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Morimont, Laure; Didembourg, Marie; Haguët, Hélène; Modaffari, Élise; Tillier, Maxence; Bouvy, Céline; Lebreton, Aurélien; Dogné, Jean-Michel; Douxfils, Jonathan

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ORIGINAL ARTICLE

Interlaboratory variability of activated protein C resistance using the ETP-based APC resistance assay

Laure Morimont PharmD^{1,2}  | Marie Didembourg MBS² | H el ene Haguet MBS^{1,2} |  lise Modaffari MLT¹ | Maxence Tillier MLT³ | C eline Bouvy MSc, PhD¹ | Aur ilien Lebreton MD, PhD³  | Jean-Michel Dogn e PharmD, PhD² | Jonathan Douxfils PharmD, PhD^{1,2} 

¹Qualiblood sa, Namur, Belgium

²Faculty of Medicine, Department of Pharmacy, Namur Research Institute for Life Sciences (NARILIS), Namur Thrombosis and Hemostasis Center (NTHC), University of Namur, Namur, Belgium

³Service d'h ematologie Biologique, CHU Clermont-Ferrand, Clermont-Ferrand, France

Correspondence

Laure Morimont, Qualiblood sa, Namur, Belgium and Faculty of Medicine, Department of Pharmacy, Namur Research Institute for Life Sciences (NARILIS), Namur Thrombosis and Hemostasis Center (NTHC), University of Namur, Rue du S eminaire 20a, 5000 Namur, Belgium.
Email: laure.morimont@qualiblood.eu

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Abstract

Background and objective: Although the endogenous thrombin potential (ETP)-based activated protein C (APC) resistance is recommended for the development of steroid contraceptive agents, one of the main limitations of this technique was its lack of standardization, which hampered study-to-study comparison. A validated methodology that meets all the regulatory requirements in terms of analytical performances has been developed recently. To ensure a wide implementation of this test, the assessment of the interlaboratory variability was needed.

Method: The assay was implemented in three testing laboratories. First, dose-response curves were performed to locally define APC concentration leading to 90% of ETP inhibition on healthy donors. Intra- and inter-run repeatability were assessed on a reference plasma and three quality controls. To investigate the variability in results among the different testing units, 60 donor samples were analyzed at each site.

Results: The APC concentration leading to 90% of ETP inhibition was defined at 1.21 $\mu\text{g/ml}$ and 1.14 $\mu\text{g/ml}$ in the two receiving units. Intra- and inter-run repeatability showed standard deviation below 3%. Analyses of the 60 donor samples showed no statistically significant difference. The sensitivity of the test in the different laboratories was maintained and subgroup analyses still reported significant differences depending on hormonal status of donors.

Conclusion: This study is the first reporting the interlaboratory variability of the ETP-based APC resistance assay. Data revealed excellent intra- and interlaboratory reproducibility. These results support the concept that this blood coagulation test provides an appropriate sensitivity irrespective of the laboratory in which analyses are performed.

KEYWORDS

activated protein C resistance, blood coagulation test, contraceptive agents, laboratories, reproducibility of results

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Essentials

- The ETP-based APC resistance assay is recommended for the development of contraceptive agents.
- The transferability of this validated methodology was assessed in three testing laboratories.
- Data revealed excellent intra-laboratory precision and high inter-laboratory reproducibility.
- In view of its screening potential, the next step is to implement the test in clinical routine.

1 | BACKGROUND

Evidence suggesting that combined oral contraceptives (COCs) were associated with an increased risk of venous thromboembolism rapidly appeared after their marketing in the 1960s.¹ Subsequently, numerous studies assessing the impact of the different estrogenic combinations on hemostasis demonstrated that a poor response to the activated protein C (APC) could explain, at least in part, the pro-coagulant state observed in users of oral contraceptive agents.²⁻⁵ Several methods were therefore developed to evaluate this acquired APC resistance, among which the endogenous thrombin potential (ETP)-based APC resistance assay. This test is based on the continuous measurement of thrombin generation in the presence and absence of exogenous APC.⁶ As a global coagulation assay, it takes into account the initiation, the propagation, and the termination phase of the coagulation providing additional information, notably on the G20210A mutation, antithrombin, and protein S deficiencies or FVIII levels.⁷⁻⁹ In 2004, this blood coagulation test was recommended by the European Medicines Agency for the evaluation of APC resistance during the development of steroid contraceptive agents in women.¹⁰ However, a major limitation of this technique was its lack of standardization, which hampered study-to-study comparison.^{11,12} Hence, our group recently proposed a standardized methodology that met all the regulatory requirements in terms of analytical performances. The ETP-based APC resistance assay was validated on the Calibrated Automated Thrombogram device using commercially available reagents to ensure batch-to-batch traceability, recovery, and reproducibility of the method over time.¹³ The transfer of this methodology to different laboratories is a next step for the wide implementation of this promising test in the routine laboratory setting.

