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Impact of apixaban on routine and specific coagulation assays: a practical laboratory guide

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Summary

Apixaban does not require monitoring nor frequent dose adjustment. However, searching for the optimal dose for the individual patient may be useful in some situations. Moreover, there is a need for clinicians to know whether coagulation assays are influenced by apixaban use. The aim of this study was to determine which coagulation assay could be used to assess the impact of apixaban on haemostasis and provide good laboratory recommendations for the accurate interpretation of haemostasis assays. Apixaban is spiked at concentrations ranging from 5 to 500 ng/ml in platelet-poor plasma. Routinely used or more specific coagulation assays are tested. Results show a concentration dependent prolongation of aPTT, PT and dilute PT. The sensitivity mainly depends on the reagent, but none of these tests is sensitive enough to ensure an accurate estimation of the pharmacodynamic effect of apixaban. FXa chromogenic assays show high sensitivity and a linear correlation depending on the reagent and/or the methodology. Immunological assays and assays acting below the FXa are not influenced by apixaban. In conclusion, PT and/or dilute PT cannot be used to assess apixaban pharmacodynamic properties. More specific and sensitive assays such as chromogenic FXa assays using specific calibrators are required. In case of thrombophilia or in the exploration of a haemorrhagic event, immunological assays should be recommended, when applicable. Standardisation of the time between the last intake of apixaban and the sampling is mandatory.

Keywords

Apixaban, monitoring, coagulation assays, assessment, laboratory recommendations

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Introduction

Apixaban, a direct factor-Xa inhibitor authorised in Europe since May 2011 for the prevention of venous thromboembolism (VTE) in patients undergoing major orthopaedic surgery (1), has just received its approval from the European Commission (EC) and the Food and Drug Administration (FDA) for the prevention of stroke in patients with non-valvular atrial fibrillation (NVAF) (2, 3). Additionally, it is being evaluated in the reduction of recurrent ischaemic events when added to antiplatelet therapy after acute coronary syndrome (4) and in the (extended) treatment of VTE (5, 6).

For the prevention of thromboembolism in patients undergoing major orthopaedic surgery, the dose of apixaban is 5 mg once daily, i.e. two tablets of 2.5 mg (1), while for the prevention of stroke in patients with NVAF, apixaban is given as one tablet of 5 mg taken twice daily (1). However, in patients with NVAF and at least two of the following characteristics: age ≥ 80 years, body weight ≤ 60 kg, or serum creatinine, ≥ 1.5 mg/dl; the recommended dose of apixaban is 2.5 mg taken orally twice daily.

The biological monitoring of apixaban is not recommended since pharmacokinetic and pharmacodynamic properties are predictable. However, some situations may require an assessment of apixaban anticoagulant activity. These include recurrent thrombosis, bleedings, urgent surgery, estimation of patient's compliance and bridging with other anticoagulants. In addition, some pharmacological interactions (P-gp and CYP3A4/5) (7) and underlying physiopathological states (moderate or severe renal impairment and mild or moderate hepatic impairment) may importantly influence the pharmacokinetic profile of apixaban, although no relevant data on drug levels associated with approximate therapeutic and harmful ranges are currently available (8). Finally, monitoring may also be useful in extreme body weight or to know if and when an invasive surgery may be safely performed since a standardisation of the delay between the last intake of the drug and the sur-

gery is subject to debate in the literature for these new oral anticoagulants (9, 10).

To our knowledge, this is the first extensive study that discusses how to manage and interpret haemostasis assays from patients treated with apixaban. The aim of the present study was to assess and compare the sensitivity, specificity and reproducibility of routinely used and more specific coagulation assays to measure the pharmacodynamic effects of apixaban and estimations of its plasma concentration. We also aimed at providing good laboratory recommendation for the accurate interpretation of haemostasis laboratory assays that may be influenced in patients treated with Eliquis® (apixaban Pfizer, New York, NY, USA).

Materials and methods

Apixaban was spiked at increasing concentrations in pooled citrated normal human platelet-poor plasma (PPP) to assess its impact of apixaban on a large series of haemostasis assays (►Table 1).

Testing solutions of apixaban

Apixaban was extracted from commercially available Eliquis® tablet obtained from the clinical pharmacy at the University Hospital of Mont-Godinne (Yvoir, Belgium). A stock solution at 1.0 mg/ml

Table 1: Overview of the different assays performed in this study. †Thromboplastin reagents are diluted with $CaCl_2$ 25 mM ($CaCl_2$ 20 mM for RecombiPlasTin 2G®) to obtain an initial clotting time of approximately 30 sec. The dilutions are expressed as [one part of reagent/ x parts of $CaCl_2$ solution].

Coagulation assay	Reagent	Manufacturer	Method	Coagulation Analyzer (Manufacturer)
Prothrombin time	Triniclot PT Excel S®	Trinity Biotech, Bray, Ireland	Chronometric	STA-R Evolution® (Diagnostica Stago)
	Triniclot PT Excel®			
	Triniclot PT HTF®			
	Neoplastin R®	Diagnostica Stago, Asnieres, France		
	Neoplastin CI+®			
	Innovin®	Siemens Healthcare Diagnostics, Deerfield, IL, USA		
	RecombiPlasTin 2G®	Instrumentation Laboratory, Lexington, KY, USA		ACL-TOP® (Instrumentation Laboratory)
Dilute prothrombin time†	Triniclot PT Excel S® 1/60	Trinity Biotech, Bray, Ireland	Chronometric	STA-R Evolution® (Diagnostica Stago)
	Neoplastin R® 1/256	Diagnostica Stago, Asnieres, France		
	Neoplastin CI+® 1/128			
	Innovin® 1/100	Siemens Healthcare Diagnostics, Deerfield, IL, USA		
	RecombiPlasTin 2G® 1/64	Instrumentation Laboratory, Lexington, KY, USA		ACL-TOP® (Instrumentation Laboratory)
Activated partial thromboplastin Time	STA-CKPrest®	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
	STA-Cephascreen®			
	Actin FS®	Siemens Healthcare Diagnostics, Deerfield, IL, USA		
	SynthASil®	Instrumentation Laboratory, Lexington, KY, USA		ACL-TOP® (Instrumentation Laboratory)
Thrombin time	STA-Thrombin®	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
Reptilase time	STA®-Reptilase	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
Lupus anticoagulant	STA®-DRVV Screen	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
	STA®-DRVV Confirm			
	STA®-Staclot LA			
	PTT-LA®			
Protein C	Hemosil® Protein C	Instrumentation Laboratory, Lexington, KY, USA	Chromogenic	ACL-TOP® (Instrumentation Laboratory)

