Characterisation of Direct Acting Oral Anticoagulants and Their Pharmacodynamics on Thrombin Generation and Coagulation Parameters.

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Matthew Halliday
Abstract

Introduction: Vitamin K antagonists (VKA) are historically used to treat thrombosis but newer direct acting oral anticoagulants (DOAC) have been released. DOACs influence laboratory methods that monitor haemostasis, leading to false positives and prolonged clotting results. Trough DOAC concentrations may have less influence at than at peak, but this is unknown. There is speculation that obesity also affects DOAC efficiency. This study sought to validate the effects of two common DOACs, rivaroxaban and apixaban, on 12 laboratory assays at peak and trough concentrations, as well as the influence of obesity on the drugs and assays. Methods: Rivaroxaban (n=20) and apixaban (n=21) patients had samples collected immediately before their next dose, and 3 hours after to provide peak and trough. Healthy controls (n=20) provided a sample at one-time point. Relevant medical histories and demographics, including BMI, were collected after obtaining informed consent. Samples underwent coagulation and thrombophilia panels as well as thrombin antithrombin complex, prothrombin fragments 1+2, and thrombin generation assays (TGA). Statistical analysis was performed between peak and controls, peak and trough, and all BMI groups. Results: Significant prolongations were found with the prothrombin time (PT), activated partial thromboplastin time (APTT), TGA, and false positives for the dilute Russell viper venom time (DRVVT) by both rivaroxaban and apixaban. Apixaban was less potent than rivaroxaban with significant differences only between peak and trough for PT, and APTT. Only two results were influenced by weight; the APTT with rivaroxaban and free protein S for the controls. Conclusion: Rivaroxaban and apixaban are similar drugs but impact the assays differently due to dosage, half-life, and rate activity. Both influence assays more at peak than trough, but trough is still notably influenced compared to the controls. This study highlights the influence of these drugs on most tests performed in a haemostasis laboratory.
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1. Introduction

1.1 Introduction to Coagulation

1.1.1. Primary Haemostasis

Primary haemostasis is characterised by the formation of an aggregate platelet plug which begins as soon as endothelial tissue is damaged or exposed. The aggregation of platelets occurs from the binding of endothelial von Willebrand factor to collagen, which then binds and activates platelets. Further, fibrinogen circulating within the plasma binds to activated platelets and forms a soft platelet plug that is reinforced from insoluble fibrin generated from secondary haemostasis (1).

1.1.2 Secondary Haemostasis, and Fibrinolysis.

Secondary haemostasis was described in 1964 as a coagulation cascade, which was initiated after activation of either the extrinsic (initiated by tissue factor) or intrinsic (initiated by contact factors) pathways, leading to the activation of the common pathway (2,3). The extrinsic pathway begins with exposed tissue factor from trauma reacting with Factor VII (FVII) and calcium ions to activate Factor X (FX). The intrinsic pathway begins when Factor XII (FXII) is activated via contact with negatively charged surfaces. FXIIa then activates Factor XI (FXI) which in turn activates Factor IX (FIX) leading to FX activation with Factor VIII as a cofactor, phospholipids, and calcium ions. Both intrinsic and extrinsic pathways activate the common pathway which begins with the activation of FX with Factor V as a co-factor which cleaves prothrombin into thrombin. Thrombin then cleaves fibrinogen into fibrin and forms fibrin monomers, and then a fibrin based gel. Thrombin also activates FXIII which interacts with the fibrin gel to form the stable fibrin clot, a series of crosslinked polymers that prevents further bleeding (4).

Whilst the traditional coagulation cascade is useful for the evaluation of coagulation assay results, the model is no longer the most physiologically accurate model for coagulation (5). The simple biochemical pathway does not take in account the presence of anticoagulant proteins
such as protein C and tissue factor pathway inhibitor, the interactions of the fibrinolysis pathways, or the interactions of both intrinsic and extrinsic pathways on each other. A cell based model of the coagulation cascade has become the current gold standard for our understanding of *in vivo* blood coagulation with initiation, amplification, and termination phases providing coagulation. Initiation begins with vascular endothelium and circulating blood cells being disturbed by trauma or disease revealing cellular bound tissue factor. This tissue factor interacts with plasma derived FVIIa which activates lesser amounts of FIXa and FXa, with the cofactor FVa, to produce the prothrombinase complex. The prothrombinase complex cleaves prothrombin into active thrombin and leads to the amplification phase. Active thrombin binds to platelets previously activated from primary haemostasis, as well as propagate and activate the release of Factors XI, IX, VIII, and V from internal platelet stores (6). The activated FIX and FVIII leads to the formation of the tenase complex in copious quantities. Large numbers of factors migrate to the surface of platelets and produce the rapid formation of prothrombinase and the generation of a large thrombin burst. Thrombin then cleaves fibrinogen to fibrin monomers and forms the fibrin clot (7).

1.1.3. Regulation of Coagulation.

Termination is the last phase of coagulation and is regulated by anticoagulant feedback mechanisms which inhibit the proteins and cease the formation of the clot. Thrombin is regulated by thrombomodulin, a protein co-factor in activating the anticoagulant protein C, catalysed by protein S. Activated protein C proteolytically cleaves peptide bonds in FVa and FVIIIa, the critical precursors in generating thrombin, causing the inactivation of both factors, and inhibiting further thrombin generation and coagulation. Antithrombin, a protein produced in the liver inhibits factors Xa, IXa, Xla, XIIa and thrombin. The final regulatory protein is tissue factor pathway inhibitor (TFPI), a single chain polypeptide that binds to FVIIa within a tissue factor:FVIIa complex, FXa and FV and to FVa when present in the prothrombinase complex. The inhibition of these factors by TFPI serve to prevent the conversion of prothrombin to thrombin.
during the initiation phase of coagulation. Without these key mechanisms in place the cascade will produce excessive generation of thrombin and fibrin, which will significantly increase the chances of developing unnecessary clotting events known as thrombosis.

![Figure 1.1](image.png)

**Figure 1.1.** A model of secondary haemostasis with both intrinsic, extrinsic, and common pathways. Green arrows represent positive feedback mechanisms and red arrows represent negative feedback mechanisms (8)

1.2. What is Thrombosis?

1.2.1. Global Burden of Thrombosis

Thrombosis is a condition characterised by the formation of blood clots within the vascular system of a living animal. These obstructions of blood vessels can also lead to stroke, myocardial infarction, foetal wastage syndrome and localised ischemia within individual organs or limbs (9). Thrombosis is most noticeable when occurring within deep veins around the pelvic region or inner legs which is named Deep Vein Thrombosis (DVT) (10), or within a major artery which can lead to ischemia and tissue necrosis from vessel occlusion and platelet aggregation (11). Thrombi within vessels can be dislodged and block smaller vessels throughout the body in an event called a thromboembolism, and especially become entrapped within the vascular mesh within the
lungs leading to a potentially fatal event called a Pulmonary Embolism (PE). The combination of DVT and thrombotic or pulmonary embolisms is collectively called Venous Thromboembolism (VTE). The development and presence of thrombotic disorders is the leading cause of death within the Western world, with 350000 patients affected every year in the United States alone (12). VTEs are highly common events with 201 VTE episodes occurring per 100,000 persons in 2010/2011 across Europe, which marks a steady increase of VTE incidents from 1990 to 2011 (13,14).

1.2.2: Risk Factors of Thrombosis.

Risk factors of thrombosis include trauma, surgery, cancer, age, pregnancy and use of oral contraceptives, lupus anticoagulants and immobilization during travel or lifestyle choices (15-20). Whilst the risk factors are many, the cause of thrombosis is generally defined by Virchow’s triad which is the stasis of blood, endothelial cell injury, and altered blood coagulation (21).

**Figure 1.2.** A model of Virchow’s Triad displaying the three key events to trigger a thrombotic event. (22)
1.3 How To Detect And Measure Thrombosis

1.3.1 Coagulation Assays

As the risk of thrombosis is common and can be triggered by a variety of factors, there is a clinical imperative to monitor the coagulation cascade to evaluate the risks of haemostatic disruption. Laboratories have a set of assays to measure individual processes within the cascade. These assays include coagulation tests such as the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time (TCT), D-dimer levels and fibrinogen assays. Thrombophilia assays include, antithrombin levels (AT), dilute Russell’s viper venom test (DRVVT), protein C activity, free protein S activity, anti-Xa activity, prothrombin fragments 1+2 (PF1+2), thrombin/anti-thrombin complexes (TAT) and the thrombin generation assay (TGA).

The different assays and how their results are interpreted are detailed below.

1.3.2 Coagulation Panel

The coagulation panel contains a series of five routine assays that evaluates an individual’s ability to clot which include the PT, APTT, TCT, and quantitative assays measuring fibrinogen and D-dimer levels.

Figure 1.3. Intrinsic and extrinsic pathways are the area of interest for the coagulation panel.
The prothrombin time measures the activities of the extrinsic and common pathways of coagulation based on the time required for a fibrin clot to develop. The assay measures the time taken for a sample of platelet poor plasma (PPP) to clot in the presence of phospholipids, tissue factor and calcium. The assay is dependent on the activity of FVIIa, FXa, FVa, prothrombin and fibrinogen (23).

The activated partial thromboplastin time is used to measure the intrinsic and common coagulation pathways based on the amount of time required for a fibrin clot to develop. The APTT assay consists of the clotting time taken from citrated PPP and phospholipids on a negatively charged surface, after the addition of calcium chloride which initiates for fibrin clot formation.

The thrombin clotting time evaluates the time taken for thrombin to form a clot in human citrated plasma, and detects the presence of thrombin inhibitors. The assay places a concentration of human thrombin into PPP and measures the time taken before a fibrin clot is formed. This reaction does not need calcium, unlike the APTT or PT, as the thrombin present in the sample can readily cleave fibrinogen and continue the reaction to the formation of a fibrin clot.

The fibrinogen assay is used for the quantification of the protein fibrinogen in human plasma, usually using the Clauss method on an automated analyser. The Clauss fibrinogen assay uses diluted PPP (1:10 in buffer) together with phospholipids and high concentrations of thrombin (24). This is to ensure clotting times are independent of thrombin concentration over a wide range of fibrinogen levels found in samples. The reaction begins with the addition of thrombin to diluted plasma and the time taken for fibrinogen to produce a fibrin clot is recorded. The time is then compared to a calibration curve produced using a series of plasma dilutions produced from commercially available standard plasmas. The clotting time is inversely proportional to the plasma fibrinogen concentration.
The final assay within the coagulation panel is the D-dimer assay, which is used for the quantitative determination of cross-linked fibrin degradation products, or D-dimers, in human plasma. Fibrinogen consists of three pairs of polypeptide chains that meet to form a central E domain and two D domains. When fibrinogen is activated by thrombin and becomes fibrin, the D-domains can bind to other fibrin E domains to form a fibrin clot. Adjacent D-domains are then covalently bound by FXIIIa. D-dimers are formed when cross-linked fibrin polymers covalently bound to each other are degraded by plasmin and broken down into the individual E and D domain fragments. These D-domain fragments or D-dimers can be measured as an indicator of coagulation activity via the use of an immunoturbidimetric assay containing polystyrene particles covered in monoclonal antibodies to bind D-dimers from the plasma onto the polystyrene surface.

The coagulation panel can aid in diagnosing genetic or acquired factor deficiencies within the extrinsic and intrinsic pathways, liver failure, fibrinogen deficiency or disorders, disseminated intravascular coagulation (DIC), dysfibrinogenaemia and can also be used to rule out the risks of a thromboembolic event. The coagulation panel can monitor different therapies such as oral anticoagulants, fibrinolytic therapies, or the effects of heparin and direct thrombin inhibitors, such as dabigatran (25-28).

1.3.3 Thrombophilia Panel

The thrombophilia panel is used to determine an individual’s potential to clot and the risk of abnormal blood coagulation. The panel is comprised of four assays that measure the anticoagulant aspects of clotting. These assays include quantitative measurements of antithrombin III, protein S, protein C, and the Dilute Russell’s Viper Venom Time (DRVVT).
The Antithrombin III (ATIII) assay is used for the quantitative determination of the functional activity of ATIII in human plasma samples. ATIII is a natural anticoagulant which acts by inhibiting thrombin and FXa by forming an irreversible inactive complex. The ATIII assay uses plasma diluted in saline with heparin and excess thrombin to form a AT-Thrombin-Heparin complex. The residual thrombin then reacts and cleaves a chromogenic substrate to produce a colour change. The absorbance of the reaction is measured at 405nm and the rate of change in absorbance is inversely proportional to the activity of ATIII. With the use of a reference curve the rate of change in absorbance is used to determine the level of ATIII in the plasma (29).

The DRVVT is used for the detection of lupus anticoagulants (LA) in human plasma using a two-step screening and confirmation process containing Daboia russelli (Russell Viper) snake venom. Lupus anticoagulants are antibodies formed against negatively charged phospholipids or clotting
factors, including prothrombin, and are a significant risk factor in patients with unexplained thrombosis or women suffering recurrent foetal loss (30,31). The DRVVT screening test measures the amount of time taken for plasma to clot after the direct activation of FX. A prolonged clotting time could result from the presence of LA. The confirmatory test is nearly identical to the screening test but includes a high concentration of phospholipids to counteract the effects of LA. The screening test and the confirmatory test results are placed within a ratio (LA screen ratio/LA confirm ratio= Result ratio) to produce a normalised DRVVT ratio. If the plasma produces a normalised ratio more than 1.25 the sample is considered to have LA present, and if the ratio is less than 1.25 then the sample is confirmed to be LA negative.

