

## ORIGINAL ARTICLE

# Accuracy and consistency of anti-Xa activity measurement for determination of rivaroxaban plasma levels

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## Essentials

- Accurate determination of anticoagulant plasma concentration is important in clinical practice.
- We studied the accuracy and consistency of anti-Xa assays for rivaroxaban in a multicentre study.
- In a range between 50 and 200  $\mu\text{g L}^{-1}$ , anti-Xa activity correlated well with plasma concentrations.
- The clinical value might be limited by overestimation and intra- and inter-individual variation.

**Summary.** *Background:* Determining the plasma level of direct oral anticoagulants reliably is important in the work-up of complex clinical situations. *Objectives:* To study the accuracy and consistency of anti-Xa assays for rivaroxaban plasma concentration in a prospective, multicenter evaluation study employing different reagents and analytical platforms. *Methods:* Rivaroxaban 20 mg was administered once daily to 20 healthy volunteers and blood samples were taken at peak and trough levels (clinicaltrials.gov NCT01710267). Anti-Xa activity was determined in 10 major laboratories using different reagents and analyzers; corresponding rivaroxaban plasma concentrations were

measured by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS). *Findings:* Overall Pearson's correlation coefficient of anti-Xa levels and HPLC-MS results was 0.99 for Biophen<sup>®</sup> Heparin (95% CI, 0.99, 0.99), Biophen<sup>®</sup> DiXaI (95% CI, 0.99, 0.99) and STA<sup>®</sup> anti-Xa liquid (95% CI, 0.99, 1.00). Correlation was lower in rivaroxaban concentrations below 50  $\mu\text{g L}^{-1}$  and above 200  $\mu\text{g L}^{-1}$ . The overall bias of the Bland-Altman difference plot was 14.7  $\mu\text{g L}^{-1}$  for Biophen Heparin, 17.9  $\mu\text{g L}^{-1}$  for Biophen DiXaI and 19.0  $\mu\text{g L}^{-1}$  for STA anti-Xa liquid. Agreement between laboratories was high at peak level but limited at trough level. *Conclusions:* Anti-Xa activity correlated well with rivaroxaban plasma concentrations, especially in a range between 50 and 200  $\mu\text{g L}^{-1}$ . However, anti-Xa assays systematically overestimated rivaroxaban concentration as compared with HPLC-MS, particularly at higher concentrations. This overestimation, coupled with an apparent interindividual variation, might affect the interpretation of results in some situations.

**Keywords:** blood coagulation tests; drug monitoring; factor Xa inhibitors; reproducibility of results; rivaroxaban; sensitivity and specificity.

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## Introduction

Measuring plasma levels of direct oral anticoagulants (DOACs) reliably may be desirable in the work-up of complex clinical situations. DOACs have been developed to overcome major drawbacks of vitamin K antagonists (VKA), including their need for routine laboratory

monitoring. All substances demonstrated favorable characteristics, namely fast onset of effect, predictable pharmacokinetics and pharmacodynamics, a large therapeutic window, and a low risk of interaction with food [1–5]. Thus, all phase II/III clinical studies have been successfully conducted with a fixed dose and without laboratory monitoring [6–8]. In clinical practice we are, however, confronted with a variety of situations where knowledge of the drug level is important [6,9–11], particularly in the case of bleeding, thrombotic events or unplanned surgery. Renal impairment, liver failure or old age may facilitate accumulation of DOACs and drug interactions may occur, such as with antifungals. Other circumstances where knowledge of the DOAC plasma level is of interest include, for example, extreme bodyweight or alcohol intoxication. Its determination can therefore be crucial to clinical decision making.

The clinical value of any laboratory assay critically depends on its accuracy, precision and reproducibility. Chromogenic anti-Xa assays, based on the inhibition of a defined amount of exogenous factor Xa by the anticoagulant, were developed for the determination of rivaroxaban plasma level [12–14] and evaluated in a number of studies. Spiked samples were used to assess the correlation with known rivaroxaban plasma concentrations, determined by dilution techniques or high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS) [12,14,15]. The correlation of anti-Xa activity and rivaroxaban plasma concentration has also been studied in samples of patients or healthy volunteers [16–25]. However, important issues such as the reproducibility between laboratories, the consistency between different reagents and analyzers, as well as the variability within and between individuals, have not been fully elucidated.

