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Full Length Article

A comparative *in vitro* study of the anticoagulant effect of branded *versus* generic rivaroxaban

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ABSTRACT

Background: Several generic formulations of rivaroxaban were recently marketed to be used interchangeably with their branded equivalent. However, there have been no previously published studies that directly compared the *in vitro* anticoagulant effect of branded *vs.* generic rivaroxaban. The aim of this *in vitro* study was to compare the effects of three raw rivaroxaban materials, obtained from the branded (Xarelto®) and two generic (Rivarolto® and Rivaroxaban Sandoz®) rivaroxaban formulations on an array of coagulation assays.

Methods: A pool of normal plasma was spiked with several concentrations of the three rivaroxaban (range 50–750 ng/ml). The concentrations were assessed with a rivaroxaban calibrated anti-Xa assay and confirmed by ultra-high-performance liquid chromatography-mass spectrometry coupled with tandem mass spectrometry (UHPLC-MS/MS). The following assays were performed: Prothrombin time (PT), activated Partial Thromboplastin time (aPTT), Diluted Russell's Viper Venom Test (dRVVT), Thrombin time (TT), Clauss Fibrinogen, Factor VII, VIII and IX assays, and thromboelastography.

Results: The results obtained by the three rivaroxaban at similar concentrations were comparable. Increasing concentrations of the three rivaroxaban showed a strong positive correlation with the PT, aPTT and dRVVT assays ($r > 0.95$, $p < 0.01$ for all), and a strong negative correlation with the Factors assays ($r < -0.95$, $p < 0.01$ for all). TT and Clauss Fibrinogen were not affected by rivaroxaban. No significant difference was identified in the mean assays' results obtained by the three rivaroxaban.

Conclusion: This study showed that the branded and generic rivaroxaban exert an identical *in vitro* anticoagulant effect across a wide range of concentrations.

1. Introduction

Rivaroxaban is a direct oral anticoagulant (DOAC) widely used for the treatment and prevention of several thromboembolic disorders [1]. It is a small molecule which selectively binds with high affinity to the active site of activated factor X (FXa) in a concentration-dependent manner, without the need for any co-factors. The interaction of rivaroxaban with the active site is both competitive and reversible [2]. Rivaroxaban directly inhibits both free and clot-associated FXa, and can also inhibit FXa in the prothrombinase complex, unlike the indirect FXa inhibitors [3]. FXa is an ideal target for effective anticoagulation since it is positioned at the point where the intrinsic and extrinsic pathways converge into the common pathway of the coagulation cascade [4].

In 2020, the first generic formulations of rivaroxaban obtained

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marketing authorisation by the European Medicines Agency (EMA). The recent availability of the generic versions of rivaroxaban is set to increase the use of the DOACs [5], leading to more competitive prices and decreasing the costs associated with treatment and prevention of thromboembolic disorders [6]. Both the branded type and the generic versions contain the same active substance, rivaroxaban. They are considered bioequivalent since bioavailability studies showed that the generic versions have the same pharmacokinetic properties as the branded version (Xarelto®) [7]. In particular, the maximum plasma concentration (Cmax) and the area under the concentration time curve (AUC), and their 90 % confidence interval, should be within the conventional acceptance range of 80–125 % [8,9].

Due to its anticoagulant activity, rivaroxaban can impact both routine and specialised coagulation assays which are affected by the function of FXa [10–12]. However, it was still unclear whether the anticoagulant effect of the generic rivaroxaban on coagulation assays was identical to the branded rivaroxaban, since no previous studies have been published on the matter.

The aim of this study was to compare the *in vitro* anticoagulant effect of three raw rivaroxaban materials, obtained from the branded rivaroxaban (Xarelto®) and two recently approved generic rivaroxaban (Rivarolto® and Rivaroxaban Sandoz®) at several concentrations (range 50–750 ng/ml), on an array of coagulation assays.

2. Materials and method

2.1. Plasma collection and preparation

Plasma from six blood donors was collected at the National Blood Transfusion Service centre (Pieta, Malta). All donors signed a consent form prior to the donation procedure. The plasma bags contained citrate phosphate dextrose as an anticoagulant preservative. The plasma bags (average platelet count $<10*10^9$/L) were initially frozen at −30 °C, as per standard preparation procedure for the fresh frozen plasma, and subsequently stored at −80 °C until further processing.

On the day of pooling, the six plasma bags were thawed in the water bath at 37 °C for 15–20 min. The thawed plasma was pooled in a polypropylene container and gently mixed for homogeneity. A pool of normal platelet poor plasma (NPPP) was obtained with the following characteristics: platelet count $6*10^9$/L, prothrombin time ([PT], Dade® Innovin®, Siemens Healthcare Diagnostics, Marburg, Germany) 10.8 s, activated partial thromboplastin time ([aPTT], Dade® Actin® FS, Siemens Healthcare Diagnostics, Germany) 27.1 s, rivaroxaban anti-Xa (Biophen® Heparin LRT with Biophen® Rivaroxaban Plasma Calibrator, Hyphen BioMed, Neuville-sur-Oise, France) lower than the limit of detection (<30 ng/ml). Anti-Xa values below the threshold concentration 30 ng/ml are not considered clinically significant [13].

2.2. Rivaroxaban preparation and spiking

Xarelto® (Bayer HealthCare, Germany; lot number ITA3LKA), Rivarolto® (TAD Pharma, Germany; lot number V31421) and Rivaroxaban Sandoz® (Sandoz, Slovenia; lot number V0RF01A) were purchased as 10 mg tablets. One tablet (*i.e.* 10 mg) for each rivaroxaban formulation was crushed into powder form using a pill crusher. The powder was initially dissolved in 10 ml of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Hamburg, Germany) and then diluted at 1:9 in deionised water to obtain a stock solution at 0.1 mg/ml.

The stock solution of each formulation was diluted with NPPP aiming to obtain a concentration of 1000 ng/ml. Further dilutions were performed by adding NPPP to obtain five predetermined concentrations: 750, 500, 300, 150, 50 ng/ml. A concentration difference of ±50 ng/ml from the target concentration was accepted. The concentration of each formulation was measured twice with the chromogenic anti-Xa assay (Biophen® Heparin LRT, Hyphen BioMed, Neuville-sur-Oise, France) using a rivaroxaban specific calibrator (Biophen® Rivaroxaban Plasma Calibrator, Hyphen BioMed, Neuville-sur-Oise, France). It was then adjusted accordingly, by adding either the same unspiked NPPP or the same NPPP spiked with a higher concentration of rivaroxaban. The target concentrations were selected to represent a broad range of concentrations, ranging from supratherapeutic to subtherapeutic. There is no universally established therapeutic range for rivaroxaban, but the peak levels were reported to be 90–190 ng/ml following a 10 mg prophylactic dose and 180–340 ng/ml following a 20 mg therapeutic dose of rivaroxaban [14].

The same unspiked NPPP was used as comparison (concentration: 0 ng/ml). Spiked and unspiked plasma were divided into 500 μl aliquots and frozen at −80 °C until analysis. On the day of testing, the aliquots were thawed for 2 min in a water bath at 37 °C. All the plasma used in this study underwent three freeze-thaw cycles. A similar study stated that the maximum acceptable times that a freeze-thawing cycle can be performed without significantly reducing the factor VIII levels is three, on the condition that the plasma is stored at −80 °C [15]. In the current study, the factor VIII level in the NPPP was confirmed to be stable at a level above 100 % after the three freeze-thaw cycles.

