DEPARTMENT OF CLINICAL SCIENCES,
DANDERYD HOSPITAL
Division of Cardiovascular Medicine
Karolinska Institutet, Stockholm, Sweden

EFFECTS OF DIRECT ORAL ANTICOAGULANT TREATMENT ON HEMOSTASIS IN PATIENTS WITH ATRIAL FIBRILLATION

Viktor Schutz Taune, M.D.

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Effects of direct oral anticoagulant treatment on hemostasis in patients with atrial fibrillation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Viktor Schutz Taune

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Principal Supervisor:
Mika Skeppholm MD, PhD
Karolinska Institutet
Department of Clinical Sciences, Danderyd Hospital
Division of Cardiovascular Medicine

Co-supervisors:
Professor Håkan Wallén, MD, PhD
Karolinska Institutet
Department of Clinical Sciences, Danderyd Hospital
Division of Cardiovascular Medicine

Associate Professor Anna Ågren, MD, PhD
Karolinska Institutet
Department of Clinical Sciences, Danderyd Hospital
Division of Internal Medicine

Opponent:
Professor Hugo ten Cate, MD, PhD
Maastricht University Medical Centre
Department of Internal Medicine
Division of Vascular Medicine

Examination Board:
Associate Professor Eva Zetterberg, MD, PhD
Lund University
Department of Translational Medicine
Clinical Coagulation Research Unit

Professor Anders Själander, MD, PhD
Umeå University
Department of Public Health and Clinical Medicine

Professor Riitta Lassila, MD, PhD
University of Helsinki
Faculty of Medicine

POPULAR SCIENCE SUMMARY OF THE THESIS

Hemostasis refers to the ability of blood to form clots (coagulate) and prevent blood loss when blood vessels are injured while still maintaining the ability to remain fluid in intact vessels. Atrial fibrillation (AF) is a very common heart rhythm disorder where the atria (heart chambers that receive blood from the circulation) do not contract properly. The resulting stasis of blood in the atria disrupts hemostasis with an increased tendency to form clots which can cause stroke. The increased risk of stroke is reduced by treatment with blood thinning medications called oral anticoagulants (OAC). Until the last decade a medication called warfarin, which blocks formation of several of the proteins involved in hemostasis, was the only OAC available. Warfarin is now rapidly replaced by a new type of anticoagulants called direct oral anticoagulants (DOAC). These act on blood coagulation in a different way from warfarin, i.e by directly inhibiting either factor Xa (rivaroxaban, apixaban and edoxaban) or thrombin (dabigatran), two of the central proteins in blood coagulation. Compared to treatment with warfarin, the blood thinning effects of DOAC treatment are more predictable, and there is no need to routinely monitor the effect and adjust the dose, as is the case with warfarin. However, in certain situations there is a need to determine the blood thinning effect of these medications, e.g prior to emergency surgery or in severe bleeding, altogether situations where rapid reversal of the drug effect may be needed. The blood thinning effect of DOACs is thought to be best assessed by measuring the concentration of DOAC in the blood. Since it with the available tests to determine DOAC concentration usually takes almost an hour to provide test results, there is a need to provide data more rapidly, especially in urgent clinical situations. Such tests that can be used bedside, e.g. in an emergency room, are called point-of-care (POC) tests. Blood thinning properties of DOACs assessed by methods other than those that determines the drug concentration in blood, can also provide other valuable information on drug-related effects on hemostasis.

The aims of this thesis were to evaluate and improve the use of POC-tests to rapidly determine blood thinning effects of DOACs and to further increase knowledge about the blood thinning effects of DOACs compared to warfarin treatment by analyzing their effect with methods that reflect different parts of hemostasis.

We found that the ability of the POC-test rotational thromboelastometry (ROTEM) to measure DOAC effect depends on the "trigger" used to start the clotting process (**study I-III**). Commercially available triggers were not able to adequately detect effect of DOACs. Using specific triggers for each DOAC we could however increase the test performance. With specially designed triggers based on thrombin (detects dabigatran) and factor Xa (detects apixaban), ROTEM could accurately discriminate between samples with or without significant DOAC present.

We found that patients treated with dabigatran had more permeable clots than patients treated with rivaroxaban, apixaban or warfarin. Clot formation was slower in patients on dabigatran compared to patients on apixaban and rivaroxaban (**study IV**).

In conclusion, we have found that by modifying the ROTEM assay with specific triggers we could detect the effect of DOACs on blood clotting. The test could be performed quickly and should be suitable as a POC-test in various emergency situations where the detection of DOACs is needed. We also found that the blood thinning effect was stronger in dabigatran treated patients compared to patients treated with other OACs.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hemostas kallas blodets förmåga att levra sig (koagulera) och skydda mot blodförlust vid skada på ett kärl, samtidigt som förmågan att hålla sig flytande i intakta kärl bibehålls. Förmaksflimmer är en mycket vanlig hjärtrytmrubbning som innebär att förmaken (utrymmen i hjärtat som tar emot blod från cirkulationen) inte kontraherar som de ska. Blodet i förmaken blir då stillastående vilket stör hemostasen och ger blodet en ökad tendens att koagulera vilket kan orsaka stroke. Den ökade stroke-risken minskas med blodförtunnande mediciner som kallas orala antikoagulantia (OAK). Fram till för cirka ett årtionde sedan var warfarin, som motverkar bildning av flera av de proteiner som är involverade i hemostasen, det enda OAK som fanns tillgängligt. Warfarin ersätts nu i snabb takt med en ny typ av blodförtunnande mediciner som kallas direkta orala antikoagulantia (DOAK). Dessa verkar på hemostasen på ett annat sätt är warfarin, genom att direkt hämma antingen faktor Xa (rivaroxaban, apixaban och edoxaban) eller trombin (dabigatran), två av de centrala proteinerna i hemostasen. Jämfört med warfarin-behandling så är den blodförtunnande effekten av DOAK mer förutsägbar så det finns inget behov av att rutinmässigt mäta den blodförtunnande effekten och justera dosen som man gör med warfarin. I vissa situationer behöver man dock veta den blodförtunnande effekten av DOAK, till exempel innan akut kirurgi eller när en patient blöder, dvs situationer när man överväger att ge antidot som reverserar den blodförtunnande effekten. Den blodförtunnande effekten av DOAK anses bäst uppskattas genom att mäta koncentrationen av DOAK i blodet. Eftersom det med tillgängliga metoder för koncentrationsbestämning av DOAK vanligtvis tar nästan en timme att få ett svar, så finns det ett behov av nya tester som ger svar snabbare än så, särskilt i väldigt akuta situationer. Sådana tester som kan användes patientnära, till exempel i ett akutrum, kallas ”point-of-care” (POC) tester. Utvärdering av blodförtunnande effekt av DOAK med andra metoder än koncentrationsbestämning kan också ge annan värdefull information om deras påverkan på hemostasen.

Målen med den här avhandlingen var att utvärdera och förbättra användning av POC-tester för att snabbt bedöma den blodförtunnande effekten av DOAK samt att öka kunskapen om de blodförtunnande effekterna av DOAK jämfört med warfarin genom tester som speglar olika delar av hemostasen.

Vi har visat att POC-testet rotationstromboelastometris (ROTEM) förmåga att mäta DOAK-effekt beror på vilket reagens som används för att starta koagulationen (**studie I-III**). Kommersiellt tillgängliga reagens kunde inte detektera DOAK-effekt på ett tillräckligt bra sätt. Genom att använda reagens specifika för respektive DOAK kunde vi öka precisionen i testet. Med specialdesignade reagens baserade på trombin (detekterar dabigatran) och faktor Xa (detekterar apixaban) så kunde ROTEM skilja på blodprov med och utan signifikant mängd DOAK med stor säkerhet. Resultaten var tillgängliga inom 20 minuter.

Vi visade att patienter med dabigatran-behandling bildade mer genomsläppliga blodproppar än patienter behandlade med rivaroxaban, apixaban eller warfarin. Bildningen av blodproppar var långsammare hos dabigatran-behandlade patienter än hos patienter behandlade med rivaroxaban och apixaban (**studie IV**).

Sammanfattningsvis så har vi visat att genom att modifiera ROTEM-testet med specifika reagens för DOAK så kunde vi detektera de blodförtunnande effekterna av DOAK. Tester kunde genomföras snabbt och borde vara användningsbart som ett POC-test i olika akuta situationer när bedömning av DOAK-effekt är nödvändig. Vi visade också att den blodförtunnande effekten var starkare hos dabigatran-behandlade patienter än hos patienter behandlade med andra OAK.

ABSTRACT

The direct oral anticoagulants (DOAC) – dabigatran, rivaroxaban, apixaban and edoxaban – were approved without the need for routine monitoring. However, in parallel with clinical implementation, studies on how to measure the effect of DOACs have become a research area of increasing interest during the last decade. The obvious need to assess the anticoagulant effect of DOACs in emergency situations such as bleeding and before emergency surgery has driven this research. Further characterization of the effects of DOACs on different parts of hemostasis has also been in focus of research.

The aims of this thesis were to evaluate and improve the use of point-of-care (POC) tests to rapidly determine DOAC effects, and to further increase knowledge of different DOACs' effect on hemostasis by analyzing their effect on both global and more specific coagulation assays.

In **study I**, we evaluated two whole blood-based tests, the viscoelastic test rotational thromboelastometry (ROTEM), and the flow-based Total Thrombus-Formation analysis system (T-TAS) in patients with atrial fibrillation (AF) on dabigatran treatment. We found that dabigatran concentration correlated strongly with ROTEM clotting time (CT) when activated with two commercial triggers (r-values 0.92 and 0.93), but CT could still be within normal reference interval at significant dabigatran concentrations. There were weak correlations however, between dabigatran concentration and T-TAS data, both time-related thrombus formation and total thrombus formation (r-values 0.39-0.41). In **study II**, we used ROTEM-CT to show that a thrombin-based trigger was more sensitive than commercial triggers for detection of dabigatran, both in vitro and in samples from patients with AF. The thrombin-based trigger accurately discriminated between samples with dabigatran concentrations above or below 20 ng/mL (100 % sensitivity and specificity). In **study III**, we went on to investigate the most commonly used DOAC, i.e apixaban. Using ROTEM-CT we showed that a factor Xa (FXa)-based trigger was more sensitive than a snake venom-based or commercial trigger for detection of apixaban, both in vitro and in samples from AF patients. The FXa-based trigger accurately discriminated between samples with apixaban concentration above or below 20 ng/mL (100 % sensitivity and specificity). Results were available within 20 min, considerably faster than emergency analyses of drug-concentrations determined at the chemistry lab of the hospital. In **study IV**, we investigated the effects of DOACs by analyzing both direct and global methods of hemostasis. For comparison, plasma from warfarin-treated patients were also analyzed. Dabigatran treatment was associated with a more permeable fibrin network, delayed thrombin generation and fibrin formation and a lower fibrin turn-over compared to rivaroxaban and apixaban. Warfarin tended to have stronger effects than the FXa-inhibitors. There were no significant differences between apixaban and rivaroxaban in any of the tests performed.

In conclusion, ROTEM with DOAC-specific triggers is a promising method to rapidly detect DOAC effect in emergency situations. In patients treated with OACs according to clinical routine, dabigatran treatment was the drug associated with the broadest anticoagulating effects.

LIST OF SCIENTIFIC PAPERS

I. **Viktor Taune**, Håkan Wallén, Anna Ågren, Gunilla Gryfelt, Carolina Sjövik, Anna M. Wintler, Rickard E. Malmström, Agneta Wikman, Mika Skeppholm: Whole blood coagulation assays ROTEM and T-TAS to monitor dabigatran treatment. *Thrombosis Research, May 2017; 153: 76-82.*

II. **Viktor Taune**, Mika Skeppholm, Anna Ågren, Gunilla Gryfelt, Rickard E. Malmström, Agneta Wikman, Joanne van Ryn, Håkan Wallén: Rapid determination of anticoagulating effects of dabigatran in whole blood with rotational thromboelastometry and a thrombin-based trigger. *Journal of Thrombosis and Haemostasis, Oct 2018; 16(12): 2462-2470.*

III. **Viktor Taune**, Mika Skeppholm, Anna Ågren, Agneta Wikman, Andreas Hillarp, Håkan Wallén: Rapid detection of apixaban by a ROTEM based approach and reversibility with andexanet alfa or DOAC-Stop. *TH Open, Aug 2022; 6(3):e238-e247.*

IV. **Viktor Taune**, Michal Zabczyk, Shu He, Anna Ågren, Margareta Blombäck, Håkan Wallén, Mika Skeppholm. Effects of dabigatran, rivaroxaban and apixaban on fibrin network permeability, thrombin generation and fibrinolysis. *Manuscript.*

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LIST OF ABBREVIATIONS

AA	Andexanet alfa
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AF	Atrial fibrillation
ANOVA	Analysis of variance
aPTT	Activated partial thromboplastin time
AUC	Area under curve
BID	Twice daily dosing (latin: bis in die)
CAT	Calibrated automated thrombogram
CFR	Clot formation rate
CFT	Clot formation time
CT	Clotting time
CT_{diff}	CT difference
DMSO	Dimethylsulfoxide
DOAC	Direct oral anticoagulant
DS	DOAC-Stop
dTT	Dilute Thrombin Time
DVT	Deep venous thrombosis
ECA	Ecarin chromogenic assay
ECT	Ecarin clotting time
eGFR	Estimated glomerular filtration rate
ETP	Endogenous thrombin potential
FXa	Factor Xa
GI	Gastrointestinal
ICH	Intracranial hemorrhage
IDZ	Idarucizumab
INR	International normalized ratio
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LDL	Low density lipoproteins
MCF	Maximum clot firmness
NOAC	Non-vitamin K oral anticoagulant / New oral anticoagulant
NPP	Normal pool plasma
OAC	Oral anticoagulant
OT	Occlusion time
PAI-1	Plasminogen activator inhibitor-1

PCC	Prothrombin complex concentrate
PE	Pulmonary embolism
POC	Point-of-care
PPL	Phospholipids
PT	Prothrombin time
QD	Once daily dosing (latin: quaque die)
RCT	Randomized controlled trial
ROC	Receiver operating characteristics
ROTEM	Rotational thromboelastometry
RVV	Russel viper venom
T-TAS	Total Thrombus-Formation Analysis System
T10	Time to reach 10 kPa
TAFI	Thrombin activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complex
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
tPA	Tissue plasminogen activator
TT	Thrombin time
uPA	Urokinase-type plasminogen activator
VKA	Vitamin K antagonist
VTE	Venous thromboembolism
vWF	Von Willebrand factor

1 BACKGROUND AND LITERATURE REVIEW

1.1 HEMOSTASIS

Hemostasis means “arrest of bleeding” (1) and is the process by which a fibrin clot is formed to seal off injuries to blood vessels and prevent blood loss. As important as this process is, it is equally important to limit formation of clots to places of injury. The hemostatic system involves platelets, coagulation factors, fibrinolytic factors and the vessel wall with the endothelium (2, 3). These components constantly interact in an intricate system of feed-back and inhibitory loops to maintain blood fluidity in physiological conditions while still forming clots rapidly at places of vessel injury (2, 3). Any imbalance in this system may cause thrombotic- or bleeding disorders (2, 3).

1.1.1 Vascular endothelium

Endothelial cells line the inside of the vessel wall and interact with blood components. It is a central component in the hemostatic system as the undamaged endothelium acts to maintain blood fluidity whereas damage to the endothelium signals the start of clot formation and constriction of the vessel wall (4-6). The endothelial cells are protected by a carbohydrate-rich layer called the endothelial glycocalyx (6-8). The vascular endothelium and the glycocalyx exert antithrombotic properties through the actions of e.g. various glycosaminoglycans, proteoglycans, and glycoproteins constituting the glycocalyx, together with antithrombin, thrombomodulin with protein C and S, tissue factor pathway inhibitor (TFPI), nitric oxide and prostacyclin (4, 5, 8). Disruption of the glycocalyx causes capillary leak, oedema and inflammation and further damage to the endothelium exposes collagen, von Willebrand Factor (vWF) and, tissue factor (TF) in the subendothelial matrix, which start activation of hemostasis (4-6).

1.1.2 Platelets

The formation of a platelet plug is called primary hemostasis and starts with adhesion of platelets to the area of damage through the action of vWF which binds to collagen and to the platelet glycoprotein Ib receptor (5, 9-11). Upon activation, platelets release various compounds with biological effects, e.g. adenosine diphosphate (ADP) and thromboxane A_2, which in turn increase expression and induce a conformational change of the receptor glycoprotein IIb/IIIa (5, 9-11). This receptor mediates the aggregation phase in which platelets are linked to each other by binding to fibrinogen, thereby creating the clot (5, 9-11). Of note, platelets act together with the coagulation system to create the clot, providing a surface of phospholipids (PPL) for coagulation reactions while also being potently activated by thrombin (5, 9-12).

1.1.3 Coagulation system

The coagulation cascade culminating in the formation of a fibrin network is sometimes referred to as secondary hemostasis, but the process is simultaneous with the formation of

the platelet plug (5, 13). Like the platelets, the coagulation factors circulate the blood in an inactive form. When TF is exposed in the subendothelium, circulating factor VII binds to it and forms a complex which activates factor IX (5, 13). Factor IXa with its cofactor VIIIa activate factor X (5, 13). Factor X is also activated directly by the TF-factor VIIa complex (5, 13). Factor Xa (FXa) acts together with factor Va to generate thrombin from prothrombin (II) (5, 13). Thrombin converts fibrinogen into fibrin, the final step of the coagulation cascade (5, 13). Thrombin also participates in multiple positive (activation of platelets, thrombin activatable fibrinolysis inhibitor (TAFI), factors V, VIII, XI, XIII) and negative (activation of protein C and fibrinolysis) feedback loops (5, 13).

The TF-initiated start of the coagulation cascade is referred to as the extrinsic pathway. There is also an intrinsic pathway, or contact activation pathway, so called as it can be activated on surfaces, such as the glass of a test tube or on medical devices such as catheters, heart valves or stents (5, 13, 14). The intrinsic pathway is initiated by activation of factor XIIa, followed by activation of factor XI and then factor IX which, as described above, activate factor X together with factor VIIIa (5, 13, 15). It is also active in the process of thrombus growth and stabilization through positive feedback of thrombin on factor XI (15, 16). The understanding of the role of the intrinsic pathway in hemostasis is still not complete (15, 16). Patients with deficiencies in factor XI and factor XII usually do not have severe bleeding disorders but are less prone to thrombotic disease (15, 16).

