Thrombin generation and rotational thromboelastometry in the healthy adult population

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Keywords
Thrombin generation, calibrated automated thrombogram, rotational thromboelastometry

Summary
Aim: Published data on thrombin generation variables and their correlation with thromboelastometry in the healthy population are scarce. This study aimed at assessing thrombin generation in adults and its correlation to classical rotational thromboelastometry (ROTEM). Methods: Thrombin generation was measured in platelet-poor plasma from healthy volunteers using the calibrated automated thrombogram (CAT) with 1 and 5 pmol/l tissue factor and ROTEM. ETP (p = 0.001), although with a moderate regression slope. Regarding ROTEM, there was a positive correlation between age and maximum clot firmness and alpha angle (p = 0.001), but a negative correlation between age and clotting time (p = 0.039). Comparing both assays, thrombin peak and ETP measured with a final tissue factor concentration of 5 pmol/l correlated significantly with alpha angle and maximum clot firmness. Conclusion: The age-related changes in CAT and ROTEM variables among adults are not linear. There is a significant correlation, although with a moderate slope, between data from CAT measured with 5 pmol/l tissue factor and ROTEM.

Schlüsselwörter
Thrombingenerierung, Calibrated Automated Thrombogram, Rotationsthromboelastometrie

Zusammenfassung
Es gibt nur wenige Studien zur Korrelation zwischen den Parametern Thrombingenerierung und Thromboelastometrie an Gesunden. Ziel der Studie ist die Thrombingenerierung an gesunden Erwachsenen zu untersuchen und die Daten mit denen der klassischen Rotationsthromboelastometrie (ROTEM) zu vergleichen. Methoden: Thrombingenerierung wurde in plättchenarmem Plasma mittels Calibrated Automated Thrombogram (CAT) mit 1 und 5 pmol/l Gewebefaktor-Endkonzentration analysiert. Die Parameter Lag-time, Thrombin-Peak, Time-to-thrombin-Peak und endogenes Thrombinpotenzial (ETP) wurden ausgewertet. ROTEM wurde ohne Aktivator (NATEM) durchgeführt und die Daten für Gerinnungszeit, Alpha-Winkel, Gerinnelbil- dungszzeit und maximale Gerinnelfestigkeit dokumentiert und mit denen der CAT korreliert. Ergebnisse: Insgesamt nahmen 132 Personen (72 Männer, 60 Frauen; medianes Alter: 48,0 Jahre) teil. Eine signifikante positive nicht-lineare Korrelation für Alter versus Lag-time (p < 0,001) und Time-to-peak (p = 0,001) und nahezu lineare Korrelation für Alter versus Thrombin-Peak (p = 0,024) und ETP (p = 0,001) wurde beobachtet, allerdings mit moderater Regressionsteigung. Im Bezug auf ROTEM gab es eine positive Korrelation zwischen Alter und maximaler Gerinnelfestigkeit und Alpha-Winkel (p = 0,001), jedoch eine negative Korrelation zwischen Alter und Gerinnungszeit (p = 0,039). Thrombin-Peak und ETP, gemessen mit 5 pmol/l Gewebefaktorkonzentration, zeigten eine signifikante Korrelation gegenüber maximaler Gerinnelfestigkeit und Alpha-Winkel, allerdings mit moderater Regressionsteigung. Schlussfolgerung: Altersabhängige Änderungen der CAT- und ROTEM-Parameter bei Erwachsenen sind nicht linear. Die Korrelation zwischen CAT und ROTEM ist je nach Gewebefaktorkonzentration im Testansatz unterschiedlich.

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The thrombin generation (TG) assay is frequently applied in experimental and clinical studies in the preceding decades. The fundamental hypothesis behind this assay is that the amount and the kinetics of thrombin generation describe the haemostatic potential of a subject (10).

The TG assay is generally considered as the laboratory means of defining the cell-based model of haemostasis.

It displays four variables, mainly the
• lag time,
• thrombin peak,
• time to thrombin peak and
• endogenous thrombin potential (ETP).

