Fibrin Clot Properties and Clinical Outcomes in Patients with Acute Coronary Syndrome: An additional therapeutic target?

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ABSTRACT

Acute coronary syndrome (ACS) results from thrombotic occlusion of an atherosclerotic coronary artery. Platelets are pivotal in arterial thrombosis and, as a result, preventative therapy has focussed on antiplatelet drugs. Currently, dual antiplatelet therapy with aspirin and a $P2Y_{12}$ antagonist is the recommended treatment following ACS. New-generation $P2Y_{12}$ inhibitors (ticagrelor and prasugrel) offer potent and consistent antithrombotic effect. Despite this, recurrent events remain common. Mechanisms driving those events are not entirely clear.

The protein arm of coagulation also plays an important role in arterial thrombosis, leading to fibrin formation and stabilisation of thrombus. Although the cellular arm of coagulation may be optimally inhibited with contemporary therapy, the protein arm remains largely unaffected. Adverse fibrin clot dynamics have been associated with vascular conditions and with conditions closely related to worse outcome such as diabetes mellitus (DM). It is, therefore, plausible that adverse fibrin-rich clots that resist lysis are responsible for some of the recurrent events.

In my PhD, I have studied fibrin clot properties in 4,354 plasma samples, collected at hospital discharge, and 4,032 plasma samples, collected at 1-month, from ACS patients in the PLATelet inhibition and patient Outcome (PLATO) trial. I found that adverse fibrin clots that resisted lysis at hospital discharge independently predicted the combined outcome of cardiovascular (CV) death and spontaneous myocardial infarction with no association with PLATO-defined major bleeding events.

Following resolution of inflammation at 1-month, fibrin clot density (determined by maximum turbidity) was attenuated but fibrin clot lysis potential remained relatively stable, showing consistent relationships with high-risk conditions such as DM, peripheral artery disease and chronic kidney disease.

Moreover, increased fibrin density and lysis inefficiency strongly correlated with inflammatory and prognostic biomarkers (leukocyte count, high-sensitivity C-reactive protein, high-sensitivity troponin T, cystatin C, N-terminal pro B-type natriuretic peptide and growth differentiation factor-15) giving us mechanistic insights into worse outcomes. With the cellular arm effectively targeted by contemporary dual antiplatelet therapy, fibrin lysis inefficiency constitutes a therapeutic target to mitigate the residual risk post ACS.

I have also studied the ability of different anticoagulants to modulate fibrin clot properties as a novel approach to establish treatment effects. Fibrin clot lag time appeared sensitive to very low concentrations of rivaroxaban, apixaban and fondaparinux and high concentrations promoted fibrinolysis. Therefore, fibrin clot lag time seems promising as a novel assay to monitor treatment but *ex-vivo* studies are needed to confirm these findings.

In an attempt to optimise antithrombotic therapy in ACS patients, I have investigated enoxaparin infusion in ST-elevation myocardial infarction (STEMI) patients undergoing primary angioplasty as a novel approach to mitigate acute thrombotic risk. Absorption of oral $P2Y_{12}$ inhibitors is delayed in opiate-treated STEMI patients and this may constitute a risk for acute stent thrombosis. In 20 patients undergoing primary angioplasty, a novel regimen consisting of a bolus of enoxaparin 0.75 mg/kg followed by an infusion of 0.75 mg/kg/6 hours offered sustained anti-Xa levels and improved fibrin clot lysis potential throughout the infusion. No patient suffered a thrombotic complication despite a high prevalence of poor $P2Y_{12}$ inhibition in opiate-treated patients. This study offers preliminary pilot data to support the hypothesis that this regimen would be sufficient to circumvent the delayed onset of action of oral $P2Y_{12}$ inhibitors.

DEDICATION

I would like to dedicate this thesis to my parents. From a very young age, they have inspired me by their dedication to science and education. Throughout my education and career, they have been there to offer all the support they could possibly offer.

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ABBREVIATIONS

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AF	Atrial fibrillation
CAD	Coronary artery disease
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcomes Study
CKD	Chronic kidney disease
COX	Cyclooxygenase
CRP	C reactive protein
CV	Cardiovascular
DAPT	Dual antiplatelet therapy
DES	Drug eluting stents
DM	Diabetes mellitus
ENT	Equilibrative nucleoside transporter
GDF-15	Growth differentiation factor 15
GP	Glycoprotein
GPI	Glycoprotein IIb/IIIa inhibitor
HsTnT	high-sensitivity troponin T
IL1ra	Interleukin 1 receptor antagonist
IL	Interleukin
IFNγ	Interferon γ
KM	Kaplan-Meier
LMWH	Low-molecular-weight heparins
MACE	Major adverse cardiovascular events

MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MI	Myocardial infarction
MMP	Metalloproteinase
NET	Neutrophil extracellular traps
NO	Nitric oxide
NOACS	Non-vitamin K antagonist oral anticoagulants
NSTE-ACS	Non-ST-elevation acute coronary syndrome
NT-proBNP	N-terminal pro B-type natriuretic peptide
PAR	Protease-activated receptors
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PLATO	PLATelet inhibition and patient Outcome
PMA	Platelet-monocyte aggregates
PAD	Peripheral artery disease
RCT	Randomised clinical trial
ROS	Reactive oxygen species
SE	Side effects
SMC	Smooth muscle cell
STEMI	ST-elevation myocardial infarction
TAFI	Thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor-α
tPA	Tissue plasminogen activator
TXA_2	Thromboxane A_2

UFH	Unfractionated heparin
VCAM	Vascular cell adhesion molecules
VKA	Vitamin K antagonist
vWF	Von Willebrand factor
WCC	White cell count

1 Acute coronary syndromes: pathophysiology and optimising antithrombotic therapy

Publications resulting from this chapter:

1) Sumaya W, Storey RF. The challenges of antithrombotic therapy in patients with left ventricular thrombosis. Eur Heart J. 2018;39:209-211.

2) Sumaya W, Storey RF. Dual antiplatelet therapy following acute coronary syndromes: optimal regimens and duration of therapy. Br J Cardiol. 2017;24(suppl 1):S10-S15.
3) Sumaya W, Storey RF. Ticagrelor: Effects Beyond the $P2Y_{12}$ Receptor. Interv Cardiol Clin. 2017;6(1):49-55.

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1.1 Abstract

In the 1980s, we learnt that thrombosis in atherosclerotic coronary arteries is the usual cause of acute coronary syndrome (ACS). Since then, our understanding of the disease process has evolved, leading to the development of effective antithrombotic treatments. This has revolutionised therapy, facilitating revascularisation with percutaneous coronary intervention (PCI) and greatly improving clinical outcomes. However, despite this revolution, approximately 20% die or suffer recurrent events in the first year following ACS. The downside to antithrombotic treatment is the increased risk of bleeding and striking the right balance between ischaemia and bleeding is challenging. Detailed understanding of the pathophysiology is key to tackling this issue. Markers of the disease process could enhance our understanding and may guide treatment. In this chapter, I will consider the pathophysiology of ACS, currently available antithrombotic treatments and how markers of increased thrombotic risk could guide treatment in the future.

1.2 Background

Cardiovascular disease remains amongst the biggest killers in the world with over 30% of global deaths secondary to cardiovascular disease and approximately 7.4 million

deaths attributable to coronary artery disease (CAD) (1). Acute presentation of CAD in the form of ACS is usually caused by thrombus formation in an atherosclerotic coronary artery, secondary to a complex process that involves activation of both the cellular and protein arms of coagulation. Antithrombotic treatment has a key role in the management of ACS and consists of anticoagulants and antiplatelet therapy in the acute setting with preventative antiplatelet therapy continued in the long term (2, 3).

Despite contemporary medical treatment, ACS continues to carry significant mortality and morbidity, with a large proportion of patients experiencing recurrent events (4). In recent randomised controlled trials (RCTs) using potent antiplatelet regimens, approximately 10% of patients died or had a cardiovascular event within one year of ACS (5, 6) with even a higher rate of adverse clinical outcomes in real-life settings and outside RCT conditions (7, 8).

One option to improve outcomes is the introduction of other potent antithrombotic treatments and low-dose of rivaroxaban, a direct factor Xa inhibitor, has shown promising results but the increased risk of bleeding has caused concern and limited widespread use (9).

One caveat to antithrombotic therapy is the desire to have “one therapy for all”, when such therapy may have differing effects in various individuals. The challenge is to choose the right patient cohort in whom escalated treatment is necessary. Understanding the disease process is essential to help us identify those individuals. Certain disease markers could also help risk stratify patients. In this section, I will review the pathophysiology of ACS, currently available treatment options and some of the thrombotic and inflammatory markers of worse outcome.

1.3 Pathophysiology

1.3.1 Atherosclerosis

Atherosclerosis is a complex pathological process where endothelial dysfunction, inflammation and thrombosis play inter-related roles (10).

Though a single layer of cells forming the inner lining of blood vessels, the endothelium plays many important roles including making a barrier, maintaining vascular tone, and regulating thrombosis and inflammation. Endothelial cells produce nitric oxide (NO) and prostacyclin (prostaglandin-I_2: PG-I_2). Both inhibit platelets and lead to vasodilation. (11-14). Traditional CAD risk factors, including hypertension, smoking, hyperlipidaemia and diabetes, have all been associated with endothelial dysfunction (15-19).

Cytokines like tumour necrosis factor-α (TNFα) and interferon–γ (IFNγ) can also influence endothelial function. They change the distribution of endothelial cadherin-catenin complexes and increase intra-cellular calcium levels, thereby activating myosin light chain kinase (20, 21). This facilitates leukocyte migration to the intima. Circulating leukocytes (mainly monocytes and some lymphocytes) bind to vascular cell adhesion molecules expressed on activated endothelial cells and then migrate to the intima (22). Chemokines such as monocyte chemoattractant protein–1 (MCP-1) enhance leukocyte migration by activating G-coupled protein receptors (23). Circulating cholesterol in the form of low-density lipoprotein migrates to the intima where it is oxidized (24). In the intima, complex inflammatory events take place mediated by different cytokines (Figure 1-1). Macrophage colony-stimulating factor (M-CSF) helps monocytes mature into macrophages (25). Macrophages engulf oxidized LDL through scavenger receptors to form foam cells. Platelet-derived growth factor (PDGF) and other chemokines stimulate smooth muscle cell (SMC) migration from the tunica media to the intima. SMCs express tissue factor (TF). Interleukin (IL)1, TNFα and IFNγ further amplify inflammatory

responses and promote apoptosis of foam cells and SMCs. They also have an impact on endothelial cells, inducing production of pro-coagulant factors and inhibiting release of antithrombotic factors (26). As plaques progress further, debris from apoptotic SMCs and foam cells form the necrotic core of the plaques. Macrophages also produce TF and metalloproteinases (MMPs). MMPs degrade collagen, leading to thinning of the fibrous cap. (Figure 1-1)

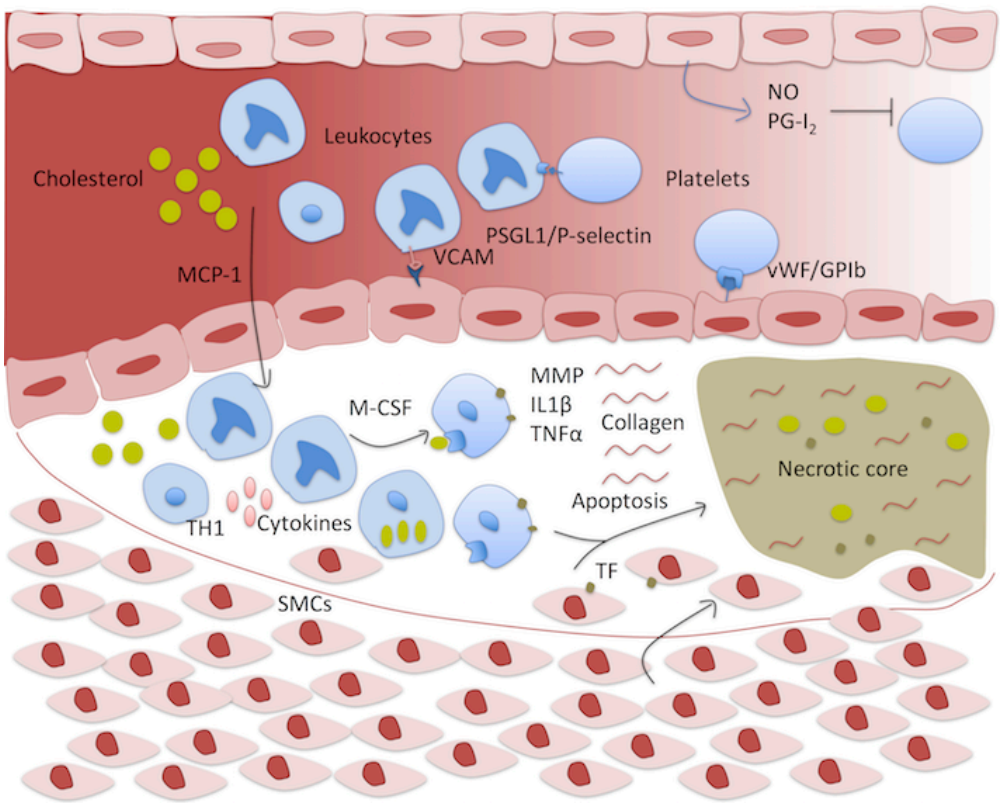


Figure 1-1: Schematic drawing illustrating the pathophysiology of atherosclerosis

Under normal circumstances, the endothelium (top) forms a barrier and secretes nitric oxide (NO) and prostaglandin-I_2 (PG-I_2) and these inhibit platelets. Cytokines interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα) contribute to the activation of endothelial cells (ECs). Activated ECs express vascular cell adhesion molecules (VCAMs) and these bind to circulating leukocytes. Leukocytes then migrate to the intima. Monocyte chemoattractant protein-1 (MCP-1) enhances leukocyte migration. LDL migrates to intima where it is oxidised. Monocytes mature into macrophages through the action of macrophage colony stimulating factor (M-CSF). Macrophages express scavenger receptors, taking in oxidised LDL to form foam cells. They also express the pro-coagulant molecule tissue factor (TF). Monocytes and macrophages produce multiple cytokines, in particular interleukin-1 (IL1) and TNFα, and these have direct effect on endothelial function, induce apoptosis and promote T helper 1 cell responses (TH1). The few TH1 cells produce IFNγ and TNFα, both of which influence ECs and amplify the inflammatory response. Smooth muscle cells (SMCs) migrate from the intermediate layer to the intima in response to chemokines such as platelet-derived growth factor (PDGF). They too express TF and produce collagen which forms the fibrous cap. Macrophages produce metalloproteinases (MMPs). MMPs remodel collagen structure leading to thin fibrous caps. Debris from apoptotic cells (SMCs and foam cells) accumulates to form the necrotic core in atherosclerotic plaques.

1.3.2 Arterial thrombosis

Platelets assume the driving seat when it comes to arterial thrombosis. Subsequent to plaque rupture, they adhere to exposed sub-endothelial agonists, such as collagen and von Willebrand factor (vWF) through platelet glycoprotein (GP) VI and Ib receptors, respectively, in order to produce an initial platelet plug. Integrin $\alpha 2\beta 1$ also plays a role mediating platelet adhesion to collagen (27). The sub-endothelial ligands activate platelets, which change in shape, express multiple receptors and secrete soluble agonists including thromboxane A_2 (TXA_2) and adenosine diphosphate (ADP). TXA_2 activates platelets further and ADP amplifies and sustains platelet activation predominantly through platelet $P2Y_{12}$ receptors, leading to further expansion of the platelet-rich thrombus (Figure 1-2) (28). In parallel to platelet activation, exposed TF binds to activated factor VII and this activates factors IX and X. Activated factor X cleaves prothrombin to form thrombin (Figure 1-2). Thrombin has multiple actions including: 1) further activation of platelets through its action on platelet protease-activated receptors (PAR1/PAR4); 2) activation of the coagulation system resulting in the conversion of soluble fibrinogen into insoluble fibrin network (28-32). Thrombin can also be produced on the surface of activated platelets through phospholipid scramblase activity (33). However, pre-clinical data suggest that thrombin may play a greater role in stabilisation of the core of arterial thrombus rather than its expansion (34).

Additionally, activated platelets lead to further recruitment of inflammatory cells through release of chemokines and other cytokines from their α-granules (28, 35, 36). Activated platelets cross-link by binding with formed fibrin through platelet integrin receptor $\alpha_{IIb}\beta_3$, otherwise known as GPIIb/IIIa.

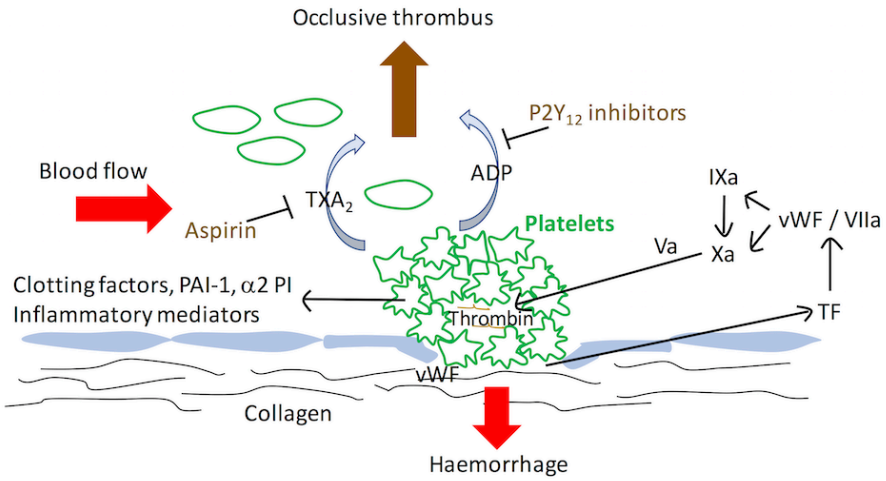


Figure 1-2: Schematic drawing of arterial thrombosis

Following plaque rupture, platelets adhere to von Willebrand factor (vWF) and collagen. Activated platelets undergo shape change, express multiple receptors, release adenosine diphosphate (ADP) from dense granules and produce thromboxane A_2 (TXA_2). ADP amplifies platelet activation and together with TXA_2 lead to further expansion of the platelet plug. Thrombin is also produced but primarily stabilises the core thrombus. Multiple inflammatory mediators and clotting factors are also released from platelet α-granules. Serpins that limit fibrinolysis including plasminogen activation inhibitor-1 (PAI-1) (37), α2 plasmin inhibitor (α2 PI) (38) and protease nexine 1 (39) (not shown) are also secreted. Platelet derived PAI-1 accounts for a great proportion of plasma PAI-1 (37); 'a' denotes activated clotting factor.

1.3.3 Regulatory mechanisms

Despite the systemic nature of atherosclerosis risk factors, atherosclerotic plaques in the coronary arterial tree tend to be localized, at least at early stages of the disease. This is likely to be secondary to local variation in haemodynamic factors and shear stress levels; in areas of steady laminar flow, vasoprotective genes are up-regulated in contrast to areas exposed to turbulent flow (40). Anti-inflammatory cytokines include IL10, IL33, IL35, IL1 receptor antagonist (IL1ra) and transforming growth factor β (TGFβ) (26).

Intrinsic mechanisms to control thrombosis include protein C, thrombomodulin, heparin/antithrombin, tissue factor pathway inhibitor (TFPI) and tissue plasminogen activator (tPA) (Figure 1-3). Protein C inhibits activated factors V and VIII. TFPI inhibits

TF and factor VII dependent pathway. Heparin inhibits thrombin and other activated factors (IX, X, XI). Thrombomodulin redirects thrombin activity to activate protein C and thrombin-activatable fibrinolysis inhibitor (TAFI). Plasminogen is cleaved by tPA to produce plasmin and this degrades fibrin, initiating fibrinolysis (32). Plasmin acts on the carboxy termini in fibrin, cleaving the αC domain and subsequently cleaving more fragments moving towards the E domain (Figure 1-4) (41). This produces fibrin degradation products (42) and D-dimer is the most commonly used as a biomarker. D-dimer also interacts with monocytes promoting IL6 production inducing hepatocytes' production of both fibrinogen and C-reactive protein (CRP) (43).

Plasminogen activation inhibitor-1 (PAI-1) is the main enzyme limiting tPA's activity. α2-antiplasmin forms a complex with plasmin and limits lysis activity. TAFI breaks the positive feedback loop between fibrin and plasmin by cleaving off lysine residues in the C terminal of fibrin (44).

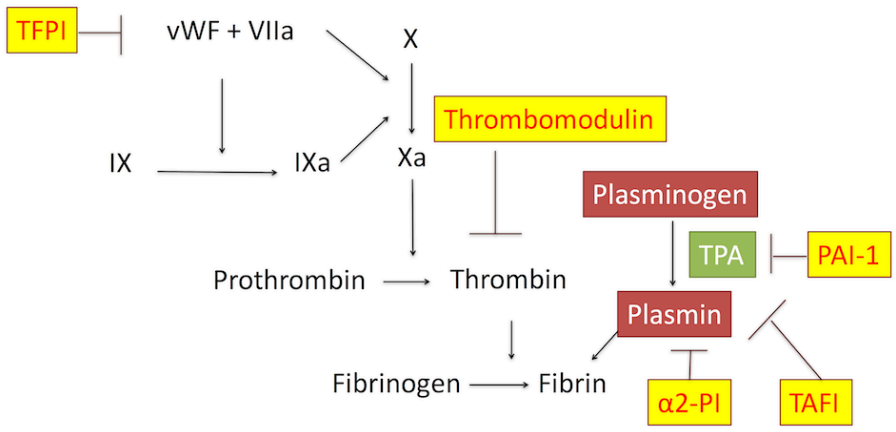


Figure 1-3: Fibrin clot formation, lysis and antifibrinolytic pathways

Tissue factor pathway inhibitor (TFPI) inhibits the pathway activated by factor VII and von Willebrand factor (vWF). Tissue plasminogen activator (tPA) initiates internal fibrinolysis by cleaving plasminogen to produce plasmin. Thrombomodulin redirects thrombin activity to activate protein C (not shown) and thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI inhibits plasmin and its production. α2 plasmin inhibitor (α2-PI) inhibits plasmin and plasminogen activator inhibitor-1 (PAI-1) inhibits plasminogen conversion by inhibiting the action of tPA.

1.4 Diagnosis

The diagnosis of ACS has three elements: clinical assessment, electrocardiograph (ECG) assessment and measurement of markers of myocardial necrosis (primarily troponin). Patients usually present with ischaemic chest pain at rest or minimal exertion. Once ACS is suspected, the ECG is indispensable. ST-elevation myocardial infarction (STEMI) is usually diagnosed by persistent ST-segment elevation (≥ 1 mV) in two contiguous leads. In the absence of persistent ST-segment elevation, certain ECG changes (transient ST-segment elevation, ST-segment depression, T wave inversion) could indicate a diagnosis of non-ST-elevation ACS (NSTE-ACS). NSTE-ACS can be classified into unstable angina or non-ST-elevation myocardial infarction (NSTEMI) on the basis of troponin measurements (45).

1.5 Preventative antithrombotic treatment

It was the 1980s when the paradigm shift in our understanding of ACS took place. The hypothesis of coronary artery spasm leading to ACS was predominant when Dr. Michael

Davies confirmed, for the first time, the presence of thrombotic lesions in the coronary arteries of those who died of ACS (46). This was the milestone that prompted a body of research to focus on antithrombotic treatment. In this section, I will review currently available treatment.

1.5.1 Antiplatelet therapy

Aspirin

Aspirin (acetylsalicylic acid) irreversibly acetylates platelet cyclooxygenase (COX) -1, blocking its activity. COX-1 converts arachidonic acid to PGH_2/G_2 and these form substrates for thromboxane synthase, leading to the production of TXA_2. TXA_2 is a potent vasoconstrictor and also activates platelets through thromboxane prostanoid receptors (47). Aspirin indirectly exerts paradoxical effects on platelets at higher doses; as well as inhibiting platelet COX-1 enzyme, it also acetylates endothelial cell COX-2, thereby inhibiting $PG\text{-}I_2$ production (48) and reducing platelet inhibition through the $PG\text{-}I_2$ pathway.

Evidence of efficacy of aspirin emerged in 1983 when a significant 51% risk reduction of recurrent ACS or death was demonstrated in unstable angina patients receiving aspirin as compared to placebo (49). These results were further echoed in several other studies (50, 51) and, in patients with STE-ACS, aspirin reduced vascular death significantly by 23% (52).

A wide range of doses of aspirin has been used but the Antithrombotic Trialists' Collaboration completed a meta-analysis of randomised trials and concluded that low maintenance doses of 75 to 150 mg are as effective as higher doses but higher doses were needed for loading in the hyper-acute setting (53).

The OASIS–7 trial compared lower maintenance dose aspirin (75 – 100 mg) to higher doses (300 – 325 mg) and found no difference in clinical efficacy or safety (at 30 days) when combined with clopidogrel in ACS patients referred for invasive treatment (54).

Gastrointestinal bleeding was found to be marginally higher in the higher-dose aspirin group (0.4% vs. 0.2%, p = 0.04).

Although aspirin's pharmacodynamic effects are evident with "extra low" doses (20-40 mg daily) (55, 56), clinical efficacy at those levels has not been assessed.

$P2Y_{12}$ receptor antagonists

Ticlopidine is a 1st generation thienopyridine. Its active metabolite (thiolactone) irreversibly blocks platelet $P2Y_{12}$ receptors (57). Ticlopidine showed promising results in reducing ischaemic events following PCI, especially in ACS patients, and dual antiplatelet therapy (DAPT) was first tested with ticlopidine (58-61). Its success, however, was hampered by a profile of side effects including thrombotic thrombocytopenic purpura, which carries a high mortality (62).

Clopidogrel is a prodrug that also belongs to the thienopyridine family. Its active metabolite irreversibly inhibits platelet $P2Y_{12}$ receptors. Clopidogrel metabolism is complex, requiring conversion to the active metabolite by the cytochrome P450 (CYP) enzymes in the liver, and the majority (85%) of clopidogrel is catabolised to inactive metabolites by plasma carboxylesterases (63). This is largely responsible for the wide inter-individual variability in platelet reactivity during treatment with clopidogrel and "poor response" to clopidogrel has been shown to increase ischaemic risk (64).

In NSTE-ACS patients, clopidogrel in addition to aspirin was tested in the CURE study. Clopidogrel, for 3 to 12 months post NSTE-ACS, reduced major adverse cardiovascular events (MACE) compared to placebo (9.3% vs. 11.4%, p < 0.001). Despite a trend towards better cardiovascular mortality with clopidogrel, MACE reduction was primarily driven by a reduction in non-fatal myocardial infarction (MI). There was no significant difference in terms of life-threatening or intracranial bleeds but clopidogrel resulted in a significant increase in major bleeding events (3.7% vs 2.7%, p = 0.001) (65). Patients received clopidogrel at randomisation (within 24 hours of presentation, pre-PCI) and

those requiring PCI in the placebo arm received open-label clopidogrel or ticlopidine for ~ 4 weeks post PCI. Patients pre-treated with clopidogrel had a 31% reduction in the risk of MI or cardiovascular death ($p = 0.002$) (66).

Clopidogrel is the only $P2Y_{12}$ inhibitor tested in conjunction with aspirin and thrombolysis in STEMI patients. The addition of clopidogrel offered a significant 36% risk reduction in occlusion of the infarct-related artery, recurrent MI or death (67). In medically-treated STE-ACS patients, clopidogrel in addition to aspirin reduced MACE by 9% ($p = 0.002$) (68).

Prasugrel is another thienopyridine prodrug. Its active metabolite is chemically similar to that of clopidogrel and also inhibits the $P2Y_{12}$ receptor irreversibly. It provides more rapid and potent platelet inhibition as compared to clopidogrel due to more efficient active metabolite formation, starting with intestinal esterase followed by a one-step conversion in the liver by multiple CYP enzymes (69). Prasugrel, compared to clopidogrel, reduced MACE (9.9% vs. 12.1%, $p < 0.001$) in PCI-treated ACS patients in the TRITON-TIMI 38 trial (6), an outcome that was predominantly driven by a reduction in MI (7.4% vs. 9.7%, $p < 0.001$). Patients with NSTE-ACS were randomised only if PCI was indicated following coronary angiography. STE-ACS patients were included either following initial medical management and subsequent coronary angiography or if they were planned to receive primary PCI. Patients with diabetes and STE-ACS had the biggest reduction in ischaemic events. The downside was an increase in TIMI-defined major bleeding, including life-threatening bleeds, in the prasugrel-treated group (2.4% vs. 1.8%, $p = 0.03$). There was also a marginal but significant increase in fatal bleeding with prasugrel (0.4% vs. 0.1%, $p = 0.002$). Despite this, subgroup analyses suggested net clinical benefit except for those with history of stroke, low body weight (<60 kg) or age > 75 years (6).

The superior efficacy of prasugrel compared to clopidogrel was not replicated in medically-treated ACS patients in the TRILOGY trial (70). Interestingly, major bleeding was similar in both clopidogrel- and prasugrel-treated patients. This difference in bleeding profile compared to the previous trial might be explained by a reduction in prasugrel dose in patients with low body weight (< 60 kg) and those > 75 years old (70).

The ACCOAST trial compared prasugrel pre-treatment in NSTE-ACS patients to treatment post coronary angiography only for those undergoing PCI and found no difference in ischaemic endpoints but significantly higher rates of major bleeding (2.6% vs. 1.4%, p = 0.006) (71). Although this trial had limitations (over 50% of procedures were performed through the femoral approach, the study protocol did not recommend a wash-out period prior to coronary artery bypass surgery (CABG) and the majority of bleeding events were related to access site and surgery), this has put an end to prasugrel treatment in NSTE-ACS patients prior to coronary angiography (2).

Ticagrelor belongs to a different class (cyclopentyl-triazolopyrimidines). It acts directly and reversibly blocks platelet $P2Y_{12}$ receptors in a non-competitive pattern (allosteric inhibition). Being an active drug, ticagrelor achieves rapid platelet inhibition and minimises any chance of inter-individual variance in pharmacodynamic response (72). Ticagrelor is metabolised via hepatic CYP3A and consequently concomitant use of strong CYP3A inducers or inhibitors is contraindicated (73). Ticagrelor also has a more rapid and consistent offset of action compared to clopidogrel – a desired effect should serious bleeding occur or if major surgery is planned (72).

Ticagrelor (90 mg twice daily) reduced MACE in moderate- to high-risk ACS patients compared to clopidogrel in the PLATO trial (5) (9.8% vs. 11.7%, p < 0.001). This was driven by a reduction in MI (5.8% vs. 6.9%, p = 0.005) and cardiovascular death (4% vs. 5.1%, p = 0.001). Death from any cause was also reduced in ticagrelor-treated patients (4.5% vs. 5.9%, p < 0.001). Although overall major, fatal and life-threatening bleeding

rates were similar in both groups, non-CABG major bleeding was more with ticagrelor (4.5% vs. 3.8%, p = 0.03). Intracranial haemorrhage was also marginally increased with ticagrelor (0.3% vs. 0.2%, p = 0.06). Other important adverse effects of ticagrelor included dyspnoea (13.7% vs. 8.7%, p < 0.001) and more frequent ventricular pauses in the first week of treatment (5.8% vs. 3.6%, p = 0.01). Despite this, there was no increase in syncope or pacemaker implantation and the majority of dyspnoea events were mild or moderate with a minority having to discontinue treatment (74). More reassuringly, ticagrelor treatment did not have negative effects on cardiac or pulmonary function (75, 76). Subgroup analyses confirmed consistent benefit with ticagrelor, including patients with STE-ACS (77), diabetes mellitus (DM) (78) or chronic kidney disease (CKD) (79) and the elderly (80). The benefit of ticagrelor was attenuated in patients recruited in the United States (US). Although this might have been due to chance (the US cohort was only ~ 8% of the PLATO patients), the majority of patients in the US were receiving a higher maintenance dose of aspirin (> 300 mg daily) (81). This raised the hypothesis of a negative interaction between higher doses of aspirin and ticagrelor, which remains to be further explored.