Factor deficiencies can also affect the DRVVT as a lack of factors can prolong the clotting times and can be mistaken for LA influences. To avoid mistakes, a prolonged sample can be mixed with normal plasma and undergo the DRVVT test again. The test is identical as the one before but the only difference is the addition of mixed pooled plasma to counteract the effects of factor deficiencies. The final ratio is calculated as previously described and LA is deemed to be present if the ratio over 1.25. If the ratios are below 1.25 for both tests with the mixed plasma, LA can be eliminated and factor deficiencies are suspected (32).

The protein C assay uses a chromogenic substrate for the quantitative determination of active protein C in PPP. Protein C is a vitamin K-dependent coagulation inhibitor that degrades with FVa and FVIIIa, with protein S as its co-factor. The protein C assay uses snake (Akistrodon contortrix contortrix) venom to initiate protein C activation within sample plasma, with activated protein C cleaving a chromogenic substrate to produce a coloured substrate which is measured at 405nm. The level of absorbance from each sample is calculated using a standard curve produced from reference commercial plasma. The standard curve then provides a measurement of the amount of protein C present within the plasma.

The free protein S assay used for the quantitative determination of free protein S in plasma by measuring the turbidity before and after inactivating FVa and FVIIIa in PPP (33). The assay uses
polystyrene particles coated in two different monoclonal antibodies to aggregate free protein S. The degree of aggregated particles is directly proportional to the level of free protein S in the plasma and is measured from the increase in turbidity over the course of the reaction. The assay is calibrated using commercial plasma standards, which provides a standard curve of known values and can be used to determine the level of protein S directly from the curve.

The thrombophilia panel can be used for the determination of ATIII, free protein S, and protein C deficiencies which are linked to increased risk of VTE. The prevalence of ATIII deficiency in VTE patients is 1-2% which is higher compared to 0.02-0.2% of the normal population (34). Protein C deficiencies can also lead to thrombosis if genetically heterozygous and are non-compatible with life if homozygous (35). Free protein S deficiency, which is associated with an increased risk of VTE, can be caused by hepatic disorders, warfarin, viral infections, disseminated intravascular coagulation, oral contraceptives, and pregnancy (36).

1.3.4 Anti-Xa Activity

The anti-Xa activity assay is a measurement of the direct anti-Xa anticoagulants in human plasma. FXa cleaves prothrombin into thrombin and is the direct target for current anti-Xa direct acting oral anticoagulant drugs (DOACs), as inhibiting FXa inhibits thrombin generation and clotting. The chromogenic assay uses undiluted plasma with a chromogenic substrate and adds FXa into the mixture, triggering a competitive reaction between FXa hydrolysis of the substrate and FXa inhibition by a DOAC. The amount of coloured substrate produced is inversely proportional to the mass concentration of drug present in the plasma, which can be calculated using a calibration curve from known standards (37). Anti-Xa assays use a drug specific standard curve produced from the use of commercial standards as a method to determine the levels of drugs present in the plasma (38).
1.3.5 Coagulation Enzyme-Linked Immunosorbent Assays (ELISA)

Enzyme-linked immunosorbent assays, or ELISAs, are used within a haematology laboratory to aid in quantifying individual molecules of interest. Two ELISA assays of interest would include the Prothrombin fragments 1+2 (PF1+2) and the Thrombin/antithrombin complex (TAT) assays.

The prothrombin fragment 1+2 assay (PF1+2) can be used to quantify the amount of thrombin generated in a reaction in PPP. The conversion of prothrombin into thrombin releases a peptide called prothrombin fragment 1+2 which has been linked to thrombosis if found in elevated levels. The assay uses specific PF1+2 antibodies to attach to PF1+2 antigen on the surface of a micro titre plate. Additional peroxidase-conjugated antibodies are added and bind to free PF1+2 before being rinsed, leaving only the bound PF1+2. The attachment of PF1+2 to its antibody in the presence of peroxide releases a coloured substrate, proportional to the PF1+2 concentration.
present which is measured to provide the amount of PF1+2 present, and subsequently the amount of thrombin present in the sample which is useful to diagnosis the risk of thrombosis (40).

The thrombin/anti-thrombin complex (TAT) assay is a quantitative ELISA kit specifically aimed at quantifying the presence of TAT within PPP. After thrombin is activated and initiated fibrin clotting the process is inhibited by antithrombin to prevent unnecessary clot formation. Antithrombin binds to thrombin to form an inactive proteinase/inhibitor complex known as the thrombin/anti-thrombin complex, which is measured by this assay. The assay contains monoclonal antibodies for TAT and binds the complexes to a microtitre plate. The antibody bound TAT enzymes then reacts with an added chromogenic substrate to produce a measurable coloured substrate which is directly proportional to the concentration of TAT present (41). This assay is useful for the diagnosis of thrombotic events as higher TAT leads to a greater chance of developing a thrombus, or the incidence of DIC. The assay can also uncover if anti-thrombin or thrombin is low from excessive use or underproduction in patients with liver dysfunction, multiple trauma or septicaemia and is significantly more sensitive than other routine coagulation assays (42,43).

1.3.6 Thrombin Generation Assay (TGA)

The thrombin generation assay is a global assay used to monitor the complete formation and inhibition of thrombin within plasma with the use of a fluorogenic substrate. The global assay differs from a basic clotting time assay as the TGA measures the entire process of haemostasis and not just clot formation. The reaction is generated with the use of tissue factor and calcium to activate the coagulation cascade to cleave prothrombin into thrombin and generate a thrombin burst. The TGA is usually an automated procedure run by the Calibrated Automated Thrombogram (CAT) or the Ceveron Alpha® with TGA analyser. Both methods measure the entire space of thrombin activation which is represented by five parameters forming a TGA graph. These parameters include the lag time, the Endogenous Thrombin Potential (ETP), the
time-to-peak, the peak height, and the velocity index. The results received from different parameters are informative as the assay reveals the entire process of thrombin generation and inhibition within a plasma sample, not just the clotting time as other coagulation assays describe. Clotting times alone are insensitive to mild haemostatic disorders and increased coagulability, which is vital to diagnosing the increased risk of thrombosis (44).

The lag time is the measurement of time when tissue factor is added to a platelet poor plasma sample until there is a thrombin burst observed. The lag time is 4-6 minutes on average, or one sixth of the total reaction time and is defined as when more than 95% of the overall thrombin is still to be formed (46). Many other clotting time assays measure only this brief period and stop at the thrombin burst and do not provide a measurement of a patient’s overall coagulation potential, unlike the TGA. The area under the curve, or Endogenous Thrombin Potential (ETP), contains a measurement of the amount of thrombin generated overall within the reaction. With a patient’s ETP known, a clinician can accurately predict an individual’s coagulation potential which indicates if they are at risk of developing thrombosis, or to monitor the effects of anti-

Figure 1.6. A typical thrombin generation assay curve produced using the Calibrated Automated Thrombogram. Parameters measured include Lag time, Endogenous Thrombin Potential (ETP), Velocity index (VI), Time to peak and Peak height (Peak). Tail represents termination of the reaction. (45)
coagulant drugs on the patient (47). The peak height is the largest thrombin concentration detected during the reaction and time to peak is the minutes taken to reach that concentration. The final parameter is the velocity index which is the rate of the thrombin generated during the thrombin burst per minute which is useful to compare how intense the generation of thrombin is for the patient.

1.4 Treatment of VTE and Review of DOACs and Coagulation Assays.

The most common prophylactic treatment for thrombosis is the anticoagulant drug warfarin, approved by the FDA in 1954. It is a vitamin K antagonist (VKA) that inhibits coagulation by preventing the formation of vitamin K-dependent coagulation factors (48). Whilst warfarin is very effective for the treatment and prevention of thrombosis, there is a major disadvantage with the substance interacting with foods containing prominent levels of vitamin K as well as having constant fluctuations in drug levels which requires constant monitoring. These interactions decrease drug effectiveness and increase adverse interactions (49). New anticoagulant drugs have been developed to counter act the disadvantages of warfarin by acting directly on a specific clotting factor within the coagulation cascade instead of acting as a vitamin K antagonist.

The new Direct Acting Oral Anticoagulant (DOAC) class is gaining greater representation for the treatment of thrombosis as global DOAC use has steadily increased within the last decade. DOACs, previously referred to as Novel Oral Anticoagulants (NOACs) are rapidly replacing traditional treatments due to their direct action, significantly less food/drug interactions, fixed dosage, and non-inferior efficacy (50). The current DOACs being prescribed include rivaroxaban, apixaban and edoxaban; direct FXa inhibitors and dabigatran; a direct thrombin inhibitor. With FXa or thrombin inhibited the conversion of fibrinogen into fibrin is blocked and clotting ability is significantly reduced.
DOACs are currently used to treat VTE and unlike VKA, do not requiring routine monitoring. However, there are occasions that monitoring DOACs would be advantageous such as during VTE events, serious bleeding, prior to urgent surgery or in suspected DOAC overdose (53). When monitoring drug concentrations, the two standard measurements taken include the peak and the trough levels. The peak level is the highest drug concentration available within the patient’s system after drug administration whilst the trough is the lowest concentration of the drug before the next dose. The difference between the two levels is the time taken since the last dose with peak usually around 2-4 hours and trough can be up to 24 hours since administration. The peak and trough measurements are important as they can identify a patient’s risk of overdosing, occurrence of drug toxicity, or if the drug concentration is still within therapeutic levels.

<table>
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</tbody>
</table>

Table 1.1. Characterising the pharmacological properties of warfarin and the DOACs (50-52)

*OD- Once Daily, BD- Twice daily*

The adaption of DOACs since 2010 has been rapid with medical guidelines undergoing change to preference DOACs as a frontline treatment option for VTE and atrial fibrillation (54). However,
with the rapid adaption to DOACs there are still several questions within the literature on the effects of DOACs that have not been addressed. These include the effects of DOACs on routine and non-routine coagulation tests, such as false positives or prolonged clotting times within the DRVVT and APTT assays for LA testing (55). The effects of the concentration of the DOACs, at either peak or trough level is also very limited with some studies reporting DOAC effectiveness decreasing at trough concentrations (56). Also, it remains unknown whether the efficacy of DOACs are affected by obesity and if the practice of fixed dosage for DOACs is affected.

1.5. DOAC Effects on Coagulation Assays.

One of the largest advantages for DOAC therapies is that routine monitoring is non-essential, but this has led to unknowns regarding the effects of DOACs on routine and non-routine coagulation assays. There are circumstances where monitoring the effects of DOACs are important such as assessing which drug a patient is on and its concentration during emergency events such as bleeding, trauma, urgent surgery, and strokes (57), or accidental overdose. According to a study by Gosselin et al. (2016), less than 1% of laboratories that run routine coagulation tests perform additional quantitative FXa assays. This assumes that the routine tests such as PT and APTT can be used to accurately assess DOAC drug presence and plasma concentration (58). Gosselin et al. (2016) conducted a study which assessed the sensitivity of these methods to determine the DOACs anti-Xa effects on routine assays and some non-routine coagulation assays. The study revealed that the PT and APTT are sensitive to interference from edoxaban and rivaroxaban but less for apixaban with longer clotting times experienced with increasing dosage of the drug. A study by Martinuzzo et al. (2013) ran silica clotting time assays as confirmation tests for rivaroxaban and dabigatran prolonged APTT samples but this test proved to be negative for any false positives (55). They concluded that the PT and APTT can be used as a screening test for the presence of anti-Xa DOACs but not to accurately provide their concentration.
The Gosselin et al. (2016) paper also studied a series of assays to evaluate if there are significant influences from the anti-Xa DOACs, which is recorded as changes in the baseline values by more than 15%. The study tested the Fibrinogen, ATIII activity, D-dimers, TCT, reptilase time, DRVVT, protein C, protein S, plasminogen, von Willebrand factor activity and antigen, as well as FVIII activity assays. The study concluded that there were no significant effects on these assays, with the exceptions of the ATIII, DRVVT, protein C and S, and Factor VIII assays. The ATIII, protein C and S assays produced falsely elevated results whilst the FVIII assay was also falsely decreased by the presence of DOACs, depending on the reagents and DOACs used (58).

The DRVVT test for the presence of lupus anticoagulants produced increased normalised ratios which led to false positives for the presence of LA, a result that has been reported repeatedly for rivaroxaban, dabigatran and edoxaban (59-61), but interestingly very little for apixaban (62). A study conducted by Arachillage et al. (2015) investigated the influence of rivaroxaban on LA detection using in vitro and ex vitro samples by testing DRVVT at both peak and trough concentrations. The results demonstrate a concentration dependent increase of clotting time and increased rate of false positives for the DRVVT in 90% of their normal control and 92% in LA-negative in vivo samples when using peak (250ng/ml) rivaroxaban concentration with commercial assays (59) and led to ex vivo false positives for LA negative patients. However, false positives were not observed when the rivaroxaban plasma was at its trough (50ng/ml). It was concluded that peak, but not trough rivaroxaban concentrations will cause false positives in the DRVVT method (59). Acquiring only trough samples for testing is difficult however, as in most cases laboratories do not know if their samples are taken at peak or trough unless an anti-Xa assay is also performed, which has already been reported to be less than 1% of routine cases (54).

Arachillage et al. (2015) also used Taipan venom time/Ecarin clotting time (TVT/ECT) assays for the detection of LA which reported no false positive results even at peak rivaroxaban
A previous study by Parmar et al. (2009) was similar in showing that TVT/ECT was more effective for determining the presence of LA in plasma containing VKAs compared to the DRVVT, but also stated that the assay is insensitive for individuals not receiving oral anticoagulants when compared to DRVVT (63). The results of the Parmar et al. (2009) study highlighted that the influence of VKA on DRVVT assays corresponds with the Arachillage et al. (2015) results for DOACs affecting the DRVVT assays. The reason TVT and ECT is unaffected by DOACs is due to the action of the venoms occurring independently from phospholipids and plasma concentrations of FV, FVII and especially FX. The DRVVT, however, is dependent on phospholipids for confirmation testing as the venom relies directly on the activation of FX which relies on the surface of phospholipids to continue the coagulation cascade, which means the assay is affected by DOACs. TVT/ECT was therefore stated to be reliably used for the detection of LA in patients undergoing DOAC therapy due to the absence of inference, whilst the 1st line LA detection method of DRVVT will produce false positives at peak concentrations.

