The Prothrombotic Phenotypes in Familial Protein C Deficiency Are Differentiated by Computational Modeling of Thrombin Generation

Kathleen E. Brummel-Ziedins1*, Thomas Orfeo1, Peter W. Callas3, Matthew Gissel1, Kenneth G. Mann1, Edwin G. Bovill2
1 Department of Biochemistry, University of Vermont, College of Medicine, Burlington, Vermont, United States of America, 2 Department of Pathology, University of Vermont, College of Medicine, Burlington, Vermont, United States of America, 3 Department of Mathematics and Statistics, University of Vermont, Burlington, Vermont, United States of America

Abstract

The underlying cause of thrombosis in a large protein C (PC) deficient Vermont kindred appears to be multicausal and not explained by PC deficiency alone. We evaluated the contribution of coagulation factors to thrombin generation in this population utilizing a mathematical model that incorporates a mechanistic description of the PC pathway. Thrombin generation profiles for each individual were generated with and without the contribution of the PC pathway. Parameters that describe thrombin generation: maximum level (MaxL) and rate (MaxR), their respective times (TMaxL, TMaxR), area under the curve (AUC) and clotting time (CT) were examined in individuals with PC mutation, prothrombin G20210A polymorphism and thrombosis history (DVT or PE). This family (n = 364) is shifted towards greater thrombin generation relative to the mean physiologic control. When this family was analyzed with the PC pathway, our results showed that: carriers of the PC mutation (n = 81) had higher MaxL and MaxR and greater AUC (all p<0.001) than non-carriers (n = 283); and individuals with a DVT and/or PE history (n = 13) had higher MaxL (p = 0.005) and greater AUC (p<0.001) than individuals without a thrombosis history (n = 351). These differences were further stratified by gender, with women in all categories generating more thrombin than males. These results show that all individuals within this family with or without PC deficiency have an increased baseline procoagulant potential reflective of increased thrombin generation. In addition, variations within the plasma composition of each individual can further segregate out increased procoagulant phenotypes, with gender-associated plasma compositional differences playing a large role.

Introduction

Determining who is at risk for thrombotic events is difficult because thrombosis is a multicausal disorder. Venous thromboembolism (VTE) has an annual incidence of >1 per 1,000 person years [1]. VTE mainly consists of deep venous thrombosis (DVT) and its complication, pulmonary embolism (PE). VTE is lethal due mostly to PE [2], which is considered an independent predictor of reduced survival [3]. Methods to reliably identify individuals at risk for VTE would be an important advance.

A genetic risk factor can be detected in approximately 50% of patients with a first episode of VTE [4]. Well-established genetic risk factors for VTE comprise deficiencies or functional abnormalities in two natural anticoagulant pathways: the antithrombin (AT)-heparin sulphate pathway (antithrombin deficiency) and the protein C (PC) pathway, in which protein S (PS) serves as a cofactor (PC deficiency, PS deficiency and resistance to activated PC (APC)) [5,6,7,8]. Another mutation (prothrombin G20210A) has been associated with a 30–70% increase in prothrombin levels and has been weakly correlated with VTE risk [9]. The PC pathway provides a dynamic inhibitory system to regulate thrombin production [10]. If one looks at the prevalence of thrombophilic risk factors, defects in the PC system taken together (PC, PS and FVLeiden) are the single most prevalent (28.8%) abnormality [11]. In the EPCOT study [12] of first venous thrombotic events in carriers of familial thrombophilic defects, the majority of first events were associated with abnormalities of components of the PC system. Thus, defects in the PC system are the most prevalent thrombophilic risk factors in thrombophilia. Understanding what occurs in individuals with defects in this pathway may help in understanding potential mechanisms of VTE risk as a multicausal disease.

Homozygous PC deficiency is associated with severe thrombotic tendencies and can result in fatal neonatal thrombotic events [13]. Heterozygous PC deficiency is also associated with an increased risk of thrombosis [14,15,16]. The prevalence of PC deficiency is estimated to be 0.5% in the general population [17,18]. Studies of selected PC deficient families have shown that heterozygous individuals have a 50% chance of experiencing a first venous thromboembolic event by the age of 45...
[19,20], but their overall mortality is not affected [21]. A population with familial type I PC deficiency, first described by Bovill et al. [22] identified not only a high incidence of VTE in PC deficient individuals, but also a strong relationship between PC deficiency and venous thrombosis in the family (relative risk = 11.7, p<0.001). About 15% of those with venous thrombosis were not PC deficient. The phenotypic pattern in this family led to the conclusion that they have another genetic risk factor which interacts with PC deficiency to increase the risk of thrombosis [15]. Thus, this family is an ideal cohort for investigating what other hemostatic variables along with PC deficiency might account for thrombosis.

