Thrombin generation in paediatric patients with congenital heart disease*

Determination by calibrated automated thrombography

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Summary
Thrombin generation was studied in paediatric patients with congenital heart disease (CHD) undergoing cardiac surgery using the calibrated automated thrombography (CAT) in terms of the lag time until the onset of thrombin formation, time to thrombin peak maximum (TTP), endogenous thrombin potential (ETP), and thrombin peak height. The suitability to determine the coagulation status of these patients was investigated. *Patients, material, methods:* CAT data of 40 patients with CHD (age range from newborn to 18 years) were compared to data using standard coagulation parameters such as prothrombin fragment 1.2 (F1.2), antithrombin (AT), tissue factor pathway inhibitor (TFPI), activated partial thromboplastin time (aPTT), and prothrombin time (PT). *Results:* A significant positive correlation was seen between ETP and F1.2 (p < 0.05, r = 0.349) and between peak height and F1.2 (p < 0.01, r = 0.463). A significant negative correlation was seen between ETP and TFPI values (p < 0.05, r = −0.225) while no significant correlation was seen between peak height and TFPI. A significant negative correlation was seen between F1.2 generation and ETP (p < 0.05, r = −0.23) and between F1.2 generation and peak height (p < 0.05, r = −0.236). No correlation was seen between AT and ETP or peak.

Conclusions: CAT is a good global test reflecting procoagulatory and inhibitory factors of the haemostatic system in paediatric patients with CHD.

Keywords
Calibrated automated thrombography, thrombin generation, endogenous thrombin potential, prothrombin, cardiac surgery, congenital heart disease

Schlüsselwörter
Kalibrierte automatisierte Thrombographie, Thrombinentstehung, endogenes Thrombinpotenzial, Prothrombin, Herzoperation, angeborene Herzfehler

Zusammenfassung
Wir untersuchten die Thrombinentstehung bei Kindern mit angeborenen Herzfehlern unter Herzoperationen. Es eignet sich daher als globale Test. *Patienten, Material, Methoden:* Wir untersuchten 40 Kinder mit angeborenen Herzfehlern vom Neugeborenen- bis ins Jugendalter. Unsere CAT-Daten haben wir verglichen mit Messgrößen wie AT, TTP und ETP. *Ergebnisse:* Wir fanden eine positive Korrelation zwischen ETP und F1.2 sowie zwischen der maximalen Höhe des Thrombinbursts und F1.2. Eine negative Korrelation zeigte sich zwischen F1.2 und ETP sowie zwischen der maximalen Höhe des Thrombinbursts und AT. *Schlussfolgerung:* Das neue CAT spiegelt die pro- und antikoagulatorischen Faktoren des Gerinnungssystems bei Kindern mit angeborenen Herzfehlern gut wider. Es eignet sich daher als globaler Test.

Thrombinbildung bei Kindern mit angeborenem Herzfehler
Bestimmung durch kalibrierte automatisierte Thrombographie


Cardiac surgery is associated with the risk of either significant postoperative bleeding or thrombotic complications in paediatric patients. Using cardiopulmonary bypass (CPB) requires heparin administration to prevent clot formation within the extracorporeal circuit, and normal AT levels to effectively catalyze this antithrombotic action of heparin (1). This complex is known to inhibit thrombin, the pivotal enzyme in haemostasis (2). As a major regulator of haemostasis thrombin plays several roles such as modulating the cleavage of fibrinogen to fibrin, activating platelets and the process of fibrinolysis and also stimulation of the vascular endothelium to release inflammatory mediators. Indeed, some of the morbidity following cardiac surgery may be the consequence of mediators released due to incomplete inhibition of thrombin during CPB (3).

We evaluated the thrombin generation (TG) in our paediatric patients undergoing cardiac surgery. Several studies showed elevated postoperative levels of markers of thrombin generation such as TAT and F 1.2 generation for days (4, 5) after heart surgery. It has been shown that some coagulation parameters return to normal within days whereas others return to preoperative levels within 24 hours after surgery (6).

