ORIGINAL ARTICLE

ST Genesia reference values of 117 healthy donors measured with STG-BleedScreen, STG-DrugScreen and STG-ThromboScreen reagents

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Abstract

Introduction: The ST Genesia is a benchtop, fully automated thrombin generation (TG) device. It is completely standardized and ensures a uniform heat distribution throughout the measurement. We aimed to determine reference values and to compare TG in men and women with and without the use of oral contraceptives (OCs).

Materials and Methods: Plasma from 117 healthy donors was measured on the ST Genesia with the available reagent kits: STG-BleedScreen, STG-DrugScreen, and STG-ThromboScreen. All kits include at least two quality controls and a reference plasma to normalize data. STG-ThromboScreen has a second trigger containing thrombomodulin (TM) to include the effect on the protein C pathway. Means were compared with one-way analysis of variance and reference ranges were established with 2.5th to 97.5th percentiles on absolute TG parameters.

Results: Mean age of the donors was 35 years (SD ± 12); 49.6% were men, 37.6% women without OCs, and 12.8% women with OCs. Men and women without OCs had, respectively, a mean peak height of 167 nM and 164 nM with STG-BleedScreen, 335 nM and 351 nM with STG-DrugScreen, and 192 nM and 198 nM with STG-ThromboScreen. Women taking OCs had a mean peak height of 263 nM, 473 nM, and 312 nM, respectively (P < .05 compared to men/women without OCs). TM decreased endogenous thrombin potential by 54% in men, 47% in women without OCs, and only 25% in women with OCs (P < .05 compared to men/women without OCs).

Conclusions: TG in men and women without OCs was similar; however, women taking OCs had significantly higher TG values, and the effect of TM was also less pronounced in these women.

Keywords
oral contraceptives, protein C, reference values, thrombin, thrombomodulin
1 | INTRODUCTION

The thrombin generation (TG) assay is an established research tool in the research field of thrombosis and hemostasis. It mirrors a significant part of the overall function of the blood-clotting system. The assay has been used since the early 1950s, but back then it was performed by subsampling, which was very labor intensive. Nowadays, more (semi) automated assays are commercially available such as the Calibrated Automated Thrombography (CAT) assay (Diagnostica Stago SAS, Asnières sur Seine, France), the Technothrombin (Technoclone, Wien, Austria) and the Innovance ETP assay (Siemens Healthineers, Erlangen, Germany). Numerous studies have already demonstrated the added value of measuring TG in a patient sample, for example, in patients with rare inherited coagulation disorders, patients with hypercoagulability, or patients with hemophilia. The TG assay was revealed to be instrumental in the elucidation of the coagulopathy of chronic liver diseases. Moreover, TG can also be used to monitor or predict the outcome of blood product transfusion. Unfortunately, the assay is still not available in the clinic due to a lack of standardization or reagents), which makes it difficult to compare data between laboratories, as well as a lack of clear cutoff values for clinical treatment decisions.

The newly developed ST Genesia (Diagnostica Stago) is a bench-top, fully automated TG assay, related to the previous CAT assay. The ST Genesia method is more standardized, has specific kits for specific experimental aims, and ensures a stable and uniform heat distribution throughout the measurement and the measuring cuvette. Other differences include the presence of a reference plasma and quality controls in the reagent kits and a different calibration method. The necessary quality controls for clinical use of the ST Genesia are available and comply with local legislation or guidelines, which recommend the use of at least two levels of controls. The use of a reference plasma to normalize the data is specifically advantageous, as plasma samples do not have to be measured all at once anymore and the need for a single batch of reagents also becomes unnecessary. In addition, it enables comparison of data between laboratories, and it reduces the interlaboratory variation.

