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Journal of Thrombosis and Haemostasis, **3**: 2497–2505

ORIGINAL ARTICLE

Thrombin generation profiles in deep venous thrombosis

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To cite this article: Brummel-Ziedins KE, Vossen CY, Butenas S, Mann KG, Rosendaal FR. Thrombin generation profiles in deep venous thrombosis. *J Thromb Haemost* 2005; **3**: 2497–505.

Summary. *Background:* Reliable markers and methods to predict risk for thrombosis are essential to clinical management. *Objective:* Using an integrated approach that defines an individual's comprehensive coagulation phenotype might prove valuable in identifying individuals at risk for experiencing a thrombotic event. *Methods:* Using a numerical simulation model, we generated tissue factor (TF) initiated thrombin curves using coagulation factor levels from the Leiden Thrombophilia Study population and evaluated thrombotic risk, by sex, age, smoking, alcohol consumption, body mass index (BMI) and oral contraceptive (OC) use. We quantitated the initiation, propagation and termination phases of each individuals' comprehensive TF-initiated thrombin generation curve by the parameters: time to 10 nM of thrombin, maximum time, level and rate (MaxR) of thrombin generated and total thrombin. *Results:* The greatest risk association was obtained using MaxR; with a 2.6-fold increased risk at MaxR exceeding the 90th percentile. The odds ratio (OR) for MaxR was 3.9 in men, 2.1 in women, and 2.9 in women on OCs. The association of risk with thrombin generation did not differ by age (OR:2.8 ≤ 45 years > OR:2.5), BMI (OR:2.9 ≤ 26 kg m^{-2} > OR:2.3) or alcohol use. In both numerical simulations and empirical synthetic plasma, OC use created extreme shifts in thrombin generation in both control women and women with a prior thrombosis, with a larger shift in thrombin generation in control women. This suggests an interaction of OC use with underlying prothrombotic abnormalities. *Conclusions:* Thrombin generation based upon the individual's blood composition is associated with the risk for thrombosis and may be useful as a predictive marker for evaluating thrombosis on an individual basis.

Keywords: coagulation proteins, numerical simulations, oral contraceptives, thrombin generation, thrombosis risk.

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Received 15 March 2005, accepted 22 June 2005

Introduction

Determining who is at risk for thrombotic events (venous and arterial) is difficult because thrombosis is a multicausal disorder. Risk predictions are now based upon genetic factors (antithrombin [1,2], protein C [3,4] and protein S [5,6] deficiencies; factor V Leiden [7,8] and prothrombin G20210A [9,10]), increases in coagulation factors [notably factor VIII (FVIII) [11], FIX [12], FXI [13], fibrinogen] and environmental factors (obesity [14,15], oral contraception [16,17], hormone replacement therapy [17], age [18], alcohol use [19] and potentially smoking [20]). The heterologous presence of any of these circumstances in asymptomatic individuals does not ordinarily necessitate clinical intervention.

The coagulation and fibrinolytic systems are composed of pro- and anticoagulant and fibrinolytic components that maintain the balance of blood fluidity. Qualitative or quantitative alterations in this hemostatic balance can result in hemorrhagic or thrombotic diseases. Current laboratory evaluation techniques cannot fully identify subjects with an increased risk for venous thrombotic disease and the ranges considered normal for blood coagulation and fibrinolytic protein concentrations are broad (> 100%). We hypothesize that an integrated approach that defines an individual's comprehensive coagulation phenotype might prove valuable in understanding the dynamics of hemostasis and potentially identify individuals at risk for experiencing a thrombotic event.

Thrombin generation is essential to hemostasis [21]. Formation of this key enzyme can be described as occurring in three phases: initiation, propagation, and termination. During the initiation phase, exposed or expressed tissue factor (TF) binds circulating factor (F) VIIa and activates FIX and FX [22]. Minute amounts of thrombin are formed by direct activation of prothrombin by FXa [23]. Thrombin activates the essential components (platelets, procofactors FV and FVIII) needed to produce the major burst of thrombin generation. The resulting assembly of the membrane bound coagulation enzyme complexes yields the majority of thrombin (~96%) during the propoagation phase [24]. Clot formation occurs when approximately 10 nM of thrombin–antithrombin is generated and coincides with the onset of the propagation phase. Any thrombin formed after this is not detected by standard

clot-based assays (e.g. the prothrombin time, the partial thromboplastin time or the activated clotting time [CT]).

Each individual's blood composition yields a unique clotting profile [25–27]. Hemostatic disorders, whether genetic, environmental or therapeutically induced [27–31], produce major alterations in these profiles. Several empirical technologies have been developed to directly or indirectly measure the comprehensive thrombin generation profile [25,32–34]. However, the utility of any thrombin generation profiles in clinical diagnosis remains hypothetical.

In this study, we analyzed the theoretical thrombin generation profiles from a case–control study of deep venous thrombosis (DVT), the Leiden Thrombophilia Study (LETS) [35] using a numerical simulation model of thrombin generation [36]. The phases of thrombin generation obtained from the numerical simulations are evaluated relative to risk of DVT and compared with an empirical synthetic plasma model using protein concentrations obtained from the LETS population of interest.