In this study we investigated the TG by means of calibrated automated thrombography (CAT). CAT is the latest derivative of a global thrombin generation assay. This method allows detection of the influence of procoagulants and anticoagulants on the formation of thrombin (7, 8). The data were investigated with respect to lag time preceding the thrombin burst, time to peak (TTP), peak height, and endogenous thrombin potential (ETP). Measurements were carried out in the presence of low amounts (~0.35 pmol/l final concentration) of tissue factor (TF), a condition that allows sensitive detection of the influence of anticoagulants on thrombin formation (9–11). This procedure not only allows us to measure the effects of both pro- and anticoagulant agents at various concentrations, with greater sensitivity (2), but probably reflects in vivo conditions more closely (7). The TG was further investigated by means of the key parameters prothrombin (FII), antithrombin (AT),...
tissue factor pathway inhibitor (TFPI),
- thrombin antithrombin complex (TAT),
- prothrombin fragment 1.2 (F 1.2),
- prothrombin time (PT), and
- activated partial thromboplastin time (aPTT).

The aim of our study was to investigate the possible suitability of the CAT for determination of TG in pediatric heart patients undergoing open heart surgery when compared to standard coagulation assays that are conventionally used.

Patients, material, methods

Collection of blood samples, ethics

Samples of 40 paediatric patients with CHD (age ranged from newborn to 18 years) were obtained from the blood that was collected for the routine coagulation screening before cardiac surgery and furthermore in a post-operative control 7 days following open heart surgery. Blood was collected into plastic tubes containing sodium citrate (0.1 mol/l end concentration) using S-Monovet® (DMSO), which was purchased from Sigma, St. Louis, Mo., USA. Calcium chloride was purchased from Merck, Darmstadt, Germany. Testkit F 1.2 micro and TAT micro, for determination of F 1.2 and TAT respectively, were purchased from Dade Behring (Marburg, Germany). Stopping solution for F 1.2 and TAT determination consisted of 80 ml sodium citrate (3.8%), 10 ml 0.2 mol/l EDTA, 10 ml Trasylol® (from Bayer; Vienna, Austria), and 110 µmol/l dPhe-Pro-Arg chloromethyl ketone from Sigma (Vienna, Austria). Actichrome™ TFPI activity assay was obtained from American Diagnostica Inc. (Greenwich, CT, USA). The coagulation factor II deficient plasma, for determination of prothrombin, was purchased from Dade Behring (Marburg, Germany). The platelet-poor plasma (PPP) reagent with a content of 5 pmol/l tissue factor and 4 µmol/l phospholipids and the thrombin calibrator was purchased from Thrombinoscope BV, Maastricht, Netherlands.

Thrombin generation

Measurement of the thrombin generation was performed using the CAT (8). For each experiment a fresh mixture of 2625 µl fluorobuffer and 300 µl of 1 mol/l CaCl₂ solution was prepared and incubated for 5 minutes at 37°C. After 5 minutes 75 µl of the Fluor-DSMO-solution were added, mixed and incubated for 5 minutes again. The resulting cleavage solution was referred to as FluCa. PPP-reagent was solubilized with 2 ml deionised water.

Twenty µl of this TF containing trigger solution were put into each sample well of a 96-well round-bottom microtiter plate made of polypropylene, purchased from Nunc, Roskilde, Denmark. After reconstitution with 1 ml deionised water, the thrombin calibrator was used in each experiment to compare the simultaneously measured thrombin activity in the sample to that from a known and stable concentration in the calibrator well. Finally, 80 µl of PPP were put into each well. All reagents were warmed up to 37°C before starting the experiment. The 96-well-plate was put in the fluorometer (Fluoroskan Ascent, Thermolabsystems OY, Helsinki, Finland) with an excitation filter at 390 nm and an emission filter at 460 nm. The automated dispensing of 20 µl FluCa started the measurement process. During 60 minutes each well was measured every 20 seconds. Each experiment was performed six times. Upon completion of the measurement we used the Analysis Software from Thrombinoscope BV to analyze our results. The inter-assay coefficient of variance was lag time 10%, ETP 7%, TTP 12%, and peak thrombin 8%.

AT, F 1.2, TAT, TFPI, prothrombin

Determination of the AT content of plasma was performed by means of a standard chromogenic method on a BM/Hitachi 917 from Boehringer Mannheim, Vienna, Austria.