Ticagrelor also blocks cellular uptake of adenosine by erythrocytes and other cells through inhibition of the equilibrative nucleoside transporter 1 (ENT-1) (82, 83). It remains uncertain whether this explains some of ticagrelor's beneficial effects as well as some of its side effects (84).

One caveat to oral $P2Y_{12}$ inhibitors is the delayed onset of action in patients presenting with STE-ACS. In STE-ACS patients, especially those pre-treated with opiates, slow gastric transit might delay absorption and as a result pharmacodynamic effects can be delayed by up to 6-8 hours (85-87). This can increase the risk of stent thrombosis (88, 89). Approaches to deal with this issue include GP IIb/IIIa inhibitors (GPIs) or cangrelor (an intravenous $P2Y_{12}$ inhibitor). However, GPIs increase the risk of major bleeding (90)

and a 2-hour infusion of cangrelor might not be sufficient in all patients. Furthermore, costs associated with either of these approaches might limit wide-spread use. Studies to assess alternative approaches to deal with this issue are needed.

Landmark clinical trials discussed above are summarised in table 1-1.

Table 1-1: Summary of landmark dual antiplatelet therapy trials

Study and population	Intervention	Outcomes
CURE: 12,562 NSTE-ACS patients *within 24 hrs of presentation*	Clopidogrel 300 mg (at presentation) followed by 75 mg OD for 3-12 months vs. placebo	MACE: 9.3% vs. 11.4%; P < 0.001 CV death: 5.1% vs. 5.5%; MI: 5.2% vs. 6.7% Major bleed: 3.7% vs. 2.7%; P = 0.001
TRITON-TIMI 38: 13,608 PCI-treated ACS patients	Prasugrel 60 mg followed by 10 mg OD vs. clopidogrel 300	MACE: 9.9% vs. 12.1%; P < 0.001

(Following angiography in NSTE-ACS and planned PPCI in STE-ACS)	mg followed by 75 mg OD for 6-12 months	CV death: 2.1% vs. 2.4%; MI 7.3% vs. 9.5% Major bleed: 2.4% vs. 1.8%; P = 0.03 Fatal bleed: 0.4% vs. 0.1%; P = 0.002
TRILOGY-ACS: 9,326 NSTE-ACS patients treated conservatively	Prasugrel 60 mg followed by 10 mg OD* vs. clopidogrel 300 mg followed by 75 mg OD up to 30 months. **In patients < 60 kg or ≥ 75 yrs – Prasugrel dose adjusted to 30 mg followed by 5 mg OD.*	MACE: 13.3% vs. 13.9%; P = 0.45 Major bleed: 1.9% vs. 1.5%; P = 0.1
PLATO: 18,624 ACS patients *within 24 hrs of presentation*	Ticagrelor 180 mg followed by 90 mg BD vs. clopidogrel 300-600 mg followed by 75 mg OD up to 12 months.	MACE: 9.8% vs. 11.7%; P < 0.001 CV death: 4% vs. 5.1%; MI 5.8% vs. 6.9% Major bleed: 4.5% vs. 3.8%; P = 0.03 Fatal bleed: 0.3% vs. 0.3%; P = 0.66

MACE: major adverse cardiovascular events; NSTE-ACS: non-ST-elevation acute coronary syndrome; STE-ACS: ST-elevation acute coronary syndrome; CV: cardiovascular; OD: once daily; BD: twice daily.

Dual antiplatelet therapy beyond the 12 months

The PEGASUS-TIMI 54 trial (91) assessed the efficacy and safety of ticagrelor 60 mg or 90 mg twice daily in patients with MI 1 to 3 years previously. 21,162 patients were randomised to placebo, ticagrelor 60 mg or ticagrelor 90 mg. All patients received aspirin (75-150 mg daily). Patients were at least 50 years of age with one additional marker of increased risk (age ≥ 65 years, DM requiring treatment, an additional previous MI, multivessel CAD or CKD not requiring dialysis). Patients with stroke (ischaemic or haemorrhagic), central nervous system tumour, or intracranial vascular abnormality, or those treated with anticoagulants, cilostazol or dipyridamole were excluded. The trial also excluded patients with gastrointestinal bleed within 6 months and those who had undergone major surgery within 4 weeks. Both the 60-mg and 90-mg twice-daily doses

of ticagrelor reduced 3-year MACE rates compared to placebo (7.77% vs. 9.04%, p = 0.004 and 7.85% vs. 9.04%, p = 0.008, respectively). Pooled analysis of both ticagrelor groups showed a non-significant trend towards improved cardiovascular mortality with ticagrelor compared to placebo (2.9% vs. 3.39, p = 0.06). There was no increase in rates of fatal or intracranial bleeding with ticagrelor. There was, however, a significant increase in TIMI-major bleeding with both doses (60 mg and 90 mg) compared to placebo (2.3% vs. 2.6% vs. 1.1%, respectively). Dyspnoea was significantly more frequent in ticagrelor-treated patients and this led to higher discontinuation rates. Ticagrelor 60 mg was associated with numerically lower rates of dyspnoea compared to 90 mg but nevertheless resulted in discontinuation of therapy in 4.55% of cases. However, it should be borne in mind that very few patients in PEGASUS-TIMI 54 had been exposed to ticagrelor previously so it is highly likely that lower rates of discontinuation would be seen in patients continuing ticagrelor after tolerating this for 1 year post-ACS. Gout was also marginally increased in ticagrelor-treated patients attributable to the effect of ticagrelor on serum uric acid levels (91).

From the trial results overall, it is estimated that 42 major cardiovascular events per year would be prevented for every 10,000 patients treated with ticagrelor 60 mg at the cost of 31 additional non-fatal TIMI major bleeds per year (91). A more favourable net benefit might be seen when ticagrelor therapy is extended without interruption after initial 1 year of treatment.

Pharmacodynamic assessment showed ticagrelor 60 mg to provide potent and consistent platelet inhibition that is comparable to ticagrelor 90 mg in all compliant patients, explaining why the efficacy of the 60-mg and 90-mg doses was almost identical in PEGASUS-TIMI 54 (92).

DM and peripheral artery disease (PAD) patients accrued greater absolute risk reduction of MACE with ticagrelor (1.5% and 4.1% respectively), including a 22% relative

reduction in cardiovascular mortality in patients with DM (93, 94). Absolute excess risk of TIMI-major bleeding was also attenuated in patients with PAD (0.12%) (94). Patients with CKD (GFR < 60 ml/min) also had a robust absolute risk reduction of MACE (2.7%) with similar bleeding risk to those without kidney dysfunction (95).

The DAPT study (96) assessed the safety and efficacy of 30-months' treatment with DAPT (aspirin + clopidogrel/prasugrel) following PCI with drug-eluting stents (DES). 9961 patients, who had received 12 months of DAPT post PCI with DES, were randomised to either continued DAPT with clopidogrel (65%) or prasugrel (35%) for a further 18 months or monotherapy with aspirin. Only 26% of patients had PCI for ACS and the trial excluded patients with MACE or significant bleeding in the 12 months' period post PCI. Prolonged DAPT resulted in a reduction in rates of definite stent thrombosis (0.3% vs. 1.2%) and MACE (4.3% vs. 5.9%, $p < 0.001$) (96). This was primarily driven by a reduction in MI, both related and unrelated to stent thrombosis. There was no effect on cardiovascular mortality but all-cause mortality was numerically higher in the continued therapy group (2% vs. 1.5%, $p = 0.05$), mainly due to excess non-cardiovascular mortality with prolonged therapy.

Prolonged thienopyridine-based DAPT had previously been assessed in multiple smaller trials with no clear benefit of prolonged therapy (97-99). However, a collaborative meta-analysis that included the subgroups with an index event of MI and the 21,162 patients from the PEGASUS-TIMI 54 study showed that prolonged therapy reduced MACE (6.4% vs. 7.5%, $p = 0.001$) and reduced cardiovascular death (2.3% vs. 2.6%, $p = 0.03$) with no significant increase in non-cardiovascular death (100).

Shorter duration dual antiplatelet therapy

In cases of heightened bleeding risk or when interruption to DAPT is anticipated, it might become necessary to stop DAPT. Trials assessing short durations of DAPT (3-6 months)

post PCI with modern second-generation DES have shown comparable safety with 12-months' therapy (101, 102). Furthermore, polymer-free DES were superior to bare-metal stents with 1-month DAPT post PCI (103). These trials, however, have primarily recruited low-risk stable patients and were designed to confirm the safety of these devices in terms of late stent thrombosis. They have also used clopidogrel-based DAPT and therefore the results cannot be generalised to ticagrelor-based DAPT in patients with ACS.

Switching from a potent $P2Y_{12}$ inhibitor to clopidogrel after one month of therapy in ACS patients undergoing PCI resulted in reduced major bleeding risk in the TOPIC trial (104). However, the trial only recruited patients who were stable during the first month after PCI and was not powered to detect a difference in ischaemic outcomes. A similar, but guided approach was trialled in the Tropical-ACS trial (105). One week of prasugrel-based DAPT following PCI in ACS patients, followed by de-escalation of treatment to clopidogrel (to switch back to prasugrel in non-responders based on platelet function testing), was non-inferior to standard treatment with prasugrel. Major bleeding events did not significantly differ in the two treatment approaches raising the question of benefit of such approach. Taking these two trials into consideration, de-escalation of therapy may be a useful approach in patients with high bleeding risk following ACS.

1.5.2 Anticoagulants

The notion of using anticoagulants following ACS to offer long-term secondary prevention is far from new. Anticoagulation with warfarin (a vitamin K antagonist) following ACS has significantly reduced death and recurrent events compared to placebo by 34% and 24% respectively (106). Compared to aspirin, warfarin offered a significant 19% risk reduction in MACE following ACS at the expense of a significant 25% increase in risk of major bleeding, which greatly limited its routine use (107).

Rivaroxaban is an oral, directly-acting factor Xa inhibitor (108). Factor Xa is the penultimate step in the coagulation pathway, which culminates in the production of thrombin and fibrin clot formation. It has predictable pharmacokinetics and does not usually require monitoring. Anticoagulation (with 20 mg daily) with rivaroxaban in atrial fibrillation (AF) patients, yielded similar efficacy in terms of stroke prevention to warfarin with comparable rates of major bleeding (109).

Low-dose rivaroxaban (2.5 mg twice daily and 5 mg twice daily) has been tested in ACS in the ATLAS ACS2-TIMI 51 trial (9). Investigators randomised moderate- to high-risk ACS patients (after stabilisation following the acute event including revascularisation if needed) to placebo, rivaroxaban 2.5 mg twice daily or 5 mg twice daily. This was in addition to standard medical therapy that included DAPT (aspirin + clopidogrel/ticlopidine). MACE was reduced with rivaroxaban compared to placebo (8.9% vs. 10.7%, $p = 0.008$): a reduction driven by recurrent MI and cardiovascular mortality. Non-CABG major bleeding events were significantly higher with rivaroxaban (2.1% vs. 0.6%, $p < 0.001$). There was a trend towards less bleeding events with the lower dose (2.5 mg) compared to the higher dose (5 mg) with improved all-cause mortality with the lower-dose rivaroxaban as compared to placebo (2.9% vs. 4.5%, $p = 0.002$).

Apixaban is another directly-acting factor Xa inhibitor. Anticoagulation with apixaban (5 mg twice daily) is superior to warfarin in reducing stroke in AF patients (110). The same dose (5 mg twice daily) was tested in ACS patients in the APPRAISE-2 trial (111) against placebo on top of standard medical therapy including DAPT (aspirin + clopidogrel). Due to increased bleeding events including fatal and intracranial bleeds with no clear benefit, the trial was stopped prematurely.

These two trials indicate that a small dose of anticoagulant might have a role to play in secondary prevention. Currently the more potent $P2Y_{12}$ inhibitors (ticagrelor/prasugrel)

are preferred to clopidogrel in ACS and adding anticoagulants to these is unlikely to be tolerated in all patients. However, the residual ischaemic risk despite ticagrelor/prasugrel treatment might indicate that a selected cohort of patients would benefit from further optimisation of antithrombotic therapy. Markers of increased thrombotic risk might be best placed to help identify patients who are suitable for more intensive therapy.

In this section, I will discuss possible markers that indicate increased propensity to thrombosis and how they correlate with clinical outcome.

1.6 Potential markers of increased thrombotic risk

1.6.1 Coagulation factors

As most clotting factors are implicated in arterial thrombosis, it is no surprise that patients with bleeding disorders such as haemophilia A (Factor VIII deficiency) and B (Factor IX deficiency) have some protection against MI (112, 113). Even mild factor VIII and IX deficiencies in relatives of haemophilia patients conferred some cardiovascular mortality benefit (114). Contrary to this, factor XI deficiency showed no protection against ACS (115) whereas high factor IX and XI levels have been identified as possible predictors of MI (116). Factor VIII was also demonstrated to be elevated in ACS patients (117). Although this suggests that these factors may play an important role in thrombus formation in ACS, high levels of factor VIII have not been clearly associated with increased risk of ACS (118). Most of these observations originate from relatively small epidemiological studies and therefore it is difficult to reach a firm conclusion regarding the prognostic value of these measurements. Furthermore, clot formation is complex and involves multiple interactions between many different proteins and cells. Therefore, levels of individual clotting factors may not be successful in identifying patients with increased risk.

However, fibrinogen is the only factor that has been consistently shown to predict risk of arterial thrombosis irrespective of its relationship with conventional risk factors.

Fibrinogen molecules consist of 2 pairs of 3 non-identical aminopeptide chains Aα, Bβ and γ (Figure 1-4). These chains are connected through disulfide bonds. The N termini form the central E region whilst the carboxy termini of Bβ and γ chains form the D regions (119). Thrombin cleaves fibrinopeptide A (FpA) and fibrinopeptide B (FpB) in the E nodule, exposing the positively charged A and B sites. A and B sites bond with the negatively charged a and b sites in the D region of other fibrin monomers to form protofibrils that further aggregate to form fibrin fibres (32, 120, 121). The arterial clot consists of a network of fibrin fibres with embedded platelet aggregates, red blood cells and leukocytes.

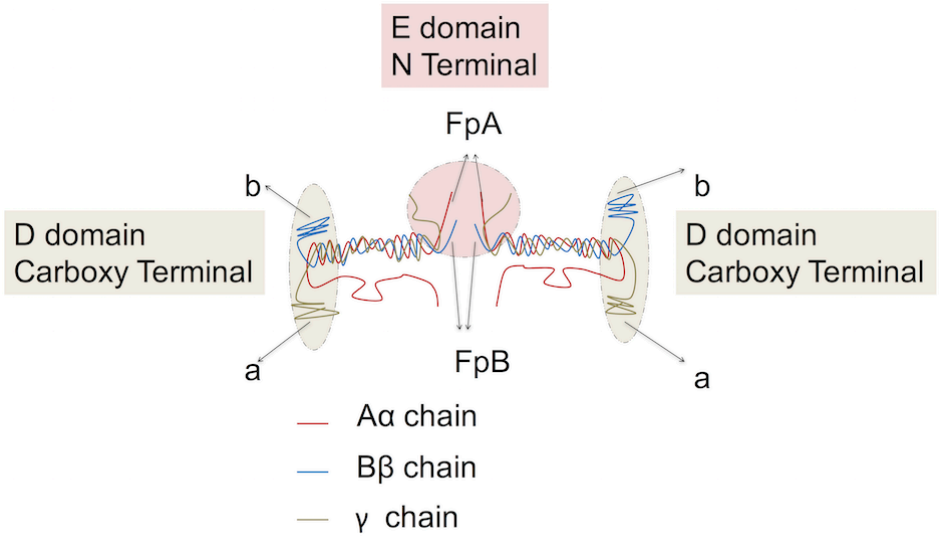


Figure 1-4: Schematic drawing of the fibrinogen molecule

3 different chains named Aα, Bβ and γ. N termini of the chains form the central E domain. Carboxy termini of Bβ and γ chain form the D domain. Thrombin acts by cleaving fibrinopeptide A (FpA) and fibrinopeptide B (FpB) in the E domain exposing the positively charged sites named A and B and forming fibrin monomer. Both A and B bind to negatively charged a and b sites respectively in the D domain of another fibrin monomer forming protofibrils. Protofibrils aggregating together form fibrin fibres.

Fibrinogen levels are strongly related to the risk of ACS. The Fibrinogen Studies Collaboration have completed an individual participant meta-analysis of 31 studies involving over 150,000 healthy participants showing an adjusted hazard ratio for CAD of 2.42 (95% CI, 2.24 – 2.46) for every 1 g/L increase in fibrinogen levels (122).

In patients with CAD, high fibrinogen levels were related to the extent of disease and also found to be an independent predictor of worse outcome (123, 124).

Both fibrinogen and D-dimer were found in tissue samples of atherosclerotic plaques, raising the hypothesis that fibrinogen plays a role in the pathogenesis of atherosclerosis through the formation of mural thrombi in the vessel walls (125).

1.6.2 Lysis markers

Thrombotic occlusion of a coronary artery could be viewed as failure of the protective fibrinolytic activity to effectively lyse the clot. In patients presenting with STEMI, approximately 15 – 20 % have spontaneous reperfusion (126, 127). This probably indicates an efficient fibrinolytic activity, which translates into better outcomes.

Interpreting the significance of blood levels of lysis markers is challenging. Intuitively, it could be argued that greater levels of tPA and D-dimer reflect efficient lysis, which might be beneficial. However, thrombosis will inevitably lead to tPA activation and subsequently D-dimer release and therefore these could be indirect markers of increased thrombotic burden. Similar analogy could apply to levels of inhibiting factors such as TAFI, PAI-1 and α2-PI. Furthermore, these markers do not reflect the overall thrombolytic potential of the fibrin clot, which is influenced by interactions between multiple complex pathways.

This might explain the conflicting data in the literature when it comes to the prognostic value of these different markers in predicting the incidence of CAD or in the context of ACS (128-136).

1.6.3 Fibrin clot network

The fibrin network forms the skeleton of blood clots and the structure of fibrin clots can determine stability and susceptibility to lysis.

Fibrin clots in patients with advanced and premature CAD have demonstrated "unfavourable" properties of increased fibrin density with thinner fibres that are more difficult to lyse (131, 137, 138). ACS patients also display similar unfavourable fibrin characteristics (139-141). Patients with DM, CKD and PAD are at higher risk of thrombotic complications (78, 142) and prothrombotic changes in fibrin clot networks might be one mechanism for the increased risk (143).

Quantitative and qualitative changes in coagulation, lysis and other plasma proteins can influence fibrin clot properties. For instance, fibrinogen levels might affect fibrin clot density (138) and increased levels in animal models have resulted in more rapid thrombosis and resistance to lysis (144). High concentrations of prothrombin and thrombin can also result in more compact and thinner fibrin fibres (145). At fibre level, thick fibres are more difficult to lyse; however, fibrin clots made of densely packed thin fibres are less permeable to lytic protein and therefore take longer to lyse (146).

Post-translational modifications to the fibrinogen molecule can also lead to lysis inefficiency. Increased fibrinogen oxidation is one mechanism for reduced lysis potential (147). Protein glycation, particularly glycation of fibrinogen lysine residues as a result of hyperglycaemia associated with DM, inhibits plasmin generation and results in prolonged lysis (148).

Factor XIII also plays an important role in fibrin clot stability. Following activation by thrombin, it promotes cross-linking of fibrin fibres (α and γ chains) and incorporation of α2-PI to fibrin clots, leading to compact clots that resist lysis (149-151). Activated factor XII also contributes to fibrin clot density and stability by binding the N termini of fibrinogen molecules (152).

As discussed earlier, PAI-1 limits tPA activity and therefore elevated levels might contribute to inefficient lysis. Moreover, TAFI breaks the feedback cycle between fibrin formation and activation of plasmin and therefore limits lysis potential.

Inflammation unfavourably modulates fibrin clot characteristics (138, 153). Incorporating complement C3 in fibrin clots could be one mechanism for prolonged lysis, an effect that is enhanced in patients with DM (132). Factor XIII might be responsible for the incorporation of complement C3 into the fibrin clot (154).

Common genetic variations of both fibrinogen and factor XIII also determine fibrin clot structure (155, 156). A twin study has demonstrated a significant 40% heritability in the overall fibrin clot structure (157).

Studying fibrin clot formation and lysis potential appears attractive as it offers a global assessment of thrombus formation. It has the potential to give us a holistic assessment of complex and dynamic processes, exploring the qualities of fibrin clot as opposed to measuring different factors leading to fibrin clot formation.

1.6.4 Tissue factor and platelet-monocyte aggregates

TF ignites the coagulation cascade at the site of plaque rupture by binding to and activating factor VII. Although most TF is tissue-bound, circulating TF can also be measured in plasma. Patients with CAD and ACS have increased levels of TF (158, 159). A few studies have demonstrated a positive relationship between rising levels of circulating TF and worse outcomes following ACS. In a study involving 523 ACS patients followed up for over 2 years, cardiovascular death was significantly higher in the highest quartile of TF (HR 2.06, CI 1.24-3.45, $p = 0.006$) (160). Other smaller studies reached similar conclusions (161, 162). Although the functional capacity of circulating TF is not clear, higher levels might indicate more extensive disease and increased thrombotic potential.

Activated platelets express the adhesion molecule CD62P (P-selectin) through which they bind to monocytes, thus forming platelet-monocyte aggregates (PMAs). PMAs are found in stable CAD but levels significantly increase in ACS (163, 164). TF is also expressed on PMAs, potentially increasing thrombotic potential in CAD (165).

1.6.5 Von Willebrand Factor

vWF is a glycoprotein predominantly produced by endothelial cells (166). It promotes platelet adhesion and activation at the site of vessel injury. It also acts as a carrier to clotting factor VIII, protecting it from deactivation by protein C (167). Therefore, vWF can be a thrombotic marker as well as a marker of endothelial dysfunction (168).

Traditional CAD risk factors have been associated with increased levels of vWF (169-172), likely reflecting the relationship with endothelial dysfunction. Consequently, any relationship observed between high vWF levels in healthy volunteers and incidence of CAD largely disappears after adjustment for other risk factors (173).

Numerous other studies have demonstrated a stronger relationship between vWF levels and worse outcome in CAD patients. In a study where 2960 stable CAD patients were followed up for 2 years, patients with recurrent coronary events tended to have marginally higher vWF levels than those with no recurrent events and vWF levels were strongly related to the extent of CAD (174). In another study, amongst 1212 ACS patients, patients with recurrent events had marginally but significantly higher vWF levels compared to a control group matched for age and sex (86). Similar signals were observed in other studies, indicating increased risk with higher levels of vWF (175-178). However, they were all relatively small epidemiological studies.

Therapeutic interventions to target vWF have been developed (179). However, upstream inhibition of both coagulation arms is likely to be risky. For instance, combining a high dose anticoagulant with dual antiplatelet therapy resulted in unacceptable increase in major bleeding risk (111). Concerns regarding increased risk of bleeding and the

availability of treatments targeting specific pathways are likely to hinder the success of such treatment strategies.

1.7 Inflammatory markers

1.7.1 Leukocytes

A relationship between elevated leukocytes and CAD is well established. Increasing levels of leukocytes translate into marginal increase in risk but likely through the association with traditional risk factors (180).

As discussed earlier, inflammation contributes to the increased thrombotic risk. Leukocytes appear to play a critical role in thrombotic risk modulation. Under normal circumstances, leukocytes exert an anti-thrombotic effect by expressing endothelial protein C receptor (181), thrombomodulin (182) and TFPI (183).

Once activated, as could happen when PMAs form, their action reverses to a pro-thrombotic effect by producing coagulation factors. Neutrophils primarily produce cathepsin G and elastase (184). These are serine proteases that can activate factors V, VIII and X as well as inactivating antithrombotic factors (185-188).

Activated neutrophils also secrete nuclear material (chromatins) that form neutrophil extracellular traps (NETs). NETs offer a platform for pro-thrombotic factors to accumulate and can activate factors XI and XII (189). Elevated markers of NETs have been associated with severe CAD (190).

Monocytes also express TF and have been identified in atherosclerotic plaques (191, 192). There are three different subsets of monocytes: classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$) and non-classical ($CD14^{+}/CD16^{++}$). In 951 patients undergoing elective PCI, levels of intermediate monocytes independently predicted MACE (193).

1.7.2 C-Reactive Protein and cytokines

CRP is an acute phase reactant protein, produced primarily from hepatocytes in response to IL6. It is a marker commonly used to detect acute or chronic inflammatory processes. CRP promotes the complement pathway and macrophage phagocytosis of apoptotic cells (194).

In a meta-analysis of over 160,000 participants, high CRP levels were independently related to CAD (Risk ratio 1.44; CI 1.32-1.57) and vascular mortality (Risk ratio 1.55; CI 1.37-1.76) (195).

Upstream of CRP in the inflammatory cascade are cytokines such as IL6, TNF-α and IL1.

A recent meta-analysis involving over 29 prospective studies showed a significant relationship between different cytokines, including IL6, TNF-α and IL18, and the risk of CAD (196).

Of particular interest is IL6, with some studies suggesting a causal relationship with CAD. Most convincing of all are the Mendelian genetic studies: certain polymorphisms of alleles implicated in IL6 and its receptor signalling pathways resulted in lower CRP levels and conferred some lifetime cardiovascular protection (197). This is in contrast to CRP Mendelian randomisation studies that did not indicate a causal relationship between alleles implicated in high CRP levels and incidence of CAD (198).

Growth differentiation factor (GDF) 15 belongs to the family of TGFβ proteins. It has emerged as a cardio-protective protein in cases of induced myocardial ischaemia, limiting macrophage adhesion to endothelial cells and reducing the inflammatory response (199, 200). It has been shown to be a predictor of recurrent events following ACS (201, 202). Intriguingly, high levels of GDF-15 were shown to be an independent predictor of major bleeding in ACS patients (202). The exact mechanisms for this are

unclear but it might be a combination of associations with other risk factors and possible inhibition of platelet integrin receptors (203).

1.8 Future perspectives and discussion

The driving forces behind recurrent ischaemic events are complex and require careful consideration. In the PLATO trial (5), 5.8% of patients receiving ticagrelor suffered recurrent MI and about 9% of patients with raised inflammatory markers had recurrent MI or cardiovascular death in the first year (4). This is despite potent and consistent $P2Y_{12}$ inhibition levels achieved with ticagrelor (204-207). The mechanisms leading to these ischaemic events are difficult to pin down but some possible explanations might be adherence to antiplatelet therapy, a prothrombotic state or increased bleeding events. Higher levels of inflammatory markers at the time of ACS presentation are associated with higher risk of recurrent ischaemic events and it is possible that a "prothrombotic state" is partially responsible for this observation. Further studies are needed to shed some light on the mechanisms leading to recurrent events and potentially identify patients who might benefit from intensive treatment, thus mitigating ischaemic risk without increasing bleeding events. Balancing risks of ischaemia and bleeding is important as there is a clear relationship between major bleeding events and worse outcome (208). In a meta-analysis of PCI studies involving over 500,000 patients, major periprocedural bleeding was independently related to risk of mortality (Odds ratio 3.31; CI 2.86 – 3.82) and also independently related to major adverse cardiovascular events (OR 3.89; CI 3.26 - 4.64) (209). It is likely that many factors contribute to these observations, including a tendency to discontinue antithrombotic treatment, increased inflammatory state associated with bleeding (210) and possibly also increased erythropoietin secretion (211, 212).

The availability of potent and consistent $P2Y_{12}$ inhibitors have led to question whether there is any scope for additional therapy. Some have questioned the role of aspirin (213). Recently, ticagrelor compared to aspirin failed to reduce cardiovascular events in both stroke and PAD patients (214, 215). These surprising findings indicate that $P2Y_{12}$ inhibition alone is unlikely to offer sufficient protection in high-risk ACS patients. Moreover, although platelets are optimally inhibited with contemporary treatment with aspirin and ticagrelor, the protein coagulation arm remains unaffected. Trials will be needed to choose the optimal antiplatelet regimen in addition to anticoagulant therapy. GEMINI-ACS study showed low-dose rivaroxaban to have similar safety profile to aspirin when combined with $P2Y_{12}$ inhibition post ACS (216). Ongoing trials, including Global Leaders (213) and TWILIGHT (9), are investigating ticagrelor monotherapy after PCI.

Targeting the inflammatory pathway might also play a role in the future. Anakinra (IL1 receptor antagonist) successfully reduced inflammatory markers in ACS patients; however, pilot data raised concerns regarding its safety (217). Strategies inhibiting upstream inflammatory pathways, such as lipoprotein-phospholipase A_2 and p38 mitogen-activated protein kinase (MAPK), have failed to improve outcomes and, in some cases, resulted in increased risk (218-220). These failures could indicate that inflammation is an important response to vascular injury and to some extent could be protective. Moreover, inflammation is a physiological response to many illnesses and dampening this response could increase the risk of other conditions such as sepsis and malignancy. An ideal inflammatory target is therefore likely to be a specific pathway that is negatively implicated in and probably unique to atherosclerosis.

Inhibiting IL1β with canakinumab in MI patients who have CRP levels > 2 mg/L has resulted in a modest but significant reduction in recurrent events but resulted in increased risk of fatal infections in the CANTOS trial (221). The benefit appeared greatest in those

who responded to treatment with a drop in CRP to < 2 mg/L (222). These interesting findings have opened the gate to new possibilities in managing high-risk ACS patients.

Furthermore, epidemiological studies have identified potential triggers that are associated with ACS. As well as certain physical and emotional attributes, smoking and cocaine use, air pollution was identified as a major contributor at the population level (223). Public health interventions aiming at improving pollution levels, may therefore help improve outcomes following ACS (223).

In conclusion, there remains a need to further optimise therapy in patients with ACS. Understanding some mechanisms behind recurrent events will guide further research to improve prognosis following ACS.

1.9 Rationale

As discussed above, mechanistic insights into recurrent events following ACS are needed to identify potential treatment targets that may improve prognosis. We have discussed how patients with high-risk conditions have dense fibrin clots with impaired lysis. We therefore hypothesize that adverse fibrin clot properties independently predict worse outcomes following ACS. Confirming inefficient lysis as a prognostic biomarker may aid clinicians to choose patients likely to benefit from additional therapy.

Low-dose anticoagulation with rivaroxaban, in addition to aspirin and clopidogrel, successfully improved ischaemic outcomes following ACS (9). The ability of different anticoagulants, including rivaroxaban, to modulate fibrin clot formation and lysis will be explored. This may be a novel approach to assess treatment effects, when necessary, and may help us understand if additional anticoagulant therapy is a viable option to target fibrin clot lysis.

We have also discussed the limitations of oral $P2Y_{12}$ inhibitors in STEMI patients undergoing primary angioplasty. Delayed absorption could result in acute thrombotic

complications and strategies to deal with this issue are needed. We will explore a strategy of continuous anticoagulation with enoxaparin for 6 hours and study the pharmacodynamic effects of this regimen. The results of this study will guide future research. Furthermore, we will be able to understand how treatment with enoxaparin influences fibrin clot properties (*ex-vivo*).

