Fibrin clot properties in patients with atrial fibrillation: associations with clinical characteristics, biomarkers, and clinical outcomes

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Abstract

Atrial fibrillation (AF) is a common disorder of heart rhythm. While the pathological mechanisms are not completely understood, AF is associated with a substantially elevated stroke risk. Once AF is recognised, the risk of stroke can be estimated and mitigated with anticoagulant medication, such as the factor Xa inhibitor, apixaban. These medications substantially reduce stroke risk, but they also increase bleeding risk. Adjusting treatment intensity depending on an individual's likelihood of either thrombosis or bleeding may allow clinicians to optimally balance these competing risks.

The propensity of fibrin clots to form in plasma can be measured using various techniques. In this thesis, I iteratively refined a protocol for a tissue factor fibrin clot assay, aiming to maximise its utility in a cohort of patients with AF. I then used this, together with a thrombin assay, to measure fibrin clot properties in 4,350 plasma samples from Apixaban for Reduction In Stroke and Other ThromboembоLic Events in Atrial Fibrillation (ARISTOTLE) participants, with samples taken both before (n = 1,891) and during (n = 2,459) apixaban therapy. Clot properties were then compared with clinical characteristics, biomarkers, outcomes, and treatment effect.

I found that delayed clot formation was associated with diabetes mellitus, renal impairment, and inflammatory biomarkers. Tissue factor-induced lag time was profoundly longer after apixaban was started, but this increase was heterogenous, with a greater difference observed in older individuals and those with increased body mass index.

Additionally, prolonged fibrin clot lysis was associated with female sex, diabetes, increasing BMI, and decreasing age. Lysis time also strongly correlated with inflammatory biomarkers. Of note, people with delayed lysis had fewer bleeding events.

I discuss how these findings provide insight into mechanisms of stroke in patients with AF and how they may influence risk stratification and treatment in the future.

Dedication

I dedicate this work to my family, particularly my wife, Rachel. They have sustained me throughout this journey which would not have been possible without their love and support.

Acknowledgement

I would like to express my gratitude to my supervisors Professor Rob Storey and Dr Justin Lee. They encouraged me to think creatively about the subject whilst providing a supportive foundation for research. Rob's meticulous nature and enthusiasm for cardiovascular research is inspiring.

My PhD was always likely to be challenging but became doubly so with the advent of Covid-19. No one could foresee the impact the Coronavirus pandemic would have on hospital medicine and research. During the many lockdowns of 2020, being unable to access the lab, I turned my focus onto methods of analysing data. Throughout this, Rob was supportive of my efforts while juggling his clinical commitments.

I would also like to thank the research teams in Sheffield and Uppsala. In Sheffield, Heather Judge provided insightful evaluation of lab methods and was particularly helpful when experiments were going awry. The Uppsala Clinical Research Centre (UCR), led by Professor Lars Wallentin, facilitated access to an incredibly powerful repository of plasma samples associated with clinical data, and statistical support from Johan Westerberg.

Finally, none of this would have been possible without the generous support from the British Heart Foundation. The clinical research training fellowship funded my PhD studies and provided a necessary steppingstone to the world of academia. I am indebted to the organisation.

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Declaration

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university. Details of my contributions to all studies are as follows:

a) *In vitro* studies & anti-Xa studies: I planned and performed all experiments, and analysed all data.

b) Fibrin clot studies in patients with atrial fibrillation: I was involved in study inception, and designed and planned the study in conjunction with my supervisors and Uppsala Clinical Research Center (UCR). I performed all experiments and analysed all the fibrin clot data myself. I produced a statistical analysis plan, subsequently executed by biostatisticians at UCR.

Chapter 1: Introduction; Predicting and managing the stroke risk, and measuring clot function in patients with atrial fibrillation

1.1 Background

Atrial fibrillation (AF) is a common disturbance of heart rhythm, with an estimated 33.5 million affected individuals worldwide, whose prevalence increases with advancing age.[1] Its presence is associated with substantial morbidity and mortality.[2] A major cause of this mortality is the approximately 5-fold increased stroke risk associated with AF compared with individuals without the disease, even when adjusting for other risk factors.[3,4] From the alternative perspective of patients who have had a stroke, approximately 20% are found to have AF, which is frequently asymptomatic until the time of the stroke.[5] AF-related strokes tend to result in more disability and worse clinical outcomes when compared with non-cardioembolic strokes, presumably due a larger burden of thrombus affecting a larger downstream arterial bed within the cerebral circulation (when compared with strokes resulting from cerebrovascular thrombosis).[6–8] Unsurprisingly, given its association with advancing age and common risk factors, AF is frequently seen in combination with other forms of cardiovascular disease, such as coronary artery disease (CAD), valvular heart disease, and heart failure.[9]

1.1.1 Mechanisms of stroke in atrial fibrillation

Stroke resulting from thromboembolism (TE) from the left atrial appendage (LAA) was first described by London neurologist William Gowers in 1875, a paradigm that was unchallenged for a century.[10] In this model of disease, uncoordinated atrial myocyte activity in AF results in impaired mechanical contraction of the atria, causing stasis of blood within the (left) atrial appendage, ultimately leading to thrombus formation which may then embolise. However, 'Virchow's triad' of pathology (stasis, endothelial injury and hypercoagulability) is widely-taught as the mechanism for thrombosis and may be relevant in AF.[11] Indeed, more recent work suggests complex multifactorial mechanisms are responsible for thromboembolic complications in AF, including hypercoagulability and atrial wall abnormalities.[12]

It therefore follows that, whilst AF is clearly correlated with stroke, a true causal link has been more challenging to demonstrate as it does not satisfy the Bradford Hill criteria for

causation.[13] For example, in AF, a successful rhythm control strategy does not significantly reduce the likelihood of TE and temporal association between stroke and AF episodes is not always evident.[14–17] An alternate explanation exists where common pathophysiological processes are associated with development of both an 'atrial myopathy' and stroke, with AF being a marker of disease. The term atrial myopathy describes atrial structural and functional alterations which may include cardiomyocyte damage / impairment, fibrosis, fat infiltration or other inflammatory changes that are prevalent in AF.[18] In this instance, co-morbidities such as age, hypertension, cigarette smoking, diabetes mellitus and chronic kidney disease may increase the risk of both stroke and AF. Finally, a link between stroke and subsequently detected AF could plausibly be explained if that AF burden was modulated by stroke, perhaps via autonomic influences. For example, the 'sympathetic surge' observed following acute stroke may result in adverse atrial remodelling.[19]

Lim *et al* demonstrated haemostatic differences in response to atrial pacing in human subjects attending for AF ablation after AF was induced in patients by rapid atrial pacing.[20] Measures of thrombin generation and platelet aggregation were increased in those with AF or atrial pacing, but not in control subjects. Similarly, local platelet activation has been demonstrated within minutes of AF onset[21] and plasma D-dimer increases after AF onset.[22] These studies suggest that the AF rhythm itself can affect coagulability and thus cause stroke, rather than merely being a marker of a prothrombotic state.

1.1.2 Prothrombotic states

Thrombophilia

Several mutations causing an inherited thrombophilia have been identified and include Factor V Leiden mutation (FVL), prothrombin 20210A, antithrombin deficiency and protein C or S deficiency.[23,24] Up to a third of patients with venous thromboembolism (VTE) will have such an identifiable genetic abnormality. However, clinical manifestations of these mutations vary. For example, the presence of FVL predicts deep vein thrombosis (DVT) but, paradoxically, not pulmonary embolism.[25] The mechanism for this paradoxical effect is not known; one potential explanation is that FVL increases clotting tendency but is protective against embolism due to an antifibrinolytic effect of the FVL mutation.[26]

A chronic thrombophilia can be acquired such as in the antiphospholipid syndrome and human immunodeficiency virus (HIV).[27] A prothrombotic state also occurs in many clinical conditions such as sepsis and malignancy.[28,29] Inflammation is associated with development of atherosclerosis and this interaction has been explored recently with anti-inflammatory agents targeting cardiovascular disease.[30–32] Myocardial infarction occurs more frequently in the 30 days after sepsis hospitalisations, compared with non-sepsis admissions.[33] The mechanism behind this observation are not completely understood, but is likely to include the effects of inflammation on platelet reactivity, plaque stability and coagulation factors.[34–36] Coagulation factors such as fibrinogen, prothrombin, tissue factor and plasminogen activator inhibitor-1 (PAI-1) are upregulated in inflammatory conditions, shifting the coagulation-fibrinolysis equilibrium towards a thrombotic state.[37–40]

Prothrombotic states in arterial thromboembolism

Whilst genetic and acquired thrombophilia are strong risk factors for VTE, their effect on rates of arterial thrombosis is less pronounced. Several studies have examined the risk of arterial strokes in those with genetic thrombophilia. In a large meta-analysis of these predominantly case-control studies, FVL, prothrombin 20210A, protein C and protein S deficiency were all associated with a modestly increased risk of arterial strokes (Figure 1-1, taken from [41]).

Amongst young patients (< 40 years), those with unprovoked VTE had a nearly 4-fold increased risk of acute myocardial infarction, though this association was not seen in older individuals suggesting that inherited thrombophilia may be an important mechanism in young patients presenting with arterial emboli, but that risk is superseded by atherosclerotic risk factors with advancing age.[42]

Relationship between arterial and venous thrombosis

There appears to be limited overlap in acquired risk factors for venous and arterial thrombosis. The strongest risk factors for arterial atherothrombotic disease are hyperlipidaemia, smoking, hypertension and diabetes, while for VTE the strongest risk factors

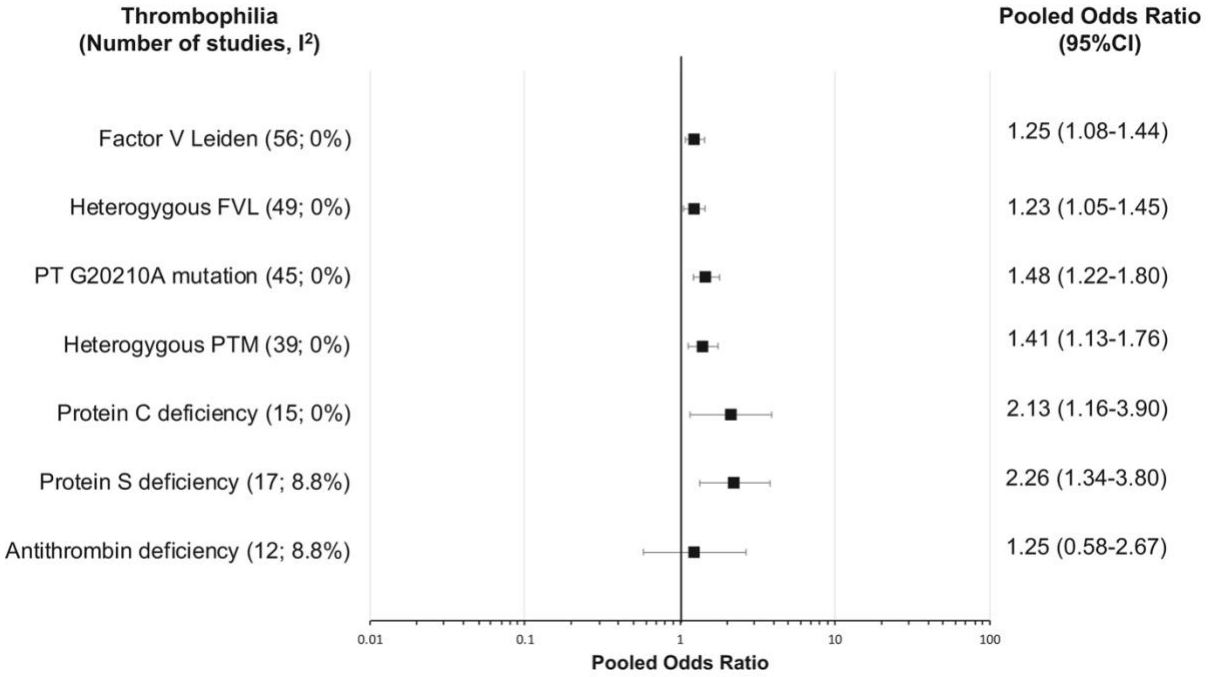


Figure 1-1 – Forest plot showing association between inherited thrombophilia and risk of arterial ischemic stroke. The forest plot shows the results from the meta-analysis for each type of thrombophilia and its association with arterial ischemic stroke. The pooled odds ratio (OR) is represented by the square box. The whiskers represent 95% CIs. The I2 statistic was used to evaluate study heterogeneity. FVL indicates factor V Leiden; PTM, prothrombin G20210A mutation.

are trauma, surgery (particularly oncological or major orthopaedic surgery), pregnancy and oral contraception.[43] This suggests broadly different mechanisms are responsible for arterial and venous thromboses. However, counterintuitively, stable atherosclerotic disease also appears to be more common in people with VTE[44,45] and cardiovascular events occur more frequently in those with unprovoked, rather than secondary, VTE.[46] Mechanisms for this relationship have not been elucidated, but it has been suggested that unprovoked VTE may be a symptom of the pro-inflammatory / hypercoagulable metabolic syndrome that characterises atherothrombotic risk.[43] For example, in the JUPITER clinical trial, patients taking rosuvastatin to target vascular inflammation had fewer deep vein thrombosis events than those taking placebo.[47] Inflammation may therefore be a risk factor common to both arterial and venous thrombosis.

Relationship between AF stroke and arterial or venous thrombophilia

The mechanism for initiation of thrombosis depends on the stroke mechanism. In arterial strokes, vulnerable atherosclerotic plaques in large blood vessels rupture due to inflammatory processes that weaken the cap overlying the plaque as well as repeated shear stress, exposing the plaque core to blood, which results in platelet activation and

intravascular thrombosis. Conversely, VTE results from slow flow in lower limb veins allowing thrombin to accumulate in sufficiently high concentrations, while the activated endothelium downregulates anti-thrombotic proteins and upregulates prothrombotic components.[48] Thrombi formed by VTE can cause stroke by 'paradoxical' embolisation through the heart (for example, through an atrial septal defect). For both venous and arterial embolisms, the levels of coagulation factors dictate the propensity for fibrin clot to form and dissolve. Treatment of these events is targeted towards the underlying mechanism. Typically, antiplatelet medications are in use for arterial stroke, whereas anticoagulants are used to treat VTE and cardiac thromboembolism.

In a study examining the histology of stroke thrombectomy samples, clots from cardioembolic strokes were typically not platelet-rich, whereas those retrieved in large artery or cryptogenic stroke subtypes were commonly platelet-rich.[49,50] Thrombi in AF may form and mature slowly before embolisation, affecting their composition by causing a mature clot with dense fibrin networks. This phenomenon has also been observed in myocardial infarction, where a greater ischaemic time resulted in greater fibrin proportion within clot, whereas platelet-rich clots are seen earlier in the disease process.[51]

Risk factors such as hypertension, diabetes and age are common to both AF stroke and atherosclerotic conditions. Because of this overlap, many patients with AF will have a non-atrial stroke mechanism, in keeping with observations from the EMBRACE and CRYSTAL-AF trials of cardiac rhythm monitoring for AF after cryptogenic stroke; episodes of AF were not temporally related to recurrent stroke.[52,53] Nevertheless, evidence for the superior efficacy of anticoagulants, rather than aspirin, for AF stroke prophylaxis suggests that, despite the overlap in risk factors, the mechanism of AF strokes in the majority is different from cryptogenic strokes. Attribution of AF stroke to exclusively either arterial or venous mechanisms may be misleading. AF stroke can instead be viewed as a distinct clinical entity with its own risk factors and management.

Inherited thrombophilia and risk of stroke in AF

An association between inherited thrombophilias and stroke or thromboembolism (TE) in AF has not been demonstrated. In a large case-control series, FVL was not associated with an

increased risk of stroke in AF.[54] In a separate series, neither FVL nor prothrombin G20210A was associated with TE in AF, though the presence of the prothrombin G20210A mutation was seen more frequently in those with AF than controls.[55]

Prothrombotic State in AF

In addition to prothrombotic structural and mechanical changes in the left atrium that increase stroke risk, AF is itself also associated with a prothrombotic state. The mechanisms for this may include platelet activation[56] and high circulating levels of D-dimer,[57] tissue factor and vascular endothelial growth factor.[58] Fibrosed atrial myocardium overexpresses von Willebrand factor,[59] while, in a small study of atrial histopathology samples, endothelial tissue factor expression was associated with local inflammatory infiltrates in the atrial myocardium.[60]

Elevated PAI-1 levels in AF patients result in a hypofibrinolytic state.[61] There is evidence from a rat model that the prothrombotic state observed in AF may itself induce fibrotic and pro-inflammatory changes in the atrial myocardium.[62] In a rabbit model, thrombin-induced delayed afterdepolarisations in pulmonary vein tissue were completely inhibited by the direct thrombin inhibitor, dabigatran.[63] Differential effects on atrial electrophysiological properties were also observed when comparing warfarin and apixaban, with warfarin-suffused pulmonary vein preparations exhibiting decreased action potential duration and increased early and delayed afterdepolarisations.[64] In a human cell preparation, dabigatran attenuated thrombin-mediated cleavage of protease-activated receptor 1 (PAR1) responsible for adverse atrial remodelling,[65] while, in a rat model, dabigatran prevented left atrial remodelling.[66] Similarly, factor (F) Xa mediates pro-inflammatory signalling in atrial myocardium (again via PAR1) and the FXa inhibitor, rivaroxaban, attenuates this.[67] These mechanisms suggest a plausible ‘two-way’ association between the coagulation system and atrial fibrillation; AF causes a prothrombotic state and this may itself drive further atrial remodelling. These findings could partly explain how “AF begets AF”,[68] and suggest a potential role for anticoagulation in preventing AF progression.[69]

Despite these associations, a clinical relationship between inflammation and stroke is more challenging to demonstrate. Nevertheless, high levels of C-reactive protein (CRP) have been

correlated with LA thrombus and spontaneous echo contrast at transoesophageal echocardiography.[70,71] In the SPAF-III clinical trial cohort, CRP was positively associated with stroke risk and, in ARISTOTLE, the highest quartile of CRP was associated with cardiovascular death, but *not* stroke.[72,73]

To summarise, strokes in patients with AF are predicted by established risk factors, with substantial overlap with other forms of cardiovascular disease. This risk is likely to be governed by changes in coagulation factors, inflammatory markers, macroscopic structural changes in the left atrium and expression of factors at the endothelial level. The relative contribution of each of these mechanisms is not known, while other mechanisms are undoubtedly yet to be discovered.

1.1.3 Quantifying stroke risk

Despite the early descriptions of TE, AF as a risk factor for stroke is a relatively recent discovery. Rheumatic heart disease (with or without concomitant AF) has been recognised as a risk factor for TE since at least the 1950s,[74] although it was only in the late 1970s, after analysis of data from the Framingham registry, when 'non-valvular' AF (AF not associated with severe, often rheumatic, mitral valve disease) began to be recognised as a risk factor for stroke.[4] However, it was unclear whether non-valvular AF (NVAF) is a true independent risk factor or simply a marker of risk resulting from comorbid conditions such as hypertension, advancing age, CAD and heart failure.[75,76] Further analysis of the Framingham data meant that, by the early 1990s, AF was recognised as an independent risk factor for stroke.[3] However, it was also established that AF in the absence of these other risk factors was associated with a low overall TE risk, but that risk increased substantially with the presence of one or two risk factors.[3] Accordingly, the contemporaneous trials of antiplatelet agents and anticoagulation often required the presence of these other risk factors.[77–79]

Risk stratification in AF can be done in several ways. Guidelines have so far mandated stratification based upon the presence of clinical risk factors, although other metrics, including AF burden, biomarkers and imaging data, are discussed.[80,81]

Clinical risk factors

The Stroke Prevention in AF (SPAF) investigators reported predictors of strokes in the cohorts studied in the first two SPAF trials.[82] The following were found to be independent risk predictors of TE:

- Female aged > 75 years
- Systolic blood pressure > 160 mmHg
- Impaired left ventricular function (defined as either echocardiographic fractional shortening < 25% or recent congestive heart failure episode)
- Prior TE

Those patients without any risk factors had an event rate of 1.9%/year, compared with 5.9%/year in the high-risk group (any of the above risk factors). This is compared with a rate of ischaemic stroke in the general age-matched population of approximately 0.8-1.0%/year.

Meanwhile, the Atrial Fibrillation Investigators (AFI) pooled data from the AFASAK, SPAF, BAATAF, SPINAF and CAFA trials and identified the following independent stroke risk factors: [83]

- Increasing age
- Hypertension
- Diabetes mellitus
- Previous stroke or transient ischaemic attack

Importantly, this study also examined the effect of anticoagulant (warfarin) and antiplatelet therapy (aspirin) for each of these risk factors. Warfarin therapy reduced the rate of subsequent ischaemic stroke to <2%/year in all risk factor subgroups, implying that treatment is likely to be beneficial whichever risk factor is present. The treatment effect of aspirin was less clear, in keeping with the various trials of aspirin showing limited benefit.

These clinical risk factors are combined in the $CHADS_2$ and CHA_2DS_2-VASc risk scores which are discussed in detail in subsequent sections.[84,85] Briefly, these scores allow rapid assessment of stroke risk based on simple addition of clinical risk factors (For CHA_2DS_2-VASc, these are: Congestive heart failure, hypertension, age, diabetes mellitus, prior cerebral

ischaemia, vascular disease and female sex). These scores have been adopted clinically and remain the benchmark for other methods of risk stratification.

AF burden

If TE in AF occurred as a direct result of blood stasis in the LAA, stroke risk should increase linearly with time spent in AF. However, the association between AF burden and stroke risk is more complex. For example, in the IMPACT trial, the remote monitoring function of patients' pacemakers was exploited, with anticoagulation being started when AF was detected, and stopped if sinus rhythm returned.[86] This strategy did not reduce strokes or major bleeding and moreover no temporal relationship between episodes of AF and stroke was observed. As a result, commonly used risk scores such as $CHADS_2$ and CHA_2DS_2-VASc do not make a distinction based on AF burden.

Nevertheless, duration of AF does affect stroke risk. In non-anticoagulated patients in ACTIVE-A, stroke rates for paroxysmal AF, persistent and permanent AF were 2.1%, 3.0% and 4.2% respectively.[87] In anticoagulated patients in ROCKET-AF, stroke was significantly less common in those who had paroxysmal AF (defined as lasting < 7 days at a time) compared with those who had persistent AF (>7 days), although the absolute difference was small with 1.73 and 2.18 events per patient-year respectively.[88] Similar results were seen in the ARISTOTLE, ENGAGE and RE-LY trials.[89–91]

Whilst these studies compared the type of AF reported at baseline, it is more challenging to measure the duration of AF. Cardiac implanted electrical devices (CIEDs) such as pacemakers and defibrillators can monitor AF duration and several studies have associated device-documented AF duration with stroke risk.[92–94] However, patients with these CIEDs necessarily have other forms of heart disease and may not be representative of the wider population with AF. Moreover, device-detected AF is frequently asymptomatic and may be identified earlier in the disease process so applicability of these findings to unselected patients with AF is not clear.

Although patients with more advanced (permanent) AF may be expected to have associated risk factors, Botto *et al* combined duration of AF (< 5 minutes, > 5 minutes, > 24 hours) and

$CHADS_2$ score to identify a truly low risk group.[95] Essentially, those with two risk factors but < 5 minutes of AF had a similar stroke rate to those with persistent AF and no risk factors, suggesting an important role of AF duration in risk stratification, even after adjustment for traditional risk factors. When added to CHA_2DS_2-VASc risk factors, AF duration improved the risk stratification C-statistic in another cohort of patients with pacemakers.[96]

In a retrospective study of 1,965 symptomatic patients found to have AF while wearing 14-day cardiac monitors, those in the highest tertile of AF burden (median 27% of recording time in AF) had a 3-fold higher (hazard ratio 3.13, 95% CI 1.50 – 6.56) rate of stroke when compared with the two lower tertiles of AF burden (median 0.56% and 4.7% of recording time in AF, respectively) after adjustment for CHA_2DS_2-VASc risk factors.[97] A limitation of this study is that even those in the highest tertile of AF duration had relatively low AF burden – presumably because patients attending clinic with persistent AF did not require ambulatory monitoring – so results may not apply to patients with a higher burden of AF.

In summary, AF burden appears to act as an additional independent risk factor for AF. However, the relationship is non-linear and even short durations of AF are associated with substantially increased stroke risk compared with patients who do not have AF. With people increasingly wearing smart watches and other technology,[98] our identification of subclinical AF is certain to rise rapidly so understanding how to treat these patients is likely to play an increasingly important role in prevention of cardiovascular events.

Biomarkers

Inflammation

Atherosclerosis is an inflammatory disease, and thus markers of inflammation are an attractive possibility for risk stratification for a number of cardiac conditions.[99] Perhaps the simplest of these measures is the white cell count (WCC). Studies have demonstrated an association between WCC and incident atrial fibrillation,[100] stroke,[101,102] acute coronary syndromes (ACS),[103,104] and mortality.[105–107] The Anticoagulation and Risk Factors in Atrial Fibrillation (ATRIA) score for stroke risk stratification in AF investigated WCC. In the cohort from which this score was derived, the univariate rate of stroke in those patients with WCC < 8000 /µL was 1.85 /100 person-years compared with 3.00 /100 person-years in

those with a WCC > 10,000 /μL.[108] However, this was not seen to be significant after adjusting for other risk factors and the validated score does not include WCC.

The neutrophil-to-lymphocyte ratio (NLR) has been shown to be more closely linked with cardiovascular outcomes in some situations and is emerging as a potentially useful biomarker. NLR predicts new-onset AF,[109] presence of left atrial (LA) thrombus [110] and TE in patients with established AF.[111,112] However, data for this biomarker are currently sparse, limiting its use in risk stratification schema.

Another marker of inflammation, C-reactive protein (CRP), has also been examined as a risk factor for cardiovascular disease. Highly-sensitive CRP measurements have been shown to correlate with stroke events, either in quartile-based analyses or using absolute CRP thresholds of 1 or 3 mg/L.[113–117] However, adjustment for other risk factors such as age and sex attenuates the predictive value of CRP. After adjustment for other risk factors, it was not seen to be an independent predictor of risk.[115]

Kidney disease & R-CHADS$_2$

Kidney disease can be both a cause and effect of cardiovascular disease and has been identified as a risk factor for cardiovascular events, such that more people with chronic kidney disease (CKD) will die from cardiovascular events than end-stage kidney disease.[118] Traditional risk factors for the development of cardiovascular disease (older age, diabetes, hypertension etc) are highly prevalent in patients with CKD. Additionally, factors specific to CKD may also increase cardiovascular risk (e.g., hyperhomocysteinaemia, oxidant stress, hypercoagulability, and higher levels of inflammation). Kidney function is most commonly measured by the estimated glomerular filtration rate (eGFR).

Those with end-stage kidney disease on haemodialysis have a 10- to 30-fold increased risk of cardiovascular mortality compared with the general population, even after adjustment for age, sex, race and diabetes [119]. At the other end of the severity spectrum, relatively mild CKD (eGFR <60 ml/min/1.73m^2 body surface area) remains an independent risk factor for cardiovascular events.[118] In meta-analysis of observational data, stroke risk in all-comers

with CKD (defined as eGFR < 60 ml/min/1.73m^2) was 43% higher than those with a normal eGFR, and the risk of stroke was significantly greater still with eGFR < 40 ml/min/1.73m^2.[120]

AF is both more common at lower eGFR (two to three times higher risk than the general population) and is associated with double the mortality risk in patients with CKD, although this may be at least in part due to the presence of other underlying conditions such as heart failure.[121] The Anticoagulation and Risk Factors in Atrial Fibrillation (ATRIA) group examined the risk associated with CKD in patients with AF and found a graded increased risk of stroke at progressively lower eGFR strata.[122] In the ROCKET AF trial population, stroke rates increased with a relative hazard of +12% (95% confidence interval 7 – 16%) for each 10ml/min reduction in eGFR.[123] In a Chinese population with AF, the adjusted hazard ratio for stroke with eGFR < 60 ml/min/1.73m^2 was 1.79 (95% confidence interval 1.33 – 2.41).[124] The addition of an eGFR score to the existing $CHADS_2$ schema to form the R-$CHADS_2$ score (two points if eGFR < 60 ml/min, plus the original $CHADS_2$ score) did not substantially improve the model's predictive capability compared with either $CHADS_2$ or CHA_2DS_2-VASc,[123,125] although, in a small Chinese cohort, R_2-CHA_2DS_2-VASc (CHA_2DS_2-VASc with additional points for eGFR) outperformed the traditional CHA_2DS_2-VASc.[124]

B-type natriuretic peptide

B-type natriuretic peptide (BNP) and N-terminal pro-BNP (NT-pro-BNP) are markers of cardiac wall tension that are used clinically to detect heart failure. Atrial fibrillation also causes a rise in levels of NT-pro-BNP (in one cohort, 154.4 pg/ml (IQR 41.7 - 303.6) for those with atrial arrhythmia versus 52.8 (IQR 30.4 - 178.0) for those without), and it has therefore been proposed as a potential screen for AF.[126] Amongst people with stroke, BNP is higher in those with a cardioembolic source,[127] and high levels of BNP after transient ischaemic attack (TIA) predict subsequent strokes, presumably at least in part due to detection of occult paroxysmal AF.[128]

Recognised causes of raised BNP include TE risk factors such as left ventricular systolic dysfunction, increasing age, hypertension, and female sex. Despite these risk factors making up a substantial part of the CHA_2DS_2-VASc score, BNP has been shown to substantially improve the prediction of TE in patients with AF.[129] In the RE-LY trial, stroke event rates

increased across the quartiles of BNP, with a significant hazard ratio of 2.40 (95% confidence interval 1.41 – 4.07) when comparing the highest quartile (> 1402 ng/L) with the lowest quartile (< 387 ng/L). In the ARISTOTLE trial, BNP levels were associated with increased risk of TE and mortality. When added to the CHA_2DS_2-VASc score, the C-statistic improved from 0.62 to 0.65 for stroke and 0.59 to 0.69 for mortality, suggesting a role for BNP as a relevant adjunct for risk stratification in AF.[72]

Troponin

In myocytes, the troponin protein is an integral part of sarcomeric contraction. When myocytes are damaged, troponin is released. Troponin assays are used clinically in the diagnosis of myocardial infarctions.[130] In ARISTOTLE, troponin at baseline predicted stroke and cardiac death. Adding troponin to the CHA_2DS_2-VASc risk score increased the C-statistic from 0.629 to 0.653 for stroke and from 0.591 to 0.731 for cardiac death.[131] Similarly, in the RE-LY trial, which used the highly-sensitive troponin I assay, a significant hazard ratio of 1.99 for stroke (95% confidence interval 1.17 – 3.39) was observed when comparing the highest (≥ 0.040 µg/L) with the lowest quartile (< 0.010 µg/L) of troponin. Troponin tertiles were observed to predict both TE and major bleeding in the ENGAGE trial of edoxaban.[132]

Growth differentiation factor 15

Growth differentiation factor 15 (GDF-15) is a member of the transforming growth factor β (TGF-β) cytokines and is produced in response to cellular stress.[133] It is a strong predictor of all-cause mortality in patients with heart failure.[134] In vitro studies have shown that local myocardial GDF-15 expression correlates with the presence of atrial fibrillation and atrial fibrosis.[135]

In the ARISTOTLE trial, GDF-15 quartiles correlated with stroke, systemic embolism and mortality, even after model adjustment for clinical risk factors, BNP and troponin.[136] Moreover, GDF-15 also predicted major bleeding events, leading to its inclusion in the ABC-bleeding score.[137]

D-dimer

D-dimers are produced when fibrin clot is degraded and are measured using widely available assays. D-dimer levels are increased in conditions that involve the formation and subsequent breakdown of fibrin, such as venous thromboembolism, infections, cancer, surgery or ACS.[138] In AF, D-dimer levels are associated with clinical risk factors for TE,[139] and can predict the presence of left atrial thrombus to a moderate degree.[140] D-dimer levels are reduced by anticoagulant treatment and, in warfarin-treated patients with AF, D-dimer may predict subsequent thromboembolic events.[141] In the RE-LY cohort, D-dimer was a risk factor for stroke, cardiovascular death and bleeding, including after adjustment for CHA_2DS_2-VASc risk factors.[142] In the ENGAGE cohort, D-dimer improved the capability of risk prediction models that included clinical risk factors, troponin and BNP.[143]

Fibrin clot properties

The properties of occlusive clot in stroke may depend on its origin. Traditional teaching states that arterial sources of stroke produce fibrin- & platelet-rich 'white' thrombus, whereas clot that forms in slow-flow areas, such as in venous thromboembolism or in the left atrial appendage, have higher concentrations of red blood cells (RBCs) and appear 'red'. Indeed, histology of thrombi retrieved during cerebral thrombectomy can, with modest accuracy, identify cardioembolic causes in patients who suffer a cryptogenic stroke, with thrombi from the LAA having the highest proportion of RBCs.[50,144]

Adverse fibrin clot permeability, a marker of impaired susceptibility to plasmin-mediated clot lysis, has been demonstrated in patients with both ischaemic and haemorrhagic stroke, left ventricular thrombus, heart failure, end-stage kidney disease and diabetes.[145–149] Prolonged fibrin clot lysis time has been associated with recurrent MI and death in patients after ACS.[150]

Patients with paroxysmal AF, even while in sinus rhythm, had denser fibrin clot than controls,[151] while denser fibrin clot is independently associated with both TE and bleeding in patients with AF.[152,153] This is in keeping with evidence that AF frequently occurs in patients with a prothrombotic milieu that is independent of the actual cardiac rhythm.

Other biomarkers

Other biomarkers, including fibrinogen, tissue plasminogen activator (tPA), plasminogen activator inhibitor (PAI-1), prothrombin fragments, antithrombin-III and von Willebrand factor, have been associated with onset of AF and stroke or systemic embolism.[154] However, these biomarkers have not been analysed in conjunction with clinical risk factors and their additive value for risk stratification is unclear.

To summarise, WCC, NLR and CRP are potentially useful biomarkers. They predict TE events in patients with AF although do not appear to add incremental benefit over clinical risk factor scores such as CHA_2DS_2-VASc. Biomarkers of renal disease may improve risk stratification, although there is conflicting evidence of additive benefit over clinical risk factor scoring.

Troponin, BNP and D-dimer appear to act as independent risk factors that have the ability to add predictive accuracy in models incorporating the $CHADS_2$ / CHA_2DS_2-VASc clinical risk factors (Figure 1-2) when predicting TE.[155] Biomarkers may also predict competing causes of death, such as major bleeding in patients with AF, which is potentially useful given the low rates of stroke & systemic embolism experienced by contemporary cohorts of patients taking direct-acting oral anticoagulants (DOACs; apixaban, dabigatran, edoxaban, rivaroxaban and ximelagatran).[156]

Imaging markers of stroke risk in AF

Echocardiography

Echocardiography has been used to attempt to risk stratify patients in AF. Spontaneous echo contrast (SEC) is a swirling phenomenon of blood flow within the heart, seen echocardiographically, that has been linked to thrombotic risk. SEC has been associated with left atrial appendage thrombus and TE in non-anticoagulated patients.[157]

In the SPAF cohort, investigators identified heart failure (defined as *either* fractional shortening < 25% and / or recent clinical episode of heart failure) as a predictor of thromboembolism in AF.[82] Pooled data from SPIN-AF, BAATAF and SPAF-I found that left ventricular function was a strong predictor of stroke (odds ratio for moderate or severe left

ventricular systolic dysfunction vs normal ejection fraction 2.5 (95% confidence interval 1.5 – 4.4), but left atrial (LA) size was not.[158]

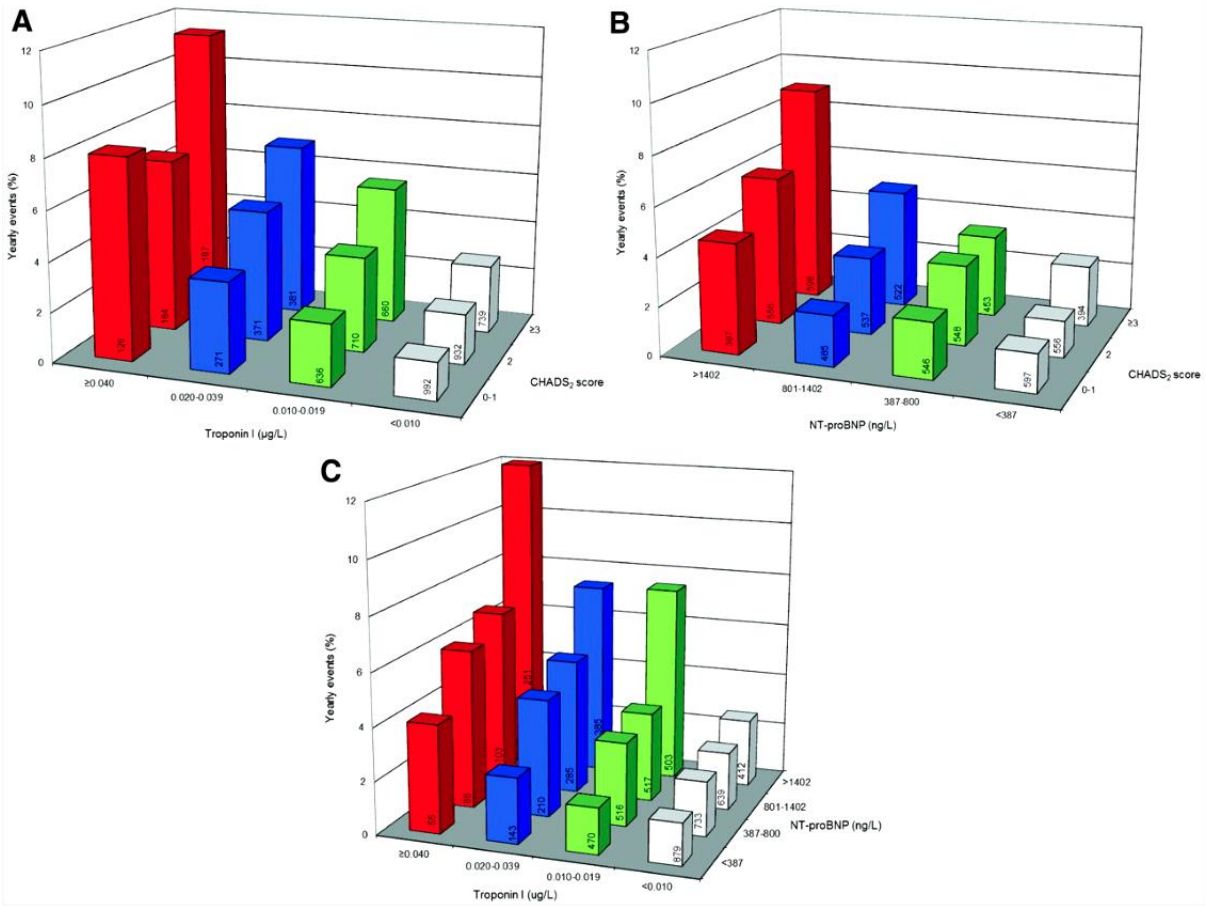


Figure 1-2 – Stroke, systemic embolism, pulmonary embolism, myocardial infarction, vascular death (excluding haemorrhagic death) in relation to troponin I levels and CHADS2 scores (A), NT-pro-BNP levels and CHADS2-scores (B), and troponin I and NT-pro-BNP levels (C). Total number of patients given in each bar. Taken from Hijazi et al, 2012.

In anticoagulated patients in ARISTOTLE, imaging evidence of SEC or left atrial (appendage) thrombus were not associated with TE,[159] although, in a recent study analysis of patients undergoing catheter ablation for atrial fibrillation, SEC was a risk factor for stroke after adjustment for CHA_2DS_2-VASc factors.[160] In the prospective ENGAGE echocardiographic substudy, no risk factors for TE were identified, although left ventricular diastolic indices such as E/e' were an independent predictor of death.[161] In AFFIRM, another predominantly-anticoagulated cohort was studied with no echocardiographic predictors of stroke being identified, although larger atrial size corresponded with more episodes of AF.[162] In RAF, a large prospective trial of patients with acute stroke and AF, increased LA size measured with transthoracic echocardiography was associated with recurrent stroke events.[163] LA size

also predicted cardiovascular events in patients with 'lone' AF (those patients without cardiac comorbidity or clinical risk factors for stroke), suggesting that this simple echocardiographic metric could help identify truly low risk patients.[164]

LA size and left ventricular diastolic function are intrinsically linked; in the Olmsted County echocardiography registry of 2,042 patients, both diastolic function and LA size predicted mortality. However, when controlling for diastolic dysfunction, LA size was not an independent predictor of mortality.[165] Measures of left ventricular diastolic function as estimated by E/A ratio,[166] E/e', [167–169] LA strain,[170,171] and left ventricular mass [172] are significantly associated with stroke rates in patients with AF, in some cases after adjustment for CHA_2DS_2-VASc risk factors.[170] Closely related to left ventricular filling pressure, LA size (≥ 33 ml/m^2) was related to stroke risk in a cohort having undergone atrial surgery.[173] Providência *et al* used transthoracic echocardiography-derived indices (LA volume and left ventricular function) to predict transoesophageal echocardiographic (surrogate) markers of thrombosis risk.[174] In this study, a hybrid risk score using CHA_2DS_2-VASc risk factors and echocardiographic parameters modestly outperformed CHA_2DS_2-VASc alone.

3D imaging - cardiac CT & MRI

Cardiac computed tomography (CT) has been used to identify LA thrombus, for example prior to cardioversion. However, its ability to predict future thrombus (and stroke) is less well-established. Di Biase *et al* studied 932 LA images (either CT or magnetic resonance imaging, MRI) and described four distinct LA morphologies - Chicken Wing, Cactus, Cauliflower and Windsock (Figure 1-3, taken from [175]).[176] The authors found that patients with a 'Cauliflower' LAA had the highest risk of prior stroke / TIA, and that dichotomising patients into a low-risk 'Chicken Wing' phenotype and 'Non-Chicken Wing' morphologies gave an odds ratio of 2.95 (95% confidence interval 1.75 – 4.99) for prior stroke / TIA. In patients with low TE risk (CHADS2 ≤ 1), this difference was even more stark, with an odds ratio of 10.1 (95% confidence interval 1.25 – 79.7), suggesting that imaging of the LAA may provide additional information alongside traditional risk scores, particularly in identifying 'truly low-risk' individuals. Meta-analysis of 2,596 patients in this and 7 similar studies confirmed that patients with chicken wing LAA morphology had a 54% lower risk of TE.[177]

Patients with chicken wing LAA morphology have smaller LAA orifice area, higher LAA velocities (measured by transoesophageal echocardiography) and fewer trabeculations, which may explain how LAA morphology affects TE risk.[178–180] In order to streamline the classification of LAA morphology and improve inter- and intra-observer variability, a simpler classification scheme is proposed where an acute angle from the proximal to mid portions of the LAA is defined as low risk but this is not yet validated.[181]

Figure 1-3 – Computed Tomography images of four common shapes of left atrial appendage.

Delayed-enhancement cardiac MRI images can identify atrial fibrosis, which can be graded by the percentage of atrial wall affected.[182] LA fibrosis quantified in this manner predicts atrial arrhythmia onset, LAA thrombus and AF ablation outcomes.[182–184] The degree of LA fibrosis is strongly determined by clinical risk factors,[185] yet King *et al* retrospectively reviewed 1,228 MRI images and showed that the degree of atrial fibrosis independently predicted stroke outcomes (hazard ratio 3.94 for stage IV vs stage I fibrosis, 95% CI 1.72 – 8.98).[186] This finding was in keeping with earlier work by Daccarett and colleagues who first described an association between degree of LA fibrosis and stroke in a smaller cohort.[187]

Imaging - conclusions

Left ventricular systolic and diastolic function indices predict stroke in patients with AF. In early trials examining other echocardiographic parameters, SEC was associated with TE. However, these imaging features may be less important in anticoagulated patients, and more recent trials of anticoagulated patients do not demonstrate this finding. These findings could also be explained by the low overall TE rates in treated patients in the ENGAGE and ARISTOTLE cohorts, with subtle differences in TE rates being difficult to detect, or due to underreporting of negative results. LA size measured echocardiographically does not appear to be a reliable predictor of TE, though LAA morphology appears to independently predict stroke rates, with the common 'chicken wing' LAA having the lowest rates of TE. Other markers, such as LA fibrosis, are closely correlated with stroke rates. The degree to which all these imaging parameters act independently of one another is not established.

Risk scores

Risk factors identified by the SPAF and AFI investigators were amalgamated to form the $CHADS_2$ risk score.[188] Developers of this score were motivated to generate a bedside scoring tool that was easy use without the need for further investigations. In this schema, patients are assigned a point for each of congestive heart failure, (history of) hypertension, age > 75 years, and diabetes mellitus, with two points assigned for history of cerebral ischaemia. Scores were internally validated against a Medicare database, with the $CHADS_2$ score performing well (C-statistic 0.82). However, in subsequent validation studies, the $CHADS_2$ score performed less well, with C-statistics between 0.58 and 0.72.[189–194]

Similar risk scores were proposed by the UK National Institute for Health and Care Excellence (NICE) [195] and the American Heart Association/European Society for Cardiology,[196] with small differences in the way the same risk factors were used to define low, medium or high risk individuals. A more precise risk stratification method using multiple age and systolic blood pressure strata was devised by the Framingham Heart group, though this is harder to calculate and not more accurate than $CHADS_2$.[193,194]

The $CHADS_2$ score was later refined with the addition of two other risk factors (vascular disease and female sex) and a third age strata (0 points for age < 65, 1 point for age 65-74, 2 points for >75), forming the CHA_2DS_2-VASc score.[85] The rationale for this was to produce a clinically useful score that would identify truly low-risk patients (who do not require anticoagulation), even if this resulted in a reduced ability to differentiate moderate- from high-risk patients - a clinically less important distinction, at least when making anticoagulation decisions.

QSTROKE was developed from UK general practice databases, aiming to risk stratify individuals for stroke using risk factor data that had already been collected for the QRISK2 score for predicting cardiovascular disease risk.[197] Although the derivation database included those with prior stroke, these individuals were excluded. It is notable that, unlike other risk scores, it includes deprivation and ethnicity indices. It performed marginally better than CHA_2DS_2-VASc in its own validation study, although due to its complexity has not been adopted outside of primary care.

The ATRIA score was derived from a population of non-anticoagulated patients with NVAF.[108] It combines clinical variables (age, prior stroke, female sex, diabetes, heart failure and hypertension) with biomarker data for renal function (proteinuria and eGFR). In external head-to-head validation, it performed similarly or slightly better when compared to CHA_2DS_2-VASc.[108,125,198–201] In meta-analysis, the ATRIA score slightly outperformed CHA_2DS_2-VASc overall, although, for identifying truly low-risk patients, CHA_2DS_2-VASc was superior.[202]

With the aim of refining risk stratification using biomarkers, the ABC scores (Age, Biomarkers, Clinical history) were developed.[203] They were derived using the ARISTOTLE study population and validated using the STABILITY, ENGAGE and RE-LY cohorts.[132,137,203] The ABC-stroke score uses weighted values for age, previous stroke / TIA, cardiac troponin T and BNP. The ABC-bleeding score uses age, previous bleeding event, GDF-15, cardiac troponin T and haemoglobin (Table 1-1). The ABC-stroke score outperformed CHA_2DS_2-VASc (c-index of 0.66 compared with 0.58) while the ABC-bleeding score outperformed the ORBIT bleeding score [204] and the commonly-used HAS-BLED score.[205] However, it is not as easily

calculated as CHA_2DS_2-VASc, and a limitation is that, in each of the derivation cohorts, patients had risk factors for TE and were anticoagulated, therefore using this score to classify someone as low risk (below a threshold for anticoagulation) requires extrapolation of the data.

Table 1-1 – Components of the ABC-stroke and ABC-bleeding scores

	Age	Biomarkers	Clinical Features
ABC-stroke	Age	NT-pro-BNP Cardiac troponin T	Previous stroke / TIA
ABC-bleeding	Age	GDF-15 Cardiac Troponin T Haemoglobin	History of bleeding

NT-Pro-BNP, N-terminal pro B-type natriuretic peptide. TIA, transient ischaemic attack. GDF-15, growth-derived factor 15.

Another risk model, GARFIELD-AF, was generated using stepwise regression of multiple clinical risk factors in a large cohort of 39,898 patients with NVAF.[206,207] Its web-based tool allows input of risk factors to calculate a score for ischaemic stroke risk, bleeding risk and mortality. When validated in an external population, it outperformed CHA_2DS_2-VASc (C-statistic 0.69 for stroke / systemic embolism vs 0.64).

The CHA_2DS_2-VASc schema can predict TE in patients without a history of AF. This has been observed in populations with ACS,[208] chronic obstructive pulmonary disease,[209] systemic lupus erythematosus,[210] implanted cardiac devices,[211] interatrial conduction delay [212] or high cardiovascular risk,[213] suggesting that the CHA_2DS_2-VASc stroke risk factors are not specific to AF itself.

To summarise, multiple scoring schema have been devised using clinical risk factor information ($CHADS_2$, CHA_2DS_2-VASc, Framingham, ESC, GARFIELD-AF). All have limited ability to predict TE with C-statistics mostly < 0.7, depending on the population being studied. Of these schema, GARFIELD-AF is the most accurate but is complex to calculate. CHA_2DS_2-VASc performs accurately, particularly for identifying truly low-risk individuals who do not require anticoagulation. However, most patients with AF will be classified as moderate or high risk according to CHA_2DS_2-VASc. The ability to accurately predict stroke rates at every threshold of a risk stratification schema, as reflected by the C-statistic, is arguably less important when

making individual decisions regarding anticoagulation. Nevertheless, for wider decision-making there is a clear interest in refining risk stratification for patients with AF, particularly for individuals where the bleeding risk is substantial [194]. Scores such as ABC-stroke, which utilise biomarker data, are inherently more time-consuming to calculate but provide more accurate risk assessment.

Aside from D-dimer, an *indirect* measure of fibrin clot breakdown, it is notable that no measures of either coagulation or bleeding are used in established risk stratification schema for TE or bleeding events. A summary of the pathological processes and risk stratification metrics discussed is provided in Figure 1-4.

1.1.4 Managing stroke risk

Once AF has been identified, potential options for managing the increased stroke risk include antiplatelet or anticoagulant medications. In general, these agents typically result in lower rates of thrombotic events at the expense of bleeding, with the net effect being of paramount interest. Various agents have been trialled, including antiplatelet drugs (aspirin, clopidogrel, dipyridamole and indobufen), vitamin-K antagonists (VKA, such as warfarin), and DOACs.

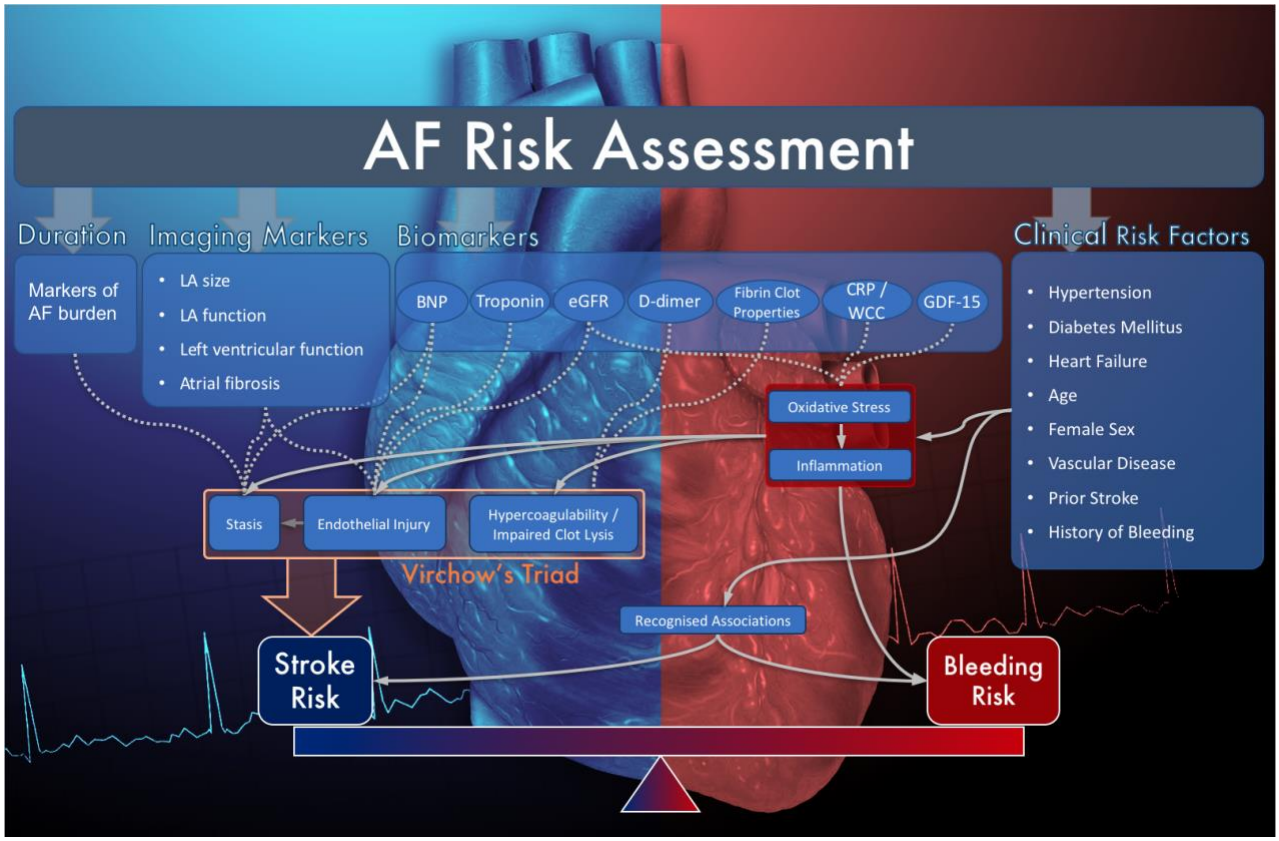


Figure 1-4 – Representative diagram of risk factors for stroke and bleeding in AF. Bold lines indicate pathophysiological processes, dashed lines represent associated risk markers. AF, atrial fibrillation. LA, Left atrium. eGFR, estimated glomerular filtration rate. CRP, C-Reactive Protein. WCC, White Cell count. GDF-15, Growth-differentiation factor 15. Cardiac image adapted from: Patrick J. Lynch, medical illustrator; C. Carl Jaffe, MD, cardiologist; used under Creative Commons Attribution 2.5 license.

Warfarin vs placebo & warfarin vs aspirin

The first randomised controlled trial of VKA in atrial fibrillation was AFASAK, which, although small (a total of 46 ischaemic events in 1,009 participants) and non-blinded, showed a statistically significant benefit of warfarin over both aspirin and placebo.[214] Similarly, in the 1990 Boston Area Anticoagulation for AF (BAATAF) trial, 'low-dose' warfarin (prothrombin time ratio target 1.2 – 1.5, corresponding to an international normalised ratio (INR) of approximately 1.5 – 2.7) was seen to be superior to a control group taking either aspirin or placebo.[215]

The SPAF-I trial compared aspirin (an antiplatelet medication) or warfarin (an anticoagulant) to placebo and found both were effective in reducing rates of stroke.[216] In this trial, the magnitude of stroke reduction was greater in those taking warfarin, though no head-to-head comparison was performed since the two treatment cohorts were not directly comparable.

However, the later SPAF-II trial showed warfarin to be more effective than aspirin in a direct comparison.[217] In the Veterans' Affairs Stroke Prevention in Non-Rheumatic AF trial (SPIN-AF), warfarin was superior to placebo in men with AF.[218] The European Atrial Fibrillation (EAFT) trial randomised individuals with a past history of stroke or TIA to either warfarin, aspirin or placebo.[219] Anticoagulation with warfarin was superior to both aspirin and placebo. The Canadian AF Anticoagulation (CAFA) trial also compared warfarin with placebo, but was terminated early after previous trials conclusively demonstrated substantial benefit of warfarin therapy.[220] Nevertheless, it too demonstrated a significant reduction in TE events in those treated with warfarin.

The Birmingham AF Treatment in the Aged (BAFTA) trial specifically compared warfarin to aspirin in an elderly population (age over 75 years), in whom there had been lingering concerns regarding the safety of warfarin.[221] This again demonstrated superiority of warfarin over aspirin in the reduction in stroke events, although no mortality benefit was shown.

Meta-analysis of these warfarin vs placebo trials (with the exception of the later BAFTA trial) found a 64% relative risk reduction in stroke (95% confidence interval 49 – 74%).[222]

Rates of haemorrhagic stroke were higher in the earlier trials of warfarin vs placebo than in the more recent trials. This may be because higher INR ranges were chosen, with more contemporary trials using the narrower range of 2.0 – 3.0. Additionally, several studies have demonstrated that haemorrhagic stroke risk is greatest in the first 90 days or 1 year of treatment,[223,224] perhaps because INR levels are initially unstable or an underlying bleeding diathesis is identified. Therefore, it follows that the earliest studies of anticoagulation typically enrolled warfarin-naïve individuals and had higher rates of bleeding. This is contrasted with the lower bleeding rates in BAFTA, in which 40% of participants were already taking warfarin at enrolment, despite similar time in the therapeutic range to the earlier trials.[221] Current guidelines suggest switching to a DOAC if warfarin cannot be well-controlled, but a large cohort study in Sweden demonstrated that well-controlled warfarin was non-inferior to DOAC for stroke prevention.[80,225] This suggest that although patients with a new diagnosis of AF may usually start a DOAC, patients already established on warfarin

with good control (an older population by default) may continue to take it, rather than switch to a DOAC.

