

## ORIGINAL ARTICLE

# Thrombin generation measured on ST Genesis, a new platform in the coagulation routine lab: Assessment of analytical and between-subject variation

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**Abstract**

**Background:** The thrombin generation (TG) assay, which measures global coagulation, has mainly been used as a research tool to investigate thrombotic and bleeding disorders. Recently, Diagnostica Stago launched the ST Genesis, a fully automated system to perform “routine version” of this assay. The objectives of this study were to evaluate the imprecision compared with the previous method, Thrombinoscope CAT, and to establish reference intervals.

**Methods:** Thrombin generation was measured in platelet-poor citrated plasma from 20 normal controls (fresh and after freezing at  $-80^{\circ}\text{C}$  up to 12–13 weeks) on CAT and ST Genesis in duplicate to estimate the total variation, and within and between variations. The reference intervals were estimated nonparametrically in 30 men, 30 women taking combined oral contraceptives (COCs), and 30 women not taking COCs. These were sampled in both Vacutainer and Monovette tubes (i.e., tubes with a high and minimal contact activation, respectively).

**Results:** Freezing had minimal effects. Imprecision was comparable between the ST Genesis and CAT, with a strong correlation between the two methods. TG was higher when sampled in Vacutainer than in Monovette. We observed a distinct difference between women taking and not taking COCs, whereas men and women not taking COC were quite similar.

**Conclusions:** Thrombin generation on ST Genesis showed an analytical variation similar to that of CAT. The results depended on the type of sample tubes; thus, reference intervals must be established for the collection tubes used in each laboratory. Furthermore, a considerable difference was observed between women using and not using COCs.

**KEYWORDS**

CAT, contact activation, imprecision, reference intervals, ST genesis, thrombin generation

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## Essentials

- A new routine instrument for thrombin generation assay, the ST Genesia, was evaluated.
- Imprecision and reference intervals (tubes with high and low contact activation) were estimated.
- ST Genesia showed analytical variation similar to that for the CAT instrument
- The reference intervals depend on the sample tube; for women, use of combined oral contraception.

## 1 | INTRODUCTION

In the routine coagulation laboratory, the global analyses activated partial thromboplastin time (APTT) and prothrombin time (PT) have been used for almost a century and remain widely used. These tests measure the time from the initiation of the coagulation process until the first fibrin formation. However, it has been shown that most of the thrombin is actually formed after the start of fibrin formation; therefore, these analyses provide only a small glimpse of coagulation activity and do not measure the activity of coagulation inhibitors. In the 1950s, MacFarlane and Biggs<sup>1</sup> reported measurements of the entire thrombin generation (TG) to provide a more complete picture of the coagulation process. The method was greatly improved by Hemker at the advent of the 20th century<sup>2–4</sup> with the introduction of a fluorogenic substrate for thrombin to allow measurements in plasma with fibrinogen.

This methodology has been widely used as a research tool on various platforms: calibrated automated thrombin (CAT) generation performed on the Thrombinoscope CAT (Diagnostica Stago; Asnieres sur Seine, France); on Technothrombin (Technoclone; Vienna, Austria); or Innovance ETP (Siemens; Erlangen, Germany). Patients with thromboses show a higher level of TG, whereas those with bleeding show lower TG,<sup>5–8</sup> although there is a substantial between-subject variation.<sup>9</sup> Furthermore, several new causes of bleeding have been identified and described that include other mechanisms than APTT and PT measure.<sup>10,11</sup> However, this methodology has not been introduced in routine laboratories because the current equipment was mainly developed for research. Recently a new generation of analyser the ST Genesia was launched by Diagnostica Stago to introduce the TG assay to routine laboratory investigations. The ST Genesia is based on the CAT platforms but was developed with full automation with improved temperature control. This TG platform is further enhanced by the inclusion of specialized kits, including samples for quality control and for specific clinical conditions, namely Bleedscreen (BS) with a low concentration of tissue factor (TF) sensitive for measuring factor deficiencies (reportedly all factors included in APTT and PT<sup>12</sup>); Thromboscreen (TS), with a medium concentration of TF sensitive for measuring thrombophilia (reportedly the natural anticoagulants<sup>12</sup> and possibly also Factor V Leiden and Prothrombin G20210A<sup>13</sup>); and Drugscreen with a high concentration of TF sensitive for the measurement of anticoagulants. However, the exact concentrations of TF and phospholipids are not available. The calibration also differs between CAT and Genesia: in the CAT measurements, a calibrator is added to all samples, whereas for the ST Genesia, calibration is only performed once daily with a solution of thrombin and a fluorogenic substrate, and each plasma sample is spiked with a fluorophore to correct for plasma color.