Therefore, the aim of this study was to demonstrate the robustness of the method through an evaluation of the interlaboratory transferability, ensuring that any laboratory implementing the method obtains similar results in its environment compared with those obtained by the originating laboratory. To this end, the ETP-based APC resistance assay was implemented in two different laboratories and results were compared with those obtained by the reference laboratory.

2 | MATERIALS AND METHODS

2.1 | ETP-based APC resistance assay

The ETP-based APC resistance assay was performed as previously described.¹³ Briefly, the activator reagent (STG ThromboScreen) (TS) contains a mixture of phospholipids (\pm 4 μ mol/L) and tissue

factor (\pm 5 pmol/L) to initiate coagulation through the extrinsic pathway in the tested plasma. The addition of exogenous APC (Enzyme Research Laboratories, Swansea, UK) to this reagent enhances the APC-protein S anticoagulant complex in the tested plasma. The resulting effect is a reduction of the ETP (corresponding to the area under the curve) in comparison with the non-APC condition. The quantity of APC to introduce in the test is defined per APC/TS batch and targets a decrease of 90% of the ETP of a reference plasma.

The ETP values measured with TS (-APC) and TS (+APC) are then used to compute the percentage of inhibition of the ETP (inhibition %) (Equation 1), and the normalized APC sensitivity ratio (nAPCsr) (Equation 2). The obtained ratio stands between zero and 10 and the higher the nAPCsr, the more resistant the donor is to APC.

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

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

2.1.1 | Reference plasma

The use of a reference plasma is dedicated to the normalization of thrombin generation test (TGT) parameters. It also determines the quantity of APC to introduce in the test, to obtain 90% of ETP inhibition. In our study, we used two different reference plasmas: an in-house healthy pooled plasma (HPP) and a commercially available reference plasma dedicated to a thrombin generation application (Diagnostica Stago). The latter ensures batch-to-batch recovery because the HPP cannot be produced at large scale. Indeed, previous publications demonstrated that the use of local plasma (i.e., a reference plasma made at the laboratory facilities) was generally reported as unable to improve the interlaboratory variability. The choice of a commercially available and certified plasma is therefore more appropriate.^{11,13-15} On the other hand, mostly because of their large-scale production requiring specific manufacturing processes, the production conditions of these reference plasmas are probably far from the best recommendations of blood sample collection for thrombin generation testing. Therefore, one solution is to compare the response of the commercial reference plasma with a smaller pool of plasma from healthy donors collected in ideal conditions (i.e., our in-house HPP) and then to apply a correction factor to the commercial plasma to compensate for these differences. This approach has already been proven to be efficient in this and other settings.^{16,17}

2.1.2 | Quality controls

Three levels of quality controls (QC) were used to validate each experiment. These are lyophilized citrated human plasma designed for TGT (Diagnostica Stago) and are described as hypocoagulable (QC low), intermediate (QC intermediate), or hypercoagulable (QC high). Acceptability ranges of these controls are based on 10 consecutive runs and are defined as the mean \pm 2 standard deviations (SD). These acceptability ranges were defined for each batch by the originating unit. However, as the residual ETP of QC low in presence of APC is usually zero, this results in 100% inhibition; thus, no range for this QC level can be determined. If this QC level is slightly below 100% inhibition, but the other QC levels and the reference plasma are within their acceptability ranges, the run is validated.

2.2 | Method transferability

The transferability of the methodology was investigated at the department of pharmacy of the University of Namur (Namur, Belgium) (named receiving unit 1) and at the hematology laboratory of CHU Estaing (Clermont-Ferrand, France) (named receiving unit 2). The originating unit, i.e., QUALblood sa (Namur, Belgium) was responsible for providing the analytical procedures, reagents, and samples to the receiving laboratories. One operator at each site, already familiar with TGT, has been trained remotely before the transfer.

The transferability assessment was performed in three steps (Figure 1): (1) the determination of the APC concentration at each site, (2) the assessment of the method precision, (3) the assessment of the interlaboratory variability.

2.2.1 | Step 1: determination of 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 with 6 solutions of TS containing increasing concentrations of APC: 0.5 $\mu\text{g/ml}$; 0.75 $\mu\text{g/ml}$; 1.25 $\mu\text{g/ml}$; 2.0 $\mu\text{g/ml}$; 2.75 $\mu\text{g/ml}$; and 3.5 $\mu\text{g/ml}$. The chosen APC concentration was the one leading to a target inhibition % of the commercial reference plasma defined by the originating unit. For the receiving unit 1, the targeted inhibition of the reference plasma (batch 202983) was 57.8% and for the receiving unit 2, the targeted inhibition % of the reference plasma (batch 202984) was 75.0%. To ensure this concentration was properly determined, an additional verification step has been set up and dose-response curves were performed in parallel on the HPP. The inhibition % of the HPP should stand between 87.5% and 92.5%.

