosphate (ADP) channel immediately prior to and 6 hours after administration of a 600mg dose of clopidogrel in 28 patients on low dose aspirin undergoing PCI

3.3.5 Correlation between Short TEG and VerifyNow

There was good correlation between AUC15 of the AA Channel and VN ARU measured at 370 time points (R^2= 0.491, correlation coefficient r=0.701, p<0.001) and between AUC15 of the ADP channel and VN PRU measured at 296 time points (R^2=0.371, correlation coefficient r=0.609, p<0.001). This is illustrated in figures 11A and 11B respectively.

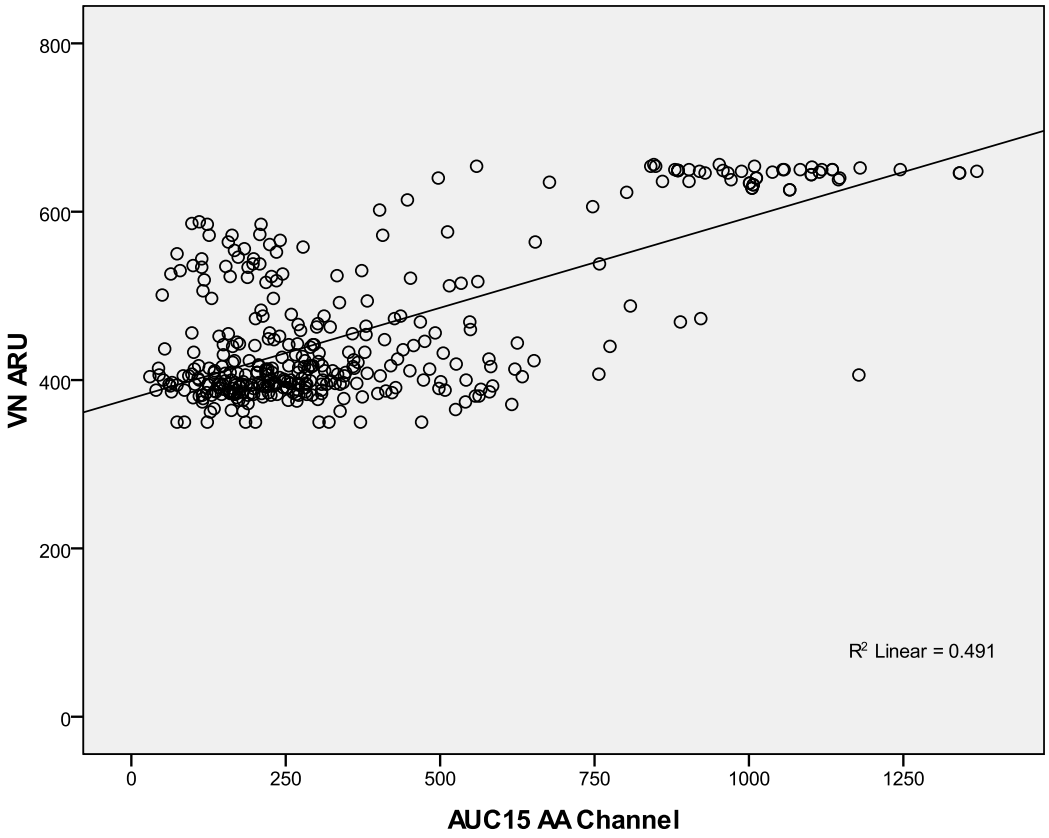

Figure 11A. Correlation between VerifyNow Aspirin Response Units (VN ARU) and TEG Area Under the Curve at 15 minutes (AUC15) of the arachidonic acid (AA) channel (n=370, R^2= 0.491, correlation coefficient r=0.701, p<0.001)

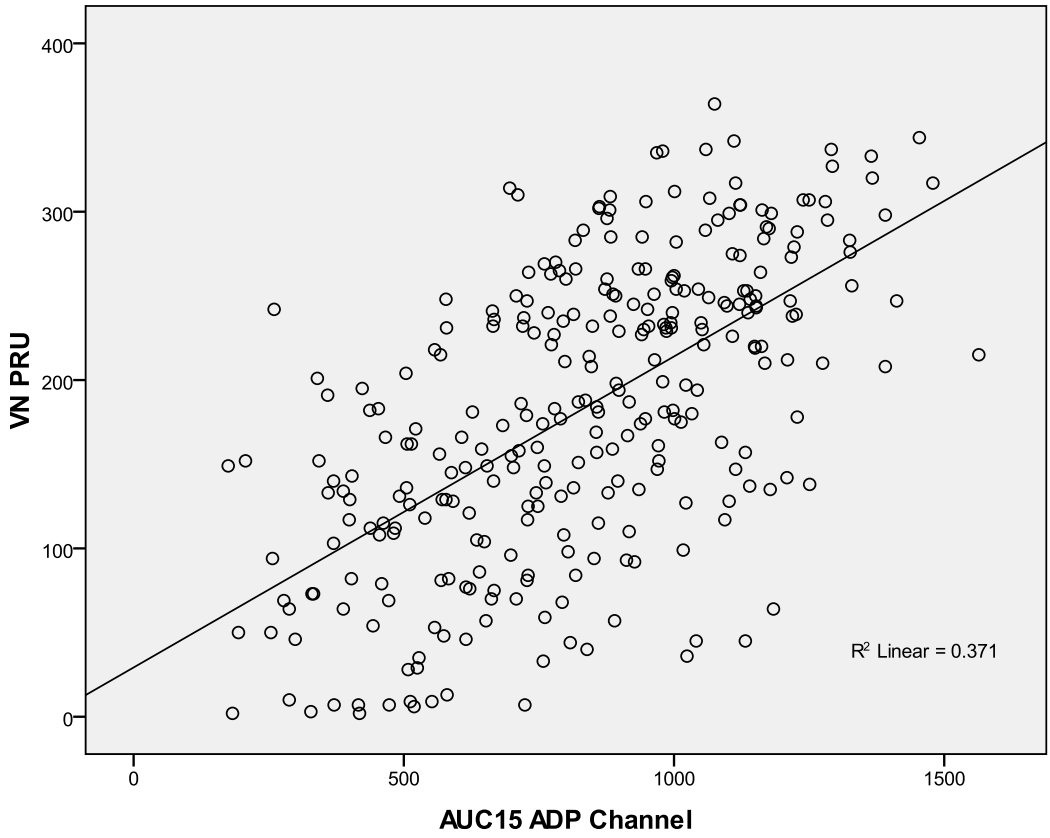

Figure 11B. Correlation between VerifyNow Platelet Response Units (VN PRU) and TEG Area Under the Curve at 15 minutes (AUC15) of the adenosine diphosphate (ADP) channel (n=296, R^2=0.371, correlation coefficient r=0.609, p<0.001)

3.3.6 Agreement between Short TEG and VerifyNow

The level of agreement between Short TEG and VerifyNow in determining response to APT was determined: (i) in 27 patients at 6hrs following a 600mg loading dose of clopidogrel, (ii) in 25 patients at 6hrs following a 900mg loading dose of clopidogrel, and (iii) in 79 patients following maintenance therapy with aspirin 75mg.

The cut-off values used to define hyporesponsiveness to aspirin and clopidogrel are as follows:

Short TEG: A percentage clotting inhibition of <50 and <30 in the AA and ADP channels indicate hyporesponsiveness to aspirin and clopidogrel respectively. The

derivation and validation of percentage clotting inhibition is described in detail in section 1.9.

VerifyNow: ARU ≥ 550 and PRU ≥ 230 indicate hyporesponsiveness to aspirin and clopidogrel respectively. These cut-off values are derived from data from previous clinical studies (149, 150).

Using the above cut-off values:

(i) The raw agreement between PRU ≥ 230 and a percentage clotting inhibition < 30 in defining hyporesponsiveness to clopidogrel 600mg was 59% and the level of agreement according to the Kappa statistic was fair ($\kappa = 0.29$, $p = 0.033$).

(ii) The raw agreement between PRU ≥ 230 and a percentage clotting inhibition < 30 in defining hyporesponsiveness to clopidogrel 900mg was 76% and the level of agreement according to the Kappa statistic was fair ($\kappa = 0.39$, $p = 0.014$).

(iii) The raw agreement between ARU ≥ 550 and a percentage clotting inhibition < 50 in defining hyporesponsiveness to aspirin 75mg was 78% and the level of agreement according to the Kappa statistic was poor ($\kappa = 0.10$, $p = 0.014$).

Of note, the use of the Kappa statistic to assess level of agreement between Short TEG and VerifyNow is limited by the fact that Kappa is very sensitive to the choice of cut-off value used to differentiate responders from non-responders. Hence, a small change in cut-off value would result in a significant change in the level of agreement (for example, some clinical studies have used a PRU ≥ 208 (rather than 230) to define clopidogrel hyporesponsiveness with VerifyNow (149, 150)). The use of arbitrary and binary cut-off values to differentiate responders from non-responders may be inappropriate for various reasons outlined in detail in section 1.4.1. These factors need to be taken into account when interpreting the level of agreement between Short TEG and VerifyNow based on the Kappa statistic.

3.4 Discussion

This study yields results that suggest that Short TEG represents a plausible test for measurement of responses to APT in clinical practice. Firstly, we have shown that, in

healthy volunteers, Short TEG is a reproducible and reliable test of clotting responses to APT with minimal intra- and inter-individual variability. These findings are consistent with results from a previous study on healthy, APT naïve blood donors which showed an analytical variation of approximately 5% in TEG platelet mapping variables (204). Secondly, we have shown that Short TEG reliably measures responses to both aspirin and clopidogrel as demonstrated by the overall significant decline in AUC15 (as well as MA) of the AA and ADP channels following loading doses of aspirin and clopidogrel respectively. The patients who received a loading dose of clopidogrel were also on low dose aspirin therapy. As expected, aspirin did not have any effect on ADP-induced platelet aggregation as demonstrated by the high MA and AUC values pre-clopidogrel therapy. Thirdly, we have demonstrated that the novel TEG parameter AUC15 is not only easily reproducible but also exhibits a good correlation with VerifyNow in the assessment of responses to aspirin and clopidogrel. This observation is in keeping with findings from a previous study that showed a strong correlation between AUC15, VerifyNow and VASP in assessment of responses to loading doses of clopidogrel therapy in ACS patients (201). Both VerifyNow and VASP assays have been shown to predict adverse clinical outcomes (95-97,162,205). Increasingly, it is accepted that there would be important clinical value in a reliable and reproducible platelet function test that could be utilised at the point of patient contact to rapidly detect responses to APT with a view to patient-specific tailoring of treatment. However, it is also generally accepted that *"tailored treatment cannot yet be recommended in daily clinical practice because the best laboratory method to monitor the effects of clopidogrel on platelet function still needs to be identified, standardised, and validated in the clinical setting"* (206). Nonetheless, individualised patient-specific treatment may be of particular clinical value in those patients at highest risk of cardiovascular events by virtue of elevated residual platelet reactivity. This hypothesis may be unavoidable given the now well-established association between poor response (or "resistance") to aspirin or, more commonly, clopidogrel and serious adverse events. The lack of routine clinical testing of responses to aspirin and clopidogrel is, therefore, illogical but is partially due to a lack of an easy-to-use, rapid, reliable and comprehensive test requiring minimal blood preparation. Most currently available assays of platelet reactivity are time consuming, expensive and technically demanding and therefore do not fulfil the criteria of an ideal point-of-care test that can be utilised in the acute setting. Previous studies have demonstrated the

ability of Short TEG to detect responses to APT in a time-dependent manner (116) and this study now also confirms the reproducibility of the test.

These findings demand further investigation from large scale clinical trials to evaluate whether Short TEG can be utilised as a clinical tool to guide APT prescribing strategies that will ultimately lead to reduced risk of adverse ischaemic events. This is of particular importance in high risk patient groups such as those presenting with ST and is of particular relevance in the current era of newer, more potent antiplatelet agents such as prasugrel and ticagrelor and their associated increased bleeding risks. However, the ability to test individual responses to aspirin and clopidogrel to provide personalised therapy may have wider clinical relevance in the field of cardiovascular medicine. For example, in the field of stroke, the reported prevalence of resistance to aspirin is up to 60% (125) which indicates that this could represent a therapeutic target. Furthermore, recent data suggest that the interaction between clopidogrel and "aspirin-specific" pathways of platelet reactivity may be clinically relevant (178,181) and this demands further investigation.

What may become mandatory in the future is careful selection of the most appropriate, safe and effective antiplatelet agent for each patient based on individualised assessment of response to therapy, but for now what is urgently needed is a standardised and widely accepted point-of-care test. What this group has demonstrated here, consistent with data from previous studies, is the feasibility of Short TEG. These data suggest that, with refinement in terms of ease of use, Short TEG may represent a plausible candidate as a point-of-care test with which tailored therapy can be delivered.

This study has several limitations. Firstly, the sample sizes are relatively small. Second, there is marked variability in the fibrin channel, both intra- and inter-individual. The reason for this is unclear but has informed the development of a "snapshot" tool for describing a time-specific figure to express the response of an individual to aspirin or clopidogrel. Thus, Hobson et al (116) have described the percentage clotting inhibition (described in Section 1.9.8), a parameter that eliminates the fibrin component and uses the thrombin channel as an invaluable internal control. This is in contrast to other groups who have tried to take account of fibrin variability (79).

3.5 Conclusion

Short TEG represents a plausible candidate to provide a rapid, reproducible and relatively near-patient test of response to APT. This may have importance in the clinical environment as the case for patient-level tailored therapy becomes stronger.

ORIGINAL ARTICLE

Personalised antiplatelet therapy in stent thrombosis: observations from the Clopidogrel Resistance in Stent Thrombosis (CREST) registry

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Heart 2012;**98**:706—711

ABSTRACT

Objective Previous studies have demonstrated significant heterogeneity in responses to antiplatelet therapy (APT), and high residual platelet reactivity is associated with the risk of ischaemic events, including stent thrombosis (ST). The prevalence of APT hyporesponsiveness in a 'real world' registry of ST patients and the feasibility of personalising APT are reported.

Patients and setting 39 consecutive patients admitted to a single regional cardiothoracic centre with definite ST were prospectively evaluated.

Interventions Response to aspirin and clopidogrel was measured following discharge using short thrombelastography (TEG), a rapid, well validated near patient platelet function test. Treatment modification in hyporesponders comprised an increase in aspirin dose and/or changing clopidogrel to prasugrel or ticagrelor. Short TEG was repeated following treatment modification to ensure an adequate response had been achieved.

Results 12 (31%) patients had an adequate response to both aspirin and clopidogrel, 16 (41%) were hyporesponsive to clopidogrel alone, one (3%) was hyporesponsive to aspirin alone and 10 (26%) were hyporesponsive to both aspirin and clopidogrel. Following treatment modification, an adequate response to aspirin and P2Y12 agent was achieved in 10 (91%) and 22 (85%) patients, respectively. None has presented with a further ST episode.

Conclusions There is a high prevalence of hyporesponsiveness to APT in patients with ST. Improved APT efficacy can be achieved by tailored therapy. Short TEG is a plausible platelet function test that can be used to deliver point of care personalised APT.

Study objectives: to investigate the feasibility and outcome of using Short TEG in the acute clinical setting to measure responses to APT with a view to providing tailored treatment in a 'real-world' registry of patients with ST

4.1 Introduction

ST continues to dominate the profile of adverse events to which patients undergoing PCI are susceptible. Administration of APT is universal and subject to international guidelines (207,208). These guidelines reflect the concern about the ongoing attrition rate for ST events that runs at approximately 0.6% per year for DES (209), although the disparate and ever-changing nature of the clinical trial data mean that the optimal duration of dual APT remains uncertain. Whilst ST is likely to be multifactorial in most cases, it is now well described that an absence of, discontinuation or inadequate response to antiplatelet drugs are dominant risk factors in many patients. Specifically, hyporesponsiveness to either aspirin or clopidogrel that results in high residual platelet reactivity has been shown in multiple studies to be associated with post PCI ischaemic events including ST (82,115,135,158,159). Despite this, in routine clinical practice, no assessment of individual responses to these agents is made and patients are prescribed standard doses of therapy.

There are limited and discrepant data in the literature regarding the ability to tailor therapy to individual responses and thereby improve clinical outcome (147,148,210). This is particularly true in the case of clopidogrel and has stimulated interest in replacing it with more potent inhibitors of the ADP P2Y12 receptor. However, recent reports of higher than expected prevalence of hyporesponsiveness to prasugrel (211,212) raise logical concern about this blunderbuss approach.

In this section, I describe the outcome of assessing responses to APT in a large series of patients who have experienced an episode of ST, and then altering therapy where their response was deemed inadequate. This series demonstrates the feasibility of assessing individual responses to APT and modifying therapy accordingly in order to achieve adequate responses.

4.2 Methods

The Clopidogrel Resistance in Stent Thrombosis (CREST) Registry is a 'real-world' registry of consecutive patients admitted to a single regional cardiothoracic centre with definite ST. This condition is defined, according to the Academic Research Consortium (ARC) criteria (157), as a clinical presentation with an ACS associated with angiographic evidence of stent occlusion or thrombus. All patients were treated in accordance with local clinical guidelines and subsequently discharged on dual APT with aspirin at a dose of either 75 or 150mg depending on physician preference and clopidogrel 75mg once daily. Response to APT was measured at least 2 weeks following hospital discharge using Short TEG. Changes to APT were directed by Short TEG and repeat testing was undertaken following treatment modification to ensure an adequate response had been achieved.

4.2.1 Blood sampling and analysis

Venesection and sample analysis were performed as specified in the Study Methods in section 2.4.

4.2.2 Percentage clotting inhibition

Based on the novel Short TEG parameter AUC15, the percentage clotting inhibition, which describes the absolute effect of antiplatelet medication on the overall *ex-vivo* clotting response of an individual, has been derived and validated (116). It is calculated by comparing AA- or ADP-induced clotting responses with response to thrombin, which represents an invariable internal control. A percentage clotting inhibition cut-off value of <50 in the AA channel and <30 in the ADP channel was used to define hyporesponsiveness to aspirin and P2Y12 receptor inhibitors respectively in this registry. The derivation and validation of percentage clotting inhibition is described in detail in section 1.9.

As described previously, percentage clotting inhibition (%CI) is calculated using the following formula:

%CI = 100-((AUC15 of ADP* or AA‡ channel/AUC15 of thrombin channel) x100)

(*AUC15 ADP channel is used in the formula when measuring response to P2Y12 agent and ‡AUC15 AA channel is used in the formula when measuring response to aspirin)

4.2.3 Treatment modification

Following the acute episode of ST, patients were recalled for platelet function testing using Short TEG at between 14 and 186 days (median 34 days). Patients demonstrating an inadequate response had their APT changed in the following algorithms:

(i) aspirin hyporesponders: the dose of aspirin was increased up to a maximum of 300mg

(ii) clopidogrel hyporesponders: prasugrel was commenced instead of clopidogrel at a dose of 10mg in patients <75years of age and 5mg in patients ≥75years of age. The dose of prasugrel was subsequently increased to 10mg in the latter group according to level of response

(iii) prasugrel hyporesponders: ticagrelor 90mg twice daily was commenced instead of prasugrel

Following treatment modification, further platelet function testing was performed to assess response.

4.3 Results

4.3.1 Demographics

Forty three consecutive patients were admitted with definite ST from October 2009 to October 2011. Baseline patient characteristics and demographics are provided in Table 3. They were predominantly male (88%) with a mean age of 64.7 (9.6) years.

Cardiac risk factors included hyperlipidaemia (60%), hypertension (60%), positive family history (44%), active smoking (35%) and diabetes (19%).

The majority of patients (81%) presented with very late ST; the median time from index PCI to ST was 919 days (range 0.02 to 3379 days). APT at the time of ST comprised dual therapy with aspirin and clopidogrel in 10 (23%) patients, aspirin alone in 30 (70%) patients, clopidogrel alone in 1 (2%) patient, and no antiplatelet agent in 2 (5%) patients due to non-compliance.

Index procedural characteristics are provided in Table IV. DES were used at index PCI in 81% of cases. Mean stent diameter (SD) and stent length (SD) was 2.9 (0.46) mm and 18.8 (6.28) mm respectively.

