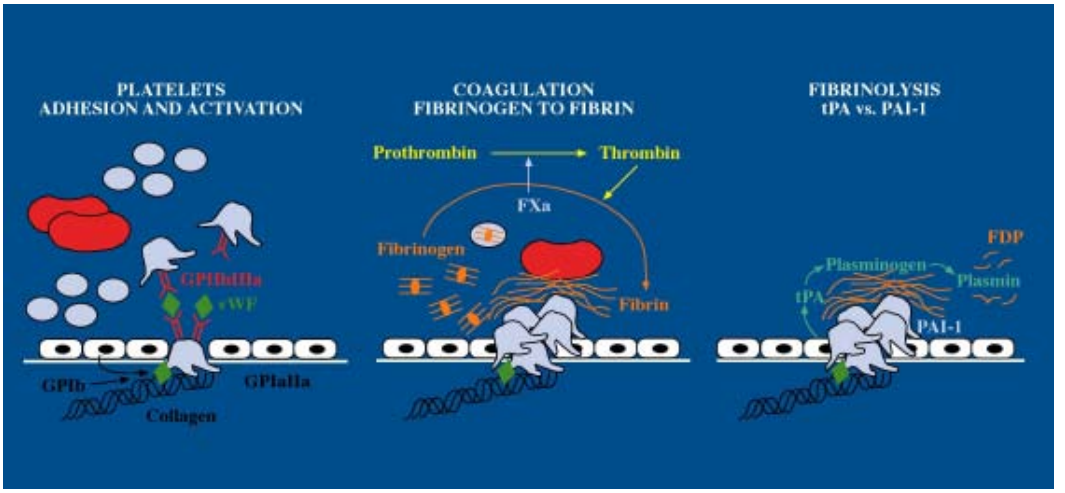
SPECIAL ISSUE 2008 – *Coagulation*

Klinisk Biokemi i Norden



Nordisk Forening for Klinisk Kemi

Introduktion

Detta nummer av Klinisk Biokemi i Norden är förbehållet artiklar som behandlar olika aspekter av blodets koagulation. Artiklarna illustrerar den dramatiska utveckling som ägt rum under senare decennier. På sextiotalet tillkom koagulationskaskaden, som innebar ett stort steg framåt, även om man kan hävda att den i bästa fall beskrev koagulationen i ett provrör. Den gav dock form och konkretion åt koagulationsprocessen, vilket stimulerade biokemiskt inriktade forskare att rena och kemiskt karakterisera de olika koagulationsfaktorerna. Framstegen har givit dagens forskare möjligheter att studera hur koagulationssystemet och protein C systemet balanserar varandra – livets fortbestånd beror att balansen vidmakthålls (slås protein – C systemet ut koagulerar hela kapillärsystemet inom loppet av ett par minuter och döden blir omedelbar). Att förstå processerna in vitro är svårt men om man också måste ta hänsyn till effekten av olika celltyper: trombocyter och andra celler, intakta såväl som skadade; samt att allt äger rum in vivo så blir vidden av problemet uppenbart.

Svårigheterna till trots måste den som är kliniskt verksam besluta om behandling av trombos och blödning och kanske än mer intrikat, ta ställning till om en patient bör få profylaktisk behandling efter en trombos eller om riskerna med en dylik behandling är större än fördelarna. Lägg därtill att utvecklingen mot nya/bättre läkemedel inte gått framåt som man hoppats; både Waran och heparin har många decennier på nacken. Waran användes i Sverige första gången av Jörgen Lehman vid Sahlgrenska Sjukhuset redan1941.

Låt oss hoppas att detta nummer av Klinisk Kemi i Norden blir en inspiration för unga forskare vid våra laboratorier att ta sig an problemen – kanske tar utvecklingen ett nytt steg framåt, ett steg mot bättre behandlingar och bättre diagnostiska metoder.

Johan Stenflo

Dear colleague

In connection with the arrangement of the XXXIX Nordic Coagulation Meeting In Malmö, Sweden May 4-6, 2006, the editorial board of "Klinisk Biokemi i Norden" initiated the thought of an educational issue of the journal covering different aspects of the coagulation field.
We have now put together this issue covering the field from a Nordic perspective. The various topics have been chosen with the ambition to give the non-specialized clinical chemist an update and a valuable guide to the area. We are very thankful that so many distinguished and experienced colleagues contributed! With their manuscripts they give both the historical overview and an update of current knowledge of their respective field.
We hope that this issue will be pleasant reading and of use in the education of new colleagues and students many years to come.

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Contents

Modern cell-based coagulation-overview of the system

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- Coagulation is a cell controlled process
- Two cell types are required; TF-bearing cells and platelets
- The coagulation process includes three overlapping phases; initiation, amplification and propagation
- To regulate the process, the two cell types has to be separated until activation of coagulation is needed at a site of injury

The coagulation process has for more than 40 years been considered as a cascade system or waterfall modell where activation of one proenzyme to an active enzyme, eg one coagulation factor, led to activation of another coagulation factor and finally resulting in thrombin formation and a fibrin clot[1]. This cascade modell included the intrinsic pathway or contact activation and the extrinsic pathway or tissue factor-dependent pathways initiated by coagulation factor XII and factor FVIIa bound to tissue factor (TF), respectively. The two pathways were thought to converge, in a Y-shaped modell, in a common pathway with formation of the prothombinase complex consisting of FXa/FVa. Phospholipids and calcium are required for the activity of the factors. These pathways are assayed separately by the prothrombin-time (PT) for the extrinsic pathway and activated partial thromboplastin time (APTT) for the intrinsic pathway. However, in the clinical situation deficiency of FXII and FXI seldom leads to bleeding problems despite prolonged APTT. In contrast, patients with equally prolonged APTT dependent on deficiency of FVIII or FIX have serious bleeding tendency. In 1977 Osterud and Rapaport published data showing that FVIIa/TF complex beyond activation of FX to FXa also activated FIX to FIXa and thus proposed an additional way for the initiation of coagulation[2]. These new data clearly indicated that the two pathways were linked rather than separated from each other.

Hemostasis

The main functions of the hemostatic system are to prevent loss of blood upon damage of a blood vessel and to keep the blood in a fluid phase. The site of blood vessel injury requires the formation of an impermeable platelet and fibrin plug. However, to inhibit clot propagation through the vascular tree, localizing of activated platelets and the coagulation process at the site of injury is necessary. Accumulating data during recent years clearly demonstrate that coagulation is a highly regulated reaction that takes place on specific cell surfaces[3;4]. In this process platelets support procoagulant reactions and endothelial cells provide anticoagulant properties. The clot is later dissolved by the activation of the fibrinolytic system, which also prevents the vessel from being occluded by the clot during its formation. In order for the blood to stay fluid within the circulation, a delicate balance between coagulation and fibrinolysis is needed. An imbalance in either system will result in a propensity for bleeding or thrombosis[3;4].

Tissue factor

Hemostasis starts in arteries or veins when TF-bearing cells come into contact with blood at a site of tissue injury. Tissue factor (TF), a 47 kDa transmembrane glycoprotein, is a member of the class II cytokine receptor superfamily and functions as the receptor and essential cofactor for factors VII and VIIa. Upon binding to TF, FVII is rapidly converted to FVIIa by FXa, FVIIa itself and/or noncoagulation proteases. The activation mechanism is today not fully understood. Assembly of the TF/FVIIa complex on cellular surfaces initiates coagulation by activating FX in the presence of Ca^{2+} ions. TF is constitutively expressed in extravascular cells found in and surrounding blood

vessels and large organs to form a haemostatic barrier. TF can also be induced in monocytes in response to a number of inflammatory stimuli, such as the adhesion molecules P-selectin and CD 40 ligand expressed by activated platelets, endotoxin, cytokines, growth factors and oxidized LDL[5]. Total lethality in homozygous TF knock-out mice embryos demonstrate that TF is indispensable for life.

Beyond its role in hemostasis, the TF/FVIIa-complex triggers intracellular signal transduction, resulting in the expression of various genes and explaining its role in various biological functions, such as embryonic development, angiogenesis, inflammation, cell migration and apoptosis[6;7].

Cell-based model and control of coagulation

The cell surface-based coagulation process can be currently described in 3 overlapping phases: initiation, amplification and propagation. The process starts on TF-expressing cells, and continues on the surfaces of activated platelets[8-10].

Initiation Phase

The initiation phase is localized to the TF-bearing cells that are exposed from subendothelial tissue upon vascular injury. The proteolytic TF/FVIIa-complex activates both FIX to FIXa and FX to FXa. On TF-bearing cells, the prothrombinase complexes form by the interaction of FXa and FVa (**Figure 1**). FVa derives from several sources, including activated platelets adhering at sites of injury, which release partially activated FV, and plasma, where FV can also be activated by FXa. The prothrombinase complexes then cleave prothrombin on the TF-exposing cells to generate small amounts of thrombin, the enzyme responsible for clot formation. This amount of thrombin is not enough for the clotting of fibrinogen. It is, however, sufficient to prime the coagulation system for a burst of thrombin during the next steps of the process. The concentration of TF/FVIIa complexes and of the inhibitor tissue factor pathway inhibitor (TFPI) regulates the duration of this initiation phase. When a certain amount of FXa is formed, TFPI is bound, forming a quaternary complex with TF and FVIIa. TF is essential to trigger thrombin generation, but after 120 s most of the initially formed TF/FVIIa complexes are found in inactive complexes with FXa-TFPI. In contrast to FXa, FIXa is not inhibited by TFPI, and only slowly inhibited by antithrombin. FIXa does neither act on the TF-exposing cells or during the initiation phase of coagulation. At the site of vessel injury FIXa diffuses in the fluid phase from TF-bearing cells to nearby activated platelets. It binds to a specific platelet surface receptor, interacts with FVIIIa and this complex than activates FX to FXa directly on the platelet surface[11].

Amplification phase

Upon a vessel injury platelets adhere to the tissue through binding between platelet expressed GPIa and GPVI receptors and collagen. They also bind through the von Willebrand factor. The adherence induces a partial activation of the platelets. In the amplification phase, low concentrations of thrombin fully activate platelets adhering to the injury site. A positive feedback loop is initiated, whereby low concentrations of thrombin activates FV to FVa. It also activates FVIII and dissociates FVIII from von Willebrand factor. Such activated factors bind to platelet surfaces, which provide enough scaffolding for the large-scale thrombin generation that occurs during the propagation phase. Thrombin also activates FXI bound to platelets[12] (**Figure 1**). The activation of FXI by thrombin on the platelet surfaces explains why FXIIa is not needed for activation of the clotting process. The role of FXIa, a member of the intrinsic pathway of coagulation, can be considered as a booster of FIXa-production on the platelet surface and thus increases thrombin generation.

The propagation phase

During this phase the activated platelets provide the necessary surface for large production of thrombin and play a major role to localize the clotting process at the site of vessel injury. In the propagation phase, the phospholipid surface of activated platelets acts as a cofactor for the activation of the FVIIIa-FIXa complex (termed "Xase") and of the FXa-FVa complex ("prothrombinase"), which accelerate the generation of FXa and thrombin, respectively. In addition, FXIa bound to the platelet surface activates FIX to form more Xase. FXa associates rapidly with FVa on the platelet surface, resulting in a burst of thrombin, ultimately leading to the bulk cleavage of fibrinogen to fibrin. Soluble fibrin is finally stabilised by FXIIIa, also activated by thrombin, to form a stable fibrin clot (**Figure 1**). Formed thrombin is also incorporated in the clot.

Thrombomodulin (TM), a transmembrane molecule expressed on endothelial cells, also binds thrombin, and the thrombin-TM complex activates the protein C anticoagulation system. Probably, the role of activated protein C is to limit the FXa-FVa activity on the endothelial surface of the injured vessel and thus the propagation of coagulation reactions. However, the burst of thrombin also induces activation of the carboxypeptidase thrombin-activatable fibrinolysis inhibitor (TAFI), which removes terminal potential binding sites on fibrin for fibrinolytic enzymes and thereby increases the resistance of the clot. The three phases of the cell-based coagulation model should be understood as overlapping continuing steps.

Interplay between coagulation and inflammation

Recently obtained data have demonstrated that coagulation and inflammation are integrated processes through a network of components. This interplay contributes to diseases, as illustrated by the thrombus formation on ruptured atherosclerotic plaques, which contain abundance of inflammatory cells. Inflammation is modulated by thrombin, FXa and TF/FVIIa complex by activation of protease activated (PAR) receptors and also by thrombomodulin and binding of activated protein C to endothelial protein C receptor[13]. PARs are seven transmembrane domain, G-coupled receptors expressed on numerous cells, such as platelets, endothelial cells and leukocytes. Thrombin activates PAR1 and 4 on platelets and thereby induces the expression of P-selectin and CD40 ligand (CD40L) and the release of inflammatory cytokines and growth factors. Cross-talk of the cells in platelet-leukocyte complexes via P-selectin and CD40L and the binding to their counterreceptors leads to production of TF, metalloproteinases and cytokines. The ternary complex TF/FVIIa/FXa can also bind to PAR1. PAR2 cannot bind thrombin, but TF/FVIIa complex and FXa can activate this receptor. Binding of the different coagulation proteases to the PARs results in cell signal events leading to induction of a number of genes involved in inflammation, including interleukin (IL)-8.

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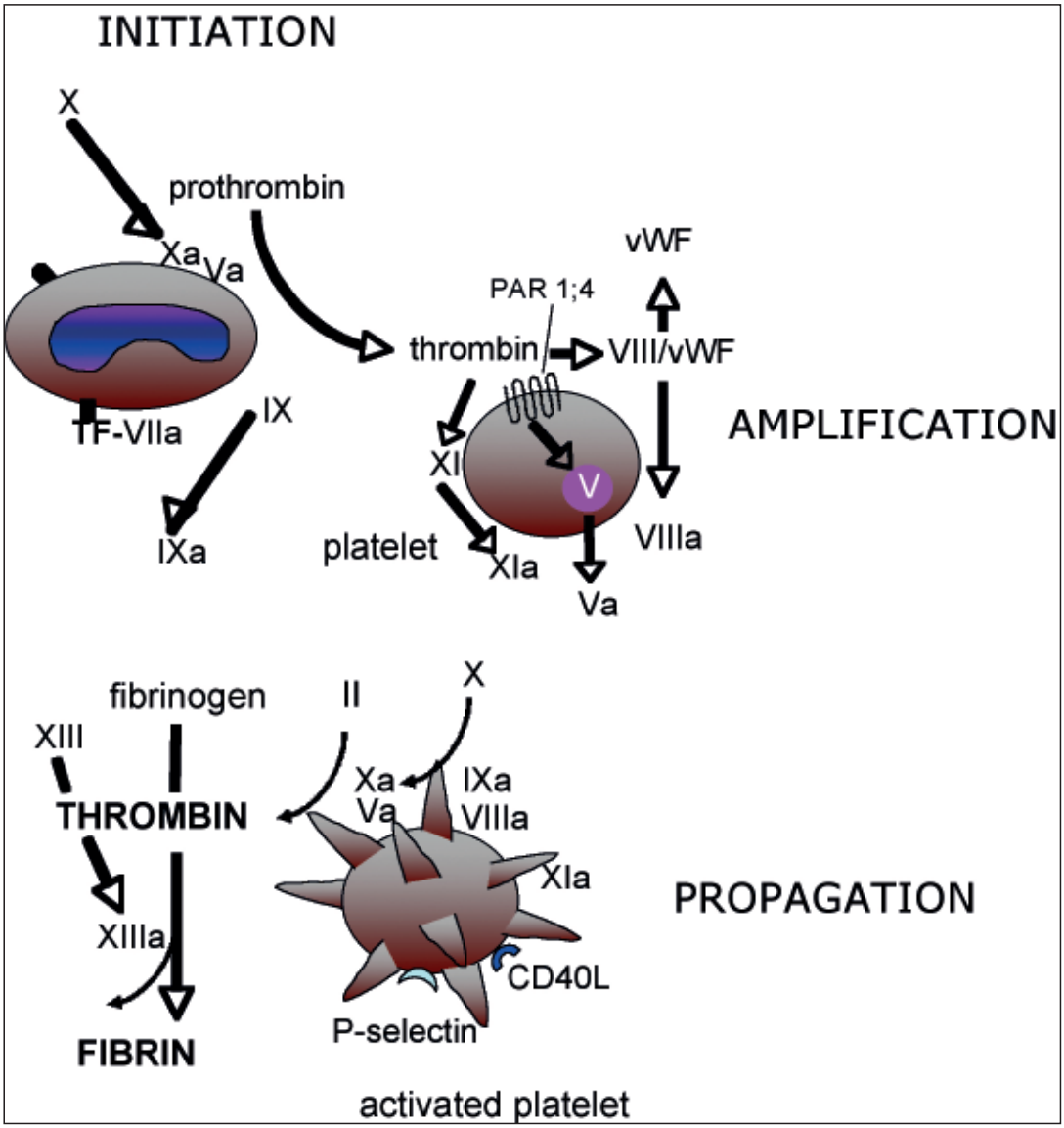

Figur 1. Scheme of the cell-based model of blood coagulation

The coagulation process includes three overlapping phases. In the initiation phase TF- expressing cells come in contact with the circulating blood, FVIIa binds to TF and the complex initiates coagulation leading to the generation of trace amounts of thrombin. In the amplification phase the small amount of thrombin fully activates platelets adherent to the site of injury, FV, FVIII and FXI. In the propagation phase, FVIIIa forms complexes with FIXa and FVa with FXa on the platelet surfaces, thus generating FXa and thrombin, respectively. In these phase a burst of thrombin is generated. Thrombin then cleaves fibrinogen to fibrin and the fibrin network is stabilized by FXIIIa.

Measuring primary haemostasis

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Virchow's triad

The factors influencing the fate of haemostatic or thrombotic response on injured vessel wall include the triad of 1) blood with its elements, i.e. red blood cells, platelets and coagulation and fibrinolytic factors and their regulators 2.) blood flow introducing shear forces, i.e. the smaller the vessel lumen the larger forces, and 3) vessel wall components, i.e. the condition of endothelial cells and subendothelial constituents, i.e. collagen, laminin, thrombospondin and tissue factor. The deeper the injury the more thrombogenic material is exposed, including platelet -activating species of collagen, type I and III and adventitial tissue factor expressed by the smooth muscle cells (see Figure - cover page). Often the contribution of these baseline factors is overlooked when the impact of platelets or coagulation factors, such as von Willebrand factor (VWF) or FVIII and IX are analysed or discussed.

Measurements of platelet activation

Measuring platelet activation is a demanding task as isolating platelets from their natural environment introduces several sources of artefacts. The important contributing factors in primary haemostasis include shear forces where blood viscosity impacts platelet adhesion with the critical elements of haematocrit and fibrinogen (1-3). The extreme role of these contributors can be demonstrated in the case of massive transfusion -related coagulopathy (3). Anaemia is a risk factor for bleeding complications in association with strong platelet inhibitors used due to unstable angina or non-ST elevation myocardial infarction (4). The previous situation resembles compromised primary haemostasis due to severe impairment of platelet-activating mechanisms due to antithrombotic combination therapy. Anaemia is also a risk factor for bleeding events related to outpatient anticoagulation treatment as a part of a bleeding risk index (5). It is thus selected also as exclusion criteria for new antithrombotic drugs entering the phase III for a long-term use. However, the phenotypic impact of possible anaemia per se is not generally considered when bleeding problems are discussed.

The important contribution of haematocrit on primary haemostasis will be excluded in studies which exclude red blood cells and blood flow in the setup. Developing or persistent anaemia may thus be a serious phenotypic factor that may impair primary haemostasis and cause worsening bleeding tendency in patients with bleeding disorders, especially in those affecting platelet vessel wall interaction, i.e. in von Willebrand disease (VWD) (1). Also, the quantity and quality of platelets affect the final haemostatic response, i.e. the primary adhesive mechanism in addition to VWF function. As an obvious example of this is the role of collagen receptor glycoprotein (GP) Ia/IIa; the activity of which has been shown to influence the bleeding phenotype in patients with VWD (6). Thus, an optimal test measuring primary haemostasis should include whole blood and flow conditions and a measure of platelet – vessel wall interaction.

Most common studies to assess platelet function measure their aggregation. This method isolates their capacity from adhesion, though. With regard to adhesion the adhesive agonist - whether von Willebrand factor (vWF), laminin, thrombospondin, collagen, or fibrin(ogen) - triggers both distinct and general signalling events (7). Thus, measuring platelet aggregation may well differ from the activation mechanisms induced by the adhesion events (8). The major concern is how well in the presence of a specific exogenous platelet agonist the assay reflects the physiological situation under the static conditions. On the other hand, the properties of the immobilized ligands for platelet adhesion may well have an impact on the platelet activation, distinct from the activation brought by a soluble ligand (7, 8). A clear example of this is both VWF and

fibrinogen, which as soluble ligands do not recognize unactivated platelets, but upon immobilisation bind platelets and are activated, obviously even more clearly if fibrinogen has been converted to fibrin with bound thrombin to activate platelets in the vicinity (7, 8).

Preanalytical factors

Before platelet analysis the patient should have been resting and not used medication for at least a week to impair platelet function (i.e. NSAID, aspirin, clopidogrel, omega-3-fatty acids) if native conditions are to be tested (11). Avoidance of stress, recent fatty meal, smoking, alcohol and coffee are recommended close to blood collection. Platelets should be handled with caution and used within 2 hours of their preparation, whether studied in whole blood or processing to platelet-rich plasma (PRP). Temperature should be room temperature or 37°C and pH should not increase, which occurs during storage. Haemolysis liberates ADP and falsely causes refractoriness.

The main differences in experimental versus physiological conditions relates to native blood versus anticoagulation, usually with citrate. Citrate causes chelation of calcium and also magnesium which - despite their specific roles and delicate balance in physiology – a fact which is often completely overlooked. The signalling events introduced by the adhesive receptors under appropriate conditions are likely to be influenced with the cations (8-10). Thus, by comparing different anticoagulation, i.e. citrate with thrombin or factor Xa inhibitor could provide interesting novel insights into platelet-activating mechanisms. Also, shear force by itself can be considered as a platelet agonist. This measurement is called low or high shear-induced platelet aggregation (SIPA). However, SIPA does not operate alone but acts in concert with other agonists, whereas low and high SIPA are often studied as such without additional agonists. Ristocetin is used as an agonist to mimic the shear force-induced interaction between VWF and platelet glycoprotein (GP) Ib in agglutination experiments with fixed platelets. Thus, either VWD or Bernard Soulier syndrome can be causative for impaired agglutination. On the other hand, aggregation involves GP IIb/IIIa in addition to GPIb and thus both receptors and their cooperation are involved in the final outcome. Furthermore, it is intriguing that both FIXa and thrombin interact with GPIb creating a microenvironment to enhance coagulation activity. The procoagulant role of platelets and again shear forces are underlined by the process of microvesiculation., which can be inhibited at least by GPIb and GPIIb/IIIa antagonists (8).

Optical and chemiluminescence-related aggregation of PRP

This approach provides tools to diagnose rare platelet function disorders and lack of specific granules. As the alpha-granules contain in addition to the agonists also adhesive ligands such as P-selectin and VWF, their contribution to platelet aggregation may well exert an unanticipated role in human donors, while animal data are available supporting these interactions in enhancing the haemostatic response (12).

Impedance-induced aggregation of whole blood.

Multiplate is a new standardized method to assess aggregation on platelets which have adhered on a rod by impedance-induced mechanism in whole blood. It is similar to the previous impedance technology, but it is made more user-friendly. The assay urges to use in addition to citrate, also lepirudin as an anticoagulant to provide physiological cations for the aggregation response (9,10). Standardized doses of ristocetin, ADP, arachidonic acid, collagen and thrombin receptor-activating peptide (TRAP) are used as agonists. The method has mostly been initially aimed at screening responses to acetylsalicylic acid and clopidogrel. There are limited data about the utility in VWD, although the ristocetin responses appear interesting in this regard and include the action of both GPIIb and GPIIb/IIIa.

Platelet Function Analysis, PFA100

PFA100 has been developed by the group of Dominique Meyer and it provided early on very coherent information with regard to the contribution of shear forces, number of platelets and red cells, of VWF and GP Ib and GP IIb/IIIa. Its advantage is that it is a standardized method including whole blood and shear forces and it assesses occlusion time in small capillaries where blood enters into a transverse collagen mesh and platelet adhesion leads to subsequent aggregation and clots the channel stopping the blood flow. Its disadvantges are the price and also considerations about standardization; the CV being around 15%. PFA100 does not differentiate between VWF and platelet disorder, thus additional assessments are needed in both scenarios. In case of platelet granule defects PFA100 correlates well with transmission electron microscopic data (personal

communication, Dr Gines Escolar). However, when comparing this to the much more extensive variations reported in some VWFR:Co methods for example, the CV is acceptable. It would be important to get increasing information on the associations with PFA100 and various tests of VWD, as has already been reported in the case of collagen binding assay (VWF:CB) (13). The PFA100 has been shown to predict bleeding complications.

Ultegra rapid platelet function assay

Ultegra is a point-of-care method (VerifyNow) to assess the effects of platelet antagonists, i.e. more recently acetylsalicylic acid (Aspirin Assay) and clopidogrel (P2Y12 Assay), as well as earlier GPIIb/IIIa antagonists (IIb/IIIa Assay). The method is mainly used in cardiology settings in the catherization laboratory to evidence the effects of antiplatelet antagonist (14). It is a turbidometric detection system which utilizes whole blood and platelet agonist (arachidonic acid or ADP), and is based on fibrinogen-coated beads and the reactivity of platelet GPIIb/IIIa (TRAP) under conditions targeted to find the responses specific to each condition. In the P2Y12 assay PGE_1 is added and that excludes the role of P2Y1 receptor. The same approach is used in Multiplate when sensitivity to clopidogrel is evaluated.

In all, however, the finding of aspirin or clopidogrel resistance has been shown to be test-specific and therefore currently there is no consensus on the matter and the suggestion is that the tools are for research use only (15)

ImpactR

Dr David Varon has developed this cone- and plate viscometry which is suitable for VWD diagnostics. It utilizes whole blood under shear force and thus assesses VWF-mediated aggregation on surface (polystyrene) –adherent platelets. Image analysis measures both surface coverage and the average size of the aggregates. Also, fibrinogen and platelet receptors GPIb and IIb/IIIa are involved in the reaction. The test has the advantage of small blood volumes, simplicity and it has been used to detect platelet hyper- and hypofunctions in transfusion medicine (11). There is limited data on its usefulness in diagnosis of platelet function disorders, though.