Evidence for aspirin monotherapy

Several of the large trials described above also had arms that compared aspirin monotherapy with placebo for stroke prevention in AF.[214,216,219,226] Other trials have only compared aspirin with placebo or no treatment.[227,228] Of these trials individually, only the original SPAF-I trial demonstrated any statistically significant benefit of aspirin over placebo. In meta-analysis of seven trials comparing aspirin with placebo, a 19% relative risk reduction in stroke was observed with aspirin but this was not statistically significant (95% CI, -1% to 35%) and was substantially less than the 64% (95% CI, 49 – 74) relative risk reduction observed with warfarin.[222,229]

Aspirin had been posited as a safer option in patients at high risk of bleeding complications, for example the very elderly. However, bleeding risk factors overlap substantially with thrombotic risk factors,[188,205] while the rates of bleeding with aspirin monotherapy are similar compared with warfarin.[221,230] Moreover, as age increases, the absolute thrombotic risk increases at a greater rate than the absolute bleeding risk;[231] therefore, even in those at the highest risk of bleeding, no evidence supporting the preferential use of aspirin monotherapy has been established.[80,232]

In published guidelines, the ESC do not recommend aspirin at any level of stroke risk, although the AHA guidelines recommend either aspirin or no antithrombotic therapy for low-risk individuals with AF.[80,81]

Warfarin + SAPT

Since both warfarin and aspirin monotherapy (single antiplatelet therapy, SAPT) have been individually shown to reduce stroke events in AF patients, combination therapy has been trialled.

SPAF-III found that, in AF patients deemed to be at high risk of TE, low-dose warfarin (target international normalised ratio (INR) of 1.2 – 1.5) in combination with aspirin was substantially less effective than warfarin with a target INR of 2.0 – 3.0.[77] AFASAK-II also compared aspirin & 'mini-dose' warfarin (warfarin with a standardised daily dose of 1.25mg, median INR 1.10) alongside warfarin with a target INR of 2.0 – 3.0 and an aspirin-only arm.[233] This trial was terminated with only 45% of the estimated sample size recruited and only limited interpretation of these results is possible. Nevertheless, rates of adverse events were numerically higher in the mini-dose warfarin arms. In SAFT, low-dose warfarin plus aspirin was again compared with placebo, this time in an intermediate-risk cohort.[234] No significant stroke reduction was observed in those taking aspirin and warfarin, although bleeding complications were more common.

Another regimen of warfarin with SAPT was trialled in the high-risk arm of National Study for Prevention of Embolism in AF (NASPEAF).[235] Here, high-risk patients with AF were randomised to receive either warfarin (with a target INR of 2.0 – 3.0), or combination therapy with triflusal (a salicylate antiplatelet agent) and warfarin (target INR 1.4 – 2.4). In contrast to SPAF-III and AFASAK-II, combination therapy *was* seen to be superior to intensive warfarin therapy, suggesting that warfarin regimens with target INR < 1.4 are ineffective at reducing rates of stroke.

Other antiplatelet options

Until recently, aspirin and warfarin were the mainstay of stroke prevention for patients with AF. However, alternative regimens have also been trialled with combinations of either two antiplatelet drugs or an antiplatelet drug and an anticoagulant. For patients who require antiplatelet medication for CAD, rather than AF, the use of dual antiplatelet therapy (DAPT) is well-established,[236–238] suggesting that dual-pathway (thromboxane A_2 and $P2Y_{12}$ receptor) platelet inhibition is effective at reducing arterial thrombotic events. However, the differing pathophysiology of stroke in AF – that is, strokes that result from thrombus originating within the heart – means that results may not be applicable to a population with AF.

In the European Stroke Prevention Study (ESPS) 2, aspirin monotherapy, dipyridamole monotherapy and combination therapy with aspirin and dipyridamole respectively showed 18%, 16% and 24% relative risk reduction of stroke events, although this trial was not restricted to individuals with AF.[226]

The ACTIVE trials enrolled patients with AF and ≥ 1 risk factor for TE. Patients who were considered to be eligible for VKA (i.e., warfarin) were enrolled into the ACTIVE-W arm, comparing warfarin with DAPT (in this case aspirin & clopidogrel).[239] Those ineligible for VKA were enrolled in a separate arm (ACTIVE-A) comparing aspirin monotherapy with aspirin & clopidogrel. In ACTIVE-W, DAPT was inferior to oral anticoagulation with warfarin with respect to stroke events and stroke severity, leading to the study being terminated early.[78] A numerically worse (but not statistically significant) rate of major bleeding in the DAPT arm was also observed. In ACTIVE-A, the addition of clopidogrel to aspirin reduced the number of ischaemic events (6.8%/year vs 7.6%/year) but this was offset by an increase in the rates of major bleeding (2.0%/year vs 1.3%/year) such that no overall benefit was seen in a composite outcome of ischaemic events and major bleeding.[79]

In the Studio Italiano Fibrillazione Atriale (SIFA), the platelet inhibitor indobufen provided similar efficacy and safety compared with warfarin for the prevention of stroke in patients with AF and a previous stroke episode.[240]

DOAC randomised controlled trials

More recently, DOACs have been used to treat AF. The first DOAC, the direct thrombin inhibitor ximelagatran, demonstrated similar efficacy when compared with warfarin,[241] although its development was halted due to hepatotoxicity. Subsequently, four other drugs have been developed for use in AF. Those licensed for use in AF include apixaban, rivaroxaban, dabigatran and edoxaban, all of which have been shown to be non-inferior to warfarin in large-scale clinical trials.[242–245] Moreover, meta-analysis of these four trials found an overall mortality benefit of DOACs compared with warfarin, predominantly due to a reduction in major bleeding, particularly intracranial haemorrhage.[246] DOACs have the additional benefit over warfarin in that they do not require blood test monitoring of INR and are thus more convenient. As a result, the most recent European Society of Cardiology (ESC) and

American Heart Association (AHA) guidelines both recommend the use of DOACs preferentially over warfarin in eligible patients, although a substantial number of patients are still ineligible on the grounds of body weight, renal function, liver disease or those with mechanical prosthetic heart valves.[80,247]

Network meta-analysis & observational data

With a role for anticoagulation in patients with AF firmly established, it is unethical to enrol patients with AF (and ≥1 risk factor for TE) in new trials of anticoagulation vs placebo. Therefore, while trials comparing DOACs with placebo have been performed in patients with venous thromboembolism,[248,249] no randomised controlled trials have compared DOAC with placebo in patients for stroke prevention in AF. Similarly, no randomised trials have performed direct head-to-head comparisons of the various DOACs available.

Network meta-analysis (NMA) can be performed to overcome this limitation and to provide limited head-to-head comparison. Briefly, this statistical method allows comparison of interventions across different trials based on a common comparator.[250] For example, where data exists for placebo vs warfarin and warfarin vs DOAC, an NMA allows the indirect comparison of DOAC vs placebo, although it is not possible to completely adjust for confounders between groups. NMA has been performed showing similar performance of apixaban, rivaroxaban, edoxaban and dabigatran for both safety and efficacy endpoints, while aspirin and VKA perform less well.[251,252]

Comparison of DOACs and warfarin in healthcare registry data has shown similar 'real world' efficacy and safety of DOACs as in the DOAC randomised trials.[253–256] Whilst direct head-to-head comparison using this observational technique is inherently less robust than data from RCTs, these data reassuringly confirm that DOACs appear to be safer than warfarin, although Lip *et al* found different rates of stroke and major bleeding between DOACs.[253]

Left atrial appendage closure

Left atrial appendage closure (LAAC) devices occlude the LAA and thus prevent TE from the LAA entering the systemic circulation. Evidence from two prospective trials (PROTECT-AF and PREVAIL) comparing the Watchman LAAC device with warfarin suggests that these devices

offer similar protection from ischaemic stroke and have similar safety profiles.[257,258] LAAC is invasive with substantial 'up-front' procedural risk, although, since there is no long-term requirement for anticoagulation, a potential reduction in long-term bleeding risk may be expected. Using 5-year follow-up data from PROTECT-AF and PREVAIL, Reddy *et al* demonstrated a reduction in haemorrhagic stroke and all-cause mortality with LAAC compared with warfarin, with non-inferiority for ischaemic stroke.[259] In subgroup analysis, LAAC performed better in those with higher bleeding risk (HAS-BLED >2).

A weakness of the PROTECT-AF and PREVAIL trials is that they compare LAAC with VKA rather than the current standard of care in anticoagulation - DOACs. In NMA of LAAC vs DOAC treatment, LAAC was associated with fewer gastrointestinal bleed events.[260,261] Overall mortality, ischaemic stroke and major bleed rates were similar between the two treatments. However, confidence intervals were wide, and the LAAC trials may have been underpowered to show subtle differences in bleed rates – it is noteworthy that a total of 1,114 patients were enrolled in PROTECT-AF and PREVAIL, compared with 71,683 patients in the four large DOAC trials (ROCKET-AF, ARISTOTLE, RE-LY, and ENGAGE-AF).

LAAC offers an alternative to anticoagulation for patients with AF and high stroke risk. It is particularly useful for patients who have a contraindication to anticoagulation or those with high bleeding risk. However, superiority over DOACs has not been demonstrated and it remains unclear whether LAAC will become a useful treatment for a majority of patients with AF.

1.1.5 Summary

The presence of AF increases the risk of stroke 5-fold, but this risk can be reduced by approximately 64% with the prescription of anticoagulants. To address this risk, there is good evidence that warfarin is superior to placebo, using a dose titrated to INR. However, DOACs have now largely superseded warfarin due to their improved safety profile. Although widely prescribed, there is limited evidence of a much more modest benefit of aspirin for stroke prevention in AF, with similar bleeding risks seen when compared with warfarin. Other antiplatelet regimens have been trialled but are not currently recommended. ESC guidelines and AHA guidelines now both recommend DOAC treatment for those with stroke risk

factors.[80,81] For those with the lowest stroke risk, the ESC recommends DOAC or no antithrombotic treatment, whereas the AHA recommends aspirin or no treatment.

With current stroke prophylaxis treatments, the side-effect of bleeding is prevalent, so being able to accurately risk stratify individuals for both stroke and bleeding risk is of paramount importance. Many of the risk factors for stroke and bleeding overlap, meaning those with the highest stroke risk also frequently have a high bleeding risk with net benefit of anticoagulation remaining. Nevertheless, the perception of high bleeding risk may lead to under-anticoagulation in high-risk groups, particularly the elderly.

Risk stratification for individuals with AF is complex and our understanding is incomplete. Comprehensive risk stratification could involve clinical risk factors, duration of AF, biomarkers measurement, and imaging techniques. While multiple metrics are individually useful for risk stratification, it remains unclear how these markers of risk can be combined to tailor an individual's treatment.

1.2 Assays to measure fibrin clot properties in patients with atrial fibrillation

1.2.1 Fibrin clot

Fibrin clots are temporary cross-linked fibrous structures that form in order to attenuate bleeding.[262] Fibrin clot is the end-product of the coagulation cascade, a tightly-regulated homeostatic mechanism where a series of consecutive reactions between proteases and co-factors result in the conversion of prothrombin to thrombin.[263] Thrombin then catalyses the conversion of fibrinogen to fibrin and activates factors V, VIII and XIII. The properties of the fibrin clot depend upon this thrombin concentration, with higher thrombin concentrations resulting in impermeable clots with relatively thinner fibrin strands.[264] Factor XIII acts to stabilise the clot by forming crosslinks between fibrin strands, which increase clot elasticity and stability.[265]

To enable healing, these clots are subsequently broken down in a process called fibrinolysis. The major pathway responsible for fibrinolysis is the plasminogen-plasmin system.[266] Tissue plasminogen activator (tPA) and/or urokinase activate plasminogen, forming plasmin which hydrolyses peptide bonds in the fibrin molecule, resulting in clot lysis.[267] While free tPA has weak affinity for plasminogen, when bound to the surface of fibrin its activity is increased several-fold.[268] This has the effect of restricting its activity to the site of clot formation. Plasmin generation exhibits positive feedback mechanisms, with plasmin cleaving tPA to a more active form, further increasing plasmin generation, amplifying clot lysis.[269]

The process of coagulation-fibrinolysis is a dynamic equilibrium with a natural tendency for clot formation followed by fibrinolysis (negative feedback). Increased fibrinolysis is observed in FXI insufficiency and acquired disease states such as liver cirrhosis, trauma and Dengue fever.[270] Conversely, hypofibrinolysis may increase thrombotic risk in other disease states such as malignancy, myocardial infarction and chronic thromboembolic pulmonary hypertension.[150,271,272]

1.2.2 Measuring fibrin clotting function

Activated partial thromboplastin time (APTT) and prothrombin time (PT) are clotting tests used in routine clinical practice. These tests have found widespread clinical utility in monitoring drug activity – heparin and vitamin K antagonists respectively.

For these assays, the endpoint is fibrin clot generation, overlooking the ongoing thrombin production ('propagation') and fibrinolysis. This is potentially important since clotting is a dynamic equilibrium between the opposing elements of fibrin generation and fibrinolysis. Indeed, clotting occurs well before the majority of thrombin is produced, typically after only 5% of the total thrombin response.[273,274] From a practical viewpoint, clinicians typically have access to information from clotting – not fibrinolysis – assays so have limited access to personalised data to weigh up thrombotic and bleeding risks when considering anticoagulant, antiplatelet or thrombolytic treatments, or prothrombotic treatments such as tranexamic acid. Since substantial heterogeneity of fibrinolysis has been observed in healthy volunteers and several disease states, understanding an individual's capacity for fibrinolysis may offer a way to guide treatment.[150,275,276]

Viscoelastic measures

Measuring global coagulation function via viscoelastic tests was established with thromboelastography (TEG) in 1948.[277] In this initial description, a torsion wire sits within an oscillating cup containing whole blood in which clotting is induced. As clot is formed, the movement of the wire is proportional to the clot strength. Since this assay uses whole blood, results are affected by the activity of platelets and other constituents of whole blood. Rotational thromboelastometry (ROTEM) is a refined, semi-automated, implementation of the TEG technology and has found a clinical role as a point-of care test to identify coagulopathy in trauma and surgical settings.[278] These assays have predominantly been used to determine coagulation metrics, although it is possible to identify lysis parameters. Hyperfibrinolysis during acute trauma or surgery can be detected and treated, for example with tranexamic acid. These assays have a modest ability to detect anticoagulation.[279,280] While typically used on whole blood samples, TEG and ROTEM can be used on plasma samples. Although this application has not seen widespread use, it has been used to demonstrate the effects of inflammation on fibrin clot strength.[281] White blood cells

promote fibrinolysis via neutrophil elastase,[282] while platelets are an integral part of the fibrin clot structure *in vivo*, with increasing evidence available to suggest that they modulate fibrinolysis.[283,284] Indeed, TEG measures of fibrinolysis in whole blood demonstrate a different response to endotoxaemia when compared with plasma-derived TEG measurements.[285] TEG and ROTEM require for specialist equipment and have poor assay reliability (high coefficient of variation), but are used in specialist situations, for example after cardiac surgery.[286]

Plasma turbidity and lysis

The plasma turbidity and lysis assay (PTL) involves plasma which is made to clot (with the addition of thrombin or tissue factor) and then lyse (with addition of tissue plasminogen activator (tPA)). Photometry measures the light transmission through the sample, with light absorption being approximately proportional to clot density at each timepoint. Several parameters can be computed from the absorption-time plot, including clot lag time, peak turbidity, and lysis time. Since PTL relies on light transmission, this technique cannot be used on whole blood as haemoglobin readily absorbs light.

Since clot lysis is induced by the addition of exogenous tPA, the concentration of endogenous plasminogen activators is not critical for lysis. Thus, PTL lysis parameters may represent the ability of clot to withstand exogenous tPA rather than measure intrinsic lysis potential. Secondly, this technique necessarily excludes the effects of other components of whole blood such as white blood cells and platelets which may, for the reasons listed above, limit its ability as a global assay of clotting and lysis. A widely-accepted protocol for PTL has not yet been developed.[287]

On the other hand, a substantial benefit of this technique is that it can be performed on high-throughput devices, making it ideal for large-scale research projects. As a result, it has been used to demonstrate heterogeneity of fibrinolysis and clotting across a number of clinical conditions including arterial thrombosis, venous thrombosis and ACS.[150,288,289]

Global fibrinolysis capacity assay

Using similar methods to the PTL assay, global fibrinolysis capacity (GFC) examines only the fibrinolysis phase.[290,291] Clotting is induced in plasma, then light transmission detects the timing of clot dissolution. This has the benefit that it is less reliant on the concentration of exogenous tPA. This technique has been used to demonstrate hypofibrinolytic states in polycystic ovarian syndrome and hypothyroidism, and a hyperfibrinolytic state in chronic liver disease but has not seen widespread use.[292–294] A more complex implementation of GFC in whole blood has been developed but has also not been widely adopted.[295]

Calibrated automated thrombography

The endpoint of the coagulation cascade is thrombin production, so coagulation capacity can be inferred by the amount of thrombin produced. The endogenous thrombin potential (ETP) is the amount of thrombin that can be produced by a sample after clotting has been initiated (typically with tissue factor).[273] Practically speaking, this can be determined by calculating the area under a thrombin concentration – time curve. Measuring thrombin concentration during clotting is challenging, but the calibrated automated thrombogram (CAT) does so using a fluorogenic thrombin substrate which is activated in the presence of thrombin resulting in a measurable fluorescence.[296] This assay has been adapted for use in whole blood.[297] It is technically challenging, requires reference plasma and is sensitive to changes in sample preparation.[298] It has been used to demonstrate hypercoagulability in stroke, pulmonary hypertension, venous thromboembolism, malignancy, and the antiphospholipid syndrome.[299–303] However, like PT and APTT, it measures only thrombin production, not fibrinolysis, limiting its scope. It has been further adapted to simultaneously measure both plasmin (a marker of fibrinolysis) and thrombin, either in one or multiple wells, but this method adds significant complexity and is not (yet) well-validated.[304] In its standard form, CAT appears to have a relatively narrow scope and in modified implementations is technically very challenging.

Conclusion

Several assays examining fibrin clotting and lysis exist, each with inherent benefits and drawbacks. No clear leader has yet emerged. Currently, these assays exist as non-standardised research tools, although efforts are underway to create global consensus which may aid their clinical adoption. I intend to use the PTL assay, which has advantages including being able to identify heterogeneity in fibrinolysis while being highly scalable for high-throughput projects.

Our group has previously examined fibrin clot properties in a large cohort of patients with cardiovascular disease, demonstrating that individuals with impaired fibrin clot lysis characteristics after ACS have a higher risk of cardiovascular death and recurrent MI[150]. This study used a thrombin-containing solution to initiate clot formation, measurable by a process called turbidimetry.

1.2.3 Thrombin assay design

In brief, patients' plasma is thawed and brought to 37°C in a water bath, before being aliquoted into a 96-well plate in duplicate wells. Including four control samples (to ensure quality control and reliability), this allows 46 plasma samples to be analysed on one plate. An activation mix is then added column-wise at timed intervals to the plasma to initiate fibrin clot formation. The activation mix comprises a buffer solution (tris(hydroxymethyl)aminomethane (TRIS), sodium chloride), procoagulant factors (e.g., thrombin or tissue factor), tissue plasminogen activator (tPA) and calcium chloride ($CaCl_2$, citrated plasma samples must be recalcified to reverse the anticoagulant effect of citrate). The plate is then added to a high-throughput MultiSkan FC Microplate Photometer (ThermoFisher Scientific) for measurement of each well's light absorbance at regular intervals (typically every 10-30s). Light absorbance is used to estimate plasma turbidity, a marker of clot density over time. An example turbidimetry plot from a healthy volunteer is shown in Figure 1-5. Several parameters can be calculated from the light absorbance-time plot and are explained in detail in Chapter 2. Briefly, the lag time is the time (in seconds) from the beginning of the experiment to the initial rise in turbidity due to clotting. The peak turbidity is self-explanatory, while the 50% lysis time (LT_{50}) is the time from the peak of the curve to a 50% drop in turbidity.

1.2.4 Tissue factor assay concept

Our group has previously studied fibrin clot properties in patients following ACS. However, I wanted to explore fibrin clot associations in another cardiovascular condition, atrial fibrillation (AF). AF is associated with an increased risk of stroke and as discussed previously, there is substantial overlap in risk factors for AF strokes and MI. However, the assay previously used is not optimised for use in AF. This is predominantly because many patients with AF take anticoagulant medications – indeed, the proposed study will use plasma samples from the Apixaban for Reduction in Stroke and Other Thromboembolic Events in AF (ARISTOTLE) clinical trial, which compared the effects of two anticoagulant medications (warfarin and apixaban).[242] Moreover, I wished to examine individuals' response to apixaban. Since apixaban is a FXa inhibitor, it acts 'upstream' of thrombin and thus we may expect a thrombin-

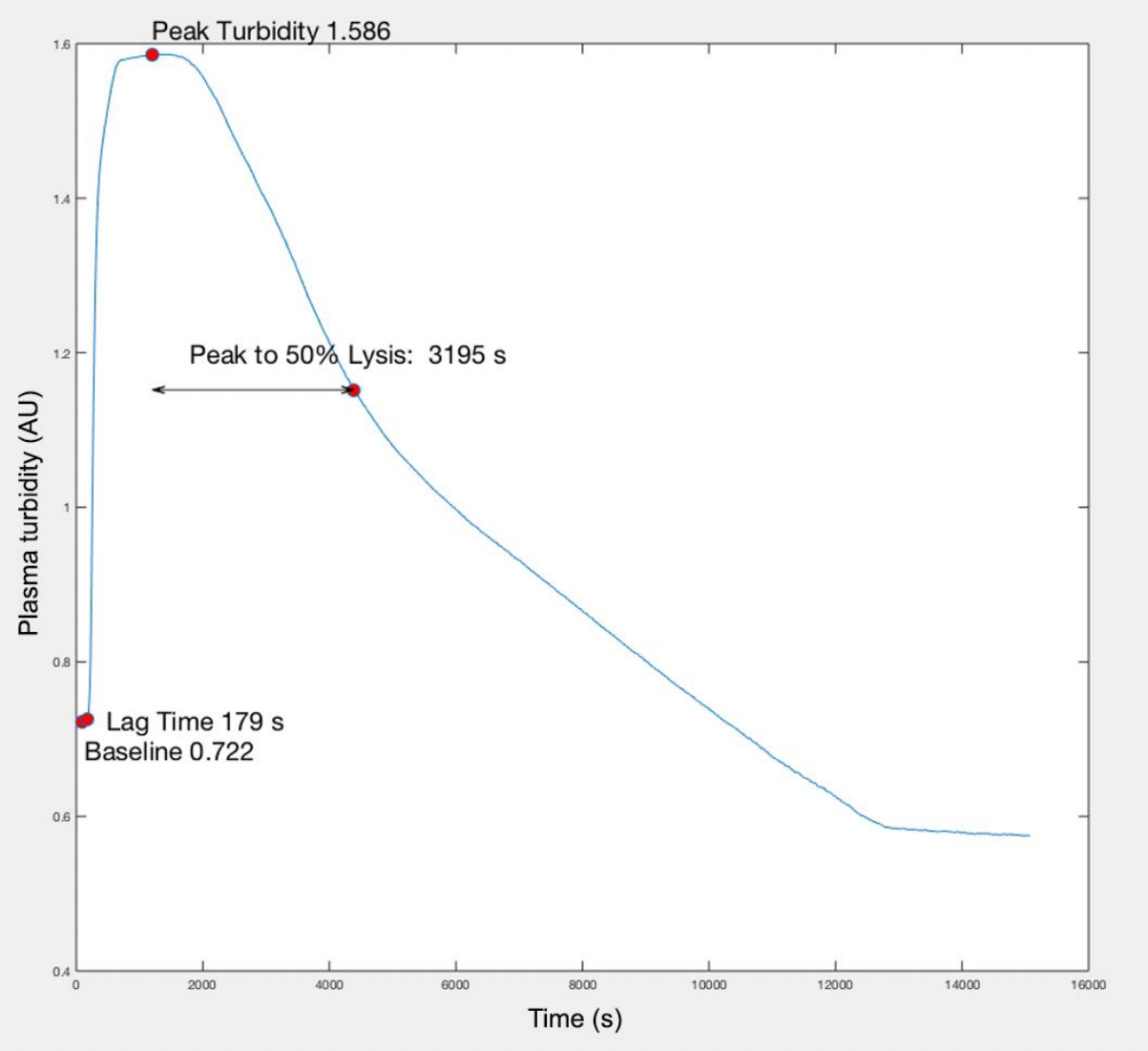


Figure 1-5 – Example turbidimetry plot demonstrating the initial lag phase, then rapid increase in turbidity during clot formation, followed by clot lysis.

based assay to be insensitive to the effects of an FXa inhibitor (Figure 1-6). Our group have previously shown that FXa inhibitors prolong fibrin clot lag time *in vitro* [unpublished data]. I hope to leverage that phenomenon to identify heterogeneity of drug levels amongst individuals as an additional marker of thrombosis or bleeding risk. This may be, for example, due to medical comorbidity that affects apixaban metabolism or excretion such as chronic kidney disease.

Alternative thrombotic agents, such as tissue factor (TF) or thromboplastin (a mixture of TF and phospholipids) activate the coagulation cascade upstream. Their use in fibrin clot assays has been described previously.[305,306] I intend to use a TF-based assay to examine fibrin

clot lag time, peak turbidity and lysis time. However, a protocol for the use of TF for this is not fully described and requires optimisation for my proposed patient population.

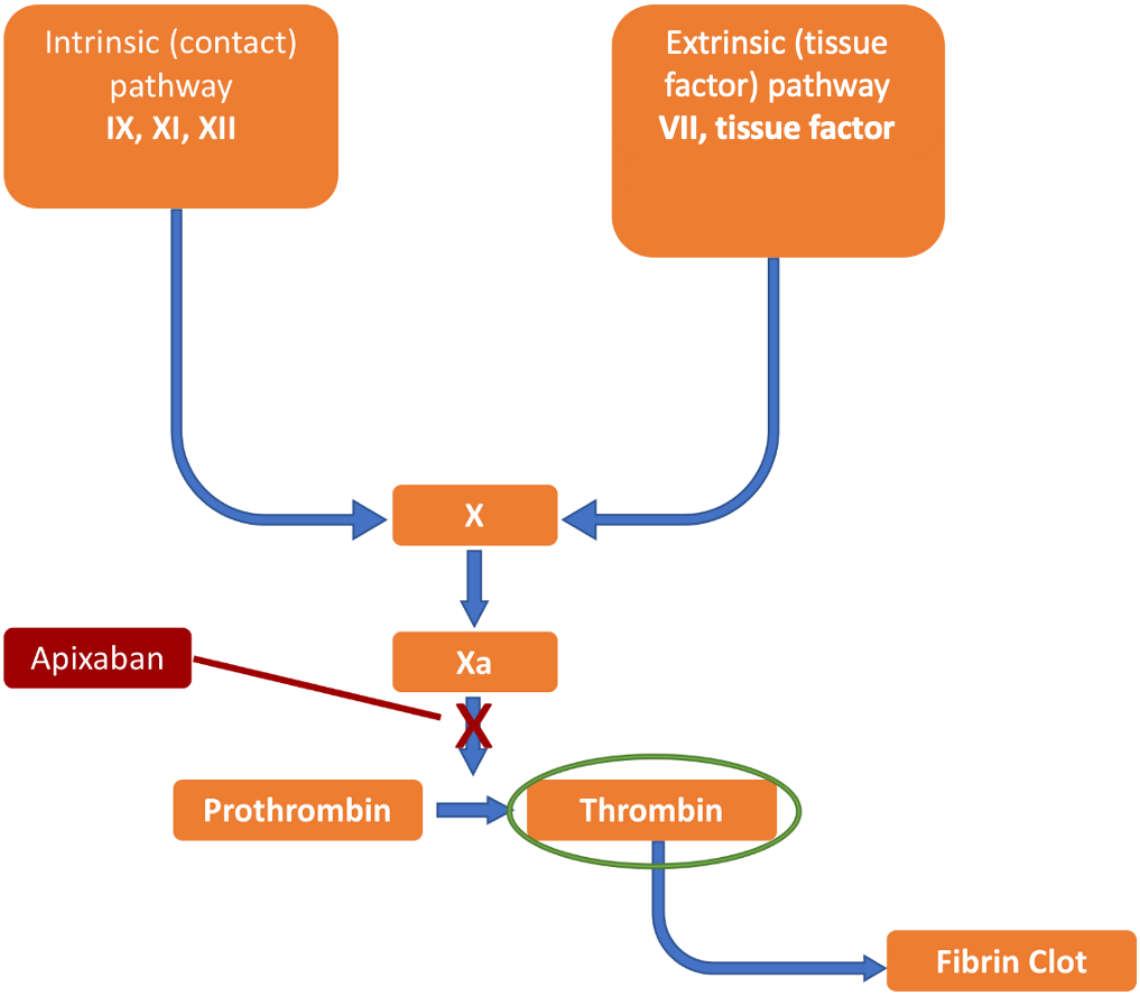


Figure 1-6 – Simplified coagulation cascade demonstrating the sites of action of apixaban, thrombin and tissue factor.

1.3 ARISTOTLE clinical trial

In Apixaban for reduction in stroke and other ThromboembolIc events in atrial fibrillation (ARISTOTLE), the then novel anti-Xa antithrombotic medication, apixaban, was compared with the standard of care anticoagulant, warfarin, in a large multicentre randomised, double-blind controlled trial.[242] The inclusion criteria are given in Table 1-2.

Table 1-2 – ARISTOTLE trial inclusion and exclusion criteria

Inclusion criteria	Atrial fibrillation or atrial flutter on two ECGs at least 2 weeks apart in the prior year and ≥ 1 stroke risk factor: • Age ≥ 75 years • Prior stroke, transient ischaemic attack, or systemic embolism • Symptomatic heart failure in prior 3 months or left ventricular ejection fraction < 40% • Diabetes mellitus • Hypertension requiring medications
Exclusion criteria	• AF from reversible cause • Moderate or severe mitral stenosis • Need for anticoagulation other than AF • Stroke in prior week • Need for aspirin dose ≥ 165 mg/day or aspirin and clopidogrel • Creatinine > 2.5 mg/dL or creatinine clearance < 25 mL/min

ECG, electrocardiogram.

1.3.1 Patient population

18,201 patients consented to participate in ARISTOTLE, of which 14,892 additionally consented to biomarker substudies and had blood samples taken for plasma storage. ARISTOTLE and biomarker substudy participants provided informed consent. Protocols were subject to ethical committee review at enrolling centres.

1.3.2 Outcomes

Outcomes in ARISTOTLE were adjudged by a clinical events committee blinded to study-group assignment, according to definitions in Table 1-3.

Table 1-3 – ARISTOTLE trial outcomes

Primary efficacy outcome	Stroke (focal neurological deficit from a nontraumatic cause lasting at least 24 hours) OR systemic embolism
Secondary efficacy outcome	Myocardial infarction
Primary safety outcome	ISTH Major bleeding[307] Clinically overt bleeding accompanied by a decrease in the haemoglobin level of at least 2 g per decilitre or transfusion of at least 2 units of packed red cells, occurring at a critical site, or resulting in death.
Secondary safety outcome	Composite of: • major bleeding AND • clinically relevant non-major bleeding (clinically overt bleeding that did not satisfy the criteria for major bleeding and that led to hospital admission, physician-guided medical or surgical treatment, or a change in antithrombotic therapy).

ISTH, International Society on Thrombosis and Haemostasis.

1.4 Hypotheses and Aims

I hypothesise that variance in measurable fibrin clotting dynamics predicts the risk of thrombotic and bleeding tendencies, in both individuals and populations with atrial fibrillation. Moreover, that patient characteristics may explain some of the variance in fibrin clot dynamics in patients with atrial fibrillation.

The specific hypotheses in this thesis are as follows:

1. Fibrin clot lysis time and lag time are associated with bleeding in patients with atrial fibrillation treated with oral anticoagulants.
2. Fibrin clot lysis time and lag time are associated with thrombotic events in patients with atrial fibrillation treated with oral anticoagulants.
3. Fibrin clot lysis time, lag time and maximum turbidity are associated with inflammatory biomarkers in patients with atrial fibrillation.
4. The treatment effect of apixaban, determined by fibrin clot lag time with a tissue factor-based assay, is influenced by renal function and other patient characteristics.

To examine the above hypotheses, I have developed the following objectives:

1. Develop and refine thrombin-based and tissue factor-based assays to measure fibrin clot properties in patients with atrial fibrillation.
2. Examine fibrin clot lysis time in patients with atrial fibrillation using thrombin-based and tissue factor-based assays, examining correlations with baseline demographics, comorbidity, comedication, clinical outcomes and other biomarkers.
3. Examine fibrin clot lag time in patients with atrial fibrillation using thrombin-based and tissue factor-based assays, examining correlations with baseline demographics, comorbidity, comedication, clinical outcomes and other biomarkers.
4. Examine fibrin clot maximum turbidity in patients with atrial fibrillation using thrombin-based and tissue factor-based assays, examining correlations with baseline demographics, comorbidity, comedication, clinical outcomes and other biomarkers.
5. Examine the association between fibrin clot lysis time and bleeding events in patients with atrial fibrillation treated with oral anticoagulants.
6. Examine the association between fibrin clot lysis time and ischaemic events in patients with atrial fibrillation treated with oral anticoagulants.

7. Examine the associations between increase in fibrin clot lag time with a tissue factor-based assay during treatment with apixaban, compared with prior to initiation of apixaban treatment, and clinical characteristics, including renal function.

Chapter 2: Measuring fibrin clot properties and estimating anti-Xa levels in patients with atrial fibrillation – materials and methods.

2.1 Participant samples and general study design

2.1.1 Plasma samples

Plasma was produced by centrifugation of citrate-anticoagulated venous blood samples and was stored at -20°C at participating centres before shipping to Uppsala Clinical Research Center (UCR) for aliquoting and storage at -70°C. Prior to the fibrin clot analyses, all samples were shipped to the Sheffield Cardiovascular Research Unit in a single batch under freezing conditions and stored at -80°C.

2.1.2 Sample selection

Participants who had plasma samples at the randomisation and/or 2-month study timepoint were included. At the time of enrolment in ARISTOTLE, warfarin was the standard of care anticoagulant for patients with atrial fibrillation (AF). Accordingly, 57% of trial participants were either taking or had previously taken vitamin-K antagonists (VKA) such as warfarin. Patients were randomised according to whether they had previously received VKA, resulting in a marginally asymmetric randomisation with 49.9% of patients randomised receiving warfarin.

When planning my experiments in 2019, DOACs such as apixaban had become a standard of care in cardiological practice. For this reason, I did not study plasma from patients taking VKA at the time of sampling. Instead, I elected to focus on plasma samples from patients without history of VKA use at the randomisation timepoint, and, for the 2-month samples, those randomised to apixaban.

2.1.3 Cohorts

As a result of the sample selection procedure, some participants had samples at the enrolment, some at 2-month, and some at both timepoints. In almost all cases, sufficient plasma was available at both timepoints to perform both thrombin and TF assays. Subsequently, totals for participants depends on the assay used, timepoint and available

clinical data. Nevertheless, all data were used where available. This gave rise to three distinct cohorts (Table 2-1).

Table 2-1 – Sample cohorts for the fibrin clot studies

Non-treatment cohort	Samples taken at study enrolment. Participants with a history of VKA use were excluded. This allowed us to compare fibrin clot properties with demographics, biochemical information, and clinical outcomes in the absence of any confounding influence of anticoagulant medication.
Apixaban cohort	Samples taken at the 2-month follow-up timepoint, from patients randomised to apixaban treatment.
Paired cohort	Participants with samples available at both enrolment (no anticoagulant treatment) and the 2-month timepoint (during apixaban treatment). This allowed us to examine demographic and biochemical factors that may influence apixaban activity by comparing fibrin clot properties before and during apixaban treatment.

4,372 samples were tested. Results were available for 4,350 samples participants from 3,419 participants. These comprised 1,891 samples at the randomisation timepoint (hereby referred to as the 'non-treatment cohort') and 2,459 at the 2 month follow up ('apixaban cohort'). Of these, 931 had a result for both timepoints and results could be compared ('paired cohort').

2.1.4 Experimental Series

The fibrin clot studies were performed across three experimental series:

- Thrombin assay, randomisation samples
- Thrombin assay, 2-month samples
- Tissue factor assay, randomisation and 2-month samples

Reagents for each series were reconstituted in advance of all experiments within that series where possible. Identical plasma standards were used in each experiment within a series but differed between series. Accordingly, results for the quality control of experiments are comparable within each series but cannot accurately be compared between different series.

2.2 Assay

As discussed previously, different methods exist for studying fibrin clot properties using turbidity and lysis experiments. Here, I used two assays, a thrombin-based assay, and a tissue

factor (TF) assay. The development of the latter is discussed in Chapter 3. In both cases, a mixture of procoagulant agents and agents that promote clot lysis are added to participants' plasma in a 96-well plate. The light absorbance (as a surrogate of plasma turbidity) of each sample is measured periodically as the samples first clot, then lyse over a period of approximately 30-240 minutes.

The thrombin assay is established and validated in a similar cohort of patients. The TF assay has the additional advantages of potentially being able to measure the apixaban drug effect but is less well-established and potentially less reliable, due to phospholipid bilayer clumping in TF–phospholipid solutions.

2.2.1 Reagents

A tris (Fisher Scientific)-containing buffer solution (pH 7.4, NaCl 100 mmol/L, Tris 50 mmol/L) was used for dilution, while 1 mol/L calcium chloride (Sigma-Aldrich), tPA (Technoclone), thrombin (Calbiochem) and tissue factor (PPP-Reagent HIGH, Diagnostica Stago) were used.

Normal pooled plasma for quality control was created by mixing equal volumes of plasma from 11 healthy volunteers who had given their written consent for venepuncture and blood sample processing for the purposes of research.

2.2.2 Plasma sample preparation

In both the thrombin and TF versions of the assay, sample preparation was similar. Plasma samples are removed from storage at -80°C and thawed in a water bath at 37°C for 10 minutes, before inverting several times to ensure mixing. Samples were then aliquoted in duplicate into a flat-bottomed polystyrene 96-well plate (25 µL plasma for the thrombin assay or 50 µL for the TF assay). Quality control (QC) plasma was used on each plate (in positions A1, B1, G12 and H12).

Thrombin assay

Separate lysis and then activation mixes were added sequentially to the plasma samples in the 96-well plate. 75 µL lysis mix containing 167 ng/mL tPA was added prior to 50 µL activation mix (90 x 10^{-3} IU/mL thrombin, 22.5 mmol/L $CaCl_2$).

Tissue factor assay

100 µL of a mixture containing 17.7 mmol/L $CaCl_2$, 2.52 mg/mL PPP-Reagent-high and 124 ng/mL tPA was added to each plasma sample in the 96-well plate.

Final concentrations of reagents for both assays are given in Table 2-2.

Table 2-2 – Final concentration of reagents in each form of the assay

	Thrombin assay	Tissue factor assay
Thrombin	30 x 10^{-3} IU/mL	N/A
Tissue factor	N/A	1.68 mg/mL
tPA	83.5 ng/mL	82.6 ng/mL
$CaCl_2$	7.5 mmol/L	11.8 mmol/L
Plasma ratio	1 : 5	1 : 2

Concentrations after plasma added to well. Plasma volume given as ratio – e.g. for the thrombin assay, one part of plasma is added to five parts of the reagent mix. tPA, tissue plasminogen activator. $CaCl_2$, calcium chloride.

2.2.3 Measurement

High-throughput turbidimetric analysis was performed using a plate-reader (MultiSkan FC, ThermoFisher Scientific) measuring light absorbance at a wavelength of 340 nm. After shaking each plate for 5 seconds to remove bubbles, absorbance was measured every 15 seconds at 37°C for 1000 iterations (250 mins) or until >50% clot lysis was achieved in all samples.

2.2.4 Fibrin clot assay post-processing

Automated calculation of lag time, lysis time, baseline turbidity and peak turbidity were performed with manual correction (if timepoints were annotated incorrectly), and samples were repeated if no result was available. The mean of duplicate results was used where available. At all stages during analysis, observers were blinded to subjects' clinical characteristics and outcomes.

2.3 Anti-Xa levels

I observed that anticoagulation with apixaban influences fibrin clot lag time *in vitro*. The activity of apixaban can be monitored using anti-Xa levels.[308] I therefore sought to understand the relationship between fibrin clot lag time and anti-Xa levels.

Apixaban inhibits the activity of free and clot-bound FXa, preventing conversion of prothrombin to thrombin. Levels of anti-Xa activity are measurable using commercial assays and are sometimes used in the monitoring of DOAC activity.[308] Various assays exist to assess anti-Xa activity, but the general principle is the same in each; a known concentration of FXa is added to a plasma sample. Any FXa inhibitor present in the sample will neutralise FXa, while the remaining FXa can cause a measurable reaction. I used a chromogenic anti-Xa assay (Technochrom, Diapharma, Ohio) in which any remaining FXa cleaves a tripeptide-*p*-nitroaniline substance, releasing the chromogenic *p*-nitroaniline (p-NA). This results in a colour change measurable with a light absorptiometer[309].

Anti-Xa levels were estimated on a subset of the total fibrin clot cohort. I identified a subset of the month 2 (apixaban-treated) plasma samples on which to test anti-Xa activity. A sample size of 40 was chosen due to the practicalities and cost of the available testing kits. To identify samples with a relevant range of lag times, a selection was made from the median value of each of 40 quantiles of the lag time data (Figure 2-1). Controls were created with 0, 20, 50, 120, 180, 300, 350 and 500 ng/mL of apixaban (Cayman chemicals) in normal pool plasma and were tested contemporaneously.

The chromogenic anti-Xa assay was used according to its instructions. This contains an inhibitor that binds a known concentration of FXa, while any residual Xa reacts with a chromogenic substrate resulting in a compound (p-nitroanilide hydrochloride) whose concentration can be estimated using absorptiometry. For each participant, 200 µL anti-Xa buffer was added to 50 µL plasma and vortexed. 50 µL of the resulting mixture was added to a 96-well plate. At 60 s intervals, first 50 µL bovine factor Xa, then 50 µL of the chromogenic substrate, then 50 µL 2% citric acid was added to retard the reaction before reading the absorption using 405 nm wavelength light. Apixaban control plasmas were used to construct

a standard curve (Figure 2-2). Estimates of anti-Xa activity were then generated from the absorption data using the standard curve.

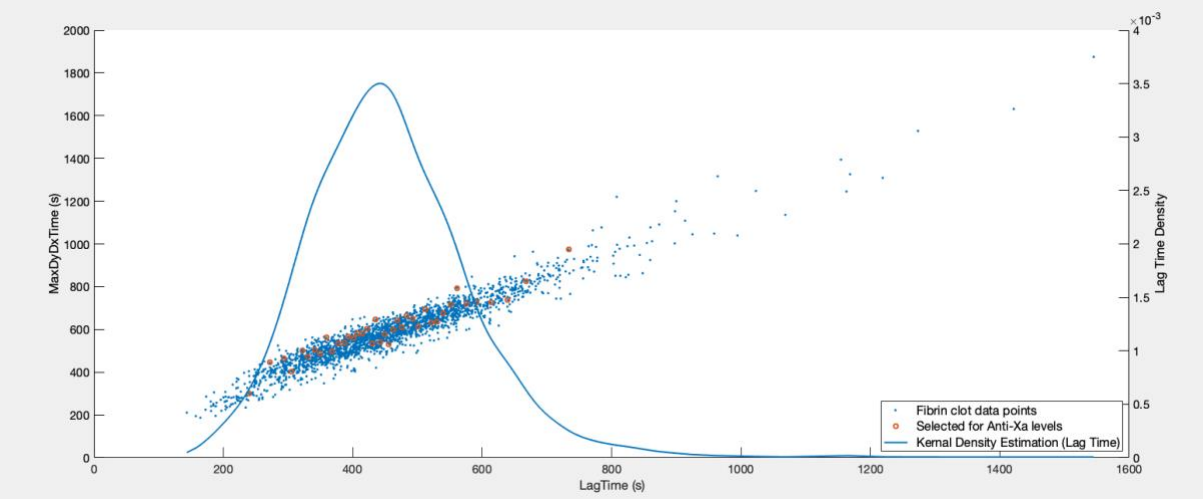


Figure 2-1 – Fibrin clot lag time of participants from the apixaban cohort, plotted against the related variable, maximum Dy/Dx Time (the time at which clotting is occurring most rapidly), with lag time kernel density estimate overlayed, to demonstrate sample selection. Datapoints from samples selected for anti-Xa estimation circled.

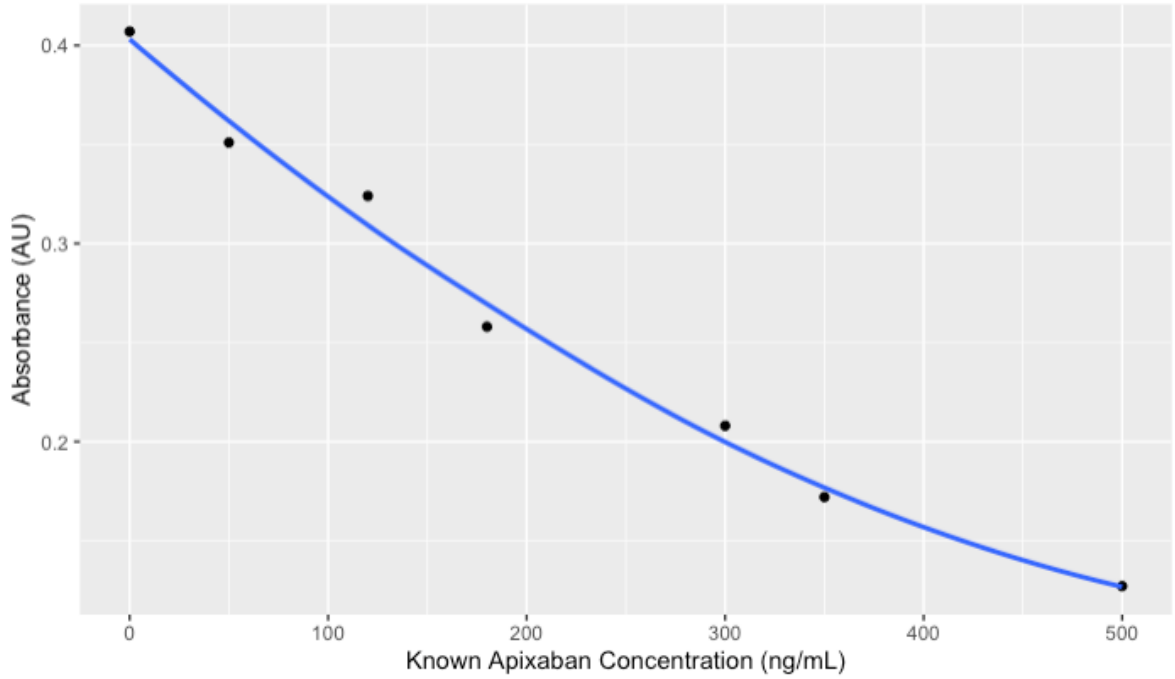


Figure 2-2 – Standard curve used to estimate apixaban concentration from absorbance data. AU, absorption units.

2.4 Statistical analysis

A statistical action plan covering all aspects of the subsequent analyses was devised prior to experiments by me and Professor Storey in conjunction with the statistical team at Uppsala Clinical Research (UCR). Analysis for the QC and reliability of the fibrin clot assay, and comparisons between assays were performed by me. Baseline characteristics, co-medication and clinical outcome data were processed by UCR in accordance with the statistical action plan.

Analysis was performed by quantiles of fibrin clot biomarker. For the thrombin assay, four quartiles were used. Given the limited number of participants in the paired cohort (TF assay only), three tertiles were used during the analysis of TF results.

2.4.1 Definitions

Stroke was defined as a non-traumatic focal neurological deficit lasting for more than 24 hours. Retinal arterial occlusion was classified as stroke. Systemic embolism was defined as a clinical history consistent with acute loss of blood flow to a peripheral artery (or arteries), supported by objective imaging, or histological or autopsy evidence of embolism. Due to a limited number of thrombotic complications in the cohort studied, results are given for the composite endpoint of stroke, systemic embolism (SE), myocardial infarction (MI) and cardiovascular (CV) death.

The prespecified safety endpoint in ARISTOTLE was the composite of major bleeding and clinically-relevant non-major bleeding; this was the endpoint used for bleeding in this analysis.[242] The definitions used are given in Table 2-3.

2.4.2 Survival analysis

A Cox proportional hazards model was used in the survival analysis. Unadjusted models used only the fibrin clot biomarker. An adjusted model included a propensity scoring estimate of fibrin clot variable based on the randomisation variables listed in Table 2-4.

Table 2-3 - Bleeding definitions used

Major bleeding	Acute or sub-acute clinically overt bleeding accompanied by ≥ 1 of the following: • a decrease in haemoglobin level of ≥ 2 g/dL • a transfusion of ≥ 2 U of packed red blood cells • bleeding that was fatal or occurred in the following critical sites: intracranial, intra-spinal, intra-ocular, pericardial, intra-articular, intra-muscular with compartment syndrome, retroperitoneal.
Clinically relevant non-major bleeding	Acute or sub-acute clinically overt bleeding that did not satisfy the criteria for major bleeding and led to hospital admission for bleeding, physician-guided medical or surgical treatment for bleeding, or a change in antithrombotic therapy (including study drug) for bleeding.
Minor bleeding	All acute clinically overt bleeding events not meeting criteria for major bleeding or clinically relevant non-major bleeding

ISTH bleeding definitions. Adapted from Granger *et al* (Supplementary material). ISTH, International Society on Thrombosis and Haemostasis.

2.4.3 Spline plots

Plots were constructed for each fibrin variable demonstrating the (log) hazard ratio and its confidence interval across the range of biomarker values. These are given as unadjusted and adjusted splines fitted using four knots corresponding to biomarker quartiles. Adjustment used the variables in Table 2-4. P values for the spline plots are the probability that the spline differs from a horizontal line. Hazard ratios for the spline plots are given comparing the highest value in Q1 to the highest value in Q3 (and thus the P value given may not always correspond with the HR confidence interval).

Table 2-4 – Parameters used in multivariate analyses

Demographic	Age, sex, region.
Clinical	Weight at randomisation, systolic blood pressure, history of myocardial infarction, history of spontaneous or clinically relevant bleed, type of atrial fibrillation, history of stroke, transient ischaemic attack or systemic embolism, heart failure, left ventricular ejection fraction <40%, diabetes mellitus, history of hypertension, creatinine at randomization.
Medications	ACE inhibitor or ARB, amiodarone, beta blocker, aspirin, clopidogrel, digoxin, calcium channel blocker, statin, NSAIDS, acid-suppressing drug at randomisation, warfarin/VKA treatment within 7 days of randomisation
Biomarkers	CRP, IL-6, leukocytes, troponin T, NT-pro-BNP, GDF-15, D-dimer

ARB, angiotensin receptor blocker. VKA, vitamin-K antagonist. CRP, C-reactive protein. IL-6, Interleukin-6, NT-pro-BNP, N-terminal pro-B-type natriuretic peptide. GDF-15, growth differentiation factor 15.

2.5 Optimising a workflow for analysing results from a turbidity and lysis assay

2.5.1 Background

The plasma turbidity and lysis (PTL) experiments are performed by recording light absorbance in a well. The raw data consist of a light absorbance measurement every 15 seconds for each of 96 wells on a plate, generating a large volume of data. For my protocol, each experiment generates 960 timepoints (every 15 seconds for 4 hours) for each of 96 wells, giving a total of 92,160 data points. I planned to perform at least 160 such experiments for the proposed study.

Software for the plate reader (SkanIt for Microplate Readers[310]) exports these results in a Microsoft Excel spreadsheet format. Previously, the user was required to reorder the data and copy it into a third software package (for example, the Shiny Clot Lysis software[311]) for analysing the raw data to generate the calculated measurements such as lag time, peak turbidity and lysis time. This was time consuming and introduced the potential for error since results were copied manually between software packages.

To address these concerns, I designed and developed a software package, written in the MATLAB programming language, to standardise and streamline the data import, calculations, plotting, database storage and export. Some of the considerations for analysing the data reproducibly and efficiently are discussed here.

2.5.2 Dependencies

The software requires MATLAB (MathWorks, Natick, MA, USA). It was written using MATLAB versions R2019a, R2019b and R2020a. It requires the Signal Processing and Image Processing Toolboxes (MathWorks).

2.5.3 Metadata

The SkanIt output spreadsheet contains six lines of experiment metadata detailing the protocol used, data and time of the experiment, wavelength of light used and plate number. In addition, I adapted this metadata to include the sample ID numbers and ‘offset’ so that

these could be all be stored and imported from the same results file, thus reducing the chance of transcription errors. (Figure 2-3).

Offset

All turbidimetry timings are calculated relative to the addition of the activation mix. The crude elapsed time (from the start of the automated plate reading) is corrected by the total 'offset' - the time difference between adding the activation mix to the first column of wells and the beginning of automated plate reading. In my protocol, activation mix is sequentially added to each of twelve columns in 10 second increments. Each column's offset time is therefore adjusted for these time differences.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Measurement results	Row \ Col	1	2	3	4	5	6	7	8	9	10	11	12	
2	ARISTOTLE (21)	AB	NP	5457168	5344760	4665995	5012629	5290421	5058140	4766228	5314169	5159156	5156587	52777653	
3	17/09/2020 09:57:44	CD	5164697	5137802	5166947	5277758	5273128	5287810	5369257	5166949	5493133	4930936	5166953	53787587	
4	128	EF	5344763	5284481	5378759	5256696	5386394	5332104	5175470	5156583	5290334	0175032	5104968	49455551	
5	Absorbance 1	GH	5233666	5214610	5166950	5314172	5354262	5371978	5290333	5348559	5306941	4987227	5562357	NP	
6	Wavelength: 340 nm														
7	1000														
8	Plate 1														
9															
10	Reading	avg. time [s]	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B0
11	1	0.00	0.340	0.313	0.380	0.276	0.378	0.226	0.311	0.284	1.102	0.267	0.427	0.261	0.3
12	2	15.00	0.341	0.314	0.381	0.275	0.384	0.225	0.308	0.283	1.097	0.267	0.426	0.262	0.3
13	3	30.00	0.341	0.315	0.380	0.274	0.382	0.224	0.308	0.283	1.090	0.265	0.428	0.262	0.3
14	4	45.00	0.341	0.314	0.380	0.274	0.380	0.224	0.308	0.283	1.089	0.265	0.423	0.262	0.3
15	5	60.00	0.341	0.315	0.379	0.273	0.380	0.224	0.308	0.283	1.087	0.265	0.428	0.261	0.3
16	6	75.00	0.341	0.314	0.379	0.273	0.380	0.223	0.307	0.282	1.084	0.264	0.427	0.261	0.3
17	7	90.00	0.341	0.314	0.379	0.273	0.383	0.224	0.307	0.282	1.082	0.264	0.427	0.261	0.3
18	8	105.00	0.343	0.314	0.380	0.272	0.386	0.223	0.306	0.282	1.079	0.263	0.436	0.261	0.3
19	9	120.00	0.346	0.315	0.380	0.275	0.394	0.223	0.306	0.282	1.078	0.263	0.433	0.261	0.3
20	10	135.00	0.358	0.316	0.382	0.276	0.420	0.223	0.307	0.284	1.076	0.263	0.430	0.261	0.3
21	11	150.00	0.394	0.319	0.385	0.277	0.478	0.224	0.308	0.287	1.076	0.263	0.433	0.262	0.4
22	12	165.00	0.445	0.325	0.395	0.281	0.574	0.225	0.311	0.294	1.077	0.261	0.429	0.262	0.4

Figure 2-3 – Example header from SkanIt output file demonstrating saved metadata. Additionally, fields for sample ID and offset were added.

2.5.4 Measured parameters

Light absorbance (analogous to plasma turbidity) is plotted as a function of time. The time – absorbance plot can be analysed in several ways to find markers of clotting and / or lysis (Figure 1-5).

Baseline turbidity

The light absorbance at the start of the experiment before any clotting has occurred.

Lag time

The time elapsed from the addition of activation mix to the initiation of clot formation. This can be defined in several ways, though the method our group has used previously is the first of three (or five) consecutively rising absorbance values. Alternatively, the time taken to attain an absolute rise of 0.015 absorbance units (AU) has been proposed to define the lag time [287].

Peak turbidity & corrected peak turbidity

The greatest light absorbance, reflecting the fibre width in fibrin clots.[312] The peak turbidity value also has an associated timing value (T_{peak}). The peak turbidity is corrected by subtracting the baseline turbidity to define the change in turbidity that occurs. From here on, 'peak turbidity' refers to this corrected value.

$$Corrected\ peak\ turbidity = peak\ turbidity - baseline\ turbidity$$

Lysis time (LT)

The lysis point is calculated as the earliest point after the peak at which the absorbance drops by a chosen amount (typically 50%) of the (corrected) peak turbidity, representing the amount of clot lysis that has occurred.

$$Lysis\ Point_n = baseline\ turbidity + \left(corrected\ peak\ turbidity * \left(1 - \frac{n}{100}\right)\right)$$

The lysis time (LT) for the clot to partially lyse is the time elapsed between the peak turbidity and the lysis point. LT_{50} is typically used.

$$LT_{50} = T_{Lysis\ Point\ 50} - T_{peak}$$

2.5.5 Other potential metrics

In addition to the commonly used metrics, other methods of measuring clotting or lysis phenomena are available. Some of these have been described previously.

tPCR

In my experiments, clotting occurred rapidly (after a short lag period), with a steep 'upstroke' in the turbidity plot. Because this increase in turbidity occurs over a short time period, I was

able to design a metric to identify the point at which turbidity (and therefore, clot density) is increasing most quickly. This is the peak of the differential (dy/dx) of the turbidity-time plot (See also Chapter 8). The time to peak clotting rate (tPCR) is the elapsed time from the start of the experiment until this point. In my experiments, this occurred soon after the lag point, and is a useful metric that would potentially be less sensitive (than lag time) to noise in the signal.

AUC

The area under the absorbance-time curve (AUC) can be used to describe PTL results. Typically, absorbance is adjusted for baseline light absorbance before calculation of the sum of a series of trapezoids defined by adjacent datapoints and the baseline. AUC is theoretically a measure of both the clotting and lysis phase, although because with my protocol the lysis phase is typically longer than the clotting phase, AUC is primarily determined by the clot lysis kinetics. AUC will be truncated if 100% clot lysis does not occur before the end of the recording.

