NEW HEMOSTASIS TESTS

EVALUATION OF THE OVERALL COAGULATION POTENTIAL BY THE THROMBIN GENERATION ASSAY

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Test for the evaluation of the overall coagulation potential in plasma. Given the central role of thrombin in blood clotting, the tendency of a plasma sample to generate thrombin might contain useful information about thrombotic or haemorrhagic risk. In this presentation I will talk about the thrombin generation (TG) assay by the Calibrated Automated Thrombogram (CAT), developed in Maastricht by Dr. Hemker. In this assay, the formation of thrombin in full plasma is induced by a mixture of TF, phospholipids (PL) and $CaCl_2$, and it is monitored by measuring the fluorescence signal generated by a fluorogenic thrombin substrate. The result of the test is a thrombin generation curve (thrombogram) characterized by the following parameters: a lag phase (initiation), a propagation phase, which reaches the maximum at the peak of the curve , and the termination phase due to the inhibition of thrombin activity by plasma protease inhibitors. The area under the curve, i.e. the endogenous thrombin potential (ETP), represents the total amount of thrombin formed. Each parameter is dependent on specific procoagulant/anticoagulant proteins. The analytical conditions of the test can be modified to increase the sensitivity of the assay by varying the TF, PL and substrate concentrations. The pre-analytical conditions of the test (i.e. blood withdrawal and plasma preparation) are very important to achieve reliable results. An increased ETP has been described in prothrombotic conditions, while reduced ETP was found in hemorrhagic disorders. When the test is performed in the presence of activated Protein C (APC) (i.e. ETP-based APC resistance test), the APC-resistant phenotype, secondary or not to FVL, can be detected. With this test, an acquired APC resistance has been described in women on oral contraceptives or hormonal replacement therapy, in patients with lupus anticoagulant and in cancer.

NEW ROTATION THROMBOELASTOGRAPHY (ROTEM) IN THROMBOTIC AND HAEMORRHAGIC DISORDERS: LIGHTS AND SHADOWS

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Thrombelastography (TEG) was first described by Hartert in 1948.[1] This method records the viscoelastic changes that occur during clot initiation, formation and lysis providing a continuous graphical representation of fibrin polymerization and stability. Thrombelastographic analysis of coagulation can be carried out on plasma as well as on whole blood (WB). Recently, the modified rotation thrombelastogram analyzer (ROTEM®; Pentapharm, Munich, Germany) has overcome some of the limitations of classical TEG. ROTEM® assay is based on a pin rotating within a heated cup containing WB samples. An optical system detects the impedance of the rotation of the pin and a plot system describes the trace produced by viscoelastic changes associated with fibrin polymerization. Respects to classical TEG, ROTEM® is very robust and not susceptible to vibrations or mechanical shocks. By using an electronic pipette, reproducibility and performance has increased. Also, depending on the parameters measured, ROTEM® results are available as early as 15 min up to 1 hour. In addition, coagulation is triggered by different activators making it possible to evaluate different activation pathways. Four standard ROTEM® assays, named INTEM, EXTEM, NATEM, and FIBTEM, can be performed according to protocols supplied by the manufacturer.[2] The INTEM and EXTEM represent assays in which the intrinsic or the extrinsic coagulation pathways are triggered, respectively. NATEM (Non-Activated TEM) was used to assess WB clot formation in the absence of activation of the clotting cascade other than recalcification and spontaneous contact activation. Finally, the FIBTEM assay was used for the assessment of the specific role of fibrinogen in clot formation following inhibition of the platelets by Cytochalasin D. As for INTEM and EXTEM, 300 μL of citrated blood are recalcified with 20 μL of $CaCl_2$ 0.2 M (star-TEM® reagent, Pentapharm GmbH, Munich, Germany) and the coagulative cascade is activated by partial thromboplastin phospholipids made of rabbit brain plus ellagic acid (in-TEM® reagent, Pentapharm GmbH, Munich, Germany) and thromboplastin from rabbit brain (ex-TEM® reagent, Pentapharm GmbH, Munich, Germany), respectively. Regarding NATEM, 300 μL of citrated blood is recalcified with star-TEM® reagent. As for FIBTEM, 300 μL of citrated blood is mixed to 20 μL of ex-TEM® reagent plus 20 μL of cytochalasin D/DMSO solution, 0.2 M $CaCl_2$ (fib-TEM® reagent, Pentapharm GmbH, Munich, Germany).

