
**ASSESSMENT OF THROMBOTIC AND THROMBOLYTIC STATUS IN
PATIENTS WITH CORONARY ARTERY DISEASE AND ITS RELATION
TO CLINICAL OUTCOMES**

Dr Smriti Saraf

**A Thesis submitted to the University of Hertfordshire for partial fulfilment of the
requirements of the degree of Doctorate of Medicine, University of Hertfordshire**

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September 2013

Acknowledgements

I am grateful to my Principal supervisor, Dr. Markos Klonizakis, Honorary Clinical Lecturer at University of Hertfordshire, for his support, supervision and guidance which has helped me in the completion of this project and write up of this thesis.

I would like to take this opportunity to greatly thank my Clinical supervisors, Prof. Diana Gorog, Visiting Professor at University of Hertfordshire and Consultant Interventional Cardiologist at East and North Hertfordshire NHS Trust, and Prof. Kenneth Farrington, Professor at University of Hertfordshire, and Consultant Nephrologist at East and North Hertfordshire NHS Trust, without whose support it would not have been possible to execute this study. They have been excellent mentors, and have given sound advice and support at every stage of this study.

I am extremely grateful to Dr. Dave Stott, Dr. David Wellsted and Sam Norton, Statisticians at University of Hertfordshire, who have helped me perform statistical analysis on the data, without which it would not have been possible to analyse and publish our results.

I would like to thank all the staff in the Cardiology Department at East and North Hertfordshire NHS trust for their valuable support offered to me during the duration of this study.

I would also like to thank Prof. Junichiro Yamamoto and his team, from Kobe Gakuin University, Kobe, Japan for collaborating with us and helping us compare results in different ethnic groups.

Last but not least, special thanks go to my family for their constant support and encouragement during the duration of the study.

<u>TABLE OF CONTENTS</u>	PAGE NO
<i>Abstract</i>	9
<i>Objectives, Hypothesis and Outline of this thesis</i>	12
<i>Abbreviations</i>	14
<i>Chapter 1: Introduction</i>	16
<i>Background</i>	16
<i>Pathophysiology of thrombus formation</i>	19
<i>Determinants of thrombus formation (Occlusion time) - Role of calcium and agonists in platelet aggregation</i>	23
<i>Antiplatelet agents: a review of the evidence</i>	27
<i>Antiplatelet Resistance: Review of the evidence and extent of the problem</i>	43
<i>Prevalence of Antiplatelet resistance in different populations</i>	57
<i>Determinants of endogenous lysis (Lysis time), mechanism of fibrinolysis and plasma fibrinolytic markers</i>	66
<i>Endogenous Thrombolysis- A neglected entity, with prognostic usefulness in Acute Coronary Syndrome</i>	74
<i>Tests to assess endogenous plasma fibrinolysis</i>	78
<i>Platelet function tests</i>	43
<i>Limitations of current platelet function tests, Rationale behind assessment of platelet reactivity and need for newer testing modalities</i>	80
<i>Chapter 2: Methodology</i>	83
<i>Chapter 3: Platelet function in Healthy Volunteers and comparison with Japanese volunteers</i>	88
<i>Chapter 4: Role of ADP, Aspirin and Clopidogrel on thrombus formation</i>	103
<i>Chapter 5: Platelet function in Acute Coronary Syndrome, stable angina and Healthy volunteers</i>	144
<i>Chapter 6: Discussion, Future Research and Study Limitations</i>	198
<i>General Discussion</i>	198
<i>Future Research: Fibrinolytic markers and therapeutic modulation of fibrinolysis</i>	202
<i>Study Limitations</i>	203
<i>Appendices</i>	207
<i>Ethical approval documents</i>	207
<i>Good clinical practice certificate</i>	213
<i>Case report form</i>	214
<i>Publications from this thesis</i>	215
<i>References</i>	217

<u>LIST OF FIGURES</u>	PAGE NO
<i>Figure 1 – Platelet receptors</i>	22
<i>Figure 2.1: GTT tube demonstrating 2 metal balls and space in between the balls and wall of the plastic tube detects the time interval [d] between consecutive blood drops</i>	53
<i>Figure 2.2: Principle of the GTT – Shear stress activates platelets and platelet aggregation begins in the space between the 2 metal ball bearings</i>	54
<i>Figure 3.1: Distribution of OT in healthy Western volunteers</i>	91
<i>Figure 3.2: Distribution of LT in healthy Western volunteers</i>	93
<i>Figure 4.1: Distribution of OT in Western (W) and Japanese (J) volunteers</i>	95
<i>Figure 4.2: Box and Whiskers plot demonstrating minimum, maximum and median difference in OT between Japanese and Western volunteers.</i>	96
<i>Figure 4.3: Distribution of LT in W and J volunteers</i>	97
<i>Figure 4.4: Box and Whiskers plot demonstrating minimum, maximum and median difference in LT.</i>	96
<i>Figure 5.1: Accelerated GTT</i>	110
<i>Figure 5.2: OT before and after water priming (aGTT)</i>	111
<i>Figure 5.3: Box plot demonstrating minimum, maximum and median OT pre and post distilled water.</i>	112
<i>Figure 5.4: LT before and after water priming (aGTT)</i>	113
<i>Figure 5.5: OT before and after saline priming</i>	115
<i>Figure 5.6: LT before and after saline priming</i>	116
<i>Figure 5.7: OT before and after ADP priming</i>	117
<i>Figure 5.8: OT percentage inhibition before and after ADP priming</i>	118
<i>Figure 5.9: LT before and after ADP priming</i>	119

<i>Figure 6.1: Ladder plot demonstrating OT pre and post aspirin</i>	121
<i>Figure 6.2: Box plot demonstrating minimum, maximum and median OT pre and post aspirin</i>	122
<i>Figure 6.3: Box plot: Paired analysis demonstrating difference in OT pre and post aspirin</i>	123
<i>Figure 6.4: Box plot demonstrating minimum, maximum and median LT pre and post Aspirin</i>	124
<i>Figure 6.5: Box plot: Paired analysis demonstrating difference in LT pre and post aspirin</i>	125
<i>Figure 7.1: OT pre and post clopidogrel</i>	128
<i>Figure 7.2: Box plot demonstrating minimum, maximum, and median OT pre and post clopidogrel</i>	129
<i>Figure 7.3: LT pre and post clopidogrel.</i>	130
<i>Figure 7.4: Comparison of normal OT (pre-clopidogrel) and the accelerated OT after water-priming following clopidogrel</i>	131
<i>Figure 7.5: Box plot demonstrating minimum, maximum and median OT of normal OT (pre clopidogrel) and accelerated OT after water priming following clopidogrel</i>	132
<i>Figure 7.6: Effect of clopidogrel on aGTT (accelerated GTT with water-priming). $OT.W=OT$ after waterpriming and $OT.W.C=OT$ after water-priming, and after clopidogrel ingestion. Clopidogrel prevented the acceleration OT produced by water-priming.</i>	133
<i>Figure 7.7: Box plot demonstrating minimum, maximum and median OT in a GTT pre and post clopidogrel</i>	134
<i>Figure 7.8: GTT and aGTT results before after clopidogrel loading</i>	135
<i>Figure 7.9: OT pre and post clopidogrel in stable angina patients</i>	136
<i>Figure 8.1: OT in healthy volunteers demonstrating normal distribution</i>	156
<i>Figure 8.2: OT in ACS patients</i>	156
<i>Figure 8.3: LT in healthy volunteers</i>	157
<i>Figure 8.4: LT in ACS patients</i>	157

<i>Figure 8.5: ROC Curves for OT and LT</i>	158
<i>Figure 8.6: KM curve demonstrating probability of event free survival using OT as a predictor of adverse events at 180 days</i>	159
<i>Figure 8.7: KM curve demonstrating probability of event free survival using OT as a predictor of adverse events at 1 year</i>	160
<i>Figure 8.8: KM curve demonstrating probability of event free survival at 180 days using LT ≥ 3000s as cut-off</i>	161
<i>Figure 8.9: KM curve demonstrating probability of event free survival at 1 year using LT ≥ 3000s as cut-off</i>	162
<i>Figure 9.0: KM curve demonstrating probability of event free survival at 180 days using LT ≥ 5000s as cut-off</i>	163
<i>Figure 9.1: KM curve demonstrating probability of event free survival at 1 year using LT ≥ 5000s as cut-off</i>	164
<i>Figure 9.2: MACE at 6 months using LT ≥ 3000s as cut off</i>	165
<i>Figure 9.3: MACE at 1 year using LT ≥ 3000s as cut off</i>	166
<i>Figure 9.4: MACE at 6 months using LT ≥ 5000s as cut off</i>	167
<i>Figure 9.5: MACE at 1 year using LT ≥ 5000s as cut off</i>	168
<i>Figure 9.6: HR at 6 months for CV death, Non-fatal MI, and Stroke by LT</i>	176
<i>Figure 9.7: HR at 1 year for CV death, Non-fatal MI, and Stroke by LT</i>	177
<i>Figure 10.1: OT in Stable Angina patients</i>	184
<i>Figure 10.2: Box plot comparing minimum, maximum and median OT in Healthy Volunteers, Stable Angina and ACS patients</i>	185
<i>Figure 10.3: LT in Stable Angina patients</i>	186
<i>Figure 10.4: Box plot comparing minimum, maximum, and median LT in Healthy Volunteers, Stable Angina and ACS patients</i>	187
<i>Figure 11.1: Scatter plot demonstrating no correlation between GTT OT and PRU</i>	189

Figure 11.2: Kaplan –Meier Curve demonstrating probability of event free survival using Verify Now Assay at 6 months 190

Figure 11.3: Kaplan –Meier Curve demonstrating probability of event free survival using Verify Now assay at 1 year 191

LIST OF TABLES

PAGE NO

Table 1: Randomized controlled trials demonstrating reduction of MACE with Clopidogrel 31

Table 2.1: Studies demonstrating prevalence of Aspirin non-responsiveness or resistance 61

Table 2.2: Studies demonstrating prevalence of Clopidogrel non-responsiveness or resistance 62

Table 2.3: Studies demonstrating clinical implications of Aspirin non-responsiveness or resistance 63

Table 2.4: Studies demonstrating clinical implications of Clopidogrel non-responsiveness or resistance 63

Table 3.1: OT and LT results (in seconds) as measured in the healthy female volunteer 91

Table 3.2. Subgroup analysis to eliminate the effects of age and sex. 97

Table 4: GTT results before and after priming the GTT tube with saline 114

Table 5.1: GTT results in Group 1 before and after priming the GTT tube with water, and post administration of clopidogrel 137

Table 5.2: GTT results in Group 2 before and after priming the GTT tube with water, and post administration of clopidogrel 138

Table 5.3: Baseline demographics of stable angina patients 138

Table 6.1: Exclusion criteria 147

Table 6.2: Baseline demographics of ACS patients 154

Table 6.3 - Angiographic, Interventional and Echocardiographic characteristics of ACS patients 155

<i>Table 6.4: Multivariate analysis using Cox regression to demonstrate HR for LT $\geq 3000s$ at 6 months</i>	<i>169</i>
<i>Table 6.5: Multivariate analysis using Cox regression to demonstrate HR for LT $\geq 3000s$ at 1 year</i>	<i>169</i>
<i>Table 6.6: Multivariate analysis using Cox regression to demonstrate HR for LT $\geq 5000s$ at 6 months</i>	<i>170</i>
<i>Table 6.7: Multivariate analysis using Cox regression to demonstrate HR for LT $\geq 5000s$ at 1 year</i>	<i>170</i>
<i>Table 6.8: Total number of MACE in ACS patients, at 6 months and at 1 year</i>	<i>171</i>
<i>Table 6.9: Hazard ratio at 6 months and 1 year according to OT (seconds)</i>	<i>173</i>
<i>Table 6.10: Hazard ratio at 6 months and 1 year according to LT (seconds)</i>	<i>175</i>
<i>Table 6.11: Variable and Hazard ratio at 6 months and 1 year. The 95% confidence interval is shown in brackets</i>	<i>178</i>

Abstract

Background: Platelets provide the initial haemostatic plug at sites of vascular injury. They also participate in pathological thrombosis that leads to myocardial infarction, stroke and peripheral vascular disease.

The outcome of an acute myocardial infarction depends not only on the formation and stability of an occlusive thrombus, but also on the efficacy of the endogenous thrombolytic process, which allows reperfusion of the infarct related artery and prevents recurrent ischaemic episodes. Various platelet function tests are available to measure the thrombogenic potential of an individual, but the sensitivity of these tests remain questionable as most of these tests use citrated blood and measure response to a particular agonist. Endogenous thrombolysis has been a neglected entity, and its beneficial effects on cardiovascular outcomes has not been studied in depth in the past, possibly as until recently there has been no available technique to measure spontaneous thrombolytic activity in native blood. The Global Thrombosis Test (GTT) is a new point of care tests that allows us to measure time to thrombus formation (Occlusion time: OT) using native blood, avoiding the use of agonists and making the test results more physiological. The GTT also measures the time to lyse this formed thrombi without use of any lytic agents (Lysis time: LT), allowing us to measure the patient's endogenous thrombolytic potential.

Aim: Our aim in this study was to detect patients who are at risk of future thrombotic events despite dual antiplatelet therapy, either due to prothrombotic tendency or due to impaired endogenous thrombolysis, and to determine if these two parameters were correlated.

Methods: GTT was used to assess the thrombotic and thrombolytic activity in healthy volunteers, and in different patient populations. 100 healthy volunteers were tested using the GTT, and a normal range was established. 300 patients admitted to hospital with a diagnosis

of acute coronary syndrome (ACS) were included in the study, and tested using the GTT after they had been stabilized on dual antiplatelet therapy (Aspirin and Clopidogrel). All these patients were followed up for a year, to determine if their baseline GTT results were a predictor of recurrent cardiac events. The primary endpoint of the study was major adverse cardiovascular events (MACE), which was a composite of cardiovascular death, nonfatal myocardial infarction, or stroke at 12 months.

Results: All results were analysed using statistical package SPSS version 16.0 (SPSS Inc., Chicago, Illinois).

The 100 healthy volunteers were all non-smokers, and were not taking any medications. There were 55 males and 45 females, and mean age was 38 ± 11 years (range 22-76, IQR 11). OT was normally distributed with mean OT 377.80s, and using mean \pm 2SD, we derived a normal range of 185-569s (200-550s). LT demonstrated a skewed distribution with values ranging between 457 – 2934s. Using log transformation, a normal range of 592 – 1923 (600-2000s) was established for LT.

OT and LT were both prolonged in ACS patients compared to normal volunteers ($p < 0.001$). No association was observed between OT and risk of major adverse cardiovascular events. LT was noted to be a significant and independent predictor of MACE in a multivariate model adjusted for cardiovascular risk factors. $LT \geq 3000$ s was the optimal cutoff value for predicting 6 month MACE [hazard ratio (HR): 2.48, 95% CI: 1.2-4.8, $P = 0.008$] and cardiovascular death [HR: 4.04, 95% CI : 1.3-12.0, $P = 0.012$] and 12 month MACE [HR:1.9, 95% CI: 1.04- 3.5, $P = 0.03$] and cardiovascular death [HR: 3.9,95% CI: 1.34-11.9, $P = 0.013$]. $LT \geq 3000$ s was observed in 23% of ACS patients.

Conclusions: Our study suggests that endogenous thrombolytic activity based on lysis of platelet rich thrombi can be assessed by the point of care GTT assay, which can help in

identification of ACS patients at high risk of future cardiac events. Prolongation of OT may be explained by the antiplatelet effects of Aspirin and Clopidogrel, as both these drugs prolong time to thrombus formation and hence increase OT. Further large studies are required to study factors which can reduce thrombogenic potential, and improve endogenous thrombolytic activity, which can be monitored using the GTT to improve cardiovascular outcomes.

Objectives, Hypothesis and Outline of this thesis

Objective

The main objective of this thesis was to identify patients who are at recurrent risk of thrombotic events despite being on dual antiplatelet therapy, using a new point of care system – the GTT. It was our aim to determine if OT or LT was a significant predictor of adverse cardiac events, and if there was a correlation between these two variables.

Hypothesis

Among patients receiving dual antiplatelet therapy with standard doses of aspirin and clopidogrel following an acute coronary syndrome, those with enhanced platelet reactivity or impaired endogenous thrombolysis are at increased risk of future major adverse cardiac events.

Outline

In Chapter 1, I will discuss the mechanism and determinants of thrombus formation and endogenous thrombolysis, the different antiplatelet agents available, the mechanism of antiplatelet resistance and role of different platelet function and plasma fibrinolytic tests in identification of patients at risk of future cardiac events.

Chapter 2 explains the methodology, and in Chapter 3, I will discuss the development of normal range for OT and LT in healthy volunteers in the Western and Japanese populations.

Chapter 4 explores the role of ADP in thrombus formation, and its effect on OT and LT in healthy individuals. It also studies the effect of Aspirin on prolongation of OT, with a moderate effect on LT in patients with stable angina. A significant effect is observed with clopidogrel on prolongation of OT, with no significant effect on LT in healthy individuals, and in patients with stable angina. In Chapter 5, the relationship between OT and MACE,

LT and MACE and effect of other variables on LT and MACE in the ACS group of patients is demonstrated. It also studies the effect of dual antiplatelet therapy on OT and LT in stable angina patients, and comparison is made with healthy volunteers and ACS patients. Comparison is then made with the results of two platelet function tests, the GTT and the Verify now assay. Chapter 6 discusses the limitations of our methodology, with future research ideas. This is followed by Appendices, which includes the Ethical approval documents, Case Report Forms, publications from this study, and references.

Abbreviations

AA	Arachidonic acid
ACS	Acute coronary syndrome
ADP	Adenine diphosphate
AHA	American heart association
AMI	Acute myocardial infarction
ATP	Adenosine triphosphate
CAD	Coronary artery disease
CI	Confidence interval
CV	Cardiovascular
CVA	Cerebrovascular accident
DM	Diabetes mellitus
Fig	Figure
GP	Glycoprotein
GTT	Global Thrombosis Test
GPHR	Global high platelet reactivity
HR	Hazard ratio
ISR	Instant restenosis
IHD	Ischaemic heart disease
J	Japanese
LMWH	Low molecular weight heparin
LT	Lysis time
LTA	Light transmittance aggregometry
MACE	Major adverse cardiac events
mg	Milligram
µg	Microgram
NSTEMI	Non ST elevation myocardial infarction

NS	Non significant
OT	Occlusion time
PAI	Plasminogen activator inhibitor
PCI	Percutaneous coronary intervention
PFA	Platelet Function Analyser
PRU	Platelet reactive unit
PVD	Peripheral vascular disease
RRR	Relative risk reduction
SAT	Subacute stent thrombosis
SCAI	Society for cardiovascular angiography and Interventions
Sec	Seconds
STEMI	ST elevation myocardial infarction
TxA2	Thromboxane A2
TxB2	Thromboxane B2
t-PA	Tissue type plasminogen activator
TIA	Transient ischaemic attack
TAFI	Tissue-type plasminogen activator
TRAP	Thrombin receptor activating peptide
UA	Unstable angina
uPA	Urokinase type plasminogen activator
VASP	Vasodilator-mediated phosphoprotein
vWF	Von Willebrand factor
W	Western

Chapter 1: Introduction

Background

Aspirin and clopidogrel are the antiplatelet agents most commonly used in patients with coronary artery disease. However, 5-60% of patients taking aspirin and 4-30% of those taking clopidogrel continue to experience thrombotic events despite treatment with these agents (Nguyen et al. 2005). Evidence from some studies suggests that reduced responsiveness or “resistance” to antiplatelet agents is associated with subsequent major adverse cardiac events (MACE). The definition of antiplatelet drug resistance largely depends on the method used to measure platelet function. As a result, no standard definition has been universally accepted. However, it remains unknown whether altering therapy based on platelet function tests is beneficial to patients. There are currently no guidelines for the treatment of antiplatelet drug resistance. Although point-of-care platelet-function testing makes screening for resistance feasible, routine screening has not been recommended in clinical practice due to lack of standardized measures of platelet function.

Platelet function tests are used to detect patients with abnormal platelet function, which may be inborn or acquired, and is used to detect platelet activation in patients at risk of thrombotic events. They are also used to monitor the effect of antiplatelet agents such as aspirin, clopidogrel or membrane glycoprotein IIb/IIIa inhibitors. Incorrect blood sampling is a major source of error in measuring platelet function. Most tests lack sensitivity and have low positive predictive value for clinical events and are difficult to perform in the clinical setting. There has been no suitable bedside test of thrombotic function, all prior tests showing clopidogrel resistance have been done in a haematology laboratory, away from the patient, and most of these tests either used platelet rich plasma or citrate-anticoagulated blood, or supra high doses of agonists to induce platelet aggregation and thus were not “physiological”

in terms of truly representing the in vivo situation (Zucker et al. 1978). Recent data from the Reclose 2-ACS study demonstrated high platelet reactivity to both clopidogrel and aspirin in 9% of ACS patients. 1,789 patients with ACS were enrolled in the study, and tested using both arachidonic acid and ADP as agonists. This phenotype was known as global high platelet reactivity (GHPR), and GHPR was significantly associated with cardiovascular ischemic events and cardiac death in a Cox regression analysis (MACE: HR=1.5[1.0-2.2], p=0.02; cardiac death: HR= 1.9[1.2-3.2], p=0.008). These results suggest that global high platelet reactivity is a more effective parameter for identifying ACS patients at high risk of ischaemic cardiac events (Marcucci et al. 2012).

There are various global assays of fibrinolysis, but none of the tests are really used in the clinical setting (Stief et al. 2007). They are time consuming, labour intensive and most importantly there is uncertainty with regards to which biomarker should be measured, as limited data is available on fibrinolytic marker levels and cardiac outcomes. Furthermore, most of the tests available measure clot lysis as opposed to thrombolysis.

Thrombotic events not only depend on the propensity to thrombus formation, but also on the efficacy of endogenous thrombolytic activity. Endogenous thrombolysis is a protective mechanism against lasting arterial occlusion, and acute myocardial infarction (AMI) has been considered a result of failure of timely spontaneous thrombolysis. A large number of patients with occluded arteries had coronary angiography weeks to months after their initial AMI, and patency of the culprit artery was demonstrated, suggesting endogenous thrombolysis had a key role to play in the dissolution of the thrombus (Swan et al. 2003). This entity has been neglected so far, more so because until recently, there has been no physiological test available to assess endogenous thrombolytic activity.

The Global Thrombosis Test is a point of care test that allows the measurement of dynamic coagulation and spontaneous thrombolysis, which is lysis of an autologous platelet-rich thrombus in the absence of added plasminogen activators. The test is performed on non-anticoagulated native blood without added external agonists. In this technique, an occlusive thrombus is formed using high shear stress, analogous to that in a stenosed coronary artery. The first phase of the test (Occlusion time: OT) is used as a marker of platelet function, the more reactive the platelets the faster the occlusion will occur. The restart of blood flow following occlusion is due to spontaneous thrombolysis (Lysis time: LT). This is a near patient test, which provides a result on the patient's thrombotic and thrombolytic status, and is applicable to acute clinical situations, as well as more general screening (Yamamoto et al. 2003).

Pathophysiology of thrombus formation

Platelets are small discoid cells present in blood, produced by fragmentation of megakaryocytes and play a significant role in haemostasis. They are 1-2µm in diameter, and have a life span of 5-10 days. Normal platelet count is 150,000-350,000 per microlitre of blood. The vascular endothelium is responsible for maintaining the integrity of the vessel wall. Intact endothelium releases nitric oxide, prostacyclin, ectonucleotidase CD39, thrombomodulin and tissue factor pathway inhibitor, all of which inhibit thrombus formation. CD39 is an ectonucleoside triphosphate diphosphohydrolase enzyme and an integral component of the endothelium. It degrades ADP by neutralizing prothrombotic releasase and thus prevents thrombus formation. It also reduces norepinephrine release in the heart and prevents serious cardiac arrhythmias (Marcus et al. 2005).

Disruption of the endothelium by flowing blood results in exposure of prothrombotic substances such as collagen, vWF, fibrin, fibronectin and oxidized LDL which activate platelet monocyte complex formation and activates platelets resulting in platelet aggregation. Neutrophils represent 40-60% of the leucocytes and monocytes represent 5% of the leucocyte concentration. Both neutrophils and monocytes have a very short half-life of upto 20 hours, but their life span increases approximately three times in inflammation. Leucocyte activation results in release of mediators of inflammation such as elastase, cathepsin G, lactoferrin and cytokines. The different types of cytokines released are interleukins, TNF α , G-CSF and GM-CSF. These mediators of inflammation cause leucocyte adhesion to the endothelium, initiate release of oxidant radicals O₂ and H₂O₂ which result in tissue damage which in turn cause platelet activation and aggregation. Tissue factor is also present on the surface of monocytes and macrophages, which together with Factor VIIa aids in the coagulation cascade. Tissue factor forms a complex with Factor VIIa and converts inactive Factor X to active Factor Xa.

Activated Factor X then combines with Factor V in the presence of phospholipid and calcium, generates thrombin and results in thrombus formation (Gorbet et al. 2004).

Collagen is a protein of the vessel wall that maintains tissue integrity, and allows matrix constituents to adhere to the vessel. The platelet-reactive collagens I–IV support platelet adhesion up to relatively high shear rates and also induce platelet aggregate formation at shear rates found in the small vessels upto a shear of 2000s. (Farndale et al.2004). Platelet accumulation on collagen at shear rates of 300s to 1250s is enhanced by coprefusion with plasma fibronectin (Nievalstein et al. 1988). Fibronectin is a glycoprotein dimer and component of the subendothelium, and helps in stabilization of the platelet aggregates after vascular injury.

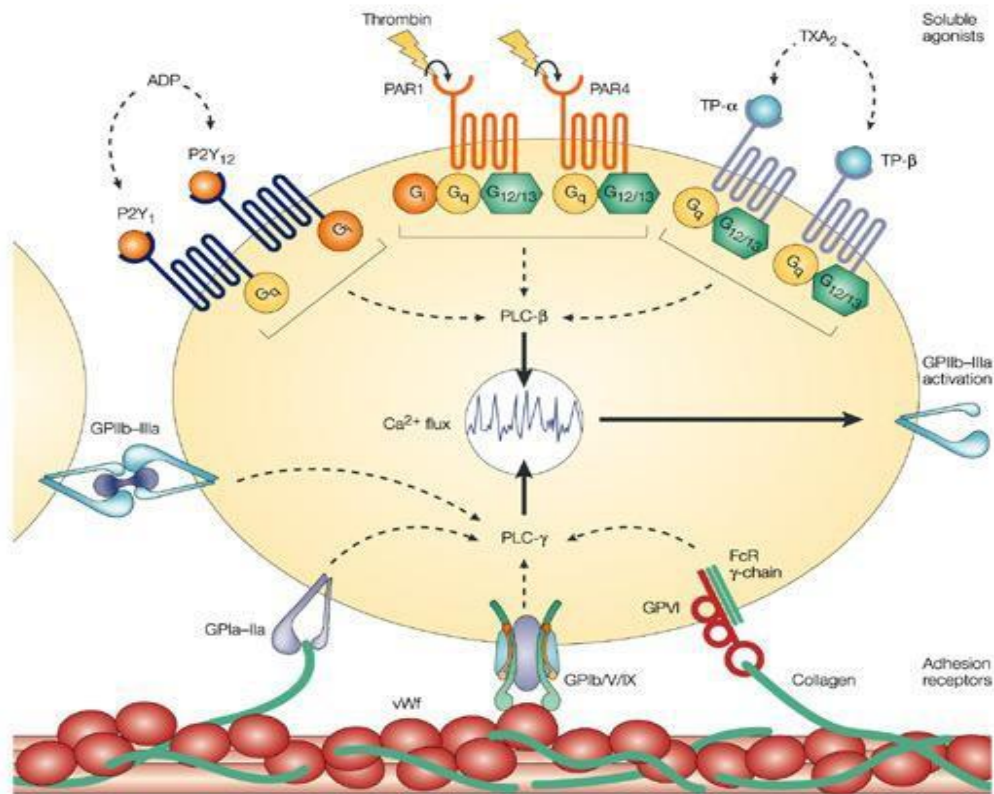
vWF is a multimeric glycoprotein synthesized in the endothelial cells and megakaryocytes. It is present in the subendothelial matrix, blood plasma and platelets. It binds and transports Factor VIII and collagen and promotes platelet adhesion, aggregation and thrombus formation (Ruggeri et al. 2007). Oxidized LDL is cytotoxic to endothelial cells and enhances thrombus formation by free radical formation and impairment of nitric oxide synthase activity (Mehta et al. 2001). It also activates CD40 and increases activity of metalloproteinases. Alternative pathway involves tissue factor release which forms a complex with Factor VIIa and activates Factor IX and generates thrombin. Protease activated receptor PAR4 is the main thrombin receptor in platelets and thrombin is responsible for cleaving this receptor on the platelet surface resulting in thromboxane A₂, serotonin and ADP release which help in the formation of thrombus. ADP is stored in the dense granules of platelets, and activates platelets by binding to P₂Y₁ and P₂Y₁₂ receptors (Fig 1). Thus thrombus formation occurs in 2 stages – initial stage involves glycoprotein VI and glycoprotein Ia/IIa mediated platelet activation following collagen

exposure, followed by stabilization of the thrombus by formation of thrombin and fibrin triggered by tissue factor release (Colman et al. 2006).

Disruption of an atherosclerotic plaque results in platelet aggregation and eventually formation of an intra-coronary thrombus. This thrombus obstructs the lumen of the vessel, resulting in either partial or complete occlusion of blood flow. This imbalance in myocardial demand and supply results in coronary ischemia, and presents clinically as an acute coronary syndrome. The atherosclerotic plaque is composed of a central lipid core, surrounded by a fibrous cap. The plaque is composed of various inflammatory cells which result in disruption of the fibrous cap. Various enzymes such as metalloproteinase are also produced by the plaque, and result in further plaque disruption. Thin capped fibrous atheroma with fibrous cap thickness < 65 μ m are more prone to disruption and result in acute coronary syndromes.

Endothelial dysfunction is an important determinant of thrombus formation. Intact vascular endothelium release nitric oxide and prostacyclin which relax blood vessels and inhibit platelet activation. In endothelial dysfunction, the release of these substances is reduced resulting in platelet activation and aggregation, and increased amounts of thrombus formation.

Figure 1 – Platelet receptors



Nature Reviews | Drug Discovery

Nature Reviews Drug Discovery 2, 775-789

Determinants of thrombus formation (Occlusion time) - Role of calcium and agonists in platelet aggregation

Intracellular calcium plays an important role in the coagulation process. It activates phospholipase A₂ which results in release of arachidonic acid, thromboxane A₂ and subsequently enhances platelet aggregation. It plays a role in both the intrinsic and the extrinsic clotting pathway, as it is required for activation of Factors VII, IX, X, XI and XIII. It is also important for conversion of glycoprotein IIb/IIIa (GPIIb/IIIa) complex into the functional fibrinogen receptor, and assists in binding of fibrinogen to its receptor (Shattil et al. 1985). The calcium concentration required for aggregation to occur is in the range of 10-100µM, below which no platelet aggregation occurs (Ataullakhanov et al. 1994). Most platelet function tests use citrated blood and test platelet activation and aggregation. Citrate is known to reduce the plasma calcium concentration significantly from 0.94-1.33mM to 40-50 µM, and hence results in suboptimal platelet aggregation. Use of trisodium citrate in citrated blood reduces the calcium concentration to significantly low levels (Rebello et al. 2000), altering the platelet response to agonists and antagonists, and renders the tests non physiological by eliminating the effects of thrombin on platelet aggregation. Minimum calcium concentration required for platelet aggregation is 10 µM and at levels greater than 250 µM, thrombin is generated by activated platelets and eventually coagulation occurs at levels over 330 µM concentration (Scarborough et al. 1999).

Available platelet function tests use agonists like arachidonic acid, collagen, epinephrine and ADP in citrated blood. All these agonists result in release of platelet granule contents and subsequently increase production of thromboxane A₂ which accelerates aggregation. Furthermore, the concentrations of these agonists in vitro are significantly higher than that measured in native blood. There is also intraindividual variability in release of the granule contents and TXA₂, questioning the reliability of the results obtained. At physiological

calcium concentration, none of these agonists cause granule or TXA₂ formation (Gorog et al.2009). A study by Patel et al compared the amount of platelet aggregation using different quantities of ADP (1, 5, 10, and 20 μM), and no significant difference in aggregatory response was seen with 10 μmol/l when compared to 20 μmol/l ADP. This study also demonstrated reduced platelet aggregation in citrate anticoagulated plasma. The non calcium chelating anticoagulant used in the study was PPACK (D-Phenylalanyl-L-propyl-L-arginine chloromethyl ketone), and platelet aggregation was measured using a Light Transmission Aggregometer (Patel et al. 2006).

Thrombin is important in the platelet activation and aggregation pathway. It is a serine protease protein that cleaves soluble fibrinogen to insoluble fibrin and causes platelet activation and aggregation. It activates the G protein-coupled protease receptors PAR1 and PAR4 on platelets, which in turn activate heterotrimeric and monomeric G proteins leading to increase in cytosolic calcium concentration, platelet shape change and enhances platelet aggregation. In native blood, at physiological doses of calcium, all agonists induce thrombin release and activate the intrinsic coagulation system resulting in granule content release, platelet aggregation and thrombus formation. Thrombin converts fibrinogen to fibrin, which stabilizes the thrombus to the vessel wall. In citrated blood, calcium is below the threshold concentration of 250μM, which suggests thrombin is not generated. Hence, most platelet function tests do not assess the role of thrombin in platelet aggregation, and exclusion of such an important mediator of thrombosis makes the results of these tests unreliable in the clinical setting. Direct thrombin inhibitors, PAR1 antagonists such as Atopaxar and Vorapaxar have recently been developed to inhibit thrombosis (Brass et al. 2003, O'Donoghue et al. 2011, and Morrow et al. 2012).

The plasma protein **vWF** is essential for adhesion and activation of platelets, and regulates release of thrombin upon binding to platelet membrane receptors glycoprotein Ib-IX-V and IIbIIIa (Crary et al.1995). Moake and colleagues demonstrated the binding of vWF to glycoprotein Ib in vitro, and this binding ultimately assists in the cross linking of platelets to form an aggregate (Moake et al. 1986). A study by Nishida et al revealed inverse correlation between vWF and occlusion time using the GTT in 132 healthy volunteers, suggesting vWF is essential in the formation of thrombus (Nishida et al. 2006). Inverse correlation was also noted between RBC, haemoglobin, haematocrit and occlusion time in this study. Another Japanese study by Ikarugi et al has also reported inverse correlation between haematocrit and occlusion times (Ikarugi et al. 2003).

It has been mentioned that **Shear stress** is required for platelet activation and this stress can vary between 50-3000 dyne/cm² depending on the degree of arterial stenosis. On activation by high shear of at least 250dynes/cm², platelets release ADP and contribute to thrombus formation. Strony et al demonstrated that platelets are activated by high shear stress levels in stenosed coronary arteries, and adhere at sites of vessel wall damage eventually forming a thrombus (Strony et al. 1993).

ADP is a nucleoside diphosphate stored in platelet granules and released upon platelet activation. It binds with the G-protein coupled platelet receptors P2X1, P2Y1 and P2Y12 resulting in release of intracellular calcium, changes the shape of the platelet, and plays an important role in platelet activation and aggregation through inhibition of the enzyme adenyl cyclase. It also generates thromboxane A₂ by hydrolysis of arachidonic acid from phospholipid, which enhances the aggregation process (Jianguo et al. 2002).

Collagen is a naturally occurring protein found in the subendothelium of the vessel wall, and gets exposed on disruption of the vessel wall. It binds to glycoprotein VI and glycoprotein

Ia/IIa receptors on platelets, and results in thromboxane A₂ generation which enhances platelet aggregation. Glycoprotein Ib-V-IX binds to the vWF present in collagen and further activates platelets (Lodish et al. 2000).

Epinephrine, also known as adrenaline is a catecholamine released by the adrenal glands from the amino acids phenylalanine and tyrosine. Tyrosine is oxidized to L-Dopa which is decarboxylated to Dopamine. Dopamine β -hydroxylase converts Dopamine to Norepinephrine, which is eventually methylated to form epinephrine. Epinephrine binds to the α_2 -adrenergic receptor on platelets inhibiting adenylyl cyclase and releases calcium ions. It induces fibrinogen receptor expression and fibrinogen binding and activates platelet aggregation (Shattil et al. 1989).

Arachidonic acid is a polyunsaturated omega -6 fatty acid. It is converted to prostaglandin G₂ and H₂ and thence to thromboxane A₂ by cyclooxygenase and thromboxane synthase. Thromboxane A₂ is a potent vasoconstrictor and induces platelet activation and aggregation.

Antiplatelet agents: a review of the evidence

Aspirin

Aspirin, also known as acetylsalicylic acid inhibits prostaglandin and thromboxane A₂ production by acetylation of COX1, the enzyme that produces the cyclic endoperoxide precursor of thromboxane A₂. The action of aspirin on platelet cyclooxygenase is permanent, lasting for the lifetime of the platelet (7-10 days) and repeated doses of aspirin produce a cumulative effect on platelet function.

In addition to being an antiplatelet agent, it also works as an anti-inflammatory drug with analgesic properties. A metanalysis by the Antiplatelet Trialists collaboration analysed data from 25 randomized trials, and demonstrated that aspirin reduced vascular mortality by 15% and non-fatal vascular events (stroke or myocardial infarction) by 30% (Antiplatelet Trialists' Collaboration .1998). Another metanalysis by the Antithrombotic Trialists collaboration examined data from from 195 clinical trials involving more than 135000 patients, and demonstrated a 40% reduction in cardiac events in unstable angina patients on aspirin (Antiplatelet Trialists' Collaboration . 2002). The ISIS-2 study showed a significant reduction in non-fatal reinfarction and stroke in patients with prior myocardial infarction on aspirin (ISIS-2 Collaborative Group.1988). 17187 patients with AMI were randomized with placebo control, to aspirin, streptokinase, both or none of the medications. A significant reduction in vascular mortality was observed in the aspirin group when compared to placebo (9.4% vs. 11.8%, $p < 0.001$). Patients admitted with myocardial infarction, on aspirin the week prior to admission have also demonstrated a reduction in ischaemic episodes and reinfarction rates (Garcia-Dorado et al.1995). In a study of 539 patients admitted with an ACS, 214 were taking aspirin prior to admission. AMI occurred in 52 (24%) of these patients, compared to unstable angina in 162 (76%), and AMI was significantly lower in the cohort of patients who had been

on aspirin prior to admission compared to those not on prior aspirin (24% vs 54%, $p < 0.0001$). The Swedish Angina Pectoris Aspirin Trial (SAPAT) was the first prospective study of aspirin in stable angina patients established on a beta-blocker, demonstrating a significant reduction in the incidence of first myocardial infarction in patients with symptoms of stable angina pectoris (Juul-Moller et al. 1992). 2035 stable angina patients on sotalol were randomised double-blind to treatment with aspirin 75 mg daily or placebo. There was a 34% reduction in primary outcome events which included myocardial infarction and sudden death; (95% CI 24-49%; $p = 0.003$). In a Veterans Administration Cooperative Study by Lewis et al, 1266 men with unstable angina were randomized to aspirin or placebo. The primary endpoint was death and acute myocardial infarction, the incidence of which was 51 per cent lower in the aspirin group than in the placebo group (5% vs. 10.1%, $p = 0.0005$) (Lewis et al. 1983). However, aspirin does have its limitations as it only inhibits TXA₂ synthesis, and has little or no effect on other platelet agonists. It inhibits some platelet inhibitors like prostacyclin and important side effects include bleeding events and gastric irritation. Resistance to aspirin is seen in approximately 30-40% of patients who continue to experience adverse cardiac events inspite of being on aspirin.

Clopidogrel

Clopidogrel is a potent, oral antiplatelet agent. It is a thienopyridine derivative, and inhibits both exogenous ADP dependent platelet activation and aggregation. It is a prodrug oxidized by the hepatic cytochrome P450 system to its active metabolite, which irreversibly binds to the ADP-coupled P₂Y₁₂ receptor. P₂Y₁₂ inhibition thus inhibits ADP-induced platelet activation and resultant aggregation. Clopidogrel has been used as an “add-on” therapy to aspirin, and has shown significant reduction in thrombotic events in patients with atherosclerotic disease (MI, CVA and PVD). The CURE study (The Clopidogrel in Unstable Angina to Prevent

Recurrent Events Trial Investigators.2001), showed a 20% relative risk reduction (RRR) in cardiovascular death, non-fatal MI and CVA in NSTEMI patients on dual antiplatelet therapy (aspirin and clopidogrel). 12,562 patients with NSTEMI were randomized to clopidogrel (300 mg immediately, followed by 75 mg once daily) or placebo in addition to aspirin for 3 to 12 months. The primary outcome was a composite of death from cardiovascular causes, nonfatal myocardial infarction, or stroke and occurred in 9.3 percent of the patients in the clopidogrel group and 11.4 percent of the patients in the placebo group. (RR with clopidogrel as compared with placebo, 0.80; 95 percent confidence interval, 0.72 to 0.90; $P < 0.001$). The COMMIT (CLOpidogrel and Metoprolol in Myocardial Infarction Trial collaborative group. 2005) and CLARITY TIMI 28 (Sabatine et al. 2005) study demonstrated a significant reduction in death, non-fatal MI and CVA in STEMI patients. 45,852 patients admitted with STEMI were randomized in the COMMIT study, and allocated clopidogrel 75 mg daily or placebo in addition to aspirin 162 mg daily. A significant reduction in death was seen in the clopidogrel group compared to placebo group (9.2% vs. 10.1% , $p = 0.002$). In the CLARITY TIMI 28 study, 3491 patients with STEMI were randomized to clopidogrel (300-mg loading dose, followed by 75 mg once daily) or placebo. Primary endpoint occurred in 21.7 % of the placebo group and 15.0% in the clopidogrel group, $p < 0.001$. Further benefit with clopidogrel was seen in the CREDO (Steinhubl et al. 2002) study, with a 27% RRR in patients undergoing PCI. 2116 patients who were to undergo elective PCI were recruited and randomly assigned to receive a 300-mg clopidogrel loading dose or placebo pre PCI. Thereafter, all patients received clopidogrel, 75 mg/d, through to day 28. From day 29 through 12 months, patients in the loading-dose group received clopidogrel, 75 mg/d, and those in the control group received placebo. Both groups received aspirin throughout the study. Clopidogrel pretreatment did not significantly reduce the combined risk of death, MI, or urgent target vessel revascularization at 28 days (reduction, 18.5%; 95% CI, -14.2% to

41.8%; $P = .23$), but significantly reduced the composite end point of death, MI, or stroke (relative risk reduction [RRR] 26.9% [$p = 0.02$; 95% CI 3.9% to 44.4%]) at one year. In the CAPRIE study (CAPRIE Steering Committee. 1996), there was an 8.7% RRR with clopidogrel in patients with atherosclerotic disease (MI, CVA and PVD). 19 185 patients with atherosclerotic vascular disease manifested as either recent ischaemic stroke, recent myocardial infarction, or symptomatic peripheral arterial disease were randomized and treated with either clopidogrel or aspirin. Patients on clopidogrel had an annual 5.32% risk of ischaemic stroke, myocardial infarction, or vascular death compared with 5.83% with aspirin resulting in a relative-risk reduction of 8.7% in favour of clopidogrel (95% CI 0.3—16.5, $p = 0.043$). The MATCH (Diener et al. 2004) study was a double-blind, placebo-controlled trial to compare aspirin (75 mg/day) with placebo in 7599 high-risk patients with recent ischaemic stroke or transient ischaemic attack and at least one additional vascular risk factor who were already receiving clopidogrel 75 mg/day. There was 6.4 % RRR with clopidogrel plus aspirin in ischaemic CVA, MI, vascular death or rehospitalization for acute ischaemia. 15.7% patients receiving aspirin and clopidogrel reached the primary endpoint compared to 16.7% in the clopidogrel alone group (RRR 6.4%, [95% CI -4.6 to 16.3, $p = NS$]). Recent data suggests higher doses of clopidogrel exhibit greater and more rapid onset of platelet aggregation. The ARMYDA-2 (Giuseppe et al. 2005) trial compared 600mg and 300 mg loading doses in patients undergoing PCI, and a significant reduction in the clinical endpoint of death or non fatal MI was seen in the 600 mg group (12% vs. 4%, $P = 0.041$) at 30 days. Major side effects limiting use of clopidogrel include bleeding events and intolerance in a small proportion of patients. Data from various studies including CREDO and CAPRIE recommend use of clopidogrel for at least a year in ACS patients on medical management and in stented patients. Table 1 summarizes different randomized controlled trials demonstrating reduction in MACE with clopidogrel.

Table 1: Randomized controlled trials demonstrating reduction of MACE with Clopidogrel

Study	Study population	Trial arms	Primary endpoint (%); significance
CURE	12,562 patients with UA/NSTEMI	Clopidogrel plus Aspirin vs Placebo plus Aspirin	9.3 vs. 11.4 ; p<0.001
CAPRIE	19,185 patients with atherosclerotic disease (CVA/MI/PAD)	Aspirin vs Clopidogrel	5.8 vs. 5.2 ; p =0.043
CREDO	2,116 patients undergoing PCI	Clopidogrel plus Aspirin vs Placebo plus Aspirin	8.5 vs. 11.5 ; p = 0.02
CLARITY TIMI 28	3,491 patients < 75 years with STEMI presenting within 12 hrs of symptoms	Clopidogrel plus Aspirin vs Placebol plus Aspirin	15 vs. 21.7; p<0.001
COMMIT	45,852 patients with suspected ACS with STEMI	Clopidogrel plus Aspirin vs Placebo plus Aspirin	9.2 vs. 10.1; p =0.002
MATCH	7,599 patients with recent ischaemic CVA/TIA	Clopidogrel plus Aspirin vs Placebo plus Aspirin	15.7 vs. 16.7; p=NS

Ticlopidine

Ticlopidine is a thienopyridine derivative that irreversibly inhibits the P₂Y₁₂ receptor. It is a prodrug that requires conversion to the active metabolite by the hepatic cytochrome P450 enzyme. It is rapidly absorbed, highly bioavailable and has a prolonged effect. It permanently inhibits the P₂Y₁₂ receptor by forming a disulfide bridge between the thiol on the drug and a free cysteine residue in the extracellular region of the receptor and thus has a prolonged effect. Like aspirin it has a short half-life with a long duration of action. Maximal inhibition of platelet aggregation is not seen until 8 to 11 days after starting therapy (McTavish et al. 1990). The loading dose is 500 mg followed by 250 mg twice per day. In a number of randomised trials like the FANTASTIC, MATTIS and the ISAR study (Martin et al. 1999), aspirin was compared with aspirin and ticlopidine and a reduction in recurrent cardiovascular events was observed in the combination group. In the STAMI trial (Scrutinio et al. 2001), 1470 patients post AMI were allocated to receive aspirin 160 mg/day or ticlopidine 500 mg / day. No significant difference was seen between aspirin or ticlopidine in the rate of the primary combined end point of death, recurrent AMI, stroke or angina. The primary end point was recorded in 59 (8.0%) of the 736 aspirin-treated and 59 (8.0%) of the 734 ticlopidine-treated patients (p = 0.966). In another study by Tanuicchi et al, clopidogrel conferred similar protection as ticlopidine against subacute stent thrombosis and major adverse cardiac events in 1016 patients 2 weeks post coronary stenting, but clopidogrel was better tolerated than ticlopidine with less adverse reactions (Tanuichi et al. 2001). Within 30 days, stent thrombosis of the stent occurred in 1.92% of the patients in the ticlopidine group and in 2.02% of the clopidogrel group (P=0.901). A MACE occurred in 4.60% of patients receiving ticlopidine and in 3.85% of patients receiving clopidogrel (P=0.551). Ticlopidine's unfavourable side-effect profile with risk of bone marrow suppression has led to the withdrawal of this drug in some countries (e.g. United Kingdom).

Dipyridamole

Dipyridamole is a phosphodiesterase inhibitor. It increases the cellular concentration of platelet adenosine 3, 5-monophosphate (cAMP) levels by interfering with platelet function and inhibits its breakdown. This effect is mediated by inhibition of cyclic nucleotide phosphodiesterase and/or by blockade of available uptake of adenosine, which acts at adenosine A₂ receptors to stimulate platelet adenylyl cyclase. High cAMP levels lead to a reduction in intracellular Ca²⁺, and low Ca²⁺ levels inhibit events leading to platelet activation and granule excretion. In the European Stroke Prevention Study. 2, 6602 patients were recruited and randomized to treatment with ASA alone (50 mg daily), modified-release dipyridamole alone (400 mg daily), the two agents in a combined formulation, or placebo. Dipyridamole plus aspirin combination resulted in a 37% reduction in stroke ($p < 0.001$), compared to 8% with aspirin alone ($p = 0.013$); and 16% with dipyridamole alone ($p = 0.039$) (Diener et al. 1996).

Cilostazol

Cilostazol is a reversible cAMP phosphodiesterase inhibitor with antiplatelet and antithrombotic properties. In a review of 8 trials by Chapman et al, patients on cilostazol demonstrated an increase in walking distances and improved quality of life compared with placebo. In six of eight well designed clinical trials, cilostazol was significantly more effective than placebo in increasing walking distances and improving the quality of life of patients with moderate to severe intermittent claudication (Chapman et al. 2003).

Glycoprotein IIb/IIIa Inhibitors

Glycoprotein IIb/IIIa receptors on the platelet surface bind fibrinogen, and are the final common pathway of platelet activation. The GP IIb/IIIa receptor may be activated by any platelet agonist, and can be inhibited by GPIIb/IIIa receptor inhibitors, which block platelet aggregation. Three such agents approved for use at present are abciximab, eptifibatid and tirofiban. All are effective but need to be given intravenously and are only approved for short-term use (Lippi et al. 2007).

Abciximab is a long acting, reversible GPIIb/IIIa receptor inhibitor with a half life of 30 minutes. It is a monoclonal antibody and is eliminated by protease degradation. It has shown significant risk reduction in the 30 day composite endpoint of death, myocardial infarction, and need for urgent repeat revascularization procedures in various trials. In the EPIC trial (Marmur et al. 2006), abciximab resulted in a 35% reduction in ischaemic events at 30 days in 2099 high risk ACS patients undergoing PCI. Six months follow up data from this trial also showed a significant reduction in the rate of instent restenosis. In the EPILOG study (Roe et al. 1998), abciximab was used in 2792 elective and urgent PCI patients, and resulted in a 68% reduction in ischaemic events at 30 days. In the EPISTENT study (Topol et al. 1998), there was a 60% RRR in mortality in the abciximab plus stent group at 1 year as compared to the other treatment groups. In the CAPTURE trial (Umans et al. 1997), abciximab showed a 29% RRR in ischaemic events in 1265 high risk unstable angina patients at 30 days, but this benefit was not maintained at 6 months follow up. In the RAPPORT study, abciximab resulted in a 48% reduction in ischaemic events in 483 STEMI patients undergoing PAMI. In the ISAR REACT II study (Ndrepepa et al. 2008), there was a RR of 0.71, $P=0.02$, with a significant reduction in ischaemic events in 2022 ACS patients with elevated troponin. Eptifibatid is another GPIIb/IIIa inhibitor, and produces a dose-dependent

inhibition of platelet aggregation and has been shown to reduce the frequency of acute ischemic complications following percutaneous coronary revascularization. In the IMPACT II study (The IMPACT-II Investigators.1997), eptifibatide was used in suboptimal doses, 135 µg/kg bolus plus 0.5 or 0.75 µg/kg/min infusion. Among patients who received eptifibatide treatment, the reductions in the composite end point at 30 days were 22% (P = .035) and 14% (P = 0.178) for the 135/0.5 and 135/0.75 doses, respectively. The efficacy of increased doses was tested in the PURSUIT trial (The PURSUIT Trial Investigators. 1998), where a bolus of 180 µg/kg and infusion of 1.3 µg/kg/min or a bolus of 180 µg/kg and infusion of 2.0 µg/kg/min was given to patients undergoing PCI. This infusion rate was significantly higher than the doses used in the IMPACT II trial, and resulted in a statistically significant reduction in the incidence of death or MI. Similarly, in the ESPRIT study (The ESPRIT investigator. 2000), the dose of eptifibatide was fourfold higher than in the IMPACT II study, with two intravenous boluses of 180mcg/kg followed by a continuous infusion of 2 mcg/kg/min for 18-24 hours post-intervention. In this trial, a significant reduction in the incidence of the composite endpoint was seen in patients who received eptifibatide at 48 hours, 30 days, 6 months, and was maintained at 1 year. Recent results from the EARLY ACS study (Giugliano et al. 2009) do not support use of Eptifibatide as upstream therapy in high risk ACS patients undergoing PCI. The EVA-AMI study (Zeymer et al. 2010) compared eptifibatide vs abciximab as an adjunctive treatment for patients undergoing primary PCI, and no significant difference was noted between the two agents, suggesting eptifibatide may be used as an alternative to abciximab in PAMI. There is however, a risk of major bleeding and thrombocytopenia with GP IIb/IIIa inhibitors, limiting its use in all high risk ACS patients undergoing PCI.

