Detection of procoagulant imbalance
Modified endogenous thrombin potential with results expressed as ratio of values with-to-without thrombomodulin

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Summary
Each individual possesses his/her own endogenous-thrombin-potential (ETP) (i.e. the ability to generate thrombin) which depends on the relative strength of the pro- and anticoagulant drivers operating in plasma. This ability depends in turn on the clinical conditions in which the balance between the two drivers is variably affected. One of the major determinants of this balance is the factor (F)VIII-protein C (PC) axis and its effect can be conveniently explored by the thrombin generation procedures with results expressed as ETP ratio with/without thrombomodulin (TM) (ETP-TM ratio). Furthermore, owing to the many feedback mechanisms mediated by thrombin (e.g. activation of PC, FXI, FV, FVIII, platelets etc.) it is also possible that any perturbation of the balance between pro- and anticoagulants that may occur in plasma even outside the FVIII-PC axis could result in an increased ETP-TM ratio and therefore may suggest a procoagulant imbalance. In deed, other non-coagulation moieties (e.g. microparticles, neutrophil extracellular traps, pro-inflammatory cytokines and others) circulating in blood of patients with various clinical conditions may also contribute to the procoagulant imbalance even when FVIII and/or PC are apparently normal. It can be postulated that dual ETP measurements performed in the presence and absence of TM with results expressed as their ratio may be the candidate procedure to detect subtle procoagulant imbalance in many clinical conditions characterised by an increased risk of thromboembolism. This article aimed at reviewing the clinical conditions in which evidence for the value of the ETP-TM ratio has been provided.

Keywords
Hypercoagulability, thrombosis, protein C/S pathway, factor VIII

Introduction
Coagulation has been historically investigated by the basic and time-honored tests prothrombin and activated partial thromboplastin times (PT and APTT). These tests have been instrumental for improving our understanding of the coagulation mechanisms and for the diagnosis of congenital haemorrhagic diseases such as haemophilia and allied disorders, conditions in which they are abnormally prolonged. PT and APTT are however much less suitable to investigate the other face of the coin (i.e. thrombosis). They are in fact within normal limits in patients with congenital deficiency of the naturally occurring anticoagulants [antithrombin, protein C (PC) and protein S (PS)], conditions in which they should be shorter than normal as thrombin production in these conditions is heightened because of the deficiency of the anticoagulants. It should therefore be concluded that PT and APTT are responsive to the procoagulant factors, but much less to their anticoagulant counterpart. Possible explanations for this conclusion rest on the design of the two assays. PT and APTT are static tests in which plasma clots soon after 5% of the total amount of thrombin is generated (1), thus leaving the remaining 95% unnoticed. Furthermore, owing to the relatively short time interval between coagulation ignition and clot formation (a few seconds) the most important naturally occurring anticoagulant systems cannot express their full anticoagulant potential and cannot therefore contribute to downregulate thrombin generation. This is valid for both the antithrombin and PC systems that require conformational changes brought about by heparin-like substances and activation by thrombomodulin (TM), respectively (2, 3). These moieties are located on endothelial cells and much less in plasma.

A procedure based on the continuous registration of thrombin generation (mediated by the procoagulants) and decay (mediated by the anticoagulants) was described in the 1950s by Macfarlane and Biggs (4) and was later modified by Hemker et al. (5) to generate the so-called thrombogram, which is described by various parameters. Among them, the endogenous thrombin potential (ETP) defines the area under the thrombin generation (and decay) curve recorded upon activation of coagulation with small amounts of tissue factor and negatively charged phospholipids. The ETP represents the net amount of thrombin that can be generated by the test plasma under the experimental conditions and is driven by the balance between the pro- and anticoagulants. Other parameters of
the thrombogram are the lag-time, defined as the time elapsing from coagulation ignition to the formation of the first amounts of thrombin; the thrombin-peak height and the time to reach the peak. Over the last two decades, ETP and allied parameters have been extensively used as laboratory tools to evaluate thrombin generation in many clinical conditions characterised by increasing risk of venous or arterial thromboembolism. Furthermore, thrombin generation was also used to help elucidate coagulation in clinical conditions in which the mechanisms were poorly understood. Although the thrombin generation procedure should be considered as the closest approximation to what occurs in vivo, it presents with some limitations. The time interval for the procedure to be completed is much longer than that required for PT/APTT and therefore antithrombin and PC can substantially be activated even in the absence of their respective endothelial activators (heparin-like substances and TM). However, the activation is not optimal, especially for PC and therefore a modification of the procedure has been introduced by the addition of exogenous soluble TM (6, 7). The addition of TM makes the procedure to mimic much better than ever the conditions operating in vivo and made possible to reevaluate the coagulopathy of cirrhosis, a condition where both pro- and anticoagulants factors are concomitantly decreased, making the balance of coagulation to be restored (8, 9). The addition of TM into the test system showed that thrombin generation in these patients is normal if measured by a testing procedure responsive to both pro- and anticoagulants, in spite of the fact that the PT and APTT are prolonged (8, 9). However, based on pathophysiological considerations and evidence from the literature it can be surmised that the dual ETP measurement with and without TM addition and the results expressed as their ratio could be more important than that of the single procedure alone in detecting the procoagulant imbalance in many clinical conditions at increased risk of thromboembolism. This article reviews the pathophysiological considerations and the evidence stemming from the literature so far accumulated.