Velocity index

This has been used in thrombin generation assays to assess the rapidity of thrombin formation. Whilst thrombin generation assays are not directly comparable to PTL assays, the curves are visually similar and share parameters (E.g. Lag time, peak height, Figure 2-4).[313] Therefore, the velocity index could be applied to PTL outputs as a compound measure of both the speed of formation and density of clot formed.

$$Velocity\ index = \frac{Peak\ turbidity}{(Time\ to\ peak - Lag\ time)}$$

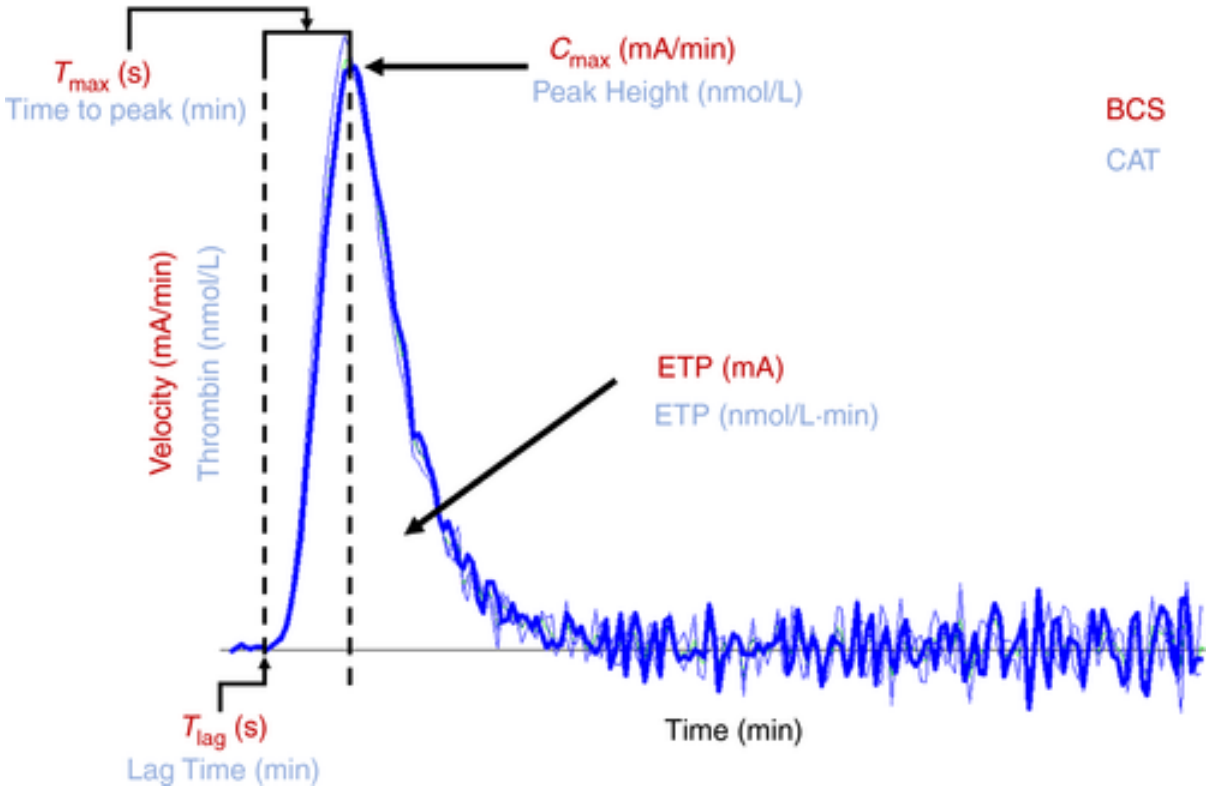


Figure 2-4 – Measurements from a thrombin generation assay. Whilst not directly comparably to turbidity and lysis assays, several of the parameters are similar. Taken from Kintigh et al.

2.5.6 Correcting for noise

The absorption signal is inherently susceptible to noise. This can be due to physical issues within the well, either the movement of small air bubbles or potentially movement of clot in or out of the detection beam. Additionally, the light absorbance measure has a degree of expected variability; the stated accuracy of the Thermofisher MultiSkan FC plate reader is +/- 1%.[314] This results in an input signal with a variable amount of apparently random noise, which can affect measurements. An example of this is shown in Figure 2-5.

To reduce the effect of noise on calculated values, the input signal (measured light absorbance) was filtered. Several algorithms exist for this purpose, each with strengths and weaknesses.

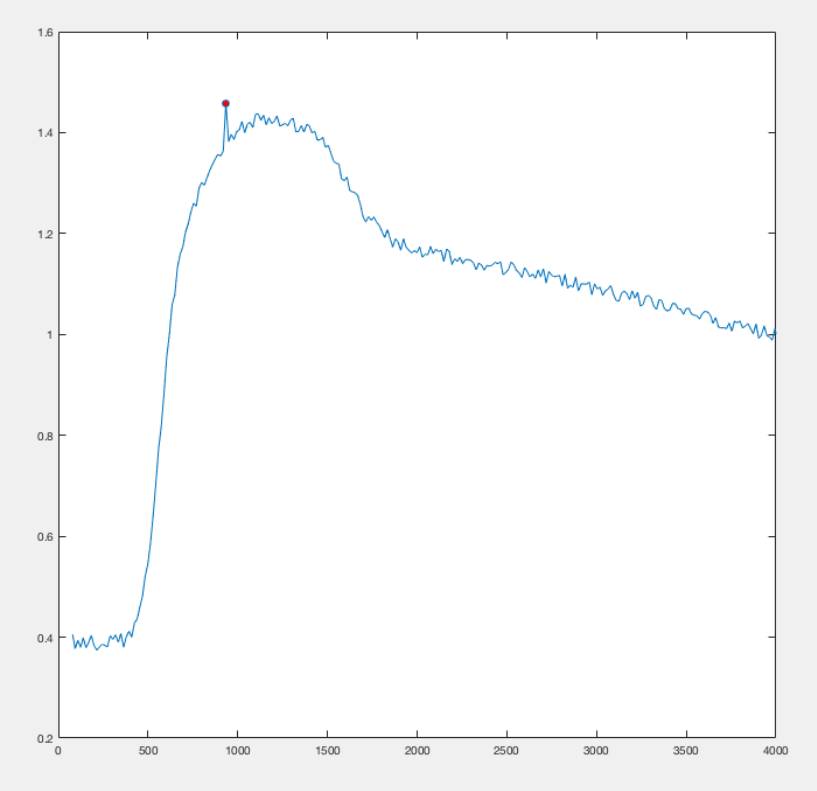


Figure 2-5 – Example of noisy input signal affecting the determination of the peak turbidity. Here, the peak has been erroneously annotated earlier and at a greater amplitude than would be expected because of noise in the signal. Since calculation of the lysis time depends on the peak time, this artefact compounds when calculating other parameters.

Types of filters

An ideal filter would remove the artefactual noise without affecting the true signal. In practice, excessive filtering results in artefacts which can also affect results. The degree of filtering required is dependent on the parameter being observed. For example, when determining lag time, I was seeking to identify an inflection point in the initial part of the turbidity curve. Here, smoothing is undesirable since the abrupt inflection marking the onset of clotting may be blunted. Several types of filtering one-dimensional data exist, with differing advantages and disadvantages.

Moving average filter

The moving average filter takes the mean value of a 'window' within the data. The size of this window can be altered to determine the degree of smoothing applied. Excessive moving average filtering results in a reduction in the maximum turbidity. Moreover, moving average filtering is prone to 'phase delay' artefact whereby the filtering affects not only the magnitude but also the timing of peaks (Figure 2-6). For time-based parameters such as lag time or LT_{50},

any amount of phase delay is undesirable, theoretically making moving average filtering a poor choice. In practice, for the small degree of filtering required for simple processing of turbidimetry data, this phenomenon is unlikely to affect results significantly.

Median filter

Median filtering works similarly to moving average filtering, except that it uses the median value rather than the mean. This is useful since the output is not skewed by outlying values. Moreover, unlike moving average filtering, it is unaffected by phase delay, so timing values can be measured with accuracy.

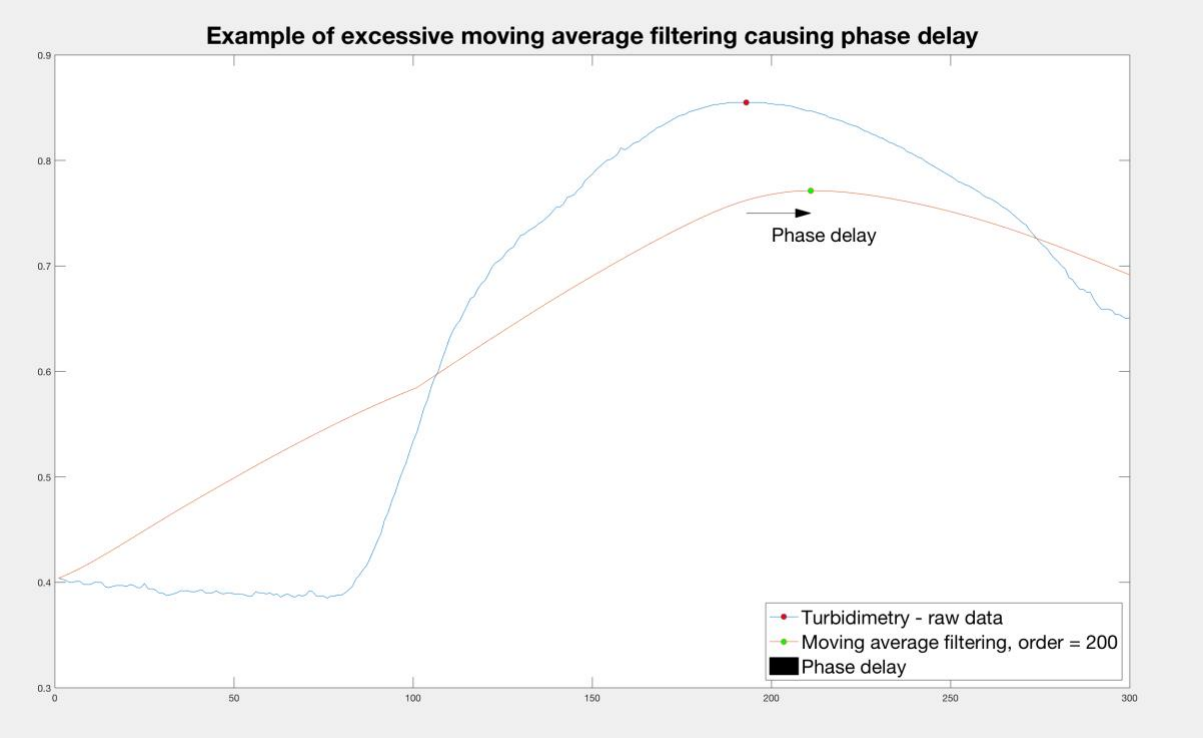


Figure 2-6 – Representative example of the raw signal (blue line) and excessive moving average filtering (orange line) applied to a turbidity data series, resulting in phase delay. The peak value in both series is marked, with the over-filtered signal demonstrating a reduction and a rightward shift of the peak value.

More sophisticated methods of filtering used for calculations based on derivatives of the line were also investigated. However, these techniques were ultimately not used in my analyses of fibrin clot data but are discussed in appendix 8.2.

Summary

Multiple filter types were tested. To remove noisy values that can adversely affect peak values, a low order median filter is a simple, computationally efficient option that generates good results and is not susceptible to phase shift. Median filtering was implemented into the automated analyses of results.

Chapter 3: Development and optimisation of a tissue factor assay to measure fibrin clot properties and comparison with a thrombin-based assay

3.1 Background & general considerations

Our laboratory has used a thrombin-based fibrin turbidity assay in previous work. As previously discussed, a tissue factor (TF)-based assay has potential advantages. Our group has previously used a TF assay to examine drug effects *in vitro* [unpublished data] and *in vivo,*[315] while similar assays have previously been used by other groups to measure fibrin clot turbidity and lysis in anticoagulated patients,[316] and patients with factor XI deficiency,[317] aortic aneurysm,[318] or AF.[153]

The aim of the study is to identify inter-individual heterogeneity in fibrin clot parameters and to determine if this heterogeneity is associated with clinical risk factors and cardiovascular outcomes. Secondly, I hope to use the assay to examine the effect of anticoagulant medication on fibrin clot properties in patients with AF.

In this chapter, the general considerations and the process for optimising such an assay for use in patients with atrial fibrillation (AF) are discussed.

3.1.1 Considerations for optimisation

General principles

The primary aim of the assay optimisation process was to maximise the accuracy and reproducibility of the assay, by minimising its coefficient of variance (CoV). The second aim was to highlight inter-individual variation in coagulation parameters by maximising the differences observed *between* individuals. I primarily used healthy volunteer plasma for assay optimisation since it is easily obtained in our laboratory. However, plasma from patients with cardiovascular disease was later used to ensure compatibility.

Scale

4,476 plasma samples from participants in the ARISTOTLE trial are anticipated. With a maximum of 46 samples analysed per plate, a total of 98 plates is required to analyse all samples. Given this substantial number, firstly it is important to maximise the throughput of the experiment. Secondly, the volume of reagents required will have significant cost implications to be considered.

Variables to be assessed

Potential variables for the assay include:

- Plasma volume
- Activation mix volume
- Calcium concentration
- Tissue factor concentration
- tPA concentration

Other considerations

Total volume in the well should not be too low

At low volumes of liquid in the well, inconsistent results are possible due to an adverse signal to noise ratio (SNR) when the attenuation of absorbed light is low. Additionally, electrostatic effects and surface tension may also play a role when very small volumes (< 50 µL) are used in each well.[319,320]

Ratio of plasma to reagent

A potential criticism is that by using too much buffer solution (relative to plasma volume), the resulting mixture may be homogenised, reducing the inter-individual variation. I therefore sought to minimise the dilution of plasma where practical.

Plasma volume

Plasma for the proposed study has been collected previously and stored. For each patient, between 100 and 500 µL of plasma is available. Therefore, the maximum plasma volume

possible in each well is limited by its availability. In order to test each patient's plasma in duplicate, the plasma volume in the well should be less than 50 µL.

tPA concentration

As previously discussed, the concentration of tPA is critical for the lysis period of the assay. With high exogenous tPA concentrations, the degree of inter-individual heterogeneity identifiable may be limited. Therefore, where practical, lower concentrations of tPA were thought to be optimal.

Duration of the assay

We have previously observed that different reagent concentrations greatly affect the time required to achieve clot and subsequent lysis in all samples. To maximise throughput,I was mindful of the time required to prepare each experiment and the plate read time.

Simplicity

An assay that is simpler to perform may reduce the potential for errors and thus increase reliability of the results obtained. Accordingly, a simpler assay design is favoured where practical.

Cost of reagents

Many of the assay constituents are costly. Since I plan to assess multiple plates using this technique, the costs are substantial. Accordingly, a small reduction in reagent volume may have a significant cost saving, particularly for tissue factor (the most expensive component of the assay).

Technical considerations

There are various technical reasons why it may not be possible to obtain a result for a given parameter. For example, clotting that happens too rapidly may occur before plate reading starts and it may not be possible to obtain a result for lag time. Similarly, if the lysis phase is excessively prolonged, lysis may not occur before the end of the recording window.

3.2 In vitro experiments used to refine the assay protocol

3.2.1 Plasma volume

Previous work in our lab has used a protocol with a 25 µL plasma volume. The thrombin assay we have previously used required 25 µL plasma with 125 µL activation mix. I sought to reduce this ratio for the reasons listed above, if it is possible to do so without compromising the reliability. For the proposed study the maximum plasma volume per well was limited by the volume of patient plasma available. In order to test every sample in duplicate, a maximum plasma volume of 50 µL/well was required. However, if I decided to relax the requirement for duplication, up to 100 µL per patient would be available.

Hypotheses

With higher volumes of plasma:

1. Lag time may decrease, since in-well concentrations of coagulation factors will necessarily be higher
2. The peak turbidity as measured by photometry will be higher, since the resultant mixture is more opaque
3. The lysis time (e.g., LT_{50}) will not change since this is dependent upon the tPA added in the activation mix rather than the concentration of coagulation factors

Methods

In this experiment, a plate with healthy volunteer plasma (n=6) in two volumes (25 µL and 50 µL) was used to examine if this results in a difference in the clotting or lysis phase. 75 µL of activation mix was then added (final concentrations: tPA 73.8 ng/mL, TF 4.6 mg/mL, $CaCl_2$ 15 mmol/L) before photometry was performed.

Results

Normalised and supernormalised turbidimetry is shown (Figure 3-1). In the 50 µL samples (compared with the 25 µL samples), the lag time was similar, the peak turbidity was greater while the 50% lysis time (LT_{50}) was longer (Figure 3-2). Coefficient of variance for the assay was lowest at 50 µL (Figure 3-3).

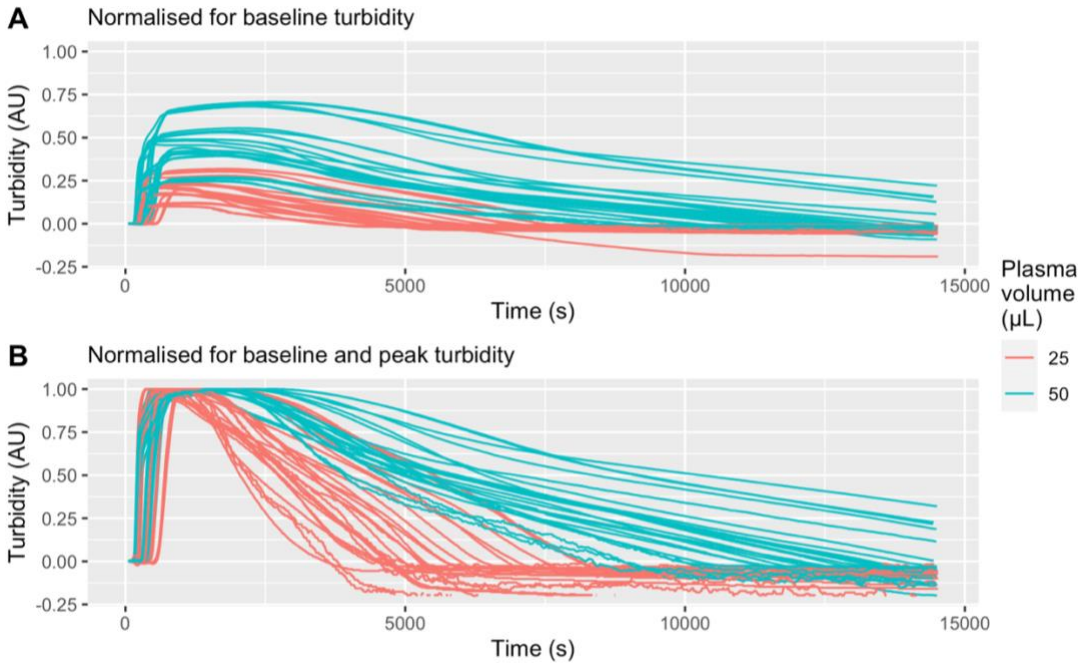


Figure 3-1 – Turbidimetry using 25 or 50 µL of healthy volunteer plasma (A) normalised for baseline plasma turbidity and (B) normalised for both baseline and peak plasma turbidity. AU, absorbance units.

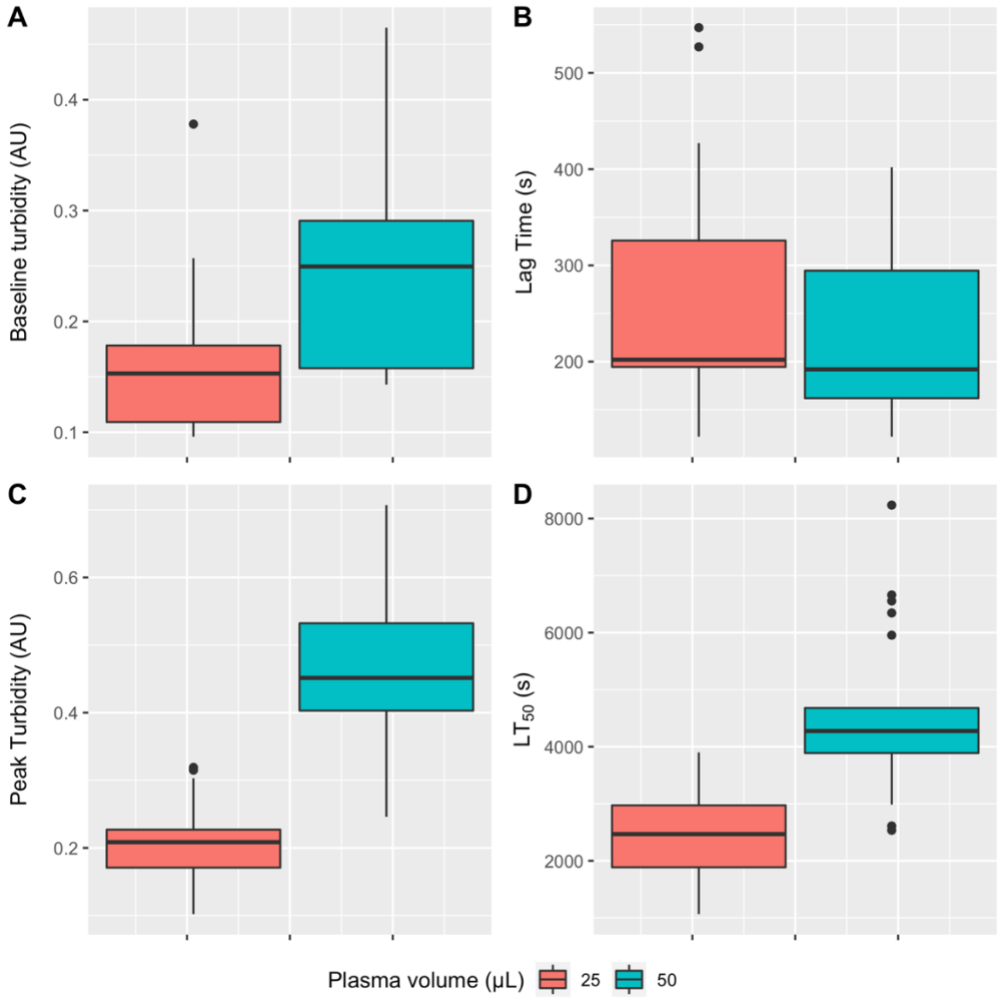
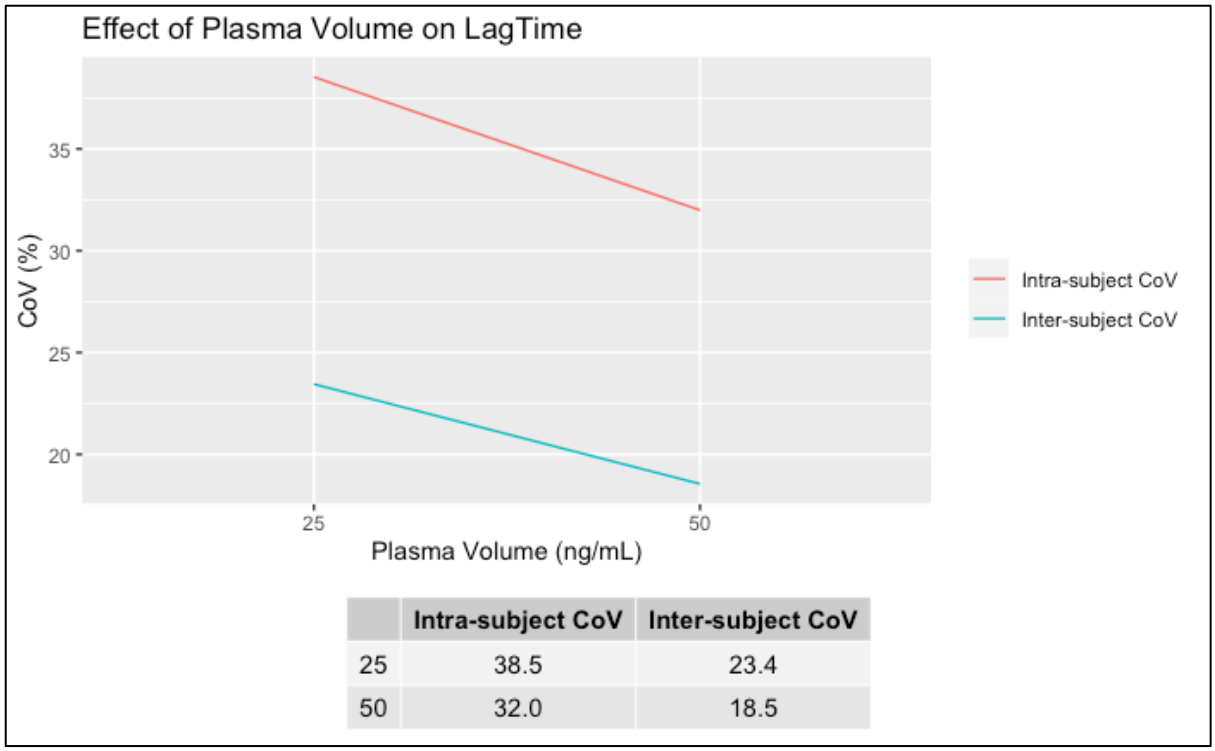


Figure 3-2 – Effect of volume of plasma on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time. AU, absorbance units. LT_{50}, time to 50% clot lysis.



	Intra-subject CoV	Inter-subject CoV
25	38.5	23.4
50	32.0	18.5

Figure 3-3 – Inter- and Intra- volunteer Coefficients of Variation at 25 and 50 µL plasma volume.

Conclusions

Lag time is not significantly affected by plasma volume. Peak turbidity and LT_{50} values are increased with increased volume of plasma. This difference, particularly in LT_{50} suggests that accurate dosing of plasma into the well is critical. Experiments run times are shorter using smaller volumes of plasma.

3.2.2 Plasma : activation mix volume ratio

Having demonstrated that results are affected when different volumes of plasma are used, experiments to determine the optimum plasma volume within a range of feasible volumes were planned.

Keeping a constant activation mix volume in the well, with a changing volume of plasma, the ratio of plasma volume to activation mix volume changes. Accordingly, the relative concentrations of the activation mix constituents have also been affected. Therefore, this experiment is a test of the ratio of plasma to activation mix volume.

Methods

A plate with varying aliquots of plasma was prepared to determine the optimum plasma volume. 25 µL, 50 µL, 75 µL, 100 µL and 150 µL aliquots were used. 75 µL of the standard activation mix was then added (final concentrations: tPA 74.9 ng/mL, TF 7.5 mg/mL, $CaCl_2$ 15.3 mmol/L) before photometry was performed.

Results

Results from turbidimetry are shown in Figure 3-4. At higher ratios of plasma volume to activation mix, measurements of peak and baseline turbidity were higher, while lag time and LT_{50} were prolonged (Figure 3-5). The coefficient of variance was similar across the different ratios (except at 25 µL, Figure 3-6).

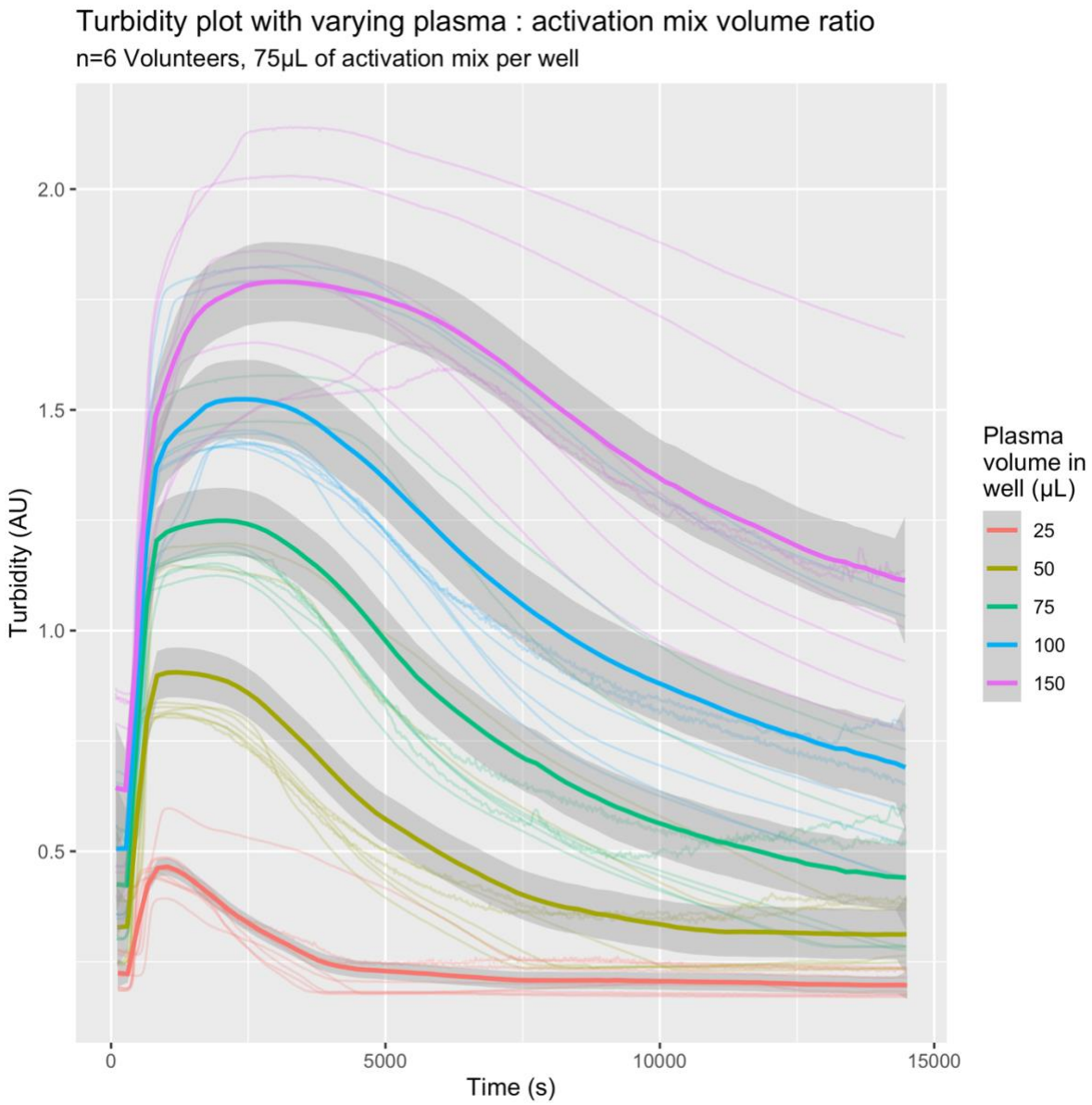


Figure 3-4 – Turbidimetry plots using varying plasma volumes with a constant activation mix volume. Fine lines represent individual volunteers. Bold lines (with confidence intervals) represent the mean of values, grouped by plasma volume. AU, absorbance units.

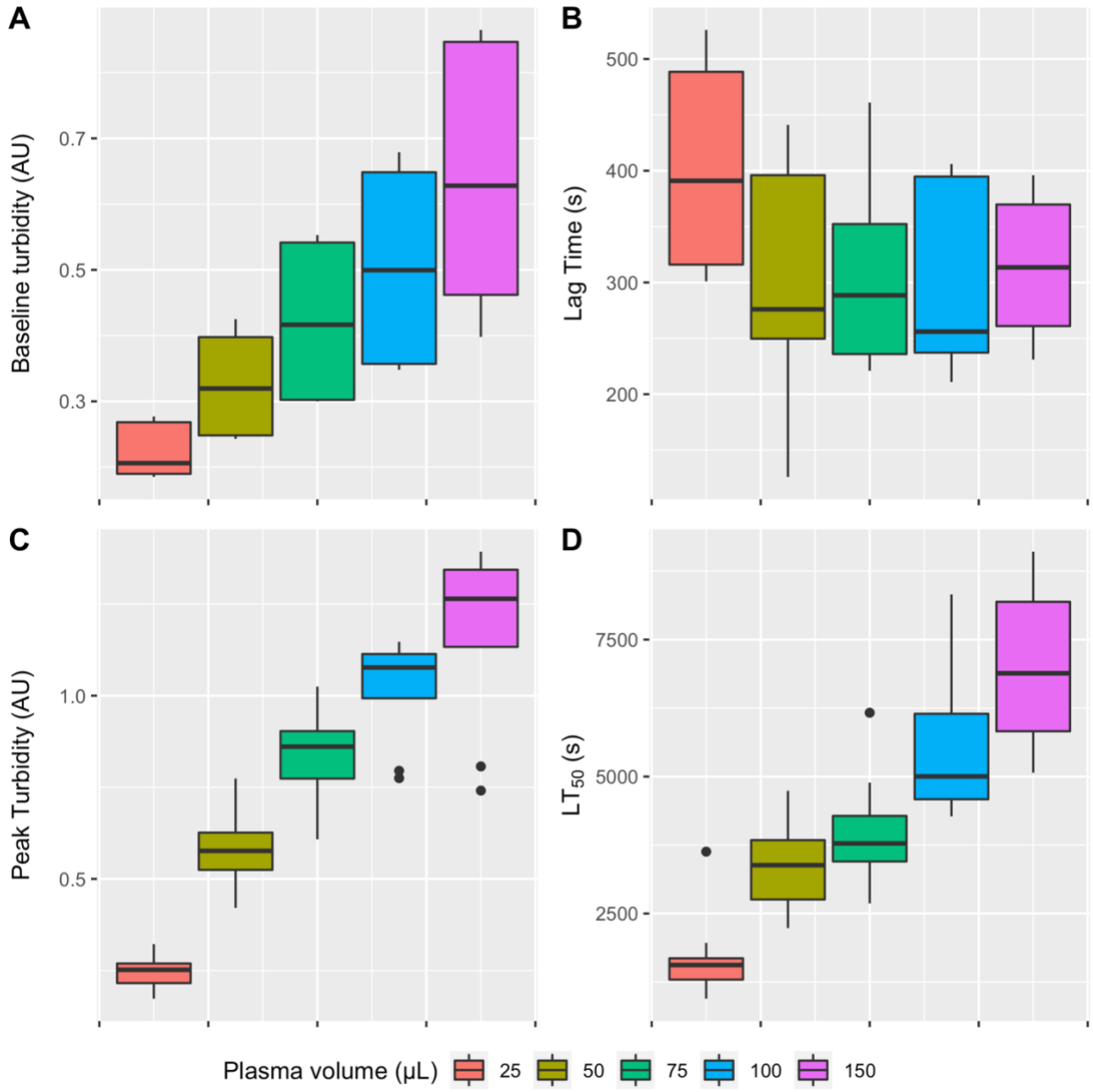
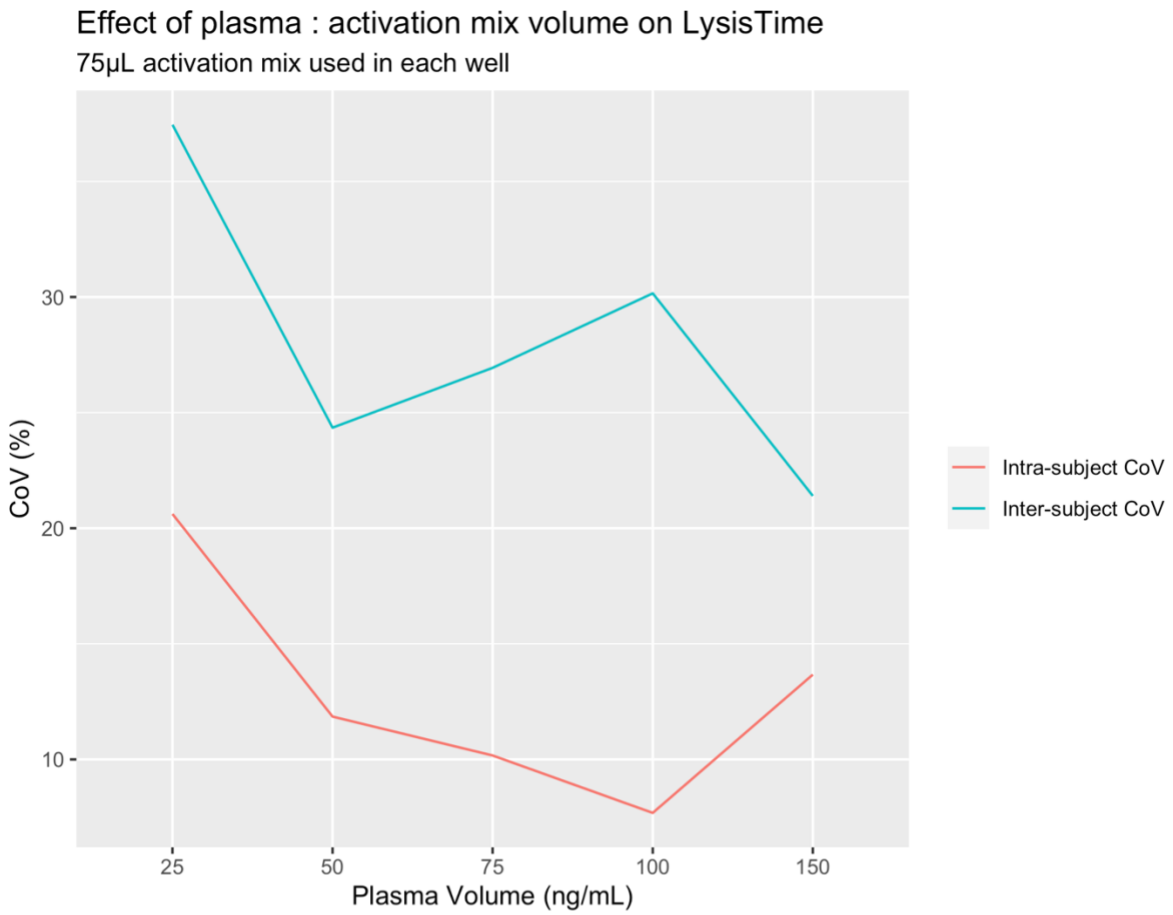


Figure 3-5 – Effect of plasma volume on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time. $LT_{50,}$ time to 50% clot lysis.



	Intra-subject CoV	Inter-subject CoV
25	20.62	37.5
50	11.85	24.4
75	10.17	26.9
100	7.69	30.2
150	13.67	21.4

Figure 3-6 – Effect of plasma : activation mix volume on the inter- and intra-individual CoV of the lysis time parameter. CoV, coefficient of variation.

Conclusions

Small volumes of plasma (e.g., 25 µL) are challenging to accurately pipette and may be more prone to technical issues such as introducing bubbles, surface tension in the well or electrostatic effects. This is perhaps reflected in the higher CoV observed for low plasma volumes. No significant differences were observed for ratios greater than 50 µL plasma : 75 µL activation mix.

3.2.3 Activation mix volume

As an alternative to varying the plasma volume, I sought to find the optimum diluent volume, keeping the concentration of other reagents constant.

Methods

A plate was prepared with 50 µL of volunteer (n=6) plasma and varying volumes of activation mix. 25 µL, 50 µL, 75 µL and 125 µL levels were used. The concentration of the activation mix was adjusted such that the final concentrations of reagents in the well were constant, except for the buffer solution (containing TRIS and sodium chloride). Final concentrations: tPA 74.9 ng/mL, TF 7.49 mg/mL, $CaCl_2$ 15.3 mmol/L. Photometry was then performed.

Results

The plasma turbidity plot (Figure 3-7), and results for baseline turbidity, lag time, peak turbidity and lysis time are shown (Figure 3-8). Both lag time and lysis time were lower with progressively greater activation mix volumes.

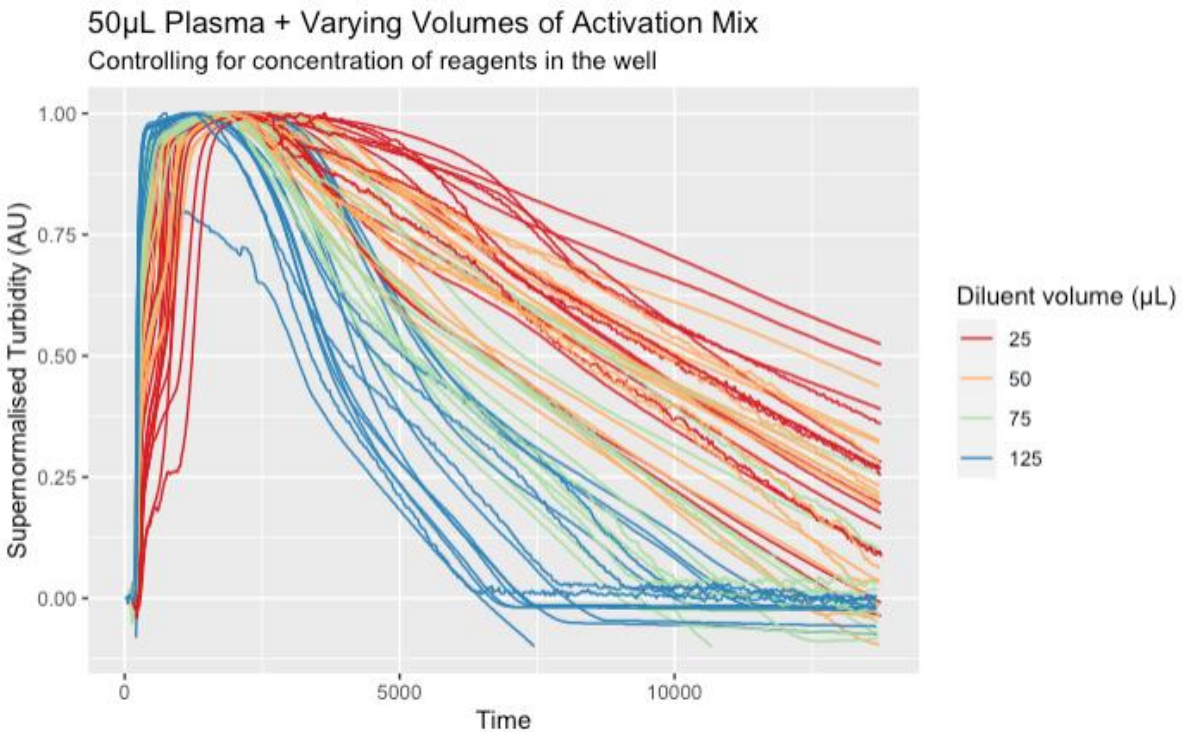


Figure 3-7 – Effect of activation mix volume on the turbidity plot (normalised for both baseline and peak turbidity.

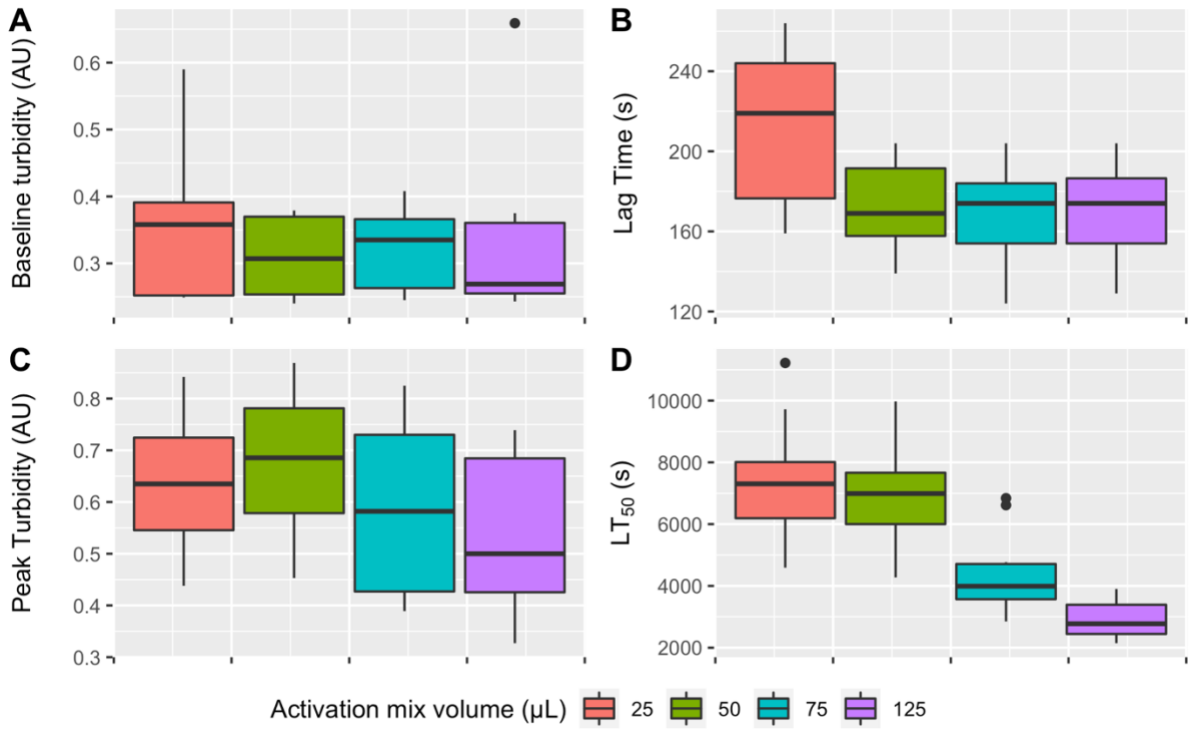
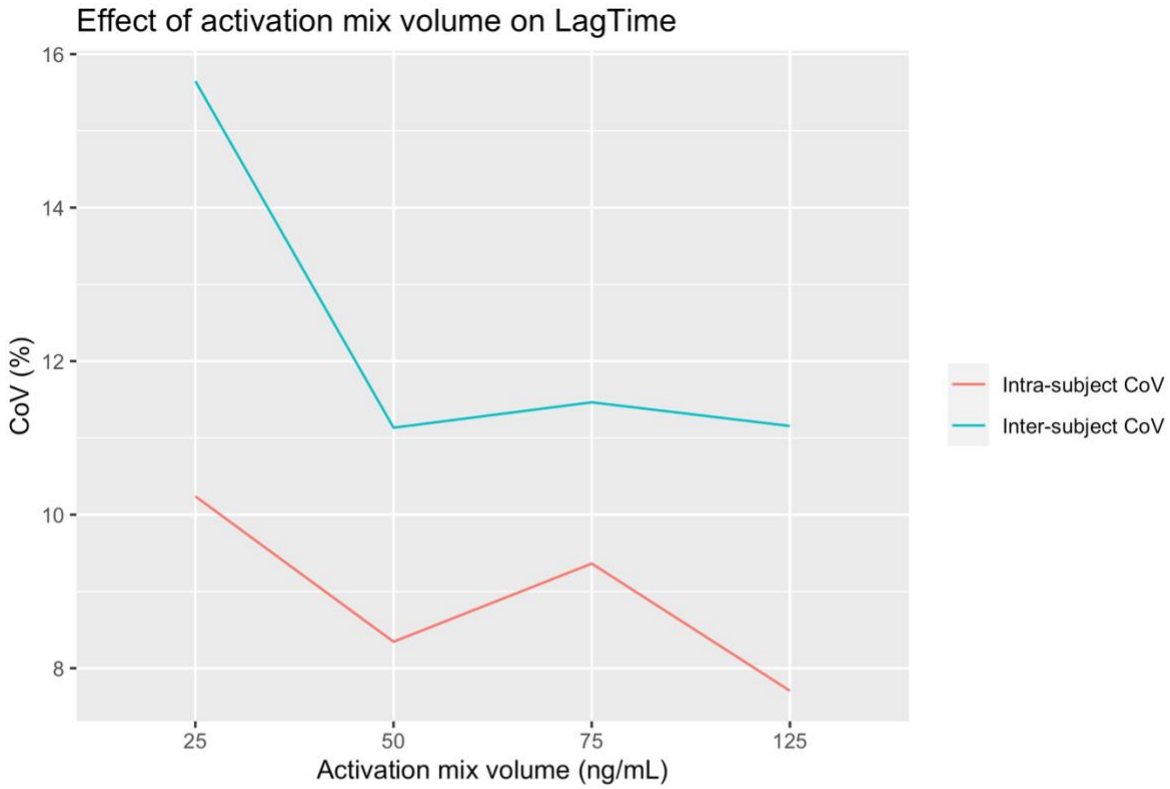


Figure 3-8 – The effect of varying the activation mix volume on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time. LT_{50}, time to 50% clot lysis.

Conclusions

Greater diluent volume appears to be correlated with shorter lag and lysis times, but (more importantly) reduced CoV. Given that the concentrations of each constituent were controlled for, it may be that consumption of factors within the activation mix is an important determinant of the rate of clot formation and lysis.

We opted for a higher activation mix volume since that was associated with the lowest CoV. There is a small cost implication with this decision since the higher volumes of activation mix use a slightly greater amount of tPA and TF.



	Intra-subject CoV	Inter-subject CoV
25	10.24	15.6
50	8.34	11.1
75	9.36	11.5
125	7.71	11.2

Figure 3-9 – Effect of activation mix volume on the CoV of results

3.2.4 tPA concentration

In previous work, we have observed that the concentration of tPA in the activation mix has a substantial effect on the lysis phase of the turbidity and lysis assay. The duration of this lysis phase scales inversely with the tPA concentration. Thus, the overall experiment length is governed predominantly by the tPA concentration.

tPA (1)

To establish the effect of tPA on various parameters, I first wanted to observe the effect of omitting tPA entirely from the experimental protocol. The hypothesis was that tPA concentration would affect the lysis phase of the experiment (I.e., LT_{50}) but not the coagulation phase (I.e., baseline turbidity, lag time or peak turbidity).

Methods

A plate was prepared with 50 µL of volunteer (n=6) plasma. 75 µL of activation mix was added with either tPA (at a concentration of 94 ng/mL) or no tPA. Other concentrations: TF 7.49 mg/mL, $CaCl_2$ 15.3 mmol/L. Photometry was then performed.

Results

No difference in baseline turbidity, peak turbidity or lag time was observed. Lysis did not occur in the absence of tPA. Since LT_{50} requires lysis to occur to generate a result, no results are available for this parameter.

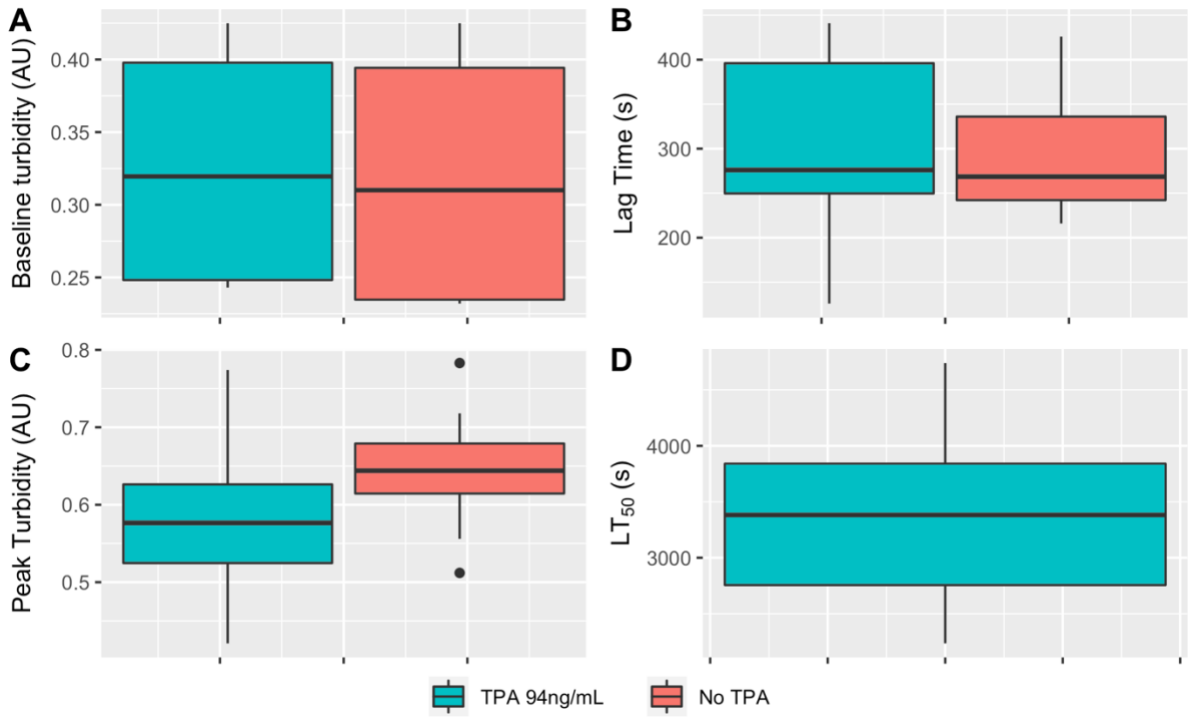


Figure 3-10 – Effect of tPA presence on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time. N.b. No results for lysis time are available in absence of tPA. tPA, tissue plasminogen activator, LT_{50}, time to 50% clot lysis.

Conclusions

The presence of tPA only affects the lysis phase parameters. It is useful to note that lag time and peak turbidity are not affected. However, it is possible that tPA concentrations in excess of 94 ng/mL may have an effect.

tPA (2)

Having established the effect of tPA presence or absence on certain measured parameters, I sought to identify the optimum concentration of tPA to use.

Methods

50µL of volunteer plasma was added to a standard photometry plate. 75 µL of an activation mix containing one of several concentrations of tPA (79, 120, 178 or 270 ng/mL) was added before photometry. Other constituents of the assay were kept constant (TF 0.74 mg/mL, $CaCl_2$ 24 mmol/L).

Results

Turbidity plots are shown in Figure 3-11. Higher concentrations of tPA are associated with more rapid lysis. Also noteworthy is the broad peak observed at low tPA concentrations. This affects the timing of the peak value with a subsequent effect on the lysis time calculation. No significant differences were observed for baseline turbidity, peak turbidity, or lag time, although there is a trend for lower peak turbidity at higher tPA concentrations (Figure 3-12). As tPA concentration increases, lysis time is reduced.

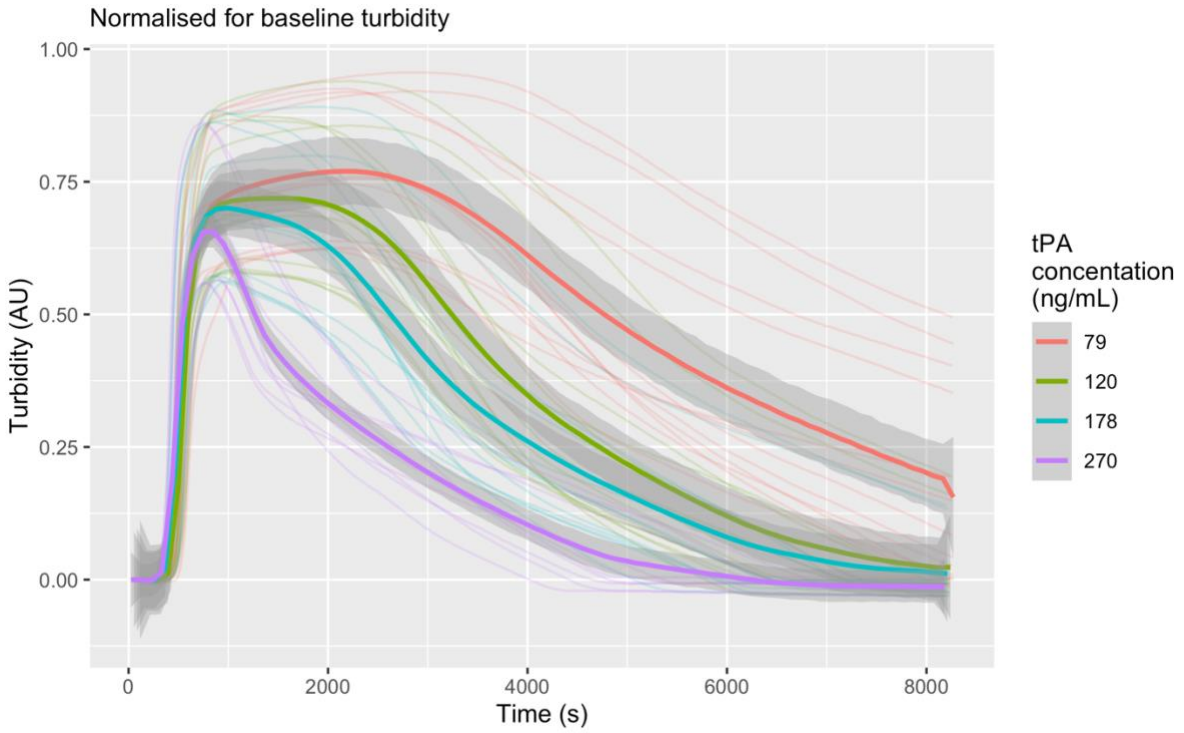


Figure 3-11 – Turbidity plots at varying concentrations of tPA. Fine lines represent individual volunteer-tPA concentrations, bold lines are a smoothed average of all samples at a given tPA concentration. tPA, tissue plasminogen activator.

Conclusions

At high concentrations of tPA, lysis time is reduced. The timing of the peak value is affected by tPA concentration, which has a significant knock-on effect on the lysis time which is calculated from this measurement.

