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ORIGINAL ARTICLE

Analytical performance of the endogenous thrombin potential–based activated protein C resistance assay on the automated ST Genesia system

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Abstract

Background: The evaluation of activated protein C (APC) resistance based on the endogenous thrombin potential (ETP) is recommended during the development of steroid contraceptives in women. In 2019, this assay was validated on the calibrated automated thrombogram (CAT) device. However, in view of its screening potential, its automation is essential.

Objectives: To transfer the ETP-based APC resistance assay on the ST Genesia system using reagent STG-ThromboScreen with exogenous APC added.

Method: Dose-response curves were performed to define APC concentration leading to 90% ETP inhibition on healthy donors. Intra- and interrun reproducibility was assessed. The normal range was defined on the basis of 56 samples from healthy individuals. The sensitivity was assessed on 40 samples from women using combined oral contraceptives (COCs). A method comparison with the validated ETP-based APC resistance on the CAT system was performed. Results were expressed in normalized APC sensitivity ratio (nAPCsr).

Results: The APC concentration leading to 90% ETP inhibition was 652 mU/mL. Intra- and interrun reproducibility showed standard deviation <4%. The nAPCsr normal range stood between 0.00 and 2.20. Analyses of 40 samples from women using COCs confirmed the good sensitivity of the assay. Compared to the CAT system, nAPCsr values were slightly higher on the automated system.

Conclusion: This study is the first reporting the analytical performances of the ETP-based APC resistance assay on an automated platform. Results support the concept that this test, when incorporated into clinical routine, could become a promising regulatory and clinical tool to document on the thrombogenicity of female hormonal therapies.

KEYWORDS
activated protein C resistance, combined oral contraceptives, reproducibility of results, thrombin, venous thromboembolism

Essentials

- The endogenous thrombin potential (ETP)–based APC resistance assay is validated and standardized on the calibrated automated thrombogram (CAT) system.
- This validated methodology was transferred on an automated platform, the ST Genesia system.
- Results were similar between the CAT (validated platform) and the ST Genesia system.
- In view of its screening potential, a complete validation on the ST Genesia could be considered.

1 | INTRODUCTION

Throughout the course of their lifetime, women are exposed to hormonal changes, thus modulating the risk of venous thromboembolic events (VTEs). These hormonal changes are either endogenous, as during pregnancy and in the postpartum period, or due to the intake of exogenous hormones, as with the use of combined hormonal contraceptives (CHCs) or hormone replacement therapies (HRTs) during early menopause.

The first case of VTE associated with the use of a hormonal therapy dates back to the 1960s, shortly after the approval of the first combined oral contraceptive (COC), called Enovid, a combination of 150 μg of mestranol and 10 mg of norethynodrel.[1] Over the years, many estrogen-progestogen associations have been marketed, modulating the dose and the nature of the estrogenic component as well as the nature of the progestogen with the aim of reducing the associated risk of VTE.[2] Numerous studies have assessed the impact of these products on hemostasis and shown that a poor response to activated protein C (APC) could explain, at least in part, the procoagulant state observed in women using hormonal therapies (eg, CHCs or HRTs).[3-6] In 2004, the European Medicines Agency therefore recommended the evaluation of APC resistance during the development of steroid contraceptive agents in women.[7] Initially, the main purpose of APC resistance testing was to detect the presence of a factor V Leiden (FVL) mutation.[8] The original test proposed a ratio between a baseline activated partial thromboplastin time (aPTT) and the aPTT after purified exogenous APC has been added. However, many interferences on the aPTT assay could falsely lead to APC resistance. Consequently, over the years, this coagulation test evolved, with the aim of making it less or even not sensitive to interferences (eg, the use of CHCs).[8] Nowadays, the measurement of APC resistance in women on hormonal therapies should thus rely on the endogenous thrombin potential (ETP)-based APC resistance assay.[9,10] This test is a global coagulation test aiming to assess the resistance toward APC based on the measurement of thrombin generation over time. Results are expressed as normalized APC sensitivity ratio (nAPCsr) standing between 0 and 10, and scores that are closer to 10 show higher resistance to APC suggesting a higher risk of VTE.[11] This test was developed more than 20 years ago by Nicolaes et al,[12] but due to the lack of standardization and harmonization, results were laboratory dependent, hampering study-to-study comparison.[13,14] In 2019, in the light of its potential as a global biomarker of thrombogenicity, our group proposed a standardized methodology to perform the assay that met all the regulatory requirements in terms of analytical performance.[11] The ETP-based APC resistance assay was validated on the calibrated automated thrombogram (CAT) device using commercially available reagents to ensure batch-to-batch traceability, recovery, and reproducibility of the method over time.[11] Recently, the interlaboratory variability study supported the concept that the ETP-based APC resistance, performed with a validated and standardized methodology, provides an adequate sensitivity and an excellent interlaboratory reproducibility.[15] Nevertheless, the CAT mostly remains a research platform, and the implementation of the ETP-based APC resistance on an automated analyzer is of paramount importance to have this innovative biomarker available for the clinicians in their daily practice.

The aim of this study was therefore to implement the ETP-based APC resistance assay on the automated thrombin generation instrument, the ST Genesia system (Diagnostica Stago, Asnières-sur-Seine, France). This instrument offers the capacity to continuously load patient samples for unitary testing, which highly facilitates its use in clinical routine.[16]

2 | MATERIALS AND METHODS

2.1 | General procedure of the ETP-based APC resistance assay

The ETP-based APC resistance assay is based on the global potential of plasma to form thrombin after triggering of the coagulation with (or without) addition of exogenous APC and has been extensively described elsewhere.[11] In this assay, the activator reagent, the STG-ThromboScreen (TS) is tested in absence and presence of APC (Diagnostica Stago) to trigger the protein C anticoagulant pathway in the tested plasma. The resulting reduction of the ETP (corresponding to the area under the curve) in the presence of APC is compared to the non-APC condition. The reference method for this test is performed on a CAT system (Diagnostica Stago), using the Thrombinoscope software, version 5.0.[14]

The amount of APC to introduce in the test is defined for each APC/TS batch and targets a decrease of 90% of the ETP of a healthy pooled plasma (HPP). This decrease corresponds to the percentage of inhibition of the ETP (inhibition %) computed as the ratio of the ETP value measured with TS (+APC) and the ETP value measured with TS (−APC), as depicted in Equation 1. The lower the ETP inhibition % compared to the HPP, the more resistant to APC the subject is. The normalization of these results based on a reference plasma (ie, HPP) leads to the normalized APC sensitivity ratio (nAPCsr) (Equation 2). Since the inhibition induced by APC on HPP is targeted to be 90%, the obtained nAPCsr stands theoretically between 0 and 10 and, in contrast with the ETP inhibition %, the higher the nAPCsr, the more resistant the donor is to APC.