1.10 Aims

- Study fibrin clot properties in ACS patients and explore the relationships between fibrin clot density/lysis potential and clinical characteristics and inflammatory markers.
- Study the independent association between fibrin clot properties at hospital discharge and clinical outcomes in patients with ACS.
- Explore whether studying fibrin clot properties in ACS patients with DM would yield additional prognostic value.
- Study the variability of fibrin clot phenotype over time by comparing results of fibrin clot properties at hospital discharge and at 1-month follow-up as well as exploring how these properties change in relation to change in inflammatory markers.
- Study the *in-vitro* effects of different concentrations of factor Xa inhibitors on fibrin clot properties as a novel approach to monitoring therapy and a possible approach to positively modulate fibrin clot structure.
- Complete a clinical study (PENNY PCI) of the pharmacodynamic effects of a novel anticoagulant regimen (bolus enoxaparin + 6-hour infusion) in the context of primary PCI as a safe and inexpensive solution to the delayed absorption of oral $P2Y_{12}$ inhibitors in the acute setting.

1.11 Declaration

I declare that work presented in this thesis is the result of my own work and has not been submitted for any other degree. Details of my contributions to all studies and publications are as follows:

a) PLATO Fibrin Studies: I was involved in study planning and performed all fibrin clot experiments myself. I produced a statistical analysis plan, which was executed by biostatisticians based at Uppsala. I analysed data and wrote the manuscript, which is now published at the European Heart Journal.
b) *In-vitro* studies: I planned and performed all experiments. I analysed data and wrote the chapter, which I intend to submit for publication.
c) PENNY PCI: I planned the study including protocol development and obtaining all approvals. I recruited patients and performed all laboratory analyses with some technical help only when available. I analysed data and wrote the manuscript which is now accepted for publication in Thrombosis and Haemostasis.

2 Chapter 2: Methods

2.1 Establishing the association between fibrin clot properties and clinical outcomes following ACS

2.1.1 Patient population and samples

The PLATelet inhibition and patient Outcomes (PLATO) trial was an international multi-centre, double-blind, RCT of ticagrelor compared with clopidogrel in 18,624 moderate- to high-risk ACS patients. The study design and results have previously been published (5, 224). Baseline patient characteristics, including medical and medication history, were recorded at baseline. Study visits were performed at 1, 3, 6, 9 and 12 months. In the PLATO biomarker sub-study (225), a subset of 4,354 patients provided blood at hospital discharge, and 4,032 provided blood at 1-month follow-up. Hospital discharge samples were used in order to minimise the effects of anticoagulant therapy used as part of initial ACS management and 1-month samples were used in order to assess the effect of inflammation resolution on fibrin clots. Plasma was obtained from citrate-anticoagulated venous blood samples and stored initially at –20 °C prior to transfer to Uppsala Clinical Research Centre for storage at –80 °C. All study patients provided written informed consent according to a protocol approved by local research ethics committees at

participating centres. For our analyses, plasma samples were transferred to the University of Sheffield in freezer conditions and stored at –80 °C until analysis.

To assess the relationship between fibrin clot properties and fibrinogen levels, we used 37 stored plasma samples collected from a local ACS cohort at day 7 after the ACS event (207, 226).

2.1.2 Fibrin clot assessment

Human thrombin was obtained from Merck Biosciences (Calbiochem), recombinant tPA from Technoclone**,** calcium chloride dehydrate ($CaCl_2$) and Tris from Fisher Scientific, and sodium chloride (NaCl) from Sigma Aldrich. High-throughput turbidimetric analysis was performed in flat-bottomed, polystyrene 96-well plates (Greiner) using a dedicated Multiskan FC (Thermo Scientific) plate reader. Permeation buffer solution (100 mM NaCl, 50 mM Tris, pH 7.4) was used for dilution. 25-µL of plasma (in duplicates) were mixed with 75 µL lysis mix and clots were formed by adding 50 µL activation mix (tPA 83 ng/ml, $CaCl_2$ 7.5 mM, thrombin 0.03 U/ml; final concentrations). After shaking for 2 seconds, plates were read at 340 nm every 12 seconds at 37 °C until lysis in all samples was achieved. Quality control samples were included in all plates. Although, there is no standard method for fibrin clot assessment, this method has previously been validated in multiple studies (139, 227-229). It is possible that the sensitivity of this assay is improved by reducing tPA concentrations, however, lower tPA concentrations may be insufficient to lyse all samples. Studying fibrin clot with different methodologies may be an ideal option but this approach is not possible when analysing > 4000 samples. Studied variables included lag time, lysis time (a measure of lysis potential) and maximum turbidity (turbidity refers to the scatter of light as a measure of fibrin clot density) (Figure 2-1). All analyses were performed blinded to clinical outcomes and clinical characteristics.

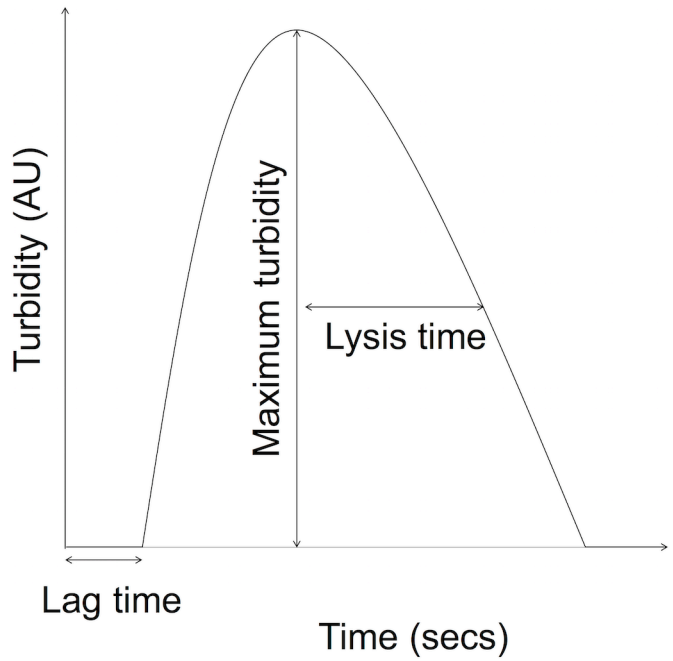


Figure 2-1: Fibrin clot turbidimetric parameters

Lysis time is measured from time of maximum turbidity till the time turbidity drops by 50%. AU: arbitrary units.

2.1.3 Determination of fibrinogen levels

Reference plasma was obtained from Biomereux. Sodium acetate trihydrate and sodium barbital were obtained from Sigma Aldrich.

KC10 clotting analyser was used for determination of fibrinogen levels using Clauss methodology: Plasma was diluted 1:10 with Veronal buffer solution (2.6 mM Sodium acetate trihydrate, 2.6 mM sodium barbital, pH 7.35) and thrombin was used to initiate clotting (5 U/ml; final concentration). When clotting was achieved in less than 7 seconds, plasma was further diluted 1:20.

2.1.4 Statistical methods

Cumulative rates of cardiovascular (CV) death or spontaneous MI (sMI) in the PLATO trial in patients in the highest quartile group of CRP levels were 12.3% compared to 7.3%

in the lowest quartile group (adjusted hazard ratio 0.57). We hypothesized fibrin clot properties to have a similar impact on this outcome measure. Allowing for events that occur pre-hospital discharge, we estimate a rate of 9% for the combined outcome measure of CV death or sMI in the highest quartile groups of lysis time and maximum turbidity. Including 4000 patient samples (1000 per quartile group) will give this study 90% power to detect a 43% risk reduction between the highest and lowest quartile groups.

Histograms were used to visually assess data distribution. Non-normally distributed data (all variables and biomarkers) were natural log-transformed. Baseline patient characteristics, medical history and biomarkers were compared across quartile groups of each of the fibrin variables. Continuous data are presented as medians and interquartile ranges (IQRs) and compared using Kruskal-Wallis tests. Categorical data are presented as numbers and percentages and compared using Chi-square tests. The primary outcome of interest was the composite of CV death and sMI. Secondary outcomes were CV death alone, sMI alone, all-cause mortality, stroke, definite or probable stent thrombosis according to Academic Research Consortium criteria, PLATO-defined major bleeding, and PLATO-defined major bleeding unrelated to coronary artery bypass graft surgery (CABG). Kaplan-Meier curves were derived to visually compare cumulative event rates across the four quartile groups of each of the fibrin clot variables. Cox proportional hazards models were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). HRs are expressed per 50% increase in value when assessed as continuous variables or compared to the lowest quartile group when assessed as categorical variables. Two models were used for adjustment. Model 1 included randomized treatment, type of ACS, age, gender, body mass index (BMI), smoking history, hypertension (HTN), dyslipidaemia, DM, CKD and previous MI, congestive heart failure (CHF), revascularisation, ischaemic stroke or PAD. Model 2 included all variables in

model 1 (excluding CKD) and the following inflammatory and prognostic biomarkers: N-terminal pro B-type natriuretic peptide (NT-proBNP), high-sensitivity troponin T, cystatin C, CRP, growth differentiation factor-15 (GDF-15) and white cell count (WCC). The choice of these variables is based on our knowledge of factors that have been related to clinical outcome in the PLATO cohort (202, 230-233). The assumptions of proportional hazards were assessed visually by calculating Schoenfeld residuals. To assess the prognostic value of fibrin clot properties, Harrell's C-index was estimated and compared to a clinical predictive model (Model 1) with and without the addition of fibrin clot variables using likelihood ratio tests. The efficacy and safety of ticagrelor compared with clopidogrel according to fibrin clot properties was assessed using a Cox proportional hazards model that included transformed fibrin clot variables using restricted cubic splines and randomized treatment by fibrin clot variables interaction. Interaction analyses using restricted cubic splines have been performed to study the interaction between presentation, treatment strategy, treatment with low-molecular-weight heparins and the prognostic value of fibrin clot parameters.

Subgroup analysis of patients with DM was performed to explore the prognostic value of fibrin clot properties at hospital discharge following ACS in patients with DM. Due to the exploratory nature of this study, we did not adjust for multiple testing.

Hospital discharge and 1-month fibrin clot properties were compared using Wilcoxon signed-rank tests. Spearman correlation and Bland-Altman plots were used to study the relationships between the two time-points. The relationship between change in fibrin clot properties and change in inflammatory biomarkers has also been assessed.

All statistical analysis was performed using R statistics software (Version 3.3.2; R Foundation for Statistical Computing, Vienna, Austria).

2.2 Modulation of the fibrin clot network with different anticoagulants

2.2.1 Reagents and fibrin clot assessment

Rivaroxaban and apixaban were obtained from Cayman Chemical, fondaparinux from Aspen, enoxaparin from Sanofi Aventis, dimethyl sulfoxide (DMSO) from Sigma-Aldrich and TF from Stago.

Fibrin clot dynamics were assessed using the same validated turbidimetric assay described above (227, 228) but with TF to induce thrombosis. 25 μL of plasma (in duplicates) was mixed with 75 μL of the activation mix (83 ng/ml tPA, 1 pM TF, 17 mM $CaCl_2$; final concentrations). Plates were maintained at 37°C and read every 30 seconds at 340 nm, until lysis of all samples. Clotting only assays were performed by excluding tPA in the activation mix. Variables included lag time, maximum turbidity and lysis time.

2.2.2 Plasma samples

Citrate-anticoagulated venous samples were collected from healthy volunteers and ACS patients (1-month post event) as per protocols approved by the local ethics committee. Blood was centrifuged at 3000 x g for 15 mins and plasma samples were obtained and stored at - 80° prior to analysis. Written informed consent was obtained from all individuals donating blood for these studies.

Rivaroxaban and apixaban were dissolved in DMSO, further diluted with permeation buffer and mixed with plasma (1:10) to achieve final concentrations of 1 μg/L, 3 μg/L, 10 μg/L, 33 μg/L, 100 μg/L, 330 μg/L and 1000 μg/L with final concentrations of DMSO of 1/1000. Fondaparinux was diluted with permeation buffer and mixed with plasma (1:10) to achieve final concentrations of 33 μg/L, 100 μg/L, 330 μg/L, 1000 μg/L and 10,000 μg/L. For clotting and lysis assays, fondaparinux was mixed with plasma (2:10) to achieve a final concentration of 500 μg/L. Enoxaparin was also diluted with permeation buffer and mixed with plasma (1:10), achieving final concentrations of (0.5 IU/ml and 1 IU/ml). Change in fibrin clot parameters was compared to control plasma

diluted with permeation buffer ± DMSO by the same ratios. These concentrations cover the clinically relevant range for these anticoagulants as summarised in table 2-1.

2.2.3 Statistical analysis

Data is presented as mean ± standard error of the mean (SEM). Paired/unpaired t tests and repeated measures analysis of variance (ANOVA) were used to determine significance of effect as appropriate. Multiple comparisons against control samples were performed using Dunnett's multiple comparison tests. Significance levels were drawn at $p < 0.05$. All statistical analyses were performed on GraphPad Prism 7.

Table 2-1: Summary of pharmacokinetic data for fondaparinux, rivaroxaban and apixaban

Anticoagulant	Dose	C_{max} μg/L	C_{trough} μg/L	Reference
Fondaparinux	2.5 mg OD	530 (200-830)[a]	210 (80-530)[a]	(234, 235)
Fondaparinux	5 – 7.5 – 10 mg OD (weight adjusted)	(1200-1260)[b]	(460-620)[b]	(236, 237)
Rivaroxaban	2.5 mg BD	47 (28-70)[a]	17 (6-37)[a]	(238)
Rivaroxaban	20 mg OD	249 (184-343)[c]	44 (12-137)[c]	(239)
Apixaban	2.5 mg BD	62.3[d]	21[d]	(240)
Apixaban	5 mg BD	128.5[d]	49.6[d]	(240)

All values represent steady-level state. [a] Medians (5^{th} – 95^{th} percentile range). [b] Range of means. [c] Geometric means (5^{th} – 95^{th} percentile range). [d] Geometric means. OD: once daily; BD: twice daily.

2.3 Prolonged ENoxapariN in primarY Percutaneous Coronary Intervention: PENNY PCI trial

2.3.1 Trial design

This is a single-centre, single-arm pharmacodynamic pilot study of an intra-arterial (IA) bolus of enoxaparin (0.75 mg/kg) followed by an intravenous (IV) infusion of enoxaparin (0.75 mg/kg) over 6 hours in STEMI patients undergoing primary percutaneous coronary intervention (PPCI). We planned to stop the infusion at 3 hours in patients with significant renal impairment, defined by an estimated glomerular filtration rate (eGFR) of < 30 ml/hr (cumulative dose of 1.125 mg/kg).

2.3.2 Study population

We have recruited 20 patients presenting acutely with STEMI according to the following inclusion and exclusion criteria:

Inclusion criteria

1. Age ≥ 18
2. Confirmation of the diagnosis of STEMI on the basis of a classical history, ECG changes and angiographic findings
3. Pre-treatment with either ticagrelor or prasugrel
4. Intention to proceed with PPCI
5. Feasibility to obtain informed verbal consent pre PPCI

Exclusion criteria

1. Active bleeding that cannot be controlled by local measures
2. Pregnant patients
3. Patients with end-stage renal failure requiring renal replacement therapy
4. Known thrombocytopenia (platelet count < 100,000/μL)
5. Known history of intracranial haemorrhage
6. Known current treatment with oral anticoagulants

7. Known history of major surgery or trauma or history of GI/GU haemorrhage within the last month
8. Known intracranial malignancy or aneurysm
9. Known allergy to enoxaparin
10. Known hypersensitivity to benzylalcohol
11. Patients with acute bacterial endocarditis
12. Active gastric or duodenal ulceration
13. Inability to easily understand verbal information given in English for any reason
14. Inability to give informed consent due to either temporary or permanent mental incapacity
15. Current participation, or participation within the last month, in an interventional clinical trial.

2.3.3 Study procedures

Patients admitted to the catheter laboratory or coronary care unit with STEMI and accepted for PPCI were screened. Figure 2-2 summarises the study procedures.

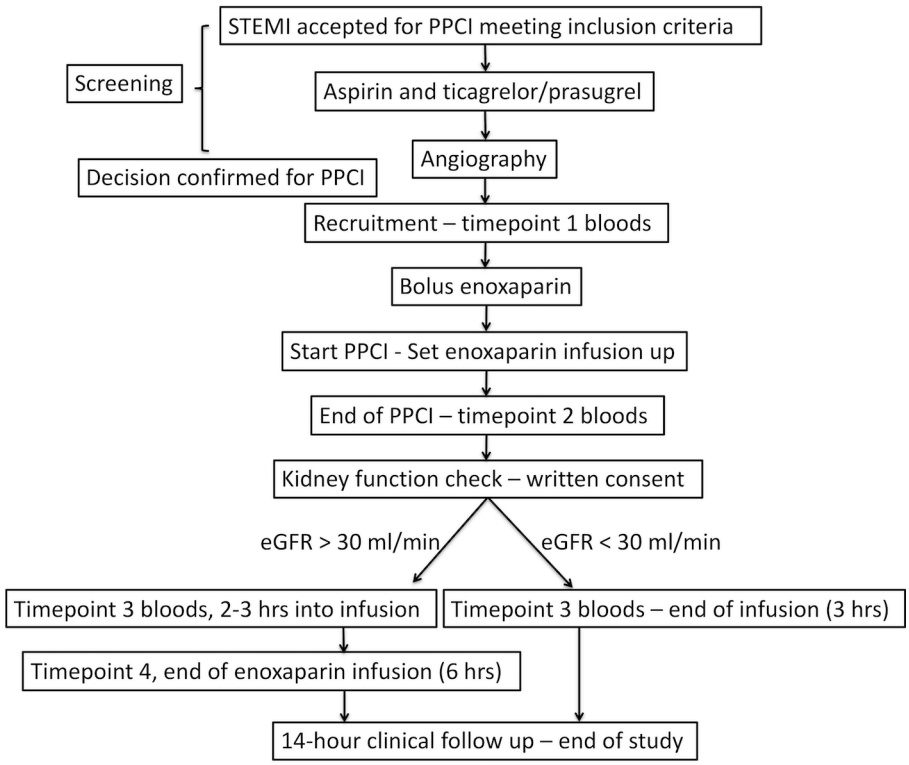


Figure 2-2: PENNY PCI flow chart

STEMI: ST-elevation myocardial infarction; PPCI: Primary percutaneous coronary intervention; eGFR: estimated glomerular filtration rate

Blood samples for pharmacodynamic assessment were collected at the following timepoints:

1. Timepoint 1 (T1) prior to anticoagulation – at the start of PPCI procedure.
2. Timepoint 2 (T2) at the end of PPCI.
3. Timepoint 3 (T3) 2-3 hours from the start of enoxaparin infusion.
4. Timepoint 4 (T4) at the end of enoxaparin infusion.

As PPCI is time-critical and delay in treatment can be detrimental to clinical outcome, informed verbal consent using an abbreviated patient information sheet was obtained prior to enrolment followed by informed written consent as soon as possible after PPCI. The consent process was performed by a qualified medical practitioner according to the

principles of Good Clinical Practice (GCP) and the declaration of Helsinki. Following consent, details of patient participation were sent to their general practitioner.

Clinical outcomes and adverse events were determined at least 14 hours after initiation of the enoxaparin regimen. The half-life of enoxaparin is 1.5-2 hours when given intravenously to patients undergoing PCI (12) and, therefore, adverse events related to the study medication are unlikely to arise beyond 14 hours.

Concurrent treatment with unfractionated heparin, bivalirudin, fondaparinux or glycoprotein IIb/IIIa inhibitors (GPIs) was prohibited. If GPI was used for bailout indications or to deal with thrombotic complications, enoxaparin infusion was to be stopped.

2.3.4 Study objectives

Primary objective

The primary objective was to assess anti-Xa activity (IU/ml) at the three time-points after initiation of enoxaparin.

Secondary objectives

Secondary objectives were to assess the following:

1. Fibrin clot properties (lag time, lysis time and maximum turbidity) at each of the timepoints.
2. Clotting times and clot firmness as determined by thromboelastometry at each of the timepoints.
3. Platelet $P2Y_{12}$ % inhibition and Platelet Reactivity Units determined by VerifyNow $P2Y_{12}$ assay at each of the timepoints.
4. Rates of safety and efficacy events (any bleeding and acute stent thrombosis events) in order to obtain pilot data for guiding further studies.

2.3.5 Laboratory analyses

Anti-Xa levels were determined using the commercially-available Coamatic kit

(Chromogenix) and Sysmex CS-5100 analyser (241). Fibrin clot properties were determined using the same methodology described in 2.2.2.1 but with 10 pM TF (final concentration). Clotting times and clot firmness in whole blood was determined using thromboelastometry (ROTEM) with intem-S (Tem Innovations) reagents. $P2Y_{12}$ inhibition was determined using the VerifyNow analyser and $P2Y_{12}$ assay cartridges at least 20 mins post-sampling, as per manufacturer's instructions.

2.3.6 Sample size and statistical methods

Since this is a novel regimen, a formal sample size calculation was not possible prior to the start of the study. Following determination of anti-Xa levels at the end of PPCI, we have determined that we have >90% power to detect at least 25% drop in anti-Xa levels throughout the infusion. This was based on one-way ANOVA test.

Continuous data are presented as mean ± SEM or median (interquartile range) as appropriate. Categorical data are presented as numbers and proportions. One-way ANOVA with Dunnett's multiple comparison tests were used for assessment of continuous variable. Amongst opiate-treated patients, two-way ANOVA was used to assess the interaction between $P2Y_{12}$ inhibition and antiemetic treatment. Results with P-values < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 7 for Mac OS X.

2.3.7 Trial organisation

This trial has been sponsored by Sheffield Teaching Hospitals NHS Foundation Trust and managed by the Cardiovascular Research Unit, University of Sheffield. Data was recorded using dedicated case report forms (Appendix) and subsequently transferred to an electronic database for analysis and archiving purposes.

An independent data monitoring committee (DMC) reviewed data on safety and adverse events after the 10^{th} patient had been recruited and at the end of the study.

2.3.8 Ethical considerations

The study has been conducted according to European Union Clinical Trials regulations. The study protocol has been approved by the UK Medicines and Healthcare Products Regulatory Agency (MHRA) and the local research ethics committee (17/YH/0103).

3 Chapter 3: Fibrin Clot Properties Independently Predict Adverse Clinical Outcome Following Acute Coronary Syndrome: A PLATO Substudy

Publications and abstracts arising from this chapter:

1) The abstract has been presented at the American Heart Association 2017 conference in Anaheim, California.
2) Sumaya W, Wallentin L, James SK, Siegbahn A, Gabrysch K, Bertilsson M, Himmelmann A, Ajjan R, Storey RF. Fibrin clot properties independently predict adverse clinical outcome following acute coronary syndrome: A PLATO Substudy. European Heart Journal 2018;39:1078-85.

Inclusion of this research in this thesis does not violate copyright agreements and is in agreement with co-authors.

3.1 Abstract

Introduction: Compact fibrin clots that resist lysis have been implicated in thrombotic conditions but large outcome studies are lacking. We investigated fibrin clot properties in a longitudinal study of patients with ACS.

Methods: Plasma samples were collected at hospital discharge from 4,354 ACS patients randomized to clopidogrel or ticagrelor in the PLATO trial. A validated turbidimetric assay was employed to study plasma clot lysis time (a measure of fibrinolytic efficiency) and maximum turbidity (a measure of clot density). One-year rates of cardiovascular (CV) death, spontaneous myocardial infarction (sMI) and PLATO-defined major bleeding events were assessed after sample collection. Hazard ratios (HR) were determined using Cox proportional analysis.

Results: After adjusting for CV risk factors, each 50% increase in lysis time was associated with CV death/sMI (HR 1.17; 95% CI 1.05-1.31; P<0.01) and CV death alone (HR 1.36; 1.17–1.59; P<0.001). Similarly, each 50% increase in maximum turbidity was associated with increased risk of CV death (HR 1.24; 1.03–1.50; p = 0.024). After

adjustment for other biomarkers (leukocyte count, high-sensitivity C-reactive protein, high-sensitivity troponin T, cystatin C, N-terminal pro B-type natriuretic peptide and growth differentiation factor-15), the association with CV death remained significant for lysis time (HR 1.15; 1.01–1.30; P=0.032) but not for maximum turbidity. These associations were consistent regardless of randomized antiplatelet treatment (all interaction P > 0.05). Neither lysis time nor maximum turbidity was associated with major bleeding events.

Conclusions: Fibrin clots that are resistant to lysis independently predict adverse outcome in ACS patients. Novel therapies targeting fibrin clot properties might be a new avenue for improving prognosis in patients with ACS.

3.2 Background

Recurrent events, including CV death, remain common following ACS. Intensive antithrombotic therapies, including potent $P2Y_{12}$ inhibitors and the addition of low-dose anticoagulant therapy (rivaroxaban), have all resulted in improved outcomes but increased the risk of major bleeding events (5, 6, 9).

There is marked overlap between risk factors for ischaemic and bleeding events (7, 242). Consequently, tailoring therapy to achieve the "sweet spot" of mitigating ischaemia whilst maintaining effective haemostasis is an ongoing challenge and readily-available biomarkers to aid the decision process are lacking. Spontaneous major bleeding events are associated with a similar prognosis to ischaemic events (243) and, in patients undergoing PCI, major bleeding events independently predict MACE (209).

Following ACS, treatment includes aspirin and a $P2Y_{12}$ inhibitor (244, 245). Although contemporary therapy effectively targets platelets (206, 246), around 20% of patients

suffer recurrent events within 12 months (7). This dual antiplatelet treatment strategy largely spares the protein arm of coagulation, which leads to fibrin formation.

Patients with thrombotic conditions often demonstrate unfavourable fibrin clot structure (139-141). High-risk conditions, such as DM, CKD and PAD, have all shown associations with compact fibrin clots and resistance to fibrinolysis (143, 247, 248). However, the majority of studies in this area used a cross-sectional retrospective design and large-scale longitudinal studies are lacking. We therefore aimed to study fibrin clot properties in plasma samples collected from ACS patients at hospital discharge and explore the relationship between those characteristics and clinical outcomes.

3.3 Results

3.3.1 The relationships between fibrin clot properties, clinical characteristics and biomarkers

A total of 4,354 patients were included in this study. Tables 3-1 and 3-2 summarize the clinical characteristics and biomarkers across the four quartiles of both lysis time and maximum turbidity. The prevalence of DM, HTN, CKD and female sex significantly increased with increasing lysis time quartiles. Although the differences were not significant, the highest quartile of lysis time appeared to have the highest prevalence of PAD and CHF.

Patients in the lowest lysis time quartile tended to be older but the absolute difference in mean age was small. Strong associations were also observed between lysis time and other biomarkers: with increasing quartiles, the levels of HsTnT, NT-proBNP, CRP and WCC increased. GDF-15 and cystatin-C levels were also highest in the highest quartile.

Similar to lysis time, the prevalence of DM and CKD was highest in the highest quartile of maximum turbidity. There was a higher percentage of female patients in the lowest maximum turbidity quartile. The relationship between maximum turbidity and the other biomarkers was also pronounced (Table 3-2).

Neither lysis time nor maximum turbidity appeared to be affected by haemoglobin or haematocrit (Tables 3-1, 3-2).

Maximum turbidity and lysis time had a modest correlation (Spearman correlation co-efficient 0.375, $P < 0.001$) (Figure 3-1).

Table 3-1: Clinical characteristics and biomarkers across lysis time quartile groups

Variables	Lysis time (secs) quartile group				P value
	Q1 (<564) n = 1098	Q2 (564-696) n = 1108	Q3 (696-888) n = 1066	Q4 (>888) n = 1082	
Demographics and medical history					
Age (years)	63 (55 – 72)	61 (54 – 70)	61 (53 – 70)	61 (53 – 70)	< 0.001
Female	242 (22%)	318 (28.7%)	271 (25.4%)	442 (40.9%)	< 0.001
BMI (kg/m^2)	27 (24.5-29.7)	27.3 (24.9-30.1)	27.8 (25.3-30.8)	28.6 (25.6-31.8)	< 0.001
Current smoker	392 (35.7%)	450 (40.6%)	404 (37.9%)	349 (32.3%)	< 0.001
Hypertension	673 (61.3%)	717 (64.7%)	711 (66.7%)	764 (70.6%)	< 0.001
Hyperlipidaemia	453 (41.3%)	462 (41.7%)	456 (42.8%)	469 (43.3%)	0.744
Diabetes mellitus	206 (18.8%)	221 (19.9%)	242 (22.7%)	305 (28.2%)	< 0.001
Previous MI	216 (19.7%)	215 (19.4%)	214 (20.1%)	201 (18.6%)	0.843
Previous CHF	53 (4.8%)	59 (5.3%)	58 (5.4%)	79 (7.3%)	0.068
Previous stroke	34 (3.1%)	42 (3.8%)	35 (3.3%)	40 (3.7%)	0.783
PAD	62 (5.6%)	68 (6.1%)	63 (5.9%)	80 (7.4%)	0.345
CKD	29 (2.6%)	42 (3.8%)	27 (2.5%)	49 (4.5%)	0.028
Type of ACS					
STE-ACS	504 (45.9%)	522 (47.1%)	510 (47.8%)	486 (44.9%)	0.536
Biomarkers					
Troponin T (ng/L)	129 (35-453)	151 (37-511)	177 (46-582)	210 (47-755)	< 0.001
NTproBNP(pmol/L)	387 (119-992)	386 (129-1088)	389 (131-1033)	469 (139-1433)	0.009
Cystatin C (mg/L)	0.80(0.65-0.97)	0.80 (0.66 – 0.96)	0.81(0.66– 0.97)	0.86(0.69-1.05)	< 0.001
GDF-15 (ng/L)	1503(1095-2058)	1446(1108-2092)	1509(1182-2064)	1584(115-2254)	0.003
CRP (mg/L)	2.4 (1.1-5.8)	3.1 (1.4-7.6)	3.9 (1.8- 9.8)	5.2 (2.2-13.5)	< 0.001
WCC (x 10^9/L)	8.7 (6.9-10.9)	9.1 (7.3-11.4)	9.7 (7.8-11.9)	9.9 (7.8-12.5)	< 0.001
Haemoglobin (g/L)	141 (131-151)	142 (132-151)	142 (132-152)	141 (130-151)	0.144
Haematocrit (L/L)	0.41(0.39-0.44)	0.42 (0.39-0.44)	0.42 (0.39-0.45)	0.42(0.38-0.44)	0.265

Values are medians (IQRs) for continuous data and n (%) for categorical data. BMI: body mass index; MI: myocardial infarction; CHF: congestive heart failure; PAD: peripheral artery disease; CKD: chronic kidney disease; STE-ACS: ST-elevation acute coronary syndrome; NTproBNP: N-terminal pro b-type natriuretic peptide; GDF: growth differentiating factor; CRP: C-reactive protein; WCC: white cell count. P values calculated using Chi-square test (categorical variables) or Kruskal-Wallis test (continuous variables)

Table 3-2: Clinical characteristics and biomarkers across maximum turbidity quartile groups

Variables	Maximum turbidity (au) quartile group				P value
	Q1 (≤ 0.38) n = 1091	Q2 (0.38-0.5) n = 1089	Q3 (0.5-0.62) n = 1085	Q4 (>0.62) n = 1089	
Demographics and medical history					
Age, (years)	62 (54-71)	61 (53-70)	61 (54-70)	62 (54-70)	0.27
Female	366 (33.5%)	334 (30.7%)	327 (30.1%)	246 (22.6%)	< 0.001
BMI (kg/m^2)	27 (24.6-29.9)	27.8 (25.2-30.8)	27.8 (25.3-30.8)	28 (25.2-31.1)	< 0.001
Current smoker	358 (32.8%)	405 (37.2%)	409 (37.7%)	423 (38.8%)	0.02
Hypertension	747 (68.5%)	690 (63.4%)	715 (65.9%)	713 (65.5%)	0.09
Hyperlipidaemia	496 (45.5%)	492 (45.2%)	432 (39.8%)	420 (38.6%)	< 0.001
Diabetes mellitus	222 (20.3%)	225 (20.7%)	256 (23.6%)	271 (24.9%)	0.03
Previous MI	259 (23.7%)	214 (19.7%)	194 (17.9%)	179 (16.4%)	< 0.001
Previous CHF	90 (8.2%)	54 (5.0%)	48 (4.4%)	57 (5.2%)	< 0.001
Previous stroke	43 (3.9%)	41 (3.8%)	29 (2.7%)	38 (3.5%)	0.38
PAD	79 (7.2%)	63 (5.8%)	55 (5.1%)	76 (7%)	0.12
CKD	28 (2.6%)	37 (3.4%)	33 (3.0%)	49 (4.5%)	0.08
Type of ACS					
STE-ACS	392 (35.9%)	467 (42.9%)	553 (51%)	610 (56%)	< 0001
Biomarkers					
Troponin T (ng/L)	90.6 (21.3 – 301)	129 (30.6 – 410)	163 (43.8 – 494)	375 (97.1 – 1161)	< 0.001
NTproBNP(pmol/L)	335 (124 – 867)	332.5 (111.8 – 904.2)	390.5 (117.8 – 989)	662 (191 – 1774)	< 0.001
Cystatin C (mg/L)	0.82 (0.66 – 0.99)	0.79 (0.65 – 0.96)	0.81 (0.66 – 0.98)	0.84 (0.68 – 1.02)	0.002
GDF-15 (ng/L)	1447(1084-2007)	1417 (1065-1993)	1504 (1144-2076)	1692 (1243-2414)	< 0.001
CRP (mg/L)	2.4 (1.1 – 5.2)	2.7 (1.4 – 6.0)	3.9 (1.7 – 8.6)	6.8 (2.7 – 22)	< 0.001
WCC ($*10^9/L$)	8.2 (6.6 – 10.3)	8.9 (7.2 – 11.1)	9.7 (7.7 – 11.8)	10.7 (8.7 – 13.1)	< 0.001
Haemoglobin (g/L)	141 (130-150)	142 (132-151)	141 (131-150)	142 (132-152)	0.12
Haematocrit (L/L)	0.42 (0.38-0.44)	0.42 (0.39-0.44)	0.41 (0.39-0.44)	0.42 (0.39-0.45)	0.05

Values are medians (IQRs) for continuous data and n (%) for categorical data. AU: Arbitrary units; other abbreviations and P values calculations as per table 3-1.