2.2.2 | Step 2: assessment of precision

After defining the right concentration of APC to introduce in the test, the receiving units had to be able to replicate with an acceptable

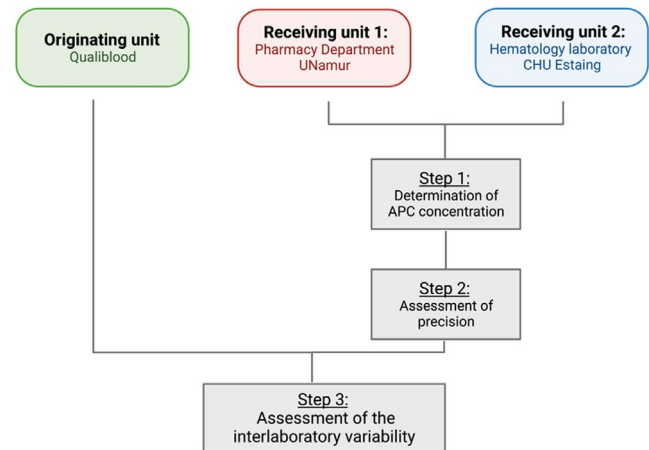


FIGURE 1 Study scheme for the transferability of the endogenous thrombin potential (ETP)-based activated protein C (APC) resistance assay

level of performance the methodology. To this end, the precision (i.e., the intra- and inter-run repeatability) was assessed. The intra-run repeatability was assessed by analyzing the three QC levels and the reference plasma on the same plate, five times in duplicates. For the inter-run repeatability, those plasmas were analyzed in duplicate over three independent runs. Results were expressed as inhibition % and the acceptance criteria were the same as for the method validation (i.e., SD values lower than 10%).

2.2.3 | Step 3: assessment of the interlaboratory variability

This step consisted of investigating the variability in results of donor samples among the originating unit and both receiving units. Sixty individual plasma samples were analyzed at each site. A comparison between four subgroups according to sex and the use of hormonal contraception in women was performed as these parameters are known to impact the resistance toward the APC. Results were expressed as nAPCsr values.

2.3 | Sample description

2.3.1 | Healthy pooled plasma

This pool was used to confirm that the chosen APC concentration, in both receiving units, led to $90\% \pm 2.5\%$ inhibition. This in-house HPP was constituted of 20 healthy individuals (10 men and 10 women not taking contraceptive agents). Exclusion criteria were history of thrombotic and/or hemorrhagic events, treatment by antiplatelets or anticoagulants medication or other drugs potentially affecting platelets or coagulation, pregnancy, use of hormonal therapy (i.e., contraceptive or hormone replacement therapy), and carrier of factor V Leiden or prothrombin G20210A mutations.

The absence of factor V Leiden and prothrombin G20210A mutations was confirmed by a CE-IVD-approved technique (Lamp Human FII & FVL Duplex kit, reference LC-FII & FVL-LP-24, LaCAR). The mean age of the participants was 25 years (range, 18–56 years) and the mean body mass index (BMI) was 22 kg m⁻² (range, 19–26 kg m⁻²).

2.3.2 | Individual plasma

To assess the interlaboratory variability, 60 healthy donors were recruited and stratified in four groups of 15 individuals, namely men (mean age, 21 years; mean BMI, 22 kg m⁻²), women not using hormonal therapy (mean age, 21 years; mean BMI, 22 kg m⁻²), women using second-generation COCs (i.e., containing levonorgestrel as progestin) (mean age, 22 years; mean BMI, 23 kg m⁻²), and women using third-generation COCs (i.e., containing desogestrel or gestodene as progestin) (mean age, 23 years; mean BMI, 22 kg m⁻²). The demographic characteristics (age and BMI) of the four groups were well matched. Exclusion criteria were the same as the previously mentioned except for the use of hormonal therapy.

2.3.3 | Blood sample collection and plasma preparation

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. All the volunteers were recruited at the University of Namur, Namur, Belgium. Written informed consent was obtained from each donor. All samples were stored and managed by the Namur Biobank-eXchange, the registered biobank from the University of Namur.

Blood was taken by venipuncture in the antecubital vein and collected into 0.109 M sodium citrate tubes (9:1 v/v) (Vacuette Greiner) without corn trypsin inhibitor using a 21-gauge needle (BD Vacutainer Eclipse). The first tube was always discarded. The platelet-poor plasma was obtained from the supernatant fraction of blood tubes after double centrifugation for 15 min at 2500g at room temperature. The first centrifugation was performed within 30 min after blood sampling. Immediately after centrifugation, platelet-poor plasma were pooled for the constitution of the HPP or aliquoted as individual plasmas. 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 maximum 10 min and mixed gently just before the experiment. All tests were performed within 4 h after thawing.

2.4 | Statistical analysis

Statistical analysis was performed using GraphPad version 9.0 (GraphPad Prism Software, Inc.). Descriptive statistics were used to

analyze the data. The APC concentrations were determined using nonlinear regressions (one-phase association – least square fit) with no constrain on the Y-axis. Because the SD is stable along the whole range of measurement, the intra- and inter-run repeatability were expressed in terms of SD instead of coefficient of variation. A pairwise multiple comparison Friedman test was performed to compare nAPCsr values from each donor between units. Linear regressions and Spearman correlations were used to assess the correlation between the originating unit versus receiving units. Derived Bland-Altman analysis was performed by plotting differences (nAPCsr unit – mean nAPCsr) against mean nAPCsr. Differences were expressed in absolute values as well as percentages of mean results. Within each subgroup, ordinary one-way analysis of variance (ANOVA) was performed to compare nAPCsr values between units. Finally, grouped analysis, including all nAPCsr values obtained at the three units, was performed to compare subgroups with each other.