We comprehensively investigated analytical variation and practicality of this new coagulation instrument compared with the previous model CAT. We have also described reference intervals for both BS and TS reagents.

## 2 | MATERIALS AND METHODS

### 2.1 | Blood collection

Venous blood was collected from the antecubital fossa using a 21-gauge needle in 20 healthy volunteers into 3.2% (w/v) trisodium citrate Monovette tubes (Sarstedt, Nümbrecht, Germany). For estimation of reference intervals, blood was obtained from blood donors (healthy people without any apparent illness) using a 16-gauge needle and both Monovette and Vacutainer tubes (BD, Plymouth, UK) containing 3.2% (w/v) trisodium citrate. The first tube was discarded. Platelet-poor plasma (PPP) was obtained by centrifugation twice at 2500g for 15 min at 20°C immediately after collection in accordance with current guidelines.<sup>14</sup> After centrifugation, plasma samples from each individual and type of tube were pooled and stored at -80°C until analysis, except the fresh samples were analyzed within 2–3 h. For the variation study, one-half of the plasma was diluted 9 + 1 in HEPES buffer, pH 7.35 (containing 300 mM HEPES and 5 mg/ml bovine serum albumin) as described previously.<sup>15</sup>

All samples were obtained after obtaining informed consent. According to Danish legislation, this kind of measurement is classified as quality control; therefore, approval from the local ethical committee was not required.

### 2.2 | Thrombin generation

TG was measured using the ST Genesia (Diagnostica Stago, Asnieres sur Seine, France) and the CAT method (Thrombinoscope BV, Maastricht, The Netherlands). For the imprecision study, fresh and frozen samples were measured, and the frozen samples were thawed for 5 min at 37°C and analyzed after 2–3, 5–6, and 12–13 weeks as duplicates each day. In the CAT system, the PPPLow reagent (1 pM TF, 4 μM PL) (Thrombinoscope BV) was used. For the ST Genesia, the BS kit was used. All validation measures were performed using the same batch of reagents. For estimation of the reference intervals, all plasma samples were frozen before analysis with the BS and TS used on the ST Genesia. When using TS, the TG was measured with and without the addition of thrombomodulin (TM).

## 2.3 | The calibrated automated thrombogram

Thrombin generation measurements were conducted according to the manufacturer's instructions using 80  $\mu$ l PPP mixed with 20  $\mu$ l of trigger solution denoted PPPLow; subsequently coagulation was initiated by the addition of 20  $\mu$ l FluCa buffer containing a fluorescent substrate (Z-Gly-Gly-Arg-AMC) and  $\text{CaCl}_2$  (all reagents from Thromboscope BV). All measurements were performed in duplicates. To correct for differences in plasma color, each plasma measurement was calibrated against the same plasma mixed with 20  $\mu$ l thrombin calibrator (Thromboscope BV), also in duplicate. The fluorescence of AMC (7-amino-4methylcoumarin) was measured using a Fluoroskan Ascent (Thermo Scientific, Waltham, MA, USA) equipped with a 390 nm excitation and 460 nm emission filter set. The dedicated Thromboscope software (Thromboscope BV) was used to calculate: lagtime, endogenous thrombin potential (ETP), peak, and time to peak (ttPeak).

## 2.4 | ST Genesis

The ST Genesis (Diagnostica Stago) measures the development of fluorescence of AMC to detect thrombin activation as in the CAT system but differs from the CAT system by performing one overall calibration representing the activity of a known thrombin concentration once daily rather than a plasma-specific calibration. A separate fluoSet containing a fixed amount of the AMC fluorophore was added to each plasma to correct for the plasma color. The ST Genesis performs four measurements for each sample, namely, TG in samples in duplicate and fluoSet in duplicate. The samples are preheated at 37°C with precise temperature control and the fluorescence registered with a 377/440 filter set (excitation/emission). The kits contain a standardized STG-QualiTest, Low and Normal control set, and reference plasma (STG-RefPlasma) for normalization of the TG value.