As mentioned above, there are proteins that inhibit the coagulation system at different levels, called endogenous anticoagulants. Their role is to limit clot formation to places of injury, and they are generally activated by the actions of a healthy endothelium and through negative feedback loops. Antithrombin inactivates several of the enzymes in the coagulation cascade, among others factor X and thrombin (17). Its function is augmented by heparan sulfate in the endothelium or by administered heparins (17). TFPI binds and forms a complex with FXa that inhibits the actions of FXa as well as the actions of the TF and factor VIIa complex (5, 18). Thrombomodulin is an endothelial cell receptor that forms a complex with thrombin that activates protein C. Protein C and its cofactor protein S bind and inactivates factors Va and VIIIa (5, 19).

1.1.4 Fibrinolytic system

The fibrinolytic system degrades the clot through the action of plasmin on the fibrin network (5, 20). Plasmin circulates as an inactive precursor, plasminogen (5, 20). It is activated by tissue plasminogen activator (tPA) produced by endothelial cells and by urokinase-type plasminogen activator (uPA/urokinase), which is produced by several cell types and is not only active in fibrinolysis but also in degradation of extracellular matrix and cell migration (5, 20). Fibrinolysis is inhibited through direct inactivation of plasmin by $Alpha_{2}$-antiplasmin circulating in plasma, by removal of plasminogen binding sites on fibrin by TAFI and by inactivation of tPA by plasminogen activator inhibitor (PAI-1) (5, 20).

1.2 THROMBOTIC DISORDERS

When the prothrombotic factors dominate, thrombosis may occur, leading to occlusion of blood vessels. This causes ischemia, tissue inflammation and necrosis and in cases of arterial thrombosis ultimately leading to loss of function in the organ the blood vessels supply (21). Thrombosis is the leading cause of mortality in the world and can occur in veins (venous thromboembolism (VTE)), arteries (e.g. myocardial infarction, ischemic stroke) and in the cardiac chambers (e.g. cardio-embolism due to atrial fibrillation (AF)) (21). Most pathological processes leading to thrombosis may be grouped into one or several of three categories described by German pathologist Rudolf Virchow in the 19th century (Virchow's triad); (1) irritation of the blood vessels and its surroundings (endothelial dysfunction), (2) hypercoagulability (such as in patients with inherited thrombophilia, cancer, pregnancy) and (3) disturbed blood flow (blood stasis or turbulence) (22, 23).

1.2.1 VTE

Thrombosis in veins most frequently occur in lower extremities (superficial and deep venous thrombosis (DVT)) but may occur in almost all venous vessels. Pulmonary embolism (PE) is most frequently a complication of DVT, occurring when part of the thrombus breaks away and travels with venous blood through the right atrium and ventricle to the pulmonary arteries (23, 24). The thrombi that form in low flow conditions in veins are rich in fibrin and trapped red blood cells and are usually referred to as "red thrombi" (25). VTE is common, with an incidence of 1-4/1000/year and is the 3rd leading cause of cardiovascular related death in developed countries (26, 27). The main risk factors for VTE – major surgery and trauma, active cancer, immobilization, pregnancy and use of oral contraceptives – all affect one or several of the factors in the explanatory model offered by Virchow's triad (24, 26, 27). The main treatment for venous thrombosis is anticoagulants, i.e. medications targeted against the coagulation system (23, 24, 27).

1.2.2 Arterial thrombotic disorders

Arterial thrombosis, mainly myocardial infarction and ischemic stroke, is the leading cause of cardiovascular related death and is in most cases a consequence of atherosclerosis (21). Atherosclerosis starts with lipoprotein deposits in the vessel wall that cause inflammation and endothelial damage (6, 28). The inflammation attracts leukocytes, platelets, smooth muscle cells and connective tissue cells that eventually remodels the vessel wall into the atherosclerotic plaque (28). The build-up of atherosclerotic plaques is considered to be slow, starting at a young age and is asymptomatic until the vessel is sufficiently narrowed to reduce blood flow or until a plaque ruptures (28). As the integrity of the endothelium is compromised when the plaque ruptures, a clot is quickly formed that might completely occlude the vessel and cause sudden and dramatic symptoms (28). Atherosclerosis is associated with various risk factors like hypertension, diabetes mellitus, smoking, obesity, lack of exercise, high levels of low density lipoproteins (LDL) among others (28). The thrombi formed in the high flow conditions of the arteries are rich in platelets and are

traditionally referred to as “white thrombi” (25). The main antithrombotic treatment target in arterial thrombosis today is the platelet, i.e. antiplatelet medication (24, 29).

1.2.3 Cardio-embolism due to AF

Cardioembolic stroke due to AF is estimated to cause around 25 % of ischemic strokes (30). AF is the most common reason for anticoagulant treatment, with a prevalence of around 3 % (31, 32). Prevalence is expected to increase due to increasing life expectancy, increasing prevalence of risk factors for AF (obesity, hypertension, diabetes and obstructive sleep apnea) and increasing screening efforts and opportunities (33, 34). AF is defined as a supraventricular atrial tachyarrhythmia characterized by uncoordinated atrial activity resulting in ineffective atrial contractions (33). This leads to blood stasis in the atria, especially the left atrial appendage, where most of the thrombi are formed. With time, AF also leads to enlargement of the atria, which further increases blood stasis and stroke risk (35). There is also evidence to suggest that atrial cardiomyopathy and prothrombotic alterations in the blood constituents contribute to thrombus formation in AF, thus including the whole of Virchow’s classical triad (35). The thrombi that form in AF are similar to the red thrombi formed in venous thrombosis and the most efficient treatment is treatment with anticoagulants (25, 36). Thromboembolic risk in patients with AF can be estimated using the CHA_2DS_2-VASc score (Congestive heart failure, Hypertension, Age > 75, Diabetes, prior Stroke/transient ischemic attack, Vascular disease, Age > 65, Sex category), in which points are given for risk factors of thromboembolic risk (37). In male patients with 2 or more points and female patients with 3 or more points anticoagulant treatment is indicated. In male patients with 1 point and female patients with 2 points anticoagulant treatment should be considered (37).

1.3 ORAL ANTICOAGULANTS (OAC)

1.3.1 Vitamin K antagonists (VKA)

VKAs, e.g. warfarin, are OACs that inhibit the enzyme vitamin K epoxide reductase, resulting in reduced metabolism of vitamin K that is necessary for carboxylation of glutamic residues on coagulation factors thrombin, factor VII, factor IX and factor X as well as anticoagulants protein C, protein S and protein Z (38, 39). VKAs have been used clinically since the 1940’s and until recently they were the only available OACs (40). VKAs are dosed individually and monitored using the prothrombin time (PT) assay and standardization through international normalized ratio (INR). For patients with AF, warfarin treatment has been shown to reduce stroke risk by approximately 60% compared to antiplatelet treatment that reduces stroke risk by approximately 20% (41).

1.3.2 Direct oral anticoagulants (DOAC)

DOACs - i.e. dabigatran, rivaroxaban, apixaban and edoxaban – have been introduced during the last decade as a new class of OAC that act through direct inhibition of coagulation factors thrombin or FXa (42-45). A few different terms have been used when referring to this group of anticoagulants. When introduced the acronym NOAC stood for

'new/novel oral anticoagulants'. After a while this was changed to 'non-vitamin K oral anticoagulants'. Now the acronyms NOAC and DOAC are used interchangeably (46, 47). In contrast to VKAs, they are prescribed with fixed doses without the need for routine monitoring (48-51). Each of the DOACs have been evaluated against warfarin in large randomized controlled trials (RCTs) with AF patients, which have demonstrated equal or better protection against ischemic stroke without increases in major bleeding and above all, a considerably lower risk of intracranial bleedings (ICH), the most dreaded complication to anticoagulant treatment (42-45). Their convenience of use together with their appealing safety profile has led to them being preferred over warfarin treatment for stroke prevention in AF patients, both in guidelines and in real world practice, and many countries are also reporting increasing use of anticoagulants in AF patients after introduction of DOACs (33, 52, 53). The replacement of warfarin by DOACs in Sweden is evident by looking at how many patients collect a prescription of each anticoagulant each year, although those numbers include other indications than AF (Figure 1) (54). In fact, both apixaban and rivaroxaban are high on the list of the best-selling medications worldwide (55). Nevertheless, there are some patient groups in which warfarin treatment is still preferred over DOACs. The DOACs have been approved under the term "non-valvular AF" (48-51). However, most patients with AF and valvular heart disease can be treated with DOACs with the exception being patients with mechanical heart valves or rheumatic heart disease in which treatment with warfarin seem superior (56-59). Other AF patients in which warfarin treatment may be preferred are patients with end-stage renal disease and patients with concomitant treatment with medications with known interactions with DOACs (60-62).

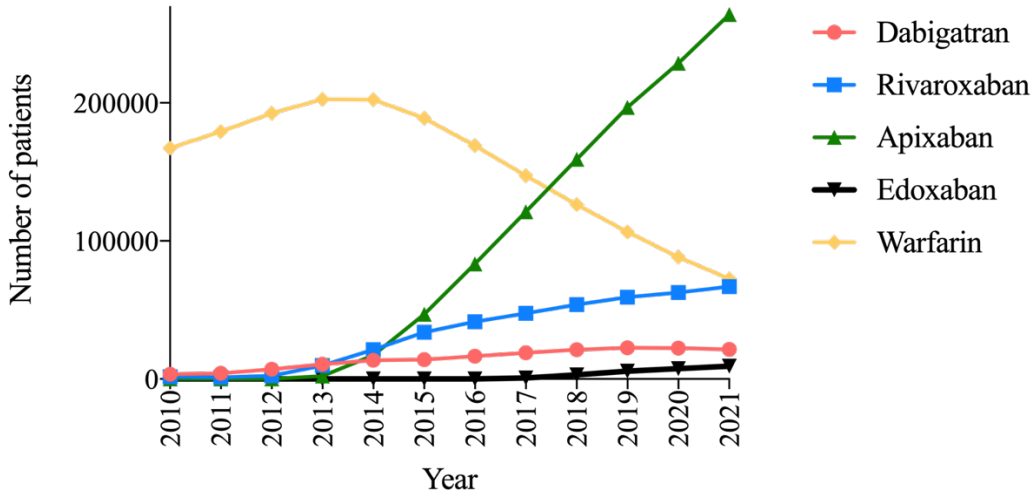

Figure 1. Number of patients who collected a prescription of each OAC in Sweden between the years 2010-2021 (54).

1.3.2.1 Dabigatran

Dabigatran is a direct thrombin inhibitor and was the first of the DOACs to be approved for use in AF patients after the phase III trial RE-LY (42). 18 113 patients were randomly assigned to treatment with either dabigatran (110 or 150 mg twice daily (BID)) or warfarin.

Dabigatran compared favorably with warfarin with patients treated with 150 mg BID having less ischemic strokes than warfarin treated patients with similar rates of major bleeding while patients treated with 110 mg BID had similar rates of ischemic stroke with lower rates of major bleeding. ICH was markedly reduced with both doses of dabigatran compared to warfarin while gastrointestinal (GI) bleeding was increased in patients treated with 150 mg BID. There was a trend towards mortality benefit with dabigatran treatment. Interestingly, the rate of myocardial infarction was higher in dabigatran-treated patients than in warfarin-treated patients in the RE-LY study (42). This has been the subject of some controversy and discussion but although other results of the RE-LY trial compare well to real world data of dabigatran treatment, rates of myocardial infarction have not been observed to be higher in dabigatran treated patients (63, 64).

Dabigatran is administered as a pro-drug - dabigatran etexilate - which is converted to dabigatran by esterases in the gut, plasma and liver (65). It acts as a competitive reversible inhibitor of thrombin by binding to its active site. Bioavailability is around 6 % and compared to other DOACs it is eliminated through the kidneys to a higher degree (80 %). The half-life is around 12-14 hours after multiple doses in patients with preserved renal function, but since the elimination is dependent on renal clearance, the half-life can be considerably longer in patients with renal failure. In Europe, patients with AF are treated with the doses 150 or 110 mg BID, with the lower dose recommended in older patients (age > 80 years), patients with reduced kidney function (estimated glomerular filtration rate (eGFR) < 50 ml/min) and patients with a high bleeding risk (48).

1.3.2.2 Rivaroxaban

The direct FXa inhibitor rivaroxaban was compared to warfarin for prevention of stroke in patients with AF in the phase III trial ROCKET AF (43). It was administered 20 or 15 mg once daily (QD), with the lower dose chosen in patients with eGFR < 50 ml/min (43). Rivaroxaban was non-inferior to warfarin at preventing ischemic strokes and had similar rates of major bleeding. There were less ICH and fatal bleedings in the rivaroxaban treated group but more GI bleedings. Observational data generally support the findings in ROCKET AF but in comparison to both dabigatran and apixaban, rivaroxaban treated patients seem to have more bleedings (63, 66). Bioavailability is 80-100 % and half-life is 7-11 hours (67). Elimination is mainly via the liver but around 1/3 is excreted unchanged via the kidneys (67).

1.3.2.3 Apixaban

The direct FXa inhibitor apixaban was compared to warfarin for prevention of stroke in patients with AF in the ARISTOTLE trial (44). The doses used in the study were 5 or 2.5 mg BID, with the lower dose chosen for patients with at least two of the following: s-creatinine > 133 μmol/L, weight < 60 kg or age > 80 years (44). Apixaban treated patients had similar rates of ischemic strokes, less major bleedings, less ICH and lower mortality than warfarin treated patients. Notably, it is the only DOAC that did not increase the risk of GI bleedings when compared to warfarin (68). These findings are supported by real world

data in which apixaban seem to have less bleedings compared to warfarin as well as other DOACs without an increased rate of ischemic stroke (63). Bioavailability is around 50 %, half-life is around 12 hours and elimination by renal excretion is lower than with other DOACs at 25 % (69).

1.3.2.4 Edoxaban

The direct FXa inhibitor edoxaban was approved in 2015 after the phase III trial ENGAGE AF where AF patients were randomized to warfarin treatment or a high (60 mg QD) or low (30 mg QD) dose of edoxaban (45). Both doses were halved in patients with either reduced kidney function (eGFR 30-50 ml/min), low body weight (< 60 kg) or concomitant treatment with verapamil or quinidine. The rate of ischemic stroke was similar to warfarin with the high dose of edoxaban but higher than warfarin with the low dose. Patients treated with both doses of edoxaban had lower rates of major bleeding and ICH as well as lower mortality from cardiovascular causes than warfarin treated patients, but GI bleedings were more frequent in the high dose group compared to warfarin treatment. Based on these data, it was the higher of the doses that was approved for clinical use (70). Bioavailability is around 60 %, half-life is 10-14 hours and elimination by renal excretion is around 35 % (71).

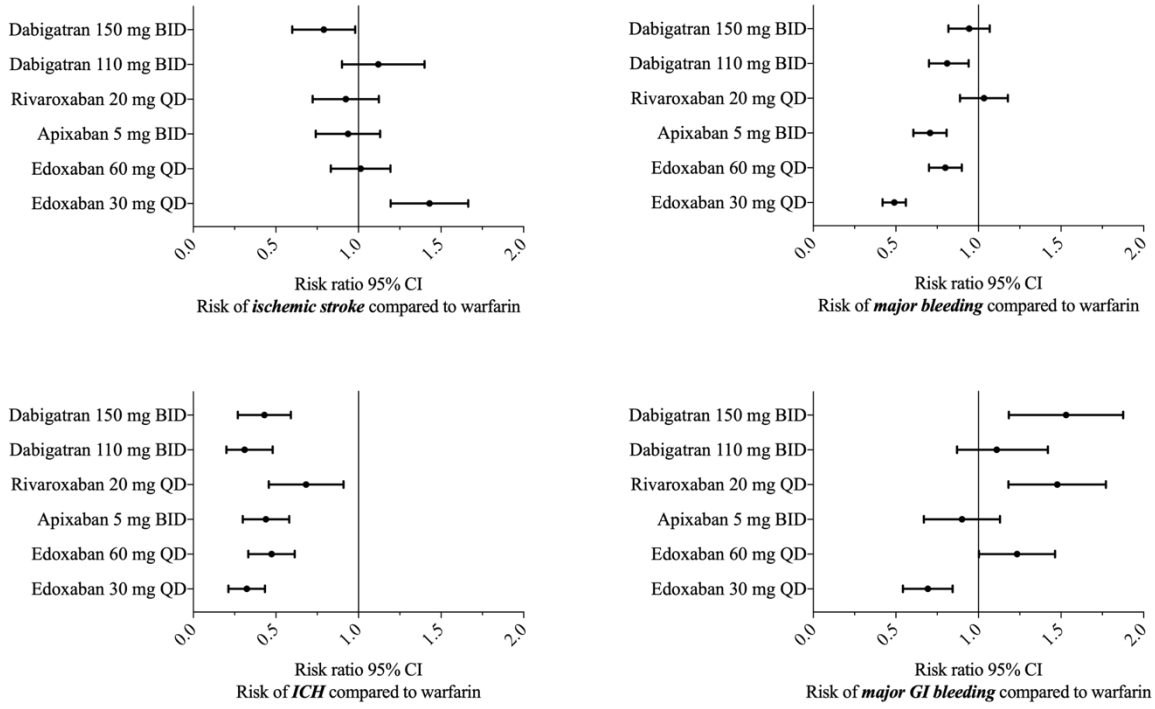

Figure 2. Risk ratios with 95 % CI of outcomes (ischemic stroke, major bleeding, ICH and major GI bleeding) of the phase III trials comparing DOACs with warfarin for prevention of stroke in patients with AF (68).

1.3.3 Factor XI-inhibitors

Factor XI-inhibitors are a new class of anticoagulants that are currently being evaluated in clinical trials (72). Based on the observation that factor XI-deficiency decrease the risk of thrombotic disorders but only lead to limited bleeding problems, the hope is that inhibition of factor XI will prevent thrombosis but reduce the problem with bleedings seen with other anticoagulants (72). Phase II trials have shown promising results for both safety and efficacy for the prevention of VTE in patients undergoing orthopedic surgery and as an anticoagulant in hemodialysis (73-78). In patients with AF, oral factor XI-inhibitor asundexian was compared to apixaban in a phase II trial powered for safety but not for efficacy (79). There was less bleeding with asundexian but there were 3 patients with ischemic stroke in the asundexian group (505 patients) and none in the apixaban group. Further clinical trials on this new class of anticoagulants will give more information on whether they are effective in patients with AF.

