Evaluation of platelet dependent thrombosis and the role of dual antiplatelet therapy on blood thrombogenicity in patients with type 2 diabetes mellitus and coronary artery disease

Thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Evaluation of platelet dependent thrombosis and the role of dual antiplatelet therapy on blood thrombogenicity in patients with type 2 diabetes mellitus and coronary artery disease

The prevalence of type 2 diabetes mellitus (T2DM) is rapidly increasing. Recurrent thrombotic events and cardiac mortality are higher in T2DM.

Aims:

My overall aim was to identify abnormalities in blood thrombogenicity in T2DM and assess the response to dual antiplatelet therapy.

Hypotheses:

Study 1 ACS study: Patients with T2DM have increased blood thrombogenicity following non ST-elevation acute coronary syndromes (NSTE-ACS).

Study 2 CAD study (double blinded RCT): Addition of clopidogrel to standard treatment will reduce blood thrombogenicity in T2DM and stable coronary artery disease (CAD).

Methods:

Study 1 ACS study: Eighty patients (40 with T2DM) with troponin positive NSTE-ACS on aspirin and clopidogrel underwent thrombogenicity studies. Study 2 CAD study: Ninety patients with T2DM and proven but stable CAD were randomised to either clopidogrel 75 mg od or placebo and were studied at base line and one week after therapy. I performed i) Badimon chamber study, ii) thromboelastography, iii) VerifyNow® aggregometry, iv) scanning electron microscopy (SEM), v) Multiplate® aggregometry, vi) biomarkers of platelet reactivity assays and vii) serum pro-inflammatory cytokines assays.

Results:

In T2DM patients with NSTE-ACS, there was higher thrombus area compared to non-diabetic patients. Diabetic thrombus showed lower viscoelastic tensile

strength and was more resistant to autolysis. On SEM, fibrin fibres in diabetic thrombus were thinner, with higher lateral interlinkage and mesh-like organisation. Thrombus correlated inversely with the rate of thrombus retraction. P selectin, CD40 ligand and inflammatory cytokines were higher in T2DM. Clopidogrel decreased thrombus area in stable CAD patients with T2DM, lowered platelet content of thrombus and increased fibrin diameter and density. Fibrin fibre diameter correlated negatively with shear elastic force of the thrombus. In a post hoc analysis, thrombus area of non diabetic ACS patients' was similar to that of T2DM patients with stable CAD. Thrombus area and point of care tests showed no correlation.

Conclusions:

T2DM had higher blood thrombogenicity after NSTE-ACS and platelet dependent thrombus was reduced in stable CAD patients after clopidogrel therapy. Clopidogrel resulted in favourable ultra structural changes to thrombus. Patients with T2DM and stable CAD were "ACS equivalent" and addition of clopidogrel may improve their clinical outcomes. Novel pharmacotherapy to target fibrin and inflammation in T2DM may reduce blood thrombogenicity.

Dedications

I dedicate this work to my beloved parents, respectful teachers and patients who volunteered to help.

Acknowledgements

I would like to thank wholeheartedly everyone who helped me with this project and I could only acknowledge a handful of those names in this section. It would have not been possible to complete this work without the kindness of those who offered me guidance and support with pure altruism and passion for science.

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I would like to thank the Chief Executive of Newcastle Upon Tyne Hospitals NHS Foundation trust and the regulatory authorities both at local and national levels for their approval of this project. I express my sincere thanks to British Heart Foundation for funding the study. I would also like to thank The Northumberland, Tyne and Wear Clinical Research Network for their assistance. Last but not the least; I thank all the patients who volunteered for this study. I would have served my due if this research could advance the knowledge and understanding of cardiac illness in patients with diabetes and eventually improve their care.

Declaration

I declare that this thesis submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy to Newcastle University is a product of my original work and not submitted elsewhere for a degree or diploma. The studies were conducted by me at the premises of Newcastle upon Tyne Hospitals NHS Foundation trust, and Institute of Cellular Medicine, Newcastle University. I was responsible for the design and organisation of the clinical studies, recruitment of participants and their care, conduct of the studies, collection and analysis of the samples, data management, data analysis and research governance. I have correctly acknowledged the specific contributions by others in the relevant sections of the thesis.

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Abbreviations

ACC	American College of Cardiology
ACS	Acute coronary syndrome
ADAPT-DES	Assessment of Dual AntiPlatelet Therapy With Drug Eluting Stents
ADP	Adenosine diphosphate
AGE	Advanced glycation end products
AHA	American Heart Association
AMI	Acute myocardial infarction
APTT	Activated partial thromboplastin time
ARCTIC-DES	Assessment by a Double Randomization of a Conventional Antiplatelet Strategy versus a Monitoring-guided Strategy for Drug-Eluting Stent Implantation versus Continuation One Year after Stenting
ARU	Aspirin reactive units
ASCET	Aspirin non-responsiveness and Clopidogrel Endpoint Trial
ATC	Antithrombotic Trialists' Collaboration
ATP	Adenosine triphosphate
AUC	Area under the curve
BARI-2D	Bypass Angioplasty Revascularization Investigation 2 Diabetes
BMI	Body mass index
BT	Blood thrombogenicity
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CAPRIE	Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events
CCS-2	Second Chinese Cardiac Study
cGMP	Cyclic guanosine monophosphate

CHARISMA	Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance
CI	Clot index
CLARITY	The Clopidogrel as Adjunctive Reperfusion Therapy
CLEAR-PLATELETS	Clopidogrel With Eptifibatide to Arrest the Reactivity of Platelets
COMMIT	ClOpidogrel and Metoprolol in Myocardial Infarction Trial
COX-1	Cyclo-oxygenase -1
COX-2	Cyclo-oxygenase -2
CREDO	Clopidogrel for the Reduction of Events During Observation
CRP	C reactive protein
CRUSADE	Can Rapid Risk Stratification of Unstable Angina Patients Suppress ADverse Outcomes with Early Implementation
CURE	Clopidogrel in Unstable Angina to Prevent Recurrent Events
CURRENT-OASIS	Clopidogrel and Aspirin Optimal Dose Usage to Reduce Recurrent Events-Organization to Assess Strategies in Ischemic Syndromes
CVD	Cardiovascular disease
CYP450	Cytochrome P450
DAG	Diacylglycerol
DAPT	Dual Anti Platelet Trial
DECADES	Discontinuation Effect of Clopidogrel After Drug Eluting Stent
ECL	Electro-chemiluminescence
ELAPSE	Evaluation of Long-term clopidogrel AntiPlatelet and Systemic anti-inflammatory Effects
EPC	Endothelial cell protein C
EQC	Electronic Quality Control

EUROASPIRE	European Action on Secondary and Primary Prevention by Intervention to Reduce Events
EVASTENT	Évaluation coût/efficacité du stent actif au sirolimus chez les patients diabétiques et non diabétiques
FRISC II	Fragmin and Fast Revascularization During Instability in Coronary Artery Disease
GLUT-3	Glucose transporter protein 3
GpIb	Glycoprotein Ib
GPIIb IIIa	Glycoprotein IIb IIIa
GRACE	Global Registry of Acute Coronary Events
GRAVITAS	Gauging Responsiveness With A VerifyNow® P2Y12 Assay: Impact on Thrombosis and Safety
HOTR	High On Treatment platelet Reactivity
HS-CRP	High sensitivity C reactive protein
IFNγ	Interferon gamma
IL-1	Interleukin-1
IL-6	Interleukin-6
IMP	Investigational medicinal product
IPA	Impedance platelet aggregometry
kV	Kilo Volts
LDL	Low density lipoprotein
LTA	Light transmission aggregometry
MA-A	Maximum amplitude-Activated factor F
MA-AA	Maximum amplitude-Arachidonic Acid
MA-ADP	Maximum amplitude- Adenosine diphosphate
MCP-1	Monocyte chemoattractant protein-1
MHRA	Medicines and Health Regulatory Authority
MI	Myocardial infarction
MNC	Mononuclear phagocytic cells
MRL	Maximum rate of lysis

MRTG	Maximum rate of thrombus generation
NFKB	Nuclear factor kappa B
NSAID	Nonsteroidal antiinflammatory drugs
NSTE-ACS	Non ST elevation acute coronary syndrome
NSTEMI	Non ST segment elevation myocardial infarction
NYHA	New York Heart Association
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptors
PBS	Phosphate buffer solution
PCI	Percutaneous coronary intervention
PDT	Platelet dependent thrombosis
PGE1	Prostaglandin E1
PGI2	Prostaglandin I2
PKC	Protein Kinase C
PLATO	Study of Platelet Inhibition and Patient Outcomes
PLUTO-DIABETES	The PLavix Use for Treatment Of Diabetes
PPA	Platelet procoagulant activity
PROCLAIM	A Pilot Study to Examine the Effects of Clopidogrel Compared to Placebo on Markers of Inflammation in Subjects with Metabolic Syndrome Who Are Receiving Background Therapy, including Low-Dose Aspirin
PRUb	P2Y12 reactive units – baseline
PRUz	P2Y12 reactive units – activated
PSGL	P-selectin glycoprotein ligand
QALY	Quality adjusted life years
RAGE	Receptors for advanced glycation end products
RCT	Randomised control trial
REACH	REduction of Atherothrombosis for Continued Health

REC	Research Ethics Committee
SCAAR	Swedish Coronary Angiography and Angioplasty Registry
sCD40	Soluble CD40 ligand
SEM	Scanning electron microscope
STEMI	ST elevation myocardial infarction
SUSAR	SUspected Serious Adverse Reaction
SWAP	SWitching Anti Platelet
SYNTAX	Synergy between Percutaneous Coronary Interventions with Taxus and Cardiac Surgery
T2DM	Type 2 diabetes mellitus
TEG	Thromboelastography
TEG-PM	Thromboelastography-Platelet Mapping
TEM	Transmission electron microscopy
TFPI	Tissue factor pathway inhibitor
TG	Total thrombus generated
TIMI	Thrombolysis In Myocardial Infarction
TMRL	Time to maximum rate of clot lysis
TMRL	Time to the maximum rate of lysis
TMRTG	Time to maximum rate of thrombus generation
TNFα	Tumour necrosis factor alpha
TRAP	Thrombin receptor activating peptide
TRITON–TIMI	Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction
TTP	Thrombotic thrombocytopenic purpura
TXA2	Thromboxane A2
UA	Unstable angina
UKCRN	United Kingdom Clinical Research Network
UKPDS	United Kingdom Prospective Diabetes Study

VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cells
vWF	von Willebrand Factor
WHR	Waist hip ratio

Chapter 1 Introduction

The prevalence of diabetes mellitus is rising at an exponential rate worldwide (Wild *et al.*, 2004). It is estimated that the number of people with diabetes will double by 2030 (www.rcplondon.ac.uk/2009, Type 2 diabetes: national clinical guideline for management in primary and secondary care (update). accessed on 20th August 2013, diabetes.niddk.nih.gov/dm/pubs/statistics, accessed on 20th August 2013).

Type 2 Diabetes Mellitus (T2DM) is the most common form of diabetes, accounting for ninety percent of patients with diabetes mellitus (www.who.int/mediacentre/factsheets, accessed on 20th August 2013). T2DM is defined as a metabolic disorder of multiple aetiologies and characterised by chronic hyperglycaemia secondary to impaired insulin action and or insulin secretion resulting in disturbances of carbohydrate, fat and protein metabolism, and accompanied by clinical features such as increased thirst, polyuria and weight loss.

The World Health Organisation biochemical diagnostic criteria for diabetes are:

i. fasting plasma glucose ≥ 7.0mmol/l and
ii. plasma glucose two hours after ingestion of 75 grams of oral glucose load ≥ 11.1mmol/l

In the presence of clinical features of diabetes, one of the above is sufficient to make a diagnosis of diabetes. (www.who.int/diabetes/publications/en/index.html, accessed on 20th August 2013)

1.1 Cardiovascular disease in type 2 diabetes mellitus (T2DM)

1.1.1 Cardiovascular mortality and morbidity in type 2 diabetes mellitus

Patients with T2DM have a fivefold risk of developing cardiovascular disease compared to those without (Preis *et al.*, 2009). Eighty percent of patients with T2DM die of a cardiovascular cause (Fox *et al.*, 2007). More than half of the individuals with T2DM have evidence of cardiovascular disease at the time of diagnosis of diabetes. Mortality in patients with diabetes but without overt coronary artery disease is similar to those without diabetes but with a history of myocardial infarction (Haffner *et al.*, 1998; Haffner, 2000; Schramm *et al.*, 2008). This has led diabetes mellitus to be labelled as a 'cardiovascular equivalent' (Laakso, 2008). Diabetes mellitus accounts for 6% of global mortality and reduction of life expectancy by a decade (Mulnier *et al.*, 2006).

Acute myocardial infarction is twice as common in individuals with diabetes compared to those without (Schramm *et al.*, 2008). Despite current aggressive secondary prevention therapies, mortality after acute myocardial infarction is four times higher in patients with diabetes (Ford, 2005; Huxley *et al.*, 2006). The prevalence of cardiac disease and age specific cardiovascular mortality is lower in women generally. However, women with diabetes not only lack this gender related cardiac protection but also have a poorer prognosis from cardiovascular illness; their mortality rates are 50% higher than in men (Mulnier *et al.*, 2006; Kautzky-Willer *et al.*, 2010).

There has been a significant reduction in cardiovascular mortality in patients with and without diabetes over the last two decades. However, patients with diabetes mellitus remain at higher risk than non diabetic patients and the relative risk of cardiovascular mortality in patients with diabetes remains around 2.5, unchanged over the last fifty years (Hanefeld *et al.*, 1996; Fox *et al.*, 2007) .

Cardiovascular morbidity in patients with diabetes is difficult to estimate. Cardiovascular disease is responsible for significant numbers of hospitalisations, longer duration of in-hospital stay and loss of quality adjusted life years (QALY) in patients with diabetes (Sampson *et al.*, 2006). Ten percent of patients admitted to

hospital at any time in the United Kingdom have diabetes as a co-morbidity (Moore, 2000). In 2004, more than 10% of the National Health Services budget was spent on treatment of the complications of diabetes mellitus, with cardiovascular complications as the leading cause for hospitalisation (Bottomley, 2001). The enormity of this problem was reflected from a Cardiologists' perspective in a large North American registry of patients with coronary artery disease and one third of those needing percutaneous coronary artery revascularisation had diabetes mellitus (Venkitachalam *et al.*, 2009). Patients with diabetes experience prolonged stays in hospital, resulting in about 80,000 additional bed days per year in the UK (Sampson *et al.*, 2006). In addition, diabetes accounts for 2.4 times higher medical expenditure compared to a similar population in the absence of diabetes (Roglic *et al.*, 2005)

1.1.2 Coronary Artery Disease (CAD) in T2DM

Coronary artery disease (CAD) accounts for the majority of cardiovascular events in patients with T2DM. Coronary artery disease manifests as acute myocardial infarction (ST elevation and non ST elevation acute coronary syndrome), and chronic stable angina (Berry *et al.*, 2007). A significant number of patients with T2DM do not survive their first myocardial event (Cho *et al.*, 2002). However, due to improved secondary prevention methods, the cardiovascular mortality rate in patients with T2DM has fallen over the last two decades (Fox *et al.*, 2007). The health and socioeconomic benefits of this improving survival of patients with T2DM after an acute cardiac event have been offset by the incidence of higher recurrent cardiovascular events (Grundy *et al.*, 1999; Lee *et al.*, 2004). In a large scale cohort of patients followed up in the European Action on Secondary and Primary Prevention through Intervention to Reduce Events (EUROASPIRE) study, T2DM was a strong predictor of mortality and recurrent cardiovascular events even after adjustment for conventional risk factors such as hypertension and dyslipidaemia (Kotseva *et al.*, 2010). In large scale registries such as The Global Registry of Acute Coronary Events (GRACE) and global Reduction of Atherothrombosis for Continued Health (REACH) Registry, Can Rapid Risk Stratification of Unstable Angina Patients Suppress ADverse Outcomes with

Early Implementation of the ACC/AHA Guidelines (CRUSADE), recurrent cardiovascular event rates remained higher in diabetes despite optimal secondary prevention therapy(Franklin *et al.*, 2004; Koek *et al.*, 2007; Bhatt *et al.*, 2010). In the Swedish Coronary Angiography Angioplasty Registry (SCAAR), recurrent cardiac events occurred in one fourth of the T2DM patients who had percutaneous coronary revascularisation (Norhammar *et al.*, 2010). Results from the Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI-2D) study in T2DM patients who underwent coronary bypass surgery also yielded similar results (Rutter and Nesto, 2010). After adjusting for baseline and treatment differences in the pooled Thrombolysis In Myocardial Infarction (TIMI) trials from 1997 to 2006, 30-day mortality was 78% higher in unstable angina (UA) and non ST segment myocardial infarction (NSTEMI) patients with T2DM and 40% higher in those with ST elevation myocardial infarction (STEMI). At 1 year, the excess mortality risk independently attributed to diabetes was 65% and 22%, respectively for UA/NSTEMI and STEMI (Donahoe JAMA 2007). There is less reduction in recurrent cardiovascular events with aggressive coronary revascularisation strategies such as percutaneous and surgical revascularisation in those with T2DM compared to those without T2DM (Franklin *et al.*, 2004). In addition, significantly higher rates of stent thrombosis were seen in patients with T2DM who received percutaneous coronary revascularisation treatment, even after adjusting for vessel diameter and implanted stent length. In the large multicentre EVASTENT registry of largely on-label sirolimus drug eluting stent use, the rate of drug-eluting stent thrombosis was 4.3% among T2DM vs. 3.0% in non diabetic patients in those with multivessel disease and 3.2% vs. 1.7% in those with single vessel disease at one year follow-up (Machecourt *et al.*, 2007).

1.1.3 Factors Contributing to CAD in T2DM

Higher cardiovascular events in T2DM were traditionally attributed to the increased prevalence of the cluster of conventional risk factors such as hypertension, smoking and dyslipidaemia (Bhatt *et al.*, 2010). However, large scale studies have confirmed significantly higher event rates in T2DM and coronary artery disease after adjustment for conventional risk factors (Buse *et al.*,

2007). The presence of other factors such as visceral obesity and impaired endothelial function are associated with higher event rates in patients with T2DM and coronary artery disease. Despite improvement of obesity and vascular endothelial function by multifactorial lifestyle interventions, cardiovascular mortality and non fatal cardiac events remained higher in patients with diabetes compared to those without diabetes (Eberly *et al.*, 2003; Griffin *et al.*, 2011). Tighter control of hyperglycaemia reduces cardiovascular mortality over many years when begun in individuals with short-duration diabetes in United Kingdom Prospective Diabetes Study (UKPDS) follow up study (Home, 2008; Gore and McGuire, 2009) but not when commenced in individuals with longer duration of T2DM (Gerstein *et al.*, 2008; Patel *et al.*, 2008; Duckworth *et al.*, 2009; Skyler *et al.*, 2009). This has led to search for novel risk factors unique to the diabetes population (Hayden and Reaven, 2000).Pathophysiology of coronary atherosclerosis in T2DM

1.1.4 Overview of coronary atherosclerosis

Pathophysiological mechanisms contributing to atherosclerosis are complex and inter linked. The definitions of atherosclerosis in general have changed in parallel with an ever expanding knowledge base. In the nineteenth century, one eminent pathologist, Virchow, called this condition a local proliferative disease, whilst another, Rokitansky, referred to it as recurrent thrombotic disease (Mayerl *et al.*, 2006). Multiple experimental data in the early 20th century supported the significant role played by lipids in atherosclerosis. Late 20th century studies have revealed inflammation and genetic propensity to be important in various stages of atherosclerosis. Our current knowledge of atherosclerosis encompasses all these mechanisms, with inflammation occupying a significant role in the initiation and progression of atherosclerotic lesions.

No single definition of atherosclerosis can explain the nature and mechanism of the condition. The definition which I favour is *"atherosclerosis is defined as a systemic, dysfunctional endothelial, focal or diffuse, stenotic, chronic inflammatory, fibro-proliferative, prothrombotic, angiogenic, multifactorial disease*

of the arterial tree caused by the retention of modified low-density lipoproteins, and hemodynamic and oxidative stress" (Hayden and Tyagi, 2004).

The mechanism of coronary atherosclerosis is well studied. The earlier view of atherosclerosis as a localised narrowing of the arteries secondary to lipid deposits is being replaced as a widespread vascular inflammatory disorder with acute (e.g. acute myocardial infarction), chronic (e.g. chronic stable angina) and acute on chronic (e.g. acute myocardial infarction in those with angina pectoris) clinical manifestations (Libby, 2002). A focal atherosclerotic lesion in a coronary artery is known as an atheromatous plaque, which is in constant flux. It responds to changes internal and external, mainly mediated by the inflammatory, vasomotor and lipid environment in the intra and extra plaque milieu.

1.1.5 Coronary artery: anatomical considerations

Our knowledge of coronary artery anatomy has changed significantly since the days of Virchow. An understanding of the anatomical ultra structure is crucial in interpreting the pathophysiology of atherothrombosis.

A normal coronary artery is made up of:

i. a thin inner layer, the tunica intima consisting of a single layer of endothelial cells
ii. a thick middle layer, the tunica media, made up of smooth muscle cells and elastin rich extra cellular matrix
iii. an outer layer, the tunica adventitia, rich in collagen fibrils and with the neurovascular bundle with vasa vasorum.

The endothelial cells form a continuous sheet in the inner most layer of the arteries which is non thrombogenic in healthy individuals. It covers the highly thrombogenic collagen in the tunica media, preventing exposure to flowing blood (Fuster *et al.*, 1992).

1.1.6 Initiation and progression of coronary atherosclerosis

The mechanism of very early atherosclerotic changes remains conjectural. However, evidence from autopsy studies and multiple experimental studies has provided a unifying theory pointing towards structural and functional changes in the endothelial layer as a marker of initiation of atherosclerosis. Damage to vascular endothelium in the form of 'fatty streak' has been identified in infants as early as 21 days old. Fatty streaks are intracellular and extracellular deposits of cholesteryl esters and other lipids in the tunica media layer of the artery (Strong, 1995). Fatty streaks progress to frank atheromatous lesions later in life.

The growth of an atheromatous lesion does not occur in a linear fashion with an inexorably progressive course with age, but in a step wise fashion with periods of spurts in growth interspaced with periods of slower progression related to age. Such periods of rapid growth of a plaque are precipitated by episodes of plaque rupture, sub-total luminal thrombosis and smooth muscle cell proliferation (Gutstein and Fuster, 1999).

1.1.6.1 Fatty streak

The progression of minor fatty streaks to frank atheromatous lesions is facilitated by impairment in the endothelial repair process. The coronary endothelium suffers continuous damage from external and systemic factors throughout the life span of an individual. When inherent protective mechanisms of the coronary endothelium in the form of functional antagonism to injurious products and the damage-repair process is overwhelmed, the fatty streak progresses to extensive atherosclerotic changes (Chien, 2003).

In a lipid rich milieu, small lipoprotein molecules in circulating blood tend to enter the tunica media layer and reside in the sub endothelial region where they bind to proteoglycan molecules of the tunica intima. Lipoprotein particles accumulate over a period of time and undergo oxidative modification in the presence of free radicals. Low density lipoproteins (LDL) that reside in the sub endothelial region undergo modification by oxidation and glycation and become oxidised LDL cholesterol which is more atherogenic and immunogenic (Tegos *et al.*, 2001).

1.1.6.2 Laminar flow and protection against atherosclerosis

Atheromatous changes occurring in coronary arteries can be focal or diffuse. Early atheromatous lesions in certain anatomical parts of the vascular tree, especially at bifurcations and branch points, are more susceptible to progression to significant atherosclerotic plaque (Nigro *et al.*, 2011). Rapid changes to shear stress and non laminar flow at bifurcation points and tortuous parts of the coronary vasculature trigger and maintain damage to endothelial function mediated via the nuclear factor kappa B (NFKB) pathway (Tegos *et al.*, 2001; Cunningham and Gotlieb, 2005; Berk, 2008; Chiu and Chien, 2011) .

1.1.6.3 Progression of atheromatous lesion

Endothelial injury accelerates the progression of coronary atheromatous lesions. The damaged endothelial cell initially responds by expressing adhesion molecules for leucocytes such as vascular cell adhesion molecule-1 (VCAM- 1) and P-selectin as a repair mechanism. However, upon continuous insult by both endogenous (high cholesterol) and exogenous (smoking) factors, these adhesion molecules are over expressed in coronary endothelium. The circulating leucocytes, mainly mononuclear phagocytic cells (MNC), attach to damaged endothelium via these molecules (Linton and Fazio, 2003). Once in contact with endothelial surface adhesion molecules, the MNC's undergo morphological and functional transformation to tissue macrophages inside the tunica media layer. MNC adhesion is mediated by cell membrane receptors and protein molecules such as monocyte chemo attractant protein-1 (MCP-1) (Chien, 2003; Boyle, 2005).

Once inside the tunica intima layer of vascular tissue, the modified MNC's releases various inflammatory mediators including cytokines, leukotrienes, prostenoids and histamine like substances. These act via membrane receptors to impair the functional and regenerating capacity of the endothelium (e.g. impaired production of nitric oxide). MNC's that are transformed to macrophages digest the lipoproteins to become 'foam cells'. In the presence of macrophage stimulating

factors, these foam cells undergo mitotic cell division and thus constitute enlargement of atheromatous plaque (Libby and Theroux, 2005).

Smooth muscle cells elaborate the matrix structure of the sub endothelial layer by synthesis of collagen and proteoglycans (Robbie and Libby, 2001; Libby *et al.*, 2010). The resultant initial outward remodelling preserves the luminal diameter of the arteries (Clarkson *et al.*, 1994). Some of these smooth muscle cells secrete cytokines and sequester calcium into the atheromatous plaque, thus enabling calcific mineralisation of the plaque. When the parallel apoptotic processes of macrophages and smooth muscle cells are overwhelmed, growth of plaque occurs both abluminally and inside the lumen, thereby resulting in a focal stenotic lesion (Bjorkerud and Bjorkerud, 1996; Stoneman and Bennett, 2004).

1.1.7 Atherosclerotic plaque vulnerability and rupture

Coronary atherosclerotic plaque morphology has recently been classified into 7 types based on lipid core, ratio of fibrous tissue to lipid core, fibrous tissue lining of the plaque and presence of thrombus (Figure 1.1). This pathological classification has put more emphasis on the vulnerability and rupture of the plaque (Farb *et al.*, 1996; Virmani *et al.*, 2000). The final common end point in the life cycle of a coronary atherosclerotic plaque is often plaque rupture and thrombosis (Arai *et al.*, 2010).

Fibrous tissue forms the cap of the atheromatous lesion. The architecture of the fibrous cap is weakened in the presence of matrix degrading enzymes such as metalloproteinases and cathepsins, resulting in rupture or erosion of the plaque. Programmed cell death of the smooth muscle cells can also result in reduced production of collagen and extracellular matrix, thus rendering the plaque vulnerable for rupture. Plaque rupture exposes thrombogenic lipids and collagen to flowing blood and can result in either occlusive or non occlusive thrombus leading to myocardial necrosis (Lafont, 2003). Plaque erosion without overt rupture can also occur secondary to similar mechanisms and can result in thrombosis. Most plaque erosions do not result in clinically apparent myocardial infarction but are responsible for bursts in growth of atheromatous plaques, (Fuster *et al.*, 2005; Virmani *et al.*, 2006a).

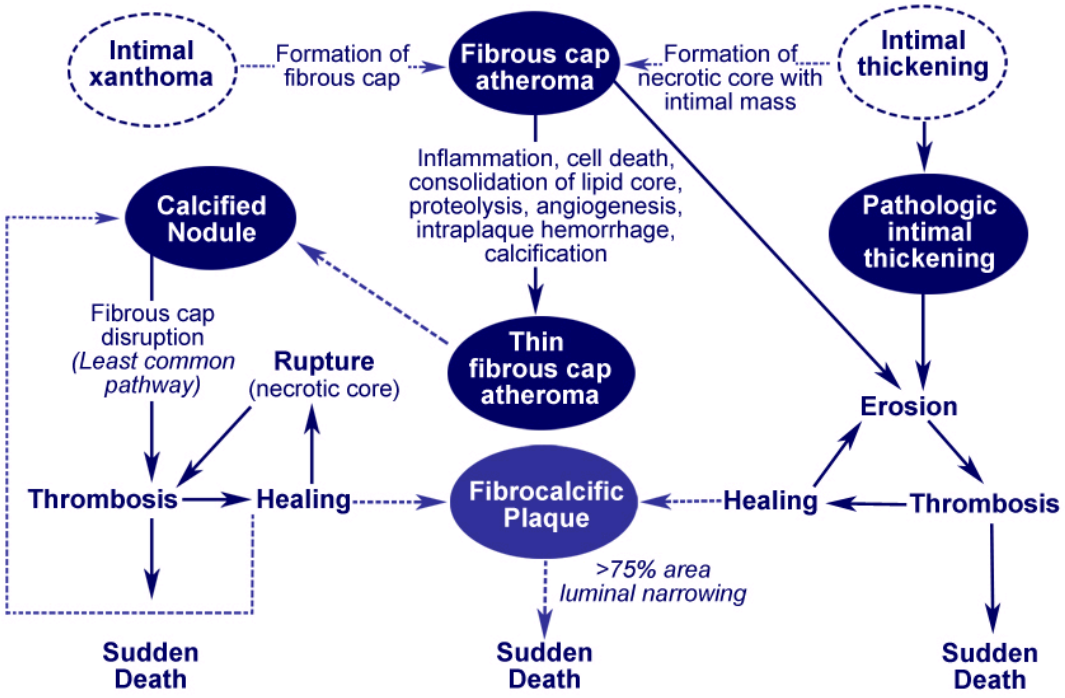


Figure 1.1 Classification of atherosclerotic plaques.

The boxed areas represent the 7 categories of lesion. Dashed lines were used for two categories because there is controversy over the role that each of them plays in the initial phase of lesion formation, and both "lesions" can exist without progressing to a fibrous cap atheroma (i.e., AHA type IV lesion). The processes leading to lesion progression are listed between categories. Lines (solid and dotted, the latter representing the least-established processes) depict current concepts of how one category may progress to another, with the thickness of the line representing the strength of the evidence that these events do occur. From Virmani R et al, Arterioscler Thromb Vasc Biol, 2000; 20: 1262 - 1275.

Vulnerable plaque is currently defined not as a plaque prone to rupture, but as a plaque prone to thrombus formation. There is a strong association of thrombus with plaque rupture and plaque erosion (Lafont, 2003). Autopsy studies of patients who died suddenly due to cardiac causes have revealed the presence of multiple layers of thrombi of different ages, thereby indicating this process is continuous and often silent (Lafont, 2003). In addition, recurrent episodes of thrombus in vulnerable plaques drive further atherosclerosis via platelet derived growth factors and cytokines (Fu *et al.*, 2001).

The pathophysiological mechanisms in the progression of stable plaques to vulnerable plaques can be summarised as:

i. biomechanical
ii. biochemical

The traditional model of Davies et al (Davies and Thomas, 1984) describes the plaque prone to rupture as consisting of a large lipid core composed of foam cells, apoptotic and necrotic cells, and debris, and which is separated from the lumen by a fibrous cap (made up of collagen, proteoglycans, and smooth muscle cells) which is actively weakened both by continuous lysis of extra cellular matrix and absence of repair. This results in plaque fissuring, often at the shoulder region of the plaque, exposing platelet activating substances such as collagen, and ultimately leading on to occlusive thrombus formation (Davies and Thomas, 1984).

The eccentric plaques are subjected to more mechanical shear stress and rupture at their shoulder region (Garcia-Pavia *et al.*, 2007). A myriad of scientific evidence confirms the significant role played by biochemical factors in plaque rupture. Cytokines released by macrophages play a central role in plaque rupture through extra cellular matrix damage induced by matrix metalloproteinases (Libby, 2009; Fishbein, 2010), suppression of collagen synthesis (Libby, 1995) and enhanced tissue factor generation (Mach *et al.*, 1997). Plaque erosion due to fracture of the fibrous cap and or superficial erosion of the intimal layer with or without alterations in the extracellular matrix of the tunica media of coronary arteries account for two-thirds of fatal acute myocardial infarctions (AMI)

(Sugiyama *et al.*, 2004). Biomechanical and biochemical processes are not separate but complementary to each other (Shah, 2003).

1.1.8 Coronary atherosclerosis in T2DM

Coronary atherosclerotic changes of some degree are observed in almost all patients with T2DM. Every stage in the process of atherosclerosis namely initiation, progression and plaque rupture is widespread and accelerated in T2DM. A greater atherosclerotic burden is consistently seen in medium sized arteries in T2DM, along with microvascular disease (Goraya *et al.*, 2002). Atherosclerotic changes are more dynamic from initiation to progression in T2DM, resulting in early plaque disruption and thus heralding more frequent occlusive thrombotic events resulting in AMI compared to those without diabetes (Waller *et al.*, 1980). In addition, mild to moderate sized atherosclerotic plaques known as 'non-flow limiting coronary atherosclerotic lesions' occur more frequently in T2DM and are associated with the majority of the plaque rupture events and thrombosis resulting in non ST elevation acute coronary syndrome (NSTE-ACS) in T2DM (Goraya *et al.*, 2002).

In patients with T2DM, the endothelial damage – repair cycle loses its 'balancing act' earlier than in those without T2DM. In the presence of frequently associated cardiovascular risk factors in T2DM such as hypertension, dyslipidaemia and obesity, damage to endothelium is more pronounced and the repair mechanisms are rapidly exhausted, thereby resulting in early and widespread atheromatous disease (Moreno and Fuster, 2004).

T2DM is associated with small coronary arteries and diffuse irregularity in the coronary architecture (Spinetti *et al.*, 2008). This anatomical feature would result in non laminar flow and increased LDL permeability due to a high cell turnover rate and the widening of the intercellular junction (Figure 1.2). In addition, non laminar flow also favours an increased tendency for more monocyte accumulation due to NFKB mediated cytokine generation in T2DM compared to non diabetic individuals (Chien, 2008).

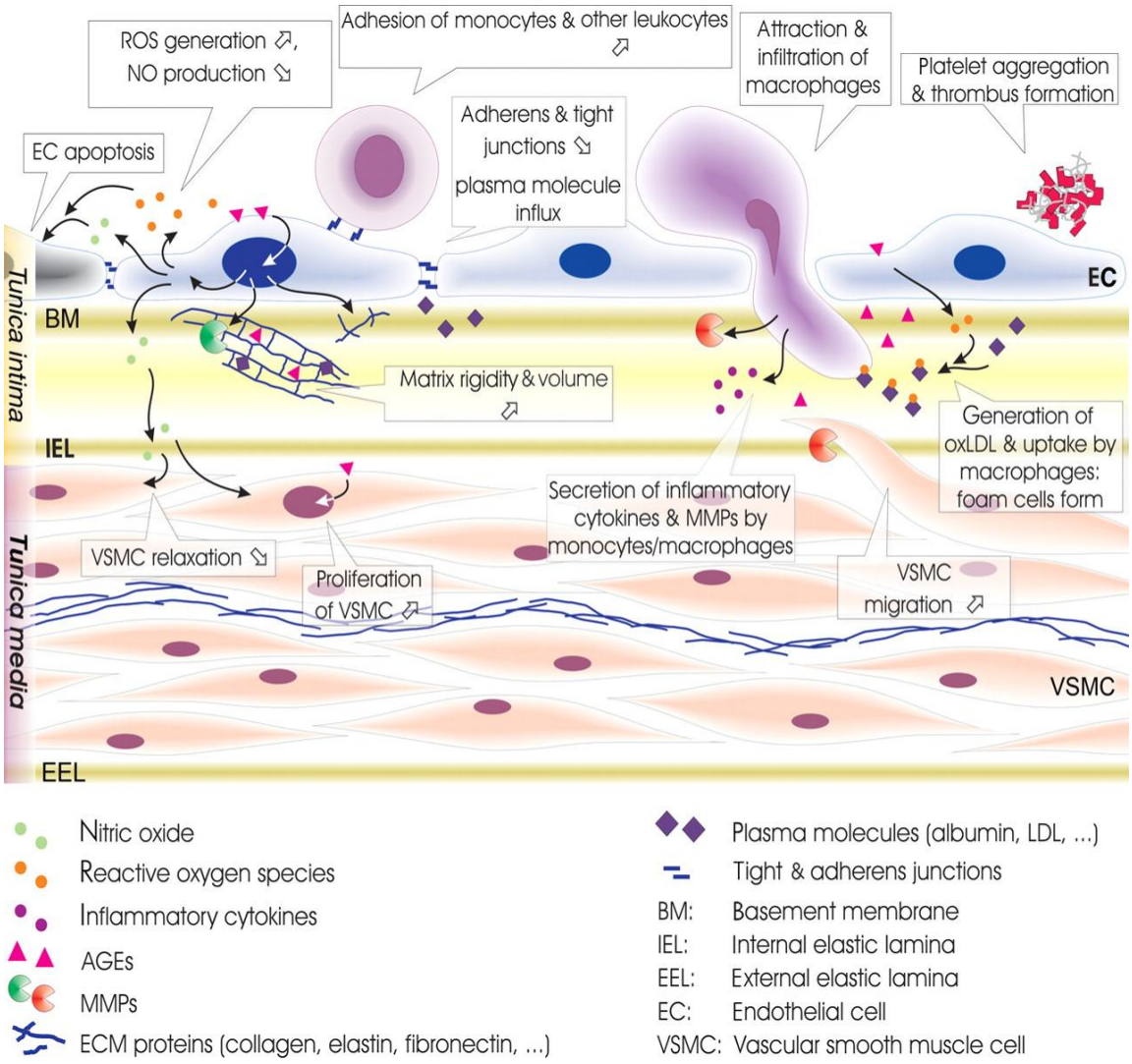


Figure 1.2 Atheroma formation in type 2 diabetes mellitus.

Endothelial cell apoptosis, reduced generation of nitric oxide (NO), and loss of endothelial cell junctions allow infiltration of macrophages and extravasation of plasma proteins. Leukocyte adhesion is facilitated by endothelial expression of adhesion molecules, aiding monocyte/macrophage infiltration and ultimately foam cell generation. Increased protein entrapment in the extracellular matrix, together with increased matrix deposition, and reduced degradation result in higher matrix volume. Low NO levels promote vascular smooth muscle cell (VSMC) proliferation and impede relaxation. Infiltrating macrophages and VSMC further increase intima/media (IM) thickness. Secretion of thrombogenic factors accelerates platelet adhesion and thrombus formation. From: Spinetti G et al, Cardiovasc Res, 2008; 78(2): 265-273.

1.1.9 Inflammation in T2DM and CAD - a common soil hypothesis

An inflammatory hypothesis for CAD and diabetes has been put forward which advocates shared molecular mechanisms between inflammatory mediators and insulin signalling pathways. Every step in the 'life cycle' of an atheromatous plaque in T2DM from endothelial dysfunction to plaque rupture and clinical events is associated with increased amounts of inflammatory constituents compared to those without diabetes (Yan, 2008, (Meng *et al.*, 2010). Coronary atherectomy specimens from patients with T2DM show larger areas of monocyte-macrophage infiltration compared to those from patients without diabetes (Figure 1.3) (Moreno *et al.*, 2000).

The presence of low grade inflammation which is characteristic of T2DM favours monocyte activation, migration and foam cell formation (Moreno *et al.*, 2000). The changes in MNC's are mediated by chemo attractant cytokines such as macrophage colony stimulating factor and interleukin 1(IL-1) which are abundant in T2DM compared to those without T2DM. Metabolic abnormalities in T2DM, such as higher concentrations of diacylglycerol (DAG) and advanced glycation end products (AGE), initiate and further intensify humoral, cellular and subcellular responses in coronary arteries that lead to vascular inflammation and atherothrombosis (Linton and Fazio, 2003; Zhang *et al.*, 2003). The damaged endothelial cell initially responds by expressing adhesion molecules for leucocytes such as vascular cell adhesion molecule-1 (VCAM- 1) and P-selectin. The circulating leucocytes, mainly mono nuclear phagocytic cells (MNC) attach to damaged endothelium.

The changes in MNC's are mediated by chemo attractant cytokines such as macrophage colony stimulating factor which are augmented in T2DM (Meng *et al.*, 2010). There is enhanced retention of macrophages in the sub endothelial layer in T2DM and interaction with medial vascular smooth muscle cells (VSMC). This vicious cycle increases further MNC accumulation in the tunica media. Both the VSMC and MNC show hyper response to various oxidative, immunogenic and haemodynamic factors in T2DM and secrete mediators of vascular damage such as matrix metalloproteinases and leukotrienes. The recent identification of

receptors for advanced glycation end products (RAGE) in monocytes provides the missing link between diabetes and hyper response of cytokines on sub-endothelial macrophages and VSMC's. Secretary (paracrine) products along with local endogenous tissue factors increase activation, proliferation and migration of vascular cells resulting in neointimal vascular formation (Yan *et al.*, 2008). Changes in the constituents of extracellular matrix of the coronary artery in T2DM delay the transfer of accumulated lipoproteins back into the vascular lumen, which promotes foam cell formation in the tunica media of patients with T2DM (Schmitz and Grandl, 2008; Mooradian, 2009).

1.1.10 Adventitial neovascularisation in T2DM

The coronary artery adventitial layer derives the majority of its oxygen by a process of diffusion from the intimal layer. The vessel wall thickens due to atheroma formation and leads on to the proliferation of new adventitial vasa vasorum into the inner layers of the vessel wall (Fuster *et al.*, 2005). It has been identified that T2DM patients have complex morphology of neovascularisation including sprouting, and red blood cell and monocyte extravasation with macrophage erythrophagocytosis eventually resulting in intraplaque haemorrhage (Kockx *et al.*, 2003; Moreno *et al.*, 2006; Orasanu and Plutzky, 2009). A vicious cycle is set up following intraplaque haemorrhage by recruitment of more inflammatory cells and delayed clearance of the oxidation prone iron radical (Virmani *et al.*, 2005; Michel *et al.*, 2011).

1.1.11 Plaque vulnerability in T2DM

Plaque vulnerability as determined by various stages of plaque disruption is higher in T2DM. Increased shear stress in patients with T2DM leads to damage of proteoglycans and results in a poorly intact extra cellular matrix. Furthermore, the higher levels of cytokines seen in T2DM disturb the interlinking of collagen by vascular smooth muscle cells (VSMC) that form a vital link in stability of the plaque (Shah, 2003; Hess and Grant, 2011). Greater apoptosis and matrix metalloproteinase levels in T2DM result in increased matrix degradation and destabilization of the plaques, and trigger thrombus formation (Figure 1.4)

(Sayeeda *et al.*, 2007). Intravascular ultrasound (Nicholls *et al.*, 2008), coronary computed tomography scanning (Yun *et al.*, 2009) optical coherence tomography (Feng *et al.*, 2010) and angioscopic studies (Ueda *et al.*, 2003) have shown a higher proportion of plaque rupture events occurring in patients with Acute Coronary Syndrome (ACS) and T2DM. Autopsy studies also confirm this, along with the presence of excess cellular infiltrates in vulnerable plaques (Figure 1.5) (Virmani *et al.*, 2006b).

1.2 Thrombogenicity in T2DM and CAD

1.2.1 Blood haemostasis and thrombus formation

Thrombus formation is the most effective protective mechanism by which haemostasis is maintained in a closed, high-pressure circulatory system after vascular damage. Haemostasis is Nature's first and often the most efficient response to control bleeding. This process must remain 'dynamically' inactive but should be activated immediately to minimise extravasation of blood from the vasculature following tissue injury. After thrombogenesis is activated, it is critical to contain thrombus formation so that it is localised to the site of injury and thrombus size is appropriate to the injury (Figure 1.6). Thus, there exists a very fine balance between the pathways that initiate thrombus formation and regulate or modulate thrombus formation (Furie, 2009).

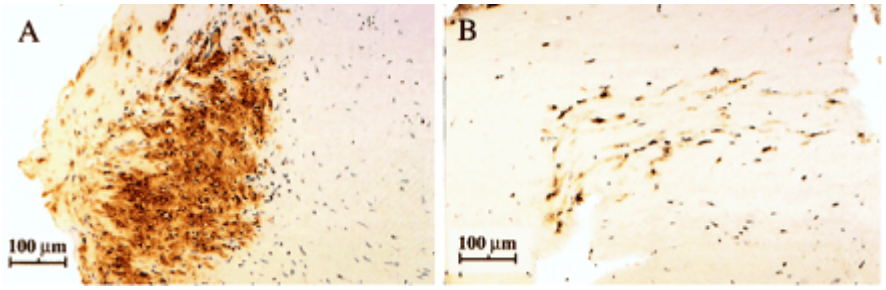


Figure 1.3 Macrophage content in coronary atheroma of type 2 diabetes mellitus.

Increased percent macrophage area in coronary tissue from patients with diabetes mellitus (left) versus tissue from patients without diabetes (right). Photomicrographs of coronary atherectomy tissue were immunostained with antihuman pan-macrophage antibody. Greater macrophage content is seen in coronary tissue from a patient with diabetes mellitus (A) than in coronary tissue from a patient without diabetes (B). From Moreno PR, et al. Circulation 2000; 102: 2180–4.

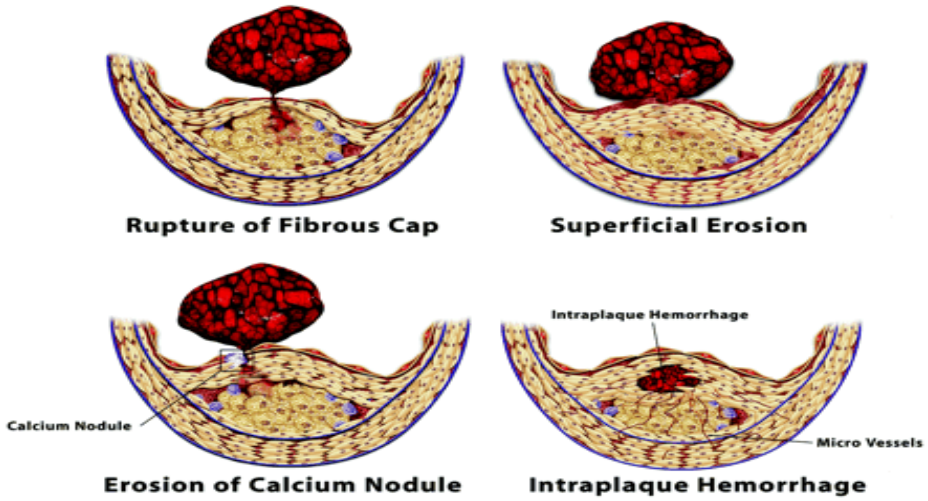


Figure 1.4 Plaque rupture and thrombosis in type 2 diabetes mellitus.

In type 2 diabetes mellitus, rupture of fibrous cap (upper left) causes majority of fatal coronary thrombosis. Superficial erosion (upper right) occurs in one fifth to one quarter of all cases of fatal coronary thrombosis. Diabetic patients are more susceptible to superficial erosion as the mechanism of plaque disruption and thrombosis. Erosion of a calcium nodule may also cause plaque disruption and thrombosis (lower left). In addition, friable micro vessels in the base of atherosclerotic plaque may rupture and cause intraplaque haemorrhage. Consequent local generation of thrombin may stimulate smooth muscle cell proliferation, migration, and collagen synthesis, promoting fibrosis and plaque expansion on a subacute basis. Severe intraplaque haemorrhage can also cause sudden lesion expansion by mass effect acutely. Modified from Libby et al Circulation. 2005;111:3481-3488.

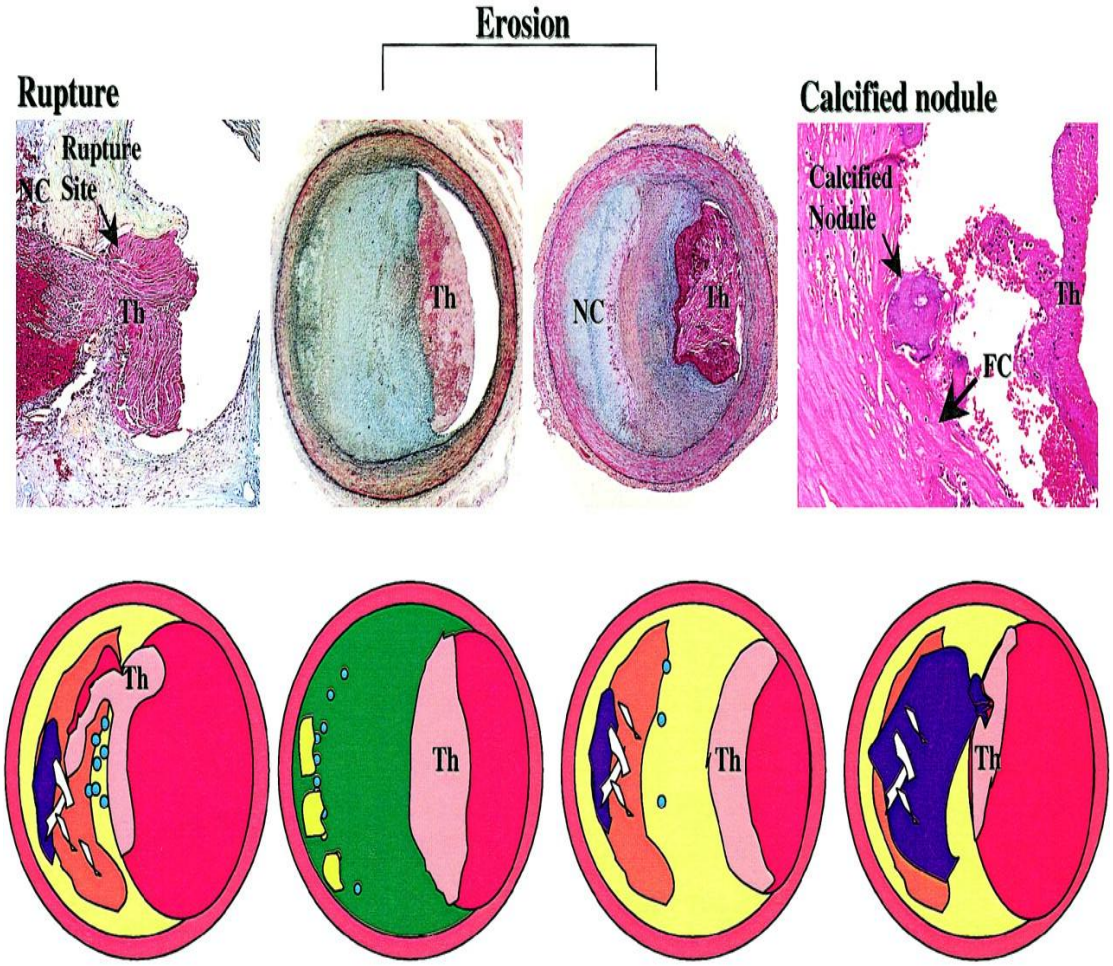


Figure 1.5 Atherosclerotic lesions with luminal thrombi.

Ruptured plaques are shown as thin fibrous cap atheroma lesions with luminal thrombi (Th). These lesions usually have an extensive necrotic core (NC) containing large numbers of cholesterol crystals and a thin fibrous cap (< 65 μm) infiltrated by foamy macrophages and a paucity of T lymphocytes. The fibrous cap is thinnest at the site of rupture and consists of a few collagen bundles and rare smooth muscle cells. The luminal thrombus is in communication with the lipid-rich necrotic core. Erosions occur over lesions rich in smooth muscle cells and proteoglycans. Luminal thrombi overlie areas lacking surface endothelium. The deep intima of the eroded plaque often shows extracellular lipid pools, but necrotic cores are uncommon; when present, the necrotic core does not communicate with the luminal thrombus. Inflammatory infiltrate is usually absent, but if present, is sparse and consists of macrophages and lymphocytes. Calcified nodules are plaques with luminal thrombi showing calcific nodules protruding into the lumen through a disrupted thin fibrous cap (FC). There is absence of an endothelium at the site of the thrombus, and inflammatory cells (macrophages, T lymphocytes) are absent. From Virmani R et al, Arterioscler Thromb Vasc Biol, 2000; 20: 1262 – 1275.

1.2.2 Mechanisms of thrombosis

At sites of vascular injury, platelets rapidly adhere to the exposed subendothelial extracellular matrix, become activated and, together with the coagulation system, form a thrombotic plug that seals the lesion (Furie and Furie, 2008). A highly specialised and efficient haemostatic system consisting of cellular (platelets) and non cellular (coagulation factors) components is responsible for three main components of thrombosis, namely:

i. Initiation of thrombosis
ii. Propagation of thrombus
iii. Autolysis of thrombus

1.2.2.1 Initiation of thrombosis

Endothelial cells play a crucial role in the delicate balance between activation and inhibition of the haemostatic system. Under normal circumstances, endothelial cells produce and release the two powerful soluble inhibitors of platelet activation, nitric oxide and prostacyclin, and express high levels of CD39. The major platelet feedback agonists such as ADP are rapidly metabolised (Davi and Patrono, 2007). Anatomically and functionally intact endothelium is key to deter thrombogenesis. Loss of endothelial cells due to injury or plaque rupture exposes the subendothelial extracellular matrix, rich in collagen, to flowing blood. This activates, in the presence of tissue factor, powerful cell signalling molecules that result in platelet adhesion and activation. Circulating independent platelets are subjected to a phenomenon called ‘rolling’ to the exposed endothelium which results in platelet adhesion. Platelet adhesion to the subendothelial structure results in a ‘platelet plug’ that forms the hallmark of the events resulting in initiation of thrombosis. Exposed collagen in the subendothelial region, von Willebrand Factor (vWF) and glycoprotein Ib (GpIb) play a key role in the initiation of thrombus formation (Jennings, 2009).

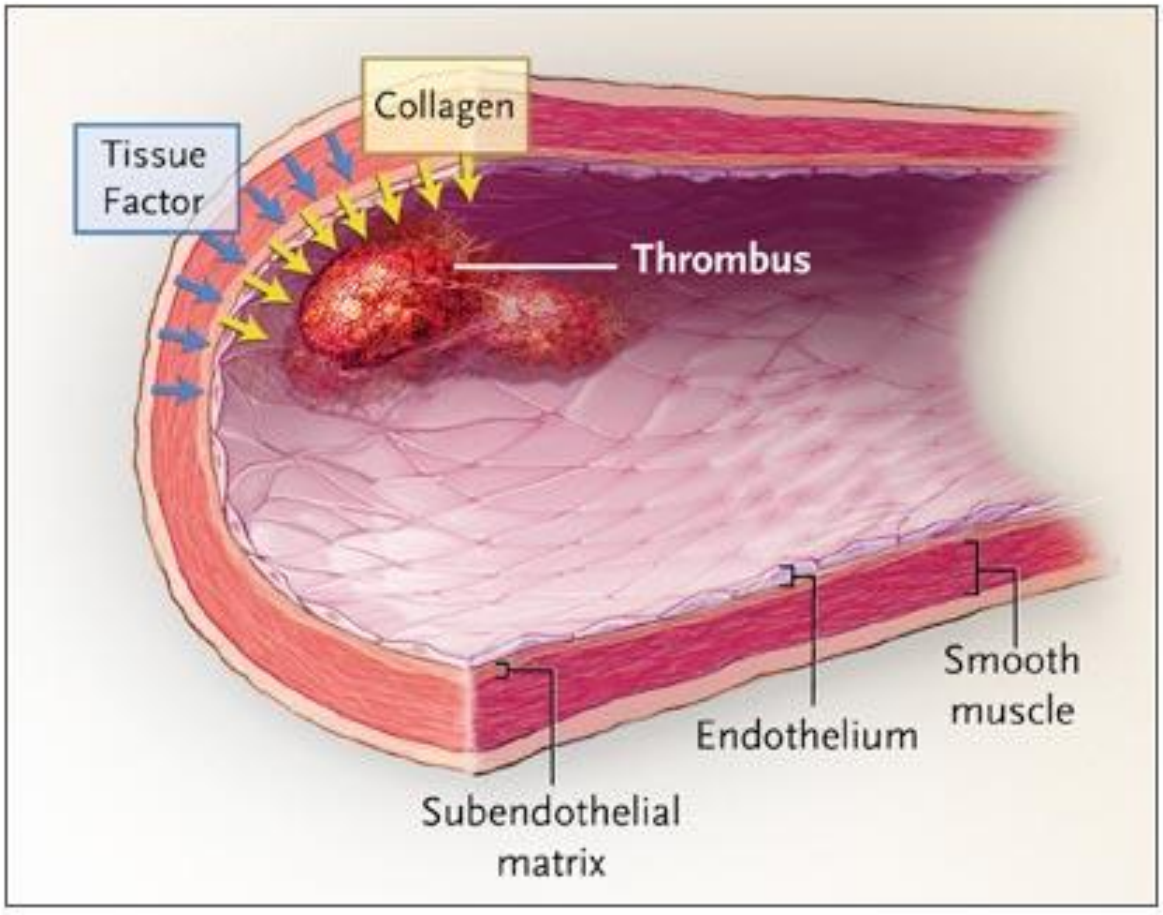


Figure 1.6 Thrombus formation in the vessel wall.

Collagen and tissue factor associated with the vessel wall provide a haemostatic barrier to maintain the high-pressure circulatory system. Collagen (yellow arrows), located in the subendothelial matrix beneath the endothelium, is not exposed to flowing blood under normal conditions. Tissue factor (blue arrows), located in the medial (smooth muscle) and adventitial layers of the vessel wall, comes in contact with flowing blood when the vessel is disrupted or punctured. Both collagen and thrombin initiate thrombus formation. Collagen is a first line of defense, and tissue factor is a second line of defense. From Furie et al. N Engl J Med 2008;359:938-49.

Platelets contain a surface bound molecule known as glycoprotein VI. This interacts with collagen of the exposed vessel wall, vWF and GpIb, resulting in adhesion of platelets to the vessel wall injury site. In addition to its role in the adherence of platelets to collagen, glycoprotein VI is the major agonist for initial platelet activation and granule release. The platelet integrin $\alpha_2\beta_1$ plays a supportive but not an essential role in the interaction between platelets and collagen (Jurk and Kehrel, 2005). There exists an equally powerful, parallel pathway involving tissue factor from the vessel wall and or flowing blood which generates thrombin, and results in activation and aggregation of platelets (Mackman, 2006; Meerarani *et al.*, 2006). Tissue factor triggers a second independent pathway that initiates platelet activation. Tissue factor forms a complex with coagulation factor VIIa, the enzymatically active form of factor VII, and this tissue factor–factor VIIa complex activates coagulation factor IX, thereby initiating an enzymatic and proteolytic cascade that generates thrombin (Steffel *et al.*, 2006). Thrombin cleaves protease-activated receptor 1 (PAR 1) on the platelet surface and eventually results in activation and aggregation of platelets. Thrombin also promotes platelet adhesion to the endothelial surface, via an unknown mechanism (Andrews and Berndt, 2004).

1.2.2.2 Progression of thrombus formation

Further development of thrombus is mainly mediated by platelets and fibrin. There is a continuous activation of platelets influenced by the shear force of flowing blood and coagulation factors. Platelet activation is positively reinforced by the release of granules and microparticles from already activated platelets. The platelet integrin $\alpha_{2b}\beta_{3a}$, when activated, mediates recruitment of platelets to the thrombus as well as platelet–platelet interactions. Activation of platelets bound to the wall of the injured vessel causes a conformational transition in $\alpha_{2b}\beta_{3a}$ integrin of the platelet cell membrane that increases its affinity for fibrinogen and von Willebrand factor (Lefkovits *et al.*, 1995). At low shear rates, fibrinogen is the predominant ligand promoting thrombosis, whereas von Willebrand factor plays an important role at higher shear rates. Platelet activation releases the contents of platelet alpha granules and dense granules, each of

which is critical for thrombus formation (Nesbitt *et al.*, 2009). Proteins in various subpopulations are released from alpha granules, whereas ADP and calcium ions are released from the dense granules. The release of ADP stimulates platelet activation through two ADP receptors, P2Y1 and P2Y12. The role of these receptors in platelet function and the pharmacology of drugs directed against these receptors are described in detail *vide infra*.

Following adhesion, rapid signal transduction leads to platelet activation and structural changes including cytoskeletal changes associated with shape change, spreading and pseudopodia formation and functional changes such as secretion of thrombogenic substances. There is an inside–out activation of integrins that support adhesion and aggregation. Activated platelet aggregates thus accelerate the coagulation cascade, leading to stabilization of the clot by fibrin and $\alpha_{2b}\beta_3$-dependent platelet surface contraction. Activated platelets also interact with circulating leukocytes and facilitate platelet–leukocyte–endothelial cell adhesion (Libby, 2009). In addition, platelets promote the interaction of inflammatory leukocytes with the vessel wall, thus accelerating the formation of atherosclerotic plaques (vide infra). The role of platelets in thrombus formation and leukocyte adhesion is illustrated in figure 1.7.

The next important step in propagation of thrombus is its stabilisation by interlinkage of platelets and leucocytes with fibrin polymers. Fibrinogen in its soluble form is converted to insoluble fibrin by thrombin and other coagulation factors. The insoluble fibrin provides the ultraskeletal framework for the thrombus. Fibrinogen binds to activated $\alpha_{2b}\beta_3$ integrin on the platelet surface, forming bridges responsible for platelet aggregation in haemostasis, and also has important adhesive and inflammatory functions through specific interactions with other cells (Weisel *et al.*, 1983). The significant mechanical strength or viscoelastic property to thrombus is derived from fibrin to fibrin cross linkage and overlapping of longitudinal fibrin fibres. By forming a mesh-like structure due to cross linking of the individual fibres, cellular elements in blood are trapped in the thrombus, resulting in cellular thrombus (Collet *et al.*, 2003a; Weisel, 2005).

Circulating monocytes are attracted towards the organising thrombus. The adhesion of leukocytes to platelets is mediated by inflammatory cytokines in blood, microparticles shed from activated platelets and high levels of P-selectin ligands expressed on the surface of activated platelets. Monocytes release tissue factor which augments the coagulation response (Zarbock *et al.*, 2007). Platelet monocyte interaction in thrombus is described in detail elsewhere.

1.2.2.3Autolysis of thrombus

Autolysis of the formed thrombus is Nature's mechanism by which the balance in haemostasis is restored by removal of excessive clot formation. This consists of an early but weaker thrombus retraction mediated by platelets to be followed by a stronger process of fibrinolysis mediated by fibrinolytic enzymes. Thrombus retraction is the earliest step in regulation of excessive thrombus formation and organisation of the thrombus. This step consists of rearrangement of internal actin cytoskeletons in the platelet cell wall and results in platelet contraction. Shape changes in platelets during this phase are combined with rearrangement of fibrin to platelet and fibrin to fibrin cross linkages. Exact stimuli for initiation of this process are still unknown (Carroll *et al.*, 1981).

Fibrinolysis follows thrombus retraction immediately. The fibrinolytic system consists of plasminogen activators which convert plasminogen to plasmin. Plasmin converts insoluble fibrin to soluble fibrin degradation products and thereby results in thrombolysis. Plasmin activity is mediated by two kinds of powerful plasminogen activators, namely tissue plasminogen activator, produced in the endothelium, and urokinase-type plasminogen activator produced in the liver (Cesarman-Maus and Hajjar, 2005). Tissue plasminogen activator mediates intravascular fibrinolysis and urokinase plasminogen activator mediates extravascular proteolysis (Medcalf, 2007).

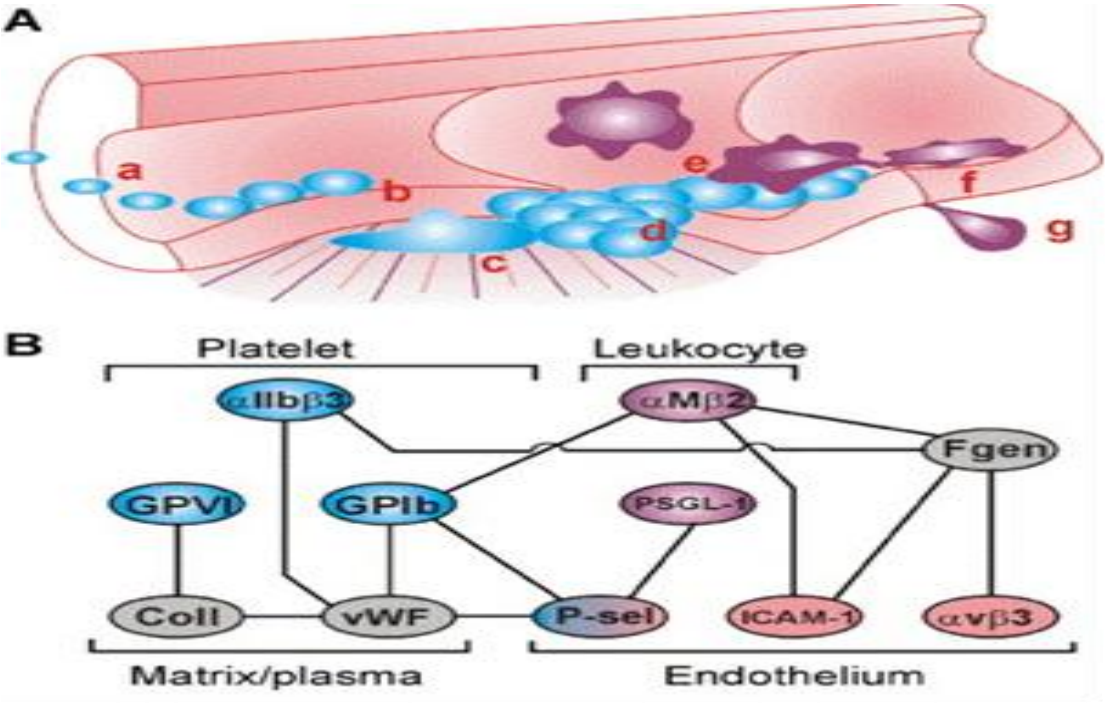


Figure 1.7 Vascular cell interactions and adhesion receptors.

(A) Vascular cell interactions: circulating platelets (a) interact with activated endothelial cells (b) or subendothelial matrix (c) to form mural thrombi (d) providing a substrate for adhesion of leukocytes (e) which also adhere to activated endothelium (f) prior to extravasation through the vessel wall (g).

(B) Vascular cell adhesion receptors: adhesive interactions in the vasculature [(A), a–g] are mediated by specific receptors on platelets, leukocytes, and/or endothelial cells, and their ligands in plasma or subendothelial matrix, such as fibrinogen, von Willebrand factor, or collagen. The interaction of platelet GPIbα (the major ligand-binding subunit of GPIb-IX–V) or GPVI with von Willebrand factor or collagen, respectively, initiates activation of the integrin and αIIbβ3. This will then bind to von Willebrand factor or fibrinogen and mediate platelet aggregation. GPIbα can also mediate platelet–endothelial cell adhesion by binding to P-selectin, or P-selectin-bound von Willebrand factor. GPIbα can mediate platelet–leukocyte adhesion by binding to the leukocyte integrin, αMβ2 (Mac-1). The leukocyte receptors PSGL-1 and αMβ2 are involved in leukocyte adhesion to endothelium by binding P-selectin or ICAM-1, respectively, on endothelial cells, or by binding to P-selectin or GPIbα, respectively, on adhered and activated platelets. This network of receptor–counter receptor or ligand interactions provides intricate regulation of platelet–leukocyte–endothelial cell cross-talk. Andrews RK, Berndt MC. Thromb Res. 2004; 114(5-6): 447-53.

Plasminogen activator inhibitor-1 (PAI-1), a serine protease enzyme produced by the liver and endothelial cells, binds to the active site of both tissue plasminogen activator and urokinase plasminogen activator and neutralizes their activity. There is a dynamic equilibrium between tissue plasminogen activator and PAI-1 on the luminal surfaces of vessels that determines net local fibrinolytic activity. Either increased expression of PAI-1, or decreased expression of tissue plasminogen activator, or both can lead to decreased fibrinolytic activity and predispose to thrombosis (Vaughan, 2005).

Macrophages which are often seen in formed thrombus are involved in its resolution. Platelets and other cellular elements are resorbed by macrophages once the fibrinolytic process is completed. However, some of these cellular elements engulfed by macrophages undergo calcification and result in giant cell formation. Calcification and a layering effect are seen in human coronary arteries and are the hallmark of old and recurrent thrombus and are mediated by macrophages (Fuster *et al.*, 2005; Medcalf, 2007).

1.2.3 Platelets in thrombogenesis

Platelets are derived from megakaryocytes in the bone marrow and are anucleate and discoid cells which circulate in blood for 7-10 days. Platelets contribute to haemostasis by activation, adhesion and aggregation. Platelets exhibit extreme flexibility and change of shape (Jennings, 2009). They are activated by vessel wall damage and agonists such as arachidonic acid, collagen, adenosine diphosphate (ADP), fibrinogen and epinephrine via trans-membrane receptors (outside-in activation). This leads to intraplatelet events mediated by substances such as adenyl cyclase and calcium, resulting in conformational changes to integrins such as alpha IIb/beta3 (glycoprotein IIb/IIIa receptor). The integrins are translocated to the outer membrane from inside the platelets and they form high affinity sites for molecules such as fibrinogen (inside-out activation) (Lefkovits *et al.*, 1995). This results in binding of fibrinogen and leads on to platelet to platelet interaction. Activated platelets are identified by up regulation of cell surface markers such as p-selectin, CD63 and CD40 ligands. Platelets also bind to

leucocytes via integrin type membrane receptors (Biondi-Zoccai *et al.*, 2003; Antoniades *et al.*, 2009; Ferroni *et al.*, 2009).

Upon activation, platelets respond with:

i. change of shape from a discoid to a pseudopodial structure
ii. aggregation with other platelets and other blood-derived cells such as monocytes
iii. release of substances from secretory granules (α-granules), dense bodies, and lysosomes
iv. liberation of arachidonic acid, which is rapidly converted to prostaglandins and lipoxygenase (Jennings, 2009)

The end result of platelet activation is conversion of soluble fibrinogen into insoluble fibrin and also activation and recruitment of other platelets. Platelets, along with other cells such as erythrocytes and monocytes, are trapped in the fibrin mesh formed by fibrin polymerisation. This results in growth and stabilisation of thrombus.

1.2.4 Platelet procoagulant activity

Once activated, platelets promote thrombogenesis further by interaction with other coagulant factors and activation of other resting platelets by paracrine and endocrine mechanisms. This eventually will lead on to exponential amplification of coagulation cascade (Ilveskero *et al.*, 2001). This inherent property is known as platelet procoagulant activity (PPA) and is shown to play a key role in thrombogenicity.

Platelet activation often begins upon attachment of platelets to the damaged endothelium and exposed collagen. This process is mediated by vWF and glycoprotein Ib (vide supra). This step initiates PPA which comprises of changes in platelet membrane architecture, intra cytoplasmic enzyme activation and release of powerful procoagulants from the platelets. Interaction between circulating coagulation factors and platelets sustain this platelet procoagulant state.

Activation of factor X and formation of thrombin constitute the most powerful step in coagulation cascade. This process requires a catalytic surface on which the enzyme complexes can be assembled. This catalytic surface is provided by the phospholipids of the activated platelet plasma membrane, which are turned inside out and exposed to flowing blood and collagen. This results in the loss of normal asymmetric distribution of phospholipids on the inner surface of the platelet and formation of a procoagulant surface (Bevers *et al.*, 1991). Activated platelets further accelerate coagulation cascade by releasing coagulation factor V and thrombin and ultimately results in insoluble fibrin. These exert a positive effect on the resting platelets and thus PPA is magnified in whole blood. This prothrombotic tendency of the activated platelets is kept in balance by the inherent inactivation of coagulation factors V and VIII via an activated protein C dependent pathway (Solum, 1999).

