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Rivaroxaban pharmacodynamics in healthy volunteers evaluated with thrombin generation and the active protein C system: modeling and assessing inter-individual variability

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ESSENTIALS

- Data on interindividual rivaroxaban variability assessed with thrombin generation are scarce
- We assessed thrombin generation (3 conditions) in 60 healthy volunteers after rivaroxaban intake
- The relationships between peak height and rivaroxaban concentrations were sigmoidal (models).
- The low rivaroxaban PD variability contrasts with the substantial PK variability

ABSTRACT

Background: Rivaroxaban is a direct factor Xa inhibitor with substantial inter-individual pharmacokinetic (PK) variability. Pharmacodynamic (PD) variability, especially assessed with thrombin generation (TG), has been less documented.

Objectives: i/to assess TG parameter time-profiles in healthy volunteers, TG being studied under different conditions; ii/to model the relationship between rivaroxaban concentrations and TG parameters, and subsequently estimate inter-individual variability

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Methods: Sixty healthy male volunteers (DRIVING-NCT01627665) received a single 40-mg rivaroxaban dose. Blood sampling was performed at baseline and 10 pre-defined time-points over 24-hours. TG was investigated with the fully automated ST-Genesia system (Stago), using two tissue-factor (TF) concentrations, in absence (-), or presence (+) of thrombomodulin (TM) for the lowest one. PD-models were built to characterize the relationships between plasma rivaroxaban concentrations and endogenous thrombin potential (ETP) or peak-height induced by the lowest TF concentration.

Results: TG parameter time-profiles with the lowest TF concentration showed a good sensitivity to rivaroxaban, especially +TM (active protein C negative feed-back). The relationship between rivaroxaban concentrations and TG parameters was modeled with a sigmoidal relation. Mean rivaroxaban concentrations halving the baseline value of ETP and peak-height (-TM) (C_{50}) were of 284 and 33.2 ng/mL, respectively: +TM, C_{50} declined to 19.4 and 13.8 ng/mL, reflecting a powerful inhibitory effect. The estimated C_{50} population coefficients of variation were of 12.2% (-TM) and 31.3% (+TM) with the peak-height models, 34.8% (+TM) with the ETP-model.

Conclusions: This low-to-moderate rivaroxaban PD variability in healthy volunteers contrasts with the substantial PK variability, and deserves to be studied in different patient settings.

KEY WORDS

rivaroxaban, thrombin generation, pharmacodynamic model, thrombomodulin, healthy volunteers

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INTRODUCTION

Rivaroxaban was the first oral direct factor Xa (FXa) inhibitor to be developed and approved for the prevention and treatment of thromboembolic disease. Rivaroxaban competitively and selectively inhibits free, prothrombinase and clot-associated FXa [1,2]. FXa plays a central role in the physiological coagulation process, at the crossroad between tissue factor and contact phase coagulation pathways. Thrombin generated by FXa is first produced in small amounts at the initiation phase of the coagulation cascade, but in much greater amounts during the propagation and amplification phases, and then down regulated by natural inhibitors [3].

In contrast to traditional vitamin K antagonists (VKA), direct oral anticoagulant (DOAC) pharmacokinetics (PK) and pharmacodynamics (PD) are predictable. Given their wide therapeutic margin, DOACs are administered at fixed doses without laboratory monitoring except in specific situations [2,4]. Rivaroxaban bioavailability is approximately 80 % after oral intake, and the maximal plasma concentration is rapidly reached. It is metabolized by cytochrome P450 3A4 and is a substrate of P-glycoprotein (P-gp). About 66 % of rivaroxaban quantities are eliminated by the kidney, half of which is eliminated unchanged [1,2]. However, substantial rivaroxaban PK inter- and intra-individual variability has been documented in different settings, including healthy volunteers, patients enrolled in clinical trials, or in real world cohorts, with variability not fully explained [5–14]. In the DRIVING study conducted in 60 healthy male volunteers after a single 40 mg rivaroxaban intake [13], we found a coefficient of variation (CV) for the rivaroxaban concentration area under the curve of 51 %, in agreement to that reported in patients included in clinical trials [10,11]. In addition, DRIVING volunteers were selected on their 2677-3435 haplotype of *ABCB1* gene encoding P-gp and we found no significant effect of this haplotype on rivaroxaban PK [13].

A more limited number of studies investigated *ex vivo* inter-individual rivaroxaban PD

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variability [9,11-12,15–22]. Among assays measuring clot formation, prothrombin time was the most commonly used in prior published studies, showing a poor suitability to assess rivaroxaban PD, especially at low concentrations. Moreover, prothrombin time sensitivity varies widely across reagents [4,8,11,24-25). In addition, prothrombin time is only reflective of the initiation phase of coagulation. Other assays based on viscoelastometry have also been shown to be poorly sensitive to FXa inhibitors (18,24-25).

Thrombin generation (TG) has been proposed as an attractive assay to assess *in vitro* or *ex vivo* direct FXa inhibitor effect on coagulation based on studies with small series of healthy volunteers or patients, after single or repeated direct Xa inhibitors dose exposure (rivaroxaban, apixaban, edoxaban, or otamixaban) (15-22,26-32). However, some persistent issues remain regarding the most appropriate experimental conditions among different methods, including TF concentrations (33). Moreover, data regarding the magnitude of TG parameter inter-individual variability in subjects receiving direct Xa inhibitors are scarce (15,20,29).

In the present study, we sought to: 1) characterize plasma TG parameter time-profiles in DRIVING healthy volunteers after intake of a 40 mg-rivaroxaban single dose, 2) measure TG under several relevant experimental conditions, and 3) develop PD models to characterize the relationship between plasma rivaroxaban concentrations and several TG parameters, independently from time of drug intake to estimation of population inter-individual variability.