2.3. Ultra-high-performance liquid chromatography coupled with tandem-mass spectrometry

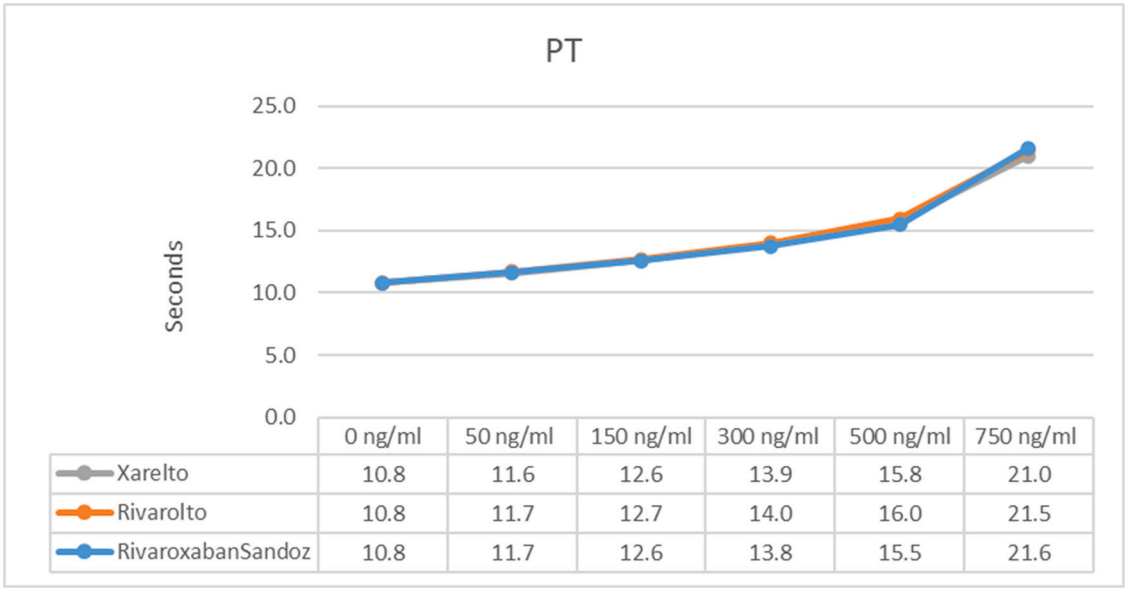
In order to obtain a more accurate measurement of the concentration of rivaroxaban in the spiked samples, frozen aliquots were sent in dry ice to Qualiblood laboratories (Namur, Belgium) for ultra-high-performance liquid chromatography coupled with tandem-mass spectrometry (UHPLC-MS/MS) analysis. This technique was performed on the Acquity UHPLC H-class system® (Waters Corporation, Massachusetts, USA) attached to a Xevo TQ-S tandem mass spectrometer (Waters Corporation, Massachusetts, USA). The MassLynx 4.1 software (Waters Corporation, Massachusetts, USA) was used to obtain the data and control the instrument. UHPLC-MS/MS is considered as the gold standard to measure the plasma concentration of DOACs since it is capable of measuring rivaroxaban concentrations between 1 and 500 ng/ml with excellent accuracy [16,17]. The lower limit of quantification (LLOQ) is 3 ng/ml and the range of measurement extend until 500 ng/ml without additional sample dilution. The validation is in accordance with the ICH guideline M10 on bioanalytical method validation. The intra-run CV was below 10 % and the inter-run CV was below 15 % for all quality control levels.

2.4. Coagulation assays

The coagulation assays were performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) on Sysmex® CS-2500 analysers (Sysmex Corporation, Kobe, Japan). Internal quality control was conducted before the commencement of sample analysis with each assay or when there was a reagent change. Each sample was analysed in duplicate, and the mean value was recorded. A difference < 10 % between the two results was accepted (except for PT-derived fibrinogen, for which a difference < 20 % was considered acceptable).

For each assay, unspiked pooled plasma was analysed first, followed by the spiked samples. The following routine and specialised coagulation assays, which are known to be affected by the anticoagulant activity of FXa inhibitors [10,18–20] were performed:

- PT and PT-derived fibrinogen (PT-Fg): The PT reagent utilised was Dade® Innovin® (Siemens Healthcare Diagnostics, Marburg, Germany), which is known to have a low sensitivity for direct FXa inhibitors [18,21]. The CV% in this study were 0.5 % for the PT and 1.2 % for the PT-derived fibrinogen.
- aPTT: The aPTT reagents utilised were Dade® Actin® FS and Dade® Actin® FSL (Siemens Healthcare Diagnostics, Marburg, Germany). The CV% were 0.4 % with Actin® FS and 0.2 % with Actin® FSL. A ratio between the Actin® FSL and the Actin FS® was also calculated.



	0 ng/ml	50 ng/ml	150 ng/ml	300 ng/ml	500 ng/ml	750 ng/ml
Xarelto	10.8	11.6	12.6	13.9	15.8	21.0
Rivarolto	10.8	11.7	12.7	14.0	16.0	21.5
RivaroxabanSandoz	10.8	11.7	12.6	13.8	15.5	21.6

Fig. 1. Relationship between the prothrombin time (PT) values and the concentrations of the three rivaroxaban.

- Diluted Russell's Viper Venom Test (dRVVT): Hemoclot® LA-S and Hemoclot® LA-C (Hyphen BioMed, Neuville-sur-Oise, France) were used to perform the screen and confirm tests, respectively. The CV% in this study were 0.7 % for the dRVVT screen and 0.8 % for the dRVVT confirm. A ratio between dRVVT screen and dRVVT confirm (lupus anticoagulant [LA] ratio) was also calculated.
- One-stage PT-based Factor VII: Dade® Innovin® and Coagulation Factor VII Deficient Plasma (Siemens Healthcare Diagnostics, Marburg, Germany) were utilised to determine the level of Factor VII. The CV% in this study was 2.5 %.
- One stage aPTT-based Factor VIII and IX: Dade® Actin® FS, Coagulation Factor VIII and IX Deficient Plasma, and $CaCl_2$ (Siemens Healthcare Diagnostics, Marburg, Germany) were used to determine the activity of Factor VIII and IX. The CV% in this study were 1.4 % for the one-stage FVIII and 1.8 % for the one-stage FIX.
- Chromogenic Factor VIII and IX: The Biophen® FVIII and Biophen® FIX kits (Hyphen BioMed, Neuville-sur-Oise, France) were used to chromogenically measure the factor activity of FVIII and FIX. The CV % in this study were 4.3 % for the Chromogenic FVIII and 2.4 % for the Chromogenic IX.

The following assays, which were not expected to be influenced by direct FXa inhibitors [10,18–20,22,23], were performed as control:

- Thrombin time (TT): Thromboclotin® (Siemens Healthcare Diagnostics, Marburg, Germany) was the reagent used to measure the thrombin time. The CV% in this study was 2.3 %.
- Clauss Fibrinogen: Dade® Thrombin Reagent (Siemens Healthcare Diagnostics, Marburg, Germany) was used to conduct this test. The CV% in this study was 1.4 %.

2.5. Thromboelastography

The thromboelastography (TEG) was performed using the TEG® 5000 analyser (Haemonetics®, Salt Lake City, USA), and TEG Analytical Software Version 4.2.3 (Haemonetics®, Salt Lake City, USA). Internal quality control was conducted daily before the sample analysis. Since the native TEG was performed on plasma, 330 μl of plasma and 30 μl of 0.2 M $CaCl_2$ were used, as previously reported [24]. The reaction time (R time), kinetics time (K time), alpha angle (α angle) and maximum amplitude (MA) were recorded. Each sample was analysed in duplicate, and the mean value was recorded. The CV% were 14.3 % for the R-time, 38.8 % for the K-time, 26 % for the α-angle and 8.2 % for the MA.