Prasugrel

Prasugrel is an oral, irreversible P₂Y₁₂ receptor blocker, and is deemed to be 10 times more potent than other thienopyridine derivatives. It is a prodrug and is hydrolysed to a thiolactone in the intestine with the help of the Cytochrome P450 pathway. It achieves peak plasma concentration within 30 minutes, and has been shown to achieve greater platelet inhibition when compared to clopidogrel. The Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis in Myocardial Infarction (TRITON-TIMI 38) study (Wiviott et al. 2007) was an international, randomised double blind study that compared clopidogrel (300mg loading dose, 75 mg maintenance dose) with prasugrel (60 mg loading dose, 10 mg maintenance dose) in 13,608 ACS patients. Patients in the prasugrel group demonstrated greater platelet inhibition when compared to the clopidogrel group. Significant reduction in clinical endpoints of CV death, non-fatal MI or stroke was observed in the prasugrel group (9.4% vs. 11.5%, p< 0.001) irrespective of baseline characteristics such as age and sex. There was 24% reduction in myocardial infarction and 52% relative reduction in stent thrombosis in ACS patients. Patients greater than 75 years age and less than 60 kgs body weight demonstrated less benefit with prasugrel when compared to clopidogrel due to bleeding side effects. Bleeding complications were more frequent with prasugrel than clopidogrel (2.4% versus 1.8%, P=0.03), including fatal bleeding (0.4% versus 0.1%; P = 0.02). The Trilogy-ACS study compared prasugrel and clopidogrel in ACS patients with NSTEMI or UA who were medically managed without any intervention. 9,326 patients were recruited, and no significant difference in primary outcome of cardiovascular death, myocardial infarction or stroke was noted in either the prasugrel or clopidogrel group at 30 months (13.9% vs. 16%, p =0.21, HR 0.91). The maintenance dose of prasugrel was reduced to 5 mg in the > 75

years of age group patient, with no significant difference in primary outcome (18.7% vs. 20.3%, $p = \text{NS}$) (Gurbel et al. 2012).

The PRINCIPLE-TIMI 44 study showed that high dose clopidogrel (600 mg loading followed by 150mg/day) resulted in less inhibition of platelet aggregation than prasugrel (60 mg loading followed by 10 mg/day) in patients with stable coronary disease undergoing planned PCI (Wiviott et al. 2007). The primary end point of this study was inhibition of platelet aggregation (IPA) with 20- $\mu\text{mol/L}$ ADP measured after six hours. 201 patients were randomized, and IPA at 6 hours was significantly higher in subjects receiving prasugrel (mean \pm SD, 74.8 \pm 13.0%) compared with clopidogrel (31.8 \pm 21.1%; $P < 0.0001$). In the ACAPULCO study, which was a randomised double blind cross over study, greater platelet inhibition was observed with prasugrel 10 mg maintenance dose compared to clopidogrel 150 mg maintenance dose in ACS patients. Patients who were loaded with 900 mg clopidogrel and switched to prasugrel 10 mg for maintenance also demonstrated further platelet inhibition suggesting prasugrel was a more potent platelet inhibitor (Montalescot et al. 2010). The study showed that the 10 mg prasugrel dose produced a lower level of platelet aggregation compared to the 150 mg clopidogrel dose. The primary endpoint of the study was maximum platelet aggregation (MPA with 20 micromoles of ADP) as assessed by light transmission aggregometry at 14 and 28 days. MPA was 26.2% for prasugrel 10 mg and 39.1% for clopidogrel 150 mg ($p < 0.001$).

Ticagrelor

Ticagrelor or AZD6140 is an oral, reversible P_2Y_{12} receptor blocker. It is an ATP derivative and belongs to the cyclopentyltriazolopyrimidine group of drugs. It does not require conversion to an active metabolite for its action, and has a half-life of 12 hours. Being a reversible agent,

Ticagrelor provides greater flexibility with regards to timing of bypass surgery. In a study of 200 patients with stable atherosclerosis (DISPERSE), ticagrelor (50mg, 100mg, 200mg, 400mg) was compared to clopidogrel 75 mg. Although higher platelet inhibition was achieved with higher doses of ticagrelor (100mg, 200mg and 400mg) compared to clopidogrel, ticagrelor was associated with a higher incidence of bleeding and dyspnoea. In the DISPERSE-2 study (Cannon et al. 2007), ticagrelor was compared to clopidogrel in 990 patients with ACS.

Although there were fewer adverse events in the ticagrelor group, the study was underpowered to detect a statistically significant difference. In the PLATO study (Cannon et al. 2010), 13 408 ACS patients were randomized to receive either ticagrelor (180 mg loading dose followed by 90 mg twice a day) or clopidogrel (600 mg loading, followed by 75 mg maintenance). The primary endpoint of cardiovascular death, non fatal MI, or stroke occurred was significantly lower in the ticagrelor group than the clopidogrel group (9% vs. 10.7%, hazard ratio 0.84, 95% CI 0.75-0.94; p=0.0025). In this study, no significant difference was observed in bleeding rates in between the two groups (11.6% vs. 11.5%, p =0.88).

Cangrelor

Cangrelor is a nonthienopyridine adenosine triphosphate analogue, and a potent, short acting intravenous P₂Y₁₂ receptor antagonist. It is an ATP analogue with a very short half-life (3-5 mins), with recovery of platelet function within an hour of discontinuation of the drug. The CHAMPION-PCI (Harrington et al. 2009) , and the CHAMPION-PLATFORM study were large multicentre trials comparing clopidogrel with cangrelor in ACS patients undergoing PCI , but the studies were prematurely terminated as no significant differences in measures of clinical effectiveness was seen in with cangrelor. Cangrelor did not reduce the composite endpoint of death, MI or ischemia-driven revascularization when compared to clopidogrel, in the CHAMPION PCI study. In the Champion – Phoenix study, 11 145 patients undergoing urgent or elective PCI were randomized in double-blind fashion to receive cangrelor in a

bolus plus infusion or a 600-mg or 300-mg loading dose of clopidogrel. Patients in the cangrelor group had a significantly lower rate of the composite of all-cause death, myocardial infarction (MI), ischemia-driven revascularization, and stent thrombosis at 48 hours compared with clopidogrel (4.7% versus 5.9%, $p < 0.01$) (Bhatt et al. 2013).

Atopaxar

Atopaxar is a protease activated receptor antagonist and blocks the PAR 1 receptor. In phase 1 studies, it has shown inhibition of platelet aggregation, without any significant increase in bleeding time. It also suppresses the effects of inflammatory markers, which have been linked to adverse outcomes in ACS patients. The LANCELOT ACS study was a randomized, double blind, placebo controlled study, assessing the safety and tolerability of -Atopaxar in ACS patients, in addition to standard therapy. In this study, 603 patients admitted to hospital with ACS were randomized to placebo or to a 400-mg loading dose of atopaxar followed by a daily dose of 50 mg, 100 mg, or 200 mg for 12 weeks. All patients were on aspirin and greater than 75% were taking aspirin in combination with clopidogrel or ticlopidine. The incidence of the primary endpoint of cardiovascular death, myocardial infarction, stroke, or recurrent ischemia was similar between the atopaxar and placebo arms (8.03% versus 7.75%; $P=0.93$). Larger trials are required to further investigate the efficacy and safety of atopaxar (O'Donoghue et al. 2011).

Vorapaxar

Vorapaxar is another novel antiplatelet agent. It is a novel thrombin receptor (PAR 1) antagonist. The TRACER study (Tricoci et al. 2012) was a multinational, randomized, double-blind, placebo-controlled study. 12,944 patients with NSTEMI were randomised to either Vorapaxar or placebo, in addition to dual antiplatelet therapy. A loading dose of 40 mg

of vorapaxar was given followed by 2.5 mg maintenance dose for a year. The TRACER study did not show a reduction in its primary endpoint which included cardiovascular death, myocardial infarction, stroke, recurrent ischemia with re-hospitalization and urgent coronary revascularization. There was a non-significant ($p=0.72$) 8% reduction in MACE. In the TRA-PCI study (The TRA-PCI Investigators et al. 2009), a phase II trial, 1031 patients scheduled for angiography and possible elective stenting were randomised to either vorapaxar or placebo. This was in addition to dual antiplatelet therapy with aspirin and clopidogrel. Fewer adverse events were seen in the vorapaxar group, with no significant increase in bleeding over a 4 month follow up period. The TRA 2P –TIMI 50 was a multinational, randomised, double blind trial comparing vorapaxar to placebo in patients with history of prior MI, stroke or PAD. 26,449 patients who had a history of myocardial infarction, ischemic stroke, or peripheral arterial disease were randomized to receive vorapaxar (2.5 mg daily) or placebo and followed up for a median of 30 months. At 3 years, the primary endpoint occurred in 9.3% in the vorapaxar group and in 10.5% in the placebo group (HR for the vorapaxar group, 0.87; 95% confidence interval [CI], 0.80 to 0.94; $P<0.001$). There was an increase in the rate of intracranial hemorrhage in the vorapaxar group (1.0%, vs. 0.5% in the placebo group; $P<0.001$) (Morrow et al. 2012).

BM573

BM573 is a combined thromboxane receptor antagonist and synthase inhibitor. It has shown promising results in mice and rat models, inhibiting platelet aggregation without increasing bleeding time. It is a torsemide derivative, but without any diuretic effect. In pig models, it has shown reduction in MI induced by coronary thrombosis, and has also shown reduction of pulmonary vascular resistance. It is yet to be tried in humans, but animal model results suggest this could be a promising antiplatelet and antithrombotic agent.

Clinicians are currently able to partially improve the responsiveness to antiplatelet therapy by acting on extrinsic factors involved in the aetiology of resistance. This includes compliance to treatment, drug drug interactions and good control of blood pressure, glycaemia and lipid levels. Studies have shown that clopidogrel loading with 600 mg has a stronger and faster inhibitory effect on platelet reactivity than the 300 mg loading dose (Patti et al. 2005). Increasing the loading dose to 900 mg has not been shown to be of benefit, indicating a threshold to the platelet inhibitory effect of clopidogrel (Von Beckerath et al. 2005). The CLEAR-PLATELETS study showed that Clopidogrel loading combined with eptifibatide resulted in reduced myocardial necrosis compared to standard or high loading dose of clopidogrel alone (Gurbel et al. 2005). The ISAR-CHOICE-2 study demonstrated the beneficial effect on platelet inhibition of increasing the maintenance dose of clopidogrel to 150 mg (Von Beckerath et al. 2006). In the ARMYDA-4 study, reloading with 600 mg clopidogrel pre PCI did not confer any additional benefit in patients on chronic clopidogrel therapy. The ARMYDA-5 study, which compared Clopidogrel loading with 600 mg in lab vs. 4–8 hours pre PCI, did not show any significant difference in outcome in the two groups, but this study was underpowered to detect a significant difference (Germano et al. 2010). Results from the recent ARMYDA-PRO study suggest high pre PCI platelet reactivity using the VerifyNow P2Y12 assay may predict MACE at 30 days (Patti et al. 2008). Use of point of care platelet function tests may help in identification of these high-risk patients, and assist the clinician in optimising their antiplatelet medications. Results from the TRITON-TIMI 38 study showed that prasugrel significantly reduced the rates of recurrent ischemic events, including stent thrombosis, although this was offset by an increase in major bleeding (Wiviott et al. 2007). The GRAVITAS study did not demonstrate improved clinical outcomes with tailoring of doses of clopidogrel based on the results of the VerifyNow assay. Whether the newer antiplatelet agents like Prasugrel, Ticagrelor and Direct thrombin inhibitors will be sufficient to overcome the HPR seen in some individuals and improve clinical outcomes remains unknown. The relative bleeding risks with these regimens will also need to be

evaluated in detail to understand the risks and benefits associated with assessment of high platelet reactivity using platelet function tests with these novel antiplatelet agents.

Platelet function tests

Discussed below is the mechanism of some platelet function tests used in clinical practice.

Bleeding time

Dating back as far as 1901, this very simple test measures the time it takes for a small skin cut to stop bleeding. It has very poor reproducibility and no study so far has shown it to correlate with bleeding or thrombotic risk. It is dependent on operator technique, and demonstrates significant variability with age, gender and body temperature (Rodgers et al.1990).

Light transmittance aggregometry (LTA)

LTA has been regarded as the “gold standard” test for measuring platelet function (Cattaneo et al. 2009). It is often used to validate newer platelet function tests. It measures light transmittance in whole blood or platelet rich plasma. Platelet rich plasma is stirred in a cuvette at 37°C, and this cuvette sits between a light source and a photocell. When an agonist is added, the platelets change shape from discoid to tiny spheres, aggregate and absorb less light and transmittance increases. Agonists used to activate and aggregate platelets are arachidonic acid, ADP, Thrombin receptor activating peptide, collagen or epinephrine. Platelets clump in response to these agents and an increase in light transmittance is noted. Subjects whose platelet aggregation is more than 20% with arachidonate are considered aspirin resistant. Light transmittance is inversely proportionate to platelet clumping. It is a time consuming test performed on citrated blood, and variability in results has been noted (Ohmori et al. 2006).

Urinary thromboxane

Urinary thromboxane is a simple test to assess platelet activation through urinary metabolites. Activated platelets synthesize 11-dihydroxy thromboxane B2 (TxB2), an active metabolite of TxA2, and this is detected in urine with an ELISA assay. However, although detection of 11-dihydroxy thromboxane B2 in urine reflects systemic TxA2 formation, 30% is derived from non-platelet sources, and thus falsely high readings may be observed in inflammatory conditions. Increasing urinary thromboxane levels are associated with increased risk of death, MI, and stroke. Studies measuring TxB2 levels in aspirin treated patients have reported a prevalence of aspirin resistance in the range of 1-1.7% (Catell et al. 1987).

Flow cytometry

Flow cytometry uses a flow cytometer, through which red blood cells labelled with fluorescently conjugated monoclonal antibody are passed. The rate at which cells pass through the cytometer is 1000 to 10000 cells per minute. The cells are then made to pass through an active laser light, which activates the fluorophore that is conjugated to the monoclonal antibody. The intensity of fluorescence is directly proportional to the antigen being studied. P Selectin (CD62) is expressed on the surface of activated platelets, and helps in formation of monocyte-platelet aggregates, which are considered to be the most sensitive marker of platelet activation (Michelson et al.2000).

Platelet Function Analyzer (PFA -100)

The PFA-100TM system (Dade Behring, Germany) is a platelet function test that uses collagen and epinephrine as agonists. It is a semi-automated dual channel device and is based on a closure time caused by occlusion of an aperture by platelet aggregates. Blood is inserted in a citrate primed tube, and left for 30 minutes to 4 hours. This blood is then passed through twocartridges coated with collagen, and epinephrine and ADP are used as agonists. Blood is drawn into a tube

containing 3.2% citrate and allowed to stand for between 30 min and 4h, after which 800 µl of citrated whole blood is added to each of two pre-prepared cartridges to wet the filters. Both cartridges contain a membrane coated with type I equine collagen together with an agonist to induce platelet aggregation. In one cartridge the membrane is coated with 10 µM epinephrine and the other with 10 µM ADP. The measurement begins by drawing the blood through a capillary tube and a single aperture (150 µm diameter) into a collagen coated cellulose-acetate filter. This results in the platelets being pre-activated by shear stress of 190 dynes/cm² even before reaching the filters and the agonists. As platelets come into contact with the collagen, they will adhere, aggregate and form the primary hemostatic plug, which occludes the aperture (closure time, CT). Closure time is inversely proportional to platelet inhibition (Hayward et al. 2006).

Verify Now assay

The Verify Now system (Accumetrics, San Diego, California) is a turbidometry-based optical detection device that utilizes light source to detect the amount of platelet aggregation. The assay cartridge has fibrinogen-coated beads to which platelets adhere to, aggregate and eventually fall out of solution. This results in change of light transmittance in the cartridge, and light transmittance is inversely proportional to amount of platelet aggregation. Results are obtained in 5 minutes. It is a rapid, easy to use, point of care system used to assess platelet reactivity. There are 3 types of Verify Now Assay – Aspirin assay utilizes arachidonic acid as an agonist, P2Y₁₂ assay utilizes ADP to assess effect of Clopidogrel, Ticlopidine or Prasugrel, and IIB/IIIa assay utilizes a thrombin receptor activating peptide as an agonist to assess response of GPIIb/IIIa inhibitors like Abciximab, or Eptifibatide The instrument measures the increase in light transmittance over time, thus a blood sample that is prothrombotic produces low light transmittance, whereas a sample with normal platelet function produces high light transmittance. The system reports two results for each assay: P2Y₁₂ reaction units (PRU) report

the amount of ADP-mediated aggregation, and higher PRU's are associated with worse outcomes in reported studies; and % inhibition (%) is the percent change from baseline aggregation, and is calculated from the PRU result and the BASE result, which is based on the rate and extent of platelet aggregation in the TRAP channel, where $\% \text{ Inhibition} = (I - \text{PRU}/\text{BASE}) \times 100$. The GRAVITAS (Gauging Responsiveness with A Verify Now assay—Impact on Thrombosis And Safety) study compared the effect of high dose clopidogrel (600mg loading dose,150mg maintenance dose) versus standard dose clopidogrel (75 mg maintenance dose)in patients with high on- treatment platelet reactivity using the Verify Now assay (Price et al. 2011). 2214 patients undergoing PCI were included in this double blind randomized study, and no significant reduction in end point was noted in either of the groups. Patients who were treated with PCI, and achieved low platelet reactivity and were tested using the Verify now assay at discharge and at 30 days post PCI, demonstrated a 50% reduction in CV death, MI and stent thrombosis. The TRIGGER-PCI (Testing platelet reactivity in patients undergoing elective stent placement on clopidogrel to guide alternative therapy with prasugrel) study comparing prasugrel to clopidogrel for patients with high on-clopidogrel platelet reactivity following PCI has been prematurely terminated due to relatively few occurrences of primary endpoint in the study at six months. High on-clopidogrel platelet reactivity (>208 PRU by VerifyNow P2Y12 test) was observed less frequently than expected, and prasugrel was seen to reduce platelet reactivity greater than clopidogrel (Trenk et al. 2012).

Thromboelastograph Haemostasis System (TEG 5000)

Thromboelastography (Haemoscope, USA) is a platelet function test that provides global information on clot development, stabilization and dissolution in vitro. The original TEG involved native blood, but modified versions now utilize citrated blood to assess clot formation and lysis. Whole blood sample is placed in a cup that oscillates at an angle of 4°45'. A

stationary pin attached to a torsion wire is immersed in this blood, and monitored for motion. The strength of fibrin-platelet bond during clot formation affects magnitude of pin motion, and gives a measure of haemostasis. It provides measurements of clotting time, clot strength and kinetics, and clot lysis. Software is required to interpret results that helps assess the risk of ischaemia and bleeding, and determine need for antiplatelet therapy (Donahue et al. 2005)

Plateletworks

Plateletworks (Helena Laboratories, Texas, US) is a point of care platelet function test that provides information of platelet activation and aggregation. Platelet numbers are counted before and after activation with an agonist using collagen, ADP or arachidonic acid. Blood can be drawn from any existing indwelling line or by venepuncture. The change in platelet count is measured using an electronic impedance-cell based counter, and results expressed as percentage inhibition. The test can be performed in 2-5 minutes, and tests efficacy of aspirin, clopidogrel, and glycoprotein IIb/IIIa inhibitors. In the POPULAR trial, six platelet function tests were compared head to head to predict a composite of death, MI, stent thrombosis, and stroke at one year in 1069 stable patients on clopidogrel undergoing elective PCI. Of the six tests, only three tests - light transmittance aggregometry (LTA 5 $\mu\text{mol/L}$ ADP ($n = 1,049$; $P = 0.0002$), LTA 20 $\mu\text{mol/L}$ ADP ($n = 1,051$; $P = 0.0003$), VerifyNow ($n = 1,052$; $P = 0.0002$), and the Plateletworks assay ($n = 606$; $P = 0.054$) were noted to have a modest effect on predicting cardiovascular outcomes. None of these tests were able to predict bleeding risk in PCI patients (Breet et al. 2010).

Multiplate Analyser

The Multiplate analyser (Multiplate, Roche Diagnostics, Switzerland) or multiple platelet aggregometry (MEA) is a platelet function analyser that utilises different reagents like ADP,

TRAP, Collagen and ristocetin to determine high platelet reactivity. It has multiple channels and uses multiple electrodes to accurately assess platelet function. It utilizes the principle of impedance aggregometry. Hirudin anticoagulated blood is stirred for a few minutes in a test curvette at 37°C, and ADP in a concentration of 6.4 µmol/l (ADP test), or a combination of ADP (6.4 µmol/l) and PGE1 (9.4 nmol/l) (ADP test high sensitivity – ADPtest HS) are added and aggregation recorded for 5 minutes (Johnson et al. 2008). Platelets attach to the multiplate sensors and increase impedance which is transformed into arbitrary aggregation units (AAU) and plotted against time. In a large study by Sibbing et al, 1608 patients undergoing PCI were enrolled. Clopidogrel non responders were identified using the multiplate analyser, and the composite of death or stent thrombosis was higher in low responders compared to normal responders (3.1% vs. 0.6%; CI: 2.2-11.6; P< 0.001) and significant correlation was observed between the MEA and LTA (rho= 0.71; P<0.0001) (Sibbing et al. 2008). In another study by Sibbing et al, MEA has been shown to predict thrombotic and bleeding events (Sibbing et al. 2010). Based on another study, MEA test value of 468 AAU has been taken as a cut off for prediction of stent thrombosis (Freyenhofer et al. 2011).

Vasodilator-stimulated phosphoprotein assay (VASP)

Vasodilator-stimulated phosphoprotein is an intracellular platelet protein, phosphorylation of which is regulated by the cAMP cascade. ADP inhibits this cascade and Prostaglandin E1 activates it. The Platelet VASP test (Biocytex, Marseille, France) is a flow cytometry assay that can assess the effect of the P2Y12 antagonists. It is reproducible, uses citrated blood, and samples can be stored at room temperature for upto 48 hours for analysis. Like most platelet function tests, the first few mls of blood collected must be discarded. The optimum time to perform the P2Y12 assay is 6 hours after a loading dose, or at least 7 days after commencing maintenance therapy with clopidogrel. The

assay measures the suppression of VASP phosphorylation due to the ADP–P2Y₁₂ interaction, and results are expressed as platelet reactivity index (PRI, %). Normal individuals who are not on any P2Y₁₂ antagonist have a PRI > 69%, and studies have demonstrated PRI < 50% to have a very high negative predictive value. Lower PRI is indicative of good response to clopidogrel, and higher PRI is indicative of a poor response to clopidogrel. Recent studies have demonstrated a correlation between PRI and clinical outcomes in PCI patients (Bonello et al. 2007), stent thrombosis (Morel et al. 2007), and recurrent ischaemic cardiac events (Frere et al. 2007).

Global Thrombosis Test

The Global Thrombosis Test (GTT, Montrose Diagnostics, London, UK) is a point of care test that is designed to assess platelet reactivity, thrombotic status and thrombolytic activity. The equipment comprises 4 channels, into which four plastic tubes can be inserted. The top of the tube consists of a detachable conical plastic tube which contains 2 metal ball bearings (Figure 2.1). Native blood is withdrawn with a 21 gauge butterfly cannula, first 2 mls discarded and subsequent 3-5 mls injected into the plastic GTT tubes within 15 seconds of venepuncture. Blood flows at 37 degrees by gravity through the narrow gap between the large metal ball and the conical plastic tube, and high shear stress (175 dynes/cm^2) in this space activates the platelets (Figure 2.2). Blood then flows through the space between the two ball bearings, where low shear and turbulent flow favour large platelet aggregation. Thrombin is generated by the platelets, and blood coagulation initiated. Large fibrin stabilized platelet aggregates are formed which flow in the space below the lower ball bearing and the conical end of the plastic tube, and eventually results in arrest of flow displaying Occlusion time (OT: seconds). Increased or decreased OT indicates inhibition or enhancement of platelet reactivity respectively. The instrument measures the time (d) between two consecutive blood drops. There is a sensor at the base of the unit, which generates a signal whenever a drop of blood interrupts the light path.

Blood collects in the bottom collecting tube, and the time interval increases gradually as the flow slows down, and when $d > 15$ seconds the end point of the initial measurement is displayed on the instrument as occlusion time (OT; seconds). This time coincides with the time it takes for the fibrin stabilized platelet aggregate to occlude the conical end of the tube. There is also a preset “thrombi stabilization time” following OT (200 sec), during which the sensors are inactive. This time allows stabilization of the formed thrombi, lasting occlusion and ignores small re-bleeds. Subsequently, blood flow restarts due to spontaneous thrombolysis of the formed thrombus, displaying a second reading Lysis time (LT; seconds). LT is the time difference between the last drop before thrombus formation, and the first drop after occlusion during restart of blood flow. When this time (d) exceeds 200 seconds, lysis time is displayed. $LT = (\text{time of first drop with } d > \text{lysis} - d) - (\text{time of last drop with } d < \text{lysis} - d)$. If lysis does not occur within 6000s, no lysis is recorded. Increase or decrease in LT indicates inhibition or enhancement of spontaneous thrombolysis, respectively.

The unique features of this test are that it uses native non- anticoagulated blood, uses high shear stress to activate platelets and appears to be more physiological than other platelet function tests that utilize agonists in citrated blood. It measures the effect of thrombin on platelet aggregation, which is inhibited in citrated blood due to low calcium levels. In a normal coronary artery (Stepp et al. 1999), shear stress is upto 19 cm^2 whereas in stenosed arteries (Ikeda et al. 1991) the shear stress can be up to 1500 dyne/cm^2 . The shear stress created in between the plastic tube and big ball bearing of the GTT tube is 175 dynes/cm^2 . The shear rate (G) for the GTT was initially calculated by Prof. Yamamoto and colleagues (Yamamoto et al. 2003) with the equation $4Q/pR^3$ where Q was the flow rate (ml/s) calculated by measuring the weight (and converting it to volume) and timing of the first three blood drops and R was the radius of the gap (cm). Shear rate was converted to shear stress by multiplying it with blood viscosity. This initial

shear stress (178 dynes/cm²) corresponds to and is even a bit higher than that which exists in a critical stenosis of 70% luminal stenosis (150 dynes/cm²) where thrombosis is likely to occur (Merino et al. JACC 1994). Shear stress as high as 3000 dyne/cm² has been reported at high levels of stenosis (Strony et al. 1993). There is data to suggest that high shear stress does not necessarily favour large platelet aggregate formation, as such aggregates get fragmented at this high force. At high shear, low shear is required immediately below the high shear level at the post stenotic level for aggregation to take place. There is also some evidence to suggest minimal platelet activation may be required for platelet aggregation and this shear induced process may be a result of mechanical cross linking of platelets (Zhang et al. 2002).

The principle of the GTT technique has been supported by experiments performed by Prof Yamamoto and colleagues who concluded that there it was the initial pressure that determined the rate of thrombus formation and there was no need to maintain the applied pressure accurately. The optimal initial pressure was 50-90 mmHg. They also demonstrated large platelet aggregates in between the lower ball bearing and conical end of the tube, but not in the space between the two balls. As it was difficult to ascertain some of the morphology due to small space between some gaps, they carried out specific experiments using tubes with none, one ball bearing or two ball bearings to confirm the mechanism of occlusion. In a tube with one ball bearing, occlusion time was greatly prolonged. Although aggregation occurred distal to the ball, the blood flow was not obstructed suggesting delayed occlusion was secondary to coagulation. On the contrary, in a tube with two ball bearings, shear activation of platelets in the first space in between the two balls resulted in generation of thrombin, and eventually arrest of blood flow. If platelet aggregation occurred in between the two balls, the aggregates would disintegrate due to the high shear in between the second ball and the conical lumen of the tube, and occlusion would not occur. Once the thrombus is formed, there is reduction of pressure and the thrombus is allowed to stabilise for a period of 200 seconds known as “thrombi stabilization time” during which the sensors are

inactive. This time allows stabilization of the formed thrombi, lasting occlusion and ignores small re-bleeds. The pressure then reverses and there is restoration of flow by dislodgement of the thrombus i.e. lysis occurs (Yamamoto et al. 2003). Fibrinolysis of the platelet thrombus requires shear stress of at least 250 dynes/cm^2 (Brown et al. 1975), hence when blood passes through the space between the big metal ball and the tube, platelets get activated and start aggregating, whereas lysis is minimal during that stage.

In a study by Taomoto et al, 185 stroke patients were tested using the GTT. Their OT and LT were compared to 195 healthy volunteers not taking any medication. Occlusion time was significantly shorter in stroke patients (mean age 65.5 years) compared to healthy volunteers (mean age 39.7 years) (OT: $210.3 \pm 140.8\text{s}$ vs. $284.9 \pm 92.2\text{s}$, $P = < 0.0001$) suggesting a prothrombotic state existed in the patient population. LT was significantly prolonged in stroke patients when compared to healthy volunteers (LT: $3159 \pm 1549\text{s}$ vs. $2231 \pm 1223\text{s}$, $P < 0.0001$) suggesting stroke patients had impaired endogenous thrombolytic activity (Taomoto et al. 2009).

All patients had been admitted with an acute cerebrovascular event, and MRI and MRA were the initial investigations performed for establishing the diagnosis. All 185 patients received Aspirin or Cilostazol, a thromboxane A₂ synthesis inhibitor such as Ozagrel, or anticoagulants agents such as Heparin or Warfarin. OT and LT were assessed again after patients had been stabilized on medication for at least 14 days, and OT in stroke patients on medication was significantly prolonged, and LT shortened suggesting medication reduced the thrombogenic potential in stroke patients.

Figure 2.1: GTT tube demonstrating 2 metal balls and space in between the balls and wall of the plastic tube (Saraf et al. 2009) - A flat segment created along the inner wall of a conical plastic tube forms the basis of the technique, since it prevents the round steel ball bearing from occluding the lumen. When blood is added, it flows through the narrow gaps by the ball and exits in droplets into an adjacent collecting tube. The latter is trans-illuminated and a light sensor generates a signal whenever a drop of blood interrupts the light path. The instrument detects the time interval [d] between consecutive blood drops.

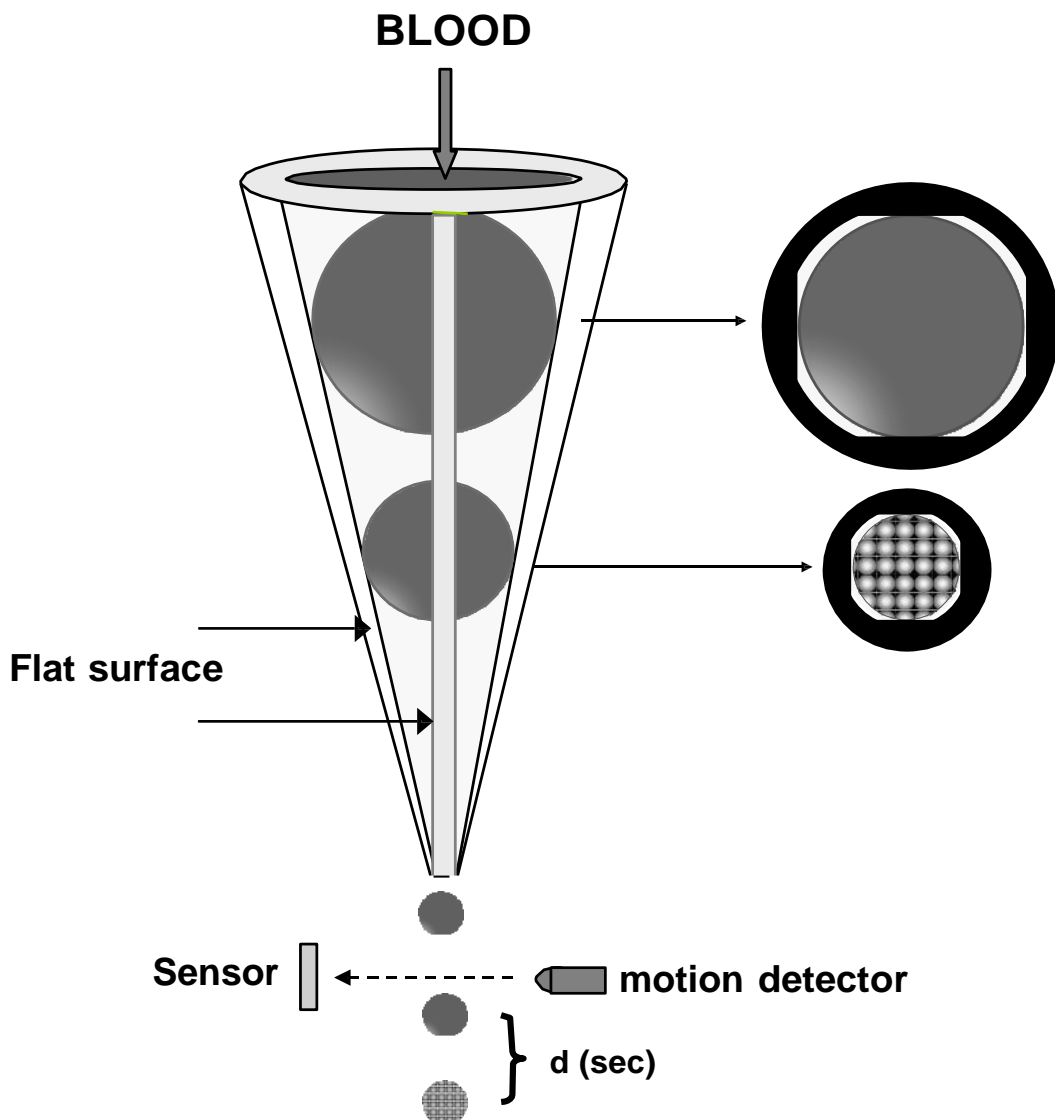
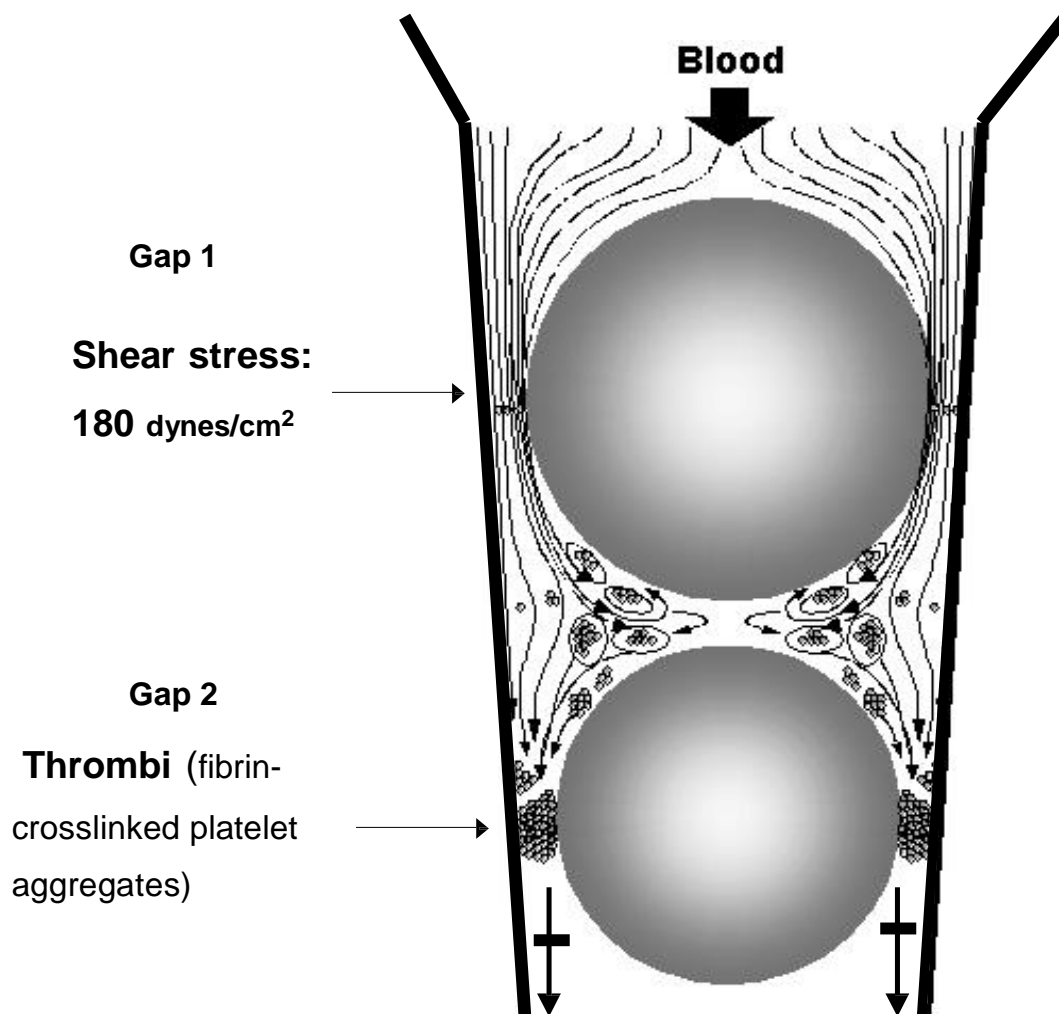


Figure 2.2: Principle of the GTT – Shear stress activates platelets and platelet aggregation begins in the space between the 2 metal ball bearings (Saraf et al. 2009)- Blood flows at 370C under the influence of gravity through a narrow gap [1]formed between the larger ball bearing and the inner wall of the tube, where high shear stress (175 dynes/cm²) activates platelets. These activated platelets remain single, since the very short transit time and high shear prevent aggregation. In contrast, in the space downstream, low shear and turbulent flow favour large platelet aggregate formation. The activated platelets generate thrombin and initiate coagulation. Flow then carries these fibrin-stabilised platelet aggregates into the gap [2] resulting in occlusion of the gap and arrest of flow.



Antiplatelet Resistance: Review of the evidence and extent of the problem

Platelet activation and aggregation play an important role in development of atherosclerosis and result in manifestation of conditions such as acute coronary syndrome, stroke and peripheral vascular disease. A significant percentage (8-60%) of atherothrombotic patients, despite being on optimum antiplatelet therapy continue to experience recurrent adverse cardiac events such as ACS, stroke, peripheral vascular disease and are considered to be resistant to antiplatelet medications (Hovensal. 2007). Patients resistant to aspirin or clopidogrel continue to experience thrombotic events and fail to demonstrate platelet inhibition, as measured in the laboratory using different platelet function tests (Cattaneo et al. 2004). Several studies have been carried out under different settings to determine the level of resistance and its relation with adverse events. There has been poor correlation so far amongst the various different platelet function tests and it is extremely difficult for clinicians to determine which method to use and in which cohort of patients. Therefore, new tests and treatments are being studied to help overcome this problem.

The proposed mechanism of antiplatelet resistance is multifactorial and depends on various intrinsic and extrinsic factors, and is outlined in Table 1.

Non compliance with aspirin accounts for 3% of aspirin resistant patients, and this resistance could be overcome by a monitored treatment program in hospital (Tantry et al.2005). Bioavailability of aspirin is increased with food, and enteric coated aspirin may affect absorption. Non steroidal antiinflammatory drugs (NSAIDS) seem to interact with aspirin by interfering with the COX pathway and increasing thromboxane A₂ production. Inflammation increases COX-2 activity and release of TXA₂ by platelets, resulting in prothrombotic events in patients on aspirin (Awtry et al. 2003). The Antithrombotic Trialists' Collaboration demonstrated 33.3% reduction in occurrence of non fatal MI, a 25% reduction in stroke and

16% reduction in vascular mortality in atherosclerotic patients on aspirin (Antiplatelet Trialists' Collaboration. 2005).

Similarly, clopidogrel resistance can be multifactorial and various mechanisms for resistance have been proposed in the literature. Non compliance, inadequate dosing and reduced gastrointestinal absorption are common causes. Various studies have demonstrated greater platelet inhibition with loading dose clopidogrel 600 mg, when compared to 300 mg (Muller et al. 2001). Clopidogrel is a prodrug that is converted to its active thiol metabolite by cytochrome (CYP) P enzymes CYP3A4 and CYP2C19, and CYP3A4 inhibitors such as erythromycin, clarithromycin, and antifungals such as ketoconazole, fluconazole, itraconazole affect clopidogrel conversion to its active metabolite (Taubert et al. 2004). Omeprazole is a CYP2C19 inhibitor, and various studies have demonstrated inhibition of clopidogrel with this proton pump inhibitor (Sibbing et al. 2009). Some patients demonstrating clopidogrel resistance have reduced CYP2C19 metabolic activity, which can be genetically determined (Boulenc et al. 2012). Enzyme inducers such as rifampin, carbamazepine, barbiturates, and St. John's wort increase CYP3A4 activity, and enhance the antiplatelet effects of clopidogrel resulting in increased bleeding risk in some patients.

Although variably defined, resistance to antiplatelet therapy is a serious emergent clinical entity for which an efficient diagnostic test as well as a management plan becomes imperative. The clinician is currently able to partially improve the responsiveness to antiplatelet therapy by acting on extrinsic factors, involved in the aetiology of resistance, including compliance to treatment, drug-drug interactions and good control of blood pressure, glycaemia and lipid levels. Antiplatelet resistance remains a major cause of concern, as antiplatelet agents are the most important therapeutic options in patients with coronary artery disease

Prevalence of Antiplatelet resistance in different populations

Stable Coronary artery disease

A prospective study of 326 patients with stable CAD were followed up for 2 yrs. 5.5% were resistant to aspirin by Light Transmission Aggregometry and 9.5 % resistant to aspirin by Platelet Function Analyzer 100 (Gum et al. 2001). Resistance to aspirin was defined as mean aggregation greater than 70% with 10 microM ADP, and mean aggregation greater than 20% with 0.5 mg/ml arachidonic acid. Another study in a cohort of 98 subjects showed 29.6% resistance in patients on Aspirin 160 mg/d. Resistance to aspirin by PFA-100 was defined as having normal collagen or epinephrine closure time less than 193 seconds. (Macchi et al. 2003).

Acute Coronary syndrome

In a study involving 204 patients (104 with ACS and 100 with stable CAD) 40.3% were resistant to aspirin by PFA 100 compared to 27% with stable CAD. The Warfarin Aspirin Reinfarction II Study (WARIS-II) study involved 202 patients allocated to receive either Aspirin, Warfarin or both. Aspirin resistance was observed in 35% taking aspirin alone and in 40% taking aspirin and warfarin. There was an increased CV event rate in aspirin non responders compared to responders (36% vs 24%) (Andersen et al. 2002).

Primary percutaneous coronary intervention

60 patients with acute MI undergoing primary PCI were loaded with 300 mg clopidogrel followed by 75 mg/d, and 300 mg aspirin followed by 200 mg /d. They were divided into 4 quartiles based on reduction in platelet aggregation. Patients in the first quartile had platelet aggregation 103+/- 8%, whereas those in the 2nd, 3rd and 4th quartile had platelet aggregation

of 69, 58 and 33 % of their respective baselines. After 6 months' follow up, 7 patients in the first quartile and 1 patient in the 2nd quartile had a CV event (Matetzky et al. 2004).

Elective percutaneous coronary intervention

In a study involving 151 Asian patients undergoing elective PCI, aspirin resistance was assessed by the Verify Now assay. All patients had been taking 80-325 mg aspirin for at least a week prior to the procedure and clopidogrel for 12-24 hrs prior to the PCI. 29 (19.2%) patients were deemed aspirin resistant (Chen et al. 2004). Another study looked at antiplatelet resistance to IIbIIIa inhibitors in 485 patients undergoing elective PCI using the Verify Now assay. Patients whose platelet function was inhibited by 90% or more had an event rate of 2% compared with 10% for patients with inhibition of less than 90% (Hochholzer et al. 2006).

Stent thrombosis

The CREST trial (Clopidogrel Effect on Platelet Reactivity in patients with stent thrombosis) showed high platelet reactivity in patients with stent thrombosis (ST) when assessed by LTA and VASP methods. Another study by Gurbel et al compared 20 patients with ST to 100 patients with patent stents. Platelet function was assessed by LTA and the results suggested that high post treatment platelet reactivity and incomplete inhibition of P2Y12 are risk factors for stent thrombosis (Gurbel et al. 2005). Another prospective study by Muller et al followed up 105 patients undergoing elective PCI and found that 2 patients who developed ST were resistant to clopidogrel (Muller et al. 2003). A study by Azenberg et al compared 10 patients with ST to 22 controls using the shear induced platelet

aggregation method and found that resistance to antiplatelet therapy and increased shear induced platelet aggregation correlated well with stent thrombosis (Ajzenberg et al. 2005). The interval between stent thrombosis and assessment of antiplatelet resistance in all the above studies was variable. Also, the patient population was diverse hence further trials are required to assess the relation between stent thrombosis and antiplatelet resistance mechanisms.

Cerebrovascular disease

In a study involving 180 post stroke patients, 33% patients were found to be aspirin resistant. All patients were followed up for 2 years and major end points were observed in 40% of aspirin resistant patients compared with 4% of aspirin responders ($p < 0.0001$) (Grottemeyer et al. 1993). Another small study compared 35 patients with symptoms (ischaemic stroke or TIA) in previous 3 days to 18 patients without symptoms (no CVA symptom for > 24 mths). All patients had been on aspirin for at least 5 mths prior to being tested for aspirin resistance. 34% of symptomatic patients were identified as aspirin resistant compared to none of the asymptomatic patients (Grundmann et al. 2003).

Peripheral vascular disease

A study involving 100 patients with intermittent claudication undergoing elective iliofemoral percutaneous balloon angioplasty were assessed for aspirin resistance. Corrected whole blood aggregometry (CWBA) was assessed at baseline and at regular intervals for up to a year post angioplasty while they were on aspirin 100 mg/d. AA, ADP and collagen were used as main stimulants, and significant platelet inhibition to AA was

noted. Inhibition to platelet aggregation using CWBA was reduced in male patients, who were noted to be at significant risk of reocclusion at site of angioplasty ($p= 0.009$). None of the female patients had reocclusion, and statistical analysis demonstrated significant difference in risk profile in the male patient group, including levels of smoking and HDL levels. 65 % patients were detected to be aspirin resistant with 8 male patients having a repeat vascular event during a 1 year follow up (Mueller et al. 1997).

Table 2.1 and 2.2 summarize prevalence of Aspirin and Clopidogrel resistance in different patient populations respectively.

Table 2.3 and 2.4 summarize clinical implications and relevance of Aspirin and Clopidogrel resistance in different patient populations

Table 2.1: Studies demonstrating prevalence of Aspirin non-responsiveness or resistance

Study	Number of subjects	Patient subgroup	Aspirin dose	Platelet function test	Prevalence of resistance (%)
Gum et al	325	Stable CAD	325 mg	ADP and AA induced optical aggregation	5.2
Mueller et al	100	PVD	100 mg	Corrected whole blood aggregometry	60
Grotemeyer et al	180	CVA	1500 mg	Platelet reactivity	33
Chen et al	151	Elective PCI	80-325 mg	RPFA	19
Anderson et al	202	Post MI	160 mg aspirin vs. 75 mg aspirin plus warafarin	PFA-100	35 % in aspirin only patients compared to 40% in patients on aspirin plus warfarin
Macchi et al	72	Stable CAD	160 MG	PFA-100	29.2
Helgason et al	306	CVA	300-325 mg	ADP induced platelet aggregation	25
Alberts et al	129	CVA	81 vs. 325	PFA-100	37 % overall 56% in patients on 81 mg vs. 28% in patients on 325 mg

Table 2.2: Studies demonstrating prevalence of Clopidogrel non-responsiveness or resistance

Study	Number of patients	Patient subgroup	Clopidogrel dose : loading (mg)	Clopidogrel dose: maintenance (mg)	Platelet function test	Prevalence of resistance
Gurbel et al. 2003	92	PCI	300	75	LTA	31-35%
Angiolillo et al.2005	52	Diabetics	300	75	LTA and Flow cytometry	38% in Diabetics , 8% in non-diabetics
Angiolillo et al.2005	48	PCI	300	75	LTA	44%
Lepantal o et al. 2004	50	PCI	300	75	LTA and PFA100	40%
Jaremo et al.2002	18	PCI	300	75	LTA	28%
Lev El et al.2006	150	PCI	300	-	LTA	24%
Moble et al.2004	50	PCI	300	75	LTA	30%
Muller et al.2003	119	PCI	600	75	LTA	5-11%
Barragan et al.2003	48	ISR (16) vs no ISR (32)	-	Clopidogrel 75bd vs Ticlopidine 250 mg bd	Flow Cytometric Assay	63.28 +/- 9.56% (in ISR) vs 39.8 +/- 10.9% (in non ISR)
Ajzenberg et al.2005	32	ISR (10) vs no ISR (22)	300	75	Shear induced platelet aggregation (SIPA)	41% cases vs. 18% controls at shear rate of 200/s. 57% cases vs. 23% controls at shear rate of 4000/s
Miatzky et al.2004	60	STEMI	300	75	LTA	25%
Dziewierz et al.2005	31	CAD	300	-	LTA	23%

Table 2.3: Studies demonstrating clinical implications of Aspirin non-responsiveness or resistance

Study	Gum et al.2003	Substudy ofHOPE.	Mueller et al.1997	Panukou et al.2006	Grottemeyer et al.1993
Patients and Follow up period	326 patients with stable CAD 2 years follow up	488 patients with MI, stroke or CV death 5 years follow up	100 patients with intermittent claudication who underwent percutaneous peripheral angioplasty 18 months follow up	105 patients with ACS 12 months follow up	174 patients with cerebrovascular disease 2 years follow up
Platelet function test	Optical aggregometry	Urinary thromboxane metabolite levels	Corrected whole blood aggregometry	PFA-100	Platelet reactivity test
Clinical implications of Aspirin Resistance	5.2% resistance, associated with increased risk of CV death, MI or stroke (HR: 3.12).	Patients in the upper quartile had 1.8 times higher risk than those in the lower quartile (p=0.009).	Risk of reocclusion of the site of angioplasty 87% higher in patients with failed inhibition of aggregation upon collagen and ADP	MACE occurred in 45% of patients with aspirin resistance and in 11.7% in Aspirin sensitive patients.	Current stroke, MI or vascular death was more likely to occur in aspirin non-responders compared with responders (40% vs 4.4% respectively) (p<0.001)

Table 2.4: Studies demonstrating clinical implications of Clopidogrel non-responsiveness or resistance

Study	Patients and Follow up period	Platelet function test	Clinical implications of Clopidogrel resistance
Matetzky et al .2004	60 patients with STEMI undergoing PCI 6 months follow up	ADP induced aggregation using LTA	Higher ADP induced platelet aggregation in recurrent cardiac events group (91±21 vs. 62±21%, p< 0.001)
Barragan et al.2003	16 patients with stent thrombosis compared to 30 patients with no stent thrombosis Retrospective study	Enhanced platelet reactivity using VASP assay	Stent thrombosis patients had higher VASP assay measured platelet reactivity (63.2±.6 vs. 39.8±10.9%, p<0.0001)
Ajzenberg et al.2005	49 patients (10 patients had stent thrombosis, remaining 39 were control group) Retrospective study	Shear induced platelet aggregation (SIPA)	Patients with Stent thrombosis had higher SIPA (41±12 vs. 18±8%, p=0.013 at shear rate of 200/s)
CREST study. Gurbel et al. 2005	120 Retrospective study	High post-treatment reactivity assessed by LTA and incomplete P2Y12 receptor inhibition assessed by VASP	Greater incidence of Stent thrombosis in patients with greater ADP induced aggregation (5µmol/l ADP: 49±4 vs. 33±2%,p<0.05 , 20 µmol/l ADP: 65±3 vs. 51±2%, p<0.001)
Cuisset et al. 2006	106 patients treated with PCI 1 month follow up	LTA assessed at the time of the intervention	Recurrent events in patients with greater ADP induced aggregation (p <0.0001)
EXCELSIOR. Hochholzer et al. 2006	802 undergoing PCI pre-treated with 600 loading dose of clopidogrel 1 month follow up	ADP induced platelet aggregation assessed by LTA immediately before intervention	MACE increased with increase in ADP induced aggregation (0.5% in first 2 quartiles,3.1% in 3 rd quartile, and 3.5% in 4 th quartile, p=0.034)
PREPARE POST-STENTING. Gurbel et al.2005	192 patients undergoing PCI 6 months follow up	ADP induced platelet aggregation assessed by LTA	Aggregation greater in patients with recurrent ischaemic events (63±2 vs. 56±15,p=0.02)
Lev et al.2006	150 patients (aspirin resistant and aspirin sensitive) undergoing elective PCI Assessed at discharge	LTA	Aspirin resistant patients had lower response to clopidogrel than aspirin sensitive patients (20µmol/l ADP:19 vs. 73%, p=0.001, 5µl/l ADP:18 vs. 79%,p=0.001)
Geisler et al.2006	379 (206 stable angina and 173 ACS) undergoing PCI, treated with 600 mg loading dose. 3 months follow up	Assessment of response to clopidogrel using LTA	Significant higher risk of major cardiovascular events in non-responders compared to responders (22.7% vs 5.6%; p=0.004)

There are many tests to assess platelet reactivity and these have demonstrated a large variability in the response to antiplatelet medication, with variable prevalence of “resistance”. The definition of “resistance” is fraught with difficulty as the different methods report different prevalences, depending on the test used, the cut-off value used to define resistance, the timing with respect to medication and the population studied. A metaanalysis by Hovens et al found heterogeneity in the prevalence of aspirin resistance, and this was due to the variability in results using different platelet function tests (Hovens et al. 2007). It is extremely difficult for clinicians to determine which method to use to assess platelet function and how to interpret the results. There has been no good correlation so far amongst the various different platelet function tests. Many are time consuming, and not applicable to a clinical setting. None fulfills the “ideal” criteria described in our introduction. Furthermore, to date, there is very little data to suggest that altering antiplatelet medication based on the results of laboratory tests of “resistance” improves clinical outcomes. A metaanalysis by Snoep et al (Snoep et al. 2007) suggests patients with laboratory aspirin resistance are more likely to experience adverse cardiac events, but it is important to point out that no prospective, well-powered clinical trial has assessed the benefit of tailoring antiplatelet medication specifically to populations with increased platelet reactivity. This is partly because we do not know which test or tests best define antiplatelet resistance and which medications best improve outcome in these patient populations. The approach to the problem of antiplatelet resistance has been to develop newer drugs to further inhibit platelet reactivity or increase the dose and timing of treatment with currently available antiplatelet agents. However, both these approaches have been targeted at “allcomers”, rather than specifically tailoring either of these approaches to those patients identified as being non-responders. Importantly, the common side effect of bleeding with all antiplatelet medications means that the risk vs. benefit ratio needs to be carefully balanced, and it may be more important

to individualize such medications to subjects identified as “resistant” rather than giving stronger medication or higher doses to allcomers. Furthermore, the prevalence of “resistance” to these newly developed antiplatelet medications has not been evaluated. We believe a simple, rapid, near-patient test, which is affordable and useful in the clinical (not just laboratory) setting needs to be validated in a large scale clinical trial, to identify patients with impaired response to antiplatelet medication. This would allow risk stratification and individualization of antiplatelet medication to improve outcome in these patients, with novel treatments or optimised doses of currently available drugs (Saraf et al. 2009).