Mean age (yrs) ±SD	64.7 (9.6)
Male gender, n (%)	38 (88)
Cardiac risk factors, n (%)	
Diabetes	8 (19)
Current smoker	15 (35)
Hypertension	26 (60)
Hyperlipidaemia	26 (60)
Family history of CAD	19 (44)
Past medical history, n (%)	
Previous MI	36 (84)
Previous PCI in other vessel	8 (19)
Previous CABG	5 (12)
Previous stroke	0 (0)
Malignancy	1 (2)
APT at the time of ST, n (%)	
Aspirin alone	30 (70)
Clopidogrel alone	1 (2)
Aspirin and clopidogrel	10 (23)
None	2 (5)
Other medication at the time of ST, n (%)	
Statin	40 (93)
Proton pump inhibitor	9 (21)
ACE- inhibitors/ A-2RBs	34 (79)
Betablockers	29 (67)
Calcium channel blockers	9 (21)
ST presentation	
NSTEMI	5 (12)
STEMI	38 (88)
Timing of ST event, n (%)	
Acute ST	2 (5)
Sub-acute ST	5 (12)
Late ST	1 (2)
Very late ST	35 (81)

Table III. Baseline patient characteristics (n=43)

Lesion location, n (%)	
Left anterior descending artery	20 (47)
Circumflex/ obtuse marginal artery	6 (14)
Intermediate artery	1 (2)
Right coronary artery	14 (33)
Saphenous vein graft	2 (5)
Type of stent(s), n (%)	
Bare-metal stent	10 (23)
Paclitaxel-eluting stent	29 (67)
Sirolimus-eluting stent	4 (9)
Everolimus-eluting stent	1 (2)
Zotarolimus-eluting stent	2 (5)
Mean stent length (mm) ± SD	18.8 ± 6.28
Mean stent diameter (mm) ± SD	2.9 ± 0.46

Table IV. Index Procedural characteristics (n=43)

4.3.2 Short TEG results and response to treatment modification

Fourteen (33%) patients underwent short TEG testing within 30 days of their ST episode and the median time from ST to initial short TEG testing was 34 days. As described previously, a percentage clotting inhibition cut-off value of <50 and <30 was taken to indicate hyporesponsiveness to aspirin and P2Y12 receptor inhibitor respectively. Based on these criteria, of the total cohort, 12 (28%) patients had an adequate response to both aspirin and clopidogrel, 20 (47%) were hyporesponsive to clopidogrel alone, 1 (2%) was hyporesponsive to aspirin alone and 10 (23%) were hyporesponsive to both aspirin and clopidogrel at the first Short TEG test. Thus, a significant proportion of patients (91%) who were hyporesponsive to aspirin were also hyporesponsive to clopidogrel. Following treatment modification, an adequate response to aspirin was achieved in 10 (91%) patients and an adequate response to P2Y12 receptor inhibitor was achieved in 26 (87%) patients. Of note, 3 out of 6

(50%) prasugrel hyporesponders achieved an adequate response to ticagrelor. None of the patients in this registry have so far presented with a further ST episode. Short TEG results are summarised in table V and response to treatment modification is illustrated in Figures 12 and 13.

	n (%)
Adequate response to aspirin and clopidogrel	12 (28)
Hyporesponsive to aspirin alone	1 (2)
Hyporesponsive to clopidogrel alone	20 (47)
Hyporesponsive to both aspirin and clopidogrel	10 (23)
Total number of aspirin hyporesponders	11 (26)
Total number of clopidogrel hyporesponders	30 (70)

Table V. Summary of initial Short TEG results

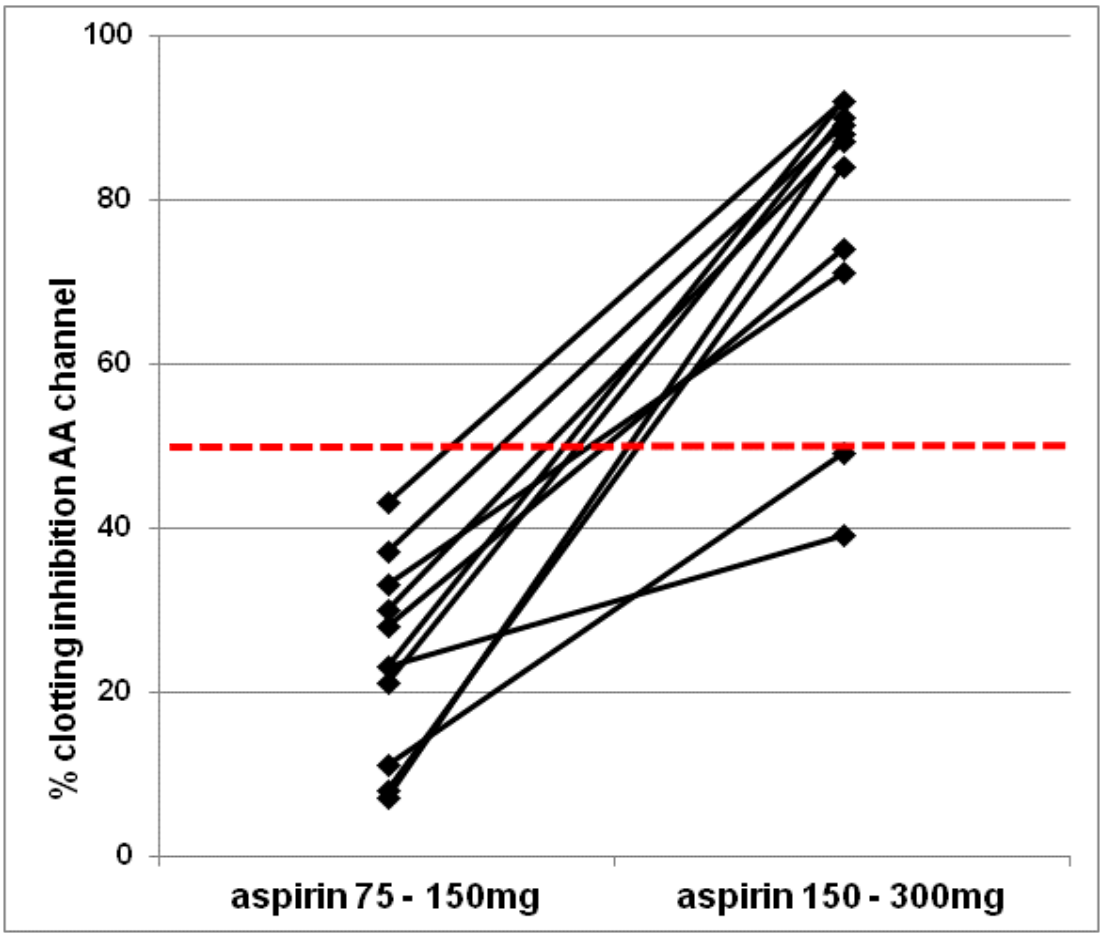

Figure 12. Change in percentage clotting inhibition following an increase in aspirin dose in patients found to be hyporesponsive at first Short TEG test following stent thrombosis episode (Horizontal dotted line represents the percentage clotting inhibition cut-off value of >50 which is indicative of an adequate response to aspirin).

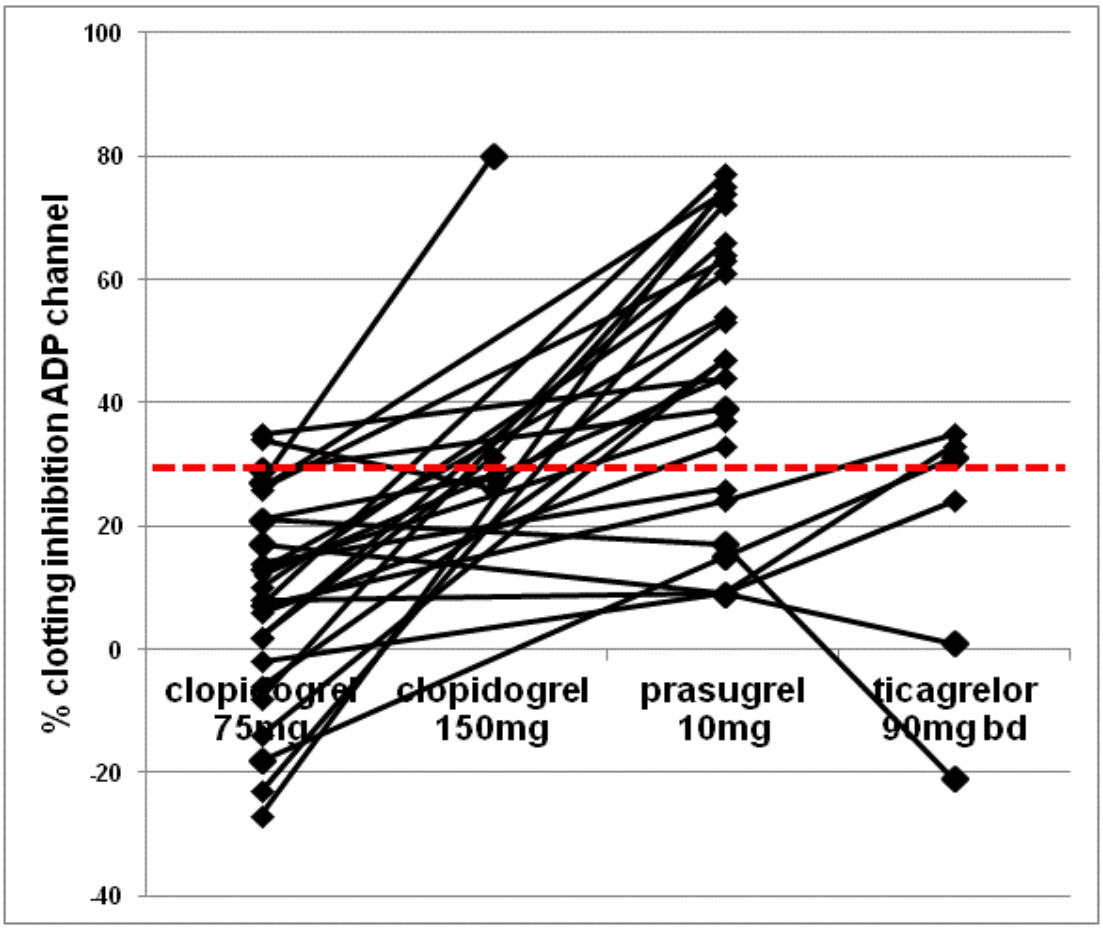

Figure 13. Change in percentage clotting inhibition following P2Y12 receptor inhibitor modification in patients found to be hyporesponsive at first and subsequent short TEG tests following stent thrombosis episode (Horizontal dotted line represents the percentage clotting inhibition cut-off value of >30 which is indicative of an adequate response to P2Y12 agent).

4.4 Discussion

These observational data demonstrate the feasibility of providing tailored APT to patients who have experienced definite ST using Short TEG, a novel point-of-care assay that has previously been shown to correlate well with VerifyNow (201,213). The data also highlight the prevalence of hyporesponsiveness to APT in this selected high risk group.

Our data from this 'real-world' registry raise four important questions with regards to clinical practice that warrant further investigation. Firstly, there is a high prevalence of hyporesponsiveness to APT, particularly clopidogrel (70%). These findings are consistent with previous studies (163,164) that observed significantly higher rates of hyporesponsivenes to clopidogrel in patients with ST compared with controls, measured using VerifyNow P2Y12 assay (40% vs. 14%, p=0.02 and 69% vs. 3%, p<0.001 respectively). Similarly, Hobson et al (165) have also previously demonstrated a higher incidence of clopidogrel hyporesponsivess in ST patients versus matched controls measured using both Verifynow P2Y12 and Short TEG assays. These data suggest that ST patients may possess a prothrombotic tendency, consistent with the significant proportion found to have high on-treatment platelet reactivity. This supports the concept of routine use of platelet function testing to tailor APT in this high risk patient group. In our registry, platelet function testing was undertaken at least 2 weeks following hospital discharge, thereby eliminating the effect of the prothrombotic milieu on platelet reactivity at the time of the acute presentation with ST.

Second, the majority of cases (81%) were very late ST and, as a result, 32 (74%) patients were not on clopidogrel at the time of their ST presentation. In fact, clopidogrel was discontinued more than 3 months prior to the ST event in 90% of cases. Despite this, the prevalence of clopidogrel hyporesponsiveness was remarkably high in the CREST registry (70%). This result is particularly striking given that 23 out of 30 patients in whom we demonstrated high platelet reactivity on clopidogrel were not on the drug at the time of ST. These findings raise further questions with regards to (i) the mechanism(s) of ST and particularly its timing in relation to the prevalence of hyporesponsiveness to APT, and (ii) whether responses to aspirin and clopidogrel are dynamic and vary significantly over time. The latter has been suggested by Pulcinelli et al (214) and Helgason et al (215) who observed that long term treatment with aspirin is associated with progressive reduction in platelet sensitivity to this drug. This hypothesis demands further investigation.

Third, the majority of aspirin hyporesponders in this registry were also hyporesponsive to clopidogrel (91%). This finding is consistent with previous observations (63) which showed that 47% of aspirin resistant patients also exhibited hyporesponsiveness to clopidogrel. This is clearly important since dual APT hyporesponsiveness has been shown to be an independent predictor of ST and cardiac

death (134), thus identifying a unique group of patients who may particularly benefit from tailored APT and in whom the more potent antiplatelet agents such as prasugrel and ticagrelor could play a crucial role. However, the underlying mechanism behind dual APT hyporesponsiveness remains to be elicited**.** Previous data (178,181,216,217) have suggested that clopidogrel influences AA pathways, thereby potentiating the effect of aspirin. Thus, the term "aspirin hyporesponder" defined by the extent of AA-induced platelet aggregation, may actually be indicative of a profound inadequate response to clopidogrel instead. Thus, the definition of aspirin "hyporesponsiveness" based on a functional test of AA-induced clotting should be interpreted with caution as it may overestimate the true prevalence of aspirin hyporesponsiveness. Ideally, an assessment of response to aspirin should include both a functional test as well as serum TXB2 measurements in parallel. The latter is considered the most specific pharmacologic test to evaluate the effect of aspirin on platelet COX-1 activity. These findings demand further investigation by way of large scale clinical studies.

Fourth, the current observational data support the notion that hyporesponsiveness to APT can be overcome with tailored therapy by way of dose or agent adjustment. However, it is interesting to note that 7 (16%) patients who were treated with prasugrel for clopidogrel hyporesponsiveness were also apparently hyporesponsive to this agent. These data are consistent with those from other groups who reported an incidence of prasugrel hyporesponsiveness of up to 25% in ACS patients, measured using the VASP assay (211,212). Three patients in our registry who were hyporesponsive to prasugrel, responded adequately to ticagrelor. Although patient compliance was not objectively assessed, this is an interesting finding in its own right that warrants further investigation. To date, there are no head-to-head studies that directly compare the antiplatelet effect and/or clinical benefit of prasugrel versus ticagrelor. These observations lend further support to the concept of individual platelet function testing in *all* PCI patients, a theory that requires further large scale clinical investigation.

This study has limitations. Firstly, it describes a clinical observation in a small number of patients. Having said that, this represents a relatively large series of patients with definite ST. Secondly, the use of Short TEG as the platelet function test is a further potential limitation. However, this method has now been extensively validated and its results have been shown to correlate with VerifyNow, as well as being highly reproducible (201,213,218). Thirdly, we are obliged to use cut-off values

for “response” and “hyporesponse” in order to make our observations binary. Clearly in a biological system, such cut-off values are artificial. Fourth, although two patients admitted to being non-compliant with their APT at the time of their ST episode, we did not objectively assess for APT compliance by measuring (i) serum TXB2 levels in the case of aspirin, or (ii) inactive carboxyl metabolite levels in the case of clopidogrel.

4.5 Conclusion

In addition to raising the above four clinically relevant questions, the CREST registry has also demonstrated that improved APT efficacy can be achieved by tailored therapy and that Short TEG is a plausible platelet function test that can be used to deliver point-of-care personalised APT. None of the patients in this registry have so far presented with recurrent ST; long term clinical outcome data following tailored APT are clearly needed. Furthermore, the data also raise more far-reaching questions regarding the case that *all* patients undergoing PCI should have personalised APT, particularly because the current randomised trial data suggest that for patients who respond adequately to clopidogrel the ischaemic event rate is low, and the risk of bleeding is less than with either prasugrel or ticagrelor.

CHAPTER 5: RESULTS

ORIGINAL ARTICLE

Effect of clopidogrel withdrawal on platelet reactivity and vascular inflammatory biomarkers 1 year after drug-eluting stent implantation: results of the prospective, single-centre CESSATION study

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ABSTRACT

Background The optimal duration of clopidogrel treatment, particularly following drug-eluting stent (DES) implantation, remains contentious. Previous studies have observed a clustering of adverse events following clopidogrel cessation 1 year after DES, the aetiology of which is poorly understood.

Objective To investigate, in the prospective CESSATION study, the effect of clopidogrel withdrawal at 1 year after DES implantation on (i) arachidonic acid (AA)- and adenosine diphosphate (ADP)-induced platelet aggregation, and (ii) biomarkers of vascular inflammation, including soluble CD40 ligand (sCD40L), high-sensitivity C-reactive protein (hsCRP) and interleukin 6 (IL-6).

Methods and results The prospective CESSATION study was undertaken in 33 patients receiving aspirin and due to discontinue clopidogrel 1 year after DES. Platetet reactivity was measured using short thromboelastography, and compliance with aspirin determined from serum thromboxane B_2 (TXB_2) levels. Venesection was performed at 4 weeks and 24 h before, and at 24 h, 48 h, 1, 2 and 4 weeks after, clopidogrel cessation. Following clopidogrel withdrawal, there was (i) a predictable increase in ADP-induced platelet aggregation (ii) an unexpected significant increase in AA-induced platelet aggregation (iii) a decline in IL-6 and hsCRP at 1 week and 4 weeks respectively; and (iv) a non-significant increase in sCD40L at 4 weeks TXB_2 levels were consistently suppressed, indicating complete inhibition of cyclo-oxygenase-1 by aspirin.

Conclusion An aspirin-independent, time-dependent increase in AA-induced platelet activation following clopidogrel withdrawal in patients with a DES has been described. New insights into a potential mechanism for the observed clustering of adverse events that occur early after clopidogrel cessation have been provided. These findings raise the question as to whether AA-induced clotting is an appropriate test of aspirin sensitivity.

Study objectives: to investigate the effect of clopidogrel withdrawal 1 year after DES implantation on ADP- and AA-induced platelet reactivity and on biomarkers of vascular inflammation, thereby exploring a potential mechanism for the observed clustering of adverse events that occur early after clopidogrel cessation

5.1 Introduction

Dual APT with aspirin and clopidogrel is recommended in all patients undergoing PCI and following ACS to reduce the risk of ischaemic events and cardiovascular mortality. Current clinical guidelines recommend aspirin lifelong and clopidogrel for up to 1 year, after which time clopidogrel is abruptly withdrawn. The optimal duration of clopidogrel therapy, particularly following DES implantation, remains a contentious issue and is the focus of ongoing clinical studies (42,168). Ho et al (169,170) reported a clustering of adverse clinical events within the first 90 days of clopidogrel withdrawal in ACS patients treated either medically or with PCI. It has been suggested that this may be due to a "rebound" prothrombotic and/or pro-inflammatory response that occurs following cessation of chronic clopidogrel therapy. However, the causative mechanism behind this observed response and therefore a potential strategy to attenuate this effect is not fully elucidated.

Previous clinical studies have investigated the effect of clopidogrel withdrawal on (i) inflammatory biomarkers alone (175) (ii) platelet reactivity alone (176) and (iii) inflammatory biomarkers and platelet reactivity in a diabetic population alone (174). The results were conflicting and warrant further investigation. Furthermore, previous data also suggest that there is an interaction between clopidogrel and the AA-induced pathway of platelet reactivity (178,181,217). Such a mechanism could be of considerable importance at a time when clopidogrel is abruptly withdrawn, because it may enhance the inevitable increase in ADP-mediated platelet reactivity.

Thus, the effect of clopidogrel cessation on both biomarkers of inflammation and platelet reactivity at multiple time points is unknown. Importantly, the effect of clopidogrel cessation on AA-induced pathways of platelet reactivity, currently thought to be predominantly influenced by aspirin, is unknown. Therefore, the CESSATION study investigated the effect of clopidogrel withdrawal at 1 year after

DES implantation on (i) AA- and ADP-induced platelet aggregation, and (ii) biomarkers of vascular inflammation including sCD40L, hsCRP and IL-6.

5.2 Methods

5.2.1 Study population

Study participants were identified by review of a database from the Wessex Cardiothoracic centre. Between February and June 2010, 38 patients on low dose aspirin therapy who had undergone PCI with DES 11 months earlier and were due to stop clopidogrel at 1 year were prospectively enrolled. Subjects were excluded if they were taking non-steroidal anti-inflammatory medication, steroids or anticoagulant therapy.

5.2.2 Study design

Blood samples were taken at the following pre-specified time points during the study period: (i) 4 weeks and 24 hours pre-clopidogrel cessation, and (ii) 24, 48 hours, 1, 2 and 4 weeks post clopidogrel cessation. At each time point, platelet reactivity was measured using Short TEG. In addition, serum TXB2 and inflammatory biomarkers were also measured.

5.2.3 Blood sampling and analysis

Venesection and sample analysis were performed as specified in the Study Methods in section 2.4. Platelet reactivity was measured using Short TEG and serum TXB2 and inflammatory biomarkers IL-6, hsCRP and sCD40L levels were also measured.

5.2.4 Statistical analysis

This is an exploratory study. There are no data available on the effect of clopidogrel cessation on all three parameters (i.e. platelet reactivity, TXB2 and inflammatory biomarkers) in this study population. Data are presented as the mean change from baseline with 95% confidence intervals, unless otherwise stated. The baseline time point refers to 24 hours pre-clopidogrel cessation. Significance between time points

was determined using paired t-tests with a p value of <0.05 considered to represent statistical significance. Due to the exploratory nature of the study no adjustments for multiple testing were made. Statistical analysis was performed using SPSS version 17.0 software, Microsoft Excel and Prism v5.0 (GraphPad software, La Jolla, CA, U.S.A.).

5.3 Results

A total of 38 patients were enrolled in the study. Four patients withdrew prematurely due to the inconvenience associated with multiple hospital visits required for venesection and 1 patient was excluded because he chose not to discontinue clopidogrel as required. Thus, study data are presented on 33 patients. Baseline characteristics and demographics of the study population are provided in Table VI. The participants were predominantly male (82%) with a mean age (SD) of 65.9 (8.2) years. Cardiac risk factors included hyperlipidaemia (58%), family history of ischaemic heart disease (52%), hypertension (30%), active smoking (12%) and diabetes (3%). All participants were on aspirin 75mg, clopidogrel 75mg and statin therapy. Eight (24%) patients were on proton pump inhibitors and 6 (18%) were on calcium channel blockers. Mean total duration (SD) of clopidogrel therapy following PCI was 373 (7.1) days.

Mean age (yrs) ±SD	65.9 ±8.2
Male gender, n (%)	27 (82)
Risk factors/ medical history, n (%):	
Diabetes	1 (3)
Hypertension	10 (30)
Hyperlipidaemia	19 (58)
Current smokers	4 (12)
Family history of CAD	17 (52)
Previous CABG	0 (0)
Previous other PCI	1 (3)
Previous stroke	1(3)
Indication for PCI, n (%):	
STEMI	7 (21)
NSTEMI	11 (33)
Unstable angina	6 (18)
Stable angina	9 (27)
LV systolic function (LVEF), n (%):	
Good (LVEF >50%)	27 (82)
Moderate (LVEF 30-50%)	3 (9)
Poor (LVEF <30%)	2 (6)
Unknown	1 (3)
Type of drug-eluting stent:	
Paclitaxel-eluting stent	13 (39)
Sirolimus-eluting stent	7 (21)
Everolimus-eluting stent	1 (3)
Zotarolimus-eluting stent	12 (36)
Number of stents (no of vessels), n (%):	
1 (1)	18 (55)
2 (1)	7 (21)
2 (2)	6 (18)
3 (1)	2 (6)
Antiplatelet therapy:	
Aspirin 75mg	33 (100)
Clopidogrel 75mg	33 (100)
Other medication:	
Statins	33 (100)
Beta blockers	29 (88)
ACE-inhibitors/ A2RBs	30 (91)
Diuretics	2 (6)
Calcium channel blockers	6 (18)
Oral hypoglycaemics	1 (3)
Proton pump inhibitors	8 (24)

Table VI. Baseline demographics (n=33)

5.3.1 Clinical events

One patient was admitted with ST 8 days after clopidogrel cessation. Clopidogrel was therefore recommenced as per ACS treatment guidelines. Subsequent coronary angiography confirmed an occluded right coronary artery that was successfully treated with PCI. We have included study data up until the fifth time point in this patient (i.e. just prior to his ST presentation).