We have therefore conducted a prospective multicenter evaluation study comprising a variety of reagents and analyzers, to investigate the accuracy and reproducibility of anti-Xa activity for determining rivaroxaban plasma concentration and to observe factors that affect the results.

## Methods

### Study design

This prospective multicenter evaluation study is the second study of the Swiss RIVAMOS study group. It focuses on anti-Xa measurements in individuals treated with therapeutic-dose rivaroxaban (20 mg), whereas a previous study was conducted using *ex vivo*-spiked samples [12]. The RIVAMOS study group comprises the hemostasis laboratories of the nine largest Swiss hospitals and one private laboratory (in alphabetical order, Cantonal Hospital Aarau, University Hospital of Basel, Inselspital University Hospital of Berne, University Hospitals of Geneva, University Hospital of Lausanne, Regional Hospital of Locarno, Cantonal Hospital of Lucerne,

Cantonal Hospital of St. Gallen, Unilabs Zurich, and University Hospital of Zurich). The protocol was approved by the responsible ethical committee (Kantonale Ethikkommission Luzern; #12041) and the Swiss surveillance authority for medicines and medical devices (Swissmedic; #2012DR1207), and was registered with clinicaltrials.gov (NCT01710267).

### Study subjects

Rivaroxaban 20 mg was administered for 10 consecutive days to 21 healthy male volunteers aged 18–65 years. Blood samples were taken at peak level (exactly 3 h after each daily dose) and at trough level (immediately before ingestion of the subsequent daily dose). Volunteers were recruited from hospital staff. Inclusion criteria were: no known comorbidities, no malabsorption, no regular medication intake, absence of hemostatic disorders, normal liver function tests and normal serum creatinine. One participant was excluded during the study because of an acute illness independent of rivaroxaban intake. All participants gave their informed consent. To ensure accurate results, a detailed protocol was followed for rivaroxaban intake and blood sampling. In particular, peak samples were drawn exactly 3 h after ingestion and subjects were instructed to take rivaroxaban with an adequate food intake. In summary, 10 daily doses of rivaroxaban were administered to each participant, a total blood volume of 300 mL was taken, and the entire observation period was 14 days. Rivaroxaban tablets were provided by the manufacturer (Bayer Healthcare).

### Collection and handling of samples

Samples of venous blood were taken centrally in one institution (Cantonal Hospital of Lucerne) at the time-points specified above and in a standardized fashion. An in-house protocol was followed to ensure adequate preanalytical conditions [26]. Samples were collected in plastic syringes containing 1 ml trisodium citrate ( $0.106 \text{ mol L}^{-1}$ ) per 9 mL of blood (Monovette<sup>®</sup>, Sarstedt, Nümbrecht, Germany). Samples were snap-frozen and shipped in one batch at a constant temperature of  $-20 \text{ }^{\circ}\text{C}$  (delivery time 2–4 h).

### Determination of anti-Xa activity and rivaroxaban plasma concentration

All laboratories implemented strict quality management guidelines that include regular internal and external quality assessment, and evaluated the assays thoroughly. All are accredited to the Swiss Accreditation Service (SAS).

Anti-Xa activity was measured according to current guidelines [27,28]. Assays were calibrated using commercially available calibrators (Biophen Rivaroxaban Plasma calibrators, Hyphen BioMed, Neuville-sur-Oise, France).

**Table 1** Assays and analytical platforms used for anti-Xa activity measurements by the participating laboratories

Analyzer*	Anti-Xa assay (Reagent)*		
	Hyphen Biophen® Heparin	Hyphen Biophen® DiXal	STA® anti Xa liquid
ACL Top 500†	1		
ACL Top 700†	1		
BCS XP‡	1	3	
CS 5100‡	1		
STA-R§	1	1	1

\*Numbers indicate the number of laboratories applying the particular combination of assay and analyzer. †Instrumentation Laboratory; ‡Siemens Diagnostics; §Roche Diagnostics.