## 2.5 | Routine assays

APTT (seconds), international normalized ratio (INR) (ratio, [no unity]), fibrinogen ( $\mu\text{mol/L}$ ), and antithrombin (IU/ml) assays were performed on the ACL Top 500 (Instrumentation Laboratory, Milano, Italy) with dedicated reagents for INR, fibrinogen, and antithrombin, and the reagent Pathromtin (Siemens, Erlangen, Germany) for APTT.

## 2.6 | Statistical analysis

Calculations of the total variation ( $\text{CV}_T$ ), within variation ( $\text{CV}_W$ ), and the variation between runs ( $\text{CV}_B$ ) were performed essentially as described before.<sup>9</sup> It is also described in the Supporting Information.

Values are generally described as mean  $\pm$  SEM. When appropriate, differences were tested with Student's *t* test and were considered significant at  $p < 0.05$ . The associations between tests were assessed using Pearson's correlation coefficients and are described as  $R^2$ .

The reference intervals were determined as 2.5 and 97.5 percentiles (nonparametrically) for each group as recommended.<sup>16</sup> Furthermore, the 25th and 75th percentiles were calculated and are included in the figures.

## 3 | RESULTS

The imprecision of the methods was calculated by measuring fresh samples and samples frozen for 2–3, 5–6, and 12–13 weeks from 20 volunteers. The measurements were performed in duplicates on both CAT and the ST Genesis. First, the effect of freezing was examined by comparing the results obtained from the fresh samples to the mean of the three frozen samples and to each of the days. All differences and SEM results are shown in Table S1. The samples measured on CAT showed no differences in ETP and Peak, whereas there was a small, but significantly shorter lagtime and ttPeak in fresh samples. The ST Genesis showed slightly different results, with a trend toward a higher ETP. Peak and lagtime were not affected, whereas ttPeak was slightly reduced (i.e., only ttPeak was slightly shorter for both methods in fresh samples). However, because the differences between fresh and frozen samples were very small, although significant, we chose to use all these results, fresh and frozen samples, for the calculation of variations. The total ( $\text{CV}_T$ ), between ( $\text{CV}_B$ ), and within assay ( $\text{CV}_W$ ) variations were measured as described in the Methods section, and as shown in Table 1.

We previously reported that pH in frozen samples may increase during the storage,<sup>15</sup> which can be avoided by adding HEPES buffer to the sample. We also tested this procedure in the present study by mixing some of the fresh samples with HEPES buffer, which were analyzed as fresh samples and after storage for 2–3 and 12–13 weeks. This reduced the small difference in ttPeak between the fresh and frozen samples. Table S2 shows the  $\text{CV}_T$ ,  $\text{CV}_B$ , and  $\text{CV}_W$  of these samples. Although the TG level was higher in these samples, the variation was comparable to that in samples without HEPES.

The ST Genesis has a reference plasma that can be used for normalization of the samples to reduce the variation. The variations calculated based on these values are also shown in Table 1.  $\text{CV}_W$  was unchanged, but  $\text{CV}_T$  and  $\text{CV}_B$  were slightly higher when the normalized values are used.

Finally, we included controls at the beginning and at the end of each series of analyses. We observed no trend of change in the level during each of the series or from the start to the end of all the series. Moreover, the imprecision for the ST Genesis and CAT was similar to the other samples.

The results for CAT and ST Genesis were compared by calculating the correlations between the means of each of the 20 samples (four duplicates of each) on each system (Figure 1). Generally, there was a rather strong correlation between the two measurements with  $R^2$  values between 0.67 and 0.81. However, lagtime and ttPeak were considerable shorter on the ST Genesis with BS, indicating that the TF concentration is higher in BS than in PPPLow. The peak was quite similar, whereas the ETP was slightly lower on the ST Genesis.

We calculated reference values for the reagents: BS (low TF), TS (medium-high TF), and TS with the addition of TM. We included 30 men (age [M ± SD] 46 ± 15 years, range 22–69 years) and 60 women, 30 of whom were taking combined oral contraceptives (COCs) (age: 26 ± 5 years, range 19–35 years) and 30 women who were not taking COCs (age: 36 ± 12 years, range 19–55 years), because COCs may affect TG,<sup>17,18</sup> based on limited numbers of individuals in the previous reports. Sample tubes also reportedly had a substantial effect on TG<sup>19</sup>; therefore, we used two types of tubes, one with a high degree of contact activation (i.e., activation via surface activation of Factor XII/Kallikrein) (Vacutainer) and one with a minimal contact activation (Monovette). If coagulation is initiated through contact activation in addition to TF in the reagents, the coagulation response will be higher than that without