Determinants of endogenous lysis (Lysis time), mechanism of fibrinolysis and plasma fibrinolytic markers

Fibrinolysis plays an important role in lysis of a platelet rich thrombus, and disturbances in the lytic system may result in thrombotic or haemorrhagic complications. Plasminogen is the main component of the fibrinolytic pathway, and it is converted to plasmin by tissue type plasminogen activator t-PA and urokinase type plasminogen activator uPA. Plasmin assists in degradation of fibrin and extracellular matrix proteins such as fibronectin, laminin, proteoglycan, and type IV collagen, and PAI-1 and α 2-antiplasmin help in regulation of the fibrinolytic system.

The most common determinants of endogenous fibrinolysis and fibrinolytic markers are: tissue type plasminogen activator (t-PA), urokinase type (u-PA) plasminogen activator, plasminogen activators inhibitors type 1 (PAI-1), alpha2-antiplasmin (alpha2-AP), plasmin-alpha2-antiplasmin (PAP) complexes, thrombin activable fibrinolytic inhibitors (TAFI), D-dimer and fibrin/fibrinogen degradation products (FDP).

Various markers of plasma fibrinolysis as mentioned above have been studied, but no conclusive evidence is available to suggest use of any particular fibrinolytic marker. Studies have used the different biomarkers with conflicting results, and the role of different fibrinolytic markers in predicting outcome of coronary artery disease remains undetermined. This is primarily due to variability in plasma levels of these proteins between and within subjects, and other non fibrinolytic properties of these proteins.

Tissue type plasminogen activator (t-PA)

t-PA is a serine protease found in endothelial cells, and activates fibrinolysis by converting plasminogen to plasmin. High plasma levels of t-PA antigen and activity have been reported

during the acute phase of myocardial infarction, and high levels during the subacute phase have been predictive of future adverse cardiac events (Soeki et al. 2002). An association between elevated t-PA concentrations and risk of subsequent cardiac events has also been noted in patients with stable angina (Jansson et al.1996). In the MIRACL (Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering) study, higher levels of t-PA were associated with recurrent adverse cardiac events. This may be due to an association between t-PA and sex, body mass index or smoking or due to formation of t-PA/PAI-1 complex (Kinlay et al. 2009). t-PA levels in healthy individuals (Gram et al. 2000) and post menopausal women are also predictive of future coronary artery disease (Pradhan et al. 2004).

Recombinant t-PA is used in clinical setting to treat STEMI, large pulmonary infarcts and ischaemic stroke. PAI-1 and PAI-2 are specific t-PA inhibitors produced by endothelial cells, smooth muscle cells, fibroblasts and hepatocytes. t-PA forms a complex with PAI-1 which has inhibitory effect on fibrinolysis and thus paradoxically reflects impaired endogenous fibrinolysis (Jansson et al. 1993).

Plasminogen activator inhibitor -1 (PAI-1)

PAI-1 is a serine protease inhibitor secreted primarily by endothelial cells, and inhibits fibrinolysis by inhibition of t-PA and Urokinase –type plasminogen activator (u-PA). Majority of PAI-1 is secreted by vascular endothelium, but it is also present in platelets, liver and vascular smooth muscle cells (Hinsberg et al.1991). It has the ability to bind to fibrin resulting preventing fibrin degradation, and its ability to bind to t-PA results in inhibition of fibrinolysis promoting thrombus formation. There are studies suggesting high levels of PAI-1 are independently associated with occurrence of first myocardial infarction in healthy young

individuals and subsequent increased risk of future cardiac events in patients with coronary artery disease (Thøgersen et al.1998). PAI-1 accumulates in vessels, and its accumulation is known to be associated with atherosclerosis resulting in plaque formation. Diabetic patients have been known to have higher PAI-1 levels, which may partly explain the increased incidence of coronary disease in this subgroup (Sobel et al. 1998).In the Stockholm Heart Epidemiology Program (SHEEP), plasma concentration of tPA/PAI-1 complex was significantly associated with risk of MI in both genders (Nordenhem et al. 2005). On the contrary, in the ADVANCE (Action in Diabetes and Vascular Disease) and a subgroup of the Framingham study, no significant correlation was seen between PAI-1 levels and adverse outcomes (Wang et al. 2007). Nevertheless, in the Caerphilly Study, elevated levels of PAI-1 were associated with higher incidence of cardiovascular events (Smith et al.2005).

Several factors stimulate release of PAI-1 and include the activated renin angiotensin system (RAS), hypertriglyceridaemia, hyperglycaemia, and hyperinsulinaemia. Oestrogen production in females is known to maintain PAI-1 at normal levels.

The **Renin angiotensin system** is primarily concentrated in the vascular and endothelial cells. Angiotensin II stimulates production of PAI-1 by binding to these endothelial cells. Angiotensin converting enzyme (ACE) converts Angiotensin I to Angiotensin II stimulating production of PAI-1 (Rakugi et al.1994). Bradykinin is a vasodilator that promotes t-PA production and promotes fibrinolysis. ACE increases Bradykinin degradation thus inhibiting fibrinolysis and promoting thrombus formation (Brown et al. 1999).

Elevated glucose and Insulin levels are known to stimulate release of PAI -1. Glucose stimulates PAI-1 release by stimulating transcription of PAI-1 gene in vascular smooth muscle cells. Good control of diabetes by antidiabetic medications thus helps in reduction of PAI-1 activity reducing thrombogenicity, and reduces risk of future cardiac events (Pandolfi et al. 1996).

Endogenous Insulin and its precursors promote PAI-1 activity and use of exogenous Insulin helps in reducing and PAI-1 mRNA expression, inhibiting PAI-1 mediated thrombosis (Jain et al. 1993).

Hypertriglyceridaemia is associated with coronary artery disease, and this can be explained by the association between triglycerides and PAI-1. VLDL and LDL triglyceride stimulates release of PAI-1 from endothelial cells, inhibiting fibrinolysis (Mussoni et al. 1992).

Thrombotic events are more commonly seen in post menopausal women. This is primarily due to the protection provided by oestrogen in premenopausal women and in post menopausal women on oestrogen containing Hormone replacement therapy (HRT). Oral oestrogen reduces production of PAI-1 from the liver and promotes fibrinolysis. Nitric oxide also inhibits PAI-1 expression, and oestrogen exerts a protective effect by increasing production of nitric oxide (Brown et al. 2002).

Pharmacological agents have also been shown to influence levels of PAI-1 and have a beneficial effect on the fibrinolytic system. Angiotensin converting enzyme inhibitors (ACE-i), Hormone replacement therapy agents (HRT), Biguanides like metformin, and HMG coenzyme-A reductase inhibitors like statin have shown to have a beneficial effect on the fibrinolytic system by altering levels of PAI-1.

In both human and animal models, **ACE-I** have been shown to significantly attenuate PAI-1 expression and activity. High dose quinapril demonstrated a 26% reduction in PAI-1 activity, $p= 0.08$ in normotensive patients who had no underlying cardiovascular, renal, pulmonary or endocrine disease (Brown et al. 1998). A study by Vaughan et al demonstrated a 44% reduction of PAI-1 antigen in 120 patients who were started on ramipril within 24 hours of an acute myocardial infarction (Vaughan et al.1997). Captopril provided a significant reduction of PAI-1 antigen level when initiated after 2 days ($p= 0.02$) and 1 month ($p< 0.001$) of an

AMI (Moriyama et al. 1998). In another study, captopril reduced PAI-1 antigen level by 46% when initiated 8 weeks after an AMI ($p= 0.001$) (Wright et al.1994).

Hormone replacement therapies have various beneficial effects when taken in postmenopausal women. They improve carbohydrate metabolism by controlling hyperglycaemia and hyperinsulinaemia, and regulating body fat distribution. They decrease LDL cholesterol and increase HDL cholesterol levels, and play an important role in control of insulin resistance syndrome. Oral oestrogen reduces PAI-1 antigen levels and activity in contrast to transdermal oestrogen. This is possibly due to suppression of hepatocyte production and enhancement of hepatic clearance of PAI-1. HRT also increase t-PA activity by reducing t-PA Antigen levels and hence promote fibrinolysis. They increase nitric oxide bioavailability which in turn inhibits expression of PAI-1. In a study of 288 postmenopausal women, Shahar et al demonstrated a 28% reduction in PAI-1 antigen ($p= 0.04$) and 18% reduction in t-PA antigen level ($p= 0.004$) in post-menopausal women on HRT when compared to post-menopausal women not on oestrogen replacement. Oestrogen is also known to elevate triglyceride levels in post-menopausal women, but PAI-1 activity reduction was independent of this effect as demonstrated in the Atherosclerosis Risk in Communities (ARIC) study (Shahar et al. 1996). A study by Katz et al demonstrated 45% reduction of PAI- 1 levels only in the early hours of the morning in postmenopausal patients on oestrogen replacement, suggesting circadian rhythm plays an important role in fibrinolysis (Katz et al. 1996).

Biguanides like metformin are used in the treatment of Type 2 Diabetes Mellitus. These drugs improve insulin sensitivity; reduce glucose production by the liver and increase it's utilization in peripheral tissues. They reduce cholesterol levels and aid in weight reduction. They act as a fibrinolytic by reducing PAI-1 levels. Several studies have demonstrated significant reduction in the levels of PAI-1 antigen in diabetic patients on

metformin. Grant et al demonstrated significant reduction in PAI-1 antigen levels in 75 diabetic patients on different doses of metformin. Reduction of PAI-1 was independent of the dose of metformin used, and a significant reduction was noted when compared to placebo group who were not on biguanide treatment (Grant et al. 1996). In the BIGPRO -1 study, 457 non diabetic obese patients were randomized to metformin or placebo. There was 30-40 % reduction in PAI-1 antigen and activity level in both groups, but no added benefit was seen in the metformin group compared to placebo (Charles et al. 2000).

Thiazolidinediones lower blood glucose levels and improve insulin sensitivity. In a small study by Kato et al, high doses of troglitazone were shown to reduce PAI-1 antigen and activity level in diabetic patients, but no reduction in PAI-1 was seen in non-diabetics or healthy lean patients (Kato et al.2000).Larger studies are required to determine the true effect of this medication on fibrinolysis.

Insulin therapy is used in diabetic patients to lower glucose levels. Hyperinsulinaemia results in elevated PAI-1 levels, and insulin therapy improves insulin sensitivity and helps in reduction of PAI-1 and aids fibrinolysis in diabetic patients (Lormeau et al. 1997).

Statins are HMG-Coenzyme A reductase inhibitors that help lower cholesterol levels in patients with hypercholesterolaemia, but also in patients with normal cholesterol levels. Several small studies have been carried out comparing effect of statins on PAI-1 activity. A study by Weisbauer et al demonstrated reduction of PAI-1 production by all statins but pravastatin (Weisbauer et al.2002). On the contrary,a study by Dangas et al demonstrated a significant 22% reduction in PAI-1 levels in 57 hyperlipidemic patients on pravastatin when compared to placebo. This effect was seen independent of cholesterol reduction (Dangas et al.2000). A study by Isaacsohn et al showed a significant decrease in PAI-1 activity with lovastatin (Isaacsohn et al.1994), whereas another small study by Zambrano et al

demonstrated no effect on PAI-1 with lovastatin in hyperlipemic heart transplant patients (Zambrano et al.1997).

Urokinase type (u-PA) plasminogen activator

Urokinase type plasminogen activator is a serine protease found in the blood stream plasma and extracellular tissue. It is a plasminogen activator, which when activated to plasmin facilitates fibrinolysis. It is found in plasma concentrations of 2 to 4 ng/ml. Recombinant urokinase is used in treatment of pulmonary embolism, and in flushing haemodialysis catheters.

Plasmin-alpha2-antiplasmin (PAP) complex

PAP is a serine protease that inactivates plasmin. There have been several studies to demonstrate relationship between PAP levels and future coronary events. In the Cardiovascular health study, PAP levels were predictive of myocardial infarction in healthy individuals > 65 years age (OR: 3.1) (Cushman et al. 1999). Similarly, association was noted between PAP levels and death in the large Multiethnic study of Atherosclerosis (HR: 2.0) (Folsom et al.2009). By contrast, in the AtheroGene study, PAP levels had no association with future adverse cardiac events (Morange et al.2006).

Thrombin activable fibrinolytic inhibitors (TAFI)

TAFI is a carboxypeptidase B-like proenzyme identified in platelets at a concentration of 50 ng/l x 10⁹. It is activated by thrombin, either alone or in combination with thrombomodulin to form TAFIa. TAFIa is the active form of TAFI, and is associated with down regulation of fibrinolysis by removal of C-terminal lysine's from fibrin. It is synthesized in the liver, and active form has a half-life of several hours. People deficient in TAFI tend to suffer with bleeding diathesis. A study by Cloucci et al demonstrated increased bleeding in cirrhotic

patients was secondary to hyperfibrinolysis due to deficiency of TAFI. TAFI antigen is measured using ELISA and Clot Lysis assay (Colucci et al. 2003). There is data to suggest that both high and low levels of activated TAFI antigen are seen in patients with coronary artery disease. High TAFIa levels were seen in the Athero Gene study (Tregouet et al.2009) and were independently associated with a high risk of cardiovascular death (HR: 1.7). On the contrary, in the ATTAC (The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age) and SMILE study (Study of Myocardial Infarctions Leiden), low levels of TAFIa were associated with a higher incidence of cardiovascular disease (Meltzer et al.2009).

Endogenous Thrombolysis- A neglected entity, with prognostic usefulness in Acute Coronary Syndrome

Endogenous thrombolysis is considered a protective mechanism against lasting arterial occlusion, and acute myocardial infarction has been considered a result of failure of timely spontaneous thrombolysis (Swan et al. 1989).

As discussed in the previous chapter, the vascular endothelium cells synthesize various thrombotic and thrombolytic agents, and these agents are activated and released during vessel wall injury and are regulated by nitric oxide release. The common agents that stimulate thrombosis are PAI-1, PAI-2, TAFI, D-dimer and Protein C and the common fibrinolytic agents are t-PA and u-PA. A balance between the activity of these pro and anti fibrinolytic factors is crucial in determining the efficacy of endogenous thrombolysis. In various clinical conditions like diabetes mellitus, multiple sclerosis, severe sepsis and advanced cirrhosis, endogenous thrombolysis is impaired resulting in a prothrombotic state. Great individual variation is seen in the efficacy of the fibrinolytic system in individuals, and is hugely dependent on activity of PAI-1 antigen, TAFI and t-PA levels. Several factors can result in failure of spontaneous lysis, common factors being excess circulating PAI-1 antigen, lack of lytic elements, increasing thrombogenicity of the plaque secondary to increase in size, composition or location. Marked variation in the level of plasma fibrinolytic proteins levels both between and within subjects, and its antifibrinolytic effects may explain reduced interest in this important clinical entity. Below, we discuss the various studies that have demonstrated beneficial effects of endogenous thrombolysis in atherothrombotic patients.

In a study by Swan et al (Swan et al. 1989), patients with occluded arteries had coronary angiography few weeks to months after their initial AMI, and patency of the culprit artery was demonstrated during angiography, suggesting endogenous thrombolysis had a key role to play in the dissolution of the thrombus. In another study, greater than 90% AMI patients had

complete occlusion of the affected coronary artery secondary to thrombus within 4 hours of cardiac chest pain, and repeat angiography 12 hours later demonstrated 30-40% spontaneous recanalization of this artery (DeWood et al. 1980). Hackett et al demonstrated spontaneous intermittent coronary reperfusion in a small study of 45 AMI patients. Continuous ECG and serial coronary angiography was performed in these patients, and in 8 patients, the ST elevation on ECG returned to baseline prior to the coronary arteriogram being performed suggesting spontaneous reperfusion was common in early stages of an AMI (Hackett et al. 1987). In a study by Rentrop et al, 122 acute MI patients were randomized to intracoronary streptokinase, intracoronary nitroglycerin, intracoronary streptokinase and intracoronary nitroglycerin, or conventional therapy without initial angiography. In 67% patients, baseline angiography demonstrated complete occlusion of the infarct related artery (Rentrop et al. 1984). However, at 10-14 days repeat angiography demonstrated patency of culprit vessel in 74% patients irrespective of treatment received, demonstrating the positive effect of endogenous thrombolysis. Evidence also suggests spontaneous reperfusion occurs within 40 minutes in unstable angina, at 60-90 minutes in partial thickness myocardial infarction, and greater than 3 hours in full thickness myocardial infarction. Late spontaneous lysis also occurs in a significant proportion of patients, especially those developing a left ventricular aneurysm (Forman et al. 1986). In a study by De Wood et al, 29 of 36 patients with AMI had completely occluded arteries, of which 17 demonstrated spontaneous recanalization on repeat angiography. Left ventricular function was measured in all patients, at baseline and during repeat angiography. Although no significant difference was observed in the completed occluded group ($55\pm 8\%$ to $52\pm 8\%$, $P=NS$), a significant improvement in ejection fraction was noted in the spontaneous recanalization group ($44\pm 15\%$ to $56\pm 10\%$, $P= 0.05$). No significant difference was observed in the ejection fraction difference in the 2 groups during repeat angiography ($52\pm 8\%$ versus $56\pm 10\%$, $P=NS$), nor

did all patients with spontaneous reperfusion demonstrate improvement suggesting larger studies are required to study the beneficial effects of recanalization (DeWood et al.1985).

Myocardial salvage using technetium -99m sestamibi perfusion imaging was determined in AMI patients treated with aspirin and heparin, and patency of the infarct related artery was seen to increase from 16-24% during the first 24 hours to 57-64% after 3 days. Heparin is an anticoagulant, and prevents conversion of prothrombin to thrombin preventing further clot formation and propagation. It does not act as a thrombolytic, and increase in perfusion noted was presumed to be secondary to spontaneous reperfusion of the infarct related artery (Christian et al. 1991). Myocardial salvage was detected in 21 patients, and noted to be around $6\pm 11\%$ of the left ventricle, and was significantly greater with patent infarct related arteries, compared to arteries that were completely occluded ($P= 0.001$)(Christian et al.1998). More data from 8 studies have suggested that patency of the infarct related artery ranged between 9-28% before thrombolytic therapy or angioplasty, and increased to 36-78% by 72 hours on heparin infusion in the absence of any other reperfusion therapies (Granger et al. 1992). Significant symptomatic benefit with resolution of chest pain was noted in patients with patent infarct related arteries (100% vs 55%, $P=0.003$).

Lee et al demonstrated that reperfusion secondary to spontaneous fibrinolysis resulted in faster coronary blood flow and significantly improved clinical outcomes. The study included 199 patients with STEMI who underwent primary angioplasty. 6 weeks post angioplasty, patients with spontaneous reperfusion in this subset of patients had a higher rate of TIMI 3 flow, and mortality, reinfarction and congestive heart failure were significantly lower in this group of patients (4.5% vs 18.4%, $P <0.05$) suggesting spontaneous reperfusion was a prognostic indicator in AMI patients undergoing primary angioplasty (Lee et al. 2001).

There are case reports on spontaneous fibrinolysis of thrombi in internal carotid arteries (Calleja et al. 2004), and peripheral arteries (Weiner et al. 1984) suggesting spontaneous

reperfusion secondary to endogenous thrombolysis can occur both in the central and peripheral vasculature.

A Japanese group led by Ikarugi et al. investigated the extent of platelet reactivity and spontaneous thrombolysis in men and women using the GTT, and significantly reduced thrombolytic activity were noted in men > 51 years age. 145 normal subjects without any significant cardiac history or risk factors were tested using the GTT. No significant difference was observed in the OT values in the different age groups in either men or women, but LT was significantly prolonged in older men > 51 yrs age when compared to middle aged men aged 31-50 years (LT: $3657 \pm 461s$ vs $2398 \pm 236s$, $P= 0.0002$) (Yamashita et al. 2005). Coronary artery disease is more prevalent in men, and these findings are in agreement with this observation. Another Japanese study in 64 subjects demonstrated significantly longer LT's in elderly men compared to the young men (mean age 64.5 ± 1.1 years in the elderly, LT: 4555.0 ± 187.2 vs $3134.2 \pm 249.3s$, $P < 0.001$) using the GTT. In elderly smokers, LT was prolonged when compared to elderly non-smokers (LT 5407.1 ± 83.4 vs $2910.4 \pm 404.6S$, $P < 0.001$), and may be secondary to age related endothelial dysfunction and reduced nitric oxide release (Ikarugi et al. 2003).

Tests to assess endogenous plasma fibrinolysis

Global assays of fibrinolysis have been in place for a while, but none of the tests are really used in the clinical setting. They are time consuming, labour intensive and most importantly there is uncertainty with regards to which biomarker to be measured, as limited data is available on fibrinolytic marker levels and cardiac outcomes. Furthermore, most of the tests available measures clot lysis as opposed to thrombolysis. Below, I discuss the different available tests for assessing fibrinolysis below, with their merits and shortcomings.

Fibrin plate method

The fibrin plate assay was introduced in 1952 by Astrup and Mullertz. Plasminogen was added to the fibrin plates, and was incubated with urokinase or streptokinase for few hours. This assay has been reported to be complicated, time consuming, unreliable and has low reproducibility (Marsh et al. 1972).

Euglobulin Clot Lysis time (ELT)

The ELT is a test that measures overall fibrinolysis. It uses addition of acid to citrated platelet-poor plasma to precipitate clotting factors in the euglobulin fraction. The euglobulin fraction also contains fibrinolytic factors like fibrinogen, PAI-1, t-PA, Plasminogen, Factor VIII, and alpha 2 antiplasmin. Addition of calcium is required to activate clotting, and subsequently there is clot lysis. The test is sensitive to temperature and pH, and data suggests results are not reproducible and it is not a reliable test for measurement of fibrinolysis (Katz et al. 1970).

Fibrinolysis Parameters Assay (FIPA)

This assay of fibrinolysis measures the amount of plasmin activity in blood. The FIPA reagent consists of urokinase, tranexemic acid, and albumin and is added to citrated blood. Further reagents are added to determine plasmin activity. At fibrinolysis reaction time (FRT)

of 10 mins at 37°C, maximally inducible plasmin activity is measured. Normal range is 100±15% (MV ±1 SD). This assay is not sensitive to physiological concentrations of pro-urokinase and tissue type plasminogen activator, and has its limitations in clinical practice (Stief et al. 2000).

Intrinsic Oxidative Clot Lysis Assay (INOXLA)

Granulocytes are mediators of fibrinolysis, and release prourokinase, chloramine and NADPH - oxidase, which in turn release superoxide anion and play a role in fibrinolysis. Chloramine generates O₂ which enhances urokinase mediated fibrinolysis. Plasma is incubated with various agonists like calcium chloride to which chloramine is added. Urokinase is then added to this microtitre plate, and fibrinolysis occurs. Normal range for INOXLA has been reported as 100% ±25% (mean ±SD). If the urokinase activity is quenched by PAI -1 no clot lysis occurs. Based on this principle, the INOXLA has been developed where clot turbidity helps in determination of clot mass or clot lysis (Steif et al.2007).

Limitations of current platelet function tests, Rationale behind assessment of platelet reactivity and need for newer testing modalities

Current European Society of Cardiology Guidelines do not recommend routine use of platelet function testing as dose adjustments of clopidogrel have not demonstrated any clinical benefit (Price et al. 2011). A IIb indication has been issued for testing of high platelet reactivity in selected NSTEMI patients who are on clopidogrel. They also recommend use of novel antiplatelet agents Prasugrel or Ticagrelor in PCI treated NSTEMI patients, provided there is no contraindication (Hamm et al. 2011). The AHA/SCAI Guidelines 2011 recommend use of platelet function testing in patients who are at high risk of clinical events after PCI and switching them to novel antiplatelet agents if their results are suggestive of high on treatment platelet reactivity (Levine et al. 2011).

The platelet function tests that have shown to predict clinical outcomes are the Verify now assay, Multiplate assay, VASP and the LTA (Freyenhofer MK et al. 2011, Stone et al. 2013).. However, due to the lack of standardization with the LTA, use of LTA is only recommended if other assays are not available (Bonello et al. 2010).

In a study of 297 patients with ACS (Pannicia et al. 2009), residual platelet reactivity was assessed using the MEA, LTA and the PFA-100 and significant correlation was noted between the MEA and the LTA ($P < 0.0001$) and between MEA and the PFA-100 ($P < 0.0001$). On the other hand, in another study by Gaglia et al (Gaglia et al. 2011), 200 patients undergoing PCI and on clopidogrel were assessed for HPR using the VASP, LTA and the Verify now assay. The percentage of patients with high HPR using the VASP was 39.3%, 27.3% with Verify Now, and 23.1 % (ADP 5 μ M and 16.2 % (ADP 20 μ M) with the LTA. No significant correlation was noted between the three tests (Bonello et al. 2010). Another study of 201 stable coronary artery disease patients did not demonstrate any significant correlation using different platelet function tests. The prevalence of aspirin was 10.3–51.7% using the LTA, 6.7% for Verify now assay,

18.0% for whole blood aggregometry, 59.5% for PFA-100, and 22.9% by measuring urinary 11-dehydro-thromboxane B2 concentrations (Lordkipanidzé et al. 2007).

The ADAPT-DES registry was a large multicenter registry that demonstrated early (HR: 3.00, 95% CI: 1.39–6.49, $P = 0.005$) and late stent thrombosis (HR: 2.49, 95% CI: 1.43–4.31, $P = 0.001$) in patients with HPR using the Verify now assay (Stone et al. 2013). In the TRILOGY-ACS study, medically managed NSTEMI patients on prasugrel demonstrated greater platelet inhibition compared to clopidogrel, but this did not translate into improved clinical outcomes (Gurbel et al. 2012). In the GRAVITAS study, despite higher platelet inhibition using the Verify now assay no significant difference in MACE was noted between high dose and standard dose clopidogrel groups (HR: 1.01, 95% CI: 0.58–1.76, $P = 0.98$) (Price et al. 2011). Similarly, in the RECLOSE-2 ACS study, although patients with HPR had an increased risk of cardiac death and stent thrombosis, increasing the dose of clopidogrel did not reduce their risk of an adverse event (Parodi et al. 2011). Although higher doses of clopidogrel did not improve outcomes in these studies, it remains to be seen if switching these HPR patients to newer antiplatelet agents will result in improved outcomes.

The major limitation of the above mentioned available platelet function tests are that they are all performed on citrated blood. Citrate reduces the plasma Ca^{2+} concentration from 0.94–1.33 mM to 40–50 μM . Platelet aggregation is optimum at levels of 100 μM Ca^{2+} , and at levels below 10 μM , platelet aggregation does not occur. In citrated blood, thrombin is not generated, and Ca^{2+} levels are significantly reduced to 40–50 μM , resulting in suboptimum platelet aggregation (Ataullakhanov. 1994, Moore et al. 1970,).

In most platelet function tests, the citrated sample is tested between 30 mins to 48 hours of collection. During this duration, the platelet behavior and response to aggregating stimuli change resulting in minimal response to aggregation, as maximum aggregation is demonstrated upto an hour after collection of blood (Rossi et al. 1975).

Above mentioned platelet function tests in clinical use measure response of platelets to only one particular agonist, either ADP, epinephrine, arachidonic acid or collagen. The effect of shear stress and thrombin is not measured due to reasons mentioned above. Also, overall fibrinolytic status is difficult to ascertain using these platelet function tests as most of these tests measure response to only one or few, but not all fibrinolytic markers. Hence, it is difficult to ascertain the individual's true thrombotic and thrombolytic status using currently available platelet function tests, and hence the need for a more global test of thrombosis and thrombolysis.

The GTT is performed on native blood immediately after collection; is shear induced, and is able to assess the effect of thrombin on thrombosis. It is also able to assess endogenous thrombolytic activity and hence seems to be a more physiological test than currently available platelet function tests in clinical use.

In the last few years there has been a significant reduction in the rate of recurrent events after an ACS due to routine use of antiplatelet therapy and interventional procedures. However, the risk of a recurrent event remains high. Recent studies suggest most of these events occur after discharge in the first year after the initial event (Fox et al. 2010), with a recurrence rate of approximately 10% per annum (Wallentin et al. 2009, Wiviott SD et al. 2007).

Dual antiplatelet therapy is currently recommended for upto 1 year after an ACS (Wright et al. 2011). Although tailoring clopidogrel therapy based on platelet function assay results is currently not recommended, Expert groups recommend platelet function testing in patients at high risk of events post PCI or at risk of stent thrombosis (Aradi et al. 2013). It remains to be seen if newer and novel antiplatelet agents such as prasugrel, ticagrelor, and direct thrombin inhibitors in due course can improve clinical outcomes in patients with HPR.

Chapter 2: Methodology

Platelet function was assessed in healthy volunteers and in patients with coronary artery disease using the Global Thrombosis Test. A detailed description of this test has been described in Chapter 1. Testing was performed in healthy volunteers to develop a normal range, and then assessed and compared to various different patient populations. It was our aim to assess whether impaired thrombotic and thrombolytic status in patients with coronary artery disease had any relationship to adverse outcomes.

Sampling procedure

Blood samples were taken from an antecubital vein using an 18 –G butterfly cannula using a 2- Syringe technique. The first 3 mls of blood was either discarded or used for routine blood tests and the next 2 ml blood samples were used to assess thrombotic and thrombolytic status. This blood sample was injected with force into the GTT tube, and the shear force between the small aperture between tube and ball bearings resulted in platelet activation and subsequent aggregation. Measurement was started within 15 seconds, and the instrument indicator changed colour once the analysis was complete. Each instrument had a memory card, which could be connected to a computer to download and view the measurement graphs.

Healthy Volunteers

A normal range was established using the GTT in 100 healthy volunteers. The study was approved by the local Research Ethics Committee, and all volunteers gave written informed consent. Advertising for healthy volunteers was done via the Hospital trust bulletin, and posters were put up in hospital wards and outpatient units. A brief summary of the study and need for control group was included in the poster. Recruited volunteers were nonsmokers, not on any regular medication, and were advised not to take any medication with known platelet effect in the preceding 7 days (aspirin or the oral contraceptive pill). None of the volunteers recruited were

heavy alcohol consumers and all were asked to abstain from alcohol the night before sampling. Blood pressure and ECG were not performed prior to recruitment, but none of the volunteers had any known underlying illness, history of hypertension or cardiac disease. All healthy volunteers were tested in the morning, between the hours of 0900 and midday.

ACS patients

Patients admitted to hospital with ACS were included in the study. ACS was defined by the presence of at least two of the following: ischaemic chest pain, elevation of cardiac enzymes (troponin or creatine kinase isoenzyme at least twice the upper limit normal limits), or dynamic electrocardiographic changes (ST elevation, ST depression or T wave inversion). All patients received dual antiplatelet therapy for a year since index admission. Exclusion criteria are listed in Table 6.1. All patients were loaded with Aspirin 300 mg and Clopidogrel 300 mg on admission, and were taking 75 mg each of Aspirin and Clopidogrel thereafter as maintenance dose. 300 patients admitted with ACS were included in the study and sampled during their index admission. Most ACS patients receive unfractionated low molecular weight heparin (LMWH) on admission, and all these patients were sampled a minimum of 48 hours, (5 ± 3 days after admission, Mean \pm SD) after discontinuation of LMWH to avoid any effect of the anticoagulant on OT and LT. Fasting was not required. All patients received dual antiplatelet medication for a whole year for follow-up as per normal clinical care. Informed written consent was obtained from all subjects, and the study was approved by the local REC.

Stable angina patients pre and post Aspirin

We measured OT and LT using the GTT in 10 stable angina patients. All patients were reviewed and recruited in the outpatient clinic, and none of these patients had been on Aspirin prior to being tested. None of these patients were on any other antiplatelet medications. Baseline samples were taken prior to starting Aspirin. Post aspirin samples were taken at

least a week after each patient had been taking 75mg aspirin. None of these patients had been loaded with higher doses of aspirin.

Healthy volunteers pre and post Clopidogrel

Thirteen normal healthy volunteers were tested before and 8 hours after a loading dose of 300 mg clopidogrel. Volunteers were non-smokers, not taking any regular medications and in particular, did not take any medication with known platelet effect (such as aspirin or the oral contraceptive pill) in the preceding 7 days. Testing was performed at the same time of the day by the same operator, under similar conditions.

Effect of Clopidogrel on OT and LT in Stable angina patients on Aspirin

We examined the effect of clopidogrel in Stable angina patients. Ten patients with stable angina were tested on 75mg Aspirin (pre clopidogrel), and retested at least a week later on aspirin 75mg and clopidogrel 75 mg. Testing was performed by the same operator, under similar conditions.

Western and Japanese volunteers

We collaborated with Professor Yamamoto and his colleagues in Kobe Gakuin University, Kobe, Japan. Our aim was to compare thrombotic and thrombolytic activity in the native Japanese and native Western population, to determine if ethnicity had any effect on thrombotic and thrombolytic status. 100 volunteers in each group were tested. Advertising for healthy volunteers was done via the Hospital trust bulletin, and posters were put up in hospital wards and outpatient units. A brief summary of the study and need for control group was included in the poster. Recruited volunteers were nonsmokers, not on any regular medication, and were advised not to take any medication with known platelet effect in the preceding 7 days (aspirin or the oral contraceptive pill). None of the volunteers recruited were heavy alcohol

consumers and all were asked to abstain from alcohol the night before sampling. Blood pressure and ECG were not performed prior to recruitment, but none of the volunteers had any known underlying illness, history of hypertension or cardiac disease. All healthy volunteers were tested in the morning, between the hours of 0900 and midday.

The Accelerated Global thrombosis test (a GTT)

The aim of this modification was to induce ADP release from RBC, and assess the effect of this on shear-induced thrombosis. Due to the osmotic gradient, contact between RBC/platelets and water would result in haemolysis causing localised release of ADP from RBC and platelets, which we postulated would accelerate the thrombotic process and reduce OT. Prior to injection of blood in the GTT tube, 0.5 ml distilled water was added to the tube. Water was added 5-10 minutes before injection of blood to the tube, and filled the space between the two balls (100 μ l) and the upper water level was constantly 1-2 mm above the upper ball (Fig 5.1). Due to the osmotic gradient, on contact of water with blood there was cell destruction, haemolysis and release of ADP from RBC and platelets which we postulated would accelerate the thrombotic process and result in shortening of OT. This method was used to assess the effect of endogenous ADP release on OT and LT. To assess the effect of exogenous ADP, we preloaded a GTT tube with ADP solution (5 μ M ADP in saline; 0.5 ml) prior to injecting native blood from normal volunteers.

Clopidogrel in aGTT

Thirteen healthy volunteers were tested using the aGTT before and 8 hours after a loading dose of clopidogrel. Before introducing the blood sample into the GTT, a small volume of distilled water was placed into the tube, which remained at the site where platelet activation occurred. Normally, when the inflowing native blood would come in contact with distilled water, haemolysis would occur with release of ADP resulting in shortening of OT. Clopidogrel is an ADP antagonist, and would prevent this shortening of OT, demonstrating sensitivity of the individual to this thienopyridine derivative.

In clopidogrel resistant individuals, the shortening of OT would not be neutralised by the administration of clopidogrel.

Saline priming of GTT tubes

The aim of saline priming of the GTT tube was to avoid RBC haemolysis and ADP release that may be caused due to contact of blood with the plastic surface of GTT tube, or due to contact of air with blood. Saline 0.5 ml was added to the GTT tube 5-10 minutes before addition of blood. The upper level of saline was maintained 1-2 mm above the upper metal ball.

Verify Now assay

In a subgroup of 71 patients, thrombotic status was assessed using the Verify Now assay (Accumetrics, San Diego, California) and compared with thrombotic status measured using the GTT. Venous blood samples were taken and anticoagulated with sodium citrate 0.109 mol/l (ratio 9:1). The VerifyNow system is a turbidometry-based optical detection device that measures platelet-induced aggregation in a system containing fibrinogen-coated beads (9). The instrument measures changes in light transmission and thus the rate of aggregation in whole blood. In the cartridge of the VerifyNow P2Y12 assay, there is a channel in which inhibition of the adenosine diphosphate (ADP) P2Y12 receptor is measured. This channel contains ADP as platelet agonist and prosta- glandin E1 as a suppressor of intracellular free calcium levels, to reduce the nonspecific contribution of ADP binding to P2Y1 receptors. Results are expressed as P2Y12 reaction units (PRU). Recently, in patients with ACS, a cutoff level of PRU 240 was shown to be predictive of major cardiovascular events. The VerifyNow system does not assess thrombolytic status.

Chapter 3: Platelet function in Healthy Volunteers and comparison with Japanese volunteers

Background: The Global Thrombosis Test is a point of care platelet function test, that assesses the time to form a thrombus (OT: seconds), and subsequently determines the time to lyse the formed thrombus (LT: seconds). Our aim in this study was to develop a normal range in healthy Caucasian volunteers, and to determine if OT or LT was normally distributed in healthy individuals, who were not on any medication.

We also collaborated with Professor Yamamoto and his colleagues in Kobe Gakuin University, Kobe, Japan. Our aim here was to compare thrombotic and thrombolytic activity in the native Japanese and native Western population, to determine if genetic; lifestyle and environment factors influenced platelet activation and predisposed certain ethnic groups to higher risk of thrombotic diseases.

The incidence of and mortality from acute myocardial infarction is significantly lower in the Japanese population in Japan, has been on the decline over the last 40 years and is four times lower than the risk of coronary disease in USA (Kitamura et al. 2008). This does not hold true for Japanese living in the Western countries, as their risk of an AMI is much higher, and is comparable to the Western population. A study by Takeya et al demonstrated significantly higher risk of coronary disease in Japanese men living in Hawaii and California (Takeya et al 1984). Classical risk factors such as genetic background, smoking, dietary habits and lifestyle measures do not explain these inter racial differences in risk, and it is possible thrombotic and fibrinolytic markers play an important role in determination of this risk.

The most important thrombotic factors that are associated with an increased risk of cardiovascular disease are fibrinogen, vWF levels, plasma viscosity, and fibrin D-dimer in women. In a study by Yano et al, plasma fibrinogen levels were higher in elderly Japanese men living in Hawaii compared to elderly Japanese men in Japan. The MONICA Optimal Haemostasis study (Yarnell et al. 2005) demonstrated a strong association between thrombotic

factors and coronary disease. Levels of fibrinogen (clottable and nephelometric), vWf, tissue plasminogen activator antigen, PAI inhibitor activity, fibrin D-dimer, plasma viscosity, C-reactive protein, and total cholesterol were measured in 3996 subjects. Significant correlations were noted between adverse cardiac events and these thrombotic factors when adjusted for age, smoking habits and body mass index, most significant being vWF antigen in both sexes, nephelometric fibrinogen in men, and D-dimer in women. The PRIME study (Scarabin et al. 2003) was a prospective cohort study in which 10,600 men aged between 50-59 years were recruited from Northern Ireland and France. The risk of future coronary events was 1.9 times higher in the Irish than in the French population (95% confidence interval: 1.5–2.4). Fibrinogen levels accounted for 30 % of the increased risk of coronary heart disease in the Irish population, and conventional risk factors together accounted for 25 % increased risk in the Irish population.

Hypothesis:

We postulated that healthy Western volunteers would have shorter OT and LT when compared to healthy Japanese volunteers as the incidence of coronary artery disease is lower in the Japanese population.

Methods: Thrombotic and thrombolytic status of 100 healthy Western and Japanese volunteers was tested using the GTT. All volunteers gave informed consent and the study was approved by the local Research Ethics Committee. Volunteers were non-smokers, not taking any regular medications and in particular, did not take any medication with known platelet effect (such as aspirin or the oral contraceptive pill) in the preceding 7 days. All patients were tested in the morning, between the hours of 0900 and midday.

Advertising for healthy volunteers was done via the Hospital trust bulletin, and posters were put up in hospital wards and outpatient units. A brief summary of the study and need for control group was included in the poster. Recruited volunteers were nonsmokers, not on any regular medication, and were advised not to take any medication with known platelet effect in the preceding 7 days (aspirin or the oral contraceptive pill). None of the volunteers recruited were heavy alcohol consumers and all were asked to abstain from alcohol the night before sampling. Blood pressure and ECG were not performed prior to recruitment, but none of the volunteers had any known underlying illness, history of hypertension or cardiac disease. All healthy volunteers were tested in the morning, between the hours of 0900 and midday.

100 volunteers in each group were tested and all volunteers gave written informed consent.

Results:

Results were analysed using SPSSv16 (SPSS Inc., Chicago, Illinois). Continuous variables were not normally distributed, hence median and interquartile range (IQR) statistics were evaluated using non parametric methods (Spearman's rho, Mann-Whitney), and dichotomous variables evaluated using cross tabulation. Effect size of non-parametric comparison was evaluated using Somer's d.

Repeatability of GTT test

Repeatability was assessed by testing 8 healthy Western subjects with two separate blood draws. The coefficients of variation (CV) for OT was 12%, and for LT 20%. One healthy female subject was tested twice a week for 4 weeks by the same person (Table 3.1), under similar conditions, and at the same time of the day. The CV for OT was 6.2%, and for LT 20.9%.

Table 3.1: OT and LT results (in seconds) as measured in the healthy female volunteer

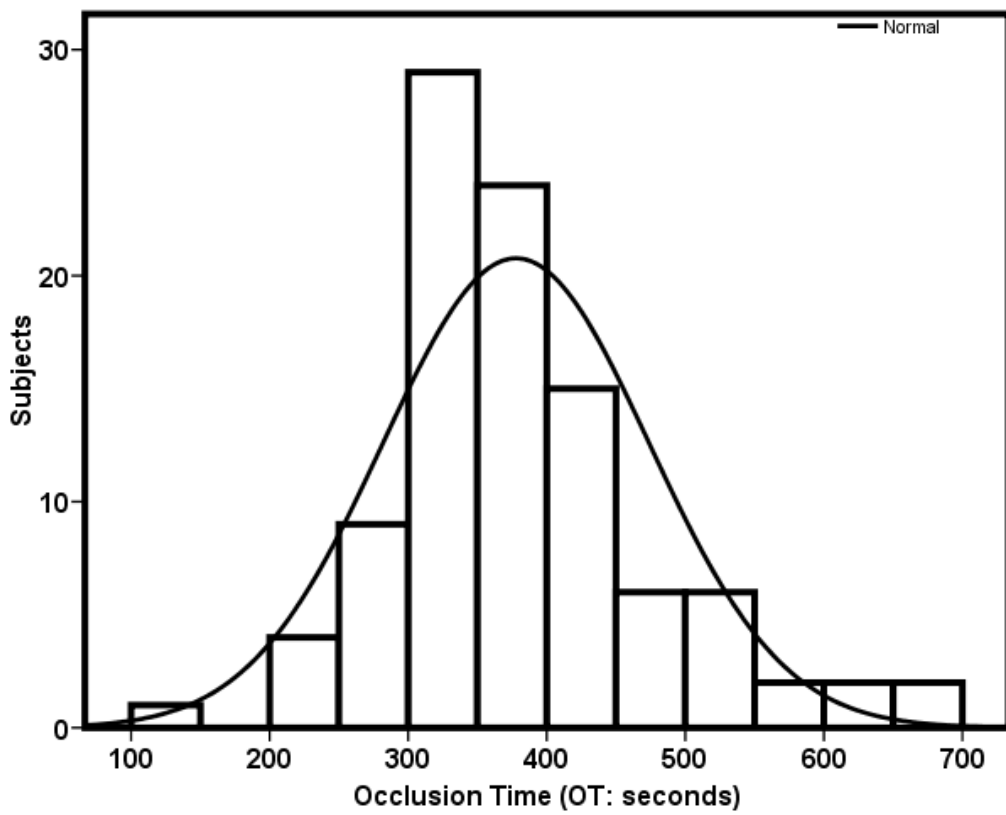
Week	Occlusion Time (sec)	Lysis Time (sec)
1.1	318	1741
1.2	343	1058
2.1	361	1094
2.2	383	1166
3.1	342	1732
3.2	368	1244
4.1	348	1519
4.2	383	1176
Coefficient of variation (%)	6.2	20.9

Distribution of OT in Western volunteers

The Western group consisted of 55 males and 45 females. The age of the volunteers was 38 ± 11 years (range 22-76, IQR 11).

OT was normally distributed in the healthy volunteer population (Fig 3.1). Mean OT was 377.80s, and using mean \pm 2SD, we derived a normal range of 185-569s (200-550s). There was no relationship between OT and gender, even after excluding post-menopausal women from the analysis. There was no relationship between OT and age in the population.

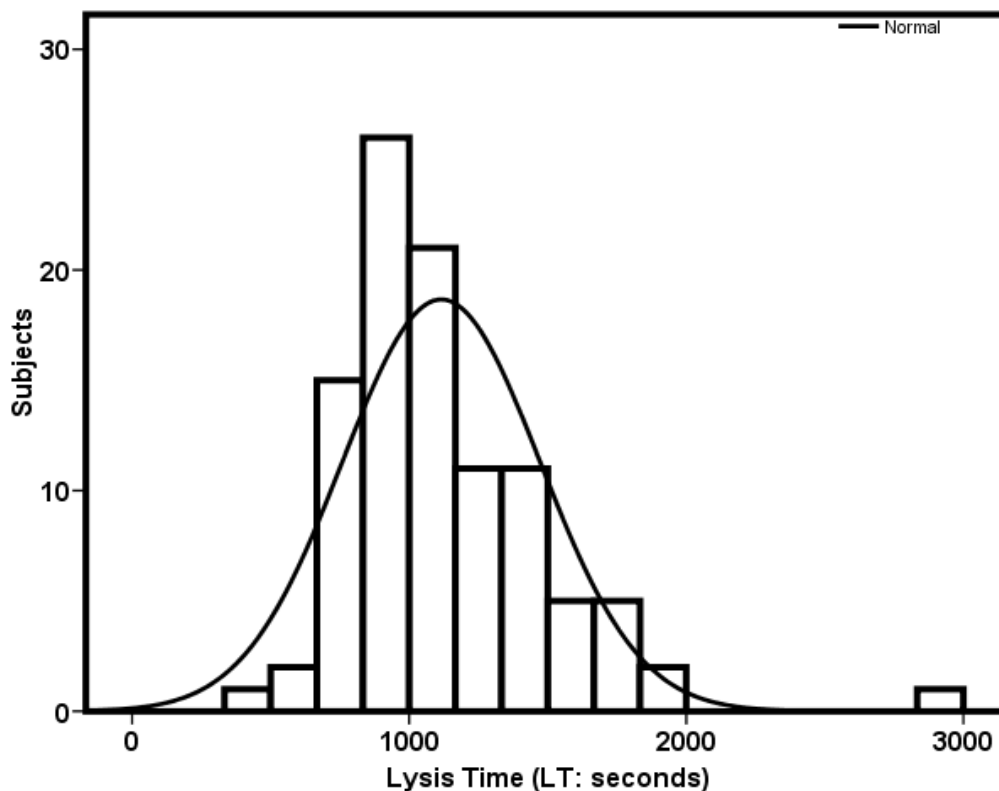
Figure 3.1: Distribution of OT in healthy Western volunteers (X axis: OT in seconds; Y axis: Number of subjects)



Distribution of LT in healthy Western volunteers

Median LT was 1052s (range 457-2934, IQR 405), and was not normally distributed in the healthy volunteers (Fig 3.2). The distribution was skewed, with values ranging between 457 – 2934s. Using log transformation, a reference range of 600-2000s was developed. There was no relationship between LT and gender even after excluding post-menopausal women from the analysis. There was no relationship between LT and age.

Figure 3.2: Distribution of LT in healthy Western volunteers. (X axis: LT in seconds. Y axis: Number of subjects)



The Japanese population consisted of 28 men and 72 women and median age was 50 ± 9 years (range 22-76, IQR 11). Western volunteers were younger compared to the Japanese volunteers ($z = 7.25$, $P < 0.001$), and there were more men in the Western population ($X^2 = 16.2$, $P < 0.001$). There was no correlation between age and OT or LT ($\rho < 0.1$ in all cases). No difference was noted between men or women in either population ($P > 0.05$ in all cases). Median OT in the Western cohort was 365 (range 109-674, IQR 102) and median OT in the Japanese cohort was 545 (range 189-713, IQR 149) (Fig 4.1). OT in the Japanese cohort was significantly longer than OT in the Western cohort (OT: 545 vs. 365s, $P < 0.0001$, $z = 8.83$, $d = 3.76$). Median LT in the Western cohort was 1052 (range 457-2934, IQR 405) and median LT in the Japanese cohort was 1753 (range 205-5555, IQR 1036) (Fig 4.2). LT was significantly longer in the Japanese population than LT in the Western cohort (LT: 1753 vs 1052s, $P < 0.0001$, $z = 8.94$, $d = 3.99$) (Fig 4.3). Markedly impaired lytic status was noted in 18% Japanese subjects, with LT > 3000 s compared to none of the Westerners ($P < 0.0001$) (Fig 4.4).

