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Betrixaban

Siriez, Romain; Evrard, Jonathan; Dogné, Jean-Michel; Pochet, Lionel; Gheldof, Damien; Chatelain, Bernard; Mullier, François; Douxfils, Jonathan

Published in:

Thrombosis and Haemostasis

DOI:

[10.1055/s-0038-1657772](https://doi.org/10.1055/s-0038-1657772)

Publication date:

2018

[Link to publication](#)

Citation for published version (HARVARD):

Siriez, R, Evrard, J, Dogné, J-M, Pochet, L, Gheldof, D, Chatelain, B, Mullier, F & Douxfils, J 2018, 'Betrixaban: Impact on Routine and Specific Coagulation Assays-A Practical Laboratory Guide', *Thrombosis and Haemostasis*, vol. 118, no. 7, pp. 1203-1214. <https://doi.org/10.1055/s-0038-1657772>

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Betrixaban: Impact on Routine and Specific Coagulation Assays—A Practical Laboratory Guide

Romain Siriez¹ Jonathan Evrard¹ Jean-Michel Dogné¹ Lionel Pochet¹ Damien Gheldof^{1,2}
Bernard Chatelain³ François Mullier³ Jonathan Douxfils^{1,2}

¹University of Namur, Department of Pharmacy, Namur Thrombosis and Hemostasis Center (NTHC), Namur Research Institute for Life Sciences (NARILIS), Namur, Belgium

²Qualiblood s.a., Namur, Belgium

³Université catholique de Louvain, CHU UCL Namur, Namur Thrombosis and Hemostasis Center (NTHC), Namur Research Institute for Life Sciences (NARILIS), Hematology Laboratory, Yvoir, Belgium

Address for correspondence Jonathan Douxfils, PharmD, PhD, University of Namur, Department of Pharmacy, Namur Thrombosis and Hemostasis Center (NTHC), Namur Research Institute for Life Sciences (NARILIS), B-5000, Namur, Belgium (e-mail: jonathan.douxfils@unamur.be).

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Abstract

Introduction Betrixaban is a novel direct oral factor Xa inhibitor approved by the Food and Drug Administration for prophylaxis of venous thromboembolism in adult patients hospitalized for an acute illness at risk for thromboembolic complications. Assessment of the anti-coagulant effect of betrixaban may be useful in some situations. Also, clinicians need to know how routine coagulation assays are influenced.

Objective The aim of this study is to determine which coagulation assay(s) should be used to assess the impact of betrixaban on haemostasis and provide laboratory guidance for their interpretation.

Materials and Methods Betrixaban was spiked at final concentrations ranging from 0 to 250 ng/mL in platelet-poor plasma. Different reagents from several manufacturers were tested and the impact of betrixaban on pro-thrombin time (PT), activated partial thromboplastin time (aPTT), dilute Russel viper venom time (dRVV-T), chromogenic anti-Xa assays, thrombin generation assay (TGA), and a large panel of haemostasis diagnostic tests has been assessed.

Results A concentration-dependent prolongation of aPTT, PT and dRVV-T is observed. The sensitivity mainly depends on the reagent. Chromogenic anti-Xa assays show high sensitivity depending on the reagent and/or the methodology. These assays applicable for other direct factor Xa inhibitors have to be adapted to obtain a relevant range of measurement. TGA may also be attractive to assess the anti-coagulant activity of betrixaban.

Conclusion Adapted chromogenic anti-Xa assays are the most appropriate assays to estimate the concentration of betrixaban. Betrixaban significantly affects several haemostasis diagnostic tests and this needs to be taken into consideration when requesting and interpreting such tests.

Keywords

- ▶ betrixaban
- ▶ drug measurement
- ▶ coagulation tests
- ▶ guidance
- ▶ factor Xa inhibitors

received
February 13, 2018
accepted after revision
May 1, 2018

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DOI <https://doi.org/10.1055/s-0038-1657772>.
ISSN 0340-6245.

Introduction

Betrixaban, a novel direct oral factor Xa (FXa) inhibitor developed by Portola Pharmaceuticals Inc., has received its market authorization under the brand name of BevyxXa on the 23rd of June 2017 in the United States for the prophylaxis of venous thromboembolisms (VTE) in adult patients.¹ The approval was based on the data from the Acute Medically Ill Prevention with Extended Duration Betrixaban Study, a randomized, double-blind, multi-national clinical trial comparing extended duration betrixaban to short duration enoxaparin in the prevention of VTE in an acutely medically ill hospitalized population with risk factors for VTE.²⁻⁶ It has been developed to have a low renal clearance (< 7% of administered dose), a minimal hepatic metabolism (< 1%) and a long half-life (± 37 hours).⁷ Although direct oral anti-coagulants (DOACs) do not require routine monitoring, the assessment of their effect on coagulation may be useful in some clinical situations (e.g., detection of drug accumulation in acute renal or hepatic failure; planning the timing of urgent invasive procedure; recurrence of stroke or bleedings, etc.).^{3,8} In this study, spiked plasma has been used to realize calibration curve. This technique has already been used in the past for the assessment of other DOACs.⁹⁻¹² It has been found that we can reliably apply these results in real-life samples. Indeed, some studies revealed similarities between *in vitro* and *ex vivo* data for rivaroxaban.¹³⁻¹⁵

The primary aim of this study is to assess and compare the performance of routinely used and more specific coagulation assays to measure the anti-coagulant effect of betrixaban and estimate its plasma concentration. In addition, good laboratory recommendations for the interpretation of haemostasis diagnostic tests that may be affected by betrixaban will be provided.

