Emerging Technologies in Hemostasis Diagnostics: A Report from the Australasian Society of Thrombosis and Haemostasis Emerging Technologies Group

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ABSTRACT

Technology in hemostasis laboratories has evolved enormously during the last 30 years. Although many scientists and clinicians will remember the traditional tilt-tube techniques to screen for coagulation abnormalities and to monitor anticoagulant therapy, the hemostasis laboratory today uses a variety of modern technologies. These include flow cytometry, chromogenic assays, molecular typing (e.g., polymerase chain reaction), immunologic assays (e.g., enzyme-linked immunosorbent assays), functional assays of specific coagulation proteins, and platelet function analyzers. Although these advances in technology have resulted in greater capability, productivity, sensitivity, specificity, and ultimately, improvement in the clinical care of patients, controversies and limitations remain. This article highlights new and emerging technologies in hemostasis and discusses whether they have improved or are likely to improve laboratory diagnostics by specifically addressing the following: (1) Can new technologies help predict likelihood of thrombosis recurrence? (2) Has an understanding of the role of a disintegrin-like and metalloprotease with thrombospondin type 1 motifs (ADAMTS13) in microangiopathy resulted in improved diagnostic methods for this disorder? (3) Does thrombelastography allow better definition of bleeding risk than conventional hemostasis assays, especially in settings of acute hemostatic pathology?

KEYWORDS: Hemostasis, laboratory testing, thrombin generation, thrombophilia, bleeding

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CAN NEW TECHNOLOGIES HELP PREDICT LIKELIHOOD OF THROMBOSIS RECURRENCE?

Conventional thrombophilia testing has helped to explain thrombophilic mechanisms, but has been less helpful in defining ongoing thrombotic risk. Despite the effectiveness of current anticoagulation therapy, defining the optimal duration of therapy remains a problem for clinicians and patients alike. Given the bleeding risk of vitamin K antagonists (VKAs) such as warfarin, indefinite anticoagulation should be restricted to patients with a high risk of recurrent venous thromboembolism (VTE). Follow-up of a large patient cohort after a first symptomatic deep vein thrombosis (DVT) demonstrated that more than 20% will suffer a recurrent event within 5 years.[1] However, our inability to define which patients are in the 20 to 30% at high risk exposes the whole cohort to risk of complications from continued therapy with VKA. Clinical parameters, such as an idiopathic thrombosis, past history of VTE, or even being a male, are markers of increased risk of recurrence, but none of these factors alone are indications for indefinite warfarin. There is a clear need for a laboratory assay of hypercoagulability that predicts recurrent thrombosis.

Thrombophilia Screening

The ability of conventional thrombophilic factors to predict recurrence remains controversial. Earlier studies reported higher rates of recurrent VTE in the presence of antiphospholipid antibodies[2,3] and elevated levels of factor (F) VIII:coagulant activity (C).[4,5] However, the high prevalence of these abnormalities in the normal population and uncertainty about laboratory criteria for identifying abnormal results have hampered the clinical application of these tests. In reviewed retrospective case-control studies,[6] heterozygous carriage of either the FV Leiden or prothrombin 20210A mutations was not a significant risk factor for recurrence, but compound heterozygotes had higher hazard ratios (HRs) of 2.4 to 4.8. Several recent prospective studies have addressed this question. One or more thrombophilic markers (deficiencies of antithrombin, protein C and S, acquired protein C resistance, FV R506Q and PT20210A mutations, hyperhomocysteinemia, and antiphospholipid antibodies) were found in 30% of 195 patients with a first episode of idiopathic proximal DVT and treated for either 3 or 12 months.[7] After 46 months follow-up, recurrence rates were higher in the thrombophilic cohort (35.1%) than in those with normal test results (21%; $p = 0.046$). However, the excess of recurrent events was only seen in patients who received 3 months of anticoagulation; recurrence rates were similar in both the thrombophilic and normal cohorts after 12 months of treatment. The risks of stopping warfarin in patients with familial thrombophilia was addressed in 180 patients with either factor V Leiden or deficiencies of antithrombin, protein C, or protein S who did not receive long-term anticoagulation after a first event.[8] Recurrent VTE occurred at a rate of 5%/year (during a mean 5.6-year follow-up) in this cohort, compared with a rate of 1%/year in those remaining on anticoagulation. Although this study found a higher risk in males compared with females, the recurrence rates seen here may not be high enough to justify indefinite anticoagulation in all patients with a thrombophilic defect. Therefore, testing for individual thrombophilic factors, with the possible exception of antiphospholipid antibodies, is unlikely to determine the duration of anticoagulation.

