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Full Length Article

Concomitant assessment of rivaroxaban concentration and its impact on thrombin generation☆

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ABSTRACT

Background: Reliable assays to measure direct oral anticoagulant (DOAC) levels and their activity in critical situations are needed. Drug levels alone are not representative of the effect of DOACs on an individual's coagulation. We developed a technique that provides direct assessment of the global effect of rivaroxaban on the individual's coagulation in addition to plasma concentrations.

Methods: DOAC concentrations were determined in fifty patients using rivaroxaban, with the new assay, Xross-CAT. The effect of rivaroxaban on coagulation (activity) was measured with thrombin generation (TG) in platelet poor plasma using 5 pM tissue factor on the same device. The levels were validated with the Biophen DiXal assay. The prothrombin time (PT) and dilute Russell viper venom time (dRVVT) were performed to estimate the effect on coagulation.

Results: The variability of Xross-CAT was below 12%. Xross-CAT correlates well with Biophen DiXaI (r_s = 0.885). The bias, determined by Bland-Altman analysis, was 4.9% and the Passing-Bablok equation was y = 1.1x − 2.1. The correlation of plasma levels with TG was moderate (ETP r_s = −0.548; Peak r_s = −0.559), as for the PT (r_s = 0.739) and the dRVVT (r_s = 0.692).

Conclusions: Xross-CAT shows a good correlation with Biophen DiXaI that was previously confirmed to accurately assess rivaroxaban levels. Bleeding and thrombotic complications are not necessarily associated with drug levels and could be influenced by concomitant risk factors. The main benefit of Xross-CAT is that it can be performed simultaneously with thrombin generation, providing an overview of the global anticoagulation status of a patient in relation to circulating DOAC levels.

1. Introduction

Direct oral anticoagulants (DOACs) are currently prescribed according a fixed dose regimen in routine clinical practice. Their pharmacokinetic and pharmacodynamic profiles are more predictable and less prone to drug-drug interactions than those of vitamin K antagonists (VKAs). Therefore, DOACs are not monitored regularly. There are multiple clinical scenarios in which laboratory assessment of drug concentrations can be important (e.g. at the occurrence of a thrombotic or hemorrhagic event, before an invasive procedure, before thrombolysis therapy, in patients with a high bleeding risk, an extreme body weight, a decreased renal function, or when drug accumulation or an

Abbreviations: DOAC, direct oral anticoagulant; VKA, vitamin K antagonist; FXa, factor Xa; VTE, venous thromboembolism; AF, atrial fibrillation; PT, prothrombin time; α_2M, α_2-macroglobulin; CAT, calibrated automated thrombinography; TF, tissue factor; PL, phospholipids; ZGGR-AMC, Z-Gly-Gly-Arg aminomethylcoumarin; α_2M-IIa, α_2-macroglobulin-thrombin; BSA, bovine serum albumin; PPP, platelet poor plasma; NPP, normal pooled plasma; dRVVT, dilute Russell viper venom time; SD, standard deviation; CLSI, Clinical and Laboratory Standards Institute; LOD, limit of detection; LOQ, limit of quantification; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; CV, coefficient of variation; CI, confidence interval; ETP, endogenous thrombin potential; ttPeak, time-to-peak; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; wr, within run; rr, between run; dd, between day; t, total; FU, fluorescence units

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overdose is suspected) [1–5]. Furthermore, measuring DOACs may be helpful in assessing antidote requirement and around antidote administration [2], to estimate the effect of drug-drug interactions or to guide the transition between VKAs and DOACs. In the future, specialized measurement of DOAC levels may even support clinicians in choosing an appropriate anticoagulant for individual patients.

While drug levels can be useful to assess the individual's pharmacokinetic response, correlations to clinical outcome are still uncertain. Patients have developed thrombosis with DOAC levels in the "on-therapy" range [6,7]. Similarly, cases of spontaneous bleeding have been reported within the "on-therapy" range or even below this threshold, demonstrating that the drug level alone may not be sufficient to identify those who are more at risk [8,9]. Additionally, there is a tendency to undertreat patients by choosing a lower dose of DOACs, out of fear of inducing bleeding complications [10–12]. This scenario increases the risk of ischemic stroke without diminishing the risk is of bleeding, and should therefore be avoided by all means. Reliable tools to measure DOAC concentrations and their anticoagulant effect can help to prevent these situations and provide reassurance to the physician.

Rivaroxaban was the first direct factor Xa (FXa) inhibitor that was approved for clinical use e.g. to treat and prevent venous thromboembolism (VTE), and in patients with atrial fibrillation (AF). Moreover, rivaroxaban in a low dose combined with asprine, is the first DOAC that can be used for secondary cardiovascular prevention, in patients with stable atherosclerosis [13]. Assays to determine the rivaroxaban levels that are based on clotting times (e.g. prothrombin time (PT)) turned out to have limited sensitivity, high inter-reagent variability and poor comparability with specific assays for DOAC levels [3,14–19]. Standardized rivaroxaban-calibrated, chromogenic anti-FXa assays are currently the most reliable for the quantification of rivaroxaban levels in the routine laboratory [20]. However, multiple studies have shown that drug levels in real-life patients can differ extremely between patients, [5,19,21,22], resulting in broad ranges of trough and peak levels (6–87 ng/ml at T_{trough} and from 189 to 419 ng/ml at T_{max} in VTE patients, and 12–137 ng/ml and 184–343 ng/ml for AF patients, respectively) [15]. Moreover, the variability of rivaroxaban levels within individuals over time has not yet been sufficiently investigated.

Besides this large inter-individual variation, meaningful interpretation of drug levels is complicated by a lack of guidance on extrapolating anti-Xa activity to bleeding and thrombosis. In other words, levels alone do not provide sufficient information about the influence of rivaroxaban on the coagulation of individual patients. Of note, this not only applies to anticoagulants, but also to antiplatelet agents. Different trials demonstrated that adjusting the dose of P2Y12 inhibitors based on platelet-function monitoring did not reduce bleeding nor ischemic events [23,24]. For the abovementioned purpose, a functional test, which is able to measure both rivaroxaban levels as well as their effect on the coagulation process, is needed. The rivaroxaban test described in the current study, the Xross-CAT, is based on the capacity of rivaroxaban to inhibit both free FXa as well as bound FXa. Rivaroxaban can still bind to the active site of FXa when it is bound to α_2-macroglobulin (α_2M) or when it is in the prothrombinase complex, due to its small size and lack of necessity for a cofactor [25]. It is known that direct thrombin inhibitors (e.g. dabigatran) interfere with the measurement of calibrated automated thrombinography (CAT) by inhibiting the calibrator (α_2M-thrombin complex) [26,27]. We speculated that the α_2M-FXa complex will be inhibited by small molecule FXa inhibitors such as rivaroxaban. We used this principle to develop a test to measure plasma rivaroxaban levels. Since this test is based on the CAT method, it is possible to determine not only drug levels but also the overall coagulation activity (by determining thrombin generation) within the same setup. We hypothesize that this novel approach will accurately measure rivaroxaban concentrations together with the possibility to measure the effect on the coagulation system in the same CAT setup, and will therefore provide a major advantage over currently used level assays.