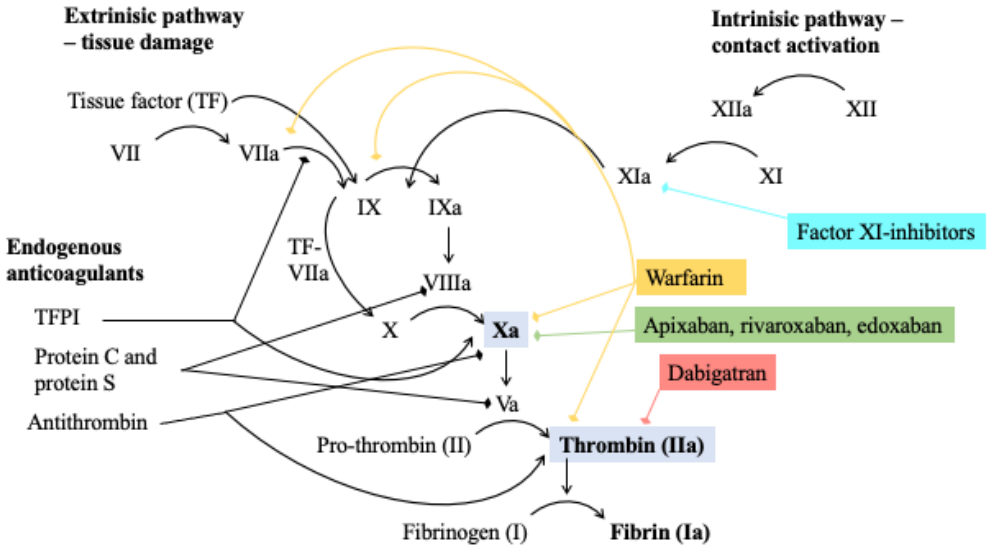

Figure 3. The actions of the coagulation factors in the coagulation system and the targets of endogenous- and OACs as well as factor XI-inhibitors.

1.3.4 Reversal of OAC effects in emergency situations

The inherent side effect of anticoagulant treatment is an increased risk of bleeding, in which case reversal of the anticoagulant effect might be needed. Additionally, anticoagulated patients might require urgent surgery that is not possible if the anticoagulant effect is not reversed. In the phase III-trials comparing DOACs with warfarin, major bleeding occurred at a rate of around 3 % per year but was higher in patients with chronic kidney disease (80). In a real-world setting, patients are generally older with more comorbidities and so higher rates of bleeding are expected. Similar bleeding rates as in the RCTs has been observed in Sweden whereas higher bleeding rates of 4 and 5 % per year have been reported in US populations, and a study on a Korean population reported major bleeding rates of around 8

% (81-84). Urgent surgical procedures occurred at a rate of around 1 % per year in the RE-LY trial (85). Considering the high, and increasing, prevalence of anticoagulant treatment, these should be rather common situations for clinicians to deal with.

1.3.4.1 Reversal of VKA effect

Reversal of VKA treatment can be guided by measurement of PT-INR which is available in most emergency rooms as a POC-test (86). If indicated, VKA effect can be reversed rapidly using prothrombin complex concentrates (PCC) and less rapidly using vitamin K (38).

1.3.4.2 Reversal of DOAC effect

When the DOACs were introduced, there were no established reversal strategies and no consensus on how to estimate DOAC effect in emergency situations. Thus, this has been an area of intense research. Laboratory evaluation of DOAC effect will be further discussed below. The mortality rate in patients with severe bleeding treated with reversal agents is reportedly high (17 %) and thromboembolic complications occur in almost 5 % of patients (87).

In 2015, the monoclonal antibody fragment idarucizumab (IDZ) was approved for reversal of dabigatran effects in patients with uncontrolled or life-threatening bleeding or in need of urgent surgery (88). When indicated, it is given at a fixed dose of 5 grams, binds dabigatran with high affinity and effectively neutralizes its effect within minutes without any evident prothrombotic effects (89). The standard dose of 5 g is sufficient for most patients except in cases with exceptionally high concentrations of dabigatran. Of note, IDZ has a relatively short half-life and dabigatran effect can reappear after 12 hours (88, 90, 91).

In 2018, andexanet alfa (AA) was approved for reversal of apixaban and rivaroxaban effect in patients with uncontrolled or life-threatening bleeding (92, 93). It is a recombinant protein, similar to FXa but not enzymatically active, that acts as a decoy for FXa inhibitors (94, 95). Depending on when and how much DOAC was last ingested, a high (800 mg bolus followed by 8 mg/min continuous infusion during 120 min) or low (400 mg bolus followed by 4 mg/min continuous infusion during 120 min) dose of AA is administered (93). Of note, this regimen does not achieve complete reversal of anticoagulant effect. Median FXa activity was reduced by 92 % in apixaban- and rivaroxaban treated patients but remained high in some patients. Additionally, the anticoagulant effect of the FXa-inhibitors returns after the infusion is stopped and is back at about 50 % of pre-treatment value 4 hours post-infusion (92). A prothrombotic signal with a transient increase in prothrombin fragments 1 and 2, thrombin-antithrombin complex and D-dimer has been detected after treatment with AA (92). The clinical relevance of this is yet to been determined but in a meta-analysis of reversal of DOACs there were more thromboembolic complications in patients treated with AA compared to IDZ and PCC (87).

PCC has been, and is still, used for DOAC reversal when antidotes are not available and it appears to be somewhat effective, at least for patients on FXa-inhibitors (87, 96-102). Per

oral administration of activated carbon can also be used in patients with recent ingestion of DOACs to prevent further uptake (103).

Another reversal agent that is under development but has shown promise in animal experiments and phase II trials is ciraparantag, a small, synthetic molecule that binds and blocks the effects of both DOACs and heparins (104-106).

1.3.5 Laboratory evaluation of DOAC effect

1.3.5.1 When should we assess DOAC effect?

DOACs have been studied and approved without the need for routine monitoring and real world data confirm the safety and efficacy of this approach compared to warfarin treatment (42-45, 63). However, there are certain situations when assessment of the anticoagulant activity in a DOAC treated patient is probably useful to help guide appropriate management. These include emergency situations such as bleeding, before urgent surgery, guiding reversal therapy and determining suitability for thrombolysis in patients with ischemic stroke (107-111). Non-emergent testing to confirm therapeutic effect in some patient groups with possibly different pharmacokinetic profiles from the patients largely included in the RCTs (elderly patients, patient with extreme body weights, patients with renal failure and patients with possible drug interactions) may also be considered (106-111).

1.3.5.2 What do we measure when we assess DOAC effect?

DOACs are direct inhibitors of either thrombin or FXa and it seems intuitive that the anticoagulant effect is directly related to the plasma concentration of the actual drug. Clinical data support this notion, as there were clear correlations between through concentration of both dabigatran and edoxaban and the risk of ischemic stroke and major bleeding (Figure 4) (112, 113). Consequently, assessing DOAC effect for clinical decision-making means estimating DOAC concentration. In non-emergent situations, to confirm that a patient is adequately treated, trough concentration is determined and compared to the range provided by the large RCTs (110). In emergency situations however, the question is whether there is a significant DOAC effect present that needs to be considered that would warrant reversal therapy in a bleeding patient or that would permit emergency surgery or thrombolysis. The evidence for what DOAC concentration should be considered clinically significant is rather weak but guidelines documents have suggested the following thresholds, largely based on expert opinion: a DOAC concentration > 50 ng/mL in patients with a major bleeding and a DOAC concentration > 30 ng/mL in patients requiring emergency surgery with high bleeding risk or thrombolysis (110, 114). 20 ng/mL has also been suggested as a limit of significant anticoagulant effect (115).

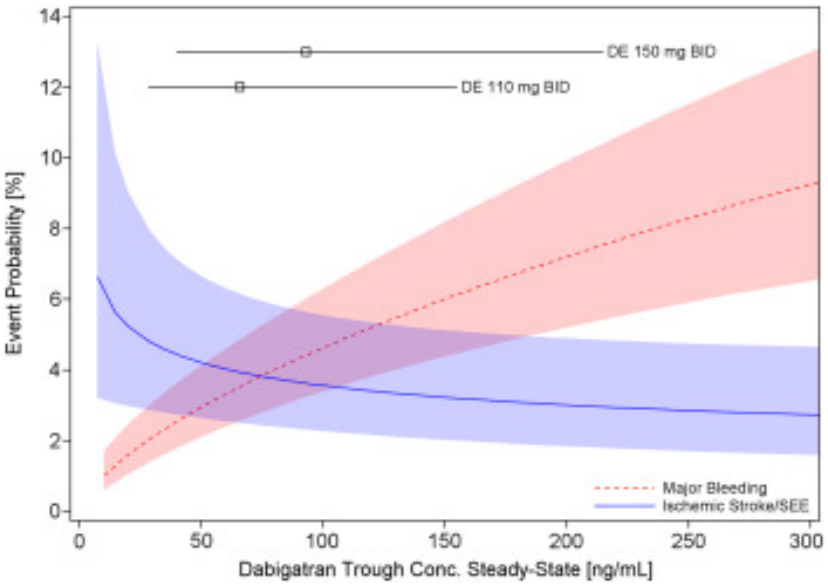

Figure 4. Probability of major bleeding and ischemic stroke depending on trough dabigatran concentration for patients in the RE-LY trial. Figure reprinted with permission from Elsevier (112). © 2014 by the American College of Cardiology Foundation.

1.3.5.3 What tests can we use?

1.3.5.3.1 LC-MS/MS

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is considered the gold standard for determination of DOAC concentration (116-124). It is a highly sensitive method which determines the exact concentration. However, it is quite laborious, time consuming and requires instruments that are not available at all hospitals. Therefore, this method is not suitable for use in emergency situations.

1.3.5.3.2 Routine coagulation tests

DOACs affect routine coagulation tests to a varying degree depending on type of DOAC, its concentration and which test and reagent is used (111, 125-129). The most used coagulation tests are PT, which activates the extrinsic coagulation pathway through TF, and activated partial thromboplastin time (aPTT), which activates the intrinsic/contact coagulation pathway through kaolin, ellagic acid or silica (130). All DOACs increase PT and aPTT if the concentrations are sufficiently high, but a normal value of either test does not exclude a clinically significant concentration of any of the DOACs (107-111, 125-129, 131). The FXa-inhibitors generally prolong PT more than they prolong aPTT, with the least effect seen with apixaban, while dabigatran generally prolongs aPTT more than it prolongs PT (107-111, 125-129). Thrombin time (TT) measures clotting time after activation with thrombin. It is highly sensitive to dabigatran treatment - a normal value basically excludes presence of dabigatran, but TT is prolonged even by clinically irrelevant concentrations of dabigatran (<30 ng/mL) (108, 131, 132). TT is not affected by FXa-inhibitors (108).

Of note, DOACs also affect the results of coagulation assays used for thrombophilia testing (lupus anticoagulants, antithrombin, protein S and protein C) (109, 127-129, 133-139). The

general recommendation is to make an interruption of DOAC treatment for 3 days or more before performing these tests (110). As an alternative to this approach, products based on activated carbon (DOAC-Stop (DS) and DOAC-Remove) have been developed for in vitro removal of DOACs before testing (110, 140-145).

1.3.5.3.3 Functional DOAC specific assays

As the LC-MS/MS method of estimating plasma concentration of DOACs is not suitable for use in emergency situations, plasma-based coagulation assays with drug specific calibrators have been developed – dilute Thrombin Time (dTT), ecarin clotting time (ECT) and ecarin chromogenic assay (ECA) for dabigatran and the anti-FXa assay for FXa-inhibitors (116-119, 146-153). The assay results are converted to an estimated mass concentration, and they accurately measure DOAC concentration down to 30-50 ng/mL which is around the threshold for clinically relevant concentration of DOACs (110, 114, 116-119). When expediated, turnaround times (time from registration of sample in the laboratory until a test result is provided) of 30 min has been described but at most places and in most situations, it is probably considerably longer (107).

1.3.5.3.4 Point-of-care (POC) tests

The need for POC tests that provide rapid assessment of DOAC effect and can help guide treatment in emergency situations has been highlighted, but as of now, there are none in clinical use (110).

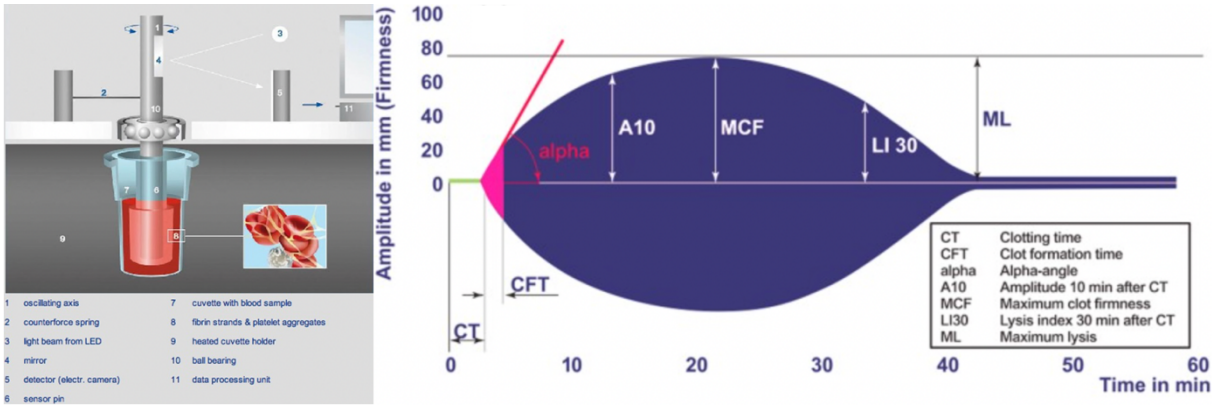

Figure 5. The ROTEM device (left) consists of a pin rotating in a small cup where whole blood and reagents are mixed. As the clot is formed, the strength of the clot is measured over time by the rotation of the pin and is depicted as a graph (right). Figure reprinted with permission from John Wiley and Sons (154). © 2013 Wiley Periodicals, Inc.

Viscoelastic tests such as rotational thromboelastometry (ROTEM®), thromboelastography (TEG®) and ClotPro® are widely used POC to guide transfusions in perioperative and trauma settings (Figure 5) (154-158). The methods assess the strength of a forming clot in real time, providing information that can help diagnose factor deficiencies, hyperfibrinolysis, thrombocytopenia and hypofibrinogenemia (155, 156). As these methods are already used POC in the clinic, there has been a growing interest in evaluating their usefulness to detect treatment with DOACs (159-180).

Other technologies being evaluated as POC tests for DOACs include urine dipsticks and microfluidic assays (110, 181-184).

1.3.5.3.5 Coagulation assays used primarily in research

Total Thrombus-formation analysis system (T-TAS®) is an instrument that allows the quantification of thrombus formation under variable flow conditions which in theory is more physiological than other coagulation tests (Figure 6) (185, 186). T-TAS has been shown to detect effects of DOACs and results have been shown to predict bleeding events (187-189).

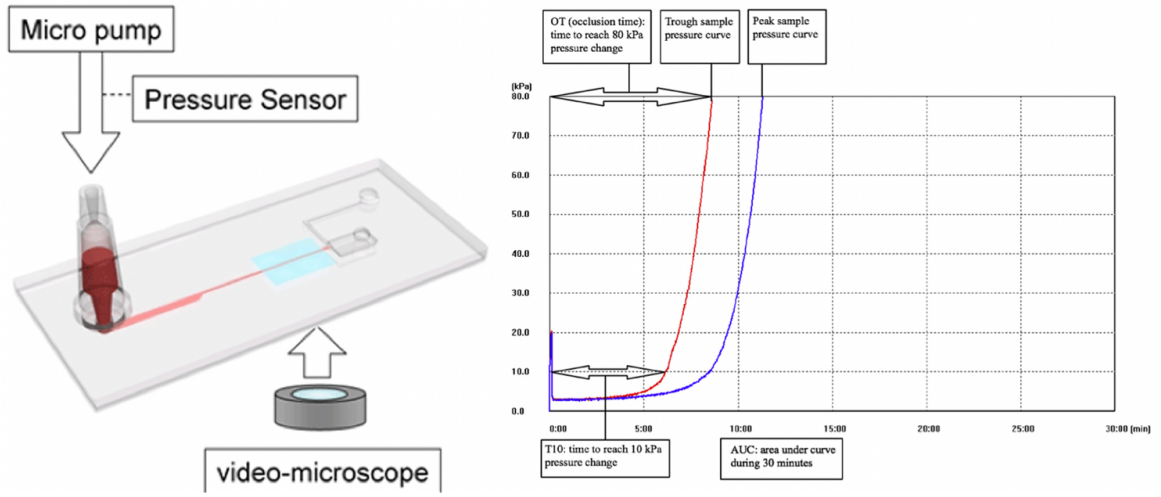

Figure 6. The T-TAS device (left) consists of a micropump with a pressure sensor that injects whole blood through a disposable chip with artificial capillaries. As thrombi are formed, pressure in the capillaries increase. This pressure build-up is registered over time as a graph (right). Two curves are seen in the graph, trough and peak samples of dabigatran – the peak sample curve is displaced to the right as thrombus formation is delayed compared to the trough sample. Left image reprinted with permission of Fujimori Kogyo CO., LTD. Right image reprinted with permission from Elsevier (190). © 2017 Elsevier Ltd.

Calibrated automated thrombogram (CAT) measures thrombin concentration over time using a fluorogenic substrate, usually after clotting activation with TF (Figure 7) (191). CAT results have been linked to various thrombotic disorders, including acute coronary syndrome (ACS), ischemic stroke and VTE (192-194). DOAC treatment increases lag time and FXa-inhibitors reduce peak thrombin and endogenous thrombin potential (ETP) (195, 196). A paradoxical increase in peak thrombin and ETP has been described with dabigatran treatment (197-199). The reason for this is that the method for calculating thrombin concentration includes an algorithm to subtract the activity of α_2-macroglobulin-thrombin complex, which becomes incorrect in the presence of dabigatran due to interaction between dabigatran and the α_2-macroglobulin-thrombin complex (200, 201).

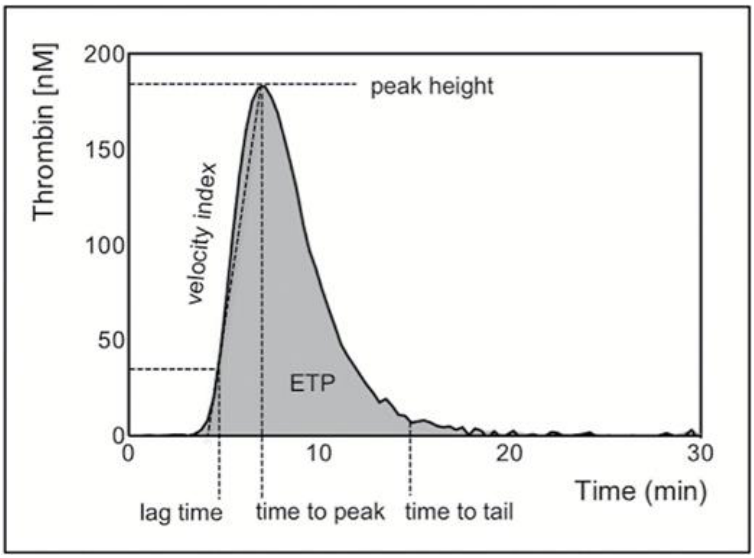

Figure 7. The output of the CAT assay is a "thrombogram" - a graph that depicts thrombin concentration over time in a clotting sample. Figure reprinted with permission from Oxford University Press (202). © Oxford University Press.