5.3.2 Thromboxane B2 levels

Serum TXB2 was suppressed consistently at all time points confirming effective inhibition of COX-1 by aspirin throughout the study. Mean (SD) serum TXB2 concentrations were 122.7±203.8 pg/ml at 4 weeks pre, 137.7±317.9 pg/ml at 24 hours pre, 94.3±74.4 pg/ml at 24 hours post, 61.8±47.6 pg/ml at 48 hours post, 129.7±125.4 pg/ml at 1 week post, 164.3±232.7 pg/ml at 2 weeks post and 103.5±85.4 pg/ml at 4 weeks post clopidogrel cessation. The change in mean serum TXB2 from baseline was -43.4 (95% CI, -156.2 to 69.4, p=0.439) at 24 hours post, -75.9 (95% CI, -187.5 to 35.7, p=0.176) at 48 hours post, -8.1 (95%CI, -103.3 to 87.2, p=0.864) at 1 week post, 26.5 (95% CI, -111 to 164.1, p=0.697) at 2 weeks post and -36.5 (95% CI, -155.8 to 82.8, p=0.537) at 4 weeks post clopidogrel cessation.

5.3.3 ADP-induced platelet aggregation

Following clopidogrel withdrawal, there was a significant and time-dependent increase in ADP-induced platelet aggregation measured using Short TEG AUC15 (Figure 14). The change in mean AUC15 from baseline was 128.3 (95% CI 71.9 to184.7, p<0.001) at 24 hours post, 193.2 (95% CI 134 to 252.4, p<0.001) at 48 hours post, 271.3 (95% CI 194 to 348.6, p<0.001) at 1 week post, 295.8 (95% CI 212.7 to 378.9, p<0.001) at 2 weeks post and 280.4 (95% CI 203.4 to 357.4, p<0.001) at 4 weeks post clopidogrel cessation (Table VII). The percent change in mean AUC15 from baseline was 15.6% at 24 hours post clopidogrel cessation and increased to 33% at 1 week post clopidogrel cessation.

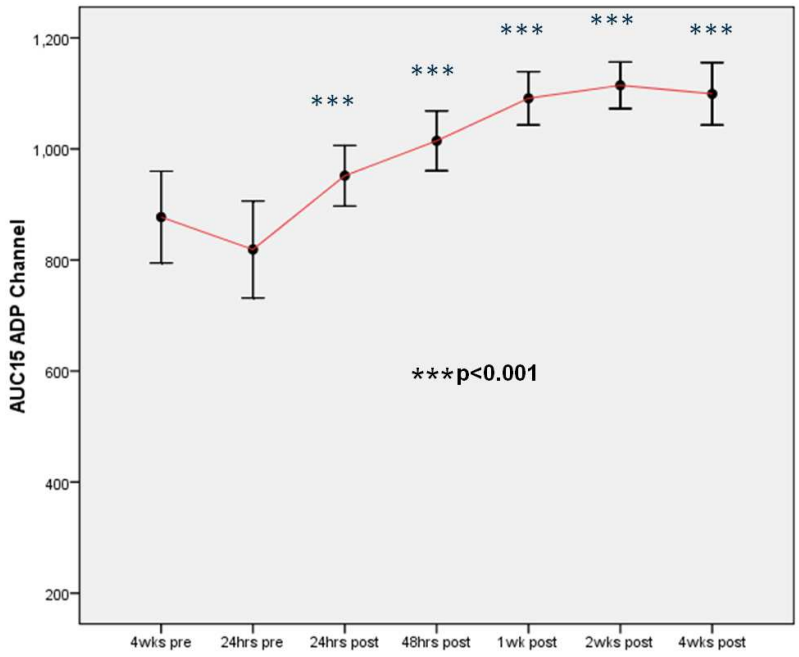

Figure 14. Area Under the curve at 15 minutes (AUC15) of the adenosine diphosphate (ADP) channel. Error bars represent mean with 95% confidence intervals. The p values given (***p<0.001) are for comparison to the baseline time-point at 24hrs pre clopidogrel cessation. Although the x-axis time points are equally spaced on the graph, the actual time intervals between any two points vary.

ADP Channel	Baseline (24hrs pre)	24hrs post	48hrs post	1wk post	2wks post	4wks post
Mean AUC15	823.8	952.1	1017.0	1095.1	1114.7	1099.3
95% CI of mean AUC15	741.9 to 905.7	901.3 to 1002.9	966.7 to 1067.3	1049.8 to 1140.4	1074.2 to 1155.2	1045.4 to 1153.2
Median	841	946.8	1030.8	1107.0	1136.3	1073.1
Change in mean AUC15 from baseline		128.3	193.2	271.3	295.8	280.4
95% CI of change in mean AUC15		71.9 to 184.7	134 to 252.4	194 to 348.6	212.7 to 378.9	203.4 to 357.4
Percent change in mean AUC15 from baseline		15.6	23.5	33	35.3	33.4
P value		<0.001	<0.001	<0.001	<0.001	<0.001

Table VII. Change in ADP induced platelet aggregation following clopidogrel cessation measured using Short TEG Area Under the Curve at 15 minutes (AUC15)

5.3.4 AA-induced platelet aggregation

Following clopidogrel withdrawal, there was a significant increase in AA-induced platelet aggregation measured using Short TEG AUC15 (Figure 15). The change in mean AUC15 from baseline was 129 (95% CI 46.2 to 211.8, p=0.005) at 24 hours post, 171.6 (95% CI 74.9 to 268.3, p=0.002) at 48 hours post, 242.9 (95% CI 146.7 to 339.1, p<0.001) at 1 week post, 316.2 (95% CI 200.7 to 431.7, p<0.001) at 2 weeks post and 235.7 (95% CI 115.3 to 356.1, p=0.001) at 4 weeks post clopidogrel cessation (Table VIII). The percent change in mean AUC15 from baseline was 31.5% at 24hrs post clopidogrel cessation and increased to 59.4% at 1 week post clopidogrel cessation.

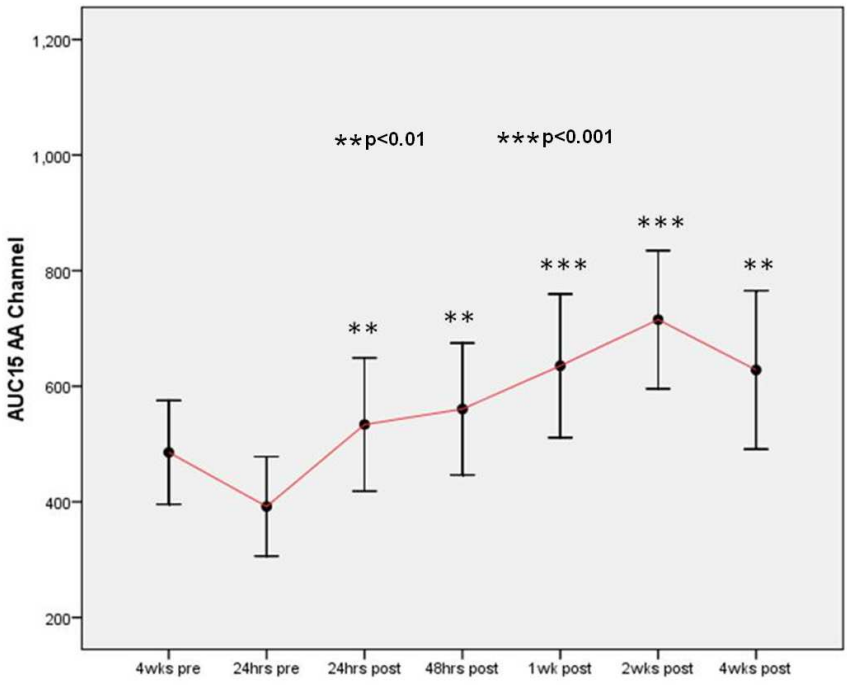

Figure 15. Area Under the curve at 15 minutes (AUC15) of the arachidonic acid (AA) Channel. Error bars represent mean with 95% confidence intervals. The p values given (**p<001; ***p<0.001) are for comparison to the baseline time point at 24hrs pre clopidogrel cessation. Although the x-axis time points are equally spaced on the graph, the actual time intervals between any two points vary.

AA Channel	Baseline (24hrs pre)	24hrs post	48hrs post	1wk post	2wks post	4wks post
Mean AUC15	409.0	538.0	561.4	651.9	727.9	646.4
95% CI of mean AUC15	322.7 to 495.3	431.7 to 644.3	455.2 to 667.6	535.8 to 768	614.1 to 841.7	513.6 to 779.2
Median	323.1	545.1	558.3	683.8	783.8	729.8
Change in mean AUC15 from baseline		129	171.6	242.9	316.2	235.7
95% CI of change in mean AUC15		46.2 to 211.8	74.9 to 268.3	146.7 to 339.1	200.7 to 431.7	115.3 to 356.1
Percent change in mean AUC15 from baseline		31.5	37.3	59.4	78	58.3
P value		0.005	0.002	<0.001	<0.001	0.001

Table VIII. Change in AA induced platelet aggregation following clopidogrel cessation measured using Short TEG Area Under the Curve at 15 minutes (AUC15)

5.3.5 Concomitant medical therapy and platelet reactivity

Patients on regular proton pump inhibitors (n=8) and calcium channel blockers (n=6) did not show a trend towards higher residual AA- or ADP-induced platelet reactivity prior to clopidogrel cessation. Furthermore, the increase in platelet aggregation following clopidogrel withdrawal did not amplify in this group.

5.3.6 Inflammatory biomarkers

There was a decline in mean IL-6 at 1 week post clopidogrel cessation (p=0.006), a decline in hsCRP at 4 weeks post clopidogrel cessation (p=0.033) and an insignificant increase in sCD40L at 4 weeks post clopidogrel cessation (p=0.32) (Figures 16-18 and Tables IX-XI). Of note, there was a solitary inexplicably high IL-6 value at 2 weeks post clopidogrel cessation in one patient that was approximately 100-fold greater than all other IL-6 measurements. There was no accompanying significant rise in any of the other inflammatory markers at this time point and, furthermore, the patient did not report any illness or adverse event at any time during the study period. Thus, this solitary IL-6 measurement is likely to be spurious and has been excluded from the analysis.

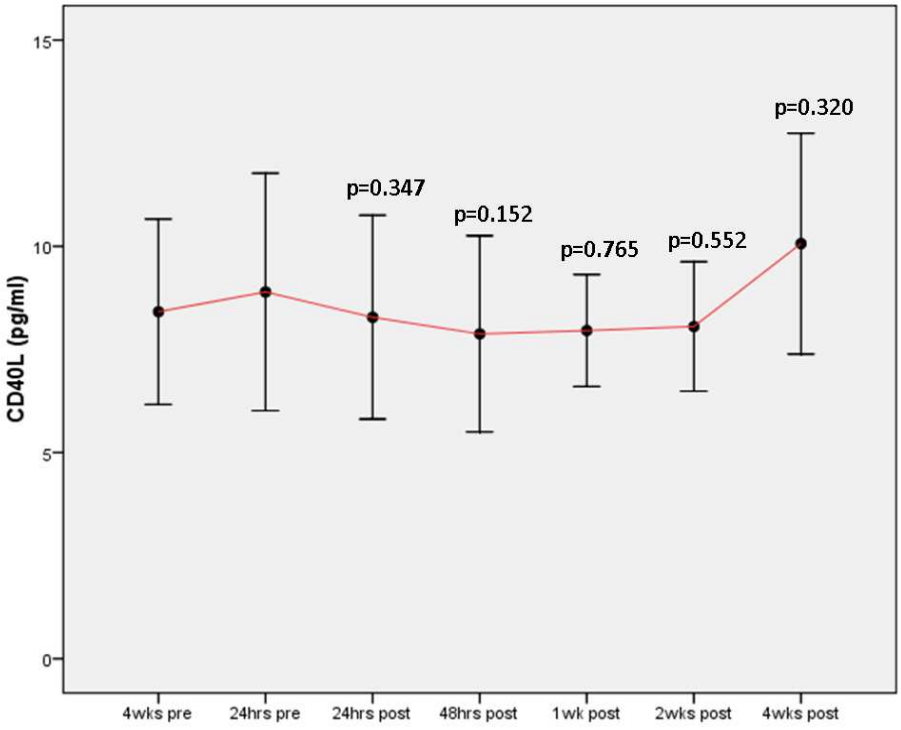

Figure 16. Soluble CD40 ligand pre and post clopidogrel cessation. Error bars represent mean with 95% confidence intervals. The p values given are for comparison to the baseline time point at 24hrs pre clopidogrel cessation. Although the x-axis time points are equally spaced on the graph, the actual time intervals between any two points vary.

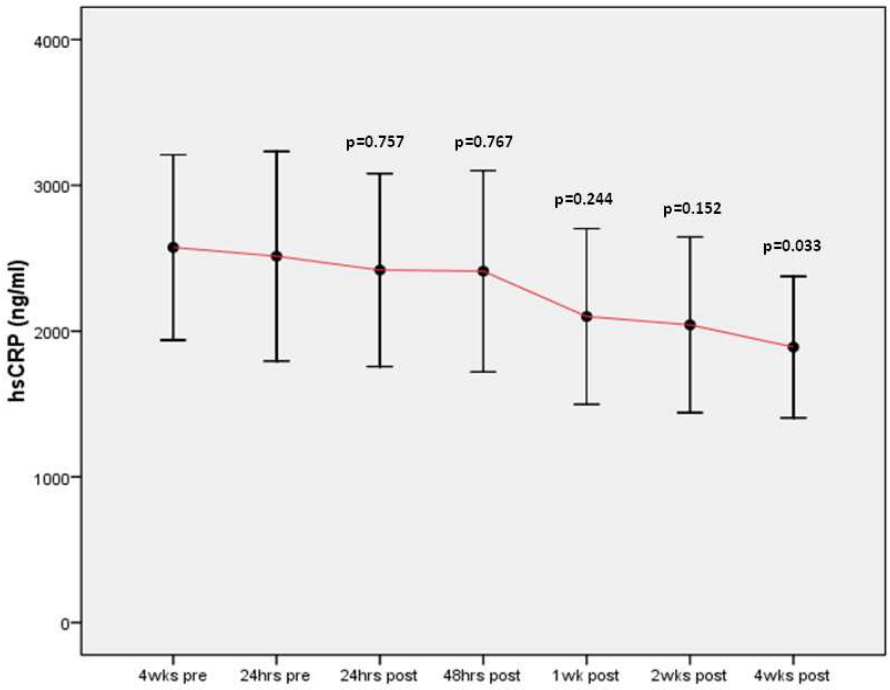

Figure 17. High sensitivity C-reactive protein (hsCRP) pre and post clopidogrel cessation. Error bars represent mean with 95% confidence intervals. The p values given are for comparison to the baseline time point at 24hrs pre clopidogrel cessation. Although the x-axis time points are equally spaced on the graph, the actual time intervals between any two points vary.

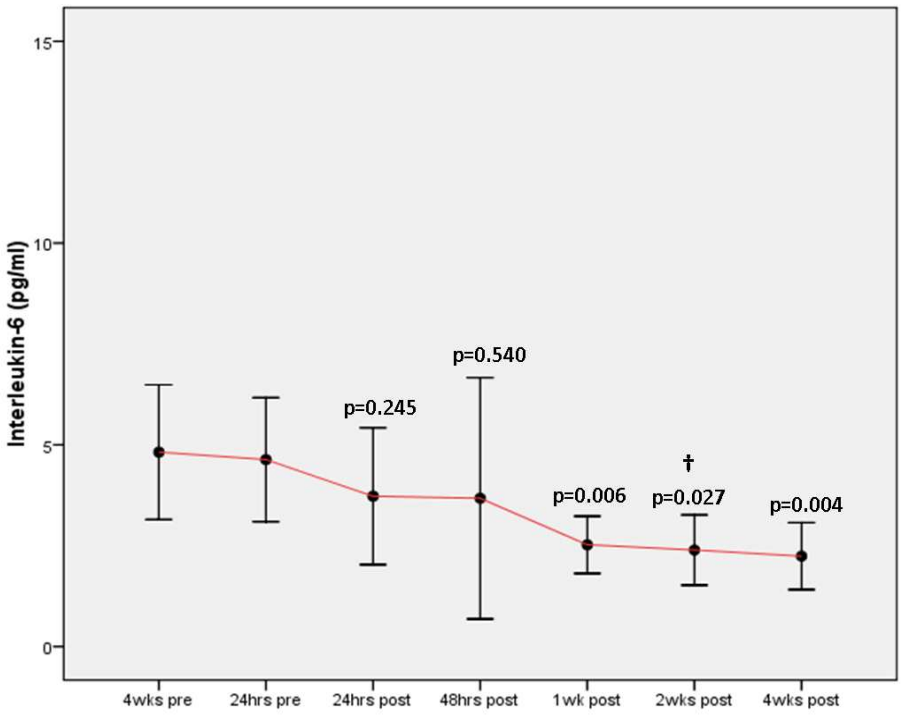

Figure 18. Interleukin-6 pre and post clopidogrel cessation. Error bars represent mean with 95% confidence intervals. The p values given are for comparison to the baseline time-point at 24hrs pre clopidogrel cessation. Although the x-axis time points are equally spaced on the graph, the actual time intervals between any two points vary (+At this time point there was a solitary inexplicably high IL-6 value observed in one patient. This was approximately 100-fold greater than all other IL-6 measurements. As described in the results section of the text, this is likely to be a spurious result and has therefore been excluded from the data analysis)

hsCRP	Baseline (24hrs pre)	24hrs post	48hrs post	1wk post	2wks post	4wks post
Mean	2513.7	2418.6	2410.9	2100.2	2042.8	1890.4
95% CI of mean	1822 to 3205.4	1782 to 3055.2	1746.5 to 3075.3	1520.5 to 2679.9	1462.5 to 2623.1	1424.1 to 2356.7
Median	2006	1867.4	1796.5	1361	1721.8	1616.4
Change in mean from baseline		-95.1	-102.9	-413.5	-471	-556
95% CI of change in mean		-693.7 to 503.5	-779 to 573.2	-1095.9 to 268.9	-1100.8 to 158.8	-1043.7 to -68.3
Percent change in mean from baseline		-3.8	-4.1	-16.5	-18.7	-24.8
P value		0.757	0.767	0.244	0.152	0.033

Table IX. Change in inflammatory marker high sensitivity CRP (hsCRP) (ng/ml) following clopidogrel cessation

sCD40L	Baseline (24hrs pre)	24hrs post	48hrs post	1wk post	2wks post	4wks post
Mean	8.8	8.1	7.8	8.3	8.1	10.2
95% CI of mean	6.2 to 11.4	5.9 to 10.3	5.7 to 9.9	6.9 to 9.7	6.6 to 9.6	7.7 to 12.7
Median	6.8	6.9	6.8	6.7	7.2	8.3
Change in mean from baseline		-0.7	-1	-0.4	-0.6	1
95% CI of change in mean		-2.1 to 0.7	-2.3 to 0.3	-3.2 to 2.4	-2.5 to 1.3	-1 to 3
Percent change in mean from baseline		-8.3	-12.1	-5.7	-8	15.7
P value		0.347	0.152	0.765	0.552	0.32

Table X. Change in inflammatory marker soluble CD40 ligand (sCD40L) (pg/ml) following clopidogrel cessation

IL-6	Baseline (24hrs pre)	24hrs post	48hrs post	1wk post	2wks post	4wks post
Mean	4.5	3.7	3.8	2.5	2.6	2.2
95% CI of mean	3.1 to 5.9	2.2 to 5.2	1.1 to 6.5	1.9 to 3.1	1.7 to 3.5	1.4 to 3
Median	2.9	2.5	2.4	2.3	2.1	1.4
Change in mean from baseline		-0.9	-0.7	-2	-2	-2.3
95% CI of change in mean		-2.3 to 0.5	-3 to 1.6	-3.3 to -0.7	-3.7 to -0.3	-3.7 to -0.9
Percent change in mean from baseline		-19	-16.2	-45	-43.3	-50.8
P value		0.245	0.54	0.006	0.027	0.004

Table XI. Change in inflammatory marker interleukin-6 (IL-6) (pg/ml) following clopidogrel cessation

5.4 Discussion

The novel finding in this study is that, when clopidogrel is withdrawn 1 year after DES implantation, there is a significant aspirin-independent time-dependent increase in AA-induced platelet reactivity in addition to a predictable time-dependent increase in ADP-induced platelet reactivity as measured by Short TEG. Further, clopidogrel withdrawal is associated with a decline in IL-6 and hs-CRP, results which are unexpected given its anti-inflammatory reputation. These findings provide new

insights into a potential mechanism for the observation that adverse events cluster early after clopidogrel withdrawal. Also, our results further support accumulating evidence that clopidogrel influences pathways of AA-induced clotting and thereby potentiates the effect of aspirin (178,181,217).

A secondary, but important, issue that arises is whether AA-induced platelet aggregation is an appropriate test for aspirin sensitivity. It is well established that aspirin achieves its antithrombotic effects through inactivation of COX-1 and thus prevents generation of TXA2 and its stable metabolite TXB2. Serum TXB2 analysis is the most specific pharmacologic test to evaluate the effect of aspirin on platelets and, furthermore, is a marker of compliance with aspirin therapy. In this study, despite a significant increase in AA-induced platelet reactivity following clopidogrel cessation, TXB2 levels were consistently suppressed indicating complete inhibition of platelet COX-1 by aspirin. This observation therefore excludes aspirin treatment failure as an explanation for the increase in AA-induced clotting and suggests that there is an alternative clopidogrel-mediated mechanism responsible for the observed AA pathway response. Similar observations were reported by Frelinger et al (179) who demonstrated a P2Y12- dependent but COX-independent pathway of residual AA-induced platelet activation in a cohort of 700 consecutive aspirin-treated patients undergoing PCI. These findings demand further investigation by way of large scale clinical studies to elicit the precise mechanisms by which clopidogrel achieves its antithrombotic effect. Specifically, the extent to which an effective P2Y12 inhibitor could achieve blockade of the AA-pathway requires further assessment. It is conceivable that, if such effects were potent enough, the addition of aspirin would be obsolete. This remains speculative, pending further data (216).

At the very least, our data suggest that measurement of AA-induced platelet aggregation may not be a reliable test for measuring clinical response to aspirin, given that this parameter has changed in the patients in this study in whom there was complete suppression of TXB2 levels. This is potentially an explanation for the apparently high levels of functional aspirin “resistance” reported in studies using AA-mediated tests. For example, a study investigating the incidence of aspirin “resistance” in 45 stroke patients reported a rate of 67% measured using the AA-specific unmodified TEG method (125). Serum TXB2 levels were not measured in this study.

Elevated inflammatory biomarkers have been linked to poor outcome after PCI and increased risk of adverse cardiovascular events in ACS patients (219-221). Although the anti-inflammatory properties of clopidogrel in the acute context are well described (222-225), the data with regards to its anti-inflammatory effects in patients with stable CAD on chronic therapy are inconsistent and conflicting. For example, one randomised controlled study showed that long term clopidogrel in patients with stable CAD significantly inhibited the production of sCD40L but had no effect on hsCRP levels (226). Similar, apparently discordant, findings in a different patient group were also observed in the ELAPSE study (227) which showed a significant decline in sCD40L one year after PCI in patients on chronic clopidogrel, as well as an increase in soluble P-selectin and serum IL-18 levels but no change in hsCRP compared to baseline.

