

**Interactions between antiphospholipid antibodies,
oral anticoagulants and haemostasis activation in
thrombotic antiphospholipid syndrome**

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Declaration

I, Deepa Jayakody Arachchillage, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that has been indicated in the thesis.

Abstract

Antiphospholipid antibodies (aPL) are associated with an increased thromboembolic risk but the mechanism is unclear. Warfarin anticoagulation has traditionally been employed in thrombotic antiphospholipid syndrome (APS), but direct oral anticoagulants such as rivaroxaban have recently become available.

The aims of this thesis were to:

1. Investigate the frequency, mechanism and clinical associations of activated protein C resistance (APCr) in thrombotic APS
2. Assess the effects of rivaroxaban on lupus anticoagulant tests
3. Investigate the efficacy of rivaroxaban in terms of thrombin generation (TG) and haemostasis activation markers in thrombotic patients with or without APS

Thrombotic APS patients had greater APCr than disease controls. Nearly 50% of APS patients had anti-protein C (anti-PC) antibodies; those with high avidity antibodies had significantly greater APCr using either exogenous activated PC or activation of endogenous PC with Protac®. High avidity anti-PC antibodies were strongly associated with a severe thrombotic phenotype in APS. There was a wide variation in the sensitivity of thromboplastin reagents to rivaroxaban. Of the six commonly used thromboplastin reagents studied, Neoplastin®R was the most sensitive while Innovin® and Thromborel®S were the least sensitive. False positive dilute Russell's viper venom time occurred in patients with therapeutic levels of rivaroxaban. Taipan /Ecarin venom clotting time ratio and Textarin time were not affected irrespective of the rivaroxaban level, enabling accurate detection of LA. *In vitro* studies showed that aPL did not influence the anticoagulant activity of rivaroxaban as measured by TG and anti-Xa assays. Both rivaroxaban and warfarin achieved effective anticoagulation, as assessed by inhibition of TG and *in-vivo* coagulation activation markers in patients with and without APS.

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List of abbreviations

ACCP	American College of Chest Physicians
aCL	anticardiolipin antibodies
AF	Atrial fibrillation
APC	activated protein C
APCr	activated protein C resistance
aPL	Antiphospholipid antibodies
APS	Antiphospholipid syndrome
APS ACTION	Antiphospholipid Syndrome Alliance for Clinical Trials and International Networking
APTT	Activated partial thromboplastin time
aβ2-GPI	anti- β 2-glycoprotein-I
BCSH	British Committee for Standards in Haematology
BSA	Bovine serum albumin
CaCl₂	Calcium chloride
CAPS	Catastrophic antiphospholipid syndrome
CAT	calibrated automated thrombogram
CI	Confidence interval
CV	Coefficient of variation
DMSO	Dimethyl sulfoxide
dPT	Diluted prothrombin time
DRVVT	Dilute Russell's Viper Venom Time
ECT	Ecarin clotting time
ELISA	enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor

ETP	Endogenous thrombin potential
F1.2	Prothrombin fragment 1.2
FDA	Food and drug administration
FET	Fisher's exact test
FEU	Fibrinogen equivalent unit
FIIa	thrombin (FIIa)
FIX	Factor IX
FPS	Free protein S
FV	Factor V
FVII	Factor VII
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXII	Factor XII
FXIII	Factor XIII
H₂SO₄	Sulphuric acid
HIT	Heparin induced thrombocytopenia
HPLC	High-performance liquid chromatography
INR	International Normalised Ratio
ISI	International Sensitivity Index
ISTH	International Society on Thrombosis and Haemostasis
KCT	Kaolin clotting time
LA	Lupus anticoagulant
LDA	Low dose aspirin

LMWH	Low molecular weight heparin
NaCl	Sodium chloride
NC	Healthy normal control
NICE	National Institute for Health and Care Excellence
OVB	Owren's Veronal buffer
PBS	phosphate buffered saline
PC	Protein C
PE	Pulmonary embolism
PF4	Platelet factor 4
pNA	Para-nitro aniline
PPP	platelet poor plasma
PT	Prothrombin time
PTR	Prothrombin time ratio
QC	Quality control
RAPS	Rivaroxaban in Antiphospholipid Syndrome
RCT	Randomised controlled trial
RCT	Randomised controlled trial
RITAPS	RITuximab AntiphosPholipid Syndrome
RPL	Recurrent pregnancy loss
SCR	Screen confirm ratio
SLE	systemic lupus erythematosus
SPC	Summary product characteristics
SSC	The Scientific and Standardization Committee
TAT	Thrombin Antithrombin complex
TC	Thrombin calibrator

TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
TIA	Transient ischaemic attack
TMB	3,3',5,5'-Tetramethylbenzidine
TVT	Taipan venom time
UFH	Unfractionated heparin
VDRL	Venereal Disease Research Laboratory
VKA	Vitamin K antagonists
VKOR	Vitamin K epoxide reductase

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Chapter 1 Introduction

1.1 Normal haemostasis

The fine balance between procoagulant and anticoagulant factors, fibrinolytic plasma proteins, platelets, leucocytes and the vascular endothelium maintain the blood fluidity in the circulation without thrombosis or bleeding in normal circumstances. Disturbance of this fine balance of the normal haemostatic mechanism results in either bleeding or thrombosis. When the activated haemostatic system overwhelms the normal regulatory mechanisms that localise the thrombus formation to areas of injury, pathological thrombus occurs. In a cell based model of haemostasis, formation of fibrin clot occurs on different cell surfaces by three overlapping steps of initiation, amplification and propagation (Hoffman & Monroe, III, 2001).

1.1.1 Initiation

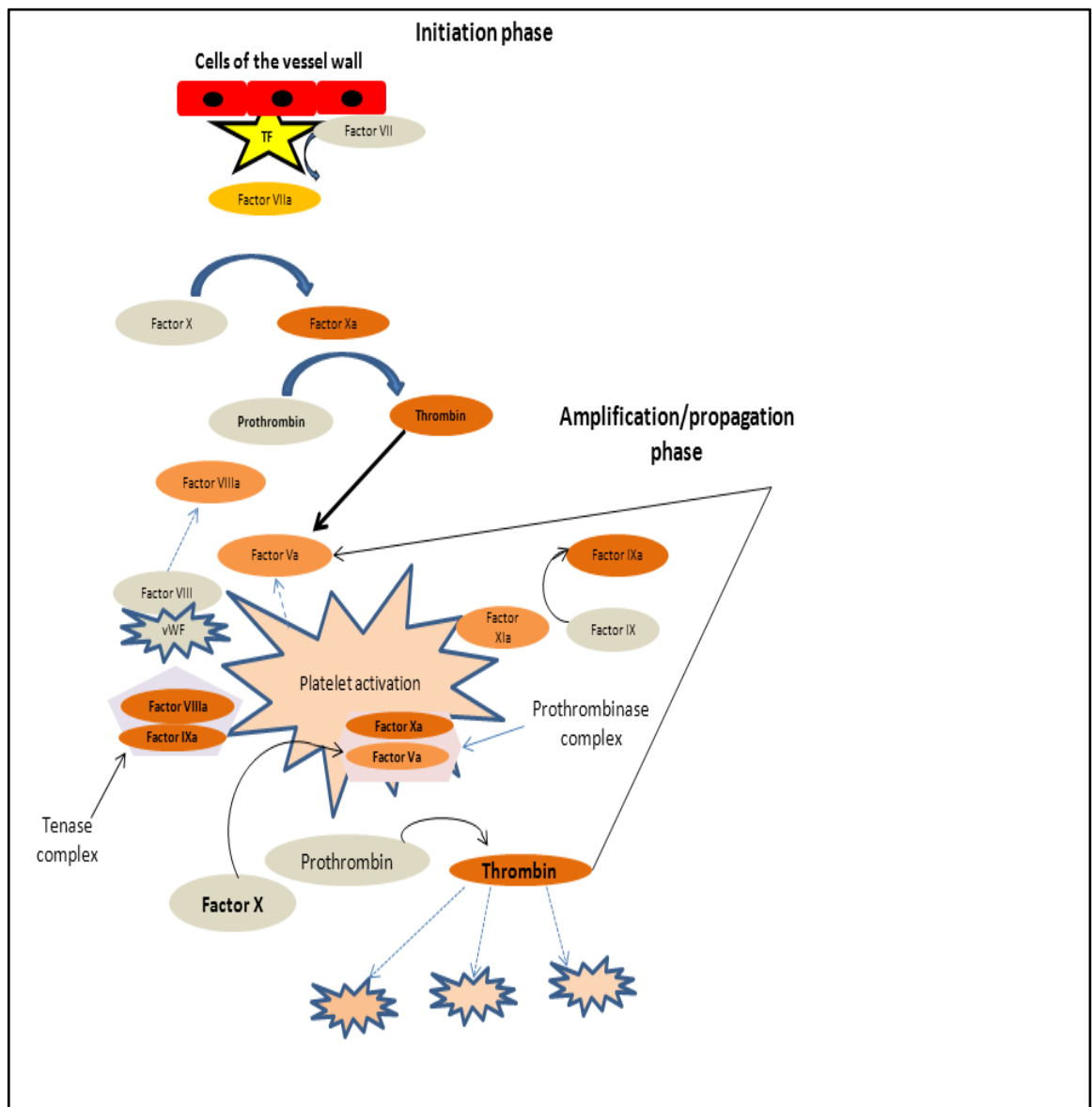
When injury occurs to a blood vessel leading to expression of collagen and tissue factor (TF), the zymogens of the procoagulant proteins are converted sequentially to enzymes that initiate thrombus formation (Furie & Furie, 2007). Formation of the TF-factor VIIa (FVIIIa) complex activates additional FVII to FVIIa allowing to generate more TF-FVIIa complex activity. The TF-FVIIIa complex activates a small amount of factor IX (FIX) and factor X (FX)(Hoffman & Monroe, III, 2001). Tissue factor pathway inhibitor (TFPI) and antithrombin rapidly inactivate activated FX (FXa) that escapes into the cell surface environment, whereas FXa that moves onto the surface of nearby cell surfaces (platelets or other cells) is not inhibited by TFPI. FXa activates prothrombin (factor II) to form small amount of thrombin (FIIa) which is an essential step for the activation of platelets, FVIII, FV and the formation of large amount of thrombin by a positive feedback mechanism.

1.1.2 Amplification

A small amount of thrombin generated at the initiation phase of coagulation, diffuses away from the TF-bearing cells to activate the platelets that leak from the blood

vessels at the site of injury (Monroe *et al*, 1996). Binding of thrombin to the platelet surface receptors causes conformational changes, expose procoagulants on the platelet membrane surface and release platelet granular contents that are required for the clotting reactions. Platelet granules contain a large number of proteins and agonists to induce further platelet activation (Smith, 2009). Furthermore, thrombin generated in the initiation phase of the coagulation process, converts Factor XI (FXI) to FXIa and factor V (FV) to FVa on the platelet surface.

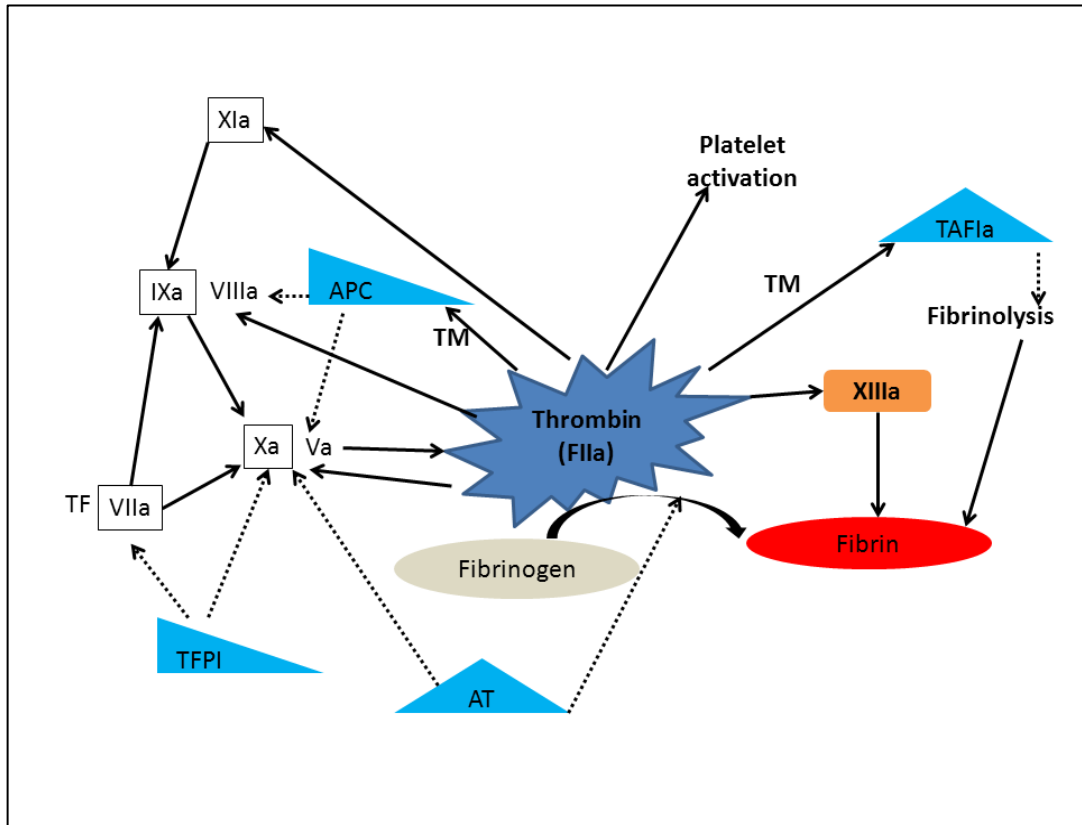
Figure 1-1 Cell based model of the coagulation cascade



Adapted from: (URL: www.discoverymedicine.com/Ingo-Ahrens/2012/06/23/development-and-clinical-applications-of-novel-oral-anticoagulants-part-i-clinically-approved-drugs, accessed 23rd February 2015).

In addition, thrombin cleaves von Willebrand factor (vWF) and the factor VIII complex, releasing vWF to mediate platelet adhesion and aggregation. Thrombin activates FVIII to FVIIIa (Hoffman, 2003).

Figure 1-2 The central role of thrombin in haemostasis



Thrombin is generated in small quantities during the initiation phase which occurs on the TF-bearing cell, then in larger quantities on the platelet surface in the propagation phase. Thrombin becomes anticoagulant via thrombomodulin-mediated activation of the protein C system. Thrombin also impacts on clot structure through activation of cross-linked FXIII, and thrombin activatable fibrinolysis inhibitor [adapted from (Smith, 2009)].

1.1.3 Propagation

Release of granular contents from the small number of platelets activated in the amplification phase results in recruitment of more platelets to the site of injury, and the propagation phase occurs on the surface of these platelets. FVIIIa combines with FIXa (generated by TF-FVIIa complexes in the initiation phase) to form intrinsic ‘tenase’ complexes on the activated platelet surfaces. The FVIIa/FIXa complex is a potent and major activator of FX. FXa in amalgamation with its co-factor FVa and calcium ions forms prothrombinase complexes, which is 300,000 fold more active than FXa alone in activating prothrombin to thrombin, thus generating large amount

of thrombin locally (thrombin burst)(Hoffman & Monroe, III, 2001;Mann *et al*, 2003).

1.2 Role of phospholipids in haemostasis

The central role of phospholipids in the coagulation cascade has been well established for many years (Mann *et al*, 1990). The negative charges on the phospholipids are recognised as necessary for the binding of the vitamin K-dependent enzymes and substrate through their N-terminal Gla domains. During post-translational processing, vitamin K-dependent proteins undergo carboxylation of the glutamic (Glu) acid residues at their N-terminus, by a vitamin K-dependent carboxylase. This process is required for biologically active proteins and results in the unique and characteristic presence of nine to thirteen γ -carboxyglutamic (Gla) residues consisting of the Gla domain (Freedman *et al*, 1995).

The Gla domain is essential for establishing a strong association with Ca^{2+} , which is crucial for both the interaction of the Gla-containing proteins with negatively charged phospholipids and for their correct structural folding. Intra-chain interactions between Ca^{2+} and Gla residues induce a structural transition of the Gla domain, transforming it from an unfolded and non-functional unit to a tight folded and essentially functional domain. Folding of the Gla domain also exposes a hydrophobic patch of three residues within the Gla domain, termed as the ' ω loop', which enables interaction with cell membranes. Positively charged Ca^{2+} -bound Gla residues form ion bridges with the negatively charged phosphate head groups of phospholipids, facilitating localisation of the vitamin K-dependent proteins on the surface of cell membranes where assembly of procoagulant and anticoagulant complexes takes place (Huang *et al*, 2003). Although the Gla domains of all vitamin K-dependent proteins are highly conserved, their affinities and binding rates for membranes vary considerably between the different members of the family. For instance, protein S has one of the highest affinities for negatively charged phospholipids (dissociation constant (KD) ~ 10 nM), whereas protein C exhibits a ~ 100 -fold weaker affinity (KD ~ 1 μM) (Nelsestuen *et al*, 1978;Sun *et al*, 2003).

1.3 Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is characterized by thrombosis (venous and/or arterial or microvascular) and/or pregnancy loss or morbidity in association with persistent positivity of a heterogeneous group of autoantibodies known as antiphospholipid antibodies (aPL) (Miyakis *et al*, 2006). The International consensus criteria (Sydney; updated Sapporo) for APS were designed for scientific clinical studies, however, these clinical and laboratory diagnostic criteria have been adapted for the diagnosis of APS in routine clinical practice. The primary targets of aPL are phospholipid-binding proteins, although antibodies directed against phospholipids and other proteins also occur. One or more of the non-criteria features of APS such as heart valve disease, livedo reticularis, thrombocytopenia and nephropathy may present in association with thrombosis and/or pregnancy morbidity or as isolated features. aPL are a heterogeneous group of autoantibodies, which include lupus anticoagulant (LA), IgG and/or IgM anticardiolipin antibodies (aCL) and anti- β_2 -glycoprotein-I ($\alpha\beta_2$ -GPI) antibodies, of moderate or high positivity (>40 GPL or MPL units, or exceeding the 99th centile). The International consensus classification criteria now define persistently positive aPL as those being present on two or more consecutive occasions at least 12 weeks apart (Miyakis *et al*, 2006).

The prevalence of aPL in the form of LA or aCL is 1–5% of healthy individuals. The prevalence increases in the elderly and in those with chronic disease (Keeling *et al*, 2012). Conditions that are associated with production of aPL are listed in Table 1-1. APS has been described as secondary if there is an associated autoimmune disorder, however, the international consensus classification advises against using the term ‘secondary’ on the basis of that the relationship between APS and systemic lupus erythematosus (SLE) is undefined (Miyakis *et al*, 2006). Several studies have shown that the prevalence of aPL in SLE patients is variable (15–86%). The frequency of antibody positivity is likely to be around 30%, with the wide variation found in the literature explained by study variations, ethnicity, and extent of autoimmune disease activity. Up to an estimated 40% of patients with SLE and aPL will eventually develop clinical features consistent with APS, whereas under 5% of patients with APS will develop SLE.

Table 1-1 Associations of antiphospholipid antibodies

Conditions associated with production of antiphospholipid antibodies

Systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, Behçet's disease, temporal arteritis, Sjögren's syndrome

Infections – HIV, varicella, hepatitis C, syphilis, malaria, leprosy

Drugs – phenothiazines, procainamide, phenytoin, quinidine, hydralazine

Lymphoproliferative disease (lymphoma, paraproteinaemia)

1.3.1 Pathogenesis

Despite clear associations between aPL and thrombosis as well as with pregnancy morbidity, the pathophysiology of these complications is not well understood, with their heterogeneity suggesting that more than one pathogenic aetiological process is involved. Proposed mechanisms of aPL-mediated thrombosis and pregnancy complications are listed in Table 1-2 and Table 1-3. The clinical significance of any one or more of these hypotheses remains unclear and reflects the likely multifactorial complex nature of this condition, as is generally the case in acute thrombosis. Despite the persistent presence of aPL in the systemic circulation, thrombotic events occur only occasionally, suggesting that the presence of aPL alone may not be sufficient to cause thrombosis and/or pregnancy morbidity. The development of aPL is probably only one step towards the development of APS, and it is likely that other factors play a role. Such ‘second hits’ or ‘triggers’ may tip the thrombotic/haemostatic balance in favour of a prothrombotic state, and include infection, endothelial injury, and other nonimmunological procoagulant factors (Pengo *et al*, 2011). The patient’s genetic make-up, in relation to candidate genes for inflammatory mediators, may also be a critical variable for the development of clinical APS manifestations. Data from several studies and systematic reviews suggest that positive LA are a stronger risk factor for the development of thrombosis than are aCL or a β_2 -GPI (DE Groot *et al*, 2005;Galli *et al*, 2003;Boey *et al*, 1983). In addition, a first thromboembolic event is considered rare in individuals positive for aPL but the increased risk in those who are ‘triple positive’ for all of LA, aCL and

a β ₂-GPI appears to be considerable, showing a 30% recurrence rate over a 6-year follow up period (Pengo *et al*, 2010).

Patients with APS inherently differ from other patients with venous and arterial thrombosis due to the presence of aPL, which are known to interfere with a number of haemostatic mechanisms. There is some evidence that patients with APS may have antibodies which recognize protein C, protein S and thrombomodulin, which are naturally occurring anticoagulants, thus generating a prothrombotic state (Malia *et al*, 1990). Generation of thrombin is a pivotal component of the haemostatic mechanism, with increased *ex vivo* thrombin generation a key marker of thrombogenic potential with predictive value for the development of recurrent VTE (Eichinger *et al*, 2008; Hemker & Beguin, 2000). Limited data suggest an activated protein (APC) resistant phenotype, demonstrated in a thrombin generation-based test, is seen in individuals with APS exhibiting LA with a history of thrombotic events. In these patients, aPL are associated with a stronger resistance to the anticoagulant effects of APC (Liestol *et al*, 2007b).

Whilst studies in animals, employing administration of aPL from humans, tend to suggest that the antibodies are directly pathogenic in pregnancy failure and thrombosis (Branch, 1990; Blank *et al*, 1991). However, no single mechanism of action has been convincingly demonstrated. Among those postulated, the more persuasive include accelerated thrombin generation through antibody-mediated concentration of prothrombin on anionic phospholipids (Rao *et al*, 1996) or increased cellular expression of tissue factor (Branch & Rodgers, 1993; Amengual *et al*, 1998) and through displacement of annexin V from phospholipid (Rand *et al*, 1997b; Rand *et al*, 1997a; Rand *et al*, 1998). Annexin V is a potent anticoagulant that serves a thrombomodulatory function in the placental circulation (Rand *et al*, 2003). It may also play a thrombomodulatory role in the systemic circulation. Annexin V is highly expressed by human endothelial cells and is present on the surface of endothelial cells (Rand *et al*, 1997a). It has been demonstrated that aPL reduce the quantity of annexin 5 on the surface of cultured placental trophoblasts (Rand *et al*, 1997b; Rand *et al*, 1997a; Rand *et al*, 1998) and endothelial cells (Rand *et al*, 1997a), where they

accelerate coagulation reactions. This has been proposed as mechanisms for pregnancy losses and thrombosis in APS (Rand *et al*, 2003). Understandably, early studies concentrated on pathogenic mechanisms involving the coagulation and haemostatic systems; however in a ground breaking publication, Girardi *et al* demonstrated activation of complement by aPL, through the classical pathway (Girardi *et al*, 2004). Despite these uncertainties regarding pathogenesis there is no doubt that, although APS is an immune-mediated condition, the principal manifestation is a prothrombotic state which leads frequently to arterial, venous and microvascular thrombosis, and even ischaemic multiorgan failure. It is unsurprising therefore that clinical management has centred around the use of various antithrombotic compounds.

There is evidence that other pathogenic factors, such as complement activation, with excess C3a and C5a generation, may contribute to thrombotic manifestations in APS, as has been observed in several animal models. Complement is implicated in APS via generation of the potent inflammatory mediator C5a which contributes to vascular inflammation and complement inhibition may ameliorate aPL-induced thrombosis (Girardi *et al*, 2003;Pierangeli *et al*, 1999). Murine models and human studies suggest involvement of the complement system in thrombotic APS (Misita & Moll, 2005;Oku *et al*, 2009). It has also been suggested that complement activation is a central mechanism in aPL-induced pregnancy loss and intrauterine fetal growth restriction (Cohen *et al*, 2011;Salmon *et al*, 2003;Salmon & Girardi, 2008). Also to further strengthen this hypothesis, low complement levels (C3, C5) have been demonstrated in patients with APS (Breen *et al*, 2012). Widespread complement activation may also play a role in catastrophic APS (CAPS) as some case reports described patients with CAPS successfully treated with complement inhibition with eculizumab (Lonze *et al*, 2010;Shapira *et al*, 2012).

Table 1-2 Proposed mechanisms for development of thrombosis in APS

Interference with the components of the coagulation cascade
Inhibition of protein C activity (acquired protein C resistance)
Inhibition of protein S cofactor activity
Inhibition of antithrombin activity
Inhibition of protein Z pathway
Activation of contact pathway
Inhibition of tissue factor pathway inhibitor
Activation of factor XI
Induction of platelet aggregation
Induction of microparticle formation
β_2 GPI-thrombin interaction
Impairment of fibrinolysis
Complement activation
Cell interactions
-Induction of proinflammatory phenotype on endothelial cells
-Induction of procoagulant activity of activity on endothelial cells and monocytes
Oxidant-mediated endothelial injury
Disruption of annexin V shield
ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif, member 13) dysfunction

Table 1-3 Proposed mechanisms for aPL-mediated fetal loss or complications

Intraplacental thrombosis
Complement activation
Inflammation
Inhibition of syncytium-trophoblast differentiation
Disruption of annexin V shield

1.3.2 Diagnosis of antiphospholipid syndrome and the identification of relationships between assay results and clinical manifestations

In the years which followed the first identification of APS in the 1980s as an important acquired prothrombotic condition there were extensive attempts to improve the sensitivity and specificity of the essential diagnostic laboratory assays, both coagulation- based and solid phase, as well as investigations into the mechanism of the prothrombotic state. Detection of aCL and a β_2 -GPI are commonly done by radioimmunoassays, enzyme-linked immunosorbent assays (ELISAs) (Loizou *et al*, 1985). In relation to assay performance, despite considerable efforts to improve sensitivity, specificity and reproducibility there remain limitations (Favaloro & Silvestrini, 2002;Pengo *et al*, 2007) although the implementation of guidelines, both laboratory and clinical, has improved the accuracy of diagnosis (Miyakis *et al*, 2006;Jennings *et al*, 2002;Keeling *et al*, 2012). Whilst the identification of the most important antigen, β_2 glycoprotein I, offered hope of improved assay specificity and performance, this has been achieved only partially (Santiago *et al*, 2001;Favaloro *et al*, 2007). It has been shown by Galli and colleagues in a systematic review, that LA is a stronger risk factor for thrombosis than aCL (Galli, 2003), and, subsequently by Pengo and colleagues that those subjects who react positively in all three of LA, aCL and a β_2 GPI assay systems have a particularly high rate of thrombosis (Pengo *et al*, 2012;Pengo *et al*, 2011)

Detection of LA requires provision of evidence that results of phospholipid-dependent screening tests are prolonged above the 99th centile of the normal healthy people (Pengo *et al*, 2009; Tripodi *et al*, 2011b). A large number of tests have been suggested for LA screening and confirmation, which have interference with different parts of the coagulation pathway. It is generally recommended that evidence must be provided that the phospholipid dependent clotting time from the screening procedure is not corrected upon the addition of equal volume of pooled normal plasma (PNP) to patient plasma. If the clotting of the 1:1 patient and PNP is higher than 99th centile of the normal healthy people, a confirmatory assay should be performed. Confirmatory tests involve repeating the abnormal screening test with an increased phospholipid concentration. The presence of LA is confirmed when the percentage of correction (screening versus confirmatory test) of the clotting time is above the local cut-off value (Pengo *et al*, 2009). A large number of tests have been suggested for LA screening and confirmation, which cause interference with different parts of coagulation pathway. The British Committee for Standards in Haematology (BCSH) recommended that two test systems employing different principles should be used to ensure that weak LA are detected and to improve specificity (Keeling *et al*, 2012). Clinical evidence based on associations with thrombosis suggest that the DRVVT (diluted Russell's viper venom test) has good value and should be one of the tests (Keeling *et al*, 2012). The other tests are usually an activated partial thromboplastin time (APTT) with a reagent with proven LA sensitivity, a modified APTT or a dilute prothrombin time.

The Kaolin clotting time (KCT) is another test used for detection of LA (Exner *et al*, 1978), however this test has poor sensitivity and specificity and is more liable to variation due to poor sample quality (Jennings *et al*, 1997). For snake venom assay, screening tests involve Taipan venom time (TVT) or Textarin time and confirmatory test, Ecarin clotting time (ECT) (Moore *et al*, 2003; Moore, 2007). The utility of these tests to detect LA in the presence of non-vitamin K antagonist oral anticoagulants (NOAC), mainly the direct factor Xa inhibitor rivaroxaban, will be discussed further in relevant chapters.

1.3.3 Clinical manifestations of APS

APS is a multisystem disorder affecting almost all the organs in the body. Clinical manifestations in patients with aPL are listed in Table 1-4 .

Table 1-4 Clinical manifestations in patients with aPL

Thrombotic- venous/arterial thromboembolic disease and microvascular thrombosis

Obstetric – recurrent miscarriage, intrauterine foetal death (IUFD), stillbirth, early severe pre-eclampsia, HELLP* syndrome, placental insufficiency, prematurity, intrauterine growth restriction (IUGR)

Cardiovascular- valvular heart disease, sterile endocarditis with embolism

Neurological – cerebral ischaemic events, chorea, dementia, psychiatric disorders, transverse myelopathy, seizures, Guillain–Barré syndrome, Sneddon's syndrome

Haematological – autoimmune thrombocytopenia, autoimmune haemolytic anaemia

Dermatological – livedo reticularis

1.3.3.1 Thrombosis

The association between aPL and thrombotic events is well established. Venous thromboembolism (VTE) is a common disorder, occurring in 1 per 1000 people per year (White, 2003) with APS accounting for approximately 10% of these acute VTE cases. Thrombosis of the deep veins of the lower limbs (deep venous thrombosis; DVT) is the most common site of venous thrombosis in APS and nearly half of patients presenting with DVT also have pulmonary embolism (PE). The most frequent site of arterial occlusion is in the cerebral vasculature, which may be thrombotic or embolic, resulting in transient cerebral ischaemic attacks (TIAs) and/or stroke. Estimates vary for the true frequency of aPL in stroke. A study by the AntiPhospholipid Syndrome Alliance For Clinical Trials and InternatiOnal Networking (APS ACTION), based on analysis of 120 full-text papers and calculation of the median frequency for positive aPL tests for clinical outcome, has

estimated the overall frequency of aPL in stroke and TIA to be 13.5% and 7%, respectively (Andreoli *et al*, 2013;Sciascia *et al*, 2014). Furthermore, more than 20% of strokes in patients younger than 45 years of age are probably associated with APS. However, the association between aPLs and stroke recurrence in older patients are conflicting. Limitations of the literature were that 60% of the papers were published before 2000, all 3 criteria aPL tests were performed in only 11% of the papers, 36% of papers used a low-titer aCL cutoff, a β 2GPI cutoff was quite heterogeneous, aPL confirmation was performed in only one-fifth of papers, and the study design was retrospective in nearly half of the papers. Therefore, authors stated that appropriately designed population studies are required to determine the true prevalence. The British Committee for Standards in Haematology (BCSH) guidelines recommend that young adults (<50 years) with ischaemic stroke should be screened for aPL (level 2C) (Keeling *et al*, 2012).

1.3.3.2 Neurological manifestations

Ischemic stroke due to arterial thrombosis is the most common neurological manifestation (>50% of central nervous system complications) in APS. Recurrent stroke can lead to multi-infarct dementia. aPL have also been linked to Sneddon's syndrome (recurrent stroke and livedo reticularis). The revised international consensus (Sydney) classification criteria do not include other neurological manifestations which may be associated with aPL such as cognitive dysfunction, headache or migraine, multiple sclerosis-like disease, transverse myelitis, epilepsy, psychiatric disorders, ocular symptoms or chorea (Miyakis *et al*, 2006). Headache is one of the most often described neurologic manifestations in patients with APS presenting with either chronic headache or episodes of migraine, although there are conflicting data on a possible relationship between migraine and aPL (Nunez-Alvarez & Cabiedes, 2011).

1.3.3.3 Pregnancy morbidity

The association between aPL and pregnancy loss was probably first noted in the 1970s (Nilsson *et al*, 1975). There is considerable evidence linking aPL to recurrent pregnancy loss (RPL) (Laskin *et al*, 1997; Rai *et al*, 1995), however the evidence is not robust for late pregnancy complications (Branch *et al*, 2001; Out *et al*, 1992). Furthermore, evidence suggests that fetal death is most specific for APS and recurrent early miscarriage may be the most sensitive manifestation of obstetric APS. However, the specificity of recurrent early miscarriage is probably low due to difficulty in fully excluding other potential causes (Miyakis *et al*, 2006) and the commonest cause of early miscarriage is a chromosomal abnormality (Porter & Scott, 2005; Stephenson, 1996).

LA is the most frequent aPL in thrombotic APS and in a systematic review, LA appears to be the strongest risk factor for thrombosis (Galli *et al*, 2003). Furthermore, a meta-analysis of the association between aPL and recurrent fetal loss in women without autoimmune disease, demonstrated that LA was associated with late RPL (OR 7.79, 95% CI 2.30-26.45), by which time the placenta is well formed. However, data were insufficient to analyse the association of LA with early miscarriages (<13 weeks) (Opatrny *et al*, 2006). IgG aCL, when combining both low and moderate to high antibody levels, were associated with both early (OR 3.56, 95% CI 1.48-8.59) and late RPL (OR 3.57, 95% CI 2.26-5.65). Restricting analysis to include only women with moderate to high aPL levels (>99th centile) increased the strength of the association (OR 4.68, 95% CI 2.96-7.40). IgM aCL was also associated with late RFL (OR 5.61, 95% CI 1.26-25.03). However, there was no association found between early RPL and a β 2GPI (OR 2.12, 95% CI 0.69-6.53) (Opatrny *et al*, 2006). Gardiner *et al*, (Gardiner *et al*, 2013) demonstrated that over 50% of women with clinical features of obstetric APS, but no thrombosis, had weak aCL and/or a β 2GPI in the absence of LA. Detection of positive or negative LA might depend on how LA testing is performed (i.e. the combination of tests used), for example the APTT and KCT or Kaolin cephalin clotting time would detect anti-FXII, which appears to be more frequent in obstetric APS, whereas the diluted prothrombin time and dilute Russell viper venom time might be more sensitive to prothrombin antibodies (Fleck

et al, 1988). In the study by Gardiner *et al*, showed IgG / IgM aCL levels and IgM a β 2GPI levels were significantly higher in patients with a history of thrombosis than in women with a history of purely obstetric APS ($p < 0.05$), while the rate of LA positivity was significantly higher in patients with a history of thrombosis compared with those with obstetric APS alone (50.5% v 15%; $p < 0.0002$).

1.3.3.4 Catastrophic APS (CAPS)

Catastrophic antiphospholipid syndrome (CAPS) is a rare but potentially fatal variant of APS, which is characterized by sudden multiple sites extensive microvascular thrombosis leading to multiorgan failure (Cervera *et al*, 2011). Clinically CAPS is characterised by clinical evidence of multiorgan involvement developing over a very short period time period, histological evidence of multiple small vessel occlusions, and laboratory confirmation of aPL (Cervera, 2010). Widespread complement activation may play a role. Although less than 1% of patients with APS develop CAPS, currently the outlook for these patients is very poor with the mortality rate as high as 50%, even with intensive treatment (Cervera *et al*, 2002). The evidence base to guide management of CAPS is weaker due to the low frequency of the condition.

1.3.3.5 Other clinical manifestations associated with APS

Although thrombosis and pregnancy morbidities are the hallmark of APS, there are other clinical manifestations associated with the syndromes, which are collectively referred to as non-criteria manifestations of APS. Thrombocytopenia, heart valve disease, chorea nephropathy and livedo reticularis are all likely to be associated with APS but none of them are specific to the syndrome (Miyakis *et al*, 2006)

1.3.4 Management of thrombosis in antiphospholipid syndrome

Typically, the initial treatment of acute thrombosis has been with heparin and vitamin K antagonists such as warfarin rather than immunosuppressive agents. For example, an review of APS by Bingley and Hoffbrand contains a report of two patients with recurrent arterial events treated with warfarin, one of whom had

initially received steroids and azathioprine with no reduction in anticardiolipin levels (Bingley & Hoffbrand, 1987). Warfarin continued to be the mainstay of treatment and in the 1990's the perception that the re-thrombosis rate was particularly high compared with other prothrombotic states led to the promotion of high intensity oral anticoagulation for the prevention of thrombosis in APS (target INR higher than the usual 2.5). However this was based upon observational data from case series at tertiary referral centres (Khamashta *et al*, 1995;Rosove & Brewer, 1992), and subsequently two important randomised controlled trials demonstrated that high-intensity warfarin is not superior to moderate-intensity warfarin (INR 2.0-3.0) for the prevention of recurrent thrombosis (Crowther *et al*, 2003;Finazzi *et al*, 2005). When the results of the 2 studies were combined in a meta-analysis, a significant excess of minor bleeding was evident in patients allocated to high-intensity warfarin (Finazzi *et al*, 2005). Use of moderate intensity anticoagulation with warfarin reduces the risk of recurrent VTE by 80% to 90% as compared to no treatment (Lim *et al*, 2006).

Despite the above, the optimal intensity of anticoagulation following arterial, as opposed to venous, thrombosis in patients with antiphospholipid syndrome remains controversial. Patients with arterial thrombosis and patients with recurrent thrombosis whilst on therapeutic anticoagulation were poorly represented in these trials of Crowther and Finazzi. Patients with arterial thrombosis represented only, 24% and 32% respectively in the two trials. Of note, of 6 patients who develop recurrence of thrombosis in the high intensity arm in the study by Crowther *et al*, 3 had subtherapeutic INRs and one had been off warfarin for a considerable period; the Finazzi study did not report on this issue. However, patients with arterial thrombosis and patients with recurrent thrombosis whilst on therapeutic anticoagulation were poorly represented in these trials. An earlier prospective cohort study, the Antiphospholipid Antibodies and Stroke Study (APASS), had found no benefit of warfarin anticoagulation (target INR 1.4-2.8) over aspirin (325mg/day) in stroke prevention (Levine *et al*, 2004). However, the study had important limitations, not least antiphospholipid antibody testing being performed only on entry to the study raising the possibility that some recruits may have had transient antibody positivity, especially in this elderly cohort (average age 60 years). More recently, Ruiz-Irastorza

and colleagues recommended, following a systematic review of cohort studies, that APS with arterial thrombosis and/or recurrent venous events should be treated with warfarin at an INR >3.0 (Ruiz-Irastorza *et al*, 2007). The task force at the 13th International Congress on Antiphospholipid Antibodies also recommended that patients with definite APS and arterial thrombosis should be treated with warfarin at an INR >3.0 or combined platelet inhibitor-anticoagulant (INR 2.0–3.0) therapy. (Ruiz-Irastorza *et al*, 2011). However this was a non-graded recommendation due to lack of consensus. In current practice many clinicians opt for a target INR of 2.5, with escalation to a higher target INR should thrombosis recur despite this.