Thrombin has long been recognized for its multiple functions in blood coagulation and platelet aggregation as well as its roles in tissue repair, development and pathogenic processes [23,24]. Methods that profile thrombin generation, either directly or indirectly, have potential utility in the realm of clinical testing [25], since these methods provide a significant increase in the information collected relative to that available with standard clotting time tests designed for the evaluation of deficiencies in coagulation factors. However, there is great diversity in

Figure 1. Variation of thrombin generation in familial PC deficiency. Thrombin generation profiles were generated from each individual's plasma composition (n = 364), containing fII, fV, fVII, fVIII, fIX, fX, AT, TFPI and PC and a 5 pM TF initiator. The mean thrombin curve is shown in maroon with the standard deviation in salmon. A control curve, representing mean physiologic concentrations of each factor is illustrated in gold as a comparison.

doi:10.1371/journal.pone.0044378.g001

Table 1. Plasma composition within familial PC deficiency.

| Protein | Mean (SD) | Range  | Physiological mean | Clinically accepted normal range |
|---------|-----------|--------|--------------------|----------------------------------|
| FII, mM | 1.8 (0.4) | 0.7–2.9| 1.4                | 0.8–2.0                          |
| FV, nM  | 20.1 (6.7)| 7.0–60.0|20.0              | 12–28                            |
| FVII, nM| 10.0 (2.5)| 4.0–20.5|10.0            | 6–14                             |
| fVIII, nM| 0.8 (0.3)| 0.24–1.8| 0.7             | 0.4–1.6                          |
| FIX, nM | 93.3 (27)| 47.6–225|90.0             | 62–135                           |
| FX, nM  | 167 (38)  | 79.8–274|160               | 96–224                           |
| AT, mM  | 3.4 (0.6) | 1.5–5.6 | 3.6              | 3.2–6.3                          |
| TFPI, nM| 2.3 (0.6) | 1.1–5.0 | 2.5              | 1.1–4.3                          |
| PC, nM  | 96 (38)   | 16.0–207| 65               | 50–119                           |

doi:10.1371/journal.pone.0044378.t001
experimental hematology protocols, resulting in the widely-
recognized inter-laboratory variability in the results of thrombin
generation studies [26]. One of the approaches to evaluating
thrombin generation is to use mathematical models [27,28,29,30,31,32,33,34]. In addition to several studies from
our laboratory [31,35,36] presenting empirical validation of our
computational model, it was recently evaluated independently
against datasets from different laboratories, and showed
reasonable agreement with the experimental data [33].

One focus of our group has been to use computational
modeling to study the effects that normal range compositional
differences in the coagulation proteomes of individuals have on
their thrombin generation profiles [36,37,38] and test whether
differences in predicted thrombin generation segregate with
potential risk factors [36,37,39,40]. In this study we evaluated the
plasma composition derived thrombin generation profiles from
individuals within familial protein C deficiency. To do so, we
combined our previously described model of tissue factor
initiated thrombin generation [41] with an empirically validated
description of the protein C pathway [42]. The potential
promoters of thrombotic risk that were evaluated in this family,
included the presence of the PC mutation, the presence of the
prothrombin G20210A polymorphism, a past history of throm-
bosis (DVT or PE) and gender.

Results

The Variation in Thrombin Generation within this Family

Thrombin generation within this entire family shifted towards
greater thrombin generation relative to the mean physiologic
control (Figure 1). The MaxR varied from 0.02–2.75 nM/s with a
mean of 0.65 (0.52) nM/s. The MaxL varied 78 fold (6–478 nM)
with a mean of 112 (88) nM. The AUC varied 83 fold, 3–
250 μM·s with a mean of 35 μM·s. The TMaxL varied 2 fold
(426–825 s), and the TMaxR varied 3.3 fold (212–716). This large
variation of thrombin generation is shown as the standard
deviation in grey in Figure 1.

