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Impact of centrifugation on thrombin generation in healthy subjects and in patients treated with direct oral anticoagulants

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Abstract

Introduction: Double centrifugation before freezing is recommended before thrombin generation assays (TGA). However, this procedure is not mandatory for routine hemostasis tests, precluding the use of these samples for TGA. The aim of this study is to assess the impact of single and double centrifugation on TGA performed on frozen samples from healthy volunteers (HVs) and patients receiving direct oral anticoagulants (DOACs).

Methods: Forty HVs and 57 patients receiving a DOAC (dabigatran, rivaroxaban, apixaban, or edoxaban) were included in this prospective double-center observational study. Blood was collected into 109 mmol/L citrated tubes and frozen at −70°C before TGA using ST Genesia with STG-DrugScreen reagent. Four pre-analytical conditions were studied: (A) single centrifugation (2000 *g*, 15 minutes) before freezing; (B) one centrifugation before freezing and another after thawing (2000 *g*, 15 minutes for both); (C) one centrifugation before freezing(2000 *g*, 15 minutes) and another after thawing (2000 *g*, 10 minutes); (D) double centrifugation (2000 *g*, 15 minutes) before freezing (reference). Centrifugation conditions (A), (B), and (C) were compared with the reference condition (D). Acceptable relative differences were defined at 6%, 8%, and 10% for normalized lag time, endogenous thrombin potential, and peak height, respectively.

Results: Centrifugation conditions had a small but acceptable impact on HVs samples, but single centrifugation always resulted in unacceptable reductions in normalized lag times for DOAC samples. A second centrifugation after thawing permitted the recovery of acceptable differences for the three TGA parameters for edoxaban but not for apixaban, rivaroxaban, nor dabigatran.

Conclusion: Double centrifugation before freezing should remain the recommended pre-analytical condition before TGA.

KEYWORDS

centrifugation, direct oral anticoagulant, pre-analytical, thrombin generation

Michael Hardy, Claudia Dellanoce, Sophie Testa and François Mullier equally contributed.

1 | INTRODUCTION

Thrombin generation (TG) is a global coagulation assay evaluating hemostatic balance resulting from procoagulant and anticoagulant activation. It has been proposed for the evaluation of bleeding and thrombotic risk in many clinical situations such as monitoring of anticoagulant drugs, evaluation of thrombotic recurrence risk after an episode of arterial or venous thromboembolism, or to assess bleeding risk in patients with congenital bleeding disorders.[1,2] However, until now, its implementation in the clinical laboratory has been limited by the lack of standardization both of method and reagents showing a high interlaboratory variability.[1] A new fully automated analyzer, the ST Genesia (Diagnostica Stago), has been developed, with the aim of enhancing the reproducibility of the method for its implementation in clinical practice.[3] Improvement of TG standardization has been obtained through a strict standardization of analytical conditions such as temperature, plasma volumes, reagents, and normalizing the expression of the results by the use of a reference plasma.[4] However, the lack of clinical studies evaluating its clinical performances is a limitation for its use as a daily clinical decision-making test.

Pre-analytical variables have an important impact on TG and must be mastered in order to reduce the variability of the measurement.[5] Double centrifugation at 1500-2500 *g* for 15 minutes is usually recommended before TG assays to eliminate residual platelets.[6] However, the requirements of this pre-analytical procedure are time-consuming and not mandatory for routine hemostasis tests, precluding the subsequent use of the same samples for thrombin generation measurement. In addition, the majority of plasma samples are often tested in series after freezing, usually at minimum −70°C. Knowing that the freeze-thawing process may fragment and activate residual platelets, it is recommended that double centrifugation should be performed before freezing to ensure minimal residual platelet count. On the other hand, few data are available regarding the impact of centrifugation on TG measurement on healthy subjects samples, especially after TG activation with high picomolar tissue factor (TF) concentration,[5,7,8] and no data are available on pathological samples.

The aim of this study is to evaluate the impact of single or double centrifugation on thrombin generation results measured on frozen samples from healthy subjects and patients treated with four direct oral anticoagulants: dabigatran, rivaroxaban, apixaban, and edoxaban.