There are contradictions, however, regarding trough level assay validity and on the use of the TVT/ECT. Whilst all DOAC and LA studies have concluded that peak levels of DOACs led to false positives, there has been the recommendation that trough levels are safe to use, such as found in the Arachillage et al. (2015) and Gosselin et al. (2016) papers (58,59,61). A new study conducted by Ratzinger et al. (2016) focused solely on the effects of trough concentrations on LA assays and has reported false positives at even the lowest trough levels of all DOACs present for the DRVVT assay (56). At lower concentrations rivaroxaban and apixaban had a minor effect on APTT-LA confirmatory testing, whilst dabigatran caused major alterations with minimal dosage (56). Apixaban produced a false positive rate of 20% for the DRVVT which is unacceptably high, and is a contradiction to the earlier Hillarp et al. (2014) paper that stated apixaban does not cause false positive results (62). As there were discrepancies with the DRVVT, the TVT/ECT was recommended as an alternative LA assay by several papers (58,64,65) but the assay is
reportedly influenced by dabigatran at even trough levels (66). However, the current 2014 guidelines for lupus anticoagulant detection support the TVT/ECT assays use for detecting LA when patients are undergoing anticoagulant therapies (67). The literature ultimately concludes that a DOAC neutralising agent for LA testing or the availability of a consensus based alternative testing system is highly desired and until these are a reality, LA testing for patients on DOAC should be avoided entirely.

1.6 Effects of Obesity with DOACs on Coagulation Testing.

There are unanswered questions involving DOACs and their effects on obese patients. It is well known that obesity is a risk factor for thrombosis, but only recently have the underlying pro-thrombotic mechanisms been uncovered. Obesity is an epidemic with WHO estimating 1.9 billion overweight and nearly 600 million obese adults globally, with an imbalance of energy intake and expenditure the main cause (68). Body Mass Index (BMI) estimates obesity and is classified as six weight groups which consist of underweight (<18.5), healthy (18.5-24.9), overweight (25.0-29.9), obesity I (30.0-34.9), obesity II (35-39.9) and obesity III (>40) as per National Health and Medical Research Council guidelines (69). Obesity is linked to the dysregulation of metabolic homeostasis which leads to systemic inflammation caused by inflammatory cytokines produced by adipocytes (70). The inflammation encourages activated macrophage recruitment which leads to further inflammation and systemic release of Tumour Necrosis Factor-α and interleukin 6 and 1β. These cytokines maintain the systemic inflammatory state which activates prothrombin signalling pathways leading to increased thrombin and tissue factor expression and ultimately the initiation of thrombosis (70). Normal anticoagulant mechanisms such as ATIII and protein C systems are also disrupted which further increase the risks of thrombosis (72). Platelets are also associated with obesity as mediators, with increased BMI leading to higher amounts of activated platelets which amplify the inflammation response via a range of mircoRNAs found within platelets (73).
Current dosing strategies for DOACs do not take BMI into consideration. However, there are studies that question the effectiveness of this practice. A major review of the published DOAC clinical trials revealed that most trials have not investigated obesity amongst their cohorts with no statistical comparisons, or have omitted the BMI and weight information from the results altogether (74). The trials that have recorded the BMI have shown there is a relationship between the plasma drug concentration and body weight, but their conclusions have stated that there are no clinically relevant differences (74). A rivaroxaban clinical trial in 2007 examining the effects at extremities of weight stated there is no clinically related differences and that rivaroxaban is unlikely needed to be adjusted for weight (75). The RELY-ABLE long term clinical trial for dabigatran was a 2.8-year long trial to assess the additional safety of dabigatran but did not provide weight information (76). Three studies have published cases where obese patients experienced a VTE whilst on a regular regimen of dabigatran, because the dosage fell below therapeutic levels (77-79). Whilst the case reports suggested increased creatinine clearance as a possible cause, they also state that fixed dosage of dabigatran may be ineffective for obese patients. The dabigatran was replaced with warfarin or rivaroxaban which was stated to have stronger pharmacotherapeutic properties than dabigatran in obese patients but there are currently no recommendations for the replacement of DOACs (79). With the limitations of the data provided from most studies regarding obesity and the growing evidence of ineffective treatment with fixed dosage, further study of the effects of obesity on fixed DOACs dosage is required.

1.7 Proposed Research

With limitations of knowledge on the effects of DOACs on coagulation assays, the effectiveness of fixed dosage with peak and trough concentrations, and the effects obesity may play on DOACs and coagulation assays, we aim to expand our understanding of DOAC interference. With this study, we are aiming to characterise the effects of rivaroxaban and apixaban on coagulation
assays as there are limited studies, especially with apixaban. Apixaban’s relationship with coagulation testing has only been studied by the conflicting Hillarp et al. (2014) (62) and Ratzinger et al. (2016) (56) papers. This study will provide valuable information on how to interpret apixaban coagulation results. By comparing the peak and the trough concentrations it will be possible to characterise their effects as well as determine the amount of variation between peak and trough levels. This would lay the foundations for future guidelines within emergency medicine regarding DOAC monitoring and would either support or discredit the use of the DRVVT assay as the 1st line test for LA whilst on peak or trough rivaroxaban and apixaban levels. Peak and trough samples are also useful for assessing obesity as a factor as studies have indicated that trough levels from fixed dosed obese patients may be ineffective for not only dabigatran but also apixaban and rivaroxaban (78,79). Characterising if there are additional statistical differences between weight with coagulation assay results would also be sought after as this area does not have any current published data. If additional undocumented effects between obese and non-obese patients are discovered, this study can be used to help prevent medical emergencies by improving management and monitoring of this high-risk group.

1.8 Statement of Aims

The DOAC influence on coagulation and thrombin generation assays still requires further research as there are contradictions in the literature, especially their impact on peak and trough levels. Whilst current guidelines and studies have reported similar discrepancies with DOACs affecting assays, there is a lack of consensus on how much they are affected and their final interpretations. There is also a lack of statistical information on the significance of obesity with patients taking DOACs, as many studies are now questioning the current recommendation of fixed dosage.
It is vital to have rigorous, accurate and reproducible methods to monitor DOACs efficacy and therapeutic effects, especially considering the current limitations of knowledge within this field. This study will be important to provide clinicians future recommendations on fixed dosages for obese patients experiencing thromboembolic disorders. Therefore, the further understanding of how these drugs relate to coagulation assays will allow for the improved management and monitoring of patients, especially obese, prescribed DOACs. With improved management, it can be expected to lead to a reduction of adverse effects associated with this class of pharmacotherapeutic intervention.

To gain further understanding within this field we hypothesise that the DOACs rivaroxaban and apixaban exert a dose dependent effect on coagulation parameters and thrombin generation.

To prove this hypothesis this study aims to;

1. Characterise and compare the effects of rivaroxaban and apixaban on routine and non-routine coagulation parameters.
2. Determine whether samples taken at trough levels have less interference on coagulation assays than at peak levels.
3. Determine the effects obesity has on coagulation parameters and its interferences on rivaroxaban and apixaban levels.
2. Materials:

2.1 Reagents and Suppliers

2.1.1 Blood Collection

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Catalogue No., Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic citrate tube, .109 M/3.2%</td>
<td>363095; Becton Dickinson PTY Ltd.</td>
</tr>
<tr>
<td>Plastic whole blood tube with spray-coated K2EDTA</td>
<td>367839; Becton Dickinson PTY Ltd.</td>
</tr>
<tr>
<td>BD SST tube with silica clot activator, polymer gel, silicone-coated interior</td>
<td>367954; Becton Dickinson PTY Ltd.</td>
</tr>
<tr>
<td>21-G x .75-in needle with 12-in tubing and luer adapter</td>
<td>367365; Becton Dickinson PTY Ltd.</td>
</tr>
<tr>
<td>Norma icon 3 Haematology Analyzer</td>
<td>Norma Diagnostika GmbH</td>
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</table>

2.1.2 Thrombin Generation Assay

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Catalogue No., Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA LowRC (Rc low conc. of phospholipid micelles containing rhTF in Tris-Hepes-NaCl Buffer )</td>
<td>5006224; Technoclone TGA GmbH</td>
</tr>
<tr>
<td>TGA Sub (1mmol Z-Gly-Arg-AMC)</td>
<td>5006237; Technoclone® TGA GmbH</td>
</tr>
<tr>
<td>TGA Reaction Buff (Tris-Hepes-NaCl Buffer)</td>
<td>5006370; Technoclone® TGA GmbH</td>
</tr>
<tr>
<td>TGA Control High (Human Plasma with increased thrombin generation, lyophilized)</td>
<td>5006322; Technoclone® TGA GmbH</td>
</tr>
<tr>
<td>TGA Control Low (Human Plasma with decreased thrombin generation, lyophilised)</td>
<td>5006332; Technoclone® TGA GmbH</td>
</tr>
<tr>
<td>25mL CaCl2 (25mmol/L)</td>
<td>5277017; Technoclone® TGA GmbH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equipment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro tube 1.5mL PP</td>
<td>72.692.005; Sarstedt,</td>
</tr>
</tbody>
</table>
2.1.3 TAT and PF1+2 ELISA

Reagents
Enzygnost® TAT micro ELISA Kit
Enzygnost® F 1+2 micro ELISA Kit

Equipment
ART ™ Pre-sterilized barrier pipette tips (10,20,200, 1000 uL)
Ceveron Alpha® with TGA analyser
Thermo shaker PHMP (V.5GW)
Multipette® (X)stream
Eppendorf Combitips advanced® (1mL, 10mL)
Ovation Pipette E8 25-1250 µL
VistaRak™ 1250 µL pipette tips
FLUOstar Omega microplate reader
FLUOstar Omega MARS Data Analysis Software (V3.10R6)

2.1.4 Sysmex CS-5100 Assays

Reagents
Thromborel® S Reagent
Dade® Actin® FSL Activated PTT Reagent
Thromboclotin® Reagent
Dade® Thrombin Reagent

Catalogue No., Suppliers
OWMGG15E0503 (971); ©Siemens AG
OPBDG03E0503 (1445); ©Siemens AG
OWMGG15E0503 (971); ©Siemens AG
OUHP47; ©Siemens AG
B4219-2; ©Siemens AG
2810; ©Siemens AG
B4233; ©Siemens AG

2140-05-HR, 2149-P05-HR, 2270,
2179-05-HR; Thermo Scientific
9820010; Technoclone® TGA GmbH

2140-05-HR, 2149-P05-HR, 2270,
2179-05-HR; Thermo Scientific
Grant-Bio, Keison Products
4986000831; Eppendorf
0030089430, 0030089464; Eppendorf
1160-1250; VISTALAB TECHNOLOGIES, INC.
4060-3004; VISTALAB TECHNOLOGIES, INC.
0415F0005B; BMG LABTECH GmbH
BMG LABTECH GmbH
Matthew Halliday

Innovance® D-Dimer
LA1 screening reagent/ LA2 Confirmation Reagent
Innovance® Free PS Ag
Berichrom® Protein C
Berichrom® Antithrombin III (A)
STA®- Rivaroxaban Calibrator
STA®- Rivaroxaban Control
STA®- Apixaban Calibrator
STA®- Apixaban Control

Equipment
ART ™ Pre-sterilized barrier pipette tips (10,20, 200,1000 uL)
Sysmex® CS-5100 System

2.2 Location of Suppliers
BMG LABTECH GmbH, Ortenberg, Germany
Becton Dickinson PTY Ltd., Franklin Lakes, USA
DIAGNOSTICA STAGO, Paris, France
Eppendorf, Hamburg, Germany
Keison Products, Chelmsford, England
Norma Diagnostika GmbH, Untertullnerbach, Austria
SARSTEDT AG & Co, Nümbrecht, Germany
©Siemens AG, Munich, Germany
Sysmex® Corporation, Kobe, Japan
Technocline GmbH, Vienna, Austria
Thermo Scientific, Waltham, USA
VISTALAB TECHNOLOGIES INC, Brewster, USA
3. Methods

3.1 Ethics

This project was approved by the Hollywood Private Hospital Research Ethics Committee on the 2nd of May 2017 (Reference Number: HPH408), and received reciprocal approval by the Murdoch University Human Ethics Committee (Reference Number: 2017/115) on the 2nd of June 2017.

3.2 Description of the Research Design:

This study was conducted with the recruitment of 40 VTE patients and 1 AF patient currently undergoing anticoagulant therapy with either rivaroxaban (20mg), or apixaban (2.5mg or 5mg) oral tablets at their routine haematologist appointments. Twenty healthy volunteers were also recruited to serve as a control group. The relevant medical history of all the patients were checked for internal influences such as factor/protein deficiencies. The medical histories of the patients included 9 cases of Factor V Leiden, 6 cases of increased Factor VIII, 2 with anti-B2GP1, 2 with Anticardiolipin antibodies present, 1 Prothrombin gene mutation, and a protein S deficiency.

The Inclusion Criteria includes;

- Identified individuals already being treated with anticoagulant therapy with rivaroxaban or apixaban for prevention or treatment of venous thromboembolism associated with non-valvular atrial fibrillation or previous venous thromboembolism.
- A small group (n=20) of healthy age and gender matched volunteers will also be included in this study.