contact activation, especially when the TF concentration is low.<sup>19</sup> The absolute results are shown as box-whisker plots in Figure 2 (BS) and 3 (TS ± TM). The results of the normalized values are shown in Figures S1 and S2, respectively. The ETP and peak values were higher and the ttPeak values shorter in samples in Vacutainer for all participants and analyses compared with samples in Monovette tubes. The differences were more pronounced using BS than for TS. The reference intervals for women taking COCs differed from the reference intervals for women not taking COCs, with higher ETP and peak and shorter ttPeak, whereas lagtime was only slightly shorter in women taking COCs. The differences between Vacutainer and Monovette tubes were less distinct for women using COCs than for men and women not taking COCs.

We observed a strong correlation between the results using BS and TS (without TM) (Table 2). We examined the correlation with age, which generally was very weak ( $R^2 < 0.05$ ) for peak and ETP, whereas it was slightly higher for lagtime and ttPeak ( $R^2 = 0.003$ – $0.13$ ), with unchanged to slightly higher values for the elder persons, but the differences were minor (Table S3).

We investigated correlations of these analyses with the routine analyses, APTT, INR, fibrinogen, and antithrombin (Figure S3). The correlations with INR and fibrinogen were very weak to weak and slightly higher in Vacutainer than in Monovette tubes. Correlations with APTT were higher and more marked in Vacutainer tubes than in Monovette tubes. On the contrary, the correlations to antithrombin were moderate in Monovette tubes, but lower in Vacutainer tubes.

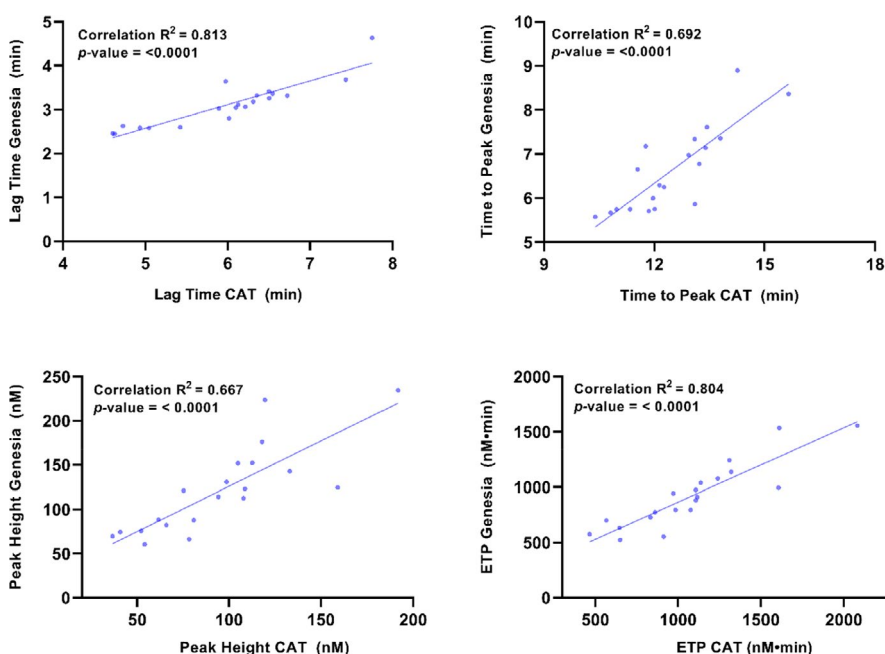
**TABLE 1** Coefficients of variation for TG performed on CAT and ST Genesis, as well as normalized values on the ST Genesis

	Lagtime	ttPeak	Peak	ETP
CAT				
CV <sub>W</sub> (%)	2.7	2.2	8.3	6.8
CV <sub>B</sub> (%)	5.5	3.3	5.6	5.9
CV <sub>T</sub> (%)	6.1	4.0	10.0	9.0
ST Genesis				
CV <sub>W</sub> (%)	3.3	2.6	5.1	3.4
CV <sub>B</sub> (%)	2.9	3.8	9.5	7.1
CV <sub>T</sub> (%)	4.4	4.6	10.8	7.9
Normalized				
CV <sub>W</sub> (%)	3.3	2.6	5.3	3.6
CV <sub>B</sub> (%)	3.6	3.2	10.2	9.1
CV <sub>T</sub> (%)	4.9	4.1	11.5	9.8

Abbreviations: CAT, calibrated automated thrombin; ETP, endogenous thrombin potential; TG, thrombin generation; ttPeak, time to peak.