Fibrin network permeability can be measured using variations of a technique described by Blombäck and Okada in 1982 (203, 204). Decreased fibrin network permeability has been described in many different conditions associated with thrombosis including e.g. ischemic stroke, VTE and ACS (205-210). Increased permeability has been described with anticoagulant treatment (211-216). Previous studies from our group have demonstrated that fibrin network permeability is dose-dependently increased by VKAs, direct thrombin inhibitors and direct- and indirect FXa-inhibitors in *in vitro* experiments (212, 213, 215).

Turbidimetric clotting and lysis assays describes clotting and lysis of a sample in response to a reagent, usually thrombin or TF, by measuring its optical density over time (217). Results have been linked to VTE, ischemic stroke and adverse outcome after ACS (205, 218-220). Previous studies have reported more pronounced effects of dabigatran compared to FXa-inhibitors as well as dose dependent shortening of lysis time with dabigatran treatment (221-223).

2 RESEARCH AIMS

The aims of this thesis were:

- To evaluate the use of whole blood point-of-care tests ROTEM (viscoelastic method) and T-TAS (measures flow-based thrombus formation) to assess the anticoagulant effect of the direct oral anticoagulant (DOAC) dabigatran (study I)

- To improve the accuracy of the ROTEM assay (the point-of-care test with most promising results in study I) by using modified triggers (study II and III)

- To further characterize the anticoagulant effects of DOACs by analyzing their effect with both global and specific coagulation assays (study IV)

3 MATERIALS AND METHODS

3.1 STUDY DESIGNS AND POPULATIONS

3.1.1 Study I

In this observational study on AF patients we assessed the anticoagulant effect of dabigatran with the coagulation assays ROTEM and T-TAS and compared the results to plasma dabigatran concentration measured by LC-MS/MS. 30 patients (30 % female gender, median age 71 years (range 62-78), median CHA_2DS_2-VASc score 2 (range 1-5)) treated with dabigatran 150 mg BID due to AF were included from the Hemostasis Center at Danderyd Hospital. Blood samples were drawn at estimated trough (mean 12.2 hours after last capsule intake) and peak (mean 2.8 hours after last capsule intake) levels of dabigatran and collected in 3.2 % citrate tubes for ROTEM- and T-TAS analyses, determination of dabigatran concentration by LC-MS/MS as well as routine coagulation tests aPTT and PT-INR. As a result of time and labor limitations, T-TAS analyses could only be performed in 23 patients and ROTEM with commercial triggers Ex-tem, Fib-tem and In-tem in 15 patients.

3.1.2 Study II

In study II, we evaluated if the ROTEM assay could work as a POC-test to rapidly determine dabigatran effect in whole blood samples and if the accuracy of the assay could be improved by using a modified trigger based on thrombin and by reversing the effect of dabigatran in the samples with the antidote IDZ.

35 patients (17 % female gender, median age 69 years (range 53-73), median CHA_2DS_2-VASc score 2 (range 0-5)) treated with dabigatran 150 mg BID due to AF were included from the Hemostasis Center at Danderyd Hospital. Blood samples were drawn at any time in relation to capsule intake and collected in 3.2 % citrate tubes for ROTEM analyses and determination of dabigatran concentration by LC-MS/MS. Before ROTEM analysis, half of the blood from each donor was incubated with IDZ for 5 minutes at a final whole blood concentration of 1 mg/mL (estimated to be similar to the concentration in a patient receiving the standard dose of IDZ (5 g)).

In addition, 20 healthy donors were recruited from the local blood donor center at Danderyd Hospital. Blood samples were collected in 3.2 % citrate tubes. 10 of these donors served as controls for the patients and these samples were analyzed with ROTEM with and without incubation with IDZ.

Blood from the other 10 donors was used for in vitro experiments with dabigatran. Whole blood was incubated at 37 °C for 15 minutes with 1 % dabigatran solution (diluted in dimethylsulfoxide (DMSO) and acetonitrile-water) at estimated plasma concentrations of 0, 20, 50, 100, 300 and 500 ng/mL, assuming a hematocrit of 40 %. These samples were then analyzed with ROTEM, with and without incubation with IDZ.

3.1.3 Study III

In study III, we evaluated if the ROTEM assay could work as a POC-test to rapidly determine apixaban effect in whole blood samples and if the accuracy of the assay could be improved by using modified triggers based on FXa or Russel viper venom (RVV; a snake venom that contains a potent activator of factor X (224)) and by reversing the effect of apixaban in the samples with the antidote AA or the charcoal-based agent DS.

40 patients (38 % women, median age 74 years (range 55 - 85), median CHA_2DS_2-VASc score 3 (range 0-6)) treated with apixaban 5 mg BID (32) and 2.5 mg BID (8) due to AF (39) and VTE (1) were recruited during hospitalization (due to AF (n=25), heart failure (n=9), ACS (n=3), ventricular tachycardia (n=1), bleeding (n=1) and benign chest pain (n=1)) at the Department of Cardiology at Danderyd Hospital. Blood samples were drawn and collected in 3.2 % citrate tubes for ROTEM analysis and determination of apixaban concentration with LC-MS/MS and with a chromogenic anti-Xa assay.

1/3 of the blood intended for ROTEM analyses was incubated with antidote AA (0.16 and 0.64 mg/mL – corresponding to maximum concentration of the high dose of AA in healthy volunteers as well as a supratherapeutic concentration) and 1/3 of the blood was incubated with DS (one minitab of DS was added to 4.5 ml of whole blood) for at least 10 minutes before analysis. As AA was not available to us at the start of the study, fewer of the samples were analyzed in the presence of AA (only 10 of the patients).

In addition, blood samples were collected from 14 healthy donors and collected in 3.2 % citrate tubes for in vitro experiments with apixaban. Whole blood was incubated at 37 °C for 15 minutes with 1 % apixaban solution (diluted in DMSO and acetonitrile-water) at estimated plasma concentrations of 0, 20, 50, 100, 300 and 500 ng/mL, assuming a hematocrit of 40 %. These samples were then analyzed with ROTEM, with and without incubation with AA and DS. As AA was not available to us at the start of the study, fewer of the samples were analyzed in the presence of AA (only 4 of the donors).

3.1.4 Study IV

In study IV, we assessed the anticoagulant effect of dabigatran, rivaroxaban and apixaban, in comparison to each other and to warfarin treatment, with a fibrin network permeability assay, the CAT assay, a turbidimetric clotting and lysis assay and levels of coagulations markers thrombin-antithrombin complex (TAT) and D-dimer and compared the results to plasma DOAC concentration measured by LC-MS/MS.

96 patients treated with dabigatran (150 mg BID (23)), rivaroxaban (20 mg QD (26)), apixaban (5 mg BID (18) and 2.5 mg BID (2)) and warfarin (27) were recruited from coagulation centres in the Stockholm County (Danderyd Hospital, Karolinska University Hospital and Stockholm Heart Centre). The indication for DOAC treatment was AF whereas the indications for warfarin treatment were VTE (23), AF (1), arterial embolism (1) and mechanical heart valve (2). Patients' clinical characteristics are provided in Table 1. Blood samples were drawn and collected in 3.2 % citrate tubes and immediately

centrifuged at 2000 x g for 20 minutes at room temperature. Plasma was then frozen at –70°C in aliquots of 0.5 mL and thawed before analysis with a fibrin network permeability assay, the CAT assay, a turbidimetric clotting and lysis assay, PT-INR and aPTT and determination of DOAC concentration with LC-MS/MS and levels of D-dimer, TAT and fibrinogen. D-dimer level and turbidimetric assays were not performed in warfarin samples as these samples had been lost in a freezer accident at the time of analysis.

In addition, fibrin network permeability was determined in purchased normal pool plasma (NPP) incubated with different concentrations of dabigatran, rivaroxaban and apixaban (5 µl of DOAC solution to 200 µl of NPP for concentrations ranging from 0-1000 ng/mL) and purchased plasma from patients with known PT-INR. NPP was also analyzed for reference in the turbidimetric assay in five repeated runs.

Table 1. Clinical characteristics of patients included in study IV. Values presented as median and quartiles or percentages.

	Dabigatran (n=23)	***Rivaroxaban (n=26)***	***Apixaban (n=20)***	***Warfarin (n=27)***
Female gender (n (%))	7 (30)	9 (35)	9 (45)	12 (44)
Age (years)	67 (58-70)	69 (62-76)	76 (67-77)	54 (46- 64)
Weight (kg)	84 (74-97)	84 (81-89)	82 (66-92)	86 (78-97)
Smokers/ex smokers (%)	0/57	12/31	22/30	22/41
Diabetes mellitus (n (%))	2 (9)	5 (19)	5 (25)	1 (4)
Hypertension (n (%))	18 (78)	16 (62)	12 (60)	12 (44)
Myocardial infarction (n (%))	3 (13)	2 (8)	3 (15)	3 (11)
Heart failure (n (%))	4 (17)	2 (8)	2 (10)	2 (7)
Ischemic stroke/Transitory ischemic attack (n (%))	4 (17)	8 (31)	2 (10)	1 (4)
Venous thromboembolism (n (%))	0	0	1 (5)	23 (85)
Aspirin treatment (n (%))	0	2 (8)	0	1 (4)

3.2 LABORATORY METHODS

3.2.1 ROTEM

All ROTEM analyses were performed at 37 C° within 60 minutes after sampling and in accordance with the manufacturer's recommendations. Briefly, 300 µL of citrated whole blood was mixed with 20 µL of trigger solution and recalcified with 20 µL of Star-tem before starting the analysis.

3.2.1.1 Study I

ROTEM analyses were performed with commercial triggers Ex-tem (based on recombinant TF), In-tem (based on ellagic acid) and Fib-tem (based on recombinant TF but with addition of cytochalasin D to inhibit platelet contribution) as well as a trigger developed by us with low concentration of TF (ROTEM Low TF; Innovin, final concentration 5 pM). Variables recorded from the graphs were CT (clotting time), CFT (clot formation time), alfa angle and MCF (maximum clot firmness).

3.2.1.2 Study II

ROTEM analyses were performed with the commercial trigger Ex-tem as well as a trigger developed by us that consisted of thrombin (final concentration in the cup 2 IU/ml) and PPL (final concentration in the cup 2 µM). Based on the results from study I, the only

variable recorded from the graphs was CT. The difference in CT between samples with and without IDZ was evaluated as a separate variable, CT difference (CT_{diff}).

In 10 patients, we performed our ROTEM analyses as expediently as possible and recorded the turnaround time.

3.2.1.3 Study III

ROTEM analyses were performed with the commercial trigger Ex-tem (which seems to be the most sensitive to apixaban treatment of the commercial triggers (176, 225)) as well as two triggers developed by us, one based on a FXa (final concentration the cup 0.05 IU/ml) and one based on RVV (final concentration in the cup 1500 ng/mL) and both with the addition of PPL (final concentration in the cup 2 μM). The only variable recorded from the graphs was CT. The difference in CT between samples with and without DS was evaluated as a separate variable, CT difference (CT_{diff}). Analyses with AA was not used for calculation of CT_{diff} as there were too few analyses available.

In 5 patients, we performed our ROTEM analyses as expediently as possible and recorded the turnaround time.

3.2.2 T-TAS

Thrombus formation was evaluated by perfusing whole blood through artificial capillaries in a disposable chip (the atheroma chip (AR-chip) was used, in which the capillaries are lined with collagen and TF). Citrated whole blood was recalcified and treated with corn trypsin inhibitor that inhibits contact activation of coagulation before perfusion at a flow of 10 μL/min, which corresponds to a shear rate of 600 s^{-1} (corresponding to flow in large arteries). All analyses were performed at 37 C° until pressure change reached 80 kPa, or during maximum 30 minutes, in accordance with the manufacturer's recommendations. The variables recorded from the graph were T10 (time to reach 10 kPa), OT (occlusion time, time to reach 80 kPa) and AUC (area under the pressure time curve during 30 min). The difference between OT and T10 (OT –T10), which represents the rate of thrombus growth, was also calculated.

3.2.3 Fibrin network permeability

Fibrin network permeability was determined according to the approach originally described by Blombäck et al. (203) and later modified by our group (211, 226). Briefly, fibrin gels were formed in plastic cylinders by mixing plasma with recombinant TF (Innovin, final concentration 5pM), PPL (final concentration 4μM) and $CaCl_2$ (final concentration 20mM) and allowing Tris-NaCl buffer to percolate trough the gel at a known hydrostatic pressure. The permeability coefficient was calculated by using the following formula:

$$Ks\ (\times 10^{-9}, cm^2) = [Q \times L \times \eta] / [t \times A \times \Delta P]$$

where Q ($cm^{3)}$ is the flow rate at time t (sec), L (cm) is the length of the fibrin gel, η (dyne x sec/cm^2) is the viscosity of the liquid, A (cm^2) is the cross-sectional area and Δp (dyne/cm^2)

is the differential pressure. Fibrin network permeability measured in patient samples was normalized against values obtained from NPP and reported as "% of control".

3.2.4 CAT

A coagulation trigger solution was prepared by adding TF and PPL to tris buffer, giving the concentrations 30 pmol/L and 24 μmol/L, respectively. In a well of the microplate, 20 μL of above trigger solution was mixed with 80 μl of the plasma sample, to which 20 μL of FluCa (a commercial reagent containing $CaCl_2$ and the fluorogenic substrate) was then added to start the reaction, giving final concentrations 5 pmol/L of TF, 4 μmol/L of PPL and 20 mmol/L of $CaCl_2$. The fluorescence in the plasma was read every 30 seconds for 60 minutes using a Fluoroscan Ascent fluorometer (Fluoroscan Ascent, Thermo Scientific, Vanta, Finland), providing a graph of estimated thrombin concentration at each time point, from which the variables lag time, peak thrombin and ETP were recorded.

3.2.5 Turbidimetric clotting and lysis assay

From each sample, turbidimetric clotting and lysis assays were performed according to the method previously described by Carter et. al (217). Both were performed in transparent flat bottom 96-well plates (Sigma-Aldrich, St-Louis, MO, United States) and optical density as a measure of clotting of the sample was read by a Tecan Sunrise (Männedorf, Switzerland) absorbance microplate reader every 12 s for 1 hour. Briefly, 25 μl of plasma was diluted with 75 μl of tris buffer and clotting was initiated by adding 50 μl of an activation mix containing, for the clotting assay, thrombin (final concentration 0.03 IU/mL) and $CaCl_2$ (final concentration 7.5 mmol/L). For the lysis assay, the same activation mix was used but with the addition of tPA (final concentration 83 ng/mL). The variables recorded were lag time (time until clotting initiation in clotting assay; when there is an exponential increase in optical density), max absorbance (maximum clotting of clotting assay; difference in optical density between time of clotting initiation and maximum absorbance), CFR (clot formation rate of clotting assay; maximum absorbance divided by time between start of clotting initiation and maximum absorbance) and lysis time (time between maximum absorbance of lysis assay and 50% lysis).

3.2.6 LC-MS/MS

Plasma concentrations of apixaban, rivaroxaban and dabigatran were determined with LC-MS/MS at the Department of Clinical Pharmacology, Karolinska University Hospital as previously described in detail elsewhere (116-118).

3.2.7 Anti-FXa assay

Determination of apixaban concentration using a chromogenic anti-FXa assay (STA® Liquid Anti-FXa; Diagnostica Stago, Asnieres sur Seine, France) was performed at the Department of Clinical Chemistry at Karolinska University Hospital as previously described (118).

3.2.8 D-dimer

D-dimer concentrations were measured by a rapid particle-enhanced immunoturbidimetric assay, Siemens INNOVANCE® D-dimer on the instrument Sysmex CS5100.

3.2.9 TAT

An enzyme immunoassay was used to determine TAT complex levels in plasma (Enzygnost TAT Micro; Behringwerke AG, Marburg, Germany).

3.2.10 Fibrinogen

Fibrinogen was quantified immunologically using reagents from Siemens Healthcare Diagnostics (Deerfield, IL, USA). Nephelometric measurement was performed on a BN ProSpec (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The locally established reference interval used is 2.0-4.2 g/L.

3.2.11 PT

PT using the Owren reagent SPA+® (DiagnosticaStagoAsnieres, France) was performed at the Department of Clinical Chemistry at Karolinska University Hospital on a Sysmex® CS2100i (Sysmex, Kobe, Japan). Results are presented as INR with normal range<1.2.

3.2.12 aPTT

aPTT using the Automate® reagent (DiagnosticaStagoAsnieres, France) was performed at the Department of Clinical Chemistry at Karolinska University Hospital on a Sysmex® CS2100i (Sysmex, Kobe, Japan). Results are given in seconds with normal range ≤ 40 seconds.

3.3 STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, Inc., USA) and IBM SPSS version 22 (IBM Inc., USA). Normality distribution was evaluated through visual interpretation of histograms as well as testing with either D'Agostine –Pearson or Shapiro-Wilks. P-values <0.05 were considered significant for all tests. Pearson's or Spearman's correlation coefficients were used for estimation of simple correlations between variables as appropriate depending on normality distribution. Differences between two dependent samples were evaluated using the paired t-test or the Wilcoxon sign rank test for related samples as appropriate depending on normality distribution. Differences between two independent samples were evaluated using the unpaired t-test or the Mann-Whitney U-test as appropriate depending on normality distribution. Differences between three or more independent samples were analyzed using ordinary one-way analysis of variance (ANOVA) followed by post hoc testing (Tukey's or Dunnett's) if significant, or Kruskal Wallis followed by post hoc testing (Dunn's) as appropriate depending on normality distribution. Differences between three or more dependent samples were analyzed using repeated measures one-way ANOVA followed by post hoc testing (Tukey's or Dunnett's) if significant, or Friedman followed by post hoc

testing (Dunn's) as appropriate depending on normality distribution. For comparison of distribution of nominal variables Fisher's exact test was used.

Analysis of receiver operating characteristics (ROC) was used to determine CT and CT_{diff} with maximum specificity and sensitivity for discrimination between samples with and without dabigatran and apixaban respectively in study II and III.

3.4 ETHICAL CONSIDERATIONS

All studies were performed in accordance with the Declaration of Helsinki and were approved by the Ethical Review Board in Stockholm, Sweden. Oral and written informed consent was obtained from all study participants. The voluntariness of participation and the right to demand to cancel the participation at any time without any explanation was stressed. Blood samples and patient data were handled and stored in accordance with the Biobanks in Medical Care Act and General Data Protection Regulation.