Flow cytometry, VASP

With flow cytometry information can be obtained mainly about the quantity, but also with the help of specific antibody tools recognising only the active conformations also the quality of the platelet receptors (11). Whole blood can be used to avoid processing and platelet activation in vitro. Platelets can be fixed for later analysis and also thrombocytopenic samples can be studied. Also, subpopulations and microvesicles can be assessed and with annexin V binding also procoagulant quality and release reactions can be evaluated (11,12). A specific vasodilator-stimulated phosphoprotein, VASP can be used to analyze the function of i.e. P2Y12 receptor (11,16).

Bleeding time

This method has been largely abandoned because of its impracticality and variations related to the vascular tissue and implementation and failure to predict bleeding tendency (11). Bleeding time, as PFA100, does not differentiate between VWF and platelet function disorder.

Conclusions

As the global thrombin generation also the primary haemostasis remains challenging to the laboratory. At the moment a general overview of haemostatic capacity or thrombogenic property is not achieved by a single test, but a battery of tests the results of which need to be associated with the clinical phenotype, including blood flow conditions and the contribution of red cells. To comparatively analyze the role of anticoagulation on platelet activation needs ongoing efforts and may provide crucial insights into platelet disorders.

Figure legend

The three steps of haemostasis or thrombosis. Primary haemostasis occurs under blood flow as a response to vessel injury. The role of red cells is to enhance platelet adhesion and provide ADP to foster platelet activation. The matrix which is revealed upon injury affects the haemostatic response demanding counterparts on the platelet surface as responsive elements for adhesion. On the adhered platelets coagulation will ensue and initiate the fibrinolytic system to regulate fibrin formation. Normally platelets provide strong mechanisms to resist fibrinolysis. All the three steps of haemostasis regulate the outcome of platelet-vessel wall interaction.

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Common screening test for coagulation - APTT, ACT and PT

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Activated partial thromboplastin time (APTT)

The method activated partial thromboplastin time (APTT) was developed to solve problems with the older screening method for coagulation factor deficiencies, the partial thromboplastin time. The cephalin (brain extract without tissue factor, i.e. "partial thromboplastin") used in different laboratories varied in potency and, most important, plasma was activated to various degree by contact with foreign surfaces. Proctor and Rapaport (1) bypassed the problem by maximally activating the citrated plasma with a suspension of kaolin powder for 3 minutes at +37°C followed by recalcification and determination of the clotting time. Other materials used as contact activators are silica and ellagic acid. The preincubation step initiates contact activation in which factors XII and XI are activated by prekallikrein and high-molecular weight kininogen, facilitated by the phospholipids. By adding adsorbed plasma to the APTT one could detect specific deficiencies.

The analysis procedure consists of two steps;

Step 1: Citrated plasma + phospholipids + contact activator → activation of fXI and XII
Step 2 Recalcification → Fibrin clot

APTT is thus dependent on the intrinsic pathway of the coagulation system, i.e. factors VIII, IX, XI, XII, prekallikrein and high-molecular weight kininogen and factors of the common pathway, i.e. fibrinogen, prothrombin, factor V and X. Factors VII and XIII have no influence at all on the APTT. APTT is used as screening test for deficiencies in coagulation factors, to monitor therapy with unfractionated heparins and to detect lupus anticoagulant.

It is not possible to standardize the APTT and introduce common units because different reagents and instruments vary widely in responsiveness to factor levels and heparins. Some reagents are more sensitive for factor deficiencies and other less but more sensitive for lupus anticoagulant. The reference interval for healthy controls must be determined locally. Causes of falsely prolonged APTT are contamination of heparin used for flushing indwelling catheters, too high citrate concentration in the sample due to under-filling and partially clotted samples due to insufficient mixing.

APTT is most sensitive for factor deficiencies at the start of the intrinsic system. Mild factor deficiencies (> 25 %) in factor VIII or IX usually go undetected, but for some reagents even a factor concentration of only 10% might go undetected! For example, some years ago the correct diagnosis of hemophilia in a young boy was delayed more than two months following a bleeding in the knee joint, partly due to an insensitive reagent and an APTT within reference range. Most reagents are less sensitive to factor IX deficiencies than to other factor deficiencies. It must be emphasized that many patients with mild, and in worst case even moderate, bleeding disorders will have a normal APTT.

The responsiveness to heparin varies at least two-fold between reagents and thus results in a prolongation of the APTT of 1.5-2.5 or 2-3 -fold compared to the control value. However, the dose of heparin will vary widely depending on the local reagent-instrument combination. APTT may vary considerably even between instruments of same make and between reagent lots. An important cause of falsely short APTT is leakage of platelet factor 4 from the platelets in the blood sample neutralising the heparin, thus it is important to separate the plasma shortly after blood sampling (within one hour).

However, intravenous treatment of venous thromboembolism with un-fractionated heparin (UFH) has diminished very much in recent years and has been substituted with low molecular weight heparins (LMH) given weight-adjusted subcutaneously without laboratory monitoring with APTT. LMH treatment prolongs

the APTT, at prophylactic doses only a few seconds but at doses used for treatment of venous thrombosis the APTT usually will be some seconds above the upper limit of the reference interval. Treatment with warfarin also prolongs APTT, at INR 2-3 commonly 5-15 sec above the upper limit of the reference interval, but when heavily overdosed the APTT may become prolonged to >180 sec.

Activated clotting time (ACT)

Hattersley adjusted the APTT test for samples of fresh whole blood using diatomite as activating reagent. He named the test activated coagulation time (ACT) (2). Nowadays kaolin and celite are the two most used contact activators, sometimes used in combination. Celite is more responsive to aprotinin than is kaolin (3). ACT has a wider dose-range response than APTT so it is used for monitoring high-dose UFH therapy. In contrast to APTT, ACT is still one of the most frequently performed coagulation analyses and the number of tests are increasing, at point-of-care to control treatment with intravenous UFH in open-heart surgery and in percutaneous coronary intervention. Commonly a heparin dosage aims at a coagulation time of >420-480 sec in order to avoid clotting when connecting the patient to the extra-corporeal circuit. Depending on reagents and instrument, the ACT for a plasma sample from a patient on high dose heparin may vary considerably, even between duplicate samples. The variation is worse than for APTT (4). Some manufacturers have introduced algorithms in the software of the instrument converting the measured clotting time to become equal to the old manual method at the decision limit for open heart surgery in order to improve customer acceptance.

Prothrombin time (PT, PT-INR, PK-INR)

PT is used for three purposes; to monitor oral anticoagulant therapy with coumarins, to asses liver function in severe liver disease, and to screen for deficiencies in the extrinsic and common pathways. Two different assay types are used. The assay type invented by Quick in the 1930s (5, 6) is characterized by the use of undiluted citrated plasma mixed with equal volumes of thromboplastin reagent (= plain thromboplastin) and calcium chloride solution or two volumes of a mixture of thromboplastin and calcium chloride. Thromboplastin is an extract containing tissue factor and phospholipids, usually from rabbit brain or human placenta – sources rich in tissue factor. In recent years, recombinant tissue factor has been introduced. The Quick PT result depends on the activity of the vitamin-K-dependent factors II, VII, X and of factor V and fibrinogen. Factor V is unstable and thus blood samples has to be analysed within one hour after blood sampling or plasma has to be separated and frozen.

Schematically, the analysis procedure is as follows;

Citrated plasma + thromboplastin + Ca^{2+} → Fibrin clot

A more specific assay type was described by Owren almost two decades later (7, 8). It is characterized by mixing the patient sample with a reagent containing thromboplastin, factor V, fibrinogen and calcium chloride (=combined thromboplastin). Due to the addition of factor V and fibrinogen, often in the form of factors II, VII and X depleted bovine plasma, this assay is more specific for the coagulation factors II, VII and X.. If PT is analyzed by the Owren method samples from patients on warfarin may be stored at room temperature for 48 hours. PT in samples from patients on oral anticoagulation depends mostly on prothrombin and factor VII.

In brief, the analysis procedure for the Owren PT is as follows;

Citrated plasma prediluted in buffer 1/7 + bovine depleted plasma + rabbit thromboplastin
+ Ca^{2+} → Fibrin clot

The Owren method has been modified and in the Owren-type PT assay procedure currently performed in the Nordic and Baltic countries, there is an initial plasma sample dilution (1 part +6 parts) with buffer containing citrate before addition of the combined reagent (2 parts) to the diluted sample (1 part), giving a final sample dilution of 1/21. This can be contrasted with the original Quick PT assay which has a final sample dilution of 1/3. In Quick PT assays modified for point-of-care utilising lyophilised thromboplastin dissolved by the native blood sample, the patient plasma is not diluted at all, for example in the CoaguChek instrument (Roche Diagnostics, Basel, Switzerland).

The advantage of the current Owren method is that a minimal amount of sample is needed for the PT test, and the test is therefore suitable for automated

laboratory analysis of paediatric and capillary blood samples. The dilution also reduces interference, which is desirable particularly when lupus anticoagulant or other inhibitors such as direct thrombin inhibitors are present (9, 10). With the Quick method, and in particular if applied to point-of-care instruments, lupus anticoagulant may increase the INR-value considerably and patients are at risk for under-treatment and thrombosis.

With PT the primary result is clotting time in seconds, for normal plasma with the Quick method about 12 seconds, with the Owren method about 20 seconds. The fibrin formation is usually detected by increase in absorbance by turbidometry or nephelometry, alternatively by detection of the increase in viscosity by mechanical devices. Different thromboplastins exhibit variable sensitivities to factor deficiencies. Mild factor deficiencies (>40%) may go undetected. The most common causes of spontaneously prolonged PT are liver disease, hereditary factor VII deficiency (usually mild without bleeding symptoms) and insufficient vitamin K intake. Therapeutic doses of UFH prolong the PT by a few seconds unless the reagent contains a heparin neutralizer. Capillary drawn citrated whole blood is conveniently analyzed by the Owren method thanks to the low blood volume required, but very high or low hematocrit will cause erroneous results unless the instrument measures and compensates for the hematocrit, done for example by the point-of-care instrument Simple Simon™ (Zafena AB, Borensberg, Sweden).

For many years, the PT results were expressed in seconds, prothrombin index, prothrombin activity, or prothrombin ratio (11). Depending on reagent, the results and consequently the mean doses of coumarins varied widely between hospitals. In order to standardize the PT the International Normalized Ratios (INR) was adopted by the World Health Organisation 1983 (12).

$$INR = (PT/MNPT)^{ISI}$$

PT is the prothrombin time in seconds, MNPT is the geometric mean of PT of plasma samples from at least 20 normal subjects and ISI is the International Sensitivity Index of the thromboplastin.

The idea of ISI is to convert the ratio obtained with local working method to the ratio which would have been obtained with the first international reference preparation (IRP) of thromboplastin designated 67/40, using the manual tilt tube technique. The ISI of the working method is determined by the split-sample PT testing against the reference method and an IRP. Fresh plasmas from at least 20 normal subjects and 60 patients on stable oral anticoagulation must be tested by both methods. The ISI is the slope of the orthogonal regression line of the reference method log-PT on the working method log-PT. The original stock of the IRP 67/40 has been exhausted and chains of secondary IRPs have been assigned ISI-values in a hierarchical mode. Depending on which secondary IRP is used the results will deviate remarkably, due to errors introduced in each assignment and minute changes of properties during storage of the IRPs. The WHO protocol is cumbersome, the stocks of IRPs are limited and expensive and calibration has to be done in each laboratory some times each year (when changing reagent lots, instruments or after service of instrument). Very few labs uses the WHO protocol but relies instead on ISI-values provided by manufacturers. However, ISI values assigned to classes of instruments or models are not necessarily valid for specific instruments. Due to the limitations of calibration procedure described above there is an increasing interest in using plasma calibrators with assigned INR-values. However, different IRPs, analytical methods and production methods of calibrators may result in significant different assigned INR values. Two examples of additional unresolved questions are how many calibrators are needed and matrix effects of calibrators of calibrators versus fresh patient plasmas.

In Sweden and Norway, where the Owren type PT is used, an alternative calibration procedure was introduced in 1999 (13). The method utilised dilutions of normal plasma instead of IRP and the percent of normal activity (PT%) was converted to INR by using an equation derived from a regression analysis based on split-sample analysis of plasma samples from normal subjects and patients on oral anticoagulation using the Owren method and the manual tilt-tube technique and an IRP (INR = (1/PT% + 0.018) / 0.028). For example 100%= INR 1, 25%=INR 2.07, 20%=INR 2.43, 10%=4.21. Using this algorithm any dilution of normal plasmas has an INR value, valid for ever, which is used to assign INR-values to lyophilized calibrators. A reference thromboplastin (IRP) is therefore not required any longer. At the local laboratory level only two calibrators are needed, one in the normal range and one with INR in the therapeutic range. A three-year follow-up in Sweden where several laboratories had participated in an external quality control programme, reported that

the intra- and inter-laboratory variations had become markedly improved with this local INR calibration (14). Despite different modes of calibration used for the Owren-PT in Denmark, Finland and Iceland, the inter-laboratory variation in the Nordic countries is very small in an international perspective (15).

In order to obtain correct results with the Owren type PT is it important to mix the blood sample to ensure anticoagulation and homogenous plasma, to have samples and reagents equilibrated to correct temperature before analysis, to dissolve the reagent the required time before analysis (to avoid changes in ISI and/or MNPT during the working day), to perform a correct calibration, to run control samples and adhere to rules for QC.

Despite PT has been used for more than 60 years in control of oral anticoagulation, there is still need for improvement of reagents and calibration procedures. In countries using the Quick-method the between laboratory variation is considerably and for a patient sample clinically significantly different result may be obtained due to reagent properties. A significant improvement would be achieved if countries still using the Quick PT-method changed to the more specific Owren PT-method.

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Coagulation factor and inhibitor analyses

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Introduction

Historically, assays of single coagulation factors have generally attracted interest only in laboratories with a specialised repertoire, including research laboratories and those providing laboratory assistance to haemostasis units such as haemophilia centres. Since recent epidemiological research has revealed, that high levels of factor VIII is recognised as a risk factor for recurrent arterial thrombosis and venous thromboembolism (1,2) the present author felt that the presentation should focus on the various methods available for assessment of factor VIII in plasma.

Coagulation factor	Procoagulant Function	Protein quantity	Activated form
Factor VIII	F VIII:C	F VIII:Ag	F VIIIa
Factor IX	F IX:C	F IX:Ag	F IXa

Table1. *Acronyms used in coagulation factor assays. Here exemplified in the case of coagulation factor VIII and factor IX.*

Single coagulation factors may be determined using different approaches, mainly focusing on function or mass. Today, mono-specific immunological reagents are available that can be utilised in ELISA assays for measurement of single coagulation factors, superseding the formerly used electro-immunoassays. In some instances, commercial assays may also be obtained for recording of activation peptides cleaved off from a single factor during its conversion from zymogen to the active form of clotting factor. Determination of the antigenic content of a single coagulation factor is often requested in characterisation of a single coagulation factor deficiency state, and where the antigen concentration is equal to the relative function of the protein (Cross Reactive Material, CRM) the patients phenotype is classified as CRM^-, while in instances where the antigen concentration is normal (as opposed to the function), the phenotype is denoted CRM^+.

However, most frequently a single coagulation factor assay is synonymous with a functional coagulation test that measures the factor in an environment that is completely devoid of the factor in question but completely normal in all other respects. The ideal testbase for measurement of a specific coagulation factor would be a plasma sample delivered by a patient with severe deficiency of the factor investigated, e.g. a plasma from a patient suffering from severe haemophilia A in the case where we wish to record F VIII:C.

In a few instances, assays are also available in which the serine protease enzyme reaction of the factor is recorded enzymatically by its ability to cleave a substrate specific for the factor whereby a coloured split product is released. In some cases, e.g. in measurement of F VIII:C, a factor not holding enzymatic properties itself, a coupled reaction takes place activating factor X, the enzymatic activity of which is measured. Methods of this kind are called chromogenic or amidolytic assays, and commercial kits are available for recording of some single coagulation factors such as thrombin (F II:C), F VIII:C, F X:C, and F VII:C.

Since profound single factor deficiencies are rare, and patients are unfortunately often chronically infected with hepatitis, industrially prepared deficiency plasmas are utilised in which the single coagulation factor has been removed by immunoaffinity chromatography or by other means.

In practice, the single factor assays most commonly used in the haemostasis laboratory are those adopted in haemophilia laboratory service to diagnose, classify, and monitor therapy. With the recently introduced automation in coagulation factor assays, manufactur-

ers of coagulation instruments and reagents provide users with detailed protocols for optimal recording on the specific instrument.
The fundamentals in biometry of factor VIII and factor IX have previously been reported in detail in an excellent monograph published back in 1984 (3).

Coagulometric analysis of factor VIII and IX

The factor VIII molecule is notoriously unstable. It displays a tendency to adsorption to any foreign surface and it degrades quickly when exposed to ambient temperature in particular if the pH value falls outside a rather narrow range around pH 7.4, or when the factor VIII molecule is exposed to any naturally occurring proteolytic plasma enzymes that cause irreversible inactivation of factor VIII. Further, factor VIII is the single coagulation factor in plasma with the lowest concentration of around 100 ng/mL. Another issue of interest is, that a sample may record differently with different reagents and instruments. In comparison, coagulation factors such as F IX, F VII, F X are much more robust and less influenced by storage and assay conditions. Hence, a precise and accurate coagulometric assay for factor VIII is a major challenge to the coagulation laboratory, which is further underscored by the rather strikingly high inter-laboratory variances reported by various external quality assessment organisations such as ECAT (EU) and NEQAS (UK).

Clinical utility of coagulation analyses

In the following the laboratory work-up utilised in diagnosis of haemophilia is described in detail.

APTT

In all laboratory management of haemophilia, the APTT plays a central role in the screening procedure as well as in assessment of the residual level of F VIII:C and FIX:C. A prolonged APTT raises suspicion of a coagulation factor VIII or IX deficiency when detected in an otherwise healthy male person demonstrating an increased propensity to spontaneous bleeds or increased bleeding after trauma or surgery. It should be mentioned that a multitude of different APTT reagents exists on the market, showing some variance in their response to lowered levels of F VIII or F IX, depending on the type of activator as well as the type and concentration of reagent phospholipids (see also section on common screening tests). The versatility of the reagent must be challenged against the various classes of severity of haemophilia, and its usefulness should be documented in functional assays of single coagulation factors. The laboratory protocol for the APTT should adhere to the recommendations given by the manufacturer, including the time of pre-activation before starting the reaction with Ca^{2+}-ions. For practical reasons laboratories usually prefer to use the same brand of APTT reagent for all routine work, that is: APTT in screening as well as activity determination of any intrinsic pathway coagulation factor (XII, XI, IX, VIIII). The phospholipid is a key element to obtain correct measurements of f.i. recombinant factor VIII in post-infusion plasma samples from patients with haemophilia (3).
For analysis of the APTT and its application in measurement of factor VIII or other single coagulation factors, no standardised systems have been produced.

Which kind of information may we extract from the APTT?

The raw APTT is prolonged in most persons with haemophilia, and there is some degree of correlation between the actual APTT value and the level of critical factor in haemophilia, in particular amongst persons with moderate and mild haemophilia. In contrast, APTT's in severe haemophilia are much more diverse. Following treatment with a factor VIII or factor IX concentrate, the APTT will usually return a value in or near the normal range.

Factor VIII activity (F VIII: C) determined by the one-stage clotting technique.

Recording of factor VIII:C in plasma by the one-stage (APTT-based) assay is a simple extension of the crude APTT assay. The assumption is, that the time of clotting is a function of the level of factor VIII:C activity in a reaction system where factor VIII is the only variable, while all other coagulation factors are constant and normal. The test-base is deficiency plasma from a patient with severe haemophilia A or a comparable artificial deficiency plasma, and the current International Standard for Plasma Factor VIII should represent the primary standard. Secondary standards (in-house plasma pool or commercial standard) are often employed in the daily routine assays. Re-calibration is recommended when analytical conditions change which include shifting to a

new batch of APTT reagent, changing the secondary standard, pool of SHP, buffers etc.

Deficiency plasmas
In the routine laboratory, severe deficiency plasma is best obtained from a well-characterised patient with severe haemophilia A (residual F VIII:C level < 0.01 IU/ml, F VIII:Ag < 0.01 IU/ml, a genetic defect that excludes secretion of factor VIII, and no detectable inhibitors), who has not received factor VIII concentrate for the previous 2 weeks. Such ideal conditions may be difficult to meet. Commercial F VIII or F IX deficient plasmas may hold small amounts of residual factor VIII:C or FIX:C activity or contain a small residue of inactive F VIII or F IX that may influence the results.

Calibration
Today's automated instruments have a built-in memory function saving the calibration curves for various lengths of time. In measurement of factor VIII this approach is not recommended.
The calibration procedure is critical for recording of factor VIII:C by the one-stage assay. Freshly prepared calibrator samples are made up by several dilution steps of standard in deficiency plasma and utilised in any new series of factor VIII:C measurements. True double determinations are highly recommended and the test sample should be tested in two or more different dilution steps to ensure linearity of the assay.

Factor VIII:C measured by chromogenic assays

The principles of reaction steps of the chromogenic assay are outlined in Fig. 1, showing that the activity of activated factor X (factor Xa) is recorded as a result of the action of factor VIII (thrombin-) activated into factor VIIIa together with an excess of reagent factor IXa in an environment containing phospholipids. Factor VIIIa is thus the rate limiting factor in the chromogenic reaction.
The chromogenic assay characteristics are quite similar to those of the clotting assay. A multiple-dilution point standard curve is recommended, and if very low levels of factor VIII:C are to be recorded, an extended sensitivity calibration curve should be adopted for the lower part of the measurement range (below 0.20 IU/ml of factor VIII:C). The recommended preactivation time (activation of factor VIII to factor VIIIa by thrombin) should be followed. In principle, standardisation and calibration of the chromogenic assay follows the same rules as governed for the F VIII:C clotting assay. The chromogenic assay displays improved precision compared to the clotting assay, imprecision values most often found at 5% (CV%) compared to around 10% (CV%) with the one-stage assay.

Quality control systems in the haemostasis laboratory

Besides adopting in-assay control procedures that are equally mandatory in clotting assays as in all other laboratory procedures, the services of national and international quality assessment programmes are important. The ECAT Foundation (www.ecat.nl) circulate four annual sets of samples for proficiency testing of most haemostasis components. Participation in this programme is highly valuable since national programmes often only partly cover the quantities of haemostasis.

Assay discrepancy

In some cases of mild haemophilia, inconsistent results are found when comparing results of recordings of factor VIII:C by the one-stage assay to those obtained with the chromogenic assay, formerly the two-stage assay (4,5). This difference is often referred to using the term assay discrepancy.
The most likely explanation for the assay discrepancy in these patients is ascribable to differences in the way factor VIII is activated with the two types of assays, and the particular missense mutation. While factor VIII activation during the APTT-based one-stage assay most likely depends on endogenous formation of thrombin, the chromogenic assay reagents contain exogenous thrombin that directly activates factor VIII, while further thrombin formation is quenched by a specific thrombin inhibitor. In mild haemophilia with assay discrepancy, the mutations are mostly found in the intersection between the A-domains the linkage of which is thought to disrupt prematurely when exposed to thrombin (8.9). In such cases, the F VIII:C value obtained with the chromogenic method renders lower than the one-stage value.
Assay discrepancy has also been reported in haemophilia A patients undergoing treatment with recombinant factor VIII products. Most markedly discrepant results have been found with a B-domain depleted (B-DD) recombinant factor VIII concentrate.

Models for studying the entire process of coagulation

In routine laboratory monitoring of haemophilia the test-base is citrated platelet-free plasma, and the time is recorded until the initial signal of small amounts of fibrin appear in the test tube. Similarly, when recording of factor VIII:C activities by a clotting assay identical end-point measurements are utilised. For numerous years speculations have been raised whether a description of the entire course of coagulation might contribute with valuable information on the pathology of coagulation in various haemorrhagic disorders. An early finding showed that considerable amounts of thrombin activity is being generated in plasma during a period of several minutes after the initial signs of fibrin formation (10) demonstrable in continuously collected sub-samples from the reaction mixture. Another method, the thrombelastograph may characterise entire course of fibrin formation in whole blood by recording the mechanical elasticity changes occurring during fibrin polymerisation. Recent developments have led to an all-automated method for study of thrombin generation (11,12). Although not routinely utilised in the study of haemophilia, this method has a high potential to be used in clinical research of these bleeding disorders.

Recent developments on the thrombelastographic method have shown that severe haemophilia A is highly heterogeneous, as judged from since fibrin formation, and that the response to ex vivo substitution with factor VIII is highly variable amongst patients (13). The response to treatment with these methods is still at the research level.

Determination of the antigens of factor VIII and factor IX

In order to fully characterise haemophilia patients, the antigen concentration of the lacking factor may be useful, in particular in phenotyping of the patient. A CRM- status (low or no recordable antigen) is often associated with a severe bleeding condition linked with a genetic defect that may predict a high risk inhibitor formation, whereas a CRM+ condition (excess of antigen over activity) is rare in cases of severe haemophilia but often seen in milder cases.

Home-made ELISA assays using polyclonal or monoclonal antibodies are used in a few laboratories.