The simulations of thrombin generation are dependent on each
individual's plasma composition. As shown in Table 1, a large
variation is observed: specifically fV (8.5 fold), fVIII (7.5 fold) and
PC (13 fold).

Investigating the Range of Thrombin Generation within
Familial PC Deficiency

Effect of PC mutation. The contribution of the PC mutation
to simulated thrombin generation is shown in Figure 2 panel A
and in Table 2. Individuals that are grouped as positive for the PC
mutation (n = 81) have greater thrombin generation than individ-
uals within the family that do not have the PC mutation (n = 283).
The MaxL, MaxR and AUC were significantly different between
the groups. The plasma composition in individuals with the PC
mutation had significantly lower levels of fII, fV, fIX, TFPI and
PC (Table 3).

For all thrombin parameters measured, thrombin generation
was greater than the mean physiologic control. The percentage
of subjects with the PC mutation for whom the thrombin
generation parameters (CT, MaxL, MaxR, AUC) exceeded
those for the mean physiologic control ranged between 84%
and 95%. Without the PC mutation, this percentage was still
67–70%. The mean PC concentration in the group with the PC
mutation was 47 (29) nM. In the group without the PC
mutation, the mean concentration of PC was 110 (28) nM. The
mean physiologic control has a PC concentration of 65 nM.
Thus, the increased thrombin generation within this family is
not due to PC alone.

Effect of prothrombin G20210A polymorphism. Although
the prothrombin concentration was higher in the subjects with this

Figure 2. Investigating the range of thrombin generation
within familial PC deficiency. Individuals within the family were
segregated by A) Protein C mutation, B) Prothombin G20210A
polymorphism, and C) Thrombosis history. The groups with the
mutation/history are shown in grey (-SD). A control curve, representing
mean physiologic concentrations of each factor is illustrated in gold as a
comparison.
doi:10.1371/journal.pone.0044378.g002
Table 2. Thrombin generation parameters within groups and stratified by gender.

|                | All Subjects (Mean (SD)) | Females (Mean (SD)) | Males (Mean (SD)) |
|----------------|--------------------------|---------------------|------------------|
|                | Yes N = 81 | No N = 283 | P value | Yes N = 54 | No N = 159 | P value | Yes N = 27 | No N = 124 | P value |
| **Clot Time (s)** | 313 (93) | 340 (93) | 0.07 | 287 (98) | 334 (97) | 0.005 | 358 (85) | 349 (84) | 0.52 |
| **Max Rate (nM/s)** | 1.01 (0.47) | 0.55 (0.46) | <0.001 | 1.15 (0.50) | 0.59 (0.50) | <0.001 | 0.76 (0.41) | 0.50 (0.40) | 0.003 |
| **Max Level (nM)** | 182 (78) | 93 (77) | <0.001 | 208 (85) | 97 (84) | <0.001 | 135 (65) | 86 (64) | 0.11 |
| **AUC (μM*s)** | 59 (29) | 28 (28) | <0.001 | 70 (32) | 30 (32) | <0.001 | 41 (21) | 25 (21) | 0.08 |

|                | Definite N = 13 | No history N = 351 | P value | Definite N = 8 | No history N = 205 | P value | Definite N = 5 | No history N = 146 | P value |
|----------------|-----------------|---------------------|---------|----------------|---------------------|---------|----------------|---------------------|---------|
| **Clot Time (s)** | 321 (95) | 334 (93) | 0.40 | 292 (101) | 323 (99) | 0.42 | 359 (87) | 350 (84) | 0.81 |
| **Max Rate (nM/s)** | 0.89 (0.51) | 0.65 (0.50) | 0.11 | 1.06 (0.56) | 0.72 (0.55) | 0.14 | 0.67 (0.43) | 0.55 (0.41) | 0.53 |
| **Max Level (nM)** | 182 (86) | 110 (84) | 0.005 | 226 (96) | 122 (95) | 0.006 | 115 (69) | 94 (67) | 0.49 |
| **AUC (μM*s)** | 67 (31) | 34 (30) | <0.001 | 88 (35) | 38 (35) | <0.001 | 34 (23) | 28 (22) | 0.56 |

mutation (2.1 (0.4) μM vs. 1.7 (0.4) μM), there were no differences between any thrombin parameters between the two groups (Figure 2 Panel B).

