

ORIGINAL ARTICLE

Coagulation factors and the protein C system as determinants of thrombin generation in a normal population

A. W. J. H. DIEELIS,* E. CASTOLDI,† H. M. H. SPRONK,* R. VAN OERLE,‡ K. HAMULYÁK,‡
H. TEN CATE* and J. ROSING†

*Department of Internal Medicine, Laboratory for Clinical Thrombosis and Haemostasis, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht; †Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht; and ‡Department of Internal Medicine, Division of Haematology, University Hospital Maastricht, Maastricht, the Netherlands

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Summary. *Background:* Thrombin generation is a powerful tool to probe overall plasma coagulability. *Objective:* To determine which plasma factors influence the various parameters of the thrombin generation curve, for example lag time, peak height and endogenous thrombin potential (ETP), under different experimental conditions. *Patients and methods:* Plasma levels of coagulation factors and inhibitors, as well as thrombin generation at 1 pM tissue factor (TF) ± thrombomodulin (TM) and at 13.6 pM TF ± activated protein C (APC), were determined in plasma from 140 healthy individuals. Data were analysed by multiple regression models. *Results:* Thrombin generation increased with age and was higher in females than in males. Under all conditions, the lag time was mainly dependent on the levels of free tissue factor pathway inhibitor (TFPI), free protein S (PS), factor VII (FVII), FIX and fibrinogen. The major determinants of thrombin generation (ETP and peak height) at 1 pM TF were fibrinogen, FXII (despite inhibition of contact activation), free TFPI and antithrombin (AT), both in the absence and in the presence of TM. Thrombin generation in the presence of TM was also dependent on protein C levels. At 13.6 pM TF, thrombin generation was determined by prothrombin, AT, fibrinogen, free TFPI and FV levels in the absence of APC, and by free TFPI, free PS and FX levels in the presence of APC. *Conclusions:* The lag time, ETP and peak height of thrombin generation depend on the levels of multiple coagulation factors and inhibitors. The specific assay determinants vary with the experimental conditions.

Correspondence: Arne W. J. H. Dielis, Laboratory for Clinical Thrombosis and Haemostasis, Department of Internal Medicine, Cardiovascular Research Institute Maastricht, Maastricht University, P.O. Box 616, 6200 MD Maastricht, the Netherlands.
Tel: +31 433884263; fax: +31 433884159; e-mail: a.dielis@bioch.maastricht.nl

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Introduction

Thrombin, the central enzyme in blood coagulation [1], is the end product of the coagulation cascade which is initiated after the exposure of tissue factor (TF) on the sub-endothelium. Binding of circulating activated factor VIIa (FVIIa) to TF leads to the sequential activation of coagulation factors, eventually resulting in the conversion of prothrombin to thrombin. Once formed, thrombin converts fibrinogen into fibrin, activates platelets and stimulates the generation of more thrombin by enhancing upstream reactions by positive feedback loops (e.g. activation of coagulation FV, FVIII and FXI). After binding to thrombomodulin (TM) at the endothelial cell surface, thrombin also initiates the anticoagulant pathway by activating protein C. Activated protein C (APC) and its cofactor protein S (PS) inhibit thrombin formation by proteolytically inactivating FVa and FVIIIa [2]. Thrombin generation is further down-regulated by the tissue factor pathway inhibitor (TFPI)/PS system, which inhibits FXa and the TF–FVIIa complex [3,4], and by antithrombin (AT) which irreversibly blocks FXa and thrombin [5].

Given the central role of thrombin in blood clotting, the tendency of a plasma sample to generate thrombin might contain useful information about thrombotic or haemorrhagic risk. Recently, an automated method (Calibrated Automated Thrombogram, CAT) has become available to follow *in vitro* thrombin generation in plasma after activation of coagulation with TF, phospholipids and CaCl₂ [6,7]. The thrombin generation curve is characterized by a lag phase (initiation), followed by a thrombin burst (propagation) which is eventually completely inhibited by plasma protease inhibitors (termination). The area under the curve, known as the endogenous thrombin potential (ETP), represents the total amount of active thrombin formed.