The exact mechanism of PPA remains uncertain. Mechanical factors play a significant role in PPA. Firstly, shape change occurs upon activation of the platelets as described vide supra. This would expose the circulating coagulation factors to the pseudopodia and membrane channels of the platelets. This is accompanied by changes in the membrane receptors and ligands like $\alpha_2\beta_3$ in activated platelets (Swords *et al.*, 1993). Platelet activation also causes mobilisation of intracellular fibrinogen-glycoprotein IIb/IIIa complexes to the platelet surface in a secretion-dependent manner. This would facilitate binding of fibrinogen and vWF (Sims *et al.*, 1991) and thereby lead on to platelet to platelet and platelet to fibrin binding. In addition, the membrane phospholipids contributes to the formation of microparticles whose significance is described elsewhere (Chapter 1.3.8, page 47).

PPA can result from biophysical or biochemical mechanisms. Physical shear force can increase platelet procoagulant activity. In the presence of high shear condition, platelet activation was still demonstrated after blocking vWF and GpIb receptors suggesting direct effect of shear stress on the procoagulant activity. High shear force has been shown to increase the release of ADP and accumulation of cytosolic calcium in activated platelets. This will sustain further procoagulant activity and thus will enhance thrombogenesis (Goto *et al.*, 2003;

Chen *et al.*, 2010). Thrombin is a powerful substance released by platelets upon activation. Thrombin sustains platelet activation by augmenting the release of procoagulant factors like calcium and ADP. In addition, upon activation platelets contribute to coagulation activity by releasing several compounds, factor V, factor XIII, fibrinogen, vWF, calcium ions, and ADP from their alpha granules and dense granules (vide supra). These compounds in turn activate the resting platelets and thereby sustain and amplify procoagulant tendency of the platelets (Jennings, 2009).

Every aspect of PPA is enhanced in patients with T2DM. Excess production of cytosolic factors like ADP, thrombin, calcium, thromboxane and fibrinogen were noted in diabetic platelets. These prothrombotic substances are released even at sub threshold stimuli in T2DM. In addition, the plasma membrane changes are more pronounced in diabetic platelets as evidenced by higher levels of microparticles and $\alpha_2\beta_3$ integrins on the platelet surface compared to non diabetics. These changes were attributed to the reduction in the antiplatelet effects of aspirin and clopidogrel in T2DM (Davi *et al.*, 1990; Carr, 2001; Angiolillo, 2009).

1.2.5 Coagulation factors

Historically, the coagulation cascade is divided into the extrinsic (tissue factor mediated) pathway and the intrinsic (factor XII, XI initiated) pathway (Davie *et al.*, 1991; Gailani and Renne, 2007; Meerarani *et al.*, 2007). Both these pathways stimulate a final common pathway which mediates the conversion of prothrombin to thrombin and ultimately results in production of insoluble fibrin and thrombus (Figure 1.8). The coagulation cascade is tightly regulated by a series of inhibitors. Tissue factor pathway inhibitor (TFPI) inhibits coagulation by binding with activated factor X, thereby inhibiting the tissue factor- activated factor VII complex. Activated protein C and protein S inactivate factor V and factor VIII whereas antithrombin III inhibits thrombin (Girard *et al.*, 1990; Esmon, 1992).

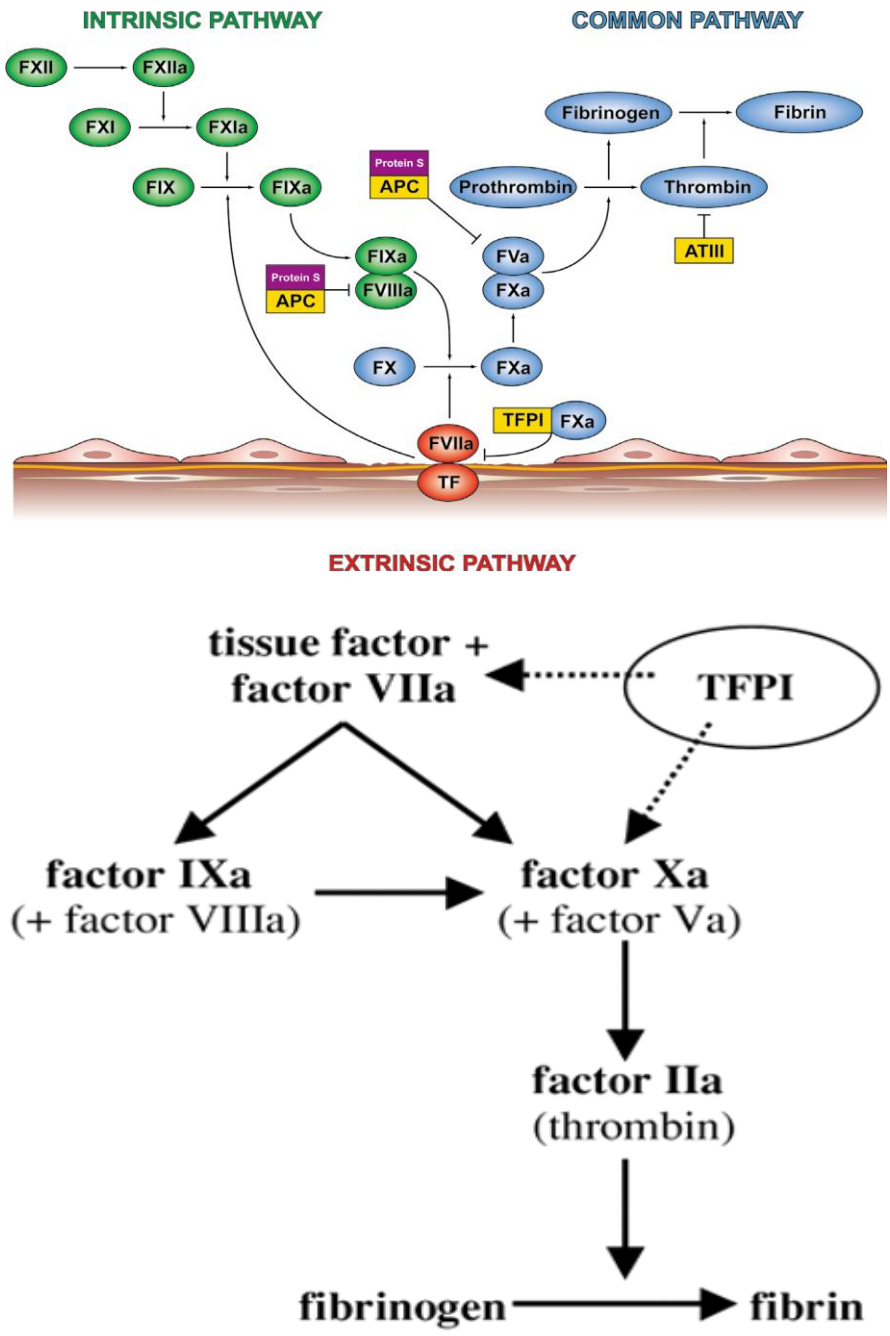


Figure 1.8 Coagulation cascade.

F represents factor, “a” represents activated, APC- activated protein C, TFPI tissue factor pathway inhibitor, ATIII anti-thrombin III, TF tissue factor. Modified from Breitenstein A, et al Circ Journal 2011; 74:3-12.

Tissue factor (TF) is a key trans-membrane protein involved in the initiation of the coagulation pathway. The name tissue factor is a misnomer as it is derived from both vascular tissue sources such as endothelium, vascular smooth muscle cells, and fibroblasts (Day *et al.*, 2005) and blood borne cells including platelets and monocytes (Giesen *et al.*, 1999; Steffel *et al.*, 2006). TF is a 263-residue membrane-bound glycoprotein which is not normally present in or exposed to the circulation. The location of TF on plasma membranes of endothelium and smooth muscle cells enables the process of coagulation to remain fixed at the site of vessel wall injury. The exposure of cell surface TF to plasma leads to the binding of factor VII to TF. The complex TF-factor-VII is activated by free factor VIIa and or pre-existing TF-factor VIIa complexes. The TF-factor VIIa complex rapidly activates factor X and, at a lower rate, factor IX. Activated factor IX, in the presence of its co-factor VIII, cleaves further factor X to activated factor Xa. Eventually, these processes culminate in the generation of thrombin, which converts fibrinogen to fibrin (Butenas *et al.*, 2009).

Tissue factor pathway inhibitor (TFPI) is a serine protease formed in endothelial cells which circulates in inactive form. Upon activation of the coagulation cascade, TFPI is activated and inhibits conversion of factor X into activated factor X (Xa) of the coagulation cascade by binding to tissue factor-factor VII complex. It also inhibits the conversion factor IX (Broze *et al.*, 1993; Lu *et al.*, 2004). Levels of TFPI correlate to the levels of tissue factor in patients with dyslipidaemia (Hansen *et al.*, 2001) and acute coronary syndrome (Falciani *et al.*, 1998; Hansen *et al.*, 2001; Gori *et al.*, 2002). A rise in TFPI is a marker of hyperactivation of the coagulation system (Lwaleed and Bass, 2006) and offers some but not full protection against future thrombotic events (Viles-Gonzalez and Badimon, 2004). For example, high levels of tissue factor seen in T2DM are insufficient to protect against future thrombotic events (Leurs *et al.*, 1997; Badimon *et al.*, 1999).

von Willebrand factor (vWF) is synthesized and secreted by endothelial cells and megakaryocytes. vWF promotes platelet adhesion to the vascular subendothelium which is exposed following plaque rupture mediated by platelet GPIb receptor. Elevated levels of vWF are an indication of endothelial cell

damage, and correlate with cardiovascular risk factors including T2DM and dyslipidaemia (Kessler *et al.*, 1998). vWF is the carrier of procoagulant factor VIII in circulating blood and plays a crucial role at high shear states.

vWF plays a crucial part in thrombosis by the following mechanisms:

i. Binding of vWF to components of the sub endothelium (various forms of fibrillar collagen, non-fibrillar collagen, heparin and sulfatide)
ii. Conformational change of vWF, an essential step in its interaction with platelet GPIb receptor
iii. Initial platelet adhesion through binding of fibrinogen and vWF to platelet GPIb
iv. Transduction of an intraplatelet signal triggering the expression of GPIIb/IIIa at the platelet membrane surface
v. Binding of vWF to GPIIb/IIIa, leading to the spreading of platelets and their irreversible adhesion and aggregation (Kessler *et al.*, 1998; Reininger *et al.*, 2006)

Fibrinogen is a polypeptide synthesised by liver which influences thrombogenesis, rheology of blood flow, blood viscosity and platelet aggregation. Fibrinogen is an acute phase protein and a marker of systemic inflammation. Fibrinogen is present in blood in the soluble state at a concentration of 2.0 to 5.0 g/dl. Each end of a fibrinogen molecule can bind with high affinity to the $\alpha_{2b}\beta_3$ integrin receptor on activated platelets, and thus act as a bridge to link platelets. The fibrinogen molecule is made up of alpha, beta and gamma peptide chains, figure 1.9 (Weisel, 2004).

Serum fibrinogen concentration is a well known independent risk factor for coronary artery disease and is frequently used as a surrogate marker for cardiovascular risk. The relative risk for coronary artery disease in individuals in the top compared to the bottom tertile of plasma fibrinogen is 1.8. In a longitudinal observational study over 13 years, elevated plasma fibrinogen was associated with higher rates of sub clinical cardiovascular disease (Danesh *et al.*, 1998).

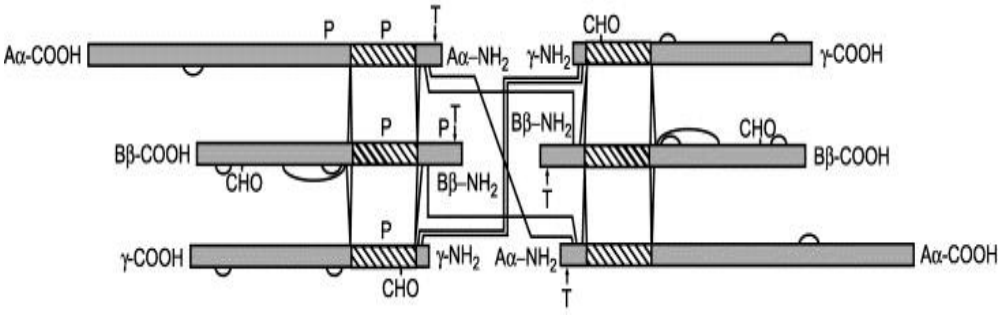


Figure 1.9 Schematic diagram of the three pairs of polypeptide chains of fibrinogen.

The Aα, Bβ, and γ chains are represented by bars with lengths proportional to the number of amino acid residues in each chain and the N- and C-terminal ends of the chains are labelled. The coiled-coil regions are indicated by the diagonally striped boxes, while the intra- and interchain disulfide bonds are indicated by solid lines. Carbohydrate attachment sites are labelled with CHO, while thrombin and major plasmin cleavage sites are indicated by T and P, respectively. From Weisel, J Thromb Haemost, 2007;5,116-124.

Fibrin is formed by polymerisation of fibrinopeptides. Soluble fibrin is converted to insoluble fibrin by thrombin. The fibrin monomers are formed from a molecule of fibrinogen in the form of fibrinopeptide A and B which subsequently bind to themselves after the exposure of the binding sites within these monomers in the presence of factor XIII (Figure 1.10). Staggered arrangement of fibrin monomers in this fashion result in protofibrils. Once protofibrils reach sufficient length (600-800 nm), they begin to elongate laterally by binding to their adjacent fibril units to form mesh like fibres (Weisel, 1986; Weisel *et al.*, 1987; Ryan *et al.*, 1999). The intermolecular interactions that occur in lateral aggregation are specific, so that the fibres have a repeat of 22.5 µ, or about half their molecular length (Cohen *et al.*, 1983). The molecular structure and packing in fibrin revealed that fibres are par crystalline structures with the molecules precisely aligned in the longitudinal direction, but only partly ordered in the lateral direction. The length and lateral growth of fibrin is limited by the twisting of individual fibres, which reduces the availability of binding sites. Increased thickness of fibrin strands in this way limits the growth of fibre, as the energy to stretch these twisted fibres to expose additional binding sites exceeds the energy stored in the unbound individual fibrin monomers (Wolberg, 2007).

Fibrin is a viscoelastic polymer, characterised by stiffness (representing its elastic properties) and compliance (representing its inelastic properties). Viscoelasticity determines the response of the clot to the shear force of flowing blood. A clot with a greater inelastic component will deform permanently with stress, while one with a greater elastic component will return to its original shape. Detailed physical principles behind clot viscoelasticity are still largely unknown (Weisel, 2007).

1.2.6 Platelet monocyte interaction and thrombosis

Platelet monocyte interaction forms a crucial link between thrombosis and inflammation and is mediated by P-selectin, a membrane glycoprotein contained within platelet alpha granules and Weibel-Palade bodies of endothelial cells.

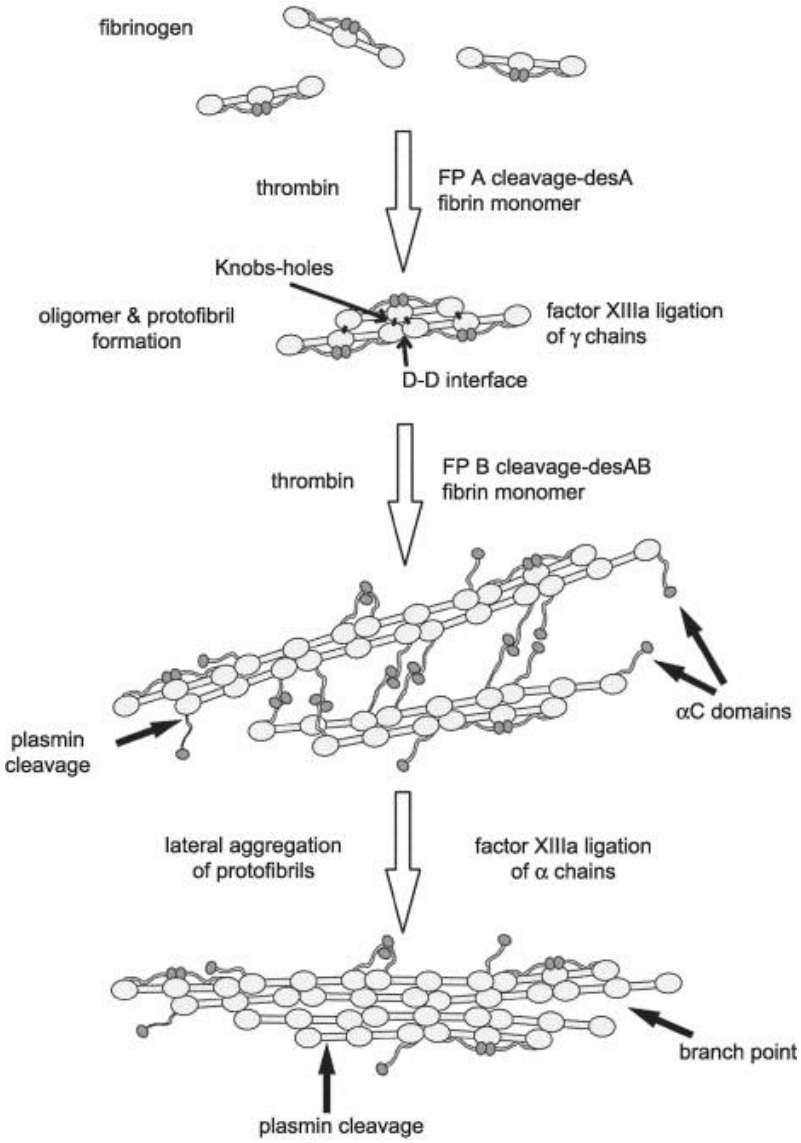


Figure 1.10 Schematic diagram of fibrin polymerisation.

Fibrinopeptide A is cleaved from fibrinogen, producing desA fibrin monomers, which aggregate via knob-hole interactions to make oligomers. Fibrinopeptide B is cleaved primarily from polymeric structures. The oligomers elongate to yield protofibrils, which aggregate laterally to make fibres, a process enhanced by interactions of the αC domains. Factor XIIIa crosslinks or ligates γ chains more rapidly than α chains. Plasmin cleaves the αC domains and Bβ1-42 and then cuts across the fibrin in the middle of the coiled coil. At the bottom of the diagram, a branch point has been initiated by the divergence of two protofibrils. From Weisel, J Thromb Haemost, 2007;5,116-124.

P selectin is rapidly translocated to the plasma membrane following cell activation. P-selectin interacts with its natural ligand, P-selectin glycoprotein ligand-1 (PSGL-1), present on neutrophils and monocytes, thus providing an anchoring source for inflammatory cells on activated platelets and endothelial cells (McEver *et al.*, 1995). Neutrophil rolling on platelets is mostly mediated by platelet P-selectin binding to P-selectin glycoprotein ligand (PSGL)−1 on leukocytes. Blocking one of these molecules with a monoclonal antibody completely inhibits neutrophil rolling on platelets. Firm adhesion of leukocytes to platelets is facilitated by molecules such as CD11b/CD18 and CD11a/CD18 (Esmon, 2005). Platelets also activate monocytes via cytokines such as IL-6. Activated platelets increase monocyte binding to inflamed endothelium, and thus indirectly accelerate progression of atheromatous plaque. Monocytes also secrete tissue factor, and thus enhance thrombogenicity (Pamukcu *et al.*, 2011). Antiplatelet agents including clopidogrel suppress platelet monocyte interaction (Zarbock *et al.*, 2007).

1.2.7 Blood thrombogenicity in T2DM

Higher cardiovascular mortality has been attributed to higher thrombogenicity in patients with T2DM (Osende *et al.*, 2001; Berry *et al.*, 2007). An inherent prothrombotic state has been established by invitro studies in patients with T2DM. Blood thrombogenicity in patients with T2DM results from a complex interplay of coagulation factors, hyperactive platelets and impaired fibrinolysis. Antiplatelet therapy reduces this thrombogenicity in those with T2DM less than in those without diabetes (Natarajan *et al.*, 2008a). Hyporesponse to the currently available antiplatelet agents may be responsible for excess mortality seen in patients with T2DM (Angiolillo, 2009).

1.2.7.1 Hyperactive platelets in T2DM

Platelet function is significantly altered, favouring a thrombotic tendency in patients with T2DM (Vinik *et al.*, 2001; Natarajan *et al.*, 2008b; Ferroni *et al.*, 2009). Platelets of patients with T2DM have been referred to as ‘angry platelets’ (Bhatt, 2008) as they constantly remain in a state of hyperactivation. Flow

cytometry studies have revealed that a subpopulation of large, hyperactive platelets circulates in patients with T2DM, at a level similar to that seen in patients who have had an MI but do not have T2DM. This suggests that platelets in T2DM respond to sub threshold stimuli and have high potential for aggregation (Trovati and Anfossi, 2002).

Platelet hyperactivity could itself be a marker of diabetes mellitus. Animal studies showed enhanced platelet aggregation within a week after onset of diabetes (Stratmann and Tschoepe, 2009). Platelet dysfunction in T2DM is due to a combination of innate platelet abnormalities such as enhanced aggregation to stimulus, excess platelet agonists such as arachidonic acid and reduced platelet inhibitory substances like prostaglandin I_2 (PGI2) (Sobel and Schneider, 2004). Platelets in diabetic subjects have higher surface expression of the glycoprotein IIb IIIa receptor in the resting state (Tschoepe *et al.*, 1990).

Persistent hyperglycaemia enhances platelet activation. Activated platelets require glucose to supply energy rich adenosine triphosphate (ATP) for regulation of transmembrane function. Glucose entry into the platelet occurs by a gradient driven process due to translocation of glucose transporter protein 3 (GLUT-3) from alpha granules inside the platelets. This process of translocation is enhanced by thrombin (Ferreira *et al.*, 2005). Unlike most human cells, glucose entry into platelets is not directly facilitated by insulin. New evidence suggests that insulin promotes glucose entry into platelets indirectly by activation of protein kinase B (PK-B) in normoglycaemic states (Randriamboavonjy and Fleming, 2009). Hyperglycaemia results in an increase in the intraplatelet concentration of the platelet agonist thromboxane (Davi *et al.*, 1999) and short bursts of hyperglycaemia have been associated with platelet hyperactivity (Assert *et al.*, 2001; Osende *et al.*, 2001). Platelets of patients with T2DM respond to hyperglycaemia by translocation of membrane receptors such as protein kinase C (PKC) and increase in intracellular calcium, which mediate the enhanced activity to platelet agonists (Assert *et al.*, 2001). Hyperglycaemia also increases oxidation of LDL, which facilitates calcium entry into platelets.

This cytosolic hypercalcaemic state is responsible for enhanced adhesion and aggregation of platelets, reduction of intracellular nitrate production and changes in anti-aggregatory properties of the platelet membrane (Ferretti *et al.*, 2002). Risk factors such as obesity and hypertension independently augment platelet hyperactivity irrespective of glycaemic status (Stratmann and Tschoepe, 2009). Platelet leucocyte interaction is responsible for propagation and stabilisation of thrombus. T2DM is associated with higher platelet monocyte linkage and is mediated by P-selectin and cytokines (Elalamy *et al.*, 2008). Leukocyte-platelet heterotypic aggregates (involving mainly monocytes) produce procoagulant, oxidative, and mitogenic substances which potentially play a role in capillary micro embolism and arterial thrombosis (Kaplar *et al.*, 2001).

Platelet synthesis is also hyperactive in patients with T2DM. As diabetic platelets respond more frequently to sub-threshold stimuli, they soon become exhausted, consumed and finally incorporated into thrombus. This relatively excess consumption of platelets results in accelerated thrombopoiesis in the bone marrow mediated by thrombopoietin and release of 'fresh' hyper-reactive platelets. The presence of 'young' active platelets in T2DM reduces the response to antiplatelet agents and augments the response to naturally occurring platelet agonists (Watala *et al.*, 1999; Watala *et al.*, 2005). These platelets also exhibit altered membrane fluidity which promotes a vicious cycle of hyperactivity and sustains the prothrombotic state in T2DM (Papanas *et al.*, 2004). Platelet structural and functional alterations seen in T2DM are summarised in Table 1.1.

1.2.7.2 Coagulation factor activity in T2DM

Higher thrombogenicity in T2DM is facilitated by coagulant factor activity. Patients with T2DM have increased quantities of prothrombotic factors, to the extent that T2DM is called a 'hyper-coagulable' state (Carr, 2001). In addition to conventional screening tests such as shorter prothrombin and partial thromboplastin times (PT and APTT), T2DM is associated with elevated tissue factor levels (Meerarani *et al.*, 2007), relative reduction of tissue factor pathway inhibitor activity (Lwaleed and Bass, 2006), higher prothrombin activating factor and higher thrombin-antithrombin complexes compared to individuals without

diabetes (Davì *et al.*, 1992). Increases in the levels of factor VIII, plasma fibrinogen and Von Willebrand factor and factor XI found in T2DM favour thrombus formation (Ferroni *et al.*, 2004). In T2DM, ***Tissue factor (TF)*** expression is increased directly by hyperglycaemia (Stegenga *et al.*, 2006), and hyperinsulinaemia (Boden *et al.*, 2007) and indirectly by stimulation of receptors of advanced glycation end products (RAGE) (Min *et al.*, 1999), nuclear factor kappa B (NFkB) and reactive oxygen species (Herkert *et al.*, 2004). Tissue factor pathway inhibitor (TFPI) levels are higher in patients with T2DM and correlates with hyperglycaemia and insulin resistance (Leurs *et al.*, 1997; Vambergue *et al.*, 2001) but functionally these higher levels do not offer protection from excess thrombus formation. Experimental administration of more than double the usual levels of TFPI reduced platelet dependent thrombogenicity in T2DM, confirming that the levels of TFPI seen in T2DM are insufficient to protect against future thrombotic events (Badimon *et al.*, 1999).

Factor VII is a pro-coagulant protein which is elevated in patients with T2DM, in pre-diabetes and relatives of patients with T2DM (Heywood *et al.*, 1996; Mansfield *et al.*, 1996). There is a strong association between Factor VII levels and LDL cholesterol and triglyceride levels, particularly in T2DM (Hoffman *et al.*, 1992; Zito *et al.*, 2000). ***von Willebrand factor (vWF)*** is elevated and correlates with other cardiovascular risk factors such as T2DM and dyslipidaemia (Frankel *et al.*, 2008; Jax *et al.*, 2009). Recent reports from the Framingham heart study have confirmed the association between high levels of vWF and T2DM and correlation of vWF and cardiovascular events in patients with T2DM. These suggests that vWF may represent a unique risk factor in patients with T2DM and coronary artery disease (Frankel *et al.*, 2008).

- Increased aggregating responses
- Decreased responses to anti-aggregating substances
- Increased membrane viscosity
- Increased protein tyrosine phosphorylation
- Increased calpain activity
- Increased calcium content and reverse mode of sodium/calcium exchanger
- Increased calcium-induced presentation of the thrombospondin receptor CD36
- Reduced L-arginine uptake
- Reduced endothelial-type nitric oxide synthase activity
- Enhanced homocysteine ability to reduce nitric oxide availability
- Trend to leukocyte-platelet heterotypic aggregation
- Increased expression of adhesive proteins
- Decrease of nitric oxide availability
- Protein kinase C translocation to the membrane

Modified from Trovati et al. Current Diabetes Reports 2002;2:316–322

Table 1.1 Platelet alterations in type 2 diabetes mellitus.

Elevation of **fibrinogen** levels may provide the link between inflammation, thrombosis and T2DM as it is an acute phase protein synthesised in response to inflammatory mediators (Takebayashi *et al.*, 2006). Poor glycaemic control is associated with high fibrinogen levels which are reversed upon restoration of normoglycaemia in T2DM (Emanuele *et al.*, 1998). Multiple epidemiological studies such as the Insulin Resistance Atherosclerosis study (IRAS) and Framingham heart study (Kannel *et al.*, 1990) confirm that elevated serum fibrinogen is a strong and independent cardiovascular risk factor in T2DM (Danesh *et al.*, 1998; Kaptoge *et al.*, 2007; Ang *et al.*, 2008; Bhatt, 2008; Guardado-Mendoza *et al.*, 2009). Fibrinogen levels are associated with a higher incidence of silent myocardial infarction in patients with T2DM (Guardado-Mendoza *et al.*, 2009) and hypo-response to antiplatelet agents including clopidogrel in patients with T2DM (Ang *et al.*, 2008). Levels of coagulation factors such as ***factor XII*** and ***factor XIII*** were higher in individuals with T2DM both in the baseline state and after acute myocardial infarction compared to those without T2DM (Miller *et al.*, 1997; Mansfield *et al.*, 2000).

1.2.7.3 Impaired fibrinolysis in T2DM

The procoagulant state in DM is further sustained by an imbalance of prothrombotic and fibrinolytic mechanisms. In T2DM, reduced fibrinolysis potentiates thrombogenicity and also promotes atherogenesis by extended fibrin deposition (Sobel and Schneider, 2004). The fibrinolytic system is less effective in T2DM because of abnormalities in clot structure, such as increased side to side fibrin polymer chains, making it more resistant to degradation, and elevated levels of fibrinolytic inhibitors such as PAI-1 (Aso *et al.*, 2002; Alzahrani and Ajjan, 2010). Aggressive management of cardiovascular risk factors including dyslipidaemia and hypertension reduces the levels of PAI-1, thereby providing one mechanistic link for reduction of thrombotic events with risk factor management (Colwell, 2001; Mertens *et al.*, 2008). PAI-1 is consistently elevated in the vessel walls of patients with T2DM (Figure 1.11) (Sobel *et al.*, 1998; Pandolfi *et al.*, 2001). Genetic polymorphisms, hyperglycaemia and inflammatory

cytokines are responsible for high PAI-1 levels seen in patients with T2DM (Jokl *et al.*, 1994; Mansfield *et al.*, 1995; Dellas and Loskutoff, 2005; Saely *et al.*, 2008). Current evidence suggests that increased PAI-1 production is linked not only to increased thrombotic events seen in T2DM but also may be important in the development of widespread atheroma (Raghunath *et al.*, 1995; Carmeliet *et al.*, 1997; Eitzman *et al.*, 2000).

1.2.8 Microparticles in T2DM

Microparticles (MPs) are small membrane prothrombotic structures of size 0.02 to 0.10 µm shed from platelets and endothelial cells. They are released into the circulation by exocytosolic budding of plasma membranes of the cells and are highly thrombogenic (Horstman and Ahn, 1999; Nieuwland *et al.*, 2007). MP levels are consistently higher in T2DM. It is still unclear if an increase in platelet MPs serves as an independent marker for future coronary events or is just a reflection of platelet hyperactivity in T2DM (Nomura *et al.*, 2008). Interestingly, elevated endothelial MP levels were useful in identifying a subpopulation of diabetic patients without typical anginal symptoms but with angiographic evidence of coronary artery disease. Monocyte derived MP were associated with microvascular and macrovascular complications of T2DM. (Omoto *et al.*, 2002; Koga *et al.*, 2005).

1.2.9 Inflammation and thrombosis in T2DM

Type 2 diabetes mellitus has been associated with chronic low grade inflammation. Persistent hyperglycaemia, advanced end glycation products and hyperinsulinaemia are associated with greater inflammation. Recent evidence has categorically shown a link between inflammation and thrombosis leading on to coronary events (Hotamisligil, 2006). As mentioned earlier, it is possible that inflammation is the hidden but potent “common soil” for the prothrombotic state in T2DM (Stern, 1995; Hess and Grant, 2011).

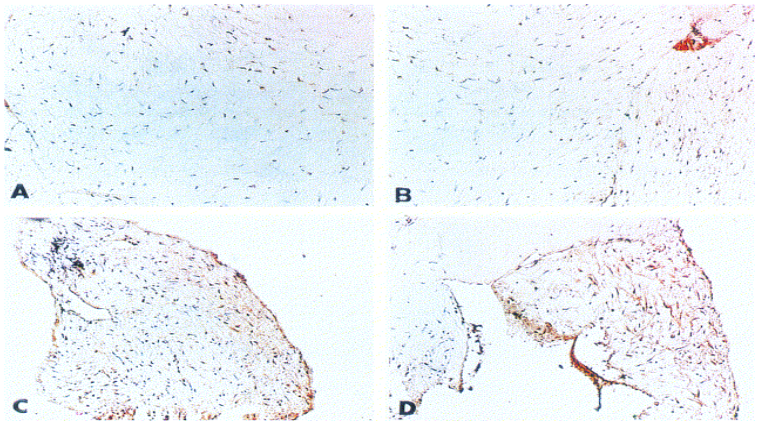


Figure 1.11 Immunohistochemistry of Plasminogen activator inhibitor-1.

Intensity of immunohistochemical staining for plasminogen activator inhibitor-1 (PAI-1) from patients without diabetes (*A and B*) compared with those from patients with diabetes (*C and D*). Intensity and distribution of PAI-1 were generally greater in samples from subjects with diabetes than in those without. Magnification ×100. From Sobel, Am J Med,2007; 120(9, Supplement 2), S3-S11.

The exact mechanism by which inflammation contributes to thrombogenicity is unknown. However, it has been widely accepted that inflammation up-regulates procoagulants, and down regulates anticoagulants and fibrinolysis (Figure 1.12) (Willerson and Ridker, 2004; Levi and van der Poll, 2010).

Tumour necrosis factor α (TNFα) levels are higher in T2DM and have been linked to insulin resistance and complications of T2DM such as diabetic nephropathy and diabetic retinopathy (Mavridis *et al.*, 2008). Suppression of TNFα either by biological antagonists or physical measures such as weight reduction and exercise improve glycaemic control in T2DM. It is plausible but yet remains to be proven that similar benefits can be achieved in alleviating the prothrombotic state in T2DM by physical or pharmacological modulation of the TNFα pathway (Alexandraki *et al.*, 2006).

Elevated C reactive protein (CRP) correlates with the increased risk of myocardial infarction (Ballantyne *et al.*, 2004). A recent meta-analysis of large scale clinical and epidemiological studies confirmed the strong association between elevated CRP and cardiovascular events (The Emerging Risk Factors Collaboration, 2010). Numerous data exist that high sensitivity CRP (hs-CRP) also can predict, independently of conventional risk factors, coronary heart disease (CHD) and cardiovascular disease (CVD) mortality in patients with T2DM and metabolic syndrome (Ridker *et al.*, 2004). The American Heart Association suggested in their guidelines on cardiovascular risk factors that higher levels of CRP are a hallmark of T2DM and may warrant preventive therapy for cardiovascular events even in patients with pre-diabetes (Sabatine *et al.*, 2007). However the Norfolk - EPIC study and a recent meta-analysis have shown that the association between T2DM and CRP is weak and is often confounded by visceral adiposity (Boekholdt *et al.*, 2006; The Emerging Risk Factors Collaboration, 2010).

In patients with T2DM the levels of IL-6 and IL-8 are significantly higher than those without T2DM even after adjustment for confounding factors including visceral obesity. Inflammatory mediators, such as interleukin IL-6 increase platelet production. As newly released platelets are more thrombogenic

compared to the older ones, IL-6 indirectly increases thrombogenicity (Lim *et al.*, 2004). Inflammatory mediators induce expression of protease activated receptors (PARs) on endothelium of patients with T2DM which in turn increases leukocyte adhesion molecules on the cell surface (Dangwal *et al.*, 2011).

1.2.10 Insulin resistance and thrombogenicity

Insulin resistance augments the abnormalities of coagulation, including platelet aggregation and platelet adhesion in T2DM. Hyperinsulinaemia increases levels of thromboxane, von Willebrand factor, factor VIII, tissue plasminogen activator and fibrinogen. Levels of plasma insulin, proinsulin, modified lipoproteins and cytokines, which are elevated in metabolic syndrome and hyperinsulinaemic states, are directly correlated to platelet activating factor inhibitor type 1 (PAI-1) (McBane *et al.*, 2010). Insulin has inherent anti-platelet effects in healthy volunteers. In vitro and in vivo studies have demonstrated that insulin inhibits platelet aggregation in healthy non-obese subjects. However, in obese and T2DM subjects with insulin resistance, this anti-aggregating effect of in vitro insulin is blunted (Sobel, 1999a; Colwell, 2001; Pandolfi *et al.*, 2001; Randriamboavonjy and Fleming, 2009).

Platelet dysfunction in T2DM is modulated by insulin resistance at the molecular level by:

i. blunting of the insulin effects on the nitric oxide / cyclic guanosine monophosphate (NO/cGMP) system and on platelet aggregation (Sobel and Schneider, 2004)
ii. resistance to the anti-aggregating effects of nitrates and adenosine mediated via cGMP and cyclic adenosine monophosphate (cAMP) pathway respectively (Trovati *et al.*, 1994; Anfossi *et al.*, 1998)
iii. increased platelet cytosolic calcium (Watala *et al.*, 2005)

Restoration of insulin sensitivity by experimental weight loss has restored these molecular abnormalities to normal, thereby improving platelet function (Baalbaki and Bell, 2007).

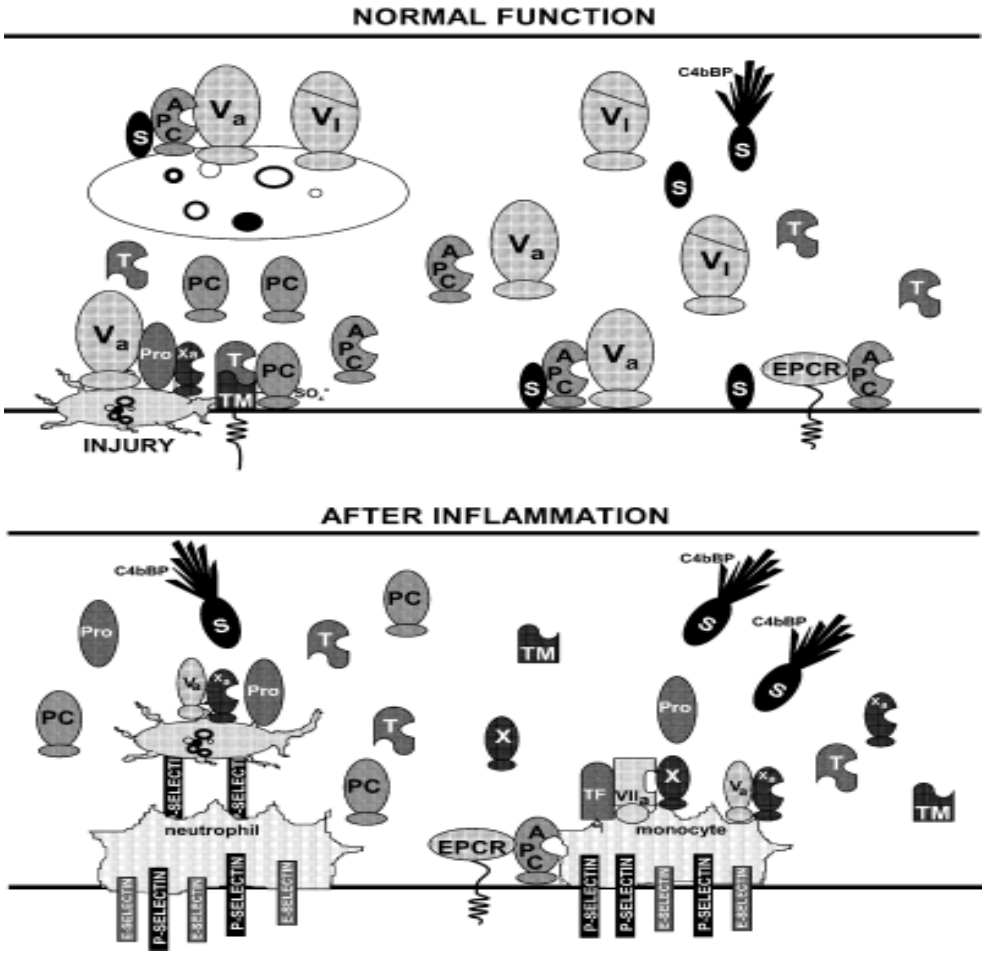


Figure 1.12 Differences between coagulation in normal versus inflamed vasculature.

Top: The protein C anticoagulant pathway under normal conditions. Vascular injury initiates prothrombin (Pro) activation, which results in thrombin (T) formation. Prothrombin activation results in formation of factor Va (Va) and factor Xa (Xa) complex. Thrombin then binds to thrombomodulin (TM) on the lumen of the endothelium, illustrated by the heavy line, and the thrombin-TM complex converts protein C (PC) to activated protein C (APC). The APC then binds to protein S (S) on cellular surfaces. The APC protein S complex then converts factor Va to an inactive complex (Vi), illustrated by the slash through the larger part of the two-subunit factor Va molecule. Protein C and APC interact with an endothelial cell protein C receptor (EPCR).

Bottom: The protein C pathway after inflammation. In this model, inflammatory mediators lead to the disappearance of thrombomodulin from the endothelial cell surface. Endothelial cell leucocyte adhesion molecules, P-selectin or E-selectin, are synthesised or expressed on endothelial or platelet surfaces. Tissue factor (TF) is expressed on monocytes and binds factor VIIa (VIIa), and this complex converts factor X (X) to factor Xa (Xa), which forms complexes with factor Va

(Va) to generate thrombin (T) from prothrombin (Pro). Because little APC is formed and the little that forms does not function well because of low protein S (S), factor Va is not inactivated and prothrombin activation complexes are stabilised. From Remick DG, Pathophysiology of sepsis, Am J Pathol. 2007; 170(5): 1435-44.

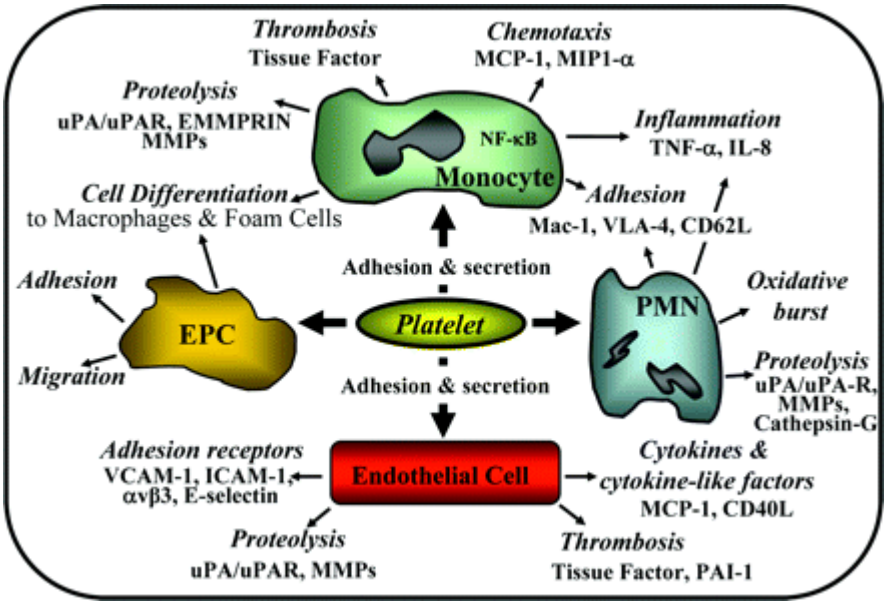


Figure 1.13 Platelet leukocyte interaction.

Activated platelets recruit inflammatory cells and amplify vascular inflammation. In addition, they induce synthesis of adhesion molecules in endothelium and tilt the balance towards thrombogenicity. Inflammatory cytokines are released from activated platelets, monocytes and polymorpho nucleated neutrophils (PMN).This inflammatory pathways interact with each other and augment the thrombotic milieu. From Esmon, C. T., Br J Heamatology, 2005: 131: 417–430.

1.3 Antiplatelet therapy in patients with T2DM and CAD

Platelets play a central role in thrombosis and antiplatelet agents are the cornerstone in prevention of coronary arterial thrombosis in high risk patients.

Antithrombotic action of antiplatelet agents can be summarised via two major mechanisms:

i. Enzyme inhibition and
ii. Receptor blockade

Commonly used antiplatelet agents in patients with CAD and T2DM are ***aspirin*** and ***clopidogrel***.

1.3.1 Structure of aspirin

Aspirin is the most commonly used antiplatelet agent for prevention of atherothrombotic events. Low dose aspirin (e.g. 75 mg daily) has been used as an antiplatelet agent since 1960 (Varon and Spectre, 2009). The routine use of aspirin for primary prevention has recently been questioned, but clear cut benefits are well established in secondary prevention trials (Antithrombotic Trialists Collaboration, 2009), (Vane and Botting, 2003; Patrono and Rocca, 2009). Aspirin is prepared by chemical synthesis from salicylic acid, by acetylation with acetic anhydride and the structure of acetyl salicylic acid, the chemical name for aspirin is given below (Figure 1.14) (Vane, 1971).

1.3.2 Mechanism of action of aspirin

Aspirin exerts its antithrombotic effect by irreversible inhibition of thromboxane A2 synthesis in platelets. Upon activation by a variety of stimuli such as vascular tissue injury, inflammation and stress, arachidonic acid (a 20 carbon chain fatty acid) is liberated from platelet membrane phospholipids by enzymes known as phospholipases. Arachidonic acid is then metabolised by cytosolic prostaglandin H synthase (PGH) enzymes, which act by two distinct pathways namely cyclo-oxygenase (COX) and hydroperoxidase activity.

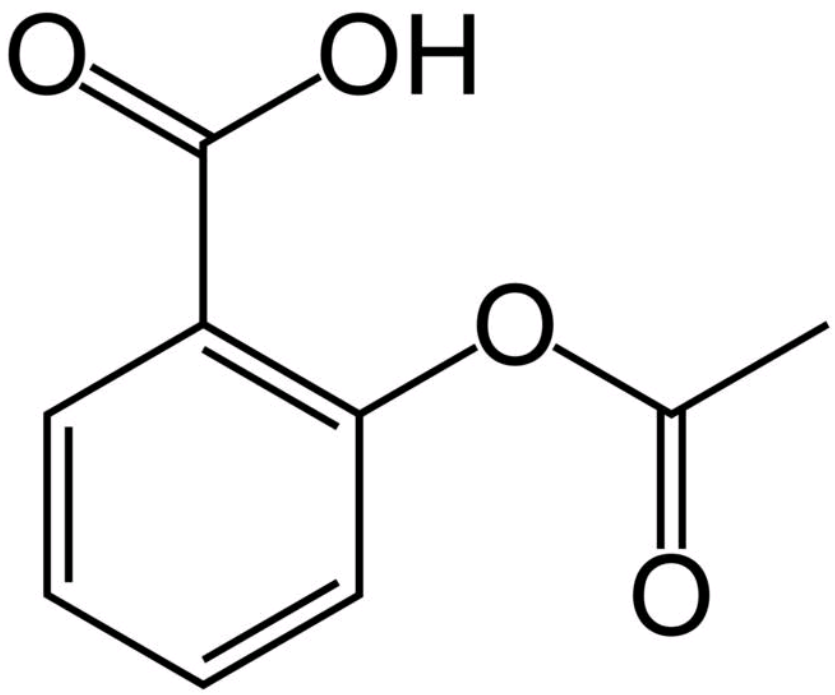


Figure 1.14 Structure of aspirin.

The final common products of this reaction are thromboxane A2 (TXA2) by the COX pathway and prostaglandin I2 (PGI2) by the hydroperoxidase pathway (Patrono *et al.*, 2005). TXA2 induces potent platelet aggregation and vasoconstriction, whereas PGI2 inhibits platelet aggregation and induces vasodilation. In addition, TXA2 augments the actions of platelet agonists and is pro atherogenic and a potent stimulus for vascular smooth muscle cells to express adhesion molecules (Vane and Botting, 2003). Aspirin irreversibly inhibits the cyclo-oxygenase enzyme in human platelets (Vane, 1971). Cyclo-oxygenase enzyme exists in two forms called COX-1 and COX-2. Low dose aspirin (30-100mg) selectively and irreversibly inhibits COX-1, and high dose aspirin (more than 1000mg) inhibits both COX-1 and COX-2 (Reilly and FitzGerald, 1987; Vane, 2000).

1.3.3 Pharmacokinetics of aspirin

Aspirin is absorbed mainly from the stomach and duodenum by passive diffusion. Plasma levels peak at 30-40 minutes for standard preparations of aspirin whereas it may take up to three or four hours for enteric coated preparations. Aspirin undergoes hydrolysis in gastrointestinal mucosa and liver, and forms salicylic acid (Pedersen and FitzGerald, 1984). Aspirin has a half life of 15–20 minutes in the circulation but its antiplatelet effects last for the entire lifespan of platelets due to the irreversible inhibition of COX-1, allowing the use of a once a day regimen for antiplatelet therapy despite the very short half life of the drug (Reilly and FitzGerald, 1987; Maree and Fitzgerald, 2004). Platelet function recovers after stopping aspirin faster than the predicted 10 days and is due to the non-linear relationship between TXA2 biosynthesis and COX-1 inhibition (Perneby *et al.*, 2006), (Antithrombotic Trialists Collaboration, 2002).

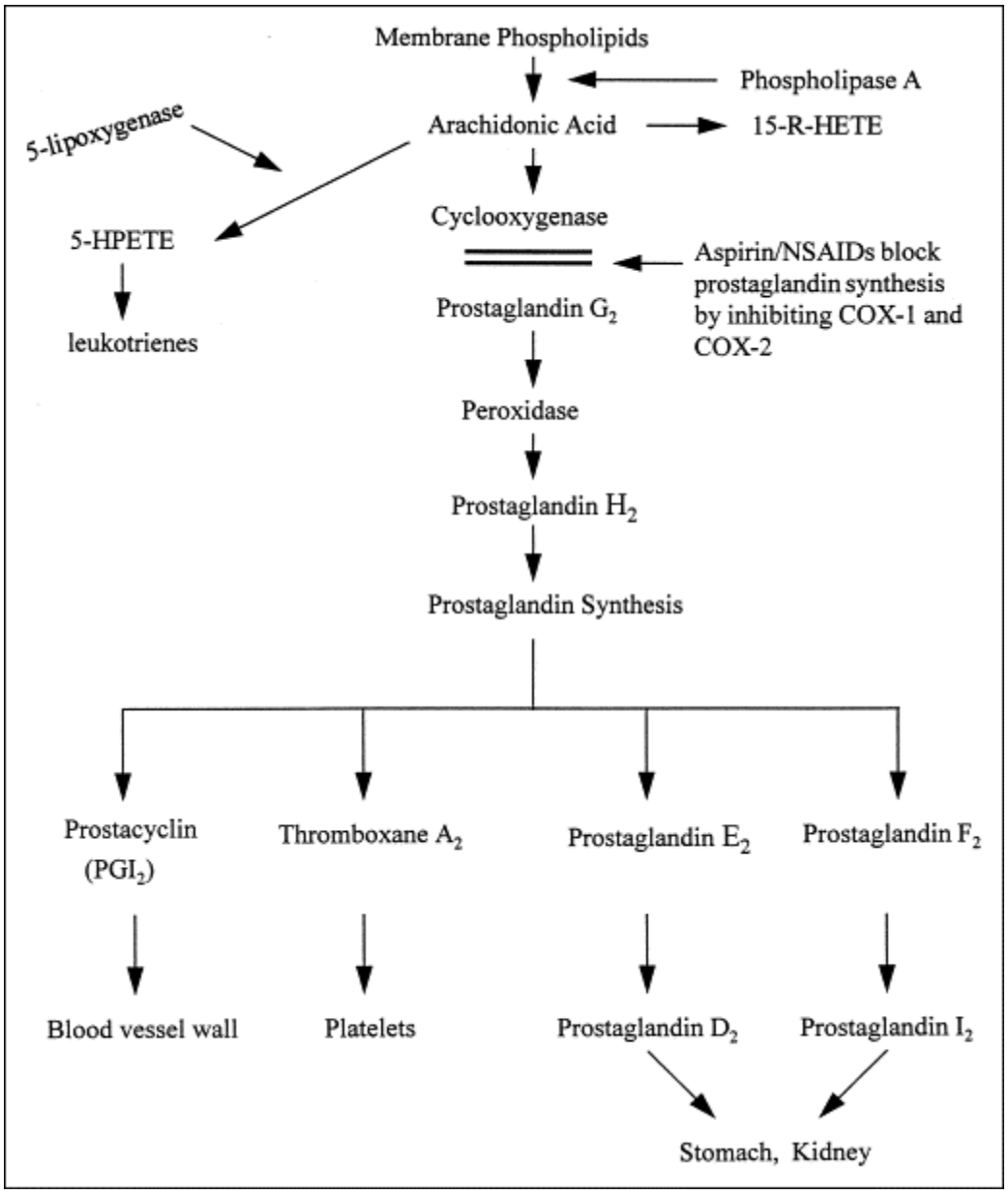


Figure 1.15 Cyclo-oxygenase pathway and aspirin. From Bjorkman DJ, AM J Med 1998;105,1,8S-12S.

1.3.4 Dosage of aspirin for coronary artery disease

Aspirin is commonly used at doses 75 mg to 100 mg for primary and secondary prevention of cardiovascular diseases as this dosage has maximum antiplatelet effect and reduces major cardiac events without excess bleeding risk. Complete suppression of COX-1 mediated platelet activity was achieved at doses 50-81 mg (Patrono *et al.*, 2005; Lordkipanidzé *et al.*, 2007; Qayyum *et al.*, 2008).

Three factors support the use of low dose aspirin in patients with CAD:

i. saturation of antiplatelet effects of aspirin at low doses
ii. lack of higher dose related clinical benefits
iii. dose dependent side effects (Patrono *et al.*, 2008)

1.3.5 Side effects of aspirin

The most common side effect of aspirin is gastrointestinal toxicity mediated by gastric mucosal erosion. Concomitant use of NSAIDS, *Helicobacter pylori infection,* history of previous gastric ulcer and age more than 70 years increase the risk of gastro intestinal side effects of aspirin (Lanas and Scheiman, 2007; Yeomans *et al.*, 2009). The estimated excess risk of upper gastrointestinal complications attributable to aspirin is around 5 extra cases per 1,000 aspirin users per year (Garcia Rodriguez *et al.*, 2001). Meta-analysis by Antithrombotic Trialists' Collaboration (ATC) found an odds ratio of 1.6 for major extracranial bleeding, i.e. gastric bleeding, in patients taking aspirin (Antithrombotic Trialists Collaboration, 2009).

1.3.6 Aspirin resistance

There is no consensus on the definition of aspirin resistance. In general terms, aspirin resistance is defined as failure to inhibit platelet activation and aggregation upon stimuli or ‘platelet hypo responsiveness to aspirin’ (Hankey and Eikelboom). In addition, non-compliance of aspirin is more common (up to 40 %) than true aspirin resistance and any laboratory or clinical assessment of aspirin resistance should be adjusted for compliance to aspirin therapy (Cotter *et al.*, 2004; Schwartz *et al.*, 2005).

Clinical resistance or 'real life resistance' to aspirin is revealed only after the individual who is taking aspirin suffers from a major thrombotic event such as acute myocardial infarction. Patients with type 2 diabetes mellitus, high platelet turnover and chronic kidney disease and those taking non steroidal anti-inflammatory drugs are at risk of aspirin resistance. Laboratory aspirin resistance (also known as biochemical resistance) has recently been studied intensively. The reported prevalence of biochemical aspirin resistance ranges widely from 1% to 61% (Hankey and Eikelboom, 2006). Three studies followed individuals with failure to suppress thromboxane production by aspirin and found they were 2.2 to 4.3 times at risk of developing cardiovascular events (Dragani *et al.*; Sciulli *et al.*, 2006; Santilli *et al.*, 2009). Routine measurement of serum or urinary thromboxane is not practical because of its wide day to day variability secondary to the inherent ability of platelets to augment thromboxane production under high shear state (Tantry *et al.*, 2009).

1.3.7 Aspirin and T2DM

Patients with T2DM have hyperactive platelets and enhanced platelet regeneration with biochemical evidence of persistent thromboxane-dependent platelet activation *(vide supra).* Aspirin at doses of 75mg-100 mg is the antiplatelet drug of choice for a secondary prevention strategy in T2DM with a history of CVD, whereas direct evidence for the efficacy of low dose aspirin in the primary prevention in T2DM is inconclusive (Antithrombotic Trialists Collaboration, 2009; De Berardis *et al.*, 2009). It has been argued that patients with T2DM may need higher doses of aspirin. In a study by Mortenson et al, patients with T2DM and CAD on aspirin 75mg od had higher platelet reactivity as measured by two point of care tests (Mortensen, Larsen et al. 2010). Geisler et al have shown that T2DM remained a significant risk factor for residual platelet reactivity in patients treated with aspirin after adjustment of confounding factors and this has been correlated with clinical cardiovascular events (Geisler *et al.*, 2010).

In a meta-analysis of more than 45,000 patients with diabetes mellitus, the incidence of vascular events was reduced, from 23.5% in the control group to

19.3% in the group treated with aspirin, compared to 17.2% to 13.7% in the 42,000 non diabetic patients. There were 42 vascular events prevented for every 1000 diabetic patients and 35 events for every 1000 non diabetic patients (Antithrombotic Trialists Collaboration, 2002).

Thromboxane inhibition by low dose aspirin is less in T2DM. Potential mechanisms underlying low dose aspirin insensitive thromboxane biosynthesis in T2DM include:

i. reduced cyclo-oxygenase-1 (COX-1) binding capacity of aspirin due to glycation of COX-1 (Watala *et al.*, 2005)
ii. faster recovery of COX-1 activity because of accelerated platelet release from bone marrow (DiMinno *et al.*, 1986).
iii. enhanced platelet COX-2 expression and recovery (Rocca *et al.*, 2012)
iv. increased production of thromboxane by diabetic platelets (Davi *et al.*, 1990)
v. extra platelet sources of thromboxane, uninhibited by aspirin (Santilli *et al.*, 2011)

1.3.8 Pharmacology of clopidogrel

Clopidogrel is a thienopyridine class antiplatelet agent and acts by inhibition of P2Y12 receptors in the membrane of platelets. This is the most widely used and well studied medication of this class. Platelet P12Y12 receptor is stimulated by adenosine diphosphate (ADP) released by platelet granules. Activation of P2Y12 receptor augments platelet activity by further secreting more platelet activator substances. Clopidogrel is absorbed well in the upper small intestine and enters the portal circulation. Clopidogrel is converted to its active metabolite by a group of cytochrome P450 enzymes in the liver. The platelets that come in contact with the active metabolite of clopidogrel in the portal circulation will be inactivated permanently by blocking their P2Y12 receptor.

Only 15% of the absorbed drug is metabolised by cytochrome P450 (CYP450) enzymes in the liver into an active metabolite. About 85% of the drug is hydrolyzed by an esterase to create an inactive carboxylic acid derivative.

Cytochrome enzymes produce an unstable active metabolite of clopidogrel by a two step process (Figure 1.17):

i. CYP450 oxidizes the thiophene ring of clopidogrel to 2-oxoclopidogrel, which will undergo further hydrolysis by CYP450. Oxidation of the thiophene ring of clopidogrel by a CYP450 dependent mechanism, involving enzymes like CYP3A4, CYP3A5 and CYP2C19, is necessary to generate 2-oxo-clopidogrel (Lau *et al.*, 2004)
ii. The second reaction results in opening of the thiophene ring to form a thiol and carboxyl group. This short lived thiol derivative of clopidogrel binds with cysteine residues (cys17 and cys270) of the P2Y12 receptor of human platelets with high affinity (Ding *et al.*, 2003),(Savi *et al.*, 2000) and results in permanent irreversible platelet inhibition.

Clopidogrel by inhibiting platelet activation via P2Y12 receptor blockade prevents expression of GP IIb llla receptors and thereby inhibits fibrin mediated platelet aggregation. Clopidogrel also inhibits collagen and thrombin induced aggregation with human platelets (Patrono *et al.*, 2008). In addition to specific inhibition of the effects of ADP, Clopidogrel reduces platelet–leukocyte aggregate formation, and the levels of CRP, *p*-selectin and CD 40L, and the rate of thrombin formation (Gurbel *et al.*, 2007).

Platelet inhibition occurs within 1–2 h after a single loading dose of clopidogrel. The maximal level of platelet inhibition (approximately 30%) is achieved within 4–5 h after a 300 mg dose and is maintained for at least 24 h (Jernberg *et al.*, 2006). Platelet inhibition decreases to pre-treatment levels one week after the drug is discontinued (Wallentin, 2009). Doubling the loading dose to 600mg achieves platelet inhibition at 2-3 hours and a further increase in platelet inhibition is reported at this dose. Only a limited increase in platelet inhibition occurs at doses higher than 600mg (Taubert *et al.*, 2004) (Cuisset *et al.*, 2006; Price *et al.*, 2006). Antiplatelet response to clopidogrel is highly heterogeneous. Variability in clopidogrel response is widely recognised and is associated with higher adverse clinical events (vide infra) (Angiolillo *et al.*, 2007a; Vila *et al.*, 2009).

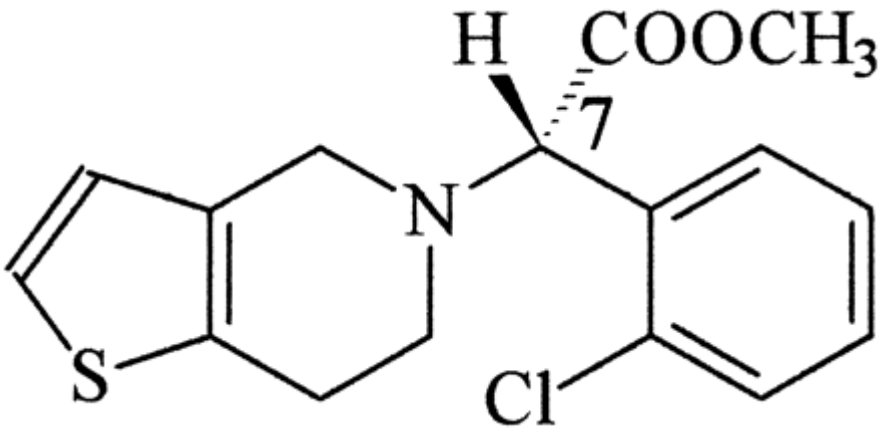


Figure 1.16 Structure of clopidogrel

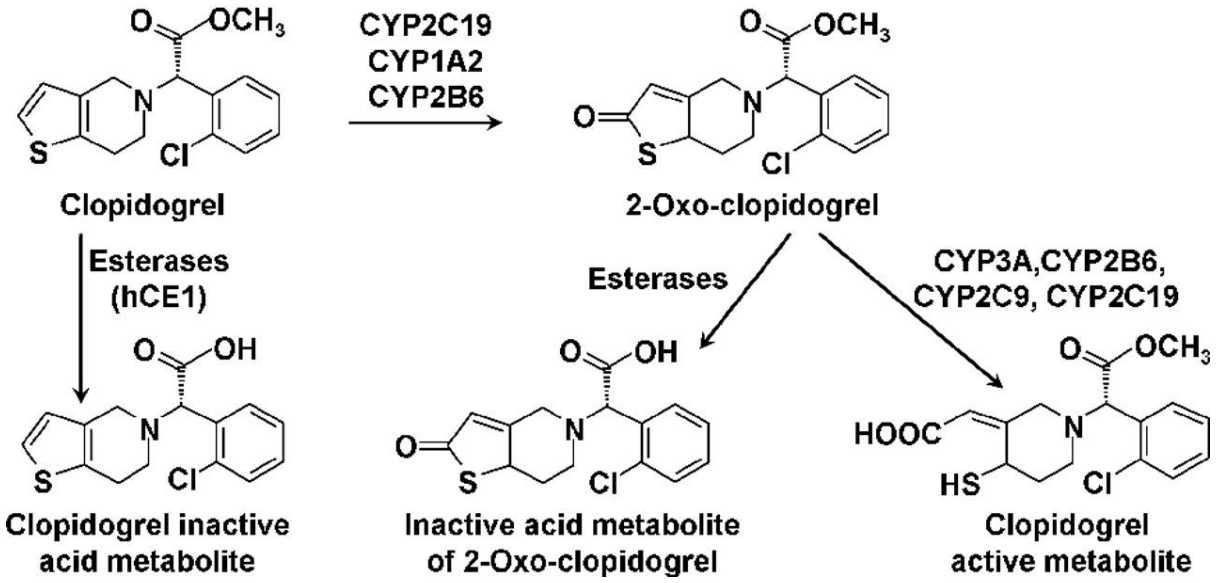


Figure 1.17 Metabolism of clopidogrel

Cytochrome enzymes produce an unstable active metabolite of clopidogrel by a two step process: CYP450 oxidizes the thiophene ring of clopidogrel to 2-oxoclopidogrel, which will undergo further hydrolysis, to generate 2-oxo-clopidogrel. The second reaction results in opening of the thiophene ring to form a thiol and carboxyl group. This short lived thiol derivative of clopidogrel results in permanent irreversible platelet inhibition. From Gurbel et al, Thromb Res 2007, 120: 311 – 321.

1.3.9 Clinical studies of clopidogrel in CAD

The Clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE) study randomised patients to receive either clopidogrel 75 mg or aspirin 325 mg. The study population comprised of patients with a previous history of symptomatic atherothrombotic disease or with major risk factors such as diabetes mellitus or hypercholesterolaemia. There was a significant reduction in the primary endpoint (death, myocardial infarction or stroke) with a relative risk reduction of 8.7% (95% confidence interval 0.3% - 16.5%) between clopidogrel and aspirin. The overall incidence of haemorrhagic events did not differ between treatment groups (CAPRIE steering Committee, 1996) .

The Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) study randomised patients with non-ST-segment elevation acute coronary syndrome to receive either clopidogrel (a loading dose, 300 mg orally, followed by 75 mg daily) or placebo in addition to standard therapy with aspirin (dose 75-325 mg). After a mean follow-up of 9 months, in the group receiving clopidogrel, there was a significant reduction in the primary end point including death from cardiovascular causes, non-fatal acute myocardial infarction, or stroke (ischaemic and haemorrhagic) [all cardiovascular events: OR 0.84, 95% CI 0.77 to 0.93]. There was an increase in major bleeding events in the clopidogrel group [OR 1.39, 95% CI 1.14 to 1.70]. This study was a landmark study which resulted in widespread use of clopidogrel as a dual antiplatelet agent in patients after non ST elevation acute coronary syndrome (Yusuf S, 2001).

The Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance trial (CHARISMA, 2006) randomised patients at high risk for a cardiovascular event (multiple atherothrombotic risk factors, documented coronary disease, or cerebrovascular disease, or symptomatic peripheral arterial disease) either to clopidogrel (75 mg daily) plus low dose aspirin (75 to 162 mg per day) or to a placebo plus low dose aspirin. There was a non significant reduction in the primary end point of myocardial infarction and death from cardiovascular causes (OR 0.92, 95% CI 0.81 to 1.04). There was a

non significant increase in major bleeding (OR 0.92, 95% CI 0.81 to 1.04) (Bhatt *et al.*, 2006).

The Clopidogrel as Adjunctive Reperfusion Therapy (CLARITY)–Thrombolysis in Myocardial Infarction (TIMI) 28 trial randomised patients who underwent thrombolysis and aspirin therapy for ST elevation myocardial infarction, to receive a 300 mg clopidogrel loading dose, followed by 75 mg daily, or matching placebo for 30 days. The primary composite end point (death, myocardial infarction, and infarct related occluded artery flow) was lower in the clopidogrel arm (odds ratio 0.64, 95%CI 0.53 -0.76). There was no difference in bleeding between the two groups. Clopidogrel improved patency of the infarct related artery compared to placebo (odds ratio of 1.36, 95%CI 1.18 to 1.57) (Gibson *et al.*, 2008).

The ClOpidogrel and Metoprolol in Myocardial Infarction Trial (COMMIT) also known as the Second Chinese Cardiac Study (CCS -2) is the largest study to test the effectiveness of aspirin and clopidogrel. Patients with suspected acute myocardial infarction were randomised to receive aspirin and clopidogrel 75 mg each daily or aspirin and placebo for four weeks or until hospital discharge. The clopidogrel group had a 9% (95%CI 3-14%) relative risk reduction in the composite end point of death, reinfarction, and stroke (p=0.002; odds ratio 0.91, 0.86 to 0.97). There were no excess major bleeding events or cerebral haemorrhage in the clopidogrel group (COMMIT (ClOpidogrel and Metoprolol in Myocardial Infarction Trial) collaborative group, 2005).

The Clopidogrel and Aspirin Optimal Dose Usage to Reduce Recurrent Events−Seventh Organization to Assess Strategies in Ischemic Syndromes (CURRENT–OASIS 7) trial was a complex design and showed that there was no difference in the primary outcome (cardiovascular death, myocardial infarction and stroke) in patients assigned to double dose clopidogrel (150mg) as compared to standard dose clopidogrel (75mg) (hazard ratio, 0.94; 95% CI 0.83 to 1.06). Major bleeding occurred at similar rates in both the groups and 150mg of clopidogrel was associated with a significant reduction in stent thrombosis (CURRENT-OASIS7 Investigators, 2010).

Trial	Population	Study treatments	Sample size	Mean study duration	Primary composite outcome	Primary outcome: clopidogrel vs. comparator	Primary outcome: clopidogrel vs. comparator (diabetes subset)
CURE	USA or NSTEMI	ASA + clopidogrel vs. ASA + placebo	12,562	9 months	CV death/MI/stroke	HR 0.80; 95% CI 0.72–0.90	HR 0.85; 95% CI not reported
CAPRIE	Recent stroke, MI, or PAD	ASA vs. clopidogrel	19,185	1.9 years	Vascular death/ MI/stroke	HR 0.91; 95% CI 0.84–0.98	HR 0.87; 95% CI 0.76–0.99
CHARISMA	CVD or multiple CVD risk factors	Clopidogrel + low dose ASA vs. placebo + low dose ASA	15,603	28 months	CV death/MI/stroke	HR 0.93; 95% CI 0.83–1.05	HR 1.2; 95% CI 0.91–1.59
PCI-CURE	NSTEMI	ASA + pre treatment with clopidogrel followed by long term therapy vs. ASA + no pre treatment and short-term therapy with clopidogrel	2658	9 months	CV death/MI/urgent revascularisation	HR 0.70; 95% CI 0.50–0.97	HR 0.77; 95% CI 0.48–1.22
CREDO	Elective PCI	Clopidogrel loading dose pre-PCI + long term therapy post PCI vs. placebo	2116	12 months	CV death/MI/stroke	HR 0.73; 95% CI 0.56–0.96	HR 0.89; 95% CI 0.54–1.47
CURRENT-OASIS 7	ACS undergoing PCI	Increased dose clopidogrel vs. standard clopidogrel plus high dose ASA vs. Low dose ASA	25,086	30 days	CV death/MI/stroke	HR 0.94; 95% CI 0.83–1.06	HR 0.86; 95% CI not reported
COMMIT	Suspected ACS	Aspirin and clopidogrel Vs aspirin alone	45,852	4 weeks	CV death/MI/stroke	HR 0.91; 95%CI 0.86-0.97	Not reported

Table 1.2 Clinical trials on aspirin and clopidogrel use in patients with coronary artery disease.

1.3.10 Dosage of clopidogrel

Clopidogrel achieves better platelet inhibition after a loading dose of 300mg or higher followed by a daily dose of 75mg or above compared to aspirin (Steinhubl *et al.*, 2002). Dosage regimes of clopidogrel differ based on the timing of percutaneous intervention and clinical indications.

1.3.10.1 Loading dose of clopidogrel

Current guidelines recommend loading dose of clopidogrel as follows:

i. In patients who are undergoing elective PCI, a dose of 300mg is advised, to be administered at least 24 hours before PCI
ii. In patients who have acute coronary syndrome, a loading dose of 600mg is advised
iii. In patients who are undergoing non elective PCI and have never had clopidogrel (clopidogrel naïve), a loading dose of 600 mg is advised. If the patients are already preloaded with 300mg of clopidogrel, a further dose of 300mg is recommended
iv. In patients who are undergoing elective PCI, a loading dose of 600mg can be considered when glycoprotein IIb IIIa inhibitor usage is either contraindicated or to be avoided
v. Doses higher than 600 mg are not currently recommended (Bassand *et al.*, 2007; Hamm, 2011; Hamm *et al.*, 2011; Patrono *et al.*, 2011; Wenger, 2012)

1.3.10.2 Maintenance dose of clopidogrel:

Clopidogrel 75 mg once daily achieves optimal platelet inhibition in 5 days when administered without a loading dose (Wenger, 2012), (Hamm, 2011). Therefore, daily dose of clopidogrel 75 mg od is advised for maintenance therapy.