METHODS

Study design

The DRIVING study was a randomized, open, crossover study with four treatment sequences. The protocol has been described elsewhere [13]. Briefly, 60 healthy male

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volunteers were recruited at two academic clinical investigation centers in Paris (Hôpital Européen Georges Pompidou and Hôpital Pitié-Salpêtrière). Caucasian males aged 18 to 45 years were eligible if body mass index was between 18 and 28 kg/m². The 60 participants were selected based on their *ABCB1* genotype: 20 were homozygous wild-type (P-gp 0 group) for the haplotype 2677-3435, 20 were heterozygous for the variant (P-gp 1 group), and 20 homozygous for the variant (P-gp 2 group). Only the DRIVING sequences corresponding to the rivaroxaban treatment alone were considered for the present study. Rivaroxaban (Xarelto®, Bayer Pharma AG, 13342 Berlin, Germany) was administered as a single oral dose of 40 mg at 9:00 am under fasting conditions, followed by a standardized breakfast at 10:15. The single drug dose was selected to obtain plasma concentrations in the same order of magnitude as those observed in patients with atrial fibrillation [11]. All volunteers adhered to a standardized diet throughout the 24-hour study period. The study (NCT 01627665) was approved by the regional Ethics Committee (#P100507-DRIVING, CPP Île-de-France 10). All participants gave their written informed consent to participate.

Sample collection

Blood samples for analysis (4.5 mL) were collected by venipuncture into 5 mL tubes containing sodium citrate (3.2 % 0.105 M; 1:9 v/v; Greiner-Alcyon, Nancy, France). Tubes were gently inverted five times to ensure adequate mixing. Tubes were immediately double centrifuged at 2000g for 15 min at 20°C and platelet poor plasma (PPP) was aliquoted, frozen and stored at −80°C until use [13]. All PPP samples were thawed 3 min in a 37°C water bath just before use.

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Rivaroxaban plasma concentrations

Pharmacokinetic parameters were derived from rivaroxaban plasma concentration-time profiles obtained by serial blood sampling at pre-defined sampling time points (T): at baseline (T0), and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 hours after rivaroxaban intake. Plasma rivaroxaban concentrations were quantified using an ultra-high-pressure liquid chromatography (UPLC) system coupled with a Quattro micro triple quadrupole mass spectrometer (Waters, Saint-Quentin en Yvelines, France) [13].

Thrombin generation

TG was investigated for the 11 PPP samples prepared from blood drawn at different time points over the 24 hour period (see above) in 18 out of 60 volunteers, which were selected to obtain six out of the 20 homozygous wild-type volunteers (P-gp 0 group), six out of the 20 heterozygous variants (P-gp 1 group), and six out of the 20 homozygous variants (P-gp 2 group). In each P-gp group, the six volunteers were chosen as follows: two subjects with low rivaroxaban peak concentration profile, two subjects with intermediate peak concentration profile and two subjects with high peak concentration profile. For the remaining 42 volunteers, we performed TG at three time-point PPP samples, *i.e.* at baseline (T0), at peak level time (Tmax) and at T24. Tmax was defined for each volunteer as the time associated with the highest concentration following rivaroxaban intake. If two concentration peaks were observed, the earliest one was chosen.

TG was performed on a ST-Genesia system (Stago, Asnières-sur-Seine, France). The ST Genesia system is a dedicated platform to enable fully automated quantitative and standardized evaluation of TG, using dedicated reagents, calibrator, quality controls, and reference plasmas. The assay principle of the ST Genesia system is similar to the previous generation, semi-automated Calibrated Automated Thrombogram (CAT) system (Stago) [34],

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but differs with respect to improved 37°C temperature control at and a unique calibration performed for each series. Briefly, STG-ThrombiCal, a buffered solution containing a known fixed amount of human thrombin, is incubated for 10 min in a cuvette precisely pre-warmed at 37°C; a solution containing the Z-Gly-Gly-Arg-7-amino-4-methylcoumarin (Z-GGR-AMC) fluorogenic substrate together with calcium chloride (STG-FluoStart) was then added**.** Upon substrate cleavage by thrombin, fluorescence increases, and is monitored at 37°C with measurement every 15 seconds at 377 nm excitation / 450 nm emission wavelengths. In parallel, STG-ThrombiCal was incubated in another cuvette with a solution containing a fixed concentration of AMC (STG-FluoSet), enabling adjustment of the calibration curve for the optical characteristics of the milieu on subsequent plasma measurements, correcting for the inner filter effect. Once the calibration had been validated, individual plasma samples (80 µL) are run in duplicate under different experimental conditions upon final addition of STG-Fluostart, but always in parallel to the STG-FluoSet determination. ST-Genesia embedded software calculated from fluorescence traces the concentration of active thrombin generated over time. Results are displayed as mean of duplicate results.

TG was investigated according to the manufacturer's recommendations using two TF concentrations (STG-DrugScreen and STG-ThromboScreen), in absence (-), or presence (+) of thrombomodulin (TM). STG-DrugScreen contains a mixture of phospholipids and recombinant human TF at a relatively high picomolar concentration, referred *infra* to as "high picomolar TF concentration", whereas STG-ThromboScreen contains the same mixture of phospholipids and human TF at an intermediate picomolar concentration, referred *infra* to as "intermediate picomolar TF concentration", both reagents adjusted for each batch by the manufacturer to obtain the desired TG profile ("reagents manufacturer undisclosed data"). TM concentration is present in the reagent in order to inhibit 50 % of the ETP in absence of TM on frozen normal pooled plasma.

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STG-DrugScreen and STG-ThromboScreen reference plasmas, and TG dedicated quality controls, were run for each series, at low and normal levels for STG-DrugScreen, and at low, normal and high levels for STG-ThromboScreen as recommended by the manufacturer.