There was no relationship between OT and LT, in either the Japanese or the Western cohorts. As outlined above, neither age nor sex had a significant impact on the differences observed between Western and Japanese participants. Although age and sex are potential confounders in this sample, there was no obvious bias introduced in the observed differences in OT and LT between the Western and Japanese samples, but this was further interrogated as follows.

Subgroup analyses to investigate effects of age and sex.

To eliminate the effects of age and sex, we also compared 56 Western subjects (28 M, 28 F) with 56 Japanese subjects (28 M, 28 F) matched for age. Since it is accepted that pre-menopausal women have a low incidence of cardiovascular events and post-menopausal women are at higher risk, only premenopausal women were included in these groups.

The mean age in the Western cohort was 31 ± 6 yrs and in J was 31 ± 8 yrs. OT was significantly prolonged in Japanese compared to Western subjects (537 vs. 363, $P<0.0001$). LT was also prolonged in Japanese compared to Western subjects (1660 vs. 1115, $P<0.0001$) (Table 3.2). This subgroup analysis confirms that the observed differences were not attributable to differences between the groups in terms of age or sex. This subgroup analysis in age-matched Western and Japanese men and women showed consistently longer OT in both Japanese men and women, compared to Western, with no difference between men and women, only between the two racial groups. Lysis time was prolonged in Japanese compared to Western subjects. This again was not confined to men or women, but interestingly, premenopausal Japanese women exhibited even longer LT than Japanese men.

Figure 4.1: Distribution of OT in Western (W) and Japanese (J) volunteers. (X axis: OT in seconds. Y axis: Percentage of total number of subjects)

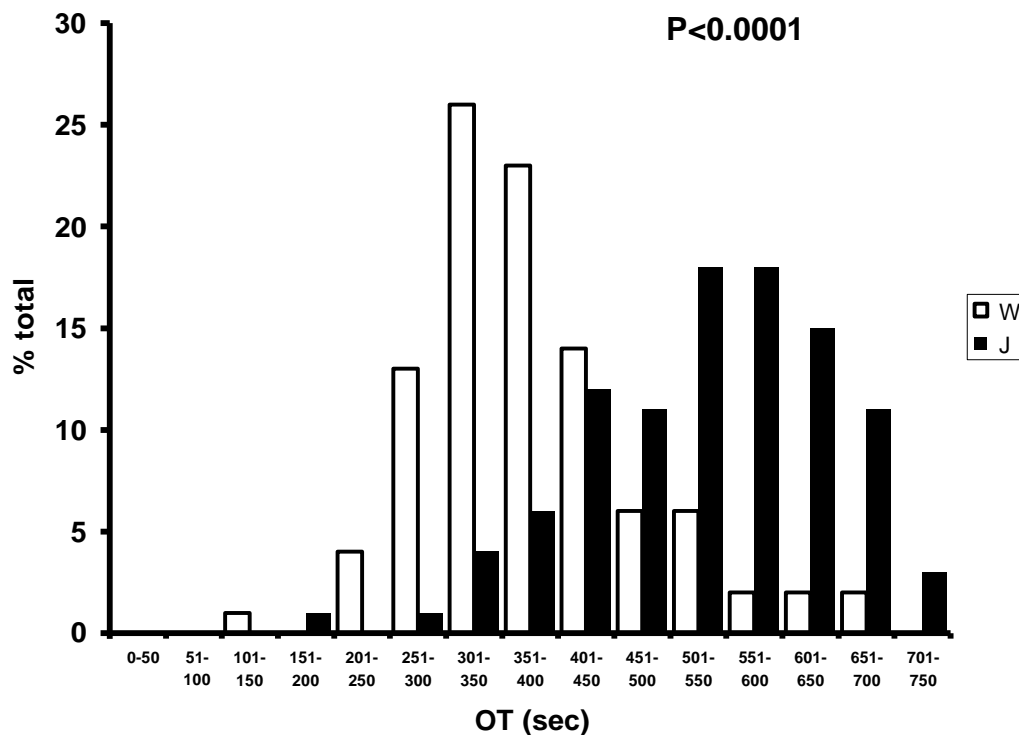


Figure 4.2: Box and Whiskers plot demonstrating minimum, maximum and median difference in OT between Japanese and Western volunteers.

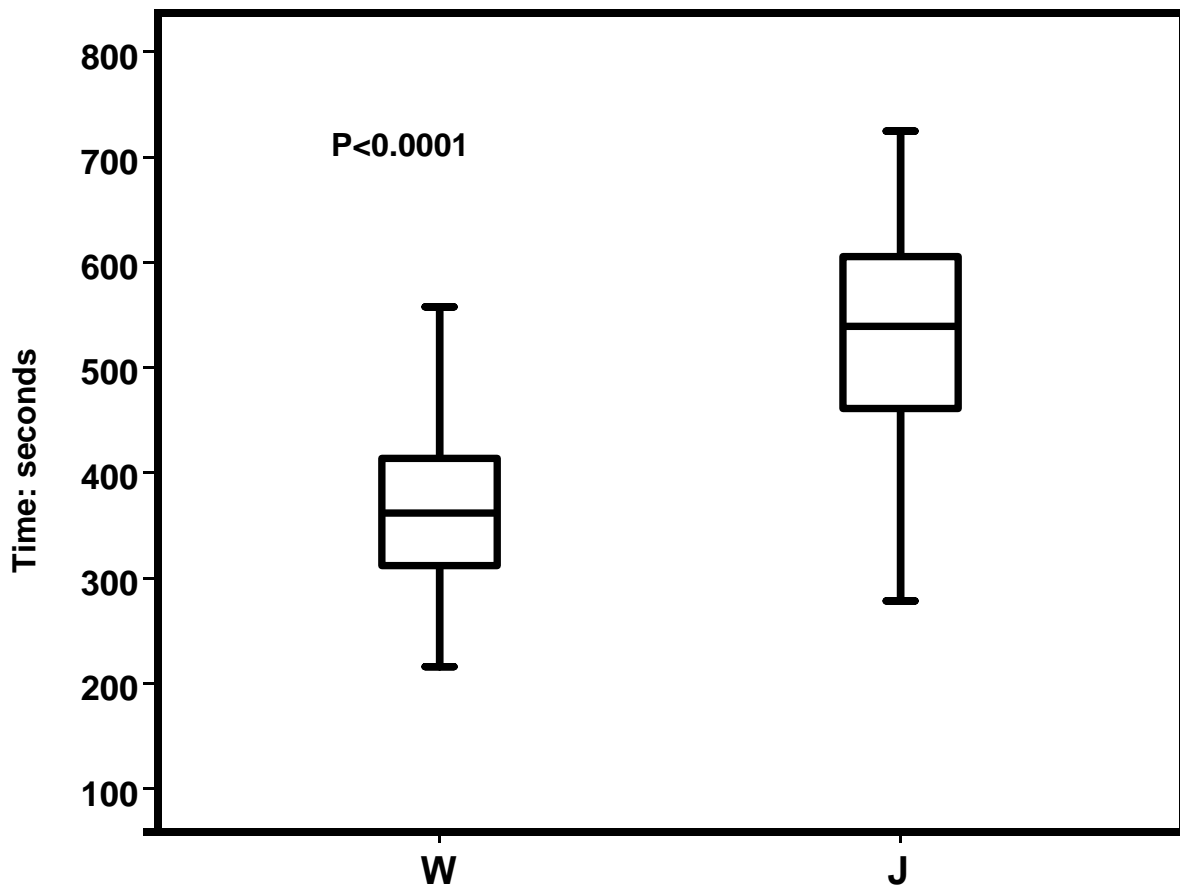


Figure 4.3: Distribution of LT in W and J volunteers (X axis: LT in seconds. Y axis: Percentage of total number of subjects).

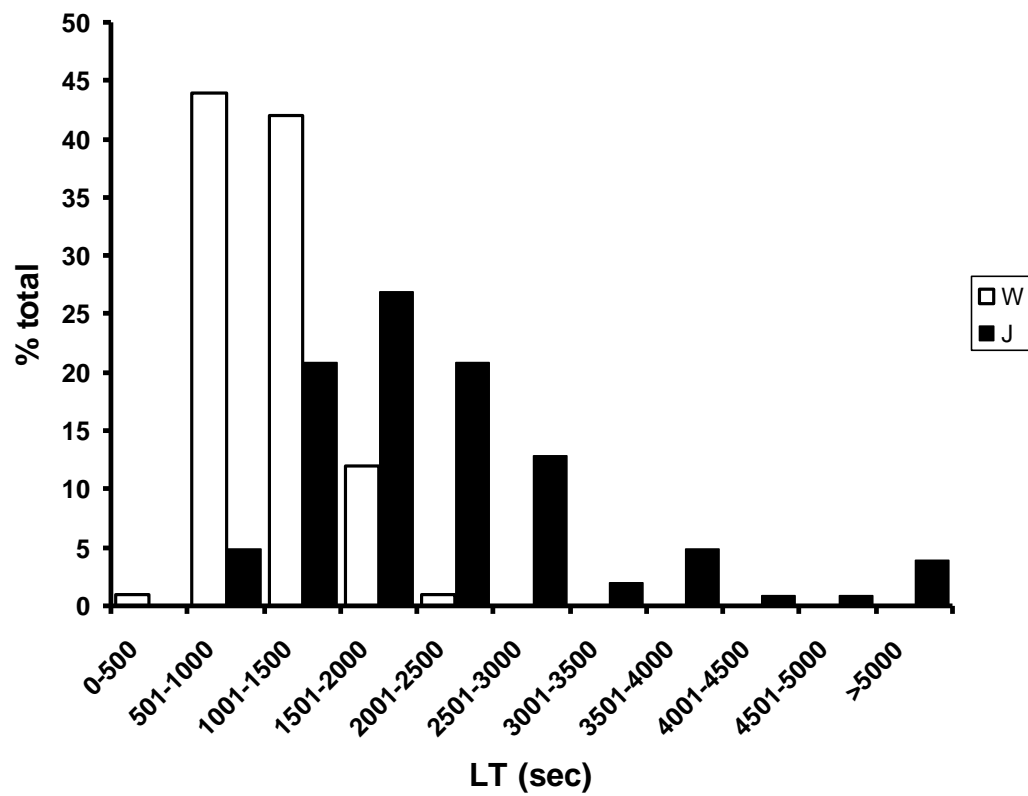


Figure 4.4: Box and Whiskers plot demonstrating minimum, maximum and median difference in LT.

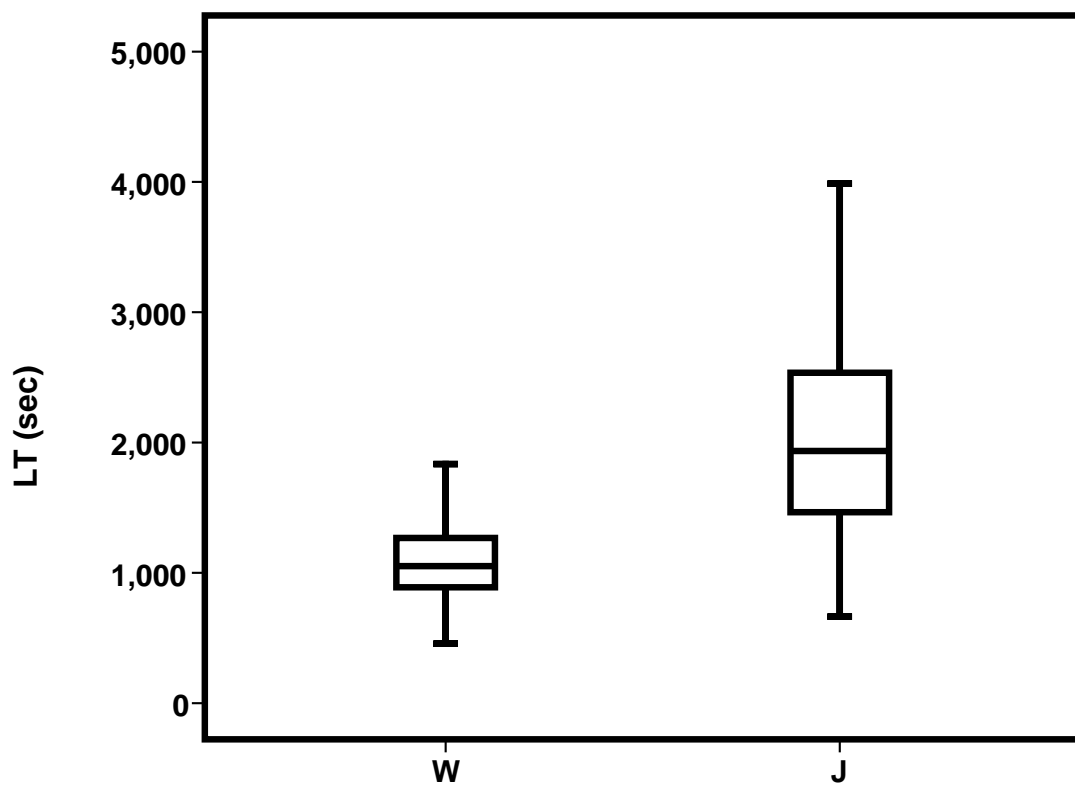


Table 3.2. Subgroup analysis to eliminate the effects of age and sex. Distribution of occlusion time (OT, sec) and lysis time (LT, sec) in Japanese (J) and Western (W) volunteers.

Sample	N	Japanese	Western	Significance
Median OT (min-max, IQR)	56	537 (310-872, 161)	363 (225-674, 146)	$z=5.9, p<0.0001$
Median LT (min-max, IQR)	56	1660 (1036-5555, 919)	1115 (635-2934,2001)	$z=6.6, p<0.0001$

Discussion, Limitations and Conclusions

We developed our own reference range in the healthy Western population. The idea was to characterize the distribution of OT and LT measurements in healthy individuals who had no underlying disease condition, and then test it in patients with coronary artery disease. Reference limits were generated, and using the GTT, we developed a normal range for OT: 200-550s, and for LT: 600-2000s. OT was normally distributed, whereas LT showed a skewed distribution with a tail at either end. This may be partially explained by the small sample size, there also remains a possibility that some outliers had underlying clinical conditions which they were unaware of which could potentially result in impaired lysis. As there is limited data on endogenous thrombolysis and factors that affect it, it was beyond the scope of this study to accurately determine and investigate the factors contributing to this. This remains a limitation of our study. Data from larger studies will help in determination of factors resulting in impaired endogenous thrombolysis.

Reliability was assessed by assessing repeatability and measuring coefficient of variation. CV for OT ranged between 6-12%, and for LT- 20%. Although our small sample size remains a limitation, similar variations have been reported using the GTT in other studies. In one study CV for OT was 9.3% and LT was 19.9% (Yamamoto et al. 2003), and in another CV for OT was 10% and LT was 11.1% (Yamashita et al. 2005). We think the reason of the relatively high variation of lysis times can be explained by the technique. The GTT measures the first drop of blood after the complete arrest of flow and a thrombus stabilization period of 200 sec. At the beginning of thrombolysis the re-established flow is extremely slow and it takes >100 sec for the first blood drop to detach and to be detected by the sensor. In addition to the low atmospheric pressure, several other factors affect the speed of the detachment of the first blood drop and this may account for the relatively high variation. Thus despite the excessive variation,

this technique is able to differentiate between rapid, existing thrombolysis and lack of thrombolysis.

It is important to note that even using well established techniques, the coefficient of variation in patients can be as high, or even higher, than that which we measured for lysis times. Measuring aspirin resistance in patients with ischemic heart disease, a recent study found the CV for the PFA-100 technique to be 31.2% for arachidonic acid induced platelet activation, which is considered to be the gold standard for detecting aspirin resistance (Muir et al.2009). Furthermore, the CV for measurement of PAI-1 activity, considered to be the most reliable marker of fibrinolysis, is reported to be 21.4% (Sakkinen et al. 1999).

The results in the Japanese population suggest Japanese population are less prothrombotic with longer OT's, and have less favourable endogenous thrombolytic activity with longer LT's. The prolonged OT in this group indicates that thrombosis is less likely to occur, and therefore the clinical significance of the impaired lysis may be lessened in case of cardiac events. On the contrary, the incidence of haemorrhagic stroke is much higher in Japan compared to incidence in Western countries (Kitamura et al.2008). In Japan, haemorrhagic stroke (intracerebral haemorrhage or subarachnoid haemorrhage) accounted for approximately 33% of all strokes, whereas in USA it accounts for 10-13% of all strokes (Robertson et al. 1977).

Stroke is one of the major causes of mortality in Japan (Turin et al. 2010). A large registry in Kyoto, Japan analysed 13788 stroke patients over 10 years. The majority of strokes were due to cerebral infarction (65.4%), but a significant proportion suffered with intracerebral haemorrhage (25.7%) and subarachnoid haemorrhage (8.7%). On analysis of risk factors, hypertension seemed to be the most important risk factor as greater than 60% stroke patients were deemed to be hypertensive (Shigematsu et al.2013). Despite the high prevalence of hypertension in Japan, the mortality from ischaemic heart disease is quite low and Japanese population has the longest

life expectancy in the world. Although blood pressure levels have decreased over recent years and other lifestyle changes may explain the reduced incidence of coronary disease in Japan (Ueshima et al. 1987), it does not explain the continued high incidence of haemorrhagic stroke. This suggests there are other factors which are involved; this remains a subject for further research.

Our results of prolonged OT in the Japanese volunteers may support this finding, and may explain why Japanese have a reduced incidence of coronary artery disease, but increased incidence of haemorrhagic stroke. LT was prolonged in the Japanese population, suggesting if a thrombus does occur, it would take more time to lyse and final outcome may be less favourable in case of myocardial or cerebral infarction. The Japanese stroke data bank results demonstrate an increase in incidence of cerebrovascular infarction in Japan, and cerebral infarcts account for 78% of all acute stroke.

In a study by Taomoto et al, 185 stroke patients in Japan were tested using the GTT. Their OT and LT were compared to 195 healthy volunteers not taking any medication. Occlusion time was significantly shorter in stroke patients (mean age 65.5 years) compared to healthy volunteers (mean age 39.7 years) (OT: 210 ± 140 s vs. 284 ± 92 s, $P < 0.0001$) suggesting a prothrombotic state existed in the patient population. LT was significantly prolonged in stroke patients when compared to healthy volunteers (LT: 3159 ± 1549 s vs. 2231 ± 1223 s, $P < 0.0001$) suggesting stroke patients had impaired endogenous thrombolytic activity.

These observed racial differences in the Japanese and Western population could not be attributed to the effects of age or sex. Further large population studies are required to directly assess the relationship between thrombotic and thrombolytic profile and the occurrence of cardiovascular and cerebrovascular events, in different ethnic groups. . Furthermore, the modern Japanese diet

is identical to a Western diet, thus the effects of diet are likely to be insignificant, if any. Cholesterol levels were not compared in our two groups, but the population cholesterol levels are reported to be similar by the WHO. Impaired fibrinolysis is a known risk factor for cardiovascular events. The impaired fibrinolytic activity observed in the Japanese cohort in our study is in contrast to the findings of Iso and co-workers, who reported higher tPA and PAI-1 levels in Caucasians than in Japanese (Iso et al. 1990, Iso et al. 1993). However, our results are supported by Takayima and co-workers who observed significantly lower PAI-1 levels in American than in Japanese men (Takamiya et al. 2006).

However, approximately 90% of the total amount of PAI-1 present in blood is stored in the alpha granules of platelets and released only from the platelet aggregates in the thrombus mass. For this reason, measurement of plasma PAI-1 content in these earlier studies (Iso et al. 1990) does not reflect true potential thrombolytic status. In contrast, the GTT employed in our study measures the lysis of an autologous thrombus, a process in which PAI-1 released from platelets plays a decisive role and therefore this test provides a far more accurate reflection of the global thrombolytic status. Furthermore, our finding of impaired thrombolytic status in Japanese, who are an ethnic group with an unusually high incidence of hemorrhagic stroke, is in keeping with the finding that in a well conducted, prospective study, tPA/PAI-1 complex, a marker of impaired fibrinolysis, was independently associated with the development of a first-ever stroke, especially hemorrhagic stroke (Johansson et al. 2000).

In conclusion, Westerns were more prothrombotic than Japanese. The thrombolytic profile of Westerns however, was more favourable than that of Japanese. Although OT and LT status probably both contribute to thrombotic risk, since thrombotic events are more frequent in the Western population, we postulate that OT is likely to be more predictive of thrombotic events

than LT. Although LT is less favorable in Japanese, the prolonged OT in this group indicates that thrombosis is less likely to occur, and therefore the clinical significance of the impaired lysis is lessened. However, if thrombosis does occur, its outcome may be less favorable in Japanese persons. Further large population studies are required to directly assess the relationship between thrombotic/thrombolytic profile and the occurrence of cardiovascular events, in different ethnic groups.

Chapter 4: Role of ADP, Aspirin and Clopidogrel on thrombus formation

Background: ADP is an important component of the thrombotic pathway and promotes platelet activation and aggregation. Minute concentrations of ADP can result in platelet aggregation, and most platelet function tests utilize ADP as an agonist to determine the efficacy of an antiplatelet agent. Most tests use supra high doses of ADP which make the test results physiologically difficult to interpret. The GTT is a shear induced platelet function test, in which the shear force causes haemolysis and releases minute concentrations of ADP (up to 0.5 μM) which are not only adequate to cause platelet aggregation, but also increase the sensitivity of the test and help in monitoring of aspirin mediated platelet inhibition.

There are 2 main ADP receptors on the platelet surface: P2Y1 and P2Y12. The two P2Y1 and P2Y12 receptors are G-protein coupled receptors which utilize ADP as agonist. The P2Y1 receptor binds to $G_{q/11}$ activates phospholipase C and mobilizes intracellular calcium while P2Y12 receptor binds to G_i , inhibits adenylate cyclase, activates PI3-kinase and reduces cAMP intracellular levels. These receptors also inhibit the N-type voltage-gated calcium channels, and activate the G protein gated inward rectifier potassium channels in neurons and endocrine cell lines, and activate the chloride channels in airway epithelium (Ralevic et al. 1998, Burnstock et al. 2004). ADP mediated activation of the P2Y12 receptor leads to a series of intracellular signals that activate the GP IIb/IIIa receptor, resulting in granule release and platelet activation and aggregation. Clopidogrel selectively and irreversibly inhibits the P2Y12-receptor by forming a disulphide bridge between the two cysteine residues of the P2Y12 receptor.

Using various platelet tests at high shear (Haemostatometry, GTT, Cone-and-plate analyzer, PFA-100, whole blood impedance aggregometry), significant correlation was found between occlusion times and haematocrit. Further, the presence of red blood cells (RBC) was shown to be critical, as from platelet rich plasma (PRP) occlusion could not be achieved. Recent

studies on the mechanism by which shear promotes platelet aggregation *in vivo* has revealed a two-stage process. The first stage is the formation of an unstable aggregate of platelets attached by membrane tethers. Conversion of these initial aggregates into stable thrombus requires the release of ADP. The ADP receptors P2Y (1) and P2Y (12) were shown to have distinct and determinant roles in von-Willebrand factor (vWF)-mediated initial platelet adhesion and aggregation P2Y (1), in the formation of larger aggregates, in activation of intrinsic coagulation and thrombus stabilization P2Y (12) under high shear stress. Platelet aggregation at high shear was impaired in a patient with congenital defect of platelet response to ADP. It is known that even minute concentrations of ADP can induce rigid platelet aggregate formation. Such low concentrations of ADP can exist in shear-induced *in vitro* tests as a result of the unavoidable haemolysis during blood sampling, release from platelets and RBC at the blood-air interface and through interaction of these cells with the dry plastic surface of the test-tube or cartridge. The functional importance of such low ADP concentrations is further supported by the observation that only low ADP concentrations (up to 0.5 μM), much lower concentrations than those commonly employed as agonists to induce platelet aggregation, can be considered a useful platelet agonist for monitoring aspirin-mediated platelet inhibition, since higher concentrations reduce the sensitivity of the test (Saraf et al. 2009). Using the experiments we performed using the GTT, it was not possible to determine the exact source and amount of ADP release from RBC and platelets. Alkhamis et al using the cone-and-plate viscometer have previously demonstrated the amount of ADP release from RBCs and percent decrease of platelets in their study during laminar shear flow at a low shear stress of 200 dyne/cm². ADP release from RBCs was about twice that of platelets in their study (Alkhamis et al. 1990). Further experiments need to be performed using the GTT to determine the exact source and amount of ADP release.

GTT is a platelet activation test, in which high shear stress induces platelet activation and aggregation. Recent studies have revealed that platelet aggregation is a two stage process (Maxwell et al. 2007) - initial stage involves formation of an unstable aggregate of platelets which then subsequently form a stable thrombus on release of ADP. The ADP receptor P2Y (1) plays an important role in von-Willebrand factor (Vwf) mediated initial platelet adhesion and aggregation, and P2Y (12) receptor plays an important role in the formation of larger aggregates, in the activation of intrinsic coagulation system and stabilization of the thrombus (Mazzucato et al.2004). Data suggests that in patients with congenital defect of platelet response to ADP, platelet aggregation at high shear is impaired (Cattaneo et al. 1994).

It is known that contact of blood with air at the blood-air interface promotes damage to blood cells and furthermore, contact of blood with foreign surfaces results in release of ADP from RBC and platelets. We aimed to exclude these factors by priming the GTT tube with saline, to avoid the contact of blood with the plastic surface of the GTT cartridge and avoid air in the system, thus avoiding the effect of haemolysis at the blood-air interface.

The aim of addition of water to the GTT tube was to induce ADP release from RBC and platelets, and assess the effect of this on shear-induced thrombosis. Due to the osmotic gradient, contact between RBC/platelets and water would result in haemolysis causing localised release of ADP from RBC and platelets, which we postulated would accelerate the thrombotic process and reduce OT. Using the GTT, it is not possible to determine the exact source and amount of ADP release from RBC and platelets, but the main determinant of the observed changes in our experiments is likely to be platelet-derived ADP.

Aspirin is an antiplatelet agent, and acts by irreversibly acetylating COX1 enzyme, and inhibits platelet aggregation by inhibiting prostaglandin and TxA2 production. It is the most common

antiplatelet agent, and has been in use for greater than 100 years. A metanalysis by Eideleman (Eidelman et al. 2003), which included 5 major studies and 55,580 patients, a significant 32 % reduction in risk of first MI was observed in people who were on aspirin and had underlying risk factors for coronary artery disease. Aspirin reduces this risk by inhibiting platelet activation and aggregation. Our aim in the Aspirin study was to demonstrate the antiplatelet effect of aspirin. We hypothesized that inhibition of platelet aggregation would prolong time to thrombus formation, and hence result in prolongation of OT. There is limited evidence on the fibrinolytic properties of aspirin, and it was our aim to determine if there was any effect of aspirin on LT.

Clopidogrel is an antiplatelet medication, and acts by binding to the P2Y₁₂ receptor. It is a prodrug, oxidized by the hepatic cytochrome P450 system to its active metabolite and irreversibly binds to the ADP P2Y₁₂ receptor. P2Y₁₂ inhibition thus inhibits ADP-induced platelet activation and resultant aggregation. Inhibition of platelet activation and aggregation prolongs time to thrombus formation. Our aim in this study was to examine the effect of clopidogrel on OT and LT. We will demonstrate in this chapter that addition of distilled water to the GTT tube prior to injection of native blood resulted in acceleration of the thrombotic reaction and shortening of OT (a GTT). It was also our aim to observe the effect of clopidogrel on aGTT, to observe its effect on ADP mediated accelerated thrombotic reaction to determine if the GTT was capable of monitoring the efficacy of ADP-inhibitor antiplatelet medications. This would allow us to individualise antithrombotic therapy to achieve optimal thrombotic status, as well as allow us to monitor individual response to antiplatelet therapy.

To summarize, our aim in this study was to observe the effect of endogenous and exogenous ADP on OT, the effect of Aspirin and Clopidogrel and to investigate the effect of these in monitoring the efficacy of ADP- antagonist medication.

Hypothesis:

We postulated that ADP would shorten OT by promoting platelet activation and aggregation, with no significant effect on LT. We also postulated that aspirin and clopidogrel would prolong OT due to its antiplatelet effect, with no significant effect on LT.

Methods:**Endogenous ADP using the Accelerated Global thrombosis test (a GTT)**

Prior to injection of blood in the GTT tube, 0.5 ml distilled water was added to the tube. Water was added 5-10 minutes before injection of blood to the tube, and filled the space between the two balls (100 µl) and the upper water level was constantly 1-2 mm above the upper ball (Fig 5.1). Due to the osmotic gradient, on contact of water with blood there was cell destruction, haemolysis and release of ADP from RBC and platelets which we postulated would accelerate the thrombotic process and result in shortening of OT.

As the water was limited to the small part of the tube where platelet reaction took place, the bulk of the sample was not diluted by the water and exited the system in less than 20 seconds. Thus, the dilution affected only the very start of the measured thrombotic reaction maintaining physiological conditions. Due to this short contact between blood and water, minute amounts of ADP are released which can be inhibited more sensitively than higher concentrations of ADP which are generally employed as agonists in various platelet function tests (Dobaczewski et al. 2008). Neither water nor clopidogrel had any effect on lysis times.

Saline priming of GTT tubes

Saline 0.5 ml was added to the GTT tube 5-10 minutes before addition of blood. The upper level of saline was maintained 1-2 mm above the upper metal ball. The aim of saline priming of GTT tube was to avoid RBC haemolysis and ADP release that may be caused due to contact of blood with the plastic surface of GTT tube, or due to contact of air with blood.

Exogenous ADP

We preloaded a GTT tube with ADP solution (5 μ M ADP in saline; 0.5 ml) prior to injecting native blood from normal volunteers. Our aim was to examine the effect of exogenous ADP on OT and LT in normal healthy volunteers, using the GTT.

Statistical analysis was done using SPSS version 16. As OT is an interval variable, paired comparisons such as a paired t-test were used. In situations where results were skewed, non parametric test such as Wilcoxon signed rank's test was used. Data are presented as mean \pm 2SD and $p < 0.05$ is considered significant.

Stable angina patient's pre and post Aspirin

We measured OT and LT using the GTT in 10 stable angina patients. All patients were reviewed and recruited in the outpatient clinic, and none of these patients had been on Aspirin prior to being tested. None of these patients were on any other antiplatelet medications. Baseline samples were taken prior to starting Aspirin. Post aspirin samples were taken at least a week after each patient had been taking 75mg aspirin. None of these patients had been loaded with higher doses of aspirin.

Healthy volunteers pre and post Clopidogrel

Thirteen normal healthy volunteers were tested before and 8 hours after a loading dose of 300 mg clopidogrel. Volunteers were non-smokers, not taking any regular medications and in particular, did not take any medication with known platelet effect (such as aspirin or the oral contraceptive pill) in the preceding 7 days. Testing was performed at the same time of the day by the same operator, under similar conditions.

Effect of Clopidogrel on OT and LT in Stable angina patients on Aspirin

We examined the effect of clopidogrel in Stable angina patients. Ten patients with stable angina were tested on 75mg Aspirin (pre clopidogrel), and retested at least a week later on aspirin 75mg and clopidogrel 75 mg. Testing was performed by the same operator, under similar conditions.

The Accelerated Global thrombosis test (a GTT)

The aim of this modification was to induce ADP release from RBC, and assess the effect of this on shear-induced thrombosis. Due to the osmotic gradient, contact between RBC/platelets and water would result in haemolysis causing localised release of ADP from RBC and platelets, which we postulated would accelerate the thrombotic process and reduce OT.

Prior to injection of blood in the GTT tube, 0.5 ml distilled water was added to the tube. Water was added 5-10 minutes before injection of blood to the tube, and filled the space between the two balls (100 μ l) and the upper water level was constantly 1-2 mm above the upper ball (Fig 5.1). Due to the osmotic gradient, on contact of water with blood there was cell destruction, haemolysis and release of ADP from RBC and platelets which we postulated would accelerate the thrombotic process and result in shortening of OT. This method was used to assess the effect of endogenous ADP release on OT and LT. To assess the effect of exogenous ADP, we preloaded a GTT tube with ADP solution (5 μ M ADP in saline; 0.5 ml) prior to injecting native blood from normal volunteers.

Clopidogrel in aGTT

Thirteen healthy volunteers were tested using the aGTT before and 8 hours after a loading dose of clopidogrel. Before introducing the blood sample into the GTT, a small volume of distilled water was placed into the tube, which remained at the site where platelet activation occurred.

Normally, when the inflowing native blood would come in contact distilled water, haemolysis would occur with release of ADP resulting in shortening of OT. Clopidogrel is an ADP antagonist, and would prevent this shortening of OT, demonstrating sensitivity of the individual to this thienopyridine derivative. In clopidogrel resistant individuals, the shortening of OT would not be neutralised by the administration of clopidogrel.

Results:

Accelerated GTT: Reduction of OT was observed with addition of distilled water (OT.W) in all 13 volunteers (OT vs. OT.W = 379 ± 30 vs. 177 ± 26 , $n=13$, Wilcoxon signed rank's test $z= -3.1$, $p<0.01$) (Fig 5.2, 5.3). There was no effect on LT (LT vs. LT.W = 1390 ± 206 vs. 1420 ± 153 , $n=13$, Wilcoxon signed rank's test $z= -0.105$, $p= NS$) (Fig 5.4).

Figure 5.1: Accelerated GTT - 0.5 ml distilled water was added to the tube, some 5-10 min before the start of the test. The added water fills up the space between the two balls and the upper water level is constantly 1-2 mm above the upper ball. Due to the osmotic gradient, contact between RBC/platelets and water would result in haemolysis causing localised release of ADP from RBC and platelets, which we postulated would accelerate the thrombotic process and reduce the occlusion time (Saraf et al 2009).

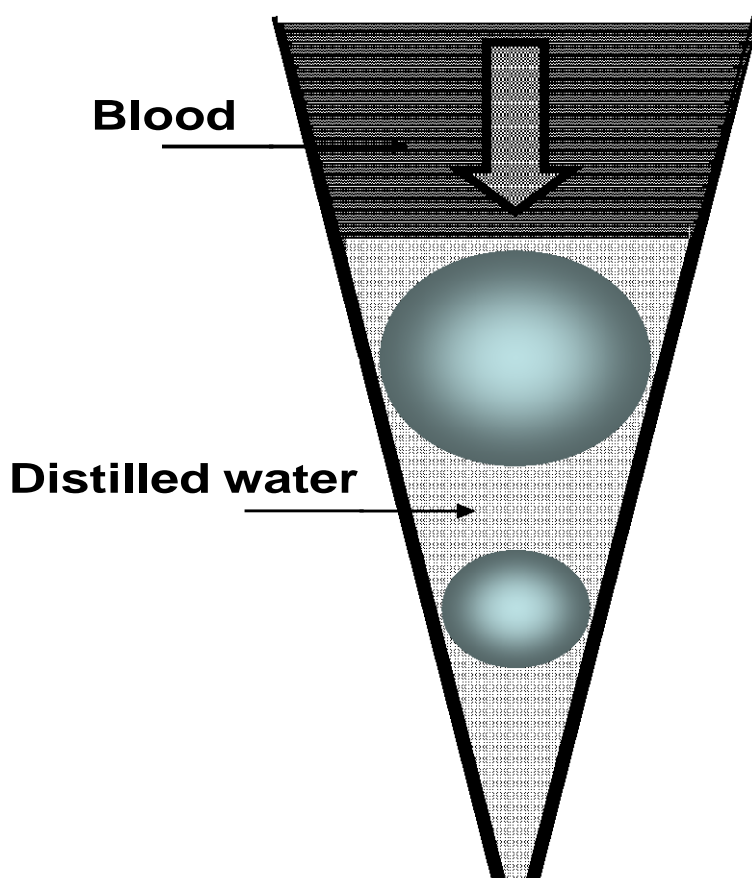


Figure 5.2: OT before and after water priming (aGTT) - OT (sec) - time is shown in seconds, OT= occlusion time. Reduction of OT was observed with addition of distilled water (OT.W) in all volunteers

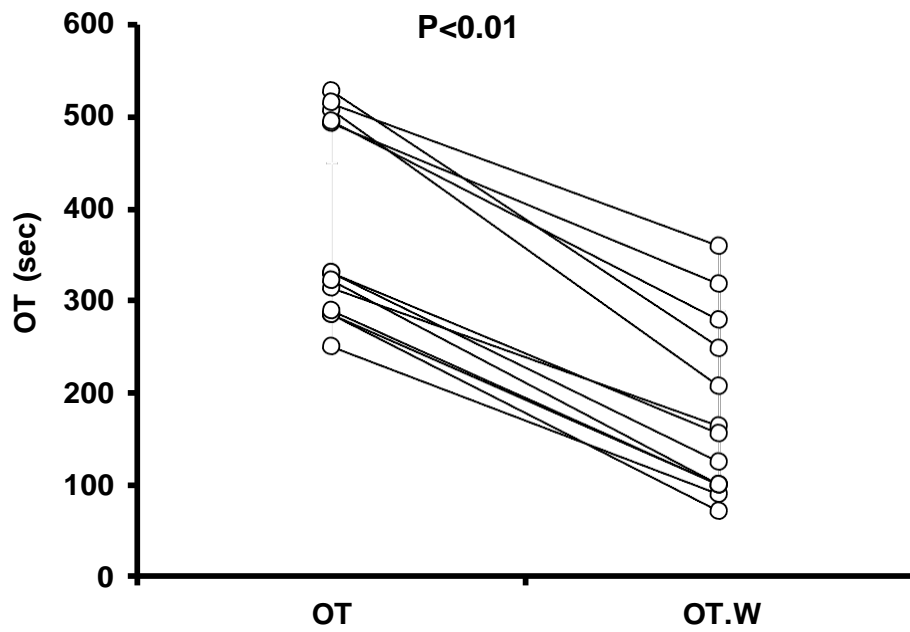


Figure 5.3: Box plot demonstrating minimum, maximum and median OT pre and post distilled water. OT (sec) - time is shown in seconds, OT= occlusion time. Reduction of OT was observed with addition of distilled water (OT.W) in all volunteers

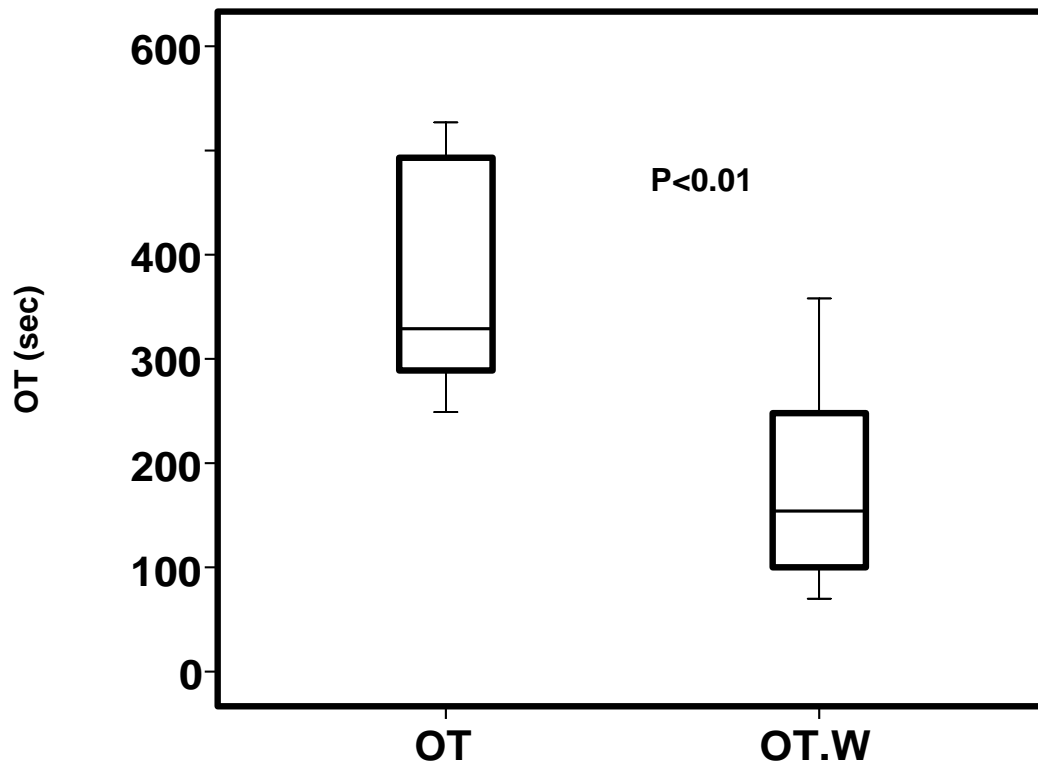
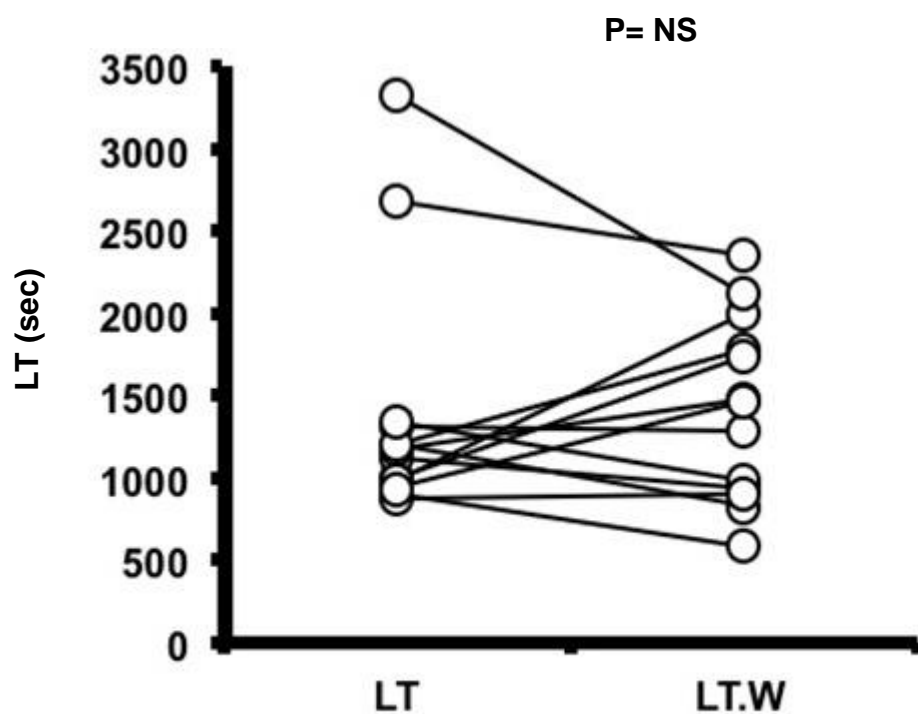


Figure 5.4: LT before and after water priming (aGTT) - LT (sec) - time is shown in seconds, LT= Lysis time. Addition of distilled water had no effect on LT.



Saline priming of GTT tubes

13 healthy volunteers were tested using the GTT and addition of saline was noted to increase the OT (OT.S) (Fig 5.5) in all volunteers (OT vs. OT.S = 391 ± 40 vs. 489 ± 37 , $n = 13$, paired t-test, $df = 12$, $t = -4.428$, $p < 0.001$). Saline had no effect on LT (LT.S) (Fig 5.6) (LT vs LT.S = 1400 ± 99 vs. 1320 ± 94 , $n = 13$, Wilcoxon signed rank's test $z = 0.561$, $p = \text{NS}$).

Table 4: GTT results before and after priming the GTT tube with saline (OT= occlusion time, LT= lysis time, OT.S = OT after saline priming and LT.S = LT after saline priming, sec= time in seconds, SEM= standard error of mean, IQR= Interquartile range)

	<i>OT(sec)</i>	<i>OT.S(sec)</i>	<i>LT(sec)</i>	<i>LT.S(sec)</i>
Mean \pm SEM	391.80 ± 40.2	489.20 ± 36.8	1400.9 ± 99.0	1320.7 ± 93.8
Median (IQR)	334 (204)	483 (182)	1312 (490)	1374 (503)
P value	P<0.001		P=NS	

Figure 5.5: OT before and after saline priming. OT (sec) - time is shown in seconds, OT=occlusion time. Addition of saline was noted to increase the OT (OT.S) in all volunteers.

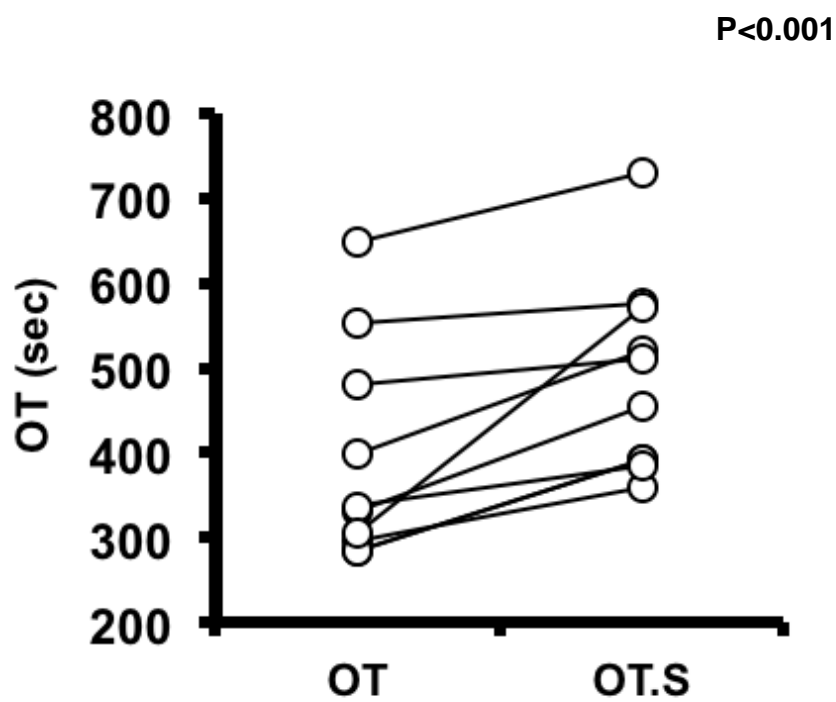
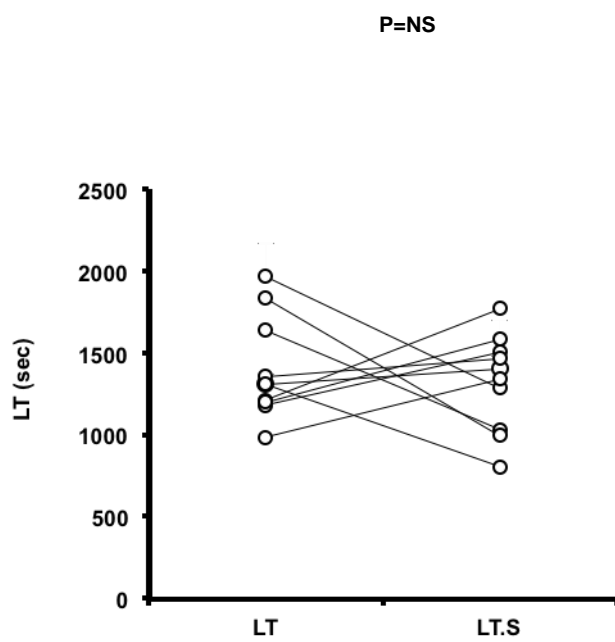


Figure 5.6: LT before and after saline priming. LT (sec) - time is shown in seconds, LT= lysis time. Addition of saline did not have any effect on LT (LT.S).



Exogenous ADP

ADP resulted in acceleration of thrombotic reaction and a reduction of 53% was noted in OT (Fig 5.7, 5.8) (OT vs. OT.ADP = 441 ± 32 vs. 254 ± 38 , $n=5$, Wilcoxon signed rank's test $z = -2.0$, $p < 0.05$). There was no effect on LT (Fig 5.9). (LT vs. LT.ADP = 2955 ± 1036 vs. 1989 ± 990 , $n=5$, Wilcoxon signed rank's test $z = -0.67$, $p = \text{NS}$).

The effect noted with exogenous ADP was similar to the effect noted with addition of distilled water, suggesting ADP played an important role in the thrombotic process. Mean Hct was 0.41, and no significant correlation was noted between Hct levels and OT.ADP or LT.ADP. The small sample size remains a limitation of this substudy.

Figure 5.7: OT before and after ADP priming. OT (sec) - time is shown in seconds, OT= occlusion time. Reduction of OT was observed with addition of ADP (OT.ADP) in all volunteers

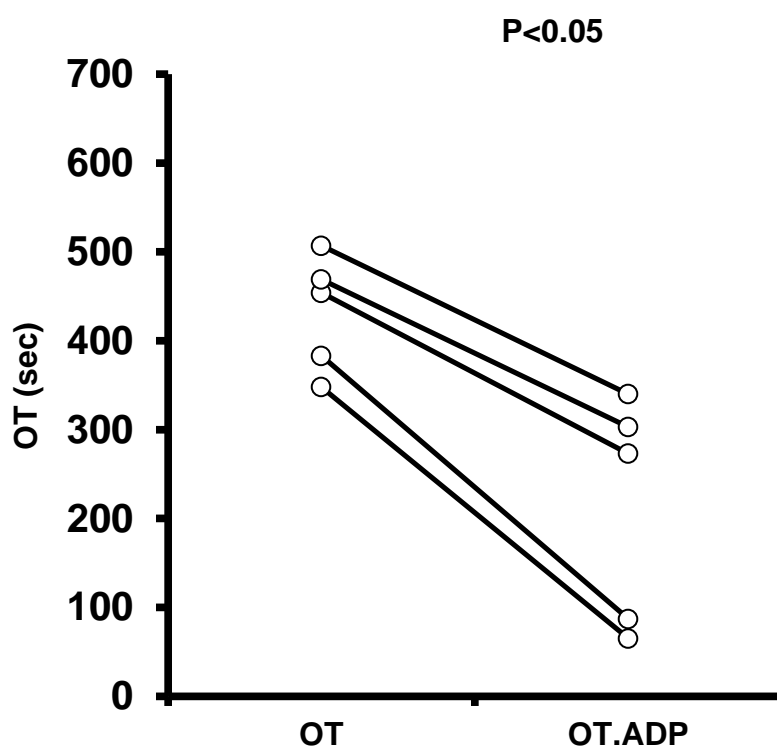


Figure 5.8: OT percentage inhibition before and after ADP priming (Percent inhibition defined as percent decrease of measurable response as a consequence of compound treatment)

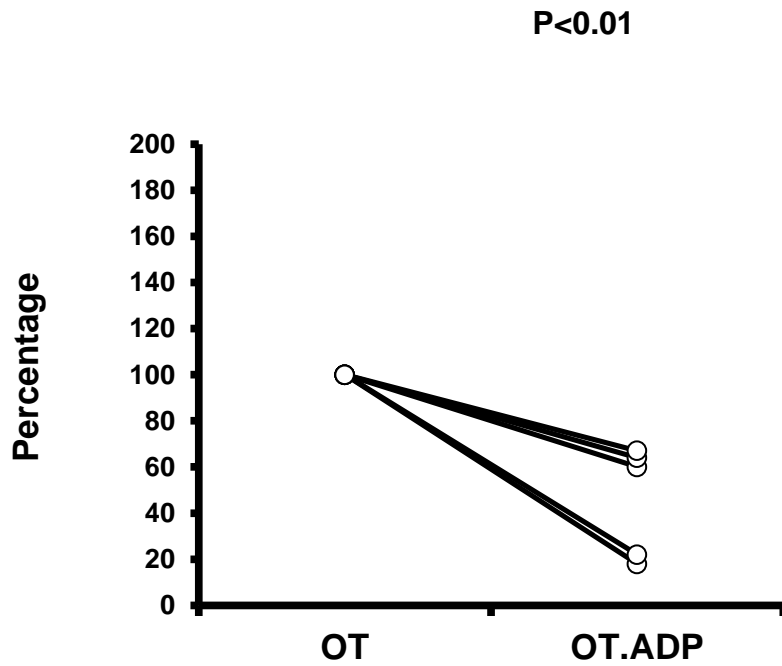
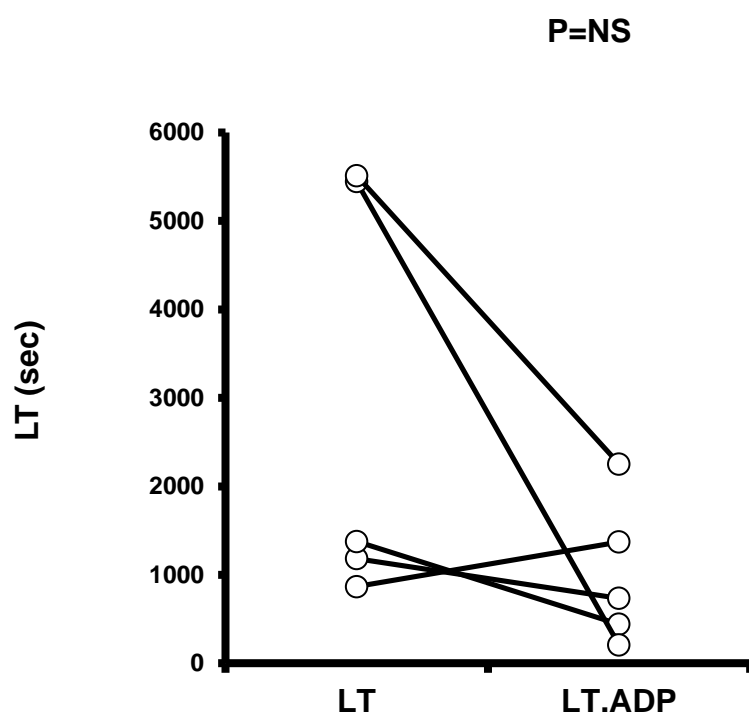


Figure 5.9: LT before and after ADP priming. LT (sec) - time is shown in seconds, LT= lysis time. Addition of ADP had no effect on LT (LT.ADP).