1.3.11 Side effects of clopidogrel

Clopidogrel was well tolerated in clinical trials and serious side effects at loading doses of 300mg or maintenance doses of 75 mg are rare in clinical practice. The most serious adverse drug reactions occurred in less than 1% and were gastrointestinal haemorrhage, haemorrhagic ulcer and haemothorax. Common, non serious side effects of clopidogrel include diarrhoea, dyspepsia, abdominal pain and epistaxis.

The incidence of major or minor bleeding with clopidogrel was 2.5% and was similar to aspirin in the CAPRIE study (CAPRIE steering Committee, 1996). However, there were more bleeding events in the clopidogrel combined with aspirin group compared to aspirin alone (1.6% vs. 1.0%) in CURE study (Yusuf S, 2001). Contemporary evidence from a large scale registry warns that bleeding complications in patients on dual antiplatelet therapy after acute MI are 3.7%, twice the risk of bleeding compared to aspirin alone (Sorensen *et al.*, 2009). Thrombotic thrombocytopenic purpura (TTP), a severe multisystem thrombotic microangiopathy, occurs secondary to clopidogrel therapy in 1 in 100,000 patients.

Dyspeptic symptoms were more frequent in clopidogrel therapy in CAPRIE study (27.1%) but resulted in discontinuation of the drug in very few cases (3.2%) (CAPRIE steering Committee, 1996). Gastrointestinal haemorrhage requiring hospitalisation was less frequent with clopidogrel compared to aspirin in CHARISMA trial (2.0% vs. 2.7%) (Berger *et al.*, 2010).

1.3.12 Variability of clopidogrel response

Clopidogrel has a significant variability in its antiplatelet effects across a wider range of subjects. The variability is attributable to patient related clinical factors, pharmacokinetic mechanisms and genetic variability. Table 1.3 lists major mechanisms behind the heterogeneous response of clopidogrel. (O'Donoghue and Wiviott, 2006; Holmes *et al.*, 2010).

Clinical factors

- Failure to prescribe/poor compliance
- Under dosing
- Poor absorption
- Drug–drug interactions involving intestinal P-glycoprotein
- Drug–drug interactions involving CYP3A4
- Acute coronary syndrome
- Diabetes mellitus/insulin resistance
- Elevated body mass index

Pharmacokinetic factors

- Accelerated platelet turnover
- Reduced CYP3A metabolic activity
- Increased ADP exposure
- Up-regulation of the P2Y12 , P2Y1 and P2Y–independent pathways

Genetic factors

- Polymorphisms of Cytochrome P450 enzymes
- Polymorphisms of GPIa, P2Y12, GPIIb/IIIa receptors

(Adopted from Angiollilo DJ, et al; J Am Coll Cardiol 2007; 49, 1505-16)

Table 1.3 Mechanisms of clopidogrel response variability.

1.3.13 Pharmacogenomics of clopidogrel

The response to clopidogrel is influenced by pharmacokinetic variables such as intestinal absorption and metabolic activation in the liver, both of which are affected by genetic polymorphisms (Giusti and Abbate, 2010). Three types of genetic polymorphisms are of clinical interest in patients taking clopidogrel, namely, CYP2 series, CYP3 series and ABC series polymorphisms (Figure 1.18a and 1.18b). Genetic variation of these cytochrome 450 enzymes have been associated with variability in clopidogrel response and this has resulted in a special warning issued by the FDA regarding low or poor response to clopidogrel (Holmes *et al.*, 2010). CYP2C19 polymorphism involving CYP450 enzyme was present in 28.6% individuals who had a significantly increased risk of cardiovascular death (hazard ratio 1.84) and stent thrombosis (hazard ratio 2.81) (Mega *et al.*, 2010).

1.3.14 Clopidogrel and T2DM

Evidence supports the superiority of clopidogrel compared to aspirin in patients with diabetes mellitus. The Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial included 4,000 patients with T2DM. The primary endpoint (combined incidence of vascular death, MI, or ischemic stroke) occurred less in the clopidogrel group compared to the aspirin group (15.6% vs. 17.7%) (Bhatt *et al.*, 2002). This has been confirmed by a sub group analysis of CHARISMA trial (Bhatt *et al.*, 2007). In the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) study, a sub group analysis of patients with T2DM showed that addition of clopidogrel to aspirin therapy resulted in fewer cardiovascular events (14.2% vs. 16.7%). In a smaller randomised control study, (The PLavix Use for Treatment Of Diabetes (PLUTO-Diabetes) Trial), combination therapy with aspirin and clopidogrel has resulted in greater suppression of biomarkers of platelet activation compared to aspirin alone (Serebruany *et al.*, 2008).

Nevertheless, clinical benefits of clopidogrel are lower in T2DM compared to those without diabetes (Angiolillo, 2009). The variability of clopidogrel response

is more pronounced in T2DM (Angiolillo *et al.*, 2007a). Lower response to standard dose of clopidogrel therapy (75 mg once daily), also known as ‘clopidogrel resistance’, a term widely used but not yet clearly defined, is more common in patients with T2DM compared to those without (Angiolillo *et al.*, 2007b). In a recent study by El Ghannundi et al, impaired response to clopidogrel in T2DM was an independent marker of cardiac mortality after adjusting for confounding factors (El Ghannudi *et al.*, 2011).

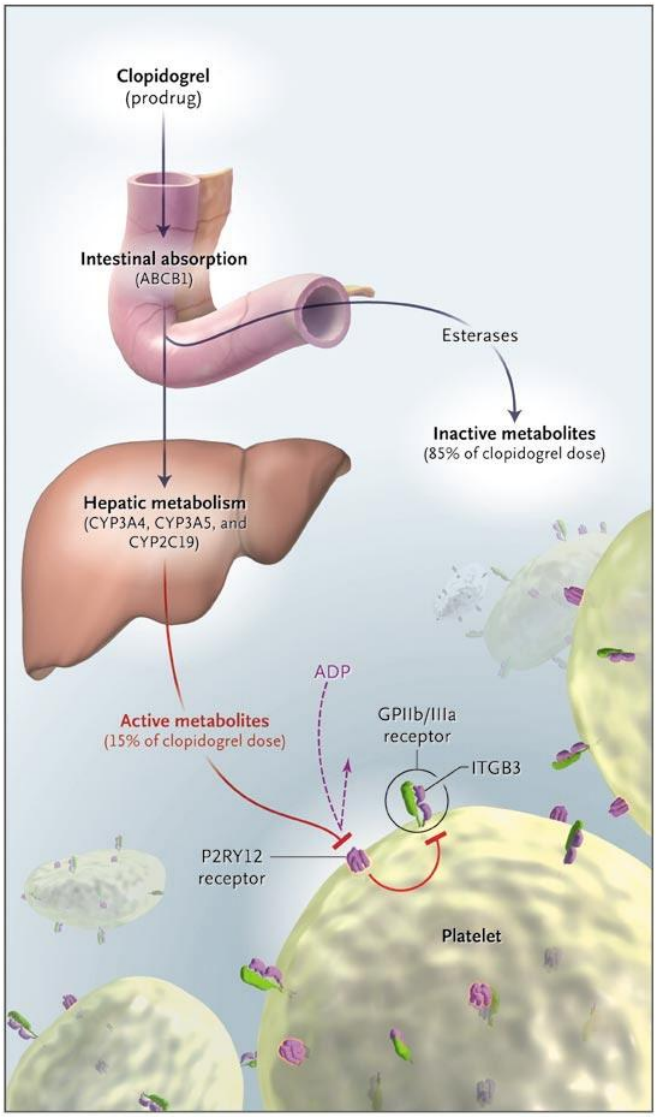


Figure 1.18a Pharmacogenomics of clopidogrel intestinal absorption.

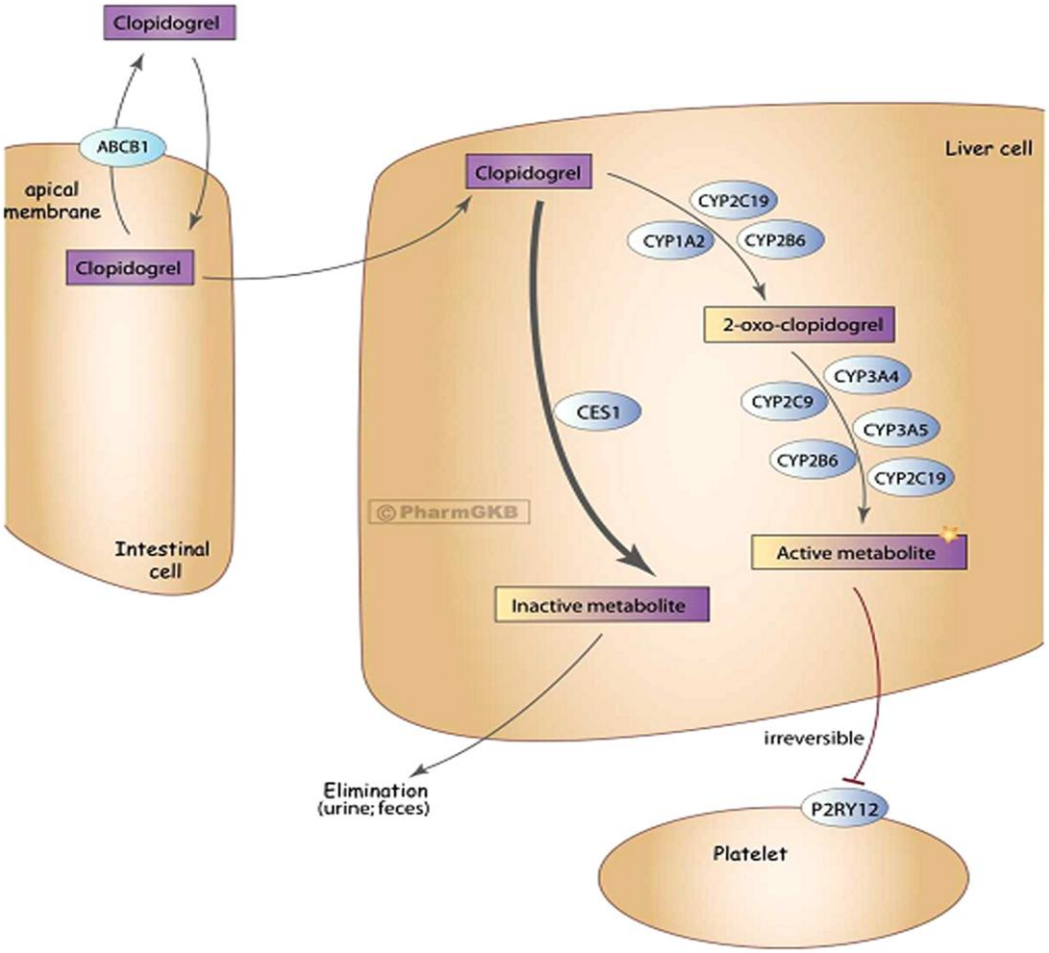


Figure 1.18b Pharmacogenomics of clopidogrel.

Pharmacogenomics of clopidogrel intestinal absorption of the prodrug clopidogrel is limited by an intestinal efflux pump P-glycoprotein coded by the ABCB1 gene. The majority of the prodrug is metabolised into inactive metabolites by ubiquitous esterases. The minority is bioactivated by various cytochrome P450 (CYP) isoforms into active metabolites. These metabolites irreversibly antagonise the adenosine diphosphate (ADP) receptor (coded by the P2RY12 gene), which in turn inactivates the fibrinogen receptor (the glycoprotein GPIIb IIIa receptor coded by the ITGB3 gene) involved in platelet aggregation. From Simon et al, N Engl J Med 2009; 360:363-37

Chapter 2 Hypothesis and aims

2.1 Study 1: ACS study

2.1.1 Hypothesis

Blood thrombogenicity is higher in patients with type 2 diabetes mellitus (T2DM) after non ST-elevation acute coronary syndrome (NSTE-ACS) despite currently recommended therapy with dual antiplatelet agents, aspirin and clopidogrel.

2.1.2 Aims

i. To quantify platelet dependent thrombus in patients 7-10 days after NSTE-ACS (two groups: T2DM and non DM)
ii. To study the association between platelet dependent thrombus and
    a. on treatment platelet reactivity upon stimulation with platelet agonists
    b. inflammation
    c. metabolic factors
    d. platelet activation markers
iii. To evaluate viscoelastic properties of thrombus after NSTE-ACS
iv. Clinical relevance of platelet dependent thrombus in T2DM after NSTE-ACS
    a. to evaluate the association between coronary atheroma burden and platelet dependent thrombus

2.2 Study2: Stable CAD study

2.2.1 Hypothesis

Dual antiplatelet therapy with aspirin and clopidogrel reduces blood thrombogenicity compared to aspirin monotherapy in patients with type 2 diabetes mellitus (T2DM) and stable coronary artery disease (CAD).

2.2.2 Aims

i. To quantify platelet dependent thrombus in patients with T2DM and stable CAD after treatment with aspirin and clopidogrel
ii. To study the association of platelet dependent thrombus in patients with T2DM and stable CAD to
    a. on treatment platelet reactivity upon stimulation with platelet agonists
    b. inflammation
    c. metabolic factors
    d. platelet activation markers
iii. To evaluate viscoelastic properties of thrombus in patients with T2DM and stable CAD
iv. To assess the ultrastructural changes to thrombus after initiation of dual antiplatelet therapy
v. To evaluate the relationship between platelet reactivity measured by “point of care” platelet function assays and platelet dependent thrombus

Chapter 3 Methods

3.1 General methods

3.1.1 Patient selection

I conducted two hypothesis led clinical studies, namely study 1 ACS study (observational cohort study) and study 2 stable CAD study (randomised, double blind, placebo controlled study).

3.1.1.1Study 1 ACS study

Patients who had non ST elevation acute coronary syndrome (NSTE-ACS) with elevated serum cardiac troponin levels were studied one week following their index coronary event. Patients with type 2 diabetes mellitus (T2DM) and those without T2DM, who were treated with secondary prevention therapy according to current international society guidelines (including aspirin 75mg once daily and clopidogrel 75mg once daily), underwent blood thrombogenicity studies including the ex-vivo Badimon chamber. The period of study was between June 2008 and May 2011.

i. Patient selection

Eligibility Criteria

a. Inclusion Criteria

1. T2DM and NSTE-ACS as defined below:
   T2DM: Diagnosed according to the WHO criteria (whqlibdoc.who.int/publications/2006/9241594934_eng.pdf, accessed on 01st August 2013), and on treatment
   NSTE-ACS: Patients admitted to hospital with cardiac chest pain and who had electrocardiographic evidence of ST segment depression and / or T wave inversion accompanied by elevated serum cardiac troponin levels, measured 12 hours after the onset of symptoms
2. Aged between 18 and 75 years
3. Provided written informed consent for participation in the trial prior to any study-specific procedures or requirements

4. Patients who had aspirin 300 mg and clopidogrel 300 mg loading doses
5. Patients who had maintenance doses of aspirin 75 mg and clopidogrel 75 mg
6. Patients with angiographic evidence of coronary artery disease, with at least one major vessel having more than 50% stenosis
7. Patients who were treated with a beta blocker (or a rate limiting calcium channel blocker if beta blockade was contraindicated), angiotensin converting enzyme inhibitor (or an angiotensin receptor blocker) and a hydroxy methyl co-enzyme A reductase inhibitor (statin)

b. Exclusion Criteria

1. Patients with ST elevation acute coronary syndrome
2. Patients who were on anti thrombotic agents other than aspirin and clopidogrel e.g. warfarin, prasugrel
3. Current smokers (as smoking could increase clopidogrel responsiveness)
4. Malignancy (currently diagnosed or under evaluation, as it could increase the prothrombotic state and bleeding risk)
5. Haematological disorders (anaemia, malignancy, bleeding disorders)
6. Use of corticosteroids or non steroidal anti-inflammatory drugs as they could increase bleeding risks
7. Chronic liver disease (cirrhosis, malignancy and patients with abnormal liver function tests, as clopidogrel bioavailability will be unpredictable)
8. End stage renal disease on dialysis as clopidogrel can be removed from the blood by haemodialysis
9. Patients who were waiting for urgent coronary artery bypass surgery, as they were considered clinically unstable
10. Patients with unstable atrial and ventricular rhythms
11. Ongoing septic illness

12. Patients who were taking aspirin at doses other than 75 mg once daily
13. Use of glycoprotein IIb/IIIa inhibitors within the previous 5 days
14. Heparin within the previous 48 hours
15. Patients with NYHA class III or IV symptoms
16. Patients who are unable to consent
17. Patients who had unstable blood glucose levels and were at high risk of fasting hypoglycaemia
18. Intercurrent gastrointestinal illness as drug absorption may be unpredictable
19. Serum cardiac troponin rise secondary to causes other than NSTE-ACS, such as cardiac failure, pulmonary embolism and sepsis
20. Use of other investigational study drugs within 1 year prior to study entry
21. Patients who are already taking part in another investigational clinical trial
22. Poor venous access

ii. Screening and recruitment

All participants were recruited from the cardiology departments of the Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne. Potential participants were identified by review of inpatient hospital records and eligibility was confirmed after obtaining permission from the responsible clinicians. Details of the study were discussed with eligible in-patients and a patient information sheet was provided. After a 24 hour “cooling off” period, written informed consent was obtained. I arranged them a single visit to the Clinical Research Facility, Royal Victoria Infirmary, Newcastle upon Tyne for the study specific procedures including Badimon chamber experiment 7 to 10 days after their index cardiac event. The study was approved by Regional Ethics Committee, Sunderland. No financial reimbursements

were offered to the participants. The study was conducted according to general principles of clinical research as per Helsinki declaration, and the patient's right to refuse to participate in the study without giving reasons was respected.

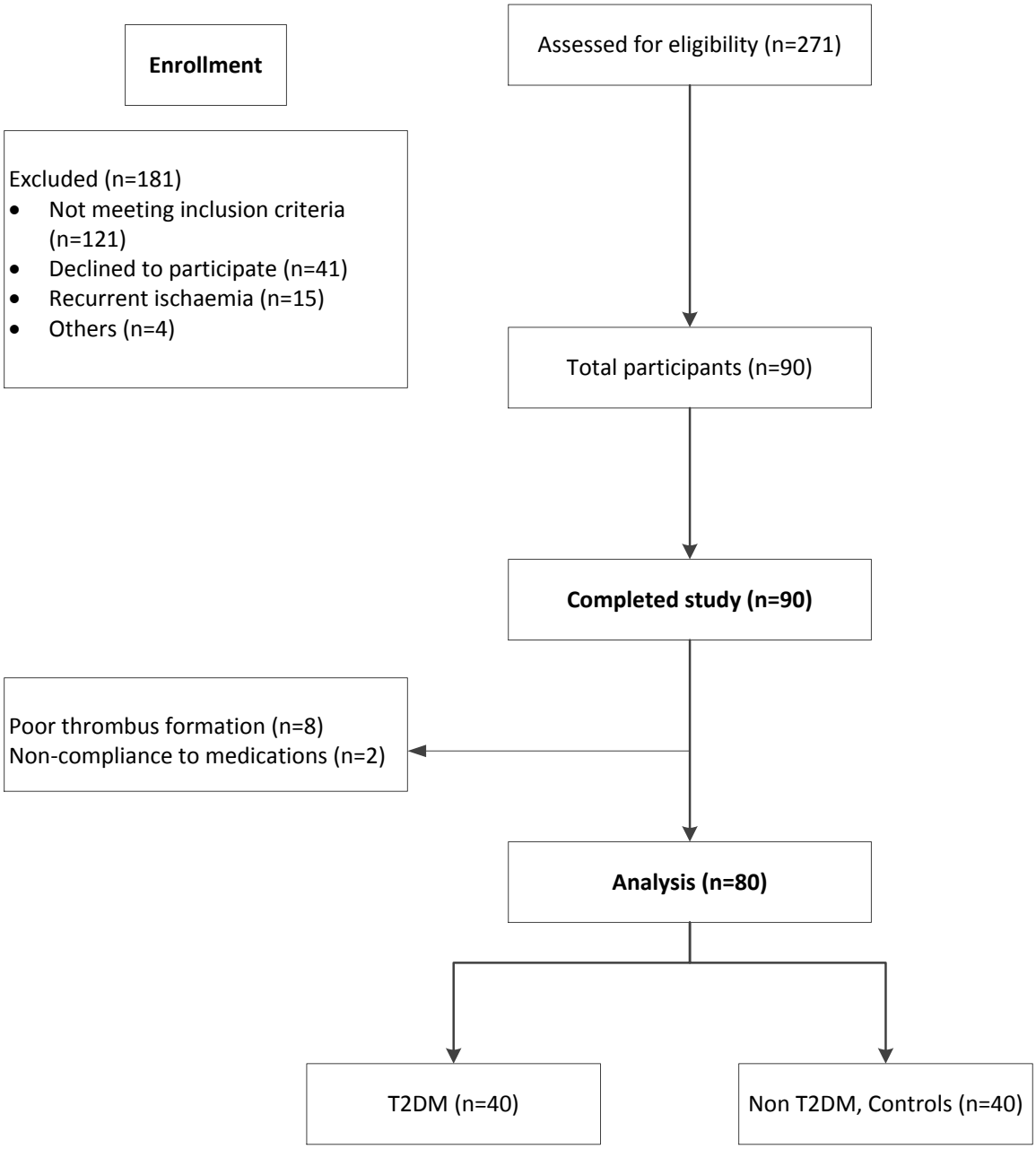


Figure 3.1 Flow chart for Study 1 – ACS study**.**

3.1.1.2 Study 2: stable CAD study

Patients who had type 2 diabetes mellitus (T2DM) and stable, established coronary artery disease (CAD) were recruited. This was a randomised, double-blind, 1:1 placebo-controlled, parallel design study, comparing efficacy of clopidogrel versus placebo in reduction of thrombogenicity in patients with type 2 diabetes mellitus and coronary artery disease, who were already taking aspirin 75mg daily. The objective of this study was to compare the effect of clopidogrel and placebo on platelet dependent thrombosis in patients with T2DM and CAD. Briefly, eligible patients with T2DM and stable CAD were studied on day 1 to assess baseline thrombogenicity. They were randomised to either clopidogrel 75mg once daily or matching placebo for 7 days and studied again on eighth day. All patients were on their routine medications as per standard practice.

i. Patient selection

Eligibility Criteria

a. Inclusion Criteria

1. Patients with T2DM and CAD as defined below

   T2DM: Diagnosed according to the WHO criteria, and on treatment, as in Study 1

   CAD: Presence of any one of the following: history of angina and positive exercise tolerance test, history of (enzyme and/or Q wave) positive myocardial infarction, angiographic evidence (>50% stenosis of one vessel) or prior history of percutaneous or surgical coronary revascularisation
2. Patients aged between 18 and 75 years
3. Patients who were taking aspirin 75 mg once daily
4. Patients who could provide written informed consent for participation in the trial prior to any study-specific procedures or requirements

b. Exclusion Criteria

1. Contraindications to clopidogrel, as per Summary of Product Characteristics (www.medicines.org.uk/emc/medicine/9483/SPC,

accessed May 5th 2013) such as history of hypersensitivity and major bleeding

2. Current smokers
3. Malignancy (currently diagnosed or under evaluation)
4. Haematological disorders (anaemia, malignancy, bleeding disorders)
5. Women of child-bearing potential as clopidogrel is contraindicated in pregnancy
6. Use of corticosteroids or non steroidal anti-inflammatory drugs as it could increase bleeding risks
7. Chronic liver disease (cirrhosis, malignancy and patients with abnormal liver function tests, as clopidogrel bio-availability will be unpredictable)
8. End stage renal disease on dialysis as clopidogrel can be removed from the blood by haemodialysis
9. Patients who were already on clopidogrel for standard clinical indications e.g. stroke and coronary stent insertion
10. Patients who were taking aspirin at doses other than 75 mg once daily
11. Patients who were taking other antithrombotic agents like dipyridamole, warfarin and low molecular weight heparin
12. Unable to consent
13. Use of other investigational study drugs within 1 year prior to study entry

ii. Screening and recruitment

All participants were recruited from the Newcastle Diabetes Centre, Diabetes clinics in general practice of Tyneside PCTs and cardiology outpatient clinics and day case unit of the Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne. Potential participants were identified by me and eligibility was verified from information documented in the case records. The patient information sheet and consent form were posted to eligible patients with a letter of

invitation at least 1 week prior to their planned outpatient appointment or cardiac day case procedure. Eligible patients then were approached in person at a suitable time point during this visit and I provided detailed verbal information regarding the study and answered any questions. Patients who provided written consent were given a further minimum of 24 hours to reflect on their agreement to participate in the study. The study was approved by Regional Ethics Committee, Sunderland. Participants voluntarily agreed to take part in the studies and no financial reimbursements were offered to them.

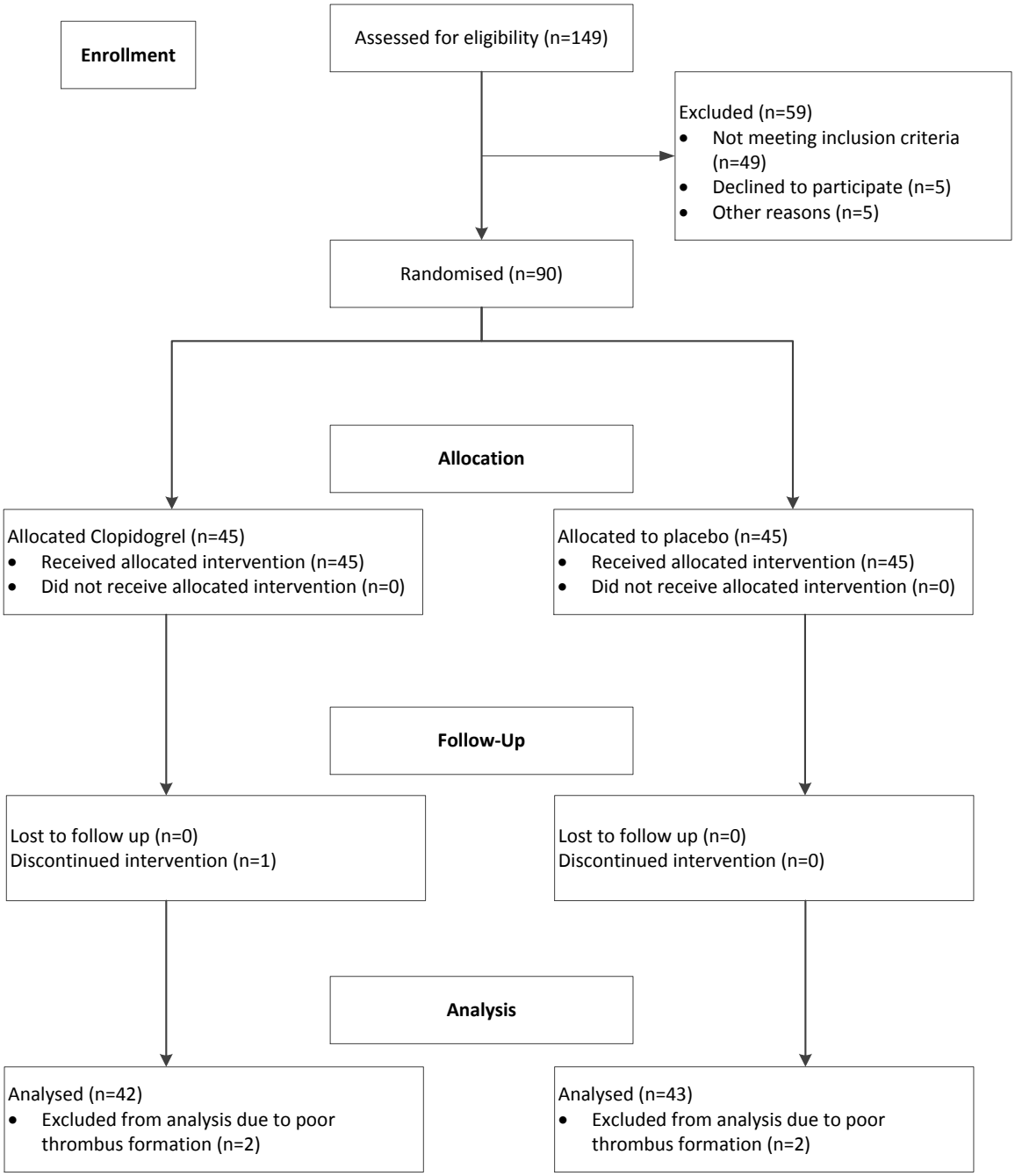


Figure 3.2 Flow chart for Study 2 – CAD study.

3.1.2 Study specific procedures undertaken by participants

3.1.2.1 Study 1: ACS study

Participants of ACS study attended their early morning appointment, (usually between 8 am to 9 am) at Clinical Research Facility, Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS foundation trust for the study after 12 hour fasting. I checked their compliance with medications by pill counting and confirmed their eligibility prior to the study. Participant's demographic data were entered in a clinical research report form. A well validated Rose angina questionnaire (Heyden *et al.*, 1971; Haywood *et al.*, 1998; Graff-Iversen *et al.*, 2008) was completed by the patient. I recorded height, weight, heart rate, blood pressure, waist and hip circumference prior to the Badimon chamber study (vide infra).

An 18G cannula was inserted in the forearm. The first 2 ml blood was discarded to avoid platelets activated by venepuncture. Ten ml blood was then collected for serum separation and then the Badimon chamber experiment was conducted. Effluent blood sample (5 ml) was collected for serum separation. Venous blood (5 ml) was collected from the antecubital vein at the end of the chamber study using the same cannula for platelet function assays and biomarker studies. The cannula was then removed. Participants were discharged from the Clinical Research Facility after consuming breakfast.

3.1.2.2 Study 2 CAD study

Participants attended two morning appointments (usually between 8 am and 10 am), one week apart at Clinical Research Facility, having fasted overnight and delayed taking their usual medications till the chamber studies were completed. At visit 1 (baseline visit), I reconfirmed their eligibility and understanding of the study specific procedure. The random number (randomisation procedure explained vide infra) assigned to the patient (sequential numbering beginning from 501) was checked independently by two members of the staff at Clinical Research Facility. Participants' demographic data including height, weight, waist and hip circumference, heart rate and blood pressure were recorded. Participants

completed the Rose angina questionnaire on visit 1 and their up-to-date routine prescription was checked.

Badimon chamber experiment was performed as described above during visit 1 and haematology, clinical biochemistry, serum and plasma samples were collected as described for ACS study. The bottle containing the investigational medicinal product (IMP), i.e. either clopidogrel 75 mg or placebo was rechecked to match the randomisation number assigned to the participant. Instructions were given to the patient to take one capsule a day starting on the day of visit 1. We provided spare capsules for compliance check with pill counting and to be used in the event of spillage or damage to the IMP. A wallet sized card with information regarding the IMP, emergency contact details of myself and principal investigator, randomisation number and sponsors of the study was provided. Participants were given breakfast and then were observed to swallow their IMP with at least 100ml water. Participants were then discharged from Clinical Research Facility. The above investigations were repeated on study day 8, after 7 daily doses of IMP. Adverse events were documented and compliance was checked by pill counting.

3.1.3 Demographic data

Demographic data was collected during patients visit to Clinical Research Facility for ACS study and during their first visit for stable CAD study. Data were collected in a clinical research proforma and stored electronically in a password operated computer in the premises. Participant's age was calculated as total number of completed years on the day of chamber study. Weight and height were measured with light clothing and without shoes, using calibrated and certified scales. BMI was calculated as body weight (kg) divided by height squared (m^2). Waist circumference (cm) was measured at the midpoint between the lower rib margin and the iliac crest. Hip circumference (cm) was measured at the level of the widest circumference over the greater trochanters. Waist hip ratio (WHR) was computed as waist circumference divided by hip circumference. I measured heart rate and blood pressure in the reclining position after 5 minutes of rest. The left arm was used for blood pressure measurements.

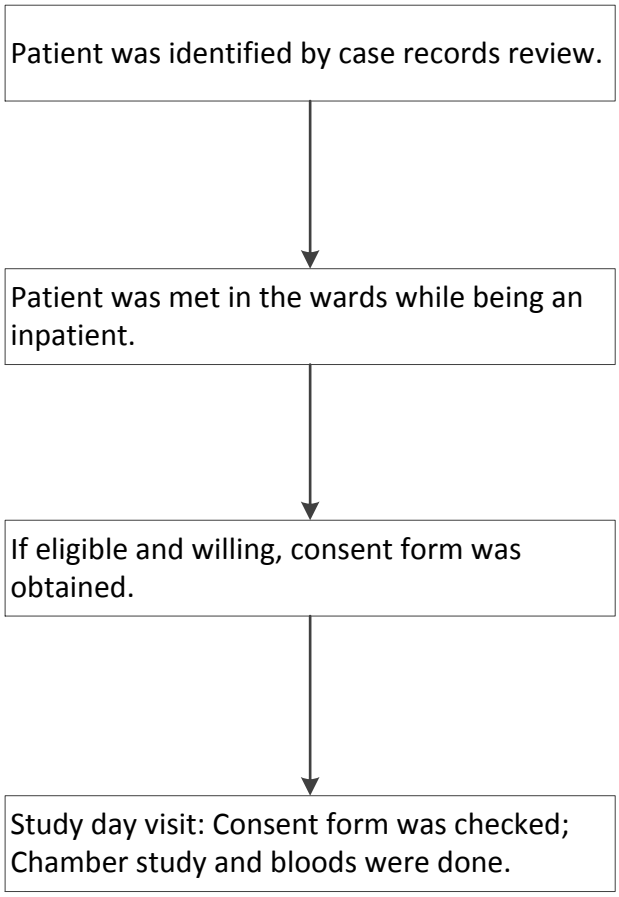


Figure 3.3 Participant Pathway for study 1 – ACS study.

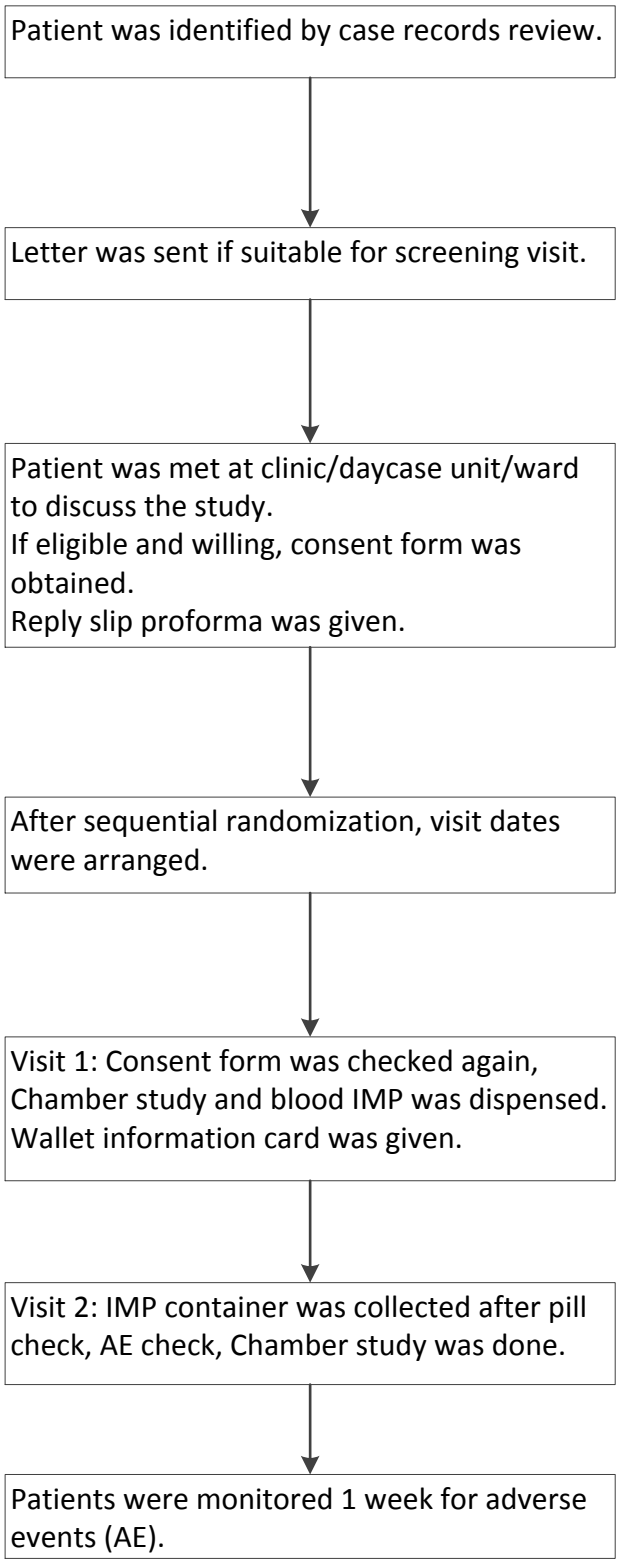


Figure 3.4 Participant Pathway for study 2 – CAD study.

3.1.4 General biochemistry and haematology

3.1.4.1 General Biochemistry

Patient's blood samples were analysed at nationally accredited clinical biochemistry laboratory at Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne (www.cpa-uk.co.uk, CPA 0925). Samples were identified by study numbers only, so that all staff were blinded to the patient's details. For the stable CAD study (double blinded RCT), statistical analysis was performed only after unblinding.

The following tests were performed by standard routine laboratory methods:

i. Serum creatinine µmol/L
ii. Blood urea mmol/L
iii. Serum high sensitivity C reactive protein, mg/dl
iv. Lipid profile (fasting) including total cholesterol, LDL cholesterol, triglycerides and HDL cholesterol, mmol/L
v. Fasting plasma glucose, mmol/L
vi. HbA1c (DCCT aligned),%
vii. Troponin I (for ACS study only), mg/dl

3.1.4.2 General Haematology

Patients' blood samples were analysed at the nationally accredited general haematology and coagulation laboratories at Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne (www.cpa-uk.co.uk, CPA 0926). Blood samples were collected at patients' only visit for the ACS study and on both visits for the stable CAD study. Samples were collected and processed according to local laboratory protocols. I preferred citrate tubes over heparinised tubes, as citrate preserves labile coagulation factors V and VII, thus ensuring better reproducibility of coagulation parameters including fibrinogen. Plasma fibrinogen was measured using von Clauss quantitative method, by measuring the time taken for clot formation after mixing with excess thrombin to plasma (Clauss, 1957). Clauss fibrinogen assay is "the most reliable

method for use in clinical laboratories" and has been standardised according to the guidelines of British Society of Haematology (Mackie *et al.*, 2003). Quality control and quality assurance were performed according to the local laboratory protocols.

Following haematology tests were performed by standard methods:

i. Red blood cell count, 10^{12}/L
ii. Haematocrit, %
iii. Haemoglobin, g/L
iv. Platelet count, 10^{9}/L
v. Plasma fibrinogen (Clauss) g/L
vi. Prothrombin time, sec
vii. Activated partial thromboplastin time, sec

3.1.5 Preparation of investigational medicinal product

For the double blinded RCT study (Stable CAD study) we used clopidogrel (Plavix®, Sanofi-Aventis) and placebo. Clopidogrel was defined as the "active" investigational medicinal product (IMP) and placebo was defined as "comparator" IMP. Both the IMP's were prepared as "look alike" to ensure blinding in accordance with the specifications approved by Medicine Health Regulatory Authority (MHRA), UK.

Tablets of clopidogrel bisulphate 75 mg (Plavix®, Sanofi-Aventis) were over-capsulated with a brownish orange coloured capsule at the pharmacy production unit at Royal Victoria Infirmary, Newcastle upon Tyne (MHRA Unit IMP license number 17736). Methyl cellulose was used as a filler to avoid movement of the clopidogrel tablet inside the capsule. Another set of capsules are filled with methylcellulose alone and labelled as placebo IMP. Both clopidogrel and placebo capsules looked alike and had similar weight.

In order to ensure uniform disintegration and gastrointestinal absorption, I conducted drug disintegration experiments in the Pharmacy production unit for both the capsules using special product testing equipment. The solution used was acidified to mimic the gastric milieu and a mechanical stirrer was used to

mimic gastric peristaltic movements. I conducted experiments for four time periods, namely 5 min, 10 min, 15 min and 30 min to document capsule disintegration and dissolution of methylcellulose. At 15 minutes, there was complete dissolution of the capsule. At 30 minutes, both the capsule and clopidogrel tablet dissolved completely. These experiments were repeated 3 times, with the same results. The experiments were supervised and certified by the Pharmacy Production unit, Royal Victoria Infirmary, Newcastle upon Tyne. The Production unit then prepared and labelled both the IMP's (clopidogrel and placebo). I was blinded to randomisation code and the pharmacy department placed 8 of the individual IMP's in the medication container labelled with study ID. I chose to use 8 capsules for the following reasons: i) pill counting to monitor compliance ii) spare IMP to be used if patients lost any capsules and iii) the volume of the container would allow maximum of 8 capsules only.

3.1.6 Randomisation and blinding

The randomisation schedule was generated by an independent team at Newcastle Clinical Trials Unit (NCTU, Newcastle upon Tyne) using the web-based service at http://www.randomization.com. The master copy of the codes was held at NCTU in a sealed envelope to facilitate unblinding at the end of the study. Two sets of sequentially numbered 100 code break envelopes were also prepared, to be held by the Pharmacy Clinical Trials and Medicines Information, Royal Victoria Infirmary and the Newcastle Clinical Trials Unit. This was done to facilitate urgent unblinding in case of a clinical emergency (in which case, pharmacy unit assumed responsibility for unblinding) or for unblinded reporting of suspected serious adverse reaction (SUSARs), in which case NCTU assumed responsibility for unblinding. Fortunately there were no SUSAR's and there was no need for emergency unblinding during the study.

A randomly permuted block system with variable block size was used to allocate patients to clopidogrel or placebo (2x2 block size but I was blinded to the block size). Randomisation on a 1:1 ratio was generated for 50 participants in each arm of the study to allow a 10% drop out rate. The randomisation schedule comprised a list of sequential study numbers (Patient Study Number: 501 to 600), and the

random treatment allocation to Clopidogrel or placebo. The open randomisation schedule was provided to the production unit at Newcastle upon Tyne Hospitals NHS Foundation Trust, who assembled and labelled study drug according to the "Patient Study Number" and "treatment allocation".

3.1.7 Pharmacovigilance and data monitoring

An adverse event (AE) was defined as any untoward medical occurrence which does not necessarily have a causal relationship with the treatment. "Treatment" included all investigational agents administered during the course of the study. Medical conditions/diseases present before starting study treatment were only considered adverse events if they worsened after starting study treatment. During the study two AE's occurred:

i. deterioration of glycaemic control

ii. self terminating minor gingival bleeding

Both were reported to the authorities and the study safety committee has decided that no further action was necessary.

As per the delegation log set for this study, I was responsible for monitoring and reporting adverse events. Any serious adverse event was deemed to be recorded throughout the duration of the trial until 5 days after cessation of study drug. Non-serious adverse reactions were monitored and recorded throughout duration of trial until 5 days after cessation of study drug either clopidogrel or placebo. Participants were provided with a wallet sized contact card to enable them to contact an appropriate member of the research team if they experienced any further events after the final visit. I was delegated with the task of informing the MHRA and main REC of all SUSARs occurring during the study. A study data monitoring committee and adverse event monitoring committee oversaw the safety of the study.

3.1.8 Statistical methods

Statistical analyses were performed using SPSS version 17.0 (SPSS Inc, NY, USA) and Stata version 12 (StataCorp LP, TX, USA) with the help of a senior statistician. Study 1 ACS study was an observational study and the dependent variable in all analyses presented was high shear and low shear thrombus. The key independent variable was type 2 diabetes mellitus. In addition to a crude hypothesis test, I performed analyses adjusting for the effects of age, sex, body mass index, waist-hip ratio, basic laboratory tests (including lipids and measures of glycaemic control), and indicators for three modalities of treatment for diabetes (diet, oral agents, insulin). Also due to the highly skewed distribution of the outcome variable, analysis was repeated with log-transformation. An analysis including fibrinogen was carried out, not to "control" for its effects, but to determine to what extent the effect of diabetes on thrombus levels is mediated through increased fibrinogen levels.

Study 2 was a randomized study, and the two study groups were compared on all study variables. For continuous variables, means and standard deviations were calculated, and student t test was used to measure statistical significance and Cohen's d was calculated to evaluate the size of group differences. For dichotomous variables, the percentage in each group was measured and chi-square estimates were used to calculate the statistical significance. I also examined the Pearson correlations of each of the variables with the thrombus measure outcomes to identify any variables which might need to be controlled for.

In the second study, because of the 2X2 block randomisation design, no potential confounding variables were identified; therefore the effect of the intervention on thrombus formation was studied without inclusion of covariates in the analysis. I estimated mixed effects models with high shear and low shear thrombus as the dependent variables. The independent variables were indicators for study group, time (baseline vs. post treatment) and the interaction between group and time. The effect of the intervention on thrombus formation is represented by the group X time interaction term (The coefficient of this term estimates the average

difference between groups of the pre-post treatment difference in outcome). A random effect at the individual patient level was included to account for the nesting of observations within patients. A p value of less than 0.05 with two tailed measurement was considered statistically significant.

For correlation analysis, Pearson's method and linear regression analysis were employed for normal data and Spearman's method and non linear regression analysis method were employed for non-normal data. Graphpad prism version 6 (Graphpad software, CA, USA) was used to analyse the data prior to constructing graphs.

Using data from our previous study (Natarajan *et al.*, 2008a), in order to detect a 20% reduction in thrombus area (I predicted a 10% higher amount in ACS patients) between the groups, with 80% power, we estimated recruitment of 45 patients with T2DM and 45 controls. For study 2 (stable CAD patients), in order to detect a 20% difference in reduction in thrombus from baseline between the two groups, (i.e. 20% reduction in active group versus 0% reduction in placebo group, alpha = 0.05 and a 2 tailed test) with 80% power, I estimated a sample size of 90 (45 patients on placebo and 45 patients on clopidogrel), each supplying data on two occasions (baseline and follow up visits).

3.1.9 Permission from regulatory authorities

1. Study 1 ACS study:

i. Main Research Ethics Committee (North East), Sunderland Reference number: 08.H0904.4
ii. Research and development department, NUTH NHS Foundation trust, Newcastle Upon Tyne, Reference number 3639b

2. Study 2 Stable CAD study

i. Main Research Ethics Committee (North East), Sunderland Reference number: 08.H0904.6
ii. Medicines and Health Regulatory Authority, London. Eudract number: 2006-003745-16; MHRA number: 31088/0006/001-0001
iii. Research and development department, NUTH NHS Foundation trust, Newcastle Upon Tyne, Reference number 3639a

3. Clinical trial registries

i. Clinical trials.org: ACS study Ref: www.clinicaltrials.gov, NCT00728286
ii. Clinical trials.org: Stable CAD study www.clinicaltrials.gov, NCT00728156
iii. UKCRN: Portfolio study ACS study number www.ukcrn.org.uk, 7338
iv. UKCRN: Portfolio study Stable CAD study number www.ukcrn.org.uk. 5159

3.2 Special methods

3.2.1 Badimon chamber

3.2.1.1 Principles of the Badimon chamber

The Badimon chamber mimics rheological conditions that favour coronary thrombosis using a deep arterial injury model. The perfusion chamber has been designed to "retain the cylindrical shape typical of the vasculature, to be flexible enough to accept a variety of biologic and prosthetic materials, and to simulate a broad range of physiologic flow conditions" in either an ex vivo or in vitro perfusion system (Badimon *et al.*, 1987). The Badimon chamber used in my study is an ex vivo model which mimics:

i. the flow conditions of normal arteries and arteries with moderate stenosis
ii. deep arterial injury (Napoli *et al.*, 2006)

This well validated chamber has been in use for over fifteen ye*ars and* recently re-validated for consistency and reproducibility in a cohort of 24 healthy volunteers (Lucking *et al.*, 2010)

Generation of platelet dependent thrombus (PDT) by Badimon chamber involves the basic physiological principles of arterial thrombosis in coronary vasculature namely,

i. collagen mediated
ii. shear induced platelet activation.

I used freshly slaughtered pig's aorta as a source of collagen in Badimon chamber experiments. The thin endothelium was surgically dissected and the tunica media, which is rich in collagen, was exposed to flowing blood. The second principle involved in Badimon chamber experiments is shear mediated platelet activation. Platelet activation occurs at high shear conditions present in the coronary circulation at the site of luminal narrowing produced by atheromatous plaques. The laminar flow of blood is disrupted and the shear rate of flowing blood increases at these sites. Thrombus generation in Badimon

chamber is favoured by the laws of physics governing Newtonian motion of particles in moving fluid, as reduction of luminal diameter increases the velocity of flowing blood in the chamber. In addition, the disruption of the laminar flow results in redistribution of platelets in a moving column of blood. Platelets are being pushed towards the periphery from the centre. This enables contact activation of the platelets with the tunica media and to one another (Ahlqvist, 2001).

3.2.1.2 Standardisation of the Badimon chamber

A series of experiments were carried out in our reference laboratory at Mount Sinai Medical School, New York, USA to assess the extent of thrombus generation at varying shear forces and duration of blood flow through the Badimon chamber. Iridium 111 labelled platelets were used to assess platelet dependent thrombus burden at

i. time intervals from 1 minute to 20 minutes
ii. flow rates at 5 to 20 ml/minute (blood velocity of 2.65 to 42.3 cm/sec)
iii. shear rates from 106 per sec to 3380 per sec (Badimon *et al.*, 1986)

Thrombus formation increased steeply from 1 to 5 minutes but reached a plateau after 10 minutes. Thrombus generation increased when flow rates increased from 5 ml/minute to 10 ml/minute but not at higher rates. The response of thrombus generation to changes in shear rates was studied both by radio labelled platelets and by image analysis. At low shear states (e.g. 100-200 s^{-1}, Reynolds number 30), fibrin rich thrombus was formed, whilst at high shear rates (1500-3000 s^{-1}, Reynolds number 60) platelet rich thrombus was formed. Overall quantitative assessment of thrombus (platelet and fibrin content combined) showed less thrombus at low shear states compared to high shear state. I standardised my Badimon chamber experiments to obtain adequate amount of thrombus (at least three fourths of the aorta substrate covered in thrombus) but using the lowest possible volume of blood. I used chambers with inner diameter of 1.0 mm (high shear chamber) and inner diameter of 2.0 mm (low shear chamber) to generate Reynolds number 60 (measure of non laminar flow), shear rate of 1920 per sec and Reynolds number 30 and shear rate of 500 per sec respectively. I generated a flow rate of 10 ml/min for 5 minutes (total of 50 mgs of

blood). 18 G cannula (inner diameter 1.2 mm, maximum flow rates 90-100 ml/min) and Tygon tube (number 14.0, inner diameter 1.6 mm) were used in all subjects to maintain the same shear force in the Chamber. The same peristaltic pump (Masterflex® pump, Thermo-Fisher Scientific Inc, MA, USA) was used in all the experiments at a fixed flow rate of 10 ml/min. Quality control of the set up was checked at fixed time intervals (vide infra in quality control section).

3.2.1.3Set up for Badimon chamber experiment

The assembly unit of the Badimon chamber consists of

i. one low shear plexi glass chamber
ii. two high shear plexi glass chambers
iii. three plexi glass over chambers with a screw to secure the substrate inside the chambers respectively
iv. four plastic connectors
v. a plexiglass container designed to accommodate the chambers connected in line with one another

Each of the chambers contain two split individual units, called the upper lid unit and lower core unit and are placed one over the other. The lower core unit has cylindrical channels carved out in the rectangular plexiglass blocks with a dimension of 1.0 mmX25.0 mm (high shear) and 2.0 mmX25.0 mm (low shear) respectively. The upper lid unit contains a rectangular plexiglass unit with a central projection of thickness 1.0 mm and 2.0 mm respectively.

Substrate for thrombus formation (i.e. porcine aorta) is held in place by the pressure of the upper lid on to the lower core. The over chamber with a screw holds together the upper and lower unit with the substrate sandwiched in between (Badimon *et al.*, 1986). The channels of the chamber are connected by plastic connectors with matching diameters. For my study I arranged the chambers in the following order, proximal to distal, low shear unit ⇨ first high shear unit ⇨ second high shear unit. This whole assembly was kept as a unit inside a plexiglass container and placed in a circulating water bath maintained at 37 degrees Celsius (Figure 3.5).

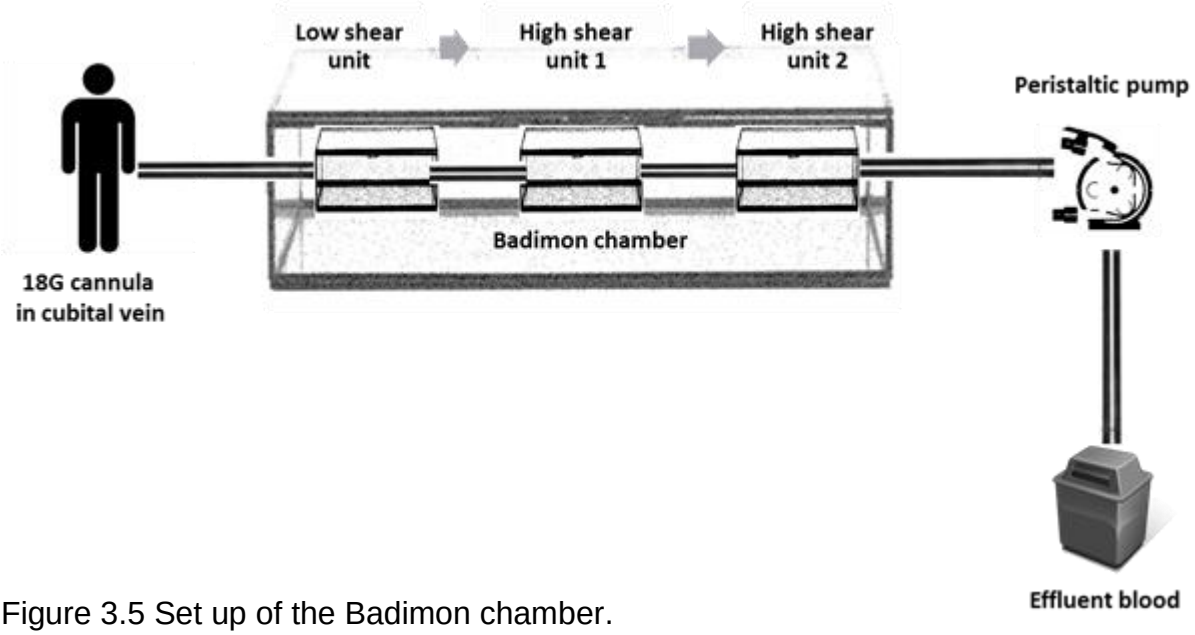


Figure 3.5 Set up of the Badimon chamber.

Blood from patients' antecubital fossa is allowed to flow through the Badimon chamber with aorta substrate via tygon tubing. The chamber was kept in a circulating water bath and was connected to a peristaltic pump at flow rate of 10ml/min. The three units of the chamber were connected in the order as low shear, high shear 1 and high shear 2 and thrombus was obtained at uniform flow conditions without any sluggish flow. High shear chamber has an inner diameter of 1.0 mm and generates shear rate of 1920 s^{-1} and Reynolds number of 60. Low shear chamber has an inner diameter of 2.0 mm and generates a shear rate of 500 s^{-1} and Reynolds number 30. The effluent blood was discarded except for few ml to perform biochemical analysis.

Aortas of untreated and freshly slaughtered pigs were purchased from a local abattoir. The thin endothelial layer was stripped off to expose the underlying collagen rich tunica media. The aortas were then surgically dissected into pieces of 15X35 mm size. Excess tunica adventitia layer was gently trimmed to accommodate the aorta inside the chamber. The aortas were inspected to rule out presence of any flaps or damage to the inner layers using 5X magnification glass. They were then immediately submerged and stored in 0.01M phosphate buffer solution at minus 80° Celsius. Aortas were slowly thawed overnight at 2-8 degrees and brought to room temperature slowly just before the chamber studies. The aortas were visualised with a 2.5X magnifier glass and examined for any flaps or damage to media layer. Only aortas without dissection artefacts were selected to be used in the chamber. The rest of the aorta pieces were disposed according to our local research facility and laboratory guidelines. The flow chamber unit was constructed as a tubular channel with upper hemisphere covered by aorta tissue with exposed tunica media and lower hemisphere by the plexiglass groove in the lower core unit. Segments of aorta are fed in to the top core of the chamber unit and approximated with bottom core cylindrical unit. The outer chamber was used to hold the halves in place, using a screw on its top. This enables the flowing blood in the channel to come in contact with the exposed tunica media of the porcine aorta.

The chamber was maintained in a water bath at 37 °C. Using 14.0 Tygon® tubing (Cole Palmer, IL, USA), the output of the chamber was connected to a peristaltic pump (Masterflex, model 7013) which regulated flow at a rate of 10ml per minute. Initially, to flush and prime the system, 0.01M phosphate buffer solution (PBS) was drawn through the system. Then, native (non-anticoagulated) blood was allowed to flow from the ante cubital venous cannula into the perfusion chamber for exactly 5 minutes. The effluent blood was collected during the 3rd minute, into plain and 3.2N sodium citrate tubes. After 5 min, PBS was again drawn through the chamber for 30 seconds under identical flow conditions to remove unattached cells and blood overlying the formed thrombus on the substrate. The changes from buffer to blood and vice versa were achieved manually by a three-way *valve* without stasis in the chamber.

3.2.1.4Connectors of Badimon chamber

Connectors used in the Badimon chamber have satisfied the following principles:

i. Non thrombogenic
ii. Able to generate and sustain the expected shear rates of (200-300 per sec and 1900-2000 per sec)
iii. Fit tightly in to the peristaltic pump.

I used Tygon® tubing (Cole Palmer, IL, USA) which satisfies all these conditions. Venous cannula inserted to patients forearm was connected via a three way tap (Alaris®,MFX2280E, Cardinal Health Inc, Rolle, Switzerland) to the low shear chamber.

3.2.1.5Phosphate buffer solution

0.01 M Phosphate buffer solution (PBS) was prepared from commercially available RNAse and DNAse free PBS concentrate in powder form (Cole-Palmer inc, MA, USA). I reconstituted the powder to prepare fresh 0.01M solution using deionised water.

3.2.1.6Histopathology methods

Aorta tissue with thrombus was carefully removed from the chamber units, to avoid any dislodgement of fresh thrombus. Individual aorta segments were then preserved in 10% buffered formalin. After 72 hours of fixing in formalin, the aorta segments (one low shear and two high shears) are sectioned into eight pieces each approximately of 1mm width (Natarajan *et al.*, 2008a). The segments are labelled as follows and placed in separate histocassettes:

i. Low shear chamber unit : A1,A2,A3,A4,B1,B2,B3,B4
ii. High shear chamber unit 1: C1,C2,C3,C4,D1,D2,D3,D4
iii. High shear chamber unit 2: E1,E2,E3,E4,F1,F2,F3,F4

The aorta samples were then embedded in liquefied wax. The tissue blocks in wax were sectioned to 0.4 µm width sections using microtome and stained using modified Masson trichrome stain (method vide infra) and placed in a glass slide.

Sectioning and staining procedures were carried out in Cellular Pathology department, Royal Victoria Infirmary.

3.2.1.7Staining of the thrombus

The thrombus in the aorta was stained with Masson trichrome method, a three colour staining procedure. After staining, the thrombus with fibrin appears red, the elastin of the aorta substrate appears green and the aortic smooth muscle appears pink. The original Masson trichrome staining was first described in 1929 (Bancroft JD and M, 2002). The technique was modified to enhance the thrombus over the aorta and also to clearly demonstrate the plane of cleavage between the thrombus and tunica media of the aorta substrate. This modification has been validated by Thrombosis Research Laboratory at Mount Sinai Hospital, New York (Osende *et al.*, 2001).

Step by step method of the staining is given below. Briefly, the slides were serially rinsed in xylene and alcohol of varying strengths. They were then stained with Weigert's haematoxylin solution to stain the cellular nuclei and then Ponceau red to stain fibrin. After staining with Light Green to stain elastin fibres, the slides were quickly rinsed through alcohol of varying strengths and xylene. The specimens were then air dried and cover slip was mounted.

3.2.1.8Image acquisition

The slides were viewed using a high precision light microscope (Leica DM 2000, Leica Microsystems, Wetzler, Germany). The microscope was calibrated according to manufacturer's standards and cleaned and inspected for the integrity of the components with the help of a senior technician twice a year. A standard template image was inspected every six months and matched to ensure correct optimal illumination and precision. The thrombus images (10X magnification) were identified (pink to red) on the top of the raised section of the tunica media of the aorta tissue (green). A digital camera with a very high definition resolution of 1360X1024 (KY-F1030, JVC, Japan) was used to capture the images electronically. This camera was connected using an IEEE1396 electronic link and an inbuilt TWAIN driver. This allowed the images to be

digitised without losing precision, using special software (What You See Is What You Get software (WYSIWYG) ®, JVC, Japan).

3.2.1.9 Thrombus area measurement

Image analysis of the thrombus image was carried out using the specialist software, Image-Pro Plus®, Version 4.0 (Media cybernetics Inc, MD, USA), calibrated using the standard micron calibration scale supplied by the manufacturer. Acquired images in .jpeg format were opened using this software. Brightness and contrast of the images were standardised using an "automated best fit algorithm" of the software. This enhanced the contrast of the thrombus portion of the image. The thrombus was identified as 'the area of interest'. Using the tool 'irregular area of interest (AOI)' in the software, the thrombus was carefully traced with the help of a cursor. The thrombus aorta interface was delineated and utmost care has been taken not to include the tunica media of aorta as an area of interest and to correctly identify the plane of cleavage between the thrombus and the underlying aorta. Once the area of interest has been distinguished from the rest of the aorta tissue, the thrombus area has been measured using the dark object identification algorithm. Briefly, the thrombus in the delineated section of the image appeared darker than the plain light background image and was counted as multiple individual dark objects. The area of the thrombus was quantified as the sum area of the multiple individual dark objects. The thrombus areas of each of the 24 sections of aorta (8Xlow shear and 16Xhigh shear) were counted. The mean thrombus area was obtained for low shear and high shear sections of the thrombus and expressed as μ^2 per millimetre aorta surface area (μ^2/mm).

Modified Mason trichrome staining

1. Mix 150 ml of Weigart's haematoxylin solutions A and B.
2. Take the slides serially to solutions of XyleneX2, 99% alcoholX2, 95% alcohol and 75% alcohol.
3. Leave in Weigart's solution (A and B) for 10 minutes.
4. Run in tap water.
5. Dip in acid alcohol and then run in tap water.
6. Dip in Scotts tap water and then run in tap water.
7. Dip in 2% Poncaeu Red in acetic acid for 10 minutes.
8. Wash in distilled water.
9. Leave in 2% light green for 40 seconds.
10. Dip in distilled water and then 2% acetic acid
11. Run in tap water and dehydrate quickly, 70% alcohol, 95% alcohol, 99% alcoholX2, XyleneX2.
12. Put in slide holder and paste cover slip.
13. Blot with tissue and arrange the slides with cover slip.

Figure 3.6 Stepwise protocol for modified Mason trichrome staining.

The images were checked for quality control (vide infra) by two observers, myself and the senior experienced investigator Prof Azfar Zaman. We excluded the sections of aorta deemed unsuitable for analysis as follows:

i. Sections with flaps: may lead to sluggish flow and over estimation of the thrombus
ii. Sections which were damaged by tissue microtome: may underestimate the thrombus
iii. Sections with dye artefacts and cover slip artefacts
iv. Sections with very negligible thrombus (less than 500 μ^2/mm), wash artefact
v. Sections with red cell thrombi (appears orange in colour), indicative of sluggish flow in the chamber

We excluded 3.75% of the thrombus images from final analysis (162/4320 images).

3.2.1.10 Quality control

I performed assessment of the circuit using 0.01M phosphate buffer solution. Every day before the chamber study, I checked the integrity of the circuit by priming with the solution at 10 ml/min (standard laminar flow) and 150 ml/min (high shear, non laminar flow).

I performed quality control of the chamber study once every four months using 30 g of coffee powder mixed with 50 ml of 4% cream and 50 ml of water (1:1) to mimic the flow conditions of viscous blood. Deposition of the coffee powder particles was examined during uniform flow and shear conditions under magnifying glass (X4) and the presence of coffee powder in places other than the central three fourths of aorta was considered non uniform flow. Thirdly, a control study with a volunteer (myself) was performed once a year, in exact similar conditions to the patient study. Thrombus area in three studies was not statistically different (mean thrombus area μ^2/mm, 9218, 8878, 9881).

I standardised and performed quality control exercises during image analysis. Two independent observers (myself and Dr Azfar Zaman) analysed 10% images

chosen at random. Images were labelled with dummy numbers by a third observer, to ensure blinding. The inter-observer co-efficient of variation of thrombus area was 5.8 %. I also quantified thrombus area in these same images on three occasions on three consecutive days, with an intra-observer coefficient of variation of 4.5 %. To ensure uniformity of measurements, I measured thrombus area of these standard images every four months and the variability was 3.6% (range 0.4 to 5.0). I also performed duplicate thrombus area measurement for each set of aortas. Four sections were randomly chosen from each set of images (four sections out of 24, from every Badimon chamber assessment). The median co-efficient of variation was 1.6 percent (for 4230 images, range of variability was 0.4 to 3.2). In addition, another two independent but in-experienced observers (Prof Sally Marshall and Dr Kath White) have analysed the 24 images with a co-efficient of variation of 9.6% Besides, I used two different versions of Image Pro-Plus software (version 4.0 and version 7.0) held in two different computers to assess inter software variability and the coefficient of variation was 4.9 %.

3.2.2 Ultrastructural assessment of thrombus using Scanning electron microscopy (SEM)

3.2.2.1 Introduction

Ultrastructure assessment of thrombus includes studying the architecture of cellular component (platelet) and fibrinous component (fibrin fibres) of the formed thrombus. Weisel et al have first documented fibrin fibre arrangement of thrombus using electron microscopy as early as 1981(Weisel *et al.*, 1981). Badimon et al have shown thrombus structure in a small cohort of patients who were given iridium labelled platelets (Badimon *et al.*, 1987). Not much progress has been made in quantitative electron microscopic assessment of thrombus due to difficulties in sample preparation and time consuming and error prone quantitative assessment methods (Collet *et al.*, 2005). Current techniques therefore focus mainly on fibrin structure derived from platelet poor plasma rather than whole blood. Use of semi automated image analysis software (e.g. J software, http://rsbweb.nih.gov/ij) has given new impetus in quantitative assessment of whole blood thrombus. However, the quantitative assessment of thrombus from SEM images is still not yet formally standardised and remains in its infancy.

3.2.2.2 Operational characteristics of SEM

Electron microscopy (EM) uses a “beam of energetic electrons to examine objects on a very fine scale”. EM was developed due to the limitations of light microscopes, which cannot generate images of higher magnification than 1000X. Scanning electron microscopy (SEM) uses the principles of signal generation when high voltage electron beams are focussed on a smaller area on the surface of the solid biological sample. The signals that derive from electron sample interactions reveal information about the sample including external morphology, components of the sample, crystalline structure and orientation of individual subunits of the sample. The electron beam receiver collects data over a selected area of the surface of the sample, and a 2-dimensional image is generated that displays spatial variations in these properties. Areas ranging from 1 cm to 5

microns in width can be imaged using conventional SEM techniques (magnification ranging from 20X to approximately 30,000X, spatial resolution of 50 to 100 nm).

Components of SEM include i) electron optical column with a source to produce electrons and electromagnetic coils to control and modify the beam ii) vacuum systems within a chamber which “holds” vacuum to minimise artefacts and iii) signal detection and display unit which consists of detectors that collect the signal and electronics to produce an image from the signal.

The physics behind the image acquisition and analysis is beyond the scope of this thesis. Briefly, accelerated electrons in an SEM carry significant amounts of kinetic energy, and when the incident electrons hit and decelerate at the surface of the sample, this energy is dissipated as a variety of signals. These signals mainly include secondary electrons which are refracted and reflected back to the detector. SEM generates powerful electric and magnetic fields for separating trajectories of backscattered electrons and secondary electrons generated from a specimen. A backscatter electron detector covers the trajectory of the backscattered electrons. Since secondary electrons and backscattered electrons can be detected efficiently in a separate fashion at a low accelerating voltage and the detector is strategically placed to avoid the pathway of the primary electron beam, backscattered and secondary electron images of high resolution can be obtained.

Transmission EM (TEM) is another high resolution EM and can be used to image very smaller objects and magnify up to 450,000X. I decided to use SEM rather than TEM for the following reasons:

i. SEM is better suited to study surface of the thrombus formed in Badimon chamber as TEM may focus on a very minute area of the thrombus
ii. thrombus preparation on a very thin slice of aorta for TEM will be technically challenging
iii. fibrin to fibre interactions are best studied using SEM

iv. cross sectional imaging of thrombus by TEM will be very time consuming and less likely to yield more information than what is already known

3.2.2.3My study protocol

Detailed step by step process has been explained below. With the help of the senior operators at Electron Microscopy services, Newcastle University, I performed a series of pilot images acquisition. For better reproducibility and speed of processing, I standardised the pilot image at approximately 60X magnification using an 8 kilo Voltage electron beam and the sample kept at 6mm distance from the electron gun. For high power I chose 3,200X magnification and for ultra high power, 15,000X magnification as standard for this study. I chose to analyse 20 random patient samples (10 in each group) on both visits. The samples were identified by the study identification numbers, chosen randomly by an independent member of our team and I was kept blinded to the choice of therapy of the patients during the analysis. There was no published literature available on SEM analysis of thrombus on which to base a power calculation. I chose 20 as my sample size as I estimated image analysis of at least 8 patients per treatment group per visit accounting for at least 20% sample artefacts. Each patient had three aorta specimens, one from low shear and two from high shear and therefore I anticipated a minimum of 48 individual thrombus specimens can be used for analysis for each group. Thrombus laden aorta samples from the Badimon chambers were fixed in 2% glutaraldehyde. A sample of 1mm width is taken from each chamber (1Xlow shear and 2Xhigh shear) at the median point of the aorta. After 72 hours of fixing, the thrombus specimen was serially treated with varied concentration of ethyl alcohol. After critical point drying, the samples were mounted using silver adhesive. It was then gold coated in a specialised chamber and the samples were loaded to SEM. Standard pilot images were taken at 60X magnification. Six images were taken at high power $3.2X10^3$ magnification and further six images were taken at $15X10^3$ magnification. The areas of interest were identified using a validated random grid model using 2X3 squares (Silvain *et al.*, 2011). At least 80 fibrin fibres and 200 individual platelets

were analysed per sample per visit for diameter analysis. Total numbers of fibres were counted in a square grid model with area of 36 μ^2. All the images were analysed by me completely blinded to treatment allocation.

The images were analysed using Image ProPlus® software 4.0.1 (Media Cybernetics Inc, MD, USA). I first focussed on the standard pilot image (60X magnification) and identified platelet rich areas and fibrin areas using a simple grid analysis model and calculated proportional content of each of them. This did not provide the absolute value of platelet or fibrin content of thrombus but percentage area occupied by each of the components. Internal validation was performed and intra-observer's COV (GV) was 11.5% and inter-observer's COV (GV vs KW) was 16.5% for platelet-fibrin content of thrombus.