Table 1: Continued

Coagulation assay	Reagent	Manufacturer	Method	Coagulation Analyzer (Manufacturer)
Protein S	STA®-Staclot® Protein S	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
Free protein S antigen	Hemosil® Free Protein S	Instrumentation Laboratory, Lexington, KY, USA	Immunoturbidimetric	ACL-TOP® (Instrumentation Laboratory)
Activated protein C resistance	Hemosil® Factor V Leiden	Instrumentation Laboratory, Lexington, KY, USA	Chronometric	ACL-TOP® (Instrumentation Laboratory)
Fibrinogen Clauss method	STA® Fibrinogen	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
Fibrinogen PT-derived	RecombiPlasTin 2G®	Instrumentation Laboratory, Lexington, KY, USA	Chronometric	ACL-TOP® (Instrumentation Laboratory)
Antithrombin	STA®-Stachrom® ATIII	Diagnostica Stago, Asnieres, France	Chromogenic	STA-R Evolution® (Diagnostica Stago)
	Hemosil® Liquid antithrombin	Instrumentation Laboratory, Lexington, KY, USA		ACL-TOP® (Instrumentation Laboratory)
Extrinsic clotting factors	STA-CKPrest®	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
Intrinsic clotting factors	Innovin®	Siemens Healthcare Diagnostics, Deerfield, IL, USA	Chronometric	STA-R Evolution® (Diagnostica Stago)
Ecarin Clotting Time	Ecarin	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution® (Diagnostica Stago)
Anti-Xa activity	Biophen Direct FXa Inhibitor®	Hyphen Biomed, Neuville-sur-Oise, France	Chromogenic	STA-R Evolution® (Diagnostica Stago)
	Biophen Heparin®			
	Biophen Heparin LRT®			ACL-TOP® (Instrumentation Laboratory)
	Liquid Anti-Xa®	Diagnostica Stago, Asnieres, France		STA-R Evolution® (Diagnostica Stago)
	Stachrom Heparin®			
	Rotachrom Heparin®			
	Liquid Heparin®	Instrumentation Laboratory, Lexington, KY, USA		ACL-TOP® (Instrumentation Laboratory)
Thrombin Generation Assay	PPP-Reagent Low	Thrombinoscope BV, Maastricht, The Nederlands	Chromogenic	Calibrated Automated Thrombogram Analyser® (Thrombinoscope BV)
	PPP-Reagent			
	PPP-Reagent High			

†Thromboplastin reagents are diluted with $CaCl_2$ 25mM ($CaCl_2$ 20 mM for RecombiPlasTin 2G®) to obtain an initial clotting time of approximately 30 sec. The dilutions are expressed as [one part of reagent/ x parts of $CaCl_2$ solution].

in DMSO was obtained and intermediate solutions at 50 ng/ml, 100 ng/ml, 200 ng/ml, 500 ng/ml, 1000 ng/ml, 2000 ng/ml and 5000 ng/ml diluted in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} were prepared. Working solutions of 5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml and 500 ng/ml of apixaban were obtained by mixing these stock solutions with normal pooled plasma (NPP). The DMSO concentration in plasma was ≤ 0.05% (v/v) which did not influence the coagulation (11).

Preparation of platelet-poor plasma

Forty-two healthy individuals were included in the study. The exclusion criteria were thrombotic and/or haemorrhagic events, antiplatelet and/or anticoagulant medication, hormonal therapy, pregnancy and uptake of drugs potentially affecting the platelet and/or coagulation factor functions during the two weeks prior to the blood drawn. The study protocol is in accordance with the Declaration of Helsinki. Blood was taken by venipuncture in the antecubital vein and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Heverlee, Belgium) using a 21-gauge

needle (Terumo). PPP was obtained from the supernatant fraction of the blood tubes after a double centrifugation for 15 minutes (min) at 1500 g at room temperature. Immediately after centrifugation, PPP from the 42 donors was brought together to obtain the NPP which was frozen at – 80°C without any delay. Frozen NPP samples were thawed and heated to 37°C for 5 min just before the experiment.

Routinely used and specific coagulation assays

A summary of the tested reagents in this study is provided in ►Table 1. All procedures were performed according to the recommendation of the manufacturer. For dPT, thromboplastin reagents were diluted with $CaCl_2$ 25 mM to obtain an initial clotting time of approximately 30 seconds (sec). For all chromogenic anti-Xa assays, we asked the system to give results expressed in OD/min. For STA®-Liquid Anti-Xa we used the methodology provided by the manufacturer for the assessment of rivaroxaban and we asked the system to give results expressed in OD/min.

For the following anti-Xa chromogenic assays, an adapted method was proposed with the aim of increasing the dynamic range of quantitation.

For Biophen Heparin® (Hyphen Biomed, Neuville-sur-Oise, France) and Rotachrom Heparin® (Diagnostica Stago, Asnieres, France), 30 µl of spiked NPP diluted ¼ in Owren-Koller was mixed with 150 µl of chromogenic substrate and incubated 240 sec at 37°C. Then, the addition of 150 µl of bovine FXa started the measurement on STA-R®.

For Biophen Heparin LRT® (Hyphen Biomed), 50 µl of spiked NPP diluted 1/10 in physiological saline was mixed with 125 µl of chromogenic substrate (SXa-11) and incubated 240 sec at 37°C. Then, 125 µl of bovine factor Xa pre-warmed at 37°C was added, starting the measurement on STA-R Evolution®.

For Liquid Heparin® (Instrumentation Laboratory, Lexington, KY, USA) 10 µl of spiked NPP was mixed with 100 µl of chromogenic substrate and incubated for 180 sec at 37°C. Thereafter, 75 µl of bovine FXa are added, starting the measurement on ACL-TOP®.

The calibrated automated thrombogram (CAT) measurement is performed according to previously reported procedures (12, 13).