The main descriptive parameters associated with ROTEM® are the following (Figure 1):

- Clotting Time (CT) corresponding to the time in seconds from the beginning of the reaction to an increase in amplitude of thrombelastogram of 2 mm.
- Clotting Formation Time (CFT), the time in seconds between an increase in amplitude of thrombelastogram from 2 to 20 mm.
- Maximum Clot Firmness (MCF), the maximum amplitude in millimetres reached in thrombelastogram which correlates with platelet count and function as well as with the concentration of fibrinogen.
- Alfa (α) angle, the tangent to the clotting curve through the 2 mm point.
- Maximum Lysis (ML), the ratio of the lowest amplitude after reaching of the MCF and the MCF.
- Maximum Velocity (maxVel), the maximum of the 1st derivative of the clot curve.
- Time to maximum velocity (t-maxVel), the time to reac-

tion start to reach maximum velocity.
- The Area Under Curve (AUC), defined as the area under the velocity curve, i.e. the area under 1st derivative curve ending at a time point that corresponds to MCF.

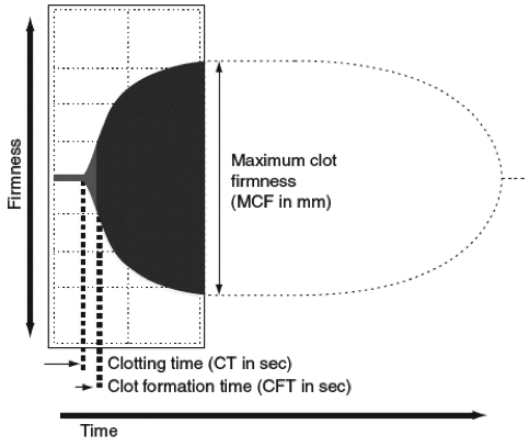

Figure 1. Thrombelastograph® tracing showing the dynamics of clot development (clotting time and clot formation time) and clot firmness (maximum clot firmness).

CT reflects the initiation phase of the clotting process; CFT, α-angle, maxVel and t-maxVel are measures of the propagation phase of WB clot formation; MCF and AUC quantify the maximum clot firmness of the established WB coagulum and correlate with the platelet count and function as well as with the concentration of fibrinogen.[3,4] ROTEM® is already utilized in several clinical settings e.g. monitoring haemostasis during liver transplantation[5] and in patients who underwent cardiac[6,7] or abdominal surgery.[8] ROTEM®-guided component replacement after hepatic and cardiac surgery has lead to a significant reduction in blood transfused. On the other hand, a number of studies have failed to find a correlation between preoperative haemostasis findings and intraoperative blood loss. Moreover, ROTEM® has been shown to be a point of care device for rapid diagnosis in various bleeding disorders and for global assessment of coagulation status in patients with hypercoagulable conditions. In particular this method has the potential to illustrate hypocoagulation typically found in disorders such as haemophilia[9,10] miscellaneous coagulation factor deficiencies[11] and platelet disorders.[12] Furthermore, experimental *ex vivo* substitution with various haemostasis-promoting components have revealed that ROTEM® is useful to illustrate the correction of a compromised haemostatic capacity. Little is known whether the thrombelastographic method might be able to visualize the characteristics of clotting pattern of hypercoagulation.[13,14] So in patients with acute deep vein thrombosis[15] as in subjects with a prior arterial or venous thromboembolic event[16] a hypercoagulable ROTEM® profile was detected. In conclusion, WB thrombelastography performed by ROTEM® is an attractive laboratory tool for studying the simultaneous and integrated effects of the different components involved in the dynamic process of clot formation: plasmatic factors, platelets, leukocytes, and red blood cells. Large prospective trials should be performed to clarify the clinical implications of ROTEM® assays in evaluating the therapeutic approach and prognostic significance of different states ranging from hypo- to hypercoagulation.