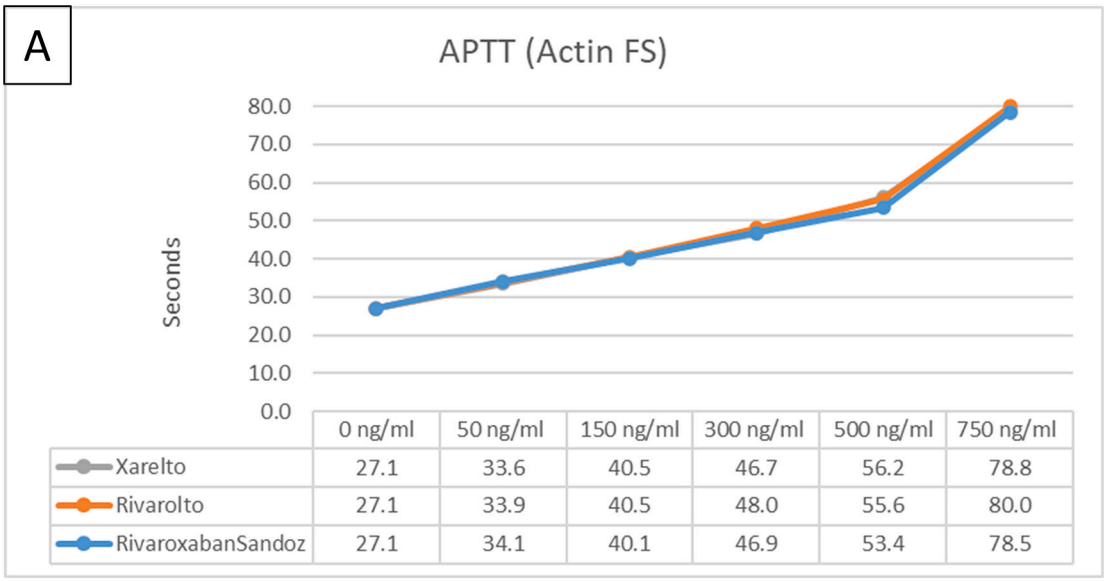
2.6. Ethical approval

This study was approved by the Faculty of Health Sciences Research Ethics Committee and the University of Malta Research Ethics Committee (UREC Form V_15062020 8553).

2.7. Statistical analysis

The data was analysed using the IBM® SPSS® Statistics software for Windows, Version 27 (IBM Corp., USA). Normality was assessed using the Kolmogorov-Smirnov and the Shapiro Wilk tests. Since all the variables were found to follow a normal distribution, parametric tests were performed, and results were expressed as mean ± standard deviation (SD). The Pearson's correlation test was utilised to measure the strength of the relationships between the concentrations of the three rivaroxaban (measured with the chromogenic Anti-Xa) and the results of each coagulation assay, and to determine whether these relationships are significant. Using the Bonferroni correction method, a 0.017 level of significance was adopted to adjust for multiplicity of comparisons. After checking equality of variances using the Levene's test, the One-way ANOVA test was utilised to identify any difference in the mean values of the three rivaroxaban. Moreover, the Tukey *post hoc* test was used for pairwise comparison of the three rivaroxaban. This *post hoc* analysis, similar to Bonferroni correction method, makes adjustments based on the number of comparisons; however, it makes adjustments to the test statistic when running the pairwise comparisons.

The coefficient of variation (CV%) of each assay was established to determine the intra-assay variability. Two aliquots from each of the six plasma bags were tested and the CV% between the two values was calculated using the following formula inputted into Microsoft Excel®: CV% = (SD / mean) * 100. Subsequently, the mean of the six CV% obtained from the six plasma bags was calculated and presented as the CV % of the assay.



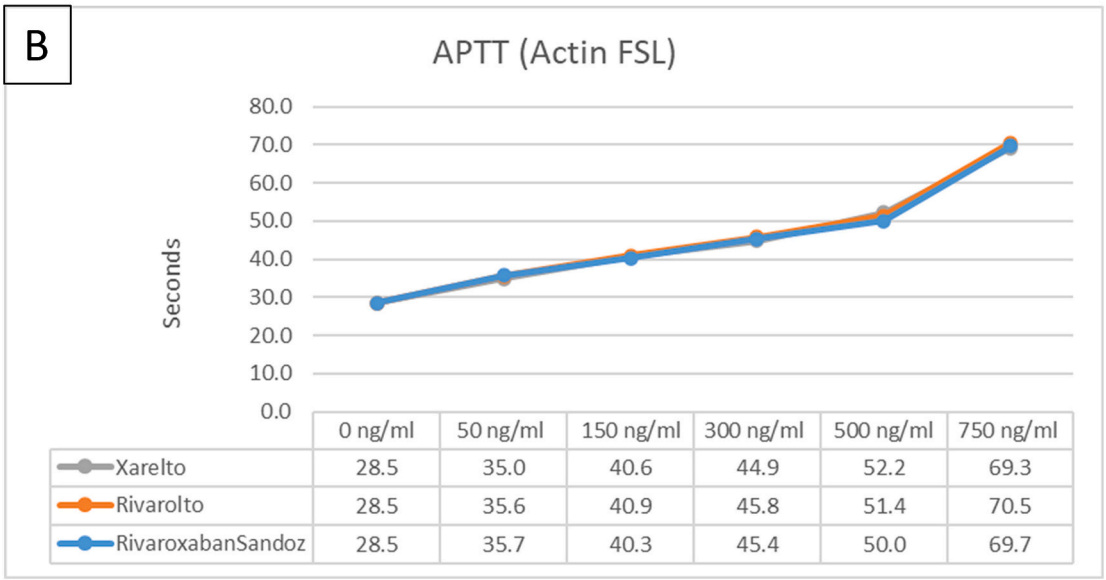


Fig. 2. Relationship between the activated partial thromboplastin time (aPTT) values, measured with Actin® FS (panel A) and Actin® FSL (panel B), and the concentrations of the three rivaroxaban.

3. Results

3.1. Chromogenic anti-Xa and ultra-high-performance liquid chromatography coupled with mass spectrometry

Using the rivaroxaban-calibrated chromogenic anti-Xa assay, all the measured concentrations of the three rivaroxaban were within ±50 ng/ml from the planned concentrations and they were similar for the three rivaroxaban (Table 1). Results obtained with the UHPLC-MS/MS were slightly higher than the chromogenic anti-Xa assay for concentrations 50–500 ng/ml, while for the 750 ng/ml concentration the UHPLC-MS/MS technique gave slightly lower measurements. However, the latter was outside the upper limit of measurement of this assay and additional dilution of the samples was required. There was a strong positive correlation between the chromogenic anti-Xa and the UHPLC-MS/MS for all three rivaroxaban ($r > 0.95$, $p < 0.01$ for all; *Supplementary table*).

3.2. Prothrombin time assay

The three rivaroxaban prolonged the PT in a similar concentration-dependent manner (Fig. 1). There was a strong positive correlation between the concentrations of the three rivaroxaban and the PT assay results ($r > 0.95$, $p < 0.01$ for all) and no statistically significant difference among the mean PT values ($p = 0.997$; *Supplementary table*).

3.3. Activated partial thromboplastin time assay

The three rivaroxaban prolonged the aPTT in a similar concentration-dependent manner. Comparable results were obtained when using both Actin® FS and Actin® FSL reagents (Fig. 2a-b). There was a strong positive correlation between the concentrations of the three rivaroxaban and the aPTT assays results ($r > 0.95$, $p < 0.01$ for all) and no statistically significant difference among the mean aPTT values ($p \geq 0.996$; *Supplementary table*).