$$\text{Inhibition } \% = 1 - \frac{\text{Sample ETP}(+\text{APC})}{\text{Sample ETP}(-\text{APC})}\ \% \quad (1)$$

$$\text{nAPCsr} = \frac{\text{Sample ETP}(+\text{APC})/\text{Sample ETP}(-\text{APC})}{\text{Ref ETP}(+\text{APC})/\text{Ref ETP}(-\text{APC})} \quad (2)$$

Nonetheless, as the homemade reference plasma (ie, the HPP) cannot be produced at a large scale, while it has to be analyzed at each run, a commercially available reference plasma (STG-RefPlasma TS) is used to compute the nAPCsr. The downside of using a commercial plasma is its large-scale production, which is probably not compliant with the best recommendations of blood sample collection for thrombin generation testing. Therefore, one solution is to compare the sensitivity of the commercial reference plasma with a smaller pool of plasma from healthy donors collected in ideal conditions (ie, our in-house HPP) and then to apply a correction factor to the commercial plasma to compensate for these differences. This correction factor is determined by computing the nAPCsr of the commercial reference plasma using the in-house reference plasma (ie, HPP) as the reference plasma (Equation 3). The adjusted nAPCsr is detailed in Equation 4:

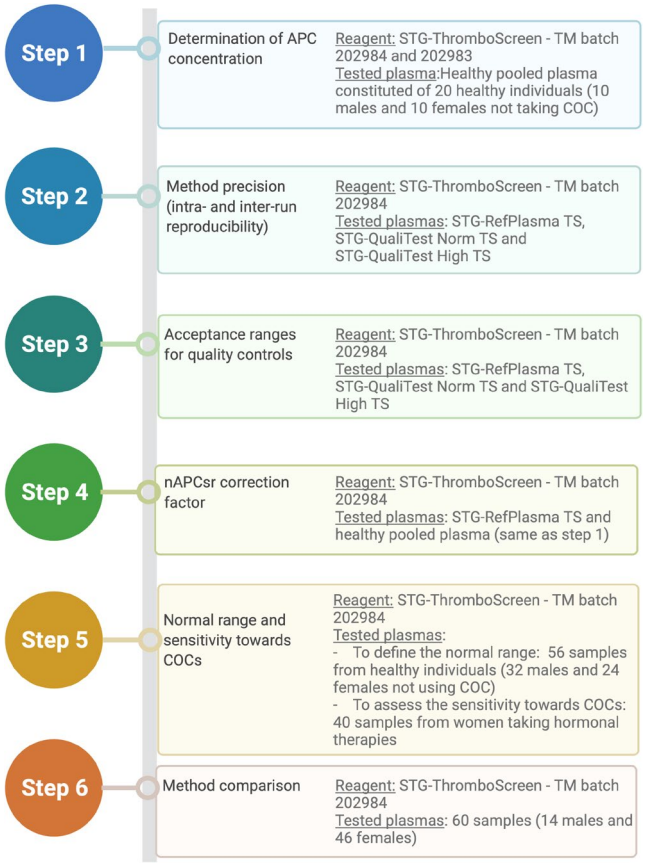

FIGURE 1 Flowchart of the transferability of the ETP-based APC resistance assay on the ST Genesia system. APC, activated protein C; COC, combined oral contraceptive; ETP, endogenous thrombin potential; nAPCsr, normalized activated protein C sensitivity ratio

$$\text{nAPCsr correction factor}\,(F) = \frac{\text{STG} - \text{RefPlasma TS ETP}(+\text{APC})/\text{STG} - \text{RefPlasma TS ETP}(-\text{APC})}{\text{HPP ETP}(+\text{APC})/\text{HPP ETP}(-\text{APC})} \quad (3)$$

$$\text{Adjusted nAPCsr} = \frac{\text{Sample ETP}(+\text{APC})/\text{Sample ETP}(-\text{APC})}{\text{STG} - \text{RefPlasma TS ETP}(+\text{APC})/\text{STG} - \text{RefPlasma TS ETP}(-\text{APC})} \times F \quad (4)$$

2.2 | Transferability of the ETP-based APC resistance assay on the ST Genesia system

The transferability, as described in Figure 1, was performed as follows: (i) determination of the APC concentration to achieve 90% of ETP inhibition of the HPP, (ii) assessment of the method precision, (iii) definition of acceptance ranges for the reference plasma (STG-RefPlasma TS) and quality controls (STG-QualiTest Norm TS and STG-QualiTest High TS), (iv) computation of the nAPCsr correction factor, (v) definition of the normal range and assessment of the sensitivity toward different oral contraceptive therapies, and (vi) comparison of the methods between the CAT and the ST Genesia system.

2.2.1 | Determination of the APC concentration

The first step consisted of defining the amount of exogenous APC to be added in the TS reagent for the +APC condition. To this end, dose-response curves were performed using the in-house reference plasma (ie, the HPP), and two different batches of TS (batches Nr 202983 and Nr 202984). To constitute the HPP, 20 healthy volunteers (10 men and 10 women not taking hormonal therapy) were enrolled and displayed the following characteristics: mean age of 25 years (range, 18-56 years) and mean body mass index (BMI) of 22 $kg.m^{-2}$ (range, 19-26 $kg.m^{-2}$). Five solutions of TS containing

increasing concentrations of APC, that is, 200, 400, 600, 700, and 1034 mU/mL (final concentration in the reagent) were prepared to generate dose-response curves, all concentrations being derived from the APC labeled activity titer provided by the manufacturer. The concentration leading to 90% of ETP inhibition of the HPP was then intrapolated for both batches of TS. Analyses were performed in triplicates on each batch of TS. It was then validated by analyzing the HPP, five times within the same run, with both batches of TS in the presence of the determined adequate APC concentration. The mean ETP inhibition % of the HPP obtained with each batch of TS had to stand between 87.5% and 92.5%.

2.2.2 | Assessment of the method precision

Intra- and interrun reproducibility was assessed on three samples: the STG-RefPlasma TS, the STG-QualiTest Norm TS, and the STG-QualiTest High TS. The third level of quality control (STG-QualiTest Low TS) included in the commercial kit was not analyzed, as its thrombin generation was completely inhibited in presence of APC. These plasmas are lyophilized, citrated human plasmas designed for thrombin generation testing, providing distinct levels of response in the ETP-based APC resistance assay. The intrarun reproducibility was assessed by performing five individual measurements of the three above-mentioned plasmas, within the same run. For the interrun reproducibility, those plasmas were analyzed over 10 independent runs. Results were expressed as ETP inhibition %, and acceptance criteria were the same as for the method validation on the CAT system,[11] that is, standard deviation (SD) values <10%. For this performance and subsequent steps, only one batch of STG-ThromboScreen was used (batch Nr 202984).