For the determination of F 1.2 and TAT plasmas were prepared and activated as described above. At timed intervals, 10-µl aliquots were withdrawn from the plasma and subsampled into 490 µl stopping solution. Subsamples were divided into two aliquots, 1 and 2. After subsequent 1:10 dilution of aliquot 1 in stopping solution, the amount of F 1.2 generated was quantified with a standard immunoenzymatic test kit. After 1:20 dilution of aliquot 2 in stopping solution, TAT generation was quantified with an immunoenzymatic kit.

TFPI antigen levels in plasma were determined by means of the Imubind™ Total TFPI ELISA Kit.

Determination of the prothrombin values in plasma were determined using coagulation factor II deficient plasma. 50 µl of a complete thromboplastin (Thromborel STM) were added to 50 µl of the citrated plasma samples. For determination of the prothrombin values we used the Behring Coagulation Timer, from Behring Diagnostics GmbH (Marburg, Germany).

aPTT, PT

For the determination of activated partial thromboplastin time (aPTT) 50 µl of a partial thromboplastin (Pathrombin SL™) were added to 50 µl of the citrated plasma samples and then the mixture was recalculated by addition of 50 µl CaCl₂. For determination of the aPTT we used the Behring

Reagents and devices

Fluobuffer contained 20 mmol/l HEPES and 60 mg/ml bovine serum albumin (both Sigma, St. Louis, Mo., USA). Working buffer consisted of 140 mmol/l NaCl (Merck, Darmstadt, Germany), 20 mmol/l HEPES and 5 mg/ml human serum album (both Sigma, St. Louis, Mo., USA). The fluorogenic substrate Z-Gly-Gly-Arg-amino-methyl-coumarin was purchased from Bachem, Bubendorf, Switzerland, and was solubilised in pure dimethylsulfoxide (DMSO), which was purchased from Sigma, St. Louis, Mo., USA. Calcium chloride was purchased from Merck, Darmstadt, Germany. Testkit F 1.2 micro and TAT micro, for determination of F 1.2 and TAT respectively, were purchased from Dade Behring (Marburg, Germany). Stopping solution for F 1.2 and TAT determination consisted of 80 ml sodium citrate (3.8%), 10 ml 0.2 mol/l EDTA, 10 ml Trasylol® (from Bayer; Vienna, Austria), and 110 µmol/l dPhe-Pro-Arg chloromethyl ketone from Sigma (Vienna, Austria). Actichrome™ TFPI activity assay was obtained from American Diagnostica Inc. (Greenwich, CT, USA). The coagulation factor II deficient plasma, for determination of prothrombin, was purchased from Dade Behring (Marburg, Germany). The platelet-poor plasma (PPP) reagent with a content of 5 pmol/l tissue factor and 4 µmol/l phospholipids and the thrombin calibrator was purchased from Thrombinoscope BV, Maastricht, Netherlands.
To determine the prothrombin time (PT) 100 µl of a complete thromboplastin (Thromborel S™) were added to 50 µl of the citrated plasma samples. For determination of the PT we used the Behring Coagulation Timer, from Behring Diagnostics GmbH.

**Statistics**

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, Illinois, USA). Correlation between the standard coagulation assays and the thrombin generation values using CAT were calculated using Pearson’s correlation. P-values less than 0.05 were considered as significant.

**Results**

**Thrombin generation**

The characteristics of a typical CAT resultant curve are described as follows: The time until to the onset of TG during the curve remains flat is referred to as lag time. After a steep increase the thrombin generation curve arrives at its peak, the maximum concentration of thrombin. The inclination of the curve until the thrombin peak is reached is described as time to peak (TTP). The start tail marks the endpoint of the decay of thrombin formation. The area under curve (ETP) represents the total amount of thrombin built over the time during the whole process of TG and stands for a real time monitoring of the coagulability of blood (7, 12).

Figure 1 shows a typical curve in one of our patients (8 years old patient with tricuspid atresia undergoing the Fontan procedure) before and after the cardiac surgery. The patient was under oral anticoagulation in the measurements after the cardiac surgery.

**Congenital heart defects**

The different cardiac defects of our paediatric patients are shown in Table 1. The patients were divided into age groups:

- Infants below 1 year of age,
- Children up to an age of 10 years, and
- Adolescents (age: 10–18 years).