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MULTIPLATE® PLATELET FUNCTION ANALYZER – A NEW APPROACH TO THE MONITORING OF ANTI-PLATELET THERAPY IN WHOLE BLOOD

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Cardiovascular and cerebrovascular diseases continue to be leading causes of death throughout the world. Blood platelets play a pivotal role in the pathogenesis of arterial thrombosis and atherosclerosis. The benefits of various antiplatelet therapies ranging from aspirin, ticlopidine, Clopidogrel, and intravenous platelet GPIIb/IIIa antagonists are well documented. However non-responsiveness to aspirin and clopidogrel is reported in 5-30% of treated patients. In addition a high level of variation in the pharmacodynamics of GpIIb/IIIa antagonists has been shown. Methods for the monitoring of anti-platelet therapy need to be simple to use, standardized and responsive to the commonly used therapeutic strategies. The Multiplate® platelet function analyser is a relatively new instrument, which is based on the assessment of platelet aggregation by the change of impendance on electrodes immersed in whole blood. Activated platelets adhere and aggregate on the electrodes and thus enhance the electrical resistance between them. In contrast to previously applied methods the Multiplate® uses a single-use measuring cell with an improved reproducibility. For the assessment of anti-platelet drugs the specific activators arachidonic acid, ADP and thrombin-receptor activating peptide (TRAP-6) are applied. Simultaneous measurements can be performed on 5 channels. For ensuring a high level of standardization automatic pipetting and full computer analysis are provided. Multiplate is today widely used throughout Europe, with an installed base of more than 250 systems. The main application is the monitoring of the anti-platelet drugs Aspirin, Clopidogrel and GpIIb/IIIa antagonists. Other applications include the screening for platelet disorders, peri-operative analysis of platelet function and research applications including animal studies.

ASSESSMENT OF UFH, LMWH AND DIRECT THROMBIN OR FXa INHIBITORS USING THE PICT, A NEW FXa-AND RVV-V-BASED ASSAY

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UFH and direct thrombin inhibitors are usually monitored using the activated partial thromboplastin time (aPTT), which has many known limitations. These include non-linear-dose response-relationships as well as a high variability among different reagents. In addition the aPTT has a low sensitivity towards low molecular weight heparin (LMWH). Newer methods such as chromogenic substrate analysis, ecarin clotting time or the Heptest assay have provided improved sensitivity and specificity, but still have reached limited application in routine or emergency laboratories. The performance of a new universal monitoring method (Pefakit® PiCT®, Pentapharm, Basel) in detecting anticoagulant effects of anti-Xa and/or arti-IIa agents was evaluated. *Methods.* 50 µL of platelet poor plasma are added to 50 µL of a reagent containing a combination of FXa, the snake venom RVV-V and phospholipids. During an incubation time of 180 sec the RVV-V activates FV to FVa. The added FXa is inhibited by antithrombin and antithrombin-heparin complexes. Thereafter 50 µL of $CaCl_2$ (25 mM) are added to reverse the calcium chelation of the citrated sample. Mediated by the free Ca^{++} ions, the prothrombinase complex is formed on the phospholipid surfaces and thrombin is generated. The generation of thrombin is faster when high amounts of active FXa are left in the solution, while the thrombin generation is slow when most FXa is bound by antithrombin. The formed thrombin is also bound by antithrombin, antithrombin-heparin or other endogenous or exogenous thrombin inhibitors. As soon as free thrombin is formed the sample clots and the clotting time is detected. By its assay principle is sensitive towards anti-Xa and anti-IIa agents. For the analysis of direct Xa inhibitors (e.g. Rivaroxaban) the PiCT assay is performed without an incubation step. As a clotting test with pipetting volumes and an incubation regimen identical with the aPTT, the test can be used in routine or emergency laboratories, thus facilitating 24-hour availability of the method with short turn-around times. Allowing the assessment of UFH, LMWH as well as direct thrombin and Xa inhibitors therapy with one method may simplify their management in clinical practice.