Calibrators were re-assessed using HPLC-MS (nominal concentration vs. measured concentration [deviation]: 0 µg L<sup>-1</sup> vs. < 5 µg L<sup>-1</sup> [0%]; 51 vs. 52. 2 [2.5%]; 109 vs. 109.5 [0.5%]; 266 vs. 239.4 [-10%]; 490 vs. 466.3 [-4.8%]). In addition, all laboratories were provided with *in-vitro* spiked samples in a preparatory phase of the study (rivaroxaban concentrations: 405 µg L<sup>-1</sup>, 236 µg L<sup>-1</sup>, 133 µg L<sup>-1</sup>, 53 µg L<sup>-1</sup>, 31 µg L<sup>-1</sup>, 0 µg L<sup>-1</sup>). Anti-Xa activity was determined in all samples using the same batch of reagents in each laboratory. As a reference standard, rivaroxaban plasma concentrations were measured by HPLC-MS at peak level on day 1 and at trough level on days 9 and 10 [29].

Table 1 shows the reagents and analytical platforms used by the participating laboratories. Results of one analyzer (ACL Top 500) were excluded because of technical issues (abnormal internal quality control on the day of determination and inability to repair the device within a reasonable time period).

### Statistical analysis

We calculated Pearson's correlation coefficients between anti-Xa results and rivaroxaban plasma concentrations to assess the accuracy of anti-Xa activity for every reagent. In addition, we conducted a regression analysis according to Deming's procedure and created Bland-Altman difference plots [30,31]. We reported overall results (Figures, text) and created clinically relevant strata (< 50 µg L<sup>-1</sup>; 50–200 µg L<sup>-1</sup>; > 200 µg L<sup>-1</sup>; Table 2) [32]. To quantify the impact of different reagents and analyzers on anti-Xa results, we performed a mixed linear regression analysis using anti-Xa levels as a dependent variable, type of reagent (or analyzer, respectively) as a predictor variable, and the volunteer as the random variable. An analysis studying individual combinations of reagents and analyzer was not possible because of a limited number of combinations.

Reproducibility *between* laboratories was assessed by calculating intraclass correlation coefficients (ICC) for peak and trough level (two-way random-effects model treating anti-Xa levels as a dependent variable, volunteers

**Table 2** Accuracy of anti-Xa activity measurements if compared with HPLC-MS\*

Reagent	Hyphen Biophen® Heparin			Hyphen Biophen® DiXal			STA® anti-Xa liquid		
	< 50 µg L <sup>-1</sup> n = 96	50–200 µg L <sup>-1</sup> n = 51	> 200 µg L <sup>-1</sup> n = 29	< 50 µg L <sup>-1</sup> n = 70	50–200 µg L <sup>-1</sup> n = 52	> 200 µg L <sup>-1</sup> n = 32	< 50 µg L <sup>-1</sup> n = 18	50–200 µg L <sup>-1</sup> n = 13	> 200 µg L <sup>-1</sup> n = 8
Pearson's correlation coefficient (95% CI)	0.82 (0.74, 0.88)	0.94 (0.90, 0.98)	0.78 (0.59, 0.89)	0.46 (0.25, 0.62)	0.96 (0.93, 0.98)	0.88 (0.77, 0.94)	0.94 (0.83, 0.98)	0.99 (0.96, 1.00)	0.92 (0.59, 0.99)
Deming regression									
Slope (95% CI)	1.10 (0.95, 1.26)	1.20 (1.09, 1.33)	1.72 (1.29, 2.15)	4.20 (2.34, 6.16)	1.15 (1.05, 1.26)	1.18 (0.95, 1.41)	0.86 (0.74, 0.99)	1.26 (1.09, 1.43)	1.56 (0.73, 2.38)
Y-intercept (95% CI)	5.7 (1.7, 9.8)	-17.5 (-34.4, -0.58)	-128.75 (-226.79, -30.70)	-63.5 (-104.7, -22.4)	1.11 (-16.00, 18.22)	-1.64 (-54.06, 50.76)	7.56 (5.06, 10.06)	-25.5 (-54.58, 3.54)	-68.25 (-255.05, 118.54)
Bland-Altman difference plot									
Bias (95% CI)	7.93 (6.76, 9.10)	13.80 (9.02, 18.60)	38.60 (31.29, 45.91)	3.25 (-0.82, 7.32)	24.44 (20.51, 28.37)	39.32 (35.42, 43.22)	4.75 (3.23, 6.28)	14.21 (6.34, 22.08)	58.98 (46.71, 71.25)
Lower limit of agreement	-3.66	-20.25	0.16	-30.88	-3.79	17.70	-1.38	-11.84	29.62
Upper limit of agreement	19.51	47.87	77.04	37.38	52.67	60.94	10.88	40.26	88.34

