

Ex vivo effects of low-dose rivaroxaban on specific coagulation assays and coagulation factor activities in patients under real life conditions

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Summary

Global coagulation assays display variable effects at different concentrations of rivaroxaban. The aim of this study is to quantify the *ex vivo* effects of low-dose rivaroxaban on thrombophilia screening assays and coagulation factor activities based on the administration time, and to show how to mask possible interferences. Plasma samples from 40 patients receiving rivaroxaban 10 mg daily were investigated to measure activities of clotting factor II, V, VII, VIII, IX, XI, XII and XIII; protein C- and protein S-levels; lupus anticoagulants; anticardiolipin IgG and IgM; D-dimer, heparin-platelet factor 4 (HPF4) antibodies and screening tests for von Willebrand disease (VWD). Two hours after rivaroxaban administration, the activities of clotting factors were significantly decreased to different extents, except for factor XIII. Dilution of plasma samples resulted in neutralisation of these interferences. The chromogenic protein C activity assay was not affected by rivaroxaban. Depending on the timing of tablet intake in relation to blood

sampling protein S activity was measured falsely high when a clotting assay was used. False-positive results for lupus anticoagulants were observed depending on the assay system used and the administration time of rivaroxaban. ELISA-based assays such as anticardiolipin IgG and IgM, D-dimer, HPF4-antibodies and the turbidimetric assays for VWD were not affected by rivaroxaban. Specific haemostasis clotting tests should be performed directly prior to rivaroxaban intake. Assay optimisation in the presence of rivaroxaban can be achieved by plasma dilution. Immunologic assays are not influenced by rivaroxaban, while chromogenic assays can be used, when they do not depend on factor Xa.

Keywords

Clotting factor activity, D-dimer, lupus anticoagulants, protein C, protein S, rivaroxaban, von Willebrand disease

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Introduction

Rivaroxaban, the first orally administered direct factor Xa inhibitor, binds competitively and reversibly to free factor Xa and factor Xa bound in the prothrombinase complex (1–3). This mode of action induces a prolongation of prothrombin time (PT) and of activated partial thromboplastin time (aPTT) which correlates with rivaroxaban plasma concentrations, as has been demonstrated in several studies (4–8). Global clotting assays are performed to screen the functions of extrinsic and intrinsic pathways to provide an overview of coagulation factor activities. In addition, factor assays may be necessary if pathological PT and aPTT are discovered during pre- and postoperative screening. Factor activity is often determined in a modified PT or aPTT assay system in combination with corresponding factor-deficient plasma. Therefore measurement of factor activities can be influenced by rivaroxaban, which has recently been demonstrated (8, 9).

The influence of vitamin K antagonists on thrombophilia screening is well documented. For instance, accurate detection of

lupus anticoagulant (LA) in patients given vitamin K antagonist therapy is difficult due to the coagulation defect induced by the anticoagulants, which leads to prolongation of all phospholipid-dependent clotting times.

The influence of rivaroxaban on thrombophilia screening assays including determination of protein C, protein S and lupus anticoagulant activity depending on the methods used is described in few studies (9–11). Merriman et al. (11) demonstrated that patients receiving rivaroxaban yielded false-positive LA test results.

When these alterations in hospitals are observed, concerns may arise regarding a potential bleeding risk in the case of reduced factor activities or an increased risk for developing thrombosis in the case of positive lupus anticoagulants.

Clinicians should be able to accurately interpret coagulation parameters in patients taking new anticoagulants. However, by focusing on rivaroxaban, the influences on thrombophilia screening assays and clotting factor activities have mainly been documented by *in vitro* studies and may be underestimated in clinical practice.

Therefore, we investigated in this study the time-dependent effects after oral intake of low-dose rivaroxaban on factor activities and thrombophilia screening assays in patients under real life conditions.

Methods

Patients

Between December 2009 and Mai 2010 *ex vivo* plasma samples were obtained from 40 consecutive patients treated with 10 mg of rivaroxaban once daily postoperatively after hip or knee replacement surgery. This patient population was chosen since – at the time the study had been performed – rivaroxaban was only licensed for postoperative thrombosis prophylaxis after major orthopaedic surgery in Europe.

According to our previously published study protocol (7), blood samples were collected at different time points with respect

to drug intake. Blood samples were collected when rivaroxaban had reached a steady-state level (four to five days after surgery). In this study we included the plasma samples collected exactly 2 hours (h) after rivaroxaban dosing, 12 h after dosing, and 24 h after dosing (immediately prior to the next administration of rivaroxaban).

The study was performed in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the University Hospital Frankfurt. The unique ClinicalTrials.gov identifier is NCT00986635.

Platelet-poor plasma (PPP)

PPP was obtained by two immediate sequential centrifugations executed at room temperature at 1,500 x g for 15 minutes. The resultant supernatant plasma samples were stored at –80°C until use.

Table 1: An overview of the various assays used in this study.