Materials and Methods

Betrixaban was spiked at increasing concentrations in pooled citrated normal human platelet-poor plasma (PPP). The tested concentrations cover the on-therapy range (from ± 9 ng/mL at C_{trough} to ± 122 ng/mL at C_{max} for the 40 and 120 mg once daily doses, respectively, according to the clinical pharmacology and biopharmaceutics reviews of the Center for Drug Evaluation and Research).¹⁶

Testing Solutions of Betrixaban

Betrixaban (molecular weight: 451.90 g/mol; purity = 92.35%) was obtained from ChemScene (Monmouth Junction, New Jersey, United States). A stock solution at 1.0 mg/mL in dimethyl sulfoxide (DMSO) was obtained and intermediate solutions at 100, 300, 500, 1,000, 1,500 and 2,500 ng/mL diluted in phosphate-buffered saline without Ca^{2+} and Mg^{2+} were prepared. Working solutions of 10, 30, 50, 100, 150 and 250 ng/mL of betrixaban were obtained by mixing these stock solutions with normal pooled plasma (NPP) (1:9 v/v). According to purity, these concentrations represented 9.2, 27.7, 46.2, 92.4, 138.5 and 230.9 ng/mL of active betrixaban, respectively, in plasma. The DMSO

concentration in plasma was $\leq 0.05\%$ (v/v) which did not influence the coagulation.¹⁷

Preparation of Platelet-Poor Plasma

Fifty-six individuals were included in the study. The exclusion criteria were thrombotic and/or haemorrhagic events, anti-platelet and/or anti-coagulant medication, hormonal therapy, pregnancy and uptake of drugs potentially affecting the platelet and/or coagulation factor functions during the 2 weeks prior to the blood drawn. The study protocol is in accordance with the Declaration of Helsinki. Blood was taken by venipuncture in the antecubital vein and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe, Terumo, Belgium) using a 21-gauge needle (Terumo). The PPP was obtained from the supernatant fraction of blood tubes after a double centrifugation for 15 minutes at $1,500 \times g$ at room temperature. Immediately after centrifugation, PPP from the 56 donors were brought together to obtain the NPP which was frozen at -80°C without any delay. Frozen NPP samples were thawed and heated to 37°C for 5 minutes just before experiment. All tests were performed within 4 hours after thawing.

Routinely Used and Specific Coagulation Assays

A summary of the different assays and their reagents performed in this study is provided in **Table 1**. Several reagents have been used for some tests. All tests were performed in accordance with the recommendations of the manufacturers, or otherwise this is specified in the sections below. Depending on the test and reagent, evaluations were performed on a STA-R Evolution (Diagnostica Stago, Asnières-sur-Seine, France), a STA-R Max (Diagnostica Stago), an ACL-TOP 700 (Instrumentation Laboratory, Lexington, Kentucky, United States) or an Ascent Fluorocan (Thermo Fisher Scientific, Waltham, Massachusetts, United States) using the calibrated automated thrombogram software (CAT, Thromboscope BV, Maastricht, The Netherlands) (**Table 1**).

Pharmacodynamics Assays

To evaluate the anti-coagulant effect of betrixaban, prothrombin time (PT), activated partial thromboplastin time (aPTT), dilute Russell viper venom time (dRVV-T), chromogenic anti-Xa assays and the thrombin generation assay (TGA) were assessed. PT, aPTT, dRVV-T and TGA were performed in accordance with the recommendations of the manufacturer.

For all chromogenic anti-Xa assays, the results were collected as optical densitometry (OD)/min by using several reagents and methodologies (**Table 1**).

For STA-Liquid Anti-Xa, DiXaI and Technochrom Anti-Xa assays, the methodologies provided by the manufacturer for the assessment of rivaroxaban were used but the tests were not calibrated with rivaroxaban calibrators and results were reported as OD/min.

For the following anti-Xa chromogenic assays, an adapted method was proposed with the aim of increasing the dynamic range of quantitation. For Biophen Heparin LRT, 50 μL of spiked NPP diluted at 1:10 or 1:2 in physiological

Table 1 Overview of the different assays performed in this study

Coagulation assay	Reagent	Manufacturer	Method	Coagulation analyser (manufacturer)
Pro-thrombin time	TriniCLOT PT Excel S	Tcoag, Bray, Ireland	Chronometric	STA-R Evolution (Diagnostica Stago)
	TriniCLOT PT Excel			
	TriniCLOT PT HTF			
	STA-NeoplastineR	Diagnostica Stago, Asnieres, France		
	STA-Neoplastine CI+			
	Dade Innovin	Siemens Healthcare Diagnostics, Deerfield, Illinois, United States		
RecombiPlasTin 2G	Instrumentation Laboratory, Lexington, Kentucky, United States			
Activated partial thromboplastin time	STA-C.K.Prest	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution (Diagnostica Stago)
	STA-Cephascreen			
	STA-PTT Automate			
	Actin FS	Siemens Healthcare Diagnostics, Deerfield, Illinois, United States		
	SynthASil	Instrumentation Laboratory, Lexington, Kentucky, United States		ACL-TOP (Instrumentation Laboratory)
	SynthAfax			
Thrombin time	STA-Thrombin	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution (Diagnostica Stago)
Lupus anti-coagulant	STA-Staclot DRVV Screen	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution (Diagnostica Stago)
	STA-Staclot DRVV Confirm			
Reptilase time	STA-Reptilase	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Evolution (Diagnostica Stago)
Anti-thrombin	STA-Stachrom ATIII	Diagnostica Stago, Asnieres, France	Chromogenic (thrombin-based)	STA-R Evolution (Diagnostica Stago)
	HemosIL Liquid Antithrombin	Instrumentation Laboratory, Lexington, Kentucky, United States	Chromogenic (FXa-based)	ACL-TOP (Instrumentation Laboratory)
Chromogenic anti-Xa assays	Biophen Direct Factor Xa Inhibitors	Hyphen BioMed, Neuville-sur-Oise, France	Chromogenic	STA-R Evolution (Diagnostica Stago)
	Biophen Heparin LRT			
	STA-Liquid Anti-Xa	Diagnostica Stago, Asnieres, France		
	Technochrom Anti-Xa	Technoclone, Vienna, Austria		
	HemosIL Liquid Heparin	Instrumentation Laboratory, Lexington, Kentucky, United States		ACL-TOP (Instrumentation Laboratory)
Thrombin generation assay	PPP-Reagent Low	Thrombinoscope BV, Maastricht, The Nederland's	Fluorimetric	Calibrated Automated Thrombogram (Thrombinoscope BV)
	PPP-Reagent			
	PPP-Reagent High			

(Continued)

Table 1 (Continued)