2. Materials & methods

2.1. Reagents

Recombinant tissue factor (TF) was Innovin (Dade-Behring, Marburg, Germany). Synthetic phospholipids (PL) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and used in the form of vesicles consisting of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine (1:1:3, mol:mol:mol). The fluorogenic substrate Z-Gly-Gly-Arg aminomethylcoumarin (ZGGR-AMC) was obtained from Bachem (Basel, Switzerland). The calibrator (α_2M-thrombin (α_2M-IIa)) was prepared according to Hemker et al. [28]. Hepes buffers containing 5 mg/ml or 60 mg/ml bovine serum albumin (BSA5 and BSA60) were prepared as described by Hemker et al. [29]. Rivaroxaban (Xarelto®) was obtained by crushing tablets (Bayer, Berlin, Germany) or as powder (100% purity) from Alsachim (Illkirch Graffenstaden, France).

2.2. Study population

Fifty plasma samples from different patients on rivaroxaban were collected from September 2014 until October 2015 in the Centre Hospitalier Universitaire UCL Namur, Yvoir, Belgium. The study was performed in accordance with the Declaration of Helsinki and sample collection was approved by the Medical Ethics Committee of the Centre Hospitalier Universitaire UCL Namur in Yvoir, Belgium (number of approval BU039201422406). The inclusion criteria were patients receiving rivaroxaban for secondary prevention of thromboembolic events in atrial fibrillation or treatment of venous thromboembolism, as well as collection of blood samples at trough (24 ± 2 h after last pill intake) or peak (2–3 h after last pill intake) concentrations. In this case, the included patients were inpatients.

2.3. Plasma preparation

Blood was taken by venipuncture in the antecubital vein using a 21-gauge needle (Terumo) or through a peripheral venous catheter (BD Insyte-W, 18- or 16-gauge) and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe, Terumo, Heverlee, Belgium). Platelet poor plasma (PPP) was obtained from the supernatant fraction after double centrifugation at 1500*g* for 15 min at room temperature. Samples were then aliquoted and frozen immediately at −80 °C. Plasma sample aliquots were thawed and heated to 37 °C for at least 5 min before running the experiment. Normal pooled plasma (NPP) was prepared from blood collected from 116 healthy volunteers. Blood was centrifuged at 2500*g* for 5 min after which plasma was pooled. The pooled plasma was then ultracentrifuged at 100,000*g* for 10 min. (This speed was mainly used to remove microparticles. Since this plasma is only used as a medium for α_2M-FXa, rivaroxaban and the substrate, for the concentration determination; or as a control for experimental variation in thrombin generation, the high speed did not affect experimental results.) Aliquots of 500 µl were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

2.4. Calibrated automated thrombinography

Thrombin generation was performed in platelet poor plasma using CAT as described previously by Hemker et al. [28]. In summary, calibrator wells consisted of 20 µl calibrator with 80 µl of plasma. Thrombin generation wells consisted of 20 µl TF/PL reagent and 80 µl of PPP. The final concentration of TF was 5 pM and 4 µM PL. After 10 min of incubation at 37 °C 20 µl of $CaCl_2$ solution containing the fluorescent substrate (final concentration: 416.7 µM) were dispensed into the wells. Measurements were performed on a Fluoroskan Ascent fluorimeter (Thermo Labsystems, Helsinki, Finland). Data were analysed with specialized software from Thrombinoscope (version 5.0,

Maastricht, The Netherlands). In each run, NPP was included in order to control for experimental variation.

2.5. α_2M-FXa preparation

The preparation of α_2M-FXa consists of two steps. Firstly, semi-pure α_2M was prepared and in the second step the α_2M was incubated with FXa to form the α_2M-FXa complex. The α_2M-FXa was purified further by running it on a Ni-Sepharose column.

In step 1, α_2M was prepared by adding 3% (w/v) PEG 20,000 to bovine plasma containing 10 mM tri-sodium citrate and 4 mM citric acid and incubating the suspension for 30 min at room temperature (RT) while stirring. The formed precipitate was spun down (25 min at 5277 $\times g$) and discarded. Then, an additional 3% (w/v) PEG 20,000 was added to the supernatant containing α_2M and again stirred for 30 min at RT. The precipitate was spun down as before. The pellet containing α_2M was dissolved in 68.4 mM NaCl, 8.5 mM Na-citrate (pH 7.0). Residual fibrinogen was removed by adding 200 nM thrombin, stirring for 20 min at RT and spinning down the formed fibrin at 5277 $\times g$ during 30 min. The fibrin pellet was discarded. The traces of α_2M-thrombin and free thrombin in the supernatant were inactivated by addition of 400 nM PPACK. The raw α_2M was put at 4 °C overnight in order to allow the excess PPACK to decay and then filtered (0.22 μm) before further use.

In the second step, for further preparation and purification of α_2M-FXa, 8 ml FXa (70 μM) were added to 62 ml α_2M (10 μM) and incubated for 3.5 h at 37 °C. Twenty-three milliliters of α_2M-FXa were applied to a 150 ml Ni-Sepharose column (5 cm^2 × 30 cm). Unbound material was removed by eluting the column with 350 ml 500 mM NaCl, 20 mM HEPES, 2 mM imidazole, 0.02% NaN_3 (pH 7.35). Then the α_2M-FXa was eluted in a 750-ml gradient of 2–32 mM imidazole and the obtained material was concentrated by precipitation in 40% saturated ammonium sulfate (= 242 g/l) by stirring for 30 min at RT and centrifuging for 20 min at 5277 $\times g$. The pellet was dissolved in calibrator buffer (20 mM HEPES, 100 mM Na_3-citrate, 0.02% NaN_3 (pH 7.35)) and centrifuged (20 min at 5277 $\times g$) to remove insoluble material before gel filtration. To the preparation 200 nM AT and 2 U/ml heparin were added to inactivate free FXa.

