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Thrombin generation

Development and validation of a thrombin generation-based APC resistance assay

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Resumen

Introducción: Trombosis venosa es una enfermedad multifactorial causada por condiciones genéticas y/o adquiridas. La resistencia del plasma a la acción anticoagulante en la Proteína C Activada (resistencia a APC), es el principal factor de riesgo. La resistencia a APC puede ser determinada midiendo la generación de trombina (TG) en plasma; siendo desencadenado con factor tisular, fosfolípidos y $CaCl_2$, en la ausencia y presencia de APC.
Propósito: Desarrollar un test de resistencia a APC basado en TG usando reactivos comerciales; y validando este en sujetos con trombofilia heredada y adquirida.
Materiales y Métodos: Reactivos (trigger, fosfolípidos y APC) fueron evaluados para estabilidad y reproducibilidad. Titulaciones fueron presentadas para identificar las concentraciones óptimas que serán usadas en el test. Para validar el test, nosotros medimos TG con y sin APC en 61 sujetos con condiciones trombofílicas y en 43 controles. Parámetros de TG fueron comparados entre grupos con el Student's t-test.
Resultados: Las mejores condiciones del test fueron: 5 pM TF, 25 μM lípidos, 16 mM $CaCl_2$ y 2.8 nM APC. Sujetos con condiciones trombofilicas como FV Leiden, deficiencia de proteína S, uso de anticonceptivos orales y embarazo (solo o en combinación) tuvieron una alta resistencia de APC en relación a los controles. Sin embargo, los portadores de la mutación de protrombina no difirieron de los controles.
Conclusiones: Nosotros hemos desarrollado un test de resistencia de APC basado en TG, el cual identifica muchos factores de riesgo para trombosis venosa.

Abstract

Background: Venous thrombosis is a multifactorial disease caused by acquired and/or genetic conditions. Resistance of plasma to the anticoagulant action of activated protein C (APC resistance) is the main risk factor. APC resistance can be determined by measuring thrombin generation (TG) in plasma triggered with tissue factor, phospholipids and $CaCl_2$ in the absence and presence of APC.
Purpose: To develop a TG-based APC resistance assay using commercial reagents and to validate it in subjects with inherited and acquired thrombophilia.
Materials and Methods: Reagents (trigger, phospholipids and APC) were tested for stability and reproducibility. Titrations were performed to identify the optimal concentrations to be used in the assay. To validate the assay, we measured TG with and without APC in 61 subjects with thrombophilic conditions and in 43 controls. TG parameters were compared between groups with the Student's t-test.
Results: The best assay conditions were: 5 pM TF, 25 μM lipids, 16 mM $CaCl_2$ and 2.8 nM APC. Subjects with thrombophilic conditions like FV Leiden, protein S deficiency, Oral Contraceptives use and pregnancy (alone or in combination) had a higher APC resistance than controls. However, prothrombin mutation carriers did not differ from controls.
Conclusions: We have developed a TG-based APC resistance assay which identifies several risk factors for venous thrombosis.