Inhibitors to factor VIII and factor IX

The Bethesda assay and the Nijmegen modified assay variants

The standard test system for determination of inhibitors against factor VIII and factor IX is the Bethesda assay, based on the well-known principle that inhibi-

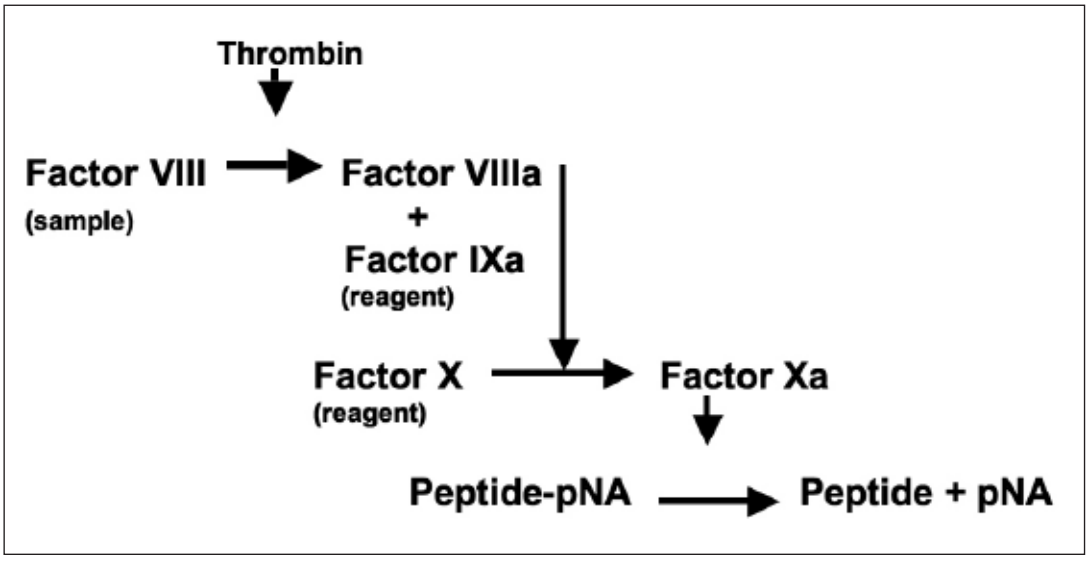

Fig.1 Reaction scheme steps in the chromogenic substrate method for measurement of factor VIII:C. The release of yellow colour pNA molecules is depends on the amount of factor VIII in the sample

tors, when present, inactivate the factor VIII or factor IX molecules in normal plasma. Several different dilutions of patient's plasma are added to a normal plasma sample and incubated for two hours at 37° C. All samples are recorded for their residual content of factor VIII:C. A positive inhibitor result is concluded if the patient sample causes a significant loss of factor VIII:C. Twenty years later a modified assay was devised in which the natural instability of factor VIII caused by change in pH during the incubation period was ruled out by buffering of the normal plasma used (14). A number of false-positive inhibitors could now largely be excluded.

Concluding remarks

Today, haemophilia treatment is based on sufficient amounts of concentrates of high safety. The haemostasis laboratory is still in focus providing a correct phenotype of patients and assisting in ensuring optimal substitution treatment. There still is a need for some kind of point-of-care methods in haemophilia to assist in tailoring individualised treatment based on the response.

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von Willebrand disease – Diagnosis and treatment

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Introduction

von Willebrand disease (VWD) is a bleeding disorder, affecting both males and females, caused by deficiency of von Willebrand factor (VWF). VWD is probably the most common inherited bleeding disorder[1,2]. Rare acquired forms exist. Congenital VWD is divided into several subtypes. Treatment or prevention of bleeding episodes in VWD is achieved by normalizing the level of functional VWF and FVIII, either by stimulating the endogenous factor release with desmopressin (DDAVP, 1-desamino-8-D arginine vasopressin) or by substituting the VWF with infusion of a VWF concentrate. The diagnosis and management of patients with VWD is centralized to national hemophilia centers.

Background to the VWF

The VWF is a large multimeric protein synthesized in endothelial cells and megakaryocytes. VWF circulates in plasma and in platelet alpha-granules. VWF has two main functions in hemostastis[3]. It participates in the formation of the platelet plug, and it is a carrier protein for coagulation factor VIII (FVIII), protecting it from inactivation. The multimers range in size from dimers of 500 kDa to large multimers of 40 monomers or more, to a size of >10 million Da. After release from endothelial cells, the multimers are cleaved in plasma by the enzyme ADAMTS13, at a specific cleavage site, resulting in a typical multimeric pattern. Mutations in the VWF gene may affect different functions depending on the site of the mutation. A registry on VWF mutations is available at http://www.vwf.group.shef.ac.uk/mutations/mutreferences.html.

Epidemiology of VWD

The prevalence of VWD in population studies has been about 1%[1,2] whereas the referral-based prevalence (including only patients diagnosed at specialized centers) is significantly lower, with estimates ranging from 23 to 113 cases per million inhabitants[4,5]. The relatively frequent bleeding symptoms and positive family histories in the general population involve a risk of overestimating the prevalence of VWD and may contribute to the high prevalence seen in population studies. In selected populations, the prevalence has been even higher. A high frequency (overall 13%) of VWD has been described in some studies of women with menorrhagia[6].

Symptoms and family history

VWD is characterized by a bleeding tendency. Mucocutaneous bleeds, e.g. nose bleeds and menorrhagia are common. There is also a tendency to bleed in connection with surgery and other invasive procedures. Rare patients with severe VWD may also have joint bleeds due to low levels of FVIII. The International Society of Thrombosis and Haemostasis (ISTH) has set up criteria for a significant bleeding history (Table 1)[7]. A scoring system for objective quantification of the severity of bleeding symptoms has been developed within a European multicenter collaboration[8].

Criteria for family history have also been presented in the ISTH guidelines for diagnosis of VWD type 1[7]. A positive family history compatible with VWD type 1 requires that at least one first-degree relative, or at least two second-degree relatives, have a personal history of significant mucocutaneous bleeding and laboratory tests compatible with VWD type. When available, the use of VWF mutations or genetic markers linked to the VWF locus may permit the analysis of more remote relatives, and may allow asymptomatic relatives with low VWF levels to provide evidence for inheritance.

Laboratory tests

Low levels of functional VWF in plasma characterize VWD. FVIII in plasma may also be decreased. The diagnosis of VWD involves several different tests of VWF activity, structure and concentration (Table 2)[9].

Other causes of bleeding diathesis should be ruled out before going into the complete laboratory investigation of VWD. The APTT is usually normal in VWD, but may be slightly prolonged in the most severe cases, due to FVIII deficiency. The PT or INR is normal, as the vitamin K dependent coagulation factors II, VII and X, are not affected. Screening tests for primary hemostasis, e.g. bleeding time and platelet function analyzer 100 (PFA-100), may be prolonged, but these tests are hampered by low specificity and sensitivity[10-12]. Screening for VWD should involve VWF activity testing, as the antigen level may be normal in some cases. The golden standard for VWF activity is the VWF ristocetin cofactor activity test (VWF:RCo)[13]. The FVIII coagulation activity (FVIII:C) should be measured, as it may be low in some cases. The ABO blood group should be tested as persons with blood group 0 have about 30% lower VWF levels in plasma.

Subtypes of VWD

Type 1

Type 1 is a quantitative defect without functional abnormalities in the VWF. The diagnosis of VWD type 1 should be based on bleeding symptoms (Table 1), a positive family history, and VWF deficiency. VWF levels in plasma should preferably be <0.35 kIU/L to avoid overlap with the normal population. VWF:RCo and VWF:Ag are equally decreased, and the VWF:RCo/VWF:Ag ratio close to 1, (typically not <0.6-0.7). The multimeric structure should be normal[14]. The sensitivity to ristocetin in the RIPA test should not be increased. Numerous mutations in families with a historical diagnosis of type 1 VWD have recently been identified. As the mutations are not localized in any certain area of the VWF gene, it is still demanding to apply mutation screening to the routine investigation of VWD type 1 [15,16].

Type 2

Type 2 VWD is characterized by functional defects in the VWF. The VWF levels are usually low and the VWF:RCo/VWF:Ag ratio is usually <0.6-0.7. Type 2 is further subdivided into subtypes 2A, 2B, 2M and 2N, depending on the type of dysfunction. Mutations in functional domains can be identified in most families. Inheritance is usually dominant, except type 2N and rare families with other subtypes. Most patients are non-responders to DDAVP. The high molecular weight multimers (HMWM) of the VWF are lacking in types 2A and 2B. All multimers are present in types 2M and 2N, but in 2M the pattern of the multimeric bands may be aberrant. The RIPA is decreased in 2A and 2M, but increased in 2B. In type 2B, the VWF show increased affinity for the platelet receptor GPIb[17], and stress, infections and pregnancy may cause platelet aggregation and thrombocytopenia. DDAVP provokes or aggravates thrombocytopenia, and should not be used in patients with classical type 2B. Type 2N is characterized by decreased affinity of VWF for FVIII. The VWF is often normal, but FVIII levels are low due to increased clearance.

Type 3

Type 3 VWD is characterized by total or almost total deficiency of VWF. FVIII levels are low due to the lack of VWF, usually <0.10 kIU/L. Patients may encounter hemophilia like symptoms, like joint bleeds. Inheritance is recessive. Mutations are usually nonsense or frameshift. Larger deletions or missense and splice site mutations also occur. There is no response to DDAVP.

Treatment

Bleeds are treated with a normalization of the VWF and FVIII levels. VWF levels are generally considered to be most important for mucocutaneous bleeds, whereas FVIII levels are more important for joint bleeds and soft tissues bleeds. Factor levels can be raised by stimulating the endogenous release of FVIII and VWF with DDAVP (DDAVP, 1-desamino-8-D arginine vasopressin)[18]. Most patients with type 1 respond to DDAVP. Some patients with type 2 may respond sufficiently, but most type 2 patients will have an insufficient response due to functional abnormalities in the VWF. In the classical form of type 2B, DDAVP cause platelet aggregation and thrombocytopenia, and is therefore contraindicated. Type 3 patients do not respond to DDAVP. Patients who are unresponsive to DDAVP require concentrates containing large amounts of VWF in case of bleeds or surgery. Some type 1 patients may also require VWF concentrates if they need prolonged hemostatic cover due to major surgery, or present with contraindications to DDAVP, such as cardiovascular disease.

Summary

VWD is the most common inherited bleeding disorder, but it is heterogeneous and consists of several subtypes. The diagnosis may be complicated espe-

cially in mild cases that may be difficult to differ from normal individuals with low VWF levels in plasma. The diagnosis should be suspected in persons with mucous membrane bleeds and a positive family history. VWD is usually not revealed by routine screening tests, i.e. the APTT and INR, but require specific testing of the VWF level. Bleeds are treated either with DDAVP which stimulates the endogenous VWF and FVIII release, or the infusion of a concentrate containing VWF. Patients should be referred to a national hemophilia center for diagnosis.

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Table 1[7] Significant mucocutaneous bleeding symptoms (ISTH criteria):

Significant mucocutaneous bleeding symptoms:

- Nose bleeding, ≥2 episodes without a history of trauma not stopped by short compression of <10 min, or ≥1 episode requiring blood transfusion.
- Cutaneous hemorrhage and bruisability with minimal or no apparent trauma, as a presenting symptom or requiring medical treatment.
- Prolonged bleeding from trivial wounds, lasting ≥15 min or recurring spontaneously during the 7 days after wounding.
- Oral cavity bleeding that requires medical attention, such as gingival bleeding, or bleeding with tooth eruption or bites to lips and tongue.
- Spontaneous gastrointestinal bleeding requiring medical attention, or resulting in acute or chronic anemia, unexplained by ulceration or portal hypertension.
- Heavy, prolonged, or recurrent bleeding after tooth extraction or other oral surgery such as tonsillectomy and adenoidectomy, requiring medical attention.
- Menorrhagia resulting in acute or chronic anemia, or requiring medical treatment, not associated with structural lesions of the uterus.
- Bleeding from other skin or mucous membrane surfaces requiring medical treatment (e.g. eye, ear, respiratory tract, genitourinary tract other than uterus).

A significant mucocutaneous bleeding history requires at least two symptoms in the absence of a blood transfusion history, or one symptom requiring treatment with blood transfusion, or one symptom recurring on at least three distinct occasions.

Table 2. Laboratory methods for the VWF

Abbreviation	Method	Comment
VWF:RCo	Ristocetin cofactor activity	The ability of the VWF to bind to the platelet receptor gpIb and cause agglutination of normal formalin fixed platelets. The main diagnostic and monitoring method
VWF:Ag	Von Willebrand factor antigen	Determines the concentration of VWF protein
VWF:CB	Von Willebrand factor collagen binding	The binding capacity of VWF to collagen
RIPA	Ristocetin induced platelet aggregation	Detects increased sensitivity to ristocetin in type 2B. RIPA is performed in patient platelet rich plasma
VWF:MS	VWF multimeric sizing	The analyzis of the multimeric structure of the VWF
VWF:FVIIIB	The FVIII binding capacity of von Willebrand factor	Determination of the VWF capacity to bind FVIII

Hemophilia. Diagnosis and therapy

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Diagnosis

Hemophilia has different degrees of severity (Table 1) and therefore time of symptoms and diagnosis may range from birth up till adulthood. Symptoms are very similar for hemophilia A (factor VIII deficiency) and hemophilia B (factor IX deficiency) with joint bleeds as the most typical manifestation. Occasionally diagnosis is established even at old age. When there is a family history diagnosis is straightforward.

If a family history is present, diagnosis usually is made shortly after birth. However, about half of the cases do not have a known family history and time of diagnosis is dependent upon when symptoms occur. In the severe form of hemophilia bruising or even joint bleeds may occur around the age of 6-12 months when the child is becoming more physically active. Joint bleeds typically occur in elbows, knees or ankles and when the child has started to walk. Symptoms close to delivery and during the first months of life are rare but may include brain hemorrhage in connection with delivery, intra-abdominal bleeds, and bleeds occurring in connection with blood sampling. In mild hemophilia the first symptom may be disclosed in connection with surgery later in life.

Typically, diagnosis in severe hemophilia is made in a boy at the age of around 6 months. A thorough bleeding history should be taken in order to understand type of potential coagulation defect in a bleeding child. In hemophilia, having a defect plasma coagulation, tumor-like soft tissue bleeds (subcutaneous and muscular) are typical as well as joint bleeds whereas e.g. the child with von Willebrand disease (VWD) is more prone to present with mucosal bleeds such as bleedings from nose and the gastrointestinal tract. It is important to stress that bleeding symptoms due to a plasma coagulation defect or a defect in primary hemostasis may show big overlap. Joint bleeds are very typical for hemophilia but may e.g. also occur in type 3 VWD. In summary the suspicion of hemophilia is strong if male gender and clinical signs of a plasma coagulation defect are present, especially the manifestation of a joint bleed. In a series of 140 boys diagnosed as having hemophilia A or B during the years 1960-1987 mean age at diagnosis was 9 months for the severe cases and 22 months for the moderate cases[1] . Of the presenting symptoms, subcutaneous bleedings constituted 41 % while joint and muscle bleedings were uncommon. 9 % were diagnosed in the post-neonatal period, but 20 % had shown abnormal bleeding tendency already in the neonatal period. Intracranial hemorrhage occurred in 5 %, with a majority in the neonatal period. Thus, awareness of hemophilia among physicians taking care of children, not least in the neonatal period, should make early diagnosis possible.

When the diagnosis of hemophilia is suspected from clinical grounds or heredity a relevant laboratory check-up is done. For the non-specialized physician this includes a full blood count including hemoglobin level, white cell count, differential count and a platelet count. These parameters are normal in hemophilia provided anemia is not present due to chronic bleeding problems or an acute hemorrhage. An APTT and a prothrombin time (PT) are also compulsory for the investigation. In hemophilia the PT is normal whereas the APTT usually is clearly prolonged although in mild hemophilia, the prolongation may be so discrete that the inexperienced physician can by mistake neglect the prolongation. When the clinical situation and the APTT indicate the possibility of hemophilia a blood sample should be referred to a coagulation laboratory, where competence and experience for check-up of bleeding disorders are present. The specialized coagulation investigation always includes analysis of factor VIII and IX. A finding of a low factor VIII level is followed by determination of VWF to rule out the presence of

VWD. In cases of suspected mild hemophilia A, where the diagnosis is not fully evident e.g. by a family history including both genders, the possibility of VWD type Normandy should be considered. Low factor IX values may be caused by non-genetic abnormalities such as vitamin K deficiency but normal PT concords with hemophilia. Another differential diagnosis for factor VIII deficiency could be a combined factor V/VIII deficiency [2]. These patients have, however, a prolonged PT and an autosomal inheritance.

Therapy

General aspects

If not optimally treated, hemophilia is a disorder with severe symptoms and complications. An important basis for a good outcome of hemophilia treatment is the organization of care. Experience from several countries, as well as recommendations from WHO and WFH (World Federation of Hemophilia), state that there must be a structure of comprehensive care. Hemophilia centers are organized by doctors with specialist qualifications in hematology and pediatric hematology. In addition there is specially trained staff including nurses, physiotherapists, orthopedic surgeons, dentists, geneticists and social workers. Degree of centralization and exact staffing varies among centers and countries. In the Nordic countries centralized comprehensive care has been the model since decades in Denmark, Sweden and Norway whereas Finland has had a more split model. Comprehensive care includes responsibility not only for the patient but also for the impact hemophilia has on the rest of the family. When a diagnosis is made in the young child the family is thoroughly informed and educated and this is later on extended by staff members also to day care center and school. A hemophilia card is issued in order to secure information about the severity of the disease to other health care institutions and to ensure communication to the hemophilia center. A program for routine check-ups is implemented where the child with severe hemophilia is seen at the center every 6 month and the adult once a year. An example of a check-up program is given in Table 2.

Specific therapy

As hemophilia is caused by deficiency of a specific coagulation factor the rationale for therapy is replacement of factor VIII or IX. In mild hemophilia A, especially in cases where the factor VIII level is well above 0.05 kIE/L, a good response to desmopressin, given intravenously, subcutaneously or as a convenient nasal-spray can be seen resulting in an increase to which hemostatic levels can be achieved [3]. Tranexamic acid is also a useful hemostatic agent which especially is recommended in connection with mucosal bleeds. The drug is often used in connection with desmopressin.

In severe forms of hemophilia and also in mild hemophilia B replacement therapy must be given in case of bleedings or as prophylactic therapy. The era of factor concentrates is rather young and the first product was Cohn fraction I-0, later manufactured as AHF-Kabi during the 1950s up till the 1980s [4]. Factor IX concentrates were developed a few years later. With the introduction of more purified concentrates during the 1970s home treatment could be implemented. Thus the patients or the patient´s guardian could easily give the treatment at home or during travelling. The patient was no longer bound to a health care facility. The concentrates had a small reconstitution volume and were very effective and convenient to use. They were however produced from large plasma pools and the problem with blood transmitted agents causing liver disease due to hepatitis became evident during the 1970s [5]. Transmission of HIV struck the hemophilia population during the early 1980s and the problems with HIV and the discovery of hepatitis C virus prompted a rapid development of factor concentrates [6]. Careful screening of donors and implementation of viral reduction methods dramatically increased safety and since 1992 recombinant factor VIII concentrates have become available and since 1999 a recombinant factor IX concentrate is available [7]. The current view is that factor concentrates used for hemophilia is entirely safe with regard to transmission of disease agents.

Surgery

Modern, safe treatment has also opened up the possibility of joint replacement surgery. During the last decades many older patients have get rid of their disabling joint pain thanks to replacement of hips and knees. Other important interventions are ankle arthrodesis, resection of capitulum radii and also radioactive synovectomy. Modern prophylaxis since early age will probably make orthopedic surgery in hemophiliacs a very rare event in the future.

Treatment of acute bleeds

Treatment of acute bleeds in severe hemophilia is based on one to two infusions of factor concentrate usually about 20-40 IU per kg body weight per dose. This administration will increase factor VIII levels to 0.4-0.8 kIU/L or factor IX to 0.2-0.4 kIU/L which means that factor IX often needs to be given in higher doses than factor VIII. On the other hand factor IX has a longer biological half-life (18-20 hrs) and hemostatic effect compared to factor VIII (11-16 hrs) given as terminal half-lives in a two-compartment pharmacokinetic model [8].

Prophylaxis

Prophylactic treatment of hemophilia has revolutionized the well being of patients with severe hemophilia and today it is almost possible to prevent the disabling hemophilic arthropathy, sequel from brain hemorrhage etc. In Sweden, prophylactic treatment of boys with hemophilia was started already in 1958. During the 1960s and the beginning of 1970s, only limited supplies of factor VIII were available and prophylactic treatment could not be given as effectively as desired. Since the 1980s prophylaxis has been given to virtually all patients in need of the treatment and it has become more optimized both in terms of efficacy, cost-effectiveness and convenience. The traditional Swedish dosing regimen has been based on infusion of around 20-40 IU per kg body weight three times per week often trying to keep the trough VIII:C/IX:C level not to fall below 0.01 kIU/L. The first long term results were published in 1992 where 65 patients (56 hemophilia A and 9 hemophilia B) aged 3-35 years were reported [9]. The results were striking and showed that early start and intense prophylactic treatment could virtually prevent all joint disease. Since then a number of studies from Sweden and other countries have shown the benefit of prophylaxis. In a recent study performed in the USA patients were randomized between on demand treatment and prophylaxis [10]. The medical outcome was strongly in favour of prophylaxis which concords with what has been shown in cohort studies in Europe since decades[11].

Prophylaxis has evolved with years and regimens used today are not exactly the same as a decade ago. Also there are different strategies described from different geographic areas as outlined in Table 3. The trend in Sweden is to give very frequent, often daily, small doses of concentrate based on individual patient pharmacokinetics. In the Netherlands a less frequent, intermediate dosing regimen is used whereas in Canada a so called dose escalation model has been suggested and studied. The different regimens have their pros and cons and there are several open issues in prophylaxis as when to start, when/if to stop and dosing [12]. The issue of venous access is also very important for early start of prophylaxis [13] as well as health-economic and quality of life issues [14]. The benefit of prophylaxis has been clearly shown in a comparative health-economic study between Sweden and Norway [15].

Side effects of therapy

Viral transmissions

Virus transmission via plasma-derived factor concentrates has thrown shadows over hemophilia treatment during several decades. Around 80 % of patients treated before 1985 have become hepatitis C infected [16] and more than half have attracted HIV [17]. Since the advent of safer products during the second half of the 1980s new viral transmissions have eventually ceased.

Inhibitors

Development of antibodies to factor VIII or IX replacement therapy causing neutralization of the factor is today the most challenging problem in modern hemophilia care [18]. These so called inhibitors may occur in all types and severities of hemophilia although they pose the major problem in severe hemophilia A where around 30 % will develop inhibitors, already after a median of about 10 infusions. In hemophilia B the incidence is reported to be considerably lower, around 5 %. Thus the typical inhibitor patient is a small child with severe hemophilia A, who just has started prophylaxis. Some patients and families are more prone to develop inhibitors and there seems to be several genetic and circumvention factors which increase (or protects) the risk of inhibitor development [19-23]. Among these can be mentioned type of mutation in the causative gene, polymorphisms in the CTL-A4, IL-10 and TNF-α genes, age at start of treatment, type of concentrate, prophylaxis, inflammatory reactions and surgery. More studies are needed, and ongoing, to establish the importance

of these and other factors. Inhibitors may disappear with time, so called transient inhibitors, but many persist and respond briskly in titer when challenged with factor. Obtaining sufficient hemostasis in such patients is difficult and the patient in addition bleeds as frequent as patients who are not on prophylaxis. Therefore the main goal of treatment is to induce immune tolerance.

Immune tolerance induction (ITI) is nowadays usually started in connection with, or early after development of an inhibitor. The regimen used often comprises very high doses of factor concentrate and the treatment course spans over several months or even years. Other regimens are also in use with lower doses or combined with immunosuppressive agents and extracorporeal inhibitor adsorption [24]. The high cost of the treatment makes it controversial and a comparison between two different regimens using different dose levels is now ongoing [25].

The reported success rates of ITI in hemophilia A is around 60-80 %. In hemophilia B the lack of experience with ITI makes figures very unsure but using the so-called Malmö protocol in a small series the success rate was as high as in hemophilia A [26]. In therapy resistant patients episodic treatment with so called by-passing agents is the state of the art treatment when acute bleeds occur [27]. Even if the success rate is high, there is a fraction of patients who do not respond to treatment (around 10 %). In addition some patients respond differently to the two available products, factor eight inhibitor bypassing agent (FEIBA) and recombinant activated factor VII (NovoSeven) and there is no possibility to predict this different response. Much work remains to improve treatment of inhibitor patients.

Future

Hemophilia is a monogenic disease and should be ideal for gene therapy but previous optimism has now turned into more realism and much basic research needs to be done before going into clinical trials again [28]. Much research and focus is now devoted to construction of factor VIII and IX molecules of formulations with improved pharmacokinetic properties with the potential to improve cost-effectiveness as well as convenience [29].

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Table 1.
Degrees of severity in hemophilia

		Coagulation activity VIII:C (%)	IX:C (%)	Joint bleeds
Hemophilia A	Severe	<1	100	+++
	Moderate	1-4	100	+
	Mild	5-40	100	(+)
Hemophilia B	Severe	100	<1	+++
	Moderate	100	1-4	+
	Mild	100	5-40	(+)

Table 2.
Routine check-up program of a patient with severe hemophilia.
Items may differ due to age and medical situation.

Medical history with focus on bleeding symptoms
Sociomedical aspects (absence from school/work, leisure activities, quality of life)
Physical examination including joint score
Orthopedic evaluation
Laboratory surveillance (VIII/IX:C values, inhibitor testing, liver enzymes)
Future treatment plan

Tabel 3.
Dose regimen comparisons

	Convenience	Overall efficacy	Cost
Dutch	+/-	+	-/+
Swedish	+/-	++	--
Canadian	+	+	+
PK	-	+++	+++

+ = superior; - = inferior

PK, pharmacokinetic (Swedish) regimen

Screening tests for bleeding disorders

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1.1 Introduction

The hemorrhagic disorders include primary hemostatic disorders (PHD), i.e. thrombocytopenia, platelet dysfunction (PD) and von Willebrand disease (VWD), secondary hemostatic disorders (coagulation disorders) or, rarely, hyperfibrinolysis ("tertiary" hemostatic disorders). Additionally, vascular disorders such as hereditary telangiectasia may cause abnormal bleeding but will not be discussed here. It is the role of the physician in cooperation with the coagulation laboratory to accurately identify those individuals that truly have an abnormal bleeding tendency caused by disorders of hemostasis from those that do not. The diagnostic process should always begin with symptom analysis. However, it complicates the process that in unselected populations 25% of individuals report abnormal bleeding. Since self-reported bleeding may be exaggerated and clinically irrelevant and mildly lowered laboratory values are found in normal individuals, together this may lead to false positive diagnoses[1-6].