D-Dimer

At present, the most promising assay for the prediction of recurrence is an old assay revisited. Plasma levels of D-dimer have not been demonstrated to be specific for DVT and pulmonary embolism, but are a sensitive marker of fibrin formation, and thus a potential indicator of the activity of the coagulation pathways. In a small prospective study of D-dimer levels during warfarin therapy, elevated levels at diagnosis normalized within 6 weeks in 13 patients with nonidiopathic DVT, whereas five of nine patients with idiopathic DVT showed persisting elevation.[9] Recent studies have suggested that elevated D-dimer levels, typically measured after the cessation of warfarin therapy, may be a marker for recurrent thrombosis[10,11]; however, a subsequent study[12] reported that thrombophilic carriers with normal D-dimer levels had a very low risk of recurrence, suggesting that a negative result may be a more useful predictor. The D-dimer assay used in this study (VIDAS D-Dimer enzyme-linked immunosorbent assay [ELISA]; bioMèrieux; Marcy l'Etoile, France) had a sensitivity of 62% and specificity of 65.6% across all subjects, but a negative predictive value of 94%.[12] Given that only 16% of patients with elevated D-dimer suffered a recurrence, its positive predictive value appears limited.

The contribution of D-dimers was also compared with residual venous obstruction (RVO) in a prospective cohort study of 400 patients with idiopathic proximal DVT.[13] In this study, elevated D-dimer level was an independent risk factor for recurrence at 2 years (HR, 3.32; 95% confidence interval, 1.78 to 6.75), whereas RVO at the time of warfarin cessation did not contribute significantly. Of the patients with high D-dimer levels, 25.9% had recurrent thromboembolic events, compared with 5.7% and 10.4% for those with normal D-dimer levels and the absence of presence of RVO, respectively. Taken together, these studies provide good evidence that

D-dimer levels, measured after cessation of warfarin, can identify a group at low risk of recurrence. This hypothesis is being tested in a management study (PROLONG), in which elevated D-dimer levels after cessation become an indication to recommence warfarin. From the viewpoint of both the clinician and patient, it would be preferable to identify a high-risk cohort without stopping warfarin; whether D-dimer assays can be used for this purpose remains to be proven.

Thrombin Generation

Thrombin plays a central role in coagulation, modulating both pro- and antithrombotic pathways. Our conventional clotting times are poor markers of thrombin activity, given that 95% of thrombin generation occurs after the generation of fibrin. Thrombin generation assays have evolved considerably since the 1950s, as reviewed recently.[14] Crucial advances include the use of a slow-reacting, fluorogenic substrate, allowing one-stage measurement in the presence of fibrin, and calibration against a nonclotting control. Among several competing systems, the most widely studied have been the endogenous thrombin potential (ETP)[15] and a commercial system, calibrated automated thrombography (CT), which can also measure thrombin generation in platelet-rich plasma.[16,17] Multiple groups are now using the CT or thrombogram to screen different patient groups, and it has been recognized that the CT analysis can be performed on fresh and frozen-thawed PRP, but results are dependent on tissue factor and phospholipid concentrations.[18] Further modifications, such as collection into corn trypsin inhibitor, have been proposed to prevent in vitro thrombin generation via the contact factor pathway.[18] Although the sensitivity of these assays to thrombophilic conditions has been studied in small groups of patients,[15,19–21] the recent availability of a standardized assay should enable researchers to study larger patient groups, and define the temporal relationships between thrombin potential, thrombotic events, and subsequent therapy.