Statistical analysis

Sensitivity and reproducibility of the different assays are compared using GraphPad Prism 5.01® for Windows®.

Sensitivity of the different chronometric assays is defined as the final concentration in apixaban needed to double the clotting time (2xCT [CT = Clotting Time]). For chromogenic assays, the sensitivity is defined as the final concentration in apixaban needed to halve the analytical parameter (½xOD/min [The concentration needed to halve the change in the optical densitometry reported by minute]). For the CAT, the sensitivity of the different parameters in defined as follow: C_{max} IC_{50} [The final apixaban concentration reducing the C_{max} of 50%]; Peak IC_{50} [The final apixaban concentration reducing the Peak of 50%]; mVRI IC_{50} [The final apixaban concentration reducing the mVRI of 50%]; 2xLT [The final apixaban concentration needed to double the lag time (LT)]; 2xTTP (The final apixaban concentration needed to double the Time to Peak (TTP)).

Reproducibility expressed by CV (coefficient of variation = [(standard deviation/mean)*100]) of the triplicate for each concentration and each test is determined.

The lower limit of quantitation is calculated as follow: [(10*standard deviation of Y0)/ slope] where Y0 is the baseline value of the linear regression. The upper limit of quantitation reflects the concentration from which results are unreliable (concentrations above 500 ng/ml have not been tested and are stated as the upper limit of quantitation when applicable). For aPTT, dPT, PT and chromogenic anti-Xa assays, the dynamic range presented is the mean of the individual lower and upper limit of quantitation of the different reagents.

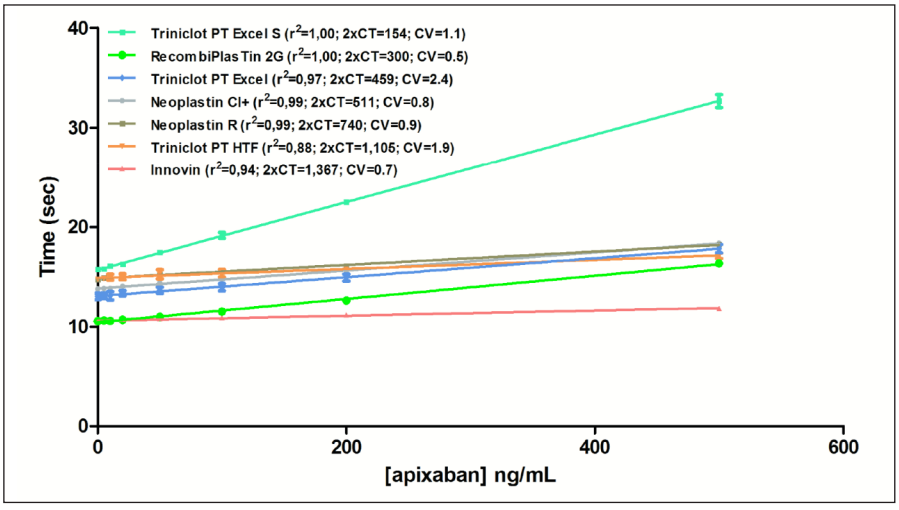


Figure 1: Impact of apixaban on prothrombin time (PT). Apixaban prolonged PT concentration-dependently. The relation was linear, and the sensitivity depended on the reagent. The sensitivity ranged from 154 ng/ml for Triniclot PT Excel S® to 1364 ng/ml for Innovin®. Nevertheless, the poor sensitivity reflects the inaccuracy of PT for the measurement of apixaban. (r^2: correlation coefficient; 2xCT: 2x clotting time (sensitivity) expressed in ng/ml; CV: coefficient of variation expressed in percentage [%]).

Results

Prothrombin time (PT)

A concentration-dependent prolongation of PT is found. The sensitivity depends on the reagent and is ranging from 154 ng/ml (Triniclot PT Excel S®) to 1354 ng/ml (Innovin®) (►Figure 1). Prothrombin time may be normal (ratio <1.2) with therapeutic concentration of apixaban, except with Triniclot PT Excel S®. The reproducibility ranges from 0.7% (Innovin®) to 2.4% (Triniclot PT Excel®).

Diluted Prothrombin Time (dPT)

A concentration dependent prolongation of dPT is shown. The sensitivity also depends on the reagent (see Suppl. Figure 1, available online at www.thrombosis-online.com). Triniclot PT Excel S® is the most sensitive reagent (2xCT = 613 ng/ml) and Innovin® the least sensitive one (2xCT = 1300 ng/ml). The reproducibility is calculated from 0.7% (Triniclot PT Excel S®) to 6.3% (Neoplastin CI+®).

Activated partial thromboplastin time (aPTT)

aPTT shows a concentration dependent prolongation of clotting time followed by a plateau at 200 ng/ml (see Suppl. Figure 2, available online at www.thrombosis-online.com). The 2xCT in the ascending phase ranges from 45 ng/ml (Synthasil®) to 112 ng/ml (Cephascreen®).

Thrombin time, ecarin clotting time and reptilase time

Both of these tests are not influenced by apixaban.

Determination of lupus anticoagulant

The PTT-LA® and the STA-Staclot-LA® are both prolonged in a concentration dependent manner in presence of apixaban (see Suppl. Figure 3, available online at www.thrombosis-online.com). Staclot®-DRVV Screen and Confirm are also prolonged dose dependently (see Suppl. Figure 4, available online at www.thrombosis-online.com). For PTT-LA® and STA-Staclot-LA® (with buffer or phospholipids) the relation is exponential and 2xCT is 52 ng/ml, 23 ng/ml and 32 ng/ml, respectively. For STA-Staclot®-DRVV Screen and Confirm the relation is linear, and the 2xCT is 205 ng/ml and 230 ng/ml, respectively.