Analyte	Method	Coagulation Analyzer (Manufacturer)
Factor II, Factor V, Factor VII, Factor X	• Innovin® (Siemens Healthcare Diagnostics, Marburg, Germany)	Clotting BCS® (Siemens Healthcare Diagnostics, Marburg, Germany)
Factor VIII, Factor IX, Factor XI, Factor XII	• SynthASil® (Instrumentation Laboratory, Lexington, MA, USA)	Clotting ACL Top® (Instrumentation Laboratory, Lexington, MA, USA)
Factor VIII	• Factor VIII Chromogenic test kit (Siemens Healthcare Diagnostics, Marburg, Germany)	Chromogenic BCS® (Siemens Healthcare Diagnostics, Marburg, Germany)
Factor XIII	• Berichrom® Factor XIII test kit (Siemens Healthcare Diagnostics, Marburg, Germany)	Chromogenic BCS® (Siemens Healthcare Diagnostics, Marburg, Germany)
Protein S	• STA Protein S (Diagnostica Stago, Asnières, France)	Clotting ACL Top® (Instrumentation Laboratory, Lexington, MA, USA)
Free Protein S Antigen	• HemosIL® Free ProteinS test kit (Instrumentation Laboratory, Lexington, MA, USA)	Immuno-turbidimetric ACL Top® (Instrumentation Laboratory, Lexington, MA, USA)
Protein C	• HemosIL® Protein C test kit (Instrumentation Laboratory, Lexington, USA)	Chromogenic ACL Top® (Instrumentation Laboratory, Lexington, MA, USA)
D-Dimer	• STA –LIATEST D-DI (Diagnostica Stago, Asnières, France)	Immuno-turbidimetric STA-R evolution® (Diagnostica Stago, Asnières, France)
Heparin-platelet factor-4 antibodies	• Stago Asserachrom® HPIA (Diagnostica Stago, Asnières, France)	ELISA -
Anti-Cardiolipin	• Anticardiolipin IgG/IgM (ORGENTEC Diagnostica GmbH, Mainz, Germany)	ELISA -
Lupus anticoagu- lants	• Mixcon-LA-test (Instrumentation Laboratory, Lexington, MA, USA) • DRVVT-LAC screen/confirm (Instrumentation Laboratory, Lexington, MA, USA)	Clotting ACL Top® (Instrumentation Laboratory, Lexington, MA, USA) Clotting
Von Willebrand Disease	• BC vWF • vWF Ag* (both: Siemens Healthcare Diagnostics, Marburg, Germany)	Platelet agglutination Immuno-turbidimetric BCS® (Siemens Healthcare Diagnostics, Marburg, Germany)

HPLC-MS/MS

The rivaroxaban concentrations of the plasma samples were validated by HPLC-MS/MS at Bayer Pharma AG, Wuppertal, Germany, according to Rohde et al. (12) as described in the Suppl. Material (available online at www.thrombosis-online.com).

Specific coagulation assays

A summary of the test reagents used in this study is shown in ► Table 1. A detailed description of the used test systems is fea-

ured in the Suppl. Material (available online at www.thrombosis-online.com). All measurements were performed by an experienced medical technician.

Clotting factor activities

The exogenous activities of factors II, V, VII and X were determined in a modified prothrombin time (PT)-test, the endogenous factor activities of factors VIII, IX, XI and XII were determined by means of a modified activated partial thromboplastin time

Table 2: Clotting factor activities and specific coagulation parameters obtained directly prior rivaroxaban administration, 2 hours and 12 hours after rivaroxaban administration in plasma samples of 40 patients receiving rivaroxaban 10 mg od (steady state).

Analyte (Normal range)	Mean ± SD Median (range)			Significance between values obtained 2 hours and 24 hours after drug administration
	day 4 – 5 postoperatively after rivaroxaban administration (10 mg/od)			
	2 hours	12 hours	24 hours (= prior drug intake)	
Rivaroxaban-concentration measured by HPLC-MS/MS [ng/ml]	155.7 ± 56.3 153.5 (24.0 – 283.0)	27.9 ± 17.2 23.4 (6.3 – 101.0)	13.6 ± 8.9 12.2 (1.8 – 43.5)	p < 0.001
Factor II (80 – 130 %)	118.6 ± 17.9 116.8 (85.1 – 156.8)	131.2 ± 18.1 130.8 (100.9 – 173.2)	128.9 ± 19.4 127.4 (97.2 – 167.1)	p < 0.05
Factor V (80 – 130 %)	117.9 ± 21.9 117.8 (72.2 – 161.5)	137.9 ± 21.7 143.9 (99.8 – 185.6)	138.5 ± 21.4 142.5 (92.4 – 179.8)	p < 0.001
Factor VII (80 – 130 %)	116.3 ± 32.1 115.2 (55.9 – 189.5)	136.2 ± 26.5 136.1 (74.4 – 206.1)	140.0 ± 29.5 139.3 (78.7 – 206.1)	p < 0.001
Factor X (80 – 130 %)	96.4 ± 21.8 94.9 (55.4 – 158.3)	116.8 ± 18.9 114.6 (74.3 – 154.3)	116.8 ± 20.5 114.2 (69.3 – 163.8)	p < 0.001
Factor VIII (60 – 150 %)	121.7 ± 26.8 120.0 (59.0 – 173.0)	145.2 ± 27.3 144.0 (90.0 – 210.0)	149.9 ± 29.4 148.5 (96.0 – 200.0)	p < 0.001
Factor IX (65 – 150 %)	127.1 ± 23.2 120.0 (90.0 – 184.0)	160.1 ± 22.6 157.0 (114.0 – 210.0)	167.5 ± 22.4 167.5 (122.0 – 236.0)	p < 0.001
Factor XI (65 – 150 %)	106.5 ± 23.6 104.0 (67.0 – 174.0)	135.6 ± 25.2 134.0 (98.0 – 200.0)	136.5 ± 25.2 136.0 (88.0 – 208.0)	p < 0.001
Factor XII (50 – 150 %)	72.5 ± 17.7 74.0 (32.0 – 109.0)	84.9 ± 19.4 88.0 (40.0 – 125.0)	86.0 ± 18.4 87.0 (40.0 – 117.0)	p < 0.001
Factor XIII (70 – 140 %)	70.8 ± 14.9 73.3 (26.7 – 103.4)	70.8 ± 16.1 71.1 (21.7 – 108.5)	71.6 ± 13.5 72.2 (34.1 – 98.2)	not significant
Protein C (chromogenic) (> 70 %)	113.5 ± 16.9 114.0 (81.0 – 168.0)	121.3 ± 17.9 122.0 (89.0 – 188.0)	112.7 ± 18.6 108.5 (81.0 – 180.0)	not significant
Free Protein S- antigen (> 68 %)	87.0 ± 18.6 83.0 (61.1 – 135.3)	88.7 ± 22.7 84.6 (36.8 – 139.2)	86.7 ± 19.7 82.0 (57.5 – 130.1)	not significant
Protein S-activity (> 72 %)	108.4 ± 23.4 108.4 (53.1 – 175.8)	86.0 ± 19.5 84.1 (53.4 – 143.5)	78.7 ± 18.9 77.0 (39.1 – 114.1)	p < 0.001
D-Dimer (< 5 ug/ml)	2.02 ± 0.97 1.89 (0.88 – 6.06)	2.31 ± 0.98 2.06 (0.95 – 5.19)	1.84 ± 0.83 1.72 (0.78 – 5.08)	not significant
HPF-4 (< 1.0 U/ml)	0.08 ± 0.05 0.07 (0.04 – 0.29)	0.09 ± 0.06 0.07 (0.03 – 0.30)	0.08 ± 0.06 0.06 (0.03 – 0.30)	not significant