Pre and post Aspirin

In this small study, there were 7 men and 3 women, and mean age was 57.5 years (range 47-83 years). None of these stable angina patients were diabetic, 50 % had underlying history of hypertension, 20% were current smokers, 30% were on ACE inhibitors and 20 % were taking statin. None of these patients were on any other antiplatelet medications.

Aspirin significantly prolonged OT ($365\pm 37s$ vs. $527\pm 31s$, $p = 0.001$) (Fig 6.1, 6.2), OT mean difference (Fig 6.3): -162.1 (95% CI -234.6 to -89.6). Effect size $162.1/101.3 =$

1.60 (large effect) suggesting patients on aspirin were less prothrombotic. There was significant variability in LT values pre and post aspirin (Fig 6.4), ($1034\pm 141s$ vs. $1443\pm 223s$, $p = NS$), LT mean difference (Fig 6.5): -408.4 (95% CI -1113.6 to $+296.8$). However, a moderate effect was noted post aspirin with an effect size $408.4/985.7 = 0.41$ suggesting Aspirin could potentially have some role in endogenous thrombolysis.

Figure 6.1: Ladder plot demonstrating OT pre and post aspirin. OT (sec) - time is shown in seconds, OT= occlusion time. An increase in OT was observed in all volunteers post aspirin (OT.A).

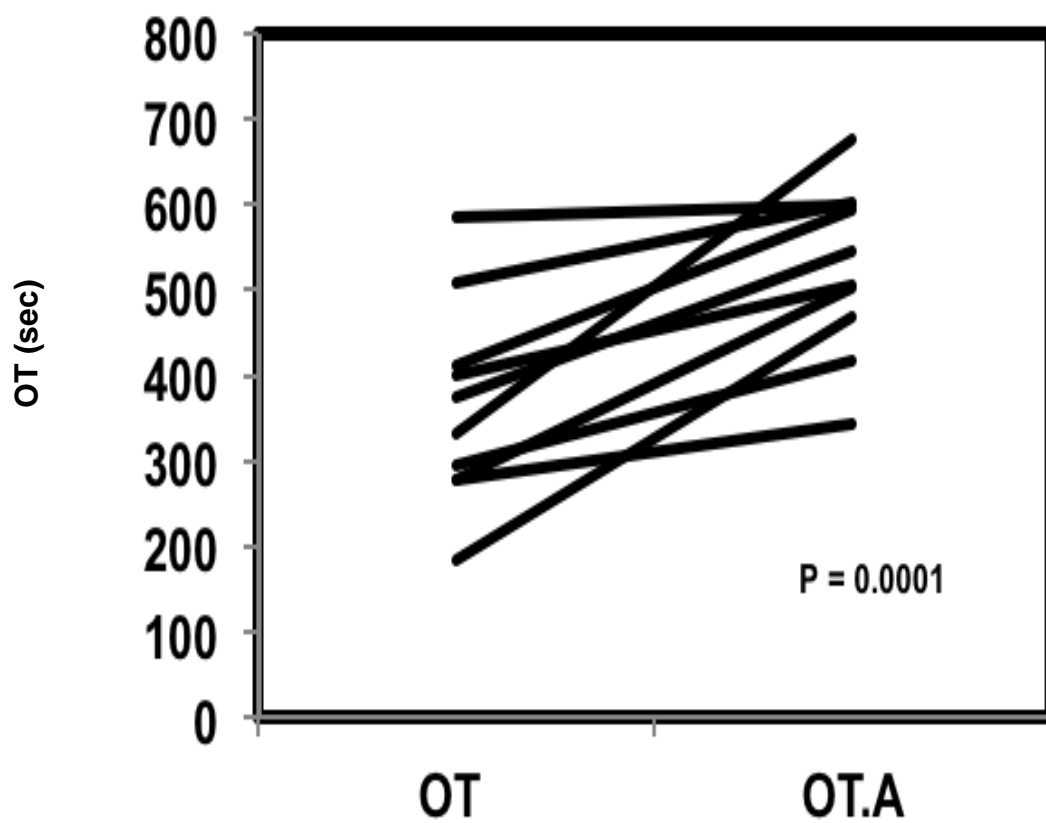


Figure 6.2: Box plot demonstrating minimum, maximum and median OT pre and post aspirin

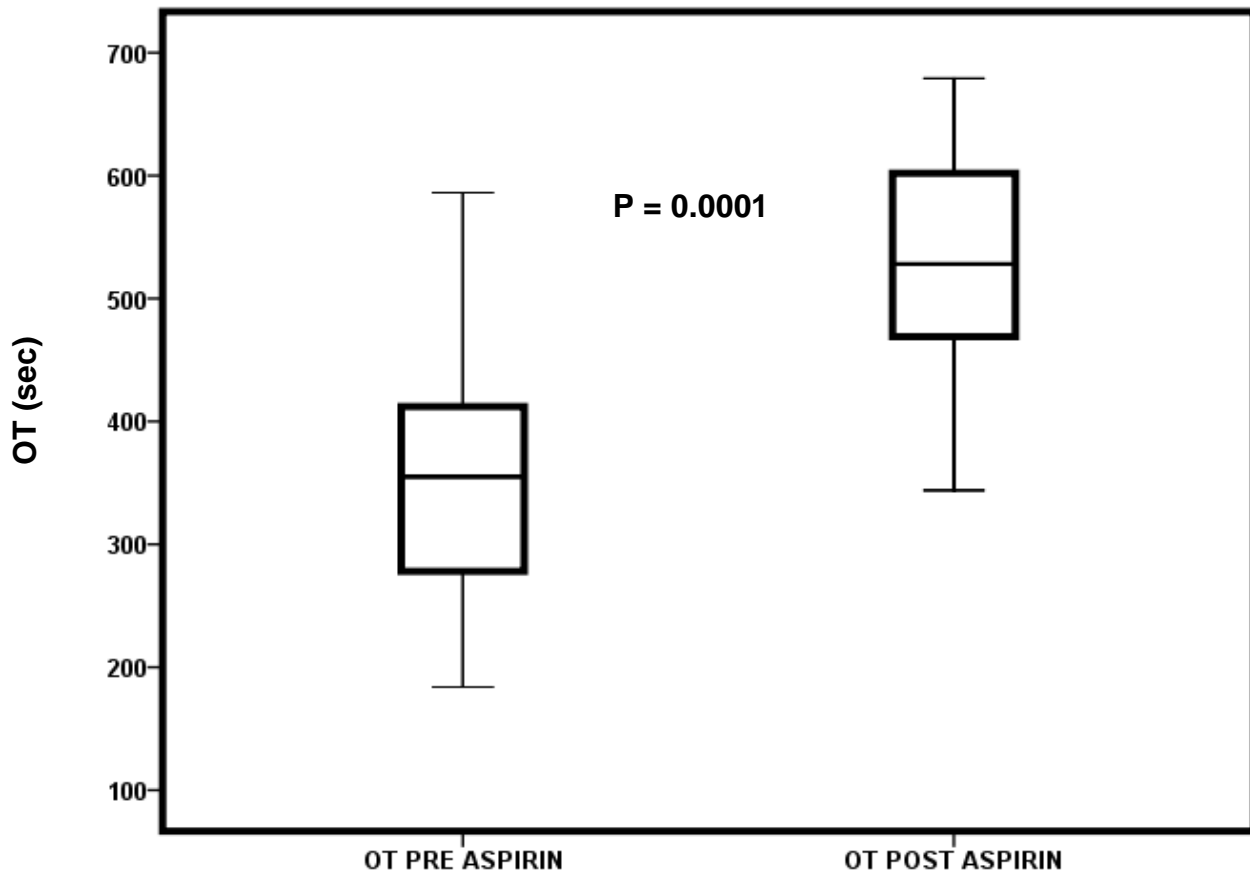


Figure 6.3: Box plot: Paired analysis demonstrating difference in OT pre and post aspirin

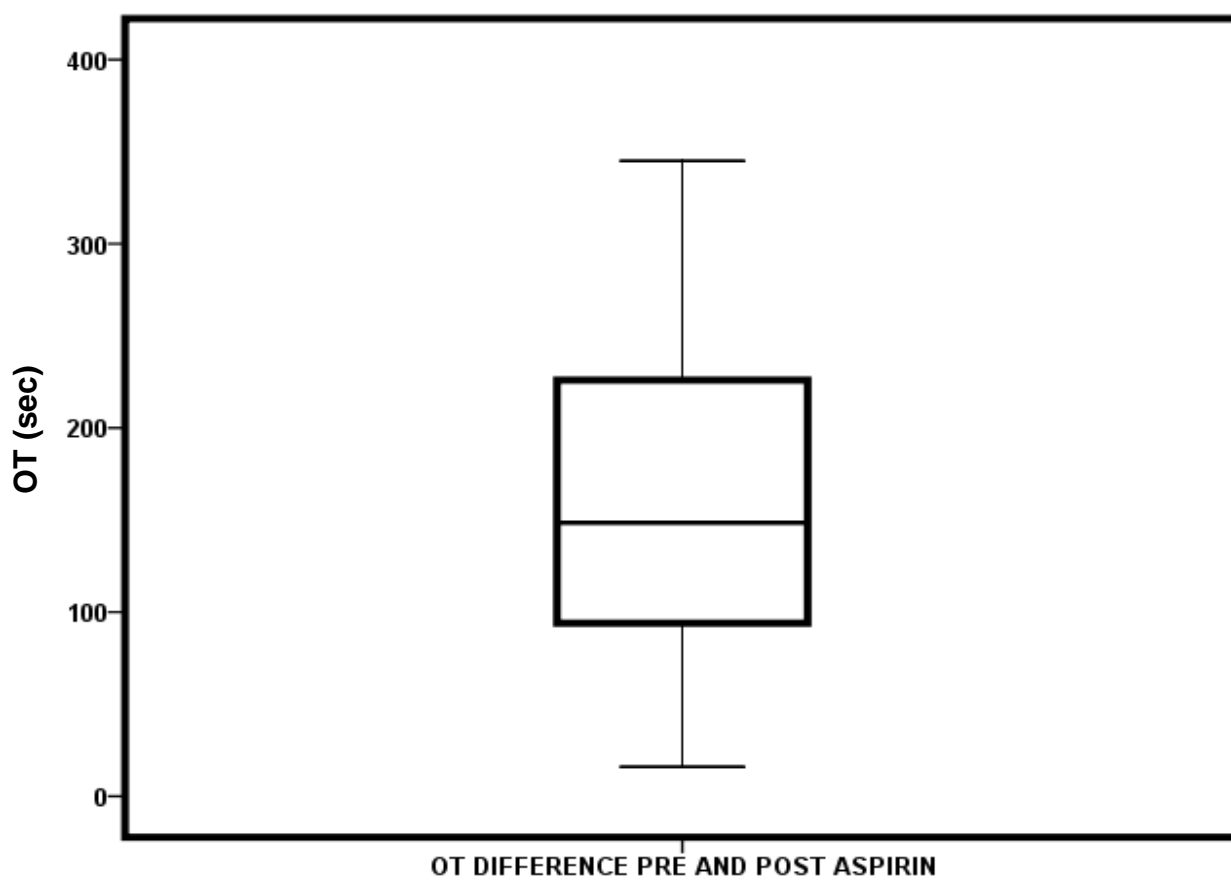


Figure 6.4: Box plot demonstrating minimum, maximum and median LT pre and post Aspirin

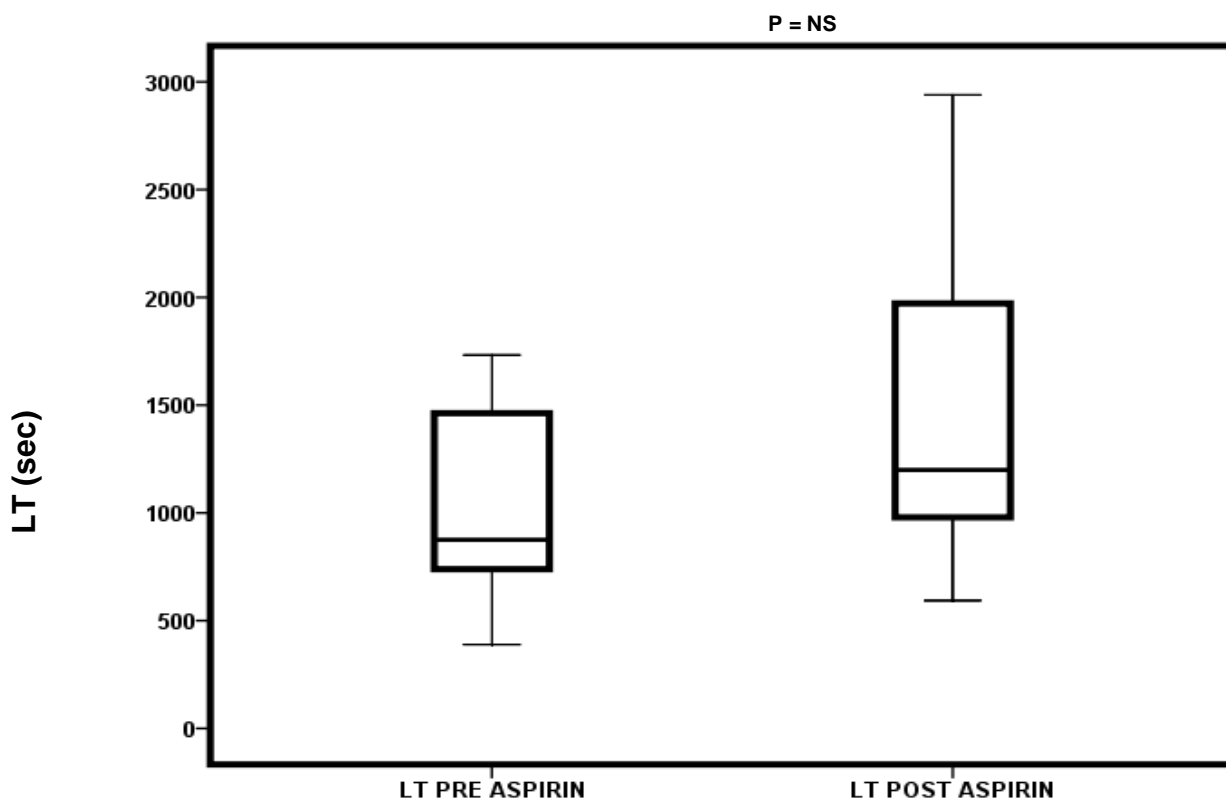
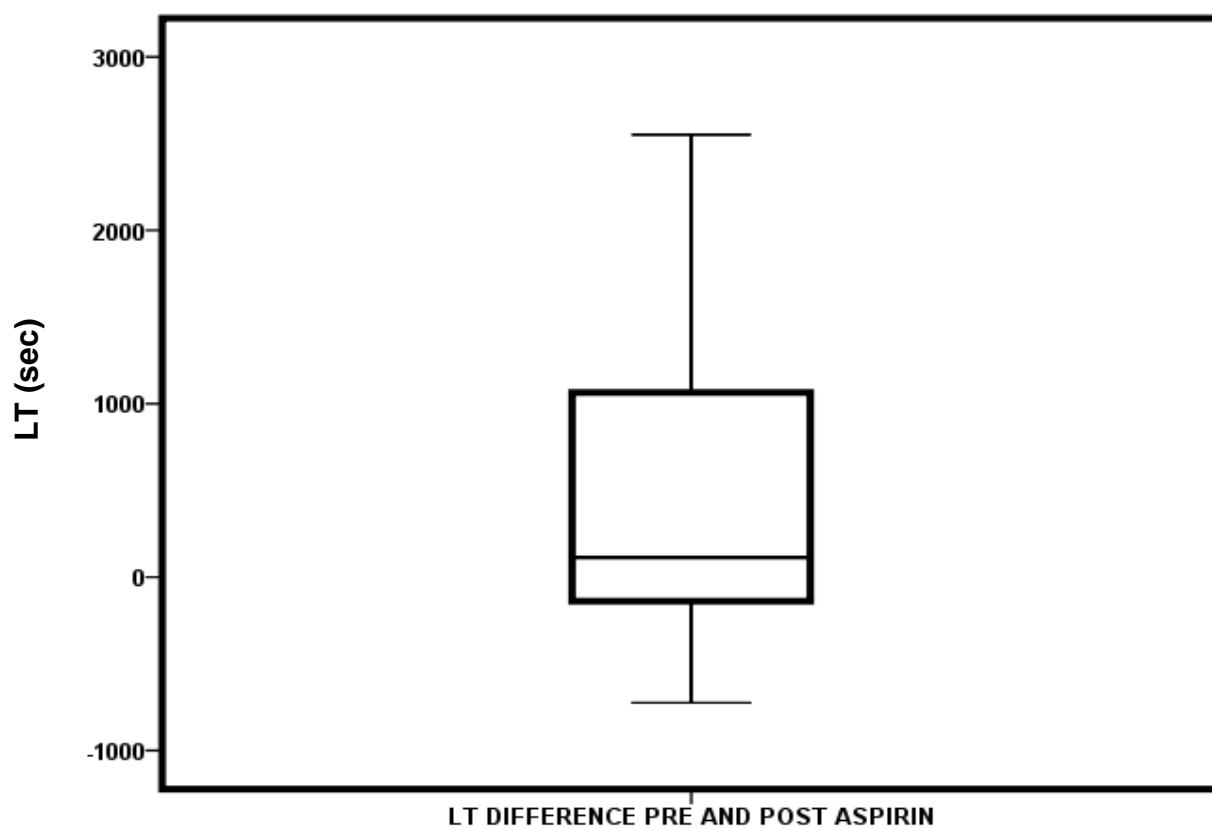


Figure 6.5: Box plot: Paired analysis demonstrating difference in LT pre and post aspirin



Clopidogrel effect on OT and LT

Mean age of the 13 healthy volunteers was 36 ± 14 years, and there were 6 males and 7 females included in the study. Results below are presented as mean \pm SEM.

Clopidogrel significantly prolonged OT (Fig 7.1, 7.2): OT vs. OTC = 379 ± 30 vs. 477 ± 29 , n=13, Wilcoxon signed rank's test $z = -3.1$, $p < 0.01$, with no measurable effect on LT (Fig 7.3): LT vs. LTC = 1390 ± 206 vs. 1345 ± 170 , n=13, Wilcoxon signed rank's test $z = -0.8$, $p = \text{NS}$.

Eight volunteers (Group 1) seemed to have a shorter OT (OT < 490s) compared to the remaining five volunteers (Group 2), (OT > 490s). Figure 7.1 suggests there is a characteristic difference in the 2 groups and they responded differently to Clopidogrel. Group 1 volunteers showed a greater rise in OT post Clopidogrel compared to Group 2 (Table 5.1, 5.2). The effect on LT was less variable. These changes were not age or sex related, and comparison between OT's was done using independent group's t-test. All volunteers were healthy non smokers, and not on any medication. The Effect ratio of Clopidogrel in Group 1 was 0.64, suggesting people with shorter OT showed a greater beneficial response to the drug, potentially achieving, and would achieve greater benefit from its use.

Due to the small sample size in this pilot study, it is difficult to clearly interpret the results, and further larger studies are required to demonstrate a greater effect size which may help in identification of high risk patients who will benefit most with use of this drug. However, the statistical power of the difference in OT in this small study is relatively strong. For $\alpha = 0.05$, the power of this study using 13 subjects was 0.8 (80% power), $P < 0.0001$.

Clopidogrel and aGTT

We have shown clopidogrel significantly prolonged OT in healthy volunteers, and we also demonstrated that water priming of the GTT tube significantly shortened OT, reflecting release of ADP secondary to haemolysis. In all 13 volunteers, aGTT was measured simultaneously alongside the GTT. Clopidogrel was seen to prevent the water-priming induced

acceleration of the thrombotic occlusion (Fig 7.4, 7.5, 7.6, 7.7) (OT.W vs. OT.W.C = 177 ± 26 vs. 362 ± 25 , n=13, Wilcoxon signed rank's test $z = -3.1$, $p < 0.01$), with no observable effect on LT (LT.W vs. LT.W.C = 1420 ± 153 vs. 1377 ± 322 , n=13, Wilcoxon signed rank's test $z = -1.0$, $p = \text{NS}$).

Effect of Clopidogrel on OT and LT in patients with Stable angina

10 patients with stable angina were tested using the GTT. Baseline demographics of these patients are listed in Table 5.3. Mean age in these patients was 72 ± 15 years and 50% were males, 50% females. All patients were known to suffer with coronary artery disease, and 30% had coexistent type 2 diabetes and 80% were known to be hypertensive. All patients were non-smokers. All patients were taking ACE inhibitors, 90% were on a form of statin therapy and 50% were on a beta blocker. Clopidogrel significantly prolonged OT (Fig 7.8), (OT vs. OTC = 404 ± 27 vs. 544 ± 35 , $p = 0.002$), with mean change in OT being an increase of 140 sec (95% CI 67.9-221.1). The standard deviation of the difference was 100.1. Median was 109.5 and reflected positive skew. Paired t test was quite robust, and the equivalent non-parametric test (Mann Whitney) gave a p value of 0.005.

There was no measurable effect of clopidogrel on LT (LT vs. LTC = 1572 ± 252 vs. 1939 ± 476 , $p = \text{NS}$). The LT data was quite skewed mean \pm SD 367 ± 1787.9 . A T test was not feasible and Mann Whitney gave a p value of 0.721. There was high level of variability in the paired differences. Larger studies are required to determine if clopidogrel significantly affects endogenous thrombolysis.

Figure 7.1: OT pre and post clopidogrel. OT (sec) - time is shown in seconds, OT= occlusion time. Clopidogrel significantly prolonged OT (OT.C) in all volunteers. Group 1 volunteers showed a greater rise in OT post-clopidogrel compared to Group 2.

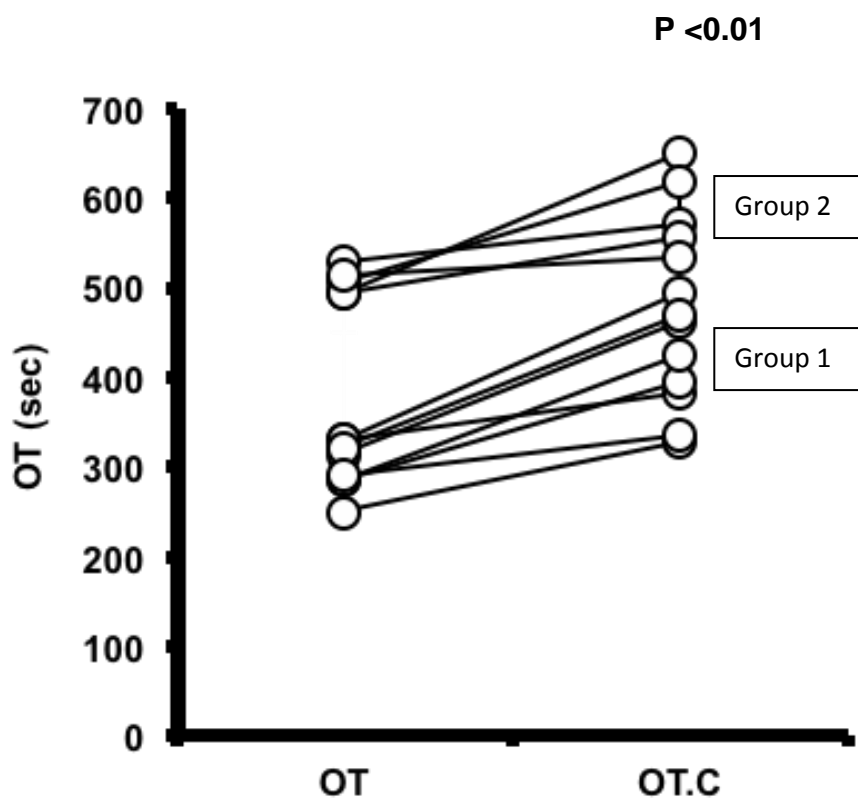


Figure 7.2: Box plot demonstrating minimum, maximum, and median OT pre and post clopidogrel

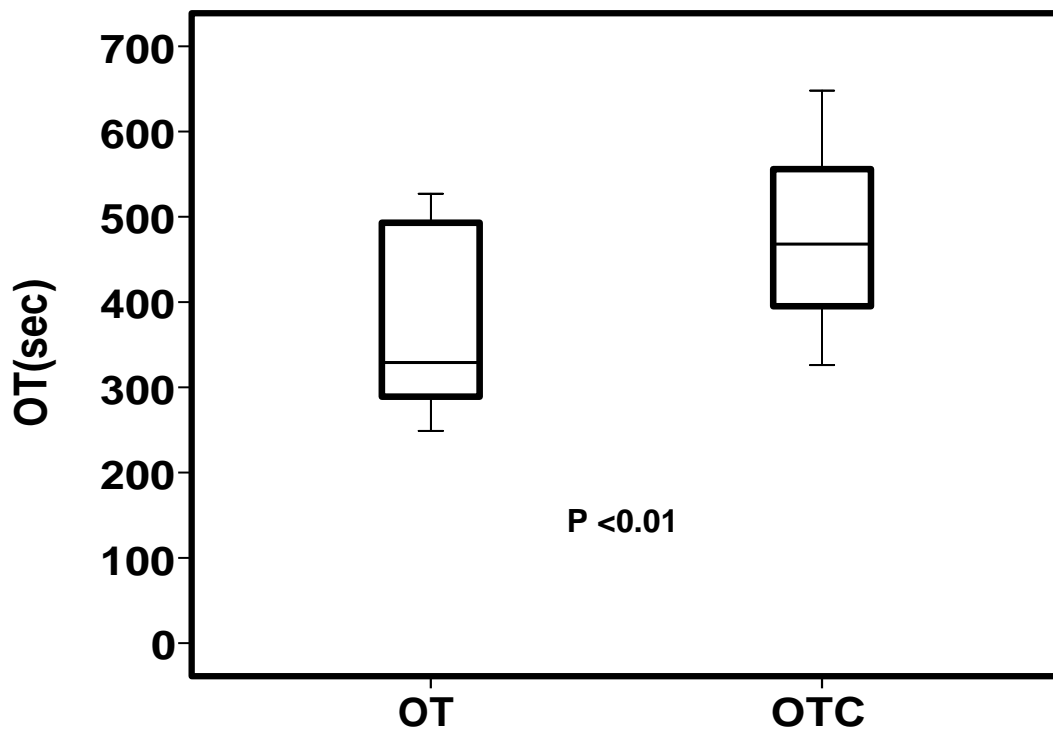


Figure 7.3: LT pre and post clopidogrel . LT (sec) - time is shown in seconds, LT= lysis time.

Clopidogrel had no significant effect on LT (LT.C)

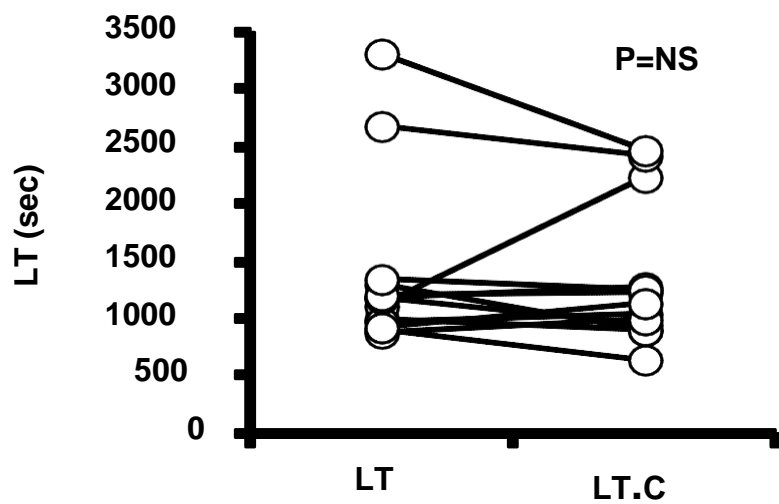


Figure 7.4: Comparison of normal OT (pre-clopidogrel) and the accelerated OT after water-priming following clopidogrel, shows that clopidogrel completely inhibits the effect of water-priming.

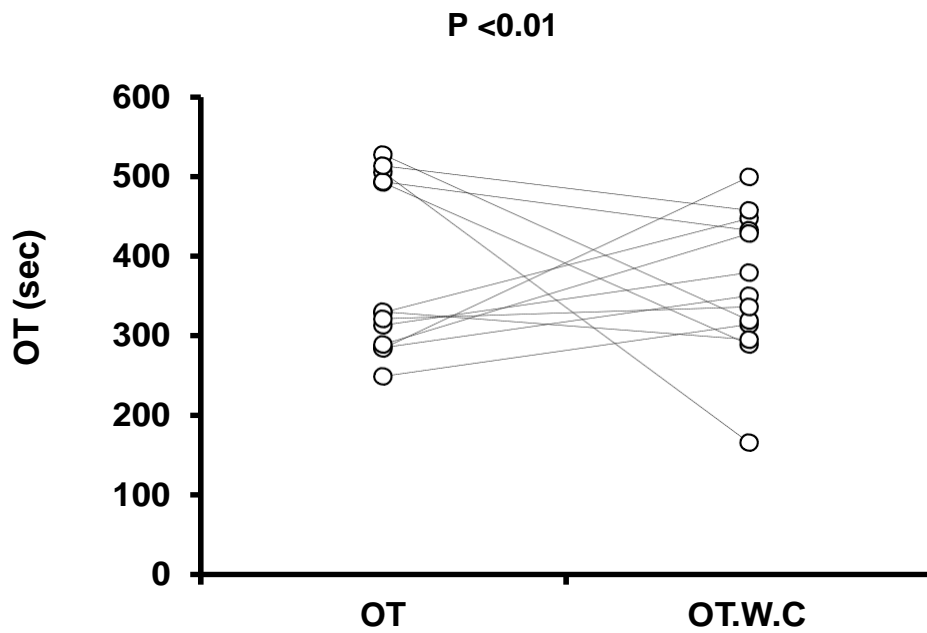


Figure 7.5: Box plot demonstrating minimum, maximum and median OT of normal OT (pre clopidogrel) and accelerated OT after water priming following clopidogrel

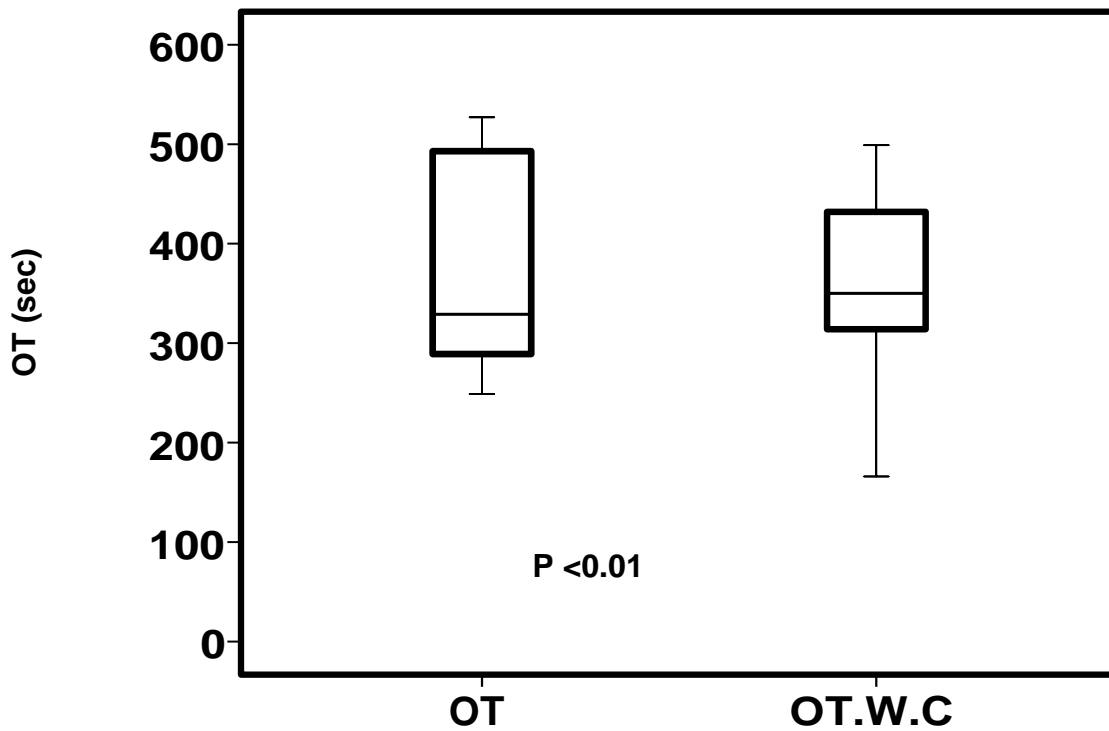


Figure 7.6: Effect of clopidogrel on aGTT (accelerated GTT with water-priming). OT.W=OT after waterpriming and OT.W.C=OT after water-priming, and after clopidogrel ingestion. Clopidogrel prevented the acceleration OT produced by water-priming.

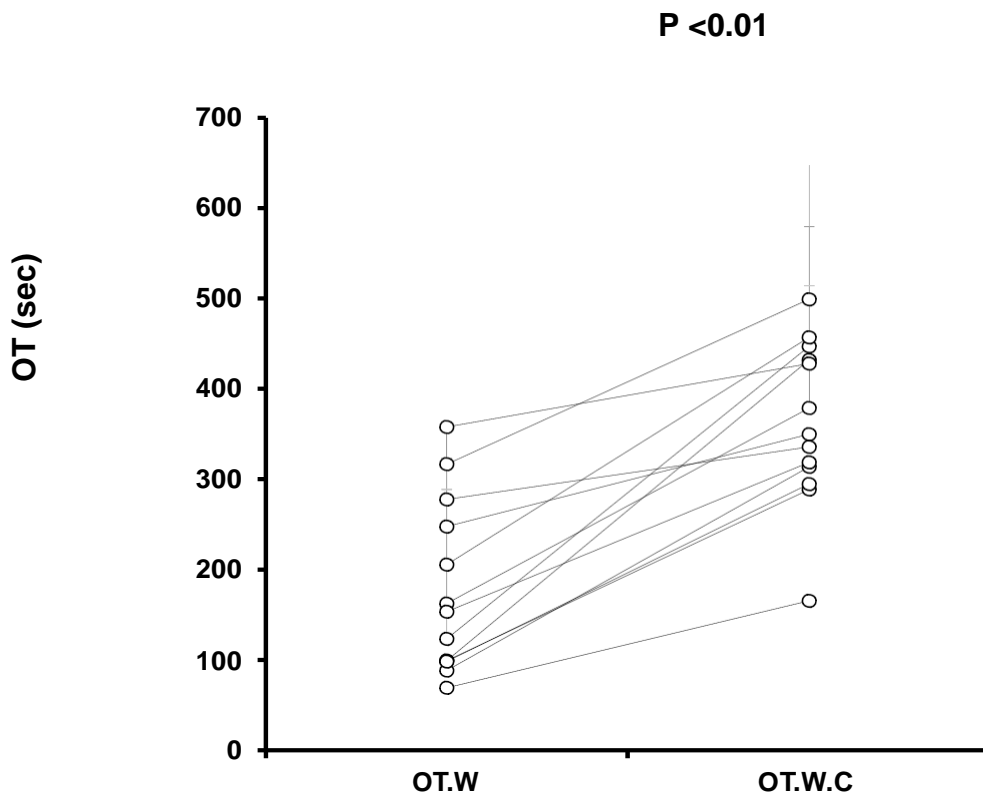


Figure 7.7: Box plot demonstrating minimum, maximum and median OT in a GTT pre and post clopidogrel

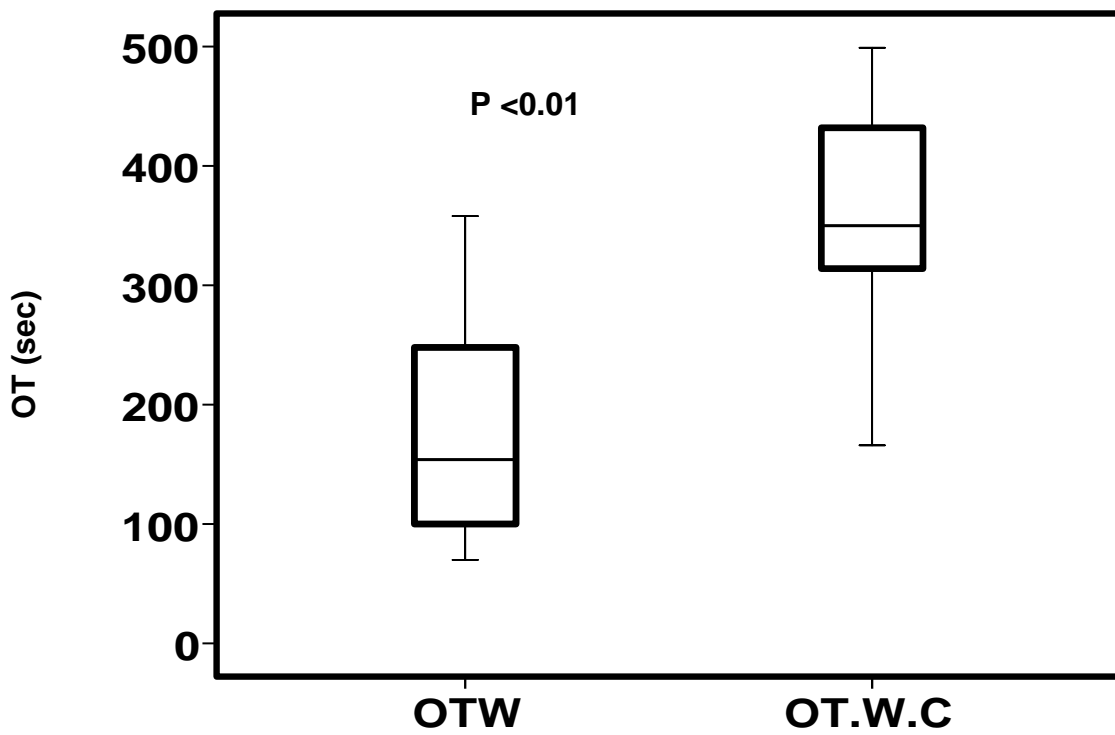


Figure 7.8: GTT and aGTT results before after clopidogrel loading. A. Effect on Occlusion Time (OT). B. Effect on Lysis Time (LT). Times are shown in seconds. (OT.W and LT.W denote the results after water-priming with aGTT= accelerated Global Thrombosis Test. [C] denotes measurements post-clopidogrel). Comparisons between treatment conditions were evaluated using Wilcoxon sign-ranked test. Columns represent the mean value and the error bar is the standard error of the mean.

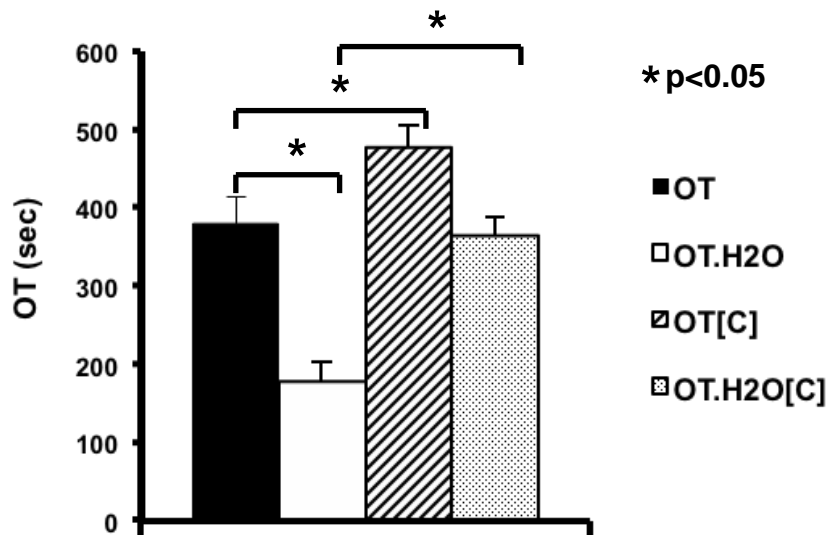
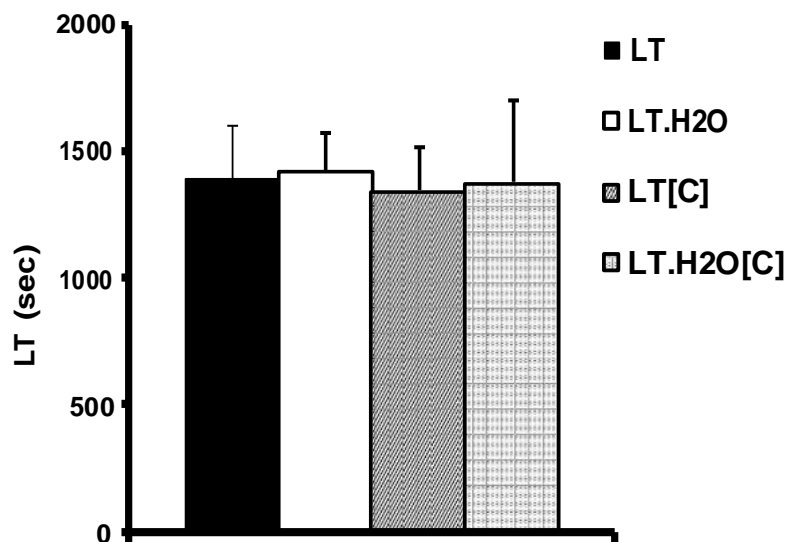
A**B**

Figure 7.9: OT pre and post clopidogrel in stable angina patients. OT (sec) - time is shown in seconds, OT= occlusion time. Clopidogrel significantly prolonged OT (OT.C) in all stable angina patients

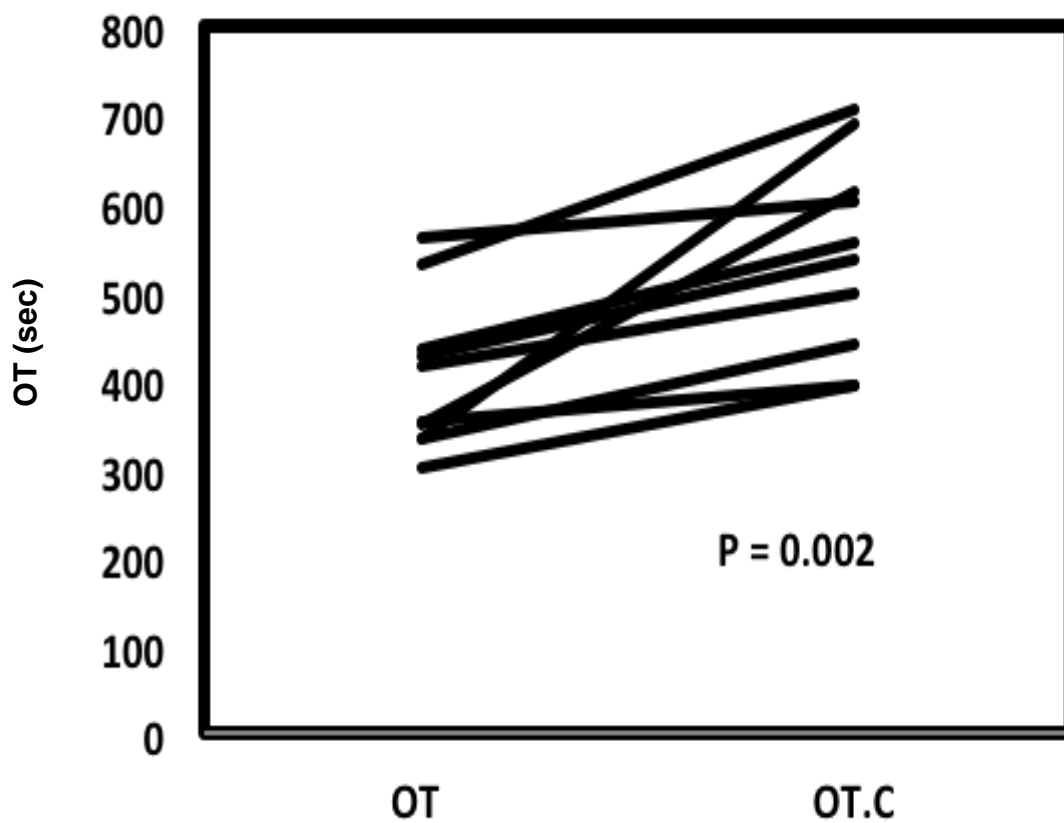


Table 5.1: GTT results in Group 1 before and after priming the GTT tube with water, and post administration of clopidogrel (OT= occlusion time, LT= lysis time, OTW = OT after water priming and LTW= LT after water priming, OTC= OT post administration of clopidogrel, LTC= LT post administration of clopidogrel, OTCW = OT post clopiogrel in aGTT, LTCW= LT post clopidogrel in aGTT, SEM= standard error of mean, IQR= Interquartile range)

Group 1: Shorter OT, Effect ratio of clopidogrel =0.64

	<i>OT</i>	<i>OTW</i>	<i>LT</i>	<i>LTW</i>	<i>OTC</i>	<i>OTCW</i>	<i>LTC</i>	<i>LTCW</i>
Mean	300	112.3	1600.3	1711.3	410.2	330.1	1526.6	1502.25
±	±	±	±	±	±	±	±	±
SEM	9.95	11.38	314.2	163.5	21.74	31.8	252.6	367.4
Median	301	100	1188	1760.5	409.5	316.5	1157.5	1078.5
(IQR)	(42.75)	(55)	(1312.25)	(755.2)	(119.25)	(128.25)	(1454)	(1170)

Table 5.2: GTT results in Group 2 before and after priming the GTT tube with water, and post administration of clopidogrel (OT= occlusion time, LT= lysis time, OTW = OT after water priming and LTW= LT after water priming, OTC= OT post administration of clopidogrel, LTC= LT post administration of clopidogrel, OTCW = OT post clopiogrel in aGTT, LTCW= LT post clopidogrel in aGTT, SEM= standard error of mean, IQR= Interquartile range)

Group 2: Longer OT, less response to clopidogrel

	<i>OT</i>	<i>OTW</i>	<i>LT</i>	<i>LTW</i>	<i>OTC</i>	<i>OTCW</i>	<i>LTC</i>	<i>LTCW</i>
Mean	506	281	1054.6	954.4	584.6	414	1056.4	1177.8
±	±	±	±	±	±	±	±	±
SEM	6.54	26.4	93.1	142.8	21	31.1	113.29	644.3
Median	505	278	933	910	570	428	1130	649
(IQR)	(27.50)	(110.5)	(379)	(517)	(88.5)	(135)	(427)	(1804)

Table 5.3: Baseline demographics of stable angina patients - Values are mean (range) or n (%).

	Overall group (n=10)
Age, yrs	72 (57-87)
Male	5 (5%)
Diabetes mellitus	3 (30%)
Smoking	0 (0%)
Hypertension	8 (80%)
Prior CAD	10 (10%)
Renal insufficiency	0 (0%)
PVD	0 (0%)
Prior CVA	0 (0%)
Beta blocker	5 (50%)
ACE inhibitor	10 (100%)
Statin	9 (90%)

Discussion, Limitations and Conclusions

Our results provide evidence that ADP may play a role in the GTT, a test of shear-induced thrombosis. It is known that air interface promotes damage to blood and contact of blood with foreign surfaces results in release of ADP from RBC and platelets (Alkhamis et al. 1990). Exclusion of these factors with saline priming significantly prolonged OT. It should be emphasized that the bulk of the blood sample was not diluted by the applied saline, since saline was restricted to the small part of the tube where platelet reaction took place. In less than 30 sec the saline-diluted blood exits the tube thus influencing only the very start of the thrombotic reaction. Although our experiments cannot distinguish between the effects of these two factors, i.e. contact with a plastic surface and blood-air interface, we regard the former to be more important. At shear stresses similar to that created in the GTT, different plastic materials were ranked according to their haemolytic effects (Offeman et al. 1979, Lafayette et al. 2007). This concurs with information obtained from the GTT manufacturer that using GTT test tubes with identical gap-sizes and thus identical shear-forces, but made from different plastic material showed great variation in blood flow rates and OT in parallel measurements of identical blood. We therefore think that the initial contact of inflowing blood with the plastic surface results in variable ADP release from platelets (Moritz et al. 1983), RBC or both. Given the observed effects of clopidogrel on OT and OT.W, the main determinant of these changes is likely to be platelet-derived ADP. The significant prolongation of OT with saline-priming demonstrates the importance of preventing the initial generation of thrombin from RBC attached to plastic surfaces (Keuren et al. 2003). Aspirin significantly prolonged OT in all 10 stable angina patients. A large Effect size of 1.60 was noted with OT measurements suggesting aspirin prolonged OT and reduced propensity to thrombus formation. Aspirin has long been used in atherothrombosis, due to its recognised antiplatelet effects which were clearly demonstrated using the GTT. LT results were variable, but a moderate effect size of 0.41 was noted. This study was a pilot study,

and there were only 10 subjects as a result of which it was underpowered to detect a statistically significant effect.

Aspirin acetylates the lysine residues in the fibrinogen molecule and alters the fibrin network structure, resulting in enhanced permeability of the fibrin network structure initiating fibrinolysis (Antovic et al. 2005). Williams et al investigated the effects of low and intermediate dose aspirin on fibrin gel porosity and fibrinolysis in 19 subjects, with 75 and 325 mg aspirin. Increased fibrin gel porosity was observed with low dose aspirin only (Williams et al. 1998).

A study by Bjornsson et al used the clot lysis assay to determine the fibrinolytic potential of aspirin. Five healthy subjects received 650 mg of aspirin twice daily for 5 days. Although the sample size was small, the results suggested shorter clot lysis times after aspirin when compared to control group (9.1 min vs. 12.4 min, $p = 0.04$) (Bjornsson et al. 1989). Reports also suggest fibrinolytic activity of aspirin through the stimulation of NO synthesis in human volunteers (Karmohapatra et al. 2007). Further larger studies are required to help us study the effect size of aspirin on endogenous thrombolysis, which if significant could prove beneficial in modulation of impaired endogenous thrombolysis, and improve outcomes.