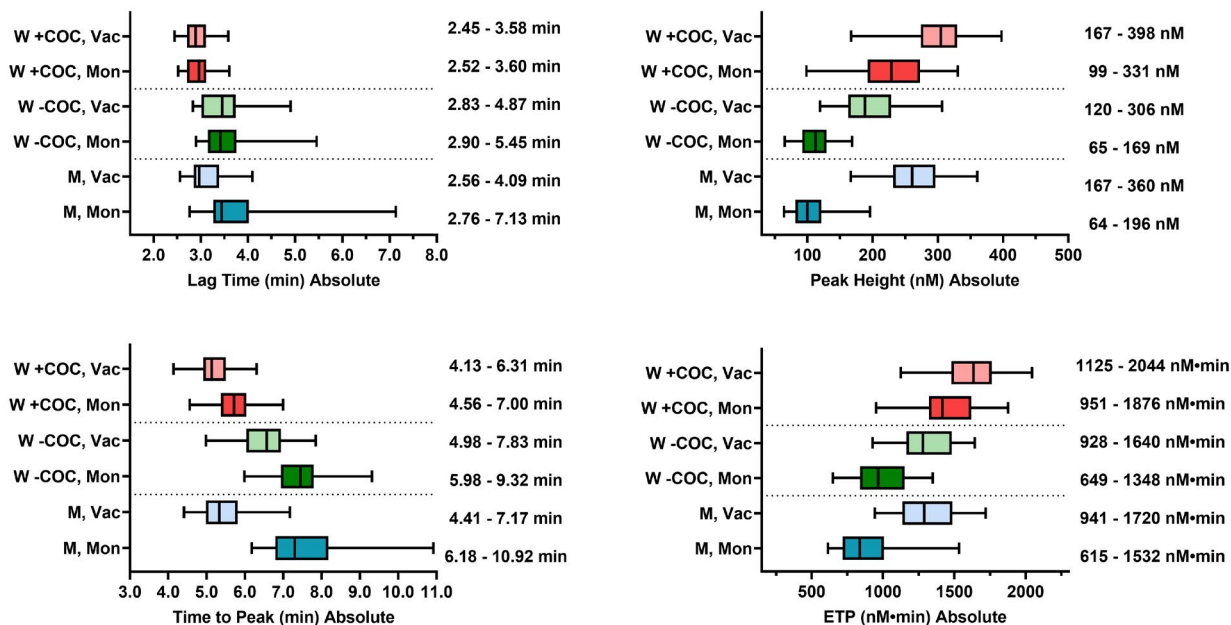
## 4 | DISCUSSION

The present study shows that the new equipment for TG assays, the ST Genesis, is comparable to the Thromboscope CAT regarding variation. We have presented suggestions for reference intervals



**FIGURE 1** Comparison between TG performed on CAT (PPPLow reagent) and on ST Genesis using the Bleedscreen reagent. The correlations between the mean of eight values (four duplicates) of each sample measured on each instrument are shown. TG, thrombin generation

## BleedScreen, Absolute



**FIGURE 2** Reference intervals for the absolute values for women (W) taking COCs, women not taking COCs, and men (M) sampled in the tubes Monovette (Mon) or Vacutainer (Vac) tubes and measured using the BleedScreen reagent. Each line shows the 95% central values, whereas the bars indicate the 50% central values and the median for lagtime, peak, ttPeak, and ETP. The values are shown to the right of each group. COCs, combined oral contraceptives; ttPeak, time to peak; ETP, endogenous thrombin potential

and highlighted that the TG levels are highly dependent on the tubes that are used for sampling. Furthermore, we show that the TG levels are substantially different between women taking COCs and women not taking COCs.