3 | RESULTS

3.1 | Determination of APC concentration

Dose-response curves of APC performed at both receiving units are shown in Figure 2. Coefficients of determination R^2 of individual and mean nonlinear regressions for HPP and for reference plasma were ≥0.98 in both receiving units. The concentration of APC leading to 57.8% inhibition (receiving unit 1) and 75.0% inhibition (receiving

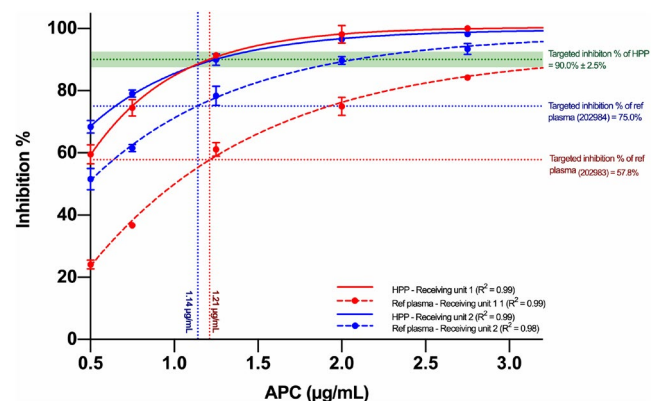


FIGURE 2 Inhibition percentage of the endogenous thrombin potential (ETP) at various concentrations of activated protein C (APC) with the healthy pooled plasma (HPP) and the commercial reference plasma ($N = 3$). The continuous lines represent the nonlinear regressions obtained with HPP. The dotted lines represent the nonlinear regressions obtained with the commercial reference plasma. The red lines refer to the receiving unit 1 and the blue lines refers to the receiving unit 2. The targeted inhibition % for the reference plasma issued from batch 202983 (used at receiving unit 1) equaled 57.8% (horizontal red dotted line) and the targeted inhibition % for the ref plasma issued from batch 202984 (used at receiving unit 2) equaled 75% (horizontal blue dotted line). The targeted inhibition % of HPP equaled 90% (horizontal green dotted line) \pm 2.5% (light green area)

unit 2) of the reference plasma was 1.21 µg/ml and 1.14 µg/ml, respectively. These concentrations led to an inhibition % of the HPP within the established ranges (87.5%–92.5%) (i.e., 90.1% inhibition at receiving unit 1 and 88.6% inhibition at receiving unit 2).

3.2 | Assessment of method precision

Intra-run and inter-run repeatability at both receiving units are shown in Tables 1 and 2, respectively. In both units, SD values for each tested plasma (i.e., the three QC levels and the reference plasma) were within acceptance criteria ($SD < 10\%$). In addition, values were within QC ranges defined by the originating unit except for QC low, at the receiving unit 2, which was below 100% inhibition for two runs.

3.3 | Interlaboratory variability assessment

Sixty samples were tested at each unit and compared to each other. However, one sample could not be analyzed at the receiving unit 2 because of insufficient volume. A nonparametric Friedman test was performed and revealed no statistically significant differences

between units ($p > 0.05$). Spearman correlations depicted in Figure 3, showed a significant effective pairing between nAPCsr values from each receiving unit and nAPCsr values from the originating unit. Correlation coefficients (rs) were 0.99 (95% CI, 0.9812–0.9935; $p < 0.0001$) and 0.99 (95% CI, 0.9851–0.9949; $p < 0.0001$) for receiving unit 1 and receiving unit 2, respectively. Linear regressions showed the following equations $Y = 1.154x - 0.2408$ ($R^2 = 0.98$) for receiving unit 1 and $Y = 0.9213x + 0.08939$ ($R^2 = 0.98$) for receiving unit 2. Differences (nAPCsr values from each laboratory – mean nAPCsr from the three units), either expressed in absolute values (Figure 4A) or as percentages of the mean nAPCsr (Figure 4B), were plotted against the mean nAPCsr. The average of the differences $\pm 95\%$ CI for the originating unit equaled 0.00 ± 0.22 in absolute values and $1.3\% \pm 11.1\%$ in percentages of the mean. The average of the differences $\pm 95\%$ CI for the receiving unit 1 equaled 0.09 ± 0.48 in absolute values and $-0.5\% \pm 20.5\%$ in percentages of the mean. The average of the differences $\pm 95\%$ CI for the receiving unit 2 equaled -0.09 ± 0.38 in absolute values and $-0.8\% \pm 16.8\%$ in percentages of the mean. The 95th percent limits of agreement of differences, when gathering the three laboratories, equaled 0.40 in absolute values and 16.5% in percentages of the mean.