The risks study participants were subjected to when included in the studies was weighed against the benefits of performing the research. The only physical risk was the risk of venous blood sampling which, when performed by an experienced person, can be considered almost negligible. There is also a risk towards the study persons' personal integrity when sensitive data is retrieved, stored and analyzed. To minimize this risk, all data was coded and anonymized in the data sets and original data was stored separately in locked spaces. Published data cannot be traced back to any individual study participants.

There were no direct benefits for study persons to participate in the studies. However, some study participants benefited from an extra opportunity to ask questions about their treatment and, by contributing to increased knowledge on DOAC effects and treatment, it is possible that they will benefit further in the future.

4 RESULTS AND CONCLUSIONS

4.1 STUDY I

4.1.1 ROTEM

Dabigatran concentration correlated to only one ROTEM variable, CT, and the degree of correlation depended on which trigger that was used (Table 2). The TF-based triggers Ex-tem and Fib-tem, which activates the extrinsic coagulation pathway, were most sensitive, and dabigatran concentration correlated strongly to them (r-values 0.92 and 0.93). The "low TF" trigger however, was least sensitive (r-value 0.36). The ellagic acid-based trigger In-tem, which activates the intrinsic coagulation pathway, was less sensitive than Ex-tem and Fib-tem (r-value 0.73). This stands in contrast to the routine coagulation tests as aPTT (activates the intrinsic pathway; r-value 0.72) is more sensitive to dabigatran treatment than PT (activates the extrinsic pathway; r-value 0.43).

Table 2
Correlations between plasma dabigatran concentration and coagulation assay variables. ** $p < 0.01$.

ROTEM Ex-tem CT (n=30)	**0.92****
ROTEM Ex-tem CFT (s), n=30	-0.17
ROTEM Ex-tem alfa angle (s), n=30	0.18
ROTEM Ex-tem MCF (s), n=30	0.19
ROTEM In-tem CT (n=30)	**0.73****
ROTEM In-tem CFT (s), n=30	-0.17
ROTEM In-tem alfa angle (s), n=30	0.17
ROTEM In-tem MCF (s), n=30	0.17
ROTEM Fib-tem CT (n=30)	**0.93****
ROTEM Fib-tem MCF (s), n=30	0.08
ROTEM Low TF CT (n=60)	**0.36****
ROTEM Low TF CFT (s), n=60	0.13
ROTEM Low TF alfa angle (s), n=60	-0.3
T-TAS OT (n=45)	**0.41****
T-TAS AUC (n=45)	**0.40****
T-TAS T10	**0.39****
T-TAS, 600 s-1, OT-T10 (s), n=45	**0.40****
aPTT (n=59)	**0.72****
PT-INR (n=60)	**0.43****

Despite the excellent correlation with dabigatran concentration of the Ex-tem trigger (r-value 0.92), we had some reservations about the applicability of the ROTEM assay with this trigger as a POC-test to assess dabigatran effect. There were quite few patients with low concentrations of dabigatran in this study, but those in the lower range (< 100 ng/mL) had CT-values within or just slightly above the reference interval provided by the manufacturer (Figure 8).

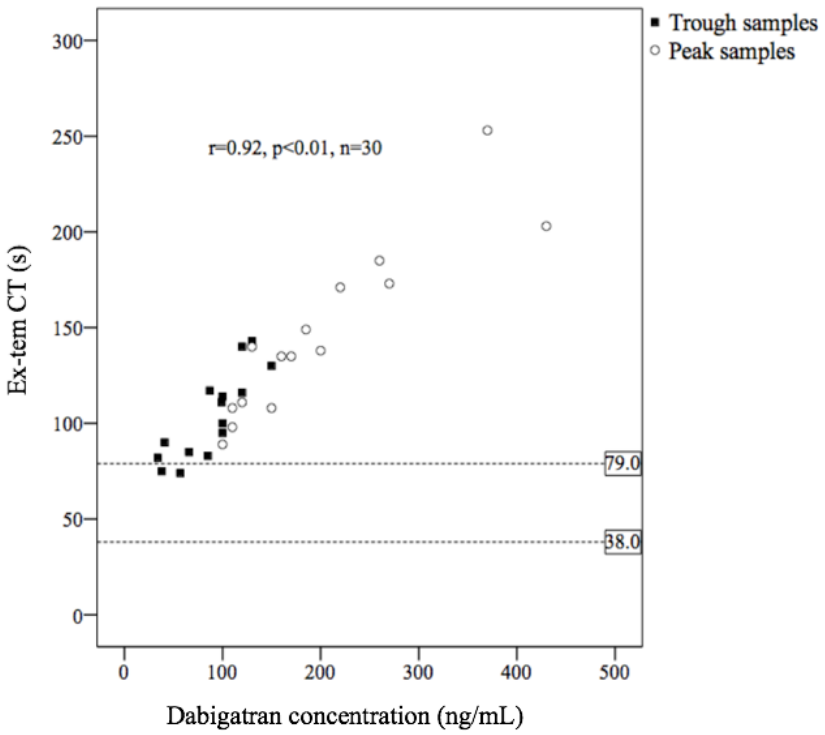

Figure 8. Correlation between dabigatran concentration and Ex-tem CT. Dotted lines correspond to reference interval provided by the manufacturer. Figure reprinted with permission from Elsevier (190). © 2017 Elsevier Ltd.

4.1.2 T-TAS

Dabigatran concentration correlated to all T-TAS variables, but the associations were rather weak (r-values 0.39-0.41; Table 2), indicating that T-TAS is less suitable to assess dabigatran effect – at least when the AR-chip is used, and at shear rate settings of 600 s^{-1}. Of note, this chip assays thrombus formation dependent on both platelet activation and activation of the coagulation system under flow conditions.

4.1.3 Conclusion

Dabigatran concentration correlates strongly to the ROTEM variable CT when it is activated with Ex-tem and Fib-tem triggers, but CT can still be within normal reference interval at significant dabigatran concentrations. Dabigatran concentration correlates weakly to T-TAS variables making them unsuitable to estimate concentration-dependent effects of dabigatran on coagulation.

4.2 STUDY II

4.2.1 Detecting dabigatran effect in vitro

Both Thrombin-CT and Ex-tem CT were concentration-dependently prolonged by dabigatran, but the thrombin trigger was more sensitive as Thrombin CT was prolonged already at 20 ng/mL ($p>0.01$), whereas Ex-tem CT was prolonged first at 50 ng/mL ($p < 0.05$; Figure 9 a-b). Results with the CT_{diff} variable were similar (Figure 9 c-d). Samples with IDZ had similar CT as control samples, regardless of dabigatran concentration,

indicating complete reversal of dabigatran effect in this concentration range (20-500 ng/mL).

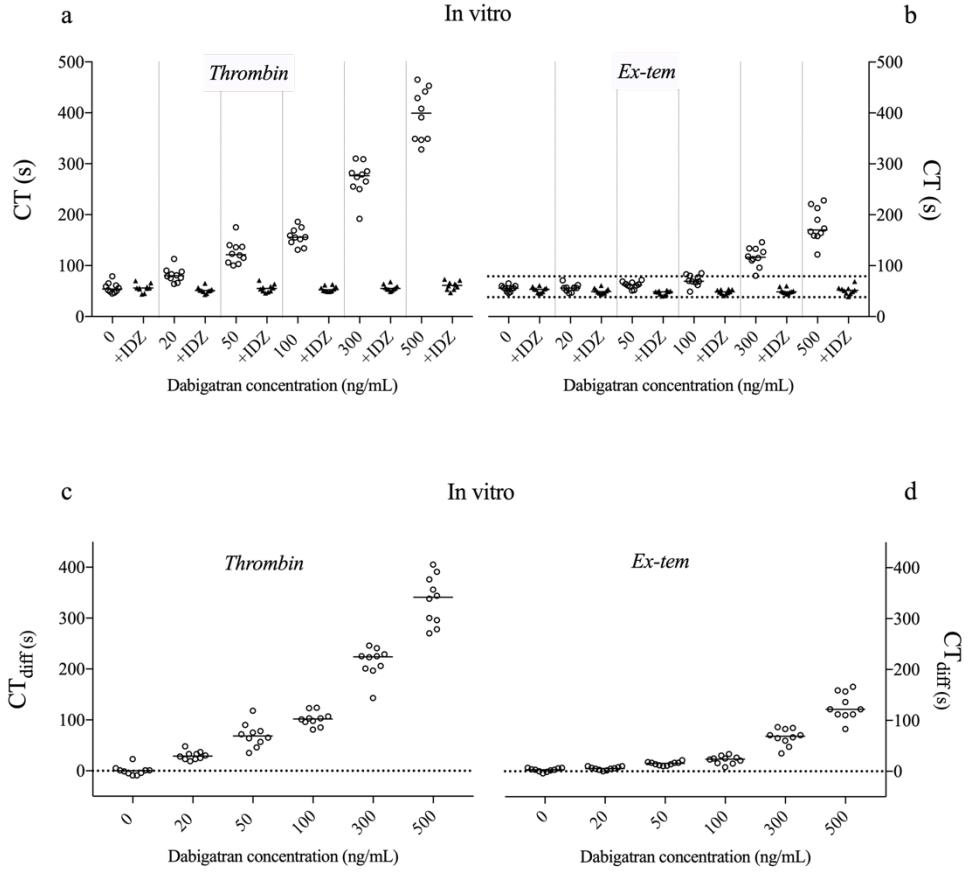

Figure 9. ROTEM analyses using a thrombin-based trigger and Ex-tem trigger in whole blood incubated with estimated dabigatran plasma concentrations 0, 20, 50, 100, 300 and 500 ng/mL, all samples analyzed with or without IDZ. a) Thrombin CT, b) Ex-tem CT, c) Thrombin CT_{diff}, d) Ex-tem CT_{diff}. Medians are indicated by horizontal lines and the dotted lines in the Ex-tem graph correspond to reference interval provided by the manufacturer. Figure reprinted with permission from John Wiley and Sons (227). © 2018 International Society on Thrombosis and Haemostasis.

4.2.2 Detecting dabigatran effect in patients

Both Thrombin CT and Ex-tem CT were significantly longer in patients than in control samples ($p>0.01$; Figure 10 a-b). However, using Ex-tem CT, a few patient samples had CT-values that overlapped with control samples and were within reference interval provided by the manufacturer. With Thrombin CT on the other hand, there was no overlap between patient- and control samples. The only patient with a CT close to control samples was a patient with very low dabigatran concentration (9 ng/mL).

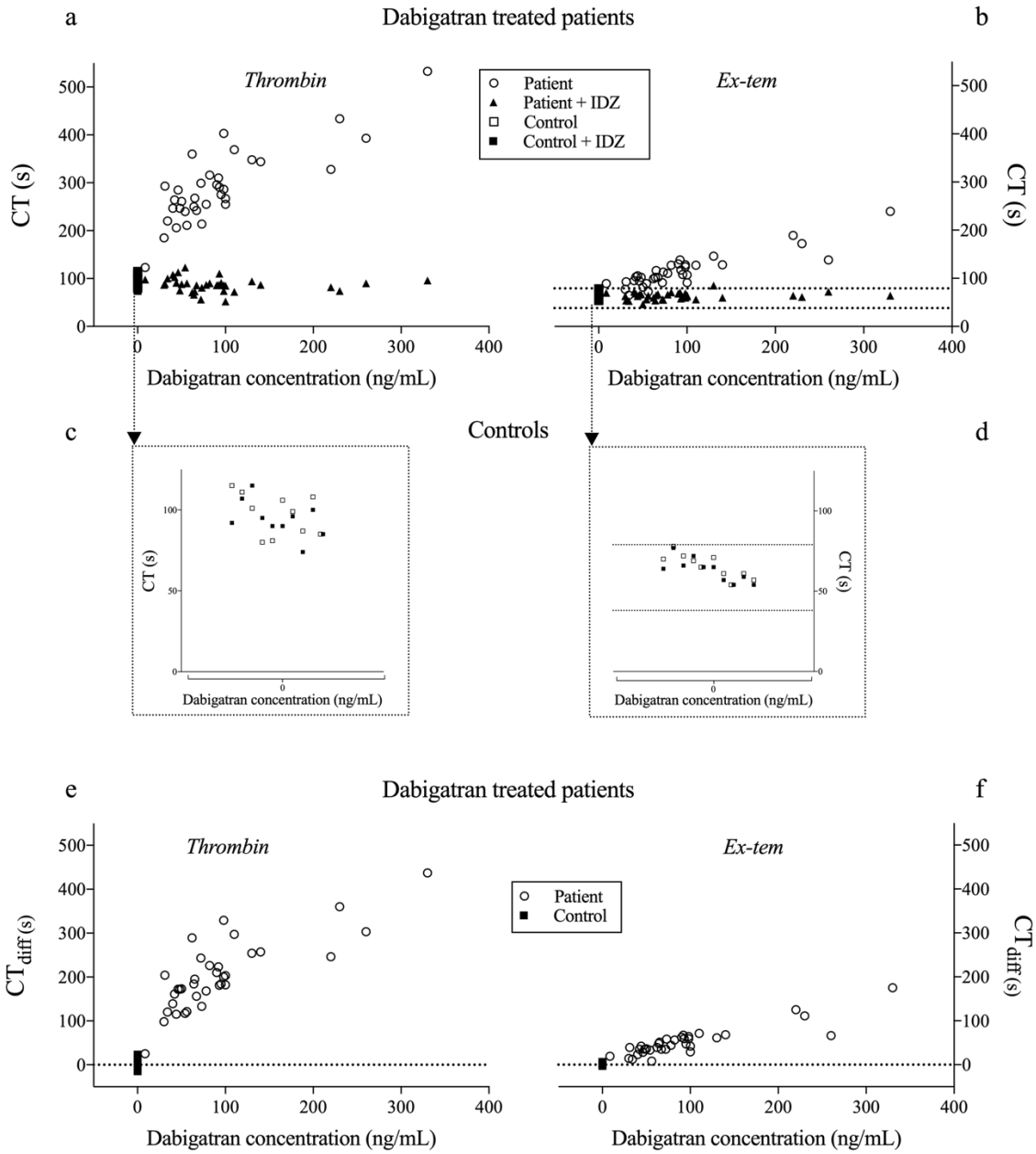

Figure 10. ROTEM analyses using a thrombin-based trigger and Ex-tem trigger in whole blood from 35 patients on dabigatran treatment, and 10 healthy donors, all samples analyzed with and without IDZ. a) Thrombin CT, b) Ex-tem CT, c and d) focused image of Thrombin and Ex-tem CT in healthy donors, all with dabigatran concentration 0 ng/mL but spread on the x-axis to make all data points visible. e) Thrombin CT_{diff}, f) Ex-tem CT_{diff}. Figure reprinted with permission from John Wiley and Sons (227). © 2018 International Society on Thrombosis and Haemostasis.

ROC analysis to discriminate between samples with dabigatran concentration above and below 20 ng/mL showed 100 % sensitivity and specificity for Thrombin CT and 85 % sensitivity and 100 % specificity for Ex-tem CT (Table 3). As in the in vitro material, samples with IDZ had similar CT as control samples, regardless of dabigatran concentration and results with the CT_{diff} variable were similar as the results with CT (Figure 10 e-f).

Table 3.
Optimal receiver operating characteristics (ROC) discriminating between samples with dabigatran concentration above or below 20 ng/mL.
Sensitivity and specificity presented as mean with 95 % CI.

	Cut-off (s)	Sensitivity (%)	Specificity (%)
Thrombin CT	154	100 (90–100)	100 (72–100)
Ex-tem CT	90	85 (69–95)	100 (72–100)
Thrombin CT_{diff}	62	100 (90–100)	100 (72–100)
Ex-tem CT_{diff}	21	91 (76–98)	100 (72–100)

In 10 patients ROTEM analyses were expediated to determine turnaround time. In these patients, ROTEM data was available within 15 min from sampling.

4.2.3 Conclusion

ROTEM with a thrombin-based trigger is more sensitive to dabigatran treatment than when the commercial trigger Ex-tem is used. ROTEM-CT with a thrombin-based trigger could accurately discriminate between samples with dabigatran concentrations above or below 20 ng/mL and results were available within 15 min, making it promising as a POC-test to detect dabigatran treatment in emergency situations.

4.3 STUDY III

4.3.1 Detecting apixaban effect in vitro

Both CT and CT_{diff} were concentration-dependently prolonged by apixaban and were significantly longer than control samples already at 20 ng/mL using all three triggers

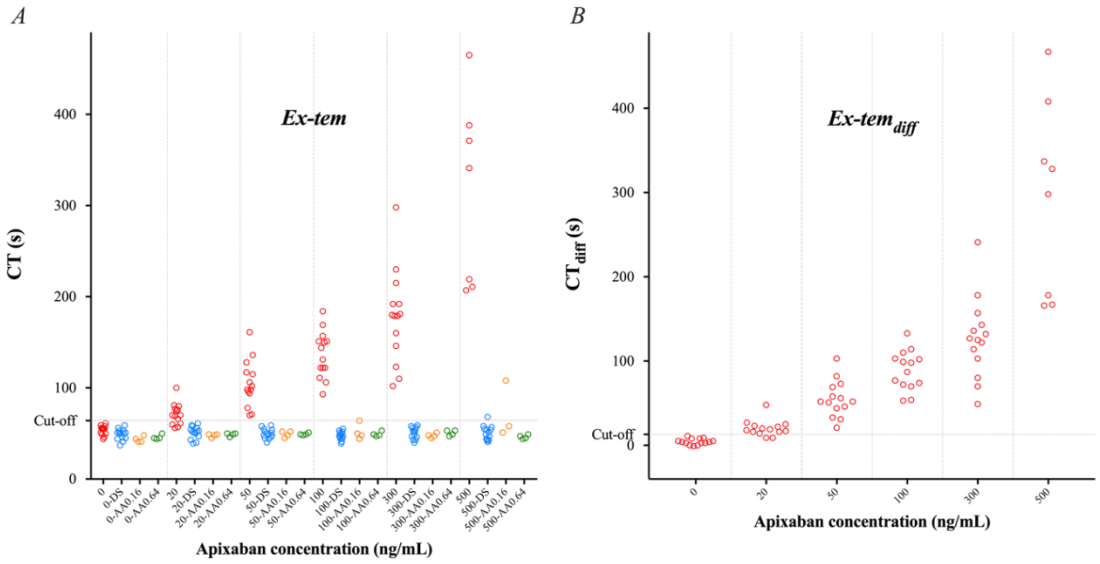

Figure 11. ROTEM analyses using Ex-tem trigger in whole blood samples from 14 healthy donors incubated with apixaban at estimated plasma concentrations 0, 20, 50, 100, 300 and 500 ng/mL (red), samples analyzed with or without DS (blue) and AA (two concentrations; 0.16 (orange) and 0.64 (green) mg/mL). a) CT, b) CT_{diff}. Horizontal line represents cut-off for discriminating between samples with apixaban concentration above or below 20 ng/mL. Figure reprinted with permission from Georg Thieme Verlag (228).