Introduction

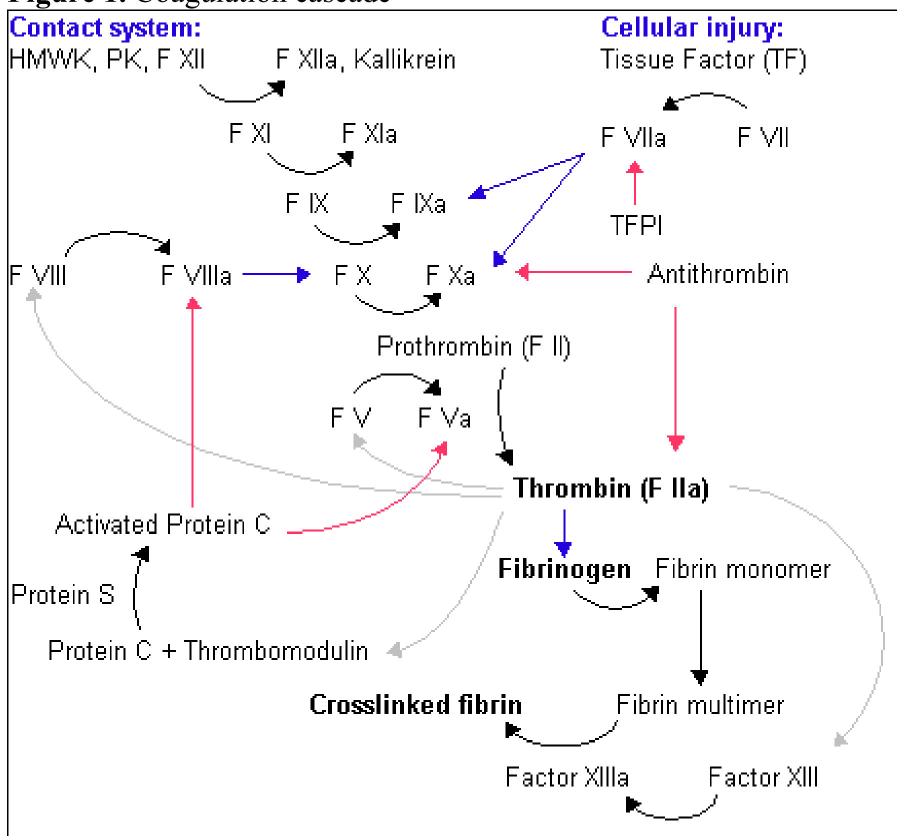
Hemostasis

The hemostatic system counteracts wound bleeding by coordinating two steps, the activation of blood platelets (primary hemostasis) and the formation of an insoluble fibrin clot by the coagulation system (secondary hemostasis) (1).

Within seconds after an injury, platelets bind to collagen in the exposed vessel wall to form a haemostatic plug (2). During this process, platelets become activated, release the contents of their granules and expose negatively charged phospholipids necessary for coagulation reactions.

Secondary hemostasis involves a cascade of coagulation factors, eventually leading to thrombin generation and the formation of a fibrin clot (Figure 1). Following damage to the vessel wall, the sub-endothelial tissues containing collagen and tissue factor (TF) become exposed to the circulating blood. TF starts the coagulation cascade by binding to FVII/FVIIa. The TF-FVIIa complex activates FIX and FX. FXa binds to FVa (FV was activated by thrombin) in the presence of calcium ions and phospholipids to form the prothrombinase complex (FXa-Va), which activates protrombin to thrombin, the key enzyme of hemostasis. Similarly, FIXa binds to FVIIIa in presence of calcium ions and phospholipids to form the tenase complex, which activates FX, and thereby reinforces prothrombinase activity.

Figure 1. Coagulation cascade



The role of tissue factor (TF) in initiating clotting; the relations between pathways; and the role of thrombin in supporting the cascade by feedback activation of coagulation factors.
HMWK: high-molecular-weight kininogen; PK: prekallikrein; TFPI: tissue factor pathway inhibitor
Joe D. http://en.wikipedia.org/wiki/Coagulation. (8)

Although the TF-FVIIa complex is rapidly inactivated by tissue factor pathway inhibitor (TFPI), the small traces of thrombin formed in the initial phase of coagulation support the generation of more thrombin by positive feedback reactions (activation of FV, FVIII and FXI). Finally, thrombin converts fibrinogen to fibrin, and the fibrin clot is stabilized by FXIIIa.

When the fibrin clot is not needed anymore, it is broken down by plasmin in a process called fibrinolysis (3).

Thrombin generation and activity are physiologically down-regulated by several endogenous inhibitors. Antithrombin and heparin cofactor II inhibit thrombin directly by occupying the

active site, and their action is enhanced by heparin. While antithrombin also inhibits FVIIa, FIXa, FXa and FXIa, heparin cofactor II inhibits only thrombin. TFPI binds to FXa to inhibit the activity of the TF-FVIIa complex. Finally, activated protein C (APC) and its cofactor protein S shut down the prothrombinase and tenase complexes by proteolytically inactivating the essential cofactors FVa and FVIIIa (1). Protein C is activated by thrombin bound to thrombomodulin at the surface of endothelial cells.

