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Department for Vascular Diseases,

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THE IMPACT OF APIXABAN ON OVERALL HEMOSTATIC POTENTIAL

Diploma thesis

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Declaration:

I declare that this thesis is my original work. All used literature and information sources are summarized in the list of references and properly cited. This work has not been submitted for equal or any other degree.

Hradec Králové 2020 Bc. Ladislava Cablíková

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ABSTRAKT

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Název diplomové práce: Vliv apixabanu na celkový hemostatický potenciál

Poruchy na určitých úrovních komplikovaného hemostatického systému mohou vést jak ke krvácení, tak i k nadměrnému srážení krve. Tyto patologické stavy jsou léčeny antikoagulačními látkami, které svým působením korigují nadměrnou koagulaci. Ovšem tradiční antikoagulační léčba nese nemálo omezení, tudíž bylo snahou vyvinout orální antikoagulancia, která by vykazovala lepší profil. Antikoagulancii nové generace jsou tzv. přímá perorální antikoagulancia - Direct Oral AntiCoagulans, zkráceně DOAC. Apixaban, jako jeden z xabanů, má předvídatelnou farmakokinetiku a farmakodynamiku, a proto nevyžaduje běžné laboratorní monitorování účinku léčby. V naléhavých klinických situacích je ovšem jeho vyhodnocení nezbytné. Klasické screeningové testy koagulace, jako je PT (protrombinový test) a APTT (aktivovaný parciální tromboplastinový test) plně neodrážejí skutečný stav léčiva. Proto je snahou nalézt relativně jednoduchý a rychlý hemostatický test korelující se skutečným stavem pacienta. Jedním z nich by mohla být metoda celkového hemostatického potenciálu (OHP) založena na spektrofotometrické registraci tvorby fibrinu a jeho degradaci v plazmě. V této práci byla metoda použita u pacientů léčených apixabanem. Byla vyhodnocena její reprodukovatelnost (CV = 24,9 %) a vztah mezi celkovým hemostatickým potenciálem a koncentrací apixabanu v plazmě (R = -0,418), vykazující mírnou korelaci. Slabá korelace byla pozorována mezi časem koagulace a koncentrací apixabanu (R = 0,277) a také se projevila u PT (R = 0,501) a APTT (R = 0,360). Na základě našich výsledků jsme dospěli k závěru, že test celkového hemostatického potenciálu nelze použít pro monitorování pacientů léčených apixabanem. I přes to OHP představuje metodu s velkým potenciálem, která může být odrazovým můstkem při hledání nových přístupů k pochopení hemostázy jakožto vysoce komplexního celku.

ABSTRACT

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Thesis title: The Impact of Apixaban on Overall Hemostatic Potential

Disorders at certain levels of the complicated haemostatic system can lead to either bleeding or excessive blood coagulation. These pathological conditions are treated with anticoagulants, which aim to correct excessive coagulation. However, traditional anticoagulant therapy has many limitations, which initiated efforts to develop oral anticoagulants with a better profile. These new-generation anticoagulants are called DOAC - Direct Oral AntiCoagulans. Apixaban, as one of xabans, has predictable pharmacokinetics and pharmacodynamics and therefore does not require a routine laboratory monitoring of the treatment effect. Nevertheless, it still requires evaluation in urgent clinical situations. Standard coagulation screening assays, e.g., PT (prothrombin test) and APTT (activated partial thromboplastin test), do not fully reflect the actual status of the drug. Therefore, researchers aim is to find a relatively simple and fast hemostatic assay that would correlate with the actual condition of the patient. One of them could be the method of overal hemostatic potential (OHP), which is based on spectrophotometric registration of fibrin formation and its degradation in plasma. In our study, OHP has been used in patients treated with apixaban. This thesis evaluated its reproducibility (CV = 24.9 %) and the relationship between the total hemostatic potential and apixaban plasma concentration (R = -0.418), showing a weak correlation. Weak correlation was also observed between the time of coagulation and apixaban concentration (R = 0.277) as well as in PT (R = 0.501) and APTT (R = 0.360). In view of our results, we conclude that the total hemostatic potential assay cannot be used to monitor patients treated with apixaban. Yet, OHP presents a method with great potential that can serve as a starting point for the search for new approaches to understanding haemostasis as a highly complex whole.

CONTENT

LIST OF ABBREVIATIONS

Abs ..... absorbance

ADP ..... adenosine diphosphate

ALT ..... alanine transaminase

APTT ..... activated partial thromboplastin time

ASA ..... acetylsalicylic acid

AST ..... aspartate transaminase

AT III ..... antithrombin III

AUC ..... area under the curve

CNS ..... central nervous system

CrCL ..... creatinine clearance

CV ..... coeffition of variation

CVI ..... cerebrovascular incident

DIC ..... disseminated intravascular coagulation

DOAC ..... direct oral anticoagulant

dTT ..... dilute thrombin time

FXa ..... activated factor X

FDA ..... The Food and Drug Administration

FDP ..... fibrin degradation products

GIT ..... gastrointestinal tract

GPIb/IX ..... glycoprotein Ib/IX

HIT ..... heparin induced thrombocytopenia

HMWK ..... high molecular weight kininogen

INR ..... international normalized ratio

ISI ..... interantional sensitivity index

IU ..... international unit

LA ..... lupus anticoagulans

LC-MS/MS ..... liquid chromatography with tandem mass spectrometry

LMWH ..... low-molecular weight heparin

MW ..... molecular weight

NPP ..... normal pool plasma

OCP ..... overall coagulation potential

OFP ..... overall fibrinolytic potential

oGF ..... glomerular filtration rate

OHP ..... overall hemostatic potential

PAI – 1 ..... plasminogen activator inhibitor type 1

PIVKA ..... protein induced by vitamin K absence

pNA ..... paranitroaniline

PPP ..... platelet poor plasma

PT ..... prothrombin test

RES ..... reticuloepithelial system

TF ..... tissue factor

TFPI ..... tissue factor pathway inhibitor

tPA ..... tissue type plasminogen activator

TRIS ..... tris(hydroxymethyl)aminomethan

TT ..... thrombin time

UFH ..... unfractioned heparin

uPA ..... urokinase type plasminogen activator

UPLC-MS/MS ... ultra high-performance liquid chromatography combined with tandem mass spectrometry

VTE.....venous thromboembolism

vWF ..... von Willebrand factor

1. INTRODUCTION

Haemostasis is undoubtedly one of the most important self-regulatory systems in the human body. It belongs to basic vital mechanisms that are crucial for the individual's life. This process, which is responsible for blood viscosity, is characterized by complex interaction of cellular elements and specific proteins, enzymes, and other biological agents. The very purpose of haemostasis is to stop bleeding in case of injury. This involves recognition of the injured site and its precise targeting, very rapid activation of the clotting process (coagulation) that leads to the closure of the damaged vessel and its repair, and eventually restoration of the physiological blood circulation. The activation of haemostasis is concurrent with the activation of fibrinolysis, which helps to remove the blood coagulum after it has served its function. There is a delicate balance between these two closely related processes and this balance guarantees prevention of adverse bleeding conditions or, on the other hand, unstoppable blood coagulation.

Many dysfunctions and disorders of haemostasis exist, both congenital and acquired. A great portion of these pathologies involve excessive blood clotting. Thrombotic and thromboembolic complications pose a serious problem in individuals with a congenital predisposition to thrombosis (for example factor V Leiden), however, surgical procedures requiring long-term immobilization, cardiovascular diseases and associated unhealthy and sedentary lifestyles, use of hormonal contraception, pregnancy, or higher age also increase the risk. In view of the ever-increasing prevalence of these disorders, the objective is to provide the patient with an effective yet comfortable treatment and prophylaxis without serious side effects. In thrombophilic conditions, the treatment consists in anticoagulant drugs. UFH (unfractionated heparin), which had long been used for these conditions, is now being replaced by its fractionated derivatives. Compared to UFH, low-molecular-weight heparin (LMWH), e.g. enoxaparine (Clexane) has a lower incidence of side effects and does not require regular laboratory control monitoring. Another classic anticoagulant preparation is warfarin, whose advantage lies in the possibility of oral administration, however, it requires regular laboratory monitoring due to a wide range of factors that affect its efficacy, e.g., great inter-individual dose variability and drug and food interactions.

The current trend in the development of new anticoagulants is the search for the "perfect anticoagulant". Such a preparation would be easy to administer, selective, and

free of serious side effects, thus safe for the patient. It would also eliminate the need for laboratory monitoring owing to its predictable pharmacokinetics. Partly, these parameters are met by the new-generation direct oral anticoagulants - DOACs (gatranes – direct thrombin inhibitors, xabans – direct factor Xa inhibitors), which have been intensively developed since the beginning of the new millennium and which have been increasingly indicated in the past few years to patients for the clinical conditions associated with accelerated blood clotting. They remove the need for routine monitoring. However, they still require assessment in situations that lead to significant, even life-threatening, variations in blood concentration of the drug.

Measuring the concentration or coagulation activity of individual DOACs in patients has proved to be rather problematic. So far, there are no clearly defined therapeutic ranges for individual DOACs, even though assays to determine their concentration and activity are available. Common screening tests used in classic anticoagulants (PT, APTT, etc.) provide only a rough indication of the status of the activity of DOAC, however, they are certainly not suitable for their precise monitoring. For gatranes (dabigatran), there is a specific plasma concentration test – dilute thrombin time (dTT), which is performed using a calibrated Hemoclot assay. The activity of xabanes (apixaban, rivaroxaban) is measured using a calibrated quantitative chromogenic analysis of anti-Xa activity.

The objective is to find better yet undemanding tests that will be able to respond sensitively to both changes in haemostasis and the course of anticoagulant, antithrombotic, or antifibrinolytic therapy and prophylaxis. It appears that the overall haemostatic potential (OHP) could meet these criteria. OHP measures the ability of the proper function of procoagulant, anticoagulant, and fibrinolytic factors, which means that its parameters provide results for the evaluation of coagulation and fibrinolysis of the examined plasma sample. So far, it has been demonstrated that the OHP essay can be used to evaluate hypercoagulable states and fibrinolysis disorders, which are difficult to define by assays commonly available in routine laboratory testing. However, in order to introduce the assay into standard laboratory practice, more detailed and comprehensive studies need to be conducted while modifying the assay to a more relevant form.

2. THEORETICAL PART

2. 1. Haemostasis

It is the body's physiological mechanism to stop bleeding and restore the integrity of a damaged blood vessel. Haemostasis is a set of highly complicated processes in which cellular elements of the vascular wall, platelets, the system of plasmatic haemocoagulation factors, blood coagulation inhibitors, and fibrinolytic factors all interact together. Its objective is to prevent excessive blood loss and restore the disturbed balance between procoagulation and anticoagulation processes (Chottová Dvořáková and Mistrová, 2018). Disruption of this haemostatic balance may be expressed either as bleeding or in the form a pathological thrombophilic condition with an increased tendency to clotting and thrombosis (Salaj, 2017). As a result of activation of the haemocoagulation systems, the clot formed mends the damaged place and prevents further blood loss. The entire process of haemostasis can be divided into four partially overlapping stages. These are initiating vasoconstriction, primary platelet plug formation, haemocoagulation, and final removal of thrombus by fibrinolysis (Chottová Dvořáková and Mistrová, 2018).

2. 2. Haemostasis stages

2. 2. 1. Vasoconstriction

This stage with an extremely rapid onset, in fractions of a second, is very effective in preventing bleeding from small vessels. It is a complex interaction and cooperation between the nervous system, muscle cells of the damaged vascular wall, and fixed mediators such as serotonin, adrenaline, or noradrenaline. Damage to the vascular wall and the muscle cells that create it results in the contraction of these cells and in the activation of platelets at the place of damage. The platelets release vasoconstriction facilitators (serotonin, thromboxane A_2) and ensure the process of primary haemostasis (Chottová Dvořáková and Mistrová, 2018).

2. 2. 2. Primary platelet plug formation

The process of forming a primary precisely localized haemostatic platelet plug consists in the contact of the platelets with the damaged vascular wall or a foreign surface and involves four consecutive coordinated reactions. The platelets attach themselves to the wetting area of the inner surface of the damaged vascular wall or of another non-

physiological surface (adhesion) and begin to aggregate with one another (aggregation). Adhesion is mediated by the bond between the vascular wall basement membrane collagen receptor and the platelet membrane receptor GPIb/IX by means of the von Willebrand factor. Already at this stage, platelet activation partially occurs which is fully manifested in the aggregation process. Platelets are activated by a number of variety of agents, such as ADP, adrenaline, serotonin, thrombin, or thromboxane A. These activated platelets then also release other pro-aggregating agents that stimulate the activation of surrounding platelets, which accelerates both the adhesion and aggregation processes (Indrák et al., 2006).

Based on their activation, the platelets change their shape and internal arrangement of the organelles. On the membrane surface, the processes are expressed, and the cell becomes spherical. At the same time, changes are also taking place within the platelet, including the release of Ca^{2+} from the granules into the cytoplasm and the passage of the organelles to the centre of the cell. The granules move closer to the cytoplasmic membrane and channel system and release their contents outside the cell. There is a formation of an unstable platelet structure, the so-called primary thrombus. During the degranulation, ADP is also excreted, which stimulates other platelets and enhances the activation of the original platelets. Together with the release reaction, there is also platelet aggregation due to the bond of vWF and fibrinogen to the platelet membrane glycoprotein receptor. Platelet aggregation continues until the resulting thrombus closes the defect of the vascular wall. The platelet plug is reinforced with fibrin fibres, which make it firm and definitive. Finally, the coagulum retracts, which restores the patency of the damaged vessel (Chottová Dvořáková and Mistrová, 2018).

2. 3. Haemocoagulation

Haemocoagulation (secondary haemostasis) is a sequence of enzymatic reactions leading to the arrest of bleeding. Physiologically, it takes place only at the site of damage and its objective is to prevent blood loss and to close the affected area of the vessel wall as quickly as possible. As stated above, the core principle is to create a solid fibrin network that captures thrombocytes, leukocytes, and erythrocytes from the bloodstream and to form a durable definitive thrombus that replaces the primary unstable thrombus. This process is aided by a number of coagulation factors and other substances. Simply put, this process can be conceived as a set of consecutive enzymatic reactions that create

a so-called coagulation cascade (Chottová Dvořáková and Mistrová, 2018). The cascade is divided into the initial outer and inner pathways that interact with each other in a feedback manner and coalesce into a common pathway of transformation of soluble fibrinogen to insoluble fibrin. The haemocoagulation process must be regulated by natural blood coagulation inhibitors (e.g., antithrombin, protein C…) as well as by fibrinolysis. Inactive precursors (proenzymes) of the blood coagulation factors present in the bloodstream are gradually activated by enzymatic cleavage with the aid of the previous active enzyme (coagulation factor) until a stable resulting fibrin network is formed. Except for factor XIII, all coagulation factors with the serine amino acid incorporated in the active site are referred to as serine prostheses. Factors V, VIII, and HMWK (high molecular weight kininogen) are referred to as coagulation cofactors accelerating enzyme processes (Sakalová, 2010).