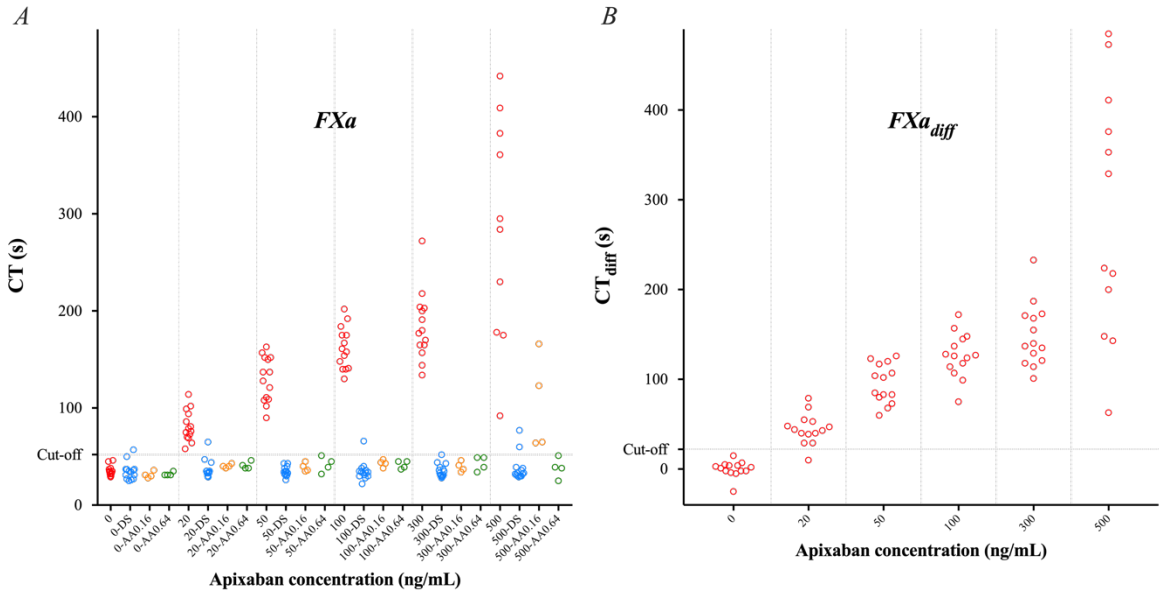

Figure 12. ROTEM analyses using FXa trigger in whole blood samples from 14 healthy donors incubated with apixaban at estimated plasma concentrations 0, 20, 50, 100, 300 and 500 ng/mL (red), samples analyzed with or without DS (blue) and AA (two concentrations; 0.16 (orange) and 0.64 (green) mg/mL). a) CT, b) CT_{diff}. Horizontal line represents cut-off for discriminating between samples with apixaban concentration above or below 20 ng/mL. Figure reprinted with permission from Georg Thieme Verlag (228).

(Figure 11-13; $p<0.01$ for all). ROC analyses to discriminate between samples with apixaban concentration above and below 20 ng/mL showed high sensitivity and specificity for all three triggers but the FXa trigger stood out with 100 % sensitivity and specificity (Table 4). Samples with DS had similar CT as control samples with the FXa trigger, but with the Ex-tem and RVV trigger CT was slightly shorter than control samples, indicating a procoagulant effect of DS. Samples with AA had similar CT as control samples using the Ex-tem and FXa trigger for all samples except those with the highest concentration of apixaban (500 ng/mL) and the lower concentration of AA (0.16 ng/mL; high therapeutic concentration), indicating that very high concentrations of apixaban might not be completely reversed by AA in therapeutic doses ($p<0.01$ for both triggers). Using the RVV trigger, samples with AA reversed effects of apixaban but CT was still longer than in control samples and there seemed to be a dose-dependent prolongation of CT by AA (Figure 13; $p<0.05$), suggesting an inhibitory effect of AA on RVV-induced coagulation.

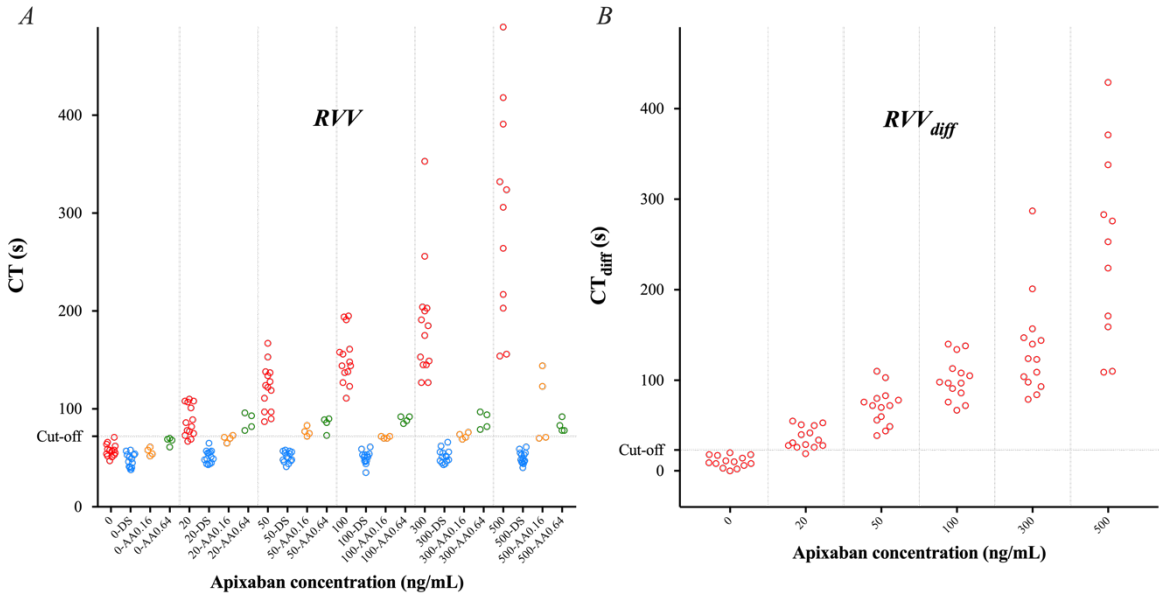

Figure 13. ROTEM analyses using RVV trigger in whole blood samples from 14 healthy donors incubated with apixaban at estimated plasma concentrations 0, 20, 50, 100, 300 and 500 ng/mL (red), samples analyzed with or without DS (blue) and AA (two concentrations; 0.16 (orange) and 0.64 (green) mg/mL). a) CT, b) CT_{diff}. Horizontal line represents cut-off for discriminating between samples with apixaban concentration above or below 20 ng/mL. Figure reprinted with permission from Georg Thieme Verlag (228).

4.3.2 Detecting apixaban effect in patients

Both CT and CT_{diff} were longer in patient samples than in control samples using all three triggers (Figure 14-16; $p<0.01$ for all). ROC analysis to discriminate between samples with apixaban concentration above and below 20 ng/mL showed high sensitivity and specificity for all three triggers but, as in the in vitro experiments, the FXa trigger stood out with 100 % sensitivity and specificity (Table 4). In 5 patients, we expediated analysis of the ROTEM assay and compared turnaround time with emergency analysis of apixaban concentration at the local clinical chemistry lab using a chromogenic anti-Xa assay. Turnaround time for the ROTEM assay was 19 (95 % CI 16-22) min and for the chromogenic anti-Xa assay 65 (95 % CI 52-77) min.

Table 4.
Optimal receiver operating characteristics discriminating between samples with apixaban concentration above and below 20 ng/mL.
Sensitivity and specificity presented as mean with 95 % CI.

		Cut-off (s)	Sensitivity (%)	Specificity (%)
In vitro data	Ex-tem CT	64	94 (86-98)	100 (77-100)
	FXA CT	52	100 (95-100)	100 (77-100)
	RVV CT	72	97 (90-100)	100 (77-100)
	Ex-tem CT_{dif}	13	97 (90-100)	100 (77-100)
	FXA CT_{diff}	22	99 (92-100)	100 (77-100)
	RVV CT_{diff}	23	99 (93-100)	100 (77-100)
Patient data	Ex-tem CT	62	92 (79-98)	100 (79-100)
	FXA CT	57	100 (91-100)	100 (79-100)
	RVV CT	89	97 (86-100)	94 (70-100)
	Ex-tem CT_{dif}	9	87 (73-96)	88 (62-98)
	FXA CT_{diff}	21	97 (87-100)	100 (79-100)
	RVV CT_{diff}	22	97 (87-100)	94 (70-100)

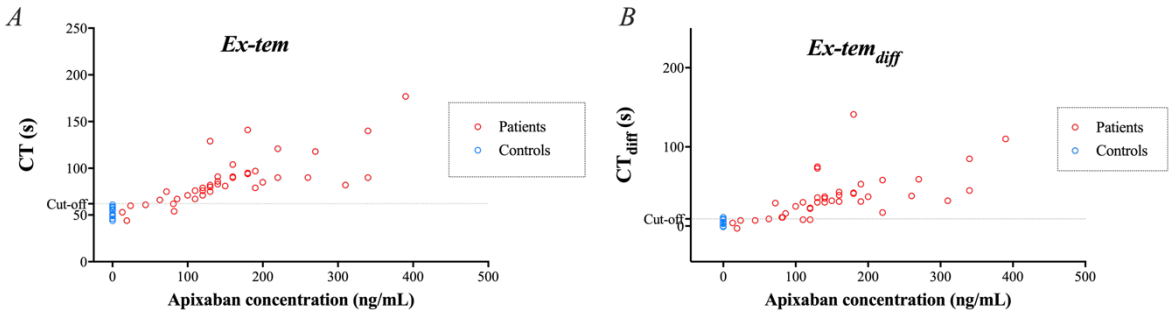

Figure 14. ROTEM analyses using Ex-tem trigger in whole blood samples from 40 patients on apixaban treatment. a) CT, b) CT_{diff}. Horizontal line represents cut-off for discriminating between samples with apixaban concentration above or below 20 ng/mL. Figure reprinted with permission from Georg Thieme Verlag (228).

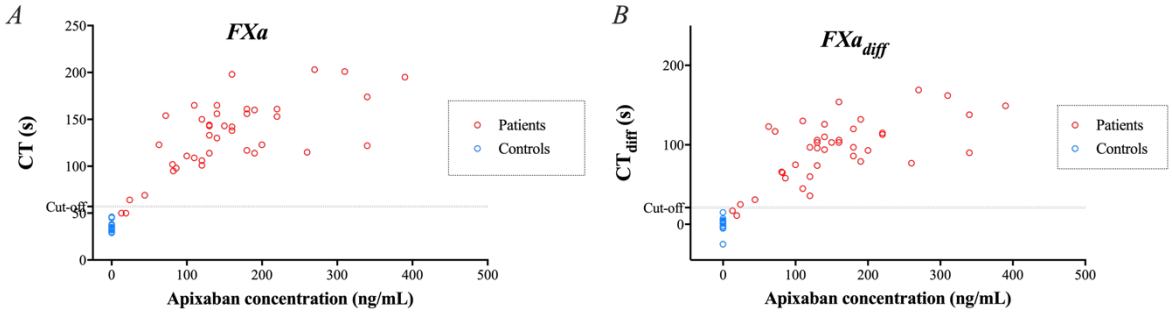

Figure 15. ROTEM analyses using FXa trigger in whole blood samples from 40 patients on apixaban treatment. a) CT, b) CT_{diff}. Horizontal line represents cut-off for discriminating between samples with apixaban concentration above or below 20 ng/mL. Figure reprinted with permission from Georg Thieme Verlag (228).

4.3.3 Conclusion

ROTEM with a FXa-based trigger is more sensitive to apixaban treatment than commercial trigger Ex-tem and a trigger based on RVV. It could accurately discriminate between samples with apixaban concentrations above or below 20 ng/mL and results were available within 20 min, considerably faster than emergency analysis using a chromogenic anti-Xa assay at the local chemistry lab, making it promising as a POC-test to detect apixaban treatment in emergency situations.

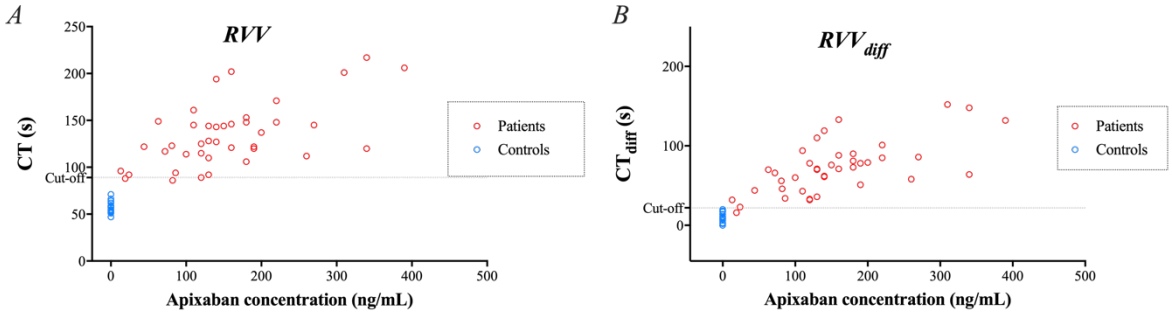

Figure 16. ROTEM analyses using RVV trigger in whole blood samples from 40 patients on apixaban treatment. a) CT, b) CT_{diff}. Horizontal line represents cut-off for discriminating between samples with apixaban concentration above or below 20 ng/mL. Figure reprinted with permission from Georg Thieme Verlag (228).

4.4 STUDY IV

4.4.1 Fibrin network permeability

Fibrin network permeability (Ks) was higher in dabigatran-treated patients than in each of the other treatment groups (Figure 17; $p<0.01$) and patients on apixaban had lower permeability than warfarin-treated patients ($p<0.01$). All treatment groups had higher permeability than NPP ($p<0.01$). Images of the clots formed during the assay using scanning electronic microscopy provide a visual presentation of the structure of the fibrin network in samples with different Ks (Figure 18).

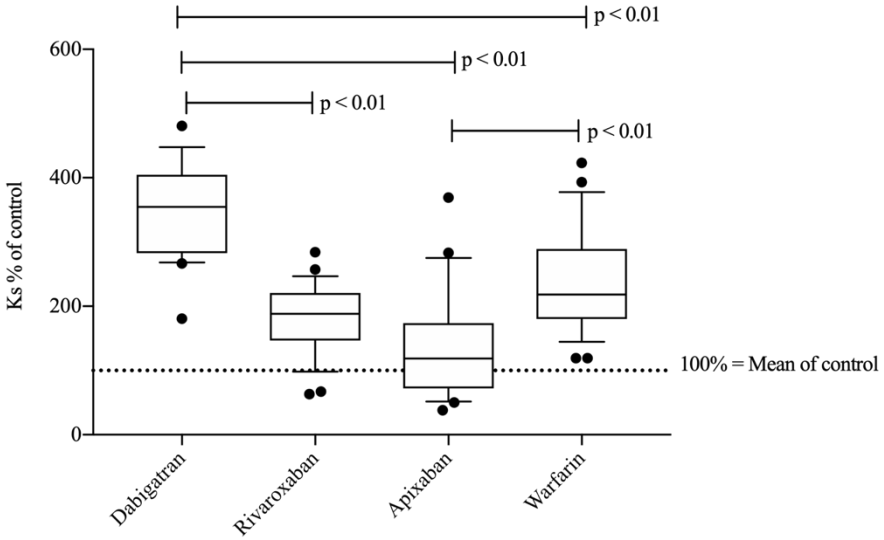

Figure 17. Fibrin network permeability (Ks – see method section – clotting triggered with TF) in samples from patients treated with dabigatran (n=23), rivaroxaban (n=26), apixaban (n=20) and warfarin (n=27) – results presented as Ks % of normal pool plasma (NPP). Differences between groups evaluated with Kruskal Wallis test followed by Dunn's post hoc testing if significant. P-values represent significance level of post hoc testing.

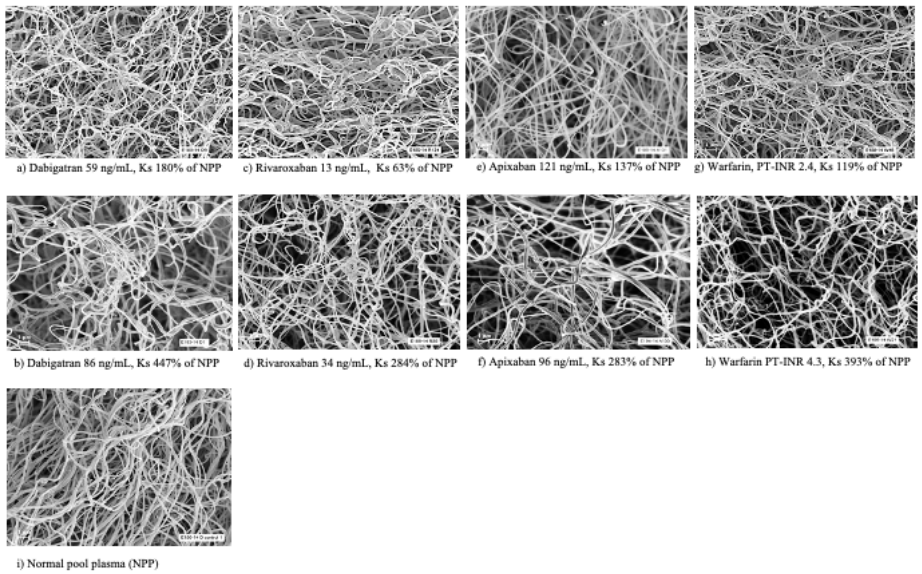

Figure 18. Scanning electronic microscope images showing the fibrin network structure in selected dabigatran- (a, b), apixaban- (c, d), rivaroxaban- (e, f) and warfarin-treated (g, h) patients with different fibrin network permeability (Ks) as well as NPP for comparison (i). The respective DOAC concentration or PT-INR is provided below each image.

There was no significant correlation between DOAC concentration and Ks in patient samples, but there was a significant correlation between PT-INR and Ks in patients treated with warfarin ($r=0.40$; $n=27$; $p<0.05$). However, in experiments with NPP spiked with DOACs there were strong correlations between DOAC concentrations and Ks (Figure 19; $r=0.97$; $n=8$; $p<0.001$ for dabigatran- and apixaban treated samples respectively and $r=0.93$; $n=8$; $p<0.001$ for rivaroxaban treated samples). In purchased plasma from warfarin-treated patients (PT-INR 1-9) correlation between PT-INR and Ks was $r=0.96$ ($n=7$; $p<0.001$). In these experiments, there is only one analysis per concentration, which don't allow for a statistical comparison of the relative effects of each DOAC on fibrin network permeability but at similar concentrations, dabigatran had the most porous fibrin networks followed by rivaroxaban and then apixaban with the densest networks.