The large Austrian Study on Recurrent Venous Thromboembolism[22] recently has confirmed an association between thrombin generation and VTE recurrence. Plasma samples were collected from 914 patients at a median of 13 months after ceasing anticoagulation for a first spontaneous VTE. It is important to note that this study excluded those with deficiencies of antithrombin III, protein C, protein S, lupus anticoagulants, cancer, or pregnancy. Peak thrombin was higher in women than in men, and in those with the prothrombin 20210G mutation, but not in carriers of FV Leiden. By tertiles of peak thrombin, the risk of recurrence increased, so that the highest tertile (>400 nM) had a 20% risk at 4 years compared with 6.5% for the remaining two thirds ($p < 0.001$). Mean peak thrombin levels were higher in those with recurrence (420 nM) than in those without (349 nM), but the assay levels were similar for spontaneous and precipitated recurrent events. The authors propose that the threshold of 400 nM can be used to identify a group at low risk of recurrence, for whom long-term warfarin is not appropriate. This is consistent with the D-dimer studies, but the low-risk group here comprises a larger proportion. Comparative studies are needed, using the widely used thrombin generation methods (ETP/CT) to determine if these are also predictive. Finally, there is now the potential to study the contribution of platelets to thrombin generation in these patients.

Other Novel Assays For Predicting Risk of Thrombosis

Other global assays of coagulation are under development that could identify high- or low-risk cohorts among VTE patients. These have been reviewed recently,[14] and include indirect measures of thrombin measurement, such as thromboelastography (also discussed later in this review) and studies of fibrin polymerization. The overall hemostatic potential (OHP) developed by He et al[23] quantitates fibrin generation and lysis in vitro, in response to trace amounts of thrombin/tissue factor and tissue plasminogen activator. Hypercoagulable OHP patterns may persist long after a thrombotic event[24] and also were observed in a subset of patients receiving therapeutic doses of warfarin.[25] As with the D-dimer assays, testing of patients for recurrence risk ideally would be done before stopping warfarin. Additional modifications of the OHP assay may increase its sensitivity for thrombophilic defects.[19] The role procoagulant phospholipid plays in clotting can also be assessed by a simple and easily automated technique that measures the ability of plasma, platelet-rich plasma, or whole blood to enhance the activity of activated FX.[26] This may prove to be a useful and rapid screening test for hypercoagulable patients.

There is growing consensus that the so-called reductionist approach to VTE (testing for an ever-increasing number of thrombophilic factors) is of limited utility in predicting recurrence risk.[14] The reductionist approach is also associated with a greater chance of generating false-positive results.[27] Instead, it is hoped that global coagulation assays, which integrate multiple inherited, acquired, and temporary influences, will be more useful. Thrombin-generation assays are reproducible enough for clinical studies, and one system at least has shown a similar low recurrence risk in patients with low/normal levels of peak thrombin.[6] The sensitivity of many of these

assays to known thrombotic risk factors is not well defined. Patients with natural anticoagulant deficiencies and antiphospholipid antibodies often have been excluded from studies on the premise that they require indefinite warfarin after a first event. However, as a recent review noted,[14] to be clinically useful, global assays of coagulation must do more than just detect the inherited thrombophilias. Until we have a greater range of treatment options, a first priority is to identify low-risk patients and limit their exposure to anticoagulation.

HAS AN UNDERSTANDING OF THE ROLE OF ADAMTS13 IN MICROANGIOPATHY IMPROVED DIAGNOSTIC METHODS FOR THIS DISORDER?

Mortality from thrombotic thrombocytopenic purpura (TTP) has declined substantially since early intervention with plasma exchange has become routine therapy. In addition, the pathophysiology of many cases of acute and chronic relapsing TTP has been defined following the recognition of the cleaving protease ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs). Mortality from TTP was common until the 1970s, when it was discovered experimentally that treatment by plasmapheresis combined with replacement fresh frozen plasma was often an effective means of overcoming the acute phase.[28] Early diagnosis and rapid initiation of this treatment has resulted in a dramatic improvement in survival of greater than 80%,[29] highlighting the need for clear diagnostic criteria.