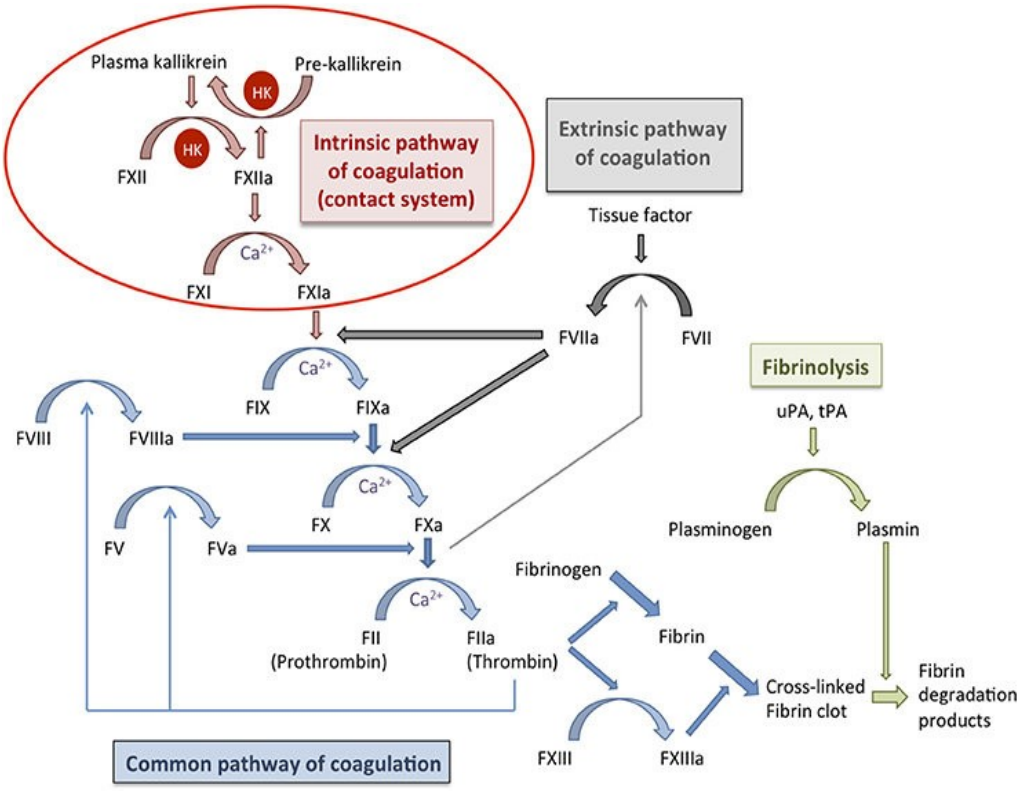

Fig 1 Diagram of the classic coagulation model;
Source: Loof et al., 2014

This classical model of coagulation (see **Fig 1**) is today being slowly replaced by the so-called cell-based model of blood coagulation based on the finding that, physiologically, coagulation occurs only on the surface of some cells, especially on cells expressing the tissue factor or on activated platelets (with the concurrent action of a variety of blood coagulation mechanisms). It follows from this that coagulation in

a healthy individual does not occur freely in the bloodstream but only at the site of vascular damage or extravasally. The interaction of the tissue factor, platelets, and plasma factors is crucial (Salaj, 2017). This revised model, which is based on the original model, can rather accurately explain the in vivo processes of haemocoagulation in the human body.

The new coagulation cell-based model comprises of three main steps: initiation, amplification, and propagation.

Initiation – the primary signal needed to develop the coagulation process is the contact of the tissue factor (TF) and FVIIa. In the blood, FVIIa is physiologically present in a small amount and, when there is damage and release of the tissue factor, FVIIa forms a complex with it (external tenase). The formed complex then activates other FVII molecules as well as factors IX and X on the surface of tissue factor-carrying cells. Activated factor FXa binds to FVa and forms prothrombinase on the surface of TF-expressing cells. Moreover, FXa generates a minute amount of thrombin, which is still sufficient to continue the coagulation events (Chottová Dvořáková and Mistrová, 2018).

Amplification – can be considered as the key coagulation process. At this stage, thrombin activates FV, VIII, and XI located on the surface of the adhered and thrombin-activated platelets at the place of damage. This increases procoagulant activity. Factor XIa is an initiator of the intrinsic blood coagulation pathway. In result of amplification, a greater amount of thrombin is generated which leads to the formation of reaction surfaces with bound activated factors that serve as a basis for the follow-up coagulation stage (Chottová Dvořáková and Mistrová, 2018).

Propagation – takes place on the activated platelets and represents the final stage of the formation of a fibrin plug. FXa, which was formed during the initiation stage, binds to the internal tenase complex (FIXa, FVIIIa, calcium ions, and phospholipids). This leads to activation of other FX molecules, which then form prothrombinase complexes with FVa. This catalyses massive production of thrombin, so-called “thrombin burst”. For illustration, one FXa molecule can produce up to 1000 thrombin molecules. Given the steep increase in thrombin concentration, there is extensive activation of fibrinogen, which is effectively transformed into fibrin. By the action of FXIIIa, this polymeric unstable fibrin is reinforced and allows the formation of the final thrombus. It is important to note that the mechanism of action of today’s anticoagulants targets the very FXa, either

inhibiting its synthesis or activation (anti-Xa assay) (Chottová Dvořáková and Mistrová, 2018).

2. 4. Fibrinolysis

As a regulatory system of haemocoagulation, fibrinolysis ensures the removal of the blood coagulum after it has served its role and restoration of the blood flow after repair. Its essence is the cleavage of the insoluble fibrin network into soluble fibrin degradation products (FDP) with the help of the action of enzyme plasmin. Enzyme plasmin occurs in the body as an inactive plasminogen and enzymatically affects the fibrin clot after its activation by proteolytic cleavage. The final product of the disintegrated coagulum is D-dimers. The resulting cleavage products and fibrin particles are removed by the reticuloepithelial system (RES) (Sakalová, 2010).

Activation of plasminogen to plasmin is a highly regulated process mediated by plasminogen activators, the most important of which is tissue plasminogen activator (tPA) released from the cells of the damaged endothelium. Others include urokinase-type plasminogen activator (uPA) produced in the kidneys and intrinsic coagulation pathway factors (FXIa, FXIIa, and kallikrein). In order to maintain the balance of the fibrinolysis process, the presence of inhibitors is necessary. The acute-stage protein α2-antiplasmin is a plasmin inactivator, while the plasminogen activator inhibitor (PAI-1), which ranks among serpins, prevents the activity of plasminogen activators (Kvasnička, 2003).

2. 5. Anticoagulant treatment

The essence of the anticoagulant therapy is blocking of the formation and action of thrombin in order to prevent blood coagulation and thrombus formation. Thrombin is a serine protease that is actively involved in the haemostasis process. Its function lies in the activation of platelets and factors V and VIII, catalysation of the transformation of fibrinogen into fibrin, and induction of the release of a specific fibrinolysis inhibitor (TFPI) (Kvasnička, 2003).

Anticoagulants themselves cannot remove the thrombus, but can prevent it from spreading and expanding. Anticoagulants are the core medications for prevention and treatment of VTE, in the prophylaxis of cerebral stroke, atrial fibrillation, and pulmonary and systemic embolisms in oncological patients, in patients after extensive operations, and in pregnancy or puerperium (Kvasnička, 2012).

The currently used or newly introduced anticoagulants can be divided into four basic groups:

Heparins: unfractionated heparin (UFH), low-molecular weight heparin (LMWH), synthetic pentasaccharides (fondaparinux) – selectively acting inhibitors of activated F Xa; injection, intravenous, or subcutaneous administration.

Coumarin preparations: vitamin K antagonists (warfarin), oral administration

Direct thrombin inhibitors: hirudin and other similar substances (e.g., bivalirudin), injection or oral administration

New anticoagulants-DOAC: direct oral inhibitors of factor Xa (rivaroxaban and apixaban) and thrombin (dabigatran etexilate)

Heparins and coumarins can be termed so-called indirect inhibitors of coagulation factors as they need cofactors for their anticoagulant effect. In heparins and pentasaccharides, this cofactor is antithrombin. Due to their action, coumarins induce the formation of ineffective coagulation factors (PIVKA – protein induced by vitamin K absence). Substances of the third group belong to direct thrombin inhibitors as they do not require cofactors and act selectively on given coagulation cascade factors. They also affect platelets when they inhibit their activation. The fourth group includes new-generation synthetic oral anticoagulants that should gradually replace the existing anticoagulant therapy. They do not need antithrombin for their action and are closely focused on specific components of the coagulation cascade. Rivaroxaban and apixaban are direct inhibitors of activated factor Xa, preventing thrombin generation; dabigatran etexilate is a direct inhibitor of thrombin (Kvasnička, 2012).

2. 5. 1. Unfractionated heparin (UFH)

Heparin (a mixture of acidic mucopolysaccharides) is present in the mast cells of parenchymatous organs. Commercial heparin preparations are produced by extraction from bovine lungs and pig intestinal mucosa. For this reason, heparin is biologically standardized, and its doses are given in IU. Heparin acts as an anticoagulant, i.e., reduces blood clotting and prevents the clumping and spreading of blood clots in the body. Its anticoagulant effect is given by the bond to ATIII (antithrombin III) and consists in accelerating and potentiating the inhibiting effects of this natural inactivator against

factors IIa, Xa, IXa, XIa, and XIIa. Heparin is therefore used as a catalysing cofactor for ATIII. It also has an anti-aggregation effect on blood platelets and reduces their adhesion to the endothelium. Its effects can be induced both in vitro and in vivo. There are two forms of heparin – unfractionated heparin (UFH, MW 5000–40000 d) and low-molecular weight heparin (LMWH, MW <5000 d). If there is ATIII deficiency or elevated factor VIII or another heparin-binding proteins (this may occur in inflammatory diseases or cancer) in the patient, the heparin therapy must factor in the patient's resistance to the preparation (Martínková et al. 2018).

Monitoring APTT (activated partial thromboplastin time) allows checking and adjustment of the patient's individual dose, which depends on the extent and type of injury and the degree of failure of the relevant body system. Heparing is primarily indicated for prevention and treatment of deep vein thrombosis and thrombembolic complications, pulmonary embolism, or DIC (disseminated intravascular coagulation). APTT should be 2–4 times the control value (35–45 s as a standard). Dosage is crucial heparin therapy, and it may not exceed critical dose limit as this could lead to life-threating bleeding. Heparin is administered parenterally by continuous or intermittent intravenous infusion. After intravenous administration, the effects of the preparation are immediate and last for 12-18 hours. Heparin does not pass through the placenta or into breast milk, is not absorbed from the GIT, its biotransformation occurs mainly in the liver, and is excreted via the kidneys. Distribution is only intravascular (Martínková et al., 2018).

Heparin treatment may be associated with serious adverse effects. In the event of intense bleeding complications, the patient must be intravenously administered protamine sulfate as an antidote. Heparin-induced thrombocytopenia (HIT) is another serious condition that may occur during the therapy. If heparin is administered for more than a few days, it is necessary to monitor the patient's blood platelets on a daily basis. Type 1 HIT, usually in a transient form, occurs approximately from the fourth day of administration. It is not immunological, and the number of blood platelets does not drop critically, which means the heparin therapy does not have to suspended. Heavy forms of type 2 HIT develop between 2 and 14 days from therapy initiation; the mechanism is immunological. The patient develops IgM and IgG antibodies to the platelet factor 4-heparin complex; these circulating immunocomplexes bind to blood platelets, which they activate and then lyse (Martínková et al., 2018). In consequence, the number of blood platelets usually drops below 100,000 and the patient is then at risk of thrombosis and

thrombembolic complications. If so, heparin therapy must be discontinued and further procedure considered. Under no condition, the patient may be administered platelets. In heparin therapy lasting over six months, osteoporosis or spontaneous fractures may occur as another adverse effect. Last but not least, there is also a risk of allergic reactions and manifestations of hypoaldosteronism and hyperkalaemia (Goldemund and Reif, 2012).

2. 5. 2. Low-molecular weight heparin (LMWH)

Contrary to unfractionated heparin, the use of low-molecular weight (fractionated) heparin is in many aspects more advantageous, and it finds application in range of medical fields. The concept of fractionating heparin was established based on the discovery that its antithrombotic properties are due to only a part of the molecule of very low weight (about 5000 daltons). The isolation of the active ingredient of heparin resulted in reduction or even complete elimination of adverse reactions observed in UFH (bleeding, thrombus formation, etc.) and enabled easier dosing to the benefit and convenience of the patient (Kvasnička, 2003). This greatly reduced the rate of complications, but not completely.

LMWH is used in transplantology, surgery, orthopaedics, in patients with thromboembolism, in prophylaxis and therapy of deep venous thromboses in patients with ischemic heart disease, post-operative venous thromboses, and in the treatment and prophylaxis of vascular occlusions in neoplastic diseases (2004). It binds to antithrombin III and thus promotes its inhibitory effects especially against factor Xa, less against IIa (thrombin). Its affinity for AT III is four times greater than that of UFH, which produces lower anticoagulant response of UFH compared to LMWH. It also activates fibrinolysis, promotes the release of TFPI (tissue factor pathway inhibitor) from the endothelium of blood vessels, and inhibits the external coagulation system (Kvasnička, 2003).

A major advantage of LMWH is its use for outpatient and home treatment. In fact, studies show that LMWH does not require regular laboratory control as is the case of UFH (Bounameaux and De Moerloose, 2004). One reason is the difficulty in examining the efficacy of LMWH using APTT or thrombin time assays. Theoretically, a special blood plasma assay could be used to target the level of inhibition of activated factor Xa, which is, however, not performed in practice (Kvasnička, 2003).

Dosage is adjusted according to the patient's weight, and the preparation is injected subcutaneously. The LMWM plasma half-life is not dependent on the dose but on renal clearance as it is excreted by the kidney. Therefore, it is advisable to perform clinical and laboratory monitoring of antiXa in patients with renal impairment. LMWM has better biological availability, which allows its administration once a day, while its stable plasma activity enables accurate prediction of its anticoagulant effect (Malý, 2004). In the Czech Republic, LMWM is available, for instance, as dalteparin, enoxaparin, tinzaparin, or nadroparin (Martínková et al., 2018).