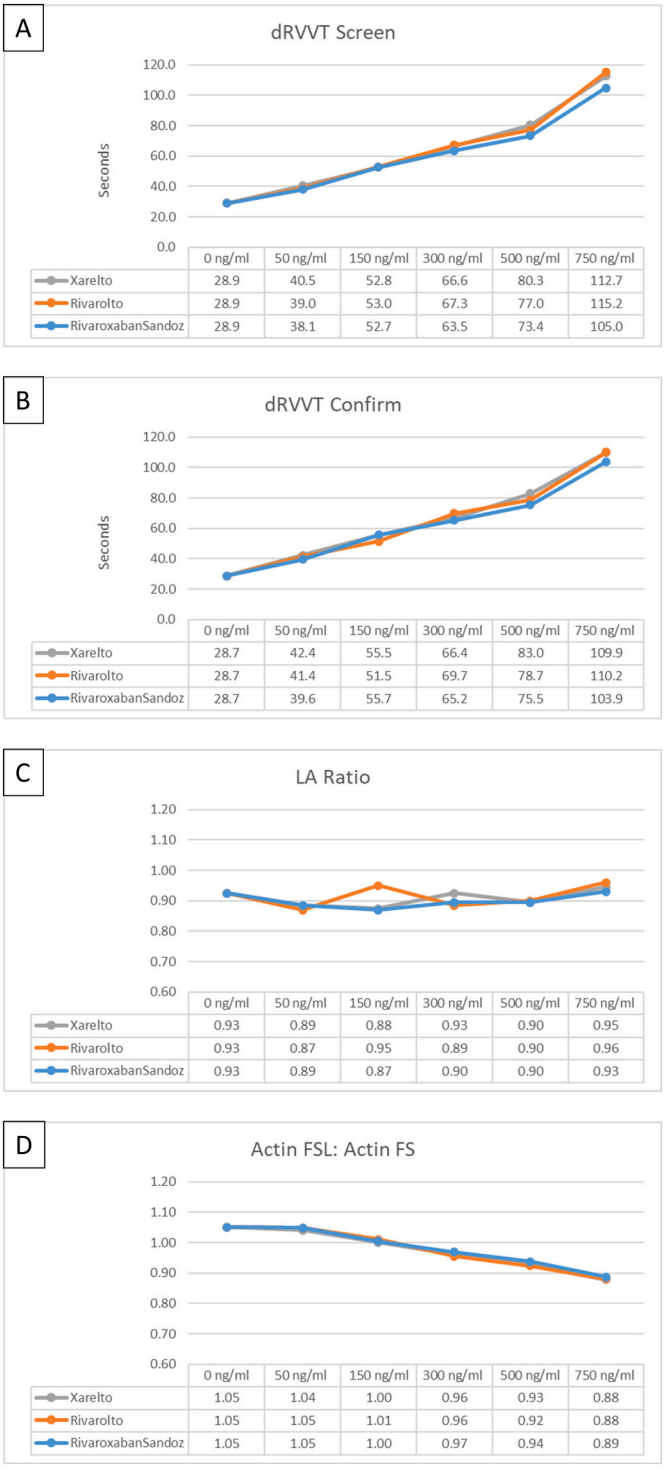


Fig. 3. Relationship between the diluted Russell viper venom time (dRVVT) screen (panel A), dRVVT confirm (panel B) values, lupus anticoagulant (LA) ratio (panel C), Actin® FSL: Actin® FS ratio (panel D) and the concentrations of the three rivaroxaban.

3.4. Diluted Russell's viper venom test and lupus anticoagulant ratio

The three rivaroxaban prolonged the dRVVT screen and confirm tests in a similar concentration-dependent manner (Fig. 3a-b). There was a strong positive correlation between the concentrations of the three rivaroxaban and the dRVVT screen and confirm assays results ($r > 0.95$, $p < 0.01$ for all) and no statistically significant difference among the mean dRVVT values ($p \geq 0.966$; *Supplementary table*).

The LA ratio remained relatively constant as the rivaroxaban concentration increased (Fig. 3c), while the Actin® FSL: Actin® FS ratio, which can be used for LA screening, decreased as the concentration increased (Fig. 3d).

Table 1
The measured concentrations of the three rivaroxaban using the chromogenic anti-Xa and the ultra-high-performance liquid chromatography-coupled with tandem mass spectrometry.

Predetermined concentrations (ng/ml)	Xarelto®		Rivarolto®		Rivaroxaban Sandoz®	
	Anti-Xa (ng/ml)	UHPLC-MS/MS[a] (ng/ml)	Anti-Xa (ng/ml)	UHPLC-MS/MS[a] (ng/ml)	Anti-Xa (ng/ml)	UHPLC-MS/MS[a] (ng/ml)
50	49.8	66.3	49.0	64.5	48.9	64.0
150	172.0	210.6	166.4	209.5	168.3	200.4
300	322.9	378.0	333.1	382.2	313.5	350.9
500	527.9	531.7	540.3	550.3	482.4	506.4
750[b]	*796.5*	*641.8*	*797.7*	*746.1*	*795.5*	*660.9*

Legend: UHPLC-MS/MS = ultra-high-performance liquid chromatography-coupled with tandem mass spectrometry.
[a] Each UHPLC-MS/MS value is a mean out of six measurements; however, for the highest concentration only two measurements were available.
[b] For both the Anti-Xa and the UHPLC-MS/MS, all measurements of the highest concentration (in italics) were calculated with the dilution method.

3.5. Thrombin time assay

There was no significant correlation between the concentrations of the three rivaroxaban and the TT assay results (Fig. 4) and no statistically significant difference among the mean TT values ($p = 0.277$; *Supplementary table*).

3.6. Clauss Fibrinogen assay

There was no significant correlation between the concentrations of the three rivaroxaban and the Clauss Fibrinogen assay results (Fig. 5a) and no statistically significant difference among the mean Clauss Fibrinogen values ($p = 0.427$; *Supplementary table*).

3.7. Prothrombin time-derived fibrinogen assay

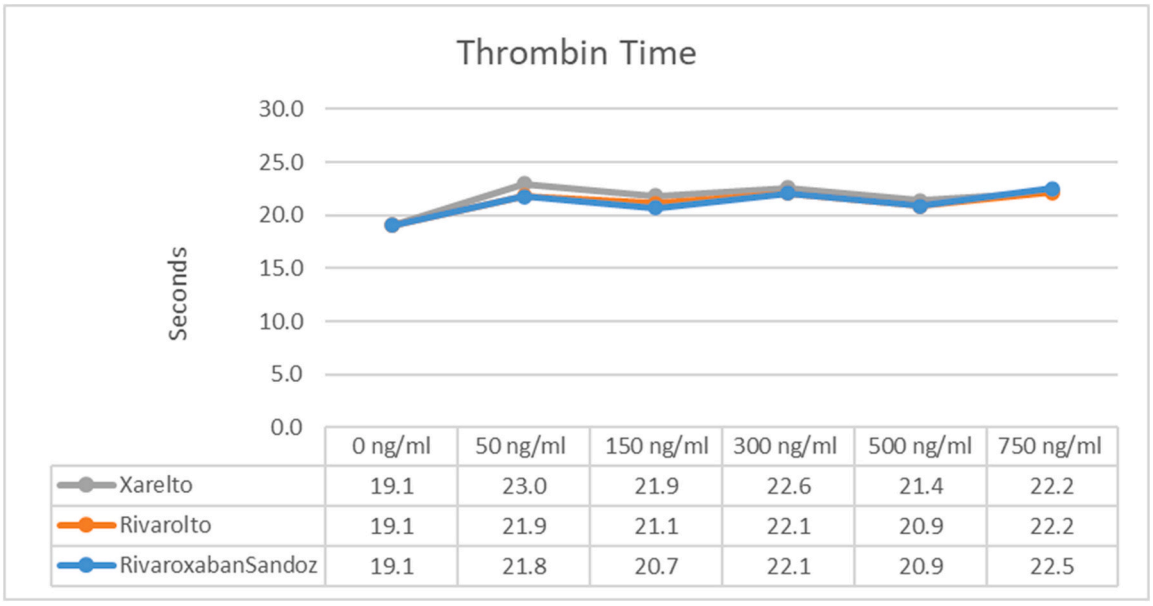
The three rivaroxaban prolonged the PT-derived fibrinogen assay in a similar concentration-dependent manner (Fig. 5b). However, the values of the PT-derived fibrinogen were almost identical for the sub-therapeutic and therapeutic concentrations. There was a strong positive correlation between the concentrations of the three rivaroxaban and the PT-derived fibrinogen assay results ($r > 0.95$, $p < 0.017$ for all) and no statistically significant difference among the mean PT-derived fibrinogen values ($p = 0.980$; *Supplementary table*).