The results of thrombin generation measurements in different age groups of the 40 patients with CHD are shown in Table 2.

**Parameters before and after open heart surgery**

The PT, aPTT, FII and AT levels were within the normal age specific ranges before and after surgery in our patients, therefore showing no signs of an acquired deficiency state. FII levels were slightly higher in the postoperative measurements, TFPI values slightly but not significantly lower in the postoperative measurements when compared to the preoperative measurements (data not shown).

In nine of our patients after cardiac surgery an oral anticoagulation was necessary. In those patients we observed a prolonged lag time, a prolonged TTP, a reduced peak height and a reduced ETP postoperatively when compared to preoperative measurements (one representative example is shown in Fig. 1).

**Correlations**

A significant positive correlation was seen between ETP and FII (p < 0.01; r = 0.369) (Fig. 2a), as well as between peak height and FII (p < 0.01; r = 0.483) (Fig. 2b) in our pediatric heart patients. A significant negative correlation was seen between ETP and the TFPI values (p < 0.05; r = -0.225) (Fig. 2c).

**Tab. 1 Congenital heart defects of the patients (n=40)**

<table>
<thead>
<tr>
<th>age (years)</th>
<th>≤1</th>
<th>1–10</th>
<th>&gt;10</th>
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<tr>
<td>atrial septal defect</td>
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<td>4</td>
<td>3</td>
</tr>
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<td>3</td>
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<td>0</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>0</td>
</tr>
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<td>tetralogy of Fallot</td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>TAPVR</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>single ventricle*</td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td>MS/MR</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>AS/Ca/o/Al</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>PA/PS/TA</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

* Children included who required an atrial septectomy, Glenn anastomosis, or Fontan procedure.

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Fig. 1 Thrombin generation (TG) curves in a patient with tricuspid atresia undergoing the Fontan procedure before (■) and after (▲) its cardiac surgery.
while no significant correlation was seen between peak height and TFPI values (Fig. 2d). A significant negative correlation was detected between F1.2 generation and ETP (p < 0.05; r = -0.254) (Fig. 3a) and between F1.2 generation and peak height (p < 0.05; r = -0.236) (Fig. 3b). No significant correlation could be detected between ETP as well as peak and AT (data not shown).

**Discussion**

Paediatric patients with CHD have a high risk of developing bleeding and/or thrombotic complications following heart surgery with a broad spectrum within the different heart diseases (13, 14). Heying et al demonstrated a marked imbalance between prothrombotic and antithrombotic activity in paediatric heart patients undergoing cardiac surgery (15). In routine clinical practice, clinicians often rely on the PT and aPTT to assess the patient’s haemostatic status. These routine parameters were checked and were altogether to be normal in the measurements before and after the cardiac surgery in all of our patients. This is in agreement with the observed clinical fact that none of our patients suffered from a serious postoperative bleeding or thromboembolic event. However, prolongation of PT and aPTT gives only a simplified picture of the relative importance of various components of the coagulation process. Davie et al. (16) suggests that coagulation activation via the extrinsic pathway with low amounts of tissue factor (TF) is probably more physiological than the plasma activation commonly used in standard assays. Low TF concentrations have been shown to be suitable for sensitive detection of the effects of different levels of pro- and anticoagulants on thrombin generation (9). We have previously demonstrated (17–19) that the low anticoagulant capacity of activated protein C, TFPI, and AT in cord plasma allows enhanced thrombin formation, which is associated with shorter clotting times compared with adult plasma, when low amounts of TF are applied to initiate clot formation. Butenas et al (9) demonstrated that the most limiting factors for the TG are the levels of FII and AT present. Additionally to F II and AT we determined the values of TFPI, F1.2 generation, TAT complex formation, PT, and aPTT in our paediatric heart patients before and after their cardiac surgery. In the preoperative evaluation none of our patients had FII or AT levels below the age specific normal ranges in the preoperative evaluation and therefore no signs of an acquired deficiency state. However, to date there is no haemostatic assay reflecting the haemostatic status of paediatric patients with CHD. We therefore investigated the usefulness of the CAT for clinical practice. The CAT developed by Hemker (12) allows simultaneous analysis of several samples. Importance of TG measurement lies in the key enzyme role of thrombin, so this method may become a new tool better reflecting overall haemostasis than global tests such as PT and aPTT or specific factor assays. The CAT reflects the influence of all plasmatic pro- and anticoagulant factors and is appropriate to detect hypocoagulable states due to various forms of anticoagulant treatment like oral anticoagulants, and heparins of all types (8, 20). Beyond this, the influence of alterations in the APC-pathway is reflected (21, 22). In recent studies we show a strict age dependency of TG measured by CAT and therefore specific reference ranges for infants, children, and adolescents (23, 24). In the current study the lag times and time to peak were significantly shorter in infants