2. 5. 3. Coumarin anticoagulants

The best-known product among indirect vitamin-K-dependent oral anticoagulants is warfarin. It belongs to coumarin derivatives. These are vitamin K antagonists whose mechanism of action consists in inhibition of important enzymes of the cycle of this vitamin leading to reduction of the synthesis of vitamin K-dependent coagulation factors in the liver. These include, in particular, factors of the prothrombin complex II, VII, IX, and X. This also reduces the formation of coagulation inhibitors, i.e., protein C and protein S (Martínková et al., 2018).

Warfarin is widely used in the prophylaxis and long-term therapy of thrombosis, in the prevention of stroke in atrial fibrillation, or in the prevention of peripheral embolism where it is necessary to monitor the preparation’s efficacy (Indrák, 2014). The maximum effect is reached after 36–48 hours, and the mechanism of its action is highly complex where the dose-response relationship is greatly individual depending on the patient’s metabolic condition and difficult to predict. The ideal therapeutic effect of warfarin is achieved only after a few days of administration (4–5 days) and therefore the treatment should be initiated with the concomitant administration of low-molecular weight heparin. Otherwise, there is a risk of increased thrombophilia (so-called coumarin necrosis is a typical phenomenon) due to the suppression of the effects of natural blood coagulation inhibitors, protein C, and protein S (Kvasnička, 2003).

The efficiency of warfarinization is evaluated using the International Normalized Ratio (INR). It expresses the proportion between the prothrombin time of the patient and

the prothrombin time of normal pooled plasma of healthy non-treated raised to the power of the International Sensitivity Index (ISI), (see **Fig 2**).

$$\text{INR} = \left(\frac{\text{PT of patient}}{\text{PT of normal range mean}} \right)^{\text{ISI}}$$

Fig 2 Formula for the expression of INR
Source: Bhutta, 2013

The prothrombin test (PT) reflects the overall changes in the external coagulation system (FV, FII, FX, FVII, fibrinogen). PT is conducted on a blood plasma sample to which appropriate reagents important for initiating the reaction are added, i.e., thromboplastin reagents containing the tissue factor and calcium ions eliminating the effect of citrate. It measures the time from the moment of reagent addition until the formation of the clot (Pecka, 2002).

INR serves as a tool for standardization and better comparison of PT results among different laboratories since it is not affected by differences between tissue factor lots from different manufacturers, which is the basis for the production of thromboplastin reagent. Manufactures are required to assign the ISI value to their thromboplastin, and the value informs about the comparability of the lot with the internationally standardized thromboplastin (Kvasnička, 2003). Individual dosage of warfarin is then predicted using the prothrombin time (PT, Quick test). This test determines the decrease in coumarin-sensitive coagulation factors (factor II, VII, and X) is the primary test to monitor coumarin therapy. The normal value of prothrombin time is usually 12–15 s; the normal range of INR is 0.8–1.2 (McGlasson, 2003). The value of INR, as an indicator of the efficacy of warfarin treatment, ranges from 2 to 3.5 (Tuka and Janota, 2011).

Prolongation of PT time is observed in factor II, V, VII, X, and fibrinogen deficiency, both congenital and acquired. The cause may be liver impairment, disseminated intravascular coagulopathy (DIC), or the presence of autoantibodies against coagulation factors and of Lupus anticoagulant antibodies (Pecka, 2006).

Complications of warfarin treatment include, in particular, a significant risk of bleeding even in minor dose changes, which is due to the low therapeutic index where a slight dose change may result in major change in effect. For this reason, it is crucial that the treatment is monitored and INR checked and optimized while excluding food

interactions (alcohol, grapefruit, etc.) or interactions with other drugs, which could be severe in this situation. The antidote for warfarin overdose with subsequent bleeding is vitamin K, fresh frozen plasma, prothrombin complex concentrate, or the more expensive but more effective recombinant activated factor VIIa (Kessler, 2006).

Warfarin is absorbed from the gastrointestinal tract (GIT) and eliminated by the liver and kidneys. It binds to plasma proteins, which means it is necessary to pay increased attention to their decrease, for instance in inflammation or diarrhoea, due to greater availability of the free unbound and effective form of warfarin in the body. It is degraded by several forms of cytochrome P450, while CYP450 2C9 (most commonly CYP2C9*2 or CYP2C9*3) is the most important for its metabolism. Patients with genetic polymorphisms have different sensitivity to warfarin and their metabolism degrades it more slowly (Kessler, 2006).

2. 6. Direct oral anticoagulants (DOACs)

DOACs have been developed as a replacement for classic anticoagulant therapy with heparins and warfarin. In view of the many shortcomings of the traditional therapy, the effort aims at creating a so-called ideal anticoagulant. Essential factors in this respect are greater safety, predictable pharmacokinetics, easier use and dosing without the need for routine monitoring, comparable efficacy, and economic acceptability. DOACs are competitive targeted inhibitors that do not need antithrombin to function (see **Fig 3**) (Indrák, 2014). They are classified into two groups. Gatrans, which are direct inhibitors of thrombin (factor IIa) and xabanes which act as direct inhibitors of factor Xa. Presently, these substances are registered: dabigatran (Pradaxa®), rivaroxaban (Xarelto®) apixaban (Eliquis®), and edoxaban (Lixiana®) (Hlusi et al., 2015). Despite their demonstrated safety in extensive controlled clinical trials, it is still a novelty treatment some aspects of which have not yet been fully examined. It has so far been established that the most common complications of DOACs include gastrointestinal haemorrhage, incompatibility with patients with severe renal dysfunction and hepatic diseases, and absence of the defined dose for patients with moderate renal dysfunction for most of these preparations (Ruff et al., 2014, Heidbuchel et al., 2017, Küpper et al., 2018). Yet, in view of their generally better properties, their fast growth and deployment in clinical practice can be expected.

DOACs are used for primary and secondary prevention of venous thromboembolism in extensive orthopaedic surgeries (hip or knee replacement), pulmonary embolism, and as a prophylaxis of cardioembolizing stroke and systemic embolism (Indrák, 2014).

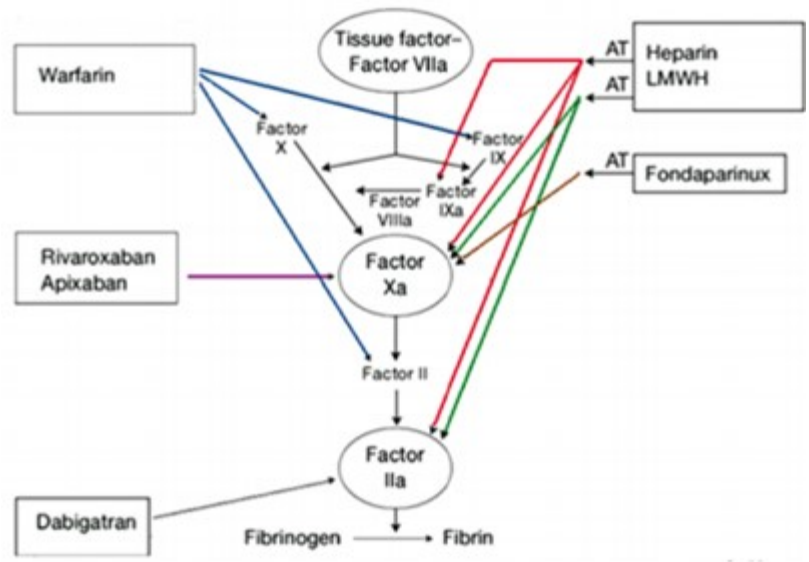

Fig 3 Diagram of the effects of anticoagulants on target molecules
Source: Hlusi et al., 2015

DOACs do not require routine monitoring of coagulation parameters in routine clinical practice thanks to their predictable anticoagulant effect, provided the recommended dosing is used. However, as mentioned above, in case of certain specific risk situations, such as increased risk of bleeding, renal insufficiency, or in elderly polymorbid patients with extensive medication, such monitoring is desirable (Connors, 2018) In laboratory practice, basic screening laboratory tests – activated partial thromboplastin test (APTT), thrombin test (TT), and prothrombin test (PT) – may be used in acute conditions. DOACs prolong these coagulation times depending on their plasma concentration. TT and APTT are particularly recommended for the evaluation of anticoagulant activity; PT does not provide sufficient sensitivity. INR measurements are not recommended neither as the values may be falsely increased. APTT is significantly prolonged by dabigatran, less by apixaban and rivaroxaban (Krcova et al., 2012).

APTT is one of the global screening tests for haemocoagulation. It monitors the intrinsic and common coagulation pathways – factors XII, XI, IX, VIII, prekallikrein, and high-molecular-weight kininogen. Also, APTT is a generally accepted test for monitoring heparin therapy where prolongation of coagulation time is proportional to heparin

concentration. In the presence of non-specific inhibitors, such as LA, APTT prolongation can be expected, however, this variable effect is often due to the composition of the APTT reagent used (Sakalová, 2010).

The principle of the APTT test is to activate coagulation via the intrinsic pathway. Partial thromboplastin (cephalin – a source of phospholipids) and Ca^{2+} are added to a decalcified plasma sample at 37 °C. To speed up the activation, an activator (kaolin, silicates, ellagic acid) is added. The measurement results are expressed in seconds or as a ratio of the times of the tested and control plasma. Prolongation of APTT may most often be due to a congenital or acquired deficiency of intrinsic coagulation pathway factors, presence of a specific or non-specific inhibitor, or presence of heparin. APPT is also prolonged in case of coagulation disorders (Penka and Tesařová, 2011).

According to some recommendations and current studies, it is not advisable to use APTT and PT as monitoring tests in DOACs altogether since there is poor correlation between the patient's DOACs plasma concentrations and APTT or PT prolongation. The variability of the results is given by the APTT reagents used and the specific DOAC. Latest studies have shown that patients with therapeutic DOAC plasma concentrations may have normal APTT and/or PT depending on the DOAC administered and the agent used. For this reason, APTT and PT should no longer be used as a general indicator of the patient's level of anticoagulation and associated bleeding in patients who are or could be treated with DOAC (Adcock, 2018, Patel et al., 2019, Connors, 2018).

Time of taking the last dose is an essential factor for evaluating all of these tests since the anticoagulant response depends on the relationship between the time of blood sampling and the time of the last dose. For example, concentration of the drug in the blood will differ after two hours and 10 hours. Blood sampling at the stage of minimum drug plasma concentration, i.e., before the next dose, is most important for assessing bleeding or overdose. However, for clinical use, the use of screening tests is not recommended since there are special tests available to assess the effect of DOACs quantitatively. Specifically, there is a special calibrated thrombin inhibitor assay for dabigatran named HEMOCLOT (trombin inhibitor assay, Hyphen, Bio-Med, France) or dilute thrombin test (dTT) assay that allows direct quantitative determination of dabigatran plasma levels (Krcova et al., 2012, Božič-Mijovski M et al., 2016). To assess the efficacy of rivaroxaban and apixaban treatment, an anti-Xa assay is used (Martínková et al., 2018, Douxfils et al.,

2018, Zotz and Weißbach, 2017). The issue of interpretation of coagulation tests in these new anticoagulants is subject of further examination by laboratory methods.

Today, newly developed antidotes are available against all direct oral anticoagulants (Dobesh et al., 2019). In an overdose without bleeding, the short half-life of DOACs may be advantageous. In addition to the patient's data indicating an increased risk of bleeding in the treatment with DOACs, also a scoring system, such as a HAS-BLED score or Q-Bleed, a validated scoring system to calculate a higher individual bleeding risk into the GIT or CNS, may help in decisions to control the patient control and adjust dosing (Gutierrez and Blanchard, 2016, Kvasnička et al., 2015). Naturally, laboratory testing, especially serum creatinine, should also be performed; with apixaban, liver tests (AST, ALT, bilirubin) should also be performed and coagulation tests at the time of maximum and minimum effect (Krcova et al. 2012). Bleeding manifestations must be thoroughly clinically evaluated to determine whether it is a life-threatening, severe, or mild bleeding.

2. 6. 1. Dabigatran (Pradaxa)

Of the DOACs group, dabigatran was the first one approved (in 2008). It is a direct, selective, reversible inhibitor of thrombin, both free and fibrin-bound. Thrombin is a key enzyme at the end of the coagulation cascade which converts fibrinogen to fibrin and activates factors V, VIII, XI, XIII and protease-activated platelet receptors.

Dabigatran inhibits free thrombin, fibrin-bound thrombin, and also thrombin-induced platelet aggregation. Dabigatran etexilate, as a prodrug, is rapidly absorbed after oral administration and converted enzymatically to the active form of dabigatran inside the liver. Its onset is rapid, after about half an hour to two hours. Its plasma half-life is about 12–14 hours. It is primarily excreted by the kidneys, and therefore any renal dysfunction of the patient must be considered. Ideally, the patient's renal function should be evaluated prior to the treatment by calculating creatinine clearance (CrCL) using the Cockcroft-Gault equation (Indrák et al., 2014). The treatment with dabigatran etexilate is contraindicated for patients with severe liver impairment. Dabigatran is indicated for the prevention of stroke and systemic embolism in adult patients with non-valvular atrial fibrillation and is used therapeutically in patients with deep vein thrombosis or pulmonary embolism.

As already mentioned, routine monitoring is unnecessary given the predictable pharmacokinetic effect. However, in overdose, acute haemorrhage, or severe renal or impairment, laboratory control associated with discontinuation of treatment is recommended. Other risk factors, that may be associated with severe gastrointestinal bleeding, include concomitant administration of platelet aggregation inhibitors, including acetylsalicylic acid (ASA) or clopidogrel. This naturally excludes concomitant treatment with other anticoagulants (UFH, LMWH, etc.) or strong inhibitors of glycoprotein P (ketoconazole, cyclosporine, dronedarone, rifampicin, ritonavir, etc.).

Until 2016, there was no known antidote, however, unlike the duration of action of warfarin (2–5 days), the anticoagulant activity of dabigatran subsided within 17 hours, and so the "waiting strategy" was applied. Since 2016, there has been an antidote for dabigatran available in the Czech Republic. It is the specific preparation Praxbind with the active substance idarucizumab, which is indicated in urgent and life-threatening bleeding conditions when fast intervention is required to reverse the effects of anticoagulant therapy. Idarucizumab binds tightly to dabigatran and forms a complex with it, thus stopping its anti-clotting effect within few minutes (Goldemund and Reif, 2012, Cortese et al. 2018).

In connection with dabigatran and its effects, it is worth mentioning one of the largest randomized studies in patients with non-valvular atrial fibrillation. This three-year, multicentre, international study titled RE-LY (Randomized Evaluation of Long-Term Anticoagulation Therapy Trial) involved 18,113 patients who were administered 150 mg or 110 mg of dabigatran twice a day. The preparation's anticoagulant activity and safety were compared with warfarin therapy (INR 2.0–3.0). The results showed that dabigatran 150 mg twice a day reduced the risk of stroke and systemic embolism by 35 % compared to warfarin while 110 mg twice a day did not differ much in the frequency of stroke or systemic embolism from warfarin therapy, however, while showing a statistically significantly lower incidence of severe bleeding. Both doses of Pradaxa thus had a statistically significantly lower risk of bleeding compared to warfarin (Connolly et al., 2013, Eikelboom et al., 2011). In the perspective of this and other less extensive trial, dabigantran was indicated in 2010 and 2011 as a prophylaxis of stroke and systemic embolism (Krcova et al., 2012).