2 | PATIENTS AND METHODS

The study has been conducted in two different sites: the Azienda Ospedaliera di Cremona (Cremona, Italy, site 1) and at the CHU UCL Namur (Yvoir, Belgium, site 2) after approval of both local ethics committee (institutional review board numbers 0019980 and B039201939103). All patients and healthy volunteers provided written informed consent.

2.1 | Study population

Twenty healthy volunteers aged between 18 and 60 years were included in each site. Exclusion criteria for the healthy individuals were abnormal prothrombin time or activated partial thromboplastin time, history of coagulation disorders, or recent (within 1 week) intake of drugs affecting hemostasis (eg, non-steroidal anti-inflammatory drugs and antithrombotic drugs) or the fluorescence measurement (eg, curcumin supplementation).

Twenty-four adult patients treated with a DOAC for non-valvular atrial fibrillation (6 for each drug (ie, dabigatran, rivaroxaban, apixaban, and edoxaban)) were also recruited in each site.

2.2 | Blood collection and processing

Blood was drawn in conformity with local procedure for hemostasis testing and between 1 and 6 hours after the last DOAC intake for DOAC-treated patients. Blood samples were collected into three trisodium citrate 109 mmol/L (9:1 v/v) plastic tubes (BD Vacutainer® 2.7 mL tubes, Becton Dickinson and Company®, Franklin Lakes, United States in site 1; Vacuette® 3.5mL tubes, Greiner Bio One®, Kremsmünster, Austria in site 2), after drawing the first milliliters of blood into a discard tube. Blood samples were centrifuged at 2000 *g* for 15 minutes at 20°C within 1 hour of blood collection. Supernatants were collected, pooled and four 1mL aliquots (aliquots A, B, C, and D) were prepared (Figure 1). Aliquots A, B, and C were directly frozen at minimum −70°C within 4 hours after centrifugation. Aliquots D underwent a second centrifugation (at 2000 *g* for 15 minutes at 20°C) before freezing the supernatant and was considered as the reference plasma.

After at least 5 days of freezing and within six months after blood draw, the 4 aliquots of plasma from the same donor/patient were rapidly thawed at 37°C in a thermostatic water bath for 5 minutes (Figure 1) and were then kept at room temperature (RT). Aliquots B and C underwent a second centrifugation after thawing (2000 *g* for 15 minutes at 20°C for aliquots B and 2000 *g* for 10 minutes at 20°C for aliquots C). Supernatants were collected. All four aliquots were then analyzed simultaneously within 2 hours after thawing.

2.3 | Laboratory methods

Anti-Xa or anti-IIa levels were measured once in each subject after single centrifugation and before freezing on a STA-R Max analyzer with STA—Liquid anti-Xa or STA—ECA II reagents (Diagnostica Stago, Asnières-sur-Seine, France) in both sites. Results were reported as estimated ponderal concentration after conversion using a calibration curve calculated with the batch of reagent used in the study.

Thrombin generation was measured on two ST Genesia analyzers (Diagnostica Stago) using the STG-DrugScreen reagent as described before (Diagnostica Stago; high picomolar TF concentration; same batch in both sites).[4] Calibrations were performed using the