**Thrombosis history.** Individuals within this family, not on any anticoagulants at the time of the blood draw, with a previous history of thrombosis (n = 13) were compared to the larger group without a previous thrombosis history (n = 351). Thrombin generation was greater in this small subset (Figure 2, panel C). Specifically, the MaxL of thrombin was greater (192 (96) nM vs. 110 (84) nM, p = 0.005) and the total amount of thrombin generated (AUC: 67 (31) μM*s vs. 34 (30) μM*s, p = <0.001). Plasma composition within individuals with a previous thrombosis, showed greater IV and VIII suppressed PC and fIX (Table 3). These lower levels of PC stem from the fact that 8 out of the 13 individuals within this category had the PC mutation.

**Gender Effect**

Our data was further stratified by gender to determine if there is any relationship between increased thrombin generation in the subsets analyzed. Gender does appear to further segregate women (Figure 3, panel A and Table 2). Women possessing the PC mutation have a significantly faster clot time and higher maximum rate, maximum level and AUC (all p<0.005). Surprisingly, only the maximum rate was significantly different in the males when those with and without the PC mutation were compared.

Having a previous history of thrombosis was also further segregated in women (Figure 3, panel C), a higher MaxL (226 (96) nM vs. 122 (95) nM, p = 0.006) and greater AUC (88 (35) μM*s vs. 38 (35) μM*s, p<0.001) were observed.

In comparing males to females with an additional category of risk (either PC or PT mutation or a previous history of thrombosis, Figure 4), thrombin generation parameters in nearly all cases trend higher in women. This is most notably seen in the PC mutation group (Figure 4, panels E and F), where all parameters are significantly different.

**Plasma factor compositional differences by gender.** Gender plasma compositional differences were seen in all cases evaluated (Table 3). Higher levels of IV, VII, VIII, and IX were seen in women with a thrombosis history versus without, and with men only increases in III and VIII were seen in men with a previous history of thrombosis.

In women with the PC mutation versus women without, fII, TFPI and PC were significantly lower. Whereas in men with the mutation versus without, fIX and PC were significantly lower and AT was significantly higher. In women with the prothrombin G20210A mutation versus women without, only III was significantly elevated. This phenomenon was also observed in men.

**Discussion**

In this study we show that thrombin generation derived via each individual’s concentrations of pro- and anticoagulant factors identifies groups within familial PC deficiency and in comparison to unrelated controls. Individuals within the family containing risk factors that include the PC mutation, the prothrombin G20210A mutation and having a past history of thrombosis show increased thrombin generation. Females who have any of the evaluated risk factors generate more thrombin than males with the same risk factors. These studies suggest that within this family gender might further influence the risk of thrombosis.

The PC anticoagulant pathway plays a major role in the balance of procoagulation and anticoagulation, by providing a dynamic inhibitory system to regulate thrombin production. APC produced by the thrombin–thrombomodulin complex, inactivates the cofactors IVa and VIIla [43], thereby down-regulating further generation of thrombin and stopping clot propagation. In PC deficiency, the down-regulation of thrombin production is compromised. Therefore, thrombin generation as a measure of a
thrombosis potential is a good marker in evaluating familial PC deficiency. In a previous study of this family, the genetic basis for coagulation factor hereditability was evaluated and the results showed that the heritability correlated best with measures of thrombin activity [44]. In our current study, we show that by using each individual’s plasma composition to simulate thrombin generation, we are able to identify that the increased thrombin generation in this family is significantly greater than the mean physiologic value. If PC was the only contributing factor, at increasing PC concentrations, thrombin generation would be suppressed.

This family has been extensively studied, and the observed phenotypic pattern led to the conclusion that they have another genetic risk factor which interacts with PC deficiency to increase the risk of thrombosis [15,45]. Several candidates for the interacting factor have been ruled out [46], including the prostaglandin H synthase 1 gene [47] and platelet-activating factor acetyl-hydrolase Ib [48]. The G20210A prothrombin polymorphism was not found to be associated with risk of venous thrombosis in the family [49], although, in this current study we show that individuals that possess the prothrombin mutation have increased thrombin generation. Factor VLeiden is rare in the family (2% affected) and thus cannot explain the observed inheritance pattern. Recent genotyping and resequencing have provided some promising evidence of a possible interacting gene, cell adhesion molecule 1 [50]. In this current study, using each individual’s plasma composition to evaluate thrombin generation, we are able to identify that the increased thrombin generation in this family is also not directly related to their level of PC. For example, individuals without the PC mutation display greater thrombin generation despite the fact that their mean PC level was significantly lower than the mean physiologic value. If PC was the only contributing factor, at increasing PC concentrations, thrombin generation would be suppressed.