Many publications have shown that the TG assay can characterise
• bleeding diathesis (2, 4, 15, 24) and
• thrombophilic states (5, 7, 11, 18, 23) as well as
• monitoring anticoagulant treatment (1, 22).

The relevance of the TG variables in different clinical states is still not fully understood. The widely used assay is the CAT (calibrated automated thrombogram). Studies concerning the reference range in the healthy adult population are scarce. One study included mainly children (9), another one a relatively small number of healthy adults (8). A detailed investigation on healthy adults showed a good intra- and inter-assay variation, concluding the clinical applicability of CAT using platelet-poor plasma (PPP) (25).

ROTEM (rotational thromboelastometry) is based on an old test principle, which is basically the observation of clot development and firmness, particularly the formation of fibrin polymers and their interaction with platelets (3, 17, 20). It has been reported that the three phases of the cell-based haemostasis, namely initiation, amplification and propagation can be described by ROTEM (12, 13, 14). In this context, the
• clotting time (CT) corresponds to the initiation phase,
• clot formation time and the α-angle to the amplification phase,
• maximum clot firmness to the propagation phase.

This prospective study aimed at measuring thrombin generation in a healthy adult population and to correlate the data with those from ROTEM measurements. The study was part of a project to test the predictive value of these assays in the preoperative evaluation of the coagulation system.

Study participants and methods

The study included healthy volunteers and it was approved by the local ethics committee. Subjects were included after an informed consent. Exclusion criteria were body mass index (BMI) > 30 kg/m², a known coagulopathy, liver disease, active malignancy, current anticoagulant treatment, sign of active infection or bleeding, pregnancy, use of hormonal contraception or hormone replacement therapy and age below 18 years. Clinical history regarding bleeding diathesis and medication was recorded using the questionnaire published by Koscielny et al. (16).

Blood samples were drawn in the morning hours between 9 and 11 a.m. by an antecubital venipuncture using a 19 Gauge cannula and collected into Sarstedt tubes containing 3.8% citrate. Data for haematocrit, platelet count, activated thromboplastin time (aPTT) and the Quick test were collected. Samples for the CAT assay were centrifuged within an hour after collection at 20°C at 170 g for 10 min and then at 1800 g for 20 min to prepare a platelet-poor plasma (PPP). PPP aliquots were then immediately stored at −70°C until measurement. The thrombin generation (TG) assay was carried out on a Fluoroscan Ascent (Helsinki, Finland) at 390/460 nm wave length using the calibrated automated thrombogram (CAT) with commercially available test kits (Thrombinoscope BV, Maastricht, NL). In short, in every test run, 20 µl of the thrombin calibrator and 80 µl PPP of the patient were transferred into a well of a 96-well microtiter plate (Greiner Bio One, Germany). Simultaneously, 20 µl of the PPP reagent containing tissue factor (TF) were transferred into another well and mixed with 80 µl of PPP of the patient, the final solution in this well containing 5 pmol/l TF and 4 µmol/l phospholipids. Then, 20 µl of a solution containing a fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and buffer prepared according to the instruction of the manufacturer was added into each well. The test was also run with 1 pmol/l TF in the final solution. Data for lag time, thrombin peak, time to thrombin peak and ETP were calculated from the signals derived using the computer program provided by the manufacturer.

ROTEM was conducted using the commercially available equipment according to the standard protocol supplied by the manufacturer (tem International, Munich, Germany). Blood samples collected as described above were analyzed immediately. The non-activated thromboelastometry (NATEM) assay was run for at least 30 minutes. This is a semi-quantitative assay used to monitor the coagulation process after contact activation by the surface of the measurement cell. The NATEM reagent contains 0.2 mmol/l CaCl₂ in HEPES buffer with pH 7.4 and 0.1% sodium azide.