\*Rivaroxaban 20 mg was administered once daily to 20 healthy volunteers. Blood samples were taken at peak (day 1) and trough level (day 9 and day 10), and analyzed in 10 routine laboratories.

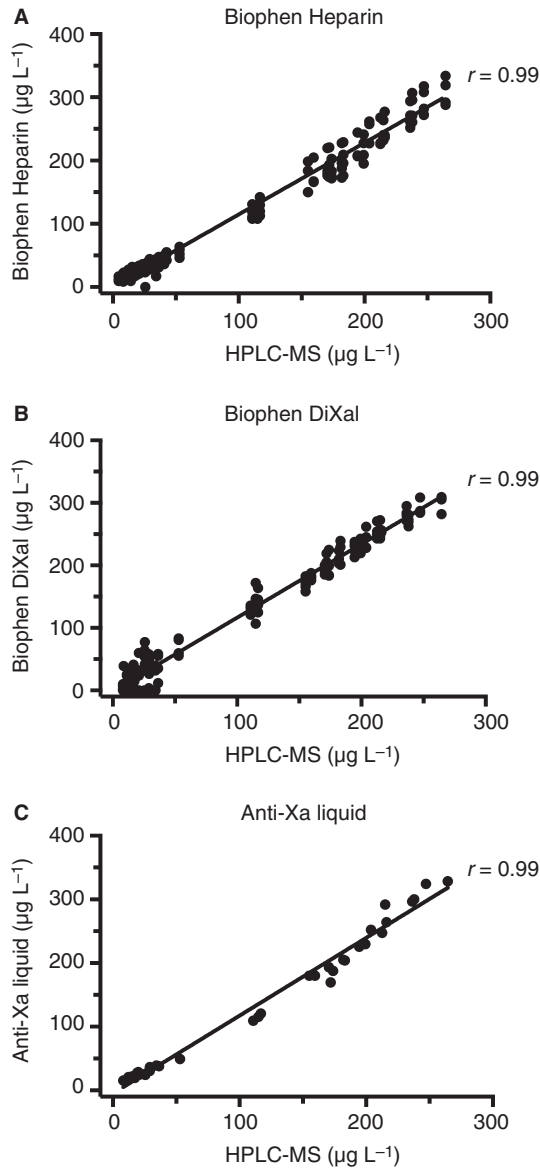
[targets] as a random variable and laboratories [raters] as a random variable) [33]. Variability *between* individuals was observed by describing median and range of HPLC-MS results at peak (day 1) and trough level (day 10). Variability *within* individuals was studied by describing median and range of differences between HPLC-MS

results at trough level on days 9 and 10, respectively. The Stata 14.1 statistics software package was used (Stata-Corp. 2015. Stata Statistical Software: Release 14. Stata-Corp LP, College Station, TX, USA).

