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Doctoral Programme in Clinical Research

LABORATORY MONITORING OF DIRECT ORAL ANTICOAGULANTS

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ACADEMIC DISSERTATION

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To my father

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, referred to in the text by their Roman numerals:

I.   Helin TA, Pakkanen A, Lassila R, Joutsi-Korhonen L. Laboratory assessment of novel oral anticoagulants: method suitability and variability between coagulation laboratories. *Clinical Chemistry* 2013;59:807-814

II. Helin TA, Pakkanen A, Lassila R, Joutsi-Korhonen L. Effects of apixaban on prothrombin time, activated partial thromboplastin time and anti-Xa assays: a European survey. *Clinical Chemistry and Laboratory Medicine* 2016 Dec 17 [EPub ahead of print]

III. Helin TA, Lemponen M, Hjemdahl P, Rönquist-Nii Y, Lassila R, Joutsi-Korhonen L. From laboratory to clinical practice: Dabigatran effects on thrombin generation and coagulation in patient samples. *Thrombosis Research* 2015;136:154-160

IV.   Helin TA, Virtanen L, Manninen M, Leskinen J, Leppilahti J, Joutsi-Korhonen L, Lassila R. Effects of thromboprophylactic doses of apixaban and rivaroxaban on coagulation and thrombin generation in association with total hip replacement. *Journal of Thrombosis and Thrombolysis* 2017;43:562-569

In addition, some unpublished data are presented.

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LIST OF ABBREVIATIONS

AF	atrial fibrillation
ALT	alanine aminotransferase
AST	aspartate transaminase
APTT	activated partial thromboplastin time
ASA	acetylsalicylic acid
BMI	body mass index
CAT®	Calibrated Automated Thrombogram®
CRP	C reactive protein
Dabi-TT	functional dabigatran concentration
DIC	disseminated intravascular coagulation
DOAC	direct oral anticoagulant
DTI	direct thrombin inhibitor
dTT	diluted thrombin time
DVT	deep vein thrombosis
ECA	ecarin clotting assay
ECT	ecarin clotting time
EMA	European Medicines Agency
EQA	external quality assessment
ETP	endogenous thrombin potential
F1+2	prothrombin fragments assay
FDA	Food and Drug Administration
FDP	fibrin degradation product
FFP	fresh frozen plasma
FX	coagulation factor X
FXa	activated coagulation factor X
FXaI	factor Xa inhibitor
GFR	glomerular filtration rate
HR	hazard ratio
HPLC	high-performance liquid chromatography
INR	international normalised ratio
ISI	international sensitivity index
LC-MS/MS	liquid chromatography tandem mass spectrometry
LMWH	low molecular weight heparin
PCC	prothrombin complex concentrate
PE	pulmonary embolism
PiCT	prothrombinase-induced clotting time
PPP	platelet-poor plasma
POC	point-of-care
PT	prothrombin time

RVVT	Russel's viper venom time
SD	standard deviation
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TG	thrombin generation
THA	total hip arthroplasty
TT	thrombin time
TTR	time in therapeutic range
tPA	tissue plasminogen activator
VKA	vitamin K antagonist
VKORC	vitamin K epoxide reductase complex
VTE	venous thromboembolism
vWF	von Willebrand factor

CONTENTS

ABSTRACT

Direct oral anticoagulants (DOACs), the thrombin inhibitor dabigatran and the anti-Xa inhibitors rivaroxaban and apixaban are currently licensed for thromboprophylaxis after orthopaedic surgery, in non-valvular atrial fibrillation, and treatment of deep vein thrombosis and uncomplicated pulmonary embolism. The drugs are easy to use, as standard dosing is recommended and no routine monitoring of coagulation is advocated. However, in cases of acute thrombosis or bleeding, emergency surgery, renal or hepatic failure, overdose and suspected non-adherence, assessing anticoagulant bioactivity is essential for safe and effective treatment.

The aims of this study were to firstly assess the effects of these DOACs in commonly used coagulation screening tests (PT, INR and APTT) using both spiked samples and samples collected from patients on treatment. Secondly, the availability of specific methods for drug effect assessment, namely drug-calibrated thrombin time (dabigatran) and anti-Xa (rivaroxaban, apixaban) assays were assessed in surveys with European laboratories (n=86). Thirdly, the effects of DOACs on these specific assays were explored using patient samples. Finally, the effect of DOACs on the global coagulation assay thrombin generation (TG) was assessed in patients using dabigatran, rivaroxaban or apixaban.

The spiked sample laboratory survey included 73 laboratories for dabigatran, 22 laboratories for rivaroxaban and 21 laboratories for apixaban. The laboratories performed coagulation assays in samples spiked with varying concentrations of the drug. A variety of different analysers and reagents were observed, with 24 different coagulation analysers, 13 different PT reagents and 10 different APTT reagents used in the investigated laboratories. The effects of all DOACs on INR were modest, but large reagent variability was noted in the responses, with the Quick-type reagents being more sensitive to the effects of dabigatran ($p<0.001$). In APTT, rivaroxaban and apixaban prolonged the APTT only modestly, but with dabigatran there was a clear prolongation, albeit the variability between laboratories was large (CVs 13-19% for all DOACs). Only about a fourth of these (in the surveys ranging from 15-36% of the participants) were able to provide more specific methods, TT or anti-Xa assays for drug quantification at that time.

Dabigatran effects in patient samples were different than what was observed in the surveys using spiked samples. We studied dabigatran effects in 241 unselected patient samples. The effect on PT was very modest, with little prolongation. In APTT, there was a curvilinear relationship ($R^2 = 0.71$) with dabigatran. However, the sensitivity effects among patients varied, with some patients having a normal APTT at dabigatran levels up to 160 ng/mL. In the specific assays, diluted thrombin time, ecarin clotting assay and anti-IIa assay accurately quantified dabigatran concentrations as confirmed by mass

spectrometry (R^2 = 0.81, 0.96 and 0.90, respectively). In the RVVT and PiCT assays, the correlations with diluted thrombin time were modest (R^2 = 0.49 and 0.73, respectively). In TG, a paradoxical increase was observed in the endogenous thrombin potential (ETP) and Peak TG, while lag time also prolonged with increasing concentrations of dabigatran.

Rivaroxaban and apixaban effects on coagulation in patient samples were further assessed in a well-characterised group of patients using these drugs for thromboprophylaxis after orthopaedic surgery. In this study, a noticeable inflammatory response after the surgery was evident, with a mean C reactive protein (CRP) of over 100 mg/L at one week after surgery, while haemoglobin and albumin were clearly below the reference intervals. This inflammatory setting was also reflected by the increases in FVIII:C and fibrinogen at one week after surgery. We observed clear differences between responses to rivaroxaban and apixaban. Rivaroxaban peak levels were higher and trough levels lower than with apixaban. This reflects the fact that rivaroxaban is dosed only once daily (10 mg once daily), while apixaban twice daily (2.5 mg twice daily), with corresponding steady state peak levels of 184 ng/mL and 135 ng/mL, respectively. It is noteworthy that the difference between these drugs extended to responses in TG; the ETP response at peak drug levels was strong with rivaroxaban, whereas with the trough drug levels, the TG parameters were close to the baseline levels. With apixaban the responses were more stable. The RVVT, used as a qualitative test, detected prolongation only at rivaroxaban peak levels.

In conclusion, from the surveys performed we encountered large diversity in different reagents used and assays offered among European laboratories, with a wide variability in results. In the studies with patient samples, it became evident that many factors other than drug concentration influence the responses in coagulation assays and TG, which reflects the coagulation status of the patients. In clinical practice, care and diligence is required when assessing and interpreting the effects of DOACs on patients.

1 INTRODUCTION

Anticoagulation is required when an individual has thromboembolism or deep vein thrombosis (DVT). Anticoagulants affect the coagulation cascade by diminishing feedback activation of coagulation factors, diminishing thrombin, and subsequently, fibrin formation. This reduced thrombin formation also supports fibrinolysis.

Novel anticoagulant drugs have emerged; the direct oral anticoagulants (DOACs) include the direct thrombin inhibitor (DTI) dabigatran and the direct FXa-inhibitors (FXaIs) rivaroxaban, apixaban and edoxaban. These agents have the advantage of relatively predictable pharmacokinetics and pharmacodynamics, making routine laboratory monitoring, as practised with traditional vitamin K antagonist (VKA) anticoagulation unnecessary.[1-4] However, to assess compliance, as well as acute situations, e.g. thrombosis, bleeding events, surgery, hepatic or renal failure, DOAC bioactivity measurement is crucial. DOAC concentrations can be accurately measured by drug calibrated assays—thrombin time (TT) for DTI, anti-Xa for FXaIs.[5]

The availability of drug-calibrated assays is not yet widespread, and clinical laboratories should be aware of the effects of DOACs on the coagulation assays they are using. Some activated partial thromboplastin time (APTT) assays are sensitive to dabigatran, whereas other APTT assays show little sensitivity. Prothrombin time (PT) is usually not very sensitive to dabigatran effects.[6] For FXaIs, both PT and APTT are usually prolonged only with significant drug concentrations.[7,8] One must also be aware of the potential of these drugs to interfere with coagulation assays, one of the more critical assays being the Russel's viper venom time (RVVT), commonly used as a test to screen for lupus anticoagulant.[9]

Even though these drugs have predictable pharmacokinetics and pharmacodynamics, the same dose does not apply for all patients. Renal insufficiency is important to recognise, especially with dabigatran. In this situation, the effective dose for stroke prevention in atrial fibrillation (AF) or treatment of DVT should be reduced from the usual 150 mg twice daily dose to 110 mg twice daily. The prophylactic doses for all the DOACs are smaller than the treatment dosages. Risk groups for bleeding complications include patients with renal insufficiency, patients with a high number of comorbidities and medications and patients over the age of 75.[10] The decision on which anticoagulant to use and at what dosage is complex and each anticoagulant carries advantages and disadvantages. Indeed, further dose tailoring based on the patient's risk profile might be in order.

The aim of this study was to examine the availability of laboratory tests to measure the DOAC effects on screening tests PT and APTT in two quality control surveys in European laboratories. The effects of dabigatran, rivaroxaban and apixaban in patient samples were next studied using a

comprehensive panel of coagulation tests, including global coagulation assays, such as thrombin generation (TG). This allowed improved understanding of the biological effects of these drugs in a real-life setting, in both unselected and selected (in association with hip replacement surgery) patients

2 REVIEW OF THE LITERATURE

2.1 OVERVIEW OF COAGULATION

Rapid haemostasis is needed in response to injury. The function of the haemostatic system is traditionally modelled with Virchow's triad.[11] Three components, namely blood, vessel wall and flow are all crucial for maintenance of a normal haemostatic process. Disturbances in any of these components can lead to thrombosis or bleeding. Blood coagulation occurs in three overlapping stages: 1) primary haemostasis, 2) coagulation cascade leading to fibrin formation and 3) fibrinolysis. For the purposes of this study, primary haemostasis and fibrinolysis are only briefly covered, with more emphasis on the coagulation cascade and fibrin formation, as these are the basis of most coagulation laboratory tests.

Vascular collagen and von Willebrand factor (vWF) are crucial for haemostasis initiation. *Primary haemostasis* occurs within seconds of tissue injury, by platelet activation as they encounter collagen and vWF in the subendothelium. The platelet glycoprotein Ib-V-IX mediates binding between platelets and vWF, and glycoprotein VI with collagen and platelets. Activated platelets bind to one another via glycoprotein IIb/IIIa. They secrete procoagulant factors, including factor V (FV), FXIII, calcium, thromboxane and serotonin, which stimulate platelets further, thus amplifying the reaction. Platelets express multiple chemokines, e.g. P-selectin and platelet factor 4, which recruit leukocytes to initiate an inflammatory response in order to curtail possible pathogens, promote angiogenesis and healing.[12]

Coagulation with insoluble fibrin as the end product has been extensively studied with basic laboratory screening tests measuring time to formed fibrin. Early on, it was recognised that essential components for blood coagulation are prothrombin, fibrinogen and calcium.[13] With the discovery of coagulation factors and the crucial role of factors FVIII and FIX in haemophilias, a coagulation cascade model was independently suggested by two groups in 1964.[14,15] In the classic blood coagulation cascade model, the extrinsic pathway is triggered by tissue factor (TF), and the intrinsic pathway is triggered by the contact pathway and proceeds from FXII. The common part of the cascade starts with FX, with both pathways eventually leading to conversion of fibrinogen to fibrin in a thrombin-mediated reaction (Figure 1). The coagulation cascade model is not physiological, but it is crucial to understand when interpreting laboratory coagulation tests. In coagulation tests, factor activation is based on this model.

Physiologically, blood coagulation is more complex. The coagulation factors interact in complexes on the platelet phospholipid cell membrane. Coagulation is divided into three stages. In the *initiation phase*, TF and FVII form a complex, which activates FX and leads to small amounts of thrombin

formation. This is rapidly counteracted by tissue factor pathway inhibitor (TFPI). However, in the *amplification phase* the small amount of thrombin is sufficient to activate platelets, vWF and FVIII, with concurrent activation of FV, FIX and FXI. In the *propagation phase*, FIX complexes with FVIII to activate an FV/FX-complex, which leads to greatly increased thrombin formation. Thrombin forms a feedback loop perpetuating the amplification phase to further propagate thrombin formation.[16,17]

Inhibitory feedback loops are required to prevent an uncontrolled coagulation response. Both unspecific and specific natural inhibitors occur. Alpha-2-macroglobulin is an unspecific serine protease inhibitor with a wide array of functions. It functions as an inhibitor by trapping the protease.[18] Specific inhibitors include the protein C complex and antithrombin. Protein C is a natural anticoagulant, which inhibits FVa and FVIIIa. Protein S acts as a cofactor for the reaction. Protein S and protein C are vitamin K dependent.[19] Protein S circulates in protein-bound and free forms, with about 60% bound to the complement inhibitor and 40% in a biologically active free form.[20] Antithrombin is a specific inhibitor of many coagulation factors, most importantly thrombin (FIIa) and activated factor X (FXa).[21] The role of antithrombin is crucial for the function of heparin anticoagulants, as they exert their anticoagulant effects through antithrombin binding. The aforementioned TFPI is an important inhibitor of the extrinsic pathway, inhibiting the TF-FVIIa complex.[22]

With formation of the fibrin network, FXIII strengthens the clot by forming crosslinks between fibrinogen D subunits. *Fibrinolysis*, or dissolving of the clot, starts immediately after clot formation and is in interaction with primary haemostasis and the coagulation cascade. Urokinase and tissue plasminogen activator (tPA) form plasmin from plasminogen. Plasmin cleaves the fibrin network between the molecule subunits D and E, leading to formation of fibrin degradation products (FDPs). D dimer is a commonly measured FDP and it is used as a marker of fibrinolysis and clot formation status. Fibrinolysis inhibitors include plasminogen activator inhibitors 1 and 2, which inhibit urokinase and tPA, and alpha-2-macroglobulin and alpha-2-antiplasmin, which in turn inhibit plasmin.[23]

2.2   LABORATORY COAGULATION ASSAYS

Coagulation assays are among the most requested laboratory assays in the clinical laboratory. Mostly due to the prevalence of warfarin use, the international normalised ratio (INR) is the bulk assay. Many of the coagulation laboratory tests are required under emergency situations, thus requiring a rapid turnaround time and fluent stat test processing to attain satisfactory service. The screening assays PT, INR and APTT are most frequently performed, with more specialised assays usually utilised when there is clinical

suspicion of a coagulation problem. Abnormalities in the screening tests strongly suggest further investigation.

2.2.1 SCREENING TESTS PT, INR, APTT AND THROMBIN TIME

PT measures the extrinsic pathway coagulation factors VII, X, II and sometimes, depending on the method, fibrinogen and FV (Figure 1). PT was the first coagulation screening assay measuring the formation of fibrinogen. Earlier, coagulation assessment was based on bleeding time.[24] PT was developed by Dr. Armand J. Quick in the 1930s. The method principle is simple: anticoagulated blood (originally sodium oxalate was used, the current state-of-the-art is to use sodium citrate) is mixed with thromboplastin solution obtained from rabbit brain. Finally, calcium chloride is added and time in seconds to formation of a blood clot is recorded. The sample volume is 1/3 of the total volume.[25] Most of the modern PT assays are based on the original Quick method. With the use of photometers (turbidimetric principle), the formation of first fibrin strands can be readily assessed for accurate results. In the Nordic Countries, Benelux and Japan, the Owren method, first introduced by Dr. Paul Owren in the 1950s, is the most commonly used PT assay. In the Owren method, the plasma is heavily diluted to a ratio of 1:20, and fibrinogen and FV are added to the reagent, to avoid the effect of those factors in Owren-type PT assays.[26,27] Abnormal PT results are expected in liver dysfunction—a rapid change in PT is observed due to the short half-life of FVII of 6-7 hours.[28] Most of the factors measured in the PT, i.e. FVII, FX and FII are vitamin K dependent, as their synthesis requires vitamin K activity as a cofactor.[29] PT is consequently prolonged in vitamin K deficiency and with VKA anticoagulants such as warfarin. Other causes for prolonged PT include FVII deficiency, other anticoagulants such as heparin in high concentrations and consumption of coagulation factors, in e.g. disseminated intravascular coagulation (DIC).

The PT alone has inter-laboratory variation depending on the thromboplastin reagent used. Previously, common practice was to use a standard preparation from which the PT was calibrated.[30] Nevertheless, different laboratories exhibited different sensitivities despite the standards depending on the reagent and instrument used. In the 1980s, however, it was discovered that a linear relationship exists between the PT and the effect on PT due to a particular thromboplastin reagent or instrument. On this basis, it was possible to establish a standard that would harmonise results between different laboratories, the INR.[31] The INR is calculated as follows:

$$INR = \left(\frac{PT_{patient}}{PT_{normal}}\right)^{ISI}$$

Where ISI is the International Sensitivity Index, which accounts for the differences in sensitivity in different thromboplastin reagents. The INR offers

many benefits: laboratories can collaborate more easily on research as the standardized values are comparable. Most importantly, the standardised INR means that all warfarin patients receive the dose optimised for most effective treatment. However, it should be noted that the ISI is calculated using plasma samples from healthy individuals and on individuals on warfarin therapy. Therefore, the INR can only be reliably used on warfarin patients. Monitoring of other anticoagulants or detection of coagulation disturbances using INR is unreliable and not recommended.
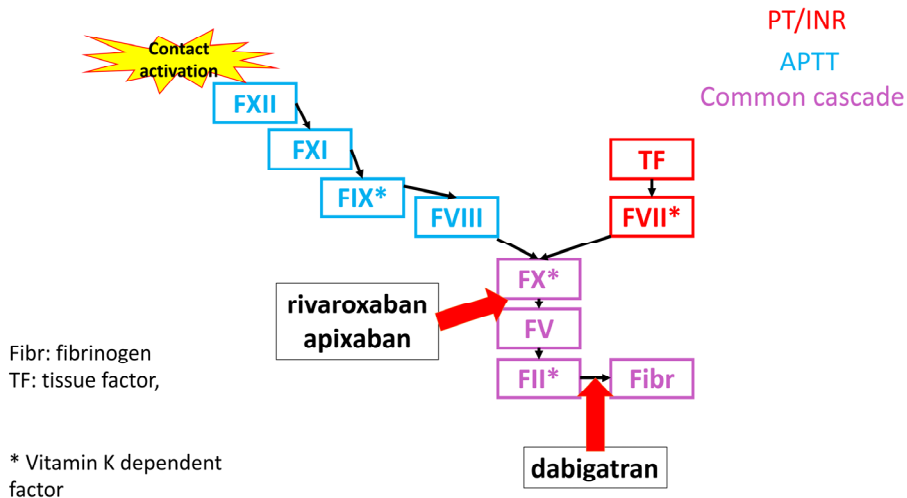

Figure 1   Extrinsic and intrinsic pathways of coagulation, with PT/INR and APTT measuring extrinsic and intrinsic pathways, respectively. DOACs directly inhibit factor X or thrombin, whereas vitamin K antagonists affect all the vitamin K-dependent factors (*).