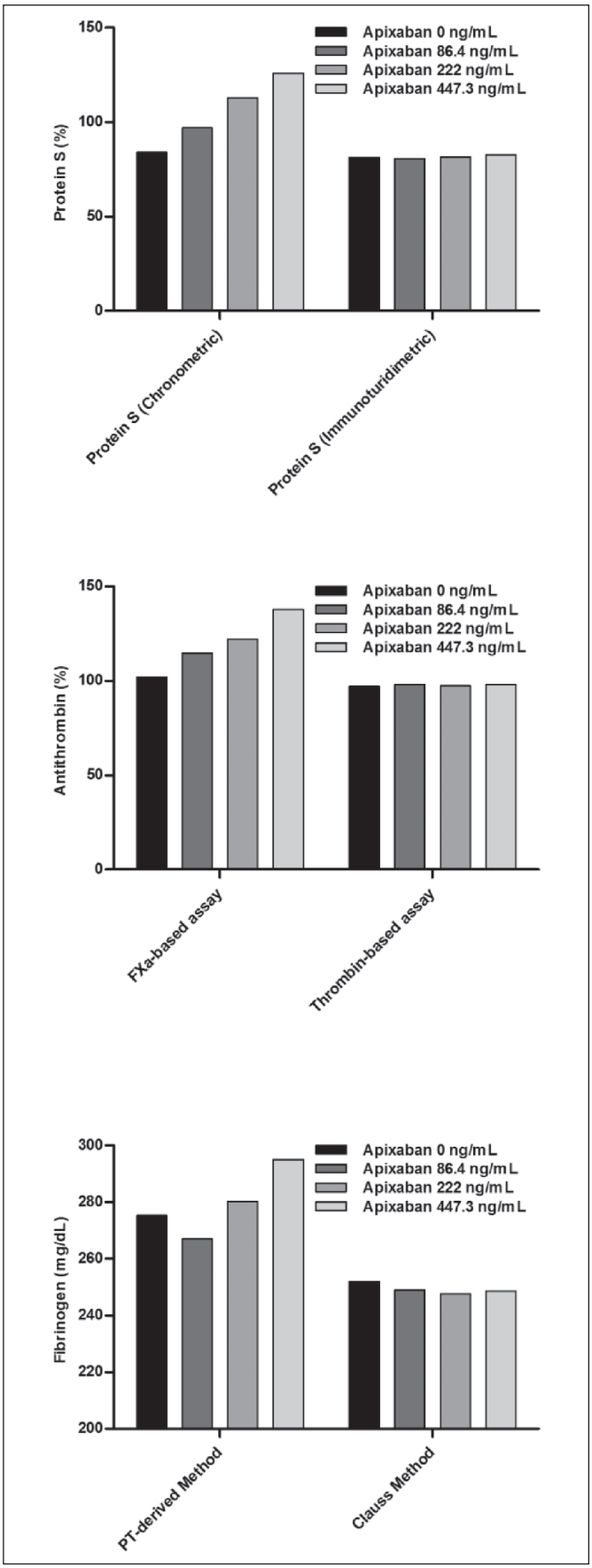
Activated protein C resistance, antithrombin measurement, fibrinogen measurement (Clauss and PT-derived methods), clotting factor measurement, protein-C and protein-S measurement

The influence of apixaban on these tests is summarised in ►Table 3. ►Figure 2 and Suppl. Figure 5 (available online at www.thrombosis-online.com) summarise the impact of apixaban on protein S, antithrombin and fibrinogen measurement and on clotting factor activities measurement, respectively. For factors of the intrinsic pathway (FVIII, FIX, FXI, and FXII), the aPTT-based clotting method shows a mean decrease of approximately 12% per 100 ng/ml of apixaban. However, the relation is not linear, and for higher

Table 2: Summary of assays for the monitoring of apixaban in plasma. aPTT: activated partial thromboplastin time; dPT: dilute prothrombin time; DRVVT: dilute Russell Viper Venom time; ECT: ecarin clotting time; PT: prothrombin time. * 205 ng/ml and 230 ng/ml for Staclot®-DRVV Screen and Confirm, respectively.

	Useful for monitoring	Reliable but requires laboratory experience			Not recommended						
	Chromogenic anti-Xa assays (linear)	Chromogenic anti-Xa assays (not linear)	mPT	TGA	PT	dPT	aPTT	TT	ECT	RT	DRVVT
Sensitivity (ng/ml)	7 to 20	2 to 9	N.A	Peak: 17 to 60 mVRI: 18 to 45	154 to 1,367	613 to 1,300	45 to 112	Insensitive			205 to 230*
Dynamic range of quantitation (ng/ml)	48 to 500	46 to 200	N.A	Peak: 22 to 500 mVRI: N.A	53 to 500	153 to 500	119 to 200	N.A			38 to 500
Reproducibility (%)	1.4 to 2.9	0.9 to 9.8	N.A		0.5 to 2.4	0.8 to 6.3	0.5 to 2.2	N.A			1.8
Dependence of reagent	Yes	Yes	Further investigation required	Yes	Yes	Yes	Yes	No			Yes
Linearity of the response	Yes	No	Yes	No	Yes	Yes	No	N.A			Yes

* 205 ng/ml and 230 ng/ml for Staclot®-DRVV Screen and Confirm, respectively.



concentration of apixaban (447 ng/ml), the maximal decrease is 48%. For FV, FVII and FX, a maximal decrease of 11%, 6% and 15% is noted (at apixaban concentration of 447 ng/ml), while thrombin measurement does not seem to be affected.

Chromogenic anti-Xa assays

A concentration-dependent decrease in OD/min is shown. Depending on the reagent, the reaction is fitted by a linear or an exponential model (►Figure 3A, B). The sensitivity (½xOD/min) ranges from 2 ng/ml (Stachrom Heparin®) to 20 ng/ml (Biophen Heparin: adapted method). The reproducibility ranges from 0.9% (Liquid Heparin®) to 9.8% (Biophen Heparin®).

Thrombin generation assay (TGA)

The most influenced parameters are the peak and the mVRI, while the lag time, the time to peak and the ETP are less affected as shown in ►Figure 4. The sensitivity towards the lag time and the time to peak depend on the reagent. The sensitivity towards the time to peak increases with the amount of tissue factor (TF) in the reagent.