Materials and methods

Materials

HEPES and EDTA were purchased from Sigma Chemical Co (St Louis, MO, USA). Brain phosphatidyl serine (PS) and egg phosphatidyl choline (PC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Recombinant TF was a gift from Drs Roger Lundblad and Shu-Len Liu (Hyland division, Baxter Healthcare Corp, Duarte, CA, USA) and was relipidated (1:2000) in PCPS (25% PS, 75% PC) vesicles by a previously described protocol [37,38]. Human coagulation FVII, FX, FIX and FII were isolated from fresh frozen plasma by using methods described by Bajaj *et al.* [39] and were purged of trace contaminants and active enzymes as previously described [40]. Recombinant full-length tissue factor pathway inhibitor (TFPI) was provided by Dr K. Johnson (Chiron, Emeryville, CA, USA). Spectrozyme TH was from American Diagnostica (Greenwich, CT, USA).

Study population

The LETS is a case–control study where 474 patients with an objectively diagnosed first DVT (enrollment Jan. 1, 1988 to Dec. 31, 1992) were contrasted to the same number of sex and age-matched controls [35]. The patients were selected from three anticoagulation clinics in The Netherlands. The controls were acquaintances of the patients or partners of other patients. Patients with known malignancies were excluded and all patients were younger than 70 years. Blood samples were taken from 6 to 56 months after thrombosis was diagnosed.

Our thrombosis population for this study included 426 individuals (48 individuals were excluded for being on oral anticoagulation) and a control population of 473 individuals (1 individual excluded for being on oral anticoagulation). For risk investigation, we subdivided the populations into groups: men and women; women without oral contraceptives (OC), women with OC, age $\leq$45 years old and $>$45 years old, smokers and non-smokers, alcoholic beverages day^{-1}, 0-, $\leq$1-, 2–4-, 5–10-drinks day^{-1} and body mass index (BMI) of $\leq$26 kg m^{-2} and $>$26 kg m^{-2}. When we investigated the effect of OC (as noted in Table 1), we excluded individuals who were pregnant, postmenopausal, within 30 days *post partum* or had a recent miscarriage at the index date (i.e. the time of thrombosis for patients). As the median time between occurrence of DVT and venipuncture was 18 months, we only evaluated women who either used OC at the index date and the venipuncture date or did not at both dates. Seven individuals were excluded from analyses involving alcohol as information was missing, and three individuals were excluded from analyses involving BMI who were not 18 at the time of the blood draw.

Blood collection and coagulation protein analyses

Whole blood (0.9 Vol.) was collected as previously described [35] from the antecubital vein into Sarstedt Monovette tubes (Nümbrecht, Germany) containing 0.106 M of trisodium citrate (0.1 Vol.). Plasma was prepared by centrifugation for 10 min at 2000 *g* at room temperature and stored in aliquots at −70 °C until assayed.

All protein factor assays were previously performed and are either activity or antigen-based clinical assays [11,12,35,41–43]. In brief, FII activity was measured by a chromogenic assay using *Echis carinatus* venom as an activator [44]. FV:Ag was measured by an in-house-developed sandwich-type enzyme-linked immunosorbent assay (ELISA) with two different monoclonal antibodies, both with a high affinity for the light chain of activated FV [42]. The FVII and FVIII activities were measured by one-stage coagulation assays [11,43]. FIX and FX antigen levels were measured by sandwich ELISA's using commercial polyclonal antibodies (Dako A/S, Glostrup, Denmark) [12,41]. TFPI total and free antigen was measured with a commercial ELISA (Asserachrom Total TFPI and Free TFPI, Diagnostica Stago, Asnieres, France) [45]. The TFPI free antigen assay is specific for free-circulating TFPI and does not detect lipid-bound TFPI. TFPI activity was measured using a two-stage chromogenic substrate assay as previously described. All three concentrations of TFPI were evaluated in our model as we do not know which one is clinically the most significant. All of these percentages were translated into molar concentrations based upon mean published plasma values [46]. The mean factor levels for the populations are shown in Table 2. Note the broad range in concentrations observed for the 899 subjects of this study.

Numerical model of thrombin generation

The numerical model as previously described [36,47] is composed of selected species that are exposed to picomolar concentrations of TF and yield active thrombin generation profiles. The mixture of zymogens, cofactors and inhibitors