Thrombosis

Venous thrombosis is a multifactorial disease which affects about 1 in 1000 people in developed countries (4). It is caused by combinations of genetic and/or acquired risk factors. Table 1 gives a list of inherited and acquired conditions that have been associated with an increased risk of venous thrombosis (thrombophilic conditions). Three elements contribute to the pathogenesis of venous thrombosis: hypercoaulability, venous stasis and vascular damage (Virchow's triad). Although most known thrombosis risk factors cause hypercoagulability, obesity and immobilization are associated with venous stasis.

Table 1. Risk factors of venous thrombosis

Inherited	**Acquired**
Antithrombin deficiency	Trauma
Protein C deficiency	Immobilization
Protein S deficiency	Surgery
APC resistance Factor V Leiden	Oral contraceptives - pregnancy - hormone replacement therapy
Prothrombin G20210A	Cancer
	Antiphospholipid syndrome

By Juan Pablo Escobar

Theoretical Framework

In this project, we are going to focus on APC resistance, FV Leiden, prothrombin G20210A, protein S deficiency, pregnancy and oral contraceptives as risk factors for venous thrombosis. APC resistance is defined as poor sensitivity of plasma to the anticoagulant action of APC. It is the most common risk factor for venous thrombosis and it occurs in 5% of individuals in the general population and in 25-40% of patients with family history of thrombosis. APC resistance is often, but not always, associated with a FV gene mutation (Factor V Leiden) which replaces arginine at position 506 with glutamine. As a consequence FVa Leiden is resistant to cleavage by APC, while it has the same procoagulant activity as normal FVa (5).

Prothrombin (factor II) is the precursor of thrombin. The G20210A mutation in the 3'-untranslated region of the prothrombin (FII) gene results in a ~30% increase in the plasma level of prothrombin. This is associated with a hypercoagulable state and a 2-3-fold increased risk of venous thrombosis (4).

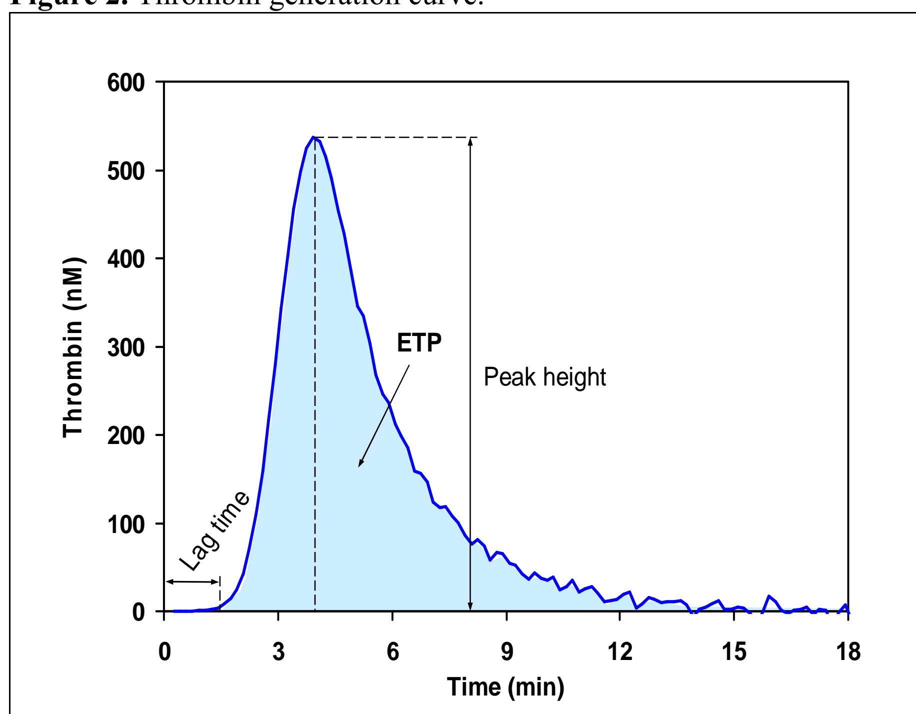
Protein S is a cofactor of APC in the inactivation of FVa and FVIIIa. Protein S deficiency is a rare coagulation disorder inherited as an autosomal dominant trait. Protein S deficiency is associated with a hypercoagulable state and a 5-10-fold increased risk of venous thrombosis (4).