2.6. DOAC concentration assay (Xross-CAT)

The new Xross-CAT assay is based on the known CAT setup. Twenty microliters of α_2M-FXa were incubated with 80 μl of plasma that was diluted 8 times in Hepes buffer containing bovine serum albumin (BSA) (BSA5 buffer) [29]. Upon addition of 20 μl of ZGGR-AMC substrate in BSA 60 buffer [29] (final concentration: 416.7 μM), the activity of rivaroxaban was gauged kinetically by the conversion of the fluorescent AMC-substrate on a Fluoroskan Ascent fluorimeter (Thermo Labsystems, Helsinki, Finland) using Ascent software (version 2.6). Because of the similar set-up as thrombin generation determinations via the CAT assay, this could be assessed in the same 96-well plate. Since it is usually unknown in what range the plasma concentration is situated, the samples had to be measured at two concentrations of α_2M-FXa (30 nM and 90 nM, as further explained in the Results section). The lower concentration was appropriate for determining levels between 22 and 218 ng/ml (which corresponds to 50–500 nM) and the higher concentration for levels between 218 and 436 ng/ml (corresponding to 500–1000 nM). These concentrations of α_2M-FXa were selected based on an inhibition of the slope by rivaroxaban at different concentrations in the range of 15–85%. The reference curves were prepared by spiking NPP with rivaroxaban. The samples were compared to two reference curves in each experiment (22, 44, 87, 131, 174 and 218 ng/ml for the lower concentration of α_2M-Xa and 218, 262, 305, 349, 392 and 436 ng/ml for the higher concentration). Fluorescence was measured during 15 min. The conversion of the ZGGR-AMC substrate by α_2M-FXa will result in straight lines (over a period of time of at least 10 min, see Fig. 1). The slope of these lines will decrease upon addition of increasing concentrations of rivaroxaban. The data were analysed by calculating the slope of the fluorescent curve (0–10 min) and comparing those values to the reference curves. Concentrations that were quantified below 44 ng/ml were not included in the analysis, as explained in the Results section.

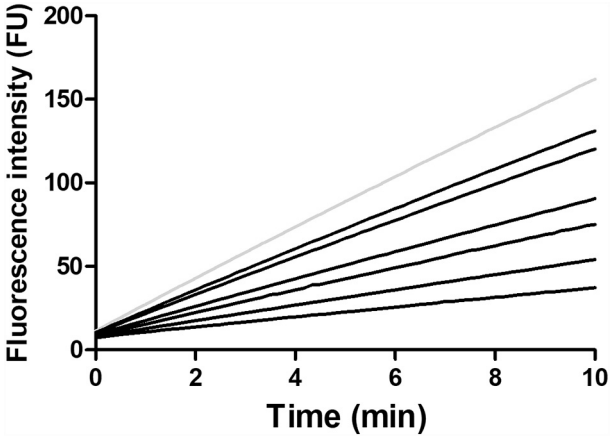

Fig. 1. Dose dependent effect of increasing rivaroxaban concentrations on α_2M-FXa. The effect of 0, 22, 44, 87, 131, 174 and 218 ng/ml (lines from top to bottom) of rivaroxaban (mean of two curves) on α_2M-FXa is illustrated. FU, fluorescence units.

2.7. PT Triniclot PT Excel S®

The prothrombin time was measured using Triniclot PT Excel S® (TrinityBiotech, Bray, Ireland) on a STA-R Evolution® coagulometer. Triniclot PT Excel S® is derived from rabbit brain. The PT was performed as previously described [30]. We have chosen these reagents as they were known to be the more sensitive one [30].

2.8. Dilute Russell viper venom time (dRVVT)

Briefly, 100 μl of plasma sample was incubated during 240 s at 37 °C [31]. Thereafter, 100 μl of STA®-Staclot®DRVV-Confirm (containing high PL concentration) was added, starting the reaction on a STA-R Evolution® coagulometer. Results are given in seconds.

2.9. Biophen direct factor Xa inhibitors

Two hundred microliters of diluted plasma (1:50 with Tris-NaCl-EDTA buffer at pH 7.85) were incubated with 75 μl of human FXa (Hyphen BioMed) for 120 s at 37 °C, then 75 μl of a specific FXa substrate [CS-11(65)] (Hyphen BioMed) were added to start the reaction on a STA-R Evolution® coagulometer (Diagnostica Stago) [32]. The concentrations of calibrators for the normal therapeutic range of rivaroxaban (Biophen Rivaroxaban Plasma Calibrator, Hyphen BioMed) were 50, 250 and 500 ng/ml in the initial samples after reconstitution. The low concentration procedure (Biophen DiXaI LOW) was the same as the normal one except that plasma was diluted 1:8 in buffer and the calibration was performed with standards for low plasma concentrations of rivaroxaban (Biophen Rivaroxaban Calibrator Low). The standard rivaroxaban concentrations were 0, 52 and 110 ng/ml.

2.10. Statistical analysis

Statistical analyses were performed with Graphpad Prism software (version 5.00). Passing-Bablok regression was performed with MedCalc (version 17.7.2). Data are represented as mean with standard deviation (SD). Assay variation coefficients were calculated according to the

Clinical and Laboratory Standards Institute (CLSI) guidelines [33]. Normality of the data was assessed using the Shapiro-Wilk test. Correlation analysis was performed with the Spearman test. A two-sided *p*-value of 0.05 was considered statistically significant. Bland-Altman analysis was executed by analysing the percentage difference of the two methods ($100 * (A - B) / average$) versus the average ($(A + B) / 2$) of the two methods. Method A was the new rivaroxaban assay and method B was the Biophen DiXaI assay. Passing-Bablok regression analysis was used to analyse the comparability of measurements, with the new assay on the y-axis and the established assay on the x-axis. The limit of detection (LOD) and limit of quantification (LOQ) were assessed according to ICH Q2R1 guideline. The LOD and LOQ were determined based on 5 measurements of the reference curve and 5 measurements of the blank as follows: LOD = ((3 * SD of Y0) / slope) and LOQ = ((10 * SD of Y0) / slope); with Y0 being the y-axis intercept.

3. Results

3.1. Optimal α_2M-FXa concentrations

In order to set up this assay, the effect of different concentrations of rivaroxaban on α_2M-FXa was evaluated and found to be dose-dependent (Fig. 1). Dose response curves were constructed to determine the concentrations of α_2M-FXa at which a linear relation with rivaroxaban concentrations was found. The curves were constructed at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 nM of α_2M-FXa, in the presence of eleven concentrations of rivaroxaban (between 22 ng/ml and 436 ng/ml). The two α_2M-FXa concentrations were selected in which the inhibitory effects of all rivaroxaban concentrations were between 15 and 85%. Thirty nM of α_2M-FXa was the most fitting concentration for rivaroxaban levels from 22 to 218 ng/ml (Fig. 2A) and 90 nM of α_2M-FXa for levels between 218 and 436 ng/ml (Fig. 2B).

3.2. Assay performance

The variability and accuracy of the assay are characterized in Table 1. These results suggest that the precision of the assay is adequate for the assay to be implicated in the laboratory. For the Xross-CAT the LOD was determined to be 3.3 ng/ml and the LOQ was 10.1 ng/ml. However, the variability and accuracy of the assay for samples spiked with 22 ng/ml, the coefficients of variation (CVs) increased considerably (a within-run variation of 37% and a between-run variation of 42%). For all other tested concentrations > 22 ng/ml the CVs were below 13% (Table 1). Since the CVs for 22 ng/ml were high, samples with concentrations below < 44 ng/ml were considered as unreliable and therefore excluded from analysis.

3.3. Comparison of the assay to other tests

In order to test the applicability of the new assay, it was compared to several assays that are known to be affected by rivaroxaban. Samples from 50 patients taking rivaroxaban were tested. The median time since last intake for the rivaroxaban samples was 3 h (min–max: 0–180 h, interquartile range: 2–20 h, n = 44).