Table 1 Comparison of thrombin parameters from the case and control populations

	n	MaxR OR (95% CI)	CT,s mean (SD)	MaxL, nM mean (SD)	AUC, μM s mean (SD)	MaxR, nM s^{-1} mean (SD)	TimeMaxL,s mean (SD)
Whole population							
Cases	426	2.6 (1.8–3.8)	372 (72)	450 (96)	98 (24)	3.5 (1.0)	698 (87)
Controls	473		393 (80)	412 (83)	92 (19)	3.0 (0.8)	730 (96)
Sex/men							
Cases	172	3.9 (2.0–7.5)	382 (68)	442 (89)	98 (23)	3.4 (0.9)	713 (81)
Controls	201		412 (80)	399 (81)	90 (18)	2.9 (0.8)	751 (93)
Sex/women							
Cases	254	2.1 (1.4–3.3)	365 (75)	456 (100)	98 (24)	3.6 (1.0)	688 (89)
Controls	272		379 (78)	421 (84)	93 (19)	3.1 (0.9)	714 (96)
Females without OC*							
Cases	40	8.8† (3.2–23.7)	357 (83)	464 (121)	101 (35)	3.6 (1.2)	681 (94)
Controls	90		408 (74)	388 (65)	87 (15)	2.8 (0.6)	750 (87)
Females with OC*							
Cases	30	2.9 (1.1–7.7)	310 (79)	520 (124)	112 (29)	4.3 (1.2)	623 (95)
Controls	47		311 (71)	472 (92)	100 (21)	3.8 (1.0)	625 (87)
+OC patients vs. –OC controls*							
Cases	30	27.7† (9.2–82.9)	310 (79)	520 (124)	112 (29)	4.3 (1.2)	623 (95)
Controls	90		408 (74)	388 (65)	87 (15)	2.8 (0.6)	750 (87)
Age (years)							
≤45							
Cases	209	2.8 (1.7–4.8)	366 (78)	446 (106)	98 (27)	3.4 (1.0)	697 (94)
Controls	223		398 (88)	401 (85)	90 (18)	2.9 (0.9)	738 (106)
>45							
Cases	217	2.5 (1.5–4.0)	377 (66)	455 (85)	98 (20)	3.5 (0.9	699 (79)
Controls	250		388 (72)	421 (81)	94 (19)	3.1 (0.8)	723 (87)
Smoke							
Yes							
Cases	155	2.5 (1.3–4.5)	376 (74)	449 (107)	99 (28)	3.4 (1.0)	707 (90)
Controls	168		392 (85)	412 (82)	92 (19)	3.0 (0.8)	732 (102)
No							
Cases	271	2.7 (1.7–4.3)	369 (71)	451 (89)	98 (21)	3.5 (0.9)	693 (85)
Controls	305		393 (78)	411 (84)	92 (19)	3.1 (0.8)	729 (93)
Alcohol							
None							
Cases	135	3.2 (1.6–6.3)	372 (76)	458 (91)	98 (20)	3.6 (1.0)	696 (93)
Controls	111		384 (77)	417 (79)	93 (18)	3.1 (0.8)	722 (90)
≤1							
Cases	175	2.0 (1.2–3.4)	374 (73)	439 (91)	96 (24)	3.4 (0.9)	699 (85)
Controls	222		394 (82)	410 (83)	91 (19)	3.0 (0.9)	732 (103)
2–4							
Cases	95	2.7 (1.2–6.0)	370 (68)	453 (94)	99 (23)	3.5 (0.9)	699 (84)
Controls	124		396 (82)	408 (87)	91 (19)	3.0 (0.8)	731 (90)
5–10							
Cases	13	6.3 (0.6–64.9)	349 (69)	511 (174)	119 (51)	4.0 (1.7)	682 (90)
Controls	11		395 (92)	425 (101)	96 (23)	3.1 (0.8)	736 (107)
BMI kg m^{-2}							
≤26							
Cases	206	2.9 (1.6–5.0)	386 (78)	429 (91)	93 (21)	3.3 (0.9)	712 (91)
Controls	275		409 (83)	394 (78)	88 (17)	2.9 (0.8)	748 (99)
>26							
Cases	216	2.3 (1.4–3.8)	356 (61)	473 (95)	103 (26)	3.7 (1.0)	681 (77)
Controls	193		370 (72)	437 (83)	97 (20)	3.3 (0.9)	704 (88)

OR, odds ratio calculated at the 90% cut off point using MaxR; BMI, body mass index; OC, oral contraceptives.
*These women were premenopausal, age 15–49, with no recent miscarriage, pregnancy, within 30 days *post partum* or use of depot contraceptives only and were only selected when OC use at the index date was the same as at the time of the blood draw.
†The OR was calculated at a 75th percentile, since no control individuals were present at a 90th percentile cut-off point.

are described by 27 equations and 42 rate constants [36]. These numerical simulations of thrombin generation are comparable with thrombin generation curves observed in empirical synthetic plasmas and whole blood [31,36,48–50]. For each individual in the LETS population, we used their protein factor levels for FII, FV, FVII, FVIII, FIX, FX,

Table 2 Inventory of the LETS case/control population

Protein	Case population		Control population	
	Percentage mean (SD)	Range	Percentage mean (SD)	Range
FII	108 (17)	67–178	104 (15)	63–153
FV	133 (35)	41–305	131 (33)	47–302
FVII	114 (25)	53–200	110 (22)	41–171
FVIII	141 (35)	53–318	122 (33)	49–232
FIX	109 (26)	63–209	103 (21)	52–188
FX	107 (20)	58–174	103 (17)	49–163
AT	99 (11)	67–143	99 (10)	63–125
TFPI total	94 (21)	35–159	92 (21)	46–171
TFPI active	118 (18)	62–219	117 (17)	72–198
TFPI free	89 (32)	23–211	85 (32)	20–275