The INR target range in warfarin treatment is generally 2.0-3.0. In patients with a mechanical heart valve, the INR treatment range is 2.5-3.5. In patients with a high bleeding risk, e.g. previous intracranial bleed, hepatic failure or elderly patients, an individual lower treatment range is often used. To assess warfarin therapy in clinical studies, the time in therapeutic range (TTR) parameter is used, where the INR is assumed to change linearly over two measurement points with each treatment day assigned an INR value.[32]

APTT is another coagulation screening test. It measures the effect of factors XII, XI, IX, VIII, X, V, II and fibrinogen (Figure 1). The method was first described in 1953 by Dr. Robert D Langdell for the study of haemophilia.[33] The problem with the first assays was contact activation with the glass surfaces of the test tubes, making the assay difficult to standardise. The technique was improved by Dr. J Margolis, and further refined by Drs. Robert R Proctor and Samuel I Rapaport. Kaolin was used as a contact activator. Calcium chloride-activated coagulation and time to the formation of fibrin was measured.[34,35]

Currently, both kaolin- and silica-based activators are in use. APTT, similar to PT, is currently rapidly and reliably measured with the use of commercial reagents and analysers. APTT is prolonged in haemophilias and hereditary deficiencies of factor VIII and IX. APTT is also abnormal in liver failure, during unfractionated heparin therapy and DIC.

TT is a derivative of the other coagulation screening assays, where thrombin is added in excess to the reaction, so that the coagulation time is dependent only on the conversion of fibrinogen to fibrin. TT has been used for decades as a screening test for fibrinogen abnormalities and presence of thrombin inhibitors (heparins and specific thrombin inhibitors).[36] Fibrinogen can also be assessed directly, using a clot-based method, from which fibrinogen concentration is derived.[37]

From the screening tests PT and APTT, single factor assays can be derived. The measurement principle involves using factor-deficient plasma in the reagent (containing all the other coagulation factors except the one that is measured), so that the coagulation factor in question becomes the rate-limiting step in the reaction. Accordingly, it is possible to estimate the activity of the factor as a percentage of normal. The factors that can be measured depend on the factors affecting the assay, i.e., FVII, FX, FV and FII can be measured in PT-based assays and all factors except FVII and FXIII in APTT-based assays (Figure 1). FVIII and FIX are the most important in APTT-based assays, being low in haemophilia, with high FVIII levels being a thrombophilic risk factor.[38,39] In addition to clot-based assays, chromogenic assays can be used to measure single coagulation factors.

2.2.2   NATURAL ANTICOAGULANTS

As the screening assays PT, APTT and TT are rarely abnormal in a patient with thrombophilia, other tests need to be used. Deficiencies of the natural anticoagulants are among the most devastating thrombophilias, with highly increased thrombotic tendency.[40,41] The assessment of these factors is usually performed as part of a thrombophilia screening panel. Antithrombin deficiency leads to increased incidence of venous thrombi in particular. Antithrombin activity is generally measured using a chromogenic assay using either thrombin or factor Xa inhibition by antithrombin, with antithrombin activity inversely proportional to the amount of chromophore measured. The advantage of the chromogenic assay is that only active antithrombin is measured. Antithrombin antigen can also be assessed using immunochemical assays.[42]

Protein C and protein S act physiologically as a complex. Protein S is required as a cofactor for protein C-mediated inactivation of FV and FVIII. Protein C is usually measured based on clot activation, as chromogenic methods can erroneously also measure some inactive protein C.[43] Protein S can also be measured using the activity measurement, but the approach is not without problems, as erroneously low activities are sometimes observed.[44] For

this reason, in some laboratories, either total protein S antigen or only the free, biologically active fraction of protein S is measured using an immunoassay.[45]

2.2.3   LUPUS ANTICOAGULANT

Antiphospholipid syndrome is a thrombophilic disorder characterised by the development of phospholipid antibodies. The syndrome is peculiar in that, oftentimes, the APTT is prolonged as the phospholipid antibodies bind to the phospholipids in the reagent. This explains why patients might be erroneously screened for bleeding tendency, even though they are actually at risk for thrombosis.[46] Lupus anticoagulant assay is a three-step assay. In the screening assay, an APTT- or dilute RVVT-based assay is performed. If the result is normal, no further testing is performed. If it is abnormal, a mixing study is performed, where the test plasma is mixed with normal plasma at a ratio of 1:1, so that if there is a factor deficiency, it is corrected. If a patient is receiving anticoagulants, the mixing study can also be abnormal. Finally, in the confirmation assay, excess phospholipid is added, which normalises the measurement if the prolongation is due to phospholipid antibodies. The principle is that if the prolongation is due to an anticoagulant, the assay remains prolonged.[47]

2.2.4   TESTS FOR FIBRINOLYSIS AND GENERAL COAGULATION ACTIVITY

D dimer is one of the end products of fibrinolysis and is widely used as a marker of general coagulation activity. The marker has a significant role in excluding a diagnosis of venous thromboembolism (VTE). Negative D dimer in an outpatient setting with a low risk for thrombosis excludes thrombosis with a high likelihood of over 95%.[48] In contrast, a positive D dimer does not indicate thrombosis, as inflammation, pregnancy and advanced age are associated with increased fibrin turnover and thus, increased D dimer levels. Recently, it has been suggested that the cut-off values might need to be adjusted according to patient age.[49] Currently, the clinical practice is to use a single cut-off, which can vary depending on the reagent and analyser used in the laboratory. D dimer is usually measured with immunoassays, using antibodies to detect the protein. Another method to measure general coagulation activity, currently only in research use, is the prothrombin fragments assay (F1+2), in which fragments released from FXa-activated prothrombin are measured.[50,51]

2.3 VITAMIN K ANTAGONISTS (VKAs)

In the 1920s and 1930s, veterinarians Drs. Frank W Schofield and LM Roderick discovered that feeding spoiled hay made from the sweet clover resulted in a haemorrhagic disease in cattle.[52] The blood from the affected animals failed to clot, but clotting could be achieved using prothrombin preparation from unaffected animals. Clinically, the animals could be rescued by cessation of feeding the spoiled hay followed by prompt blood transfusion.[53,54]

In February 1933, a farmer in Wisconsin drove 190 miles with a dead heifer, spoiled sweet clover fodder and uncoagulated blood from his cattle to Madison in order to find a cure for his animals. The office of the State Veterinarian was closed and he arrived at the Biochemistry building by chance.[52] The examination of the hay and the blood prompted research, which lead to isolation of the active compound dicoumarol from the spoiled sweet clover.[55] The success led to the discovery of many new VKAs used in anticoagulation (i.e., warfarin, phenindione, ethyl biscoumacetate, acenocoumarol, phenprocoumon).[52,56-59] Synthesis of warfarin first took place in 1948 – its name was derived from Wisconsin Alumni Research Foundation and coumarin – WARFarin. It was discovered to be a more potent and more rapidly acting anticoagulant than dicoumarol and its effects could be readily counteracted with vitamin K. It was used to treat US president Dwight D Eisenhower after a heart attack in 1955.[52] Currently, warfarin is the most widely used VKA, with acenocoumarol and phenprocoumon also used in Europe.[60,61]

Warfarin, as well as the other VKAs, has great interindividual variation in its metabolism. VKAs inhibit the vitamin K epoxide reductase complex (VKORC), which converts vitamin K from its inactive to active form. VKAs, by inhibiting this enzyme, diminish the synthesis of vitamin K-dependent coagulation factors II, VII, IX and X. This leads to diminished formation of fibrin. VKAs affect the extrinsic pathway of the coagulation (Figure 1) and require continuous laboratory monitoring with the INR measurement. VKA benefits include regular monitoring of INR, confirming patient adherence and drug effect. This benefit is also a drawback. The inconvenience of INR measurements every 4-8 weeks limits the lifestyle of the patients. VKA effects can be counteracted by vitamin K administration. Vitamin K is recommended with an INR of over 5.0-9.0, depending on the clinical situation. The effects of vitamin K on coagulation factor levels, however, take several hours. In acute bleeding, replacement of deficient coagulation factors is required and provides immediate restoration of coagulation. For acute reversal, prothrombin complex concentrates (PCC) are recommended. Fresh frozen plasma (FFP) can also be used, if PCC is unavailable but use of FFP can easily lead to volume overload.

2.4 DIRECT ORAL ANTICOAGULANTS (DOACs)

As VKA metabolism and dosing are highly dependent on individual factors, constant INR monitoring is required. Accordingly, agents that affect the coagulation cascade directly provide a lucrative option for anticoagulation, since their dose and effects are more easily predictable. DTIs include ximelagatran (no longer used due to unexpected hepatic toxicity) and dabigatran etexilate (Pradaxa®). FXaIs include rivaroxaban (Xarelto®), apixaban (Eliquis®) and edoxaban (Lixiana®).

DOACs are advantageous in that no routine measurement for their effects is required and dosing can be standardised due to predictable pharmacokinetics and pharmacodynamics. The development timeline of the currently available DOACs is shown in Figure 2.
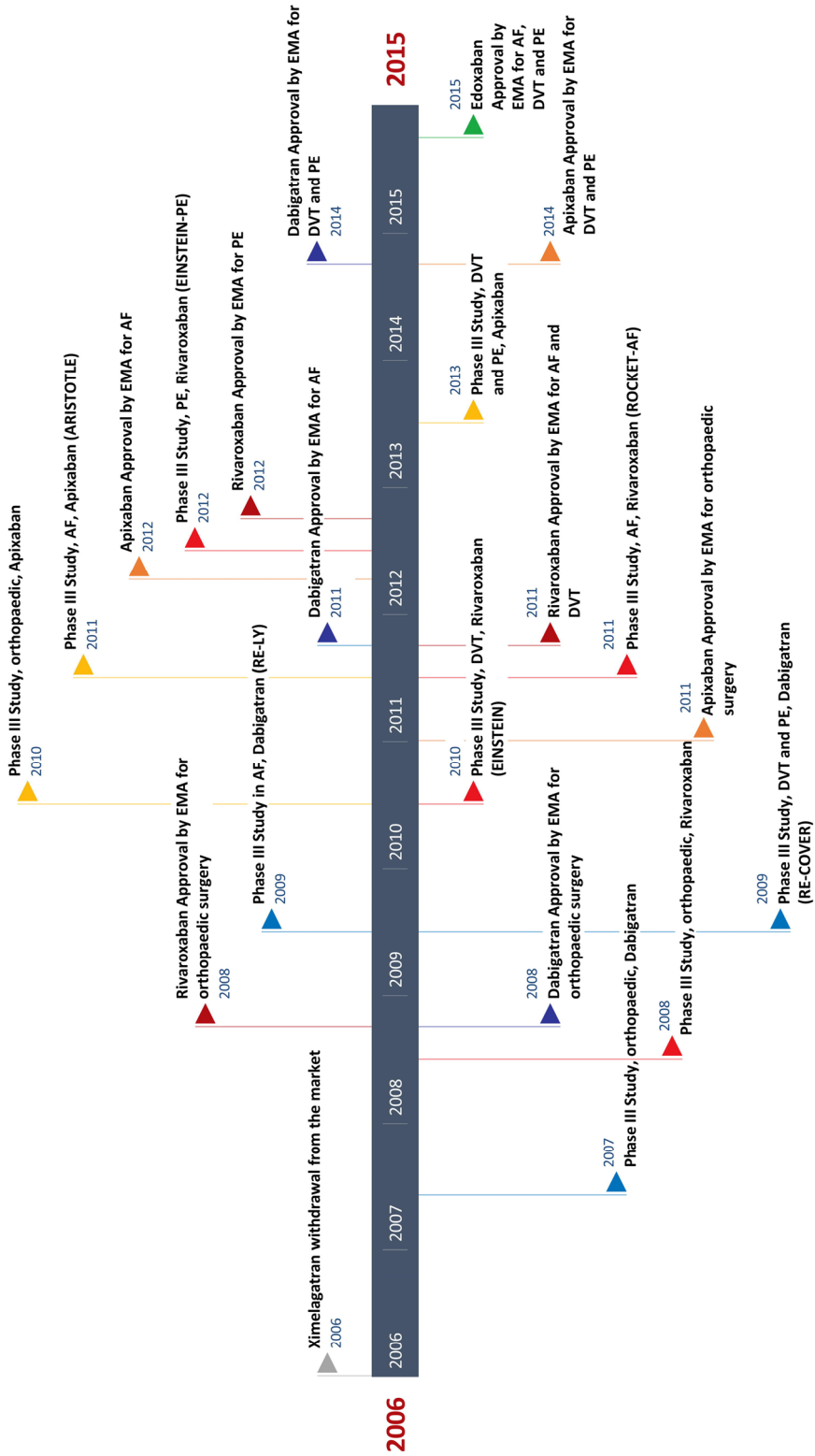
Figure 2   Timeline of studies leading to approval of the direct oral anticoagulants (DOACs)

2.4.1 XIMELAGATRAN

Ximelagatran was developed by AstraZeneca and was the first DOAC to enter the market. The active form, melagatran, showed significant variations in absorption, so a prodrug form was synthesised. Melagatran showed prolongation in PT, APTT and increased anti-Xa activity in both mouse models and healthy human volunteers. No significant side effects were observed in healthy volunteers.[62] The beneficial effects of ximelagatran were shown in a small open-label study with 12 patients with DVT or pulmonary embolism (PE). Maximum plasma concentrations of 680 ng/mL and half-life of 4 hours were observed.[63] In a large patient study including 1900 patients, the effects of ximelagatran were assessed in patients undergoing orthopaedic surgery. The effects were compared with dalteparin. The protocol was to start with subcutaneous melagatran injections, followed by oral ximelagatran once daily with dose ranging from 8 to 24 mg. Small and transient increases in liver enzymes alanine aminotransferase (ALT) and aspartate transaminase (AST) were reported, but they were more common in the dalteparin group than in the ximelagatran group.[64] Subsequently, in a trial including 3500 patients with non-valvular AF, ximelagatran showed non-inferiority to warfarin in systemic thromboembolism, bleeding and all-cause mortality. In this study, the ALT levels rose above three times the upper limit of normal in 1% of patients in the warfarin group and 6% in the ximelagatran group.[65] The drug was subsequently approved for the European market, but the US Food and Drug Administration (FDA) did not approve the drug due to the 6% of patients developing elevated liver enzymes. It was subsequently found that there was a potential of liver toxicity even after discontinuing the drug. Due to these concerns, AstraZeneca withdrew ximelagatran from the market in 2006. Ximelagatran is a cautionary tale of the potential hazards of introducing new drugs in large patient populations.

2.4.2 DABIGATRAN

Dabigatran etexilate is a DTI developed by Boehringer Ingelheim. The European Medicines Agency (EMA) first licensed dabigatran etexilate in 2008 for thromboprohylaxis after orthopaedic surgery. It was licensed for use in AF in 2011 and DVT and uncomplicated PE in 2014.

Dabigatran etexilate is a prodrug with the active compound dabigatran, which is released after hydrolysis of the drug by esterases in the liver, gut and plasma. In healthy male volunteers, dabigatran etexilate was tolerated well with no evidence of major bleeding at doses from 10 to 400 mg up to three times a day for 7 days.[66] At the highest dose of 400 mg three times daily, bruising at venipuncture sites and gum bleeding did occur. In these early studies, dabigatran was shown to consistently prolong the APTT at 8 and 12 hour dosing intervals. Renal function is essential, as 80% of dabigatran

clearance is through the kidneys. Dabigatran etexilate was initially administered in tablet form. However, as there was significant interindividual variation in dose responses, a multiparticulate pellet capsule with sealed coating was developed.[1,66]

In 2007, a study in healthy male volunteers with multiple dabigatran doses (doses ranging from 100 to 400 mg three times daily) was performed. Drug was administered after a fast and standardised meals were given over the study period (6 days).[67] Blood samples were taken 15 minutes before first drug administration and subsequently in serial samples post-administration. Urine samples were also collected 30 minutes before and serially after drug administration. The dabigatran concentration was assessed by liquid chromatography tandem mass spectrometry (LC-MS/MS). In this study, blood samples were also collected for functional coagulation measurements APTT, PT, TT and ecarin clotting time (ECT), using a Stago Compact (Roche Diagnostics, Basel, Switzerland) coagulometer for the APTT and PT and a Biomatic B10 coagulometer (Desaga, Wiesloch, Germany) for the TT and ECT. The specific coagulation reagents used were not described. The mean dabigatran peak concentrations were 128 ng/mL, 199 ng/mL and 303 ng/mL for the 100 mg, 200 mg and 400 mg thrice-daily groups, respectively. The CV was high (16-46%), depending on the concentration. The time to highest dabigatran concentration was 1.25-1.5 hours. The half-life of dabigatran was 14-17.2 hours and again the CV was high (16-32%). The steady state of drug metabolism was attained on day 3 with thrice-daily administration. There was a clear dose effect of dabigatran plasma concentration of INR, APTT, TT and ECT observed in this study. The prolongation pattern in APTT was curvilinear above a dabigatran concentration of approximately 150 ng/mL. TT, INR and ECT all showed a linear response. INR values, however, remained below 2.0 at concentrations below 400 ng/mL. TT was highly sensitive, with several-fold prolongations already at levels of 100 ng/mL of dabigatran.

The safety and effectiveness of dabigatran was first studied in orthopaedic patients. In the BISTRO I study in 2004 (Boehringer Ingelheim Study in Thrombosis), dabigatran was assessed in 314 patients undergoing total hip arthroplasty (THA) in a sequential dose-escalating study with dabigatran doses 12.5 to 300 mg twice daily.[66] The aim was to study bleeding events and occurrence of thromboembolic events at particular dose levels. The safety outcome was major bleeding and the primary efficacy outcome was VTE events confirmed by venography. No patients developed major bleeding, but at the highest concentration of 300 mg twice daily, there were minor bleeding events, leading to discontinuation of this dose level. The DVT events were lowest with the highest dabigatran dosage. The dabigatran peak level was 146 ng/mL at 150 mg twice-daily dosing. A linear correlation was observed with prolongations of ECT and APTT and dabigatran concentration. In the BISTRO II study in 2005, which included 2039 patients undergoing total hip or knee replacement, patients were randomised to receive dabigatran etexilate or enoxaparin.[68] In a subgroup of 325 patients, dabigatran concentrations were

measured preoperatively and on postoperative days 4 and 5 pre-dose and at serial intervals 0-12 hours post-dose using LC-MS/MS. There was an observed dose-response relationship with increased bleeding risk with increasing dabigatran concentration, as well as an inverse relationship with DVT risk and dabigatran concentration, suggesting a therapeutic range for dabigatran. The steady state concentration of dabigatran was 48-271 ng/mL. The time to peak dose was 2.3-2.9 hours at steady state. In comparison with enoxaparin, dabigatran appeared to be more effective than enoxaparin in preventing thrombosis, with an increase in bleeding events. Dabigatran was subsequently licensed by the EMA in 2008 for prophylaxis of thrombosis after orthopaedic surgery at doses of 220 mg once daily for most patients and a reduced dose of 150 mg once daily for patients with decreased renal function (creatinine clearance of 30-50 mL/min), patients on verapamil, amiodarone, quinine and elderly patients (over 75 years old). It is advocated, that no routine coagulation monitoring is necessary.

In a pivotal RE-LY trial in 2009, dabigatran was assessed in the prevention of stroke in non-valvular AF to demonstrate non-inferiority to warfarin.[69] The multi-centre study included 18113 AF patients randomised to receive blind doses of dabigatran 110 mg twice daily, dabigatran 150 mg twice daily or warfarin unblinded. The primary outcomes were stroke or systemic embolism and the primary safety outcome was major bleeding. The average patient age was approximately 71.5 years in all three study groups, with 63% being male. Both the 110 mg and 150 mg twice-daily doses were non-inferior to warfarin regarding stroke or systemic embolism rates at 1.53% per year, 1.11% per year and 1.69% per year, respectively. The rates of major bleeding were 2.71% per year, 3.11% per year and 3.36% per year with dabigatran 100 mg, dabigatran 150 mg and warfarin, respectively. The study established non-inferiority in the clinical setting. The major limitations were lack of dabigatran concentration measurements in the patients and the lack of reporting the warfarin arm parameters in assessment of warfarin treatment (e.g. TTR). In a subsequent analysis, in centres with poor-quality warfarin treatment of under 57.1% TTR, dabigatran was safer in preventing both bleeding and thrombosis (hazard ratio, HR 0.48-0.89). However, in a cohort with high TTR of over 72.6%, there was no clear difference between warfarin and dabigatran (HR 0.59-1.45). Interestingly, the TTR was a clear indicator for the quality of anticoagulation as a whole, since in centres with high TTR, the bleeding and thromboembolism incidence was also lower in the dabigatran groups.[70] Dabigatran was approved for treatment of non-valvular AF in 2011 with 150 mg twice-daily dosing, except for patients over the age of 80 and patients on verapamil, where the recommended dose is 110 mg twice daily. For patients 75 to 80 years old, patients with moderate renal insufficiency, patients with gastritis, esophagitis or reflux disease and patients with increased bleeding risk, individual risks need to be taken into account. These patients should use either 150 mg or 110 mg twice-daily dosing, dependent on the individual assessment. However, compared to dabigatran, warfarin was shown to be superior in patients with

artificial mechanical heart valve, and the study was terminated due to ethical reasons as there was a clear increase in risk with dabigatran for thromboses.[71]

In the RE-COVER trial in 2009, dabigatran was compared to warfarin in the treatment of DVT after an initial parenteral anticoagulation therapy.[72] The dabigatran dose was 150 mg twice daily. In this double-blind multi-centre study, 2539 patients experiencing DVT or PE were recruited, with a sample size of 1274 in dabigatran and 1265 in the warfarin group. The majority (90%) of patients were on low molecular weight heparin (LMWH) treatment before initiation of the study group, with the remainder on unfractioned heparin or fondaparinux. The TTR for INR was 60%. 30 patients (dabigatran group) and 27 patients (warfarin group) had thromboembolic events. Dabigatran was non-inferior to warfarin in terms of efficacy. 20 patients (dabigatran group) and 24 patients (warfarin group) had major bleeding episodes. Thus, dabigatran was non-inferior in terms of adverse effects. Dabigatran was approved for the treatment of DVT and uncomplicated PE in 2014, with the same dosage as with AF.