20 µl of this reagent and 300 µl of citrated whole blood were added into the prewarmed ROTEM cup. Data for clotting time (CT), clot formation time (CFT),

Tab. 1 Basic laboratory data (mean ± SD) of the study population (in brackets: 95% confidence interval)

<table>
<thead>
<tr>
<th>variable</th>
<th>total population</th>
<th>men</th>
<th>women</th>
<th>Pmen vs. women</th>
</tr>
</thead>
<tbody>
<tr>
<td>haematocrit (%)</td>
<td>41.9 ± 3.9</td>
<td>43.0 ± 4.1</td>
<td>40.4 ± 3.2</td>
<td>0.001</td>
</tr>
<tr>
<td>(41.1–42.8)</td>
<td>(41.9–44.1)</td>
<td>(39.3–41.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>platelet count (per nl)</td>
<td>249 ± 54.7</td>
<td>248 ± 57.7</td>
<td>251 ± 51</td>
<td>0.667</td>
</tr>
<tr>
<td>(238–261)</td>
<td>(233–264)</td>
<td>(234–267)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>30.7 ± 3.1</td>
<td>30.3 ± 3.1</td>
<td>30.5 ± 3.2</td>
<td>0.888</td>
</tr>
<tr>
<td>(29.7–31.0)</td>
<td>(29.4–31.1)</td>
<td>(29.4–31.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quick value (%)</td>
<td>109 ± 10.8</td>
<td>109 ± 9.4</td>
<td>110 ± 12.8</td>
<td>0.569</td>
</tr>
</tbody>
</table>
alpha angle and maximum clot firmness (MCF) were collected.

Statistical analysis was conducted using the program SPSS Version 20.0. Regression analysis was used to test the relationship between haematocrit and platelet count on the one hand and ROTEM variables on the other hand. Gender comparison was also conducted for all variables tested. Pearson correlation was applied for correlation analysis between age and TG as well as ROTEM variables. Correlation was tested for the following variable pairs: lag time vs. CT, time to thrombin peak vs. CFT, thrombin peak vs. alpha angle and ETP vs. MCF. Furthermore, assuming that a direct linearity between age and the coagulation variables would not be observed, the LOESS (locally weighted scatterplot smoothing) function was applied in computing the regression lines. A sampling proportion of 0.7 was used for defining the grade of smoothing. Data are given as median with interquartile ranges in brackets, otherwise as mean ± standard deviation with 95% confidence interval in brackets. A value for p < 0.05 was considered statistically significant.

**Results**

A total of 132 persons (72 men, 60 women) with a median age of 48.0 [30.0] years and...
mean BMI of $24.8 \pm 2.2$ kg/m$^2$ were included in the study (no age difference between men and women, $p = 0.68$). Basic laboratory data of the subjects are given (▶Tab. 1), with haematocrit the only variable with a statistically significant gender difference.

The mean intra-assay coefficient of variation for ROTEM variables was 5.0% for CT, 7.0% for CFT, 4.0% for MCF and 10.0% for alpha angle. For CAT variables, the figures were 10.0% for lag time, 5.5% for time to peak, 9.9% for ETP and 6.6% for thrombin peak. There was a significant positive correlation, although with a moderate slope, between age and all four TG variables: lag time ($p = 0.032$), time to peak ($p < 0.001$), thrombin peak ($p < 0.001$) and ETP ($p < 0.001$), both with 1 and 5 pmol/l TF test run. Controlled by LOESS, the correlation was almost linear for ETP with both 1 and 5 pmol/l TF and thrombin peak with 5 pmol/l TF. Lag time and time to peak with both TF concentrations and thrombin peak with 1 pmol/l TF showed deviation from linear correlation with age (▶Fig. 1a).

The data from the ROTEM assay showed a significant positive correlation, but with a moderate slope, for age versus MCF ($p = 0.001$) and alpha angle ($p < 0.001$), while there was a negative correlation for age versus CT ($p = 0.039$) (▶Fig. 1b). There was no significant correlation between platelet count and ROTEM variables. On the other hand, there was a significant but moderate correlation between haematocrit and CFT ($r = 0.26, p = 0.028$) and a negative correlation between haematocrit and alpha angle ($r = -0.375, p = 0.002$).