Discussion

Routinely used and specific coagulation assays: Advantages and drawbacks

Prothrombin time is broadly used to evaluate the impact of vitamin K antagonist (VKA) therapy. As shown in previous studies, the International Normalised Ratio (INR) cannot be used to reflect the anticoagulation effect of FXa inhibitors since the International Sensitivity Index (ISI) has been specifically developed for VKA therapy (ISI_{VKA}) and does not reflect the sensitivity towards direct FXa inhibitors (14, 15). Previous studies have shown that the 2xCT was 3.6 µM (± 1654 ng/ml) with Thromboplastin C+® in *in vitro* studies on human plasma (16). Another study evaluating the sensitivity of PT reagents towards apixaban showed a 2xCT of 538 ng/ml, 991 ng/ml, 1467 ng/ml and 4750 ng/ml for RecombiPlasTin®, Neoplastin CI+®, Neoplastin CI® and Innovin®, respectively (17). In these studies, the concentrations required to double the clotting time were expressed as total plasma concentrations and not in final concentrations (after the addition of the reagent), as performed in this work. By calculating the sensitivity expressed in final concentration, the 2xCT was ± 179 ng/ml, 330 ng/ml, 489 ng/ml, 551 ng/ml, and 1586 ng/ml for RecombiPlasTin®, Neoplastin CI+®, Neoplastin CI®, Thromboplastin C+ and Innovin®, respectively. These results are consistent with our data (►Figure 1). So, PT is not sen-

Figure 2: Impact of apixaban on protein S, antithrombin and fibrinogen measurement. Assays that involve FXa or upstream coagulation factors are more likely to be influenced by apixaban than immunological or thrombin-based assays.

sitive enough to estimate apixaban plasma concentrations and, depending on the reagent, may be normal with therapeutic concentration of the drug. This inter-reagent variability prevents valid recommendations of cut-offs in seconds associated with a bleeding risk applicable to all reagents. In addition, drugs or haematologic abnormalities affecting at least one factor assessed by PT could interfere and bias the conclusions. We definitively do not recommend PT to estimate plasma concentration of apixaban.

As suggested previously, **dPT** may be used to increase the sensitivity of thromboplastin reagent by mimicking physiological conditions (14). In our study, dPT proved to be similar to PT without any improvement in terms of sensitivity (►Table 2 and Suppl. Figure 1, available online at www.thrombosis-online.com). Thus, dPT is not recommended to estimate plasma concentration of apixaban.

Previous study has shown that a modified PT (mPT) which is performed by diluting 1.00 ml of Thromboplastin C+ ® with 1.25 ml of 100 mM $CaCl_2$ (4 times more concentrated in $CaCl_2$ than a traditional PT) could improve the sensitivity by 5.4-fold (18). The 2xCT was 0.37 µM (± 170 ng/ml) in total plasma concentrations, resulting in a 2xCT of ± 57 ng/ml in final concentration. Thus, mPT method could be used for the assessment of the pharmacodynamic activity, but the limitations highlighted previously for PT might remain valid. However, for research purposes, the variability of the mPT assay is reasonable (<10 %CV for inter-assay precision). With further development and standardisation, this assay could provide a potential option (18).

A concentration-dependent prolongation of **aPTT** is shown in presence of increasing doses of apixaban. This is in line with a previous study (16). The 2xCT is ranging from 45 to 112 ng/ml, but the relation is curvilinear showing a plateau at 200 ng/ml limiting