Several studies have shown that the ETP is a good overall indicator of prothrombotic [7–12] and haemorrhagic tendency

[7,13–16]. However, despite sporadic attempts to elucidate the effects of single coagulation factors on thrombin generation [17–19], it remains unclear how the levels of coagulation factors and inhibitors influence the various parameters of the thrombin generation curve (lag time, ETP and peak height). To address this issue, we have used multiple regression analysis to dissect the effect of individual coagulation factors and inhibitors on thrombin generation parameters measured under different experimental conditions in a population of healthy individuals.

Materials and methods

Study population

Blood samples were collected from 140 healthy volunteers (67 males, 47.9%, and 73 females, 52.1%). Mean age was 54 years (range 22–90 years). Women using oral contraceptives and individuals on anticoagulation therapy were not included. Six out of the 140 individuals (4.3%, four males and two females) were found to carry the factor V Leiden mutation [20] and were subsequently excluded from the analysis.

Blood collection and plasma preparation

Venous blood was collected in 3.2% citrate (w/v). Platelet-poor plasma was prepared by two centrifugation steps: the first at 2000 *g* for 15 min and the second at 11 000 *g* for 5 min. Plasma aliquots were snap-frozen, stored at –80 °C and thawed at 37 °C before analysis.

Thrombin generation measurements

Thrombin generation in platelet-poor plasma was measured using the CAT method [6], which employs a low-affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) to continuously monitor thrombin activity in clotting plasma. According to the manufacturer's instructions, measurements were conducted on 80 µL full plasma in a total volume of 120 µL and in the presence of 416 µM fluorogenic substrate and 16 mM added CaCl₂. In order to correct for inner-filter effects and substrate consumption, each thrombin generation measurement was calibrated against the fluorescence curve obtained in the same plasma with a fixed amount of thrombin-α₂-macroglobulin complex (thrombin calibrator; Thrombinoscope BV, Maastricht, the Netherlands). Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 filter set and thrombin generation curves were calculated using the Thrombinoscope software (Thrombinoscope BV). Three parameters were derived from the thrombin generation curves: lag time (min), ETP (nm.min) and peak height (nm).

Thrombin generation was determined under the following experimental conditions (final plasma concentrations): 1 pM TF and 4 µM phospholipids in the absence and presence of 1.5 nM recombinant soluble TM (Asahi Kasei Pharma Corporation, Tagata, Japan); and 13.6 pM TF and 20 µM phos-

pholipids in the absence and presence of 12 nM plasma-derived human APC (Kordia Life Sciences, Leiden, the Netherlands). The 1 pM TF trigger was a commercial product (PPP Reagent Low; Thrombinoscope BV, Maastricht, the Netherlands), while the 13.6 pM trigger was prepared in-house by mixing recombinant TF (Hemoliance RecombiPlastin; Instrumentation Laboratory, Breda, the Netherlands) and a phospholipid emulsion containing phosphatidylserine, phosphatidylcholine and sphingomyelin (TGT-lipids; Rossix, Mölndal, Sweden). The concentrations of TM and APC were chosen such as to inhibit thrombin generation in normal plasma by 70% and 90%, respectively. To prevent contact activation, the measurements at low TF (1 pM) were performed in the presence of 33 µg mL⁻¹ corn trypsin inhibitor (CTI; Haematologic Technologies Inc, Essex Junction, VT, USA), a specific inhibitor of FXIIa. In control experiments, this CTI concentration was found to completely abolish thrombin generation in plasma spiked with phospholipids only (no TF) and to reduce the ETP and thrombin peak height by 30–40% in plasma triggered with 1 pM TF (data not shown).

A TM ratio was calculated by dividing the ETP obtained in the presence of TM by the ETP in the absence of TM, both measured at 1 pM TF. Similarly, a normalized APC sensitivity ratio (nAPCsr) was calculated by dividing the ETP obtained in the presence of APC by the ETP in the absence of APC, both measured at 13.6 pM TF, and by normalizing this ratio with the ETP ratio of normal plasma in the same run.