Information on dabigatran effects in real-life patient population, i.e. patients not on randomised controlled trials has emerged. In a recent meta-analysis comprising 700000 dabigatran patients, dabigatran had a favourable profile of stroke incidence (HR 0.86, 95% CI 0.74-0.99) and major bleeding rate (HR 0.79, 95% CI 0.69-0.89) compared to warfarin. Dabigatran adherence has been shown to be significantly lower in real-life settings than in the randomised controlled trials. In a New Zealand study, 70% of patients continued dabigatran treatment at 8 months after initiation. The primary reasons for discontinuation were drug side effects (particularly dyspepsia).[73] More encouragingly, in a recent Danish cohort study, dabigatran adherence was 83.9% measured as the proportion of days covered for patients at one year of treatment, after excluding the patients discontinuing treatment during that first year. It seems that adherence is good if the patients do not encounter severe side effects.[74] In the Dresden DOAC register study, discontinuation rates with dabigatran were similar to that of warfarin (approximately 25%).[75]

2.4.3   RIVAROXABAN

Rivaroxaban was developed by Bayer and is the first FXaI in its class. The EMA licensed rivaroxaban for thromboprophylaxis after orthopaedic surgery in 2008, for thromboprophylaxis of non-valvular AF and treatment of DVT in 2011, for treatment of PE in 2012 and for thromboprophylaxis in acute coronary syndrome in conjunction with acetylsalicylic acid (ASA) alone or ASA and ticagrelol or clopidogrel in 2013.

In a 2005 study, rivaroxaban was shown to be safe and well-tolerated after a single dose in healthy male volunteers (dose ranging 5 to 80 mg). Drug concentration was measured with LC-MS/MS and functional drug effects were assessed with the coagulation assays anti-Xa inhibition, PT and APTT, among others. At the 20 mg dose, rivaroxaban reached a concentration of 173 ng/mL

with a time to highest concentration of 1.5 hours and half-life of 12.4 hours. The coagulation assay anti-Xa inhibition showed a curvilinear response with increasing rivaroxaban concentrations, whereas Quick PT showed a linear response. There were no adverse effects reported with the drug.[2] In another study, with multiple doses of rivaroxaban ranging from 5 mg once daily to 30 mg twice daily, healthy male volunteers were given the drug for four days, with monitoring of vital signs until 12 days and a full physical examination at the end of the study. Concentration was analysed by LC-MS/MS and coagulation function with anti-Xa inhibition, PT and APTT, among others. The steady-state drug concentrations reached a maximum of 158 ng/mL for the 10 mg twice-daily dosing, and the time to highest concentration was 3 hours and the half-life was 8 hours. While one patient had elevated ALT levels while on rivaroxaban, ALT elevations were also observed in the placebo group. In the coagulation assays, the response was again concentration dependent, being curvilinear in the anti-Xa inhibition assay and linear in the PT assay.[76]

In a phase II study with approximately 800 patients conducted in 2006, rivaroxaban was evaluated for thromboprophylaxis of patients with THA in both multiple-dose daily and single-dose daily regimens. In both cases, rivaroxaban at multiple doses was compared to enoxaparin 40 mg daily. Rivaroxaban performed favourably, particularly at the 10 mg once-daily dosing, where the risk of composite end point events including DVT, PE, and all-cause mortality was lowest (10.6% risk with rivaroxaban and 25.2% risk with enoxaparin, with a bleeding risk of 0.7% and 1.9%, respectively).[77,78] In a 2008 phase III trial with 4500 patients, rivaroxaban 10 mg once daily was compared to enoxaparin 40 mg daily. Rivaroxaban achieved a statistically significant risk reduction in major venous thromboembolism of 1.7%, with bleeding rates similar to enoxaparin. It was concluded that rivaroxaban is as safe and more effective than enoxaparin in this setting. There were no drug concentration measurements.[79] The EMA licensed rivaroxaban in 2008 for thromboprophylaxis after orthopaedic surgery at 10 mg once daily dosing. There is no dose reduction protocol, but caution should be exercised when prescribing to patients with renal impairment.

A major breakthrough for rivaroxaban use was the 2011 phase III randomised study ROCKET-AF, which compared rivaroxaban with warfarin in patients with non-valvular AF.[80] The study included 14000 patients. The INR was measured using a point-of-care (POC) coagulometer Alere INRatio®. Rivaroxaban was non-inferior to warfarin, with the primary endpoint of stroke and systemic embolism occurring in 1.7% per year in the rivaroxaban group and 2.2% per year in the warfarin group. Bleeding rates of 14.9% per year (rivaroxaban group) and 14.5% per year (warfarin group) were observed. It was concluded that rivaroxaban is at least as effective as warfarin in this setting. There were no drug concentration measurements. In a subsequent analysis comparing different centre TTRs, it was observed that the trend favouring rivaroxaban was present in all of the different TTR groups, but even greater when there was poor TTR control. Again, the centres with high TTR

also had lower rates of bleeding or thrombosis in the rivaroxaban group, suggesting better overall patient management.[81] In December 2014, the FDA issued a recall of Alere INRatio® coagulometers, raising doubts about the accuracy of the results in the ROCKET-AF trial. In re-analyses of the data, it was shown that the original conclusions are still valid. The EMA and FDA have reviewed these data and concluded that there exists no reason to change the original recommendation to license the drug for thromboprophylaxis in AF patients, originally granted in 2011.[82] The dose is 20 mg once daily, with a reduced 15 mg daily dose for patients with a creatinine clearance of 30-49 mL/min.

Rivaroxaban in DVT treatment was also studied in a phase III trial including 3500 patients conducted in 2010.[83] Rivaroxaban was started at 15 mg twice daily for 3 weeks, after which the dose was 20 mg once daily compared to the standard therapy of enoxaparin followed by the VKAs warfarin or acenocoumarol. The efficacy of rivaroxaban was non-inferior, with an event rate of 2.1% (rivaroxaban) and 3.0% (VKAs) and identical bleeding rates (8.1%) in both groups. In this study, the INR was measured by a central laboratory, so there was no issue with POC coagulometer. In a subsequent trial, rivaroxaban also performed favourably against VKA in the treatment of PE, with the primary efficacy outcome occurring in 2.1% (rivaroxaban) and 1.8% (standard therapy). The bleeding risk was 10.3% (rivaroxaban) and 11.4% (standard treatment).[84] As the exclusion criteria included fibrinolytic treatment and hemodynamic instability, the results can only be generalised to uncomplicated PE. Neither of the trials included drug concentration measurements. The EMA licensed rivaroxaban for treatment of DVT in 2011 and uncomplicated PE in 2012 with a dosage of 15 mg twice daily for 3 weeks and subsequently 20 mg once daily. In a subsequent Magellan trial, rivaroxaban was non-inferior to enoxaparin for prevention of VTE in medically ill patients, but had increased risk of bleeding.[85]

There are registry-based studies assessing the effects of rivaroxaban in real-life settings. In the Dresden NOAC registry study, rivaroxaban was found to have comparable rates of thromboembolism (2.0/100 patient-years) compared to the ROCKET-AF study. The major bleeding rate was also acceptable (3.0/100 patient-years). The thrombosis and also bleeding rates were higher when the 15 mg daily dose was used, probably due to the lower dose used in a patient population with a higher general bleeding risk.[86] Rivaroxaban adherence was reported to be relatively better than with dabigatran and warfarin, with approximately 80% of patients persisting with treatment. This might be due to the different side-effect profile of rivaroxaban, i.e., dyspepsia is less common than with dabigatran.[86,87]

2.4.4 APIXABAN

Apixaban, developed by Pfizer, is the second FXaI to reach the market. It was licensed by the EMA in 2011 for thromboprophylaxis in patients with hip or

knee replacement, in 2012 for thromboprophylaxis of AF and in 2014 for DVT and PE treatment.

Apixaban was found to be a potent inhibitor of FXa using models based on spiked samples published in 2007.[88,89] In a study using radiolabelled apixaban after a single 20 mg dose of the drug in male healthy volunteers, apixaban metabolism was found to be approximately 15 to 30% through the renal route (captured radioactivity in urine). Apixaban concentrations were measured using LC-MS/MS, and apixaban radioactivity reached the highest value of 509 ng-Eq/mL with a time to highest concentration of 1 hour and a half-life of 13 hours.[3] The apixaban summary of product characteristics states that the expected concentrations are 171 ng/mL at maximum and 103 ng/mL at minimum with a 5 mg twice-daily dose.[90]

In 2007, apixaban was shown to be as effective as enoxaparin and warfarin in thromboprophylaxis after knee replacement surgery.[91] The study comprised 1238 patients, with patients experiencing the primary end point of venous thromboembolism and all-cause mortality with the most benefit at the 2.5 mg twice-daily dose, rate of 9.0%, compared with 15.6% (enoxaparin) and 16.6% (warfarin). There was a relative risk reduction of 21 to 69% compared to enoxaparin. There was not a single major bleeding event at the 2.5 mg twice-daily dose; this was not observed in the enoxaparin and warfarin groups either. When all bleeding events (including minor) were analysed, the rates were 3% (apixaban 2.5 mg twice daily) and 5% (enoxaparin and warfarin). It was thus concluded that apixaban is at least as good as standard prophylaxis in this indication and that the 2.5 mg twice-daily dose seemed to outperform the others in terms of efficacy. However, as the bleeding events were few and no major bleeding events were recorded, the data was not sufficient to make any safety conclusions. No apixaban concentration measurements were included. In a subsequent 2010 study, similar effects were shown in a study of 5500 hip replacement patients using apixaban 2.5 mg twice daily compared to enoxaparin 40 mg once daily.[92] The primary efficacy endpoint, thrombosis, occurred in 1.4% (apixaban group) and 3.9% (enoxaparin group). The composite endpoint of major and clinically relevant bleeding occurred in 4.8% (apixaban group) and 5.0% (enoxaparin group). It was therefore shown that apixaban is associated with fewer thrombosis events with similar rates of bleeding as enoxaparin. Apixaban was licensed by the EMA in 2011 for thromboprohylaxis after orthopaedic surgery at a dose of 2.5 mg twice daily.

A major finding facilitating widespread apixaban use is the 2011 ARISTOTLE study on apixaban effects in AF.[93] In a large study comprising 18000 patients with non-valvular AF, apixaban performed favourably in comparison with warfarin. The apixaban dose used was 5 mg twice daily. The rate of thrombotic outcome was 1.3% per year (apixaban) and 1.6% per year (warfarin). The major bleeding rate was 2.1% per year (apixaban) and 3.1% per year (warfarin). The TTR for warfarin treatment had a median of 66% and mean of 62%. It was concluded that apixaban was superior to warfarin in thromboprophylaxis in patients with non-valvular AF. The effect was similar

in centres with different levels of TTR, with HRs for stroke (range 0.73-0.91) and bleeding (range 0.50-0.75) for centre TTR levels from 25-60% and 71-83%, respectively. Again the event rates were slightly lower in centres with a good TTR control.[94] Another study investigated potential apixaban use instead of ASA when VKA was considered unsuitable. There was a clear benefit with apixaban (dose 5 mg twice daily) in comparison to ASA (dose 81-324 mg daily), with thromboembolic rates of 1.6% per year (apixaban) and 3.7% per year (ASA). Major bleeding was 1.4% per year (apixaban) and 1.2% per year (ASA). These results established apixaban as a suitable alternative with improved outcome in comparison to ASA.[95] The EMA licensed apixaban in 2012 for thromboprophylaxis in AF, with a standard dose of 5 mg twice daily. A reduced dose of 2.5 mg twice daily is recommended for patients with at least two of the following characteristics: age over 80 years, weight below 60 kg and serum creatinine over 133 µmol/L.

Apixaban in DVT showed a favourable profile in a phase II trial in 2008.[96] In a phase III trial on 5500 patients in 2013, apixaban was shown to be noninferior to conventional treatment using enoxaparin followed by warfarin. The apixaban dose was 10 mg twice daily for 7 days, followed by 5 mg twice daily for 6 months. The thromboembolic rate was 2.3% (apixaban) and 2.7% (conventional therapy). Major bleeding was observed in 0.6% (apixaban) and 1.8% (conventional therapy). Apixaban proved to be noninferior in terms of efficacy and superior in terms of bleeding risk.[97] The EMA licensed apixaban in 2014 for treatment of DVT and uncomplicated PE with a dose of 10 mg twice daily for 7 days, followed by 5 mg twice daily.

2.4.5   EDOXABAN

Edoxaban is a FXaI developed by Daichii Sankyo. The EMA licensed edoxaban in 2015 for thromboprophylaxis in AF and treatment of DVT and PE.

Edoxaban was found to be safe in animal studies in 2008.[98] In healthy human volunteers in a 2010 study, it had peak concentrations 302 ng/mL after a single administration of the 60 mg dose, with time to peak concentration of 1 hour and a half-life of 8 hours.[4] It was subsequently shown to be noninferior to warfarin in thromboprophylaxis in AF and DVT and PE, and was licensed by the EMA in these indications in 2015.[99-101] The standard dose is 60 mg once daily, with a reduced dose of 30 mg once daily in the very elderly and in patients with a reduced creatinine clearance of 15-30 mL/min. Edoxaban effects on coagulation were not assessed in this thesis, and it will not be further discussed.

2.4.6   DOAC ANTIDOTES

If a patient experiences a serious bleed while on DOAC treatment, first the drug is discontinued. DOAC drugs have half-lives of approximately 10-15 h, in a patient with normal liver and renal function.[102] In case of serious bleeding,

non-specific reversal agents recombinant FVII or PCC are used.[103] A specific antidote, however, enables rapid reversal of anticoagulant effect without the considerable risk of thrombosis, the disadvantage of the non-specific agents. For dabigatran, a humanised monoclonal antibody fragment idarucizumab is currently available. Idarucizumab binds to and inactivates dabigatran. It normalises abnormal APTT and PT in dabigatran-treated patients. Idarucizumab was licensed for emergency reversal of dabigatran effects by the EMA in 2015. For rivaroxaban and apixaban, a specific antidote, andexanet alfa, is currently under development. Andexanet alfa mimics FXa, binding the inhibitors, and may be a potential universal antidote for FXa inhibitors. It has been shown to normalise anti-Xa and TG in rivaroxaban- and apixaban-treated patients.[103]

2.5 LABORATORY MONITORING OF DOACs

2.5.1 WHEN TO MONITOR

The consensus among experts is that no routine coagulation monitoring is needed with DOACs. However, at extremes of body weight, the very elderly, in cases of suspected non-adherence, renal or hepatic failure, acute thrombosis or bleeding and emergency surgery, laboratory testing for anticoagulant effect is recommended.[5,10, 104] When assessing renal function, one must bear in mind that in the original studies of DOACs, renal function was assessed with creatinine clearance. Numerically, creatinine clearance is similar, but not equal to the estimated glomerular filtration rate (GFR), which is commonly used to assess kidney function. GFR is an estimate calculated from plasma creatinine level.[105] Generally, also in the elderly population (over 75 years old), DOACs have shown at least non-inferiority and sometimes superiority in terms of efficacy with similar rates of bleeding. However, with declining renal function the risk for bleeding complications increases.[106,107] Extremes of body weight are likely to affect anticoagulant response. Dabigatran is lipophilic and has a very large volume of distribution, while rivaroxaban and especially apixaban have smaller volumes of distribution. In principle, the pharmacodynamics studies favour one-dose-for-all protocols, since at steady state the differences between individuals of different weight are smaller.[108-110] However, plasma concentrations might be affected at the extremes of BMI.[111] Dabigatran is the only DOAC that is hemodialysable, with a high volume of distribution causing a rebound effect in concentration if haemodialysis is ended prematurely.[112] Renal function is of crucial importance especially with dabigatran, due to dabigatran metabolising primarily (85%) through the renal route.[113] For DOAC assessment in cases of acute thrombosis, bleeding or before surgery, the key components are time of the last drug intake, renal function and functional measurement of DOAC concentration.

2.5.2 PT AND APTT

The coagulation screening tests PT and APTT are widely available in clinical chemistry laboratories, with short turnaround times facilitating stat measurements and establishing a 24-hour service. They are well known among clinicians prescribing anticoagulants, and INR (used to monitor warfarin) is a test of choice for patients with bleeding risk.

Unfortunately, for the DOACs, coagulation assessment is not as simple as measuring PT and APTT. In the early pharmacokinetic and pharmacodynamic trials, rivaroxaban and dabigatran have shown a linear response of PT values with increasing concentrations. APTT showed a curvilinear response with relatively high sensitivity for dabigatran.[67,76] Further experiments with *in vitro* spiking of DOACs have shown variable responses using different reagents for the PT and APTT.[6,114-117] INR, while standardised for VKA treatment, cannot be used to measure the effects of DOACs. Accordingly, it is difficult to use PT and APTT in clinical practice, as local laboratories should examine the effects of DOACs on the assays they are using. Apixaban is especially problematic, since both PT and APTT usually remain within the reference interval at treatment levels, with levels above 700 ng/mL required to double the values.[8] In addition, the effects observed using spiked samples cannot be generalised to patient samples. Typically, in actual patient populations, the effects are similar but express even greater variation between reagents.[9,118-120] However, most of the studies on the effects of DOACs on these assays have been performed using *in vitro* spiking.

2.5.3 SPECIFIC COAGULATION ASSAYS

For the DTI dabigatran, laboratory assays mostly depending on thrombin activation seem to be suitable alternatives for activity measurement. Indeed, TT is very sensitive to the effects of dabigatran. The problem with the test is that the upper limit of measurement is exceeded already at low dabigatran concentrations.[121] A more specific assay, diluted thrombin time (dTT), is commercially available in Europe. In dTT, the assay is diluted with reference plasma that can be specifically calibrated to quantify dabigatran (Hemoclot Thrombin Inhibitors® assay).[122] While the result is expressed as dabigatran concentration, it should be noted that what is actually measured is fibrin formation in time, instead of the absolute concentrations. The functional concentration, which might be higher or lower than the actual plasma concentration, depends on the clinical situation.