2. 6. 2. Rivaroxaban (Xarelto)

Rivaroxaban ranks among selective inhibitors of activated factor Xa to which it binds directly. FXa is a serine protease which, together with FVa, Ca^{2+}, and negatively charged phospholipids, forms an active prothrombinase complex that facilitates conversion of prothrombin to thrombin (Martínková et al., 2018).

Rivaroxaban has a rapid onset of action similar to dabigatran (2.5–4 hours), and its plasma half-life is around nine hours. It is characterized by very high bioavailability (80 %). It is excreted partly by the kidneys and partly eliminated in the liver (Indrák, 2014). Again, special care should be taken in patients with renal or hepatic disease, similarly as with dabigatran.

An antidote against rivaroxaban has been approved only recently. This substance is named andexanet alfa, and it was ratified in 2019 by the European Medicines Agency (EMA) after having being authorized in 2018 by the US Food and Drug Administration (FDA). The basis for the approval were multicentre, open-label studies ANNEXA-4, ANNEXA-A, and ANNEXA-R, which have shown safe and beneficial effects in the test group of patients using xabans. Andexanet alfa is a modified recombinant inactive form of human factor Xa intended for specific bond and sequestration of factor Xa inhibitor molecules. Andexanet alfa rapidly decreases their activity, i.e., reduces the anticoagulant effect of factor Xa inhibitors (rivaroxaban and apixaban) (Connolly et al., 2019, Tummala et al. 2016, Mujer et al., 2020).

Monitoring of the pharmacodynamics and effect of rivaroxaban is generally unnecessary. In life-threatening serious conditions (overdose, bleeding, emergency surgery, etc.), the use of a specific quantitative chromogenic test is recommended to determine direct anti-Xa inhibitors calibrated for rivaroxaban. Rivaroxaban present in the patient's plasma inactivates excessive factor Xa. Unbound factor Xa is cleaved by a specific chromogenic substrate with the formation of the coloured product paranitroaniline (pNA). Absorbance at 405 nm is measured to determine the amount of pNA. The amount of released pNA is directly proportional to the activity of the remaining factor Xa and thus indirectly proportional to the level of rivaroxaban (Harenberg et al., 2011).

2. 6. 3. Apixaban (Eliquis)

This preparation also ranks among direct, competitive, selective, and reversible inhibitors of activated factor Xa. It inhibits both free Xa and Xa bound to the prothrombinase complex or incorporated into the fibrin network of the coagulum by which it ensures the blockade of the factor and prevents the triggering of secondary activation of coagulation after the thrombus has been broken down. Its advantages include rapid onset of action (30–60 minutes) and high bioavailability (up to 80 %) compared to dabigantran (about 10 %). The maximum concentration of the preparation in the body occurs after 3–4 hours, and the plasma half-life is around 10–14 hours. It is metabolised by the liver to a large extent, less by the kidneys. Therefore, it is contraindicated for coagulopathy in liver disease, which excludes its administration. No dose adjustment is required in patients with mild or moderate renal impairment. (Bultas and Karetová, 2011).

It is normally taken twice a day orally at a dose of 5 mg or 2.5 mg. It is indicated for the treatment and prevention of VTE in patients after extensive orthopaedic surgeries, such as hip or knee replacement, and for the prevention of stroke (CVA) and systemic embolism in patients with non-valvular atrial fibrillation, etc. Based on many extensive studies, apixaban appears to be the most reliable option of the DOACs group since it has the most reliable profile. The risks of food and drug interactions are minimal compared to other anticoagulants; in the long term, concomitant administration of apixaban and potent CYP3A4 inhibitors (azole antifungals – fluconazole, HIV protease inhibitor – ritonavir, etc.) or P-glycoprotein (multi-drug resistance protein MDR1) substrates (Goldemund and Reif, 2012) should be avoided. The inter- and intra-individual variability of its effect is low thanks to its stable bioavailability. Also, its plasma levels are predictable over the entire course of the therapy (Bultas and Karetová, 2011).

A specific antidote against apixaban (rivaroxaban), andexanet alfa (Andexxa, Ondexxya), was approved by the FDA in 2018. Andexanet alfa is a modified variant of factor Xa, which, thanks to its similarity to the human factor Xa, allows very effective and successful binding to its inhibitors (Mujer et al., 2020, Tummala et al., 2016) Reduction of the anticoagulant activity then occurs within two minutes after intravenous administration of the preparation. Given its minimum rate of adverse events, andexanet alfa can be considered a rapid and relatively safe preparation for reversing life-threatening

bleeding complications in the treatment with apixaban or rivaroxaban (Momin et al., 2019).

Laboratory monitoring of the effect of apixaban in urgent or emergency situations during the treatment is the same as for rivaroxaban. It is performed by a modified chromogenic anti-Xa assay calibrated for apixaban. As mentioned above, the standard APTT and PT assays are not suitable due to the large variability of the measured values (Bultas and Karetová, 2011).

Since DOACs rank among drugs with high pharmacokinetic variability and a narrow therapeutic index and since it is difficult to measure their pharmacodynamic parameters directly, therapeutic monitoring is not recommended. Besides coagulation assays, which are susceptible to interference due to various factors, liquid chromatography is used to quantify apixaban in conjunction with tandem mass spectrometry (LC-MS/MS). The technique is characterized by much better selectivity and specificity, thus allowing very accurate quantification and detection of anticoagulants, especially at low concentrations (<50 ng/mL) where, for instance, anti-Xa assays could fail. Based on recently published studies, it appears that the best accuracy is provided by a combination of ultra high performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS) (Lindahl et al., 2018). The disadvantage of simple routine use of these analytical methods is their complexity and the need for highly specialized expensive equipment. The very sample preparation presents certain imperfections that contribute to reduced sensitivity and matrix effects. Phospholipids may accumulate on the analytical column that have not been removed from the sample by coagulation, which can contaminate the mass spectrometer. The trend in the development is to remove these problems and modify the method to achieve the most sensitive and correct results. In consequence, LC-MS/MS is still the gold standard as a reference method for measuring the concentration of all DOACs (Slavik et al., 2018).

In 2011, a comprehensive international, multicentre study ARISTOTLE was published, which monitored over 18,000 patients with non-valvular atrial fibrillation, stroke, or heart failure. The outcome of the study confirmed the better efficacy of apixaban compared to warfarin. Even though the primary objective of the study was to demonstrate that apixaban is not inferior to warfarin, which is the reason for the selection of a relatively low and safer dose of apixaban of 2×2.5 milligrams. Enrolment of patients

into the study started in 2006 and continued to 2010. There were two groups of subjects – one administered apixaban and the other warfarin, both monitored for nearly two years. The evaluation brough pleasantly surprising results. Apixaban therapy reduced the incidence of CVI and systemic embolism by 21 % ($p < 0.01$) compared to warfarin. From a safety point of view, apixaban performed better than warfarin, with reduced bleeding complications by 31 % ($p < 0.001$) and even mortality by 11 % ($p = 0.047$). Apixaban exhibited an acceptable side effect profile, was more effective than warfarin in preventing stroke, and achieved this goal at a significantly lower risk of bleeding and at lower doses. Based on these results, apixaban was approved in 2012 as prophylaxis for patients after stroke and as from 2014 it could be applied to prevent VTE in patients after hip or knee replacement. Research suggests that the treatment with apixaban and other DOACs is in fact much more sophisticated in all aspects for the patient compared to current standards, and the only downside of their wide use is their higher financial cost (Granger et al., 2011).

2. 7. Overall haemostasis potential

In view of the complexity of testing hemostatic abnormalities, researchers are trying to develop new approaches and methods that would provide a comprehensive view of the entire haemostatic system and could detect changes in the level of coagulation or fibrinolysis. Haemostasis imbalance, which involves multiple activation and inhibition of proteases, can be difficult to measure if only individual components of the system are evaluated and analysed.

One of novelty tests for this purpose is the so-called overall haemostasis potential (OHP). This method is based on the formation of a fibrin-aggregation curve in platelet-poor plasma, which contains minute amounts of exogenous thrombin, tissue-type plasminogen activator (tPA), and calcium (Antovic, 2010). Unlike other screening tests, which are not able to detect all possible pathological abnormalities in the haemostatic process since they focus only on a certain part of coagulation, OHP can be used to assess both total coagulation and fibrinolysis. According to recent studies, the assay can be used to assess conditions associated with hypercoagulation in pregnant women, thrombophilic patients, and individuals with cardiovascular disease, stroke, diabetics or in hypocoagulation conditions. Also, its is envisaged for monitoring in anticoagulant and antithrombotic treatments (He et al., 2001).

A study of pregnant women with deep vein thrombosis treated with LMWH showed that OHP successfully reflected changes in the haemostatic system after drug administration. Although the anti-Xa activity reflected the amount of LMWH in plasma, the OHP test was able to assess the overall haemostatic profile of patients better. In another study group in patients who were taking standard anticoagulant therapy (heparin, warfarin), a significant sensitive response of OHP to a specific phase of treatment was demonstrated. Based on these results, it might be that the OHP poses another suitable tool not only for assessing the course of anticoagulant treatment, but also for determining haemostasis imbalance. However, introduction of the assay into clinical practice will require larger controlled clinical trials focusing on all available anticoagulants (Antovic, 2010).

The principle of the OHP assay is in the repeated spectrophotometric measurement of the resulting fibrin-aggregation curves in platelet-poor plasma (PPP), to which thrombin and phospholipids are added. tPA is present only in the first plasma sample and is not added to the second one. This generates two fibrin aggregation curves showing the process of gradual conversion of fibrinogen in the plasma into fibrin with concurrent generation of thrombin; at the same time, the production of plasmin, which consumes fibrin, is activated. Changes in absorbance are recorded every minute at 405 nm for 40 minutes. Each absorbance value indicates the fibrin level at a particular time. The output of the measurement are graphs of the dependence of absorbance on time. The balance between generation and consumption of fibrin during the measurement is shown by the area under the curve (AUC). The AUC is determined by adding together the absorbance values recorded during the test (Abs-sum) and then subtracting the background absorbance, which corresponds to the initial optical density of the plasma sample before the start of coagulation. The AUC is a crucial laboratory parameter. In a plasma sample with added tPA, the AUC is represented by the values for OHP, while in plasma without tPA, the AUC is represented by the values for OCP (overall coagulation potential) (see **Fig 4**). The difference between these two parameters reflects the OFP (overall fibrinolysis potential) using the relationship:

$$OFP = [(OCP-OHP) / OCP] \times 100\ (\%)$$

The simplicity of the measurement and evaluation ranks the OHP test among time-saving and inexpensive methods for many coagulation and routine analytical laboratories, which causes interest in examining the test for its benefits in controlling dosing of anticoagulant therapy for various hyper- and hypocoagulation abnormalities and in monitoring persisting effects of the therapy without secondary thrombotic events or bleeding (Antovic, 2010).

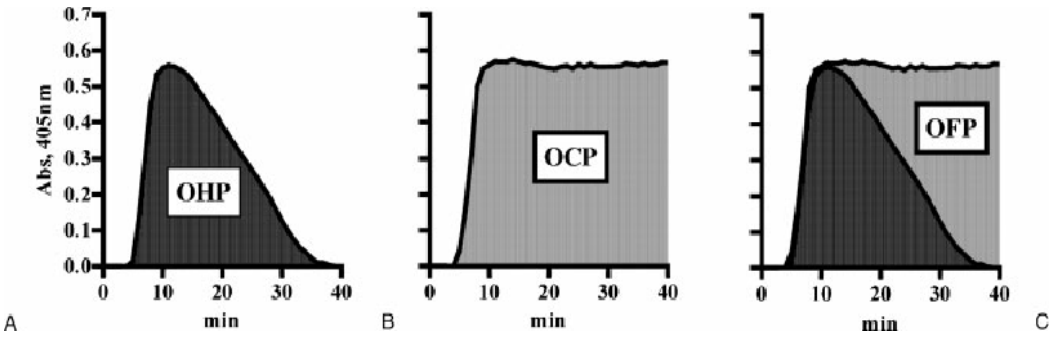

Fig 4 Graphical output of fibrin-aggregation curves. A: Overall haemostasis potential. B: Overall coagulation potential, C: Overall fibrinolytic potential
Source: Antovic, 2010

3. EXPERIMENTAL PART

3. 1. Aim of the Thesis

The purpose of the practical part of the diploma thesis was to measure OHP in *ex vivo* plasma samples of patients receiving apixaban, statistical evaluation of the results and their comparison with other performed haemostatic tests, and final evaluation of the reliability of the OHP assay. The thesis was prepared under the auspices of the Faculty of Pharmacy in Ljubljana, Slovenia, as part of the CEEPUS - Central European Exchange Programme for University Studies (www.ceepus.info). The measurements were performed in the Laboratory for Haemostasis and Atherothrombosis, University Medical Center Ljubljana, Slovenia

3. 2. Objectives of the Thesis

The practical part of the thesis focused on the evaluation of the following problems:

- identification of the relationship between measured OHP parameters and apixaban plasma concentrations;
- verification of the correlation between apixaban concentration and coagulation onset time;
- evaluation of the reproducibility of the OHP test;
- comparison of the OHP test with PT and APTT assays;
- evaluation of the suitability of the OHP test in the treatment monitoring and of the possibility of predicting thrombotic or haemorrhagic events

3. 3. Patients

The study included 75 citrate plasma samples from 13 patients treated with apixaban. The cohort comprised of seven men and six women. Nine patients received a 5 mg dose of apixaban every 12 hours, four patients 2.5 mg per 12 hours. Six venous blood samples were taken from each patient for three consecutive months, meaning that two samples were taken once a month on the same day. The first sample was taken just before the next dose of apixaban when the plasma concentration was the lowest (trough concentration), and the second sample three hours after administration of the drug at the maximum concentration (peak concentration) of apixaban in the organism. Venous blood was collected into standard 4.5 mL plastic collection tubes with anticoagulant sodium citrate (0.109 mol/L, Becton Dickinson, USA) with the anticoagulant-to-blood ratio of 1:9.

Immediately after collection, blood samples were centrifuged at 2.000 × g for 20 minutes at room temperature. After centrifugation, plasma was aliquoted (cca 500 μL) into cryovials, frozen in liquid nitrogen and stored in a freezer at -75 °C.