3.8. One-stage Factor VII, VIII and IX assays

The three rivaroxaban caused a similar concentration-dependent decrease in the levels of FVII, FVIII and FIX, measured by the respective one-stage factor assays (Fig. 6). There was a strong negative correlation between the concentrations of the three rivaroxaban and the one-stage FVII, FVIII and FIX assays results ($r < -0.95$, $p < 0.01$ for all) and no statistically significant difference among the mean coagulation factor values ($p \geq 0.951$; *Supplementary table*).

3.9. Chromogenic FVIII and FIX assays

The three rivaroxaban caused a similar concentration-dependent



	0 ng/ml	50 ng/ml	150 ng/ml	300 ng/ml	500 ng/ml	750 ng/ml
Xarelto	19.1	23.0	21.9	22.6	21.4	22.2
Rivarolto	19.1	21.9	21.1	22.1	20.9	22.2
RivaroxabanSandoz	19.1	21.8	20.7	22.1	20.9	22.5

Fig. 4. Relationship between the thrombin time (TT) values and the concentrations of the three rivaroxaban.

decrease in the levels of FVIII and FIX, measured by the respective chromogenic assays (Fig. 7). There was a strong negative correlation between the concentrations of the three rivaroxaban and the Chromogenic FVIII and FIX assays results ($r < -0.95$, $p < 0.01$ for all) and no statistically significant difference among the mean coagulation factor values ($p \geq 0.971$; *Supplementary table*).

3.10. Thromboelastography

For all three rivaroxaban, the R-time increased in a concentration-dependent manner (*Supplementary fig. A*). Xarelto® and Rivaroxaban Sandoz® showed a strong positive correlation with the R-time ($r > 0.95$, $p < 0.01$ for both), while the correlation for Rivarolto® was not statistically significant ($r = 0.932$, $p = 0.021$; *Supplementary table*).

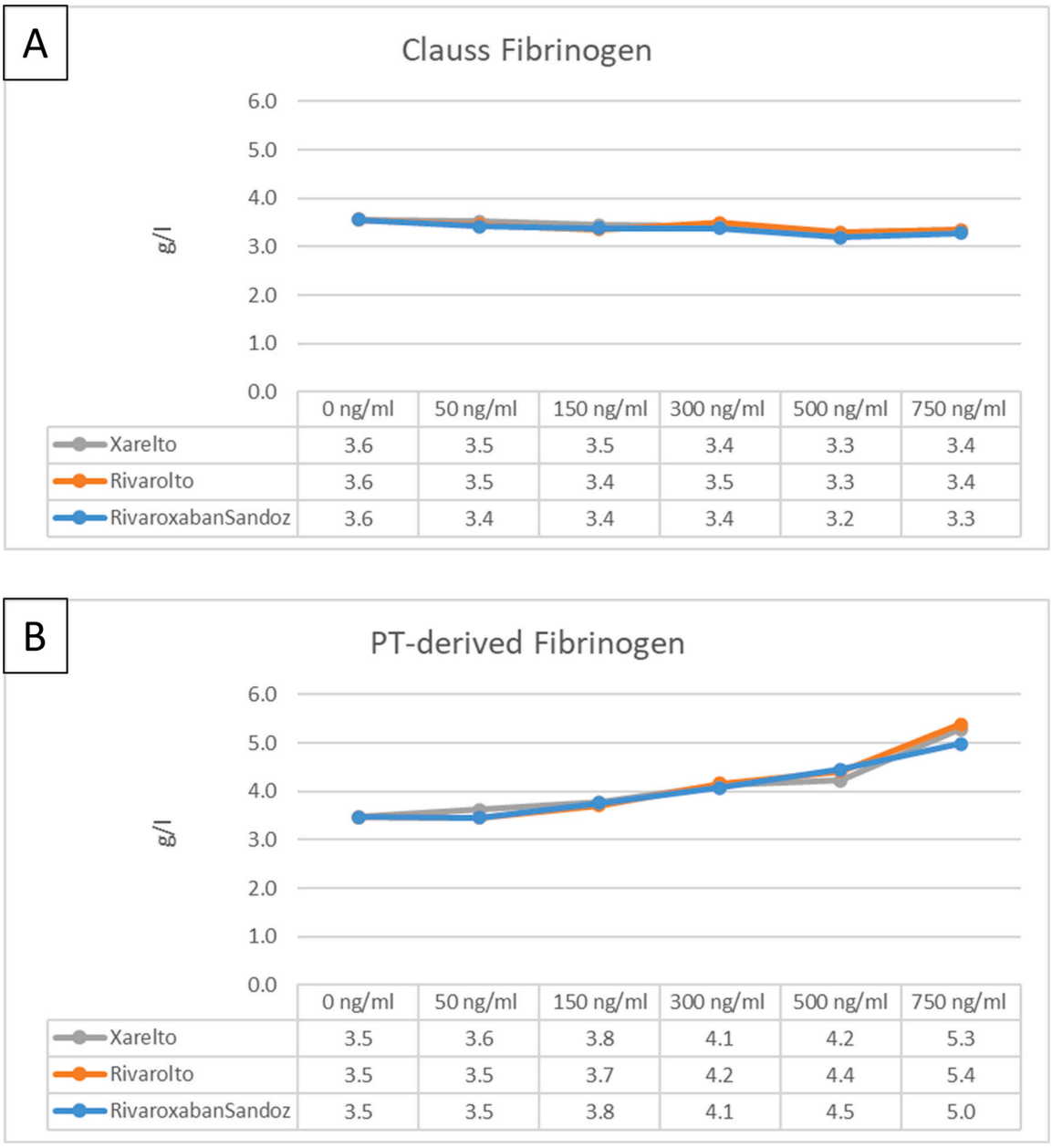
The patterns of the K-time, α-angle and MA were more erratic (*Supplementary figs. B-C-D*). Of note, the CV% of the four TEG parameters were relatively high compared to the other assays utilised in this study. No statistically significant difference was identified among the mean values of the four TEG parameters obtained by the three rivaroxaban ($p \geq 0.165$; *Supplementary table*).

4. Discussion

This study showed that three raw rivaroxaban materials, obtained from the branded (Xarelto®) and two generic (Rivarolto® and Rivaroxaban Sandoz®) rivaroxaban formulations, exerted an identical *in vitro* anticoagulant effect. A concentration range of 50–750 ng/ml was achieved by *in vitro* spiking of rivaroxaban into normal plasma, thus our results show that under controlled laboratory conditions the three rivaroxaban have the same impact on an array of coagulation assays. As summarised in Fig. 8, increasing concentrations of the three rivaroxaban resulted in increasing values of PT, aPTT, dRVVT screen, dRVVT confirm, and PT-derived fibrinogen, and decreasing values of the one-stage and chromogenic factor assays. Previously, several studies have observed the same anticoagulant effect produced by the branded rivaroxaban [10,11,18–20,22,25–34]. However, the current study, for the first time, compared the effect of similar concentrations of branded *vs.* generic rivaroxaban on these coagulation assays.