Other assays that are generally not in use in routine coagulation laboratories can be used to measure dabigatran effects. The ecarin clotting assay (ECA) is a chromogenic assay derived from ECT, in which a chromogenic substrate instead of clotting time (with fibrin formation) is measured. The substrate is cleaved by meizothrombin, which has biologically similar effects to thrombin.[123,124] ECA can measure the effects of DTIs, as shown in 2003 for hirudin, and later also for dabigatran.[104,125,126] Another assay that can be used

is the anti-IIa assay, which detects thrombin inhibition with the use of a specific chromophore (paranitroaniline) and known amounts of added thrombin. The assay is used during unfractionated heparin therapy, but also has the potential to measure thrombin inhibitors, such as dabigatran.[127,128]

While the FXaIs apixaban and rivaroxaban affect routine anti-Xa results, the problem lies with the LMWH-calibration of the routine assay, rendering direct assumptions about DOAC concentrations unreliable. However, the anti-Xa assay can be calibrated with known rivaroxaban and apixaban concentrations to establish functional concentrations of these drugs.[114,115,129]

Another potential assay for DOAC measurement is the dilute RVVT, which uses snake venom from Russel's viper, a snake native to India. Native Russel's viper venom activates both factor V and factor X. Some commercial preparations use the native venom, and some use only the factor X activating RVV-X, which is more specific to factor X inhibition.[130] As previously discussed, the RVVT assay is commonly used in coagulation laboratories for the detection of lupus anticoagulant.[131] It has the potential to detect both thrombin and FXa inhibitors.[104] In a recent study, RVVT was prolonged with increasing dabigatran and rivaroxaban concentrations, while the type of DOAC (FXa or thrombin inhibition) could not be elucidated.[132]

The prothrombinase-induced clotting time (PiCT) has been assessed for monitoring of thrombin inhibitors shortly after its introduction. PiCT is also based on Russel's viper venom, but utilising the compound activating FVa, and with a defined amount of added FXa.[133-135] PiCT has recently been studied for measurement of dabigatran, but also rivaroxaban and apixaban, with clear prolongations in spiked samples.[116,136,137] The one-step protocol, without the standard 180-second incubation after activator reagent (but before calcium reagent addition), is recommended for rivaroxaban assessment. The standard approach seems insensitive to low rivaroxaban concentrations. Instead, a modified approach without incubation period is recommended.[138]

2.5.4   MASS SPECTROMETRY

Mass spectrometry is the gold standard for drug concentration measurements. Its advantages are that it is based on the molecular mass and chemical properties of the drug and has the potential to measure any molecule, regardless of function. To achieve this specificity, substances need to be introduced in the mass spectrometer in as pure a form as possible to enable detection of the desired substance at low levels, which would otherwise disappear in the background noise. To enable any meaningful mass spectrometry findings, chromatography is initially used to separate the compounds. In chromatography, a stationary phase binds to the substances introduced in the mobile phase at different strengths based on the chemical composition of the substance. Different substances pass through the stationary phase at different time intervals, so each substance has a specific retention time. The stationary phase can be gas, solid or liquid. High-

performance liquid chromatography (HPLC) is commonly used in clinical laboratories.[139]

HPLC used as a separator technique before mass spectrometry enables only relatively pure substances to enter the mass spectrometer. In the mass spectrometer, molecules are detected based on their mass-charge ratio (m/z). Substances entering the device are first ionised using e.g. heat or an electrical field. The ions are then accelerated in an electrical field, and the time to strike the detector is based on their mass-charge ratio (time-of-flight mass spectrometry). To gain higher specificity with a lower background signal, tandem mass spectrometry is often used. In this method, compounds of specific mass-charge ratio are firstly selected with a quadrupole mass spectrometer, where only the specific compound can pass through. These ions are then accelerated again, and collided with an inert gas molecule (often nitrogen, argon or xenon). The molecule subsequently fragments based on its chemical properties and the fragments strike the detector. With this method, molecules having the same retention time and mass-charge ratio can be separated. The result is a highly specific and sensitive assay, able to reliably determine drug concentrations at the 1 ng/mL level. With selective reaction monitoring, the reaction can be further sensitised to include only the molecule with the specific fragmentation pattern. This comes at the expense of potentially not detecting other molecules, but is highly specific to the molecule studied.[140]

LC-MS/MS to measure DOACs was validated early in the drug development process and used in pharmacokinetic studies to assess concentrations.[3,67,76] Several groups have set up mass spectrometry methods to enable comparison of coagulation assays and functional concentrations to the actual plasma concentration, with very low limits of detection at approximately 1 ng/mL.[137,141-144]

Mass spectrometry remains the gold standard for DOAC concentration measurement. The challenges with the method are the high technical skill and expertise required to set up and maintain the assay.[145] University hospitals and other large centres will have the facilities to do these assays, but longer turnaround times than the routine coagulation assays are expected. Furthermore, the functional assay might actually give a more accurate overall situation regarding patient care, as other pro- or anticoagulant factors present might be reflected in the data obtained with these assays. The strength of mass spectrometry is the ability to detect very low drug concentrations, though the clinical significance of this is debatable.

2.5.5   THROMBIN GENERATION

As opposed to the coagulation screening tests PT, APTT and TT, where the reaction is stopped at the formation of the first fibrin strands, TG assays measure the entire spectrum of thrombin formation. This includes not only the initiation phase, but also the propagation and amplification of coagulation.

The principle of TG measurement is simple. In a solution containing fibrinogen, the amount of thrombin is related to clotting time. When calibration is done with known amounts of added thrombin, the amount of thrombin in the unknown sample can then be deduced from the clotting time. In blood samples, when samples are taken from the plasma aliquot at serial time intervals, the TG curve as clotting progresses can be obtained.[146] However, this is a highly laborious process demanding approximately 1 hour per sample analysis time of a skilled laboratory technician. A great innovation was the use of a fluorogenic substrate, which releases a fluorophore after activation by thrombin. The fluorophore is longer lasting and enables measurement of thrombin formation using a fluorometer. The measurement is done on a well plate in parallel with a calibrator. The alpha-2-macroglobulin-thrombin complex is used as a calibrator to provide a constant thrombin-like activity, i.e. a fluorescence signal. The TG is then calculated from the fluorescence signal corrected with the calibrator signal. This can be done in a semi-automated fashion, using the Calibrated Automated Thrombogram® (CAT) assay. The acquired parameters include lag time, i.e. time to the start of thrombin formation, time to peak, i.e. time when maximum concentration of thrombin is reached, peak thrombin concentration (Peak) and endogenous thrombin potential (ETP), which is the area under the curve, reflecting the total amount of thrombin formed. The assay generally takes approximately 1 hour, of which only a small proportion is hands-on time. The drawback is that platelet-rich or platelet-poor plasma needs to be prepared.[147] Recently, a new method was developed using whole blood, but most of the research so far has been performed using the plasma-based assay.[148]

Diminished TG is seen in haemophilia; lower TG associated with a more severe bleeding phenotype.[149] Increased TG is seen in thrombophilia, and it has even been proposed that increased TG induces a risk of venous thrombosis, regardless of the cause.[150,151] Vice versa, in a prospective cohort study, it was observed that after discontinuation of anticoagulation therapy, patients with lower TG have a significantly smaller risk of recurrence.[152] Furthermore, consistent with the prothrombotic state in normal pregnancy, TG parameters were increased significantly according to gestational weeks in healthy women.[153]

With the DOACs, the expected effect on TG is diminished Peak and ETP with prolonged lag time. This has been shown in studies with *in vitro* spiking of pooled plasma and also in some studies including patient samples.[154-156]

2.5.6 POINT-OF-CARE ASSAYS

POC assays are increasingly used to monitor a patient's coagulation status on-site. They are frequently used in emergency rooms when rapid assessment of coagulation status is vital, e.g. when considering ischemic stroke thrombolysis or emergency surgery. They also offer access to coagulation tests in remote

locations, where the cost of a laboratory haematology analyser might be excessive.

POC coagulation assays generally use whole blood instead of plasma. The sample is added directly to the measurement strip or cuvette, so anticoagulant use is not necessary upon immediate processing. No sample dilutions take place either, as with standard PT and APTT protocol. The dilution factor is probably the key to differences of the behaviour of POC coagulometers and central laboratory assays.

With DOACs, it was observed first with dabigatran that the POC INR can be significantly prolonged. In a patient case report, a 59-year-old woman who had recently switched from warfarin to dabigatran 150 mg twice daily had a point-of-care INR of 7.2 (with a Hemochron Jr. Signature+® device, International Technidyne Corporation) and a central lab INR of 1.6. In another patient, a 52-year-old man, a POC INR of 1.6 was observed 16 hours after a single dose of dabigatran.[157] In a study of whole blood from healthy volunteers spiked with dabigatran, a POC INR (measured with a Hemochron Jr. Signature+® device) was increased from 1.7 to 4.0 with concentrations of approximate trough to peak levels of dabigatran from 60-275 ng/mL. The central lab recorded an INR increase from 1.1 to 1.5.[158] In actual patients treated with rivaroxaban or dabigatran, the same phenomenon was observed, with a PT prolongation in whole-blood measurement of 65%.[159] One coagulometer (Coaguchek XS Pro®, Roche Diagnostics) showed an almost linear correlation of rivaroxaban (R=0.82), but was less specific for apixaban and had no correlation with dabigatran.[160] One must realise that the findings are not generalizable to all POC assays, but must always be interpreted only in the context that they were performed. However, if a laboratory supplies a specific POC analyser e.g. at the emergency department and local studies were performed, it might be possible in the future to use the POC assays as a qualitative test to detect some of the DOACs.

2.5.7 INTERFERENCES OF DOACS ON COAGULATION ASSAYS

DOACs can interfere with many commonly used coagulation assays albeit the abnormal assay result may refer to functional impairment anyway. Although APTT and PT as methods to test the agents are unreliable, the drugs can interfere with factor assays based on these measurements. Indeed, APTT-based factor assays in particular might show erroneously low results. However, PT-based assays might also be modestly affected with large concentrations of the drug.

Some fibrinogen assays show diminished responses when dabigatran is present, leading to underestimation of fibrinogen. Dabigatran can also overestimate antithrombin activity in thrombin-based assays, potentially leading to a deficiency state characterised as normal. In APTT-based assays, dabigatran also showed erroneously high APC resistance ratios, which might make heterozygous factor V Leiden patients appear normal.[161] Rivaroxaban

and apixaban have been shown to interfere with protein S activity and FXa-based antithrombin activity. Also, as previously mentioned, the effect of FXa inhibitors to RVVT is pronounced and might cause a false suspicion of lupus anticoagulant.[9]

In light of these findings, it is critical to know the presence of a DOAC also when interpreting thrombophilia tests. Very often the patients have started anticoagulation or were already on anticoagulation therapy before the sample was collected. Care should be taken when interpreting the results with a DOAC present. Even though the lupus anticoagulant confirmation test should limit the interference with anticoagulants, a substantial, 20-40% false positive rate with DOACs present exists.[162] As the use of DOACs is becoming widespread and experience has accumulated, the limitations for interpretations can be better appreciated, analogous to the well-known interferences of warfarin and heparins. Studies on international forums are needed, as well as local testing with the specific assays to aid in their interpretation. The problems with one assay might be surmountable by choosing an assay with different methodology. This was shown by the recent study in which rivaroxaban surprisingly had little effect on the RVVT-based activated protein C resistance assays, despite reports of interference with the APTT-based assay.[163]

2.6 DOAC CONCENTRATIONS IN HEALTHY INDIVIDUALS AND IN PATIENT POPULATIONS

During DOAC development, the effects of the drugs were tested in healthy individuals, which amounted to concentration data. In Table 1, concentration data by LC-MS/MS most relevant to the currently recommended maximum dosages of the DOACs are presented. These early studies, as discussed with the individual DOACS, often used concentrations not in the current summary of product characteristics for safety assessment, while the concentrations used in the patient studies were simulated in pharmacokinetic models.[108,121] Indeed, the expected concentrations of DOACs at peak and trough levels are often derived from these studies.[164] The small sample size and mostly male population in the small series using actual concentration measurements make it difficult to draw conclusions on the concentrations encountered in patients.

Table 1. *DOAC concentrations in pharmacokinetic studies in healthy volunteers*

DOAC	Number of patients	Age range	Male/ female	Dose (mg)	Peak level (ng/mL)		Trough level (ng/mL)		Time to peak level (h)
					mean	SD	mean	SD	
Dabigatran[165]	17	65-87	9/8	150 mg BID	256	58	79	23	3
Rivaroxaban[76]	7	20-45	7/0	10 mg BID	158	30	-	-	3
Apixaban[166]	6	24-41	6/0	5 mg BID	129	13	50	10	4

BID, twice daily; SD, standard deviation

Since DOACs have been approved for use in thromboprophylaxis and thrombosis treatment, several studies have been performed to assess the

actual DOAC concentrations in patient populations to ascertain that the responses seen in the pharmacokinetical models are realised in a real-life setting. Dabigatran has the most studies published thus far, as this drug has been on the market the longest. Table 2 lists studies where DOAC concentrations were assessed in actual patient samples post-authorization. The patients were recruited at phase IV, with the expection of the Reilly 2014 study conducted in the phase III RE-LY trial for dabigatran.

Most of the studies were performed on patients undergoing orthopaedic surgery, where the lower DOAC dose was used. It is likely that the drug peak concentrations encountered in AF and DVT patients might be higher due to the higher treatment dose. However, even in the patients where the higher DOAC dose was used with dabigatran peak levels at the 150 mg BID dose approximately 120-180 ng/mL and trough levels of 70-120 ng/mL reached, significantly lower than the 256 ng/mL peak and 79 ng/mL trough levels in healthy volunteers.[119,165,167,168] With rivaroxaban, the trough levels were estimated to be approximately 30 ng/mL in the pharmacokinetic estimations for the 20 mg OD dose, corresponding to the actual measured concentrations with that dose.[108,168] With the orthopaedic prophylactic dose of 10 mg OD, trough levels as low as 5 ng/mL were observed.[169] With apixaban in a patient study with 5 mg BID dosing, a significantly higher peak (160 ng/mL) and trough level (100 ng/mL) were reached than in healthy volunteers (129 ng/mL peak, 50 ng/mL trough).[166,168] A trend of higher concentrations emerges in studies with mixed indications for DOACs as opposed to use in thromboprophylaxis in the setting of orthopaedic surgery.

Table 2. *DOAC concentrations observed in patient samples in previous studies*

DOAC	Study	DOAC indication	DOAC dose	number of patients	Peak level (ng/mL)			Trough level (ng/mL)			Measurement method
					mean	median	range	mean	median	range	
Dabigatran	Antovic et al. 2013[141]	Atrial fibrillation	not reported	70	-	-	-	-	-	<0.5-586	LC-MS/MS
	Reilly et al. 2014[170]	Atrial fibrillation	110 mg BID	4583	126	133	1-745	65	66	1-608	LC-MS/MS
	Reilly et al. 2014[170]	Atrial fibrillation	150 mg BID	4600	175	184	2-1000	91	93	1-809	LC-MS/MS
	Skeppholm et al. 2014[167]	Atrial fibrillation	110 mg BID	17	-	-	-	-	60	23-168	LC-MS/MS
	Skeppholm et al. 2014[167]	Atrial fibrillation	150 mg BID	73	-	-	-	-	52	8-188	LC-MS/MS
	Chan et al. 2015[171]	Atrial fibrillation	110-150 mg BID	100	-	155	-	-	-	<30-722	dTT
	Shimomura et al. 2015[119]	Atrial fibrillation	110 mg BID	259	-	79	-	-	-	-	dTT
	Shimomura et al. 2015[119]	Atrial fibrillation	150 mg BID	57	-	128	-	-	-	-	dTT
	Freyburger et al. 2011[172]	Hip or knee replacement	150-220 mg OD	40	105	-	-	-	-	-	dTT
	Herrmann et al 2013[169]	Hip or knee replacement	150 mg BID	17	129	-	-	68	-	-	dTT
	Samama et al 2013[173]	Hip or knee replacement	150-220 mg OD	65	51	-	0-324	-	-	-	dTT
	Schellings et al. 2016[144]	Hip or knee replacement	220 mg OD	40	-	81	-	-	12	-	LC-MS/MS
	Testa et al. 2016[168]	Various	110 mg BID	90	170	-	30-650	120	-	10-400	dTT
	Testa et al. 2016[168]	Various	150 mg BID	70	180	-	30-550	120	-	10-500	dTT
Rivaroxaban	Freyburger et al. 2011[172]	Hip or knee replacement	10 mg OD	40	117	-	-	-	-	-	DC-anti-Xa
	Herrmann et al 2013[169]	Hip or knee replacement	10 mg OD	15	133	-	-	5	-	-	DC-anti-Xa
	Freyburger et al. 2015[174]	Hip or knee replacement	10 mg OD	51	-	113	-	-	7	-	LC-MS/MS
	Samama et al 2013[173]	Hip or knee replacement	10 mg OD	41	142	-	0-412	-	-	-	DC-anti-Xa
	Schellings et al. 2016[144]	Hip or knee replacement	10 mg OD	40	-	125	-	-	17	-	LC-MS/MS
	Arachillage et al. 2016[175]	Thromboembolism	20 mg OD	105	280	-	-	-	-	-	DC-anti-Xa
	Testa et al. 2016[168]	Various	20 mg OD	37	210	-	50-450	30	-	5-140	DC-anti-Xa
	Testa et al. 2016[168]	Various	15 mg OD	34	180	-	60-410	20	-	5-120	DC-anti-Xa
Apixaban	Freyburger et al. 2015[174]	Hip or knee replacement	2.5 mg BID	51	-	76	-	-	45	-	LC-MS/MS
	Testa et al. 2016[168]	Various	5 mg BID	73	200	-	110-440	140	-	30-400	DC-anti-Xa
	Testa et al. 2016[168]	Various	2.5 mg BID	26	160	-	50-300	100	-	20-280	DC-anti-Xa

BID, twice daily; DC-anti-Xa, drug-calibrated anti-Xa; dTT, diluted thrombin time; LC-MS/MS, liquid chromatography tandem mass spectrometry; OD, once daily

3 AIMS

DOACs are increasingly used for patient anticoagulant therapy and the need for laboratory coagulation testing for these drugs will increase. Specific assays should be widely available, on a 24 hours/7 days per week basis, to support management at on-call hours. We firstly assessed DOAC effects in *in vitro* samples spiked with dabigatran, rivaroxaban or apixaban. Their effects on screening assays and effects of different reagents used in laboratories were analysed. We then examined their effects in patient samples on a wide variety of coagulation parameters.

The specific study aims were to determine:

1) The effects of dabigatran, rivaroxaban and apixaban on coagulation screening tests INR and APTT in a European laboratory survey conducted in conjunction with an external quality assessment (EQA) round (I-II).

2) The effects of dabigatran (I, III) and rivaroxaban (I) on the screening coagulation assays PT, INR and APTT as well as specific coagulation assays and TG (III) in unselected patient samples.

3) The effects of apixaban and rivaroxaban on basic laboratory variables, screening and specific coagulation assays, including TG, in patients undergoing THA (IV).

4 MATERIALS AND METHODS

4.1 SPIKED SAMPLES

Lyophilised pooled human plasma samples spiked with dabigatran, rivaroxaban or apixaban were sent to European laboratories participating in the EQA rounds of Labquality Ltd for INR, PT and APTT (dabigatran, rivaroxaban) and anti-Xa (apixaban). The total number of laboratories was 86. The EQA rounds were conducted in July 2011 (dabigatran), January 2012 (rivaroxaban) and September 2015 (apixaban) and the results were published in studies I and II. The dabigatran, rivaroxaban and apixaban samples were a generous gift from Boehringer Ingelheim, Bayer and Pfizer, respectively. Dabigatran samples were sent to 115 European laboratories (Poland n=49, Finland n=35, Lithuania n=17, Latvia n=9, Estonia n=3 and Iceland n=2), rivaroxaban samples to 38 laboratories (Finland n=18, Norway n=9, Ireland n=5, Denmark n=2, Sweden n=2, Estonia n=1 and Lithuania n=1) and apixaban samples to 28 laboratories (Finland n=20, Denmark n=1, Estonia n=1, Greece n=2, Iceland n=1, Ireland n=2, Norway n=1). In the apixaban round, it was determined that 10 of the laboratories were university centres, among which 5 were in Finland. The laboratories were instructed to reconstitute the samples adding 1.0 mL distilled water, to mix thoroughly and to incubate for 30 minutes at room temperature before analysis.

The lyophilised samples had a final drug concentration of 120 and 300 ng/mL (Aniara) for dabigatran, 60, 146 and 305 ng/mL (Technoclone) for rivaroxaban and 80 (Diagnostica Stago), 120 (Technoclone), 270 (Stago) and 300 ng/mL (Technoclone) for apixaban. Laboratories were asked to use screening tests INR, PT and APTT, as well as specific methods for drug-concentration assessment: TT and calibrated dTT for dabigatran and calibrated anti-Xa for rivaroxaban and apixaban, respectively. For apixaban, laboratories were also specifically asked to assay the samples using their routine anti-Xa assay calibrated for LMWH monitoring as well. The number of laboratories reporting results was 73 (63.5%), 22 (57.9%) and 21 (75%) for the dabigatran, rivaroxaban and apixaban rounds, respectively. For rivaroxaban, in addition to the samples, calibrators with rivaroxaban concentrations of 0, 14, 60, 100 and 150 ng/mL were sent to the 18 participating Finnish laboratories to help set up a calibration of the local anti-Xa assay of rivaroxaban, with 8 laboratories (44%) reporting results with rivaroxaban calibration. For dabigatran, TT was reported by 11 (15%) laboratories and drug calibrated-dTT was reported by only one (1%) university laboratory. For apixaban, 15 laboratories (71%) reported LMWH-calibrated results, but only 7 (33%) reported apixaban-calibrated anti-Xa results.

A multitude of different reagents and analysers were in use in the laboratories surveyed; there were 13 different PT/INR reagents, 4 of the Owren type, 10 different APTT reagents, 3 different TT reagents and 6 different anti-Xa reagents (Table 3). The number of different coagulometers used for analysis was 24 (Table 4).