Consistent with our prior studies [37,51], our current findings using a computational model which includes a PC pathway

### Table 3. Plasma composition comparison within groups and stratified by gender.

| 1. PC mutation | All Subjects (Mean (SD)) | Females (Mean (SD)) | Males (Mean (SD)) |
|----------------|--------------------------|---------------------|------------------|
| FII, μM        |                          |                     |                  |
| Yes N = 81     | 1.7 (0.4)                | 1.7 (0.4)           | 1.8 (0.4)        |
| No N = 283     | 1.8 (0.4)                | 1.8 (0.4)           | 1.8 (0.4)        |
| P value        | 0.01                     | 0.05                | 0.05             |
| FV, nM         |                          |                     |                  |
| Yes N = 54     | 19.4 (6.8)               | 18.7 (6.5)          | 19.9 (6.5)       |
| No N = 159     | 20.0 (7.0)               | 21.2 (6.9)          | 21.2 (6.9)       |
| P value        | 0.18                     | 0.42                | 0.42             |
| FVII, nM       |                          |                     |                  |
| Yes N = 27     | 9.9 (2.5)                | 10.0 (2.8)          | 10.3 (2.8)       |
| No N = 124     | 9.9 (1.9)                | 9.8 (1.8)           | 9.8 (1.8)        |
| P value        | 0.64                     | 0.86                | 0.86             |
| FIX, nM        |                          |                     |                  |
| Yes N = 74     | 88 (27)                  | 94 (26)             | 97 (29)          |
| No N = 159     | 83 (23)                  | 93 (22)             | 93 (22)          |
| P value        | 0.26                     | 0.03                | 0.03             |
| FX, nM         |                          |                     |                  |
| Yes N = 160    | 160 (39)                 | 161 (38)            | 170 (38)         |
| No N = 292     | 160 (38)                 | 168 (38)            | 168 (38)         |
| P value        | 0.08                     | 0.60                | 0.60             |
| AT, μM         |                          |                     |                  |
| Yes N = 8      | 3.5 (0.6)                | 3.4 (0.6)           | 3.3 (0.6)        |
| No N = 159     | 3.7 (0.5)                | 3.3 (0.5)           | 3.5 (0.5)        |
| P value        | 0.79                     | 0.004               | 0.004            |
| TFPI, nM       |                          |                     |                  |
| Yes N = 4      | 2.2 (0.6)                | 2.0 (0.6)           | 2.3 (0.5)        |
| No N = 118     | 2.4 (0.6)                | 2.5 (0.6)           | 2.5 (0.6)        |
| P value        | 0.04                     | 0.59                | 0.59             |
| PC, nM         |                          |                     |                  |
| Yes N = 47     | 47 (29)                  | 110 (28)            | <0.001           |
| No N = 292     | 45 (28)                  | 112 (27)            | <0.001           |
| P value        | 0.17                     | 0.001               | 0.001            |