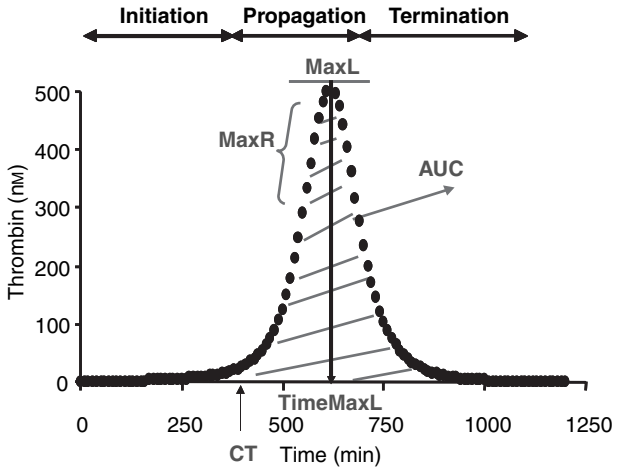

Fig. 1. A numerical simulation of an active thrombin profile illustrating the thrombin parameters. The clinical profile on an individual containing the concentration for FII, FV, FVII/VIIa, FVIII, FIX, FX, AT and TFPI was used to initiate coagulation in the presence of a 5 pM TF stimulus. Active thrombin was evaluated using the parameters, time to 10 nM of thrombin (CT), MaxR, MaxL, TimeMaxL and AUC.

antithrombin (AT) and TFPI (active, free, and total) and generated active thrombin profiles that represent how much dynamic thrombin would have been generated in each individual at the time of their blood draw. Each individual's blood factor concentration was entered into the computer database, Clot Speed II [36]. Simulated reactions were initiated with a 5 pM TF stimulus and solved for active thrombin over 1200 s. Active thrombin is displayed as a combination of thrombin and meizothrombin weighted according to meizothrombin's activity toward synthetic thrombin substrates. The outputs of these active thrombin curves are evaluated by parameters that describe the initiation, propagation and termination phases of thrombin generation: maximum level of thrombin generation (MaxL), maximum rate of thrombin generated (MaxR), time to 10 nM of thrombin (clot time, CT), time to maximum level of thrombin generated (TimeMaxL) and total thrombin generated (area under the curve, AUC) (Fig. 1).

Synthetic plasma analyses of thrombin generation

The procedure used is a modification of Lawson *et al*. [51] and van't Veer and Mann [40] and involves mixing proteins, lipids and relipidated TF to produce a synthetic equivalent of *in vivo* thrombin generation. *I. Procofactor solution*. Relipidated TF (10 pM; molar ratio PCPS:TF = 5000) is incubated with 4 μM PCPS in HBS (20 mM of HEPES and 150 mM of NaCl, pH 7.4) and 2 mM of $CaCl_2$ for 10 min at 37 °C. FV and FVIII at selected mean concentrations from the LETS population are added prior to the initiation of the reaction. *II. Zymogen-inhibitor solution*. FII, FVII/FVIIa, FX, FIX, FXI, TFPI, and AT at selected mean concentrations from the LETS population are preheated in HBS, 2 mM of $CaCl_2$ at 37 °C for 3 min. The reaction is started by mixing equal volumes of both solutions resulting in the desired concentration of the zymogens, pro-cofactors and inhibitors, 5 pM TF, 2 mM of $CaCl_2$ and 2 μM of PCPS. Following the start of the reaction, at selected time points, a 10 μL aliquot is withdrawn from the reaction mixture and quenched in 20 mM of EDTA in HBS (pH 7.4) containing 0.2 mM of spectrozyme TH and assayed immediately for thrombin activity. The hydrolysis of the substrate is monitored by the change in absorbance at 405 nM by using a V_{max} spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Four experiments were performed: (a) control women not on OC (mean of $n = 90$; FII: 1.4 μM, FV: 25 nM, FVII: 10 nM, FVIII: 0.82 nM, FIX: 83 nM, FX:153 nM, AT: 3.4 μM, TFPI:2.9 nM); (b) control women on OC (mean of $n = 47$; FII: 1.5 μM, FV: 22 nM, FVII: 12 nM, FVIII: 0.87 nM, FIX: 103 nM, FX:189 nM, AT: 3.2 μM, TFPI:2.3 nM); (c) women with DVT not on OC (mean of $n = 40$; FII: 1.5 μM, FV: 27 nM, FVII: 12 nM, FVIII: 0.98 nM, FIX: 104 nM, FX:183 nM, AT: 3.3 μM, TFPI:2.9 nM); (d) women with DVT on OC (mean of $n = 30$; FII: 1.6 μM, FV: 22 nM, FVII: 12 nM, FVIII: 0.96 nM, FIX: 108 nM, FX:194 nM, AT: 3.1 μM, TFPI:2.4 nM).

Statistical analysis

We evaluated the effect of variables used to describe the initiation, propagation and termination phases of thrombin generation on the risk of developing DVT by determining the odds ratios (OR). We calculated ORs and their 95% confidence intervals (95% CIs) using the 90th percentile levels for MaxL, MaxR, and AUC. The time variables, TimeMaxL and CT, were investigated at the 10th percentile in order to investigate the faster CTs, which are prothrombotic. To adjust for confounding by the other propagation variables (MaxR, MaxL, AUC, TimeMaxL), we used a logistic regression model including all other propagation variables (except CT) in the model as covariates when calculating the OR for MaxR, MaxL, AUC and TimeMaxL. Subsequently, we calculated crude ORs and 95% CIs using the 90th percentile levels for MaxR for subgroups i.e. men, women, women with or without OC, individuals older or

younger than 45 years, smokers or non-smokers, and different classes of alcohol use or BMI.