The test

The thrombin generation procedure reported here is performed as described by Hemker et al. (5) and detailed by Chantarangkul et al. (10). Briefly, plasma samples are dispensed into wells of a microplate and coagulation is initiated through small amounts of tissue factor (1 pM) (Recombiplastin, Werfen, Orangeburg, MA, USA) and negatively charged phospholipids (1 µM) (Avanti Polar, Alabaster, AL, USA) as triggers. Testing is simultaneously performed (in different wells of the same microplate) after adding soluble TM (Haematologic Technologies, Essex, VT, USA) at a final concentration of 4 nM. The generated thrombin is continuously recorded with an automated fluorometer by means of a slow acting fluorogenic substrate (Z-Gly-Gly-Arg-AMC HCl, Bachem, Bubendorf, Switzerland) and thrombin is quantified in comparison with an internal thrombin calibrator (Thrombinoscope BV, Maastricht, the Netherlands). Readings are recorded and analysed with a dedicated software (Thrombinoscope\textsuperscript{TM}, Thrombinoscope BV), which displays the thrombogram and calculates the lag-phase that follows the addition of the trigger and the initiation of thrombin generation, the thrombin peak (nM), the time to reach the peak and the area under the curve, defined as ETP and expressed as nM thrombin times minutes (nM•min). Results are then expressed as ratio of the ETP measured in the presence of TM to the ETP measured in its absence. The ETP-TM ratio represents the resistance to the anticoagulant activity of TM and should be taken as an in vitro index of the procoagulant imbalance (the higher the ratio, the greater the procoagulant imbalance) (see below). The thrombin generation procedure needs careful standardisation as it is affected by the temperature (11) and by the concentration of the reactants, tissue factor and negatively charged phospholipids (10). The ETP-TM ratio also needs careful standardisation. Although the laws behind it are largely unexplored, the ratio is likely dependent upon the concentrations of tissue factor and TM that are used for its determination. The concentration of tissue factor or negatively charged phospholipids are usually in the range of 1–5 pM or 1–5 µM, respectively. However, concentrations towards the lower limit seems to improve sensitivity of the procedure, although reproducibility decreases as well. TM concentrations are usually in the range of 4–6 nM, but should be such to inhibit the ETP of the pooled normal plasma at about 50%.

Normalisation of the ETP-TM ratio by the following equation

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\text{n-ETP-TM ratio} = \text{Patient}[\text{ETP}\_\text{TM}/\text{ETP}\_\text{noTM}] / \text{Normal}[\text{ETP}\_\text{TM}/\text{ETP}\_\text{noTM}]
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seems to improve both the sensitivity and reproducibility of the procedure (12). Results interpretation requires special attention and it is recommended whenever possible to carry out case-control studies by testing equal numbers of cases and controls within the same microplate in order to reduce the between-assay variation.