We compared the Xross-CAT to the PT and the dRVVT, which are both able to detect rivaroxaban but have limited sensitivity. The PT and dRVVT, displayed a relatively good correlation with our assay (r_s = 0.739 and 0.692, respectively) (Fig. 3). To evaluate the applicability of the new assays to detect concentration levels, we compared the Xross-CAT with the Biophen DiXaI assay, which is currently used in the clinic as the most reliable assay to detect DOAC levels. Our assay showed the best correlation with the Biophen DiXaI assay (r_s = 0.885) (Fig. 4). As it can be expected that no addition of rivaroxaban would result in any detection by the Xross-CAT or the Biophen DiXaI assay, the trend line was forced through zero. A Bland-Altman analysis was performed to analyse the agreement between our assay and the Biophen DiXaI assay, which showed a bias of 4.9% between the two tests (95% limits of agreement: −34.2%–44.0%). The Passing-Bablok regression equation was y = 1.1 (95% CI: 0.9 to 1.2)x − 2.1 (95% CI: −41.8 to 27.8).

We also evaluated the correlation between the Xross-CAT levels with the four main TG parameters: endogenous thrombin potential (ETP), peak, lag time and time-to-peak (ttPeak). As shown in Fig. 5, TG is very sensitive to rivaroxaban. When comparing thrombin generation parameters to the Xross-CAT levels (Fig. 6), we found a moderate correlation between rivaroxaban levels and the lag time and time to peak (r_s = 0.646 and r_s = 0.601, respectively). As anticipated, no linear correlation between the ETP and the peak was found. As is displayed in Fig. 5, there was an indication for a hyperbolic relation, which can be explained by the fact that low concentrations of rivaroxaban can already suppress the peak considerably. Above a certain concentration the peak is inhibited completely (a plateau phase is reached). This applies to the ETP as well.

4. Discussion

In the past years, DOACs have become the primary choice as anticoagulants for patients with atrial fibrillation or venous thrombosis. Rivaroxaban is also proven to be useful in patients with coronary artery disease and/or peripheral arterial disease (with stable atherosclerotic vascular disease) for the reduction of ischemic events [13]; since arterial thrombi also contain fibrin (not only platelets), showing the involvement of the coagulation pathway [34]. One of the most important advantages of DOACs is that they can be given in a fixed dose and do not require regular monitoring. However, there are certain clinical scenarios in which laboratory assessment of DOAC levels is needed to optimize treatment and increase patient safety. As bleeding or thrombotic complications in patients receiving DOACs may not be systematically associated to the drug levels, a combined measurement of drug levels and global haemostatic activity might be more informative than

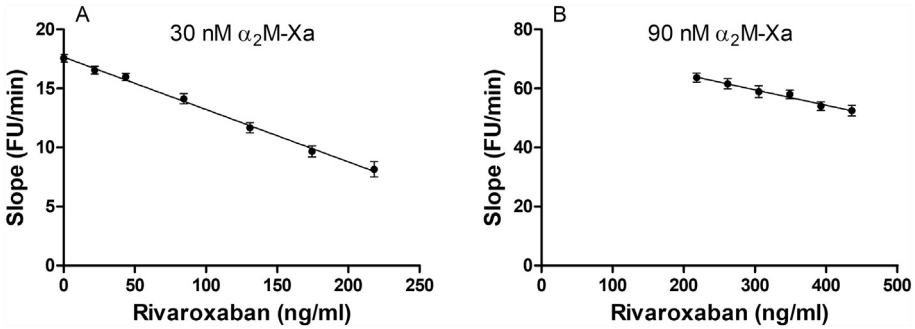

Fig. 2. Dose-response of increasing rivaroxaban concentrations at chosen α_2M-FXa concentrations. Increasing concentrations of rivaroxaban measured at the 30 nM [A] and 90 nM [B] α_2M-FXa; n = 5. Data are presented as mean ± SD. FU, fluorescence units.

Table 1
Assay variability and accuracy.

Added concentration (ng/ml)	Mean concentration (ng/ml)	SDwr	SDrr	SDdd	SDt	CVwr	CVrr	CVdd	CVt
43.6	51.0	5.1	1.1	0.2	5.3	10.0	2.2	0.4	10.4
174.4	187.9	17.6	15.5	0.0	23.5	9.4	8.2	0.0	12.5
436.0	464.3	16.2	23.4	24.5	37.5	3.5	5.0	5.3	8.1

SD, standard deviation; CV, coefficient of variation; wr, within run; rr, between run; dd, between day; t, total. SDs are presented in ng/ml. CVs are presented in %.

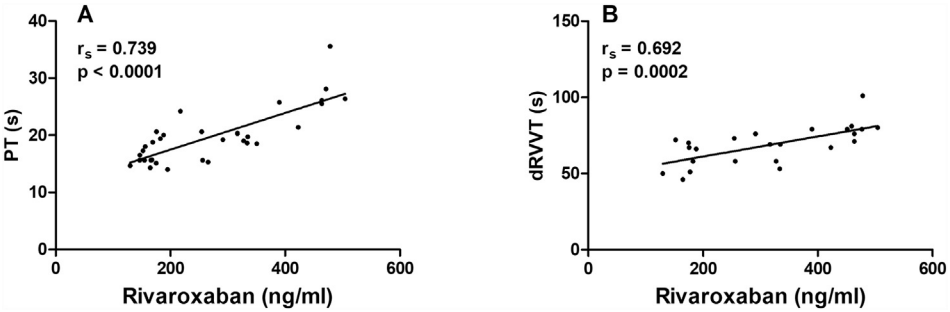

Fig. 3. Correlation of rivaroxaban concentration (as determined by the Xross-CAT assay) with [A] prothrombin time (PT) and [B] dilute Russell Viper Venom time (dRVVT). The correlations were determined via Spearman correlation analysis. PT, n = 33, dRVVT, n = 24.

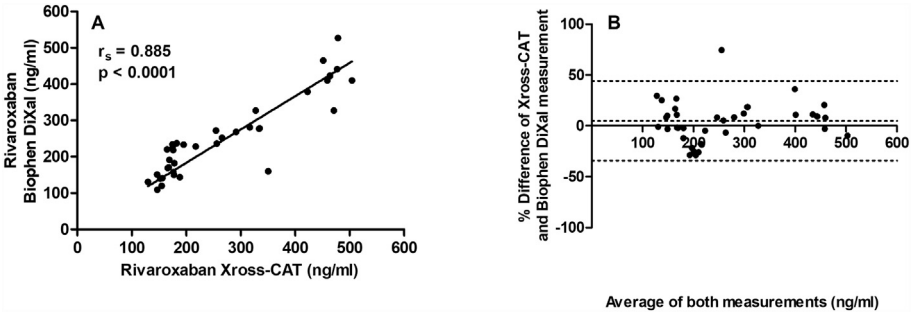

Fig. 4. Correlation and Bland-Altman analysis between rivaroxaban concentration as determined by the Xross-CAT assay and the concentration determined by the Biophen DiXaI assay. [A] The correlations were determined via Spearman correlation analysis, n = 38. [B] The % difference in the Bland-Altman analysis is calculated as: $(100 * (A - B) /$ average) with A being the concentration determined by the Xross-CAT and B the concentration determined by the Biophen DiXaI assay, n = 38. The mean difference was 4.9% (95% limits of agreement: −34.2% to 44.0%).