Oral contraceptive (OC) use is associated with acquired APC resistance and a 2-6-fold increased risk of venous thrombosis (5). APC resistance and thrombosis risk are higher in users of third-generation contraceptives (containing desogestrel or gestodene) than in second-generation contraceptives (containing levonorgestrel). OC use is the most common cause of thrombosis in young women. The risk of thrombosis increases within four months of the initiation of therapy and decreases to previous levels within three months of cessation (7).

Thrombin generation assay

The Calibrated Automated Thrombogram (CAT) is a recently developed *in vitro* technique designed to follow the generation and subsequent inhibition of thrombin in a plasma sample activated with TF, phospholipids and $CaCl_2$. The thrombin generation curve (Figure 2) is characterized by a lag phase, which corresponds to the clotting time, followed by a thrombin peak, which reflects the propagation and subsequent termination of the coagulation cascade. The area under the curve, also called the endogenous thrombin potential (ETP), is a measure of the total amount of active thrombin formed in plasma (8).

Figure 2. Thrombin generation curve.



A typical thrombin generation curve obtained at high TF is shown. The output parameters relevant to the present study are indicated. By Elisabetta Castoldi.

APC resistance and thrombin generation

Reduced sensitivity of plasma to the anticoagulant action of activated protein C (APC resistance) is a common risk factor for venous thrombosis. Recently, we have developed a thrombin generation-based APC resistance assay using home-made reagents. In this assay, thrombin generation is measured in the absence and presence of APC. The APC concentration is chosen such as to inhibit thrombin generation in normal plasma by ~90%. The outcome of the assay is expressed as normalized APC-sensitivity ratio (nAPCsr), which is the ratio between the ETPs measured in the presence and absence of APC (%rest), normalized to the %rest of normal plasma (NP):

$$nAPCsr = \frac{(ETP_{+APC} / ETP_{-APC})_{sample}}{(ETP_{+APC} / ETP_{-APC})_{NP}} = \frac{\%rest_{sample}}{\%rest_{NP}}$$

This assay detects most forms of inherited and acquired thrombophilia (Factor V Leiden, Protein S deficiency, high prothrombin levels, APC resistance induced by oral contraceptive use and pregnancy) and correlates with thrombosis risk. To make it possible for other laboratories to perform this assay, we want to develop a similar assay with commercially available reagents.

Aim of the project

General Objective:

1. To develop a thrombin generation-based APC resistance assay and to test it in individuals with inherited and acquired thrombophilia vs. normal individuals.

2. Specific Objectives:

1. To develop a thrombin generation-based APC resistance assay using commercially available reagents.

2. To test the stability of the reagents over time and the reproducibility of the measurement.

3. To validate the assay in subjects with thrombophilic conditions and in normal individuals.

Materials and methods

Materials

To develop a thrombin generation-based APC resistance assay, the following commercial reagents were employed:

- PPP Reagent 5 pM from Thrombinoscope BV (Maastricht, The Netherlands). This lyophilized reagent contains a mixture of tissue factor and lipids and can be used as a trigger for thrombin generation reactions. When the powder is dissolved in 0.5 ml water, and 10 µl of the solution are used in a final volume of 120 µl, final concentrations of 5 pM TF and 4 µM lipids are obtained.
- 0.5 mM Phospholipids TGT from Rossix (Mölndal, Sweden). This phospholipid suspension was used to supplement extra phospholipids for better APC action.
- Plasma-purified human APC from Innovative Research (Southfield MI, USA).

- I-1140 (Z-Gly-Gly-Arg-AMC) from BACHEM (Bubendorf, Switzerland). This low-affinity fluorogenic substrate for thrombin was used to continuously monitor thrombin activity.

Methods

Thrombin generation assay

Thrombin generation was measured with the CAT method, which employs a low-affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) to continuously follow thrombin activity in clotting plasma (8). The measurements were performed on 80 μL plasma in a total volume of 120 μL. Specifically, plasma (80 μl) was pipetted in the wells of a 96-well plate, the TF/phospholipid trigger (10 μl) was added and the plate was pre-warmed at 37 °C for 5 min. Just before starting, buffer and/or lipids and/or APC (10 μl) were added and the reaction was started with 20 μl of a solution containing $CaCl_2$ and fluorogenic substrate. Final concentrations were: 5 pM TF, 16 mM added $CaCl_2$ and 300 μM substrate. The final concentrations of phospholipids and APC varied from experiment to experiment. Each thrombin generation measurement was calibrated against the fluorescence curve obtained in the same plasma with a fixed amount of thrombin activity. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems, Helsinki, Finland) and thrombin generation curves were calculated using the Thrombinoscope software. Three parameters were derived from the thrombin generation curves: lag time (min), ETP (nM.min), and peak height (nM) (6).