In 1996, two groups independently isolated a protease that cleaved von Willebrand factor (vWF) multimers.[30,31] This protein was later shown to be absent in congenital TTP[32] as well as autoantibody-induced, acquired TTP.[33] The enzyme, then known as von Willebrand factor cleavage protease, was subsequently sequenced and identified as a metalloprotease and designated ADAMTS13.[34] The discovery that ADAMTS13 deficiency was the cause of most cases of TTP led to an understanding of the probable pathophysiology. It had been known for some time that very large multimers of vWF were present in the plasma of many patients with TTP,[35] and a series of in vitro experiments, using both recombinant and human-derived ADAMTS13, demonstrated that vWF multimers are cleaved by this enzyme, probably under conditions of high shear stress. It is likely that deficiency of ADAMTS13 leads to accumulation of highly procoagulant ultra large multimers of vWF in vivo with resultant thrombus formation in the microvasculature.[36,37] In cases of microangiopathic hemolytic anemia where TTP seems likely, the laboratory now has several options to help establish the diagnosis. These are the demonstration of very high multimers of vWF, a reduction in the functional or antigenic levels of ADAMTS13, or the presence of antibodies to the enzyme.

vWF Multimer Assays

Multimeric patterns of vWF traditionally have been used to help categorize the clinical types of von Willebrand disorder. Multimers are separated by electrophoresis through a low-concentration agarose gel, followed by immunoblotting. The bands can be visualized by either radioactive[38] or enzyme-linked antibody to vWF.[39] The assay is complex and time consuming and therefore usually is only performed in specialized centers, with results rarely available quickly enough to be of value in reaching a diagnosis of TTP.

ADAMTS13 Functional Assays

These were reviewed in detail by Veyradier and Girma in 2004.[40] At that time the available techniques were technically demanding, and were based on electrophoresis,[30,31,41–43] measurement of residual vWF antigen,[44,45] or measurement of residual vWF activity[46–49] after incubation of vWF with test plasma. Since then, there have been several major improvements, including an automated activity assay[49] that can be completed within a few hours,[50] a technique using the Impact-R (DiaMed, Cresier, Switzerland) cone and plate analyzer to measure the effect of TTP plasma on the adherence of normal platelets to a polystyrene well under conditions of high shear stress,[51] and a fluorescent resonance energy transfer assay for ADAMTS13 activity that can be completed within 2 hours.[52]

ADAMTS13 Antigen Assays

Following an initial abstract reporting an ELISA in 2004,[53] the last 12 months have seen a major breakthrough in laboratory testing for ADAMTS13 and its antibodies. A technique, published in January 2006, used a peptide containing the ADAMTS13 cleavage site linked to a horseradish peroxidase. The method is rapid, sensitive, and reproducible.[54] Rieger et al[55] reported a sandwich ELISA using polyclonal rabbit anti-ADAMTS13 as the capture antibody and peroxidase-complexed rabbit antihuman immunoglobulin (Ig) G as the detection antibody. This method was sensitive to low levels of ADAMTS13. In cases of inherited TTP, the authors demonstrated that circulating ADAMTS13 antigen/antibody complexes could be detected by the antibodies, producing normal antigenic levels and rendering the assay of limited value in such cases. The latest reported technique used a monoclonal ADAMTS13 as the capture antibody with two biotinylated anti-ADAMTS13 antibodies for detection.[56]

With this method, all 11 patients with acquired TTP had low ADAMTS13 levels. There are now commercially available ELISA kits from American Diagnostica (Stamford, CT) and Technoclone (Vienna, Austria).