Testing for abnormal hemostasis usually includes both screening (global) tests and more specific tests such as factor assays and genetic testing. There is considerable ongoing debate on the appropriate use of screening tests of hemostasis, especially those used to screen for PHD. This chapter briefly discusses the relationship between bleeding symptoms and screening tests used in the diagnostic process. More detailed discussion on coagulation screening tests, specific assays and platelet function is found in other chapters of the current issue.

1.2 Bleeding symptoms and bleeding disorders

Although all bleeding disorders are associated with abnormal bleeding following surgery and trauma, other specific symptoms point to certain hemostatic disturbances and should help the clinician select patients for appropriate testing[6]. Thus, the hallmark of PHD (PD, mild/moderate/severe VWD) is abnormal mucocutaneous bleeding (frequent or recurrent nosebleeds, gum bleeding, primary menorrhagia and iron deficiency anemia, excessive bruising, petechiae) and primary bleeding, i.e. prolonged bleeding from small cuts and/or poor healing of small lacerations 7. The typical symptoms of disorders of secondary hemostasis (e.g. hemophilia A and B) are related to abnormal fibrin formation and include secondary (late onset) hemorrhage, large subcutanous and muscle hematomas and recurrent hemarthroses. Symptoms associated with hyperfibrinolysis are similar to those of abnormal fibrin formation. Severe and to less degree moderately severe VWD has both primary and secondary bleeding symptoms since it is associated with low coagulation factor VIII activity (FVIIIc) into the moderate hemophilia range.

1.3 The initial laboratory approach

Once an appropriate patient has been selected for laboratory testing the direction of testing is usually based on the results of initial complete blood count and screening tests. Coagulation screening test include the activated partial thromboplastin time (APTT) and the prothrombin time (PT), which when applied together are sensitive to decreased function of all coagulation factors except factor XIII. Screening tests intended to measure platelet function (adhesion and aggregation) include the template bleeding time (BT) and the more recent PFA-100® closure times (CT). Since neither the CT nor the BT are sensitive to mild forms of VWD or mild PD, many laboratories will include VWF assays and platelet aggregation studies in the initial work-up.

1.4 Impact of definition of bleeding disorders on screening test results

The sensitivity of screening assays for certain bleeding disorders is highly dependent on the severity and definition of the bleeding disorder. Thus, abnormal

screening tests do accurately separate patients with the more severe bleeding disorders from healthy individuals (e.g. APTT in hemophilia, CT in severe VWD or Bernard-Soulier syndrome), but are not as sensitive to the more common mild PHD. According to some studies, mild PHD may be present in 1-2 percent of the general population (i.e. mild type 1 VWD, mild PD)[6 8 9]. The prevalence, however, is dependent on how abnormal laboratory results are defined (and, hence, how disease is defined). Interestingly, the prevalence is much lower in referred patients as evident by data from the Nordic Hemophilia Council[10]. It has been argued that the high reported population prevalence of mild type 1 VWD in population studies is a consequence of false positive diagnoses[1]. Many patients with this diagnosis have VWF activity in the 40-50 kIU/L range, mainly related to blood group O, and it is now known that most of them do not have mutations in the VWF gene whereas those below 40 kIU/L are likely to carry such mutations[11-13]. However, a recent case-control study has shown that randomly selected teenagers with marginally lowered VWF (35-45 kIU/L) do have excessive bleeding[6]. Should the definition of VWD then be based on VWF activity level, severity of clinical symptom or on the mutational status? Which condition should the screening test reflect or reasonably be expected to reflect? For clinical purposes abnormal screening tests should be sensitive to "clinically meaningful lowered VWF" but not simply to a statistically defined abnormal cut-of limit of VWF. Unfortunately, there is no current agreement on the definition of this condition.

Similar considerations apply to PD. The diagnosis of mild PD is probably even more controversial than that of mild type 1 VWD. Although rare PD´s (e.g. Glanzmann´s, Bernard-Soulier) are well defined disorders, this is not the case for most mild PDs. There is no single accepted gold standard diagnostic method for PD and PD includes many conditions that are diagnosed by different methods, including platelet aggregation in platelet rich plasma (PRP) or whole blood, platelet granule release assays, flow cytometry, abnormal CT or BT[14-18]. Although platelet aggregometry is a cornerstone in the diagnosis of PD, it´s application varies markedly between laboratories and so far only rare unsuccessful attempts have been made to standardize it[19 20 21]. Also, there remains no consensus yet on which platelet agonists to apply, which concentrations to use, or even on how to interpet the aggregometric patterns[19 22 23]. Hence, the clinical significance of mild PD diagnoses often is uncertain[24-27]. Based on these considerations it is hard to judge the exact value of screening tests in light of the many poorly defined platelet syndromes.

1.5 Screening tests for primary hemostatic disorders

The BT, the oldest test for primary hemostasis and the only in vivo screening test, is time consuming, invasive and operator dependent[14 16 18 28]. It is also influenced by age, sex, skin texture and temperature, hematocrit, drugs, venostasis and the site and direction of the incision[20]. It is a poor predictor of surgical bleeding in low risk individuals and not recommended for pre-operative screening[28 29]. On the other hand it may be useful for identifying patients with severe PHD such as severe VWD or Glanzmann´s thrombasthenia[20 30]. Due to the invasiveness and the lack of sensitivity and specificity, as well as poor positive and negative predictive value, the BT has been abandoned as a screening test or even as a complimentary test in many laboratories.

The PFA-100 CT is an in vitro test that uses buffered citrated whole blood to simulate platelet function in a wounded vessel as blood is drawn through a microaperture on a coated membrane with a constant vacuum. The platelets gradually adhere and aggregate to plug the hole and the instrument records the time until the flow stops[14 31]. The test is somewhat sensitive to the citrate concentration and there is an inverse correlation between platelet count and hematocrit as with the BT[32 33]. It is insensitive to coagulation factor deficiency and to heparin and warfarin[14 34]. The CT is more sensitive in detecting congenital PD than the BT[34] and it is very sensitive to moderately severe and severe VWD[35 36]. However, the sensitivity to mild (or marginal) VWD is only about 50%. Currently, there is consensus that the CT cannot rule out mild hemostatic disorders and that it should under no circumstances be used as a screening tool that supplants specific testing for PHD[14 32 37 38]. The author, however, finds the CT useful as complementary test during the initial work-up when rapid results are needed to give an early indication of the presence of clinically important moderate or severe PHD before specific test results become available.

1.6 Screening tests for secondary hemostatic disorders

The APTT and PT can become prolonged once a coagulation factor activity is < 40-50 % of normal

activity. Since clinically relevant coagulation factor deficiencies usually have a factor activity of < 25%, there is little overlap with normals (mostly over 50 kIU/L). Therefore, these tests are sensitive screening tests for most coagulation factor deficiencies and there is good evidence for their usefulness in patients with bleeding symptoms. However, screening for bleeding disorders with APTT or PT is not warranted in asymptomatic individuals, e.g. random pre-operative screening[39]. When an abnormal result is found in the absence of bleeding symptoms, it is usually caused by lupus anticoagulant or factor XII deficiency, conditions that are either thrombophilic or not associated with bleeding.

1.7 Conclusions

Only symptomatic patients should be screened for hemostatic disorders. The BT should be abandoned. The applications of the PFA-100 are currently debated. The PFA-100 CT should not be used as an exclusive screening test for PHD but when used as complementary test often can predict clinically relevant moderate or severe PHD while results of more specific tests are pending. A normal CT does not exclude mild PHD. The screening tests of secondary hemostasis, however, are useful in the diagnosis of most clotting factor deficiencies with the exception of factor XIII deficiency.

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Tests used in the clinical evaluation of the fibrinolytic system

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Introduction

The fibrinolytic system is of importance in many different physiological or patophysiological processes, such as haemostasis, tissue remodelling, reproduction and cellular invasiveness. Regarding its role in the haemostatic system an over-function of the fibrinolytic system results in a bleeding tendency, such as in patients with antiplasmin deficiency or in patients with PAI-1 deficiency. A decreased function of the fibrinolytic system, on the other hand, may result in a thrombotic tendency, both on the arterial and venous sides of the circulatory system. Most commonly, increased levels of PAI-1 is the cause of an impaired fibrinolytic function. A few individuals have been shown to have a plasminogen deficiency and some have a decreased capacity to release or store tPA. To monitor clinically significant deviations within the fibrinolytic system a number of tests are available today. Some of these tests are of the global type, developed in order to investigate any type of problems within the full fibrinolytic system. Such tests are euglobulin clot lysis, fibrin plate clot lysis, whole blood clot lysis among others. Specific tests to measure the individual components of the fibrinolytic system have eventually been developed, using either immunochemical procedures or functional techniques. All tests have advantages or disadvantages as will be discussed in this review. In addition to these analyses also some DNA tests of possible clinical interest have been developed for some of the compounds. In this article only the most important aspects of tests within the fibrinolytic system will be covered. For those who want a deeper understanding of the field I recommend some review articles (1, 2).

Tests in use – *Global tests*

Most of the so called global tests are only of historical interest. Tests like euglobulin clot lysis time, fibrin plate clot lysis or whole blood clot lysis are laborious and difficult to automate and will therefore not play an important role in a modern clinical laboratory. In the early days they were definitely important and mainly used to find a correlation between an impaired fibrinolytic function and thrombosis. The studies were mostly retrospective and it was really difficult to evaluate the results, especially regarding the question of cause or consequence. Thromboelastography is one method used today in the clinical evaluation of haemostatic problems. It is probably better in the evaluation of the clotting process itself, rather than of the fibrinolytic process. The rationale in using global tests to pinpoint problems within the fibrinolytic system is according to my view difficult to understand, since it will slow down the diagnostic process. This will in fact be more costly, because even complicated biochemical tests are relatively cheap, in comparison to a prolonged diagnostic process. The various global tests used within the area of fibrinolysis will not be covered in this review.

Specific tests

Specific tests for the individual components within the fibrinolytic system are available for most of these factors, both by immunochemical methods and by functional techniques. However, the clinical usefulness in measuring some of the components is questionable.

The specific compounds of the fibrinolytic system that will be discussed here are plasminogen, antiplasmin, plasminogen activators, plasminogen activator inhibitors, the fibrin degradation product D-dimer and TAFI. Plasminogen is the central compound in the fibrinolytic system, which can be activated to the aggressive proteolytic enzyme plasmin (figure 1). Plasmin is able to degrade virtually any protein, but in the circulatory system its action is mainly directed towards fibrin, by several complicated regulatory mechanisms. This includes a selected activation of fibrin-bound plasminogen by tPA and protection of fibrin-bound plasmin against its

inhibitor antiplasmin (3). The two physiological plasminogen activators tPA and uPA seems to have different physiological functions: tPA has its main function within the circulatory system, while uPA mainly exerts its function in exocrine glands, including kidneys, and at the pericellular level (2). Regulation by inhibition occurs at two levels: inhibition of plasmin by antiplasmin and inhibition at the activation level by PAI-1 (tPA and uPA) or PAI-2 (only uPA) in pregnant women. Fibrin itself has an important function in regulating the fibrinolytic process or rather keeping it localized at the fibrin surface (3). Measuring the degradation product D-dimer (4) is a common test of fibrinolytic function, but reflects activation of the coagulation system and fibrin formation, rather than the fibrinolytic capacity. TAFI is an abbreviation for thrombin activatable fibrinolysis inhibitor, which is a strange name, because it is not an inhibitor at all. It is a procarboxypeptidase, which after activation can remove COOH-terminal lysine-residues (5). Since COOH-terminal lysine-residues formed by plasmin action on fibrin normally stimulates the fibrinolytic activation, this stimulatory action might theoretically be lost. However, it has not yet been fully proven that this really occurs in vivo. In addition, not only thrombin, but a number of other proteolytic enzymes may activate TAFI.

Plasminogen, antiplasmin and plasmin-antiplasmin complex

Determination of plasminogen is mostly performed as a functional test, by activation of plasminogen with streptokinase and then measuring formed plasmin with a chromogenic peptide substrate (6). Several kits are commercially available, some of which works well even with highly automated equipment. However, the clinical need to measure plasminogen routinely is not strong. A few families with plasminogen deficiency in connection with thrombotic problems have been described. Except for the fact that low levels of plasminogen are typically found in severe liver disease, or due to consumption in connection with thrombolytic treatment, the need to measure plasminogen in different clinical conditions is low.

Functional chromogenic peptide substrate methods measuring antiplasmin are also commercially available. The concentration of antiplasmin in plasma samples is typically about 70 ± 7 mg/L, which equals about 1μmol/L (7). A few families with antiplasmin deficiency have been found. These have a severe bleeding tendency comparable and similar to patients with haemophilia. However, once correctly diagnosed, they are easily treated with antifibrinolytic drugs. Since antiplasmin like many other proteins are synthesised in the liver, decreased plasma levels are seen in severe liver disease. Also, decreased levels are typically found in connection with thrombolytic therapy. Under those circumstances it seems that at the point when the antiplasmin concentration in plasma decreases and free plasmin may occur in the circulatory system, the fibrinogen concentration decreases and the risk of bleeding increases in parallel. Otherwise the clinical need for measuring antiplasmin in plasma is small.

Measuring plasmin-antiplasmin complex in plasma is one way to really monitor and follow the activity of the fibrinolytic system, which might be important in certain research projects. It is quite easily done with two-site ELISA techniques (8). Unfortunately, no commercial kits are presently available on the market.

tPA and uPA

Regarding measurement of tPA, immunochemical and functional methods are very deviating. The reason is that active tPA only constitutes a small portion of the tPA antigen present in plasma. The major portion is tPA in complex with various inhibitors, such as C1-inhibitor, antiplasmin, and in particular with PAI-1 (9). The different compounds included in tPA antigen are demonstrated schematically in figure 2. Therefore, increased tPA-antigen rather reflects an impaired fibrinolytic activity, than an increased activity. In fact, the correlation between tPA- antigen and PAI-1 activity is typically quite high. Thus, high tPA-antigen levels indicate a risk of thrombosis. To measure tPA activity precautions have to be made to avoid inactivation during sample handling and storage (10). This can be achieved by collecting the samples into an acidic buffer (acetic acid/acetate). Such vacuum tubes are present on the market, but are typically not included in normal routines. Therefore tPA activity is rarely included in routine analysis of the fibrinolytic process, although several kits are commercially available. From investigations of different research materials it is known that tPA activity is inversely correlated to PAI-1 activity.

Regarding measurement of uPA in plasma, immunochemical assay procedures (ELISA) are typically used, but the clinical usefulness is doubted. However, determination of uPA-antigen has been used in extracts of various solid tumours and in several patient materials it has been demonstrated that increased levels is connected with a poor prognosis (11).

PAI-1 and tPA-PAI-1 complex

PAI-1 is probably one of the most important factors to measure within the fibrinolytic system (13). However, there are certain problems in doing so. First, the concentration of PAI-1 in plasma is very low (5-60 µg/L). Secondly, PAI-1 exists in several different forms with different properties. Moreover, the active form is labile and converted to so called latent forms under physiological conditions. Therefore it is a relevant task to decide which type of assay should be used in clinical research projects or for routine purposes. The immunochemical assays measure active PAI-1, latent PAI-1 and PAI-1 in complex with tPA or uPA (figure 2). Under normal circumstances most of the PAI-1 in freshly collected plasma is active. However, as a result of preanalytical factors, such as inadequate anticoagulation, latent PAI-1 may leak from the platelets and the PAI-1 antigen concentration may increase up to ten-fold (12). Measurement of PAI-1 activity is on the other hand connected with other types of problems. As already mentioned above, PAI-1 is labile and at pH 7.3 and 37 °C in a plasma milieu, conversion to latent PAI-1 will occur with a half-life of 4 hrs. In addition, loss of activity will occur at every thawing/freezing cycle. Therefore it is very important that blood sampling and handling is correct and smooth. Directly after sampling the sample should be put on ice, transported to the laboratory and centrifuged within 30 min. The samples should be rapidly frozen and kept at -70°C until analysis. A refrozen sample should never be used for PAI-1 activity measurement. Frequently, it seems that the PAI-1 concentration in plasma seems to be the determinant of the fibrinolytic potential (8). Indeed, increased PAI-1 levels are connected with increased risk of both deep vein thrombosis (13) and myocardial infarction (14). On the other hand, PAI-1 deficiency is connected with a mild bleeding tendency. There are different commercially available kits on the market, utilizing different principles. The principle of having microtiter plate wells coated with tPA and subsequently measuring bound PAI-1 by an immunochemical technique seems to be very reliable.

It has been demonstrated that tPA antigen is a risk factor for myocardial infarction. Since tPA-PAI-1 complex constitutes an important part of tPA antigen, it seems that this complex is an even better predictor of a myocardial infarction. A classical two-site ELISA seems to work well (15). Today there is one commercially available kit on the market.

PAI-2 is another plasminogen activator inhibitor, which typically is found only in pregnant women (16). Occasionally it has been found in plasma in connection with malignancies.

D-dimer

The fibrin degradation product, D-dimer, is a result of plasmin action. Nevertheless, the presence of increased levels of D-dimer does not reflect the status of the fibrinolytic system, but rather an activation of the coagulation system, which have resulted in fibrin formation. It is extremely rare that the fibrinolytic system is so depressed that fibrin formation does not result in an increase in D-dimer. Therefore, a negative or normal concentration of D-dimer is a safe way of excluding the presence of a fresh deep vein thrombus. Most assays used today are latex enhanced immunological precipitation quantitative assays. However, the detection limit of these assays is typically not good enough to accurately measure D-dimer concentrations in plasma from healthy individuals. For this purpose ELISA methods needs to be used. Another problem with D-dimer analysis is standardization. The reason is that D-dimer constitutes a heterogeneous mixture of compounds that differ between patients. The immunochemical assays in use will always measure these variant compounds with different efficiency.

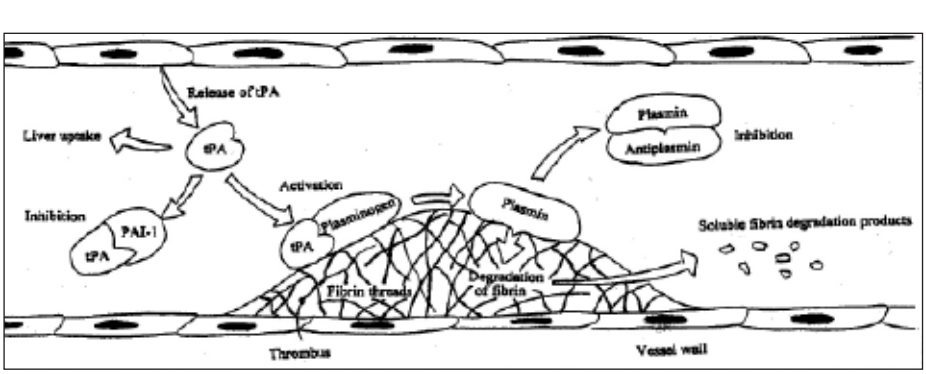

Figure 1. A schematic representation of the fibrinolytic system within a blood vessel.

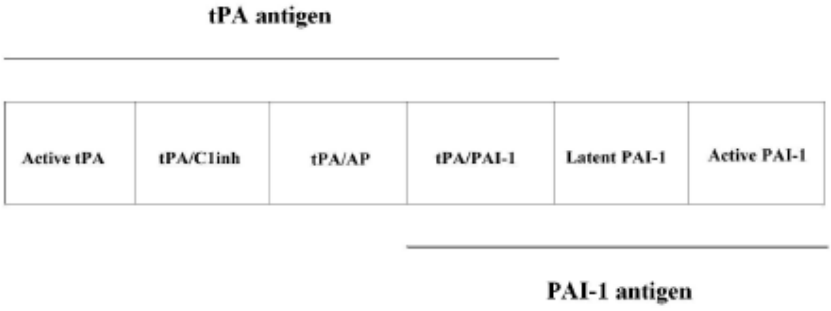

Figure 2.
A schematic picture on what is measured with tPA antigen and PAI-1 antigen methods. C1inh = C1 inhibitor; AP = antiplasmin.

TAFI

There are several commercial kits available for determination of TAFI. However, the correlation between them is not good. Thus, the methods available to measure TAFI seems not yet to be very reliable. In addition, there is no strong data, suggesting correlation to any particular clinical condition. However, knock-out mice seems to have an impaired wound healing, indicating that pericellular fibrinolysis is affected (5).

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The Anticoagulant Protein C Pathway

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The protein C system is one of several important anticoagulant pathways. Activated protein C (APC) is a serine protease that exerts its anticoagulant effect by regulating the activity of two important cofactors in blood coagulation: factors VIIIa (FVIIIa) and Va (FVa), which function in the tenase and prothrombinase complex, respectively [1-4]. APC has a plasma concentration of ≈ 1µg/L – its vital importance is illustrated by the severe thrombotic disease, purpura fulminans, that affects homozygous protein C deficient individuals already in the neonatal period. Heterozygous protein C deficiency and other genetic defects that disturb the function of the system are common genetic risk factors for venous thrombosis in adults.

Initiation of the protein C pathway on endothelial cells

In the vascular system, thrombin is converted to the physiological activator of protein C when it binds to its cofactor thrombomodulin (TM) on the surface of endothelial cells. TM is an integral membrane protein on vascular endothelial cells. In the capillaries, the ratio between the cell surface and blood volume reaches its peak and as a consequence, the high concentration of TM in the capillary circulation ensures that thrombin binds to TM (Kd≈0.5 nM) and activates protein C [4-6]. TM not only functions as an efficient cofactor to thrombin in the activation of protein C but also inhibits the procoagulant properties of thrombin. Thus, TM transforms thrombin from a pro- to an anticoagulant enzyme. A recently identified membrane protein on endothelial cells, endothelial protein C receptor (EPCR), provides a further stimulation of the thrombin-TM-mediated activation of protein C in vivo (figure) [7]. The half-life of APC in circulation is relatively long (around 20 minutes) as the active APC is slowly inhibited by one of three protease inhibitors, the protein C inhibitor (PCI), α1-antitrypsin, and α2-macroglobulin.

Activated protein C inhibits coagulation through cleavage of FVa and FVIIIa

FV and FVIII share the domain structure A1-A2-B-A3-C1-C2, with the A-domains arranged in a triangular pattern [3, 8]. Activation of FV and FVIII by thrombin releases the carbohydrate-rich B domains from the active FVa and FVIIIa that are thus composed of the A1-A2-A3-C1-C2 domains. In human FVa, three sites are cleaved by APC, at positions Arg306, Arg506, and Arg679 (figure) [3]. The cleavage at Arg506 results in partial loss of FXa cofactor activity, whereas Arg306 cleavage gives complete loss of FVa activity due to dissociation of the A2 fragments [3]. The regulation of the activity of the tenase complex by APC encompasses cleavage of human FVIIIa at Arg336 and Arg562. This reaction is more complex than the degradation of FVa because, in addition to protein S, the non-activated form of FV serves as an APC-cofactor [3]. In vivo, the concentration of FVIII is much lower than that of FV. The complicated task of regulating the highly efficient tenase complex in the presence of a large molar excess of the competing APC substrate FV/FVa may explain the requirement of two APC cofactors [3].

Multiple functions of the vitamin K-dependent protein S

Protein S, which is a vitamin K-dependent protein thus containing an N-terminal Gla domain, functions as an anticoagulant cofactor to APC (figure) [9, 10]. Protein S also has anticoagulant activities that are independent of APC. According to one proposed hypothesis, direct binding of protein S to FXa and FVa inhibits the prothrombinase complex. Recently, it was shown that protein S also functions as a cofactor to tis-

sue factor pathway inhibitor (TFPI) in the regulation of the tissue factor pathway [11].

In human plasma, approximately 30-40% of protein S circulates in complex with the complement regulatory protein C4BP suggesting that protein S plays a role also in the control of the complement system [10, 12]. C4BP has an octopus-like structure with seven identical α-chains and a single β-chain (figure). Each α-chain can bind a C4b (activated complement protein C4) molecule and convert it into a substrate for factor I, a complement regulatory enzyme in blood. This makes C4BP an important regulator of the classical complement pathway. The binding site for protein S is located in the β-chain of C4BP [13]. During apoptosis, negatively charged phospholipid is exposed on the surface of cells to which protein S binds. The bound protein S is found to be important for the phagocytosis of the apoptotic cell [14, 15]. The binding of protein S and protein S-C4BP complexes to the surface of apoptotic cell may be important for local control of coagulation and complement pathways.

Anti-inflammatory and anti-apoptotic effects of the protein C pathway

The protein C pathway exerts other biological effects than those strictly referred to as anticoagulant. Anti-inflammatory and anti-apoptotic effects of APC have been demonstrated both in vivo and in vitro. These effects are reported to depend on the presence of EPCR and to APC-mediated cleavage of PAR-1 (protease activated receptor-1) [4, 7, 16-18].

Severe sepsis and the protein C pathway

During sepsis, the blood coagulation system is activated by TF expressed on endothelium and monocytes/macrophages [18-20]. In severe cases, protein C in plasma is consumed, which can worsen the disease if the concentration of APC decreases. Beneficial effects of APC infusion have been demonstrated in a sepsis model in baboons [21]. APC has also been tried for treatment of sepsis in humans. In the PROWESS study recombinant APC gave a 19.4% reduction in the relative risk of death and an absolute reduction of 6.1% [22]. Further evidence for the efficacy of APC in the treatment of severe sepsis was obtained in an open-label trial called ENHANCE [23]. However, in the recent ADDRESS study of patients with severe sepsis and low risk of death (defined by an Acute Physiology and Chronic Health Evaluation (APACHE II) score <25 or single organ failure) no beneficial treatment effects were observed arguing against the use of APC. Thus, it appears that APC treatment is efficient in cases with very severe sepsis (APACHE >25 or multiple organ failure) when protein C and APC levels reach very low levels, whereas there are no beneficial effects of treatment in milder forms of sepsis. The critical factor for the therapeutic effect of APC may be the endogenous level of APC, suggesting that methods to determine the generation of APC in vivo, e.g. by measuring APC directly [24] or indirectly as APC-PCI complexes [25], may have a role in deciding which patients will benefit from the treatment with APC.