1) Development of the APC resistance assay

During one and a half months we worked at the development of the assay to find the best possible conditions in the laboratory of CARIM (Cardiovascular Research Institute of Maastricht). We tested the stability of the trigger over time, the APC concentration needed to inhibit thrombin generation by 90% (APC titration), the reproducibility of the thrombin generation curves in the absence and presence of APC, and the effect of extra lipids on the activity of APC (lipid titration). All this experiments were performed in normal pooled plasma.

2) Validation of the APC resistance assay

To validate the APC resistance assay, we measured thrombin generation in the absence and presence of APC in plasma samples from subjects with genetic and acquired thrombophilic conditions. Before each measurement we did an APC titration to verify the APC concentration needed to reduce thrombin generation to 10%.

Study Population. The study population consisted of 104 individuals. There were 43 controls and 61 subjects with different thrombophilic conditions: Factor V Leiden (n = 14), prothrombin 20210 G/A mutation (n = 19), Factor V Leiden + prothrombin 20210 G/A (n = 10), protein S deficiency (n = 8), oral contraceptive use (n = 4), prothrombin 20210 G/A + oral contraceptive use (n = 3), and pregnancy (n = 3).

Blood collection and plasma preparation. Venous blood was drawn by venipuncture in 3.8% sodium citrate (w/v) and centrifuged at 2000 x g for 15 minutes. Platelet – poor plasma was aliquoted, snap – frozen and stored at –80° C until use;

Thrombin generation. Coagulation in plasma was triggered with 5 pM TF and 25 μM lipids (4 μM from the commercial trigger and 21 μM added exogenously) in the absence and

presence of 2.8 nM APC. Thrombin generation curves were calculated with the Thrombinoscope software. From the thrombin generation curves we derived the following parameters: lag time, ETP and peak. Moreover, we calculated the normalized APC-sensitivity ratio (nAPCsr).

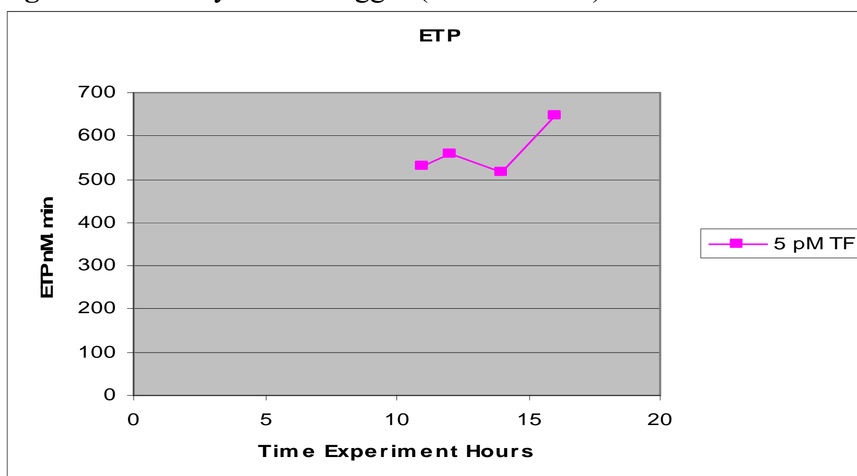
Statistical analysis. Data are expressed as mean ± standard deviation. Thrombin generation parameters were compared between groups using the Student's t-test.

Results

Stability of the trigger over time

In the development of the thrombin generation – based APC resistance assay, initially we tested the stability of the Thrombinoscope trigger. To do so we measured thrombin generation at different times during the day: 10 am, 12 pm, 2 pm and 4 pm (Figure 3). The obtained ETPs are shown in Figure 3. Although there is some scattering, the ETP does not seem to decrease in time. The trigger was stable for 6 hours.