Exclusion Criteria includes;

- Patients who are unable or unwilling to provide informed consent.
Patients on any other anticoagulant treatment (e.g. heparin, warfarin), or are currently taking aspirin and/or fish oils.

The participants provided written informed consent to the study for the collection of clinical and laboratory information, and to provide 16.2mL of blood (six 2.7ml citrate tubes) once if in the control cohort, or at trough and three hours later at peak for DOAC patients. A screening log was kept and the reasons documented for any non-participation from eligible subjects. The participant’s height and weight was measured and recorded and the BMI was calculated (Weight (kgs)/Height (cms)/Height (cms) *10000). Between the 2nd of June 2017 to the 25th of August 2017 61 participants were recruited consisting of 33 females and 28 males, with a median age and weight of 55 years and 75.8kg, and 57.5 years and 90.4kgs respectively. The median BMIs of males and females was 28.4 and 27.9 respectively. Participants were sorted into rivaroxaban, apixaban or control cohorts.

Patients undergoing apixaban treatment were prescribed with 2.5mg or 5mg oral tablets, so the apixaban cohort further split into 2.5 or 5mg sub-cohorts to represent the differences between the two doses. The rivaroxaban and apixaban cohorts were required to provide a blood sample immediately before their daily dosage time for the drug trough concentration, and then three hours later for the peak. The control cohort provided only the one sample. The ethnic background of the participants consisted of 93.6% Caucasian, 1.6% African and 4.8% Indian. The cohorts were also organised into weight sub-groups (Table 3.1).
Blood was collected into evacuated siliconized glass Vacutainer tubes (BD Vacutainer System, Plymouth, United Kingdom) containing 3.2% sodium citrate in a ratio of 1:9 with blood and centrifuged at 822 x g for 12 minutes, followed by repeat centrifugation of the separated plasma at 822 x g for 12 minutes. Plasma was aliquoted into 1.5ml polypropylene tubes.
(Eppendorf, Hamburg, Germany) and samples were frozen at -80°C until testing. Blood processing was completed within 30 minutes of collection.

3.3 Testing Methods.

3.3.1. Coagulation and Thrombophilia Panels.

The Prothrombin time (Thromborel® S), Activated Partial Thromboplastin Time (Dade® Actin® FSL Activated PTT Reagent), Thrombin clotting time (Thromboclotin®), Fibrinogen (Dade® Thrombin Reagent), D-Dimer (Innovance® D-Dimer), DRVVT (LA1 screening reagent/ LA2 Confirmation Reagent), Protein S (Innovance® Free PS Ag), Protein C (Berichrom® Protein C) and Antithrombin III (Berichrom® Antithrombin III (A)) assays and Liquid Anti-Xa (Stago, Paris, France) were prepared before loading within the Sysmex® CS-5100 analyser (Sysmex®)
Corporation, Kobe Japan) after samples were thawed in a 37°C water bath. Samples were loaded ten at a time into the Sysmex® CS-5100 analyser and automatically processed as described by Flieder et al. (2016) (80).

3.3.2. Thrombin Generation Assay:

The Thrombin Generation assay was performed using the Ceveron Alpha® with TGA analyser (Technoclone GmbH, Vienna, Austria) using TGA TechnoThrombin reagents (Technoclone GmbH, Vienna, Austria). TGA reagents were first prepared with the addition of 1mL ultrapure water to TGA LowRC, TGA Reaction Buffer, TGA Control High, TGA Control Low, and 3mL to the TGA Substrate and left for 10 minutes at 15-25°C. The reagents were scanned into the Ceveron analyser before up to 36 plasma samples were thawed in a 37°C water bath and loaded into the analyser. The Ceveron Alpha measures the complete thrombin formation and inhibition within plasma via the cleavage of a fluorogenic substrate by thrombin. The analyser records and measures the thrombin generation kinetics continuously to produce a thrombogram which is represented by the five parameters.

3.3.3. ELISA Assays

The Enzygnost thrombin/antithrombin complex micro, and Enzygnost prothrombin fragments 1+2 micro ELISA kits contain 96 well plates and employ reagents provided by Siemens (Siemens, Newark Germany). The Enzygnost prothrombin fragments 1+2 micro ELISA protocol included the preparation of reagents including the formation of; washing solution POD, Anti-Human Prothrombin/POD Conjugate Reagent, Chromogen working solution (protected from sunlight), Human prothrombin fragment F 1+2 standards containing 20,80,400 and 1200 pmol/L, and lyophilised human plasma as a positive control. Testing began by adding 50 μL of Tris buffer solution (100mM) followed by PF1+2 Standard / Sample to each well in duplicate, before agitation and incubation at 30 minutes at 37°C. All wells were aspirated and filled with...
wash Buffer (300μl/well) and aspirated again. Process was repeated once and 100 μL/well of conjugate solution was added before being covered with fresh adhesive foil and incubated for 15 minutes at 37°C. Sample wells were washed 3 times with Wash Buffer (300 μL/well) before Chromogen Buffer/Substrate (100 μL/well) was added. Wells were covered with fresh adhesive foil, and incubated for 15 minutes at 15-25°C, protected from light. 100 μL/well of Stop Solution (0.25 M sulphuric acid) was added and the plate was read at 450 nm absorbance within one hour using the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) and results were calculated using Omega Mars analysing software (BMG Labtech, Ortenberg, Germany).

The Enzygnost Thrombin/Anti thrombin complex ELISA protocol included the preparation of; washing solution POD, Anti-AT III/POD Conjugate Reagent, thrombin/anti-thrombin complex standards containing 2,6,20 and 60 μg/L, and lyophilised human plasma as a positive control. 50 μL of Tris buffer solution (100 mM) followed by 50 μL TAT standard/ plasma sample was added to each well in duplicate before agitation, covered with adhesive foil and incubated for 15 minutes at 37°C. All wells were aspirated and filled with Wash Buffer (300 μl/well) and aspirated. Washing process was repeated twice and conjugate solution (100 μL/well) was added before being covered with fresh adhesive foil and incubated for 15 minutes at 37°C. Working chromogen solution was then prepared during this incubation step by transferring 10 mL of buffer/substrate POD (hydrogen peroxide (0.3 g/L)) in a citrate buffer solution into a vial of chromogen POD (o-phenylenediamine dihydrochloride), and was shaken to dissolve. The plate was washed 3 times with wash buffer (300 μL/well) and freshly made chromogen Buffer/Substrate (100 μL/well) was added. Plate was then covered with fresh adhesive foil and incubated for 15 minutes at 15-25°C, protected from light. 100 μL/well of stop solution (0.5 N sulphuric acid) was added and the plate was read at 492 nm absorbance within one hour using
the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) and results were calculated using Omega Mars analysing software (BMG Labtech, Ortenberg, Germany).

3.4 Statistical Analysis:

The results of the assays were analysed using three different methods. The results were first analysed as a comparison of the peak DOAC cohort and the controls to determine if rivaroxaban or apixaban influence the various assays. This was statistically performed using a 2-sample unpaired t-test assuming equal variances using Microsoft excel analysis ToolPak software (Microsoft ver.1707). Apixaban and rivaroxaban cohorts peak and trough drug concentrations were then analysed using a paired two sample for means t-test. As the apixaban cohort consists of patients taking 2.5mg tablets or 5mg tablets, the cohort was split into sub-cohorts of peak and trough 2.5mg and 5mg for analysis purposes. The 2.5mg and 5mg apixaban sub-cohorts at both peak and trough were compared to the normal cohort using the 2-sample t-test assuming equal variance and a paired t-test when comparing peak and trough 2.5mg or 5mg apixaban results.

The peak rivaroxaban, apixaban, and control cohorts were also evaluated to establish whether there is a relationship between the results and the patient’s weight, via the use of the Kruskal Wallis test, also known as the single factor ANOVA method. The significance level for all statistical testing is $\alpha < 0.05$.

4. Results:

4.1 Effects of Peak and Trough Rivaroxaban and Apixaban Concentrations on Coagulation Parameters

The rivaroxaban cohort consisted of 20 patients prescribed a daily dosage of 20mg oral tablets for the treatment and/or prevention of VTE. The average concentration of rivaroxaban at peak
dose was 233.06ng/ml. The Apixaban cohort consisted of 21 patients currently prescribed either 2.5mg or 5mg oral tablets with the average concentration of 86.77ng/ml with peak 2.5mg apixaban, and 165.31 ng/ml with peak 5mg apixaban for the treatment and/or prevention of VTE, or atrial fibrillation.

4.1.1 Prothrombin Time

The Prothrombin time assay result was influenced by rivaroxaban as there was prolongations in clotting time between the rivaroxaban and control cohort (p<0.0001). The mean and range of the prothrombin time was also prolonged. When rivaroxaban trough and peak cohorts were analysed using a paired t-test the peak concentrations had significantly more impact on the assay than at trough (p<0.0001 Table 4.1). Comparison of the trough to the normal cohort was found to not have significant differences (p=0.73 Table 4.1).

The peak apixaban cohorts were not found to have a difference in prothrombin time (2.5mg: p=0.16, 5mg: p=0.87 Table 4.1). However, there were differences between peak and trough concentrations for both doses, as the peak prothrombin time was prolonged compared to the trough (2.5mg: p<0.001, 5mg: p<0.01 Table 4.1), but was still contained within the range of the controls.

<table>
<thead>
<tr>
<th>Table 4.1</th>
<th>The mean Prothrombin time, range and drug concentration for each cohort. p values for unpaired and paired t-tests are provided. Bold figures represent significance. * represents comparison of cohort to control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Mean Prothrombin Time (Seconds)</td>
<td>12.71</td>
</tr>
<tr>
<td>Mean Drug concentration (ng/ml)</td>
<td>-</td>
</tr>
<tr>
<td>Unpaired t-test</td>
<td>-</td>
</tr>
<tr>
<td>Paired t-test</td>
<td>-</td>
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</tbody>
</table>
4.2.2 Activated Partial Thromboplastin Time

The Activated Partial Thromboplastin time assay was found to be influenced by rivaroxaban at peak concentrations (p<0.001 Table 4.2). Comparison of the effects of peak and trough rivaroxaban levels described prolonged clotting times, with greater assay interference at peak levels than at trough (p<0.0001 Table 4.2). The 2.5mg and 5mg Apixaban cohorts did not considerably influence the APTT assay at peak concentrations or at trough time (Table 4.2). The comparison of influence between
peak and trough concentrations were not significantly different for the 2.5mg tablets, however there were differences between peak and trough 5mg tablets (p<0.001 Table 4.2).

**Table 4.2.** The mean Activated Partial Thromboplastin time, range and drug concentration for each cohort. $p$ values for unpaired and paired t-tests are provided. Bold figures represent significance. * represents comparison of cohort to control.

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<tr>
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<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
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</thead>
<tbody>
<tr>
<td><strong>Mean Activated Partial Thromboplastin Time (Seconds)</strong></td>
<td>28.49</td>
<td>32.78 *</td>
<td>27.57</td>
<td>26.68 *</td>
<td>26.29</td>
<td>27.72 *</td>
<td>26.49</td>
</tr>
<tr>
<td><strong>Mean Drug concentration (ng/ml)</strong></td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
<td>65.92</td>
</tr>
<tr>
<td><strong>Unpaired t-test</strong></td>
<td>-</td>
<td>0.00037</td>
<td>0.34368</td>
<td>0.183228</td>
<td>0.127573</td>
<td>0.507745</td>
<td>0.099797</td>
</tr>
<tr>
<td><strong>Paired t-test</strong></td>
<td>-</td>
<td>-</td>
<td>1.50E-09</td>
<td>-</td>
<td>0.35989</td>
<td>-</td>
<td>0.00016434</td>
</tr>
</tbody>
</table>

**Figures 4.2.** The Activated Partial Thromboplastin time for each cohort. Error bars represent mean with standard error of the mean. ** represents statistical significance $p<0.001$, *** represents statistical significance $p<0.001$. 
4.1.3 Thrombin Clotting Time

The thrombin clotting assay was not affected by the peak presence of rivaroxaban or apixaban. The trough concentrations also did not affect the assay in a significant way.

When peak and trough were compared there was no major changes between rivaroxaban and both apixaban cohorts (Table 4.3).