2 | MATERIALS AND METHODS

2.1 | Samples

Personnel of the university campus of Maastricht were approached and asked to participate to our study. In total, 120 healthy donors volunteered to participate. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre and conducted according to the Declaration of Helsinki (2013). Blood samples were taken after the informed consents were signed. Exclusion criteria included the use of drugs interfering with coagulation, known coagulation disorders, and/or being younger than age 18 years or older than age 65 years. Twenty-seven milliliters of blood was drawn aseptically in vacuum blood collection tubes (Greiner Bio-One, Kremsmünster, Upper Austria) containing 3.2% sodium citrate (in a 9:1 ratio), from the antecubital vein of healthy subjects. Directly after blood drawing, platelet-poor plasma was prepared by centrifuging the blood for 10 minutes at 2630 g at room temperature. Immediately thereafter, the supernatant was collected and centrifuged for 10 minutes at 2630 g at room temperature. The platelet-poor plasma samples were stored anonymously at −80°C until further use. The samples were measured at the latest 11 months after blood collection.

2.2 | Thrombin generation

TG was measured on the ST Genesia (Diagnostica Stago SAS). The first step was to run a calibration curve. This calibration curve was performed with the STG-Cal&Fluo kit, which contained STG-ThrombiCal, STG-FluoStart, and STG-FluoSet. The STG-ThrombiCal (lyophilized powder) contained the calibrator and had to be reconstituted with 2 mL of distilled water. The STG-FluoStart (liquid) contained the substrate and calcium, while the STG-FluoSet (liquid)
TABLE 1  Patient characteristics

|                | Men       | Women –OCs | Women + OCs | Total |
|----------------|-----------|------------|-------------|-------|
| Number (%)     | 58 (49.6) | 44 (37.6)  | 15 (12.8)   | 117 (100) |
| Mean age, y (SD) | 33 (12)   | 40 (13)    | 25 (4)      | 35 (12)   |
| Median age, y (IQR) | 29 (25-39) | 38 (27-52) | 25 (21-27)  | 30 (25-42) |

Note: Data are expressed as indicated. Abbreviations: IQR, interquartile range; OCs, oral contraceptives; SD, standard deviation.

3 | RESULTS

3.1 | Study population characteristics

TG was measured with the ST Genesia in 117 of 120 healthy donors, as 3 samples had to be discarded due to very low plasma volume. One sample did not contain enough plasma for measuring TG with all triggers (STG-ThromboScreen with TM was annulled). Table 1 shows the number of healthy donors per subgroup: men (49.6%), women without OCs (women –OCs, 37.6%) and women taking OCs (women + OCs, 12.8%). The mean age of the whole population was 35 ± 12 years (mean ± SD), and the median age was 30 years (IQR, 25-42). The female group taking OCs was statistically younger than the men or women without OCs (P = .029 and P < .00, respectively). No statistical difference in age was observed between men and women without the use of OCs.

3.2 | Thrombin generation measured with the STG-BleedScreen, STG-DrugScreen, and STG-ThromboScreen

Tables 2, 3, and 4 show the data of all TG parameters measured with STG-BleedScreen, STG-DrugScreen, and STG-ThromboScreen, respectively. The absolute data are shown in the upper panels (N = 116-117), while the normalized data are in the lower or middle panels (N = 93-102). The reason for the lower number of subjects in the normalized data panel is that normalization did not occur in all samples due to human error. Of all data sets, median with IQRs, as well as mean (SD) and interindividual variation are given. Overall, the lowest interindividual variation was for the start tail, while the highest variation was for velocity index. The variation for peak height decreased with increasing TF concentration in the reagent, while the variation in ETP did not vary. The interindividual variation of the normalized data was similar to the absolute data but somewhat lower. Tables S1-S3 contain the same data.
excluding the values of women taking OCs. Irrespective of the trigger used, TG parameters did not differ significantly when these women were omitted from the data set.