Coagulation assay	Reagent	Manufacturer	Method	Coagulation analyser (manufacturer)
Evaluation of extrinsic clotting factor levels	RecombiPlasTin 2G	Instrumentation Laboratory, Lexington, Kentucky, United States	Chronometric	STA-R Max (Diagnostica Stago)
Evaluation of intrinsic clotting factor levels	STA-C.K.Prest	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Max (Diagnostica Stago)
Fibrinogen Clauss method	STA-Liquid Fib	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Max (Diagnostica Stago)
Fibrinogen PT-derived	RecombiPlasTin 2G	Instrumentation Laboratory, Lexington, Kentucky, United States	Chronometric	ACL-TOP (Instrumentation Laboratory)
Protein S	Protein S Activity	Instrumentation Laboratory, Lexington, Kentucky, United States	Chronometric	ACL-TOP (Instrumentation Laboratory)
Protein C	STA-Stachrom Protein C	Diagnostica Stago, Asnieres, France	Chromogenic	STA-R Max (Diagnostica Stago)
Activated protein C resistance	STA-Staclot APC-R	Diagnostica Stago, Asnieres, France	Chronometric	STA-R Max (Diagnostica Stago)

Abbreviations: PPP, platelet-poor plasma; PT, pro-thrombin time.

saline were mixed with 125 µL of chromogenic substrate (SXA-11) and incubated for 240 seconds at 37°C. Then, 125 µL of bovine FXa pre-warmed at 37°C was added. For HemosIL Liquid Heparin, 200µL of spiked NPP was mixed with 100 µL of chromogenic substrate (S2732) and incubated for 180 seconds at 37°C. Thereafter, 75 µL of bovine FXa was added. For DiXaI, two procedures were used (normal or low). Briefly, 200 µL of spiked NPP diluted at 1:50 (normal procedure) or 1:15 (low procedure) in Tris-NaCl-ethylenediaminetetraacetic acid (EDTA) reaction buffer provided by the manufacturer was mixed with 75 µL of human FXa and incubated for 120 seconds at 37°C. Then, 75 µL of FXa-specific chromogenic substrate (CS11(65)) pre-warmed at 37°C was added. For STA-Liquid anti-Xa, 30 µL of spiked NPP diluted at 1:4 in physiological saline was mixed with 150 µL of chromogenic substrate (CBS 02.44) and incubated for 240 seconds at 37°C. Then, 150 µL of bovine FXa pre-warmed at 37°C was added. Finally, for Technochrom Anti-Xa, 85µL of spiked NPP diluted 1:15 or 1:5 in Tris-EDTA buffer provided by the manufacturer was mixed with 85 µL of bovine FXa and incubated for 240 seconds at 37°C. Then, 85 µL of chromogenic substrate pre-warmed at 37°C was added. All measurements were performed on STA-R Evolution.

Diagnostic Assays

The influence of betrixaban on the following diagnostic tests has also been investigated. Thus, in addition to PT, aPTT and dRVV-T, which could be useful for the assessment of betrixaban as described above, Fibrinogen (Fib), clotting factors, protein C and S and anti-thrombin (AT) measurements, thrombin time (TT) and reptilase time (RT) have also been

performed. Investigations for lupus anti-coagulant (LA) and activated protein C resistance were also assessed.

Determination of Lupus Anti-Coagulant: Dilute Russell Viper Venom Time

The diagnosis of anti-phospholipid syndrome can be done by the estimation of the percentage of correction obtained with the dRVV-T: $(\text{Screen} - \text{Confirm})/\text{Screen} \times 100$. The cut-off of 5% of correction is proposed by the British Committee for Standards in Haematology guidelines for the investigation of LA (reference ratio is 0.9:1.05).¹⁸

Anti-Thrombin Measurement

Antithrombin measurement was performed using the STA-Stachrom ATIII (Diagnostica Stago), a chromogenic assay based on the inhibition of bovine FIIa, and the HemosIL Liquid Antithrombin (Instrumentation Laboratory), a chromogenic assay based on the inhibition of bovine FXa.

Fibrinogen Measurement (Clauss Method and PT-Derived)

For the Clauss method, 150 µL of plasma diluted at 1:20 with saline solution was incubated for 240 seconds. Then, 50 µL of STA-Liquid Fib (Diagnostica Stago) was added to trigger the coagulation. For the PT-derived method, 50 µL of plasma was mixed with 100 µL of RecombiPlasTin 2G (Instrumentation Laboratory) to trigger the coagulation.

Thrombin Time

Thrombin time was investigated with the STA-Thrombin (Diagnostica Stago) according to the recommendations of the manufacturer.

Reptilase Time

Reptilase time was investigated using STA-Reptilase (Diagnostica Stago) according to the recommendations of the manufacturer.

Protein C Activity Measurement

The activity of protein C was investigated using STA-Stachrom Protein C (Diagnostica Stago) according to the recommendations of the manufacturer.

Protein S Activity Measurement

The activity of protein S was investigated using Protein S Activity (Instrumentation Laboratory) according to the recommendations of the manufacturer.

Assessment of Activated Protein C Resistance

The activity of protein C resistance was investigated using STA-Staclot APC-R (Diagnostica Stago) according to the recommendations of the manufacturer.

Measurement of Clotting Factors

Intrinsic clotting factors activity (VIII, IX, XI and XII) was assessed using STA-C.K.Prest (Diagnostica Stago). Fifty microlitre of spiked NPP diluted at 1:10, 1:20 or 1:40 in physiological saline was incubated for 240 seconds at 37°C after adding 50 µL of deficient plasma (Diagnostica Stago) and 50 µL of STA-C.K.Prest. Coagulation was then triggered by adding 50 µL of CaCl₂ 0.025 M. Extrinsic clotting factors activity (II, V, VII and X) was assessed using RecombiPlasTin 2G. Fifty microlitre of spiked NPP diluted at 1:10, 1:20 or 1:40 in physiological saline was incubated for 240 seconds at 37°C after adding 50 µL of deficient plasma. Coagulation was then triggered by adding 100 µL of RecombiPlasTin 2G. All measurements were performed on a STA-R Max coagulometer.

Statistical Analysis

Statistical analyses and graphics were computed using GraphPad Prism 5.04 for Windows 7 Professional (GraphPad Software, La Jolla, California, United States).

To compare the sensitivity of the different clotting assays, the concentration of betrixaban needed to double the clotting time ($2 \times CT$ [CT = clotting time]) was used. For chromogenic assays, the sensitivity is defined as the concentration of betrixaban needed to halve the analytical parameter ($1/2 \times OD/min$ [the concentration needed to halve the change in the OD reported by minute]). For the CAT, the sensitivity of the different parameters is defined as follow: $C_{max} IC_{50}$ (the concentration reducing the C_{max} of 50%); peak IC_{50} (the concentration reducing the peak of 50%); mVRI IC_{50} (the concentration reducing the mVRI of 50% [the mean velocity rate index (mVRI) represents the effective rate of thrombin generation (i.e., the slope) between lag time and time to peak]); $mVRI = Peak/(time\ to\ peak - lag\ time)$; $2 \times LT$ (the concentration needed to double the lag time [LT]); $2 \times TTP$ (the concentration needed to double the time to peak [TTP]).