Results

Thrombin generation profiles

The mean thrombin generation and the 95% CI in the control and thrombosis population are seen in Fig. 2, panel A. Active TFPI values, rather than total or free TFPI, were used in the simulation of these curves. The control population had a mean CT of 393 s (SD:80), a mean MaxL of 412 nM (SD:83 nM), a mean MaxR of 3.0 nM s^{-1} (SD:0.8 nM s^{-1}), a mean AUC of 92 μM (SD:19 μM) and a mean TimeMaxL of 730 s (SD:96 s) (Table 1). The thrombosis population was shifted toward a slightly faster CT of 372 s (SD:72 s), TimeMaxL of 698 s (SD:87 s) and MaxR of 3.5 nM s^{-1} (SD:1.0 nM s^{-1}), and a slightly higher MaxL 450 nM (SD:96 nM) and AUC 98 μM (SD:24 μM).

Thrombin parameters as predictors of thrombosis

We investigated the thrombin generation parameters for their effect on the risk of developing DVT. Using a 90% cut off point for each of the individual variables, the crude OR for developing a DVT was 2.6 (95% CI: 1.8–3.8) for MaxR, 2.4 (95% CI: 1.7–3.5) for MaxL, 2.2 (95% CI: 1.5–3.2) for AUC, 1.6 (95% CI: 1.1–2.4) for CT at a 10% cut-off point and 1.6 (95% CI: 1.1–2.3) for TimeMaxL. When we adjusted the propagation variables MaxR, MaxL, AUC and TimeMaxL for the effect of the other propagation variables, MaxR still displayed the highest OR at 2.6 (95% CI: 1.4–4.7) when adjusted for MaxL, AUC and TimeMaxL. The adjusted OR was 1.0 (95% CI: 0.5–1.9) for MaxL, 1.5 (95% CI: 0.9–2.5) for AUC and 0.8 (95% CI: 0.5–1.3) for TimeMaxL. When total or free TFPI values were used, rather than active TFPI concentrations, MaxR also yielded the highest OR (1.8 in the model with total TFPI and 2.9 in the model with free TFPI).

Association of thrombin generation parameters with risk in relation to classical risk factors

Individuals with DVT of both sexes had a faster CT, TimeMaxL and MaxR, a higher MaxL and a greater AUC than controls of the same sex (Table 1). Using MaxR at the 90% cut off point, men showed an OR of 3.9 (95% CI: 2.0–7.5) and women an OR of 2.1 (95% CI: 1.4–3.3). We studied the effect of OC use by selecting women who were premenopausal, aged 15–49, with no recent miscarriage or pregnancy and within 30 days *post partum*. In this group, the OR for MaxR exceeding the 90th percentile was 2.9 (95% CI: 1.1–7.7). In women who did not use OC, cases clotted faster than controls (mean difference 51 s, 95% CI: 21–81 s), had higher levels of thrombin (mean difference 76 nM, 95% CI: 36–116 nM) generated at a faster rate and time (mean difference 69 s, 95% CI: 35–103 s) and more total thrombin was present (mean difference 14 μM s, 95% CI: 3–25 μM s) (Fig. 2, Panel B). As none of the controls had levels of MaxR above the 90th percentile, we calculated the OR for developing DVT at the 75th percentile where the OR was 8.8 (95% CI: 3.2–23.7) for women without OC use.