The GTT sensitively detected the effect of haemolysis and endogenous ADP release. Turbulent flow of blood just below the upper ball may initiate haemolysis (Kamaneva et al. 2004). The importance of cell destruction, haemolysis and ADP release in the thrombotic occlusion were shown by the effect of water priming, which greatly enhanced the thrombotic reaction. The great individual variation in the shortening of OT with water-priming may be attributable to variable response of platelets to ADP or variable osmotic damage to RBC. Clopidogrel significantly prolonged OT in the GTT, and furthermore, greatly reduced or rather prevented the accelerated OT (aGTT) indicating that haemolysis and ADP release are

responsible for the acceleration of the thrombotic reaction. We have to emphasize that GTT OT measurements reflect not only platelet reactivity to aggregate but also the coagulant activity of platelets i.e. thrombin generation.

In all 13 healthy volunteers, clopidogrel resulted in a significant prolongation of OT with no observable effect on LT (Fig 6.8). Clopidogrel is an ADP antagonist and exerts this effect by inhibiting ADP release, providing us evidence that haemolysis and ADP release are essential in the shear induced thrombotic reaction. We have demonstrated shortening of OT due to haemolysis induced by distilled water in the aGTT. We postulated that due to the osmotic gradient between water and blood, there is cell destruction, haemolysis and release of ADP from RBC and platelets which in turn activates platelet adhesion and aggregation resulting in the accelerated thrombotic reaction. Clopidogrel was able to inhibit this accelerated thrombotic reaction, providing evidence that ADP released during haemolysis is responsible for the enhanced thrombotic reaction. Clopidogrel prevented shortening of OT in most individuals in the aGTT, allowing the GTT to sensitively determine the efficacy of ADP antagonist therapy which can prove to be an invaluable tool in optimizing dose or determining resistance to antiplatelet agents. Parallel measurements of GTT and aGTT can allow assessment of global thrombotic status and monitor response and efficacy of ADP antagonists in cardiac patients, and help optimize antiplatelet therapy to improve outcomes.

Neither water nor clopidogrel had any effect on lysis times. This concurs with earlier findings that clopidogrel does not induce fibrinolysis in healthy subjects (Taher et al. 2004).

In relation to our findings, we have to discuss the pathological significance of RBC damage in arterial thrombus formation. The contribution of RBC to arterial thrombogenesis is supported by clinical and experimental evidence. Thrombotic complications occur in conditions such as haemolytic anaemias, and RBC haemolysis is reported around valve

replacements. Formation of thrombi on cardiovascular devices is partly attributed to the significantly higher osmotic sensitivity of RBC exposed to turbulent shear forces (Michelson et al. 2007) and the haemolytic effect of turbulent flow (Aziz et al. 2007). Thrombin is a key factor in arterial thrombogenesis and RBC lysates are strong promoters of thrombin generation (Quinlan et al. 2007). The extent of RBC damage by very high turbulent shear forces around prosthetic valves or around deep vessel wall injury (such as plaque rupture) should not be underestimated. The accelerated thrombosis test used here simulates conditions where ADP is released mainly from RBC by high shear forces and turbulent flow in an artery of severely reduced lumen, or at the site of plaque rupture. The test can be criticized for not using a well defined concentration of ADP, however in individuals the extent of haemolysis and hence the released ADP concentration is variable. We regard this as an advantage of this test over using fixed ADP concentrations, as it reflects the *in vivo* situation of variable RBC damage during an arterial thrombotic event. In addition, the minute amount of ADP released during the very short contact between blood and water can be inhibited more sensitively, than the much higher concentrations of ADP commonly employed as the agonist in various platelet function tests.

As a "global test", GTT has the obvious limitation that it is not specific for any particular agonist. It reflects true thrombotic status but cannot reveal the causes of enhanced or inhibited thrombotic activity. Contribution from potential plasma thrombotic/inflammatory factors to the results obtained with the GTT should also be considered. Among all such factors, only vWF, which reflects endothelial damage/dysfunction is regarded to be significant. Although the importance of vWF in the pathogenesis of myocardial infarction is undeniable, the association between high plasma vWF levels and arterial thrombosis has been controversial (Spencer et al. 2007, May et al. 2007). An earlier report of increased vWF

levels and platelet reactivity after coronary angioplasty only in blood samples taken from the coronary sinus but not in peripheral venous samples may explain the above controversy (Gorog et al. 2003). Nevertheless, a good correlation was found between GTT OT measurements and plasma vWF levels (Nishida et al. 2006).

Although the normal GTT detected a significant antiplatelet effect of clopidogrel, the test in this form is not sensitive enough to monitor individuals for clopidogrel effect or resistance.

The accelerated thrombosis test presented here allows a more physiological assessment of thrombotic risk; since it employs native blood, high shear akin to that in a significantly stenosed vessel and detects the contribution of endogenous ADP release to occlusive thrombus formation. Furthermore, it sensitively detects the effect of ADP-antagonist medication on shear-induced thrombus formation and may allow individualised tailoring of such therapy. Ongoing studies in patients with coronary disease will reveal the clinical usefulness of using the normal and accelerated GTT in detecting antiplatelet resistance .

Chapter 5: Platelet function in Acute Coronary Syndrome, stable angina and Healthy volunteers

OT and LT in Acute Coronary Syndrome Patients

Background: Spontaneous thrombolysis is a determinant of the outcome of arterial thrombosis. The risk of future events after an acute myocardial infarction depends not only on occlusive thrombus formation, but also on the physiological process of spontaneous thrombolysis, preventing lasting occlusion and prolonged ischaemia of the myocardium.

Thrombotic events continue to occur despite treatment of high risk patients with dual antiplatelet medication. Nonresponsiveness to antiplatelet drugs remains a major limitation in the prevention of future thrombotic episodes in patients who experience acute coronary syndrome (ACS). Previous studies estimated that 5.5-56.8% of the population are aspirin resistant (Kim et al. 2008), while the prevalence of clopidogrel nonresponsiveness was 21% (Snoep et al. 2007). Although much fewer published data are available for dual (aspirin and clopidogrel) nonresponsiveness, the incidence in a recent study in 746 patients was only 6% (Gori et al. 2008).

In a study using Verify now assay and five other platelet function tests (Lordkipanidze et al. 2007), poor correlation was observed between the tests suggesting there is low agreement between assays. In the ARMYDA-PRO (Antiplatelet therapy for Reduction of Myocardial Damage during Angioplasty-Platelet Reactivity Predicts Outcome) study, PRU was tested in 160 patients undergoing PCI and high platelet reactivity was a significant predictor of MACE (Marcucci et al. 2009). Using ROC curve analysis, the optimal cut-off for the primary end point was $PRU \geq 240$ (area under the curve: 0.69; 95% CI: 0.56 to 0.81, $p=0.016$). Similarly, in a study by Price et al, 380 patients undergoing PCI with DES were tested using the Verify now assay, and the optimal cut-off for MACE was a $PRU \geq 235$ (area under the curve

0.711; 95% CI: 0.529–0.893, $P = 0.03$) (Price et al.2008). Based on the results of these studies, $PRU \geq 240$ was considered a significant determinant of high platelet reactivity.

Our aim in this study was to determine OT and LT in patients admitted to hospital with a diagnosis of Acute Coronary Syndrome (ACS), and to determine if either of these variables were a significant predictor of future adverse cardiac events.

It was also our aim to compare OT and LT in healthy volunteers, stable angina and ACS patients to determine if the GTT was able to identify individuals at high risk of thrombus formation, due to prothrombotic status or impaired endogenous fibrinolysis .This would allow us to monitor the high risk stable angina patients, and modify risk factors to prevent development of subsequent thrombus that could result in acute myocardial infarction.

We also compared platelet reactivity using the GTT and Verify Now Assay, to determine if there was any correlation between these two tests.

Hypothesis:

We postulated that OT would be prolonged in coronary artery disease patients due to the antithrombotic effect of antiplatelet medications. We also postulated that ACS patients with prolonged LT would be at increased risk of future major adverse cardiac events due to impaired endogenous thrombolysis.

Methods:

Study Population of ACS patients

Patients admitted to hospital with ACS were included in the study. ACS was defined by the presence of at least two of the following: ischaemic chest pain, elevation of cardiac enzymes (troponin or creatine kinase i s o e n z y m e at least twice the upper limit normal limits), or

dynamic electrocardiographic changes (ST elevation, ST depression or T wave inversion). All patients received dual antiplatelet therapy for a year since index admission. Exclusion criteria are listed in Table 6.1. All patients were loaded with Aspirin 300 mg and Clopidogrel 300 mg on admission, and were taking 75 mg each of Aspirin and Clopidogrel thereafter as maintenance dose. 300 patients admitted with ACS were included in the study and sampled during their index admission. Most ACS patients receive unfractionated low molecular weight heparin (LMWH) on admission, and all these patients were sampled a minimum of 48 hours, (5 ± 3 days after admission, Mean \pm SD) after discontinuation of LMWH to avoid any effect of the anticoagulant on OT and LT.

The coefficient of variation was assessed in ten patients with ACS twice, at 24 h intervals. The CV for OT was 7% and for LT was 19%, similar to the CV obtained in normal volunteers.

Table 6.1: Exclusion criteria

Inability to consent

Current participation in another study

More than 90 years of age or <18 years of age

Cardiogenic shock

Sepsis

Malignancy

Bleeding diathesis

Thrombolysis, warfarin, or glycoprotein IIb/IIIa inhibitor before sampling

Concomitant medication with erythromycin, dipyridamole

Blood dyscrasia (platelets <100, hemoglobin <8 g/dl, international normalized ratio >1.4, activated partial thromboplastin time more than twice the upper limit of normal, leukocyte count <3.5 X 10⁹/l, neutrophil count <1 X 10⁹/l)

Intolerance to or contraindication to aspirin or clopidogrel

Complete follow-up over 1-year period not likely

Other disease shortening life expectancy to <12 months

Sampling procedure

Blood samples were taken from an antecubital vein using an 18 –G butterfly cannula using a 2- Syringe technique. The first 2 mls of blood was either discarded or used for routine blood tests and the next 3-5 ml blood samples were used to assess thrombotic and thrombolytic status. This blood sample was injected with force into the GTT tube, and the shear force between the small aperture between tube and ball bearings resulted in platelet activation and subsequent aggregation. Measurement was started within 15 seconds, and the instrument indicator changed colour once the analysis was complete. Each instrument had a memory card, which could be connected to a computer to download and view the measurement graphs.

Data collection and Follow up

All ACS patients were recruited into the study during their index admission, and baseline demographics recorded on the case report forms (CRF). GTT measurements of OT and LT were taken pre PCI, and results documented for all 300 patients. Clinical or telephonic follow up was done at 3 monthly intervals, and source documentation of all major adverse cardiovascular events (MACE) obtained. After ascertainment of the time of event, blood results including cardiac biomarkers were obtained from the electronic pathology database, and inpatient admission notes screened to determine the sequence and details of the adverse events.

Admission ECGs, echocardiogram, angiogram and other radiology reports were accessed, and medication history determined. Compliance to antiplatelet and other medications was determined by direct communication with the patient where possible. After accumulation of all data required for determination of MACE, analysis was performed with the use of statistical package SPSS v16.

Primary and Secondary Outcome

The primary endpoint of the study was a composite occurrence of cardiovascular death, non-fatal myocardial infarction or stroke (MACE) at 1 year. The endpoints of the study were: 1) cardiovascular death, defined as death in the presence of ACS, significant cardiac arrhythmia, refractory congestive heart failure, or death attributed to cardiovascular cause at post mortem; 2) nonfatal MI (a rise in troponin I or an increase in creatinine kinase – myocardial band isoenzyme at least twice the upper limit normal limits with atleast 1 of the following : acute onset of prolonged (≥ 20 minutes) ischaemic sounding chest pain; ST- segment elevation of atleast 1 mm in 2 or more contiguous electrocardiographic leads, or ST- segment depression ≥ 0.5 mm in ≥ 2 contiguous leads; or T- wave inversion > 1 mm in leads with predominant R waves; or 3) stroke, defined as the presence of new neurological deficit though to be vascular in origin, with signs or symptoms lasting more than 24 hours and supported by an imaging procedure such as a computed tomography (CT scan) or magnetic resonance imaging (MRI). The secondary endpoints of the study included MACE at 30 days, bleeding complications and the need for repeat revascularization at 1 year. Bleeding complication was defined as intracranial or intraocular bleeding, access site haematoma > 5 cm diameter, significant bleeding resulting in haemodynamic compromise, or a drop in Hb ≥ 3 g/dl.

Statistical Analysis

SPSS version 16 (SPSS Inc., Chicago, Illinois) was used for all analyses. Normally distributed variables were analysed using unpaired *t* test and non-normally distributed variable were analysed using Mann - Whitney *U* Test. Dichotomous variables were compared using Chi – Square or Fisher’s Exact tests. Correlations were analysed using Spearman’s coefficient of rank correlation and Log transformation was used, where appropriate. Receiver operating

curve (ROC) was used to discriminate between patients with and without MACE. A cut-off value was determined by identifying an LT value providing greatest sum of sensitivity and specificity. Cox regression was used to investigate relationship between LT and MACE, and LT was divided into bands of 1000's, and Kaplan - Meier curve with log-rank test was used to compare survival curves.

Univariate and Multivariate hazard regression model of Cox were used to determine risk factors for clinical end points, and to adjust for potential confounders that were associated with clinical endpoints in Univariate analysis. A significance level was defined as $p < 0.05$.

OT and LT were found to be separately related to MACE. To establish whether OT or LT outside the normal range may be predictive of MACE, a normal range was established from Mean \pm 2SD s of normal volunteers.

Comparison of platelet function in ACS, Stable angina and Healthy volunteers

We evaluated OT and LT using the GTT in seventy five stable angina patients. Stable angina patients were reviewed and recruited in cardiac outpatient clinic, and all patients with underlying evidence of coronary artery disease were recruited in the study. All patients were on aspirin and clopidogrel at the time of recruitment. OT and LT of normal volunteers, SA and ACS patients were then compared to demonstrate if there was any significant difference in the three groups.

SPSSv16 was used for statistical analysis. Comparison between groups was done using ANOVA, and non-parametric Mann -Whitney test was used to demonstrate any statistically significant difference ($p < 0.05$).

Comparison between GTT and Verify now assay in ACS patients

In a cohort of 71 ACS patients, we assessed thrombotic status using the GTT and the Verify now assay. Venous blood samples were taken from the antecubital vein and anticoagulated with sodium citrate 0.109 mol/l (ratio 9:1). We used the Verify Now P2Y (12) cartridge which measures ADP (P2Y12) inhibition.

Results from the Verify Now P2Y (12) assay are measured in P2Y (12) reaction units (PRU), higher the PRU worse the outcome in reported studies. Based on normal range of OT in healthy volunteers (200-550s, we compared OT < 200s to Verify now PRU \geq 240 units as an indicator of prothrombotic status in ACS patients.

Results

Demographics of ACS patients are listed in Table 6.2.

Completed follow-up was available in 297 patients. It has been mentioned in previous chapters that OT was normally distributed in healthy volunteers (Fig 8.1), and LT distribution was skewed (Fig 8.2). In ACS patients, OT was significantly prolonged compared to that of normal volunteers (428 ± 155 s vs. 378 ± 96 s, $p < 0.001$) (Fig 8.3, 8.4). The ROC curve analysis for OT and LT demonstrated that LT level significantly discriminated between patients with and without MACE with an area under the curve of 0.63 (95% CI: 0.51 to 0.69; $p < 0.05$). LT ≥ 3000 was identified as the optimal cut point to predict MACE outcome, with sensitivity of 60% and specificity of 80%. (Fig 8.5).

Survival analysis did not demonstrate any relationship between OT and MACE (Fig 8.6, 8.7).

Different variables were interrogated for effects on OT, including patient demographics,

echocardiographic and angiographic characteristics, medications and a significant effect was observed only with the use of statin. Patients on statin therapy were noted to have a longer OT (less prothrombotic) compared to those not on statin therapy (439 ± 157 s vs. 359 ± 120 s, $p=0.02$). Patients on recent LMWH (< 48 hrs earlier) and recent thrombolysis (< 5 days earlier) were excluded from this analysis to avoid any confounding results.

LT was positively skewed in both healthy volunteers and ACS patients. LT was significantly prolonged in ACS patients compared to healthy volunteers [median 1053 (978 to 1125) s vs. 1362 (1240 to 1514) s, $p < 0.001$]. 23 % ACS patients had $LT > 3000$ S, compared to none of the healthy volunteers, with 15.3% patients demonstrating markedly impaired thrombolytic status with $LT \geq 5000$ s.

LT was noted to be significantly predictive of MACE at 180 days and at 1 year.

At $LT \geq 3000$ s, the HR 2.48 (CI = 1.2 -4.8, $p= 0.007$) at 6 months (Fig 8.8), and HR 1.85 (CI= 1.01- 3.4, $P=0.04$) at 1 year (Fig 8.9). Above this LT level, the HR increased as the LT increased. With $LT \geq 5000$ s HR was 2.57 (CI = 1.2-5.2, $P=0.009$) at 6 months (Fig 9.0), and HR: 2.08 (CI=1.07-4.01, $p=0.02$) at 1 year (Fig 9.1).

Using Univariate Cox regression analysis, $LT \geq 3000$ s was associated with a significantly higher risk of cardiovascular death at 6 months (Fig 9.2) (HR: 4.04, CI = 1.3-12.0, $P=0.012$), and at 1 year (HR: 3.9, CI= 1.3-11.9, $P= 0.013$) (Fig 9.3). The risk of CV death did not increase significantly with increase in LT ($LT \geq 5000$ s; CV Death HR: 3.6, CI = 1.18-11.1, $P= 0.024$) but $LT \geq 5000$ s was a significant predictor of recurrent ACS (HR: 2.2, CI=1.08-4.6, $P= 0.02$) both at 6 months and at 1 year (Fig 9.4, 9.5).

Variables were interrogated for effects on LT: patient demographics, echocardiographic and angiographic features, and medications. In patient demographics, only age was related to LT, and LT increased with age ($p=0.008$). A trend was noted between troponin and LT ($p= 0.05$). A weak association was also noted between OT and LT ($r = -0.2$, $p=0.07$).

Univariate analysis suggested some variables were related to MACE: age, diabetes mellitus, metformin, low haemoglobin, low haematocrit, C-reactive protein, peripheral vascular disease, angiotensin-converting enzyme inhibitor, and oral nitrate therapy. Very few patients were known to suffer with peripheral vascular disease in the study.

Variables were then inserted into the final model of multivariate logistic regression analysis; age, sex, diabetes mellitus, angiotensin-converting enzyme inhibitor, and nitrates. Multivariate analysis demonstrated LT to be an important independent predictor of MACE, after adjustment for all these risk factors at 6 months and at 1 year (LT ≥ 3000 s at 6 months: HR 2.4, 95% CI: 1.2-4.8, $p = 0.008$, LT ≥ 3000 s at 1 year: HR 1.9, 95% CI: 1.04-3.5, $p = 0.003$, LT ≥ 5000 s at 6 months: HR 2.69, 95% CI: 1.3-5.5, $p = 0.007$, LT ≥ 5000 s at 1 year: HR 2.1, 95% CI: 1.12-4.2, $p = 0.002$). Hazard ratio was noted to increase slightly as LT increased.

Table 6.2: Baseline demographics of ACS patients

	Overall group (n=300)	LT<3000 s (n= 231)	LT≥3000s (n=69)	P value
Age, yrs	65 (40-90)	64.2	66.7	NS
Male	216(72.3)	170(73.6)	47(68.1)	NS
Diabetes mellitus	50(16.7)	35(15.2)	15(21.7)	NS
Smoking	68(22.7)	54(23.4)	14(20.3)	NS
Hypertension	142(47.5)	111(48.1)	31(44.9)	NS
Prior CAD	129(43.0)	105(45.5)	24(34.8)	NS
Renal insufficiency	24(8.1)	17(7.4)	7(10.1)	NS
PVD	8(2.7)	6(2.6)	2(2.9)	NS
Prior CVA	16(5.4)	13(5.6)	3(4.3)	NS
STEMI	73(24.5)	55(23.8)	19(27.5)	NS
Troponin positive	226(79.0)	171(74.0)	55(79.7)	NS
Dynamic ECG changes	203 (68.0)	135(58.4)	39(56.5)	NS
Beta blocker	234(78.3)	181(78.4)	53(76.8)	NS
ACE inhibitor	216(72.2)	168(72.7)	48(69.6)	NS
Statin	273(91.3)	212(91.8)	61(88.4)	NS
Nitrate	73(24.4)	59(25.5)	14(20.3)	NS
Insulin	15(5.0)	10(4.3)	5(7.2)	NS
Metformin	15(5.0)	12(5.2)	3(4.3)	1.0

Values are mean (range) or n (%). Renal insufficiency was defined by creatinine levels >2.0 mg/dl. ACE= angiotensin-converting enzyme; CAD =coronary artery disease; CVA = cerebrovascular accident; LT=Lysis time; NS= not significant; PVD= peripheral vascular disease; STEMI= ST-segment myocardial infarction

Table 6.3 - Angiographic, Interventional and Echocardiographic characteristics of ACS patients

	Overall group (n=300)	LT<3000 s (n=231)	LT≥3000 s (n=69)	P value
Angiogram performed	263(87.6)	202/231 (87.4)	61/69(88.4)	NS
1-vessel disease	107 (36)	87/202 (43)	20/61(32.7)	NS
2-vessel disease	57 (19)	41/202 (20.2)	16/61(26.2)	NS
3-vessel disease	39 (13)	30/202 (14.8)	9/61(14.7)	NS
PCI performed	103 (34)	80/202 (39.6)	23/61 (36)	NS
DES only	46 (15)	31/80 (38.7)	15/23(65)	NS
BMS only	47 (16)	40/80(50)	7/23 (30.4)	NS
DES and BMS	93 (31)	71/80 (88.75)	22/23 (95.6)	NS
GPIIb/IIIa antagonist	0 (0)	0 (0)	0 (0)	NS
Echo performed	197 (66)	152 (65.8)	45 (65.2)	NS
LVEF >50%	136 (45)	109/152 (71.7)	27 /45(60)	NS
LVEF 40-50%	23 (7.7)	15/152 (9.86)	8 /45(17.7)	NS
LVEF 30-40%	22 (7.3)	17/152 (11.18)	5 /45(11.1)	NS
LVEF <30%	16 (5.3)	11/152(7.2)	5 /45(11.1)	NS
Referred for CABG	34 (11)	26/202 (12.8)	8 /61(13.11)	NS

Values are n (%). PCI=percutaneous coronary intervention; DES= drug-eluting stent (s);

BMS=bare metal stent(s); GP=glycoprotein; LVEF=left ventricular ejection fraction;

CABG=coronary artery bypass grafting.

Figure 8.1: OT in healthy volunteers demonstrating normal distribution

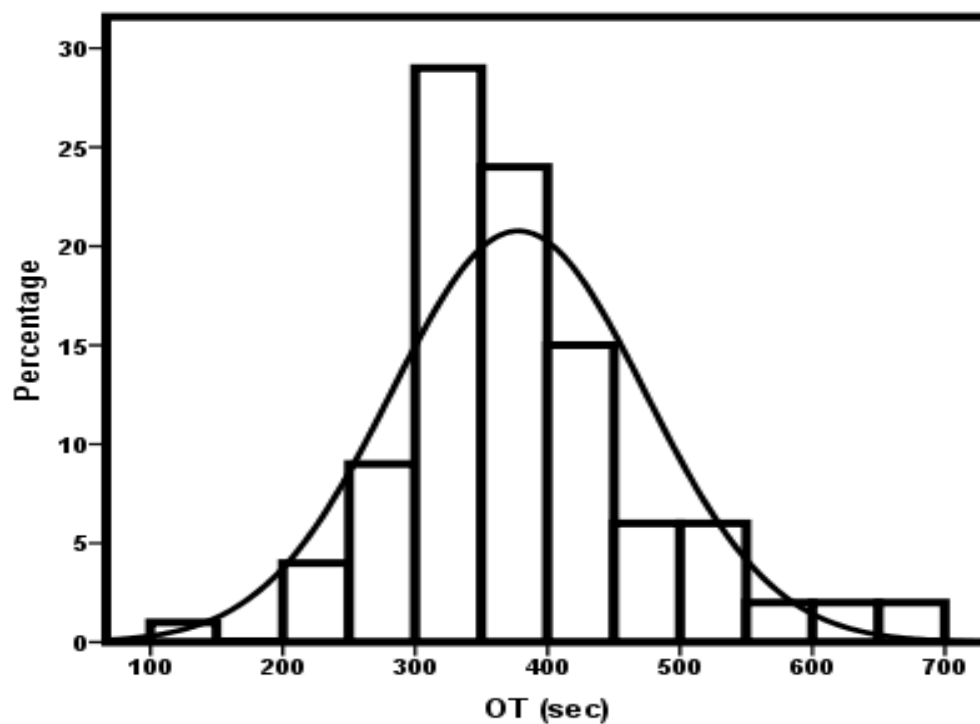


Figure 8.2: OT in ACS patients - In ACS patients taking dual-antiplatelet medication, OT was significantly prolonged (showing reduced platelet reactivity) compared with that of normal volunteers.

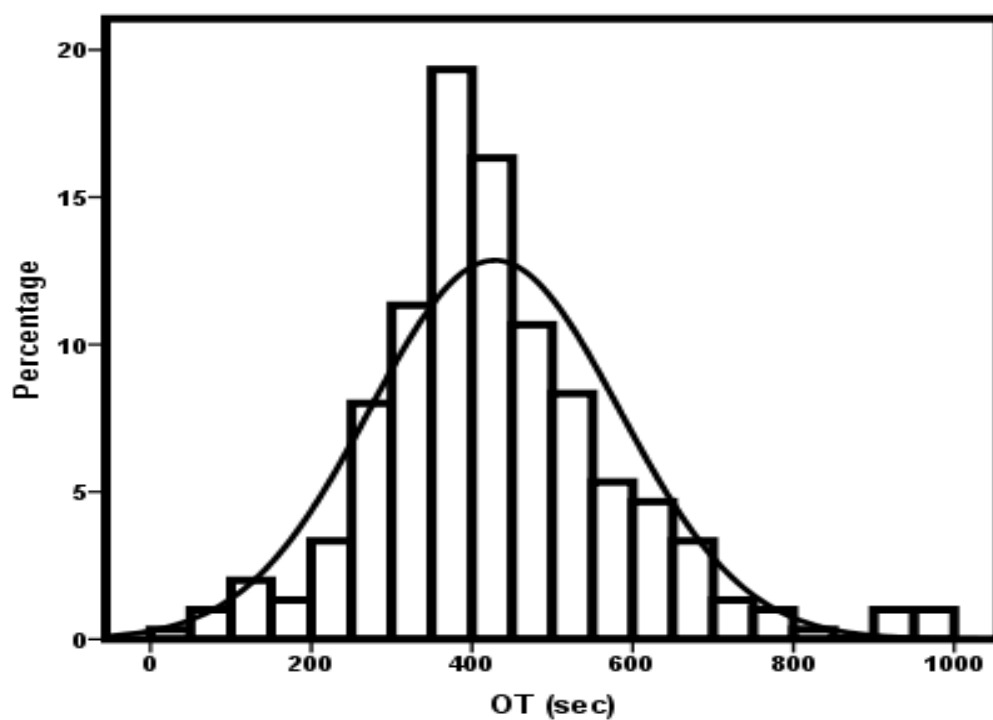


Figure 8.3: LT in healthy volunteers

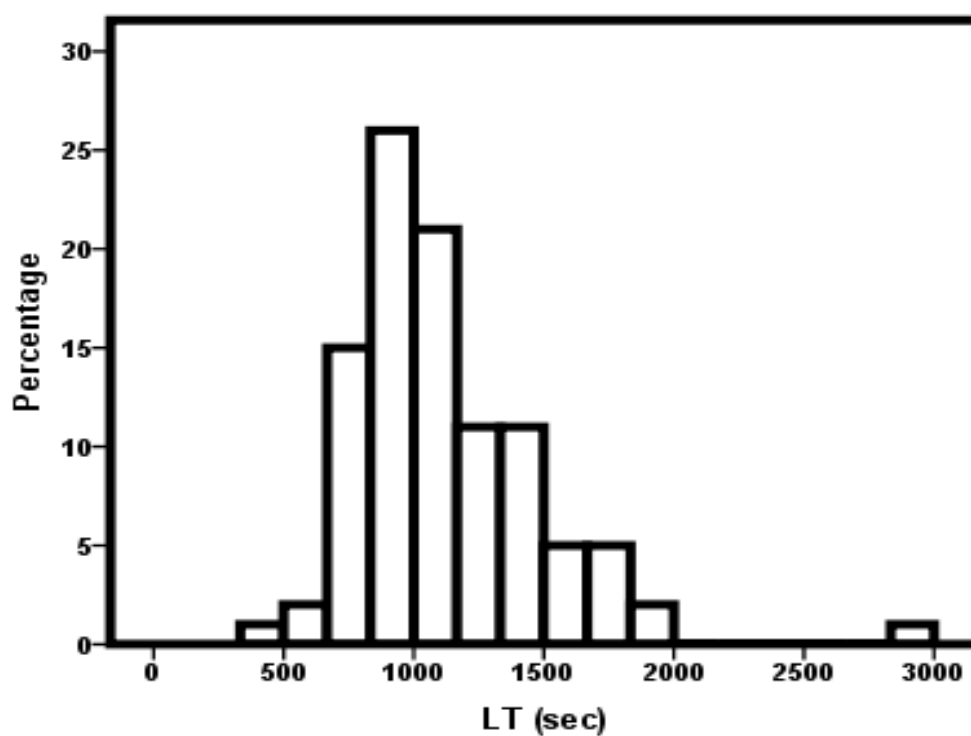


Figure 8.4: LT in ACS patients- In ACS patients, LT was significantly prolonged (impaired endogenous thrombolysis) compared with that of healthy volunteers.

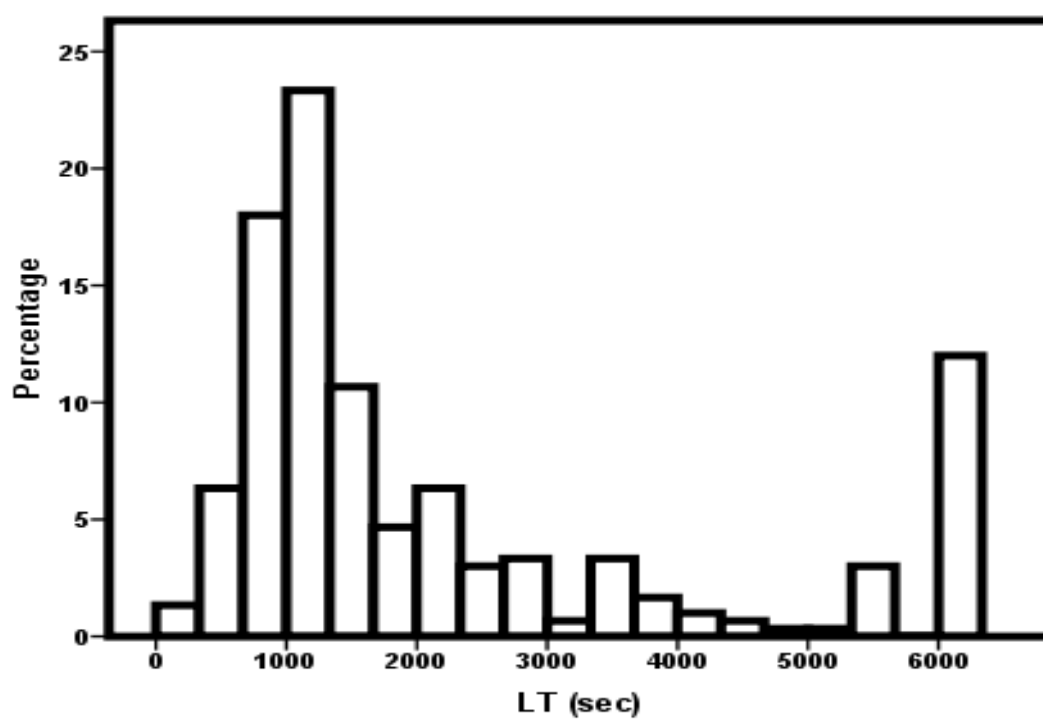


Figure 8.5: ROC Curves for OT and LT (Receiver-operating characteristic (ROC) curves for (A) occlusion time (OT) and (B) lysis time (LT). The LT level significantly discriminated between patients with and without major adverse cardiovascular events (MACE) with an area under the curve of 0.63 (95% confidence interval: 0.51 to 0.69; $p < 0.05$). An LT $>3,000$ s was identified as the optimal cut point to predict MACE outcome, with sensitivity of 60% and specificity of 80%).

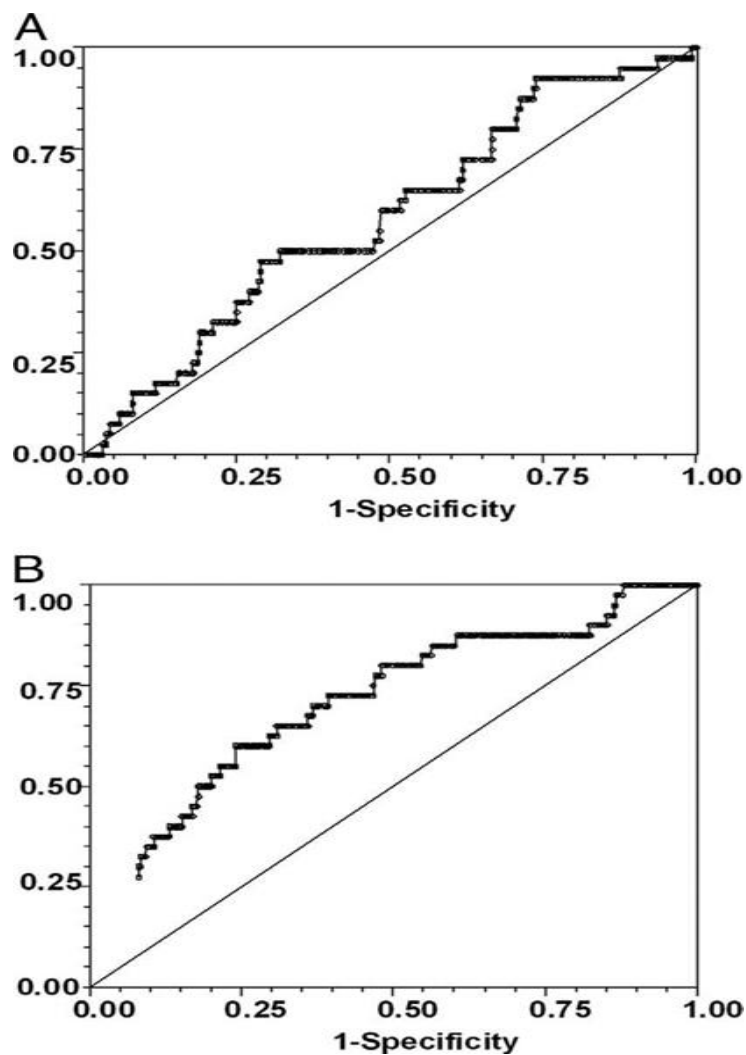


Figure 8.6: KM curve demonstrating probability of event free survival using OT as a predictor of adverse events at 180 days. Survival analysis did not demonstrate any relationship between OT and MACE.

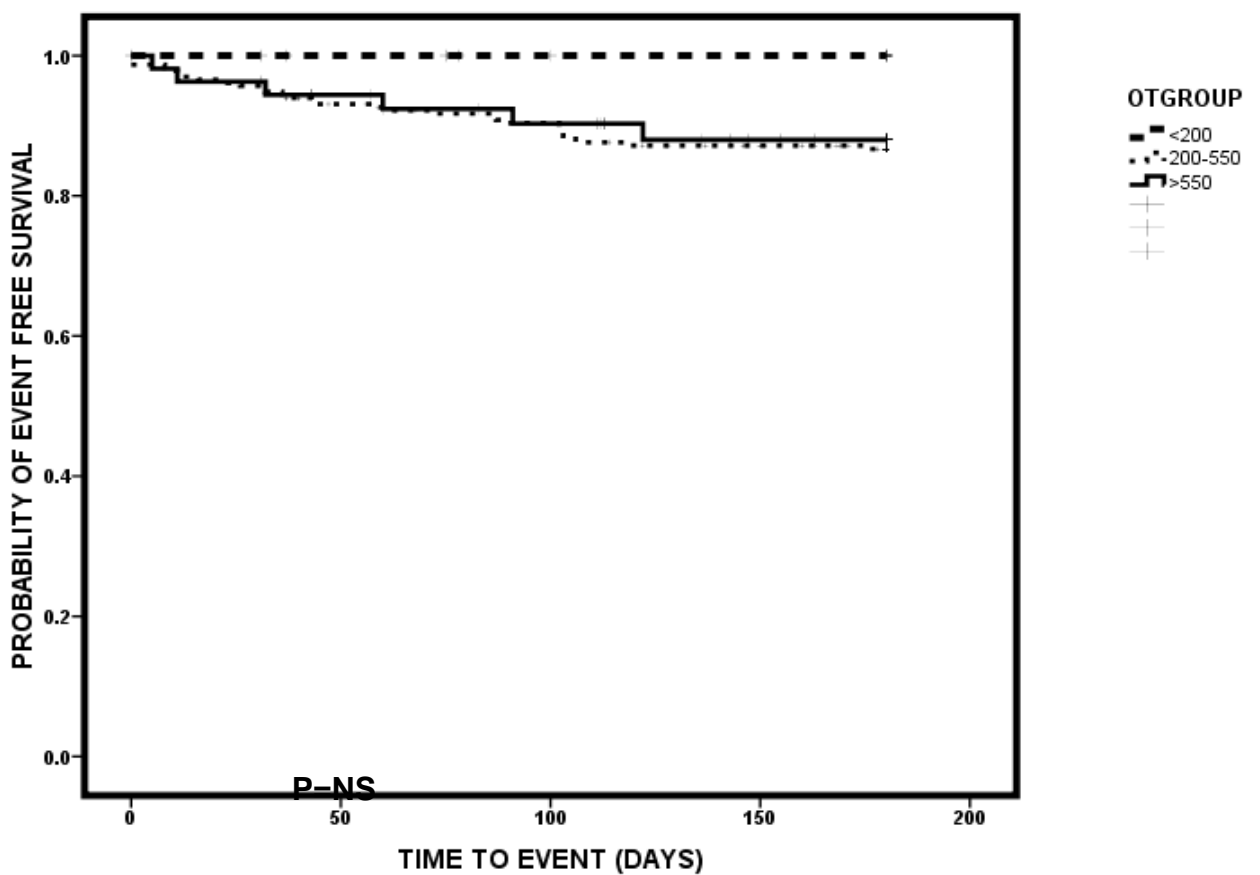


Figure 8.7: KM curve demonstrating probability of event free survival using OT as a predictor of adverse events at 1 year. Survival analysis did not demonstrate any relationship between OT and MACE.

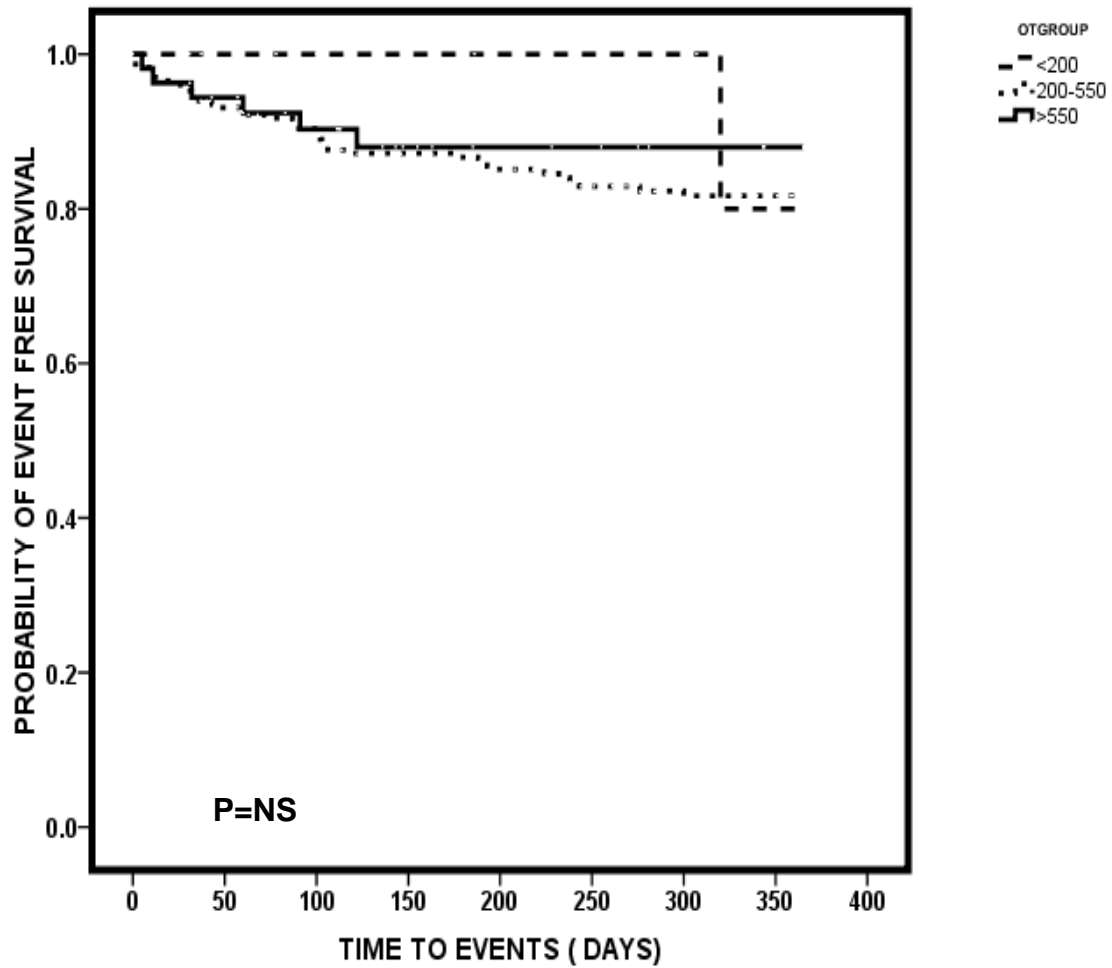


Figure 8.8: KM curve demonstrating probability of event free survival at 180 days using $LT \geq 3000s$ as cut-off. LT was noted to be significantly predictive of MACE at 180 days with HR 2.48 (CI = 1.2 -4.8, $p= 0.007$) at 6 months.

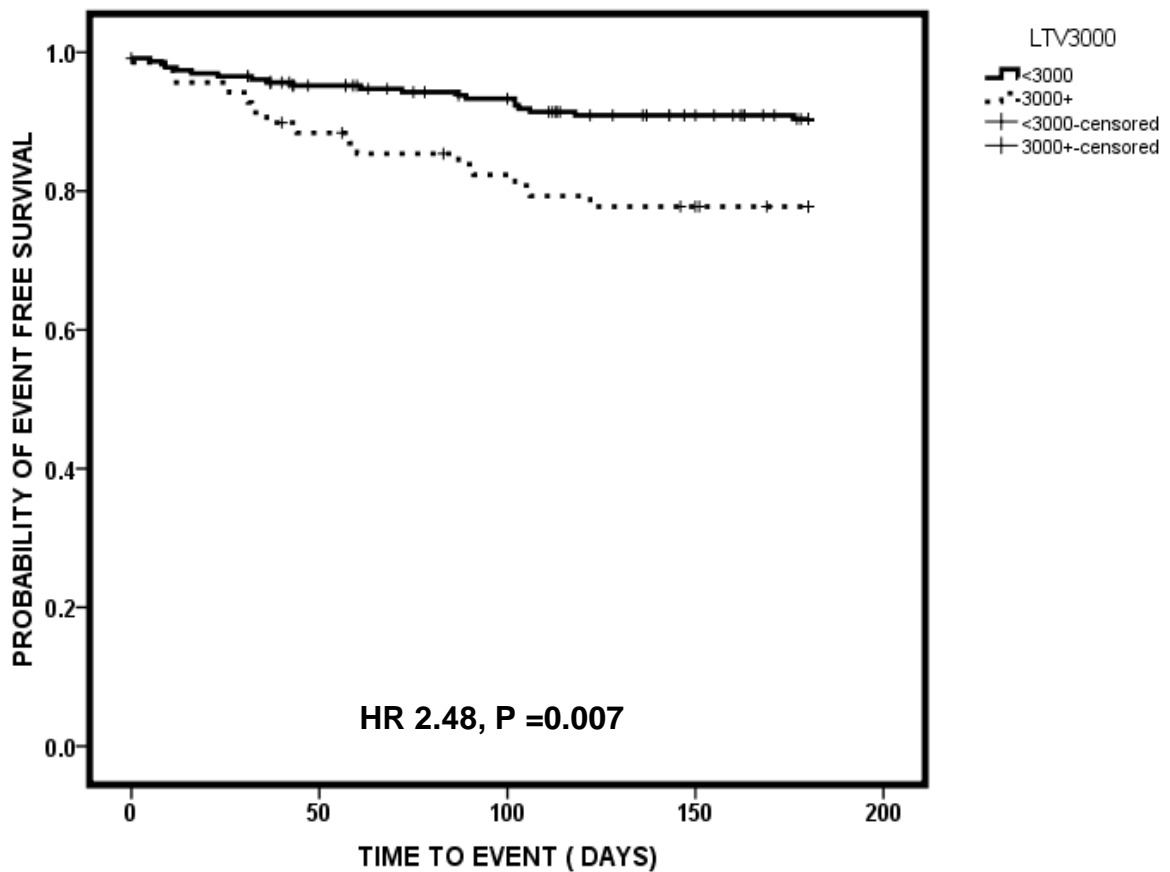


Figure 8.9: KM curve demonstrating probability of event free survival at 1 year using LT ≥ 3000 s as cut-off. LT was noted to be significantly predictive of MACE at 1 yr with HR 1.85 (CI= 1.01- 3.4, P=0.04).

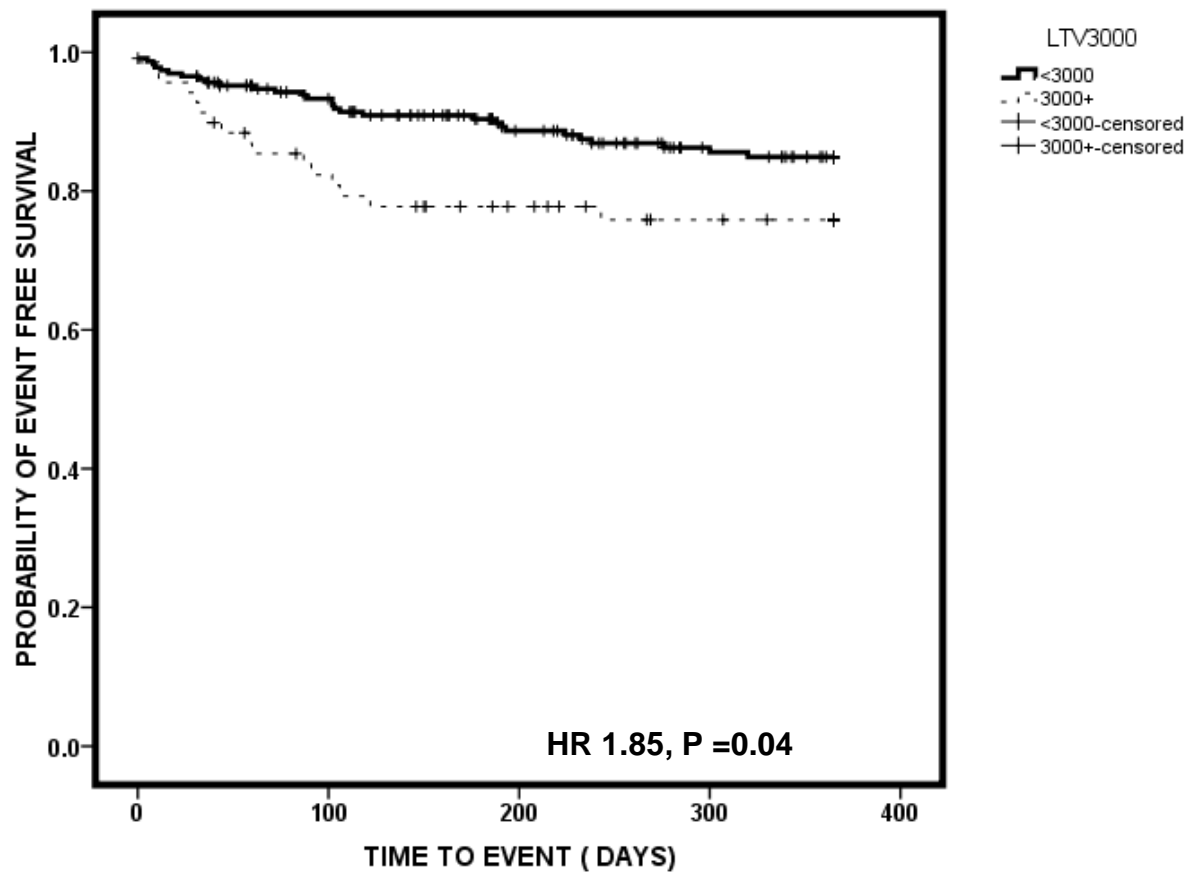


Figure 9.0: KM curve demonstrating probability of event free survival at 180 days using $LT \geq 5000$ s as cut-off. LT was noted to be significantly predictive of MACE at 180 days with HR 2.57 ($CI = 1.2-5.2, P=0.009$).