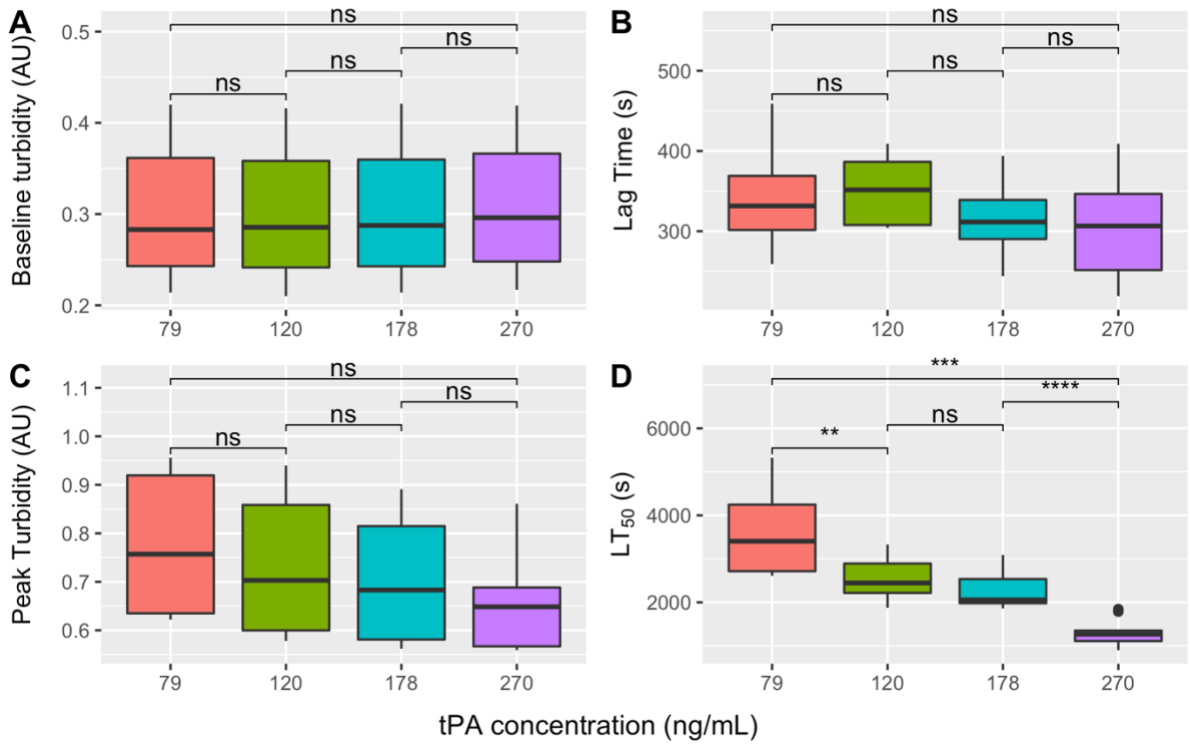


*Figure 3-12 – Effect of tPA concentration on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time with selected pairwise comparisons. tPA, tissue plasminogen activator. LT_{50}, time to 50% clot lysis. ns, not significant. **, p<0.01. ***, p<0.001. **** p< 0.0001.*

3.2.5 Tissue factor concentration (1)

A further variable that can be altered is the TF concentration. Concentrations of TF for plasma turbidity and lysis measurement have been used ranging between 2 pmol/L and 10 pmol/L.[315,316] The specific TF preparation may also have an effect on their activity.

We would expect a greater concentration of TF to lead to more rapid onset of clotting (i.e., shorter lag time) and potentially a prolonged lysis time. If the coagulation initial activity is too rapid it can affect the ability to automatically detect lag time, since the rapid increase in plasma turbidity may have begun before the plate can be loaded into the analyser. Secondly, TF is the costliest component of the assay, therefore I sought to reduce the volume required where possible. Finally, the TF concentration may have an unacceptable effect on the overall experiment duration.

Note on TF concentration nomenclature

Methods for tissue factor turbidity and lysis assays used by our lab previously reference tissue factor concentrations as molar values. However, the reagent I used, PPP-Reagent High (Diagnostica Stago), is a proprietary combination of TF and phospholipids and the concentration of TF within this mixture is not made available by the manufacturer. I therefore calculated a weight-by-volume concentration from the measured mass of lyophilised PPP-Reagent High. The mean mass ten vials of PPP-Reagent (0.0618g, SD 0.012g) was used. Each vial is reconstituted with 1ml water giving a concentration of 61.8mg/ml. This corresponds to previous methods quoting a 10pM concentration of tissue factor, but since I was unable to confirm a molar concentration with PPP-Reagent High, I have used the w/v concentration in this manuscript.

Methods

Volunteer plasma (25 µL/well, n=6) was added to a 96-well plate. 75 µL of activation mix containing one of five concentrations of PPP-Reagent (0, 0.57, 1.7, 2.7 and 5.2 mg/mL) was added to the well before photometry. Except for the TF concentration, the activation mixes were the same ($CaCl_2$ 16.7 mmol/L, tPA 83.3 ng/ml).

Results

No significant differences were observed for baseline or peak turbidity (Figure 3-13). Interestingly, clotting still occurred in the absence of TF, albeit with a far greater lag time (Figure 3-13 (B)). Unfortunately, lysis did not occur reliably therefore no result for lysis time is available. A significant trend towards shorter lag times was observed with increasing concentrations of TF. With either no TF or low concentrations of TF, the intra-individual CoV for lag time is high (undesirable), at times higher than the inter-individual CoV (Figure 3-14).

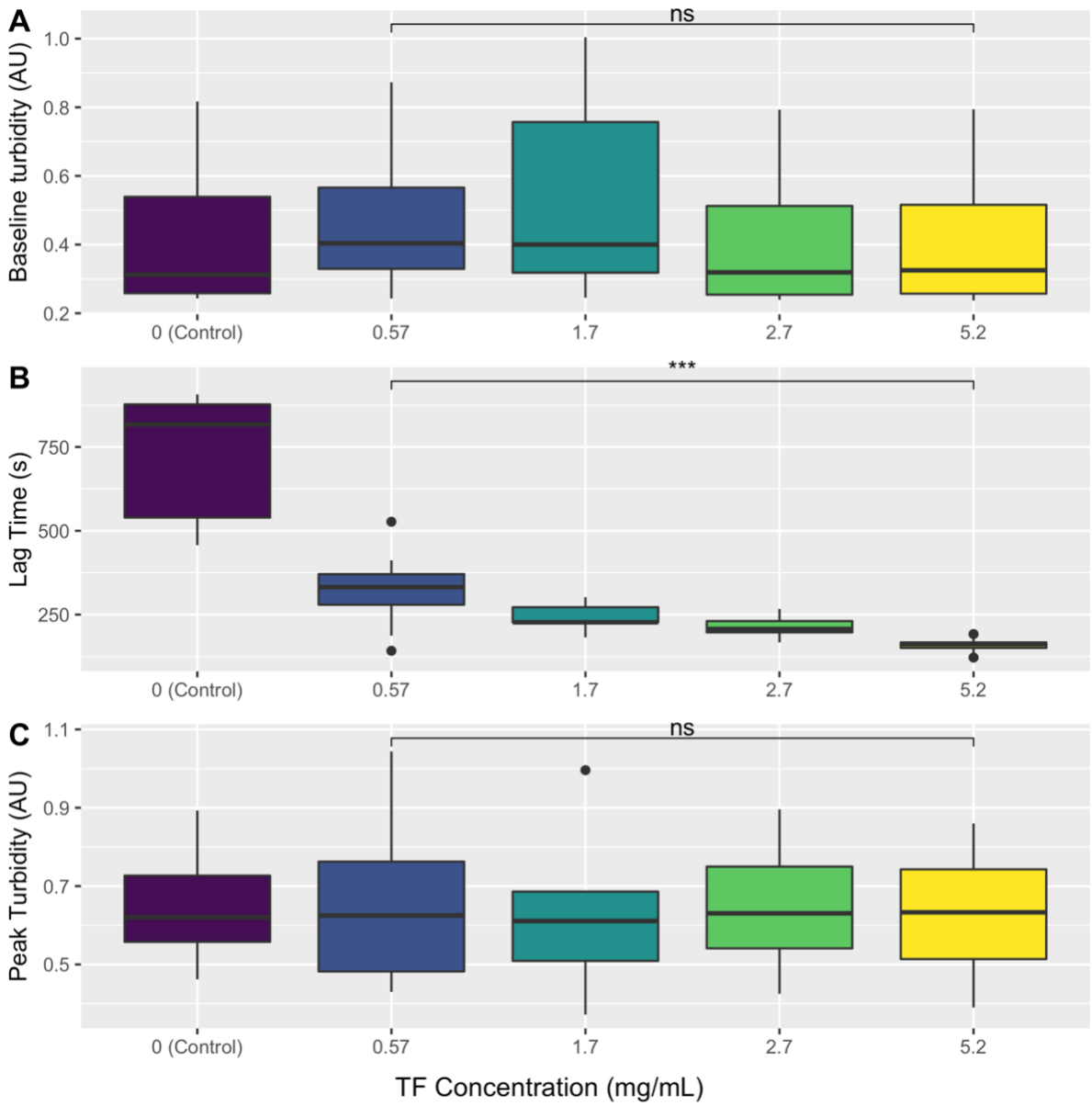


*Figure 3-13 – Effect of TF concentration on (A) Baseline turbidity, (B) Lag Time and (C) Peak turbidity with a selected pairwise comparison. TF, tissue factor. LT_{50}, time to 50% clot lysis. ns, not significant. ***, p<0.001. **** p< 0.0001.*

TF conc.	Intra-subject CoV	Inter-subject CoV
0 (Control)	14.15	22.66
0.57	26.18	7.89
1.7	12.75	20.78
2.7	5.52	12.77
5.2	4.80	11.71

Figure 3-14 – Effect of TF concentration on the inter and intra-individual CoV of the lag time parameter. CoV, coefficient of variation.

Conclusions

Plasma will clot *in vitro* without the requirement for TF. The concentration of TF has a significant and substantial effect on the lag time. At low concentrations of TFs, the CoV is higher. Based on this, concentrations of TF in the range 2.7 – 5.2 mg/mL are optimal.

Tissue factor concentration (2)

The above experiment was repeated to confirm results and establish the effect on lysis time. This time, the experiment was performed with pooled normal plasma rather than multiple volunteers. This was done to provide a high number of datapoints, with a view to analysing the true assay CoV (intra-individual CoV) more accurately.

Methods

50µL normal pool plasma was aliquoted into a standard 96-well plate. 100µL of an activation mix containing one of three TF concentrations (0.46, 2.32, 4.64 mg/mL) was added. All other constituents were controlled for (tPA 71.2 ng/mL, $CaCl_2$ 8.9 mmol/L).

Results

At higher concentrations of TF, lag time was reduced (Figure 3-15-A, Figure 3-16-A). Lysis time also reduced with increasing TF concentrations.

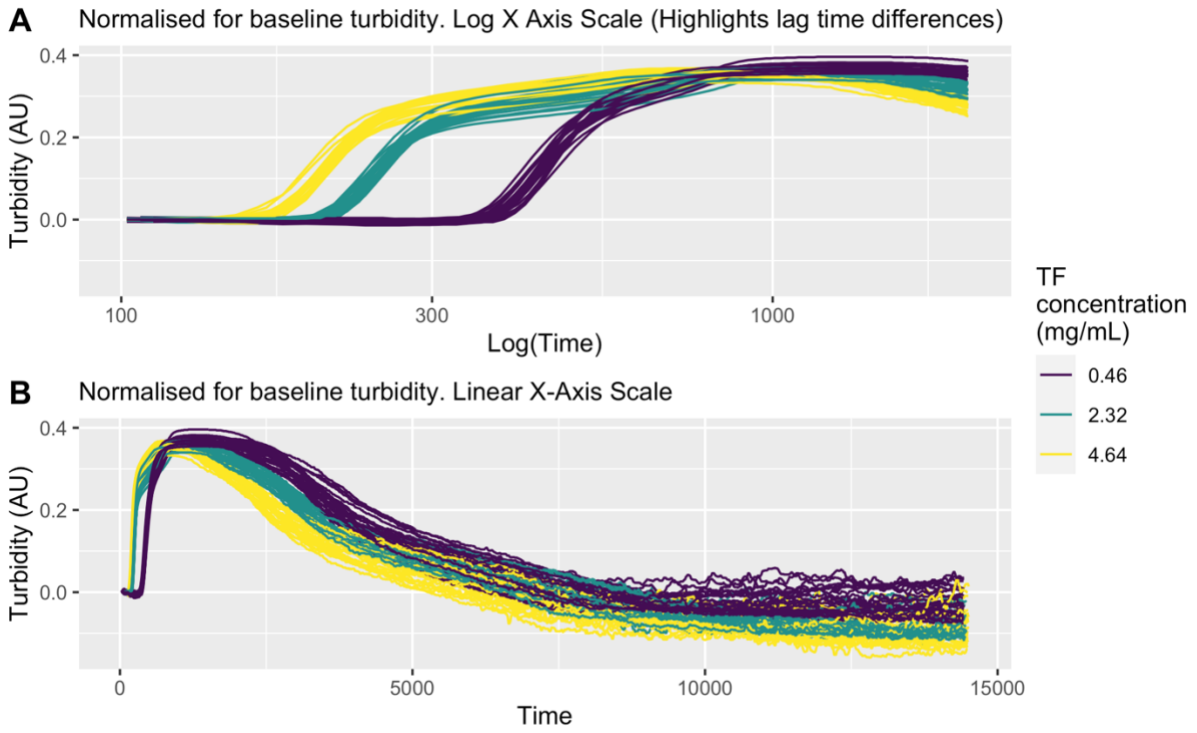


Figure 3-15 – Turbidity plots demonstrating the effect of varying the TF concentration in the activation mix (A) logarithmic x-axis scale to highlight differences in lag time, (B) linear x-axis scale. TF, tissue factor

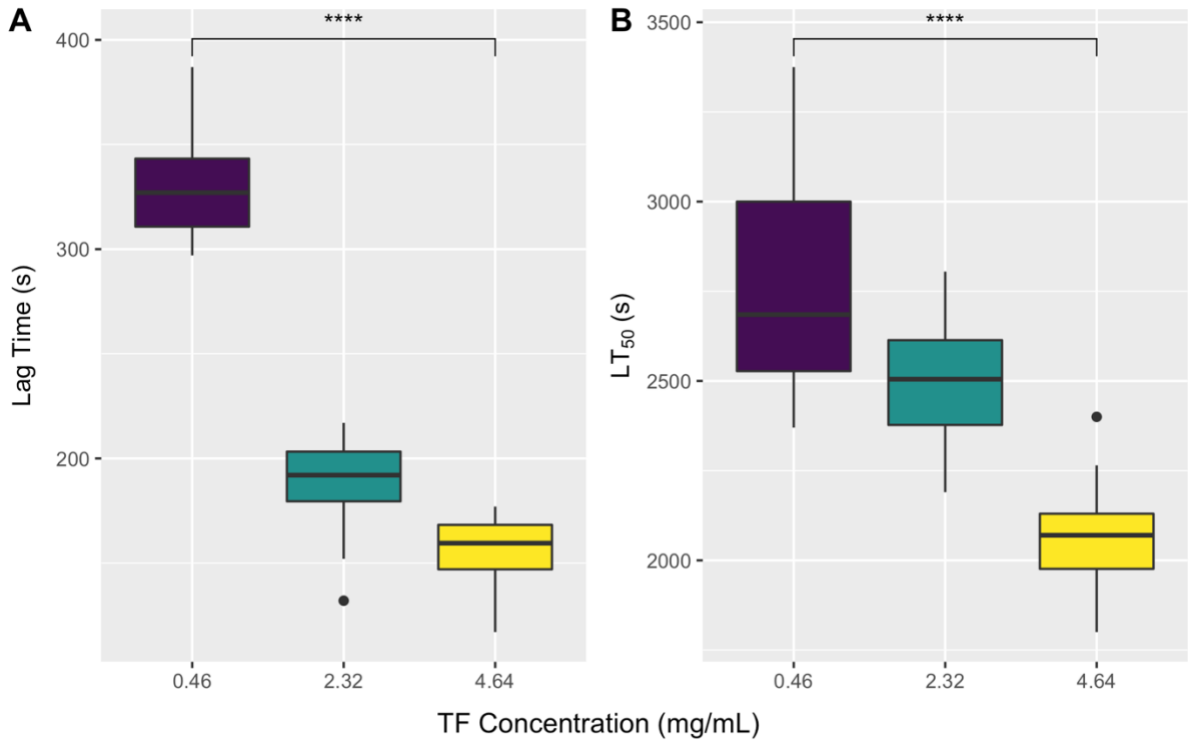


*Figure 3-16 – Effect of TF concentration on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time with a selected pairwise comparison. TF, tissue factor. $LT_{50,}$ time to 50% clot lysis. ns, not significant. ***, p<0.001. **** p< 0.0001.*

Conclusions

As I have noted previously, increasing the TF concentration initiates clot formation more rapidly. However, it is not clear why lysis occurs more rapidly with higher concentrations of TF. It is possible that more vigorous thrombin production and coagulation may trigger a more potent clot lysis response via negative feedback mechanisms.

3.2.6 Calcium concentration

During venepuncture for clotting studies, blood is drawn into tubes containing citrate. The citrate acts as an anticoagulant by chelating calcium, a necessary reagent for clotting to occur. Commonly-used venesection systems such as Vacutainer (BD medical technology, New Jersey, USA) and Vacuette (Greiner Bio-One GmbH, Bad Haller, Austria) contain 3.2% sodium citrate (109 mmol/L) which is, in optimally-filled samples, typically diluted 9:1 by whole blood giving a final concentration of 10.9 mmol/L. Two citrate molecules can bind three calcium ions giving a potential for citrate to remove 16.35 mmol/L calcium before it is saturated, in excess of the total calcium concentration in plasma (2.2 – 2.7 mmol/L).[321] Samples can be 'recalcified' by adding $CaCl_2$ to the activation mix. This calcium saturates the citrate binding sites resulting in free calcium, allowing coagulation to occur.

Methods

25µL of volunteer plasma (n=6) was aliquoted row-wise into each well of a 96-well plate. 75µL of activation mix containing one of several concentrations of $CaCl_2$ (0, 5, 10, 20, 40, 100 mmol/L) was added. Concentration of other reagents were kept constant (TF 4.64 mg/mL, tPA 100ng/mL).

Results

Turbidimetry results are shown (Figure 3-17). For low (0 mmol/L) or very high (100 mmol/L) concentrations of $CaCl_2$, no clotting occurred. At the 40 mmol/L concentration of calcium chloride, the turbidimetry plot was irregular. At this concentration lag time was extremely prolonged, while the peak turbidity and lysis time were reduced. Results were broadly consistent within the range 5-20 mmol/L. At the 20 mmol/L concentration, a small but significant increase in lag time was observed (mean (SD) of lag time 258 s (44.0 s) at 20 mmol/L compared with 199 s (16.7 s) at 10 mmol/L, Figure 3-18).

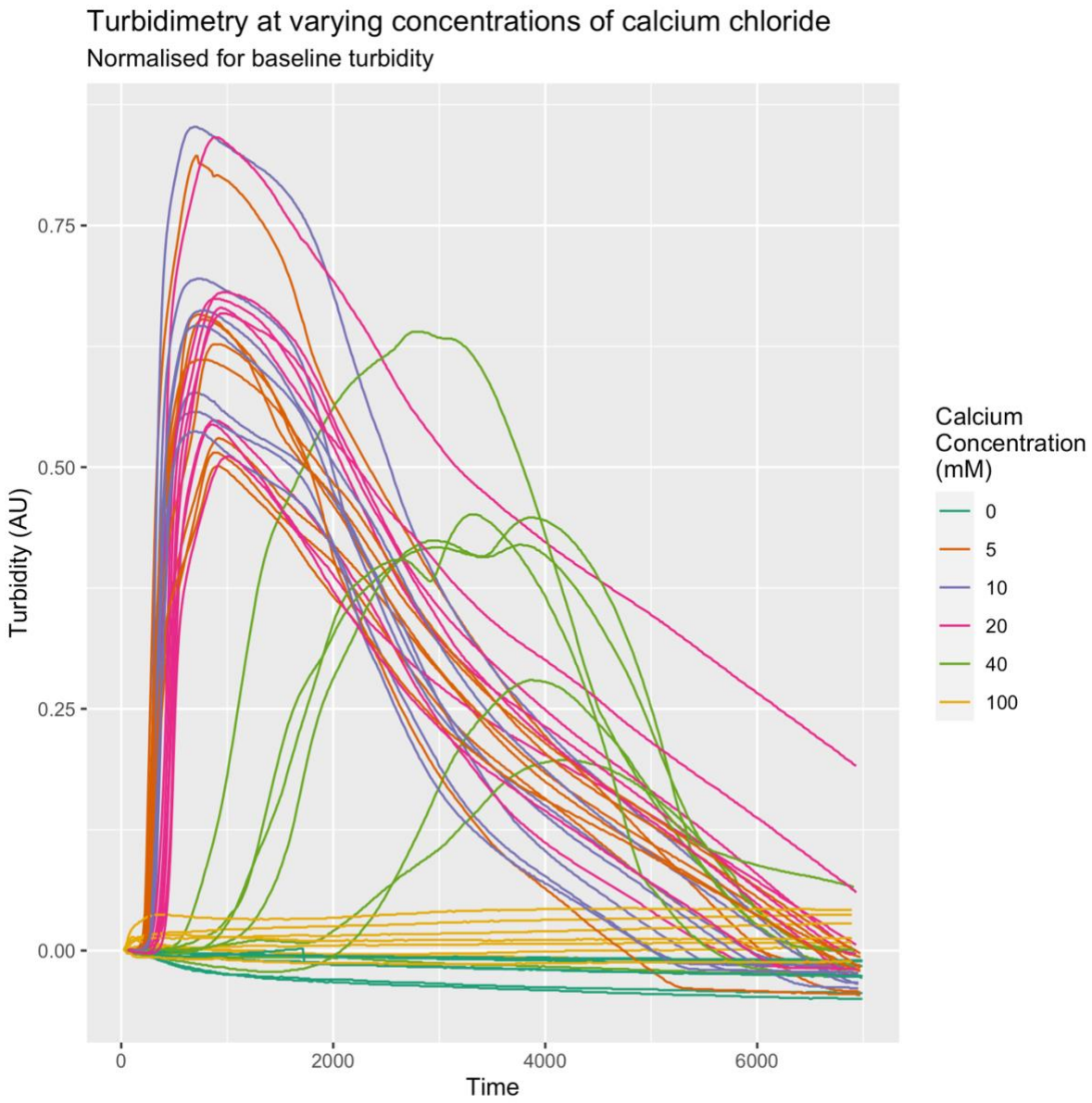


Figure 3-17 – Turbidimetry at varying concentrations of calcium chloride.

Conclusions

Calcium chloride is a necessary component of the assay. At the extremes of calcium chloride concentrations, coagulation and lysis does not occur predictably. However, I have observed that, across a wide range of intermediate values (5-20 mmol/L), the precise calcium concentration does not appear to be critical for the accuracy of this assay.

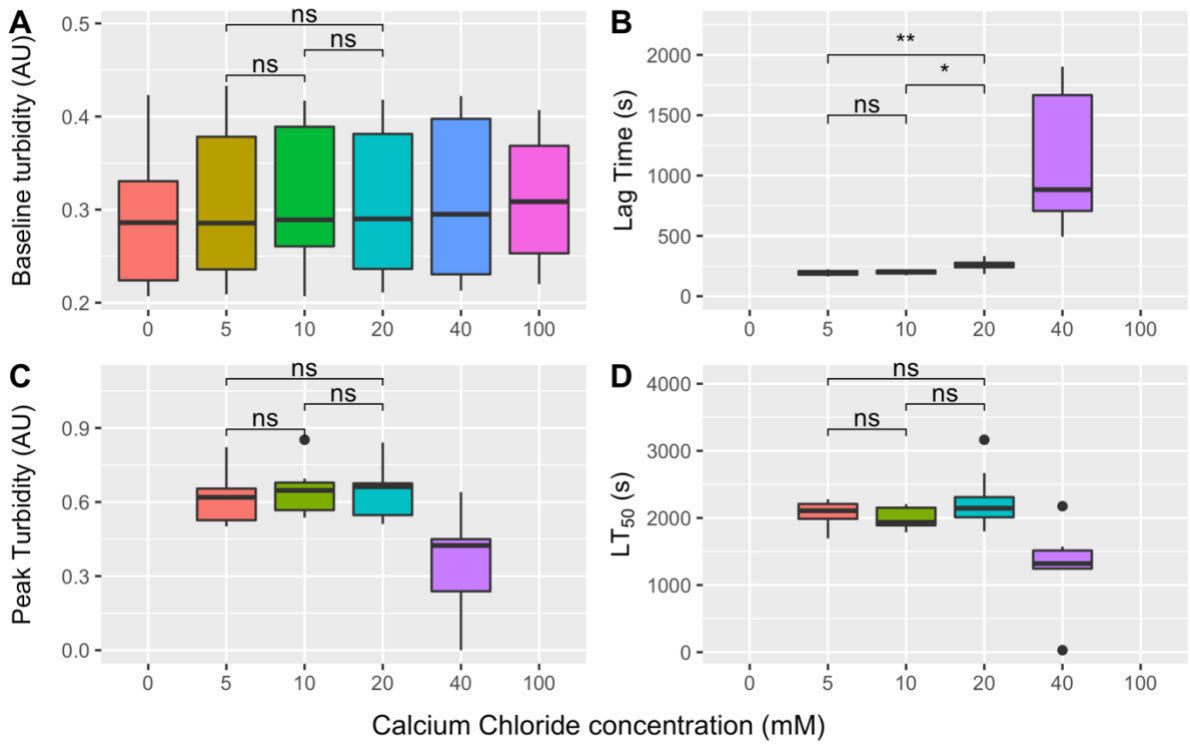


*Figure 3-18 – Effect of $CaCl_2$ concentration on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time. $CaCl_2$, calcium chloride. LT_{50}, time to 50% clot lysis. ns, not significant. *, p<0.05, **, p<0.01.*

Since the sample collection tubes have a fixed volume of citrate, the final concentration in the collection tube depends on the volume of blood collected, I.e., underfilled tubes will have higher citrate concentrations. It is typically not possible to *overfill* vacuum sampling tubes therefore lower-than-standard citrate concentrations are unlikely. The experimental protocol assumes that all samples have been filled optimally. Using a higher concentration towards the upper end of the 5-20 mmol/L range will, to an extent, reduce the effect of underfilled samples. A final concentration of 12.4 mmol/L was chosen partly for this reason but also because that requires precisely 200 µL of $CaCl_2$ (at 1 mol/L), a volume that is easily and accurately aliquoted.

3.2.7 Stability of reagents

Plasma samples are stored at -80 °C, while the TF and tPA reagents are refrigerated until reconstituted, before being frozen at -80 °C. This is to minimise the effect of any errors. For example, a low concentration of TF is required for each experiment, and this requires accurate pipetting of a small volume. Since precise pipetting is more challenging with small volumes, I sought to minimise this potential source of error by reconstituting all reagents *a priori* into single-experiment aliquots. I will analyse plates over the course of several months, so I wanted to assess the stability of reagents over a longer timeframe. However, I wanted to be assured that this process was not introducing any error.

We wanted to explore whether any difference in activity was evident between batches of TF concentrate and tPA and the effect of freezing these (reconstituted) reagents.

Methods

50 µL of plasma from volunteers (n = 4) was added to a 96-well plate. Activation mix containing either freshly reconstituted TFs ('Fresh'), TFs from short-term frozen storage (approximately 48 hours, 'Short freeze'), TFs from a 3-month freeze ('Long freeze') and freshly-reconstituted TFs from an expired batch ('Fresh (Exp batch)') was added. Final concentrations for other reagents were constant: tPA 74.9 ng/mL, TF 7.49 mg/mL, $CaCl_2$ 15.3 mmol/L. Photometry was then performed.

Results

Turbidity and lysis parameters are presented (Figure 3-19). No significant differences between groups were observed for any of these parameters. The lag time was numerically shorter (not significant) for the expired batch ('Fresh (Exp batch)') of TFs.

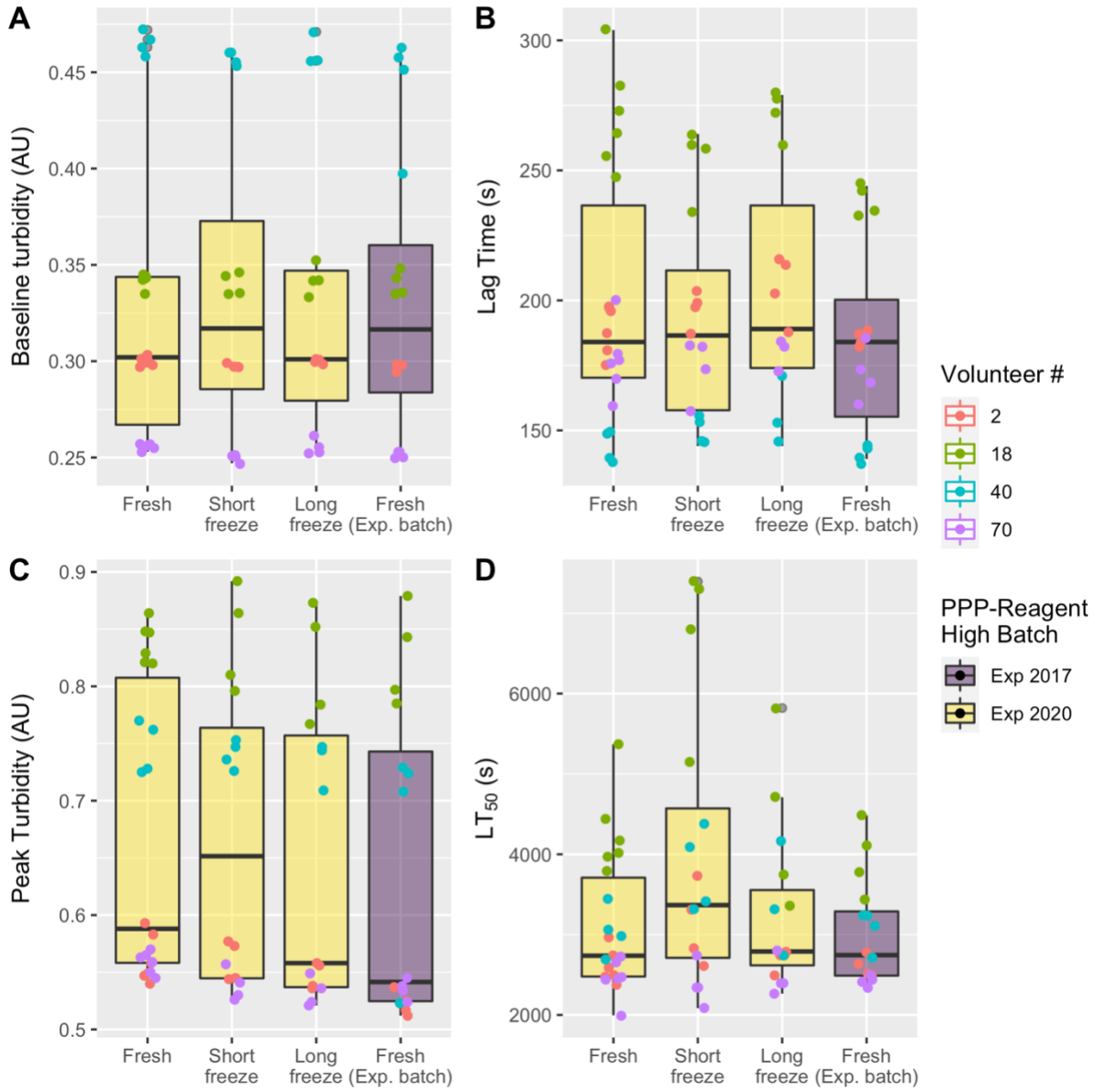


Figure 3-19 – The effect of using different TF preparations in the activation mix on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time.

Conclusions

Although the numbers are small, I have not observed any effect of freezing the TF aliquots suggesting that frozen storage (at least to 3 months) does not affect activity. A small difference in activity between batches of PPP-Reagent High (TF) is possible. Thus, reconstituting and mixing all the TF *a priori* and freezing aliquots for each plate does not reduce accuracy and may reduce the potential for batch-related error.

3.2.8 Stability of plasma

Samples for the proposed study have been stored at -80°C since their collection. However, the effects of this frozen storage on this version of the PTL assay are unknown. I therefore wished to establish the effect, if any, of freezing and thawing plasma samples multiple times.

Methods

A single blood draw was collected from healthy volunteers (n=6). Plasma was then spun and aliquoted into small volumes. Pooled normal plasma was also aliquoted. All samples were then frozen at -80°C, before being subjected to several excessive freeze-thaw cycles (0, 3 or 10). On a single plate on a single day, samples were then analysed using a turbidity and lysis protocol with a standardised activation mix (tPA 87.5 ng/mL, TF 1.80 mg/mL, $CaCl_2$ 18.8 mmol/L). 50 µL of plasma per well and 100 µL of activation mix was used.

Results

Similar results were seen for baseline turbidity, lag time and peak turbidity irrespective of the number of excessive freeze-thaw cycles (Figure 3-20). Lysis time was unavailable due to technical issues with the assay. Results from individuals were compared, with the mean of the 0 excessive freeze-thaw cycles being used as a reference value (Figure 3-21).

Conclusions

No significant differences were observed overall, suggesting that, for the purposes of my assay, thawing and refreezing does not affect the results. In this experiment, lysis unfortunately did not occur reliably. This may have been due to an inadvertent reduction in the tPA concentration. Since the sample preparation was performed over several weeks, it was not feasible to repeat this to obtain lysis time results. Nevertheless, I have shown that baseline turbidity, lag time and peak turbidity remain reliable markers.

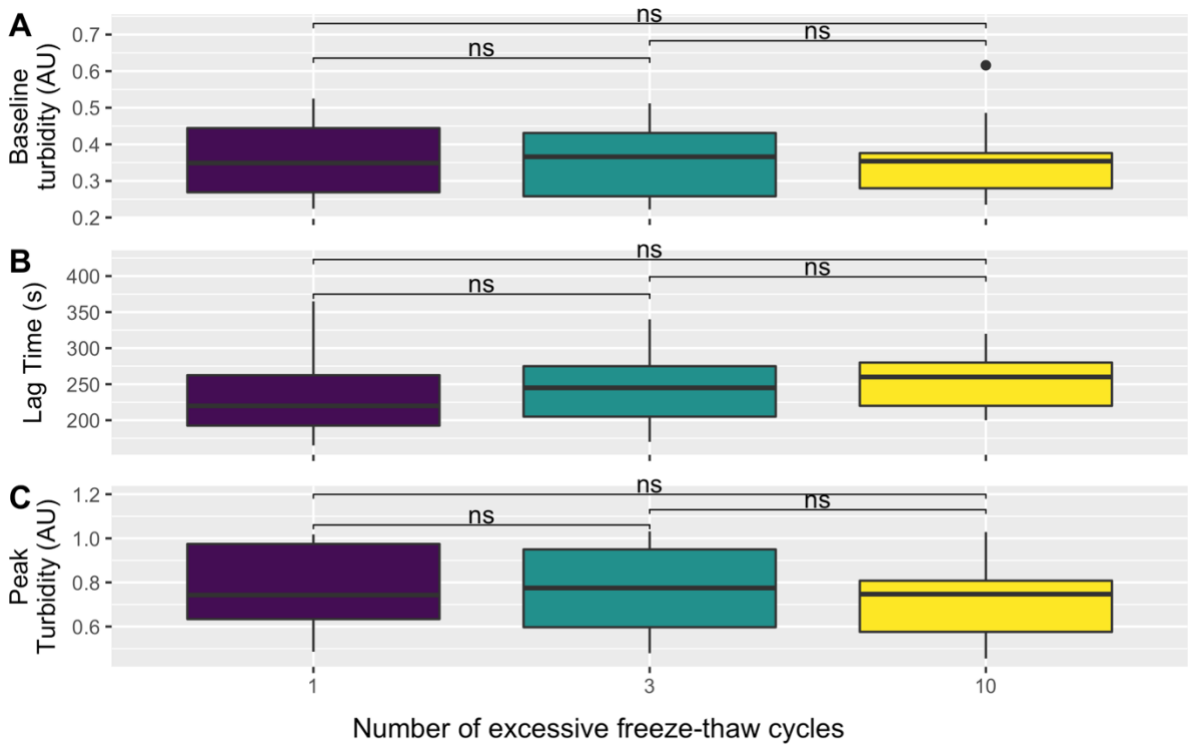


Figure 3-20 – The effect of excessive freezing and thawing of plasma samples on (A) Baseline turbidity, (B) Lag Time and (C) Peak turbidity. ns, not significant.

Although a difference can be detected per-individual over increasing numbers of freeze-thaw cycles, the magnitude of the difference is small (substantially less than <1%) and within the expected CoV of the assay. Moreover, this effect does not appear to be directional.

The number of freeze-thaw cycles did not significantly affect results, but I was not able to determine whether the duration of frozen storage of plasma affects the analysis.

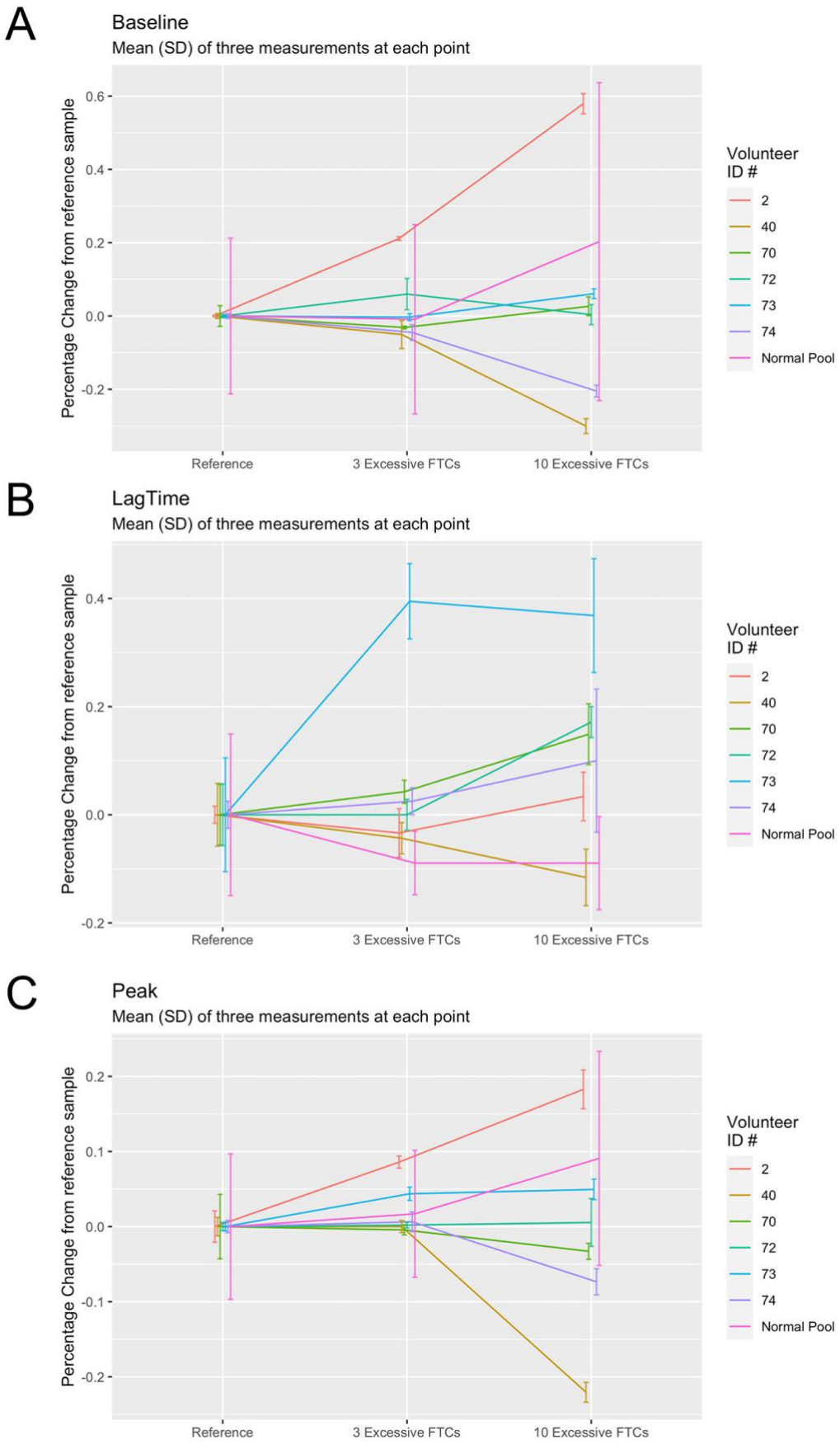


Figure 3-21 – Effect of excessive plasma sample freeze-thaw cycles on (A) Baseline turbidity, (B) Lag time and (C) Peak turbidity. FTCs, Free-thaw cycles.

3.2.9 Anticoagulation

One of the aims of the assay was to assess if differences in anticoagulant activity could be detected. To accurately assess the concentration-response of FXa inhibitor anticoagulants, I assessed the effect of the drug *in vitro*. My aim is to use this assay for patients taking the factor Xa inhibitor apixaban. At the standard clinical doses of apixaban (5mg twice daily, orally), mean plasma concentrations at steady state have been reported as 129 ng/mL (peak) and 49.6 ng/mL (trough).[322]

Methods

50µL volunteer plasma was added to a standard 96 well plate. 100µL activation mix was added with apixaban at varying concentrations to simulate a range of physiologically plausible concentrations (0, 50, 100, or 150 ng/mL). Concentrations of other reagents were kept constant (TFs 1.76 mg/mL, tPA 83.4 ng/mL, $CaCl_2$ 12.4 mmol/L).

Results

Apixaban has a clear effect on the lag phase of the experiment (Figure 3-22). The mean (SD) for lag time was 155 s (14.0), 226 s (16.8), 278 s (25.8), 276 s (15.1) at 0, 50, 100 and 150 ng/mL concentrations respectively. A trend towards shorter LT_{50} with increasing apixaban concentrations was observed, though the reduction was only significant between the 50 and 100 ng/mL concentrations (Figure 3-23 D). Peak turbidity was lower at the highest two levels of apixaban concentration (Figure 3-23 C).

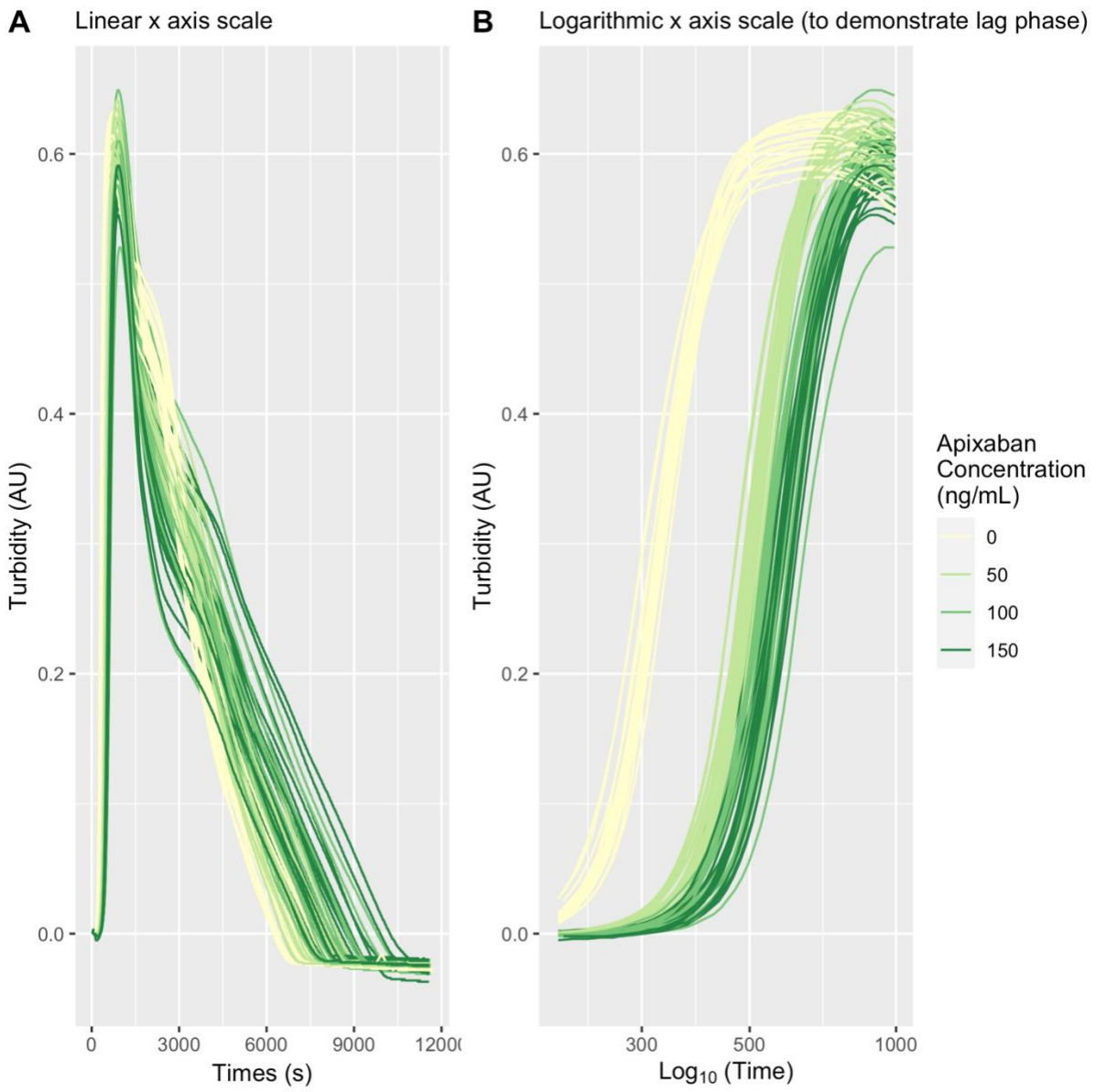


Figure 3-22 – Turbidity plots demonstrating the effect of varying the apixaban concentration in vitro. (A) Linear x-axis scale, (B) logarithmic x-axis scale to highlight differences in lag time. TF, tissue factor.

Conclusions

Apixaban concentration affects the lag time and, to a lesser extent, peak turbidity and LT_{50}. I hope to be able to utilise this effect on lag time as a marker of plasma concentration of apixaban.

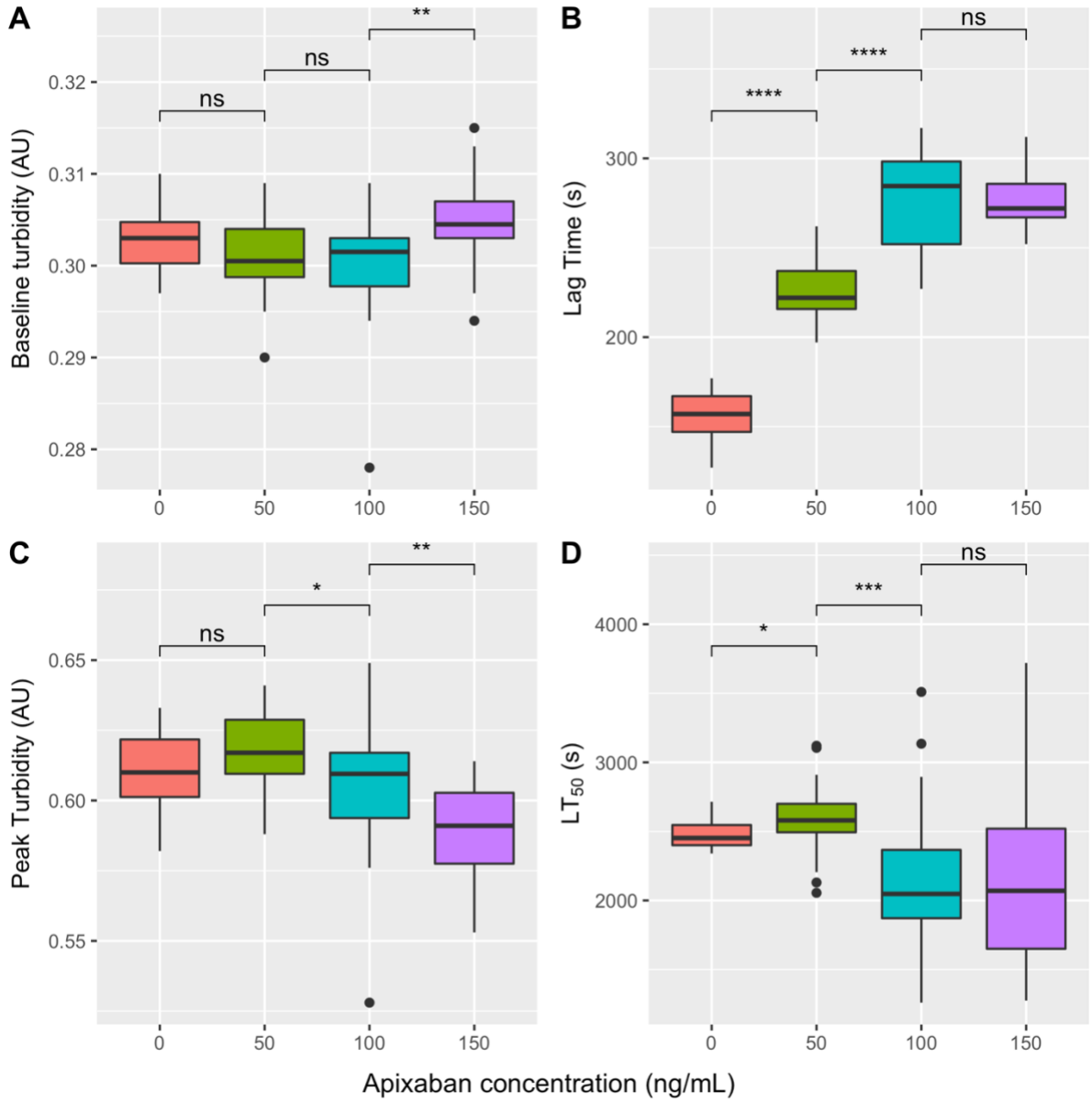


*Figure 3-23 – Effect of apixaban concentration on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time with selected pairwise comparisons shown. LT_{50}, time to 50% clot lysis. ns, not significant. *, p<0.05, **, p<0.01. *** p<0.001. ****, p<0.0001.*

3.2.10 Testing the assay on plasma from patients with cardiovascular disease

Until this point, the assay refinement process had been performed using healthy volunteer plasma, since it was abundant. However, we have previously observed significant differences, particularly in lysis time, in patients with cardiovascular disease (CVD). Since the proposed study will examine patients with CVD, I wanted to ensure that there were no unforeseen effects when testing in these patients.

Methods

A plate was prepared with 50 µL plasma per well using samples from patients with established chronic coronary syndromes (CCS, n=12) and healthy volunteers (n=4). 100 µL of an activation mix was added, containing one of three possible tPA concentrations (83, 110 or 150 ng/mL). All other constituents were kept constant (TF 1.80 mg/mL, $CaCl_2$ 12.0 mmol/L).

Results

Peak turbidity was greater and lysis time longer amongst individuals with CCS (Figure 3-24, Figure 3-25). Lag times and baseline turbidity were similar. At the end of the recording time, the majority of HV, but not CCS samples had completely lysed (Figure 3-24). The inter-individual CoV was highest at lower concentrations of tPA (Figure 3-25).

Discussion

Substantial differences exist for results between patients with CCS and healthy volunteers, most notably for the lysis time parameter. The peak turbidity is much higher in CCS individuals, though this may represent relative dehydration in the context of medication or fasting. The lysis time was greater for individuals with CCS across all tPA concentrations.

The patient cohort from which the plasma samples were taken (patients with CAD) may be different compared with the atrial fibrillation cohort I intend to study. However, as previously discussed, many of the risk factors overlap substantially so I expect results to be similar.

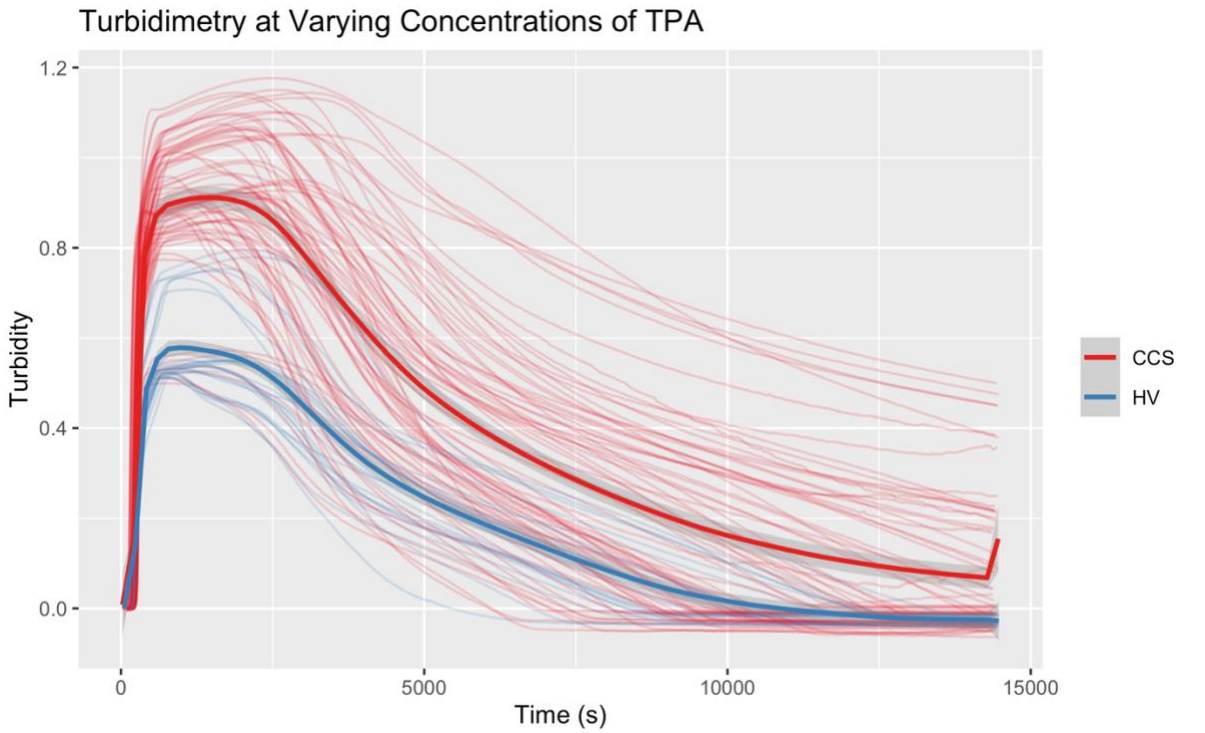


Figure 3-24 – Turbidity plots comparing patients with chronic coronary syndromes to healthy volunteers. Fine lines represent individual samples, bold lines are a smoothed average of all values from that group across several tPA concentrations. HV, healthy volunteer. CCS, chronic coronary syndrome (patient). tPA, tissue plasminogen activator.

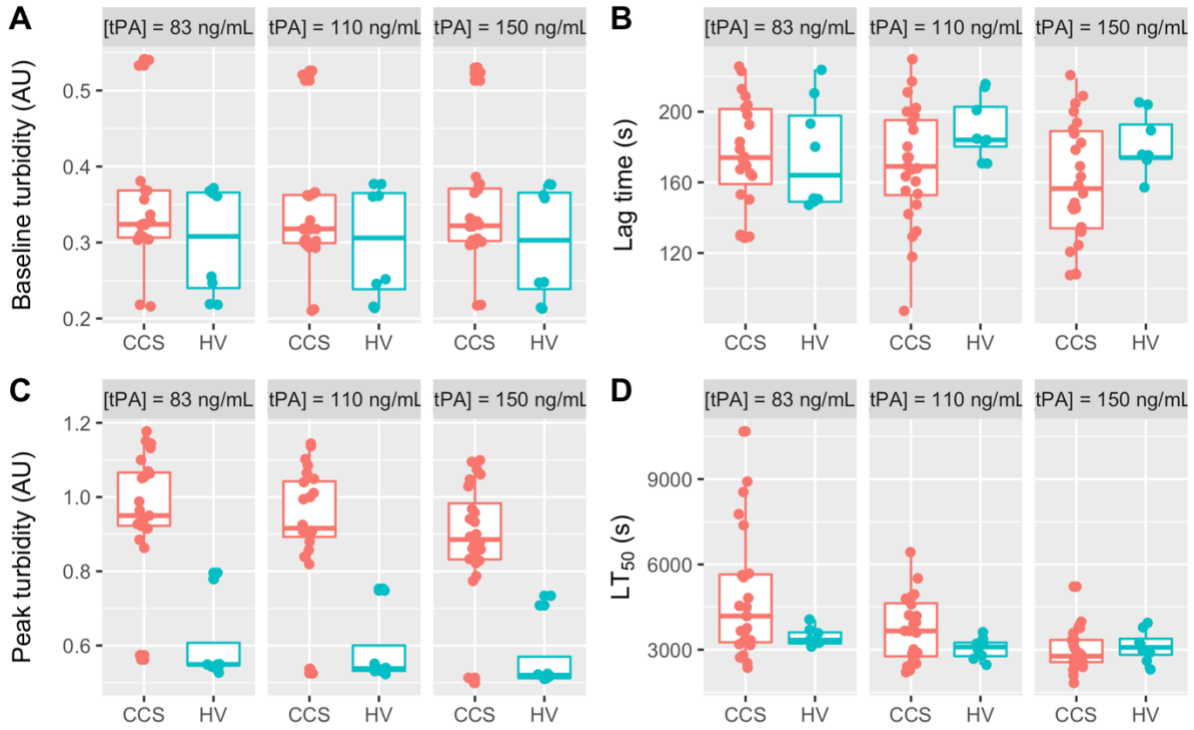
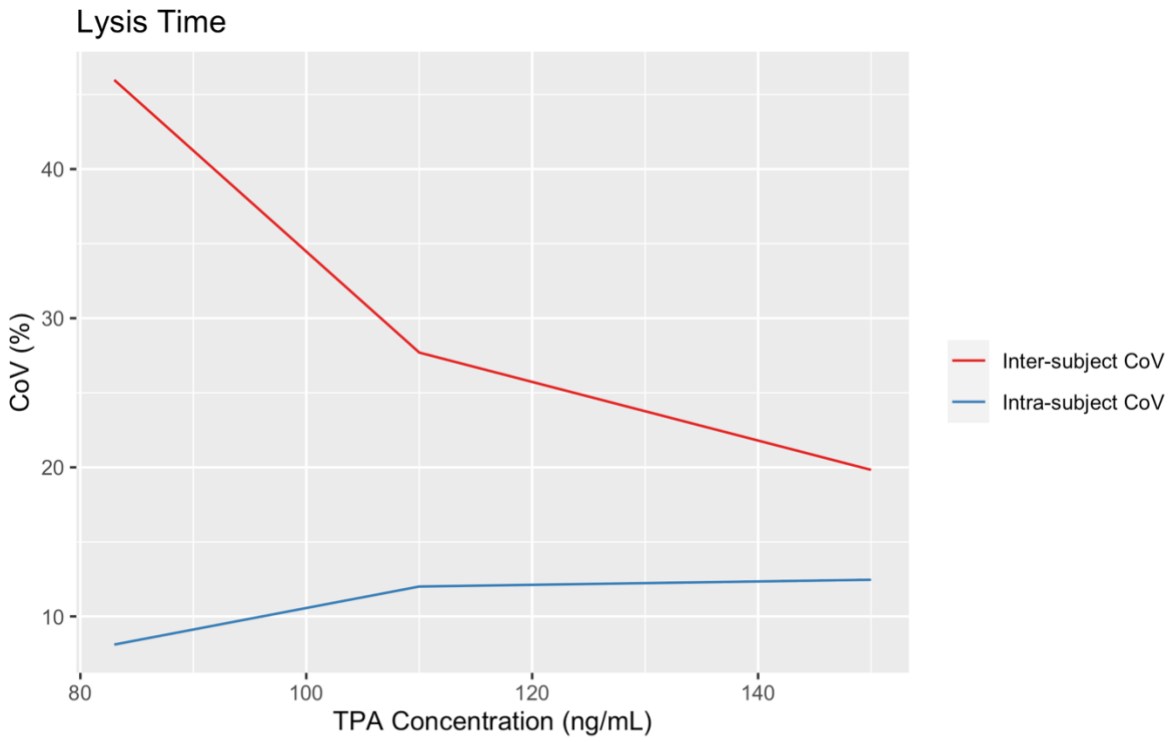


Figure 3-25 – The effect of using differing tPA concentrations between plasma samples from healthy volunteers and patients with CCS on (A) Baseline turbidity, (B) Lag Time, (C) Peak turbidity and (D) Lysis Time. [tPA], concentration of tissue plasminogen activator. HV, healthy volunteer. CCS, chronic coronary syndrome (patient). LT_{50}, time to 50% clot lysis.