We recorded and analyzed the following parameters: lag time (in min: time from test triggering to signal detection), time to peak (in min: time necessary for thrombin concentration to reach its maximal value), peak height (in nM: maximal thrombin concentration) and endogenous thrombin potential (ETP; in nM•min: area under the thrombin time-concentration curve).

We determined there were no substantial differences in results from the ST-Genesia system *vs*. the extensively documented CAT system (24). We tested 47 DRIVING samples covering a wide range of rivaroxaban concentrations on both ST-Genesia system using STG-ThromboScreen without TM and the Calibrated Automated Thrombogram (CAT) using PPP-reagent (5 picomolar TF) (Stago). Comparisons between both systems showed acceptable agreements using Bland-Altman difference plots and Passing-Bablok equations, without biases of clinical relevance within the measuring range as also confirmed in another comparison study (Suppl Fig 1) (35-36).

For STG-DrugScreen, inter-series coefficients of variation (CV) of 2.1 and 2.7% for lag time, 3.0 and 1.6% for time-to-peak, 14.5 and 11.5% for peak height, 12.0 and 13.4% for ETP were obtained with low and normal quality control levels, respectively (15 runs). For STG-ThromboScreen, in the absence of TM, CVs of 5.4, 4.5 and 5.0% for lag time, 4.3, 3.8 and 3.0% for time-to-peak, 10.0, 5.8 and 2.8% for peak height, 6.3, 4.2 and 5.5% for ETP, were obtained with low, normal and high quality control levels (STG-QualiTests), respectively (15 runs); in the presence of TM, CV of 3.9, 2.9 and 4.2% for lag time, 3.7, 3.0 and 2.6% for time-to-peak, 9.8, 6.9 and 3.8% for peak height, 8.4, 6.5 and 4.3% for ETP,

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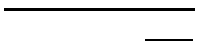
were obtained with low, normal and high quality control levels (STG-QualiTests), respectively (15 runs).

Statistical analysis

All statistical analyses were run using R software (version 3.5.1) using the nlme package (37-38). The assessed PK parameters were peak plasma concentration (Cmax), and time to peak plasma concentration (Tmax). The assessed PD parameters were lag time, time-to-peak, peak height and ETP. Data were described as median or mean values (± standard deviation), interquartile ranges, minimal and maximal values. We performed univariate analyses to test the association between TG parameters and covariates, using Spearman correlation test for quantitative variables (age, BMI and creatinine clearance), and Kruskal-Wallis test for qualitative variables (*ABCB1* genotypes).

The PD relationship between plasma rivaroxaban concentration and TG parameters (ETP or peak height in the presence or absence of TM) was modeled by a sigmoidal relationship (derived from Hill's relation) with the constrain of a null ETP or peak height for infinite rivaroxaban concentrations (no residual effect) and between-subject variability on the PD parameter baseline value and the half-concentration, leading to the following model:



where $Y_{i,j}$ is the PD parameter (peak height or ETP) for patient i at time j, the baseline value of this parameter for patient i, γ is the Hill coefficient (sigmoidicity parameter), $C_{50,i}$ is the rivaroxaban concentration, which when divided by two yields the baseline value for patient , and $\varepsilon_{i,j}$ is the residual error at time j for patient i. Residual errors and random effects on baseline and C_{50} values were assumed to follow a normal distribution in the log scale. Errors were assumed to be independent, identically distributed between them and from the

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random effects. Random effects were assumed to be independent and identically distributed between all subjects. This mixed-effects model was fitted to the data by maximizing its likelihood, using the nlme function within the nlme package for R. Initial values for the minimization algorithm were obtained on the average curve. Assumptions were checked and verified graphically.

RESULTS

DRIVING volunteer characteristics

Among the 60 volunteers enrolled in the DRIVING study [13], 17 out of 18 subjects had TG analyzed at all 11 time-points. In the case of one volunteer, (P-gp 2 group, low Cmax), TG could not be assessed due to insufficient plasma sample quantity. The remaining 42 volunteers were tested at three time-points (T0, Tmax and T24). Mean age of the 59 analyzed volunteers was 31 ± 8 years (min 19-max 45), mean BMI 23.6 ± 2.5 kg/m^2, mean creatinine clearance (Cockcroft-Gault) 122 ± 22 mL/min. Rivaroxaban concentration at peak level (Cmax) was reached at a median time (Tmax) of 1.5 hours (interquartile range IQR 1.25-1.5 - min 0.5 - max 4). Rivaroxaban concentrations at Tmax and T24 for the 59 volunteers are displayed in Table 1. We checked that distributions of rivaroxaban concentrations at both Tmax and T24 were comparable in the 17 volunteers with 11 time-points and in the 42 volunteers with three time-points (Suppl Fig 2).

TG parameter time-profiles

Using three conditions, high and intermediate picomolar TF concentrations, the latter in absence and presence of TM, TG parameter time-profiles were determined for the 17 volunteers with 11 time-points (Figures 1, 2 and 3). The individual ETP- and peak height-time profiles are displayed in Suppl Figure 3, along with plasma rivaroxaban concentration time-profiles. When TG was studied with no TM added, progressive prolongations of

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temporal parameters, *i.e.* lag time and time-to-peak, were observed during the first two hours following rivaroxaban intake, as expected. Then, lag time and time-to-peak slowly decreased up to T24 with both high and intermediate picomolar TF concentration, without returning back to baseline values (Figures 1 and 2). The greatest parameter variability was observed between T0.5 and T4, reflecting the inter-individual variations of both Tmax and Cmax. In contrast, the variability of temporal parameters was lower at baseline and at T24. ETP and peak height rapidly decreased, *i.e.* within 90 min after drug intake, and then progressively increased without return to baseline, with both high (Figure 1) and intermediate picomolar TF concentration (Figure 2). As expected, temporal parameters were shorter. In contrast, ETP and peak height values were greater, when using high TF concentration vs. intermediate TF concentration.