### 3.3 The reference ranges for the three reagent kits

The reference ranges (2.5th to 97.5th percentiles) of the TG parameters for the three reagent kits are depicted in Table 5. Overall, the

| TABLE 2 | Absolute and normalized TG parameter values and interindividual variation measured with STG-BleedScreen |
|----------------|--------------------------------------------------|
| Absolute data (N = 117) | Median | IQR | Mean (SD) | Interindividual variation (%) |
| Lag time, min | 2.9 | 2.6-3.4 | 3.0 (0.7) | 23.3 |
| Peak, nM | 167 | 140-204 | 178 (57.9) | 32.5 |
| Time to peak, min | 6.4 | 5.8-7.3 | 6.5 (1.2) | 18.1 |
| ETP, nM/min | 1128 | 938-1325 | 1141 (266) | 23.3 |
| Velocity index, nM/min | 60.2 | 45.2-81.6 | 70.9 (37.6) | 53.0 |
| Start tail, min | 19.6 | 16.6-21.6 | 19.6 (3.5) | 18.0 |

| Normalized data (N = 102) | Median | IQR | Mean (SD) | Interindividual variation (%) |
| Lag time, ratio | 1.0 | 0.9-1.2 | 1.1 (0.2) | 20.1 |
| Peak, % | 131 | 105-155 | 136 (43.8) | 32.1 |
| Time to peak, ratio | 1.1 | 0.9-1.2 | 1.1 (0.2) | 17.8 |
| ETP, % | 115 | 95.8-129 | 115 (25.2) | 22.0 |
| Velocity index, % | 115 | 86.8-156 | 136 (73.2) | 53.9 |
| Start tail, ratio | 0.8 | 0.8-1.0 | 0.9 (0.2) | 17.8 |

Note: Data are expressed as indicated.
Abbreviations: ETP, endogenous thrombin potential; IQR, interquartile range; SD, standard deviation; TG, thrombin generation.

| TABLE 3 | Absolute and normalized TG parameters values and interindividual variation measured with STG-DrugScreen |
|----------------|--------------------------------------------------|
| Absolute data (N = 117) | Median | IQR | Mean (SD) | Interindividual variation (%) |
| Lag time, min | 1.3 | 1.2-1.5 | 1.4 (0.3) | 18.7 |
| Peak, nM | 349 | 301-409 | 359 (85.6) | 23.8 |
| Time to peak, min | 2.8 | 2.5-3.2 | 2.9 (0.6) | 20.9 |
| ETP, nM/min | 1225 | 1089-1432 | 1288 (299) | 23.2 |
| Velocity index, nM/min | 329 | 242-423 | 350 (163) | 46.5 |
| Start tail, min | 11.2 | 10.3-12.3 | 11.5 (1.5) | 13.1 |

| Normalized data (N = 93) | Median | IQR | Mean (SD) | Interindividual variation (%) |
| Lag time, ratio | 1.0 | 0.9-1.1 | 1.0 (0.2) | 17.4 |
| Peak, % | 76.1 | 66.7-88.3 | 77.8 (18.2) | 23.4 |
| Time to peak, ratio | 1.1 | 1.0-1.3 | 1.2 (0.2) | 20.3 |
| ETP, % | 70.1 | 63.0-81.0 | 73.0 (16.2) | 22.2 |
| Velocity index, % | 55.1 | 40.2-67.2 | 58.1 (26.3) | 45.2 |
| Start tail, ratio | 0.9 | 0.8-0.9 | 0.9 (0.1) | 13.2 |

Note: Data are expressed as indicated.
Abbreviations: ETP, endogenous thrombin potential; IQR, interquartile range; SD, standard deviation; TG, thrombin generation.
for STG-ThromboScreen with TM. The number of subjects varied throughout the table, as outliers were excluded from the data set.