Measurement of plasma factor levels

Plasma fibrinogen levels were measured using the Clauss method. All other coagulation factor levels were determined by one-stage PT-based (prothrombin, FV, FVII, FX) or aPTT-based (FVIII, FIX, FXI, FXII) clotting assays. AT levels (FXa inhibitory activity) and protein C levels (amidolytic activity after activation with Protac) were measured chromogenically. All assays were performed on a Sysmex CA-7000 Automated Coagulation Analyzer with reagents obtained from Dade Behring (Liederbach, Germany). Total PS was measured using a home-made ELISA, as described [21]. Free PS and free TFPI antigen levels were determined with the respective Asserachrom ELISA kits (Diagnostica Stago, Asnières sur Seine, France), and TFPI activity with the Actichrome TFPI activity assay (American Diagnostica, Stamford, CT, USA). Factor levels are expressed as U dL⁻¹, except for fibrinogen (expressed as mg mL⁻¹) and free TFPI antigen and activity (expressed as ng mL⁻¹). All plasma factor level measurements employed commercially available standards (Dade Behring) calibrated to WHO standards.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between parameters of the different thrombin generation measurements (1 pM and 13.6 pM TF with and without added TM or APC) were analyzed using paired

Student's *t*-test, whereas differences between genders were analyzed using Student's *t*-test. Correlations were expressed as Pearson's coefficients. A two-tailed probability value $P < 0.05$ was considered statistically significant.

To assess the determinants of thrombin generation parameters, multiple linear regression analysis was performed with lag time, ETP and peak height as dependent variables and plasma levels of coagulation factors and inhibitors as independent variables, as previously described [22]. For each model, the adjusted R^2 and the standardized regression coefficients (beta) of the independent variables were calculated. The beta indicates the change of the dependent variable, expressed in SD, when the independent variable increases 1 SD and all other variables in the model remain unchanged. The higher the beta, the larger the effect of the particular plasma factor on thrombin generation for variations of that factor level within its physiological range.

Statistics were computed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Thrombin generation parameters

After exclusion of FV Leiden carriers ($n = 6$), there were 134 individuals available for analysis. Thrombin generation parameters determined at 1 μM TF with and without added TM, and at 13.6 μM TF with and without added APC are presented in Table 1.

Thrombin generation curves measured at 1 μM TF showed longer lag times and lower ETPs and peaks than at 13.6 μM TF ($P < 0.001$). Addition of TM to the 1 μM TF assay resulted in a significant decrease of the ETP and peak height ($P < 0.001$), and in a small but significant reduction of the lag time

Table 1 Parameters of thrombin generation determined at 1 μM tissue factor (TF) in the absence and presence of thrombomodulin (TM), and at 13.6 μM TF in the absence and presence of activated protein C (APC)

	Lag time (min)	ETP (nm.min)	Peak height (nm)
All samples ($n = 134$)			
1 μM TF	5.84 \pm 1.65	995 \pm 289	116 \pm 50.8
1 μM TF + TM	5.57 \pm 1.85	299 \pm 143	59.6 \pm 34.2
13.6 μM TF	2.67 \pm 0.38	1490 \pm 224	335 \pm 40.7
13.6 μM TF + APC	4.34 \pm 0.88	152 \pm 107	31.8 \pm 27.1
Males ($n = 63$)			
1 μM TF	6.06 \pm 1.52	948 \pm 300	113 \pm 55.2
1 μM TF + TM	5.81 \pm 1.79	280 \pm 150	56.1 \pm 36.4
13.6 μM TF	2.72 \pm 0.39	1480 \pm 239	328 \pm 38.0
13.6 μM TF + APC	4.62 \pm 1.01	99.9 \pm 73.9	18.9 \pm 17.8
Females ($n = 71$)			
1 μM TF	5.66 \pm 1.74	1030 \pm 277	119 \pm 47.3
1 μM TF + TM	5.38 \pm 1.89	315 \pm 137	62.5 \pm 32.2
13.6 μM TF	2.62 \pm 0.36	1490 \pm 212	342 \pm 42.1*
13.6 μM TF + APC	4.11 \pm 0.69*	194 \pm 111*	42.2 \pm 28.9*

Data are expressed as mean \pm SD. * $P < 0.05$ compared with males.

($P < 0.001$). Addition of APC to the 13.6 μM TF measurement prolonged the lag time and decreased both the ETP and peak height ($P < 0.001$).