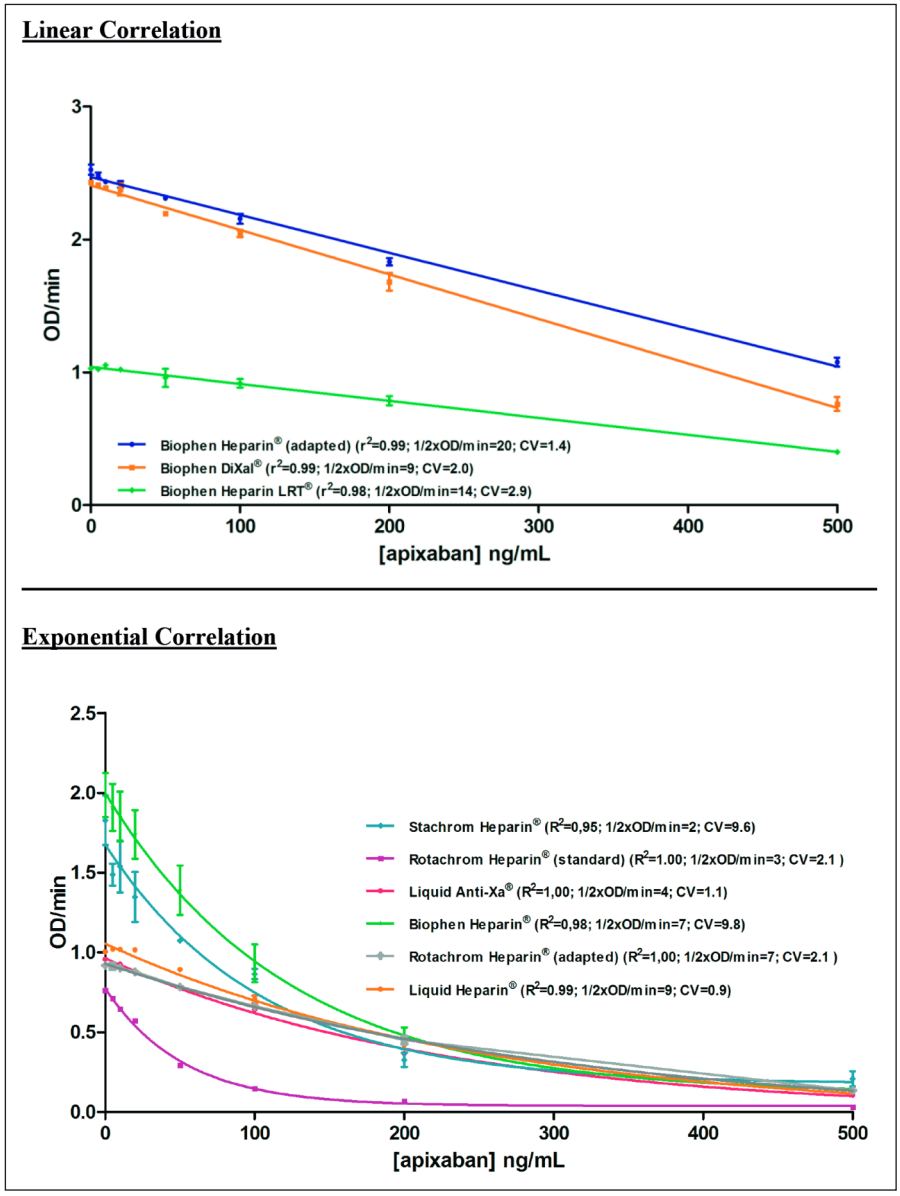
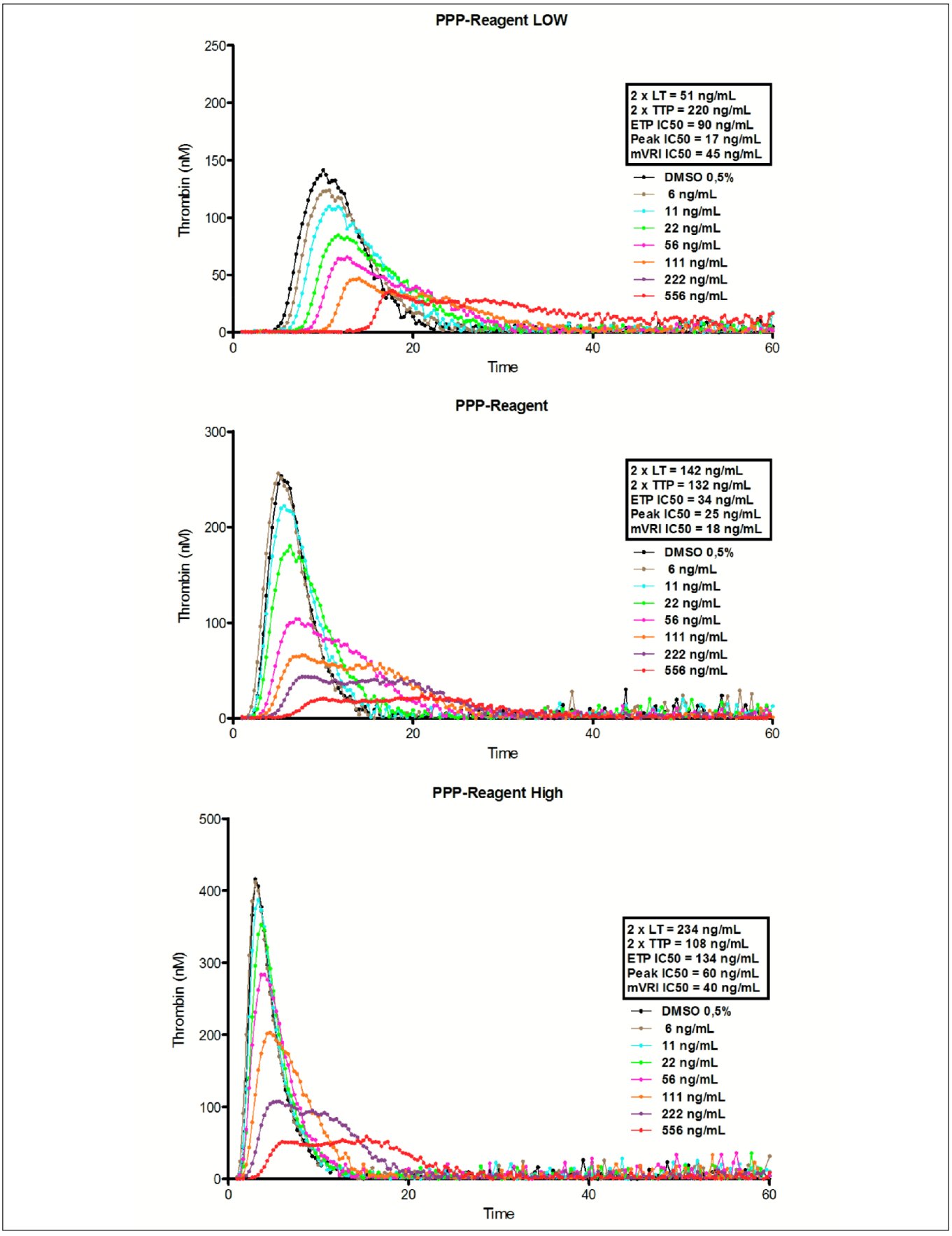


Figure 3: Impact of apixaban on chromogenic anti-Xa assays. There was a concentration-dependent decrease of the OD/min. The most sensitive reagents showed an exponential correlation but the dynamic range of quantitation was lower. Thus, linear correlation seemed to be favourable due to the wide dynamic range of quantitation and the relatively good sensitivity. (r^2: correlation coefficient; ½xOD/min: Halve in optical density by minute (sensitivity) expressed in ng/ml; CV: coefficient of variation expressed in percentage [%]).



the interest in aPTT as a parameter to estimate plasma concentration of apixaban.

The concentration-dependent prolongation of **DRVV-T** showed a linear correlation, but the sensitivity is not sufficient to ensure an accurate measurement of plasma drug concentration.

For a particular chronometric assay, the between-reagent variability could be explained by the differences in the composition of the reagent. The source of phospholipids (phosphatidylserine/ phosphatidylethanolamine/ phosphatidylcholine), their proportion in the reagent, as well as other factors such as the ionic force, the pH, the source of TF (for PT and dPT) or the activator (for aPTT) could be some explanations.

Chromogenic anti-Xa assays have already been described as accurate assays for the measurement of plasmatic drug level of direct FXa inhibitors (17, 19, 20). The different chromogenic assays available on the market used in this study showed linear or exponential relations depending on the reagent and the methodology applied (►Figure 3A, B). Even if a $\log_{10}$ transformation of the Y-axes may be performed to give a linear regression, anti-Xa assays which directly give a linear relation such as Biophen DiXaI®, Biophen LRT® and our adapted method of Biophen Heparin® should be preferred since there is no decrease of sensitivity for higher plasma concentration of apixaban. Nevertheless, the sensitivity of these two tests can be five-fold lower than with the more sensitive assay Stachrom® Heparin. In fact, the kinetic of the reaction is linear until the maximal competition between the inhibitor and the substrate. Therefore, there is a need to dilute the sample, or to increase the amount of FXa brought into the test, reducing the sensitivity. In this study, we aimed at adapting the methodology to increase the dynamic range of quantitation as clearly suggested by our adapted procedure of Rotachrom Heparin® and Biophen Heparin® (►Figure 3A, B). The addition of exogenous antithrombin has not been assessed in this study since the addition of exogenous antithrombin resulted in falsely high concentrations of rivaroxaban in a previous study (21). Compared to other chromogenic anti-Xa assays, Biophen DiXaI® is specific for direct FXa inhibitors such apixaban. Thanks to its Tris/EDTA/NaCl buffer at pH= 7.85, this assay is insensitive to the presence of antithrombin-dependent FXa inhibitors (22).