I then proceeded with high power images of $3.2X10^3$ magnification and focussed on two distinct structures, platelets (disc shaped) and fibrin (filament shaped)

Platelets in the sample were identified by the following validated structural features, Figure 3.7 (Williams Hematology, 8th edition, McGraw Hill, New York, USA):

i. disc shaped structure
ii. presence of pseudopodia
iii. presence of filaments
iv. presence of exosomal globular structures (exocytosis of golgi apparatus and granules)
v. presence of clumping

Fibrin fibres were identified by the following validated features, figure 3.8:

i. long filamentous architecture
ii. presence of interlinkage between filaments
iii. attachment to platelets
iv. twisting of filaments
v. entrapment of platelets

The "hub and spokes" or "neps" model is routinely used in commercial cotton industry to identify the quality of individual cotton fibres and standardisation of the quality of the cotton yield. Entanglement of cotton fibres known as "neps" is

equivalent of the “hub and spokes” arrangement of fibrin fibres seen in the thrombus. Presence of fewer “neps” is deemed as a pre-requisite for premium quality of yield and thus used in pricing of wholesale cotton and forms a part of essential benchmark tool and a specific cotton industry standard known as Uster® statistics (www.ars.usda.gov/main/docs.htm?docid=5260&page=10, www.uster.com/en/service/uster-statistics accessed on 23rd Aug 2013). I elaborated this concept of “neps” and hypothesised that the presence of higher number of “hub and spokes” would limit the energy to be used in individual fibrin fibre polymerisation and thus make them more susceptible for autolysis. I visited cotton standardisation laboratories in Liverpool, UK and Coimbatore, India and the “hub and spokes” model of thrombus assessment was created following the discussion with the senior laboratory technicians.

Dendrite identification algorithm of Image Proplus version 4.0.1 was used to study the fibrin to fibrin interlinkage. This semi automated software selects the presence of fibrin filaments in the thrombus and can be used to count the numbers and measure the size of individual fibres. More specifically, the dendrite function algorithm identifies fibrin-fibrin interlinkage in a predefined area of thrombus with multiple fibrin fibres. The “hub and spokes model” was overlapped with the image to study individual fibrin to fibrin interaction. “Hub” was defined as presence of three or more individual fibrin fibres intersecting each other at three different angles with at least 20 degrees in between. Those with angles less than 20 are defined as parallel fibrin fibres. This model was introduced to study the compactness of the thrombus. Individual fibrin fibres were known as spokes. In addition, as the thrombus was heterogeneous in any given field in SEM, I measured clusters of hub and spokes per square micron of the field, providing a quantitative assessment of compactness of the thrombus. For quality control, every fourth sample was re-analysed and if the differences in fibrin diameter or platelet diameter were more than 10%, analysis was performed for the third time and the results of the third analysis was included in the analysis.

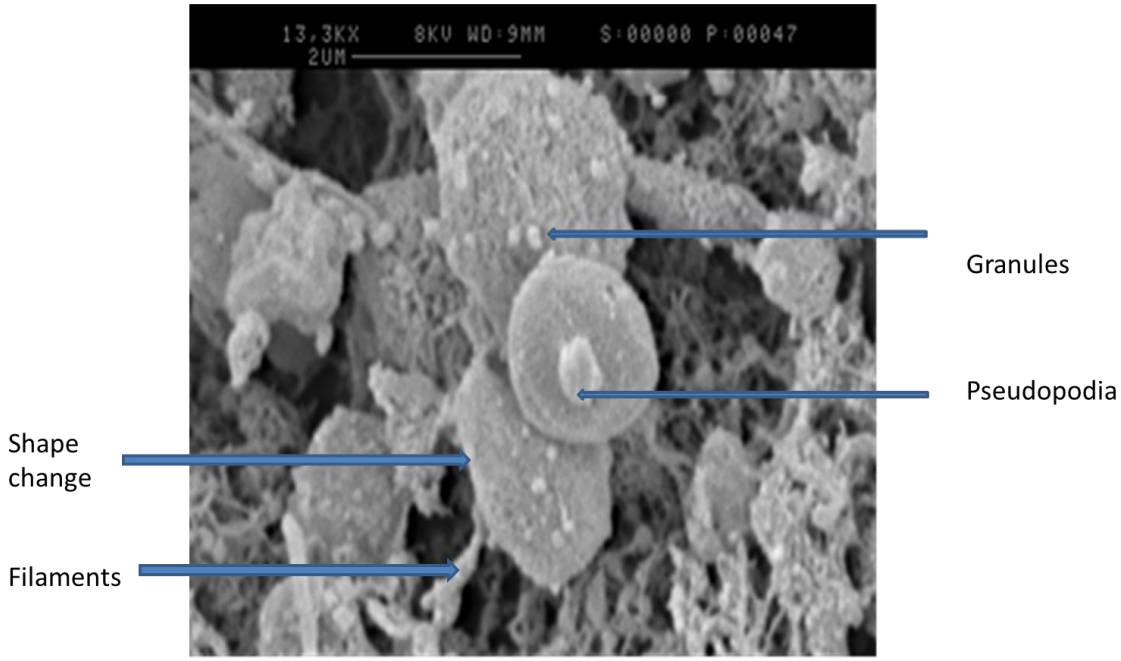


Figure 3.7 Electron microscopy appearance of platelets.

Electron microscopy appearance of platelets in various stages of activation was seen at magnification of 13,300 times and at 8kV energy. Platelets were characterised by the presence of pseudopodia at the middle of the figure which was seen as an “end on” appearance. The presence of granules was seen as small projections on the surface of the platelets. The platelets were seen here at various stages of changes in shape. The fibrin filaments are seen bridging the adjacent platelets there by resulting in a platelet clump.

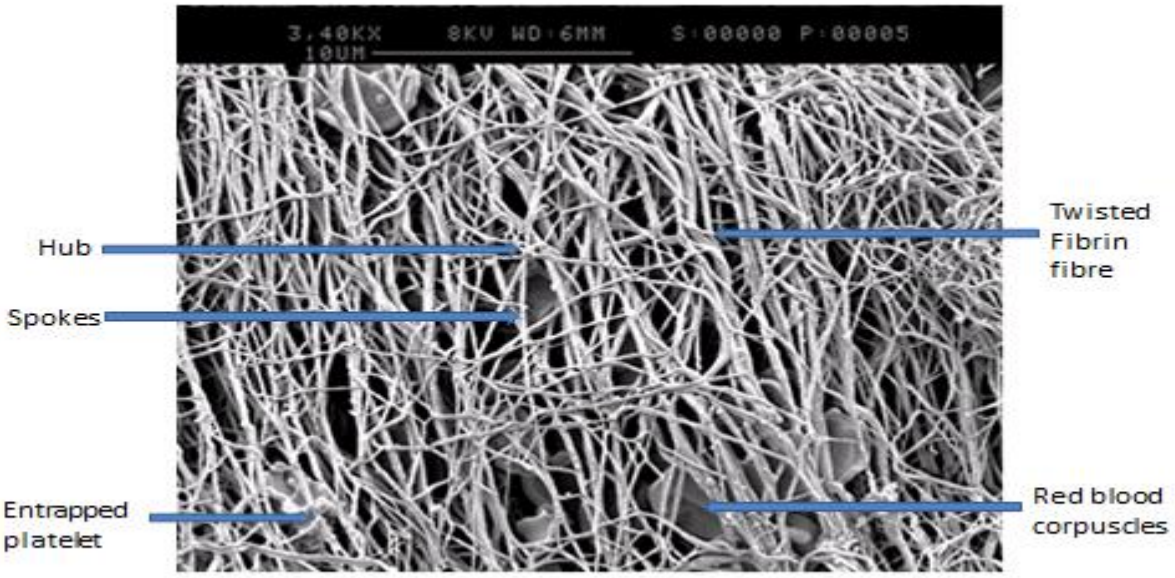


Figure 3.8 Electron microscopy appearance of fibrin fibres.

Fibrin fibres were viewed at 3,400 times magnification by scanning electron microscopy. Fibrin fibres were arranged in longitudinal pleated structure with trapped RBC's and platelets. The presence of hub and spoke appearance was also seen in this section with lateral fibrin fibre interlinkages.

3.2.2.4Step by step study protocol

SEM analysis of the thrombus consisted of three distinct stages, sample preparation, image acquisition and image analysis. My protocol is given below.

i. Sample preparation

1. After Badimon chamber study has finished, carefully detach the chambers
2. Dissect 1 mm width of aorta laden with thrombus at the median point, using a precision blade/scalpel. Take care not to disturb freshly formed thrombus. Repeat for the two other chambers, so that three specimens will be obtained (one low shear, 2Xhigh shear). Fix immediately in 2% glutaraldehyde in Sorensons phosphate buffer
3. Preserve the specimen at 5°C for at least 72 hours. Take the fixed thrombus sample and rinse twice for 15 minutes using 0.01M phosphate buffer solution
4. Proceed to step by step dehydration using various strengths of alcohol as follows: (gradual increase in ethanol concentration to minimise artefacts)
   a) 25% ethanol for 30 minutes
   b) 50% ethanol for 30 minutes
   c) 75% ethanol for 30 minutes
   d) 100% ethanol one hour
   e) 100% ethanol for one hour
5. Perform critical point drying using Baltec critical point dryer. Once dried, samples are mounted on an aluminium stub with sample number written on the bottom surface. Use Achesons silver Electrodag as an adhesive on the adventitial side of the aorta
6. Using Poloron SEM coating unit, with argon vapour, colloid gold was spray coated (up to 15 nm thickness) on the surface of the thrombus

ii. Image acquisition

1. Load six gold coated samples in the aluminium stubs in the SEM tray
2. Open the door and load one tray in to the holder
3. Adjust the electron detector
4. Turn on SEM machine to generate vacuum
5. Adjust the green flicker from tungsten filament to be a sharp image
6. Turn on image detector and the sample will appear in the screen
7. Use the recommended settings to get a magnification of 60-72X times as a basal image
8. Acquire the image to the folder in the PC using Olympus software
9. This image is the pilot image and check patients ID number
10. Divide the sample to 6 squares as a major grid. One image to be obtained from each major grid
11. Re focus to the square of interest, start with left top square
12. Divide the grid to 4 squares as high power grid. Choose one square at random and focus at 3,200 to 3,600X magnification. Fine tune the image to get better contrast. Acquire and store in the PC as in step 8
13. Divide the high power image as a 4 square ultra power grid. Choose one square at random and focus at approximately 15,000X magnification. Fine tune the image and acquire the image. Store in PC as in step 8
14. Return to low power at 60-70X magnification and move to next square in pilot grid, bottom left corner. Repeat steps as above
15. Repeat image acquisition for middle top, middle bottom, right top and right bottom squares. There should be one high power and one ultra power image for each major square in the pilot grid
16. In total each sample will have six high power (X3200 times magnification) and six ultra high power (X15000 magnification). There will be six samples for each patients (3 from visit 1 and 3 from visit 2) generating 36 samples for each patient

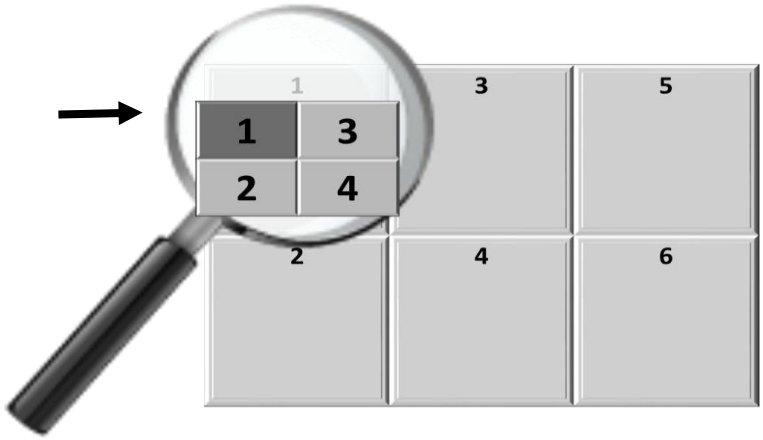


Figure 3.9 Image acquisition in SEM – Major Grid

The sample is divided into 6 squares as a major grid. One square is chosen and divided into four high power grids (squares). One square was chosen at random for high power image (3200 to 3600 times magnification) per one major grid of the pilot image. Similar steps were repeated for ultra power image (15000 times magnification) per one major grid of pilot image. Solid arrow indicates the area of interest in the pilot image of high power grid.

17. Once completed, back up the images in the portable hard drive
18. Turn down filament, and leave SEM under vacuum

iii. SEM image analysis

1. Open image Proplus and SEM image
2. Open pilot image (usually called as LP1 image).
3. Open measure ⇨ Calibration ⇨ Spatial ⇨ Select SEM pilot ⇨ Select
4. Open Process ⇨ Grid mask ⇨ GV1 grid ⇨ Apply
5. Count the stars in the dark areas (or fibrin rich thrombus) and total number dots. Calculate the percentage
6. Proceed to open HP1
7. Classify the image as fibrin rich or platelet rich image
8. If PLATELET RICH, Open process ⇨ GV2grid ⇨ Select lines ⇨ Apply
9. Choose 4 random squares
10. Calibrate the scale
11. Count number of cells and record the diameter of the cells. Use open L rule
12. If FIBRIN RICH, Open process ⇨ GV2grid ⇨ Select lines ⇨ Apply
13. Choose 4 random squares
14. Select using a rectangular square
15. Open measure and count size
16. Select measurements--called dendrites- and click OK
17. Select automatic bright objects, 8 hole filler, accumulate counts
18. Select count
19. Select view -statistics - and total number of cells and valid counts (last two lines)
20. Select view and look for measurement data then select sort descending-and count hub numbers (those with over 30 spokes aka dendrites)
21. Select measure and cluster and note down cluster values
22. Copy data in Microsoft Excel sheet (four values: All, spokes, hub, cluster)

23. Repeat for three more squares
24. Follow similar procedure for all HP images
25. Proceed to open UP1 image
26. Open process ⇨ GV3Grid ⇨ Select lines ⇨ Apply
27. Choose 1 random square from 4
28. Count number of vertical and horizontal fibres
29. Calibrate ⇨ Spatial calibration ⇨ New
30. Choose Unit ⇨ µm ⇨ Calibrate the scale
31. Click ⇨ Measure ⇨ Calliper ⇨ Single line image ⇨ calibrate
32. Before measuring, check with reference line and measure its thickness
33. Use clip board function and store as note pad
34. Convert note pad files using .csv function to Microsoft Excel format

3.2.3 Assessment of viscoelasticity by Thromboelastography® (TEG) and Platelet Mapping™

3.2.3.1 Principles and technique

Thromboelastography is a point of care technique which measures the viscoelastic property of whole blood. It provides a global measurement of haemostatic system function and can be used to identify defects in thrombus formation and auto lysis. TEG® is used to assess bleeding tendency and to monitor blood and blood product replacement after major cardiac and hepatobiliary surgery. TEG® provides a dynamic and real time assessment of the elastic property of the thrombus (Reikvam *et al.*, 2009).

The TEG® consists of two mechanical parts: a heated (37 °C) oscillating cuvette or cup, and a stationary pin which is suspended in the centre of the cup and connected to a torsion wire (Figure 3.10). Blood is placed in the cuvette and, whilst the sample remains liquid as unclotted blood, the motion of the cuvette does not affect the pin. However, when thrombus starts to form, the fibrin strands "couple" the motion of the cup to the central pin. The viscoelastic force of the blood is thus transmitted via the pin to the torsion wire. The cup is rotated in an oscillating fashion, 4.45 degrees every 10 seconds both clockwise and anticlockwise. As fibrin and platelet aggregates interlink, they connect the inside of the cup with the metal pin, transferring the energy used to move the cup to the central pin. The torsion wire, connected to the pin, measures this kinetic energy as viscoelastic strength of the thrombus over time, and converts the data to a digital form which is displayed as a graph. TEG® also evaluates clot lysis or retraction by measuring the decrease in the strength of the thrombus, causing the amplitude (A) of the TEG® tracing to decline from its maximum strength (MA). By measuring the tensile strength of the thrombus continuously, TEG® results reflect dynamic whole blood thrombosis including platelet aggregation, platelet to fibrin interaction and fibrin polymerization. However, TEG® also measures the velocity at which the thrombus forms known as clot kinetics (Figure 3.10).

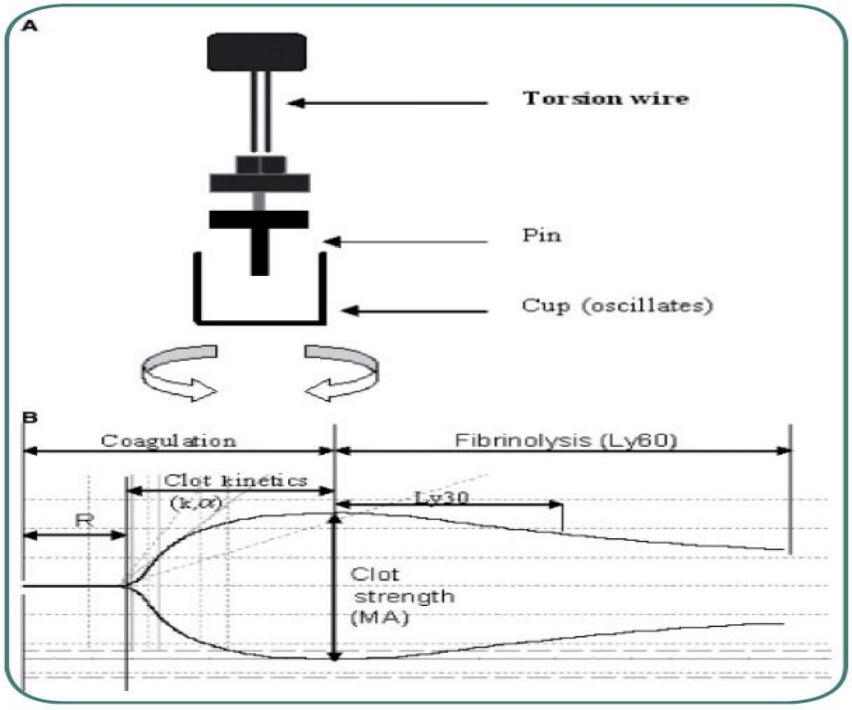


Figure 3.10 Thromboelastography

The top panel shows the transmission of the torque to the inner central pin. The elastic force is recorded digitally and displayed as a graph. The bottom panel shows a 4 channel TEG® with reagents mixed to the cuvette.

TEG® measurements are derived from a graph and various parameters are explained in the table below. Thrombus initiation begins with activation of the enzymatic cascade and ends with formation of fibrin polymers (R time). This R value reflects fibrinogen levels in blood. TEG® also measures the speed at which the thrombus forms (K time) and is calculated from the graph as the time taken for amplitude of the clot to increase from 2 mm to 20 mm. Maximum amplitude of the thrombus (MA) provides a measure of overall structural interactions of platelets interlaced with fibrin polymers is measured as the distance between both the diverging limbs of the graph on either side of the y-axis. TEG® measurements include α angle, which represents the slope between R and K values of the graph and this represents the fibrin to fibrin interlinkage. Early autolysis, which is secondary to thrombus retraction, is mainly contributed by rearrangement of fibrin to fibrin linkage. Autolysis is measured by percentage of lysis at 30 minutes and one hour respectively (CL30 and CL60) and the rate of loss of thrombus strength as measured by lysis parameter (L mm/min). L parameter is calculated using a complex first derivative integral calculus equation by continuously measuring loss of maximum amplitude in mm per minute.

Platelet Mapping™ is an advancement of TEG® technique and it is used to measure the platelet suppressant effect of individual antiplatelet agents such as aspirin, clopidogrel and glycoprotein IIb IIIa inhibitor. Heparin was used in Platelet Mapping™ to neutralise the effect of thrombin, and enabled us to measure the effects of particular platelet agonists such as arachidonic acid and ADP. Commercially available activator F (labelled A-P1) was used to stimulate conversion of fibrinogen to fibrin and activation of Factor XIIIA to crosslink the fibrin network, thereby bypassing the effect of heparin. In addition, platelet agonists, ADP (2 µM) and arachidonic acid (1 mM) were used to study the effect of clopidogrel and aspirin respectively in individual cuvettes. Then TEG® data was collected over 60 minutes. Platelet inhibitory effect of clopidogrel and aspirin were calculated by measuring maximum amplitude using the formula:

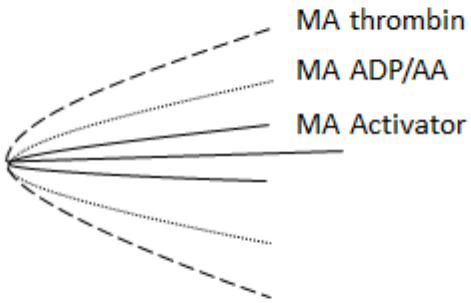
Percentage maximum amplitude reduction, MA reduction

$$= 100 - (\mathbf{(}[MA_{AA \text{ or } ADP} - MA_{activator}]/[MA_{thrombin} - MA_{activator}]\mathbf{)x}100 \mathbf{)}$$

There are published data to validate the above formula by using

i) heparinase-coated cups in patients on heparin therapy
ii) non citrated blood (Zmuda *et al.*, 2000; Wasowicz *et al.*, 2008)

In addition to standard TEG® parameters, changes in the viscoelstic strength were depicted as a real time graph known as V curve. This quantifies the velocity of both generation and autolysis of thrombus based on either the changes in amplitude (A) or the elastic modulus (G) of the elastic force over time. Since clot development occurs in series of steps strongly influenced by inherent thrombin activity, the speed at which thrombus gains or loses its elastic force in the stage of thrombogenesis is directly proportional to the speed of thrombin generation. Thus, by measuring the velocity changes in TEG®, thrombin generation can be quantified indirectly. These values are represented as V curve parameters and are not yet published in diabetic individuals. (Rivard *et al.*, 2005).



Thromboelastography® Platelet Mapping®

Figure 3.11 TEG® Platelet Mapping™ tracings

The tracings are derived from the changes in viscoelastic force of the thrombus from three cuvettes upon activation by kaolin (MA thrombin), ADP or arachidonic acid (MA ADP) and Activator F (MA Activator). Individual platelet inhibitory values are calculated using the formula, percentage maximum amplitude reduction, MA reduction = 100 – **«(**[$MA_{AA\ or\ ADP}$ – $MA_{activator}$]/[$MA_{thrombin}$ – $MA_{activator}$]**)x**100**»**. The higher the reduction in MA, better is the antiplatelet response of clopidogrel.

Name of the parameter	***Definition***	***Clinical relevance***	***Normal values***
R time, in min	Time from the beginning of the TEG® trace until amplitude of 2 mm is reached.	This measures the time taken from the placement of the blood sample in the cuvette to the initial fibrin formation	2-8
K time, in min	Time from the end of R until a fixed level of thrombus strength is reached i.e. amplitude of the trace is 20 mm.	This measures the time taken to form a firm thrombus, corresponds to fibrinogen levels	1-3
α- angle (thrombus formation rate)	The angle formed from the horizontal to a point on the trace until amplitude is 20 mm	This measures the rate of fibrin polymerisation and fibrin to platelet interaction, thereby assesses the speed of thrombus formation	55-78
MA, maximum amplitude, mm	Measures the maximum distance between the two arms of the graph at their peak amplitude	This measures maximum viscoelastic strength of the thrombus. This has been the most studied and consistent measurement among all the TEG® indices. MA is influenced by fibrinogen and platelet fibrin interaction. Using the modification of TEG® assay in Platelet Mapping™ assay individual effects of aspirin	51-69

Name of the parameter	***Definition***	***Clinical relevance***	***Normal values***
		and clopidogrel are studied.	
G, dynes/cm^2	Measure of the shear elastic force of the thrombus, G= (5000A)/(100-A)/1000, A is the amplitude of the continual tracing till MA, at which point G parameter measurement will stop.	G parameter measures the elastic force and a small increase in A will reflect an exponential increase in G. These values are indicative of firmness of the thrombus.	4,600 – 10,900
CI, clot index	Dimensionless parameter calculated by the formula, CI **=** - *(0.1227)R + (0.0092)K + (0.1655)MA – (0.041)α - 5.0220.*	Overall measure of thrombus strength and it is a composite measure and a dimensionless index. CI measures the combined effect of fibrinogen and platelets on thrombus	-3 to 3
LY30	Measures the area under the curve 30 min after MA has been achieved	Indicative of thrombus retraction at 30 minutes, signifies early autolysis.	0-8
LY60	Measures the area under the curve 60 min after MA has been achieved	Indicative of thrombus retraction at 60 minutes.	0-15

Name of the parameter	***Definition***	***Clinical relevance***	***Normal values***
L parameter, mm/min	This is a thrombus lysis parameter, calculated as the average reduction in amplitude per unit of time. This is the first derivative calculus measurement of thrombus strength and is calculated for 90 minutes in standard TEG® tracings.	This represents the changes in viscoelastic property due to ongoing clot retraction or autolysis.	30-60

Table 3.1 Thromboelastography measurements.

Based on the V curve graph and using the area under the curve, the following parameters are calculated. L parameter, the composite measure of autolysis has been described before. Maximum rate of thrombus generation, (MRTG, mm/min) measures the maximum velocity at which viscoelastic strength develops within a developing thrombus clot. Time to maximum rate of thrombus generation (TMRTG, in seconds) measures the time taken for the initiation of thrombus formation (i.e. lag time) plus the time it takes for the thrombus to reach its maximum strength. Total thrombus generated (TG, mm/min) was measured indirectly as a derived parameter from the area under the curve (AUC). It is derived purely on the total elastic force of thrombus generated. Once thrombus is formed, it undergoes autolysis. Time to maximum rate of clot lysis (TMRL, in seconds) measures the time from the start of the coagulation process until the maximum rate of thrombus lysis is achieved. This reflects clot stability and persistence of the thrombus. Maximum rate of lysis (MRL, mm/min) is a thrombus autolysis parameter which represents the maximum rate of clot breakdown. The standard V curve graph is shown in Figure 3.12.

3.2.3.2 My study protocol

My step by step study protocol, as per manufacturer's guidelines is given below (Hemoscope, CA, USA). Briefly, citrated and heparinised bloods were collected from the patient and 340-360 µl of blood was placed in each cuvette labelled as Kaolin, F, ADP and AA respectively. After addition of calcium chloride (for Kaolin sample) or heparinise activator (Reagent F, AP1), respective agonists either ADP or arachidonic acid were added. TEG® was allowed to run for up to 90 minutes and the data was collected and displayed graphically and electronically incorporating individual parameters. A total of 100 patients completed the study (due to delays in installation of the machine and supply chain of the distributors, not all patients were able to complete the sub study). Patients were identified only by their study numbers and the data was stored anonymously in the dedicated computer attached to TEG® machine.

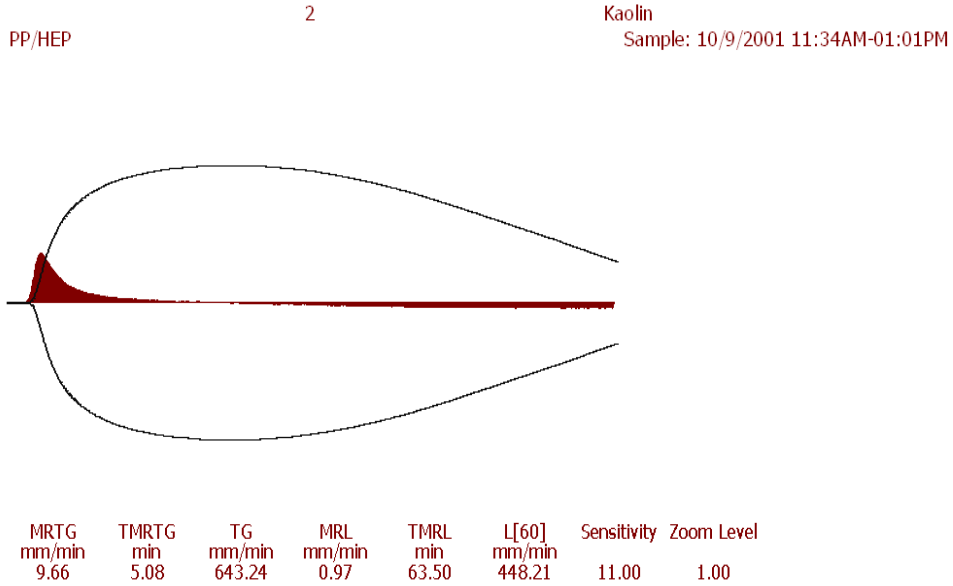


Figure 3.12 V curve as displayed in TEG®
See legend below

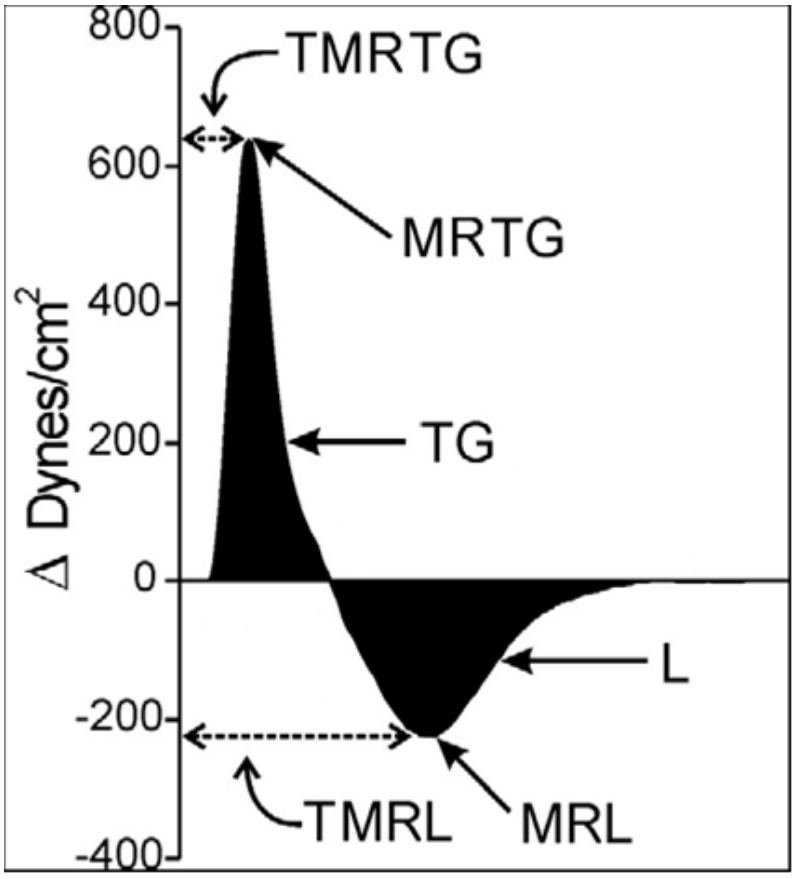


Figure 3.13 V curve parameters, raw data of Thromboelastography®.

The strength of the thrombus as it forms and undergoes autolysis is represented in a graphical format in both the top panel (graph displayed in TEG®) and the bottom panel shows the real time graph of changes in elastic strength of the thrombus . Thrombus formation is dictated by thrombin generation and thus the thrombin can be quantified indirectly by measuring thrombus kinetics.

MRTG (Maximum rate of thrombus generation) – This parameter reflects the rate at which viscoelastic strength develops during thrombogenesis.

TG (Thrombus generated) – This parameter is derived from the positive area under the curve (AUC) and represents the total amount of viscoelastic strength generated during thrombogenesis. It is directly proportional to the amount of thrombin generated during thrombus generation.

MRL (Maximum rate of lysis) – This parameter represents the maximum rate of thrombus autolysis.

TMRL (Time to the maximum rate of lysis) – This is the time from the start of the study till the maximum rate of thrombus lysis is achieved. This parameter reflects the stability of the thrombus.

L (Lysis parameter) – This gives the composite measure of autolysis or clot retraction.

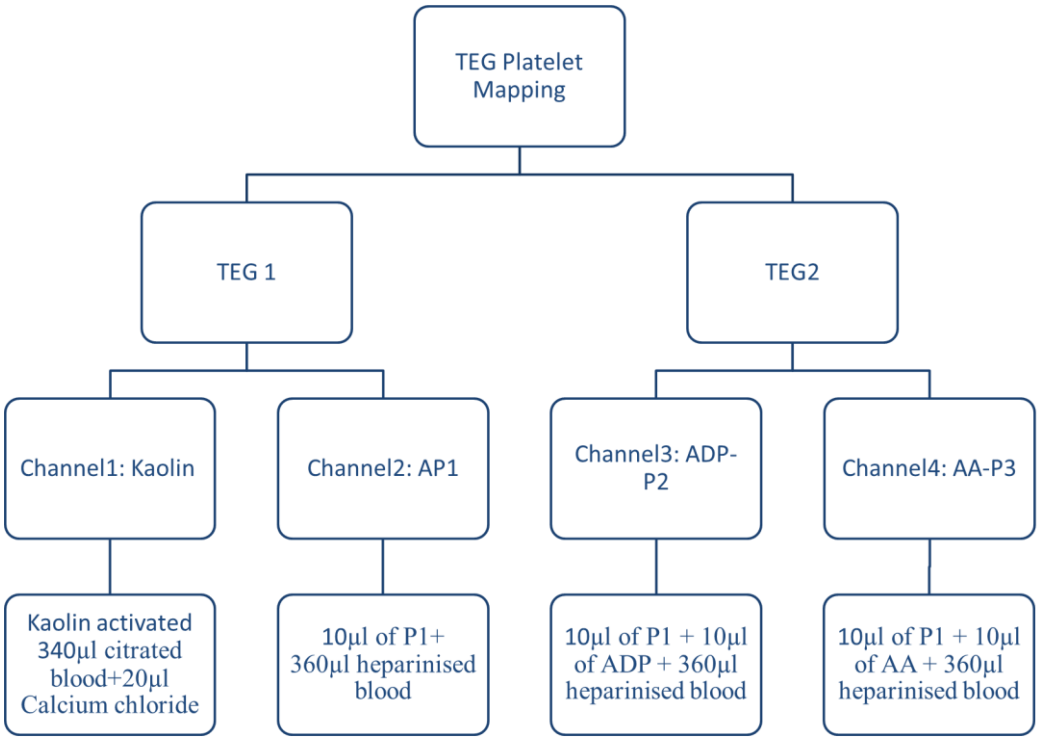


Figure 3.14 Schematic diagram of the four channels of the Thromboelastography (TEG).

• Activator F (labelled A-P1, measured in channel 2) – No platelet agonist added. MA results from fibrin contribution only. Results are referred to as MAP1 or MA-FIBRIN.
• ADP (labelled ADP-P2) and Activator F (in channel 3) – ADP activates non-inhibited P2Y12 and GPIIb IIIa receptors. Addition of ADP gives an assessment of P2Y12 receptor inhibiting agent clopidogrel. Activator F is added to catalyse the conversion of fibrinogen to fibrin and to add Factor XIIIa. MA results from non-inhibited platelets and fibrin. Results are referred to as MAP2 or MA-ADP.
• AA (labelled AA-P3) and Activator F (Channel 4) – Arachidonic acid stimulation gives an assessment of the effect of aspirin. Activator F is added to catalyse the conversion of fibrinogen to fibrin and then arachidonic acid is added. The MA results from non-inhibited platelets and fibrin. Results are referred to as MAP3 or MA-AA.

3.2.3.3 Quality control for TEG® Platelet Mapping™

I performed electronic quality control every day before starting the test. This ensured equilibrium of the central pin and torsion wire related to baseline surface. This was recorded and stored digitally. Once every week, I performed quality control using the control sample provided by the manufacturer. This was repeated prior to start of each new batch of reagents, and for every 30 studies performed (which ever being earlier). The results were stored digitally and plotted in a graph. In addition, I assessed reproducibility using 8 volunteers' samples. The coefficient of variation of MA thrombin was 3.7%. I operated the TEG® machine for all my study samples and received assistance in quality control and monitoring from the Coagulation services unit, Freeman Hospital, Newcastle upon Tyne.

My study protocol:

1. Load the plain disposable cups & pins into the TEG® analysers.

2. The lyophilised vials containing activator F(A), adenosine diphosphate (ADP) and arachidonic acid (AA) are taken out from storage at 6 degrees Celsius and gently brought to room temperature.

3. From the cannula after the Badimon chamber study is over, blood is directly filled in to a sodium citrate tube (1.75 ml of blood into the tube containing 0.25 ml of sodium citrate) and 2 ml to lithium heparin tube (100 units).

4. Pipette out 1 mL blood into the kaolin vial (supplied by Haemoscope) at 30 min after drawing blood gently by letting it run down the side of the vial until it reaches the 1mL graduation mark. Re-stopper the vial. Mix by gentle inversion five times. Do not shake the sample.

5. Transfer 340 µl blood from the kaolin vial (provided) into a cuvette in CHANNEL 1.

6. Add 20 µl 0.2M of calcium chloride (supplied by the manufacturer).

7. Immediately slide the carrier up and move the lever to Test position.

8. Click on the Start button in the software main toolbar to begin sample run.

9. Add 1ml of distilled water (supplied by manufacturer) each to the aliquot vials containing reagents A-P1, ADP-P2 and AAP3. Gently mix it by rolling between the hands. Avoid foam formation.

10. Pipette 10 µL of prepared A-P1 into each plain cup.

11. Add 10 µL of ADP-P2 into plain cup (for ADP assay) in CHANNEL3.

12. Add 10 µL of AA-P3 into plain cup (for AA assay) in CHANNEL 4.

13. Pipette 360 µL of heparinised whole blood into A-P1 cup in CHANNEL 2. Pipette sample up and down three times in cup to mix. Slide the carrier up, move the lever to test and click start button.

14. Pipette 360 µL of heparinised whole blood into ADP-P2 cup in CHANNEL 3. Pipette sample up and down three times in the cup to mix.

15. Pipette 360 µL of heparinised whole blood into AA-P3 cup in CHANNEL 4. Pipette sample up and down three times in the cup to mix. Slide the carrier up, move the lever to test and click start button.

16. The TEG® machine will start showing graphs from four of the channels in four different colours.

17. Recording will automatically stop once the graph lines reach a steady state. It usually takes up to 90 minutes for plain kaolin added sample and 60 minutes for other channels. Once the study is completed, obtain a print out of the tracings.

18. *The results are digitally stored in the computer attached to the TEG® machine.*

19. *After the study is over, obtain percentage inhibition. Select the Multi button located in the software Main toolbar to select tracings for computation.*

20. *For the ADP assay, select the three samples with A (A-P1), ADP (ADP-P2), and kaolin (K) sample types. Select the Done button located in the software main toolbar. For the AA assay, select the three samples with A (A-P1), AA (AA-P3), and kaolin (K) sample types.*

21. *Select the Done button located in the software Main toolbar. The percentage inhibition will be displayed in the screen.*

Table 3.2 Study protocol for Thromboelastography® (TEG) and Platelet Mapping™.

3.2.4 Measurement of Optical aggregation by VerifyNow® assay

3.2.4.1 Physical principles of VerifyNow®

VerifyNow® (Accumetrics, CA, USA) is a point of care platelet function assay which measures platelet aggregation employing the principles of optical aggregation and turbimetry. VerifyNow® is a microprocessor-controlled, cartridge-based, software driven automated assay that measures the antiplatelet effect of aspirin, P2Y12 antagonists and glycoprotein IIb IIIa antagonists in whole blood. The results are displayed in a digitised format.

VerifyNow® uses turbimetric principles to measure platelet aggregation in response to various agonists such as ADP, prostacyclin E1 and thrombin receptor activating peptide (TRAP). It uses similar principles employed in "gold standard" light transmission aggregometry (LTA). The VerifyNow® System consists of a cartridge with four wells containing various agonists of platelet aggregation. After adding whole blood to the wells, using a light source and an optical sensor with the cartridge placed in between, platelet induced aggregation is measured as decrease in light transmittance. The assay device contains a lyophilised preparation of human fibrinogen coated beads and a platelet agonist, either arachidonic acid or adenosine diphosphate (ADP). Light scattering will occur depending on the ability of platelet glycoprotein IIb IIIa receptors to bind to fibrinogen coated beads. The effective platelet inhibition with antiplatelet therapy is measured by the changes in light transmittance using arbitrary (dimensionless) units. The greater the effect of antiplatelet therapy there will be more platelet inhibition. This will lead on to less platelet aggregation and less light transmittance and thereby will record lower reactive units.

I used different types of cartridges to measure aspirin induced platelet suppression and clopidogrel induced platelet suppression respectively. Cartridges were purchased from the manufacturer and the whole assay has been performed according to the manufacturer's instruction. Due to unforeseen problems with the distributors and supply chain of the cartridges, the assay was performed for half of ACS study participants but all of the stable CAD study participants.

In one channel of the assay cartridge, prostaglandin E1 (PGE1) (22 nM) is incorporated with ADP (20 µM). The values measured are reported by the device as P2Y12 reactive units – activated (PRUz). In a separate channel iso-thrombin receptor activating peptide (iso-TRAP) is used as an agonist to measure platelet aggregation independent of the effects of P2Y12 receptors; this value is regarded as baseline or PRUb. The VerifyNow® P2Y12 assay reports the results as P2Y12 Reaction Units ("PRUz"), "% Inhibition" and "baseline PRUb". Percentage inhibition due to clopidogrel (P2Y12 antagonist), "% Inhibition" is calculated as (1-PRUz/PRUb) X100. If clopidogrel has produced significant platelet suppression, the PRUz values will be lower and the percentage inhibition will be higher. There is no universal agreement on the cut off used to measure platelet inhibition. I have chosen PRUz of 240 as a cut off and percentage inhibition of less than 40% as a cut off for high on treatment platelet hyperactivity to clopidogrel ("hypo responders") based on a landmark paper (Marcucci *et al.*, 2009). Patients with ARU ≥495 were classified as high on treatment platelet reactivity to aspirin ("hyporesponders") (Paniccia *et al.*, 2007).

3.2.4.2 Performance of VerifyNow® assay

Blood was collected in sodium citrate tubes (0.105M, 3.2% sodium citrate + citric acid, pH 5.2, Catalogue number 367691, BD Vacutainer®) and gently mixed by inverting 5 times. After leaving the sample static for 30 minutes, the cartridges were taken out of the sealed pouch and inserted into the well. The citrated blood tube was inverted gently again 5 times and fixed to the needle inside the cartridge. The assay device assembly was closed with a cover. Platelet aggregation usually proceeded for 3 to 6 minutes and the results were displayed as respective units (PRU or ARU). My detailed study protocol was given below.

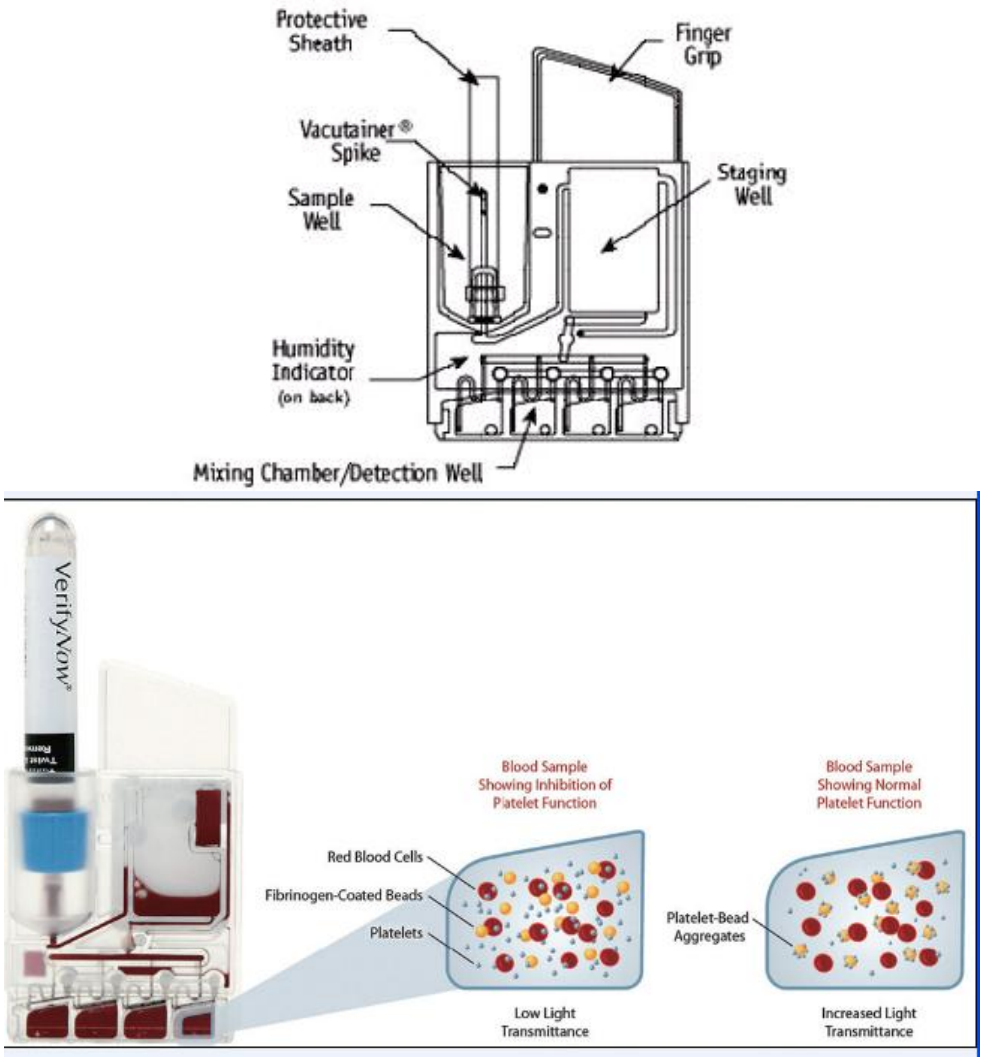


Figure 3.15 Verify Now® assay principles.

The cartridge used in VerifyNow® assay consists of fibrinogen coated beads in the four chambers at its bottom (top picture). Blood drawn from the patient mixes with these beads and produces aggregation stimulated by a specific agonists like ADP or arachidonic acid. The amount of platelet bead aggregation depends on the level of residual antiplatelet activity and will result in differential light transmittance. The emitted light is read by a sensor and the results will be displayed as a dimensionless unit (e.g. ARU, PRU).

My study protocol:

1. After performing electronic QC, enter patients study ID in the device and press OK.

2. Draw 2ml of blood into two 3.2% (0.105M) citrated plasma glass tubes (BD vacutainer, Oxford, UK) after the Badimon chamber experiments are over.

3. Mix the blood gently by turning upside down five times.

4. At 30 minutes, open the aspirin assay device (cartridge) from the pack. Check purple colour indicator for product quality control. If there is any decolourisation, discard the assay device.

5. Handle aspirin device from the top taking care not to touch the sensor portion at the bottom. Insert the device firmly into the column.

6. Remove the needle cover by single outward motion. This will avoid needle injuries.

7. Mix the blood sample gently 5 times and then insert the tube upside down into the well where the needle is located.

8. Close the cover of the assay device to make it lightproof.

9. After 180 seconds, results will be displayed as ARU.

10. Remove aspirin assay device and discard in a sharps container.

11. Remove P2Y12 assay device (cartridge) from the pouch taking care not to touch the bottom panel.

12. Check for the purple colour to ensure quality control. If there is any decolourisation, discard the device.

13. Handle P2Y12 device from the top taking care not to touch the sensor portion at the bottom. Insert the device firmly inserted to the column.

14. Remove the needle cover by single outward motion. Mix the blood

sample gently 5 times and then insert the tube upside down into the well where the needle is located. *15. Close the cover of the assay device to make it lightproof.* *16. At 360 seconds, results will be displayed as PRUz, PRUb and percentage inhibition.* *17. Note the results and record them in a folder named as 'accumetrics only'.* *18. Remove the assay device with vacutainer tube and discard it in a sharps box.* *19. Clean the surface with 70% alcohol wipes and close the cover. Switch off the device.*

Table 3.3 Study protocol for VerifyNow® assay.

3.2.4.3 Quality control

VerifyNow® incorporates its own manufacturer specified quality control. An automatic error message would be displayed as “attention” with a diagnostic code, if the results fall outside the wider range for ARU or PRU. I performed an electronic quality control (EQC) every day using the standard assay device supplied by the manufacturer. I performed a wet quality control assay (supplied by the manufacturer) for both aspirin and clopidogrel cartridges once a month and at every time a new batch of assay devices was used. In addition, I tested reproducibility annually using 10 healthy volunteers measured twice on the same day. The coefficient of variation of ARU was 3.7% and for PRUz 4.2% and the results of ARU were (median, *IQR) 602*, *624-596* and PRUz were 324, *306- 342*.

3.2.5 Electrical impedance analysis of thrombus using Multiplate® impedance aggregometry

3.2.5.1 Physical principles

The Multiplate® whole blood aggregometer evaluates platelet aggregation by measuring the changes in impedance of whole blood during the process of thrombogenesis. The Multiplate® assay uses the original principles of whole blood impedance platelet aggregometry (IPA) introduced in 1980 by Cardinal and Flower (Francis, 1987). Even though the earlier devices have been widely used in coagulation laboratories, the reproducibility was affected by the short life of the electrodes and presence of residual thrombus after each test. The Multiplate® device overcomes these problems by using individual disposable test cells with integrated electrodes and use of twin electrodes.

Blood is a very good conductor of electricity in its fluid state. When electricity is passed across the blood via metal electrodes, the electrical resistance is almost negligible when it is in fluid state. However, when thrombus forms, platelets tend to form a sheet over the electrodes and produce resistance to the flow of electrons from cathode to anode plates. When more thrombus is formed, the electrical impedance increases proportionately to the platelet aggregation. A change in electrical impedance which is a reflection of platelet aggregation is measured as a continuous variable over time (Figures 3.15 and 3.16). The maximum increase in electrical impedance thus reflects platelet activity and can be plotted as a graph. When a specified quantity of platelet agonist is added to the whole blood of patients taking antiplatelet agents, the changes in impedance will reflect residual platelet reactivity and can be used to assess the response of these agents.

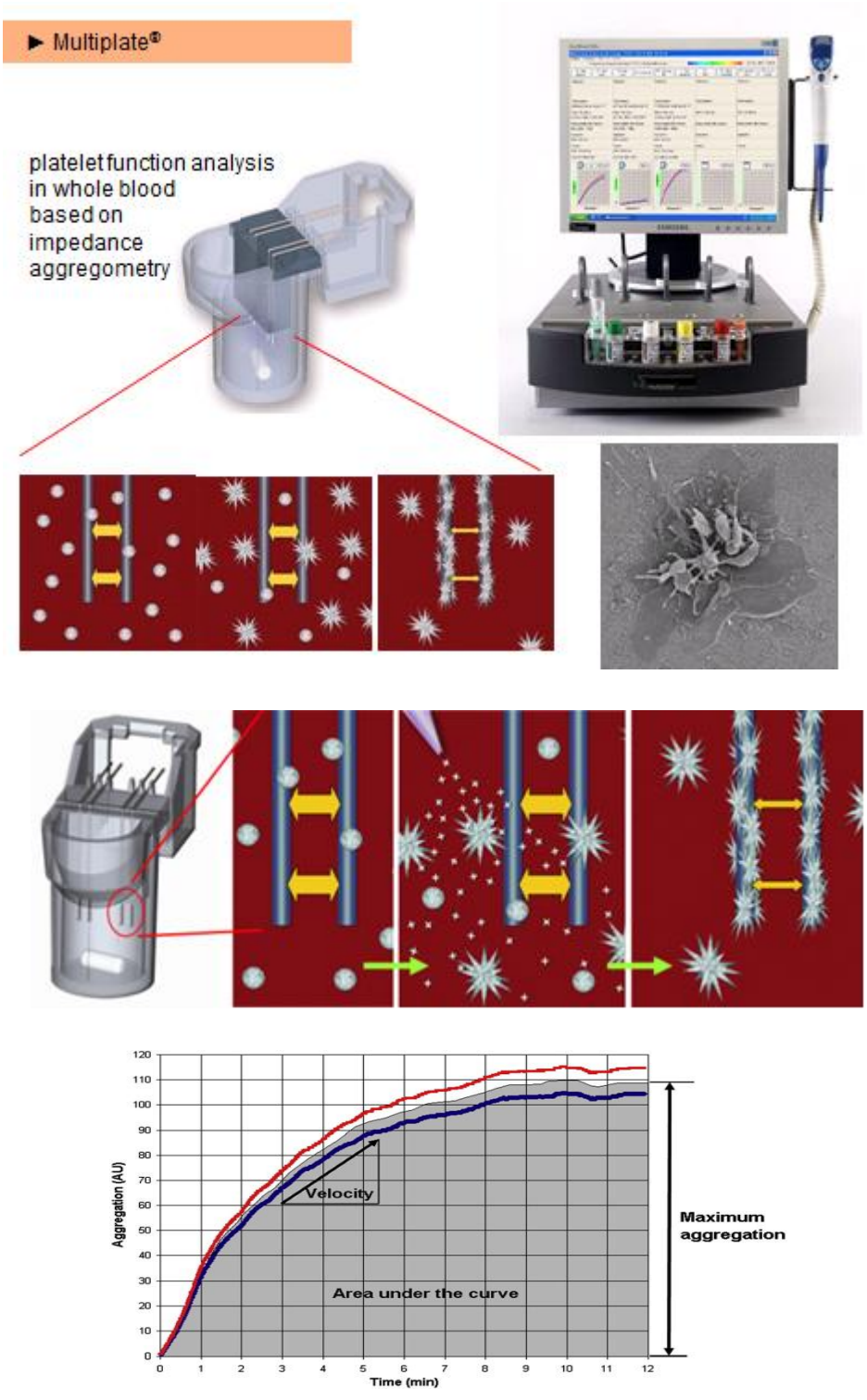


Figure 3.16 Multiplate® impedance assay.

Schematic diagram of the cartridges and platelet aggregation inside the cuvette is presented here. When electricity is passed across the blood via metal electrodes, the electrical resistance is almost negligible when it is in fluid state. However, when thrombus forms, platelets tend to form a sheet over the electrodes and produce resistance to the flow of electrons from cathode to anode plates. When

more thrombus is formed, the electrical impedance increases proportionately to the platelet aggregation. A change in electrical impedance which is a reflection of platelet aggregation is measured as a continuous variable over time. The maximum increase in electrical impedance thus reflects platelet activity and displayed as a graph.

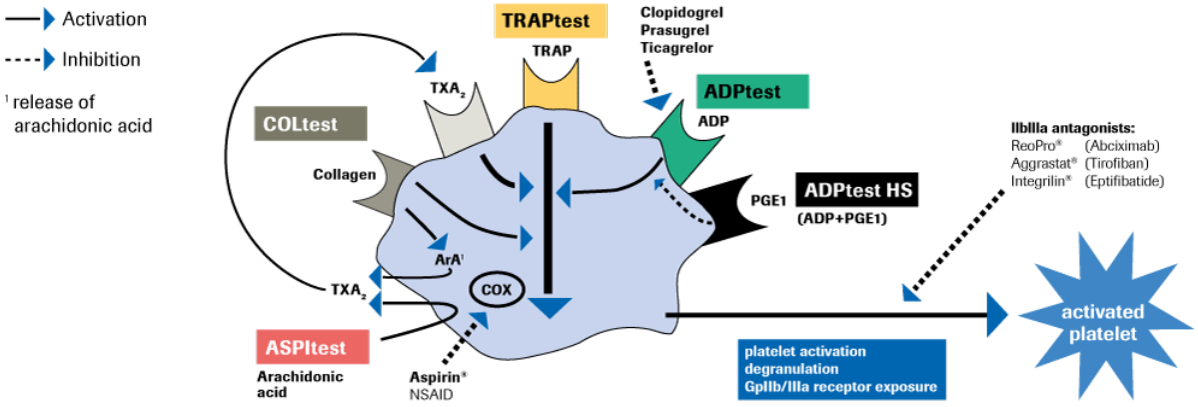


Figure 3.17 Platelet function tests performed by Multiplate® aggregometry.

Schematic diagram showing various platelet function tests performed by Multiplate® aggregometry is presented here. Most commonly used test, ADPtest measures platelet inhibition of P2Y12 antagonists such as clopidogrel. ASPItest measures the activity of aspirin using arachidonic acid as a stimulus. COLtest measures the platelet inhibitory effects of either of the agents upon stimulation with collagen. TRAPtest measures the platelet reactivity upon stimulation by thrombin receptor activating peptide.

3.2.5.2Multiplate® assay procedure

Whole blood impedance aggregometry was carried out using the novel Multiplate® analyser (Dynabyte medical, Munich, Germany) according to manufacturer's guidelines. Twenty eight patients from stable CAD study underwent assessment as a pilot study. Whole blood sample collected from patients in hirudin tubes was first incubated to 37 °C in a cuvette supplied by the manufacturer called the 'test cell'. Each disposable test cell has a pipette intake, a cup portion with the dual electrode sensor unit protruding into the blood which is stirred by a teflon coated magnet at 800 rpm and a jack portion which connects the test cell to the device via an electronic cable and records the electrical impedance between the sensor wires. The dual sensor units consist of silver-coated, highly conductive copper sensor wires of 0.3 mm diameter and 3.2 mm length. When thrombus begins to form, platelets become attached to the metal electrodes of the sensor unit, leading to a change in the electrical conductivity (or impedance), which is continuously recorded. The study was continued for 6 minutes till steady state electrical impedance was reached. Results are displayed as a graph in arbitrary units namely:

i) platelet aggregation (AU, maximal aggregation)
ii) velocity of aggregation (slope of the curve)
iii) area under curve (AUC)

Of these three parameters, I chose to use maximal aggregation (AU) as it was the most reproducible parameter and has been studied in clinical context (Sibbing *et al.*, 2010b; Kim *et al.*, 2011). The Multiplate® analyser has five test cells, thus enabling us to study the effect of five different platelet agonists.

Step by step study method is described below. Briefly, 154 mmol/l sodium chloride solution (300 µL) was pre-heated to 37 °C and pipetted into the test cells and 300 µL of blood sample anticoagulated with hirudin, was added to achieve a 1:1 dilution. After 3 min incubation and stirring using a magnetic stirrer at 37 °C, 20 µL of the agonist solution (e.g. ADP, arachidonic acid, collagen or thrombin receptor activating peptide) was added. The results were displayed as a graph with dual lines each representing a single pair of electrodes and displayed as two

aggregation curves, serving as an internal control. I used duplicate measurements as my internal quality control as recommended by the manufacturer. If the area under the curve (AUC) differed by >20% for the two curves and or their correlation coefficient was <0.98, the study results were discarded and the whole process was repeated. The mean value from the graphs was displayed as ‘aggregation units’ (AU). Whole blood samples of 1.2 ml from each patient were used to study the effects of four agonists, namely arachidonic acid, ADP, collagen and thrombin.

My study protocol

1. Draw 2ml of blood in hirudin vacutainer supplied by the manufacturer.

2. Gently mix the blood and leave it to stand.

3. Start the study 35 minutes after blood collection.

4. In the meantime, switch on the Multiplate® analyser. It will take 20 minutes to warm up.

*5. Perform electronic quality control every day. Follow the commands "**Multi Plate"** ⇨ **"Electronic Control"** ⇨ **"Electronic monitoring"** ⇨ **"Run".** The values should match the controls in all the five channels.*

6. Enter patient ID and select tests, ADPtest, ASPtest, Coltest, TRAPtest.

7. Bring the lyophilised vials of the reagents ADP, ASP, Col and thrombin to room temperature.

8. Mix each with 1 ml of distilled water supplied by the manufacturer. Gently swirl for thorough mixing.

9. Divide the reagents to small aliquot tubes each 100 µl and label them with name and date. AA, ADP and thrombin mixture is stored at -20 deg C and collagen mixture is stored at 5-6 deg C. All the reagents are labelled with an expiry date, one week from the date of preparation.

10. Insert four test cells into the machine and connect them to the sensor cable firmly.

11. Using autopippete (or change to manual) put 300 µl of blood from hirudin tube.

12. Add 300 µl 154 mmol/l saline.

13. For ADP test, add 20 µl of the reconstituted ADP reagent (5 µM of ADP). *14. Allow 3 minutes of incubation.* *15. Add 20 µl of activator to the test cell.* *16. The reaction starts and platelet aggregation will be displayed as a graph with two lines.* *17. The test will run for 6 minutes and final results are printed out.* *18. For ASP (aspirin), TRAP (Thrombin) and Coll(collagen) reagents, repeat steps 11 to 16 in respective channels with a different test cell.* *19. Once the test is completed, remove the test cells after disconnecting the cables and dispose them in the sharps disposal container.* *20. If the COV between the recorded curves is more than 2% or there was >20% difference in AUC readings, discard the results and repeat again. The repeat recording was taken for analysis.*

Table 3.4 Study protocol for Multiplate® impedance aggregometry.

3.2.6 Biomarker assay in serum and plasma samples

3.2.6.1 Sample collection and storage

Serum and plasma samples for special biomarker assays were collected at the end of Badimon chamber study from venous cannula and effluent blood from the chamber.

Following biomarkers were assayed on the stored samples

i. Soluble P selectin
ii. Soluble CD40 ligand
iii. Interferon gamma (IFNγ)
iv. Interleukin 1
v. Interleukin 6
vi. Tumour necrosis factor alpha (TNF α)
vii. Plasminogen activating factor inhibitor 1 (PAI-1)

For all samples other than PAI-1, the following methods of collection and storage were used.

i. Serum samples (pre chamber venous sample)
   Venous blood (5ml) was collected from the antecubital vein at the end of the chamber study using the same cannula in plain glass test tubes. The sample was allowed to settle for 10 minutes and serum was separated and stored in two aliquots of 1 ml each after centrifuging the sample at 1550g (3000 rpm) for 10 minutes. The samples were stored at minus 80 degrees and were thawed just before the analysis
ii. Activated serum samples (post chamber activated blood)
   Blood samples were collected from the effluent of the post chamber. Effluent blood from the chamber was traditionally discarded. However, I hypothesised that the effluent blood consists of coagulation markers secondary to shear and collagen activated platelets and is more likely representative of the blood in the local milieus than the standard venous blood. The difference in levels of blood cytokines in flowing

and effluent blood is likely to reflect the cytokines used in the organising thrombus. Samples were collected exactly between 3rd and 5th minute of Badimon chamber experiment and were stored in a similar fashion to pre chamber-standard venous samples

For assessment of plasma PAI-1 levels, I used 3.2 N sodium citrate tubes (Grainer-bio one, GmbH) instead of plain glass tubes and used same collection and storage methods as above.

3.2.6.2Measurement of P selectin

P selectin was measured in serum samples according to manufacturer's instructions (R&D systems, Abington, UK, Human sPselectin/P62, SBBE6). Quality control for P selectin was performed using the standards supplied by the manufacturer. I used 4 parameter logistic curve fitting method (Masterplex software, Hitachi Solutions America, Ltd., CA, USA) to derive soluble P selectin values from optical density values. Studies were done with duplicates and samples from normal controls to ensure assay precision. In addition, using 30 volunteer samples analysed on two separate plates randomly chosen, the mean inter plate variability was 3.6%. Eight volunteer samples collected on three occasions on three different days were analysed, giving a day-to-day coefficient of variability of 2.4%.

3.2.6.3Measurement of soluble CD40 ligand

Soluble CD40 ligand was measured in serum samples according to manufacturer's instructions (R&D systems, Abington, UK, Human sCD40L, DCDL40 and quality control kit lot number 1242552-54). I used 4 parameter logistic curve fitting method (Masterplex software, Hitachi Solutions America, Ltd.CA, USA) to derive soluble CD40L values from optical density values. Studies were done with duplicates and samples from normal controls to ensure assay precision. Quality control for CD40 ligand was performed using the standards supplied by the manufacturer. Standard values matched with my assay values in all the plates. I also tested 30 volunteer samples and the mean inter plate variability was 5.4% as paired samples. Eight samples were repeated on three

different days and the results had a coefficient of variability of 8.6% for day to fluctuations. Patient samples were studied in duplicates to improve accuracy.

3.2.6.4Measurement of Plasminogen activator inhibitor -1 (PAI-1)

I performed PAI -1 antigen ELISA assay on plasma samples using Technozym® PAI-1 antigen ELISA kit, TC12075, Technoclone GmbH, Vienna, Austria as per manufacturer's instructions. All samples were collected between 08.00 am and 10.00 am to minimise variations in PAI-1 due to circadian rhythm. I did my experiments in the nationally accredited coagulation laboratory at Freeman Hospital, Newcastle upon Tyne under the supervision of the chief technician and my protocol was tested with standard samples to ensure reproducibility. 30 volunteer samples were assayed with a mean COV of 2.3%.

3.2.6.53.2.6.5Measurement of serum cytokines

I used a novel multi array platform with electro-chemiluminescence (Meso Scale Devices, Gaithersburg, MD, USA). Electro-chemiluminescence (ECL) is a well studied alternative to conventional ELISA colorimetric methods. It is highly sensitive but unaffected by residual serum factors or plasma proteins in the test cell matrices. (Blackburn *et al.*, 1991; Swanson *et al.*, 1999; Yuan *et al.*, 2003; Horninger *et al.*, 2005; Chowdhury *et al.*, 2009; Rhyne *et al.*, 2009; Thway *et al.*, 2010). Background signals are minimised in ECL because the stimulation mechanism (electricity) is decoupled from the signal (light) which also improves its detection range (Deaver, 1995). *N*-hydroxy-succimide biotin and ruthenium are used to generate chemiluminescence upon stimulation by electrodes. In addition, use of multiplex technology enables the analysis of large panels from small volume samples (Toedter *et al.*, 2008; Fu *et al.*, 2010). The intricate details of this assay are beyond the scope of this thesis.

MSD platform used in this study was a 96-well plate-based assay that incorporated electro-chemiluminescence as the basis for detection. Following the capture of the cytokine by the spotted antibody, labelled detection antibodies were bound to the cytokines in a sandwich manner. The detection antibodies were attached to electro-chemiluminescent labels that emitted light when

electrochemically stimulated via carbon-coated electrodes in the bottom of the array wells. A detection buffer was added prior to the reading of the assay to enhance the electrochemical signal. The resulting signal was read using a photo detector in a charge-coupled device (sector reader) available in Institute of Cellular Medicine, Newcastle University. Duplicates were run as 1:1 and the reproducibility of the results was excellent with a COV of 2.1% in 30 volunteer samples tested in all the six plates. Eight volunteer samples were repeated on three different days and the results showed a COV of 3.8%.

3.2.7 Quantification of coronary atheroma using Gensini scoring system

3.2.7.1 Gensini score

Coronary atheroma is routinely quantified using diagnostic clinical angiography in clinical practice. I aimed to study the association between atheroma burden and blood thrombogenicity after an acute coronary event. In order to assess coronary atheroma burden, various scoring systems based on luminal stenosis in epicardial coronary arteries as visualised by angiography have been devised. I chose the Gensini scoring system to quantify coronary atheroma as it is time tested, easy to score manually and well validated (Austen *et al.*, 1975; Falciani *et al.*, 1998; Blanco-Colio *et al.*, 2007). Coronary angiographic images of patients who were included in the ACS study were reviewed and Gensini scores were assigned to each patient (Figure 3.18).

3.2.7.2 Calculation of Gensini score

Diagnostic coronary angiographic images of both the left and right coronary arteries were retrieved from the archives for each patient, and each segment was systematically examined. A score was assigned based on the degree of luminal obstruction, which included both concentric and eccentric lesions. Points were assigned starting at 25% occlusion, with values doubled for each increasing level of occlusion. The point assignment was based on the angiographic estimate of percentage occlusion. The score for each segment was then multiplied by a weighting factor, which reflected the relative importance of that particular coronary segment. The Gensini score is the summative value of the scores of the individual coronary segments.

Gensini score = SUM [(points for each segment) X (weighting factor)]

I devised modified Gensini score by giving equal points to each segment with lesions.

Modified Gensini score = Total Number of segments with more than 25% occlusive lesions.

Ten randomly selected angiograms were assessed for both inter and intra observer variability. Inter-observer co-efficient of variability was 12.4% and intra-observer CV by myself was 1.8%.

Level	Percent Occlusion	Points
0	None	0
1	25%	1
2	50%	2
3	75%	4
4	90%	8
5	99%	16
6	100%	32

Table 3.5 Gensini Score points

Coronary Artery	Artery	Weighting Factor
right	proximal	1
	mid	1
	distal	1
Left	main	5
	LAD proximal	2.5
	LAD mid	1.5
	primary diagonal	1
	secondary diagonal	0.5
	apical	1
	circumflex proximal	2.5
	circumflex distal	1
	obtuse marginal	1
	posterolateral	0.5
left or right	posterior descending	1

Table 3.6 Gensini score weighting factor.

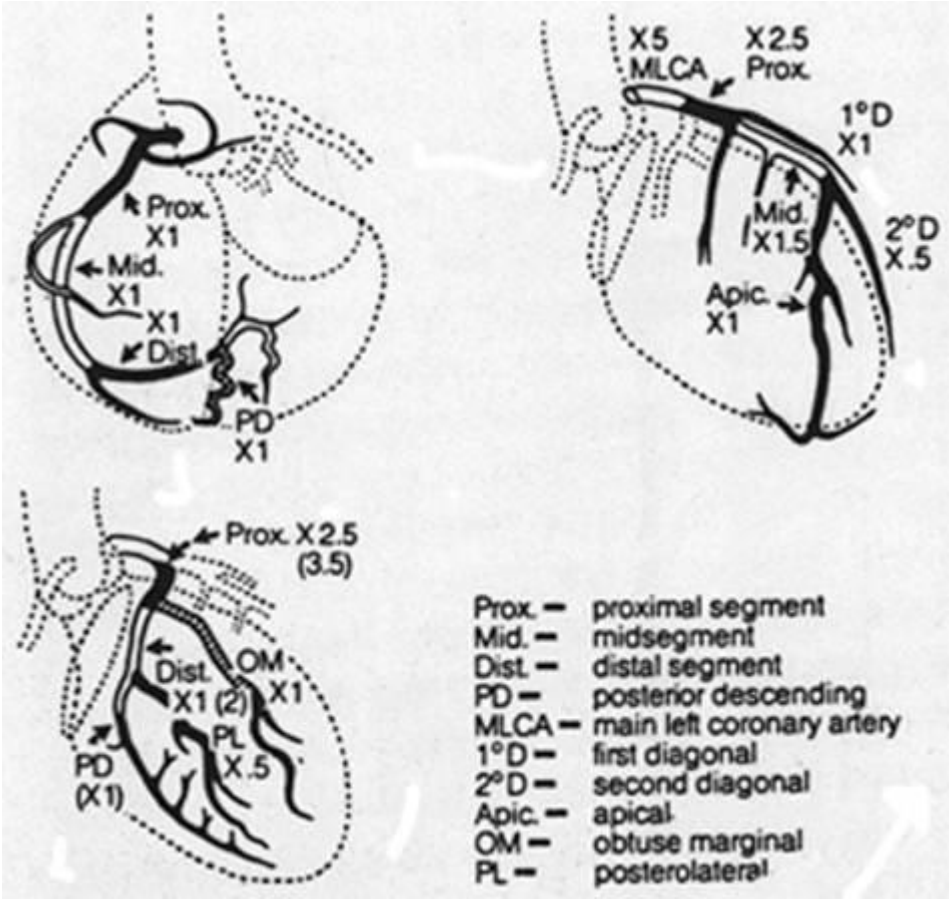


Figure 3.18 Gensini scoring system for coronary atheroma.

Coronary arterial tree is sub divided in to various segments as proximal, mid and distal part of the artery. Weightings are given according to the amount of myocardium supplied by that segment of the artery. Any segment with luminal narrowing more than 25% is taken as significant stenosis. After multiplying with the weightage factor, a segmental score is assigned. Summation of individual segmental scores will give the final Gensini score.

Chapter 4 Results and discussion

4.1 Platelet dependent thrombosis in T2DM

4.1.1 Results – Platelet dependent thrombosis in ACS study

For this observational cohort study, I screened 271 patients who were admitted with a diagnosis of NSTE-ACS between 2008 and 2011. I invited 90 eligible patients to take part in the study and 80 patients (40 T2DM and 40 non DM controls) who were treated as per current AHA/ESC guidelines including aspirin 75mg od and clopidogrel 75mg od for one week, have completed the Badimon chamber experiments (Figure 3.1). Details of patient selection and patient's journey through the study protocol were explained in chapter 2: General methods section.

Baseline data is shown in Table 4.1. Baseline characteristics showed no statistical differences between the groups despite numerically higher values of blood pressure and serum creatinine in those with T2DM. By virtue of their selection, T2DM had higher HbA1c, plasma glucose, serum triglyceride levels and BMI. There was a higher prevalence of treated hypertension and dyslipidaemia among those with T2DM reflecting their metabolic syndrome phenotype. LDL cholesterol levels were lower in T2DM compared to those without DM.