Figure 3 Stability of the Trigger (ETP vs Time)



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APC titration

In order to develop the APC resistance assay, the next step was the APC titration (Figure 4 and 5). The purpose was to find the APC concentration that reduces the thrombin generation to 10%. In Figure 4 we can see the inhibition power of APC; thrombin generation is highest in the absence of APC (dark blue line – No APC), and progressively decreases as APC concentration increases (other lines). In Figure 5 we can see that the ETP decreases with increasing APC. The concentration of APC that reduces thrombin generation to 10% is approximately 5-6 nM.

Figure 4 APC titration (Thrombin vs Time)

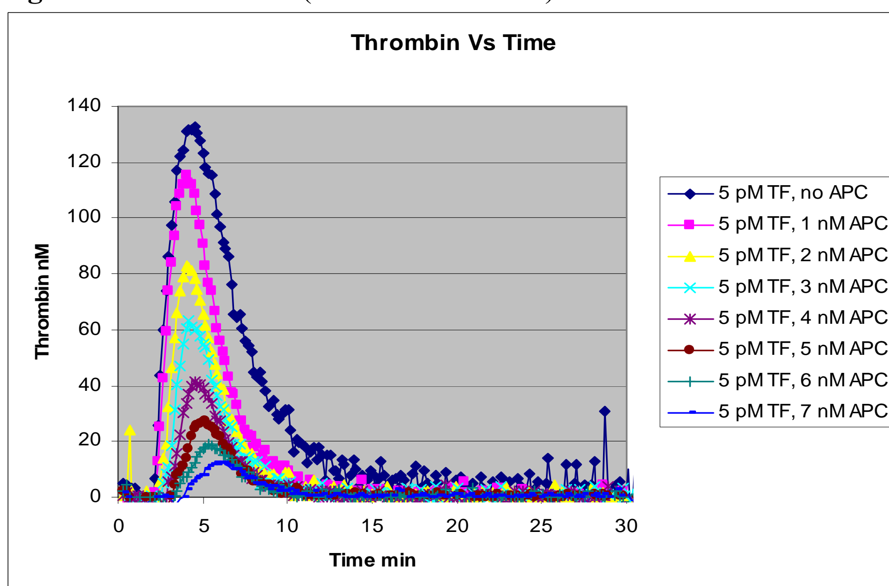

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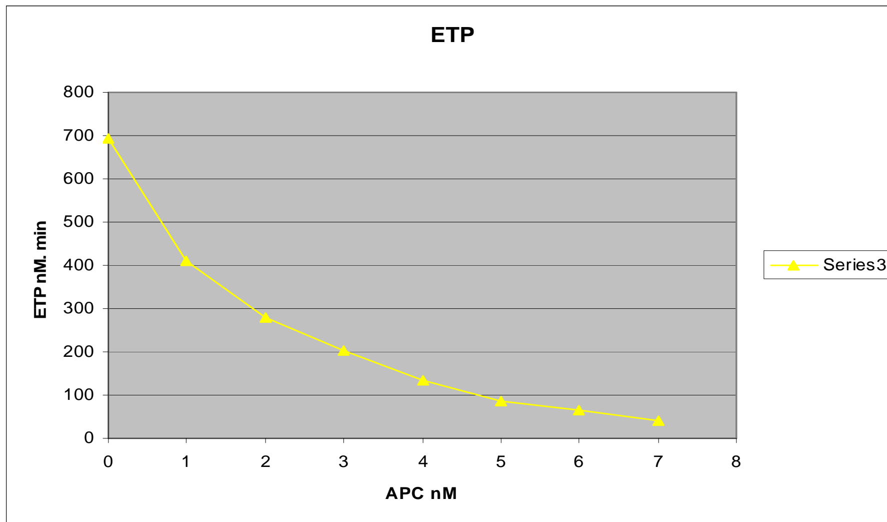
Figure 5 APC titration (ETP vs APC)