3.3.2 Fibrin clot properties and inpatient treatment

Proportions of patients treated with inpatient invasive treatment did not vary significantly across the four quartile groups of lysis time. However, significantly higher proportions of patients received invasive treatment in the highest quartile groups of maximum turbidity (Tables 3-3 and 3-4).

A subset of 1637 patients received LMWH the day before sampling and 1229 patients continued to receive it on the day of sampling. However, the proportion of patients who received LMWH did not vary significantly across quartile groups of lysis time or maximum turbidity.

Details of other inpatient treatments are summarised in tables 3-3 and 3-4.

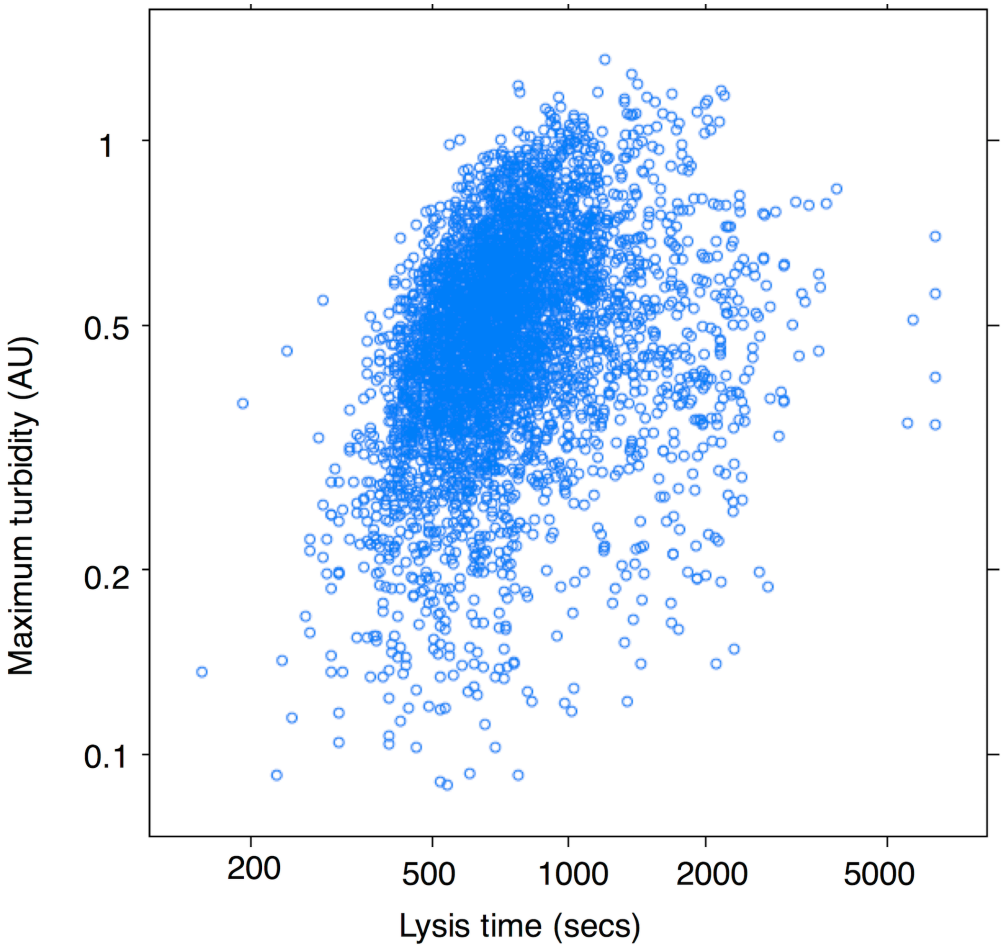


Figure 3-1: Relationship between maximum turbidity and lysis time

Scatter plot of maximum turbidity and lysis time. AU: arbitrary units. N = 4354.

Table 3-3: Inpatient treatments across the four lysis time quartile groups

Variables	Lysis time (secs) quartile group				P value
	Q1 (<564) n = 1098	Q2 (564-696) n = 1108	Q3 (696-888) n = 1066	Q4 (>888) n = 1082	
Invasive treatment	769 (70%)	793 (72%)	784 (74%)	742 (69%)	0.07
Aspirin	1081 (99%)	1094 (99%)	1055 (99%)	1057 (98%)	0.085
LMWH	608 (55%)	608 (55%)	593 (56%)	573 (53%)	0.59
LMWH on day before sampling	414 (38%)	403 (37%)	422 (40%)	398 (37%)	0.43
LMWH on day of sampling	311 (29%)	303 (28%)	321 (30%)	297 (28%)	0.47
Fondaparinux	16 (1.5%)	17 (1.5%)	17 (1.6%)	24 (2.2%)	0.5
ACE-I or ARB	954 (87%)	978 (88%)	925 (87%)	944 (87%)	0.71
Statins	1051 (96%)	1060 (96%)	1004 (94%)	998 (92%)	<0.001
Beta blockers	959 (87%)	969 (88%)	929 (87%)	938 (87%)	0.95

LMWH: low-molecular-weight heparins; ACE-I: angiotensin converting enzyme inhibitor; ARB: angiotensin II receptor blocker. P values calculated using Chi-square test.

Table 3-4: Inpatient treatments across the four maximum turbidity quartile groups

Variables	Maximum turbidity quartile				P value
	Q1 (≤ 0.38) n = 1091	**Q2 (0.38-0.5) n = 1089**	**Q3 (0.5-0.62) n = 1085**	**Q4 (>0.62) n = 1089**	
Invasive treatment	637 (58.4%)	765 (70.2%)	820 (75.6%)	866 (79.5%)	<0.001
Aspirin	1077 (99%)	1074 (99%)	1065 (98%)	1071 (98%)	0.7
LMWH	613 (56%)	613 (56%)	584 (54%)	572 (53%)	0.21
LMWH on day before sampling	436 (40.3%)	401 (37.1%)	408 (37.9%)	392 (36.3%)	0.26
LMWH on day of sampling	351 (32.4%)	303 (28%)	285 (26.5%)	293 (27.2%)	0.01
Fondaparinux	15 (1.4%)	19 (1.7%)	19 (1.8%)	21 (1.9%)	0.79
ACE-I or ARB	935 (86%)	946 (87%)	961 (89%)	959 (88%)	0.18
Statins	1010 (93%)	1027 (94%)	1038 (96%)	1038 (95%)	0.007
Beta blockers	923 (85%)	957 (88%)	965 (89%)	950 (87%)	0.02

LMWH: low-molecular-weight heparins; ACE-I: angiotensin converting enzyme inhibitor; ARB: angiotensin II receptor blocker. P values calculated using Chi-square test.

3.3.3 Fibrin clot properties and clinical outcome

During follow-up, 145 patients (3.3%) died from any cause, 125 (2.9%) died from CV causes ('CV death'), 183 (4.2%) had sMI and 41 (0.94%) had stroke. Only 38 (0.88%) had definite or probable stent thrombosis. PLATO-defined major bleeding occurred in 256 patients (5.9%) with 96 (2.2%) having non-CABG-related major bleeding.

Cumulative rates of the composite outcome of CV death and spontaneous MI were higher in the highest quartile groups of both lysis time and maximum turbidity compared with rates in the lowest quartile groups (Figure 3-2). This was primarily driven by higher rates of CV death in the highest quartile groups (Figure 3-3). After adjustment for CV risk factors (model 1), the highest quartile group of lysis time was associated with increased risk of CV death/spontaneous MI (HR 1.48; 95% CI 1.06-2.06; P = 0.027) and CV death alone (HR 1.92; 95 % CI 1.19-3.1; P < 0.001). As a continuous variable, each 50% increase in lysis time was associated with increased risk of CV death/spontaneous MI (HR 1.17; 95% CI 1.05-1.31; P = 0.006) and CV death alone (HR 1.36; 95% CI 1.17 – 1.59; P < 0.001). This association remained significant for lysis time after adjustment for inflammatory and prognostic biomarkers. Similarly, each 50% increase in maximum turbidity was associated with CV death alone (HR 1.24; 95% CI 1.03 – 1.50; P = 0.024) but this association was no longer significant after adjustment for inflammatory and prognostic biomarkers. Findings for all-cause mortality were similar to those for CV death (Figure 3-4 and tables 3-5 and 3-6).

There was no clear association with rates of stent thrombosis and stroke but event rates were low (Figures 3-6, 3-7). Neither lysis time nor maximum turbidity was able to predict major bleeding events (Tables 3-5, 3-6). Further characterisation of bleeding events is provided in tables 3-7 and 3-8.

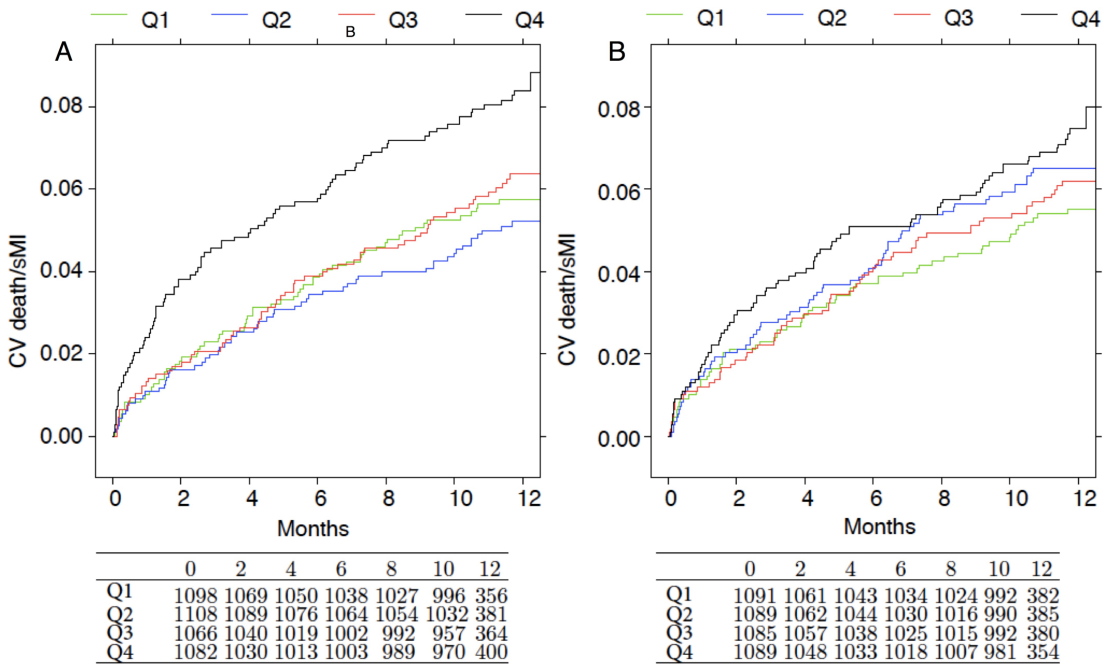
There was no significant impact of fibrin clot properties on the CV mortality reduction with ticagrelor compared with clopidogrel (interaction P values > 0.7) (Figure 3-5; Panels A,B). There was also no significant impact of fibrin clot properties on the association

between randomized treatment and major bleeding (Figure 3-5; Panels C,D). Similarly, the association between fibrin clot properties and CV death was present irrespective of subtype of ACS presentation (all interaction P > 0.4). The predictive value of fibrin clot parameters was not affected by LMWH treatment in the last 2 days, being consistent amongst those who received LMWH in the last two days compared with those who did not (Figure 3-8).

The predictive value of fibrin clot lysis time was not affected by treatment strategy. However, the relationship between maximum turbidity and CV death appeared more pronounced in invasively-treated patients (Figure 3-9).

3.3.4 Prognostic value of lysis time

Model performance to predict the composite outcome of CV death/sMI significantly improved when lysis time was added to a clinical predictive model: C-index 0.67 (0.637 - 0.703) for model 1 + lysis time vs. 0.665 (0.631 – 0.698) for model 1 only, P = 0.007. Prediction of CV death also significantly improved: C-index 0.7 (0.649 - 0.75) for model 1 + lysis time vs. 0.69 (0.642 – 0.741) for model 1 only, P < 0.001.

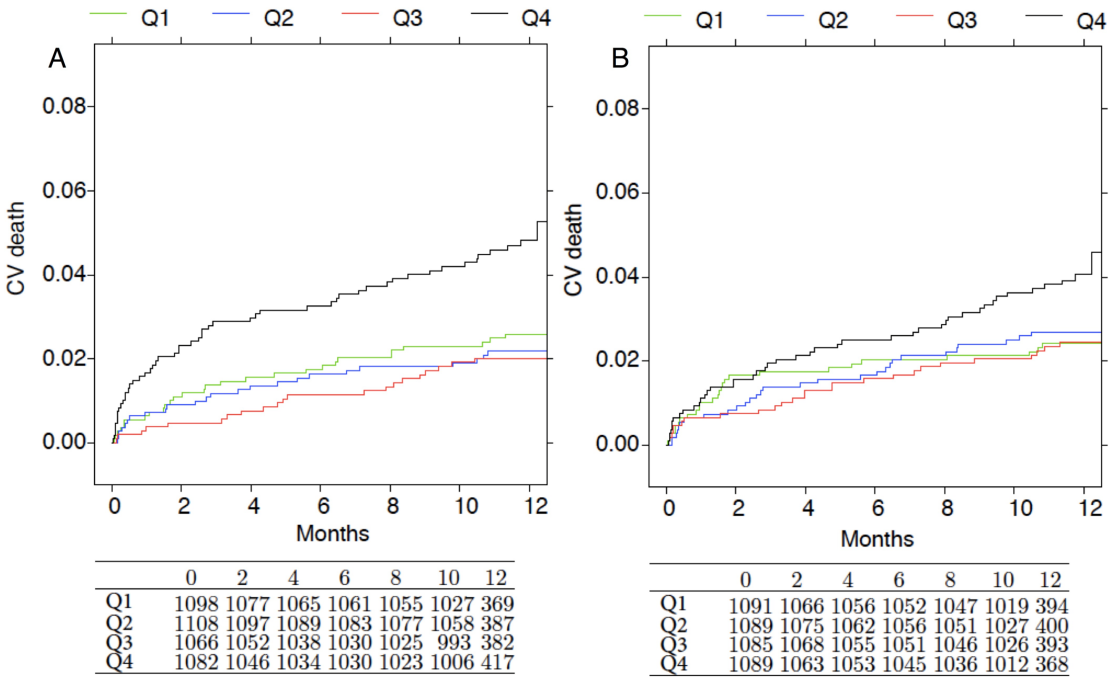


	0	2	4	6	8	10	12
Q1	1098	1069	1050	1038	1027	996	356
Q2	1108	1089	1076	1064	1054	1032	381
Q3	1066	1040	1019	1002	992	957	364
Q4	1082	1030	1013	1003	989	970	400

	0	2	4	6	8	10	12
Q1	1091	1061	1043	1034	1024	992	382
Q2	1089	1062	1044	1030	1016	990	385
Q3	1085	1057	1038	1025	1015	992	380
Q4	1089	1048	1033	1018	1007	981	354

Figure 3-2: Relationship between fibrin clot parameters and 1-year rates of CV death or sMI

Kaplan-Meier curves for rates of the combined outcome of cardiovascular (CV) death/spontaneous myocardial infarction (MI) per quartile groups of lysis time (Panel A) and maximum turbidity (Panel B).



	0	2	4	6	8	10	12
Q1	1098	1077	1065	1061	1055	1027	369
Q2	1108	1097	1089	1083	1077	1058	387
Q3	1066	1052	1038	1030	1025	993	382
Q4	1082	1046	1034	1030	1023	1006	417

	0	2	4	6	8	10	12
Q1	1091	1066	1056	1052	1047	1019	394
Q2	1089	1075	1062	1056	1051	1027	400
Q3	1085	1068	1055	1051	1046	1026	393
Q4	1089	1063	1053	1045	1036	1012	368

Figure 3-3: Relationship between fibrin clot parameters and 1-year rates of CV death

Kaplan-Meier curves for 1-year rates of cardiovascular (CV) death per quartile groups of lysis time (Panel A) and maximum turbidity (Panel B).

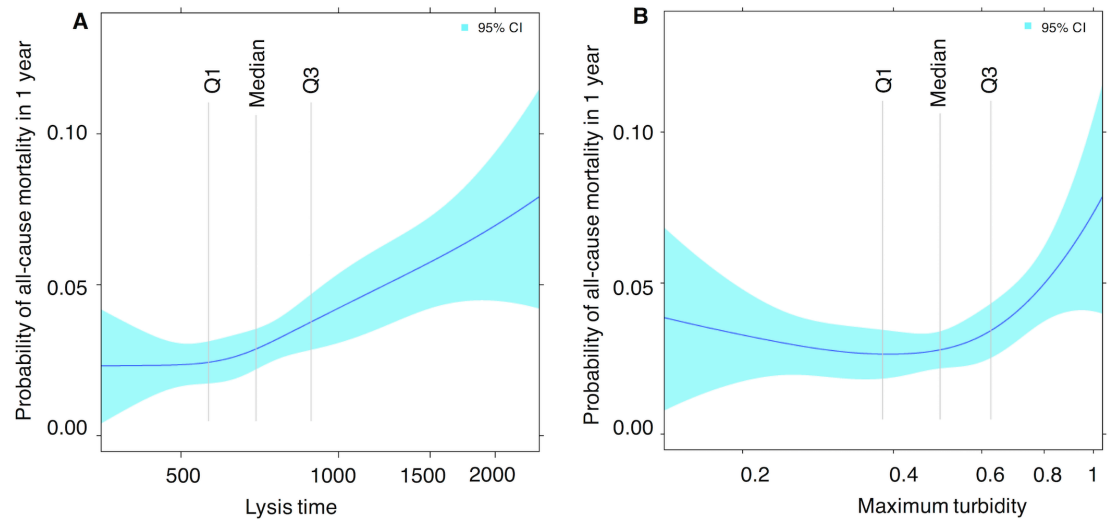


Figure 3-4: Relationship between fibrin clot parameters and 1-year all-cause mortality

1-year rates of all-cause mortality in relation to lysis time (secs) (A) and maximum turbidity (AU) (B) transformed using restricted cubic splines. Shaded areas represent 95% confidence intervals. Vertical lines indicate quartiles.

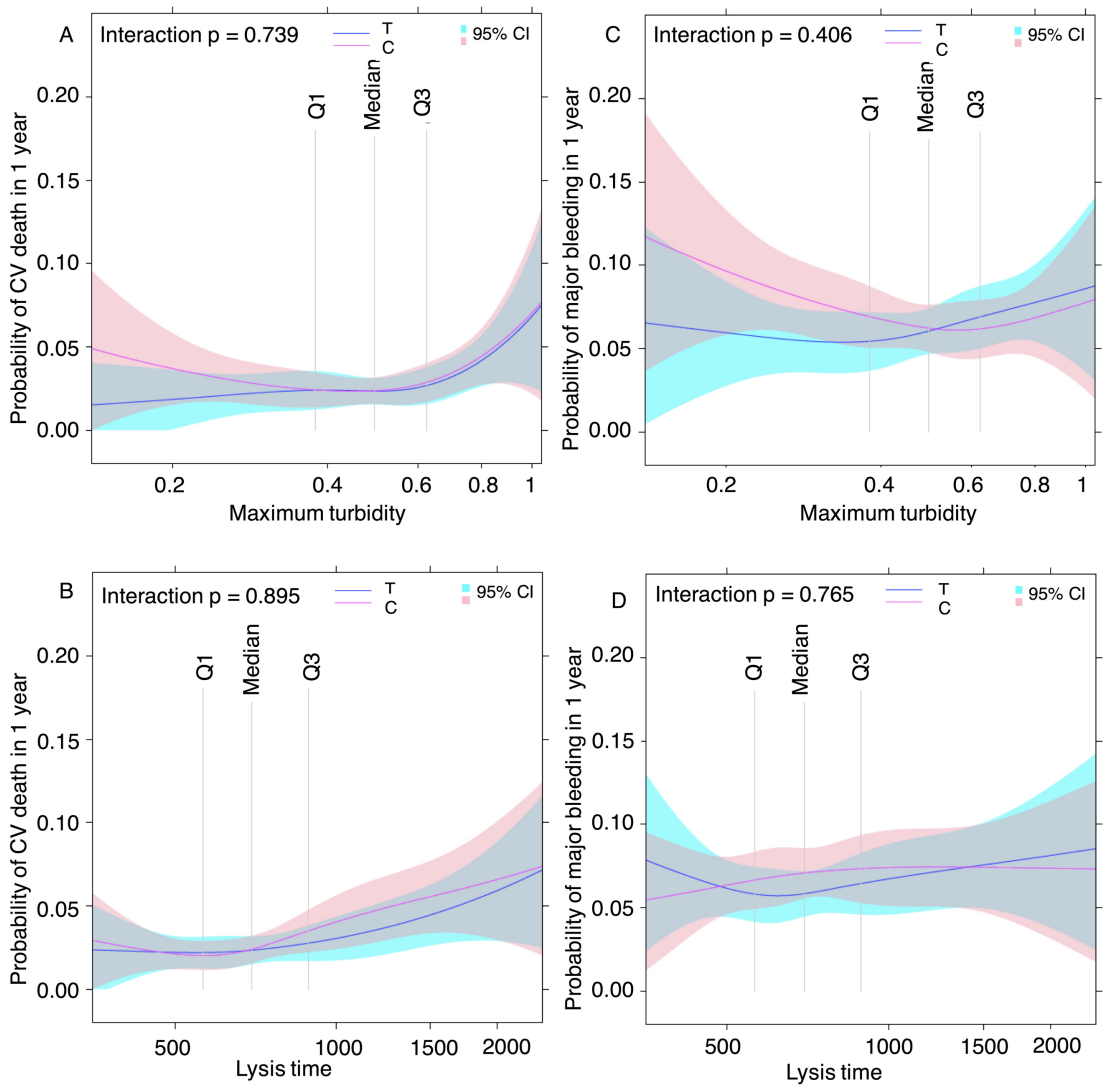


Figure 3-5: Relationship between fibrin clot parameters and 1-year rates of CV death or major bleeding according to randomized treatment group

1-year rates of CV death (Panels A and B) or major bleeding (Panels C and D) in relation to maximum turbidity (AU) (Panels A and C) or lysis time (secs) (Panels B and D), transformed using restricted cubic splines, according to randomized treatment with clopidogrel (C, pink lines) or ticagrelor (T, blue lines). Shaded areas represent 95% confidence intervals. Vertical lines indicate quartiles limits.

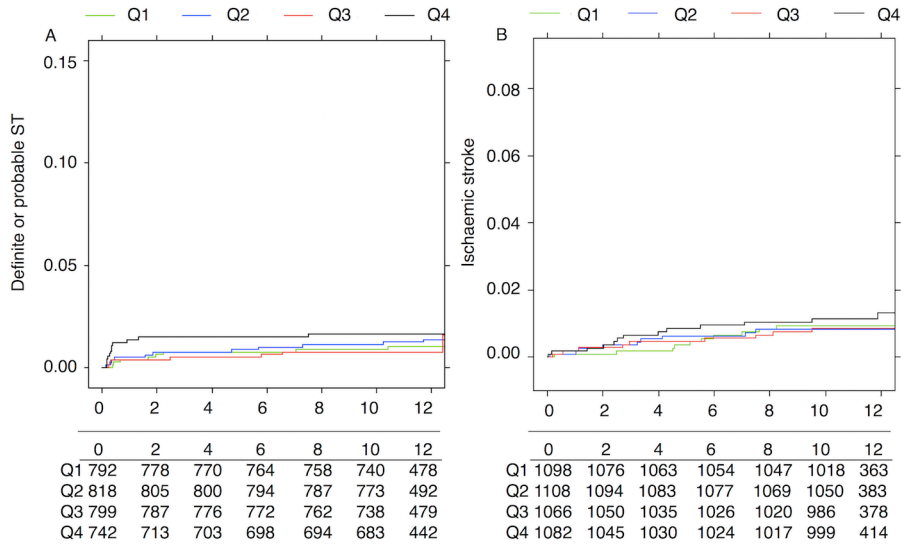


Figure 3-6: Relationship between lysis time and 1-year rates of stent thrombosis (A) and ischaemic stroke (B)

Kaplan-Meier curves for 1 year rates of definite or probable stent thrombosis (ST, panel A) and ischaemic stroke (Panel B) per quartile groups of lysis time

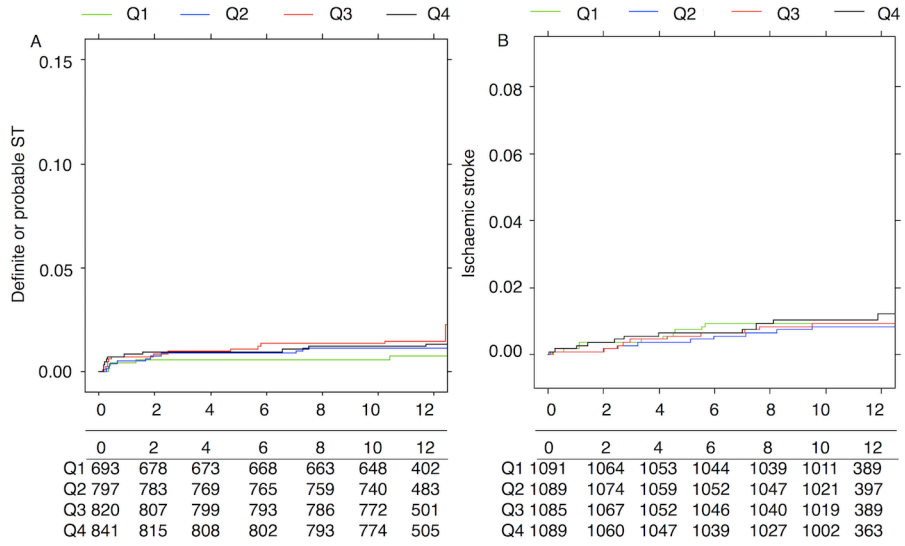


Figure 3-7: Relationship between maximum turbidity and 1-year rates of stent thrombosis (A) and ischaemic stroke (B)

Kaplan –Meier curves for 1 year rates of definite or probably stent thrombosis (ST, panel A) and ischaemic stroke (Panel B) per quartile groups of maximum turbidity

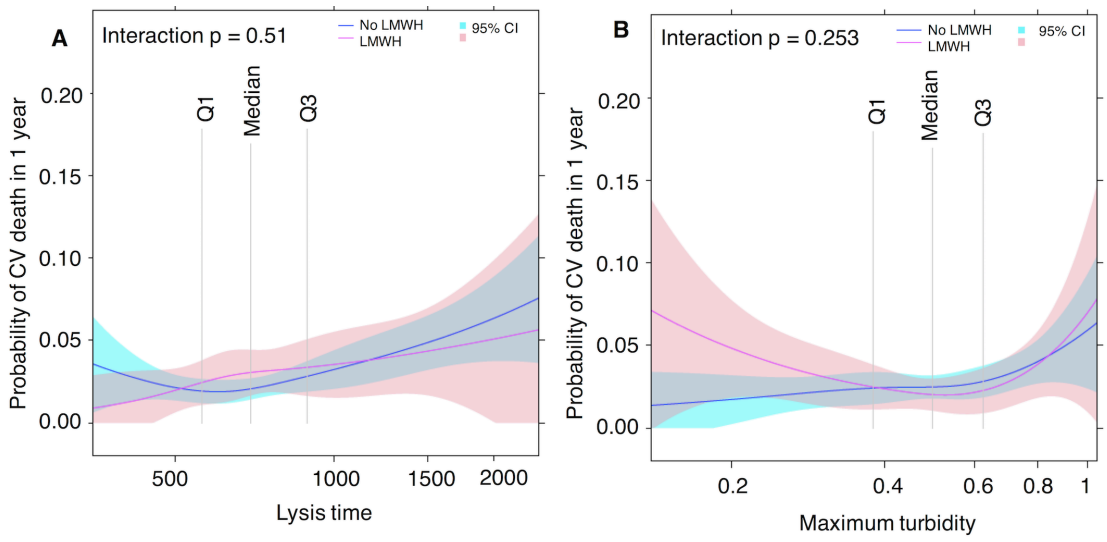


Figure 3-8: Relationship between fibrin clot parameters and 1-year rates of CV death according to treatment with LMWH within the last 2 days

1-year rates of CV death in relation to lysis time (secs) (Panel A) and maximum turbidity (AU) (Panel B), transformed using restricted cubic splines, according to LMWH treatment in the last 2 days with LMWH (pink lines) or no LMWH (blue lines). Shaded areas represent 95% confidence intervals. Vertical lines indicate quartiles.