Patients with T2DM had higher thrombus compared to those without T2DM, total thrombus area from high shear chamber was (in μ^2 per mm, mean±SD) 20,414±12,344 vs. Non DM 14,933±8,415 p=0.023, 95% CI of the difference between the two groups was 811 – 10674 (Table 4.2, Figure 4.1, Figure 4.2a). In view of the skewed nature of the data, I log transformed the values (Figure 4.2b) for statistical analysis. Higher thrombus was also seen in low shear chamber (μ^2 per mm, mean±SD, 11313±4526 vs. 8755±3492, p=0.006). Fibrinogen was higher in T2DM and had a significant correlation to thrombus (r=0.283, p=0.013) which was lost when multiple regression analysis was performed.

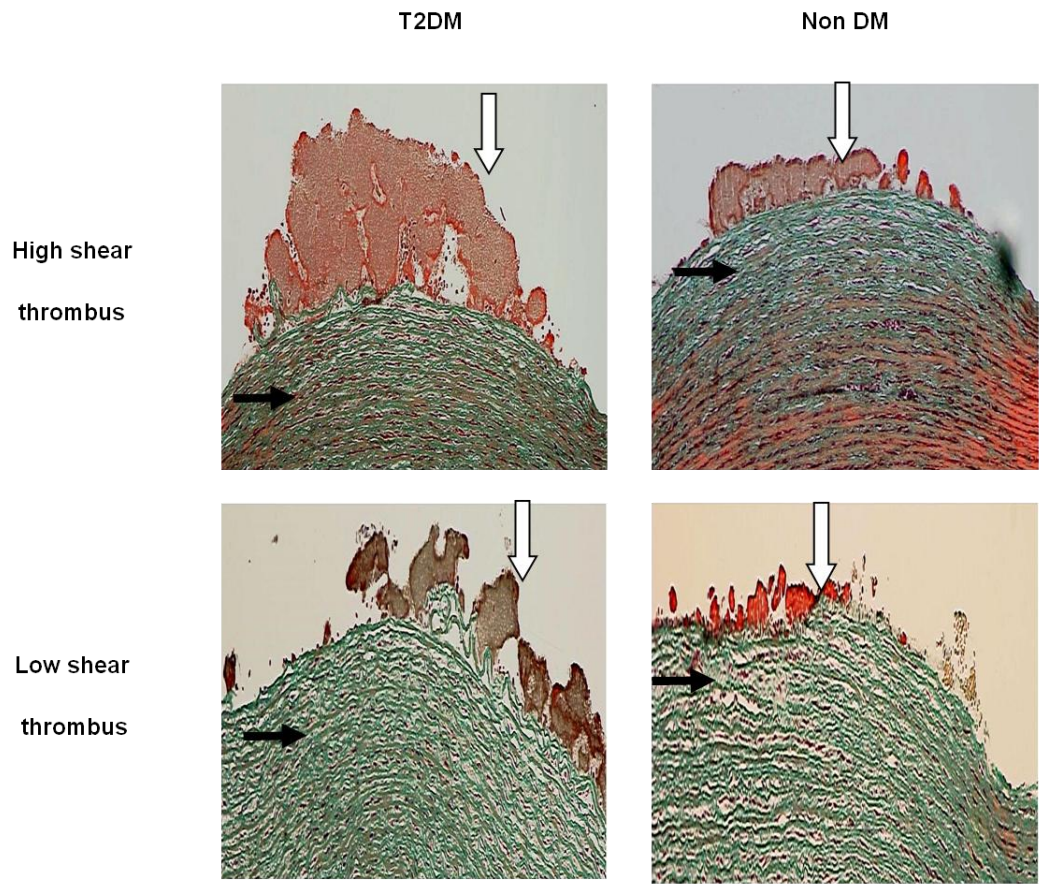


Figure 4.1 Thrombus images from ACS study

Thrombus was higher in T2DM in both high and low shear states. Thrombus images were stained with modified Mason trichrome stain. Platelet dependent thrombus was stained in red (solid white arrows) and tunica media was stained with green and pinkish hue (solid black arrows). Thrombus was seen adherent to the tunica media which was exposed to the flowing blood in the Badimon chamber

	T2DM (n=40)	Non T2DM (n=40)	P value
Demographic data Mean±SD or %(n)			
Age, years	63.8±12.2	62.2±11.4	0.553
Male gender, % (n)	67.5 (27)	90.0 (36)	0.027
Body mass index, kg/m^2	33±8.3	30±5.0	0.029
Waist to hip ratio	1.0±0.05	1.0±0.05	0.422
Systolic BP, mmHg	130±18.9	125±19.7	0.212
Diastolic BP, mm Hg	73±11.6	69±8.0	0.137
Risk profile: **%(n)**			
History of hypertension	77.5 (31)	45.0 (18)	0.003
History of dyslipidaemia	90.0 (36)	42.5 (17)	0.001
History of chronic kidney disease	27.5 (11)	15.0 (6)	0.172
History of acute myocardial infarction	27.5 (11)	12.5 (5)	0.094
Medications: **%,(n)**			
B blockers	82.5 (33)	97.5 (39)	0.057
Calcium channel blockers	37.5 (15)	17.5 (7)	0.055
Laboratory data: mean±SD			
HbA1c, %	7.5±1.5	5.8±1.4	
Random plasma glucose, mmol/l	11.2±6.8	6.3±1.7	
Fasting plasma glucose, mmol/l	7.8±3.1	5.2±0.8	
Haemoglobin, g/dl	13.2±1.8	14.0±1.3	0.027

	T2DM (n=40)	Non T2DM (n=40)	P value
Platelets, x1000/mm^3	254±89.9	246±68.2	0.685
Fibrinogen, g/l	4.2±0.8	3.5±1.4	0.012
S Creatinine, µmol/l	111±65.0	104±28.3	0.560
HsCRP, mg/l	10.9±9.7	3.6±3.2	0.001
Total cholesterol, mmol/l	3.4±1.0	3.8±0.9	0.135
LDLc, mmol/l	1.6±0.6	1.9±0.9	0.023
HDLc, mmol/l	1.0±0.3	1.1±0.4	0.301
Triglyceride, mmol/l	1.9±1.2	1.5±0.6	0.048
Troponin I, µg/l	6.9±10.2	7.0±12.7	0.974

Table 4.1 Baseline characteristics for ACS study.

Mean thrombus area, μ^2/mm, Mean±SD	T2DM (n=40)	Non T2DM(N=40)	P value
High shear chamber	20414±12344	14933±8415	0.023
Low shear chamber	11313±4526	8755±3492	0.006

Table 4.2 Thrombus area for ACS study.

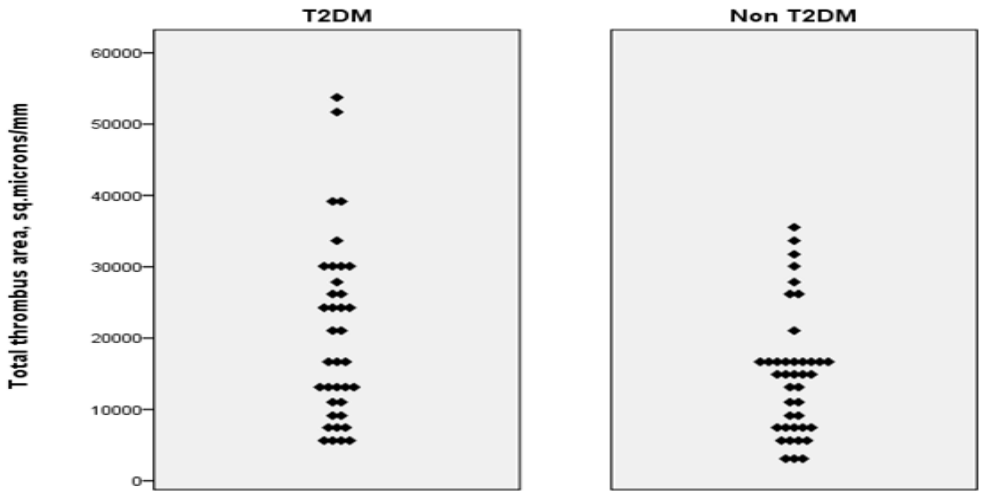


Figure 4.2 a Thrombus area study 1 (ACS patients).

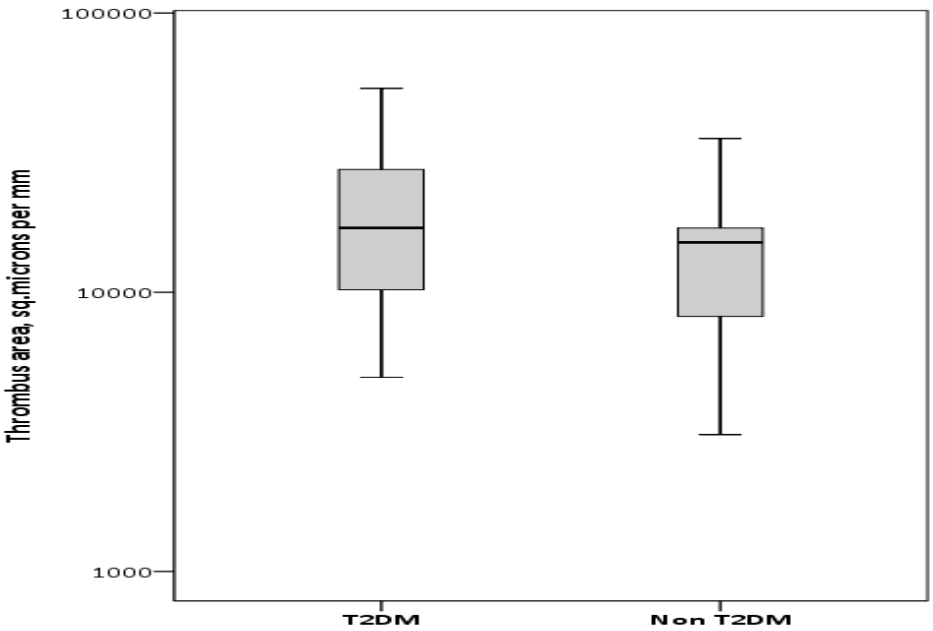


Figure 4.2 b Log transformed values.

Mean area of thrombus, measured in μ^2/mm. T2DM = Type 2 diabetes mellitus. Patients with T2DM had higher thrombus after NSTE-ACS despite current optimal secondary prevention therapy. Total thrombus area in T2DM [(μ^2/ mm, mean±SD,) 20,414±12,344 vs. Non DM 14,933±8,415 p=0.023, 95%CI of the mean difference was 811 – 10674]. In view of the skewed nature of the data, we log transformed the values before analysing and Figure 4.2b represents bar chart with log transformed values.

4.1.2 Discussion – Platelet dependent thrombosis in ACS study

Patients with T2DM have more thrombotic events and poorer prognosis compared to those without T2DM despite current aggressive secondary prevention therapy both in NSTE-ACS setting and stable CAD setting. Current international society guidelines (AHA/ACC/EHA) recommend similar secondary prevention therapies to these high risk patients compared to those without T2DM (Anderson *et al.*, 2007; Fraker *et al.*, 2007; Wright *et al.*, 2011).

The findings from ACS study are important as

i. This is the largest cohort of patients studied after NSTE-ACS using the Badimon chamber

ii. Participants were on all recommended secondary prevention therapy and were chosen based on a strict eligibility criteria to minimise confounding variables

iii. Data provides a focus for studies of mechanisms underlying the observational data of higher recurrent thrombotic event rates in T2DM following NSTE-ACS (Cho *et al.*, 2002),(Lee *et al.*, 2004; Bhatt, 2008)

My findings argue against the current identical recommendations for secondary prevention therapy in patients with and without T2DM, and suggest that either a more targeted and or more aggressive approach to antithrombotic treatment is required in T2DM patients after NSTE-ACS. Studies have confirmed higher recurrent thrombotic event rates in T2DM following NSTE-ACS, despite optimal secondary prevention therapy but the underlying mechanisms are not clearly defined (Bartnik *et al.*, 2005; Donahoe *et al.*, 2007). In trials of more potent antiplatelet therapies, the outcomes in patients with diabetes and acute coronary syndromes have not improved to levels seen in those without T2DM: in trials of newer antiplatelet agents ticagrelor and prasugrel, The Study of Platelet Inhibition and Patient Outcomes (PLATO) and Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction (TRITON–TIMI), the active arm still had a one year mortality of 7.2% and 12.2%, respectively, in patients with diabetes. This suggests that in

T2DM we may need to target therapy beyond platelets (Wiviott *et al.*, 2008a; James *et al.*, 2010).

The failure of more potent antiplatelet drugs, and tight glycaemic, lipid and blood pressure control in T2DM to reduce vascular event rates to levels seen in the non-diabetic population is testament to the need for ongoing studies specifically in this patient population (Sobel, 2007; Balasubramaniam *et al.*, 2012). The recently presented trial on intensive weight reduction and risk factor control in T2DM did not reduce cardiovascular events(The Look Ahead Research Group, 2013). Prothrombotic tendency of those with T2DM has been supported by studies on individual platelet and coagulation factor abnormalities but their contribution to increased thrombus has been speculative (Carr, 2001; Grant, 2007; Tufano *et al.*, 2011; Vazzana *et al.*, 2012).

My findings suggest that persistently raised blood thrombogenicity ("vulnerable blood") after discharge from hospital following NSTE-ACS is an important contributory factor for recurrent events in T2DM and a focus for targeted therapy given the failure of glycaemic and risk factor control to reduce macrovascular events in the diabetic population (Skyler *et al.*, 2009). Patients with T2DM had higher levels of fibrinogen, a consistent finding reported previously (Kannel *et al.*, 1990; McBane *et al.*, 2010; Zhao *et al.*, 2011) and this association was lost when regression analysis was performed. My findings were from a smaller cohort and the association between fibrinogen and whole blood thrombus in T2DM is yet to be shown in a large cohort. I studied the effects of a prototype P2Y12 antagonist clopidogrel. Nevertheless, even in trials of more potent oral antiplatelet agents (prasugrel and ticagrelor), patients with T2DM had a 20% increased risk of recurrent cardiac events in the first year after the index coronary event, suggesting that more potent antiplatelet agents may not completely neutralise the higher thrombus burden in T2DM (Wiviott *et al.*, 2008b; James *et al.*, 2010).

Overall, there is very little published data on PDT in the diabetic population with ACS and in that aspect my findings are new and so should be confirmed by another large scale study. The finding of greater thrombus in T2DM may not be entirely surprising as many of the published studies on surrogate haematological

markers such as P selectin, tissue factor and thromboxane have suggested the possibility of greater whole blood thrombus in this high risk population(Lim *et al.*, 2004; Mason *et al.*, 2005; Meerarani *et al.*, 2007; Stellos *et al.*, 2010; Badimon *et al.*, 2011). It is possible that hyperglycaemia could have been the major factor responsible for high thrombus burden in T2DM but only a few studies have shown that improvement in glucose control reduces the markers of thrombogenicity (Becker *et al.*, 2003; Lemkes *et al.*, 2010; Neubauer *et al.*, 2010). A small study from Badimon et al showed good correlation with glycaemic control and reduction in platelet dependent thrombosis (Osende *et al.*, 2001). Persistence of high thrombogenicity in T2DM could be due to either the relative lack of efficacy on “hyperactive platelets” with current antiplatelet therapy or the presence of powerful non-platelet mediated factors (e.g. fibrinogen, inflammation). Nevertheless, my findings are supported by a significant proportion of the literature which argues that current secondary prevention therapies for patients with T2DM after ACS are sub optimal (Cayla *et al.*; Angiolillo *et al.*, 2008b; Fuster and Farkouh, 2008).

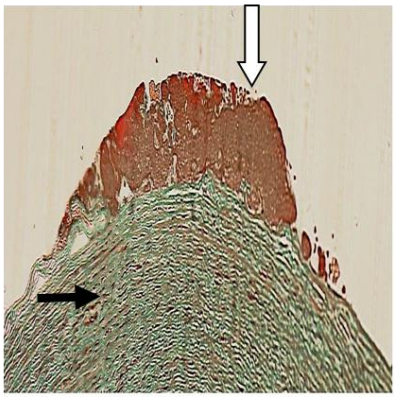
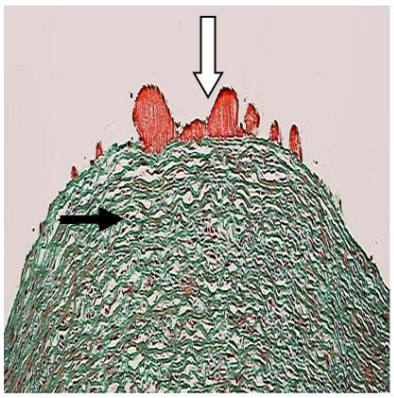
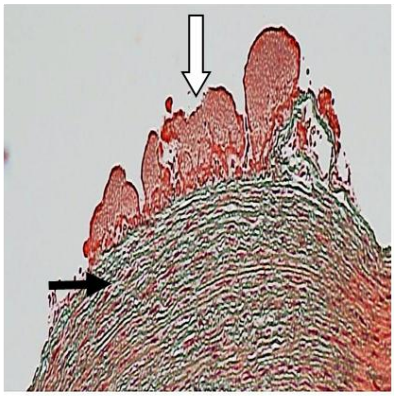
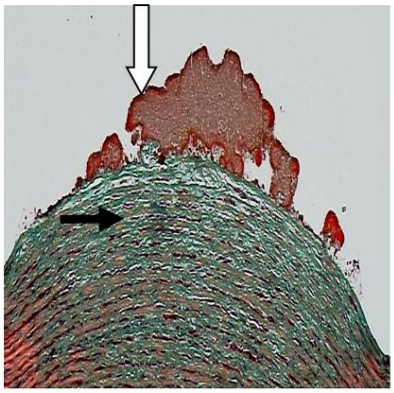
4.1.3 Results – Platelet dependent thrombosis in CAD study

The stable CAD study was a double blinded placebo controlled randomised control study. Of 149 patients assessed for eligibility, 90 were found eligible and completed the study (Figure 3.2). Details of patient selection were explained in general methods section.

Differences in baseline data between the clopidogrel and placebo group were not statistically different despite lower BMI and higher number of insulin treated individuals in the clopidogrel group (Table 4.3). Duration of T2DM was not statistically higher in patients who had clopidogrel (10 years vs. 7 years). Two thirds of patients in both the groups received metformin and one fifth of the patients received insulin as a part of glucose lowering treatment. For my study, T2DM was managed by clinicians with special interest in diabetes and I have not influenced their choice of therapies. Mean HbA1c was 7.5% (DCCT aligned) and mean fasting blood glucose was 8.0 mmol/l.

Thrombus measurements at baseline (visit 1) and 1 week after 75mg od clopidogrel therapy (visit 2) are shown in Table 4.4, Figure 4.3a and Figure 4.3b. At baseline, both clopidogrel and placebo groups had similar thrombus area in high shear chamber (thrombus area in μ^2/mm, mean±SD, 13978±5502 vs. 13959±7038 p = 0.988). In patients who had clopidogrel therapy, the thrombus area reduced from (thrombus area in μ^2/mm, mean±SD) 13978±5502 to 11192±3764 (mean change in the thrombus area 3035 μ^2/mm, 95% confidence interval 1437- 4633, p<0.0005). Thrombus area remained unchanged in placebo group (13959±7038 vs. 14208±6780, p=0.519, Figure 4.4). Similar findings were seen in low shear chamber. No major bleeding events were noted during the study period and two patients in clopidogrel group and one patient in placebo group reported minor bleeding from gums.

In a post hoc analysis combining both the studies, the thrombus area was greatest in patients with ACS and T2DM and lowest in patients with stable CAD and T2DM who had aspirin and clopidogrel therapy (μ^2/mm, mean±SD 24414±12344 vs. 11192±3764, p=0.001). Mean thrombus area of non diabetic ACS patients was not different to that of diabetic patients with stable CAD and treated with aspirin only (μ^2/mm, mean±SD, 14933±8415 vs. 14208±6780, p=0.742) (Table 4.5, Figure 4.5).

High shear thrombus (a)	Baseline	After therapy (visit 2)
Clopidogrel group		
Placebo group		

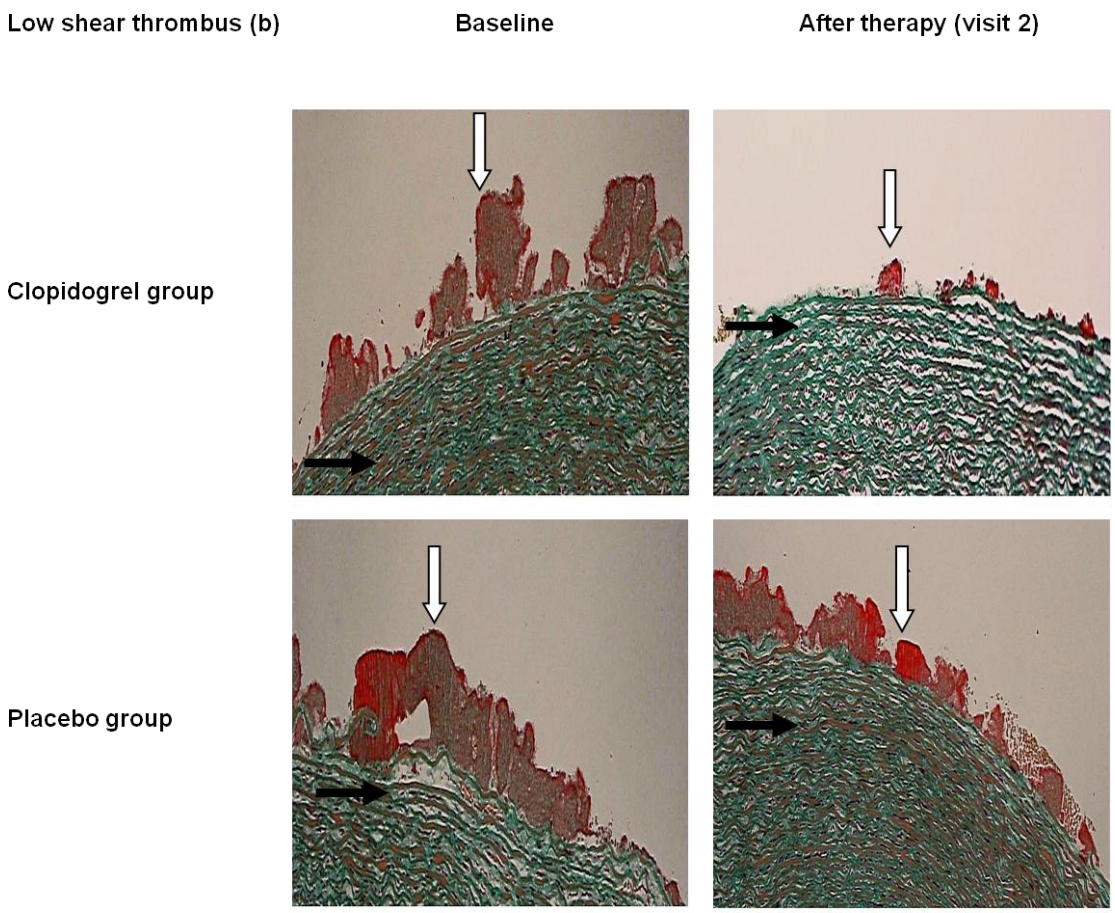


Figure 4.3 a and b Thrombus images from stable CAD study.

Thrombus was reduced after clopidogrel therapy in both the high and low shear states. Thrombus remained unchanged in placebo group.

Thrombus images were stained with modified Mason Trichrome stain (vide supra). Platelet dependent thrombus was stained in red (solid white arrow) and tunica media was stained with green and pinkish hue (solid black arrow). Thrombus was seen adherent to the tunica media which was exposed to the flowing blood in the Badimon chamber.

	Clopidogrel + aspirin (n=45)	**Placebo + aspirin (n=45)**	P value
Demographic data: Means or %(n)			
Age, years	65±8.1	64±7.2	0.546
Male gender, % (n)	82.2 (37)	84.4 (38)	0.777
Body mass index, kg/m^2	31.8±4.8	33.2±6.3	0.260
Waist to hip ratio	1.0±0.1	1.0±0.1	0.139
Systolic BP, mmHg	143±18.4	139±20.7	0.257
Diastolic BP, mmHg	77±10.2	78±9.3	0.897
Duration of diabetes, years	10±7.0	7±4.3	0.712
***Risk profile:* %(n)**			
Angina	68.9 (31)	64.4 (29)	0.655
Previous MI	40.0 (18)	42.2 (19)	0.830
PCI	28.9 (13)	28.9 (13)	1.000
CABG	28.9 (9)	20.0 (9)	0.327
Chronic kidney disease	11.1 (5)	22.2 (10)	0.157
***Medications:* %,(n)**			
Sulphonylurea	26.7 (12)	26.7 (12)	1.000
Metformin	64.4 (29)	64.4 (29)	1.000
Insulin	24.4 (11)	20.0 (9)	0.800

	Clopidogrel + aspirin (n=45)	**Placebo + aspirin (n=45)**	P value
Laboratory data: mean±SD			
Haemoglobin, g/dl	13.6±1.4	13.7±1.3	0.817
Platelets X1000 cells/ mm^3	227±55.5	225±56.6	0.880
Fibrinogen, g/ml	3.4±0.6	3.4±0.8	0.560
HbA1c, %	7.5±1.2	7.5±1.1	0.957
Fasting plasma glucose, mmol/l	7.9±2.6	8.3±2.4	0.947
eGFR, ml/min/1.73m^2	72±20.6	76±19.4	0.332
Total cholesterol, mmol/l	3.4±1.0	3.8±0.9	0.542
LDLc, mmol/l	1.9±0.7	1.9±0.7	0.910
HDLc, mmol/l	1.1±0.3	1.1±0.3	0.847
Triglyceride, mmol/l	1.6±0.8	1.8±1.0	0.269
HsCRP, mg/l	4.4±4.5	3.4±3.6	0.293

Table 4.3 Baseline characteristics for study 2 (stable CAD patients).

	Aspirin+Clopidogrel			Aspirin+Placebo		
Mean±SD μ^2/mm	**Visit 1 (baseline)**	**Visit 2**	**P value**	**Visit 1 (baseline)**	**Visit 2**	**P value**
Thrombus in high shear chamber	13978±5502	11192±3764	0.001	13959±7038	14208±6780	0.519
Thrombus in low shear chamber	8426±4080	6561±2925	0.001	7317±2906	6990±2482	0.443

Table 4.4 Thrombus area in patient with stable CAD study.

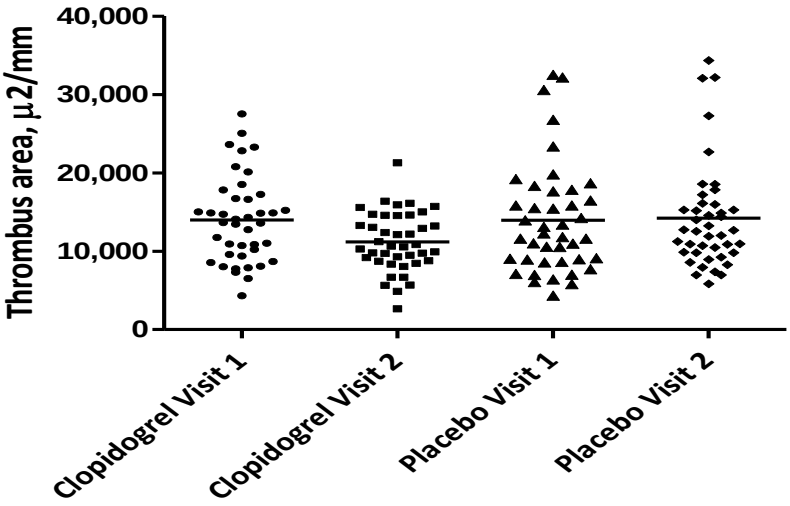


Figure 4.4 High shear thrombus area of patients in stable CAD study.

High shear thrombus area measurements in patients with T2DM and stable CAD are shown at baseline and one week after therapy with aspirin+clopidogrel vs. aspirin+placebo. In clopidogrel group, thrombus area (μ^2/mm, mean±SD), reduced from 13978±5501 to 11192±3764 whereas it remain unchanged in placebo group from 13959±7038 to 14208±6780, $p<0.0005$. Mean difference in thrombus area of clopidogrel group was (μ^2/mm) 3035 with 95% confidence interval was between 1437 and 4633.

Thrombus area, μ^2/mm	Mean	Std. deviation
ACS and T2DM (n=40)	**20414**	12344
ACS and Non DM (n=40)	**14933**	8415
T2DM with stable CAD on aspirin (n=45)	**14208**	6780
T2DM with stable CAD on aspirin and clopidogrel (n=45)	**11192**	3764

Table 4.5 Thrombus area of the combined study population.

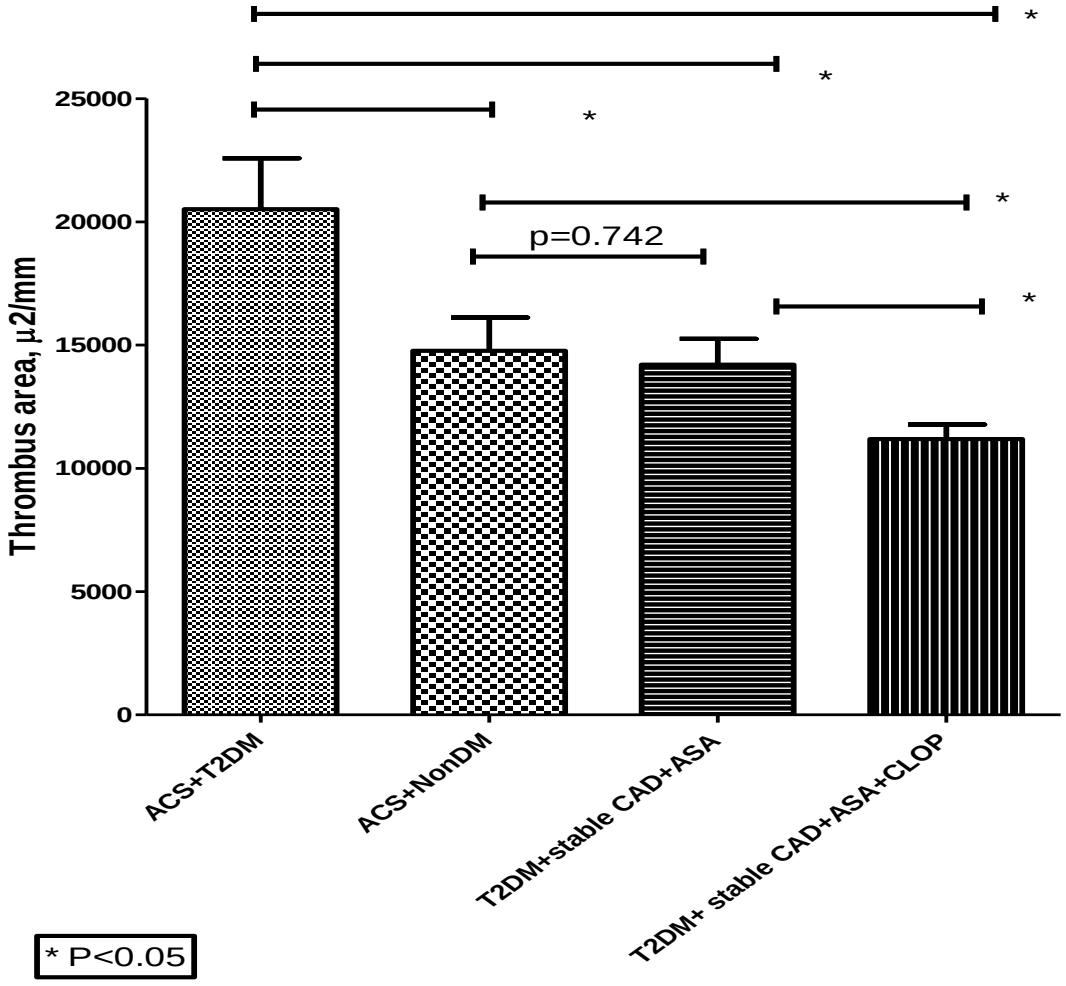


Figure 4.5 Thrombus area of combined study population.

ACS and T2DM had highest thrombus and patients with stable CAD and T2DM who had aspirin and clopidogrel therapy had the lowest thrombus (μ^2/mm, mean±SD 20414±12344 vs. 11192±3764). Thrombus area of non diabetic ACS patients was not different to that of patients with stable CAD and treated with aspirin only (μ^2/mm, mean±SD, 14933±8415 vs. 14208±6780). One way ANOVA: F statistic 8.277, P<0.001. The individual p values are ACS-T2DM vs. ACS non DM 0.023, ACS-T2DM vs. T2DM+stable CAD+ aspirin p= 0.006, ACS-T2DM vs. T2DM+stable CAD+ aspirin and clopidogrel p=0.001, ACS non DM vs. T2DM+stable CAD+ aspirin and clopidogrel p=0.016, ACS non DM vs. T2DM+stable CAD+aspirin p=0.742, and T2DM + stable CAD+aspirin and clopidogrel vs. T2DM+stable CAD+aspirin p<0.001.

4.1.4 Discussion - Platelet dependent thrombosis in CAD study

In the study on patients with stable CAD and T2DM, clopidogrel reduced thrombus area significantly when added to aspirin 75 mg. In this double blinded RCT, those treated with placebo had no changes in thrombus. Our group has earlier shown higher thrombus area in diabetic compared to non-diabetic individuals with stable CAD taking aspirin (Natarajan *et al.*, 2008a). This current study was aimed at exploring thrombus when clopidogrel is added to aspirin in patients with T2DM and stable CAD.

These findings can have significant implications in the management of patients with T2DM and CAD. To achieve a significant reduction of thrombus with DAPT in the chosen population with stable CAD which has been very well treated with lipid control, hypertension control and good (but not very tight) glycaemic control is clinically important. Clopidogrel and aspirin therapy (DAPT) reduced thrombogenicity without any increase in bleeding complications. The benefits are likely to be sustained if DAPT is continued beyond the protocol duration and it remains to be seen if long term benefits may outweigh the bleeding risks. My findings are in contrast to the findings of a similar but large scale, multi centre, CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance) study and its subsequent T2DM sub group analysis (Bhatt *et al.*, 2006). My study population differed from CHARISMA population by a number of ways. CHARISMA population had a 20% discontinuation rate for clopidogrel, nearly one sixth of them were current smokers and only three fourths had statin therapy. However, in their sub study on patients with established cardiovascular disease, clopidogrel showed benefits by reducing MI, stroke or death (relative risk, 0.88; 95% CI, 0.77 to 0.998; P=0.046). In addition, a subgroup analysis of the CHARISMA study showed that the benefits of clopidogrel are greater in population with T2DM and a previous cardiovascular event (relative risk reduction 17.1%, 95% CI: 4.4-28.1) (Bhatt *et al.*, 2007).

Of interest, among those treated with current guideline based antiplatelet therapy, I found that patients with T2DM and CAD (treated with aspirin only) had

numerically almost equal thrombus area compared to those non diabetic ACS patients (treated with aspirin and clopidogrel). When stratified as four groups, patients with T2DM and ACS had the greatest thrombus, whereas those with stable CAD and treated with DAPT had the lowest thrombus (Table 4.5, Figure 4.5). Haffner et al's land mark study showed that patients with T2DM but no overt CAD had similar risks of cardiac events when compared to patients without T2DM but established CAD resulting in the term "T2DM is a coronary artery disease equivalent"(Haffner, 2000). My findings take this assumption further, suggesting that patients with T2DM and established stable CAD have similar thrombogenicity as non diabetic patients with ACS and thus suggesting "T2DM and clinically stable CAD is ACS equivalent". This is a post hoc analysis of the data and should be interpreted with caution as 'hypothesis generating' and needs further validation. If these findings are proven in large scale studies, it strengthens the argument that T2DM patients with stable CAD should be managed with more powerful antiplatelet therapy to achieve similar clinical benefits seen in non diabetic population. These results show increased thrombus quantity in patients with T2DM and the qualitative aspects of diabetic thrombus are discussed vide infra.

4.2 Coagulation biomarkers

4.2.1 Results – ACS study

After NSTE-ACS, serum fibrinogen levels were higher in patients with T2DM (4.2±0.8 vs. 3.5±1.4, p=0.012). P selectin values were nearly 1.5 higher and CD40 ligand levels were twice as high in T2DM compared to non T2DM controls (Table 4.6). Post chamber effluent blood levels of P selectin and CD40 ligand (Figure 4.6) showed similar consistent results. PAI-1 levels were higher in T2DM both in pre and post chamber plasma samples. There was a significant difference between pre and post chamber values of soluble CD40 ligand (p=0.009). As mentioned before, serum fibrinogen had a significant correlation to thrombus (r= 0.283, p=0.013) but the correlation was lost when multiple regression analysis was performed. Interestingly, there were no significant correlations seen between soluble P selectin, soluble CD40 ligand and PAI-1 levels and thrombus area in venous blood samples and post chamber effluent samples (Table 4.7).

4.2.2 Results – Stable CAD study

In patients with established and stable CAD, levels of coagulation biomarkers, P selectin, CD40 ligand, PAI-1 and fibrinogen showed no statistically significant differences between the groups at baseline. Levels did not change following one week's therapy with clopidogrel (Table 4.8).

	T2DM (n=40)		**Non T2DM (n=40)**		
	Mean±SD	Median (IQR)	Mean±SD	Median (IQR)	P value
Fibrinogen,g/l	4.2±0.8	4(4-5)	3.5±1.4	3(1-5)	0.012
Venous P selectin, ng/ml	76.1±25.6	76 (57-93)	56.7±20.5	52(42-69)	0.001
Post chamber P selectin, ng/ml	73.3±22.9	73 (57-87)	57.3±17.6	60(42-67)	0.001
Venous CD40 ligand, ng/ml	5991±5258	4208(2042-7186)	2268±2960	1233(593-2839)	0.001
Post chamber CD40 ligand, ng/ml	4490±2851	4272(2446-5582)	2074±1772	1402(958-2659)	0.001
Venous PAI-1, ng/ml	96.9± 45.7	93.7 (58.3-136.6)	69.7± 31.3	68.2 (43.9-91.8)	0.016
Post chamber PAI-1, ng/ml	108.3 ± 55.2	126.9 (55.2-155.2)	67.9± 31.5	80.2 (30.8-86.8)	0.001

Table 4.6 Coagulation biomarkers – ACS study.

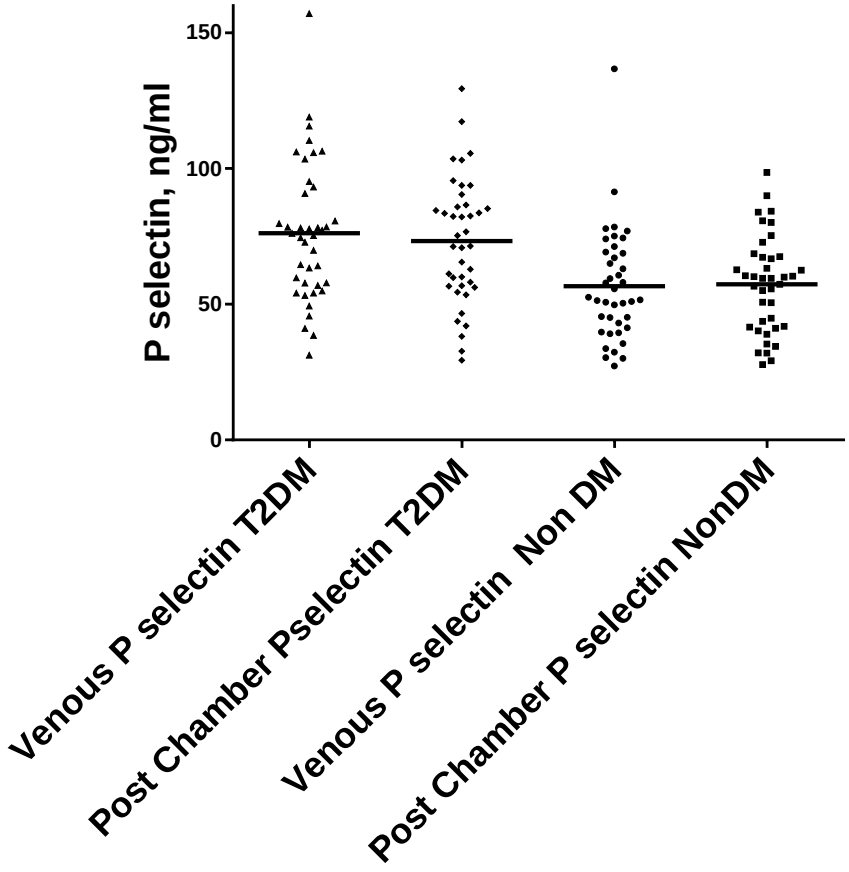


Figure 4.6 P selectin levels in ACS Study.

P selectin levels were higher in T2DM both in venous samples and post chamber “effluent” blood in T2DM compared to non DM one week after NSTE-ACS. The solid lines represent mean values.

Spearman rho, * p<0.05	Thrombus area
Serum fibrinogen g/l	0.283*
Venous P selectin, ng/ml	0.067
Post chamber P selectin, ng/ml	0.101
Venous CD40 ligand, ng/ml	0.063
Post chamber CD40 ligand, ng/ml	0.129
Delta P selectin, ng/ml	0.038
Delta CD40 ligand, ng/ml	0.086
Venous PAI-1, ng/ml	0.071
Post Chamber PAI-1, ng/ml	0.170
Delta PAI-1, ng/ml	0.179

Table 4.7 Correlations between coagulation biomarkers and thrombus area- ACS study.

	Clopidogrel+aspirin (n=45)			**Placebo+aspirin (n=45)**		
Mean±SD	Visit 1	Visit 2	P value	Visit 1	Visit 2	P value
Fibrinogen, g/l	3.4±0.6	3.3±0.6	0.091	3.4±0.8	3.3±0.7	0.910
Venous P selectin, ng/ml	77.0 ±30.2	70.5 ±29.6	0.251	75.0 ±28.7	69.9 ±29.5	0.256
Post chamber P selectin, ng/ml	72.9 ±26.1	74.1 ±27.3	0.752	71.3 ±32.1	68.1 ±21.8	0.455
Venous CD40 ligand, ng/ml	1039 ±620	939 ±707	0.343	1175 ±862	1143 ±972	0.672
Post chamber CD40 ligand, ng/ml	1149 ±709	1050 ±747	0.409	995±689	1039 ±630	0.621
Venous PAI-1, ng/ml	82.1 ±41.6	74.3 ±39.3	0.733	79.2 ±37.4	81.4 ±40.8	0.423
Post chamber PAI-1, ng/ml	57.8 ±41.7	71.7 ±46.5	0.797	55.2 ±41.4	66.7 ±43.9	0.652

Table 4.8 Coagulation biomarkers- Stable CAD study.

4.2.3 Discussion - Coagulation biomarkers and platelet dependent thrombosis

To explore the underlying mechanisms of PDT in T2DM, I studied coagulation markers namely fibrinogen, P selectin and CD40 and found that they were higher in T2DM after ACS despite current optimal secondary prevention therapy. In patients with stable CAD, these levels were unaffected by clopidogrel treatment.

In ACS study, there was a significant reduction of these biomarkers in both the groups in “effluent” post chamber blood also. These novel findings are supported by the fact that these biomarkers play an important role in enhancing PDT and stabilisation of the thrombus. It is possible that the gradient between pre and post chamber blood levels of soluble CD40 ligand reflects the incorporation of these biomarkers into the thrombus formed in Badimon chamber (vide infra).

P selectin is a well studied surrogate marker of pro thrombotic state in CAD and T2DM. Raised levels of P selectin were reported as early as 1993 in patients immediately after acute MI and ischaemic stroke (Wu *et al.*, 1993). Soluble P-selectin was raised in patients with stable CAD and peripheral arterial disease (Blann *et al.*, 1995) and unstable angina(Ikeda *et al.*, 1995; Tomoda and Aoki, 1998; Parker *et al.*, 2001). T2DM influences P selectin levels. Newly diagnosed diabetic patients, who were treated with insulin, had five times increased levels of P selectin compared to healthy controls at baseline and remained higher despite insulin therapy(Tschoepe *et al.*, 1995). Hyperglycaemia is an independent predictor of P selectin in patients with T2DM (Santilli *et al.*, 2010)(Yngen *et al.*, 2001; Gresele *et al.*, 2003; Neubauer *et al.*, 2010) and these findings were confirmed in a recent study of patients with T2DM on aspirin therapy (Evangelista *et al.*, 2007). Improvement in glycaemic control lowers P selectin levels, thus indicating a direct relationship between platelet activation and hyperglycaemic stress on platelets (Eibl *et al.*, 2004). However, these findings were challenged by a study evaluating multifactorial intervention in patients with T2DM and CAD (Lim *et al.*, 2004). In another study of 667 patients who underwent coronary stenting, platelet P-selectin was higher in patients with ACS, particularly in those with STEMI compared to those with stable CAD and correlated with myocardial

necrosis markers creatinine kinase (CK-MB) and troponin I. There were no differences seen in patients with or without T2DM who presented with ACS in that study (Stellos *et al.*, 2010). In a landmark subgroup analysis of BARI-2D trial, only patients with T2DM and obesity were found to have higher P selectin thus suggesting that raised BMI contributes to heightened platelet activation (Schneider *et al.*, 2009). A prospective case control study with 16 years of follow-up showed higher serum P selectin levels in diabetic patients without prior history of CAD compared to non diabetics, but after adjusting for conventional risk factors such as age, sex, smoking, lipid profile and socio-economic status, this difference ceased to exist (Sharma and Berger 2011). With the shift in definition and diagnostic criteria of T2DM recently, (American Diabetes Association, 2010) results from these long term follow up studies have to be interpreted with caution till the post hoc analysis data using current definitions of T2DM are available.

The role of CD40 ligand (CD40L) has been extensively investigated in patients with coronary artery disease and the current literature supports my findings of higher soluble CD40L in patients with T2DM after ACS. In my cohort, these findings suggest that soluble CD40L is a surrogate marker of high thrombogenicity after ACS but not an independent risk factor (Tousoulis *et al.*, 2010). Levels of CD40L were higher in patients with CAD compared to healthy controls and the levels were higher in patients with unstable coronary artery disease compared to stable CAD. CD40L levels correlated with severity of atherosclerosis and the trans coronary gradient (levels in aorta –levels in coronary sinus) in patients with ACS (Wang *et al.*, 2007). Multiple interventions to reduce the coronary artery risk factors in patients with CAD were associated with reduction in CD40L levels suggesting a causal link between reduction of platelet activation and inflammation and prognostic benefit. However, in a large cohort of healthy volunteers, sCD40L levels correlated poorly with both the individual risk factors and the calculated Framingham Coronary Heart Disease Risk Score(Verma *et al.*, 2005), suggesting the need for long-term follow-up studies to answer the question if CD40L could be an additional biomarker of cardiovascular risk. In large and representative multi ethnic population studies, soluble CD40L was not associated with conventional atherosclerotic risk factors, questioning its

global utility as a screening tool (de Lemos *et al.*, 2005). Findings from CD40 ligand studies are summarised in a table modified from Antoniades et al (Table 4.9) (Antoniades *et al.*, 2009).

T2DM is an independent factor associated with elevated CD40L (Varo *et al.*, 2003; Jinchuan *et al.*, 2004; Cipollone *et al.*, 2005; Gokulakrishnan *et al.*, 2006). Literature on soluble CD40 ligand (CD40L) in patients with T2DM after NSTE-ACS is minimal and my study findings are significant in that respect (Undas *et al.*, 2008). In patients with T2DM without overt CAD, CD40L levels correlated with urinary excretion rate of 11-dehydro-thromboxane B2 which is a marker of platelet hyperactivity (Santilli *et al.*, 2006). Significant reduction in plasma CD40L was noted after 7 days of aspirin and intensive diabetes risk factor control (Desideri and Ferri, 2003; Cipollone *et al.*, 2005). In addition, the strong association between tissue factor release, interleukin 6 and levels of CD40L in T2DM with CAD suggests that CD40L perpetuates thrombogenicity in T2DM (Lim *et al.*, 2004).

Addition of clopidogrel did not reduce the levels of CD40L in my study of patients with stable CAD and T2DM. These findings are in contrast to some of the earlier studies. Administration of clopidogrel for one week after ACS reduced platelet monocyte interactions and CD40L levels (Xiao and Théroux, 2004). In patients who underwent percutaneous coronary intervention (PCI), clopidogrel pre-treatment with 300-600mg was associated with lower serum CD40L levels immediately after PCI and these changes were more marked in flow cytometry assay after stimulation with ADP (Quinn *et al.*, 2004).

My study findings were also different to PROCLAIM study (A Pilot Study to Examine the Effects of Clopidogrel Compared to Placebo on Markers of Inflammation in Subjects with Metabolic Syndrome Who Are Receiving Background Therapy, including Low-Dose Aspirin), a pilot study to examine the effects of clopidogrel on inflammatory markers in patients with metabolic syndrome receiving low-dose aspirin.

Author	Population and methods	Outcome/Conclusion
Aukrust et al. (Aukrust *et al.*, 1999)	26 patients with unstable angina, 29 with stable angina, and 19 controls	Higher sCD40L levels and CD40L expression on T cells in patients with unstable angina; enhanced sCD40L release after platelet activation
Wang et al. (Wang *et al.*, 2007)	20 patients with ACS, 10 patients with stable angina, and 10 controls who underwent coronary angiography	Higher sCD40L levels in ACS and stable angina; higher trans-coronary gradient of sCD40L in ACS; higher sCD40L in patients with complex lesions
Yan et al. (Yan *et al.*, 2006)	32 patients with ACS, 24 with stable angina, and 16 controls	Elevated levels of sCD40L in stable angina and ACS; marked increase in sCD40L after percutaneous intervention
Peng et al. (Peng *et al.*, 2002)	27 patients with ACS, 23 with stable angina, and 30 controls	Higher levels of sCD40L, in ACS; sCD40L positively correlated with cellular adhesion molecules like sICAM-1 and sVCAM-1, triglycerides, and lipoprotein B, and negatively correlated with HDL-C
Garlichs et al. (Garlichs *et al.*, 2001)	40 patients with ACS, 15 with stable angina, and 12 controls who underwent coronary angiography or PCI	Higher sCD40L levels and CD40L platelet expression in ACS and values reduced after 6 months
Antoniades et al. (Tousoulis *et al.*, 2007)	109 patients with acute MI, 201 patients with stable CAD, and 286 controls	Higher sCD40L and IL-6 levels in acute MI; higher adhesion molecules in AMI and stable angina group; IL-6 was independently associated with sCD40L only in controls

Author	Population and methods	Outcome/Conclusion
Malarstig et al.(Malarstig *et al.*, 2006)	24-month follow-up of 2,359 patients with NSTEMI FRISC II trial, randomized to receive early invasive or conservative treatment with low molecular weight heparin, dalteparin or placebo	In placebo group, sCD40L levels were associated with increased risk of myocardial infarction; dalteparin reduced this risk in patients with sCD40L levels above median.
Ohashi et al.(Ohashi *et al.*, 2006)	17 patients with acute MI and 10 patients with unstable angina who underwent PCI. Thrombectomy was performed in MI patients	Transcoronary gradient of sCD40L increased at 9 h in both groups; high CD40L expression in thrombi; association between sCD40L and MMP-9 activity in AMI; aspirating thrombectomy reduced sCD40L levels.
Tayebjee et al. (Tayebjee *et al.*, 2005)	204 patients with stable CAD	Higher sCD40L in CAD; no relation with severity of the CAD
Rondina et al.(Rondina *et al.*, 2008)	909 patients undergoing angiography; 303 patients with CAD with a cardiac event within 1 year, 303 with CAD without event, 303 without CAD or event	Higher sCD40L levels associated with decreased risk of CAD in non-ACS patients
Martins et al. (Martins *et al.*, 2006)	865 patients with chest pain, with or without angiographically confirmed CAD, with or without AMI	There was no difference in sCD40L levels between CAD and non-CAD patients
Antoniades et al. (Antoniades *et al.*, 2006)	219 patients with premature MI and 389 controls; followed up for 1 year	Patients with MI had higher sCD40L levels compared with controls; sCD40L levels were significantly reduced after 1 year in the same subjects
Plaikner et al.(Plaikner *et al.*, 2009)	1,089 consecutive patients in an emergency department for medical or neurologic emergencies	sCD40L was not useful as a diagnostic marker for acute cardiac, cerebrovascular ischemic, or thromboembolic events

Author	Population and methods	Outcome/Conclusion
Schonbeck et al.(Schonbeck *et al.*, 2001)	130 healthy women, followed up for 4 years for CV events	CD40L levels were higher in women who subsequently developed cardiovascular event and women with CD40L levels above 95% percentile had three times risk of cardiovascular events after adjustment of conventional risk factors
De Lemos et al.(de Lemos *et al.*, 2005)	2811 subjects from the Dallas Heart Study, a multi ethnic population based cross-sectional study assessed by CT coronary calcium score and MRI of aortic media thickness	No association was found between CD40L levels and atheroma severity. There was lack of correlation between CD40L and conventional CV risk factors
Heeschen et al. (Heeschen *et al.*, 2003)	1088 patients with ACS and 626 patients with non cardiac chest pain admitted to emergency unit	Higher sCD40L levels were associated with increased risk of death or MI in ACS group and controls. Abciximab(GpIIb IIIa inhibitor) reduced cardiac risk in patients with higher sCD40L levels
Manenti ER et al. (Manenti *et al.*, 2006)	172 patients with non-ST-elevation ACS	No additional prognostic value of CD40L to TIMI risk score
Apple FS et al.(Apple *et al.*, 2009)	457 patients with ACS were studied for a multiple biomarkers based risk strategy	No differences in mortality rates were observed between those with increased versus normal concentrations of CD40L

Table 4.9 Summary of clinical studies on soluble CD40 ligand and CAD.

With dual antiplatelet therapy, there was a reduction in the levels of CD40L over a six week period without changes in P selectin and inflammatory markers (Willerson *et al.*, 2009). The contrasting findings from my study can be explained by lack of prolonged course of clopidogrel therapy (my study cohort took one week of clopidogrel therapy) and the differences in study population as I included patients with and without metabolic syndrome. There is heterogeneity of methods in evaluating CD40 levels and most of the studies which showed significant reduction after antiplatelet therapy have employed either serum or plasma samples. I used serum samples and employed ELISA method. It is unknown if the results would be different if I used plasma or novel flow cytometric analysis of CD40 (Halldórsdóttir *et al.*, 2005). In a study by Dunzendorfer et al, which looked at cytokine triggered priming of endothelial cells, and a study by Hermann et al, which assessed platelet expression of CD40L, there were no significant effects of aspirin treatment on CD40L levels. The data on reduction of CD40L with clopidogrel treatment still remains controversial in stable CAD (Azar *et al.*, 2006; Yip *et al.*, 2006; Stellbaum *et al.*, 2007).

In patients with T2DM and NSTE-ACS, venous plasma plasminogen activation inhibitor-1 (PAI-1) levels were higher than those without diabetes. Similar findings were seen in post chamber (effluent) blood. It has been well recognised that PAI-1 levels are elevated in T2DM (McGill *et al.*, 1994),(Sobel *et al.*, 1998). Elevated PAI-1 levels in T2DM not only favour persistence of thrombus but also are responsible for conversion of micro thrombus to macro thrombus and further propagation of thrombus (Schneider and Sobel, 2012). In their landmark IRAS (Insulin Resistance Atherosclerosis Study) study Festa et al showed that higher levels of PAI-1 predicted the incidence of T2DM and insulin resistance(Festa *et al.*, 2002) and they concluded that PAI-1 in T2DM independently lead to an increased risk of coronary heart disease (Festa *et al.*, 2006). These findings were confirmed in Framingham offspring study (Ingelsson *et al.*, 2007) and there are suggestions that PAI-1, along with pro inflammatory mediators play a significant role in development of metabolic syndrome and its cardiovascular complications (Alessi and Juhan-Vague, 2006).

Higher PAI-1 levels in patients with acute coronary syndromes is well known (Yazici *et al.*, 2005). Elevated PAI-1 levels in acute coronary syndrome was associated with higher major adverse cardiac events like (acute MI, stroke, death) (Collet *et al.*, 2003b; Marcucci *et al.*, 2006). Presence of higher concentration of PAI-1 in atherectomy specimen of patients with T2DM has led studies confirming its role in conversion of stable to unstable plaque. It has been proposed that PAI-1 plays a significant role in evolution and outcomes in patients after acute coronary syndrome in T2DM (Sobel *et al.*, 1998; Sobel, 1999b; Schneider and Sobel, 2012).

Various strategies were aimed in reducing the PAI-1 levels and thus reduce cardiac events in patients with T2DM. Improved glycaemic control, use of insulin sensitising agents and weight reduction were known to reduce the levels of PAI-1 (Schneider and Sobel, 2012). There are studies showing reduction in PAI-1 levels after treatment with aspirin and clopidogrel (Sakata and Kario, 2011). My study on patients with stable CAD and T2DM (stable CAD) failed to show any effect of clopidogrel therapy on PAI-1 levels. My sample size was small and the duration of therapy was shorter (one week). It is possible that clopidogrel add on therapy (75mg od) can reduce thrombus formation in better controlled T2DM on regular aspirin but may be ineffective in reducing the hypofibrinolysis in these individuals.

Overall, platelet activation markers were higher in T2DM after NSTE-ACS despite aspirin and clopidogrel therapy. This partly explains higher thrombus seen in these individuals. However, in stable CAD patients with T2DM, dual antiplatelet therapy has made no changes to these biomarkers.

4.3 Biomarkers of inflammation

4.3.1 Results – ACS study

Serum inflammatory biomarkers HsCRP, IL-6 and TNFα levels were significantly higher in diabetic compared with non-diabetic patients in standard venous blood samples. In the “activated-effluent” post chamber blood samples, only IL-6 values were higher in patients with T2DM (Table 4.10). IFNγ and IL-1 levels were numerically higher but not statistically different between both the groups. Venous levels of IL-6 (rho=0.251, p=0.27) and TNFα (rho=0.319, p=0.005) significantly correlated with thrombus area (Table 4. 11, Figure 4.7). The delta values of pre and post chamber blood, which may represent the amount of inflammatory cytokines consumed in the formed thrombus had significant correlation to thrombus (IL-6 delta: rho=0.371, p=0.001 and TNFα: rho=0.389, p=0.001, Table 4.11). The association of inflammatory cytokines and coagulation markers with thrombus from Badimon chamber persisted after adjusting for diabetic state and body mass index.

4.3.2 Results – Stable CAD study

Clopidogrel therapy in T2DM with stable CAD failed to reduce inflammatory cytokines TNFα, IFNγ and IL-6. However, there was a very small but significant reduction in IL-1 levels (mean±SD, ng/ml, 0.12±0.22 vs 0.08±0.15, p=0.021, table 4.12).

	T2DM (n=40)		Non T2DM (n=40)		
	Mean±SD	**Median (IQR)**	**Mean±SD**	**Median (IQR)**	**P value**
HsCRP mg/l	10.94 ±9.69	10.76 (3.05-11.22)	3.55±3.20	2.77 (2.00-3.90)	0.001
Venous IFNγ ng/ml	7.35 ±10.76	4.68 (3.19-7.89)	4.60±3.81	3.51 (2.88-4.72)	0.131
Post chamber IFNγ ng/ml	4.56 ±6.53	3.47 (1.89-5.09)	3.73±3.27	2.79 (2.46-3.29)	0.473
Venous IL-1 ng/ml	3.10 ±13.01	0.40 (0.11-2.21)	0.90±0.60	0.80 (0.50-1.30)	0.284
Post chamber IL-1 ng/ml	0.65±0.45	0.56 (0.31-0.86)	0.71±0.65	0.48 (0.28-1.06)	0.636
Venous IL-6 ng/ml	3.30±2.37	2.24 (1.70-4.91)	1.82±0.80	1.61 (1.46-2.16)	0.001
Post chamber IL-6 ng/ml	3.05±2.05	2.29 (1.68-4.27)	1.86±3.05	1.84 (1.31-2.19)	0.001
Venous TNFα ng/ml	9.48±3.77	9.43 (7.03-10.90)	7.65±3.31	7.89 (4.86-10.03)	0.024
Post chamber TNFα ng/ml	5.98±2.20	5.83 (4.34-6.96)	5.4±5.98	5.24 (4.43-6.09)	0.167

Table 4.10 Inflammatory biomarkers – ACS study.

	HsCRP	Venous IL-6	Venous TNFα	Post chamber IL-6	Post chamber TNF	Delta IL-6	Delta TNFα
Correlation with thrombus area	0.151	0.251	0.319	0.126	0.073	0.371	0.389
P value	.189	.027	.005	.276	.527	.001	.001

Table 4.11 Correlations between cytokines and thrombus area –ACS study.

	Clopidogrel+aspirin (n=45)			**Placebo+aspirin (n=45)**		
Mean±SD	Visit 1	Visit 2	P value	Visit 1	Visit 2	P value
Venous IFNγ, ng/ml	2.32±1.5	2.51 ±1.6	0.053	3.28 ±2.79	3.14±3.20	0.154
Post chamber IFNγ, ng/ml	2.90±1.7	2.91 ±2.2	0.112	3.56±2.7	3.18±2.55	0.512
Venous IL1, ng/ml	0.12±0.22	0.08 ±0.15	0.021	0.20 ±0.25	0.12±0.21	0.167
Post chamber IL1, ng/ml	0.19±0.25	0.16 ±0.31	0.026	0.37 ±0.54	0.17±0.31	0.705
Venous IL6, ng/ml	2.40±1.9	2.60 ±1.4	0.274	2.74 ±2.19	2.59±1.73	0.865
Post chamber IL6, ng/ml	3.00±2.5	2.71 ±1.5	0.904	2.91 ±2.30	2.74±1.81	0.881
Venous TNFα, ng/ml	10.20±5.3	10.51 ±4.9	0.835	10.31 ±5.20	10.41±4.87	0.865
Post chamber TNFα, ng/ml	10.71±5.1	10.9 ±5.0	0.245	11.26 ±5.14	11.84±3.82	0.210

Table 4.12 Inflammatory cytokines - Stable CAD study.

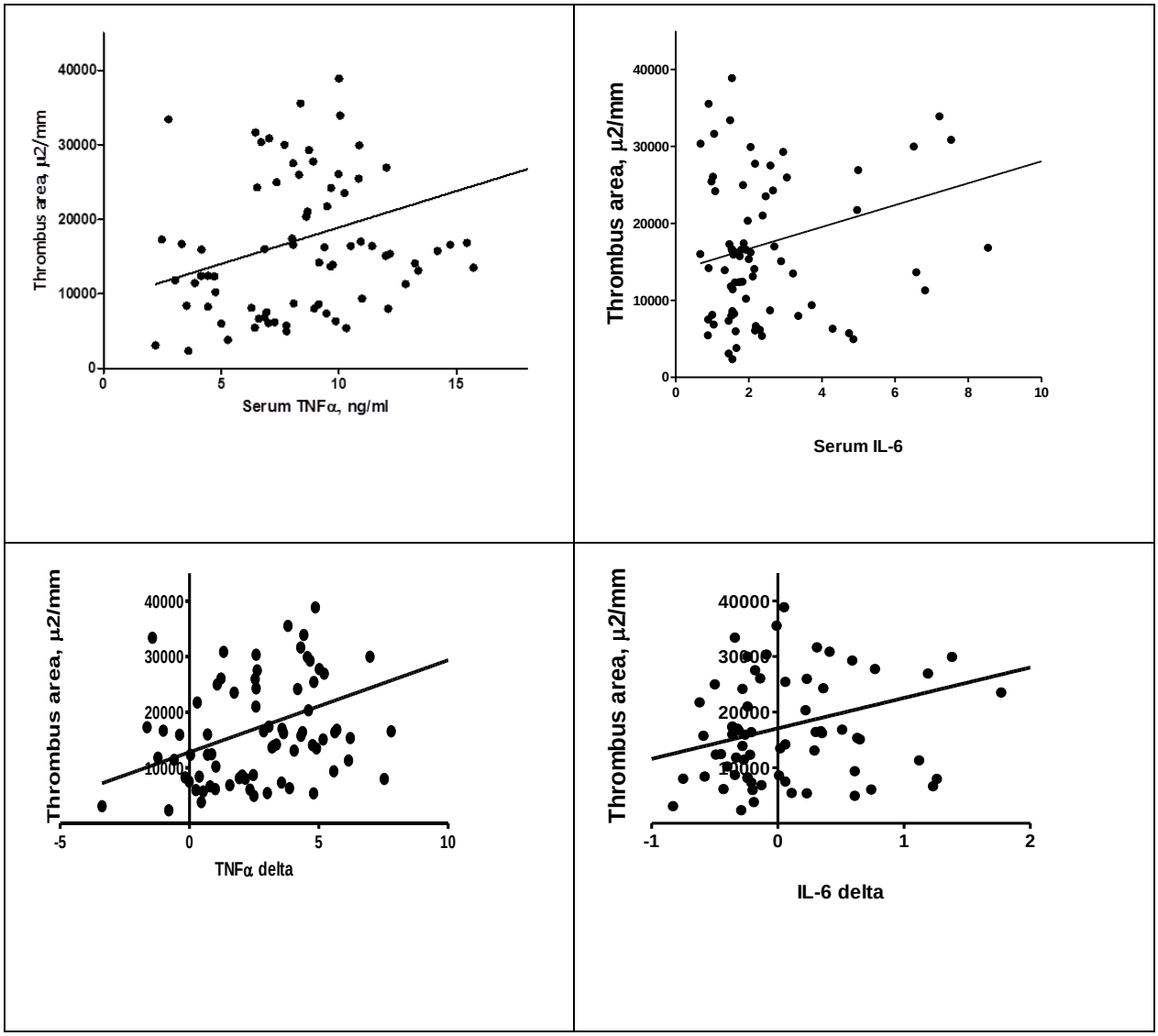


Figure 4.7 Correlation of TNFα and IL-6 levels and thrombus area in ACS study.

Serum levels (Pre chamber-venous samples) of TNFα (rho=0.251, p=0.027) and IL-6 (rho=0.319, p=0.005) correlated with thrombus area. The delta values (pre chamber- effluent blood) levels also correlated with thrombus area (TNFα delta rho=0.389, p=0.001, IL-6 delta rho=0.371, p=0.001).

4.3.3 Discussion - Inflammation and platelet dependent thrombus

I explored the role of inflammation, which is linked to both T2DM and CAD, in thrombosis as a "common soil" factor (Stern, 1995). High sensitivity CRP (HSCRP) and inflammatory cytokines TNFα and IL-6 were higher in patients with T2DM after ACS compared to non-diabetic patients and were directly correlated to thrombus. Clopidogrel therapy failed to reduce the levels of hsCRP, TNFα and IL-6 but reduced the levels of IL-1.

Indirect evidence shows TNFα promotes thrombogenicity by inducing expression of tissue factor in monocytes, and platelet and leucocyte adhesion to endothelium (Lindmark *et al.*, 2000). TNFα also increases the production of large multimers of van Willebrand factor (vWF) which in turn enhances thrombogenicity. TNFα plays a positive feedback role in the synthesis of fibrinogen and thereby indirectly increases thrombogenicity (Zhao *et al.*, 2011). IL-6 induces synthesis of new platelets which are more thrombogenic and contribute to thrombogenicity in T2DM (Stratmann and Tschoepe, 2005). In addition, high levels of IL-6 delay the clearance of vWF and thus sustain the thrombogenicity (Bernardo *et al.*, 2004). TNFα and IL-6 also down regulate naturally occurring antithrombotic substances such as protein C and thrombomodulin (Esmon, 2005). Inflammatory cytokines and hsCRP levels appear to fall after aspirin and clopidogrel therapy, but the majority of evidence was derived from patients who had NSTEMI and coronary stent procedures (Steinhubl *et al.*, 2007; Antonino *et al.*, 2009; Muhlestein, 2010). Findings from my ACS study confirm the available evidence of a pro-inflammatory and pro-thrombotic state in T2DM and ACS (Dovio and Angeli, 2001; Tousoulis *et al.*, 2006) but also add that these findings remain unaltered despite current aggressive secondary prevention and there is a direct association between inflammatory cytokines and thrombus formation.

My study on stable CAD showed that cytokine levels remain unchanged after a week of clopidogrel therapy in T2DM. Failure of DAPT to reduce these biomarkers of inflammation and thrombosis in patients with T2DM and CAD has been noted in two separate one year follow up studies namely Aspirin non-responsiveness and Clopidogrel Endpoint Trial (ASCET) study and ELAPSE

(evaluation of long-term clopidogrel antiplatelet and systemic anti-inflammatory effects) study, both of which included patients with and without T2DM (Solheim *et al.*, 2006; Saw *et al.*, 2008). It is also possible that T2DM is associated with greater inflammation and 75mg of clopidogrel therapy is insufficient to suppress the levels of biomarkers.

I found that the levels of TNFα and IL-6 were significantly associated with thrombus formed in Badimon chamber (IL6: rho=0.251, p=0.027, TNFα: rho=0.319, p=0.005). These findings are novel and may imply a simple association secondary to ACS (Tousoulis *et al.*, 2006) or a causal factor for increased thrombogenicity in these high risk individuals (Young *et al.*, 2002).

4.3.4 Post chamber blood ("effluent" blood) from the Badimon chamber

To explore the causal association of these biomarkers in patients with ACS and T2DM, I studied post chamber effluent blood. In simplistic terms, post chamber effluent blood represents "activated blood" after being subjected to high shear force inside the chamber and contact activation with collagen. As the chamber recreates the milieu of coronary vasculature, this may be equivalent to the blood sample from coronary sinus in these individuals. Similarly, for P selectin and CD40 ligand, the gradient between pre and post chamber blood might represent the amount of biomarkers used in the thrombus which was derived from venous (pre chamber) blood and those released from activated platelets inside the chamber. Delta values of P selectin and CD40L showed no correlation to thrombus area, perhaps because of the differential release of these substances from the cells in flowing blood.

Since cytokines are neither synthesised nor released from the flowing blood inside the chamber, the delta values would solely represent the cytokines consumed in the chamber and probably been used in the formed thrombus. My findings are interesting as it validates a role of cytokines in the thrombus formed in Badimon chamber and also represents a novel model to assess the role of various biomarkers directly in the production of thrombus. As Badimon chamber represents flow conditions of coronary arteries, if my findings are validated in different clinical settings, it will be possible to substitute Badimon chamber

studies for highly invasive coronary sinus sampling for certain coronary flow experiments. The effluent blood can be used to quantitatively study the interplay of cytokines and thrombus after pharmacological intervention.

Levels of inflammatory cytokines (TNFα, IL6, IL-1 and INFγ) remain unchanged in pre and post chamber samples before and after clopidogrel therapy in patients with stable CAD and T2DM. This negative finding can be due to i) inflammatory markers were lower in T2DM with stable CAD ii) addition of clopidogrel did not reduce venous cytokines in patients with T2DM and stable CAD and iii) there was a lack of association between cytokines in venous blood and thrombus in patients with stable CAD and T2DM.

For P selectin and CD40 ligand, the gradient between pre and post chamber blood may represent the amount of these biomarkers used in the thrombus. P selectin in the thrombus is likely derived from venous (pre chamber) blood and that released from activated platelets inside the chamber. However, I could not find any correlation between the venous soluble P selectin to thrombus. Delta values of P selectin and CD40L showed no correlation to thrombus area. The negative values may be due to smaller sample size in my study. Soluble P selectin measured by ELISA methods (used in my study) show more heterogeneous spread compared to the measurements from flow cytometric methods (Ritchie *et al.*, 2000). It is possible that the P selectin and CD40ligand levels may have shown better correlation to thrombus if flow cytometric methods were used in the study.