Table 2: Continued

Analyte (Normal Range)	Mean \pm SD Median (range)			Significance between values obtained 2 hours and 24 hours after drug administration
	day 4 – 5 postoperatively after rivaroxaban administration (10 mg/od)			
	2 hours	12 hours	24 hours (= prior drug intake)	
Anti-Cardiolipin IgG (< 20 U/ml)	1.83 \pm 2.4 0.80 (0.1 – 9.6)	1.83 \pm 2.4 0.60 (0.1 – 8.7)	1.98 \pm 2.9 0.90 (0.1 – 15.5)	not significant
IgM < 20 U/ml)	1.46 \pm 1.6 1.10 (0.1 – 8.4)	1.53 \pm 1.6 0.95 (0.20 – 8.3)	1.46 \pm 1.6 0.95 (0.1 – 7.7)	not significant
Lupus Anticoagulants				
DRVVT Ratio (0.94– 1.30)	1.68 \pm 0.2 1.71 (1.18 – 2.30)	1.32 \pm 0.15 1.29 (1.14 – 1.67)	1.24 \pm 0.12 1.22 (1.04 – 1.58)	p < 0.001
MIXCON Ratio (0.95 – 1.12)	0.99 \pm 0.09 0.99 (0.78 – 1.27)	1.02 \pm 0.10 1.01 (0.82 – 1.29)	0.98 \pm 0.08 0.99 (0.80 – 1.21)	not significant
vWD vWF Ag* (50–160 %)	251.5 \pm 74.8 259.0 (89.7 – 452.6)	256.0 \pm 66.5 260.0 (89.1 – 457.4)	252.9 \pm 71.3 252.9 (78.8 – 432.9)	not significant
BC vWF (58–172 %)	246.3 \pm 68.3 253.3 (80.9 – 434.5)	248.7 \pm 63.4 253.6 (85.3 – 410.9)	256.4 \pm 88.6 251.7 (35.4 – 578.4)	not significant

(aPTT)-test, both in combination with the respective coagulation factor-deficient plasma.

The factor parallelism feature was applied for factors VIII and X on the coagulation analyser ACL Top® (Instrumentation Laboratories, Lexington, MA, USA). Factor parallelism is a procedure developed to allow the increase of quality of clotting factor test results by detection of interference to the test measuring at least three dilutions of the plasma samples. The standard plasma dilution for determination of clotting factor activities was 1:10 in Factor Diluent (HemosIL, Instrumentation Laboratories; 0.1% sodium azide in saline solution). For factor VIII and X activity tests, the plasma samples were measured at subsequent two, respectively three dilutions: 1:40; 1:80 and 1:160.

For these factor parallelism investigations plasma samples a subgroup of 10 consecutive patients were included.

Additionally, we determined factor VIII activity using a chromogenic assay from Siemens Healthcare Diagnostics (Marburg, Germany). In this test kit, the factor VIII in the sample is activated by thrombin. Activated factor VIII activates factor X and accelerates the conversion of factor X in the presence of factor IXa. The factor Xa activity is assessed by hydrolysis of a chromogenic substrate, which is specific for factor Xa.

This assay was performed by measuring a 1:62 and 1:124 dilution of plasma samples of 10 consecutive patients.

Factor XIII activity was determined by a chromogenic assay using a substrate, which is specific for factor XIII.

Protein S and protein C activities

Protein S, the cofactor of activated protein C, was measured using a clotting assay.

The free protein S antigen concentration was determined using an immunoturbidimetric assay. Protein C activity was determined using a chromogenic assay.

D-dimer

For the quantitative determination of D-dimer an immuno-turbidimetric test system was used.