For chromogenic anti-Xa assays, the variability could be explained by the difference in chromogenic substrate as well as by the ratio between the substrate and the factor Xa brought into the test (23). The ionic force and the pH of the buffer solution could also be parameters that impact the sensitivity of a particular assay.

Figure 4: Impact of apixaban on Calibrated Automated Thrombogram®. The most influenced CAT® parameters are the peak and the mean velocity rate index. There is an inter-reagent variability. Thanks to their high sensitivity, the better resolution and their large range of application, PPP-Reagent and PPP-Reagent High seemed to be the best reagents to monitor patients on apixaban. (ETP: endogenous thrombin potential; IC_{50}: half-maximum inhibitory concentration; LT: lag time; mVRI: mean velocity rate index; TTP: time to peak). mVRI was defined as follow: (peak) / (time to peak – lag time).

What is known about this topic?

- Therapeutic monitoring is generally not necessary with novel oral anticoagulants (NOACs), but may be required in some clinical situations like recurrence of stroke and bleeding.
- No guideline/guidance documents are currently available on the assay to use to perform such a monitoring.
- Chromogenic assays are more accurate than the chronometric one's for the monitoring of apixaban.
- NOACs may interfere with routinely used coagulation assays which can lead to misdiagnosis.

What does this paper add?

- Our study provides a comparison of the impact of broad plasma concentrations of apixaban on specific and routinely used coagulation assays with a large panel of reagents.
- We recommend chromogenic anti-Xa assays for the monitoring of apixaban using appropriate calibrator and controls.
- We assessed the impact of apixaban on diagnostic tests and provided recommendations on how to deal with possible interferences.

The **calibrated automated thrombogram®** gives more information than traditional coagulation assays (13). By its mode of action, apixaban mainly acts on the amplification phase of the thrombin generation (►Figure 4) by affecting mostly the peak and the mean velocity rate index. The sensitivity towards the lag time and the time to peak depends on the reagent. So, the sensitivity towards the lag time decreases with the amount of TF in the reagent since all reagents tested in this study contain 4 μM of phospholipids. Inversely, the sensitivity towards the time to peak increases with the amount of TF in the reagent. With PPP-Reagent Low, the sample is not enough stimulated preventing accurate resolution in the profile (tailing). Thus, we recommend using PPP-Reagent or PPP-Reagent High to assess apixaban plasma samples with the CAT analyser.

Delay between the drug intake and the blood sampling

Another important point to consider is the delay between the last intake of the drug and the blood sampling since assays are influenced proportionally to apixaban concentration. C_{max} is reached within 1-3 hours depending of the results of different studies (24, 25). In opposition to other novel oral anticoagulants (NOACs), peak and trough plasma concentrations varied modestly by 15 to 20% (20).

Recommendation for an accurate monitoring of patients on apixaban

Pharmacokinetic studies revealed that apixaban plasma concentrations varied modestly between peak and trough and are mainly

comprised within the range of 100–300 ng/ml (20, 26). ▶Table 2 shows that PT is inappropriate to ensure an accurate quantitative measurement of apixaban. For the most sensitive reagents it may only inform the clinician if the patient is taking the drug. Consequently, chromogenic anti-Xa assays calibrated with specific apixaban calibrators should be performed, and the results should be compared to the plasma drug concentration obtained in pharmacokinetic studies. Another advantage of chromogenic anti-Xa assays is that they are less sensitive than PT to sample collection conditions and variations in the amounts of clotting factors among patients (17). Nevertheless, confirmation of the accuracy of the quantitative measurement of apixaban by chromogenic anti-Xa assays using specific calibrators and controls should be confronted to the reference HPLC-MS/MS measurement in patients treated by Eliquis®. In addition, chromogenic anti-Xa assays are not widely available, and their use may be difficult in emergency situations. The mPT could be a promising screening test (18), but further investigations are required to show whether the methodology could be applicable to different thromboplastin reagents. Finally, the TGA gives important information of the coagulation process, but the turnaround time, the lack of standardisation and the inter-individual variability are still limitations that restrict its use in clinical practise (27).

Impact of apixaban on haemostasis diagnosis assays

As other anticoagulant drugs, apixaban may affect the results of a series of coagulation assays routinely used in case of thrombophilia or in the exploration of a haemorrhagic event. For thrombophilia, an APC-R, AT, protein-C, protein-S and clotting factor assays may be required. On the other hand, when exploring a haemorrhagic event, specific tests such as RT, fibrinogen (Clauss or PT-derived method [dFib]), TT and clotting factor activity may be used. There is a need for the clinicians to know whether these tests are influenced by apixaban.

The Hemosil® Factor V Leiden, an aPTT-based assay, shows a slight influence in the ratio between the two conditions (in the presence or absence of aPC) in presence of high concentration (447ng/ml) of apixaban (▶Table 3). Nevertheless, such high concentrations will normally not be encountered with the doses used in clinical studies, and at lower concentration the ratio is not influenced. Thus, in most cases (i.e. random sampling), the Hemosil® Factor V Leiden might be applicable. The antithrombin measurement using FXa-based chromogenic assays is overestimated in presence of therapeutic concentration of apixaban (▶Figure 2). Thus, in patients treated with apixaban, thrombin-based chromogenic assays should be used for the assessment of antithrombin rate as stated in ▶Table 3. The Hemosil® Protein C is not affected by apixaban and could be used for the assessment of protein C. The Hemosil® Free Protein S, an immunological method, is not influenced compared to the STA®-Staclot® Protein S, a clotting method, where an over-estimation of approximately 13% per 100 ng/ml of apixaban is found (▶Figure 2). In clinical routine practice it is preferable to use immunological assays since they are not influenced by apixaban (▶Table 3).