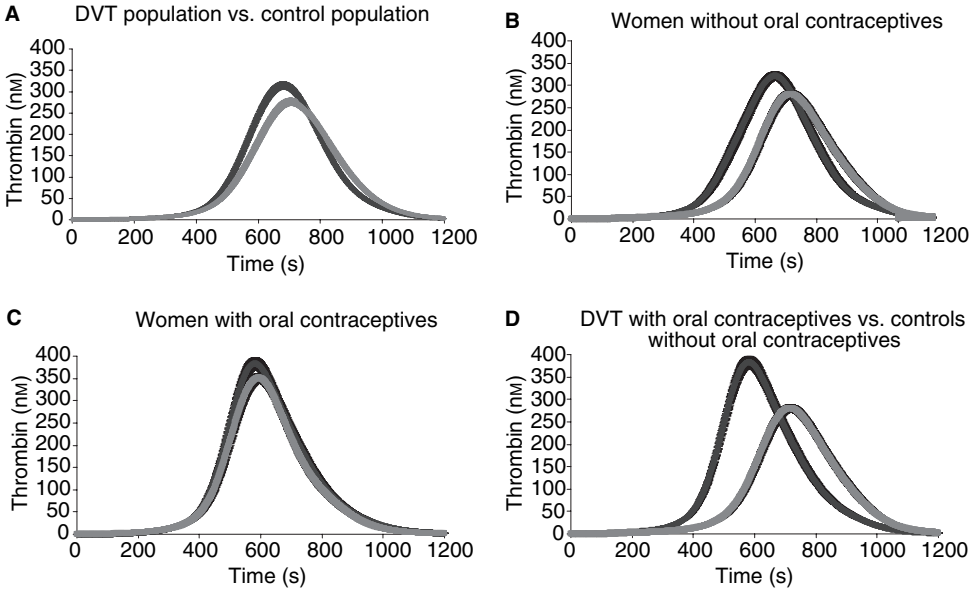

Fig. 2. Comparison of selected active thrombin profiles for the control and deep vein thrombosis (DVT) populations. These active thrombin generation curves are shown as the mean and 95% confidence interval of the population selected. (A) The DVT population ($n = 426$, left curve) is compared with the control population ($n = 473$, right curve). (B) Women without OCs are compared between the DVT population ($n = 40$, left curve) and the control population ($n = 90$, right curve). (C) Women with OCs are compared between the DVT population ($n = 30$, left curve) and the control population ($n = 47$, right curve). (D) Women with DVT on OCs ($n = 30$, left curve) and the control population not on OCs ($n = 90$, right curve) are compared.

Contraceptive use consistent between the index date and the venipuncture date affected both the DVT and control populations by increasing the thrombin parameters. However, in women with a DVT (Fig. 3B), thrombin parameters were not as increased upon OC use as in the control population (Fig. 3A) upon OC use. The increase in thrombin generation parameters with OC use in women with DVT was as follows: CT by 47 s (95% CI: 9–85 s), MaxR by 0.7 nM s^{-1} (95% CI: 0.1–1.3 nM s^{-1}) and TimeMaxL by 58 s (95% CI: 13–103 s). No significant decrease was seen for MaxL (mean difference 56 nM, 95% CI: −2 to 114 nM) and AUC 11 μM s (95% CI: −4 to 26 μM s). In the control women upon OC use, the thrombin parameters increased as follows: CT by 97 s (95% CI: 72–122 s), MaxL by 84 nM (95% CI: 54–114 s), AUC by 13 μM s (95% CI: 6–20 μM s), MaxR by 1.1 nM s^{-1} (95% CI: 0.8–1.4 nM s^{-1}) and TimeMaxL by 125 s (95% CI: 94–156 s).

The OR for DVT of MaxR was 2.9 in women on OC hormones with an increase in MaxR of 19% in women with DVT on OC vs. those not on OC and 36% in control women on OC vs. those not on OC. As well, women with a DVT who used OCs had a 54% faster MaxR than control women who did not use OC (Fig. 2, panel D). Their coagulation profiles were increased over the controls by: CT: 98 s (95% CI: 66–130 s), MaxL: 132 nM (CI: 86–178 nM), AUC: 25 μM s (CI: 14–36 μM s), MaxR: 1.5 nM s^{-1} (95% CI: 1.1–1.9 nM s^{-1}), TimeMaxL: 127 s (95% CI: 89–165 s). The OR at the 75th percentile for women with DVT on OC use vs. control women not on OC use was 27.7 (95% CI: 9.2–82.9).

The mean factor levels of the OC populations (thrombosis and control) shown in Fig. 3A and B were used in empirical reconstructed plasma experiments with the same TF stimulus used in the numerical simulations. These results are shown in panel B and C of Fig. 3. By recapitulating the mean procoagulant and anticoagulant protein profiles of the LETS individuals identified for each curve on a phospholipid surface (see Materials and methods), we obtain similar patterns of OC effect on thrombin generation that is seen in the numerical simulations. In both the control and women with DVT, thrombin generation is accelerated in women that used OC. For the control women not on OC use vs. the control women on OC use, the thrombin parameters increased from: 312 s to 243 s for CT, 1 nM s^{-1} to 1.9 nM s^{-1} for MaxR, 236 nM to 262 nM for MaxL, 54 μM s to 62 μM s for AUC and 540–420 s for TimeMaxL (Fig. 3, panel C). In comparing women with a DVT not on OC use vs. women with DVT on OC use, the thrombin parameters increased from: 219 s to 195 s for CT, 1.6 nM s^{-1} to 1.8 nM s^{-1} for MaxR, 289 nM to 299 nM for MaxL, 69 μM s to 68 μM s for AUC and 420–360 s for TimeMaxL (Fig. 3, panel D). The most prothrombotic profile was seen in women who had had a DVT and were still using OCs at the time of the blood draw, followed by healthy control women using OCs at the index and blood draw date. The least prothrombotic curves were in healthy women not using OCs.