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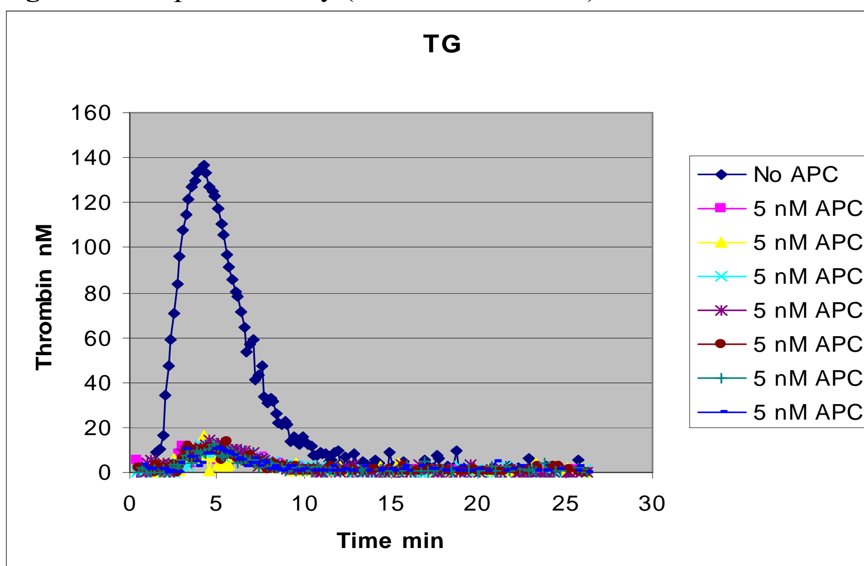
Reproducibility

Figure 6 shows an essential step in the development of the thrombin generation – based APC resistance assay, the reproducibility of the measurement in the presence of APC (5 nM). The measurement was indeed reproducible.

Figure 6 Reproducibility (Thrombin vs Time)



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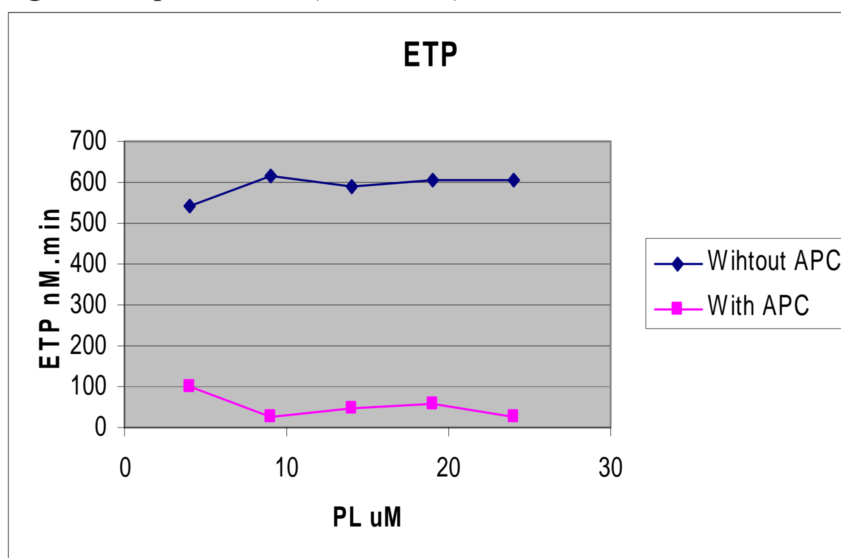
Lipid titration

Since APC action is lipid-dependent, we also tested the effect of increasing the lipid concentration on thrombin generation with and without APC. As shown in Figure 7, in the absence of APC (blue line) the ETP increases when the phospholipids (PL) concentration increases, while in the presence of APC (pink line) the ETP decreases when the phospholipid concentration increases. This means that APC works better at higher lipid concentrations. At 25 µM lipids, less APC (~3 nM) is enough to reduce the ETP to 10% of its value without APC.

Figure 7 Lipid titration (ETP vs PL)



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In summary, the best conditions for the APC resistance assay are: 5 pM TF, 25 µM lipids and ~3 nM APC. These conditions were employed in the subsequent validation of the assay.

Assay validation

To validate the newly developed APC resistance assay, thrombin generation in the absence and presence of APC was measured in 104 individual plasmas: 61 from individuals with thrombophilic conditions (FV Leiden, the prothrombin 20210 G/A mutation, protein S deficiency, oral contraceptive use and pregnancy) and 43 from healthy controls. Relevant parameters (lag time, ETP and peak) were derived from the thrombin generation curves and the nAPCsr was calculated for each sample. Results are presented in Table 2 (mean ± standard deviation per group). Unfortunately, 24 samples (mainly controls) had to be excluded from the statistical analysis, because their thrombin generation curves in the presence of APC were too low and could not be calculated by the Thrombinoscope software.