### 2. PT mutation

| FII, μM        | Yes N = 43               | 2.1 (0.4)           | 1.7 (0.4)        |
| FV, nM         | 20.3 (6.8)               | 20.4 (6.9)          | 19.1 (6.7)       |
| FVII, nM       | 10.3 (2.5)               | 10.0 (2.5)          | 10.2 (2.8)       |
| FIX, nM        | 86 (27)                  | 94 (27)             | 95 (29)          |
| FX, nM         | 178 (38)                 | 165 (39)            | 176 (39)         |
| AT, μM         | 3.3 (0.6)                | 3.4 (0.6)           | 3.3 (0.6)        |
| TFPI, nM       | 2.5 (0.6)                | 2.3 (0.6)           | 2.2 (0.6)        |
| PC, nM         | 91 (37)                  | 100 (38)            | 92 (38)          |
| P value        | 0.10                     | 0.001               | 0.001            |
| Definite N = 5 | No history N = 146       |                     |                  |
| FII, μM        | 2.0 (0.4)                | 1.8 (0.4)           | 2.1 (0.4)        |
| FV, nM         | 25.2 (6.7)               | 20.1 (6.7)          | 19.3 (6.3)       |
| FVII, nM       | 11.1 (2.5)               | 10.0 (2.5)          | 12.4 (2.8)       |
| FIX, nM        | 78 (27)                  | 94 (27)             | 96 (29)          |
| FX, nM         | 186 (39)                 | 167 (38)            | 199 (38)         |
| AT, μM         | 3.3 (0.6)                | 3.4 (0.6)           | 3.3 (0.6)        |
| TFPI, nM       | 2.6 (0.6)                | 2.3 (0.6)           | 2.5 (0.6)        |
| PC, nM         | 69 (39)                  | 97 (38)             | 67 (40)          |
| P value        | 0.10                     | 0.20                | 0.20             |

### 3. Thrombosis history

| FII, μM        | Definite N = 13           | 2.0 (0.4)           | 1.8 (0.4)        |
| FV, nM         | 25.2 (6.7)                | 20.1 (6.7)          | 19.3 (6.3)       |
| FVII, nM       | 11.1 (2.5)                | 10.0 (2.5)          | 12.4 (2.8)       |
| FIX, nM        | 78 (27)                  | 94 (27)             | 96 (29)          |
| FX, nM         | 186 (39)                 | 167 (38)            | 199 (38)         |
| AT, μM         | 3.3 (0.6)                | 3.4 (0.6)           | 3.3 (0.6)        |
| TFPI, nM       | 2.6 (0.6)                | 2.3 (0.6)           | 2.5 (0.6)        |
| PC, nM         | 69 (39)                  | 97 (38)             | 67 (40)          |

Consistent with our prior studies [37,51], our current findings using a computational model which includes a PC pathway...
component suggest that it is not one factor alone that contributes to thrombin generation dynamics in this subject group, but a combination of each individual's other plasma composition factors (as seen in Table 1). If individuals possess higher normal procoagulant and anticoagulant factors, they will generate thrombin faster than individuals with the same PC levels at lower normal procoagulant and anticoagulant levels. Thus, an individual could potentially be at a better hemostatic advantage over another even though the PC levels are equivalent.

In our study, we identify that gender appears to play a large role in this family in that women have increased thrombin generation over men. As well, plasma composition differences were identified in segregating women from men. Previously, we have shown that simulated thrombin generation was increased in healthy women,
women with a DVT [37] and women on oral contraceptives [36,46,52]. A recent study by Christiansen et al. [53] showed that approximately half of the thrombotic recurrences in women were provoked and were mainly related to oral contraceptive use. In earlier research on this family, we found that PC deficiency increased risk of thrombosis in female family members when taking oral contraceptives and during pregnancy [15,52]. Because of those studies, women in the family who are PC deficient were strongly advised against use of oral contraceptives and are almost always given prophylactic heparin during pregnancy. Further studies regarding plasma compositional differences to elucidate the mechanism behind the increased thrombin generation in women and the effect from additional thrombotic risk factors are warranted in this family.

Figure 4. Thrombin generation stratified by gender and additional risk. Males to females with an additional category of risk were compared for either PC mutation (panels A and B), prothrombin G2010A polymorphism (panels C and D), or previous history of thrombosis (panels E and F). Female profiles are shown in pink (+SD). Male profiles are shown in blue (−SD). doi:10.1371/journal.pone.0044378.g004
Although, sex differences in thrombosis have been described previously [34,35,36], their underlying mechanisms are not completely understood. Since our study involves changes in plasma composition (gender dependent) and increased procoagulant potential, one link between these two (coagulation factors and gender) can be the liver. Coagulation proteins are synthesized in the liver, and liver gene expression is sex specific and depends on sex differences in growth hormone secretion. A study by Wong et al. [57] proposed a novel mechanism whereby sex specific growth hormone patterns mediate sex differences in thrombosis through coordinated changes in the expression of coagulation inhibitor genes in the liver. It has also recently been suggested by Tripodi and Mannucci [58] that changes in the balance of pro and anticoagulants in chronic liver disease account for their coagulopathic state. Therefore, changes in liver function in relationship to gender and deficiency state should be further investigated in this family.