CAT has been used for many years, and is well-known to show a considerable variation, especially between laboratories.<sup>20</sup> The present findings (Table 2) are consistent with previous results.<sup>9</sup> We observed a similar variation in the ST Genesis; that is, this more automated instrument did not perform better than the older one. The manufacturer has introduced a reference sample to allow “normalization” of the results. However, we did not observe any improvement in variation after using this system; in fact, the variation may be slightly higher. A plausible explanation is probably that two analyses are needed for these results, and, therefore, you have variation on both. Thus, it is not an improvement in the daily practice using a specific lot, but it is probably an advantage when lots are changed, as shown by Cornette et al.<sup>7</sup> Also in cases where results from different laboratories are compared, this normalization may reduce the substantial inter-laboratory variation.<sup>20,21</sup> However, it is also crucial that the standardization material (i.e., STG RefPlasma) is robust and validation of new lots is performed thoroughly to maintain the level (cf use of material for the calculation of international sensitivity index values to maintain a stable INR calculation).

We previously demonstrated that pH changes of plasma may affect TG results<sup>15</sup>; therefore, we also measured the variation in plasma with HEPES added. Although this slightly improved the imprecision, the difference was not marked. This addition always

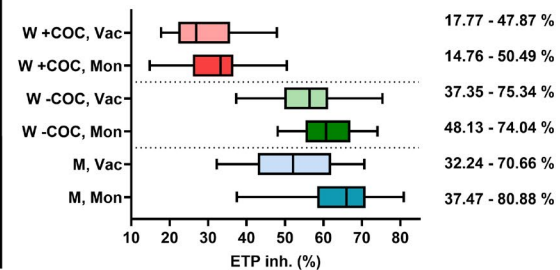
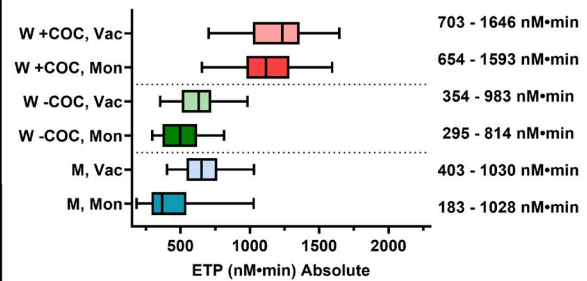
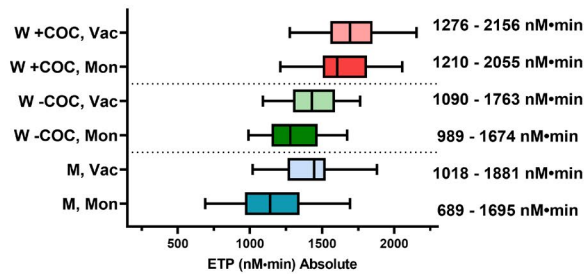
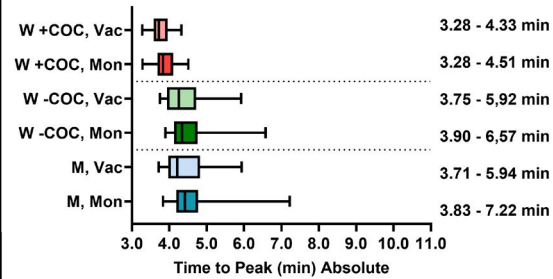
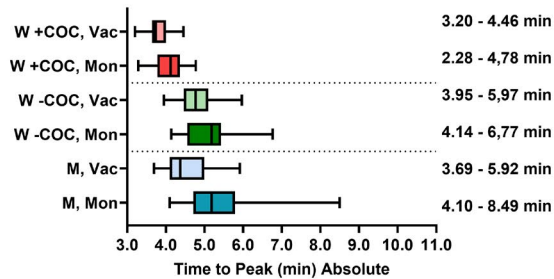
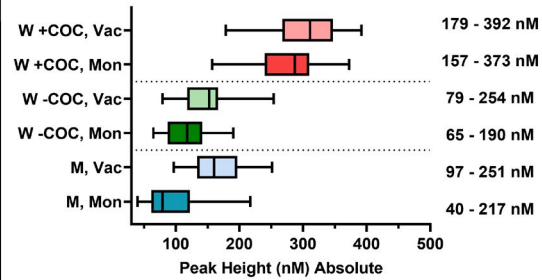
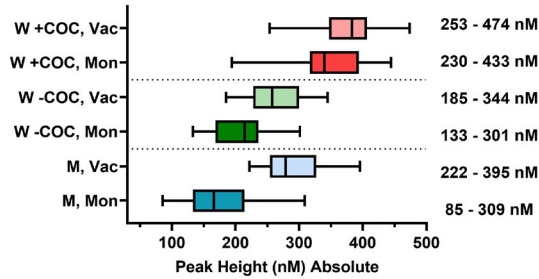
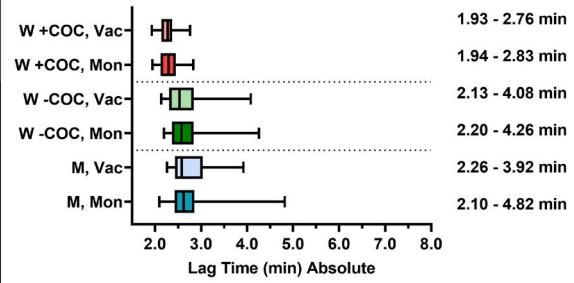
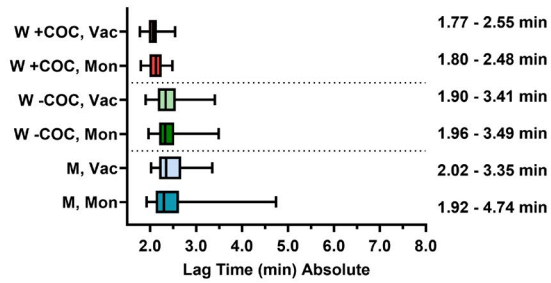
resulted in higher TG, although the difference between the results with and without HEPES was much smaller using ST Genesis. The reason for this is not known; however, because of this difference, if HEPES is used, it must be used in all samples in a specific project, even though it is smaller on the ST Genesis. When samples are stored for shorter periods, this addition is not beneficial but can be considered if samples must be stored for longer periods although this is a time-consuming step in a routine laboratory. We previously recommended filling vials to the maximum volume to avoid pH changes.<sup>15</sup>