<table>
<thead>
<tr>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Thrombin clotting Time (seconds)</td>
<td>16.72</td>
<td>16.99 *</td>
<td>17.02</td>
<td>17.3 *</td>
<td>17.53</td>
<td>16.9 *</td>
</tr>
<tr>
<td>Mean Drug concentration (ng/ml)</td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
</tr>
<tr>
<td>Unpaired t-test</td>
<td>-</td>
<td>0.56243</td>
<td>0.53017</td>
<td>0.4248</td>
<td>0.20601</td>
<td>0.7229</td>
</tr>
<tr>
<td>Paired t-test</td>
<td>-</td>
<td>-</td>
<td>0.58478</td>
<td>-</td>
<td>0.41288</td>
<td>-</td>
</tr>
</tbody>
</table>

* represents comparison of cohort to control.
4.1.4 Fibrinogen

The fibrinogen assay was not affected by the peak presence of rivaroxaban or by the apixaban cohorts. The trough concentrations also did not affect the assay in a significant way (Table 4.4). When peak and trough were compared there was no significant differences between rivaroxaban (p=0.64 Table 4.4), and the 5mg apixaban cohorts (p=0.83 Table 4.4), but there were statistically significant differences between the 2.5mg cohort (p<0.05 Table 4.4).
**Table 4.4.** The mean fibrinogen level, range and drug concentration for each cohort. *p* values for unpaired and paired t-tests are provided. Bold figures represent significance.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Fibrinogen concentration (g/L)</td>
<td>4.2325</td>
<td>3.469</td>
<td>3.498</td>
<td>3.269</td>
<td>3.402</td>
<td>4.08</td>
<td>4.16</td>
</tr>
<tr>
<td>Range</td>
<td>0-10.16</td>
<td>1.75-4.78</td>
<td>1.63-5.36</td>
<td>1.74-4.8</td>
<td>1.86-4.94</td>
<td>2.22-5.95</td>
<td>2.15-6.17</td>
</tr>
<tr>
<td>Mean Drug concentration (ng/ml)</td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
<td>65.92</td>
</tr>
<tr>
<td>Unpaired t-test</td>
<td>-</td>
<td>0.28152</td>
<td>0.29698</td>
<td>0.32415</td>
<td>0.39444</td>
<td>0.87304</td>
<td>0.93821</td>
</tr>
<tr>
<td>Paired t-test (0.05)</td>
<td>-</td>
<td>-</td>
<td>0.64507</td>
<td>-</td>
<td>0.0267</td>
<td>-</td>
<td>0.83345</td>
</tr>
</tbody>
</table>

**Figures 4.4.** The fibrinogen level for each cohort. Error bars represent mean with standard error of the mean. *represents statistical significance *p*<0.05
4.1.5 D-Dimer

The D-Dimer assay was not affected by the peak or trough presence of rivaroxaban or apixaban. There were also no differences between the peak and the trough concentrations for rivaroxaban and both apixaban cohorts (Table 4.5).

| Table 4.5. The mean D-dimer level, range and drug concentration for each cohort. *p values for unpaired and paired t-tests are provided. * represents comparison of cohort to control |
|---|---|---|---|---|---|---|---|
| Cohort | Mean D-Dimer concentration (μg/mL) | Range | Mean Drug concentration (ng/ml) | Unpaired t-test | Paired t-test |
| Control | 0.303 | 0-0.64 | 234.94 | - | - |
| Peak 20mg Rivaroxaban | 0.359* | 0-1.41 | 0.429 | 0.07-0.43 | 0.70839 | 0.20271 |
| Trough 20mg Rivaroxaban | 0.262 | 0-1.86 | 0.254* | 0.07-0.45 | - | - |
| Peak 2.5mg Apixaban | 0.558* | 0-1.73 | 0.44982 | 0.07-0.43 | - | 0.39936 |
| Trough 2.5mg Apixaban | 0.582 | 0-1.84 | 0.4844 | 0.07-0.45 | - | 0.43955 |
| Peak 5mg Apixaban | 0.429 | 0-1.86 | 0.262 | 0.07-0.45 | - | 0.39936 |
| Trough 5mg Apixaban | 0.254* | 0-1.41 | 0.429 | 0.07-0.45 | - | - |

Figures 4.5. The D-Dimer level for each cohort. Error bars represent mean with standard error of the mean.
4.1.6 Dilute Russell Viper Venom Test

The DRVVT normalised ratio (LA screen ratio/LA confirm ratio) is significantly influenced by rivaroxaban at both peak (p<0.0001 Table 4.6) and at trough (p<0.001 Table 4.6) concentrations. The ratio increases when increasing amounts of rivaroxaban are present, with a maximum concentration of 446.04 ng/ml producing a ratio of 2.34. The difference between peak and trough rivaroxaban concentrations was also significant (p<0.0001 Table 4.6). The DRVVT normalised ratio was also influenced by both peak apixaban cohorts but to less than half the extent of rivaroxaban (2.5mg: p<0.05, 5mg: p<0.01 Table 4.6). The trough concentration of the 2.5mg apixaban was not found to be different than the controls (p=0.08 Table 4.6), but the 5mg trough concentration was (p<0.01 Table 4.6). It was also found that there were no notable differences between the peak and trough influences of apixaban for both 2.5 and 5mg doses (2.5mg: p=0.26, 5mg: p=0.36 Table 4.6).

If the DRVVT normalised ratio is over 1.25, it is recorded as positive for the presence of LA. The participants had not previously recorded the presence of LA before partaking in the study, so ratios over 1.25 are considered false positives. The results of the rivaroxaban cohort included false positives for the majority of the trough and all the peak samples. Apixaban was not as influential as rivaroxaban and included five false positive at peak apixaban concentration and four false positives within the trough concentration between both doses.
Table 4.6. The mean DRVVT normalised ratio, range and drug concentration for each cohort. * p values for unpaired and paired t-tests are provided. Bold represents significance. * represents comparison of cohort to control.
4.1.7 Protein S

The Free Protein S assay was not found to be notably influenced by the peak presence of rivaroxaban or 2.5 or 5mg apixaban dosage when compared to the controls. The troughs also were not remarkably different to the controls for rivaroxaban or both apixaban doses. The
peak and trough concentrations for both DOACs also did not differ in their influence on the assay (Table 4.7).

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Mean % Free Protein S present</th>
<th>Range</th>
<th>Mean Drug concentration (ng/ml)</th>
<th>Unpaired t-test</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>113.79</td>
<td>76.82-150</td>
<td>234.94</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20mg Rivaroxaban Trough</td>
<td>106.20 *</td>
<td>69.07-144.15</td>
<td>27.095</td>
<td>0.19434</td>
<td>0.25781</td>
</tr>
<tr>
<td>20mg Rivaroxaban Peak</td>
<td>102.26</td>
<td>51.92-152.59</td>
<td>86.77</td>
<td>0.10678</td>
<td>0.44311</td>
</tr>
<tr>
<td>2.5mg Apixaban Trough</td>
<td>105.51 *</td>
<td>51.9-159</td>
<td>31.924</td>
<td>0.32855</td>
<td>-</td>
</tr>
<tr>
<td>2.5mg Apixaban Peak</td>
<td>111.54</td>
<td>70.96-150</td>
<td>165.31</td>
<td>0.76257</td>
<td>-</td>
</tr>
<tr>
<td>5mg Apixaban Trough</td>
<td>104.25 *</td>
<td>67.03-141.48</td>
<td>65.92</td>
<td>0.18074</td>
<td>-</td>
</tr>
<tr>
<td>5mg Apixaban Peak</td>
<td>104.59</td>
<td>69.1-140.08</td>
<td>0.2556</td>
<td>0.2556</td>
<td>-</td>
</tr>
</tbody>
</table>

* represents comparison of cohort to control

**Figure 4.7.** The Percentage level of Free Protein S for each cohort. Error bars represent mean with standard error of the mean. Highlighted area is discussed further (5.1.2).
4.1.8 Protein C

The Protein C assay was found to not be notably influenced by the peak presence of rivaroxaban or 2.5 or 5mg apixaban dosage when compared to the controls. The troughs also were not remarkably different to the controls for rivaroxaban or both apixaban doses. The peak and trough concentrations for both DOACs also did not differ in their influence on the assay (Table 4.8).

<table>
<thead>
<tr>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean % Protein C present</strong></td>
<td>121.02</td>
<td>125 (*)</td>
<td>125.55</td>
<td>131.5 (*)</td>
<td>135.8</td>
<td>141.09 (*)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>60-182.03</td>
<td>100.43-151.78</td>
<td>95.73-155.36</td>
<td>71.34-191.66</td>
<td>72.65-199</td>
<td>92.48-189.7</td>
</tr>
<tr>
<td><strong>Mean Drug concentration (ng/ml)</strong></td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
<td>65.92</td>
</tr>
<tr>
<td><strong>Unpaired t-test</strong></td>
<td>0.59854</td>
<td>0.55426</td>
<td>0.3805</td>
<td>0.22639</td>
<td>0.07092</td>
<td>0.06682</td>
</tr>
<tr>
<td><strong>Paired t-test</strong></td>
<td>-</td>
<td>-</td>
<td>0.70072</td>
<td>-</td>
<td>0.06123</td>
<td>-</td>
</tr>
</tbody>
</table>

* represents comparison of cohort to control
4.1.9 Antithrombin III

The antithrombin III assay was not notably influenced by the presence of peak rivaroxaban or apixaban cohorts. Trough concentrations of both apixaban doses and rivaroxaban also did not influence the assay. There were also no statistically significant differences between the peak and trough concentrations for both DOACs (Table 4.9).

<table>
<thead>
<tr>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean AT concentration</td>
<td>113.10</td>
<td>109.22*</td>
<td>108.4</td>
<td>110.75*</td>
<td>113.82</td>
<td>109.9*</td>
</tr>
<tr>
<td>Range</td>
<td>93.12-133.08</td>
<td>88.11-130.18</td>
<td>89.19-127.62</td>
<td>81.06-140.44</td>
<td>76.64-151</td>
<td>90.74-129.08</td>
</tr>
<tr>
<td>Mean Drug concentration (ng/ml)</td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
</tr>
<tr>
<td>Unpaired t-test</td>
<td>-</td>
<td>0.23385</td>
<td>0.13767</td>
<td>0.60949</td>
<td>0.89117</td>
<td>0.39460</td>
</tr>
<tr>
<td>Paired t-test</td>
<td>-</td>
<td>0.58579</td>
<td>0.58579</td>
<td>0.15805</td>
<td>0.15805</td>
<td>0.6348</td>
</tr>
</tbody>
</table>

Figure 4.8. The Percentage level of protein C for each cohort. Error bars represent mean with standard error of the mean.

Table 4.9. The mean percentage of ATIII, range and drug concentration for each cohort. p values for unpaired and paired t-tests are provided. * represents comparison of cohort to control.
Antithrombin III

Healthy

20mg Rivaroxaban Trough
20mg Rivaroxaban Peak
2.5mg Apixaban Trough
2.5mg Apixaban Peak
5mg Apixaban Trough
5mg Apixaban Peak

0
80
100
120
140
160

Cohorts

Antithrombin III (%)

Figure 4.9. The Percentage level of Antithrombin III for each cohort. Error bars represent mean with standard error of the mean.

4.1.10 Thrombin/Antithrombin complex

The Thrombin/Antithrombin (TAT) complex assay was found to not be notably influenced by the peak presence of rivaroxaban or 2.5 or 5mg apixaban dosage when compared to the controls. The troughs also were not remarkably different to the controls for rivaroxaban or both apixaban doses. The peak and trough concentrations for both DOACs also did not differ in their influence on the assay (Table 4.10).

Table 4.10. The mean percentage of TAT, range and drug concentration for each cohort. $p$ values for unpaired and paired $t$-tests are provided. * represents comparison of cohort to control
Figure 4.10. The quantity of Thrombin-Antithrombin complexes for each cohort. Error bars represent mean with standard error of the mean.
4.1.11 Prothrombin Fragment 1+2

The prothrombin fragment 1+2 (PF1+2) assay was found to not be notably influenced by the peak presence of rivaroxaban or 2.5 or 5mg apixaban dosage when compared to the controls. The troughs also were not remarkably different to the controls for rivaroxaban or both apixaban doses. The peak and trough concentrations for both DOACs also did not differ in their influence on the assay (Table 4.11).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean PF1+2</strong></td>
<td>160.87</td>
<td>191.39*</td>
<td>203.2</td>
<td>164.2*</td>
<td>185.53</td>
<td>173.07*</td>
<td>191.8</td>
</tr>
<tr>
<td><strong>Mean Drug</strong></td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
<td>65.92</td>
<td></td>
</tr>
<tr>
<td><strong>concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(pmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unpaired t-test</strong></td>
<td>0.23983</td>
<td>0.12788</td>
<td>0.892</td>
<td>0.43137</td>
<td>0.56751</td>
<td>0.16405</td>
<td></td>
</tr>
<tr>
<td><strong>Paired t-test</strong></td>
<td>0.39793</td>
<td>0.39793</td>
<td>0.33149</td>
<td>0.33149</td>
<td>0.19351</td>
<td>0.19351</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.11. The mean percentage of PF1+2, range and drug concentration for each cohort. *p values for unpaired and paired t-tests are provided. * represents comparison of cohort to control.
4.1.12 Thrombin Generation Assay

The thrombin generation assay is recorded as five interlinked parameters which provide a model of a patient’s overall haemostatic potential.

**Lag Time:**

The Lag time was found to be prolonged with the presence of peak concentrations rivaroxaban when compared to the healthy controls (p<0.001 Table 4.12). The apixaban cohorts did not produce a difference between the peak concentrations results and the healthy controls (2.5mg: p=0.49, 5mg: p=0.58 Table 4.12). The trough concentrations of both rivaroxaban or apixaban did not affect the assay significantly (Table 4.12). There was a marked increase in lag time for the peak concentrations.
compared to the trough concentration for rivaroxaban \((p<0.01 \text{ Table 4.12})\), but this was not seen for both apixaban doses \((2.5\text{mg}: p=0.14, 5\text{mg}: p=0.34 \text{ Table 4.12})\).