### 3.4 The effect of the use of OCs on thrombin generation

The effect of OC usage on TG parameters was also investigated. Table 6 shows the TG parameters obtained with the three reagent kits for the three groups of healthy donors: men, women without OCs, and women with OCs. TG parameters (except for lag time) did not differ significantly between men and women without the use of OCs. Lag times were significantly longer in men compared to women without the use of OCs for all reagents. Peak height, ETP, and velocity index values were statistically higher in women taking OCs compared to the other groups (men, women without OCs, and the total population of healthy donors). This observation was irrespective of the reagent used to activate the samples. The only exception here is the ETP measured with STG-DrugScreen of women without OCs versus women with OCs. The ETP% inhibition was also significantly lower in women with OCs (25%) compared to men (54.2%) and women without OCs (47.4%). Women taking OCs had shorter lag time, time to peak, and start tail compared to men and to women without the use of OCs. However, statistically, women with OCs had only significantly shorter lag times compared to men for all reagents. Time to peak was also significantly shorter in women taking OCs compared to the other groups (except between women with and without OCs activated with STG-DruDrugScreen and STG-ThromboScreen + TM. Start tail was significantly shorter in women with OCs compared to men and women without OCs when activating with STG-BleedScreen and STG-ThromboScreen –TM but not with STG-DrugScreen and STG-ThromboScreen + TM. The ratios of the three groups versus the total population for peak height values and ETP% inhibition are illustrated in Figure 1.
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STG-DrugScreen, and STG-ThromboScreen reagent kits. The activator of the STG-BleedScreen reagent kit contains TF at a low picomolar level that is balanced for sensitivity to factor deficiencies (eg, hemophilia A and B), while the STG-DrugScreen activator contains TF at a high picomolar level to assess the anticoagulant effect at prophylactic and therapeutic doses (eg, direct oral anticoagulants, heparin, vitamin K antagonists). The activator of the STG-ThromboScreen reagent contains TF at a medium picomolar level balanced for sensitivity to deficiencies in natural anticoagulants (eg, protein C). To date, this is the first study that used the three reagent kits to simultaneously measure TG in a large population of healthy donors.

TG interindividual variation is similar to the one obtained by the CAT-TG assay (ie, 23% for ETP). The interindividual variation of the peak height and velocity index decreased with increasing TF concentration of the reagent kit, but the one of ETP remained stable. This is probably due to the ETP being less dependent on the TF concentration but more dependent on the overall coagulation potential of the sample. On the contrary, peak height and velocity index are highly dependent of the TF concentration as the TF will affect the rate at which thrombin is being formed and therefore also the propagation of TG.

One of the advantages of ST Genesia kits is the use of a reference plasma to normalize the data and the use of quality controls to ensure qualitative results. The use of normalized data makes it possible to compare data between laboratories and reduces inter-laboratory variation. One of the ST Genesia reagent kits, the STG-ThromboScreen, also includes an activator that contains TM. The addition of TM activates the protein C pathway and in that way also involves this major coagulation regulation loop. The data of the samples measured with TM are not normalized, but they do reveal the percentage of ETP inhibition in comparison to the measurement without TM. The chosen TM concentration in this reagent kit is based on the TM concentration needed to inhibit 50% of the ETP in a pooled normal plasma. If we then compare this ETP% inhibition to the data obtained in this study, we indeed observed an overall ETP inhibition of 48.3%.

The ST Genesia is based on its precursor the CAT assay. They both use the same thrombin-sensitive substrate, but one of the major differences between the CAT assay and the ST Genesia is the method used for calibrating the samples. For the CAT assay, a thrombin calibrator is used (α2-macroglobulin–thrombin complex) that is added to every sample. Calibration of samples on the ST Genesia occurs in two steps: (i) Once daily a calibration curve is obtained using purified human thrombin in buffer with the fluorogenic substrate and also the activity of a fluorophore product is measured in the same buffer; and (ii) every plasma sample is spiked with a fluorophore product and measured. By taking the ratio between the fluorophore product measured in the plasma sample and in the buffer system, the plasma sample can be calibrated correctly. The CAT

### Table 5: Reference ranges for the ST Genesia

| Reference ranges, absolute data (%) | Lag time (min) | Peak height (nM) | Time to peak (min) | ETP (nM/min) | Velocity index (nM/min) | Start tail (min) | ETP% |
|------------------------------------|----------------|-----------------|-------------------|-------------|------------------------|----------------|------|
| STG-BleedScreen (N = 111-117)      | 1.9            | 4.4             | 92                | 320         | 4.2                    | 9.0            | 706  |
| STG-DrugScreen (N = 115-117)       | 1.0            | 2.0             | 205               | 620         | 2.0                    | 4.3            | 759  |
| STG-ThromboScreen (N = 110-117)    | 1.6            | 3.3             | 108               | 372         | 3.5                    | 7.5            | 657  |
| STG-ThromboScreen + TM (N = 109-116) | 1.5        | 3.4             | 51                | 319         | 3.3                    | 6.2            | 209  |