The overall assay duration was much longer in patients with CVD, which is notable since premature ending of the analysis may result in a failure to record the lysis time. The inter-individual CoV demonstrated a much greater heterogeneity identifiable amongst patients with CCS, than HV, whilst maintaining an intra-individual CoV less than 10%.



indvar	Intra-subject CoV	Inter-subject CoV
83	8.11	46.0
110	12.00	27.7
150	12.46	19.8

Figure 3-26 – Effect of TF concentration on the inter and intra-individual CoV of the lysis time parameter in patients with CCS and healthy volunteers. CoV, coefficient of variation. CCS, chronic coronary syndromes.

3.2.11 Effect of apixaban on lag time in vitro

We had seen previously that rivaroxaban (an anti-Xa antithrombotic medication in widespread clinical use) prolonged lag time *in vitro* [unpublished data], but I had not measured the effect of apixaban on fibrin clot properties using the optimised TF assay. Since some of my subjects would be taking apixaban, and not taking rivaroxaban, I wished to measure the effect of apixaban on lag time *in vitro*. Apixaban is typically administered twice daily, with a peak plasma concentration appearing 3-4 hours after dosing.[322] Even after 10 days, there is substantial variability in plasma concentration during the day with approximately 3-fold difference between peak and nadir values (Table 3-1).

Table 3-1 – Maximum (C_{max}) and minimum (C_{min}) plasma concentration of apixaban observed in healthy volunteers

Regime		C_{max} (ng/mL) Mean (%CoV)	C_{min} (ng/mL) Mean (%CoV)	T_{max} (h) Median (min, max)
Day 1	Apixaban 2.5 mg BD	51.0 (27)	14.2 (53)	3.5 (2.0, 12.0)
	Apixaban 5 mg BD	81.9 (18)	25.3 (20)	3.5 (3.0, 6.0)
Day 7	Apixaban 2.5 mg BD	62.3 (37)	21.0 (17)	3.0 (3.0, 9.0)
	Apixaban 5 mg BD	128.5 (10)	49.6 (20)	4.0 (2.0, 4.0)

Adapted from Frost *et al.* BD, twice daily. C_{max}, maximum concentration. C_{min}, minimum concentration, T_{max}, time to maximum concentration. CoV, coefficient of variance.

Methods

At the doses used in ARISTOTLE (2.5 or 5 mg, twice daily*), the expected plasma concentration of apixaban at steady state is 21.0 – 128.5 ng/mL.[242,322] Apixaban (Cayman chemicals) was reconstituted with Dimethyl sulfoxide (DMSO) and diluted with permeation buffer to create apixaban-containing solutions with the following concentrations (0, 1, 3, 10, 30, 100, 300, 1000 ng/mL), controlling for the concentration of DMSO. PPP-reagent HIGH (2.52 mg/mL), calcium chloride (17.7 mmol/L) and tissue plasminogen activator (124 ng/mL) were added to these solutions to create an apixaban-containing activation mixture. A plate was prepared with 50 µL volunteer plasma (n=6) per well. 100 µL of the apixaban-containing activation mixture was added.

* The standard dose of 5 mg twice daily was reduced to 2.5 mg twice daily in a subgroup of patients with criteria expected to provide higher drug levels (elderly patients, those with low body weight and those with renal dysfunction). This dose reduction was only used in 4.7% of the ARISTOTLE cohort.

Results

Clotting and subsequent lysis was achieved in all samples. Fibrin clot lag time increased with increasing apixaban concentration (Figure 3-27). Lysis time (LT_{50}) was variable but appeared to reduce with concentrations of apixaban in excess of 300 ng/mL (Figure 3-28).

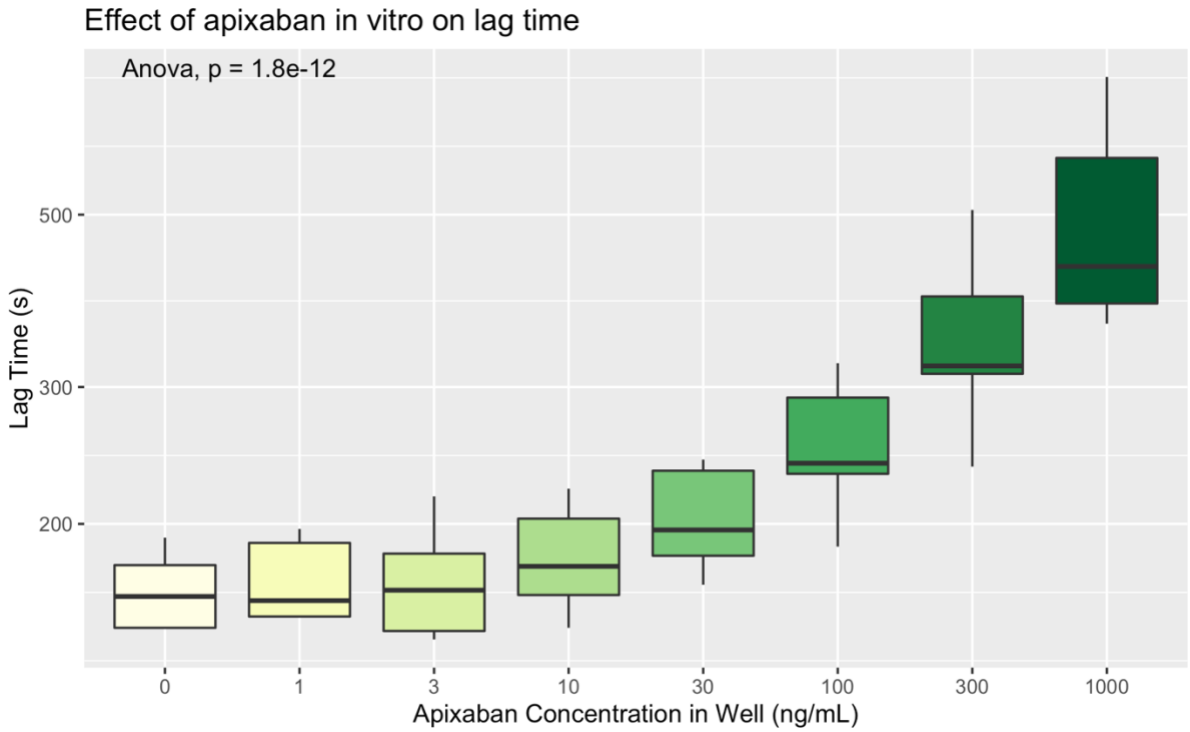


Figure 3-27 – Lag time of healthy volunteer samples at varying concentrations of apixaban in vitro.

Conclusion

Apixaban prolongs fibrin clot lag time *in vitro* when tissue factor is used as a reagent. This effect is detectable at the plasma concentrations expected during apixaban therapy, suggesting that tissue factor lag time should detect the presence of apixaban or even estimate its concentration.

The effect, if any, of apixaban on lysis time was more difficult to detect. I observed a significant reduction in lysis time at higher concentrations of apixaban, but this was only significant at supra-therapeutic concentrations of apixaban.

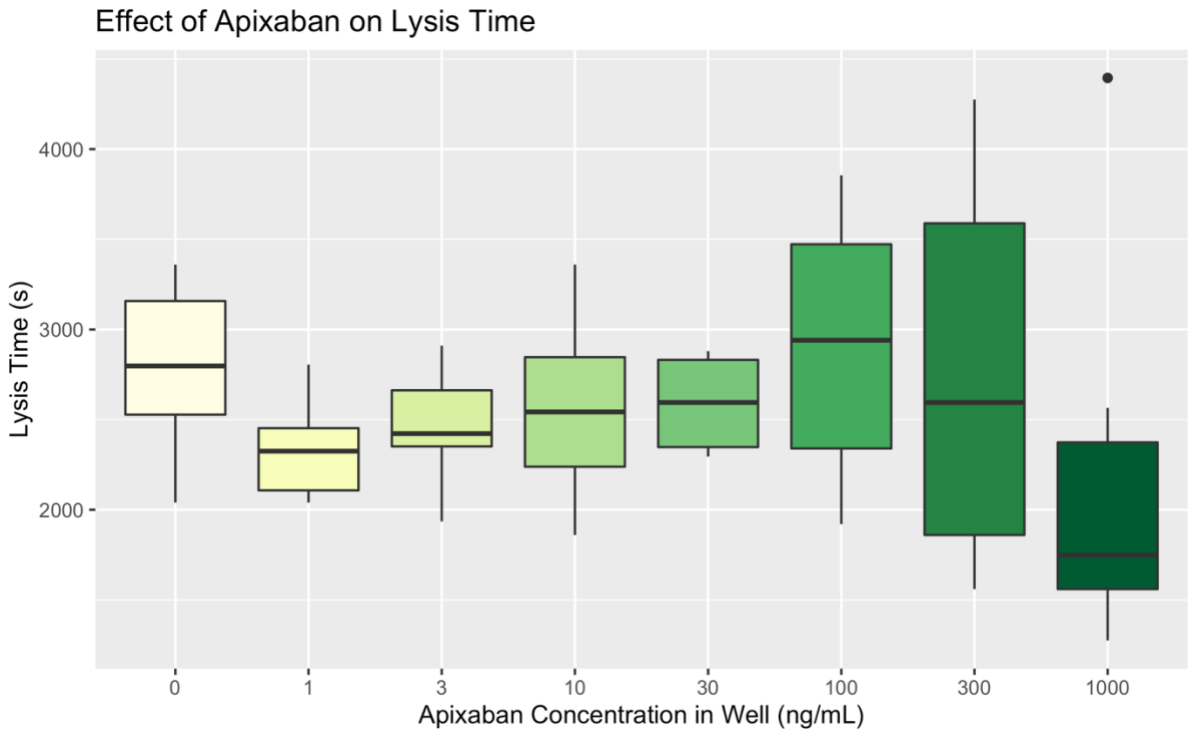


Figure 3-28 – Lysis time of heathy volunteer plasma samples at varying concentrations of apixaban in vitro.

3.2.12 Conclusions of *in vitro* experiments

Through multiple experiments and iterations, the importance of each variable has been determined. For some parameters, an optimum level is evident but for others, a decision based on experimental results and practical considerations is made.

Based on the above experiments, I have concluded that:

- A wide range of acceptable calcium concentration was found
- Generally, lower tPA concentrations were associated with lower intra-individual and higher inter-individual variability
- An optimal volume of plasma is at least 50 µL (but potentially more)
- At fixed concentrations of reagents, the volume of activation mix in the well does affect the results obtained, suggesting that consumption of reagent and coagulation factors may be an important determinant of the rate of coagulation and lysis
- The concentration of TF influences both the lag time and lysis time substantially
- The number of freeze-thaw cycles does not significantly affect TF activity, though maintaining the TF batch as a constant is likely to be important
- The number of freeze-thaw cycles for plasma samples does not affect the results

- Therapeutic levels of apixaban *in vitro* affect the lag time and, to a lesser extent, the lysis time. The lag time may be a useful proxy for plasma apixaban levels when analysing samples from patients who have taken apixaban
- Turbidimetry demonstrates a much greater heterogeneity in results from those with cardiovascular disease than from healthy volunteers
- Apixaban concentration has a measurable effect on fibrin clot lag time *in vitro* at therapeutic concentrations, suggesting that it should be possible to observe differences between apixaban concentration *in vivo* using this assay

Conflicting results & practical considerations

In some circumstances, compromises were made. For example, although a concentration of tPA greater than 83 ng/mL resulted in a more rapid lysis (i.e., it allowed the experiment to conclude sooner), this still did not permit the analysis of more than two plates per working day. Since the 83ng/mL concentration of tPA was associated with the lowest CoV, I chose that concentration for the final assay.

Measuring very small volumes of reagents or plasma is challenging. Moreover, repeated measures using a pipette, water and a balance have demonstrated that, in our laboratory, measurement of very small volumes (< 20 µL) is less reliable (data not shown). When attempting to aliquot small volumes of plasma (<40 µL), bubbles occurred more frequently. Since bubbles adversely affect the accuracy of any measurements of light transmission and may render results unusable, it is beneficial to avoid small plasma volumes. For the proposed study, higher volumes (>50 µL/well) are impractical given the samples available. I have seen that, occasionally, samples do not produce an accurate result. This is often due to inadvertently introducing air bubbles into the well. Although more common when pipetting small volumes, it can occur at any volume. For this reason, I decided to a) conserve plasma where possible and b) duplicate each result. Therefore, a practical decision was made to use 50 µL plasma per well (in duplicate), rather than 100 µL.

Frozen storage of reagents and plasma is convenient and safe. I have not observed any detrimental effect of this form of storage on the turbidity and lysis experiments and will continue to use this method.

To control the concentration of reagents as closely as possible, minimising potential sources of error, the TF and tPA were prepared in advance and aliquoted into small volume vials, each suitable for a single experiment. This way, any inter-batch variability or potential for inaccurate measurement was reduced.

We saw a wide range of possible calcium concentrations. I have chosen a level towards the upper end of this to reduce the effects of potential underfilled tubes as previously discussed.

The final concentrations of reagents for the optimised TF assay are given in Table 3-2.

Table 3-2 – Constituents of the activation mix of the optimised tissue factor assay after refinement

Reagent	Starting Concentration	Volume (µL)	Concentration in mix	Concentration in well (2:1 Mix with plasma)
Permeation Buffer	N/A	10500	N/A	
Tissue factor	61.8 mg/ml	460	2.52 mg/mL	1.68 mg/mL
Calcium chloride	1 mol/L	200	17.7 mmol/L	11.8 mmol/L
Tissue plasminogen activator (tPA)	10 µg/ml	140	124 ng/mL	82.6 ng/mL
	Total:	11300		

The concentrations of reagents in the activation mix (column 4) and in the well of the 96-well plate, after mixing with plasma (column 5) are given.

3.3 Investigating the reliability of fibrin clot metrics

There are several ways used to calculate lag time, peak turbidity and LT_{50}. I sought to find the most reliable metrics for each phase of the turbidimetry (clot onset, peak, lysis) in my PTL assay.

Interaction of peak time and lysis time

Measurement of clot lysis in the PTL assay is challenging. Traditionally, LT_{50} (the time from the peak turbidity to when the turbidity reduces to less than 50% of its (corrected) peak value) is used. However, since this is a compound measure consisting of two variables (peak time and lysis point time), it inherently has more potential for variation than other measures that have a fixed start point, such as lag time. Accordingly, determining where to annotate the first marker is of paramount importance. If, for example, a wide plateau at the peak of the PTL curve exists, substantial variation in the algorithmic placement of a peak marker can occur.

Therefore, I investigated several ways to define the peak:

- $Peak_{abs}$ – The absolute peak; the position of the greatest turbidity
- $Peak_{2c}$ – The point at which two consecutive values do not increase (i.e., a plateau has been reached)
- $Peak_{3c}$ – As above, but requires three consecutive values
- $Peak_{0.9}$ – Time to reach a value > 90% of the absolute peak

Since the lysis time measure (e.g., LT_{50}) depends on the peak timing, it is possible to construct a lysis time from each of these peak variables. A further measure of lysis, clot lysis time (CLT) has been proposed, measuring the time elapsed from the point of 50% rise in turbidity, to the point of a 50% decrease.[287]

3.3.1 Methods

Turbidimetric results from 24 wells with identical normal pool plasma samples (50 µL) and 100 µL activation mix (Calcium chloride 17.7 mmol/L, TF 2.52 mg/mL, tPA 124.0 ng/mL) were compared. All output variables were computed for each well. The mean, standard deviation, and coefficients of variation of each variable were calculated.

3.3.2 Results & discussion

Clotting occurred in all wells. Automated placement of markers for lag time, peak and lysis time were confirmed visually, with no errors identified. Results for the mean, standard deviation, and coefficient of variation for the lag time parameters are shown in Table 3-3.

For lag time, high CoV values are seen when three consecutive values are required, but this reduces when five or seven consecutively rising values are required. However, using an absolute rise of 0.015 AU appears to be a more reliable marker with a lower CoV. Lower still is tPCR which demonstrates the lowest CoV of all the parameters studied, though occurs slightly later than the lag time and is not a true measure of clot lag time, since the timing point is triggered by the maximal rate of turbidity increase (I.e., the steepest portion of the upslope), rather than the onset of detectable clotting. Since clotting occurs rapidly with my protocol, the time difference between lag time and tPCR is small (mean lag $time_{0.015\ AU}$ 180s compared with 185 s for tPCR). However, if clotting occurs more slowly (for example in the presence of anticoagulants) it may be important to differentiate between lag time and tPCR.

For the timing of peak turbidity, all methods showed substantial variance with CoV greater than 10%. The lowest CoV was seen by detecting the absolute peak. The 50% lysis point was annotated more reliably than the 90% lysis point, and accordingly compound measures that used the 50% point as their marker performed better than those using 90%. CLT, tPCR-to-$lysis_{50}$ and LT_{50} performed similarly. The minimum d(turbidity)/d(time) remains very challenging to annotate correctly despite the filtering techniques described above (and in the appendix) and thus has poor reliability.

The velocity index is a compound measure of both the speed of clot formation and its density, while AUC provides an overall measure of both clotting and lysis, so these provide slightly different ways to analyse the data. AUC performed slightly better than the velocity index in terms of reliability in this experiment.

Whilst some measures (for example tPCR) may provide slightly improved reliability, they are novel and thus less widely understood. Terms such as lag time are inherently understood and

widely published. In the absence of a substantial improvement, a decision was made to use the conventional measurements in preference.

Limitations

These filtering algorithms were applied to data generated on a plate reader with a fixed time interval (15 s) between sampling points. Using a substantially different time interval or different equipment may affect these findings and would require revalidation.

3.3.3 Conclusions

Software to analyse the results from the PTL assay was designed and developed according to current standards in measurement of PTL variables. Several challenges are created with the complexity and volume of data recorded for these experiments. Measures were taken to ensure data integrity was maintained at each step of the process, annotating the results directly from the sample barcode, hard coding this information into a results file, and ensuring that the results were visualised and checked. By streamlining the data acquisition process, any potential for transcription or calculation errors is reduced, while the automated analyses are time efficient. This software has potential applications in any future study using the PTL assay.

We investigated commonly used metrics and some novel parameters to attempt to find the optimum way of analysing PTL data. Lag time (0.015 AU rise) or tPCR are the best ways to identify the onset of clotting. The velocity index has a relatively poor CoV. Although annotation of the peak turbidity can appear variable, nevertheless I found that using a minimally filtered signal, the time to the absolute peak ($peak_{abs}$) was more reliable than other ways of annotating the peak time. LT_{50} is amongst the best measures of lysis, although CLT is similarly reliable. However, using a 90% lysis point (rather than 50%) worsened the reliability of the assay. Both CLT and AUC are non-specific for clot lysis (since they both include time for clot formation), so I elected to use LT_{50} as the primary metric for clot lysis.

Ultimately, having extensively investigated some commonly used and some novel metrics, I found that the traditionally used markers of lag time, peak turbidity and LT_{50} were among the

Table 3-3 – Comparing ways of calculating fibrin clot parameters from absorbance data

Variable	Mean	SD	CoV (%)
Lag Time (seconds)			
Lagtime (rise over 3 consecutive values)	141	17.2	12.1
Lagtime (rise over 5 consecutive values)	174	15.5	8.89
Lagtime (rise over 7 consecutive values)	174	15.5	8.89
Lagtime (0.015 AU rise)	180	7.94	4.42
tPCR	185	4.89	2.65
Peak time (seconds)			
Time to $peak_{abs}$	780	80.9	10.4
Time to $peak_{2c}$	756	94.3	12.5
Time to $peak_{3c}$	791	89.8	11.4
Time to $peak_{0.9}$	513	95.2	18.6
Lysis point timings, relative to start of experiment (seconds)			
Minimum of d(turbidity)/d(time)	5603	3807	67.9
50% lysis point	2845	180	6.33
90% lysis point	5616	692	12.3
Turbidity measures (Absorbance units)			
Baseline	0.50	0.02	3.28
Baseline (average of first 3 values)	0.50	0.02	3.31
$Peak_{abs}$	0.85	0.02	1.87
$Peak_{corrected}$	0.36	0.01	2.30
$Peak_{2c}$	0.35	0.01	2.31
$Peak_{3c}$	0.36	0.01	2.32
$Peak_{0.9}$	0.32	0.01	2.29
Lysis point (50%) turbidity	0.18	0.00	2.36
Lysis point (90%) turbidity	0.03	0.00	5.88
Compound measures (seconds, except where stated)			
LT_{50} ($Peak_{abs}$ to 50% lysis)	2065	146	7.05
LT_{90} ($Peak_{abs}$ to 90% lysis)	4836	636	13.2
Lag time to 50% lysis	2671	180	6.73
tPCR to 50% lysis	2660	179	6.75
tPCR to 90% lysis	5431	690	12.7
$Peak_{2c}$ to 50% lysis	2088	130	6.21
$Peak_{2c}$ to 90% lysis	4859	618	12.7
$Peak_{3c}$ to 50% lysis	2054	135	6.59
$Peak_{3c}$ to 90% lysis	4825	624	12.9
$Peak_{0.9}$ to 50% lysis	2332	129	5.55
$Peak_{0.9}$ to 90% lysis	5103	618	12.1
CLT	2724	178	6.51
Velocity index (*10^5 AU/second)	59.6	7.93	13.3
AUC (AU·seconds)	1048	94.1	8.96

Table shows the mean, standard deviation (SD) and coefficient of variation (CoV) of several computed indices in the plasma turbidity and lysis assay. See main text for definitions of parameters. AU, absorbance units. tPCR, time to peak clotting rate. $LT_{50,}$ time to 50% clot lysis. $LT_{90,}$ time to 90% clot lysis. CLT; clot lysis time. AUC, area under the curve.

best performing. Moreover, they require little explanation of methods and are more likely to be accepted by readers.

3.4 Comparison of optimised tissue factor assay with established thrombin assay

3.4.1 Correlations between parameters measured with thrombin and TF assay

Baseline and peak absorbance characteristics were similar between the TF and thrombin assays, with R of 0.96 and 0.88 respectively (Figure 3-29, Figure 3-31). This suggests excellent correlation between the assays for these parameters.

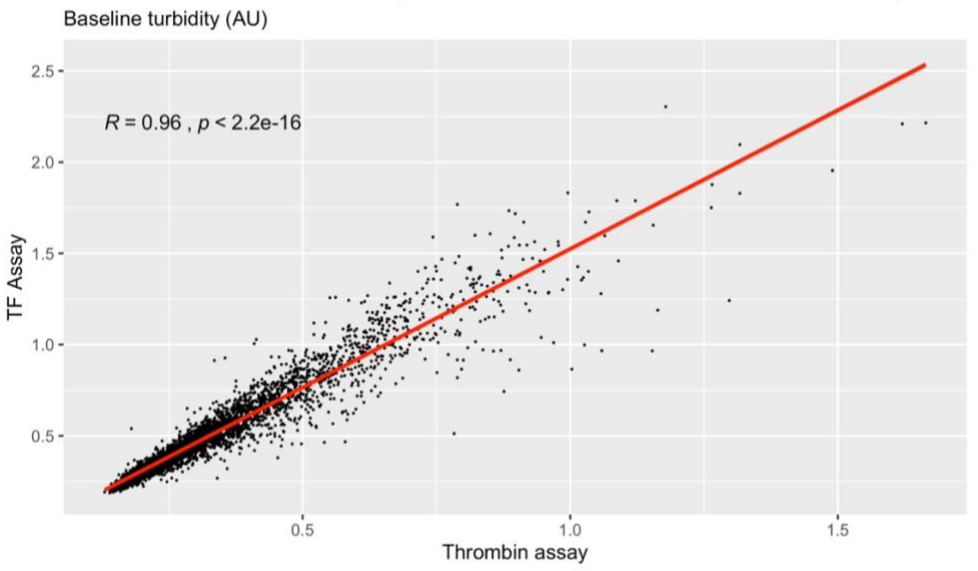


Figure 3-29 – Correlation between fibrin clot baseline turbidity in two assays. TF, tissue factor. AU, absorbance units.

Lag time results were weakly correlated between the thrombin and TF assays (R=0.54, p<0.001, Figure 3-30). This is expected, since I hoped to be able to identify more heterogeneity of lag time with TF than with thrombin, and this supports the notion that there is a difference between the assays.

LT_{50} saw a stronger correlation between TF and thrombin assays (R=0.66, p<0.001, Figure 3-32) than was observed for lag time. Nevertheless, the correlation was lower than for baseline and peak turbidity, suggesting either detectable differential lysis mechanisms or potentially unwanted variance in the assay(s).

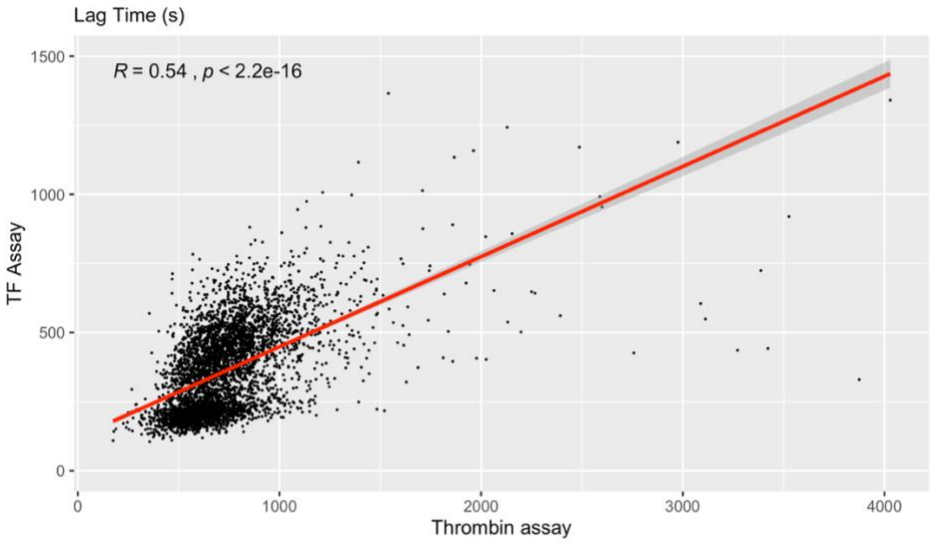


Figure 3-30 - Correlation between fibrin clot lag time in two assays. TF, tissue factor. AU, absorbance units.

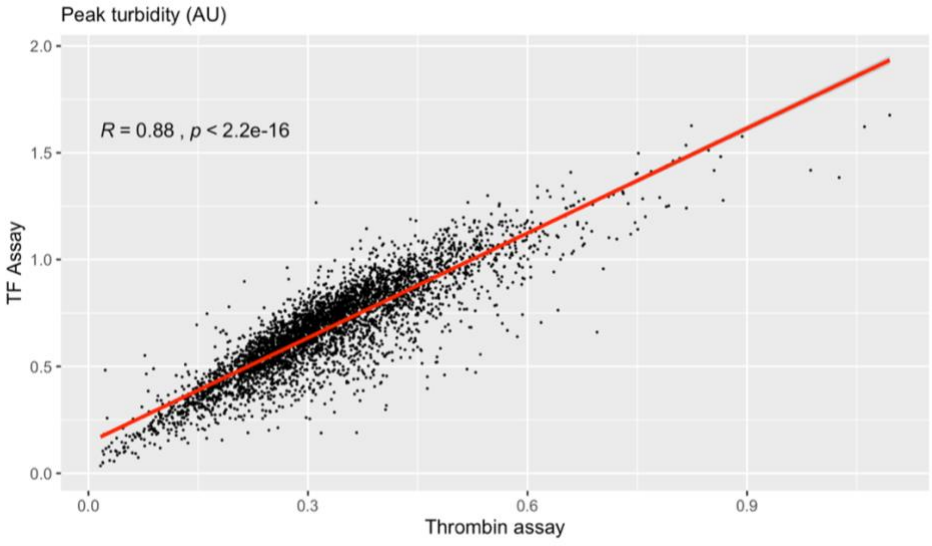


Figure 3-31 - Correlation between fibrin clot peak turbidity in two assays. TF, tissue factor. AU, absorbance units.

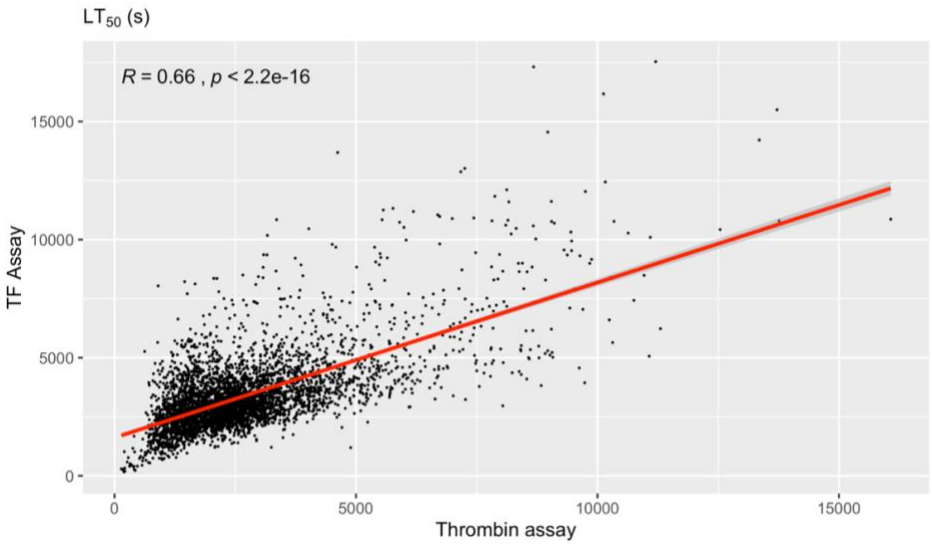


Figure 3-32 – Correlation between fibrin clot lysis time in two assays. TF, tissue factor. LT_{50}, time to 50% clot lysis.

3.4.2 Differential ability of assay to discriminate between fibrin clotting parameters

In the TF assay, the mean (SD) lag time for the non-treatment cohort was 231 s (89.8) compared with 464 s (140) in the apixaban cohort, whereas in the thrombin assay, the difference was less marked at 666 s (272) in the non-treatment cohort compared with 781 s (244) in the apixaban cohort. This is demonstrated in density plots of the lag times seen across both cohorts, with greater lag time differential evident in the TF assay (Figure 3-33, Table 3-4).

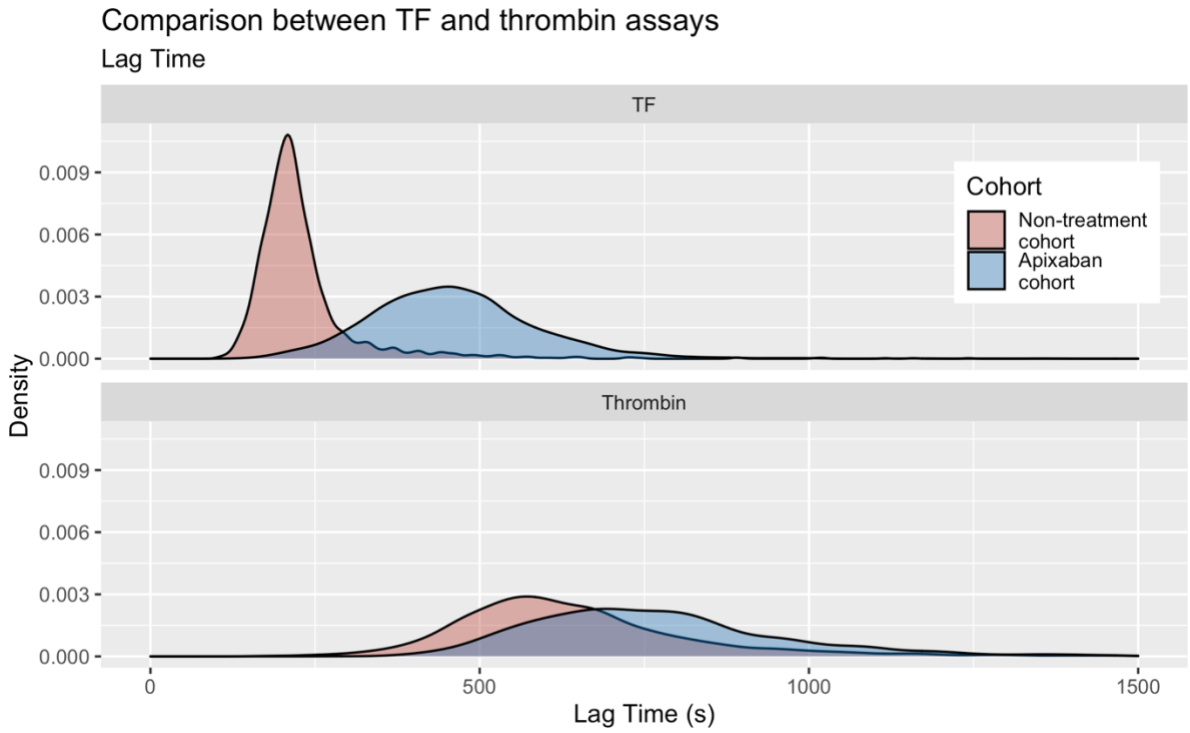


Figure 3-33 – Density plots demonstrating the distribution of lag times across the non-treatment and apixaban cohort. Greater separation of lag time is seen in the TF assay. TF, tissue factor.

At the 2-month timepoint with participants taking apixaban anticoagulation (apixaban cohort), lag time was increased in the TF assay. When patients were matched (paired cohort), the mean difference was 209.5 s (Figure 3-34). Although in almost all cases, the lag time was prolonged, in some cases there was a negative $\Delta_{\text{lag time}}$. This could have occurred due to change in the patient's circumstances or medication but is likely due to the variability in the measurement of clot lag time using the TF assay.

Table 3-4 – Mean and SD of values observed for patient samples across each assay and experimental series

	Tissue factor assay (randomisation)		Tissue factor assay (2-month)		Thrombin assay (randomisation)		Thrombin assay (randomisation)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Baseline	0.45	0.25	0.54	0.28	0.30	0.17	0.35	0.17
Lag Time	231.0	89.8	463.8	139.7	666.1	271.9	781.5	244.3
Peak	0.69	0.23	0.67	0.23	0.31	0.11	0.34	0.13
LT_{50}	3107.6	1344.4	3612.3	1765.0	2613.7	1237.5	2814.2	1849.6

LT_{50}, time to 50% lysis. SD, standard deviation.

In contrast with lag time, Figure 3-35 demonstrates that apixaban-treated patients appeared to have shorter clot lysis (compared with those in the non-treatment cohort) in the thrombin assay, but not the TF assay (see also Table 3-4).

In the non-treatment cohort, there was reasonable correlation between lag time in the thrombin assay and lag time in the TF assay (red, R=0.64, Figure 3-36), but this reduced in the apixaban cohort (blue, R=0.54), suggesting that apixaban may be having a differential effect on clot lysis depending on the assay used.

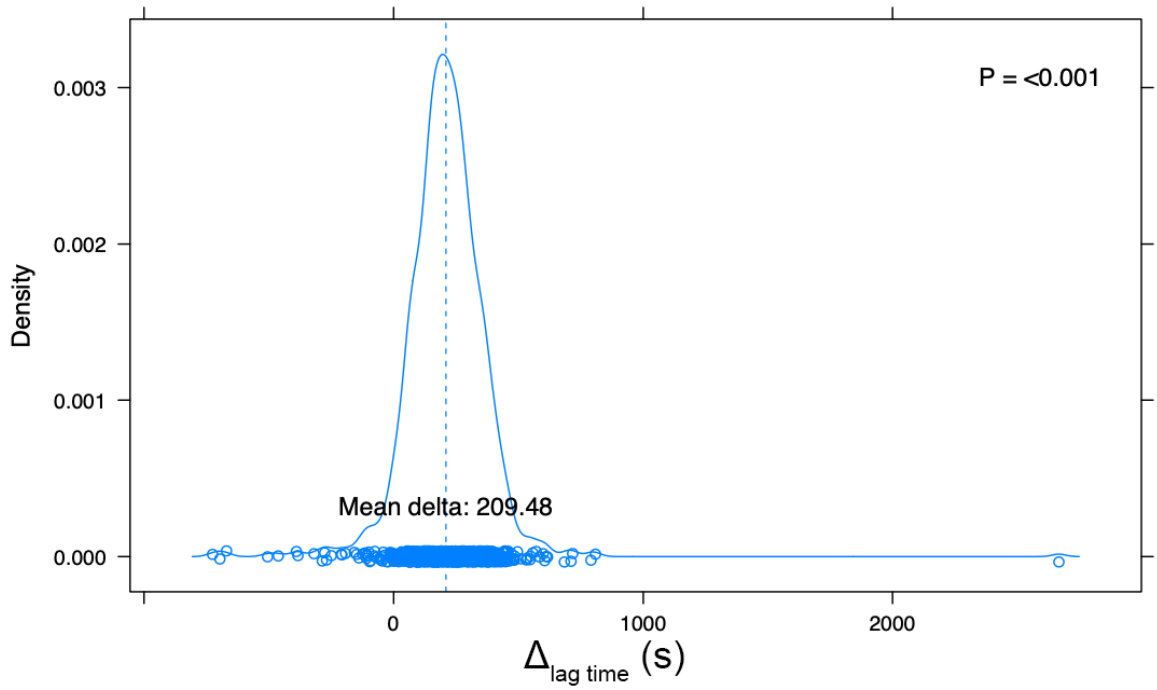


Figure 3-34 – $\Delta_{lag\ time}$ in the paired cohort. The mean delta between (randomisation) and 2-months was 209s.

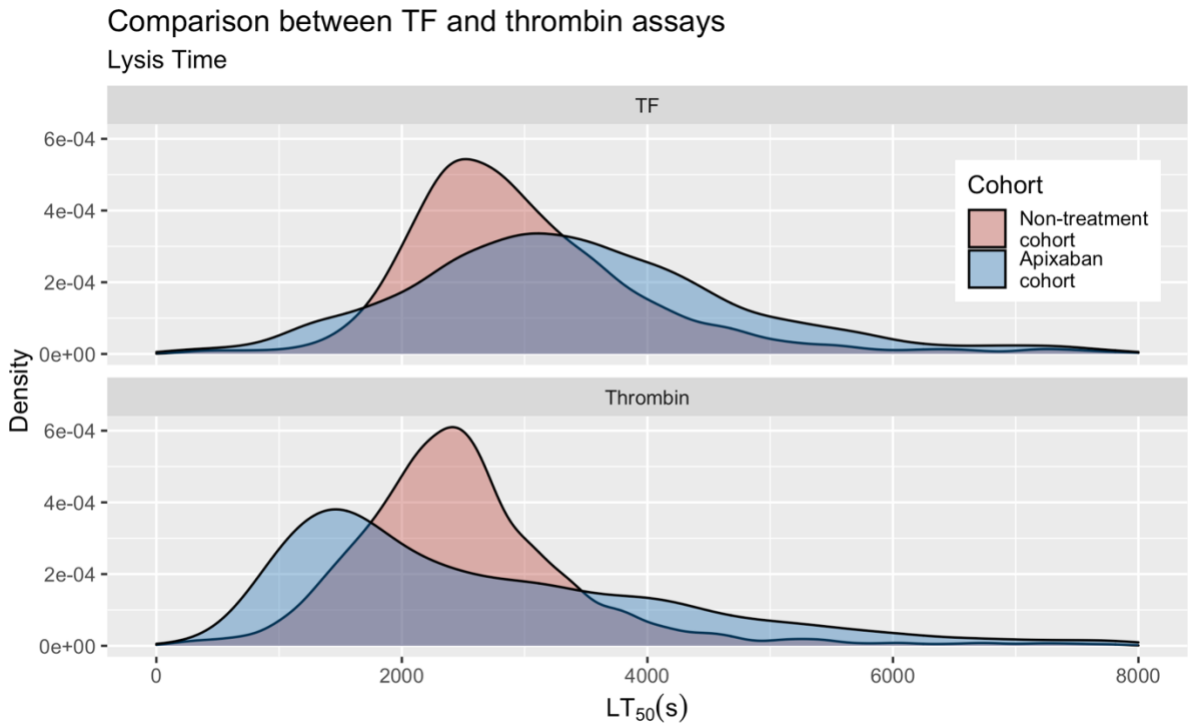


Figure 3-35 – Density plots demonstrating the distribution of lysis times across the non-treatment and apixaban cohort. Greater separation of lag time is seen in the TF assay. TF, tissue factor. LT_{50}, time to 50% lysis.

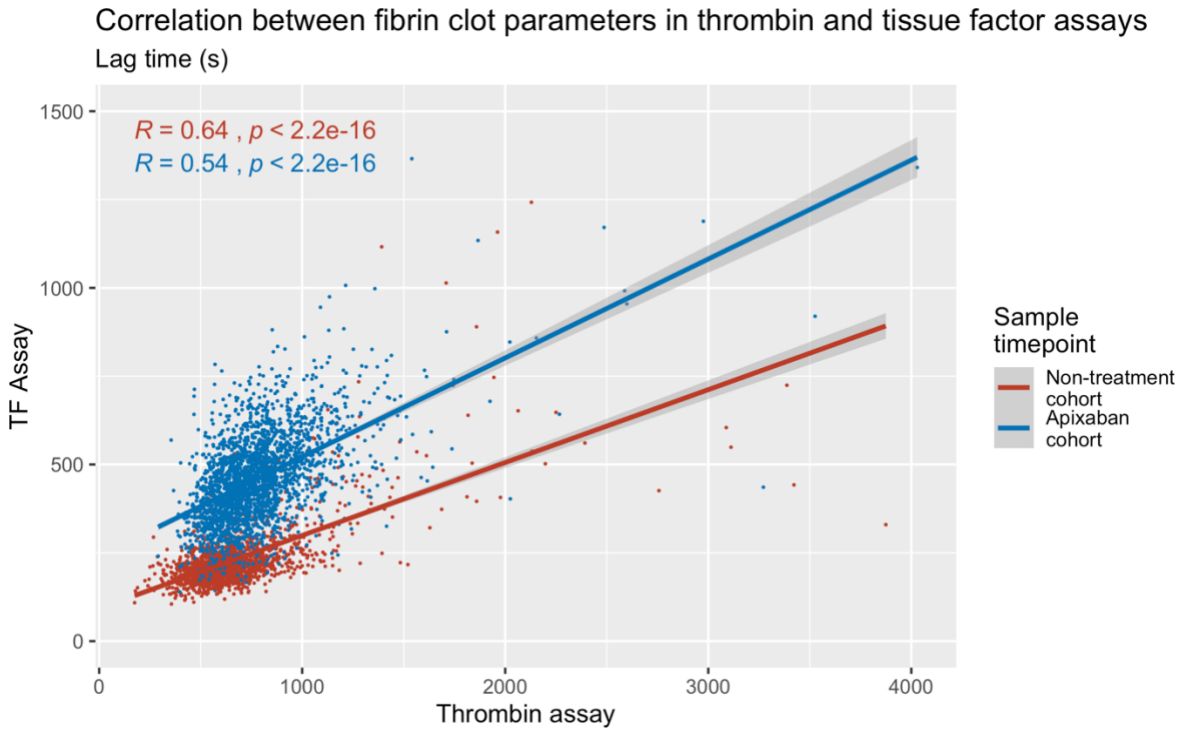


Figure 3-36 – Correlation between fibrin clot lag time in two assays, with and without apixaban treatment. TF, tissue factor. AU, absorbance units.

Equivalent plots for the baseline and peak plasma turbidity, and LT_{50} are given in the appendix (Tables 8-11 to 8-13). These did not demonstrate this difference between the correlations at the different timepoints for variables other than lag time.

3.4.3 Discussion

Previously, our group has used thrombin-based assays to measure fibrin clot dynamics in patients with cardiovascular disease. However, I hypothesised that individuals' response to antithrombotic medication may influence clinical outcomes, with this heterogeneity of response to medication representing a potential biomarker in clinical practice. There is precedence for this in contemporary cardiovascular medicine; the antiplatelet medication clopidogrel displays substantial heterogeneity of activity across people taking it, at least in part due to genetic variability in the isoform of the enzyme used to convert it to its active metabolite (CYP2C19), and there remains debate about whether we should test for this genetic variation before prescribing the medication[323]. In contrast to traditional anticoagulant regimes, such as warfarin (where regular blood testing is required to measure its effect), with DOAC treatment such as apixaban, no such testing is used, and minimal dose adjustment is required.

We set out to design an assay that aims to measure heterogeneity in apixaban concentration (in addition to other parameters of clotting). I aim to use lag time as a surrogate for the activity of apixaban *in vivo*. Moreover, I will assess whether clinical response to antithrombotic medication may influence clinical outcomes. For example, if certain comorbidities increased exposure to apixaban, we may expect this to correlate with bleeding events.

Baseline and peak turbidity

There was excellent agreement in baseline turbidity between the TF and thrombin assays (R=0.96), though this is perhaps expected since this datapoint represents the starting turbidity of plasma, prior to any clotting. Nevertheless, it has potential utility as a biomarker so its reproducibility between techniques is welcome.

Similarly, peak turbidity from the TF assay correlated well with its analogue in the thrombin assay (R=0.88). This marker represents the fibre width of clots formed in stimulated plasma and elevated maximum turbidity is associated with cardiovascular risk.[324,325] Using the thrombin assay, our group has previously shown an increase in cardiovascular death associated with elevated plasma turbidity in a cohort of patients following ACS.[150] As discussed in Chapter 4, in my cohort of individuals with a different form of cardiovascular disease (AF) I observed associations between increasing peak turbidity and several biomarkers suggesting that peak turbidity is a useful measure.

Lag time

In the TF assay, the mean lag time was 231 s in the non-treatment cohort and 464 s in the apixaban cohort while a similar increase was also seen in the (smaller) paired cohort analysis. This 2-fold increase suggests a measurable *in vivo* effect of apixaban anticoagulation on fibrin clot lag time using the TF assay. Using the thrombin assay, the difference was much smaller – approximately 1.2-fold difference between apixaban-treated and non-anticoagulated patients, suggesting that the TF assay is a better discriminator of apixaban activity *in vivo*. Moreover, the reduced correlation between the TF assay and thrombin assay in the apixaban cohort (compared with the non-treatment cohort) suggests that the optimised TF assay may be capable of detecting differences in apixaban effect not detected by the thrombin assay. This is intuitively accurate, since apixaban exerts its effect on the coagulation cascade 'upstream' of thrombin, by inhibiting the activated factor X-mediated conversion of prothrombin to thrombin and further clot propagation. Accordingly, one would expect heterogeneity in anti-Xa concentration or activity to be identifiable using TF assays, whereas a thrombin assay effectively bypasses this common step in the coagulation cascade.

It is perhaps surprising that *any* difference in lag time was detectable using the thrombin assay, with thrombin acting downstream of apixaban. This observation suggests the presence of positive feedback loops within the coagulation cascade which are, at least partly, modified by apixaban treatment.

Apixaban activity is monitored clinically using anti-Xa assays, though this is not performed routinely.[308] The ability of my assay to detect the presence (and potentially activity) of

apixaban while also examining clot lysis makes it a useful tool for examining clotting in patients taking anticoagulants.

We observed a shorter lag time in the TF assay compared with the thrombin assay. One might expect the TF lag time to be longer since more steps are required for clot formation with TF than with thrombin. However, it is unlikely that this observation represents a mechanistic difference between thrombin and TF, since the lag time measurement is highly dependent on the concentration of reagents (TF and thrombin, respectively).

LT_{50}

Both TF and thrombin produced similar LT_{50} with a higher variance than that seen for lag time or baseline and peak turbidity. This occurs because the comparatively slow lysis phase (compared to clot formation) means more variability is possible in the detection point for 50% lysis. Nevertheless, there was reasonable agreement in LT_{50} between the TF and thrombin assays (R=0.66).

When comparing the non-treatment and apixaban cohorts, it appears that apixaban treatment was associated with a modestly longer LT_{50} in TF assays, whereas LT_{50} was shorter in apixaban-treated individuals when tested using the thrombin assay. The randomisation and apixaban cohorts are not directly comparable since they do not contain the same individuals, although, given the large number of participants, it is likely that these observations are representative of the population.

However, as discussed previously (see Section 2.1.4), samples from the randomisation and 2-month timepoints were analysed separately for the thrombin assay so may not be directly comparable since different reagent batches were used. Nevertheless, the thrombin experiments suggest that apixaban modestly reduces fibrin LT_{50}, a finding in keeping with work from thromboelastography assays, so the surprising result here is that I did not observe the same effect with the TF assay.[326] This is further discussed in Chapter 6.

Limitations

The variances seen in both the thrombin and TF assays make this technique potentially more useful in study using a large dataset, such as the present study, than for use in individuals. However, further refinement of methods aimed at improving the reliability of results may widen the assay's scope.

Conclusions

The TF assay has been shown to be feasible and reproducible, while potentially revealing more heterogeneity of fibrin clot information, particularly in the case of lag time. In this respect, it has the potential to be a simple assay that provides information about several different aspects of clotting.

Based on the variances observed, I found that lag time in the TF assay was more reproducible compared with the thrombin assay. However, when measuring LT_{50}, the opposite was true, with higher variance seen in the TF assay. This suggests that each assay may be useful for examining different aspects of clotting, with the TF assay more useful for clot formation (lag time) and the thrombin assay potentially more reproducible for clot lysis (LT_{50}). Together, they form a broad assessment of fibrin clotting dynamics.

Chapter 4: Relationships between fibrin clot properties, clinical characteristics, and other biomarkers in patients with atrial fibrillation

4.1 Background

In patients with atrial fibrillation (AF), stroke is a devastating complication that remains common despite contemporary treatment. As discussed previously, risk stratification methods for stroke in AF, such as CHA_2DS_2-VASc, use a patient's history and comorbidity to determine their stroke risk, which can be used to make decisions about antithrombotic treatment.[85] Other risk scores, such as HAS-BLED, can estimate a person's risk of bleeding.[205] Unfortunately, risk factors common to both scores, such as hypertension, age and history of stroke, mean individuals with an elevated CHA_2DS_2-VASc stroke risk score often have an elevated bleeding risk, so it remains challenging to identify patients who will benefit, or be harmed, by anticoagulant treatment.

We sought to examine the relationship between fibrin clot properties and baseline clinical characteristics to help explain how these demographic features might contribute to thrombotic and/or bleeding complications and explore whether more detailed phenotyping of patients in the future might be useful in guiding anticoagulant dosing regimens

For these analyses, I used the randomisation (non-anticoagulated) cohort, with results from the thrombin assay (n=1,840) and attempted replication in the tissue factor (TF) assay (n=1,891).

4.2 Results

4.2.1 Age of participants

No differences in age were seen across the quantiles for lag time or peak turbidity, but mean age was significantly lower in the highest quantile of LT_{50} (Table 4-1). In keeping with results from the thrombin assay, there was no apparent effect of age on lag time in the TF assay, but age decreased across quantiles of LT_{50}. I observed a subtle trend for an increase in age across quantiles of peak turbidity (p=0.049).

Table 4-1 – Age of participants across quantiles of measured fibrin clot biomarker

Thrombin assay	N	Q1	Q2	Q3	Q4	p-value
Lag time (s)	1840	70.0 (62.0 – 75.0)	69.5 (62.0 – 75.0)	68.0 (61.0 – 75.0)	69.0 (62.0 – 75.0)	0.39
Peak turbidity (AU)		69.0 (61.0 – 75.0)	68.0 (61.0 – 74.0)	69.0 (62.0 – 74.0)	70.0 (62.0 – 75.0)	0.29
LT_{50} (s)		70.5 (63.0 – 77.0)	69.0 (62.0 – 74.0)	70.0 (62.5 – 76.0)	66.0 (60.0 – 72.0)	< 0.001
Tissue factor assay	**N**	**Q1**	**Q2**	**Q3**		**p-value**
Lag time (s)	1891	69.0 (61.0 – 74.0)	69.0 (61.0 – 75.0)	70.0 (62.0 – 76.0)		0.44
Peak turbidity (AU)		68.0 (60.0 – 75.0)	69.0 (62.0 – 75.0)	70.0 (63.0 – 75.0)		0.049
LT_{50} (s)		71.0 (64.0 – 76.0)	69.5 (62.0 – 75.0)	67.0 (60.0 – 73.0)		< 0.001

AU, absorbance units. LT_{50}, time to 50% clot lysis. m (a - b) represents median (Q1 - Q3). p values calculated using Kruskal-Wallis one-way analysis of variance.

4.2.2 Sex

Of the 1,840 participants in the non-treatment cohort (thrombin assay), 1,149 (62.4 %) were male. Male sex was more prevalent in lower quantiles of LT_{50} in both the thrombin and TF assays (68.5 % vs 52.5 %, p<0.001 and 69.4 % vs 56.2 %, p<0.001 respectively, Table 4-2). Similarly, male sex was more common in the lower quantiles of peak turbidity in both assays with a prevalence of 66.2 % in Q1 compared with 58.7 % in Q4 (p=0.037) in the thrombin assay and 67.7 % in Q1 compared with 57.5 % in Q3 (p<0.001) in the TF assay.

Table 4-2 – Prevalence of male sex across quantiles of measured fibrin clot biomarker, thrombin assay

Thrombin assay	N	Q1	Q2	Q3	Q4	p-value
Lag time (s)	1840	268 (58.1%)	307 (66.5%)	300 (65.4%)	274 (59.8%)	0.020
Peak turbidity (AU)		305 (66.2%)	300 (65.2%)	274 (59.7%)	270 (58.7%)	0.037
LT_{50} (s)		319 (68.5%)	326 (70.9%)	263 (57.8%)	241 (52.5%)	< 0.001
Tissue factor assay	**N**	**Q1**	**Q2**	**Q3**		**p-value**
Lag time (s)	1891	403 (63.6%)	412 (65.3%)	370 (59.1%)		0.066
Peak turbidity (AU)		427 (67.7%)	396 (62.9%)	362 (57.5%)		< 0.001
LT_{50} (s)		438 (69.4%)	393 (62.4%)	354 (56.2%)		< 0.001

AU, absorbance units. LT_{50}, time to 50% clot lysis. p values calculated using Fisher's exact test.

4.2.3 Diabetes mellitus, body mass index and weight

An overall prevalence of 23.0 % for diabetes mellitus (DM) was seen at randomisation. The median (IQR) body mass index (BMI) was 28.5 (25.2-32.3) kg/m^2, while the median body weight was 81.2 (70.0 – 94.0) kg.

The prevalence of DM increased across lysis time (LT_{50}) quantiles with 20.4 % of Q1 having diabetes at randomisation, compared with 29.2 % in Q4 (p<0.001, Table 4-3). This finding was mirrored in the TF assay, where DM was significantly more prevalent (28.4%) in the highest than lowest (18.9%) quantile of LT_{50} (p<0.001). For lag time, DM was seen more frequently (25.1 %) in the highest quantile in the TF assay, than in lower quantiles (Q1, 18.6 %, p=0.008) and the highest quantile of peak turbidity (Q1 23.8 % vs Q3 25.1 %) but these findings were not mirrored in the thrombin assay.

Like the trends for DM, BMI increased across quantiles for peak turbidity and LT_{50} in both the thrombin and TF assays. For example, median (IQR) BMI was 31.1 (27.7-35.2) kg/m^2 in Q4 compared with 26.6 kg/m^2 in Q1 (thrombin assay, p<0.001, Table 4-4). BMI was also highest in the highest quantiles of peak turbidity, but no difference was observed across lag time quantiles with either assay.

Body weight was analysed separately to BMI, though, as expected, trends were similar. Results for these observations are seen in tables 8-1 to 8-3 in appendix 8.4.