2.2.3 | Definition of acceptance ranges of the commercial reference plasma and quality controls

Acceptance ranges have been defined in ETP inhibition %. For quality controls (ie, STG-QualiTest Norm TS and STG-QualiTest High TS), ranges were computed as the mean ETP inhibition % (of 10 independent runs) $\pm 2 \times$ SD and $3 \times$ SD to follow Westgard rules. On the other hand, for the commercial reference plasma, the acceptance range was defined as the ETP inhibition % that corresponded to 87.5% and to 92.5% of ETP inhibition of the in-house HPP. This narrow acceptance range was defined to ensure a ratio close to 0.1 at the denominator, when computing the nAPCsr, to limit the nAPCsr score up to 10.[17]

2.2.4 | Determination of the nAPCsr correction factor

To compute nAPCsr values of plasma samples, the correction factor was determined based on the 10 runs of the commercial reference plasma performed during the interrun reproducibility and on the 5 runs of the HPP performed during the APC concentration validation.

2.2.5 | Definition of the normal range and assessment of the sensitivity toward different oral contraceptive therapies

To define the normal range, 56 samples from healthy individuals (32 men and 24 women not using hormonal therapy) were analyzed. The mean age of participants was 23 years (range, 18-56 years), and the mean BMI was 23 $kg.m^{-2}$ (range, 17-32 $kg.m^{-2}$). Results were expressed in nAPCsr values. The lower and upper limit of the normal range corresponded to the 10th to 90th percentile of nAPCsr values.

To assess the sensitivity of the test in a subset of real-life samples, 40 healthy young women taking hormonal therapies were recruited and stratified in three subgroups, namely, users of second-generation COCs (ie, containing 20 or 30 μg of ethinylestradiol (EE) with 100 or 150 μg of levonorgestrel; n = 18; mean age, 21 years; mean BMI, 22 $kg.m^{-2}$), users of third-generation COCs (ie, containing 20 or 30 μg of EE with 150 μg of desogestrel or 75 μg of gestodene; n = 13; mean age, 22 years; mean BMI, 22 $kg.m^{-2}$), and users of the so-called "other" COCs (ie, containing 20, 30, or 35 μg of EE with 3 mg of drospirenone, 2 mg of cyproterone acetate, or 2 mg of dienogest; n = 9; mean age, 21 years; mean BMI, 21 $kg.m^{-2}$). The demographic characteristics (age and BMI) of the three groups were well matched. Comparisons between healthy individuals (men and women separated) and the three subgroups according to the use of hormonal therapies were performed. Results were expressed as nAPCsr values and ETP values (for the latter, in the absence and presence of APC).

2.2.6 | Comparison between the CAT system and the ST Genesia system

Sixty samples from individuals (14 men and 46 women) were analyzed with the validated assay on the CAT system and on the ST Genesia system. The mean age of participants was 21 years (range, 18-30 years) and the mean BMI was 22 $kg.m^{-2}$ (range, 17-31 $kg.m^{-2}$). To cover a wide range of nAPCsr values, of the 46 women, 13 were not using any contraception, 33 were on various hormonal contraceptives, and, finally, one participant was carrying a heterozygous FVL mutation and another a heterozygous prothrombin (PT) G20210A mutation. The comparison between both platforms was performed using nAPCsr results.

2.3 | Blood sample collection and plasma preparation

All the volunteers included in this study were recruited at the University of Namur, Belgium. Biological samples were collected in

accordance with the Declaration of Helsinki after approval by the Ethical Committee of the CHU UCL Namur (Yvoir, Belgium) under the approval number B03920096633. Written informed consent was obtained from each donor. Exclusion criteria were history of thrombotic and/or hemorrhagic events, treatment by antiplatelets or anticoagulant medications or other drugs potentially affecting platelets or coagulation, pregnancy, use of hormonal therapy (only for the recruitment of healthy volunteers and for the constitution of the HPP), and carriers of FVL or PT G20210A mutations (except for the comparison between platforms). The absence/presence of FVL and PT G20210A mutations was identified by a CE-IVD-approved technique (Lamp Human FII & FVL Duplex kit, reference LC-FII & FVL-LP-24, LaCAR MDx Technologies, Ougrée, Belgium). All samples were stored and managed by the Namur Biobank-eXchange, the registered biobank from the University of Namur. Regarding sampling procedures, blood was taken by venipuncture in the antecubital vein and collected into 0.109 M sodium citrate tubes (9:1 v/v) (Vacuette(R), Greiner Bio-One, USA) without corn trypsin inhibitor using a 21-gauge needle (BD Vacutainer Eclipse, Becton Dickinson, Franklin Lakes, NJ, USA). The first tube was always discarded. The platelet poor plasma (PPP) was obtained from the supernatant fraction of blood tubes after double centrifugation for 15 minutes at 2500 *g* at room temperature. The first centrifugation was performed within 30 minutes after blood sampling. Immediately after centrifugation, PPP was pooled for the constitution of the HPP or aliquoted as individual plasma. The aliquots were snap-frozen in liquid nitrogen within 4 hours after the sampling and stored at ≤ −70°C. Frozen plasma samples were thawed in a water bath at 37°C for a maximum of 10 minutes and mixed gently just before the experiment. All tests were performed within 4 hours after thawing.

2.4 | Statistical analyses

Statistical analyses were performed using GraphPad Prism version 9.0.0 for MacOs (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Descriptive statistics were used to analyze the data. The APC concentration was determined using nonlinear regressions (one-phase association–least square fit) with no constrain on the *y*-axis. As the SD is stable along the whole range of measurements, intra- and interrun reproducibility was expressed in terms of SD instead of coefficient of variation. To compare subgroups with each other, analysis of variance (ANOVA) with Tukey's multiple comparison tests were performed. A linear regression and a Pearson correlation were used to assess the correlation between the CAT system versus the ST Genesia system. A derived Bland-Altman analysis was performed by plotting nAPCsr differences (CAT − ST Genesia) against nAPCsr values obtained on the CAT system, considered as the gold standard. Within each subgroup, unpaired *t* tests were performed to compare nAPCsr values between platforms. Finally, a two-way ANOVA with multiple comparison tests was performed to compare subgroups with each other.

3 | RESULTS

3.1 | Determination of APC concentration

Dose-response curves of APC, performed on two different batches of TS, are shown in Figure 2. Coefficients of determination R^2 of individual nonlinear regressions were ≥.98 with both batches of TS. The concentration of APC leading to 90% of ETP inhibition on both batches of TS was the same (ie, 652 mU/mL). The intrarun reproducibility of the HPP tested using this APC concentration revealed a mean ETP inhibition percentage (±SD) of 91.3% (±4.8%) and 90.9% (±2.7%) on batches Nr 202984 and Nr 202983, respectively. Thrombin generation curves of HPP, in the absence and presence of 652 mU/mL of APC, with both batches of TS, are shown in Figure 3.