**Table 4.12.** The mean lag time, range and drug concentration of the TGA for each cohort. \(P\) values for unpaired and paired \(t\)-tests are provided. Bold represents significance. * represents comparison of cohort to control.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Mean Lag Time (Minutes)</th>
<th>Range</th>
<th>Mean Drug concentration (ng/ml)</th>
<th>Unpaired t-test</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.46</td>
<td>3.22-5.7</td>
<td>234.94</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20mg Rivaroxaban Peak</td>
<td>5.55*</td>
<td>1.4-9.6</td>
<td>27.095</td>
<td>0.00055</td>
<td>-</td>
</tr>
<tr>
<td>20mg Rivaroxaban Trough</td>
<td>4.96</td>
<td>2.6-7.31</td>
<td>86.77</td>
<td>0.10437</td>
<td>0.00290</td>
</tr>
<tr>
<td>2.5mg Apixaban Peak</td>
<td>4.69*</td>
<td>2.3-7</td>
<td>31.924</td>
<td>0.49786</td>
<td>0.1458</td>
</tr>
<tr>
<td>2.5mg Apixaban Trough</td>
<td>4.35</td>
<td>3-5.65</td>
<td>165.31</td>
<td>0.64073</td>
<td>-</td>
</tr>
<tr>
<td>5mg Apixaban Peak</td>
<td>4.6*</td>
<td>3-6.2</td>
<td>65.92</td>
<td>0.58183</td>
<td>-</td>
</tr>
<tr>
<td>5mg Apixaban Trough</td>
<td>4.83</td>
<td>2.5-7.2</td>
<td>2.5-6.2</td>
<td>0.25549</td>
<td>0.3417</td>
</tr>
</tbody>
</table>

**Figures 4.12.** The TGA lag Time for each cohort. Error bars represent mean with standard error of the mean. ** represents statistical significance \(p<0.01\), *** represents statistical significance \(p<0.001\).
**Time to Peak:**

The Time to peak (tPeak) was found to be prolonged with the presence of peak rivaroxaban concentrations when compared to the healthy controls (p<0.0001 Table 4.13). The 5mg apixaban cohort was not found to be prolonged (p=0.93 Table 4.13), but the peak 2.5mg concentration result has a notable prolongation when compared to the healthy controls (p<0.05 Table 4.13). The trough concentrations of rivaroxaban also displayed a prolongation for tPeak (p<0.01 Table 4.13). The trough apixaban doses did not affect the assay significantly (2.5mg: p=0.16, 5mg: p=0.5 Table 4.13). There was a marked increase for the time to peak for the peak concentrations compared to the trough concentration for rivaroxaban (p<0.0001 Table 4.13), but this was not seen for both apixaban doses (2.5mg: p=0.07, 5mg: p=0.53 Table 4.13).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Time to Peak (minutes)</td>
<td>10.98</td>
<td>19.23*</td>
<td>15.93</td>
<td>15.31*</td>
<td>12.93</td>
<td>10.9*</td>
<td>11.88</td>
</tr>
<tr>
<td>Range</td>
<td>5.97-16</td>
<td>1.48-37</td>
<td>2.45-29.41</td>
<td>0.18-30.4</td>
<td>2.95-22.9</td>
<td>4.14-17.65</td>
<td>2.1-21.66</td>
</tr>
<tr>
<td>Mean Drug concentration (ng/ml)</td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
<td>65.92</td>
</tr>
<tr>
<td>Unpaired t-test</td>
<td>-</td>
<td>1.6E-07</td>
<td><strong>0.00385</strong></td>
<td><strong>0.02624</strong></td>
<td>0.16264</td>
<td>0.93692</td>
<td>0.50216</td>
</tr>
<tr>
<td>Paired t-test</td>
<td>-</td>
<td>-</td>
<td><strong>8.838E-07</strong></td>
<td>-</td>
<td>0.0765</td>
<td>-</td>
<td>0.53791</td>
</tr>
</tbody>
</table>

*Table 4.13. The mean Time to peak, range and drug concentration of the TGA for each cohort. p values for unpaired and paired t-tests are provided. Bold represents significance. * represents comparison of cohort to control.
Peak Height:

The Peak Height (PH) was found to be depressed with the presence of peak rivaroxaban concentrations when compared to the healthy controls ($p<0.0001$ Table 4.14). The 5mg apixaban cohort was not found to be different ($p=0.12$ Table 4.14), but the peak 2.5mg concentration result has a notable decrease when compared to the healthy controls ($p<0.05$ Table 4.14). The trough concentrations of rivaroxaban and
apixaban were not notably decreased for the PH (Table 4.14). There was a marked
decrease in the PH for the peak concentration compared to the trough concentration
for rivaroxaban (p<0.001 Table 4.14), but this was not seen for either apixaban doses
(2.5mg: p=0.1, 5mg: p=0.73 Table 4.14).

<table>
<thead>
<tr>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Peak Height (nM)</td>
<td>139.71</td>
<td>72.7*</td>
<td>101.58</td>
<td>71.97*</td>
<td>93</td>
<td>95.83*</td>
</tr>
<tr>
<td>Range</td>
<td>0-311.8</td>
<td>0-229.68</td>
<td>0-276.8</td>
<td>0-184.05</td>
<td>0-264.6</td>
<td>9.65-182</td>
</tr>
<tr>
<td>Mean Drug concentration (ng/ml)</td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
</tr>
<tr>
<td>Unpaired t-test</td>
<td>-</td>
<td>0.00001</td>
<td>0.17293</td>
<td>0.03236</td>
<td>0.17156</td>
<td>0.12546</td>
</tr>
<tr>
<td>Paired t-test</td>
<td>-</td>
<td>-</td>
<td>0.000471</td>
<td>-</td>
<td>0.10398</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.14. The mean Peak height, range and drug concentration of the TGA for each cohort. p values for unpaired and paired t-tests are provided. Bold represents significance. * represents comparison of cohort to control.
Endogenous Thrombin Potential:

The ETP was found to be significantly reduced with the presence of peak rivaroxaban concentrations when compared to the healthy controls ($p<0.001$ Table 4.15). Both apixaban doses were not found to be different from the healthy controls. The trough concentrations of rivaroxaban and apixaban were not notably decreased for the ETP (Table 4.15). There was a major reduction in the ETP for the peak concentration compared to the trough concentration for rivaroxaban ($p<0.0001$ Table 4.15), but this was not seen for either apixaban doses (2.5mg: $p=0.67$, 5mg: $p=0.36$ Table 4.15).

Table 4.15. The mean ETP, range and drug concentration of the TGA for each cohort. $p$ values for unpaired and paired t-tests are provided. Bold represents significance. * represents comparison of cohort to control.

<table>
<thead>
<tr>
<th>Control</th>
<th>Peak 20mg Rivaroxaban</th>
<th>Trough 20mg Rivaroxaban</th>
<th>Peak 2.5mg Apixaban</th>
<th>Trough 2.5mg Apixaban</th>
<th>Peak 5mg Apixaban</th>
<th>Trough 5mg Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ETP (nM/min)</td>
<td>1685.51</td>
<td>1282.73*</td>
<td>1583.18</td>
<td>1384.67*</td>
<td>1442.85</td>
<td>1753.52*</td>
</tr>
<tr>
<td>Range</td>
<td>526.3-2844.7</td>
<td>0-2644</td>
<td>317.86-2848.49</td>
<td>397.8-2371.5</td>
<td>458.4-2427.3</td>
<td>720.7-2786.33</td>
</tr>
<tr>
<td>Mean Drug concentration (ng/ml)</td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
</tr>
<tr>
<td>t-test</td>
<td>-</td>
<td><strong>0.0001</strong></td>
<td>0.59686</td>
<td>0.17140</td>
<td>0.26685</td>
<td>0.74804</td>
</tr>
<tr>
<td>Paired t-test</td>
<td>-</td>
<td>-</td>
<td><strong>9.362E-08</strong></td>
<td>-</td>
<td>0.67033</td>
<td>-</td>
</tr>
</tbody>
</table>
The velocity Index (VI) was found to be reduced with the presence of peak rivaroxaban concentrations when compared to the healthy controls (p<0.001 Table 4.16). Both apixaban doses were not found to be different from the healthy controls. The trough concentrations of rivaroxaban and apixaban were not notably decreased for the VI (Table 4.16). There was a notable difference in the VI for the peak concentration.
compared to the trough concentration for rivaroxaban (p<0.01 Table 4.16), but this was not seen with the apixaban doses (2.5mg: p=0.14, 5mg: p=0.83 Table 4.16).

Table 4.16. The mean VI, range and drug concentration of the TGA for each cohort. p values for unpaired and paired t-tests are provided. Bold represents significance. * represents comparison of cohort to control

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Peak 20mg</th>
<th>Trough 20mg</th>
<th>Peak 2.5mg</th>
<th>Trough 2.5mg</th>
<th>Peak 5mg</th>
<th>Trough 5mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Velocity Index (nM/Min)</strong></td>
<td>29.47</td>
<td>11.86*</td>
<td>18.325</td>
<td>14.13*</td>
<td>20.36</td>
<td>19.3*</td>
<td>20.63</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>0-88.67</td>
<td>0-54.3</td>
<td>0-67.75</td>
<td>0-50.67</td>
<td>0-78.4</td>
<td>0-44.34</td>
<td>0-75.26</td>
</tr>
<tr>
<td><strong>Mean Drug concentration (ng/ml)</strong></td>
<td>-</td>
<td>234.94</td>
<td>27.095</td>
<td>86.77</td>
<td>31.924</td>
<td>165.31</td>
<td>65.92</td>
</tr>
<tr>
<td><strong>t-test</strong></td>
<td>-</td>
<td>0.00047</td>
<td>0.20373</td>
<td>0.14590</td>
<td>0.43044</td>
<td>0.28828</td>
<td>0.42028</td>
</tr>
<tr>
<td><strong>Paired t-test</strong></td>
<td>-</td>
<td>-</td>
<td>0.00613</td>
<td>-</td>
<td>0.14434</td>
<td>-</td>
<td>0.8396</td>
</tr>
</tbody>
</table>

Figure 4.16. The VI for each cohort. Error bars represent mean with standard error of the mean., ** represents statistical significance p<0.001, *** represents statistical significance p<0.001
4.2 The Influences and Interferences of Obesity on Coagulation Assays

The BMI was calculated for each participant (Weight (kgs)/Height (cms)/Height (cms) *10000) and the results were split into four categories which includes healthy weight, overweight, obese I and obese II/III. The Kruskal Wallis test, or single factor ANOVA was employed to evaluate if there were significant differences (\(\alpha=0.05\)) between the weight groups at peak concentration. The obese II and obese III categories were grouped together for the analysis as both cohorts did not contain adequate sample numbers for individual analysis. As the apixaban 2.5mg and 5mg doses contained too little samples for statistical purposes, both apixaban doses were compared to act as a single apixaban cohort.

**Tables 4.17.** The \(p\) values from the Kruskal Wallis tests comparing the cohorts BMI categories with each assay and each TGA parameter. Bold numbers are significantly different between weight groups (\(p<0.05\)).

<table>
<thead>
<tr>
<th>Coagulation Assays</th>
<th>PT</th>
<th>APTT</th>
<th>Fibrinogen</th>
<th>TCT</th>
<th>D-Dimers</th>
<th>ATIII</th>
<th>Protein S</th>
<th>Protein C</th>
<th>DRVVT</th>
<th>TAT</th>
<th>PF1+2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0.5191</td>
<td>0.2392</td>
<td>0.4224</td>
<td>0.3053</td>
<td>0.3679</td>
<td>0.4302</td>
<td><strong>0.0080</strong></td>
<td>0.3187</td>
<td>0.8027</td>
<td>0.1081</td>
<td>0.4171</td>
</tr>
<tr>
<td>Rivaroxaban</td>
<td>0.2668</td>
<td><strong>0.0099</strong></td>
<td>0.7185</td>
<td>0.3165</td>
<td>0.3882</td>
<td>0.4665</td>
<td>0.2821</td>
<td>0.3962</td>
<td>0.7767</td>
<td>0.4402</td>
<td>0.4088</td>
</tr>
<tr>
<td>Apixaban</td>
<td>0.7952</td>
<td>0.2047</td>
<td>0.5514</td>
<td>0.1158</td>
<td>0.2839</td>
<td>0.3274</td>
<td>0.8159</td>
<td>0.2600</td>
<td>0.2639</td>
<td>0.2495</td>
<td>0.1964</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TGA</th>
<th>Lag Time</th>
<th>Time to Peak</th>
<th>Peak Height</th>
<th>ETP</th>
<th>Velocity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0.5050</td>
<td>0.6646</td>
<td>0.4860</td>
<td>0.9086</td>
<td>0.2919</td>
</tr>
<tr>
<td>Rivaroxaban</td>
<td>0.6259</td>
<td>0.4077</td>
<td>0.4405</td>
<td>0.2044</td>
<td>0.5738</td>
</tr>
<tr>
<td>Apixaban</td>
<td>0.7888</td>
<td>0.2842</td>
<td>0.5873</td>
<td>0.6218</td>
<td>0.6320</td>
</tr>
</tbody>
</table>

The Kruskal Wallis tests found there was significant differences between weight groups for the APTT assay when patients underwent rivaroxaban therapy (\(p<0.01\) Tables 4.17),
and for the free protein S assay for the healthy controls (p<0.01 Tables 4.17). The apixaban cohort and majority of the rivaroxaban and healthy controls did not measure a significant difference between the weight groups for the other assays, including all parameters of the TGA.

![Rivaroxaban APTT compared with Cohort BMI](image)

**Figure 4.13.** The Mean APTT level per each weight category between Healthy controls and rivaroxaban. Error bars represent standard error of the mean.

![Protein S levels compared with control BMI](image)

**Figure 4.14.** The mean Protein S level per each weight category for the healthy cohort. n= Number of participants within each BMI group. Error bars represent standard error of the mean.
5. Discussion:

5.1 Effects of Rivaroxaban and Apixaban on Coagulation Assays

This study hypothesised that the DOACs rivaroxaban and apixaban exert a dose dependent effect on coagulation parameters. To validate or refute this claim we tested three aims which included; characterising and comparing the effects of rivaroxaban and apixaban on routine coagulation parameters, determining whether samples taken at trough levels have less interference than at peak levels, and determining the effects obesity has on coagulation parameters and its interferences on FXa inhibitor treated samples. The strength of this study is that we have matching real world patients instead of the commonly used normal pooled spiked plasma samples, or in silico simulations as used in previous studies. Our study has been effective as we confirm that rivaroxaban and apixaban revealed statistically significant differences for the PT, APTT, DRVVT, fibrinogen (2.5mg apixaban), and TGA assays. There were differences in the magnitude of influence from rivaroxaban as peak results were more potent than trough doses for the PT, APTT, DRVVT and TG assays. There were also significant differences between the influence of trough apixaban concentration to peak with the PT, DRVVT, TG assays at 2.5mg and 5mg doses, APTT assay at 5mg concentrations only and the fibrinogen assay at 2.5mg only. Finally, the influence of obesity on DOAC patient samples when measured was found to be non-significant with obesity exerting no effects on these parameters for the apixaban, rivaroxaban (apart from the APTT assay), and the controls excluding the free PS assay.