Clinically relevant concentration of apixaban may interfere with the measurement of clotting factors. However, the relation is not linear, as suggested by the lack of sensitivity of aPTT at higher concentration of apixaban. High dilution of the sample tends to reduce the decrease between baseline and high apixaban concentration (see Suppl. Figure 5, available online at www.thrombosis-online.com). Thus, appropriate dilution of the sample may reduce this variability (▶Table 3), as already stated for rivaroxaban (28, 29). Importantly, the sensitivity of the PT or aPTT reagent must be taken into account and we also recommend using the less sensitive aPTT and PT reagents to minimise the influence of apixaban of clotting factor measurement.

Thrombin time, ecarin clotting time and reptilase time are understandably not influenced by apixaban. As stated above, DRVV-T is affected by apixaban, and sensitivity depends on the amount of phospholipids (Screen is more sensitive than Confirm). Therefore, DRVV-T should be avoided to assess a lupus anticoagulant in patients treated with apixaban since the ratio between the Screen and the Confirm will be increased giving possible false positive results. Silica clotting time (Screen and Confirm), i.e. STA®-Staclot® LA, do not have significant difference in term of sensitivity, reducing the risk of false positive results. Further studies are, however, required to know whether the accuracy of this test is modified in the presence of apixaban. Thus, as for rivaroxaban (30), specific tests using Taipan venom snake or Ecarin clotting time could be proposed to assess lupus anticoagulant even if international standardisation of the procedure is still required. Finally, measurement of fibrinogen using the Clauss Method is not affected by apixaban while the dFib shows an overestimation at higher concentration (447 ng/ml) in apixaban (▶Figure 2).

Limitations of our study

One limitation of this study is the use of NPP spiked with apixaban. In addition, the present study is mono-centric. These results should be confirmed in patients treated with Eliquis®. Moreover, the relation between the results of coagulation assays and bleeding risk can be found only with appropriate clinical studies. It seems, however, to be not ethically acceptable to expose patients to high apixaban concentrations to assess the impact on coagulation assays. Similarities between *in vitro* and *ex vivo* data have been demonstrated for rivaroxaban (28, 31, 32), and this should be applicable to apixaban as well, but further investigations in the field are required to confirm these data and evaluate the inter-individual variability.

Conclusion

In this study we showed that chromogenic anti-Xa assays are the most appropriate assays to measure the pharmacodynamics of apixaban. Broadly used coagulation assays such as PT or aPTT are not appropriate to efficiently estimate plasma drug concentration due to several limitations and a lack of sensitivity. TGA gives further information on the coagulation process, but its use in a clinical setting may be limited due to a lack of standardisation, an

Table 3: Summary of the influence of apixaban on haemostasis assays. †Appropriate dilutions are those from which normal value are recovered.

Coagulation assay	Reagent	Method	Coagulation Analyzer (Manufacturer)	Influenced (Y/N)	Recommandations
Thrombin time	STA®-Thrombin	Chronometric	STA-R Evolution® (Diagnostica Stago)	N	/
Reptilase time	STA®-Reptilase	Chronometric	STA-R Evolution® (Diagnostica Stago)	N	/
Lupus anticoagulant	STA®-DRVV Screen	Chronometric	STA-R Evolution® (Diagnostica Stago)	Y	DRVVT testing should be avoided. STA®-Staclot® LA could be used since the ratio is not affected at therapeutic concentration
	STA®-DRVV Confirm				
	STA®-Staclot LA				
	PTT-LA®				
Protein C	Hemosil® Protein C	Chromogenic	ACL-TOP® (Instrumentation Laboratory)	N	/
Protein S	STA®-Staclot® Protein S	Chronometric	STA-R Evolution® (Diagnostica Stago)	Y	Overestimation of 13% per 100 ng/ml of apixaban. This test should be avoided.
Free protein S antigen	Hemosil® Free Protein S	Immunoturbidimetric	ACL-TOP® (Instrumentation Laboratory)	N	/
Activated protein C resistance	Hemosil® Factor V Leiden	Chronometric	ACL-TOP® (Instrumentation Laboratory)	Y (at concentration higher than 447 ng/ml)	The ratio is slightly influenced (2.4 for baseline and 2.6 for 447 ng/ml). In most cases aPTT-based test could be used.
Fibrinogen Clauss method	STA®-Fibrinogen	Chronometric	STA-R Evolution® (Diagnostica Stago)	N	/
Fibrinogen PT-derived	RecombiPlasTin 2G®	Chronometric	ACL-TOP® (Instrumentation Laboratory)	N	The variability is greater with an overestimation of the fibrinogen rate at higher concentrations (>200 ng/ml).
Antithrombin	STA®-Stachrom® ATIII	Chromogenic	STA-R Evolution® (Diagnostica Stago)	N	FXa-based chromogenic assays should be avoided (increase of 13% per 100 ng/ml) and thrombin-based chromogenic assays should be preferred.
	Hemosil® Liquid Antithrombin	Chromogenic	ACL-TOP® (Instrumentation Laboratory)	Y	
Extrinsic clotting factors	STA®-CKPrest	Chronometric	STA-R Evolution® (Diagnostica Stago)	Y	Appropriate dilution of the sample must be performed: FVII (1/80); FIX (1/160); FXI (1/160); FXII (1/80)†
Intrinsic clotting factors	Innovin®	Chronometric	STA-R Evolution® (Diagnostica Stago)	Y	Appropriate dilution of the sample must be performed: FII (1/10); FV (1/20); FVII (1/20); FX (1/80)†
Ecarin clotting time	Ecarin	Chronometric	STA-R Evolution® (Diagnostica Stago)	N	/

†Appropriate dilutions are those from which normal value are recovered.

inter-individual variability and unfavourable turn-around time. Thus, we recommend chromogenic anti-Xa assays for the estimation of apixaban plasma concentrations using appropriate calibrators and controls. Apixaban interferes with chronometric or chromogenic assays that involve FXa or upstream coagulation factors in different ways and therefore, immunological assays, thrombin-based or less sensitive assays should be used, when applicable, for the haemostasis diagnosis assays. As for all NOACs the delay between the last drug intake and the blood sampling is mandatory to give accurate information and avoid misinterpretation.

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Conflicts of interest
None declared.

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