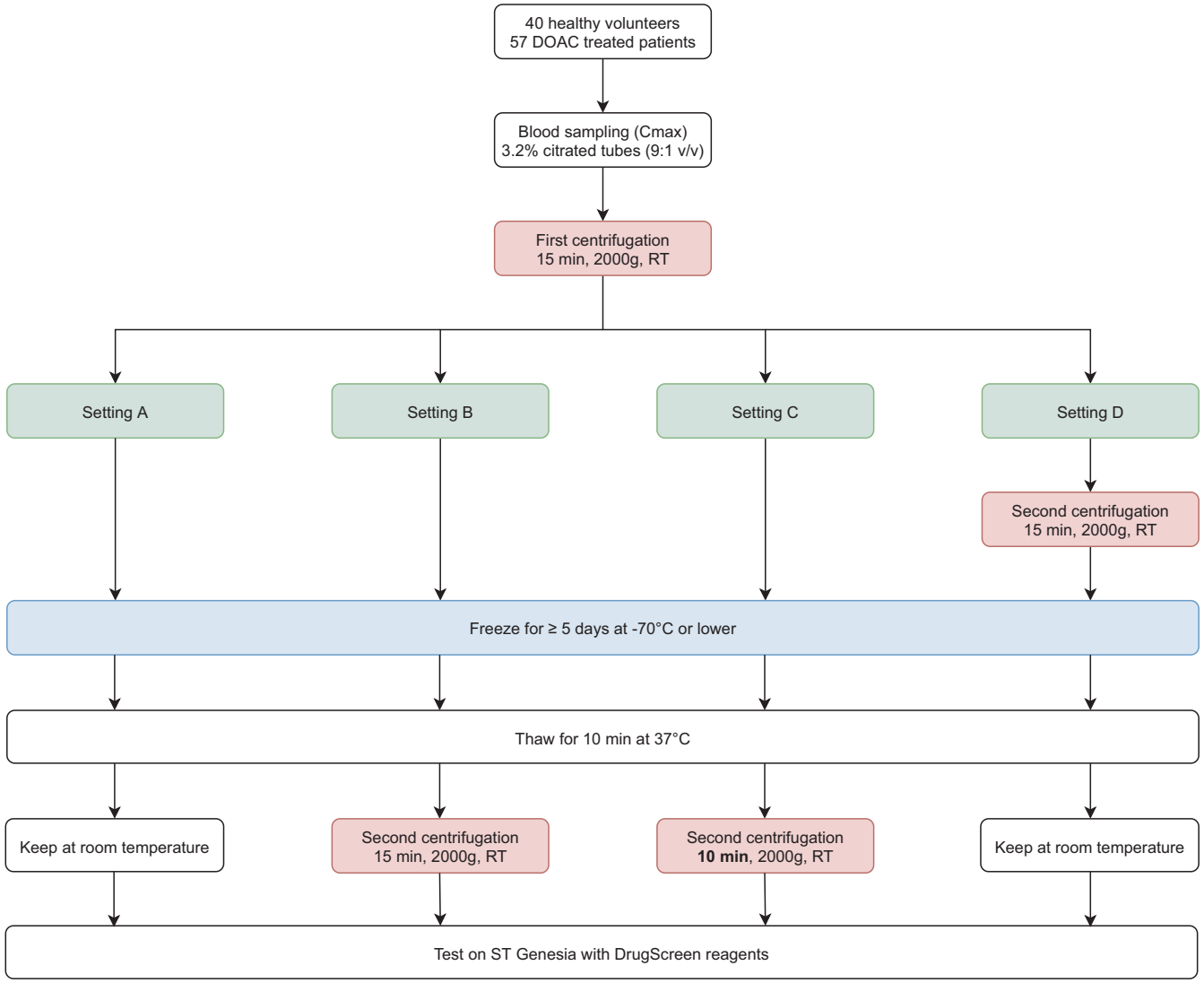

FIGURE 1 Study flowchart. DOAC, direct oral anticoagulant; RT, room temperature (20°C)

STG-Cal&Fluokit of reagents (Diagnostica Stago; different batch in each site) and two levels of quality controls supplied with STG-DrugScreen reagents were performed before each test series. Lag times, peak heights, and endogenous thrombin potential (ETP) were measured. These parameters were normalized using the lyophilized reference plasma supplied in the STG-DrugScreen kit, and results were expressed as ratios for lag times and as percentages for peak heights and ETP (the software calculates ratios and multiplies them by the assigned activity of the STG-RefPlasma lot used, calibrated against a "gold standard RefPlasma lot" labeled as 100%). Only normalized parameters will be considered in the manuscript.

2.4 | Statistical methodology

Based on internal precision data from the manufacturer and analytical variability calculated by our group on the same conditions of analyzer and reagents,[4] we estimated that a minimum of 20 healthy volunteers on each site would allow achieving a statistical power of at least 80% for comparison of healthy volunteers between study sites. For paired comparison of centrifugation conditions of a same sample, 9 pairs would be needed to achieve a statistical power of 80%. For subgroup analysis within each DOAC (n = 6 for each DOAC if results from both sites not pooled), the power would remain above 50%.