| Reference ranges, normalized data | Lag time (min) | Peak height (nM) | Time to peak (min) | ETP (nM/min) | Velocity index (nM/min) | Start tail (min) | ETP% |
|----------------------------------|----------------|-----------------|-------------------|-------------|------------------------|----------------|------|
| STG-Bleedscreen (N = 96-102)     | 0.7            | 1.5             | 75                | 255         | 0.7                    | 1.5            | 75   |
| STG-Drugscreen (N = 90-93)       | 0.8            | 1.4             | 44                | 106         | 0.9                    | 1.8            | 43   |
| STG-ThromboScreen (N = 97-102)   | 0.8            | 1.6             | 48                | 163         | 0.8                    | 1.7            | 47   |

Note: The reference ranges are obtained from the whole population of healthy donors excluding outliers. Data are expressed as raw and normalized values of the 2.5% - 97.5% range for all three reagents.

Abbreviations: ETP, endogenous thrombin potential; TM, thrombomodulin.
The assay remains a very important tool in the hemostatic research field, as it can be modified to investigate a specific part of the coagulation cascade, for example, by using a specific activator or by including the presence of the platelets.30,31 The ST Genesia also offers the possibility of using an in-house activator, although this could result in the same problems as with the other TG assays: no standardization of the reagent and therefore the data will not be comparable between laboratories.

Even though the number of women taking OCs was low in this study (n = 15), and their inclusion did not alter TG parameters significantly, we did observe significantly higher TG values between those women and men and women without the use of OCs. This

**Table 6**  TG parameter comparison between men and women with and without the use of oral contraceptives

|                      | Men (N = 58) | Women –OCs (N = 44) | Women + OCs (N = 13-15) | P value |
|----------------------|-------------|---------------------|------------------------|---------|
|                      | Median IQR  | Median IQR          | Median IQR             |         |
| STG-BleedScreen      |             |                     |                        |         |
| Lag time, min        | 3.1 2.8-3.6 | 2.7 2.5-3.2         | 2.5 2.1-2.6            | .0082   |
| Peak height, nM      | 162 140-194 | 164 120-194         | 271 223-312            | .0001   |
| Time to peak, min    | 6.8 6.1-7.6 | 6.3 5.8-7.2         | 5.2 4.3-5.9            | .0102   |
| ETP, Nm/min          | 1092 937-1229 | 1070 906-1312 | 1374 1147-1543         | NS      |
| Velocity index, nM/min | 58.4 42.1-74.8 | 56.9 43.3-77.3 | 130 89.4-157           | .0001   |
| Start tail, min      | 19.8 17.8-21.9 | 20.1 16.7-22.7 | 16.0 14.6-18.9         | .0062   |
| STG-DrugScreen       |             |                     |                        |         |
| Lag time, min        | 1.4 1.3-1.6 | 1.3 1.2-1.4         | 1.2 1.1-1.3            | .0181   |
| Peak height, nM      | 334 296-378 | 338 301-402         | 483 410-539            | NS      |
| Time to peak, min    | 3.1 2.6-3.4 | 2.7 2.4-3.0         | 2.4 2.1-2.5            | .0246   |
| ETP, nM/min          | 1185 1081-1402 | 1224 1059-1413 | 1409 1183-1851         | NS      |
| Velocity index, nM/min | 299.0 206-367 | 338.0 277-425 | 585 418-782            | .0004   |
| Start tail, min      | 11.4 10.6-12.8 | 11.3 10.2-12.3 | 10.9 10.1-11.7         | NS      |
| STG-ThromboScreen -TM|             |                     |                        |         |
| Lag time, min        | 2.5 2.2-2.8 | 2.2 2.0-2.5         | 2.0 1.8-2.2            | .0390   |
| Peak height, nM      | 190 169-219 | 194 161-229         | 280 240-376            | NS      |
| Time to peak, min    | 5.7 5.1-6.4 | 5.1 4.7-6.1         | 4.4 3.6-4.7            | .0077   |
| ETP, nM/min          | 1130 974-1362 | 1100 969-1332 | 1414 1154-1774         | NS      |
| Velocity index, nM/min | 71.9 59.6-95.2 | 83.7 63.1-112 | 191 118-259            | .0001   |
| Start tail, min      | 17.6 14.9-19.3 | 16.8 15.2-19 | 14.6 13.6-15.4         | .0153   |
| STG-ThromboScreen + TM|             |                     |                        |         |
| Lag time, min        | 2.5 2.2-2.8 | 2.1 2.0-2.5         | 2.0 1.8-2.2            | .0435   |
| Peak height, nM      | 114 83-137  | 133 91-180          | 309 197-339            | NS      |
| Time to peak, min    | 4.8 4.3-5.1 | 4.4 4.1-4.8         | 3.9 3.4-4.2            | .0003   |
| ETP (nM/min)         | 499 373-643 | 594 425-799         | 1124 900-1397          | NS      |
| Velocity index, nM/min | 64.7 45.7-78.9 | 78.9 50.5-118 | 217 123-295            | .0001   |
| Start tail, min      | 13.3 12.6-14.8 | 13.5 12.8-14.1 | 13.2 12.1-14.3         | NS      |
| ETP inhibition, %    | 57 43-66 | 45 35-63         | 25.4 19-31             | .0005   |