In the present CESSATION study, clopidogrel withdrawal 1 year after DES resulted in a decline in mean 1L-6 at 1 week post ($p=0.006$), a decline in hsCRP at 4 weeks post ($p=0.033$) but a non significant increase in sCD40L at 4 weeks post clopidogrel cessation ($p=0.32$). Our findings are consistent with observations from the DECADES study (175) which also investigated the effect of clopidogrel cessation on biomarkers of inflammation 1 year after DES but did not examine the effect on platelet reactivity. This study reported a significant increase in sCD40L levels 4 weeks after clopidogrel withdrawal ($p<0.001$) but an unexplained and apparently inconsistent *decrease* in hsCRP levels 1 week after cessation of clopidogrel ($p=0.008$). Although the sCD40L findings from this and the DECADES study suggest that clopidogrel may have important anti-inflammatory properties, there are novel questions generated by our data: does chronic clopidogrel treatment up-regulate certain inflammatory biomarkers and is the observed declining trend in hsCRP and IL-6 directly related to loss of the platelet inhibitory effect on the ADP- and AA-pathways associated with clopidogrel cessation?

This study has limitations. Firstly, sample size is small. Secondly, due to the design of study we were unable to measure baseline pretreatment levels of platelet reactivity and inflammatory biomarkers. Thirdly, although it may have been useful to measure platelet reactivity using more than one laboratory assay of platelet function, previous studies from this group and others have shown strong correlation between TEG AUC15 and VerifyNow (201,213) and between TEG and the historical “gold standard” method LTA (79,114) for measurement of response to APT.

5.5 Conclusion

Our study demonstrates a significant time-dependent increase in ADP- and AA-induced platelet reactivity and a decline in IL-6 and hs-CRP following clopidogrel withdrawal at 1 year in patients with DES. This raises important clinical questions that demand further investigation. Firstly, AA-induced clotting may not be an appropriate test to measure the antiplatelet effect of aspirin, which means the relatively high rates of aspirin "resistance" reported in previous clinical studies should be interpreted with caution and, at the very least, should include serum TXB2 measurements in parallel. It was this observation led to our subsequent study in patients with ischaemic stroke (see overleaf). Second, the time-dependent increase in AA-induced clotting observed in this study following clopidogrel withdrawal lends support to the accumulating evidence which suggests that clopidogrel exerts some of its antiplatelet effects via the AA-pathway in an aspirin-independent fashion. However, it remains uncertain as to what extent clopidogrel potentiates aspirin and whether the anti-thrombotic effect of aspirin is rendered partially or even completely redundant in the presence of clopidogrel or other more potent P2Y12 agents. This study raises the question as to whether clopidogrel should be withdrawn abruptly 1 year after DES or whether tapered withdrawal or discontinuation of aspirin instead may offer greater clinical benefit. Large scale clinical outcome studies are clearly needed to answer these clinically relevant questions.

CHAPTER 6: RESULTS

"Aspirin Resistance" in Ischemic Stroke: Insights Using Short Thrombelastography

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Aims: Aspirin achieves its antithrombotic effect through inactivation of cyclooxygenase (COX)-1, thereby preventing generation of thromboxane (TX)A_2 from arachidonic acid (AA). The reported prevalence of aspirin "resistance" varies significantly and is usually based on platelet function tests (PFTs) that use AA-induced platelet reactivity as a surrogate measure of the effect of aspirin, rather than specific assessment of its effect on its therapeutic target (ie, COX-1 inhibition). The reported rates are not only assay specific but also condition specific, with particularly high rates (up to 70%) previously reported in the stroke population. We investigated whether pharmacological responses to aspirin can be reliably determined from a functional test of AA-induced whole-blood clotting. *Methods and Results:* A prospective study included 35 patients admitted with ischemic stroke and commenced on 300 mg aspirin. AA-induced whole-blood clotting was measured using short thrombelastography, a previously extensively validated near-patient PFT. Serum TXB_2 and inflammatory biomarkers were also measured. The prevalence of apparent aspirin resistance measured using AA was high (range from 49% to 67%). However, serum [TXB_2] was consistently low, thereby confirming adequate inhibition of COX-1 by aspirin. Mean inflammatory biomarker levels were elevated throughout. *Conclusion:* This study demonstrates that although COX-1 activity is adequately and consistently suppressed by aspirin in stroke patients, this effect is not reliably indicated by whole-blood clotting in response to AA. These data help to explain why the reported prevalence of aspirin resistance in stroke from studies employing AA-induced platelet reactivity is high and cast doubt on the veracity of such reports. **Key Words:** Aspirin resistance—ischemic stroke—thrombelastography—platelet function testing—thromboxane B2.

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Study objectives: to determine whether the pharmacological response to aspirin in the acute stroke population can be reliably determined from a functional test of AA-induced whole blood clotting (Short TEG) and whether this is associated with vascular inflammation

6.1 Introduction

The clinical efficacy of aspirin in secondary prevention of CVD is well established (13,14). Aspirin achieves its antithrombotic effect by irreversibly blocking platelet COX-1 enzyme activity, thereby inhibiting synthesis of TXA2. However, despite aspirin therapy a significant number of patients experience recurrent ischaemic events, which are often attributed to aspirin treatment failure or aspirin "resistance". Strictly, this term should be reserved for a specific failure of aspirin to inhibit its primary target, i.e. COX-1-dependent TXA2 production. This endpoint can be determined by measuring serum TXB2 (the stable metabolite of TXA2) which is considered the "gold standard" biochemical index. Nonetheless, aspirin "resistance" is frequently described in the literature based upon *ex vivo* platelet function tests that utilise AA as the stimulant agonist. Thus, labelling individuals as aspirin "resistant" purely based on high on-treatment platelet reactivity is potentially flawed because these tests do not specifically assess the effect of aspirin on its therapeutic target. Furthermore, tests of platelet function are not standardised, correlate poorly and utilise arbitrary and binary thresholds to differentiate "responders" from "non responders" (123,124,228). Given this background, it is not surprising that the reported prevalence of aspirin resistance based on functional assays using AA as an agonist varies significantly, ranging from 2% to 67% (125,229-231). For example, the prevalence of aspirin resistance in stable CAD is reported to be 20 to 30% measured using VerifyNow and Multiplate (95,232) and as low as 2 to 5% on LTA (130,233). By contrast, reported rates of aspirin resistance in ischaemic stroke vary from 28% with LTA (234) to over 60% with PFA-100 and TEG (125,235,236).
Data from this group and others suggests that AA-induced clotting can be mediated via aspirin-independent pathways (178,181,237,238). For example, in patients stopping clopidogrel after one year of dual APT, we observed an unexpected and significant aspirin-independent increase in AA-induced clotting in addition to the predictable effects of clopidogrel on ADP-induced clotting. Importantly, serum TXB2

levels were consistently suppressed at all time points in this study (238). These data raised the question: is AA-induced clotting actually an appropriate test of aspirin response in patients, given this obvious discrepancy?

The hypothesis for the current study is whether the true biochemical response of patients with ischaemic stroke can be reliably determined from a functional test of AA-induced whole blood clotting. In addition, we seek to investigate whether responses to aspirin vary as a function of time and are associated with level of vascular inflammation. We specifically chose to study this hypothesis in patients with ischaemic stroke for two reasons. Firstly, the literature describes an apparently high prevalence of aspirin "resistance" in this patient group (125,234-236,239). Secondly, we wished to address the reproducibility of our previous observation, that AA-induced clotting does not represent a test for sensitivity to aspirin, in a discrete population with CAD who had undergone stenting (*see CESSATION study in section 5 above*).

The present study has employed the whole blood test Short TEG for the assessment of platelet function. As described in section 1.9, Short TEG has been extensively validated by this group (69,113,116,201,213,240) and correlates with both optical aggregometry (79,82,114,115) and VerifyNow (201,213) in assessment of responses to APT.

6.2 Methods

6.2.1 Study population

This single centre, prospective study was undertaken between February and June 2011. Thirty six consecutive patients admitted to a tertiary centre with acute ischaemic stroke were prospectively enrolled. All participants underwent computed tomography (CT) brain imaging within 24 hours of hospital admission and were commenced on aspirin 300mg once daily after intracranial or subdural haemorrhage had been effectively ruled out on CT in accordance with local clinical guidelines. Study exclusion criteria were as follows: onset of stroke symptoms greater than 72 hours prior to hospital admission; fibrinolytic therapy administered at presentation; evidence of haemorrhage on CT brain imaging; on regular non steroidal anti-inflammatory medication, steroids, anticoagulant treatment or other APT besides

aspirin. Stroke syndrome subtype was categorised according to the Oxfordshire Community Stroke Project classification system as Total Anterior Circulation (TAC), Partial Anterior Circulation (PAC), Posterior Circulation (POC) and Lacunar (LAC) Stroke. Stroke severity at presentation was defined according to Modified Rankin Scale classification (MRS) (241).

6.2.2 Study design

Blood samples were taken at the following pre-specified time points: (i) within 72 hours of onset of stroke symptoms, (ii) day 6 of stroke event, and (iii) day 8 to 10 of stroke event. At each time point, platelet reactivity was measured using Short TEG. In addition, serum TXB2 and inflammatory biomarkers were measured. All blood samples were taken within 12 hours of aspirin dosing.

6.2.3 Blood sampling and analysis

Venesection and sample analysis was performed as specified in the Study Methods in section 2.4. Platelet reactivity was measured using Short TEG and serum TXB2 and inflammatory biomarkers IL-6, TNF alpha, hsCRP and sCD40L levels were measured.

6.2.4 Percentage clotting inhibition

A percentage clotting inhibition cut-off value of <50 in the TEG AA channel is used to define hyporesponsiveness to aspirin. The derivation and validation of percentage clotting inhibition is described in detail in Section 1.9.8.

6.2.5 Statistical analysis

For continuous data, differences in means between two time points were assessed using paired samples t-test or the Wilcoxon-signed Rank test for normally and non-normally distributed data respectively. Differences in frequencies between groups were examined using Chi-squared test and differences in measurements between two groups were assessed by unpaired t-tests. Correlations between test results were calculated using the Pearsons or Spearmans rank correlation tests for normally and

non-normally distributed data respectively. A p value of <0.05 was considered to represent statistical significance. Statistical analysis was performed using SPSS version 17.0 software and Microsoft Excel.

6.3 Results

A total of 36 patients were enrolled in the study. One participant commenced low molecular weight heparin following study recruitment and was therefore excluded. Thus, study data are presented on 35 patients. This study was conducted in an acute tertiary Stroke Unit with rapid turnover and, as such, a significant number of patients were discharged to various peripheral stroke rehabilitation hospitals prior to completion of all 3 study time points. Thus, 23 patients completed 2 study time points and 15 patients completed all 3 study time points.

Baseline characteristics and demographics of the study population are provided in Table XII. Eighteen (51%) participants were female, with a mean age (SD) of 72 (15.7) years and an age range of 43 to 100 years. Cardiovascular risk factors included diabetes (11%), hypertension (46%), hyperlipidaemia (49%), active smoking (23%) and previous stroke or TIA (14%). APT prior to admission included aspirin 75mg alone in 8 (23%) patients, aspirin 300mg alone in 1 (3%) patient and dual APT with aspirin 75mg and dipyridamole in 4 (11%) patients. Thirteen (37%) patients were on lipid lowering therapy on admission. Following hospital admission, the total number of once daily dose of aspirin 300mg administered to the participant prior to taking the first blood sample was 1 dose in 11 (31%) patients, 2 doses in 20 (57%) patients and 3 doses in 4 (11%) patients.

Stroke syndrome subtype at presentation comprised TACS in 3 (9%) patients, POCS in 4 (11%) patients, PACS in 15 (43%) patients and LACS in 13 (37%) patients. Stroke severity at presentation, defined according to MRS, was as follows: MRS 1 in 4 (11%) patients, MRS 2 in 6 (17%) patients, MRS 3 in 6 (17%) patients, MRS 4 in 10 (29%) patients and MRS 5 in 9 (26%) patients.

Mean age (years) ±SD	72 ± 15.7
Female gender, n (%)	18 (51)
Risk factors/ medical history, n (%):	
Diabetes	4 (11)
Hypertension	16 (46)
Hyperlipidaemia	17 (49)
Current smoker	8 (23)
Previous stroke/TIA	5 (14)
Known CAD	11 (31)
Medication prior to admission, n (%):	
Aspirin 75mg	8 (23)
Aspirin 300mg	1 (3)
Aspirin 75mg and dipyridamole	4 (11)
Other antiplatelet agent	0 (0)
Statin	13 (37)
Proton pump inhibitor	9 (26)
Atrial fibrillation at presentation	8 (23)

Table XII. Baseline demographics (n=35)

6.3.1 AA-induced platelet aggregation

As described previously, a percentage clotting inhibition cut-off value of <50 was taken to indicate HRPR on aspirin or aspirin "hyporesponsiveness". Based on this criterion, 21 (60%) patients were hyporesponsive to aspirin at time point 1 (n=35), 17 (49%) at time point 2 (n=23) and 10 (67%) at time point 3 (n=15). Mean (SD) percentage clotting inhibition was 35(33), 32(27) and 31(37) at time points 1, 2 and 3 respectively. There was no significant change in mean percentage clotting inhibition between time points 1 and 2 (n=23, 95% CI -3.9 to 13.9, p=0.26) and between time points 1 and 3 (n=15, 95% CI -18.9 to 14.7, p=0.798). The scatterplot (figure 19) shows the distribution of percentage clotting inhibition measurements at each time

point. Stroke severity at presentation, defined according to MRS, was not related to aspirin responsiveness measured on Short TEG. Specifically, mean MRS (SD) was 3.6 (1.2) (range 1 to 5) in aspirin "hyporesponders" and 3.1 (1.6) (range 1 to 5) in aspirin "responders" (p=0.367).

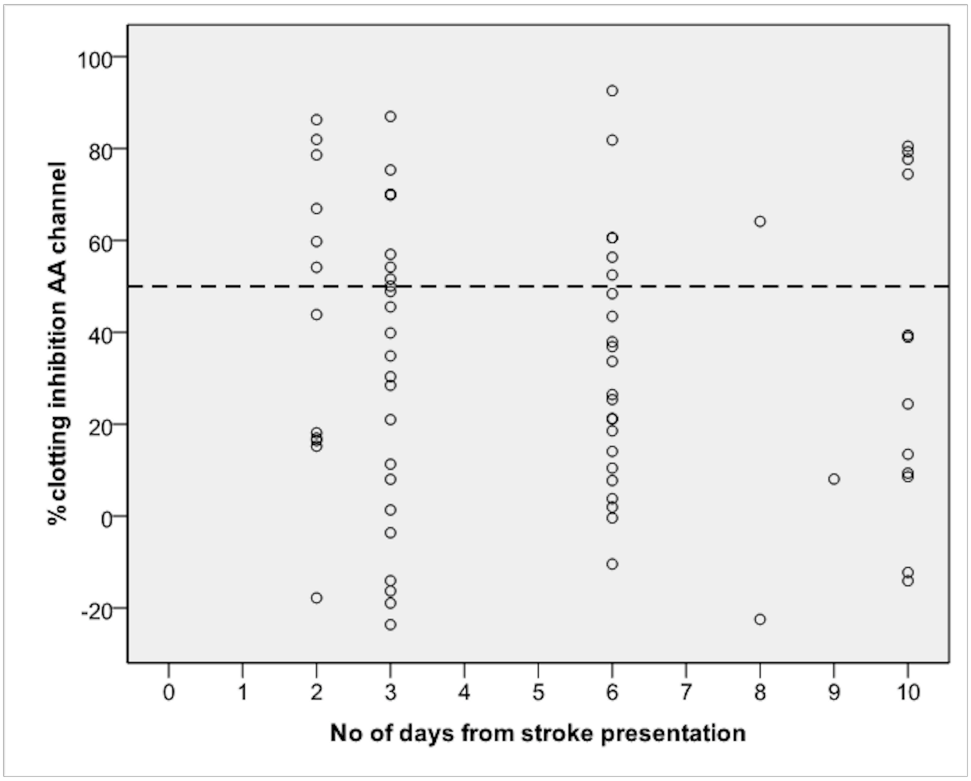

Figure 19. Scatterplot demonstrating the distribution of percentage clotting inhibition of the arachidonic (AA) channel (horizontal dashed line indicates the cut-off for high residual platelet reactivity or "aspirin hyporesponsiveness")

Out of the 15 patients who completed all 3 study time points, 2 (13%) exhibited a change in their aspirin responder status between study entry (time point 1) and study completion (time point 3) (figure 20).

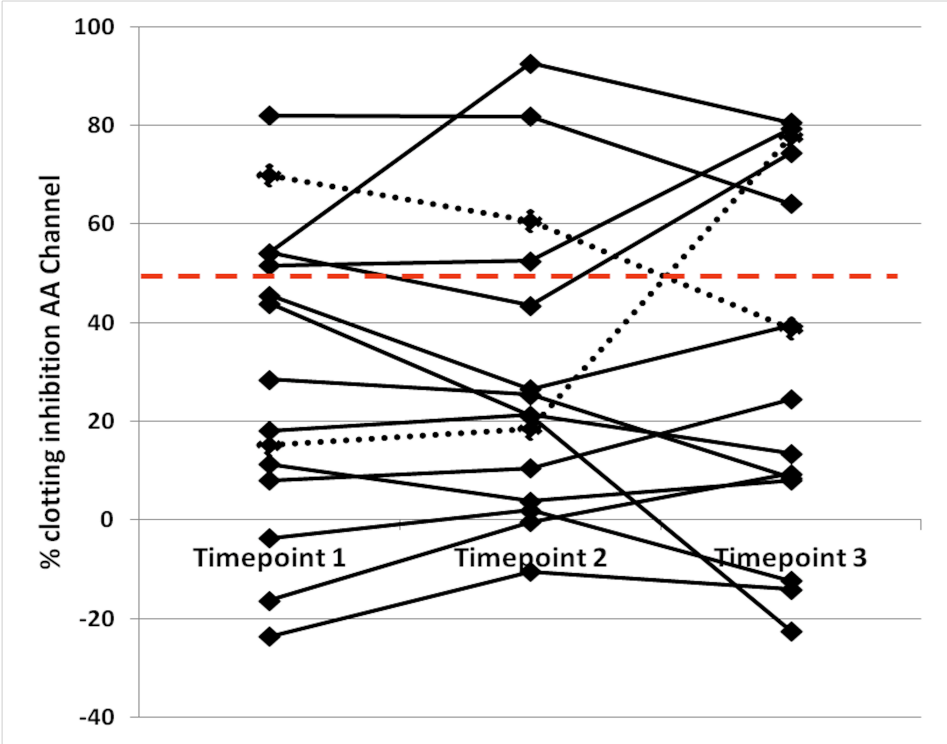

Figure 20. Percentage clotting inhibition of the arachidonic acid (AA) channel in the 15 patients who completed all 3 study time points (red horizontal dashed line represents the cut-off for high residual platelet reactivity or "aspirin hyporesponsiveness"; time point 1 is within 72 hours of stroke, time point 2 is day 6 of stroke, time point 3 is day 8 to 10 of stroke; dotted lines indicate the 2 patients who exhibited a change in their aspirin responder status from study entry (time point 1) to study completion (time point 3)).

6.3.2 Thromboxane B2 levels

Serum TXB2 was steadily suppressed at all time points confirming effective inhibition of platelet COX-1 by aspirin (absolute values were consistently <10ng/ml). Mean (SD) serum TXB2 was 2.8±2.2 ng/ml, 0.5±0.4 ng/ml and 0.4±0.3 ng/ml at time points 1, 2 and 3 respectively (Figure 21). Although there was a significant change in mean TXB2 between time points 1 and 2 (n=23, 95% CI 1.4 to 3.1, $p<0.001$) and between time points 1 and 3 (n=15, 95% CI 1.2 to 3.5, $p<0.001$), the absolute values remained below 10ng/ml. There was no significant correlation between serum TXB2

measurements and Short TEG percentage clotting inhibition in assessment of response to aspirin (r=0.005, n=73, p=0.965) (Figure 22).

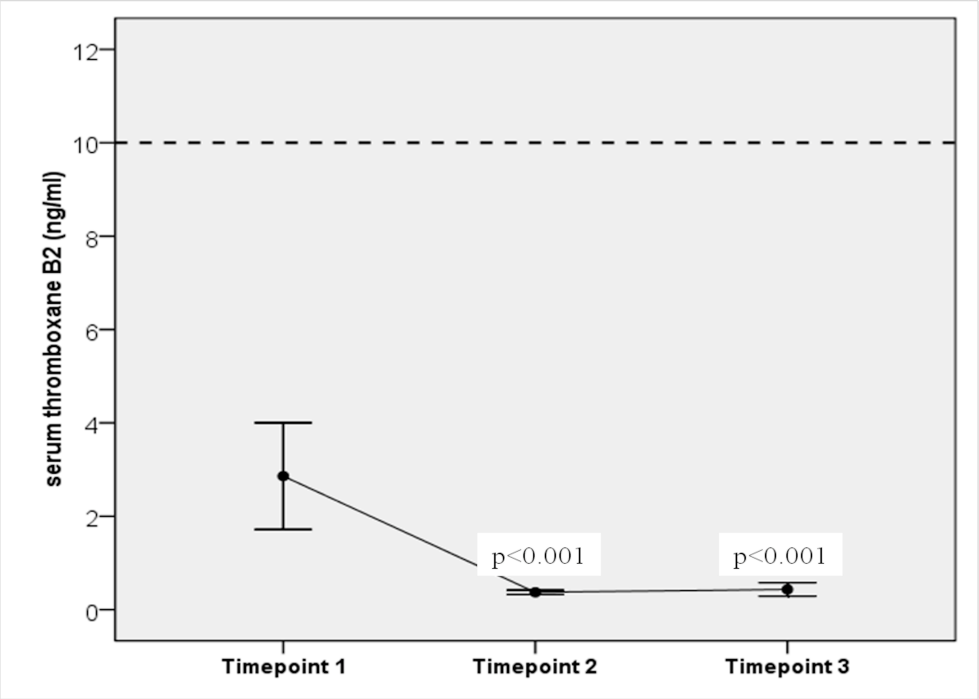

Figure 21. Mean serum thromboxane B2 levels (ng/ml) (error bars represent mean and 95% confidence intervals; horizontal dashed line represents the cut-off for adequate TXB2 inhibition; time point 1 is within 72 hours of stroke (n=35), time point 2 is day 6 of stroke (n=23) and time point 3 is day 8 to 10 of stroke (n=15). The p values given are for comparison with time point 1).