Materials and Methods

Participation of all individuals within the familial PC family was approved by the University of Vermont Human Studies Committee. All participants gave informed written consent.

Subjects

Our study population is a family with a history of high incidence of VTE (Kindred Vermont II) which was discovered to be PC deficient in the 1980s [22]. The cause of PC deficiency was determined to be a 3363 inserted C mutation in exon 6 of the PC gene [59]. Blood was drawn from 514 members of the extended family. Of family members, 33% are PC deficient and 9% have a gene [59]. Blood was drawn from 514 members of the extended family. Of family members, 33% are PC deficient and 9% have a verified history of deep vein thrombosis and/or pulmonary embolism. Of the 514 family members drawn, 364 (71%) were included in the current analysis. Reasons for exclusion included: were on coumadin at the time blood drawing (n = 35); unknown coumadin status at the time of blood drawing (n = 5); self-reported but unconfirmed history of DVT or PE (n = 11); history of superficial venous thrombosis but not DVT or PE (n = 18); insufficient sample for analysis (n = 6); and insufficient composition data (n = 73).

Plasma Composition Analyses

The blood collection procedure and the measurements of the levels of the coagulation proteins II, VII, IV, VIII, IX, X, IX, tissue factor pathway inhibitor (TFPI) and antithrombin (AT) from citrated plasma were described in detail in earlier studies within this family [44]. In brief, II, VII, and IX were measured by in-house-developed sandwich-type enzyme linked immunosorbent assays and IV was performed using a commercial assay (Enzyme Research Laboratories, South Bend, IN, USA). The VII, VIII, IX, IX, X and total TFPI antigen levels were measured by sandwich ELISAs using commercial kits (Asserachrom, Diagnostica Stago, Parsippany, NJ, USA). PC was measured as an activity assay. The mean (SD) of each of the factor levels are shown in Table 1.

Numerical Simulations

Our numerical model of the extrinsic coagulation system [41,60,61] provides a platform for investigating thrombin generation profiles and patterns in a large group of individuals. In this study, we are incorporating a module of equations describing the protein C pathway. This description primarily derives from a recently published study [42] combining empirical and computational analysis of central elements of this pathway. The complete model (Tables S1, S2,S3) also includes thrombomodulin (Tm) binding to thrombin and meizothrombin and the activation of PC by these complexes [62,63] and AT inhibition of thrombin-soluble thrombomodulin complexes. The computational inputs included: actual factor levels from each individual in the PC family (n = 364) for II, IV, VII, VII, IX, IX, AT, TFPI and PC that were translated into molar concentrations using the mean plasma concentration as 100% and a 5 pM tissue factor (Tf) trigger to correlate with our empirical studies [31,64]. Thrombomodulin was modeled at 1 nM which is an estimate of the concentration that would be found in medium veins and muscular arteries [65]. These estimates are however, completely based on the diameter of the vessel and the assumption of uniform levels of thrombomodulin expression on endothelial cells throughout the vasculature.

Total active thrombin was simulated at 1 s intervals over 20 minutes and the output was evaluated using the thrombin parameters (maximum level (MaxL) and rate (MaxR) and the corresponding times (TMaxL and TMaxR, respectively) and area under the curve (AUC)). Clot time (CT) was taken to be the time at which 10 nM thrombin is generated [64]. A mean physiologic control was used that sets all the factor levels at mean physiologic concentrations.

Statistics

Thrombin generation parameters were compared using variance component analysis methods described by Almasy and Blangero [66]. In this approach, models are compared using likelihood-ratio tests, with relatedness of study subjects accounted for as polygenic heritability. In our analysis age and sex were adjusted for by including them as covariates in the models.

Supporting Information

Table S1 Reaction mechanism of the computational model (list of equations). For equilibrium expressions denoted by \(-1 \rightarrow 2\), the first number listed describes the reverse/dissociation reaction (k_d); the second number listed describes the association reaction (k_a). Notation and the accompanying rate constants are listed in separate tables beneath the list of equations. Complexes are represented with an equal sign between the components. Active enzymes are listed as the zymogen followed by an “a”.

Table S2 Abbreviations used in the computational model.