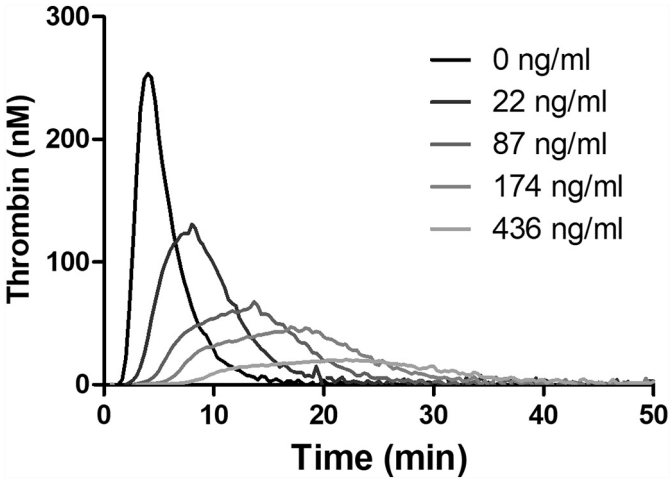

Fig. 5. Effect of different rivaroxaban concentrations on thrombin generation curves. Increasing concentrations of rivaroxaban were added to NPP in the thrombin generation assay, measured at 5 pM TF.

drug levels alone.

We developed an assay, the Xross-CAT, to measure plasma rivaroxaban levels via the dose-dependent inhibitory effect on the α_2M-FXa complex. We have optimized the test with a large range of rivaroxaban concentrations (44 to 436 ng/ml). These concentrations include in vivo rivaroxaban levels of patients with atrial fibrillation (12–343 ng/ml) and VTE (6–419 ng/ml) [4]. Although not shown in the current manuscript, the principle of this test is also applicable to other direct FXa inhibitors (apixaban or edoxaban).

Many approaches have been proposed to measure rivaroxaban levels [3,14,15,30]. Some assays are based on clotting times, others on the anti-FXa activity [35]. In order to specify which coagulation assays may be recommended for the measurement of rivaroxaban levels, they need to be compared to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) or to the assays with the best relation to LC-MS/MS. At the moment, rivaroxaban-calibrated anti-FXa assays, such as the Biophen DiXaI assay, showed the best correlations with LC-MS/MS [20,36]. However, it should be taken into account that the quality of calibrators can differ [37]. Xross-CAT showed a high agreement with the Biophen DiXaI assay in the Bland-Altman and the Passing-Bablok analysis. This indicates that this newly developed assay reliably reflects the patient plasma concentrations. However, plasma levels assessed with the Xross-CAT are slightly higher compared to the Biophen DiXaI assay, which can also be concluded from the Bland-Altman and the Passing-Bablok analysis. This is likely due to the use of different calibrators for the two assays. In the Xross-CAT, the use of homemade calibrators may introduce an unwanted bias. The use of the same sets of calibrators within the two assays could reduce this discrepancy. However, as samples in the Xross-CAT were measured retrospectively, this was not possible in the present study. Further validations are thus required to confirm this hypothesis. At this moment we were not able to reliably detect low concentrations of rivaroxaban (22 ng/ml) with the concentration of calibrator α_2M-FXa used, although this is not in agreement with the found LOD and LOQ of the assay. Therefore, we would not recommend using this assay to measure concentration lower than 44 ng/ml.

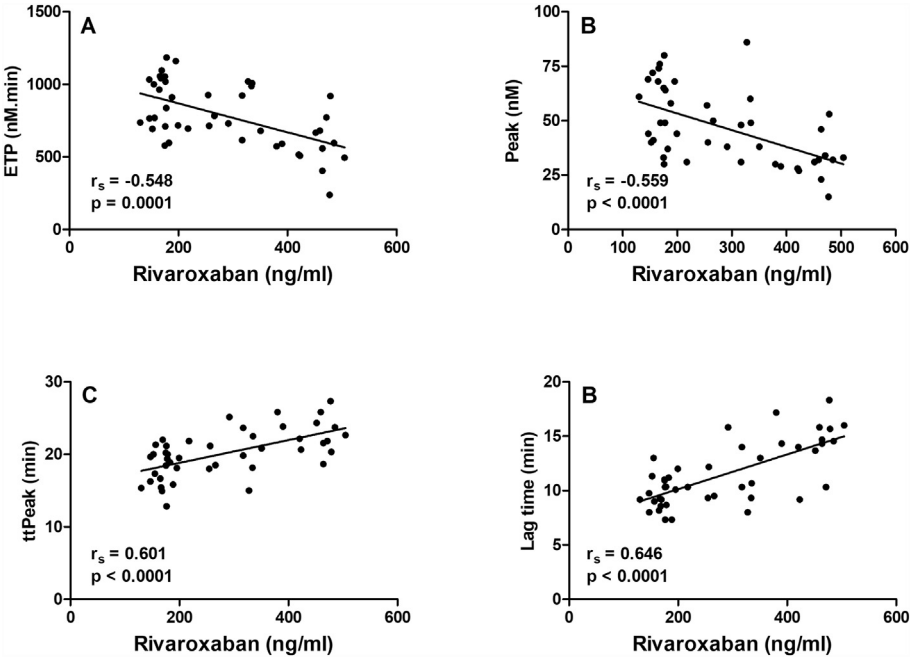

Fig. 6. Correlation of rivaroxaban concentration and thrombin generation parameters. The correlations of the rivaroxaban concentration, as determined by the Xross-CAT assay, with [A] endogenous thrombin potential (ETP), [B] peak, [C] time-to-peak (ttPeak) and [D] lag time were determined via Spearman correlation analysis, n = 44.