The level of interference exerted by rivaroxaban on a coagulation assay mainly relies on the assay's level of dependence on FXa's function, due to rivaroxaban being a direct FXa inhibitor. The chromogenic rivaroxaban-calibrated Xa assay is the most indicated test to routinely measure rivaroxaban plasma levels [18]. We observed a strong correlation between the anti-Xa and the UHPLC-MS/MS results, as previously reported [18,35–38]. For concentrations ranging from 50 to 500 ng/ml the variation between the anti-Xa and UHPLC-MS/MS results was low, with the anti-Xa assay being slightly lower. For the predetermined 750 ng/ml concentration, the anti-Xa provided higher concentration values compared to the UHPLC-MS/MS. Both assays are accurate up to concentrations of approximately 500 ng/ml, and the measurement of higher concentrations requires additional dilution of the sample and re-testing. The dilution method provides estimated concentration values that are less reliable, considering that at such supratherapeutic concentrations the calibration curve for the anti-Xa does not remain linear [39]. Another study testing the 750 ng/ml concentration showed similar results [37]. In the current study, when the ~750 ng/ml concentration was removed, the positive correlation between UHPLC-MS/MS and the chromogenic anti-Xa results of the three rivaroxaban was stronger (*Supplementary table*). The UHPLC-MS/MS is considered as the gold standard to measure the rivaroxaban plasma concentration. However, the chromogenic anti-Xa assay is more suitable for laboratory assessment since it is cheaper, simpler, faster and requires less technical expertise, thus being more convenient for routine clinical practice [36].

The chromogenic rivaroxaban-calibrated Xa assay is the most indicated test to routinely measure rivaroxaban plasma levels [18]. We observed a strong correlation between the anti-Xa and the UHPLC-MS/MS results, as previously reported [18,35–38]. For concentrations ranging from 50 to 500 ng/ml the variation between the anti-Xa and UHPLC-MS/MS results was low, with the anti-Xa assay being slightly lower. For the ~750 ng/ml concentration, the anti-Xa provided higher concentration values compared to the UHPLC-MS/MS. Both assays are accurate up to concentrations of approximately 500 ng/ml, and the measurement of higher concentrations requires additional dilution of the sample and re-testing. The dilution method provides estimated concentration values that are less reliable, considering that at such supratherapeutic concentrations the calibration curve for the anti-Xa does not remain linear [39]. Another study testing the 750 ng/ml concentration showed similar results [37]. In the current study, when the ~750 ng/ml concentration was removed, the positive correlation between UHPLC-MS/MS and the chromogenic anti-Xa results of the three rivaroxaban was stronger (*Supplementary table*). The UHPLC-MS/MS is considered as the gold standard to measure the rivaroxaban plasma



	0 ng/ml	50 ng/ml	150 ng/ml	300 ng/ml	500 ng/ml	750 ng/ml
Xarelto	3.6	3.5	3.5	3.4	3.3	3.4
Rivarolto	3.6	3.5	3.4	3.5	3.3	3.4
RivaroxabanSandoz	3.6	3.4	3.4	3.4	3.2	3.3

	0 ng/ml	50 ng/ml	150 ng/ml	300 ng/ml	500 ng/ml	750 ng/ml
Xarelto	3.5	3.6	3.8	4.1	4.2	5.3
Rivarolto	3.5	3.5	3.7	4.2	4.4	5.4
RivaroxabanSandoz	3.5	3.5	3.8	4.1	4.5	5.0

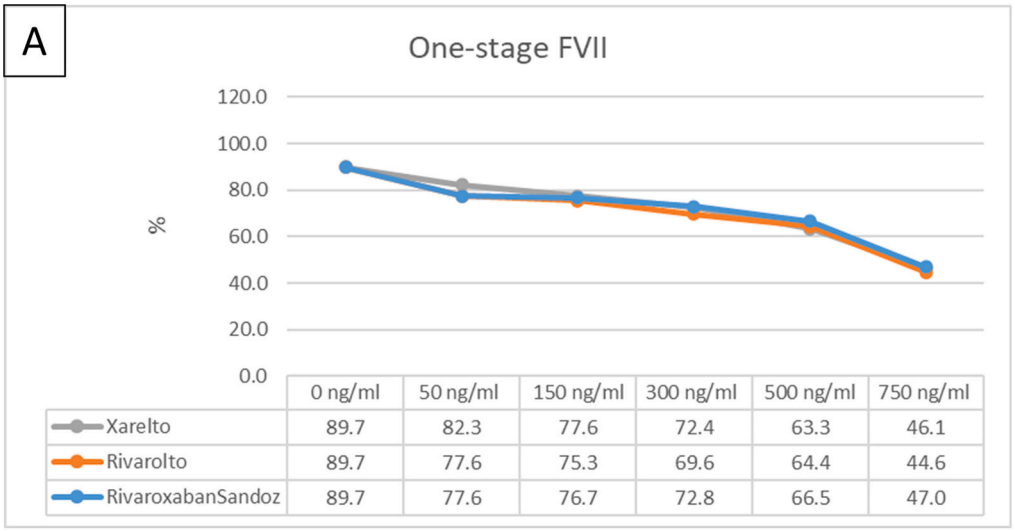
Fig. 5. Relationship between the Clauss Fibrinogen (panel A) and prothrombin time (PT)-derived Fibrinogen (panel B) values and the concentrations of the three rivaroxaban.

concentration. However, the chromogenic anti-Xa assay is more suitable for laboratory assessment since it is cheaper, simpler, faster and requires less technical expertise, thus being more convenient for routine clinical practice [36].

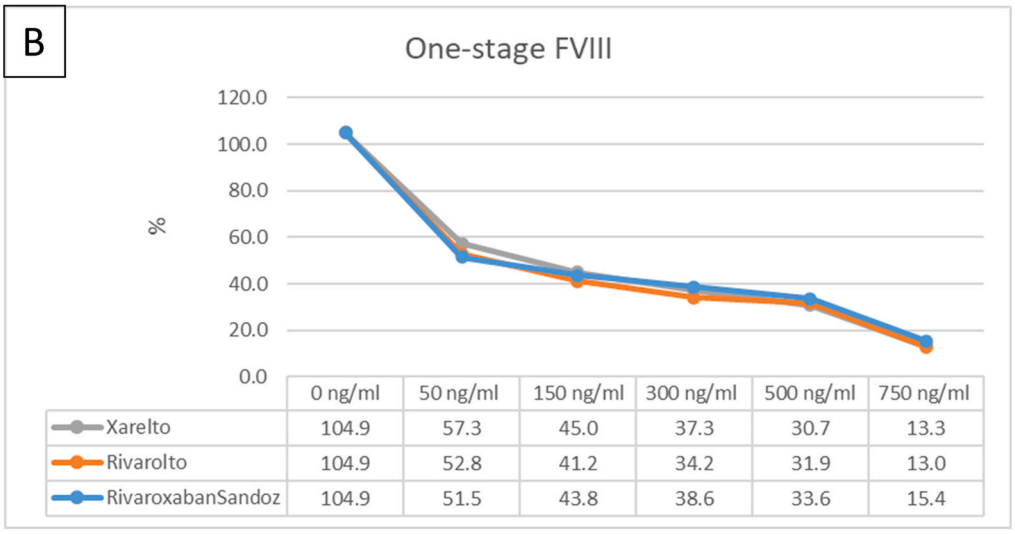
The PT and aPTT assays are less sensitive to DOACs [40] and they cannot be used for rivaroxaban monitoring, since they do not correlate reliably with drug concentrations. The PT is generally more affected by rivaroxaban; however, there is significant variability based on the thromboplastin reagent used [18,20,21]. In this study, the aPTT was more sensitive to rivaroxaban than the PT, due to the different reagents used. Actin FS® and Actin FSL® are some of the most sensitive aPTT reagents, while Innovin® is one of the least sensitive PT reagents [18,21].