Study	Intervention	Results
ALBION study, NSTEMI patients (n=103)(Montalescot *et al.*, 2006)	Loaded with 300-600-900mg clopidogrel in addition to aspirin	No difference between HSCRP, CD40L in three arms
NSTEMI, RCT and followed up for one month (n=115)(Chen *et al.*, 2006)	Aspirin vs. aspirin and clopidogrel	HSCRP and TNF alpha reduced significantly in DAPT group
NSTEMI patients, single blinded study, followed for 9 months (n=86)(Vavuranakis *et al.*, 2006)	Aspirin vs. aspirin and clopidogrel	P selectin levels reduced with DAPT but only in those with higher baseline CD40L and hsCRP levels.
Stable CAD, non randomised study, followed for 5 weeks (n=103)(Heitzer *et al.*, 2006)	Aspirin vs. aspirin and clopidogrel	CD40L and hsCRP reduced after DAPT
Stable CAD, RCT (n=73)(Azar *et al.*, 2006)	Aspirin vs. aspirin and clopidogrel	CD40L reduced significantly but hsCRP remain unchanged

Table 4.13 Biomarker studies on aspirin and clopidogrel therapy

4.4 Obesity and platelet dependent thrombus in T2DM

4.4.1 Results

The effect of obesity in platelet dependent thrombus was studied in this sub group analysis. Patients were classified as obese if their BMI was >30.0 kg/m^2.

In ACS study, obese patients with T2DM had the highest thrombus (in μ^2/mm, 22801±13840, one way ANOVA p=0.02). Both HsCRP (11.2±3.5) and TNF-α levels (13.1±10.0) were the highest in this group (one way ANOVA p=0.009 and p=0.018, respectively, Table 4.14).The individual differences remained significant between the obese T2DM vs. non-obese DM and obese T2DM vs. non-obese controls upon applying Bonferroni's correction. There were significant correlation between TNF-α levels and thrombus area in obese individuals (rho 0.413, p=0.015) and it persisted after adjusting for diabetic status (rho=0.361, p=0.042).

In stable CAD study, thrombus area did not differ between obese and non obese individuals after clopidogrel therapy. There were no differences in inflammatory markers between the groups (Table 4.15).

	Obese+ T2DM (n=23)		**Non obese+ T2DM (n=17)**		**Obese+ non DM (n=16)**		**Non obese+ non DM (n=24)**		**P value**
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D	
Thrombus area, μ^2/mm	22801	13840	20801	7111	12477	7001	14405	7507	0.002
HSCRP mg/l	13.1	10.0	7.7	9.2	9.7	14.2	3.5	3.6	0.009
TNF-α, ng/ml	11.2	3.5	9.1	2.2	8.9	2.1	9.3	1.8	0.018

Table 4.14 Inflammation and thrombus of obese patients in ACS study

Obesity was defined as BMI ≥ 30 kg/m^2.

T2DM +stable CAD	**Aspirin+Clopidogrel**					**Aspirin+Placebo**				
	Obese (n=28)		**Non Obese (n=17)**			**Obese (n=30)**		**Non Obese (n=15)**		
	Mean	S.D	Mean	S.D	P value	Mean	S.D	Mean	S.D	P value
Thrombus area, baseline, μ^2/mm	13287	5521	15175	5442	0.296	13345	7564	15327	5729	0.406
Thrombus area, visit 2, μ^2/mm	10446	3790	12485	3467	0.095	13789	7126	6101	15142	0.556
Fibrinogen, g/l	3.4	0.7	3.4	0.6	0.977	3.4	0.6	2.9	0.9	0.544
HsCRP, mg/l	4.8	4.9	3.7	3.6	0.469	3.4	3.3	3.5	4.2	0.885
TNF-α, ng/l	10.7	5.4	9.7	5.0	0.540	10.0	4.1	11.2	6.3	0.469

Table 4.15 Inflammation and thrombus in patients with T2DM and obesity (Stable CAD study).

4.4.2 Discussion

Obesity and metabolic syndrome are independently associated with higher cardiovascular events in T2DM. Obesity increases the thrombotic tendency in T2DM by direct and indirect means. Higher levels of platelet activation, inadequate response to antiplatelet agents (e.g. aspirin) and reduced fibrinolysis were documented in T2DM (Santilli *et al.*, 2012),(Das and Mukhopadhyay, 2011),(Schneider *et al.*, 2009),(Meerarani *et al.*, 2007). Obesity is an independent risk factor for poor outcomes in patients after NSTE-ACS(Bhatt, 2008). My study has shown for the first time a direct relationship between whole blood thrombus and obesity in patients with T2DM after NSTE-ACS.

Studies have shown a significant association between obesity and inflammation. It has been claimed that inflammatory mediators are responsible for the initiation and perpetuation of insulin resistance and islet cell damage in T2DM (Gregor and Hotamisligil, 2011). As discussed earlier, inflammation increased blood thrombogenicity (Levi and van der Poll, 2010). In obese individuals, it is possible that inflammation could have been the missing link between T2DM diabetes and resultant prothrombotic state (Doupis *et al.*, 2011). Recently a long term follow up study on patients with obesity and T2DM failed to show significant benefits with strict weight reduction and it is possible that inflammation plays a crucial role in determining their cardiovascular outcomes (The Look Ahead Research Group, 2013). Although my study was not aimed at establishing the causative role of inflammation in obese T2DM and blood thrombogenicity, my data may serve as a hypothesis generating tool which has to be confirmed by future studies.

4.5 VerifyNow® platelet reactivity indices and platelet dependent thrombus

4.5.1 Results – ACS study

I analysed platelet reactivity in 28 patients with T2DM and 14 patients without DM after NSTE-ACS, for VerifyNow® sub study to measure effects of aspirin and clopidogrel. Baseline characteristics of the patients were similar as seen in the main study (Table 4.16). Patients with T2DM had higher prevalence of treated hypertension and dyslipidaemia. On treatment platelet hyperactivity to aspirin was not statistically different between both the groups (ARU, mean±SD: T2DM 411±31.1 vs. Non DM 423±63.9, p=0.584, Figure 4.8). All but one patient (in T2DM group) showed good response to aspirin using a cut off of 495 units. On treatment platelet hyperactivity index for clopidogrel therapy, PRUz, was numerically higher but showed no statistical significance between diabetic and non-diabetic patients (Mean±SD: 246±58.9 vs. 220±82.3, p=0.427, Table 4.17, Figure 4.8). Percentage inhibition as calculated by using the manufacturer's formula for clopidogrel was 24% in T2DM and 31% in non DM (p=0.096). The distribution of PRUz values was highly skewed in both the groups (Figure 4.9). Using a PRUz cut off of ≥ 240 units, 43% (n=12) of T2DM and 29% (n=4) of those without DM were "hyporesponders" to clopidogrel but this difference was not statistically significant (p=0.505). PRUz values did not correlate with thrombus area (rho 0.269, p=0.089, Figure 4.11. There was also no correlation between thrombus area and ARU values (rho 0.034, p=0.849). Thrombus area was numerically lower between those who had a good response to clopidogrel (PRUz less than 240) and those who had low response to clopidogrel (thrombus area, µ2/ mm, mean±SD, 17017 ±11000 vs 20744±9132, p=0.263), but the difference lacked statistical difference. When stratified by clopidogrel response (cut off value of ≥ 240 PRUz) and diabetic status, patients with T2DM and low response to clopidogrel showed a trend for the highest thrombus whereas thrombus was lowest in those without T2DM and good response to clopidogrel (one way ANOVA F=2.177, p=0.107, Figure 4.10).

4.5.2 Results – Stable CAD study

Baseline characteristics of patients who had clopidogrel and placebo were discussed earlier (Table 4.3). Based on a PRUz cut-off value of ≥240, there were 18 patients (44%) in whom a good response to clopidogrel was detected. After one week of clopidogrel 75 mg once daily, the absolute mean PRUz values fell from 321±40.2 at baseline to 258±61.5, p=0.001 and were unchanged in the placebo arm (mean±SD) 320±78.1 to 336±48.5, p=0.123 (Table 4.16). The mean absolute reduction of PRUz values in clopidogrel group was 78.2 (95% CI 52.8 to 103.5, Figure 4.12, Figure 4.13). Platelet inhibition to clopidogrel, as measured by VerifyNow® showed a significant change from 1.5% at baseline to 20% after one week of clopidogrel therapy. For aspirin, ARU values at baseline for the treatment and placebo arms were (mean±SD), 456±53.9 vs. 468±73.2 and were not significantly altered in either group on the second visit 440±76.8 vs. 452±57.3 respectively (Table 4.18). Thrombus area had no correlation with PRUz (rho 0.08, p=0.49, Figure 4.14) and was numerically higher in clopidogrel hyporesponders but not statistically different when compared to those who had good response to clopidogrel (mean thrombus area±SD, μ^2 per mm: 12186±4294 vs. 10438±3401; p=0.17, figure 4.15). In patients who received clopidogrel, change in thrombus did not correlate with change in PRUz (rho 0.172, p=0.30). After one week of clopidogrel therapy, there were significant associations between PRUz and HsCRP, CD40 ligand and TNFα, respectively [Figure 4.16]. Platelet count and fibrinogen levels had no relationships to PRUz values. BMI levels correlated with PRUz (r=0.383, p=0.012), percentage of platelet inhibition after clopidogrel therapy (r=-0.327, p=0.037) and delta values of PRUz (-0.309, p=0.047) but fibrinogen and diabetes control as assessed by HbA1c levels showed no association to PRUz values in stable CAD patients with T2DM.

	T2DM (n=28)		Non DM (n=14)		
	Mean±SD	**Median (IQR)**	***Mean±SD***	**Median (IQR)**	P value
Demographic data					
Age, years	64±11	66 (53-74)	58±8	57(52-64)	0.310
Male gender, % (n)	50.0(14)		85.7(12)		0.207
Body mass index, kg/m^2	35±9.9	31(27-40)	31±5.1	30(29-33)	0.989
Waist to hip ratio	1.0±0.05	1.0 (0.9-1.1)	1.0±0.05	0.9 (0.9-1.1)	0.986
Systolic BP, mmHg	130±15.2	128(122-140)	121±12.1	119(112-130)	0.090
Diastolic BP, mm Hg	71±11.1	71(60-78)	71±6.2	72(68-74)	0.566
***Risk profile:* %(n)**					
History of hypertension, %(n)	17.9(5)		14.3 (4)		0.005
History of dyslipidaemia	64.3 (18)		42.9 (6)		0.007
History of chronic kidney disease	17.9(5)		21.4 (3)		0.869
History of acute myocardial infarction	17.9(5)		7.1 (1)		0.055
***Medications:* %,(n)**					
B blockers, %,(n)	71.4 (20)		100 (14)		0.407
Calcium channel blockers, %(n)	32.1(14)		7.1 (1)		0.051
Laboratory data:mean±SD					
HBA1c, %	7.5±1.6	7.3(6.4-8.2)	5.7±0.3	5.7(5.5-5.9)	

	T2DM (n=28)		Non DM (n=14)		
	Mean±SD	**Median (IQR)**	***Mean±SD***	**Median (IQR)**	P value
Random plasma glucose, mmol/l	10.8±7.7	7.8(5.8	5.3±0.6	6.9(5.3-8.1)	
Fasting plasma glucose, mmol/l	8.3±3.5	7.4(5.9-9.7)	5.7±0.3	5.2(4.9-5.6)	
Haemoglobin, g/dl	12.9±1.9	13.8(11.6-14.1)	13.6±1.5	13.7(12.4-15.1)	0.946
Platelets, x1000/mm^3	272±101	254(206-316)	287±105	252(211-347)	0.695
Fibrinogen, g/l	4.5±0.7	4.4(4.2-5.0)	3.7±1	3.6(3.0-4.3)	0.071
eGFR (ml/min/1.73 m^2)	67±23	63(57-78)	74±16	73(57-91)	0.462
Total cholesterol, mmol/l	3.1±0.9	3.2(2.6-3.7)	3.6±1	3.4(3.0-4.1)	0.659
LDLc, mmol/l	1.4±0.6	1.6(1.0-1.9)	2.1±0.8	2.0 (1.4-2.5)	0.071
HDLc, mmol/l	0.9±0.2	0.9(0.8-1.1)	2.1±0.8	0.9(0.8-1.1)	0.967
Triglyceride, mmol/l	1.8±1.1	1.4(1.0-2.1)	1±0.2	1.3(1.1-1.6)	0.320
Troponin I, µg/l	3.1±3.6	1.7(0.1-4.8)	1.7±0.7	0.6(0.2-2.2)	0.172
hsCRP mg/l	14.2±14.1	5.1(2.1-28.9)	3.4±2.5	2.8(2.0-4.0)	0.390

Table 4.16 Baseline characteristics of VerifyNow® sub study (ACS study).

Mean±SD	T2DM (n=28)	Non DM (n=14)	P value
ARU	411±31.1	423±63.9	**0.584**
PRUz	246±58.9	220±82.3	**0.427**
Platelet inhibition, %	24±15.8	30.9±21.3	**0.096**

Table 4.17 VerifyNow® indices for ACS study.

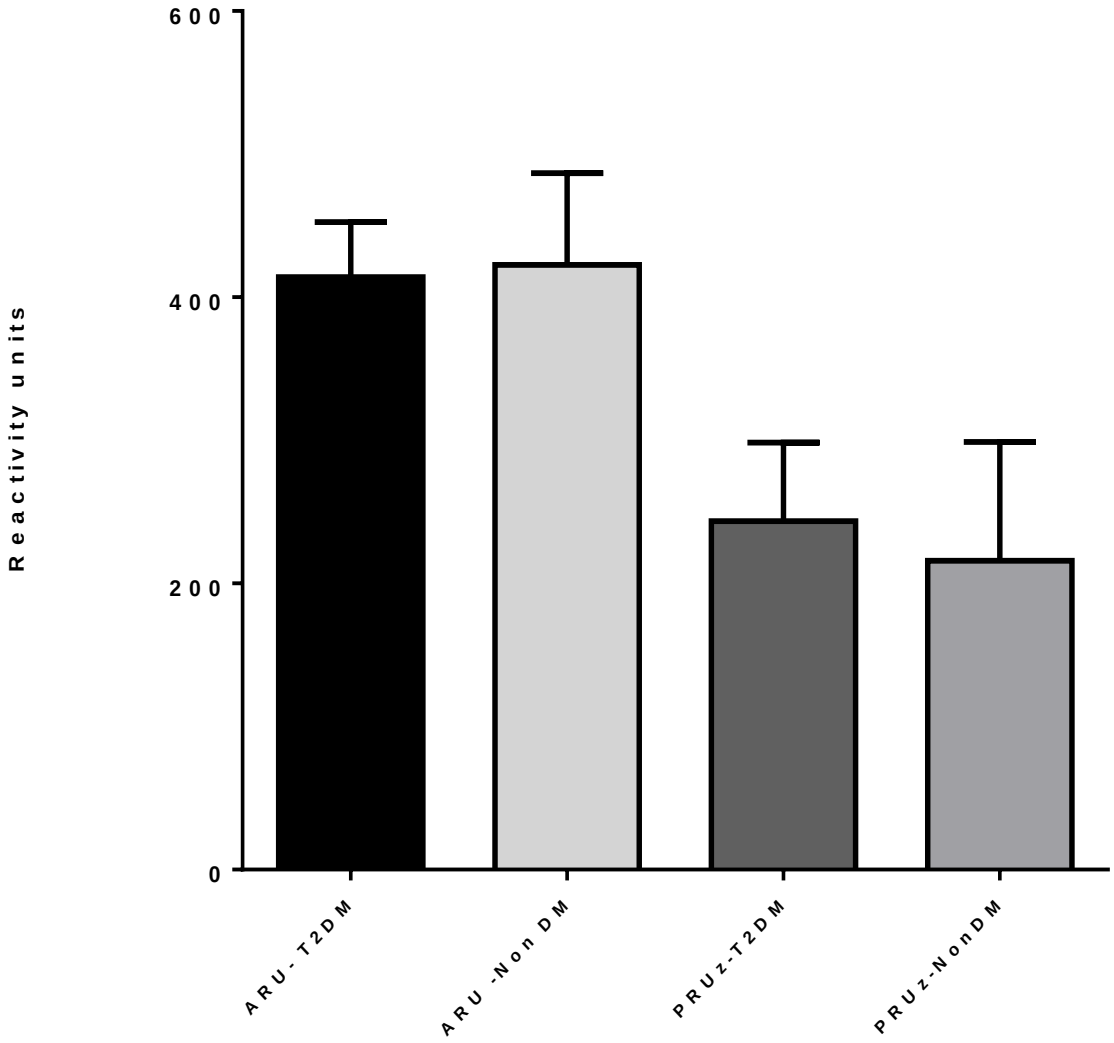


Figure 4.8 VerifyNow® platelet reactivity indices - ACS study

The aspirin on treatment platelet reactivity was measured by a dimensionless unit ARU and clopidogrel on treatment platelet reactivity was measured by a similar index PRUz. ARU values were nearly equal between the groups (mean±SD: T2DM 411±31.1 vs. Non DM 423±63.9, p=0.584). The mean values of PRUz was numerically higher but showed no statistical difference between T2DM and non-diabetic participants (mean±SD: 246±58.9 vs. 220±82.3, p=0.427). Error bars represent 1X standard deviation.

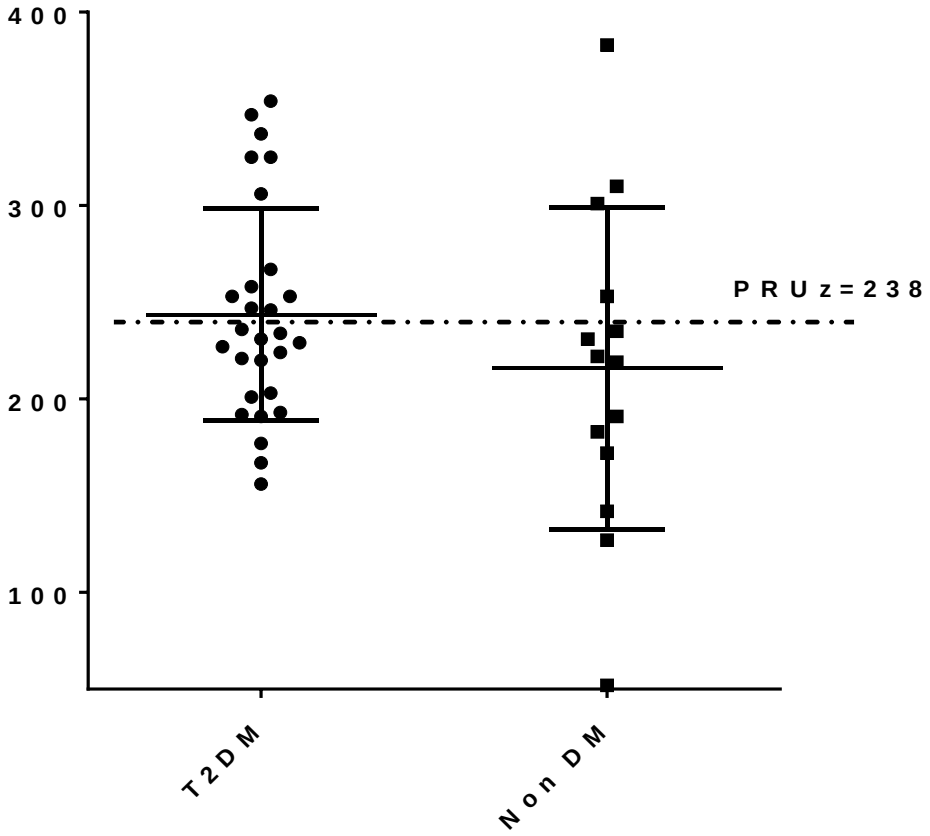


Figure 4.9 PRUz levels in patients after NSTE-ACS.

On treatment platelet hyperactivity to clopidogrel was measured by using a PRUz cut off of 240 units. 43% (n=12) of T2DM and 29% (4) of those without DM were “hyporesponders” to clopidogrel but this difference was not statistically significant (p=0.505). The clopidogrel response was heterogeneous in both the groups with a highly skewed distribution. The dotted line represents the cut off of PRUz units for high on treatment platelet hyperactivity. The solid bars represent mean values of PRUz and 2Xstandard deviation.

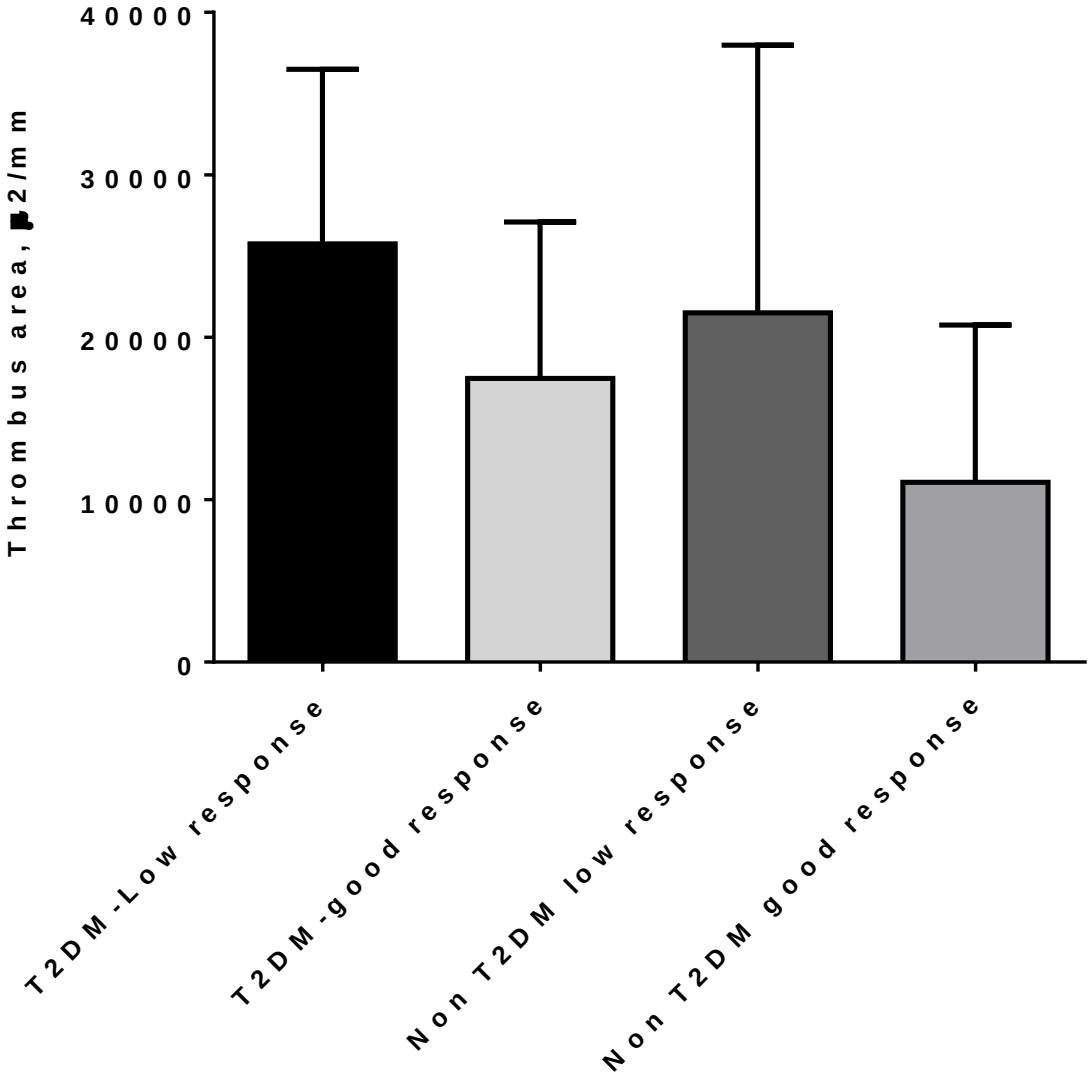


Figure 4.10 Thrombus area according to diabetic status and clopidogrel response after NSTE-ACS.

When stratified according to clopidogrel response (cut off value of 240 PRUz) and diabetic status, patients with T2DM and low response to clopidogrel had the highest thrombus area whilst those without T2DM and good response to clopidogrel had the lowest area. However, this trend was not statistically significant. (One way ANOVA F=2.177, p=0.107). The solid bars represent 1X standard deviation.

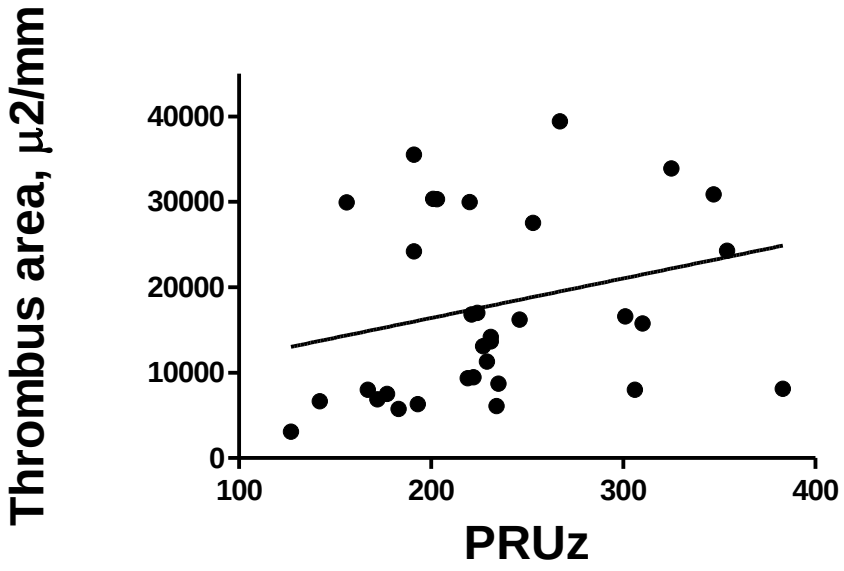


Figure 4.11 Correlation between thrombus area and VerifyNow® PRUz values in ACS study. The correlation was not significant (rho 0.269, p=0.089).

	Clopidogrel (n=45)			**Placebo (n=45)**		
Mean±SD, units	Baseline	Visit 2	P value	Baseline	Visit 2	P value
ARU	456(53.9)	440±76.8	0.124	468(73.2)	452±57.3	0.426
PRUz	321±40.2	258±61.5	0.001	320±78.1	336±48.5	0.123
% inhibition	2.3±5.9	20.4±16.1	0.001	1.5±3.4	1.14±2.5	0.380

Table 4.18 VerifyNow® indices of stable CAD study.

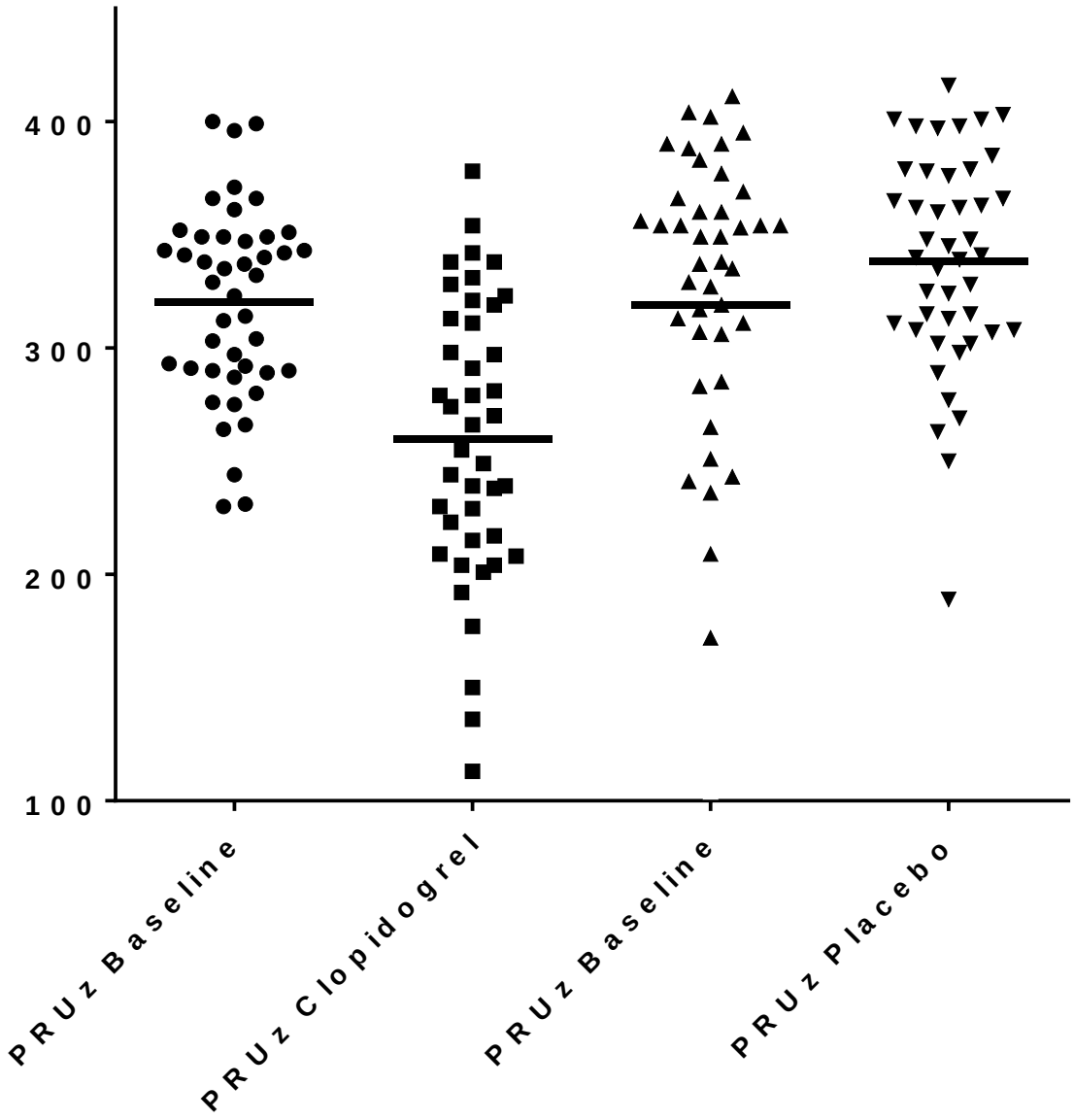


Figure 4.12 PRUz values of patients in stable CAD study.

Baseline values of patients in placebo and clopidogrel groups were similar. After one week of therapy, PRUz values were lower in clopidogrel group and remained unchanged in placebo group. (Clopidogrel, (mean±SD) 321±40.2 to 258±61.5, p=0.001; placebo 320±78.1 to 336±48.5, p=0.123). Response to clopidogrel showed a heterogeneous spread of PRUz from 46 (very good response) to 382 (no response). 44% in clopidogrel group had PRUz values lower than 240 (good clopidogrel response) and there was a considerable variation in reduction of PRUZ values. Solid lines represent mean of the values.

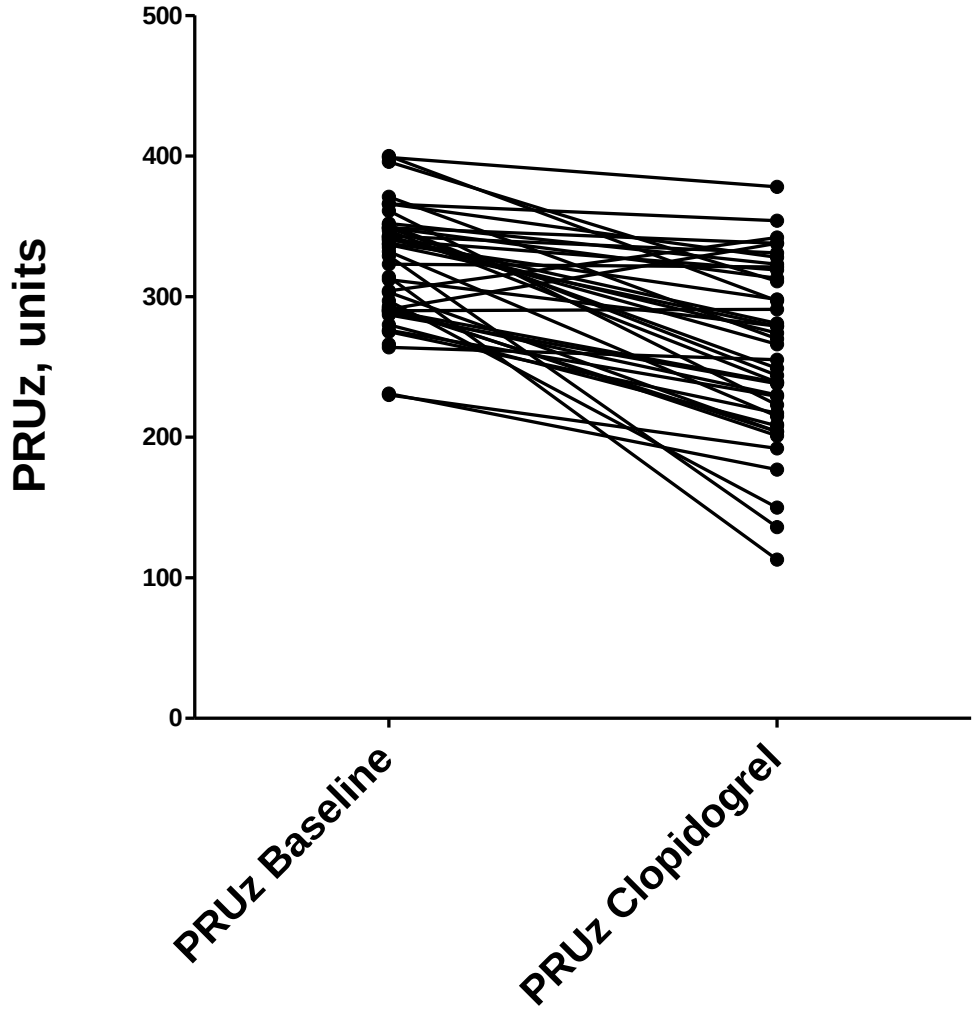


Figure 4.13 Changes in PRUz after clopidogrel therapy in stable CAD study.

Mean (mean±SD) PRUz values reduced from 321±40.2 to 258±61.5 in clopidogrel group. There was a considerable variability in reduction of PRUz values, mean reduction was 78.2 (95% CI 52.8 to 103.5).

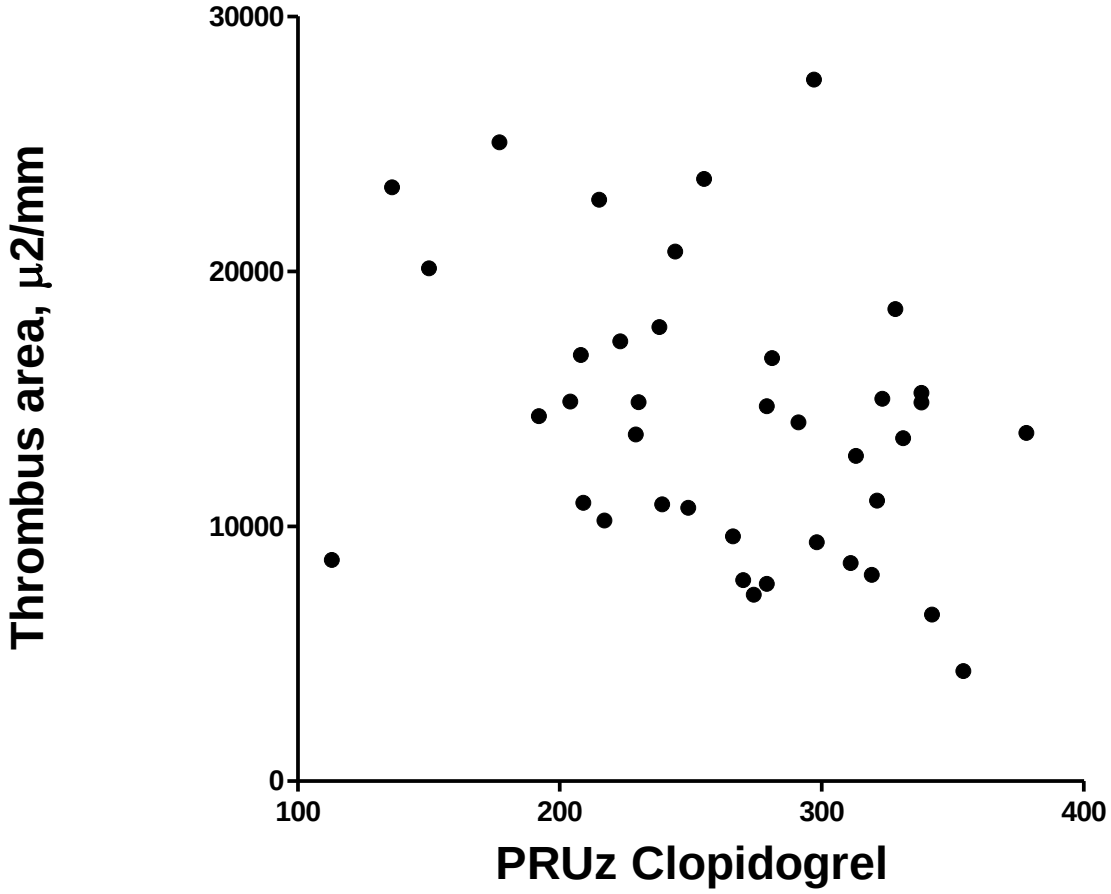


Figure 4.14 Correlation between PRUz values and thrombus area in stable CAD study.

PRUz values and thrombus area in patients with T2DM and CAD who had clopidogrel in addition to routine aspirin therapy showed no correlation (rho= 0.08, p=0.49).

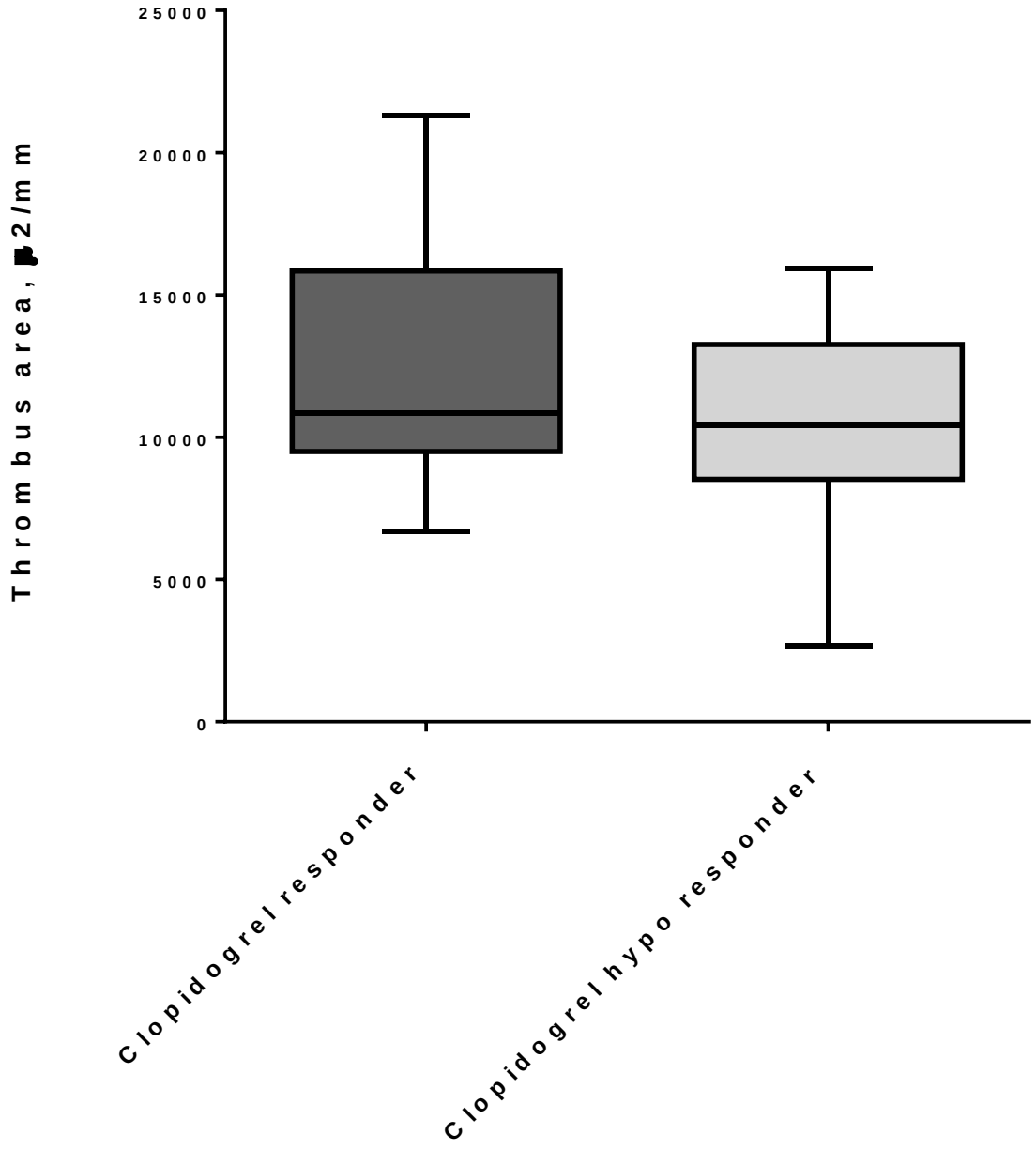


Figure 4.15 Thrombus area based on PRUz values in stable CAD study

Patients with T2DM who had one week of clopidogrel therapy have been defined as clopidogrel hyporesponders if their PRUz values were ≥ 240. Thrombus area did not differ between clopidogrel hypo responders and clopidogrel responders (mean thrombus area±SD, μ^2 per mm: 12186±4294 vs. 10438±3401; p=0.17). Whisker plot shows median (central line), 25-75 interquartile range and the solid bars represent the range between minimum and maximum values.

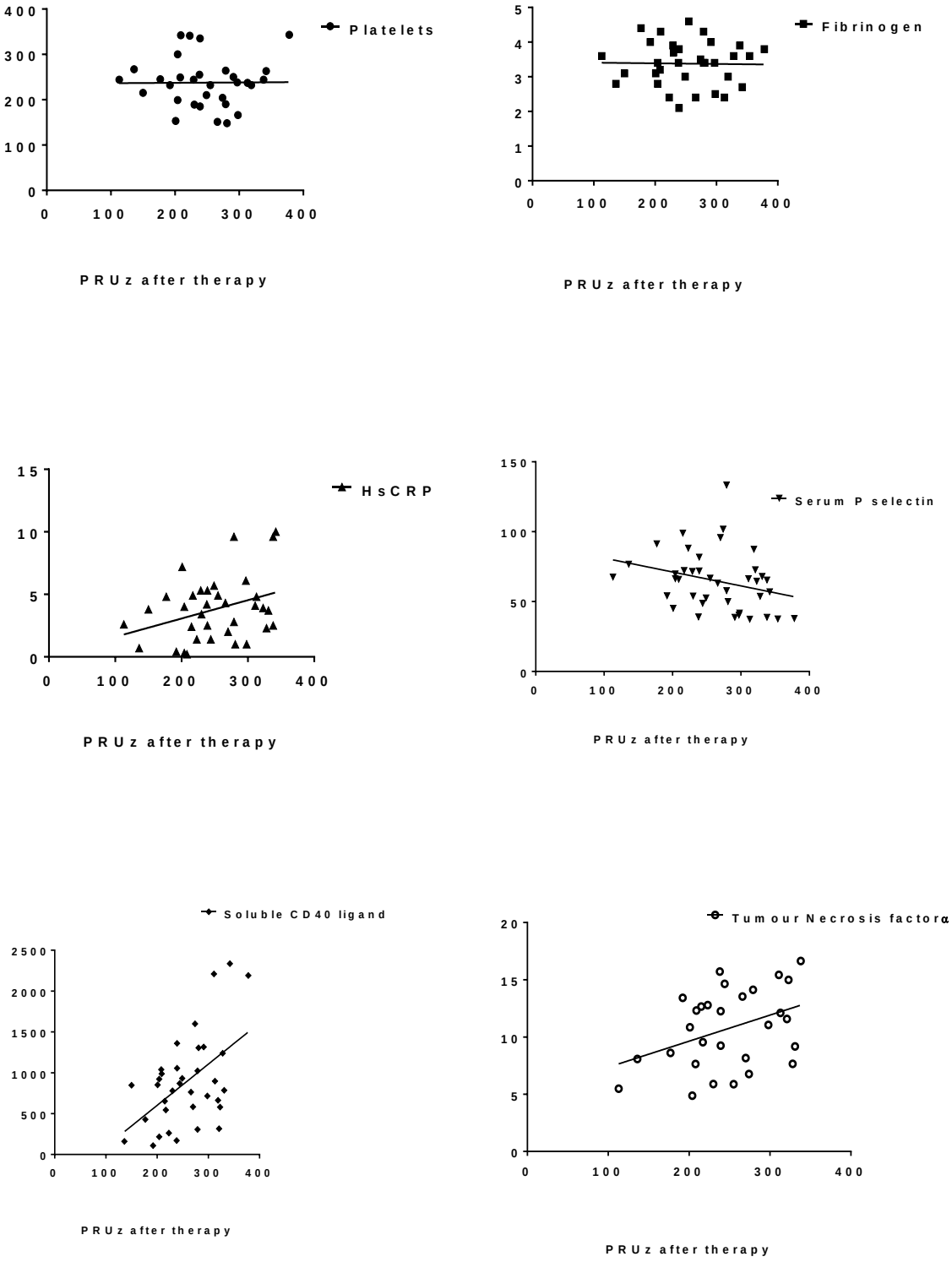


Figure 4.16 PRUz values and biomarkers in stable CAD study.

There were significant associations between HSCRP (r= 0.1168, p=0.0446), soluble CD40 ligand (r=0.2676, p=0.0015) and tumour necrosis factor α (r=0.2676, p=0.0369). Platelet count, plasma fibrinogen and serum P selectin valued had no significant correlation to PRUz.

4.5.3 Discussion

My study showed a trend towards higher PRUz index in patients with T2DM who had NSTE-ACS treated with aspirin and clopidogrel (75mg of each daily) and a significant reduction in PRUz platelet reactivity index in patients with T2DM and stable CAD following one week of clopidogrel (75mg od) therapy. For the first time, VerifyNow® indices were compared to platelet dependent thrombus (PDT) from the Badimon chamber. Comparison of point of care device indices to whole blood thrombus generated from the ex vivo chamber (mimicking flow conditions in coronary arteries) is novel and logical and will be of interest to clinicians considering using these devices to tailor antiplatelet therapy.

Thrombus (measured as total thrombus area) failed to show any correlation to PRUz levels and there was no correlation to changes in PRUz values (delta PRUz) to changes in thrombus (delta thrombus). These data suggest that PRUz values cannot serve as a surrogate marker for reduction in thrombus area in T2DM. When stratified according to presence or absence of T2DM and response to clopidogrel, there was a non-significant trend showing that those with T2M and “hyporesponse” had greater thrombus.

Numerous studies have evaluated the use of VerifyNow® in routine clinical practice to guide the choice and monitoring of antiplatelet therapy in patients with coronary artery disease. My data confirms that on treatment platelet hyperactivity to clopidogrel as measured by PRUz has high variability in patients with T2DM after NSTE-ACS and in those with stable CAD. Mean PRUz values were non-significantly higher in T2DM after ACS reflecting inadequate antiplatelet response in these high risk individuals. Majority of the studies showed 20-25% patients had adequate platelet inhibition after clopidogrel therapy in the ACS and stable CAD groups. Difficulty in comparing data from various studies arises from their heterogeneity in study design, varying dosing regimens (e.g. 75mg, 150mg), and different baseline characteristics. International cardiac societies, for the same reasons, have urged a note of caution in adopting VerifyNow® assessment as standard care in patients with cardiovascular disease (Bonello *et al.*, 2010).

Platelet reactivity to clopidogrel as measured by PRUz was normally distributed in my cohort in both the studies but with a heterogeneous spread. Only 44% patients showed a good response to clopidogrel in my cohort based on PRUz cut off of < 240 units with a trend towards higher PRUz in T2DM after NSTE-ACS. BMI, fibrinogen and HbA1c showed no correlation to PRUz in patients after NSTE-ACS but BMI showed good correlation to PRUz in stable CAD patients. Previous studies have shown that in T2DM, elevated fibrinogen and raised BMI were independent predictors of high on treatment platelet reactivity in individuals with stable CAD (Ang, Palakodeti et al. 2008).

Dose of clopidogrel other than 75mg is controversial in patients with T2DM. A small study tested the role of 150mg of clopidogrel in T2DM and found that platelet inhibition increased from 27.1 ± 12% to 40.6 ± 18% (p = 0.009). However, a significant number of patients still had PRUz values above 240 at this dosage (Angiolillo *et al.*, 2008a). Newer agents such as ticagrelor or prasugrel which showed superiority over clopidogrel in VerifyNow® sub-studies(Alexopoulos *et al.*, 2013) might have resulted in better platelet inhibition in my subjects but these agents were not available when the study protocol was approved.

To address the variability and “hyporesponse” to clopidogrel, The SWAP (SWitching Anti Platelet) study examined the role of changing patients to prasugrel instead of clopidogrel and found that prasugrel achieved earlier and better platelet inhibition in those who exhibited high on treatment platelet reactivity to clopidogrel (Angiolillo *et al.*, 2010). Similar results were shown by Gurbel et al when they used ticagrelor in patients who showed hypo responsiveness to clopidogrel. In addition to overcoming hypo responsiveness to clopidogrel, its antiplatelet effects were greater in both clopidogrel responders and hyporesponders. Nearly all clopidogrel hyporesponders and responders treated with ticagrelor had PRUz values less than 235 (Gurbel *et al.*, 2010a)and the variability and low response to clopidogrel were attributed to its metabolite. Attempts made to assess levels of the thiol derivative active metabolite of clopidogrel and its relationship to PRUz of clopidogrel yielded inconsistent results due to its pharmacokinetic properties. However, Bouman et al showed thiol active metabolite levels correlated well with PRUz values (Bouman *et al.*, 2010).

VerifyNow® has been evaluated for prediction of clinical events in many studies. Patients with T2DM and high on treatment platelet reactivity (HOTR) as measured by PRUz had the highest incidence of peri-procedural MI compared to those with low HOTR (p for trend = 0.0008). PRUz value of ≥240 was an independent predictor of peri-procedural MI (odds ratio 8.34, 95% confidence interval 2.60 to 26.76, p = 0.0003) (Migliorini *et al.*, 2009). In the Gauging Responsiveness With A VerifyNow® P2Y12 Assay: Impact on Thrombosis and Safety (GRAVITAS) trial, patients who had PRUz ≥230 were given 150mg of clopidogrel instead of 75mg od. At 6 months, high-dose clopidogrel did not reduce cardiovascular events compared with standard-dose clopidogrel (Price *et al.*, 2009). This negative study cautioned clinicians not to use VerifyNow® to tailor clopidogrel treatment. It is possible that high on treatment platelet reactivity to clopidogrel is an independent marker of future cardiovascular events which may not be reduced simply by dose adjustment. In a post hoc analysis of the study, the investigators found that after adjustment for other significant predictors of outcome, PRUz <208 units remained independently protective of the primary end point at 60 days (hazard ratio 0.23; 95% CI, 0.05 to 0.98; P=0.047) and at 6 months (hazard ratio, 0.54; 95% CI, 0.28 to 1.04; P=0.065) (Price *et al.*, 2011). In a study by Park et al, patients with ≥235 PRUz units had significantly higher rates of cardiac death and spontaneous MI (2.5% vs. 0.5%, p = 0.022) and multivariate-adjusted analysis showed that it was an independent predictor of the composite of cardiac death and nonfatal MI at one year. Interestingly, the predictability of this test was stronger in those without risk factors for cardiovascular disease, such as hypertension, diabetes mellitus, or dyslipidaemia compared to those with conventional CV risk factors. Conversely, in a larger study by the same group on more than 2000 patients who had drug eluting stents for CAD followed for 2 years showed, the occurrence of the primary endpoint (cardiac mortality, fatal or non fatal myocardial infarction) did not significantly differ among patients with and without high PRUz values (HOTR, PRUz ≥235) [2.8% vs. 2.4% at 2 years; hazard ratio [HR]: 1.33, 95% confidence interval [CI]: 0.88 to 2.01; p = 0.18] (Jeong *et al.*, 2011; Oh *et al.*, 2012; Park *et al.*, 2012). The same group showed that predictability of VerifyNow® with regard to future cardiac events was better in patients after ACS (Park *et al.*, 2013). Recently

published landmark ARCTIC trial (Assessment by a Double Randomization of a Conventional Antiplatelet Strategy versus a Monitoring-guided Strategy for Drug-Eluting Stent Implantation versus Continuation One Year after Stenting) studied the role of routine platelet monitoring and dose adjustment in those who are hyporesponders to clopidogrel. 2440 patients who underwent elective stent implantation for CAD had been randomised to a VerifyNow® based dose adjustment for clopidogrel or conventional treatment arm. There were no differences in composite end point of death, acute myocardial infarction, stent thrombosis, stroke, or need for urgent revascularisation (OR 1.13; 95% CI, 0.98 to 1.29; P=0.10) and bleeding in both the arms at one year. Interestingly 37.5% patients were hyporesponders to clopidogrel using a cut off of PRUz 208 and 7.5% to aspirin using a ARU value of 550 (Collet *et al.*, 2012). ADAPT-DES study (Assessment of Dual AntiPlatelet Therapy With Drug Eluting Stents) showed that among 11,000 patients who were entered on a registry, 42.7 % had poor response to clopidogrel based on a VerifyNow® cut off value of 208 PRUz. Stent thrombosis was higher (1.3% vs. 0.5%, p=0.005) but bleeding events were lower (5.6% vs. 6.7%, p=0.04) in those who had high platelet reactivity after clopidogrel therapy. Incidence of acute MI was higher in these patients (3.9% vs. 2.7%, p=0.001). One year mortality was higher in patients (2.4% vs. 1.5%, p=0.001) who had high platelet reactivity after clopidogrel therapy but in multivariable analysis, no independent association was seen between clopidogrel hyporesponsiveness and mortality (Stone *et al.*, 2013). However, in a meta-analysis of 6 studies, incremental PRUz values predicted higher cardiovascular events and on a continuous scale, every 10-U increase in PRU was associated with a significantly higher rate of the composite primary endpoint death, myocardial infarction or stent thrombosis.(HR: 1.04; 95% CI: 1.03 to 1.06; $p < 0.0001$). PRUz value ≥230 appeared to best predict the combined end-point of death, MI, or stent thrombosis ($p < 0.001$). A PRUz value ≥230 was associated with individual endpoints of death (HR: 1.66; 95% CI: 1.04 to 2.68; $p = 0.04$), MI (HR: 2.04; 95% CI: 1.51 to 2.76; $p < 0.001$), and stent thrombosis (HR: 3.11; 95% CI: 1.50 to 6.46; $p = 0.002$). These findings suggest that VerifyNow® may be useful in predicting short to medium CAD risk in patients up to 1 year after

they had percutaneous coronary intervention but not after 2 years (Brar *et al.*, 2011).

In contrast to clopidogrel, aspirin induced platelet inhibition as measured by ARU units showed a very narrow range of values and less variability in T2DM. In my study, ARU values were similar between T2DM and non DM and were unaffected after clopidogrel therapy in stable CAD patients. Only one patient had HOTR with aspirin after NSTE-ACS (non DM) and 13.4% (12 patients out of 90) had HOTR in stable CAD study. Using an ARU cut off value of ≥550, in a small study of healthy volunteers and those with stable CAD, no patient was hyporesonsive to aspirin (Nielsen *et al.*, 2008). In an another study of patients with stable CAD on 75mg of aspirin, T2DM patients showed significantly higher levels of platelet aggregation compared to non-diabetic patients, where increased levels of platelet activation was measured by P selectin (Mortensen *et al.*, 2010). Aspirin use at standard doses of 75-100mg per day has been detected by VerifyNow® in all the subjects (100%) and its levels highly correlated with 'gold standard' LTA assay in a study by Blais et al (Blais *et al.*, 2009). Gurbel et al showed that in patients with stable CAD with T2DM had higher on treatment platelet reactivity (PRUz values) when treated with aspirin 81mg which was partly overcome at dose of 150mg, and 325mg dose showed even greater responsiveness. VerifyNow® ARU values correlated with traditional light transmission aggregometry data (LTA) and urinary thromboxane levels. Interestingly, for each aspirin dosage (e.g. 81mg, 150mg, 325mg) ARU levels were higher in T2DM compared to those without DM (DiChiara *et al.*, 2007). Multiple measurements of platelet reactivity were tested with arachidonic acid in healthy individuals taking 75mg of aspirin. VerifyNow® results when compared to LTA showed significantly less coefficient of variation but correlated well with serum thromboxane levels (Grove *et al.*, 2010). However, there was no correlation between ARU and thrombus area in both my studies, implying that ARU at levels <495 is a poor marker of thrombogenicity in those on aspirin therapy and may explain some of these variability observed in previous studies.

I studied the role of inflammation in predicting platelet reactivity using VerifyNow®. I found that there was a strong association between inflammation

and VerifyNow® PRUz values after clopidogrel therapy. There was a significant relationship between inflammation and thrombus as discussed before (vide supra: 4.3.3, Discussion chapter on platelet dependent thrombosis). One week therapy with clopidogrel did not reduce the levels of inflammatory cytokines TNFα, IL-1, IL-6 and IFNγ. It is possible that persistent inflammation in clopidogrel treated individuals can play a significant role in future thrombotic event in patients with T2DM irrespective of PRUz values. My results underscore the point that VerifyNow® results are very "platelet centric" and I suggest caution should be exercised when using the values to tailor individualised therapy. VerifyNow® did not account for non platelet mediators of thrombus and thus lacked correlation to thrombus in my cohort. My findings re-iterate the concerns expressed in the current guidelines which suggest VerifyNow® should remain as a research tool and is not for routine clinical practice (Bonello *et al.*, 2010).

4.6 Thromboelastography® (TEG) and Platelet Mapping™

4.6.1 Results - Viscoelastic properties and thrombus architecture in ACS study

Baseline characteristics are presented in Table 4.19. By virtue of selection, T2DM patients demonstrated the typical metabolic syndrome phenotype with higher triglycerides, HbA1c and systolic blood pressure and lower LDL cholesterol in T2DM.

T2DM was associated with formation of higher thrombus in both rheological conditions in the ex-vivo chamber. More specifically, at high shear rate conditions thrombus area were (median, µ2/mm, interquartile range), 14861 (8003-30161) vs. 8908 (6812-11996) for T2DM and control groups, respectively p=0.045 and low shear thrombus was 10715 (6562 – 15932) v 6062 (3865-6312), p=0.007. A significant association was seen between fibrinogen plasma levels and thrombus in the combined cohort (rho 0.551, p=0.002) (Figure 4.17). There were no significant correlations between thrombus and HbA1c, LDL cholesterol, body mass index and duration of diabetes.

In thromboelastography, the time to form stable thrombus (a marker of initiation of fibrin polymerisation) was prolonged in T2DM whilst clot index (CI), a measure of the thrombus strength, was lower [median (inter quartile range), -0.2(-1.7 to 0.7) vs. 1.0(-0.9 to 3.3), p=0.044). On the other hand, the rate of thrombus retraction, measured over 90 minutes (L parameter) in T2DM was slower, [rate of thrombus retraction mm/min, median (inter quartile range), 27.8(11.7 – 70.7) vs. 78.8(68.5-109.6) p=0.002)]. All other TEG® parameters showed no statistically significant differences between the groups (Table 4.20). There was a negative correlation between rate of thrombus retraction and thrombus area in low shear state in the combined cohort (rho -0.450, p=0.016, Figure 4.17), but not in the high shear chamber (rho -0.153, p=0.436).

VerifyNow® studies were performed for these patients and aspirin reactive units (ARU) were almost similar between the groups. However, P2Y12 reactive units were higher in T2DM [PRUZ: median (IQR) 259 (222-311) vs. 205 (189-232),

p=0.028]. One patient (with T2DM) had high on treatment reactivity (HOTR) to aspirin. 13 patients [9 (64.2 %) with T2DM and 4 (28.6 %) non DM] had HOTR to clopidogrel. The biochemical markers of platelet reactivity, P selectin was numerically higher and CD40 ligand was numerically lower in T2DM and the differences lacked statistical significance (Table 4.20). There was no correlation between PRUz values and thrombus area (rho=0.153, p=0.544)

Scanning electron microscopy (SEM) showed thrombus architecture was different in T2DM. In the non T2DM, thrombus appeared well organized with thick fibrin fibres were arranged longitudinally in one direction, with few side branches. In contrast, in patients with T2DM, thrombus appeared disorganised. Fibrin fibres were thinner, with more side branches, giving a tangled, web or mesh like appearance (Figure 4.18).

Median (interquartile range) or %[n]	T2DM (n=14)	Non T2DM (n=14)	P value
Demographic data:			
Age, years	62 (51-68)	54 (51-63)	0.401
Male gender,	71.4[10]	92.9[13]	0.331
Body mass index, kg/m^2	33.4(29.3-43.4)	30.6 (27.4-30.7)	0.114
Waist to hip ratio	1.0(0.9-1.0)	1.0(0.9-1.0)	0.781
Systolic BP, mmHg	134 (124-147)	120 (112-136)	0.031
Diastolic BP, mmHg	77(72-82)	72 (68-80)	0.227
Risk profile %(n):			
Hypertension	64.3[9]	21.4[3]	0.052
Chronic Kidney Disease	21.4[3]	7.1[1]	0.601
Medications:			
B blockers	85.7[12]	100 [14]	0.483
Calcium channel blockers	28.6 [4]	7.1 [1]	0.331
Laboratory data:			
HbA1c, %	6.6 (6.2 -8.2)	5.6 (5.3-6.1)	--
Random plasma glucose, mmol/l	8.2 (6.3-11.4)	6.9 (4.9 -7.8)	--
Fasting plasma glucose, mmol/l	7.3 (5.6-10.9)	5.1 (4.6-5.5)	--
Haemoglobin, g/dl	13.8 (12.8-15.4)	13.7 (12.9-15.3)	0.865
Platelets, x1000/mm^3	252 (191-309)	257 (209-354)	0.448
Fibrinogen, g/l	4.4 (4.1-4.6)	3.6 (2.9-4.4)	0.095
Creatinine, µmol/l	100(86-110)	99 (81-106)	0.603

Median (interquartile range) or %[n]	T2DM (n=14)	Non T2DM (n=14)	P value
Total cholesterol, mmol/l	3.2 (2.6-4.4)	3.4 (3.0-4.0)	0.667
LDLc, mmol/l	1.3 (1.0-1.7)	1.9 (1.6-2.5)	0.023
HDLc, mmol/l	0.9 (0.7-1.1)	0.9 (0.8-1.2)	0.769
Triglyceride, mmol/l	1.8 (1.4-3.4)	1.1 (0.9 -1.5)	0.002
Troponin I, µg/l	1.2 (0.1 – 10.1)	2.0 (1.1-4.1)	0.408

Table 4.19 Baseline characteristics of Thromboelastography in ACS sub study.

Median (interquartile range)	**T2DM (n=14)**	**Non T2DM (n=14)**	**P value**
Thrombus kinetics R time, min	7.3 (6.6 to 8.5)	6.6 (4.6 to 7.1)	0.021
Maximum amplitude, MA, mm	67.7(62.0 to 70.1)	67.4 (63.8 to 74.1)	0.573
K time, min	1.8 (1.7to 2.2)	1.6 (1.2 to 2.3)	0.476
α-angle	64.5 (54.5 to 73.1)	68.6 (58.0 to 66.8)	0.306
Clot index	-0.2 (-1.7 to 0.7)	1.0 (-0.9 to 3.3)	0.044
Rate of thrombus retraction (L) mm/min	27.8 (11.7 to 70.7)	78.8 (68.5 to 109.6)	0.002
Platelet Reactivity			
ARU	419(398-422)	403(390-404)	0.623
PRUz	259(222-311)	205 (189-232)	0.028
Percentage inhibition, %	20(9-32)	32(22-34)	0.083
P selectin, µg/ml	74.8(59.3 – 77.0)	53.1 (39.7 – 68.9)	0.062
CD40 ligand, µg/ml	2934 (2046-3520)	3512 (1674-4220)	0.711

Table 4.20 Thromboelastography parameters and biomarkers in ACS study.

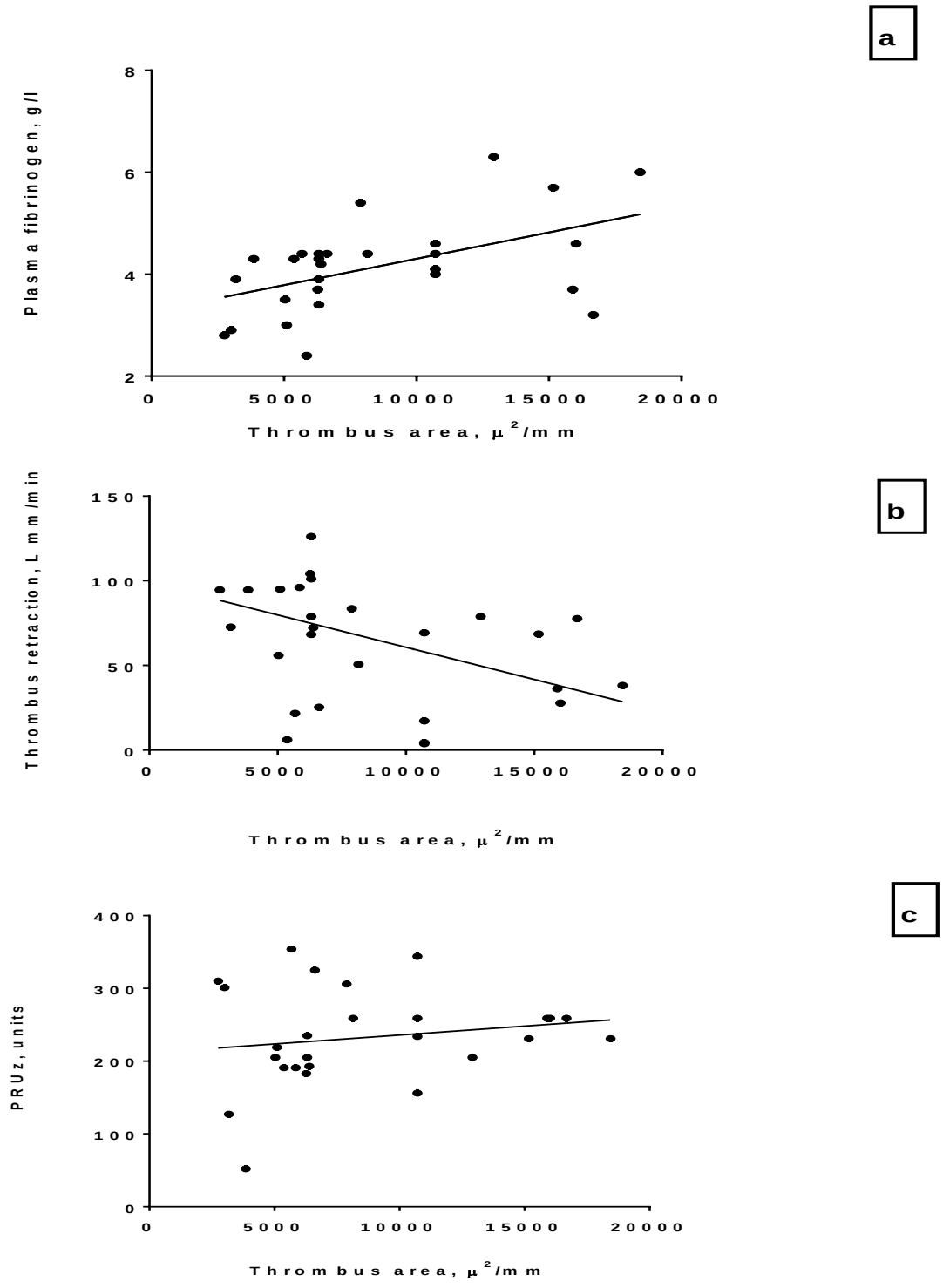


Figure 4.17 Fibrinogen, thrombus retraction and PRUz correlations to thrombus area.

Plasma fibrinogen was positively correlated to thrombus area (rho= 0.551, p=0.002). Thrombus retraction (autolysis) had a negative correlation (rho= -0.450, p=0.016). There was no correlation between VerifyNow® values and thrombus area (rho=0.153, p=0.544).

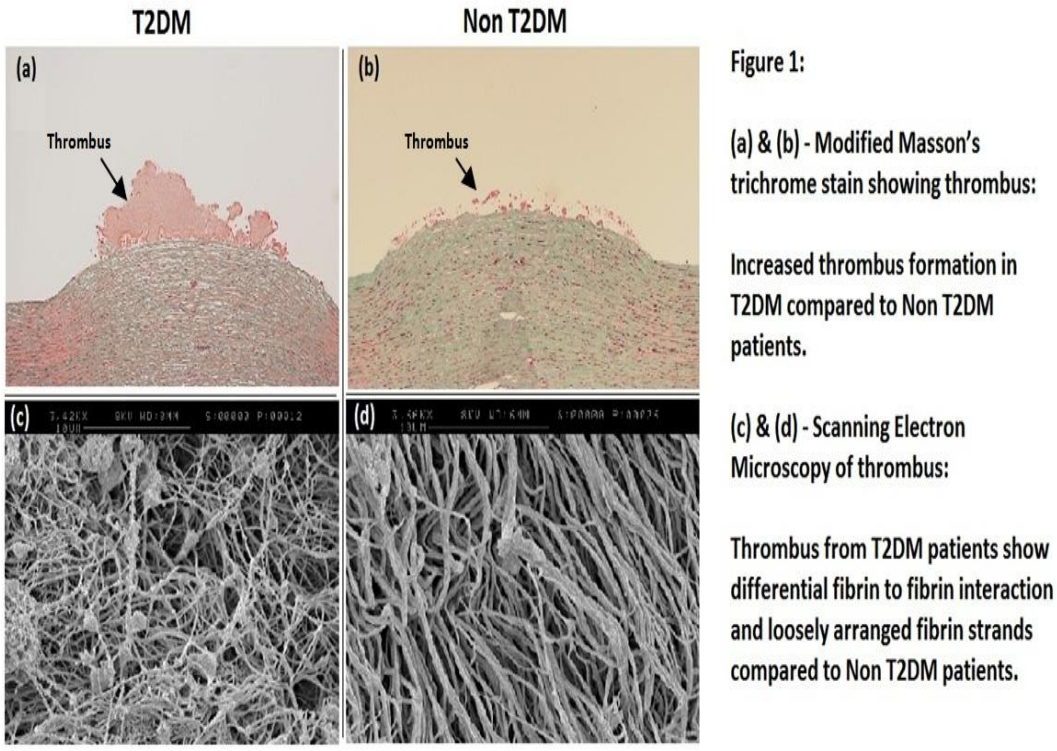


Figure 4.18 Thrombus images from Badimon Chamber and scanning electron microscopy in ACS study

4.6.2 Discussion - Viscoelastic properties and thrombus architecture in ACS study

In this pilot study I studied the impact of T2DM on the magnitude as well as structural and mechanical characteristics of thrombus from NSTE-ACS patients. I found increased thrombus formation, and altered thrombus kinetics in patients with T2DM after NSTE-ACS, a finding not hitherto reported in whole blood testing. Studies undertaken with the perfusion chamber rendered larger thrombus area both at high and low local shear rate condition in T2DM patients. Of interest, the observations on the different structural and kinetic characteristics of the thrombus have not been previously reported in whole blood testing. The most striking difference in thrombus kinetics was an approximately three fold slower rate of clot retraction in diabetic participants and the rate of retraction correlated inversely with thrombus area. The time to form stable clot was also increased whilst clot index (a measure of strength) decreased. The rate of thrombus retraction was negatively correlated to thrombus quantity in this study which suggests that thrombus, in addition to being higher in quantity, also persists longer after initial formation. In vivo, persistence of thrombus would provide a nidus for more thrombus formation and this may lead to a pathophysiological cycle of 'thrombus begetting thrombus' in patients with T2DM. Although in vitro studies show impaired fibrinolysis (later phase of autolysis) in T2DM (Grant, 2007), little is known of thrombus retraction which represents the early phase of autolysis.