Table 4-3 – Prevalence of diabetes mellitus across quantiles of measured fibrin clot biomarker

Thrombin assay	N	Q1	Q2	Q3	Q4	p-value
Lag time (s)	1840	102 (22.1%)	96 (20.8%)	104 (22.7%)	122 (26.6%)	0.18
Peak turbidity (AU)		102 (22.1%)	96 (20.9%)	106 (23.1%)	120 (26.1%)	0.28
LT_{50} (s)		95 (20.4%)	85 (18.5%)	110 (24.2%)	134 (29.2%)	< 0.001
Tissue factor assay	N	Q1	Q2	Q3		p-value
Lag time (s)	1891	118 (18.6%)	156 (24.7%)	157 (25.1%)		0.008
Peak turbidity (AU)		150 (23.8%)	123 (19.5%)	158 (25.1%)		0.046
LT_{50} (s)		119 (18.9%)	133 (21.1%)	179 (28.4%)		< 0.001

AU, absorbance units. LT_{50}, time to 50% clot lysis. p values calculated using Fisher's exact test.

4.2.4 Type of AF

Inclusion in the trial required a diagnosis of AF, which can be either paroxysmal (PAF) or non-paroxysmal (nPAF, an umbrella term containing persistent and permanent forms). Overall, 18.9 % of individuals had paroxysmal AF with the remaining 81.1% therefore having nPAF. Frequencies are given for PAF.

Table 4-4 – Body Mass Index (BMI) across quantiles of measured fibrin clot biomarker

Thrombin assay	N	Q1	Q2	Q3	Q4	p-value
Lag time (s)	1831	28.2 (24.8 – 32.4)	28.3 (25.5 – 32.0)	28.4 (25.2 – 32.4)	28.9 (25.4 – 32.4)	0.59
Peak turbidity (AU)		28.0 (24.7 – 31.2)	28.1 (25.5 – 31.6)	28.7 (25.2 – 32.8)	29.2 (25.2 – 33.2)	0.003
LT_{50} (s)		26.6 (23.4 – 30.0)	27.8 (24.8 – 31.2)	28.7 (25.8 – 32.6)	31.1 (27.7 – 35.2)	< 0.001
Tissue factor assay	**N**	**Q1**	**Q2**	**Q3**		**p-value**
Lag time (s)	1882	28.1 (25.0 – 32.2)	28.4 (24.9 – 32.0)	28.9 (25.8 – 32.4)		0.066
Peak turbidity (AU)		28.1 (25.4 – 31.6)	28.4 (25.0 – 31.9)	29.0 (25.2 – 32.9)		0.020
LT_{50} (s)		27.4 (24.1 – 30.9)	28.1 (25.0 – 31.6)	30.2 (26.8 – 33.9)		< 0.001

AU, absorbance units. LT_{50}, time to 50% clot lysis. m (a - b) represents median (Q1 - Q3). p values calculated using Kruskal-Wallis one-way analysis of variance.

PAF was significantly more common in Q4 of LT_{50} compared with Q1 in the thrombin assay (24.4 % vs 11.6 %, p<0.001) but this was not replicated using TF (20.0% in Q3 vs 17.1% in Q1, p=0.36, (Table 4-5). Similarly, PAF was more common in the highest quantile of lag time in the thrombin assay (22.1 % compared with 18.2 % in Q1, p=0.028) but this was not observed using the TF assay (21.2 % vs 18.5 %, p=0.17).

Table 4-5 – Prevalence of paroxysmal AF across quantiles of measured fibrin clot biomarker

Thrombin assay	N	Q1	Q2	Q3	Q4	p-value
Lag time (s)	1840	84 (18.2%)	68 (14.7%)	93 (20.3%)	101 (22.1%)	0.028
Peak turbidity (AU)		90 (19.5%)	92 (20.0%)	81 (17.6%)	83 (18.0%)	0.76
LT_{50} (s)		54 (11.6%)	76 (16.5%)	104 (22.9%)	112 (24.4%)	< 0.001
Tissue factor assay	**N**	**Q1**	**Q2**	**Q3**		**p-value**
Lag time (s)	1891	117 (18.5%)	108 (17.1%)	133 (21.2%)		0.17
Peak turbidity (AU)		120 (19.0%)	129 (20.5%)	109 (17.3%)		0.36
LT_{50} (s)		108 (17.1%)	124 (19.7%)	126 (20.0%)		0.36

AU, absorbance units. LT_{50}, time to 50% clot lysis. p values calculated using Fisher's exact test.

4.2.5 Reduced left ventricular ejection fraction

Left ventricular function is measured by its ejection fraction (LVEF). Typically, ≥ 55% would be considered normal (depending on imaging modality used); LVEF of ≤ 40% represents moderate or severe left ventricular impairment.

Reduced left ventricular ejection fraction (rEF) was seen less commonly in the highest quantile of LT_{50} in both the thrombin assay (13.1 % vs 18.9 %, p=0.007) and the TF assay (11.9 % vs

18.5 %, p=0.004, Table 4-6). Those in the highest quantile of TF lag time had a greater frequency of rEF (18.2 % in Q3 vs 13.1 % in Q1, p=0.017) but this difference was not seen in the thrombin experiments, nor were other associations between fibrin clot variables and rEF seen.

Table 4-6 – Prevalence of reduced LVEF across quantiles of measured fibrin clot biomarker

Thrombin assay	N	Q1	Q2	Q3	Q4	p-value
Lag time (s)	1840	70 (15.2%)	54 (11.7%)	76 (16.6%)	72 (15.7%)	0.16
Peak turbidity (AU)		70 (15.2%)	65 (14.1%)	58 (12.6%)	79 (17.2%)	0.27
LT_{50} (s)		88 (18.9%)	73 (15.9%)	51 (11.2%)	60 (13.1%)	0.007
Tissue factor assay	N	Q1	Q2	Q3		p-value
Lag time (s)	1891	83 (13.1%)	84 (13.3%)	114 (18.2%)		0.017
Peak turbidity (AU)		100 (15.8%)	79 (12.5%)	102 (16.2%)		0.13
LT_{50} (s)		117 (18.5%)	89 (14.1%)	75 (11.9%)		0.004

LVEF, left ventricular ejection fraction. AU, absorbance units. LT_{50}, time to 50% clot lysis. p values calculated using Fisher's exact test.

4.2.6 Renal function

Estimated glomerular filtration rate (eGFR) is calculated using the Cockcroft-Gault method, based on the serum creatinine, body weight, age, and sex of patients.

Median eGFR was highest in the higher quantiles of LT_{50} in both the thrombin assay (83.6 ml/min in Q4 compared with 68.9 ml/min in Q1, p<0.001) and TF assay (81.5 ml/min in Q1 compared with 71.0 ml/min in Q4, p<0.001, Table 4-7). Patients in higher quantiles of lag time had lower eGFR in the TF assay (72.8 ml/min in Q3 compared with 77.2 ml/min in Q1, p=0.006) but this was not observed using the thrombin assay, and no other associations between fibrin clot biomarker and renal function were seen.

Table 4-7 – eGFR across quantiles of measured fibrin clot biomarker

Thrombin assay	N	Q1	Q2	Q3	Q4	p-value
Lag time (s)	1833	73.1 (56.6 – 94.0)	75.4 (59.3 – 93.2)	77.1 (59.6 – 98.1)	75.2 (57.9 – 94.0)	0.37
Peak turbidity (AU)		76.2 (59.8 – 95.1)	76.5 (59.3 – 96.0)	75.0 (58.2 – 93.7)	74.2 (56.8 – 94.0)	0.61
LT_{50} (s)		68.9 (53.8 – 89.2)	76.2 (59.8 – 92.7)	73.3 (55.1 – 91.5)	83.6 (66.6– 107.7)	< 0.001
Tissue factor assay	N	Q1	Q2	Q3		p-value
Lag time (s)	1884	77.2 (62.1 – 97.4)	75.5 (58.2 – 95.6)	72.8 (55.5 – 91.8)		0.006
Peak turbidity (AU)		77.0 (60.0 – 95.9)	75.5 (59.3 – 93.4)	73.6 (56.8 – 95.3)		0.31
LT_{50} (s)		71.0 (55.6 – 90.1)	73.9 (56.8 – 92.5)	81.5 (63.4 – 104.3)		< 0.001

eGFR, Estimated glomerular filtration rate, AU, absorbance units. LT_{50}, time to 50% clot lysis. m (a - b) represents median (Q1 - Q3). P values calculated using Kruskal-Wallis one-way analysis of variance.

4.2.7 Biomarkers

As with clinical characteristics, associations of fibrin clot markers from the non-treatment cohort were tested in their quantiles against other biomarkers. Similar trends were observed for the thrombin and TF assays, except that there was more heterogeneity in the lag time results observed with the TF assay. For clarity and to reduce the number of tables, only the TF results are shown here, with the thrombin results available in appendix Section 8.4.

Higher quantiles of lag time were significantly associated with higher CRP, IL-6, troponin T and GDF-15 (Table 4-8). The strongest association was observed for GDF-15 where the median (Q1-Q3) was 1447.0 ng/L (1011.0 – 2094.0) for Q3 of lag time compared with 1249.0 (918.0 – 1805.0) for Q1 ($p<0.001$).

Strong correlations were seen with increasing CRP, IL-6, leucocyte count, NT-pro-BNP, GDF-15, and D-dimer and increasing quantiles of peak turbidity, while the association between increasing cardiac troponin T and peak turbidity quantile was weaker but still nominally significant (Table 4-9). Relationships between fibrin clotting markers and other biomarkers are shown in Figure 4-1.

Table 4-8 – Associations of biomarkers with by quartiles of fibrin clot lag time (tissue factor assay, non-treatment cohort)

	N	Q1	Q2	Q3	P value
CRP (mg/L)	1873	2.0 (1.0 – 4.2)	2.1 (1.0 – 4.5)	2.6 (1.1 – 5.4)	*0.006*
IL-6 (ng/L)	1885	1.9 (1.2 – 2.9)	1.9 (1.3 – 3.3)	2.1 (1.3 – 3.5)	*0.008*
*Leucocytes (x10*9 c/L)*	1835	6.7 (5.5 – 8.0)	7.0 (5.8 – 8.2)	6.9 (5.9 – 8.3)	*0.053*
Troponin T (ng/l)	1872	10.5 (7.4 – 15.2)	10.5 (7.6 – 16.2)	11.6 (7.6 – 17.7)	*0.008*
NT-pro-BNP (ng/L)	1874	714.0 (322.0 – 1225.0)	648.0 (335.0 – 1228.8)	731.0 (330.0 – 1340.0)	*0.56*
GDF-15 (ng/L)	1857	1249.0 (918.0 – 1805.0)	1333.0 (965.0 – 2113.0)	1447.0 (1011.0 – 2094.0)	*< 0.001*
D-dimer (µg/L)	1885	574.0 (393.2 – 947.5)	584.5 (380.5 – 949.8)	559.0 (348.0 – 997.0)	*0.55*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Kruskal-Wallis one-way analysis of variance. CRP, C-reactive protein. IL-6, Interleukin-6. NT-pro-BNP, N-terminal pro-B-type natriuretic peptide. GDF-15, growth differentiation factor 15.

Table 4-9 – Associations of biomarkers with by quartiles of fibrin clot peak turbidity (tissue factor assay, non-treatment cohort)

	N	Q1	Q2	Q3	P value
CRP (mg/L)	1873	1.5 (0.7 – 3.1)	1.9 (0.9 – 3.7)	4.1 (1.9 – 9.1)	*< 0.001*
IL-6 (ng/L)	1885	1.6 (1.1 – 2.6)	1.7 (1.2 – 2.8)	2.6 (1.7 – 4.5)	*< 0.001*
*Leucocytes (x10*9 c/L)*	1835	6.5 (5.5 – 7.9)	6.8 (5.6 – 8.0)	7.2 (6.0 – 8.6)	*< 0.001*
Troponin T (ng/l)	1872	10.2 (7.1 – 16.1)	10.7 (7.6 – 15.6)	11.4 (7.9 – 16.5)	*0.031*
NT-pro-BNP (ng/L)	1874	671.0 (293.5 – 1240.5)	668.0 (336.0 – 1215.0)	750.0 (352.5 – 1348.5)	*0.051*
GDF-15 (ng/L)	1857	1283 (916.8 – 1904)	1268 (938 – 1850)	1441 (1028 – 2232)	*< 0.001*
D-dimer (µg/L)	1885	492.0 (317.8 – 879.0)	526.0 (360.0 – 843.0)	713.0 (455.0 – 1169.0)	*< 0.001*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Kruskal-Wallis one-way analysis of variance. CRP, C-reactive protein. IL-6, Interleukin-6. NT-pro-BNP, N-terminal pro-B-type natriuretic peptide. GDF-15, growth differentiation factor 15.

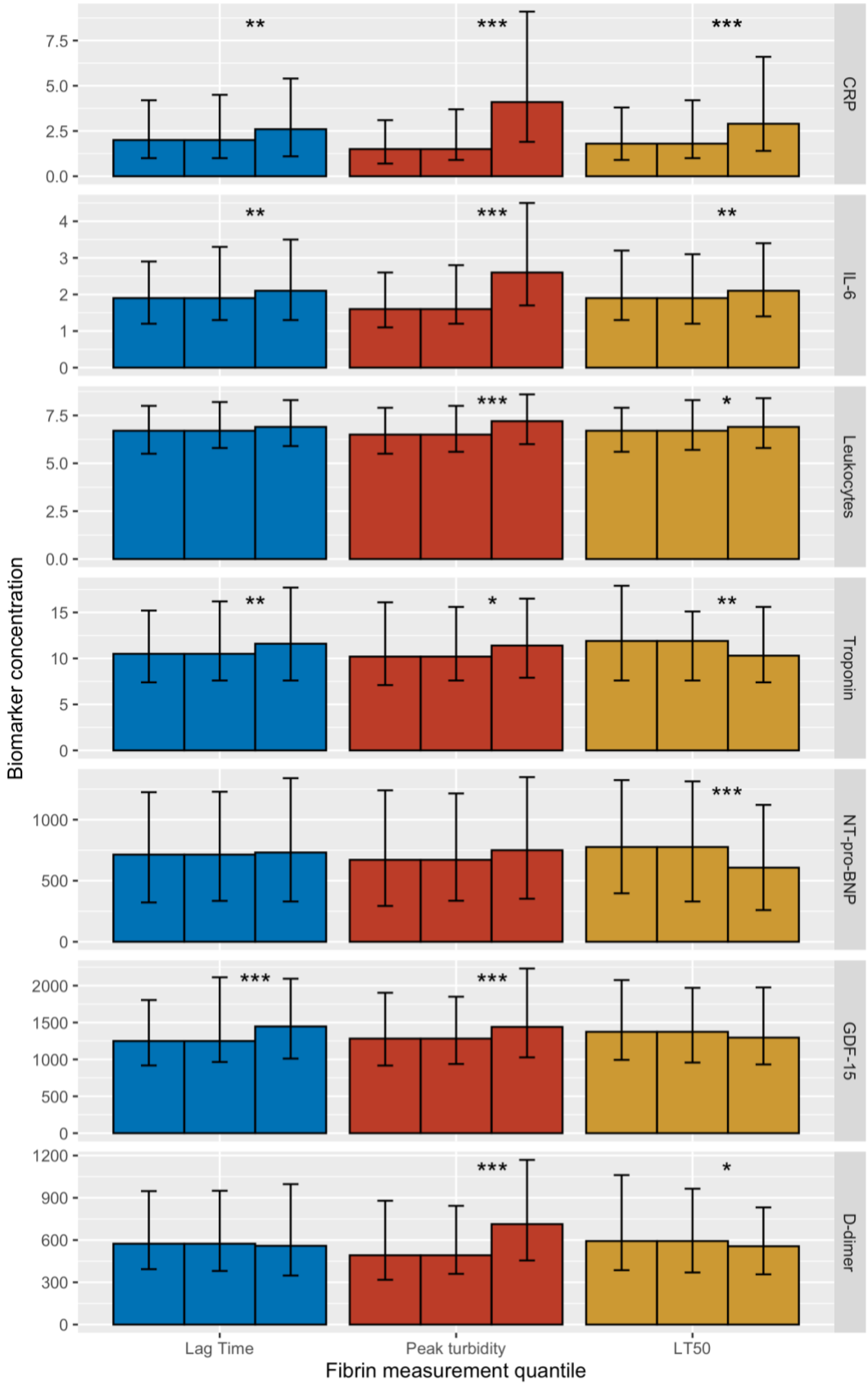


*Figure 4-1– Biomarker concentration in each quantile of fibrin clot parameter. Solid bars represent median biomarker values and bars represent the interquartile range of the biomarker values. Please see tables for units. P values calculated with Kruskal-Wallace one-way analysis of variance. LT50, time to 50% lysis. ***, p < 0.001. **, p < 0.01. *, p < 0.05.*

Prolonged LT_{50} was associated with increasing CRP and IL-6. However, troponin T, NT-pro-BNP, GDF-15, and D-dimer all decreased across the LT_{50} quantiles (Table 4-10). A weakly positive association was seen between leucocyte count and LT_{50}.

Table 4-10 – Associations of biomarkers with by quartiles of fibrin clot lysis time (tissue factor assay, non-treatment cohort)

	N	Q1	Q2	Q3	P value
CRP (mg/L)	1873	1.8 (0.9 – 3.8)	2.0 (1.0 – 4.2)	2.9 (1.4 – 6.6)	*< 0.001*
IL-6 (ng/L)	1885	1.9 (1.3 – 3.2)	1.8 (1.2 – 3.1)	2.1 (1.4 – 3.4)	*0.003*
*Leucocytes (x10*9 c/L)*	1835	6.7 (5.6 – 7.9)	7.0 (5.7 – 8.3)	6.9 (5.8 – 8.4)	*0.017*
Troponin T (ng/l)	1872	11.9 (7.6 – 17.9)	10.6 (7.6 – 15.1)	10.3 (7.4 – 15.6)	*0.009*
NT-pro-BNP (ng/L)	1874	776.0 (397.0 – 1324.0)	696.0 (330.0 – 1313.8)	607.0 (259.5 – 1121.0)	*< 0.001*
GDF-15 (ng/L)	1857	1374.0 (993.5 – 2075.5)	1308.5 (957.2 – 1970.5)	1295.5 (931.5 – 1976.2)	*0.17*
D-dimer (µg/L)	1885	593.0 (386.0 – 1061.0)	585.0 (369.8 – 964.5)	556.0 (356.5 – 831.8)	*0.036*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Kruskal-Wallis one-way analysis of variance. CRP, C-reactive protein. IL-6, Interleukin-6. NT-pro-BNP, N-terminal pro-B-type natriuretic peptide. GDF-15, growth differentiation factor 15.

4.3 Summary

4.3.1 Associations with lag time

Fibrin clot lag time represents the time taken for detectable clot to form after the addition of thrombin or TF. During this time, thrombin induces polymerisation of fibrin protofibrils, but these polymers are not yet long enough to aggregate laterally so remain undetectable by absorptiometry methods. Fibrin lag time has been shown to be a useful *in vitro* and *in vivo* marker of anticoagulant effect.[327] However, while studies measuring the partly-analogous thrombin generation lag time have demonstrated that this correlates with coagulation factor levels (factors VII, IX and Protein S), few studies have shown associations between clinical conditions and either fibrin clot lag time or thrombin generation lag time.[328]

We saw strong associations for a higher prevalence of DM and lower eGFR in higher quantiles of lag time in the TF assay. For DM, a similar (but non-significant) trend across lag time quantiles was also observed with the thrombin assay.

Using the TF assay, I also observed more rEF in patients with longer lag time, while, with the thrombin assay, a relatively weak association between prolonged fibrin clot lag time and PAF was observed. However, these findings were not replicated in the parallel assay, so it is difficult to draw firm conclusions based on this.

4.3.2 Associations with peak turbidity

Mechanistically, peak turbidity correlates with fibrin fibre diameter in clotted samples.[327] However, the main determinant of this appears to be fibrinogen concentration, and peak absorbance correlates better with fibrinogen levels than with fibre diameter.[325] Fibrinogen levels have been correlated with cardiovascular diseases, including stroke and myocardial infarction, while risk factors for elevated fibrinogen include age, female sex, obesity and smoking.[329,330] Female sex was more prevalent in higher quantiles of peak turbidity in both the thrombin and TF assays. This association has previously been described in patients with CAD both with and without DM and is in keeping with higher fibrinogen in females.[331]I observed a subtle difference in age across peak turbidity quantiles in the TF assay only. The observed effect was small and was not observed using the thrombin assay, although is in keeping with published data on fibrinogen levels increasing with age.[330] Given the strong association between plasma fibrinogen concentration and plasma fibrin clot peak turbidity, it is unsurprising that my observations follow risk factors for hyperfibrinogenaemia.

Higher BMI was observed in the higher quantiles of peak turbidity in both the thrombin and TF assays, but associations with DM were weaker (also being more prevalent in higher quantiles of peak turbidity, but in only the TF assay). Even in the context of a strong correlation between obesity and DM, this observation suggests that BMI may influence fibrin clot dynamics beyond that caused by impaired glucose control.

Peak turbidity correlates with inflammatory states and increases with elevated CRP.[150,325] Chronic elevation of fibrinogen is seen in inflammatory diseases, and fibrinogen itself appears to modulate the inflammatory response.[36,332] My results for CRP, IL-6 and GDF-15 were in keeping with this, suggesting that inflammation is a key determinant of peak turbidity.

4.3.3 Associations with LT_{50}

In vivo clotting and fibrinolysis exist in a dynamic equilibrium with prothrombotic and fibrinolytic factors working to maintain haemostasis in response to injury on the one hand, and blood vessel patency on the other. If this equilibrium is disturbed by an excess of fibrinolytic activity, bleeding is more likely while the opposite can cause pathological clotting. In clots, high local concentrations of the anti-fibrinolytic agents plasminogen activator inhibitor-1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI) prevent fibrinolysis, while factor XIII forms cross-links in the fibrin network, increasing resistance to lysis.[270] In my assay, exogenous tPA is responsible for activation of plasminogen by forming ternary fibrin-tPA-plasminogen compounds, generating plasmin that in turn degrades fibrin.[333]

Lysis time (LT_{50}) measures the time taken for clots to break down and is prolonged when dense clots are resistant to lysis. Lysis resistance is defined not only by fibre width in the established clot (itself measured by peak turbidity) but also by other metabolic and biochemical conditions, for example the degree of cross-linking inhibiting plasminogen-fibrin interactions.

We saw strong associations with higher quantiles of LT_{50} in both the thrombin and TF assays for female sex, DM, and BMI. Reduced age and increased eGFR were also associated with higher LT_{50} quantiles, while rEF was less common in higher quantiles of LT_{50}. An association with PAF and increasing quantiles of LT_{50} was observed, but only in the thrombin assay.

4.4 Discussion

4.4.1 Differences between results from tissue factor and thrombin assays

We observed some differences between results from the thrombin assay and those from the TF assay. Whilst some of the variance may be due to random chance compounded by the multiple comparisons used, differential effects of thrombin and TF in these assays are mechanistically likely, particularly for lag time (which is strongly dependent on the reagent used to induce clotting). Comparing the TF assay to the thrombin assay, several additional upstream steps such as cofactor activation are required for thrombin generation to occur with TF, so heterogeneity in components of the coagulation system that occur upstream of

thrombin generation may only be elucidated by the TF assay. Therefore, TF-based assays potentially have greater power to identify differences in lag time between participants or groups.[264] In keeping with this, I found that I could identify differences in clinical and biochemical parameters more robustly across the lag time quantiles with TF than with thrombin.

The initiating agent may also affect measurement of clot lysis. Differential effects based on reagents used have previously been seen elsewhere, with Ilich *et al* proposing reagent choices dependent on whether attempting to identify hyper- or hypofibrinolysis.[270] Imperfect correlation between LT_{50} results from TF and thrombin assays were observed suggesting there may be observable differences between the two assays used (see Chapter 3). With TF-induced clotting, most of the thrombin generation in fact occurs after the peak turbidity is achieved.[334] This ongoing thrombin generation in TF assays, compared with exogenous thrombin in the thrombin assay, is one potential mechanism of differences in LT_{50} trends observed using the two assays. Given the absence of a 'gold standard' test, I was unable to determine whether either assay was more accurate in my dataset. Nevertheless, I found greater strength of association between clinical factors and LT_{50} using the thrombin assay than with the TF assay, perhaps because LT_{50} with thrombin is measuring a less-complex series of reactions with less potential for heterogeneity that may cloud associations. This suggests lysis time may be best investigated using thrombin rather than TF.

4.4.2 Condition-specific considerations

Age

We observed a lower mean age in higher quantiles of LT_{50} in both the thrombin and TF assays, suggesting a negative association between age and LT_{50} in trial participants, although the absolute difference in age was small. A potential explanation for this is that those who develop AF at a comparatively young age are more likely to have adverse metabolic and clotting profiles. This seems plausible if conditions that associate with cardiovascular risk and adverse clotting profiles (e.g., DM, increased BMI) also cause AF onset at a younger age.[335] Whether these individuals should be treated more aggressively with anti-thrombotic medication is a potential future avenue to explore. Alternatively, since the ARISTOTLE trial

included ‘age ≥ 75 years’ as one of the clinical risk inclusion criteria, older patients may be less likely to have other risk factors such as DM that also served as inclusion criteria.

Sex

Female sex is an established stroke risk factor in AF and forms part of the CHA_2DS_2-VASc risk score.[205] However, the mechanism behind this increased risk is not known. Proposed mechanisms include anatomical factors, hormonal differences and variance in treatment uptake or compliance.[336] Alternatively, since a higher burden of inflammation is seen in women (higher CRP is seen in women compared to men), higher stroke rates may be due to inflammatory processes, though this is complex since development of AF itself is associated with inflammation.[337,338]

Here, I have shown that female sex is associated with higher peak turbidity and LT_{50}. Taken together, these suggest that female patients with AF form denser fibrin clots that are more resistant to lysis. Higher peak turbidity, as discussed, may be due to elevated fibrinogen seen in females. Prolonged lysis time in women compared with men has been shown previously in healthy populations and those with CAD,[150,339] while delayed fibrin clot lysis in women (albeit measured using a different technique) has been described in a small AF cohort.[340]

The reasons underlying the prolongation of LT_{50} and peak turbidity in females in my cohort were not examined. Although I saw a strong link between adverse fibrin clotting parameters and female sex that could plausibly explain some of the sex differences in AF stroke rates, I did not observe a strong correlation between these parameters and ischaemic events in this cohort (see Chapter 5). These relationships between sex and fibrin parameters are noteworthy but further work is required to elucidate the mechanisms behind these observations and whether these explain the excess stroke risk in women with AF.

Diabetes mellitus, body mass index and weight

DM is a component of the CHA_2DS_2-VASc scoring and is a risk factor for stroke in AF. Significant associations were observed with DM and prolonged fibrin clot lysis time in both the thrombin and TF assays. Denser fibrin networks have been observed in patients with DM, with a greater

quantity of thin fibrin fibres within the fibrin mesh.[341] Hypofibrinolysis has been observed in several cohorts previously including a small cohort of patients with co-existing DM and AF,[326,342] and predicts poor outcomes in patients with DM following ACS.[343]

DM is a procoagulant condition and therefore might be expected to shorten lag time, and this has been observed in cohort studies comparing patients with DM and healthy controls.[344] However, others have described a prolongation of lag time in patients with DM compared to propensity-matched healthy volunteers.[345] In each of these studies, TF was used as the reagent, though differential effects on lag time have been observed depending on TF concentration.[346] My findings suggest that DM is associated with prolonged lag time, at least with the TF concentration studied.

The major risk factor for DM is obesity, so it is likely that fibrin clot properties for these two factors are strongly correlated. No association was seen for lag time across quantiles of BMI or weight, though BMI was higher in the highest quantiles of peak turbidity and LT_{50}. Both (non-DM) overweight and patients with DM are characterised by a pro-inflammatory, pro-thrombotic state that predisposes to atherothrombotic complications. The observations for BMI were similar to those seen for diabetes; this is not surprising since it is likely that there is substantial overlap between raised BMI and the presence of diabetes in individuals. Moreover, since the mechanism for prolonged clot lysis in patients with diabetes is, whilst not fully understood, likely multifactorial, it follows that some hypofibrinolytic changes seen in fibrin clot structure in patients with DM will also be observed in non-DM overweight patients.

Our findings add to the weight of evidence suggesting that hypofibrinolysis may be particularly important in patients with DM and overweight patients and may be an additional therapeutic target.[342]

Type of AF

Atrial fibrillation is, superficially, a disturbance of heart rhythm. Previously it was thought that stasis of blood within the left atrial appendage was responsible for the risk of stroke. Understanding of the pathophysiology of stroke in AF has progressed substantially with some

characterising AF as a chronic progressive condition with atrial wall fibrosis where the dysrhythmia is merely the observable endpoint.[14] In this model, AF is a marker of fibrotic cardiovascular disease and stroke associations may be, at least partly, due to upstream vascular risk factors common to both stroke and atrial disease. This is supported by the finding that strokes in patients with paroxysmal AF often do not follow arrhythmic events.[347]

Risk factors for the development of AF include modifiable factors such as obesity, diabetes and smoking, and non-modifiable risk factors including age, sex, race and specific genetic loci.[348,349] Whilst AF is associated with sedentary lifestyles, paradoxically those who exercise to extremes are also at risk of developing AF, often without any other traditional risk factors.[350] This suggests different pathophysiological mechanisms may be responsible for AF in different populations. Some people may have an isolated form of atrial disease with dysrhythmia, while others develop the dysrhythmia because of wider metabolic derangement and fibrosis within the cardiovascular system.

AF can be classified as persistent or paroxysmal, based on whether a patient experiences AF-free periods. PAF often becomes persistent over time and, ultimately, persistent AF can become permanent. PAF may represent people at the less severe end of the disease spectrum, though the relationship between atrial fibrosis and AF burden is disputed.[351,352] Nevertheless, other studies have shown that the duration of AF predicts stroke risk independently of $CHADS_2$ score, suggesting that the type of AF (in the present study classified as either paroxysmal or non-paroxysmal) could be a marker of thrombotic risk.[95]

Our findings regarding the type of AF are surprising in the context of AF as a degenerative disease, since I observed more of the paroxysmal form of AF in those with hypofibrinolysis (prolonged LT_{50}). In this context, it is plausible that cardiovascular risk factors such as diabetes and obesity may cause a milder form of atrial disease with a lower dysrhythmic burden (i.e., PAF rather than nPAF). This may explain the apparently paradoxical relationship between weight and survival observed in this cohort of patients with AF.[353]

Reduced left ventricular ejection fraction

Previous studies using similar methods have observed shorter lag times and similar LT_{50} in patients with chronic heart failure compared with controls.[145] In contrast, I observed less rEF in those in higher quantiles of LT_{50}. This is, superficially, an unexpected finding, since DM, CAD and renal disease are all associated with both hypercoagulability and, separately, rEF.[354] One possible explanation for these findings is the circulating blood volume expansion seen with heart failure may reduce the concentration of clotting proteins in these samples, meaning that clots were slower to form and more readily lysed by a fixed concentration of tPA. Other possibilities include confounding from other conditions in this univariate analysis, perhaps on the basis of the inclusion criteria or medication differences.

Renal function

Renal impairment is common in patients with cardiovascular disease and is associated with an increased risk of cardiovascular death and all-cause mortality.[355] The interaction between renal disease and cardiovascular disease is complex due to shared risk factors and interactive pathophysiology in the cardio-renal syndrome.[356] Chronic kidney disease (eGFR< 60ml/min) increases bleeding risk approximately 1.5-fold but also increases arterial and venous thrombotic risk, complicating treatment decisions in these high-risk patients.[357] Kidney disease is an established risk factor for stroke and, as discussed in Chapter 1, the predictive value of the $CHADS_2$ risk system can be improved by the addition of a renal function risk factor.[125]

Higher levels of fibrinogen and prolonged lysis time have been observed in patients with kidney disease compared with controls.[358] In patients with end-stage kidney disease, chronic activation of clotting pathways leads to elevated levels of prothrombin fragments and decreased levels of the anticoagulant proteins C and S.[359] Thrombosis in patients with chronic kidney disease is thought to be substantially due to endothelial dysfunction and cellular dysfunction,[360] neither of which my methods would accurately characterise. Additionally, patients with severe forms of renal disease (serum creatinine > 2.5 mg/dL or 221 µmol/L) were excluded from ARISTOTLE so the study population population includes patients with modest renal dysfunction only. Therefore, factors that affect thrombotic risk in severe

renal impairment may not be applicable to this cohort. Accordingly, strong associations between renal dysfunction and fibrin clot properties were not expected.

However, I observed higher eGFR in the higher quantiles of LT_{50} (both thrombin and TF assays) and lower lag time quantiles (TF assay only) so, in my cohort, patients with reduced renal function and AF appeared to have greater clot lysability and delayed thrombus formation.

Biomarkers

The CRP and IL-6 markers of inflammation were both strongly associated with higher quantiles of lag time, peak turbidity and LT_{50}, while leucocyte count showed a similar trend with reduced statistical significance, suggesting that inflammation is associated with dense clots that are resistant to lysis in patients with AF. Inflammation appears to be a powerful modulator of cardiovascular risk, with inflammation associated with increased cardiovascular mortality.[361] Among patients with AF, higher levels of CRP and IL-6 correlate with prothrombotic markers during echocardiography,[71] while the addition of CRP to the $CHADS_2$ score modestly improved its predictive capability.[362] Anti-inflammatory agents reduce cardiovascular events in high risk individuals with CAD,[31,363] and further work is underway to determine whether similar treatments can reduce ischaemic stroke.[364]

GDF-15 is a biomarker associated with cardiac fibrosis, adverse remodelling, and heart failure, but its associations are not completely understood. In my experiments, increased GDF-15 was associated with longer lag times, higher peak turbidity but more rapid clot lysis, suggesting a more complex relationship with clotting than for inflammatory markers. These trends were in keeping with the fibrin clotting dynamics observed in patients with rEF. In previously-published analyses of the cohort, GDF-15 predicted bleeding and heart failure mortality.[156] My outcome analyses (discussed later in Chapter 5) demonstrate associations between lower LT_{50}. and bleeding events. These findings combined suggest that the elevated bleeding risk seen with GDF-15 may be at least partly explained by adverse fibrinolytic processes. However, it remains unclear whether GDF-15 is a marker or a driver of disease.[365]

4.4.3 Limitations

The ARISTOTLE trial included patients with a diagnosis of AF and at least one additional stroke risk factor. These included age > 75 years, prior stroke, heart failure, hypertension, and diabetes mellitus. Full inclusion criteria are described in Section 1.3. As a result of these criteria, risk factors may be dependent on each other, for example making diabetes more likely in those aged under 75. The result of this phenomenon may bias some of the associations between fibrin clot properties and demographic features. Since inclusion in the trial required only the presence of AF and one additional risk factor (e.g., hypertension, which is common), it is likely that many participants did not have multiple cardiovascular risk factors. The mean number of qualifying risk factors ($CHADS_2$ score) for participants was 2.1.[96,242], while individuals with no risk factors were not included. Accordingly, my results may not be generalisable to truly low risk populations with AF. However, the results are relevant for those patients with AF who require anticoagulation.

As already discussed, I would expect clustering of some risk factors such as DM and raised BMI. Conversely, some risk factors (such as older age and DM) may segregate by default due to the requirement for one or more risk factors for inclusion. In this univariate analysis, no adjustment was performed, meaning that these interactions between variables were not assessed.

Conditions such as DM, type of AF and LVEF were dichotomised, but this fails to consider the severity of each condition. For example, in DM, there is likely to be a spectrum of disease where those with good glycaemic control are at lower risk than those with chronically elevated glucose. In the case of DM, this could be examined using haemoglobin A1c (HbA1c) levels, though I did not have data for this.

This analysis used plasma samples taken from peripheral blood collections. Analysis of clotting using whole blood has some advantages but typically requires immediate analysis,[278] and the practicalities of storing and transportation of samples make whole blood analysis unworkable in large-scale trials. Moreover, there is often significant variability in results from whole blood assays.[366] Nevertheless, the cellular contribution to clotting is a potential source of heterogeneity that I was unable to examine with this experimental

design. Small studies have identified differences in blood obtained peripherally compared with that collected from the left atrium, suggesting that there may be an intracardiac prothrombotic state contributing to AF-related stroke risk.[367] This finding warrants further study that may reveal mechanistic insights into AF strokes, though such an approach is highly invasive and is not feasible for routine risk stratification.

4.5 Conclusions

In this large cohort of AF patients at risk of stroke, strong relationships between fibrin clot properties and clinical characteristics and biomarkers were observed. The strongest associations were observed for LT_{50} using the thrombin assay. Hypofibrinolysis was associated with female sex, decreasing age, increasing BMI, PAF, DM, and markers of inflammation such as CRP, IL-6, and leucocyte count. Conversely, rEF and impaired renal function were associated with faster clot lysis.

Higher peak turbidity (analogous to fibrin fibre width) was observed with increasing age, female sex, high BMI, and DM. Inflammation is a key determinant of peak turbidity, which was strongly associated with increased CRP, IL-6, and leucocyte count. GDF-15 had more complex relationships with clotting, with a longer lag time and rapid clot lysis, anticoagulant effects that may explain its relationship with bleeding outcomes, though also higher peak turbidity. Other factors that were associated with increased lag time were increased CRP, IL-6, increasing age, DM and lower eGFR.

Both the thrombin and TF assay provided valuable mechanistic insights into the clotting diatheses behind thrombosis and bleeding in AF. I observed some differences between the assays, with thrombin assays potentially more useful for measuring heterogeneity in clot lysis and TF appearing to be more useful for examining the early stages of clotting such as lag time.

Taken together, these results characterise the thrombotic tendencies of populations with AF. Inflammation appears to be associated with a prothrombotic state in these patients, which may predispose to either thrombosis or bleeding. High BMI and DM were associated with hypofibrinolysis, but only high BMI was strongly associated with higher peak turbidity,

suggesting that there may be differential effects of DM and BMI on fibrin clotting dynamics in patients with AF.

Chapter 5: Relationships between fibrin clot properties and thrombotic and bleeding outcomes in patients with atrial fibrillation

5.1 Background

Balancing the competing risks of thrombotic and bleeding complications is one of the primary challenges in the management of AF. As discussed in Chapter 1, whilst several scoring systems exist, they can predict events with only modest success. Paradoxically, despite these complications being the direct result of coagulation events, tests of clotting are not routinely used in clinical practice to assess the risk of bleeding or thrombosis in patients with AF.

We examined the relationship between fibrin clot properties and clinical outcomes to see whether a fibrin clotting assay could inform the risk stratification for patients with AF. For these analyses, I used the non-treatment cohort (see Section 2.1.3 for description), with results from both the thrombin assay (n=1,840) and replication in the tissue factor assay (n=1,891).

5.2 Results

5.2.1 Crude event rates and survival

A composite endpoint of CV death, MI, stroke, or systemic embolism was used. In the non-treatment cohort (thrombin assay, n=1,840), 86 instances of the composite endpoint were observed, at a rate of 2.36 %/year. Of these, 14 (0.38 %/year) were stroke/SE and 71 (1.92 %/year) were CV death. Due to low event rates of the thrombotic endpoints, further analyses used only the composite endpoint. There was a trend for fewer thrombotic events in participants with longer lag time, but this was not statistically significant (HR 0.48 (0.25 – 0.94)[†] for Q4 of lag time compared with Q1, p=0.052, Table 5-1). No other effect of fibrin clot biomarker on thrombotic outcomes was observed. Kaplan-Meier survival plots for thrombotic events against time for each fibrin clot biomarker are given in Figure 5-1.

[†] The HR presented is for Q4 compared with Q1. However, the P value represents the association with hazard and lag time across the complete range of lag time values. In this case, the relationship is not linear (Figure 5-3), resulting in a P value for the line of > 0.05, yet the 95% confidence interval comparing the two quartiles does not cross unity.

144 patients (4.28 %/year) met the ISTH major or clinically relevant non-major bleeding endpoint. There was a very strong association between lysis time (LT_{50}) and bleeding, with the cumulative risk of bleeding increasing progressively across the quantiles of LT_{50} (Q4 vs Q1 HR 0.33 (0.20-0.57), p=0.001, Table 5-2).

Table 5-1 – Rates of the composite thrombotic outcome by quantiles of fibrin clot biomarker

		Thrombosis		Unadjusted		Adjusted	
	n	n	Rate	HR	P	HR	P
Lag time							
174.5 – 529	461	26	2.91	REF		REF	
529 – 614.5	462	18	1.94	0.66 (0.36-1.21)		0.66 (0.36–1.21)	
614.5 – 729.5	459	29	3.21	1.09 (0.64-1.85)		1.10 (0.65–1.88)	
729.5 – 3874.5	458	13	1.42	0.48 (0.25-0.94)	0.052	0.50 (0.25–0.98)	0.060
Peak turbidity							
0.0195 – 0.24	461	21	2.32	REF		REF	
0.24 – 0.301	460	19	2.07	0.90 (0.48–1.67)		0.88 (0.47–1.64)	
0.301 – 0.37	459	24	2.63	1.15 (0.64–2.06)		1.07 (0.59–1.94)	
0.37 – 1.06	460	22	2.42	1.05 (0.58–1.91)	0.884	0.87 (0.45–1.71)	0.893
LT_{50}							
142.5 – 1972.5	466	27	3.02	REF		REF	
1972.5 – 2422.5	460	19	2.09	0.69 (0.38-1.24)		0.77 (0.42–1.39)	
2422.5 – 2940	455	21	2.3	0.76 (0.43-1.34)		0.94 (0.52–1.70)	
2940 – 11302.5	459	19	2.05	0.67 (0.37-1.21)	0.495	0.97 (0.50–1.88)	0.835
All							
All	1840	86	2.36				

Quantile range given in leftmost column. Crude event rate (%/year) and hazard ratios using Q1 used as reference value. LT_{50}, time to 50% clot lysis.

No associations were seen between peak turbidity or lag time and bleeding outcomes. Kaplan-Meier estimates for bleeding outcomes according to fibrin clot biomarker quantile (thrombin assay) are shown in Figure 5-2.

Table 5-2 – Rates of bleeding outcomes by quantiles of fibrin clot biomarker

		Bleeding		Unadjusted		Adjusted	
	n	n	Rate	HR	P	HR	P
Lag time							
174.5 – 529	461	38	4.51	REF		REF	
529 – 614.5	462	32	3.77	0.84 (0.52–1.34)		0.85 (0.53–1.36)	
614.5 – 729.5	459	33	4	0.88 (0.55–1.40)		0.95 (0.59–1.52)	
729.5 – 3874.5	458	41	4.85	1.07 (0.69–1.67)	0.697	1.25 (0.79–1.98)	0.417
Peak turbidity							
0.0195 – 0.24	461	31	3.63	REF		REF	
0.24 – 0.301	460	34	4.04	1.11 (0.68–1.81)		1.09 (0.67–1.78)	
0.301 – 0.37	459	35	4.18	1.15 (0.71–1.87)		1.08 (0.66–1.76)	
0.37 – 1.06	460	44	5.3	1.46 (0.92–2.31)	0.398	1.24 (0.74–2.07)	0.868
LT_{50}							
142.5 – 1972.5	466	51	6.31	REF		REF	
1972.5 – 2422.5	460	39	4.6	0.74 (0.49–1.12)		0.80 (0.52–1.22)	
2422.5 – 2940	455	36	4.31	0.69 (0.45–1.06)		0.80 (0.51–1.25)	
2940 – 11302.5	459	18	2.06	0.33 (0.20–0.57)	0.001	0.43 (0.24–0.77)	0.042
All							
All	1840	144	4.28				

Quantile range given in leftmost column. Crude event rate (%/year) and hazard ratios using unadjusted and adjusted models. Q1 used as reference value. LT_{50}, time to 50% clot lysis.

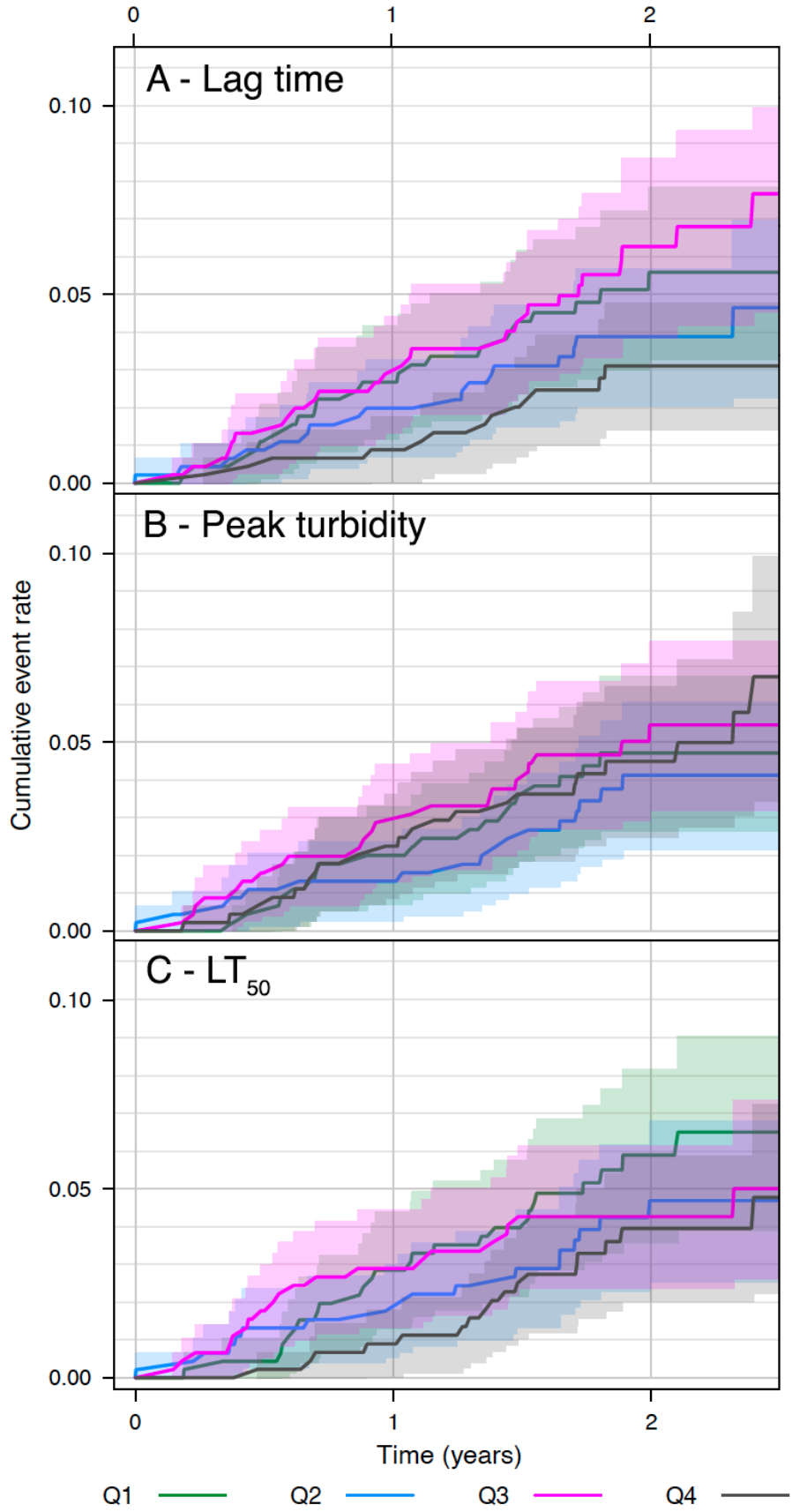


Figure 5-1 - Kaplan-Meier estimate of the cumulative event rate of CV death/MI/stroke/systemic embolism by biomarker quartile groups. Thrombin assay. LT_{50}, Time to 50% clot lysis.

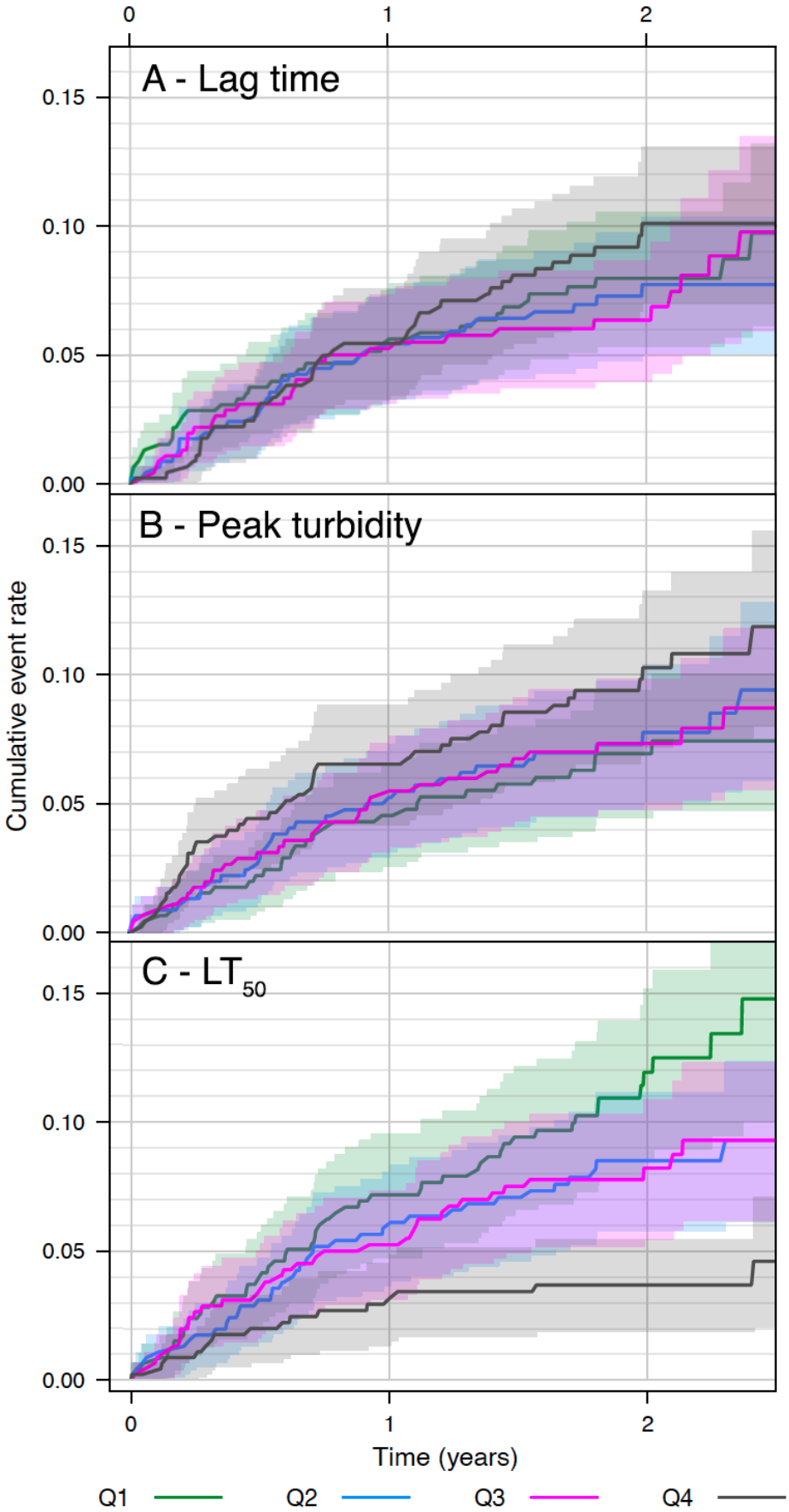


Figure 5-2 - Kaplan-Meier estimate of the cumulative event rate of bleeding by biomarker quartile groups. Thrombin assay. LT50, Time to 50% clot lysis.

5.2.2 Adjusted models

A Cox proportional hazards model was fitted, with adjustment for clinical risk factors (see Table 2-4 – Parameters used in multivariate analyses). Spline functions demonstrating the adjusted and unadjusted relationship between continuous fibrin clot variables and clinical outcomes were generated from the Cox models.

For lag time, a non-significant trend for lower thrombotic hazard was observed in the adjusted model (HR in Q4 relative to Q1 0.50 (0.25–0.98), p=0.06, Table 5-1), though no other associations were observed for the adjusted lag time model. Splines for the association with lag time and outcomes in the adjusted and unadjusted models are shown in Figure 5-3. For peak turbidity, no significant associations between the continuous fibrin variables and outcome were observed (Figure 5-4).

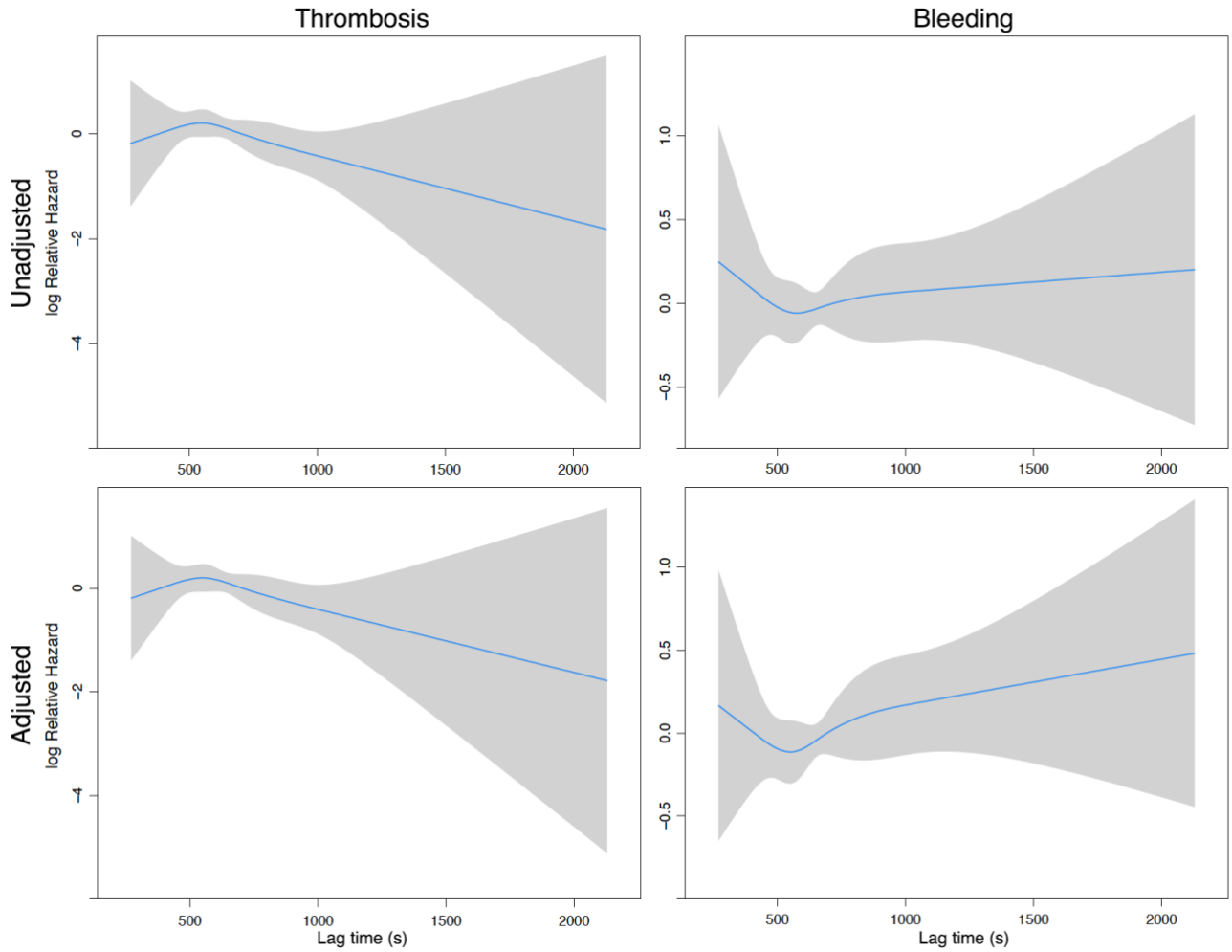


Figure 5-3 – Splines demonstrating the non-linear relationships between lag time biomarker and thrombotic and bleeding events in the unadjusted and adjusted analyses. Splines fitted using 4 knots.

The effect of LT_{50} on bleeding outcomes observed in the unadjusted analysis was attenuated when adjusted for the clinical risk factors but retained significance (Q4 vs Q1 HR 0.43 (0.24-0.77), p=0.042, Table 5-2).

The strength of association seen across the splines generated in Figures 5-3 to 5-5 can be tested statistically. For LT_{50}, the HR for the line was 0.53 (0.35-0.80[‡], p=0.001) for the unadjusted spline and 0.61 (0.39-0.94, p=0.057, Figure 5-5) for the adjusted spline. No other significant associations between fibrin clot variables and clinical outcomes were observed.

The correlations between fibrin parameters were also examined. A weak correlation was observed between LT_{50} and peak turbidity (Figure 5-6).