The data of both assays for men and women are shown (▶Tab. 2). Significant gender differences were observed for ROTEM variables.

With CAT using 1 pmol/l TF, no correlation was observed between TG and ROTEM variables, while there was a significant correlation between thrombin peak vs. alpha angle ($p = 0.015, r = 0.244$) and ETP vs. MCF ($p = 0.005, r = 0.281$) with CAT assay using 5 pmol/l TF (▶Fig. 2). Regarding lag time vs. CT and time to thrombin peak vs. CFT, no correlation was observed regardless of the TF concentration in the CAT assay.

### Discussion

Thrombin generation is supposed to reflect the overall coagulation potential of the haemostatic system. However, its application in clinical practice is still limited.

1. There is still a lack of standardisation in the test setup (6, 19, 21).
2. The need for different tissue factor and phospholipid concentrations based on the particular haemostatic issue (for example bleeding diathesis vs. thrombophilia) is not clearly defined.

3. The value of the individual TG variables for a particular clinical issue is neither fully understood nor defined.

Therefore, further understanding of the normal distribution in healthy subjects may help to define the value of the individual TG variables.

Thrombin peak and ETP show a trend towards gradual increase with increasing age (9, 25). Of note is that the LOESS function applied in this study indicates non-linearity between age and coagulation variables. Our findings support the conclusion by Spronk et al. (25) that these age-related changes would not be of any clinical importance.

Haidl et al. showed a negative correlation between lag time and time to thrombin peak on the one hand and age on the other hand, although the correlation was weak (9). In contrast, in our study that included older age groups, we have found a weak positive correlation between age and these TG variables. The reason for these conflicting findings is not clear. One explanation could be related to the method of computing these time variables in the assay. Another reason could be the inter-individual variation. On the other hand, the clotting time on ROTEM showed a negative correlation with age. One explanation for this finding is that we performed ROTEM in this study with contact activation, thus the age-associated increase in FVIII may be the cause of the shortening of CT with increasing age.

Data on the relationship between TG and ROTEM variables are scarce. One study (13) showed similar relationship between CAT using platelet-rich plasma and rotational thromboelastography while analysing stored blood samples. In our study, a positive correlation between CAT and ROTEM was observed only for thrombin peak vs. alpha angle and ETP vs. MCF, depending on the TF final concentration in the CAT assay. These findings demonstrate the in vitro manipulation of haemostatic variables depending on the reagent constitution and test procedures, making comparison impossible, at best difficult.

Limitation

In this study, two methods were compared, which suffer from lack of appropriate and generally accepted standardisation.

While CAT is still mainly a research tool, ROTEM is frequently in use as a perioperative diagnostic tool.

While CAT in this study was conducted using platelet-poor plasma, whole blood is used in ROTEM. Therefore, head-to-head comparison is difficult. Comparing CAT data in platelet rich plasma may show a better correlation with ROTEM due to the contribution of platelets in thrombin generation. However, the cumbersome and time-consuming process of TG in platelet-rich plasma makes this assay clinically, particularly in acute conditions, not feasible. Furthermore, we have applied NATEM instead of EXTEM while conducting ROTEM. The aim was to test the basic clinical utility of these assays. Using EXTEM instead of NATEM would not make the two assays more comparable, because of the markedly higher concentration of tissue factor in the EXTEM reagent than that in CAT.

Conclusion

Our study demonstrates a non-linear correlation of thrombin generation and ROTEM variables with age among the general healthy population. Although there is a significant correlation between the parameters of both assays depending on the test setup, the regression slope is moderate, which has to be considered in data interpretation. Based on our findings, the implementation of these two methods in routine clinical practice is generally questionable. They may have importance as treatment control tools, each patient being his own control.

Further research is still required on the clinical implication of any deviation from the normal range in an otherwise healthy subject and the predictive value of both methods.

Conflict of interest

The authors declare that they have no conflict of interest.

References