Except for clotting factor activities that were run only once, each test was run in triplicate on the same day. Data on

the graphics represent the mean of triplicates. The repeatability is defined as the mean of the coefficient of variation (CV) ($[(standard\ deviation/mean) \times 100]$) of the triplicate of each concentration for each test.

For tests evaluated for the assessment of the pharmacodynamics of betrixaban (i.e., aPTT, PT, chromogenic anti-Xa assays, dRVV-T and CAT), the limit of detection (LOD) and quantitation (LOQ) were calculated as follow:

- LOD: $[(3 \times standard\ deviation\ of\ Y_0)/slope]$
- LOQ: $[(10 \times standard\ deviation\ of\ Y_0)/slope]$

where Y_0 is the baseline value of the linear regression. For assays that did not fit a linear response on their entire range of measurement (i.e., Biophen Direct Factor Xa inhibitors [DiXal]; Biophen Heparin LRT; STA Liquid Anti-Xa; Technochrom Anti-Xa normal and high; and HemosIL Liquid heparin), the first points of the calibration curve were used to define a linear regression. The slope of this linear regression was then used for the calculation of the LOD and LOQ.

Results

Pharmacodynamics Assays

Pro-Thrombin Time

A concentration-dependent prolongation of PT was found. The $2 \times CT$ depended on the reagent and ranged from 198 ng/mL (STA-Neoplastiner) to 514 ng/mL (Dade Innovin). PT may be normal (ratio < 1.2) with on-therapy concentration of betrixaban depending on the reagent (**Fig. 1**; **Supplementary Fig. S1**, online only). The repeatability was always below 2%. The LOD and LOQ ranged from 5 to 36 ng/mL and from 11 to 107 ng/mL, respectively.

Activated Partial Thromboplastin Time

The aPTT showed a concentration-dependent prolongation of clotting time. The $2 \times CT$ depended on the reagent and ranged from 257 ng/mL (SynthAfax) to 479 ng/mL (STA C.K. Prest) (**Fig. 2**; **Supplementary Fig. S2**, online only). The repeatability was always below 2%. The LOD and LOQ ranged from 16 to 57 ng/mL and from 21 to 152 ng/mL, respectively.

Dilute Russell Viper Venom Time

STA-Staclot-DRVV Screen and Confirm were also prolonged dose-dependently (**Fig. 3**; **Supplementary Fig. S3**, online only). The $2 \times CT$ was 80 ng/mL for STA-Staclot-DRVV Screen and 108 ng/mL for STA-Staclot-DRVV Confirm. The repeatability ranged from 0.8% (STA-Staclot-DRVV Confirm) to 0.9% (STA-Staclot-DRVV Screen). The LOD and LOQ were 5 and 17 ng/mL for the DRVV Screen and 6 and 22 ng/mL for DRVV Confirm, respectively.

Chromogenic Anti-Xa Assays

A concentration-dependent decrease in OD/min was observed (**Fig. 4**; **Supplementary Fig. S4**, online only). The reactions were fitted by an exponential model. The $1/2 OD/min$ ranged from 120 ng/mL (Biophen Heparin LRT Low) to 569 ng/mL (Technochrom Anti-Xa High). The repeatability

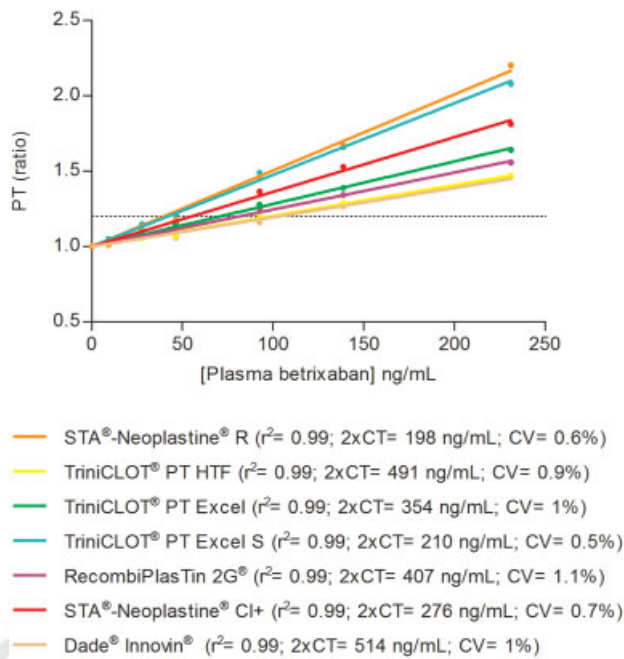


Fig. 1 Impact of betrixaban on pro-thrombin time (PT). Betrixaban showed a concentration-dependent prolongation of the PT. The relation was linear and the $2 \times \text{CT}$ depended on the reagent. The $2 \times \text{CT}$ ranged from 198 ng/mL for STA-NeoplastineR to 514 ng/mL for Dade Innovin (r^2 : correlation coefficient; $2 \times \text{CT}$: $2 \times$ clotting time expressed in ng/mL; CV : coefficient of variation expressed in percentage [%]).