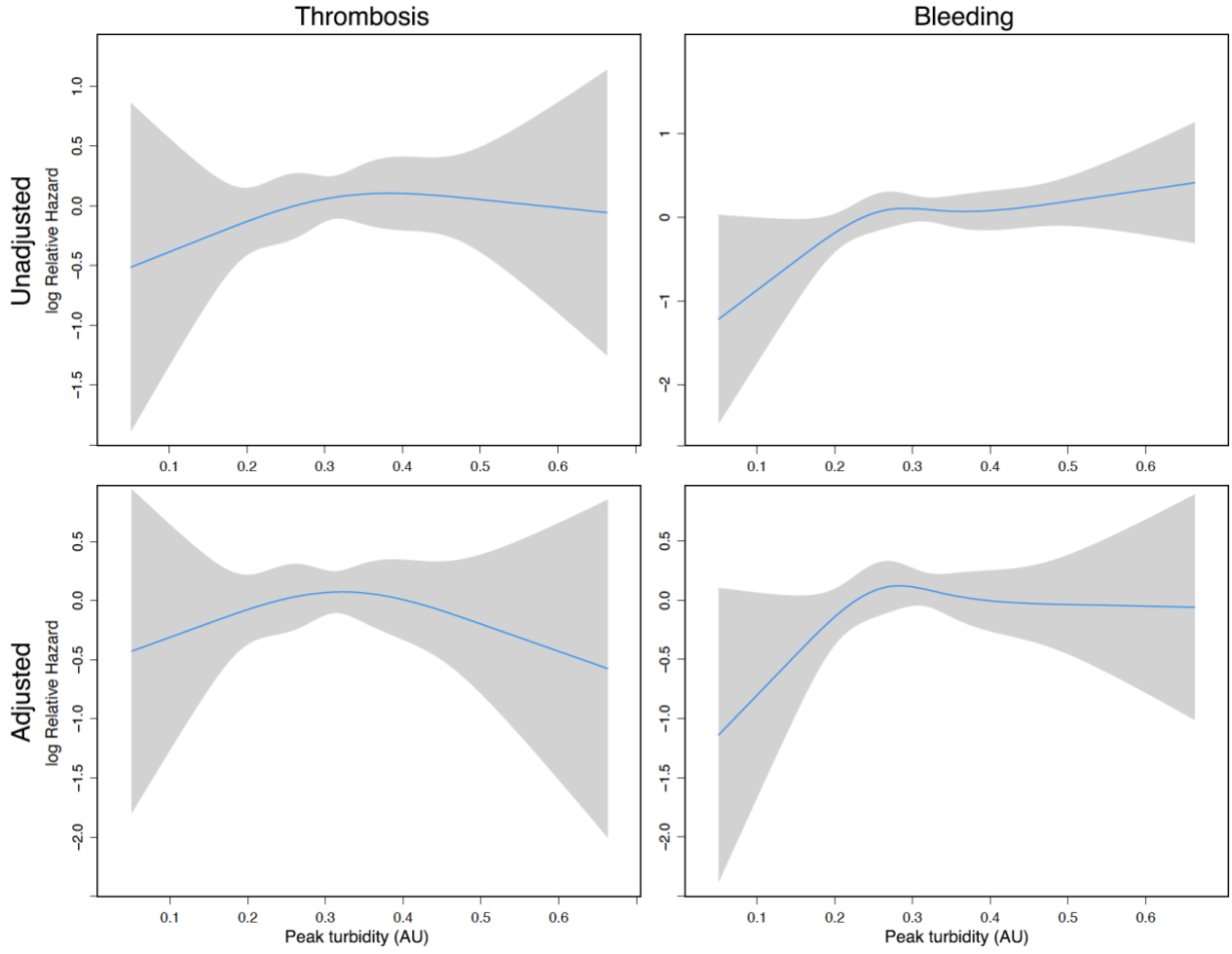


Figure 5-4 – Splines demonstrating the non-linear relationships between peak turbidity and thrombotic and bleeding events in the unadjusted and adjusted analyses. Splines fitted using 4 knots. AU, absorbance units.

[‡] This HR is demonstrative and represents the HR between the bounds of the interquartile range (i.e. the highest value in Q1 compared with the lowest value in Q4) whereas the P value assesses the strength of association of the line, essentially whether the line differs significantly from a horizontal line through 0.[368]

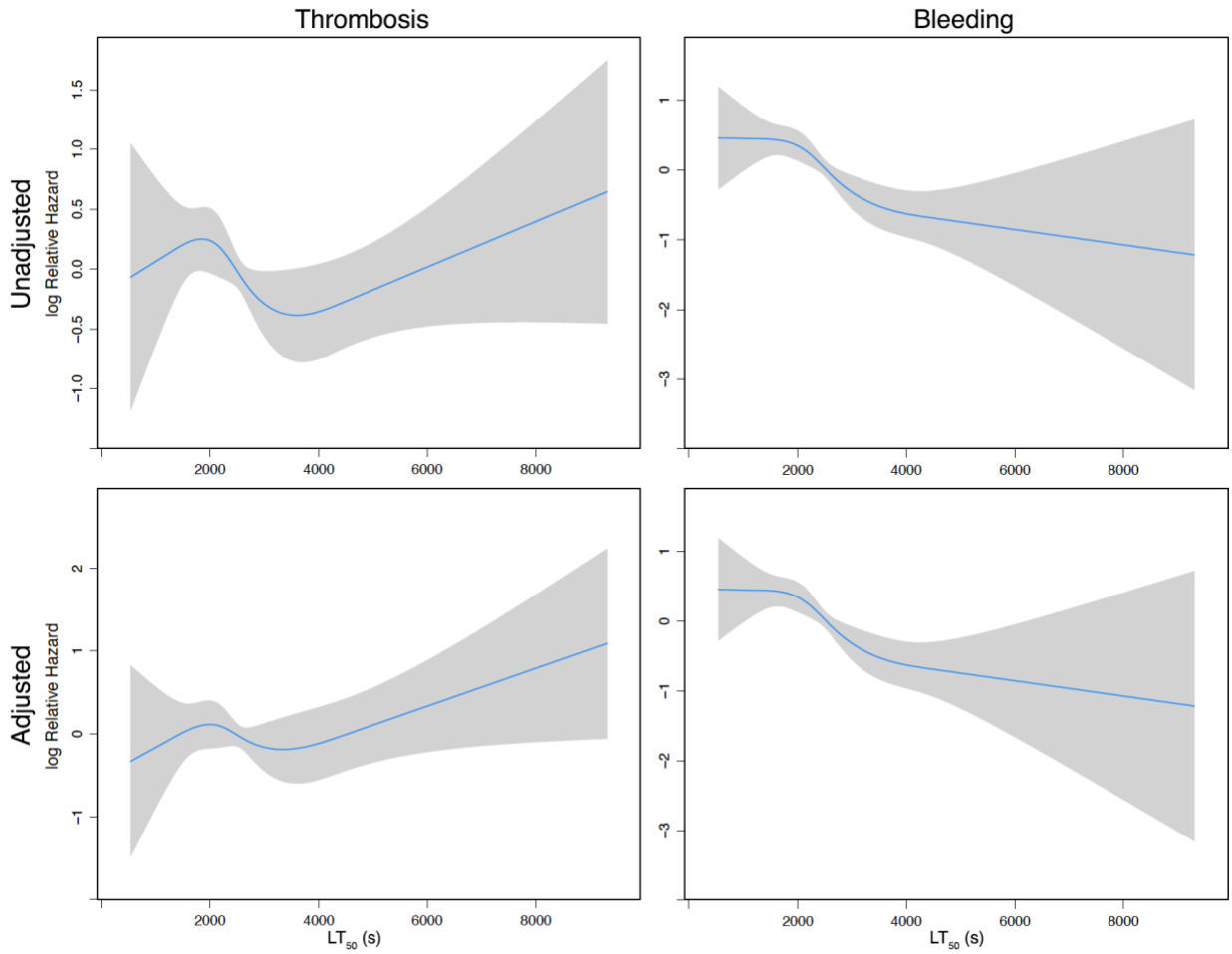


Figure 5-5 – Splines demonstrating the non-linear relationship between LT_{50} and thrombotic and bleeding events in the unadjusted and adjusted analyses. Splines fitted using 4 knots. LT_{50}, time to 50% clot lysis.

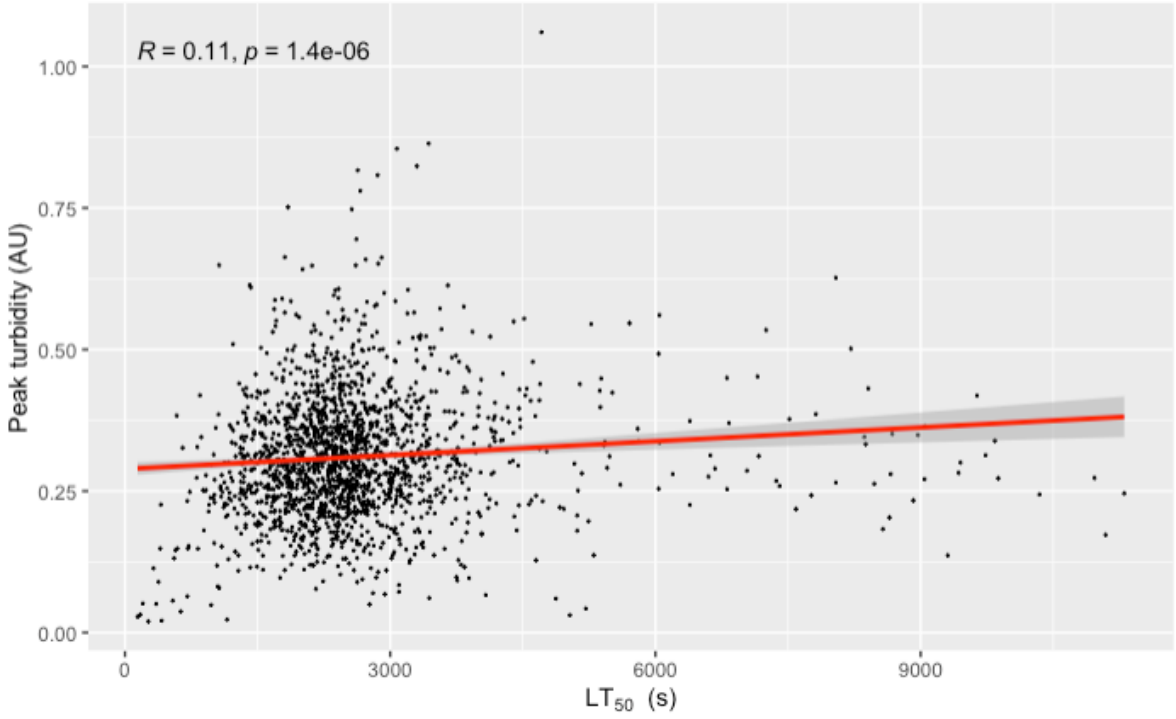


Figure 5-6 – Correlation between LT_{50} and peak turbidity in the non-treatment cohort, thrombin assay. R and P values calculated using spearman's rank correlation. LT_{50}, time to 50% lysis.

5.3 Discussion

Whilst no significant association was seen between LT_{50} and thrombotic outcomes, the trend was for more thrombotic events in those with higher LT_{50} in keeping with the hypothesis that those with the propensity to form lysis-resistant dense clots may be at higher risk of thrombotic events.[262] However, the relationships appeared complex and non-linear, with a U-shaped curve, perhaps suggesting that only those in the highest quartile of LT_{50} may have increased risk. The trends demonstrated in the spline plots for both bleeding and thrombotic outcomes compare with LT_{50} are skewed by those individuals at extremes of LT_{50}. The risk of both thrombosis and bleeding appears to only diverge from unity once LT_{50} exceeds approximately 6,000s (n = 229 patients).

These findings are consistent with results from cohorts with CAD and stroke who showed adverse thrombotic risk with increasing lysis time, particularly for individuals with the highest LT_{50}.[150,153,342] Although there is significant overlap in some risk factors for AF and CAD, the pathophysiology and risk factors for thrombotic events differ.[369,370] It is possible that LT_{50} predicts CAD events more accurately than AF events due to differential mechanisms of thrombotic occlusion. For example, CAD events predominantly result from vessel injury, acute thrombosis, and platelet aggregation, and although AF strokes are more common in those with traditional atherothrombotic risk factors, stroke mechanisms in AF may additionally include other factors such as atrial myopathy, thromboembolisation, and metabolic factors where LT_{50} may have less predictive value.

In Chapter 4 it was noted that, unexpectedly, a lower burden of AF (PAF) was associated with a hypofibrinolytic profile. I hypothesised that individuals with adverse clotting profiles may develop AF earlier than they otherwise would have. Paradoxically, this observation could additionally explain the absence of association observed between LT_{50} and thrombotic outcomes, since those patients with the most adverse clotting profiles may yet develop thrombotic complications.

A strong association was seen between the LT_{50} and cumulative bleeding outcomes; those in higher quantiles of LT_{50} had lower rates of bleeding. This makes intuitive sense, since individuals who form robust clots in response to tissue injury are less likely to suffer serious

bleeding events. The association remained significant even in a model that adjusted for other factors associated with bleeding risk, suggesting that LT_{50} is a useful predictor of bleeding risk in addition to existing risk stratification methods and may add predictive power to new risk stratification models. Indeed, my model adjusted for the majority of parameters in the commonly-used HAS-BLED bleeding risk tool.[371] Only the 'labile INR' risk factor (a challenge for dosing VKA anticoagulation) was not measured in this cohort, but this particular risk factor is becoming less important as the use of VKA anticoagulants reduces.[372] An alternative strategy could, for example, involve DOAC dose-adjustment according to LT_{50} with more aggressive anticoagulation in those with adverse clot lysis. However, no association was seen with thrombotic risk so this would need to be explored with better powered studies

Peak turbidity did not correlate with clinical outcomes in this cohort. Interestingly, those in the highest quantile of peak turbidity had numerically more bleeding events despite results suggestive of denser clot formation (though this was not a significant finding). I showed that peak turbidity was only weakly correlated with LT_{50} ($R = 0.11$), so it appears that two fibrin clot biomarkers are largely independent, but that LT_{50} is a more useful predictor of bleeding risk.

5.3.1 Limitations

In this cohort, relatively few thrombotic events occurred, leaving analysis of this endpoint relatively underpowered compared with bleeding events. This was represented by broad confidence intervals in the spline plots.

Although the inclusion criteria for ARISTOTLE were relatively broad and are likely to be representative of most patients with AF, it remains to be seen whether these results from ARISTOTLE clinical trial patients are generalisable to other populations of patients with AF, or indeed other similar populations that require anticoagulant treatment. Because the trial inclusion criteria required risk factors for stroke, patients traditionally thought to have a low thrombotic event risk were not included, though these patients still do suffer thrombotic complications. Most patients eligible for enrolment in ARISTOTLE would be candidates for antithrombotic treatment, but I did not assess whether high risk individuals without traditional risk factors could be identified with this technique.[80,247]

In this analysis, the LT_{50} of plasma samples from non-anticoagulated patients was examined. As I will discuss in Chapter 6, anticoagulation therapy may modify LT_{50}. Other groups have shown differential effects of apixaban on lysis time depending on lysis time quantile, where those with the greatest lysis time without apixaban displayed a more marked reduction while taking apixaban.[326] However, in my cohort I did not observe this large reduction in LT_{50} during apixaban treatment (see Chapter 6). Even if such a seemingly fortuitous mechanism did exist (where those who may benefit most appear to receive the greatest benefit from DOACs), I nevertheless observed less bleeding in those with prolonged clot lysis.

5.4 Conclusions

We found that participants with higher LT_{50} are at lower risk of clinically significant bleeding events while those with the very highest LT_{50} (> 6,000 s) demonstrated a trend towards a higher rate of thrombotic complications. Robust associations were not seen for the other two fibrin variables studied (lag time and peak turbidity), but a non-significant trend for fewer thrombotic events in patients with the longest lag times was observed.

Chapter 6: Fibrin clot lag time as a marker of apixaban activity and associations with clinical characteristics, biomarkers, and clinical outcomes

6.1 Background

We have shown previously that apixaban prolongs fibrin clot lag time *in vitro*, while other groups have shown similar effects *in vivo*.[327] I hypothesised that I could measure the difference between fibrin clot lag time in samples taken before and during apixaban treatment, and that this may provide insight into factors influencing the efficacy and risks of apixaban treatment in patients with AF.

As discussed in the description of the cohorts (Section 2.1.3), a subset of the patients had plasma samples available at both the randomisation visit (no anticoagulation) and at the 2-month follow-up visit (during apixaban treatment). I analysed these samples using the tissue factor (TF) assay, and calculated the difference between lag time values at each timepoint, such that:

$$\Delta_{Lag\ Time} = Lag\ time_{2\ months} - Lag\ time_{randomisation}$$

6.2 Results

Paired fibrin clot data were available for 931 participants. In this paired cohort, lag times were longer at the 2-month timepoint, with a mean $\Delta_{\text{lag time}}$ of +209.5 s ($p < 0.001$, Figure 6-1). Peak turbidity and LT_{50} both demonstrated small but significant changes with mean differences of -0.05 AU ($p < 0.001$) and +173.4 s ($p < 0.001$) respectively.

6.2.1 Clinical characteristics and biomarkers

Individuals in the third tertile of $\Delta_{\text{lag time}}$ (i.e., the largest change after starting apixaban) were older (median (IQR) age 71.0 years (64.0 – 77.0) compared with 68.0 (61.0 – 73.5) Q1, $p < 0.001$). BMI was slightly higher in Q3 (29.1 kg/m^2 (26.3 – 33.0)) compared with Q1 (28.3 (24.9 – 31.8), $p = 0.028$, Table 6-1). eGFR was lower in Q3 (71.7 ml/min (59.1 – 93.3)) than Q1 (76.7 (58.8 – 97.2)), but this was not significant ($p=0.45$). A similar trend was observed for serum creatinine (1.0 mg/dL (0.8 – 1.1) in Q1 compared with 1.0 (0.9 – 1.2) in Q3, $p = 0.076$).

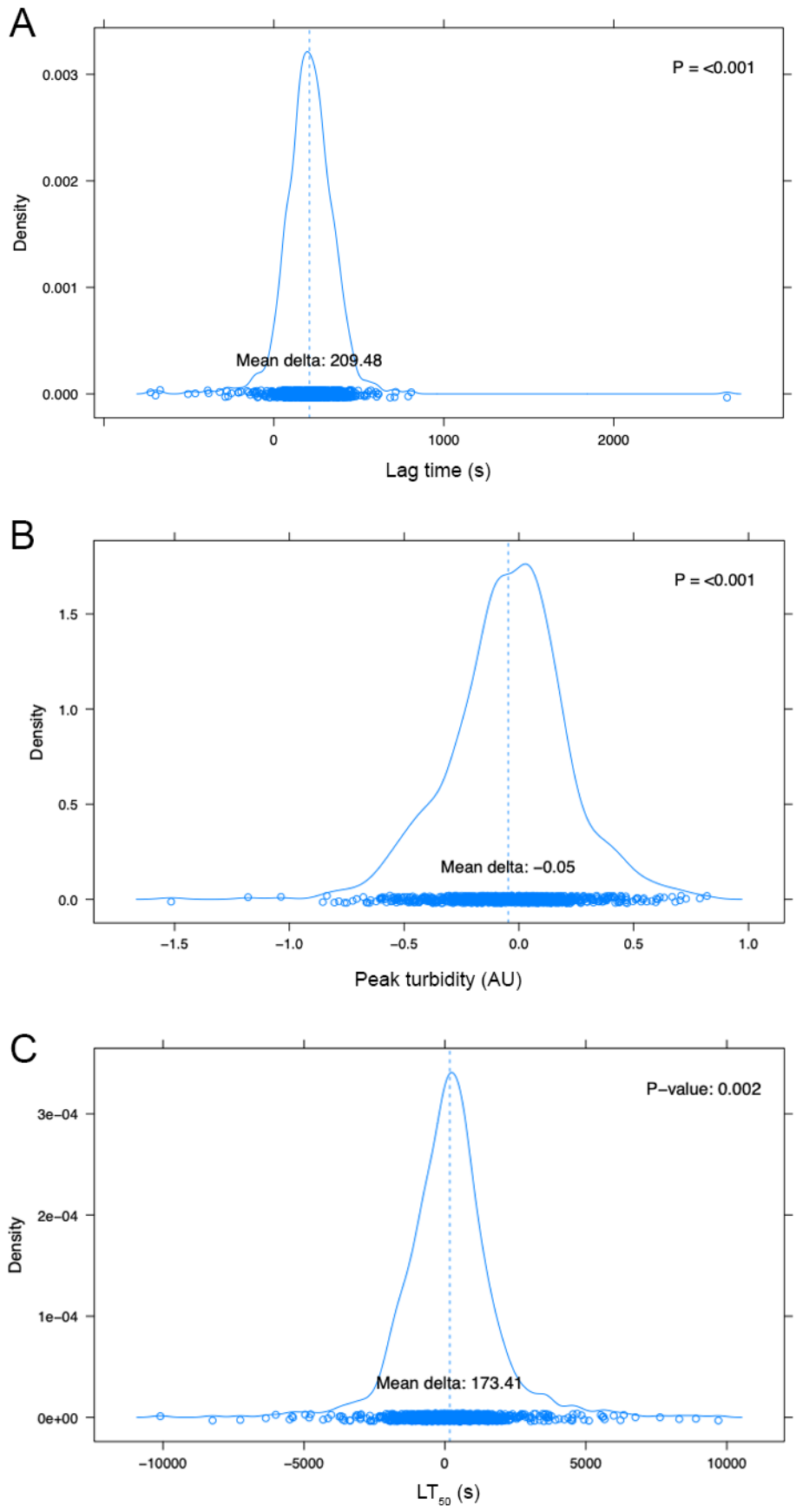


Figure 6-1 – Density plots showing the distribution of biomarkers in the paired cohort. A) Δ lag time, B) Δ peak turbidity, C) Δ LT_{50}. Mean difference shown, P values calculated using paired T-test. AU, absorbance units. LT_{50}, time to 50% lysis.

Table 6-1 – Paired cohort clinical characteristics by tertiles of $\Delta_{lag\ time}$

	N	Q1	Q2	Q3	P value
Demographic					
Age	931	68.0 (61.0 – 73.5)	68.0 (62.0 – 74.0)	71.0 (64.0 – 77.0)	*< 0.001*
Sex: Men	931	188 (60.5%)	208 (66.9%)	193 (62.5%)	*0.23*
Women		123 (39.5%)	103 (33.1%)	116 (37.5%)	
Clinical					
Weight at randomisation (kg)	926	82.0 (68.0 – 93.0)	82.0 (70.0 – 94.9)	83.0 (72.4 – 95.5)	*0.21*
BMI at randomisation (kg/m2)	924	28.3 (24.9 – 31.8)	28.1 (25.5 – 32.0)	29.1 (26.3 – 33.0)	*0.028*
History of myocardial infarction	931	46 (14.8%)	38 (12.2%)	35 (11.3%)	*0.42*
History of spontaneous or clinically relevant bleed	931	31 (10.0%)	39 (12.5%)	42 (13.6%)	*0.35*
Type of atrial fibrillation: Paroxysmal	931	66 (21.2%)	53 (17.0%)	50 (16.2%)	*0.23*
History of stroke/TIA or SE	931	52 (16.7%)	40 (12.9%)	46 (14.9%)	*0.40*
Heart failure	931	123 (39.5%)	111 (35.7%)	117 (37.9%)	*0.61*
LVEF ≤ 40%	931	46 (14.8%)	35 (11.3%)	40 (12.9%)	*0.44*
Diabetes mellitus	931	79 (25.4%)	61 (19.6%)	67 (21.7%)	*0.22*
Creatinine at Randomisation	922	1.0 (0.8 – 1.1)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	*0.076*
eGFR (Cockcroft Gault)	925	76.7 (58.8 – 97.2)	76.4 (58.0 – 95.8)	71.7 (59.1 – 93.3)	*0.45*

$\Delta_{lag\ time}$ calculated as the difference between fibrin clot lag time at randomisation (no anticoagulation) and at 2-months (during apixaban treatment). Tissue factor assay used. Clinical characteristics recorded at the randomisation visit. (a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Fisher's exact test (discrete variables) or Kruskal-Wallace one-way analysis of variance (continuous variables). LVEF, left ventricular ejection fraction. eGFR, estimated glomerular filtration rate. TIA, transient ischaemic attack. SE, systemic embolism.

Median (IQR) D-dimer levels were higher in the third tertile of lag time (608 µg/L (425 – 1091) compared with 492 (325 – 817) in Q1, p < 0.001, Table 6-2). GDF-15 was also significantly higher in Q3 (1425 ng/L (1005 – 2101)) compared with Q1 (1281 (942 – 1971), p = 0.024), as was troponin T (11.7 ng/L (8.1 – 18.0) in Q3 compared with 10.3 (7.2 – 15.7) in Q1, p = 0.024). No significant differences were observed for CRP, IL-6, leucocytes, or NT-Pro-BNP.

Table 6-2 – Paired cohort biomarkers by tertiles of $\Delta_{lag\ time}$

	N	Q1	Q2	Q3	P value
Biomarkers					
CRP (mg/L)	926	2.1 (0.9 – 4.6)	2.2 (1.0 – 4.8)	2.3 (1.2 – 4.2)	*0.59*
IL-6 (ng/L)	930	1.8 (1.2 – 3.1)	1.8 (1.2 – 2.9)	2.0 (1.3 – 3.1)	0.081
*Leucocytes (x10*9 c/L)*	902	6.9 (5.7 – 8.4)	6.9 (5.6 – 8.3)	6.8 (5.7 – 8.2)	*0.68*
Troponin T (ng/L)	926	10.3 (7.2 – 15.7)	10.8 (7.6 – 16.1)	11.7 (8.1 – 18.0)	0.024
NT-pro-BNP (ng/L)	926	602.0 (295.0 – 1219.0)	704.0 (343.0 – 1372.0)	745.5 (349.8 – 1355.5)	*0.083*
GDF-15 (ng/L)	919	1281.0 (941.5 – 1970.5)	1316.5 (919.8 – 1848.0)	1424.5 (1005.0 – 2100.5)	0.024
D-dimer (µg/L)	930	492.0 (324.5 – 817.0)	595.0 (366.5 – 1005.0)	608.0 (425.0 – 1090.8)	*< 0.001*

$\Delta_{lag\ time}$ calculated as the difference between fibrin clot lag time at randomisation (no anticoagulation) and at 2-months (during apixaban treatment). Tissue factor assay used. Biomarker levels recorded at the randomisation visit. (a – b) represents median (Q1 – Q3). P values calculated using Kruskal-Wallace one-way analysis of variance.

6.2.2 Clinical outcomes

A weak trend for fewer thrombotic events and more bleeding events in increasing quantiles of $\Delta_{lag\ time}$ was observed, but these associations were not statistically significant (Table 6-3). Using the adjusted model (described in Section 0) did not improve the strength of association.

Table 6-3 – Hazard ratios for events in the paired cohort by quantiles of $\Delta_{lag\ time}$

		Composite thrombosis				**Bleeding**			
		Unadjusted	p	Adjusted	p	Unadjusted	p	Adjusted	p
Q1	-725.5 – 132.75	Ref		Ref		Ref		Ref	
Q2	132.75 – 209.5	0.76 (0.35–1.68)		0.70 (0.31–1.57)		0.74 (0.32–1.68)		0.63 (0.27–1.46)	
Q3	209.5 – 291.5	0.59 (0.25–1.36)		0.52 (0.22–1.23)		1.31 (0.65–2.66)		1.06 (0.51–2.20)	
Q4	291.5 – 2666.5	0.83 (0.39–1.81)	0.662	0.71 (0.31–1.61)	0.521	1.20 (0.58–2.49)	0.489	0.90 (0.42–1.96)	0.594

Hazard ratios using unadjusted and adjusted models. Q1 used as reference value. Composite thrombosis defined as the composite of CV-death, stroke, systemic embolism, and myocardial infarction.

6.2.3 Correlation between lag time and anti-Xa levels

Estimates of the apixaban concentration ranged from 0 to 315 ng/ml. A weak correlation was observed between fibrin clot lag time and estimated apixaban concentration, but this was not significant (r = 0.31, p = 0.055, Figure 6-2). In 9 individuals (22.5%), apixaban was not detected using the chromogenic method in the 2-month plasma samples.

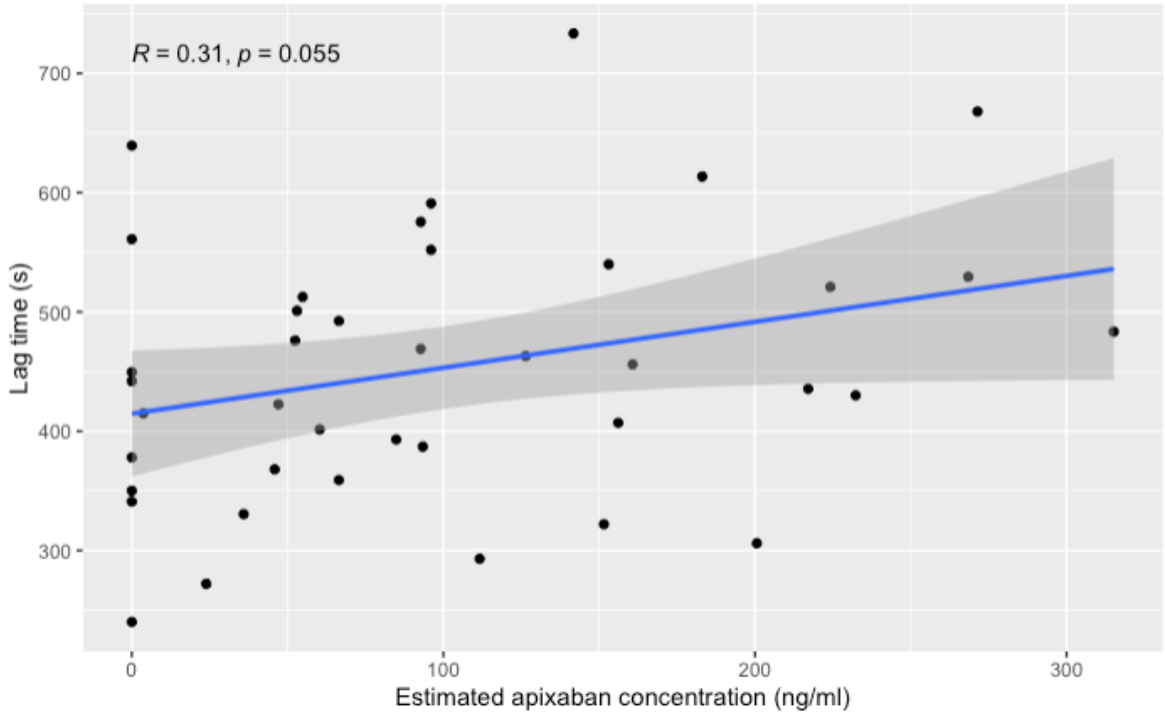


Figure 6-2 – Lag time compared with chromogenic estimation of apixaban concentration, with linear regression line and 95% confidence interval overlayed. R and p (calculated using Spearman's rank) displayed.

6.3 Discussion

Lag time was longer at the 2-month time point (during apixaban treatment), compared to the randomisation samples (when no anticoagulation was prescribed), suggesting that apixaban prolongs fibrin clot lag time *in vivo* when assessed using a TF assay. This observation is in keeping with previous studies and our *in vitro* work and is commensurate with its mechanism of action. Supporting this, apixaban affects tests of thrombin generation, also in keeping with its anti-Xa mechanism.[373,374]

Although not the focus of this analysis, I observed slightly lower mean peak turbidity and slightly higher mean LT_{50} in the apixaban-treated samples of the paired cohort. The lower peak turbidity associated with apixaban treatment is in keeping with apixaban's antithrombotic effect, though the prolongation of lysis time is surprising. Nevertheless, it is in keeping with my observations comparing the non-treatment and apixaban cohorts (Chapter 3, Figure 3-35). There is reason to suspect that apixaban would facilitate clot lysis, either by reducing the strength of fibrin clot formed through its action on Xa, or by enhancing fibrinolysis via a fibrinolytic cofactor action of Xa.[375] Other studies using similar methods to mine have reported a reduction in LT_{50} with apixaban, but the precise methods used (for

example, whether thrombomodulin is used as a cofactor) appear to be crucial.[316,326] As discussed previously, my findings suggest that LT_{50} is better assessed using a thrombin assay rather than the TF assay used here. Whether an alternative TF method would identify heterogeneity of apixaban response was not assessed.

The increase in lag time during apixaban treatment is heterogenous and is dependent on age and BMI. Some variance is expected since, unlike VKAs, there is minimal dose adjustment for apixaban and no treatment monitoring is required routinely. I hypothesised that factors that affect apixaban's pharmacokinetics, pharmacodynamics and metabolism would influence lag time.

People with the greatest $\Delta_{\text{lag time}}$ were older. The absolute difference was small (median age 68.0 years in Q1 compared with 71.0 years in Q3) but was highly significant ($p < 0.001$). This suggests that older patients may have a greater response to apixaban (at least, in terms of effect on measurable fibrin clotting function) than younger ones. Apixaban dosing is adjusted for age > 80 years but only in the presence of an additional risk factor (low body weight or impaired renal function). As discussed previously, only a small proportion of trial participants were subject to this dose reduction overall and, with a median age of the paired cohort of 69.0 years (IQR 62.0 – 75.0), the majority of my patients were below the 80-year threshold.

BMI was higher with greater $\Delta_{\text{lag time}}$, suggesting that those with greater response to apixaban had a higher BMI. Given the standardised dosing for DOACs, this is surprising. One potential explanation is that the licensed dose for apixaban for stroke prophylaxis in AF is 5 mg twice daily, reducing to 2.5 mg twice daily if two or more of the following factors are present: age ≥ 80 years, body weight ≤ 60 kg, serum creatinine ≥ 133 µmol/L. As a result, patients with low body weight are more likely to have had a dose reduction applied which may explain the differences seen. However, only relatively few participants (approximately 4.6%[§]) took the lower dose. More likely is that this relatively weak observation was a statistical anomaly; it would not remain significant if adjusted for multiple comparisons.

[§] 4.6% of all ARISTOTLE participants took a reduced dose of apixaban. The commensurate figure for the paired cohort is not known though is unlikely to differ substantially.[242]

Apixaban is predominantly metabolised in the liver, but approximately 27% is excreted via the kidneys.[376,377] However, since renal disease is relatively common, most of the variance in apixaban excretion observed in patients clinically occurs due to kidney disease, with even mild kidney disease significantly affecting its peak plasma concentration.[376] In this context, it was surprising that I did not observe a significant relationship between lag time and eGFR, although the trend was for a greater $\Delta_{\text{lag time}}$ in those with lower eGFR, as expected. eGFR is calculated from the serum creatinine and adjusted for sex, age and ethnicity.[378] Serum creatinine also trended in the expected direction across quantiles of lag time, though this trend was also not significant. As discussed previously, renal dysfunction, if present, was modest in the ARISTOTLE cohort so perhaps did not reach a threshold where it affected apixaban activity in most patients.

Overall, while I observed a substantial prolongation of lag time after apixaban was commenced, I saw relatively little heterogeneity of $\Delta_{\text{lag time}}$ across the clinical parameters analysed. This broadly supports the current dosing schedule for apixaban, which does not require adjustment based on clinical factors.

Increased $\Delta_{\text{lag time}}$ was associated with higher plasma concentrations of GDF-15, troponin T and D-dimer. GDF-15 is a marker seen in high concentrations in cancer, inflammation, aging and frailty[379–381] and, in this cohort, GDF-15 was associated with death due to bleeding.[156] Taken together, my findings suggest that part of this risk may be due to an excessive apixaban response in older, frail individuals with high levels of GDF-15. D-dimer, in addition to being a marker of specific diseases when seen at high concentrations, is a predictor of vascular death, non-vascular death and frailty.[382] In this context, it is unsurprising that the result tracks with that for GDF-15. However, D-dimer is a marker of chronic fibrinolysis and is lower in patients receiving DOACs, [383] so the positive correlation between D-dimer and $\Delta_{\text{lag time}}$ is unexpected and may represent a non-linear relationship between plasma apixaban concentration and subsequent D-dimer formation. Surprisingly, markers of inflammation such as CRP and IL-6 did not appear to influence apixaban response.

No significant effect of $\Delta_{lag\ time}$ on clinical outcomes was observed. This may, in part, be due to a relatively small sample size and event rate. However, in keeping with expectations are the trend for less thrombosis and more bleeding in the quantile where lag time increased most. A future study with a greater number of participants or higher event rate may help identify whether $\Delta_{lag\ time}$ may have clinical applicability, but the present study does not support $\Delta_{lag\ time}$ as a useful measure of bleeding or thrombosis risk.

Attempts to correlate lag time with apixaban levels measured using an anti-Xa assay unexpectedly did not demonstrate significant association between lag time and anti-Xa activity in apixaban-treated participants with AF. Other groups have seen correlations between DOAC concentration and fibrin lag time, though notably by measuring concentration using mass spectroscopy rather than an anti-Xa assay.[316] Reasons for this discrepancy are not clear. One potential explanation is that factors other than anti-Xa activity influenced lag time in apixaban-treated individuals in my cohort. Reasons for this discrepancy are not clear but possible explanations are discussed in the Limitations section below.

It is plausible that many of the patients enrolled in ARISTOTLE did so when an intercurrent illness brought them into contact with their physician. For this reason, I hypothesized that they may have been enrolled at time of increased systemic inflammation, which later settled (the biomarker data including CRP and WCC was taken at the randomisation timepoint). This could lead to a compensatory reduction in lag time, offset against the action of apixaban.

6.3.1 Limitations

The major limitation of this analysis is that the venepuncture timing is not known for the 2-month samples taken during apixaban treatment. Given the diurnal variation in apixaban concentration, when measuring anti-Xa levels to assess response to DOAC, sampling is typically timed to capture the expected peak apixaban concentration, approximately 2-4 hours post-dose.[322,384] Given my *in vitro* results, I would expect apixaban's effect on lag time to vary diurnally too, although this has not been shown experimentally.

This may explain why I was not able to demonstrate a convincing correlation between chromogenically-estimated apixaban concentration and fibrin clot lag time, for example if lag time was less sensitive to diurnal variance in apixaban concentration. In addition, data for medication compliance was not available. Apixaban was undetectable in nine individuals, perhaps due to poor compliance in these individuals (at least on the day of testing). Nevertheless, despite the undetectable apixaban, a wide range of lag times was seen in these participants.

We tested all samples available, but the relatively small number of participants who had samples at both randomisation and 2 months reduced the power of these analyses, particularly with respect to bleeding or thrombotic outcomes.

The timing of enrolment into the study may influence the paired analyses. For example, if a patient's AF was detected (and they were enrolled in the trial) during an intercurrent illness such as stroke or sepsis, there may be a detectable clotting diathesis at the first, but not the second timepoint.[385] I have observed strong relationships between markers of inflammation and fibrin lag time (see Chapter 4); any confounding effect of inflammation on these results would occur more often in samples from the first timepoint.

For the chromogenic estimated apixaban concentrations, although the apixaban-spiked control samples used for the anti-Xa assay appeared to construct an accurate standard curve (Figure 2-2), no gold standard for anti-Xa activity was available and it was not possible to verify the accuracy of the chromogenic anti-Xa assay.

Although I adjusted for potential confounders in the analysis of clinical outcomes, for the clinical characteristics and biomarkers, no adjustments were made. This was intentional since I was interested in potential underlying mechanisms and adjustment for expected bleeding or thrombosis risk factors would undermine such an analysis. However, I could not identify whether biomarker associations were due to correlated factors. For example, age correlates with GDF-15 and it was unclear whether GDF-15 would predict lag time response in a model adjusting for age. However, in terms of bleeding risk, the addition of GDF-15 to a model already controlling for clinical risk factors, including age, added to its predictive capability,

suggesting that the two risk factors are, at least to an extent, independent.[156] This observational study should be considered hypothesis generating.

6.4 Conclusions

Apixaban treatment, at the licensed dose for stroke prophylaxis in AF, prolongs fibrin lag time in patients with AF. The effects on peak turbidity and LT_{50} are small and, although statistically significant, are unlikely to be of clinical relevance. The effect of apixaban on lag time was greater in older individuals and individuals with higher BMI.

No effect of the heterogeneity of lag time on bleeding or thrombotic outcomes was observed, but my analysis was relatively underpowered to detect this. In my samples, anti-Xa activity did not correlate significantly with lag time.

Overall, $\Delta_{\text{lag time}}$ appears to be a somewhat sensitive marker of apixaban use but may lack the reliability to predict outcomes in smaller cohorts such as the one I studied.

Chapter 7: Summary and future perspectives

Stroke is a potentially devastating complication of AF. Once AF is identified, a person's stroke risk can be estimated, and anticoagulant treatment started if necessary. The most commonly-used risk score to predict stroke rates in AF (CHA_2DS_2-VASc) demonstrates a modest C-statistic of between 0.58 and 0.70, meaning a large proportion of patients assessed are incorrectly categorised.[202]

Although mechanisms of stroke in AF are complex, they typically result from thrombotic occlusion of a cerebral blood vessel, either by clots forming *in situ* or, more commonly, embolization from the left atrium. The risk of thrombotic events is substantially reduced with anticoagulation, though these treatments are associated with a risk of bleeding. While that risk is relatively low, bleeding events can be severe or even fatal. Despite the intimate relationship between blood clotting and these events, markers of clotting or bleeding potential are not used routinely when making treatment decisions.

I examined fibrin clot properties in a very large cohort of patients with AF at risk of stroke using both thrombin and tissue factor assays. I observed substantial heterogeneity in these clotting parameters, which are associated with certain clinical characteristics, other biomarkers, and clinical outcomes (bleeding and thrombotic events). For the first time, I have shown that fibrin clot lysis time correlated with bleeding events in patients with AF. This finding has potential implications for risk stratification and treatment of individuals who may require more (or less) aggressive antithrombotic therapy.

To achieve these results, I refined an existing experimental process using an iterative process, performing several experiments aimed at finding the most robust methods to maximise the reliability and utility of a tissue-factor based assay. This assay showed good reliability in the cohort used and was particularly useful for identifying heterogeneity at the start of clot formation (measured by lag time in my experiments). As with any observational study, the findings provide insights into some of the mechanisms behind these events but are unsuitable to demonstrate causality.

Fibrin clot lag time represents the time taken for detectable clot to occur before rapid amplification results in dense clot formation. In this cohort of patients with AF, prolonged fibrin clot lag time was correlated with DM, reduced left ventricular ejection fraction and renal impairment. Markers of inflammation appear to be strongly correlated with lag time, with CRP and IL-6 significantly higher for those in the highest quantile of lag time. GDF-15, a marker of tissue fibrosis and bleeding risk, was also higher in those with prolonged lag time. There was a trend towards lower thrombotic events in those with prolonged lag time, though this was not statistically significant.

Peak turbidity in fibrin clots is proportional to the maximum fibre width achieved in dense clots, though was only very weakly associated with LT_{50}, suggesting that these biomarkers may have utility independently of each other. I found that peak turbidity was also strongly associated with markers of inflammation, such as CRP, IL-6, and leucocyte count. Both BMI and DM were associated with higher peak turbidity, though this correlation was stronger for increased BMI. Given the interaction between DM and BMI, it is interesting that the latter appears to have a stronger association with peak turbidity and suggests that fibre width in clots may be associated with obesity itself rather than the impaired glucose metabolism that often accompanies it. However, no interactions between peak turbidity and clinical outcomes were seen in this cohort.

Increasing fibrin clot lysis time (LT_{50}) was associated with female sex, diabetes, increasing BMI and younger age. One explanation for this apparent paradox is that people who have adverse clotting parameters (e.g., because of a confluence of other cardiovascular risk factors, measured and unmeasured) may develop AF earlier in their lives. Markers of inflammation (CRP, IL-6, leucocyte count) were also strongly correlated with increasing LT_{50}.

As with recent work in other forms of cardiovascular disease, interest in inflammation in the pathogenesis of AF is increasing.[386] The fact that detectable inflammation appears to correlate with adverse fibrinolytic profiles in patients with AF is not surprising given what we know about the effect of inflammation on ACS.[30] However, observing this in a cohort of patients with AF is novel and of interest since modulation of inflammation is an exciting new possibility in the treatment of AF.[387]

Obesity is controversial in many cardiovascular conditions, with an apparent 'obesity paradox' where, counterintuitively, higher BMI appears to be protective.[388] In the case of AF, subgroup analyses of DOAC trials (including ARISTOTLE)[242] have demonstrated both lower stroke / SE and lower bleeding in those with increasing BMI.[389] However, these findings are difficult to interpret and, despite adjustment models, it is not possible to account for all comorbidity and comedication. In work for this thesis, I found that higher LT_{50} and higher peak turbidity were associated with increasing BMI. Indeed, the associations between peak turbidity and increasing BMI appeared stronger than for DM, suggesting that peak turbidity is influenced to a greater extent by BMI than by DM and that, from a clotting perspective, obesity should not be considered 'diabetes lite'. Future strategies to manage thrombosis risk in obesity may need to take this into consideration.

In the paired cohort analysis, I found that fibrin clot lag time was longer during apixaban treatment than before it. A very modest increase in LT_{50} with apixaban treatment was observed which, although statistically significant, is unlikely to be clinically relevant. Nevertheless, the absence of a reduction in LT_{50} after starting treatment suggests that, while apixaban has a profound effect on the rapidity of clot formation, it does not significantly affect clot lysability in patients with AF.

Although I observed strong associations between fibrin clot properties and clinical characteristics or biomarkers, I saw relatively little heterogeneity of response to apixaban across the cohort examined, though longer $\Delta_{\text{lag time}}$ was associated with increasing age and increasing BMI. The expected trend for increasing $\Delta_{\text{lag time}}$ with increasing creatinine was observed, but was not significant, perhaps because there was only mild renal impairment in this cohort. These results therefore support the current dosing schedule for DOACs with relatively minimal dose adjustment.

7.1 Potential further research

7.1.1 Lysis time and bleeding risk

The finding that bleeding events were negatively correlated with hypofibrinolysis in patients with AF is novel. However, although the finding appears to intuitively make sense, the

mechanisms behind this association remain unclear. Confirmatory work would ideally include replication in another large cohort and, if confirmed, examination of the mechanisms behind this phenomenon to determine whether this is a causal association.

7.1.2 Lag time as a marker of apixaban activity in subgroups

I showed that lag time was prolonged during apixaban treatment. The paired cohort study design precludes inferences about causality, but the strong associations seen in conjunction with my *in vitro* work (showing a strong association between apixaban concentration and lag time) suggests that causation is highly likely.

Although I found some correlations between the $\Delta_{\text{lag time}}$ and clinical conditions, these were seen within the relatively narrow window of the ARISTOTLE inclusion criteria, which excluded people with significantly impaired kidney function and extremes of body weight. Further studies of $\Delta_{\text{lag time}}$ in these populations has the potential to provide greater insight into factors influencing apixaban activity, particularly if the dosing time in relation to venepuncture could be known (or, ideally, standardised).

7.1.3 Genetic models of fibrin properties

Genetic factors have been shown to influence the development of AF, and polygenic risk scores have been used to predict both AF onset and stroke risk.[390,391] Such genome-wide association studies (GWAS) can deliver a powerful analysis of large datasets. However, one limitation of such GWAS is that they often do not provide mechanistic insight into how a gene exerts its influence on risk.

Other studies have shown that some of the variability in clotting parameters can be explained by genetic factors,[392] while heterogeneity in response to antithrombotic medication can also be dependent on genes that influence either the activation or degradation or drugs.[393] By combining the power of GWAS in large datasets with high-throughput analysis of multiple clotting parameters (i.e., lag time, peak turbidity, LT_{50}) it would be possible to identify some of the mechanisms behind a gene's increased risk. Moreover, such an approach could provide further insight into who may benefit from a personalised treatment strategy.

7.1.4 Further investigation of the obesity paradox

I have shown differential influence on peak turbidity from DM and BMI. A more detailed analysis of this phenomenon in this or another cohort, aiming to correct for the presence of DM, ideally, by controlling for HbA1c could shed light on the mechanisms behind the differential effects of DM and BMI.

7.1.5 Modification of risk scores

I found that LT_{50} was associated with bleeding risk in patients anticoagulated for AF. Future work could determine whether the predictive abilities of existing risk scores are modified by LT_{50} estimation. For example, does the addition of an LT_{50} marker to the HAS-BLED, ABC or ATRIA bleeding risk estimates improve their performance?[108,137,371]

7.1.6 Adjusting antithrombotic therapy

As an alternative to the modification of risk scores, one could examine whether implementation of differential antithrombotic strategies dependent on lysis time could improve outcomes, i.e., tailor treatment based on an individual's LT_{50}. Those patients with AF and the highest LT_{50} had the lowest risk of bleeding, so antithrombotic therapy could be intensified in those patients, presumably increasing the protection from thrombotic events.

7.2 Closing remarks

AF increases the risk of stroke by approximately 5-fold, while incidence of AF is increasing as our population ages, likely leading to an increase in stroke and associated burdens on healthcare services.[394] Recent advances in risk stratification and modern antithrombotic strategies have reduced this risk, but we have some way to go. For example, in ARISTOTLE, people with AF treated with apixaban still experienced strokes at a rate of 3.17% /year and bleeding events at 2.13% /year.[242]

I have shown a wide variance in fibrin clot properties across a large population with AF. Whilst some of this variance is associated with, and explained by, clinical risk factors (e.g., DM, age, sex) and considered by existing risk scoring systems, much of the variance is not. I have

additionally shown that this variance can predict bleeding outcomes in this cohort, suggesting a potential application of these methods in refining our management and individualising treatment strategies for patients with AF.

Chapter 8: Appendices

8.1 Protocols

TH2

Turbidity and Lysis Protocol (ARISTOTLE 2)

1) Turn on plate reader, set to incubate 37°C
2) Thaw plasma samples in 37 degrees for 20 mins then invert several times to thoroughly mix
3) **25 µL** of plasma in duplicate in 96 well plate
    a. (NB: for Matlab analysis, 1A, 1B, 12G, 12H are reserved for quality control plasma samples. Use 'vertical' pairs E.g. in first pair 1C & 1D, then 1E & 1F.

4) Lysis Mix (LM)

Reagent	Volume (µL)	Starting Concentration	Concentration in Mix	Concentration in well (1:1 Mix)
PB	**9833**	N/A	N/A	
Dil TPA	**167**	10 µg/ml	167 ng/mL	83.5 ng/mL
	10,000			

5) Activation Mix (AM)

Reagent	Volume (µL)	Starting Concentration	Concentration in Mix	Concentration in well (2:1 Mix)
PB	**5838**	N/A	N/A	
Dil Thrombin	**27**	20 u/ml	90 $x10^{-6}$ u/mL	30 $x10^{-6}$ u/mL
CaCl	**135**	1 M	22.5 mM	7.5 mM
	6,000			

6) Multichannel Pipette:
    a. **75 µL** of **LM** at 10 sec intervals – then at 3 mins
    b. **50 µL** of **AM** at 10 sec intervals.

Timepoints:

Col:	1	2	3	4	5	6	7	8	9	10	11	12
LM	0:00	0:10	0:20	0:30	0:40	0:50	1:00	1:10	1:20	1:30	1:40	1:50
AM	3:00	3:10	3:20	3:30	3:40	3:50	4:00	4:10	4:20	4:30	4:40	4:50

7) Note time plate started and subtract 3 mins for 'offset'

Thrombin: Calbiochem
TPA: Technoclone
WS with TN's efficiency adjustments

Figure 8-1 – Thrombin protocol for turbidity and lysis assay

Date: Offset:

TF

Tissue Factor Protocol March 2020 (ARISTOTLE)

Reagents used:

1. Tissue factor: PPP reagent high (Diagnostica Stago). Reconstitute with 1 ml of water for injection.
2. Calcium chloride (1 M concentration).
3. Diluted tPA (Technoclone) 10 ug/mL

Methodology

1. Thaw plasma samples in water pool at 37 degrees for 10 minutes.
2. Spin samples at 3000 rpm for 30 seconds.

Activation/lysis mix:
Make up as below:

Reagent	Starting Concentration	Volume (µL)	Concentration in Mix	Concentration in well (2:1 Mix)
PB	N/A	10500	N/A	
TF	61.8 mg/ml	460	2.52 mg/mL	1.68 mg/mL
CaCl	1 M	200	17.7 mM	11.8 mM
Dil TPA	10 µg/ml	140	124 ng/mL	82.6 ng/mL
	Total:	11300		

1. Add 50 µL of plasma samples (in duplicates in columns) to the 96 well plates.
2. Using the multichannel pipette, add 100 µL of the activation lysis mix at 10 second intervals.
3. Start reading the plate as soon as the plate is complete.

    a. Offset = time plate started. N.B. use seconds E.g. 1min32= 92s
    b. 340nm x 5 hours. 15s sampling time. Fast. (=1200 timepoints)
    c. If samples have completely lysed, can stop at 4hrs

Permeation Buffer V2
12.12g Tris
11.68g NaCl
Dissolve in 1800mls dH2O, pH to 7.4 then make up to 2000mls with dH2O.

Figure 8-2: Finalised protocol for the tissue factor turbidity and lysis experiment.

8.2 Methods of filtering turbidimetry data to analyse derivatives of the signal

The differential of the curve can be used to identify the maximum and minimum rate of change in turbidity through clotting and lysis respectively. Due to the very rapid increase in turbidity seen during the clotting phase, the maximum value of this series - the peak coagulation rate – is easily determined and thus the time from the onset of recording (plus the offset time) is the time to peak clotting rate (tPCR).

We hypothesised that the nadir of the derivative of the turbidimetry curve could also be useful as a marker of lysis. This is an estimation of the steepest (negative) portion of the turbidimetry plot i.e., the point of maximal lysis rate. However, the minimum value is much more challenging to compute due to the signal-to-noise ratio (SNR) in the plate reader outputs which is amplified when examining the derivative of the line, affecting calculated values substantially (Figure 8-3). In this typical unfiltered example, a clear maximum in the dy/dx is seen and easily identified algorithmically whereas the noise present during the lysis phase makes determining the *minimum* value of the differential signal much more challenging.

Several types of filtering methods exist to reduce the noise (high frequency oscillations in the input signal) while preserving the lower frequency component used to calculate the metrics we are interested in. Several filter types were assessed.

Moving average filter

Identifying the minimum dy/dx can be achieved with moving average filters. Different orders of median filtering were applied to determine the optimum value to reduce noise without introducing further artefact (Figure 8-4). While the unfiltered data incorrectly identified the minima, in each case the filtered data generated a plausible result. However, finding an order which reliably removed noise and identified the correct peak, without introducing phase artefact was challenging, so more sophisticated methods of filtering the data were investigated.

In each example, the absorbance (turbidity) data is filtered before the differential is calculated. The minimum and maximum values of dy / dx are annotated, with their respective points marked on the turbidity plot.

Median filter

Median filtering for the purpose of annotating the peak value has already been discussed in Chapter 2. However, to identify the maximal lysis rate, a higher order median filter is required (Figure 8-5). Values of filtering order which were too low (< 20) failed to remove sufficient noise to allow reliable results for minimum dy/dx, whereas filtering order values too high (>80) meant the data were over-smoothed and markers were placed erroneously. A filter order in the range 20-60 gave reasonable reliability and accuracy. However, even at high levels of filtering (E.g., Figure 8-5, 4th panel), noise which affected the timing of maximum and minimum values was still evident and artefact due to excessive filtering (in this case, an artificially flat section corresponding to the peak turbidity) was also seen. For the purposes of determining the minimum slope of the turbidimetry curve, median filtering proved unreliable.

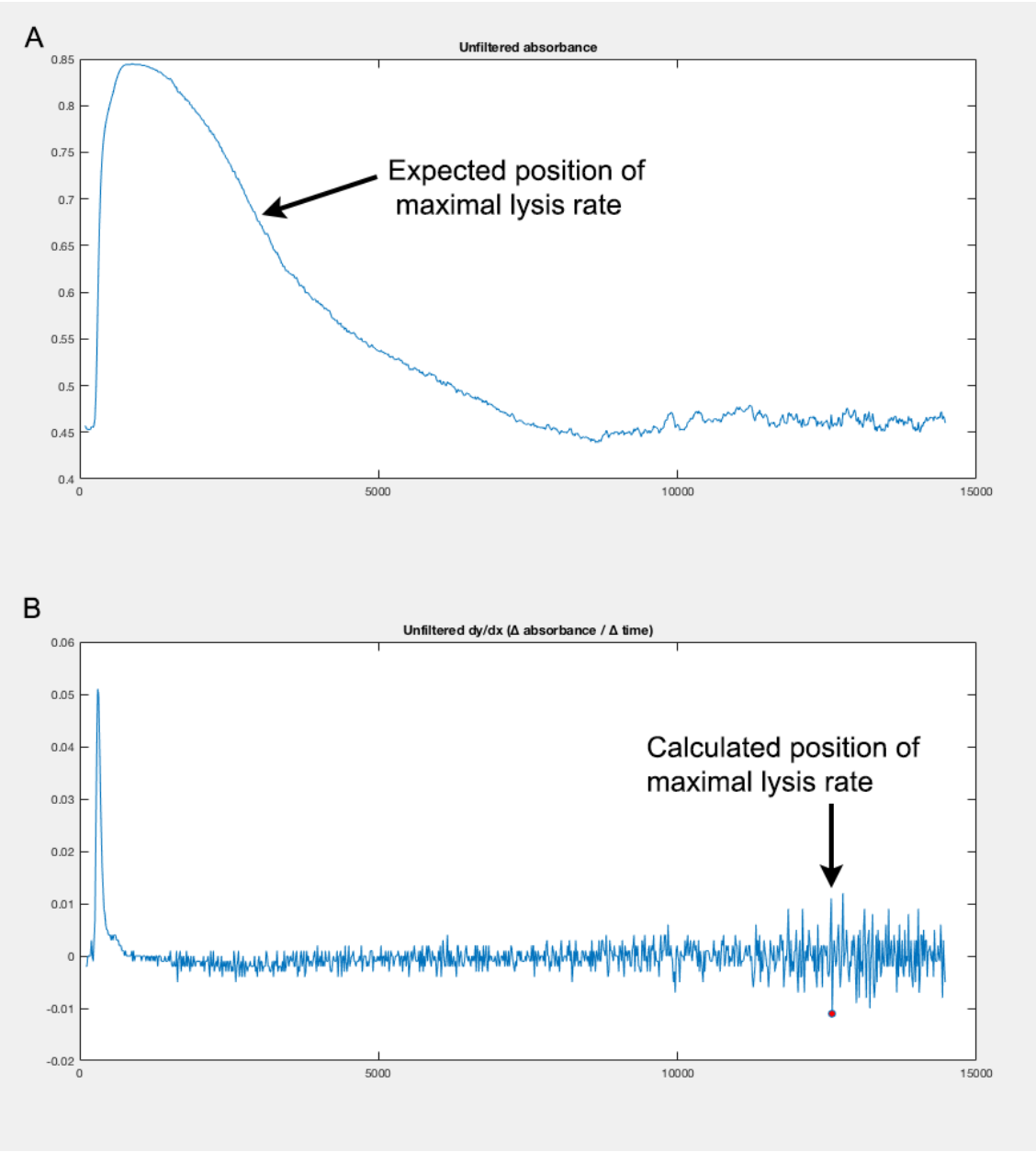


Figure 8-3 – Example of unfiltered data: a) Example absorbance signal, b) Δ Absorbance / ΔTime with the minimum value labelled.