The protein C system and venous thrombosis

In the general population, the prevalence of defective protein C alleles is around 1/600 and thus complete deficiency is affecting approximately 1/200.000-1/300.000 newborns [26]. The severe microvascular thrombotic disease that affects babies with complete protein C deficiency shows that the protein C system is vitally important to keep the blood fluid. Heterozygous protein C deficiency is associated with approximately 5-fold life-long increased risk of venous thrombosis. Heterozygous protein S deficiency have similar risk and together with protein C deficiency account for 5-10 % of cases with venous thrombosis in Western societies. The most common genetic risk factor for venous thrombosis in Caucasians is APC resistance caused by the Arg506 to Gln mutation (FV Leiden) [1, 2, 26, 27]. In thrombosis cases, it is found in 20-40%. FVa Leiden cannot be cleaved at position 506 by APC due to the mutation but cleavages at the other sites at Arg306 and Arg679 are unaffected. Moreover, the degradation of FVIIIa is affected by the FV Leiden mutation because cleavage at Arg506 in FV is important for the anticoagulant activity of FV [3]. The relative importance of these two defective reactions, i.e. the degradation of FVa and FVIIIa, for the expression of the prothrombotic phenotype is not known.

The FV Leiden mutation is absent or very rare in Asians, Australian Aboriginals and black Africans, explained by the fact that the mutation only happened once in the history of mankind, approximately 20-25,000 years ago [28]. In Europe, the prevalence of FV Leiden exhibit a north to south gradient with highest prevalence (10-15%) in the north and lowest

in the south (approximately 2%). In United States of America, the prevalence is approximately 5% [26, 27]. The relative risk of thrombosis in heterozygous individuals is increased approximately 5-fold, whereas homozygotes carry a 50-fold increased risk. The FV Leiden mutation is not a risk factor for arterial thrombosis. Women with FV Leiden have reduced risk of bleeding after delivery, which in the history of mankind have been a major survival benefit [29]. Moreover, FV Leiden is suggested to be a positive survival factor in sepsis of either man or mice [30-32]. Thus, the FV Leiden allele has provided a survival advantage during evolution, which explains its high prevalence in certain populations.

Individuals that carry FV Leiden have a lifelong hypercoagulable state and an increased risk for venous thrombosis. Considerable efforts have been put into the development of assays aiming at reflecting the increased thrombin generation associated with the hypercoagulable state, assays that would "summarize" all genetic risk factors. Examples of such assays are tests that measure the capacity for thrombin generation (TG) in patient plasma [33]. It has not been shown that differences in TG influence the occurrence of thrombosis. By comparison, measurements to identify in-vivo thrombin generation are for example prothrombin fragments, fibrinopeptides or complexes between antithrombin and thrombin. These assays have been excellent research tools but have not been found to be useful for routine clinical medicine. Recently, a novel assay which measure the concentration of the complex between APC and the protein C inhibitor (PCI) has been developed and it holds promise to accomplish the goal of reflecting the hypercoagulable state and possibly the risk of venous thrombosis [25]. Since the PCI concentration is fairly constant (≈4mg/L) the APC-PCI concentration reflects the concentration of free APC, which is dependent on the generation of thrombin. The APC-PCI complexes were measured in patients who had been treated with warfarin for venous thrombosis. One month after termination of the treatment the complex concentration was found to be higher in the thrombosis patients than in normals suggesting a remaining hypercoagulable state. Moreover, the median concentration was higher in the group of post thrombosis patients with FV Leiden than in those without this mutation [34]. With this method, it is possible to measure different degrees of APC-PCI complex formation in various hypercoagulable states. Whether it has the potential to become clinically useful, depends on confirmation of results of measurements in larger clinical materials and correlation of the APC-PCI complex concentration to thrombotic events in prospective outcome studies. It is also possible to develop the method for adopting it to random access instruments, an ongoing process.

For more information about testing in the clinical laboratory, appropriate assays and analytical performance, see also refs. [35-36].

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Legend to figure

Schematic representation of components and reactions of the protein C system. ***Top****, activation of protein C. Thrombin binds to thrombomodulin (TM) with high affinity and efficiently activates protein C. The endothelial protein C receptor (EPCR) interacts with the Gla-domain of protein C and helps orient protein C to the T-TM complex. The activated protein C dissociates and leaves room for a new protein C molecule to be activated.* ***Middle****, degradation of FVa by APC in the presence of protein S, which serves as an APC cofactor. APC cleaves FV at three sites, Arg306, Arg506 and Arg679, which results in dissociation of degradation fragments from the FVa molecule and loss of FVa activity.* ***Bottom****, multiple functions of protein S. Protein S binds to negatively charged phosphatidylserine that is exposed on the surface of cells in certain situations, e.g. during apoptosis. Protein S has important anticoagulant functions, being a cofactor to both APC and to TFPI. Both the free and the C4BP bound forms of protein S interact with the membrane surface via the Gla-domain. The octupus-like shape of C4BP is illustrated with six long α-chains and a short β-chain that interacts with protein S. This provides the potential for local regulation of both coagulation and the complement, e.g. on apoptotic cells. In addition, the bound protein S stimulates phagocytosis of the apoptotic cells.*

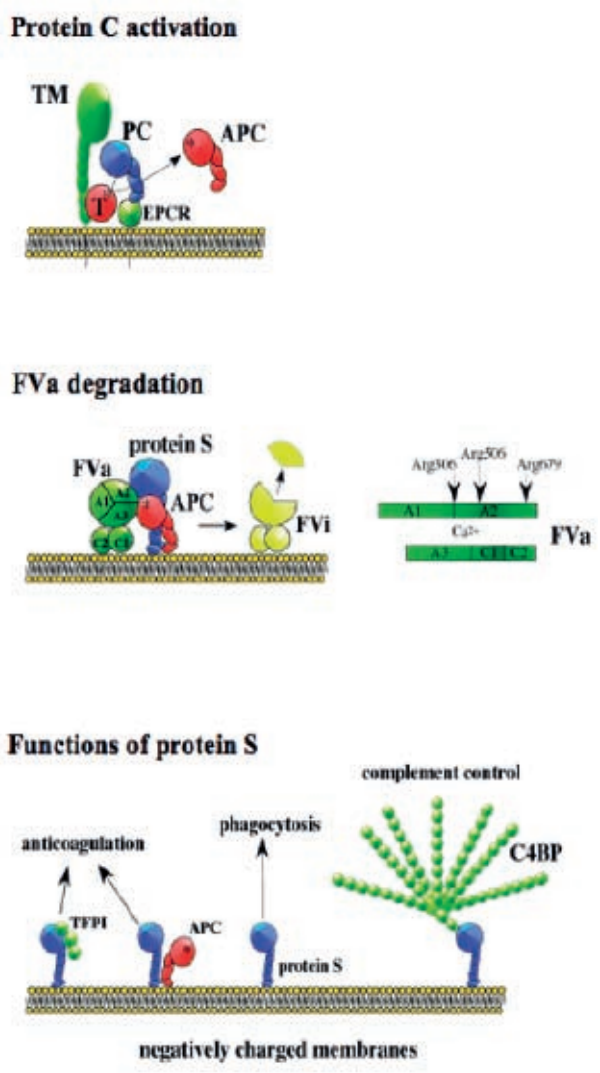

Foto: Henrik Alfthan, Island.

Arterial thromboembolism-Mechanisms, risk indicators and treatment

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Mechanisms

Cardiovascular disease states (CVD) including coronary heart disease (CHD), cerebral stroke and peripheral arterial disease, are the leading cause of death and disability in developed countries and is rapidly increasing in the developing world. The two most important processes underlying CVD are atherosclerosis and thrombosis, the first one taking place over decades, whereas the latter one may occur acutely on the basis of rupture of an atherosclerotic plaque. This is clinically most typically demonstrated in CHD where a lasting period of stable angina pectoris is interrupted by a sudden myocardial infarction (MI).

The atherosclerotic process is today looked upon as a low-graded chronic inflammatory process in response to retention and modification (mostly oxidation) of LDL-cholesterol in the arterial subendothelium, being "digested" by macrophages through their expression of the scavenger receptor (1-3). This leads to an inflammatory response, locally and systemically, which also is parallelled by an activation of the coagulation system. Especially, tissue factor produced and expressed by the macrophages (4), and fibrin have been noted in atherosclerotic plaques (5).

Upon an endothelial lesion covering an atherosclerotic plaque, or more dramatically a plaque rupture, platelets will adhere to the vessel wall, be activated by exposed von Willebrand factor and collagen, and simultaneously the subendothelium will be exposed to circulating blood and promote an immediate activation of the coagulation system. The two parts of this haemostatic process will then join in a common thrombotic development where activated platelets contribute with a suitable surface for orchestration of the necessary coagulation factors promoting thrombin generation and fibrin formation, and as thrombin itself is the strongest physiological activator of platelets this is a mutually amplifying process.

In addition, reduced fibrinolysis, mainly through increased levels of the inhibitor PAI-1, may also contribute to thrombosis due to reduced proteolysis of the fibrin threads.

The interaction of atherosclerosis and thrombosis is often summarised in the term atherothrombosis to elucidate their close relation in the pathogenesis of CVD (6,7). As the haemostatic system is mainly responsible for haemostasis to avoid bleeding, atherothrombosis may be looked upon as "haemostasis in a wrong place" potentially leading to occlusive arterial disease. These considerations also give the rationale for the different principles of antithrombotic therapy being used in CVD today.

A special form of arterial thromboembolism is the systemic embolism, mainly cerebral, accounting for up to 25% of strokes. This is mainly arising from the heart in atrial fibrillation, and the original thrombus is formed as a coagulation thrombus in the left atrial appendage or atrium. That is in a low pressure chamber more similar to the venous system where platelets play a minimal role. Thus, although the embolism occludes an artery the thrombus is mainly formed through coagulation, and this has obvious therapeutic consequences (vide infra). In artery-to-artery embolism, for example from the ascending aorta to a cerebral artery, the situation is possibly different, the embolic material consisting to a higher degree of platelets from atherothrombotic non-occlusive lesions. In cardiogenic embolism from a mural thrombus in the left ventricle generated during an acute myocardial infarction, the treatment with anticoagulants has been shown to be superior to antiplatelet therapy, possibly indicating a major component of thrombin generation and fibrin formation in this situation.

Risk indicators

In addition to the general risk factors for CVD and especially CHD as hyperlipidemia (hyper-LDL-cholesterolemia; hyper-VLDL-triglyceridemia), hyperten-

sion, diabetes mellitus, obesity, smoking and physical inactivity, several variables from the haemostatic system have been shown to be closely associated with these disease states. Their contribution to disease as independent risk factors have been hampered by the fact that most of them are also closely associated with at least one of the general risk factors. Thus, *fibrinogen* is closely related to smoking and hypercholesterolemia; *coagulation* factor VII to hypertriglyceridemia and hyperinsulinemia; PAI-1 to hypertriglyceridemia, hyperinsulinemia and obesity; and *hyperactive platelets* to hypercholesterolemia, hypertension and smoking. Nevertheless, their possible pathogenetic contribution to atherosclerosis and especially to the proneness to arterial thrombosis should not be overlooked. Thus, a low-graded continously ongoing diffuse intravascular coagulation is most likely of importance for clinical atherothrombotic events, and the circulating levels of tissue factor have been shown to be predictive for future cardiac events in patients after MI (8).

More specifically, ***fibrinogen*** was demonstrated by Korsan-Bengtsen to be related to the risk of future CVD already in 1971 in "The Gothenburg Study" of 792 men born in 1913 (9). This was later confirmed in the same population (10) as well as in other large population studies (11,12) and in meta-analysis (13). As fibrinogen is also part of an inflammatory reaction it is not definitely clear whether it is a marker of an ongoing atherothrombotic process, or by itself actively contributes to this process. As increased levels of fibrin in atherosclerotic lesions are suggested to act as a scaffold for retention of LDL-cholesterol and migration of smooth muscle cells, and that increased circulatory levels may contribute to increased thrombogenicity upon plaque rupture, a pathogenetic role of fibrinogen cannot be ruled out. However, on an individual basis the measurement of plasma fibrinogen does not seem to have any place in the risk score for CVD.

Coagulation factor VII has also been reported to be associated with the incidence of CVD and CHD (11,14). However, conflicting results exist, and the focus has recently been on the methodology used for FVII determination: The total amount of protein measured as antigen; The total coagulation potential after in vitro activation; or The circulating FVII activity. It seems to emerge that it is the total mass of FVII that is associated with CVD and CHD, whereas the active form, circulating in about 1% of the total amount, is not (15).

Of special interest is that the R353Q polymorphism in the gene coding for FVII has been linked to lower circulating levels of FVII (16), and also to some protection against CHD (17). In a Norwegian study on 560 elderly men the circulating protein FVII levels, but not the levels of active FVII were significantly lower with the Q allele. The prevalence of CHD was numerically lower in the Q allele group, although not statistically significant (18). Conflicting results have been reported on this issue, but a meta-analysis concluded with a significant protective effect of the Q allele (19). Interestingly, a reduction in FVII antigen and activity after 3 years of a "Mediterranean type" diet, *independent* of the genotype, has been reported (20).

The interpretation of FVII levels in relation to CVD and CHD is complicated by the close association with hypertriglyceridemia and hyperinsulinemia, both well known independent risk factors. On an individual basis measurements of FVII are not recommended, irrespective of the method used.

The inhibitor and important regulator of the fibrinolytic system ***PAI-1*** was first associated with the prevalence of MI in young men (21). In the same population PAI-1 was later demonstrated also to be predictive for future MI (22). Again of special interest is the observation that a polymorphism in the promoter region of the PAI-1 gene at position –675 (4G/5G) was associated with the circulating levels of PAI-1, and that the 4G allele was associated with higher PAI-1 levels. The relation to clinical thrombotic events like MI has been adressed, and a meta-analysis suggested that the PAI-1 4G genotype is associated with a marginally increased risk of MI (23). Again the close association between the levels of PAI-1 and hypertriglyceridemia, hyperinsulinemia and obesity in the so-called "metabolic syndrome" makes it difficult to depict it as an independent risk factor for CVD and CHD. Its synthesis and expression in adipose tissue make it of special interest in the ongoing rapidly increase in obesity and related CVD. This variable is not included as an independent factor in any risk score for CVD or CHD today, but may be of increasing interest in the future "obesity era".

Platelets play a central role in most cases of arterial thrombosis in contrast to its minimal role in most cases of venous thromboembolism. Thus, both increased absolute number of platelets and increased platelet size ("young active platelets") have been shown to be associated with increased tendency to arterial thrombosis (24). By activation through one or more of the glucoprotein receptors by von Willebrand factor,

collagen or thrombin, platelets adhere and aggregate to form the initial "haemostatic" platelet plug of a thrombus. In the process of atherosclerosis the platelets also contribute by being proinflammatory cells, for instance through the expression of CD40L and P-selectin, and by interaction with leucocytes. The lack of suitable laboratory tests for routine use makes evaluation of platelet hyperactivity as predictive of CVD and CHD unfashionable on an individual basis. However, for research purposes aggregometry with various agonists, flowcytometry and also measurement of circulating markers of platelet activation are of interest. At present their relation to clinical settings with arterial thrombosis is still questionable. Furthermore, the close association of platelet hyperactivity with general risk factors like hypercholesterolemia, hypertension and smoking makes the classification as independent predictor of CVD and CHD difficult.

Antiphospholipid antibodies and especially the subgroup ***lupus anticoagulant*** have been associated with arterial thrombotic disease states like stroke and MI (25,26). However, the relation is rather unspecific. Therefore, routine measurement of lupus anticoagulant is not recommended. However, in young females with MI positive testing gives a rationale for secondary prophylaxis with oral anticoagulants.

Regarding ***coagulation inhibitors*** like antithrombin, protein C and S, and TFPI, their roles in arterial thrombosis are possibly negligible, and laboratory testing in CVD and CHD is not found relevant today.

Treatment

It emerges that specific treatment of arterial thrombotic disease states should be based on thrombolysis, antiplatelet and anticoagulant principles in the acute phase, and on the latter principles in the secondary prophylaxis. Due to the serious outcome and high prevalence of CVD and CHD a large number of therapeutic clinical trials have been conducted, observationally, but also as randomised clinical trials, and a series of meta-analyses have been performed. Thus, guidelines for specific therapy have been launched both in Europe and USA. A short survey is hereby proposed.

In ***acute MI***, especially with PCI and stenting, dual antiplatelet treatment with aspirin and clopidogrel is recommended, occasionally also "triple antiplatelet therapy" with the addition of GPIIb/IIIa inhibitors during the PCI procedure. The addition of heparin or up-coming thrombin inhibitors like bivalirudin is also recommended. In cases of MI (especially ST-elevation MI, STEMI) with an estimated duration from symptom start to a PCI center of 90-180 (360) minutes, thrombolysis in addition to aspirin and heparin is still recommended in order to achieve "open index coronary artery" as early as possible.

After MI aspirin is always recommended as the basic antithrombotic. After PCI dual antiplatelet therapy is usually recommended for about 9 months. The optimal long-term secondary prophylaxis has, however, been shown to be achieved with aspirin and warfarin with an INR of 2.0-2.5 (27,28). Whether up-coming thrombin inhibitors and/or FXa inhibitors will be found more appropriate and convenient than warfarin is for the future.

In ***atrial fibrillation*** anticoagulation is significantly superior to antiplatelet drugs and should be given, aiming at INR 2.0-3.0, when additional risk for cerebral embolism is present (valvular heart disease, age, hypertension, reduced left ventricular function, previous embolism, diabetes). A problem arises in patients with atrial fibrillation and intracoronary stents after an MI who should also receive dual antiplatelet treatment with aspirin and clopidogrel. In this case anticoagulation with warfarin should be mandatory. For the addition of antiplatelet drugs no systematic experience and no guidelines exist. A safe proposal seems to be the use of all drugs ("triple antithrombotic therapy") for 3 months after the stent implantation and then to stop clopidogrel. Future registries will hopefully give a clear answer to this question.

Conclusion

In conclusion, arterial thrombosis is most often part of atherothrombosis where platelets play a major pathogenetic role in addition to thrombin generation through activation of the coagulation system. Although several risk indicators in the haemostatic system have been defined, no single marker seems suitable for routine diagnostic laboratory evaluation, although the presence of antiphospholipid antibodies may help to identify young female patients with MI for "lifelong" anticoagulation.

Treatment and secondary prophylaxis of arterial thrombosis are based on the knowledge of the joint activation of platelets and the coagulation system in CVD and CHD. In atrial fibrillation oral anticoagulation is the superior "lifelong" prophylaxis against systemic embolism.

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Venous Thrombosis – Diagnosis and Treatment

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Introduction

Venous thrombosis (VT) is a process of thrombus formation that results from excessive activation of coagulation or inhibition of anticoagulant mechanisms. Thrombus formation usually commences in the deep veins of the lower extremities, and this translates clinically to deep vein thrombosis (DVT). Frequently, fragments of a DVT dislodge and travel through the venous circulation to settle into the pulmonary vasculature, giving rise to pulmonary embolism (PE). These two conditions often co-exist and are therefore collectively referred to as VT.

The overall annual incidence of VT is approximately 1.4/1000/year (DVT 0.9/1000/year and PE 0.5/1000/year)[1]. The incidence, however, is known to increase with age from about 1/10000/year in the twenties to reach 1/100/year over the age of 75 years[2].

The causes of VT can be inherited (see section on thrombophilia) or acquired (Table 1). A risk factor for thrombosis can be identified in more than 80% of patients, but usually more than one factor is at play in a given patient [3].

Clinical features of VT

The clinical features of DVT and PE are non-specific. Only approximately 25% of patients referred with clinical suspicion of VT can be objectively verified. DVT commonly presents with pain, erythema, and swelling of the affected limb. Clinical findings include tenderness, warmth, ipsilateral oedema, or superficial vein dilatation. Differential diagnoses include a ruptured Baker's cyst, muscle tears, or cellulitis.

The clinical presentation of PE ranges from clinically silent to massively fatal[4]. Dyspnoea and chest pain are the most frequent symptoms reported by patients with PE, while tachypnoea and tachycardia are the most frequent clinical signs[5]. About one-third of the patients have clinical features of DVT. Other symptoms include haemoptysis and fainting, and the latter is a particularly important symptom of acute PE in older persons. Arterial hypoxemia is present in 80% of patients with PE.

Diagnosis

There is no single optimal test for the diagnosis of DVT or PE that is sufficiently accurate, feasible, and completely safe. Therefore, various diagnostic algorithms involving the combination of clinical probability (CP) and two or more tests have been proposed and validated to increase the diagnostic accuracy and to minimize the risk and the cost of management (Figure 1). It is recommended to commence with assessment of CP for VT, which encourages good clinical assessment and helps to reduce the need for subsequent testing. The CP can either be judged on empirical basis[6] or assessed by applying standardized prediction rules[7;8]. Several scores for predicting the clinical probability for DVT[9] or PE have been developed[10-12]. Most of these scores classify patients with suspected PE into three categories, i.e., low, intermediate, and high CP.

D-dimer

D-dimer is now widely accepted as the first step test in the management of patients with suspected VT. Plasma D-dimer is a specific cross-linked fibrin derivative produced when fibrin is degraded by plasmin. Unfortunately, elevated levels of D-dimer are found in any condition that lead to the activation of coagulation and fibrin formation[13], e.g., in patients with cancer, pregnancy, surgery, and inflammatory diseases, as well as in patients with thrombosis.

Several assays for D-dimer are commercially available (Table 2). These assays differ in their performance

and in the methodology employed to detect D-dimer. Sensitive D-dimer assays like enzyme-linked immunosorbent assays and the immunoturbidimetric latex agglutination assays ELISA can safely rule out VT in patients with low or intermediate CP[14;15]. Conversely, less sensitive assays, as whole blood agglutination tests can reliably exclude VT only in patients with low CP. However, owing to the low specificity (30-40%), their value is largely restricted to ruling out VT in outpatients, i.e., have a high negative predictive value.

Compression Ultasonography (CUS)

Compression ultrasonography is the standard imaging tool for the investigation and diagnosis of clinically suspected DVT. Although such imaging is highly sensitive for detecting thrombosis in the femoral veins of the lower extremities, it is less accurate for isolated DVT of the calf veins and the iliac veins. If DVT is still suspected despite negative CUS, it is recommended to perform contrast venography, or alternatively to repeat the examination during the following week[16].

Spiral Computed Tomography (CT)

The newer generation of spiral CT scanners, known as multi-detector CT (MDCT), has to a large extent replaced traditional imaging modalities like, ventilation/perfusion lung scanning and invasive pulmonary angiography. Several recent studies have confirmed the accuracy and reliability of MDCT in the detection or exclusion of PE[15;17]. CT provides direct visualization of the pulmonary vasculature. Moreover, its value extends to include the detection of other serious conditions in the chest, and it may provide valuable information about the severity of PE and its effect on the heart.

Treatment

The choice of initial treatment depends on the severity of VT and its effect on the haemodynamic status of the patient.

Initial Therapy

Immediate anticoagulant therapy is necessary to halt thrombus propagation. There is now compelling evidence that weight-adjusted subcutaneous treatment with either low molecular weight (LMW) heparin (including the pentasaccharide fondaparinux) once or twice daily without monitoring is at least as efficacious, safe, and cost-effective as continuous intravenous infusion with unfractionated heparin dose-adjusted according to daily APTT monitoring for the initial treatment of VT[18]. Moreover, LMW heparin therapy facilitates out-patient treatment of VT, which is now common practice in many countries. Oral anticoagulant therapy is started simultaneously with heparin, however it requires 4-5 days of treatment, before it becomes efficacious. Overlapping treatment with heparin should therefore continue for at least 5 days and until international normalised ratio (INR) is in therapeutic range (2.0-3.0) for two consecutive days. In patients with active cancer continued treatment with LMW heparin has proven superior to oral anticoagulant therapy[19].

Thrombolytic agents like streptokinase and recombinant tissue plasminogen activator, dissolve fresh clots and result in rapid restoration of blood circulation. Systemic thrombolysis is therefore recommended in patients with massive PE manifested as systolic hypotension and/or severe hypoxemia[20]. On the other hand, the value of thrombolysis in normotensive patients with signs of right ventricular dysfunction, so called sub-massive PE, is still debatable.

Although systemic thrombolytic therapy in patients with proximal DVT significantly reduces the risk of subsequent post-thrombotic syndrome (PTS), the risk of severe bleeding is unacceptably high. Systemic thrombolysis therefore no longer common practice and is only used in cases of limb-threatening thrombosis[21]. More recently, thrombolytic therapy has been given in much lower doses through catheters inserted directly into the thrombus. This treatment has shown promising results, but its efficacy and safety needs confirmation in randomised clinical trials[21].

Long-term therapy

Treatment with a vitamin K antagonist, e.g., warfarin, given at a dose that is titrated to achieve an INR of 2.0-3.0 is used for secondary prophylaxis. The optimal duration of anticoagulation is unknown. The duration of anticoagulation depends on the severity of VT and the presence of risk factors. The majority of patients should receive warfarin for at least 6 months after the first episode of an idiopathic VT. Patients with a first episode of VT secondary to a transient risk factor, e.g., immobilisation, surgery, or pregnancy, requires only 3 months of oral anticoagulation. On the other hand, longer treatment is recommended for patients with documented antiphospholi-

pid antibodies, antithrombin deficiency, or patients with combined thrombophilia, e.g., factor V Leiden and 20210A prothrombin gene mutations20. The risk of recurrence following the cessation of anticoagulation after first episode of VT is approximately 5% per year[22-24]. Indefinite treatment is recommended for patients with 2 or more episodes of VTE.

Mortality and Morbidity

The short term mortality rate following an acute episode of PE ranges from below 1% in normotensive patients with normal right ventricular function, to about 5% in normotensive patients with right ventricular dysfunction[25], and up to 30-50% in patients with massive PE[20;26].

Chronic pulmonary hypertension is a condition characterised by progressive functional dyspnoea and increased pulmonary artery pressure, and develops in approximately 4% following the first episode of PE[27]. Post-thrombotic syndrome (PTS) of the leg manifested by pain, swelling and skin discolouration, arises in half of the patients following first proximal DVT who receive standard anticoagulation. To reduce the incidence of PTS it is recommended to use below-knee graduated elastic stockings for 2 years after DVT[28].