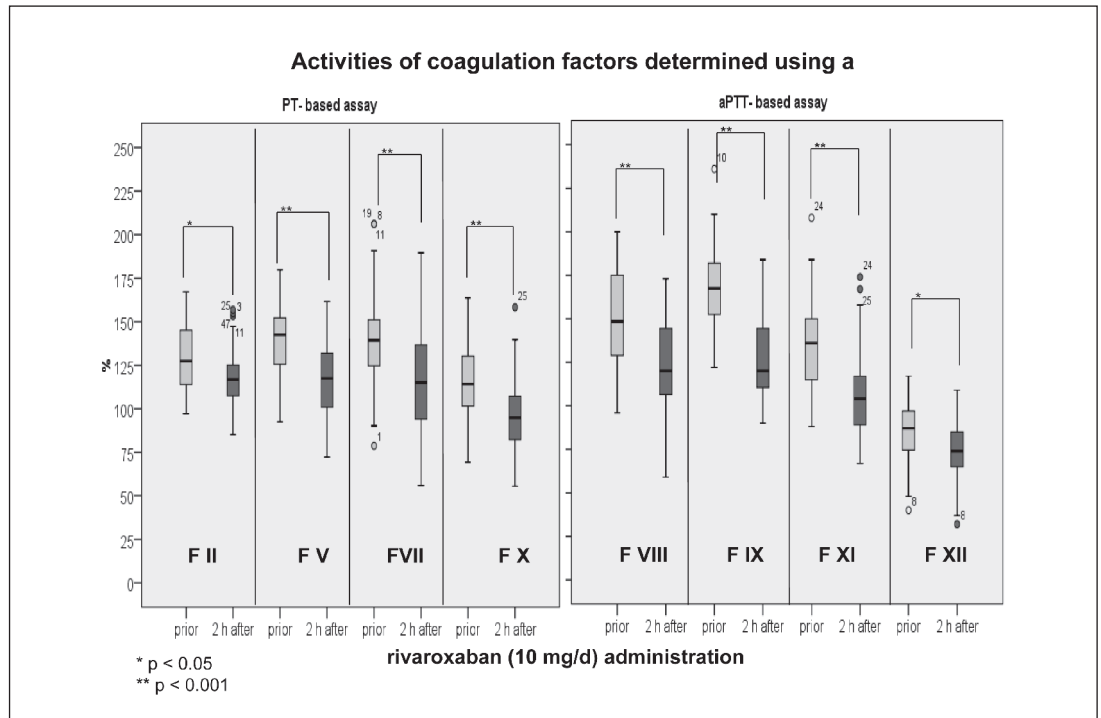
Heparin-platelet factor 4 antibodies

The presence of the HPF4 antibodies was confirmed using an enzyme-linked immunosorbent assay.

Anti-cardiolipin antibodies

Detection of antibodies against cardiolipin as an important criterion for the diagnosis of antiphospholipid syndrome (APS) and determination of IgG or IgM isotypes were performed using ELISA kits.

Figure 1: Time-dependent influence of rivaroxaban (before and 2 hours after rivaroxaban administration) on activities of clotting factors II, V, VII, X, VII, IX, XI and XII determined using modified PT and aPTT-based assay systems in plasma samples of 40 patients receiving rivaroxaban 10 mg od (steady state).



Lupus anticoagulants

Lupus anticoagulants (LA) were determined according to the criteria of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (13). The screening assays included the MIXCON-LA (Mixing and Confirmation Procedure) assay (14) and the DRVVT-LAC (Diluted Russell's Viper Venom Test) assay.

The MIXCON-LA assay uses diluted (1:1 with normal plasma) patient's plasma, activated, LA-insensitive partial thromboplastin time (SynthAFax®) and the LA-sensitive aPTT reagent (HemosIL™ aPTT-SP, Instrumentation Laboratories) for detection of LA.

The DRVVT-LAC uses undiluted plasma samples, simplified and phospholipid-rich DRVV reagents (LAC Screen and LAC Confirm) for detection of LA.

Additionally, we performed the DRVVT-LAC assay in diluted plasma samples (1:1 with normal plasma) in a subgroup of 10 consecutive patients.

Von Willebrand factor

In the presence of ristocetin, the von Willebrand factor in the plasma sample causes agglutination of stabilised platelets. This agglutination process reduces the turbidity, which can be detected by the coagulation analyser.

Samples containing vWF antigen are aggregated with small polystyrene particles to which specific polyclonal antibodies against vWF have been attached. The aggregation is detected by the change in optical density.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 17.0, Chicago, IL, USA). Descriptive statistics (frequencies, means and standard deviations, medians and ranges) were calculated, along with the paired Friedman test to compare the results obtained at different blood collection times. The criterion for statistical significance was a p-value less than 0.05. Results are also presented as box plots, with the bare length indicating the interquartile range (25th–75th percentile). Outliers are defined as values differing from 1.5–3.0 bare lengths, whereas extreme values are those differing >3.0 box lengths from the upper or lower edges of the box. In the figures, outliers are illustrated as circles and extreme values as stars. The numbers within the figures represent patient identities.

Results

HPLC–MS/MS

Plasma samples collected 2 h after rivaroxaban administration showed a great variability ranging between 24.0 and 283.0 ng/ml (see ► Table 2).

Plasma samples collected 12 h after administration contained concentrations ranging from 6.3 to 101.0 ng/ml.

Plasma samples collected directly before rivaroxaban administration exhibited the lowest concentrations of rivaroxaban ranging between 1.8 and 43.5 ng/ml.