3.2 | Method precision: Intra- and interrun reproducibility

Intrarun and interrun reproducibility are shown in Table 1. SD values for each tested plasma (ie, STG-QualiTest Norm TS, STG-QualiTest High TS and STG-RefPlasma TS) were within acceptance criteria (ie, SD ≤10%).

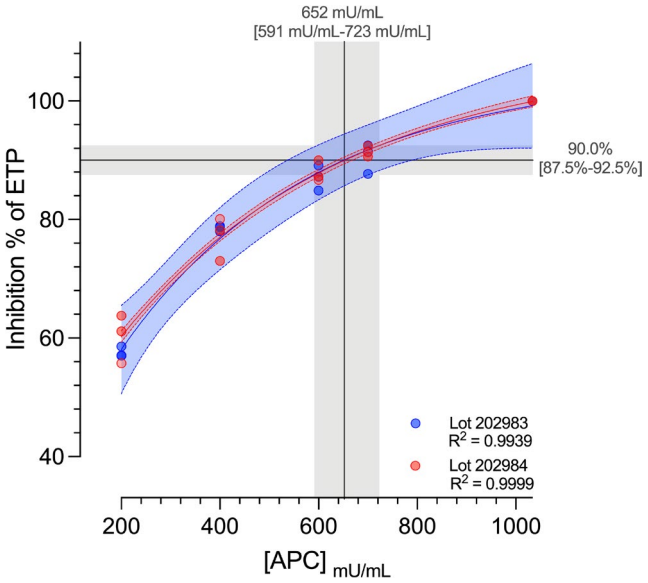

FIGURE 2 Mean inhibition percentages of the endogenous thrombin potential (ETP) of a healthy pooled plasma (HPP), at various concentrations of activated protein C (APC) on two different batches of STG ThromboScreen TS (-TM) (Nr 202983 and Nr 202984). Dose-response curves performed with batch 202983 are represented in blue, and those performed with batch 202984 are represented in red. The dark gray horizontal line and the light gray area on either side of the line represent the targeted inhibition % (lower-upper limit of acceptance), that is, 90% (87.5%-92.5%). The dark gray vertical line and the light gray area on either side of the line correspond to the APC concentration leading to 90% of ETP inhibition (upper-lower limit of acceptance), that is, 652 mU/mL (591-723 mU/mL). APC, activated protein C; ETP, endogenous thrombin potential

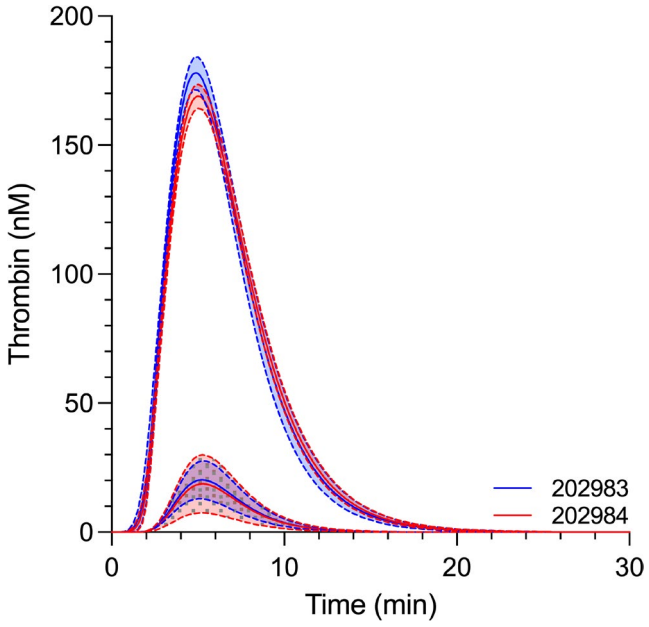

FIGURE 3 Thrombin generation curves of a healthy pooled plasma, in the absence and presence of activated protein C (APC; 652 mU/mL) with two different batches of STG-ThromboScreen -TM (TS). The blue curves were generated with TS batch 202983 and the red curves with batch 202984. The continuous lines represent the mean of the five duplicates and the dotted lines represent the 95% confidence interval. Areas in the absence of pattern represent the −APC condition, and areas with the fill gray pattern represent the + APC condition

3.3 | Acceptance ranges of the commercial reference plasma and quality controls

Acceptance ranges for the reference plasma and the two levels of quality control are shown in Figure 4. The following Westgard rules were applied to monitor the run performances throughout the entire study: (i) warning if one measurement exceeded 2 × SD for one quality control (1_{2s}) and (ii) rejection if one measurement exceeded 3*SD for one quality control (1_{3s}) or outside the acceptance range for the reference plasma. As depicted in Figure 3, no analysis was rejected throughout the course of the study. Nonetheless, on run 3, ETP inhibition % of STG-QualiTest Norm TS and STG-Qualitest High TS exceeded 2 × SD but stood within the mean ± 3 × SD.

3.4 | Computation of the nAPCsr correction factor

The correction factor, which corresponded to the nAPCsr of the commercial reference plasma (issued from STG-ThromboScreen kit batch Nr 202984), using the HPP as the reference plasma, equaled 2.45. This correction factor was applied to compute the nAPCsr of all plasma samples.

3.5 | Normal ranges and sensitivity toward different oral contraceptive therapies

In the normal-range study involving 56 healthy individuals, the mean nAPCsr was 1.03 (SD = 0.82; 95% confidence interval [CI] of the mean, 0.82-1.25). The 10th to 90th percentile, chosen as the lower and upper limit for the normal range, equaled 0.00 and 2.20. When sex was taken into account, men were significantly less resistant to APC than women, with a mean nAPCsr of 0.69 and 1.49, respectively. Among the different COC-user groups, mean nAPCsr values (±SD) equaled 3.61 (±1.04) for second-generation COC users; 3.83 (±1.03) for third-generation COC users, and 5.54 (±0.73) for the so-called other COC users. Tukey's multiple comparison tests showed significant differences between each subgroup except for women using second- and third-generation COCs ($P > .05$; Figure 5). Regarding the ETP parameter, individuals' values and means ±SDs in the absence and presence of APC of each subgroup are shown in Table S1 and Figure S1. In the absence of APC, no significant difference was observed between men and women not using COCs, as well as among women using COCs ($P > .05$). Differences between healthy individuals (men and women not using COCs) and women using COCs (regardless of the therapy) were significant. In the presence of APC, the trends remained similar, except between women using second-generation COCs and women using other COCs, for which the difference was significant ($P = .0004$) (Figure S1).