ADAMTS13 Antibody Assays

Treatment of acquired TTP differs from treatment of congenital TTP in that plasma exchange and immunosuppression are needed in the former, whereas episodes of the familial form often can be managed successfully by fresh frozen plasma infusion. Demonstration of a circulating antibody is therefore clinically useful. Most of the assays described here have been adapted for the detection of antibody in cases of acquired TTP. Prior to assay, a mixture of test and normal plasma is incubated and the residual ADAMTS13 in the normal plasma is estimated using electrophoresis,[33] the collagen-binding technique,[45] estimation of vWF:ristocetin cofactor activity by aggregometry,[47,48] or vWF:antigen by immunoradiometric analysis.[45] Park et al[57] purified patient IgG by affinity chromatography on protein A Sepharose prior to incubation with normal plasma and assessment of residual activity using the technique of Furlan et al.[33] ELISA techniques are also suitable for detection of antibody. In the first technique described, Scheiflinger et al[58] detected nonneutralizing antibodies not detected by a more traditional electrophoretic method. The more recent techniques of Wu et al[54] and Rieger[55] are also suitable for antibody detection and antibody kits are now commercially available.

Methods for the quantitation of ADAMTS13 have improved enormously in simplicity and precision since the enzyme was first described 10 years ago. It is now within the scope of most medium to large diagnostic laboratories to run these tests. In cases of congenital or acquired TTP, low ADAMTS13 levels or the detection of circulating antibodies are powerful diagnostic indicators. However, problems remain. Not all cases of clinically diagnosed idiopathic TTP have low ADAMTS13 levels.[59] Normal levels can also be found in acquired TTP by ELISA, when circulating immune complexes that cause the clinical syndrome block the activity of the protease but do not cause its removal from the circulation.[55] In these cases simultaneous measurement of antibody levels is important. Whether severe ADAMTS13 deficiency is a specific marker for TTP remains controversial[60,61]; it had been suggested that ADAMTS13 may also be reduced in other clinical conditions.[62,63]

Nonetheless, it is likely that these issues will be resolved rapidly. The disorder is rare and to date there has only been limited availability of ADAMTS13 assays. Indeed it is likely that the classification of thrombotic microangiopathies will be refined on the basis of studies during the next few years.[34,59] The newer techniques should also facilitate population studies, allow genetic counseling, optimize therapy, and help identify those at risk of recurrence.

DOES THROMBOELASTOGRAPHY ALLOW BETTER DEFINITION OF BLEEDING RISK IN ACUTE HEMOSTATIC SETTINGS THAN CONVENTIONAL TESTS?

Conventional hemostasis assays define bleeding risk, but have limitations in settings of acute hemostatic pathology. Acute bleeding in patients is a complex medical emergency, often complicated by the multifactorial nature of the bleeding. More than in any other patient, the coexistence of associated pathologies, such as sepsis, liver and/or renal failure, cardiac insufficiency, and the effects of multiple drugs can affect the hemostatic balance. The coagulopathy can often be very complex and the hemostatic balance can change rapidly due to the effects of the consumption of clotting factors, hyperfibrinolysis, defects of fibrin polymerization, hypothermia, hyper- or hypokalemia, or hypocalcemia secondary to citrate effect with calcium and magnesium chelation.[64,65]

The capacity to differentiate between surgical or hemostatic causes of bleeding is essential. Transfusion therapy is often given based on certain thresholds (hemoglobin, hematocrit, platelet count) and algorithms based on number and type of products infused. Inappropriate blood replacement can cause severe coagulopathies by further diluting depleted clotting factors. Abnormalities of laboratory tests are not specific and can be found also in nonbleeding patients. In addition, turnaround time of traditional hemostasis tests (prothrombin time, activated partial thromboplastin time, fibrinogen, thrombin time, and platelet count) results in reduced usefulness of the test because most often the patient has moved to a different hemostatic balance from the time the sample was taken. This means that traditional clotting tests will give us information only about the past and never about the present. Therapeutic decisions must be made in real time or as close to real time as possible.