Our estimation of the reference intervals showed that reference intervals must be determined for the respective sampling tubes used in the laboratory. The difference between Vacutainer and Monovette tubes was quite large, and more marked when using BS with a lower TF concentration. Contact activation in the Vacutainer tubes probably caused this difference. When measuring TG in women, it is important to know whether they use COCs because this substantially impacts TG. Therefore, separate reference intervals for these groups are necessary. The difference between Vacutainer and Monovette was smaller in women taking COCs, which may be caused by some contact activation from COCs<sup>22</sup> reducing the effect of the tube. Thrombomodulin also had a lower effect in women taking COCs, likely because COCs reduce protein S levels. Therefore, this analysis may be a tool to measure (some of) the effect of COCs on the coagulation system.

We observed a reasonably high correlation between PPPLow and BS; however, BS obviously had a higher TF concentration, and

### ThromboScreen: -TM

### ThromboScreen: +TM



**FIGURE 3** Reference intervals for the absolute values for women (W) taking COCs, women not taking COCs, and men (M) sampled in the tubes Monovette (Mon) or Vacutainer (Vac) tubes and measured with the Thromboscreen reagent without and with the addition of thrombomodulin. Each line shows the 95% central values, whereas the bars indicate the 50% central values and the median for lagtime, peak, ttPeak, and also ETP, and also ETP inhibition (the degree of inhibition after the addition of thrombomodulin). The values are shown to the right of each group. COCs, combined oral contraceptives; ttPeak, time to peak; ETP, endogenous thrombin potential

results using BS were not directly interchangeable with results from CAT using PPPLow. The correlation between BS and TS was strong; consequently, the results showing a high or a low TG will probably be revealed from both kits, and the reference values (Figures 2 and 3) are not very different. Thus, the results from the two reagents are not very different on samples from healthy individuals. However, we did not test patients with different types of coagulopathies; therefore, we cannot offer a conclusion on their applicability to pathological samples. Cornette et al.<sup>7</sup> found that CAT (PPPLow) and Genesia (BS) were almost equal in diagnosing patients with a bleeding tendency encompassing one-third of patients with a plasma coagulation defects and two-thirds without a diagnosis after routine bleeding investigations. We previously showed that the between-subject variation was higher for PPPLow with a low TF concentration compared with the PPP-reagent with a higher TF concentration.<sup>9</sup> However, using TS with and without TM will probably be an advantage be-

**TABLE 2** Correlations between BS and TS

R <sup>2</sup> values	ETP	Peak	ttPeak	Lagtime
Monovette				
All	0.88	0.92	0.92	0.94
Men	0.86	0.90	0.96	0.96
Women - COCs	0.79	0.83	0.85	0.92
Women + COCs	0.85	0.88	0.85	0.83
Vacutainer				
All	0.86	0.76	0.72	0.76
Men	0.72	0.50	0.66	0.83
Women - COCs	0.77	0.76	0.81	0.90
Women + COCs	0.94	0.77	0.86	0.83