Data are expressed as mean ± standard deviation (SD) for normally distributed variables or as median (interquartile range (IQR)) for non-normally distributed variables. Normality was checked by Shapiro-Wilk tests. Quantitative data were compared using Student's *t* tests or Wilcoxon signed rank tests, as appropriated, and qualitative data compared using chi-squared tests or Fisher's exact tests, as appropriated.

Reference range intervals were determined using the 20 healthy volunteers' results under standard centrifugation conditions (double centrifugation before freezing) by calculating 2.5th-97.5thpercentiles; women receiving oral contraceptives were excluded from calculation of reference ranges. If thrombin generation

in the populations of healthy volunteers from both sites was not statistically different (Student or Wilcoxon tests), global reference ranges would also be calculated with the 40 healthy volunteers.

For each DOAC, results from both sites were compared and pooled if not statistically different (Student's *t* or Wilcoxon tests). Anti-Xa or anti-IIa levels were measured to explore potential differences in the level of DOAC in patients recruited from the two centers. Comparisons between centrifugation conditions A, B, C, and reference condition D were performed using paired samples Student's *t* tests for normal distributions or using Wilcoxon signed rank tests for non-normal distributions. Based on internal precision data from the manufacturer and analytical variability calculated by our group on the same reagent,[4] relative differences between centrifugations conditions A, B, C, and reference condition D were judged acceptable if not superior than 6% for lag times, 8% for ETP, or 10% for peak heights. Agreement between results obtained under different centrifugation conditions was evaluated using Bland-Altman plots.

All tests were two-sided, and a p-value lower than 0.05 was considered statistically significant. All analyses were performed in R (version 3.6.2). Excel (Office 2016, Microsoft, Redmond) was used to construct Bland-Altman plots.

3 | RESULTS

3.1 | Patient's characteristics

Twenty healthy volunteers were recruited in each site (Table 1). On the first site, 24 DOAC-treated patients were included (Table S1). On the second site, 33 DOAC patients have been included (9 supplemental patients have been added to compensate for 9 patients for whom DOAC concentration has not been measured; Figure 1). Six healthy donors from site 2 were taking oral contraceptives at the time of blood collection, but none from site 1. DOAC plasma concentrations were not statistically different between patients from both sites, except for dabigatran (Table 1).

3.2 | Thrombin generation parameters

For healthy volunteers, normalized lag times and peak heights were statistically different between both sites ($P < .001$ and $P = .005$, respectively), but not normalized ETP ($P = .90$). For dabigatran samples, normalized lag times, peak heights, and ETP were statistically different between both sites ($P < .001$). These samples were thus analyzed site by site. For apixaban, edoxaban, and rivaroxaban samples, results from the two sites were not statistically different for normalized lag times, peak heights, and ETP ($P > .05$) and were pooled for subsequent analysis. Reference ranges are represented for each site in Table 2.

Differences of TG parameters between centrifugation conditions A, B, C versus the reference condition D are presented in Table 3 and on Figure 2. Agreement between results obtained under the different centrifugation conditions is represented on Figures S1-S3. In summary:

- The different centrifugation conditions had a statistically significant impact on normalized lag times, peak heights, and ETP measured on healthy volunteers' samples (Table 3). However, these differences were considered acceptable (lower than predetermined thresholds, ie, 6% for lag times, 8% for ETP, or 10% for peak heights).
- For apixaban samples, a single centrifugation before freezing was associated with statistically significant reduced normalized lag times and increased peak heights and ETP. These differences were only considered acceptable for ETP using a second centrifugation after thawing (at 2000 *g* for 10 or 15 minutes—conditions B and C).

TABLE 1 Patients' characteristics. Mean (±standard deviation) or number (%) is represented, as appropriate

	Healthy			Dabigatran		
	Site 1	Site 2		Site 1	Site 2	
	N = 20	N = 20	*P*	N = 6	N = 7	*P*
Age (y)	na	33.8 (±13.8)	/	81.4 (±4.3)	71.4 (±13.6)	.28
Female, n (%)	10 (50)	13 (65)	.52	2 (33)	2 (29)	1.00
Body Mass Index (kg/m²)	/	/	/	26.7 (±3.9)	27.9 (±3.0)	.60
Creatinine clearance (mL/min)	/	/	/	56 (±8)	88 (±46)	.12
Cirrhosis	0 (0)	0 (0)	1	0 (0)	0 (0)	1
Drug interactions						
Amiodarone	0 (0)	0 (0)	1	0 (0)	0 (0)	1
DOAC plasma levels (ng/mL)	/	/	/	246 (±109)	82 (±74)	**.01**