TG using intermediate TF concentration was further assessed in the presence of TM in the 17 volunteers (Figure 3). Adding TM markedly reduced ETP and peak height in comparison to TG performed with intermediate TF concentration without TM, especially at Tmax, without return to baseline at T24; percentages of ETP and peak height inhibition are shown on Figure 3. When adding TM, the median ETP decreased from 1059 to 548 nM•min at T0, corresponding to 48 % inhibition of ETP. At Tmax, the median ETP dramatically dropped from 654 to 54 nM•min, corresponding to more than 90 % inhibition of ETP and at T24, the median ETP raised slowly, still exhibiting 73 % inhibition of ETP when adding TM. Similar inhibition-time profiles were obtained regarding peak height (Figure 3).

Finally, in order to evaluate inter-individual variability, TG was assessed at T0, Tmax and T24 in the 42 remaining volunteers using the high or intermediate concentration of TF, the latter in absence or presence of TM. TG results for the whole cohort (n=59), i.e. ≈ 300 time-points *per* experimental condition, confirmed the higher sensitivity of peak height and ETP to rivaroxaban when using the intermediate vs. the high concentration of TF as shown by

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median Tmax/T0 ETP and peak height ratios (Table 2). Inter-individual CV values of Tmax/T0 ratios for lag time and time-to-peak, were < 20 % at both concentrations of TF in the absence of TM; regarding Tmax/T0 ratios for ETP and peak height, CV values were higher when using the intermediate TF concentration compared with a high TF concentration (25.1 and 39.1 % vs and 8.7 and 23.4 %, respectively), and were much higher when adding TM (for both ≈ 80%), but the low absolute levels ETP and peak height with the likely altered precision of the method must be taken into account. At T24, CV values were below 30 % for all parameters using both concentrations of TF.

The potential influence of the 59 volunteer characteristics on TG parameters at T0, Tmax and T24 was tested using univariate analysis. Among non-genetic (age, BMI, creatinine clearance) and genetic (*ABCB1* genotype) variables, advancing age was the only variable that was significantly associated with a higher peak height both at T0 and at Tmax ($p = 0.0135$ and $p = 0.0163$, respectively) and a higher ETP both at T0 and at Tmax ($p = 0.0409$ and $p = 0.0208$, respectively) using the intermediate concentration of TF.

ETP and peak height modeling

In order to characterize the relationships between plasma rivaroxaban concentrations and TG parameters independently of time, we sought to develop PD models. Among the different measured TG parameters, we chose ETP and peak height obtained using the intermediate TF concentration in the absence and in the presence of TM as PD parameters to build the models. Indeed, the greatest sensitivity of ETP and peak height to rivaroxaban was obtained with intermediate TF concentration compared with high TF concentration; moreover, the effect of TM on both parameters could be studied.

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The relationship between rivaroxaban concentrations and TG parameters was modeled with a sigmoidal relationship. TG parameters, assigned as *y*, were modeled as a function of the corresponding rivaroxaban plasma concentration (Figure 4). The characteristics of the four models for fitting ETP and peak height in the absence and presence of TM are given in Table 3. Model fit convergence was achieved for all models, but for the ETP without TM random effect on C_{50} had to be removed from the model. Diagnostic plots of each model suggest that the assumptions were satisfied: random effects and residuals seemed normally distributed, and residuals as a function of rivaroxaban concentration showed no remaining trend, nor apparent heteroscedasticity. There were four outliers (standardized residual > 3), related to three volunteers, but without influence on the results, thus making the results interpretable.

The model estimates a mean (μ) rivaroxaban concentration halving the baseline value of peak height (C_{50}) in absence of TM of 33.2 ng/mL (95 % confidence interval [CI]: 26.2; 40.1), with an interindividual variability standard deviation (σ) of 4.0 ng/mL; estimated mean C_{50} declined to 13.8 ng/mL (95 % CI: 10.7; 16.9) with a standard deviation σ of 4.3 ng/mL in presence of TM. The C_{50} population CVs (σ/μ) estimated with the peak height model were of 12.2 % and 31.3 % in absence and presence of TM, respectively. The mean C_{50} for ETP, of 284.2 ng/mL [244.7; 323.8] in absence of TM, was in the same order of magnitude in presence of TM, estimated at 19.4 ng/mL (95 %CI: 15.2; 23.5), and the C_{50} standard deviation was of 6.7 ng/mL, with a population CV of 34.8 %.

DISCUSSION

While numerous studies on rivaroxaban PK variability have been published [5-14], few focused on TG parameter time-profiles [11,15,19-22]. The originality of the present study lies in two main features: 1) we analyzed *ex vivo* rivaroxaban PD in a substantial number of healthy volunteers (n=59) with TG determined using an automated system under different TF

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conditions, including one in the presence of TM; ii) relevant PD models were constructed, which enabled estimation of inter-individual PD variability [2,9].