Table 3.   *Reagents used by the laboratories participating in studies I and II*

Assay	Reagent	Reagent manufacturer	Laboratories dabigatran round (I)	Laboratories rivaroxaban round (I)	Laboratories apixaban round (II)
PT/ INR	Nycotest PT	Axis-Shield	8	2	2
Owren	Owren's PT	Medirox	10	7	12
	SPA Stago	Diagnostica Stago	19	9	5
	Thrombotest	Axis-Shield	7	2	-
PT/INR	Bioksel System PT	Bio-Ksel	2	-	-
Quick	HemosIL PT	Instrumentation Laboratory	1	-	-
	Innovin	Siemens Healthcare Diagnostics	2	2	-
	RecombiPlastin 2G	Instrumentation Laboratory	5	-	2
	Roche Thromboplastin	Roche Diagnostics	1	-	-
	STA Neoplastin CIPlus	Diagnostica Stago	2	-	-
	Technoplastin HIS	Technoclone	1	-	-
	Thromborel S	Siemens Healthcare Diagnostics	12	-	-
	TriniCLOT PT HTF	Tcoag	1	-	-
	INR total		**71**	**22**	**21**
APTT	Actin FSL	Siemens Healthcare Diagnostics	16	8	7
	Bioksel System APTT	Bio-Ksel	2	-	-
	Dapttin TC	Technoclone	1	-	-
	Grifols APTT	Grifols	2	-	-
	HemosIL APTT SP	Instrumentation Laboratory	17	5	7
	HemosIL SynthasIL	Instrumentation Laboratory	-	-	1
	Pathromtin SL	Siemens Healthcare Diagnostics	9	-	-
	STA Cephascreen	Diagnostica Stago	4	2	1
	STA PTT Automate TT	Diagnostica Stago	18	7	3
	TriniCLOT aPTT HS	Tcoag	3	-	1
	APTT total		**69**	**22**	**20**
TT	Comesa Thrombin time	Comesa	1	-	-
	Siemens Thrombin Reagent	Siemens Healthcare Diagnostics	7	-	-
	STA Thrombin	Diagnostica Stago	3	-	-
	TT total		**11**		
Anti-Xa	Berichrom Heparin	Siemens Healthcare Diagnostics	-	2	3
	Chromogenix Coamatic Heparin	Instrumentation Laboratory	-	4	2
	HemosIL Liquid anti-Xa	Instrumentation Laboratory	-	-	5
	HemosIL Heparin	Instrumentation Laboratory	-	-	2
	Rotachrom Liquid Heparin	Diagnostica Stago	-	1	-
	STA Liquid Anti-Xa	Diagnostica Stago	-	1	3
	Anti-Xa total			**8**	**15**

APTT, activated partial thromboplastin time; Anti-Xa, anti factor Xa activity; INR, international normalised ratio; PT, prothrombin time; TT, thrombin time

Table 4. *Coagulation analysers used by the laboratories participating in studies I and II*

Coagulometer		Laboratories in dabigatran round (I)	Laboratories in rivaroxaban round (I)	Laboratories in apixaban round (II)
Manufacturer	**Analyser**			
Behnk	Behnk Elektronik Coagulator	1	-	-
	Thrombolyzer Compact X	2	-	-
Bio-Ksel	Bioksel 6000	1	-	-
	Bioksel Chrom 7	1	-	-
Instrumentation Laboratory	ACL Advance, IL	1	-	-
	ACL 10000	2	1	-
	ACL 9000	3	-	-
	ACL Elite Pro	2	-	-
	ACL Top 500	7	4	9
	ACL TOP Las	1	-	-
	Comesia ACL Pro Elite	1	-	-
Roche	Coasys Plus C	1	-	-
Siemens	Behring Coagulation Timer	1	-	-
	Behring Fibrintimer II	3	-	-
Stago	Amax Destiny Plus	1	-	-
	STA Compact	3	1	-
	Start 4	10	-	-
	STAR Evolution	6	8	4
Sysmex	Sysmex BCS XP	5	1	1
	Sysmex CA 1500	12	5	2
	Sysmex CA 500	5	-	-
	Sysmex CA 7000	-	2	1
	Sysmex CS 2100i	-	-	3
Teco	Coatron M2	1	-	-
Not reported		3	0	1
Total		**73**	**22**	**21**

4.2   PATIENT SAMPLES

As the results from the first EQA rounds were obtained, it became evident that comparison of *in vitro* spiked samples with patient samples would be beneficial. In study I, 10 citrate-anticoagulated plasma samples from patients using dabigatran and 10 from patients using rivaroxaban were obtained. They were unselected samples, from which a DOAC concentration assessment request was received in the Helsinki University Hospital Laboratory (HUSLAB). PT, INR, APTT and drug-calibrated dTT for dabigatran and anti-

Xa for rivaroxaban were performed. The analyser was Sysmex BCS XP® and the reagents were Nycotest PT® (for INR and PT) and Actin FSL® (for APTT). The sample collection was according to the hospital routine protocol: blood was collected into plastic tubes (BD Vacutainer) anticoagulated with sodium citrate (3.2%; 109 nM), centrifuged at 2500 x g for 15 min followed by plasma separation within 2 hours. The vacuum extraction phlebotomy is the preferred sampling method, but in difficult cases, open blood draw was performed. The ratio of sample to anticoagulant was meticulously controlled and analysis was not performed from underfilled or overfilled tubes.

To assess the effects of dabigatran on coagulation in patient samples, in study III, a retrospective analysis was performed on 241 samples from 85 patients, where dabigatran concentration assessment was requested in HUSLAB. The patient samples were accumulated over a 5-year period (2008-2013). All consecutive plasma samples were included in the study, but patient characteristics and treatment indications were not recorded. At that time in Finland, the indications for dabigatran consisted of thromboprophylaxis after orthopaedic surgery (dosage 150 mg or 220 mg once daily) and thromboprophylaxis in the setting of non-valvular AF (dosage 110 mg or 150 mg twice daily). The request of the assay was unlimited; all physicians in the hospital district could request the assay. It was, however, recommended by the laboratory that testing should be performed in special circumstances only, i.e., major bleeds, thrombosis, hepatic or renal failure or emergency surgery and to simultaneously assess PT and APTT. The samples were collected and plasma was separated according to the routine hospital protocol, with aliquots of the plasma preserved at -80 °C before analysis. In a subset of 49 samples from 35 patients, a large panel of coagulation assays was performed, including the anti-IIa, ECA, PiCT, RVVT, fibrinogen, D Dimer and TG in CAT®. In a subset of samples (n=21), the validity of the dTT method was analysed by LC-MS/MS analysis of dabigatran concentration.

Result interpretation of samples from patients using DOACs is difficult if the clinical situation of the patient and the time from last drug intake are not known. To obtain some indication-specific understanding, we conducted study IV on patients using rivaroxaban or apixaban for thromboprophylaxis after THA. This study included patients undergoing THA with the direct posterior technique, where a thromboprophylaxis of 30 days using rivaroxaban or apixaban was planned. Apixaban patients (n=22) were recruited from the Kymenlaakso Central Hospital (Kotka, Finland by orthopaedic surgeon Mikko Manninen) and rivaroxaban patients (n=20) were recruited from the Helsinki University Peijas Hospital (Vantaa, Finland, by orthopaedic surgeon Jarkko Leskinen). The doses were 10 mg once daily (rivaroxaban) and 2.5 mg twice daily (apixaban). Both rivaroxaban and apixaban were started at postoperative day 1, 24 hours after surgery. With rivaroxaban, there was a single dose of enoxaparin 40 mg administered on postoperative day 1, 6 to 10 hours after surgery. This was routine practice in the orthopaedic ward to secure the anticoagulant effect, in case the patient

experienced emesis and was unable to ingest the drugs. With apixaban, no thromboprophylaxis on the day of operation was used. The exclusion criteria consisted of active malignancy, anticoagulant (warfarin, LMWH, fondaparinux) or antithrombotic (clopidogrel, dipyridamole, prasugrel, ticagrelol) medication, with ASA being allowed at up to 100 mg once daily, bleeding tendency, inflammatory bowel, rheumatic or liver disease affecting coagulation, phospholipid antibody syndrome or positive lupus anticoagulant, severe renal insufficiency (estimated GFR < 30 mL/min), pregnancy, breastfeeding, contraindication to rivaroxaban or apixaban and age under 18 years.

The blood samples in study IV were collected at 7 time points: 1) preoperatively, 2) on postoperative day 1 before drug intake, and 3) 3 hours after drug intake, 4) on postoperative day 2 to 8 before and 5) 3 hours after drug intake and 6) on approximately postoperative day 28 before and 7) 3 hours after drug intake. The rationale for this arrangement was that the drug effects could be assessed at peak and trough levels at different time points during recovery and that these can then be compared to the preoperative baseline. The samples were collected according to the local hospital protocol. Routine laboratory assays were immediately performed, with a portion of the citrated plasma stored in aliquots at -40°C until further analysis.

Written informed consent was requested from patients participating in the study. The study protocols were approved by the Ethics Committee of Oulu University hospital, mandating a nationwide study.

4.3 LABORATORY ASSAYS

All the coagulation samples were analysed in HUSLAB (Helsinki University Hospital). The screening assays were performed according to standard hospital protocol. The specialised assays were handled and processed by a laboratory technician skilled in coagulation analyses. The different coagulation assays and their use in the studies is summarised in Table 5.

In the dabigatran study (III), the native TT was used to assess dabigatran levels below the limit of detection in the dTT assay (40 ng/mL). It was assumed that the TT changes linearly with increasing dabigatran levels. TT < 60 s was set to correspond to 0 ng/mL of dabigatran, TT 60-100 s to 10 ng/mL, TT 100-120 s to 20 ng/mL and TT 120-140 s to 30 ng/mL. These data from the native TT and the dTT were combined to obtain functional dabigatran concentration estimates (Dabi-TT) covering the entire concentration range.

In the anti-IIa assay, 25 µL of sample was mixed with 50 µL of substrate and the reaction was started with 250 µL of thrombin reagent. In the ECA, 25 µL of plasma was mixed with 100 µL prothrombin buffer and further mixed with 25 µL of ECA substrate, incubated for 1 minute at 37°C, after which the reaction was started with 50 µL of the ecarin reagent. The specific coagulation assays ECA and anti-IIa were calibrated with dabigatran concentrations of 0,

30, 250 and 510 ng/mL to enable measurement of dabigatran concentration. The PiCT was executed with a two-step protocol: 50 µl of plasma was incubated with 50 µL of the activation reagent, after which the mixture was incubated for 180 s at 37°C, before addition of 50 µl start reagent (calcium chloride).

In addition to the coagulation assays, in study III mass spectrometry was used to confirm the validity of the dabigatran assays in a subgroup of 21 plasma samples. This LC-MS/MS assay was performed by Yuko Rönnquist-Nii from plasma samples at the Karolinska Institutet, Sweden. Protein precipitation was done with acetonitrile solution containing the internal standard dabigatran-d3 (Toronto Research Chemicals), in which three deuterium molecules were added to the native dabigatran. Dabigatran was detected by positive electrospray ionization, with selective reaction monitoring to ion transitions 472 to 289 m/z for dabigatran and 175 to 292 m/z for the internal standard dabigatran-d3. The assay has a lower limit of detection of 1 ng/mL for dabigatran, with a linear range of 1.1-412 ng/mL.

In the study of rivaroxaban and apixaban effects in orthopaedic patients (IV), in addition to the coagulation tests a large panel of basic laboratory assays was included to understand the extent of inflammation and grade of organ dysfunctions. These assays included: Blood cell count, CRP, ALT, albumin, and creatinine and GFR using the MDRD formula. These assays were performed immediately after sample collection in the routine hospital laboratory with standard procedures.

Table 5   *Coagulation assays performed in the Helsinki University Hospital Laboratory (HUSLAB)*

Assay	Reagent Manufacturer	Reagent	Reference range	Analytical range	Used in studies
Screening tests					
PT/INR	Axis-Shield	Nycotest PT	70-130%	6-170%	I-IV
APTT	Siemens Healthcare Diagnostics	Actin FSL	23-33 s	18-180 s	I-IV
TT	Siemens Healthcare Diagnostics	BC Thrombin Reagent	17-25 s	12-140 s	I,III
Anti-Xa LMWH	Siemens Healthcare Diagnostics	Berichrom	NA	0.1-8.0 IU/mL	II
D dimer	Roche Diagnostics	Tina Quant D Dimer	<0.5 mg/L	0.1-9.0 mg/L	III-IV
Fibrinogen	Siemens Healthcare Diagnostics	Multifibren U	1.7-4.0 g/L	1.0-9.0 g/L	III-IV
	Siemens Healthcare Diagnostics	Dade Thrombin	1.8-3.5 g/L*	0.5-8.6 g/L*	III
FVIII:C	Siemens Healthcare Diagnostics	Pathromtin SL	52-148%	1-600%	IV
Antithrombin activity	Siemens Healthcare Diagnostics	Berichrom Antithrombin III	84-108%	10-140%	IV
Specific coagulation assays					
dTT Dabigatran	Aniara	Hemoclot Thrombin Inhibitors	NA	40-1000 ng/mL	III
Anti-IIa	Siemens Healthcare Diagnostics	Direct Thrombin Inhibitor assay	NA	0-510 ng/mL	III
ECA	JenAffin	Haemosys ECA-T	NA	0-510 ng/mL	III
PiCT*	Pentapharm	Pefakit PiCT	19-31 s	19-250 s	III
Anti-Xa Rivaroxaban	Aniara	Anti-Xa Riva	NA	15-1600 ng/mL	I,IV
Anti-Xa Apixaban	Aniara	Anti-Xa Apix	NA	25-2000 ng/mL	II,IV
RVVT**	Sekisui Diagnostics	DVVTest 10	<41 s/<35 s	NA	III-IV
F1+2	Siemens Healthcare Diagnostics	F1+2	NA	NA	IV

Anti-IIa, anti thrombin (IIa) activity; anti-Xa, anti factor Xa activity; APTT, activated partial thromboplastin time; dTT, diluted thrombin time; ECA, ecarin clotting activity; F1+2, prothrombin fragment assay; FVIII:C, FVIII clotting assay; INR, international normalised ratio; PT, prothrombin time; PiCT, prothrombinase induced clotting time; RVVT, Russel's viper venom time; LMWH, low molecular weight heparin. *Values from literature, not locally verified. ** Reference interval changed from   <41 s to <35 s between studies III and IV

TG was performed on the samples in studies III and IV. All TG tests were performed by the same researcher (TH) to ensure consistency. The TG test was performed using the CAT® device (Diagnostica Stago, Thrombinoscope, Maastricht, The Netherlands). The platelet-poor plasma (PPP) was separated using the standard laboratory protocol and subsequently frozen (-40 to -80°C). Before analysis, the sample was rapidly warmed to 37°C and examined. The reaction temperature significantly affects the output, so the water used for instrument rinsing and all the samples and reagents (except the small volumes of PPP reagent and thrombin calibrator) was warmed to 37°C. The analyses

were done using the Stago PPP reagent® with 5 pM TF and 4 μM phospholipids, without corn trypsin inhibitor addition. Firstly, 2400 μL of fluorogenic buffer (Stago Fluo-buffer®) was warmed to 37 °C in a water bath. The analyser was primed (rinsed) with room-temperature water. A 96-well plate was used for the analysis. The measurement wells were first filled with 20 μL of the reagent or calibrator, with two measurement wells per sample, containing PPP reagent and one well (III) or two wells (IV) for calibration with Stago Thrombin Calibrator®. The samples, 80 μL, were then pipetted into the wells. The pipetting was performed with the reverse pipetting technique to prevent the introduction of air bubbles into the sample. The plate was then inserted into the analyser for a 10-minute incubation, to ensure that the sample temperature was constant at 37°C. The analyser was then rinsed with 37°C distilled water (at least 5 mL) to ensure that the internal temperature of the analyser reached 37°C. The fluorogenic substrate, 60 μL, was then added to the 37 °C fluorogenic buffer and the solution was quickly vortexed. Immediately after preparing the solution, the analyser was filled with the fluorogenic substrate using the built-in dispensing system. The dispenser was then reconnected to the analyser to start the automatic measurement. The device then automatically dispensed the fluorogenic substrate with the activator into the sample, periodically measuring thrombin formation by calculating the values by comparing to the fluorogenic calibrator. The measurement time was 60 minutes. In study III, plasma from individual healthy volunteers was used as a reference material. Pooled plasma from healthy volunteers was used in study IV.

4.4   STATISTICAL ANALYSIS

For studies I and II, different reagent groups were compared to one another using analysis of variance (ANOVA) and pair-wise Student's t-test. Variations among the assays were expressed as coefficient of variation (CV). Pearson's correlation was used to determine the accuracy of the specific anti-Xa assays.

For studies III and IV, Pearson's correlation was used to determine the accuracy of the specific assays in comparison to functional concentration as determined by dTT for dabigatran and rivaroxaban- and apixaban-calibrated anti-Xa, respectively. The means for different sample groups were compared with the pair-wise Student's t-test (for normal distributions, such as routine analyses haemoglobin and albumin) and Mann-Whitney's U test (for non-normal distributions, such as drug concentration levels and TG).

The p-value significance limit was set at p=0.05, with results below this value being considered statistically significant.

5 RESULTS

5.1 DOAC EFFECTS ON COAGULATION ASSAYS IN SPIKED SAMPLES

5.1.1 PT/INR

The effects of dabigatran and rivaroxaban (study I) and of apixaban (study II) on coagulation screening tests were examined. The laboratories had 13 different thromboplastin reagents in use (Table 3). The INR was used, as some laboratories reported the PT in seconds and some as activity percentage, thus preventing direct comparisons. A total of 71 laboratories reported INR values for dabigatran. Both dabigatran concentrations (120 ng/mL and 300 ng/mL) showed that INR increased above the baseline of 1.0, with responses being stronger in the 300 ng/mL group (Table 6). The different reagents had different sensitivities. The Owren reagents as a group were less sensitive than Quick reagents to dabigatran (Figure 3A; p<0.001). The relative change in the INR between dabigatran concentrations of 120 ng/mL and 300 ng/mL sample was on average only 1.21 (range 1.11-1.55).

With rivaroxaban (plasma concentrations of 60, 146 and 305 ng/mL), 22 laboratories reported INR values. Five different thromboplastin reagents were used. The increase in INR was less clear with rivaroxaban than with dabigatran, with none of the laboratories reaching INR values of 1.9 or above. The CVs were smaller than with dabigatran (Table 6). The relative change in INR between the lowest and highest rivaroxaban concentration was on average 1.25 (range 1.14-1.43). There was a significant difference between reagent sensitivities (Figure 3C; p<0.001), but only two laboratories used a Quick-type reagent, without significant differences between the Quick and Owren groups.

With apixaban (plasma concentrations of 80, 120, 270 and 300 ng/mL), 21 laboratories reported the INR using 4 different reagents. The INR was inert, with very little prolongation between the lowest and highest concentrations (Table 6; Figure 4A). None of the laboratories reached INR of 1.6. The mean relative change in the INR was 1.10 (range 1.00-1.27).