There are limited therapeutic options for patients who have recurrent thrombotic events despite high intensity warfarin. These include addition of an anti-platelet agent or low molecular weight heparin (LMWH). However, the risk of bleeding associated with high intensity anticoagulation with warfarin and with combined anticoagulant and an anti-platelet treatment may be a concern (Keeling *et al*, 2012) .

1.3.5 Warfarin

Warfarin has been the mainstay of long term anticoagulation therapy for several decades. Warfarin inhibits vitamin K epoxide reductase (VKOR) to disrupt the vitamin K cycle, resulting in under-carboxylation of the vitamin K-dependent proteins and lowering of the functional levels of the vitamin K-dependent coagulation protein factor II, VII, IX and X and also naturally occurring anticoagulants; protein C and S levels (Furie *et al*, 1999;Stanley *et al*, 1999;Stenflo *et al*, 1974;Stenflo, 1974). Warfarin is readily absorbed from the gastro-intestinal tract. Its plasma half-life is about 40 hours. It is metabolised in the liver, and is excreted in the urine mainly as metabolites (SPC warfarin, 2014). Therapeutic doses of warfarin reduce the production of functional vitamin K–dependent clotting factors by approximately 30 to 50 percent (Horton & Bushwick, 1999). The use of warfarin is complicated by its complex pharmacokinetics and pharmacodynamics requiring frequent monitoring and dose adjustments. Warfarin has a narrow therapeutic range and care is required with all concomitant therapy. The individual product information for any new concomitant therapy should be consulted for specific guidance on

warfarin dose adjustment and therapeutic monitoring. Increased monitoring should be considered when commencing any new therapy if there is any doubt as to the extent of interactions (SPC warfarin, 2014).

Use of warfarin in patients with APS can be further complicated by the variable responsiveness of the thromboplastin reagents to LA, leading to discrepancy of INR results. Furthermore it may cause instability of INR necessitating more frequent monitoring of the INR and means the result may not accurately reflect the true level of anticoagulation. Furthermore, LA detection in patients on warfarin may be problematic because of a prolonged basal clotting time, which limits the test's diagnostic utility and ability to monitor LA status in patients with established APS (Della *et al*, 1996). However, a multicentre study of laboratory INR testing in patients with APS concluded that LA interference on the PT-INR results, measured with the majority of commercial thromboplastins is not enough to cause concern if insensitive thromboplastins, properly calibrated to assign them an instrument-specific International Sensitivity Index (ISI) are used. It was suggested that new thromboplastins, especially those made of relipidated tissue factor, should be checked for their responsiveness to LA, before they are used to monitor oral anticoagulant treatment in patients with APS (Tripodi *et al*, 2001).

1.3.6 Low molecular weight heparin

LMWHs are parenteral anticoagulants; derived from unfractionated heparin (UFH) by chemical or enzymatic depolymerization to yield fragments that are approximately one third the size of heparin. Like UFH, they are heterogeneous with respect to molecular size and anticoagulant activity. LMWHs have a mean molecular weight of 4000 to 5000, with a molecular weight distribution of 1000 to 10 000 (Weitz, 1997). LMWHs are cleared principally by the renal route, and their biological half-life is increased in patients with renal failure (Caranobe *et al*, 1985). The main mechanism of action of LMWH is the potentiation of the ability of antithrombin to inhibit coagulation factor proteases, with the main target being factor Xa. Depending on the chain length of LMWH, they have a variable factor IIa (FIIa)

activity; than its anti-Xa activity with a ratio ranging from 2:1 to 4:1 (Bendetowicz *et al*, 1994).

Routine monitoring of LMWH activity is usually not required. However, when monitoring is required in certain clinical situations or patient groups, such as with clinically significant bleeding, renal dysfunctions, extreme body weight, pregnant women, using high intensity LMWH, infants and neonates; measurement of anti-Xa, using specific calibrators is recommended (Kitchen *et al*, 2014). Heparin induced thrombocytopenia (HIT) is an immune prothrombotic disorder characterised by thrombocytopenia with or without thrombosis. IgG antibodies formed against the platelet factor 4 (PF4)/heparin complex bind to these complexes on the platelet surface and thereby cause intravascular platelet activation by cross-linking Fc γ receptor IIA; this leads to a platelet count decrease and/or thrombosis. The incidence of HIT is dependent on the degree of sulphation and the chain length of heparin, therefore incidence is higher in patients treated with UFH compared to LMWH (Martel *et al*, 2005).

1.3.7 Non-vitamin K antagonist oral anticoagulants

The non-vitamin K antagonist oral anticoagulants (NOAC) or oral direct acting anticoagulants include dabigatran etexilate (Pradaxa®) a direct thrombin inhibitor, and rivaroxaban (Xarelto®), apixaban (Eliquis) and edoxaban, (Lixiana®) which are direct anti-Xa inhibitors. These agents represent a major clinical advance as, unlike warfarin, they do not interact with dietary constituents and alcohol intake, and have few reported drug interactions which affect anticoagulant intensity (SPC Pradaxa hard capsules, 2014; SPC Xarelto film-coated tablets, 2014).

The efficacy of these anticoagulants has been demonstrated in a number of large phase III randomised clinical trials (RCT). NOAC are fixed-dose orally administered agents which exert their anticoagulant effects within hours rather than days and, due to their predictable pharmacokinetics, do not require routine laboratory monitoring with coagulation tests. Following phase III international multicentre trials in a total of about 21,500 patients, dabigatran and rivaroxaban were licensed in the UK and

Europe for the prevention of VTE in adults undergoing elective total hip replacement or knee replacement in 2009 (Eriksson *et al*, 2007;Kakkar *et al*, 2008;Lassen *et al*, 2008;Turpie *et al*, 2009).

A number of phase III clinical trials have been undertaken in conditions other than major lower limb orthopaedic surgery. Following two phase III RCT of warfarin versus single dose rivaroxaban (ROCKET-AF) (Patel *et al*, 2011) and two fixed doses of dabigatran (RE-LY) (Connolly *et al*, 2009) involving a total of 32284 patients with non valvular atrial fibrillation (AF) for the prevention of stroke or systemic embolization, rivaroxaban and dabigatran were licensed for stroke prevention in 2011. They were approved by National Institute for Health and care Excellence (NICE) and US Food and Drug Administration (FDA) for the same indication in 2012 (NICE technology appraisal guidance 249., 2012;NICE technology appraisal guidance 256., 2012;Approval of drugs by FDA available from [http: & www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs](http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs), 2014).

Rivaroxaban has been licenced for treatment and prevention of recurrent DVT and PE after an acute DVT and PE in adults following the results of EINSTEIN-DVT (Bauersachs *et al*, 2010) and EINSTEIN-PE (Buller *et al*, 2012) international multicentre randomised trials. Rivaroxaban has been approved by the US Food and Drug Administration (FDA) and NICE for the above indications in patients with non-APS. However none is currently licensed specifically for use in patients with venous thromboembolism in antiphospholipid syndrome. The use of rivaroxaban in this context is currently being evaluated in a phase II/III trial (<http://www.controlled-trials.com/ISRCTN68222801>). Owing to their structure, direct acting oral anticoagulants such as rivaroxaban and dabigatran do not interact with PF4; this has been demonstrated in *in vitro* studies, therefore HIT is unlikely (Krauel *et al*, 2012). Rivaroxaban for the treatment of HIT is currently being assessed in a phase III Open Label study (ClinicalTrials.gov Identifier: NCT01598168).

Table 1-5 Comparison of the pharmacological characteristics of NOAC

Drug	Rivaroxaban (Xarelto®)	Apixaban (Eliquis®)	Edoxaban (Lixiana®)	Dabaigatran (Pradaxa®)
Target	Factor Xa	Factor Xa	Factor Xa	Thrombin
Pro drug	No	No	No	Yes- Dabigatran etexilate
Bioavailabiliy	>80%	>50%	50%	6%
Plasma protein binding	92–95%	87%	40–59%	34–35%
Time to reach peak drug level	3 hours	3 hours	1-2 hours	2 hours
Half-life with normal creatinine clearance	9 hours	9-14 hours	9–11 hours	14-17 hours
Dosing	Fixed dose once daily	Fixed dose twice daily	Fixed dose once daily	Fixed dose twice daily
Drug monitoring	No	No	No	No
Drug interaction	CYP3A4 inhibitors, P-gp inhibitors,azole antifungals HIV protease inhibitors	CYP3A4 inhibitors, P-gp inhibitors	Potent inhibitors of both CYP3A4 and P-gpinhibitors	Proton pump inhibitors
Elimination	66% renal 33% fecal	25 renal 55% fecal	35% renal 62% fecal	Renal (80% unchanged)

Adapted from (Arachchillage & Cohen, 2013).

To date clinical trials with NOACs have been undertaken in excess of 150,000 patients. Table 1-2 summarises the clinical trial and licence status of the NOACs and as well as the situation as regards endorsement by NICE. The safety and efficacy of NOAC in children up to 18 years have not been established and no published data are yet available. Therefore, NOACs are not recommended for use in children/teenagers below 18 years of age (SPC Eliquis film-coated tablets, 2014;SPC Pradaxa hard capsules, 2014;SPC Xarelto film-coated tablets, 2014).

Table 1-6 Development status of rivaroxaban, apixaban, edoxaban and dabigatran

Indication	Rivaroxaban	Apixaban	Edoxaban	Dabigatran
Venous thromboembolism (VTE) prevention orthopaedic surgery	Phase III completed (Licensed for use) Approved by NICE and FDA	Phase III completed (Licensed for use) Approved by NICE	Phase III completed	Phase III completed (Licensed for use) Approved by NICE
Stroke prevention in nonvalvular atrial fibrillation (AF)	Approved by NICE and FDA	Approved by NICE and FDA	Use only in Japan (date)	Approved by NICE and FDA
Acute coronary syndrome (ACS)	Phase III completed	Phase II completed	No study in progress	Phase II completed
VTE prevention in medical inpatients	Phase III completed	Phase III completed	No study in progress	No study in progress
VTE treatment	Phase III completed (Licensed for use) Approved by NICE and FDA	Phase III completed (Licensed for use)	Phase III completed (Use only in Japan (date))	Phase III completed (Licensed for use)

Adapted from (Arachchillage & Cohen, 2013).

1.3.8 Management of CAPS

Anticoagulation, intravenous immunoglobulin, plasma exchange, immunosuppressive therapy, prostacyclin, fibrinolytics and defibrotide have all been used. Eculizumab is a humanised monoclonal antibody terminal complement inhibitor which is approved for the treatment of paroxysmal nocturnal

haemoglobinuria and atypical haemolytic uraemic syndrome. Individual case reports suggest possible efficacy in CAPS unresponsive to other treatments (Lonze *et al*, 2010; Shapira *et al*, 2012). Eculizumab is under evaluation in a phase II clinical trial for its ability to prevent CAPS after kidney transplantation in patients with a prior history of CAPS (<http://clinicaltrials.gov/show/NCT01029587>). Rituximab is a chimeric monoclonal antibody against CD20, which is primarily located on B cells. It is an effective treatment for B cell non-Hodgkin's lymphoma (Molina, 2008) and has been used in several autoimmune diseases including SLE and thrombotic thrombocytopenic purpura (Terrier *et al*, 2010; Scully *et al*, 2012). There are reported cases of use of rituximab in patients with CAPS. Espinosa *et al* reviewed 9 patients. Two patients died, but notably in three of the surviving patients tests for antiphospholipid antibodies became negative after rituximab treatment (Espinosa *et al*, 2011).

1.3.9 Management of asymptomatic carriers of aPL

Another unanswered question is whether primary thromboprophylaxis is indicated in asymptomatic subjects with antiphospholipid antibodies and how the risk of a first thrombosis can be stratified in order to personalise treatment. Based on limited data, unselected asymptomatic antibody positive patients have an annual thrombosis risk of around 3% (Finazzi, 2012). In a prospective multicentre Italian registry with 360 unselected individuals with antiphospholipid antibodies (>90% of them positive for lupus anticoagulant), 34 patients developed a thrombotic complication, which accounted for a total incidence of 2.5% per year, the median follow up period being 3.9 years (range 0.5-5.0) (Finazzi & Barbui, 1996). The Antiphospholipid Syndrome Study Group of the Italian Society of Rheumatology prospectively evaluated the incidence of, and risk factors for, a first vascular event in 258 asymptomatic individuals with antiphospholipid antibodies. The annual incidence rate of first thrombosis was 1.86% with a median follow up of 35 months (range 1-48). Multivariate regression analysis identified hypertension and the presence of lupus anticoagulant as independent risk factors for development of thrombosis (Ruffatti *et al*, 2011). In an important study 104 subjects positive for all three of LA, aCL and anti- β_2 GPI were followed up for a mean of 4.5 years. There were 25 first thrombotic

events (5.3% per year) with a cumulative incidence of 37.1% (95% confidence interval [CI]: 19.9%-54.3%) after 10 years suggesting that subjects with so-called triple positivity may represent a high risk group for thrombosis. Aspirin did not significantly affect the incidence of thromboembolic events (Pengo *et al*, 2011). The Antiphospholipid Antibody Acetylsalicylic Acid (APLASA) study is the first clinical trial of individuals with asymptomatic, persistently positive antiphospholipid antibodies randomised to receive a daily dose of 81mg of aspirin or placebo (Erkan *et al*, 2007). There was no significant difference in outcome between the two arms. The overall estimated rate of thrombosis was 2.75 per 100 patient-years for individuals treated with aspirin and 0 per 100 patient-years for the placebo treated subjects. A limitation of this study was small sample size. However, based on current evidence, thromboprophylaxis with aspirin and/or vitamin K antagonist is not recommended routinely for asymptomatic, antiphospholipid antibody positive individuals. Attention to additional risk factors is advisable e.g. avoidance of smoking, oestrogen-containing oral contraception and hormone replacement therapy. Whether primary prophylaxis with aspirin is useful for some subjects who may be at particularly high risk of thrombosis, such as those with specific patterns of antiphospholipid antibodies, especially triple positivity, remains to be established. Nevertheless the task force at the 13th International Congress on Antiphospholipid Antibodies suggested long-term thromboprophylaxis with low-dose aspirin in those with a high-risk antibody profile, especially in the presence of other thrombotic risk factors (Ruiz-Irastorza *et al*, 2011). Furthermore, they recommended that patients with SLE and lupus anticoagulant or persistent anticardiolipin antibody at medium–high titre receive primary thromboprophylaxis with hydroxychloroquine and low-dose aspirin (LDA). It is apparent that more high quality evidence is required.

1.3.10 Alternatives to anticoagulants in the management of the antiphospholipid syndrome

Although anticoagulant therapy has been the mainstay of management in antiphospholipid syndrome, potential alternative or additional strategies are emerging. These include hydroxychloroquine, HMG-CoA reductase inhibitors, and rituximab and complement inhibitors.

Hydroxychloroquine

Hydroxychloroquine is a well-established treatment for patients with SLE and rheumatoid arthritis due to its anti-inflammatory effects. It is currently recommended as a baseline therapy in all patients who have no contraindications (Ruiz-Irastorza *et al*, 2010). In addition to anti-inflammatory activity hydroxychloroquine may reduce blood coagulability, purportedly through reduced red cell sludging and blood viscosity and possibly through some inhibition of platelet reactivity (Madow, 1960; Ernst *et al*, 1984; Jancinova *et al*, 1994; Espinola *et al*, 2002; Petri, 2011). Because of its perceived antithrombotic activity, in the past it was employed in prophylaxis against deep vein thrombosis after hip surgery (Carter *et al*, 1971; Johnson & Charnley, 1979). More recently it has been shown to protect against thrombosis and increase survival in patients with SLE. Recent consensus guidelines for management of antiphospholipid syndrome support the use of hydroxychloroquine as an adjuvant therapy to anticoagulation in patients with recurrent thrombosis despite anticoagulation (Ruiz-Irastorza *et al*, 2011). A current phase III multicentre, international, prospective randomised-controlled trial is exploring the effect of hydroxychloroquine as primary thrombosis prophylaxis in individuals with persistently positive antiphospholipid antibodies and no history of thrombosis or systemic autoimmune diseases as a part of APS ACTION (Erkan & Lockshin, 2012). <https://clinicaltrials.gov//NCT01784523>.

Statins

Statins inhibit the enzyme HMG-CoA reductase which has a central role in hepatic cholesterol production. Statins are widely prescribed to reduce cardiovascular risk. It has become increasingly apparent that the drugs have pleiotropic effects including anti-inflammatory and antithrombotic actions (Ferrara *et al*, 2003; Halcox & Deanfield, 2004). For example statins down-regulate inflammatory cytokines in vascular endothelial cells, decrease the expression of adhesion molecules in monocytes and influence leucocyte-endothelial cell interactions (Ferrara *et al*, 2003). In addition there is evidence of increased fibrinolysis and decreased tissue factor mRNA in response to statins. It is noteworthy therefore that antiphospholipid antibodies have been shown to increase pro-inflammatory cytokine production by

vascular endothelial cells and to increase transcription and expression of tissue factor (Ferrara et al, 2004). These observations raise the question of possible benefit from statin use in APS, supported by a small study which showed a reduction in some inflammatory markers such as vascular endothelial growth factor and tissue necrosis factor alpha (Jajoria et al, 2009). A phase II clinical trial assessing the safety and efficacy of fluvastatin in reducing the risk of cardiovascular disease and thrombosis in patients with aPL or APS is currently underway (<http://clinicaltrials.gov/NCT00674297>).

Rituximab

Rituximab appears to be an effective treatment for thrombocytopenia and haemolytic anaemia associated with antiphospholipid antibodies (Erre *et al*, 2008) and may improve some other occasional manifestations of antiphospholipid syndrome such as skin ulcers (Erkan *et al*, 2013) [RITAPS: A Pilot Study of Rituximab For The Anticoagulation-Resistant Manifestations Of Antiphospholipid Syndrome] . Like all of these novel approaches, the precise role of rituximab in the management of antiphospholipid syndrome remains to be determined.

Complement inhibition

The experimental evidence that complement activation may have a role in some manifestations of APS has led to speculation that inhibition of complement may be a useful therapeutic strategy. In addition to use of eculizumab in some patients with CAPS, phase II multicentre clinical trial of evaluating the safety and tolerability of intravenous (IV) ALXN1007 (complement inhibitor) in persistently aPL -positive patients with at least one of the non-criteria manifestations of APS: aPL-nephropathy, skin ulcers and/or thrombocytopenia is underway <https://clinicaltrials.gov/NCT02128269>.

1.3.11 Treatment of obstetric APS

In a meta-analysis of data from five trials involving 334 patients (Mak *et al*, 2010), the overall live birth rates were 74.27 and 55.85% in patients who received a combination of heparin and aspirin versus that in those treated with aspirin alone. Patients who received combination treatment had significantly higher live birth rates (RR 1.301; 95% CI 1.040, 1.629) than with aspirin alone. No significant differences in pre-eclampsia, preterm labour and birth weight were found between both the groups. The American College of Chest Physicians (ACCP) guidelines (Bates *et al*, 2012) recommend treating women with obstetric APS, who meet revised Sapporo criteria, should be treated with heparin and LDA in the antepartum period as soon as pregnancy is confirmed. Use of heparin has become the standard of care in many centres and LMWH is generally favoured due to a lower incidence of HIT and low risk of osteopenia compared to UFH (Robertson & Greaves, 2006). A paradox has been the apparent efficacy of heparin, an antithrombotic, in preventing early miscarriage occurring prior to placentation. The key observations made by Girardi *et al* in 2004 that complement activation may be important in pathogenesis, and that heparin may be effective through inhibition of complement rather than as a coagulation inhibitor, provides a possible explanation for this apparent efficacy in prevention of early miscarriage in antiphospholipid syndrome (Girardi *et al*, 2004).

A small prevalence study in 20 women with obstetric APS (Rai *et al*, 1995), demonstrated that persistent weak aCL (<99th percentile) was associated with a >90% fetal loss rate in untreated pregnancies of women with recurrent miscarriage, and with significantly improved pregnancy outcome following treatment with low-dose aspirin or heparin and aspirin (Rai *et al*, 1997; Granger & Farquharson, 1997). Many obstetricians choose to treat other women with RPL, but no evidence of aPL, with LDA with or without heparin (Mekinian *et al*, 2012). Pregnancy outcome was considerably improved in women diagnosed as obstetric APS either with moderate to strong, or weak aPL when they were treated with heparin/LDA. In a retrospective observational cohort study by Cohn *et al* (Cohn *et al*, 2010), 79% of women with obstetric APS diagnosed using the aCL cut off values \geq log-transformed mean plus two SDs of results in 50 healthy adults, received aspirin and heparin during their

pregnancy had a live birth, compared to 62% of women with aPL who received aspirin only (adjusted OR 2.7, 95% CI 1.3–5.8). Given the potential devastating impact of long term disability associated with late obstetric morbidity, prospective data suggesting a benefit of standard treatment for obstetric APS in women with non-criteria manifestations, and the favourable risk/benefit ratio of heparin plus aspirin treatment during pregnancy for obstetric APS, it appears reasonable at present to offer this treatment during pregnancy to women with low-positive aPL associated obstetric morbidity as soon as the pregnancy is confirmed during the first trimester (Arachchillage *et al*, 2014b).

1.4 Aims of my thesis

The aims of my thesis were to:

1. Investigate the frequency and nature of activated protein C resistance (APCr) and resistance to activation of endogenous protein C activation using thrombin generation in patients with thrombotic antiphospholipid syndrome
2. Assess the effects of rivaroxaban on the prothrombin time and LA detection; and assess the influence of aPL on the anticoagulant activity of rivaroxaban
3. Define the impact of rivaroxaban and warfarin on thrombin generation and haemostasis activation in thrombotic patients with or without APS

The purpose of my work was to explore the role of APCr in the pathogenesis of thrombotic APS and to inform the use of the rivaroxaban in patients with thrombotic APS, which is of crucial importance for effective and safe management of these patients

1.4.1 Frequency and nature of activated protein C resistance (APCr) and resistance to activation of endogenous protein C activation

The anticoagulant protein C pathway plays a central role in the regulation of coagulation and maintenance of the fluidity of blood. Activated protein C (APC)

exerts its anticoagulant effects by proteolytic inactivation of factor Va and factor VIIIa (Kalafatis *et al*, 1994; Nicolaes *et al*, 1995). aPL interference with the anticoagulant activity of APC, resulting in acquired APC resistance (APCr) has been proposed as a key mechanism of aPL-initiated thrombosis (3), although a direct association between an aPL-dependent APCr phenotype and thrombotic events has not been established (Male *et al*, 2001; Nojima *et al*, 2005). The most common antigenic targets of aPL antibodies are β_2 glycoprotein I (β_2 GPI) and prothrombin. However, protein C, as a phospholipid-binding protein, may also be an important target (Nojima *et al*, 2002), and aPL inhibition of the thrombomodulin mediated activation of protein C, as well as the anticoagulant activity of APC (Malia *et al*, 1990), have been observed. Furthermore, β_2 GPI binding to protein C can modulate its action (Keeling *et al*, 1993) and its subsequent binding to phospholipid surfaces, thereby increasing the risk of thrombosis (Mori *et al*, 1996).

Generation of thrombin is a pivotal component of haemostasis, with increased *ex vivo* thrombin generation (TG) a key marker of thrombogenic potential (Eichinger *et al*, 2008; Hemker & Beguin, 2000). Comparison of *ex vivo* TG in the presence and absence of recombinant human activated protein C (rhAPC) or Protac®, an enzyme isolated from *Agkistrodon contortrix contortrix* venom which converts endogenous protein C into APC was performed to assess the function of the protein C system.

1.4.2 Interactions between rivaroxaban, aPL and haemostasis activation

It is clinically relevant to establish interactions between rivaroxaban with aPL and haemostasis, for the following main reasons. First, to establish anticoagulant intensity and thus efficacy in patients with aPL treated with rivaroxaban. Secondly, to enable monitoring of anticoagulant intensity in patients with thrombotic APS treated with these agents. Thirdly, it is important to establish how to detect LA in patients taking rivaroxaban. aPL are associated with multisystem clinical manifestations including haematological, dermatological, neurological and valvular cardiac lesions, and therefore continued aPL activity monitoring as a marker to assess disease activity is essential. There is a paucity of information on laboratory interactions between NOAC and aPL. Dabigatran, as a direct thrombin inhibitor,

prolongs the activated partial thromboplastin time (aPTT) and rivaroxaban does so to a lesser extent. Therefore, it is possible that these agents could modify the coagulation tests used to detect LA. In an *ex vivo* study on 19 patients, one of whom was stated to have a LA, rivaroxaban was reported to increase dilute Russell's viper venom time (DRVVT) ratio (Merriman *et al*, 2011). In addition, limited *in vitro* studies on the addition of rivaroxaban to plasma from patients with LA suggest that Taipan and Ecarin, snake venoms which directly activate prothrombin and which can be used to detect LA, are not affected by the presence of rivaroxaban (van Os *et al*, 2011). A detailed and systematic approach was undertaken in the work for my thesis to establish how best to test for LA in APS patients treated rivaroxaban.

Chapter 2 Methods

2.1 Ethical Committee Approval

The study was reviewed and given a favourable ethical opinion by the Leicester Research Ethics Committee as a proportionate review body and approved by the Research and Development office at University College London Hospitals (UCLH) NHS Trust (Reference number:13/EM/0150). Patients were provided with written information sheets and informed consent was obtained prior taking venous blood samples at the time of venepuncture for their routine clinic blood tests. All patients from Rivaroxaban in Antiphospholipid Syndrome (RAPS) trial (<http://www.controlled-trials.com/ISRCTN68222801>) had consented to samples being taken at study time point for translational research (Reference number: 12/SC/0566). Following written informed consent, blood samples from healthy volunteers (haematology staff and friends) were obtained as the controls.

2.2 Blood sample collection

Venous blood samples for measurement of haemostatic markers, thrombin generation and complement activation markers were obtained using 21 gauge butterfly needle with minimal stasis. Blood was drawn into 5ml citrated BD Vacutainers® (BD, Plymouth, UK) containing 0.5ml of 0.105M buffered tri-sodium citrate at a ratio of 1 part anticoagulant to 9 parts blood and into tubes containing EDTA. Within 1 hour of collection, citrated platelet poor plasma (PPP) and EDTA plasma were prepared by double centrifugation at ambient temperature (2000g for 15 minutes x 2) and aliquots of each type of plasma were frozen to -80°C. Immediately prior to analysis the samples were thawed for 10 minutes at 37°C in a water bath.

2.3 Prothrombin time/ International Normalised Ratio (INR)

Reagents

- Thromboplastin reagents (i.e. Innovin®, Recombiplastin 2G etc), which contain recombinant human tissue factor, Calcium Chloride (CaCl₂) and heparin neutralising reagent (hexadimethrine bromide).

Method

The PT was measured on a Sysmex CS-5100 analyser (Sysmex UK Ltd, Milton Keynes, UK)

The International Normalised Ratio (INR) was calculated using the following formula: $INR = PTR^{(ISI)}$; where PTR = Patient PT/geometric mean normal PT; ISI = International sensitivity index

The geometric mean normal PT was established by testing a minimum of 30 normal samples, and can vary from lot to lot of reagent.

2.4 Rivaroxaban anti- Xa assay (Chromogenic assay for rivaroxaban level)

Principle

Biophen DiXaI is a chromogenic assay for *in vitro* quantitative measurement of direct factor Xa inhibitors such as rivaroxaban, on citrated human plasma. The assay is based on the inhibition of exogenous Factor Xa (FXa), by the tested DiXaI, and hydrolysis of a Factor Xa specific chromogenic substrate, by the residual factor Xa. Para-nitro aniline (pNA) is released from the substrate and measured at 405nm. The amount of pNA released is inversely proportional to the concentration of the FXa inhibition in the test sample.

Reagents: Biophen DiXaI (Hyphen BioMed, NEUVILLE-SUR-OISE, France).

Method

The assay was performed on a Sysmex CS-2000i (Sysmex UK Ltd, Milton Keynes, UK)

Calibration was performed using the Biophen Rivaroxaban calibrator with a rivaroxaban range of 0 to 500ng/mL.

- Samples were tested in duplicate and two control samples with known rivaroxaban level (C1: rivaroxaban level 40-140ng/mL and C2: rivaroxaban level 220-380ng/mL) were tested.
- Rivaroxaban concentration (anti-Xa) of test samples was derived from the calibration curve
- Samples with rivaroxaban levels above the range of the standard curve were automatically re-tested at a higher dilution.

2.5 Dilute Prothrombin Time (dPT) in KC4A

Principle

Clotting is initiated by activating the tissue factor (TF) coagulation pathway with tissue factor in the presence of calcium ions. TF binds to factor VIIa resulting in the activation of FIX and X. Factor Xa converts prothrombin to thrombin which initiates clot formation by cleaving fibrinogen to fibrin. Activation of tissue factor pathway bypasses the contact (intrinsic) pathway and excludes any interference from deficiencies of factor XII.

Reagents

- ACTICLOT® dPT^{TM r} reagents (American diagnostic GmbH, Germany) which consist of LA buffer, LA phospholipids and dPT activator
- KC4A coagulometer

Method

- In the screening step, the patient plasma is mixed with LA buffer and dPT activator. The clot time is determined in semi-automated KC4A coagulometer. A positive result is indicated by a prolonged clot time relative to normal range.
- In the confirmatory step, the patient sample is mixed with LA phospholipids and dPT activator. A positive result is indicated by a significant reduction in the clot time relative to the screening step.

2.6 Dilute Russell Viper Venom Time (DRVVT) (in house method)

Principle

Russell's viper venom [RVV] isolated from the snake *Daboia russelii* contains a potent activator of factor X. In the presence of phospholipid [PL], prothrombin and calcium ions resulting FXa, causes fibrin clot formation. In the LA, clotting time is prolonged. As the RVV directly activates factor X, the test is only affected by LA and deficiencies of factors II, V and X. Substitution of the diluted phospholipid reagent with a platelet neutralisation (washed, activated platelets), demonstrates the phospholipid specificity of the antibody. If LA is present, the clotting time becomes relatively shortened in the platelet neutralisation step.

Reagents

- Russell Viper Venom (Diagnostic Reagents Ltd, Thame, UK)

2mg/ml vial of RVV was reconstituted with 2ml of isotonic saline and 30 µl of RVV further diluted in 2.5ml Imidazole /BSA buffer (Imidazole buffer (0.5M) was made using 3.4g Imidazole and 5.8g NaCl in to 900ml of water and then adjust the pH to 7.3 with final volume of 1L. Imidazole/BSA buffer was made by dissolving 1g of bovine serum albumin (A 7030, Sigma) in 100ml of Imidazole buffer)

- Phospholipid reagent (“Bell and Alton” Platelet Substitute, Diagnostic Reagents Ltd). One vial was reconstituted in 5ml of distilled water which was then diluted 1 part with 3 parts of Imidazole buffer.
- Pooled normal Plasma (platelet poor) prepared locally. Each vial of lyophilised platelet poor plasma was reconstituted in 1ml of distilled water, left for 5 minutes and mixed gently.
- 0.025M CaCl₂.

Method

- 100µL of normal plasma and 100µL of phospholipid reagent were mixed at 37C.
- 100µL of reconstituted RVV was added and incubated for 30 seconds.
- 100µL of 0.025M CaCl₂ was added and time to clot formation was recorded.
- Procedure was repeated with platelet neutralisation reagent instead of phospholipid and ratio was calculated.

Normalised screen, confirm and screen/confirm ratio are calculated as follows:

$$\text{Normalised Screen ratio} = \frac{\text{Patient Plasma Screen time}}{\text{Normal Plasma Screen time}}$$

$$\text{Normalised Confirm ratio} = \frac{\text{Patient Plasma Confirm time}}{\text{Normal Plasma Confirm time}}$$

$$\text{Normalised Test: Confirm ratio} = \frac{\text{Normalised Screen ratio}}{\text{Normalised Confirm ratio}}$$

If the normalised test: confirm ratio >1.2 it confirms the presence of LA

2.7 Dilute Russell Viper Venom Time (DRVVT) (IL ACL TOP 500

Method

Reagents

- DRVVT Screen Reagent
- DRVVT Confirm Reagent
- LA Negative Control
- LA Positive Control
- Pooled normal plasma

All reagents are from Instrumentation laboratory, Warrington, UK

Method

LA screen and confirm steps were done are per manufacture's standard protocol and normalised screen, confirm and screen/confirm ratio were calculated as described in 2.6

2.8 Dilute Russell Viper Venom Time (DRVVT) (CS-5100 / CS-2000i

analyser: Sysmex UK Ltd, Milton Keynes, UK)

- LA1 screening reagents
- LA2 confirmation reagents
- LA Negative Control
- LA Positive Control
- Pooled normal plasma

All reagents are from Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany.

Method

LA screen and confirm steps were done as per manufacture's standard protocol and normalised screen, confirm and screen/confirm ratio were calculated as described in 2.6.

2.9 Taipan venom test /Ecarin clotting time (KC4A method)

Principles

The Taipan venom time (TVT) employs a reagent isolated from the venom of the Taipan snake (*Oxyuranus scutellatus*) that directly activates prothrombin in the presence of phospholipid and calcium. Therefore it is independent of the plasma concentration of FV, FX and VII. Taipan snake venom (TSV) may be used to detect lupus anticoagulant (LA). Phospholipid, TSV and calcium ions are added to plasma, causing the conversion of prothrombin to thrombin and fibrin clot formation. By contrast, *Echis carinatus* venom (Ecarin) is able to convert native and PIVKA prothrombin to meizothrombin in the absence of Ca^{2+} and phospholipid. These characteristics make Taipan snake venom time (TSVT) and Ecarin clotting time (ECT), when performed in parallel, a useful assay in the detection of LA. If LA is present in the sample, the phospholipid dependent TSVT will be extended, while the ECT will remain normal.

Reagents

- Imidazole Buffer (0.5M)
- Taipan Snake Venom (Diagnostic Reagents). One vial was reconstituted in 5ml of distilled water and diluted with 1 in 1 0.025M CaCl_2
- Phospholipid reagent ("Bell and Alton" Platelet Substitute, Diagnostic Reagents Ltd) One vial was reconstituted in 5ml of distilled water which was then diluted 1 part with 3 parts of Imidazole buffer.

- Normal Plasma (platelet poor) prepared locally. Each vial of lyophilised platelet poor plasma was reconstituted in 1ml of distilled water, left for 5 minutes and mixed gently.
- Ecarin (*Echis carinatus*) venom (Diagnostic Reagents Ltd). Reconstitute with 2.0ml distilled water
- 0.025M CaCl₂.

Method

TVT

- 100µL of normal plasma and 100µL of phospholipid reagent were mixed at 37C.
- 100µL of TSV /CaCl₂ was added and time to clot formation was recorded.

ECT

- 100µL of Ecarin is added to 100µL of test plasma and time to clot formation is recorded.

2.10 Textarin® /Ecarin ratio to detect lupus anticoagulants

Principle

Textarin® is a serine proteinase, isolated from the venom of the Australian Brown Snake *Pseudonaja textilis*; is a phospholipid, factor V and calcium dependent prothrombin activator. It is independent of other plasma clotting factors and generates thrombin activity from prothrombin. As the action of Textarin® is affected by antiphospholipid antibodies, if the Textarin® clotting time is performed in parallel to the phospholipid –independent ecarin clotting time, , the ratio can be used to detect LA.

Instruments and reagents

- Textarin® (Pentapharm Ltd)
- 0.025M CaCl₂
- Immunoglobulin solution from patients with positive LA
- Pooled normal plasma
- Amelung-Coagulometer (KC4A)

Method

- 100µL of normal plasma and 100µL of phospholipid reagent were mixed at 37 C°.
- 100µL of reconstituted Textarin was added and incubated for 30 seconds.
- Then 100µL of 0.025M CaCl₂ was added and time to clot formation was recorded.

2.11 TVT and ECT in Sysmex CS-5100i /analyser (Sysmex UK Ltd)

Reagents

- Taipan (*Oxyuranus scutellatus*) venom (Diagnostic Reagents Ltd).

Reconstitute with 5.0ml deionised water, dilute 1:1 with Calcium Chloride/BSA and use immediately (tests to be completed within 1 hour).

- Ecarin (*Echis carinatus*) venom (Diagnostic Reagents Ltd).

Reconstitute with 2.0ml distilled water (Stable for 7 days at 2-8°C). Dilute 1/5 with Imidazole Buffer (Stable for 1 working day at 2-8°C).

- Bell and Alton Platelet Substitute (Diagnostic Reagents Ltd).

Reconstitute with 5ml deionised water and dilute 1/8 with Imidazole Buffer.

- Calcium Chloride/ bovine serum albumin (BSA) - 25 mMol/L Calcium Chloride solution (Siemens Healthcare, Ref: ORHO37); add 1g/100ml Bovine Serum Albumin (A-7030, Sigma Aldrich).

- Imidazole buffer (Siemens Healthcare, Ref: OQAA33) Ready to use.
- LA Control Low (Siemens Healthcare, Ref: OQWE11) Reconstitute with 1.0ml deionised water.
- LA Control High (Siemens Healthcare, Ref: OQWD11) Reconstitute with 1.0ml deionised water.

2.11.1 TVT

- 40µL of plasma mixed with 40µL of Bell and Alton platelet substitute and incubate for 60seconds. 80µL of Taipan + CaCl₂ was added over 130 seconds and measured the reaction time over 300seconds.

2.11.2 ECT

- 100µL of ecarin is added to 50µL of plasma over 110 seconds and the reaction time is measured over 180seconds.

2.12 D dimer

Principle of method-Immunoturbidometric assay

D dimer is generated when cross –linked fibrin is degraded by plasmin. Dilutions of standards and test plasma are mixed with a reaction buffer and latex particles coated with a monoclonal antibody to a D dimer epitope (antigen). The antigen-antibody complexes produced by the addition of samples containing D dimer causes increased turbidity.