We chose to assess TG using the novel ST-Genesia system because standardization has been improved compared with the reference CAT system and it is fully automatized. At baseline (T0), TG parameters and corresponding CVs were in the same order of magnitude as those found using the comparable TF concentrations with CAT [29,34-36]. The new system seems to be a good candidate for a wider, more convenient use in view of a potential clinical application. Regarding the TG time-profiles, the lowest concentration of TF increased TG sensitivity compared with the highest, as evidenced by Tmax/T0 ETP and peak height ratios. The normalization of TG parameter values with those of reference plasmas did not affect results (not shown) [39]. Our *ex vivo* findings reinforce those obtained *in vitro* using normal plasma spiked with rivaroxaban and 1-20 picomolar TF concentrations [24,26,40-41]. Interestingly, we obtained PD mirror-inverted time profiles to PK-time profiles for both temporal parameters (lag time and time-to-peak), markedly for peak height and to a lesser extent for ETP, peak height being consistently more affected than ETP in agreement with previous findings [24,42]. In some volunteers, a double peak pattern was observed with both PK and TG parameters on time profiles, suggesting multiphasic elimination including entero-enteric cycle of rivaroxaban in these individuals, a phenomenon already described in the literature [43]. Overall, rivaroxaban exerted a concentration-dependent effect on all TG parameters under all TF conditions, with a greater sensitivity obtained with the intermediate TF concentration. The optimal choice between these two TF conditions may depend on rivaroxaban concentration levels: intermediate picomolar TF is highly suitable for concentrations up to 300 ng/mL whereas high picomolar TF might be required for concentrations over 300 ng/mL.

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The possibility to use TM under manufacturer's conditions is obviously an advantage. Indeed, one strength of our study is that we assessed TG using intermediate picomolar TF concentration with the addition of TM, thus enabling the involvement of protein C system. *In vivo*, thrombin generation (TG) is down regulated by natural inhibitors among which the protein C (PC) anticoagulant pathway [44]. Remarkably, at baseline, we found that the addition of TM in the DRIVING volunteer plasmas generated an almost 50 % inhibition in ETP, validating the concentration of TM introduced into the commercial reagent. In the presence of increasing rivaroxaban concentrations, the percentage of inhibition markedly increased to achieve approximately 90% inhibition at peak concentration. Similar effects on TG parameters had already been observed when adding TM, using normal human plasma spiked with various rivaroxaban amounts [41].

Interestingly, we found that inhibition persisted at a high median level (73%) at T24 although the median rivaroxaban concentration was ≈ 30 ng/mL, demonstrating maintenance of the inhibitory effect. The substantial residual hypocoagulability we evidenced is consistent with the concern that invasive procedures such as neuroaxial anesthesia can be at very high risk of bleeding and should be avoided [45-46].

This persistently inhibitory effect had been previously shown in subjects analyzed in the absence of TM [5,15,19-20], but never in the presence. The involvement of the inhibitory dynamic system of activated protein C, formed in the presence of TM by thrombin, contrasts with what occurs in subjects under VKA therapy, whose hypo-gamma-carboxylated protein C and protein S are altered [47]. Interestingly, Bloemen *et al* found a significantly decreased whole blood ETP and peak height in VKA-patients with bleeding versus those without [47]. Whether the rivaroxaban response evaluated with TG should be associated with clinical events needs further investigation.

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In these young healthy male volunteers, we provided data on the TG parameter variability at baseline as well as after rivaroxaban intake, therefore minimizing inter-individual demographic and environmental factors. Indeed, only advancing age from 18 to 45 years slightly increased peak height and ETP at baseline and at Tmax using the intermediate concentration of TF, in agreement with previous data [48-49]. No effect of *ABCB1* haplotype was found in agreement with DRIVING PK results [13].

In contrast to previous studies underpowered to address this issue, we could build PD models. Modeling was made feasible due to the fact we had suitable PK data and a wide range of quantitative PD parameters (> 300 time points *per* condition), namely ETP and peak height, reflecting the effect of the drug with a good sensitivity [50]. The rivaroxaban response curve was sigmoidal, with the Hill's derived equation fitting to the experimental data. When evaluating PD with PT, a linear correlation between plasma concentrations and prolongation of PT is observed, similarly to what we found here (not shown); this is due to the lack of sensitivity of PT to low rivaroxaban concentrations [7,9,11,12]. Our approach with models enabled us to estimate a very low value of the mean rivaroxaban concentration halving the baseline peak height (C_{50}), namely 33 ng/mL *i.e.* 76 nM, with a population C_{50} CV of only 12%. These results suggest that even at low concentrations, rivaroxaban still strongly inhibits the maximal thrombin peak concentration, thus covering the 24-hour interval between two oral intakes in most subjects. Moreover, the much lower C_{50} in presence of TM suggests an *in vivo* powerful inhibitory effect even though soluble TM used here cannot be directly superimposable to endothelial TM. Regarding ETP, the mean C_{50} rivaroxaban concentration halving ETP in absence of TM (284 ng/mL) was 15-fold lower in its presence (19 ng/mL), confirming the strong inhibitory effect of rivaroxaban when the dynamic system of activated protein C was incorporated, even at low levels. Remarkably, under *in vitro* experimental conditions using normal plasmas spiked with rivaroxaban, Perzborn *et al.* reported ETP and

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peak height EC_{50} values in the absence and in the presence of TM of the same order of magnitude of those than those we found *ex vivo* [41].

In addition, our modeling approach enabled us to estimate the PD inter-individual variability independently from time of drug intake. Peak height and ETP CVs, < 20% in the absence of TM and < 35% in the presence of TM, were rather low, in comparison with PK variability, which was evaluated at 51% in these DRIVING volunteers [13]. Overall variability amounts up to 100% for ETP at Tmax when TM was added, but here, the low absolute values have to be taken into account. It is likely that PD variability as well as PK inter-individual variability were here highly minimized owing the selection of young male volunteers, providing here "baseline" variability data. The extent of PD variability in different patient settings deserve to be further investigated.

Our study has some limitations. One limitation is the use of a single dose of rivaroxaban rather than repeated dose to achieve steady state drug concentrations. Secondly, only males were included. However, gender most likely is not a factor significantly contributing to the overall variability. Third, the association between PK parameters and clinical events has been demonstrated with some direct-Xa inhibitors, including edoxaban [51]. Whether an association exists between TG parameter results and bleeding or thrombotic events in patients receiving direct-Xa inhibitors remains to be established in different settings.