Note: Quantitative data were compared using Student's *t* tests or Wilcoxon signed rank tests and categorial data using Fisher's exact test; p-values less than .05 are represented in bold. Creatinine clearance was calculated according to the Cockroft-Gault formula. Amiodarone was the only drug taken by these patients that could interfere with DOACs pharmacokinetics.

Abbreviations: DOAC, direct oral anticoagulant; na, non-available.

TABLE 2 Reference ranges calculated with healthy volunteers' samples under standard centrifugation condition (double centrifugation before freezing)

	Site 1	Site 2
LT (ratio)	0.9-1.5	0.8-1.1
PH (%)	60.3-106.5	72.0-100.3
ETP (%)	62.9-108.2	62.3-101.5

Note: Reference ranges correspond to percentiles 2.5-97.5. Women receiving oral contraceptives were excluded from the calculation.

Abbreviations: ETP, normalized endogenous thrombin potential; LT, normalized lag time; PH, normalized peak height.

- For edoxaban samples, a single centrifugation before freezing resulted in an unacceptable reduction in normalized lag times, unless a second centrifugation was performed after thawing. For ETP and peak heights, a second centrifugation after thawing was not significantly different from double centrifugation before freezing.
- For rivaroxaban samples, a single centrifugation before freezing always resulted in unacceptable reductions in lag times. However, peak heights and ETP were not statistically different when a second centrifugation was performed after thawing (at 2000 *g* for 15 minutes), in comparison to double centrifugation before freezing.
- For dabigatran samples, centrifugation conditions had no clinically relevant impact on peak heights and ETP, but the absence

TABLE 3 Differences of normalized thrombin generation parameters between pre-analytical conditions A, B, C, and D (see text for description). Results are given as percentage differences by comparison with condition D

		Healthy		Dabigatran		Rivaroxaban	Apixaban	Edoxaban
		Site 1	Site 2	Site 1	Site 2			
		(n = 20)	(n = 20)	(n = 6)	(n = 7)	(n = 14)	(n = 17)	(n = 13)
LT	A vs D	−3.7%**	−5.9%***	**−22.5%***	**−20.9%****	**−11.4%*****	**−6.1%****	**−11.1%****
	B vs D	−3.5%**	−4.1%***	−12.5%	**−10.1%****	**−8.4%*****	**−7.5%****	−5.8%**
	C vs D	−4.0%**	−5.7%***	**−25.6%***	**−12.3%****	**−7.3%****	**−6.1%***	−9.2%
PH	A vs D	+2.7%*	+1.2%*	+3.9%	+1.6%	**+13.8%*****	**+13.4%*****	+9.1%**
	B vs D	+3.9%**	+2.8%***	+4.8%	+2.3%*	+3.6%	**+14.5%****	+2.2%
	C vs D	+2.8%*	+2.3%***	+7.8%***	+2.3%*	+7.6%**	**+14.5%****	+9.6%
ETP	A vs D	+1.7%	+1.5%*	+3.3%	−1.7%	**+17.5%****	+6.1%**	+6.1%*
	B vs D	+1.6%	+1.5%*	−3.7%	+0.3%	+3.2%	+3.4%*	+1.2%
	C vs D	+1.5%	+1.1%	+1.4%	+0.8%	**+8.5%****	+4.4%*	+2.6%

Note: The statistical significant differences between centrifugations conditions were judged acceptable if ≤6% for lag times, ≤ 8% for ETP, or ≤10% for peak heights. Differences higher than these thresholds are represented in bold. Results from both sites were cumulated for apixaban, edoxaban, and rivaroxaban because not statistically different between centers.

*$P < .05$; **$P < .01$; ***$P < .001$

Abbreviations: ETP, normalized endogenous thrombin potential; LT, normalized lag time; PH, normalized peak height.