BRIDGING IN ANTICOAGULATION

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The management of patients on chronic vitamin K antagonist (VKA) therapy who need temporary interruption for an elective invasive or surgical procedure is problematic and complex. Both patient and procedure-oriented risk factors for thrombosis and bleeding have to be individually assessed and properly risk-stratified in the periprocedural period. Certain procedures, such as dental, dermatologic, ophthalmic, and endoscopic procedures can be completed without discontinuing VKA. However, most procedures with a high bleed risk – including major surgeries – will necessitate temporary discontinuation of VKA and consideration of the use of a shorter-acting anticoagulant, usually unfractionated heparin (UFH) or low-molecular-weight-heparin (LMWH), as bridging therapy to maintain functional anticoagulation in patients at intermediate-to-high risk of thromboembolism. Recently completed large, prospective, multicenter cohort studies support the safety and efficacy of mostly therapeutic doses of LMWH in the outpatient setting as periprocedural bridging therapy, provided that post-procedural bleeding risk is taken into account. Large, placebo-controlled, randomized studies are underway by Canadian and US investigators to assess safety and efficacy of various bridging strategies in specific patient populations using LMWH. Lastly, we will review evidence-based periprocedural management strategies for the at-risk patient on chronic VKA, based upon recent narrative and systematic reviews on the subject. The depth and breadth of recent clinical data has necessitated an entire chapter devoted to periprocedural management of antithrombotic therapy for the upcoming 8th ACCP recommendations.

SEVERE SEPSIS

THE PROTEIN C PATHWAY AT THE INTERFACE OF COAGULATION AND INFLAMMATION

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The protein C anticoagulant pathway serves as an on demand natural mechanism to limit thrombotic complications. In addition to being a potent inhibitor of coagulation, the pathway is involved in the regulation of inflammation and cellular death through many mechanisms. Thrombomodulin binds thrombin, thereby accelerating protein C and thrombin activatable fibrinolysis inhibitor (TAFI) activation and at the same time inhibiting thrombin activation of platelets, factors V and VIII and clotting of fibrinogen. The activation of TAFI results in the generation of a carboxypeptidase with a preference for C terminal Arg residues. While TAFI can remove lysine residues from fibrin and slow fibrinolysis, it also removes terminal Arg residues from vasoactive peptides like C5a, thereby protecting the microvasculature from injury. The N terminal lectin domain of thrombomodulin can bind and inhibit HMGB1 and can dampen MAP kinase and NF-κβ signaling. EPCR, a constitutively expressed molecule that binds protein C and factor VII, undergoes internalization and may be a mechanism for regulating the levels of these proteins. Structurally, EPCR is extremely similar to the MHC class 1 family of molecules and has a phospholipid present in the position of the antigen presenting groove of this family, making it extremely likely that it participates directly in immune surveillance. Interestingly, EPCR is a frequent target of antiphospholipid antibodies and the presence of these antibodies is associated with miscarriage. Activated protein C has multiple anti-inflammatory properties. In addition to its anticoagulant activity, it inhibits NF-κβ beta signaling, decreases leukocyte adhesion to endothelium and is cytoprotective in part through inhibition of p53 and Bax and up-regulation of Bcl2. The importance of these properties in disease is becoming apparent. The system is down regulated in atherosclerosis, sepsis, Crohn's disease, diabetes and multiple sclerosis. In most of these disease states, supplementation with activated protein C ameliorates the disease progression, at least in experimental animals. Studies with both antibodies and mutant forms of activated protein C indicate that the anticoagulant and cytoprotective properties of activated protein C can be separated.