In conclusion, the measurement of TG parameters with the ST-Genesia system enabled a reliable assessment of the PD response after rivaroxaban intake under several relevant experimental conditions. For the first time, we showed that modeling characterized the relationship between peak height / ETP and rivaroxaban concentrations as a sigmoid curve, especially in presence of TM: the low C_{50} demonstrated the powerful inhibitory effect of rivaroxaban. The concentration-effect relationship of rivaroxaban in healthy volunteers showed low-to-moderate PD variability. Further studies are required to analyze the higher

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variability reported in patients. Whether such PD models can be generalizable to patients receiving rivaroxaban for different indications deserve to be investigated.

AUTHORS'CONTRIBUTION

V. Siguret, I. Gouin-Thibault and T. Lecompte participated in the study design, analyzed data results and critically read the manuscript. J. Abdoul performed the experiments and analyzed data. V. Siguret drafted the manuscript. E. Curis made the figures, performed the statistical analyzis and built the PD models. X. Delavenne performed the PK analysis. A. Carlo, A. Blanchard, JE. Salem, P. Gaussem, C. Funck-Brentano M. Azizi, P. Mismetti and MA. Loriot critically read the manuscript.

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DISCLOSURE OF CONFLICT OF INTERESTS

The authors have no conflict of interest.

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LEGENDS TO FIGURES

Figure 1: TG parameter-time profiles using the high picomolar TF concentration. (A) Lagtime, (B) Time to peak, (C) Endogenous thrombin potential (ETP), (D) Peak height

TG was investigated in the plasma obtained from 17 volunteers over the 24-hour period (11 time-points), using the high the TF concentration (STG-DrugScreen). Box plots: bold horizontal bar within box, median; lower and upper horizontal lines of box, Q1 and Q3 quartiles, respectively; IQR = Q1 − Q3; lower horizontal bar outside box, smallest observed value greater than Q1 − 1.5 IQR; upper horizontal bar outside box, highest value smaller than Q3 + 1.5 IQR; circles represent values beyond these limits, potential outliers assuming a Gaussian distribution.

Figure 2: TG parameter-time profiles using the intermediate TF picomolar concentration without thrombomodulin as a function of time (hours): (A) Lagtime, (B) Time to peak, (C) Endogenous thrombin potential (ETP), (D) Peak height

TG was investigated in the plasma obtained from 17 volunteers over the 24-hour period (11 time-points), using the intermediate TF concentration (STG-ThromboScreen). Box plots: see Figure 1 legend.

Figure 3: TG endogenous thrombin potentials (ETP) (A) and peak height (C) in the presence of thrombomodulin and percentage of inhibition for ETP (B) and peak height (D) as a function of time

TG was investigated in the plasma obtained from 17 volunteers over the 24-hour period (11 time-points), using the intermediate TF concentration (STG-ThromboScreen) with TM. The percentage of inhibition was calculated by dividing [the result of TG parameter (peak height or ETP) with TM] by [the result without TM] × 100. Box plots: see Figure 1 legend.

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Figure 4: ETP and peak height models

ETP (A) and peak height (peak height) (B) measured using the intermediate picomolar TF concentration (STG-ThromboScreen) without TM (line) and with TM (dotted line) as a function of rivaroxaban concentration. Each symbol represents a volunteer; filled symbols correspond to values without TM; open symbols correspond to values with TM.

Supplementary Figure 1: Agreement between CAT system and ST-Genesia

Fourty-seven DRIVING healthy volunteer samples covering a wide range of TG profiles were run on CAT system and ST-Genesia. Comparisons were made using Passing-Bablok regression and Bland-Altman plots [35].

Supplementary Figure 2: Distribution of rivaroxaban concentrations at Tmax and T24

Rivaroxaban concentrations at Tmax and T24 in the 17 volunteers with the extended study over the 24hour-period and in the 42 volunteers. Box plots: bold horizontal bar within box, median; lower and upper horizontal lines of box, Q1 and Q3 quartiles, respectively; IQR=Q1-Q3; lower horizontal bar outside box, Q1-1.5 IQR; upper horizontal bar outside box, Q3+1.5 IQR; circles represent values beyond these limits.

Supplementary Figure 3: Individual PK and PD profiles of the 17 volunteers with the extended study over the 24hour-period (11 time-points)

Rivaroxaban concentrations (ng/mL) (A) measured by LC/MS-MS; Peak height (peak height) (B) and ETP (C) measured using the intermediate picomolar concentration of TF (STG-ThromboScreen) in the absence (open symbols) and in the presence of thrombomodulin (closed symbols). Patient profiles are presented according to decreasing peak rivaroxaban concentration groups.

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Table 1: Rivaroxaban concentrations measured in 59 volunteers at Tmax and T24

	Tmax	T24
Median (ng/mL) IQR range (min-max)	186.1 [143; 216] [48; 389]	31.1 [22.6; 37.1] [9.5; 68.6]

IQR: interquartile range;
Tmax corresponds to the time associated with the highest concentration following 40-mg rivaroxaban intake.