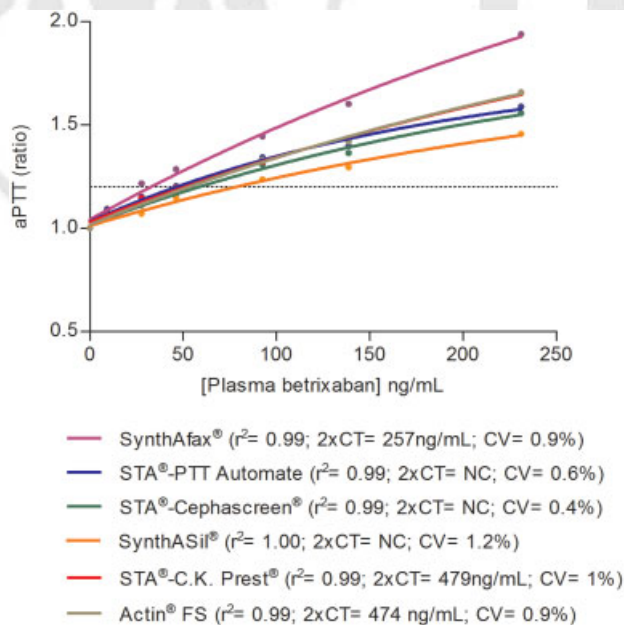


Fig. 2 Impact of betrixaban on activated partial thromboplastin time. Betrixaban showed a concentration dependent prolongation of the activated Partial Thromboplastin Time. The relation was curvilinear and the $2 \times \text{CT}$ depended on the reagent. The $2 \times \text{CT}$ was ranging from 257 ng/mL for SynthAfax® to 479 ng/mL for STA®-C.K. Prest®. (r^2 : Correlation Coefficient; $2 \times \text{CT}$: $2 \times$ Clotting Time expressed in ng/mL; CV : Coefficient of variation expressed in percentage [%]; NC : Not calculated. The plateau does not reach a ratio of 2 for the concentrations tested in this study).

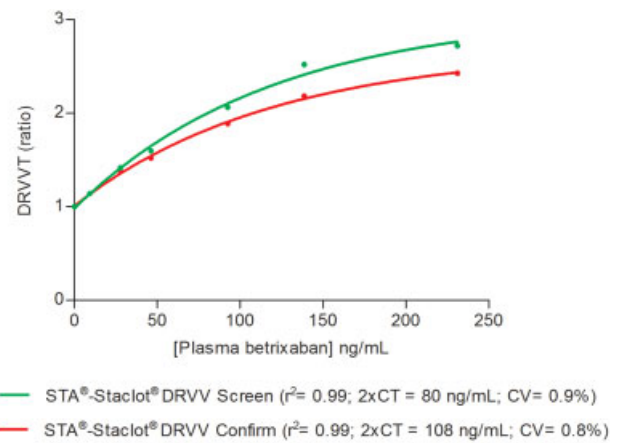


Fig. 3 Impact of betrixaban on the dilute Russell viper venom time (dRVVT). Betrixaban prolonged the dilute Russell viper venom time dose-dependently. The relation is curvilinear showing a lower sensitivity at the higher concentrations (from 150 ng/mL). However, the higher sensitivity at the lower concentrations is interesting since it provides a low limit of detection and quantitation.

was from 0.6% (Biophen Heparin LRT) to 3.1% (HemosIL Liquid Heparin). The LOD and LOQ ranged from 1 to 72 ng/mL and from 4 to 185 ng/mL, respectively.

Thrombin Generation Assay

The most influenced parameters were the peak (C_{max} IC_{50} : 8–28 ng/mL) and the mVRI (mVRI IC_{50} : 7–10 ng/mL), while the lag time, the time to peak and the endogenous thrombin potential were less affected, as reported in **Fig. 5**. The prolongation/inhibition of all parameters decreased inversely to the amount of tissue factor (TF) in the reagent. The CV of the triplicate was always below 10%. The LOD and LOQ for the peak ranged from 37 to 58 ng/mL and from 122 to 193 ng/mL, respectively. For

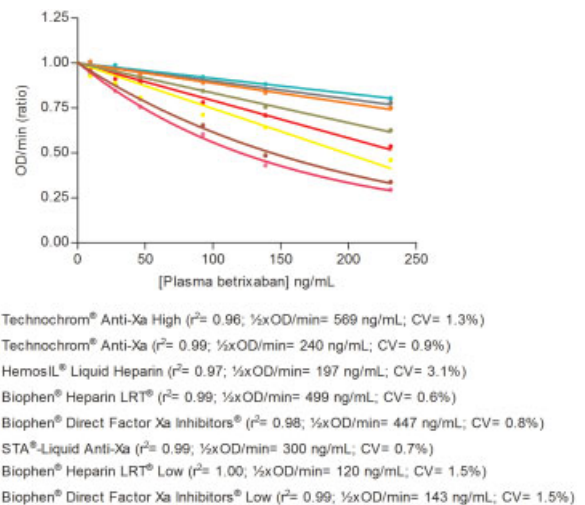


Fig. 4 Measurement of betrixaban with chromogenic anti-Xa assays. There was a concentration-dependent decrease of the OD/min. Tests with higher baseline OD/min had a wider range of quantitation compared with those starting at lower OD/min at baseline (r^2 : correlation coefficient; $1/2 \times \text{OD}/\text{min}$: halve in optical density by minute expressed in ng/mL; CV : coefficient of variation expressed in percentage [%]). Some tests are not sufficiently influenced with the current methodologies and need to be adapted.