Pathophysiological considerations

TM is the endothelial receptor for thrombin and is instrumental to accelerate the conversion of PC into activated PC (APC) (13), which in complex with its plasma cofactor PS eventually inhibits the activated form of factor V and VIII (2). Accordingly, the ETP-TM ratio is expected to be driven by the PC activity (the smaller the PC activity, the greater the ETP-TM ratio) (7). However, APC is the physiological inhibitor to activated FVIII (FVIII), hence also FVIII activity contributes to the ETP-TM ratio (the higher the FVIII activity, the greater the ETP-TM ratio) (7). From the pathophysiological standpoint, it can therefore be concluded that the ETP-TM ratio is responsive to the FVIII-PC axis. Accordingly, any perturbation of the above axis owing to decreased levels of PC or increased levels of FVIII should result in increased ETP-TM ratio and hence in procoagulant imbalance. Notably, decreased levels of PC and increased levels of FVIII are both considered risk factors for venous thromboembolism (VTE) (14, 15). Furthermore, owing to the many feedback mechanisms mediated by thrombin (i.e. ac-
tivation of PC, factor XI, V, VIII and platelets) it is also possible that any perturbation of the balance between pro- and anti-coagulants that may occur in plasma even outside the FVIII-PC axis could result in an increased ETP-TM ratio and therefore may suggest a procoagulant imbalance. Indeed, other non-coagulation moieties circulating in blood of patients with various clinical conditions may also contribute to the procoagulant imbalance even when FVIII and/or PC/PS are apparently normal. For example, microparticles that originate from endothelial cells, monocytes or platelets are known to carry considerable amounts of tissue factor or negatively charged phospholipids that may contribute to the procoagulant imbalance observed in plasma (16) and might be detected by the ETP-TM ratio. Recently, new mediators of thrombosis have been identified in the extracellular nuclear chromatin, released from dead or inflammatory cells, especially as neutrophil extracellular traps (NETs), a network of chromatin and cytoplasmic molecules released from activated neutrophils (17). Histones (e.g. negatively charged nuclear proteins that form nucleosomes) when associated with DNA, are known to influence haemostasis through activation of platelets (18) and red blood cells (19). They may also promote thrombus formation (20, 21) and derangement of the anticoagulant PC pathway by enhancing thrombin generation in plasma through the reduction of PC activation by TM (22). Evidence has also been provided that circulating extracellular DNA and histones are risk factors for both venous, microvascular and arterial thromboembolism (23–26). Myeloperoxidase that is released from activated neutrophils can oxidise TM (27) and thrombin (28), thus further contributing to the impairment of PC activation. Overall, these observations suggest that the ETP-TM ratio may be enhanced in clinical conditions associated with elevated circulating levels of NETs. Indeed, Tripodi et al. (29) have recently shown increased ETP-TM ratios in patients with Cushing disease, a condition characterised by neutrophil activation and high circulating NET levels (29). Interestingly, Cushing disease is also associated with an increased risk of VTE (30). Other studies showed procoagulant imbalance in patients with inflammatory bowel disease (IBD), a condition associated with an increased risk of VTE (31). Evidence has been provided that the procoagulant imbalance in IBD is associated with an increased release of NETs from activated neutrophil and formation of microparticles stemming from monocytes or platelets carrying tissue factor and negatively charged phospholipids (32). It can be surmised that the ETP-TM ratio would be a relatively simple laboratory procedure to detect the procoagulant imbalance in IBD.

The role played by PS in the regulation of thrombin generation should also be taken into consideration. PS does act as a naturally occurring anticoagulant both when working as the APC-cofactor, but also by mechanisms that are independent from APC (33, 34). It can therefore be concluded that also PS may contribute to the ETP-TM ratio. Likewise, the role played by FV should be considered. Indeed, elevated levels of FV and increased thrombin generation have been found in patients with gynecological malignancies (35) and high FV has been reported to affect thrombin generation in vitro (36).

**Literature observations**

There are two lines of evidence suggesting a role for the ETP ratio in detecting procoagulant imbalance. First, thrombin generation procedures modified to include the addition of exogenous APC to plasma prior to the measurement of thrombin generation and results expressed as ETP ratio with-to-without APC (ETP-APC ratio). Second, thrombin generation procedures modified to include the addition of TM prior to the measurement of thrombin generation and results expressed as ETP ratio with-to-without TM (ETP-TM ratio). Both are suitable to detect the procoagulant imbalance due to derangement of the pro- and anti-coagulants. In the following paragraphs, their relevance will be discussed.