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Table 2: TG parameters measured in the volunteers under different conditions at T0, Tmax and T24

	High picomolar TF concentration (n=59)				Intermediate picomolar TF concentration (n=59)							
					Without TM				With TM			
	T0	Tmax	Tmax/T0 ratio	T24	T0	Tmax	Tmax/T0 ratio	T24	T0	Tmax	Tmax/T0 ratio	T24
Lag time (min*)												
Median	1.0	2.1	2.15	1.3	2.0	4.9	2.5	3.0	2.2	7.7	3.3	3.6
IQR range	0.9; 1.1	1.8; 2.5	1.9; 2.5	1.2; 1.4	1.8; 2.1	4.3; 5.7	2.3; 2.8	2.8; 3.3	2.0; 2.4	5.7; 10.1	2.8; 4.4	3.2; 4.1
min-max	0.8; 1.6	1.5; 3.8	1.6; 3.4	0.3; 2.3	1.6; 2.8	3.5; 6.7	1.6; 3.6	1.4; 4.6	1.7; 3.4	0.7; 17.9	0.1; 7.3	0.7; 6.5
CV	15.5%	21.8%	18.6%	19.8%	13.0%	16.9%	15.6%	18.6%	15.3%	47.6%	43.7%	27.0%
Time to peak (min*)												
Media	2.1	6.4	3.0	3.1	4.4	11.8	2.7	7.4	3.8	10.9	2.7	5.87
IQR range	1.9; 2.2	5.4; 7.4	2.5; 3.5	2.8; 3.5	4.0; 4.7	10.6; 13.0	2.5; 3.0	6.7; 8.1	3.7; 4.1	9.6; 13.8	2.6; 3.4	5.4; 6.5
min-max	1.7; 2.7	4.3; 9.2	2.1; 4.5	0.9; 4.9	3.3; 6.0	8.4; 14.9	2.0; 4.1	4.7; 9.9	3.1; 5.3	7.1; 21.0	1.9; 5.6	2.5; 9.0
CV	9.8%	20.5%	19.1%	20.3%	12.6%	13.8%	14.4%	15.8%	10.5%	26.1%	24.7%	18.0%
ETP (nM•min*)												
Median	1272	1130	0.90	1212	1064	650	0.6	988	551	40	0.08	251
IQR range	1194; 1436	1042; 1244	0.84; 0.94	1104;1352	982; 1248	538; 830	0.5; 0.7	897; 1110	426; 741	18; 80	0.04; 0.12	177; 319
min-max	1058; 1897	873; 1876	0.69; 1.0	350; 1833	781; 1628	344; 1518	0.3; 1.1	521; 1419	278; 1108	<30**; 346	0.02 0.36	64; 520
CV	15.4%	16.9%	8.7%	19.1%	17.2%	35.3%	25.1%	17.2%	35.0%	105.8%	79.5%	41.9%
Peak height (nM*)												
Median	397	123	0.34	280	214	48	0.23	110.9	144.9	7.7	0.06	51.5
IQR range	369; 423	112; 169	0.28; 0.42	252; 306	172; 262	35; 65	0.2; 0.3	97; 134	102; 190	3.2; 14.4	0.03; 0.09	35; 66
min-max	317; 532	79; 268	0.24; 0.57	35; 437	115; 341	19; 144	0.1; 0.7	61; 201	67; 301	<5***; 53	0.01; 0.3	13; 109
CV	10.8%	29.6%	23.4%	21.4%	26.5%	50.0%	39.1%	26.3%	36.1%	97.6%	82.8%	43.0%

Tmax corresponds to the time associated with the highest concentration following 40-mg rivaroxaban intake for each volunteer.ETP: endogenous thrombin potential; TF: tissue factor; IQR: interquartile range; CV: coefficient of variation; *except for Tmax/T0 ratio (shaded columns)

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**ETP < 30 nM•min for 9 values; ***peak height < 5 nM for 3 values

Table 3: Parameters of the model equations for ETP and peak height using an intermediate TF concentration in the absence and presence of thrombomodulin, respectively

Parameter	Intermediate picomolar TF concentration - TM						Intermediate picomolar TF concentration + TM					
	Initial value	μ	95% CI	σ	95% CI	CV (%)	Initial value	μ	95% CI	σ	95% CI	CV
ETP (nM•min)												
Y_0	1109	1103	1041; 1166	179.7	142; 227.3	16.3%	562.1	576.2	511.8; 640.6	135.9	83.1; 222.3	23.6%
C_{50}	277.3	284.2	244.7; 323.8	Absent in the model			17.6	19.4	15.2; 23.5	6.7	4.2; 10.9	34.8%
γ	0.8189	0.906	0.7296; 1.082	-			1.097	1.149	1.053; 1.246	/		
Residual error	-			0.1564	0.1432; 0.1708	-	-			0.3901	0.3554; 0.428	/
Peak height (nM)												
Y_0	206.6	207.1	191.5; 222.8	39.7	27.9; 56.6	19.2%	139.6	143.2	126.3; 160	33.2	19.4; 56.7	23.2%
C_{50}	29.1	33.2	26.2; 40.1	4.0	1.2; 13.7	12.2%	12.8	13.8	10.7; 16.9	4.3	2.5; 7.4	31.3%
γ	0.7036	0.7155	0.645; 0.786	-			1.112	1.138	1.042; 1.234	/		
Residual error	-			0.2286	0.2093; 0.2497	-	-			0.4181	0.3807; 0.459	/

μ: mean value estimated by the model; σ: standard deviation estimated by the model; CV (%): coefficient of variation estimated by the model; γ: Hill coefficient; Y_0: value at baseline; C_{50}: rivaroxaban concentration associated with half maximum effect; TM: thrombomodulin