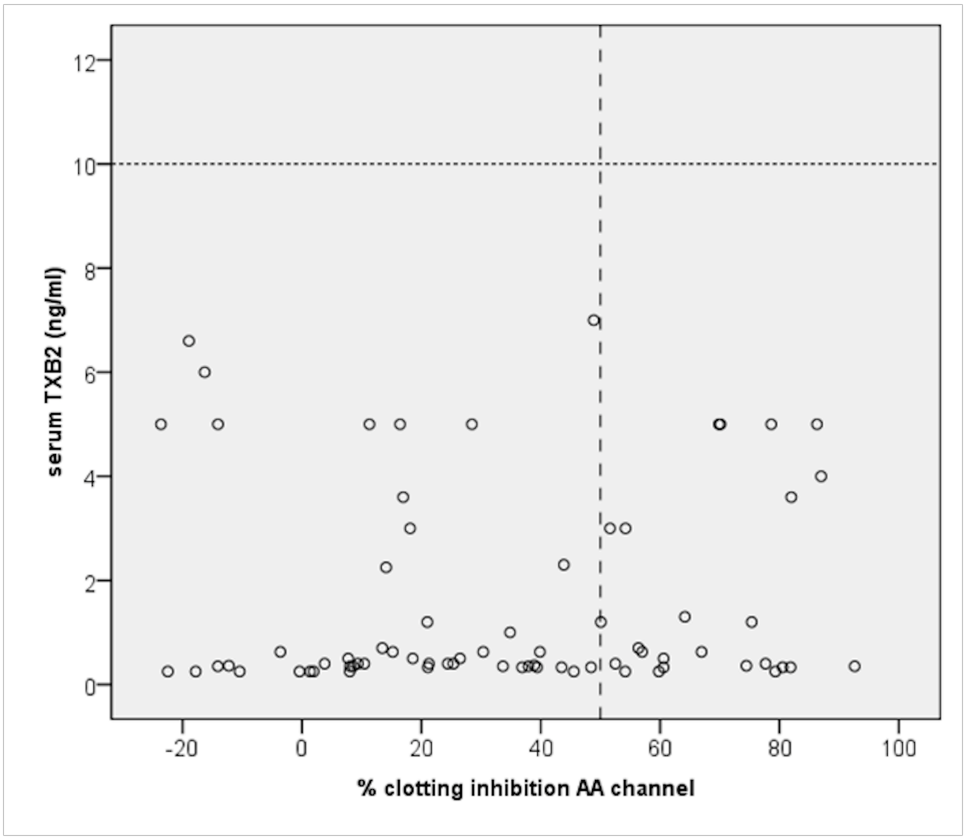

Figure 22. Correlation between serum thromboxane B2 (TXB2) and Short TEG arachidonic acid (AA)-induced clotting. The horizontal dotted line indicates the cut-off for adequate TXB2 inhibition and the vertical dashed line indicates the cut-off for high residual platelet reactivity or "aspirin hyporesponsiveness" (r=0.005, N=73, p=0.965).

6.3.3 Inflammatory biomarkers

Mean IL-6, hsCRP, TNF alpha and sCD40L were elevated at time points 1, 2 and 3 (Figure 23). There was a significant increase in mean hsCRP between time points 1 and 2 (n=23, 95%CI -1431.6 to -381.3, p=0.002) and between time points 1 and 3 (n=15, 95%CI -1755.3 to -96.5, p=0.031). By contrast, there was no significant change in mean sCD40L, IL-6 or TNF alpha across the 3 time points. There was no association between aspirin "hyporesponsiveness" measured using Short TEG and elevated inflammatory markers sCD40L (χ=0.017, df=1, p=1), hsCRP (χ=2.258, df=1, p=0.133), TNF alpha (χ=0.25, df=1, p=0.617) or IL-6 (χ=0.037, df=1, p=0.848).

There was good correlation between hsCRP and MRS at presentation (r=0.448, n=35, p=0.007) and between IL-6 and MRS at presentation (r_s=0.528, n=35, p=0.001).

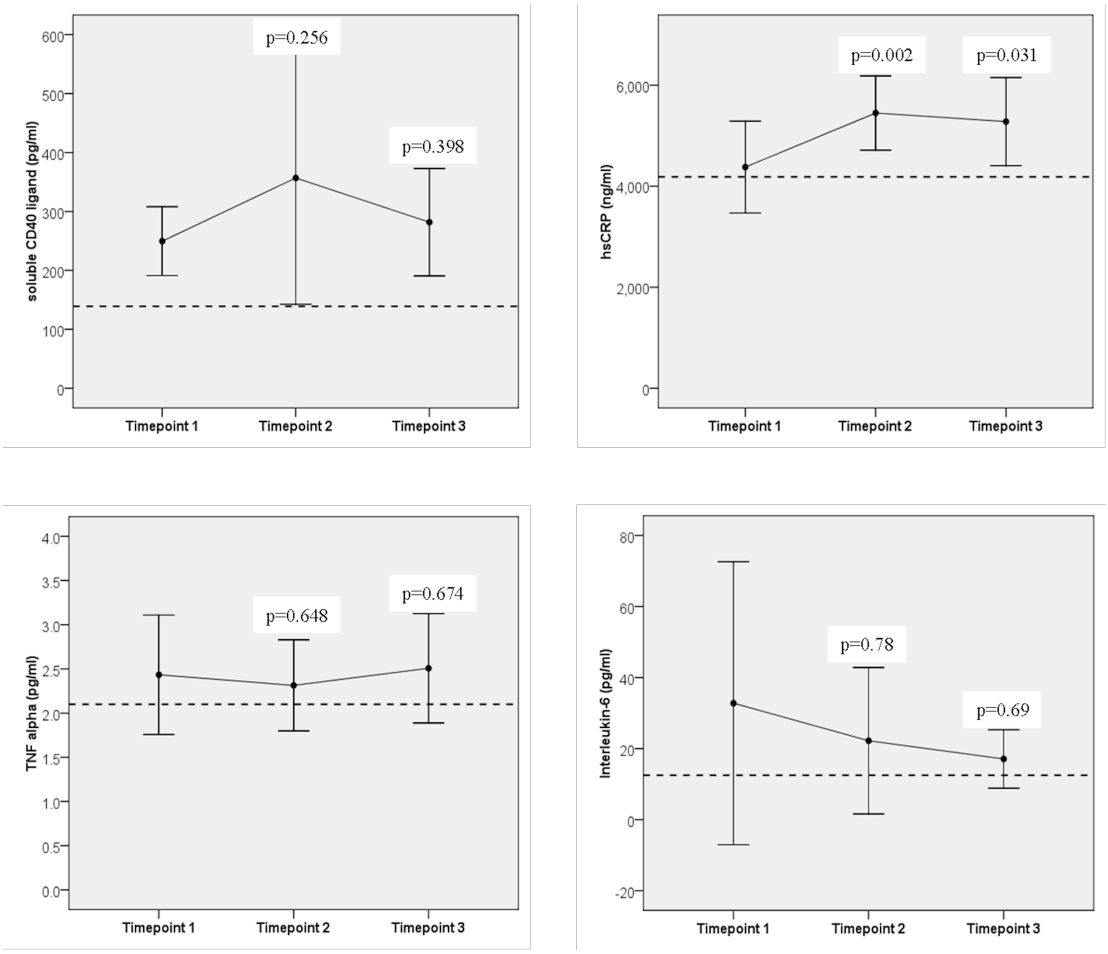

Figure 23. Inflammatory markers at all 3 time points (error bars represent mean and 95% confidence intervals; horizontal dashed line represents the cut-off values for elevated inflammatory biomarkers: >139pg/ml for sCD40L; >4185 ng/ml for hsCRP; >2.1 pg/ml for TNF alpha; >12.5 pg/ml for IL-6. Time point 1 is within 72 hours of stroke (n=35), time point 2 is day 6 of stroke (n=23) and time point 3 is day 8 to 10 of stroke (n=15). The p values given are for comparison with time point 1)

6.4 Discussion

The most important finding of this study is demonstration of significant discrepancy between functional AA-induced clotting and serum TXB2 concentration, in the assessment of responses to aspirin in patients with ischaemic stroke. Specifically, the former test suggests, as have previous studies, that there is a very high prevalence of apparent "aspirin resistance" in these patients whilst the latter demonstrates universally satisfactory activity of aspirin at its biochemical target. These findings are consistent with our previous data in CAD patients described in the *CESSATION study* in section 5.

These data raise some important and clinically relevant questions. Firstly, does platelet reactivity in response to AA agonist reflect whether aspirin is achieving its therapeutic target (i.e. COX-1 inhibition)? If not, then interpreting conventional assays of platelet function in relation to aspirin resistance may be flawed, and hence explain why such assays commonly report such high rates of apparent "resistance" to aspirin. This study, and previous data from our CAD patients, suggests that AA-induced clotting cannot in fact be reliably used to test the therapeutic response of an individual to aspirin. Secondly, by what mechanism ***does*** AA induce whole blood clotting when aspirin is actively inhibiting the COX-1 enzyme? This is an important question because the pathway(s) that are used may be dynamic and thus represent a novel therapeutic target. It is conceivable, for example, that recurrent ischaemic events in patients with CVD may be mediated via novel COX-1- (and therefore aspirin-) independent pathways or, indeed, by a pathway that is not related to platelet COX. These data demand further investigation.

The present study employed a single platelet function test that utilised a specific concentration of AA agonist, but the findings are consistent with observations from other studies that compared responses to aspirin using more than one platelet function assay in parallel with serum TXB2 measurements (124,179,242,243). These studies also found a very low prevalence of true aspirin "resistance" when based on serum TXB2 analysis but, simultaneously, a high prevalence of heterogeneity of platelet function when using AA as an agonist.

With regards to inflammatory biomarkers, previous studies have shown that elevated levels in stroke are predictive of stroke severity and long term clinical outcome (125,244-247). In the present study, we observed a significant and progressive rise in

mean hsCRP from hospital admission through to day 10 of stroke, but no evidence of time-dependent changes with any of the other inflammatory biomarkers. This is an important observation, given that previous studies in stroke patients have consistently shown that increased levels of hsCRP at hospital discharge are predictors of poor outcome and stroke recurrence (248-250). There was no association between hsCRP or any of the other inflammatory marker levels and aspirin "resistance" measured on Short TEG.

This study has limitations. Firstly, sample size is small but, nonetheless, the results are entirely consistent across the time points. Second, although we have utilised only one platelet function assay, this test has been shown to correlate well with VerifyNow in assessment of responses to aspirin (213). Third, we do not have baseline pretreatment platelet function data which would not have been feasible with this particular study design. Fourth, our study does not offer any mechanistic explanation as to how AA can induce whole blood clotting when aspirin is actively inhibiting the COX-1 enzyme.

6.5 Conclusion

This study demonstrates that: (i) platelet COX-1 activity is adequately and consistently suppressed by aspirin in patients with acute ischaemic stroke but this effect is not reliably reflected by a platelet function assay that utilises AA agonist and whole blood clotting, and (ii) the reported prevalence of aspirin "resistance" in previous studies based on AA-induced platelet reactivity is, thus, overestimated. These data have potential implications for the clinical use of whole blood clotting-based assays that employ AA as a stimulant agonist to assess responses of patients to aspirin.

Given the widespread use of aspirin in the treatment of atherothrombotic disease and consistent data to show a link between aspirin "resistance" and adverse clinical events, it is imperative to correctly identify those individuals who are truly "resistant" to the antiplatelet effects of aspirin and thus design appropriate strategies to attenuate the impact of this effect. What may be required in the future is closer scrutiny of the arbitrary cut-off values employed by platelet function tests to differentiate aspirin "responders" from "non responders". These cut-off values are not directly applicable

to a biological system and, instead, reference ranges that correlate well to serum TXB2 measurements and are linked to clinical outcome should be established. Although platelet function tests such as Short TEG and VerifyNow are currently mainly used as a research tool, the main advantage of these tests over serum TXB2 measurements is that they are simple, rapid, readily available, point-of-care tests that are ideal for use in the 'real-world' clinical setting (218,251,252).

Our study findings warrant further investigation by way of large scale clinical studies which may potentially have important implications on the way in which apparent aspirin "resistance" is diagnosed and managed in the future. Furthermore, aspirin-independent mechanisms of AA-induced platelet reactivity bear closer scrutiny as they may offer novel therapeutic targets.

CHAPTER 7: RESULTS

ORIGINAL ARTICLE

A Randomized Crossover Study Comparing the Antiplatelet Effect of Plavix Versus Generic Clopidogrel

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J Cardiovasc Pharmacol™ 2012;60:495–501

Background: Clopidogrel exists in different salt formulations. All published data that have demonstrated its beneficial effect are based entirely on the hydrogen sulphate salt contained in the branded product Plavix, which had US sales of $6.1 billion in 2010 alone. A number of cheaper generic versions of clopidogrel are increasingly being used in Europe as an alternative to Plavix, mainly for cost reasons. However, there is insufficient evidence to show that their pharmacodynamic effect is equivalent to Plavix.

Methods: This prospective study investigated whether there is any significant difference in the antiplatelet effect of Plavix versus generic clopidogrel hydrochloride in healthy male volunteers. All participants received loading and maintenance doses of both drugs, in a crossover manner, separated by a 2-week washout period. Adenosine diphosphate (ADP)–induced platelet reactivity was measured using short thrombelastography at multiple timepoints.

Results: The results showed interindividual heterogeneity in responses to clopidogrel but no significant difference in ADP-induced platelet reactivity between Plavix versus generic clopidogrel hydrochloride.

Conclusions: Our findings suggest comparable inhibition of ADP-induced platelet reactivity with Plavix and generic clopidogrel hydrochloride. This observation is particularly pertinent at a time when the patent for Plavix is expected to expire in the near future leading to the large-scale switch to cheaper generic preparations.

Study objectives: to investigate whether there is any significant difference in the antiplatelet effect of Plavix® (clopidogrel hydrogen sulphate) versus the cheaper generic clopidogrel salts which are currently in widespread clinical use in CVD despite limited data to support their efficacy.

7.1 Introduction

The beneficial effect of clopidogrel in addition to aspirin in patients undergoing PCI and following ACS is well documented (253). Preloading with clopidogrel prior to PCI is associated with significant reduction in peri-procedural MACE and subsequent maintenance therapy has been shown to reduce mortality and ischaemic events including ST (15,16,36,37). Dual APT is, therefore, recommended in international guidelines for all patients with ACS and in those undergoing PCI and, worldwide, clopidogrel remains the most commonly prescribed antiplatelet agent after aspirin. Clopidogrel exists in different salt formulations including the hydrogen sulphate, besylate, hydrochloride and resinate preparations. The specific salt formulation of a drug determines its pharmacokinetic and pharmacodynamic properties, both of which influence the extent to which it is absorbed, distributed and eliminated by the body. Modifying the salt preparation of a drug could, therefore, potentially alter its physicochemical properties and thereby have an impact on its clinical efficacy and safety (254). It is notable that all published clinical trial data that have demonstrated the beneficial clinical effect of clopidogrel are based entirely on the hydrogen sulphate salt contained in the branded product Plavix®.

Plavix® was the third highest selling pharmaceutical drug in the United States in 2010, with US sales of $6.1 billion. A number of generic versions of clopidogrel, based on the either the hydrochloride or besylate salts, have been developed and recently approved for use in Europe for the treatment of patients with ACS, ischaemic stroke and PVD, largely because they are significantly cheaper. There are, however, limited data supporting the use of generic clopidogrel in routine clinical practice. Specifically, there have been small randomised crossover studies conducted in patients (255) and in healthy volunteers (256,257) that did not show any significant difference in the antiplatelet effect of Plavix® versus generic clopidogrel measured using various platelet function tests including flow cytometry, impedance aggregometry and the VerifyNow P2Y12 assay. Similarly, with regards to

bioequivalence testing of generic clopidogrel, there is very sparse data available from small clinical studies conducted in healthy, young volunteers (258,259) (mean age 24.3 years and 33.7 years).

Despite the paucity of evidence for the efficacy of these alternative clopidogrel salts there is mounting pressure, mainly for cost reasons, to adopt these agents for use in all patients requiring long term clopidogrel. Specifically, the approval of generic clopidogrel by the European Medicines Agency in 2009 has rapidly facilitated the introduction of generic salts in the UK for financial reasons, and this was accompanied by unease amongst interventional cardiologists about the lack of data to reassure them that the newer agents shared the same degree of antiplatelet efficacy. The aim of this study was, therefore, to investigate whether there is any significant intra- and/or inter-individual variability in platelet reactivity in response to Plavix® versus generic clopidogrel (clopidogrel hydrochloride) in healthy volunteers at multiple time points. Platelet reactivity was measured using Short TEG.

7.2 Methods

7.2.1 Study population

This single centre, open-label, randomised crossover study was undertaken between March and July 2011. Seventeen healthy male volunteers aged between 18 and 60 years of age were prospectively enrolled. Females were excluded because previous data have shown significant differences in gender responses to APT using Short TEG (69), thereby introducing a potential confounding factor. Additional study exclusion criteria were as follows: current smokers; regular prescribed or over-the-counter medication; antiplatelet or non-steroidal anti-inflammatory medication administered within the previous 14 days; history of bleeding diathesis or any major alteration of full blood or platelet count; history of any recent injury; surgery planned within 4 weeks; history of liver or kidney disease; and previous stroke. All subjects underwent a full medical examination as well as screening blood tests that included full blood count, urea and electrolytes and liver function tests prior to study enrolment.

7.2.2 Study procedures

All participants received both Plavix® and clopidogrel hydrochloride, in a crossover manner, separated by a 2 week washout period. Randomisation to group A (Plavix® first, followed by clopidogrel hydrochloride) or Group B (clopidogrel hydrochloride first, followed by Plavix®) was performed using randomly selected sealed envelopes. Neither participant nor researcher was blinded to the treatment sequence. Following randomisation, the subjects received a single 300mg loading dose of Plavix® or clopidogrel hydrochloride on day 1 followed by maintenance therapy of 75mg once daily on days 2 to 8. The study design is illustrated in the flow diagram in Figure 24. Blood samples were taken at the following pre-specified time points: (i) baseline, prior to randomisation, (ii) 1, 2 and 6 hours after receiving a 300mg loading dose of Plavix® or clopidogrel hydrochloride, and (iii) on day 8 (2 to 4 hours after taking the final 75mg maintenance therapy dose). Following a 2 week washout period, identical study procedures were repeated with the second agent. At each time point, platelet reactivity was measured using Short TEG.

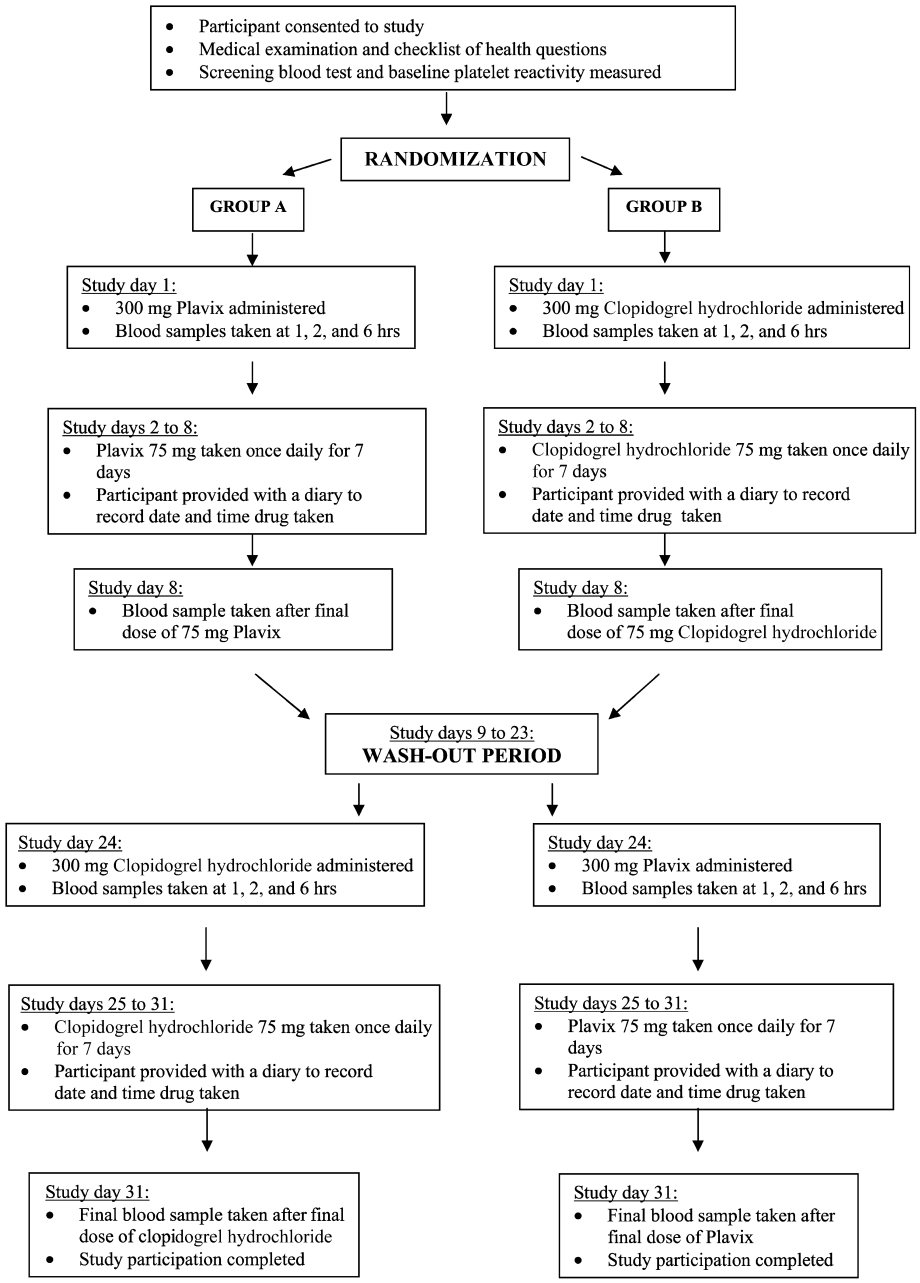

Figure 24. Flow diagram illustrating study design

7.2.3 Blood sampling and analysis

Venesection and sample analysis were performed as specified in the Study Methods in section 2.4.

7.2.4 Statistical analysis

This study was designed to be a pilot study and, in view of the lack of data comparing the pharmacodynamic effects of Plavix® versus generic clopidogrel, determination of sample size by way of power calculation was not feasible and could not be justified. Data are presented as the absolute AUC15 value as well as the mean change in AUC15 from baseline with 95% confidence intervals, unless otherwise stated. Baseline refers to the pre-clopidogrel treatment time point. Difference in mean AUC15 between Plavix® and clopidogrel hydrochloride compared to baseline was determined using paired t-tests with a p-value of <0.05 considered to represent statistical significance. Agreement of AUC15 measurements between Plavix® and clopidogrel hydrochloride was assessed using the Bland-Altman analysis with 95% ranges of agreement. Statistical analyses were performed using SPSS version 17.0 software and Microsoft Excel.