Table 6. *Studies I and II. Summary of the results reported by different laboratories for the dabigatran, rivaroxaban and apixaban in spiked plasma samples*

	INR		APTT		Anti-Xa FXaI-calibrated		Anti-Xa LMWH-calibrated	
	mean, range	CV(%)	mean, range (s)	CV(%)	mean, range (ng/mL)	CV(%)	mean, range (U/mL)	CV(%)
Dabigatran 120 ng/mL	1.3 (1.0-2.2)	17.9	68 (26-92)	19.0	-	-	-	-
Dabigatran 300 ng/mL	1.6 (1.1-3.3)	28.4	91 (59-127)	18.0	-	-	-	-
Rivaroxaban 60 ng/mL	1.1 (1.0-1.2)	6.7	35 (29-42)	14.3	67 (59-74)	8.1	-	-
Rivaroxaban 146 ng/mL	1.1 (1.0-1.4)	10.7	40 (32-49)	14.9	134 (111-154)	9.0	-	-
Rivaroxaban 305 ng/mL	1.3 (1.1-1.8)	14.6	43 (34-53)	15.5	207 (138-313)	33.8	-	-
Apixaban 80 ng/mL	1.1 (1.0-1.2)	5.4	31 (30-32)	12.8	78 (70-85)	10.8	0.7 (0.6-0.8)	48.8
Apixaban 120 ng/mL	1.0 (1.0-1.2)	5.5	36 (35-38)	13.3	120 (102-138)	16.1	1.1 (1.0-1.3)	45.2
Apixaban 270 ng/mL	1.1 (1.0.1.4)	8.0	34 (32-35)	13.1	294 (260-329)	12.5	2.3 (2.1-2.5)	20.2
Apixaban 300 ng/mL	1.1 (1.0-1.5)	10.6	38 (36-40)	14.6	320 (271-369)	14.6	2.5 (2.2-2.7)	26.5

Anti-Xa, anti factor Xa activity; APTT, activated partial thromboplastin time; CV, coefficient of variation; FXaI, Factor Xa inhibitor; INR, international normalised ratio; LMWH, low molecular weight heparin

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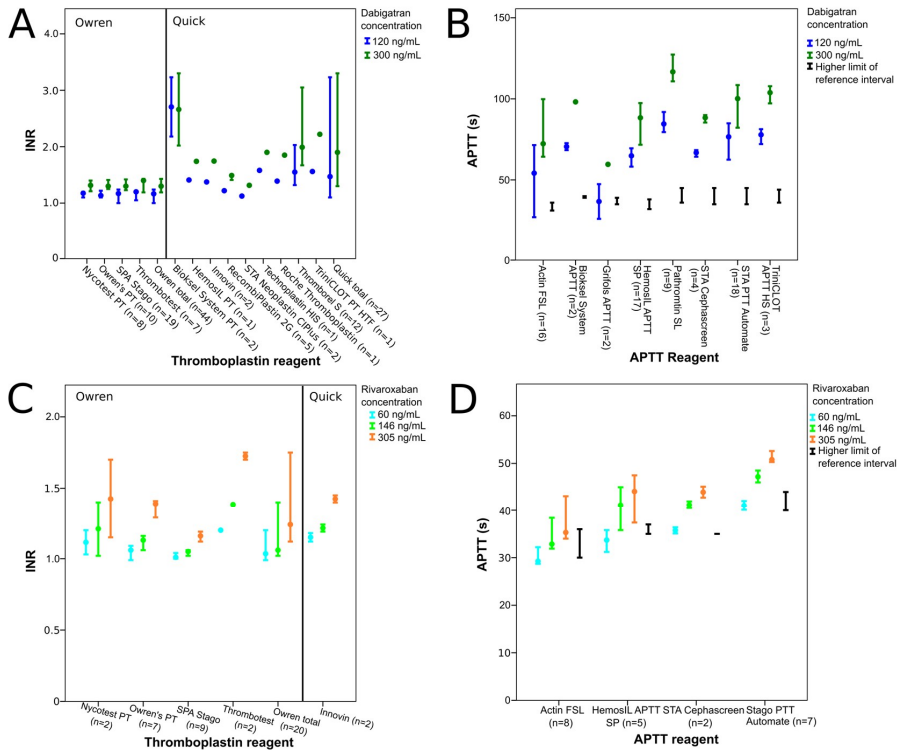
Figure 3 Study I, dabigatran and rivaroxaban. There were significant differences between Owren- and Quick-type thromboplastin reagents in the dabigatran group, with higher sensitivity and variation in the Quick-type reagents with a high variation in APTT response (3A-B). With rivaroxaban, the responses of INR were modest in all the reagents, with all values below INR 1.9, with high variation in the APTT response (3C-D). Median values and range (bars) are shown.

5.1.2 APTT

A total of 72 laboratories reported APTT values with dabigatran. Nine different reagents were used in the dabigatran round (Table 3). The APTT was sensitive to the effects of dabigatran, with 70/72 laboratories reporting prolonged APTT already at the lower concentration of 120 ng/mL. The APTT was significantly prolonged in this sample on average 23.4 s (range 6.5-41.7 s) above the reference interval. An even greater prolongation of 53.8 s (range 20.0-89.3 s) was observed at the higher dabigatran concentration of 300 ng/mL. Again, the sensitivity of APTT varied widely between laboratories (Figure 3B; p<0.001). The relative prolongation of APTT was 1.36 (range 1.31-1.62) between the samples 120 ng/mL and 300 ng/mL, and relative increase above the local reference interval was 2.45 (range 1.61-2.97) at dabigatran concentration of 300 ng/mL.
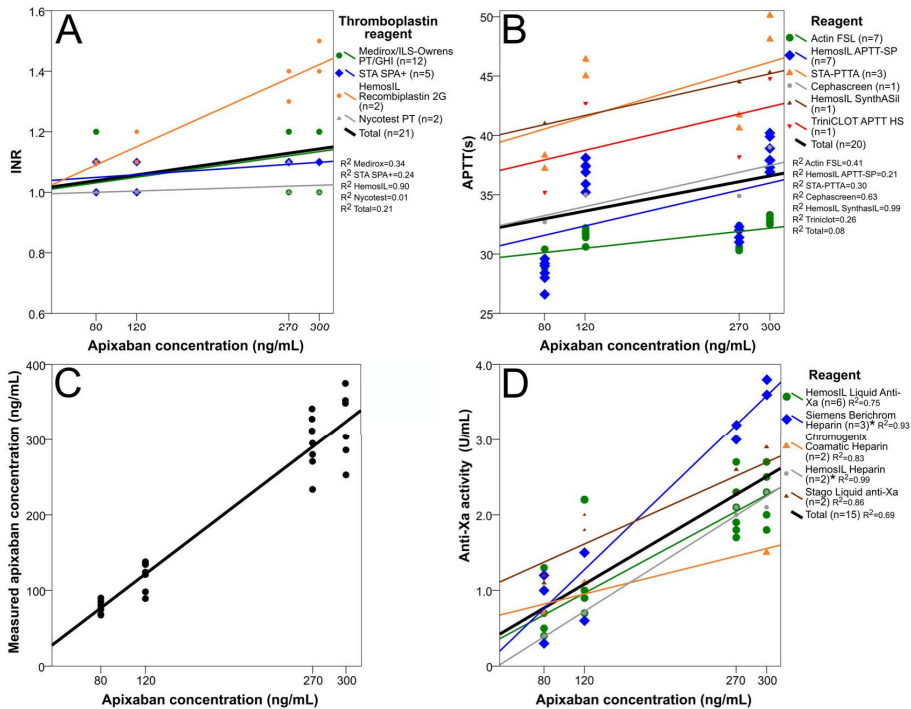
Figure 4   Study II, apixaban. INR had low sensitivity to increasing concentrations of apixaban (4A). In APTT, the responses varied widely, with modest prolongations of APTT (4B). Apixaban concentration measurement was sensitive and adequately reproducible between different laboratories (4C). The anti-Xa with LMWH calibration exhibited wide reagent-dependent variation, but there was a clear correlation with anti-Xa result and increasing apixaban concentration. Reagents with added exogenous antithrombin (asterisk) did not differ from the other reagents (4D)

With rivaroxaban, 22 laboratories reported APTT values using four different reagents. A dose-dependent prolongation of APTT was observed, but the increase seemed non-linear. The inter-laboratory variation remained large (Figure 3D; p<0.001).

With apixaban, 20 laboratories reported the APTT using 6 different reagents. The prolongation of APTT was modest (mean 7.6 s, range 4-11 s), with some significant differences between reagents (Figure 4B; p<0.001).

5.1.3 THROMBIN TIME

Although TT is a sensitive method to detect dabigatran, only 11 laboratories (15%) reported TT results. All laboratories reported TT values above the local measurement range for both the 120 and 300 ng/mL concentrations. Only one university laboratory had a dTT available to quantify dabigatran (Hemoclot

assay), and concentrations of 110 and 320 ng/mL were obtained for the 120 and 300 ng/mL samples, respectively.

5.1.4 ANTI-XA

Anti-Xa assay is sensitive and can quantify rivaroxaban and apixaban when calibrated for drug level. In the rivaroxaban round, only calibrated anti-Xa results were requested. Eight laboratories provided results (36%). Two laboratories reported accurate concentrations for all the samples, whereas six laboratories underestimated the highest sample concentration. This was probably due to the calibration, since the highest calibrator concentration sent to the laboratories was 150 ng/mL. With apixaban, both LMWH-calibrated and drug specific anti-Xa assays were requested. 15 laboratories (71%) reported LMWH-calibrated results. There was a dose-dependent increase in the LMWH-calibrated anti-Xa, but the results varied widely between laboratories, with CVs of 21-19% within reagent groups with overall CVs of 20-49% (Table 6; Figure 4C-D). The overall Pearson's correlation was moderate (R^2=0.69). Seven laboratories (33%) reported apixaban concentration with calibrated anti-Xa assays with accurate results; the correlation with the actual concentrations was excellent (R^2=0.93). With either rivaroxaban or apixaban, exogenous antithrombin addition in the anti-Xa reagent had no effect on the anti-Xa result ($p>0.05$).

5.2 DABIGATRAN EFFECTS ON COAGULATION IN UNSELECTED PATIENT SAMPLES

In study I, the effects of dabigatran on coagulation were assessed in 10 unselected clinical patient samples. The mean measured dabigatran concentration was 119 ng/mL (range 100-136 ng/mL). The mean INR was 1.08 (range 0.96-1.17), which was lower than the INR for the spiked sample of 120 ng/mL with the same reagent, Nycotest PT, 1.16 ($p<0.01$). The APTT showed significant differences in the patient samples and the spiked sample, with a mean APTT of 39.8 s (range 33-43 s) in the patient samples, while with the same Actin FSL reagents, APTT was 54 s ($p<0.001$) in the spiked sample. In one patient sample, the APTT was within the local reference interval.

In study III, the TT and dTT results were expressed as functional dabigatran concentration, Dabi-TT, to cover the full dabigatran concentration range. The mean Dabi-TT was 71 ng/mL in the 241 patient samples (Table 7). The undiluted TT was sensitive and at dabigatran levels below 40 ng/mL, values within the measurement range were obtained. 153/241 (63%) samples had TT < 140 s (Dabi-TT < 40 ng/mL). There was one outlier, with a TT of 46 s with a Dabi-TT of 160 ng/mL. APTT was normal (29 s).

Table 7.   *Study III. Original results in 241 dabigatran patient samples.*

	Dabi-TT (ng/mL)	TT (s)	APTT (s)	PT (%)
Mean	71	92	35	94
Median	10	94	33	95
Range	0-1000	15-141	20-180	23-156
SD	118	47	13	27
Normal range	NA	17-25	23-33	70-130

APTT, activated partial thromboplastin time; Dabi-TT, diluted thrombin time calibrated for dabigatran; SD, standard deviation PT, prothrombin time; TT, thrombin time

The APTT prolongation correlated with Dabi-TT, but the correlation was curvilinear (linear R^2 = 0.68, quadratic R^2 = 0.71; Figure 5A-B). One outlier sample, with APTT above 180 s and Dabi-TT of 46 ng/mL, was excluded from the analysis. Although APTT generally prolonged with increasing Dabi-TT, in 18/70 (26%) of samples with Dabi-TT values 40 ng/mL or above and 137/241 (56%) of all samples, the APTT was within the reference interval of 23-33 s. Even at Dabi-TT of 160 ng/mL, one sample had normal APTT (29 s). The PT results correlated poorly with Dabi-TT (R^2 = 0.13). The PT was abnormal (> 24 s) in only 48/241 (20 %) samples. In samples with Dabi-TT of 40 ng/mL or above, the PT was abnormal in only 26/99 (26 %).

In the 49 patient samples, a full panel of coagulation assays was performed. Dabi-TT, APTT and PT gave similar results as those performed on all patient samples (Table 8). The anti-IIa and ECA correlated well with Dabi-TT (R^2=0.90 and 0.89, respectively, Figure 6A-B). The anti-IIa and ECA results were almost identical to one another (R^2 = 0.99, bias = 3.6 %). The PiCT also correlated moderately with Dabi-TT (R^2 = 0.73; Figure 6C). The PiCT values were prolonged in 33/49 (67%) samples. With Dabi-TT above 40 ng/mL, the PiCT was often prolonged (in 98% of the samples). The RVVT also correlated somewhat with Dabi-TT (R^2 = 0.49; Figure 6D); the RVVT was normal (< 41 s) in 9/49 (18%) samples, all of which had low Dabi-TT (< 40 ng/mL). The RVVT was always prolonged when Dabi-TT exceeded 40 ng/mL. The correlation between RVVT and PiCT results was only modest (R^2 = 0.59).
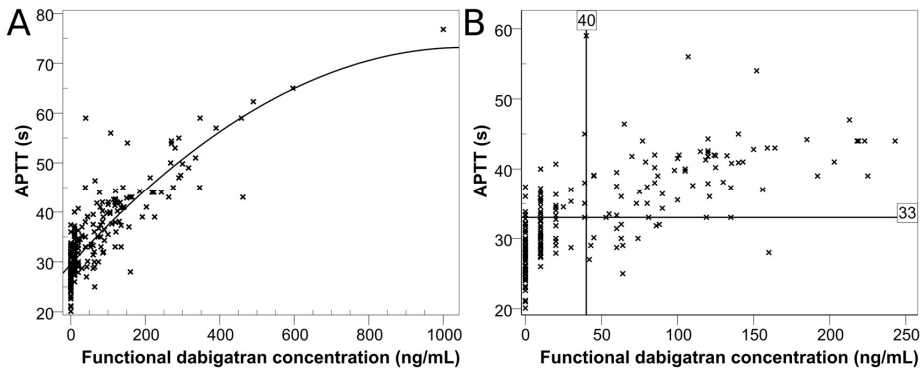
Figure 5   Study III. APTT exhibited a curvilinear relationship with increasing dabigatran levels (5A; R^2 = 0.71). Despite the correlation, the APTT was often within the normal range at dabigatran levels over 40 ng/mL. The highest dabigatran concentration in which the APTT was normal was 160 ng/mL (5B)
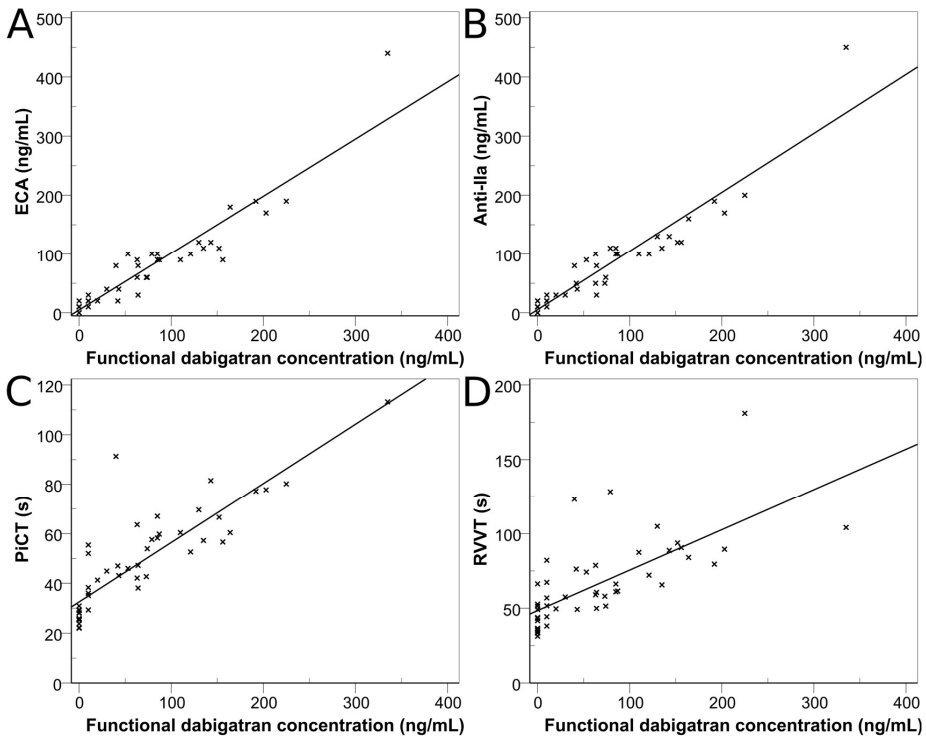
Figure 6   Study III. Excellent correlation between ECA and anti-IIa calibrated with dabigatran was seen with dTT dabigatran concentrations (R^2=0.90 and 0.89, respectively; 6A-B). The correlations with PiCT and RVVT to dabigatran concentrations were more modest (R^2=0.73 and 0.49, respectively; 6C-D).

The Dabi-TT validity was assessed using LC-MS/MS in a subset of 21 samples. The mean dabigatran level was 73 ng/mL (median 78, range 2-150 ng/mL). The correlation between Dabi-TT values was good, with Dabi-TT values being on average 10 ng/mL lower (R^2 = 0.81, bias 13.7 %). The correlation was even better with Anti-IIa and ECA assays, but the bias was larger (R^2 = 0.96 and 0.90; bias 18.4 % and 14.5 %, respectively, Figure 7A).

In TG, the lag time was prolonged with increasing Dabi-TT (R^2 = 0.51, Figure 7B). The effect of dabigatran was clear in lag time, with values below 40 ng/mL leading to shorter lag times than with Dabi-TT above 40 ng/mL ($p<0.001$). The time to peak had a similar profile. Surprisingly, however, the ETP and Peak did not behave as expected. In contrast to the expected decrease in values, ETP and Peak increased with increasing Dabi-TT levels from 40 ng/mL to 225 ng/mL (Figure 7C-D; $p<0.001$). There was a single sample with Dabi-TT of 335 ng/mL, where ETP and Peak values were again lower than in the previous samples with elevated Dabi-TT levels. In addition to these 49 samples, there were 23 samples where valid TG curves were not obtained: there was a trace of TG curve, but the alpha-2-macroglobulin calibration curve failed and the samples could not be included in the analysis. These samples had significantly higher Dabi-TT values (mean 259 ng/mL) than the included Dabi-TT values (42 ng/mL, $p<0.001$).

Fibrinogen and D dimer levels were also assessed in the 49 samples, with no correlation to Dabi-TT. Both the D dimer and fibrinogen levels were fairly high (Table 8). The two different fibrinogen reagents correlated with one another excellently at all Dabi-TT levels ($R^2=0.98$; $p < 0.001$). A correlation between fibrinogen levels and TG parameters ETP and Peak was absent

Table 8. *Study III. Results from the subsequent analyses in 49 dabigatran patient samples.*

ROUTINE COAGULATION	Dabi-TT (ng/mL)	TT (s)	APTT (s)	PT (%)	D Dimer (mg/L)	FibrMFU (g/L)	FibrThr (g/L)
Mean	63	96	33	89	1.0	4.5	4.3
Median	42	123	30	90	0.6	4.3	4.3
Range	0-335	17-141	21-59	29-140	0.0-4.0	2.4-10.6	2.2-9.0
SD	75	49	8	25	1.0	1.9	1.6
Normal range	NA	17-25	23-33	70-130	< 0.5	1.7-4.0	1.8-3.5

SPECIFIC ASSAYS	PiCT (s)	RVVT (s)	Anti-IIa (ng/mL)	ECA (ng/mL)	TG ETP (nM/min)	TG Peak (nM)	TG Lag time (min)
Mean	48	66	68	66	2912	629	8.7
Median	45	59	40	40	1856	429	6.7
Range	22-113	31-181	0-450	0-440	836-7945	165-2023	2.0-24.0
SD	21	29	78	77	2127	466	5.9
Normal range	19-31	< 41	NA	NA	682-1248	90-254	1.6-2.7

Anti-IIa, anti-thrombin (IIa) activity; APTT, activated partial thromboplastin time; Dabi-TT, diluted thrombin time calibrated for dabigatran; ECA, ecarin clotting assay; ETP, endogenous thrombin potential; FibrMFU, fibrinogen using Multifibren U; FibrThr, fibrinogen using Dade Thrombin; PiCT, prothrombinase-induced clotting time; PT, prothrombin time; RVVT, Russel's viper venom time; SD, standard deviation; TG, thombin generation; TT, thrombin time
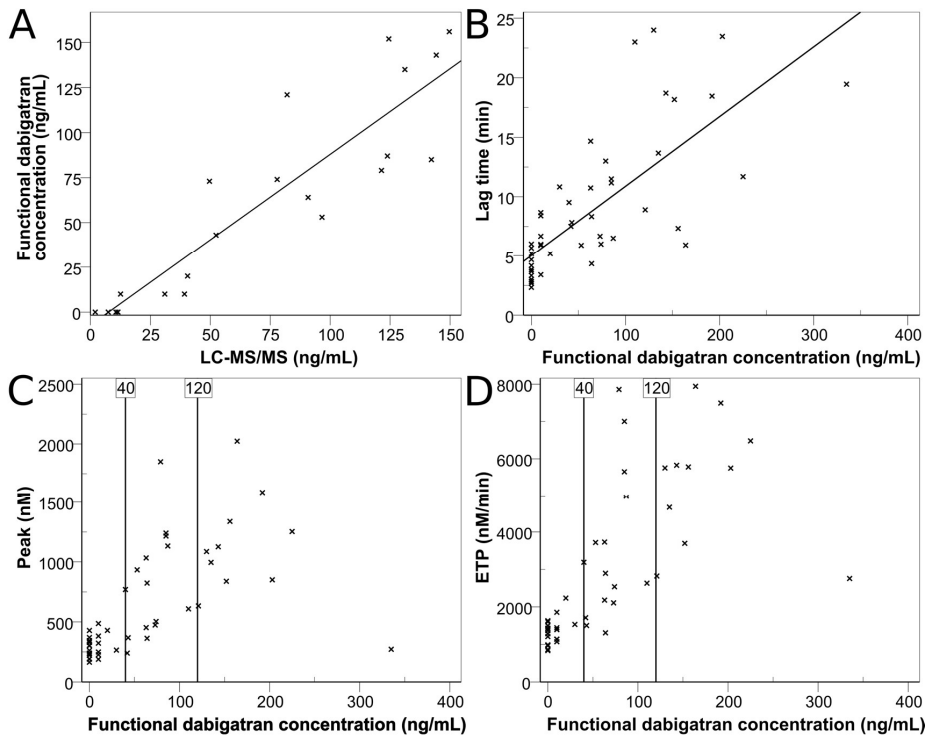
Figure 7   Study III. Functional dabigatran concentration method correlated with mass spectrometry ($R^2 = 0.81$; 7A). As expected, increasing dabigatran concentrations prolonged lag time in TG ($R^2 = 0.51$; 7B). Surprisingly, the ETP and Peak values increased with increasing dabigatran concentrations, at dabigatran levels above 40 and 120 ng/mL the average values in ETP and Peak were higher than below those concentrations ($p<0.001$; 7C-D).