Reagents: Innovance D-dimer kit

Method

The assay was performed in Sysmex CS 5100 analyse using manufacture's standard protocol. The standard curve ranging from 1.5 to 5.0mg/L FEU was generated using

the reference plasma by the analyser with its auto-dilution mode. Normal D dimer reference level is <0.55mg/L FEU.

2.13 Prothrombin fragments 1.2 (F1.2)

Principle of method –ELISA assay

Prothrombin fragment 1.2 (F1.2), is an activation peptide released from prothrombin during its factor Xa –catalysed conversion to thrombin

Reagents: Enzygnost® F1.2 (monoclonal) ELISA kit (Siemens Healthcare, Marburg, Germany)

Method

Assay was performed in an ELISA using manufacture’s standard protocol.

2.14 Thrombin-Antithrombin complexes (TAT)

TAT complexes formed following the neutralization of thrombin by antithrombin III (AT) have been used as a surrogate marker for thrombin generation

Principle of method –ELISA assay

Reagents: Enzygnost® TAT micro kit (Siemens Healthcare, Marburg, Germany)

Assay was performed in an ELISA using manufacture’s standard protocol.

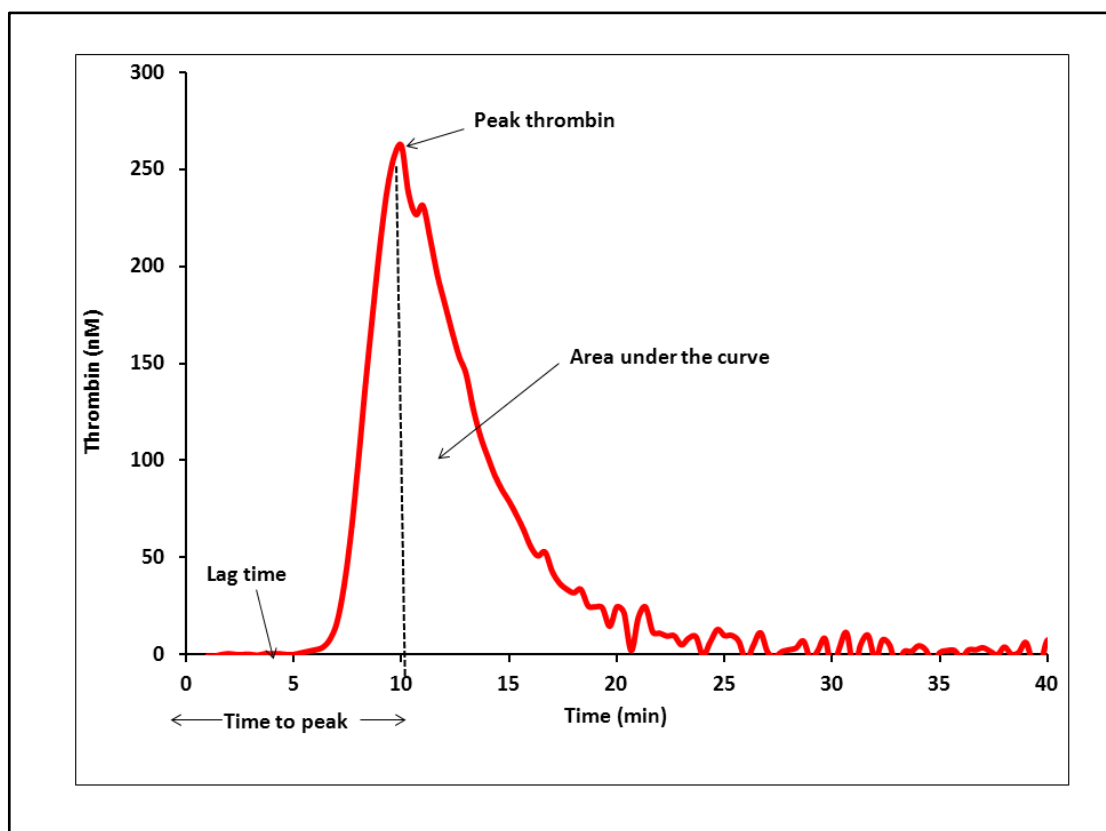
2.15 Thrombin generation test

Principle of method

TG using the calibrated automated thrombogram (CAT) method was developed by Hemker et al (2003). The CAT system allows continuous measurement of TG in clotting plasma. Thrombin generation is initiated by the addition of a combined

calcium/fluorogenic substrate (Z-Gly-Gly-Arg-AMC) in the presence of tissue factor (TF)/phospholipid trigger. The fluorescent signal generated is transmitted to and processed by a computer with specific software (Thrombinoscope™) to assess/measure thrombin generation. A thrombin calibrator (Thrombin α_2 -macroglobulin complex) is run in parallel with each sample to compensate the inner-filter effect and substrate consumption. The fluorescent signal from the sample is compared to that of the calibrator by the Thrombinoscope software. A TG curve is generated, (an example of which is shown in Figure 2-1).

Figure 2-1 A representative thrombin generation curve



Reagents

- HEPES Buffer: 20mM HEPES, 140mM NaCl, 0.02% Sodium Azide at pH 7.35
- Fluo-Buffer: 20mM HEPES buffer with 60mg/mL BSA

- PPP –reagent (Thrombinoscope BV, Maastricht, The Netherlands) 5 pM Tissue Factor and 4 μ M Phospholipids reconstituted with 1ml of H₂O
- Thrombin calibrator (Thrombinoscope BV) reconstituted with 1ml of H₂O
- Fluorogenic substrate: 100 mM solution of Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). in Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Poole, UK)
- FluCa reagent: 2.275mL Fluo-Buffer, 0.26 mL 1M CaCl₂ (incubated at 37°C). Immediately prior to use 65 μ L of 100 mM Fluorogenic substrate was added and vortex mixed.
- CAT system

Method

Six wells were allocated for each sample: three for monitoring TG and three for the thrombin calibrator (TC) in a 96- well transparent, microtitre plate (Immulon 2HB clear U-bottom, Diagnostic Stago). 20 μ L of PPP reagent was added to each TG well. 20 μ L of thrombin calibrator was added to each thrombin calibrator well and 80 μ L of plasma sample was added to each well (TG and TC). The microtitre plate was transferred to a pre-warmed Ascent Fluorskan plate reader. 20 μ L of FluCa reagent was dispensed automatically by the instrument and the reaction was monitored for 60 minutes.

Results and raw data were then transferred to a Microsoft Excel spread sheet. Peak TG and area under the curve; known as endogenous thrombin potential (ETP) were normalised relative to reference plasma, tested in each analytical run, and reported as a percentage of the reference plasma. The lag time and time to peak were also recorded.

2.16 Activated protein C resistance determined by thrombin generation

Principle of method

TG was performed in the absence and presence of activated protein (APC) C or Protac® which activates endogenous protein. Resistance to APC or Protac® was calculated as a percentage inhibition in relation to normal reference plasma.

Reagents

- Reagents as per TG
- Activated protein C (Eli Lilly) stock in 5 μ mol
- Coagulation reference plasma (Technoclone GmbH, Austria, Vienna)

Method

- 20 μ L of PPP reagent was added to each TG measurement well.
- 20 μ L of thrombin generation calibrator was added to calibrator well (as the same reference plasma was used for TG with different concentrations of APC, same calibrators were used all TG reactions)

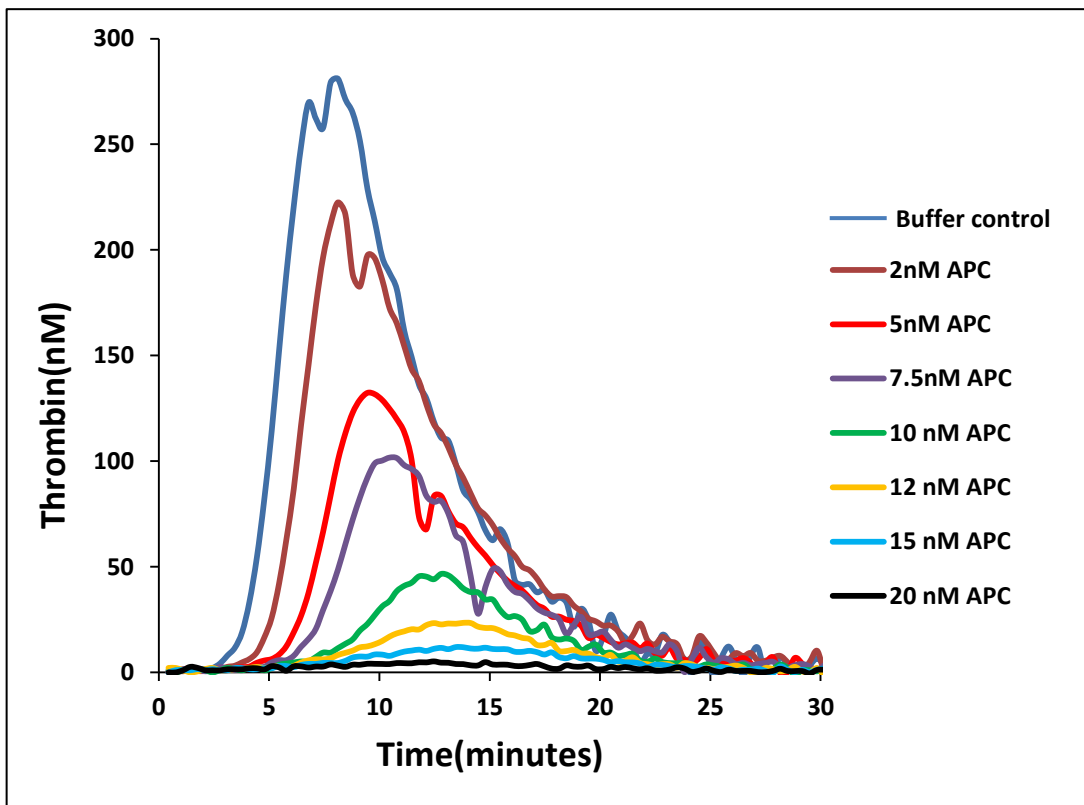
Keeping the plasma volume constant at 65 μ L, 15 μ L of APC (1 to 20nM) in buffer containing 20mM HEPES, 140mM NaCl pH 7.35 in 1% was added. Following 10 mins incubation period, 80 μ L of the plasma +APC added to TG wells which in to give a final assay concentration of APC as shown in Table 2-1.

Table 2-1 Preparation of increasing concentration of rhAPC for thrombin generation

Final rhAPC concentration in each thrombin generation test well (nM)	Final concentration of rhAPC in each 15μL of buffer (nM)
2	16
5	40
7.5	60
10	80
12	96
15	120
20	160

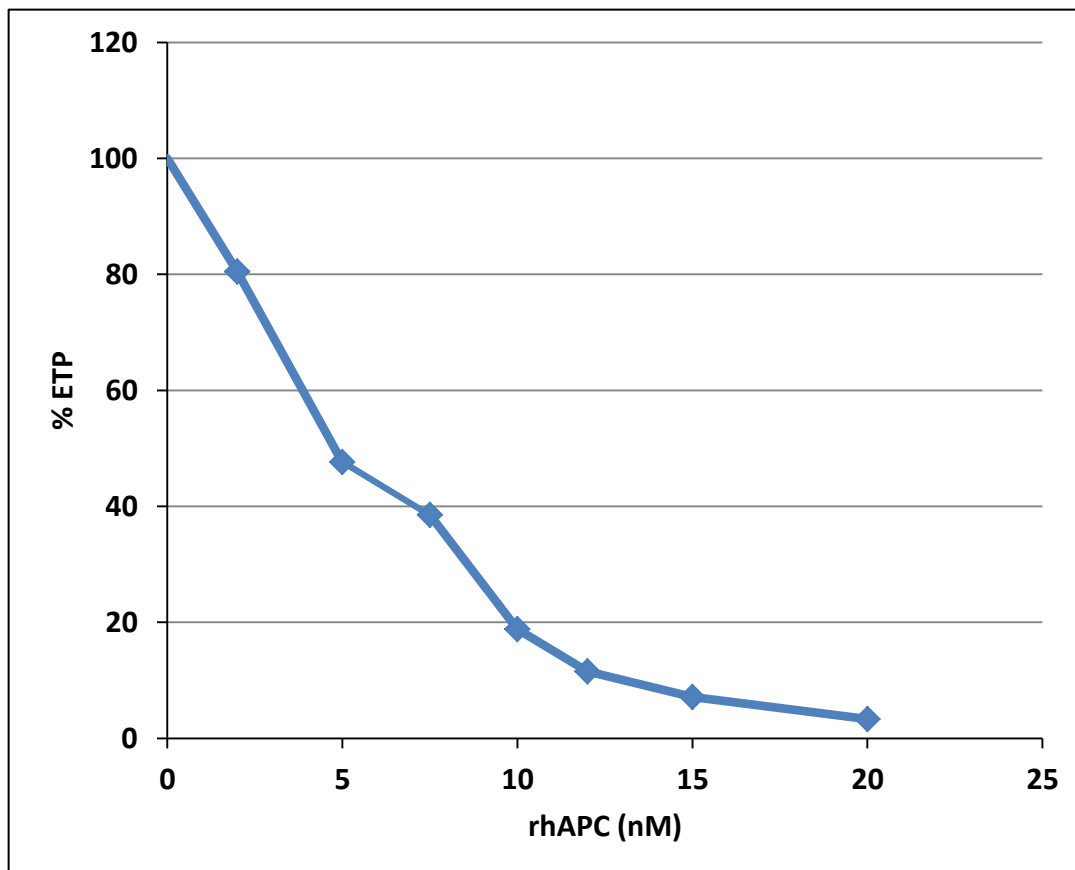
As anticipated, there was an APC concentration dependent reduction in thrombin generation. APC concentration that produced about 50% reduction in thrombin generation (5nM) was selected and repeated the assay to confirm the reproducibility and to check the inter-assay coefficient of variation (n=8).Results of the thrombin generation are shown below Figure 2-2.

Figure 2-2 Thrombin generation with increasing concentration of APC



The following graph (Figure 2.3) demonstrates the concentration dependent reduction in TG in reference plasma.

Figure 2-3 APC concentration dependent inhibition of Thrombin generation



2.16.1 The assessment of resistance to activation of endogenous protein C using Protac®

Reagents

- Reagents as per TG
- Coagulation reference plasma (Technoclone GmbH, Austria, Vienna)
- Protac® (Pentapharm), 5U per vial was dissolved in 500 μ L of distilled water to get Protac® concentration 10U/mL.

To keep the volumes of plasma (65 μ L) and Protac® (15 μ L) constant, the following concentrations of Protac® were prepared as the final volume of mixture in each well

as 120µL; plasma, Protac®, tissue factor and Fluorogenic substrate):Table 2-2
Preparation of increasing concentration of Protac® for thrombin generation

Table 2-2 Preparation of increasing concentration of Protac® for thrombin generation

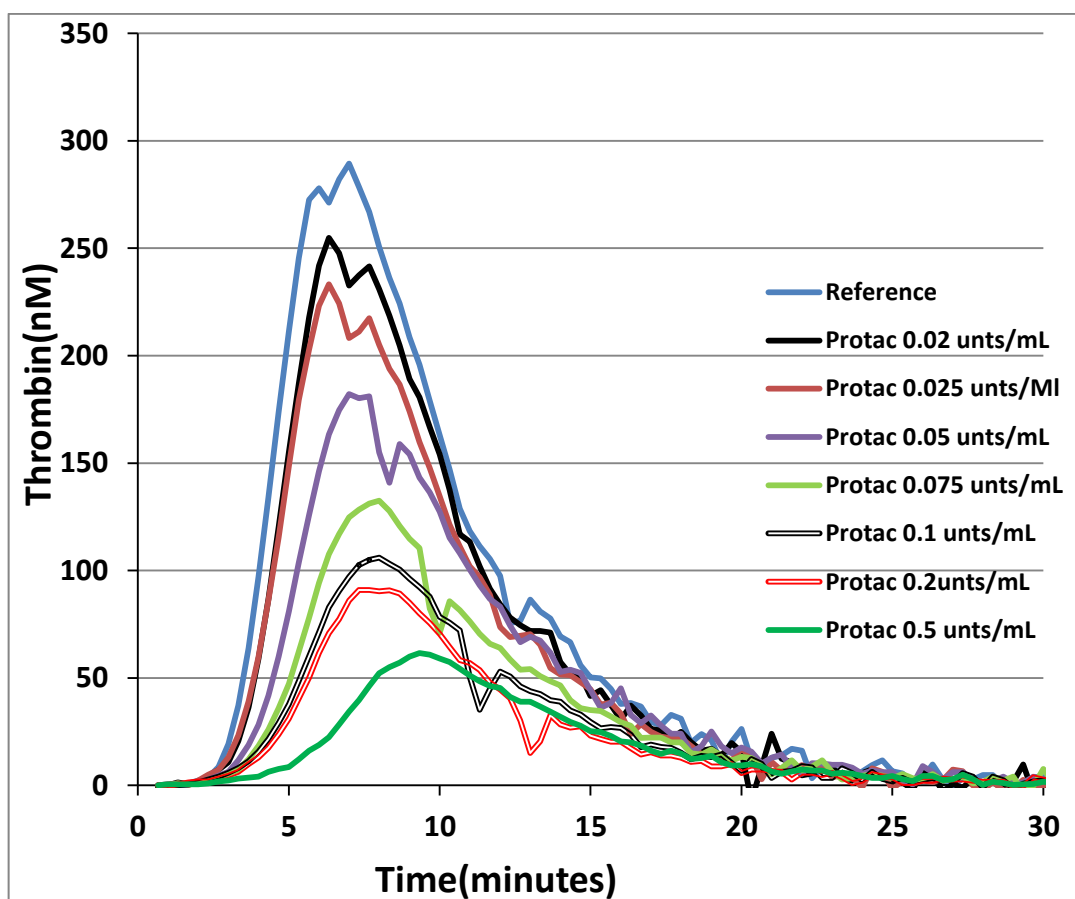
Final Protac® concentration in each thrombin generation test well (units)	Final concentration of Protac® (units) in each 15µL buffer
0.02	0.16
0.025	0.2
0.05	0.4
0.075	0.6
0.1	0.8
0.2	1.6
0.5	4.0
1.0	8.0

- 20µL of PPP reagent was added to each TG measurement well.
- 20µL of thrombin generation calibrator was added to calibrator well (as the same reference plasma was used for TG with different concentrations of APC, same calibrators were used all TG reactions)
- 65µL of reference plasma and 15µL of increasing concentration of Protac® in buffer B gently mixed with the pipette.
- Following 10 mins incubation period, 80µL of the plasma + Protac® was added TG wells which in to give a final assay concentration of Protac®as table 2.2.
- The microtitre plate was then transferred to a pre-warmed Ascent Fluorskan plate reader. Incubation and priming instructions from Thrombinoscope

Software were followed. 20 μ L of FluCa reagent was dispensed automatically by the instrument, which allows the reaction to take place and was monitored for 60 minutes.

- As expected, there was a Protac® concentration dependent reduction in thrombin generation (Figure 2.4) and Protac® that produces approximately 50% reduction in thrombin generation was selected for sample testing.
- The reproducibility was checked, intra and the inter-assay coefficient of variation were determined.

Figure 2-4 Thrombin generations with varying concentration of Protac®



Aliquots of 5 μ M rhAPC were stored at -80°C and thawed at room temperature, 10 minutes before diluting to 40nM in 20mM HEPES Buffer Saline pH 7.35 (containing 1% bovine serum albumin (BSA; A-7030; Sigma-Aldrich, Poole, UK). Protac® was stored as a 10 U/mL solution at 4°C and diluted to 1.6 U/mL in HEPES/BSA buffer

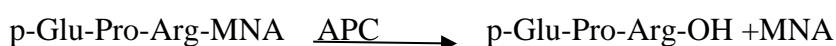
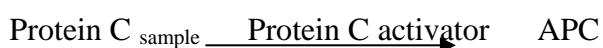
10 minutes before use. The concentrations of rhAPC and Protac® that produced approximately 50% inhibition of peak TG in PNP were selected this study. All TG reactions for samples and calibrators were tested in triplicate and PNP was run in parallel on each plate.

2.17 Chromogenic Protein C assay

Principle of the method-chromogenic assay

Protein C is activated using an extract from a snake venom (*Akistrodon contortrix contortrix*). The resulting activated PC (APC) cleaves an amidolytic substrate releasing the chromophore para-Nitroaniline (pNA). This reaction is monitored kinetically at 405nm. The amount of PC activity is proportional to the absorbance.

The assay is based on the following reactions.



Reagents and instrumentation:

- All reagents were from Siemens Diagnostics Healthcare (Marburg, Germany), and the assay was performed on a Sysmex CS-2000i (Sysmex UK Ltd, Milton Keynes, UK).
- Berichrom Protein C kit (composed of: Protein C activator; Substrate reagent and a HEPES buffer solution for reconstitution of the activator reagent)
- Calibrator (Standard Human Plasma [SHP])
- Control plasma N (normal)
- Control plasma P (pathological)
- Owren's veronal buffer (OVB)

Method

- Assay was performed using manufacture's standard protocol.
- Samples with protein C levels above the range of the calibration curve were re-tested at higher higher dilution.

2.18 Free protein S antigen assay

Principle of the method: Immunoturbidometric assay

A suspension of latex particles coated with monoclonal antibodies specific for free protein S is mixed with test plasma. The antigen–antibody reaction leads to agglutination of the latex particles, causing an increase in absorbance reaction medium. The absorbance of the medium is measured photometrically at 800nm. The degree of agglutination and hence the absorbance is directly proportional to the free protein S concentration in the test sample.

Reagents and instrumentation:

All reagents were from Siemens Diagnostics Healthcare (Marburg, Germany), and the assay was performed on a Sysmex CS-2000i (Sysmex UK Ltd, Milton Keynes, UK).

- The INNOVANCE[®] Free PS Ag assay (composed of: Free protein S (FPS) buffer and FPS reagent i.e. anti-FPS coated latex particles)
- Calibrator (Standard Human Plasma [SHP])
- Control plasma N (normal)
- Control plasma P (pathological)
- Owren's veronal buffer (OVB)

Method

- Assay was performed using manufacture's standard protocol.
- Samples with free protein S antigen levels above the range of the calibration curve were re-tested at higher dilution.

2.19 ELISA for Protein C Antibodies

Principle of the method

Microplate is coated with human purified protein C (PC). Test samples, diluted in sample buffer are added to PC coated wells in duplicate; if antibodies to P Care present they bind to well. After washing step to remove unbound antibodies and non-specific particles, PC antibodies were detected by the addition of the immunoconjugates followed by substrate for colour development.

Reagents

- Protein C concentrate 500units (42.5mg) (Baxter Healthcare Ltd, Norfolk, UK), reconstituted in 5ml of distilled water. Following reconstitution, each vial contains: 8.5mg/mL protein C, 8mg/mL human albumin, 4.4mg/mL disodium citrate dehydrate and 8.8mg/mL sodium chloride.
- Detection antibody - Horseradish peroxidase- conjugated goat antihuman IgG (α -chain specific; cat.no. A-2290, Sigma-Aldrich company Ltd, Dorset, UK)
- Coating Buffer - TBS/Ca - 0.05M Tris, 0.1M NaCl, 5mM CaCl₂, pH 7.4
- Blocking Buffer – TBS/Ca - 0.05M Tris, 0.1M NaCl, 5mM CaCl₂, pH 7.4
3% bovine serum albumin [BSA] (Product A-7030, Sigma-Aldrich company Ltd, Dorset, UK)
- Washing Buffer – TBS/Ca - 0.05M Tris, 0.1M NaCl, 2.5mM CaCl₂, pH 7.4
- Sample Buffer – TBS/Ca - 0.05M Tris, 0.1M NaCl, 5mM CaCl₂, pH 7.4, 1% BSA
- Substrate buffer – 0.1M Citrate phosphate buffer pH 5.0

Distilled water 950ml

Disodium hydrogen orthophosphate dodecahydrate (VWR product code: 102484A) 23.87g

Citric acid anhydrous (Sigma C0759) 7.3g

Make up to 1litre with distilled water; adjust to pH 5.0 with 4M Sodium Hydroxide and store at 4°C.

- Substrate solution:

Ortho phenyldiamine dihydrochloride 15mg tablet

Citrate phosphate buffer- 24ml

6% hydrogen peroxide -10µl

- Stopping agent – 2.0M Sulphuric acid

Method

- An irradiated polystyrene plate (MaxiSorp; Fisher Scientific UK Ltd, Loughborough, UK) was coated with 100µL, 10mg/L Protein C diluted in coating buffer per well in the first half of the plate and 100µL coating buffer alone, second half as a control area for non-specific binding.
- The plate was covered and incubated overnight at 4°C.
- Following 3 washes with 250µL of washing buffer; wells were blocked with 180µL of blocking buffer for 60 min and then washed 3 times.
- 100µl of each diluted test plasma samples (diluted as 1/25 in sample dilution buffer) was added to each well in duplicate.
- After 60 min incubation at room temperature, the wells were washed 3 times 100 µL of detection antibody diluted 1:1500 in sample Buffer, was added as the secondary antibody.
- After 60 min incubation period, wells were washed 3 times and colour was developed by means of 100 µl substrate.

- The enzyme reaction was stopped after suitable colour development by adding 50 μ L of 2M H₂SO₄ to each well. The absorbance was measured at 490 nm.
- Positive and negative quality control samples were run on each plate.

2.20 Detection of protein S antibodies (anti-protein S IgG ELISA)

Principle of the method

Microplate is coated with human purified protein S (PS). Test samples, diluted in sample buffer are added to PS coated wells in duplicate; if antibodies to PS are present they bind to well. After washing step to remove unbound antibodies and non-specific particles, PS antibodies were detected by the addition of the immunoconjugates followed by substrate for colour development.

- Detection of anti-PS IgG was done using anti-PS IgG ELISA kit from Stago R&D (Gennevilliers Cedex, France), which was a kind gift from Dr. Mariette Adam and Dr Bary Woodhams.
- Assay was performed essentially according to the instruction provided by the manufacture.
- In brief, all samples from patients and normal controls were diluted 1:101 in dilution buffer provided. PS standard was tested from in serial double dilution from 100% to 1.56% to obtain the standard curve. 200 μ L of each sample, the standard and the control was added to each well of the PS coated plate in duplicate.
- Following 60 minutes of incubation period at room temperature, plate was washed 5 times with washing solution and 200 μ L of immunoconjugates was added to each well. Following another 60 minutes incubation period at the room temperature, plate was washed \times 5 and colour was developed by means of 200 μ L of TMB substrate. After 5 minutes the enzyme reaction was

stopped by adding 50 μ L of 1M H₂SO₄ to each well and the absorbance was measured at 450 nm.

- Samples were considered positive for anti-PS antibodies if the absorbance was greater than 3SD above the mean 0.086 of normal controls (cut-off = 0.1. Standard, positive and negative samples were run on each plate for quality control purposes.

2.21 Determination of the avidity of IgG protein C and protein S antibodies

- The avidity of the IgG PC and PS antibodies was assessed by introducing chaotropic conditions to the ELISA using a method adapted from (Cucnik *et al*, 2004).
- Briefly, the assay was performed as above for protein C antibodies, but samples positive for IgG protein C antibodies were diluted in coating buffer containing either: 0.1, 0.15, 0.25, 0.5, 1.0, 2.0, 4.0, or 6.0M NaCl and loaded onto the microplate before incubating for 90 minutes. Bound IgG was detected as described above for detection of anti-protein C antibodies and avidity was determined by calculating the percentage of maximum binding at 0.1M NaCl.
- Samples positive for IgG PS antibodies were also tested for avidity as described above for the avidity determination for anti-protein C antibodies. Samples from 7 APS patients and the one non-APS patient positive for antibodies were diluted in sample buffer without NaCl and with the increasing concentration of NaCl (0.1M, 0.25M, 0.5M, 1M, 2M and 4M) and ELISA was performed as described above for detection of anti-PS antibodies. Avidity was determined by calculating the percentage of maximum binding in the absence of NaCl.

2.22 Purification, Dialysis, and concentration of IgG

Principle of method

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (IgG immunoglobulin) and a specific ligand (Protein A/G Agarose) covalently immobilized onto a chromatographic matrix. The sample (serum or plasma) is applied to the matrix. Unbound material is washed away, while the bound target protein is recovered by changing conditions to those favouring elution. Elution of IgG is performed by changing the matrix to acid pH. IgG is collected, dialysed and concentrated. The pH of the medium is neutralised by adding a neutralising buffer.

Instruments and reagents

- Nab protein G spin column (Pierce) Thermo Fisher Scientific UK
- Spin column (AMICON ultra, centrifugal filter units, Millipore UK or Fisher, 30 K cut off, code: UFC 803024)
- Binding (Equilibrating/Washing) Buffer - 0.1M Phosphate - pH 7.2
- Elution Buffer- 0.1M glycine pH 2.7
- Neutralisation Buffer-1M Tris pH 9 (add HCl to adjust pH)
- Dialysis solution - Phosphate buffered saline (PBS) (Sigma-Aldrich Company Ltd)
- All Buffers need to be filter sterile and this is achieved by using either vacuum filtration or a syringe and Millex-GS filters 0.22 μm (code: SLG50335S)

Method

- The column was washed with 5 ml binding buffer to pre-equilibrate, serum or plasma samples were diluted 1:1 with binding buffer and applied to the column.
- The column was washed with 15 ml of binding buffer to elute any non-specific binding proteins.
- 4 ml of elution buffer was added to the column and the eluent was collected in a single tube.
- 400 µl of neutralisation buffer was added immediately to the elution fraction and mixed in order to neutralise the pH (100 µl of neutralisation buffer for each ml of elution buffer).
- The purified IgG was added to a spin column (AMICON ultra, centrifugal filter units, (30 K cut off, code: UFC 803024) Millipore UK, centrifuged and then dialysed with PBS.

2.23 Quantification of IgG (IgG ELISA)

Principle of method

A polystyrene microtitre plate (Nunc Maxisorp) is coated with a monospecific rabbit antibody to human IgG. After incubation the plate is washed and the plate is incubated with blocking agent (3% bovine serum albumin (BSA) in PBS) and washed again. Dilutions of control and samples are applied and the plate is incubated. Subsequent to another washing step, a second antibody is applied (Horseradish peroxidase-conjugated rabbit antihuman IgG) and the plate is incubated. Excess antibody is removed by washing the plate and quantitation is done in a colourimetric enzyme substrate reaction, the colour intensity produced being directly proportional to the concentration of IgG present in sample.

Instruments and reagents

- MRX Microplate Reader DYNEX Technologies, Inc.
- Equipment Automatic pipettes 5-50, 50-200, 200-1000, and 1000-5000µl and disposable tips
- Multi-channel pipette 50-300µl and disposable tips.
- Coating antibody: Rabbit anti-human IgG (Dako Ltd A0423)
- Detecting antibody: Horseradish peroxidase-conjugated rabbit antihuman IgG (Dako Ltd, P0214)
- 6% Hydrogen Peroxide (Pharmacy)
- Concentrated sulphuric acid (~ 19M) (Merck 10276)
- Reference preparation for IgG Human serum protein calibrator (Dako Ltd X0908). Store at 4°C.
- Plate coating buffer – phosphate buffered saline (PBS), pH 7.2. Prepare using buffer tablets; store at 4°C.
- Sample and HRP conjugate antibody dilution buffer -1% BSA in PBS
- Blocking buffer: 3% BSA in PBS
- Washing buffer- 0.1% Tween 20 in PBS (PBST)
- Substrate buffer - 0.1M Citrate phosphate buffer pH 5.0

Distilled water 950ml

Disodium hydrogen orthophosphate dodecahydrate (Merck 102484A) 23.87g

Citric acid anhydrous (Sigma C0759) 7.3g

Make up to 1litre with distilled water; adjust to pH 5.0 with 4M Sodium

Hydroxide and store at 4°C.

- Substrate solution

Ortho phenyldiamine dihydrochloride 15mg tablet

Citrate phosphate buffer- 24ml

6% hydrogen peroxide -10 μ l

- Stopping agent -2.0M Sulphuric acid

Method

- 100 μ l of coating antibody diluted 1:500 in coating buffer was added to each well of a microtitre plate using a multi-channel pipette.
- The plate is covered and incubated overnight at 4°C.
- After washing the plate three times with 250 μ l of washing buffer the plate was blocked with 180 μ l of blocking buffer. The plate was covered and incubated for 1.5 hours.
- The plate was washed three times and the standard was added in duplicate with serial double dilutions in sample buffer from 1:100,000 to 1:6400, 000 (A1/2 to G1/2) and the blanks (sample buffer) to last 2 wells (H1/2). Following the placement of the test samples in duplicate (1:100,000 to 1:200,000 in sample buffer) to other wells, the plate was covered and incubated for 1 hour at room temperature.
- After 1 hour of incubation, the plate was washed three times, 100 μ L of detecting antibody diluted 1:500 in sample buffer was added to each well and incubated for another 1 hour in room temperature.
- The plate was washed three times and freshly prepared 100 μ L of peroxidase substrate was added to each well.
- The plate was incubated in the dark at room temperature until gradation of colour was seen throughout the standard curve.
- The enzyme reaction was then stopped by adding 50 μ L of stopping agent to each well. The plate was read at 490 nm on MRX Microplate Reader.

- Using the standard curve, and the absorbance of the samples IgG levels were calculated.

2.24 Rivaroxaban preparation for *in vitro* experiments

Method is based on (Molenaar *et al*, 2012).

Range of rivaroxaban concentration for experimentation is 1-1000µg/L

Reagents

- Rivaroxaban (Bayer Healthcare) 50mg (material no.80058013)
- DMSO (Sigma lot 276855)
- OVB (Siemens Haelthcare) 28.4mM Sodium Barbitone, 0.125M NaCl,pH 7.35
- Preparation of stock and working solutions:
- 50mg rivaroxaban was dissolved by gradually washing the vial contents into a beaker using the DMSO. Contents were transferred to a volumetric flask and adjusted final volume to 500mL using DMSO and stirring thoroughly. This solution has a concentration of 100µg/ml of rivaroxaban and stored in room temperature (in poisons cabinet for security reasons)
- 1 mL of 100µg/ml rivaroxaban preparation was diluted with 4ml of Owren's veronal buffer (OVB) to get 20µg/mL of rvaroxaban and at the same time 1mL of DMSO was diluted with 4mL of OVB as the control of 0µg/ml rivaroxaban. Both preparations were stored at room temperature.
- 250µl of 20µg/mL of rivaroxaban was diluted with 4750µL of normal plasma to get concentration of 1000µg/L of rivaroxaban in plasma and 250µl of DMSO/OVB solution was diluted with 4750µl of normal plasma to get 0µg/L concentration of rivaroxaban/DMSO control in plasma.

Range of rivaroxaban concentrations were prepared by mixing the 1000 μ g/L rivaroxaban and 0 μ g/L rivaroxaban /DMSO control solutions as in Table 2-3.

Table 2-3 Preparation rivaroxaban concentrations

Rivaroxaban final concentration(μg/L)	Volume of Rivaroxaban 1000μg/L in plasma(μL)	Volume of Rivaroxaban /DMSO control 0μg/L in plasma(μL)
0	0	1000
50	50	950
100	100	900
150	150	850
200	200	800
300	300	700
400	400	600
500	500	500
700	700	300
800	800	200
1000	1000	0

2.25 Effect of antiphospholipid antibodies on rivaroxaban in thrombin generation and anti-Xa assay

Reagents

- Reagents for TGT as described in section 2.3
- IgG purified from patients and normal controls as described in section 2.7, quantified by IgG ELISA as described in section 2.8.

- Different concentration of Rivaroxaban (25ng/mL, 50ng/mL and 100ng/mL) in DMSO/OVB (as described in section 2.9)
- Phosphate buffered saline (PBS) as the buffer control

Method

- Final IgG concentrations of 250µg/mL and 500µg/mL and rivaroxaban concentration of 25ng/mL to 100ng/mL were selected for the experiments.
- Pooled normal plasma was spiked with IgG and rivaroxaban to make final mixture of 10% IgG, 10% Rivaroxaban and 80% plasma of the total 80mL of plasma and incubated for 10 minutes prior to adding 20µL of 5pmol tissue factor.
- TG was performed and following parameters were recorded: Lag time, ETP, peak thrombin and the time to peak. Anti-Xa of the each sample was measured using Biophen DiXaI (Hyphen BioMed, Neuville-Sur-Oise, France) on a Sysmex CS-2000i (Sysmex UK Ltd, Milton Keynes, UK).
- Rivaroxaban anti-Xa level was assessed as described in section 5.3 2.4.

2.26 Statistical analysis

Data analysis was performed using Microsoft®Excel and Analyse-it software version 2.26 (Analyse-It Software Ltd, Leeds, UK). Data showing a normal distribution were analysed using Student's t-test and non-parametric data using Kruskal-Wallis ANOVA (for multiple groups comparisons) and the pair-wise comparisons were made using Mann-Whitney 'U' test. For multiple analyses, the p-values derived from Kruskal-Wallis ANOVA were adjusted by Bonferroni correction. Fisher's exact test (FET) was used to study associations. A p value of <0.05 was considered significant.

Chapter 3 Activated protein C resistance (APCr) and resistance to activation of endogenous protein C activation determined using thrombin generation patients in with thrombotic antiphospholipid syndrome.

3.1 Introduction

Protein C (PC), protein S (PS) and thrombomodulin are naturally occurring anticoagulants designed to regulate coagulation, maintain the fluidity of blood within the vasculature, and prevent thrombosis mainly through the regulation of thrombin generation. Antiphospholipid antibodies (aPL) may interfere with the anticoagulant activity of activated PC (APC) to induce acquired activated PC resistance (APCr). This effect of aPL on APC is one of the proposed mechanisms for the development of thrombosis in patients with APS (Rodeghiero & Tosetto, 1999). Therefore phenotypic evaluation of APC resistance has been suggested as an alternative screening strategy (Rodeghiero & Tosetto, 1999). However, direct association between an aPL -dependent APCr phenotype and thrombotic events is still not established (Male *et al*, 2001;Nojima *et al*, 2005). There are limited data on APCr in patients with APS using exogenous APC (rhAPC) (Zuily *et al*, 2013;Liestol *et al*, 2007b) with endogenous activation of protein C in these patients undefined.

The aim of this study was to investigate the frequency and nature of APCr using *ex vivo* TG with exogenous APC as well as activation of endogenous PC, in APS patients on long term warfarin for VTE.