Despite other authors reporting that rivaroxaban tends to prolong the dRVVT screen more than the dRVVT confirm, resulting in the risk of false positive LA results [20], in the current study the dRVVT screen and confirm (performed using Hemoclot® LA-S and LA-C reagents) were similarly impacted, thus the LA ratio remained approximately the same as the rivaroxaban concentration increased. A previous study [29] also found that the LA ratio (measured using Hemoclot® reagents) was not affected by increasing concentrations of rivaroxaban. However, it is recommended not to perform LA testing in patients receiving DOAC therapy, because of the risk of false positive [20] and false negative results [40,41]. An alternative is the use of DOAC stop® or the DP-Filter®, which can effectively neutralise the *in vitro* effect of all DOACs, including rivaroxaban [42,43].

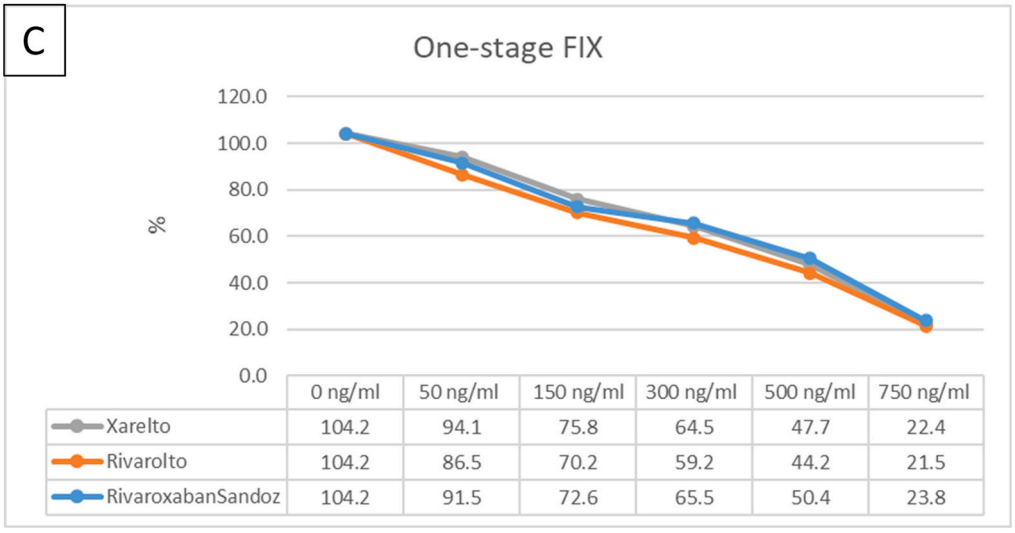
The one-stage and chromogenic factor assays (FVII, FVIII, FIX) were significantly affected by rivaroxaban, thus they should not be used to measure factor levels in patients on DOACs. Apart from the use of the abovementioned removal devices, the interference of rivaroxaban can also be reduced by using PT and aPTT reagents with a low sensitivity to rivaroxaban [34]. The PT-Fg was significantly affected by rivaroxaban, thus this assay should not be utilised to measure the fibrinogen level in patients receiving rivaroxaban, since its results can be falsely elevated



	0 ng/ml	50 ng/ml	150 ng/ml	300 ng/ml	500 ng/ml	750 ng/ml
Xarelto	89.7	82.3	77.6	72.4	63.3	46.1
Rivarolto	89.7	77.6	75.3	69.6	64.4	44.6
RivaroxabanSandoz	89.7	77.6	76.7	72.8	66.5	47.0



	0 ng/ml	50 ng/ml	150 ng/ml	300 ng/ml	500 ng/ml	750 ng/ml
Xarelto	104.9	57.3	45.0	37.3	30.7	13.3
Rivarolto	104.9	52.8	41.2	34.2	31.9	13.0
RivaroxabanSandoz	104.9	51.5	43.8	38.6	33.6	15.4



	0 ng/ml	50 ng/ml	150 ng/ml	300 ng/ml	500 ng/ml	750 ng/ml
Xarelto	104.2	94.1	75.8	64.5	47.7	22.4
Rivarolto	104.2	86.5	70.2	59.2	44.2	21.5
RivaroxabanSandoz	104.2	91.5	72.6	65.5	50.4	23.8

Fig. 6. Relationship between the one-stage FVII (panel A), FVIII (panel B) and FIX (panel C) values and the concentrations of the three rivaroxaban.

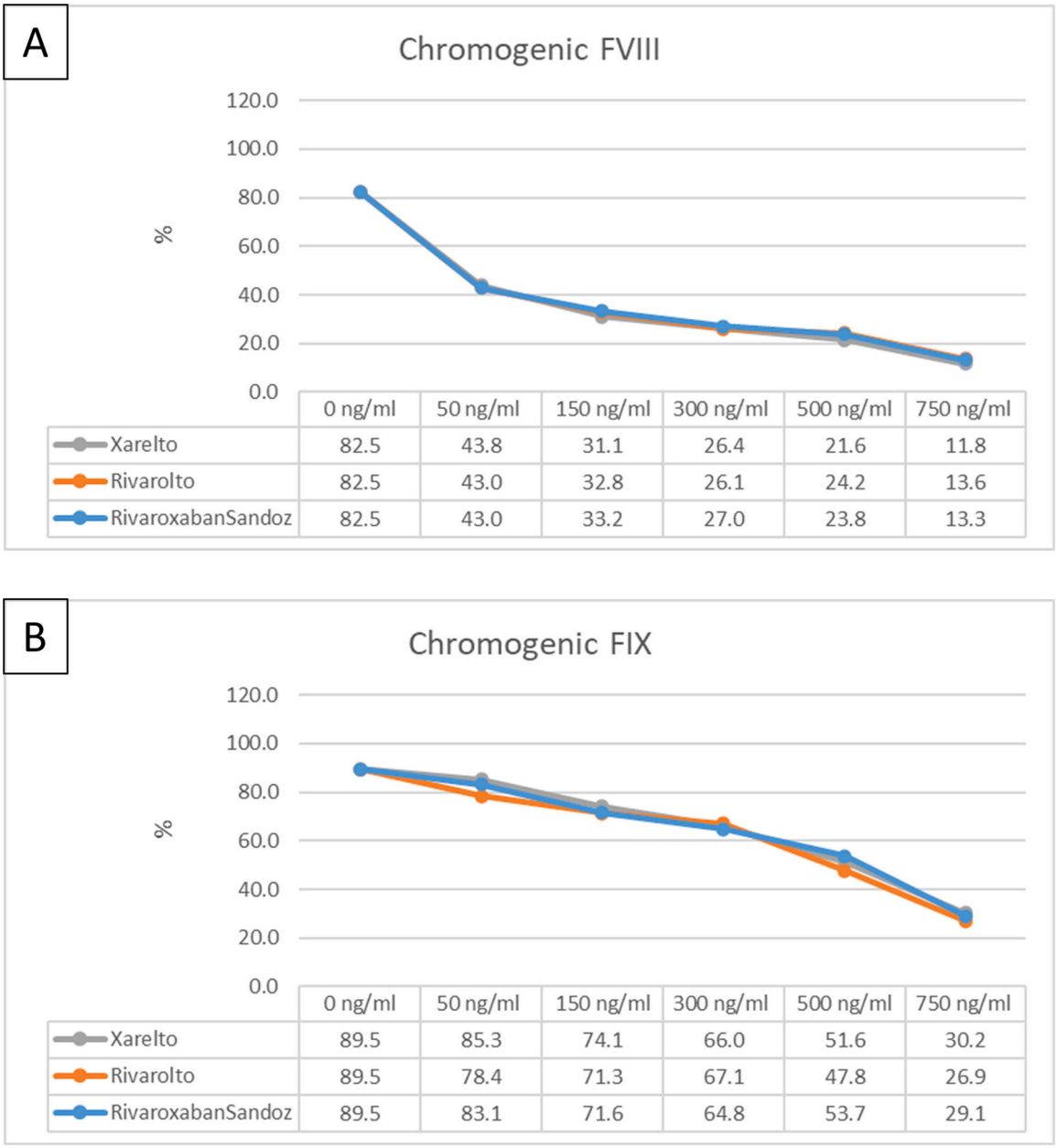


Fig. 7. Relationship between the Chromogenic FVIII (panel A) and FIX (panel B) values and the concentrations of the three rivaroxaban.