Treatment of VT in Pregnancy

Vitamin K antagonists are generally considered contraindicated in pregnancy, since it may cause harmful effects to the fetus when used throughout pregnancy. Instead, either dose-adjusted unfractionated heparin, initially given intravenously followed by subcutaneous administration, or subcutaneous weight-adjusted LMW heparin is given throughout pregnancy. We prefer to maintain a therapeutic dose of LMW heparin during pregnancy, since the risk of VT increases sharply during the last trimester and early after delivery. Monitoring of anti-factor Xa activity is recommended since pregnant women often require a higher dose to achieve the same anti-factor Xa activity. Anticoagulation should be discontinued 12-24 hours before induction of labour. After delivery, anticoagulation should be continued for 6-12 weeks, either using vitamin K antagonist or continued LMW heparin therapy[29].

Cancer and Thrombosis

The risk of cancer during the first 6 months after the diagnosis of VT is 3 times the expected level. The risk declines thereafter to a constant level that remains over one[30]. Although extensive screening for cancer results in detection of some cases with cancer, this approach is not translated into improved outcome. It is therefore only recommended to perform a thorough clinical examination, routine laboratory examinations, and chest X-ray.

Testing for Thrombophilia

At least 1/3 of the patients with idiopathic VT have an identifiable thrombophilia on laboratory testing. Although testing for thrombophilia is a routine procedure in many centres, however, there is no clear evidence that prolonging or intensifying anticoagulation in hypercoagulable patients improves the outcome[16]. It is common practice to perform thrombophilia testing patients <50 years, those with recurrent VT, and those with VT at rare localizations.

Areas of Uncertainty

Ongoing studies will hopefully clarify the uncertainty regarding the role of catheter directed thrombolysis in non limb-threatening proximal DVT[21] and the benefit of thrombolysis in sub-massive PE. However, the determination of the optimal duration of anticoagulation and the identification of patients at high risk of recurrence will remain among the most challenging issues that need to be solved in the future. It has recently been shown that patients with an abnormal D-dimer level one month after the discontinuation of oral anticoagulation have a significant incidence of recurrent VT, which is reduced by the resumption of anticoagulation[31]. Also the presence of residual DVT by ultrasound may be an important predictor for recurrence[32]. Such strategies may help to tailor anticoagulant therapy in individual patients.

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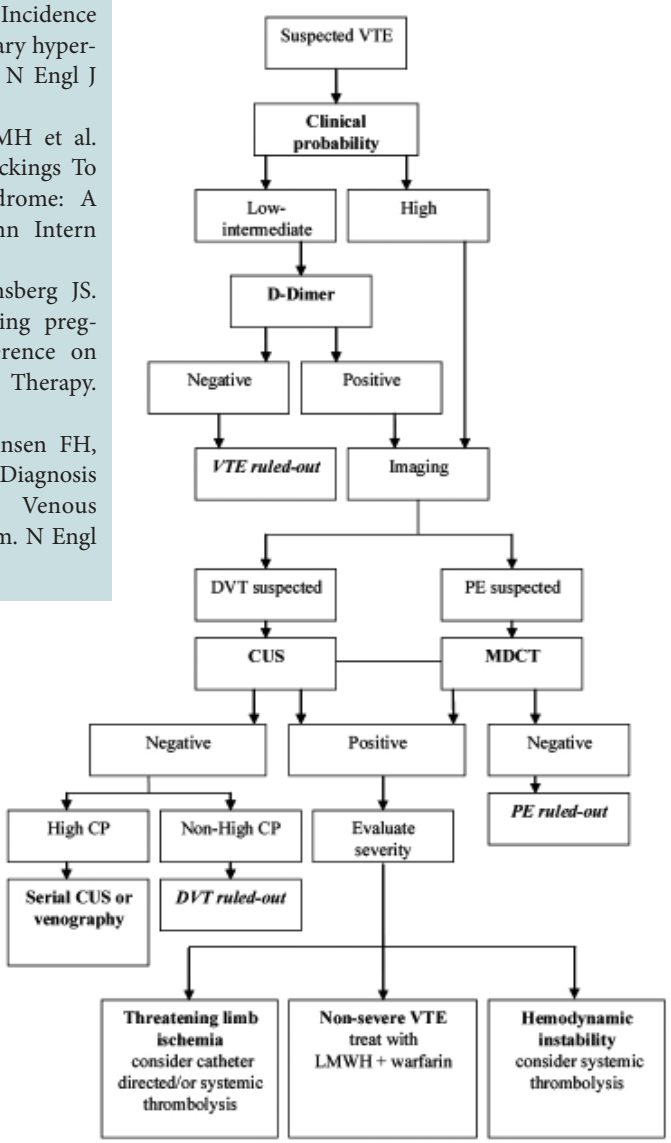

Figure 1
Algorithm for ruling out venous thrombosis (VT).
Applies only to sensitive D-dimer assays. VT can be safely ruled out by moderately sensitive D-dimer assays only in patients with low clinical probability (CP). CUS= compression ultrasonography; MDCT= multi-detector computed tomography.

Table 1. Acquired risk factors for VTE

Assay format	Commercial assay	Se%	Sp %	TAT/min
ELISA	VIDAS, Asserachrom	>95	40	35
Whole blood agglutination assays	SimpliRED	85	70	2
Latex agglutination assays	STA-Liatest, IL-test, Tinaquant	>90	40	15

Table 2. The performance of various D-dimer assays.

Major risk factors (Relative Risk 5-20)	
Surgery	Major abdominal /pelvic surgery Hip/knee replacement Post operative intensive care
Obstetrics	Late pregnancy, caesarean section and puerperium
Lower Limb problems	Fractures Varicose veins
Malignancy	Abdominal cancers Advanced metastatic disease
Reduced mobility	Hospitalisation Institutional care
Miscellaneous	Previous proven VTE
Minor risk factors (Relative Risk 2-4)	
Cardiovascular	Heart failure, superficial venous thrombosis, indwelling central vein catheter
Estrogens	Oral contraceptives and hormone replacement therapy
Miscellaneous	Antiphospholipid syndrome, occult malignancy long haul flight, chronic obstructive pulmonary disease, nephrotic syndrome, obesity, myeloproliferative diseases, paroxysmal nocturnal hemoglobinuria

ELISA=enzyme-linked immunosorbent assays; Se=sensitivity; Sp= Specificity; TAT=Turn-around time in minutes

Laboratory diagnosis of antiphospholipid antibodies

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Introduction

The antiphospholipid syndrome (APS) is characterized by arterial or venous thrombosis, or recurrent miscarriage, in patients in whom laboratory tests for antiphospholipid antibodies (APA) are positive. It is one of the most common acquired prothrombotic states with high risk of recurrences with APA playing a direct role in the pathogenesis, which makes a correct identification in the laboratory crucial. However, as the APA represents a very heterogeneous collection of autoantibodies, together with a situation of poor standardization of the diagnostic reagents, the identification process is a challenge for any laboratory. The topic of APA is extensive and sometimes confusing and controversial. This review will try to summarize current criteria for classification of APS with emphasis on the laboratory investigation.

The Sapporo criteria

The first classification criteria for APS were formulated by an international expert committee that met in Sapporo, Japan 1998, and thus called "Sapporo criteria" (1). The criteria were intended to be used in context of clinical trials and scientific investigations rather than a guide to diagnosing the syndrome in individual patients. Nevertheless, as the clinical and laboratory features of this disease are very complex and since the criteria have been shown to be specific and sensitive they are now used by clinicians for diagnosis. In brief, the main clinical criteria includes 1) one or more vascular thrombosis in arteries, veins or smaller vessels; 2) pregnancy morbidity with a) one or more unexplained deaths of a normal fetus beyond 10th week of gestation, b) or one or more premature birth of a normal neonate because of severe preeclampsia, eclampsia or severe placental insufficiency c) three or more unexplained consecutive spontaneous abortions before the 10th week of gestation. The laboratory criteria involve presence of APA through detection of lupus anticoagulans (LA) or anticardiolipin antibodies (aCL) of IgG and IgM isotype. These tests will be dealt with in more detail below. Definite APS can be considered if at least one of the clinical criteria and one of the criteria for APA are met. Moreover, the APA must be present on two or more occasions at least 6 weeks apart in order to reduce the probability to detect transient occurring antibodies associated with infection. New clinical and laboratory insights have led to a revision of the APS criteria (2). One major change involves addition of anti-ß2-glycoprotein I (aß2GPI) antibodies of the IgG and IgM isotype in the laboratory test battery. Furthermore, the time to elapse between first and second test occasions was increased from 6 to 12 weeks (Fig. 1).

Laboratory diagnosis of APS

I. Lupus anticoagulants

A circulating inhibitor of coagulation was originally described in the 1950s in patients with haemorrhagic disorders who had prolongation of the prothrombin time (PT). Later, this circulating anticoagulant was shown to be associated with mainly thrombotic manifestations in patients who also suffered from systemic lupus erythematosus (SLE) and the term lupus anticoagulant (LA) was introduced. Today, several clinical studies have shown a clear association between LA and thrombosis as well as pregnancy morbidity, with or without SLE. A large meta-analysis that focused on the risk of thrombosis found that presence of LA result in an odds ratio for thrombosis 5 to 16 times higher than controls (3). This is similar to or even higher than the risk observed in patients with genetically determined risk factors for venous thrombosis. Moreover, LA increases the risk of both venous and arterial thrombosis to the same extent.

The Sapporo criteria for APS states that LA should be detected according to the guidelines of Scientific and

Standardization Committee (SSC) on LAs and phospholipids-dependent antibodies of the International Society of Thrombosis and Haemostasis (ISTH) (4). These guidelines involves a four step procedure (Fig. 2):

a) *Prolongation of a phospholipids-dependent coagulation assay.* An assay with documented good sensitivity should be used in the screening stage. However, as no optimal assay exist it is recommended to use a combination of at least two screening assays before the presence of LA can be ruled out. Furthermore, the assays should represent different test systems with different reagent composition. There are a number of potential screening assays and some are given in the SCSC/ISTH guidelines and new assays have been developed during the recent years. Assay selection is difficult but information regarding LA responsiveness can be obtained through participation in external quality assessment schemes. A common combination of screening assays is the activated partial thromboplastin time (APTT) and the dilue Russels viper venom time (dRVVT). Preanalytical considerations involves to use plasma that is free from contaminating platelets (<10x10^9/L) prior analysis, which otherwise could compromise the result. Furthermore, common causes for prolongation of coagulation-based assays such as heparin and oral anticoagulants (OAC) must be taken into account. Unless the test system includes a heparin neutralizer, the presence of heparin must be tested by including the reptilase and thrombin time test or by measuring the anti-Xa activity. The diagnostic algorithm may be confounded by OAC and LA investigation during treatment should be avoided or dilute the patient plasma 1+1 with normal plasma before the test is performed. However, testing for LA is considered unworkable if the patient have a PT value >3.5 INR (2).

b) *Evidence of inhibition demonstrated by mixing studies.* With this means that the prolonged coagulation time seen in one (or more) of the screening assays cannot be corrected by mixing the patient plasma with normal plasma. The mixing proportion may vary, depending on the LA-sensitivity of the test, but the most common ratio used is a 1:1 mixture. The quality of the normal plasma used is crucial and most commercial available normal plasma pools are unsuitable for mixing studies. The best source is carefully prepared platelet-free citrated plasma pooled from normal donors, which is locally produced or obtained commercially as fresh-frozen pooled plasma without additives.

c) *Evidence of phospholipids dependence.* This is an important step that differentiates between inhibitors of specific coagulation factors, which are not dependent on phospholipids, and LA. It involves a confirmation test, usually the same test as the screening assay, with an excess of phospholipids. The additional phospholipid neutralizes or bypasses the prolongation effect of LA and a correction of the coagulation time is strongly indicative of LA. However, the performance and interpretation of the confirmatory step has not been standardized. There are several procedures how to add extra phospholipids. In some test systems the phospholipids (from various sources) are added as pure components to established assays and others involves use of integrated assay systems. The dRVVT test is nowadays available as a fully integrated test system from many manufacturers and the test is easy to automate on coagulation analyzers.

d) *Lack of specific inhibition of any one coagulation factor.* Specialized assays for coagulation factors should be performed whenever test results are questionable in order to exclude other coagulopathies. The worst-case scenario is the failure to detect factor inhibitors (e.g. factor VIII inhibitors). Furthermore, the addition of factor assays can sometimes be helpful in confirming the presence of LA that may manifest as simultaneous reduction of several coagulation factors determined with coagulation-based assays.

II. Anticardiolipin antibodies

Antibodies of the anticardiolipin (aCL) type are detected with immunological tests using a standardized enzyme-linked immunosorbent assay (ELISA) test principle. The Sapporo criteria specifically recommend the use of aCL ELISA tests that are dependent on the protein ß2-glycoprotein I (ß2GPI). The reason is that most clinically relevant APA do not bind directly to phospholipids but rather to protein cofactors with high affinity for phospholipids of which ß2GPI appears to be the most important protein. An extensive meta-analysis on aCL antibodies and risk for thrombosis resulted in somewhat difficult interpretations (3). Associations with thrombosis were found in only 50%

of the included studies. However, the antibody isotype and titers appears to be important factors as most of the significant associations were found for studies that utilized IgG aCL tests, especially if the cut-off were above 33-40 units.

Some of the difficulties observed in the meta-analysis can be explained by the lack of standardization of aCL ELISA reagents. There is considerable inter-laboratory variation, mainly because of differences in reagent composition, cut-off and calibration. Problems with false-positive results are also noted, especially for low titre aCL of IgM isotype (2). There have been international aCL workshops that dealt with standardization issues. The current recommendation is that the assay results should be given in GPL and MPL units (5). The primary calibrator material was obtained from patients with aCL positive sera and the definition of one GPL or MPL unit is 1 µg affinity-purified IgG and IgM, respectively. There are also monoclonal aCL calibrators in use in some laboratories and future evaluation will tell if these offer any advantage over the patient-derived GPL and MPL standards. Based on current evidences, the revised Sapporo criteria recommend that the cut-off for positive results are set at >40 GPL or MPL units, or >99th percentile of a suitable control population. Local determination of the cut-off based on at least 50 normal subjects and the use of percentiles were also proposed by the standardization committee of the European Forum on antiphospholipid antibodies (6)

Thus, there are many obstacles in obtaining reproducible aCL measurement but individual laboratories can be helped by a) choosing a validated ELISA kit; b) using a cut-off set at >40 GPL and MPL or otherwise establish the cut-off locally (>99th percentile); c) analyze samples in duplicate; d) including separate positive and negative controls in every test run, with defined values and error ranges; e) participate in external quality assessment schemes.

III. Antibodies against ß2-glycoprotein I

In 2002 the SSC/ISTH expert group suggested that the aCL test should be replaced by a specific phospholipid-independent test for anti-ß2GPI (aß2GPI). This recommendation was proven to be too hasty. Instead, available evidence indicates that the aß2GPI test merely can complement the APS investigation rather than substitute for aCL testing. In the revised Sapporo criteria the use of both IgG and IgM aß2GPI is recommended, although the committee was not unanimous in its decision (2). The decision to include aß2GPI in the test panel has been controversial and the application of the aß2GPI assays as routine diagnostic tools for the APS has been questioned (7,8).

The aß2GPI assay is based on the ELISA test principle but differs from the aCL assay that it is not dependent on phospholipid. Instead, pure ß2GPI antigen, free from phospholipid, is coupled direct to the plastic surface in the wells of the ELISA plate. However, only specific plastic surfaces can be used in order to present the immobilized antigen in a manner so it can be recognized by human immunoglobulins. Same methodology and standardizations limitations already mentioned for aCL testing also applies to aß2GPI. In order to improve agreement between laboratories the revised Sapporo criteria suggest that laboratories and manufacturers follow the procedures for measurement of aß2GPI antibodies proposed by the European Forum on antiphospholipid antibodies (9). The proposal includes establishment of local reference range, even for those using commercial kits, based on at least 50 (preferably 100) healthy normal subjects. The subjects should include mainly women as the aß2GPI antibodies are found more frequently in women. Due to lack of Gaussian distribution of values the range has to be calculated using percentiles rather than standard deviations to the mean value. For APS diagnosis, the Sapporo criteria recommends the use a cut-off >99th percentile.

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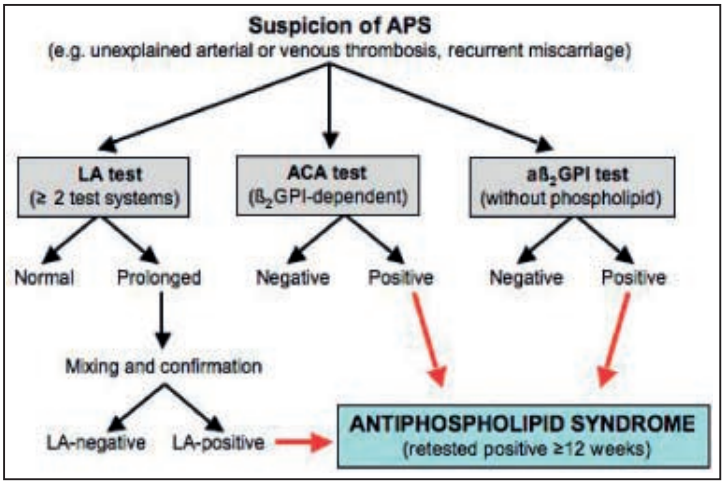

Figure 1.
Proposed test algorithm illustrating the steps of laboratory diagnosis of APS. The cut-offs for LA testing needs to be established locally. For aCL and aß2GPI tests the current recommendations is to analyze for both IgG and IgM isotypes.

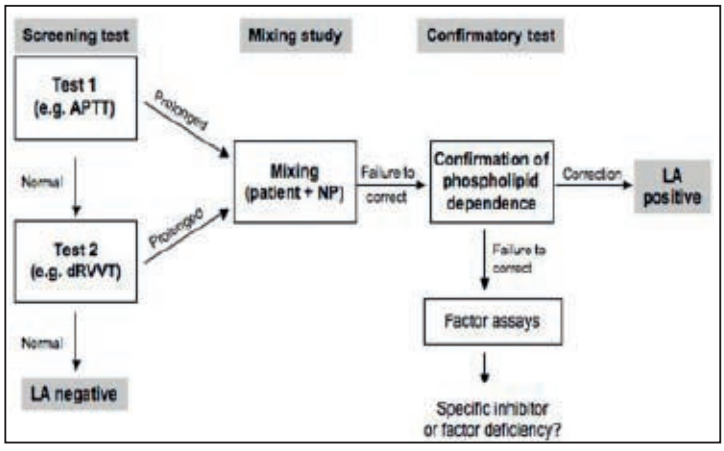

Figure 2.
Approach for LA testing. The four-step procedure begins with at least two screening assays. LA can be excluded if screening tests are normal. If any of the screening tests are prolonged, mixing studies with normal plasma (NP) should be performed. If mixing shows no (or little) correction then the phospholipid dependence is checked by a LA confirmatory test. The confirmatory test(s) should be based on the test system that resulted in an prolonged screening test. LA is present if the phospholipid dependence is confirmed. If no LA can be detected, various coagulation factor assays needs to be applied in order to identify other causes for the abnormal screening test. All steps need to have reliable cut-offs for correct identification.

Monitoring and management of anticoagulation treatment

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An increasing number of patients including elderly and children are on anticoagulant therapy alone or, justified or not, in combination with antiplatelet therapy (1). But, anticoagulant therapy may be complicated by bleeding events (2,3), individual response, renal and hepatic failure, interacting drugs, diets and herbal natural products.

Heparins, coumarins and acetylsalicylic acid are old drugs with unspecific, unpredictable anticoagulant effects, side effects and complicated pharmacokinetics. An extensive research work is going on to find new drugs; more selective, highly specific for single clotting enzymes and individual steps in the coagulation cascade, more easy to administer, with less side and rebound effects and, hopefully, with "non inferior efficiency ". The significance of individual risk-factors, however, disappears when these drugs are investigated, un-monitored, in fixed (titrated)-doses, in large clinical studies with patients from different parts of the world.

Clinical studies have shown that the antithrombotic, anticoagulative and antihemostatic properties of a certain drug do not run in parallell. For many patients a certain anticoagulant effect, which does not increase bleeding events substantially, is enough and monitoring is of little value. On the other hand there are high risk patients and high risk situations when monitoring is needed. But, a global test for monitoring and optimizing anticoagulant treatment or to measure the anticoagulant effects of all coagulation inhibitors is not available.

Anticoagulant, and antiplatelet, antithrombotic drugs inhibit, in some way or another, thrombin or the generation of thrombin (4). Automated assay for thrombin generation is now available but has not been evaluated in clinical trials (5). Most anticoagulant agents currently under investigation target thrombin (FIIa) or factor (F) Xa. These drugs, or prodrugs, can be given orally or parenterally. At the moment there are no specific methods for their reversal (6).Thrombin has, to a greater extent than F Xa, effects outside the coagulation system (there are thrombin receptors on most cells in the body) and has anti- as well as pro-coagulant effects. If early (FXa) or late (FIIa) inhibition of the coagulation cascade will be the winner can only be answered by future head-to-head clinical trials (7,8).

Heparins, low molecular weight heparins (LMH) and the synthetic pentasaccharide derivates fondaparinux and idraparinux.

Thanks to the pioneer work on heparin by Erik Jorpes and Clarence Crafoord early clinical studies were performed in Sweden (9). However, anticoagulant treatment of venous thromboembolism became accepted on the basis of reports that preceeded modern techniques of clinical evaluation and most studies were retrospective in their nature. Jorpes stated 1951 that; as necessary as determination of the prothrombinindex is on coumarins as unnecessary is determination of the clotting time on heparin. Since then there has been no randomized clinical trial comparing fixed doses of heparin with regulated doses according to heparin-concentration (protamine titration or anti-Xa assay), the activated Partial Thromboplasin Time (APTT) or any other clotting test.

Unfractionated heparin inhibits thrombin, factor Xa and other coagulation factors by catalysing antithrombin mediated inhibition and interferes with platelet function.

However, there is an important inter-individual variability requiring laboratory control.

Laboratory monitoring of heparin treatment for venous thrombosis with APTT has been widely accepted aiming at a therapeutic range of 1.5- 2.5 or 2-3 times the control value (10).

Limitations of APTT methods include non-linear and individual dose response, variability between test instruments, reagents and different lots of the same

reagent and low sensitivity for LMH. APTT tests have low sensitivity for factor Xa inhibition but are sensitive for thrombin inhibition. APTT- test (more than 300 commercial tests) is easily available and inexpensive but has low precision and is not standardized among laboratories and for different reagents. APTT reagents differ in sensibility for heparin making it impossible to use a standardized international ratio. Individual laboratories are recommended to develop their own therapeutic range using APTT values which correspond with accepted heparin levels (0.2-0.5 units/ml by the protamine titration and 0.3-0.9 units/ml by anti-Xa assay) (11).

There are recommendations against the use of a fixed target in seconds for any heparin indication (4).The anticoagulant effect is hampered in inflammatory states where antithrombin binds, more easily, to acute phase proteins than to heparin. In such situations of "heparin resistance" monitoring with a factor Xa assay is preferrable. Monitoring heparin treatment has recently been revised and the evidence supporting this APTT-range to predict thrombus extension, bleeding events or heparin concentration is weak (11). However, some studies indicate that measurement of APTT allows stratification of patients with venous thrombosis into high- and low-risk categories regarding recurrency (12,13).

The relationsship between the APTT and clinical outcome of heparin treatment has been evaluated also in patients with arterial disease, ie coronary syndromes, justifying regular APTT monitoring to minimize recurrent ischemic and bleeding events but does not support an important role (10,14). Activated Clotting Time (ACT) test is used to monitor higher doses of heparin for patients undergoing PCI (Percutanous Coronary Intervention) or cardiopulmonary by pass surgery.

Heparin is now, on many indications, replaced by LMH. However heparin is still indicated in situations where LMH has not been studied, it has a shorter half-life, is safer in patients with renal failure and can be more easily reversed by protamine than LMH. To avoid dosing and monitoring errors, it is advisable to utilise an in-hospital standard nomogram for intravenous heparin administration. The anticoagulant effect, pharmacokinetic and pharmacodynamic properties of LMH´s is more predictable but dependent of antithrombin, body weight and renal function. In most clinical studies fixed, regulated by weight, doses of LMH have been used without monitoring. The smaller molecules (3000-6000 D) of LMH have less affinity for thrombin inhibition, they are cleared by the kidneys, lack the non-specific affinities of unfractionated heparin and can be given once daily subcutanously without laboratory monitoring (15). However, LMH´s differs in molecular weight and in pharmacokinetics, the renal threshold for accumulation differs, the therapeutic range for anti-factor Xa activity depends on dosing interval, the relative anti-Xa to anti IIa activity varies (16) and all LMH are not evaluated in obese patients. Only few head to head clinical trials are performed. In addition, generic LMH´s, with marked differences in their in vivo pharmacology, have been introduced in some African and Asian countries and the suppliers have applied for approval in US and the European union.

Monitoring antifactor Xa activity is recommended in obese patients, in patients with creatinine clearance below 20-30 ml/min and in pregnant women.

It is a matter of conflict which anti-factor Xa assay is appropriate. Anti-Xa assays can be performed with and without addition of exogenous antithrombin. The rational for addition is to standardize the antithrombin effect but addition may overlook the anticoagulant effect in patients with low antithrombin concentrations (17).

The synthetic pentasaccharide derivates (< 3000 D) which are given subcutanously once daily, or once weekly, in fixed-doses for venous thrombosis or acute coronary syndrome are also dependent on antithrombin for their anticoagulant effect, have longer half life, can not be reversed by protamine and are more dependent on renal function than LMH.

If monitoring is performed with an anti-factor Xa assay a calibration curve with the pentasaccharide should be used. Like LMH, therapeutic doses, have little effect on APTT and ACT.

A rare but serious side effect of heparin, LMH and recently reported for fondaparinux (18) is Heparin Induced Thrombocytopenia (HIT). HIT is an, immunologically mediated, condition, with high mortality, which mostly appears during the first week of treatment and is most frequently seen with unfractionated heparin (19). The diagnosis, which has to be identified early, is dependent on a clinical probability score, prescense of heparinrelated platelet activating antibodies against the platelet factor 4 - heparin complex and on platelet activation tests.