Comparisons between each unit within the four subgroups (i.e., women not using COC, men, women using second-generation

TABLE 1 Intra-run repeatability of the commercial reference plasma and the three quality controls (i.e., QC low, QC intermediate, and QC high) for each receiving unit

Tested Plasmas	Duplicate	Receiving unit 1				Receiving unit 2			
		Inhibition %	Mean Inhibition %	SD	QC Ranges	Inhibition %	Mean Inhibition %	SD	QC Ranges
Reference plasma	1	79.1%	78.8%	0.4%	70%–82%	76.5%	75.3%	1.2%	67%–80%
	2	78.6%				73.4%			
	3	78.2%				75.2%			
	4	79.1%				75.0%			
	5	79.1%				76.2%			
QC low	1	100.0%	100.0%	0.0%	100%–100%	100.0%	100.0%	0.0%	100%–100%
	2	100.0%				100.0%			
	3	100.0%				100.0%			
	4	100.0%				100.0%			
	5	100.0%				100.0%			
QC intermediate	1	39.8%	40.2%	0.7%	33%–44%	39.1%	37.7%	0.9%	24%–53%
	2	40.4%				37.6%			
	3	41.1%				38.2%			
	4	39.3%				36.8%			
	5	40.3%				37.0%			
QC high	1	2.9%	2.8%	0.7%	0%–17%	2.9%	4.0%	1.5%	2%–13%
	2	2.2%				4.4%			
	3	2.4%				2.1%			
	4	4.0%				4.3%			
	5	2.6%				6.1%			

Abbreviations: QC, quality control; SD, standard deviation.

TABLE 2 Inter-run repeatability of the reference plasma and the three quality controls (i.e., QC low, QC intermediate, and QC high for each receiving unit)

Tested Plasmas	Duplicate	Receiving unit 1				Receiving unit 2			
		Inhibition %	Mean Inhibition %	SD	QC Ranges	Inhibition %	Mean Inhibition %	SD	QC Ranges
Reference plasma	1	78.8%	80.9%	1.9%	70%–82%	75.2%	78.5%	1.2%	67%–80%
	2	81.5%				79.3%			
	3	82.4%				77.6%			
QC low	1	100.0%	100.0%	0.0%	100%–100%	100.0%	97.9%	1.9%	100%–100%
	2	100.0%				97.1%			
	3	100.0%				96.5%			
QC inter-mediate	1	40.2%	42.8%	2.6%	33%–44%	37.7%	39.6%	2.3%	24%–53%
	2	42.9%				39.0%			
	3	45.4%				42.2%			
QC high	1	2.8%	5.0%	2.3%	0%–17%	4.0%	6.0%	2.1%	2%–13%
	2	7.4%				8.1%			
	3	4.9%				6.0%			

Abbreviations: QC, quality control; SD, standard deviation.

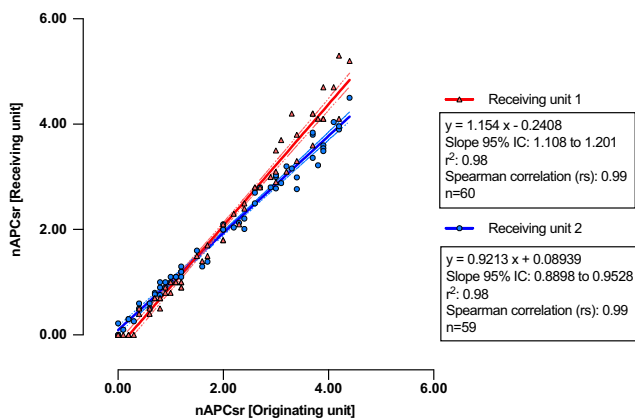


FIGURE 3 Correlation between normalized APC sensitivity ratio (nAPCsr) obtained at the receiving units and nAPCsr obtained at the originating unit. For receiving unit 1: Spearman correlation coefficient (rs) (95% CI) of 0.9890 (0.9812–0.9935); $p < 0.0001$; r^2 for linear regression = 0.9770. Linear regression (red line) with a slope (95% CI) of 1.154 (1.108–1.201) (red dotted lines) and Y-intercept of -0.2408 . For receiving unit 2: Spearman correlation coefficient (rs) (95% CI) of 0.9913 (0.9851–0.9949); $p < 0.0001$; r^2 for linear regression = 0.9837. Linear regression (blue line) with a slope (95% CI) of 0.9213 (0.8898–0.9528) (blue dotted lines) and Y-intercept of 0.08939

COCs, and women using third-generation COCs) are shown in Table 3 and Figure 5. Holm-Sidak multiple comparison tests revealed no significant difference between the three laboratories within the four subgroups ($p > 0.05$). On the other hand, the Tukey multiple comparisons test showed significant differences between each subgroup (p value < 0.0001 except for comparison between women using second- versus third-generation COC, in which p value equaled 0.0008).