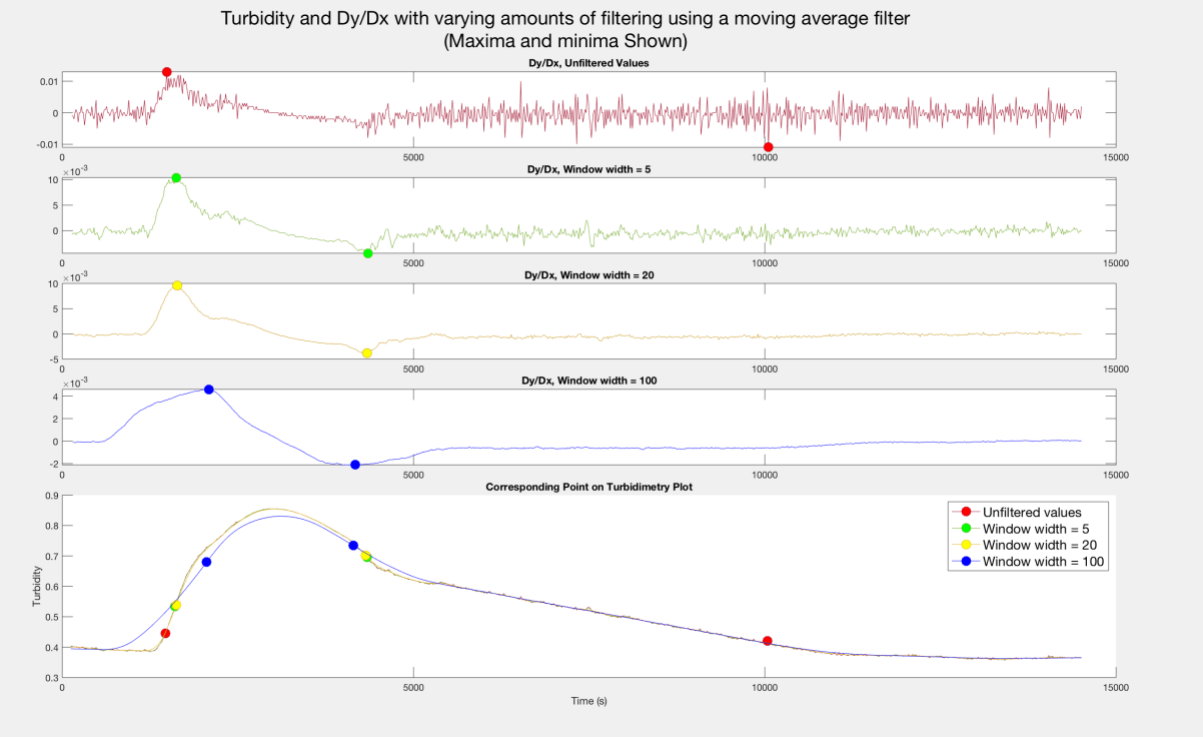


Figure 8-4 – Moving average filtering at varying orders applied to turbidimetry data. Maxima and minima annotated.

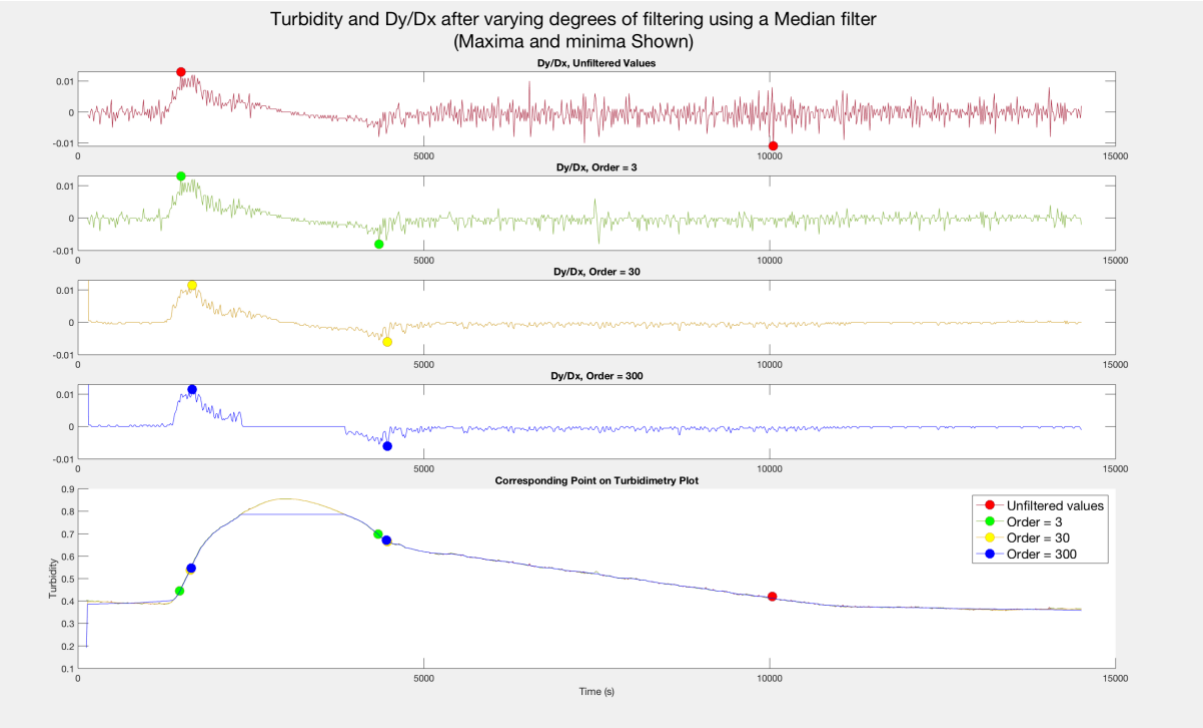


Figure 8-5 - Median filtering at varying orders applied to turbidimetry data. Maxima and minima annotated.

Savitzky-Golay filter

Savitzky-Golay filtering (SG-F) fits a polynomial line to a subset of the signal using a least-squares method.[395] This is then repeated along the length of the signal to generate a

filtered output. The framelength of filtering (which defines the number of points in the subset) and the polynomial order (a cubic polynomial, order = 3, is typical) can be varied (Figure 8-6).

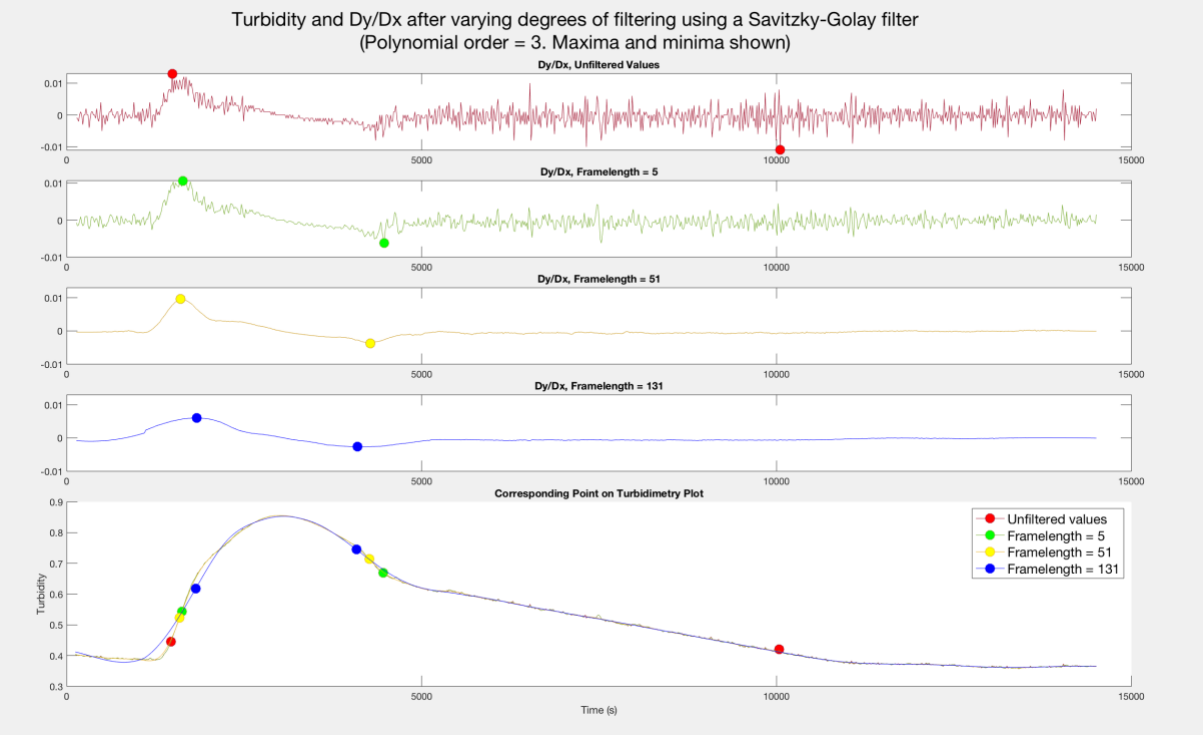


Figure 8-6 – Savitzky-Golay filtering applied to turbidimetry data with varying framelengths (Polynomial order = 3). Maxima and minima annotated.

Whilst excessive filtering results in artefacts (E.g., Figure 8-6, 4th panel), good results were obtained with framelengths in the range 21-81 without significant over-smoothing artefacts.

Low pass filter

A low-pass filter attenuates signals with frequencies above a chosen threshold. This is attractive, since the artefactual noise generated in turbidimetry plots is typically high frequency, while the true signal is low frequency. Several algorithms for low pass filtering exist, including Butterworth, Chebyshev, and Bessel.[396] Of the three, for my use-case, I have chosen to use a Butterworth filter for its ability to preserve the lower frequency components of the signal. To avoid phase distortion, the signal is parsed antegradely and then retrogradely through the filter using the 'filtfilt' command in MATLAB. The outputs can be seen in Figure 8-7.

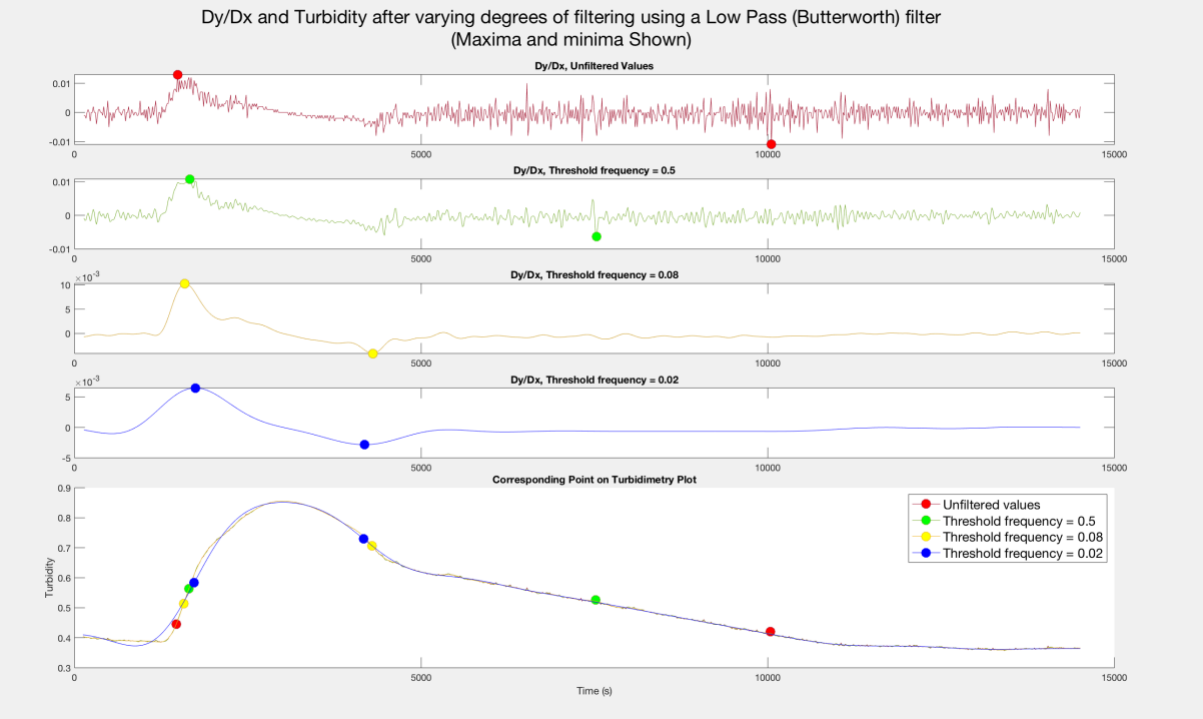


Figure 8-7 – Low pass (Butterworth) filtering applied to turbidimetry data with varying framelengths (Polynomial order = 3). Maxima and minima annotated.

A frequency threshold (normalised for the length of the signal) in the range 0.05 – 0.12 gave excellent noise reduction while preserving the shape and amplitude of the turbidimetry plot.

Choosing which signal to apply the filtering algorithm to

The filtering algorithm can be applied to either the raw signal before the differential is calculated or, alternatively, the filtering can be applied to the differential of the signal. To test which method gave the best results, optimal filtering settings were chosen for first pass Butterworth, median, moving mean and Savitzky-Golay filtering. The filtering was applied either before or after computing the differential of the signal (Figure 8-8). In each case, the timepoint corresponding to the minimum of the differential was calculated. This was repeated for each well in an 80-well experiment (16 wells of the 96 well plate were redundant) and the inter-well coefficient of variation was calculated.

With the filtering applied *before* the differential was calculated, the coefficient of variation across the different methods were generally lower than if filtering is applied to the differentiated signal. The mean coefficient of variation for filtering before computing the differential was 0.388 compared with 0.713 for filtering the differentiated signal. Averaging

the coefficients of variation in this way is subject to error since values are not directly comparable. However, the general principle shows that there is substantially less variance with the 'before' method.

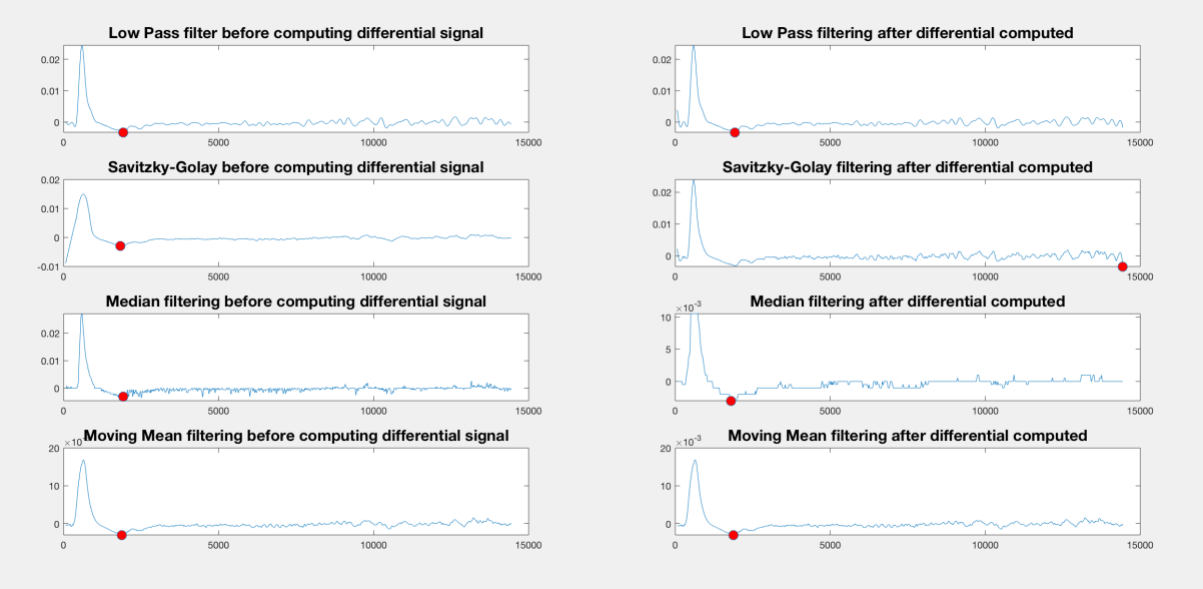


Figure 8-8 – Representative example comparing results of filtering before (left) and after (right) computing the differential. Minima annotated. Note good correlation of the minima on the left side but disagreement on the right.

Summary

For the application of attempting to determine the minimum dy/dx value – i.e., the rapid reduction in light absorbance corresponding to the point of fastest clot lysis, more complex filtering is required to remove all noise yet retain signal integrity. For this application, both SG-F and Butterworth low pass filtering produced excellent results. Ultimately, low pass filtering was chosen for measuring the maxima and minima of the differential of turbidimetry plots, on the basis that it may theoretically result in less risk of signal distortion than SG-F.

Filtering the raw signal *before* (as opposed to after) computing the differential values substantially improved the reliability when calculating the minimum dy/dx value. The result of filtering using a Butterworth-type low pass filter is shown for turbidity (Figure 8-9) and the first derivative of turbidity (Figure 8-10).

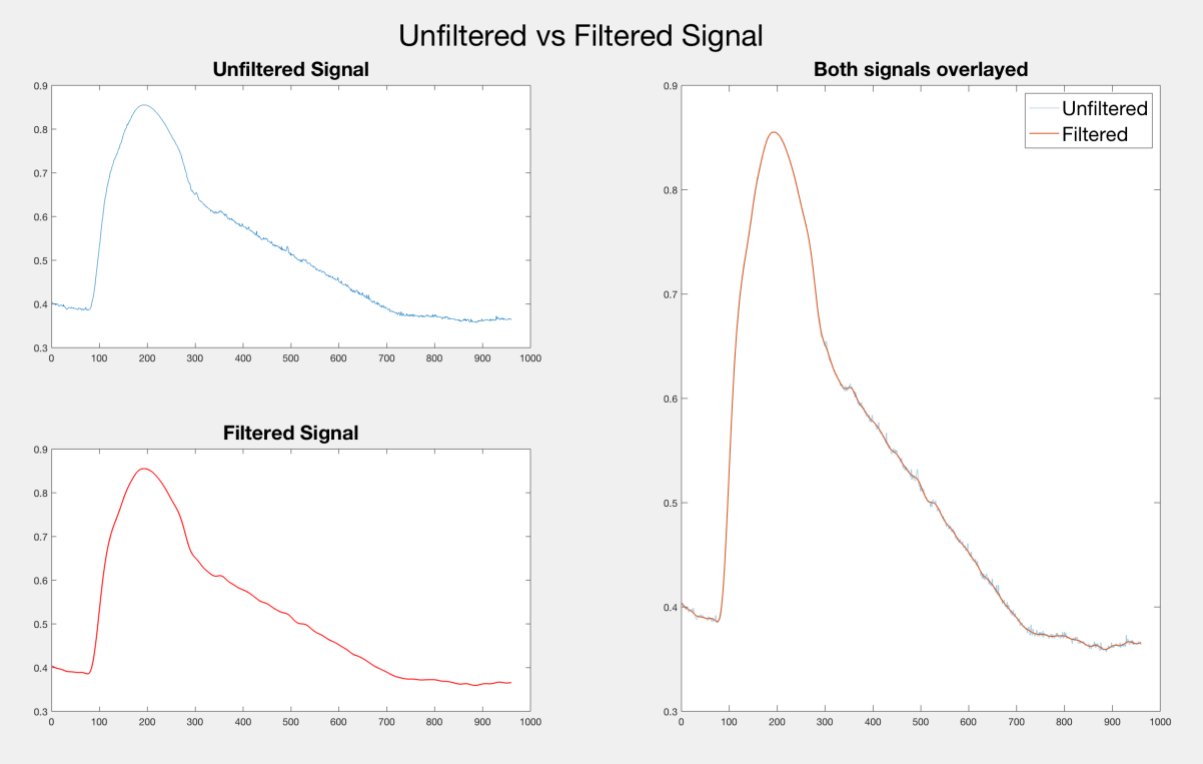


Figure 8-9 – Comparison of unfiltered signal and signal filtered with a Butterworth-type low pass filter. Note the reduction in the small amount of high frequency noise without limited or no artefactual distortion of the shape of the signal when using this filtering method.

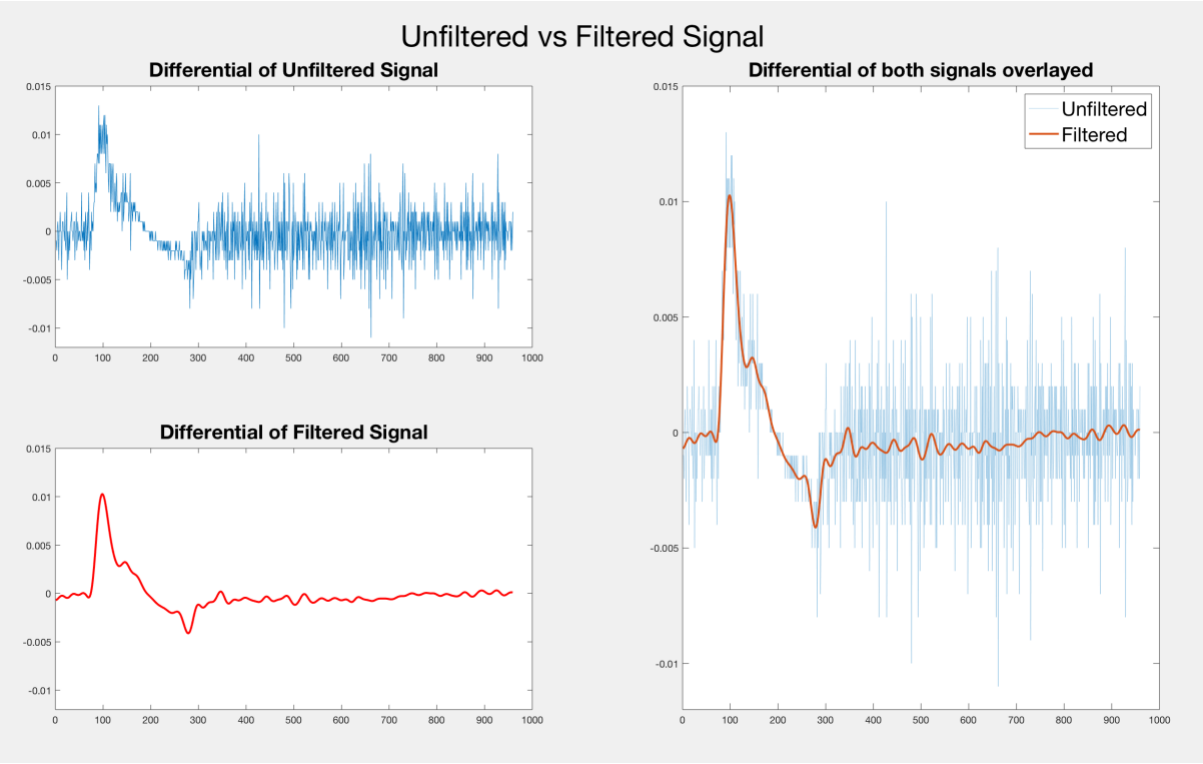


Figure 8-10 – Comparison of the differential of an unfiltered signal and the differential of a signal filtered with a Butterworth-type low pass filter. Note the substantial reduction in the amount of high frequency noise. The minimum value is easily determined from the filtered data.

8.3 Additional plots

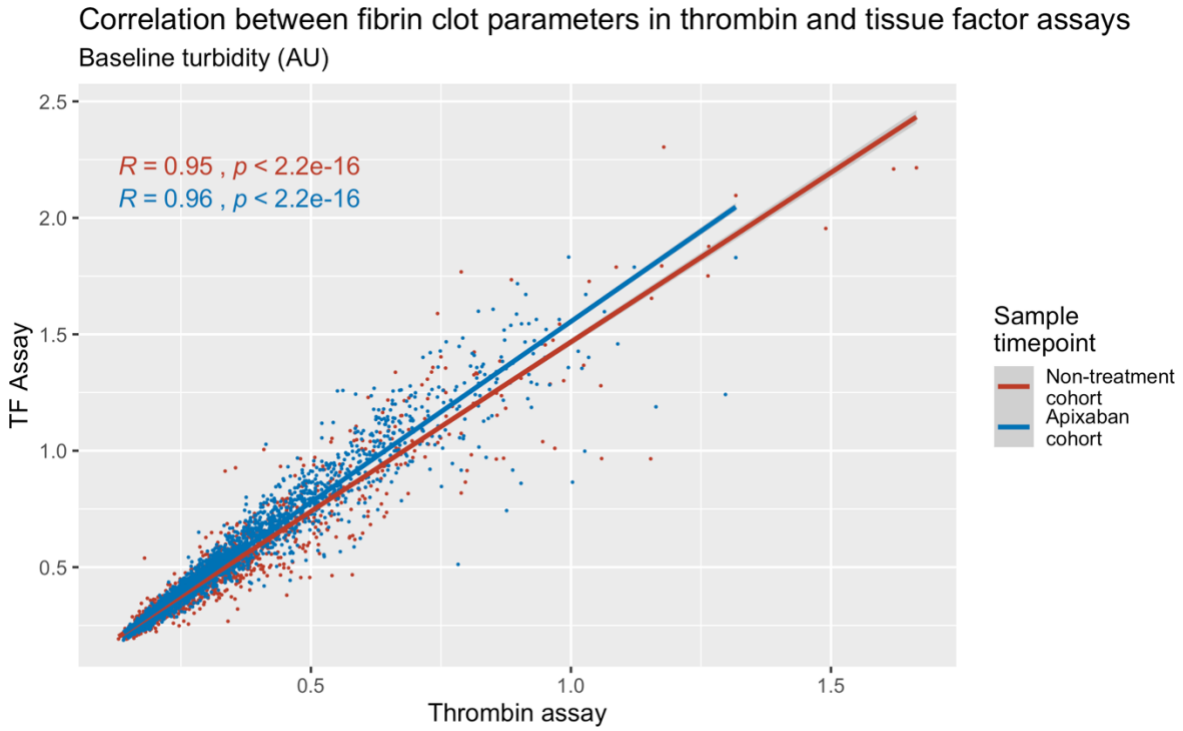


Figure 8-11 – Correlation between fibrin clot baseline turbidity in two assays, with and without apixaban treatment. TF, tissue factor. AU, absorbance units.

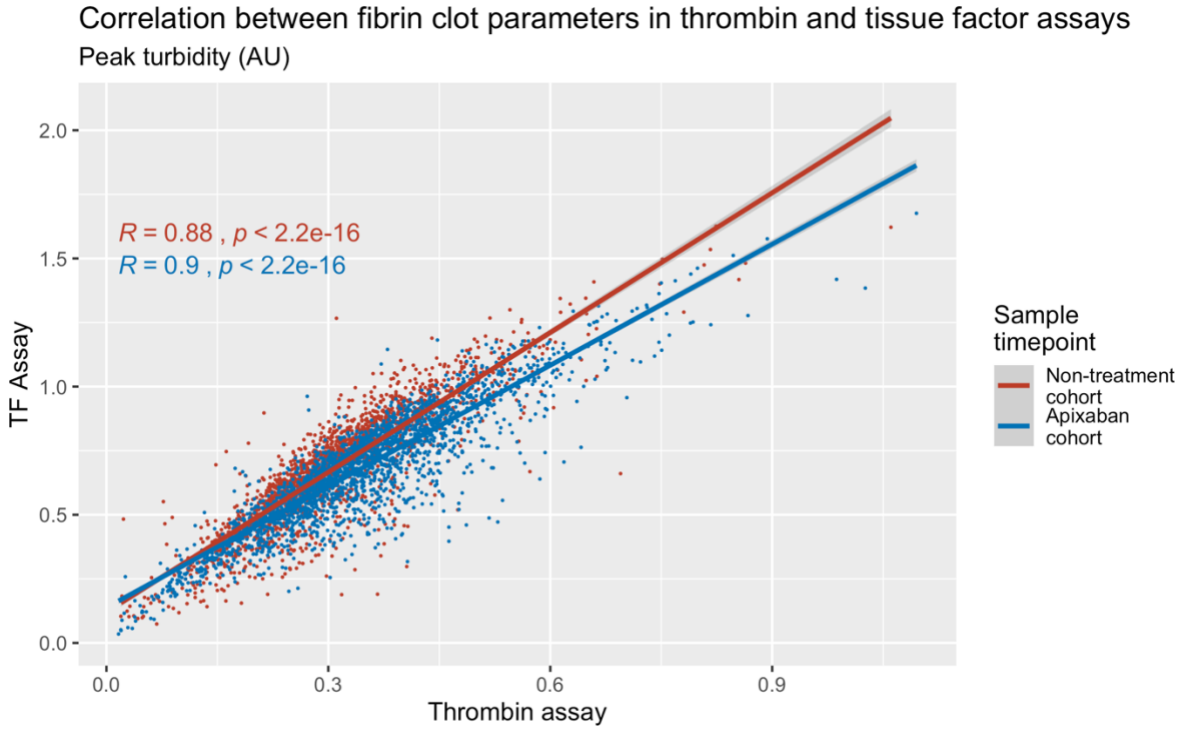


Figure 8-12 – Correlation between fibrin clot peak turbidity in two assays, with and without apixaban treatment. TF, tissue factor. AU, absorbance units.

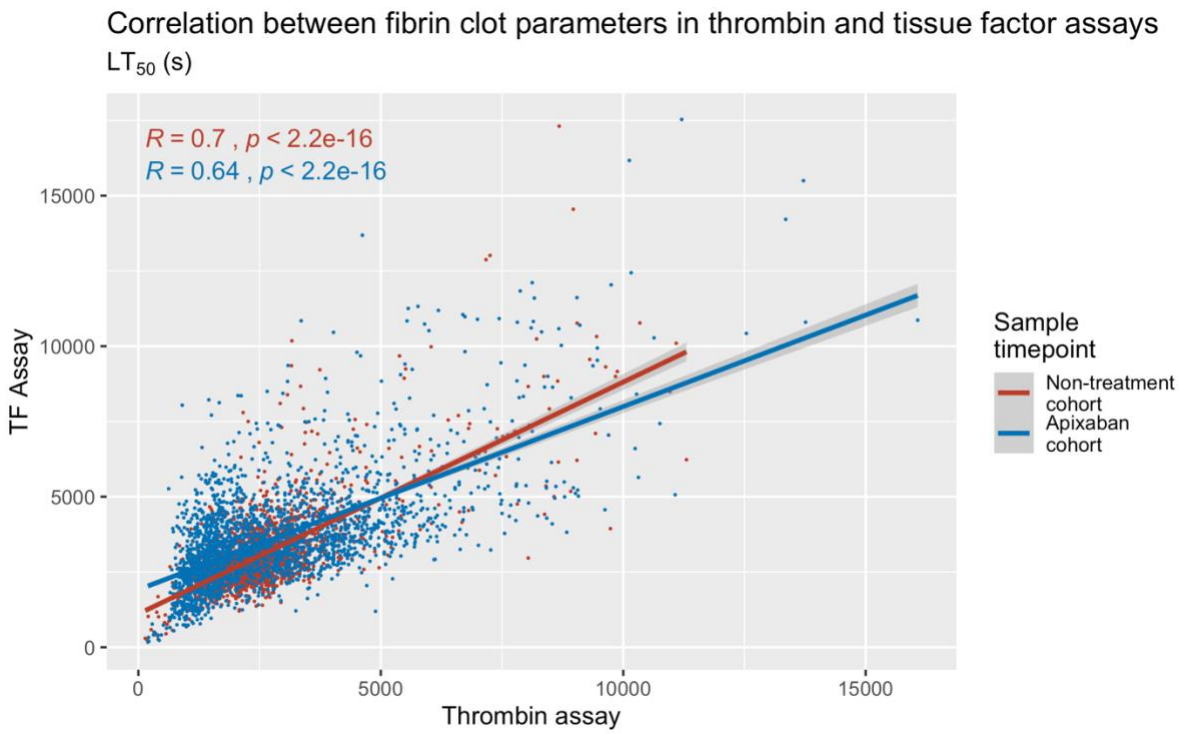


Figure 8-13 – Correlation between fibrin clot lysis time in two assays, with and without apixaban treatment. TF, tissue factor. LT_{50}, time to 50% clot lysis.

8.4 Additional tables

Table 8-1 – Non-treatment cohort clinical characteristics at randomisation, by quartiles of baseline fibrin clot lag time (thrombin assay)

	N	174.5 – 529	529 – 614.5	614.5 – 729.5	729.5 – 3874.5	P -value
Demographic						
Age	1840	70.0 (62.0 – 75.0)	69.5 (62.0 – 75.0)	68.0 (61.0 – 75.0)	69.0 (62.0 – 75.0)	*0.39*
Sex: Men	1840	268 (58.1%)	307 (66.5%)	300 (65.4%)	274 (59.8%)	*0.020*
Women		193 (41.9%)	155 (33.5%)	159 (34.6%)	184 (40.2%)	
Region: Asia/Pacific	1840	89 (19.3%)	65 (14.1%)	60 (13.1%)	60 (13.1%)	—
Europe		209 (45.3%)	263 (56.9%)	300 (65.4%)	347 (75.8%)	
Latin America		58 (12.6%)	47 (10.2%)	42 (9.2%)	26 (5.7%)	
North America		105 (22.8%)	87 (18.8%)	57 (12.4%)	25 (5.5%)	
Clinical						
Weight at randomisation (kg)	1834	80.3 (69.0 – 93.1)	82.0 (70.0 – 94.0)	81.1 (71.0 – 94.1)	81.0 (70.0 – 94.6)	*0.50*
BMI at randomisation (kg/m2)	1831	28.2 (24.8 – 32.4)	28.3 (25.5 – 32.0)	28.4 (25.2 – 32.4)	28.9 (25.4 – 32.4)	*0.59*
History of myocardial infarction	1840	52 (11.3%)	50 (10.8%)	63 (13.7%)	66 (14.4%)	*0.27*
Hist of spontaneous or clin relevant bleed	1840	49 (10.6%)	62 (13.4%)	58 (12.6%)	49 (10.7%)	*0.46*
Type of atrial fibrillation: Paroxysmal	1840	84 (18.2%)	68 (14.7%)	93 (20.3%)	101 (22.1%)	*0.028*
History of stroke/TIA or SE	1840	65 (14.1%)	68 (14.7%)	91 (19.8%)	83 (18.1%)	*0.060*
Heart failure	1840	169 (36.7%)	162 (35.1%)	186 (40.5%)	203 (44.3%)	*0.019*
LVEF ≤ 40%	1840	70 (15.2%)	54 (11.7%)	76 (16.6%)	72 (15.7%)	*0.16*
Diabetes mellitus	1840	102 (22.1%)	96 (20.8%)	104 (22.7%)	122 (26.6%)	*0.18*
Creatinine at Randomisation	1827	1.0 (0.8 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	*0.25*
eGFR (Cockcroft Gault)	1833	73.1 (56.6 – 94.0)	75.4 (59.3 – 93.2)	77.1 (59.6 – 98.1)	75.2 (57.9 – 94.0)	*0.37*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Fisher's exact test (continuous variables) or Kruskal-Wallis one-way analysis of variance (dichotomous variables). BMI, body mass index. TIA, transient ischaemic attack. SE, systemic embolism. LVEF, left ventricular ejection fraction. eGFR, estimated glomerular filtration rate.

Table 8-2 – Non-treatment cohort clinical characteristics at randomisation, by quartiles of peak plasma turbidity (thrombin assay)

	N	0.0195 – 0.24	0.24 – 0.301	0.301 – 0.37	0.37 – 1.06	P -value
Demographic						
Age	1840	69.0 (61.0 – 75.0)	68.0 (61.0 – 74.0)	69.0 (62.0 – 74.0)	70.0 (62.0 – 75.0)	*0.29*
Sex: Men	1840	305 (66.2%)	300 (65.2%)	274 (59.7%)	270 (58.7%)	*0.037*
Women		156 (33.8%)	160 (34.8%)	185 (40.3%)	190 (41.3%)	
Region: Asia/Pacific	1840	74 (16.1%)	71 (15.4%)	76 (16.6%)	53 (11.5%)	—
Europe		271 (58.8%)	262 (57.0%)	284 (61.9%)	302 (65.7%)	
Latin America		54 (11.7%)	51 (11.1%)	37 (8.1%)	31 (6.7%)	
North America		62 (13.4%)	76 (16.5%)	62 (13.5%)	74 (16.1%)	
Clinical						
Weight at randomisation (kg)	1834	80.2 (69.5 – 92.0)	80.2 (69.2 – 94.6)	82.0 (70.0 – 93.5)	82.0 (71.0 – 95.0)	*0.16*
BMI at randomisation (kg/m2)	1831	28.0 (24.7 – 31.2)	28.1 (25.5 – 31.6)	28.7 (25.2 – 32.8)	29.2 (25.2 – 33.2)	*0.003*
History of myocardial infarction	1840	51 (11.1%)	58 (12.6%)	46 (10.0%)	76 (16.5%)	*0.020*
Hist of spontaneous or clin relevant bleed	1840	51 (11.1%)	53 (11.5%)	51 (11.1%)	63 (13.7%)	*0.57*
Type of atrial fibrillation: Paroxysmal	1840	90 (19.5%)	92 (20.0%)	81 (17.6%)	83 (18.0%)	*0.76*
History of stroke/TIA or SE	1840	71 (15.4%)	71 (15.4%)	81 (17.6%)	84 (18.3%)	*0.54*
Heart failure	1840	172 (37.3%)	180 (39.1%)	195 (42.5%)	173 (37.6%)	*0.36*
LVEF ≤ 40%	1840	70 (15.2%)	65 (14.1%)	58 (12.6%)	79 (17.2%)	*0.27*
Diabetes mellitus	1840	102 (22.1%)	96 (20.9%)	106 (23.1%)	120 (26.1%)	*0.28*
Creatinine at Randomisation	1827	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	*0.34*
eGFR (Cockcroft Gault)	1833	76.2 (59.8 – 95.1)	76.5 (59.3 – 96.0)	75.0 (58.2 – 93.7)	74.2 (56.8 – 94.0)	*0.61*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Fisher's exact test (continuous variables) or Kruskal-Wallis one-way analysis of variance (dichotomous variables). BMI, body mass index. TIA, transient ischaemic attack. SE, systemic embolism. LVEF, left ventricular ejection fraction. eGFR, estimated glomerular filtration rate.

Table 8-3 – Non-treatment cohort clinical characteristics at randomisation, by quartiles of fibrin clot lysis time (thrombin assay)

	N	142.5 – 1972.5	1972.5 – 2422.5	2422.5 – 2940	2940 – 11302.5	P -value
Demographic						
Age	1840	70.5 (63.0 – 77.0)	69.0 (62.0 – 74.0)	70.0 (62.5 – 76.0)	66.0 (60.0 – 72.0)	*< 0.001*
Sex: Men	1840	319 (68.5%)	326 (70.9%)	263 (57.8%)	241 (52.5%)	*< 0.001*
Women		147 (31.5%)	134 (29.1%)	192 (42.2%)	218 (47.5%)	
Region: Asia/Pacific	1840	149 (32.0%)	71 (15.4%)	37 (8.1%)	17 (3.7%)	—
Europe		218 (46.8%)	268 (58.3%)	294 (64.6%)	339 (73.9%)	
Latin America		43 (9.2%)	38 (8.3%)	48 (10.5%)	44 (9.6%)	
North America		56 (12.0%)	83 (18.0%)	76 (16.7%)	59 (12.9%)	
Clinical						
Weight at randomisation (kg)	1834	74.0 (62.2 – 87.5)	80.5 (69.5 – 92.5)	81.4 (71.0 – 92.0)	88.2 (77.5 – 102.4)	*< 0.001*
BMI at randomisation (kg/m2)	1831	26.6 (23.4 – 30.0)	27.8 (24.8 – 31.2)	28.7 (25.8 – 32.6)	31.1 (27.7 – 35.2)	*< 0.001*
History of myocardial infarction	1840	52 (11.2%)	47 (10.2%)	63 (13.8%)	69 (15.0%)	*0.095*
Hist of spontaneous or clin relevant bleed	1840	56 (12.0%)	52 (11.3%)	56 (12.3%)	54 (11.8%)	*0.97*
Type of atrial fibrillation: Paroxysmal	1840	54 (11.6%)	76 (16.5%)	104 (22.9%)	112 (24.4%)	*< 0.001*
History of stroke/TIA or SE	1840	71 (15.2%)	83 (18.0%)	76 (16.7%)	77 (16.8%)	*0.73*
Heart failure	1840	179 (38.4%)	171 (37.2%)	169 (37.1%)	201 (43.8%)	*0.13*
LVEF ≤ 40%	1840	88 (18.9%)	73 (15.9%)	51 (11.2%)	60 (13.1%)	*0.007*
Diabetes mellitus	1840	95 (20.4%)	85 (18.5%)	110 (24.2%)	134 (29.2%)	*< 0.001*
Creatinine at Randomisation	1827	1.0 (0.8 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	*0.049*
eGFR (Cockcroft Gault)	1833	68.9 (53.8 – 89.2)	76.2 (59.8 – 92.7)	73.3 (55.1 – 91.5)	83.6 (66.6 – 107.7)	*< 0.001*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Fisher's exact test (continuous variables) or Kruskal-Wallis one-way analysis of variance (dichotomous variables). BMI, body mass index. TIA, transient ischaemic attack. SE, systemic embolism. LVEF, left ventricular ejection fraction. eGFR, estimated glomerular filtration rate.

Table 8-4 – Non-treatment cohort clinical characteristics at randomisation, by fibrin clot lag time tertiles (tissue factor assay)

	N	102 – 200.5	200.5 – 237	237 – 1138.5	P -value
Demographic					
Age	1891	69.0 (61.0 – 74.0)	69.0 (61.0 – 75.0)	70.0 (62.0 – 76.0)	*0.044*
Sex: Men	1891	403 (63.6%)	412 (65.3%)	370 (59.1%)	*0.066*
Women		231 (36.4%)	219 (34.7%)	256 (40.9%)	
Region: Asia/Pacific	1891	96 (15.1%)	95 (15.1%)	83 (13.3%)	—
Europe		407 (64.2%)	374 (59.3%)	385 (61.5%)	
Latin America		44 (6.9%)	60 (9.5%)	71 (11.3%)	
North America		87 (13.7%)	102 (16.2%)	87 (13.9%)	
Clinical					
Weight at randomisation (kg)	1885	81.0 (70.0 – 94.0)	81.0 (70.0 – 93.0)	82.0 (70.0 – 95.0)	*0.84*
BMI at randomisation (kg/m2)	1882	28.1 (25.0 – 32.2)	28.4 (24.9 – 32.0)	28.9 (25.8 – 32.4)	*0.066*
History of myocardial infarction	1891	75 (11.8%)	80 (12.7%)	86 (13.7%)	*0.60*
Hist of spontaneous or clin relevant bleed	1891	64 (10.1%)	75 (11.9%)	80 (12.8%)	*0.31*
Type of atrial fibrillation: Paroxysmal	1891	117 (18.5%)	108 (17.1%)	133 (21.2%)	*0.17*
History of stroke/TIA or SE	1891	97 (15.3%)	101 (16.0%)	121 (19.3%)	*0.13*
Heart failure	1891	242 (38.2%)	238 (37.7%)	266 (42.5%)	*0.16*
LVEF ≤ 40%	1891	83 (13.1%)	84 (13.3%)	114 (18.2%)	*0.017*
Diabetes mellitus	1891	118 (18.6%)	156 (24.7%)	157 (25.1%)	*0.008*
Creatinine at Randomisation	1877	1.0 (0.8 – 1.1)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	*0.001*
eGFR (Cockcroft Gault)	1884	77.2 (62.1 – 97.4)	75.5 (58.2 – 95.6)	72.8 (55.5 – 91.8)	*0.006*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Fisher's exact test (continuous variables) or Kruskal-Wallis one-way analysis of variance (dichotomous variables). BMI, body mass index. TIA, transient ischaemic attack. SE, systemic embolism. LVEF, left ventricular ejection fraction. eGFR, estimated glomerular filtration rate.

Table 8-5 – Non-treatment cohort clinical characteristics at randomisation, by fibrin clot peak turbidity tertiles (tissue factor assay)

	N	0.0795 – 0.596	0.596 – 0.783	0.783 – 1.63	P -value
Demographic					
Age	1891	68.0 (60.0 – 75.0)	69.0 (62.0 – 75.0)	70.0 (63.0 – 75.0)	*0.049*
Sex: Men	1891	427 (67.7%)	396 (62.9%)	362 (57.5%)	*< 0.001*
Women		204 (32.3%)	234 (37.1%)	268 (42.5%)	
Region: Asia/Pacific	1891	107 (17.0%)	98 (15.6%)	69 (11.0%)	—
Europe		369 (58.5%)	379 (60.2%)	418 (66.3%)	
Latin America		95 (15.1%)	44 (7.0%)	36 (5.7%)	
North America		60 (9.5%)	109 (17.3%)	107 (17.0%)	
Clinical					
Weight at randomisation (kg)	1885	81.5 (70.0 – 94.0)	80.3 (69.7 – 93.1)	82.0 (71.0 – 95.0)	*0.15*
BMI at randomisation (kg/m2)	1882	28.1 (25.4 – 31.6)	28.4 (25.0 – 31.9)	29.0 (25.2 – 32.9)	*0.020*
History of myocardial infarction	1891	77 (12.2%)	81 (12.9%)	83 (13.2%)	*0.88*
Hist of spontaneous or clin relevant bleed	1891	66 (10.5%)	78 (12.4%)	75 (11.9%)	*0.53*
Type of atrial fibrillation: Paroxysmal	1891	120 (19.0%)	129 (20.5%)	109 (17.3%)	*0.36*
History of stroke/TIA or SE	1891	106 (16.8%)	93 (14.8%)	120 (19.0%)	*0.13*
Heart failure	1891	258 (40.9%)	238 (37.8%)	250 (39.7%)	*0.53*
LVEF ≤ 40%	1891	100 (15.8%)	79 (12.5%)	102 (16.2%)	*0.13*
Diabetes mellitus	1891	150 (23.8%)	123 (19.5%)	158 (25.1%)	*0.046*
Creatinine at Randomisation	1877	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	*0.42*
eGFR (Cockcroft Gault)	1884	77.0 (60.0 – 95.9)	75.5 (59.3 – 93.4)	73.6 (56.8 – 95.3)	*0.31*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Fisher's exact test (continuous variables) or Kruskal-Wallis one-way analysis of variance (dichotomous variables). BMI, body mass index. TIA, transient ischaemic attack. SE, systemic embolism. LVEF, left ventricular ejection fraction. eGFR, estimated glomerular filtration rate.

Table 8-6 – Non-treatment cohort clinical characteristics at randomisation, by fibrin clot lysis time tertiles (tissue factor assay)

	N	225 – 2670	2670 – 3285	3285 – 12877.5	P -value
Demographic					
Age	1891	71.0 (64.0 – 76.0)	69.5 (62.0 – 75.0)	67.0 (60.0 – 73.0)	*< 0.001*
Sex: Men	1891	438 (69.4%)	393 (62.4%)	354 (56.2%)	*< 0.001*
Women		193 (30.6%)	237 (37.6%)	276 (43.8%)	
Region: Asia/Pacific	1891	115 (18.2%)	114 (18.1%)	45 (7.1%)	—
Europe		333 (52.8%)	371 (58.9%)	462 (73.3%)	
Latin America		56 (8.9%)	60 (9.5%)	59 (9.4%)	
North America		127 (20.1%)	85 (13.5%)	64 (10.2%)	
Clinical					
Weight at randomisation (kg)	1885	79.5 (67.9 – 90.0)	80.0 (69.9 – 92.0)	86.0 (74.0 – 100.0)	*< 0.001*
BMI at randomisation (kg/m2)	1882	27.4 (24.1 – 30.9)	28.1 (25.0 – 31.6)	30.2 (26.8 – 33.9)	*< 0.001*
History of myocardial infarction	1891	85 (13.5%)	68 (10.8%)	88 (14.0%)	*0.19*
Hist of spontaneous or clin relevant bleed	1891	78 (12.4%)	76 (12.1%)	65 (10.3%)	*0.47*
Type of atrial fibrillation: Paroxysmal	1891	108 (17.1%)	124 (19.7%)	126 (20.0%)	*0.36*
History of stroke/TIA or SE	1891	97 (15.4%)	116 (18.4%)	106 (16.8%)	*0.35*
Heart failure	1891	243 (38.5%)	226 (35.9%)	277 (44.0%)	*0.011*
LVEF ≤ 40%	1891	117 (18.5%)	89 (14.1%)	75 (11.9%)	*0.004*
Diabetes mellitus	1891	119 (18.9%)	133 (21.1%)	179 (28.4%)	*< 0.001*
Creatinine at Randomisation	1877	1.0 (0.8 – 1.2)	1.0 (0.9 – 1.2)	1.0 (0.9 – 1.2)	*0.43*
eGFR (Cockcroft Gault)	1884	71.0 (55.6 – 90.1)	73.9 (56.8 – 92.5)	81.5 (63.4 – 104.3)	*< 0.001*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Fisher's exact test (continuous variables) or Kruskal-Wallis one-way analysis of variance (dichotomous variables). BMI, body mass index. TIA, transient ischaemic attack. SE, systemic embolism. LVEF, left ventricular ejection fraction. eGFR, estimated glomerular filtration rate.

Table 8-7 – Associations of biomarkers with by quartiles of fibrin clot lag time (thrombin assay, non-treatment cohort)

		Q1	Q2	Q3	Q4	P value
CRP (mg/L)	1822	2.0 (1.0 – 5.0)	2.3 (1.0 – 4.5)	2.2 (1.0 – 4.5)	2.3 (1.0 – 4.8)	*0.97*
IL-6 (ng/L)	1834	2.0 (1.3 – 3.5)	2.0 (1.2 – 3.3)	1.9 (1.3 – 3.4)	1.9 (1.2 – 2.9)	*0.39*
*Leucocytes (x10*9 c/L)*	1784	6.8 (5.7 – 8.1)	6.9 (5.7 – 8.1)	7.0 (5.7 – 8.3)	6.8 (5.6 – 8.2)	*0.92*
Troponin T (ng/l)	1821	11.0 (7.6 – 15.5)	11.0 (7.7 – 16.2)	10.4 (7.2 – 16.2)	11.2 (7.6 – 16.6)	*0.59*
NT-pro-BNP (ng/L)	1823	709.5 (339.2 – 1225.5)	726.0 (344.0 – 1233.0)	691.5 (305.2 – 1326.0)	659.5 (293.5 – 1308.8)	*0.96*
GDF-15 (ng/L)	1806	1342.0 (962.5 – 2075.5)	1337.5 (964.5 – 2030.8)	1268.0 (952.5 – 1946.5)	1377.5 (983.2 – 2036.5)	*0.51*
D-dimer (µg/L)	1834	649.0 (415.5 – 1085.0)	576.0 (381.0 – 981.0)	551.0 (356.0 – 891.0)	524.0 (355.0 – 821.0)	*< 0.001*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Kruskal-Wallis one-way analysis of variance. CRP, C-reactive protein. IL-6, Interleukin-6. NT-pro-BNP, N-terminal pro-B-type natriuretic peptide. GDF-15, growth differentiation factor 15.

Table 8-8 – Associations of biomarkers with by quartiles of fibrin clot peak turbidity (thrombin assay, non-treatment cohort)

	N	Q1	Q2	Q3	Q4	P value
CRP (mg/L)	1822	1.3 (0.6 – 2.6)	1.5 (0.8 – 3.2)	2.3 (1.2 – 4.5)	5.1 (2.5 – 11.0)	*< 0.001*
IL-6 (ng/L)	1834	1.5 (1.1 – 2.5)	1.6 (1.1 – 2.4)	2.0 (1.4 – 3.0)	3.0 (1.9 – 4.8)	*< 0.001*
*Leucocytes (x10*9 c/L)*	1784	6.5 (5.4 – 7.6)	6.6 (5.4 – 7.9)	6.8 (5.7 – 8.2)	7.3 (6.1 – 8.8)	*< 0.001*
Troponin T (ng/l)	1821	10.6 (7.1 – 16.2)	10.5 (7.3 – 15.1)	10.9 (7.7 – 16.4)	11.6 (7.9 – 16.9)	*0.014*
NT-pro-BNP (ng/L)	1823	698.0 (280.0 – 1283.2)	656.0 (302.5 – 1109.0)	669.5 (347.5 – 1211.0)	783.0 (375.5 – 1492.0)	*0.011*
GDF-15 (ng/L)	1806	1256.0 (902.0 – 1867.0)	1245.0 (932.0 – 1791.8)	1372.0 (1018.5 – 2170.0)	1499.0 (1045.2 – 2278.5)	*< 0.001*
D-dimer (µg/L)	1834	482.5 (306.5 – 854.8)	493.0 (333.0 – 864.0)	608.0 (406.0 – 926.5)	736.5 (473.8 – 1217.2)	*< 0.001*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Kruskal-Wallis one-way analysis of variance. CRP, C-reactive protein. IL-6, Interleukin-6. NT-pro-BNP, N-terminal pro-B-type natriuretic peptide. GDF-15, growth differentiation factor 15.

Table 8-9 – Associations of biomarkers with by quartiles of fibrin clot lysis time (thrombin assay, non-treatment cohort)

	N	Q1	Q2	Q3	Q4	P value
CRP (mg/L)	1822	1.7 (0.8 – 3.9)	1.8 (0.9 – 3.9)	2.2 (1.0 – 4.7)	3.1 (1.4 – 6.2)	*< 0.001*
IL-6 (ng/L)	1834	1.9 (1.3 – 3.4)	1.8 (1.2 – 3.0)	1.9 (1.3 – 3.2)	2.1 (1.4 – 3.6)	*0.032*
*Leucocytes (x10*9 c/L)*	1784	6.6 (5.5 – 7.9)	7.0 (5.8 – 8.1)	6.8 (5.8 – 8.3)	6.9 (5.8 – 8.3)	*0.065*
Troponin T (ng/l)	1821	11.2 (7.6 – 16.8)	11.1 (7.4 – 16.2)	11.0 (7.4 – 15.9)	10.3 (7.7 – 15.5)	*0.60*
NT-pro-BNP (ng/L)	1823	828.0 (446.5 – 1485.0)	714.0 (350.0 – 1279.0)	650.5 (293.8 – 1304.2)	590.0 (250.5 – 1065.0)	*< 0.001*
GDF-15 (ng/L)	1806	1435.0 (1015.8 – 2218.0)	1274.0 (963.5 – 2012.0)	1308.0 (950.0 – 1965.0)	1318.0 (945.0 – 1966.0)	*0.026*
D-dimer (µg/L)	1834	621.0 (394.8 – 1093.2)	549.5 (366.0 – 890.0)	587.0 (391.8 – 973.5)	549.0 (345.5 – 883.5)	*0.004*

(a – b) represents median (Q1 – Q3). n (p%) represent frequency (percentage). Percentages computed by group. P values calculated using Kruskal-Wallis one-way analysis of variance. CRP, C-reactive protein. IL-6, Interleukin-6. NT-pro-BNP, N-terminal pro-B-type natriuretic peptide. GDF-15, growth differentiation factor 15.

Abbreviations

ACE	Angiotensin-converting enzyme
ACS	Acute coronary syndrome(s)
AF	Atrial Fibrillation
APTT	Activated partial thromboplastin time
ARB	Angiotensin receptor blocker
ARISTOTLE	Apixaban for reduction in stroke and other ThromboemboLic events in atrial fibrillation (trial)
AU	Absorbance units
AUC	Area under the (turbidimetry) curve
BMI	Body mass index
BNP	Brain natriuretic peptide
$CaCl_2$	Calcium chloride
CAD	Coronary artery disease
CAT	Calibrated automated thrombogram
CCS	Chronic coronary syndromes
CIED	Cardiac implanted electrical devices
CKD	Chronic kidney disease
CLT	Clot lysis time
CoV	Coefficient of variation
CRP	C-reactive protein
CT	Computer tomography
CVD	Cardiovascular disease
DAPT	Dual antiplatelet therapy
DOAC	Direct-acting oral anticoagulant
DVT	Deep vein thrombosis
eGFR	Estimated glomerular filtration rate
ETP	Endogenous thrombin potential
FTC	Freeze-thaw cycle
FVL	Factor V Leiden
GDF-15	Growth differentiation factor-15

GFC	Global fibrinolysis capacity
HV	Healthy volunteer
IL-6	Interleukin-6
INR	International normalised ratio
LA	Left atrium
LAA	Left atrial appendage
LAAC	Left atrial appendage closure
LT_{50}	Time from peak turbidity to a 50% reduction in turbidity
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
MRI	Magnetic resonance imaging
NLR	Neutrophil to lymphocyte ratio
NMA	Network meta-analysis
NSAID	Non-steroidal anti-inflammatory drug
NT-ProBNP	N-terminal pro-B-type natriuretic peptide
NVAF	Non-valvular atrial fibrillation
PAI-1	Plasminogen activator inhibitor-1
PAR-1	Protease-activated receptor-1
PT	Prothrombin time
PTL	Plasma turbidity and lysis (assay)
RBC	Red blood cell
rEF	Reduced (left ventricular) ejection fraction
ROTEM	Rotational thromboelastometry
SD	Standard deviation
SE	Systemic embolism
SEC	Spontaneous echo contrast
SG-F	Savitzky-Golay filtering
SNR	Signal to noise ratio
TE	Thromboembolism
TEG	Thromboeleastography
TF	Tissue factor

TIA	Transient ischaemic attack
tPA	Tissue plasminogen activator
tPCR	Time to peak clotting rate
TRIS	tris(hydroxymethyl)aminomethane
UCR	Uppsala Clinical Research Center
VKA	Vitamin-K antagonist
VTE	Venous thromboembolism
WCC	White cell count

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