All heparin treatment should be stopped (even flushing of catheters) and exchanged by another antithrombotic therapy. Caval filter, heparin-bounded catheters

and other devices should not be used, platelet transfusion and intravascular catheters should be avoided.

Vitamin K Antagonists (VKAs)

Dicumarol is since 1941 the only anticoagulant drug approved for oral administration.

VKA is the most effective antithrombotic therapy for atrial fibrillation and mechanic heart valve prothesis, it is as effective as heparin and LMH for venous thromboembolism and as acetylsalicylic acid after myocardial infarction (4,20). In Europe, North America and Canada about 1 % of the populations are on VKAs and this figure is increasing, mainly because of an increasing number of patients treated for atrial fibrillation.

Racemic warfarin is commonly used in Scandinavia, Italy, North America and in United Kingdom whereas phenprocoumon and acenocoumarol, which differs in plasma clearance, elimination kinetics and dependence of CYP 269 for their metabolism, are used in other European countries.

VKAs have an unpredictable dose response, requiring frequent monitoring, a narrow therapeutic range, monitoring assays that are difficult to standardize, multiple drug and food interactions and requires a complex organisation to obtain optimal outcomes.

Bleeding and thromboembolic complication rate is dependent on; intensity of treatment, measured by the International Normalized Prothrombin time Ratio (INR), gender, age, education of patients and medical staff, clinical surveillance and optimal laboratory control (3,20,21). Bleeding complication rate is increased when VKAs is combined with anti-platelet therapy (1).

Management of VKAs can be done at the general practioners, at anticoagulant clinics or by self management using point of care instruments. Policy differs in the Nordic countries.

The quality of VKA- management varies to a great extent among general practioners. It improves if patients are well educated and play an active part in the treatment, monitoring is easily available, laboratory test is given directly to the patient and there are written guidelines. The efficacy of VKA-therapy depends on maintenance INR within the designated therapeutic range. With INR above 3 the risk of serious bleeding increases exponentially and with INR below 2 the efficacy declines rapidly. In general, these factors are better controlled at anticoagulation clinics or by self management resulting in lower rates of bleeding and thrombotic events, improved quality of life and avoidance of interlaboratory variations in the prothrombin time (PT) measurements. However, even within a clinical trial setting, with enthusiatic study doctors, patients and nurses, resulted in therapeutic INR only in approximately 60% (4, 21, 22, 23).

Variations in the sensitivity for VKAs is highly dependent on a sufficient dietary Vitamin K intake and to, about 40% on genetic variation in enzyme, vitamin K epoxide reductase (VKORC1), activity (24). Supplementation of a daily low dose vitamin K (80-100 microgram) has improved stability and time within therapeutic range (25).

The optimal intensity measured by PT has changed with time. In Sweden and Norway 1973 the National Drug Information Committees recommended PT (Thrombotest) corresponding to INR values between 2 and 4.5 (26). The international recommendations, based on clinical studies and the Quick prothrombin time method, now targets INR beween 2 and 3 (4,20). In patients with low risk for recurrent venous thrombosis or in patients with high bleeding risk a less intensity (1.6-2) has been suggested and in patients with atrial fibrillation or mechanic heart valve prothesis and high thromboembolic risk a higher intensity (2.5-3.5) (4,20,27,28,29)

Inhibitors of thrombin and factor Xa

Numerous small molecule direct (non antithrombin dependent) FXa inhibitors binding in a competitively and reversible way to the active site of FXa, without prolonging the bleeding time, and FIIa inhibitors with different affinity and reversibility to exosite and active thrombin sites are manufactured and many are in clinical trials. Plasma half-life and elimination routes (renal, liver or metabolisation) varies.

Monitoring direct FIIa inhibitor with APTT, which still is mostly used, suffers from insensitivity at high concentrations and new direct prothrombin activation methods, ecarin clotting time (ECT) and prothrombinase-induced clotting time (PiCT), which also determines the anticoagulant effects of heparins, LMH, fondaparinux and idraparinux are investigated (30,31). ECT provides good linearity for lepirudin, argatroban and melagatran (FIIa inhibitors) independent of warfarin therapy. Good correlations between inhibition of a factor Xa assay, prothrombin time, PiCT and plasma concentration of the selective Xa inhibitors (rivaroxaban) have been reported.

Substance	Mode of action	AT-dependent	Administration route	Monitoring Methods
AVK´s	Vitamin K inhib	No	PO, IV	INR (Quick/Owren PT-methods)
Heparins	Heterogenous	Yes	IV, SC	APTT, anti Xa-assay, PiCT, ACT
LMH´s	Heterogenous	Yes	SC, IV	Anti Xa-assay, PiCT
Fondaparinux Idraparinux	Homogenous (FXa inhibition)	Yes	SC	Anti Xa-assay, ECT, PiCT
F IIa inhibitors*	Specific	No	IV, SC, PO	ECT, PiCT, APTT**, (INR*) ACT
FXa inhibitors*	Specific	No	PO, IV	Anti Xa-assay, PiCT, APTT**, INR**, ACT

* *Different substances have varying effects on clotting tests dependent on their affinity and molarity (32).*

** *Commercial APT and PT reagents varies considerably in sensitivity. (Owren PT method, used in the Nordic countries, is less sensitive for F IIa inhibition with argatroban than Quick method (33).*

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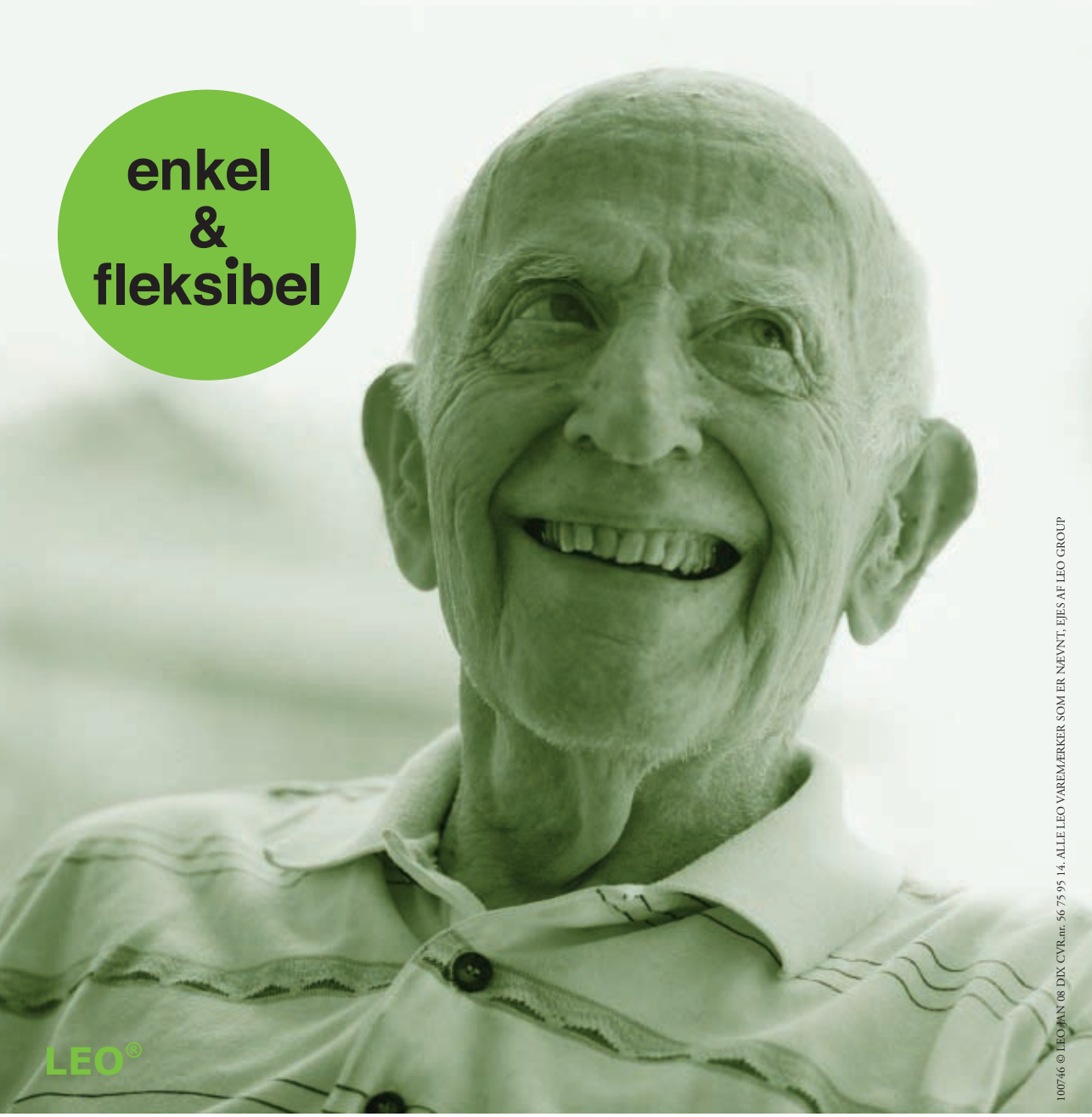

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Thrombophilia-A clinical approach to thrombophilia testing

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Introduction

The last decades we have witnessed a lot of new risk factors for venous thrombosis, of both acquired and congenital origin. The first congenital thrombophilia that was discovered was antithrombin deficency in 1965 followed by protein C and protein S deficency in the early 1980s (1, 2, 3). Together these three deficiencies of natural coagulation inhibitors could explain less than five percent of venous thrombosis. Dahlbäck discovered APC resistance 1993(4), and the factor V Leiden mutation is the most common genetic risk factor for venous thrombosis with a prevalence of around 5% in the background population and between 20-50% among patients with venous thrombosis (5). In 1996 Poort et al reported that 18% of patients with venous thrombosis had a nucleotide change (G-A transition) at base 20210 of the prothrombin gene compared to 1% in the control population (6). Antiphospholipid antibodies and lupus anticoagulants is well known to be associated with venous thrombosis and is also a risk factor for recurrence of thrombosis (7; Table 1).

Deficiencies of natural coagulation inhibitors, antithrombin, protein C and S deficiency could be both qualitative and quantitative and several mutations have been described for each deficiency which makes genetic testing unsuitable in clinical practice (6). Several studies are demonstrating a clear association between the risk of a first venous thrombosis and also for recurrence of thrombosis for deficiencies of these natural coagulation inhibitors (8) (Table 1).

APC-resistance and the factor V Leiden mutation is the most common genetic risk factor associated with venous thrombosis (4, 5, 8)(Table 1). Although factor V Leiden is a weaker risk factor than deficiency of natural anticoagulants it contributes more to the thrombotic burden in the population (10). Several authors have associated APC resistance with a higher degree of recurrence than non-carriers and Schulman noted interestingly that the effect of recurrence was higher in a longer perspective (8, 10, 11). In familial trombophilia, the factor V Leiden mutation as well as deficiencies of the natural coagulation inhibitors are associated with a higher degree of recurrence of venous thrombosis (12).

The Prothrombin 20210A mutation is a genetic risk factor for venous thrombosis associated with elevated plasma levels of prothrombin (6) (Table 1). In a recent systematic review by Ho and coworkers this mutation has been associated with a higher risk of recurrence after stopping anticoagulant treatment (10).

Elevated ***levels of clotting factors VIII, IX and XI*** are all associated with an increased risk of venous thrombosis; the risk is 2-3 fold increased in individuals exceeding the 90 percentile of the distribution of clotting factors in the general population (13, 14, 15).
However with exception of factor VIII it is unclear if these levels are acquired or genetically determined.

A clinical approach to thrombophilia testing

In a trombophilia screening it is relevant as a first step to take a careful personal and family history together with a physical examination (16). In a trombophilia panel we have today good evidence to test for deficiencies of natural coagulation inhibitors, APC-resistance and the factor V Leiden mutation, prothrombin 20210A mutation, and antiphospholipid antibodies including lupus anticoagulants. The trombophilia panel should also include a complete blood count, activated partial thromboplastin time (APTT) and an international normalized ratio (INR) to exclude an effect of ie warfarin.

Does thrombophilia testing improve the care of thrombosis patients?

Up to date the acute management of venous thrombosis in patients with inherited trombophilia or antiphospholipid antibodies and lupus anticoagulants is the same as in patients without trombophilia (17). However a growing body of evidence is supporting that trombophilia, both inherited and acquired could affect the duration of treatment with oral anticoagulants (7, 10, 11, 12, 17). The risk of recurrence of thrombosis without trombophilia is approximately 3-5% per year in patients with thrombophilia this recurrence rate could be higher and as high as 10% per year with combined defects or a homozygous state of factor V mutation or deficiencies of natural coagulation inhibitors (7, 10, 11, 12, 17). However this patient group with combined defects or a homozygous state of for instance factor V mutation or deficiencies of natural coagulation inhibitors is very small and could only be a few percent of unselected patients with thrombosis (18). In a recent systematic review by Ho both the factor V Leiden mutation and the prothrombin 20210A mutation has been associated with an increased risk of recurrence although of a low magnitude and a question has been addressed by the authors about cost-effectiveness of screening for these mild thrombophilic defects (10).

In clinical practice one approach could be trombophilia testing in the following groups of patients:

- Idiopathic VTE (first)
- Strong family history for VTE
- Age < 50 years of age
- Recurrent idiopathic or secondary non- cancer VTE
- VTE in patients on oral contraceptives or HRT
- VTE at unusual site

However, little clinical trial evidence for long term treatment with oral anticoagulants is present at the moment and most data are from non-randomized trials. The benefit of continuation of oral anticoagulant therapy beyond 12 months is uncertain and must be decided on an individual basis and taking in account the risk of major bleeding which sometimes can be of a greater magnitude than the effect of oral anticoagulants in preventing thrombosis (19).

For patients with trombophilia and family members there is some evidence that prophylaxis in high risk situations could be of value in avoiding a new thrombosis (12).

In conclusion, guidelines for thrombophilia are important especially in this field were the scientific data can be conflicting. The clinical role of thrombophilia testing requires further research in order to determine whether the results should dictate the clinical management or not of VTE patients.

Table 1.

Thrombophilia	Prevalence in the general population (%)	Prevalence in patients with VTE* (%)	Relative risk for VTE*	Recurrance (estimated)
Antitrombin deficiency	0,02	0,8	10-20	+++
Protein C deficiency	0,2	1	5-10	++
Protein S deficiency	0,1	1	5-10	++
Faktor V mutation (heterozygous)	5-10	20-25	3-5	+
FV mutation (homozygous)	0,1	3-4	30-40	+++
PT mutation (heterozygous)	2	6-7	3-5	+
PT mutation (homozygous)	0,01	?	?	+(+)
Lupus anticoagulants	1	10	10	++
Anticardiolipin antibodies	2	10	5	+

*VTE venous thrombosis**

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innohep® tinzaparin, injektionsvæske. Indikationer: Forebyggelse af dyb tromboflebit og tromboemboliske komplikationer. Forebyggelse af koagulation i venekanyler, slanger og apparatur til ekstrakorporal cirkulation og hæmodialyse. Behandling af venøs trombose og tromboembolisk sygdom incl. dyb venøs trombose og lungeemboli. Bemærk: **innohep®** er ikke indiceret til behandling af alvorlig lungeemboli, dvs. til patienter med høj risiko for hæmodynamisk instabilitet. **Dosering:** Voksne: **innohep®** *10.000 anti-Xa IE/ml: Tromboseprofylakse hos patienter med moderat risiko for trombose* (almen kirurgi): På operationsdagen 3.500 anti-Xa IE s.c. 2 timer før operationen og derefter 3.500 anti-Xa IE én gang daglig i 7-10 dage. *Tromboseprofylakse hos patienter med høj risiko for trombose (fx total hofte-alloplastik)*: På operationsdagen 4.500 anti-Xa IE s.c. 12 timer før operationen og derefter 4.500 anti-Xa IE én gang daglig i 7-10 dage. *Hæmodialyse (under 4 timers varighed):* Bolusinjektion 2.000-2.500 anti-Xa IE ved dialysens start. *Hæmodialyse (over 4 timers varighed):* Bolusinjektion 2.500 anti-Xa IE ved dialysens start efterfulgt af 750 anti-Xa IE/time som kontinuerlig infusion. *Dosistilpasning:* Om nødvendig kan bolus øges eller mindskes trinvis. *Kontrol af dosis:* Bestemmelse af plasma anti-Xa aktiviteten kan anvendes ved hæmodialyse. Plasma anti-Xa bør ligge omkring 0,5 anti-Xa 1 time efter indgift. De forskellige lavmolekylære hepariner er ikke nødvendigvis ens. Derfor skal de specifikke doseringer og instruktioner for hvert enkelt produkt følges. *Nedsat leverfunktion:* Ved udtalt nedsat leverfunktion bør en reduktion af dosis overvejes. **innohep®** *20.000 anti-Xa IE/ml: Behandling af venøs trombose og tromboembolisk sygdom inkl. dyb venøs trombose og lungeemboli:* 175 anti-Xa IE/kg legemsvægt s.c.1 gang daglig. Peroral antikoagulation bør være påbegyndt på behandlingens 2. dag. 175 anti-Xa IE/kg legemsvægt 1 gang daglig i mindst 6 dage og indtil tilstrækkelig peroral antikoagulation er igangsat. De forskellige lavmolekylære hepariner er ikke nødvendigvis ens. Derfor skal de specifikke doseringer og instruktioner for hvert enkelt produkt følges. *Børn:* Ingen erfaring. *Ældre:* Ingen dosistilpasning nødvendig. **Kontraindikationer:** Overfølsomhed over for tinzaparin, heparin eller andre **innohep®** indholdsstoffer. Aktuel eller tidligere trombocytopeni (type II) i forbindelse med heparin. Blødningstendens. Ukontrolleret svær hypertension. Septisk endokarditis. Intrakraniel eller intraokulær blødning eller anden aktiv blødning. Hæmoragisk diatese, mangel på koagulationsfaktorer, alvorlig trombocytopeni. Svær nedsat funktion af lever, nyrer eller bugspytkirtel. Aktiv gastroduodenal ulcussygdom. Hjerne-, rygmarvs- eller øjenkirurgi. Hæmoragisk apoplexi, cerebral aneurisme. Retinopati, blødning i glaslegemet. Truende abort. Uræmi med undtagelse af patienter i hæmodialyse. **innohep®** *10.000 anti-Xa IE/ml og 20.000 anti-Xa IE/ml i hætteglas:* Kontraindiceret til nyfødte, da det indeholder benzylalkohol. **innohep®** *20.000 anti-Xa IE/ml:* Lumbalpunktur, spinal- eller epiduralanæstesi. **Særlige advarsler og forsigtighedsregler vedrørende brugen:** Bør gives med forsigtighed til patienter med lever- og nyreinsufficiens, ukontrolleret arteriel hypertension, gastrointestinal ulceration, mistanke om malign tumor med blødningstendens, tidligere tilfælde af gastroduodenalt ulcus, nyre- og/eller urinledersten, samtidig brug af lægemidler, der øger serumkalium-niveauet, perorale antikoagulantia, trombocythæmmere (fx ASA). Trombocyttal anbefales før indgift af tinzaparin, på behandlingens 1. dag og derefter regelmæssigt hver 3. eller 4. dag samt ved behandlingens afslutning. Forsigtighed ved behandling af ældre. Må ikke gives som i.m. eller i.v. injektion. På grund af risiko for hæmatom under behandling med **innohep®** bør i.m. injektion af andre midler undgås. **innohep®** *20.000 anti-Xa IE/ml:* Indeholder natriummetabisulfit, bør derfor ikke benyttes til patienter med astma eller overfølsomhed over for sulfitter. **Interaktioner:** Samtidig indgift af lægemidler med virkning på hæmostasen. Interaktion mellem heparin og intravenøs nitroglycerin. Lægemidler, der øger serumkalium-koncentrationen, bør kun indtages samtidig under særlig omhyggelig lægelig overvågning. **Graviditet og amning:** Ingen erfaring med brug af LMW-heparin i første trimester. Epiduralanæstesi under fødslen er kontraindiceret. Der findes ingen data vedrørende amning. **Bivirkninger:** *Hyppigt:* Blødningskomplikationer, øgning af aminotransferaser, gamma-GT, LDH og lipase. Hæmatom og smerter på injektionsstedet. Forhøjet serumkalium-koncentration. *Lejlighedsvis:* Let forbigående trombocytopeni (type I). *Sjældent:* Hudnekrose. Antistof-fremkaldt alvorlig trombocytopeni (type II). Denne alvorlige type trombocytopeni kan ses sammen med arterielle og venøse tromboser/tromboembolismer,trombocytforbrugende koagulopatier, muligvis hudnekroser på injektionsstedet, petecchier, purpura og melaena. Anafylaksilignende reaktioner, i sjældne tilfælde anafylaktisk shock, allergiske reaktioner med symptomer som fx kvalme, opkastning, feber, hovedpine, urticaria, pruritus, dyspnø, bronchospasme, hypotension. Heparintolerance. Ved heparintolerance og antistof-fremkaldt alvorlig trombocytopeni skal brugen af **innohep®** straks afbrydes. Patienten skal oplyses om, at han/hun også i fremtiden skal undgå at tage lægemidler, der indeholder heparin. Forbigående hårtab. Alvorlige bivirkninger i form af subdural eller epidural hæmatom, intrakraniel blødning, retroperitoneal blødning, metrorrhagia, angioødem, epidermal nekrolyse, Stevens-Johnsons syndrom og priapisme er rapporteret i sjældne tilfælde. *Meget sjældent:* Hypoaldosteronisme, forbundet med hyperkaliæmi og metabolisk acidose (især hos patienter med nyresvækkelse og diabetes mellitus). **innohep®** 20.000 anti-Xa IE/ml: NB: På grund af indholdet af natriummetabisulfit ses overfølsomhedsreaktioner i enkelte tilfælde, især hos patienter, der lider af bronkial astma. Disse reaktioner kan ytre sig ved opkastning, diaré, dyspnø, akut astmaanfald, bevidsthedsforstyrrelse eller shock. **Overdosering:** Blødning er det vigtigste tegn og symptom på overdosering og tinzaparin kan neutraliseres med protaminsulfat. **Pakninger og priser:** *10.000 anti-Xa IE/ml:* 10 htgl. x 2 ml: 1.915,05 kr. Engangssprøjter i easybox: 0,25 ml: 242,80 kr.; 0,35 ml: 342,70 kr.; 0,45 ml: 517,75 kr. *20.000 anti-Xa IE/ml:* 10 htgl. x 2 ml: 3.412,20 kr. Graduerede engangssprøjter i easybox: 6 x 0,5 ml: 656,70 kr. 30 x 0,5 ml: 3.188,85 kr; 6 x 0,7 ml: 919,40 kr. 30 x 0,7 ml: 4.396,70 kr; 6 x 0,9 ml: 1.182,05 kr. 30 x 0,9 ml: 5.660,85 kr. For dagsaktuelle priser se www.medicinpriser.dk. **Udl.:** B. **Ej tilskudsberettiget.**

Diagnosis of Overt and Non-overt Disseminated Intravascular Coagulation

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Introduction

In 2001, the International Society on Thrombosis and Haemostasis (ISTH) Subcommittee of the Scientific and Standardisation Committee (SSC) on Disseminated Intravascular Coagulation (DIC) presented the following consensual definition of DIC: "DIC is an acquired syndrome characterized by the intravascular activation of coagulation with loss of localization arising from different causes. It can originate from and cause damage to the microvasculature, which if sufficiently severe, can produce organ dysfunction" (1). Furthermore, the subcommittee proposed that the microvascular system should be viewed as a distinct physiologic organ where injury may result in a stressed, but compensated haemostatic system (non-overt DIC) or a stressed and decompensated haemostatic system (overt DIC). It was anticipated that design of systems to score the presence and severity of DIC might be of importance for clinical practice as well as clinical trials on the effect of interventions directed at pathways or components of the coagulation system to improve DIC and/or the underlying disorder (e. g. sepsis). Accordingly, two scoring systems were developed: An algorithm for the diagnosis of overt DIC (table 1) and a template for scoring system for non-overt DIC (table 2). The scoring systems have now been evaluated in a number of studies and the experiences from these studies are discussed below.

Overt DIC

Use of the scoring system for overt DIC requires that the patient is suffering from a clinical condition that may be associated with overt DIC (1). If this is not obvious, the scoring system for non-overt DIC should be used instead (table 2). The scoring system for overt DIC comprises four laboratory parameters, each of which has a high sensitivity but poor specificity in the diagnosis of DIC. The aim of combining the results in a scoring system is to increase the predictive value of the parameters. All the analyses are readily available in most hospitals. Nevertheless, implementation of the scoring systems has been hampered by inter-hospital differences in the choice of analytical methods and presentation of the results. This is especially the case with the determination of fibrin-related markers (FRM) but questions has also arisen on the cut-off levels of prothrombin time (PT) and fibrinogen in hospitals where results of these analyses are given in other units than those used by the subcommittee.

Fibrin related markers

The ISTH DIC score leaves it to the user to choose method for determination of FRM (e.g. soluble fibrin monomer or fibrin degradation products) and set the cut-off values for moderate and strong increase. D-dimer is the most widely used laboratory marker for coagulation activation and numerous assays are available. There is, however, tremendous variability in the numerical results obtained with different assays (2). This was the reason that the subcommittee in 2001 left it to future studies to establish cut-off levels for FRM. Use of plasma-derived calibrators and methods for harmonisation of test results of different D-dimer assays have been suggested as tools to obtain consensus values of D-dimer (2-4).