Lag times, ETPs and peak heights obtained at 1 μM TF with and without TM and at 13.6 μM TF without APC showed a positive correlation with age (R between 0.23 and 0.39, $P < 0.01$). When thrombin generation parameters were analyzed according to gender, females showed shorter lag times ($P = 0.002$) and higher ETPs and peak heights ($P < 0.001$) than males in the 13.6 μM TF assay with APC.

Correlations between lag time, ETP and peak height

At 1 μM TF, ETP and peak height were strongly correlated ($R = 0.92$, $P < 0.001$), while the lag time showed a weak inverse correlation with the ETP ($R = -0.24$, $P < 0.01$). The same trend was observed at 13.6 μM TF, with a strong correlation between ETP and peak height ($R = 0.80$, $P < 0.001$) and a weak correlation between lag time and ETP ($R = 0.22$, $P = 0.01$).

All thrombin generation parameters measured at 1 μM TF in the presence of TM showed a positive correlation with the corresponding parameters determined in the absence of TM (R between 0.88 and 0.95, $P < 0.001$). At 13.6 μM TF, only the lag time and peak height in the presence of APC correlated with the corresponding parameters without APC ($R = 0.55$, $P < 0.001$ and $R = 0.36$, $P < 0.001$, respectively), whereas the ETPs determined in the absence and presence of APC did not correlate with each other.

Effects of age and gender on the levels of coagulation factors and inhibitors

The levels of coagulation and inhibitors in the population under study ($n = 134$, FV Leiden carriers excluded) are presented in Table 2. Females had higher levels of fibrinogen, FVII, FX, FXI, AT and protein C, but lower levels of free PS, than males.

The levels of fibrinogen, FV, FVII, FVIII, FIX, FX, FXI, protein C, total PS and free TFPI showed a positive correlation with age (R between 0.19 and 0.50, $P < 0.05$), whereas AT levels tended to decrease with age ($R = -0.16$, $P = 0.066$).

Dependence of thrombin generation parameters on coagulation factors and inhibitors

Standardized regression coefficients of the candidate determinants and adjusted R squares of the linear regression models for the 1 μM TF and 13.6 μM TF measurements are reported in Tables 3 and 4, respectively.

At 1 μM TF (Table 3), the lag time of thrombin generation was mainly determined by the levels of free TFPI, free PS, FIX and fibrinogen, both in the absence and presence of TM. Differently, the ETP and peak height were mainly dependent on fibrinogen and FXII levels (positive determinants), and free TFPI and AT levels (negative determinants). In the presence of

Table 2 Coagulation factor and inhibitor levels

	All samples (<i>n</i> = 134) Mean ± SD	Males (<i>n</i> = 63) Mean ± SD	Females (<i>n</i> = 71) Mean ± SD
Fibrinogen (mg mL ⁻¹)	3.23 ± 0.54	3.10 ± 0.55	3.34 ± 0.49*
Prothrombin (U dL ⁻¹)	119 ± 18.2	117 ± 16.5	120.5 ± 19.5
FV (U dL ⁻¹)	108 ± 18.6	105 ± 19.3	110.7 ± 17.7
FVII (U dL ⁻¹)	122 ± 22.6	116 ± 21.3	126.8 ± 22.7*
FVIII (U dL ⁻¹)	96.7 ± 20.0	93.6 ± 19.2	99.6 ± 20.4
FIX (U dL ⁻¹)	101 ± 11.5	100 ± 11.0	102.2 ± 11.9
FX (U dL ⁻¹)	126 ± 15.5	123 ± 16.6	129.3 ± 13.9*
FXI (U dL ⁻¹)	94.1 ± 12.2	90.7 ± 13.2	96.9 ± 10.6*
FXII (U dL ⁻¹)	90.9 ± 20.2	89.3 ± 19.0	92.3 ± 21.3
AT (U dL ⁻¹)	118 ± 10.1	116 ± 8.81	119.5 ± 10.9*
Protein C (U dL ⁻¹)	109 ± 19.8	103 ± 13.5	114.4 ± 22.9*
Free PS (U dL ⁻¹)	95.9 ± 17.4	101 ± 17.6	91.0 ± 15.9*
Total PS (U dL ⁻¹)	114 ± 14.9	113 ± 14.9	114.4 ± 15.1
TFPI activity (ng mL ⁻¹)	49.4 ± 22.6	50.4 ± 21.3	48.5 ± 23.8
Free TFPI antigen (ng mL ⁻¹)	11.2 ± 3.05	11.5 ± 2.51	10.9 ± 3.45

TFPI, tissue factor pathway inhibitor; PS, protein S. **P* < 0.05 compared with males.