4 | DISCUSSION

The aim of this study was to implement the ETP-based APC resistance assay in different laboratories and to assess the interlaboratory variability. Data showed that this test, when performed at different facilities, provides reproducible and sensitive results (e.g., depending on hormonal status of women), building an additional step for its implementation in clinical routine.

The critical step, in this study, was the determination of APC concentration. The amount of exogenous APC added into the TS reagent plays a major role in the sensitivity of the test and must be defined at each batch change of APC and/or TS reagent. Indeed, the activity of APC differs from one production to another, as well as TS reagent and the associated reference plasma and quality controls. For instance, the commercial reference plasma (batch 202983) was more APC-resistant compared with the reference plasma (batch 202984) and the targeted inhibition % of the reference plasma equivalent to 90% inhibition of HPP were 57.8% and 75.0%, respectively. In addition, as the reagent TS +APC is not yet ready to use and still requires the spiking of APC into the TS reagent, an inter-operator variability is nonnegligible, especially because the operators of both external units undergone a distance training, based on the documentation provided by the reference laboratory. The different APC concentrations (i.e., 1.21 $\mu\text{g/ml}$ at receiving unit 1 and 1.14 $\mu\text{g/ml}$ at receiving unit 2) did not seem to impact the final results. Indeed, inhibition % of the QC levels and the reference plasma, among the receiving units, were within the acceptability ranges defined by the originating unit except for QC low at the receiving unit 2, which was slightly below 100% inhibition compared with the reference unit. Because there is no proper range for this control based on our calculation method for definition of the range, we accepted these QCs as all other levels of controls were adequate.

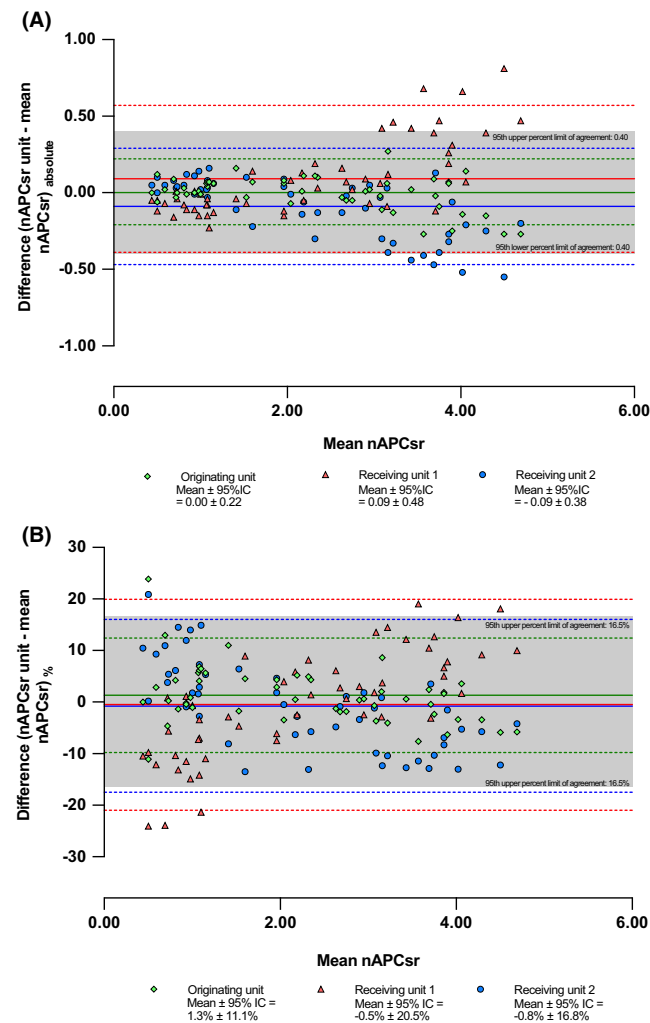


FIGURE 4 Derived Bland-Altman analysis between normalized APC sensitivity ratio (nAPCsr) values from each unit and the mean nAPCsr of the three units for the measurement of APC resistance in plasma samples. Differences (nAPCsr unit - mean nAPCsr) are expressed in (A) absolute values or (B) as a percentage of the mean. Results expressed in percent are computed as following: $\frac{\text{nAPCsr unit} - \text{mean nAPCsr}}{\text{mean nAPCsr}} \times 100$. The originating unit appears in green, the receiving unit 1 in red and the receiving unit 2 in blue. The average of the differences (continuous line) \pm 95% CIs (dotted lines) for the originating unit is 0.00 ± 0.22 in absolute values and $1.3\% \pm 11.1\%$ in percentage of the mean. The average of the differences \pm 95% CI for the receiving unit 1 is 0.09 ± 0.48 in absolute values and $-0.5\% \pm 20.5\%$ in percentage of the mean. The average of the differences \pm 95% CI for the receiving unit 2 is -0.09 ± 0.38 in absolute values and $-0.8\% \pm 16.8\%$ in percentage of the mean. The 95th percent limits of agreement (light gray area) of differences, including the three laboratories equals to 0.40 in absolute values and 16.5% in percentage of the mean