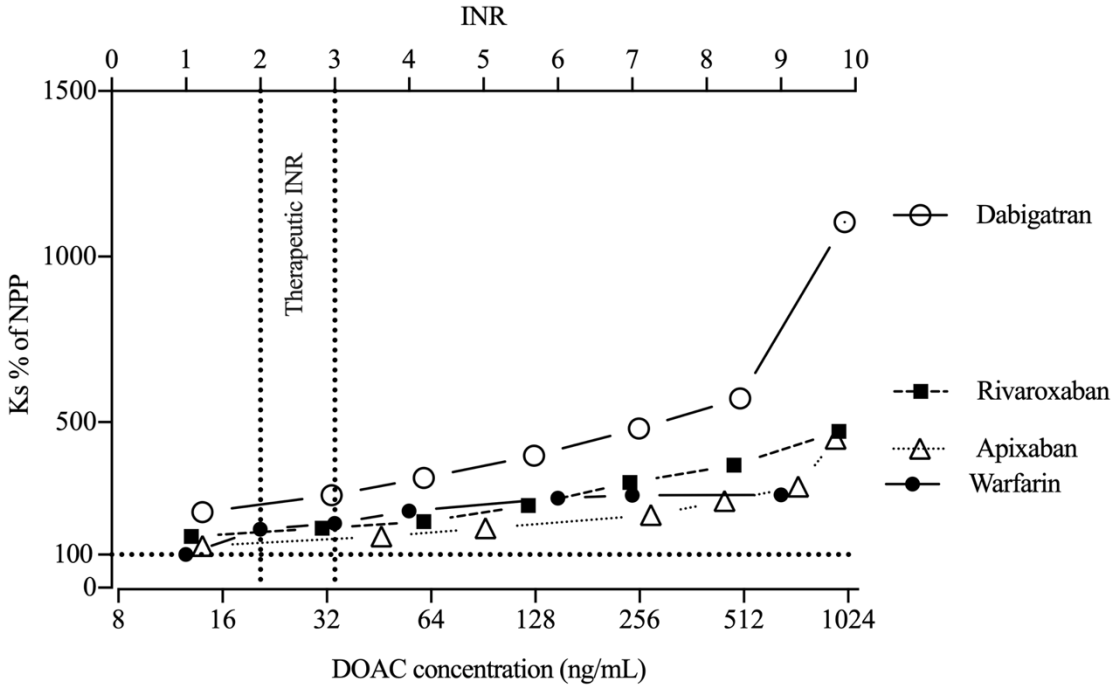

Figure 19. Fibrin network permeability in commercial NPP spiked with 8 different concentrations of DOAC (13-1000 ng/mL) and in PPP from warfarin-treated patients with PT-INR values ranging from 1-9 (n=7; each sample concentration analyzed once). Results presented as Ks % of NPP.

4.4.2 CAT

Lag time was significantly longer in patients taking dabigatran compared to apixaban- and rivaroxaban treated patients (Figure 20; $p<0.01$) and patients on warfarin had significantly longer lag time than rivaroxaban-treated patients ($p<0.05$). Peak thrombin and ETP were lower in warfarin-treated patients than in rivaroxaban- and apixaban-treated patients ($p<0.001$) whereas there were no significant differences between patients on rivaroxaban and apixaban. Peak thrombin and ETP data were not analyzed in dabigatran treated patients due to a previously described interaction between reversible thrombin inhibitors and α_2-

macroglobulin-thrombin complex, making the CAT algorithm for subtracting α_2-macroglobulin-thrombin complex incorrect (197-201).

There were significant correlations between DOAC concentration and lag time for dabigatran- (r=0.55; n=23; p<0.01) and apixaban-treated (r=0.55; n=20; p<0.01) patients and between PT-INR and lag time for warfarin-treated patients (r=0.77; n=27; p<0.001). There were significant negative correlations between DOAC concentration and peak thrombin for patients on rivaroxaban (r=-0.60; n=26; p<0.01) and apixaban (r=-0.61; n=20; p<0.01) and between PT-INR and peak thrombin for patients on warfarin (-0.92; n=27; p<0.001). There were also significant negative correlations between DOAC concentration and ETP for rivaroxaban-treated patients (r=-0.43; n=26; p<0.05) and between PT-INR and ETP for warfarin-treated patients (-0.86; n=27; p<0.001).

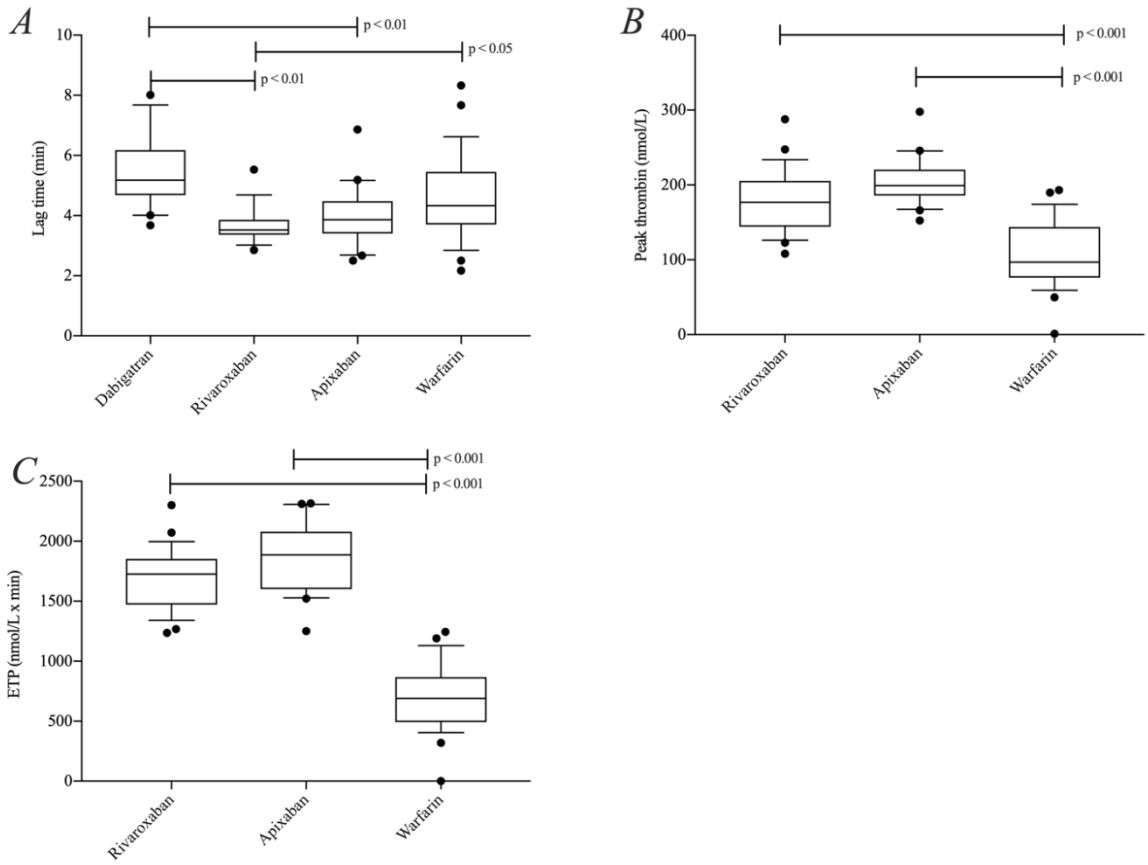

Figure 20. CAT (see method section – thrombin generation after clotting triggered with TF) lag time (A), ETP (B) and peak thrombin (C) in samples from patients treated with dabigatran (n=23), rivaroxaban (n=26), apixaban (n=20) and warfarin (n=26). Peak thrombin and ETP data were not analyzed in dabigatran treated patients due to a previously described interaction between reversible thrombin inhibitors and α_2-macroglobulin-thrombin complex (197-201). Differences between groups evaluated with Kruskal Wallis test followed by Dunn's post hoc testing if significant. P-values represent significance level of post hoc testing.

4.4.3 Turbidimetric clotting and lysis assay

Patients taking dabigatran had longer lag time and lower CFR than the other patient groups as well as NPP (Figure 21; p<0.001). Max absorbance was higher in apixaban-treated patients compared to dabigatran-treated patients and NPP (p<0.05). Lysis time was longer in patients on dabigatran compared to patients on rivaroxaban but not compared to other

treatment groups or NPP ($p<0.05$). A significant negative correlation (-0.42; $p<0.05$) was found between dabigatran concentration and CFR; other than that, there was no significant correlation between DOAC concentration and variables from the turbidimetric assays.

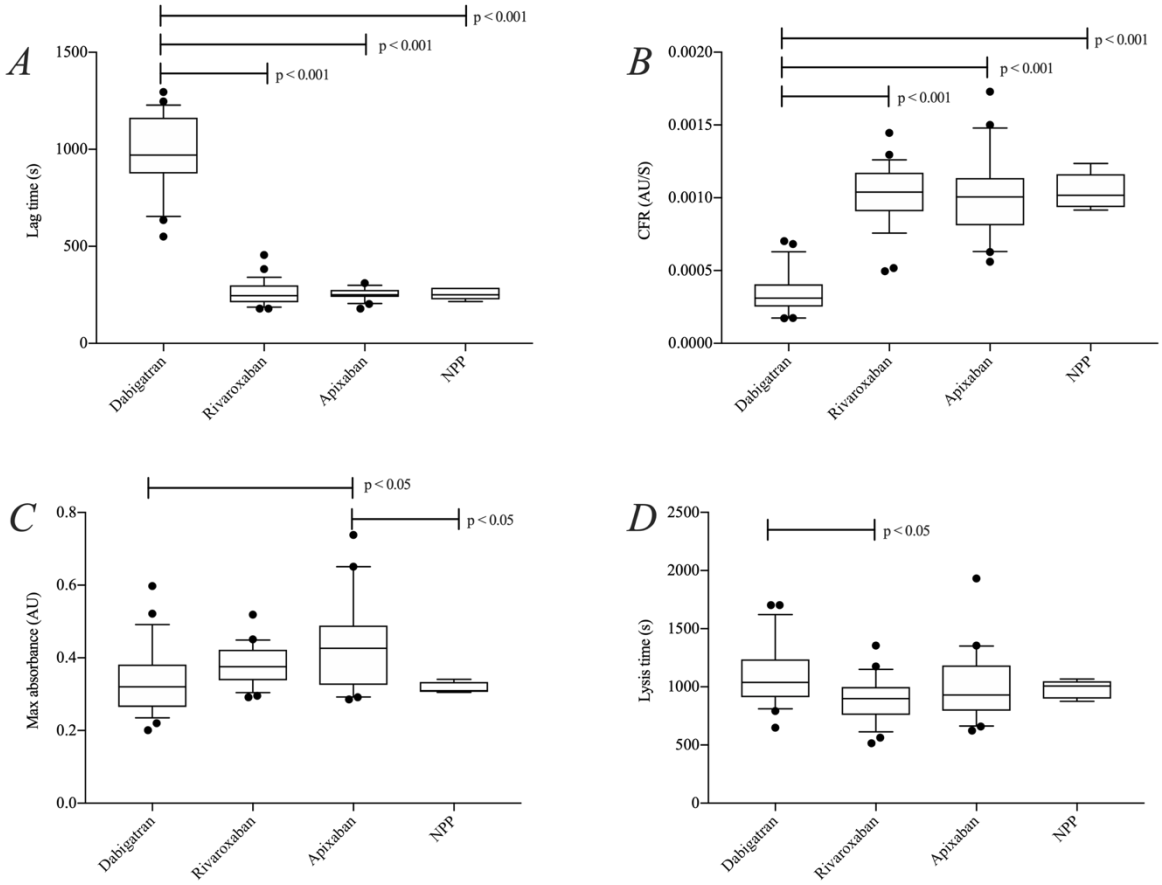

Figure 21. Results from the turbidimetric clotting and lysis assay (see method section – clotting triggered with thrombin) in samples from patients treated with dabigatran (n=23), rivaroxaban (n=26), apixaban (n=20). Commercial NPP was used for comparison (five repeated runs). (A) Lag time, (B) CFR, (C) max absorbance, (D) lysis time. Differences between groups evaluated with one-way ANOVA followed by Tukey's post hoc testing if significant. P-values represent significance level of post hoc testing.

4.4.4 TAT

There were no significant differences in TAT complex levels between treatment groups and there was no significant correlation between DOAC concentration or PT-INR and TAT levels.

4.4.5 D-dimer

D-dimer concentrations were lower in dabigatran-treated patients than in rivaroxaban- ($p<0.05$) and apixaban- ($p<0.01$) treated patients. There was no significant correlation between DOAC concentration and D-dimer.

4.4.6 Conclusion

Dabigatran treatment was associated with a stronger anticoagulative effect than apixaban- or rivaroxaban treatment and did also influence the fibrin network permeability more than warfarin treatment did. There were no significant differences between the two FXa-

inhibitors in any of the analyses performed and overall, the FXa inhibitors influenced hemostasis less than warfarin did.

5 DISCUSSION

Although DOACs have been approved without the need for routine monitoring, measuring the effect of these drugs has been an area of intense research during the last decade. A major reason for this is the need to be able to assess the anticoagulant effect in emergency situations. This allows the clinician to select patients who will benefit from reversal therapy or who are eligible for emergency surgery or thrombolysis (87, 229, 230). Withholding unnecessary antidote administration is not only cost effective, but it could also protect patients from the risk of thromboembolic complications associated with use of reversal agents (87).

Non-emergent testing of DOAC concentration to confirm therapeutic effect may be considered in patients with potentially different pharmacokinetics from those included in the RCTs (107-111). Considering the relationships between DOAC concentrations and clinical outcome, there have also been discussions on more extensive monitoring and dose-adjustments, similar to the monitoring of warfarin treatment (112, 113, 229, 231). The benefit of this approach in warfarin-treated patients has been well documented and is explained by its narrow therapeutic interval and the interindividual variability in anticoagulant effect by warfarin (232). In contrast, DOACs appear to have broad therapeutic intervals and a more predictable anticoagulant effect when prescribed at fixed doses (42-45, 112, 113). The short-acting effect of DOACs and large intraindividual variability in trough concentration also make dose-adjustments challenging and of unclear benefit (231).

Additionally, there is still substantial uncertainty how to interpret DOAC concentrations, both regarding the “optimal” therapeutic effect, and what should be considered a significant anticoagulant effect in respect to emergency surgery, thrombolysis and need for reversal therapy (103, 107, 111). Bleeding risk and clinical consequences of bleeding differs between surgical procedures, and the type of surgery likely influence which level of cut-off that can be considered “safe” (230). In patients who underwent elective surgery after standardized interruption of DOACs, residual drug level above 50 ng/mL was not associated with a higher bleeding risk (233). In a study on patients with ischemic stroke, thrombolysis was considered in all patients with rivaroxaban concentrations below 100 ng/mL and there was no increased bleeding in patients with concentrations above 20 ng/mL compared to below 20 ng/mL (234). However, the lack of complete evidence is reflected by the fact that the proposed concentration cut-offs are the same for all DOACs although they are different drugs with different pharmacology, and probably don’t have the same anticoagulant effect at equimolar concentrations. Additionally, the DOAC treatments may affect hemostasis differently, as shown in this thesis where dabigatran seemed to have broader effects on hemostasis than rivaroxaban and apixaban, when administered to AF patients according to clinical routine.

Another topic of discussion has been whether results from functional coagulation assays, such as the CAT assay, better reflect the intensity of DOAC anticoagulation than simply measuring DOAC concentration (195, 196). Results from T-TAS, turbidimetric clotting and lysis assays, CAT and the fibrin network permeability assay have all been linked to clinical end points but have not been studied in this regard in patients on DOACs (188, 192-194, 205-210, 218, 219). Prolonged PT and aPTT on the other hand, are predictors of bleeding in patients on rivaroxaban and dabigatran, respectively (235, 236). The answer to the question of what the best measure of DOAC effect is in patients, will probably have to be provided by large prospective clinical trials comparing the ability of functional coagulation assays and drug concentrations to predict outcomes such as bleeding and thrombosis.

Factor XI-inhibitors will perhaps be introduced in the clinic in the future, and there will be a need to similarly evaluate the best measure of their effect and thresholds for safe invasive procedures. Recent trials provide several different options to measure their effect: concentration of the agent, factor XI level, factor XI activity and aPTT (73-76, 79). Considering the seemingly limited bleeding profile of patients treated with factor XI-inhibitors, determining a safe cut-off for invasive procedure will probably be a future area of discussion and research.

5.1 ROTEM – A POTENTIAL POC-TEST FOR DOACS

ROTEM is a POC-test and a global coagulation assay that provides information on the strength of a clot forming in whole blood over time. In study I, CT was the only variable sensitive to dabigatran treatment, indicating that dabigatran delays clot formation but does not affect the strength of the clot once it is formed (Table 2). The reason could be a reduced "amplification signal" when FXa and thrombin is inhibited, but once enough thrombin is generated the fibrin network is formed more or less as in untreated individuals. Other studies have reported similar effects on ROTEM variables with dabigatran, as well as during treatment with other DOACs, at least at DOAC concentrations within the expected range (160, 166, 168, 237). At supratherapeutic DOAC concentrations, the strength of the clot seems to be affected as well (166, 238).

In study I, the sensitivity of ROTEM-CT to detect the dabigatran effect depended very much on which trigger we used. Regarding routine coagulation tests, aPTT, which evaluates the intrinsic pathway, is more sensitive than PT, which evaluates the extrinsic pathway (107-111). Interestingly, the TF-based triggers Ex-tem and Fib-tem were more sensitive than the ellagic-acid based trigger In-tem, whereas a trigger with low concentration of TF was least sensitive (Table 2). Dabigatran concentration correlated excellently with TEG variable R, corresponding to CT in ROTEM, using another intrinsic activator (kaolin) (169). Indeed, the importance of tailoring the trigger used in coagulation assays to the mechanism studied has been previously highlighted (239). Our studies underline that it is hard to predict the performance of a specific trigger in the detection of an anticoagulant effect as it may vary between different methods, and is dependent on the specific type of trigger and its concentration.