<table>
<thead>
<tr>
<th>Tab. 2</th>
<th>Results of thrombin generation measurements in different age groups of the 40 patients with CHD (mean values ± 2-fold SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP: endogenous thrombin potential</td>
<td></td>
</tr>
<tr>
<td>age (years)</td>
<td>lag time (min)</td>
</tr>
<tr>
<td>1–10</td>
<td>2.23 (1.67–2.73)</td>
</tr>
<tr>
<td>10–18</td>
<td>1.89 (1.18–2.57)</td>
</tr>
</tbody>
</table>
than in adolescents and the peak height as well as the ETP were significantly lower in infants than in adolescents. This is in good agreement with Kuhle et al who showed that most coagulation factors, e.g. prothrombin, are significantly lower in infants (25). The results obtained in our 40 patients with CHD are similar to the results obtained in the previous study (24). F 1.2 concentration is a specific indicator for the amount of thrombin generated and consequently a sensitive marker for coagulation activation. We found a significant negative correlation between F 1.2 generation and peak as well as between F 1.2 generation and ETP. This could indicate that in patients with an ongoing clotting activation the haemostatic potential is used and is reflected in a lower thrombin generation potential. Measurement of endogenous thrombin generation quantifies the potential to generate thrombin in response to an in vitro trigger of thrombin generation and is therefore named endogenous thrombin potential.

Tissue factor pathway inhibitor (TFPI) is the only specific inhibitor of the TF-FVIIa complex, regulating both its procoagulant and pro-inflammatory properties. It has been shown that during open heart surgery TFPI levels are increased following heparin administration and then return to normal when protamine reversal is accomplished (26). The TFPI levels in pre- and postoperative heart surgery have been described in children and adults (26). A significant negative correlation between the TFPI values and the ETP and also between the TFPI values and the peak height was seen in our study. This is in agreement with in vitro data suggesting that TFPI is an important factor for the TG and once TFPI becomes low the TG is increased. We observed a significant positive correlation between F II generation and ETP and further between F II generation and peak height. This is in good agreement with Butenas et al. (9) described that the most limiting factors for the ETP are the levels of FII and AT. AT remained stable in our preoperative and postoperative measurements. Slightly but not significant elevated FII levels 7 days after the cardiac surgery were seen and correspondingly, given the fact that FII is the most limiting factor for the ETP (9), higher peak levels and ETP in our CAT measurements, indicating that the CAT also in vivo reflects the procoagulant potential of our patients. In nine of our patients oral anticoagulant treatment had to be started after surgery.

We could not find significantly different correlations between standard coagulation assays and CAT measurements when compared to patients that did not receive oral anticoagulants after operation. Kyrle et al. (27) demonstrated for patients during the initiation of oral anticoagulant treatment a decrease up to 50% of F 1.2 levels suggesting substantial inhibition of thrombin generation during early oral anticoagulation. We found similar results in our patients and accordingly these results a prolonged lag time until the onset of thrombin generation, a longer TTP, a reduced ETP, and a reduced peak height in the CAT measurements.

**Conclusion**

CAT measurements when compared to relevant influencing factors of thrombin gener-
atation (e.g. FII, AT) and standard coagulation assays accurately reflect the coagulation status of paediatric patients with CHD undergoing cardiac surgery. Whether global assays such as the CAT is useful in clinical practice will be established when they are linked to relevant end points of bleeding, thrombosis and clinical outcome.

Conflicts of interest
All authors declare, that there is no conflict of interest.

References

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Hämostaseologie 4a/2008