**ETP procedures with/without activated protein C**

These procedures have been used over the years to detect subtle procoagulant imbalance for which the unmodified thrombin generation procedure was poorly effective. Of particular interest was the occurrence of APC-resistance not due to factor V Leiden, which is considered as an independent risk factor for VTE (37). This *acquired* APC-resistance is a typical feature of women taking oral contraceptives (OC) (38), pregnant women (39) and individuals with increased levels of such procoagulant factors as VIII or II (40, 41). The ETP-APC ratio to detect procoagulant imbalance in women taking OC has been extensively used over the last two decades. Curvers et al. (42) in their seminal paper used assays that quantified the effect of exogenous APC on ETP- or APTT-based sensitivity-APC ratio in patients with factor V Leiden and in patients on or off OC. Both procedures discriminated equally well individuals with or without factor V Leiden, but the ETP-based procedure discriminated much better the procoagulant imbalance in individuals on or off OC and between users of the second- or third-generation OC. Women on OC had procoagulant imbalance as detected by the ETP-based procedure that was higher than those who were off therapy. Furthermore, individuals who were on third-generation had higher procoagulant imbalance than those who were on second-generation OC (see Figure 1). The authors proposed that plasma factors (other than factor V Leiden) modulate differently the response to APC in the two tests and that the ETP-based procedure is not only more suitable to detect the procoagulant imbalance in patients on OC, but also to differentiate between the procoagulant imbalance observed in patients taking second- (containing levonorgestrel or lynestrenol) versus third-generation (desogestrel or gestodene) drugs. Other authors have confirmed the above results and the value of the ETP-based procedure with/without APC in women on OC (43–47). Interestingly, third-generation OC are considered to increase from 1.5 to 3.0 the relative risk of VTE compared with those of the second-generation (48–50). The reasons why OC increase the risk of VTE are not completely understood. They cause changes of hemostasis and fibrinolytic parameters (44, 51–54), but most of these changes are modest, sometimes contrasting or synergistic. Therefore, it is not surprising that no single test measuring individual components of the haemostatic system is able to give a clear picture of the phe-
nomenon. In contrast, thrombin generation procedures when modified to include anticoagulant triggers (APC or TM) are more suited to this task than those without anticoagulant triggers (47). The lesson learnt from the studies of patients on OC may pave the way for the use of these modified procedures in other clinical conditions where a procoagulant imbalance is suggested, but not yet circumstantiated.

Another area where the ETP-based procedure with/without APC has been successfully used to investigate the suspected procoagulant imbalance is the presence of myeloproliferative neoplasms such as essential thrombocythemia and polycythemia vera. Patients with these conditions are at increased risk of thrombosis, but a procoagulant imbalance was not previously detected. Studies in platelet-rich plasma with the ETP-based procedure revealed that patients with myeloproliferative neoplasms had lower ETP than controls in the absence of APC but had a higher ETP than controls in the presence of APC, thus indicating the occurrence of APC-resistance that translates into high ETP-APC ratio. Multiple regression analysis showed low free PS as the major determinant of the resistance to APC (55). Whelihan et al. (56) showed that low PS, due to increased red blood cell exposure of phosphatidylserine, increases the resistance to APC measured with ETP-based procedures in patients with sickle cell disease. Overall, the above observations demonstrate that the ETP-APC ratio is a reliable laboratory tool to investigate procoagulant imbalance.