SEM analysis of the thrombus (qualitative analysis) as a pilot study illustrated some important ultrastructural characteristics further explaining these findings. In the early stages of thrombogenesis (5 minutes of Badimon chamber study), thrombus in T2DM was composed of less compact and loosely arranged fibrin to fibrin structure. Scanning electron microscopy of thrombus revealed a markedly different architecture between the diabetic and non diabetic patients. Earlier studies using reconstituted plasma have shown altered fibrin structure in patients with T2DM, with reduced elasticity (Pieters *et al.*, 2006). This arrangement of the fibrin fibres was similar to that seen in my study using whole blood in a surrogate

model of plaque rupture. The differing ultrastructural characteristics may in part explain the differences in thrombus kinetics seen on TEG®. Thrombus in T2DM had thinner fibrin fibres, with more lateral aggregations, occurring in a disorganised, tangled or web like fashion. This may favour propagation of thrombus by trapping more cellular elements, and in addition, may make clot retraction and degradation more difficult. Thus the increased thrombus quantity in T2DM may be due to both increased blood thrombogenicity generating larger thrombus and decreased degradation. The presence of thinner fibrin fibres in T2DM may result in lower viscoelastic strength of diabetic thrombus and could be one explanation for the differences seen on TEG® (Pieters *et al.*, 2008). The finding of loosely bound but persistent thrombus in diabetes may increase the potential for embolisation to distal arteries resulting in increased end organ damage. In addition, a less compact and loosely bound fibrin fibre arrangement is a marker of poor fibrinolysis (Alzahrani and Ajjan, 2010). Presence of less longitudinal twisting in T2DM could result in lower visco elastic strength of diabetic thrombus and can thus explain the differences seen in TEG® studies (Ajjan *et al.*, 2009). T2DM is a prothrombotic state reportedly associated with higher on treatment platelet reactivity (HOTR). More patients with T2DM had HOTR with VerifyNow®, findings similar to main study.

My findings provide additional mechanistic support for the proposal that 'perpetuation and propagation of diabetic thrombus', with a pathophysiological cycle of "thrombus begets thrombus", is responsible for increased recurrent acute coronary events in T2DM patients (Hess *et al.*, 2012). It is known that patients with T2DM have 'vulnerable blood' which is responsible for more major adverse cardiovascular events after NSTE-ACS (Bhatt, 2008).There are no published studies of differences in the viscoelastic properties and fibrin structure of thrombus in patients with and without T2DM after NSTE-ACS. It is possible that loosely bound diabetic thrombus which is higher in quantity may also embolise easily to distal arteries. Nevertheless, once formed, the diabetic thrombus was slower to undergo clot retraction (early autolysis) and along with the well known defects in fibrinolysis (late autolysis), these findings support the view that 'perpetuation and propagation of diabetic thrombus' is responsible for recurrent

acute coronary events (Moreno and Fuster, 2004). Thus my findings, in a small cohort, are novel, hypothesis generating and require further validation.

This study showed the feasibility of measurement of blood thrombogenicity both qualitatively and quantitatively, in patients, optimally managed after NSTE-ACS, using a combination of point of care and laboratory assays. The differences seen in the ultrastructure and mechanistic properties of thrombus provide a focus for further studies that may lead to novel pharmacological treatments.

4.6.3 Results - Viscoelastic properties of thrombus in stable CAD study

The differences in baseline characteristics between the groups who had clopidogrel and placebo were minimal and statistically non significant (Table 4.21). Baseline TEG® and Platelet Mapping™ parameters also showed a similar trend between the groups. One week clopidogrel therapy reduced thrombus area in this sub-study (Table 4.22). Standard TEG® parameters (e.g. R, MA to kaolin, clot index) remained unchanged after a week of clopidogrel therapy, as did the results in placebo group (Table 4.23). Platelet mapping™ studies showed a significant reduction of maximum viscoelastic strength of thrombus after treatment with clopidogrel as measured by maximum amplitude (MA-ADP, in mm) upon stimulation by 10µl of ADP (59.9±7.4 vs. 54.0±10.8, p=0.007) (Table 4.24). These values remain unchanged in placebo group. Despite being on chronic aspirin therapy, response to aspirin also improved significantly in my cohort of patients prescribed additional clopidogrel therapy. Reduction in maximum amplitude of the thrombus (MA-AA, in mm) upon stimulation with 10µl of arachidonic acid (34.7±19.2 vs. 25.9±15.7, p=0.002) was seen in my cohort in those who had clopidogrel (Figure 4.19). MA-AA was unchanged in those who were randomised to placebo (37.8±19.4 vs. 32.5±19.2, p=0.399). The thrombus lysis parameters clot retraction: L parameter and time to achieve maximum rate of lysis were not statistically different between both the groups after one week of therapy (Figure 4.19).

There were significant albeit moderate correlations between thrombus area and various TEG® parameters as shown in the Figure 4.20 and Table 4.25. Importantly, platelet aggregation, as measured by the 'percentage change in maximum amplitude to ADP stimulation after 1 week of clopidogrel therapy' negatively correlated with reduction in thrombus area (delta thrombus) in clopidogrel treated individuals (rho=-0.482, p=0.009). In addition, maximum strength (MA) of the thrombus correlated with thrombus area (rho=0.260, p=0.041) and thrombus retraction (L parameter) negatively correlated with thrombus area (rho=-0.251, rho 0.047).

	Clopidogrel + aspirin (n=33)	**Placebo + aspirin (n=33)**	P value
Demographic data: Mean±SD or %(n)			
Age, years	**63.6±7.2**	**62.8±7.3**	0.662
Male gender, % (n)	**84.8 (28)**	**87.9 (29)**	1.000
Body mass index, kg/m^2	**32.5±4.9**	**33.5±6.6**	0.475
Waist to hip ratio	**1.0±0.1**	**1.0±0.1**	0.139
Systolic BP, mmHg	**142±19.9**	**138±21.8**	0.464
Diastolic BP, mmHg	**77±10.5**	**77±9.5**	0.854
Duration of diabetes, years	**10±6.0**	**8±4.6**	0.129
***Risk profile:* %(n)**			
Angina	**75.8 (25)**	**66.7 (22)**	0.589
Previous MI	**48.5 (16)**	**42.4 (14)**	0.805
PCI	**30.3 (10)**	**24.2 (8)**	0.783
CABG	**27.3 (9)**	**18.2 (6)**	0.558
Chronic kidney disease	**6.1 (2)**	**24.2 (8)**	0.082
***Medications:* %,(n)**			
Sulphonylurea	**24.2 (8)**	**30.3 (10)**	0.783
Metformin	**60.6 (20)**	**57.6 (19)**	1.000
Insulin	**33.3 (11)**	**27.3 (9)**	0.789
Laboratory data:mean±SD			
Haemoglobin, g/dl	**13.6±1.3**	**13.9±1.2**	0.369
Platelets X1000 cells/ mm^3	**228±57.2**	**225±55.0**	0.858
Fibrinogen, g/ml	**3.4±0.6**	**3.4±0.8**	0.883
HbA1c, %	**7.5±1.3**	**7.6±1.0**	0.856
Fasting plasma glucose, mmol/l	**8.6±5.1**	**8.1±2.2**	0.562

	Clopidogrel + aspirin (n=33)	**Placebo + aspirin (n=33)**	P value
eGFR, ml/min/1.73m^2	**73±22.2**	**77±21.0**	0.497
Total cholesterol, mmol/l	**3.6±0.7**	**3.6±0.6**	0.716
LDLc, mmol/l	**1.9±0.6**	**1.8±0.5**	0.395
HDLc, mmol/l	**1.1±0.3**	**1.1±0.3**	0.885
Triglyceride, mmol/l	**1.6±0.8**	**1.7±0.9**	0.815
HsCRP, mg/l	**4.3±4.5**	**3.6±3.9**	0.525

Table 4.21 Baseline characteristics of thromboelastography (stable CAD) sub study.

I compared VerifyNow® assay and TEG® Platelet mapping™ in T2DM patients. There was a significant reduction in PRUz values in those who were randomised to clopidogrel. There was a significant correlation between ARU indices of VerifyNow® and maximum amplitude of the thrombus formed in TEG® upon stimulation by arachidonic acid (rho 0.376, p=0.044). In addition, there were small but significant negative correlations seen between ARU and thrombus lysis (rho -0.389, p=0.028) and thrombin generation parameters (-0.356, p=0.045) (Figure 4.21). PRUz (which measured clopidogrel response) showed no correlation to any of the TEG® or Platelet Mapping™ parameters (Table 4.25)

	Clopidogrel+Aspirin (n=33)			**Placebo+Aspirin (n=33)**		
Mean±SD (μ^2/mm)	Baseline	Visit 2	P value	Baseline	Visit 2	P value
Thrombus in high shear chamber	14014 ±5230	11025 ±4050	0.001	13250 ±5501	13723± 5693	0.266
Thrombus in low shear chamber	9482 ±3842	6707 ±2930	<0.001	7142 ±3227	6885 ±2424	0.619

Table 4.22 Thrombus area in Thromboelastography (stable CAD) sub study.

	Clopidogrel n=33			**Placebo n=33**		
	Baseline	1-week after therapy	P value	Baseline	1-week after therapy	P value
R, min	6.1±1.5	6.6±1.7	.442	6.4±1.5	6.3±1.8	.389
K, min	1.8±0.5	2.0±0.6	.646	1.8±0.5	1.8±0.8	.134
MA, mm	65.2±4.0	64.1±4.4	.226	64.3±5.7	64.6±4.8	.304
G dynes/sec	9.6±1.7	9.1±1.7	.283	9.3±2.0	9.4±2.0	.219
CI	0.5±2.0	0.1±2.0	.403	0.3±2.0	0.2±2.3	.452

Table 4.23 Standard TEG® measurements (CAD study).

	Clopidogrel n=33			**Placebo n=33**		
	Baseline	1-week after therapy	P value	Baseline	1-week after therapy	P value
MA – CK, mm	65.2±4.0	64.3±6.0	0.151	64.1±4.4	64.3±4.9	0.389
MA-A, mm	11.4±6.6	11.8±4.1	0.703	15.1±9.2	14.3±7.7	0.968
MA –AA, mm	34.7 ±19.2	25.9 ±15.7	0.002	37.8 ±19.4	32.5 ±19.2	0.399
MA-ADP, mm	59.9±7.4	54.0 ±10.8	0.007	59.9±8.7	57.1±9.2	0.338
% Aggregation to AA	43.9 ±33.6	27.8±30. 7	0.002	49.3 ±34.4	41.5 ±37.4	0.296
% aggregation to ADP	88.6 ±12.5	80.0 ±18.2	0.018	89.3 ±12.1	83.5 ±19.3	0.205
Thrombin generation	739 ±295.2	741 ±269.3	0.954	780 ±55.7	783 ±56.1	0.428
Maximum rate of thrombin generation, min	16.8 ±27.3	12.7±3.9	0.391	11.7±2.8	12.8±4.6	0.290
Thrombus retraction, L parameter mm/min	75.3 ±22.8	78.6 ±12.9	0.150	82.4 ±16.2	75.3 ±17.6	0.153
Maximum rate of thrombus retraction, min	0.3±0.2	0.4±0.6	0.868	0.3±0.3	0.3±0.1	0.612

Table 4.24 Platelet Mapping™ and V-curve data in stable CAD study.

Rho, (2-tailed *p value)*	ARU	PRUz
R, min	0.043	-0.282
	(0.816)	*(0.119)*
K	0.257	-0.237
	(0.156)	*(0.192)*
MA for Kaolin	-0.213	0.341
	(0.242)	*(0.056)*
G dynes/sec	-0.215	0.345
	(0.238)	*(0.053)*
CI	-0.220	0.268
	(0.227)	*(0.138)*
MA for AA, mm	0.376*	0.069
	(0.044)	*(0.722)*
MA for ADP, mm	0.300	0.109
	(0.114)	*(0.572)*
Thrombin generation	-0.148	0.300
	(0.418)	*(0.095)*
Maximum rate of thrombin generation, min	-0.356*	-0.276
	(0.045)	(0.125)
Rate of thrombus retraction, L mm/min	-0.389*	0.112
	(0.028)	(0.540)
Maximum rate of thrombus retraction, min	0.124	0.160
	(0.613)	*(0.513)*
*P<0.05		

Table 4.25 Correlations between VerifyNow® and thromboelastography (CAD study).

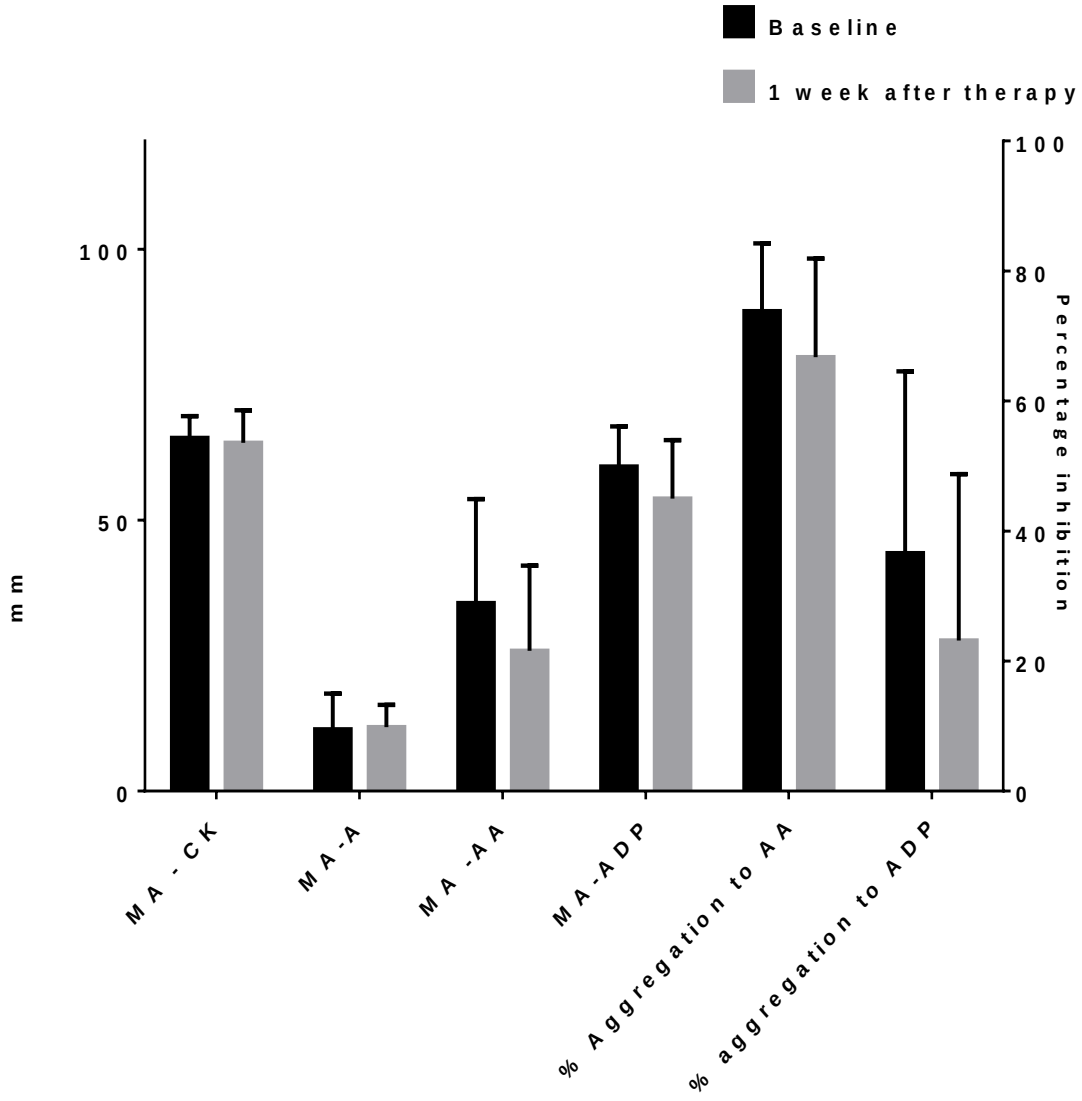


Figure 4.19 Changes in TEG®-Platelet Mapping™ platelet aggregation indices after 1 week of clopidogrel therapy

Maximum amplitude of thrombus upon stimulation with Kaolin (MA_CK) and Activator F did not change with clopidogrel therapy in stable CAD study. There were significant changes in maximum amplitude of thrombus upon stimulation with arachidonic acid (MA-AA) and ADP (MA_ADP) after clopidogrel (MA-AA, in mm: 34.7±19.2 vs. 25.9±15.7, p=0.002 and MA-ADP, in mm, 59.9±7.4 vs. 54.0±10.8 p=0.007). These changes were mirrored in the derived parameters namely percentage aggregation to arachidonic acid and ADP respectively.

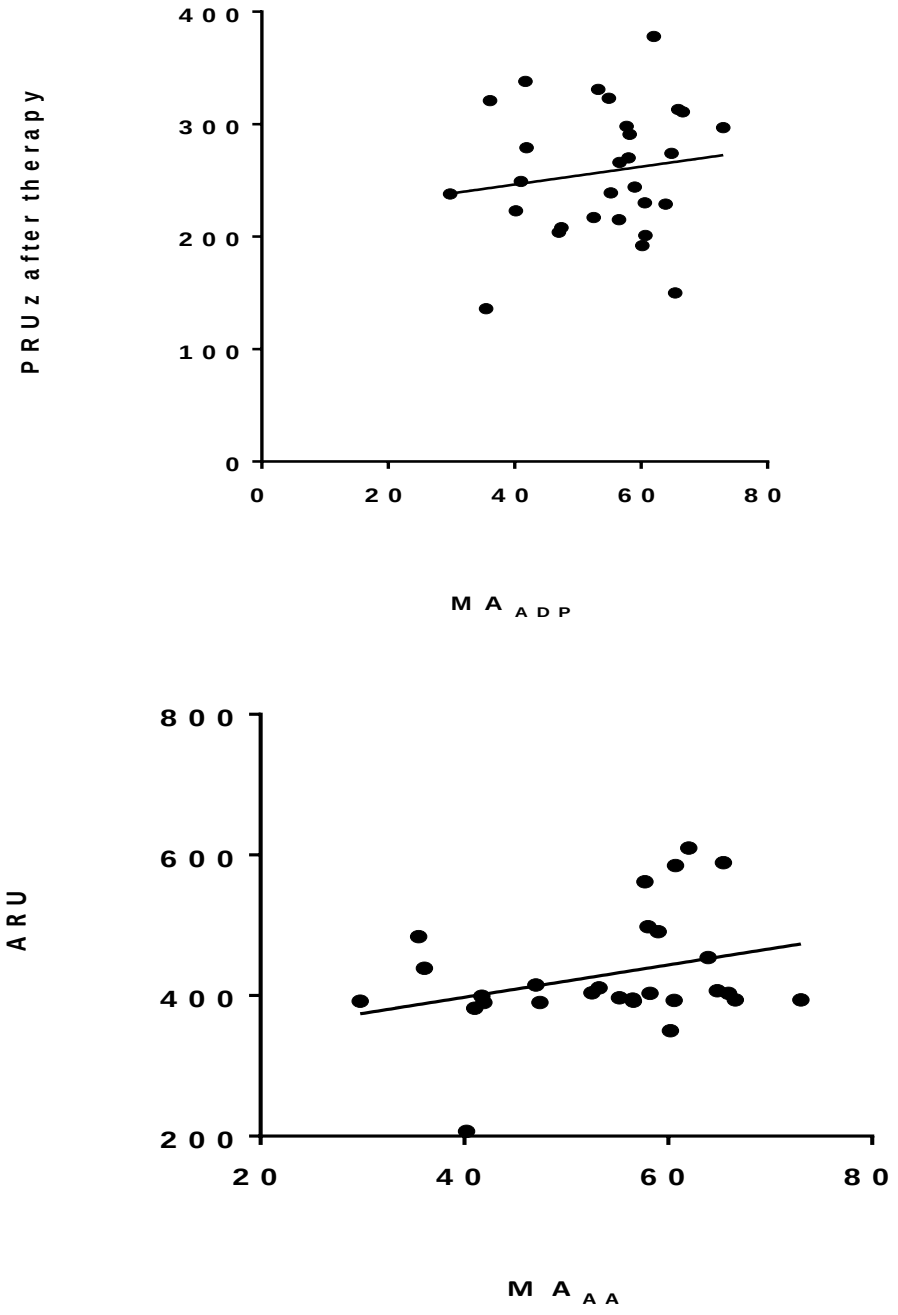


Figure 4.20 Correlation between VeifyNow® indices and TEG® Platelet Mapping™ indices.

PRUz indices did not show correlation to maximum amplitude of the thrombus formed in TEG® Platelet Mapping™ upon stimulation by ADP (MA_{ADP}, in mm), rho 0.109, p=0.572. ARU indices of VerifyNow® and maximum amplitude of the thrombus formed in TEG® Platelet Mapping™ upon stimulation by arachidonic acid (MA_{AA}, in mm) showed significant correlation, rho 0.376, p=0.044.

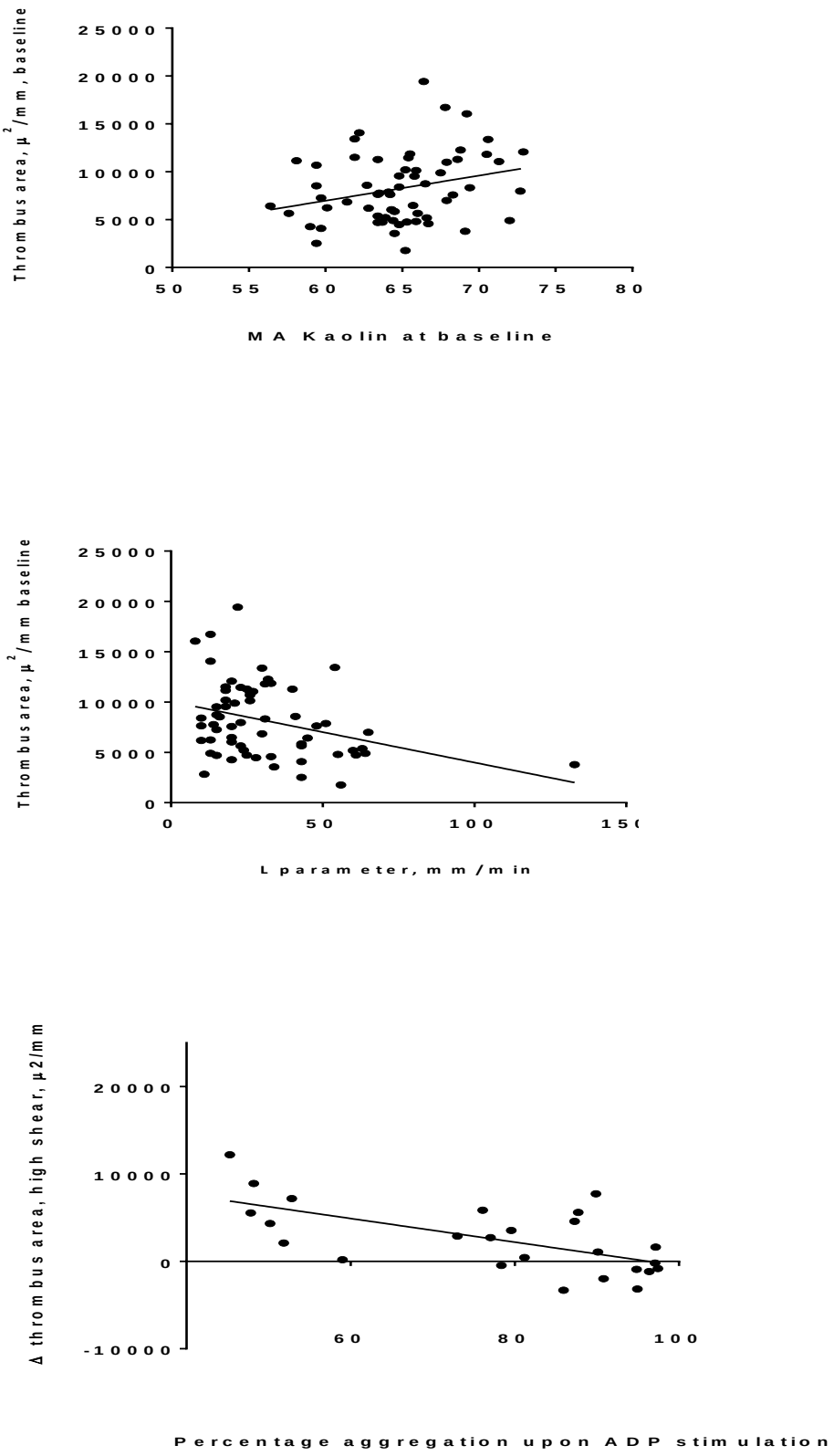


Figure 4.21 Correlation between TEG® - Platelet Mapping™ indices and thrombus after clopidogrel therapy.

Maximum strength (MA) of the thrombus correlated with thrombus area (rho 0.260, p=0.041) and thrombus retraction (L parameter) negatively correlated with thrombus area (rho=-0.251, p= 0.047). Platelet aggregation, as measured by the ‘percentage change in maximum amplitude to ADP stimulation after 1 week of clopidogrel therapy’ negatively correlated with reduction in thrombus area (delta thrombus) in clopidogrel treated individuals (rho=-0.482, p=0.009).

4.6.4 Discussion - Viscoelastic properties of thrombus in stable CAD study

I found that conventional TEG® parameters remained unaltered in T2DM with stable CAD after addition of clopidogrel 75mg OD for a week. However, upon stimulation by 10 µl of ADP, there was a significant decrease in viscoelastic strength of the thrombus. Interestingly, in those who were randomised to clopidogrel, the effect of aspirin on viscoelastic strength of thrombus was more pronounced, resulting in a decrease in maximum amplitude of the thrombus (MA, mm) upon stimulation with 10 µl of arachidonic acid.

The negative finding of unaltered kaolin stimulated TEG® parameters (standard TEG®) after clopidogrel therapy is not surprising. Kaolin stimulates thrombogenesis via the powerful pro-coagulant thrombin, which acts in the final common pathway bypassing the effects of ADP on coagulation cascade. There are independent reports showing that clopidogrel therapy reduces thrombin generation but there is no literature evidence to support the findings from kaolin stimulated TEG® assay (Wegert *et al.*, 2002). Standard TEG® assay performed by Gurbel et al in patients on dual antiplatelet therapy who underwent elective coronary stenting showed that those with higher MA and longer R values had more clinical events but this study did not report any pre and post clopidogrel values (Gurbel *et al.*, 2005). CLEAR PLATELETS-2 study (Clopidogrel With Eptifibatide to Arrest the Reactivity of Platelets) showed the usefulness of TEG® in assessing platelet reactivity in patients who received clopidogrel, bivalirudin and eptafibatide, a glycoprotein IIb IIIa inhibitor. Addition of eptifibatide to bivalirudin therapy showed an immediate and marked reduction in maximum amplitude as measured by TEG® and the values correlated with peri-procedural myonecrosis (Gurbel *et al.*, 2009). Literature on the usefulness of TEG® to guide blood product transfusion post cardiac surgery did not report any differences in standard TEG® parameters in those taking clopidogrel (Hertfelder *et al.*, 2005; Ak *et al.*, 2009). It is possible that clopidogrel is not powerful enough to demonstrate any changes in kaolin stimulated TEG® tracings and it remains to be seen if modern antiplatelet drugs (prasugrel and ticagrelor) will have any effect on standard TEG® parameters.

The results from modified TEG® assay namely Platelet Mapping™ (PM) have shown significant reduction in the maximum viscoelastic strength of thrombus upon stimulation by ADP (MA-ADP) and arachidonic acid (MA-AA). Using a cut off of 50% platelet inhibition as a marker of adequate clopidogrel or aspirin response respectively, results of the PM assay showed 89% (30/33) had inadequate response (ADP inhibition) to clopidogrel in contrast to 18% (12/66) to aspirin (AA inhibition). I also found a significant variability in individual clopidogrel response in T2DM ranging from 0% to 90%.

In a study employing various dose regimes of clopidogrel, along with aspirin 75mg od, TEG-PM showed significant reduction in MA-AA and MA-ADP (Swallow *et al.*, 2006). In a study by Bliden et al (Bliden *et al.*, 2007), 87% patients showed inadequate response to clopidogrel after elective PCI and inadequate response was a significant predictor of future cardiac events. In an unselected cohort of patients taking clopidogrel and scheduled for urgent non cardiac surgery, a wide variability of PM values were observed, confirming the heterogeneous antiplatelet effect of clopidogrel (Collyer *et al.*, 2009).

An MA(ADP) of >47 mm was identified as a predictor of future thrombotic events in a 3 year follow up study of patients with coronary stents (Gurbel *et al.*, 2010b). In a study of 18 young women with previous history of stent thrombosis, higher MA-ADP was observed compared to healthy volunteers and these patients had greater baseline MA-ADP, reduced response to clopidogrel, and higher post-treatment reactivity while on aspirin and clopidogrel therapy (Hobson *et al.*, 2009).

It is well known that aspirin response is underestimated by TEG-PM, which may explain the higher than expected aspirin resistance in my cohort (Tantry *et al.*, 2005). I found that the antiplatelet effects of aspirin therapy were enhanced upon addition of clopidogrel. This is supported by the findings in a smaller TEG-Platelet Mapping study (Alstrom *et al.*, 2006). The mechanisms underlying the augmented aspirin response as measured by a pathway specific agonist arachidonic acid remain unknown and are beyond the scope of this thesis.

In my sub-study, thrombus area as determined by Badimon chamber and platelet reactivity as measured by VerifyNow® were compared to TEG® parameters. In patients with T2DM and stable CAD, this comparison is novel, and based on post-hoc analysis. Whole blood thrombus was significantly reduced in both high shear and low shear chambers after the addition of clopidogrel and these findings are in keeping with the results of the main study. Badimon chamber evaluates predominantly platelet component of the thrombus i.e. platelet dependent thrombus and TEG® evaluates fibrin components of thrombus. The timing of thrombus assessment is different in both these tests as Badimon chamber evaluated thrombus in early phase (5 minutes) and TEG® measures thrombus properties continuously for 90 minutes. Baseline kaolin stimulated viscoelastic strength as measured by MA parameter in TEG® study showed a significantly positive but modest correlation to thrombus. Elastic force as measured by shear elastic modulus also showed similar correlation. It is possible that in early phase of thrombogenesis, higher elasticity contributed by individual fibrin fibres may allow further platelet trapping and thus can increase the thrombus quantity. There were significant correlations between rate of autolysis (thrombus retraction) and thrombus area in these individuals at baseline. Baseline platelet hyper activation is well known in patients with T2DM and this may contribute directly to fibrin synthesis. It is also possible that the findings of altered autolysis may be indicative of global impairment of fibrinolysis seen in T2DM. These interesting findings of the association between early phase of thrombogenesis and late phase of autolysis imply a potential role of TEG® in assessment of novel pharmacologic agents targeting different pathways of coagulation.

My findings showed that there was a good correlation in the assessment of aspirin response by both the studies but poor correlation with clopidogrel response between the assay methods. VerifyNow® has been shown to be superior to TEG® in measuring the responsiveness to antiplatelet therapy (Madsen *et al.*, 2010) as TEG® showed higher prevalence of aspirin resistance compared to VerifyNow® (Blais *et al.*, 2009).

Data on TEG® in patients with T2DM is scanty. TEG® results correlated with light transmission aggregometry in assessing clopidogrel hyporesponse in patients

with T2DM on 75mg maintenance therapy. R time and the time to generate maximum thrombin were prolonged. These variables improved on doubling clopidogrel dose, suggesting the usefulness of TEG® in long term monitoring of antiplatelet therapy in T2DM (Angiolillo *et al.*, 2009). My findings show that TEG-Platelet Mapping™ assay can identify response to antiplatelet therapy in patients with T2DM and CAD and testify to its use in clinical research of pharmacological agents.

4.7 Multiplate® Impedance aggregometry indices and platelet dependent thrombus

4.7.1 Results - Platelet reactivity by Multiplate® in CAD study

Baseline characteristics did not differ between those who had clopidogrel and placebo in view of the randomisation (n= 14 each). All the Multiplate® parameters showed no statistical difference between the groups at baseline (Table 4.26, 4.27). After one week of therapy, ADP aggregation units, measured upon stimulation with 10µl of ADP, showed a significant reduction in the clopidogrel group and remained unchanged in placebo group (Median IQR: Clopidogrel group, from 748 (598-905) to 412 (307-676), p=0.003; Placebo group: from 845 (677-987) to 804 (596-954), p=0.337, Figure 4.22). There was a trend towards reduction in ASP aggregation units, measured upon stimulation with arachidonic acid in the clopidogrel group. Platelet aggregations upon stimulation with collagen and thrombin receptor activated peptide (TRAP) remained unchanged in both the groups (Table 4.25).

Thrombus area measured from high shear chamber reduced from [in μ^2/mm, median (IQR)] 14662 (10231-20774) to 11489 (8712-13272), p= 0.001 in clopidogrel group and remained unchanged from 12285 (9130-15864) to 12033 (10723-15269), p=0.782 in placebo group. There was a moderate correlation between Multiplate® indices and thrombus area (For ASP AU, rho =0.574, p=0.032 and for ADP AU rho=0.556, p=0.039). There were significant correlations between VerifyNow® indices and Multiplate® indices (for ASP AU vs. ARU, rho=0.541, p=0.004 and ADP AU vs. PRUz, rho=0.557, p=0.003, Figure 4.24)

Using a published cut off of 460 AU for clopidogrel response, 8 patients (57%) had adequate response to clopidogrel. When stratified according to the cut off ADP AU values, there were no statistically differences in the reduction of thrombus area in both high shear and low shear between those who had good response and those who had low response (Δ high shear thrombus, in μ^2/mm, median (IQR), 3732 (2092-8203) vs. 3936 (402-6521); Δ low shear thrombus in μ^2/mm, median (IQR), 1640 (81-2372) vs. 2259 (573-3877), Figure 4.23).

	Clopidogrel + aspirin (n=14)	**Placebo + aspirin (n=14)**	P value
Demographic data: Median (IQR) or %(n)			
Age, years	63(60-71)	63(58-65)	0.982
Male gender, % (n)	85.7(12)	85.7(12)	1.000
Body mass index, kg/m^2	32.1(30.6-35.8)	30.9(29.2-34.2)	0.571
Waist to hip ratio	1.0 (1.0-1.1)	1.0(1.0-1.1)	0.910
Systolic BP, mmHg	130(122-139)	129(117-140)	0.603
Diastolic BP, mmHg	75(67-81)	73(70-79)	0.839
Duration of diabetes, years	8(5.8-12.8)	6.0(3.8-8.7)	0.265
***Risk profile:* %(n)**			
Angina	85.7(12)	50.0(7)	0.063
Previous MI	35.7(5)	50.0(7)	0.445
PCI	21.4(3)	28.6(4)	0.663
CABG	28.6(4)	7.1(1)	0.139
Chronic kidney disease	0	28.6(4)	0.051
***Medications:* %,(n)**			
Sulphonylurea	21.4(3)	28.6(4)	0.663
Metformin	57.1(8)	57.1(8)	1.000
Insulin	35.7(5)	21.4(3)	0.403
Laboratory data:mean±SD			
Haemoglobin, g/dl	13.8 (13.2-14.5)	13.3 (12.6-13.8)	0.512
Platelets X1000 cells/ mm^3	224 (220-249)	219 (172-225)	0.223
Fibrinogen, g/ml	3.1(2.7-3.8)	3.3 (2.8-4.7)	0.468
HbA1c, %	7.1(6.4-7.4)	7.7 (6.8-8.2)	0.320

	Clopidogrel + aspirin (n=14)	**Placebo + aspirin (n=14)**	P value
Fasting plasma glucose, mmol/l	7.6 (5.4-8.6)	7.2 (6.9-8.6)	1.000
eGFR, ml/min/1.73m^2	81 (66-98)	88 (67-98)	0.734
Total cholesterol, mmol/l	3.4 (2.8-3.9)	3.4 (3.1-3.5)	0.820
LDLc, mmol/l	1.7 (1.2-2.2)	1.6 (1.4-2.1)	1.000
HDLc, mmol/l	1.0 (1.0-1.1)	1.0 (1.0-1.2)	0.569
Triglyceride, mmol/l	1.0 (1.3-2.0)	2.0 (1.1-2.2)	0.865
HsCRP, mg/l	1.2 (0.6-3.2)	1.9 (1.0-5.4)	0.443

Table 4.26 Baseline characteristics - Multiplate® sub study.

Median (IQR)	**Clopidogrel n=14**			**Placebo n=14**		
	Baseline	1-week after therapy	P value	Baseline	1-week after therapy	P value
ASP AU	347 (222-499)	216 (176-300)	0.174	299 (185-533)	260 (124-460)	0.497
ADP AU	748 (598-905)	412 (307-676)	0.003	845 (677-987)	804 (596-954)	0.337
Collagen AU	547 (435-685)	495 (409-619)	0.537	678 (495-891)	604 (324-764)	0.082
TRAP AU	1007 (822-1141)	1022 (914-1151)	0.598	1232 (970-1411)	1162 (1000-1356)	0.649

Table 4.27 Results of Multiplate® sub study.

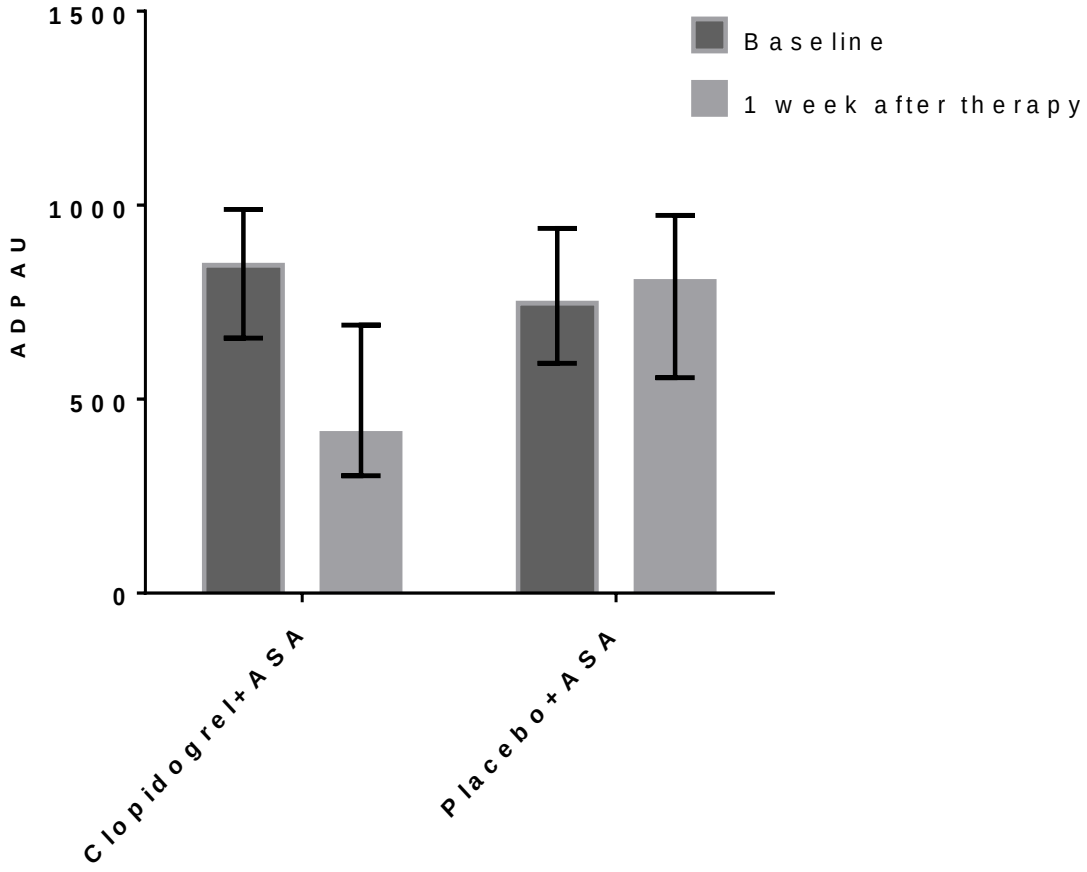


Figure 4.22 Changes in Multiplate® indices after clopidogrel therapy (stable CAD study).

After one week of clopidogrel therapy, there was a significant reduction in ADP aggregation units whereas there was no change in those who took placebo therapy. Solid bars represent median and error bars represent interquartile range. ADP AU [median (IQR)] changed from 748 (598-905) to 412 (307-676), p=0.003 in the clopidogrel group. In the placebo group, the values remain unchanged.

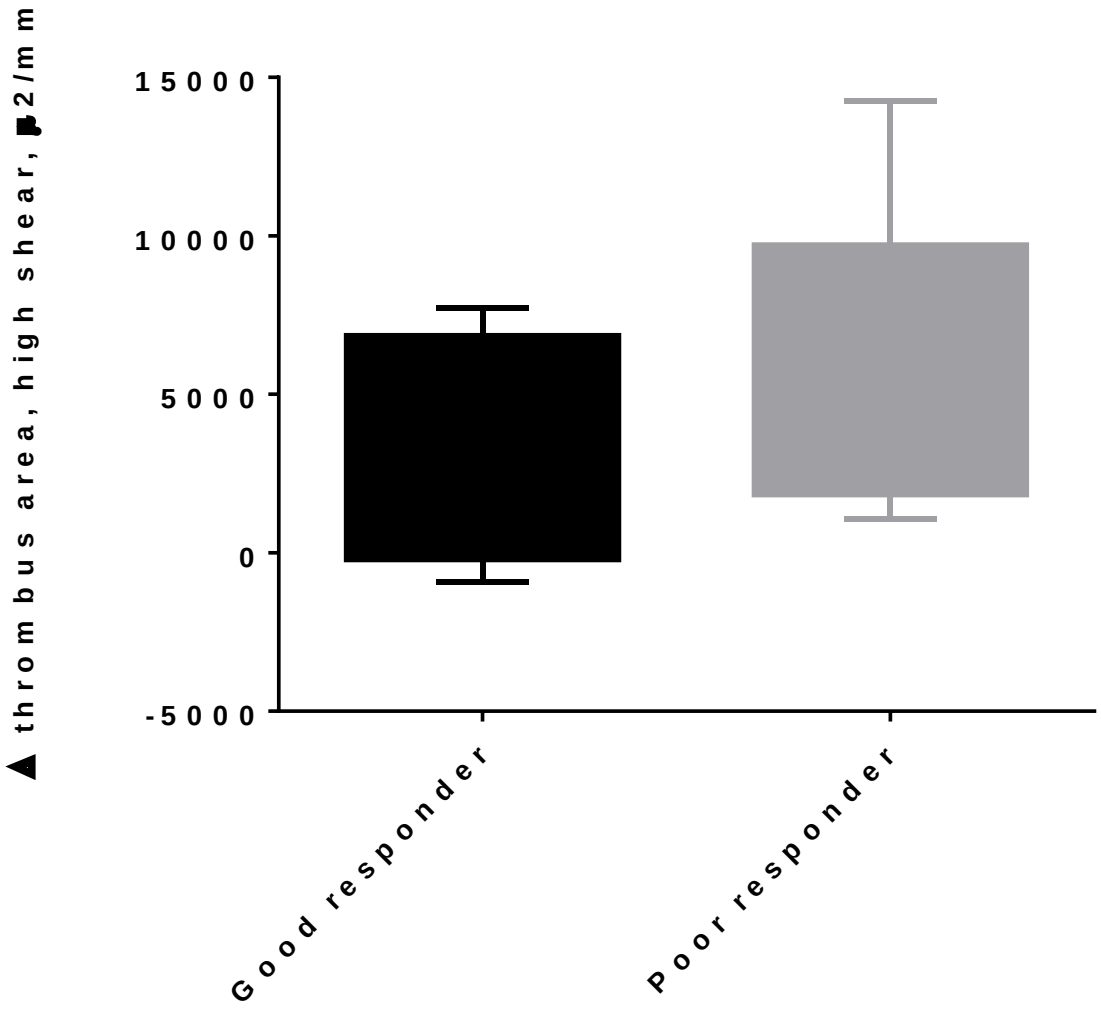


Figure 4.23 Changes in thrombus area based on response to clopidogrel in Multiplate® sub study

When stratified according to the cut off ADP AU values (good responders ≤460 AU), there were no differences in reduction of thrombus area in high shear thrombus. (Δ high shear thrombus, in μ^2/mm, median (IQR), 3732 (2092-8203) vs. 3936 (402-6521). The solid bars represent interquartile range and the error bars represent maximum to minimum range.

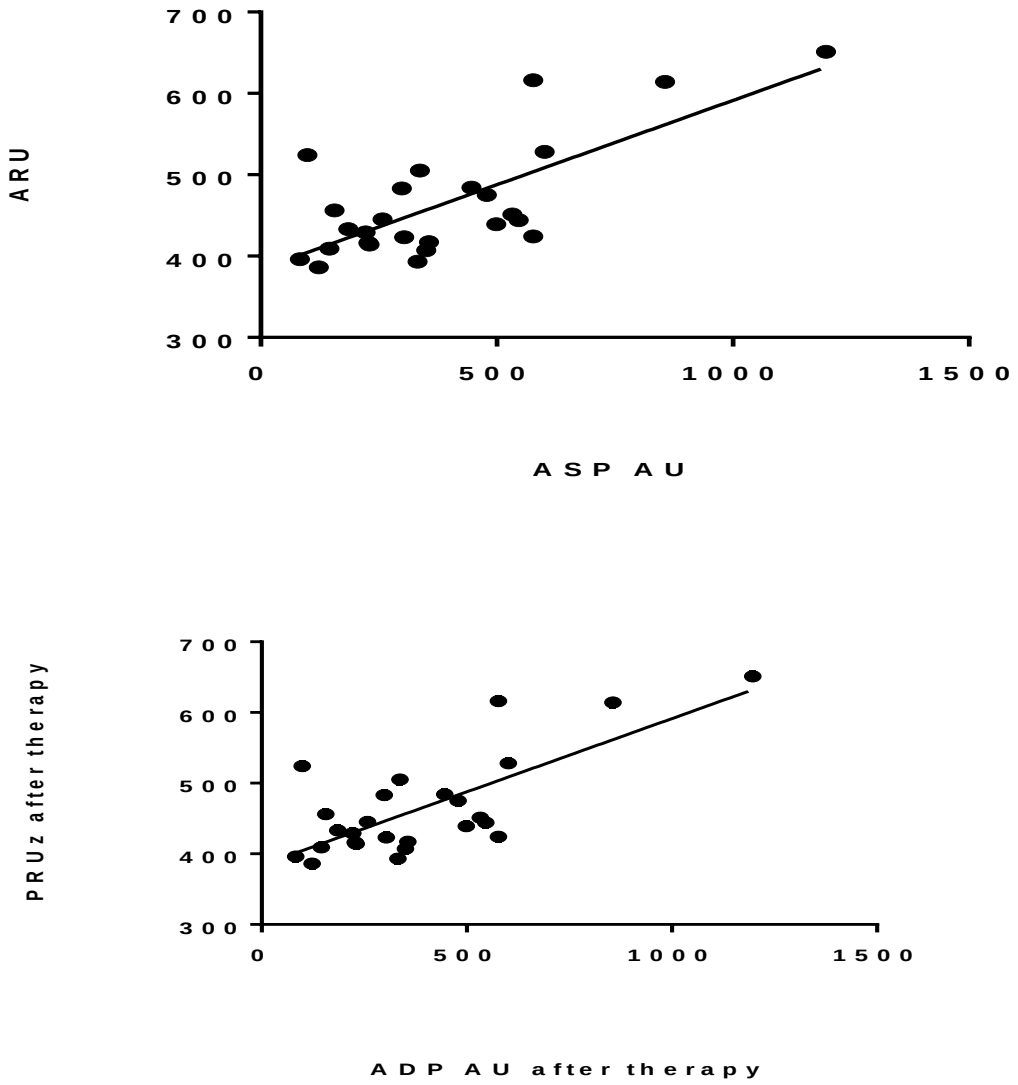


Figure 4.24 Correlations between Multiplate® and VerifyNow® indices in stable CAD study.

There were significant correlations between VerifyNow® indices and Multiplate® indices (for ASP AU vs. ARU, rho=0.541, p=0.004 and ADP AU vs. PRUz, rho=0.557, p=0.003).

4.7.2 Discussion – Platelet reactivity by Multiplate® in CAD study

I report the findings of my Multiplate® pilot study (sub study) which showed a significant reduction in platelet aggregation upon stimulation with ADP in patients with T2DM and stable CAD who were randomised to clopidogrel. I performed comparative analysis of the indices derived from Multiplate® and VerifyNow® to the whole blood thrombus generated from Badimon chamber. This comparison has not hitherto been reported.

Multiplate® has been shown to be useful in monitoring clopidogrel therapy in my small cohort of patients with T2DM, similar to the findings from a large scale study of both diabetic and non diabetic individuals. In a large study of 1000 consecutive patients who received stent implantation after 600mg of clopidogrel loading, 11.8% had low response to clopidogrel. Independent predictors of low response were acute coronary syndrome (OR = 6.54), diabetes mellitus (OR = 2.07), and male gender (OR = 1.83) (Behr *et al.*, 2011). Multiplate® was strongly correlated with light transmission aggregometry (LTA) in a study of patients who had clopidogrel therapy prior to stent insertion (rho= 0.7; P< 0.0001) (Sibbing *et al.*, 2010a). Multiplate® was used to predict bleeding risks in patients taking clopidogrel. Enhanced risk of bleeding was observed (OR 2.6) in patients who had very low AU values to clopidogrel. This was the first study to quantify hyper-response to P2Y12 antagonists as a marker for major bleeding (Sibbing *et al.*, 2010b). In a healthy volunteer study comparing various point of care tests, Multiplate® was superior to LTA and VerifyNow® to assess clopidogrel response (Chen *et al.*, 2011). Using a cut off of 460 AU, nearly half of the patients were hyporesponders to clopidogrel, mirroring findings from an earlier study (Mueller *et al.*, 2007). There was a trend towards improvement in aspirin induced platelet suppression in those who were randomised to clopidogrel. In an unselected population of patients who were prescribed aspirin 75-100mg a day for secondary prevention, using Multiplate®, high on treatment platelet reactivity was found in 26% of patients (Awidi *et al.*, 2011). However, my results were similar to another study where aspirin compliance was controlled, the prevalence of high on-treatment platelet reactivity being 3.6% (Pedersen, Grove et al. 2009),

(Mortensen, Larsen et al. 2010). This confirms the current belief that true aspirin resistance is lower in patients with good compliance. Multiplate® indices had good correlation with the findings of 'gold standard' LTA assay in larger scale studies (Siller-Matula, Delle-Karth et al. 2012).

Both arachidonic acid and ADP stimulated platelet aggregation correlated with thrombus in Badimon chamber at baseline. This is not only a testament to the usefulness of Multiplate® in assessment of thrombosis but also proof of the significant role played by platelets in the early phase of thrombogenesis in T2DM. However, post clopidogrel therapy platelet function indices did not correlate with the thrombus area in T2DM.

In addition, using a cut off of 460 ADP AU, thrombus area did not differ between those who had good and low response to clopidogrel. These findings are novel and can be explained by

i) the inadequacy of Multiplate® to assess non platelet related factors (e.g. fibrin) in thrombogenesis
ii) pleiotropic action of clopidogrel which cannot be quantified by Multiplate®
iii) reconstituted thrombus in cuvette is different from real time thrombus generated from Badimon chamber

In a meta-analysis, Multiplate® assay had higher probability of prediction of acute MI (OR 4.03 95%CI [1.16-14.00]), and stent thrombosis (OR 13.89 95%CI [2.63-73.45], p = 0.002), but only a trend was observed for its role in predicting cardiac mortality (OR 3.21 95%CI [0.86-12.00], p = 0.08) (Aradi, Komócsi et al. 2010). Because of its better reproducibility data and lower coefficient of variation, I suggest Multiplate® is a better assay to monitor pharmacodynamic status in drug development studies.

4.8 Ultrastructural analysis thrombus by scanning electron microscopy (SEM)

4.8.1 Results - SEM Analysis of thrombus in stable CAD study

I conducted a sub-study to quantify ultrastructural characteristics of thrombus in patients with T2DM and stable CAD using scanning electron microscopy (SEM). Demographic, cardiovascular and metabolic profiles are shown in Table 4.28. Briefly, those who had clopidogrel had numerically lower BMI, higher blood pressure values and a longer duration of T2DM. However, none of these reached statistical significance. Baseline characteristics of this randomly chosen study population in subgroup (n=10+10) were similar to those of main study group (n=45+45). Platelet dependent thrombus (PDT) obtained from the Badimon chamber was reduced after clopidogrel therapy and remained unchanged in placebo group (Table 4.29). SEM analysis of the thrombus showed that at baseline, thrombus was mainly composed of platelets. Quantitatively, platelet content of the thrombus, measured as the proportion of platelet rich areas to fibrin rich areas in the SEM images of high shear thrombus at 60X magnification, was similar between both the groups at the baseline visit (84.4% vs. 84.6%, Figure 4.24). After one week of clopidogrel therapy there was 9.5 % relative reduction in platelet content of the thrombus (absolute values: 84.4% to 76.4%, mean difference: 7.98% (95%CI 1.5 to 14.5, p=0.022). There was no change in platelet content of the thrombus in patients who took placebo (84.6% vs. 84.8%, p= 0.948, Figure 4.25).

Fibrin diameter was increased after clopidogrel therapy (nm, mean±SD, from 171.3±11.7 to 186.8±14.4, mean difference: 15, 95% CI 3-27, p=0.016). Fibrin diameter did not change in the placebo group (Figure 4.26). Platelet diameter remained unchanged in both the groups (Table 4.30). Clopidogrel produced significant changes to fibrin architecture of the thrombus. Qualitatively, there were more fibres in thrombus organised in a linear fashion with less sieves or mesh like arrangement (Figure 4.28). Quantitatively, individual fibrin fibre density was increased in the clopidogrel group, with higher number of hubs and spokes per μ^2 of the thrombus (Table 4.31 Figure 4.29). Platelet content of the thrombus had a

significant correlation to thrombus area in the Badimon chamber (rho=0.614, p=0.007) and fibrin diameter correlated with thrombus lysis and G value (Figure 4.30).

I then proceeded to the platelet reactivity analysis using three novel point of care assays, namely Thromboelastography (TEG®), VerifyNow® and Multiplate®. The results of this sub group analysis mirrored those seen in the whole group (Table 4.32), although because of the smaller number of patients, differences did not reach statistical significance. TEG® showed a trend towards reduction in shear elastic modulus (viscoelastic force), increase in rate of thrombus lysis and reduction in the composite measurement clot index (Table 4.32) in patients treated with clopidogrel. The maximum amplitude of the viscoelastic force upon stimulation with kaolin and ADP was lower in the clopidogrel group.

I compared the viscoelastic force of thrombus from TEG® to the fibrin data from SEM. Shear elastic force of the thrombus (G value), as measured by TEG® showed a strong negative correlation to the fibrin diameter (rho -0.609, p=0.007). Fibrin diameter had a negative correlation with the maximum amplitude of the viscoelastic force generated upon stimulation with ADP in TEG® (rho -0.491, p=0.033). The rate of thrombus lysis was similar at baseline in these randomly selected 20 patients with a trend towards improvement in rate of lysis in clopidogrel group. Thrombus lysis parameter, L, showed a moderate but significant correlation to the diameter of the fibrin fibre (rho 0.461, p=0.047).VerifyNow® results showed a significant reduction in PRUz values and platelet inhibition upon stimulation with ADP in clopidogrel group with no changes in placebo group. After clopidogrel therapy, PRUz values negatively correlated with fibrin diameter (rho= -0.601, p= 0.008), fibrin fibre density (rho -0.488, p=0.006) and number of spokes (rho= -0.476, p= 0.046). ARU both at baseline and after therapy levels remained unchanged in both the groups with no significant correlation to SEM measurements. Results from Multiplate® assay showed reduction of ADP aggregation units without any changes in arachidonic acid aggregation units in the clopidogrel group. Multiplate® indices did not show any correlations with fibrin fibre data from SEM both at baseline and after clopidogrel.

	Clopidogrel +aspirin (n=10)		**Placebo+ aspirin (n=10)**		P value
	Mean±SD	**Median (IQR)**	***Mean±SD***	**Median (IQR)**	P value
Demographic data:					
Age, years	64±8	65(56-72)	63±5	63 (61-66)	0.881
Male gender, % (n)	80.0 (8)		90.0 (9)		0.937
Body mass index, kg/m^2	31±3.5	32 (30.0-34.2)	34±6.6	34.0 (28.7-38.3)	0.274
Waist to hip ratio	1.0±0.1	1.0 (1-1)	1.0±0.1	1.0 (1-1)	0.904
Systolic BP, mmHg	135±25	133(122-139)	129±23.3	125 (113-141)	0.903
Diastolic BP, mmHg	78±16.9	69 (65-86)	72±6.8	71.5 (65.8-78.3)	0.598
Duration of diabetes, years	11.1±6.6	8 (6-19)	6.3±3.2	7 (4-9)	0.072
Risk profile: **%(n)**					
Angina	80.0 (8)		60.0 (6)		0.405
Previous MI	70.0 (7)		50.0 (5)		0.809
PCI	40.0 (4)		30.0 (3)		0.701
CABG	20.0 (2)		20.0 (2)		0.906
Chronic kidney disease	0 (0)		20.0 (2)		0.156
Medications: **%,(n)**					
Sulphonylurea	10.0 (1)		10.0(1)		0.937
Metformin	60.0 (6)		50.0 (5)		0.463
Insulin	40.0 (4)		20.0 (2)		0.252

	Clopidogrel +aspirin (n=10)		**Placebo+ aspirin (n=10)**		P value
Laboratory data:mean±SD, Median (IQR)					
	Mean±SD	**Median (IQR)**	***Mean±SD***	**Median (IQR)**	P value
Haemoglobin, g/dl	13.8±0.8	13.9 (13.2-14.5)	13.3±0.8	13.3 (12.6-13.8)	0.175
Platelets X1000 cells/ mm^3	227±38	224 (209-260)	218±61	220 (183-255)	0.750
Fibrinogen, g/l	3.0±0.5	3.0 (2.6-3.6)	3.4±0.8	3.3 (2.8-4.2)	0.326
HbA1c, %	7.4±1.6	7.1 (6.4-8.7)	7.4±0.8	7.6 (6.9-7.8)	1.000
Fasting plasma glucose, mmol/l	7.8±3.3	7.2 (5-9)	7.8±1.8	8 (7.1-9.2)	0.965
eGFR, ml/min/1.73m^2	84±26.0	76 (66-109)	81±19.6	81.5 (75.3-95.5)	0.785
Total cholesterol, mmol/l	3.2±0.6	3.05 (2.7-3.8)	3.2±0.5	3.4 (2.7-3.5)	0.978
LDLc, mmol/l	1.6±0.5	1.5 (1.2-2.2)	1.7±0.4	1.7 (1.4-2.1)	0.777
HDLc, mmol/l	1.0±0.2	1.0 (0.9-1.2)	1.0±0.2	0.9 (0.8-1.2)	0.594
Triglyceride, mmol/l	1.4±0.6	1.6 (1.2-2.4)	1.6±0.6	1.3 (1.3-2.2)	0.710
HsCRP, mg/l	1.5±1.5	0.9 (0.4-3.0)	3.0±3.3	1.7 (0.8-6.0)	0.276

Table 4.28 Baseline characteristics of SEM study (Stable CAD).

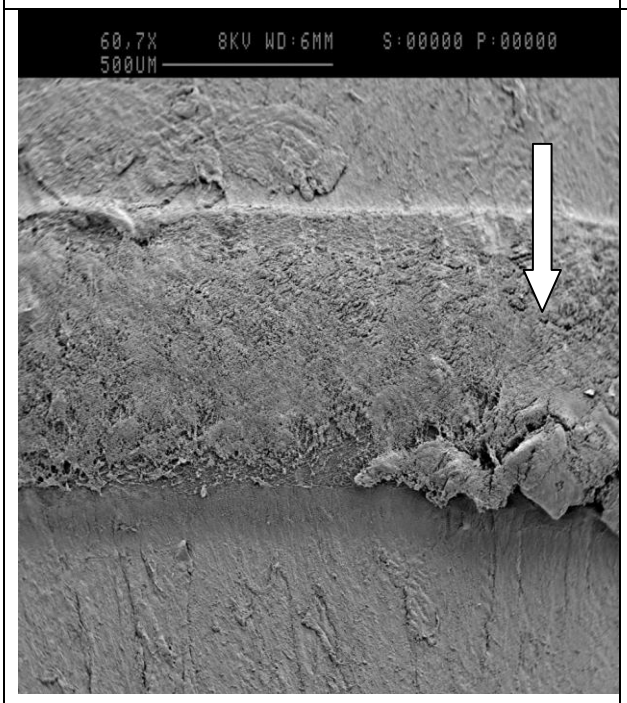
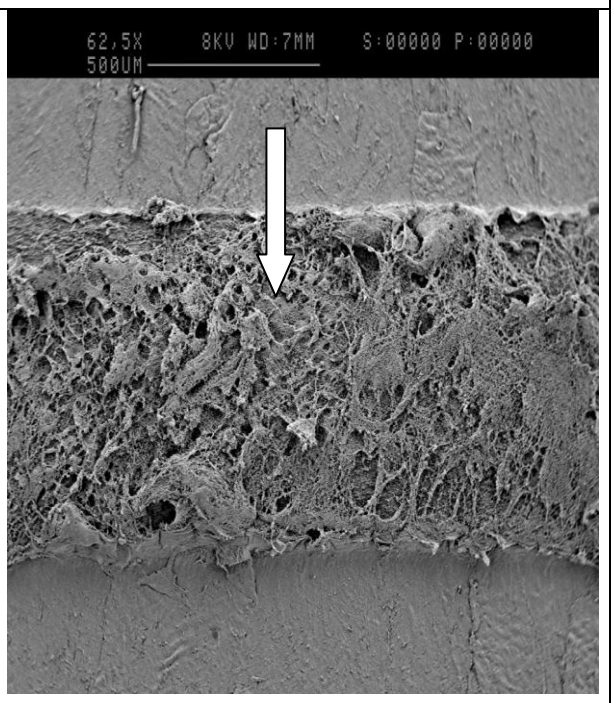
Platelet dependent thrombus at baseline	Platelet dependent thrombus after one week of clopidogrel therapy
	

Figure 4.25 Platelet content of thrombus in electron microscopy.

SEM analysis of the platelet dependent thrombus generated from Badimon chamber thrombus showed that at baseline, thrombus was mainly composed of platelets. Quantitatively, platelet content of the thrombus, measured as the proportion of platelet rich areas to fibrin rich areas in the SEM images of high shear thrombus at 60X magnification was 84.4% and after one week of clopidogrel therapy there was 9.5 % relative reduction in platelet content of the thrombus (absolute values: 84.4% to 76.4%, mean difference: 7.98%, 95%CI 1.5 to 14.5, p=0.022). Solid white arrows represent platelet rich areas.

	Clopidogrel (n=10)			**Placebo (n=10)**		
Thrombus area, μ^2/mm, Mean±SD	Baseline	Visit 2	P value	Baseline	Visit 2	P value
High shear chamber	13637 ±5252	10656 ±3586	0.013	13282 ±4756	11635 ±3127	0.128
Low shear chamber	8756 ±4565	6298 ±2578	0.018	8610 ±3631	7309 ±2451	0.126

Table 4.29 Thrombus area in SEM sub study.

	Clopidogrel (n=10)			**Placebo (n=10)**		
Mean±SD	Baseline	Visit 2	P value	Baseline	Visit 2	P value
Fibrin diameter, nm	171.3 ±11.7	186.8 ±14.4	0.016	171.6 ±7.2	176.3 ±13.3	0.406
Platelet diameter, µm	1.7 ±0.11	1.6 ±0.05	0.894	1.8±0.03	1.6 ±0.07	0.448

Table 4.30 Fibrin and platelet diameters of SEM sub study.

Fibrin at baseline

Fibrin after one week of clopidogrel therapy

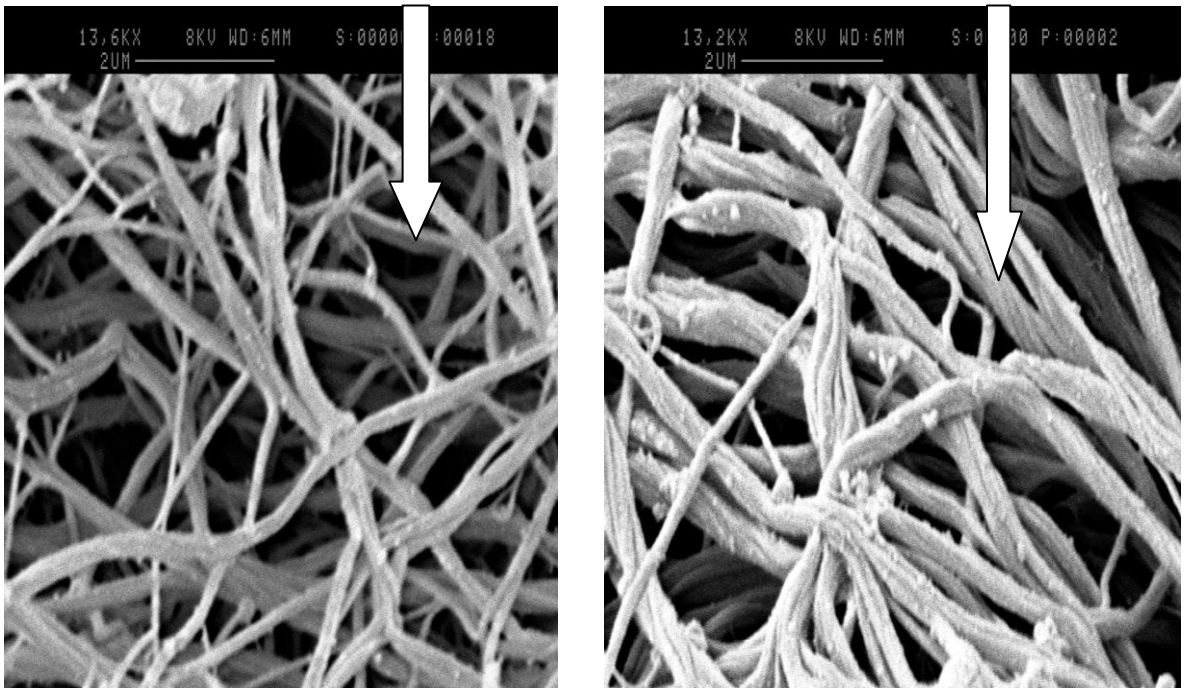


Figure 4.26 Fibrin fibre diameter after clopidogrel therapy.

Ultrastructure of fibrin derived from the thrombus in the Badimon chamber was studied at $13X10^3$ times magnification using scanning electron microscopy. Fibrin diameter increased after clopidogrel therapy, (nm, mean±SD) from 171.3±11.7 at baseline to 186.8±14.4, p=0.016. Solid arrows represent individual fibrin fibre.

Platelets at baseline

Platelets after clopidogrel therapy

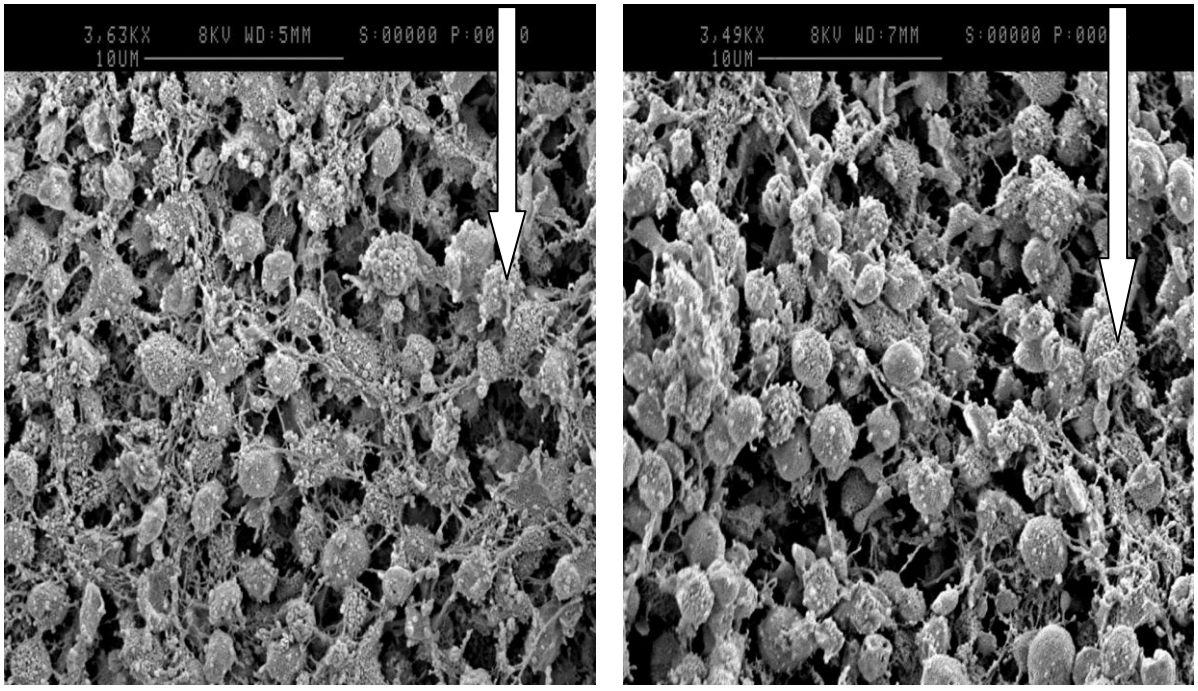


Figure 4.27 Platelet ultrastructure in thrombus after clopidogrel therapy.

Scanning electron microscopic images of platelets (at $3.5X10^3$ to $3.6\ X10^3$ magnifications) from the thrombus generated from Badimon chamber of a patient with type 2 diabetes mellitus and coronary artery disease, at baseline and after clopidogrel therapy. Platelet diameter remained unchanged after clopidogrel therapy (diameter in μm, mean±SD, from 1.7±0.11 to 1.6±0.05, p=0.894). Solid white arrows show platelets in various stages of activation, with granules and pseudopodia on their surfaces.

	Clopidogrel (n=10)			**Placebo (n=10)**		
Mean±SD, number per μ^2 of thrombus	Baseline	Visit 2	P value	Baseline	Visit 2	P value
Fibrin fibre density	32.3±4.8	41.0±10.2	0.023	29.7±6.7	29.5±9.9	0.348
Spokes of fibrin	29.5±4.4	36.6±8.3	0.030	28.9±8.9	26.9±9.5	0.852
Hubs of fibrin	15.5±1.5	16.7±1.4	0.022	14.9±3.0	13.1±4.1	0.574

Table 4.31 Quantitative analysis of Fibrin in SEM sub study.