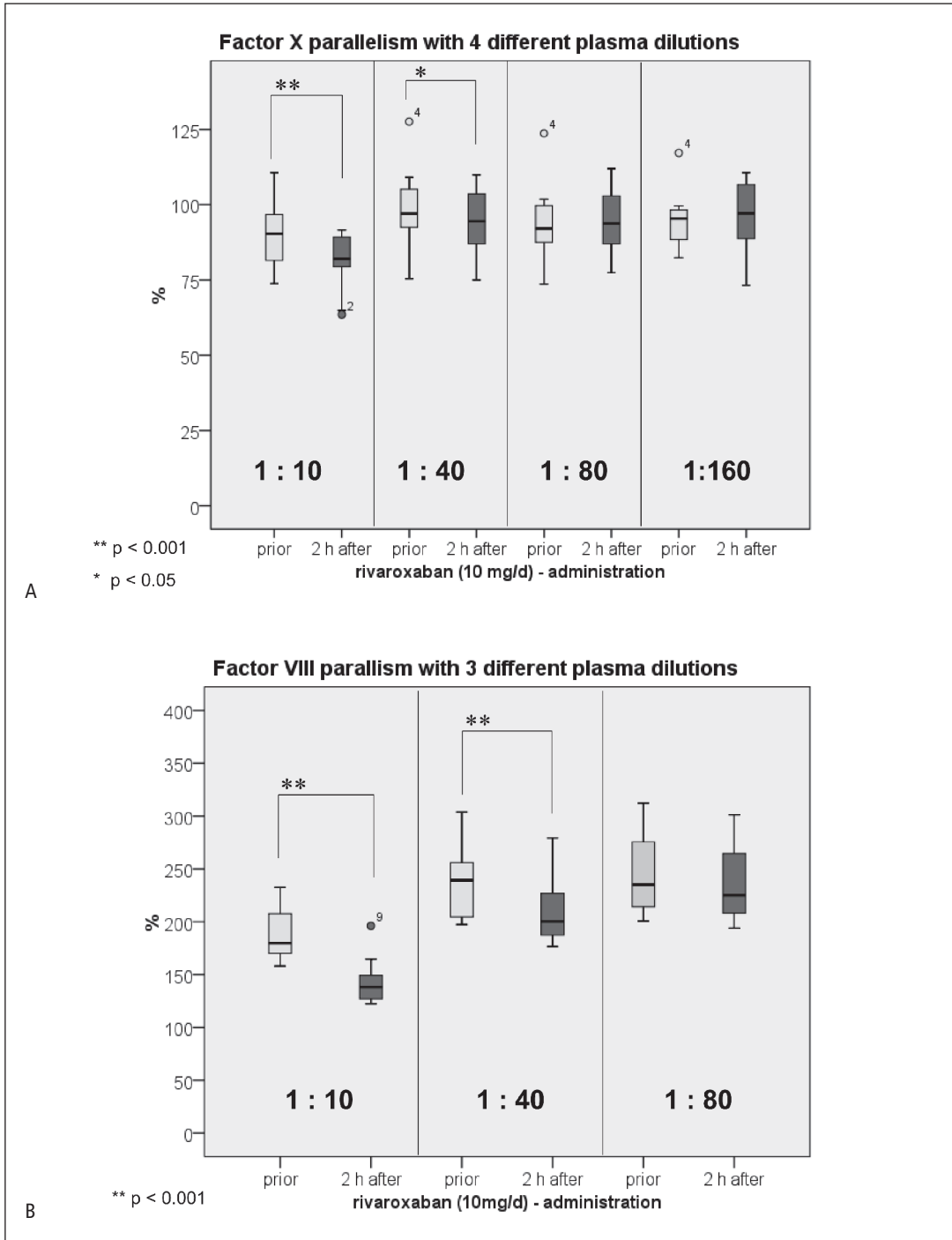


Figure 2: Influence of plasma dilutions on the interference of rivaroxaban with clotting based factor X- and factor VIII-assays in plasma samples of 10 patients receiving rivaroxaban 10 mg od (A and B). See opposite page for panel C.

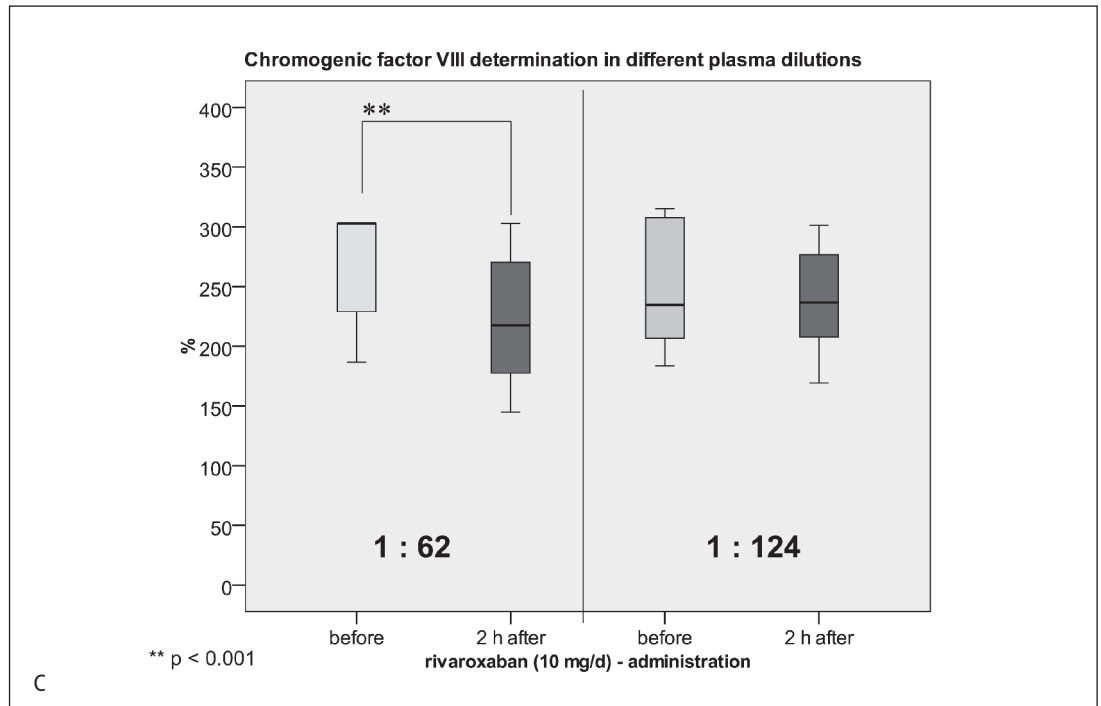
Activities of the clotting factors II, V, VII, VIII, IX, X, XI, and XII

As seen in ► Figure 1, 2 h after rivaroxaban administration, significant decreases of all investigated factors were observed. The effect of rivaroxaban diminished 12 h after drug administration (see ► Table 2).