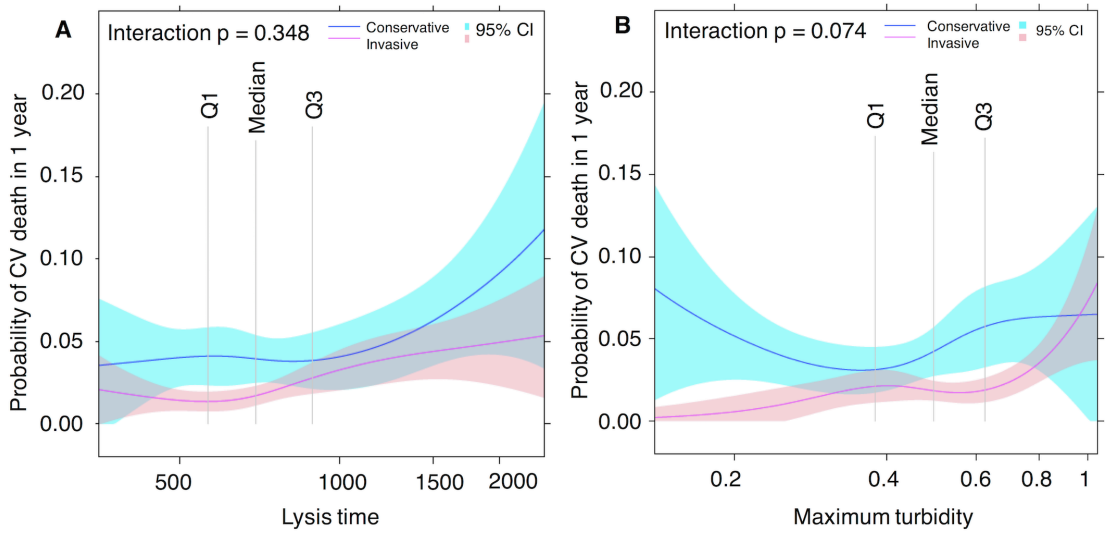


Figure 3-9: Relationship between fibrin clot parameters and 1-year rates of CV death according to treatment strategy

1-year rates of CV death in relation to lysis time (secs) (Panel A) and maximum turbidity (AU) (Panel B), transformed using restricted cubic splines, according to treatment strategy with invasive treatment (pink lines) or conservative treatment (blue lines). Shaded areas represent 95% confidence intervals. Vertical lines indicate quartiles.

Table 3-5: Associations between lysis time and clinical outcomes

Event	Model	Continuous Lysis time	P value	Lysis time quartile groups (HR compared to the lowest quartile group)			P value
		HR per 50% increase		Q2 (564-696 secs)	Q3 (696-888 secs)	Q4 (>888 secs)	
CV death/ sMI	Model 1	1.17 (1.05-1.31)	0.006*	0.92 (0.64-1.32)	1.09 (0.77-1.55)	1.48 (1.06-2.06)	0.027*
	Model 2	1.15 (1.01-1.30)	0.032*	0.88 (0.60-1.31)	1.09 (0.75-1.60)	1.40 (0.97-2.03)	0.088
CV death	Model 1	1.36 (1.17-1.59)	<0.001*	0.87 (0.50-1.51)	0.79 (0.45-1.39)	1.92 (1.19-3.10)	<0.001*
	Model 2	1.20 (1.01-1.42)	0.042*	0.82 (0.46-1.47)	0.79 (0.43-1.44)	1.44 (0.85-2.45)	0.081
All-cause death	Model 1	1.39 (1.20-1.61)	<0.001*	1.04 (0.62-1.77)	1.24 (0.74-2.06)	2.24 (1.40-3.57)	0.001*
	Model 2	1.21 (1.03-1.42)	0.021*	0.95 (0.54-1.67)	1.08 (0.62-1.88)	1.61 (0.96-2.7)	0.131
sMI	Model 1	1.08 (0.94-1.25)	0.287	0.83 (0.53-1.28)	1.14 (0.76-1.72)	1.17 (0.77-1.77)	0.36
	Model 2	1.12 (0.96-1.31)	0.15	0.80 (0.50-1.29)	1.13 (0.73-1.77)	1.24 (0.79-1.96)	0.29
Major bleeding	Model 1	1.09 (0.96-1.23)	0.18	1.03 (0.72-1.47)	1.07 (0.75-1.53)	1.27 (0.89-1.80)	0.56
	Model 2	1.07 (0.94-1.23)	0.31	1.04 (0.72-1.51)	1.04 (0.71-1.52)	1.20 (0.81-1.76)	0.81

Estimates are HRs (95% CI). Model 1 included adjustments for age, gender, BMI, Diabetes, Dyslipidemia, hypertension, chronic kidney disease (CKD), smoking, type of ACS, randomized treatment and previous myocardial infarction, revascularization, peripheral artery disease, congestive heart failure or cerebrovascular disease. Model 2 included adjustments as per model 1 (excluding CKD) and CRP, leukocyte count, cystatin C, NT-proBNP, high-sensitivity troponin T and GDF 15. CV: cardiovascular; sMI: spontaneous myocardial infarction. * indicates a statistically significant result.

Table 3-6: Associations between maximum turbidity and clinical outcomes

Event	Model	Continuous maximum turbidity	P value	Maximum turbidity quartile groups (HR compared to the lowest quartile group)			P value
		HR per 50% increase		Q2 (0.38-0.5 au)	Q3 (0.5-0.62 au)	Q4 (>0.62 au)	
CV death/ sMI	Model 1	1.13 (0.99-1.27)	0.06	1.3 (0.92-1.84)	1.21 (0.85-1.72)	1.44 (1.02-2.03)	0.2
	Model 2	1.01 (0.88-1.16)	0.87	1.25 (0.85-1.83)	1.03 (0.69-1.54)	1.14 (0.76-1.70)	0.64
CV death	Model 1	1.24 (1.03-1.50)	0.024*	1.21 (0.71-2.07)	1.04 (0.60-1.81)	1.77 (1.07-2.9)	0.08
	Model 2	0.96 (0.79–1.16)	0.65	1.04 (0.59-1.84)	0.80 (0.44-1.44)	0.88 (0.50-1.57)	0.8
All-cause death	Model 1	1.22 (1.03-1.46)	0.024*	1.17 (0.71-1.92)	1.10 (0.67-1.83)	1.71 (1.07-2.72)	0.09
	Model 2	0.96 (0.80-1.15)	0.639	1 (0.58-1.71)	0.85 (0.49-1.48)	0.93 (0.54-1.59)	0.93
sMI	Model 1	1.08 (0.93-1.25)	0.332	1.25 (0.82-1.9)	1.26 (0.83-1.93)	1.28 (0.83-1.95)	0.63
	Model 2	1.06 (0.89-1.27)	0.483	1.27 (0.80-2.03)	1.18 (0.73-1.91)	1.35 (0.82-2.23)	0.64
Major bleeding	Model 1	0.99 (0.87-1.12)	0.89	0.87 (0.61-1.24)	0.96 (0.68-1.35)	1 (0.71-1.41)	0.86
	Model 2	0.93 (0.81-1.07)	0.324	0.8 (0.55-1.17)	0.94 (0.65-1.36)	0.85 (0.57-1.27)	0.68

Estimates are HRs (95% CI). AU: arbitrary units; other abbreviations as per table 3-5. Models 1 and 2 of adjustments as per table 3-5. * indicates a statistically significant result

Table 3-7: Bleeding events per lysis time quartile groups

Type of bleeding	All	Lysis time (secs) quartile group			
		Q1 (<564) n = 1098	Q2 (564-696) n = 1108	Q3 (696-888) n = 1066	Q4 (>888) n = 1082
Fatal	4 (0.09%)	1 (0.09%)	0 (0.00%)	2 (0.19%)	1 (0.09%)
Major CABG-related	165 (3.82%)	41 (3.77%)	47 (4.28%)	38 (3.59%)	39 (3.64%)
Major Non-CABG related	96 (2.22%)	22 (2.02%)	17 (1.55%)	28 (2.64%)	29 (2.71%)
Other major non-CABG related	11 (0.26%)	5 (0.46%)	0 (0.00%)	1 (0.09%)	5 (0.47%)
Life-threatening/fatal non-CABG related	16 (0.37%)	3 (0.28%)	3 (0.27%)	6 (0.57%)	4 (0.37%)
Intracranial	5 (0.12 %)	1 (0.09%)	1 (0.09%)	2 (0.188%)	1 (0.09%)
Minor	101 (2.34%)	21 (1.93%)	28 (2.55%)	23 (2.17%)	29 (2.71%)
Major and minor	344 (7.97%)	79 (7.26%)	89 (8.11%)	82 (7.74%)	94 (8.77%)
Major, minor and minimal	381 (8.83%)	90 (8.27%)	96 (8.75%)	93 (8.78%)	102 (9.52%)

For bleeding definitions, see appendix.

Table 3-8: Bleeding events per maximum turbidity quartile groups

Type of bleeding	All	Maximum turbidity (AU) quartile group			
		Q1 (≤ 0.38) n = 1091	Q2 (0.38-0.5) n = 1089	Q3 (0.5-0.62) n = 1085	Q4 (>0.62) n = 1089
Fatal	4 (0.09 %)	0 (0.00 %)	1 (0.09%)	1 (0.09%)	2 (0.19%)
Major CABG-related	165 (3.82%)	45 (4.17%)	39 (3.61%)	41 (3.80%)	40 (3.71%)
Major non-CABG related	96 (2.22%)	24 (2.22%)	18 (1.67%)	24 (2.22%)	30 (2.79%)
Other major non-CABG related	11 (0.26%)	4 (0.37%)	2 (0.19%)	1 (0.09%)	4 (0.37%)
Life-threatening/fatal non-CABG related	16 (0.37%)	4 (0.37%)	4 (0.37%)	3 (0.28%)	5 (0.46%)
Intracranial	5 (0.12%)	0 (0.00%)	2 (0.18%)	1 (0.09%)	2 (0.18%)
Minor	101 (2.34%)	28 (2.59%)	23 (2.13%)	23 (2.13%)	27 (2.51%)
Major and minor	344 (7.97%)	92 (8.52%)	76 (7.04%)	84 (7.79%)	92 (8.54%)
Major, minor and minimal	381 (8.83%)	100(9.26%)	87 (8.06%)	94 (8.71%)	100 (9.29%)

For bleeding definitions, see appendix

3.3.5 Supplementary analysis of fibrin clot lag time

Lag time was confounded by inpatient LMWH treatment: 76% of patients in the highest quartile group received LMWH, compared to only 38% in the lowest quartile group ($p < 0.001$). Excluding patients who had received LMWH within 24 hours of blood sampling removed the imbalance in LMWH treatment between lag time quartile groups. Median (IQR) lag time was 348 (300-412) secs and there was no significant relationship between lag time measurements and clinical outcomes ($P > 0.2$ for all associations).

3.3.6 Predictive value of fibrin clot properties in patients with diabetes mellitus

972 patients had DM. Of those, 48 patients (4.9%) had CV death, 72 (7.4%) had sMI, 67 (2.9%) had major bleeding and 21 (2.2%) had non CABG-related major bleeding.

Cumulative rates of CV death or sMI increased with increasing levels of lysis time with no clear relationship between lysis time and major bleeding events (Figure 3-10). Similarly, rates of CV death or sMI increased with increasing levels of maximum turbidity. Rates of major bleeding events, however, appeared higher at lower levels of maximum turbidity (Figure 3-11).

After adjustment for clinical characteristics and CV disease risk factors (model 1 except DM), each 50% increase in lysis time was associated with increased risk of CV death/sMI (HR 1.21; 95% CI 1.02-1.44; $P = 0.026$) and CV death alone (HR 1.38; 95% CI 1.08-1.76; $P = 0.01$). Similarly, each 50% increase in maximum turbidity was associated with increased risk of CV death/sMI (HR 1.25; 95% CI 1.02-1.53; $P=0.031$) and CV death alone (HR 1.49; 95% CI 1.08-2.04; $P = 0.014$). The trend for lower bleeding rates with lower maximum turbidity levels was not statistically significant ($P = 0.15$).

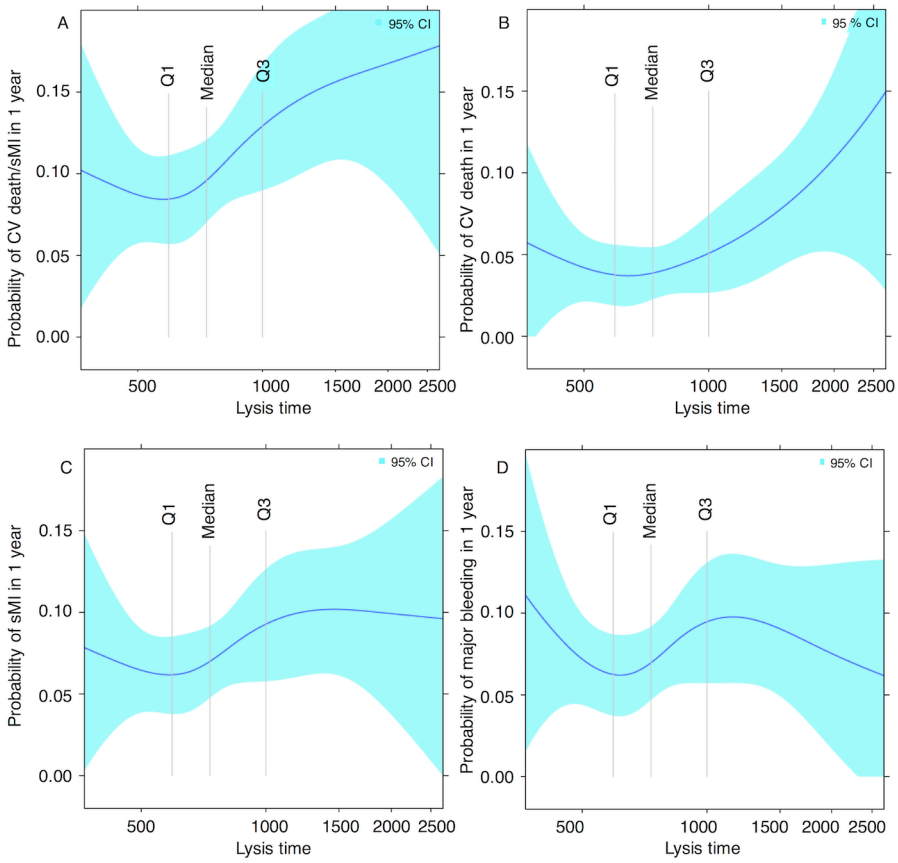


Figure 3-10: Relationship between fibrin clot lysis time and 1-year clinical outcomes in patients with diabetes mellitus

1-year rates of CV death/sMI (A), CV death alone (B), sMI alone (C) and major bleeding (D) in relation to lysis time (secs) transformed using restricted cubic splines. Shaded areas represent 95% confidence intervals. Vertical lines indicate quartiles.

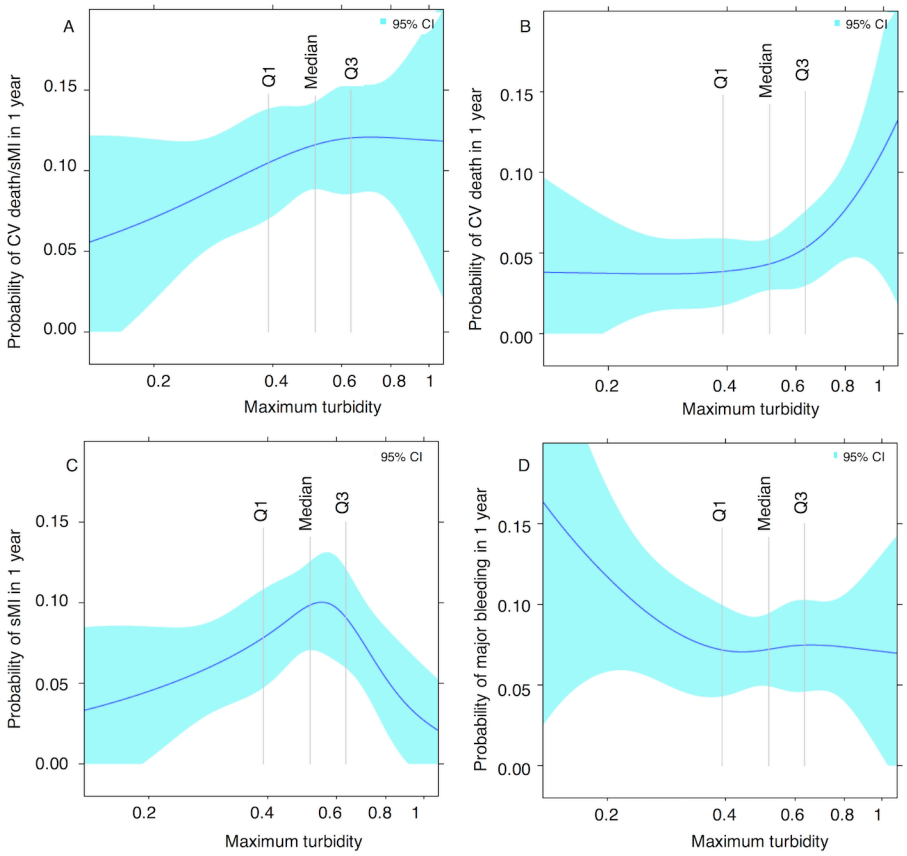


Figure 3-11: Relationship between fibrin clot maximum turbidity and 1-year clinical outcomes in patients with diabetes mellitus

1-year rates of CV death/sMI (A), CV death alone (B), sMI alone (C) and major bleeding (D) in relation to maximum turbidity (AU) transformed using restricted cubic splines. Shaded areas represent 95% confidence intervals. Vertical lines indicate quartiles.

3.3.7 Fibrinogen levels and fibrin clot properties

37 plasma samples stored locally at Sheffield from ACS patients were used for this analysis. These samples were collected at day 7 post-ACS. Significant correlations were present between fibrinogen levels and maximum turbidity but there was no clear relationship between lysis time and fibrinogen levels (Figure 3-12). These results indicate that fibrinogen levels play a more pronounced role in fibrin clot density compared to lysis potential.

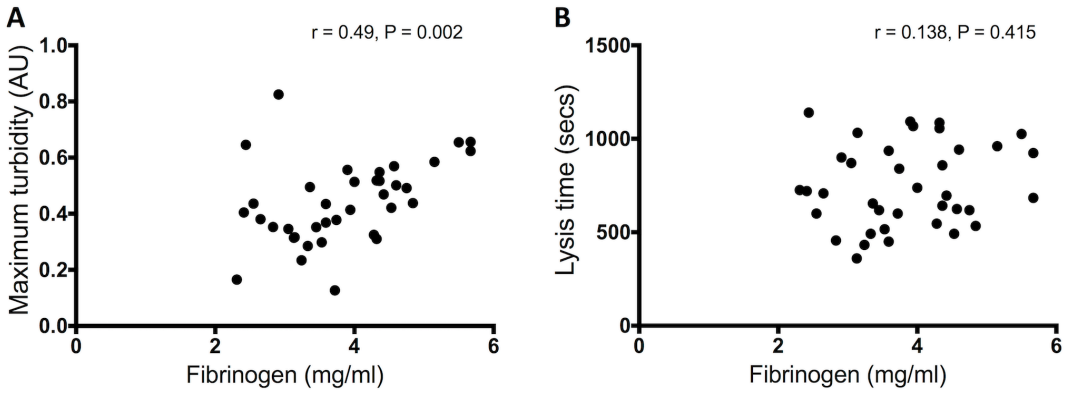


Figure 3-12: Correlations between fibrinogen levels and maximum turbidity (A) and lysis time (B)

n=37, r value refers to Spearman's correlation coefficient.

3.4 Discussion

We have shown, in a large population of ACS patients treated with contemporary therapies and followed up for up to one year, that fibrin clot properties independently predict the risk of spontaneous MI and CV death following initial in-hospital management. Importantly, lysis time predicted worse outcome after adjusting for other established or new prognostic biomarkers, thus indicating the potential for a further biomarker that provides insight into prognosis following ACS. The association between lysis time and the levels of the established biomarkers also raises the possibility that variability in fibrin clot properties might contribute to the association of these other biomarkers with thrombotic events. Given that antithrombotic therapy is mainly centred around antiplatelet agents following the acute phase, our data support the hypothesis that at least a subgroup of patients might benefit from additional therapy that aims at improving lysis potential. For example, further work could explore whether anticoagulant therapy, in combination with a platelet $P2Y_{12}$ receptor antagonist, offers an advantage over dual antiplatelet therapy in those with adverse fibrin clot properties. Other novel therapies that specifically target proteins implicated in impaired lysis, such

as complement C3 or plasmin inhibitor, is another approach that might have less impact on haemostasis, particularly if the aim was to normalise lysis potential (249).

Studying fibrin clot properties is attractive for many reasons. First, fibrinogen conversion and cross-linking of fibrin fibres to form a stable network is a key step in the formation of an obstructive vascular thrombus. Second, thrombotic occlusion of coronary arteries could represent a failure of the protective endogenous thrombolytic mechanisms to lyse clots before they become occlusive. Third, this assay provides a functional and simple assessment of the complex interactions between different clotting/lysis factors and other plasma proteins and takes into account both quantitative and qualitative changes in coagulation factors that may affect fibrinolytic efficiency (249, 250). Fourth, this is a relatively cheap, easy and reproducible test to perform and, importantly, fibrin clots that resist lysis might give us mechanistic insights into recurrent events.

Previous studies, using different assays in whole blood, have shown a positive association between prolonged lysis and CV death (251, 252). Another study using thromboelastography in plasma showed similar results (253). However, these were small studies with limited event rates and assessment of clotting and lysis in whole blood cannot reliably differentiate between the cellular and protein components of thrombus formation.

The relationships between adverse fibrin clot dynamics and some clinical characteristics demonstrated in our work are consistent with evidence obtained from smaller observational, cross-sectional studies. For example, DM, CKD and PAD, all high-risk conditions for cardiac ischaemia, have shown associations with adverse fibrin clot characteristics (143, 247, 248).

Approximately a quarter of ACS patients have DM (7, 254). DM is associated with increased ischaemic and bleeding risk and this can make treating these patients

challenging (7, 78, 242, 255). Despite the strong association between DM and adverse fibrin clots, dense clots with resistance to lysis appear to offer additional prognostic information without an association with bleeding. Therefore, studying fibrin clots in patients with DM in particular, could be a useful tool to guide antithrombotic treatments in the future.

Evidence suggests that inflammation results in prothrombotic states (138, 153). The strong correlations between fibrin clot properties and inflammatory markers support previous work demonstrating that inflammation leads to prothrombotic changes in fibrin clot dynamics and illustrates one mechanism for the higher risk of atherothrombotic events in patients with higher levels of inflammatory markers (195, 196).

The relationship between maximum turbidity and worse outcomes lost significance after adjusting for other biomarkers. In contrast to lysis time, fibrinogen levels have greater effect on maximum turbidity (229, 256) and fibrinogen levels go hand-in-hand with inflammatory markers, particularly CRP (257).

Interestingly, fibrin clot density and resistance to lysis increased with increasing levels of NT-proBNP and troponin T. The exact molecular mechanisms for these associations are difficult to ascertain. However, higher troponin T and NT-proBNP reflect larger infarcts and these are associated with a greater inflammatory response, which might account for some of the prothrombotic changes. NT-proBNP has been shown to add prognostic value regardless of the degree of necrosis after ACS (258). Our findings, therefore, point to an additional mechanism, beyond the increased risks of death from heart failure and arrhythmia associated with left ventricular systolic dysfunction, whereby NT-proBNP is associated with worse outcome, as a consequence of more dense fibrin clots that resist lysis leading to increased risk of atherothrombosis.

Turbidimetric analysis of fibrin clots requires trained laboratory personnel and therefore is not suitable as a bedside test. Similar to other clotting assays, results might be influenced by high-level anticoagulant therapy and significant liver conditions, which make results difficult to interpret in those scenarios. A limitation to this study is that it only provides a "snapshot" assessment of fibrin clot characteristics at hospital discharge (median 6 days). It is established that internal fibrinolytic activity has a circadian rhythm largely driven by variations in plasminogen activator inhibitor-1 activity (259). Unfortunately, sampling times are not available in our database but samples were collected during office working hours. The clear relationship with DM and biomarker levels, which were all measured at baseline, reassure us that variation is likely to be marginal. In Chapter 4, I seek to assess the stability of this phenotype over time and future analyses will explore how the relationship with clinical outcome could change in a stable patient cohort.

3.5 Conclusions

Despite strong relationships with clinical risk factors, particularly DM, and inflammatory and other prognostic biomarkers, the resistance of fibrin clots to lysis independently predicts CV death following ACS. These findings suggest that novel therapies targeting fibrin clot properties might be a new avenue for improving clinical outcomes in patients with ACS.

4 Prolonged Fibrin Clot Lysis Persists 1-month Following Acute Coronary Syndrome: A Plato Substudy

4.1 Abstract

Background: We have shown, in the previous chapter, that prolonged fibrin clot lysis at hospital discharge independently predicts cardiovascular death following ACS. The acute inflammatory response observed following ACS may drive some of the changes in fibrinolysis and therefore we studied fibrin clot properties 1-month post ACS and compared those to results obtained at hospital discharge.

Methods: A validated turbidimetric assay was used to determine fibrin clot lysis time and clot maximum turbidity in 4,032 plasma samples collected at 1-month post-ACS from patients in the PLATO trial. Potential differences in clot properties at hospital discharge and 1 month were investigated. Change in clot parameters between discharge and 1 month was correlated to change in CRP and WCC levels.

Results: There was little difference in mean (±SD) lysis time comparing hospital discharge with 1-month results (803±426 vs. 817±388 secs, respectively; P<0.001), the slight increase after hospital discharge being explained by in-hospital LMWH treatment. Conversely, mean maximum turbidity reduced from 0.51±0.19 AU at discharge to 0.42±0.14 AU at 1 month (P<0.001). While the relationships between change in lysis time and change in inflammatory markers were weak, the relationship between change in clot maximum turbidity and change in CRP (in particular) appeared linear. The prevalence of diabetes mellitus, chronic kidney disease and peripheral artery disease was highest in the highest quartile groups of both lysis time (>882 secs) and maximum turbidity (>0.49 AU) at 1 month.

Conclusion: Resolution of systemic inflammation at 1-month post-ACS is associated with a drop in fibrin clot density but little effect on lysis potential. This indicates that clot density has little effect on lysis time and the derangement in fibrinolysis is not related to an enhanced inflammatory environment in ACS patients. The impact of contemporary therapy on lysis efficiency is limited, which may therefore constitute a suitable target for secondary prevention therapy, particularly in individuals with multiple risk factors.

4.2 Background

We have shown fibrin clots that resist lysis to be independently related to adverse clinical outcomes following ACS. Prolonged lysis time and increased density were strongly related to acute inflammatory markers measured at baseline. In a human sepsis model, induced by endotoxin, acute inflammatory response resulted in prothrombotic changes in fibrin structure (153). Consequently, some of the adverse fibrin properties might be attenuated once inflammation settles.

On the other hand, high-risk vascular conditions have shown a clear relationship with prolonged lysis and increased density indicating a possibly stable phenotype. Large longitudinal studies, exploring the change in fibrin clots following ACS, are lacking. Understanding the extent of change in this phenotype and how this relates to changes in inflammatory markers might aid our efforts to optimise treatment.

In this chapter, I aim to study fibrin clot properties in plasma samples collected 1-month post ACS and compare measurements to results obtained at hospital discharge.

4.3 Results

4.3.1 The relationships between fibrin clot properties, clinical characteristics and biomarkers

Tables 4-1 and 4-2 summarise how different characteristics vary across the four quartile groups of lysis time and maximum turbidity.

Similar to what was observed with hospital discharge results, higher percentages of patients in the highest quartile groups of both lysis time and maximum turbidity had DM, CKD and PAD. Body mass index also increased with increasing quartile groups of both lysis time and maximum turbidity.

Interestingly, female patients had significantly longer lysis time. This observation was consistent with hospital discharge results.

The associations between lysis time and baseline cystatin C, GDF-15 and CRP remained significant, with increasing values of each of these biomarkers in the highest quartile groups.

Levels of baseline inflammatory markers and prognostic biomarkers (troponin T and NT-proBNP) also significantly increased with increasing levels of maximum turbidity.

1-month inflammatory markers were also significantly associated with both lysis time and maximum turbidity (Tables 4-3, 4-4).