7.3 Results

A total of 17 healthy male volunteers were enrolled in the study. Two participants withdrew prematurely due to epistaxis in one case and minor injuries sustained from a cycling accident in another. Thus, study data are presented on 15 male subjects with a mean age (SD) of 35 (7.3) years (range 28 to 51 years) and mean body mass index (SD) of 23.9 (3.1) kg/m^2 (range 17 to 31 kg/m^2). The results of screening laboratory blood tests were within normal ranges in all participants. Seven subjects were randomised to Group A and eight were randomised to Group B. Baseline demographics of the study groups are outlined in Table XIII.

Participant No.	Age	Race	Body mass index (kg/m^2)	Screening blood test results
Group A				
1	28	White	17	Normal
2	32	Asian	24	Normal
3	37	White	31	Normal
4	34	Asian	27	Normal
5	51	White	26	Normal
6	45	White	23	Normal
7	36	White	23	Normal
Group B				
1	31	White	23	Normal
2	35	White	23	Normal
3	41	White	20	Normal
4	28	White	27	Normal
5	29	White	25	Normal
6	28	Asian	24	Normal
7	28	White	22	Normal
8	45	White	23	Normal

Table XIII. Baseline demographics of the study groups

7.3.1 Short TEG findings

Platelet reactivity was determined from AUC15 of the ADP channel. There was significant inter-individual variability in platelet reactivity following a 300mg loading dose of Plavix® and clopidogrel hydrochloride, as demonstrated in Figures 25A and 25B respectively. However, as illustrated in Figures 26A and 26B, there were no significant differences in intra-individual AUC15 measurements in response to a 300mg loading dose of Plavix® and clopidogrel hydrochloride as well as following 7 days of maintenance therapy.

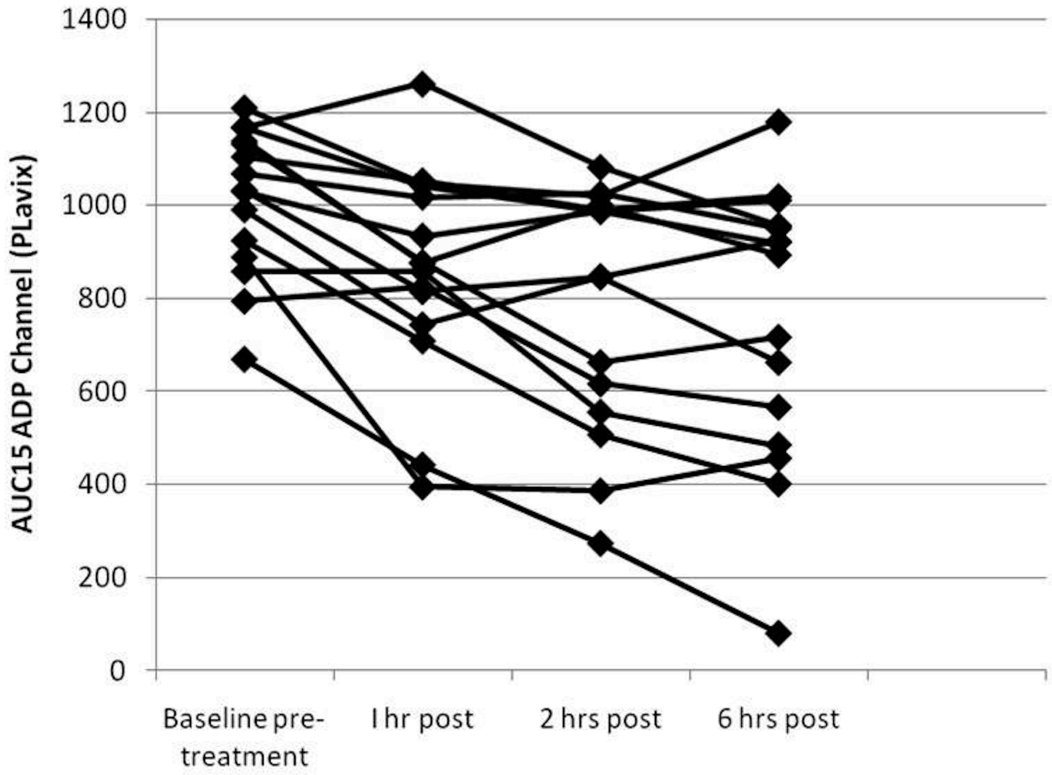

Figure 25A. Area under the curve (AUC15) of the adenosine diphosphate (ADP) channel measurements demonstrating inter-individual variability in platelet reactivity in response to a 300mg loading dose of Plavix®.

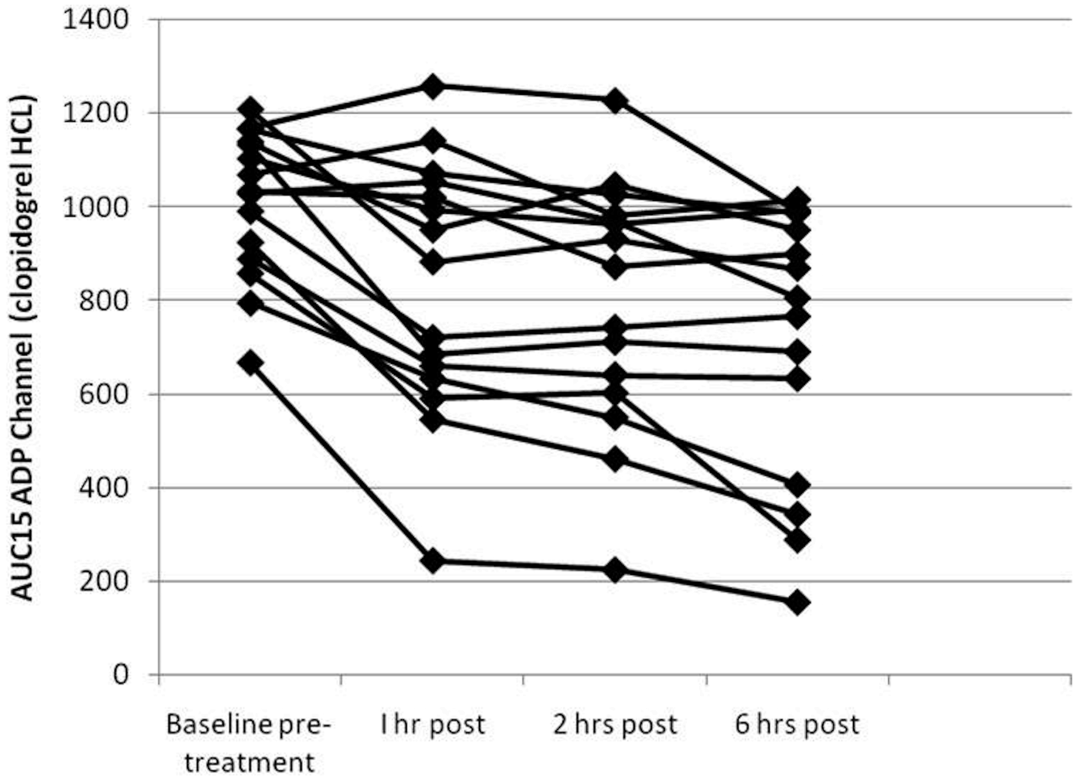

Figure 25B. Area under the curve (AUC15) of the adenosine diphosphate (ADP) channel measurements demonstrating inter-individual variability in platelet reactivity in response to a 300mg loading dose of clopidogrel hydrochloride.

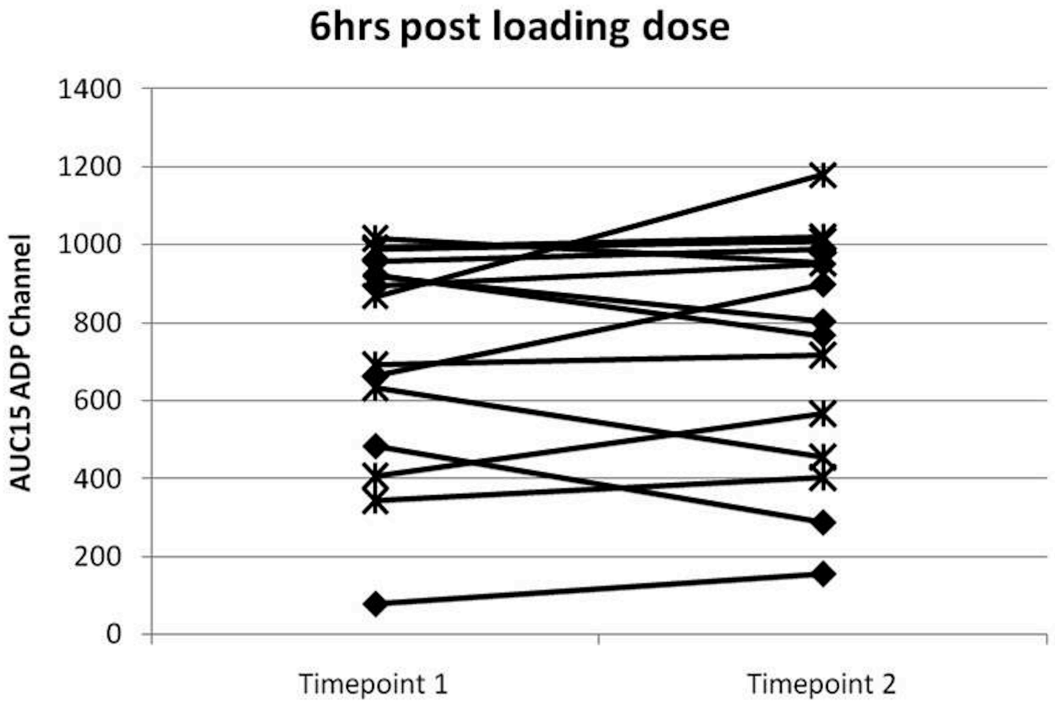

Figure 26A. Area under the curve (AUC15) of the adenosine diphosphate (ADP) channel measurements comparing the effect on platelet reactivity 6 hours after a 300mg loading dose of Plavix® and clopidogrel hydrochloride (n=15). (The ♦ connectors represent the 7 participants randomised to Group A who received Plavix® first (time point 1) followed by clopidogrel hydrochloride (time point 2) and the x connectors represent the 8 subjects randomised to Group B who received clopidogrel hydrochloride first (time point 1) followed by Plavix® (time point 2))

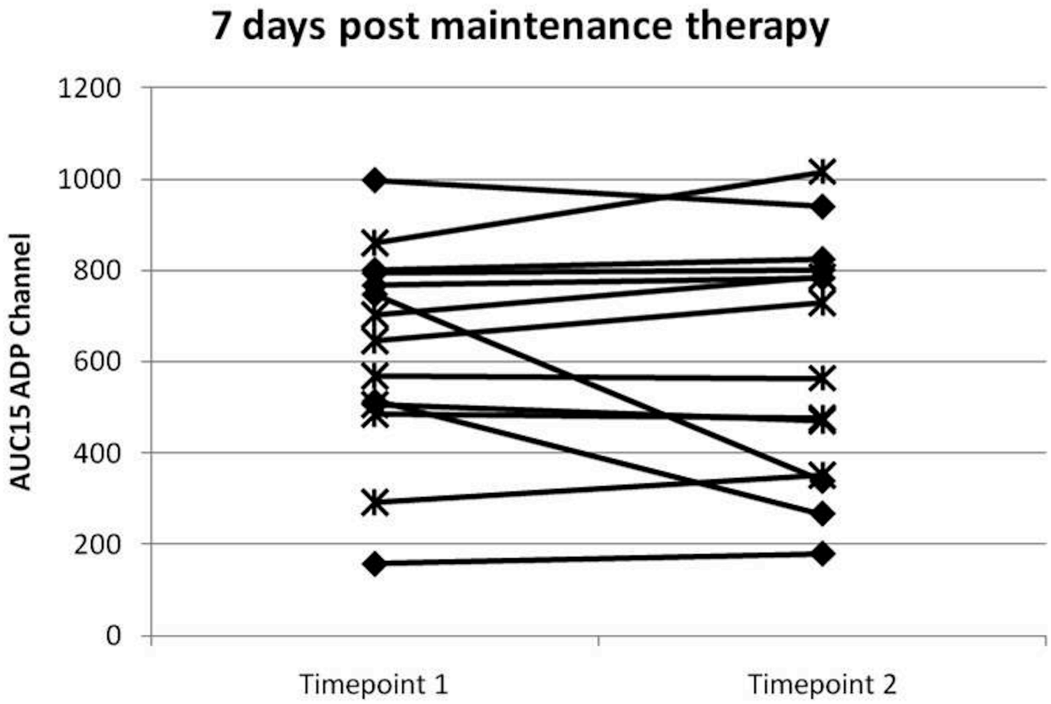

Figure 26B. Area under the curve (AUC15) of the adenosine diphosphate (ADP) channel measurements comparing the effect on platelet reactivity 7 days after receiving 75mg once daily maintenance therapy with Plavix® and clopidogrel hydrochloride (n=14). (The ♦ connectors represent the 7 participants randomised to Group A who received Plavix® first (time point 1) followed by clopidogrel hydrochloride (time point 2) and the x connectors represent the 7 participants randomised to Group B who received clopidogrel hydrochloride first (time point 1) followed by Plavix® (time point 2))

The change in mean AUC15 from baseline following a 300mg loading dose of Plavix® and clopidogrel hydrochloride respectively was 152 (95% CI 70 to 234, $p<0.001$) versus 181 (95% CI 84 to 278, $p<0.001$) at 1 hour post, 226 (95% CI 141 to 311, $p<0.001$) versus 214 (95% CI 132 to 297, $p<0.001$) at 2 hours post and 263 (95% CI 166 to 361, $p<0.001$) versus 292 (95% CI 198 to 386, $p<0.001$) at 6 hours post treatment. The change in mean AUC15 from baseline following a 75mg once daily maintenance dose of Plavix® and clopidogrel hydrochloride for 7 days was 356 (95% CI 274 to 430, $p<0.001$) and 397 (95% CI 295 to 499, $p<0.001$) respectively. (Figures 27A and 27B and Table XIV).

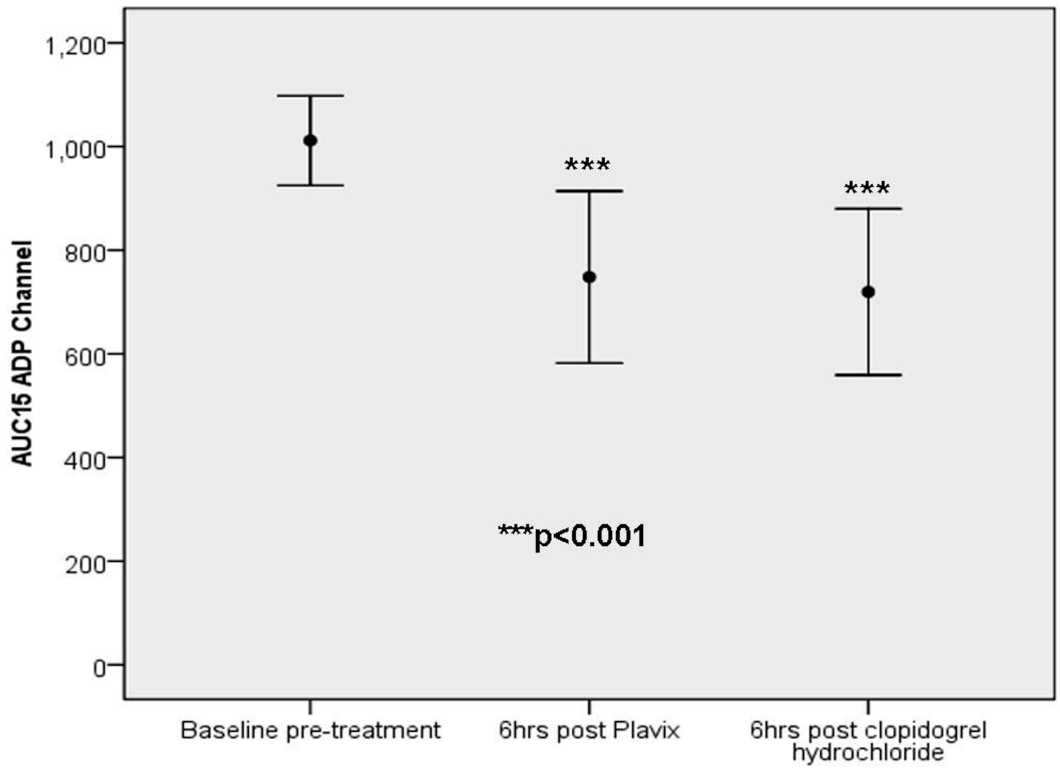

Figure 27A. Mean Area under the curve (AUC15) of the adenosine diphosphate (ADP) channel measurements at 6 hours post 300mg Plavix® and 6 hours post 300mg clopidogrel hydrochloride compared with baseline pretreatment measurement (error bars represent mean with 95% confidence intervals. The p value given (***p<0.001) is for comparison with the baseline pretreatment time point).

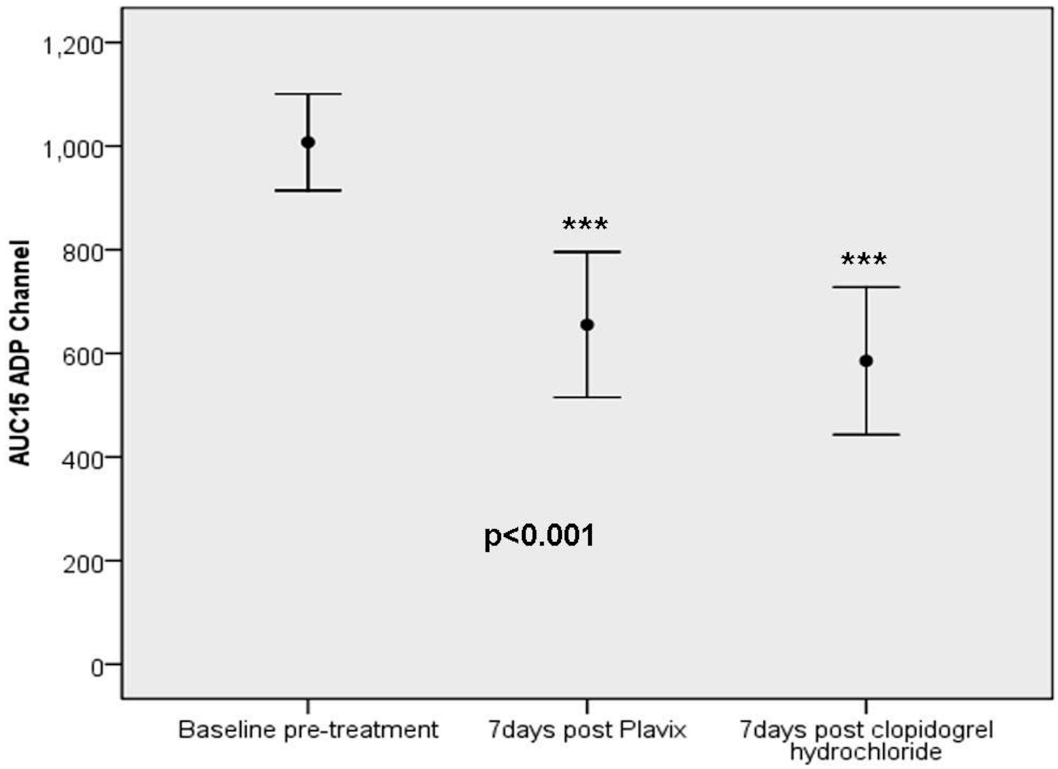

Figure 27B. Mean Area under the curve (AUC15) of the adenosine diphosphate (ADP) channel measurements at 7 days post Plavix® 75mg od and 7 days post clopidogrel hydrochloride 75mg od (error bars represent mean with 95% confidence intervals. The p value given (***p<0.001) is for comparison with the baseline pretreatment time point).

	Baseline	1 hr post loading dose		2hrs post loading dose		6 hrs post loading dose		7 days post maintenance therapy	
		Pl	CH	Pl	CH	Pl	CH	Pl*	CH
Mean AUC15 (SD)	1011.4 (156.2)	859.4 (228.3)	830.2 (273.7)	785.7 (263.7)	797 (265.5)	748.1 (299.3)	719.4 (289.5)	655.2 (242.8)	614.7 (263.1)
Change in mean AUC15 from baseline		152	181	226	214	263	292	356	397
95% CI of change in mean AUC15		70 to 234	84 to 278	141 to 311	132 to 297	166 to 361	198 to 386	274 to 430	295 to 499
p value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table XIV. Mean AUC 15 ADP channel of Plavix and clopidogrel hydrochloride compared to baseline

*n=14 (one participant did not take Plavix® for the entire 7 day period due to gastrointestinal reflux symptoms and was, therefore, excluded from this aspect of data analysis)

Pl = Plavix; CH = Clopidogrel hydrochloride

Bland-Altman analysis with 95% limits of agreement showed good agreement between Plavix® and clopidogrel hydrochloride therapies (loading dose and maintenance therapy). (Figures 28A and 28B).