Table 2 Thrombin generation parameters per group

		-APC			+APC			
Group		**Lag time**	**ETP**	**Peak**	**Lag time**	**ETP**	**Peak**	**nAPCsr**
Controls	*average*	1.56	800.5	256.4	2.37	154.8	50.6	1.9
n= 29	*SD*	0.23	105.1	48.1	0.53	103.8	35.4	1.2
FVL	*average*	1.86	709.3	220.3	2.72	218.6	67.5	**3.3***
n= 12	*SD*	0.50	329.5	95.9	1.53	213.1	63.4	2.1
PT	*average*	1.64	1185.6*	321.0*	2.19	224.8	74.1	1.8
n= 14	*SD*	0.31	326.7	67.9	0.34	238.0	79.3	1.7
FVL+PT	*average*	1.58	1150.3*	331.1*	1.96*	434.4*	126.5*	**4.0***
n= 8	*SD*	0.24	330.1	70.8	0.21	311.8	71.2	2.0
PS-def.	*average*	1.58	619.3*	201.7*	1.92	422.4*	139.0*	**5.0***
n= 8	*SD*	0.55	190.1	66.5	0.63	155.7	52.0	1.50
OC	*average*	1.56	895.7	303.4	1.89*	507.7*	157.5*	**5.6***
n= 3	*SD*	0.20	215.8	63.3	0.19	118.5	35.3	0.75
PT/OC	*average*	1.89	1106.7	339.3	2.55	693.3	189.3*	**6.2***
n= 3	*SD*	0.38	466.9	100.0	0.39	272.5	42.5	0.33

Pregnant	*average*	2.00	1296.3	392.5*	2.67	837.3*	247.9*	**6.5***
n= 3	*SD*	0.33	304.4	36.1	0.67	68.3	40.2	1.24

SD: standard deviation; * significantly different from control ($p<0.05$).
FVL: Factor V Leiden, PT: prothrombin 20210 G/A, PS.def.: Protein S deficiency.
OC: oral contraceptive use. By Juan Pablo Escobar

As shown in Table 2, individuals with thrombophilic conditions like FV Leiden, the prothrombin 20210 G/A mutation + OC use, protein S deficiency, oral contraceptive use and pregnancy have a higher nAPCsr than controls. The highest nAPCsr (6.5) was found in pregnant women.

Differently, the test did not detect the hypercoagulable state associated with the prothrombin G20210A mutation, because the nAPCsr was not different between prothrombin mutation carriers and controls. This might be due to the fact that 1) the prothrombin mutation confers only a mild prothrombotic state, and 2) the nAPCsr of controls has probably been over-estimated, because all controls with a low thrombin generation in the presence of APC had to be excluded from the analysis because their curves could not be calculated by the software. This problem might be solved by increasing the TF concentration in the assay. However, in combination with other thrombophilic conditions (FV Leiden or oral contraceptive use), the prothrombin mutation contributed to a higher nAPCsr (for example, nAPCsr = 3.3 in carriers of FV Leiden alone and nAPCsr = 4.0 in compound carriers of FV Leiden and the prothrombin mutation).

Conclusions

The thrombin generation assay is a global coagulation test appropriate for discriminating patients with thrombotic tendencies.

The commercial reagents used in this study proved suitable to develop a thrombin generation-based APC resistance assay. In particular, the trigger was stable for several hours and APC was able to reduce thrombin generation to 10% in normal plasma, especially when extra phospholipids were added to the reaction mixture.

The thrombin generation-based APC resistance assay that we have developed detects thrombophilic conditions such as FV Leiden,, protein S deficiency, oral contraceptive use and pregnancy. Also, the test reflects the interaction between multiple defects like the combination of the prothrombin mutation with FV Leiden or oral contraceptive use. However, the test did not recognise the hypercoagulable state associated with the prothrombin G20210A mutation alone.

The assay might be improved by increasing the tissue factor concentration used to trigger thrombin generation.

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