Table 4-1: Baseline clinical characteristics and biomarkers across lysis time quartile groups

Variables	Lysis time (secs) quartile group				P value
	Q1 (<582) n = 1036	Q2 (582-702) n = 1001	Q3 (702-882) n = 989	Q4 (>882) n = 1006	
Demographics and medical history					
Age (years)	62 (54 - 70)	62 (54 - 71)	62 (53 - 71)	61 (53 - 69)	0.33
Female	198 (19.1%)	263 (26.3%)	324 (32.8%)	404 (40.2%)	< 0.001
BMI (kg/m^2)	27 (24.5 - 29.4)	27.7 (24.7 - 30.4)	27.7 (25.3 - 30.8)	28.7(25.7 - 31.7)	< 0.001
Current smoker	400 (38.6%)	379 (37.9%)	388 (39.2%)	331 (32.9%)	0.01
Hypertension	583 (56.3%)	654 (65.3%)	659 (66.6%)	739 (73.5%)	< 0.001
Hyperlipidaemia	407 (39.3%)	438 (43.8%)	404 (40.8%)	439 (43.6%)	0.11
Diabetes mellitus	191 (18.4%)	204 (20.4%)	230 (23.3%)	277 (27.5%)	< 0.001
PAD	50 (4.8%)	74 (7.4%)	54 (5.5%)	75 (7.5%)	0.03
CKD	21 (2.0%)	30 (3.0%)	35 (3.5%)	45 (4.5%)	0.02
Type of ACS					
STE-ACS	524 (50.6%)	481 (48.1%)	439 (44.4%)	443 (44.0%)	0.01
Randomized treatment					
Ticagrelor	498 (48.1%)	485 (48.5%)	504 (51.0%)	510 (50.7%)	0.44
Baseline biomarkers					
Troponin T (ng/L)	149 (42 - 564)	162 (39 - 542)	146 (39 - 567)	129 (30 - 455)	0.08
NT-proBNP (pmol/L)	357(122-1038)	396 (133-941)	410(131-1099)	369(126 - 1080)	0.78
Cystatin C (mg/L)	0.8 (0.6 - 0.9)	0.8 (0.6 - 1.0)	0.8 (0.7 - 1.0)	0.9 (0.7 - 1.0)	< 0.001
GDF-15 (ng/L)	1446(1083-2044)	1505(1126-2136)	1570(1179-2154)	1536(1125-2161)	0.01
CRP (mg/L)	2.7 (1.2 - 6.3)	2.7 (1.4 - 6.9)	4.0 (1.7 - 9.0)	4.4 (1.9 - 10.0)	< 0.001
WCC ($*10^9/L$)	9.2 (7.4 - 11.5)	9.3 (7.4 - 11.6)	9.4 (7.6 - 11.9)	9.5 (7.5 - 11.9)	0.27

Values are medians (IQRs) for continuous data and n (%) for categorical data. BMI: body mass index; HTN: hypertension; DM: diabetes mellitus; MI: myocardial infarction; PAD: peripheral artery disease; CKD: chronic kidney disease; STE-ACS: ST-elevation acute coronary syndrome; NT-proBNP: N-terminal pro b-type natriuretic peptide; GDF: growth differentiating factor; CRP: C-reactive protein; WCC: white cell count. P values calculated using Chi-square test (categorical variables) or Kruskal-Wallis test (continuous variables)

Table 4-2: Baseline clinical characteristics and biomarkers across maximum turbidity quartile groups

Variables	Maximum turbidity (AU) quartile groups				P value
	Q1 (<0.33) n = 1008	Q2 (0.33-0.41) n = 1009	Q3 (0.41-0.49) n = 1009	Q4 (>0.49) n = 1006	
Demographics and medical history					
Age (years)	60 (53 - 69)	61 (53 - 70)	62 (54 - 71)	64 (56 - 71)	< 0.001
Female	276 (27.4%)	309 (30.6%)	295 (29.2%)	309 (30.7%)	0.32
BMI (kg/m^2)	27.1 (24.5 - 30)	27.5 (25.1 - 30.4)	28 (25.2 - 30.9)	28.1 (25.4 - 31.4)	< 0.001
Current smoker	356 (35.3%)	371 (36.8%)	356 (35.3%)	415 (41.3%)	0.02
Hypertension	617 (61.2%)	640 (63.4%)	672 (66.6%)	706 (70.2%)	< 0.001
Hyperlipidaemia	434 (43.1%)	420 (41.6%)	428 (42.4%)	406 (40.4%)	0.64
Diabetes mellitus	212 (21.0%)	191 (18.9%)	220 (21.8%)	279 (27.7%)	< 0.001
PAD	43 (4.3%)	54 (5.4%)	63 (6.2%)	93 (9.2%)	< 0.001
CKD	23 (2.3%)	20 (2.0%)	32 (3.2%)	56 (5.6%)	< 0.001
Type of ACS					
STE-ACS	469 (46.5%)	468 (46.4%)	465 (46.1%)	485 (48.2%)	0.77
Randomized treatment					
Ticagrelor	441 (43.8%)	471 (46.7%)	520 (51.5%)	565 (56.2%)	< 0.001
Baseline Biomarkers					
Troponin T (ng/L)	135 (30.9 - 531)	136 (34 - 443.5)	153 (42 - 502)	179 (46.1 - 632.5)	0.01
NT-proBNP (pmol/L)	305 (106 - 793)	339 (109 - 900)	398 (140 - 1108)	525 (172 - 1466)	< 0.001
Cystatin C (mg/L)	0.8 (0.6 - 0.9)	0.8 (0.6 - 0.9)	0.8 (0.7 - 1.0)	0.9 (0.7 - 1.1)	< 0.001
GDF-15 (ng/L)	1403 (1039 - 2029)	1400 (1072 -2004)	1539 (1126 - 2091)	1703 (1278 - 2374)	< 0.001
CRP (mg/L)	2.5 (1.2 - 5.8)	2.6 (1.2 - 6.4)	3.4 (1.6 - 7.5)	5.3 (2.4 - 13.0)	< 0.001
WBC ($*10^9/L$)	8.9 (7.1 - 11.3)	9.1 (7.3 - 11.4)	9.6 (7.5 - 11.6)	9.9 (8.1 - 12.1)	< 0.001

Values are medians (IQRs) for continuous data and n (%) for categorical data. BMI: body mass index; HTN: hypertension; DM: diabetes mellitus; MI: myocardial infarction; PAD: peripheral artery disease; CKD: chronic kidney disease; STE-ACS: ST-elevation acute coronary syndrome; NT-proBNP: N-terminal pro b-type natriuretic peptide; GDF: growth differentiating factor; CRP: C-reactive protein; WCC: white cell count. P values calculated using Chi-square test (categorical variables) or Kruskal-Wallis test (continuous variables)

Table 4-3: 1-month biomarker levels across maximum turbidity quartile groups

Variables	Maximum turbidity (AU) quartile groups				P value
	Q1 (<0.33) n = 1008	**Q2 (0.33-0.41) n = 1009**	**Q3 (0.41-0.49) n = 1009**	**Q4 (>0.49) n = 1006**	
NT-proBNP (pmol/L)	400 (171 – 876)	406 (187 – 1021)	506 (207 – 1111)	709 (308 – 1551)	< 0.001
CRP (mg/L)	1.2 (0.7 – 2.4)	1.7 (1.0 – 3.1)	2.6 (1.4 – 4.7)	6.2 (3.1 – 12)	< 0.001
WCC (*10^9/L)	6.7 (5.7 – 7.7)	6.9 (5.9 – 8.1)	7.2 (6.2 – 8.4)	8.1 (6.8 – 9.4)	< 0.001

Abbreviations as per tables 4-1 and 4-2. P values calculated using Kruskal-Wallis test.

Table 4-4: 1-month biomarker levels across lysis time quartile groups

Variables	Lysis time (secs) quartile group				P value
	Q1 (<582) n = 1036	**Q2 (582-702) n = 1001**	**Q3 (702-882) n = 989**	**Q4 (>882) n = 1006**	
NT-proBNP (pmol/L)	514(218– 1123)	470 (214 – 1098)	515 (205 – 1121)	501 (197 – 1107)	0.95
CRP (mg/L)	1.7 (0.8 – 3.7)	2.1 (1.1 – 4.4)	2.9 (1.4 - 6.0)	3.0 (1.4 - 6.2)	< 0.001
WCC (*10^9/L)	6.8 (5.7 – 8.2)	7.1 (5.8 – 8.4)	7.3 (6.2 – 8.6)	7.4 (6.3 – 8.8)	< 0.001

Abbreviations as per tables 4-1 and 4-2. P values calculated using Kruskal-Wallis test.

4.3.2 Change in fibrin clot properties between hospital discharge and 1-month

3,625 patients had fibrin clot measurements at both timepoints. Significant modest correlations were present between fibrin clot properties at hospital discharge and 1-month post ACS (lysis time: $r = 0.35$, $p < 0.001$; maximum turbidity: $r = 0.28$, $p < 0.001$) (Figure 4-1).

There was a slight increase in mean (±SD) lysis time at one month compared to hospital discharge (817±388 secs vs. 803±426 secs, respectively; P<0.001). This increase was more pronounced in patients who had received LMWH within two days of hospital discharge blood sampling (840±409 secs vs. 789±376 secs; P<0.001). In those who had not received LMWH within two days of hospital discharge sampling, there was no significant difference in lysis time (802±373 secs vs. 814±457 secs; P = 0.68).

In contrast, mean (±SD) maximum turbidity dropped at one month compared to hospital discharge (0.42±0.14 AU vs. 0.51±0.19 AU, respectively; P<0.001). Amongst those who had received LMWH within two days of hospital discharge blood sampling, the change was similar (0.43±0.13 AU vs. 0.51±0.19 AU; P<0.001).

To further characterise change in lysis time and maximum turbidity in paired samples, Bland-Altman plots were derived, excluding patients who had received LMWH within 2 days of sampling (Figure 4-2). Mean difference in lysis time was 12 secs; however, there seemed to be a degree of variability in lysis time in a proportion of patients, particularly those with high lysis time measurements (Figure 4-2 A). Despite this, 70% of patients in the highest quartile group at hospital discharge, remained in the highest two quartile groups at 1-month. Variation in patients with DM appeared similar to the whole cohort (Figure 4-2).

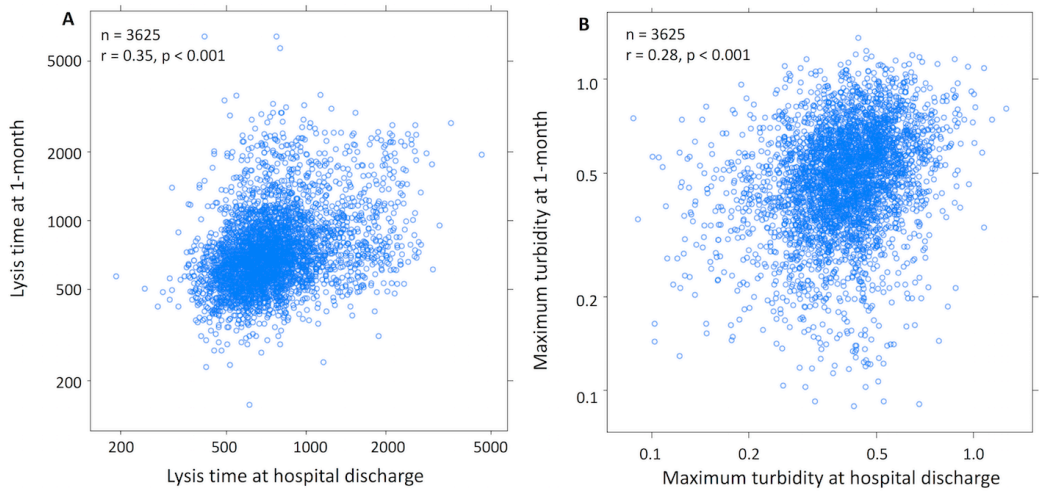


Figure 4-1: Correlation between fibrin clot properties at hospital discharge and 1-month (All patients)

A: Scatter plot of lysis time (secs) at 1-month vs. hospital discharge. B: Scatter plot of maximum turbidity (AU) at 1-month vs. hospital discharge. r: Spearman correlation coefficient.

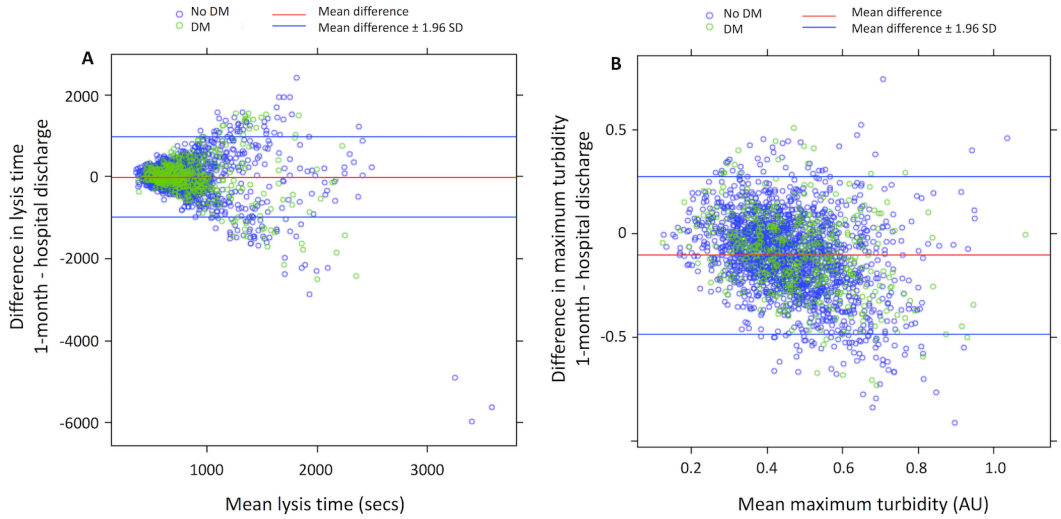


Figure 4-2: Bland-Altman plots for change in lysis time (A) and change in maximum turbidity (B)

n = 2160. This figure excludes patients who were on LMWH within two days of hospital discharge sampling. DM: diabetes mellitus.

4.3.3 The relationships between change in fibrin clot properties and change in inflammatory markers

To measure change in inflammatory biomarkers, hospital discharge and 1-month CRP measurements were used. WCC was available at baseline and 1-month.

Change in fibrin clot lysis time was only weakly related to changes in levels of inflammatory markers (Figure 4-3 A, B). When excluding patients treated with LMWH within 2 days of blood sampling, similar very weak correlations were present between change in lysis time and change in inflammatory biomarkers ($r \leq 0.08$).

However, the relationship between change in fibrin clot turbidity and inflammatory markers was more pronounced and appeared linear with change in CRP levels (Figure 4-3 C, D).

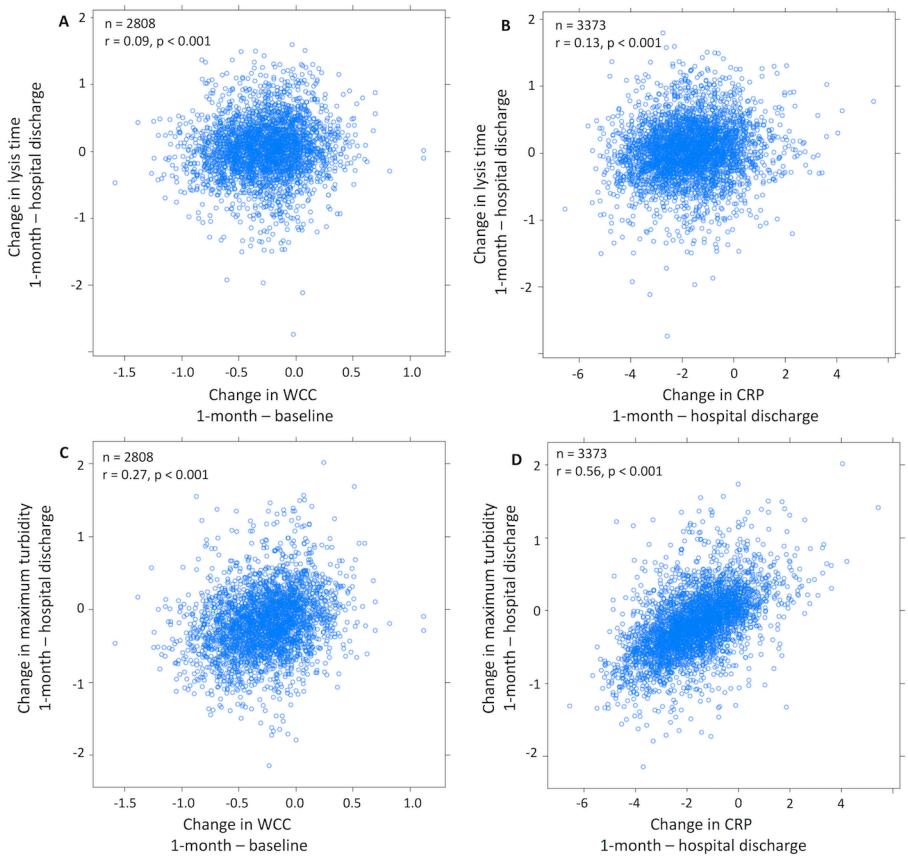


Figure 4-3: Relationship between change in inflammatory markers and change in fibrin clot parameters

A: Scatter plot of change in lysis time vs. change in white cell count (WCC). B: Scatter plot of change in lysis time vs. change in C-reactive protein (CRP). C: Scatter plot of change in maximum turbidity vs. change in WCC. D: Scatter plot of change in maximum turbidity vs. change in CRP. r: Spearman correlation coefficient. Change is calculated based on log transformed values.

4.3.4 The relationship between fibrin clot properties at 1-month and clinical outcomes

During follow-up after the 1-month timepoint, 80 patients (1.9%) had CV death, 146 (3.6%) had spontaneous MI and 192 (4.8%) had major bleeding.

There was no significant relationship between lysis time and any of the clinical outcomes. However, the cumulative event rate of the combined outcome of CV death and spontaneous MI was higher in the highest quartile groups of lysis time driven by higher rates of CV death (Figure 4-4 A, B).

After adjustment for randomised treatment, each 50% increase in maximum turbidity was associated with increased risk of CV death/spontaneous MI (HR 1.19; 95% CI 1.00-

1.41; P = 0.046). As a categorical variable, the highest quartile group was associated with increased risk of CV death/spontaneous MI (HR 1.37; 95% CI 0.95-1.97; P = 0.017) and CV death alone (HR 1.88; 95% CI 1.09-3.26; P < 0.001) (Figure 4-4 C, D). This association, however, lost significance after adjustment for clinical risk factors (model 1).

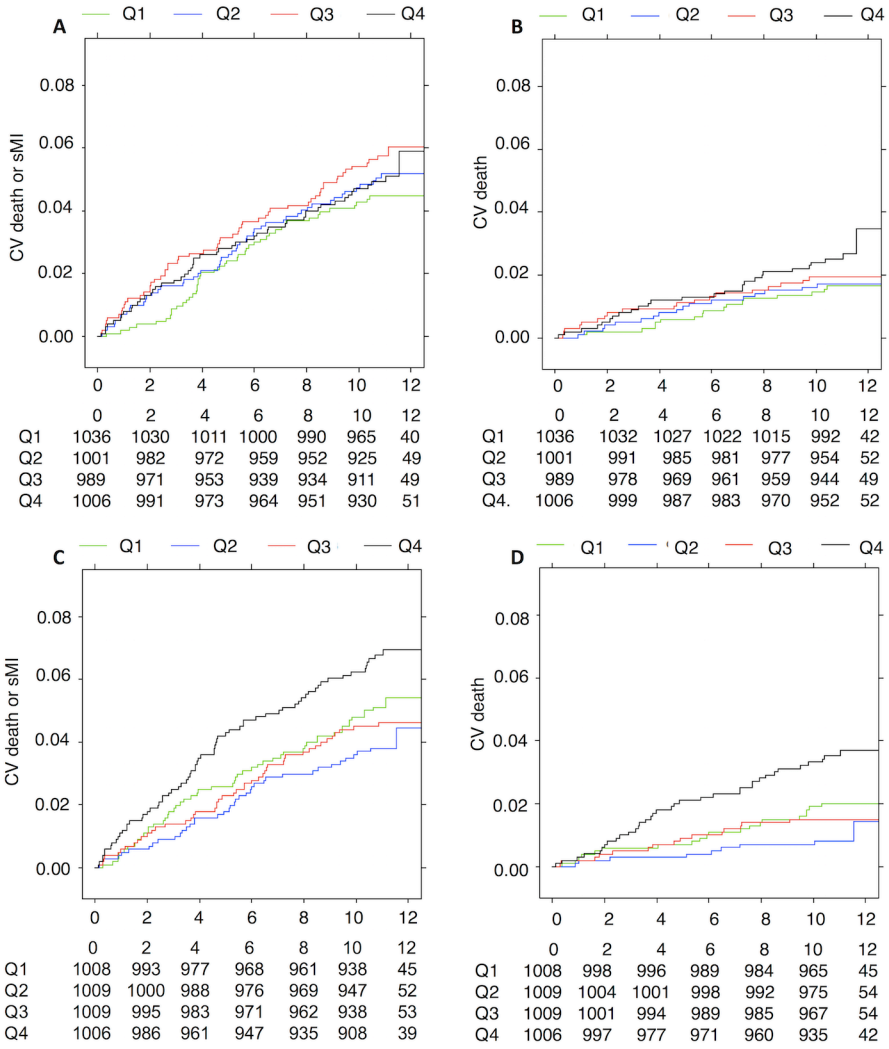


Figure 4-4: Relationship between fibrin clot parameters and 1-year rates of CV death and spontaneous MI

Kaplan-Meier curves for rates of:(A) the combined outcome of cardiovascular (CV) death/spontaneous myocardial infarction (MI) per quartile groups of lysis time, (B) CV death alone per quartile groups of lysis time, (C) combined outcome of CV death/spontaneous MI per quartile groups of maximum turbidity and (D) CV death alone per quartile groups of maximum turbidity.

4.4 Discussion

For the first time, we have demonstrated, in a large longitudinal study, that fibrin clot lysis time remains relatively stable up to 1-month post ACS. This is despite the drop in fibrin clot density that appears to be driven by resolution of systemic inflammation. This indicates that fibrin clot density and inflammation have little impact on lysis potential. The relationship between high-risk conditions and increased density/prolonged lysis persisted at 1-month, giving us mechanistic insights into worse outcomes associated with those conditions.

The relationship between female patients and prolonged lysis has also been demonstrated at both timepoints. This is consistent with previously observed results in other cohorts of high risk vascular patients (260, 261). Another study, in a younger cohort, found no difference in lysis potential between males and females (262). Prolonged lysis may be one mechanism for the loss of cardiovascular protection following menopause and further work is needed in this area to further understand mechanisms leading to this change.

In a proportion of patients, lysis time appeared to change over time. It is difficult to elucidate factors driving this change. Different diets have been shown to have an impact on the degree of clotting factors activation and levels of fibrinolytic proteins (263, 264), and this maybe one reason for the change. Diurnal variations and different levels of exercise might be another reason (265).

A limitation of this study is the lack of power to detect independent relationships between fibrin clot properties and clinical outcomes after 1 month post ACS. However, we have demonstrated these relationships for clinical outcomes following hospital discharge post ACS in chapter 3 and the trends for relationships with clinical outcomes after 1 month remained consistent with these. Notably, most events occurred early during the follow-

up period and this may indicate the need for optimisation of therapy early following ACS. The relatively stable lysis phenotype supports the suitability of this being a therapeutic target.

4.5 Conclusion

Prolonged fibrin clot lysis persists at 1-month post ACS, despite a drop in fibrin clot density in line with resolution of systemic inflammation. Current secondary preventative therapy has little impact on fibrin clot lysis and, therefore, this may constitute a suitable therapeutic target.

5 The effects of factor Xa inhibitors on fibrin clot dynamics: A novel approach for assessing anticoagulant effect?

5.1 Abstract

Introduction: Increasing numbers of patients are treated with factor Xa inhibitors. Although routine monitoring is not required, establishing treatment effects might become essential in certain scenarios. Assays sensitive to low concentrations for factor Xa inhibitors are lacking. Adverse fibrin clots predicted outcomes following ACS and it is not clear how different factor Xa inhibitors might influence this prognostic biomarker. In this study, we aimed to assess the *in-vitro* effects of increasing concentrations of different factor Xa inhibitors on fibrin clot dynamics in plasma.

Methods: Fibrin clot lag time, maximum turbidity and lysis time were determined using a validated turbidimetric assay in plasma mixed with rivaroxaban, apixaban, fondaparinux and enoxaparin. Experiments were performed in plasma collected from healthy volunteers and ACS patients. Varying concentrations were studied.

Results: All studied concentrations resulted in prolonged lag time with a clear dose-response relationship. Significant effect on lag time was evident at the ultra-low concentrations of 10 μg/L rivaroxaban and apixaban ($p < 0.01$) and 330 μg/L fondaparinux ($p < 0.01$). Lysis potential was enhanced with concentrations expected to be achieved with high anticoagulation levels with fondaparinux, enoxaparin and rivaroxaban. Maximum turbidity was not clearly affected by the different anticoagulants.

Conclusions: Fibrin clot lag time appears to be a sensitive assay for ultra-low concentrations of factor Xa inhibitors. High concentrations of factor Xa inhibitors

promote lysis potential. Fibrin clot lag time assay may be utilised in monitoring treatment but studies to confirm the observed effects *ex-vivo* are needed.

5.2 Background

The use of non-vitamin K antagonist oral anticoagulants (NOACs) is rapidly expanding. Current indications range from prophylaxis against thromboembolic disease in some orthopaedic patients, treatment of deep vein thrombosis and pulmonary embolism, stroke prevention in patients with non-valvular atrial fibrillation to secondary prevention in patients with ACS (266).

Available NOACs include dabigatran, a thrombin inhibitor, and the directly-acting factor Xa inhibitors rivaroxaban, apixaban and edoxaban. Treatment indications are comparable, but low-dose rivaroxaban (2.5 mg twice daily) is the only NOAC to be successful in patients with CAD (9, 267). Advantages of these treatments include predictable pharmacokinetics, negating the need for routine monitoring or dose adjustments (266). They have also been largely shown to reduce the risk of intracranial haemorrhage compared to vitamin K antagonists (VKAs) (268). Consequently, NOACs are quickly replacing VKAs for the aforementioned indications.

However, concerns regarding lack of reliable, sensitive and readily-available assays to monitor treatment effects of factor Xa inhibitors remain.

In cases of extreme body weights, poor adherence to therapy, deteriorating renal or liver function, bleeding complications or urgent need for surgical intervention, it might become desirable to quantify treatment effects (269). Chromogenic determination of anti-Xa activity is currently the only recommended methodology. This, however, lacks sensitivity to low concentrations and anti-Xa levels do not necessarily correlate with bleeding risk (269).

Factor Xa inhibitors reduce thrombin generation, which might favourably modulate fibrin clot network. We have shown adverse fibrin clots that resist lysis to be a prognostic marker of worse outcomes following ACS and it is not clear how different anticoagulants might affect this prognostic marker. Fibrin clot dynamics might offer a novel approach to monitoring treatment effects. In these studies, we aimed to assess the *in-vitro* effects of different concentrations of different factor Xa inhibitors on fibrin clot formation and lysis in plasma samples.

5.3 Results

5.3.1 Effect of factor Xa inhibitors on fibrin clot formation

Fibrin clot formation was studied in 6 healthy volunteers' plasma samples with each of the anticoagulants (rivaroxaban, apixaban, fondaparinux), 6 ACS patients' plasma samples with rivaroxaban, and 5 ACS patients' plasma samples with apixaban and fondaparinux.

In healthy volunteers' control samples with DMSO, fibrin clot lag time measured 449 ± 24 secs. This significantly increased to 529 ± 20 secs with 3.3 μg/L rivaroxaban (P = 0.005) and significantly increased to 564 ± 23 secs with 10 μg/L apixaban (P = 0.001). In control samples without DMSO, fibrin clot lag time measured 495 ± 26 secs and significantly increased with 100 μg/L fondaparinux to 561 ± 25 secs (P = 0.012). Increasing concentrations of each of the anticoagulants resulted in further prolongation of fibrin clot lag time with a clear linear relationship (Figure 5-1).

Similarly, fibrin clot lag time measured 432 ± 21 secs in control ACS samples with DMSO. This significantly increased to 602 ± 39 secs with 10 μg/L rivaroxaban (P = 0.003) and 529 ± 23 secs with 10 μg/L apixaban (P = 0.002). In the absence of DMSO, fibrin clot lag time measured 445 ± 16 secs in control samples and increased to 618 ± 22

secs with 330 μg/L fondaparinux (P = 0.0005). Increasing concentrations of all anticoagulants also resulted in linear increase in fibrin clot lag time (Figure 5-1).

There was no clear effect of any of the studied anticoagulants on fibrin clot turbidity (Figure 5-2).

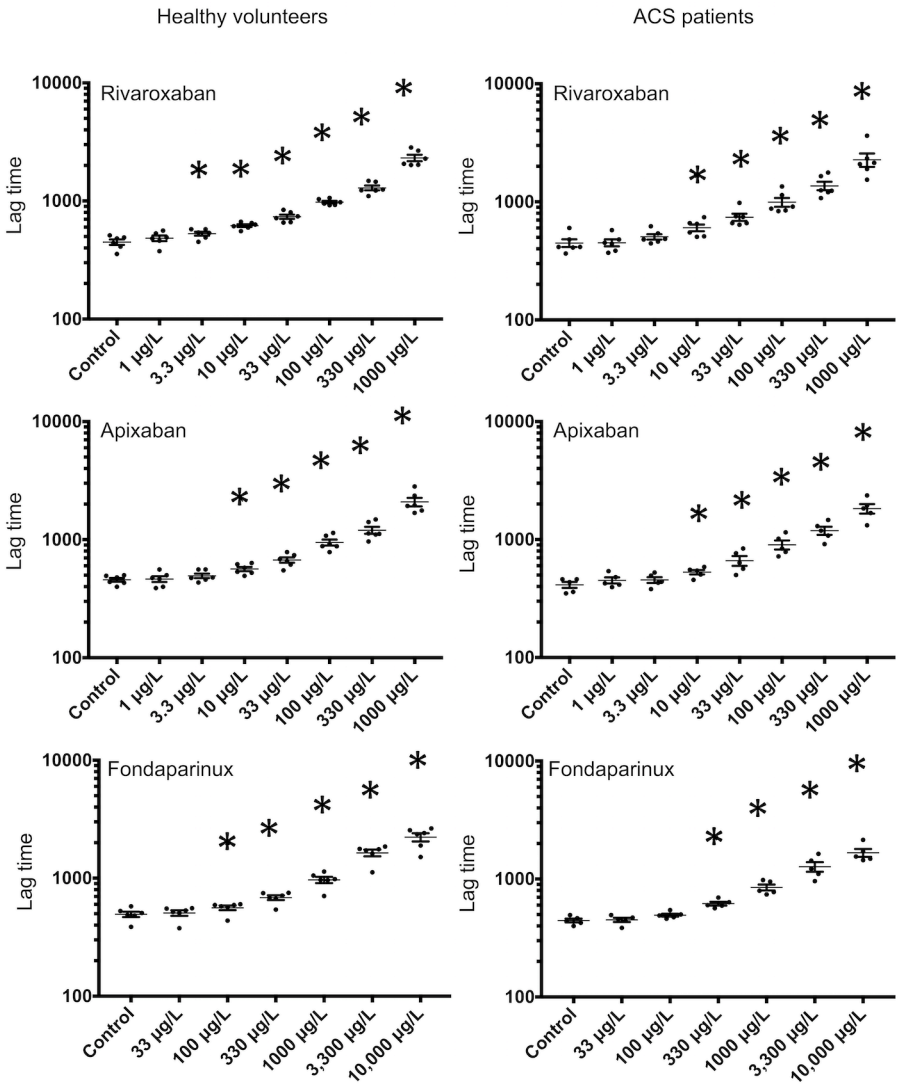


Figure 5-1: The effects of rivaroxaban, apixaban and fondaparinux on fibrin clot lag time

Y axis is $\log_{10}$ lag time (secs). Error bars represent mean ± SEM. * denotes a statistically significant result compared to control. Control samples diluted with same concentration permeation buffer and DMSO in rivaroxaban and apixaban experiments and diluted with permeation buffer only in fondaparinux experiments. All rivaroxaban/apixaban samples contained the same DMSO concentration (1/1000). n = 6 in all experiments except for apixaban/fondaparinux experiments in ACS samples (n = 5).

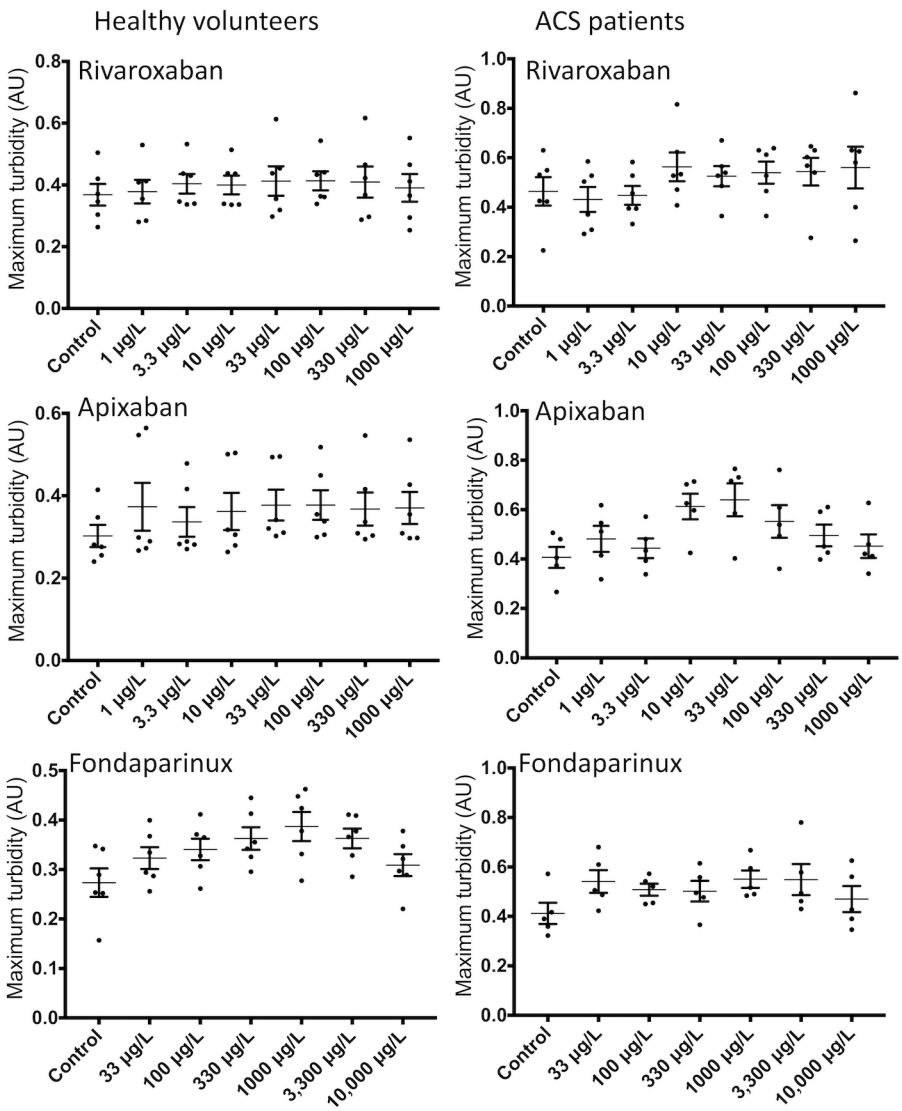


Figure 5-2: The effects of rivaroxaban, apixaban and fondaparinux on fibrin clot maximum turbidity

Error bars represent mean + SEM. AU: Arbitrary units. n = 6 in all experiments except for panels D,F (n = 5).