Rivaroxaban			Apixaban			Edoxaban		
Site 1	Site 2	p	Site 1	Site 2		Site 1	Site 2	
N = 6	N = 8		N = 6	N = 11	*P*	N = 6	N = 7	*P*
65.8 (±17.1)	74.4 (±9.4)	.35	69.4 (±9.3)	70.2 (±11.1)	0.88	74.3 (± 8.6)	73.0 (±7.2)	.77
1 (17)	4 (50)	.56	5 (83)	4 (36)	0.33	3 (50)	1 (14)	.27
26.1 (±2.9)	29.4 (±5.1)	.20	23.7 (±2.9)	28.6 (±4.3)	**0.02**	25.6 (±5.2)	31.5 (±5.9)	.10
74 (±38)	80 (±35)	.77	69 (±22)	82 (±32)	0.40	60 (±32)	78 (±36)	.41
0 (0)	0 (0)	1	0 (0)	0 (0)	1	0 (0)	0 (0)	1
1 (17)	2 (25)	1	0 (0)	4 (36)	0.24	1 (17)	2 (29)	1
236 (±95)	320 (±204)	.40	216 (±114)	181 (±106)	0.58	245 (±57)	219 (±144)	.69

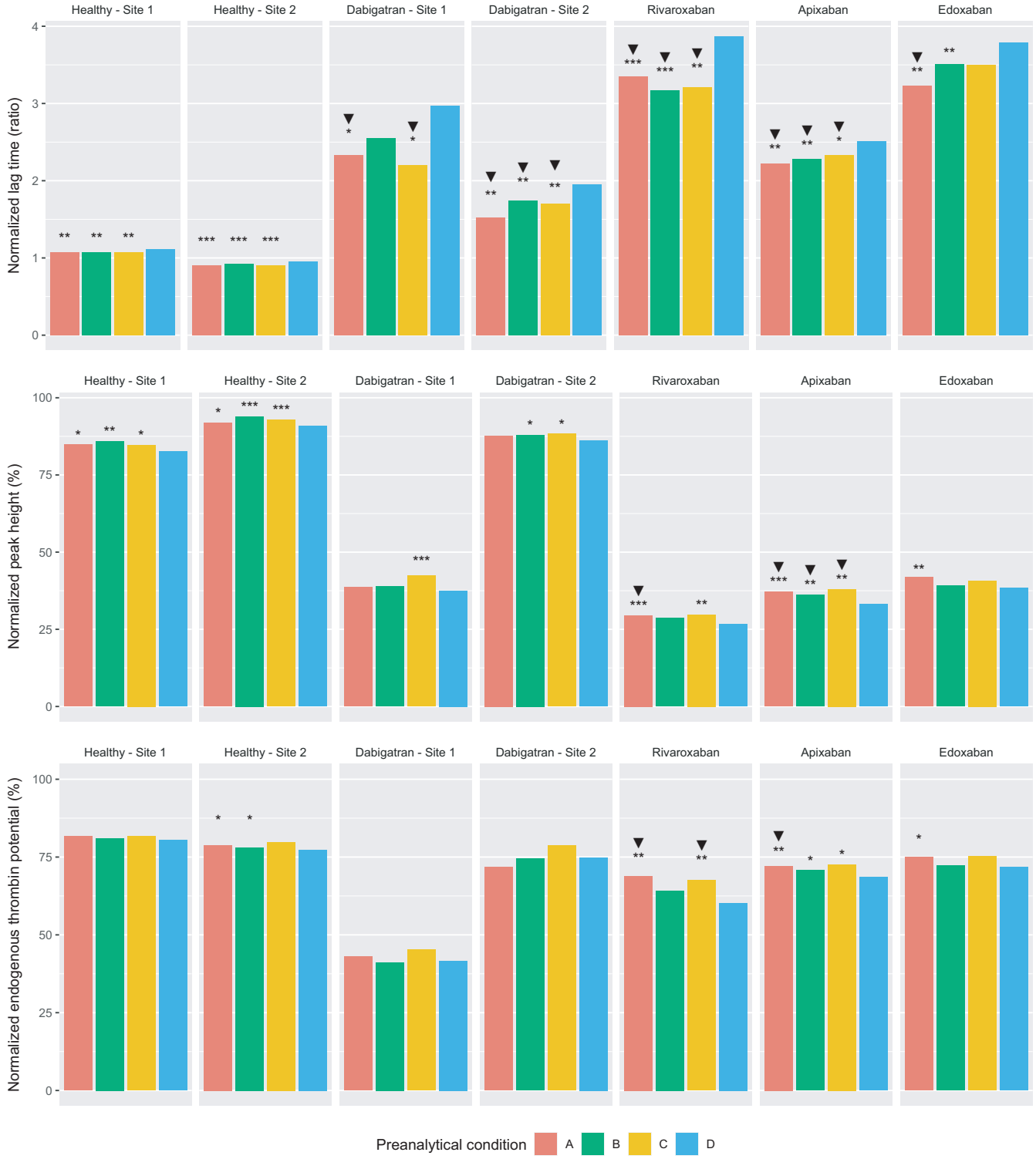

FIGURE 2 Differences between centrifugation conditions. Mean normalized lag time, peak height, and endogenous potential are represented in each centrifugation condition (A, B, C, D; see text) and for each anticoagulant. Centrifugation conditions A, B, and C were compared to reference condition (D). Stars represent statistically significant differences (*$P < .05$; **$P < .01$; ***$P < .001$); differences that are not acceptable (eg, normalized lag time relative difference >6%, normalized endogenous thrombin potential relative difference >8%, or normalized peak height relative difference >10%) are represented with triangles (▾)

of double centrifugation before freezing resulted in unacceptable reduction in lag times in samples from site 2. For site 1 samples, a second centrifugation after thawing (at 2000 *g* for 15 minutes) resulted in acceptable lag times, by comparison with double centrifugation before freezing.

4 | DISCUSSION

This study evaluates the impact of centrifugation conditions on TG measurement on frozen samples from healthy subjects and DOAC patients. Overall, single centrifugation before freezing resulted in

significantly enhanced thrombin generation in healthy volunteer's samples, but these differences remained low and acceptable (ie, lower than predefined thresholds based on analytical variability). The differences observed are certainly explained by the higher number of residual platelets after a single centrifugation.