Normal pool plasma (NPP) was prepared from the citrated plasma samples of healthy subjects and served as a control sample. This plasma was prepared in stored in the same manner as patient samples.

3. 4. Equipment

- spectrofotometer for microtiter plates Sunrise™ connected with computer, operated via software Magellan (Tecan, Austria)
- automated coagulation analyzer CS-2500 (Sysmex, Japan)
- Thermomixer Comfort, (Eppendorf, Germany) thermomixer combines shaking and heating
- rotator sample mixer HulaMixer (Thermo Fisher Scientific, USA)
- centrifuge MiniSpin® (Eppendorf, Germany)
- vibration mixer Vortex (Fisher Scientific, USA)

3. 5. Laboratory equipment

- microtiter plates (Thermo Fisher Scientific, USA)
- singlechannel automatic electronic pipettes available in volumes from 10-300 µL, 50-1000 µL, 100-5000 µL, Biohit ProlinePlus; singlechannel mechanical pipette Biohit Proline 0.5-10 µL (Sartorius, Germany)
- multichannel electronic pipette Biohit eLine e 300 (Sartorius, Germany)
- cryogenic vials CryoTubes, 4.5 mL (Sarstedt, Germany)
- plastic tubes, 1.8 mL (Sarstedt, Germany)
- plastic baths

3. 6. Reagents

- bovine thrombin (Sigma Chemical Company, USA), 996.6 NIH,
- recombinant t-PA, concentration 1 mg/mL (Actilyse, Boehringer Ingelheim, Germany)
- $CaCl_2$ 1 mol/L (Pharmacy UKCL, Slovenia)
- Tris-HCl buffer, pH = 7.5 (Pharmacy UKCL, Slovenia)
- Phospholipid - TGT, concentration 0.5 mmol/L (Rossix, Sweden)
- distilled water (Pharmacy UKCL, Slovenia)

3. 7. Preparation of reagents

Buffer solution

A Tris-HCl buffer solution was prepared by dissolving 5.0 g of Tris-HCl and 38 g of NaCl in 500 mL of distilled water. The pH of the solution was adjusted to 7.5 using 4 M HCl. The buffer was stored refrigerated (2-8 °C) for one month at the most.

Thrombin

In the first step, lyophilized bovine thrombin was dissolved in the distilled water up to a concentration 1, 000 NIH/mL. An aqueous solution was stirred by the rotary mixer about five minutes in order to mix in thoroughly. Then, a solution was diluted by mixing with distilled water to make 1:10 dilution. Final concentration of thrombin solution was 100 NIH/mL. Aliquots of 100 mL each were prepared and stored in the freezer at -75 °C.

t-PA

Tissue plasminogen activator (amount 2 mg) was diluted by adding 2 mL of distilled water and mixed in rotary mixer for 30 minutes.

Final concentration of the prepared 30 mL aliquots was 1mg/mL. They were stored in the freezer at -75 °C, as well as thrombin aliquots. Each aliquot was thawed and used only once.

3. 8. OHP method

OHP was performed according to the method of He et al (He et al., 2001). First, the spectrophotometer operating temperature was set to 37 °C and the pre-defined method was selected using Magellan computer software (method settings are described in detail in the next section). All reagents were equilibrated to room temperature. Then, 6 mL of TRIS buffer and 205 µL of $CaCl_2$ (1 mol/L) were pipetted into a plastic tube and vortexed for several seconds. 2.7 mL was pipetted from the mixture into two plastic cryotubes. One was referred to as “tPA-free solution”, the other as “tPA solution".

Meanwhile, phospholipids (0.5 mmol/L) were diluted at 1:1 with TRIS-HCl buffer (500 µL phospholipids + 500 µL TRIS-HCl) to the final concentration of 0.25 mmol/L. 20 µL of diluted phospholipids were pipetted for each test well onto the prepared microtiter plate. The volume of phospholipids was twice as large compared to the original method.

Immediately before the analysis, patient aliquots and an NPP sample were thawed at 37 °C for five minutes in a thermomixer. The thawed samples were pipetted at 70 µL into the wells of the microtiter plate. Each patient had their own six aliquots, each of which was applied six times in a row (rows marked B, C, D, E, F, G, columns marked 1 to 6). NPP was pipetted six times into the first row (marked A) as a control, also in the volume of 70 µL. A total of 42 wells were prepared for the measurement.

Thrombin and tPA were thawed in a thermomixer at 37 °C for five minutes. Subsequently, they were vortexed for several seconds. First, tPA was diluted at 1:10 (10 µL tPA + 90 µL Tris-HCl). The mixture was mixed and 22 µL (0.1 mg/mL) was pipetted into the already prepared cryotube from the first step marked “tPA solution".

The thawed thrombin (100 NIH/mL) was diluted at 1:10 (50 µL thrombin + 450 µL Tris-HCl) to the final concentration of 10 NIH/mL. The diluted thrombin was mixed and added at 30 µL portions to both the "tPA solution" tube and the "tPA-free solution" tube. The two tubes were vortexed thoroughly and poured into two equally marked plastic trays.

Using a multichannel electronic pipette, 50 µL of the mixture from the "tPA-free solution" tube to the wells of columns 1, 3, and 5 on the microtiter plate and 50 µL of the mixture from the "tPA solution" tube into the wells of columns 2, 4, and 6. Thus, the final volume in the test well was 140 µL (20 µL phospholipids + 70 µL plasma sample + 50 µL reaction solution) with the final concentration of 0.04 NIH/mL of thrombin, 285 ng/mL of tPA, 35.7 µmol/mL of phospholipids, and 11.75 mmol/mL of $CaCl_2$.

The microtiter plate was then quickly inserted into a spectrophotometer, which began to measure it immediately. The originally described method was modified since we measured the absorbance more often (at 12 second intervals, a total of 302 measurement cycles) in the total time of 60 minutes. The whole measurement was performed at 405 nm and 37 °C.

The Magellan software read the absorbance values and created the graphical dependence of the absorbance values over time (see **Fig 5**). The results were exported from Magellan to Microsoft Excel, where the OHP, OCP, and OFP parameters were calculated based on the measured values. The coagulation time was recorded at 10 % increase of the baseline absorbance.

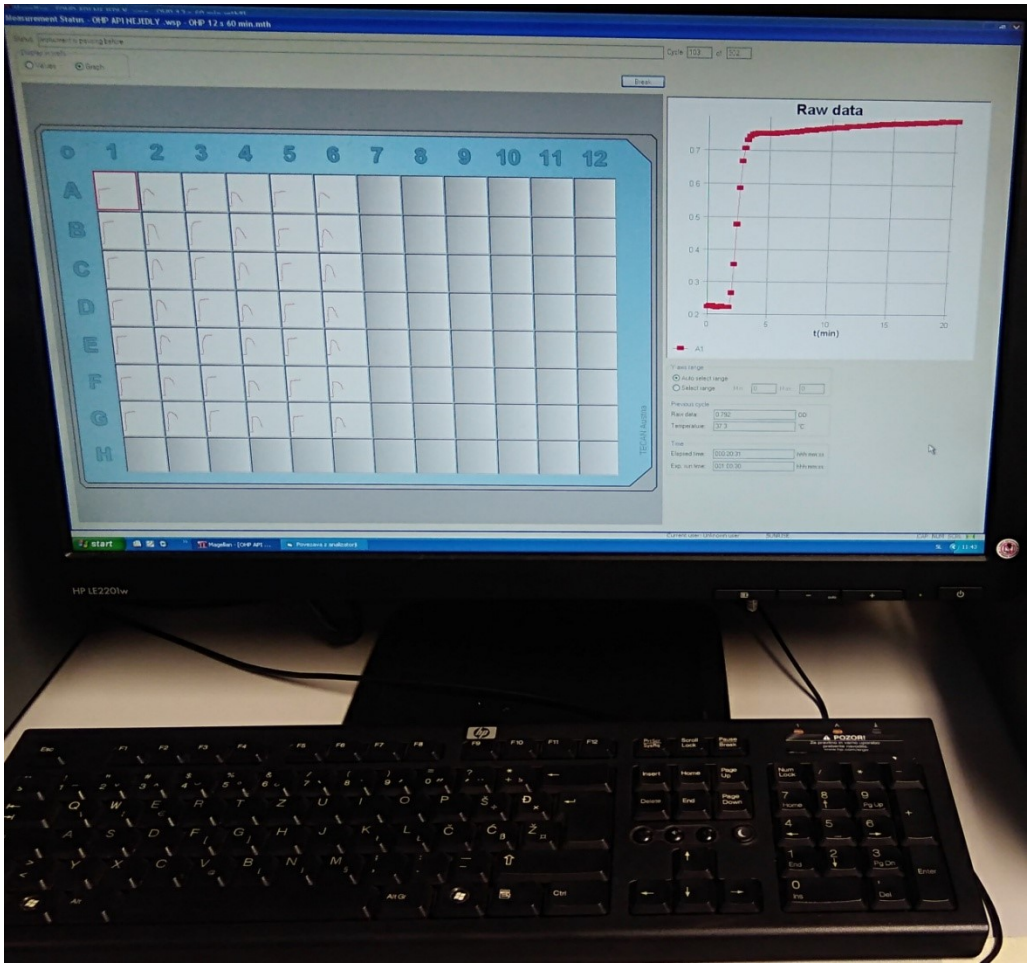

Fig 5 View of the process of measuring the absorbance of patient samples on a microtiter plate using the Magellan software

3. 9. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was used to measure apixaban plasma concentrations (Skeppholm et al., 2015). LC-MS/MS is commonly used for quantification of various drug concentrations. The advantage of the method is its high sensitivity and specificity. In our case, the method was applied to measure the concentration of apixaban in patients in whom OHP was subsequently measured. Apixaban by Selleck Chemicals, USA was used to make working solutions for quality control. Deuterated apixaban-d3 by Toronto Research Chemicals, Ontario, Canada was used as an internal standard (IS). Separation of the analytes was performed on an ACQUITY UPLC BEH C18 Column, 1.7 μm, 2.1 mm × 50 mm by Waters Corporation, USA. Electrospray ionization (ESI) was used as the interface between LC and MS, and a triple quadrupole (QQQ) was used for ion mass analysis.

3. 10. Prothrombin test (PT) and activated partial thromboplastin test (APTT)

PT and APTT, measured in patient samples included in the study, were performed on a CS-2500 automatic coagulation analyser (Sysmex, Japan). Thromborel® reagent (Siemens, Germany) was used for PT while Pathromtin® SL (Siemens, Germany) for APTT. The preparation and dilution of reagents was performed according to the manufacturer's instructions. PT value was expressed as relative, APTT in seconds.

3. 11. Statistical data processing

Data was processed using the statistical software Statistica version 12 (StatSoft, Czech Republic). Before the statistical evaluation itself, each individual patient sample was analysed, and on the basis of this measurement, a graph of absorbance versus time was created for each sample, both for parallel OHP measurements and for parallel OCP measurements. The coefficient of variation CV (in %) was calculated for each parallel measurement (see **Fig 6**). Values of OCP and OHP that exceeded 20 % were compared with the plotted curves and, based on a detailed evaluation, the most significant deviations were excluded.

$$CV = \frac{\mathrm{SD}}{\bar{x}} \times 100\ (\%)$$

Fig 6 Expression of coefficient of variation; SD = standard deviation, $\bar{x}$ = arithmetic mean

In the control samples (NPP), intra-assay, inter-assay, and total reproducibility were evaluated using the coefficient of variation. Inter-assay reproducibility was expressed as CV (%) of triplicate measurements of each NPP sample, while inter-assay reproducibility as CV (%) between the mean values of serial measurements.

The results of measuring patient samples were divided into two groups. The first group, labelled "trough", represented the values right before taking the next dose of apixaban. In the second group, labelled "peak" the values of the samples taken two hours after administration of the drug dose were pooled. The median, range between the first and third quartile, biological reproducibility and arithmetic mean were evaluated. Due to the relatively small data sample, nonparametric tests were applied, as they are not limited by the assumption of normal distribution of data and do not have such sensitivity to

extreme values. The aim was to avoid erroneous assumptions about data distribution during evaluation. The selected value of the significance level α was 0.05 (5 %); p-value < 0.05 was marked as statistically significant. The relationship between the measured parameters (OCP, OHP) was described by the Spearman correlation coefficient (r_s). The effect of the therapy on the OHP values was revealed by the ANOVA test.

4. RESULTS

4. 1. Assessment of OHP reproducibility

The assessment included 13 series NPP samples. Within each series, NPP sample was measured in six paralels and therefore three OHP values and three OCP values were obtained. The graphical representation of the OHP results of NPP measurement are shown in **Fig 7**, OCP in **Fig 8** and OFP in **Fig 9**. The median OHP in NPP was 67 ± 3 Abs - sum, OCP 163 ± 3 Abs - sum, OFP 58 ± 2 %.