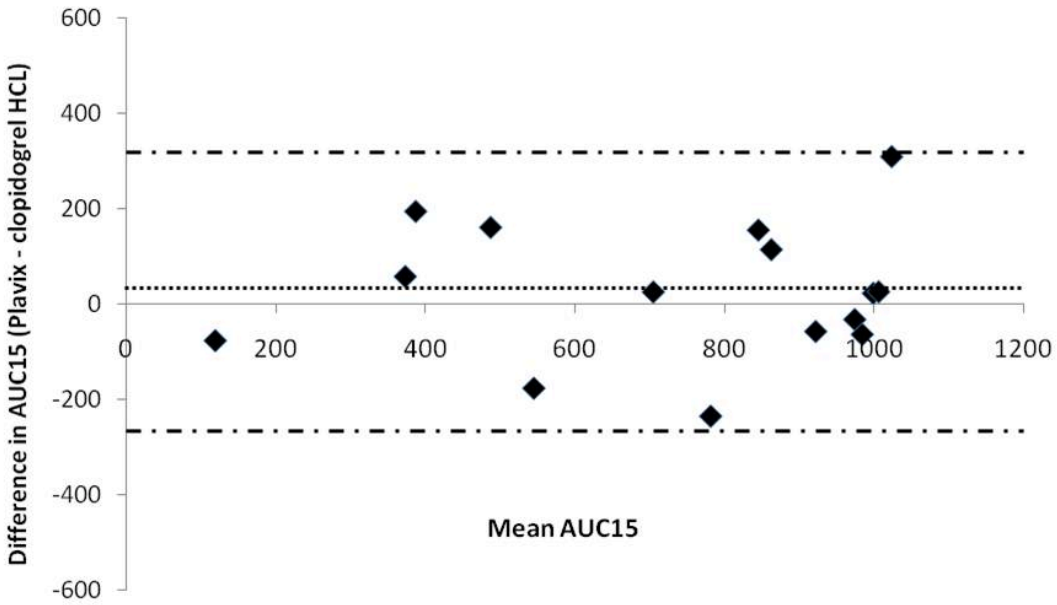

Figure 28A. Bland-Altman plot showing correlation between AUC15 Plavix and clopidogrel hydrochloride, 6 hours after a 300mg loading dose (n=15). Dotted line represents the mean difference in platelet reactivity between the two agents (i.e. average bias of one treatment relative to the other) and dashed lines represent 95% limits of agreement (i.e. 2SD of mean difference)

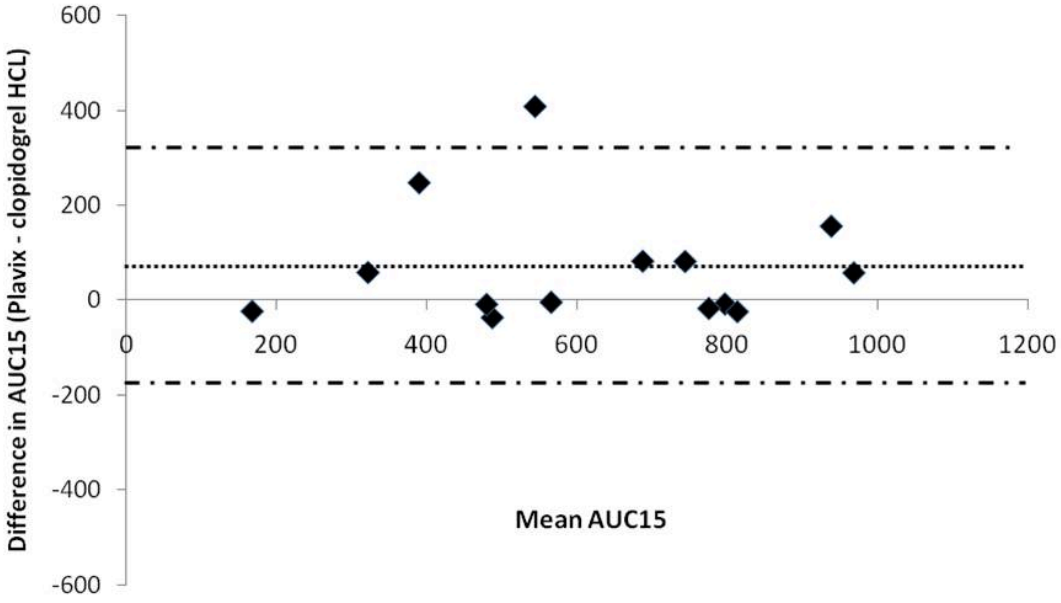

Figure 28B. Bland-Altman plot showing correlation between AUC15 Plavix and clopidogrel hydrochloride, 7 days after a 75mg once daily maintenance dose (n=14). Dotted line represents the mean difference (i.e. average bias of one treatment relative to the other) and dashed lines represent 95% limits of agreement (i.e. 2SD of mean difference).

7.4 Discussion

We have demonstrated, using Short TEG, that there is no significant difference in the effect on platelet reactivity between Plavix® versus clopidogrel hydrochloride in healthy male subjects at multiple time points. These findings are consistent following a 300mg loading dose as well as after 7 days of maintenance therapy. To our knowledge, this is the first UK-based study of its kind and our observations are consistent with findings from similar small studies undertaken in Korea (257,258) and Argentina (259) which utilised optical aggregometry to measure responses to clopidogrel. Furthermore, the present study illustrates the heterogeneity in inter-individual responses to clopidogrel, an observation that is well documented in the literature.

With Plavix® approaching the end of its patent, the routine use of less expensive generic salt formulations in patients with CVD is rapidly gaining momentum on a worldwide scale. This is despite the fact that there are few clinical studies demonstrating bioequivalence of the two agents. The findings from the present study are reassuring in relation to the comparative pharmacodynamic properties of Plavix® and clopidogrel hydrochloride. This is of value for two main reasons: firstly, it is unlikely that there will ever be a large scale randomised clinical outcome study conducted to compare Plavix® and generic clopidogrel. Secondly, the expiry of the patent for Plavix® will lead to mounting pressure to make the large scale switch to generic preparations in the United States shortly.

This study has limitations. Firstly, this was an open label design with a short duration of follow up. Secondly, it was conducted in a small population of healthy male volunteers and the results may not be directly applicable to: (i) an older age group of patients with multiple comorbidities, and (ii) the female population. Previous data have demonstrated gender-dependent differences in platelet reactivity. Specifically, Hobson et al (69) previously demonstrated that females exhibit higher baseline platelet reactivity as well as reduced responses to clopidogrel, measured using Short TEG. Thus, in a study of this size, inclusion of females would be a confounding factor that would have a significant impact on the interpretation of our findings. Inclusion of the female population would necessitate a comparative gender study.

7.5 Conclusion

This randomised crossover study demonstrates that there is no significant difference in the effect on platelet reactivity of loading and maintenance dosing of Plavix® compared to generic clopidogrel hydrochloride in healthy male volunteers. Although sample size is small, our findings are consistent at multiple time points. This is an important observation, at a time when the patent for Plavix® is expected to expire in the near future leading to the large scale switch to cheaper generic clopidogrel preparations.

CHAPTER 8: DISCUSSION

8.1 Summary of study findings

The role of platelets in the pathophysiology of CVD is now well established. The multiple pathways of platelet activation and aggregation and its complex interactions with other circulating cells and vascular endothelium lead to plaque development, plaque rupture, vascular occlusion, ischaemia and infarction. Antiplatelet agents are the cornerstone of treatment in the secondary prevention of CVD and in patients undergoing PCI, and have been unequivocally shown to improve clinical outcome. However, clinical studies have consistently demonstrated heterogeneity in responses to APT measured using *ex vivo* platelet function tests. Specifically, APT "hyporesponsiveness" that results in high residual platelet reactivity is associated with increased risk of adverse cardiovascular events. However, despite the growing body of evidence to support this link, routine testing of individual response to APT is currently not undertaken largely due to the lack of a standardised, widely available platelet function test appropriate for routine clinical use.

Short TEG is a whole blood test of platelet function that has been previously well validated and has the potential for routine use in the acute clinical care setting. In chapter 3, I demonstrate the reliability and reproducibility of Short TEG in the assessment of responses to aspirin and clopidogrel and, furthermore, show that Short TEG correlates well with the more widely used VerifyNow assay. The specific advantages of Short TEG over other historical "gold standard" platelet function tests are that: (i) it is a rapid test that provides results within 15 minutes, making it ideal for use as a point-of-care test in the acute clinical setting, and (ii) it is a whole blood assay, incorporating the interactions of all of the components of coagulation including platelets, fibrin, clotting factors and thrombin.

Thus, given the specific advantages of Short TEG and having demonstrated its reproducibility and reliability, we went on to employ this test as a clinical tool in the assessment of responses to APT in the acute clinical setting. We specifically selected patients admitted with ST because this is an important and potentially catastrophic complication following PCI. Although the aetiology of ST is multifactorial, it is now well described that hyporesponsiveness to aspirin and/or clopidogrel is a major (and potentially avoidable) risk factor for ST. In chapter 4, I describe the outcome of

assessing responses to APT using Short TEG in a prospective series of patients with definite ST, and then altering therapy where their response was deemed inadequate. The pertinent observations from this series were that: (i) there is high prevalence of hyporesponsivess to APT, particularly clopidogrel, in patients with ST, (ii) improved APT efficacy can be achieved with tailored therapy, and (iii) Short TEG is a plausible point-of-care test that can be used to deliver personalised APT.

Our next step was to employ Short TEG to investigate an entirely different group of patients requiring long term APT, i.e. stable patients on dual APT following PCI with DES and due to discontinue clopidogrel 1 year following DES implantation. Although universal guidelines recommend clopidogrel for 1 year in all patients undergoing PCI with DES, the optimal duration of therapy remains a contentious issue and is the subject of ongoing debate. This is, at least in part, due to concerns regarding the reported clustering of adverse events that occur early after cessation of long term clopidogrel, balanced with the bleeding risks that would accompany prolonged (>1 year) duration of dual therapy. In chapter 5, I demonstrate that clopidogrel cessation 1 year after DES is associated with an unexpected and significant aspirin-independent increase in AA-induced clotting in addition to the predictable effects of clopidogrel on ADP-induced clotting. Importantly, serum TXB2 levels were also measured in this study and found to be consistently suppressed indicating complete inhibition of platelet COX-1 by aspirin. These findings suggest that clopidogrel may exert some of its antiplatelet effects via the AA-pathway (traditionally thought to be exclusively influenced by aspirin) and raise the clinically important questions as to whether: (i) clopidogrel actually potentiates the antiplatelet effect of aspirin (as previously described in other studies), and (ii) abrupt cessation of clopidogrel 1 year after DES in individuals who are (unknowingly) relatively hyporesponsive to aspirin could potentially be detrimental and may be the mechanism partly responsible for the observed clustering of adverse events early after clopidogrel withdrawal.

In addition to the above, one of the more far-reaching questions that arose from our study described in chapter 5 was whether AA-induced clotting is really an appropriate test of aspirin response, given the obvious discrepancy between serum TXB2 levels and AA-induced platelet reactivity in patients who were on aspirin. Of note, the reported prevalence of aspirin hyporesponsiveness in CVD varies significantly from as little as 2% to as high as 70%, and this is based on *ex vivo* platelet function tests that utilise AA as the agonist. Particularly high rates of aspirin “hyporesponsiveness”

have been observed in the stroke population. We, therefore, went on to determine whether true biochemical response to aspirin in patients with ischaemic stroke (i.e. a population previously reported to have high prevalence of aspirin hyporesponsiveness) can be reliably determined from a functional test of AA-induced whole blood clotting. I demonstrate in chapter 6 (using Short TEG and serum TXB2 measurements in parallel), that platelet COX-1 activity is adequately and consistently suppressed by aspirin in patients with acute ischaemic stroke and this effect is not reliably detected by a platelet function assay that utilises AA agonist (Short TEG). These findings are consistent with observations from other studies that compared responses to aspirin using more than one platelet function assay in parallel with serum TXB2 measurements and also found a very low prevalence of true aspirin "hyporesponsiveness" when based on serum TXB2 analysis but, simultaneously, a high prevalence of heterogeneity of platelet function when using AA as an agonist. Finally, in chapter 7, I employed Short TEG in a small randomised crossover study of healthy volunteers to investigate whether there was any significant difference in the antiplatelet effect of Plavix® versus generic clopidogrel. Plavix® is a branded product that contains the clopidogrel hydrogen sulphate salt and was used in all published clinical trial data demonstrating the beneficial effect of clopidogrel. Our randomised study was conducted at a time when a number of significantly cheaper generic versions of clopidogrel were increasingly being used in Europe as an alternative to Plavix®, mainly for cost reasons and despite the lack of evidence to show that their pharmacodynamic effect was equivalent to Plavix®. Our study demonstrated inter-individual heterogeneity in responses to clopidogrel, but no significant difference in ADP-induced platelet reactivity between Plavix® and generic clopidogrel at multiple time points. These findings not only supported the large scale switch from Plavix® to the cheaper generic salts, but also provided further data to support the need for clopidogrel response testing in all patients given the heterogeneous responses observed in this population of healthy subjects.

8.2 Interpretation of study findings

The findings from the individual studies described in this thesis raise important clinical questions that warrant further investigation. Indeed, our study conclusions are

hypothesis-generating and may form the basis for future large scale clinical trials. Although the objective of each study was to investigate a specific clinically important issue there are clear, unifying messages derived from this work. Specifically, the move towards personalised APT across the spectrum of patients with CVD could become inevitable in the future and this is supported by our study findings. Furthermore, the role of clopidogrel (specifically its cheaper generic formulation) should be confined to those patients that are demonstrably responsive to this agent on objective testing, whereas the more expensive and potent agents should be restricted to clopidogrel hyporesponders. This practice would potentially optimise the efficacy, safety and cost effectiveness of APT prescribing in CVD. However, before this concept can be fully implemented in routine clinical practice, there remain important unanswered questions that should form the basis of future large scale clinical investigation:

a) *What is the ideal platelet function test that can be reliably used to measure individual responses to APT and what cut-off levels of platelet inhibition can be considered to adequately differentiate "responders" from "non-responders"?*

   The ideal test would be a rapid, reliable and comprehensive test that requires minimal sample preparation and can be used with relative ease in the acute clinical care setting. Furthermore, it would be a standardised assay that provides a range of values to differentiate "responders" from "non-responders" based on a clinically relevant 'gold standard' for normal, rather than using a single arbitrary cut-off value to define "responsiveness". The latter is one of the main limitations of point-of-care tests in current clinical and/or research use and is a contentious issue. This is because therapeutic response is more likely to be a continuous variable and, therefore, perceiving it in a dichotomous way may be inappropriate. The ideal, standardised platelet function test remains to be determined and is the subject of ongoing debate.

b) *In whom should platelet function be measured in routine clinical practice?*

   The heterogeneity in responses to APT is well established and the link between high residual platelet reactivity and risk of ischaemic events is well documented. The findings from our ST series clearly indicate that APT efficacy can be

improved with tailored therapy and lends support to the concept of routine testing in *all* patients requiring APT (and not just the ST group who are perceived to be at comparatively higher risk of adverse events) with a view to providing tailored therapy for *all*. This would not only achieve optimal APT efficacy, thereby reducing the risk of adverse events, but would also attenuate bleeding risk in those patients who are responsive to clopidogrel and, therefore, do not actually require the more potent agents that are associated with significantly higher bleeding complications. Large scale clinical investigation is warranted to explore these important clinical issues. Specifically, an adequately powered large randomised trial comparing default clopidogrel versus the more P2Y12 inhibitors in all-comers who are hyporesponsive to clopidogrel (and not just the stable low risk CAD populations studied in GRAVITAS and TRIGGER-PCI) is lacking. Furthermore, long term clinical outcome data following tailored APT are needed.

c) *What is the clinical significance of the accumulating evidence for the effect of clopidogrel on "aspirin-specific" AA-pathways and how could this influence APT prescribing in the future?*

There is a growing body evidence (including our own observations from the Cessation Study described in chapter 5) to suggest that clopidogrel influences the AA-pathway, thereby potentiating the effect of aspirin. As such, the antithrombotic effect of aspirin may be rendered partially or even completely redundant in the presence of clopidogrel or other more potent P2Y12 inhibitors. If this theory were examined and proven in larger studies, it could mean that patients on dual APT who are relatively hyporesponsive to aspirin would be at particular risk of adverse events when clopidogrel is discontinued and should be identified early on when dual APT is initiated. Furthermore, the influence of clopidogrel on the 'aspirin-specific' AA-pathway may indicate that routine use of aspirin in the context of dual therapy (particularly in combination with the more potent P12Y12 agents) provides little or no additional benefit over P2Y12 receptor antagonist monotherapy. The latter theory would require adequately powered clinical trials to provide answers and this would have significant implications on the future role of aspirin (which is, to date, the most widely prescribed antiplatelet agent).

Furthermore, this could potentially set in motion routine testing of response to APT in all patients in whom a prolonged period of dual APT is required.

d) *Is AA-induced clotting really a reliable test for measuring response to aspirin*?

Aspirin "hyporesponsiveness" is frequently described in the literature based upon *ex vivo* platelet function tests that utilise AA as the stimulant agonist. Thus, labelling individuals as aspirin "resistant" based on high residual platelet reactivity is potentially flawed because these tests do not specifically assess the effect of aspirin on its therapeutic target (i.e. COX-1). Previous studies (including our own observations) have shown discrepancies in the reported prevalence of aspirin "hyporesponsiveness" when determined from serum TXB2 levels in parallel with functional tests of AA-induced whole blood clotting. The latter are associated with significantly higher rates of apparent aspirin "hyporesponsiveness" raising the question as to whether these tests grossly overestimate the prevalence of this condition (which has been reported to be as high as 70% in some studies). Large scale clinical studies are clearly required to examine and clarify this issue and this could have potential implications for the clinical use of whole blood clotting-based assays that employ AA as a stimulant agonist to assess aspirin response.

8.3 Future perspectives

The unequivocal association between APT hyporesponsiveness and adverse clinical events, coupled with the advent of novel, more potent agents in the preceding decade indicate that a "one-size-fits-all" approach to APT prescribing may become obsolete in the future and the move towards individualised APT based on results of standardised platelet function tests is likely to become inevitable. This would entail careful selection of the most appropriate, safe and effective antiplatelet agent for every patient based on an individualised assessment of response to therapy. However, before this can be fully implemented in routine clinical practice, the ideal test that is reproducible, inexpensive, widely available and can be used with relative ease in the acute clinical care setting remains to be established. A further requirement of this

ideal test would be standardised and validated cut-off values that represent clinically relevant definitions of APT hyporesponsiveness.

Furthermore, the precise mechanism(s) by which clopidogrel and other more potent P2Y12 receptor antagonists elicit their antiplatelet effects (other than via the ADP pathway) and their potential interactions with aspirin remain to be fully established. Large scale clinical studies are required to determine whether the antiplatelet effect of aspirin is, indeed, rendered "redundant" when combined with these agents. If proven, this could have potentially significant implications on APT prescribing in the future, particularly in those patients requiring dual treatment with both aspirin and clopidogrel. The future role of aspirin which has, to-date, dominated the profile of APT prescribing in CVD could well be challenged.

For now, it is imperative for clinicians to adopt a dynamic approach to APT prescribing as there are likely to be significant changes in the future particularly with regards to personalised APT for *all*, pending data from large scale clinical outcome studies.

9. APPENDICES

9.1 Cessation study

9.1.1 Research participant information sheet and consent form

Cardiology Trials Office
Wessex Cardiothoracic Unit
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Research Participant Information Sheet and Consent Form

PART 1

1. Study Title

An investigation into the effects of cessation of clopidogrel therapy on vascular inflammation and platelet reactivity in patients with drug-eluting coronary stents

2. Invitation Paragraph

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

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

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

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

3. What is the purpose of the study?

Approximately 1 year ago you underwent a coronary stent procedure. Following the insertion of coronary stents it is current practice for the doctor to prescribe blood-thinning medication - aspirin and clopidogrel. After any stent procedure there is initially a small risk of clots forming on the stent (known as stent thrombosis) and these drugs help to stop this complication. You were prescribed a 12 months course of clopidogrel and are due to stop your clopidogrel in the next few weeks. The purpose of this study is to evaluate through a series of blood tests, the effect of clopidogrel on

changes in the blood of markers of inflammation and platelet activation. These are important factors in the blood clotting process.

4. Why have I been chosen?

You have been chosen because you were treated with a drug eluting coronary stent about a year ago and are coming to the end of your 12 months prescribed course of clopidogrel. About 60 patients in total are expected to participate in the study. 30 patients will be diabetic and 30 patients will be non-diabetic. These two groups will then be compared as part of the study.

5. Do I have to take part?

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

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

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

After discussing the study and having read the information sheet the research doctor will address any questions you may have regarding the study. If you would like to take part an appointment will be made for you to attend Southampton General Hospital Cardiac Unit, where the research doctor will sign a consent form with you and the first blood sample will be taken for analysis. Subsequent appointments will be made for you to attend the hospital for further blood tests. These are outlined below.

You will be followed up in the study for approximately 6 weeks. At each follow-up you will be required to visit the hospital and the research doctor will take a sample of blood for analysis. At each visit approximately 1 tablespoon of blood will be taken.

Timing of study visits to hospital	Procedure
Approximately 11 months after stent	Consent to study 1st blood sample taken
12 months after stent (immediately prior to stopping clopidogrel)	2nd blood sample taken
24 hours after stopping clopidogrel	3rd blood sample taken
48 hours after stopping clopidogrel	4th blood sample taken
1 week after stopping clopidogrel	5th blood sample taken
2 weeks after stopping clopidogrel	6th blood sample taken
4 weeks after stopping clopidogrel	7th blood sample taken

At each visit:

- You will have blood drawn for blood sampling
- You will be asked how you feel, and possible side effects will be discussed
- You will be asked about any other medication that you take while you are in the study.

For the time that you are in the study the research staff will need to carefully monitor all the other medications that you are taking. You will stop your clopidogrel medication as routine and continue on the same dose of aspirin. Please speak with your research doctor before taking any non-prescription drugs or drugs prescribed by another doctor. There are no changes required to your routine care other than a requirement for the research staff to carefully monitor all your medication.

If you decide to stop participating in the study prior to the end of the study, you will be asked to return to your doctor to have the final procedures performed.

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

7. Expenses and payments

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

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

When a needle is inserted into your vein to draw blood, you may experience pain, bruising, swelling, bleeding, irritation or infection at the site of the puncture, or you may experience dizziness or faintness. Approximately 1 tablespoon (~ 60 mls) of blood will be collected at each study visit.

In the unlikely event of an injury caused by taking part in the study, your hospital doctor will provide appropriate medical treatment.

9. Risks for Women of Childbearing Potential

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

10. What are the alternatives for diagnosis or treatment?

All standard treatments will remain available.

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

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

12. What are the possible benefits of taking part?

There is no direct benefit to you from taking part in the study. However, if your blood test results are abnormal we will inform the Consultant Cardiologist directly responsible for your care. You may feel that you benefit from the additional close follow-up during the course of the study. The results of the study may add to the understanding of your condition. It may also be helpful for future patients.

13. What if there is a problem?

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

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

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

15. Contact details:

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

Dr Nicholas Curzen
Consultant Cardiologist
Southampton General Hospital
Tel: 023 8079 4972

or

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

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

This completes Part 1 of the Information Sheet.

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

PART 2

16. What if relevant new information becomes available?

Sometimes during the course of a research study, new information becomes available about the treatment that is being studied. If this happens, your study doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your study doctor will make arrangements for your care to continue. If you decide to continue in the study you may be asked to sign an updated consent form. In addition new information may lead your study doctor to consider withdrawing you from the study if they feel it is in your best interest. They will explain the reasons and arrange for your care to continue.

If the study is stopped for any other reason, you will be told why and your continuing care will be arranged.

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

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

18. What if there is a problem?

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

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

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

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

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

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

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

20. What happens to any samples I give?

Your research doctor will collect the blood samples. Blood samples will be frozen and stored securely in the hospital Pathology Department. Access to this department is restricted by an entry key-pad. Blood samples may be stored for up to one year. This allows several samples to be tested at once making efficient use of testing equipment. The blood samples will be analysed on-site and will be destroyed following analysis. They will not be used for any additional testing.