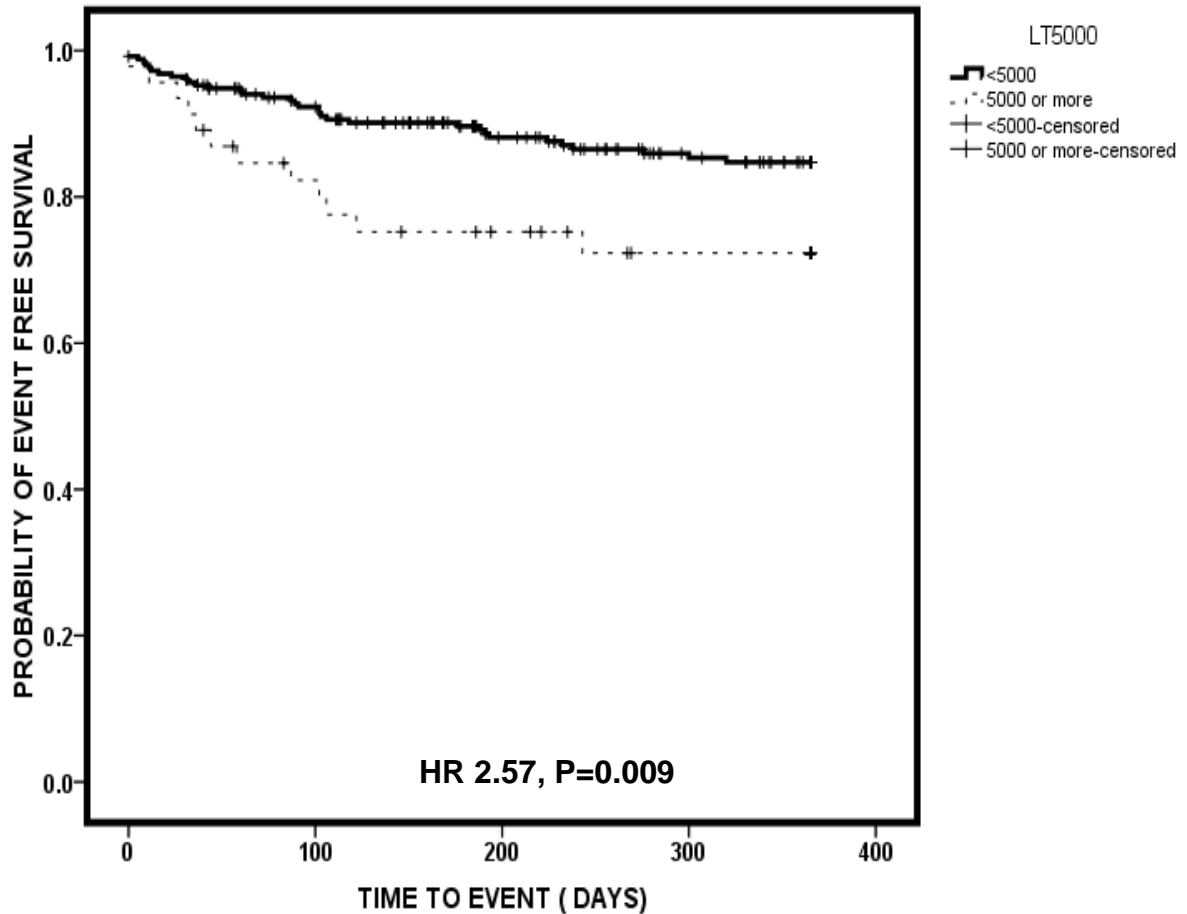
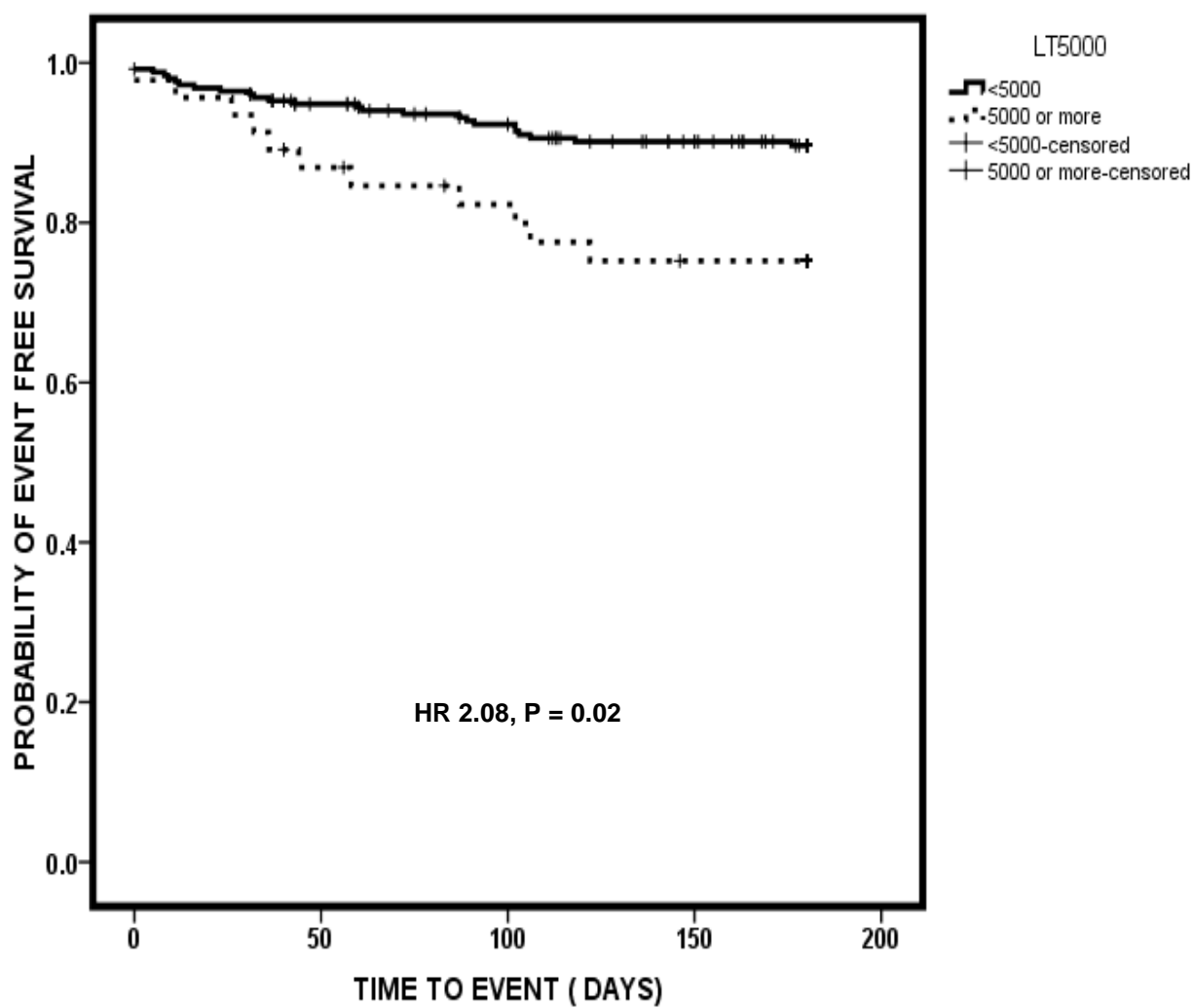


Figure 9.1: KM curve demonstrating probability of event free survival at 1 year using LT ≥ 5000 s as cut-off. LT was noted to be significantly predictive of MACE at 1 yr with HR 2.08 (CI=1.07-4.01, p=0.02).



*Figure 9.2: MACE at 6 months using $LT \geq 3000s$ as cut off ($*P < 0.05$) - prolonged LT was highly predictive of recurrent cardiovascular events, attributable to both CV death and ACS. $LT < 3000s$ is indicated by open bars; $LT \geq 3000s$ is indicated by solid bars.*

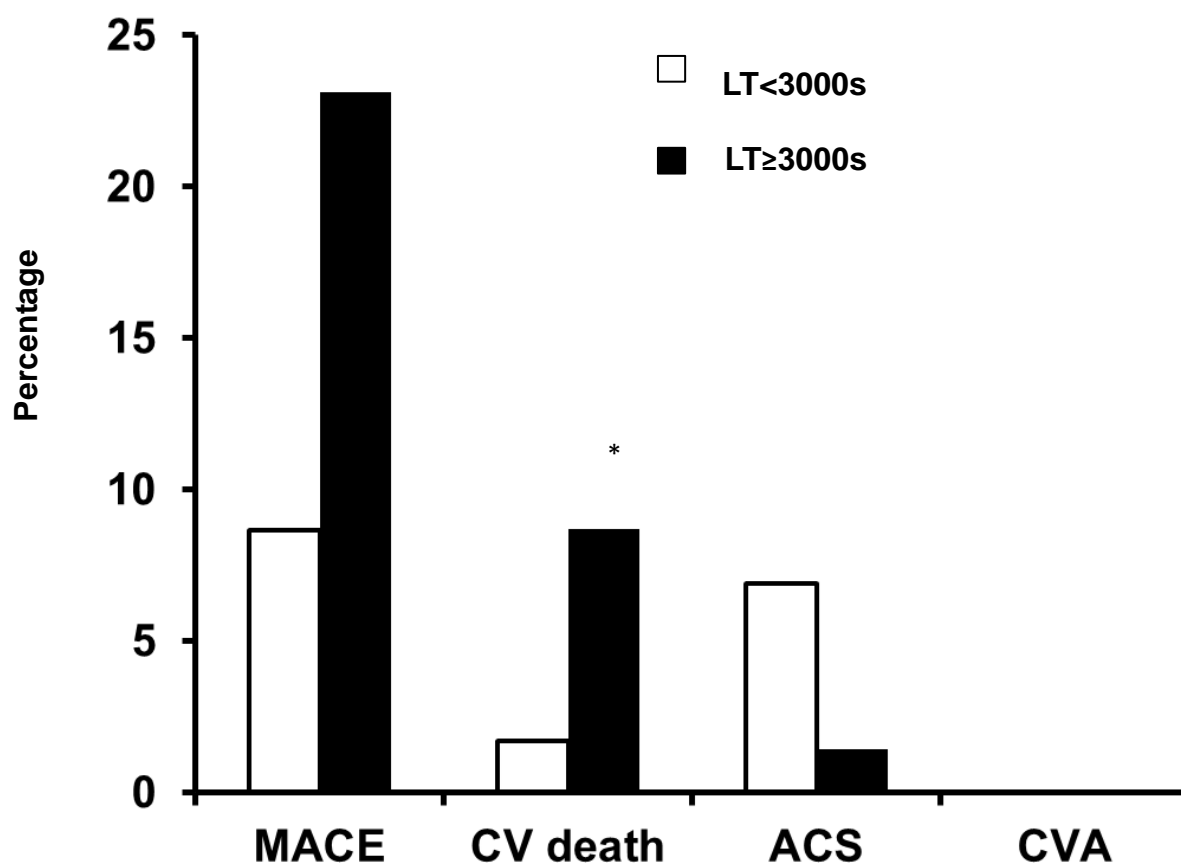
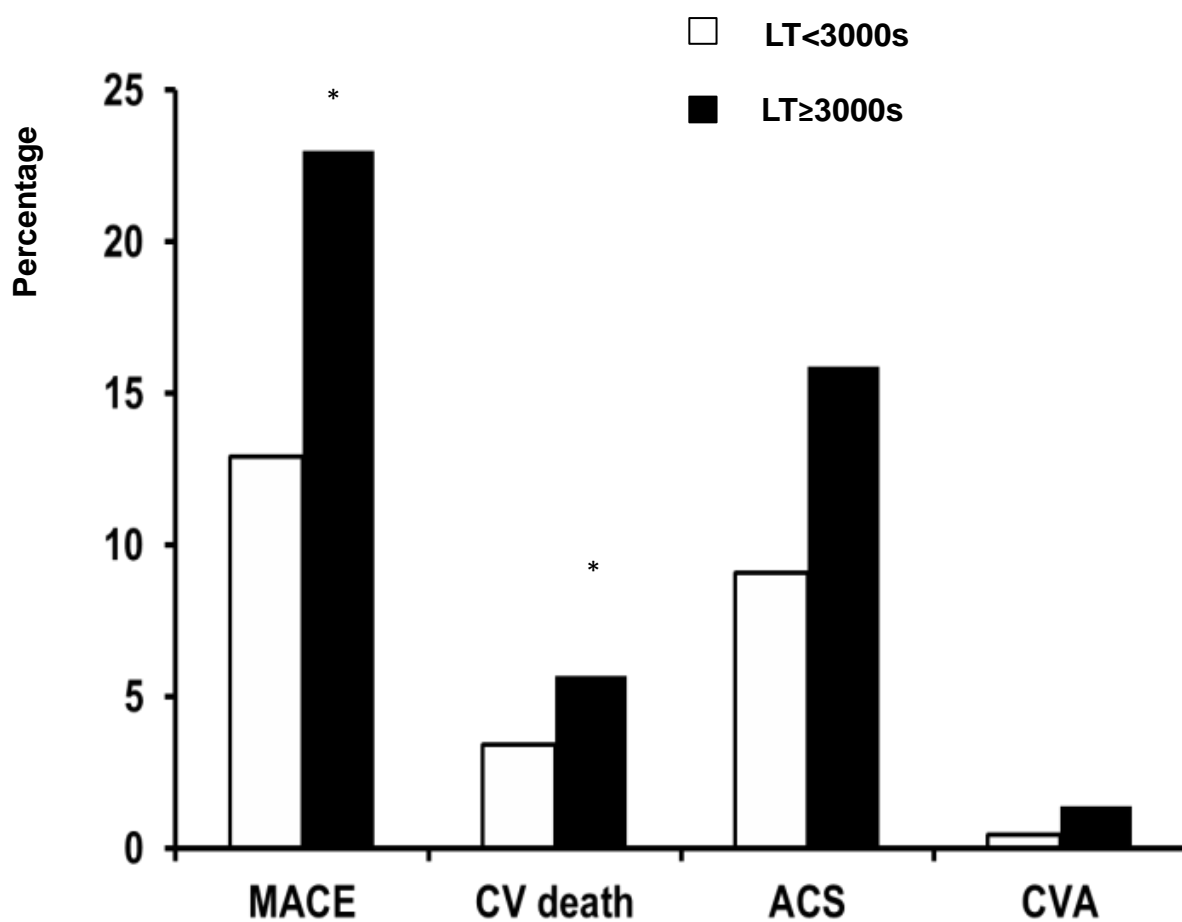
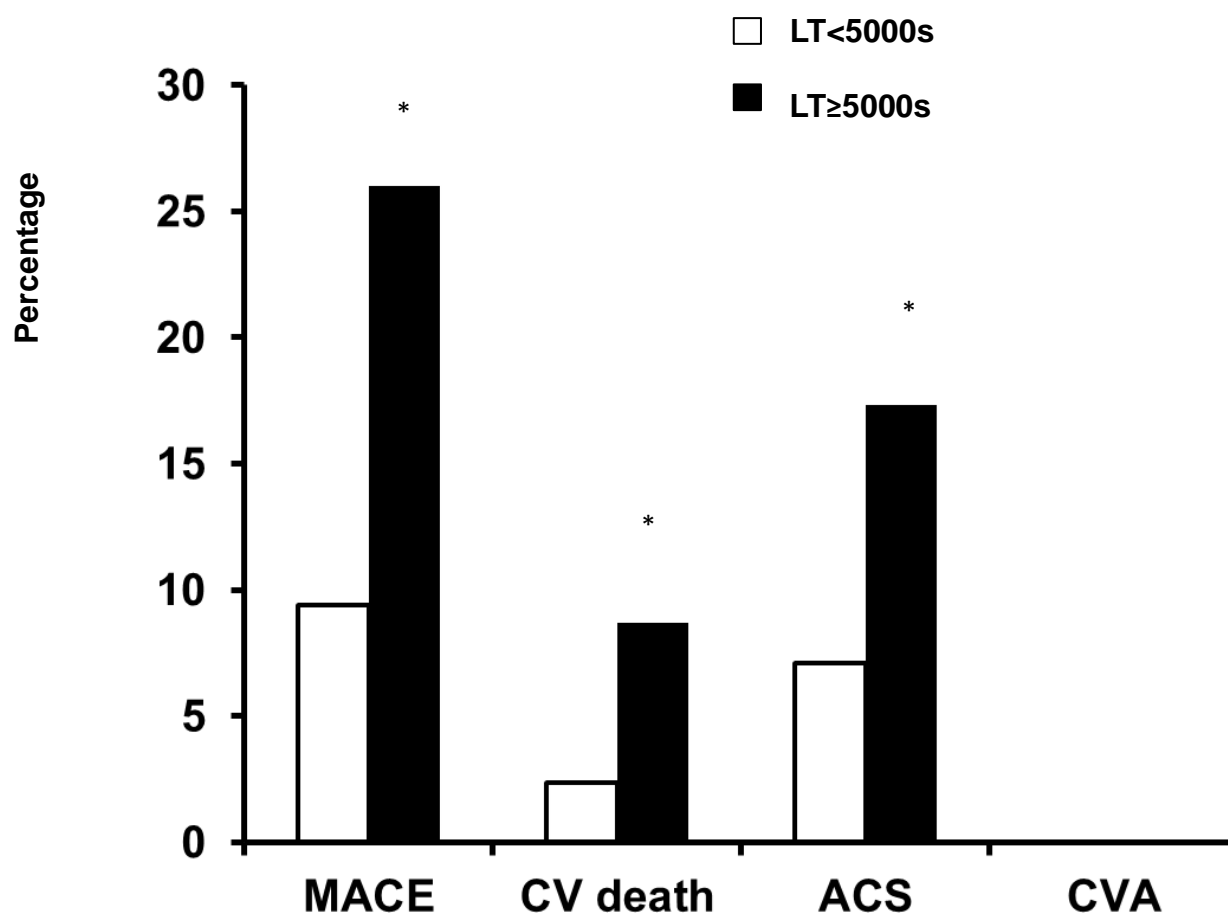


Figure 9.3: MACE at 1 year using $LT \geq 3000s$ as cut off ($*P < 0.05$) - prolonged LT was highly predictive of recurrent cardiovascular events, attributable to both CV death and ACS. $LT < 3000s$ is indicated by open bars; $LT \geq 3000s$ is indicated by solid bars.



*Figure 9.4: MACE at 6 months using $LT \geq 5000s$ as cut off ($*P < 0.05$) - prolonged LT was highly predictive of recurrent cardiovascular events, attributable to both CV death and ACS. $LT < 5000s$ is indicated by open bars; $LT \geq 5000s$ is indicated by solid bars.*



*Figure 9.5: MACE at 1 year using $LT \geq 5000s$ as cut off ($*P < 0.05$) - prolonged LT was highly predictive of recurrent cardiovascular events, attributable to both CV death and ACS. $LT < 5000s$ is indicated by open bars; $LT \geq 5000s$ is indicated by solid bars.*

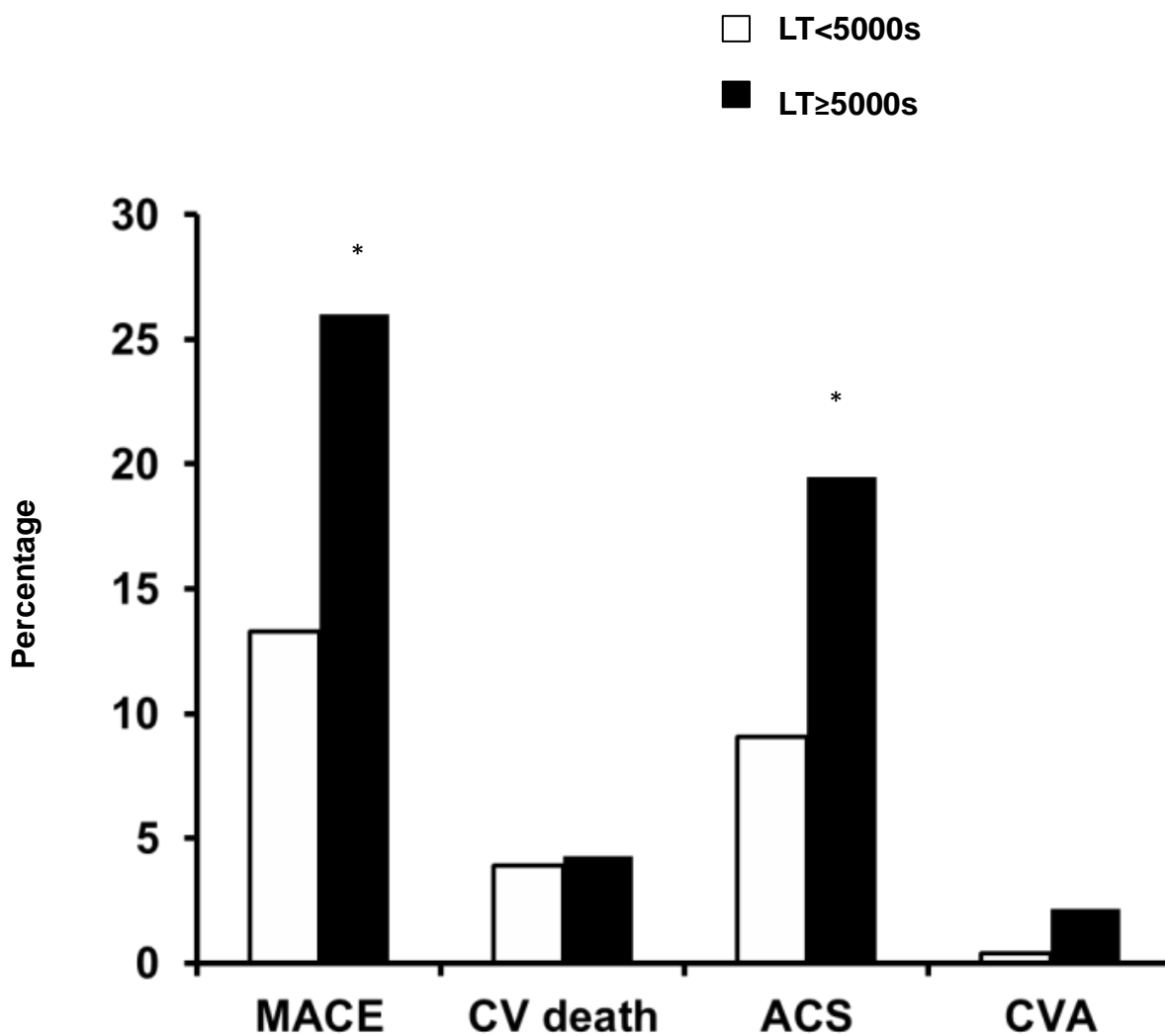


Table 6.4: Multivariate analysis using Cox regression to demonstrate HR for $LT \geq 3000s$ at 6 months (B= regression coefficient; SE = standard error of the mean; Wald= Wald statistic; Df = degrees of freedom; Sig.= significance; Exp (B) = Hazard ratio; CI = confidence interval)

	B	SE	Wald	Df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
LTV3000	.904	.342	7.008	1	.008	2.471	1.265	4.826
AGE	.030	.017	3.196	1	.074	1.030	.997	1.064
SEX	-.067	.371	.033	1	.857	.935	.452	1.934
NITR	.727	.359	4.099	1	.043	2.069	1.024	4.183
ACE	-.864	.342	6.399	1	.011	.421	.216	.823
DM	.999	.364	7.538	1	.006	2.715	1.331	5.538

Table 6.5: Multivariate analysis using Cox regression to demonstrate HR for $LT \geq 3000s$ at 1 year (B= regression coefficient; SE = standard error of the mean; Wald= Wald statistic; Df = degrees of freedom; Sig.= significance; Exp (B) = Hazard ratio; CI = confidence interval)

	B	SE	Wald	Df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
LTV3000	.654	.312	4.401	1	.036	1.924	1.044	3.546
AGE	.017	.014	1.375	1	.241	1.017	.989	1.045
SEX	-.122	.333	.134	1	.714	.885	.461	1.699
NITR	.742	.319	5.408	1	.020	2.100	1.124	3.924
ACE	-1.063	.301	12.481	1	.000	.346	.192	.623
DM	.967	.332	8.489	1	.004	2.630	1.372	5.041

Table 6.6: Multivariate analysis using Cox regression to demonstrate HR for $LT \geq 5000s$ at 6 months (B= regression coefficient; SE = standard error of the mean; Wald= Wald statistic; Df = degrees of freedom; Sig.= significance; Exp (B) = Hazard ratio; CI = confidence interval)

	B	SE	Wald	Df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
AGE	.033	.017	3.791	1	.052	1.033	1.000	1.068
SEX	-.112	.369	.092	1	.762	.894	.434	1.843
NITR	.693	.359	3.727	1	.054	1.999	.989	4.039
ACE	-.785	.339	5.351	1	.021	.456	.235	.887
DM	1.054	.362	8.487	1	.004	2.868	1.412	5.827
LT5000	.991	.367	7.292	1	.007	2.693	1.312	5.528

Table 6.7: Multivariate analysis using Cox regression to demonstrate HR for $LT \geq 5000s$ at 1 year (B= regression coefficient; SE = standard error of the mean; Wald= Wald statistic; Df = degrees of freedom; Sig.= significance; Exp (B) = Hazard ratio; CI = confidence interval)

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
AGE	.018	.014	1.576	1	.209	1.018	.990	1.047
SEX	-.156	.332	.220	1	.639	.856	.446	1.641
NITR	.727	.319	5.185	1	.023	2.069	1.107	3.867
ACE	1.005	.299	11.279	1	.001	.366	.204	.658
DM	1.010	.331	9.327	1	.002	2.747	1.436	5.253
LT5000	.783	.340	5.295	1	.021	2.188	1.123	4.264

Table 6.8: Total number of MACE in ACS patients, at 6 months and at 1 year

PRIMARY ENDPOINTS	6 months N (%)	12 months N (%)
CV DEATH	10 (3.3%)	12 (4%)
ACS	26 (8.6%)	32 (10.6%)
STROKE	0	2 (0.6%)
MACE	36 (11.9%)	46 (15.3%)

12 patients experienced an adverse cardiac event at 30 days, which included CV death in 5 patients and non-fatal MI in 7 patients. Mean OT in these 12 patients was 431 s. None of these patients had OT < 200s, 10 had OT in the range 200- 550 s, and 2 patients had OT > 550s. Mean LT in these 12 patients was 2682s, and LT \geq 5000s was observed in 3 (25%) patients of which 1 patient did not survive and 2 patients had recurrent ACS within 30 days. There was no correlation between events and OT or LT in this small group.

None of the 300 ACS patients had any bleeding complication while admitted as an inpatient, and did not report any episodes of significant bleeding at 1 year follow up.

Need for repeat revascularization at 1 year was demonstrated in 2 patients. They were both males < 55 yrs age with LT < 3000s. Both these patients continued to experience symptoms of stable angina, and repeat angiography demonstrated in stent restenosis which required further percutaneous intervention.

OT Cut off Values

Normal range for OT in our study was determined by sampling 100 healthy volunteers, who were non smokers, and not any medication. OT was normally distributed with mean OT 377.80s. Normal range was determined using Mean \pm 2SD, and OT values ranged between 185- 569.80 (200-550s).

In the ACS population, no correlation was observed between OT and outcome. We divided OT into bands of 100 s, and determined hazard ratios at 6 months and 1 year, and there was no significant increase in events at any cut off. Using survival analysis, no significant increase in events was observed using OT < 200s; 200-550s or > 550 s as cut off. ACS patients were measured using the GTT after they had been on a loading dose of Aspirin 300mg and Clopidogrel 300mg, and maintenance dose of 75 mg of each drug. There is evidence that antiplatelet effects of these medications are dose dependant, and it is possible that when the patients were tested using the GTT, steady state concentration of the drug had not been achieved on the maintenance dose of 75 mg.

All ACS patients were tested using the GTT 48 hours after LMWH was discontinued. LMWH is an anticoagulant, and its half- life is 5-6 hours. Heparin enhances agonist-induced activation and aggregation of platelets mainly in citrated blood, but significantly impairs platelet reactivity in native blood. Von Willebrand factor (vWF) is essential for platelet thrombus formation at high shear-rates and it is recognised that in addition to its effect on coagulation, heparin interferes with this platelet/vWF-mediated haemostasis, and it is possible that combination of these effects of heparin interfered with the OT result.

Table 6.9: Hazard ratio at 6 months and 1 year according to OT (seconds)

OT values (seconds)	Hazard ratio (Confidence interval and P values) at 6 months	Hazard ratio (Confidence interval and P values) at 12 months
≥200	21.1(CI:0.006-75790, P=0.465)	1.4 (CI: 0.95-10.25, P=0.73)
≥300	1.5(CI: 0.53-4.3, P=0.42)	1.05 (CI: 0.47-2.34,P= 0.905)
≥ 400	0.99 (CI: 0.51-1.9, P= 0.98)	0.88 (CI: 0.49-1.57,P= 0.67)
≥ 500	1.31(CI:0.64-2.6, P=0.44)	0.93 (CI: 0.47-1.84, P= 0.84)
≥ 600	0.67 (CI: 0.20-2.2, P=0.57)	0.53 (CI: 0.16-1.70, P=0.28)

LT Cut off Values

The results of our study suggest LT is an important novel predictor of MACE. The frequency of adverse events was greatest in the initial 6 months, following which there was relatively little additional risk noted.

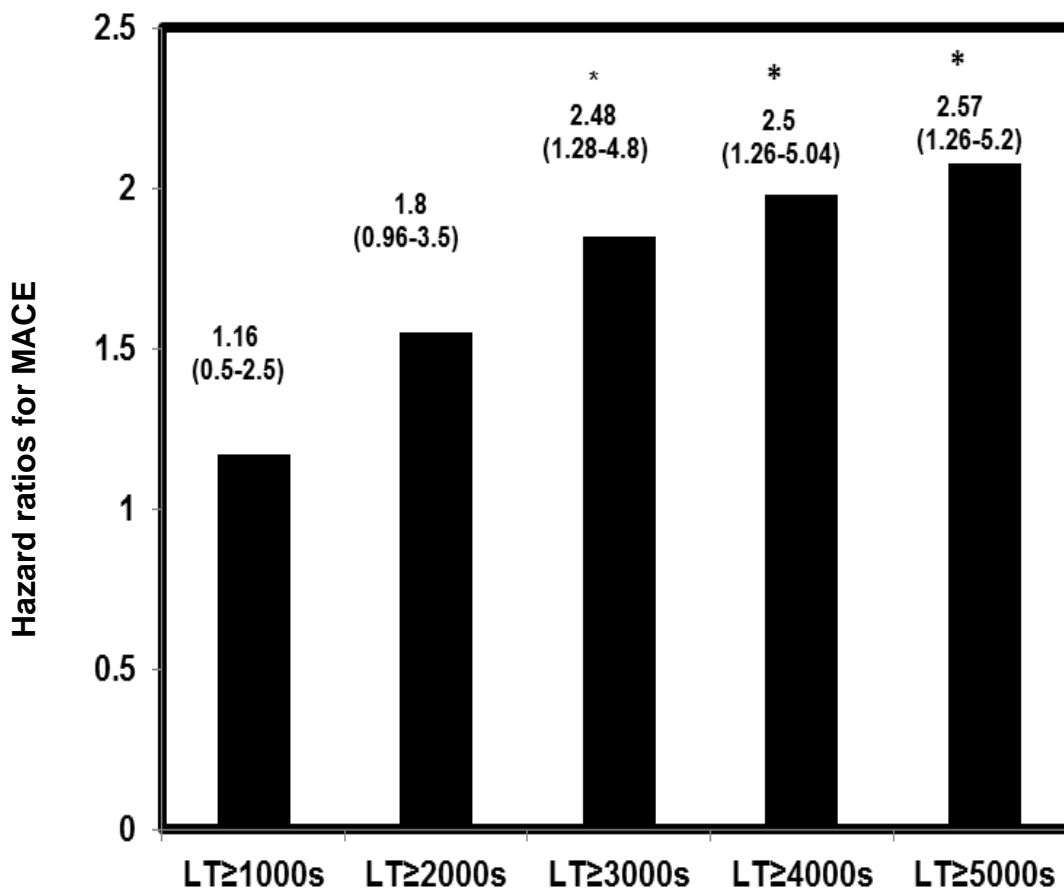
We divided LT into bands of 1000s, and optimal cut off was determined using Cox regression and a cut off LT value of 3000s was considered significant for determination of MACE both at 6 months and at 1 year. HR showed a small increase with increase in LT, and was 2.57 at $LT \geq 5000s$ at 6 months and 2.08 at 1 year follow up (Fig 9.6, 9.7).

We also calculated individual hazard ratios for CV death and ACS, and the results demonstrated $LT \geq 3000s$ to be a significant predictor of CV death at 6 months (HR 4.04, CI : 1.3-12.0, P= 0.012), and at 1 year (HR 3.9, CI: 1.34-11.9, P= 0.013). As LT increased, there was no significant increase in HR. Our primary endpoint also included ACS, and $LT \geq 3000s$ was not a significant predictor of recurrent ACS at either 6 months or 1 year. $LT \geq 5000s$ was a significant predictor of ACS at 6 months and at 1 year (HR – 2.2, CI: 1.09-4.6, P=0.028).

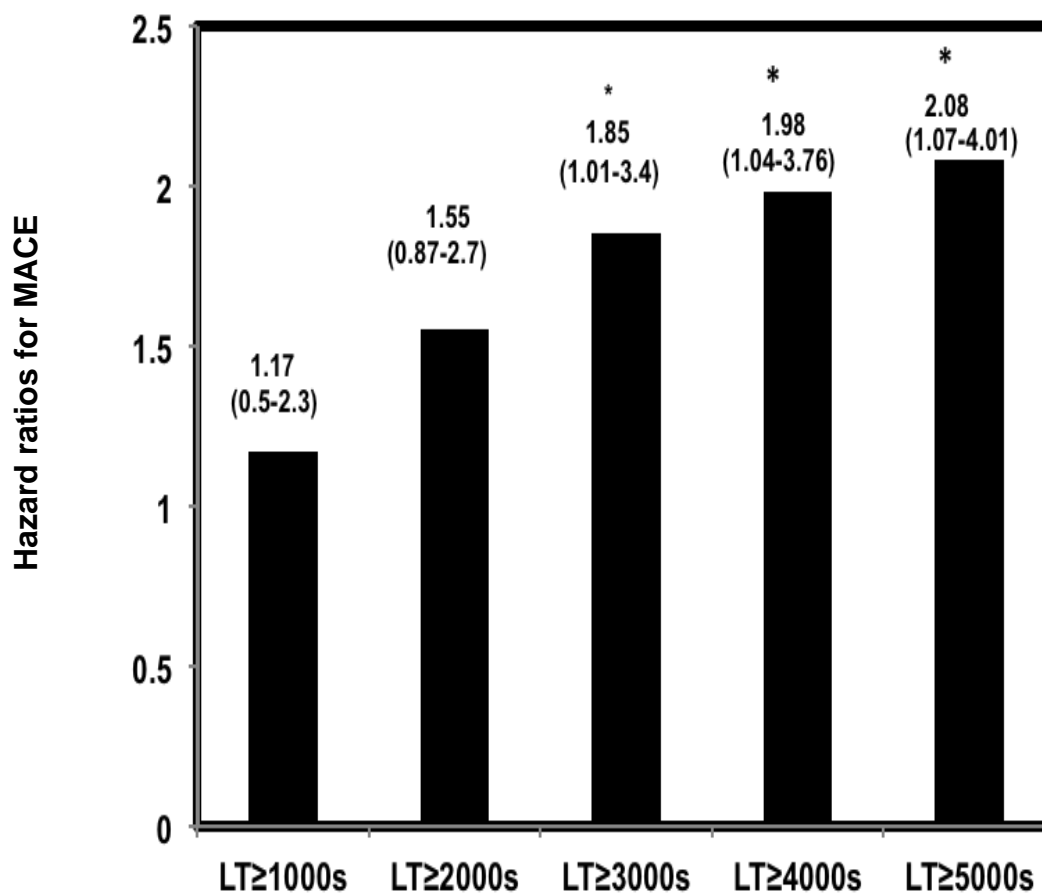
Table 6.10: Hazard ratio at 6 months and 1 year according to LT (seconds)

LT values (seconds)	Hazard ratio (Confidence interval and P values) at 6 months	Hazard ratio (Confidence interval and P values) at 12 months
≥ 1000	1.6 (CI:0.5-2.5, P=0.7)	1.17 (CI: 0.58-2.3, P=0.7)
≥ 2000	1.8(CI: 0.96-3.5, P=0.06)	1.55 (CI: 0.87-2.7,P= 0.13)
≥ 3000	2.48 (CI: 1.28-4.8, P= 0.007)	1.85 (CI: 1.01- 3.4,P= 0.046)
≥ 4000	2.50 (CI: 1.26-5.04, P=0.009)	1.98 (CI: 1.04- 3.76, P= 0.037)
≥ 5000	2.57 (CI: 1.26-5.2, P=0.009)	2.08 (CI: 1.07-4.01, P=0.02)

*Figure 9.6: HR at 6 months for CV death, Non-fatal MI, and Stroke by LT (CI shown in brackets, * $p < 0.05$) - An LT of ≥ 3000 s was identified as the optimal cut point to predict recurrent events. Above this LT level, the HR increased as the LT increased. The 95% confidence interval is shown in brackets.*



*Figure 9.7: HR at 1 year for CV death, Non-fatal MI, and Stroke by LT(CI shown in brackets, *p <0.05) - An LT of ≥ 3000 s was identified as the optimal cut point to predict recurrent events. Above this LT level, the HR increased as the LT increased. The 95% confidence interval is shown in brackets.*



Effect of other variables on survival

Using Cox regression, we determined the effect of other variables on survival. Variables that showed significant effect on survival at 6 months were Age, Diabetes mellitus, PVD, prior CAD, nitrates, ACE-I, metformin ,Hb, Hct, and CRP. Known prognostic indicators like dynamic ECG changes, troponin biomarker, ejection fraction or severity of disease on coronary angiography did not show any relationship to MACE (P=NS) in this study.

Table 6.11: Variable and Hazard ratio at 6 months and 1 year. The 95% confidence interval is shown in brackets

Variable	HR (Confidence interval , P value)	HR (Confidence interval , P
Age	HR 1.04 (CI: 1.0-1.08, P=0.004)	HR 1.03 (CI: 1.0-1.05, P=0.02)
Diabetes	HR 2.7 (CI: 1.3-5.5, P=0.004)	HR 2.4 (CI: 1.31-4.6, P= 0.005)
PVD	HR 4.09 (CI:1.2-13.3, P=0.02)	HR 4.5 (CI: 1.6-12.6, P= 0.004)
Nitrates	HR 2.3 (CI: 1.2-4.5, P= 0.012)	HR 2.14 (CI:1.18-3.8, P=0.012)
ACE – I	HR 0.45 (CI: 0.23-0.87,P= 0.018)	HR 0.38 (CI:0.21-0.69, P=0.001)
Metformin	HR 4.2 (CI: 1.7-10.3,P=0.001)	HR 3.1(CI :1.34-7.5, P=0.008)
Hb	HR 0.78 (CI:0.66-09.1, P= 0.003)	HR 0.83 (CI: 0.72-0.97, P= 0.02)
Hct	HR 0.001 (CI: 0.0-0.19, P=0.012)	HR 0.002(CI: 0.0-0.51, P=0.02)
CRP	HR 1.006 (CI: 1.0-1.012, P=0.035)	HR 1.006 (CI: 0.9-1.01, P=0.077)

All patient demographics, characteristics, comorbidities, biomarkers, echocardiography and angiography results were interrogated for effects on OT and LT. Only statin was found to have a direct effect on OT, with patients on statin therapy exhibiting a longer (less thrombotic) OT than those not taking a statin (439±157 vs. 359±120 s, P=0.02).

Of all variables, only age was related to LT, with increasing age relating to increased LT ($P=0.008$), although there was a trend toward a relationship between LT and troponin ($P=0.055$).

There was a weak negative association between OT and LT ($r = -0.2, P=0.07$).

Age is a non modifiable risk factor for cardiac disease, and was seen to be associated with MACE at both 6 months and 1 year. HR was 1.04 and 1.03 suggesting age was linearly related to outcomes, and older people were at a higher risk of adverse cardiac events.

Hypercholesterolemia is known to be associated with inflammation and prothrombotic state. Hypercholesterolaemic subjects are known to generate greater amounts of thrombin, and hence demonstrate increased platelet activation. Statins reduce cholesterol levels, and hence have the ability to reduce the prothrombotic state by reducing the amount of thrombin generated and hence reduce platelet activation (Davi et al. 1995, Notarbartolo et al. 1995).

Statins stimulate fibrinolysis by inhibition PAI-1 and increasing levels of tPA. It also helps in release of NO, and thus improves endothelial function. Statins also act as an antithrombotic by inhibiting platelet CD40 ligand and CD40L mediated thrombin generation. CRP stimulates PAI-1 secretion, and statins reduce CRP levels resulting in inhibition of PAI-1 secretion.

Diabetes mellitus is a known poor prognostic indicator of cardiac outcomes, but is a modifiable risk factor for coronary disease. HR at 6 months was 2.7 and 2.4 at 12 months, suggesting ACS patients with underlying diabetes were at a higher risk of recurrent cardiac events. Diabetic patients are known to have impaired fibrinolysis due to increased expression of PAI-1 (McGill et al. 1994). We did not find any correlation between LT and Diabetes. This may be due to the effect of Insulin or Metformin on diabetic patients, as both these drugs are known to inhibit PAI-1 activity and promote fibrinolysis (Cefalu et al. 2002). **Metformin** is a biguanide used in

patients with Type 2 diabetes mellitus. Patients on metformin had HR 4.2 and 3.1 at 6 months and 1 year respectively, suggesting poorly controlled diabetes had a significant impact on outcomes, and these diabetic patients were at a higher risk of an adverse event. Studies have demonstrated that diabetic patients have impaired fibrinolysis due to increased expression of PAI-1 (McGill et al. 1994). Metformin inhibits PAI-1 and thus promotes fibrinolysis. Studies have also demonstrated that better glycaemic and metabolic control in diabetes inhibits PAI-1 activity, suggesting other antidiabetic medications like sulfonylureas alone or in combination with metformin have a role to play in promoting fibrinolysis, thus reducing the incidence of cardiac events in the diabetic population (Cefalu et al. 2002). **ACE –I** have been shown to have a beneficial prognostic effect in patients suffering an AMI as per results of the HOPE study. The HR at 6 months in patients on an ACE –I was 0.45, and at 12 months HR was 0.38 suggesting patients on this medication were significantly protected from MACE. It is known that Angiotensin –II increases expression of PAI-1, which inhibits fibrinolysis. ACE-I inhibit conversion of Angiotensin -1 to Angiotensin-II and thus promote fibrinolysis. Inhibition of fibrinolysis results in reduced nitric oxide production, causing endothelial dysfunction with predisposition to atherosclerosis. This effect of ACE-I may explain use of ACE-I in coronary heart disease patients, reducing risk of adverse cardiovascular events (Vaughan et al. 1998).

Nitrates are generally used in cardiac patients with ongoing angina type pain with residual coronary artery disease. Following an episode of MI, it is used not only to control angina symptoms, but also for its effect as a vasodilator. At 6 months, patients on nitrates had a HR 2.3 and 2.14 at 12 months, suggesting they were at a higher risk of an event probably due to underlying residual coronary artery disease. Intravenous isosorbide dinitrate preparations have shown to have an effect on reducing platelet aggregates at low doses, using Filtragemetry

testing. Filtragemetry uses blood drawn from an antecubital vein and measures amount of platelet aggregation. No significant effect was observed with oral preparation of the medication. Slight increase in t-PA and inhibition of PAI-1 was also observed, but no significant effect was seen when compared to the control group. Nitrates also release nitric oxide promoting endothelial function, and this may well play a role in fibrinolysis and exert a positive effect on reducing thrombogenic potential in coronary disease (Wallen et al. 1993).

Patients with underlying **PVD** showed a significant relationship to outcome. HR was 4.09 at 6 months and 4.5 at 12 months, suggesting atherosclerosis in more than 1 territory was a significant predictor of adverse outcomes.

Several studies have demonstrated **anaemia** to be an important independent predictor of morbidity and mortality following an AMI (Anker et al. 2009). Patients with higher haemoglobin levels were noted to have a protective effect against MACE, and HR was 0.78 and 0.83 at 6 months and 1 year respectively. Similarly, high haematocrit values demonstrated a beneficial effect at HR 0.001 and 0.002 at 6 months and 1 year respectively.

C reactive protein is an acute inflammatory marker and high levels are noted with worse outcomes in AMI. A HR of 1.006 was noted to be of significance at 6 months, suggesting high CRP levels were associated with increase in MACE.

Cigarette smoking causes endothelial dysfunction by inhibiting substance P-induced t-PA release and increasing PAI-1 activity in humans. This results in impaired fibrinolytic activity and increases risk of thrombosis (Simpson et al. 1997). Data from another small study (Ikarugi et al. 2003) compared 11 elderly smokers to 21 elderly non-smokers (53-80yrs), and demonstrated impaired endogenous lysis in elderly smokers (LT: 5407 vs. 4147, $p < 0.001$). This may partly be

explained by endothelial dysfunction due to smoking in elderly people, but the small sample size makes it extremely difficult to interpret these findings. Endothelial dysfunction may be accelerated in elderly smokers because of the long term exposure to smoke product. However, such a mechanism cannot be demonstrated in the presently used GTT or other platelet function test in vitro systems, where endothelium is not present. In our study, we did not find an association between impaired fibrinolysis with smoking. This may be partly due to the fact that patients labelled as smokers, were sampled on average 5 days after admission, during which time they had almost universally abstained from smoking in hospital. Thus the effect of smoking on LT may be underestimated due to the late sampling after admission.

All patients admitted to hospital with ACS were sampled at a mean duration of 5 ± 3 days after admission. As smoking was not allowed in the hospital premises, all these patients had to abstain from smoking in hospital. Thus the effect of smoking on LT may be blunted due to the late sampling post admission.

Comparison of platelet function in ACS, SA and Healthy Volunteers

There were 75 stable angina patients, of which 72% were males and 28 % females, and mean age was 66 (range 40-85 years). 20% had underlying history of diet controlled diabetes, 64% were hypertensive, 10.7% had chronic kidney disease, 10.7% had peripheral vascular disease and 5.3% had history of cerebrovascular accident. 16% of stable angina patients were current smokers. 93.3% of these patients were on a statin, 82.7 % on ACE inhibitors, 70.7% were on a beta blocker, 37.8% were on nitrate therapy and 76% patients had PCI in the previous year.

Mean OT in stable angina patients (n=75) was 458.39s (range 100-752s) (Fig 10.1). We have previously determined mean OT in the normal population (n=100) was 377.80s (range 109-674s), and OT was a normally distributed variable. In the ACS patients (n=300), mean OT was 428.03s (range 29-998s). Comparison of OT was done between the three populations, and a significant difference was noted among SA and ACS patients compared with healthy volunteers (Fig 10.2). Using Anova, mean OT for healthy volunteers was significantly lower than either the SA or ACS group (p=0.001). Using post hoc bonferroni correction for multiple testing we noted that the mean OT for healthy volunteers differed significantly from both SA (p = 0.001) and ACS (p =0.007) means. No significant difference was observed between OT in the SA group compared with ACS group (p=0.292).

We have previously also determined that LT was skewed in the healthy volunteers, and in the ACS population. As such, median LT was compared between healthy volunteers, SA and ACS patients. For healthy volunteers the median LT was 1052s (95% CI: 978-1125), for SA 1243s (95% CI: 1076-1425) (Fig 10.3), and for ACS 1353s (1228-1504). Using Mann-Whitney test, a significant difference in LT measurements was noted between healthy volunteers and SA patients (p<0.001), and between healthy volunteers and ACS patients

($p < 0.001$). There was no significant difference in LT between SA and ACS patients ($p = 0.173$) (Fig 10.4). 9.3% SA patients had $LT \geq 3000s$ compared to 23% ACS patients, and in 5.3% SA patients LT was significantly prolonged at LT 6000s.

All patient demographics, characteristics, comorbidities, biomarkers, and medications were interrogated for effects on OT and LT in the SA group. None of the variables had any significant effect on OT or LT. This may be due to the small sample size of this group; larger studies will help determine the effect of these variables in SA patients.

Figure 10.1: OT in Stable Angina patients

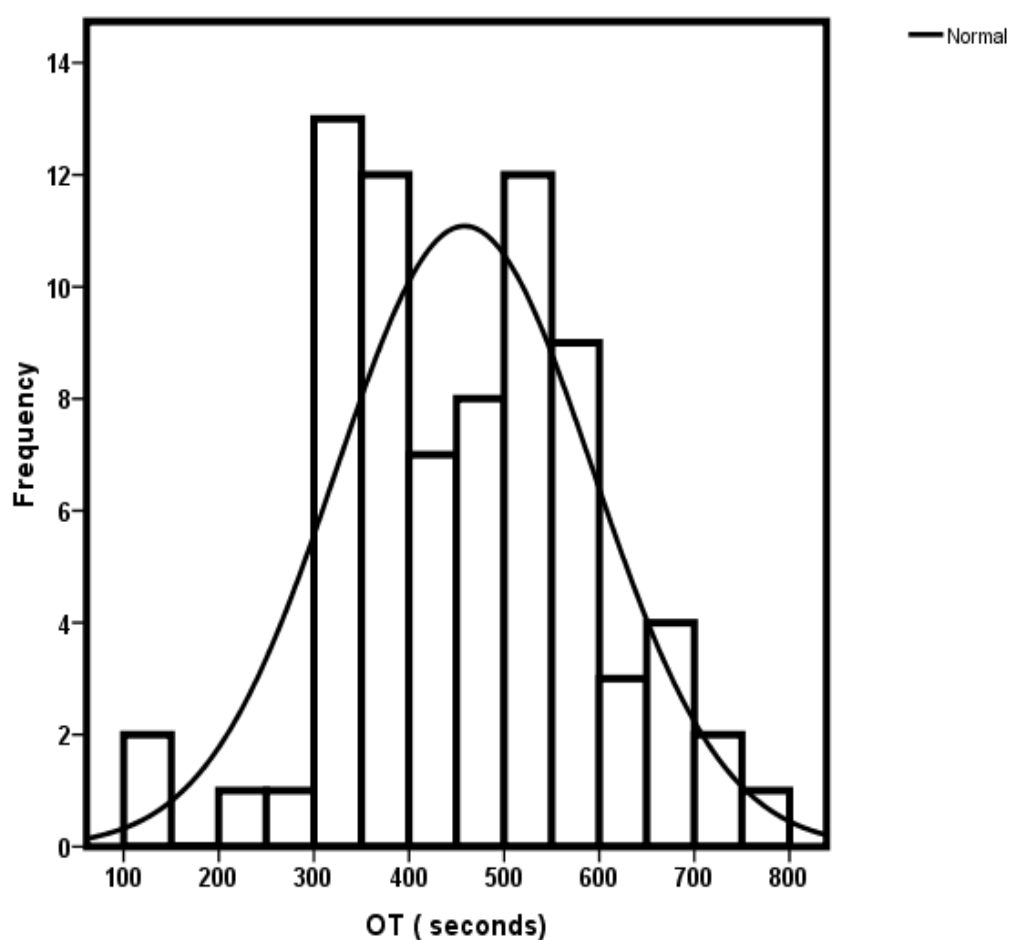


Figure 10.2: Box plot comparing minimum, maximum and median OT in Healthy Volunteers, Stable Angina and ACS patients. Mean OT for healthy volunteers differed significantly from both SA ($p = 0.001$) and ACS ($p = 0.007$) means. No significant difference was observed between OT in the SA group compared with ACS group ($p=0.292$).