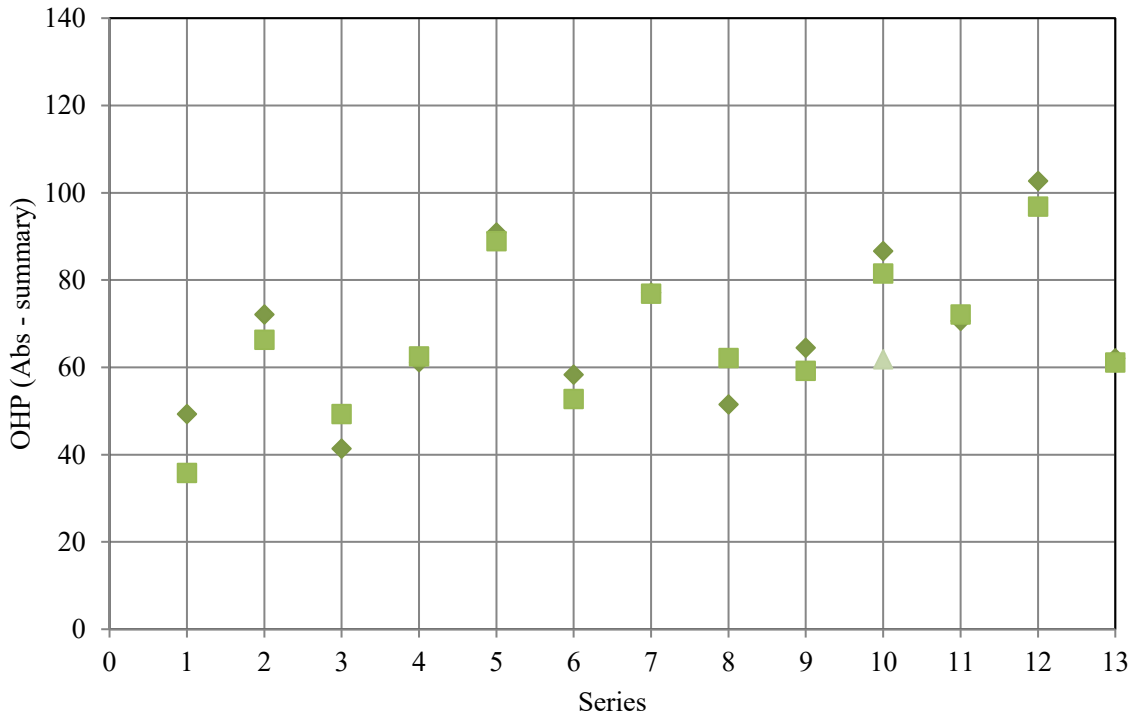

Fig 7 Graphical representation of OHP in NPP

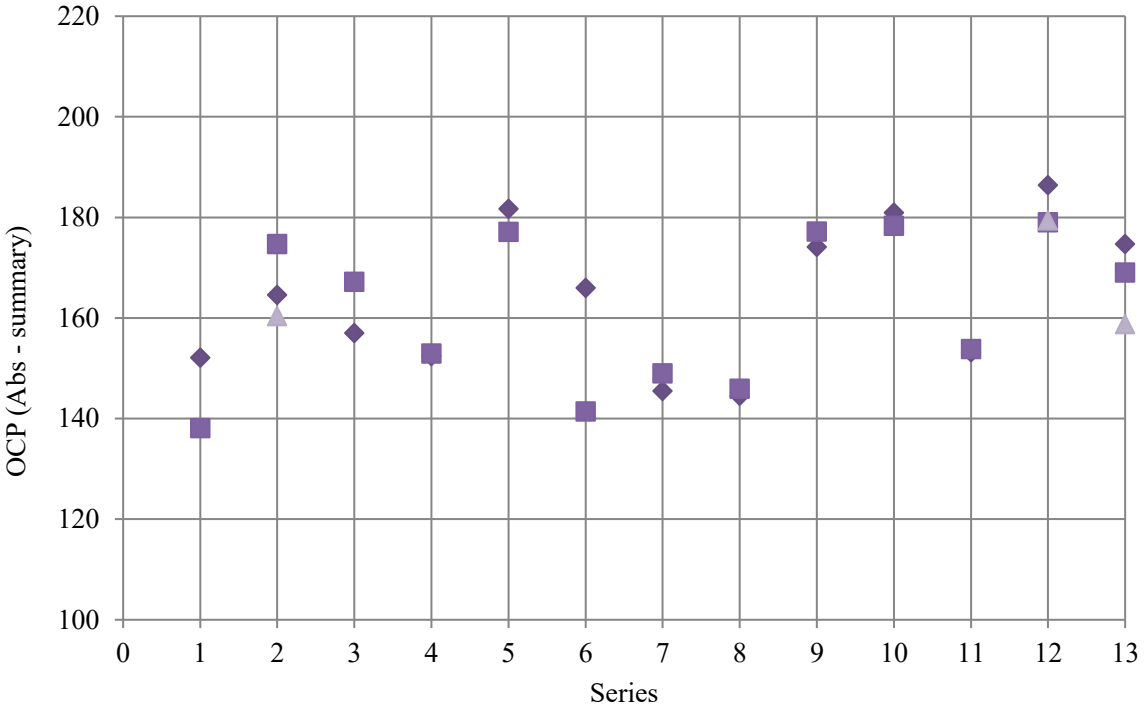

Fig 8 Graphical representation of OCP in NPP

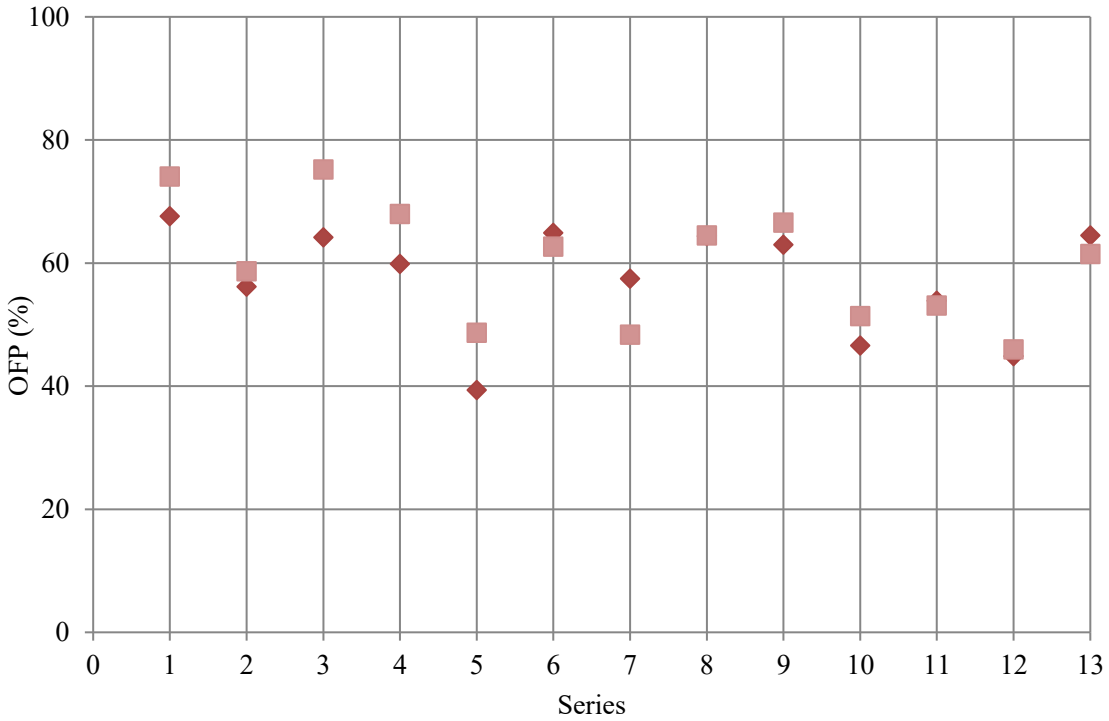

Fig 9 Graphical representation of OFP in NPP

Tab 1 shows the evaluation of intra-series reproducibility within a single assay, among series, and of the overall reproducibility. The coefficient of variation among the series was less than 15 % for OHP, OCP, and OFP.

Tab 1 Overview of reproducibility within the series, among the series, and within the whole for OHP, OCP, and OFP

	CV (%) INTRA-ASSAY	CV (%) INTER-ASSAY	TOTAL CV (%)
OHP	5.9	6.1	24.9
OCP	2.4	2.8	8.4
OFP	6.3	5.9	15.2

4. 2. Patients

The group of patients taking 5 mg of apixaban was compared with a group taking 2.5 mg of the preparation. The comparison focused on age, sex, body weight, glomerular filtration rate, creatinine concentration, and frequency of thrombotic events and bleeding. No statistically significant differences were found between the two groups.

Tab 2 Characteristics of patients treated with 5 mg of apixaban (A5) and with 2.5 mg of apixaban (A2.5), respectively.

	All	A5	A2.5	A5 to A2.5
	N = 13	N = 9	N = 4	p
Age x̄ ± SD	76 ± 8	73 ± 8	83 ± 3	0.07
Gender (w/m), N	6/7	2/7	0/4	0.85
Weight (kg), x̄ ± SD	76 ±18	85 ± 13	55 ± 7	0.19
Creatinin (µmol/L), x̄ ± SD	82 ± 19	88 ± 18	70 ± 17	0.5
oGF (mL/min), x̄ ± SD	72 ± 14	72 ± 13	73 ± 18	0.23
oGF > 50 mL/min, N (%)	11 (85)	8 (89)	3 (75)	0.39
oGF 30-50 mL/min, N (%)	2 (15)	1 (11)	1(25)	0
Thromboembolism, N (%)	0	0	0	0
Major bleeding, N (%)	0	0	0	0
Minor bleeding, N (%)	2 (15)	2 (22)	0	0

A5 = dose 5 mg, A2,5 = dose 2,5 mg, N = number of patiens, p = statistical significance, x̄ = arithmetic mean, SD = standard deviation, oGF (GFR) = glomerulal filtration rate calculated by Cockcroft-Gault equation

4. 3. Effect of apixaban on overall haemostatic potential

The mean apixaban concentration at the trough was 103 (80.7–129.3) µg/L and 232 (183–289) µg/L at the peak. At the trough, the biological reproducibility of apixaban concentration was lower than at the peak (18.5 ± 14.2 vs 24.2 ± 13.7). The biological reproducibility of OHP was comparable at the trough and peak time (18.9 ± 9.9 vs 20.8 ± 13.2), while in OCP it was better at the trough (8.6 ± 6.4) than at the peak (13.5 ± 9.9). The mean time to onset of coagulation was 10.8 ± 8.5 min. at the trough and 12.1 ± 9.2 min. at the peak (**Tab 2**).

The results of OHP measurements of patient samples and biological reproducibility of OHP are shown in **Tab 2**.

Tab 3 Biological reproducibility of apixaban concentration and coagulation assay PT, APTT and OHP

	Trough 1	**Trough 2**	**Trough 3**	**Average trough**	**Average CV (%)**	**Peak 1**	**Peak 2**	**Peak 3**	**Average peak**	**Average CV (%)**
Apixaban concentration (µg/L)	112 (96-123)	105 (73-127)	94 .5 (73-138)	102 .5 (80 .7-129 .3)	**18 .5 ± 14 .2**	260 (239-318)	207 .5 (156-247)	190 .5 (154-302)	232 .0 (183-289)	**24 .2 ± 13 .7**
PT (INR)	1 .0 (0 .98-1 .06)	1 .02 (0 .97-1 .06)	0 .99 (0 .95-1 .01)	1 .00 (0 .97-1 .04)	**2 .14 ± 1 .84**	1 .06 (1 .05-1 .13)	1 .07 (1 .02-1 .1)	1 .07 (1 .0-1 .08)	1 .06 (1 .02-1 .10)	**3 .09 ± 1 .63**
APTT (s)	36 (31 .5-28)	34 (31 .4-36 .7)	32 .8 (30 .5-36 .1)	34 .0 (31 .1-33 .6)	**3 .89 ± 2 .99**	38 .2 (34 .3-39 .8)	35 .6 (31 .7-39 .6)	35 .4 (33 .1-39 .4)	35 .4 (33 .0-39 .6)	**3 .07 ± 1 .49**
OCP (Abs - sum)	188 .6 (161 .7-239.6)	185 .3 (159 .5-213.9)	193 .4 (164 .4-204.4)	188 .3 (161 .9-219 .3)	**8 .6 ± 6 .4**	189 .1 (160 .6-229.8)	196 .9 (174 .6-207.4)	205 .6 (163 .8-212.6)	198 .4 (166 .3-216.6)	**13 .5 ± 9 .9**
Coagulation time (min)	4 .7	5 .4	5 .1	5 .1 ± 0 .3	**9 .2 ± 6 .7**	5 .5	5 .9	5 .0	5 .5 ± 0 .4	**17 .5 ± 9 .8**
OHP (Abs - sum)	62 .4 (53 .3-81 .1)	71 .8 (52 .6-85 .3)	59 .3 (44 .5-83 .9)	62 .1 (50 .1-83 .4)	**18 .9 ± 9 .9**	64 .5 (53 .0-81 .7)	54 .5 (40 .4-73 .6)	54 .1 (38 .3-65 .7)	56 .1 (43 .9-73 .7)	**20 .8 ± 13 .2**
OFP (%)	65 .6 (60 .6-72 .3)	62 .4 (58 .8-70 .5)	67 .8 (59 .3-72 .2)	65 .6 (59 .6-71 .7)	**9 .5 ± 8 .1**	66 .1 (58 .0-69 .4)	68 .9 (57 .8-73 .7)	73 .3 (63 .2-76 .3)	67 .5 (59 .7-73 .1)	**6 .2 ± 4**

Trough = time of blood collection immediately before the next dose of apixaban, peak = time of blood collection three hours after administration of apixaban,
CV = coefficient of variation,
PT = prothrombin time, APTT = activated partial thromboplastin time, Abs = absorbance

OHP values were lower during the peak time than at the trough, but this difference was not statistically significant (**Fig 10**). In OCP, the variance of values at both the peak and trough was approximately the same (**Fig 11**). OFP was significantly higher at the peak then at the trough (**Fig 12**).