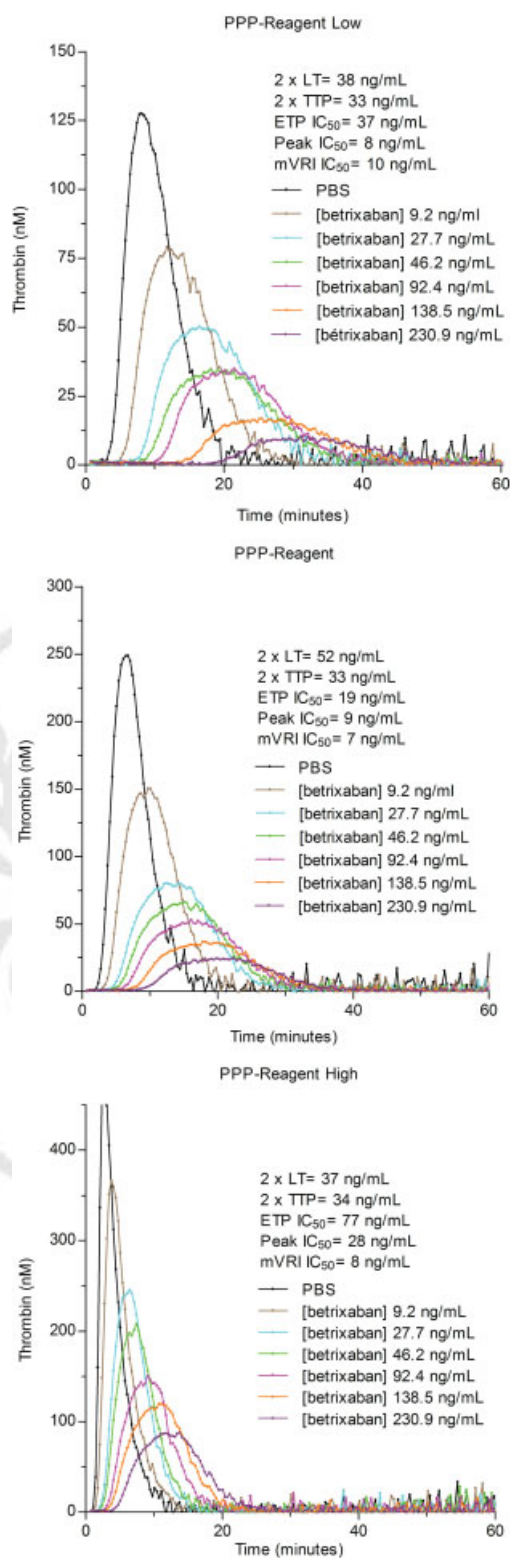


Fig. 5 Impact of betrixaban on calibrated automated thrombogram (CAT). The most influenced CAT parameters are the peak and the mean velocity rate index. There is an inter-reagent variability. Thanks to their low IC₅₀ for the peak and the mVRI, the better resolution and their large range of application, platelet-poor plasma (PPP)-Reagent Low, PPP-Reagent and PPP-Reagent High seemed to be the best reagents to monitor patients on betrixaban (ETP: endogenous thrombin potential; IC₅₀: half-maximum inhibitory concentration; LT: lag time; mVRI: mean velocity rate index; TTP: time to peak). mVRI was defined as: (Peak)/(Time to peak – Lag time).

the mVRI, the LOD and LOQ ranged from 18 to 27 ng/mL and from 66 to 88 ng/mL, respectively.

Diagnostic Assays

Determination of Lupus Anti-Coagulant: Dilute Russell Viper Venom Time

A curvilinear prolongation of % correction was observed (► **Supplementary Fig. S5**, online only). The cut-off of 5% of correction was reached at betrixaban concentration of 38 ng/mL.

Fibrinogen, Anti-Thrombin, Protein C, Thrombin Time and Reptilase Time Measurements

These assays were not influenced by the presence of betrixaban at the concentration tested in this study.

Protein S Measurement

Protein S measurement showed a concentration-dependent linear prolongation (► **Supplementary Fig. S6**, online only). The 2 × CT was 253 ng/mL and the repeatability was 1.2%. The LOD and LOQ were 12 to 22 ng/mL, respectively.

Assessment of Activated Protein C Resistance

Protein C resistance assessment showed a concentration-dependent curvilinear prolongation (► **Supplementary Fig. S7**, online only). The repeatability was 1.1%. The LOD and LOQ were 7 and 15 ng/mL, respectively.

Clotting Factor Measurement

For factors of the intrinsic pathway (FVIII, FIX, FXI and FXII), the aPTT-based clotting method showed a mean decrease of ± 7% at 10 ng/mL of betrixaban, 19% at 50 ng/mL and 42% at 150 ng/mL with a diluted sample (1:10). The impact was more pronounced for FXI and FVIII as presented in ► **Supplementary Table S1** (online only) and ► **Supplementary Fig. S8** (online only). For FV, FVII and FX, a mean decrease of 2% at 10 ng/mL of betrixaban, 10% at 50 ng/mL and 24% at 150 ng/mL was observed with a dilution of 1:10, while pro-thrombin measurement seemed to be less affected (maximal decrease of 6% at 150 ng/mL with a dilution of 1:40) (► **Supplementary Table S1**, online only, and ► **Supplementary Fig. S8**, online only). A correction of the impact of betrixaban was observed with higher dilution (i.e., 1:20 or 1:40).

Discussion

The aim of this study was to investigate the impact of betrixaban on a series of routine or more specific coagulation tests to provide good recommendations to estimate the anti-coagulant effect of the treatment as well as to correctly interpret the results of diagnostic tests, which may be altered by the presence of this novel direct FXa inhibitor. Several reagents and methodologies have been assessed on NPP from healthy volunteers spiked with betrixaban to provide good laboratory practice at the laboratory level.

Some limitations of our study have to be recognized. One limitation of this study is the use of NPP spiked with

betrixaban. This technique has already been used in the past for the assessment of other DOACs.^{9–12} It has been found that we can reliably apply these results in real-life samples. Indeed, some studies revealed similarities between in vitro and ex vivo data for rivaroxaban^{13–15} and this might be applicable to betrixaban. Further investigations are required to confirm these data and evaluate the inter-individual variability in patients treated with betrixaban.

Routinely Used and Specific Coagulation Assays: Advantages and Drawbacks

Routine Tests and Derivation of Specific Assays Acting on Factor X

Prothrombin time is broadly used to assess the impact of DOAC therapy but failed to be recommended as the reference assay for the assessment of direct FXa inhibitors.¹⁹ According to our results, PT is not sensitive enough to estimate betrixaban plasma concentrations and, depending on the reagent, may be normal in presence of concentrations observed in the on-therapy range. Thus, in addition to the biological and pre-analytical variables that may affect PT, this inter-reagent variability prevents valid recommendations of cut-offs in seconds associated with a bleeding risk applicable to all reagents. In addition, drugs or haematologic abnormalities affecting at least one factor assessed by PT could also interfere and bias the conclusions.^{10,20} Definitively, PT should not be recommended to estimate plasma concentration of betrixaban. This result corroborates previous investigations about PT and DOACs (rivaroxaban, apixaban, dabigatran and edoxaban).^{9,10,19,21}

For *aPTT*, a concentration-dependent prolongation is shown in presence of increasing doses of betrixaban. This routine test is not sensitive enough to estimate betrixaban plasma concentrations and depending on the reagent used, it may not be influenced at concentrations observed in the on-therapy range. *aPTT* should not be recommended to estimate plasma concentration of betrixaban. The dependence of the reagent has previously been investigated and observed for dabigatran, apixaban, rivaroxaban and edoxaban.^{8,22,23}

The *dRVV-T* is usually used for the assessment of anti-phospholipid syndrome.²⁴ Previous studies suggested that this test could be used for the evaluation of all DOACs but it has never been assessed on betrixaban. The concentration-dependent prolongation of *dRVV-T* showed a curvilinear correlation, suggesting a plateauing effect at higher concentrations.²⁵ The reagents used present a high sensitivity to ensure a reliable assessment of drug effect. These results are consistent with results obtained with others direct FXa inhibitors.^{19,26,27}