5.1.1 DOAC Effects on Coagulation Panel.

The prothrombin time assay is routinely used within a diagnostic laboratory for the monitoring of VKA and has been reported to be prolonged by the presence of rivaroxaban in a linear concentration dependent manner, depending on the thromboplastin reagents, instruments,
and data analysis used (81-83). Earlier papers have reported that rivaroxaban has predictable, dose dependent pharmacokinetics which can be assessed using the prothrombin time, but not by the common international normalised ratio (INR) as the INR was developed specifically for VKAs (82). Our results found that peak levels (234.94 ng/ml) of rivaroxaban significantly prolongs the prothrombin time, which is in conjunction with the literature. It has been suggested that rivaroxaban may interfere with PT-based factor activity assays (FVII, FX, FV, and FII) which leads to decrease factor levels and a prolonged prothrombin time with increasing rivaroxaban concentration (84,85,86).

Apixaban has not shown significant differences between peak concentration (2.5mg: 86.77ng/ml 5mg: 165.31ng/ml) samples and the controls, but there was an effect on the assay as significant differences in PT between peak and trough apixaban doses were reported. These differences are found to be in conjugation with other studies which state that apixaban does produce an increase in clotting times, but such a small influence that the PT would still be regarded as normal at peak levels of apixaban (87,88). The reason for this prolongation is due to apixaban having a minor influence with the same PT based clotting factors as rivaroxaban due to the similar pharmacokinetics and pharmacodynamics. However, the PT assay is not sensitive enough to detect peak levels of apixaban (89) from the controls which support our findings.

The APTT assay is affected by the presence of rivaroxaban as there was significantly prolonged clotting time at peak concentrations when compared to the healthy controls. There were also substantial differences between the peak and trough rivaroxaban clotting times, but the trough was not found to be different to the controls which would suggest that only higher concentrations adequately prolong the assay. This is reflected within the literature with research stating that rivaroxaban caused prolonged APTT results when at peak dosage (58). Interestingly, the peak apixaban cohorts did not produce a considerable influence on the assay.
when compared to the normal, but there was an effect present as there was a notable difference between peak and trough concentrations for the 5mg sub-cohort. This difference would suggest that apixaban affected the APTT assay, but at such a small influence that the APTT result can be regarded as normal at peak levels, similar to apixaban’s effect on the PT assay (87,88).

The TCT, fibrinogen and D-dimer assays were not influenced by the presence of the direct FXa inhibitors when peak and trough concentrations were compared to the control cohort. The D-dimer and TCT assays were both unaffected by the presence of either rivaroxaban or apixaban concentrations. This is seen in the literature as both DOACs have failed to prolong the TCT or the D-dimer assays (85, 90, 91). The literature has also stated that the Clauss fibrinogen assay is not affected by the presence of rivaroxaban which is consistent with our findings (90), but there is an absence of data regarding apixaban’s effect on the fibrinogen assay at trough and peak. Apixaban has not been found to be an influence on the Clauss fibrinogen assay when compared to the normal controls in this study, but there was a minor decrease in fibrinogen levels for the 2.5mg apixaban peak sub-cohort compared to the trough sub-cohort. This difference could be due to the low sample number per sub-cohort (n=11) and/or the influence of one patient diagnosed with dysfibrinogenaemia in their medical history. Whilst there was a statistical difference with the 2.5mg apixaban cohort it does not equate to a clinically relevant difference and so it can be concluded that the fibrinogen assay is not significantly affected by apixaban or rivaroxaban.

5.1.2 Thrombophilia Panel

The thrombophilia panel consists of the antithrombin III, free protein S, protein C, and the DRVVT assays which measure the presence of an individual’s anticoagulant functions. The ATIII (Berichrom® Antithrombin III (A)), PS (Innovance® Free PS Ag), and PC (Berichrom® Protein C)
assays were not found to be influenced by any means by the presence of rivaroxaban or apixaban at peak or trough concentrations within this study.

A study by Gosslin et al. (2016) regarding free PS assays has stated that the trough levels of rivaroxaban (35ng/ml) and peak doses of apixaban (471ng/ml) leads to increased PS expression to clot based free PS assays using StaClot PS reagents (58). Another study by Mani et al. (2012) found that there were significant differences using free PS clotting assays, but did not find statistical significance using an immune-turbidimetric method or reagents (90). Our results are in line with this finding as the free PS assay used was an immune-turbidimetric method with the use of two different monoclonal antibodies for free PS, not a clotting based method. The immune-turbidimetric method is not found to be affected by rivaroxaban or apixaban, which would suggest that rivaroxaban and apixaban does not directly affect free PS. There was however an interesting observation of the free PS assay results which contained three patients with unusually low free PS concentration in the rivaroxaban trough cohort. Two of these patients experienced a tremendous rise in their free PS levels at peak rivaroxaban concentration. The third patient was diagnosed with a protein S deficiency, anticardiolipin, and B2 glycoproteins and predictably did not increase their free PS levels at peak. The causation of this rise in free PS levels is not known, but it can be due to three potential causes. The first would the possibility of a pipetting error for the assay that caused a low free PS to be recorded in the samples. This would be supported by the drastic difference between peak and trough PS concentrations. Weight could also be a factor as obesity does interfere with free protein S levels (92), but both patients measured healthy BMIs. The final cause could be the effect of rivaroxaban supporting free protein S generation, or helping to reduce PS inhibition, by inhibiting thrombin generation. Reduced coagulation could place less strain on the production of free PS which could lead to increased levels of the natural anticoagulant. This could lead to patients with free PS deficiencies gaining normal PS levels during rivaroxaban treatment.
However, the TAT and PF1+2 results for these two patients are not abnormal which does not suggest DOAC influences, so these results need to be confirmed as time restraints prevented further investigation for this study.

The protein C assay is similarly reported as the same as the free PS assay, as peak levels of rivaroxaban (250ng/ml) and apixaban (>750ng/ml) caused falsely elevated clot based PC assays (58). However, both Gosslin et al. and Mani et al. studies have employed chromogenic methods for the PC assay and have reported no effects on the results by rivaroxaban or apixaban at peak or trough concentrations (58,90). Our results have also found no notable differences between the rivaroxaban or apixaban cohorts with the healthy controls regarding the PC assay.

The ATIII assay was not found to be influenced by the presence of rivaroxaban or apixaban using the Berichrom ATIII assay by this study, but this assay has been reported to be falsely increased by both apixaban and rivaroxaban (58). Another study that compared three various ATIII reagents with rivaroxaban however, stated that the Berichrom assay is not affected by the presence of rivaroxaban (88). The article states that ATIII assays can only be influenced by rivaroxaban if the type enzyme used for the reaction is susceptible to anti-Xa influence. Reagents using bovine thrombin, such as the Berichrom assay, or human thrombin are unaffected, but assays using FXa-based amidolytic activity will be inhibited in a dose-dependent manner by rivaroxaban (91). It was suggested by Hillarp et al. (2011) that rivaroxaban may raise the activity of ATIII during the screening of the thrombophilia panel, which may result in a missed diagnosis of inherited thrombophilia if the patient is on rivaroxaban therapy (91). Rivaroxaban patient samples undergoing ATIII assays need to be taken cautiously as different results will be generated based on the reagents used. However, using a thrombin-based assay over a FXa assay would lead to a reliable measurement of ATIII for rivaroxaban treated samples, as found by this study.
Apixaban may be used along the same principals as rivaroxaban due to their matching similarities but most of the encompassing studies on ATIII assays have not looked at the effects of apixaban in their analysis, and requires further data for validation. Our study however had found no effect on the ATIII assay by peak or trough concentrations of 2.5mg or 5mg tablets of apixaban, so it can be suggested that apixaban does not directly affect ATIII.

Whilst the PC, free PS and ATIII assays have not shown notable differences between the controls and drug cohorts at peak or trough, it can be noted that some assays will be influenced based on the method and reagents used. For example, the clotting methods that rely on activating factors II, V, VII, X, VIII, IX, XI, or XII have been found to be mildly influenced by rivaroxaban, which would in turn manipulate the result of the assay (90). The use of direct immune-turbiditic or chromogenic methods however are not affected which suggests that the PC, free PS and ATIII proteins are not directly affected by the FXa-inhibitors rivaroxaban and apixaban.

The DRVVT assay was found to be heavily influenced by the presence of rivaroxaban at both peak and trough concentration with the normalised DRVVT ratio increasing with rising drug concentration. This result is well documented within the literature as the DRVVT ratio is reliably increased from rivaroxaban (59,60,61,90,93,94). The increase in the DRVVT ratio has led to the prevalence of false positives for the presence of LA at peak concentrations which would be lead to the false diagnosis of LA in a LA negative patient. The earlier literature suggests using the DRVVT for trough samples as an acceptable procedure to test for LA for a patient on DOACs, but we have found that trough concentrations of rivaroxaban greatly influence the assay, leading to false positives, a result that is mirrored in only recent literature (56).
Apixaban has been shown to influence the DRVVT at peak concentrations for the 2.5mg and 5mg sub-cohorts, and at trough for the 5mg sub-cohort. Whilst there was a substantial effect, it is less than half the effect of rivaroxaban. Apixaban’s potency is displayed in our study as the lowest apixaban concentration, the 2.5mg trough sub-cohort, was not notably different when compared to the healthy controls. This result has been supported by the Hillarp et al. (2014) study which has shown that apixaban does affect the DRVVT assay at high concentrations, but has suggested that since the overall influence is smaller than rivaroxaban, apixaban cannot cause false positive results (62). From our study, we have recorded that apixaban peak levels reached past the DRVVT positive ratio threshold of 1.25 for 5 apixaban samples at peak and 4 at trough. Our results would suggest that apixaban does contain the potential to cause false positive results using the DRVVT assay. The Ratzinger et al. (2016) study on trough apixaban influences also reported false positives at 150ng/ml which further reinforced our results (56).

### 5.1.3 Non-Routine Assays (TAT, PF1+2, and TGA)

The thrombin/antithrombin complex and prothrombin fragments 1+2 ELISA assays quantitate the concentration of each molecule present within citrated plasma. The results have reported that rivaroxaban and apixaban did not influence these assays when compared to the controls. This is surprising as DOACs binding to FXa decreases the quantity of prothrombin converting into thrombin, which would in turn decrease the levels of TAT and PF1+2 produced within the system, but this reduction is not seen. The literature is scarce on information on the effects of rivaroxaban on these assays with three studies stating the effects on the TAT assay and two on the PF1+2. Two studies stated that the TAT assay is not significantly increased by the presence of rivaroxaban with patients with heart failure (95,96). However, a recent study by Horinaka et al. (2017) stated that 15mg rivaroxaban has caused a significant decrease in the amount of PF1+2 and TAT found in patient samples (97).
It should also be noted that the TAT assay in our experiment contains noticeable outliers in both the rivaroxaban and apixaban cohorts, but this burst of TAT may be due to TAT activation caused during sample collection and/or processing (98). There are also patients in both cohorts that have Factor V Leiden in their medical histories that would have affected the assay by increasing TAT levels (99) and produced the outliers.

Another study has stated that rivaroxaban patient’s PF1+2 results are significantly higher when compared to warfarin treated patients, but interestingly does not differ in its influence from peak to trough concentrations (100). It has been suggested that the reason for the lack of difference between peak and trough may be the short half-life of the PF1+2 fragment (90 minutes), or the influence of other anticoagulants such as PC, PS, AT, or TFPI (100). As our study enlisted patients already on rivaroxaban therapy, the TAT and PF1+2 concentration of the patients before rivaroxaban therapy, or on warfarin, was unknown and would require a suitable follow up trial. We do however have the difference between peak and trough doses which was found to not be significantly different, as reinforced in the literature (100), and the comparisons of peak with the control cohorts which was also not significantly different.

Apixaban was found to not cause a notable difference in TAT between the normal cohort at peak or trough in our findings, which is supported by a study by Nagao et al. (2017) (96). Apixaban also did not cause a major difference for the PF1+2 assay at peak or trough for both doses compared to the controls, or have differences between peak and trough. There are currently no other accounts of apixaban’s influence on the PF1+2 so the results should to be validated.

The five parameters of the TGA were found to all be influenced by rivaroxaban, with apixaban also influencing the tPeak and PH. The rivaroxaban cohort at peak concentration prolonged the lag time and tPeak, decreased the ETP and PH, and has slowed the thrombin generation
process by decreasing the VI. It can be shown as well that the patients experience a considerably larger effect on their TG at peak than at the trough, suggesting the effects of rivaroxaban on the TGA is dose dependent. The literature supports this as a study found similar prolongations and decreases with the TGA parameters using the CAT analyser with increasing doses (101).

Apixaban in this study has also shown to affect the TGA assay but not to the same extent to rivaroxaban. The drug produced a noticeable difference in PH and tPeak parameters for the peak 2.5mg tablets when compared to the controls, but not for the 5mg apixaban peak tablets. This is unexpected as logic would dictate that the higher the concentration of apixaban, the higher the influence on the assay. The peak 5mg apixaban cohort contained a longer tPeak and lower PH but it was not considerably different from the controls, as there are outliers pulling the data towards the normal range which results in the data to become insignificant. A study looking at the effects of apixaban on the TGA parameters have stated that apixaban influences all parameters in a similar fashion to rivaroxaban (101), but we have found only influence of the PH and tPeak with this study. Prolongations of individual TGA parameters have been found before due to variations in coagulation factor proteins (102), but the study participants did not have previous factor deficiencies recorded in their medical histories. As the TGA parameters are all linked to produce a thrombogram it can be suggested that if one parameter is affected, all the others are affected as well when taking rivaroxaban. Whilst the lag time, ETP, and VI were not statistically different with the patient apixaban concentrations in this study, it can be speculated that all parameters can be significantly influenced at higher apixaban doses.