3.2 Intra and inter-assay imprecision of thrombin generation with buffer control, rhAPC and Protac

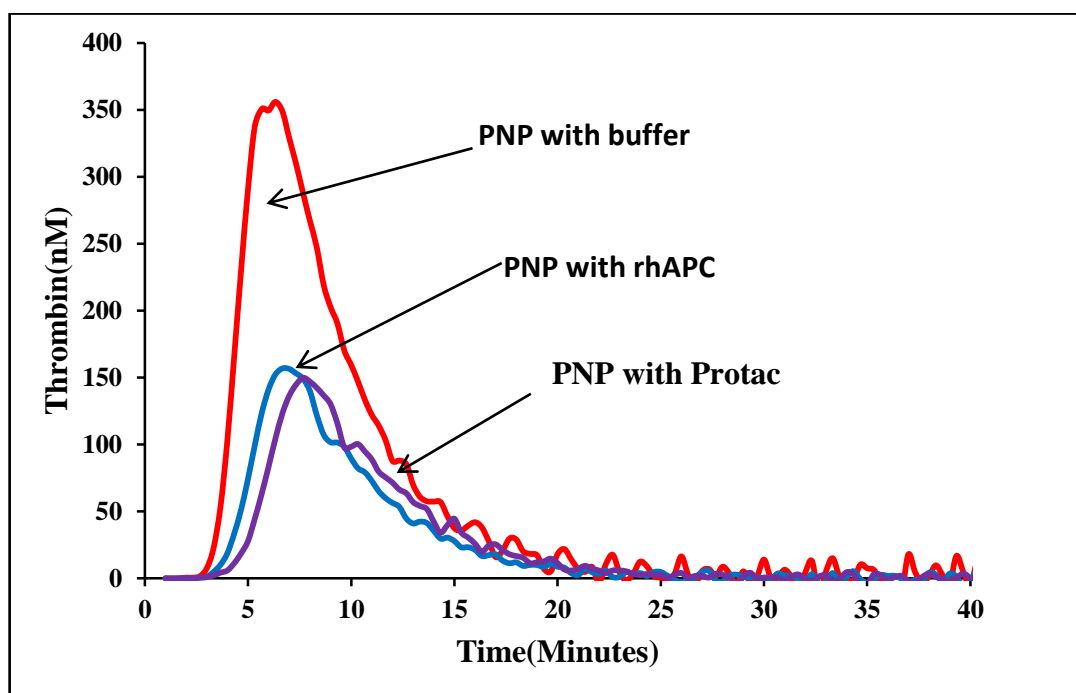
To evaluate the assay imprecision with buffer control, rhAPC and Protac in the presence of 5 pmol/L tissue factor (TF) and 4 μ M phospholipid (PPP reagent) measurement of TG was assessed in pooled normal plasma. To measure intra- assay imprecision, TG of the pooled normal plasma was tested 8 times (in triplicate for

buffer control, with rhAPC and Protac) in the same run, while inter-assay imprecision was assessed for TG using buffer control, rhAPC and Protac on 6 different days.

3.2.1 Results

Figure 3-1 shows an example on the effect of rhAPC and Protac® on thrombin generation in pooled normal plasma. The mean intra-assay coefficient of variation (CV) for ETP (n=8) using PNP with buffer control, rhAPC and Protac® were: 2.5, 3.0 and 4.5% respectively; and the inter-assay CV (daily for 6 days) was: 4.0, 4.5 and 8.3% respectively.

Figure 3-1 Example of thrombin generation of pooled normal plasma with buffer control, rhAPC and Protac



This figure demonstrates the thrombin generation of pooled normal plasma with buffer control, rhAPC (5nM) and Protac® (0.2units/mL). Both rhAPC and Protac comparable inhibition of thrombin generation in pooled normal plasma (PNP).

3.3 Patients and normal controls

Having obtained written informed consent, blood samples were obtained from 102 patients on long-term anticoagulation with warfarin for VTE (a cohort of 51 patients with APS; and 51 non-APS patients) and 51 healthy normal controls.

3.3.1 Inclusion criteria

- Patients with antiphospholipid syndrome on warfarin for venous thromboembolism
- Patients without antiphospholipid syndrome on warfarin for venous thromboembolism
- Healthy normal controls who were not on any anticoagulants or oestrogen containing oral contraceptive pills/hormone replacement therapy.

3.3.2 Exclusion criteria

- Patients with FV Leiden/ G20210A prothrombin gene mutation
- Patients with history of congenital PS and PC deficiency
- Patients on oestrogen containing oral contraceptive preparations
- Patients with history of malignancy or myeloproliferative disease
- Patients and normal controls who were on oestrogen-containing preparations or who were pregnant were also excluded.

Blood samples were collected and processed as described in section 2.2. The 51 patients with APS fulfilled the revised International consensus criteria for definite APS (Miyakis *et al*, 2006). Assays for aPL were performed as part of routine clinical management in the hospital diagnostic laboratory. All patients were on warfarin for at least 3 months following a thromboembolic event at the time of study. There are no agreed, published definitions of the clinical phenotype of thrombosis in APS patients, although those who have experienced recurrent VTE or arterial thrombosis are recognised as high risk patients (Kasthuri & Roubey, 2007; Ruiz-Irastorza *et al*, 2007). I used the term ‘severe thrombotic phenotype’ for patients who had recurrent VTE whilst receiving therapeutic anticoagulation and /or patients with both venous

and arterial thrombosis. Sixteen of the 51 APS patients (31%) were classified as having a severe thrombotic phenotype; 9/16 patients had recurrent VTE whilst on therapeutic anticoagulation and 7/16 patients had both venous and arterial thrombosis. Ten of these 16 patients (62.5%) were triple aPL positive. Five of 51 non-APS patients (10%) also had a severe thrombotic phenotype; all these had recurrent VTE whilst on therapeutic anticoagulation. Patients with a severe thrombotic phenotype were maintained at a target INR of 3.5 (range 3.0-4.0) whilst the target INR in the remainder was 2.5 (range 2.0-3.0). Table 3-1 describes demographic data and the clinical features of two patients groups and the normal controls.

Table 3-1 clinical features of the three groups of subjects studied

	APS	Non-APS	Normal Controls
Age, mean year	49.43±14.8	50±15.1	41±12.1
Sex, male/female	26/22	24/27	24/27
Vascular thrombosis			
DVT alone	17 (33%)	25 (49%)	n/a
PE alone	14 (27%)	15 (29%)	n/a
DVT+PE	4 (8%)	6 (12%)	n/a
VTE+ arterial	7 (14%)	0 (0%)	n/a
Recurrent VTE	9 (18%)	5 (10%)	n/a

APS=antiphospholipid syndrome; non-APS=Patients without APS; DVT=deep vein thrombosis; PE=pulmonary embolism; VTE= venous thromboembolism * 2/51 (4%) patients with APS had SLE

3.4 Antiphospholipid antibodies and INR

Of 51 patients with APS, 4% (2/51) also had systemic lupus erythematosus (SLE). Ninety-six percent (49/51) had lupus anticoagulant and 59% (30/51) had various combinations of aCL and/or aβ₂GPI as follows (percentage, median aPL level): IgG aCL 19.6%, 69.3 GPLU/mL; IgM aCL 27%, 58.5 MPLU/mL; IgG aβ₂GPI 27%, 51.45U/mL; and IgM aβ₂GPI 37%, 28.6U/mL. Sixteen of the 51 APS patients (31%)

were triple aPL positive; and 62.5% (10/16) of the triple aPL positive APS patients had a severe thrombotic phenotype. INR values (median (range) were 2.4 (1.8-4.2) and 2.3 (1.8-4.3) for the APS and non-APS groups respectively.

3.5 Resistance to rhAPC and activation of endogenous protein C by thrombin generation

TG was performed after mixing samples 1:1 with pooled normal plasma (PNP) to correct for warfarin induced coagulation factor deficiency, using the CAT system as described in section 2.16, resistance to rhAPC and activation of endogenous protein C using Protac® as described in section 2.16.1. APCr was expressed as percentage inhibition of ETP after normalising the results in test samples against PNP (adapted from Liestøl et al) (Liestøl *et al*, 2007b), using the following equation:

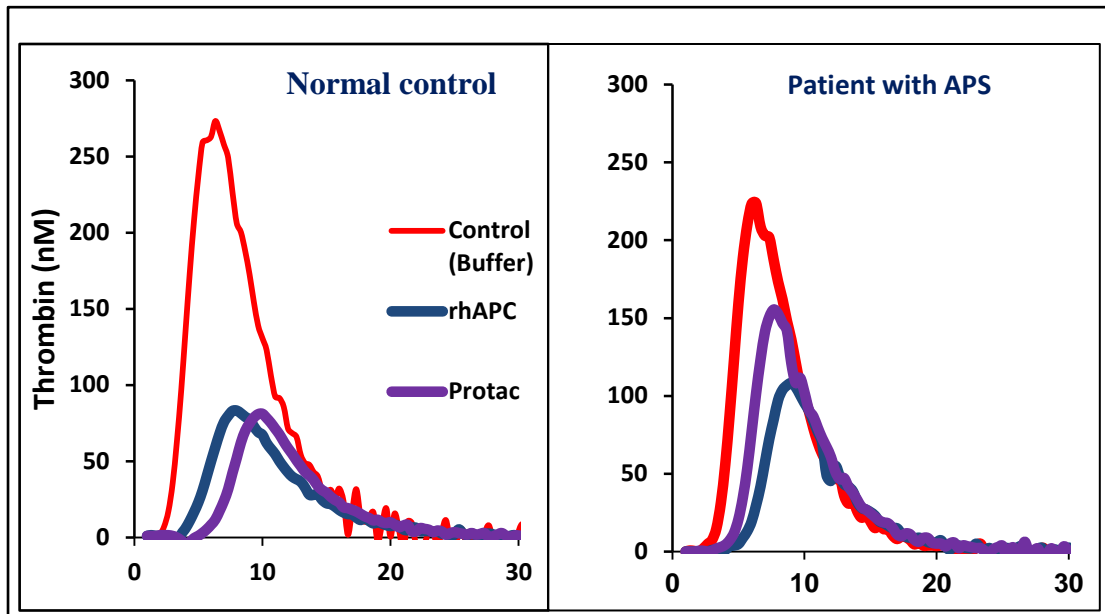
$$\begin{aligned} & \text{Percentage inhibition of ETP} \\ & = \frac{(1 - (\text{ETP sample} + \text{rhAPC or Protac} / \text{ETP sample} + \text{buffer})) \times 100}{(1 - (\text{ETP PNP} + \text{rhAPC or Protac} / \text{ETP PNP} + \text{buffer}))} \end{aligned}$$

The lower the value of percentage inhibition of ETP, the greater the APCr.

3.5.1 Results

An example of TG with buffer control, rhAPC and Protac® in a NC and a patient with APS is shown in **Figure 3-2**.

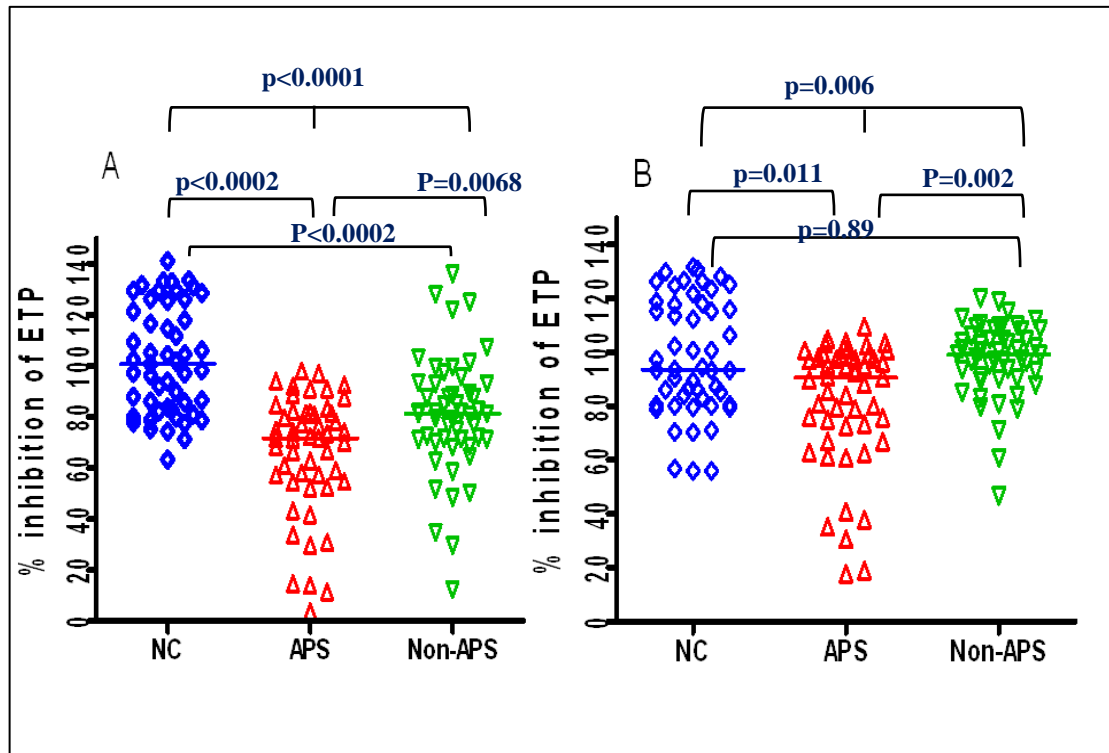
Figure 3-2 An example of TG with buffer control, rhAPC and Protac® in a NC and a patient with APS showing APCr



The normal control had >50% inhibition of thrombin generation with rhAPC and Protac and was comparable. The patient with APS demonstrated variable inhibition of thrombin generation with rhAPC and Protac and resistance to activation of endogenous PC with protac was higher compared to rhAPC.

Greater APCr (lower percent inhibition of ETP) was observed after activation of endogenous protein C with Protac® in both APS (median inhibition of ETP [95% CI]) (66.0% [59.5-72.6%]) and non-APS patients (80.7%) [74.2-87.2%], $p < 0.0001$) compared to normal controls (102.2%) [96.2-108.1%] (Figure 3-3A). Non-APS patients also showed greater resistance to activation of endogenous PC compared to NC ($p < 0.0001$), but resistance was greater in APS patients ($p = 0.0034$). APS patients also showed greater resistance to exogenous rhAPC (median [range]: 90% [18.0-109.0], $p = 0.0006$) compared to non-APS patients (99% [46.6-130.6]), and normal controls (94% [56-132]), mainly due to a sub-group of six patients who were triple positive for aPL with marked APCr (16.6-40.8%) (Figure 3-3B). Those six patients had marked resistance to activation of endogenous PC (3.7-33.8%) and also had high avidity anti-PC antibodies (see below).

Figure 3-3 Distribution of APCr in plasma samples from healthy normal controls (NC), APS and non APS patients; A: with Protac, B: with rhAPC



It is possible that patients with APS demonstrated greater resistance to activation of endogenous PC as well as rhAPC due to autoantibodies to PC or PS interfering in the PC pathway. I therefore investigated the presence of PC or PS antibodies in these patients.

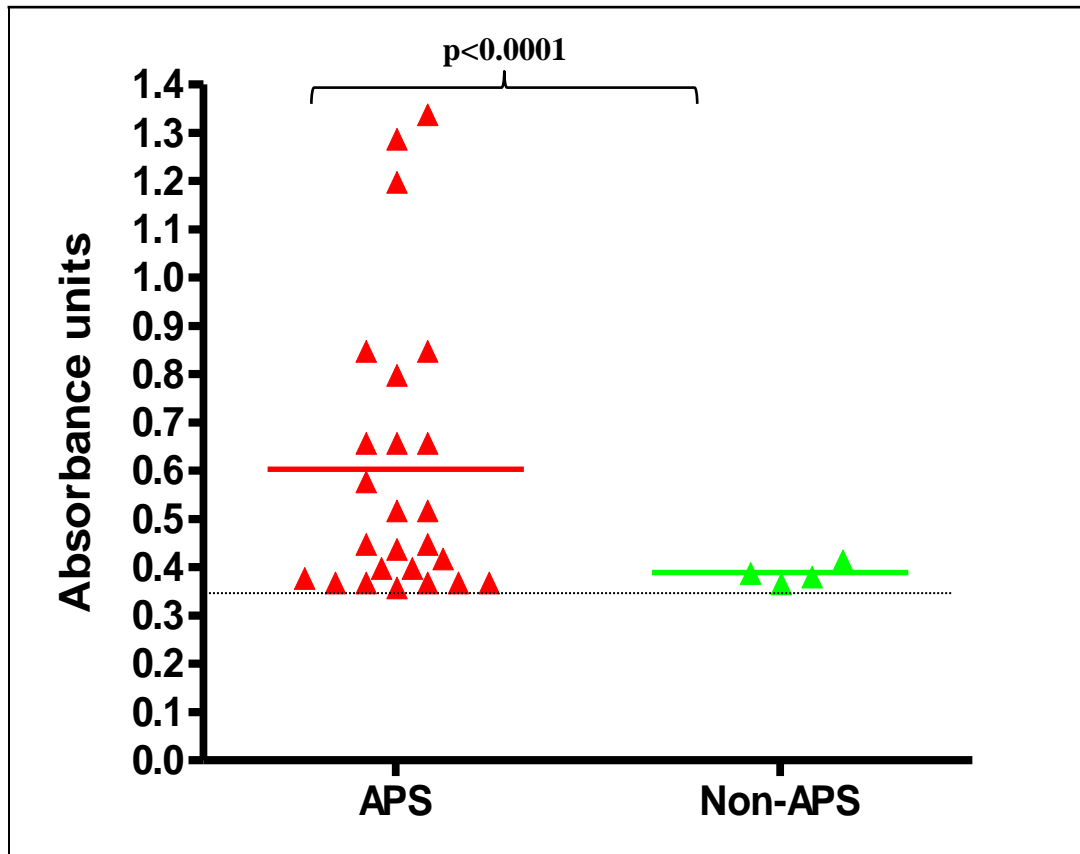
3.6 Anti-protein C antibodies

Anti-protein C antibodies were studied as described in section 2.19. I did not find any non-specific binding to the protein C uncoated plate. Samples were considered positive for anti-protein C antibodies if the absorbance was $>3SD$ above the mean (0.36 absorbance units) for normal controls (n=51). Intra-assay and inter assay CV for anti-protein C antibodies were 1.8% and 2.2% respectively.

Anti-protein C antibodies were detected more frequently in patients with APS (25/51 (49%) compared to non-APS patients (4/51 (8%), $p < 0.0001$). Furthermore patients with APS had higher anti- protein C antibody levels compared to non-APS patients;

median absorbance [range]: 0.45 [0.37-1.34] for APS and 0.39 [0.37-0.42] for non APS respectively ($p < 0.0001$) (Figure 3-4).

Figure 3-4 Anti-PC antibodies in APS and non-APS patients



The dotted line indicates 3SD above the mean (0.36 absorbance units) for normal controls (n=51).

3.7 Anti-protein S antibodies

Anti-protein S IgG antibodies were measured as described in section 2.20. Samples were considered positive for anti-protein S antibodies if the absorbance was $>3SD$ above the mean (0.1 absorbance units) for normal controls (n=51). Positive and negative quality control samples were run on each plate. Intra-assay and inter-assay CV for anti-protein S antibodies were 2% and 2.5% respectively. Anti-protein S antibodies were found in 7/51 (14%) APS patients (median absorbance [range]: 0.30 [0.19-0.61] and 1/51 (2%) non-APS (absorbance 0.2) patients respectively. Four of

the seven APS patients with anti-protein S antibodies also had anti-protein C antibodies.

3.8 Determination of the avidity of anti-protein C and anti-protein S antibodies

The avidity of the IgG anti-protein C and anti-protein S antibodies was assessed by introducing chaotropic conditions to the ELISA as described in section 2.21. Avidity was determined by calculating the percentage of maximum binding.

The avidity studies of anti-protein C antibodies in the 25 APS patients displaying them identified two clear groups (Figure 3-5): one group showed a rapid decrease in percentage of residual protein C binding as the NaCl concentration increased, with binding completely abolished at 4M NaCl (low avidity). The other group also showed a decrease in the percentage binding as NaCl increased, but a plateau was observed at approximately 1M NaCl and residual binding remained above 25% even at 6M NaCl (high avidity) (Figure 3-5).

Ten of the 25 patients had low avidity and 15 had high avidity antibodies. The difference in the percentage of maximum binding to protein C in these two groups showed a clear difference at 1M NaCl (mean residual binding 39.7% for the high avidity samples, compared to 16.5% for those with low avidity ($p < 0.0001$) and this difference remained at higher NaCl concentrations. All four patients with non-APS, who had anti- protein C antibodies, demonstrated low avidity antibodies.

Avidity studies of anti-PS antibodies demonstrated only the low avidity antibodies (Figure 3-6). Four of the seven APS patients with anti-protein S antibodies also had anti-protein C antibodies.

Figure 3-5 Avidity of anti-protein C antibodies

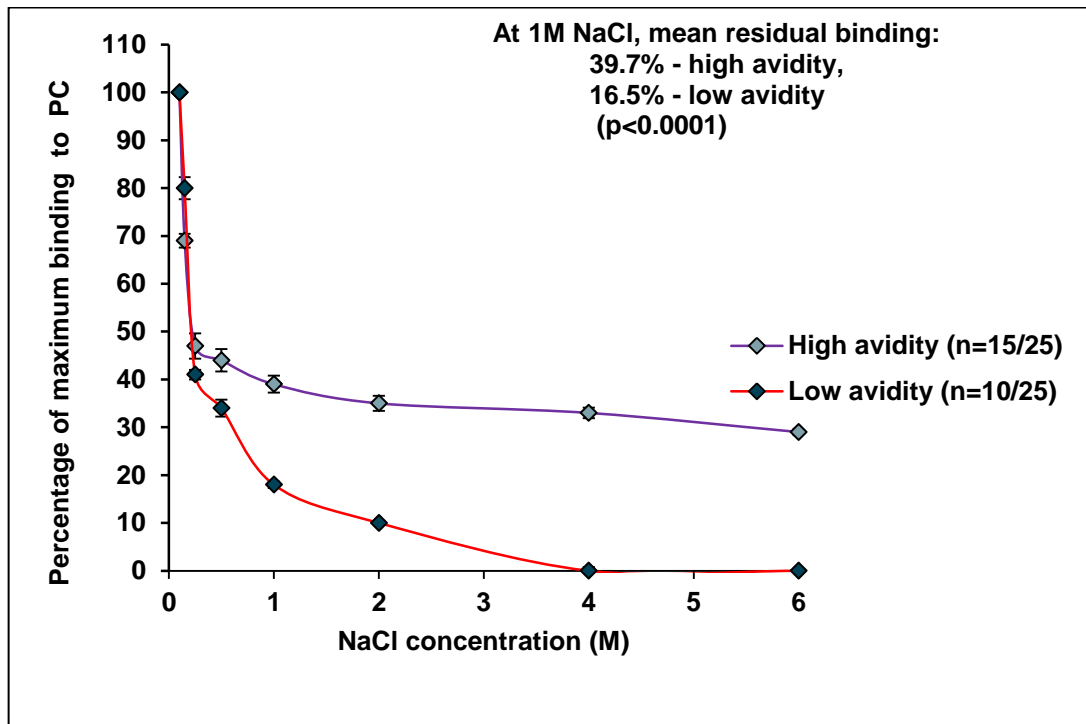
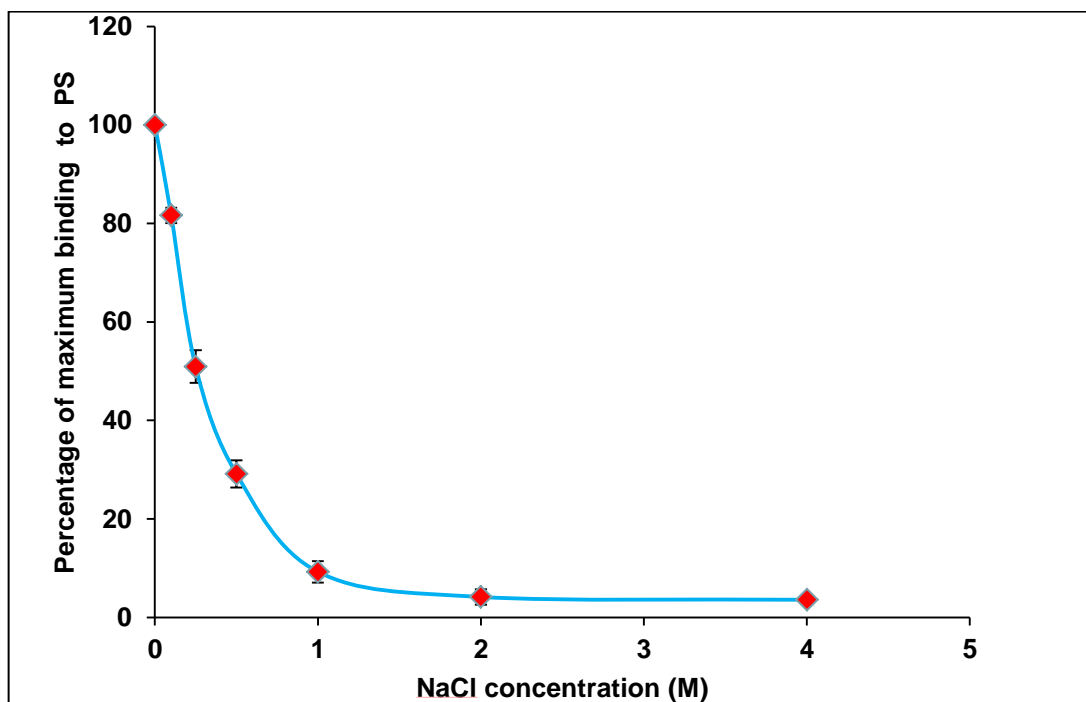


Figure 3-6 Avidity of anti-protein S antibodies



3.9 Anti-protein C antibodies and activated Protein C resistance

Patients with high avidity anti-protein C antibodies had significantly greater APCr to both rhAPC and activation of endogenous protein C with Protac® than those with low avidity anti-protein C antibodies; (median [CI]: 62.8% [35.2-74.9] and 90.8% [84.0-103.1], $p=0.0018$ for rhAPC and 43.3% [14.6-54.4] and 74.1% [66.6-87.3], $p<0.0001$) for Protac® respectively. The presence of high avidity anti-protein C antibodies in APS patients was strongly associated with a severe clinical phenotype defined as patients who had recurrent VTE whilst receiving therapeutic anticoagulation and /or patients with both venous and arterial thrombosis (section 3.3.2) (Fisher's exact test $p<0.0001$). Ninety-four percent (15/16) of the patients with a severe thrombotic clinical phenotype had high avidity anti-protein C antibodies and 62.5% (10/16) of those patients were triple positive for aPL. Nine of 15 (60%) had recurrent VTE whilst on therapeutic anticoagulation and 6/15 (40%) had experienced both venous and arterial thrombosis.

Four of 51 patients in the non-APS group with anti-protein C antibodies, which were low avidity also demonstrated marked resistance to activation of endogenous PC with Protac® (range 12.4-41.4%) and had clinically severe thrombotic disease.

3.10 Anti-protein S antibodies and activated Protein C resistance

Only 7 patients with APS and one patient with non-APS had anti-PS antibodies. The presence of anti-protein S antibodies alone appeared to have no independent effect on APCr with rhAPC or Protac® in patients with APS as there was no difference in the percentage inhibition of TG with rhAPC or Protac® in patients with anti-PS antibodies and without antibodies.

3.11 Evaluation of the presence of anti-protein C antibodies according to APS classification categories

The presence of anti-protein C antibodies was evaluated according to the APS classification category (I, IIa, IIb, IIc according to Miyakis et al, 2006) (Miyakis *et al*, 2006). Definition of APS classification categories are as follows: category I, more

than one laboratory criteria present [any combination], IIa, LA present alone; IIb, aCL antibody present alone; IIC, a β_2 -GPI alone). Twelve of 15 (80%) patients with APS who had high avidity anti-protein C antibodies were classified as APS category I and the other three patients (20%) were classified as category IIa. Forty percent (4/10) and 60% (6/10) of APS patients who had low avidity anti-protein C were classified as category I and IIa respectively.

3.11.1 PC activity and free PS antigen level

PC activity and free PS antigen levels were measured for two reasons;

1. To confirm that the observed differences in APCr with rhAPC and activation to endogenous PC with Protac® between patients with APS and non-APS was not due to differences in PC activity and free PS antigen level.
2. To assess the influence of anti-protein C and anti-protein S antibodies on PC activity and free PS antigen levels respectively.

All patients showed a reduction in PC activity and free PS antigen levels consistent with warfarin anticoagulation with no significant difference in levels between APS and non-APS patients. After 1:1 mixing of patient plasma with PNP for TG, all samples had PC and PS levels >60 IU/dL. Anti-protein C and anti-protein S antibodies had no apparent effect on PC activity or free PS antigen levels, as patients with and without anti- protein C and anti- protein S antibodies had similar levels of PC activity and free PS antigen (Table 3-2).

Table 3-2 PC activity and free PS antigen level in patients with APS and non-APS

		Original samples		1:1 mixed with PNP	
		PC activity (IU/dL)	Free PS antigen (IU/dL)	PC activity (IU/dL)	Free PS antigen (IU/dL)
APS	Mean	51.0	38.3	83.65	72.68
	Range	23.6-66.3	26.6-66.1	69.1-107.2	66.1-102.4
Non -APS	Mean	52.7	40.5	84.5	73.8
	Range	26.2-60.2	19.4-53.1	71.5-102.2	63.2-95.3
Adult reference range		PC (65–135 IU/dL)		PS antigen Males >73U/dL Females >63U/dl	

PC= protein C; PS= protein S; APS= antiphospholipid syndrome

3.12 Discussion

This study has demonstrated that APS patients with vascular thrombosis on long-term warfarin exhibit greater resistance to both exogenous rhAPC as well as to activation of endogenous PC by Protac®, using the CAT assay system, compared to non-APS patients with VTE and normal controls. Non-APS patients also showed greater resistance to activation of endogenous PC compared to healthy controls, but resistance was greater in the APS patients. Protac®, is an enzyme isolated from Agkistrodon contortrix contortrix venom which converts protein C into APC (Martinoli & Stocker, 1986). APS patients also had a significantly higher frequency and higher levels of anti- protein C antibodies: approximately half (49%) of the 51 patients with thrombotic APS had anti-protein C antibodies, compared to 8% of the non-APS patients, with 60% (15/25) of these antibodies in APS patients (29.4% (15/51 overall) with high avidity antibodies. The APS patients with high avidity anti-protein C antibodies had significantly greater APCr to both rhAPC and activation of endogenous PC with Protac® than those with low avidity anti- protein C antibodies. The presence of high avidity anti-protein C antibodies was also strongly associated

with a severe thrombotic phenotype in APS patients as defined in the methods section. Ninety-four percent (15/16) of the patients with a severe thrombotic phenotype had high avidity anti-protein C antibodies and 62.5% (10/16) of those patients were triple positive for aPL.

Previous studies have mainly focused on APCr using exogenous APC rather than on the activation of endogenous PC. Liestol *et al* (Liestol *et al*, 2007b) studied resistance to rhAPC using TG with the CAT system in 52 LA positive APS patients (29 had isolated VTE, 8 arterial thrombosis, 8 isolated obstetric complications and 7 had combined events). They found that most patients with LA showed resistance to the anticoagulant effect of exogenous APC, although a wide range of inhibitory effects were evident, and could partly have been a reflection of the heterogeneous nature of LA. Subgroup analysis suggested that a history of thrombotic events was associated with stronger APC resistance, although the comparison was made against 35 individuals on long-term warfarin therapy for non-valvular AF. In other studies using smaller patient numbers and different techniques, Devreese *et al* (Devreese *et al*, 2009) found that 83 % (40/48) non-anticoagulated LA positive subjects showed APCr to exogenous APC, however only 1/9 patients with LA on anticoagulation had APCr. Oosting *et al* (Oosting *et al*, 1991) found no effect of aPL and β_2 GPI on PC activation at the surface of endothelial cells, although Borrell *et al* found that aPL impaired APC function against FVa on the endothelial cell surface (Borrell *et al*, 1992). Previous work from our group (Green *et al*, 2012) demonstrated APCr in 11/17 APS patients (60%) when Protac was used to activate endogenous PC using an automated chromogenic TG assay. The findings of that study, which did not include assessment of clinical phenotype and aPL status of individual patients, established that the TGT exhibited good sensitivity to defects in the PC pathway.

My observation that APS patients frequently exhibit resistance to the activation of endogenous PC suggests a possible defect in the mechanism through which PC becomes activated. In many patients, a higher degree of APCr was associated with the presence of anti-protein C antibodies that may interfere with the mechanism of activation of PC or the anticoagulant action of APC. Differences observed between the APS and non-APS patients with regard to APCr with rhAPC and Protac® could

not be attributed to variation in PC, PS or procoagulant factor levels, since all samples were diluted with PNP to normalise levels of these factors.

Neither anti-protein C antibodies, nor aPL appeared to interfere with PC activity, since there was no difference in PC activity in the APS and non-APS groups and the warfarin induced PC deficiency was easily corrected by mixing with PNP as demonstrated by the chromogenic PC results. This suggests that anti-protein C antibodies or aPL do not bind directly to the active site of PC, but could bind to a site in close proximity, or induce a conformational change thus inhibiting or delaying PC activation. The explanation for the observations that anti-protein C antibodies in some patients have no obvious effect in PC chromogenic assays utilising Protac® activation, but influence APCr TG tests including this snake venom is unclear, but they could be due to the different reaction conditions. Chromogenic PC assay does not require phospholipid but effects of the endogenous activation of PC assessed through TG required phospholipids. The presence of resistance to activation of endogenous PC and /or exogenous PC in some but not all the thrombotic APS patients studied may be related to the avidity of the anti-PC antibodies or heterogeneity of their aPL phenotype. My findings that patients with high avidity anti-protein C antibodies had significantly greater APCr to both rhAPC and activation of endogenous PC with Protac® than those with low avidity anti-PC antibodies and had a severe thrombotic phenotype, suggests that these antibodies may contribute to the pathogenesis of thrombosis in APS, and extends previous observations in this area. Cucnik et al (Cucnik *et al*, 2004) reported that in 18 patients with APS, with or without SLE, high avidity a β ₂-GPI appeared to be associated with thrombosis and APS; and Lambriandides et al (Lambrianides *et al*, 2011) reported in 32 patients with APS and 29 with SLE that high avidity antithrombin antibodies, which prevent inactivation of thrombin, distinguish patients with APS from those with SLE who have aPL. The number of APS patients with SLE in my study was very small (2/51; 4%). Therefore, I did not analyse them separately. Patients with SLE/APS could behave differently to those who with APS alone due to number of reasons including presence of other auto antibodies in patients with SLE/APS. However, I do not believe my results were influenced by the

coexistence of SLE in (2/51) of patients because samples from these two patients did not behave differently from those of the other primary APS in the coagulation and binding assays examined in this chapter.

It is possible that anti-protein C antibodies represent a different subset from those which define APS: aCL, a β_2 -GPI or lupus anticoagulant. These antibodies as a whole (i.e. both high and low avidity) do not cosegregate with the presence of APS-defining aPL. However, high avidity anti-protein C antibodies were present only in patients with APS, with 80% (12/15) (versus 40% with low avidity antibodies) classified as category I APS risk stratification (i.e. the presence of more than one aPL in any combination) (Miyakis *et al*, 2006). This suggests that high avidity anti-protein C antibodies might play a role as an adjunctive risk factor in APS patients with a severe thrombotic phenotype. APS patients may have a variety of other autoantibodies with different specificities (i.e. to phosphatidyl serine (PS), prothrombin, factor XII, tissue factor pathway inhibitor, factor X, etc), and the clinical relevance of these antibodies is being unravelled. aCL/a β_2 -GPI and anti-PS/prothrombin have been shown to induce APCr in a clotting based assay (Nojima *et al*, 2005). The high avidity anti-protein C antibodies in our study were specific for human protein C, and not due to cross-reactivity with aCL/a β_2 -GPI. In our study, there was no association between APCr and aCL or a β_2 -GPI. The relevance of low avidity anti-protein C antibodies, which were also observed in some non-APS patients with a severe thrombotic phenotype, remains to be defined.

Anti-protein C antibodies were not specific to thrombotic APS, as 8% (4/51) of the thrombotic non-APS patients also had anti-protein C antibodies, although these were low avidity antibodies. However, these patients demonstrated marked resistance to activation of endogenous PC and had clinically more severe disease, i.e. recurrent VTE whilst on therapeutic anticoagulation (none had both venous and arterial thrombosis). The difference in the behaviour of the low avidity anti-protein C antibodies in the APS and non-APS patients is unclear. Possible explanations include that aPL in APS patients may require higher avidity antibodies to impair activation of endogenous PC or alternative explanation is that antibodies other than anti-protein C

antibodies and aPL are implicated in the activation of endogenous PC in these patients.

Anti-protein S antibodies were far less frequent than anti-protein C antibodies in this study (7/51 APS and 1/51 non-APS patients), and were all low avidity. Anti-PS antibodies were not independently associated with increased APCr or with a severe thrombotic phenotype. The small number of anti-PS antibodies detected in this study preclude definitive conclusion. My results concur with those of Bertolaccini et al (Bertolaccini *et al*, 2003), but in contrast, Nojima et al (Nojima *et al*, 2002), demonstrated that the presence of anti-PS antibodies was strongly associated with acquired APCr, although they did not report on avidity of the antibodies.

In conclusion, thrombotic APS patients showed greater APCr to both exogenous rhAPC and activation of endogenous PC. APS patients had a significantly higher frequency and higher levels of anti-protein C antibodies: approximately half (49%) of the 51 patients with thrombotic APS had anti-PC antibodies. High avidity anti-protein C antibodies were present in 29.4% (15/51) of these patients and associated with greater APCr to both rhAPC and activation of endogenous PC with Protac®. This could be attributed to the presence of different antibodies against PC that may interfere with the anticoagulant action of APC or with the mechanism of activation. The presence of high avidity anti-PC antibodies was also strongly associated with a severe thrombotic phenotype in APS patients. Ninety-four percent (15/16) of the patients with a severe thrombotic clinical phenotype had high avidity anti-protein C antibodies and 62.5% (10/16) of those patients were triple positive for aPL. High avidity anti-protein C antibodies, associated with greater APCr, may provide a marker for a more severe thrombotic phenotype in APS.