The strength of our assay is that rivaroxaban concentrations can be measured in combination with the coagulation state of the patient, using thrombin generation. Our measurements displayed a significant correlation between rivaroxaban concentrations and thrombin generation parameters, which turned out to be the method of choice for measuring hyper- and hypo-coagulable state [28,38–41]. The correlations between rivaroxaban levels and ETP as well as peak describe a hyperbola. This can be explained because rivaroxaban inhibits the amplification phase of thrombin formation. Hence, the effect of rivaroxaban is more pronounced on ETP and peak. Moreover, this demonstrates that low levels of rivaroxaban are sufficient to reduce the amplification phase considerably. Therefore, at high rivaroxaban concentrations, the extent of the effect on peak and ETP is relatively small [42]. Similar correlations were reported between rivaroxaban levels measured by chromogenic anti-FXa assays [43]. It should be noted that in the large trials performed with DOACs [44–46], drug levels were generally not compared to clinical outcomes, as the one-dose-fits-all principle was one of the important benefits of the new drugs. Ultimately, a large prospective clinical trial could solve the correlation between DOAC doses, levels and clinical outcomes. Because this would require a large study population and will be very costly, it is unlikely that such a trial will be performed in the near future. Therefore, the best option would be to study the relation between DOAC levels and the true effect of the DOACs on the coagulation system. Recently, an interesting observational study was published by Testa et al., which showed a relation between low trough levels of DOACs and the occurrence of thrombotic events in AF patients. Their results support the concept that measuring DOAC levels in a steady state could help identify low responders. In conjunction with clinical risk, this may help to identify those more at risk of a thrombotic event [7]. The current study is a proof of concept and larger studies are required for definite confirmation. Larger registries are currently on-going, and results are expected soon. Ideally, these studies will not solely test DOAC levels, but will also assess the effect of DOACs on coagulation.

For this purpose, a global coagulation test should be used with a good correlation to clinical outcomes. Multiple studies reported that thrombin generation is related to bleeding in patients treated with anticoagulants and in patients with hemophilia as well [39,47,48]. Other studies have shown that thrombin generation parameters are related to thrombosis [49–51]. The thrombin generation peak was shown to stay elevated in patients for up to 6 months in patients with acute coronary syndrome [52,53]. Taking this into consideration, the main advantage of the new assay is that it would provide an evaluation of rivaroxaban concentrations and the effect of rivaroxaban on coagulation (via thrombin generation) within the same measurement.

There were also limitations to this study. The turn-around time of our assay is 50 min, which is partly caused by the preparation of platelet poor plasma. The ideal DOAC test should allow near-patient measurements, ideally in whole blood, to facilitate a direct decision regarding treatment. However, these tests are currently not available. At the moment, although the determination of rivaroxaban concentrations and the effect on thrombin generation could be determined in one run, some manual calculations will be involved. Hopefully, in the future, software can be developed to integrate both determinations.

Although measuring DOAC levels is becoming increasingly available, the importance for customizing treatment of individual patients remains to be delineated. This study was retrospectively conducted. Ideally, there would be a follow-up study to clinically validate the assay, in which the Xross-CAT is prospectively measured in patients on rivaroxaban treatment and linked to potential bleeding or re-thrombosis outcomes.

In conclusion, the Xross-CAT shows a good correlation with a calibrated anti-FXa assay, which was confirmed to accurately assess rivaroxaban levels in patient samples. Measuring DOAC levels together with DOAC activity in an individuals' coagulation system provides an overview of the anticoagulation status in patients in relation to circulating DOAC levels. These findings could facilitate further developments towards personalized treatment in patients at risk of bleeding or recurrent thrombosis.

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Declaration of competing interest

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References

[1] European Medicines Agency, Product information: Xarelto, European Medicines AgencyAvailable at: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000944/WC500057108.pdf.

[2] J.H. Levy, W. Ageno, N.C. Chan, M. Crowther, P. Verhamme, J.I. Weitz, A. Subcommittee on Control of, When and how to use antidotes for the reversal of direct oral anticoagulants: guidance from the SSC of the ISTH, J. Thromb. Haemost. 14 (2016) 623–627.

[3] T. Baglin, A. Hillarp, A. Tripodi, I. Elalamy, H. Buller, W. Ageno, Measuring Oral Direct Inhibitors (ODIs) of thrombin and factor Xa: a recommendation from the Subcommittee on Control of Anticoagulation of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis, J. Thromb. Haemost. 11 (2013) 756–760.

[4] J. Douxfils, W. Ageno, C.M. Samama, S. Lessire, H. Ten Cate, P. Verhamme, J.M. Dogne, F. Mullier, Laboratory testing in patients treated with direct oral anticoagulants: a practical guide for clinicians, J. Thromb. Haemost. 16 (2018) 209–219.

[5] A. Godier, A.S. Dincq, A.C. Martin, A. Radu, I. Leblanc, M. Antona, M. Vasse, J.L. Golmard, F. Mullier, I. Gouin-Thibault, Predictors of pre-procedural concentrations of direct oral anticoagulants: a prospective multicentre study, Eur. Heart J. 38 (2017) 2431–2439.

[6] H. Ten Cate, R.H. Olie, A.J. Ten Cate-Hoek, Y.M.C. Henskens, Direct oral anticoagulants: when to consider laboratory testing? Int. J. Lab. Hematol. 40 (Suppl. 1) (2018) 30–33.

[7] S. Testa, O. Paoletti, C. Legnani, C. Dellanoce, E. Antonucci, B. Cosmi, V. Pengo, D. Poli, R. Morandini, R. Testa, A. Tripodi, G. Palareti, Low drug levels and thrombotic complications in high-risk atrial fibrillation patients treated with direct oral anticoagulants, J. Thromb. Haemost. 16 (2018) 842–848.

[8] P. Albaladejo, F. Bonhomme, N. Blais, J.P. Collet, D. Faraoni, P. Fontana, A. Godier, J. Llau, D. Longrois, E. Marret, P. Mismetti, N. Rosencher, S. Roullet, C.M. Samama, J.F. Schved, P. Sie, A. Steib, S. Susen, H. French Working Group on Perioperative, Management of direct oral anticoagulants in patients undergoing elective surgeries and invasive procedures: updated guidelines from the French Working Group on Perioperative Hemostasis (GIHP) - September 2015, Anaesth. Crit. Care Pain Med. 36 (2017) 73–76.

[9] A.L. Sennesael, A.S. Larock, J. Douxfils, L. Elens, G. Stillemans, M. Wiesen, M. Taubert, J.M. Dogne, A. Spinewine, F. Mullier, Rivaroxaban plasma levels in patients admitted for bleeding events: insights from a prospective study, Thromb. J. 16 (2018) 28.

[10] I.M. Ogilvie, N. Newton, S.A. Welner, W. Cowell, G.Y. Lip, Underuse of oral anticoagulants in atrial fibrillation: a systematic review, Am. J. Med. 123 (2010) 638–645 e634.

[11] R. Nieuwlaat, A. Capucci, G.Y. Lip, S.B. Olsson, M.H. Prins, F.H. Nieman, J. Lopez-Sendon, P.E. Vardas, E. Aliot, M. Santini, H.J. Crijns, I. Euro Heart Survey, Antithrombotic treatment in real-life atrial fibrillation patients: a report from the Euro Heart Survey on Atrial Fibrillation, Eur. Heart J. 27 (2006) 3018–3026.

[12] T. Wilke, A. Groth, M. Pfannkuche, O. Harks, A. Fuchs, U. Maywald, B. Krabbe, Real life anticoagulation treatment of patients with atrial fibrillation in Germany: extent and causes of anticoagulant under-use, J. Thromb. Thrombolysis 40 (2015) 97–107.