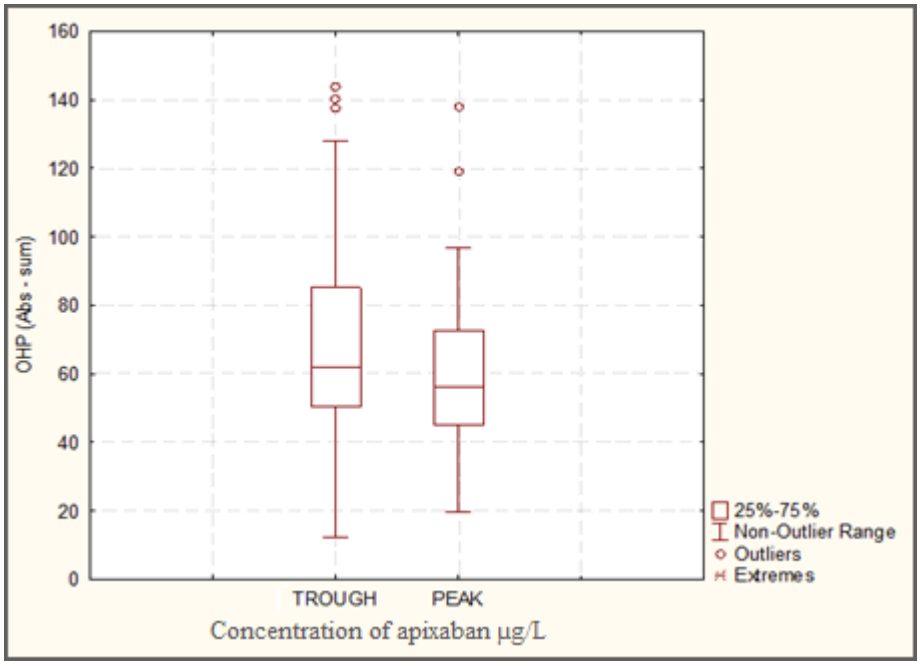

Fig 10 Dispersion of measured OHP values at the trough and at the peak

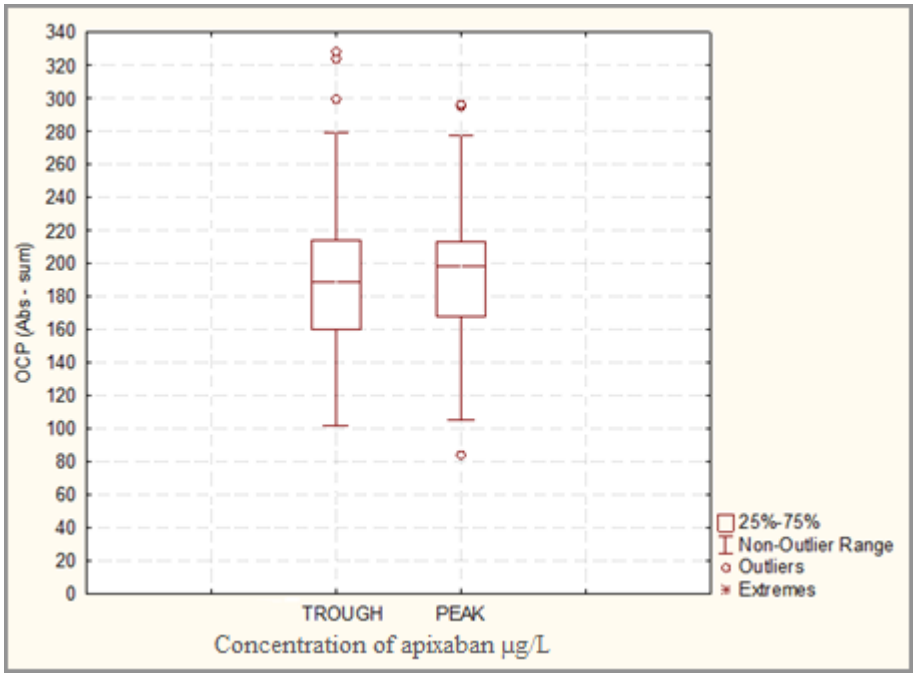

Fig 11 Dispersion of measured OCP values at the trough and at the peak

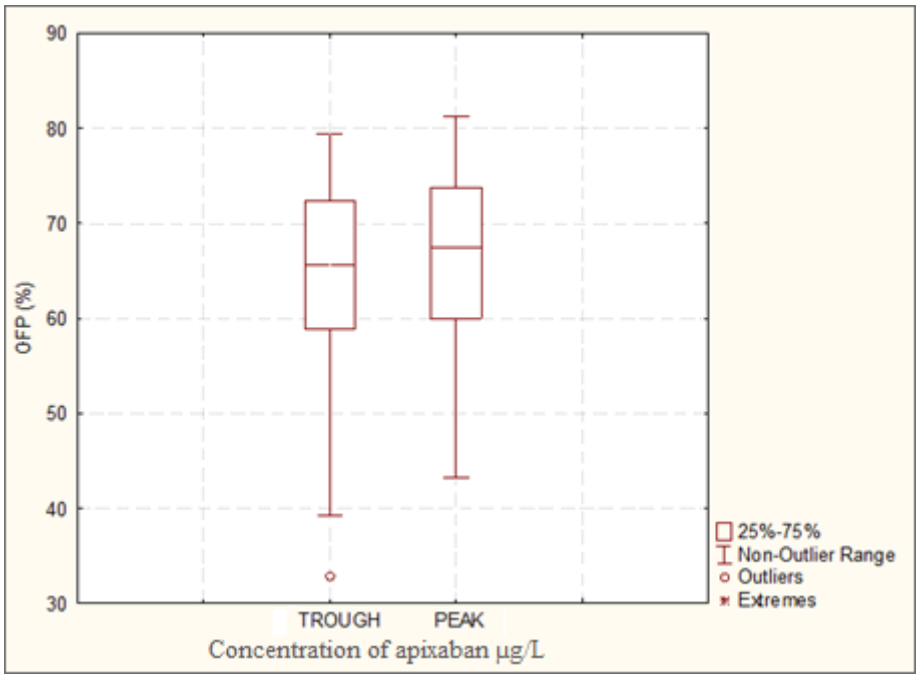

Fig 12 Dispersion of measured OFP values at the trough and at the peak

The weak correlation between apixaban and OCP concentrations was statistically insignificant, with a correlation coefficient of -0.1096 (p-value 0.3492) (**Fig 14**). The moderate correlation between apixaban and OHP concentrations was statistically significant, with a correlation coefficient of -0.4078 (p-value 0.0003) (**Fig 13**). The correlation between apixaban concentration and coagulation time was statistically significant, with a correlation coefficient of 0.2770 (p-value 0.0161). At higher concentrations of apixaban, the dispersion of the measured values was greater (**Fig 15**).

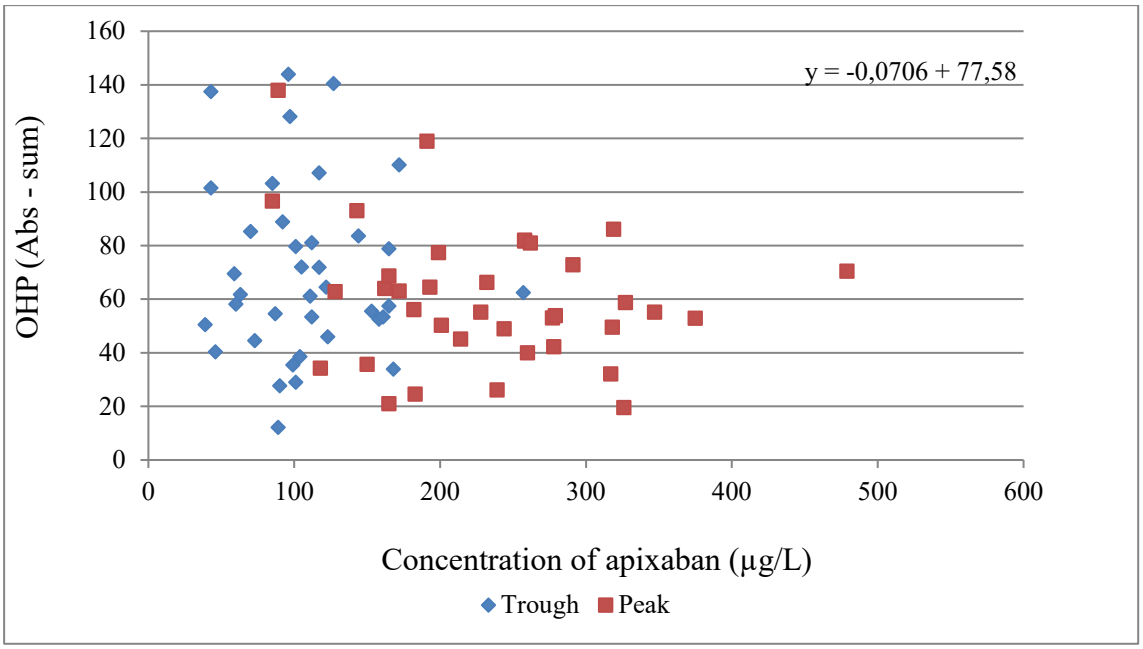

Fig 13 Correlation of OHP with apixaban concentration, the interpolation of points in the graph is expressed by the linear regression equation

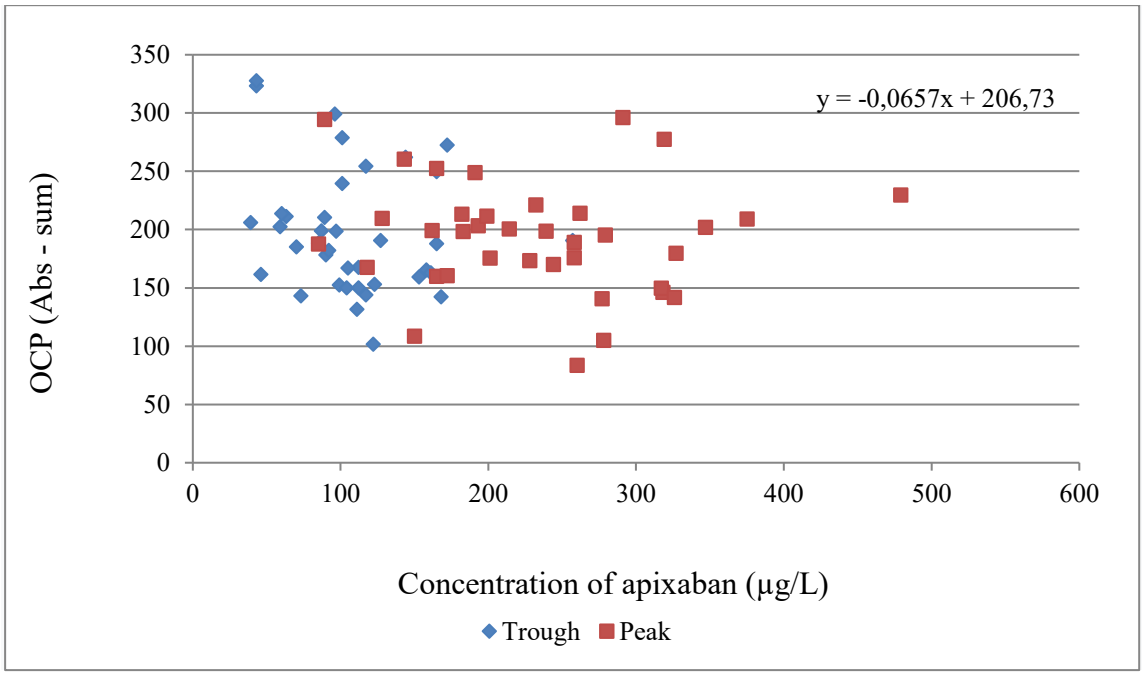

Fig 14 Correlation of OCP with apixaban concentration, the interpolation of points in the graph is expressed by the linear regression equation

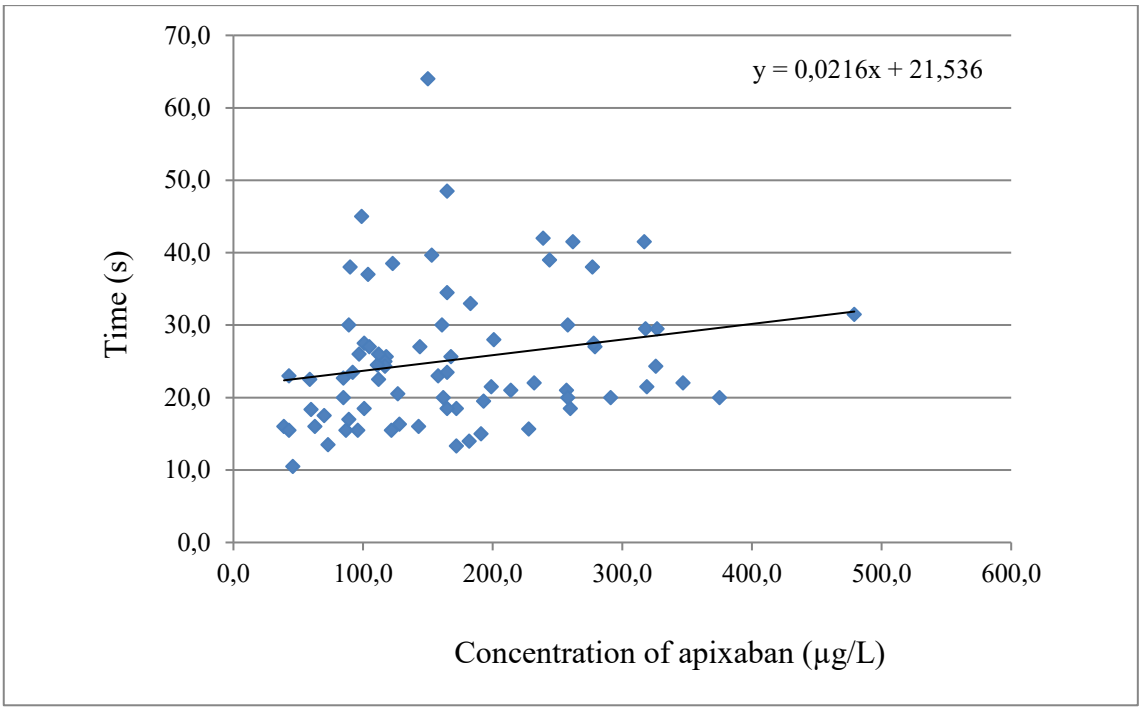

Fig 15 Correlation of coagulation time with apixaban concentration, the interpolation of points in the graph is expressed by the linear regression equation, r_s = 0,2770; p-value = 0,0161*

OCP, OHP, and OFP is shown by the number of patients in whom OCP, OHP, and OFP were above or below the median (**Tab 4**). The number of patients with OCP above the median was the same throughout the treatment, the number increased by one patient during the peak, however, the trend was statistically nonsignificant. In OCP below the median, the number of patients decreased nonsignificantly. In OHP, the number of patients with an OHP value above the median decreased during both the trough and the peak. Below the median, the number increased during both the trough and the peak. This trend was statistically nonsignificant.

Tab 4 Number of patients above and below the median OCP, OHP and OFP

		Trough 1	Trough 2	Trough 3	ANOVA p	Peak 1	Peak 2	Peak 3	ANOVA p
OCP (Abs - sum)	>191	6	6	6	SN	6	7	7	SN
	<191	7	7	6		7	5	5	
OHP (Abs - sum)	>61	8	7	6	SN	8	5	4	SN
	<61	5	6	6		5	7	8	
OFP (%)	>66	6	6	6	SN	7	7	9	SN
	<66	7	7	6		6	5	4	

p = statistic significance, SN = statistically nonsignificant

1.1 Effect of apixaban on standard screening coagulation assays

The correlation between apixaban concentration and PT (INR) was 0.5008 (p-value 0,000006). This correlation between the two variables was moderate. The relationship was statistically significant. At higher concentrations of apixaban, the measurement dispersion was greater (**Fig 16**).

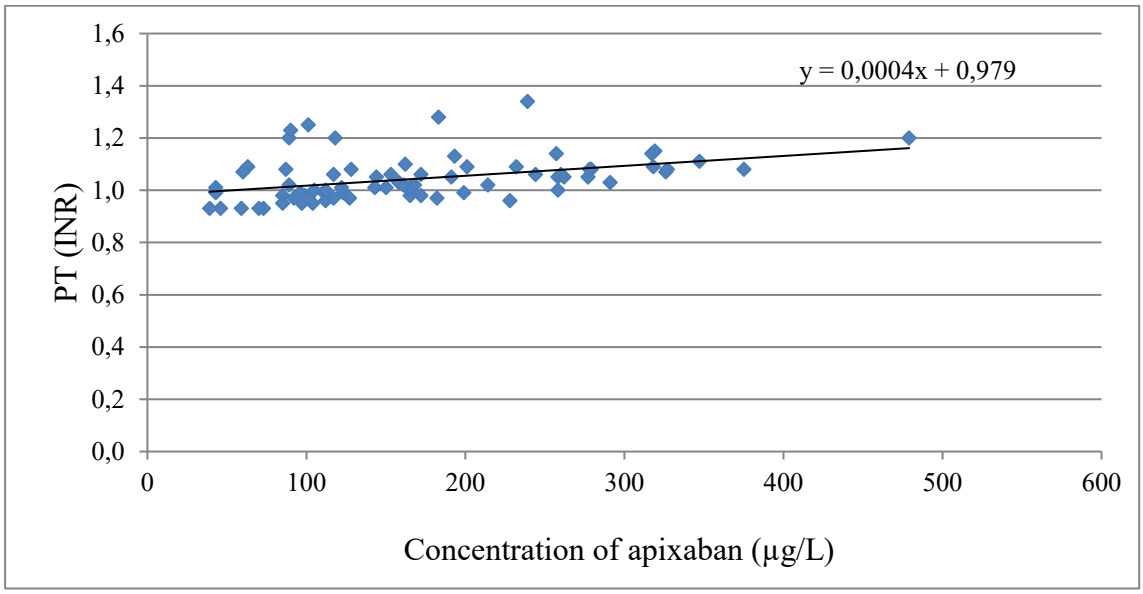

Fig 16 Correlation between PT (INR) and apixaban concentration, the interpolation of points in the graph is expressed by the linear regression equation

The correlation coefficient between apixaban concentration and APTT was 0.3597 (p-value 0.0012). The relationship was statistically significant. At higher concentrations of apixaban, the measurement dispersion was slightly greater (**Fig 17**).