Regarding the precision, the within- and between-run variability showed maximal standard deviations of 1.5% and 2.6%, respectively, well below the maximal tolerable limit of 10%. These results are in line with those obtained at the reference laboratory (during the validation of the methodology), in which a same degree of precision of the assay was obtained, irrespective of the inhibition level.¹³

Finally, to evaluate the interlaboratory variability, results of donor's samples were compared in nAPCsr values rather than inhibition %. As previously mentioned, the nAPCsr is normalized to the reference plasma, the latter being measured at each run, which allows to reduce the interlaboratory variability.¹³ As expected, the Spearman correlations (*r*s) between the nAPCsr values obtained at the receiving units versus at the originating unit showed excellent coefficients of 0.98. Based on linear regressions, the receiving unit 1 tended to provide higher nAPCsr values compared with the originating unit with a slope of 1.15, denoting a 15% upward systematic deviation. On the other hand, the receiving unit 2 tended to give lower nAPCsr values compared with the originating unit with a slope of 0.92 corresponding to a downward systematic deviation of 8%. The derived Bland-Altman analysis was performed to compare differences of nAPCsr values from each laboratory and the mean nAPCsr values from the three units. The average difference of nAPCsr for each laboratory compared with the mean of the three units was very close to zero. In addition, compared with the mean, 95% of nAPCsr values obtained in each laboratory did not differ by more than 0.4 units in absolute values or 16.5% in percent. These results demonstrate the low variability among the three units. Furthermore, subgroup's analysis confirmed that the chosen APC concentration in each laboratory, allowed differentiating between men and women not taking hormonal therapy and women using hormonal contraception. Indeed, the reference ranges based on 50 individuals (men and women not taking hormonal contraception) established during the validation were between 0 and 2.08.¹³ Regardless of the laboratory, mean nAPCsr for women without COC and men's groups were below 2.08 and above 2.08 for women using second- and third-generation COCs. In addition, no statistically significant differences between the three laboratories for each subgroup were observed which means that the methodology remains reproducible, regardless of the measurement range. Finally, pooled data maintained the expected differences between subgroup's (i.e., no significant difference between healthy individuals) and significant differences between healthy individuals, either men or women not taking COCs and women using COCs. Despite a higher mean nAPCsr for women using third-generation COCs (mean nAPCsr = 3.42) compared with women using second-generation COCs (mean nAPCsr = 2.92), the difference was not significant, certainly because of the small sample size.

5 | LIMITATIONS AND PERSPECTIVES

One limitation of our study concerns the restricted number of laboratories included due to insufficient number of aliquots for each sample. However, the between laboratory reproducibility of the method was excellent, despite the remote training of operators (i.e., instead of on-site training). From a technical point of view, there are also several limitations including the turn-around time and the absence of ready-to-use reagents. Indeed, the assay is time-consuming because of reagent preparation, stabilization time, and manual filling of the plate. Furthermore, additional experiments are required

	nAPCs _r ± SD			Mean ± SD
	Originating Unit	Receiving Unit 1	Receiving Unit 2	
Women without COC (n = 15)	1.19 ± 0.42	1.07 ± 0.38	1.20 ± 0.40	1.15 ± 0.40
Men (n = 15)	0.49 ± 0.47	0.39 ± 0.49	0.51 ± 0.41	0.46 ± 0.45
Women using second-generation COCs (n = 15) ^a	2.87 ± 0.86	3.02 ± 1.09	2.86 ± 0.79	2.92 ± 0.90
Women using third-generation COCs (n = 15)	3.39 ± 0.70	3.71 ± 0.94	3.17 ± 0.68	3.42 ± 0.80

Abbreviations: COC, combined oral contraceptive; nAPCs_r, normalized activated protein C sensitivity ratio; SD, standard deviation.

^an = 14 for receiving unit 2.