5.2 Effects of Weight

The effects of weight on DOAC pharmacodynamics has been questioned by a series of case studies on the effects of obesity with the FII inhibitor dabigatran (77-79). With most papers
omitting the data on the effects of apixaban and rivaroxaban between BMI categories, this study was aimed to evaluate if there was a relationship with weight and DOACs by measuring the effects on coagulation assays within each cohort. Using the Kruskal Wallis test we have evaluated that apixaban’s influence on coagulation or TG is not influenced by the patient’s BMI. Weight did not influence the rivaroxaban cohort, apart from the APTT assay which has recorded a significant increase in clotting time with patients with larger BMIs. The healthy control cohort contained no significant differences in results by weight, apart from the free protein S assay that contained higher free PS levels in obese controls.

The APTT assay under the influence of rivaroxaban was found to have significant differences between BMI groups with the healthy BMI group containing an average APTT of 31.58 seconds compared to the combined obese II and obese III cohort average of 36.64 seconds. There could be several reasons for this discrepancy with the most obvious cause being the differences of rivaroxaban concentrations between each of the groups. The drug levels for each of the groups varies with the averages for healthy (235.94ng/ml), overweight (157.24ng/ml), obese I (247.15ng/ml), and obese II/III (268 ng/ml) with a positive trend of increased BMI with increased drug. The use of VTE patients adds additional variability on the assay, as many patients will have several factors or influences acting on their APTT such as their age, genetics, haematocrit levels, or the timing and method of sample collection and storage (103).

There could also be physiological reasons for the differences between the healthy BMI and obesity for the APTT assay, with the literature stating that acquired obesity increases the activities of fibrinogen and the intrinsic factors FIX, FXI, FXII, and PAI-1 (104). However, as obesity would increase the amount of intrinsic clotting factors present, it would be expected that the APTT would be shortened, not prolonged as found within this study and that the fibrinogen levels would be elevated, which was also not found. Whilst there is currently no
published data for the effect of peak concentrations of rivaroxaban on the APTT of VTE obese vs lower BMI patients, this study would warrant further investigation on to the differences of rivaroxaban between obese and healthy APTT times.

The other assay that found significant differences between the BMI groups was the free protein S for the control cohort. The literature does suggest that obesity influences free protein S levels as a paper by Murakami et al. (2007) states that free protein S levels are drastically reduced in obese patients after weight loss (105). Another study by Pintao et al. (2013) has found that protein S levels were found to be higher in overweight/obese participants than healthy participants in their study (92). The free protein S assay is affected by the participant’s BMI with our study which mirrors the literature. However, our result is to be taken cautiously as there are several analytical limitations to our study. The first is the low and uneven sample numbers per BMI groups as there are ten samples within the overweight cohort compared to only four samples in the obese I, II, and III cohorts combined. As the control cohort was gathered from volunteers, previous medical histories were not acquired, so it is possible that a volunteer may not be healthy or would contain medical/lifestyle factors that would influence their free protein S levels, or the free PS assay itself. Another note is that the apixaban and rivaroxaban cohorts did not disclose significance between their results between their BMI groups for the free PS assay. The free PS assay is affected by weight so it is would be expected that other cohorts would follow suit, but this was not seen.

5.3 Comparison of Rivaroxaban and Apixaban Pharmacodynamics

Apixaban and rivaroxaban are selective, reversible direct FXa inhibitors that influence the same assays, but exert different pharmacodynamics using the same method of action. The therapeutic doses, efficacy, molecular masses (459.5 Da for apixaban, 436 Da for rivaroxaban (62)), and the pharmacokinetics of the two DOACs are comparable but Jourdi et al. (2015) has
revealed that the effects of the two drugs are not equal (89). The study stated that a discrepancy between drugs is due to a difference in the overall rates of association and dissociation to FXa which leads to difficulty in detecting apixaban. Jourdi et al. (2015) found that FXa had a higher affinity for rivaroxaban than apixaban, which results in rivaroxaban neutralising FXa four-fold more rapidly than apixaban (89). When comparing drugs using in silico PT and TG assays it was calculated that the PT is not sensitive enough to detect concentrations of apixaban, but can be used for rivaroxaban. The effect caused by rivaroxaban on the PT assay also depends on the level of thromboplastin tissue factor (TF) content as decreasing TF can increase the assay sensitivity (89). The TGA revealed all parameters in their study was affected by the two drugs, but stated that rivaroxaban is more potent than apixaban which led to a far greater rivaroxaban influence than apixaban on the TGA (89).

The study does not address however if the trough concentrations of rivaroxaban and apixaban have less of an influence than at peak. From our study, there are dose dependent responses to the four assays, with greater deviations from the normal at the peak concentrations than at trough for both rivaroxaban and apixaban.

As rivaroxaban has a 4-fold increase of rate activity compared to apixaban it can be speculated that rivaroxaban produces a rapid influence on an assay at peak but drops to a lesser influence at trough before the next dose. This is supported by the rivaroxaban’s short half-life of 7-11 hours (106). After peak, 3-4 hours after ingestion, there is rapid decrease in rivaroxaban plasma concentrations followed by approximately a 4-hour period of trough concentrations (107) before the next dose, which highlights the major difference between the peak and trough effects reported in this study.

The onset of apixaban activity can be described as more of a gradual rise that stays steady for longer before declining at trough. Apixaban contains a half-life of 10-14 hours with tablets administered at 12 hours intervals. As the time between doses had been decreased the
average trough level increases and is maintained at a steadier state throughout the duration before next the dose (105). These differences are also due to the dosing regimen of the two drugs as 20mg rivaroxaban is administered once a day, whilst apixaban is administered twice daily. Our data reflects this as the average difference between peak and trough rivaroxaban concentration was 207ng/ml, whilst 2.5mg and 5mg apixaban had an average difference of only 55ng/ml and 98ng/ml respectively. This would suggest that rivaroxaban has a greater influence at peak than at trough, whilst apixaban has a noticeably smaller influence between peak and trough.

The difference in peak over trough influence is highlighted by looking at the mean percentage difference between the rivaroxaban and the two apixaban cohorts. Mean percentage difference was used as individual paired samples will experience different variations depending on the drug level and patients baseline level. The rivaroxaban PT, APTT and DRVVT ratio mean percentage difference was 18.71%, 15.6% and 24.6% respectively. Figures 5.1 highlights the difference between means for the peak and trough daily dose of 20mg rivaroxaban.
Apixaban on the other hand displays a steadier state which can be seen when comparing the mean percentage difference of the PT, APPT, and DRVVT assays. The mean percentage differences for the assays was 3.33%, 1.62%, and 2.37% for the 2.5mg apixaban cohort, and 5.20%, 3.95%, and 3.72% for the 5mg apixaban cohort respectively. These graphs highlight the effects of bi-daily administration of apixaban over the effects of daily rivaroxaban on peak and trough.
5.4 Limitations of Study Design

We have successfully investigated the effects of apixaban and rivaroxaban on coagulation parameters in this study but there are a series of limitations that need to be addressed.

The three cohorts are limited by small sample sizes as it would be desirable to obtain larger numbers with at least 30 participants, especially with the 2.5mg and 5mg apixaban sub-cohorts as these groups only contain 10-11 participants. The healthy control cohort is limited by their unknown medical history and lifestyle influences, as more thorough documented controls would strengthen the normal ranges and reduce outliers. It should also be noted that the samples used within this study were real world patients, and not spiked normal pooled plasma, which leads to various levels for peak and trough as different participants experience different rates of pharmacodynamics. The peak sample collection time is a limitation as some samples were taken 10 minutes early or late, than exactly 3 hours which may influence the results.

When dealing with weight it would have been desirable to gather waist circumference in addition to BMI, as BMI has been criticised for being only an estimation of adiposity, and does not consider the differences in muscle mass or other lifestyle influences (108). The patient cohort should be fully representative of the weight spectrum with adequate numbers of participants in each BMI cohort especially with the obese BMI groups in the control cohort.

A key limitation found with this study would be the use of only single runs for the TGA, coagulation, and thrombophilia panels per sample, and not using duplicate or triplicate runs. The use of duplicate run would have increased accuracy and led to greater strength and statistical confidence to the results. It should be noted that the PF1+2 and TAT assays were the only assays performed in duplicate.
5.5 Summary and Future directions

This study has validated the hypothesis that the DOACs rivaroxaban and apixaban exert a dose dependent effect on coagulation parameters and thrombin generation.

The study has demonstrated that the direct acting oral anticoagulants rivaroxaban and apixaban have shown to affect the PT, APTT, DRVVT, and TG assays and do not affect the fibrinogen, D-dimers, TCT, ATIII, PC, free PS, TAT or the PF1+2 assays. It can be shown that rivaroxaban contains a more potent effect than apixaban as rivaroxaban is significantly different from the normal control cohort at peak drug concentrations for the PT, APTT, DRVVT, and TGA assays, as well as differences between peak and trough concentrations. Apixaban was shown to affect the PT and APTT assays between peak and trough but was not significant from the normal controls. Apixaban also affects the DRVVT and PH, tPeak parameters for the TGA for the 2.5mg dosage sub-cohort, but interestingly not for the 5mg apixaban sub-cohort.

An aim of this study was investigating if peak concentrations influence coagulation assays more than trough, which we have shown to be correct for affected assays. Rivaroxaban has major differences between its peak and trough, whereas apixaban has more of a steady state with slight differences between peak and trough. These differences are due to the different pharmacokinetics and pharmacodynamics of rivaroxaban and apixaban, especially the half-life and daily/bi-daily dosage routine. It is noteworthy to add that whilst the trough concentration has a considerably less of an effect than the peak, there is still an influence on these assays by the DOACs and results are manipulated at trough.

The final consideration of the study was the effect of weight, especially obesity, on rivaroxaban and apixaban influence. The effects of the weight on the assays regarding drug influence was found to be insignificant for all assays and cohorts except for the APTT for rivaroxaban, and the free PS assay for the healthy controls. Both results are subject to low, disproportioned sample
sizes and patient and drug variability, but both results are supported by the literature and other studies (92,103,104).

This study has shown promising results in the interpretation of coagulation and thrombin generation assays. The routine PT and APTT assays are both affected by rivaroxaban and apixaban which is important for clinicians to know during emergency monitoring. The effects of apixaban is underreported in the literature so this study has enhanced our understanding of the interpretation of apixaban’s influences. The effects of the DRVVT is well reported to be influenced by rivaroxaban but this study has displayed direct effects on the assay with apixaban. The TGA was also affected by rivaroxaban and apixaban by our findings but differ in their scope of influence. The effects of weight on DOAC efficiency was found to be insignificant for all but two assays.

The future direction of this study would be to expand the cohort to validate our findings and should also expand the range of assays used, particularly the inclusion of individual clotting factors and the TVT/ECT assays. The inclusion of snake venoms such as the TVT/ECT could be seen as an alternative LA assay to the DRVVT due to the venoms acting independently from phospholipids, FVa, FVIIa, and FXa, and so would not be affected by DOACs. Larger cohorts will also provide further validation on the effects of rivaroxaban and apixaban on the DRVVT and TG assays. As assays are influenced depending on the methods and reagents used, a future study with expanded assays should contain several different commercial reagents per assay to see which are affected by DOACs and which are not. Future studies may be able to build a reliable series of minimal or unaffected coagulation tests for emergency monitoring of DOAC patients. With the advancements of rivaroxaban and apixaban characterisation at peak and trough, the treatment of VTE can be improved and can improve the clinical outcomes for patients undergoing this therapy and improve DOAC management and monitoring.
Abbreviations

The following abbreviations are used in this thesis.

• (APTT): Activated partial thromboplastin time
• (ATIII): Antithrombin III levels
• (BMI): Body Mass Index
• (CAT): Calibrated automated thrombogram
• (DIC): Disseminated intravascular coagulation
• (DOACs): Direct acting oral anticoagulant drugs
• (DRVVT): Dilute Russell’s viper venom time
• (DVT): Deep Vein Thrombosis
• (ECT): Ecarin clotting time
• (ELISA): Enzyme-linked immunosorbent assay
• (ETP): Endogenous Thrombin Potential
• (PF1+2): Prothrombin fragments 1+2
• (INR): International Normalised Ratio
• (ISI): International sensitivity index
• (LA): Lupus anticoagulant
• (NOACs): Novel oral anticoagulants
• (PH): Peak Height
• (PPP): Platelet poor plasma
• (PT): Prothrombin time
• (TAT): Thrombin/anti-thrombin complexes
• (TCT): Thrombin clotting time
• (TF): Tissue Factor
• (tPeak): Time to Peak
• (TVT): Taipan venom time
• (VKA): Vitamin K Antagonist
• (VTE): Venous thromboembolism
• (VI): Velocity index
References:

Papers of interest and importance are highlighted as:
* Of Importance.
** Of Major importance.


49. Haas S. GARFIELD-AF First data on healthcare of patients with atrial fibrillation in Germany. DMW - Deutsche Medizinische Wochenschrift. 2015;140(1): 13-14.


95. Gheorghiade M, Thyssen A, Zolynas R, Nadar V, Greenberg B, Mehra M et al. Pharmacokinetics and pharmacodynamics of rivaroxaban and its effect on biomarkers of...