Factor VIII and factor X parallelism

In ► Figure 2A and B the influence of plasma dilutions on the effect of rivaroxaban is demonstrated. The decrease of factor VIII and factor X activities induced by rivaroxaban is vanishing by increasing the dilution of the patient plasma.

Figure 2C: Influence of plasma dilutions on the interference of rivaroxaban with a chromogenic factor VIII-assay in plasma samples of 10 patients receiving rivaroxaban 10 mg od.



Chromogenic determination of factor VIII activity

The activity of factor VIII determined by the chromogenic assay was significantly decreased 2 h after drug intake (see ► Figure 2C). The decrease of the factor VIII activity induced by low-dose rivaroxaban can be neutralised by increasing the dilution of the patient plasma.

Activity of factor XIII

Based on the chromogenic assay for determination of factor XIII activity, no significant differences were observed at the different time intervals after rivaroxaban administration (see ► Table 2).

Protein C and protein S activities

Based on the chromogenic assay for the determination of functional protein C activity, no significant differences were observed at the time intervals after rivaroxaban administration (see ► Table 2). Determination of free protein S antigen concentrations revealed no significant differences after rivaroxaban administration.

Whereas, the clotting assay used to determine protein S activity revealed a significant prolongation of clotting time and an increase in protein S activity two hours after rivaroxaban administration compared to the data prior to administration or 12 h after administration (see ► Table 2).

Lupus anticoagulants

The ratio of the MIXCON-LA assay demonstrated no significant differences between the blood sampling time points, whereas the ratio of the DRVVT-LAC assay revealed a significant increase in LA activity 2 h after low-dose rivaroxaban administration compared to the data prior to administration or 12 h after administration (see ► Table 2 and ► Figure 3). The interference of rivaroxaban in the DRVVT-LAC assay cannot be abolished by 1:1 plasma dilution with normal plasma performed before analysis (see ► Figure 3).

D-dimer

The immuno-turbidimetric test system used to quantitatively determine D-dimer, revealed no significant difference at the different time intervals after rivaroxaban administration (see ► Table 2).

Heparin-platelet factor 4 antibodies

No significant differences were observed at the different time intervals after rivaroxaban using the ELISA test (see ► Table 2).

Anticardiolipin antibodies

Detection of antibodies against cardiolipin and determination of IgG or IgM isotypes revealed no significant differences two hours after rivaroxaban administration (see ► Table 2).

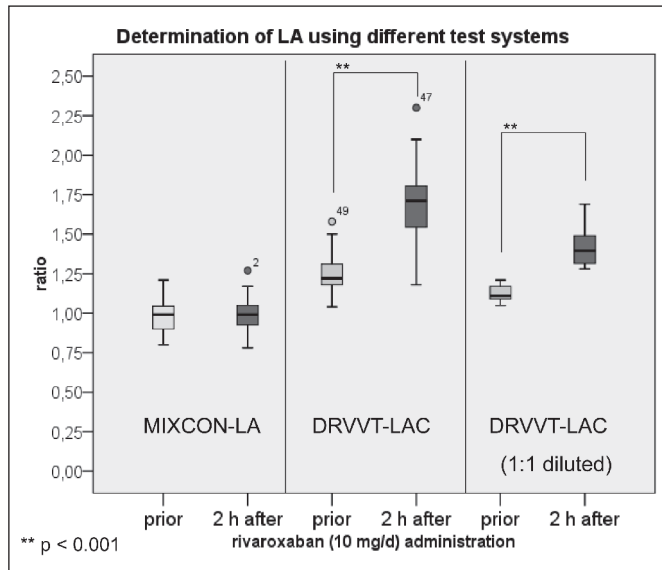


Figure 3: Time-dependent influence of rivaroxaban (before and 2 hours after rivaroxaban administration) on Lupus anticoagulants (LA) using different test methods (MIXCON-LA, DRVVT-LAC assay without and with 1:1 dilution) in plasma samples of 40 patients receiving rivaroxaban 10 mg od (diluted DRVVT-LAC assay: mixture of plasma samples of 10 patients receiving rivaroxaban 10 mg od with normal plasma).

Von Willebrand factor

The vWF activity, measured using the agglutination reagent containing stabilised platelets and ristocetin, and vWF antigen concentrations, measured using an immunoturbidimetric test kit, were not significantly influenced by rivaroxaban (see ► Table 2).

Discussion

To make accurate interpretations of global coagulation assays possible, it is essential that physicians understand exactly what impact rivaroxaban has on coagulation parameters such as PT or aPTT. Several studies have demonstrated that rivaroxaban had variable *in vitro* and *ex vivo* effects on routine coagulation tests (4–8). Different assays and even different reagents within an assay group display variable *in vitro* and *ex vivo* effects of rivaroxaban (4–8).

In case of pathological PT and aPTT during pre- and postoperative screening, additional factor assays may be performed, especially when postoperative blood loss or bleeding occurs.