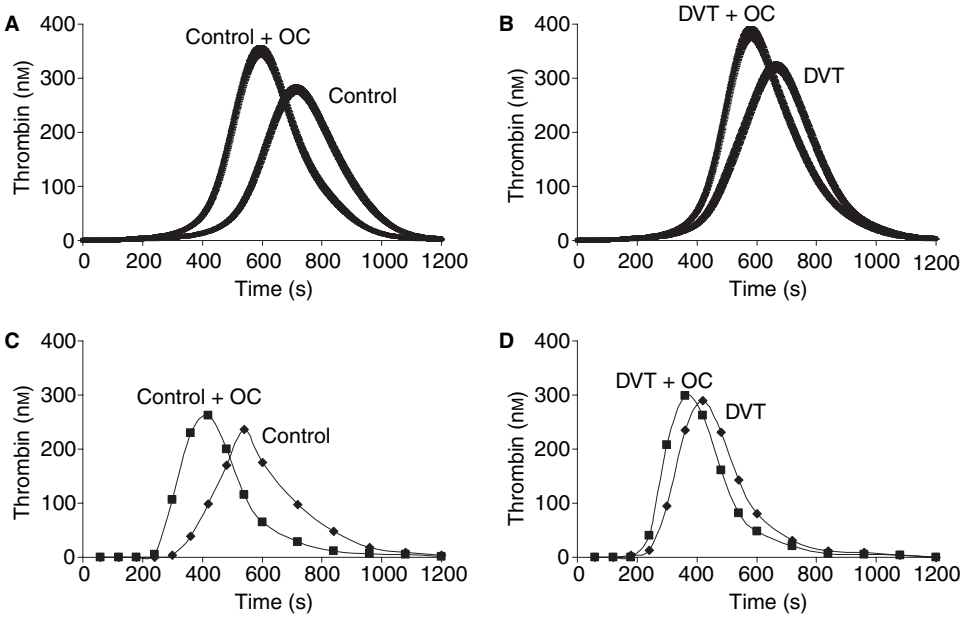

Fig. 3. Oral contraceptive (OC) effect on theoretical and empirical thrombin generation. Thrombin generation simulations under a 5 pM TF stimulus are shown as the mean and the 95% confidence interval for (A) control women with ($n = 47$) and without ($n = 90$) OC use and (B) Women with DVT with ($n = 30$) and without ($n = 40$) OC use. The mean factor levels of the selected populations shown in panel A and B were recapitulated in a synthetic plasma model with 2 μM of phospholipids and a 5 pM TF stimulus and are shown in panel C and D. (C) Control women not on OC (mean of $n = 90$; FII: 1.4 μM, FV: 25 nM, FVII: 10 nM, FVIII: 0.82 nM, FIX: 83 nM, FX:153 nM, AT: 3.4 μM, TFPI:2.9 nM) are compared with control women on OC (mean of $n = 47$; FII: 1.5 μM, FV: 22 nM, FVII: 12 nM, FVIII: 0.87 nM, FIX: 103 nM, FX:189 nM, AT: 3.2 μM, TFPI:2.3 nM). (D) Women with DVT not on OC (mean of $n = 40$; FII: 1.5 μM, FV: 27 nM, FVII: 12 nM, FVIII: 0.98 nM, FIX: 104 nM, FX:183 nM, AT: 3.3 μM, TFPI:2.9 nM) are compared with women with DVT on OC (mean of $n = 30$; FII: 1.6 μM, FV: 22 nM, FVII: 12 nM, FVIII: 0.96 nM, FIX: 108 nM, FX:194 nM, AT: 3.1 μM, TFPI:2.4 nM).

As MaxR is the most predictive parameter in the numerical model, our empirical experiments show that OC use effected MaxR in the control women by a 90% increase, whereas in the DVT population, OC use effected MaxR by only 12%. Thus, this demonstrates that OC use effects empirically generated thrombin generation to a greater degree in control women than women with DVT as was also determined with numerically simulated thrombin generation. The similarities between the empirical experiments and the numerical simulation results also validate the rate constants chosen for use in the computational model for the components that are present which include, FII, FV, FVII, FVIII, FIX, FX, AT, and TFPI.

Thrombin generation profiles were associated with thrombosis for all ages tested: using high MaxR yielded an OR of 2.8 for individuals <45 years old and an OR of 2.5 for individuals >45 years old (Table 1). Thrombin generation was associated with risk for different classes of obesity: among those with a BMI ≤ 26 kg m^{-2}, high MaxR showed an OR of 2.9, and 2.3 in those with a BMI >26 kg m^{-2}. The association between thrombin generation and the risk of developing DVT for different classes of alcohol consumption was: 6.3 (95% CI: 0.6–64.9) for 5–10-drinks day^{-1}, 2.7 (95% CI: 1.2–6.0) for 2–4-drinks day^{-1}, 2.0 (95% CI: 1.2–3.4) for ≤ 1 drink per day and 3.2 (95% CI: 1.6–6.3) for the non-drinking population. Note that the 95% CI in all of these categories is wide. The OR for developing DVT associated with thrombin generation was 2.5 in smokers and 2.7 in non-smokers.

Discussion

The predicted composition-based capacity for thrombin generation in response to a TF challenge represents an integrative method to identify an individual's propensity for developing DVT. The most influential variable was the MaxR, which, corrected for by all propagation phase variables (except CT), resulted in an OR of 2.6 (95% CI: 1.8–3.8) for the highest 10% of MaxR values. The use of the numerical simulation model allows for the combination of the results of the individual reactions into a complete ensemble describing not only the observed progress in thrombin formation but also the activation progress for each of the proteins as intermediates in the path toward thrombin formation. These results were verified by recapitulating thrombin generation based upon the protein profiles of the specified LETS population in a synthetic plasma model. Therefore, the individual's hemostatic profile is translated into a defined pattern, which can be used as an evaluation tool for thrombosis risk, potentially with the ability to influence clinical decision making. Our results show that there may exist subthreshold venous thrombosis states in individuals (i.e. OC use) that depend only on a trigger, which will create a heightened prothrombotic state that presents itself upon challenge. Thus, illustrating that evaluating comprehensive thrombin generation via an individual's protein profile can be a measure of an individual's prothrombotic state.