Chapter 4 Sensitivity of commonly used thromboplastin reagents to rivaroxaban

4.1 Introduction

Routine monitoring of the anticoagulant effect of NOAC is not required due to the predictable anticoagulant effect of these preparations. However, measurement may be needed in certain clinical situations. These include: bleeding, before surgery or an invasive procedure, identification of subtherapeutic or supratherapeutic levels in patients taking other drugs that significantly affect pharmacokinetics, extremes of body weight, deteriorating renal function, perioperative management, reversal of anticoagulation, suspicion of overdose and assessment of compliance (Baglin *et al*, 2012;Baglin *et al*, 2013)

In vitro data indicate that there is a linear concentration-response relationship between the level of rivaroxaban and prothrombin time (PT) ratio, although there is a marked variability in the sensitivity between different PT reagents (Hillarp *et al*, 2011;Samama *et al*, 2010). The Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) and British Committee for Standards in Haematology (BCSH) (Baglin *et al*, 2012;Baglin *et al*, 2013) recommend that a PT with a suitable thromboplastin can be used to determine the relative intensity of anticoagulation achieved with rivaroxaban in an emergency or urgent clinical scenario, but should not be used to quantify the plasma drug concentration. However, there is a paucity of *ex vivo* data on the sensitivity of different thromboplastin reagents to rivaroxaban. Therefore studies with *ex vivo* samples are required to determine the relative sensitivity of different PT reagents to rivaroxaban. This would provide more specific information regarding the choice of PT reagent (Baglin *et al*, 2012;Baglin *et al*, 2013).

The main objective of this chapter is to assess the sensitivity of different thromboplastin reagents, commonly used in routine hospital laboratories (based on UK National External Quality Assessment Service reports), to rivaroxaban

concentrations using blood samples from patients receiving therapeutic dose rivaroxaban.

4.2 Patients and blood sampling

Blood samples were collected from 20 patients [8 males and 12 females, median age 46 years (range 34-78)] receiving therapeutic dose rivaroxaban. They had switched to rivaroxaban therapy due to: warfarin allergy, lack of phenindione availability, lifestyle or difficulty in monitoring. Samples were taken at a single time-point, 2-4 hours after their last dose of rivaroxaban, during routine outpatient appointments. All patients had been prescribed 20mg rivaroxaban daily (to be taken with food), for the preceding 3-12 weeks, for stroke prevention in non-valvular atrial fibrillation (6/20), or for the prevention of venous thromboembolism recurrence (14/20). No patient received other anticoagulants, antiplatelet agents, or drugs known to interact with rivaroxaban; all had a creatinine clearance >50mL/min (Cockcroft and Gault) (Cockcroft & Gault, 1976) and normal liver function tests.

Venous blood samples were collected with minimal stasis into 0.105M citrate Vacutainers®. Plasma was prepared within 1 hour by double centrifugation at 2000g for 15 minutes and stored at -80°C for up to 12 weeks.

4.3 Rivaroxaban levels

Rivaroxaban levels were measured using an amidolytic anti-Xa assay (Biophen DiXaI; with Biophen rivaroxaban calibrators, covering a range of 0 to 500ng/mL, which the manufacturer validated by HPLC (Rohde, 2008) as described in section 2.4. The assay was performed as a single run on a CS-2000i analyser (Sysmex) and samples were tested in duplicate. The intra-assay CV was 7% at 100ng/mL and 4% at 300ng/mL rivaroxaban.

The median plasma rivaroxaban level in the 20 patients was 246ng/mL (range 41-457ng/mL). A detailed pharmacokinetic study showed that 20mg rivaroxaban once daily gives peak plasma concentrations of 160-360ng/mL and trough concentrations of 4-96ng/mL (Mueck *et al*, 2008). In 11 of our patients, rivaroxaban levels (200-

356ng/mL) were within the expected therapeutic peak plasma concentration range, but six patients had rivaroxaban levels less than 160ng/mL (41-156ng/mL), and three patients had levels (455-457ng/mL) above the expected peak plasma concentration.

4.4 Prothrombin time

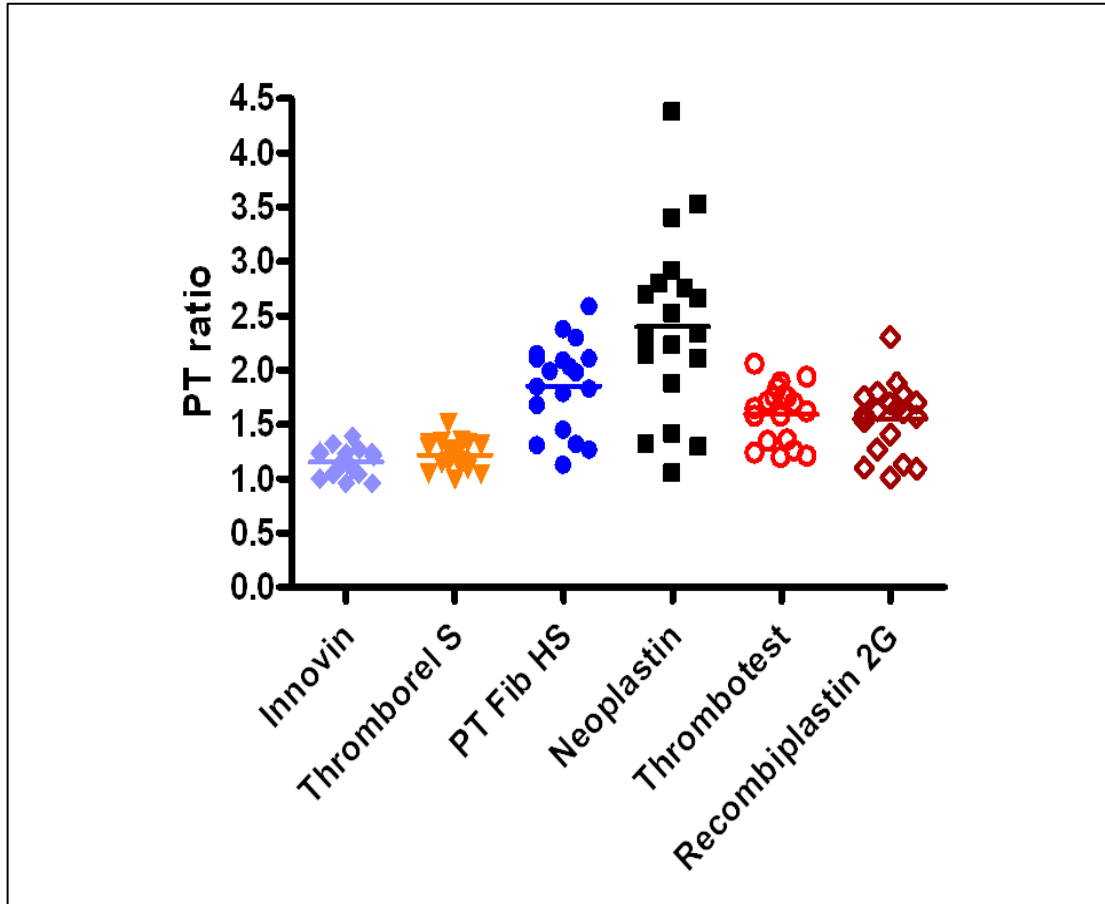
The PT was measured using a CS-5100 analyser (Sysmex) as described in section 2.3 with six different thromboplastin reagents (ISI & mean normal PT): Recombiplastin®2G (ISI 1.0 & 11.1s) and PT-Fibrinogen HS PLUS (ISI 1.2 & 16.1s) (Instrumentation Laboratory UK Ltd); Innovin® (ISI 1.0 & 11.4s) and Thromborel®S (ISI 1.0 & 12.8s) (Siemens Healthcare Diagnostics); Thrombotest™ (ISI 1.0 & 36.6s) (Alere Ltd, Stockport, UK) and Neoplastin®R (ISI 1.0 & 14.6s) (Diagnostica Stago, Asnières, France). Neoplastin R, Recombiplastin and Innovin are recombinant human tissue factor thromboplastins, Thromborel S is derived from human placenta, PT-Fibrinogen HS PLUS and Thrombotest are derived from rabbit and bovine brain, respectively. All samples were tested in the same analytical run. Results were expressed as prothrombin time ratio (PTR) = $PT_{\text{patient}}/PT_{\text{normal}}$. Geometric mean normal PT (seconds) for each thromboplastin reagent was established using samples from 30 healthy normal subjects (Table 4-1).

Table 4-1 Geometric mean normal PT for different thromboplastin reagents

Normal controls	Innovin	Thromborel S	PT Fib HS	Neoplastin R	Thrombotest	Recombiplastin 2G
1	11.4	12.8	16.2	14.6	36.3	10.4
2	11.6	12.5	16.3	14.9	38.0	11.2
3	11.2	12.4	16.3	14.9	36.2	11.3
4	11.4	12.4	15.4	14.7	36.3	10.3
5	11.0	12.3	16.8	15.0	36.6	10.8
6	11.1	12.3	16.5	14.8	36.8	11.7
7	11.1	13.1	15.0	14.9	36.7	10.6
8	11.2	14	15.8	14.8	36.6	11.4
9	11.1	11.8	15.1	14.8	36.7	10.6
10	11.4	11.2	16.8	14.7	36.7	10.7
11	11.6	12.1	15.8	14.2	37.7	10.6
12	11.7	12.3	15.3	14.9	36.8	10.8
13	11.9	12.2	15.7	14.9	36.0	11.6
14	11.4	13.1	15.9	14.5	37.0	10.0
15	11.1	13.2	15.9	14.7	36.6	11.6
16	11.2	14.0	15.8	14.4	36.1	10.9
17	12.0	13.6	17.0	14.5	36.1	11.8
18	11.9	13.5	15.8	14.4	36.2	12.3
19	11.8	14.0	16.6	14.4	37.1	12.3
20	11.1	11.0	15.9	14.4	36.2	12.3
21	12.0	14.0	15.8	14.7	36.3	11.7
22	11.0	11.2	16.8	14.9	36.6	10.6
23	11.3	13.3	16.9	14.9	36.8	13.0
24	11.2	11.2	15.8	13.0	36.7	10.6
25	11.0	13.9	15.8	14.9	36.0	10.7
26	11.9	12.8	16.7	14.8	36.7	10.6
27	10.0	12.16	16.1	14.3	36.5	10.8
28	13.0	14.0	16.8	15.0	36.7	11.6
29	10.9	13.2	17.0	14.8	36.8	10.5
30	12.9	14.0	16.9	14.7	36.0	11.6
Geometric Mean PT(S)	11.4	12.8	16.1	14.6	36.6	11.1

4.5 Prothrombin time ratio

Figure 4-1 The relative sensitivity of thromboplastin reagents in samples from 20 patients receiving rivaroxaban

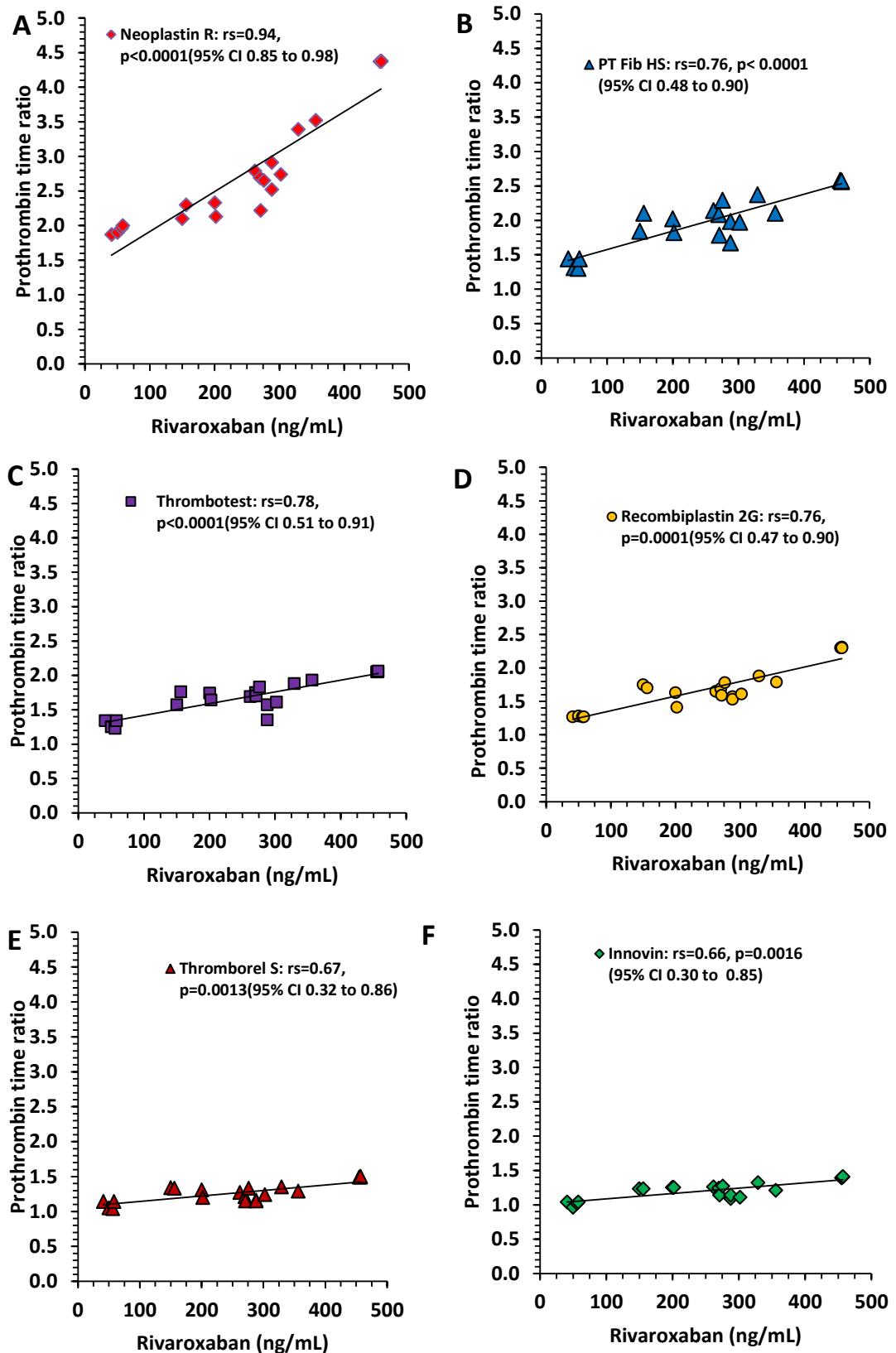


PT ratio =Prothrombin Time Ratios

The different thromboplastin reagents showed marked differences in sensitivity to rivaroxaban, as indicated by the PTR (Figure 4-1 and Figure 4-2). Neoplastin R® exhibited the widest spread of PTR values amongst the different patients and the greatest sensitivity to rivaroxaban, while Innovin® and Thromborel®S exhibited the least variability and sensitivity (Figure 4-1 and Figure 4-2). All thromboplastin reagents exhibited a significant correlation with rivaroxaban concentration (Figure 4-2).

However, in samples with therapeutic levels of rivaroxaban (as measured by the anti-Xa assay), the PTR was less than 1.5 for all samples tested with Innovin® and for 95% of the samples with Thromborel S.

Figure 4-2 Spearman correlation of Rivaroxaban levels and the prothrombin time ratio determined with six different individual thromboplastin reagent



4.6 Discussion

This study has established the sensitivity of six different thromboplastin reagents, commonly used in routine UK hospital laboratories for PT testing, in *ex vivo* samples from patients receiving therapeutic dose rivaroxaban. All thromboplastin reagents exhibited significant correlation between PTR and rivaroxaban level ($r_s >0.61$, $p < 0.005$). Neoplastin® was the most sensitive reagent to rivaroxaban, while Innovin® and Thromborel®S were the least sensitive.

Previous *in vitro* studies have suggested a wide range of sensitivity depending on the PT reagent. Limited *ex vivo* data using two PT reagents Triniclot PT Excel S® and Innovin® and an anti-Xa assay for rivaroxaban (Doux fils *et al*, 2013) suggested that the PTR using these two reagents did not correlate well with plasma levels of rivaroxaban (measured by liquid chromatography-tandem mass spectrometry, with levels ranging between 0-485ng/mL); the timing of blood samples in relation to rivaroxaban administration and the number of samples with therapeutic rivaroxaban levels were not stated. Two case reports (Patel *et al*, 2013; van Veen *et al*, 2013) have described patients with therapeutic levels of rivaroxaban, showing normal PTR with Innovin® and Thromborel®S. My findings with Innovin® concur with these observations, and in addition I have identified reagents that are more sensitive to rivaroxaban, indicating potential clinical utility.

My findings highlight that the choice of thromboplastin, based on the sensitivity of individual thromboplastin reagents to rivaroxaban, is critically important for accurate qualitative assessment of rivaroxaban levels using a PTR. The ISTH SSC (Baglin *et al*, 2013) has suggested that the PT could be used as the first line test, while an anti-Xa assay (rivaroxaban level) should be employed when an accurate anticoagulant level is required. They also suggested that all hospital laboratories should have a test that is easy to perform, readily available, and able to provide results within one hour of receiving a patient sample (Baglin *et al*, 2013). Such testing should aid decision making in the acute management of the patient by indicating whether the patient is anticoagulated in the therapeutic, subtherapeutic or supratherapeutic range. Each

laboratory should know the sensitivity of its own PT reagent and APTT tests to rivaroxaban and advice on interpretation (Kitchen *et al*, 2014).

The INR-ISI methodology currently used for vitamin K antagonists is not recommended by the BCSH. However, some studies have investigated the suitability of employing a rivaroxaban sensitivity index similar to the specific ISI in a PT/INR system to normalised and reduce the variability of the results obtained with different thromboplastin reagents (Harenberg *et al*, 2011; Tripodi *et al*, 2011a).

Assays that would provide quantitative information about the anticoagulation intensity (rivaroxaban level) are usually only available on a non-urgent basis in a limited number of clinical laboratories. Furthermore, test results are dependent on when the last dose of drug was taken and these therefore require interpretation with reference to the dose, anticipated half-life, and other factors that influence pharmacokinetics, such as renal function.

The PT can be prolonged due to a number of reasons including hepatic impairment, vitamin K deficiency and sepsis, and these factors may contribute to a prolonged PT in patients on rivaroxaban. Therefore, determining a baseline PT may be useful when later interpreting PT results during therapy. In my study, all patients had been switched from therapeutic dose warfarin to therapeutic dose rivaroxaban at least three weeks prior to sampling; a baseline PT was therefore not available. Limitations of my study were that the number of patients was relatively low and did not include patients with impaired renal function.

The use of Innovin® or Thromborel®S may give false reassurance as normal results are likely to be obtained from patients with therapeutic levels of rivaroxaban. Of note in this context, Mani *et al* demonstrated that two hours after rivaroxaban administration (10 mg once daily), the activities of clotting factors (II, V, VII, VIII, IX, XI, XII) appeared to be significantly decreased while factor XIII levels were unchanged, when performed as a single point assay (Mani *et al*, 2013). When using different dilutions of plasma samples in factor VIII and factor X assays, rivaroxaban

effects disappeared at higher plasma dilutions, demonstrating the importance of performing assays at multiple dilutions. These results were obtained with either Innovin® or the activated partial thromboplastin time (aPTT) reagent SynthASil®, which are less sensitive to rivaroxaban (Mani *et al*, 2013). Reagents which are more sensitive to rivaroxaban may exhibit an even greater interference on coagulation factor assays. These effects need to be taken into account when laboratories assess coagulation factor levels in patients taking rivaroxaban, using PT or APTT based assays especially in terms of timing and dose of rivaroxaban intake.

The median plasma rivaroxaban level in the 20 patients was 246ng/mL (range 41-457ng/mL). No patient experienced recurrence of thrombosis or clinically significant bleeding during treatment. Based on population pharmacokinetic study (Mueck *et al*, 2008), it is expected to have peak plasma concentrations of 160-360ng/mL and trough concentrations of 4-96ng/mL (Mueck *et al*, 2008) with 20mg rivaroxaban given once daily to individuals with normal renal functions. Nearly 50% (9/20) of the patients had rivaroxaban levels outside the expected therapeutic range. Six patients had rivaroxaban levels less than 160ng/mL (41-156ng/mL), and three patients had levels (455-457ng/mL) above the expected peak plasma concentration. Although patients were asked to take rivaroxaban 2-4 hours prior to outpatient attendance, nearly half had rivaroxaban levels outside the expected peak plasma concentration range. A pharmacokinetic study showed that if blood samples were taken at two or at four hours after rivaroxaban, plasma levels would vary by about 12% (Mueck *et al*, 2011), which means that therapeutic rivaroxaban levels should vary by only 60ng/mL. This suggests poor compliance in some of the patients we studied.

The sensitivity of different APTT reagents to rivaroxaban levels has also shown a similar pattern of those observed with PT reagents. In an *in vitro* study (Dale *et al*, 2014), using seven different APPT reagents (Actin FSL, Actin FS [Siemens], TriniCLOT S, TriniCLOT HS, STA-CK PREST, STA-PTT A [Stago Diagnostica], and SynthASil [Instrumentation Laboratory] has shown that all APTT reagents were sensitive to rivaroxaban but their sensitivities differed. The four most sensitive reagents

were Actin FSL, Actin FS, STA-CK PREST and STA-PTT A. These four reagents exhibited similar sensitivities with lower concentrations of rivaroxaban, with higher concentrations; Actin FSL was the most sensitive reagent.

In conclusion, there is a wide variation in the sensitivity of thromboplastin reagents to rivaroxaban. Of the six commonly used thromboplastin reagents studied, Neoplastin®R was the most sensitive while Innovin® and Thromborel®S were the least sensitive. These *ex vivo* findings, based on samples from patients on therapeutic dose rivaroxaban, are in keeping with those previously reported from *in vitro* studies. Rivaroxaban levels within the therapeutic range can give a normal PTR with the less sensitive reagents, which may be clinically misleading. In the absence of anti-Xa assays, the assessment of rivaroxaban anticoagulation should only be performed using PT reagents with good sensitivity.

Chapter 5 Detection of lupus anticoagulant in the presence of rivaroxaban and effects of antiphospholipid antibodies on rivaroxaban anticoagulant action

The thrombotic manifestations of APS correlate best with the prolongation of phospholipid-dependent clotting assays associated with LA (Galli *et al*, 2003). Patients with APS are at increased risk of recurrent thrombosis. The current mainstay of treatment for thrombotic APS is long term anticoagulation with vitamin K antagonists (VKA) such as warfarin (Keeling *et al*, 2012). However, NOAC drugs such as rivaroxaban, apixaban, edoxaban and dabigatran are increasingly being used in clinical practice for different indications, including the treatment and prevention of VTE.

Approximately 10% of patients with acute VTE have APS (Andreoli *et al*, 2013), and it is therefore likely that patients with APS were included in the phase III clinical trials of NOAC versus VKA in patients with VTE. However, aPL status was not documented in these trials (Bauersachs *et al*, 2010; Buller *et al*, 2012; Schulman *et al*, 2009). Due to increasing use of rivaroxaban, it is clinically relevant to define the appropriate methodology for *ex vivo* LA testing, firstly to enable the diagnosis of APS in patients treated with rivaroxaban; and secondly, to inform and be considered for the update of the national and international guidelines. This could be established by *in vitro* and *ex vivo* in studies of LA detection in thrombotic APS patients receiving therapeutic dose rivaroxaban. However, such studies are lacking.

It is theoretically possible that aPL could directly interfere with the anticoagulant effects of rivaroxaban, although it is hypothesised that this is unlikely due to its small molecular size (436Da) and the high specificity for its target. Rivaroxaban affects various parameters of *ex vivo* thrombin generation (TG) measured by using calibrated automated thrombography (CAT) (Arachchillage *et al*, 2015). TG as a global test of haemostasis can be used to assess the anticoagulant effects of anticoagulants including rivaroxaban. The effects of aPL on the anticoagulant action of rivaroxaban have not been clearly defined.

The aim of this chapter was to establish the influence of therapeutic dose rivaroxaban on LA detection *in vitro* and *ex vivo*, and of aPL on rivaroxaban anticoagulant action assessed using TG testing and anti-Xa assays.

5.1 Patients, sample collection and preparation

5.1.1 *In vitro* studies:

Polyclonal IgG fraction was purified (section 2.22) from venous samples collected into 0.105M citrate Vacutainers®, from 32 patients with thrombotic APS who fulfilled the revised International consensus classification criteria for APS (20 patients with persistent LA and 12 patients without LA, but positive for either aCL or a β 2GPI or both) and 20 healthy normal controls (NC), who tested negative for aPL. The presence of persistently positive LA in these 20 patients had been confirmed in accordance with the ISTH (Pengo *et al*, 2009) and BCSH (Keeling *et al*, 2012) guidelines on LA detection. Clinical features and APS classification categories of the 30 APS patients (I, IIa, IIb) (Miyakis *et al*, 2006) selected for IgG purification are shown in Table 5-1. All normal controls were tested for aPL and were completely negative.

Table 5-1 Clinical features and APS classification categories of the 30 APS patients (I, IIa, IIb, IIc according to Miyakis et al, 2006) selected for IgG purification

Clinical features and APS category of 30 APS patients	
Age, mean years	41.43± 8.6
Sex, male/female	18/12
Vascular thrombosis	
DVT alone	12 (40%)
PE alone	8(27%)
DVT+PE	6 (20%)
Arterial	4 (13%)
APS category	
I	19/30 (63%)
IIa	5/30 (17%)
IIb	3/30 (10%)
IIc	3/30 (10%)

(Category I: more than one laboratory criteria present [any combination]; IIa: LA present alone; IIb: aCL antibody present alone; IIc: aβ2GPI antibody alone. All normal controls were tested for aPL).

IgG was quantified by an in-house IgG ELISA (section 2.23). The amount of IgG purified per mL of plasma did not differ between APS and NC and ranged from 5-10mg/mL, consistent with the normal level of circulating IgG in adults. Rivaroxaban was diluted in DMSO (Sigma) and Owren's veronal buffer (OVB); Siemens Healthcare Diagnostics) (section 2.24. Following addition of required volume of rivaroxaban preparation to PNP resulted in DMSO concentration of ≤ 1% (1% for LA testing and 0.75% for TG). Both in our experiments and also work from others have shown that DMSO at this concentration has no effect on TG and other coagulation assays (Perzborn *et al*, 2014).

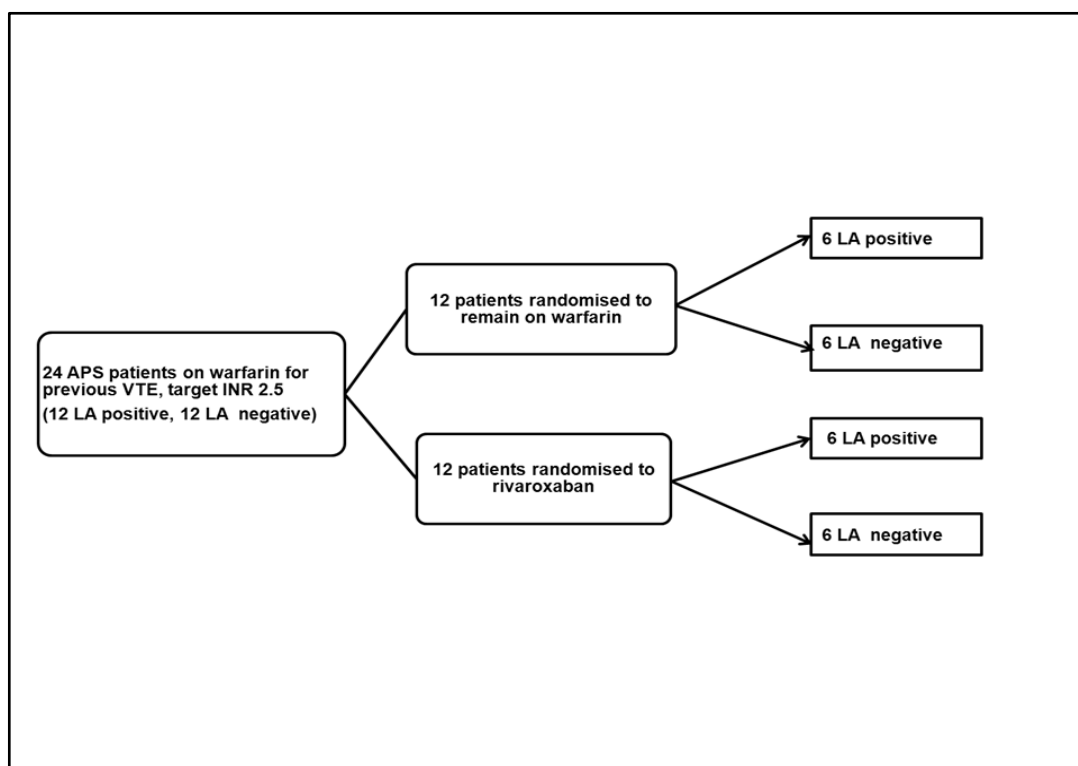
Rivaroxaban 50ng/mL, 250ng/mL and 0ng/mL (buffer control) and IgG (500ug/mL) preparations in 20mM HEPES buffer (20mM HEPES, 140mM NaCl, 0.02% Sodium Azide at pH 7.35) with 1% BSA from 32 APS patients (20 LA positive, 12 LA negative) and 20 NC were spiked into pooled normal plasma (PNP) to give the final plasma: IgG and rivaroxaban ratio 80:20 v/v 80% incubated for 10 minutes at 37°C. I established that DMSO, OVB and PBS had no influence on TG (data not shown) before proceeding with the rest of the experiments. Rivaroxaban concentrations were selected to represent the expected peak (160-360µg/L) and trough (4-96µg/L) rivaroxaban levels with rivaroxaban 20mg daily based on previous population pharmacokinetic studies (Mueck *et al*, 2011). An IgG concentration of 500ug/mL was selected based on my preliminary studies and also experience of other workers (Pericleous *et al*, 2013).

5.1.2 *Ex vivo* studies

Blood samples for *ex vivo* LA studies were collected with minimal stasis from 24 thrombotic APS patients, who were on anticoagulation with warfarin for VTE, target INR 2.5 (range 2.0-3.0) for at least 3 months, at baseline pre-randomisation to the RAPS trial. This is a phase II/III clinical trial of warfarin versus rivaroxaban (<http://isrctn.org/ISRCTN68222801>) aiming to demonstrate, in patients with APS and previous VTE that the intensity of anticoagulation achieved with rivaroxaban is not inferior to that of warfarin. The safety of rivaroxaban is assessed in terms of rates of bleeding and further thrombosis, and compare serious adverse events in patients on rivaroxaban with those on warfarin as the secondary outcome measures. These 24 patients comprised the first consecutive persistently LA positive and LA negative patients at the UCLH site randomised to the rivaroxaban arm or to remain on warfarin, respectively (12 patients in each group, comprising the first 6 consecutive persistently LA positive and 6 LA negative patients) (Figure 5-1). LA status of all patients had been confirmed previously during definition of their APS-defining aPL, in accordance with the ISTH and BCSH guidelines on LA detection (Keeling *et al*, 2012;Pengo *et al*, 2009). Of those 12 patients on rivaroxaban, (6 LA positive and 6 LA negative) were collected 2-4 hours after the morning dose of rivaroxaban to represent peak levels and at least 18 (range 18-24) hours following the last dose to

represent trough levels. All patient samples were collected after at least 30 days (range 30-90 days) of anticoagulation with either warfarin or rivaroxaban. PPP samples were stored in aliquots and the samples were thawed for 10 minutes at 37°C immediately prior to analysis.

Figure 5-1 : Patients selection for *ex vivo* LA detection studies



5.1.3 Prothrombin time (PT)

PT was performed on samples from patients on warfarin, at baseline and after at least 30 (range 30-90) days on the 12 patients randomised to warfarin, using a single lot number of Innovin (Siemens Healthcare Diagnostics), on a CS-5100 analyser (Sysmex UK Ltd) employing an instrument-specific ISI value (0.99) (section 2.3).

5.1.4 Rivaroxaban anti-Xa levels

Rivaroxaban anti-Xa levels were measured on the 12 RAPS trial patients on rivaroxaban and on *in vitro* spiked samples, which were also tested for TGT, using an amidolytic anti-Xa assay (section 2.4). *In vitro* spiked and patient samples were assayed separately with samples from each group assayed in the same analytical run.

5.1.5 Lupus anticoagulant

LA tests on *in vitro* spiked samples were performed using Textarin® time; dilute prothrombin time (dPT); DRVVT; and TVT/ECT (sections 2.10, 2.5, 2.6 and 2.11). The following DRVVT tests were performed on both *in vitro* and *ex vivo* samples: LA1 screening (low phospholipid concentration) and LA2 confirmation (high phospholipid concentration) reagents (Siemens Healthcare Diagnostics Products), and HemosIL dRVVT screen and confirm (Instrumentation Laboratory), reagents consisting of a screen and confirmatory reagents. In addition, an in-house method was undertaken in the initial *in vitro* studies. TVT/ECT were also performed in *ex vivo* studies. DRVVT on plasma samples taken from patient treated with warfarin were tested on equal volume (50:50) mixtures of patient plasma and PNP. Normal plasma, LA control high and low and a local quality control sample from a patient with confirmed LA were tested at the beginning and end of the test run. All samples were tested using the same lot numbers of reagents for each LA assay.

5.1.5.1 LA results interpretation

DRVVT results were interpreted as the normalised test/confirm ratio to correct for differences in instrument and reagent combinations. The 99th centile was used as a cut-off for normality; 1.19 (<1.2) and LA 1/LA 2 normalised ratio of >1.19 was considered to be positive for LA.

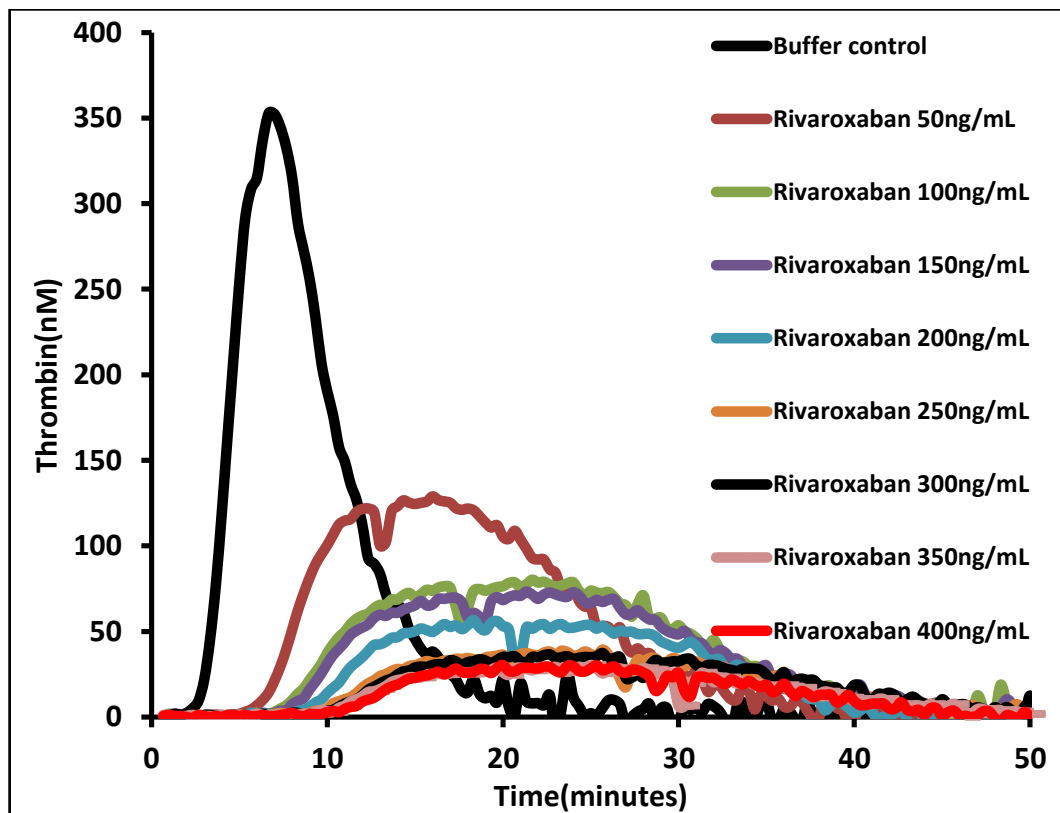
The TVT ratio (TVTR) was calculated by dividing the clotting time for each test sample by the clotting time for PNP obtained with Taipan venom. A similar ratio calculation was performed for the confirm step with Ecarin clotting time. These two ratios were used to determine the normalised TVT/ECT ratio. Normal reference

ranges for TVTR, ECT ratio and TVTR/ECT ratio are 0.96 – 1.30, 0.99 – 1.14 and 0.9 – 1.2 respectively. A TVTR/ECT ratio of >1.2 was considered to be positive for LA.

5.1.6 Studies to define the effects of aPL on rivaroxaban anticoagulant action

The effects of aPL on the anticoagulant effects of rivaroxaban were assessed using the TG test and anti-Xa assays on PNP spiked with four different concentrations of rivaroxaban (0, 25, 50 and 100ng/mL) with two concentrations of IgG (500µg/mL and 250µg/mL) from 30 APS and 20 NC. These rivaroxaban concentrations were chosen following preliminary investigation with increasing concentrations of rivaroxaban spiked into PNP on TG to demonstrate clearly measurable effects on each parameter of TG (Figure 5-2). TG was performed using the CAT system (section 2.15).