Fibrin architecture at baseline

Fibrin after clopidogrel therapy

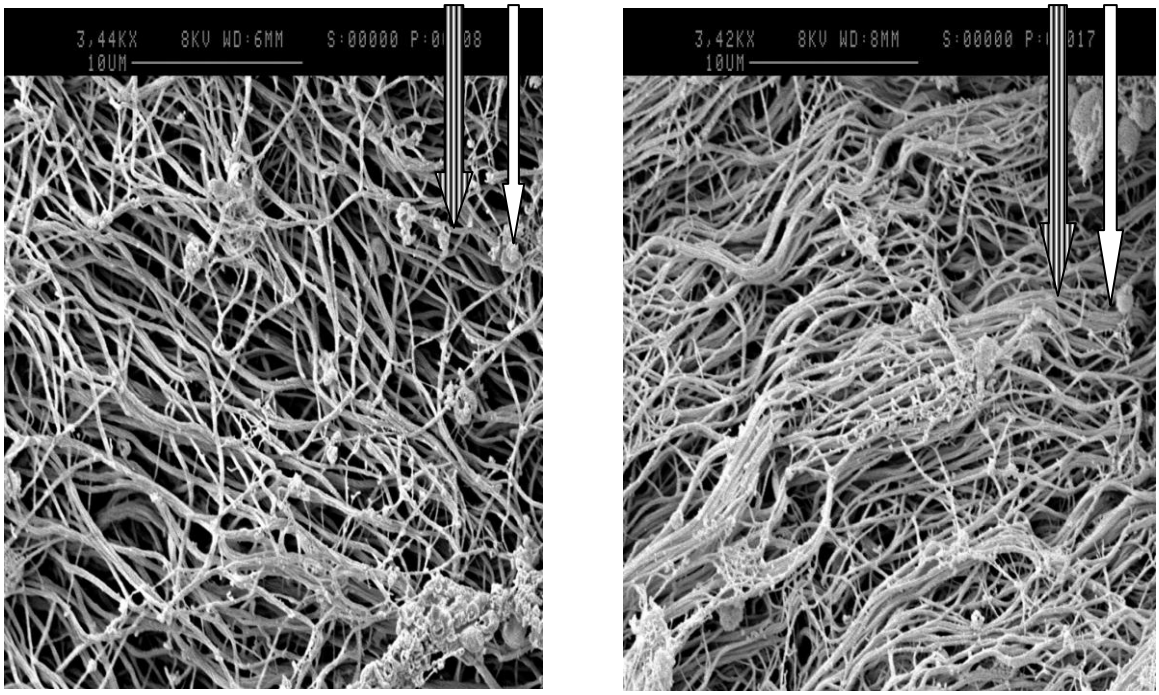
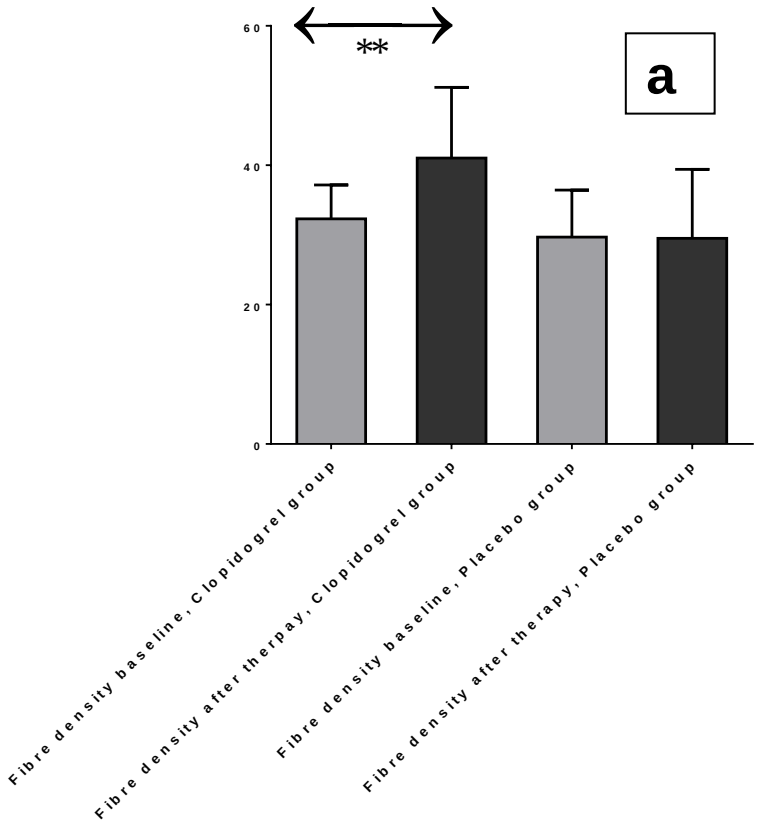


Figure 4.28 Fibrin fibre architecture after clopidogrel therapy

Fibrin fibre arrangement was studied at $3.4X10^3$ magnification using scanning electron microscopy. After clopidogrel therapy, there was more number of fibrin fibres in thrombus and fibrin arrangement showed densely packed and compact fibres. Quantitatively, individual fibrin fibre density was increased in the clopidogrel group, with higher number of hubs and spokes. Solid white arrows show individual fibrin fibres whereas patterned arrows show hub and spoke arrangement.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60
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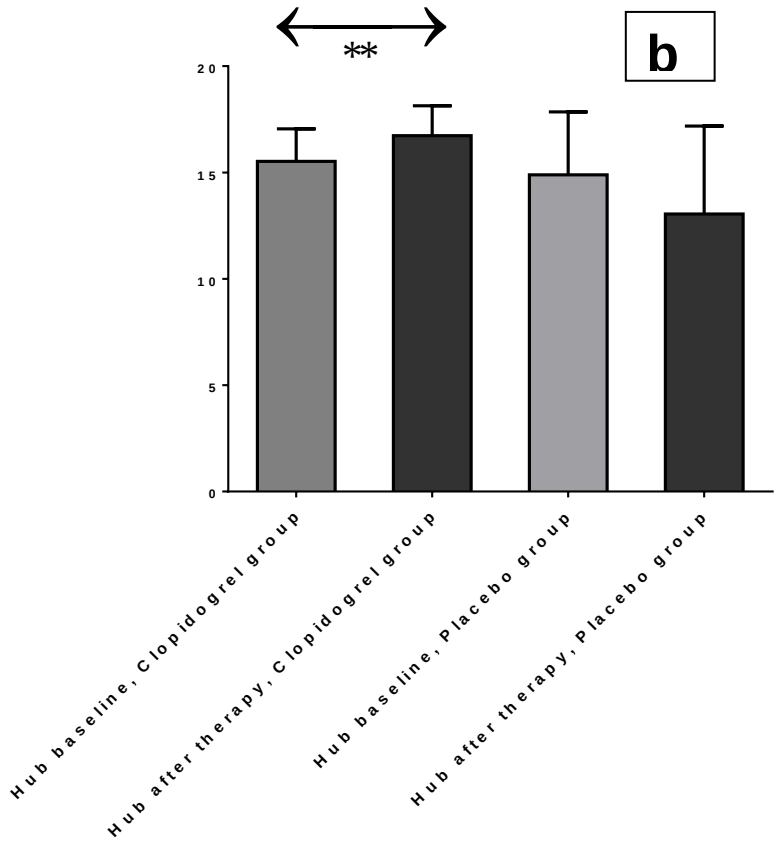
Fibre density baseline, Clopidogrel group
Fibre density after therpay, Clopidogrel group
Fibre density baseline, Placebo group
Fibre density after therapy, Placebo group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Hub baseline, Clopidogrel group
Hub after therapy, Clopidogrel group
Hub baseline, Placebo group
Hub after therapy, Placebo group

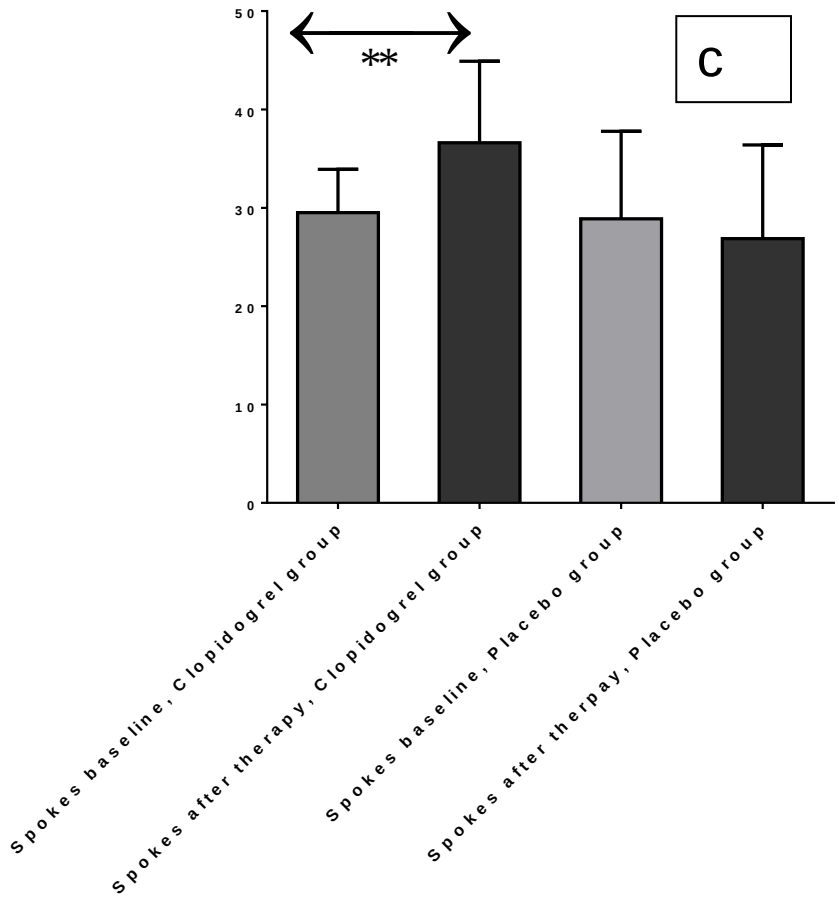


Figure 4.29 Quantitative analysis of fibrin fibre in whole blood thrombus (a,b,c).

The fibrin density (number of fibres per µ2 thrombus) was higher with increased numbers of hubs and spokes. Qualitatively, these findings represent a more organised and compact fibrin arrangement. The fibrin arrangement remained unchanged in placebo group. ** p <0.05. Error bars represent one SD. The changes seen in clopidogrel group are (mean, ±SD, per µ2 of thrombus) fibrin fibre density from 32.3±4.8 to 41.0±10.2, spokes of fibrin from 29.5±4.4 to 36.6±8.3, hubs of fibrin from 15.5±1.5 vs 16.7±1.4, all are statistically significant at p<0.05. The values remained unchanged in placebo group.

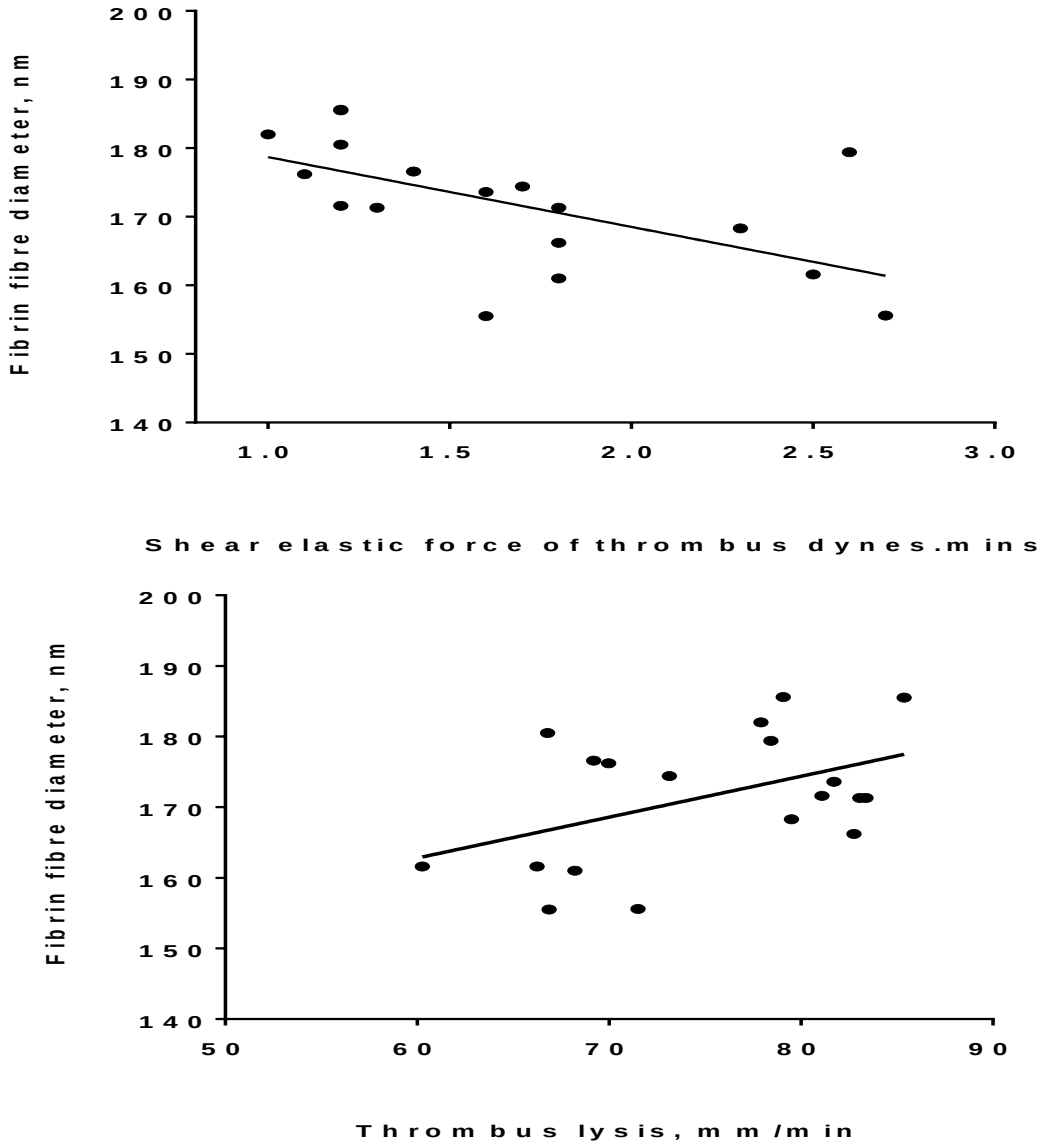


Figure 4.30 Correlations between fibrin fibre diameter and viscoelastic properties of thrombus.

Shear elastic force of the thrombus (G value), as measured by TEG® showed a strong negative correlation to the fibrin diameter (rho= -0.609, p=0.007) at baseline. Thrombus lysis parameter (L), showed a significant correlation to the diameter of the fibrin fibre (rho= 0.461, p=0.047).

	Clopidogrel (n=10)			Placebo (n=10)		
Mean±SD	Baseline	Visit 2	p value	Baseline	Visit 2	p value
Thromboelastography data:						
R, minutes	6.2±1.9	6.1±1.9	0.972	7.2±1.6	6.3±2.3	0.090
Maximum amplitude-Kaolin, mm	65.3±4.8	62.0±6.0	0.159	65.4±5.4	65.8±5.9	0.764
Maximum amplitude-AA,mm	36.3 ±13.5	30.1±17.2	0.338	34.4 ±19.9	38.0±16.7	0.575
Maximum amplitude-ADP,mm	57.4±6.2	49.3±12.7	0.114	59.2±9.3	57.2±7.7	0.424
Shear elastic force, 10^3 dynes/cm^2	9.7±2.1	8.4±2.0	0.117	8.8±2.3	10.0±2.6	0.648
L parameter, mm/min	76.1±7.2	85.2±8.1	0.079	73.9±7.8	76.0±10.0	0.440
Clot index	0.56±2.5	-0.07±2.8	0.159	0.21±2.2	1.08±2.5	0.181
VerifyNow® data:						
ARU	440±35.7	434±67.6	0.804	479 ±93.7	462±71.1	0.601
PRUz	317±43.5	251±63.1	0.007	340.7 ±48.1	340.6 ±52.8	0.996
Platelet inhibition,%	33.1 ±10.1	18.2±18.1	0.017	33.1 ±10.2	30.1±10.1	0.412
Multiplate® data:						
Arachidonic acid, AU	313±173	320±166	0.912	328±229	269±196	0.570
ADP, AU	804±460	451±211	0.050	811±227	684±338	0.178

Table 4.32 Point of care tests of SEM sub study.

4.8.2 Discussion – SEM Analysis of thrombus in stable CAD study

I report qualitative and quantitative analysis of the ultra structural properties of whole blood thrombus formed in Badimon chamber of patients with T2DM and stable CAD for the first time. Significant changes in density, diameter and arrangement of fibrin fibres in whole blood thrombus following clopidogrel therapy in T2DM with CAD were shown. The methods of assessment were novel and every step of the process has been validated internally in detail. From an earlier pilot study on patients with NSTE-ACS, I found that in patients with T2DM, qualitative SEM analysis of thrombus showed thinner fibrin fibres with predominant “lattice and sieve” arrangements when compared to non diabetic patients. This has resulted in higher thrombus, weaker viscoelastic recoil and slower thrombus autolysis. The current study quantitatively analysed the ultrastructure of the thrombus in T2DM.

After one week of clopidogrel therapy, the proportion of platelet content in thrombus decreased, paralleling my findings from the main study that platelet dependent thrombus was reduced in those who were treated with clopidogrel. Thrombus formed in Badimon chamber was heterogeneous, with fibrin rich and platelet rich areas. It is well known that thrombus formed in coronary arteries of individuals with T2DM sustaining an MI is rich in platelets which is known as “white thrombus” (Davies and Thomas, 1984). My results suggest that one of the mechanisms of reduction in thrombus quantity is by way of changes in composition of thrombus and reduction in the cellular content of the thrombus. My findings of reduced platelet rich areas in thrombus after clopidogrel therapy is novel and are supported by indirect evidence from SEM studies showing that less platelet activation, smaller platelet volume and low platelet counts altered the ultrastructure of the thrombus following antiplatelet therapy (Standeven *et al.*, 2005; Undas and Ariëns, 2011).

Fibrin fibre content and architecture were also altered by clopidogrel therapy. The diameter of individual fibrin fibres was higher after clopidogrel therapy and multiple studies have shown that this feature enables favourable fibrinolysis (Collet *et al.*, 2003a; Collet *et al.*, 2006; Weisel, 2007). Fibrin fibre density per

square micron was increased after clopidogrel therapy in patients with T2DM and CAD. There were significant differences in fibrin fibre arrangements in the form of higher number of “hub and spoke” type arrangement in patients who received clopidogrel and aspirin compared to those receiving aspirin alone. Alterations in fibrin structure in patients with T2DM have been attributed to non enzymatic glycation of fibrinogen. Glycated fibrinogen enhances thrombogenicity by increasing fibrin polymerisation and cross-linking, reducing tissue plasminogen activator and plasminogen binding, and slowing plasminogen to plasmin conversion (Dunn *et al.*, 2005). Antiplatelet therapy with aspirin improves fibrin architecture in T2DM by acetylation of the alpha chain of fibrinogen (Dunn *et al.*, 2005),(Ajjan *et al.*, 2009). Pieters et al showed improvement in glycaemic control reduced glycation of fibrinogen (Pieters *et al.*, 2007), favourably altered fibrin architecture and improved fibrinolysis in a purified fibrinogen model (Pieters *et al.*, 2008). Fibrin fibres, upon polymerisation, produce definitive ultra structural re-arrangements which include twisting, knotting, lateral linking and stretching, which have been demonstrated by SEM, TEM, confocal microscopy and polarised light microscopy. Viscoelastic force is lower when there is excess twisting of fibrin fibres around an axis (“hub”), as this structure limits elastic recoil. When fibrin fibres branch out of the hub, instead of forming straight fibres in a pleated arrangement, binding sites for the fibrinolytic peptides tissue plasminogen activator and plasmin are more exposed and therefore this arrangement favours fibrinolysis (Ajjan *et al.*, 2009). Clopidogrel, by increasing the number density of hubs and spokes in the thrombus, renders the thrombus easier to undergo fibrinolysis. This concept is supported by findings from my viscoelastic study of thrombus using thromboelastography(vide supra), and by published literature (Gabriel *et al.*, 1992).

It has been shown that the viscoelastic properties of fibrin polymers determine clot stability in healthy individuals and in patients and relatives of those with premature coronary artery disease (Collet *et al.*, 2006; Standeven *et al.*, 2007; Weisel, 2007). I measured viscoelastic properties of the platelet rich thrombus using thromboelastography (TEG®) and Platelet Mapping™. I demonstrated a significant negative correlation between fibrin fibre diameter and shear modulus

viscoelastic force of the whole blood thrombus (G parameter). Fibrin fibres use their maximum energy while undergoing polymerisation and formation of thicker fibrin fibres limits the energy available to undergo further elastic recoil. Thicker fibrin fibres formed in reconstituted thrombus using fibrinogen have reduced viscoelasticity (Collet *et al.*, 2005; Gersh *et al.*, 2009). These findings were replicated in my study by using a point of care native whole blood assay (TEG®) and directly measuring fibrin diameter from whole blood thrombus generated in Badimon chamber. I also found that there was a similar relationship between the rate of whole blood thrombus lysis (L parameter) and fibrin diameter, findings which were akin to those described in thrombus regenerated from plasma (Collet *et al.*, 2000; Hess *et al.*, 2011). Differences in the individual TEG® parameters after clopidogrel therapy did not reach statistical significance in my cohort of 33 patients (vide supra). However, the association between individual fibrin diameter and the maximum amplitude of the viscoelastic force (MA mm) measured (by stimulating native whole blood with by ADP) by TEG® is novel. If these data can be validated in further large scale experiments, this model may serve as a useful tool to study the effect of novel antithrombotic therapies including those targeting the fibrinolytic pathway in whole blood thrombus of patients with T2DM and CAD.

PRUz, the most validated measurement from VerifyNow® assay, showed a negative association between fibrin diameter and the number of fibrin fibres. Patients with higher PRUz reflecting high on treatment platelet hyperactivity, had thinner fibrin fibres and vice versa. Similarly, the number of fibrin fibres was higher in those with lower PRUz, indicating that patients with lower platelet reactivity are likely to have fibrin rich thrombus compared to platelet rich thrombus in non responders. This finding is hypothesis generating and needs validation.

Multiplate® results showed significant reduction in electrical impedance upon stimulation with ADP in those treated with clopidogrel. However, no significant associations were noted between Multiplate® indices and SEM measurements. These findings are not entirely surprising. Individual fibrin fibres in thrombus are held together in a gel which is a good conductor of electricity. Electrical

impedance is less likely to be influenced by changes in the fibrin assembly or diameter of fibrin fibres (Jung *et al.*, 2009).

Platelet diameter remained unchanged after clopidogrel therapy in T2DM (Figure 4.27). Platelets of patients with T2DM had larger platelet volume compared to published data on healthy volunteers (Rao *et al.*, 1984; Watala *et al.*, 1999). Large diabetic platelets are secondary to increased platelet turnover and larger sized “neo platelets” produced from bone marrow (Jagroop and Mikhailidis, 2005). Platelet diameter decreased after antiplatelet therapy in non diabetic subjects and healthy volunteers (Papanas *et al.*, 2004). The absence of this response in my cohort of patients with T2DM and stable CAD who were treated with clopidogrel needs to be confirmed in large scale studies. It is possible that diabetic “angry platelets” are under constant stimulation by a variety of naturally occurring agonists (Bhatt, 2008) or by pathways involving inflammatory mediators (Hess *et al.*, 2011). Treatment of T2DM by an ADP antagonist like clopidogrel for a week may not be enough to reduce their size.

Point of care assays are increasingly used by clinical researchers and I have shown for the first time an association between the ultra structural characteristics of whole blood thrombus and platelet reactivity indices measured using these instruments. As newer and more powerful ADP antagonists are licensed for use (e.g. ticagrelor, prasugrel), my data provides a focus for studying the effect of newer drugs on the structural and functional changes in thrombus.

My findings from SEM are novel and interesting but have some limitations. Firstly, the patient cohort is small in number which makes the generalisation of these findings impossible. The strict eligibility criteria, adherence to medications and cardiovascular risk factor control observed in my study population is not commonly seen in day to day clinical practice. It is unknown if similar findings can be seen with the use of newer antiplatelet agents. The computational model used in my study for quantification of fibrin architecture is yet to be validated on heterogeneous larger population cohorts including healthy volunteers and non diabetic patients with CAD.

4.9 Coronary atheroma burden and platelet dependent thrombosis

4.9.1 Results – Coronary atheroma burden and thrombus in ACS study

Coronary atheroma as quantified by the Gensini score was calculated for 80 patients (40 T2DM) in the ACS study who successfully completed Badimon chamber studies. Ten patients had previous CABG and were excluded from the analysis as the score has not been well validated in this population. Final analysis included 70 patients (34 T2DM). Coronary atheroma burden as quantified by Gensini score was higher in T2DM patients with NSTE-ACS compared to non DM (mean±SD, 46.1±20.6 vs. 37.5±13.0, p=0.042). Number of arterial segments with atheroma of more than 25% as measured by modified Gensini score was higher in T2DM [6.2±2.7 vs. 4.6±1.9, p=0.004] (Table 4.33, Figure 4.31). Gensini score did not correlate with thrombus area. However, modified Gensini score showed significant correlations with thrombus area in both the high shear and low shear chambers (High shear thrombus rho=0.293, p=0.017; low shear rho=0.325, p=0.007, Figure 4.32). Of the baseline characteristics, only age showed positive correlations to Gensini score (rho=0.240, p=0.045, Table 4.34) with no relationship observed with HbA1c, blood glucose, BMI, fibrinogen and serum creatinine.

I then classified atheroma burden in to two main groups, those with at least one occlusive plaque (more than 75% luminal narrowing) and those with non occlusive plaque disease (25-75% luminal narrowing). All the 80 subjects were included and for those who had CABG, the surgical conduits were studied for atheromatous plaque. The subjects were divided in to four groups namely:

i. T2DM and occlusive plaque (n=25)

ii. T2DM and non occlusive plaque (n=15)

iii. non diabetic and obstructive plaque (n=24)

iv. non diabetic and non obstructive plaque (n=16)

When stratified according to CAD status, thrombus area was highest in T2DM and non occlusive plaque (F=5.26, p=0.002, Table 4.35). Inflammatory cytokine interleukin 6 (F=6.759, p=0.001) and high sensitivity CRP (HSCRP, F=4.347, p=0.007) were the highest in this group. P selectin (F4.728, p=0.004) and soluble CD40 ligand (F=9.593, p=0.004) were also the highest in this group (Figure 4.33). Interleukin 1, interferon gamma and TNFα levels were numerically higher in this group but showed no statistical significance.

Mean±SD	T2DM	Non T2DM	P value
Gensini score	49.7±21.3	37.5±13.0	0.042
Modified Gensini score	6.2±2.7	4.6±1.9	0.004

Table 4.33 Gensini score in ACS study.

	Gensini Score		Modified Gensini score	
	rho	P value	rho	P value
HbA1c,%	0.193	0.123	0.334	0.006
Age, in years	0.240	0.045	0.339	0.004
BMI, kg/m^2	0.016	0.902	0.001	0.997
Random plasma glucose, mmol/l	0.004	0.972	0.037	0.731
Fasting plasma glucose, mmol/l	0.048	0.711	0.123	0.345
Serum Creatinine, mmol/L	0.062	0.614	0.042	0.732
Fibrinogen, g/L	0.054	0.658	0.071	0.558
High shear thrombus, μ^2/mm	0.117	0.351	0.293	0.017
Low shear thrombus, μ^2/mm	0.134	0.243	0.325	0.007

Table 4.34 Correlation between standard Gensini score and baseline variables.

	T2DM with non occlusive plaque (n=15)	T2DM with occlusive plaque (n=25)	Non DM with non occlusive plaque (n=16)	Non DM with occlusive plaque (n=24)	P values
Thrombus area, μ^2/mm	18645±12103	24412±11813	10927±4284	12633±3495	0.002
P selectin, pg/ml	80.2±21.6	68±28.6	52.9±12.1	62.6±13.7	0.004
sCD40 ligand, pg/ml	8020±6649	3952±3656	2250±2099	1895±1189	0.004
IL-6 ng/ml	4.4±2.6	2.9±2.0	2.1±1.1	2.2±0.7	0.001
HSCRP mg/L	12.0±14.3	7.7±7.1	2.4±2.2	11.7±7.4	0.007

Table 4.35 Biomarkers in patients with and without occlusive coronary atheromatous plaques

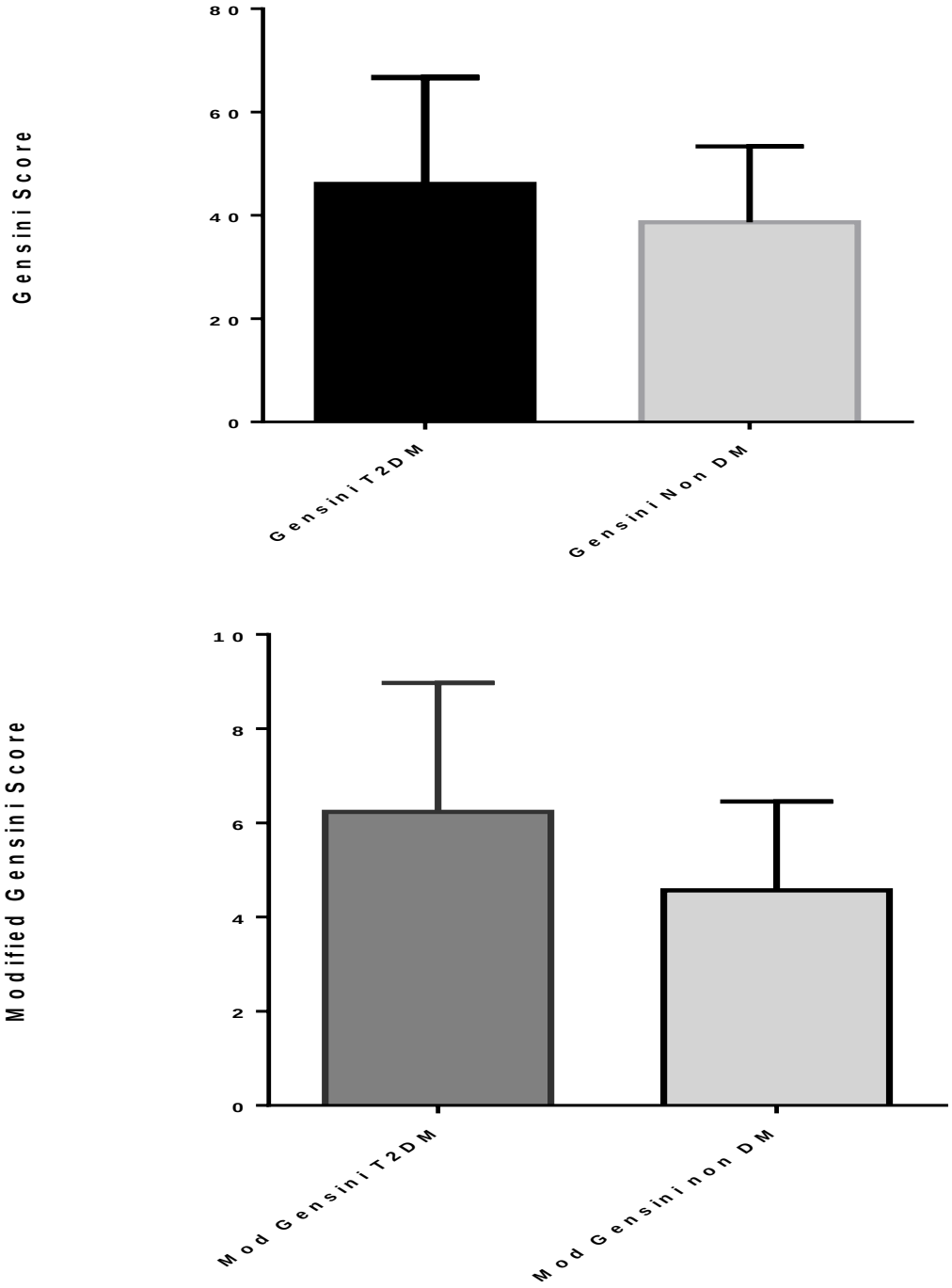


Figure 4.31 Gensini and modified Gensini scores in ACS study.

Patients with T2DM had higher Gensini (each arterial segment with lesions scored with an individual weightage factor) and modified Gensini (each arterial segment with lesions scored equally). The Gensini scores are (value±SD), T2DM 49.7±21.3 vs. non DM 37.5±13.0, p=0.042 and modified Gensini scores are T2DM (value±SD) 6.2±2.7 vs. non DM 4.6±1.9, p=0.004. Error bars represent 1XSD.

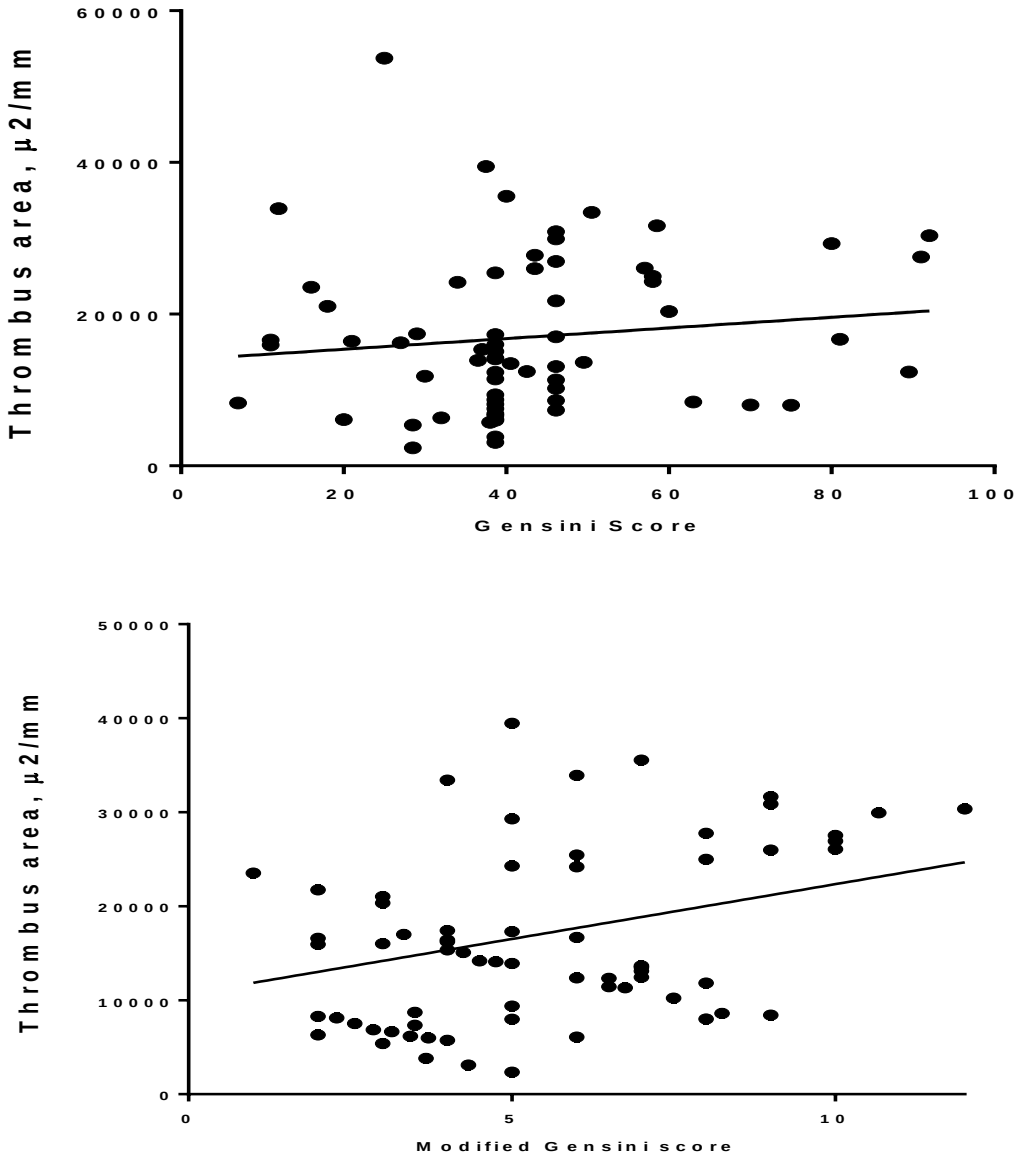
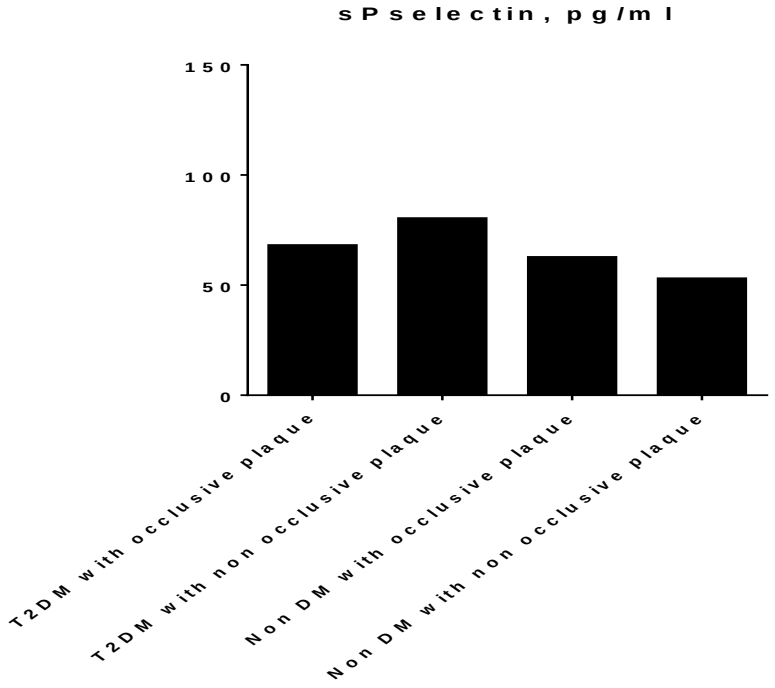
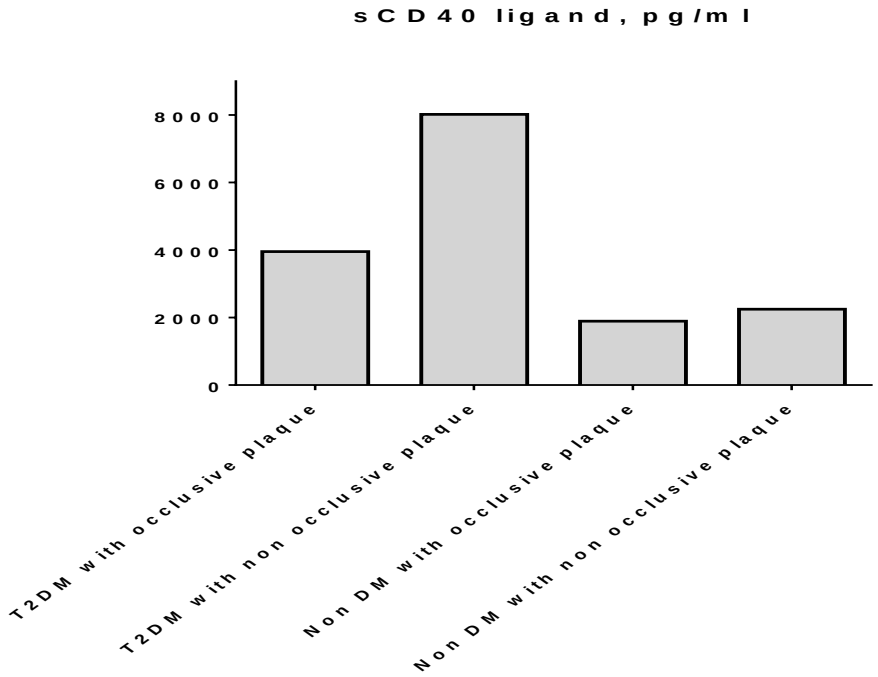


Figure 4.32 Gensini scores and thrombus area.

Scatter plot diagram of Gensini scores vs. High shear thrombus area showing no correlation with Gensini score (rho=0.117, p=0.351) and significant correlation with modified Gensini score (rho = -0.293, p=0.017).


sP selectin, pg/ml
150
100
50
0
T2DM with occlusive plaque
T2DM with non occlusive plaque
Non DM with occlusive plaque
Non DM with non occlusive plaque


sCD40 ligand, pg/ml
8000
6000
4000
2000
0
T2DM with occlusive plaque
T2DM with non occlusive plaque
Non DM with occlusive plaque
Non DM with non occlusive plaque

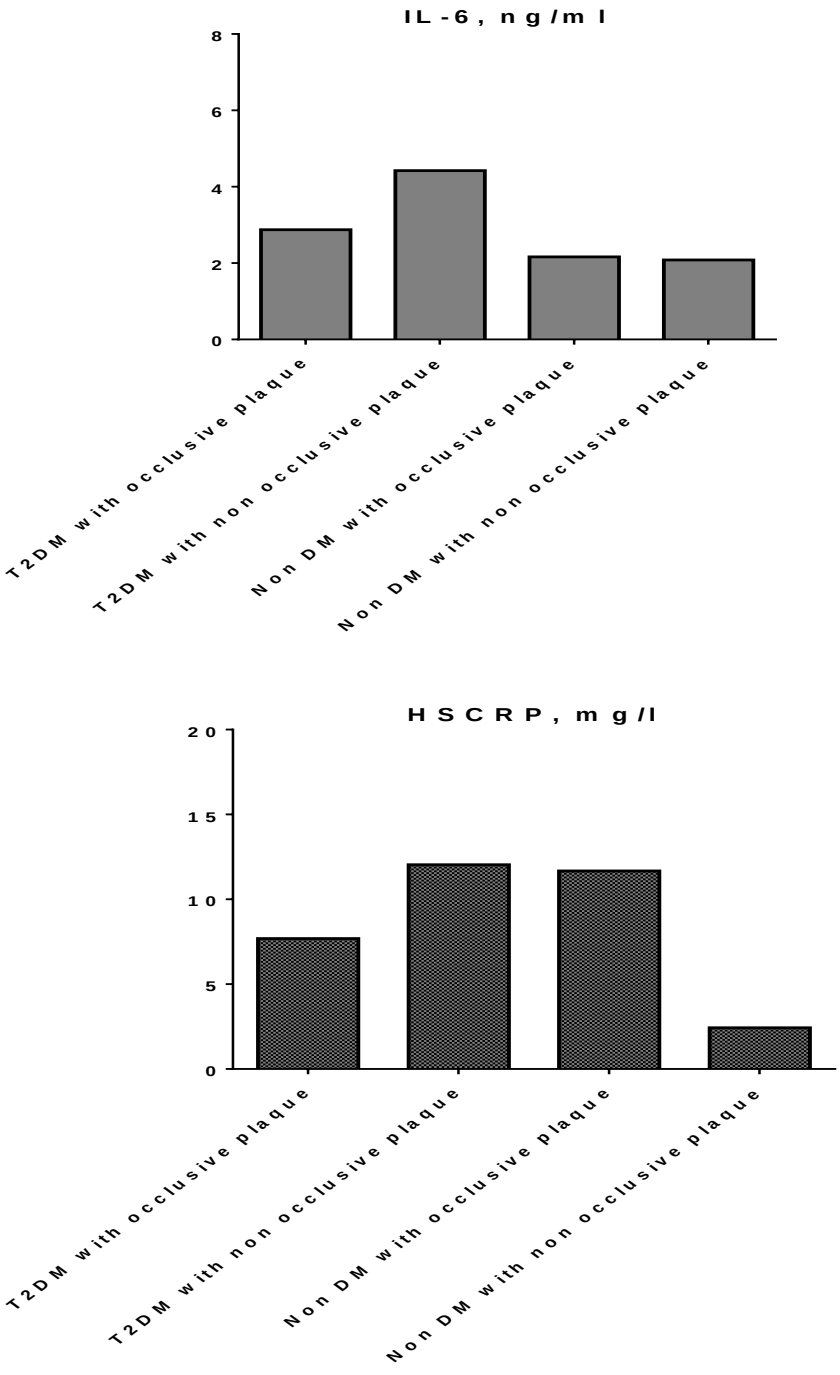


Figure 4.33 Biomarkers in patients with obstructive and non obstructive atheromatous plaques.

Patients were grouped according to the presence of obstructive plaque (>75% luminal diameter) or non obstructive (25%-75% luminal diameter). Inflammatory cytokine interleukin 6 (F=6.759, p=0.001) and high sensitivity CRP (HSCRP, F=4.347, p=0.007) were the highest in patients with non obstructive plaque and T2DM. P selectin (F=4.728, p=0.004) and soluble CD40 ligand (F=9.593, p=0.004) were also the highest in this group.

4.9.2 Discussion – Coronary atheroma burden and thrombus in ACS study

Gensini score is a very well validated tool to assess coronary artery disease severity (Austen *et al.*, 1975; Gensini, 1983). By combining luminal narrowing with site of stenosis, this score better reflects the prognostic significance of CAD. I found that Gensini score was significantly higher in T2DM. These findings are unsurprising as T2DM is associated extensive atheroma burden quantified in autopsy studies. In addition, multiple lesions in T2DM are often seen with diffuse atheromatous process. My results were similar to those published recently in patients with and without T2DM (Saleem *et al.*, 2008; Gui *et al.*, 2012). There were no correlations between the glycaemic indices such as HbA1c and blood glucose and Gensini score. This is in contradiction to earlier results which showed direct linear relationship between Gensini score and blood glucose control in stable CAD patients (Mi *et al.*, 2012). This may be due to two reasons

i. my cohort is small (T2DM n=34)

ii. glycaemic control in my study population was better than in the published literature(Saleem *et al.*, 2008)

In addition, NSTE-ACS in poorly controlled T2DM is often secondary to the rupture of non obstructive plaques and thereby glycaemic indices are less likely to reflect true flow limiting atheroma.

There was no relationship between standard Gensini score and thrombus burden but a modified Gensini score (measuring the number of diseased segments each with a 1X score) was significantly associated with thrombus. Though the relationship between atheroma burden and thrombogenicity is documented (Zaman *et al.*, 2000), there is paucity of literature on Gensini score and thrombogenicity. I speculate that any unweighted score (e.g. modified Gensini score) will predict thrombus better in view of the simplified reflection of the number of atheromatous segments whereas a weighted score is more appropriate for future events and prognosis. I propose that atheroma burden is directly related to thrombus but Gensini score may not be the right scoring system in the context of ACS to explain the relationship between atheroma and

higher blood thrombogenicity. It is possible that the results may have been different if I had used a different scoring system such as SYNTAX score (Sianos *et al.*, 2005; Valgimigli *et al.*, 2007; Farooq *et al.*, 2011). Syntax scoring system is mainly designed to evaluate the outcomes of various revascularisation strategies in patients with complex coronary anatomy rather than atheroma burden and is validated only in patients with multivessel coronary artery disease, whereas my cohort included patients with single vessel disease (Serruys *et al.*, 2009). My results in a smaller cohort of patients are novel and hypothesis generating.

Patients with T2DM and non-occlusive coronary atheromatous plaque had the highest thrombus and markers of platelet activation and inflammation. These results support the view that a “biochemical mechanism” is more often associated with acute coronary syndrome in these patients than a conventional “plaque rupture-biomechanical mechanism” (Lee and Libby, 1997; Geng and Libby, 2002). Detailed discussion on pathophysiology of inflammation, thrombosis and atheroma are presented vide supra. Presence of non obstructive plaques in T2DM in combination with higher blood thrombogenicity is associated with poorer outcomes. Moreno et al have shown extensive infiltrates of inflammatory macrophages in coronary atheromatous specimens of patients with diabetes and these have been confirmed in subsequent non invasive studies calling for a change in definition of vulnerable plaque in these individuals (Naghavi *et al.*, 2003; Moreno and Fuster, 2004). Inflammation in diabetes was proposed as a “common soil” mechanism for CAD (Stern, 1995) and inflammatory markers were associated with thrombosis in T2DM (Hess and Grant, 2011). My findings support the view that biochemical changes are responsible for thrombosis in patients with T2DM (Hess *et al.*, 2012; Libby, 2013).

A causal link, if confirmed by large-scale experimental and observation studies, may offer us an opportunity to identify therapeutic targets such as individualised antithrombotic and anti inflammatory therapy in these high risk population.

Chapter 5 Conclusion

5.1 General discussion

The studies in this thesis have upheld my two hypotheses, that blood thrombogenicity is higher in patients with T2DM after non ST-elevation acute coronary syndrome (NSTE-ACS) despite currently recommended therapy with dual antiplatelet agents, aspirin and clopidogrel and that dual antiplatelet therapy with aspirin and clopidogrel reduces blood thrombogenicity compared to aspirin monotherapy in patients with T2DM and stable coronary artery disease (CAD). The results demonstrate that in type 2 diabetes, thrombus quantity and quality are abnormal. Qualitative differences in thrombus suggested potential mechanisms for the changes in thrombus quantity in both the studies. Changes in viscoelastic property and fibrin fibre arrangement resulted in greater thrombus and these changes also reflected impairment in autolysis of the formed thrombus in T2DM. Thrombus structural analysis showed a disorganised thrombus, with thin fibrin fibres and reduced fibrin fibre density. The inverse relationship between fibrin fibre diameter and viscoelastic strength of the clot suggest that in diabetes, the abnormal structure of the thrombus alters clot strength. The physical derangement of the thrombus may make access of thrombolytic enzymes more difficult, thus slowing thrombus lysis time.

The findings in patients with NSTE-ACS and T2DM study are important for the following reasons:

i. this is the largest cohort of patients studied after NSTE-ACS using the Badimon chamber

ii. participants were on all recommended secondary prevention therapy and were chosen based on strict eligibility criteria to minimise confounding variables

iii. the data provide a focus for studies of mechanisms underlying the observational data of higher recurrent thrombotic event rates in T2DM following NSTE-ACS.

My findings argue against the current identical recommendations for secondary prevention therapy in patients with and without T2DM, and suggest that either a more targeted and or more aggressive approach to antithrombotic treatment is required in T2DM patients after NSTE-ACS.

Addition of a second antiplatelet agent, clopidogrel 75mg daily, reduced thrombus in patients with T2DM and stable CAD. To achieve a significant reduction of thrombus with dual antiplatelet therapy in population of T2DM with stable CAD which has good lipid and hypertension control and good (but not very tight) glycaemic control is clinically important. The effect of clopidogrel in suppressing platelet reactivity has been confirmed by three point of care platelet function studies.

Multiple factors could have attributed to higher platelet dependent thrombus (PDT) in T2DM. The platelets of patients with T2DM are in a constant state of hyperactivation and in the context of NSTE-ACS, aspirin and clopidogrel therapy was not adequate to reduce platelet hyperactivity, and thus PDT, to the levels seen in non DM. Similar findings were earlier shown by our group in stable CAD patients who were taking aspirin monotherapy (Natarajan et al., 2008a). Platelet dysfunction is likely the major factor contributing to higher PDT in T2DM. In my studies, platelet hyperactivation in T2DM were shown by biochemical markers, platelet reactivity indices and higher amount of thrombus. Various factors could have contributed to platelet hyperactivation in T2DM. Hyperglycaemic state reduces the threshold of the platelets to various agonists. Inflammation plays a significant role in initiation and also sustains platelet activation. Qualitative analysis of the platelets from T2DM showed various stages of activation including pseudopodia formation and change of discoid shape of platelets. These findings were observed despite dual antiplatelet therapy in T2DM after NSTE-ACS. This could have occurred in conjunction with inadequate response and impaired pharmacokinetics of antiplatelet agents. It is possible that blood thrombogenicity

would have been different if I had prescribed a more powerful agent like ticagrelor or prasugrel. It can also be argued that higher doses of antiplatelet agents could have resulted in less PDT in this population.

Mediators of inflammation were associated with PDT in T2DM and more so in obese subjects. This may have favoured higher thrombogenesis and impaired autolysis of thrombus. In obese individuals, inflammation could have been the missing link between T2DM and the resultant prothrombotic state. The significance of inflammation in thrombogenicity is yet to be fully evaluated. My findings showed a direct association of inflammatory biomarkers to thrombus and also the failure of one week of additional antiplatelet therapy (e.g. clopidogrel) in stable CAD patients with T2DM to reduce the inflammation. Presence of "vulnerable blood" in T2DM as shown by higher levels of inflammation and hyperactive platelets could have played a significant role in pathogenesis of NSTE-ACS, especially in those with non flow limiting atheromatous plaques. These results support the view that a "biochemical mechanism" secondary to pro thrombotic mediators is associated with acute coronary syndrome in these patients. T2DM is associated with persistent low grade inflammation and my findings reveal that despite good control of diabetes the levels of inflammatory cytokines remained higher in T2DM compared to non DM. I have shown for the first time a significant association between whole blood thrombus created from experimental conditions of coronary blood flow and the markers of inflammation. Persistent inflammation may harbinger thrombotic events in T2DM. Body mass index and inflammation are strongly associated and my data showed higher thrombus burden and inflammatory markers in obese T2DM individuals. Inflammation not only promotes thrombus at local vascular endothelium by plaque rupture event but also interferes with various pathways of thrombogenesis and autolysis of the formed thrombus. In addition, by studying effluent blood ("post chamber") blood, I have shown that circulating inflammatory mediators may play a direct role in thrombus formation in T2DM. A causal link, if confirmed by large-scale experimental and observation studies, may offer us an opportunity to identify therapeutic targets such as individualised anti thrombotic and anti inflammatory therapy in T2DM patients with non flow limiting atheroma in

coronary arteries. The central and powerful role played by inflammation has to be proven by mechanistic studies in patients with T2DM. It is possible that in addition to risk factor modification, targeting inflammatory pathways in these high risk patients may reduce blood thrombogenicity. Powerful anti inflammatory agents (e.g. anti TNFα agents) remain untested in a large cohort of T2DM and their therapeutic potential may be substantial.

Significant differences in viscoelastic properties, supported by changes in thrombus architecture, could have resulted in greater PDT. Increased thrombus quantity seen in T2DM may be due to both higher blood thrombogenicity generating greater thrombus quantity and decreased thrombus degradation. I showed novel qualitative changes in the thrombus of patients with T2DM after NSTE-ACS. Findings of differential ultrastructural properties in diabetic thrombus may in part explain the differences in thrombus kinetics seen on TEG. Viscoelastic properties of thrombus are determined by ultrastructural characteristics at the level of the fibrin molecule and manifest changes in individual fibrin fibres, and their branching network. I have shown for the first time, both those changes in fibrin from whole blood thrombus of patients with T2DM. Thrombus in T2DM had thinner fibrin fibres, with more lateral aggregations, occurring in a disorganised, tangled or web-like fashion. This may favour increase in thrombus quantity by trapping more cellular elements, and in addition, may make clot retraction and degradation more difficult. Altered fibrin diameter and arrangement has been documented in patients with T2DM and CAD using plasma samples and reconstituted fibrinogen (Ajjan *et al.*, 2009). I have shown for the first time, changes in fibrin after clopidogrel therapy in whole blood samples. My study documented that in patients with T2DM and stable CAD, clopidogrel therapy has changed fibrin fibre to be more favourable to autolysis. Fibrin fibres directly contribute to viscoelastic properties of the thrombus. I have also shown novel findings of viscoelastic properties of diabetic thrombus after NSTE-ACS and these findings were backed up by my electron microscopic studies on fibrin architecture. Presence of loosely bound thrombus with lower viscoelasticity, generated in flow conditions similar to those in the coronary artery, would favour distal embolisation. Lower viscoelastic property of

diabetic thrombus results from thinner fibrin fibres and inability of the fibrin to undergo further stretching. These physical properties of the thrombus result in further entrapment of cellular elements and increase the potential of micro embolisation.

The presence of thinner fibrin fibres in T2DM may result in lower viscoelastic strength of diabetic thrombus and this could be one explanation for the differences seen on TEG. SEM findings could not only explain the changes in TEG but also can provide mechanisms for changes observed in quantitative measurement of whole blood PDT. Overall my findings showed that diabetic thrombus after NSTE-ACS was greater in quantity, less compactly organised with lower viscoelastic force and thus more likely to undergo embolisation. In addition, thrombus from T2DM could have persisted longer in the vasculature as it undergoes autolysis much more slowly. Thin and highly branched fibrin fibres seen in T2DM in my study could result in less permeability of the thrombus to fibrinolytic enzymes and render thrombus less susceptible to autolysis. Conversely, after clopidogrel therapy, thrombus was made of thick fibres that favour higher permeability of fibrinolytic enzymes and better autolysis. In addition, a less compact and loosely bound fibrin fibre arrangement is a marker of poor fibrinolysis. Presence of less longitudinal twisting in T2DM could be another explanation for lower viscoelastic strength of diabetic thrombus seen in TEG studies.

Quantification of fibrin architecture in whole blood thrombus has been reported for the first time. This was possible due to a novel application of modern software (Image ProPlus®, MA, USA) based on a standard fibre quantification model used by the international cotton industry. The protocol of quantification of SEM changes to thrombus has been rigorously evaluated with internal quality control. Semi quantitative assessment of the fibrin fibre arrangement in whole blood thrombus is novel and has shown favourable response of clopidogrel to fibrin architecture. Prospective studies with long term follow up are needed to establish if these fibrin fibre parameters can predict response to antiplatelet and risk factor modification in T2DM. Moreover, studies are required to investigate if these measurements can be used as a tool to assess the future risk of thrombotic

events and clinical outcomes in T2DM and the general population. Mechanisms behind the fibrin structure and function are yet to be fully understood. My novel method of assessment of fibrin in whole blood thrombus needs validation in different patient cohorts, and can potentially help the clinicians to develop strategies to modulate fibrin structure with novel pharmacological agents. The relationship between viscoelastic properties of fibrin fibres and their interactions with platelets and other cellular components of thrombus, shear force of blood flow, and resistance to autolysis need to be explored further in this high risk population.

Point of care platelet function tests namely VerifyNow®, thromboelastography with platelet mapping and Multiplate® aggregometry devices were evaluated in my study. The pathophysiology of thrombogenesis is complex and involves a significant proportion of non platelet mediators, especially in T2DM. Platelet reactivity indices from these assays did not account for non platelet mediators of thrombus and thus lacked correlation to thrombus in my cohort. My results underscore the point that results from current platelet function tests are very "platelet centric". These findings may also explain the lack of clinical benefits observed in large scale studies attempting to tailor antiplatelet therapy based on these assays. I have used three platelet function tests measuring different aspects of thrombosis namely, platelet aggregation (Verify Now® and Multiplate®) and platelet fibrin linkage (TEG®). These platelet function tests reflect different specific pathways in thrombosis. Thrombus measurement from the Badimon chamber is a composite measure of various pathways involved in coagulation and is likely to be a superior assessment of thrombogenicity than these individual platelet function tests. However, Badimon chamber study cannot be performed as a bed side test and will only remain as a research tool. A single bed side test reflecting all the combined pathways of coagulation has so far been unavailable. Researchers in this field acknowledge these limitations (Bonello et al., 2010). A hybrid platelet function test combining thrombus structure and quality along with genotyping from a single whole blood sample can overcome the pitfalls observed in currently available tests and will prove to be of more clinical benefits. The goal of personalised antiplatelet therapy at individual levels

helped by a bedside test still remains an idealistic strategy not yet proven to be practical.

It is possible that the effects of antiplatelet therapy go beyond direct effects on platelets. Pleiotropic effects of the antiplatelet agents are recently recognised in the form of improvement in vascular endothelial function (Bundhoo *et al.*, 2011)(and anti inflammatory activity (Antonino *et al.*, 2009). My study was not aimed to explore extra thrombotic activity of these agents and the clinical relevance of the reduction in thrombus quantity needs to be studied in long term follow up studies. It is possible that persistent inflammation in clopidogrel treated individuals plays a significant role in future thrombotic event in patients with T2DM irrespective of platelet reactivity indices. One week therapy with clopidogrel did not reduce the levels of inflammatory cytokines in T2DM with stable CAD. Despite reduction in platelet function test indices, persistent inflammation could result in future thrombotic events and thus my findings may also explain the lack of clinical benefits observed in large scale studies attempting to tailor antiplatelet therapy based on these assays. I suggest caution should be exercised when using these values from point of care platelet function studies to tailor individualised therapy. My findings reiterate the concerns expressed in the current guidelines which suggest platelet function assays like VerifyNow® should remain as a research tool and are not to be used for routine clinical practice (Bonello *et al.*, 2010).

5.2 Limitations

My study was a single centre study and I studied a small cohort of 170 patients only. Though the patients were carefully selected to ensure 100% adherence to secondary prevention, these patients may not represent real world population. My study subjects were predominantly Caucasians (168 out of 170) and thus the results may not be generalised to the global diabetic population. I used clopidogrel as my study drug which is still the most widely used antiplatelet agent next to aspirin in patients with T2DM and CAD. However, clopidogrel is inferior to newer antiplatelet agents like prasugrel and ticagrelor, both of which were unlicensed at the time of the study approval. My studies were approved by the authorities in 2008. Prasugrel and ticagrelor were licensed in UK in 2009 and

2010 respectively. The study population had a week of clopidogrel therapy and effects of longer duration of therapy were not addressed. Serum and plasma assays were used in studying various biomarkers and I did not use the more accurate but time consuming flow cytometric methods. Finally, I did not record long term clinical outcomes of my participants.

5.3 Future directions

My results suggest that in addition to current secondary prevention strategies, novel approaches to reduce recurrent thrombotic events in T2DM are essential.

In patients with NSTE-ACS, newer and more potent antiplatelet agents have shown further reduction in cardiac events in T2DM but the outcomes still remained poorer than in the non-diabetic population. However the dosages used in T2DM are similar to non DM (e.g. prasugrel 10 mg and ticagrelor 90 mg bd). Findings from my ACS study showed that prescribing similar antiplatelet dosage regimes to T2DM resulted in greater thrombus. It remains to be seen if higher dosage of these agents will reduce thrombus in T2DM to levels similar to those without DM, with subsequent reduction in clinical events. Differential dosing regimes in T2DM has not been studied in large patient cohorts. This concept has been tested in my second study (patients with stable CAD), where dual antiplatelet therapy with aspirin and clopidogrel in T2DM with stable CAD reduced thrombus burden.

Personalising antiplatelet therapy has limited success at individual patient's level, but large patient cohort trials are required. Different cardiovascular risk modification regimes (e.g. lipid and blood pressure controls) for T2DM reduce cardiac events and it is possible similar success can also be achieved in antithrombotic therapy. It is logical and biologically plausible that the group of individuals with higher blood thrombogenicity (vulnerable blood) would benefit the most from aggressive pharmacotherapy. If carefully controlled studies could prove this concept in T2DM, changes to the guidelines issued by international cardiac societies on antithrombotic prescription to T2DM will become necessary.

I have shown that thrombus lysis is impaired in T2DM after NSTE-ACS and may have resulted from differential fibrin fibre arrangement. Pharmacotherapy to promote thrombus lysis is not recommended in patients outside the setting of ST elevation MI and with the widespread implementation of primary angioplasty services, thrombolytic agents are used less frequently. I have shown differential fibrin fibre arrangement in T2DM and changes in fibrin fibre ultrastructure with antiplatelet therapy are associated with reduction of thrombus in patients with T2DM. Identification and use of novel pharmacotherapy to modify these less explored pathways of coagulation in T2DM may result in better outcomes in T2DM.

A change in platelet content of thrombus was associated with reduction in thrombus quantity in T2DM. Clopidogrel which indirectly alters platelet content of the thrombus reduced thrombus quantity. The results are likely to be more pronounced with novel pharmacological agents which reduce platelet count. Rafigrelide is a newer analogue of anagrelide which suppresses platelet synthesis and results in lower platelet count. Our group has shown in a phase one study with rafigrelide that reduction in platelet count is strongly associated with reduction in thrombus quantity (In Press). It is possible that modification of platelet synthesis and reduction of platelet count to optimal levels in T2DM may reduce thrombogenicity and improve cardiac outcomes.

My thesis has made a small but a significant contribution to the understanding of complex pathophysiology of thrombosis in T2DM. My data has highlighted some of the unique features seen in this high risk population with regard to blood thrombogenicity. My findings hold the promise of development of future strategies beyond conventional antiplatelet and anticoagulant medications to improve blood thrombogenicity and thereby improve their clinical outcomes in T2DM.

I propose these following studies to carry forward my findings from this thesis:

i. Multicentre study in patients with and without T2DM who had CAD, to evaluate platelet dependent thrombogenicity using newer antiplatelet agents like prasugrel and ticagrelor will be helpful to assess the benefits of these agents over clopidogrel.

ii. A study designed to assess the long term benefits of dual antiplatelet therapy in patients with stable CAD and T2DM is needed for the international societies to issue guidelines on antiplatelet therapy specifically for T2DM with stable CAD.

iii. Interventional studies are needed to explore the role of novel pharmacotherapy to improve blood thrombogenicity in T2DM. Anti inflammatory agents, fibrin specific antagonists, agents that suppress synthesis of platelets and direct thrombin inhibitors in the ex-vivo chamber should all be studied.

iv. A prospective study on the role of blood thrombogenicity in atheroma progression and future cardiac events in T2DM with and without overt CAD will be helpful in understanding the role of “vulnerable blood” in these high risk individuals.

v. An interventional study primarily designed to assess the role of pharmacological and lifestyle interventions in improving ultrastructure characteristics and mechanical properties of whole blood thrombus in T2DM would expand our knowledge of the thrombus kinetics in this high risk population.

Appendices

Appendix 1: Prizes and Awards

Diabetes UK

Diabetes UK Type 2 Diabetes Research Award

This certificate is presented to

Girish N Viswanathan, Azfar G Zaman, Sally M Marshall

For outstanding contribution to the advancement of the understanding of Type 2 diabetes and its treatment on Wednesday 30 March 2011

at the

Diabetes UK Annual Professional Conference 2011
International Convention Centre
(ICC) London ExCeL

Baroness Young
Chief Executive

Professor Sir George Alberti
Chair

The charity for people with diabetes
A charity registered in England and Wales (no. 2...5199) and in Scotland (no. SC039136)

1. Diabetes UK type 2 diabetes research award, London, March 2011.

2. Short listed for Raftery award in Cardiology, Royal society of Medicine, London, February 2011.

3. Best researcher award, Welsh Deanery Cardiology Trainees' meeting, Clwb Calon Cymru, Cardiff, November 2011.

Appendix 2: Publications

A. Peer reviewed original research articles

1. **Viswanathan GN**, Marshall SM, Schechter CB, Balasubramaniam K, Badimon JJ, Zaman AG. Thrombus and antiplatelet therapy in type 2 diabetes mellitus: a prospective study after non-ST elevation acute coronary syndrome and a randomised, blinded, placebo controlled study in stable angina. Thrombosis and Haemostasis. 2012;108(5): 937-945
2. **Viswanathan GN**, Zaman AG. Cardiovascular disease in patients with type 2 diabetes mellitus: vulnerable plaques and vulnerable blood Clinical Medicine. 2012 (Suppl 6), s51-55.
3. **Viswanathan GN**, Marshall SM, Balasubramaniam K, Badimon JJ, Zaman AG. Differences in thrombus structure and kinetics in patients with type 2 diabetes mellitus after non ST elevation acute coronary syndrome, Thrombosis Research, TR-D-13-00687, In Press.

B. Letters and abstracts

1. **Viswanathan G** Book review: Diabetic Cardiology, Diabetic Medicine, 2009:26, 751.
2. **Viswanathan GN,** White K, Balasubramaniam K, Badimon J, Marshall SM, Zaman AG; Clopidogrel alters thrombus quantity and quality in patients with type 2 diabetes mellitus and stable coronary artery disease, J Am Coll Cardiol. 2013;61(10_S):doi:10.1016/S0735-1097(13),61154-7.
3. **Viswanathan GN,** Balasubramaniam K, Badimon J, Marshall SM, Zaman AG; Evaluation of Whole blood thrombus and platelet function tests in patients with type 2 diabetes mellitus and stable coronary artery disease, J Am Coll Cardiol. 2013;61(10_S):doi:10.1016/S0735-1097(13),61149-3.
4. **Viswanathan GN** , Balasubramaniam K, Harper AG, Bawamia B, Marshall SM, Badimon JJ, Zaman AG; *Thrombus architecture and viscoelastic property in patients with type 2 diabetes mellitus following non ST elevation acute coronary syndrome*, Eur Heart Journal 2012;33(s1):309.
5. **Viswanathan GN**, Natarajan A, Marshall SM, Badimon JJ, Zaman AG; Higher Thrombus Burden and Impaired Clot Kinetics in Patients with Type 2 Diabetes Mellitus following Non ST-Elevation Acute Coronary Syndrome, Circulation. 2010; 122: A17675.
6. **Viswanathan GN**, Nelson AJ, Thompson L, Marshall SM, Zaman AG; Thrombus burden and response to clopidogrel in patients with type 2 diabetes mellitus presenting with non ST elevation acute coronary syndrome. Eur Heart Journal 2010;131(s1):967.
7. **Viswanathan G**N, Harper A, Balasubramaniam K, Badimon JJ, Marshall SM, Zaman AG; Blood thrombogenicity is inversely related to coronary lesion severity in patients with non ST-elevation acute coronary syndrome and type 2 diabetes mellitus. Heart 2012;98:A63-A64 doi:10.1136/heartjnl-2012-301877b.113
8. **Viswanathan GN**, Marshall SM, Zaman AG; Evaluation of Thrombus in Patients with Type 2 Diabetes Mellitus After Non-ST-Elevation Acute

Coronary Syndrome (NSTE-ACS): Increased Amount and Altered Strength Atheroscl Throm Vasc Biol 2010;30(11):209

9. **Viswanathan GN**, Jardine J, Bhardwaj M, Adams P, Marshall S, Zaman A; Better clinical outcomes of participants in clinical research: Trial effect of an observational study in patients with non ST elevation acute coronary syndrome (NSTE-ACS). Eur Heart Journal 2010; 131(s1):233-234.

C. Under review

1. Blood thrombogenicity is increased in patients with subclinical hypothyroidism post non ST elevation acute coronary syndrome, Journal of American Society of Endocrinology

Appendix 3: Presentations

1. *Clopidogrel alters thrombus quantity and quality in patients with type 2 diabetes mellitus and stable coronary artery disease*. Annual Conference of American College of Cardiologists, San Francisco, March 2013.
2. *Evaluation of Whole blood thrombus and platelet function tests in patients with type 2 diabetes mellitus and stable coronary artery disease*, Annual Conference of American College of Cardiologists, San Francisco, March 2013.
3. *Thrombus architecture and viscoelastic property in patients with type 2 diabetes mellitus following non ST elevation acute coronary syndrome*, European Society of Cardiology, annual conference, Munich, August 2012.
4. *Blood thrombogenicity in patients with non obstructive coronary artery disease resulting in non ST elevation acute coronary syndrome*, British Cardiac Society, Manchester, UK May 2012.
5. *Thrombus burden, clot kinetics and response to anti-platelet therapy in Type 2 diabetes*. Annual conference, Diabetes UK 2011, London, March 2011 *(won* Diabetes UK Type 2 Diabetes Research Award).
6. *Survival and quality of life 25 years after coronary artery bypass graft surgery*, European Society of Cardiology annual conference Paris, August 2011.
7. *Thrombus burden and impaired clot kinetics despite dual antiplatelet therapy*, Medical Research Society, shortlisted for Raftery award, Royal College of Physicians, London, February 2011.
8. *Higher thrombus burden and impaired clot kinetics in patients with type 2 diabetes mellitus following non ST-elevation acute coronary syndrome (NSTE-ACS*), American Heart Association Scientific Sessions, Chicago, IL, November 2010.
9. *Evaluation of thrombus in patients with Type 2 diabetes mellitus after Non ST Elevation Acute Coronary Syndrome (NSTE-ACS): Increased Amount and Altered Strength*, Atherosclerosis Thrombosis and Vascular Biology society, San Francisco, CA, April 2010.

10. *Better clinical outcomes of participants in clinical research: 'Trial effect' of an observational study in patients with non-ST elevation – acute coronary syndrome (NSTE-ACS),* European Society for Cardiology Meeting, Stockholm, Sweden, September 2010..
11. *Thrombus burden and response to clopidogrel in patients with type 2 diabetes mellitus presenting with non ST elevation acute coronary syndrome*, European Society for Cardiology Meeting, Stockholm, Sweden, September 2010.
12. *Thrombus and inflammation in obese patients with type 2 diabetes mellitus following non ST-elevation acute coronary syndrome*, Heart Vessel Diabetes Meeting, Lisbon, Portugal, December 2010.
13. *Inflammation and thrombogenicity in obese type 2 diabetes mellitus patients after Non ST-Elevation acute coronary syndrome*, Northern Cardiac conference, Hull, April 2011.
14. *Diabetic Nephropathy: A protective factor against malignancy*? North east Postgraduate Conference, Newcastle upon Tyne, October 2009..

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