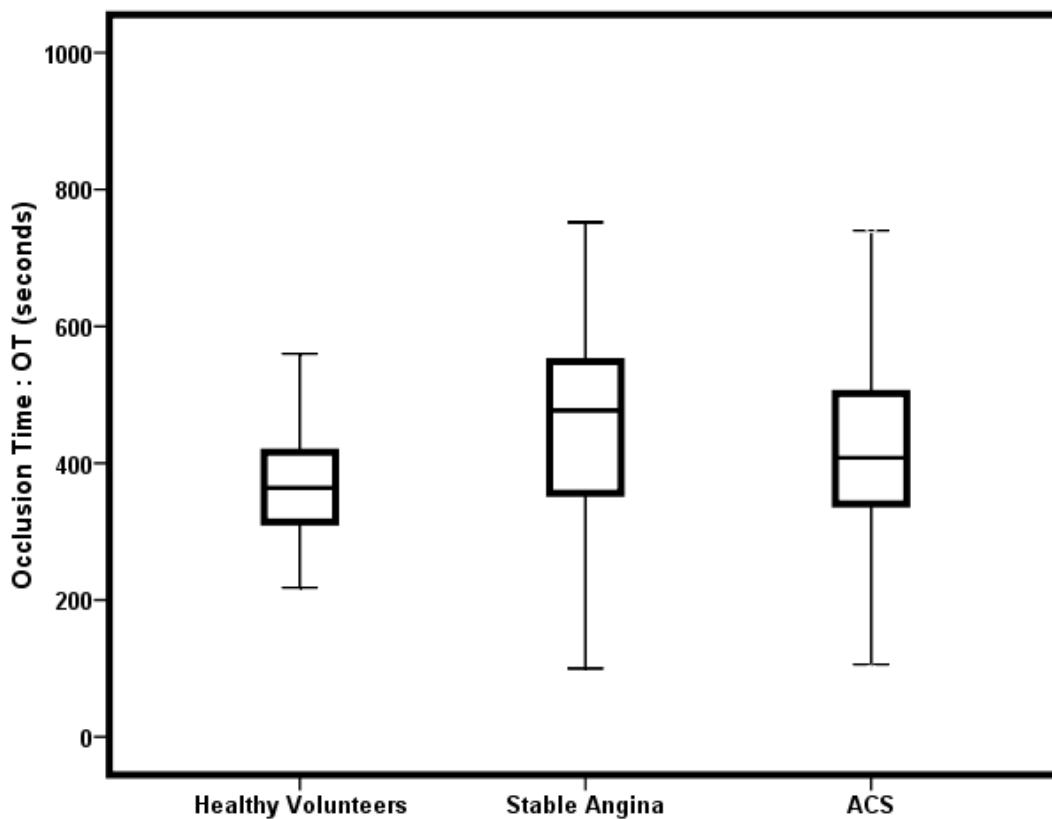


Figure 10.3: LT in Stable Angina patients

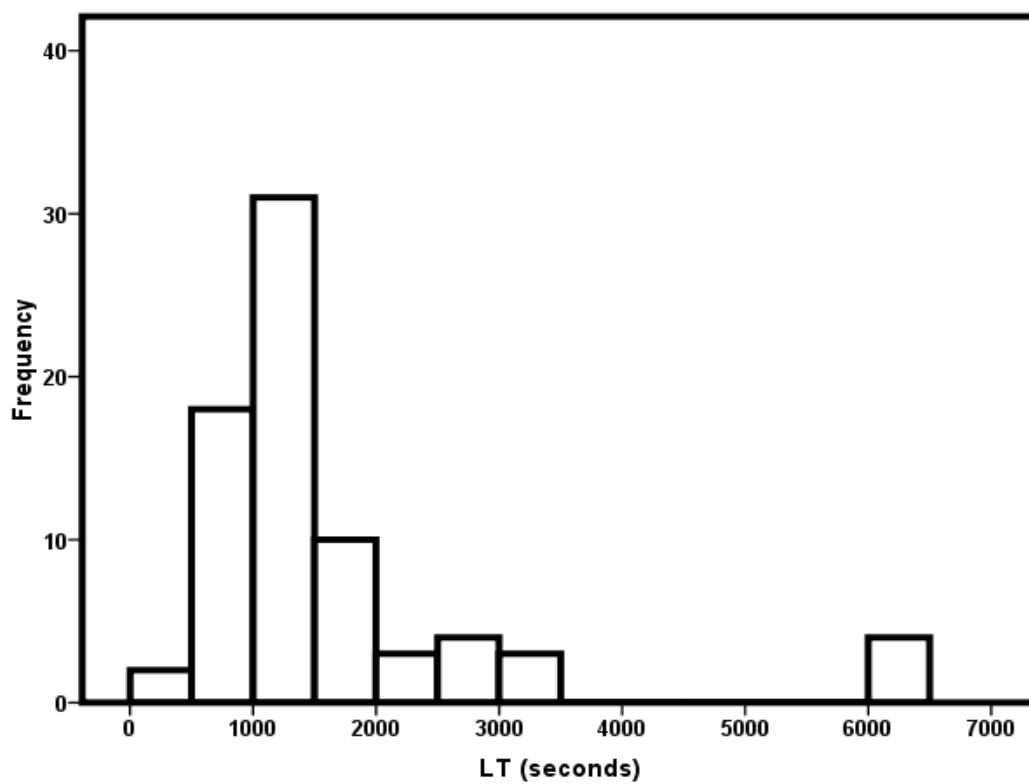
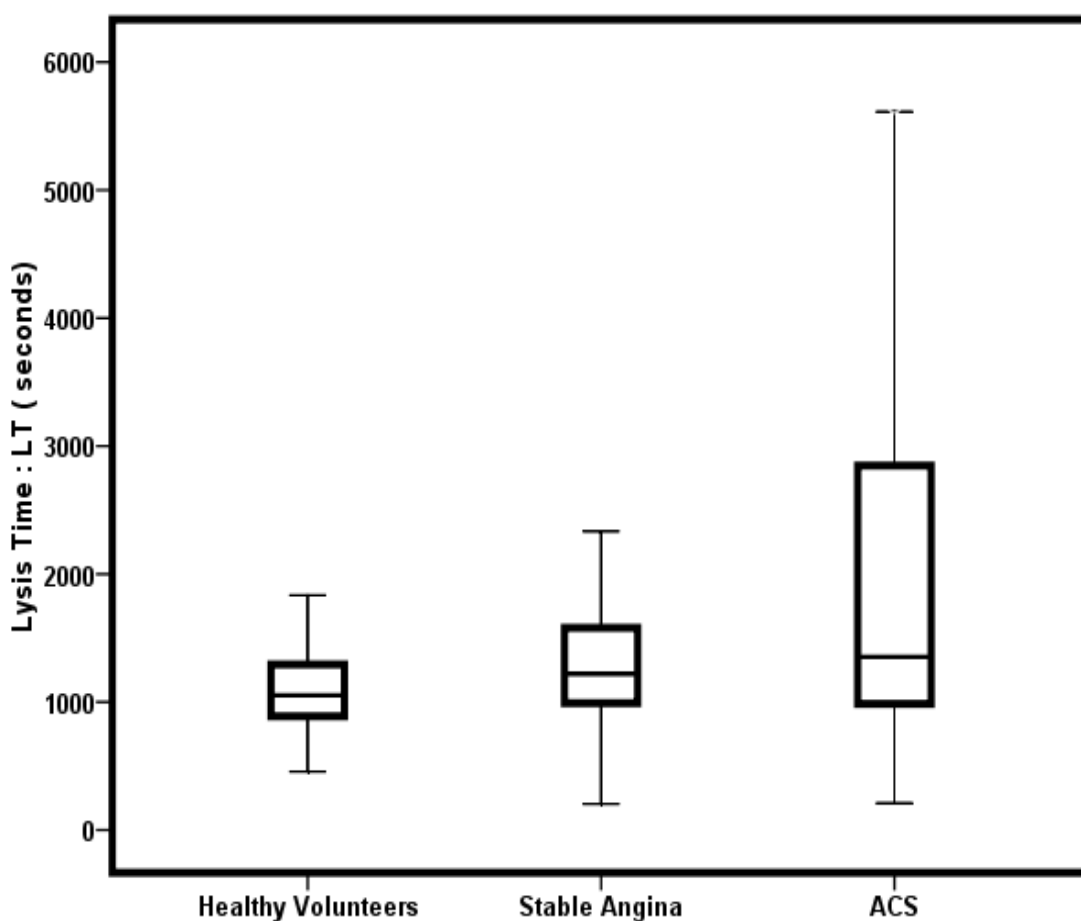


Figure 10.4: Box plot comparing minimum, maximum, and median LT in Healthy Volunteers, Stable Angina and ACS patients. Significant difference in LT measurements noted between healthy volunteers and SA patients ($p < 0.001$), and between healthy volunteers and ACS patients ($p < 0.001$). No significant difference in LT between SA and ACS patients ($p = 0.173$)



Platelet function using the GTT and Verify now assay in ACS patients

In a cohort of 71 ACS patients, we assessed thrombotic status using the GTT and the Verify now assay. Using an OT cut off < 200 s to signify prothrombotic state, no patients were deemed prothrombotic in this small subgroup of 71 patients. We have previously mentioned OT was not a predictor of MACE, and antiplatelet agents prolonged OT. Using PRU cutoff ≥ 240 , 14% ACS patients were deemed prothrombotic. No significant correlation was noted between OT or LT and PRU (OT and PRU: $\rho = -.08$, $p=0.49$) (Fig 11.1). Using Cox regression, $PRU \geq 240$ was a significant predictor of MACE at 6 months (HR: 4.7, 95% CI: 1.12-19.7, $p = 0.034$) (Fig 11.2) and showed a trend at 1 year (HR: 3.8, 95% CI: 0.96-15.4, $p=0.057$) (Fig 11.3). Of the 71 patients, 10 (14%) had an adverse cardiac event, which included 4 (5.6%) deaths and 6 (8.4%) ACS. Mean time to event was 120 ± 65 days.

Figure 11.1: Scatter plot demonstrating no correlation between GTT OT and PRU

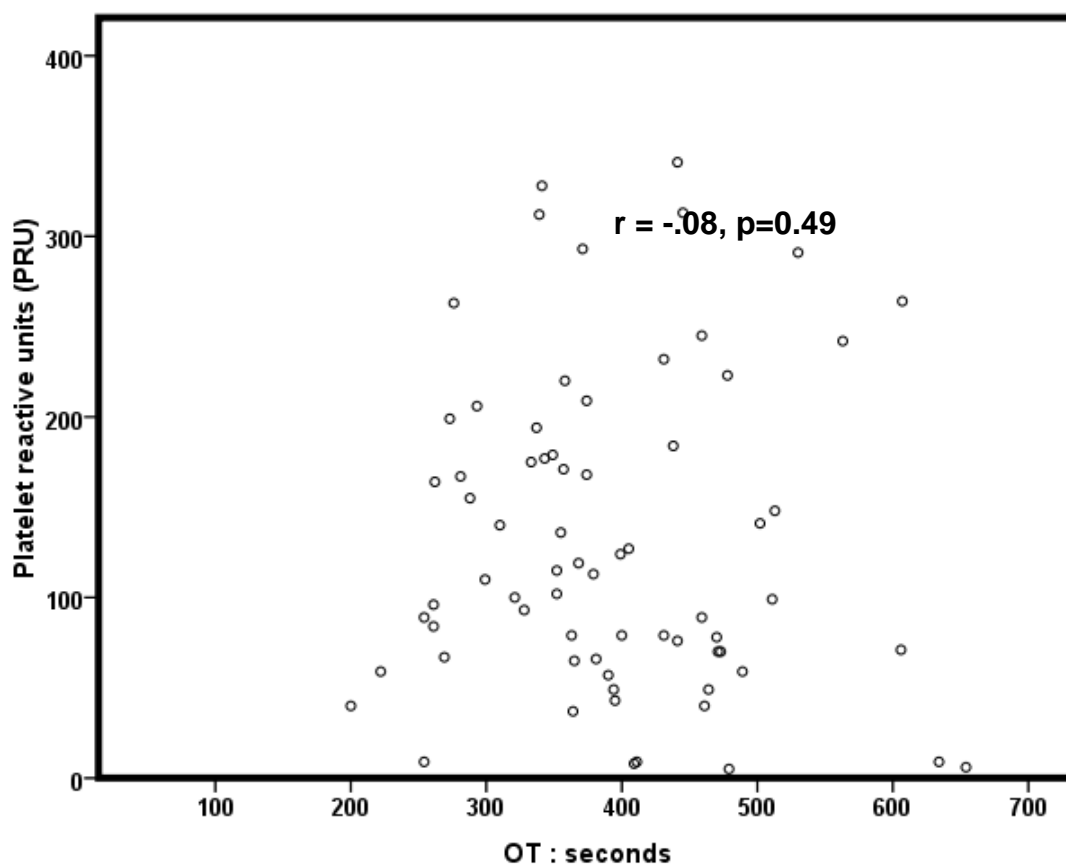


Figure 11.2: Kaplan –Meier Curve demonstrating probability of event free survival using Verify Now Assay at 6 months. Using Cox regression, $PRU \geq 240$ was a significant predictor of MACE at 6 months (HR: 4.7, 95% CI: 1.12-19.7, $p = 0.034$).

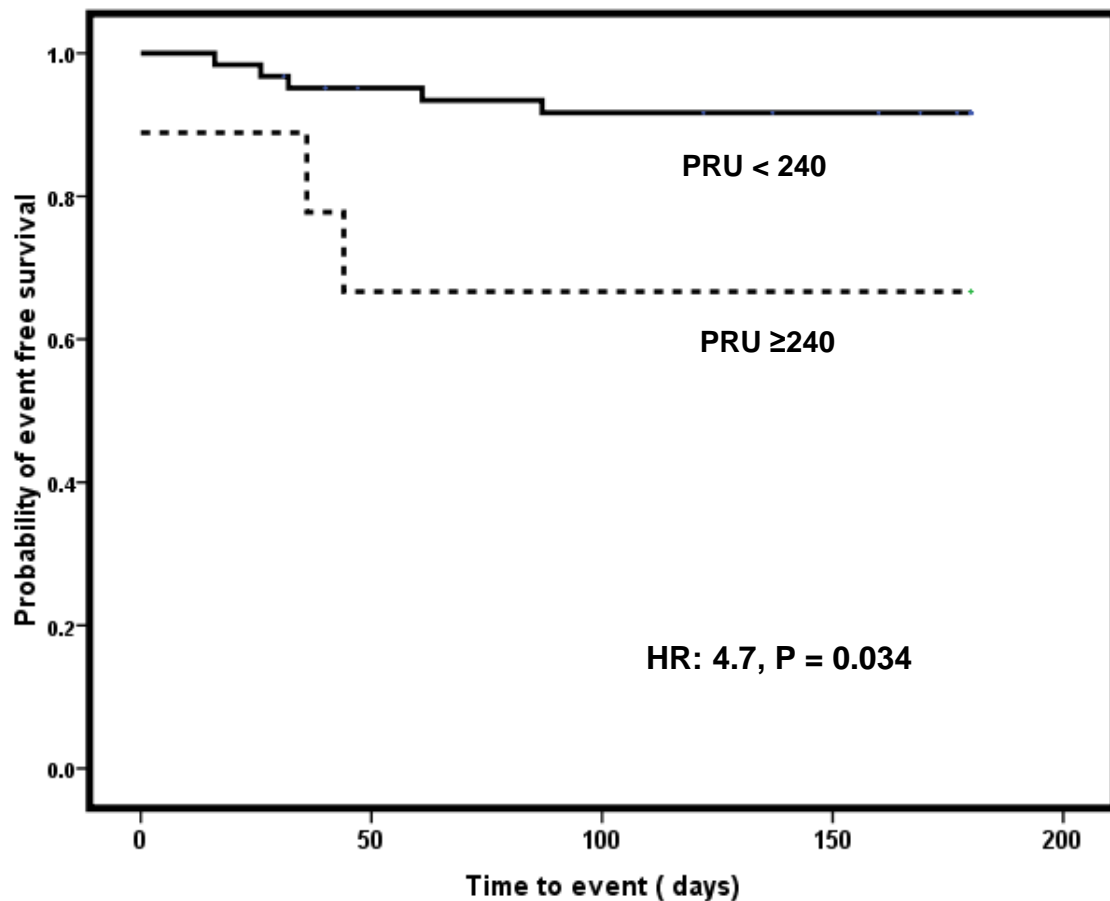
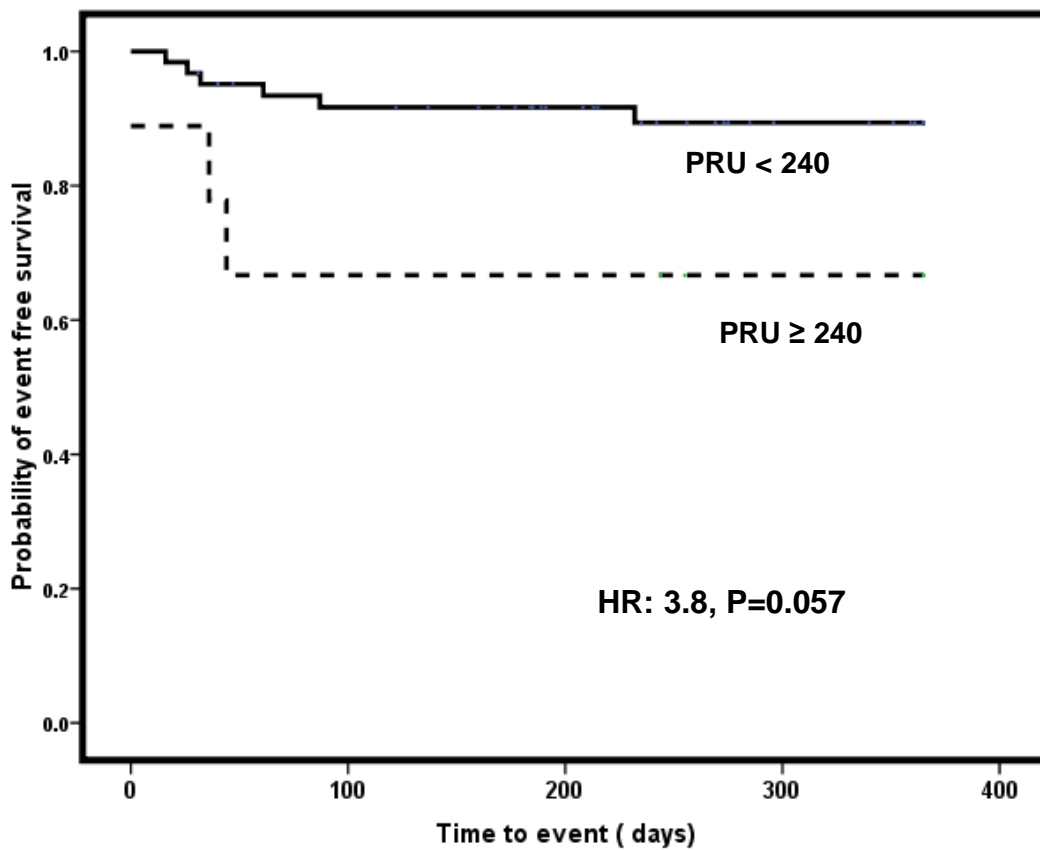


Figure 11.3: Kaplan –Meier Curve demonstrating probability of event free survival using Verify Now assay at 1 year. Using Cox regression, $PRU \geq 240$ showed a trend at 1 year (HR: 3.8, 95% CI: 0.96-15.4, $p=0.057$).



Discussion, Limitations and Conclusions

This study demonstrates that ACS patients continue to experience recurrent thrombotic events despite being on dual antiplatelet therapy. This could be secondary to non-responsiveness or resistance to antiplatelet medications, or due to impaired endogenous thrombolysis. OT in ACS patients was significantly prolonged when compared to healthy volunteers, suggesting Aspirin and Clopidogrel had an effect on prolongation of OT. However, we were unable to demonstrate any significant association between OT and MACE in the ACS population. As patients were sampled 5 ± 3 days after admission, it is possible that steady state concentration of antiplatelet agents was not achieved, and hence the lack of association between OT and MACE. It is also possible that platelet bound heparin interfered with the GTT results, even though all patients were sampled at least 48 hours after the last dose of LMWH, by which time heparin should have completely cleared from the circulation (Collignon et al. 1995).

A significant association was noted between LT and MACE. $LT \geq 3000s$ was considered a significant predictor of MACE at both 6 months and 1 year. It was also a significant predictor of CV death, and $LT \geq 5000s$ was demonstrated to predict recurrence of nonfatal MI at both 6 months and at 1 year. There were only 2 strokes in the study; hence we were unable to demonstrate any significant association between LT and stroke in our population. In a study of 585 patients with ST-segment elevation MI, electrocardiographic or angiographic spontaneous reperfusion was observed in 14.9% and 14.7%, respectively. Those with spontaneous reperfusion had lower mortality, lower composite of death/shock/congestive heart failure, and significant reduction in death or reinfarction (Bainey et al. 2008). In another study of 710 patients with ST-segment elevation MI undergoing percutaneous coronary revascularization, spontaneous reperfusion was observed in 22% of patients, and at 30 days, was associated with significantly lower incidence of death, congestive heart failure,

and recurrent ACS (Fefer et al. 2009).It has also been suggested that impaired fibrinolysis determines the outcome of coronary angioplasty (Fornitz et al. 2001). Large prospective studies provided evidence for a statistically significant association between inhibited fibrinolysis as indicated by increased levels of fibrinolysis inhibition markers such as tissue- type plasminogen activator, plasminogen activator inhibitor, thrombin activatable fibrinolysis inhibitor, plasmin-antiplasmin complex, lipoprotein (a), and an increased risk of recurrent MI or sudden cardiac death (Christ et al. 2005, Katsaros et al. 2008, Morange et al. 2007, Soeki et al. 2002, Tregouet et al. 2009). However, multivariate analysis of the measured plasma concentrations by these fibrinolysis markers showed weak or no prognostic value of these tests.

From the several global clot lysis assays described in the past few years, we have chosen the GTT technique, which has the advantage of measuring thrombolysis as opposed to clot lysis. Although worthy of further prospective confirmation, our results suggest a strong association between impaired spontaneous thrombolysis and MACE. A very recent study involving 335 young survivors of a first arterial thrombosis reported that low plasma fibrinolytic potential, measured by clot LT, and found in 10% of the population, was associated with a 2-fold increase in relative risk of arterial thrombosis (Guimaraes et al. 2009). Our findings show that in patients with ACS, the increase in risk associated with impaired lysis can even be higher.

The main limitation of our study is the fact that patients were only sampled once during their index admission, generally once they had stabilized on medical therapy. There is evidence that the antiplatelet effects of aspirin (Undas et al. 2007) and clopidogrel (Dangas et al. 2009) are dose-dependent. When we measured platelet reactivity in patients under the effect of the loading dose of 300 mg aspirin and 300 mg clopidogrel, such measurements do not necessarily reflect steady state platelet function during the maintenance doses of 75 mg/day. Secondly, heparin administered immediately after admission may have interfered with the GTT

measurement. GTT OT is determined not just by the rate of platelet aggregation but also by the formation and the effect of thrombin, generated by activated platelets. Based on published data regarding the plasma half-life of heparin, at the time of sampling, we anticipated no interference from heparin with the test. However, there is some evidence that heparin binds to platelets (Horne et al. 1989) and such membrane bound heparin may affect platelet behaviour for much longer periods. With its strong antithrombin effect (De Candia et al. 1999) such plateletbound heparin may have interfered with our GTT measurements. Had sampling been performed about 10 days after admission, when patients were established on maintenance doses of antiplatelet drugs and also interference by heparin could have been excluded, it is possible that the platelet reactivity may have been predictive of MACE and impaired thrombolysis may have been even more predictive of future events. Furthermore, it remains unknown whether the impaired thrombolysis was part of an acute phase response or whether it reflects a chronic impairment of thrombolysis that influences late outcome. Possible diurnal variation in LT was not investigated. Further studies are required to investigate the effect of medication on thrombolytic state, both in the acute and the chronic state.

Comparison of OT and LT results in healthy volunteers, SA and ACS patients demonstrated significantly prolonged OT in SA and ACS patients compared to normal volunteers. This suggests that dual antiplatelet medication normalizes platelet reactivity in patients with coronary disease, and has a beneficial effect on the patient's thrombotic status by prolonging time to thrombus formation. In previous chapters, both Aspirin and Clopidogrel have demonstrated a significant prolongation in OT in healthy volunteers. No significant difference was observed in the prothrombotic status between SA and ACS patients, despite the latter being considered more prothrombotic.

As previously mentioned, ACS patients were sampled at least 48 hours after discontinuation of heparin, but the use of heparin during the acute phase of admission may be considered as a limitation, and hence may not have demonstrated any significant difference in prothrombotic status in the two groups.

LT was significantly prolonged in SA and ACS patients compared to healthy volunteers, suggesting patients with underlying coronary artery disease had impaired endogenous thrombolytic activity. Although no significant prolongation in LT was noted in ACS patients compared to SA patients, 23% ACS patients had an $LT \geq 3000s$ compared to 9.3% SA patients.

No significant difference was noted in LT between SA and ACS patients. This may be due to small sample size, or difference in characteristics of the different populations. The healthy volunteers had no underlying medical history and were not on any medication, whereas SA and ACS patients had underlying risk factors and were on a multitude of medications. However, as a pilot study some significant observations and differences were noted in these patients thrombotic and thrombolytic status; larger studies in homogenous cohorts will help understand these differences better.

Data suggest that the extent and severity of coronary atheroma does not predict subsequent development of an unstable coronary plaque. Elevated levels of proinflammatory cytokines and vWF have been demonstrated in ACS, and predispose to thrombus formation. Studies suggest increased circulating plasma levels of proinflammatory cytokines in SA and ACS patients. Results from a small study by Simon et al (Simon et al. 2000) suggest that both Interleukin-1beta and Interleukin-6 are elevated in ACS, and contribute to increased prothrombogenicity in these patients. vWF is a glycoprotein, and plays a major role in platelet aggregation and thrombus formation, and elevated levels are an important predictor of adverse cardiac

events. Another small study by Figueras et al (Figueras et al.2000) investigated differences in thrombin generation and fibrinolytic potential in UA and AMI patients, and demonstrated high levels of thrombin-antithrombin complex, D-dimer and fibrinogen during the acute phase of the disease. These and other biomarkers were not investigated in our study, and hence remain a limitation to further analysis.

In the subgroup of 71 ACS patients, no significant correlation was noted between the GTT and Verify Now assay. Verify now was a significant predictor of MACE at 6 months, which has been demonstrated in the ARMYDA-Pro trial, and in several other studies.

The ARCTIC study randomised 2440 patients undergoing PCI to either conventional antiplatelet therapy and dose adjustment, or a strategy of platelet-function monitoring using the Verify Now assay. The dose of antiplatelet agents was increased in patients who demonstrated a poor response to the drug. Platelet inhibition was measured in the cardiac catheterization laboratory before stent deployment and in outpatient clinic 2 to 4 weeks later. The primary end point of the study was the composite of death, myocardial infarction, stent thrombosis, stroke, or urgent revascularization 1 year after stent implantation. 34.5 % patients on clopidogrel and 7.6% patients on aspirin demonstrated poor platelet inhibition, and had further boluses of the drug or were given alternative medications like prasugrel or GpIIb/IIIa inhibitors during the intervention. There was no significant difference in the primary endpoint in the monitoring or conventional group (34.6% vs. 31.1%, HR 1.13, CI 0.98 to 1.29; P=0.10), suggesting there was no improvement in clinical outcomes using this point of care test (Collet et al. 2012). The Trilogy ACS substudy used the Verify now assay to compare platelet reactivity between clopidogrel and prasugrel, in 2564 NSTEMI patients managed medically without any intervention. Although patients on prasugrel demonstrated lower platelet reactivity, this did not translate into improved clinical outcomes (17.2% vs. 18.9%, p= 0.29) (Gurbel et al. 2012).

GTT OT was not a predictor of MACE in this study. We have previously mentioned all ACS patients were on dual antiplatelet medication which may have normalized platelet reactivity, and it is possible there was formation of heparin-platelet complex even though patients were sampled 48 hours after discontinuation of heparin.

We have previously mentioned the limitations of currently available platelet function tests including the Verify now assay in Chapter 1. The Verify now assay is a turbidometry-based optical detection device that measures platelet-induced aggregation in a system containing fibrinogen-coated beads. The instrument measures changes in light transmission and thus measures the rate of aggregation in whole blood. In the cartridge of the VerifyNow P2Y12 assay, there is a channel in which inhibition of the P2Y12 receptor is measured. This channel contains ADP as platelet agonist and prosta-glandin E1 as a suppressor of intracellular free calcium levels, thereby reducing the nonspecific contribution of ADP. Using the Verify now assay, the blood sample collected is tested between 10 minutes to 4 hours of collection, and during this duration there are changes in platelet behavior. The effect of shear stress and thrombin is not measured in the Verify now assay as it uses citrated blood and low levels of calcium result in suboptimal platelet aggregation. On the contrary, the GTT is performed on native blood immediately after collection; is shear induced, and as it does not use citrated blood is able to assess the effect of thrombin on thrombosis. Although both OT and PRU are platelet driven processes, the mechanism of action of these two tests vary significantly, and no significant correlation was observed between OT and Verify now PRU levels in our study. These results may be attributable to the small sample size, or variability of the test.

Chapter 6: Discussion, Future Research and Study Limitations

General Discussion

This is the first set of studies to demonstrate a significant relationship between impaired endogenous thrombolysis and adverse cardiac outcomes in ACS patients. The results of this study suggest impaired endogenous thrombolysis is an independent predictor of cardiac events, and future research with emphasis on modulation of endogenous thrombolysis, either through control of relevant risk factors or pharmacological measures could prove to be of significant benefit. Currently, there is no reliable biomarker for determining the efficacy of the endogenous thrombolytic system, and not enough evidence is available to determine the role of drugs on the endogenous fibrinolytic system. Another limitation has been the absence of any platelet function test that could measure endogenous thrombolysis, but the use of GTT in this study provides valuable insight into use of a novel point of care test that could aid in determination of the efficacy of endogenous thrombolytic system.

In the above studies, we have demonstrated that novel platelet function tests are able to determine the thrombotic and thrombolytic status in various populations. These and newer tests can help us determine the effect of medications, and understand the magnitude of resistance in the healthy and disease populations.

Both OT and LT were significantly increased in healthy Japanese volunteers compared to healthy Western volunteers, suggesting ethnicity and other genetic and environmental factors play an important role in thrombus formation. Japanese individuals are at a lower risk of coronary artery disease, but at higher risk of haemorrhagic strokes, and prolonged OT and LT in the Japanese population would support these findings.

In 300 ACS patients, thrombotic and thrombolytic status was assessed. All patients were on dual antiplatelet therapy, and the mean OT in these patients was significantly greater than in the normal population. A significant proportion of these patients had recurrent cardiac events following their index admission suggesting they may be resistant to the effects of aspirin, clopidogrel or both. Using certain modifications, we have demonstrated that ADP and thrombin are important mediators of thrombosis, and it may be possible to identify clopidogrel non responders, allowing us to either increase dose or switch these patients to alternative antiplatelet agents. Further large studies are required to help us understand and determine the extent of clopidogrel resistance in different disease populations to improve clinical outcomes.

LT provides us with a measure of endogenous thrombolysis. LT was significantly longer in the ACS population compared with healthy volunteers, suggesting they were at a higher risk of adverse cardiac events. Healthy volunteers had significantly shorter LT's suggesting they had a functioning endogenous lytic system. Clopidogrel demonstrated no significant effect on LT in healthy volunteers or stable angina patients, suggesting it did not affect the fibrinolytic pathway. On the other hand, aspirin demonstrated a moderate effect on LT in SA patients suggesting it could have a beneficial effect on the endogenous fibrinolytic system. Larger studies using aspirin at different doses and in different subgroup of patients are necessary to study the fibrinolytic properties of aspirin.

Data from various studies suggest endogenous thrombolysis could be an important predictor of adverse cardiac events. In the ASSENT-4 PCI study (Bainey et al. 2008), in a cohort of 585 patients admitted with STEMI, spontaneous reperfusion was assessed pre PCI using ECG or angiographic result. 14.9% demonstrated ST segment resolution, and 14.7% demonstration TIMI 3 flow of the culprit artery suggesting spontaneous reperfusion. Significant reduction in CV death or reinfarction was noted in the ECG resolution group. In the Acute Coronary Syndrome Israeli Survey (ACSIS) study, 710 patients admitted with a STEMI were assessed

for SR during their admission (Fefer et al. 2009). SR was defined as greater than 70% reduction in ST elevation and chest pain. 22% patients demonstrated evidence of SR, and were conservatively treated in the initial acute stage, with some patients requiring PCI. 78% patients who did not demonstrate any significant ST segment resolution underwent primary PCI. Adverse outcome at 30 days was significantly lower in the SR group, and was a composite of CV death, congestive heart failure and recurrent ACS. Fornitz et al demonstrated the importance of fibrinolytic marker such as PAI-1, tPA and platelet-derived growth factor (PDGF) levels coronary artery disease patients pre and post PCI. 19 patients were included in this study and followed up for 6 months. Seven patients had instant restenosis, and demonstrated higher PAI-1 and lower t-PA values (Fornitz et al.2001). In a study of 106 patients (Soeki et al.2002), AMI patients who had recurrent MI or CV death over 4 years had higher t-PA levels compared to controls in the subacute phase of MI, suggesting elevated t-PA levels were a significant predictor of cardiac events ($P<0.01$). Another study by Christ et al demonstrated significant elevation of PAI-1 and t-PA levels in patients who underwent PCI (Christ et al. 2005). In patients on Ticlopidine, attenuated increase of PAI-1 in the first 24 hours was significantly predictive of ISR. In another study of 75 patients undergoing PCI (Katsaros et al. 2008), PAI-1 levels were measured pre and post PCI with a drug eluting stent. 16% patients had ISR at 6-8 months post PCI, and their pre PCI PAI-1 levels were significantly lower than the non ISR group of patients. In a case-control study in the Cardiovascular Health Study cohort (Cushman et al. 1999), 5201 individuals ≥ 65 years age were enrolled and fibrinolytic markers such as PAI-1, D-dimer and PAP studied. All 146 cases were not known to suffer from underlying coronary disease and developed subsequent MI, angina or CV death over the follow up period. D-dimer and PAP levels were independently associated with increased risk of myocardial infarction or coronary death. There was no significant correlation between PAI-1 and cardiac events. The

Atherogene study demonstrated a significant correlation between activated TAFI levels and future risk of cardiac events in 1668 patients with angiographically proven coronary artery disease (Tregouet et al.2009).

Finally, we compared GTT OT with the Verify Now assay PRU, and no significant correlation was observed between the two test results. Studies comparing high platelet reactivity using the Verify Now assay with the LTA which is considered the gold standard test have shown modest to poor correlation. In a study by Gaglia et al, 200 patients on Clopidogrel undergoing PCI were tested using Verify Now assay, LTA and VASP (Gaglia et al . 2011). Incidence of high on treatment platelet reactivity was 27.3% with Verify Now, 39.3% with VASP, and 23.1% and 16.2% with LTA ADP 5 μ M and 20 μ M respectively ($\rho= 0.60-0.86$, $p<0.001$). In another study of 222 patients undergoing PCI (Ko et al. 2011), Verify Now assay was compared with Multiple Electrode Platelet Aggregometry (MEA). Weak correlations were noted between the two tests in both the arachidonic acid-induced (Spearman $r = 0.189$, $p = .006$) and ADP-induced platelet reactivity ($\rho = 0.390$, $p < .001$). On the contrary, in a study of 801 high risk cardiac patients (Paniccia et al. 2010), MEA showed a significant correlation with the Verify Now assay ($\rho=0.62$, $p<0.0001$).

To summarize, endogenous thrombolysis has been a neglected entity, possibly as there is no reliable marker of fibrinolysis and until recently, there had been no test available to measure a patient's endogenous thrombolytic potential. This thesis has clearly demonstrated that endogenous thrombolysis is an important, independent novel predictor of adverse cardiac events. Resources need to be directed to study this entity further, to allow us to enable to control and improve the endogenous fibrinolytic activity and outcomes in patients with coronary artery disease (Saraf et al.2010).

Future Research: Fibrinolytic markers and therapeutic modulation of fibrinolysis

PAI-1 and plasminogen activation play an important role in fibrinolysis. Spontaneous thrombolysis has been a neglected entity, and there is not much data available on therapeutic modulation of fibrinolysis. Blocking PAI-1 activity seems to be the most feasible approach at enhancing lysis, and use of PAI-1 antibodies and peptides that either inhibits PAI-1 production or activity could prove beneficial in controlling thrombus formation. In animal models, use of PAI-1 monoclonal antibodies has shown to inhibit thrombus formation (Biemond et al. 1995). t-PA activates fibrinolysis, and enhancing release of t-PA from the endothelial cells would improve endogenous thrombolytic activity. Endothelial cells release nitric oxide with the help of nitric oxide synthase, and improve fibrinolysis. Several factors have been shown to improve NO synthase expression, and increase t-PA activity. They include Kruppel like Factor 2 (KLF2) (Lin et al. 2005), Dipyridamole (Kim et al. 2005) and All -Trans retinoic acid (ATRA) (Marchetti et al. 2003). KLF2 inhibits PAI-1 expression and ATRA and Dipyridamole inhibit PAI activity without altering expression.

Endothelial progenitor cells (EPCs) restore vascular endothelium increasing nitric oxide release, and have been shown to improve fibrinolysis and reduce thrombotic activity (Ozuyaman et al. 2005). Pitavastatin is a statin in development, that reduces PAI-1 antigen and activity and reduces thrombotic activity (Markle et al. 2003).

Procarboxypeptidase B (CPB) is found in plasma, and is converted by thrombin to its active form that inhibits fibrinolysis. CPB inhibitors are being developed, and may be used as a target to enhance fibrinolysis in the long term (Suzuki et al. 2004).

Study Limitations

Sampling during index admission following Aspirin 300 mg and Clopidogrel 300 mg

All patients were sampled during their index admission at a mean of 5 ± 3 days (Mean \pm SD). All patients had received a loading dose of aspirin 300 mg and clopidogrel 300 mg, and were on maintenance dose of aspirin 75 mg and clopidogrel 75 mg. As platelet reactivity is dose dependent, it is difficult to anticipate effect on thrombotic status with varying doses of aspirin and clopidogrel. It is also possible that steady state concentration of the drug was not fully reached during the time of sampling. In our study, none of these patients were sampled during subsequent cardiac related admissions. Resampling after the time of the index event may have given a better idea of impaired thrombotic and thrombolytic status, helping us understand in more detail the effects of impaired occlusion and lysis times.

As mentioned, all patients were sampled during their index admission in the acute phase of an inflammatory response. It is possible that impaired fibrinolysis was part of the acute phase response, alternatively it is possible it reflects chronic impairment of fibrinolysis that influences late outcome.

Effect of Heparin on thrombotic and thrombolytic status

Low molecular weight heparin is a depolymerized derivative of unfractionated heparin (UFH). It has the ability to inactivate coagulation Factor Xa, and has a higher anti-Factor Xa:IIa ratio compared to UFH (2:1 to 4:1). It has greater bioavailability, and a longer half life compared to UFH, hence is used commonly in ACS patients. The half life of the drug is 5-6 hours, and twice a day doses are used in ACS patients. Heparin works as an anticoagulant by binding to antithrombin III and inhibiting the coagulation cascade. Heparin does not work as a thrombolytic, but prevents further clot formation. vWF is essential for

platelet thrombus formation at high shear-rates and it is recognised that in addition to its effect on coagulation, heparin interferes with this platelet/vWF-mediated haemostasis, and forms a heparin-platelet complex that can affect platelet behaviour for longer than 48 hours, extending upto 10 days. All patients in our study were sampled atleast 48 hours after discontinuation of heparin, but there is a possibility the membrane complex could have interfered with the GTT measurements. As most ACS patients undergo coronary angiography within 10 days of admission, and are discharged in a timely manner, it was difficult to sample these patients at a later date. This might in part explain why OT was not a predictor of adverse cardiac events in this study.

Possible diurnal variation in thrombotic and thrombolytic status not investigated

There is data to suggest fibrinolytic activity in blood shows a diurnal variation, peaking in the evening with reduced trough levels early morning (Andreotti et al. 1991). PAI-1 and t-PA are also known to show this diurnal variation, which may explain the difference in trough levels. We did not sample patients at different times of the day, and LT results may hence not be reflective of this change in circadian rhythm.

Pharmacological modulation of endogenous thrombolytic status not assessed

PAI-1 and TAFI are released from activated platelets, and inhibit fibrinolysis. As thrombin is required for release of both these markers at physiological calcium concentration, thrombin inhibition would achieve the goal of adequate fibrinolysis. Direct thrombin inhibitors are being developed, and use of these drugs with measurement of PAI-1 and TAFI concentration will in due course allow us to come to a meaningful conclusion regarding effective fibrinolysis.

Other drugs shown to have some beneficial effect on fibrinolysis are aspirin at high doses, antidiabetic medications like metformin and sulfonylurea, ACE-I and nitrates. There is data to suggest Eplerenone, an aldosterone antagonist inhibits release of PAI-1 and may have a role to play in endogenous fibrinolysis.

In the absence of direct thrombin inhibitors and limited data on reliable fibrinolytic markers, it was difficult for us to measure the levels of different fibrinolysis markers in vitro. A reliable marker of fibrinolysis is required to assess the efficacy of endogenous fibrinolysis, and larger trials will help us delineate which markers and medications are most effective in enhancing fibrinolysis and improving cardiovascular outcomes.

Statistically underpowered to detect a significant effect in subgroups

The study was underpowered to investigate thrombotic and thrombolytic status in certain subgroups of patients due to the small number of patients and events in subgroups such as: STEMI, diabetes mellitus, renal impairment, and smokers. Larger studies with greater number of events would be required to study the effects of these clinical parameters in these subgroups of patients.

Future work around this subject involves use of GTT in different patient populations. Studies are currently ongoing in elective and emergency PCI patients, renal patients, in patients with atrial fibrillation and in patients on newer antiplatelet agents like direct thrombin inhibitors. Further larger studies will need to be validated in homogenous patient cohorts to avoid any bias in data interpretation, and all studies need to be adequately powered to help us reach a statistically significant conclusion. Measurement of various thrombotic and fibrinolytic markers will help understand the concept of thrombosis and thrombolysis, and microscopic observation of the injected blood at different levels in the GTT tube help in determination of

the mechanism underlying OT and LT. Comparison of various point of care assays will help determine the ideal and most reliable and physiological platelet function test.

Appendices

Ethical approval documents

Ambulance Training Centre
 VIA LOCATION CODE Q7
 QEII Hospital
 Howlands
 Welwyn Garden City
 Hertfordshire
 AL7 4HQ

Telephone: 01707 362583
 Facsimile: 01707 394475

14 September 2006

Dr Diana A Gorog
 Consultant Cardiologist
 East & North Hertfordshire NHS Trust
 Cardiology Department
 Queen Elizabeth II Hospital
 Howlands, Welwyn Garden City, HERTS
 AL7 4HQ

Dear Dr Gorog

Full title of study: Functional relationship between aspirin/clopidogrel resistance and clinical outcome in patients with acute coronary syndrome
REC reference number: 06/Q0201/38

Thank you for your letter of 21 August 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The Vice-Chair has considered the further information on behalf of the Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
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An advisory committee to Bedfordshire and Hertfordshire Strategic Health Authority

06/Q0201/38

Page 2

Application		24 May 2006
Investigator CV		24 May 2006
Protocol	1	24 May 2006
Covering Letter		24 May 2006
Statistician Comments		19 April 2006
GP/Consultant Information Sheets		24 May 2006
GP/Consultant Information Sheets		
Participant Information Sheet: PIS	1	24 May 2006
Participant Consent Form: Consent Form	1	24 May 2006
Response to Request for Further Information		21 August 2006

Research governance approval

You should arrange for the R&D department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Statement of compliance


The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0201/38

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



Mrs Linda Bone
Vice Chair

Email: jane.winter@nhs.net

Enclosures:

Standard approval conditions [SL-AC1 for CTIMPs, SL-AC2 for other studies]

Copy to:

Mrs Fiona Smith
East & North Herts NHS Trust
Lister Hospital (Location Code L57D)
Corey's Mill Lane
Stevenage, Herts
[R&D Department for NHS care organisation at lead site]

SF1 list of approved sites

An advisory committee to Bedfordshire and Hertfordshire Strategic Health Authority



National Research Ethics Service

Hertfordshire REC

East of England REC Office No 3
9th Floor, Terminus House
The High
Harlow
Essex
CM20 1XA

Tel: 01279 418 439
Fax: 01279 419 246

31 December 2008

Dr Diana A Gorog
Consultant Cardiologist
Cardiology Department, Q67
Queen Elizabeth II Hospital
Howlands
Welwyn Garden City, Herts
AL7 4HQ

Dear Dr Gorog

Study title: Functional relationship between aspirin/clopidogrel resistance and clinical outcome in patients with acute coronary syndrome
REC reference: 06/Q0201/38 (AM03)
Amendment number: 4
Amendment date: 01 October 2008

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 31 December 2008.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	4	17 December 2008
Notice of Substantial Amendment (non-CTIMPs)	4	17 December 2008
Covering Letter		17 December 2008

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet.

This Research Ethics Committee is an advisory committee to East of England Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within
the National Patient Safety Agency and Research Ethics Committees in England



R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

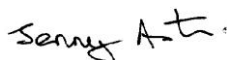
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0201/38-

Please quote this number on all correspondence

Yours sincerely



Mrs Jenny Austin
Committee Co-ordinator

E-mail jenny.austin@eoe.nhs.uk

Enclosures List of names and professions of members who were present at the meeting and those who submitted written comments

Copy to: Fiona Smith, R&D Manager, HCC
The Clock Tower
Mount Vernon Hospital
Rickmansworth Road
Northwood, Middx
HA6 2RN



National Research Ethics Service

Hertfordshire REC
 East of England REC Office No 3
 9th Floor, Terminus House
 The High
 Harlow
 Essex
 CM20 1XA

Tel: 01279 418 439
 Fax: 01279 419 246

27 January 2009

Dr Diana A Gorog
 Consultant Cardiologist
 Cardiology Department, Q67
 Queen Elizabeth II Hospital
 Howlands
 Welwyn Garden City
 Herts
 AL7 4HQ

Dear Dr Gorog

Study title: Functional relationship between aspirin/clopidogrel resistance and clinical outcome in patients with acute coronary syndrome
REC reference: 06/Q0201/38 (AM04)
Amendment number: 5
Amendment date: 12 January 2009

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 21 January 2009.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	5	12 January 2009
Email		15 January 2009
Notice of Substantial Amendment (non-CTIMPs)	5	12 January 2009
Covering Letter		12 January 2009

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Yours sincerely

Mrs Jenny Austin
Committee Co-ordinator

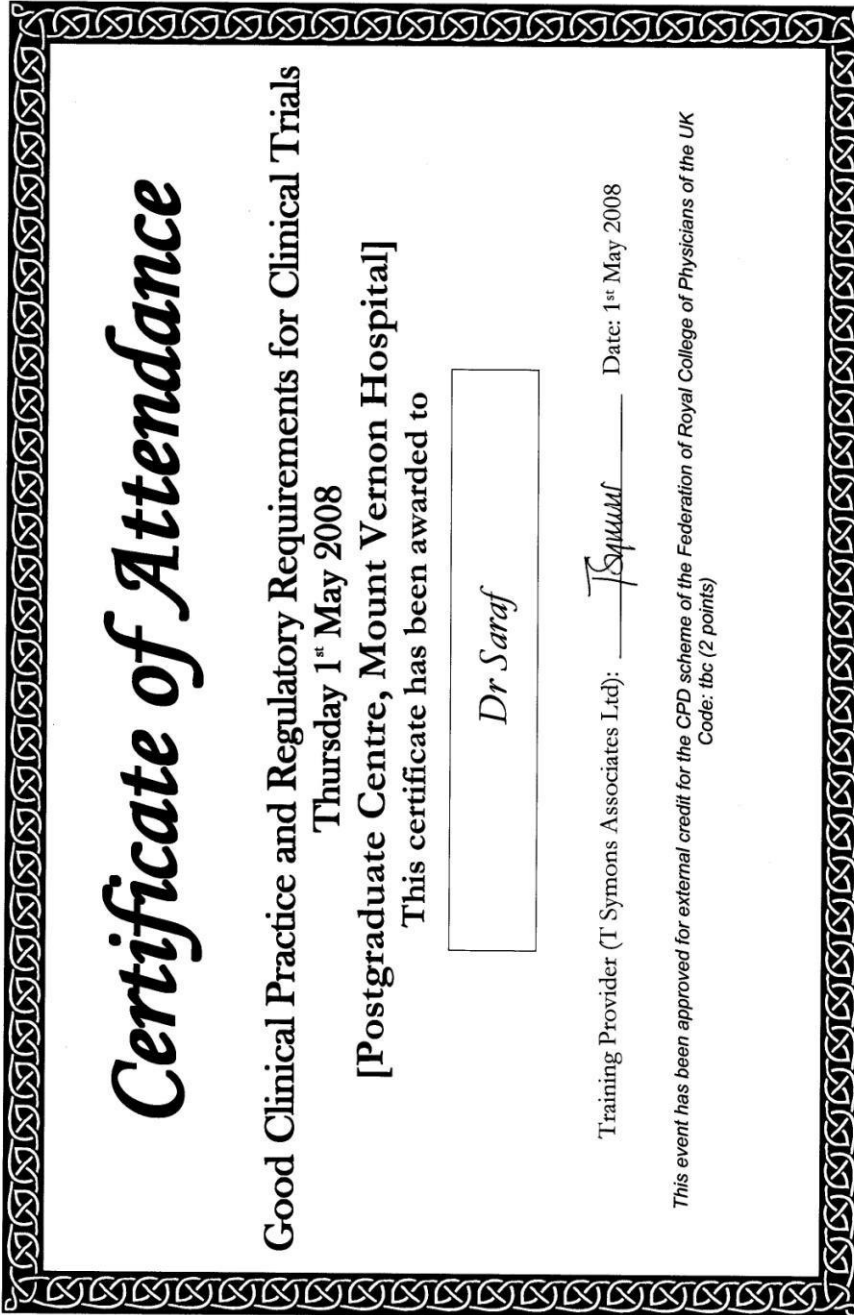
E-mail: jenny.austin@eoe.nhs.uk

Enclosures List of names and professions of members who were present at the meeting and those who submitted written comments

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Good clinical practice certificate



J

Case report form

STUDY NO		THROMBOLYSIS	
HOSPITAL NO		THROMBOLYSIS DATE/TIME	
INITIALS		DIPYRIDAMOLE	
DATE OF BIRTH		INSULIN	
AGE		METFORMIN	
SEX		OTHERS	
DIABETIC		DATE OF ADMISSION	
SMOKER		STEMI	
HYPERTENSION		THROMBOLYSED	
PRIOR CAD		TROP POSITIVE	
RENAL DISEASE		PEAK TROPONIN	
CREATININE		DYNAMIC ECG CHANGES	
PVD		LAST EPISODE CHEST PAIN	
STROKE/TIA		ANGIOGRAM RESULT	
BLEEDING DIATHESIS		INTERVENTION AND STENT	
ASPIRIN		HAEMOGLOBIN	
DATE ASPIRIN STARTED		RBC	
CLOPIDOGREL		PLATELETS	
DATE CLOPIDOGREL STARTED		CREATININE	
STATIN		UREA	
ACE I/ ARB		DATE OF TESTING	
BETA BLOCKER		TIME OF TESTING	
NITRATE		LAST DOSE ASPIRIN/ CLOP	
CLEXANE		GTT OT	
CLEXANE LAST DOSE		GTT LT	
WARFARIN		VERIFY NOW BASE	
WARFARIN LAST DOSE		VERIFY NOW PRU AND % ININHIBITION	

Publications from this thesis

Original Research Papers

Saraf S, Christopoulos C, Salha IB, Stott DJ, Gorog DA. Impaired endogenous thrombolysis in ACS patients predicts cardiovascular death and nonfatal myocardial Infarction. **J Am Coll Cardiol.**2010; 55:2107-2115

Gorog DA, Yamamoto J, **Saraf S**, Ishii H, Ijiri Y, Ikarugi H, Wellsted DM, Mori M, Yamori Y. First direct comparison of platelet reactivity and thrombolytic status between Japanese and Western volunteers: possible relationship to the “Japanese paradox”. **Int J Cardio.** 2011; 152 : 43-48

Saraf S, Wellsted D, Sharma S, Gorog DA. Shear-induced global thrombosis test of native blood: Pivotal role of ADP allows monitoring of P2Y12 antagonist therapy. **Thrombosis Res;** 124:447-451

International Abstracts

Saraf S, Sharma S, Gorog DA . Spontaneous thrombolytic activity in coronary artery disease.

POSTER presentation at the ISTH Meeting – Boston, USA – Sep 2009. **Journal of thrombosis and haemostasis** .2009; Volume 7, Supplement 2: Abstract PP-WE-751

Saraf S, Sharma S, Yamamoto J, Ishii H, Yamori Y, Gorog DA. Racial differences in thrombotic status may explain the increased incidence of haemorrhagic stroke in Japan
POSTER presentation at the ISTH Meeting – Boston, USA – Sep 2009. **Journal of Thrombosis and Haemostasis** 2009; Volume 7, Supplement 2: Abstract PP-TH-061

Saraf S, Christopoulos C, Salha IB, Stott DJ, Gorog DA. Impaired Endogenous Thrombolysis: A Novel Predictor of Recurrent Cardiovascular Events in Patients With Acute Coronary Syndrome. POSTER presentation at the British Cardiovascular Society Conference – Manchester, UK-June 2009. **Heart** 2009; 95:76

Saraf S, Ishii H, Yamamoto J, Yamori Y, Gorog DA. Marked Differences in Thrombotic and Thrombolytic Status between Japanese and Western Subjects. POSTER presentation at the American College of Cardiology - Orlando, USA – March 2009. **J Am Coll. Cardiol.** 2009; 53; A419-A458; 1013-101

Saraf S, Sharma S, Bensalha I, Wellsted D, Gorog DA. Impaired Endogenous Thrombolysis: A Novel Predictor of Recurrent Cardiovascular Events in Patients with Acute Coronary Syndrome. POSTER presentation at the American College of Cardiology Conference - Orlando, USA- March 2009. **J Am Coll Cardiol.** 2009; 53; A419-A458; 1013-1016

Saraf S, Sharma S, Bensalha I, Gorog DA. Clopidogrel resistance as assessed by P2Y (12) receptor inhibition is not reflective of global thrombotic status. POSTER presentation at the American College of Cardiology - Orlando, USA – March 2009. **J Am Coll Cardiol.** 2009; 53; A419-A458; 1013-1016

Saraf S. Gorog DA. Impaired endogenous thrombolytic status predicts future events in acute coronary syndrome patients: results using a novel thrombosis test. **Runner up** at the **British Junior Cardiologists Association Research Awards**: London, UK- Dec 2008

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