The type of information gained from these tests is also limited because none of the current tests gives insight into the fibrinolytic component of the hemostatic balance, the quality of the platelet function, the presence of fibrin polymerization disorders, the presence of substances interfering with fibrin formation, mild fibrinogen deficiencies, or FXIII activity. It is well known that these tests correlate poorly with a bleeding tendency or propensity. Furthermore, testing on plasma reveals little about the cellular interactions in the hemostatic system that we now understand are complex and drive the final event of clot formation. More specialized testing of hemostasis also has limitations, including platelet aggregometry (which is technically challenging and time

consuming) and platelet function analyzers (which provide a measure of primary hemostasis exclusively).

Clearly there is a need for more predictive tests in the acutely bleeding patient. The ideal in vitro test needs to recognize in vivo interactions between the classic elements involved in hemostasis, including the endothelium, platelets, and clotting factors, as well as other less recognized elements, including other cells (e.g., monocytes, cancer cells), metabolic effects from pathological states (e.g., uremia and paraproteinemia), medications, and fibrinolytic potential. Thrombin burst is also important, hence the intense interest in a global test of thrombin generation. Several commercial tests have been developed or are being evaluated (see previous discussion on thrombin generation assays), including thromboelastography (TEG).

Thromboelastography

TEG is a global assay in which the interaction of coagulation factors, platelets, fibrinolytic factors, inhibitors, drugs, and several other factors is assessed via the continuous registration of the stability of the forming blood clot under low shear conditions that mimic the flow conditions of venous blood. It provides a kinetic analysis of the entire clot formation and stabilization as well as clot dissolution by the fibrinolytic system. Quite distinct from traditional clotting tests, it is capable of providing information on some of the critical aspects of the clot formation process. The different parameters measured are dependent on the rate of formation, strength, elasticity, and firmness of the forming clot.[66,67]

The use of whole blood allows ex vivo investigation of the clotting process and its interactions with cellular components such as leukocytes and erythrocytes. Furthermore, TEG is influenced by the interaction of platelets with coagulation proteins, including initial contact with thrombin, platelet aggregation, cross-linking of fibrin, and clot stabilization. This technology also measures clot dissolution and hence generates quantitative and qualitative information on fibrinolytic activity. No other routine clotting test currently is capable of providing all of this information.[68] TEG can be used as a point-of-care test, such as in the operating theater where continuous monitoring of the hemostatic balance combined with a transfusion algorithm can lead to more appropriate use of blood products, resulting in significant savings.[69]

Although TEG provides a global overview and selective information about the main pathways in hemostasis, there are limitations associated with this technology. Although it is sensitive to a deficiency or excess of coagulation factors, specific clotting factor deficiencies cannot be identified. In addition, TEG does not reflect coagulation associated with endothelial cells and is sensitive only to severe, rather than mild forms of fibrinolysis. Currently, it is not standardized between laboratories due to the diversity in equipment, software, and variations in methodology,[70] has a relatively long measurement time, and requires technical expertise.

TEG has been explored extensively in surgery[71,72] and trauma.[73–76] It has also demonstrated promising results as a valuable test to monitor responses to administration of activated FVII for the treatment of patients with hemophilia and inhibitors in the setting of life-threatening acute bleeding.[77–79] Several authors have investigated the use of TEG as part of an algorithm to guide blood product replacement during surgery. The bulk of the published evidence relates to studies performed during cardiopulmonary bypass surgery. Despite the limitation that in most cases the size of the patient population analyzed is small, the overall conclusion was that TEG was useful in significantly reducing (three- to eight-fold) the use of blood products, particularly platelets and fresh frozen plasma, in the postoperative period. The reduction in use of blood products was achieved despite no differences or changes in intraoperative blood loss between patients monitored with TEG and the patients managed with standard transfusion therapy and laboratory tests.[80–82] Other possible applications of TEG include identification of patients at higher risk of bleeding during and after cardiac surgery,[83,84] monitoring the potential of FXIII administration to increase clot firmness,[85] and identification of hypercoagulability following acute bleeding and massive transfusions. The future utility of TEG in diagnostic and clinical settings will be dependent on method standardization and software developments to allow for improved raw data analysis, and application to larger prospective clinical studies.

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