Figure 5-2 Thrombin generation with increasing concentration of rivaroxaban



5.2 Results

Lupus anticoagulant studies

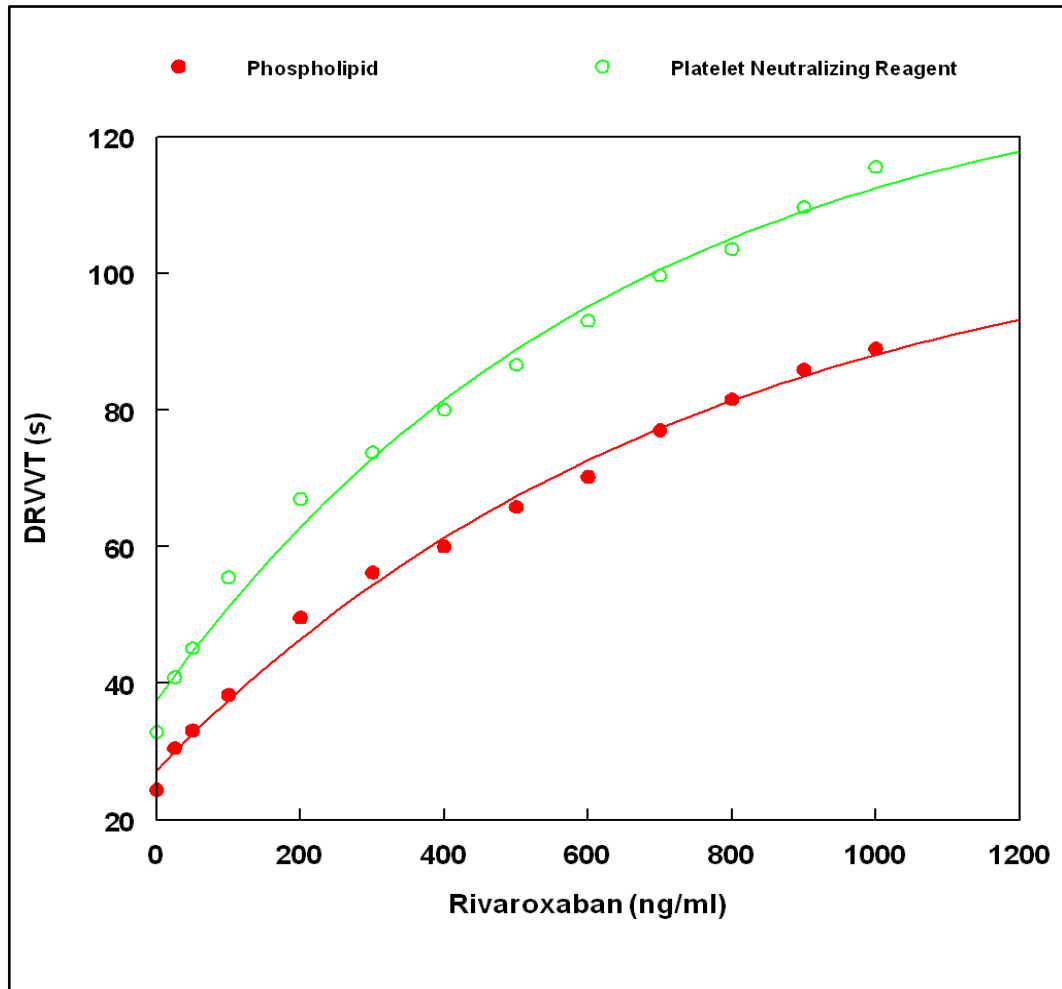
Imprecision studies using commercial QC plasmas (mean LA 1: 50.3 & 74.5s, LA 2: 34.7 & 37.7s, respectively; normalised LA 1/LA 2 ratio: 1.28 & 1.75 respectively) showed a coefficient of variation (CV) of approximately 1.0% for LA 1, 0.5% for LA 2, and 1.0% for the normalised ratio from the samples tested on 8 different days.

Imprecision studies using commercial QC plasmas (mean TVT: 50.3 & 74.5s, ECT: 34.7 & 37.7s, respectively; normalised TVTR/ECT ratio: 1.28 & 1.75 respectively) showed a CV of approximately 1.0% for TVT, 0.5% for ECT, and 1.0% for the normalised ratio from the samples tested on 8 different days.

5.2.1 *In vitro* studies: lupus anticoagulant

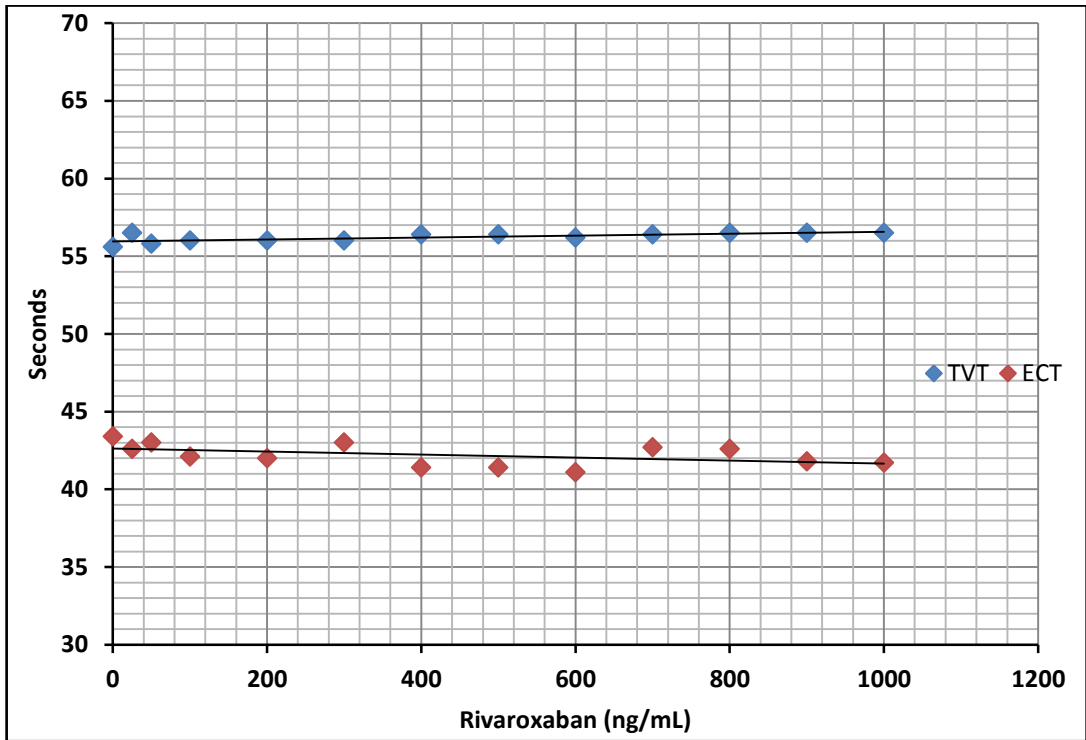
Rivaroxaban caused a concentration dependent increase in clotting times with the dPT and DRVVT (Figure 5-3), but not with the TVT, ECT (Figure 5-4) or Textarin time.

Figure 5-3 DRVVT with phospholipid and platelet neutralizing reagents with increasing concentration of rivaroxaban (in house method)



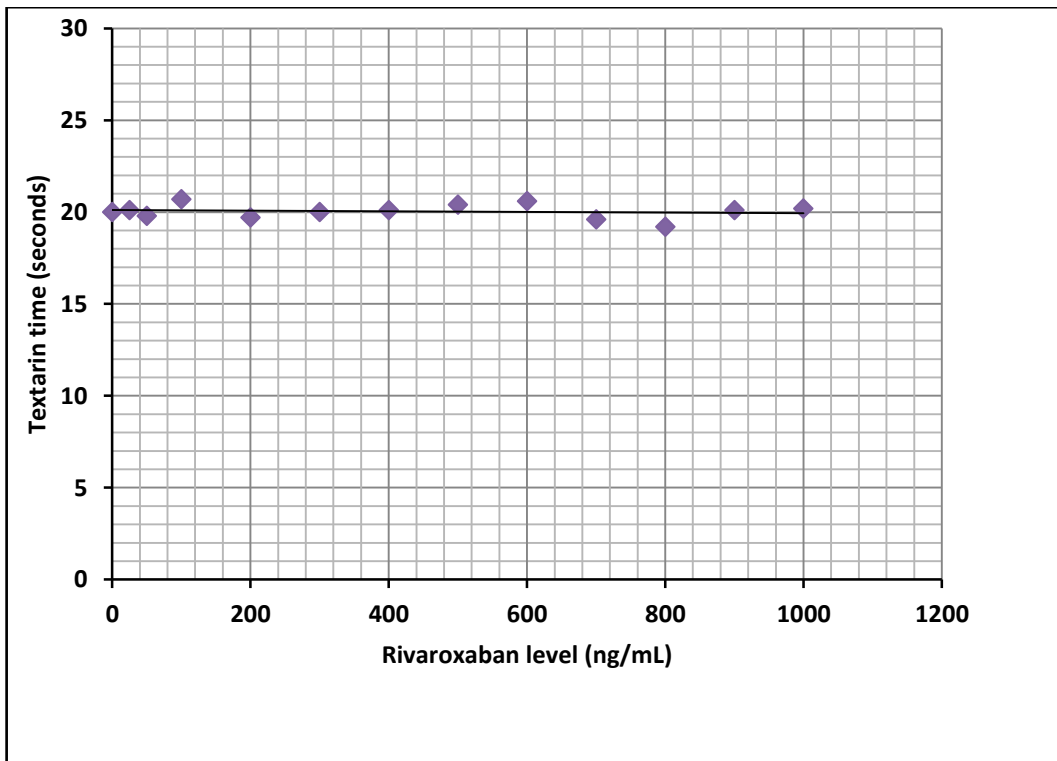
Rivaroxaban caused a concentration dependent increase in clotting times with the DRVVT with both phospholipid and platelet neutralizing reagents.

Figure 5-4 TVT and ECT with increasing concentration of rivaroxaban (KC4A method)



TVT and ECT remain unchanged with increasing concentration of rivaroxaban

Figure 5-5 Textarin time with increasing concentration of rivaroxaban (KC4A method)



Textarin time remains unchanged with increasing concentration of rivaroxaban

The dPT screen ratio became abnormal at 250ng/mL rivaroxaban. However, the dPT confirm ratio also increased in parallel, with the screen/confirm ratio (SCR) unchanged, and there were no false positives with IgG from LA negative APS patients and NC (Table 5-2). IgG spiked into PNP from 19 of the 20 LA positive APS patients remained positive for LA by dPT, irrespective of the rivaroxaban concentration (SCR mean [CI]: 1.4 [1.3-1.6] and 1.4 [1.3-1.7] for 50ng/mL and 250ng/mL respectively) (Table 5-2). The remaining patient had negative LA with dPT without spiking with rivaroxaban, and this remained unchanged at both concentrations of rivaroxaban.

The in-house DRVVT showed no false LA positive or negative LA results at either rivaroxaban level (Table 5-3). The PNP spiked with IgG from NC and rivaroxaban 50ng/ml or 250ng/mL exhibited similar results to those obtained from samples spiked with IgG from LA -ve APS patients. With the commercial DRVVT reagents, the SCR became abnormal when PNP spiked with IgG from NC or LA negative APS patients and 250ng/mL rivaroxaban, giving false positive results in 18/20 (90%) in NC and 11/12 (92%) LA negative APS (Table 5-4). This phenomenon was not observed with 50ng/mL rivaroxaban. PNP spiked with LA positive APS IgG remained LA positive at both levels of rivaroxaban (Table 5-4).

There was no effect of rivaroxaban on Textarin time (Table 5-5) or TVT/ECT ratio and LA was detected at both rivaroxaban levels for all APS IgG spiked samples (TVT/ECT ratio: 1.4 [1.3-1.5], 1.3 [1.25-1.35] and 1.3 [1.23-1.36] for 0, 50 and 250ng/mL rivaroxaban respectively (normal TVT/ECT < 1.2). There were no false LA positive results with TVT/ECT (Table 5-6).

Table 5-2 dPT ratios with APS LA+ve IgG and negative IgG with rivaroxaban 50ng/mL or 250ng/mL. (Normal Screen/confirm ratio >1.2)

dPT ratio	APS LA+VE IgG	APS LA+VE IgG+ Rivaroxaban 50ng/mL	APS LA+VE IgG+ Rivaroxaban 250ng/mL	APS LA-VE IgG	APS LA-VE IgG + Rivaroxaban 50ng/mL	APS LA-VE IgG+ Rivaroxaban 250ng/mL
Screen ratio	1.3 (1.2-1.5)	1.8 (1.5-2.27)	2.7 (2.3-3.4)	1.0 (1.0-1.1)	1.2 (1.2-1.3)	1.8 (1.8-2.0)
Confirm ratio	1.0 (0.9-1.2)	1.3 (1.2-1.6)	1.9 (1.6-2.2)	1.0 (0.9-1.0)	1.2 (1.1-1.3)	1.8 (1.8-1.9)
Screen/Confirm ratio	1.4 (1.2-1.6)	1.4 (1.3-1.6)	1.4 (1.3-1.7)	1.0 (1.0-1.1)	1.0 (1.0-1.1)	1.0 (0.9-1.1)

Data is shown as median ratio with 95% confidence interval.

Table 5-3 DRVVT ratios (in-house method) with APS LA+ve IgG and negative IgG with rivaroxaban 50ng/mL or 250ng/mL ((Normal Screen/confirm ratio >1.2)

DRVVT ratio	APS LA+VE IgG	APS LA+VE IgG+ Rivaroxaban 50ng/mL	APS LA+VE IgG+ Rivaroxaban 250ng/mL	APS LA-VE IgG	APS LA-VE IgG + Rivaroxaban 50ng/mL	APS LA-VE IgG + Rivaroxaban 250ng/mL
Screen ratio	1.5 (1.4-1.6)	2.5 (2.1-2.8)	3.6 (3.3-3.9)	1.0 (1.0-1.1)	1.5 (1.4-1.6)	2.4 (2.2-2.5)
Confirm ratio	1.1 (1.1-1.4)	1.6 (1.5-2.2)	2.7 (2.4-2.9)	1.0 (0.9-1.0)	1.4 (1.3-1.5)	2.3 (2.1-2.4)
Screen/Confirm ratio	1.3 (1.2-1.4)	1.5 (1.3-1.7)	1.4 (1.2-1.7)	1.0 (0.9-1.1)	1.0 (1.0-1.1)	1.0 (0.9-1.1)

Data is shown as median ratio with 95% confidence interval.

Table 5-4 1DRVVT ratios (Siemens reagents) with APS LA+ve IgG and negative IgG with rivaroxaban 50ng/mL or 250ng/mL (Normal Screen/confirm ratio >1.2)

DRVVT ratio	APS LA+VE IgG	APS LA+VE IgG+ Rivaroxaban 50ng/mL	APS LA+VE IgG+ Rivaroxaban 250ng/mL	APS LA-VE IgG	APS LA-VE IgG + Rivaroxaban 50ng/mL	APS LA-VE IgG+ Rivaroxaban 250ng/mL
Screen ratio	1.3 (1.2-1.4)	1.8 (1.7-2.3)	2.8 (2.6-3.6)	1.0 (0.9-1.1)	1.7 (1.6-1.8)	2.6 (2.2-2.5)
Confirm ratio	1.1 (1.0-1.1)	1.5 (1.3-1.6)	2.1 (2.0-2.2)	1.0 (0.9-1.0)	1.5 (1.4-1.6)	2.1 (2.0-2.2)
Screen/Confirm ratio	1.3 (1.2-1.4)	1.3 (1.2-1.5)	1.3 (1.2-1.8)	1.0 (0.9-1.1)	1.0 (1.0-1.1)	1.3 (1.2-1.4)

Data is shown as median ratio with 95% confidence interval.

5-5 Textarin time, Ecarin clotting time (ECT) and Textarin time/ECT ratios with APS LA+ve IgG and negative IgG with rivaroxaban 50ng/mL or 250ng/mL

	APS LA+VE IgG	APS LA+VE IgG+ Rivaroxaban 50ng/mL	APS LA+VE IgG + Rivaroxaban 250ng/mL	APS LA-VE IgG	APS LA-VE IgG + Rivaroxaban 50ng/mL	APS LA-VE IgG + Rivaroxaban 250ng/mL
Textarin time(seconds)	35.7(33.0-37.1)	36.9 (36.0-38.1)	36.2 (34.9-37.8)	23.0(22.6-24.1)	23.1 (22-23.8)	23.6 (23.1-24.1)
ECT (seconds)	25.0 (24.6-25.2)	25.6 (24.2-26.3)	25.4 (24.6-26.7)	23.4 (22.9-24.1)	23.0 (22.1-24.5)	23.9(22.9.2-25.0)
Textarin time/ECT ratio	1.4 (1.2-1.6)	1.4 (1.2-1.6)	1.3 (1.2-1.6)	0.9 (0.8-0.1.1)	0.9(0.8 -1.1)	1.0(0.9-1.1)

Textarin time/ECT ratio >1.19 confirm LA. There were no false positive or negative LA results at both rivaroxaban levels. Data is shown as median ratio with 95% confidence interval.

Table 5-6 TVT and ECT ratios with APS LA+ve IgG and negative IgG with rivaroxaban 50ng/mL or 250ng/mL

	APS LA+VE IgG	APS LA+VE IgG+ Rivaroxaban 50ng/mL	APS LA+VE IgG+ Rivaroxaban 250ng/mL	APS LA-VE IgG	APS LA-VE IgG + Rivaroxaban 50ng/mL	APS LA-VE IgG + Rivaroxaban 250ng/mL
TVT ratio	1.4 (1.3-1.5)	1.4 (1.3-1.5)	1.5 (1.3-1.5)	1.0 (0.9-1.1)	1.0 (0.9-1.1)	1.0 (0.9-1.1)
ECT ratio	1.1 (1.0-1.1)	1.2 (1.1-1.2)	1.2 (1.1-1.2)	1.0 (0.9-1.0)	1.1 (1.0-1.1)	1.1 (1.0-1.1)
TVT/ECT ratio	1.3 (1.2-1.4)	1.2 (1.2-1.4)	1.3 (1.2-1.4)	1.0 (0.9-1.1)	0.9 (0.9-1.1)	0.9 (0.9-1.1)

Normal TVT 0.93-1.30, ECT 1.03-1.14, TVT/ECT ratio >1.19 confirm presence of LA. Data is shown as median ratio with 95% confidence interval

5.2.2 Ex vivo studies

5.2.2.1 International normalised ratio (INR)

INR values on the 24 patients on warfarin pre-randomisation and in those 12 patients who remained on warfarin were; (mean [CI]) 2.4 [2.1-3.2] and 2.3 [1.9-3.1] respectively.

5.2.2.2 Rivaroxaban levels

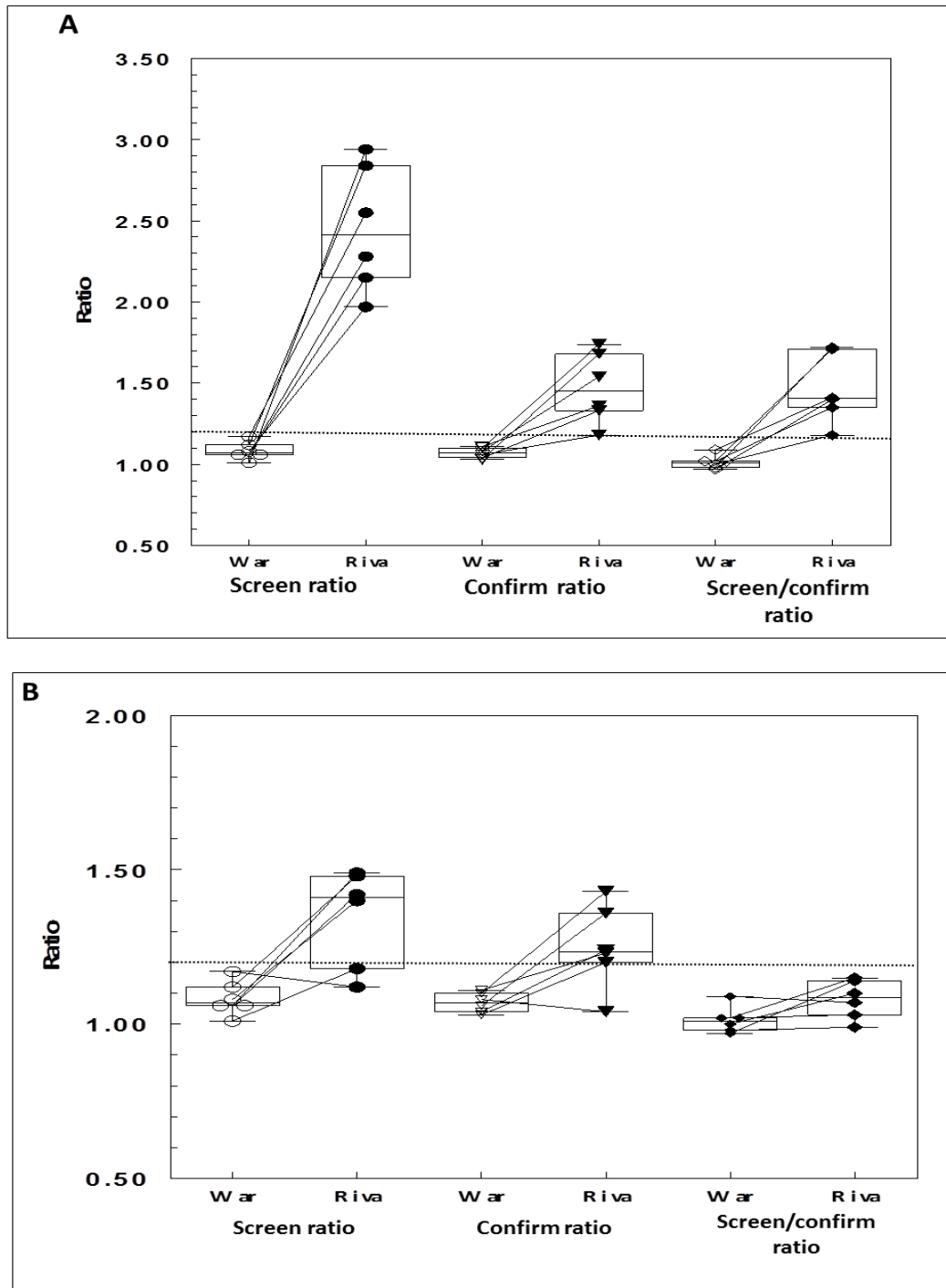
The plasma rivaroxaban levels of the 12 patients went onto rivaroxaban treatment, when samples taken at 2-4 hours following the last morning dose of rivaroxaban (mean [CI]) were 240 [165-270] ng/mL and the levels expected to be at the trough plasma rivaroxaban levels when samples were taken at over 18 (18-24) hours after the last morning dose of rivaroxaban were (mean [CI]) 55 (36-80) ng/mL. These peak and trough levels concur with those previously reported in population pharmacokinetic studies (Mueck *et al*, 2011).

5.2.2.3 Lupus anticoagulant

There were no false positive or negative LA results in the 24 RAPS patients when they were on warfarin, based on their APS-defining LA status determined prior to randomisation into RAPS trial. Six confirmed LA positive patients receiving rivaroxaban remained LA positive with TVT/ECT and DRVVT reagents at both peak (162-278ng/mL) and trough (30-85ng/mL) levels of rivaroxaban, except one patient (178ng/mL) who appeared LA negative with one commercial reagent. There was a greater prolongation of the screen DRVVT compared with the DRVVT confirm assay with both commercial reagents in patients treated with rivaroxaban at therapeutic levels, causing an increased normalised test/confirm ratio. This led to six LA negative patients becoming (apparently) LA positive with two DRVVT reagents, test/confirm ratio median [CI]: 1.6 [1.3-1.8], 1.6 [1.4-1.9] Figure 5-6A), but not by TVT/ECT (1.1 [0.8-1.2]). On samples taken at least 18 (18-24) hours after the last dose of rivaroxaban from those six patients who were previously LA negative and

randomised to rivaroxaban remained LA negative with both DRVVT reagents (Figure 5-6B), and with TVT/ECT.

Figure 5-6 Paired results on DRVVT screen, confirm and screen/confirm ratios in 6 patients negative whilst on warfarin and at least 6 weeks on rivaroxaban with therapeutic levels (A), with trough levels (B).



Normal screen/confirm ratio is <1.2. Mean rivaroxaban levels of these 6 patients that were expected to be within the therapeutic and trough levels were 240 [CI: 165-270ng/mL] and 55 [CI: 36-80ng/mL] respectively. War= Warfarin; Riva = Rivaroxaban

5.2.3 Effects of aPL on rivaroxaban anticoagulant action

5.2.3.1 Thrombin generation

Spiking PNP with IgG 500ug/mL from patients with LA positive APS caused a prolonged lag time and time to peak with LA positive IgG compared to LA negative IgG and NC IgG) (Figure 5-7): mean lag time [CI]: 5.2 [4.1-6.5], $p=0.02$ LA positive IgG compared to LA negative 3.2 [2.7-3.6], and NC 3.1 [2.7-3.5] and mean time to peak: 8.1 [7.1-9.5], $p=0.01$ compared to LA negative 6.4 [6.2-6.6] and NC 6.4 [6.1-6.6]. There were no differences in peak thrombin and ETP in PNP spiked with LA positive IgG compared to PNP spiked with LA negative or NC IgG. The differences in the lag time and time to peak between three groups remained when PNP/IgG mixtures were also spiked with increasing concentrations of rivaroxaban (25, 50 and 100ng/mL). However, there was no difference in peak thrombin (Table 5-7) and ETP (Table 5-8) between the three groups. There was no difference in the lag time or time to peak in the three groups at 250ug/mL IgG demonstrating that the LA effect is dose dependent.

5.2.3.2 Rivaroxaban anti-Xa assay

When PNP containing rivaroxaban was spiked with 250 μ g/mL and 500 μ g/mL from 20 LA positive, 12 LA negative and 20 NC with rivaroxaban 0, 25, 50 and 100ng/mL, caused no change in the rivaroxaban concentration by anti-Xa assay. Mean [CI] rivaroxaban anti-Xa levels for 0, 25, 50 and 100ng/mL PNP respectively, spiked with IgG from the three groups were: 0, 20 [19-24], 46 [44-48] and 95 [94-98] ng/mL in LA positive APS; 0, 22 [21-24], 45 [44-47] and 97 [96-98] ng/mL in LA negative APS; and 0, 23 [21-24], 46 [44-47] and 97 [96-98] ng/mL in NC.

Figure 5-7 Example of thrombin generation in pooled normal plasma spiked with IgG 500 μ g/mL, NC and LA negative APS (A), with IgG 500 μ g/mL, NC and LA positive APS (B) with increasing concentration of rivaroxaban

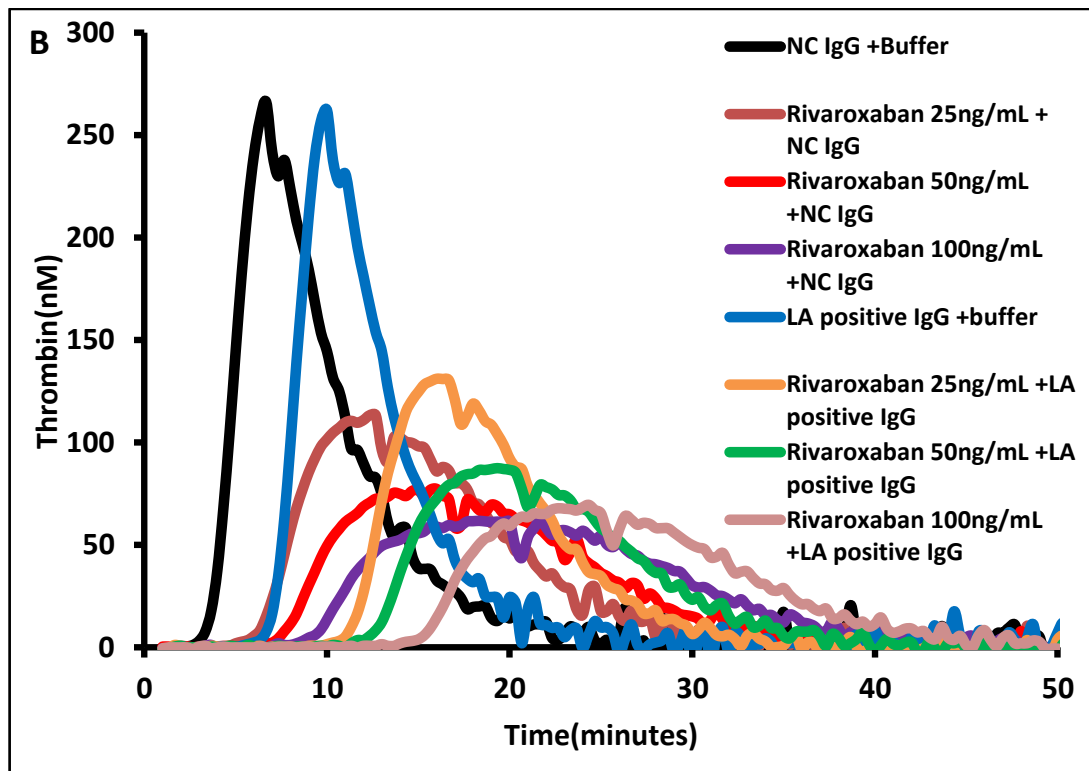
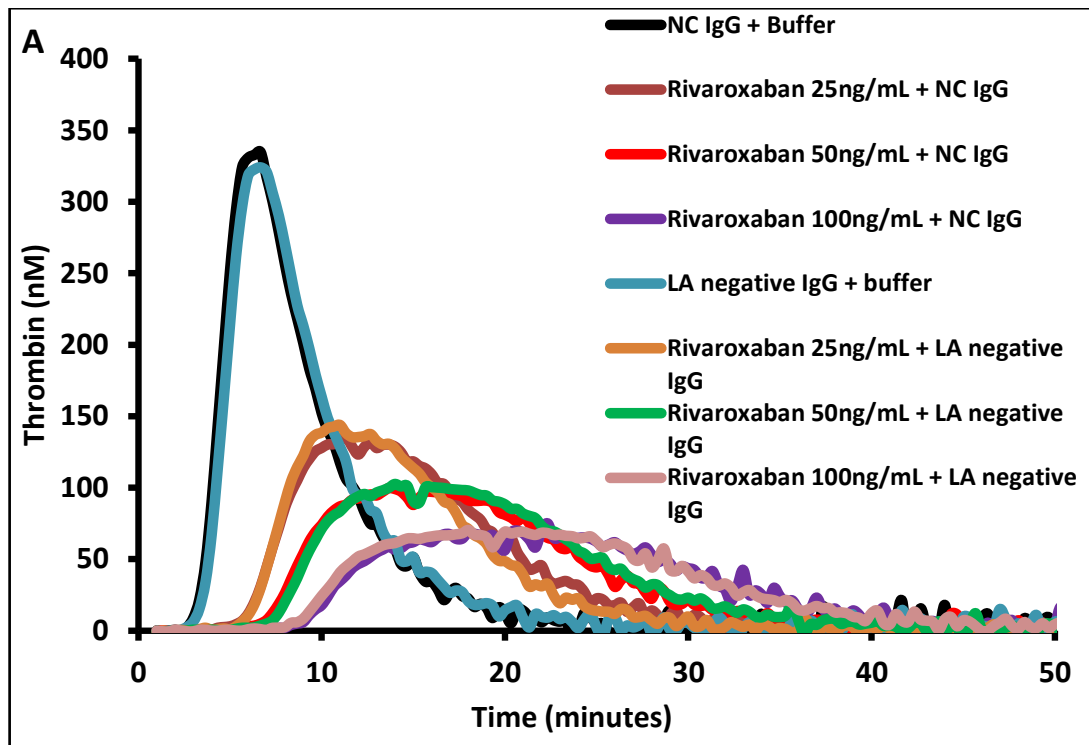


Table 5-7 Peak thrombin

Peak thrombin (nM)	LA positive	LA negative	NC	p value
0ng/mL rivaroxaban	301 [258-335]	290 [236-340]	287 [248-338]	P=0.09
25ng/mL rivaroxaban	123 [99-150]	118 [102-140]	113 [98-136]	P=0.81
50ng/mL rivaroxaban	88 76-101	78 [65-98]	83 [68=104]	P=0.72
100ng/mL rivaroxaban	65 [51-78]	63 [54-70]	65 [51-78]	P=0.62

Table 5-8 Endogenous thrombin potential (ETP)

ETP(nM.minutes)	LA positive	LA negative	NC	p value
0ng/mL rivaroxaban	1738 [1528-2188]	1690 [1470-2210]	1680 [1441-2342]	p=0.08
25ng/mL rivaroxaban	1529 [1360-1890]	1490 [1382-1790]	1472 [1376-1801]	P=0.52
50ng/mL rivaroxaban	1326 [1094-1565]	1290 [1090-1560]	1284.5 [1145-1540]	P=0.63
100ng/mL rivaroxaban	1120 [890-1320]	1145 [980-1360]	1090 [976-1356]	P=0.71

5.3 Discussion

This study has provided new insights into interactions between rivaroxaban and aPL. First, studies *in vitro* (using IgG preparations from thrombotic APS patients) and *ex vivo* (using plasma from thrombotic APS patients treated with therapeutic dose rivaroxaban), at peak and trough concentrations of rivaroxaban, established that false positive DRVVT may occur with rivaroxaban mainly at therapeutic plasma levels (165-270 ng/mL). However, the TVT/ECT ratio and Textarin time were not affected, irrespective of the rivaroxaban level, enabling accurate detection of LA. *In vitro* studies showed no false negative LA results with any of the tests used. *In vitro* studies also established, as hypothesised, that aPL do not influence rivaroxaban anticoagulant action as assessed using TG testing and anti-Xa assays. The findings in these studies should be considered for the development of future national and international guidelines on LA detection in patients treated with rivaroxaban.

My studies on LA detection in the presence of rivaroxaban represents a systematic and detailed approach to LA detection, both *in vitro* and *ex vivo*, using well characterised patients with thrombotic APS and defined LA status, and treated with rivaroxaban at both peak and trough levels of rivaroxaban. Limited previous studies provided no *in vitro* or *ex vivo* data using thrombotic APS patients, a group that can provide clinically relevant information on LA detection. An *in vitro* study (van Os *et al*, 2011), using rivaroxaban spiked into plasma from 13 patients with systemic lupus erythematosus. Six of these patients deemed to have positive LA, demonstrated that the LA ratio was not influenced by rivaroxaban when tested with TVT/ECT but activated partial thromboplastin time (aPTT) was slightly increased and a commercial DRVVT reagent was strongly influenced. In the seven remaining patients, stated to be LA negative, three became positive for LA with an aPTT, but not with TVT/ECT (the DRVVT was not assessed in the presence of rivaroxaban). Another study (Merriman *et al*, 2011) reported that 19 patients randomised to the EINSTEIN trial receiving rivaroxaban 20mg daily with no documented LA or APS, demonstrated positive LA with a DRVVT screening test, when samples were taken between 1-19.5 hours following the previous dose of rivaroxaban. However, a DRVVT confirm assay was not performed in this study, limiting the clinical

applicability of the findings. Martinuzzo et al, studied 26 patients, four on therapeutic dose, and the remainder on prophylactic dose, with no previously documented LA or history of APS for LA. Based on DRVVT screen and confirm tests with one commercial reagent, these authors reported that 75% of patient samples were apparently LA positive by DRVVT, but none had a prolonged silica clotting time (Martinuzzo *et al*, 2014).

The TVT/ECT ratio and Textarin time were not affected irrespective of rivaroxaban level, enabling appropriate detection of LA. False positive DRVVT may occur with rivaroxaban mainly at therapeutic plasma levels (165-270ng/mL). There was a greater prolongation of the DRVVT screen compared with the DRVVT confirm test with both commercial reagents, in patients treated with rivaroxaban at therapeutic levels, causing an increased normalised test/confirm ratio. This led to six LA negative patients becoming (apparently) LA positive with two DRVVT reagents, but not by TVT/ECT. *In vitro* studies of LA detection using a dPT or DRVVT with in-house reagents did not produce false positive results as there was a parallel increase in the LA screen and LA confirm DRVVT producing normal SCR. This is most likely due to the phospholipid content of the reagents (low phospholipid content in the in-house confirm reagents compared to high phospholipid content in commercial DRVVT confirm reagents). However, at trough rivaroxaban levels (30-85ng/mL), when samples were taken at least 18 (18-24) hours after the last dose of rivaroxaban, those six patients who were previously LA negative, (with LA status confirmed negative whilst on warfarin) remained LA negative with two commercial DRVVT reagents. These data support the use of the TVT/ECT to detect LA, even at peak plasma rivaroxaban levels and suggest that the DRVVT may be acceptable at trough rivaroxaban levels.

Patients with thrombotic APS are inherently different from other patients with VTE by virtue of their aPL, particularly LA and anti-prothrombin antibodies (Sciascia *et al*, 2014), which are known to interfere with a number of haemostatic mechanisms, and which could therefore modulate the actions of anticoagulants (Arachchillage *et al*, 2014a). It follows that information on the influence of aPL on rivaroxaban is

clinically relevant in thrombotic APS patients. Our *in vitro* studies demonstrated that LA led to prolonged lag times and time to peak in a TG system. These observations are in accordance with others (Liestol et al, 2007b;Regnault et al, 2003).

In vitro studies also showed that aPL did not affect rivaroxaban anticoagulant action at peak or trough levels, based on TG testing and anti-Xa levels. This was hypothesised as rivaroxaban is a small molecule with high affinity for a specific target. At the time of writing this chapter, as far as I was aware, there were no reported observations on the influence of aPL on rivaroxaban anticoagulant effects. Of note in this context, anecdotal clinical reports suggest thromboembolism recurrence following switching from warfarin to dabigatran (one patient who developed thrombotic endocarditis with symptomatic cerebral emboli) and rivaroxaban (two patients, one who experienced ischemic arterial strokes and right transverse-sigmoid sinus thrombosis, and the second, porto-mesenteric venous thrombosis) (Schaefer *et al*, 2014). There is another case report involving three APS patients who experienced recurrent thrombosis while receiving rivaroxaban or dabigatran (Win & Rodgers, 2014) Appropriately designed and powered clinical trials such as RAPS are required in patients with thrombotic APS to establish the potential use of each NOAC in these patients.

In conclusion, false positive DRVVT may occur in rivaroxaban treated patients mainly at therapeutic levels. The TVT/ECT ratio and Textarin time are not affected irrespective of the rivaroxaban levels, enabling accurate detection of LA in patients receiving rivaroxaban. In thrombotic APS patients treated with rivaroxaban, the TVT/ECT appears reliable even at therapeutic plasma levels of rivaroxaban. The DRVVT may be acceptable at trough rivaroxaban plasma levels, in samples taken at least 18 hours following the previous dose of rivaroxaban. The findings in this *in vitro* and *ex vivo* studies will guide detection of LA. This will enable diagnosis of APS in patients treated with rivaroxaban as well as monitoring of LA in APS patients, and also inform national and international guidelines. Finally, *in vitro* studies indicate that aPL do not influence anticoagulant activity of rivaroxaban as measured by TGT and rivaroxaban anti-Xa levels.

Chapter 6 Thrombin generation and *in vivo* coagulation activation markers in patients without APS, treated with therapeutic dose rivaroxaban or warfarin for venous thromboembolism

6.1 Introduction

Clinical outcome measures in patients receiving warfarin or rivaroxaban have been assessed in a number of different multicentre prospective clinical trials involving thousands of patients, including those with VTE [EINSTEIN-DVT (Bauersachs *et al*, 2010) and EINSTEIN-PE (Buller *et al*, 2012)] (196;196;196). Rivaroxaban has been shown to be non-inferior to warfarin with regard to relevant clinical efficacy and safety outcome measures. However, the *ex-vivo* effects of warfarin versus therapeutic dose rivaroxaban on TG and *in vivo* markers of coagulation activation remain undefined.