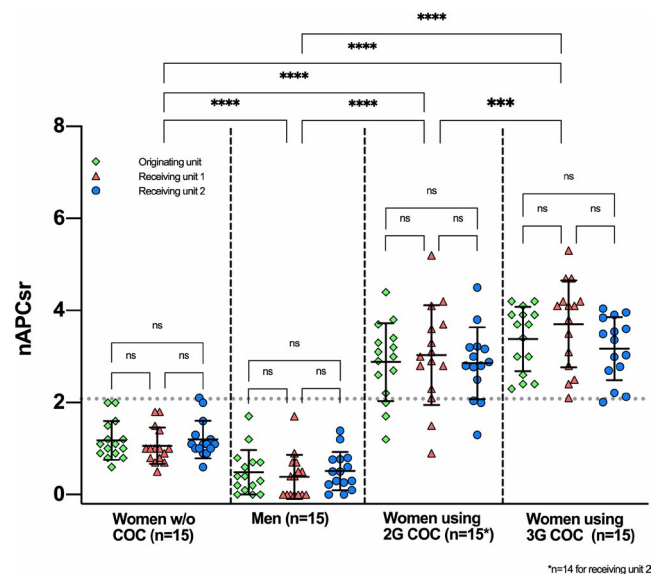


FIGURE 5 Normalized APC sensitivity ratio (nAPCs_r) values of individuals from each subgroup (i.e., women without combined oral contraceptive [COC], men, women using second-generation [2G] COC, and women using third-generation [3G] COC) obtained at the different units. Data obtained at the originating unit figure in green, data obtained at the receiving unit 1 figure in red, and data obtained at the receiving unit 2 figure in blue. Means ± standard deviation (SD) are represented. Holm-Sidak multiple comparisons tests were performed to assess the difference between units within each subgroup. On the other hand, differences between subgroups were assessed by a Tukey multiple comparison test. *p* value format is characterized as following: ns = *p* > 0.05; * = *p* ≤ 0.05; ** = *p* ≤ 0.01; *** = *p* ≤ 0.001; **** = *p* ≤ 0.0001). The gray dotted line represents the upper limit of reference ranges and equals 2.08

at each batch change to define APC concentration and to determine QC ranges. As a perspective, it is therefore of importance to consider the implementation of this assay on a clinical routine equipment and the manufacturing of ready-to-use triggering reagent with APC to throw off these technical restrictions.

TABLE 3 Mean nAPCs_r values ± standard deviation of each subgroup (i.e., women without COC, men, women using second-generation COC, and women using third-generation COC) obtained at the different units (i.e., originating unit, receiving unit 1, and receiving unit 2) and mean nAPCs_r value of each subgroup, regardless of the laboratory unit

6 | CONCLUSION

This study is the first reporting the interlaboratory variability of the validated ETP-based APC resistance assay. Data revealed excellent intra-laboratory precision and interlaboratory reproducibility. These results support the concept that the nAPCs_r, obtained with a validated and standardized methodology, provides an appropriate sensitivity irrespective of the laboratory in which the analysis is performed. This suggests that the nAPCs_r could become a promising regulatory and clinical tool to identify the thrombogenicity of COC. In views of its screening potential, the next step is therefore to implement the ETP-based APC resistance assay in clinical routine.

AUTHOR CONTRIBUTIONS

Laure Morimont, Jonathan Douxfils, Céline Bouvy, and Aurélien Lebreton designed the study. Élise Modaffari, Marie Didembourg, and Maxence Tillier performed the analyses. Laure Morimont and Jonathan Douxfils analyzed and interpreted data. Laure Morimont performed statistical analysis. Laure Morimont wrote the draft manuscript. Hélène Haguët, Céline Bouvy, Aurélien Lebreton, Jean-Michel Dogné, and Jonathan Douxfils provided input and critical review of the manuscript. All authors revised the manuscript.

RELATIONSHIP DISCLOSURE

Dr. Douxfils is chief executive officer 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. The other authors have no conflicts of interest to disclose.

ORCID

Laure Morimont <https://orcid.org/0000-0001-9195-304X>

Aurélien Lebreton <https://orcid.org/0000-0002-1650-0427>

Jonathan Douxfils <https://orcid.org/0000-0002-7644-5298>

REFERENCES

1. Jordan WM, Anand JK. Pulmonary embolism. *The Lancet*. 1961;278(7212):1146-1147.
2. 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.
3. 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.
4. Østerud B, Robertsen R, Åsvang GB, Thijssen F. Resistance to activated protein C is reduced in women using oral contraceptives. *Blood Coagul Fibrinolysis*. 1994;5:853-854.
5. 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;97:233-238.
6. 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.
7. Lavigne-Lissalde G, Sanchez C, Castelli C, et al. Prothrombin G20210A carriers the genetic mutation and a history of venous thrombosis contributes to thrombin generation independently of factor II plasma levels. *J Thromb Haemost*. 2010;8(5):942-949.
8. Duchemin J, Pan-Petesch B, Arnaud B, Blouch MT, Abgrall JF. Influence of coagulation factors and tissue factor concentration on the thrombin generation test in plasma. *Thromb Haemost*. 2008;99(4):767-773.
9. Marco A, Brocal C, Martirena F, Lucas J, Marco P. Clinical and biological factors that contribute to thrombin generation in prothrombin G20210A carriers: a case-control study in a single Thrombophilia Center. *Thromb Res*. 2012;129(5):e266-e268.
10. European Medicines Agency. *Guideline on Clinical Investigation of Steroid Contraceptives in Women - EMEA/CPMP/EWP/519/98 Rev 1 2005*. https://www.ema.europa.eu/documents/scientific-guideline/guideline-clinical-investigation-steroid-contraceptives-women_en.pdf. Accessed June 23 2021.
11. 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.
12. 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.
13. 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.
14. 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.
15. Dargaud Y, Luddington R, Gray E, et al. Effect of standardization and normalization on imprecision of calibrated automated thrombography: an international multicentre study. *Br J Haematol*. 2007;139(2):303-309.
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. Calzavarini S, Brodard J, Quarroz C, et al. Thrombin generation measurement using the ST Genesia Thrombin Generation System in a cohort of healthy adults: normal values and variability. *Res Pract Thromb Haemost*. 2019;3(4):758-768.

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