[13] J.W. Eikelboom, S.J. Connolly, J. Bosch, G.R. Dagenais, R.G. Hart, O. Shestakovska, R. Diaz, M. Alings, E.M. Lonn, S.S. Anand, P. Widimsky, M. Hori, A. Avezum, L.S. Piegas, K.R.H. Branch, J. Probstfield, D.L. Bhatt, J. Zhu, Y. Liang, A.P. Maggioni, P. Lopez-Jaramillo, M. O'Donnell, A.K. Kakkar, K.A.A. Fox, A.N. Parkhomenko, G. Ertl, S. Stork, M. Keltai, L. Ryden, N. Pogosova, A.L. Dans, F. Lanas, P.J. Commerford, C. Torp-Pedersen, T.J. Guzik, P.B. Verhamme, D. Vinereanu, J.H. Kim, A.M. Tonkin, B.S. Lewis, C. Felix, K. Yusoff, P.G. Steg, K.P. Metsarinne, N. Cook Bruns, F. Misselwitz, E. Chen, D. Leong, S. Yusuf, C. Investigators, Rivaroxaban with or without aspirin in stable cardiovascular disease, N. Engl. J. Med. 377 (2017) 1319–1330.

[14] S. Kitchen, E. Gray, I. Mackie, T. Baglin, M. Makris, B. committee, Measurement of non-coumarin anticoagulants and their effects on tests of haemostasis: guidance from the British Committee for Standards in Haematology, Br. J. Haematol. 166 (2014) 830–841.

[15] B.T. Samuelson, A. Cuker, Measurement and reversal of the direct oral anticoagulants, Blood Rev. 31 (2017) 77–84.

[16] A. Tripodi, To measure or not to measure direct oral anticoagulants before surgery or invasive procedures, J. Thromb. Haemost. 14 (2016) 1325–1327.

[17] S.T. Avecilla, C. Ferrell, W.L. Chandler, M. Reyes, Plasma-diluted thrombin time to measure dabigatran concentrations during dabigatran etexilate therapy, Am. J. Clin. Pathol. 137 (2012) 572–574.

[18] A. Cuker, D.M. Siegal, M.A. Crowther, D.A. Garcia, Laboratory measurement of the anticoagulant activity of the non-vitamin K oral anticoagulants, J. Am. Coll. Cardiol. 64 (2014) 1128–1139.

[19] S. Testa, A. Tripodi, C. Legnani, V. Pengo, R. Abbate, C. Dellanoce, P. Carraro, L. Salomone, R. Paniccia, O. Paoletti, D. Poli, G. Palareti, S.T.-L. Register, Plasma levels of direct oral anticoagulants in real life patients with atrial fibrillation: results observed in four anticoagulation clinics, Thromb. Res. 137 (2016) 178–183.

[20] J. Douxfils, A. Tamigniau, B. Chatelain, C. Chatelain, P. Wallemacq, J.M. Dogne, F. Mullier, Comparison of calibrated chromogenic anti-Xa assay and PT tests with LC-MS/MS for the therapeutic monitoring of patients treated with rivaroxaban, Thromb. Haemost. 110 (2013) 723–731.

[21] F. Al-Aieshy, R.E. Malmstrom, J. Antovic, A. Pohanka, Y. Ronquist-Nii, M. Berndtsson, F. Al-Khalili, M. Skeppholm, Clinical evaluation of laboratory methods to monitor exposure of rivaroxaban at trough and peak in patients with atrial fibrillation, Eur. J. Clin. Pharmacol. 72 (2016) 671–679.

[22] M.M. Samama, J.L. Martinoli, L. LeFlem, C. Guinet, G. Plu-Bureau, F. Depasse, E. Perzborn, Assessment of laboratory assays to measure rivaroxaban—an oral, direct factor Xa inhibitor, Thromb. Haemost. 103 (2010) 815–825.

[23] J.P. Collet, T. Cuisset, G. Range, G. Cayla, S. Elhadad, C. Pouillot, P. Henry, P. Motreff, D. Carrie, Z. Boueri, L. Belle, E. Van Belle, H. Rousseau, P. Aubry, J. Monsegu, P. Sabouret, S.A. O'Connor, J. Abtan, M. Kerneis, C. Saint-Etienne, O. Barthelemy, F. Beygui, J. Silvain, E. Vicaut, G. Montalescot, A. Investigators, Bedside monitoring to adjust antiplatelet therapy for coronary stenting, N. Engl. J. Med. 367 (2012) 2100–2109.

[24] M.J. Price, P.B. Berger, P.S. Teirstein, J.F. Tanguay, D.J. Angiolillo, D. Spriggs, S. Puri, M. Robbins, K.N. Garratt, O.F. Bertrand, M.E. Stillabower, J.R. Aragon, D.E. Kandzari, C.T. Stinis, M.S. Lee, S.V. Manoukian, C.P. Cannon, N.J. Schork, E.J. Topol, G. Investigators, Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial, JAMA 305 (2011) 1097–1105.

[25] E. Perzborn, D. Kubitza, F. Misselwitz, Rivaroxaban. A novel, oral, direct factor Xa inhibitor in clinical development for the prevention and treatment of thromboembolic disorders, Hamostaseologie. 27 (2007) 282–289.

[26] R.J. Wagenvoord, J. Deinum, M. Elg, H.C. Hemker, The paradoxical stimulation by a reversible thrombin inhibitor of thrombin generation in plasma measured with thrombinography is caused by alpha-macroglobulin-thrombin, J. Thromb. Haemost. 8 (2010) 1281–1289.

[27] I.V. Gribkova, E.N. Lipets, I.G. Rekhtina, A.I. Bernakevich, D.B. Ayusheev, R.A. Ovsepyan, F.I. Ataullakhanov, E.I. Sinauridze, The modification of the thrombin generation test for the clinical assessment of dabigatran etexilate efficiency, Sci. Rep. 6 (2016) 29242.

[28] H.C. Hemker, P. Giesen, R. Al Dieri, V. Regnault, E. de Smedt, R. Wagenvoord, T. Lecompte, S. Beguin, Calibrated automated thrombin generation measurement in clotting plasma, Pathophysiol. Haemost. Thromb. 33 (2003) 4–15.

[29] H.C. Hemker, P.W. Hemker, R. Al Dieri, The technique of measuring thrombin generation with fluorescent substrates: 4. The H-transform, a mathematical procedure to obtain thrombin concentrations without external calibration, Thromb. Haemost. 101 (2009) 171–177.

[30] J. Douxfils, F. Mullier, C. Loosen, C. Chatelain, B. Chatelain, J.M. Dogne, Assessment of the impact of rivaroxaban on coagulation assays: laboratory recommendations for the monitoring of rivaroxaban and review of the literature, Thromb. Res. 130 (2012) 956–966.

[31] J. Douxfils, B. Chatelain, P. Hjemdahl, B. Devalet, A.L. Sennesael, P. Wallemacq, Y. Ronquist-Nii, A. Pohanka, J.M. Dogne, F. Mullier, Does the Russell viper venom time test provide a rapid estimation of the intensity of oral anticoagulation? A cohort study, Thromb. Res. 135 (2015) 852–860.