5.3.2 Effect of factor Xa inhibitors on fibrin clot formation and lysis

Plasma samples from healthy volunteers were used to study anticoagulants' effects on fibrin clotting and lysis. 16 samples were used with fondaparinux, 10 with enoxaparin and 6 with rivaroxaban.

Similar to clotting-only assays, fibrin clot lag time prolonged with all studied anticoagulants (Figure 5-3 A). Sensitivity of the assay was, however, reduced and fibrin clot lag time started to significantly prolong with 100 μg/L rivaroxaban ($P = 0.0004$).

Lysis potential was enhanced to varying degrees by all anticoagulants. In control samples with DMSO, lysis time measured 2403 ± 103 secs. This was significantly reduced with 1000 μg/L rivaroxaban to 1175 ± 134 ($P = 0.0001$). A non-significant trend towards shorter lysis time started to appear with 100 μg/L rivaroxaban (Figure 5-3 B).

In enoxaparin experiments, lysis time measured 2793 ± 168 secs in control samples. This was significantly reduced to 1200 ± 156 secs with 0.5 IU/ml enoxaparin ($P < 0.0001$) and remained low with 1 IU/ml enoxaparin (Figure 5-3 B).

In fondaparinux experiments, lysis time measured 6664 ± 419 secs in control samples. This was significantly reduced to 5510 ± 350 secs with 500 μg/L fondaparinux ($P = 0.0005$).

At the studied concentrations, enoxaparin yielded greater antithrombotic effects than fondaparinux (Figure 5-3) and 4 samples did not clot with 1 IU/ml enoxaparin. Enoxaparin (0.5 IU/ml) resulted in 93 ± 10% increase in lag time. This is in contrast to 41 ± 3% increase with fondaparinux (500 μg/L) ($P < 0.0001$). Similarly, enoxaparin (0.5 IU/ml) resulted in 57 ± 5% reduction in lysis time, in contrast to 17 ± 3% reduction with fondaparinux (500 μg/L) ($P < 0.0001$).

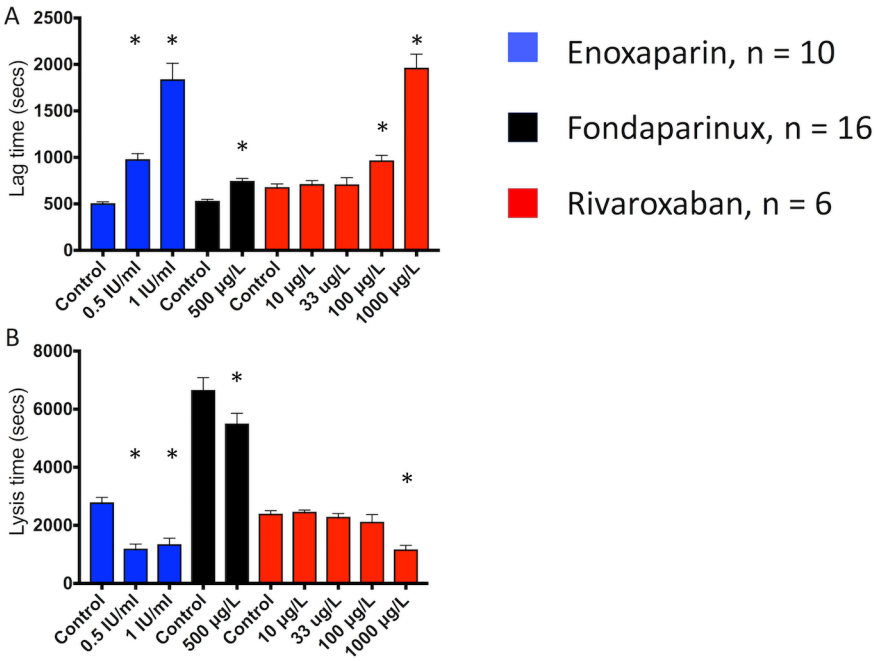


Figure 5-3: The effects of enoxaparin, fondaparinux and rivaroxaban on fibrin clot lag time and lysis

A: Anticoagulants' effects on fibrin clot lag time. B: Anticoagulants' effects on fibrin clot lysis time. Error bars represent mean + SEM. * denotes statistically significant result compared to control.

5.4 Discussion

We have shown in multiple experiments, using plasma samples from ACS patients and healthy volunteers, a linear relationship between drug concentration and fibrin clot lag time. This assay appears sensitive to ultra-low concentrations, which might be advantageous when it becomes necessary to monitor treatment effect. Furthermore, factor Xa inhibitors promoted lysis potential by reducing lysis time but this effect was only evident at high concentrations. We have shown lysis time to independently predict worse outcomes following ACS and promoting lysis might be one mechanism by which anticoagulants improve outcomes following ACS. However, targeting lysis inefficiency

with full anticoagulation long-term is unlikely to be tolerated in addition to dual antiplatelet therapy.

Fibrin clot lag time is sensitive to concentrations expected with low-dose rivaroxaban (2.5 mg twice daily) (238).

Clotting time, as measured by prothrombin time (PT), was previously shown to increase in response to rivaroxaban. However, this is hugely dependent on the reagent used and proved insensitive to low concentrations (270-272).

Modifying PT by increasing calcium chloride concentrations and diluting thromboplastin yielded promising results (273). Increasing concentrations of apixaban gradually resulted in prolongation of the modified PT. This methodology also lacked sensitivity to low concentrations (<100 μg/L) (273).

Fibrin clot lag time is relatively easy to perform and results are reproducible. Coefficient of variance (CV) in our experiments was consistently < 5%.

Insights into optimal anticoagulation levels that are needed in different scenarios might be inferred by studying the effects of different anticoagulants on fibrin clots. For instance, subcutaneous fondaparinux (2.5 mg daily) has a similar efficacy to subcutaneous enoxaparin (1 mg/kg twice daily) in patients with NSTE-ACS but results in significantly lower major bleeding events (274). At clinically-relevant concentrations (275), enoxaparin had significantly greater effects on fibrin when compared to fondaparinux. These observations might explain the excess bleeding events observed with enoxaparin and imply that lower levels of anticoagulation are needed in this scenario. Enoxaparin also inhibits factor IIa (276) and this may explain the pronounced effects of enoxaparin on fibrin clots.

The observed effects on lysis time confirm results obtained in previous studies using different methodologies to study fibrin clot lysis (277, 278).

Our studies have limitations as we have only assessed the *in-vitro* effects of these anticoagulants on fibrin clot. We have, however, used a validated assay and our results mirror some of the *ex-vivo* effects previously observed with other anticoagulants (228, 279).

This assay is also performed on plasma, which limits its potential to be used as a bedside test. Similar to other clotting assays, results might be affected in coagulopathies such as those observed with significant liver disease.

5.5 Conclusion

Factor Xa inhibitors prolong fibrin clot lag time and improve lysis potential. Lag time assays appear sensitive to ultra-low concentrations, which might offer advantages over other available assays. Studies assessing the utility of fibrin clot lag time in monitoring therapy with factor Xa inhibitors are warranted.

6 Pharmacodynamic effects of a 6-hour regimen of enoxaparin in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention (PENNY PCI study)

Abstracts and publications arising from this chapter:

1) The abstract has been presented at the Acute Cardiovascular Care Association conference in Milan 2018.
2) Sumaya W, Parker WAE, Fretwell R, Hall IR, Barmby DS, Richardson JD, Iqbal J, Adam Z, Morgan KP, Gunn JP, Mason AE, Judge HM, Gale CP, Ajjan RA, Storey RF. Pharmacodynamic effects of a 6-hour regimen of enoxaparin in patients undergoing primary percutaneous coronary intervention (PENNY PCI study). Thrombosis and Haemostasis (in press).

Including this data in my thesis does not violate copy rights agreements and is in agreement with other co-authors.

6.1 Abstract

Introduction: Delayed onset of action of oral $P2Y_{12}$ inhibitors in ST-elevation myocardial infarction (STEMI) patients may increase the risk of acute stent thrombosis. Available parenteral antithrombotic strategies to deal with this issue are limited by added cost and increased risk of bleeding. We investigated the pharmacodynamic effects of a novel regimen of enoxaparin in patients undergoing primary percutaneous coronary intervention (PPCI).

Methods: 20 STEMI patients were recruited to receive 0.75 mg/kg bolus of enoxaparin (pre-PPCI) followed by infusion of enoxaparin 0.75 mg/kg/6h. At 4 timepoints (pre-anticoagulation, end of PPCI, 2-3 hours into infusion and at the end of infusion), anti-Xa levels were determined using chromogenic assays, fibrin clots were assessed by

turbidimetric analysis, and platelet $P2Y_{12}$ inhibition was determined by VerifyNow P2Y12 assay. Clinical outcomes were determined 14 hours after enoxaparin initiation. **Results:** 19/20 patients completed the enoxaparin regimen; 1 patient, who developed no-reflow phenomenon, was switched to tirofiban after the enoxaparin bolus. All received ticagrelor 180 mg. Anti-Xa levels were sustained during enoxaparin infusion (1.17 ± 0.06 IU/ml at end of PPCI and 1.003 ± 0.06 IU/ml at 6 hr), resulting in prolonged fibrin clot lag time and increased lysis potential. Onset of platelet $P2Y_{12}$ inhibition was delayed in opiate-treated patients. No patients had thrombotic or bleeding complications.

Conclusions: Enoxaparin 0.75 mg/kg bolus followed by 0.75mg/kg/6h provides sustained anti-Xa levels in PPCI patients. This may protect from acute stent thrombosis in opiate-treated PPCI patients who frequently have delayed onset of oral $P2Y_{12}$ inhibition.

6.2 Background

Dual oral antiplatelet therapy is essential in patients presenting with ST-elevation myocardial infarction (STEMI) and undergoing primary percutaneous coronary intervention (PPCI) (3).

Potent $P2Y_{12}$ inhibition, with prasugrel or ticagrelor, resulted in improved outcomes following acute coronary syndrome (ACS) (5, 6). Although these two agents provide rapid platelet inhibition in stable patients (72, 280), their onset of action can be delayed in patients undergoing PPCI, particularly those pre-treated with opiates, by up to 6-8 hours (85-87, 281, 282). This negative interaction might increase the risk of acute stent thrombosis (88), necessitating bridging therapy with parenteral antithrombotic treatment.

Available parenteral therapies to deal with this issue include glycoprotein IIb/IIIa inhibitors (GPIs) or intravenous $P2Y_{12}$ inhibition with cangrelor. These options have several limitations. GPIs increase the risk of major bleeding events and routine use has resulted in worse outcomes in several trials (90, 283-285). Cangrelor has some advantages: It inhibits platelet $P2Y_{12}$ receptors within two minutes and has rapid offset of action over 1 hour after cessation of infusion (286). However, it has primarily been used as a 2-hour infusion and this might not be sufficient in patients with more than 2 hours delay in onset of oral $P2Y_{12}$ inhibition. Moreover, the very rapid offset of action may pose a risk should the infusion be interrupted. GPIs and cangrelor are costly, another factor that is likely to limit widespread routine use.

Enoxaparin, a low-molecular-weight heparin, targets factor Xa and, to a lesser extent, factor IIa (thrombin) (276). It has a relatively short half-life (1.5 – 2 hrs when given intravenously) (287) and provides more predictable antithrombotic effect compared to unfractionated heparin, negating the need for monitoring (288). A bolus dose of 0.5 – 0.75 mg/kg of enoxaparin can be given to support the PPCI procedure (289).

We have noted cases of acute stent thrombosis to occur at times when the effects of procedural anticoagulant are expected to be dissipating (290).

We hypothesized that an infusion of enoxaparin (0.75 mg/kg/6h) following a bolus dose of 0.75 mg/kg would provide sufficient and sustained antithrombotic effects to bridge treatment with oral $P2Y_{12}$ inhibition in STEMI patients undergoing PPCI, without the need for GPIs or cangrelor. In this study, we aimed to study the pharmacodynamic effects of this regimen.

6.3 Results

6.3.1 Baseline characteristics

20 patients were recruited; 19 patients completed the full enoxaparin regimen and 1 patient was switched to tirofiban due to no-reflow phenomenon prior to starting enoxaparin infusion. No patient had eGFR < 30 mls/min. Median age was 67 years, 80% were males, 45% had anterior STEMI, all had PPCI through the radial artery approach and 1 patient required an intra-aortic balloon pump. All patients received a loading dose of 180 mg ticagrelor pre-PPCI. Other baseline and procedural characteristics are summarised in table 6-1.

Table 6-1: Baseline and procedural characteristics

Variable	Value
Age (years – median [IQR])	67 (57-77)
Male sex (%)	16/20 (80%)
Caucasian race (%)	18/20 (90%)
Anterior STEMI (%)	9/20 (45%)
Smoking (%)	12/20 (60%)
Hypertension (%)	9/20 (45%)
Diabetes mellitus (%)	3/20 (15%)
Chronic kidney disease (%)	3/20 (15%)
Previous acute coronary syndrome (%)	3/20 (15%)
Previous PCI (%)	2/20 (10%)
Pain to balloon time (mins – median [IQR])	205 (131-364)
Call to balloon time (mins – median [IQR])	146 (114-179)
Door to balloon time (mins – median [IQR])	47 (41-65)
Stent length (mm – median [IQR])	22 (14-30)
Stent diameter (mm – median [IQR])	3.5 (3-4)
Ticagrelor loading to 1st blood sampling (mins – median [IQR])	20 (10-40)
Ticagrelor loading to 2nd blood sampling (mins – median [IQR])	60 (50-95)
Ticagrelor loading to 3rd blood sampling (mins – median [IQR])	185 (175-220)
Ticagrelor loading to 4th blood sampling (mins – median [IQR])	405 (390-425)

STEMI: ST-elevation myocardial infarction; PCI: percutaneous coronary intervention. Chronic kidney disease is defined as estimated glomerular filtration rate (eGFR) < 60 ml/min.

6.3.2 Pharmacodynamic assessments

Anti-Xa levels peaked at the end of PPCI (1.17 ± 0.06 IU/ml) and were subsequently sustained both 2-3 hours into infusion (1.01 ± 0.05 IU/ml) and at the end of infusion (1.003 ± 0.06 IU/ml) (Figure 6-1).

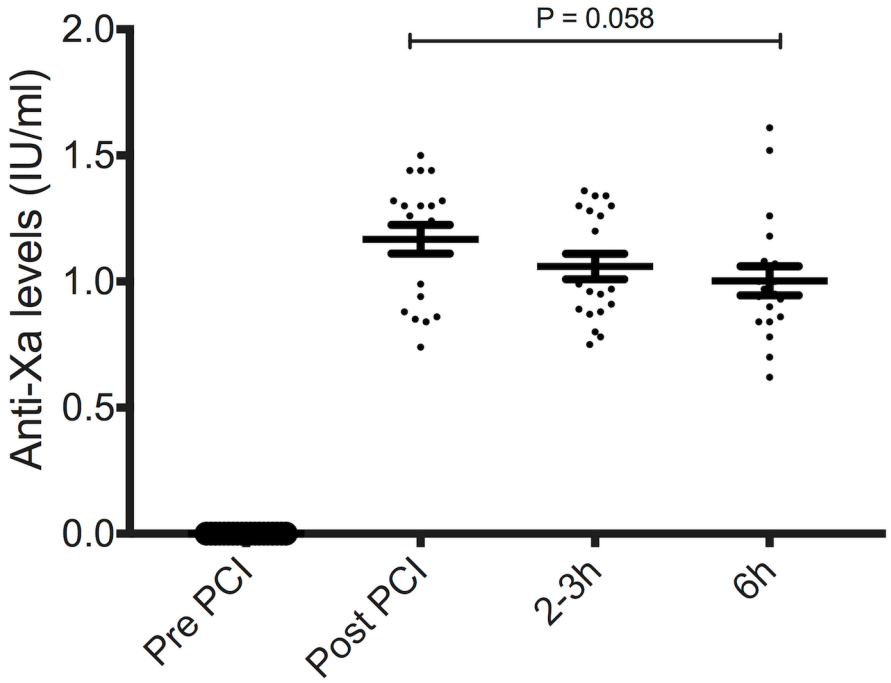


Figure 6-1: Anti-Xa levels

A scatter plot demonstrating anti-Xa levels throughout the studied time-points (n = 19). Variance during the infusion was assessed using one-way analysis of variance (ANOVA). Error bars represent standard error of the mean.

Pre-anticoagulation, fibrin clot formation was rapid with a lag time of 165 ± 4.4 secs. This significantly prolonged at the end of PPCI (431 ± 29 secs; P = 0.0001 vs baseline) and remained prolonged during the infusion at 2-3 hours (395 ± 13 secs; P = 0.0001) and at the end of infusion (337 ± 6.6 secs; P = 0.0001) (Figure 6-2 A).

Fibrin clot maximum turbidity measured 0.45 ± 0.02 AU pre-anticoagulation. This significantly dropped at the end of PPCI (0.24 ± 0.02 AU; P = 0.0001 vs baseline) and gradually increased during the infusion (0.36 ± 0.03 AU; P = 0.0012 vs baseline) returning to baseline at the end of infusion (0.43 ± 0.02 AU; P = 0.7 vs baseline) (Figure 6-2 B).

Pre-anticoagulation, lysis time measured 3161 ± 339 secs. This significantly dropped by the end of PPCI (2192 ± 489 secs; P = 0.001 vs baseline) and remained low during the infusion (2128 ± 256 secs; P = 0.014 vs baseline) and at the end of infusion (2124 ± 259 secs; P = 0.009 vs baseline) (Figure 6-2 C).

There is a degree of variability in antithrombotic effects. Dosing was based on weight estimation. This is likely to account for the outlying measurements in figures 6-1/6-2.

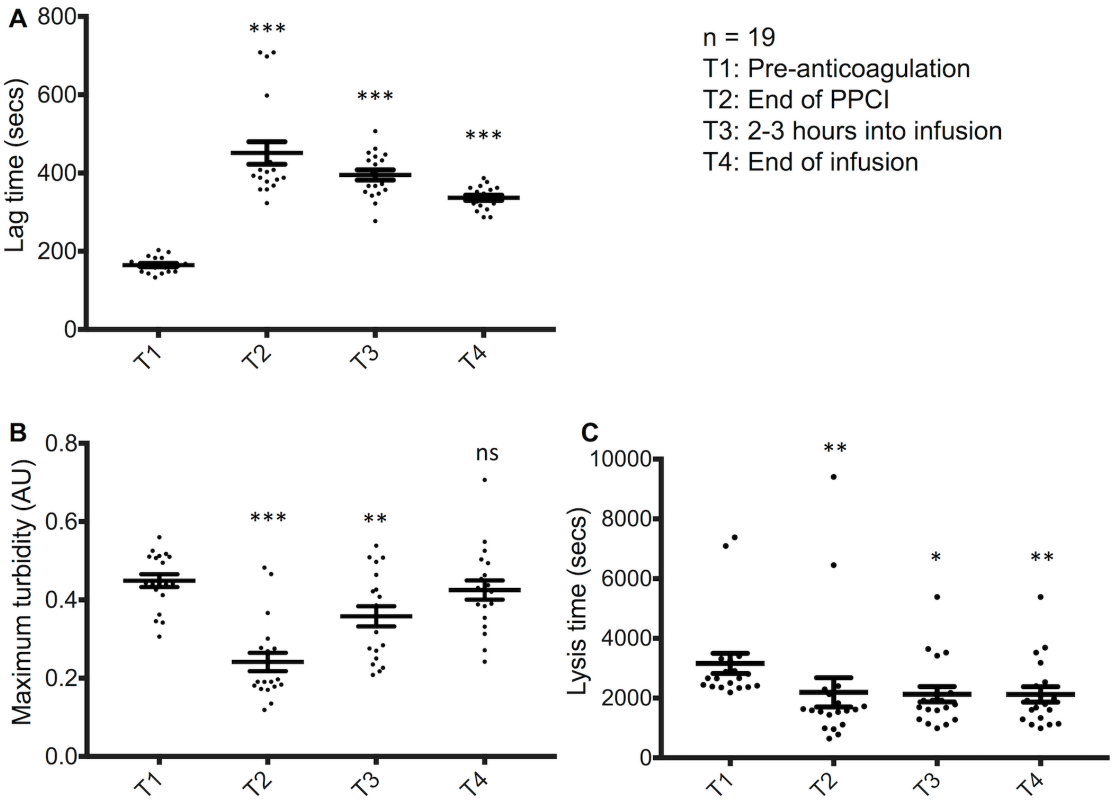


Figure 6-2: The effects of enoxaparin on fibrin clot properties

Scatter plots demonstrating fibrin clot properties pre- and throughout treatment with enoxaparin. PPCI: primary percutaneous coronary intervention; AU: arbitrary units. Error bars represent standard error of the mean. *** denotes P < 0.001, ** denotes P <

0.01, * denotes P = 0.01, ns: non-significant (P = 0.7); all compared to baseline (T1) and calculated using Dunnett's multiple comparisons tests.

A subset of 11 patients had thromboelastometry measured at all four time-points. Only clotting time was significantly prolonged throughout the infusion (Figure 6-3).

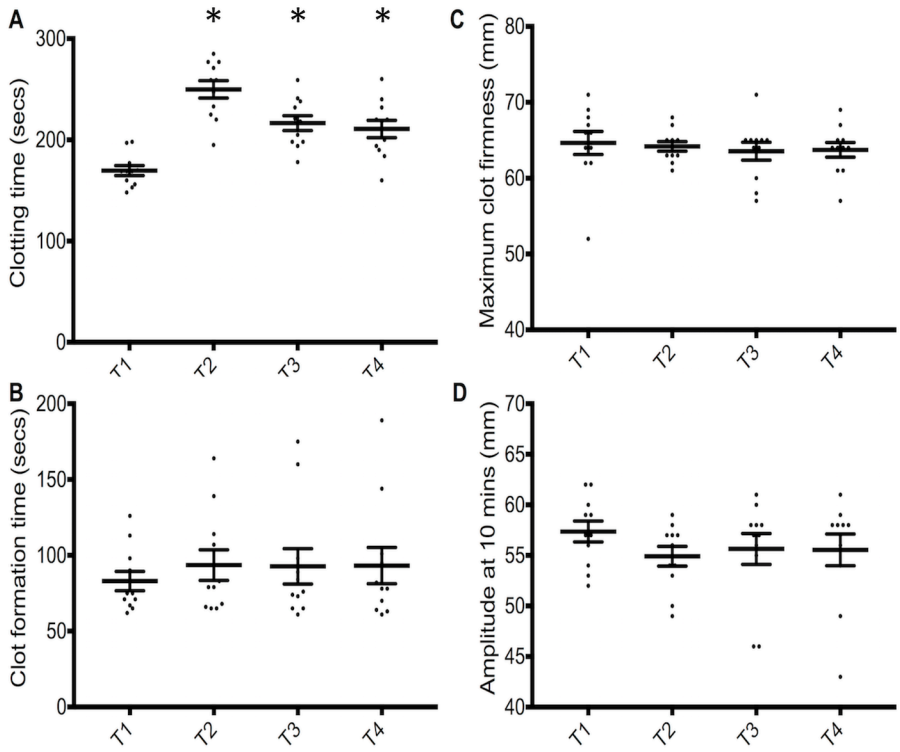


Figure 6-3: The effects of enoxaparin on thromboelastometry

Scatter plots of all thromboelastometry measurements (n = 11). T1: pre-anticoagulation; T2: end of primary angioplasty; T3: 2-3 hours into infusion; T4: end of infusion. * denotes significant difference compared to baseline (T1) (P < 0.001) calculated using Dunnett's multiple comparisons tests.

6.3.3 $P2Y_{12}$ inhibition

All patients were $P2Y_{12}$ inhibitor naive on presentation. Time from ticagrelor loading to different timepoints is summarised in table 6-1.

One patient who did not receive opiates had evidence of $P2Y_{12}$ inhibition at the beginning of PPCI (100 mins post loading with ticagrelor). The other opiate-free patient had > 20% $P2Y_{12}$ inhibition by the end of PPCI whereas the remaining 17 patients, who had received morphine, had 0% inhibition at this timepoint (Figure 6-4). Poor $P2Y_{12}$ inhibition remained common in morphine-treated patients throughout the 6-hour infusion with 8/19 patients still having 0% inhibition at 6 hours despite treatment with anti-emetics (metoclopramide ± ondansetron) in all but 3 of these patients. Treatment with anti-emetics did not affect $P2Y_{12}$ inhibition (interaction P = 0.26) (Figure 6-5). Only 1 patient vomited following ticagrelor's administration and was subsequently re-loaded with ticagrelor shortly after PPCI.

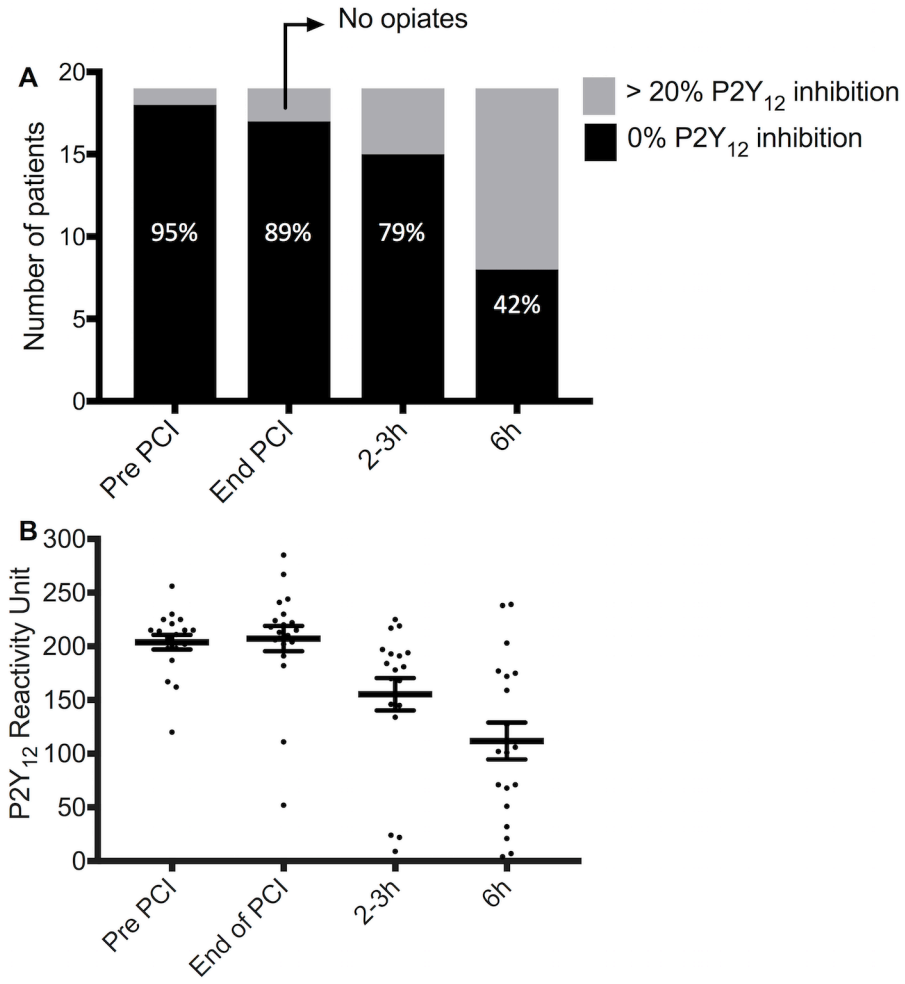


Figure 6-4: Platelet $P2Y_{12}$ inhibition throughout the infusion (VerifyNow P2Y12 assay)

A: Bar graph highlighting the percentage of patients with 0% inhibition as determined by VerifyNow. Two patients did not receive opiates and had > 20% inhibition by end of PCI (as indicated by arrow). B: Scatter plot demonstrating the corresponding $P2Y_{12}$ reaction units throughout the studied time-points.

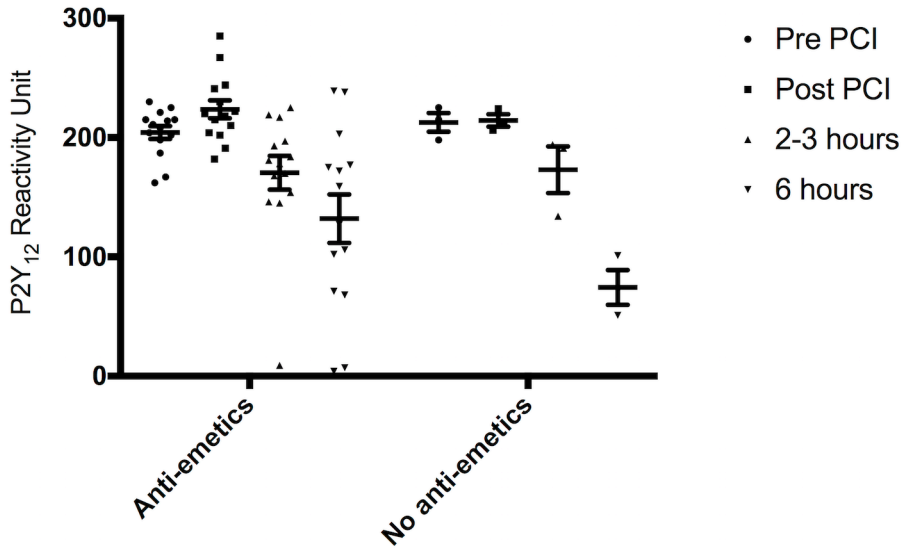


Figure 6-5: $P2Y_{12}$ reactivity units in opiate-treated patients according to co-administration of anti-emetics

VerifyNow P2Y12 results in opiate-treated patients (n = 17) according to co-administration of anti-emetics.

6.3.4 Clinical outcomes

None of the patients suffered any thrombotic or bleeding complication during the follow-up period.

6.4 Discussion

We have studied the pharmacodynamic profile of a novel enoxaparin regimen in STEMI patients undergoing PPCI and have shown that it results in sustained anti-Xa levels throughout the 6-hour infusion. This regimen positively modulated fibrin clot properties, prolonging lag time, reducing fibrin clot density and improving lysis potential, indicating the formation of less thrombotic clots (32, 143). We have identified inefficient lysis as an independent predictor of cardiovascular death following ACS and so this regimen might help to improve prognosis.