Clotting factor activities were determined in modified PT- and aPTT-tests in combination with their respective coagulation factor-deficient plasma. In our previous *ex vivo* study (7), we demonstrated that the influence of rivaroxaban differs widely between the assay reagents used, as seen with the PT reagent Neoplastin® Plus, which, 2 h after rivaroxaban dosing, demonstrated a 1.4-fold increase within seconds, whereas the Innovin® reagent only exhibited a 1.2-fold increase within seconds after rivaroxaban dosing.

In this study, we wanted to quantify the *ex vivo* effects of low-dose rivaroxaban on clotting factor activities using less-sensitive reagents to the drug; therefore, the PT reagent Innovin® and the aPTT reagent SynthASil® were used. Moreover, the influence of rivaroxaban on specific coagulation clotting assays was evaluated in plasma samples of patients treated with low-dose rivaroxaban. Despite these considerations, the factor activity values were still significantly decreased during rivaroxaban administration. Reagents, which are more sensitive to rivaroxaban, may exhibit an even greater effect on coagulation factor measurements. Moreover, the effect of rivaroxaban on coagulation assays may be more pronounced when higher concentrations of rivaroxaban are present in plasma, as seen in the *in vitro* study of Gerotziafas et al. (9), in which PT- and aPTT-based factor assays were influenced by rivaroxaban in a linear, concentration-dependent manner.

When using different dilutions of plasma samples to measure factor VIII- and factor X- activities the rivaroxaban effects on factor determination vanished with increasing dilutions *ex vivo*. This is in accordance with the *in vitro* test results of the study of Gerotziafas et al. (9), demonstrating that dilution of plasma samples containing rivaroxaban can mask possible interferences on clot-based factor assays induced by rivaroxaban.

In contrast to the clotting assays used for the determination of factor activities, no influence of rivaroxaban was observed on factor XIII activities when using a chromogenic assay that is specific for factor XIII.

On the other hand, a concentration-dependent influence of rivaroxaban based on the administration time was observed in the *in vitro* study of Tichelaar et al. (10) using a chromogenic assay for determination of factor VIII activity. In our *ex vivo* study we confirmed the interference of rivaroxaban with a chromogenic factor VIII activity assay. This influence of rivaroxaban on chromogenic factor VIII assays is explained by the chromogenic substrate used in the test systems, which are not specific for factor VIII but for factor X. To optimise such assays we could demonstrate in a subgroup of patients that the interference of low-dose rivaroxaban can be abolished by additional plasma dilutions performed before analysis.

Thrombophilic assays may also be influenced by rivaroxaban depending on the assay system used. In our study the chromogenic protein C-activity determination was not affected by rivaroxaban, whereas the clotting assay for protein S activity was sensitive to rivaroxaban. A recently published *ex vivo* study in healthy volunteers confirms our findings on the influence of rivaroxaban on protein S activities and demonstrates that measurement of protein C activity with chromogenic assays is not influenced by the presence of rivaroxaban (8).

Rivaroxaban can also falsely increase antithrombin activity time-dependently, when a factor X-based assay is used, while this influence is not observed, when a factor II-based assay is performed. These results have been previously published by our group (7).

In addition, lupus anticoagulants were measured in this study using lupus-sensitive screening assays in combination with mixing and confirmation procedures. Assay systems were based on either aPTT or DRVVT, as suggested by the Scientific and Standard-

isation Committee of the International Society on Thrombosis and Haemostasis (13). Both assays are clotting tests, but only the DRVVT assay was significantly influenced by rivaroxaban intake. The observed influence of rivaroxaban on the DRVVT assay systems is in agreement with the study results of van Os et al. (15) and Merriman et al. (11), demonstrating that the DRVVT-LAC assay is a factor X-based assay which is sensitive to rivaroxaban even in low concentrations. These influences could not be abolished by performing a 1:1 plasma dilution with normal plasma.

In contrast, the interference of low-dose rivaroxaban could be abolished by 1:1 plasma dilutions with normal plasma performed when the MIXCON-LA (Mixing and Confirmation Procedure) assay was used. This assay is a normalised Lupus Ratio test involving integrated screening, mixing and confirmatory procedures that attains high inter-laboratory agreement regarding the presence or absence of LA (14). The test is performed with a 1:1 mixture with normal plasma to allow perfect specificity when patients with acquired factor deficiencies due to the use of oral anticoagulants (such as phenprocoumon) or patients with inherited factor deficiencies (such as haemophilia A) are investigated (16). An aPTT reagent, rich in phosphatidylethanolamine, which is known to be the most relevant phospholipid for the anticoagulant function of the protein C pathway (17), and calcium chloride with polybrene were used in the assay system; therefore, heparin treatment did not interfere with the test result. As seen in our study, low-dose rivaroxaban did also not influence this test system which might be primarily due to the 1:1 mixture with normal plasma and the aPTT reagents, which are less sensitive to low-dose rivaroxaban than the DRVVT reagents which directly activate factor X.

Clotting assays are still the most commonly performed tests in coagulation laboratories. Clot formation will only occur if a sufficient reaction force of factor Xa and thrombin is available. Patients treated with direct factor Xa inhibitors have a lower proportion of active factor Xa in their plasma. Therefore, it is important to correlate the variation of clotting test results with the rate of influx and the half-life of rivaroxaban and to interpret the different test results obtained time-dependently as the effects of drug administration.

In contrast immuno-turbidimetric test systems or ELISA assays used for the determination of free protein S antigen, D-dimer, HPP4 and anticardiolipin antibodies or in von Willebrand factor diagnosis are not influenced by rivaroxaban, either because these tests are specific for their targets or because the tests use FXa-independent pathways.