The results from study I indicated that ROTEM could potentially be used to estimate the dabigatran effect. However, even though there was a strong correlation between the dabigatran concentration and Ex-tem- and Fib-tem-CT, CT could still be within reference interval or very close to reference interval in samples with dabigatran concentrations considered to have a significant anticoagulant effect (30-100 ng/mL). A thrombin-based trigger should in theory be more specific for the effects of the thrombin-inhibitor dabigatran and is used successfully to estimate dabigatran concentration in the plasma-based dTT assay. As hypothesized, we found that the ROTEM assay with a thrombin-based trigger was more sensitive to dabigatran treatment than the Ex-tem trigger (Figure 9 and 10). It was excellent in differentiating between samples with and without a clinically significant dabigatran concentration (cut-off 20 ng/mL; Table 3). Encouraged by these results, we proceeded with study III where we studied the use of ROTEM to detect apixaban treatment. This is at present the most prescribed DOAC for stroke thromboprophylaxis in AF, at least in Sweden. Of the commercial triggers we only used Ex-tem as it had been described to be the most sensitive to apixaban treatment in earlier studies (176, 225). We compared it to triggers that should be more specific to FXa-inhibition; FXa and RVV. In our material, all triggers showed excellent sensitivity to detect apixaban treatment but the FXa trigger appeared the most robust at differentiating between samples with and without significant apixaban concentration (cut-off 20 ng/mL; Figure 11-16, Table 4). At the time of publication of study III, other work had been published that similarly studied the use of specific triggers for DOACs in viscoelastic assays (178-180). Results were promising using ecarin for dabigatran and FXa for FXa-inhibitors in the TEG6s assay; ecarin for dabigatran and diluted Ex-tem for FXa-inhibitors in ROTEM; and ecarin for dabigatran and RVV for FXa-inhibitors in ClotPro (178-180).

Viscoelastic tests use whole blood and are already widely used as POC-tests, giving them the potential for very short turnaround times compared to plasma-based tests performed at clinical chemistry labs. In study III, we compared the turnaround time of our ROTEM set-up, which included a 5 min walk to our laboratory, with a chromogenic anti-FXa assay carried out as a routine emergency analysis at the hospital lab, and the ROTEM assay was considerably faster (19 vs 65 min). With a sufficiently short turnaround time, it would be possible to wait for the test result until deciding on antidote administration, and it might allow earlier decisions on safety of thrombolysis in stroke patients, as well as safety with regard to emergent surgery. The need for a POC-test for DOACs in emergency situations has been stressed in recent guidelines and several different methods are being explored (110). As for now, if it is not deemed safe to wait until the result of the plasma-based assays are obtained; the DOAC effect will have to be estimated based on exposure (time since last dose) (60). In many critically ill patients, it is not even possible to reliably acquire this information and the clinician will have to assume that there is a significant DOAC effect. Thus, a POC-test might expediate treatment in some situations where time makes a big difference for the prognosis of the patient, for example allow earlier thrombolysis in a patient with ischemic stroke, or more rapid access to surgery in a patient with an aortic

dissection. It might also help reduce unnecessary antidote administrations which is costly and may also subject patients to thromboembolic risk (87).

The need for laboratory monitoring of reversal therapies has been suggested as the anticoagulant effect may rebound (60, 111). Of note, the anti-Xa assays do not detect reversal of Xa-inhibitors with PCC (240). Additionally, they are not suitable to estimate anticoagulant effect of FXa-inhibitors after use of AA. The sample dilution required in the assay causes dissociation between AA and FXa-inhibitors which may lead to a falsely elevated anti-Xa activity in the sample (110, 241). This should not be a problem with viscoelastic tests as there is little sample dilution. Using RVV as a trigger in samples with AA present may not be suitable, as AA seems to interfere with the procoagulant effect of RVV. As previously described by others, we detected a dose dependent prolongation of CT by AA when using the RVV-trigger in study III (Figure 13). The mechanism may be competition between AA and FXa in the binding of RVV (AA and FXa have a very similar molecular structure) (242, 243). The use of the RVV trigger is also hard to standardize due to differences in venom composition between snake species and variations in its activity between batches (244).

The effect of AA is short-lasting (requires a continuous infusion for DOAC-reversal), and complete reversal of the anticoagulant effect is not seen in all patients (92). Our experiments with AA in study III give an indication of this, as a supratherapeutic concentration of AA (0.64 mg/mL) was needed to fully reverse the effects of very high (500 ng/mL) concentrations of apixaban (Figure 11-13). IDZ however, caused full reversal of dabigatran effect as measured with ROTEM in all samples in study II (highest dabigatran concentration 500 ng/mL) at a concentration that should be obtained when the standard dose of IDZ is given in clinical situations requiring drug-reversal (Figure 9 and 10) (245).

In study II and III, the difference in CT between samples before and after ex vivo reversal – with IDZ for dabigatran and DS for apixaban – was evaluated as a separate variable (CT_{diff}). The reasoning was that CT_{diff} should reflect the specific contribution of each DOAC to the sample CT, and give a more robust estimation of its anticoagulant effect. The principle is similar to the ROTEM Hep-tem assay, in which heparinase is added to the In-tem assay to reveal the heparin effect (246). In our studies, the CT_{diff} variable did not add any extra value as it detected dabigatran and apixaban effect with similar accuracy as the CT variable (Table 3 and 4). However, the patients included in our study did not suffer from trauma or major bleeding that can cause other coagulation abnormalities affecting viscoelastic test results (247, 248). In those situations, the CT_{diff} variable could still prove useful and reflect the "reversibility" of the DOAC treatment. However, this remains to be studied.

Since CT was the only variable registered in our experiments it is possible that a similar approach with DOAC specific triggers can be employed with more basic instruments that only measure whole blood clotting time. Indeed, this approach has recently been studied on dabigatran treated blood samples and with ecarin as a trigger in a small whole blood POC

coagulometer, with similar results as in viscoelastic tests (249). An advantage with the ROTEM assay is, however, that it can simultaneously use other common triggers thus providing additional valuable information and e.g. detect hyperfibrinolysis, thrombocytopenia or hypofibrinogenemia in patients with severe bleeding or trauma (155, 156).

Our studies on ROTEM with specific triggers for DOACs are small and should be considered as proof-of-concept rather than conclusive, but the results are promising. Further support of this is available in other recent studies using viscoelastic tests with specific triggers for DOACs (178-180). The results do not provide an estimated DOAC concentration as the plasma-based assays with calibrators do, but rather a qualitative estimation of whether DOACs in concentration of clinical significance is present. This approach should be sufficient for the purpose of the test in many emergency situations, i.e to determine whether DOAC reversal is needed or if emergency surgery or thrombolysis is safe. We have only tested our FXa-trigger for detection of apixaban but in other studies the same trigger was used for several FXa-inhibitors and apixaban seemed the hardest to detect (178-180). It is therefore reasonable to assume that our FXa-trigger would also be useful for detection of other FXa-inhibitors, although it would of course have to be tested. Further validation of this method is also needed in patients with emergency situations such as major bleeding and before emergency surgery, and with enough patient numbers with concentrations close to the cut-off for significant anticoagulant effect. In a not-too-distant future however, a decision-tree for managing DOAC reversal in patients with life-threating bleedings could include POC-testing with ROTEM-CT before making the decision whether to administer antidote (Figure 22).

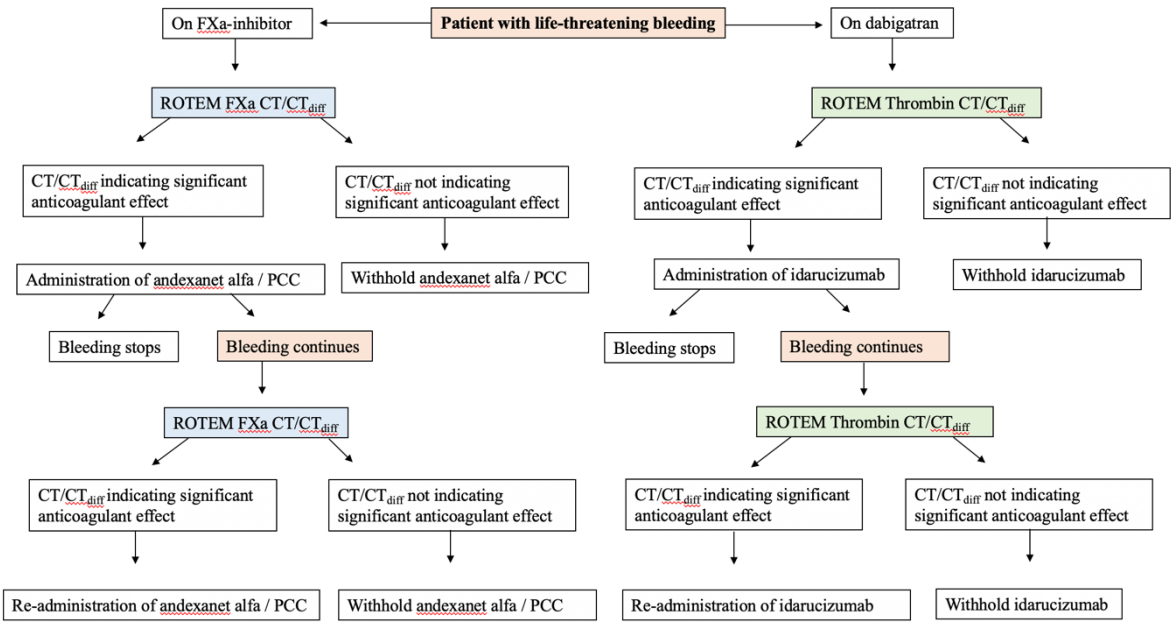

Figure 22. Proposed decision-tree for managing DOAC reversal in patients with life-threating bleedings including POC-testing with ROTEM with FXa- and Thrombin-based triggers. CT= clotting time, CT_{diff}=clotting time difference after ex vivo reversal of DOAC effect, PCC=prothrombin complex concentrate.

5.2 T-TAS

In study I, dabigatran delayed thrombus formation in a concentration-dependent manner as determined by T-TAS, but the correlation with concentration was rather weak (Table 2; r-values 0.39-0.41). We used the AR-chip, in which microcapillaries are coated with both collagen and TF. This chip evaluates both platelet function and coagulation and is therefore sensitive to both antiplatelet agents and OACs (250). This could make the method especially valuable when studying the combined antithrombotic effect of OACs and antiplatelet agents that is not easily quantified using other assays. This combination therapy is necessary in patients with AF who are treated with PCI due to ischemic heart disease to adequately protect against both ischemic stroke and stent thrombosis but is associated with a very high bleeding risk (60, 251). DOACs are preferred over warfarin in this scenario but guidelines do not recommend one DOAC over another (60, 251). Considering the central role of thrombin in activation of platelets, one might expect the thrombin inhibitor dabigatran to reduce platelet activity and protect against myocardial infarction (12). On the contrary, there was an increased rate of myocardial infarction in dabigatran-treated patients in the RE-LY trial, but as these findings have not been reproduced in observational studies, they have not been given any weight in guidelines (42, 63, 64). Nevertheless, evaluation with T-TAS could increase knowledge of the combined antithrombotic effects of different combination of DOACs and antiplatelet agents and help optimize treatment in this patient group (252).

5.3 COMPARATIVE EFFECTS OF DOACS ON VARIOUS ASPECTS OF HEMOSTASIS (STUDY IV)

5.3.1 Turbidimetric clotting and lysis assay

The turbidimetric assay, which measures fibrin formation and fibrin degradation, could not detect any significant effects in samples from patients on FXa-inhibitors, whereas in samples from dabigatran-treated patients the time to clot formation was prolonged and the clot formation rate was reduced. These findings may at least in part be due to that we used thrombin as a trigger in the turbidimetric assay, which of course should lead to a higher sensitivity to the direct thrombin inhibitor dabigatran. However, others have reported more pronounced effects of dabigatran compared to FXa inhibitors even when using a TF-based trigger in a turbidimetric clotting assay (221). Similar to the results in our study, DOACs had limited effects on max absorbance (reflects the total amount of fibrin formed) (221).

The data on clot lysis time, which is considered to reflect fibrinolysis, was not that impressive in our study. There was a small but statistically significant difference between dabigatran and rivaroxaban in this parameter, with a slightly longer clot lysis time in samples from the dabigatran patients. In contrast, previous reports from Ammollo et al. and Semeraro et al. showed a dose-dependent shortening of lysis time with dabigatran, but not with apixaban and rivaroxaban (223, 253), which would indicate an increased fibrinolytic potential during dabigatran treatment. There are some differences between our study and the studies performed by Ammolo (37) and Semeraro (65). TF-triggers were used instead of

thrombin by both Ammollo et al. and Semeraro et al. (223, 253). Furthermore, lower concentrations of tPA were used in their assays and wider range of DOAC concentrations were studied, perhaps allowing the effects of dabigatran to be more apparent (223, 253). As clot lysis time in dabigatran treated patients was prolonged only compared to rivaroxaban-treated patients and not to controls (NPP) in our study, the significance of this finding is probably limited.

5.3.2 CAT

The effects of the FXa-inhibitors were less pronounced using the CAT assay as well, with a shorter time to thrombin formation as compared to patients on dabigatran and warfarin, and the total amount of thrombin generated was higher than in patients on warfarin (Figure 20). Our data are in agreement with previous studies which have shown a more pronounced effect of dabigatran than FXa-inhibitors on the time to initiation of thrombin formation (i.e CAT lag time) (165, 171). It should be noted that the CAT data obtained in our study are based on an algorithm which cannot be used to calculate peak or total thrombin formation in the presence of dabigatran (197-201). This limitation has, however, been overcome in new refined devices for automated thrombin generation measurements (254-256). Using these new methods, FXa-inhibitors have been shown to reduce the peak concentration of thrombin formed (i.e "peak thrombin") in a dose-dependent manner, while dabigatran treatment have been shown to have a very little effect on this variable (255, 256). Of note, "peak thrombin" has been suggested as the most sensitive measure of FXa-inhibitors in the CAT assay, and the time until thrombin formation starts (i.e the lag time) as the most sensitive measure of dabigatran (196). The reason for the discrepancy between these two drug types with respect to outcome of CAT data is not entirely clear, but it reflects that direct thrombin inhibitors and FXa-inhibitors influence activation of the coagulation cascade differently.

5.3.3 Fibrin network permeability

Dabigatran-treated patients formed more permeable fibrin networks than patients on rivaroxaban-, apixaban- and warfarin and warfarin-treated patients formed more permeable fibrin networks than patients on apixaban. (Figure 17). The results on fibrin network permeability in the patient samples were supported by in vitro experiments where dabigatran concentration-dependently increased fibrin network permeability more than the other DOACs investigated (Figure 19).

There was a strong correlation in vitro between DOAC concentrations and fibrin network permeability, as well as between PT-INR and network permeability in the plasma pool from warfarin-treated patients. However, there was no correlation between DOAC concentrations and Ks in patient samples, and only a weak correlation between PT-INR and Ks in samples from warfarin-treated patients. A likely explanation is that the patient samples were collected at trough and therefore the range of DOAC concentrations was too low to detect concentration-dependent relationships. Supporting this notion are previous studies on

samples with a wider range of DOAC concentrations where dose-dependent effects of DOACs on the fibrin network permeability were reported (213, 215, 216, 257).

5.3.4 Concluding remarks on study IV

Dabigatran treatment was associated with a stronger anticoagulative effect than apixaban- or rivaroxaban treatment and did also influence the fibrin network permeability more than warfarin treatment did. There were no significant differences between the two FXa inhibitors in any of the analyses performed and overall, the FXa inhibitors influenced hemostasis less than warfarin did. The stronger anticoagulative effect in dabigatran treated patients is further supported by the fact that D-dimer level was lower in patients on dabigatran compared to the FXa-inhibitors. Of note, D-dimer can be considered as a general marker of an activated coagulation system and a reduction in D-dimer levels is associated with decreased cardiovascular events including stroke (258-260). The findings are also partly in line with clinical data as dabigatran was the only DOAC associated with less ischemic stroke compared to warfarin treatment (42). Contrary to previous reports of increased fibrinolysis with dabigatran treatment, in our studies lysis time was prolonged in dabigatran-treated patients compared to rivaroxaban-treated patients but not compared to NPP (223, 253).

The results from study IV should for several reasons be interpreted with some caution. The anticoagulant effect of DOACs can be expected to have been lower than at other timepoints during the dosing interval as samples were collected at trough, making comparisons with warfarin treatment hard to interpret. The turbidimetric assay was triggered with thrombin which should be more sensitive to dabigatran treatment and thereby might skew the comparison with the other DOACs. In the CAT assay, only the lag time could be compared between dabigatran and the other OACs whereas peak thrombin has been suggested to be the most sensitive variable to treatment with FXa-inhibitors (196); see section 5.3.2). As there was no correlation between DOAC concentrations and the fibrin network permeability in patient samples, there might be some concern as to whether differences between the treatment groups actually reflect a difference in the drug effect per se, and to what extent e.g differences in some clinical characteristics between the patient groups confound the comparisons. The same applies to lysis time, where the clinical relevance of the difference between dabigatran- and rivaroxaban-treated patients might also be questioned, as there was no difference for either of the drugs when compared to NPP. In conclusion, there are important limitations of the study, and the treatment groups are not matched for important clinical characteristics so confounding factors might affect the results, especially regarding the warfarin treatment group which mostly consisted of VTE-patients.

6 CONCLUSIONS

- The viscoelastic test ROTEM was more promising as a dabigatran point of care-test than a flow-based point of care test (T-TAS).

- Using DOAC-specific triggers, i.e. triggers which are directly inhibited by the DOAC, ROTEM became very sensitive and could accurately discriminate between samples with drug concentrations above or below 20 ng/mL, a cut-off which define low DOAC concentrations in clinical routine.

- With ROTEM, test results were available within 20 min, making it promising as a point of care-test in emergency situations.

- In patients treated with dabigatran, rivaroxaban or apixaban according to clinical routine, dabigatran treatment was the drug associated with the broadest anticoagulating effects. There were no significant differences between apixaban and rivaroxaban in any of the analyses performed.

7 FUTURE PERSPECTIVES

- Further evaluation of the ROTEM assay with DOAC specific triggers is needed to determine:
  - if the accuracy of the assay can be reproduced and if the CT_{diff} variable is useful in larger patient materials including patients in emergency situations such as bleeding or before urgent surgery
  - if the FXa-based trigger is useful to detect other FXa-inhibitors than apixaban

- The use of more basic instruments that only measure whole blood CT could probably be used in a similar way as the ROTEM assay using DOAC specific triggers. This approach could make bedside analysis even more accessible.

- The best measure of DOAC effect, and what constitutes a threshold for a significant effect, should be further examined in large clinical trials evaluating the ability of results from global coagulation assays and drug concentrations to predict outcomes such as bleeding and thrombosis.

- Factor XI-inhibitors are currently tested in randomized clinical trials and may be introduced in the clinic in the near future. There will likely be a need to evaluate the best measure of their effect, and perhaps thresholds for invasive procedures and indication for treament with reversal agents.

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