[7] Indeed, the subsequent freeze-thawing cycle results in unpredictable fragmentation of these platelets,[2] releasing phospholipids and other procoagulant factors such as platelet factor-4, coagulation factor V and fibrinogen, which could no longer be eliminated by additional centrifugations, and will increase further thrombin generation. Therefore, residuals platelets should be eliminated before freezing by adequate centrifugation protocols ensuring platelet levels <10 000 platelet/μL.[9]

Regarding DOACs, their effect on TG was underestimated in the absence of double centrifugation before freezing. Indeed, single centrifugation resulted in unacceptable reductions of lag times for all DOACs and should therefore be avoided. For edoxaban samples only, a second centrifugation after thawing appeared to be able to correct TG parameters, in comparison to double centrifugation before freezing. Among DOACs, apixaban samples were the most impacted by centrifugation conditions. This could be the result of the slower and weaker inhibition of the prothrombinase complex achieved by this drug,[10] which would make those samples more sensitive to the procoagulant effect of phospholipids and other factors released during freeze-thaw cycles. Furthermore, lag times, which reflect the initiation phase of coagulation, were more influenced by centrifugation conditions than peak heights and ETP, in both healthy volunteers and patients receiving a DOAC. This could be due to the presence of residual platelets, which contain coagulation factor V (proaccelerin) and may also contain TF, which are both important proteins for the initiation of coagulation.[11] Lag times could also be more sensitive to additional procoagulant phospholipids from residual platelets. ETP was the most stable parameter among centrifugation conditions, which is in agreement with a previous work.[7] If measuring only the ETP, a second centrifugation after thawing seemed to be an acceptable alternative to double centrifugation before freezing for both healthy volunteer's and DOAC samples using the STG-DrugScreen reagent. However, statistical power was low for subgroup analysis among DOACs so these results should be taken with caution.