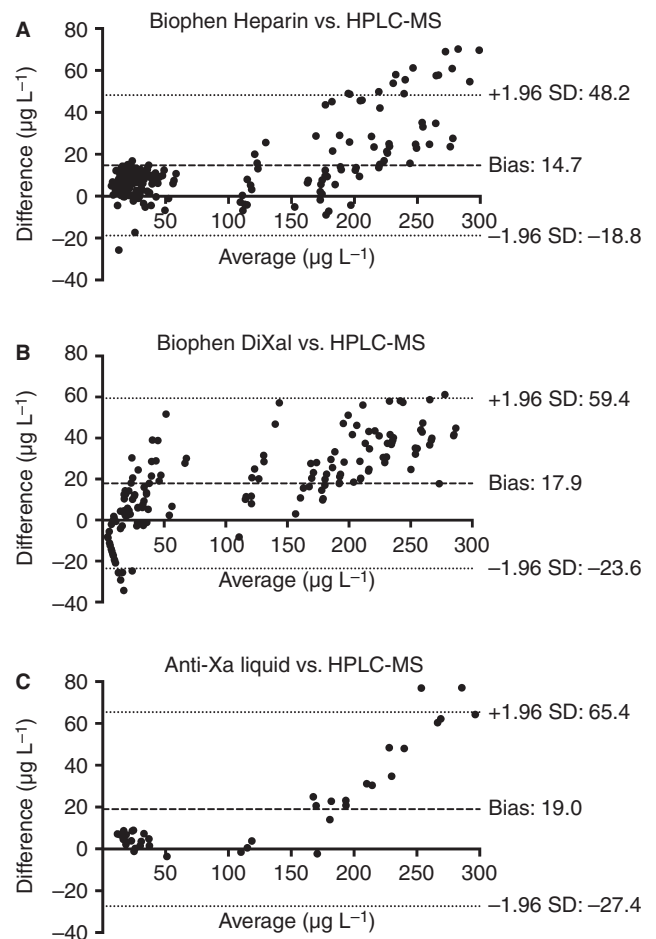
## Results

### Accuracy of anti-Xa measurements

Overall correlation between anti-Xa activity results and rivaroxaban plasma concentration as determined by HPLC-MS was high for all reagents (Fig. 1). Pearson's correlation coefficient was 0.99 for Hyphen Biophen<sup>®</sup> Heparin (95% CI, 0.99, 0.99), 0.99 for Hyphen Biophen<sup>®</sup> DiXaI (0.99, 0.99) and 0.99 for STA<sup>®</sup> anti-Xa liquid



**Fig. 1.** Correlation of anti-Xa activity and rivaroxaban plasma concentrations according to reagent (A–C). Overall Pearson's correlation coefficients and regression lines are shown; coefficients of the regression equation according to different strata of rivaroxaban plasma concentration are reported in Table 2. Blood samples were taken at peak level exactly 3 h after administration (day 1) and at trough level on day 9 and day 10 of rivaroxaban 20 mg application to healthy volunteers ( $n = 20$ ). Anti-Xa levels were determined in 10 different laboratories. Corresponding rivaroxaban plasma concentrations were determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS). (A) Hyphen Biophen<sup>®</sup> Heparin, (B) Hyphen Biophen<sup>®</sup> DiXaI, (C) STA<sup>®</sup> anti-Xa liquid.



**Fig. 2.** Bland-Altman difference plots of anti-Xa activity on rivaroxaban plasma concentrations according to reagent (A–C). Overall bias and limits of agreement are shown; parameters according to different strata of rivaroxaban plasma concentration are reported in Table 2. Blood samples were taken at peak level exactly 3 h after administration (day 1) and at trough level on day 9 and day 10 of rivaroxaban 20 mg application to healthy volunteers ( $n = 20$ ). Anti-Xa levels were determined in 10 different laboratories. Rivaroxaban plasma concentration was measured by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS). (A) Hyphen Biophen<sup>®</sup> Heparin, (B) Hyphen Biophen<sup>®</sup> DiXaI, (C) STA<sup>®</sup> anti Xa liquid.

(0.99, 1.0). However, correlation was considerably lower in rivaroxaban plasma concentrations below 50  $\mu\text{g L}^{-1}$  and above 200  $\mu\text{g L}^{-1}$ , at least with Hyphen Biophen<sup>®</sup> Heparin and Hyphen Biophen DiXal (reported in Table 2). The overall bias of the Bland-Altman difference plot is above zero for all reagents (Fig. 2). Stratified analysis revealed an increasing bias with higher rivaroxaban plasma concentrations (Table 2). This indicates a systematic overestimation of rivaroxaban plasma levels determined by anti-Xa assay as compared with HPLC-MS, particularly at higher concentrations (Fig. 2). The range between lower and higher limits of agreement was broad, but again driven by a more pronounced difference at higher concentrations. Stratified results of the Deming regression are reported in Table 2; the overall regression line is shown in Fig. 1.