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Figure 1

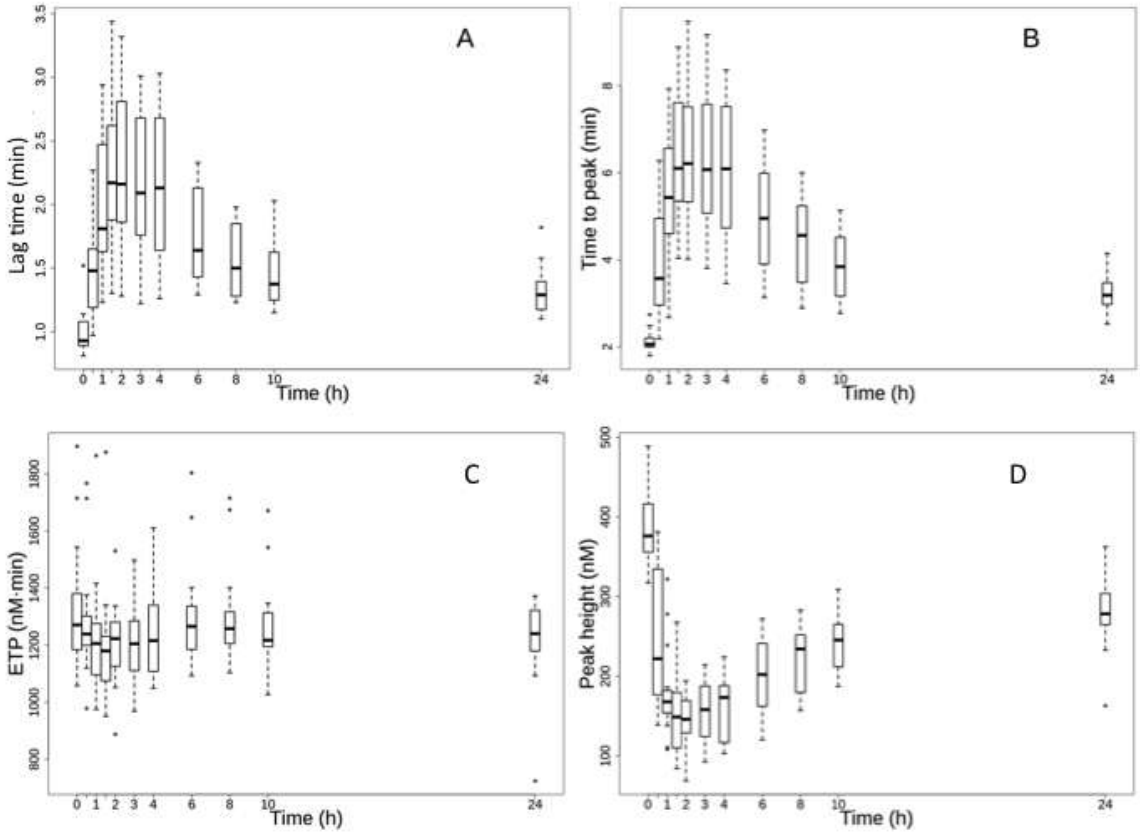

Figure 2

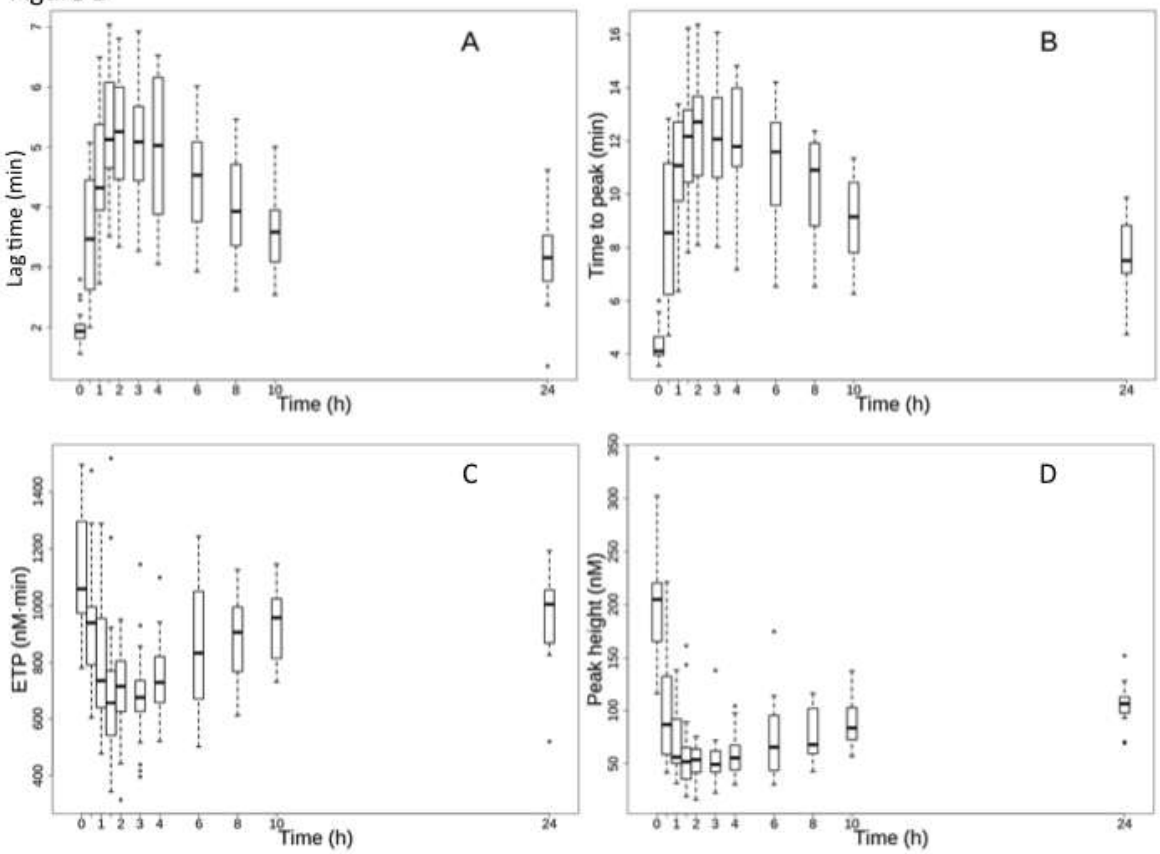

Figure 3