ETP procedures with/without thrombomodulin

The assessment of ETP with/without APC although proving remarkable in detecting the procoagulant imbalance in women on OC and other clinical situations has some theoretical limitations. It is effective whenever an acquired APC resistance is suspected and in fact, it challenges the system by including APC, thus bypassing the activation of endogenous PC. Furthermore, the evidence that the ETP with/without APC is responsive to increased levels of FVIII and other procoagulant factors, which are risk factor for VTE (57) is scanty. For instance, there are clinical conditions where either PC, FVIII or both are impaired. In these situations, direct activation of endogenous PC is arguably indicated. The prototype of these situations is chronic liver disease (cirrhosis) where PC is severely decreased and FVIII is considerably increased (58). Because of the above pattern, patients with cirrhosis are theoretically the prototype of the clinical situations in which the axis between FVIII (the procoagulant) and PC (the anticoagulant) are extremely deranged. This situation should translate into a procoagulant imbalance. Evidence has been provided that cirrhosis is in fact a condition where there is a procoagulant imbalance that may not be evident when performing the ETP procedures in the presence of TM, but becomes evident when the procedure is performed with and without TM and the results are expressed as their ratio (7, 59–61) (see Figure 2). Patients with cirrhosis display increased levels of ETP-TM ratio and these ratios mirror the de-
creasing levels of PC as well as the increased levels of FVIII (7). Furthermore, the ETP-TM ratio also mirror the ratio of FVIII-to-PC, which is increased as well in this patient population (7). Additional evidence that concurs to support the value of ETP-TM ratio is the observation that it was considerably decreased in plasma from patients with cirrhosis when plasma from these patients was supplemented with purified PC (62). Another condition where the ETP-TM ratio was useful to detect the procoagulant imbalance owing to reduced PC and increased FVIII is non-alcoholic fatty liver disease (NAFLD), a condition that affects 20% of the general population and is estimated as the most frequent cause for liver transplantation by the year 2025 (63). The ETP-TM ratio in patients with NAFLD was progressively increased from simple steatosis (the least severe form of NAFLD), non-alcoholic steatohepatitis (the intermediate form) to metabolic cirrhosis (the most severe form). PC and FVIII were decreased or increased, respectively and the ETP-TM ratio was significantly correlated with PC and FVIII when taken alone or in combination (i.e. the ratio FVIII/PC). These conclusions were also supported by results of an additional thrombin generation procedure based on the presence/absence of Protac, a non-physiologic PC activator (63). Finally, patients with ETP-TM ratios higher than the median control value had odds ratios higher than 1.0 for metabolic syndrome, fibrosis, steatosis, lobular-inflammation, and NAS-score, all considered typical features of NAFLD (63). The above results/conclusions are in line with the notion that NAFLD patients have increased cardiovascular risk as shown by clinical and epidemiological observations (63). However, it is not yet known whether the procoagulant imbalance detected by the ETP-TM ratio is causally related with the increased risk of cardiovascular disease in these patients. The ETP-TM ratio could be the candidate parameter for clinical trials assessing the causal relationship. The above results have recently been challenged by Potze et al. (64), who concluded that coagulation in patients with NAFLD is substantially normal. These authors found an increased ETP-TM ratio in patients with metabolic cirrhosis (the most severe form of NAFLD) that however did not reach statistical significance. The differences observed in the two studies can probably be explained by patients/controls selection, by the different sample size and finally by the design of the thrombin generation procedures used in the two studies (see [65] for more details). Another condition where the ETP-TM ratio proved effective in detecting a procoagulant imbalance in platelet-rich plasma due to low PS, is the presence of myeloproliferative neoplasms for which the high ETP-TM ratio (66) (see Figure 3) substantially mirrored the results obtained by Marchetti et al. (55), who used as anticoagulation trigger prior to the measurement of thrombin generation APC instead of TM (see above). Whelihan et al. (56) showed that low PS, due to increased red blood cell exposure of phosphatidylserine, increases the resistance to TM detected as ETP-TM ratio in patients with sickle cell disease. The presence of lupus anticoagulant (LA) represents a prothrombotic condition for which heightened thrombin generation has been surmised, but not conclusively shown when the procedure has been performed by measuring the ETP without triggers. In contrast, Arachchillage et al. (67) have recently shown that patients who are positive for LA display a resistance to the anticoagulant action of TM or Protac that can be detected by the ETP ratio with/without these triggers. Overall, the above observations demonstrate that the ETP-TM ratio is a reliable laboratory tool to investigate procoagulant imbalance.

Treatment with vitamin K antagonists (VKA) is another interesting area where the PC-FVIII axis is affected. PC and PS are both vitamin K-dependent coagulation factors that are considerably reduced by VKA. FVIII in contrast has been reported to be increased during this treatment (68). It might therefore be argued that the PT-based international normalised ratio that is used to control anticoagulation with VKA is not entirely suitable to this task as plasma and reagents used to perform PT do not contain sufficient amounts of TM to activate PC (69). Whether monitoring VKA may benefit from thrombin generation measured in the presence versus the absence of TM is an attractive hypothesis that remains to be investigated.
Conclusive remarks

According to Hemker et al. (70) the more thrombin is generated the higher is the risk of thromboembolism. Each and every individual possesses his/her own ETP (i.e. the ability to generate thrombin) depending on the strength of the pro- and anticoagulant drivers operating in plasma. This ability depends on the clinical condition in which the balance between the two drivers are variably affected. One of the major determinants of the balance between pro- and anticoagulants is the FVIII-PC axis. The effect of this axis on the procoagulant imbalance can be conveniently explored by the thrombin generation procedures with results expressed as ETP ratio with/without TM. Furthermore, evidence starts to accumulate that, owing to the complex feedback mechanisms that operate through thrombin signaling, it is possible that many other blood components such as microparticles, NETs, pro-inflammatory cytokines and others might be able to indirectly perturb the FVIII-PC axis even when the plasma levels of these two parameters are within normal limits. It can be postulated that dual ETP measurements performed in the presence and absence of TM with results expressed as their ratio may be the candidate procedure to detect subtle procoagulant imbalance in many clinical conditions characterised by an increased risk of thromboembolism. It cannot be excluded that in some circumstances the evaluation of the ETP in combination with the ETP-TM ratio may give more information on the thrombotic risk than either procedure alone. Clinical trials should be undertaken to see whether the in vitro procoagulant imbalance detected by the ETP-TM ratio is associated with the occurrence of clinical events even outside the use of OC.

Conflicts of interest

None declared.

References

Tripodi: ETP-TM ratio as laboratory tool to detect procoagulant imbalance