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

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

22. Who is organising and funding the research?

The study has been designed by Dr Nick Curzen who is a Consultant Cardiologist within The Wessex Cardiac Unit, Southampton General Hospital. Contact details are as follows:

Dr Nick Curzen
Consultant Cardiologist & Honorary Senior Lecturer
Wessex Cardiac Unit
Southampton General Hospital
Hants
Tel: 023 8079 8538
Fax: 023 8079 5174

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

23. Who has reviewed the study?

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

Thank you for taking time to read this sheet.

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

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

Patient Identification Number for this trial: _____

RESEARCH PARTICIPANT CONSENT FORM

Title of Study: Discontinuation Effect of Clopidogrel After Drug Eluting Stents

Name of Researcher: Dr Nick Curzen Please initial boxes

1. I can confirm that I have read and understood the information sheet (version 1 dated 01/06/2009) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I have been provided wiht the details of the known or foreseeable side effects and risks of the study procedures. ☐

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

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

4. I agree that the data collected for the study may be used for the purpose described, and to its processing and archiving in a coded form to protect the confidentiality of my personal data. This will not waive any rights that I have under local law. ☐

5. I agree to take part in the above study ☐

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

When completed, 1 for patient, 1 for researcher site file, 1(original) to be kept in medical records

9.2 Stroke study

9.2.1 Research participant information sheet and consent form

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

Research Participant Information Sheet and Consent Form

PART 1

1. Study Title

An observational study investigating the incidence of aspirin resistance in ischaemic stroke and its relationship to vascular inflammatory biomarkers, stroke severity and stroke recurrence

2. Invitation Paragraph

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

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

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

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

3. What is the purpose of the study?

Stroke is the leading cause of long term disability in the developed world and is the third most common cause of death. Current guidelines recommend aspirin lifelong in all patients with stroke to reduce the risk of recurrent stroke and adverse clinical events. However, despite aspirin treatment a significant proportion of stroke patients continue to experience recurrent adverse clinical events. Previous clinical studies have reported a high prevalence of aspirin resistance in stroke patients and this has been linked to increased risk of recurrent stroke and poor outcome.

This study will seek to determine, through a series of blood tests, the proportion of stroke patients who are resistant to aspirin and are thus potentially at increased risk of

adverse clinical events. We will also investigate whether aspirin resistance is related to inflammatory biomarker levels, stroke recurrence, stroke severity and/or death. The findings from this study may support the need for routine measurement of response to aspirin therapy in all patients admitted with stroke in the future with a view to individually tailoring treatment (i.e. increase aspirin dose or use of an alternative antiplatelet agent), thus improving long term clinical outcome.

4. Why have I been chosen?

You have been chosen because you have a confirmed diagnosis of stroke and have been commenced on aspirin treatment by your hospital doctor as per current recommended clinical guidelines.

5. Do I have to take part?

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

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

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

After discussing the study and having read the information sheet the study doctor will address any questions you may have regarding the study. If you decide you would like to take part the study doctor will sign a consent form with you and the first blood sample will be taken for analysis. Details regarding the timing of subsequent blood tests and follow up are outlined below. Approximately 3 tablespoons (30mls) of blood will be taken on every occasion.

Time period	Intervention
Within 72hrs of hospital admission	Consent to study First blood sample taken
Day 5 of aspirin treatment	Second blood sample taken
Day 10 of aspirin treatment	Third blood sample taken
6 months after hospital admission	Telephone call and/or letter from study doctor **This marks the end of study participation**

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

Six months after your hospital admission with stroke you will be contacted via telephone and/or letter by the study doctor to enquire about any recurrent episodes of stroke, quality of life and/or loss of life. The study doctor may also contact your GP to obtain this specific information.

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

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

7. Expenses and payments

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

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

When a needle is inserted into your vein to draw blood, you may experience pain, bruising, swelling, bleeding, irritation or infection at the site of the puncture, or you may experience dizziness or faintness**.** Approximately 3 tablespoons (30mls) of blood will be collected at each appointment.

In the unlikely event of an injury caused by taking part in the study, appropriate medical treatment will be provided.

9. What are the alternatives for diagnosis or treatment?

All standard treatment and care will remain available.

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

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

11. What are the possible benefits of taking part?

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

12. Contact details:

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

Dr Nicholas Curzen
Consultant Cardiologist
Southampton General Hospital
Tel: 023 8079 4972

or

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

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

This completes Part 1 of the Information Sheet.

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

PART 2

13. What if relevant new information becomes available?

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

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

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

15. What will happen if I lose capacity during the course of the study?

If you lose capacity during the course of the study, your participation in the study will continue. You can nominate a legal representative (usually someone related to you or a doctor primarily responsible for your medical treatment) to make any decisions on your behalf with regards to continued study participation.

16. What if there is a problem?

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

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

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

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

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

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

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

18. Involvement of the General Practioner/Family Doctor

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

19. What happens to any samples I give?

The study doctor will collect the blood samples. A small quantity of blood will be analysed immediately after it has been taken (to test for aspirin resistance) and the rest will be frozen and stored securely in the hospital Pathology Department. Access to this department is restricted by an entry key-pad. Blood samples may be stored for up to one year. This allows several samples to be tested at once making efficient use of testing equipment. The blood samples will be analysed for inflammatory biomarkers and will be destroyed following analysis. They will not be used for any additional testing.

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

The data collected will be used for study evaluation only. The Research Ethics Committees or other organisations required by law may review the study data. Your data will be used in publications but your identity will not be revealed in any compilations or study reports.

21. Who is organising and funding the research?

The study has been designed by Dr Nick Curzen who is a Consultant Cardiologist within the Cardiovascular & Thoracic Unit, Southampton General Hospital. Contact details are as follows:

Dr Nick Curzen
Consultant Cardiologist & Honorary Senior Lecturer
Cardiovascular and Thoracic Unit
Southampton General Hospital
Hants

Tel: 023 8079 8538
Fax: 023 8079 5174

Neither Dr. Curzen nor the hospital is paid for your participation in this study. However, financial support for a study doctor has been provided by a company called Haemonetics Ltd.

The study is being sponsored by Southampton University Hospitals NHS Trust.

22. Who has reviewed the study?

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

Thank you for taking time to read this sheet.

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

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

Research Participant Identification Number for this trial: _____

RESEARCH PARTICIPANT CONSENT FORM

Title of Study: An observational study investigating the incidence of aspirin resistance in ischaemic stroke and its relationship to vascular inflammatory biomarkers, stroke severity and stroke recurrence

Name of Researcher: Dr Nick Curzen Please initial boxes

1. I can confirm that I have read and understood the information sheet (version 1.0 dated 8th November 2010) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I have been provided with the details of the known or foreseeable side effects and risks of the study procedures. ☐

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

4. I understand that if I lose capacity during the course of the study then I am will continue with study participation and can appoint a legal representative ☐

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

6. I agree that the data collected for the study may be used for the purpose described, and to its processing and archiving in a coded form to protect the confidentiality of my personal data. This will not waive any rights that I have under local law. ☐

7. I agree to my GP being informed of my participation in the study ☐

8. I understand that either myself or my GP or my will be contacted via telephone or post in 6 months time to see how I am doing ☐

9. I agree to take part in the above study ☐

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Name of research participant	Date and Time	Signature
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Name of person taking consent	Date and Time	Signature

When completed, 1 for participant, 1 for researcher site file, 1(original) to be kept in medical record

9.2.2 Legal representative information sheet and consent form

Cardiology Trials Office
Cardiovascular and Thoracic Unit
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD
Tel 020389798538

Legal representative information sheet and consent form

Study title: An observational study investigating the incidence of aspirin resistance in ischaemic stroke and its relationship to vascular inflammatory biomarkers, stroke severity and stroke recurrence

The patient has been invited to take part in a research study. You have been asked to act as a legal representative for the patient since he/she is unable consent due to physical incapacity.

As a legal representative, you should not be related to the conduct of the trial in any way. The legal representative can be someone who is related to the patient (known as a personal representative) or can be a professional legal representative (a doctor primarily responsible for the patient's medical treatment or a person nominated by the relevant health care provider).

Before you decide to consent on behalf of the patient, it is important for you to understand why the research is being done and what it will involve. Please read the patient information sheet [version 1 dated 8th November 2010] attached. Please take time to read this 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 for the patient to take part.

As soon as the patient's condition improves and is competent to consent for themselves, he/she will be given the same information and will be asked to sign (with the date and time) a new consent form.

If you do decide to permit the patient to take part, please sign and date the attached consent form.

Thank you for taking the time to read this sheet.

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

Research Participant Identification Number for this trial: _____

LEGAL REPRESENTATIVE CONSENT FORM

Title of Study: An observational study investigating the incidence of aspirin resistance in ischaemic stroke and its relationship to vascular inflammatory biomarkers, stroke severity and stroke recurrence

Name of Researcher: Dr Nick Curzen

Please initial boxes

1. I confirm that I have read and understand the information sheet version 1.0 dated 8th November 2010, for the above study and have had the chance to ask questions. ☐

2. I have been provided with the details of the known or foreseeable side effects and risks of the study procedures. ☐

3. I confirm that I am acting as consultee for the above named patient who is currently incapacitated, and know of no reason why the patient would not want to take part in this study. In addition, I am not aware of any advanced statements that would prevent them from taking part in the study. ☐

4. I understand that my decision to permit the patient to take part is voluntary and that I am free to stop the patient from taking part at any time, without giving any reason, without the patient's medical care or legal rights being affected. ☐

5. I understand that sections of the patient's medical notes may be looked at by responsible people from the sponsor (NHS Trust) or from regulatory authorities. Where it is relevant to the patient taking part in research, I give permission for these people to look at the patient's records. ☐

6. I agree that the data collected for the study may be used for the purpose described, and to its processing and archiving in a coded form to protect the confidentiality of personal data. This will not waive any rights that the patient has under local law. ☐

7. I agree to the patient's GP being informed of their participation in the study ☐

8. I understand that the patient, the patient's legal representative or the patient's GP will be contacted via telephone or post in six months time to find out how the patient is doing ☐

9. I agree to permit the patient to take part in the above study. I understand that I will get a copy of this signed and dated consent form. ☐

10. I understand that as soon the patient's condition improves and he/she is able to consent, they will be given the same information as below and will be asked to sign (with the date and time) a new consent form. ☐

Name of participant

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Name of legal representative	Date and Time	Signature
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Name of person receiving consent	Date and Time	Signature

When completed, 1 for legal representative, 1 for researcher site file, 1(original) to be kept in medical records.

9.2.3 Patient re-consent information sheet

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

Patient re-consent information sheet

Study title: An observational study investigating the incidence of aspirin resistance in ischaemic stroke and its relationship to vascular inflammatory biomarkers, stroke severity and stroke recurrence

You have been entered into a research study without your consent. The decision of your participation in this research study was made by your legal representative because you were unable to consent at the time. Your legal representative was not related to the conduct of the trial in any way. Your legal representative was someone who was related to you (known as a personal representative) or was a professional legal representative (a doctor primarily responsible for the patient's medical treatment or a person nominated by the relevant health care provider).

Your legal representative was provided with an information sheet very similar to this one, explaining all procedures that would take place during the trial and was given plenty of time to make a decision and to ask further questions. As your condition has improved, you can now decide for yourself whether you want to remain in the trial. Please read the patient information sheet [version 1.0 dated 8th November 2010] attached. Please take time to read this 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 continue in the trial.

Once you have read this information sheet and do decide to continue in the trial you will be asked to sign (with the date and time) the consent form.

Thank you for taking the time to read this sheet.

9.3 Randomised Plavix® study

9.3.1 Research participant information sheet and consent form

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

Research Participant Information Sheet and Consent Form

PART 1

1. Study Title

A pilot study investigating the antiplatelet effect of generic clopidogrel versus Plavix™ – is there any difference in platelet reactivity in healthy subjects?

2. Invitation Paragraph

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

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

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

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

3. What is the purpose of the study?

Clopidogrel is an essential antiplatelet agent (drug used to prevent platelets from binding together and forming blood clots) that is widely used in the treatment of heart disease and in patients undergoing coronary stent implantation. It reduces the risk of adverse events including potentially fatal stent thrombosis (blood clots forming within stents). Stent thrombosis often leads to patients suffering a heart attack. Clopidogrel exists in various different salt formulations (the specific salt formulation of a drug determines its effectiveness and the extent to which it is absorbed and distributed within the body). All previous clinical studies that have demonstrated the beneficial effects of clopidogrel are based entirely on the hydrogen sulphate salt which is contained in the branded product Plavix. A number of less expensive versions of

clopidogrel have recently been introduced to the UK pharmaceutical market and are in widespread use within the NHS. However, there is limited data to support their use in routine clinical practice and insufficient evidence to show that their biological effect is equivalent to Plavix. Despite this, there is mounting pressure to adopt the use of these alternative drugs throughout the entire spectrum of heart disease including patients undergoing coronary stenting. This may potentially expose patients to an increased risk of adverse events. The purpose of this study is to evaluate, through a series of blood tests, whether there is any significant difference in the clotting effects of Plavix (hydrogen sulphate salt) versus generic clopidogrel (hydrochloride salt) in healthy subjects.

4. Why have I been chosen?

This is a small study in a total of 15 healthy subjects.
You have been chosen because you are considered to be a healthy subject. The definition of a healthy volunteer for the purposes of this study is any individual below the age of 60yrs who is currently not taking any regular prescribed or over-the-counter medication and has no significant medical history that would preclude the use of generic clopidogrel or Plavix.

Prior to study recruitment, we will ensure you fulfil the criteria of a healthy volunteer by performing a single blood test to exclude anaemia, low platelet count, liver disease and kidney disease. In addition, we will go through a checklist of questions to ensure you do not have any underlying medical conditions.

The following individuals will be excluded from the study:

- age >60yrs
- female
- smoker
- antiplatelet or non-steroidal anti-inflammatory medication administered within the previous 14 days
- on regular prescribed or over-the-counter medication
- known clopidogrel intolerance
- surgery planned within 4 weeks
- history of any recent injury
- history of peptic ulceration
- recent bleeding
- liver disease
- kidney disease
- previous stroke,
- anaemia
- low platelet count
- any blood clotting disorder

5. Do I have to take part?

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

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

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

After discussing the study and having read the information sheet the study doctor will address any questions you may have regarding the study. You will be given sufficient time to decide whether you would like to take part. If you decide to participate, the study doctor will sign a consent form with you. After receiving consent, we will confirm whether you fit the criteria of a healthy volunteer prior to study inclusion by (i) completing a checklist of questions to ensure you do not have any underlying medical conditions (ii) a medical examination will be undertaken, and (iii) a single blood sample will be taken to exclude anaemia, low platelet count, liver or kidney disease. In addition, we will measure your baseline platelet reactivity on this initial blood sample in order to reduce the total number of blood tests you undergo if you do take part in the study.

If you are eligible to take part, an appointment will be made for you to attend Southampton General Hospital Cardiac Unit, where the following interventions will take place:

- a loading dose of oral Plavix will be administered
- 3 subsequent blood samples will be taken at 1, 2 and 6 hours after the loading dose

For the first visit, your availability for a full day would be required.

The details regarding the timing of subsequent blood tests and medication administered are outlined below. Approximately 1 tablespoon (7 mls) of blood will be taken on every occasion.

You will be followed up in the study for approximately 4 weeks. On two occasions during the course of the study, you will be contacted via telephone by the study doctor to ensure that you have not experienced any side effects. At each visit, in addition to having blood drawn:

- You will be asked how you feel, and any side effects will be discussed in detail
- You will be asked about any other medication that you take while you are in the study

Time period	Procedure
First hospital visit	• Written informed consent • Medical examination • Screening blood test and baseline platelet reactivity measured • Checklist of health questions
Study Day 1 Hospital visit	• 300mg Plavix administered orally • Blood samples taken 1, 2 and 6 hours after Plavix
Study Day 2 to Day 7	• 75mg Plavix taken orally daily at home (clear written instructions will be provided) • Subject contacted once via telephone between days 2 and 4
Study Day 8 Hospital visit	• Final dose of 75mg Plavix taken orally • Blood sample taken
Study Day 9 to Day 23	• Wash-out period • No drug taken • No hospital visits planned
Study Day 24 Hospital visit	• 300mg clopidogrel hydrochloride administered orally • Blood samples taken 1, 2 and 6 hours after clopidogrel hydrochloride
Study Day 25 to 32	• 75mg clopidogrel hydrochloride taken orally daily at home (clear written instructions will be provided) • Subject contacted once via telephone between days 2 and 4
Study Day 33 Hospital visit **Your participation in the study is complete after this visit**	• Final dose of 75mg clopidogrel hydrochloride taken orally • Final blood sample

During the time that you are in the study the research staff will need to know about any medication that you are taking. Please speak with your study doctor before taking any non-prescription drugs or drugs prescribed by another doctor. In particular, you will be advised not to take any aspirin or non-steroidal anti-inflammatory drugs such as Ibuprofen during the course of the study.

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

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

7. Expenses and payments

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

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

When a needle is inserted into your vein to draw blood, you may experience pain, bruising, swelling, bleeding, irritation or infection at the site of the puncture, or you may experience dizziness or faintness**.** Approximately 1 tablespoon (~7mls) of blood will be collected at each study visit.

The potential common side effects associated with clopidogrel include dyspepsia (indigestion), abdominal pain, nose-bleeding, gastro-intestinal bleeding (bleeding into the stomach) and bruising. A package leaflet will be provided when you are given your study drugs. This is the usual information leaflet that is provided to everyone who takes this medication.

The following information is contained in the package leaflet that you will be provided with:

POSSIBLE SIDE EFFECTS:
Like all medicines, Plavix/clopidogrel can cause side effects, although not everybody gets them.

Contact your doctor immediately if you experience:
Fever, signs of infection or extreme tiredness. These may be due to rare decrease of some blood cells

Signs of liver problems such as yellowing of the skin and/or the eyes (jaundice), whether or not associated with bleeding which appears under the skin as red pinpoint dots and/or confusion
Swelling of the mouth or skin disorders such as rashes and itching, blisters of the skin. These may be the signs of an allergic reaction

The most common side effect (affects 1 to 10 patients in 100) is bleeding:
Bleeding may occur as bleeding in the stomach or bowels, bruising, haematoma (unusual bleeding or bruising under the skin), nose bleed, blood in the urine. In a small number of cases, bleeding in the eye, inside the head, the lung or the joints has also been reported.

If you experience prolonged bleeding when taking Plavix/clopidogrel:
If you cut or injure yourself, it may take longer than usual for bleeding to stop. This is linked to the way your medicine works as it prevents the ability of blood clots to form. For monor cuts and injuries e.g cutting yourself, shaving, this is usually of no concern. However, if you are concerned by your bleeding you should contact your doctor straightaway.

Other side effects reported:
Common side effects (affects 1 to 10 patients in 100): diarrhoea, abdominal pain, indigestion or heartburn
Uncommon side effects (affects 1 to 10 patients in 1000): headache, stomach ulcer, vomiting, nausea, constipation, excessive gas in stomach or intestines, rashes, itching, dizziness, sensation of tigling or numbness.
Rare side effects (affects 1 to 10 patients in 10000): Vertigo
Very rare side effects (affects less than 1 patient in 10000): Jaundice, severe abdominal pain with or without back pain, fever, breathing difficulties sometimes associated with cough, generalised allergic reactions, swelling in the mouth, blisters of the skin, skin allergy, inflammation of the mouth (stomatitis), decrease in blood pressure, confusion, hallucinations, joint pain, muscular pain, changes in the way things taste. In addition, your doctor may identify changes in your blood or urine tests results.

If any of the side effects gets serious, or if you notice any side effects not listed in this leaflet, please tell your doctor or pharmacist"

In the event of any adverse drug reactions or injury caused by taking part in the study, appropriate medical treatment will be provided.

A 24hr point-of-contact will be available to all participants - this will be a study doctor between the hours of 9am to 5pm (monday to friday) on telephone no. 023 8079 8538

At all other times the on-call cardiology registrar can be contacted using the bleep service via the hospital switchboard : 023 8079 777222.

9. What are the possible benefits of taking part?

You will have the benefit of a full medical examination including a blood test which may potentially lead to early diagnosis of as yet undiagnosed conditions. The results of the study may be of benefit patients in the future and may lead to further larger clinical studies in this field.

10. Contact details:

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

Dr Nicholas Curzen
Consultant Cardiologist
Southampton General Hospital
Tel: 023 8079 4972

or

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

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

This completes Part 1 of the Information Sheet.

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

PART 2

11. What if relevant new information becomes available?

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

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

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

13. What if there is a problem?

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

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

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

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

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

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

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

15. Involvement of the General Practioner/Family Doctor

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

16. What happens to any samples I give?

Your study doctor will collect the blood samples. The blood samples will be analysed on-site immediately after they have been taken and will be destroyed following analysis. They will not be stored or used for any additional testing. Analysing each sample will take approximately 20 minutes.

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

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

18. Who is organising and funding the research?

The study has been designed by Dr Nick Curzen who is a Consultant Cardiologist within the Cardiovascular & Thoracic Unit, Southampton General Hospital. Contact details are as follows:

Dr Nick Curzen
Consultant Cardiologist & Honorary Senior Lecturer
Cardiovascular and Thoracic Unit
Southampton General Hospital
Hants

Tel: 023 8079 8538
Fax: 023 8079 5174

Neither Dr. Curzen nor the hospital is paid for your participation in this study. However, financial support for a study doctor has been provided by a company called Haemonetics Ltd.

The study is being sponsored by Southampton University Hospitals NHS Trust.

19. Who has reviewed the study?

The Medicines and Healthcare products and Regulations Agency has approved this study. They are a national organisation who specifically assess the safety and validity of research studies. An Independent Ethics Committee, Southampton & S W Hants Local Research Ethics Committee, has reviewed the objectives and the proposed conduct of the study and has given a favourable opinion of it. It has also been reviewed and approved by the hospital's Research & Development Department and the Medicines and Healthcare products Regulatory Agency (MHRA) for the UK.

Thank you for taking time to read this sheet.

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

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

Research Participant Identification Number for this trial: _____

RESEARCH PARTICIPANT CONSENT FORM

Title of Study: The effect of Plavix versus generic clopidogrel on platelet reactivity

Name of Researcher: Dr Nick Curzen Please initial boxes

1. I can confirm that I have read and understood the information sheet (version 2 dated 19th November 2010) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I have been provided with the details of the known or foreseeable side effects and risks of the study procedures and I understand that blood samples will be taken for the study ☐

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

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

5. I agree that the data collected for the study may be used for the purpose described, and to its processing and archiving in a coded form to protect the confidentiality of my personal data. This will not waive any rights that I have under local law. ☐

6. I agree to my GP being informed of my participation in the study ☐

7. I agree to take part in the above study ☐

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Name of research participant	Date and Time	Signature
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Name of person taking consent	Date and Time	Signature

When completed, 1 for participant, 1 for researcher site file, 1(original) to be kept in medical records.

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