Note: Data are shown as median values with interquartile ranges (IQRs).
Abbreviations: ETP, endogenous thrombin potential; M, men; NS, not significant; OCs, oral contraceptives; TM, thrombomodulin; W−, women without OCs; W+, women with OCs.
finding, also demonstrated by Calzavarini et al., was seen with the three ST Genesia reagents and for almost every TG parameter. From these data an important question arises whether women taking OCs should be included when reference values are being generated. It is a well-known phenomenon that the use of OCs affects coagulation and increases the activated protein C resistance in women. Indeed, the ETP inhibition was 54.2% for men, 47.4% for women without OCs, and only 25% for women with OCs. However, these women are mostly young adults who are part of the adult healthy population. Therefore, the impasse will remain whether these women should be included in the reference values of healthy donors. Interestingly, no differences were observed between men and women without the use of OCs. Calzavarini et al showed the same findings, though only by two of the available reagents (STG-BleedScreen and STG-ThromboScreen). Therefore, we can exclude the need of separate reference values for men and women, which prevents the need for 240 healthy donors to achieve the 90% confidence limit of the upper and lower reference limits when using nonparametric statistics.

Some limitations of our study are that the data come from only one research center and only one batch of reagents was used to measure all samples. A similar study should be performed by another research lab to confirm our data, but the batch-to-batch variability can be solved by using the normalized data of this study. Additionally, differences in ethnicity should also be investigated with the ST Genesia, as they could result in differences in TG. Currently, contradicting data are published, whereby Tan et al. did not find differences in TG, while other research groups did. From our previous TG survey, we have learned that not all research groups store their plasma samples at −80°C but also at −20°C. Therefore, it could be interesting for future studies to investigate the effect of long-term sample storage at −80°C and −20°C, as well as the freeze-thaw effects on the reproducibility of the ST Genesia. Douxfils et al. tested the effect of long-term sample storage at −70°C with the STG-DrugScreen and concluded that samples were stable up to at least 11 months. As this was not tested with the other reagents, it thus generates another study limitation.

To conclude, we determined the reference ranges of the ST Genesia for the reagent kits available at the moment (STG-BleedScreen, STG-DrugScreen, and STG-ThromboScreen). These reference ranges can be applied by other laboratories using this assay. The ST Genesia finally brings the TG assay into the clinic in a standardized and qualitative manner. However, clinical validation studies will be needed in the near future to establish cutoff values that could be used in the clinic for therapeutic decisions.

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RELATIONSHIP DISCLOSURE
The authors declare no conflicts of interest.
AUTHOR CONTRIBUTIONS
BL and AC designed the study. RLK collected the samples. MN performed the experiments and wrote the first draft of the manuscript. RLK, AC, and BL critically revised the manuscript.

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

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