Thrombin is a pivotal component of the haemostatic mechanism. It has been shown that increased *ex-vivo* TG as measured by ETP is a key marker of thrombogenic potential and of predictive value for the development of recurrent VTE (Eichinger *et al*, 2008;Besser *et al*, 2008). In patients with acute thrombosis, *in-vivo* markers of coagulation activation such as prothrombin fragment 1.2 (F1.2), thrombin-antithrombin complex (TAT), and D-dimer are frequently elevated, and anticoagulation reduces the levels of these markers (Conway *et al*, 1987). One molecule of F1.2 is released with the generation of each thrombin molecule (Teitel *et al*, 1982). F1.2 level reflects thrombin activity more directly and may be a better clinical marker of the intensity of anticoagulation. All anticoagulant agents directly or indirectly inhibit TG (Adams, 2009;Eerenberg *et al*, 2011). F1.2, as the activation peptide originating from the factor Xa-mediated activation of prothrombin, has been reported to be a sensitive marker of anticoagulation (Tripodi *et al*, 1998;Millenson *et al*, 1992), and evidence supports the use of F1.2 and D-dimer in identifying patients at increased risk of VTE recurrence who need continued anticoagulation, or a different intensity anticoagulant regime (Palareti *et al*, 2006;Poli *et al*, 2008;Poli *et*

al, 2010). *In-vitro* experiments, where rivaroxaban has been spiked into normal plasma, as well as *ex-vivo* data from patients receiving prophylactic rivaroxaban, have shown that rivaroxaban affects various parameters of the TG assay using the CAT system. A greater inhibitory effect was observed on the initiation phase (lag time) and the peak TG compared to that on the area under the curve i.e. ETP (Samama *et al*, 2013; Samama *et al*, 2010) compared to fondaparinux *in vitro*; and patients on prophylactic dose rivaroxaban 10 mg daily undergoing elective total hip/knee replacement showed a greater decrease of TG, F1.2 and TAT (but not D-dimer) than those on dalteparin (Green *et al*, 2010) .

Aim of this chapter was to compare *ex-vivo* TG (using the CAT system) and *in vivo* markers of coagulation activation (F1.2, TAT complexes and D-dimer) in patients on long term anticoagulation with warfarin or rivaroxaban following VTE without APS

6.2 Patients and Methods

6.2.1 Patients

The study included 45 patients on warfarin with a target INR of 2.5 (range 2.0-3.0) and 40 patients on therapeutic dose rivaroxaban (20mg) daily for the prevention of recurrent VTE following a first episode of DVT or PE. Patients on rivaroxaban had previously switched from therapeutic dose warfarin to rivaroxaban because of: allergy to warfarin, lack of availability of phenindione, lifestyle and difficulty in monitoring warfarin due to needle-phobia. Rivaroxaban had been administered for at least 3 weeks (3-12 weeks) and taken with food, which increases the absorption of the drug (SPC Xarelto film-coated tablets, 2014). None of these patients were taking other anticoagulants, antiplatelet agents, or any other drugs known to interact with rivaroxaban. None of the patients had an episode of acute VTE within the last 3 months or acute or chronic infections/inflammation. Duration of anticoagulation in patients groups varies from 4 to 6 months. Patient demographic details are shown in Table 6-1. All patients in both anticoagulant treatment groups had a creatinine clearance over 50mL/min (Cockcroft & Gault, 1976) and normal liver function tests.

Table 6-1 Baseline characteristics of the two patients groups

	Warfarin (n=45)	Rivaroxaban (n=40)
Age in years: median (range)	47 (28-70)	46 (34-68)
Male/Female	28/17	26/14
Intensity of anticoagulation	Target INR 2.5 (range 2.0-3.0)	Rivaroxaban 20mg daily
Type of thrombosis		
DVT	25/45 (55.5%)	23/40 (57.5%)
PE	12/45 (26.7%)	11/40 (27.5%)
DVT+ PE	8/45 (17.8%)	6/40 (15%)
Creatinine clearance (mL/min)	75 (63-92)	76 (62-95)
Median (range)		

All patients were tested for aPL and were negative. As patients were already on warfarin, lupus anticoagulant (LA) tests were performed using the Taipan/Ecarin time ratio (which shows little impact of warfarin) and/or DRVVT using equal volume mixtures with normal plasma, to correct for warfarin-induced coagulation factor deficiency), in accordance with recommendations by the ISTH and BCSH guidelines on LA detection (Keeling *et al*, 2012;Pengo *et al*, 2009). All patients were tested for aPL at least 6 weeks after initiation of anticoagulation. TG was also assessed in 51 normal controls (median age 40 [range 23-70]).

6.2.2 Blood sampling

Venous blood samples were collected following informed consent at a single time-point using a 21 gauge butterfly needle, with minimal venous stasis, within 2-4 hours of the last dose of rivaroxaban, when they attended a routine clinic appointment. Blood was drawn into 5mL citrate Vacutainer® (BD, Oxford, UK) containing 0.5mL

of 0.105M buffered sodium citrate which gave a ratio of 1 part anticoagulant to 9 parts blood. Platelet poor plasma (PPP) was prepared within 1 hour of collection by double centrifugation (2000g for 15 minutes) at ambient temperature and 0.75mL aliquots were transferred to 2.0mL cryo-tubes (Sarstedt Ltd, Beaumont Leys, Leicester, UK) and stored at -80°C for up to 12 weeks. Immediately prior to analysis the samples were thawed at 37°C.

6.3 Coagulation assays

6.3.1 Prothrombin time (PT) and International normalised ratio

PT was performed only on samples from patients taking warfarin, using a single lot number of Innovin (Siemens Healthcare Diagnostics), on a CS-5100 analyser (Sysmex UK Ltd) and employing an instrument-specific ISI value (0.99) (section 2.3).

The median INR of the patients on warfarin was 2.29 (range 1.70-3.24). Seventy-one percent (32/45) of patients on warfarin had an INR within the target therapeutic range. Eight patients had INR values below 1.9 (17.7%) and five patients had INR >3.0 (11.1%).

6.3.2 Rivaroxaban levels

Rivaroxaban levels were measured using an amidolytic anti-Xa assay (Biophen DiXaI; Hyphen BioMed) (section 2.4) on a Sysmex CS-2000i analyser (Sysmex UK Ltd). Samples were tested in duplicate. All patient samples were processed on a single occasion.

The median plasma rivaroxaban level in the 40 patients was 284ng/mL (range 56-457ng/mL). In 26 of these patients (65%), rivaroxaban levels were within the expected range for therapeutic peak plasma concentrations (162-356ng/mL), as previously determined in a population pharmacokinetics study of once daily dose of rivaroxaban (Mueck *et al*, 2008). Nine patients (22.5%) had rivaroxaban levels less than 160ng/mL (56-157ng/mL), which were considered to be subtherapeutic, and in

five patients (12.5%) rivaroxaban levels were above the expected peak plasma concentration (412-457ng/mL) (Mueck *et al*, 2008).

6.3.3 *Ex vivo* thrombin generation

TG was measured using the CAT system (Thrombinoscope BV) in conjunction with PPP reagent (Stago) which gave reaction concentrations of 5 pmol/L tissue factor (TF) and 4 μ mol/L phospholipid (section 2.15). These experimental conditions were selected based on our previous experience (Lawrie *et al*, 2012) and that of others (Gatt *et al*, 2008). Since the samples tested were from patients on anticoagulation, the use of a low concentration tissue factor reagent (i.e. 1 pmol/L or less) was not appropriate as there would have been minimal or no thrombin generation. It has been demonstrated that addition of corn trypsin inhibitor (CTI) after plasma preparation had no significant influence on thrombin generation triggered with 0.5 pM TF or higher, as demonstrated by unaltered ETP and lag time values between analyses with and without CTI (Spronk *et al*, 2009). Addition of CTI before blood collection reduced thrombin generation triggered with 0.5 pM TF: both the ETP and peak height were significantly reduced compared to no CTI addition. In contrast, thrombin generation remained unaltered at a 1 pM TF trigger or above. As 5pM TF was used in this study, blood collection into CTI was not necessary.

All TG reactions for both samples and calibrators were tested in triplicate. The following parameters of the TG curve were recorded: lag-time; time to peak TG, peak thrombin and ETP. Normal ranges for TG were established using 51 normal controls (NC) none of whom were on any oestrogen preparations. The intra- and inter-assay imprecision, using normal plasma were 2.5% and 4% respectively.

6.4 Results

Figure 6-1 provides an example of the effect of warfarin and rivaroxaban on TG compared to a NC. TG was significantly lower in patients receiving rivaroxaban or warfarin (Figure 6-2) compared to NC ($p < 0.0001$ for ETP and peak thrombin). Both groups had significantly prolonged lag time ($p < 0.0001$) as well as time to peak

($p < 0.003$) compared to NC. Patients treated with rivaroxaban had significantly prolonged lag time and time to peak TG along with significantly lower peak thrombin compared with those treated with warfarin ($p < 0.0001$ for all three parameters). However, ETP was significantly lower in warfarin-treated patients compared with those receiving rivaroxaban ($p < 0.0001$). The significance levels remained unchanged even after excluding the results of eight patients in the warfarin-treated group who had subtherapeutic INR values (< 1.9).

Rivaroxaban levels were correlated with lag time and time to peak thrombin and inversely correlated with ETP and peak thrombin ($r_s = 0.61$, $p < 0.0001$, $r_s = 0.72$, $p < 0.0001$, $r_s = -0.51$, $p = 0.001$ and $r_s = -0.39$, $p = 0.013$ respectively) [Figure 6-3]. However, the correlation between INR and TG was poor in warfarin treated patients, even after removing results of samples with subtherapeutic INR values.

Figure 6-1 An example of the effect of warfarin and rivaroxaban on TG compared to a normal control

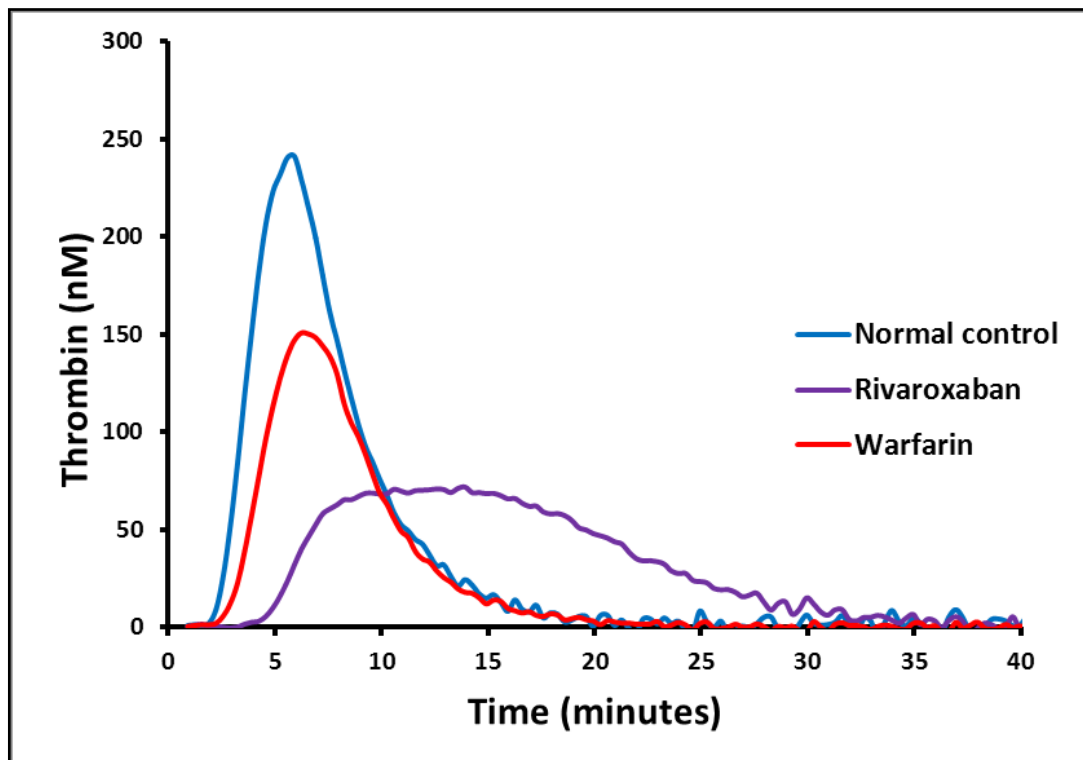
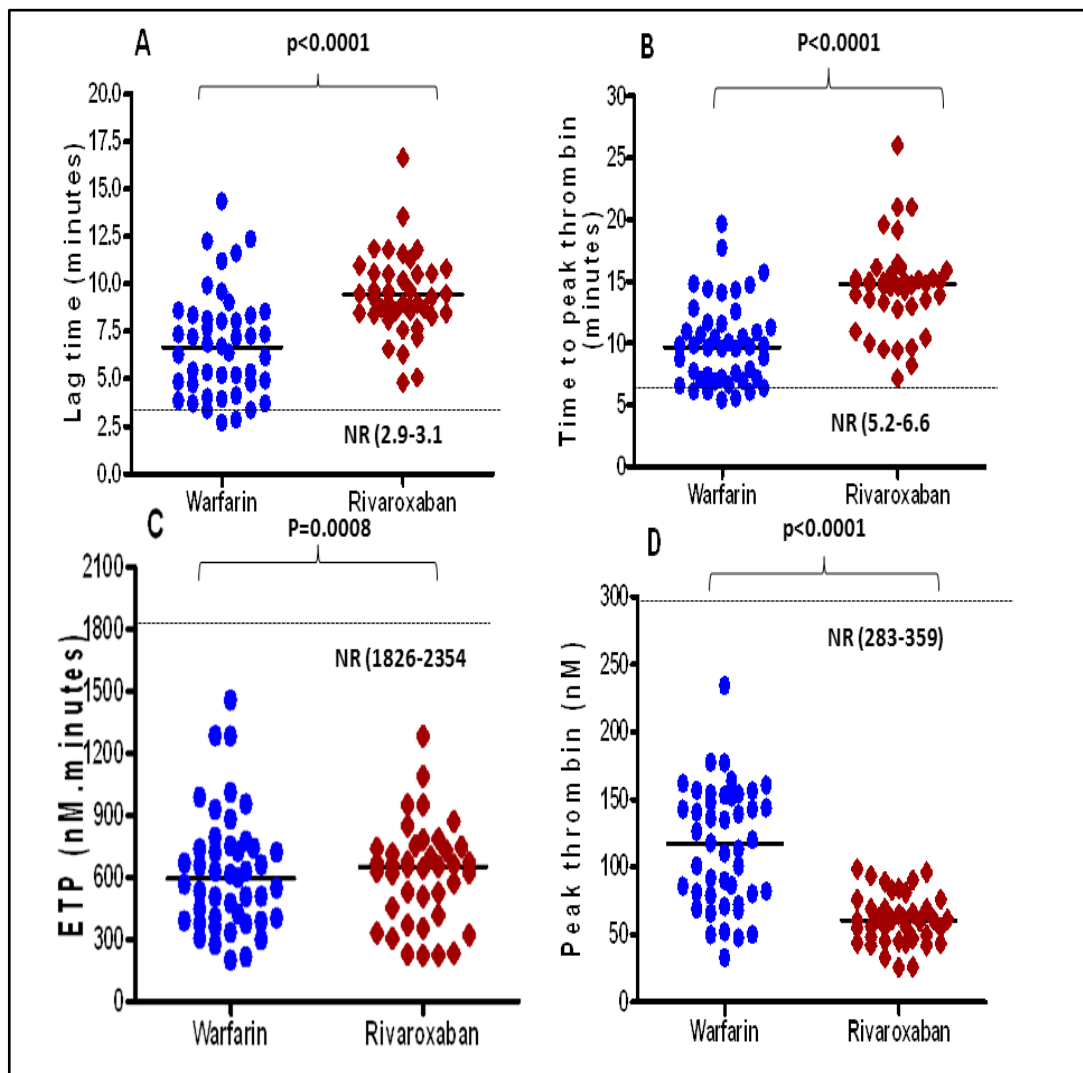
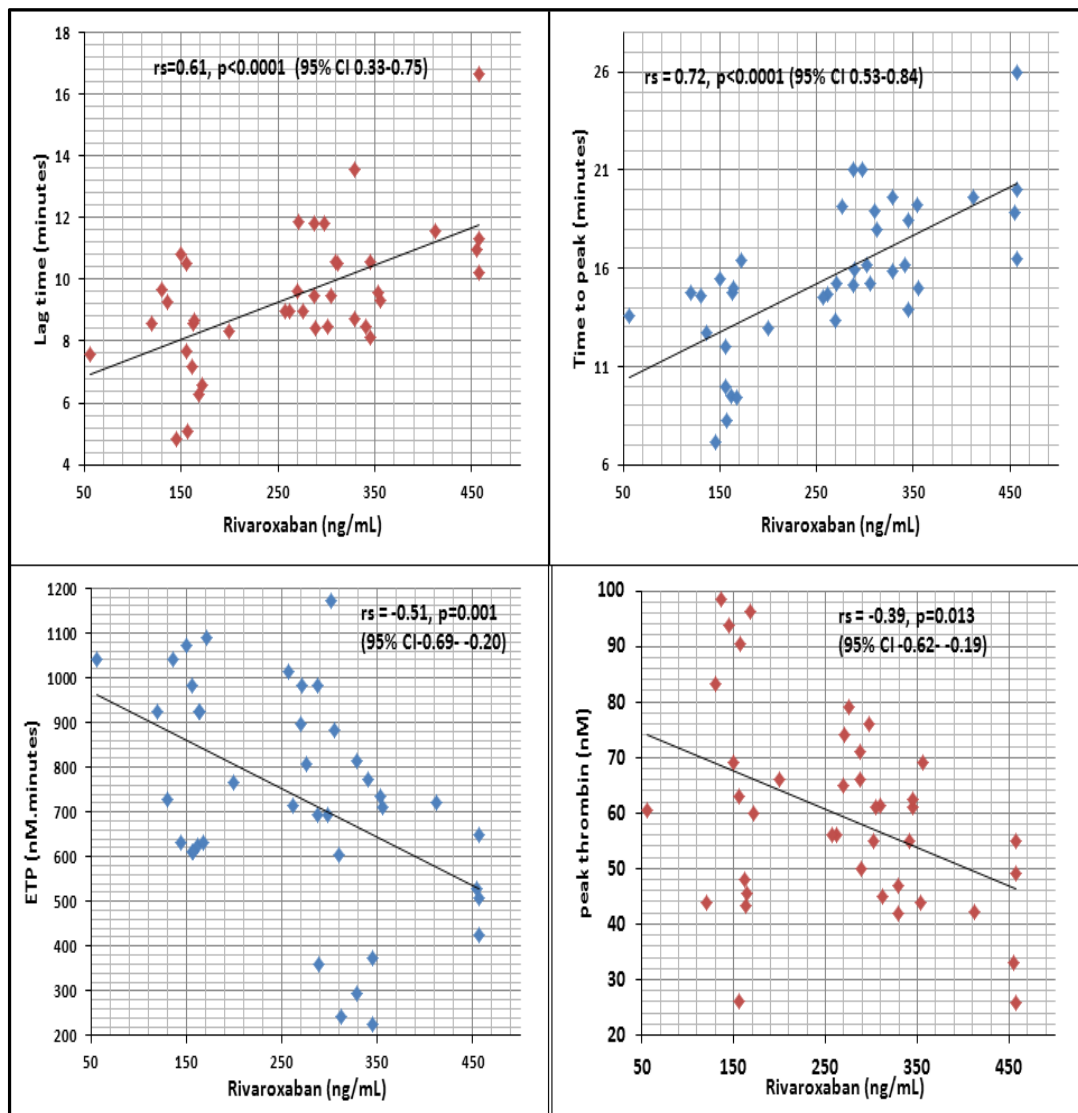


Figure 6-2 Thrombin generation parameters



Thrombin generation parameters are shown in the two study groups (warfarin n=45; rivaroxaban n=40). Normal ranges (NR) and the median for the each parameter based on results in the (unanticoagulated) normal controls is shown in brackets and the dotted lines respectively.

Figure 6-3 Spearman rank correlation between rivaroxaban level and thrombin generation parameters



6.5 In-vivo coagulation activation markers:

D-dimer was measured by an immunoturbidometric technique (Innovance D Dimer; Siemens Healthcare Diagnostics) on a CS-5100 analyser (Sysmex UK Ltd) (section 2.12). F1.2 and TAT complexes were measured by enzyme-linked immunosorbent assay (Enzygnost® F1.2 and Enzygnost® TAT respectively; Siemens Healthcare Diagnostics) (sections 2.13 and 2.14).

Table 6-2 Comparison of *in vivo* coagulation activation markers in warfarin and rivaroxaban treated patients.

	Warfarin	Rivaroxaban	Normal cut-off
D-dimer (µg/L FEU)	190 (<190-510)	190 (<190-300)	<550
F1.2 (pmol/L)	54.3 (19.9-233.3)	58.3 (18.2-121.2)	< 229
TAT (µg/L)	2.7 (1.9-4.9)	2.5* (1.6- 3.5)	<4.2

F 1.2 = prothrombin fragments 1. 2; TAT = thrombin-antithrombin complex (TAT); All values are given in median and ranges. The majority of the patients in both groups had undetectable D-dimer levels (<190µg/L FEU); * p< 0.001

In vivo coagulation activation markers were within their normal reference ranges in all the patients treated with rivaroxaban and 37/45 warfarin-treated patients with INR 2.0-3.2 (Table 6-2). Six of eight warfarin-treated patients with subtherapeutic INR values (i.e. <2.0; range 1.7-1.85) (13.3% (6/45) of the total) exhibited slightly raised TAT (n=6) and few of those also had raised F1.2 levels (Table 6-3). *In vivo* coagulation activation markers were maintained within their normal reference ranges in all the rivaroxaban-treated patients, including nine patients with subtherapeutic rivaroxaban levels (as defined above).

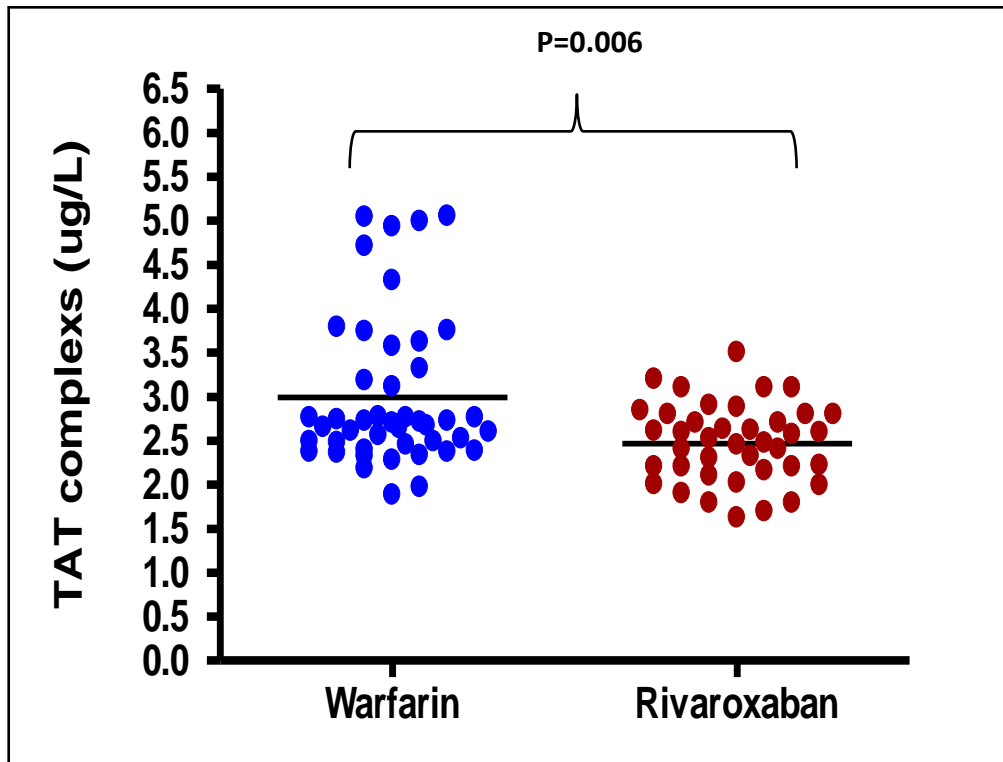
Patients treated with rivaroxaban had significantly lower TAT levels compared with those on warfarin ($p=0.006$) (Figure 6-4) and the difference remained significant even after removing the six patients with subtherapeutic INR levels ($p=0.03$).

Table 6-3 *In vivo* coagulation activation markers, ETP and peak thrombin of the warfarin-treated patients who had subtherapeutic INR values

INR	F1.2 (pmol/L)	TAT ($\mu\text{g/L}$)	ETP (nM.minutes)	Peak thrombin (nM)
1.70	260.3	5.1	1453	233.9
1.78	263.7	5.0	951	141.1
1.78	127.7	5.1	924	143.3
1.82	433.3	4.7	1282	134.2
1.83	147.8	5.0	984.4	135.7
1.85	413.8	4.3	1281	156.3
1.88	223.0	3.2	898	163.7
1.90	85.9	2.5	656	152.8
Normal cut-off/ range	229	4.2	1826-2354	283-359

INR = international normalised ratio; F 1.2 = prothrombin fragments 1. 2; TAT = thrombin-antithrombin complex (TAT); ETP=endogenous thrombin potential; INR normal range: 0.91-1.11; ETP normal range 1826-2354nM.min); peak thrombin normal range (283-359nM); values in bold indicate those above the normal reference range

Figure 6-4 Comparison of TAT level between warfarin and rivaroxaban



6.6 Bleeding and thrombotic episodes

None of the patients experienced clinically significant bleeding prior to, during, or immediately after the study. One patient, who had the highest ETP (1287nM.min) in the rivaroxaban-treated group developed arterial thrombosis in the left leg immediately after the study. However, this patient's ETP was well below the normal reference range (1826-2354nM.min). Activation markers of *in-vivo* coagulation in this patient were well within the normal range.

6.7 Discussion

The results of this study provide insight into the effects of therapeutic dose warfarin and rivaroxaban on TG and markers of coagulation activation in patients with VTE. Both rivaroxaban and warfarin significantly reduced TG, with regard to all parameters measured, compared to (unanticoagulated) NC. The effect of rivaroxaban on TG was more marked compared with warfarin for all parameters except ETP, where warfarin-treated patients had significantly lower values. *In-vivo* coagulation

activation markers were within the normal reference ranges in all rivaroxaban-treated patients and 37/45 warfarin-treated patients with INR 2.0-3.2. Six of eight (13.1% of the total) warfarin-treated patients with subtherapeutic INR values exhibited slightly raised F1.2 and/or TAT. *In-vivo* coagulation activation markers were maintained within the normal reference ranges in all the rivaroxaban-treated patients, including 9/40 (22.5%) with subtherapeutic rivaroxaban levels. None of the patients experienced clinically significant bleeding during the study period; however one rivaroxaban-treated patient, who had the highest ETP in this group, but below the range of NC, developed arterial thrombosis. This study was not designed to have statistical power for clinical outcome i.e. recurrence of thrombosis or bleeding.

The significantly reduced peak thrombin and relatively less inhibited ETP with rivaroxaban are probably explained by protraction of the thrombin generation process with rivaroxaban compared to warfarin. The ETP is considered to depend on the propagation and termination phases of the TG process and has been reported to show decreased sensitivity to direct Xa inhibitors during *in-vitro* studies (Samama *et al*, 2012; Samama *et al*, 2010). This is assumed to be due to inhibition of the positive feedback action of FXa on factor VII, which results in prolongation of the lag time and a slow start of prothrombinase complex formation and hence, a longer time to peak TG (Bloemen *et al*, 2013; Jesty *et al*, 1996). The TG test, as a global measure of anticoagulation, can assess the anticoagulant effects of both rivaroxaban and warfarin, and effects on the ETP should be assessed in combination with the other TG parameters. ETP has been shown to be a predictive marker for recurrent thrombosis (Eichinger *et al*, 2008; Besser *et al*, 2008) and in my study, the only patient who developed arterial thrombosis had the highest ETP in the group treated with rivaroxaban. However, the study was not designed to assess ETP as a marker for prediction of thrombotic risk in patients treated with rivaroxaban.

My findings of significantly inhibited peak thrombin with rivaroxaban concur with those by Samama's group, whose *in-vitro* studies compared both rivaroxaban and edoxaban with fondaparinux, and demonstrated that rivaroxaban and edoxaban were more effective in reducing peak thrombin (Samama *et al*, 2012; Samama *et al*, 2010). In that study, using molar concentrations to compare the effect of anticoagulants,

rivaroxaban and edoxaban induced more inhibition than fondaparinux of all the TG parameters, with the exception of ETP. Recently, Hermann *et al* (Herrmann *et al*, 2014) demonstrated that prophylactic dose rivaroxaban (10mg daily) significantly reduced the ETP and peak thrombin and prolonged the lag time in samples tested at two hours compared to 24 hours post dose. A strong relationship has also been demonstrated between the concentration of a given anticoagulant and the amount of thrombin generated (Bostrom *et al*, 2004). In my study all six patients on warfarin with subtherapeutic INR values had slightly raised F1.2 and/or TAT levels, and an ETP above 900nM.min (Figure 6-3C and Table 6-3). The ETP results in warfarin-treated patients agreed with other reported observations that patients on warfarin with an INR between 2.0-3.0, where an ETP range of approximately 500-700nM.min was found (Hemker & Beguin, 2000). This range is equivalent to 30%-50% of the pre-warfarin result (Brodin *et al*, 2009) or that in normal controls (Gerotziafas *et al*, 2009). In patients treated with rivaroxaban, the median ETP was 903nM.min (range 224-1287) in patients treated with rivaroxaban, and was significantly higher than in patients on warfarin.

I observed normal *in vivo* coagulation activation markers in both the warfarin and rivaroxaban treated patients, with the exception of a few patients in the warfarin group with subtherapeutic INR values who showed slightly raised TAT and F1.2 levels. However, the D-dimer level was below the normal cut-off in both groups, irrespective of anticoagulation intensity suggesting that gross fibrin deposition was not occurring. In a study to investigate the effects of low-dose rivaroxaban (10mg daily) on coagulation factor activities based on the administration time, Mani *et al* (Mani *et al*, 2013) showed that there was no significant difference in the D-dimer level at different time intervals after rivaroxaban administration. Corresponding effects of rivaroxaban on F1.2 and TAT levels are unknown.

As TAT complexes and F1.2 have much shorter half-lives (15 minutes (Pelzer *et al*, 1988) and 90 minutes respectively (Greenberg *et al*, 1994) compared to D-dimer (8 hours) (Hager & Platt, 1995), there may be differences in the levels of TAT complexes and F1.2 based on the rivaroxaban administration time. The purpose of once daily anticoagulation is to have a sustained reduction in thrombotic risk for 24

hours until the next dose of anticoagulation is administered. Although the plasma rivaroxaban level reaches a peak within 2-3 hours of ingestion, the anticoagulant effect is expected to last for 24 hours as it is given once daily. Even at trough levels of rivaroxaban, TG should be inhibited below that of the normal controls. This effect is reflected in the results of my study, which demonstrated that even at subtherapeutic levels of rivaroxaban, there was good inhibition of TG and markers of coagulation activation remained within normal limits.

This study was designed to investigate and provide information on *ex vivo* TG and markers of *in vivo* coagulation activation in a routine hospital anticoagulation clinic population. Patients were asked to attend clinics to provide blood samples 2-4 hours following their last morning dose of rivaroxaban; 65% had plasma rivaroxaban levels within the expected range for their therapeutic dose. Of the patients on warfarin, 70% had an INR within their target therapeutic range (2.0-3.0). However, even if the analysis was performed excluding patients who had INR values or plasma rivaroxaban levels lower than expected range following 2-4 hours of the last dose, the results showed no difference.

Based on TG tests from patients with congenital factor deficiencies, TG values below approximately 20% of normal (which corresponds to an ETP <350nM.min) is associated with a definite risk of bleeding (Al *et al*, 2002). Six of 45 (13.3%) patients in the warfarin group and 3 out 40 (7.5%) rivaroxaban patients had an ETP below 350nM.min. None of these patients experienced clinically relevant bleeding manifestations, suggesting that a lower ETP alone was not indicative of bleeding phenotype in the patients studied. It is possible that anticoagulated patients with a thrombotic phenotype may have a lower risk of bleeding in the presence of a similar amount of generated thrombin compared to those with inherited bleeding disorders. At the time of writing this chapter, no studies investigating relationship of D-dimer, F1.2 level and TAT complex in patients with VTE who develop clinically significant bleeding or thrombosis whilst on treatment dose rivaroxban had been published. In a study on patients given prophylactic rivaroxaban (Borris *et al*, 2008), there was a significantly lower level of urinary F1.2 in patients who developed bleeding compared to patients who developed VTE, following total hip replacement (THR).

However, urinary F1.2 levels were increased for several days after THR due to activation of coagulation factors and increased TG as a result of surgical trauma. As a result, the level of urinary F1.2 associated with bleeding or VTE following THR cannot be applied to VTE patients on long term anticoagulation with rivaroxaban.

6.7.1 Limitations of this study

Limitations of this study were that the number of subjects studied was relatively small and the study design was cross-sectional rather than a prospective cross over trial. A further limitation is that samples were taken only at a single time point from each patient, and were not designed to correlate *ex-vivo* TG parameters and markers of *in-vivo* thrombin activation with clinical outcome. Despite these limitations, the findings should inform haematologists and physicians regarding the overall coagulation response with rivaroxaban compared to warfarin and provide evidence that even at trough levels of rivaroxaban, there is no *in vivo* evidence of increased TG.

6.8 Conclusion

In conclusion, both rivaroxaban and warfarin achieve effective anticoagulation, as assessed by inhibition of TG and *in-vivo* coagulation activation markers. The effect of rivaroxaban on TG was more marked compared with warfarin for all parameters except ETP, where warfarin-treated patients had significantly lower values. The TG test, as a global measure of anticoagulation, can assess the anticoagulant effects of both rivaroxaban and warfarin, and effects on the ETP should be assessed in combination with the other TG parameters. *In-vivo* coagulation activation markers were within the normal reference ranges in all rivaroxaban-treated patients (including those with subtherapeutic levels) and in 37/45 warfarin-treated patients who had an INR ≥ 2.0 , with a minority of warfarin-treated patients with subtherapeutic INR values exhibiting slightly raised F1.2 and/or TAT.

Chapter 7 *In vivo* markers of coagulation activation in patients with antiphospholipid syndrome treated with rivaroxaban or warfarin following venous thromboembolism

Rivaroxaban is licensed for the treatment and prevention of VTE. However, use of this anticoagulant in patients with APS is not yet recommended apart from in the clinical trial setting. Rivaroxaban is currently being evaluated in a phase II/III randomised clinical trial (RAPS) for prevention of recurrent VTE in patients with APS following a first episode of DVT or PE, or recurrence of VTE after stopping therapeutic anticoagulation (<http://isrctn.org/ISRCTN68222801>).

In the previous chapter, following the study of TG and activation markers of *in vivo* coagulation in patients without antiphospholipid syndrome, treated with rivaroxaban or warfarin, I found that effective anticoagulation was achieved with both anticoagulants, as assessed by inhibition of TG and *in vivo* coagulation activation markers. Studies of the effects of rivaroxaban on TG in patients with APS are limited to *in vitro* studies and activation markers of *in vivo* coagulation have not been reported.

The aim of this chapter was to compare *in vivo* markers of coagulation activation (F1.2, TAT complexes and D-dimer) in APS patients on long term anticoagulation with warfarin or rivaroxaban (at least for 6 weeks following the switch to rivaroxaban from warfarin) for VTE.

7.1 Patients and blood sample collection

A total of 48 patients with VTE, on long term anticoagulation with warfarin, target INR 2.5 (2.0-3.0), who, fulfilled the revised international consensus criteria for definite APS (Miyakis *et al*, 2006) were studied. These patients were from the RAPS trial, and consented for the translational research at the UCLH. Out of a total of 48

patients, 25 patients remained on warfarin, whilst 23 patients switched to rivaroxaban.

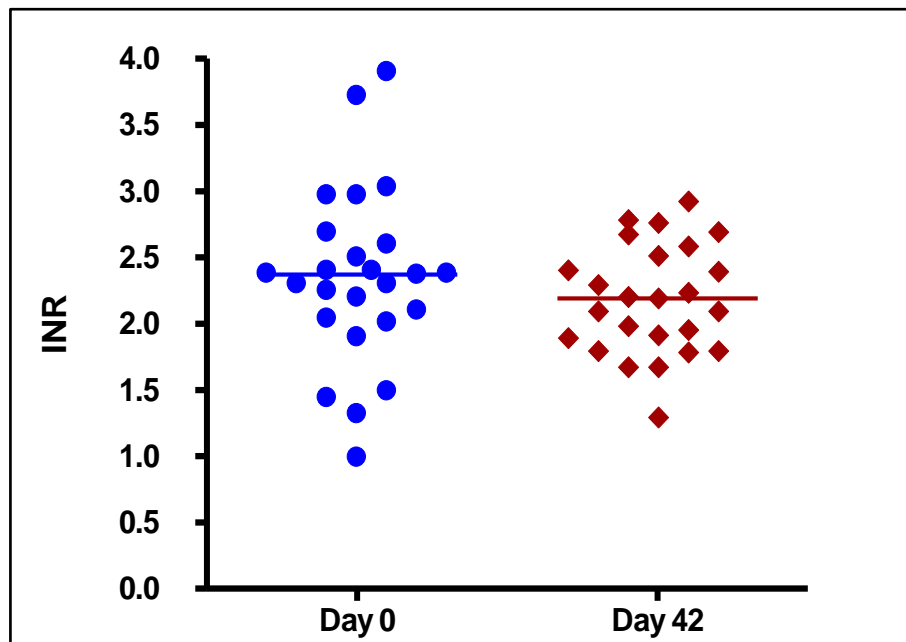
All patients in both anticoagulant treatment groups had a creatinine clearance over 50mL/min (Cockcroft & Gault, 1976) creatinine clearance < 50mL/min, rivaroxaban dose was altered to 15mg daily instead of 20mg daily. All patients had alanine transaminase level less than the twice upper limit of normal. Citrated blood samples were collected from patients receiving rivaroxaban 2-4 hours following the last morning dose (day 42 days following the randomisation). All samples were processed, stored and thawed as described in section 2.2. For each patient, day 0 and 42 samples were tested at the same time in order to avoid inter-assay variation

7.2 Results

7.2.1 Warfarin group

INR was assessed using Innovin as described in section 2.3. There was no significant difference in the INR values in the 25 patients at day 0 and day 42 following randomisation to remain on warfarin (Figure 7-1).