5.3   RIVAROXABAN AND APIXABAN EFFECTS ON COAGULATION IN PATIENTS

In study I, 10 unselected rivaroxaban samples were assessed with a mean rivaroxaban concentration of 63 ng/mL (range 32-128 ng/mL). Using the same reagents, neither the INR nor the APTT results differed from the spiked sample of 60 ng/mL, with modest prolongation of APTT and minimal effect on the INR.

In study IV, the rivaroxaban and apixaban patients undergoing orthopaedic surgery had their basic characteristics recorded. The mean age for rivaroxaban patients was 67 years (range 44-82). 15/20 patients (75 %) were women and mean body mass index (BMI) was 28.8 kg/m² (range 21.0-39.0). The mean age for apixaban patients was 68 years (range 52-82). 12/22 (55%) patients were women and mean BMI was 27.2 kg/m² (range 20.0-40.0).

Haemoglobin and albumin decreased in all patients at day 2-8, with the lowest mean values being 109 g/L (range 78-132 g/L) and 29 g/L (range 24-37

g/L), which are well below the lower reference limit. Leukocytes and CRP increased initially in all, reaching a plateau at day 2-8, and decreasing towards normal at day 28. Platelet count remained in the normal range, without any significant changes during the study, as did ALT and creatinine. (Table 9)

In the drug-calibrated anti-Xa activity, both rivaroxaban and apixaban levels varied significantly in individual patients. The mean peak concentrations after first doses were 111 ng/mL (range 7-267 ng/mL) for rivaroxaban and 19 ng/mL (range 0-73 ng/mL) for apixaban. The mean steady state highest peak level measured was for rivaroxaban, on day 28 at 184 ng/mL (range 81-355 ng/mL) and for apixaban on day 2-8 at 135 ng/mL (range 17-301 ng/mL). The lowest trough levels on day 28 were 16 ng/mL (range 0-48 ng/mL, rivaroxaban) and 46 ng/mL (range 0-103 ng/mL, apixaban) (Figure 8). Three patients with rivaroxaban and one patient with apixaban had undetectable lowest trough level. Drug concentrations did not correlate with BMI or GFR.
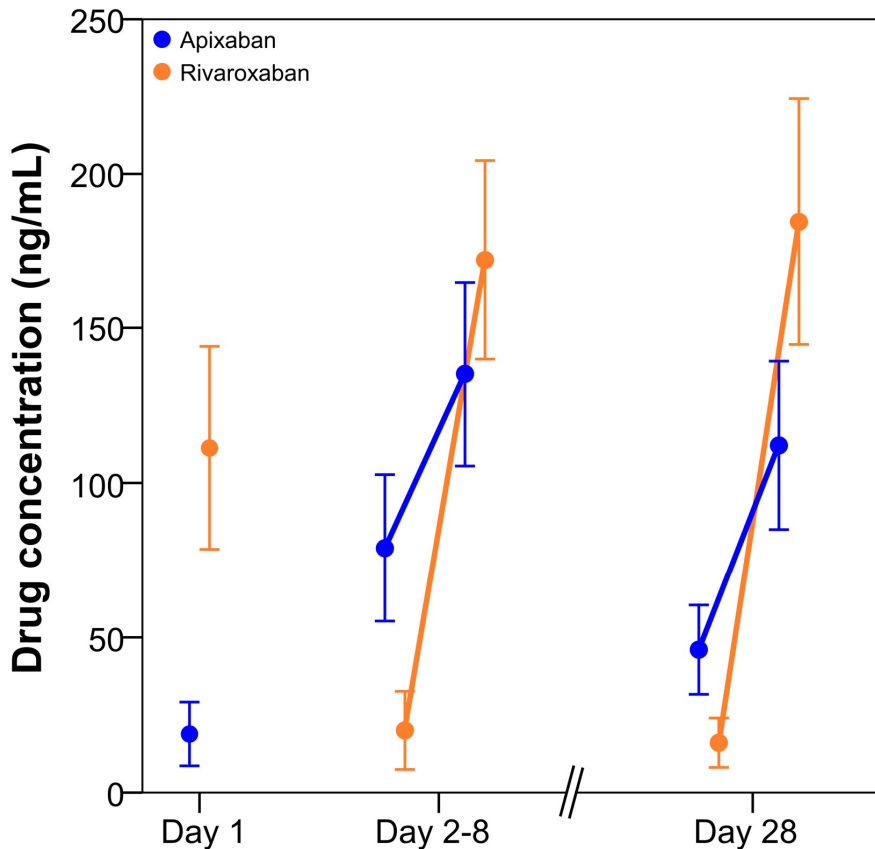
Figure 8   Study IV. Rivaroxaban and apixaban presented significant differences in their peak and trough levels. Mean values and 95% confidence intervals (bars) are shown.

The PT correlated slightly with rivaroxaban concentration ($R^2=0.18$, $p<0.001$), but not with apixaban. The APTT correlated somewhat with rivaroxaban ($R^2=0.44$, $p<0.001$), but the correlation with apixaban was poor ($R^2=0.07$, $p=0.007$). Both the PT and the APTT remained within the reference intervals for both anticoagulants at all time points. The PT and APTT data have been pooled in Table 9.

There was a distinct effect with rivaroxaban and apixaban in TG. While both drugs diminished the ETP and Peak, the response was more vigorous with rivaroxaban than with apixaban. Both drugs prolonged the lag time even at low concentrations (Figure 9A). The trough level change in ETP was marked with apixaban. ETP and Peak were close to the baseline levels at lowest trough on day 28 with rivaroxaban (Figure 9B-C). However, with apixaban, there was a significant inter-individual variation in the TG responses (Figure 10). In one 68-year old patient on postoperative day 2-8, while there was an increase in apixaban levels from 50 to 115 ng/mL (delta 65 ng/mL, 130%), ETP actually increased from 1251 to 2036 nM/min (delta 786 nM/min, 62%). The Peak value also increased (delta 210 nM, 106%) and lag time decreased (delta 30 s, 8%; Figure 10A, square). In comparison there was another patient of similar age and apixaban concentration, at the same time point, a 73 year-old patient with apixaban delta of 59 ng/mL, from 86 to 156 ng/mL (67%). In his sample there was an expected decrease in ETP from 1543 to 1140 nM/min (delta 401 nM/min, 26%, Figure 10A, triangle), with the Peak also decreasing by 95 nM (24%) and lag time prolonging by 80 s (27%). With rivaroxaban there were no patients with such a paradoxical change in TG parameters (Figure 10B).

In the special coagulation assays, there was a clear prolongation in RVVT at rivaroxaban peak levels, but not with apixaban (Figure 9D). There was a peak in fibrinogen and FVIII:C activity on day 2-8, consistent with the inflammatory state (Figure 11A-B). In contrast, D dimer levels were high throughout the study period, being borderline elevated even at baseline. D dimer levels reached a peak at day 1, nadir at day 2-8, and yet another peak at day 28 (Figure 11C). There was no discernible change in the prothrombin fragment F1+2 levels (Figure 11D). The antithrombin activity decreased slightly at day 2-8 but did not decline below the lower reference limit at any time point (Table 9).

Table 9. *Study IV. Basic laboratory tests during the study period, with mean values, 95% confidence intervals and local reference ranges. Haemoglobin and albumin were significantly decreased during the study, reaching a nadir at day 2-8. The inflammatory markers CRP and leukocytes increased with no discernible changes in platelets, ALT or creatinine. The results were pooled as the coagulation tests PT, APTT and antithrombin all remained within the reference intervals throughout the study, with minor differences between rivaroxaban and apixaban.*

	Preop	Day 1	Day 2-8	Day 28	Ref range
Hgb men (g/L)	142 (135-148)	120 (115-125)	117 (111-122)	131 (123-138)	134-167
Hgb women (g/L)	131 (127-135)	105 (101-110)	98 (94-103)	120 (115-124)	117-155
Leukocytes x 10^9/L	6.1 (5.6-6.5)	8.2 (7.5-8.8)	8.0 (7.5-8.5)	6.1 (5.6-6.5)	3.4-8.2
Platelets x 10^9/L	253 (235-270)	206 (192-220)	225 (209-242)	297 (274-320)	150-360
Albumin men (g/L)	39.7 (38.1-41.2)	31.9 (30.6-33.3)	31.9 (30.3-33.6)	39.0 (37.4-40.6)	34-45
Albumin women (g/L)	38.2 (36.9-39.5)	28.8 (27.8-29.8)	27.5 (26.4-28.5)	36.9 (35.6-38.1)	34-45
CRP (mg/L)	4 (1-7)	50 (42-58)	126 (106-147)	5 (2-8)	< 3
ALT (U/L)	25 (20-30)	21 (17-24)	24 (21-28)	22 (16-28)	< 35
Creatinine (µM)	68 (64-73)	63 (59-68)	64 (60-69)	67 (62-72)	60-90
PT* (%)	111 (106-117)	74 (69-79)	88 (82-96)	98 (91-104)	70-130
APTT* (s)	27 (26-28)	30 (29-31)	33 (32-34)	31 (30-32)	23-33
Antithrombin activity* (%)	100 (96-104)	87 (83-90)	94 (89-98)	109 (105-112)	84-108

ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; CI, confidence interval; CRP, C reactive protein; Hgb, haemoglobin; PT, prothrombin time; Preop, preoperative; Day 1, postoperative day 1; Day 2-8, postoperative day 2-8; Day 28, postoperative day 28; Ref range, reference range. * Post drug intake values
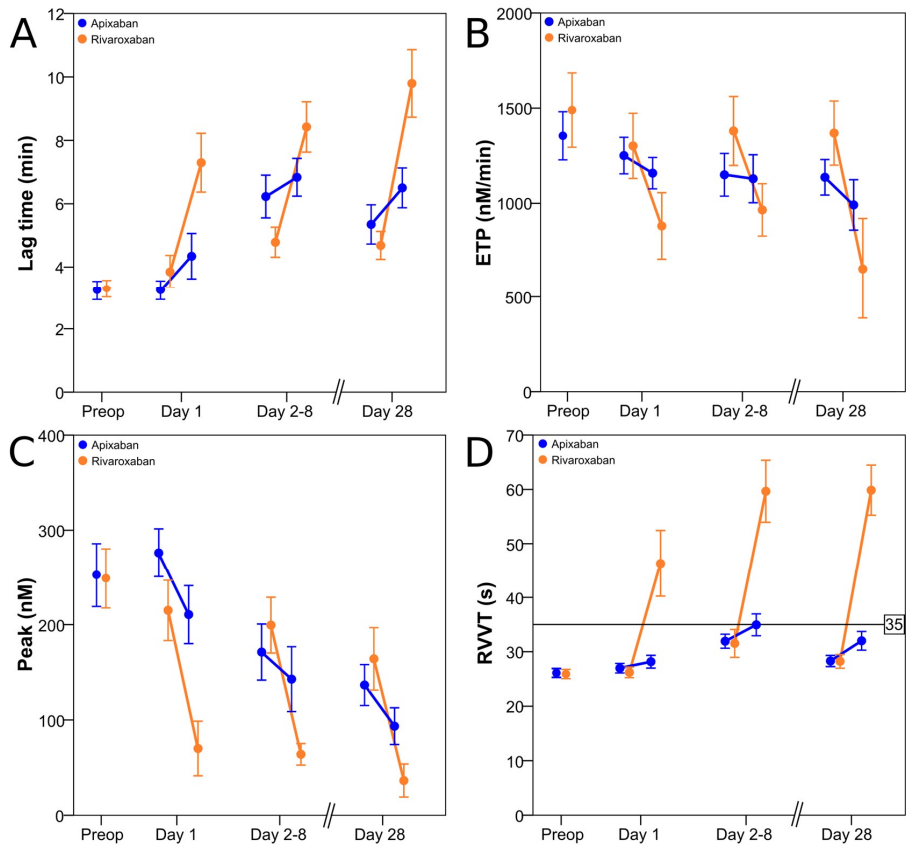
Figure 9　　Study IV. Both apixaban and rivaroxaban prolonged TG lag time and diminished ETP and Peak values. Rivaroxaban exhibited a stronger response (9A-C). With the RVVT, there was little change with apixaban, whereas with rivaroxaban, the RVVT was invariably prolonged at peak levels (9D). Mean values and 95% confidence intervals (bars) are shown. The upper limit of normal range is shown in RVVT.
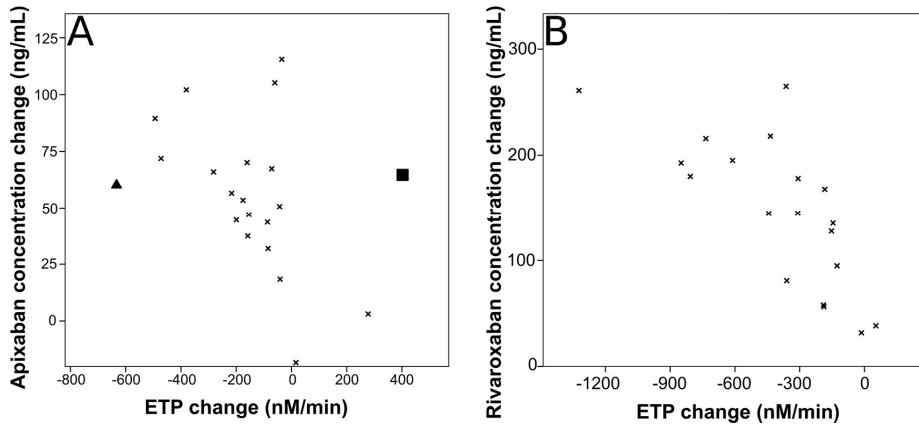
Figure 10   Individual patient responses to rivaroxaban and apixaban in TG ETP varied greatly at day 2-8. Changes in drug concentration and ETP are shown. The ETP is expected to have a negative change with increasing DOAC levels. With apixaban, however, in two different patients with a similar concentration change in the drug levels (before and after drug intake), the ETP was diminished in one patient (10A, triangle) and increased in another patient (10A, square). With rivaroxaban, ETP invariably decreased (10B).
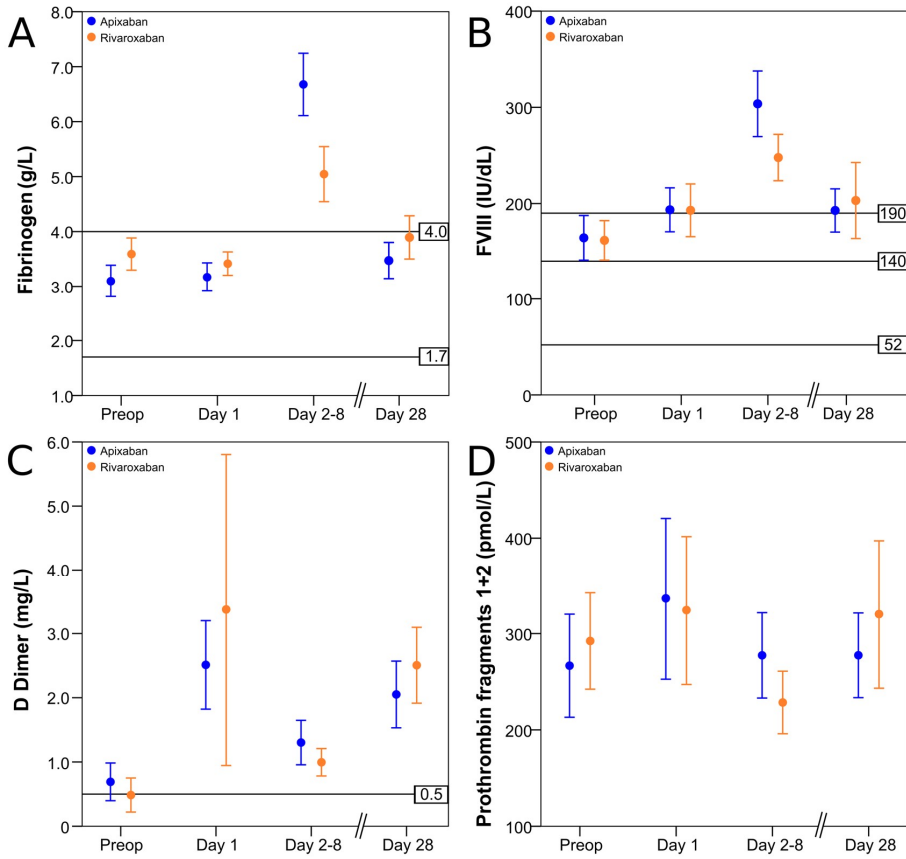
Figure 11   There was a clear peak with fibrinogen and FVIII:C in day 2-8 (values before drug intake), consistent with the proinflammatory status of the patients, with little differences between the anticoagulants (11A-B). Surprisingly, the D dimer levels were above the reference interval almost throughout the study period, with another increase in the levels at day 28 after an initial decrease at days 2-8 (11C). The prothrombin fragment levels F1+2 remained stable throughout the study (11D). Mean values, 95% confidence intervals (bars), reference intervals, and for FVIII a 190 IU/dL reference point are shown.

6 DISCUSSION

6.1 EFFECTS OF DOACs IN SPIKED SAMPLES

In general, no routine coagulation monitoring of DOACs is necessary. However, in several clinical situations (such as emergency surgery, severe thrombosis or bleeding), it is crucial to assess the effects of these drugs on coagulation, often in emergency care setting. The effects of DOACs onto laboratory coagulation assays have been extensively studied *in vitro* in spiked samples. A general consensus exists that laboratories should offer coagulation testing in this setting.[5,176,177] A total of 13 different PT reagents and 10 different APTT reagents were used in the study laboratories (studies I and II), in various combinations and in 24 different types of analysers (Tables 3-4). The laboratories should be aware that no single study or previous survey can cover the vast spectrum of different reagents and analysers used. Furthermore, DOAC responses to different coagulation assays reported in the literature are unlikely to be reproduced in the clinical laboratory setting, unless the same analyser and reagents are used. Even under these conditions, local verification of results is recommended.

In our studies I and II, the INR was assessed in the spiked samples for DOAC effects as laboratories reported the PT results diversely; some reported values in seconds and some as activity percentage compared to normal values. In these surveys, the INR was insensitive to the effects of dabigatran, rivaroxaban and apixaban, in agreement with previous studies.[5,9,121,178] The novel information obtained in our EQA surveys was that the Owren-type thromboplastin reagents, which most of the laboratories used, were significantly less sensitive to DOAC effects than the Quick assay. This was possibly due to the higher dilution factor of plasma (1:20) (Figure 3A, 3C, 4A). It is also noteworthy that the reagents used vary and recommendations on monitoring should not be based on a single reagent. It has previously been shown that Neoplastin CI Plus® reagent (Diagnostica Stago) is more sensitive to the effects of rivaroxaban than other PT reagents, but none of the laboratories participating in the rivaroxaban survey used that reagent.[7,117] It should be noted that INR was selected as a comparator assay between laboratories for practical reasons. Laboratories were able to report INR consistently as all laboratories offering coagulation services have the method available. Indeed, the PT would be the preferred assay, as the INR standards are generated using patient samples with warfarin anticoagulation. The differing ISI of the reagents can magnify the differences between reagents, even though the ISI values in the surveys conducted were close to 1.0 (with a range of 0.9-1.3 according to the manufacturers).[114,179] The PT assays might be insensitive to DOACs, due to the dilution of 1:3 (Quick method) or 1:20 (Owren

method) of plasma. In contrast, in POC assays with no dilution, there was a response in PT to various DOACs.[157,160]

Modifications to PT or INR have been suggested to monitor the effects of DOACs. It was suggested that the ISI used for INR could be calibrated using rivaroxaban instead of a VKA, with results obtained using the Quick reagents more sensitive to the effects of rivaroxaban.[179] Recently, a single-test approach was assessed in association with dabigatran, rivaroxaban and apixaban, where a diluted PT and a modified PT with plasma deficient only in FII and FX, the so-called Fiix-PT test, were used with encouraging results.[180] However, a completely novel method has the disadvantage of difficulties in widespread laboratory implementation, especially prior to the availability of commercial reagents.