Abbreviations: BS, Bleedscreen; COCs, combined oral contraceptives; ETP, endogenous thrombin potential; TS, Thromboscreen; ttPeak, time to peak.

cause the Protein C:Protein S system is included in the determination.<sup>13</sup> Roulet et al.<sup>23</sup> only observed minor differences between results using BS and TS on samples from patients who had undergone liver transplantation, whereas they observed some differences from the results of CAT that the authors could not explain.

Finally, we investigated the correlations between routine coagulation tests and TG levels. Generally, the correlations were quite low; however, interestingly, the correlations between APTT and TG were higher for Vacutainer tubes, likely indicating a higher degree of contact activation in these. On the other hand, the correlations between AT and TG were higher in the Monovette tubes, indicating that it is easier to detect the coagulation inhibitors in tubes with a low degree of contact activation. Therefore, tubes with a minimal contact activation may provide better information on global coagulation.

Calzavarini et al.<sup>17</sup> reported variation and reference intervals for the ST Genesia. However, the variation was calculated from only a few samples. The reference intervals were only described as the normalized central 50% of the population (25th–75th percentile).

The levels were comparable to our results: lagtime and ttPeak were nearly identical, whereas the peak and ETP were consequently 10%–20% lower than our values. They also reported a higher TG in women using COCs compared with women not using COCs in a limited number of individuals, as well as in elderly women, whereas we observed a minor effect of age and in the opposite direction. Ninivaggi et al.<sup>18</sup> also created reference values for BS and TS using Greiner Bio-One sampling tubes with results intermediate between those of the Monovette and Vacutainer tubes in the present study. They included a few women using COCs and reported an increased TG; however, excluding these women from the population did not change the reference values. They found similar levels for men and women without COCs, and no influence of age. This is in accordance with our findings: although we found some differences between men and women, the reference intervals were not very different. Douxfiles et al.<sup>24</sup> examined variation and sample stability but only for Drugscreen. Variation was only calculated based on controls, and they generally found smaller variation than in the present study; however, Drugscreen has a higher TF concentration. They found no effect of freezing with minimal nonsignificant differences between fresh and frozen samples for up to 11 months except for ttPeak, similar to the findings in the present study.

#### 4.1 | Practicability

The new equipment is designed for use in a routine laboratory test, whereas CAT is a research instrument. Therefore, the ST Genesia is in some ways easier to use because it is more automated with no pipetting, and samples can be measured one by one, whereas a series is usually analyzed on CAT. It can, therefore, probably be part of routine coagulation laboratory tests in the future. In this way, it functions well. However, the software in the present model is relatively slow, and the screen can frequently freeze, which slowed the analysis. This must be improved to fulfill the requirements for use in a routine laboratory setting.

#### 4.2 | Limitations

The reference intervals determined in this study were based on only 30 individuals in each group rather than the recommended 120 individuals. However, the main issue here is to describe the important effects of the type of sampling tube and use of COC in women; thus, the proposed reference intervals are suggestive of the levels. We have used tubes with a high and a minimal contact activation, but each laboratory must develop its own reference intervals based on the type of tubes used for sample collection and should recommend the use of the same tubes and the same material when samples are delivered from other laboratories. Although the range of ages in the group of women using COCs was smaller than that in women not using COCs, age is not important for the level of intervals, and COC users are generally younger women.

## 5 | CONCLUSION

The new instrument for measuring TG, the ST Genesis, showed comparable imprecision compared with that for the CAT method, and the kits contain samples for quality control and for normalization of the results, which may be an advantage. We have suggested reference intervals for men, and women using and not using COCs, and sampled in Monovette and Vacutainer tubes. The reference intervals depend on contact activation in the tubes used.

### RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest. Reagents kits for estimating variation in this project were bought with a 50% discount from Diagnostica Stago.

### AUTHOR CONTRIBUTIONS

Søren Risom Kristensen designed the study, interpreted data, and wrote the paper. Jette Nybo contributed to the design and performed the analyses and some data analysis. Shona Pedersen performed data analysis and interpretation. All authors contributed to the critical revision of the manuscript and approved the final version to be published.

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### SUPPORTING INFORMATION

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

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