Table 3 Determinants of thrombin generation at 1 pM tissue factor (TF) [in the absence and presence of thrombomodulin (TM)] and of the TM ratio

Dep. variable Mean ± SD Determinant	SD	1 pM TF			1 pM TF + TM			1 pM TF ± TM
		Lag time 5.84 ± 1.65 Beta	ETP 995 ± 290 Beta	Peak height 116 ± 50.8 Beta	Lag time 5.57 ± 1.85 Beta	ETP 299 ± 143 Beta	Peak height 59.6 ± 34.2 Beta	TM ratio 0.29 ± 0.08 Beta
(Adjusted <i>R</i> ²)		(0.430)	(0.408)	(0.421)	(0.436)	(0.409)	(0.412)	(0.647)
Fibrinogen	0.54	0.197*	0.400*	0.368*	0.270*	0.258*	0.273*	0.068
Prothrombin	18.2	0.063	-0.001	-0.181	0.002	-0.166	-0.224*	-0.301*
FV	18.6	0.109	0.116	0.134	0.108	0.241*	0.243*	0.301*
FVII	22.6	-0.232*	0.129	0.122	-0.161	0.062	0.062	0.048
FVIII	20.0	0.102	-0.013	0.119	0.129	0.099	0.146	0.114
FIX	11.5	-0.409*	0.041	0.091	-0.414*	0.125	0.111	0.173
FX	15.5	0.090	0.125	0.097	0.083	0.099	0.093	0.118
FXI	12.2	0.165	-0.153	-0.127	0.088	-0.201	-0.176	-0.169
FXII	20.2	-0.002	0.302*	0.317*	0.100	0.317*	0.328*	0.230*
AT	10.1	-0.092	-0.311*	-0.240*	-0.091	-0.247*	-0.205*	-0.121
Protein C	19.8	0.115	-0.044	-0.066	0.093	-0.229*	-0.193*	-0.331*
Free PS	17.4	0.295*	-0.101	0.012	0.355*	-0.142	-0.073	-0.192
Total PS	14.9	-0.047	0.227	0.191	-0.074	0.335*	0.292	0.284
TFPI activity	22.6	-0.012	0.062	0.032	0.071	0.061	0.065	0.060
Free TFPI	3.05	0.536*	-0.399*	-0.280*	0.479*	-0.391*	-0.332*	-0.283*

TFPI, tissue factor pathway inhibitor; PS, protein S. Data are presented as standardized regression coefficients (beta). **P* < 0.05.

TM, protein C was an additional negative determinant of the ETP and peak height.

At 13.6 pM TF (Table 4), the lag time of thrombin generation was dependent on the levels of fibrinogen, FVII and free TFPI, both in the absence and presence of APC. However, the major predictors of the lag time measured in the presence of APC were FV and FIX levels. The ETP and peak height were mainly determined by fibrinogen, prothrombin, AT, free TFPI and FV levels in the absence of APC, and by free PS, free TFPI and FX levels in the presence of APC.

The TM ratio showed an excellent correlation with the ETPs obtained both in the absence (*R* = 0.61, *P* < 0.001) and presence (*R* = 0.89, *P* < 0.001) of TM. Differently, the

nAPCsr correlated only with the ETP measured in the presence of APC (*R* = 0.97, *P* < 0.001). The determinants of the TM ratio and nAPCsr largely overlapped with those of the underlying ETPs (Tables 3 and 4).