We noticed statistically significant differences in TG parameters between centers for healthy volunteers and for dabigatran samples. For the latter, differences in plasma concentrations between patients from both sites could explain some of the differences observed in TG parameters (mostly for differences among lag times). Other uncontrolled pre-analytical variables such as differences in blood collection systems and tubes in batches of calibrators or the utilization of a brake in the centrifuge in site 1 only could also explain some of the interlaboratory variability observed.[5,12] However, the differences between the two sites remained within previously published interlaboratory variability ranges for TG measured using the calibrated automated thrombogram(CAT).[13] This emphasizes the need to locally establish reference ranges in local analytical settings. Reference ranges determined from healthy volunteers' samples are close to those previously published by Douxfils et al[4] (also measured at site 2 with STG-DrugScreen reagent), except for peak heights, which were lower in our study.

These results are consistent with previous work performed on healthy volunteers' samples only. Loeffen et al[5] identified a significant increased ETP when samples undergone simple centrifugation compared with double centrifugation before freezing. This effect was only observed when TG was triggered with low TF concentrations (1 pM vs 5 pM).[5] Lisman et al[14] also measured increased ETP and peak heights on single centrifugated samples, compared with four different double centrifugation conditions (TG triggered by TF 5 pM). Rodgers et al[7] identified reduced lag times and times-to-peak when TG (triggered by 5 pM TF) was measured on frozen samples after single versus double centrifugation at 1400 *g* for 10 minutes. However, there was no difference between single and double centrifugation when TG was performed on fresh plasma and when higher centrifugation speeds were used (2600 *g* for 15 minutes). Furthermore, they failed to identify significant differences in TG parameters between single enhanced centrifugation (2600 *g* for 15 minutes) and standard double centrifugation (1400 *g* 10 minutes). On the opposite, when lower triggering TF concentration (1 pM) was used, an enhanced double centrifugation protocol (2600 *g* for 15 minutes) was necessary to avoid the reduction of the ETP and the shortening of the lag time observed with a single enhanced centrifugation. Lacroix et al[8] compared two protocols of double centrifugation before TG measurement on healthy volunteers' samples (double centrifugation at 2500 *g* for 15 minutes vs a first centrifugation at 1500 *g* for 15 minutes followed by a second centrifugation at 13 000 *g* for 2 minutes). They identified higher mean velocities with the second protocol performed on frozen samples, but not when it was performed on fresh samples. The correlation of TG mean velocities between frozen and fresh samples was also worse with the second centrifugation protocol ($r^2 = 0.23$ vs $r^2 = 0.95$), reflecting a less effective removal of residual platelets. These results served to document the current guidelines recommending double centrifugation before TG measurement on frozen samples[6].

Our study presents several limitations. First, it included only a small number of patients on DOAC treatment. Subgroup analysis among the different anticoagulants had a low level of power to identify significant differences between centrifugation conditions or between the two study sites. Second, we only aimed at collecting DOAC samples at peak plasmatic concentrations. Trough concentration samples could be less impacted by centrifugation conditions, as suggested by the smaller differences observed between centrifugation conditions for dabigatran samples from site 2, by comparison with site 1 where dabigatran concentrations were higher.

5 | CONCLUSION

This study reinforces the current knowledge about the importance of strictly standardized centrifugation conditions before thrombin generation measurement on frozen samples. For healthy volunteers'

samples, a single centrifugation before freezing resulted in statistically different TG parameters, by comparison with double centrifugation before freezing, regardless of the performance of a second centrifugation after thawing. However, these remained "analytically acceptable" as per the limits that were defined. For DOAC samples, a single centrifugation before freezing resulted in an overall unacceptable underestimation of the anticoagulant effect, except for samples containing edoxaban provided that a second centrifugation was performed after thawing. Therefore, double centrifugation before freezing should remain the recommended pre-analytical condition for TG measurement performed with STG-DrugScreen on ST Genesia analyzer on frozen samples from both healthy volunteers and patients under DOAC therapy.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

ST and FM involved in conceptualization. MH, CD, SL, and CB involved in investigation. MH, JD, AC, and FM involved in formal analysis. MH and FM wrote the original draft. MH, CD, JD, AC, SL, CB, ST, and FM involved in writing, reviewing and editing the article. ST and FM involved in supervision.

DATA AVAILABILITY STATEMENT

Data available on reasonable request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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