3.6 | Comparison between the CAT and the ST Genesia system

To compare results obtained on the CAT system and the ST Genesia system, 60 plasma samples were analyzed on both platforms. The Pearson correlation depicted in Figure 6A, showed a significant effective pairing between both platforms. The correlation coefficient (R) was .9497 (95% CI, 0.9168-0.9697; $P < .0001$). Linear regression showed the following equation: $Y = 0.9529x + 0.2013$ ($R^2 = .90$). When nAPCsr differences (CAT system − ST Genesia system) were plotted against nAPCsr values from the CAT system (considered as the reference method as the present study assesses its transferability), the mean difference between both platforms equaled −0.1 (95% CI, −1.3% to 1.1%) (Figure 6B). Comparisons between the CAT system and the ST Genesia system within subgroups (ie, men; women not using COCs; women using second-generation, third-generation, or other COCs) are shown in Figure 7. As there was only one individual carrier of a PT G20210A mutation and another one carrier of a FVL mutation, no comparison with these groups was performed. Nevertheless, results were similar between the two systems, with nAPCsr values of 2.8 on the CAT versus 3.1 on the ST Genesia system for the PT G20210A mutation, and 4.3 on the CAT versus 4.2 on the ST Genesia system for FVL mutation.

TABLE 1 Intrarun and interrun reproducibility of STG-RefPlasma TS, STG-QualiTest Norm TS, and STG-QualiTest High TS

	Intrarun reproducibility (N = 5)				Interrun reproducibility (N = 10)			
Tested plasma	Replicate	ETP inhibition %	Mean ETP inhibition %	SD%	Replicate	ETP inhibition %	Mean ETP inhibition %	SD%
STG-RefPlasma TS	1	74.2	73.5	1.0	1	80.5	74.5	3.5
					2	73.4		
	2	74.0			3	74.8		
					4	76.4		
	3	73.2			5	68.7		
					6	75.8		
	4	74.2			7	75.1		
					8	77.8		
	5	71.9			9	72.7		
					10	70.0		
STG-QualiTest Norm TS	1	36.5	38.5	2.0	1	42.3	37.7	3.5
					2	38.9		
	2	38.0			3	38.7		
					4	32.4		
	3	37.2			5	33.3		
					6	39.6		
	4	41.6			7	40.5		
					8	39.6		
	5	39.2			9	38.7		
					10	32.7		
STG-QualiTest High TS	1	11.2	8.7	1.7	1	8.1	10.1	1.4
					2	8.1		
	2	6.8			3	10.4		
					4	11.1		
	3	7.8			5	10.7		
					6	10.0		
	4	9.7			7	9.1		
					8	11.9		
	5	8.3			9	9.6		
					10	12.0		

Abbreviations: ETP, endogenous thrombin potential; SD, standard deviation.

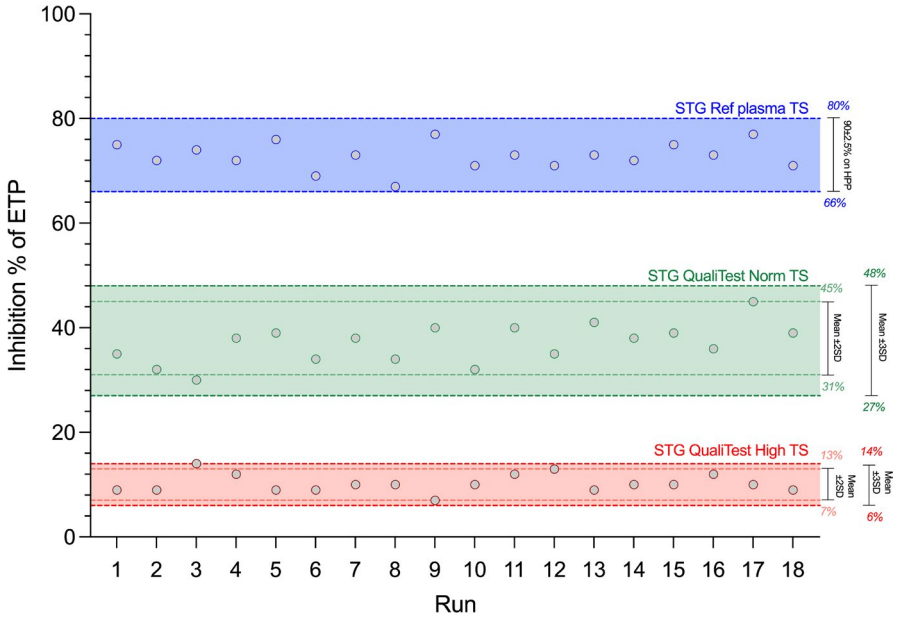

FIGURE 4 Quality controls (ie, STG QualiTest Norm TS and QualiTest High TS) and reference plasma (ie, STG-RefPlasma TS) follow-up. The acceptance range for the STG-RefPlasma TS (blue dotted lines) stood from 66% to 80% of ETP inhibition. Acceptance ranges for STG QualiTest Norm TS (green dotted lines), corresponding to the mean ±2 × SD and the mean ±3 × SD, equaled (31%-45%) and (27%-48%), respectively. Acceptance ranges for STG QualiTest High TS (red dotted lines), corresponding to the mean ±2 × SD and the mean ±3 × SD, equaled (7%-13%) and (6%-14%) respectively. ETP, endogenous thrombin potential; SD, standard deviation

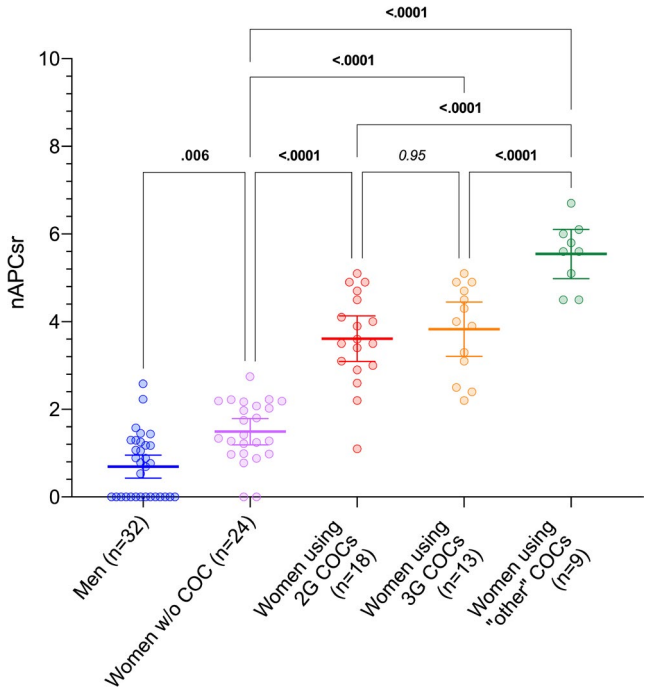

FIGURE 5 Normalized activated protein C sensitivity ratio (nAPCsr) values of individuals from each subgroup, that is, men (n = 32; in blue), women not using hormonal contraception (n = 24; in purple), women using second-generation COCs (n = 18; in red), women using third-generation COCs (n = 13; in orange), and women using the so-called other COCs (n = 9; in green). Individuals' values and means ±SDs for each subgroup are represented. Differences between subgroups were assessed by an analysis of variance with Tukey's multiple comparison tests. Threshold for significance was set at .05. COC, combined oral contraceptive; nAPCsr, normalized activated protein C sensitivity ratio; 2G, second generation; 3G, third generation