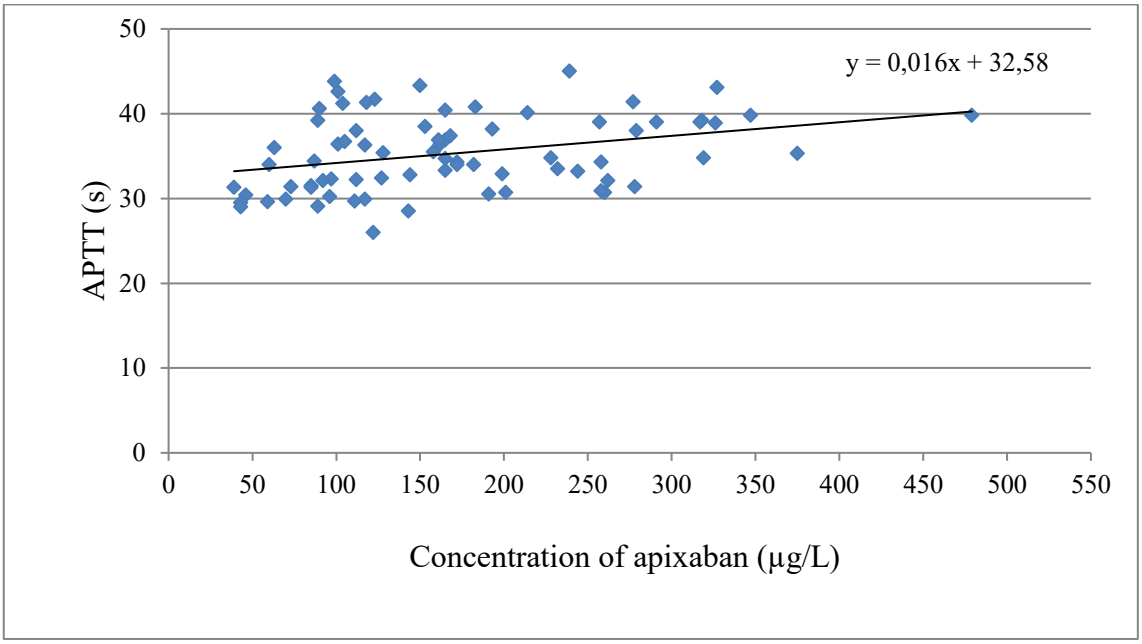

Fig 17 Correlation between APPT(s) and apixaban concentration, the interpolation of points in the graph is expressed by the linear regression equation

4. 4. Relationship between OHP and haemorrhagic events

Bleeding occurred in 2 out of 13 patients during treatment with apixaban. They were both men and each bled only once. The bleeding was not extensive and had no serious course. One patient had na occult bleeding, while the other had haematuria. None of the patients suffered a thromboembolic event. No statistically significant differences were found between bleeding and non-bleeding patients for PT, APTT, OCP, OHP, and OFP (**Tab 5**).

At peak, PT (INR) and APTT (s) values were slightly prolonged in both bleeding patients compared to the values at trough. All PT values, whether at peak or at trough, were within the reference range (0.8-1.2 INR). For APTT, two samples were above the upper limit of the reference range (upper reference limit APTT = 37.5 s) at trough, while, at the time of peak, the prolongation above the upper limit was measured for three samples out of six. Other results were within the normal range.

One of the bleeding patients had significantly higher concentrations of apixaban than the other bleeding patient at trough (approximately 50 %), while having the highest

value of apixaban concentration of all measurements (375 μg/L) at the peak time. The other patient (one with the occult bleeding) had the most prolonged APTT at peak (APTT = 43.1 s).

Tab 5 Apixaban concentration and coagulation assays in patients with and without bleeding expressed as medians with the first to third quartile

	TROUGH			**PEAK**		
	NO BLEEDING n = 11	**BLEEDING n = 2**	**p**	**NO BLEEDING n = 11**	**BLEEDING n = 2**	**p**
Concentration of apixaban (μg/L)	99 (71 .5 – 122 .5)	128 (105 – 165)	SN	221 (165 – 277 .5)	275 .5 (231 – 327)	SN
PT (INR)	1 (1 – 1 .1)	1 (1 – 1 .1)	SN	1 .1 (1 – 1 .1)	1 .1 (1 .1 – 1 .1)	SN
APTT (s)	34 (30 .3 – 37 .9)	35 .4 (33 .3 – 38)	SN	35 .9 (31 .8 – 39 .8)	36 .7 (34 .8 – 41 .7)	SN
OCP (Abs-sum)	187 .9 (152 .8 – 211 .1)	249 .2 (167 .4 – 267 .4)	SN	192 .3 (160 .3 – 212 .6)	215 .3 (194 .6 – 249 .4)	SN
OHP (Abs-sum)	61 .1 (45 .2 – 87 .1)	78 .8 (62 .6 – 96 .9)	SN	55 .2 (41 .1 – 75 .1)	62 .5 (55 .8 – 76 .1)	SN
OFP (%)	65 .6 (57 .1 – 74 .1)	68 .1 (60 .5 – 70 .2)	SN	66 .2 (57 – 74 .1)	72 .6 (70 .8 – 74 .1)	SN

p = statistic significance, SN = statistically nonsignificant

5. DISCUSSION

The use of apixaban as one of the representatives of DOACs has become increasingly important in clinical practice in recent years. Its pharmacokinetic profile is more favourable and predictable than in case of other anticoagulants, thus eliminating the need for routine monitoring of treatment efficacy. However, in certain circumstances, it is necessary to monitor the anticoagulant activity of apixaban to avoid sudden and adverse bleeding complications and to assess the current state of hemocoagulation, for example during urgent surgery. Assays for anti-FXa activity, originally calibrated to monitor the effect of low molecular weight heparins, can therefore be applied with fairly accurate accuracy to xaban measurements. However, there are efforts to develop a method that would be able to provide an overview of both coagulation and fibrinolysis and thus better predict the risk of possible haemorrhagic or thrombotic events. The assay should be simple, fast, cost-effective, and cover the entire coagulation process. The overall haemostatic potential could be one of these sought-after assays. The OHP method was first described in 1999 by He et al. in the article "A laboratory method for determination of overall haemostatic potential in plasma. I. Method design and preliminary results". However, no study evaluating OHP in patients treated with apixaban has been published.

First, the reproducibility of the OHP method was evaluated. The overall reproducibility was no longer so favourable, the value over 20 % exceeded the upper limit of acceptability. The overall reproducibility of OFP was slightly better, the value was around 15 %, which is acceptable. Intra- and inter-assay CV values for OFP were favourable, below 10 % (**Tab 1**). The reproducibility in the OHP and OCP interassay was comparable to the results of two other studies (He et al., 2001, Antovic, 2008).

Based on many studies measuring apixaban plasma concentrations using the LC-MS/MS reference method, it was confirmed that there is a significant linear correlation between the LC-MS/MS reference method and the anti-Xa chromogenic method. The LC-MS/MS method had much better selectivity than assays based on coagulation activity, which allows for the specific detection and quantification of DOAC (Jeong et al., 2019). In this way, apixaban in plasma can be quantified directly and very efficiently (Schmitz et al., 2014).

Peak and trough concentrations of apixaban were highly variable. The range of values was wide at both the peak and trough, with some values overlapping. Differences in values between medians at trough and medians at peak corresponded to current plasma drug concentrations. Reproducibility was better at trough than at peak (**Tab 3**). Not many studies have been published for apixaban to assess reproducibility during the peak and trough. The range from the minimum and maximum concentration values was greater, suggesting a significant difference between high and low doses of apixaban. This finding was confirmed by a study conducted by Skeppholm et al., which points to a statistically significant difference between the indicated doses of apixaban. On the contrary, it excludes the correlation between gender, creatinine clearance, body weight, or age, which was confirmed in this thesis. It also states that prescribing a lower dose of apixaban (2.5 mg) is recommended for patients with at least two of the following characteristics: age ≥ 80 years, body weight ≤ 60 kg, or creatinine serum ≥ 133 μmol (Skeppholm et al., 2015). These criteria were met by four patients in our cohort. However, further research and information on apixaban exposure and individualisation of treatment is still needed in order to minimize the risks of bleeding or thromboembolism.

The biological reproducibility of OHP was about 20 % with a relatively high CV (%). No statisticaly significant difference was found between trough and peak OHP. CVs for OCP and OFP were around 10 % with a percentage variation not exceeding 10 % at both peak and trough. The reproducibility of PT and APTT was much better than the reproducibility of OHP parameters, which was thanks to good standardization and automation of methods. OCP and OHP values did not correlate with apixaban concentrations, indicating low sensitivity of the OHP method to apixaban (**Tab 3**). Based on a study describing the OHP method as sensitive and able to reflect the haemostatic profile of a patient treated with dabigatran, we expected similar results (Antovic, 2010). Unfractionated heparin at low concentrations also had a significant effect on OHP compared to APTT (Božič-Mijovski et al., 2015), therefore the poor response of OHP to apixaban plasma concentrations was surprising. No other relevant studies evaluating the effects of anticoagulants on OHP were identified.

The coagulation time at trough was comparable to the peak, and there is no evidence that higher concentrations of apixaban prolong the time to clot formation (**Tab 3**). The same is true for PT and APTT, since the values do not differ significantly between

trough and peak. The relationship between apixaban concentration and coagulation time was not statistically significant.

In the next step, the relationships between the apixaban concentrations and screening coagulation times were assessed. The biological reproducibility was lower for APTT than for PT. No statistical difference in screening coagulation times was observed between trough and peak. The correlation of PT and APTT with apixaban concentration was not that strong, and PT correlated more significantly with apixaban (**Fig 16**, **Fig 17**). The correlation between apixaban concentration and PT (INR) was 0.5008. This correlation was moderate (**Fig 16**). The correlation coefficient between apixaban concentration and APTT was 0.3597 and this relationship was statistically significant (**Fig 17**). The sensitivity of PT and APTT to apixaban concentrations was insufficient. APTT and PT reagents are generally not sensitive enough to apixaban; PT may remain normal even at therapeutic plasma concentrations of apixaban (Dale et al., 2014). Therefore, PT is not recommended for estimating the plasma concentration of apixaban (Douxfils et al., 2015). There is more literature describing the poor correlation between PT, APTT, and apixaban plasma concentrations. A specific study of orthopaedic patients receiving 2.5 mg apixaban twice a day also showed a significant correlation and unreliability of the PT and APTT assays in measuring apixaban plasma concentrations compared to the anti-Xa method, which was evaluated as the most reliable clinical assay for xabans to date. (Freyburger et al., 2015). In our group of patients, PT was not significantly prolonged, and INR values were within the normal range. APTT values increased slightly in some cases, but in most cases they did not exceed the upper reference level. Recent research suggests that PT and APTT assays are not suitable for measuring therapeutic levels of apixaban (Patel et al., 2019).

Due to the wide discussion on the topic of bleeding in connection with the use of DOACs, we tried to evaluate the frequency and background of these complications. Unfortunately, due to the insufficient number of patients affected in our study, a comprehensive statistical analysis was not feasible. Of the total number of patients, only two were bleeding. There were no statistically significant differences for apixaban concentration, PT, APTT, OCP, OHP, and OFP in non-bleeding and bleeding subjects (**Tab 5**). DOAC rank among safer and more effective products in the overall range of anticoagulants. In connection with bleeding, we examined in more detail a selected large-scale meta-analysis focusing on the safety of DOAC compared to warfarin. Apixaban

together with dabigatran had a statistically lower incidence of any bleeding compared to warfarin (Nielsen et al., 2017). Currently, there is still a lack of a fixed therapeutic range for apixaban, and patient plasma concentrations may be highly variable and individual, therefore despite the generally declared safety, there is a risk of overdose and the associated occurrence of not only bleeding complications.

The OHP method was found to be insufficient to monitor apixaban treatment since no correlation with apixaban concentrations was observed. PT and APTT assays did not show a significant correlation with plasma concentrations either; on the contrary, their laboratory inapplicability was confirmed for apixaban. Further focused studies and analyses with a much larger sample of subjects are needed to increase the power of statistical comparison, and further optimization of the method should also be performed.

6. CONCLUSION

The objective of the diploma thesis was to evaluate the method of total haemostatic potential in ex vivo samples in patients treated with apixaban. We arrived at the following conclusions:

- Apixaban concentrations were highly variable between patients, both during trough and peak of plasma concentrations.

- OHP parameters are not related to apixaban concentration, therefore the OHP method is not suitable for the quantitative determination of apixaban concentration in plasma.

- OHP did not differ significantly between bleeding and non-bleeding patients, therefore the OHP method does not have a satisfactory predictive value for bleeding complications.

- APTT and especially PT have been shown to be insufficiently sensitive indicators of the anticoagulant effect of apixaban, therefore they should not be used as primary tests for anticoagulant activity.

Based on our results, we can conclude that the OHP method does not appear to be suitable for monitoring apixaban treatment based on non-correlated analysis results that are not reliably related to either apixaban concentrations or bleeding complications. The very fact that the OHP method has not been shown to be a sensitive indicator of the anticoagulant effect of the drug does not mean that the assay's principle is not suitable for evaluating coagulation and fibrinolysis of patients with a defect in haemostasis, as demonstrated by other studies. However, in the monitoring of apixaban treatment, it is also very important to define safe therapeutic limits and individualize doses for specific groups of patients different from the "normal" sample (overweight, underweight, old age, decreased oGF, etc.). Further comprehensive research must be conducted in order to identify ways to effectively monitor the treatment not only with apixaban but also with other DOACs.

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