In previous studies, routine tests have already been assessed to estimate the impact of DOACs in real-life samples⁹ and it has been demonstrated that PT and *aPTT* may not be adequate to estimate drug levels.^{19,28} For a particular chromometric assay, the between-reagent variability could be explained by the differences in the composition of the reagent. The source of phospholipids (e.g., phosphatidylserine, phosphatidylethanolamine or phosphatidylcholine), their proportion in the reagent, as well as other factors

such as the ionic force, the pH, the source of TF (for PT) or the activator (for *aPTT*) could provide some explanations. Furthermore, Smith et al demonstrated that traces of activated FVII could modulate thromboplastin sensitivity to FV, FVII, FX and pro-thrombin.^{29,30}

Chromogenic anti-Xa assays have already been described as accurate assays for the measurement of plasmatic drug level of direct FXa inhibitors.^{8,10,22,31,32} The different chromogenic assays available in the market used in this study showed linear or exponential relations depending on the reagent and the methodology applied. Compared with other chromogenic anti-Xa assays, Biophen DiXal is specific for direct FXa inhibitors such as betrixaban.^{8,33} Namely, thanks to its Tris/EDTA/NaCl buffer at pH = 7.85, this assay is insensitive to the presence of AT-dependent FXa inhibitors.³⁴ However, it has been reported that the low procedure of the Biophen DiXal may not warrant the specificity for direct FXa inhibitors anymore. This may be due to an insufficient dilution of the plasma sample in the specific buffer³⁵ (see also the “Materials and Methods” section). As the low procedure seems to be more recommended for the assessment of betrixaban, this may be a problem in case of bridging therapy. Importantly and according to the dynamic range of quantitation of the current chromogenic assays, further investigations should be done to assess the on-therapy concentration of betrixaban (– **Table 2**). The different sensitivity observed with the different chromogenic anti-Xa assays could be explained by differences in chromogenic substrate, origin of FXa, as well as by the ratio between the substrate and the FXa brought into the test.³⁶ The ionic force and the pH of the buffer solution could also be parameters that can impact the sensitivity of a particular assay.⁹ Thus, betrixaban does not respond similarly to chromogenic anti-Xa assays than do other direct FXa inhibitors. This is due to the low plasma concentrations observed in the clinical development, which require tests sensitive at very low equivalent plasma levels. Also, a different affinity for FXa compared with other direct FXa inhibitors is possible, as already observed between apixaban and rivaroxaban.³⁷ Development of specific methodologies for betrixaban are thus required, which may be a barrier to the development of a universal chromogenic anti-Xa assay applicable to DOACs and heparins.

The *CAT* gives more information than traditional coagulation assays.³⁸ By its mode of action, betrixaban mainly acts on the amplification phase of the thrombin generation, affecting mostly the peak and the mVRI. The sensitivity towards the lag time and the time to peak depends on the quantity of TF in the reagent. So, the sensitivity towards the lag time decreases with the amount of TF in the reagent since all reagent tested in this study contain 4 μM of phospholipids. Inversely, the sensitivity towards the time to peak increases with the amount of TF in the reagent.

Impact of betrixaban on Haemostasis Diagnosis Assays

As other anti-coagulant drugs, betrixaban may affect the results of a series of coagulation assays routinely used in case

Table 2 Summary of recommended assays for the measurement of betrixaban in plasma

	Useful for measurement		Reliable but requires laboratory experience	Not recommended			
	Chromogenic anti-Xa assays	dRVVT		TGA	PT	aPTT	TT
Sensitivity (ng/mL)	1–72	5–6 ^a	Peak: 37–58 mVRI: 17–27	5–36	16–57	Insensitive	
Dynamic range of quantitation (ng/mL) ^b	4–231	3–231	Peak: 122–231 mVRI: 60–231	11–231	21–231	NA	
Repeatability (%)	0.6–3.1	0.8–0.9	3.1–8.9	0.5–1.1	0.4–1.2	NA	
Dependence of reagent	Yes	Yes	Yes	Yes	Yes	No	
Linearity of the response	Yes but further investigations required	No	No	Yes	Yes	NA	

Abbreviations: aPTT, activated partial thromboplastin time; dRVVT, dilute Russell viper venom time; FDA, Food and Drug Administration; mVRI, mean velocity rate index; NA, not applicable; PT, pro-thrombin time; RT, reptilase time; TGA, thrombin generation assay; TT, thrombin time.

Note: The on-therapy range of concentration for betrixaban is from ± 9 ng/mL at C_{trough} to ± 122 ng/mL at C_{max} for the 40 and 120 mg once daily doses, respectively (according to the clinical pharmacology and biopharmaceutics reviews of the Center for Drug Evaluation and Research of the FDA¹⁶).

^a5 ng/mL and 6 ng/mL for STA-Staclot-DRVV Screen and Confirm, respectively.

^bDynamic range of quantification is defined as range covering from the lowest observed limit of quantification to the maximal concentration tested.

of thrombophilia or in the exploration of a haemorrhagic event. Our results on routine tests (i.e., PT and aPTT) suggest that sensitivity depends on the reagents used for a particular assay and, thus, there is a need for clinicians to discuss with the laboratory to know whether these tests are influenced by betrixaban to avoid misdiagnosis and useless investigations (► **Table 3**).

The AT measurement using FXa-based or FIIa-based chromogenic assays are not influenced by betrixaban. Thus, in patients treated with betrixaban, thrombin-based or FXa-based chromogenic assays might be used for the assessment of AT rate. Clinically relevant concentration of betrixaban may interfere with the measurement of clotting factors. However, the relation is not linear, as suggested by the plateauing effect observed with the aPTT or the dRVV-T. Dilution of the sample tends to reduce the drop of clotting factors between baseline and high betrixaban concentration, as already stated for rivaroxaban, apixaban and edoxaban.^{9,10,39} As mentioned, the dRVV-T is affected by betrixaban, and prolongation of the clotting time may depend on the concentration, the composition and the concentration of phospholipids (i.e., as demonstrated in this study, the screening reagent is more sensitive than the confirm).^{25,29,30} Therefore, dRVV-T should be avoided for the assessment of LA in patients treated with betrixaban since the ratio between the screen and the confirm will be increased giving possible false-positive results. This has already been reported with other direct FXa inhibitors.⁴⁰ To avoid such results, manufacturers aim to develop methods to remove DOAC from the plasma sample, without affecting the characteristics of the clotting abnormalities.^{8,41} Another possi-

bility to reduce the impact of betrixaban is to increase the dilution of the sample (i.e., at least 1:40). Measurement of protein S does not seem to be influenced until 10 ng/mL of betrixaban but show an over-estimation of approximately 10% at 30 ng/mL.