#### Impact of reagents and analyzers on anti-Xa results

We observed significant differences of anti-Xa results according to the reagent (Table 3). Compared with Hyphen Biophen<sup>®</sup> Heparin (reference), results obtained with Hyphen Biophen<sup>®</sup> DiXal were 17.4  $\mu\text{g L}^{-1}$  higher (95% CI, 11.8, 23.0;  $P < 0.001$ ), and with STA<sup>®</sup> anti-Xa liquid 17.4  $\mu\text{g L}^{-1}$  higher as well (95% CI, 10.3, 24.5;  $P < 0.001$ ). Differences were also observed dependent on the analyzer (Table 3). Higher values were obtained with the CS 5100 (12.2  $\mu\text{g L}^{-1}$ ; 95% CI, -1.0, 25.3;  $P = 0.07$ ), the STA-R (12.4  $\mu\text{g L}^{-1}$ ; 95% CI, -1.2, 26.1;  $P = 0.07$ ) and the BCS XP (19.4  $\mu\text{g L}^{-1}$ ; 95% CI, 6.7, 32.2;  $P = 0.005$ ) compared with the ACL Top 700 (reference),

#### Consistency of anti-Xa measurements

Reproducibility of anti-Xa measurements *between* laboratories as determined by ICC was comparatively high for peak levels (Table 4), 0.84 (95% CI, 0.67, 0.87;

$P < 0.001$ ), but much lower at trough values, 0.23 (95% CI, 0.08, 0.45;  $P > 0.001$ ). The differences between rivaroxaban plasma concentrations *within* individuals ranged from 0.8 to 21.5  $\mu\text{g L}^{-1}$  (median, 3.1  $\mu\text{g L}^{-1}$ ; HPLC-MS) at trough level (Table 4). Variability *between* individuals is illustrated in Fig. 3; rivaroxaban plasma concentrations as determined by HPLC-MS are shown. Concentrations ranged from 110.9 to 264.4  $\mu\text{g L}^{-1}$  (median, 188.8) at peak and from 8.8 to 46.2 at trough level (median, 20.5).

#### Discussion

This multicenter study included a variety of reagents and analyzers. A high correlation of rivaroxaban plasma concentrations determined by anti-Xa assay with those measured by HPLC-MS at peak level was found in rivaroxaban concentrations between 50 and 200  $\mu\text{g L}^{-1}$ . However, there was a systematic bias towards an overestimation by anti-Xa assays, particularly at high plasma concentrations. Reproducibility between laboratories was good at peak level, despite significant differences between reagents and analyzers. A greater inconsistency was observed at trough level. The variability of results was significant between individuals but low within individuals.

Several previous studies investigated the correlation of anti-Xa results with known rivaroxaban plasma concentrations determined by dilution studies [15] or liquid chromatography-tandem mass spectrometry (LC-MS) [12,16–19,22–25]. In line with these, correlation coefficients in our study were also high. Interestingly, correlation was better in spiked samples [12,15] as compared with samples of patients or volunteers. Instead of analyzing spiked plasma, our study determined rivaroxaban levels in plasma after intake of the drug, and should therefore better reflect assay performance under routine conditions.

Strikingly, all anti-Xa assays systematically overestimated rivaroxaban plasma levels, particularly at higher

**Table 3** Effects of reagents and analyzers on anti-Xa results\*

	Constant ( $\beta_0$ )§			Coefficient ( $\beta_1$ )¶		
	$\mu\text{g L}^{-1}$	95% CI	<i>P</i> -value	$\mu\text{g L}^{-1}$	95% CI	<i>P</i> -value
<b>Reagents†</b>						
Hyphen Biophen <sup>®</sup> Heparin	106.3	94.2, 118.5	$P < 0.001$			
Hyphen Biophen <sup>®</sup> DiXal				17.4	11.8, 23.0	$P < 0.001$
STA <sup>®</sup> anti Xa liquid				17.4	10.3, 24.5	$P < 0.001$
<b>Analyzers‡</b>						
ACL Top 700	101.1	83.4, 118.7	$P < 0.001$			
BCS XP				19.4	6.7, 32.2	$P = 0.005$
CS 5100				12.2	-1.0, 25.3	$P = 0.07$
STA-R				12.4	-1.2, 26.1	$P = 0.07$