[32] S. Lessire, J. Douxfils, L. Pochet, A.S. Dincq, A.S. Larock, M. Gourdin, J.M. Dogne, B. Chatelain, F. Mullier, Estimation of rivaroxaban plasma concentrations in the perioperative setting in patients with or without heparin bridging, Clin. Appl. Thromb. Hemost. 24 (2016) 129–138 1076029616675968.

[33] J.W. Kennedy, R.N. Carey, R.B. Coolen, C.C. Garber, H.T. Lee, J.B. Levine, I.M. Osberg, Evaluation of Precision Performance of Clinical Chemistry Devices; Approved Guideline, 19 NCCLS, 1999.

[34] C.G. Santos-Gallego, J. Bayon, J.J. Badimon, Thrombi of different pathologies: implications for diagnosis and treatment, Curr. Treat Options Cardiovasc. Med. 12 (2010) 274–291.

[35] L.J. van Pelt, M.V. Lukens, S. Testa, B. Chatelain, J. Douxfils, F. Mullier, The DaXa-inhibition assay: a concept for a readily available, universal aXa assay that measures the direct inhibitory effect of all anti-Xa drugs, Thromb. Res. 168 (2018) 63–66.

[36] J. Douxfils, R.C. Gosselin, Laboratory assessment of direct oral anticoagulants, Semin. Thromb. Hemost. 43 (2017) 277–290.

[37] R.C. Gosselin, D. Adcock, E.M. Hawes, S.J. Francart, R.P. Grant, S. Moll, Evaluating the use of commercial drug-specific calibrators for determining PT and APTT reagent sensitivity to dabigatran and rivaroxaban, Thromb. Haemost. 113 (2015) 77–84.

[38] R. Al Dieri, F. Peyvandi, E. Santagostino, M. Giansily, P.M. Mannucci, J.F. Schved, S. Beguin, H.C. Hemker, The thrombogram in rare inherited coagulation disorders: its relation to clinical bleeding, Thromb. Haemost. 88 (2002) 576–582.

[39] Y. Dargaud, S. Beguin, A. Lienhart, R. Al Dieri, C. Trzeciak, J.C. Bordet, H.C. Hemker, C. Negrier, Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B, Thromb. Haemost. 93 (2005) 475–480.

[40] A. van Hylckama Vlieg, S.C. Christiansen, R. Luddington, S.C. Cannegieter, F.R. Rosendaal, T.P. Baglin, Elevated endogenous thrombin potential is associated with an increased risk of a first deep venous thrombosis but not with the risk of recurrence, Br. J. Haematol. 138 (2007) 769–774.

[41] R. Al Dieri, B. de Laat, H.C. Hemker, Thrombin generation: what have we learned? Blood Rev. 26 (2012) 197–203.

[42] J. Graff, N. von Hentig, F. Misselwitz, D. Kubitza, M. Becka, H.K. Breddin, S. Harder, Effects of the oral, direct factor Xa inhibitor rivaroxaban on platelet-induced thrombin generation and prothrombinase activity, J. Clin. Pharmacol. 47 (2007) 1398–1407.

[43] R. Artang, M. Anderson, P. Riley, J.D. Nielsen, Assessment of the effect of direct oral anticoagulants dabigatran, rivaroxaban, and apixaban in healthy male volunteers using a thrombin generation assay, Res. Pract. Thromb. Haemost. 1 (2017) 194–201.
[44] S.J. Connolly, M.D. Ezekowitz, S. Yusuf, J. Eikelboom, J. Oldgren, A. Parekh, J. Pogue, P.A. Reilly, E. Themeles, J. Varrone, S. Wang, M. Alings, D. Xavier, J. Zhu, R. Diaz, B.S. Lewis, H. Darius, H.C. Diener, C.D. Joyner, L. Wallentin, Dabigatran versus warfarin in patients with atrial fibrillation, N. Engl. J. Med. 361 (2009) 1139–1151.
[45] M.R. Patel, K.W. Mahaffey, J. Garg, G. Pan, D.E. Singer, W. Hacke, G. Breithardt, J.L. Halperin, G.J. Hankey, J.P. Piccini, R.C. Becker, C.C. Nessel, J.F. Paolini, S.D. Berkowitz, K.A. Fox, R.M. Califf, Rivaroxaban versus warfarin in nonvalvular atrial fibrillation, N. Engl. J. Med. 365 (2011) 883–891.
[46] R.P. Giugliano, C.T. Ruff, E. Braunwald, S.A. Murphy, S.D. Wiviott, J.L. Halperin, A.L. Waldo, M.D. Ezekowitz, J.I. Weitz, J. Spinar, W. Ruzyllo, M. Ruda, Y. Koretsune, J. Betcher, M. Shi, L.T. Grip, S.P. Patel, I. Patel, J.J. Hanyok, M. Mercuri, E.M. Antman, E.A.-T. Investigators, Edoxaban versus warfarin in patients with atrial fibrillation, N. Engl. J. Med. 369 (2013) 2093–2104.
[47] Y. Bosch, R. Al Dieri, H. ten Cate, P. Nelemans, S. Bloemen, C. Hemker, P. Weerwind, J. Maessen, B. Mochtar, Preoperative thrombin generation is predictive for the risk of blood loss after cardiac surgery: a research article, J. Cardiothorac. Surg. 8 (2013) 154.
[48] Y. Dargaud, A. Lienhart, S. Meunier, O. Hequet, H. Chavanne, V. Chamouard, S. Marin, C. Negrier, Major surgery in a severe haemophilia A patient with high titre inhibitor: use of the thrombin generation test in the therapeutic decision, Haemophilia. 11 (2005) 552–558.
[49] G. Hron, M. Kollars, B.R. Binder, S. Eichinger, P.A. Kyrle, Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation, JAMA 296 (2006) 397–402.
[50] A. Tripodi, C. Legnani, V. Chantarangkul, B. Cosmi, G. Palareti, P.M. Mannucci, High thrombin generation measured in the presence of thrombomodulin is associated with an increased risk of recurrent venous thromboembolism, J. Thromb. Haemost. 6 (2008) 1327–1333.
[51] A. Tripodi, I. Martinelli, V. Chantarangkul, T. Battaglioli, M. Clerici, P.M. Mannucci, The endogenous thrombin potential and the risk of venous thromboembolism, Thromb. Res. 121 (2007) 353–359.
[52] C.G. Santos-Gallego, L. Badimon, J.J. Badimón, Perspectives: direct and specific inhibition of factor Xa: an emerging therapeutic strategy for atherothrombotic disease, Eur. Heart J. Suppl. 16 (2014) A56–A60.
[53] M. Skeppholm, A. Kallner, K. Malmqvist, M. Blomback, H. Wallen, Is fibrin formation and thrombin generation increased during and after an acute coronary syndrome? Thromb. Res. 128 (2011) 483–489.