Figure 7-1 INR values in the 25 patients on day 0 and day 42



F1.2, TAT and D-dimer

F1.2, TAT and D-dimer were performed in all patients (day 0 and day 42) as described in section 2.13, 2.14 and 2.12 respectively.

On day 0, 4% (1/25), 4% (1/25) and 16% (4/25) had raised F1.2, TAT and D dimer levels above the normal cut- off levels respectively (Table 7-1). On day 42, TAT and D-dimer were above the normal cut-off values in one (4%) and two (8%) patients respectively (Table 7-2) while none of the patients had raised F1.2 level. However, none of the patients had more than one activation marker above the normal cut-off levels and patients who had elevated activation markers at day 0 were not the same patients who had elevated markers at day 42. There were no significant differences in F1.2, TAT and D dimer levels on samples taken on day 0 and day 42 in the 25 patients who remained on warfarin anticoagulation

Table 7-1 Patients with raised activation markers treated with warfarin on day 0

Patient	INR	F1.2 (pmol/L)	TAT (µg/L)	D dimer (µg/L FEU)
A	1.3	240.7	3.9	560
B	1.5	44.2	3.1	560
C	1.1	67.2	3.1	650
D	1.5	89.3	3.7	900
E	1.9	59.2	6.2	450
Normal range	0.91-1.1	< 229	<4.2	<550

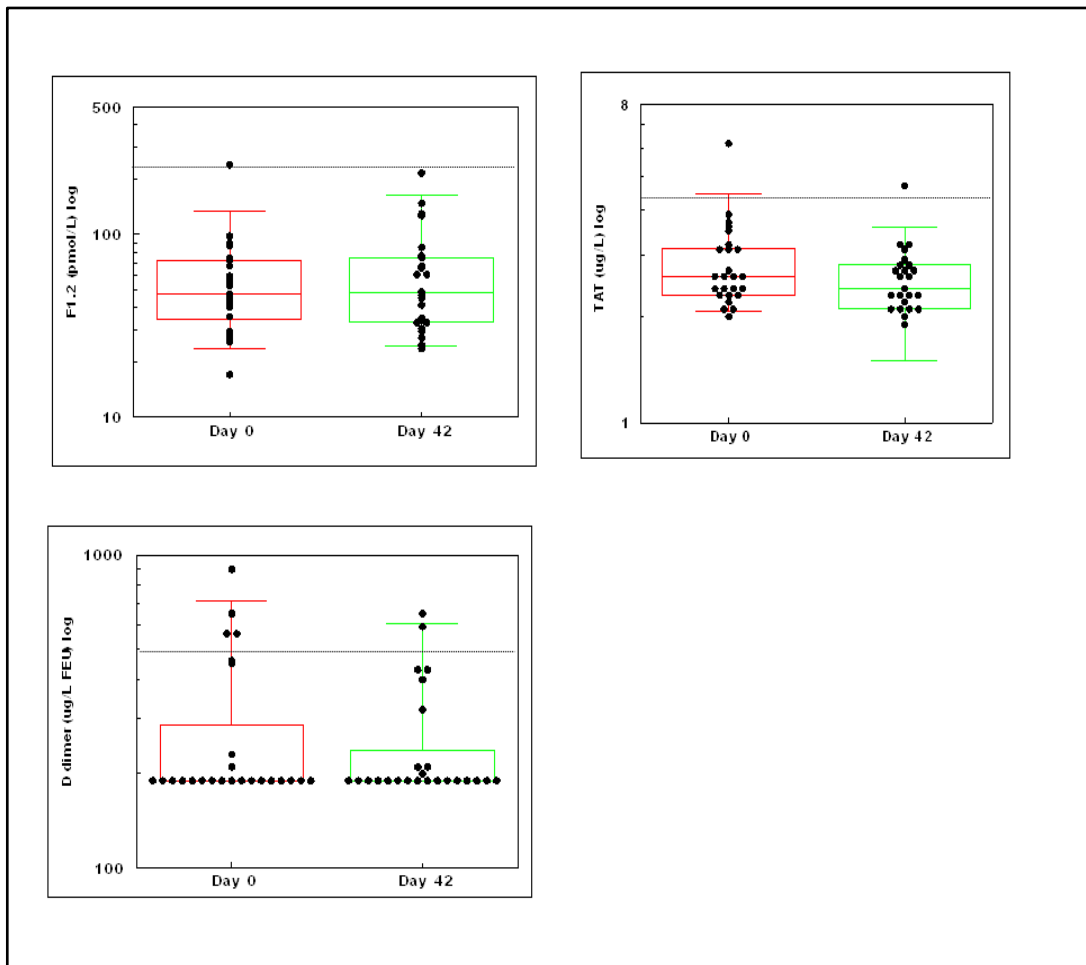
INR = international normalised ratio; F 1.2 = prothrombin fragments 1. 2; TAT = thrombin-antithrombin complex (TAT); values in bold indicate those above the normal reference range

Table 7-2 Patients with raised activation markers treated with warfarin on day 42

Patient	INR	F1.2 (pmol/L)	TAT (µg/L)	D dimer (µg/L FEU)
F	1.68	67.2	3.1	650
G	1.79	60.4	2.7	590
H	1.9	65.8	4.7	430
Normal range	0.91-1.1	< 229	<4.2	<550

INR = international normalised ratio; F 1.2 = prothrombin fragments 1. 2; TAT = thrombin-antithrombin complex (TAT); values in bold indicate those above the normal reference range.

Figure 7-2 F1.2 TAT and D-dimer level on day 0 and day 42 in warfarin group



The dotted lines indicate the normal cut off levels

INR showed a linear correlation with F1.2 at both time points; day 0: $r_s = -0.41$, 95% CI -0.69 to -0.02 , $p = 0.04$ (Figure 7-3) and day 42: $r_s = -0.61$, 95% CI -0.81 to -0.27 , $p = 0.0014$ (Figure 7-4). The correlation between INR and F1.2 remained even after removing the two outliers at day 0 and day 42.

Figure 7-3 Correlation between INR and F1.2 level day 0

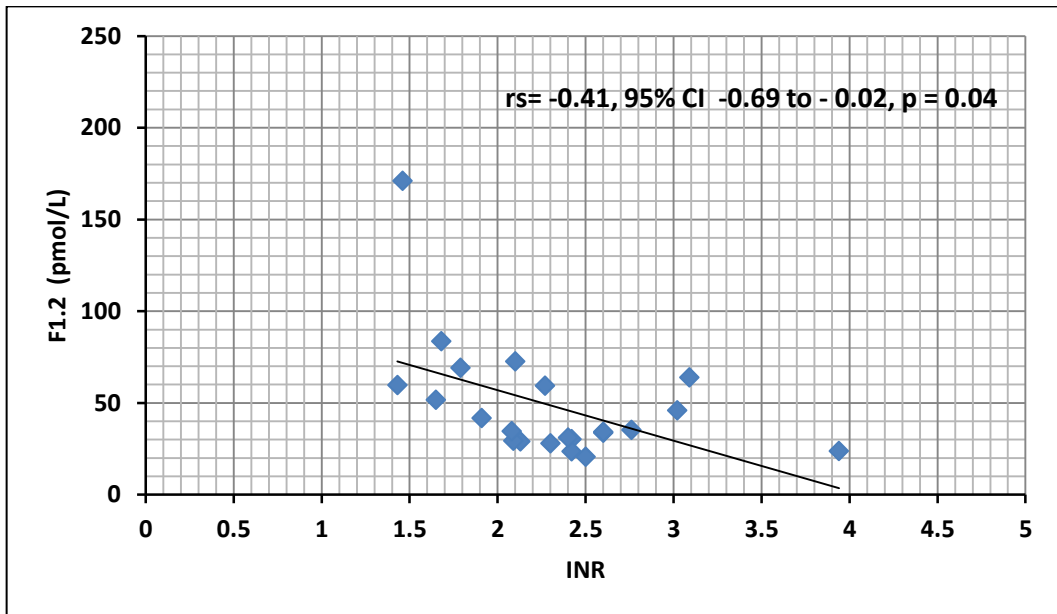
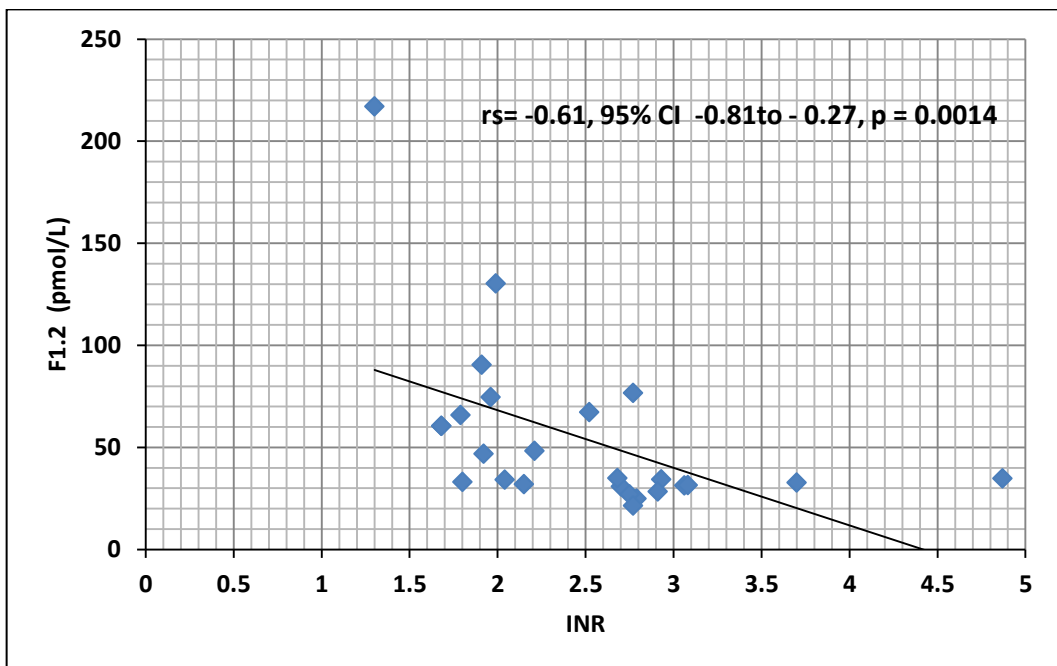


Figure 7-4 Correlation between INR and F1.2 level day 42



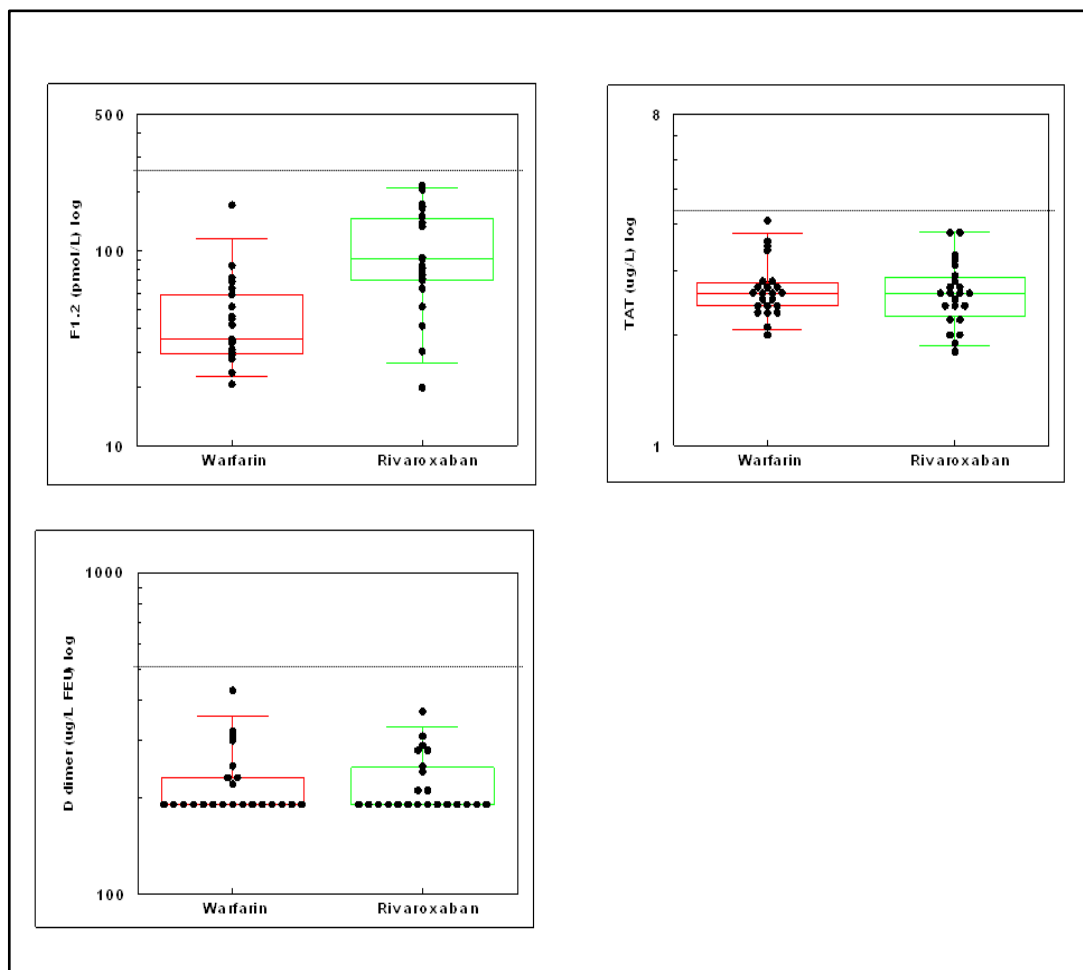
7.2.2 Rivaroxaban group

Of the 23 patients randomised to rivaroxaban anticoagulation, the median INR (95% CI) was 2.8 (2.2-3.1) while still on warfarin at day 0 and the median rivaroxaban level (CI) was 183ng/mL (121-247) at day 42.

F1.2, TAT and D-dimer

All patients had normal F1.2, TAT and D-dimer on day 0 on warfarin and day 42 on rivaroxaban. However, there was a significantly lower level of F1.2 ($p < 0.0001$) at day 0 while on warfarin compared to day 42 on rivaroxaban (Figure 7-5). There were no differences in TAT complex or D-dimer between day 0 and day 42.

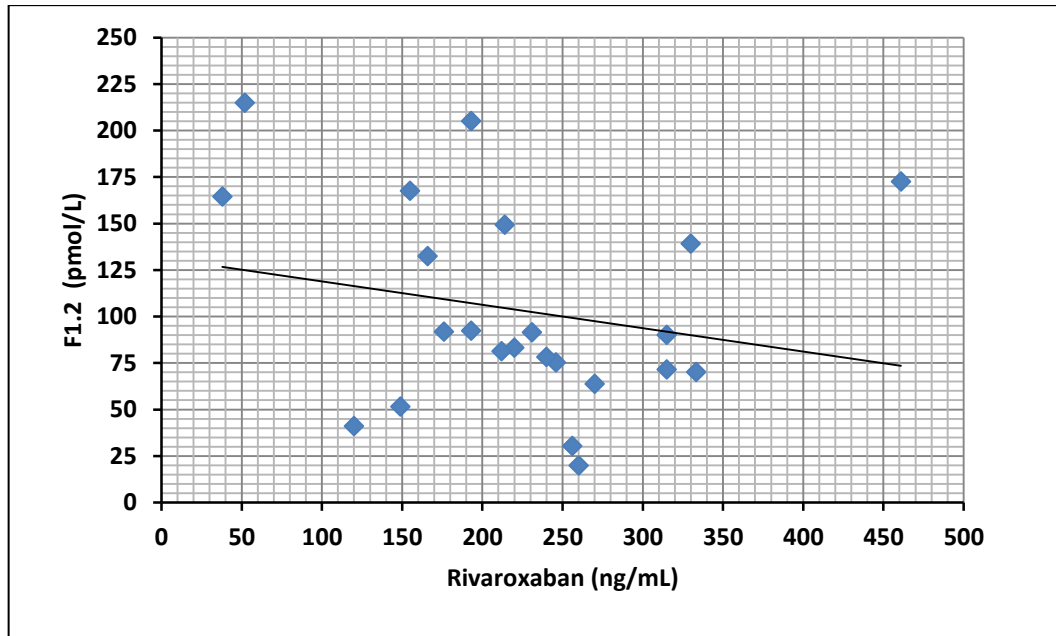
Figure 7-5 F1.2, TAT and D-dimer level on day 0 and day 42 in patients randomised to rivaroxaban



The dotted lines indicate the normal cut off levels

There was no correlation between rivaroxaban level and F1.2 (rs -0.28, 95%CI -0.82 to 0.15, p= 0.19) (Figure 7-6) (which was seen with INR in warfarin treated group) or with TAT and D-dimer.

Figure 7-6 There was no correlation between rivaroxaban level and F1.2

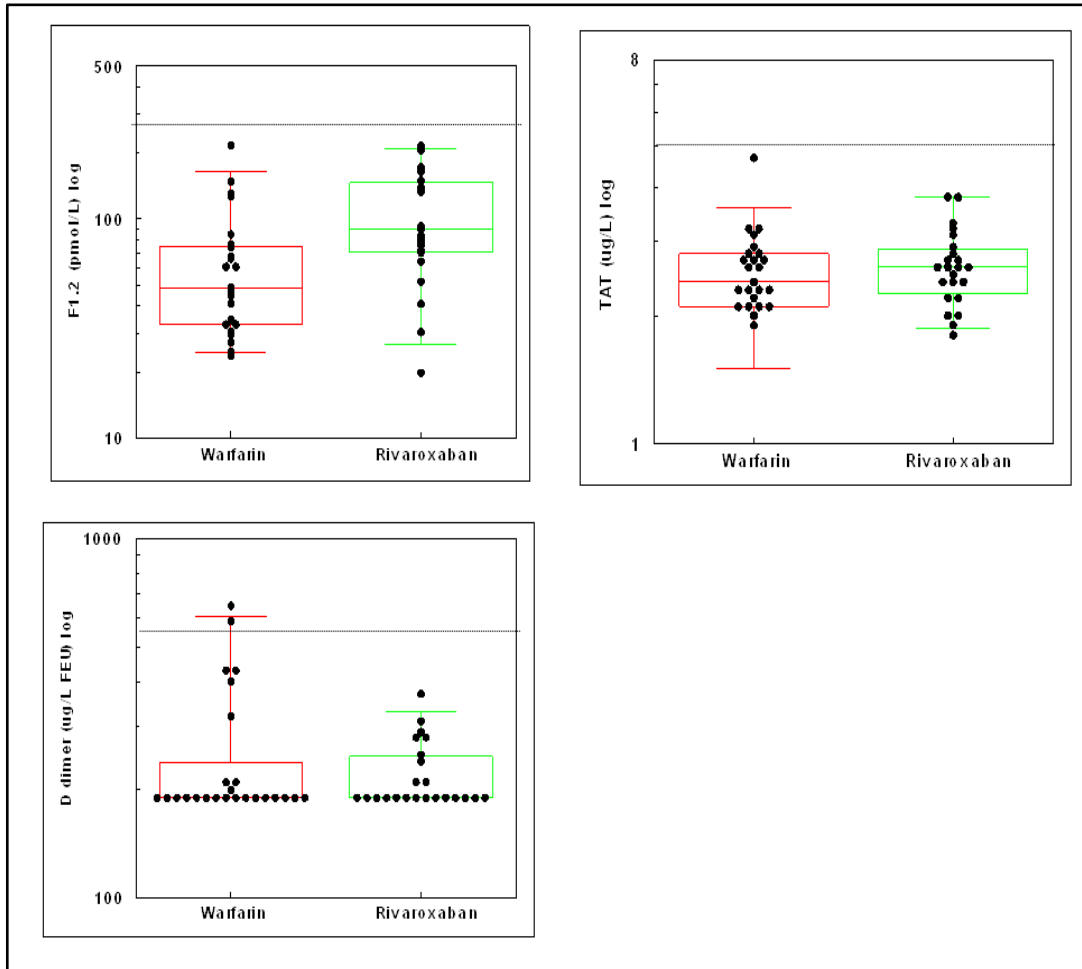


7.2.3 Comparison of warfarin and Rivaroxaban arms on day 42

There were no differences in F1.2, TAT or D-dimer levels at base line (day 0) between the two patients groups when they were on warfarin.

On day 42, the warfarin treated group had significantly lower levels of F1.2; (median [95% CI]) 48.1 pmol/L [34.2-67.2%] compared to rivaroxaban 90.1pmol/L [71.6-139.1] (Figure 7-7). However, all patients had F1.2 levels within the normal range irrespective of the anticoagulant. There were no differences in the TAT and D-dimer levels between the two groups. One patient in the warfarin treated group had slightly raised TAT level, and three patients in the warfarin treated group and two patients in the rivaroxaban treated group had D-dimer level above the normal cut-off.

Figure 7-7 Comparison of TAT, F1.2 and D-dimer levels between patients treated with warfarin vs rivaroxaban on day 42



The dotted lines indicate the normal cut off levels

7.3 Discussion

Patients with APS have an increased risk of recurrent thrombosis without anticoagulation and therefore, it is critical to deliver effective long term anticoagulation to prevent recurrence of thrombosis. I assessed and compared the effects of warfarin with a target INR of 2.5 (2.0-3.0) (n=25) and rivaroxaban 20mg daily (n=23) on *in vivo* markers of TG and D-dimer in APS patients with VTE. Although significantly lower levels of F1.2 were observed in the warfarin treated group compared to the rivaroxaban treated group at day 42, F1.2 levels in both groups were normal and hence not clinically significant. Furthermore, there were no differences in the TAT and D-dimer levels between the two groups and also only a

minority of patients (less than 5 in total in both groups) had activation markers above the normal reference at any one point of assessment, suggesting that both warfarin and rivaroxaban achieve effective long term anticoagulation in APS patients with previous VTE.

Patients with thrombotic APS inherently differ from others with thrombosis due to the presence of aPL which have various effects on the coagulation system mainly through phospholipid binding proteins. Furthermore, the INR may not be accurate in certain patients with APS because LA and other aPL may affect prothrombin time and frequently cause underestimation of the anticoagulant effect of warfarin (Rapaport & Le, 1995). In a study investigating, coagulation activation markers in patients with thrombotic APS treated with warfarin anticoagulation, it was noticed that 17%, 14% and 17% of the patients had increased levels of TAT, F1.2 and D-dimer respectively (Arkel *et al*, 2002). However, INR values were not stated for these patients at the time the samples were taken for the assessment of activation markers.

There is a limited amount of data concerning treatment failure when rivaroxaban or dabigatran have been used in thrombotic APS. One report described three consecutive APS patients who had had no recurrence of VTE whilst on warfarin, but developed thrombosis when they were switched to rivaroxaban or dabigatran (Schaefer *et al*, 2014). The three cases were as follows: A woman with primary APS who developed thrombotic endocarditis with symptomatic cerebral emboli after transition to dabigatran. A second woman with primary APS experienced ischemic arterial strokes and right transverse-sigmoid sinus thrombosis after conversion to rivaroxaban. A man with secondary APS suffered porto-mesenteric venous thrombosis after switching to rivaroxaban. A second report also described three patients with APS and a history of multiple thrombotic complications, who experienced recurrent thrombosis while receiving rivaroxaban or dabigatran (Win & Rodgers, 2014). However, the rivaroxaban and dabigatran levels were not reported in these case reports and compliance is uncertain. Conversely, another case report described the successful treatment of a patient with APS who had relapsing

superficial thrombophlebitis of the legs with rivaroxaban (Bachmeyer & Elalamy, 2014). Therefore, until results of prospective randomised trials are available, these drugs should be used with caution for the prevention and treatment of VTE in APS patients.

At the time of writing this chapter, there were no other published studies comparing the *in vivo* activation markers in patients treated with warfarin compared to rivaroxaban. A similar comparison was made in patients without APS, with VTE, in my previous chapter where I found that both anticoagulants achieved effective anticoagulation in terms of *in vivo* markers. However, VTE patients without APS showed no differences in F1.2 levels between those treated with rivaroxaban or warfarin. In contrast, in APS patients, those treated with warfarin had significantly lower levels of F1.2 compared to rivaroxaban treated patients. In addition, non-APS patients treated with rivaroxaban had significantly lower TAT complex compared to warfarin which was not seen in VTE patients with APS. The differences seen in the two groups, was not clinically relevant as nearly all the patients had normal activation markers.

In conclusion, both rivaroxaban and warfarin achieve effective anticoagulation, as assessed by inhibition of *in-vivo* coagulation activation markers. The effect of warfarin was more marked compared with rivaroxaban for F1.2 where warfarin-treated patients had significantly lower values. However, almost all patients had F1.2 levels within the normal ranges irrespective of the type of anticoagulant. *In-vivo* coagulation activation markers were within the normal reference ranges in all rivaroxaban-treated patients (including those with subtherapeutic anti-Xa levels) and in warfarin-treated patients who had an INR <2.0, with a minority of patients in both groups exhibiting slightly raised activation markers.

Chapter 8 General discussion and future directions

I have demonstrated that APS patients with vascular thrombosis on long-term warfarin exhibit greater resistance to both exogenous rhAPC as well as to activation of endogenous protein C compared to non-APS patients with VTE and normal controls using thrombin generation system. Non-APS patients also showed greater resistance to activation of endogenous protein C compared to normal controls, but resistance was greater in the APS patients. APS patients also had a higher frequency (approximately 50%) and higher levels of anti-protein C antibodies; 60% (15/25) of these were high avidity antibodies. The APS patients with high avidity anti-protein C antibodies had significantly greater APCr to both rhAPC and activation of endogenous protein C. The presence of high avidity anti-protein C antibodies was also strongly associated with a severe thrombotic phenotype in APS patients as defined as patients who developed recurrent VTE whilst receiving therapeutic anticoagulation or those with arterial as well as venous thrombosis. Ninety-four percent (15/16) of the patients with a severe thrombotic phenotype had high avidity anti-protein C antibodies and 62.5% (10/16) of those patients were triple positive for aPL.

My observations that APS patients frequently exhibit resistance to the activation of endogenous protein C suggests a possible defect in the mechanism through which protein C becomes activated. High avidity anti-protein C antibodies, present in some APS patients, may interfere with the mechanism of activation of protein C or the anticoagulant action of APC. Differences observed between the APS and non-APS patients with regard to APCr could not be attributed to variation in protein C, protein S or procoagulant factor levels, since all samples were diluted with PNP to normalise levels of these factors.

Although, I have demonstrated that patients with thrombotic APS with high avidity anti-protein C antibodies have the greater resistance to APCr and activation to endogenous protein C, whether anti-protein C antibodies are the underlying aetiology of the impairment of activation of protein C pathway is not proven. In APS,

antibodies directed against β 2GPI and particularly, domain I of the protein, possess LA activity and are associated with thromboembolic events and are thus candidates in the pathogenesis. It not clear whether anti-protein C antibodies are an adjunctive risk factor in patients with APS or anti-protein C antibodies alone are responsible for APCr and hence the thrombotic risk. There is a possibility that anti-protein C antibodies have cross reactivity with $\alpha\beta$ 2-GPI antibodies as shown in some other studies and promote APCr via β 2-GPI (Nojima *et al*, 2005). However, patients with APS can have a variety of other autoantibodies with different specificities, including anti-protein C, as demonstrated in my study (as well as other antibodies to prothrombin, factor XII, TFPI, factor X), and the clinical relevance of these antibodies is being unravelled. High avidity anti-protein C antibodies may be an adjunctive risk factor in patients with APS, and therefore associated with a severe thrombotic phenotype. Whether these antibodies cross-react with $\alpha\beta$ 2GPI antibodies is not known. In my study, there was no relationship between APCr and $\alpha\beta$ 2GPI. The only way to prove that high avidity anti-protein C antibodies promote activated protein C resistance is by demonstrating this effect in pooled normal plasma spiked with affinity purified anti-protein C antibodies. This is an important area of further investigation which is planned in the UCL Haemostasis Research Unit.

In my study, a small proportion of severe thrombotic phenotype patients without APS also had anti-protein C antibodies, which were low avidity antibodies in contrast, high avidity antibodies seen in APS patients with a severe thrombotic phenotype. The relevance of those low avidity anti-protein C antibodies in the non-APS patients remains to be defined.

The prevalence and clinical significance of APCr in association with anti-protein C antibodies have not been reported in patients with obstetric APS and those with asymptomatic aPL. However, APCr has been described in obstetric APS (Bergrem *et al*, 2011;Gardiner *et al*, 2006;Rotmensch *et al*, 1997). It is therefore plausible that APCr associated with anti-PC antibodies may also provide a marker for a severe obstetric phenotype, which may be defined as the presence of both early and late pregnancy morbidity, with these categories as defined in the Sydney clinical criteria

for APS (Miyakis *et al*, 2006) or pregnancy morbidity whilst receiving anticoagulant therapy (Gardiner *et al*, 2013), as well as for future thrombotic risk in women with obstetric APS.

Thrombomodulin (TM) is a protein cofactor expressed on endothelial cell surfaces and acts as cofactor/receptor for thrombin, accelerating the activation of protein C by thrombin ~ 2000-fold (Sadler, 1997). In addition to its role in protein C activation, TM also transforms the functional specificity of bound thrombin from procoagulant to anticoagulant. I have not investigated the thrombin/TM activation of protein C in thrombotic patients with APS in my studies, which is a key area of interest for further research.

I found a wide variation of the sensitivity of thromboplastin reagents to rivaroxaban. Of the six commonly used thromboplastin reagents studied, Neoplastin®R was the most sensitive while Innovin® and Thromborel®S were the least sensitive. My findings from *ex vivo* studies of patients on therapeutic dose rivaroxaban, were in keeping with those previously reported from *in vitro* studies. Rivaroxaban levels within the therapeutic range can give a normal prothrombin time ratio with the less sensitive reagents, which may be clinically misleading. In the absence of anti-Xa assays, the assessment of rivaroxaban anticoagulation should only be performed using PT reagents with good sensitivity. Such testing should aid decision making in the acute management of the patient by indicating whether the patient is anticoagulated in the therapeutic, subtherapeutic or supratherapeutic range. Because of the wide variation in the sensitivity thromboplastin reagents to rivaroxaban, each laboratory should know the sensitivity of its own PT reagent as recommended in the BCSH guidelines (Kitchen *et al*, 2014).

Due to the increasing use of rivaroxaban, it is clinically relevant to define the appropriate methodology for *ex vivo* LA testing, to enable the diagnosis of APS in patients treated with rivaroxaban. I established this by *in vitro* and *ex vivo* studies of LA detection in thrombotic APS patients receiving therapeutic dose rivaroxaban. I found that false positive DRVVT occurred in rivaroxaban treated patients mainly at

therapeutic levels. The TVT/ECT ratio and Textarin time were not affected, irrespective of the rivaroxaban level, enabling LA detection in patients receiving rivaroxaban. In thrombotic APS patients treated with rivaroxaban, the TVT/ECT appears reliable even at therapeutic plasma levels of rivaroxaban. However, TVT/ECT is known to be less sensitive than DRVVT for detection of LA. DRVVT may be acceptable at trough rivaroxaban plasma levels, in samples taken at least 18 hours following the previous dose of rivaroxaban. In this situation, rivaroxaban levels should always be measured to ensure that trough levels really are present, if not results should be interpreted with caution. The findings in this *in vitro* and *ex vivo* studies guide detection of LA and enable diagnosis of APS in patients treated with rivaroxaban as well as monitoring of LA in APS patients, and also inform national and international guidelines.

It is theoretically possible that aPL could directly interfere with the anticoagulant effects of rivaroxaban, although it is hypothesised that this is unlikely due to its small molecular size and the high specificity for its target. I have shown that thrombotic patients without APS treated with rivaroxaban, the anticoagulant can affect various parameters of *ex vivo* TG measured using the CAT system. Similar studies in APS patients treated with rivaroxaban have not been published at the time of writing this thesis. I demonstrated in my *in vitro* studies that aPL do not influence anticoagulant activity of rivaroxaban as measured by TGT and anti-Xa assays.

I have demonstrated that both rivaroxaban and warfarin can achieve effective anticoagulation in thrombotic patients without APS, as assessed by inhibition of TG and *in vivo* coagulation activation markers. The effect of rivaroxaban on TG was more marked compared with warfarin for all parameters except ETP (as shown in previous *in vitro* studies), where warfarin-treated patients had significantly lower values. The TG test, as a global measure of anticoagulation, can assess the anticoagulant effects of both rivaroxaban and warfarin, and effects on the ETP should be assessed in combination with the other TG parameters. *In vivo* coagulation activation markers were within the normal reference ranges in all rivaroxaban-treated patients (including those with subtherapeutic levels) and in 37/45 warfarin-treated

patients who had an INR ≥ 2.0 , with a minority of warfarin-treated patients with subtherapeutic INR values exhibiting slightly raised F1.2 and/or TAT.

In studies on thrombotic APS patients, *in vivo* markers of coagulation activation gave similar results to those of patients without APS, although some patients treated with warfarin who had subtherapeutic INR also had raised D-dimer. However, there was a significantly lower level of F1.2 levels in patients treated with warfarin compared to rivaroxaban in APS patients which was not observed in the non-APS patients, but the difference was not clinically relevant and F1.2 levels remained normal in all rivaroxaban treated patients.

In summary, I have fulfilled the aims of my thesis and believe that I have made several important contributions to the medical literature. I have established that thrombotic patients have greater resistance to both activated protein C and activation of endogenous protein C. Nearly half of the patients that I studied had anti-protein C antibodies and those who had high avidity anti-protein C antibodies had significantly greater APCr to both rhAPC and activation of endogenous protein C. The presence of high avidity anti-protein C antibodies was also strongly associated with a severe thrombotic phenotype in APS patients and could be used as a marker in future once it is validated in a larger cohort of patients. Establishing the sensitivity of commonly used thromboplastin reagents to rivaroxaban is a major clinical relevance as it aids decision making in the acute management of the patient by indicating whether the patient is anticoagulated in the therapeutic, subtherapeutic or supratherapeutic range when measurement of rivaroxaban level is not readily available. Methods of detecting LA in APS patients treated with rivaroxaban are important both nationally and internationally. Finally, I have demonstrated *in vitro* studies, that aPL do not influence the anticoagulant activity of rivaroxaban as measured by TG and rivaroxaban anti-Xa levels.

Future directions

High avidity anti-PC antibodies may be an adjunctive risk factor in patients with APS, and thus associated with a severe thrombotic phenotype. The findings in my

study are therefore potentially of major clinical relevance as they could provide a basis for early recognition of patients who may benefit from escalated therapeutic interventions, including immunomodulation. The prevalence or clinical significance of APCr in association with anti-PC has not been reported in patients with obstetric APS and those with asymptomatic aPL. It is relevant to establish the prevalence and clinical significance of APCr in association with anti-protein C antibodies with obstetric APS and those with asymptomatic aPL.

The high avidity anti-protein C antibodies detected in APS patients with a severe thrombotic phenotype may represent an underlying aetiology of thrombotic APS and this need to be confirmed by *in vitro* studies by demonstrating APCr in PNP spiked with affinity purified anti-PC antibodies.

Publications arising from this thesis

Papers

- Arachchillage,D.R., Efthymiou,M., Lawrie,A.S., Machin,S.J., Mackie,I.J., & Cohen,H. (2014) Comparative sensitivity of commonly used thromboplastins to *ex vivo* therapeutic rivaroxaban levels. *Thromb Haemost*, **112**, 421-423.
- Arachchillage,D.R., Efthymiou,M., Mackie,I.J., Lawrie,A.S., Machin,S.J., & Cohen,H. (2014) Anti-protein C antibodies are associated with resistance to endogenous protein C activation and a severe thrombotic phenotype in antiphospholipid syndrome. *J Thromb Haemost*, **12**, 1801-1809.
- Arachchillage,D.R., Efthymiou,M., Mackie,I.J., Lawrie,A.S., Machin,S.J., & Cohen,H. (2015) Rivaroxaban and warfarin achieve effective anticoagulation, as assessed by inhibition of TG and in-vivo markers of coagulation activation, in patients with venous thromboembolism. *Thromb Res.*, **135**, 388-393.
- Interactions between rivaroxaban and antiphospholipid antibodies in thrombotic antiphospholipid syndrome. Arachchillage DRJ, Mackie IJ, M Efthymiou M, Isenberg D, Machin S.J, Cohen H. *J Thromb Haemost*, 2015 **13**, 1264-73.

Abstracts

- Arachchillage DRJ, Mackie IJ, M Efthymiou M, Isenberg D, Machin S.J, Cohen H. Interactions between rivaroxaban and antiphospholipid antibodies in thrombotic antiphospholipid syndrome . Free communication BSH 2015
- Arachchillage DRJ, IJ Mackie, M Efthymiou, SJ Machin, , H Cohen .Effects of rivaroxaban on detection of lupus anticoagulant: *In vitro* and *ex vivo* studies BSHT 2014
- Arachchillage DRJ, M Efthymiou, AS Lawrie SJ Machin, IJ Mackie, H Cohen *Ex vivo* thrombin generation and activation markers of *in vivo* coagulation in patients with venous thromboembolism on therapeutic dose rivaroxaban or warfarin. Free communication BSHT 2014

- Arachchillage DRJ, M Efthymiou, AS Lawrie, SJ Machin, IJ Mackie, H Cohen Resistance to activation of endogenous protein C associated with anti-protein C antibodies may be a marker of a more severe thrombotic phenotype in antiphospholipid syndrome. EHA 19th congress, June 2014 in Milan.
- Arachchillage DRJ, M Efthymiou, AS Lawrie, SJ Machin, IJ Mackie, H Cohen Increased resistance to recombinant human activated protein C and to activation of endogenous protein C in thrombotic antiphospholipid syndrome. Free communication BSH 2014
- Arachchillage DRJ, Efthymiou M, Lawrie AS, Machin SJ, Mackie IJ, Cohen H. Increased resistance to activation of endogenous protein C in patients with thrombotic antiphospholipid syndrome.
Free communication at 14th International Congress on Antiphospholipid Antibodies and 4th Latin American Congress on Autoimmunity, 2013 in Rio de Janeiro – Brazil
- Arachchillage DRJ, Efthymiou M, Lawrie AS, Machin SJ, Mackie IJ, Cohen H. Investigation of resistance to exogenous activated protein C and activation of endogenous protein C in thrombotic patients with or without antiphospholipid syndrome. E-poster oral presentation at ISTH in Amsterdam 2013.

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activation of endogenous protein C in thrombotic patients with or without
antiphospholipid syndrome . (Abstract).*J Thromb Haemost* 2013, 11, 335-
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