\*Results of a mixed linear regression are shown using anti-Xa levels as dependent variable, type of reagent (or analyzer) as a predictor variable and volunteer as a random variable; Rivaroxaban 20 mg was administered once daily to 20 healthy volunteers. Blood samples were taken at peak and trough level and analyzed in 10 routine laboratories. †Type of analyzer was fixed. ‡Type of reagent was fixed. §Constant is corresponding to anti-Xa levels in  $\mu\text{g L}^{-1}$  if reagent = Biophen Heparin or analyser = ACL Top 700 (reference). ¶Coefficient corresponds to additional changes of anti-Xa levels in  $\mu\text{g L}^{-1}$  in comparison with the reference.



values (Table 2, Fig. 2). This was unexpected, but could be anticipated from previous publications [16,17,22,24]. By contrast, one publication reported an opposite effect with a negative bias of the Bland-Altman difference plot and a slope of the regression line just below one [19]. Although the reason for this overestimation remains unclear, it appears to result from the assay's analytical principle. Several arguments support this proposition. First, the effect was observed for all reagents, analyzers

and laboratories. Second, measurement of rivaroxaban plasma concentration by HPLC-MS was performed by an internationally recognized reference laboratory [29]. Third, the concentrations of the calibrators were reassessed using HPLC-MS. Even though some deviation exists, the degree and direction of deviation do not fit to the overestimation we found. Also, no active metabolites of rivaroxaban are known [34].

Previous studies have also reported some variability among anti-Xa reagents [20,22]. We studied differences among reagents systematically and quantified the differences using mixed linear regression (Table 3). Further to these investigations, we have been able to identify and observe differences among analyzers (Table 3).

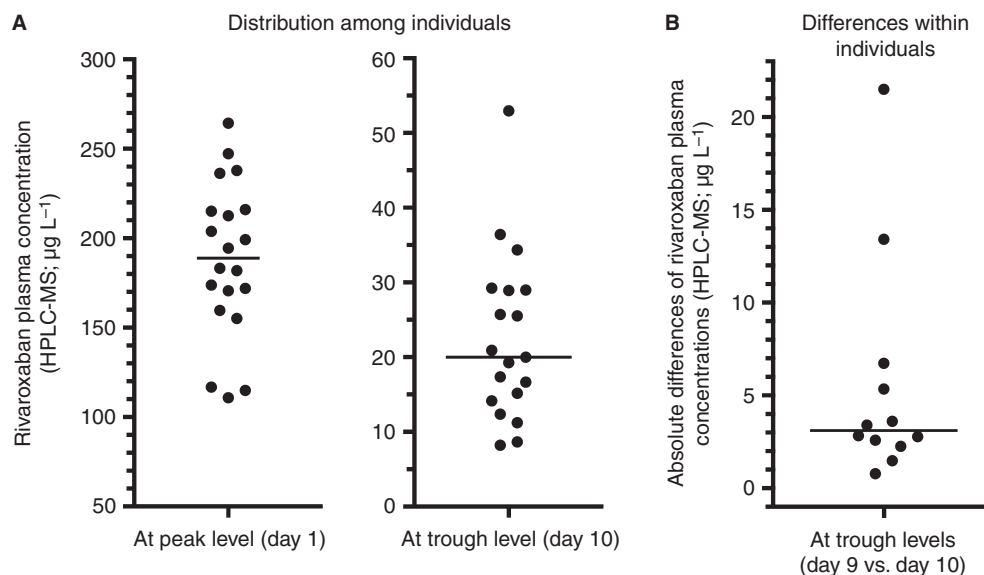
We are not aware of studies that have investigated the inter-laboratory reproducibility of anti-Xa assays for the determination of rivaroxaban plasma levels systematically, using either patient or volunteer samples. We observed adequate ICC values at peak but low ICC values at trough level (Table 4). Varying coefficients of variation were obtained in previous studies with the use of spiked samples (7–55%) [12,14,27]. In line with previous results, we observed an apparent variability of rivaroxaban plasma levels between individuals, both at peak and trough level [24,25,35], and in addition a low variability within individuals at trough level (Fig. 3).