In clinical routine practice, it is preferable to use assays that do not involve FX since they are not influenced by betrixaban like immunological assays but these do not assess the activity and have higher turn-around time.

Conclusions and Perspectives

Our results demonstrate an important lack of sensitivity for routine tests as reported for other DOACs. As it has been observed with other DOACs, chromogenic anti-Xa assays seem to be interesting candidates to assess the presence of low levels of betrixaban, below 120 ng/mL, but adapted methodologies should be evaluated to improve sensitivity of these reagents for on-therapy range. However, this study shows that adapted-chromogenic anti-Xa assays are the most appropriate assays to measure the anti-coagulant effect of betrixaban. Broadly used coagulation assays such as PT or aPTT are not appropriate to efficiently estimate plasma drug concentration due to several limitations and a lack of sensitivity. The dRVV-T could be informative in certain circumstances due to its good sensitivity and its low LOQ. CAT gives further information on the coagulation process but its use in clinical settings is limited. Finally, betrixaban significantly affects several haemostasis diagnostic tests, such as LA or other thrombophilia testing, and this needs to be taken into consideration when requesting and interpreting such tests.

Table 3 Summary of the influence of betrixaban on haemostasis assays

Coagulation assay	Reagent	Method	Coagulation analyser (manufacturer)	Influenced (Yes/No)	Availability	Recommendations
Thrombin time	STA-Thrombin	Chronometric	STA-R Evolution (Diagnostica Stago)	No	24/7 –all laboratories	/
Reptilase time	STA-Reptilase	Chronometric	STA-R Evolution (Diagnostica Stago)	No	24/7 –all laboratories	/
Lupus anti-coagulant	STA-Staclot DRVV Screen	Chronometric	STA-R Evolution (Diagnostica Stago)	Yes	Can be implemented on all coagulometers	dRVV-T testing should be avoided to determine LA
	STA-Staclot DRVV Confirm					
Anti-thrombin	STA-Stachrom ATIII	Chromogenic thrombin-based	STA-R Evolution (Diagnostica Stago)	No	Can be implemented on all coagulometers	/
	HemosIL Liquid Antithrombin	Chromogenic FXa-based	ACL-TOP (Instrumentation Laboratory)	No		
Intrinsic clotting factors	STA-C.K. Prest	Chronometric	STA-R Max (Diagnostica Stago)	Yes	24/7 –all laboratories	Appropriate dilutions of the sample have to be performed
Extrinsic clotting factors	RecombiPlasTin 2G	Chronometric	STA-R Max (Diagnostica Stago)	Yes	24/7 –all laboratories	Appropriate dilutions of the sample have to be performed. Use of less sensitive reagents could be preferred when feasible (e.g., Dade Innovin for extrinsic pathway)
Fibrinogen Clauss method	STA-Fibrinogen	Chronometric	STA-R Max (Diagnostica Stago)	No	24/7 –all laboratories	/
Fibrinogen PT-derived	RecombiPlasTin 2G	Chronometric	ACL-TOP (Instrumentation Laboratory)	No	24/7 –all laboratories	/
Protein S	Protein S Activity	Chronometric	ACL-TOP (Instrumentation Laboratory)	Yes	24/7 –all laboratories	Not influenced until 10 ng/mL. At higher concentration (from 30 ng/mL), an over-estimation of 10% is observed. This test should be avoided
Protein C	STA-Stachrom Protein C	Chromogenic	STA-R Max (Diagnostica Stago)	No	24/7 –all laboratories	/
Activated protein C resistance	Staclot APCR	Chronometric	STA-R Max (Diagnostica Stago)	Yes	24/7 –all laboratories	This test is slightly influenced and can provide false negative results

Abbreviations: dRVV-T, dilute Russell viper venom time LA, lupus anti-coagulant.

What is known about this topic?

- Therapeutic monitoring is not necessary with betrixaban but may be required in some clinical situations.
- No guidance documents are currently available to guide on the assay to use to perform measurement.
- Other DOACs have shown interferences with routinely used coagulation assays leading to misdiagnosis.

What does this paper add?

- This study provides a comparison of the impact of betrixaban on specific and routinely used coagulation assays with a large panel of reagents.
- The performance of these tests in terms of sensitivity, limit of detection, limit of quantitation, dynamic range of measurement and repeatability were assessed.
- Chromogenic anti-Xa assays with adapted methodologies are the most recommended assays for the assessment of betrixaban.

Conflict of Interest

Among the authors, J. Douxfils is CEO and founder of QUALIBlood s.a. and reports personal fees from Stago and Daiichi-Sankyo, outside the submitted work. F. Mullier reports institutional fees from Stago, Werfen, Nodia, Sysmex and Bayer. He also reports speaker fees from Boehringer Ingelheim, Bayer Healthcare and Bristol-Myers Squibb-Pfizer, all outside the submitted work. Other authors have no conflicts of interest to disclose.

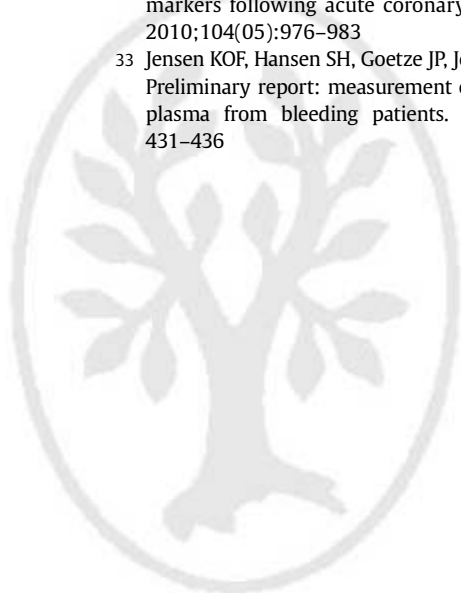
Acknowledgments

The authors would like to thank Christelle Vancraeynest, Philippe Devel, H el ene Hagu et and Celine Bouvy for their contribution to this work. Furthermore, we are grateful towards C elia Devroye and Ma it e Guldenpfenning for their invaluable assistance.

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THIEME