Discussion

Since the introduction of the CAT technique [6], which has made the measurement of thrombin generation in plasma straightforward and amenable to high throughput, thrombin generation assays have become increasingly popular in clinical laboratories [23]. Although several studies support the value of thrombin generation in detecting hyper- and hypocoagulable

Table 4 Determinants of thrombin generation at 13.6 μM tissue factor (TF) [in the absence and presence of activated protein C (APC)] and of the normalized APC sensitivity ratio (nAPCs_r)

Dep. variable Mean \pm SD Determinant	13.6 μM TF			13.6 μM TF + APC			13.6 μM TF \pm APC	
	SD	Lag time 2.67 \pm 0.38 Beta	ETP 1490 \pm 224 Beta	Peak height 335 \pm 40.7 Beta	Lag time 4.34 \pm 0.88 Beta	ETP 152 \pm 107 Beta	Peak height 31.8 \pm 27.1 Beta	nAPCs _r 1.06 \pm 0.75 Beta
(Adjusted R^2)		(0.427)	(0.471)	(0.334)	(0.235)	(0.439)	(0.402)	(0.713)
Fibrinogen	0.54	0.292*	0.280*	0.280*	0.250*	-0.031	-0.093	-0.072
Prothrombin	18.2	-0.077	0.420*	0.121	-0.171	-0.036	-0.066	-0.123
FV	18.6	0.115	-0.224*	-0.336*	0.344*	-0.168	-0.112	-0.120
FVII	22.6	-0.287*	0.048	0.074	-0.406*	0.104	0.003	0.041
FVIII	20.0	-0.031	-0.113	-0.060	0.093	-0.036	-0.016	0.002
FIX	11.5	-0.100	0.224	0.368*	-0.411*	0.243	0.186	0.178
FX	15.5	-0.010	0.100	0.199	-0.060	0.286*	0.296*	0.219*
FXI	12.2	0.209	-0.070	-0.144	0.062	-0.046	-0.040	-0.010
FXII	20.2	-0.004	0.074	0.087	0.186	0.011	0.038	0.031
AT	10.1	0.041	-0.455*	-0.221*	-0.011	0.073	0.105	0.165
Protein C	19.8	-0.033	0.156	0.111	0.014	-0.043	-0.029	-0.042
Free PS	17.4	0.331*	0.026	-0.262*	0.120	-0.470*	-0.536*	-0.475*
Total PS	14.9	0.142	0.101	0.098	0.096	-0.130	-0.108	-0.168
TFPI activity	22.6	0.017	-0.029	0.014	0.044	0.020	0.039	0.058
Free TFPI	3.05	0.254*	-0.216*	-0.297*	0.265*	-0.393*	-0.311*	-0.318*

TFPI, tissue factor pathway inhibitor; PS, protein S. Data are presented as standardized regression coefficients (beta). * $P < 0.05$.

states [7–16], the dependence of thrombin generation on the levels of individual coagulation factors and inhibitors has not been studied in detail. Therefore, we have used multiple regression analysis to dissect the contribution of individual plasma factors to thrombin generation parameters (lag time, ETP and peak height) measured under different experimental conditions in a population of healthy individuals. Thrombin generation was initiated with a commercial trigger (1 μM TF and 4 μM phospholipids) in the absence and presence of soluble TM, or with a home-made trigger (13.6 μM TF and 20 μM phospholipids) in the absence and presence of APC. Under each condition, the lag time reflects the initiation phase of thrombin generation, while the peak height and the ETP (which are highly correlated) probe the propagation and termination phases of coagulation.

In the absence of TM or APC, thrombin generation curves obtained at 13.6 μM TF were faster and higher than those obtained at 1 μM TF (Table 1), as expected from the relative trigger strengths. In accordance with a recent report [24], thrombin generation increased with age, which is largely explained by age-dependent changes in the levels of coagulation factors and inhibitors (data not shown). Moreover, plasma from females was more 'procoagulant' than that of males, as indicated by the tendency towards shorter lag times and higher ETPs and thrombin peaks, especially when thrombin generation was measured in the presence of TM or APC.