Our study has several strengths: accuracy and reproducibility of anti-Xa assays were investigated systematically in a joint assessment of routine laboratories performing these tests in everyday clinical practice, using a variety of reagents and analyzers. In addition, plasma samples taken from individuals receiving therapeutic-dose rivaroxaban were used instead of spiked samples.

**Table 4** Consistency of anti-Xa activity measurements

Measures	Results
Reproducibility of anti-Xa measurements between laboratories	
Intra-class correlation coefficient* at peak level (day 1)	0.84 (95% CI 0.67, 0.93; $P < 0.001$ )
Intra-class correlation coefficient* at trough level (day 10)	0.23 (95% CI 0.08, 0.45; $P < 0.001$ )
Variability of rivaroxaban plasma concentration <i>between</i> individuals	
Distribution of HPLC-MS results at peak level (day 1); median (range)	188.8 (110.9, 264.4)
Distribution of HPLC-MS results at trough level (day 10); median (range)	20.5 (8.8, 46.2)
Variability of rivaroxaban plasma concentration <i>within</i> individuals	
Differences between HPLC-MS results at trough level (day 9 and day 10); median (range)†	3.1 (0.8, 21.5)

Rivaroxaban 20 mg was administered once daily to 20 healthy volunteers. Blood samples were taken at peak and trough level and analyzed in 10 routine laboratories. \*Two-way random-effects model treating anti-Xa levels as dependent variable, volunteers (targets) as random variable and laboratories (raters) as random variable. †Absolute differences are shown.



**Fig. 3.** Variability of rivaroxaban plasma concentrations between and within individuals. (A) Distribution of HPLC-MS measurements among individuals at peak and trough levels (days 1 and 10 of treatment, respectively). (B) Absolute differences of HPLC-MS measurements within individuals (day 9 vs. day 10 of treatment). Blood samples were taken before (trough) and exactly 3 h after (peak) administration of 20 mg rivaroxaban to healthy volunteers ( $n = 20$ ).

Moreover, we calculated the consistency of measurements adequately by use of ICC and quantified the effects of reagents and analyzers by use of linear regression methods. Furthermore, we analyzed accuracy measures for peak and trough values separately, improving the precision of estimates.

Nevertheless, our study has also limitations. Most importantly, the study subjects were healthy volunteers and not patients, limiting the impact of potentially interfering factors such as hemolysis, drug interactions, etc.

In clinical practice, anti-Xa assays facilitate the estimation of rivaroxaban plasma concentration in particular situations, such as bleeding or unplanned surgery. High levels should be regarded with caution, however. Even though the degree of overestimation was limited (Table 2), it may exceed  $50 \mu\text{g L}^{-1}$ , potentially triggering the application of reversal agents and thereby placing patients at risk of adverse events. Future research should investigate the cause of this overestimation and improve anti-Xa assays. Of note, calibration curves should include low concentration calibrators as well (as done in our study) to appropriately determine low rivaroxaban concentrations [36].

In summary, anti-Xa activity correlated well with rivaroxaban plasma concentrations as determined by HPLC-MS for all reagents and analyzers, and should be a valuable support in clinical decision making. However, physicians must be aware that a high variability of rivaroxaban plasma levels between individuals exists, accuracy and precision are limited for concentrations below  $50 \mu\text{g L}^{-1}$ , and there is a risk of overestimation.

### Addendum

W. A. Wuillemin planned the study, wrote the study protocol, and acted as principle investigator; M. Nagler organized implementation, wrote the analysis plan, conducted the statistical analysis, wrote the manuscript, and acted as study manager; J. D. Studt wrote the manuscript; P. Schmid wrote the study protocol; all authors conducted tests, reviewed the analysis, wrote the manuscript, and approved the final version of the manuscript.

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### Disclosure of Conflict of Interests

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