[20,22,31].

The TT and the Clauss Fibrinogen assays did not significantly correlate with the rivaroxaban concentrations, as shown also by previous studies [10,18–20,22,23]. In fact, these two assays bypass the function of FXa, by using a supply of exogenous thrombin which directly converts fibrinogen to fibrin. In this study, these two assays were performed as "control" assays, to confirm the lack of effect of rivaroxaban. If fibrinogen levels need to be measured, the Clauss Fibrinogen assay is preferred to the PT-Fg, since it is not significantly affected.

Finally, the TEG results showed a concentration-dependent increase of the R-time (which did not reach the level of statistical significance for Rivarolto®). The K-time, α-angle and MA were more erratic. These findings might be due to the high intra-assay variability of the native TEG, since the results obtained for the four TEG parameters might have been less accurate and reliable than those produced by the other assays. However, the TEG results were consistent with those reported by other studies which tested the branded rivaroxaban [18,24,44].

This study has some limitations that need to be acknowledged. First, this is not a pharmacokinetic study since it does not provide information on the dissolution of generic rivaroxaban tablets in the gastrointestinal tract and their absorption into the circulation. Bioequivalence studies were previously conducted and led to the marketing authorization of several formulations of generic rivaroxaban [8,9]. The current study provides valuable information on the effect of the generic rivaroxaban on several coagulation assays. The tablets were dissolved using DMSO, which is a non-physiological strong solvent commonly used in laboratory studies evaluating anticoagulant drugs, because it does not impact the results of the coagulation assays [39,44]. By using DMSO, we dissolved the entire content of the tablets (rivaroxaban and excipients), thus our results apply to the raw rivaroxaban materials and no inference can be made on the different formulations of rivaroxaban. The identical *in vitro* anticoagulant effect suggests that the active ingredient (rivaroxaban) is likely the same in all the formulations tested. However, our *in vitro* results need to be confirmed by future research, which should either focus on creating a model similar to the gastrointestinal tract for *in vitro* dissolution testing or should test *ex vivo* samples from patients on

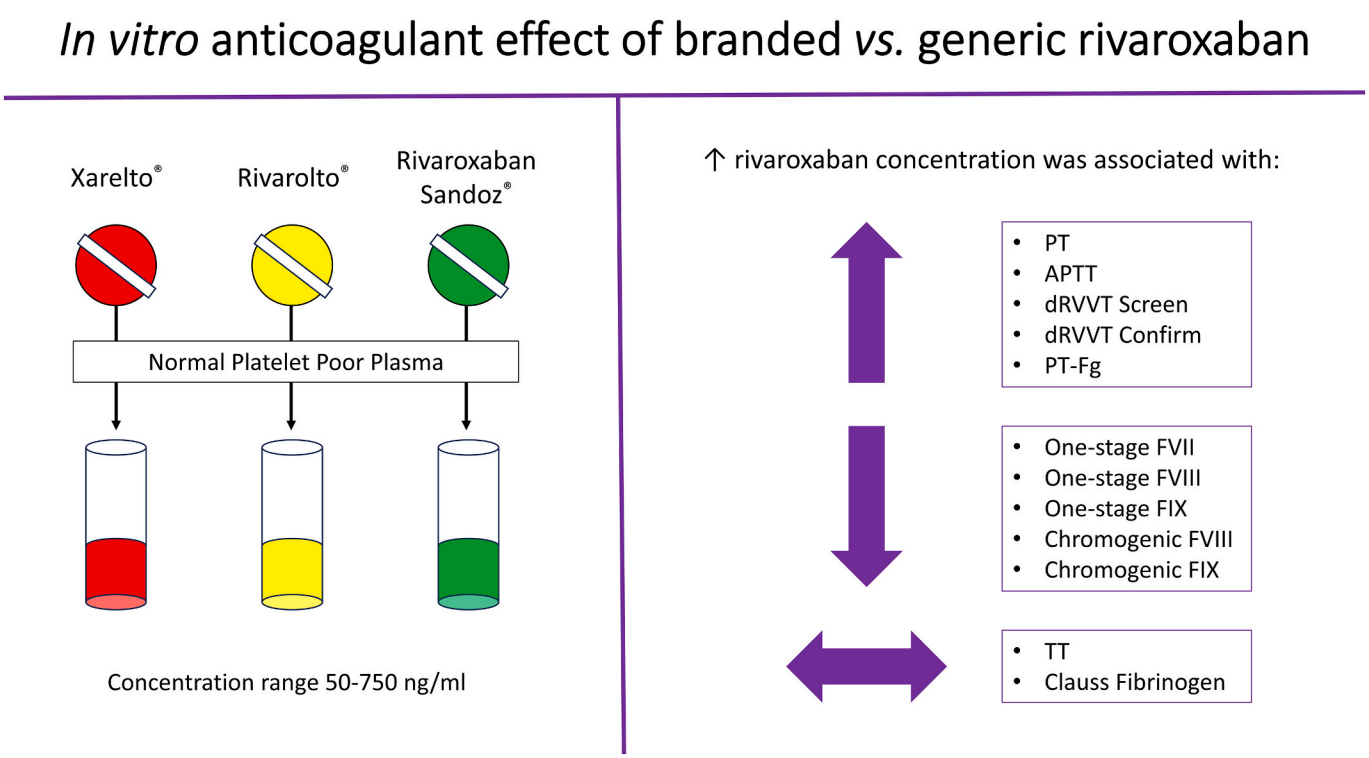


Fig. 8. Graphical abstract which summarizes the main results of this study.

treatment with generic formulations of rivaroxaban, in order to also take into consideration their release and absorption. Second, since the rivaroxaban concentrations ranged from 50 to 750 ng/ml, it is still unknown whether our findings remain valid outside this range. However, these concentrations were selected because they are clinically relevant, since they include subtherapeutic, therapeutic and supratherapeutic concentrations. Therefore, these results are likely to be relevant for practical laboratory assessment. Third, we used pooled plasma spiked with the three rivaroxaban rather than samples from patients being administered different formulations of rivaroxaban. This decision was taken since real-life patients might have other factors which can interfere with the coagulation assays. The use of samples prepared from the same pooled plasma ensures that other variables are kept constant for all samples. It has been reported that an *in vitro* approach can be utilised for research on DOACs to imitate *in vivo* conditions, since both produce consistent results [10]. The main strength of this study includes the fact that plasma concentrations of branded and generic rivaroxaban were prepared to be as similar as possible and the assay reagents were kept constant throughout testing.

In conclusion, this study showed that the branded and generic rivaroxaban exert an identical *in vitro* anticoagulant effect. These results can support the use of generic rivaroxaban, as reliable and cost-effective alternative to the branded rivaroxaban from a coagulation laboratory perspective.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2024.01.017.

CRediT authorship contribution statement

Karl Mangion: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft. **Kevin Vella:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **Alex Gatt:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Amy Marie Vella:** Investigation, Writing – review & editing. **Marica Borg:** Investigation, Writing – review & editing. **Denise Borg-Aquilina:** Investigation, Writing – review & editing. **Jonathan Douxfils:** Investigation, Writing – review & editing. **Liberato Camilleri:** Formal analysis, Writing – review & editing. **Nicoletta Riva:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – original draft.

Declaration of competing interest

The authors have no conflicts of interest to declare in relation to this study.

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