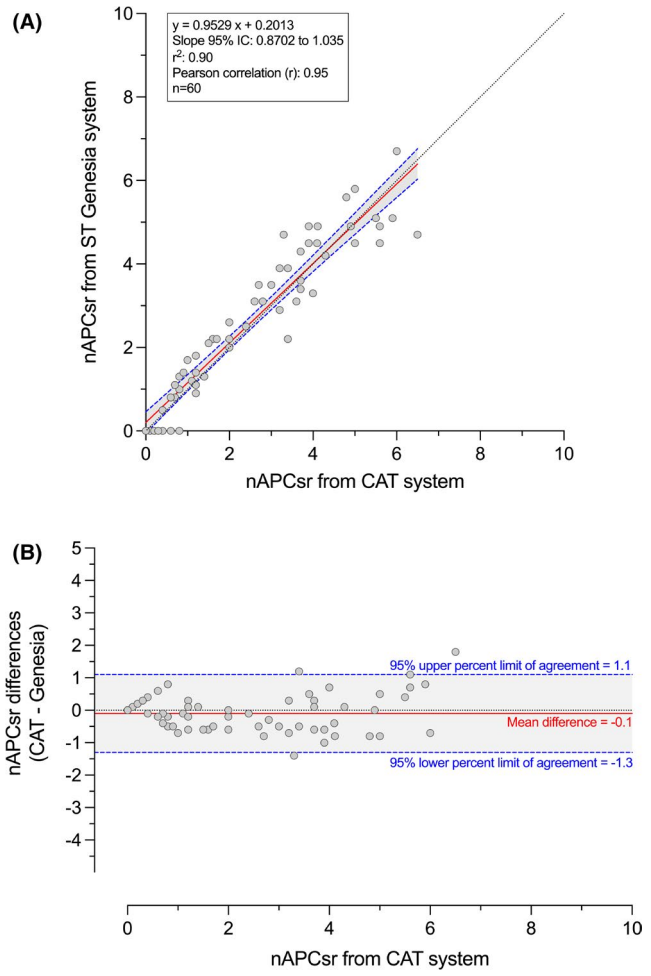

FIGURE 6 (A) Correlation between normalized activated protein C sensitivity ratio (nAPCsr) values obtained on the ST Genesia system and on the validated platform, the CAT system. Pearson correlation coefficient (*r*) [95% CI] of 0.9497 [0.9168-0.9697]; $p < 0.0001$; R^2 for linear regression = .90. Linear regression with a slope [95%] of 0.9529 [0.8702-1.035] and *y*-intercept of 0.2013. (B) Derived Bland-Altman analysis between nAPCsr values obtained on the ST Genesia system and on the CAT system. nAPCsr differences (CAT–ST Genesia) are expressed in absolute values. The mean difference (red continuous lines) ±95% CI (=1.96 × SD; blue dotted lines) equaled −0.1 ± 1.2. CAT, calibrated automated thrombogram; CI, confidence interval; nAPCsr, normalized activated protein C sensitivity ratio; SD, standard deviation

Unpaired *t* tests did not reveal any significant difference between the two systems within the five subgroups ($P > .05$). On the other hand, the two-way ANOVA with multiple comparison tests showed significant differences between subgroups except for comparison between women using second- and third-generation COCs ($P = .14$), and between women using third-generation and other COCs ($P = .23$).

4 | DISCUSSION

This is the first study evaluating the analytical performance of the ETP-based APC resistance assay on the ST Genesia system, an automated thrombin generation instrument for the routine setting.[16] To date, the CAT system is probably one of the most used thrombin generation techniques in research laboratories, but the use of microtiter plates and the manual placing of the reagent and the sample into the wells have prevented its introduction into routine practice.[18] Nevertheless, as the ETP-based APC resistance could be a potential candidate as a biomarker of thrombogenicity in women on hormonal therapy or during pregnancy,[19-21] it is crucial to facilitate its use in clinical routine, and automation is the first stage for this.

The first step, which consisted of determining the concentration of APC to be added into the TS for the APC-positive condition, was critical. Indeed, the source of the exogenous APC differed from the one used with the validated methodology on the CAT system and previously published (Enzyme Research Laboratories, South Bend, IN, USA).[11] Pretests therefore had to be performed to evaluate the inhibition capacities of the exogenous APC and to determine APC concentrations to perform dose-response curves. We decided to perform these dose-response curves with two different batches of STG-ThromboScreen to assess the interbatch variability of this reagent in the presence of exogenous APC. Results showed that the chosen APC concentration of 652 mU/mL fit for both batches