Wada et al. compared the ISTH overt DIC score with the DIC scoring system produced by the Japanese Ministery of Health and Welfare in 1987 using an assay of fibrinogen-fibrin degradation products (FDP) as FRM (5). They used 10 mg/l as the low cut-off value but suggested that a higher specificity of the DIC score might have been obtained if the low cut-off value was changed to 20 mg/l (table 3). The suggested cut-offs for FDP were used in a study by Cauchie et al. (6). In a retrospective study using data from the large database from the PROWESS clinical trial on severe sepsis Dhainaut et al. arbitrarily chose 1 and 10 times the upper normal level of the D-dimer assay as low

and high cut-off values, respectively, in assessment of a modified version (fibrinogen was omitted) of the ISTH overt DIC scoring system (7). These cut-off levels were also used by Bakhtiari et al. in a prospective validation of the ISTH overt DIC scoring system based upon expert opinion as "gold standard" (8). Dempfle et al. compared two D-dimer assays and an assay of soluble fibrin in a study evaluating the predictive value of the ISTH overt DIC score on clinical outcome in 359 surgical ICU patients (9). These authors calculated the ISTH overt DIC score retrospectively using the 25% and 75% quartiles of the 1870 plasma samples obtained from the patients during admission as low and high cut-off values. Low and high quartiles of D-dimer in patient plasma samples were also used as cut-off values in the retrospective studies by Voves et al. and by Angstwurm et al. who used the same assay as Dempfle et al. found a higher upper threshold of 5.0 mg/l (10;11). Sivula et al. defined the low cut-off as four times the upper normal level and the high cut-off as four times the low cut-off level (12).

As FRM contribute with up three of the five points requires for ISTH overt DIC, the choice of cut-off levels for FRM is expected to be of central importance to the prognostic value of the score. It is not possible from the existing studies to conclude upon the optimal cut-off levels since an ISTH overt DIC score of ≥5 in nearly all the studies identified groups of patients with significantly higher mortality than patients with a score <5. It should be noted, however, that the difference between 28-day mortality in patient with and without DIC was lower in studies using the upper normal level of D-dimer as low cut-off (median ratio: 1,48; range: 1,3-1,8) than in studies using a low cut-off of 0.9-2.0 mg/l (median ratio: 2.6; range: 2.3-2.7) (table 3).

Prothrombin time

The prothrombin time (PT) is basically the clotting time in seconds of citrated plasma after addition of thromboplastin and calcium chloride. Since the first description of the PT assay a number of modifications have been suggested and today many hospitals use calculated derivatives of PT, like PT index, PT ratio, and INR. PT is a sensitive marker of DIC as illustrated by Kinasewitz et al. who suggested a simple DIC scoring system based on PT and platelet counts (13). PT is one of the parameters in the ISTH DIC score but as it is not directly available in many hospitals cut-off values for derivatives of PT are warranted. Cut-off values for calculated derivatives of PT used in presently available studies of the ISTH overt DIC score are presented in table 3.

Fibrinogen

The cut-off level of 1 g/l is equal to 2.941 μmol/l.

Non-overt DIC

The ISTH non-overt DIC score was originally proposed as a tool to be used in the monitoring of critically ill patients with suspected DIC but not fulfilling the criteria of overt DIC, and no threshold scoring value for the diagnosis of DIC was suggested (1). In a study of 450 ICU patients Toh et al. found an ISTH non-overt DIC score of ≥5 in 90 patients (14). These patients had a mortality rate of 78% which was the same as the mortality rate in 49 patients with overt DIC (78%) compared with 29% in patients with scores <5. The authors noted that while the non-overt DIC scoring system does capture some patients that evolve onto overt DIC, it also defines those who do not but whose coagulopathy nonetheless forewarns of a potentially lethal outcome. Furthermore, they suggested that although measurement of protein C and antithrombin lend greater confidence in the diagnosis, a combination of PT, platelets and D-dimer appear to confer sufficient robustness for detecting a level of haemostatic dysfunction that has prognostic significance.

Kienast et al. used the ISTH scoring systems for overt and non-overt DIC in a retrospective study of 563 patients who did not receive concomitant heparin in the KyberSept severe sepsis trial and had sufficient data for DIC scoring (15). In these patients non-overt DIC, defined as an ISTH non-overt DIC score of ≥7, was far more common than overt DIC. At baseline, 223 patients had non-overt and 42 overt DIC, with 36 patients fulfilling the criteria for both non-overt and overt DIC. The mortality rates were 33%, 29% and 22% in patients with non-overt, overt and no DIC, respectively.

Thus, the study suggests that the prognostic value of the ISTH non-overt DIC score is at least as high as that of the overt scoring system and that non-overt DIC is more common among patients with severe sepsis than overt DIC.

Future use of the DIC scoring systems

All published studies on the use of the ISTH DIC scoring systems show that DIC scoring identifies patients with a high risk of lethal outcome. Other scoring systems used in ICU's have an equally high

prognostic value. The ISTH DIC scoring systems may, however, show to be valuable tools in the evaluation of therapeutic initiatives in patients with critical illness and suspected DIC. In the PROWESS and KyberSept clinical trials on severe sepsis DIC scoring defined cohorts of patients who were most likely to benefit from treatment with protein C and antithrombin, respectively (7;15). Further studies on harmonisation of FRM assays and calculated PT derivatives in patients with suspected DIC are warranted in order to establish scoring systems with well-defined cut-off value.

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Table 1.
ISTH overt DIC score.

Does the patient have an underlying disorder known to be associated with overt DIC? If yes: proceed. If no: do not use this algorithm.

Platelet count (109/l):
(>100 = 0; 50-100 = 1;<50 = 2)

Fibrin-related marker:
(No increase = 0; Moderate increase = 2; Strong increase = 3)

Prolonged prothrombin time:
(<3 s = 0; 3-6 s = 1; >6 s = 2)

Fibrinogen:
(>1.0 g/l = 0; <1.0 g/l: 1)

If the sum is ≥5, the patient status is compatible with overt DIC.

Table 2.
ISTH non-overt DIC score.

Does the patient have an underlying disorder known to be associated with DIC?
(Yes = 2; No = 0)

Major criteria:
Platelet count (109/l):
(>100 = 0; 50-100 = 1;<50 = 2) +
(rising = -1; stable = 0; falling = 1)

Fibrin-related marker:
(normal = 0; raised = 1) +
(falling = -1; stable = 0; rising = 1)

Prolonged prothrombin time:
(<3 s = 0; >3 s = 1) +
(falling = -1; stable = 0; rising = 1)

Specific criteria
Antithrombin:
(normal = 0; low = 1)

Protein C:
(normal = 0; low = 1)

TAT-complexes:
(normal = 0; low = 1)

Calculate the sum.

Table 3.
Overview of studies evaluating the ISTH overt DIC score.
n.d.: not described. PT: Prothrombin time. ULN: Upper level of normal.

	Prothrombin time			Fibrin-related marker			28-day Mortality		
		Cut-off			Cut-off (mg/l)				
Study	Method	Low	High	Assay	Low	High	DIC	No DIC	Ratio
Wada et al. (5)	PT (s)	ULN+3 s	ULN+6 s	Dia-Iatron, LPIA-FDP	10	40	n.d./532	n.d./752	-
Dhainaut et al. (7)	PT (s)	ULN+3 s	ULN+6 s	Stago, Liatest D-Di	0.4	4.0	166/454 = 37%	273/1114 = 25%	1.5
Bakhtiari et al. (8)	PT (s)	ULN+3 s	ULN+6 s	BioMérieux, Vidas D-dimer	0.4	4.0	33/74 = 45%	36/143 = 25%	1.8
				BioMérieux, MDA D-dimer	1.0	3.3	11/31 = 36%	47/301 = 16%	2.3
				Roche, Tinequant D-dimer	0.9	3.1	11/28 = 39%	47/304 = 16%	2.4
Dempfle et al. (9)	PT (s)	ULN+3 s	ULN+6 s	Iatro, SF	2.6	5.0	16/32 = 50%	42/300 = 14%	3.1
Toh et al. (14)	PT (s)	ULN+3 s	ULN+6 s	BioMérieux, MDA D-dimer	1.0	4.0	38/49 = 78%	105/300 = 29%	2.7
	Owren PT activity (%)	60%	30%						
Sivula et al. (12)	INR	1.2	1.9	Roche, Tinequant D-dimer	2.0	8.0	38/95 = 40%	65/399 = 16%	2.5
	PT (s)	ULN+3 s	ULN+6 s						
	Quick test (%)	70%	40%						
Kienast et al. (15)	PT ratio/INR	1.4	2.3	n.d.	0.4	4.0	12/42 = 29%	74/334 = 22%	1.3
Angstwurm et al. (10)	PT index (%)	70%	40%	BioMérieux, MDA D-dimer	1.0	5.0	15/28 = 54%	41/205 = 20%	2.7
				Stago, Liatest D-Di	n.d.	n.d.			
				Stago, FS test	n.d.	n.d.			
Cauchie et al. (6)	PT activity (%)	68%	51%	Stago, FDP Plasma	20.0	40.0	18/32 = 56%	30/116 = 26%	2.2
				Stago, Asserachrom D-Dimer	0.9	3.1	6/10 = 60%	7/30 = 23%	2.6
Voves et al. (11)	PT (s)	ULN+3 s	ULN+6 s	Roche, Enzymune-Test FM	2.6	5.0	7/12 = 58%	6/28 = 21%	2.8

Point-of-Care coagulation testing

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Introduction

Recently there has been a renewed interest in "global coagulation testing" with methods and instruments enabling bed-side measurements of coagulation and coagulum properties in whole blood. The obvious reason for this is the wish from the physicians to get rapid and continuous information on the overall coagulation status of the patient, information that hopefully might be used as a guide for treatment and care. The fact that these instruments are not only recording the time to clot formation, but also the properties of the forming coagulum, is often used as an argument for these techniques. The use of whole blood, often native blood without anticoagulants added, has been claimed to give a better picture of the situation in vivo, since all blood components are allowed to interact during the test. However, the exclusion of the endothelial component and the low shear rates in most of these devices makes the assumption of an in vivo-like situation somewhat limited. The aim of this section is to give a brief overview of the measuring principles, current applications and limitations of the instruments found on the market. Besides the instruments described in this section, there are also some point-of-care instruments that are only assessing platelet function and not coagulation, such as the PFA-100® (Dade-Behring, Marburgh, Germany), the VerifyNow® (formerly known as the Ultegra Rapid Platelet Function Analyser, RPFA), the Ichor-Plateletworks®, and the Impact® cone and plate(let) analyzer. All these have been recently reviewed (1, 2) and will not be further discussed here.

Thromboelastography (TEG®/ROTEM®)

The thromboelastograph (TEG) was first described by Hartert in 1948 (3). In 1996, the term TEG became a registered trademark for the instrument sold by Haemoscope Corp., which is using the origi-

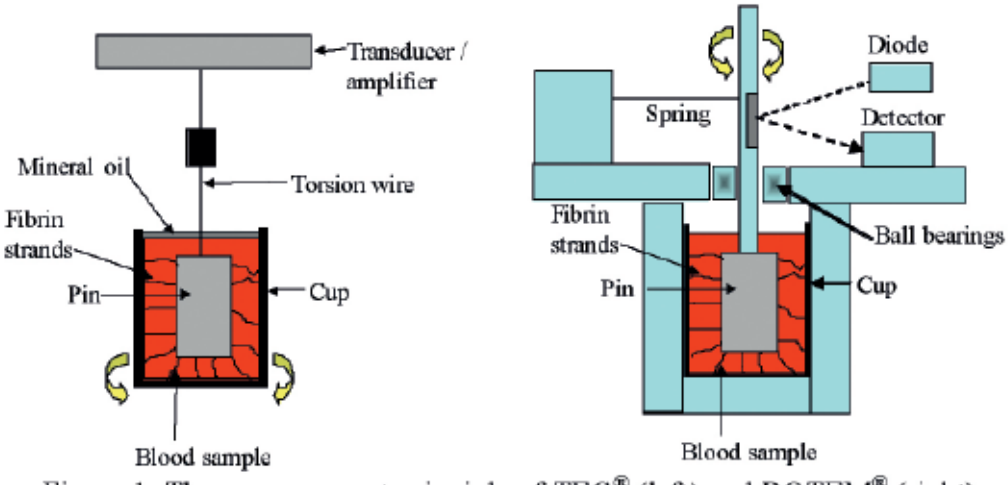

Figure 1. The measurement principle of TEG® (left) and ROTEM® (right)

nal technology. An alternative instrumentation, sold by Pentapharm GmbH, uses the term (rotational) thromboelastometry for the process and ROTEM® for the instrumentation. The measuring unit consists of a cylindrical cup, made of disposable plastic and pre-heated to 37ºC. A pin is suspended into the cup, and the pin is connected to a detector. The cup and the pin will oscillate relative each other through an angle of approximately 5°. The major difference between the instruments lies in the oscillation. In the TEG® instrument the cup oscillates and in the ROTEM® instrument the pin oscillates. The cup is filled with approximately 300 µL of blood, native or recalcified, and the pin is dipped into the blood. As fibrin strands forms in the coagulating sample, they will connect the pin with the oscillating cup (see Figure 1). In the TEG®, the motion of the pin will be transferred through the torsion wire to the transducer and amplifier, and a tracing will be recorded. In the ROTEM® instrument the impedance of the rotation of the pin is what is being detected. The ROTEM® instrument was constructed to be less sensitive towards movement or vibrations than the original TEG®. However, the TEG® manufacturer (Haemoscope Corp.) now claims that these problems are solved by vibration damping in the software. Another drawback with the TEG® is that each instrument only has two channels, making it impossible to analyse duplicate samples if several different activators are to be tested, whereas the ROTEM® instrument has four channels. The ROTEM® instrument also has an electronic pipette connected to the instrument to simplify the pipetting of the different reagents provided, and has developed software with exact step-by-step instructions and automated pipetting to make the instrument easy to use.

From the tracing obtained (Figure 2), different parameters of clot formation and lysis are measured:

The reaction time, r (TEG®) or CT (ROTEM®), is the time from the start of a sample run until the first signs of clot formation (initial fibrin formation, amplitude=2 mm in the tracing). The k (TEG®) or CFT (ROTEM®) value is defined as the time between an amplitude of 2 and 20 mm in the tracing. This value is a measure of the rapidity of fibrin build-up and fibrin crosslinking. It is shortened by increased fibrinogen level, and to a lesser extent by platelet function, and is prolonged by anticoagulants. The maximum amplitude, MA (TEG®) or MCF (ROTEM®) is a reflection of the absolute strength of the fibrin clot and depends mainly on platelet number and function but also to some extent on fibrinogen levels. The α value is measured as the slope of the curve between r and k (TEG®) or as the angle of tangent at 2 mm amplitude (ROTEM®). It denotes the speed at which the clot is being formed and crosslinked. It is increased by increasing fibrinogen levels and to a lesser extent by platelet function, and is decreased by anticoagulants that affect both. CL30 (TEG®) or LY30 (ROTEM®) is the amplitude reduction 30 min after MA. This represents clot lysis. There are also other parameters recorded, such as the time to maximum clot strength (TMA/MCF-t) and clot lysis time (TTL/CLT).

The TEG® software also combines several other parameters to calculate a "Coagulation Index", CI, which is claimed to provide a haemostasis profile of the patient. The ROTEM® software also has other parameters available for research purposes, as the analysis of the speed of coagulation using the first derivative of the curve, which has been proposed and used by Sørensen et al (4).

The original way of performing a TEG analysis was to use native blood, but citrated blood that is recalcified before analysis may also be used. Several reports does however state that the results are not comparable (5-7), a fact that might be explained by intrinsic coagulation activation occurring in some brands of blood collection tubes (8). The sample might be modified by the addition of kaolin in order to shorten the clotting time or the addition of tissue factor in the presence of the platelet fibrinogen receptor antagonist

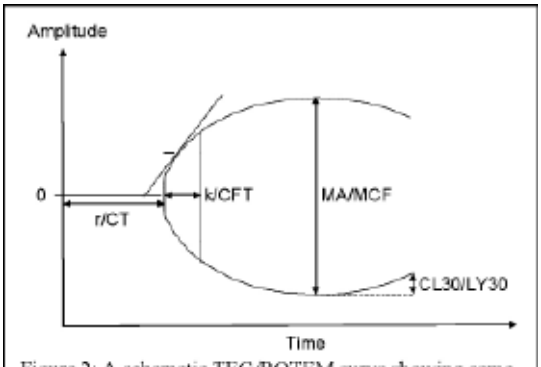

Figure 2: A schematic TEG/ROTEM curve showing some of the parameters recorded

ReoPro® in order to investigate the response caused by the fibrinogen network alone in the absence of functional platelet interactions. The use of cups and pins with heparinase enables measurements of heparin effects.

The PlateletMapping™ assay was recently introduced as a way of monitoring anti-platelet therapy by TEG® (9). A native blood sample activated by kaolin is compared with heparinised samples where the fibrin network is formed by adding reptilase and fXIIIa and the platelets are activated by ADP (2 μM) or arachidonic acid (AA, 1 mM). The use of such high doses of AA has been reported to cause platelet lysis in other studies (10), in our opinion, a critical evaluation of this approach would be of great value.

For the ROTEM® instrument, different commercial reagents are available, with tissue factor (alone (EXTEM), with aprotinin to detect hyperfibrinolysis (APTEM) or with GPIIb/IIIa antagonists to evaluate the fibrin network contribution to the clot strength (FIBTEM)), contact activator (alone (INTEM) or with heparinase for the use during heparin treatment (HEPTEM)), ecarin to monitor the use of direct thrombin inhibitors (ECATEM) or just with recalcified samples (NATEM). Correlations between EXTEM and PT, INTEM and APTT and FIBTEM and fibrinogen levels in trauma patients have been recently described (11).

TEG has been used to monitor blood component therapy during surgery for a long time (reviewed by (12)) and the first description was for the use in liver transplantation (13). Later studies describe the use of TEG in cardiac surgery (14, 15) and trauma management (16). It also has a role for monitoring the treatment by fibrinolytic drugs (17-20). The clinical use of an algorithm-based replacement therapy has however been questioned, both for in hepatic (21) and cardiac surgery (22).

In the laboratory the use of TEG®/ROTEM® has been limited, presumably due to old reports showing a poor correlation to standard laboratory parameters (23). The need for the use of citrated samples, that might not behave as the native ones (5-7) makes it important to establish a normal reference range with samples treated in the same way as for the patient samples to be analysed, instead of using the manufacturer's ranges. A low dose of tissue factor has been used for the monitoring of treatment by recombinant factor VIIa and prothrombin complex (24, 25). There have also been reports on its use for hypercoagulability screening (26, 27). In a current review, however, it was stated that no evidence exists for the utility of TEG®/ROTEM® as a screening tool for bleeding disorders (28). The current diversification in different instruments, activators and modifications also makes standardization between centres a distant issue.

Free oscillation rheometry (FOR)

The first study describing free oscillation rheometry (FOR) with the instrument ReoRox was published in 1999 (29). The instruments on the market today have one or two channels. Most of the studies published have however been using the ReoRox®4 instrument with four channels (Medirox AB, Nyköping, Sweden). The instrument uses a cylindrical sample cup which is set into free oscillation along its longitudinal axis by a forced turn of the sample cup holder every 2.5 seconds. The frequency and damping of the oscillation is recorded as a function of time. A baseline frequency in the range of 10 Hz gives a shear rate of approximately 60 s^{-1}. All measurements are performed at 37°C. The change in elasticity (G') over time in the coagulum is measured using a reaction chamber, which consists of a sample cup with a 6 mm cylinder (bob) attached to a shaft in the centre

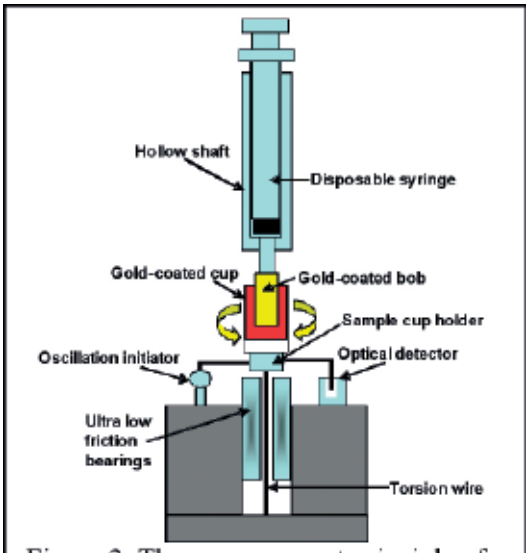

Figure 3. The measurement principle of FOR using the ReoRox® instrument

of the cup (Figure 3). The sample cup and the bob are made of disposable plastic. However, for elasticity measurements in whole blood or platelet rich plasma, gold-coated parts are used in order to avoid detachment of the clot due to clot retraction (30). The instrument's accuracy in the detection of long clotting times has been validated (31), and also how the measurements are affected by changes in different blood components (32). Until now, the instrument has mainly been used for studies of the platelet contribution to whole blood coagulation (30, 33-35). The clinical studies published so far have been of limited size (36-38) and have not been using the reagents commercially available, which consists of thromboplastin alone or together with a fibrinolysis activator or a platelet PAR1 activator.

Sonoclot®

The Sonoclot® analyzer has a tubular probe that oscillates up and down in the whole blood sample (Figure 4). The resistance to movement is measured and recorded during the coagulation process. A curve plotting the "clot signal" against time is produced. Both the curve form and some defined features of the curve are used for interpretation. Contact activators such as glass beads, celite or kaolin are used as coagulation activators. The primary use for Sonoclot® seems to be in the monitoring of coagulation status in patient treated with heparin during surgery (39). Since the introduction in the 70's (40), the use of Sonoclot® has been limited.

The Clot Signature Analyzer™

The Clot Signature Analyzer™ (CSA™; Xylum Corporation, Scarsdale, NY, USA)) is a global haemostasis screening instrument intended for use with native whole blood (41). The blood is passed through a thin plastic tube under pressure. Two holes are then punched in the tube, causing a fall in pressure. The platelet and fibrin clot will plug the holes and the time for this process to be completed is called the "platelet hemostasis time" (PHT). The coagulation then spreads though the lumen of the tube. The time this occurs is called the clot time (CT). A subsample of the blood is also passed through a second tube coated by collagen fibrils. The time to reach a 50 % reduction in pressure in this tube is called collagen-induced thrombus formation time (CITF). In a multi-center study involving 108 patients and

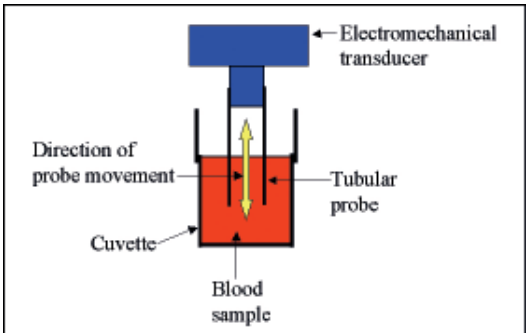

Figure 4: The measurement principle for Sonoclot®

116 normal individuals, the instrument gave at least one abnormal value in 92% of patients with coagulation factor deficiencies, 91% of patients with VWD and 63% of patients with platelet function defects, while the test results were all normal in 89% of the control individuals (42). However, despite the quite good overall sensitivity, the test could not help in distinguishing between platelet and coagulation factor defects. The need for native blood in combination with the instrument's size was also limiting its potential as a bed-side instrument, since it was not easily moved to the patient.

The Gorog Thrombosis Test

The Gorog Thrombosis Test (GTT) (43) or thrombotic status analyser (TSA) (44) (Montrose Diagnostics, London, UK) is a small, portable, four channel instrument where native blood is placed in a vertical conical tube with a hole in the bottom. The tube also contains two steel balls, one large and one smaller further down. Platelets are activated by shear stress (175 dyne/cm2) when they are passing the first large ball, and will then aggregate and initiate coagulation in the area of turbulent flow between the balls. The clot will occlude the passage between the wall and the second ball and stop the blood flow completely. A light sensor records the time between blood droplets leaving the end of the tube to determine the time for occlusion. Another parameter measured is the time when the blood flow continues again, which the manufacturer claims is due to thrombolysis, as it was inhibited by the presence of aprotinin or α2-antiplasmin (44). So far the instrument has been mainly used for experimental studies on spontaneous thrombolytic activity.

The Hemostasis Analysis system

The Hemostasis Analysis System (HAS; Hemodyne, Inc., Bethesda, MD, USA) is measuring the force developed by platelets as they undergo cellular contraction ("platelet contractile force", PCF™), and speed of clot formation in whole blood between a cup and parallel upper plate at 37°C. Force measurement is accomplished utilizing a force displacement transducer coupled to the upper plate, and is expressed in kilodynes (45). The time between assay start and PCF onset is termed the thrombin generation time (TGT™) and is used as a surrogate marker for thrombin generation (46). The instrument uses 700 µL of citrated blood for each analysis. Applications proposed are for example monitoring of treatments with enoxaparin (47) and recombinant factor VIIa (48).

HemoSTATUS™

The HemoSTATUS™ test or "platelet-activated clotting time" (Medtronic Blood Management, Parker, CO, USA) measures acceleration of kaolin-activated clotting time (ACT) by different concentrations of platelet activating factor (PAF). For a complete test, two four-channel instruments are needed. The test has mainly been used for the evaluation of patients during cardiac surgery (49, 50), even though its usefulness for predicting blood loss has been questioned (51, 52).

Concluding remarks

In conclusion, the market and alternatives for point-of-care testing of global haemostasis is rapidly expanding. However, the number of publications reporting on the use of many of the instruments is still limited, except for TEG and ROTEM with around 3000 publications together. Among the others, Sonoclot has 76 publications and all the rest has less than 35 publications.

In view of the complexity and increased knowledge in the field of haemostasis, these methods offers a logical and also potentially very important complement to the more traditional, simplified methods. However, almost all of the companies marketing these types of tests and instruments claim to be the only ones in the world "giving the whole picture". This interesting paradox and denial of the other competitors might be one explanation to the small number of comparative studies found in the field. Most instruments also use arbitrary units and special reagents which further complicates the picture.

Another problem is to produce good control materials, since the elasticity obtained in plasma control samples are much lower than the ones that will be encountered in highly elastic samples such as blood and platelet rich plasma. Many of the tests also propose the use of single samples, something that in our opinion should be avoided. Our view is that a reliable use of single samples must be verified for the actual protocol and instrument, and also that the normal reference range to be used should be established on site with samples collected, stored and treated in the same way as the patient samples to be analysed.

An objective and critical evaluation and systematic analysis on the performance of the different instruments is certainly needed to be able to advice a potential user in the choice of an instrument to study global haemostasis.

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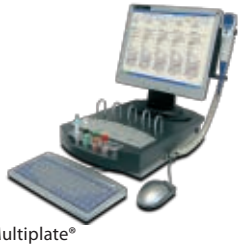

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