Irrespective of the trigger, the lag time of thrombin generation was determined by essentially the same plasma variables, for example (i) FVII, in line with the notion that the coagulation cascade is initiated by binding of TF to FVII(a); (ii) FIX (at 1 μM TF \pm TM and at 13.6 μM TF + APC), probably as a result of direct activation of FIX by the TF/

FVIIa complex at low procoagulant stimuli [25]; (iii) free TFPI and PS, reflecting inhibition of the TF/FVIIa complex by the TFPI/PS system [4]; and (iv) fibrinogen (Tables 3 and 4). The prolongation of the lag time at high fibrinogen levels is supported by the comparison between thrombin generation curves measured in full and defibrinated plasma [7] and is attributable to the ability of fibrin(ogen), particularly γ -fibrinogen, to bind to thrombin exosite II and to inhibit thrombin-mediated FVIII activation [26,27].

The most consistent determinants of the amount of thrombin formed, as quantified by ETP and peak height, were fibrinogen (positive determinant, because of the ability of fibrin to protect thrombin from inhibition by AT [28]), and AT and free TFPI levels (negative determinants). Despite the addition of CTI to prevent contact activation, FXII levels were also a major determinant of ETP and peak height in the measurements performed at low TF (1 μM), both in the absence and in the presence of TM, suggesting that some FXII might already have been activated prior to the thrombin generation measurement [29] and/or that it might have eluded inhibition by CTI. Although such minimal contact activation of coagulation may not be sufficient to exceed the threshold required for detectable thrombin generation in the absence of TF, it may well be amplified by feedback reactions from the extrinsic pathway, thereby contributing to thrombin generation at low TF concentrations.

Prothrombin level was a determinant of the ETP and peak height at 13.6 μM TF but not at 1 μM TF, possibly because only a fraction of the prothrombin present in plasma is activated at low TF. Surprisingly, FVIII level was never a determinant of the ETP, not even at 1 μM TF. In this respect, it should be noted that most individuals in the population under

study had normal FVIII levels and that the ETP is hardly affected by variations in FVIII levels within the normal range, as demonstrated by FVIII titrations in FVIII-deficient plasma (data not shown and ref. [16]). Presently, we do not have a good explanation for the negative effect of FV on thrombin generation in the absence of APC, but it cannot be excluded that FV acts a surrogate marker for a different factor that was not included in the multiple regression analysis.

When the protein C pathway was challenged by adding TM or APC to plasma, thrombin generation (ETP and peak height) was markedly reduced. The measurement at 1 μM TF in the presence of TM showed an excellent correlation with the measurement in the absence of TM. Accordingly, the two measurements were dependent on essentially the same plasma variables, with the notable exception of protein C which was a determinant only in the presence of TM (Table 3). Contrary to the expectations, free PS was not a determinant of the ETP or peak height in the measurement with TM. This may be as a result of the low phospholipid concentration (4 μM final) in the 1 μM trigger, which might be insufficient for full expression of the highly lipid-dependent APC-cofactor activity of PS [30]. The inverse relationship between prothrombin levels and thrombin generation in the presence of TM might result from increased protein C activation by the thrombin-TM complex at high prothrombin levels.

Thrombin generation measured at 13.6 μM TF in the presence of added APC showed no correlation (ETP) or only a weak correlation (peak) with the corresponding measurement in the absence of APC. The most important determinant of the measurement with APC was gender, females yielding shorter lag times and higher ETPs and peak heights than males (Table 1). Accordingly, free TFPI and PS levels, which are notably lower in females (Table 2), had the largest effects on ETP and peak height measured in the presence of APC (Tables 3 and 4). FX, which in an activated form binds to and protects FVa from APC-mediated inactivation [31], was also an important determinant of thrombin generation in the presence of APC.

The TM ratio and the nAPCsr were highly correlated with the ETPs measured in the presence of TM and APC, respectively, and were determined by virtually the same plasma variables as these ETPs (Tables 3 and 4). In the case of the nAPCsr, strong dependence on free TFPI and free PS levels, as well as on FX levels, is in good agreement with a recent study [22] on the determinants of the thrombin generation-based nAPCsr as measured using the end-point method [32].

Possible limitations of the present study are the relatively small population size and the use of a healthy population, in which all factors levels are in the normal range. Different (or additional) determinants may be identified in patient populations with factor levels far below or above the normal range (cf. for example ref. [13]).

In conclusion, this study represents the first attempt to define the determinants of thrombin generation parameters in a healthy population. We show that these determinants vary with the experimental conditions, making it possible to develop

thrombin generation assay variants targeted to specific diagnostic purposes.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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