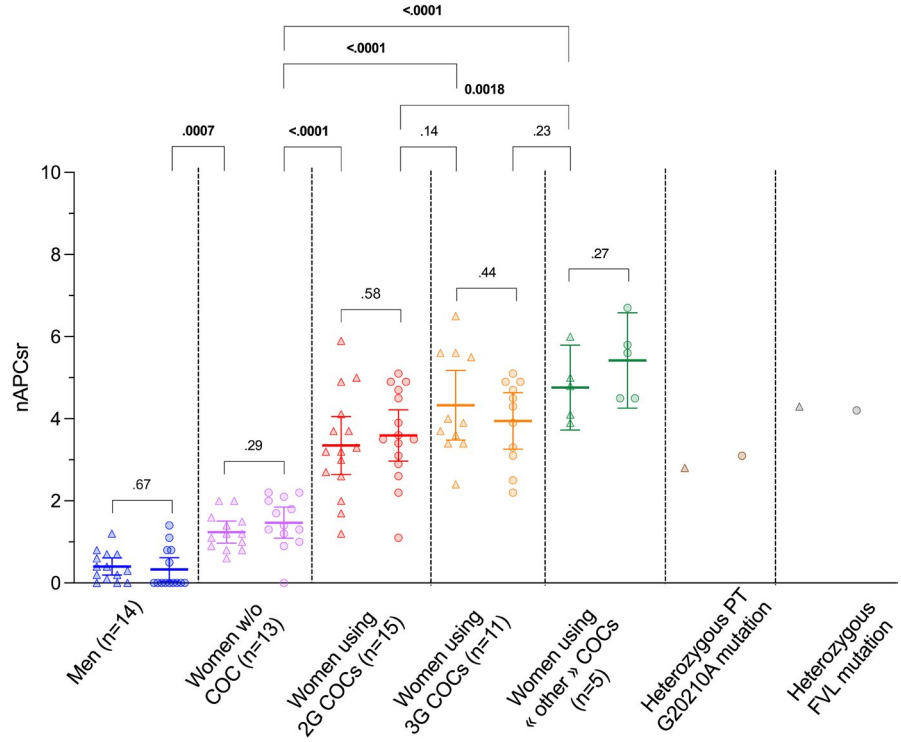

FIGURE 7 Normalized activated protein C sensitivity ratio (nAPCsr) values of individuals from each subgroup, that is, men (n = 14; in blue), women not using hormonal contraception (n = 13; in purple), women using second-generation COCs (n = 15; in red), women using third-generation COCs (n = 11; in orange), women using the so-called other COCs (n = 5; in green), the woman carrier of a heterozygous prothrombin G20210A mutation (in brown) and the woman carrier of a heterozygous FVL mutation (in gray), on both systems, that is, the CAT system (triangle pattern) and the ST Genesia system (circle pattern). Individuals' values and means ±SDs for each subgroup are represented. Differences between systems within each subgroup were assessed using an unpaired *t* test. On the other hand, differences between subgroups were assessed using a two-way analysis of variance with multiple comparison tests. Threshold for significance was set at .05. COC, combined oral contraceptive; FVL, factor V Leiden; nAPCsr, normalized activated protein C sensitivity ratio; PT, prothrombin; 2G, second generation; 3G, third generation

(Figure 2), as it led to 90.9% of ETP inhibition of the HPP with batch Nr 202983 and 91.3% with batch Nr 202984.

Regarding the method precision, the within- and between-run reproducibility showed maximal SD of 2.0% and 3.5%, respectively, well below the maximal tolerable limit of 10%. In addition, SDs were similar on the entire range of measurements demonstrating the same precision of the assay, irrespective of the level of inhibition of the sample. Compared to the application on the CAT system, these results are highly satisfactory, as maximal SDs obtained with the validated methodology were 3.0% and 7.0% for the intra- and interrun reproducibility, respectively.[11]

The daily management of the two quality control levels and the reference plasma provided acceptable results (Figure 4). Their ETP inhibition levels were within the acceptance range among the 18 experiments performed throughout the study, except run 3, for which two quality control levels stood between the mean ±2 × SD and ±3 × SD.

The correction factor corresponding to the nAPCsr of the reference plasma equaled 2.45, indicating resistance toward the APC. As already mentioned, this may be related to the manufacturing process, for example, the addition of excipients and the lyophilized form of the plasma. Indeed, it has already been reported that freeze-dried plasma has higher thrombin generation capacity than frozen plasma resulting from multiple preanalytical reasons, and this was also observed regarding the sensitivity toward added protein C.[17,22,23] Furthermore, as mentioned in previous works, the use of the STG-RefPlasma TS is useful for the normalization of the thrombin generation test parameters such as ETP, peak, lag time, or time to peak, but not for the calculation of the nAPCsr.[16,17]

The analysis of 56 samples from healthy donors had two main objectives: (i) to validate the correction factor of the commercial reference plasma used for the computation of the nAPCsr, and (ii) to provide grounds for the definition of a normal range. The mean nAPCsr (ie, 1.03) demonstrated that the correction factor for the commercial reference plasma was appropriate, as a nAPCsr value close to 1 corresponds to an ETP inhibition % close to 90%, considered as the reference value for a healthy population. The nAPCsr normal range of 0.00 to 2.20 is also very close to the range previously defined on the CAT system corresponding to 0.00 to 2.08. This similar range indicates that the test, when performed on the ST Genesia system, gives comparable results to the CAT system in a population of healthy individuals.

Concerning the sensitivity, the assay was able to discriminate healthy subjects from women treated with COCs (ie,

second-generation, third-generation, and the so-called other COCs). Of the healthy donors, we expected to observe a significant difference between men and women. Furthermore, women using COCs (ie, second-generation, third-generation, or other groups) were significantly more resistant to APC, compared to both men and women not using hormonal contraception. On the other hand, due to the low recruitment in the three COC subgroups, no significant difference was shown between second- and third-generation COCs (Figure 5).

The final step of this transferability was the comparison between the two systems, that is, the CAT system versus the ST Genesia system. Based on the linear regression and the derived Bland-Altman analysis, the ETP-based APC resistance assay, when performed on the automated platform, tended to give slightly higher nAPCsr values compared to the CAT system. Certainly, the ST Genesia system derived from the CAT principle but is not strictly equivalent.[24,25] The same fluorogenic substrate for thrombin is used on both systems, but the calibration used to obtain thrombin concentration from the fluorescent signal is different: On the ST Genesia, thrombin generation is computed in comparison to a daily calibration curve obtained with a fixed amount of human purified thrombin (STG-ThrombiCal) in buffer solution in the presence of the substrate Z-Gly-Gly-Arg-AMC and calcium (STG-FluoStart).[25] The thrombin-mediated substrate cleavage leads to a fluorescent increase (7-amino-4-methyl coumarin [AMC]), which is measured every 15 seconds at 377 nm (excitation)–450 nm (emission) wavelengths, which differs slightly from the CAT system set to 390 nm (excitation)–60 nm (emission) wavelengths.[25] Once the calibration passed, plasma samples are run individually, in duplicate. A known amount of AMC (STG-FluoSet) is added to each individual plasma sample for adjusting the calibration curve by correcting the plasma color and rectifying the inner filter effect. The thrombin generation parameters are automatically calculated on both systems, but algorithms are instrument specific, which can therefore lead to differences in the results.[24,26]

5 | LIMITATIONS AND PERSPECTIVES

This pilot study presented some limitations, among which is the absence of ready-to-use reagents. Indeed, the reagent TS +APC required the spiking of the APC into the TS reagent, which played a role in the interrun variability. Moreover, it requires to redefine the APC concentration leading to 90% of ETP inhibition of HPP at each batch change of APC and/or STG-ThromboScreen kit. Indeed, the correction factor for the STG-RefPlasma TS will have to be computed again as well as acceptance ranges for quality controls. As a perspective, it is therefore of importance to consider the manufacturing of a kit similar to the STG ThromboScreen, thus containing a ready-to-use triggering reagent with APC, a reference plasma along with a correction factor for the nAPCsr and the appropriate quality control levels. A second limitation was the low recruitment rate among women using hormonal therapies. Indeed, because of the small sample size, women were stratified by COC generation, whereas it is well documented that the risk of VTE differs within a COC generation depending on the dose of EE and the nature of the progestogen.[27] One perspective is obviously to actively collaborate with hospitals to describe APC resistance of each COC according to its composition and no longer based on the so-called generation classification. It would also be relevant to consider the extent to which thresholds could be established in a given population (eg, women using EE 20 μg with levonorgestrel 100 μg) above which the thrombotic state of the patient would be considered as abnormal.