One limitation of our study is that the blood samples were collected only from patients receiving prophylactic doses of rivaroxaban (10 mg once daily) post-surgery. Patients receiving therapeutic doses of rivaroxaban for stroke prevention in atrial fibrillation or for treatment of deep venous thrombosis were not included in this study since these indications were not licensed for rivaroxaban treatment at the time when the blood samples were collected.

Because of postoperative blood-sampling, levels of most pro-coagulant haemostasis proteins were high and did therefore not appear to become abnormal whilst on low-dose rivaroxaban. This

What is known about this topic?

- Rivaroxaban has variable *in vitro* and *ex vivo* effects on different coagulation parameters.

What does this paper add?

- The *ex vivo* effects of low-dose rivaroxaban on thrombophilia screening assays and coagulation factor activities based on the administration time in patients is quantified under routine clinical circumstances.
- Optimisation of clotting assays when measuring plasma samples containing low-dose of rivaroxaban is described.

may give the wrong impression that these tests can be performed while patients are treated with rivaroxaban. It can be expected, that abnormal results would have arisen in patients if tested either in a period way beyond the surgical period or when therapeutic doses of rivaroxaban are applied.

In conclusion, specific haemostasis tests should be avoided in patients on rivaroxaban. If test results are needed, tests should be performed directly before tablet intake.

Chromogenic or immunologic assays may be preferable, if they are confirmed to be unaffected by rivaroxaban.

Optimisation of clotting based or chromogenic factor assays may be achieved by predilution of plasma samples containing rivaroxaban before measurement.

Further improvement of specific haemostasis assays is urgently needed to make random sampling testing in blood samples containing rivaroxaban possible and accurate.

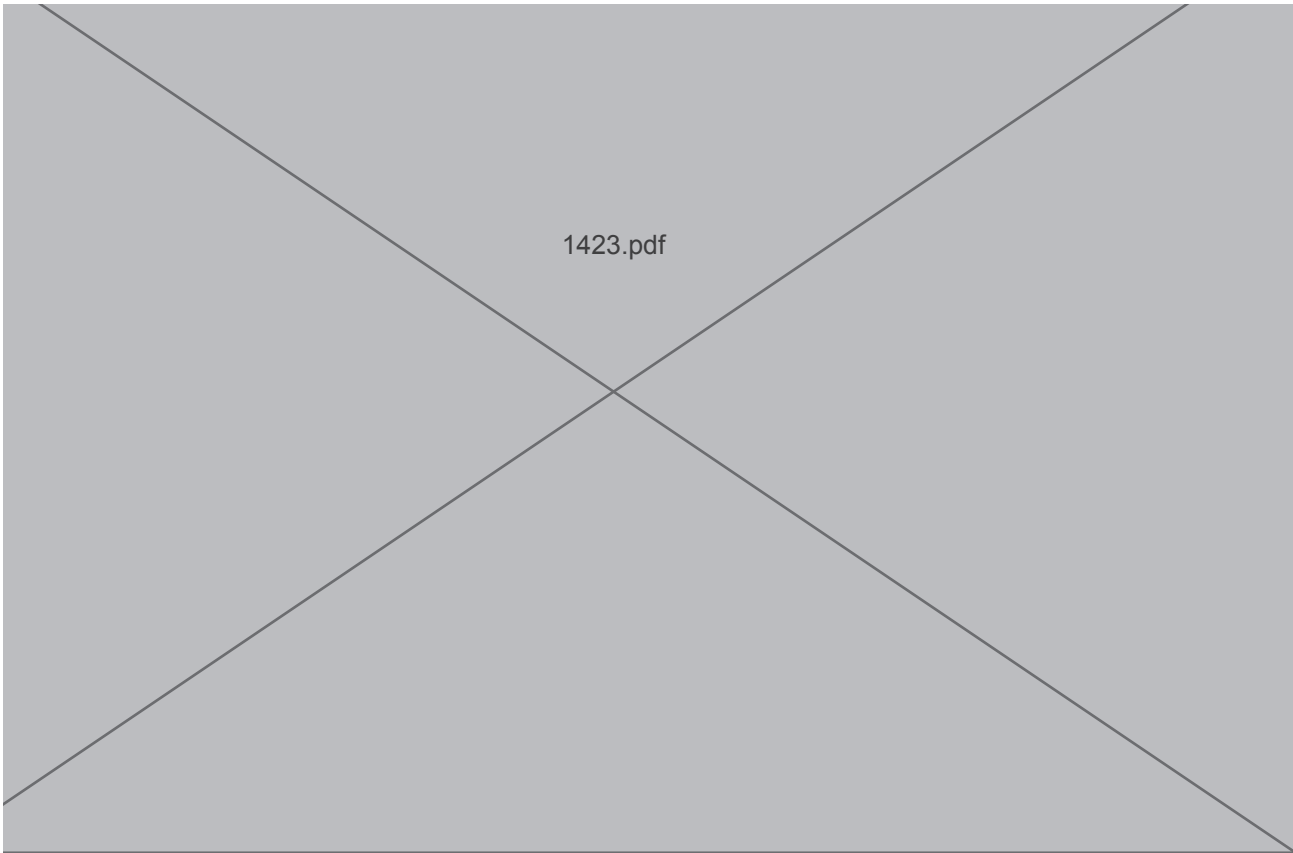
Conflicts of interest

E. Lindhoff-Last is a consultant to and has received honoraria for presentations from Bayer HealthCare and Instrumentation Laboratory. None of the other authors declare any conflicts of interest.

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