The universal prolongation of lag time following enoxaparin was prominent and consistent throughout, which may provide a useful functional measure of response to this therapy. Interestingly, fibrin clot studies provided more consistent data compared with

thromboelastometry, suggesting a higher sensitivity of this method at detecting the anti-thrombotic effects of enoxaparin. Despite high prevalence of delayed response to ticagrelor, none of our patients suffered acute stent thrombosis, providing preliminary support for the hypothesis that this regimen may be sufficient to bridge therapy with oral $P2Y_{12}$ inhibitors in opiate-treated STEMI patients.

Delayed absorption of oral $P2Y_{12}$ inhibitors in STEMI patients is increasingly recognised by interventional cardiologists as a stent thrombosis risk. Crushing tablets has been attempted as a way to accelerate absorption of oral $P2Y_{12}$ inhibitors (291-293). However, results were marginal and delayed absorption in a proportion of patients remains a risk. Our results also confirm that delayed $P2Y_{12}$ inhibition is not attenuated by concomitant treatment with antiemetic therapy. Therefore, parenteral therapies are likely to be needed in order to protect a large majority of patients.

This novel enoxaparin regimen has the advantage of being simple and easy to implement in an emergency situation. Enoxaparin is also widely used for many other indications with good tolerability. Moreover, it is inexpensive and therefore likely to offer substantial savings when compared to GPIs or cangrelor.

We should acknowledge the limitations of our study. This is a pharmacodynamic study and clinical outcomes are only reported as pilot outcomes. Larger studies are needed to establish the safety and efficacy of the studied enoxaparin regimen in STEMI patients undergoing PPCI. Furthermore, platelet $P2Y_{12}$ inhibition was only studied with one methodology, although we have found this methodology to be most discriminatory in assessment of platelet $P2Y_{12}$ inhibition (92). A high percentage of patients had poor platelet inhibition by the end of infusion, which raises the possibility that the duration of infusion might not be sufficient. However, enoxaparin's half-life is ~2 hours and more

$P2Y_{12}$ inhibition is highly likely to be established by 8 hours after loading dose of ticagrelor. This requires further assessment in future work.

6.5 Conclusion

A bolus dose of enoxaparin (0.75 mg/kg) followed by an infusion of 0.75 mg/kg/6h results in sustained pharmacodynamic effects throughout the infusion in STEMI patients undergoing PPCI. The efficacy and safety of this regimen in bridging therapy with oral $P2Y_{12}$ inhibitors should be evaluated in larger studies.

7 Discussions and Future Directions

7.1 Summary and significance of findings

As a clinician, I have often seen patients coming back with recurrent symptoms, myocardial infarctions or even dying following ACS. This is despite our best efforts including revascularisations and offering contemporary antithrombotic therapy. This drove my ambition to pursue research to understand some mechanisms behind worse outcomes and explore possible options to optimise treatment.

For the first time, in a large longitudinal study, I was able to show inefficient fibrin clot lysis to be a prognostic biomarker despite adjustment for multiple novel and established risk predictors. Although association does not conclude causation, this novel biomarker also showed clear associations with established high-risk disease states such as diabetes mellitus, chronic kidney disease, peripheral artery disease and inflammation, which may give us mechanistic insights into worse clinical outcomes following ACS.

Furthermore, we have also shown that fibrin clot density (measured by turbidity) only modestly relates to lysis tendency. Turbidity might be more a reflection of fibrinogen levels, which might explain the linear relationship between change in turbidity and change in inflammatory markers. This is in contrast to lysis potential, which showed a higher degree of stability following ACS despite resolution of inflammation.

High-risk conditions are associated with increased thrombotic potential through a variety of mechanisms. All these conditions have been associated with endothelial dysfunction, inflammation, hyperlipidaemia and increased platelet turnover. This leads to increased platelet reactivity, which is successfully targeted by dual antiplatelet therapy. However, increased levels of different coagulation factors and antifibrinolytic proteins (plasminogen activation inhibitor-1, thrombin-activatable fibrinolysis inhibitor,

complement C3, α2 plasmin inhibitor) leads to lysis inefficiency and this remains unaffected with current therapy, thus potentially constituting a therapeutic target (Figure 7-1).

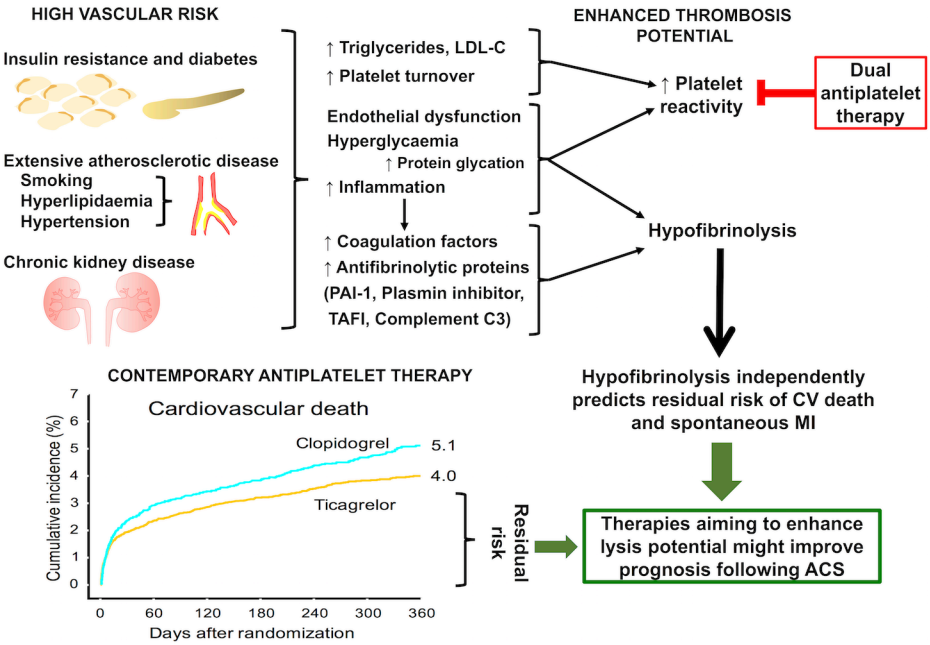


Figure 7-1: Mechanisms for increased thrombotic risk

PAI-1: plasminogen activation inhibitor-1; TAFI: thrombin-activatable fibrinolysis inhibitor. LDL-C: low-density lipoprotein cholesterol. This figure was included in a previous publication (Sumaya W et al. Fibrin clot properties independently predict adverse clinical outcome following acute coronary syndrome: A PLATO Substudy. European Heart Journal 2018;39:1078-85). Including this figure does not violate copyright agreements.

Anticoagulation might be one approach to modulate lysis tendency. However, the *in-vitro* studies suggest that this is only possible at high concentrations. Anticoagulation, in addition to the current approach of dual antiplatelet therapy, is likely to be risky, leading to unacceptable bleeding risk. However, it may be a viable option in addition to a single antiplatelet agent.

We have also seen fibrin clot lag time to be affected by treatment with low-molecular-weight heparins. This was confirmed by *in-vitro* studies and lag time appeared sensitive

to ultra-low concentrations of factor Xa inhibitors. Other assays lack sensitivity to low anticoagulant levels and therefore this assay may be advantageous. If confirmed in future *ex-vivo* studies, this has the potential of improving treatments in cases where pharmacokinetics can be unpredictable, such as in those with chronic kidney disease.

One of the caveats of current antithrombotic therapy is the delayed absorption of oral $P2Y_{12}$ inhibitors in STEMI patients and this may increase stent thrombosis risk. A novel regimen of enoxaparin infusion following a bolus dose provided consistent anti-Xa levels and consistently promoted lysis potential over 6 hours. Therefore, this may improve prognosis. Despite a high prevalence of delayed $P2Y_{12}$ inhibition, there were no thrombotic complications providing preliminary pilot data in support of this regimen.

There is no consensus on how to best deal with the delayed $P2Y_{12}$ inhibition but current treatment options are costly and can increase bleeding risk. If this intervention is successful in future trials, it has the potential of improving patient outcomes and offering substantial savings to healthcare systems around the world. The cost of enoxaparin is ~£14 per patient compared to ~£184 for glycoprotein IIb/IIIa inhibitors and ~£500 for cangrelor. In the UK, over 25,000 patients per year are treated with primary angioplasty (294). Approximately 80% of those receive opiates and therefore this approach could offer the NHS a saving of at least £3.4 million/year.

7.2 Future research

7.2.1 Lysis potential as a therapeutic target

a) Anticoagulation: Although high levels of anticoagulation are likely to be needed to promote lysis, this may be possible in a selected cohort of patients who consistently demonstrate lysis inefficacy. For instance, patients with diabetes mellitus may benefit from anticoagulation in addition to a $P2Y_{12}$ inhibitor as a secondary prevention strategy (295).

b) Inhibiting factor XIII: As discussed in chapter 1, factor XIII plays a critical role in incorporating antifibrinolytic proteins in the fibrin mesh and in the stability of thrombi by cross linking fibrin fibres. Inhibiting factor XIII may be a risky strategy since rare cases of factor XIII deficiency have resulted in significant bleeding (296). Moreover, mice deficient of factor XIII were at risk of myocardial rupture following myocardial infarction (297). However, careful inhibition may be successful and *in-vitro* studies inhibiting factor XIII with a transglutaminase inhibitor have promoted lysis (149).

c) Targeting one of the antifibrinolytic proteins: This is certainly an exciting avenue to explore. I have discussed that TAFI breaks the positive feedback loop between fibrin and plasmin by cleaving C-terminal lysine residues. Mice deficient in TAFI have normal development with no tendency towards increased bleeding (298). A novel compound, DS-1040, that targets activated TAFI has been developed. A pilot study in healthy volunteers showed improved lysis tendency without any significant adverse events (299). These studies provide reassuring data regarding the safety of such a treatment strategy. Further trials are currently ongoing in patients presenting with acute stroke (clinicaltrials.gov: NCT03198715, NCT02586233).

   We have also discussed the key role of PAI-1 in inhibiting fibrinolysis. Atherosclerotic mice deficient in PAI-1 had attenuated progression of atherosclerotic plaques and less tendency to develop occlusive thrombi following vascular injury (300). These interesting findings support the hypothesis of recurrent mural thrombi to be implicated in atherosclerosis progression and imply a pathogenic role for PAI-1 in this.

Therapeutic options to target PAI-1 have been developed (301) and inhibiting PAI-1 did not result in prolonged bleeding time in primates (302) and had favourable antithrombotic effects (303).

α2-PI is another potential therapeutic target. Mice deficient in α2-PI had smaller infarct size following ligation of their cerebral arteries (304). Similarly, inhibiting α2-PI resulted in smaller infarct sizes (305).

In a pulmonary embolism animal model, targeting α2-PI with an antibody had similar efficacy to thrombolysis with no effect on bleeding (306).

d) Identification of new molecules: It may be possible to perform genetic studies to link certain genes to the prolonged lysis phenotype. This may be successful at identifying a new pathway and a novel therapeutic approach.

e) Anti-inflammatory treatment: CANTOS was recently successful at reducing recurrent events in ACS patients with raised inflammatory markers. We have shown clear associations between inflammation and adverse fibrin clots. Therefore, attenuating fibrin clot dynamics might be one mechanism for improved outcomes with anti-inflammatory therapy. Exploring the effects of such therapy on fibrin clots could help us understand the relationship between thrombosis and inflammation and may aid selecting treatment in the future.

7.2.2 Using lysis time as a biomarker

Multiple biomarkers have been studied in the past and many failed to add additional prognostic value beyond the known clinical risk predictors (307). The only biomarker to be routinely measured is troponin. Troponin adds great value as a diagnostic and prognostic test and therefore guides therapy.

An ideal biomarker should be able to discriminate between low-, moderate- and high-risk patients regardless of their other clinical risk predictors. This is certainly challenging

to identify. Recently, GDF-15 emerged as a promising biomarker but the added benefit beyond the known clinical risk factors is small (202).

Overall, lysis time only modestly added to the accuracy of a clinical risk prediction model. However, this is a functional thrombotic assay and may be utilised in aiding clinicians to decide which coagulation arm to target (cellular component vs. protein arm). For instance, guided dual antithrombotic therapy with a $P2Y_{12}$ inhibitor and an anticoagulant (based on lysis time) could be compared to the usual approach of dual antiplatelet therapy following ACS.

7.2.3 Fibrin clot lag time as a novel approach to monitor anti-Xa effects

Clinical studies to confirm the *ex-vivo* effect of factor Xa inhibitors on fibrin clot lag time and compare this effect to chromogenic determination of anti-Xa levels are needed.

7.2.4 Enoxaparin infusion as a novel antithrombotic regimen in primary PCI

We have secured further funding to complete a second-stage feasibility study, which will hopefully aid our bid to obtain further funding for a well-powered RCT.

Overall, I believe my work towards a PhD has advanced our knowledge and understanding of risk following ACS. In particular, it uncovered the potential need to target both the protein and cellular phase of coagulation in order to optimise anti-thrombotic therapy following acute myocardial infarction. Also, my data suggest that higher risk patients, such as those with diabetes or chronic renal disease, require a more aggressive anti-thrombotic strategy. Importantly, my data indicate that anti-thrombotic therapy should perhaps be individualised in the future and the current approach, with few exceptions, of "one size fits all" requires refinement.

Many avenues could be explored in order to optimise treatments and improve prognosis following ACS.

8 Appendix

8.1 PLATO bleeding definitions

- **Major life-threatening:** Fatal, intracranial, intrapericardial with cardiac tamponade, hypovolaemic shock or severe hypotension due to bleeding and requiring pressors or surgery, a decline in the haemoglobin level of 5 g/dL or more, requiring transfusion of at least 4 units of red cells (5).
- **Other major bleeding:** Leading to clinically significant disability (eg. Intraocular bleeding with permanent vision loss), associated with a drop in haemoglobin of at least 3 g/dL and less than 5 g/dL, requiring transfusion of 2-3 units of red cells (5).
- **Minor bleeding:** Any bleeding requiring medical intervention but not meeting the above-mentioned criteria (5).

8.2 Fibrin clot data distribution in PLATO

Distribution of fibrin clot properties data was not normally distributed (Figure 8-1 A, C). We have therefore natural-log transformed our data to give a better mirror of the normal distribution and enable us to estimate Cox proportional hazards ratios (Figure 8-2 B, D).

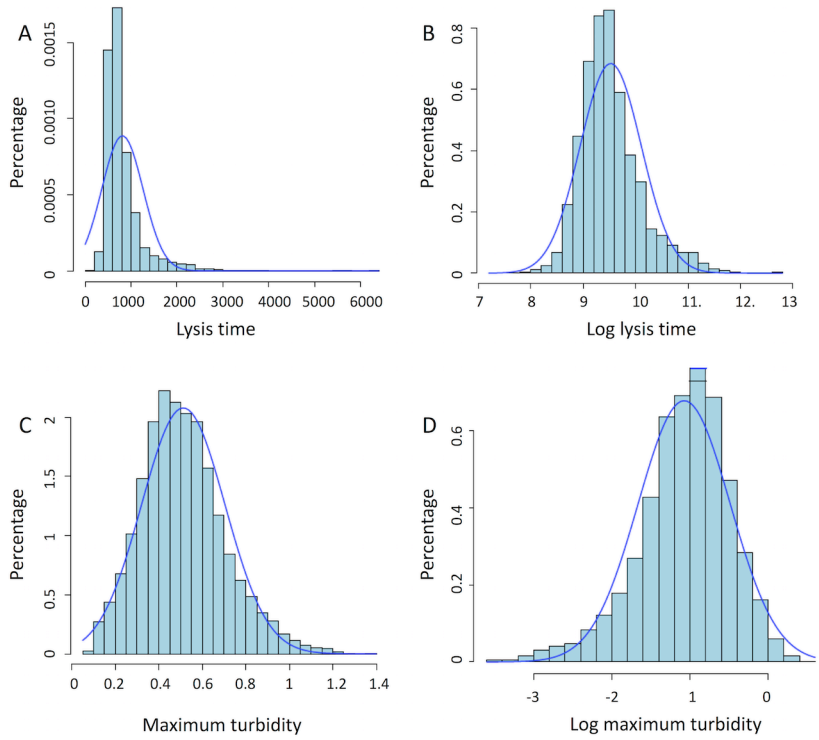


Figure 8-1: Distribution of fibrin clot properties

8.3 The effects of citrate concentrations on fibrin clot measurements

Citrated plasma was used in all our experiments. In all fibrin experiments, I have used a standardised calcium chloride concentration. Underfilling citrated blood tubes might result in increased concentrations of citrate and consequently might affect fibrin clot measurements. Haematocrit did not seem to influence fibrin clot measurements which reassures us that any effect is likely to be marginal. Furthermore, I have performed in-vitro experiments by mixing plasma samples collected from healthy volunteers with additional 3.2% citrate (1:10). Additional citrate did not result in any significant change in fibrin clot parameters (Figure 8-2).

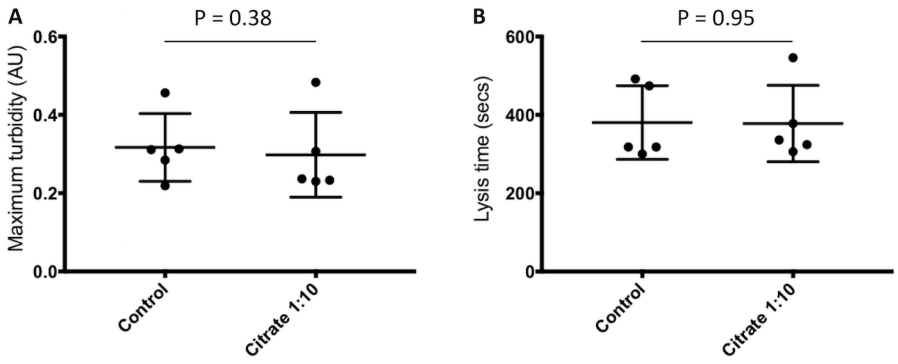


Figure 8-2: Effects of additional citrate on fibrin clot properties

A: Effect of additional citrate on maximum turbidity; B: Effect of additional citrate on lysis time. N = 5. P values calculated by using paired t tests.

8.4 Studying fibrin clot properties with tissue factor

To study the effects of factor Xa inhibitors on fibrin clot formation and lysis, thrombus formation should be induced upstream of thrombin. For this, I have used tissue factor. To optimise the assay, I have studied multiple concentrations of tissue factor (0.001 pM, 0.005 pM, 0.01 pM, 0.1 pM, 1 pM and 5 pM). Tissue factor did not have any additional effect beyond re-calcification at concentrations < 0.1 pM. Moreover, below 1 pM, the reproducibility of the assay was compromised (coefficient of variance > 20%). The effects of different concentrations are shown in figure 8-3. To select a sensitive assay, I chose 1 pM as the lowest concentration that gave reproducible results.

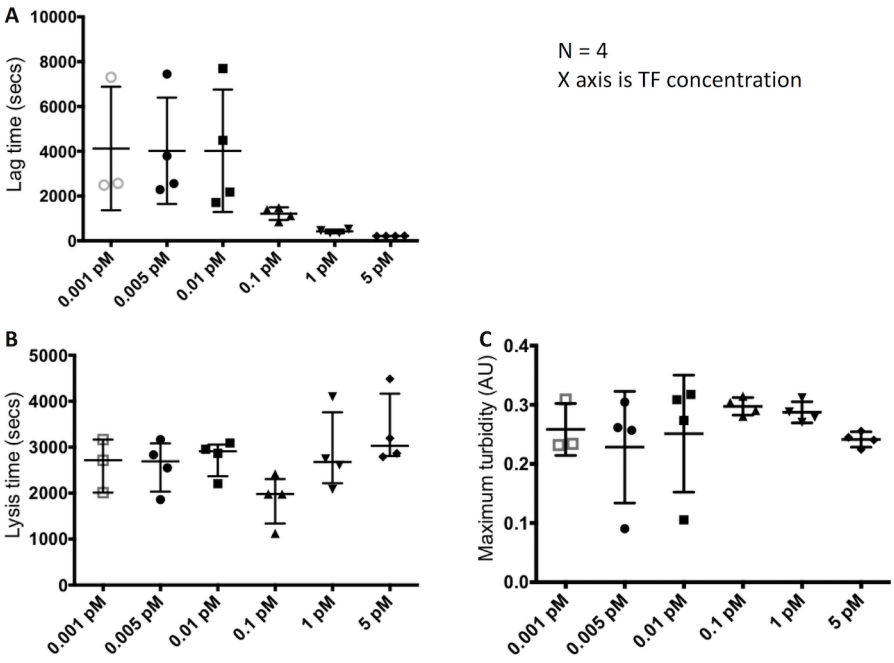


Figure 8-3: Fibrin clot assay with different concentration of tissue factor

PENNY PCI Case Report Form

Screening

Details of presentation

Date of onset of symptoms

☐☐ / ☐☐ / ☐☐☐☐

Time of onset of symptoms

☐☐☐☐

Eligibility criteria

Age ≥ 18 years	Yes ☐	No ☐
Confirmed STEMI diagnosis On the basis of ECG and history	Yes ☐	No ☐
Pre-treatment with ticagrelor or prasugrel	Yes ☐	No ☐
Intention to proceed with PPCI	Yes ☐	No ☐
Feasibility to obtain verbal consent	Yes ☐	No ☐

Exclusion criteria:

Active bleeding that cannot be controlled by local measures	Yes ☐	No ☐
Pregnant patients	Yes ☐	No ☐
Patients with end stage renal failure	Yes ☐	No ☐

requiring renal replacement therapy

Known thrombocytopenia (Platelet count < 100,000/L) Yes ☐ No ☐

Known history of intracranial haemorrhage Yes ☐ No ☐

Known current treatment with oral anticoagulants Yes ☐ No ☐

Known history of major surgery or trauma or
history of GI/GU haemorrhage within the last month Yes ☐ No ☐

Known intracranial malignancy or aneursym Yes ☐ No ☐

Known allergy to enoxaparin Yes ☐ No ☐

Known hypersensitivity to benzylalcohol Yes ☐ No ☐

Patients with acute bacterial endocarditis Yes ☐ No ☐

Active gastric or duodenal ulceration Yes ☐ No ☐

Inability to easily understand verbal information given
in English for any reason Yes ☐ No ☐

Inability to give informed consent due to either temporary
or permanent mental incapacity Yes ☐ No ☐

Current participation, or participation within the last month Yes ☐ No ☐

in an interventional clinical trial.

Verbal informed consent obtained for study (prior to procedure) on

☐☐ / ☐☐ / ☐☐☐☐ **at** ☐☐ : ☐☐

Brief PIS supplied to patient: Version ☐

Date ☐☐ / ☐☐ / ☐☐☐☐

Signature ☐

Coronary angiogram confirming diagnosis of STEMI

☐ Yes ☐ No

Person taking verbal consent and confirming eligibility ☐

Signature ☐

Patient enrolled at ☐☐ **:** ☐☐

Date of birth

☐☐ **/** ☐☐ **/** ☐☐☐☐

Age

Years ☐☐☐ Months ☐☐

Note. If <18 years old do not proceed

Male ☐ **Female** ☐

Race/Ethnicity ☐ Caucasian ☐ Black ☐ Asian ☐ other

Height ☐☐☐ cm

Weight ☐☐☐ Kg

Smoking Status Current ☐ Past ☐ Never ☐

PMH

	Yes	**No**
Hypertension	☐	☐
Dyslipidaemia	☐	☐
Heart Failure	☐	☐
Asthma	☐	☐
COPD	☐	☐
Other Lung disease	☐	☐
Peripheral arterial disease	☐	☐
Type II DM	☐	☐
Type I DM	☐	☐
CKD (eGFR <60ml/min)	☐	☐

Acute or chronic liver disease	☐	☐
ACS (excluding current episode)	☐	☐
Stable angina	☐	☐
Prior PCI (excluding current episode)	☐	☐
Prior CABG (excluding current episode)	☐	☐
TIA	☐	☐
Non Haemorrhagic stroke	☐	☐
Epistaxis in the last 12 months	☐	☐
Other non trivial bleeding in the last 12 months	☐	☐

Other Medical/Surgical history

Aspirin administered in ambulance **Yes** ☐ **No** ☐

Dose of Aspirin ☐ **mg** **Time** ☐☐ **:** ☐☐

Ticagrelor administered **Yes** ☐ **No** ☐

Dose of ticagrelor ☐ **mg** **Time** ☐☐ **:** ☐☐

Prasugrel administered **Yes** ☐ **No** ☐

Dose of prasugrel ☐ **mg** **Time** ☐☐ **:** ☐☐

Opiate treatment **Yes** ☐ **No** ☐

If yes:

Drug and dose : ☐ **mg** **Time** ☐☐ **:** ☐☐

Other concomitant medications to be recorded on page 10.

Physical examination

Vital Signs

BP ☐☐☐ **/** ☐☐☐ **mmHg**

☐☐☐

HR **beats per minute**

Body temperature ☐☐ . ☐ **˚C**

ECG ☐

ECG Interpretation

T1 bloods

Blood sample prior to anticoagulation

Taken on ☐☐ / ☐☐ / ☐☐☐☐

Taken at ☐☐ : ☐☐ (at start of PPCI)

Enoxaparin dose (0.75 mg/kg) ☐ **mg**

Person preparing the bolus dose ☐

Bolus of IA/IV enoxaparin given (0.75 mg/kg) (pre PCI)

Date ☐☐ / ☐☐ / ☐☐☐☐

Time ☐☐ : ☐☐

PPCI started Time ☐☐ : ☐☐

IV enoxaparin infusion (0.75mg/kg) started: (0.75 mg/kg added to 250 mls of saline and set to be given at a rate of 42 mls/hour)

Date ☐☐ / ☐☐ / ☐☐☐☐

Time ☐☐ : ☐☐

Person preparing enoxaparin: ☐

Person prescribing enoxaparin (Bolus + infusion) ☐

Date and time of remaining enoxaparin disposal (both vials) ☐☐ / ☐☐ / ☐☐☐☐ ☐☐ : ☐☐

Person confirming disposal of enoxaparin vials:

T2 bloods

PPCI Finished at ☐☐ : ☐☐

Blood sample (at end of PPCI)

Taken on ☐☐ / ☐☐ / ☐☐☐☐

Taken at ☐☐ : ☐☐

Written informed consent obtained on

☐☐ / ☐☐ / ☐☐☐☐ **at** ☐☐ : ☐☐

GP letter sent ☐

Blood test results

Kidney function

Na ☐ K ☐

Urea ☐ Creatinine ☐

eGFR

Full blood count

Hb WCC

Platelets RCC

MCV MCH

Clotting profile

PT secs APTT

Fibrinogen

T3 bloods

Blood sample (2-3 hours from start of enoxaparin infusion)

Taken on / /

Taken at :

If eGFR < 30 ml/min:

IV enoxaparin infusion stopped (at 3 hours):

Yes ☐ No ☐

Date Stopped ☐☐ / ☐☐ / ☐☐☐☐

Time Stopped ☐☐ : ☐☐

T4 bloods

IV enoxaparin infusion finished (after 6 hours):

Date ☐☐ / ☐☐ / ☐☐☐☐

Time ☐☐ : ☐☐

Blood sample (at end of enoxaparin infusion)

Taken on ☐☐/ ☐☐/ ☐☐☐☐

Taken at ☐☐ : ☐☐

Enoxaparin infusion dose received: ☐

If AEs occurred during the infusion please complete AEs log

If stopped early:

Reason for stopping enoxaparin ☐

If reason for stopping enoxaparin qualifies as AE – please complete AE log

Date and time of disposal of enoxaparin infusion:

☐☐ / ☐☐ / ☐☐☐☐

☐☐ : ☐☐

Person confirming disposal of remaining infusion

☐

Any adverse events: **Yes** ☐ **No** ☐

If yes adverse log completed **Yes** ☐

14 hours post PCI – End of study

Date ☐☐ / ☐☐ / ☐☐☐☐

Time ☐☐ : ☐☐

PPCI details

Access ☐ Occluded Vessel ☐

Stent (type/dimensions) ☐

Other vessels with > 50% stenosis (if applicable) ☐

Any bleeding events Yes ☐ No ☐

If yes: bleeding site? ☐

Any treatment needed? Yes ☐ No ☐

Stent thrombosis Yes ☐ No ☐

Cause of stent thrombosis: ☐

Stent thrombosis and bleeding events to be recorded as AEs as well

Any new adverse events (AEs/SAEs)

Yes ☐ No ☐

If yes, AE/SAE log completed ☐

Concomitant medication checked and any changes recorded in medication section, specifying dose and regimen for each medication. ☐

COMEDICATION SECTION

Generic drug name, dose and regimen	Date started	or >1 month	Date stopped	or continuing
	- - / - - / - - - -	☐	- - / - - / - - - -	☐
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Name of person completing form

Signature of person completing form

Date of completion

STUDY COMPLETION

PRINCIPAL INVESTIGATOR'S DECLARATION

By signing below, I declare that the information presented in this case report form accurately reflects clinical information obtained from the study participant and their medical records, including the results of tests and evaluations performed on the dates specified.

Principal investigator

Signature of principal investigator

Date __ __ / __ __ / __ __ __ __

Supplementary Laboratory CRF

Time Point 1

Date of Sample (DD:MMM:YYYY): _ _-_ _ _-_ _ _ _

Time of Sample: _ _:_ _

VerifyNow P2Y12 **Initials:** _____

PRU	Base	% Inhibition	Time of assay
			_ _:_ _
Comments:			

Fibrin

Maximum turbidity (AU)	
Lag time (secs)	
Lysis time (secs)	

TEG

In-tem S Reagent

Clotting time (CT) (secs)	
A10 (Amplitude at 10 mins) (mm)	
CFT (clot formation time) (secs)	
MCF (maximum clot firmness (mm)	

Anti Xa

Level: _____. (IU/ml)

Time Point 2

Time of Sample: _ _:_ _

VerifyNow P2Y12
Initials:_____

PRU	Base	% Inhibition	Time of assay
			_ _:_ _
Comments:			

Fibrin

Maximum turbidity (AU)	
Lag time (secs)	
Lysis time (secs)	

TEG
In-tem S reagent

Clotting time (CT) (secs)	
A10 (Amplitude at 10 mins) (mm)	
CFT (clot formation time) (secs)	
MCF (maximum clot firmness (mm)	

Anti Xa

Level: _____. (IU/ml)

Time Point 3

Time of Sample: _ _:_ _

VerifyNow P2Y12 **Initials:_____**

PRU	Base	% Inhibition	Time of assay
			_ _:_ _
Comments:			

Fibrin

Maximum turbidity (AU)	
Lag time (secs)	
Lysis time (secs)	

TEG

In-tem S reagent

Clotting time (CT) (secs)	
A10 (Amplitude at 10 mins) (mm)	
CFT (clot formation time) (secs)	
MCF (maximum clot firmness (mm)	

Anti Xa

Level: _____. (IU/ml)

Time Point 4

Time of Sample: _ _:_ _

VerifyNow P2Y12 **Initials:_____**

PRU	Base	% Inhibition	Time of assay
			_ _:_ _
Comments:			

Fibrin

Maximum turbidity (AU)	
Lag time (secs)	
Lysis time (secs)	

TEG

In-tem S reagent

Clotting time (CT) (secs)	
A10 (Amplitude at 10 mins) (mm)	
CFT (clot formation time) (secs)	
MCF (maximum clot firmness (mm)	

Anti Xa

Level: _____. (IU/ml)

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