In our *in vitro* survey, the APTT was almost uniformly prolonged when dabigatran was present. However, the prolongation varied by two-fold. The Stago PTT® emerged as the most sensitive reagent among those surveyed; Actin FSL® was the least sensitive. Both supporting and contradictory findings to this have been previously reported, possibly due to the high degree of variation among assay performance.[6,161] The CVs were almost 20% in our study. The APTT has been previously recommended as an indicator test for the presence of dabigatran.[10] While the *in vitro* data supports this, subsequent patient sample studies (among them study III) have shown that APTT can often be normal with significant amounts of dabigatran in the plasma. Rivaroxaban and apixaban had very modest prolongations in APTT with increasing concentration. These findings are consistent with previous studies.[7,8,181] From previous experience with heparins, it can be deduced that APTT reacts more strongly to thrombin inhibitors than FXa inhibitors. This is because unfractionated heparins, which prolong APTT inhibit both thrombin and FXa, while low molecular weight heparins, only inhibiting FXa, do not.[182]

Only a minority of the laboratories were able to offer DOAC-specific coagulation assays at the time studied, thus exemplifying the diverse conditions on coagulation tests. The TT for dabigatran and drug-calibrated anti-Xa for rivaroxaban and apixaban were available in 15% (dabigatran round), 36% (rivaroxaban round) and 33% (apixaban round) of the participating laboratories. In the apixaban round, the status of the laboratory (university centre or regional laboratory) was determined. Although there were 10 university hospitals, only 7 had apixaban concentration assays available. In another survey, the potential of LMWH-calibrated anti-Xa assays showed a wide variation in reported responses between laboratories, making the method suboptimal.[183] Similar results have been observed elsewhere, with approximately a third of the laboratories offering specific assays.[184] This highlights the fact that many laboratories only provide the basic coagulation assays. Thus, it is crucial for all laboratories providing coagulation testing to understand the effects of these DOACs on their particular assay-reagent-analyser combinations.

Our surveys showed that when laboratories were able to provide specific assays for DOAC measurements (namely drug-calibrated TT for dabigatran and drug-calibrated anti-Xa for the FXaIs), the obtained drug concentrations were reasonably close to the spiked concentrations. It seems that the methodology to accurately assess drug concentrations is commercially available and can be maintained with relatively low cost. The problem with laboratories is two-fold: on one hand, the paucity of the requests for these specific assays in smaller laboratories might make it difficult to justify the added cost. On the other hand, even with the specific assays available, the knowledge must be distributed in the field so that clinicians will request the appropriate assay for the patient.

6.2 DABIGATRAN EFFECTS ON COAGULATION IN UNSELECTED PATIENT SAMPLES

With the *in vitro* data obtained from the EQA rounds and discrepancies with the APTT sensitivity observed in a subgroup of 10 unselected patient samples, a larger study with 241 patient samples was performed to understand dabigatran effects on coagulation in patients. The unselected patient material provided a real-life snapshot of the samples laboratories are likely to analyse. The median levels of dabigatran were very low (10 ng/mL), suggesting that in many patients the concentration assessment was used to exclude dabigatran influence. Indeed, in almost all of the samples the dabigatran levels were below 300 ng/mL. While no routine monitoring is advocated, in patients with extremes of body weight, impaired renal function and impending surgery, concentration measurements are a valuable addition to the bleeding risk assessment, as dabigatran levels correlate with both bleeding and thrombotic complications.[170] In this study, it was clearly shown that both PT and APTT are unreliable for dabigatran analysis, with normal results observed in samples with significant concentrations of dabigatran. The findings are reagent-dependent and apply to the reagents used in the study (Actin FSL and Nycotest PT), with some assays being significantly more sensitive.[118] The thrombin-specific assays (dTT, ECA and Anti-IIa) measured dabigatran with high precision, as confirmed by LC-MS/MS. LC-MS/MS remains the gold standard, as it was observed that the correlation between dTT and LC-MS/MS was suboptimal at dabigatran concentrations below 50 ng/mL, consistent with previous studies.[141,143] However, the clinical relevance of these low drug levels might be limited.

The RVVT and PiCT also exhibited linear responses, but with higher variation. With dabigatran concentrations above 40 ng/mL, the RVVT and PiCT were almost invariably prolonged (Figure 6 C-D). These characteristics suggest these assays might be suitable for qualitative assessment of dabigatran. With the RVVT in routine use in many laboratories as part of the lupus anticoagulant screen, it could be easily adopted for this purpose. On the

other hand, presence of dabigatran should be excluded to ensure accurate assessment when detecting lupus anticoagulant in a patient.[132]

In TG, there were expected and surprising findings on dabigatran. As expected, lag time was prolonged with increasing dabigatran concentrations, as previously described.[156,169] A new and contradictory finding to many previous studies was that the ETP and Peak values were high when compared to normal samples and were further increased with increasing dabigatran concentrations.[155,156,169,185,186] However, the same phenomenon has been observed in two studies with patients undergoing orthopaedic surgery.[172,187] It should be noted that in the TG assays we performed, only a single centrifugation step was performed as all the patient samples were collected as part of a routine hospital protocol. However, this does not affect ETP and Peak values when the high TF concentration of 5 pM is used.[188] Furthermore, the reference samples from healthy volunteers were also centrifuged only once to enable comparison. The clinical situation of the patients was unknown, so its effect on the TG results cannot be directly assessed. It is likely that patients undergoing thrombosis or surgery were included; these patients probably exhibit enhanced TG. However, this still does not explain the increase in TG with increasing dabigatran concentration. It is quite possible that dabigatran exhibits different effects on patients with high risk for thrombosis, as thrombin inhibitors inhibit the thrombomodulin-mediated activation of protein C.[189,190] It has also been suggested that dabigatran impacts the alpha-2-macroglobulin-thrombin complex used for the calibration of the assay. This leads to erroneously high results, with the effect being notable at drug levels of under 200 nM (94 ng/mL).[147,172,187,191] The assay also often failed in the calibration, especially at higher dabigatran concentrations, pointing towards an issue in the assay. Due to these confounding factors, the relevance of these findings is speculative and it remains to be assessed in future studies. The effect of calibration alone is an unlikely culprit, as a similar paradoxical increase has also been observed with prothrombin fragments F1+2.[192] Recently, a modified TG test using an alternative calibration method has been assessed with dabigatran. The modified method did not show the increase in TG parameters at lower concentrations of dabigatran.[193]

Since the samples were from unselected patients, no clinical data or timing after drug intake was available. It can be assumed, however, that since the measurement of drug concentration was requested, the patients had a clinical need as routine monitoring is not performed. Both the fibrinogen levels and the D dimer levels were high in most of the patients, but there was no correlation between D dimer levels and dabigatran correlations. As dabigatran is known to affect the fibrinogen methods differently at dabigatran levels over 200 ng/mL, two methods with different sensitivities were assessed.[161] However, no conclusions could be made since so few samples (3/49 samples) had dabigatran levels over 200 ng/mL. The high fibrinogen and D dimer are relevant, as it underscores the need for clinicians to look at the overall status of the patient instead of relying on a single assay. Even the concentration

assessment does not reveal the full status of the patient undergoing anticoagulation with a DOAC.

6.3 RIVAROXABAN AND APIXABAN EFFECTS ON COAGULATION IN ORTHOPAEDIC PATIENTS

In study IV on patients undergoing orthopaedic surgery using apixaban (n=22) or rivaroxaban (n=20), there were the advantages of recorded drug intake times and known patient clinical situations. It was expected (but nevertheless important) that the patients exhibited large inter-individual variation in both rivaroxaban and apixaban drug concentrations. Consistent with their dosing, the once-daily dosed rivaroxaban exhibited higher peak levels and lower through levels than the twice-daily dosed apixaban. This was observed previously in patients undergoing orthopaedic surgery, and the drug levels were comparable to previous studies.[169,172-174] AF patients also have a similar wide variance of drug levels, as previously observed with both rivaroxaban and apixaban.[194,195] Notably, there were patients with both rivaroxaban and apixaban where the trough level drug concentration was immeasurable. However, this was observed in only one patient using apixaban. Since the peak drug levels in these same patients were detectable, it seems likely that the effect was due to inadequate dosing rather than non-compliance.

In TG, both apixaban and rivaroxaban had a clear effect of increasing lag time and diminishing ETP, using the 5 pM TF with single plasma centrifugation. There was no such paradoxical change in the variables as observed with dabigatran. There were, however, differences between the DOACs. Rivaroxaban inhibited TG vigorously already after the first dose on postoperative day 1. At this time point there was little increase in apixaban concentration with small inhibition in TG, consistent with the concentration difference between the DOACs (Figure 9A-C). However, there is a confounder with enoxaparin. Enoxaparin was used with rivaroxaban patients before the administration of rivaroxaban, which impacted both the FXa-based rivaroxaban concentration assay and the TG assay. This unfortunately makes the comparison of the initial effects of DOACs difficult to interpret. However, the differences between the DOACs remained consistent throughout the study period. With apixaban at steady state on day 2-8, there were two patients with opposite changes in TG. One exhibited an increase in the ETP and Peak, while another exhibited a decrease at similar apixaban peak levels of 115 and 156 ng/mL, respectively (Figure 10). The effects of surgery are likely to contribute to and magnify the individual differences shown in the TG assay. At day 2-8, the proinflammatory markers CRP, leukocytes and FVIII:C also peaked, suggesting the inflammatory procoagulant state might be exceeding the anticoagulant effect of apixaban in this patient. With rivaroxaban, there was a clear decrease in ETP and Peak at drug peak concentration levels, more

markedly than with apixaban. However, at trough levels the ETP and Peak were close to the preoperative, normal levels. This is consistent with the low rivaroxaban levels (under 50 ng/mL) in all the patients at trough concentrations. However, lag time was invariably prolonged even at rivaroxaban trough levels. The lag time corresponds to the initiation phase of coagulation, where very little thrombin is formed. Thus, it might be sufficient for even a small amount of the DOAC to affect this parameter, while the effect on ETP and Peak is smaller. The differences between the DOACs observed in patient samples are interesting, as in contrast, a previous study with spiked samples demonstrated that apixaban and rivaroxaban inhibited TG to a similar degree.[156,196]

The RVVT showed clear responses to rivaroxaban at peak levels. With apixaban, the assay was inert even at peak levels. There was no response in either of the DOACs at trough. It seems that the RVVT might be used as a qualitative test for rivaroxaban effects, but low levels of the drug might go undetected. The use of RVVT to assess FXa-inhibitors has been studied. Surprisingly, in our study apixaban had little effect, whereas rivaroxaban had a clear effect on the RVVT.[104,132,135,162] Differing drug concentrations at peak levels might explain this result.

With the stress of major surgery, an inflammatory response is expected. Thus, the increase in the proinflammatory markers CRP, leukocytes, FVIII:C and fibrinogen was expected and consistent with previous studies.[197,198] As previously reported, a nadir in haemoglobin and albumin was also observed, probably due to the inflammatory response and blood loss during surgery.[198,199] Simple dilution is unlikely, as leukocyte levels increased while haemoglobin and albumin levels decreased. Although expected, this finding is also relevant for the coagulation status of the patients. Haemoglobin is crucial in primary haemostasis and also provides a procoagulant membrane with microparticles and promotes TG.[200] Both drugs also bind to albumin (95% for rivaroxaban and 87% for apixaban).[113] It has been shown with both rivaroxaban and warfarin that hypoalbuminemia is an independent risk factor for intracranial haemorrhage, with the risk highest at albumin levels below 30 g/L.[201] This is relevant, as in study IV mean albumin in women was 29 and 28 g/l at days 1 and 2-8, respectively. With the normalization of inflammatory parameters at day 28, haemoglobin and albumin returned to near normal levels (Table 9).

The coagulation screening tests PT and APTT reacted minimally to apixaban and rivaroxaban, remaining within the reference interval in most cases (Table 9). This underscores the poor performance of these assays in the assessment of DOAC effects, suggesting that these assays should not be used in that setting. Surprisingly, contrary to the behaviour of inflammatory markers, D dimer levels were elevated at all time points in some individuals and remained elevated at the end of the study period on day 28 in most patients. There was a nadir in D dimer level at day 2-8 before increasing again at day 28. It has been previously shown that D dimer levels exhibit a sinusoidal

pattern after THA, with levels increasing again in postoperative days 2-3.[202] A similar finding has been previously reported in patients undergoing THA with fondaparinux thromboprophylaxis. Accordingly, D dimer remained elevated at 4 weeks postoperation.[197] It is possible that the patients are still in a prothrombotic state at 4 weeks, as fibrin formation and degradation are still ongoing.[203] Fibrin has a major impact on the healing process, and fibrin in dynamic interaction with FXIII also affects measured TG.[151,204,205]

6.4   STRENGTHS AND LIMITATIONS OF THE STUDY

When considering the effects of DOACs on individual patients, the strengths in this study are most numerous in study IV, where rivaroxaban and apixaban samples were taken at predetermined time intervals in patients in whom the clinical situation was known. This information is important when translating the results in the clinical context. The differences in TG with apixaban patients at certain time points in convalescence highlight the importance of the overall coagulation status of the patients. When assessing patient coagulation status, DOAC concentration assessment alone is not sufficient. Data from patient samples is substantially different from the *in vitro*, spiked sample data obtained from studies I and II. The spiked sample studies showed that Owren PT was insensitive to all the DOACs, a finding confirmed in the patient sample studies III and IV. However, in APTT there was a significant discrepancy between the spiked sample data, where APTT was clearly prolonged by dabigatran and the patient sample data, where it was barely prolonged. The different behaviour of the anticoagulants in TG (i.e. the paradoxical increase of TG with dabigatran and differences in rivaroxaban and apixaban responses) was evident in our patient sample data, in contrast with the previously published spiked sample data. Thus, the findings obtained in *in vitro* studies should be verified using patient samples, as significant differences in responses might occur.

A major strength of this study is the wide-ranging view of the laboratory aspects of DOAC monitoring. In studies I and II, a major inter-laboratory difference between reagents, analyser and available techniques was observed, even though the assays surveyed were relatively basic. Information on the local availability of specific assays is crucial. Even though the coagulation screening assays should not be used for DOAC effect monitoring, the absence of appropriate methods in these laboratories has the direct consequence of inexperience in these assays in the clinical settings that these laboratories serve. On the other hand, in studies III and IV, we studied patient samples where DOAC concentrations were primarily unknown. This will increase the understanding of these drugs in actual patient settings. Firstly, the range of drug concentrations observed, secondly, the characteristics of other coagulation and general laboratory parameters with DOACs on board, and

thirdly, the effects of increasing DOAC concentrations in various coagulation assays.

Study III interacts between the laboratory surveys assessing *in vitro* data and the well-characterised patients in study IV by using unselected patient samples. The limitation in this approach was that it was impossible to delve further into the paradoxical TG ETP and Peak increase to assess whether a clinical thrombogenic factor exists in these patients. The use of unselected samples can also be considered a strength, as this study represented the sample material accumulated in a central coagulation laboratory over several years, giving perspective on the actual drug concentration levels commonly encountered in laboratories. The fact that the majority of the drug concentration levels were low (median 10 ng/mL) indicates that laboratories need to consider methods that are sensitive to therapeutic and subtherapeutic levels and not rely solely on methods that are only sensitive to overanticoagulation.

One major limitation of studies III and IV is the use of the TG assay with single centrifugation of plasma instead of double centrifugation. At high TF levels, ETP and Peak are not expected to change, but there is a possibility of different sensitivities to drug inhibition possibly due to the effects of remnant platelets.[188] This means that the TG results obtained in our studies cannot be directly compared to others, where a double-centrifugation protocol was used. Another limitation in these studies is the use of the high TF concentration, which means that the role of the intrinsic activation of coagulation could not be studied.[206] This is especially relevant in study IV, as contact activation is involved in the healing process after orthopaedic surgery. Also in study IV, the early use of enoxaparin limits the interpretation of the comparative anti-Xa inhibition of rivaroxaban and apixaban after the first drug dose.

6.5 FUTURE CONSIDERATIONS

In 2017, the use of DOACs seems to be on the increase. In a recent study, as DOAC use is increasing, warfarin was used in only about half of the patients undergoing a clinic visit for AF and in two thirds of patients with DVT.[207] Rivaroxaban is the DOAC that is most widely used at the moment, followed by apixaban and dabigatran.[207] In some observational studies, these new agents were considered to have superior efficacy to warfarin in the setting of AF, with a similar bleeding risk.[103,208-211] The use of these new drugs is also increasing in VTE.[212] The development of antibodies and neutralising agents for DOACs as an antidote in emergencies will further facilitate the use of these drugs. Idarucizumab, a monoclonal antibody against dabigatran, is already on the market. The factor Xa inhibitor antidote andexanet alfa is showing promise in clinical trials.[213-217]

The increasing use of these drugs is a challenge to both clinicians and laboratory specialists alike, as specific coagulation assays will be increasingly

needed, requested and interpreted.[104,218,219] It should be noted that even though PT and APTT are not used for DOAC monitoring, assessment of baseline levels of these assays before DOAC treatment is beneficial and aids in determining the effects of possible interference of the DOAC. A single assay to detect the anticoagulant effects of all the DOACs would be an optimal strategy. The previously mentioned diluted prothrombin time and Fiix-PT-assay (a PT with only factors II and X deficient in the test plasma) are promising. However, both require rigorous validation before routine clinical use.[180] POC assays might also be of use, since the dilution step in the PT and APTT assays is omitted, leading to increased sensitivities to DOAC effects.[160]

Coagulation time testing alone is not comprehensive, even if drug-calibrated methods or other specific methods are available. TG assay measures the full spectrum of thrombin formation. However, even the semiautomated CAT® is labour intensive with no random-access option for samples. This requires sample batching, which is not always possible with urgent samples. This is the main reason this assay remains mostly in research use. Recent developments enabling TG measurement in whole blood also provides exciting possibilities for future research with DOACs.[148]

The challenge with the DOACs is the same as is with coagulation in general. A variety of factors need to be taken into account and no single variable alone is sufficient. The future of DOACs looks promising, with many patients benefiting from simple dosing. Lack of routine coagulation monitoring might improve adherence.[220] The role of the clinician is to offer the alternatives best suited to the individual patient, with adequate support from the laboratory to aid in the decision-making process.

7 CONCLUSIONS

The effects of dabigatran, rivaroxaban and apixaban on laboratory assays depend both on the characteristics of the assays as well as the individual coagulation status of the patient. We have shown that a large variety of different analysers and methods are currently available for basic coagulation tests in European laboratories. It is therefore not trivial to undertake methods for the assessment of DOACs on a wider scale.

Even if the concentration of the drug is known (functional assays dTT or drug-calibrated anti-Xa assays most commonly used, or via the gold standard method LC-MS/MS), it should be noted that there is more to coagulation in patients on anticoagulation therapy than the concentration of the DOAC. For example, with dabigatran, a paradoxical increase in TG was observed in patient samples but not in spiked samples. The relevance of this observation in patient treatment is not clear, as it is unknown at which conditions the finding observed in the TG assay would materialise in the patient setting.

Standard dosing is recommended for all the DOACs. However, it is known that the plasma concentrations in different patients vary widely. A statistical approach in large randomised controlled trials means that if the majority gains benefit, the drug is considered beneficial. A valid question is what would the individual safety profile of a DOAC be in an individual patient who has very high or very low plasma levels of the anticoagulant? We observed several-fold differences in plasma concentrations between patients using rivaroxaban and apixaban for thromboprophylaxis after orthopaedic surgery. Indeed, alarmingly, there were patients in whom the drug concentrations were undetectable at trough levels, even though the peak levels were measurable, excluding non-compliance.

In conclusion, much is known and specific tests are available to assess the DOAC levels in patients. With clinical experience and further studies, it might be feasible to establish recommended drug concentration levels for patients in treatment. With the development of new assays, it might be possible to assess TG as a global assay in the clinical context. This would be an invaluable tool in the assessment of thrombosis and bleeding risks, with applicability in all DOACs.

8 ACKNOWLEDGEMENTS

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