Understanding the relationship of TF-initiated thrombin generation to clinical outcome has been hindered by the absence of comprehensive assays and in the ability to collect blood from patients during a hemostatic challenge. The use of the numerical simulation model enables the estimation of TF-initiated dynamic thrombin generation using available tools (i.e. patients' factor levels). The numerical simulation method thus allows the evaluation of well-studied clinical databases, such as the LETS population, and compares dynamic thrombin generation retrospectively to determined clinical phenotypes. Although these studies do not include the contribution of the anticoagulant protein C pathway, the contribution of platelets, the contact pathway or the vasculature, it does include all the plasma pro- and anticoagulant proteins of the TF pathway to thrombin generation that are currently evaluated in laboratories.

In this study, we translated the active thrombin profiles into thrombin parameters that incorporate the initiation, propagation and termination phases of thrombin generation. The principal regulator of the initiation phase of thrombin generation is TFPI, the stoichiometric inhibitor of the FVIIa–TF–FXa enzyme-product complex. Thrombin generation simulations performed using either active, total or free TFPI values yielded similar risk estimates. Previously, it has been reported that for the LETS population, low levels of TFPI, especially low TFPI-free and total antigen in plasma, constituted a risk factor for DVT [45]. In the numerical system, the difference between TFPI subsets is small. In previous numerical simulations for healthy individuals (the LETS control group), the effect from any individual protein on thrombin generation outcome was $<9\%$ [27]; thus, it is the interplay between the procoagulants and the anticoagulants that determine the extent of thrombin generation.

MaxR was the most useful predictor of DVT. This parameter incorporates the velocity at which thrombin is formed, and is obtained as the slope of the thrombin generation curve (Fig. 1). The range of mean MaxR in the subpopulations was from 2.8 to 4.3 nM s^{-1}; with the cases between 3.3 and 4.3 nM s^{-1} and the controls between 2.8 and 3.8 nM s^{-1}. Previous studies of genetic bleeding disorders have shown that in hemophilia the MaxR ranges from 0.2 to 0.5 nM s^{-1} [52]. From these studies, we can begin to evaluate a thrombotic point or threshold using MaxR in healthy individuals.

During investigation of the influence of MaxR relative to risk factors, sex, age, BMI, OC use, alcohol consumption and smoking, we found that MaxR was associated with risk in all strata of these factors. MaxR was highest in patients using OCs. OC use is an established risk factor for both venous and arterial thrombosis [16,53,54]. We found that OC use strongly increases a woman's thrombin generation profile by affecting all three phases of TF-initiated thrombin generation in both case and control women. All of these women were premenopausal, age 15–49, who had no recent miscarriage, were not pregnant, nor within 30 days *post partum* and were only selected when OC use at the index date was the same as at the time of the blood draw. Previously, Bloemenkamp *et al.* [55] showed that OC use had a more pronounced hemostatic effect in women who had suffered DVT with regard to the levels of

FVII, FXII, protein C, AT, protein S and APC-sr than in healthy women, suggesting the existence of 'hyperresponders'. Heightened thrombin generation in healthy women on OC use has also been shown experimentally using special fluorogenic thrombin substrates and monitoring clotting in PPP and PRP [56]. We see comparable results, in that women with a DVT that are on OC use have the most pronounced acceleration of thrombin generation. Our results in this study also indicate that OC use had a larger impact on thrombin generation profiles in control women than women with a diagnosed DVT. As all of the simulated thrombin generation curves are initiated with the same amount of TF stimulus, shifts in the thrombin generation curve are caused by other factors that are present in these individuals. OC potentially causes a subclinical prothrombotic phenotype in these healthy control women that may become apparent when presented with a challenge. As exogenous hormones are used by more than a hundred million women worldwide as OCs or for postmenopausal hormone replacement, methods for stratification of thrombotic risk are essential.

As venous thrombosis is defined by a vasoocclusive event, the differentiation of cases and controls is of temporal quality. The accidental occurrence of precipitating environmental factors, e.g. trauma, may lead to an individual becoming a case, while another, with the same thrombin generation profile, in the absence of those factors, will remain a control. This amplification to generate thrombin in the controls may be only differentiated from the cases by time. Hemophilia patients with severe FVIII deficiency do not all have similar bleeding pathology. Potentially, the more thrombin they can generate, the lower the bleeding risk. Conversely, the more thrombin a 'healthy' individual can produce, the higher the thrombotic risk when a risk situation occurs. Our model uses the combined influence of all of the plasma pro- and anticoagulants of the TF pathway on dynamic thrombin generation. Overall, our results suggest that evaluating hypothetical thrombin generation based upon the individual's blood composition may be useful as a predictive marker for evaluating thrombosis.

Acknowledgements

We would like to thank Kihachiro Umezaki for his work on writing the computer software to handle large databases in the numerical simulation model. Supported by grants from the Program Project Grant No. HL 46703 (Project 1) from the National Institutes of Health (K. G. Mann) and the Netherlands Heart Foundation 89.063 (F.R. Rosendaal).

Conflicts of interest

The authors have no financial interests to disclose or declare.

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