6 | CONCLUSION

This study is the first reporting the analytical performance of the validated ETP-based APC resistance assay on the automated thrombin generation instrument, the ST Genesia system. Data revealed excellent intra- and interrun reproducibility, appropriate sensitivity toward hormonal therapies, and comparable results to those obtained with the validated methodology on the CAT system. This supports the concept that the nAPCsr, when incorporated into clinical routine, could become a promising regulatory and clinical tool to document on the thrombogenicity of female hormonal therapies and other coagulopathies interfering with the protein C system. In views of its screening potential, a complete validation on the ST Genesia system along with the manufacturing of ready-to-use triggering reagent with APC are essential and deserves further investigations.

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AUTHOR CONTRIBUTIONS

LM, AC, and JD designed the study. MD and LM performed the analyses. LM and JD analyzed and interpreted the data. LM performed statistical analyses. MD, AC, UG, and JD provided input and critical review of the manuscript. All authors revised the manuscript.

RELATIONSHIP DISCLOSURE

JD is CEO and founder of QUALIblood and reports personal fees from Daiichi-Sankyo, Diagnostica Stago, DOASense, Gedeon Richter, Mithra Pharmaceuticals, Norgine, Portola, Roche, and Roche Diagnostics, outside the submitted work. AC is a full-time employee of Diagnostica Stago. The other authors have no conflicts of interest to disclose.

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REFERENCES

1. Jordan WM, Anand JK. Pulmonary embolism. *Lancet*. 1961;278(7212):1146-1147.
2. Christin-Maitre S. History of oral contraceptive drugs and their use worldwide. *Best Pract Res Clin Endocrinol Metab*. 2013;27(1):3-12.
3. Henkens CM, Bom VJ, Seinen AJ, van der Meer J. Sensitivity to activated protein C; influence of oral contraceptives and sex. *Thromb Haemost*. 1995;73(3):402-404.
4. Olivieri O, Friso S, Manzato F, et al. Resistance to activated protein C in healthy women taking oral contraceptives. *Br J Haematol*. 1995;91(2):465-470.
5. Østerud B, Robertsen R, Åsvang GB, Thijssen F. Resistance to activated protein C is reduced in women using oral contraceptives. *Blood Coagul Fibrinolysis*. 1994;5(5):853-854.
6. Rosing J, Tans G, Nicolaes GA, et al. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. *Br J Haematol*. 1997;97(1):233-238.
7. European Medicines Agency. Guideline on clinical investigation of steroid contraceptives in women - EMEA/CPMP/EWP/519/98 Rev 1 2005; Available from: https://www.ema.europa.eu/documents/scientific-guideline/guideline-clinical-investigation-steroid-contraceptives-women_en.pdf. Accessed June 23, 2021.
8. Kadauke S, Khor B, Van Cott EM. Activated protein C resistance testing for factor V Leiden. *Am J Hematol*. 2014;89(12):1147-1150.
9. Curvers J, Thomassen MC, Nicolaes GA, et al. Acquired APC resistance and oral contraceptives: differences between two functional tests. *Br J Haematol*. 1999;105(1):88-94.
10. de Visser MC, van Hylckama VA, Tans G, et al. Determinants of the APTT- and ETP-based APC sensitivity tests. *J Thromb Haemost*. 2005;3(7):1488-1494.
11. Douxfils J, Morimont L, Delvigne AS, et al. Validation and standardization of the ETP-based activated protein C resistance test for the clinical investigation of steroid contraceptives in women: an unmet clinical and regulatory need. *Clin Chem Lab Med*. 2020;58(2):294-305.
12. Nicolaes GA, Thomassen MC, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. *Blood Coagul Fibrinolysis*. 1997;8(1):28-38.
13. Perrin J, Depasse F, Lecompte T, et al. Large external quality assessment survey on thrombin generation with CAT: further evidence for the usefulness of normalisation with an external reference plasma. *Thromb Res*. 2015;136(1):125-130.
14. Dargaud Y, Wolberg AS, Luddington R, et al. Evaluation of a standardized protocol for thrombin generation measurement using the calibrated automated thrombogram: an international multicentre study. *Thromb Res*. 2012;130(6):929-934.
15. Morimont L, Didembourg M, Haguet H, et al. Interlaboratory variability of activated protein C resistance using the ETP-based APC resistance assay. *Res Pract Thromb Haemost*. 2021;5(7):e12612.
16. Douxfils J, Morimont L, Bouvy C, et al. Assessment of the analytical performances and sample stability on ST Genesia system using the STG-DrugScreen application. *J Thromb Haemost*. 2019;17(8):1273-1287.
17. Morimont L, Bouvy C, Delvigne AS, Dogne JM, Douxfils J. Proof of concept of a new scale for the harmonization and the standardization of the ETP-based APC resistance. *J Thromb Haemost*. 2020;18(4):895-904.
18. Tripodi A. Thrombin generation assay and its application in the clinical laboratory. *Clin Chem*. 2016;62(5):699-707.
19. Brenner B. Haemostatic changes in pregnancy. *Thromb Res*. 2004;114(5–6):409-414.
20. Cumming AM, Tait RC, Fildes S, Yoong A, Keeney S, Hay CRM. Development of resistance to activated protein C during pregnancy. *Br J Haematol*. 1995;90:725-727.
21. Tchaikovski SN, Thomassen MC, Costa SD, Peeters LL, Rosing J. Role of protein S and tissue factor pathway inhibitor in the development of activated protein C resistance early in pregnancy in women with a history of preeclampsia. *Thromb Haemost*. 2011;106(5):914-921.
22. Dargaud Y, Luddington R, Gray E, et al. Standardisation of thrombin generation test - which reference plasma for TGT? An international multicentre study. *Thromb Res*. 2010;125(4):353-356.
23. Rodgers SE, Wong A, Gopal RD, Dale BJ, Duncan EM, McRae SJ. Evaluation of pre-analytical variables in a commercial thrombin generation assay. *Thromb Res*. 2014;134(1):160-164.
24. Talon L, Sinegre T, Lecompte T, et al. Hypercoagulability (thrombin generation) in patients with cirrhosis is detected with ST-Genesia. *J Thromb Haemost*. 2020;18(9):2177-2190.
25. Giesen PLA, Gulpen AJW, van Oerle R, Ten Cate H, Nagy M, Spronk HMH. Calibrated automated thrombogram II: removing barriers for thrombin generation measurements. *Thromb J*. 2021;19(1):60.
26. Roullet S, Labrouche S, Freyburger G. Comparison of two thrombin generation methods, CAT and ST-Genesia, in liver transplant patients. *Thromb Haemost*. 2019;119(6):899-905.
27. Morimont L, Dogné J-M, Douxfils J. Letter to the Editors-in-Chief in response to the article of Abou-Ismail, et al. entitled "Estrogen and thrombosis: a bench to bedside review" (Thrombosis Research 192 (2020) 40–51). *Thrombo Res*. 2020;193:221-223.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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