Table S3 Rate constants used in the computational model.

Acknowledgments

The authors would like to thank Maria Cristina Bravo and Stephen Everse for their work in developing and validating the model description of the protein C pathway.

A portion of these results were presented at the XXII Congress of the International Society of Hemostasis, Boston MA, July 2009.

Author Contributions

Conceived and designed the experiments: KBZ. Performed the experiments: KBZ MG TO. Analyzed the data: KBZ PWC MG. Contributed reagents/materials/analysis tools: EGB KGM. Wrote the paper: KBZ.
30. Luan D, Zai M, Varner JD (2007) Computationally derived points of fragility of protein C deficiency. J Biol Chem 282: 25383–25387.

27. Leipold RJ, Bozarth TA, Racanelli AL, Dicker IB (1995) Mathematical model of thrombin generation and fibrin clot structure. Blood Rev 9: 131–145.

26. Wolberg AS (2007) Thrombin generation assays: understanding how the method influences the results. Thromb Res 119: 663–665.

25. Dahlback B, Villoutreix BO (2005) Regulation of blood coagulation by the thrombin protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. Arterioscler Thromb Vasc Biol 25: 1311–1320.

24. Wolberg AS (2007) Thrombin formation. Chest 124: 4S-10S.

23. Mann KG (2003) Thrombin formation. J Biol Chem 278: 42021–42024.

21. Allaart CF, Rosendaal FR, Noteboom WM, Vandenbroucke JP, Briet E (1995) Defining the boundaries of normal thrombin generation: investigations into stoichiometric regulation of blood coagulation. J Biol Chem 270: 25383–25387.

20. Nagashima H (2002) Studies on the different modes of action of the anticoagulant protease inhibitors IXa/IXa and Argatroban. I. Effects on thrombin generation. J Biol Chem 277: 50439–50444.

19. Panteleev MA, Ocanosov MV, Kiriev DA, Shibeko AM, Sinauridze EI, et al. (2006) Spatial propagation and localization of blood coagulation are regulated by intrinsic and protein C pathways, respectively. Biochim Biophys Acta 1764: 1489–1500.

18. Luan D, Zai M, Varner JD (2007) Computationally derived points of fragility of a human cascade are consistent with current therapeutic strategies. PLoS Comput Biol 3: e142.

17. Miletich J, Sherman L, Broze G Jr (1987) Absence of thrombosis in subjects with heterozygous protein C deficiency in the healthy population. Thromb Haemost 73: 87–93.

16. Bovill EG, Hasstedt SJ, Callas PW, Long GL (1998) An unknown genetic defect increases venous thrombosis risk, through interaction with protein C deficiency. J Thromb Haemost 6: 131–145.

15. Hasstedt SJ, Bovill EG, Long GL (1994) Molecular mechanism for familial protein C deficiency and thrombosis in protein C V235Met (Glu202–>Ala and Val34–>Met). J Biol Chem 269: 29032–29038.

14. Lu D, Bovill EG, Long GL (1994) Molecular mechanisms for familial protein C deficiency and thrombosis in protein C V235Met. J Biol Chem 269: 29032–29038.

13. Thompson AR (1994) Molecular genetics of hemostatic proteins. In: Colman RR, Marder VJ, Hirsh J, et al., editors. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia: Lippincott. pp. 55–80.

12. Le DT, Bovill EG, Long GL (1994) Molecular mechanisms for familial protein C deficiency and thrombosis in protein C V235Met. J Biol Chem 269: 29032–29038.

11. Rosendaal FR (1999) Risk factors for venous thrombotic disease. Thromb Haemost 82: 610–613.

10. Dahlback B, Villoutreix BO (2005) Regulation of blood coagulation by the thrombin protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. Arterioscler Thromb Vasc Biol 25: 1311–1320.

9. Segal JB, Brotman DJ, Necochea AJ, Emadi A, Samal L, et al. (2009) Predictive modeling of in vivo anticoagulation using protein C antithrombotic pa thway: novel insights into structure-function relationships and molecular recognition. Arterioscler Thromb Vasc Biol 29: 136–142.

8. Vossen CY, Conard J, Fouchtencb J, Makris M, BJ VDM, et al. (2005) Risk of a first venous thrombotic event in carriers of a familial thrombophilic defect. The European Prospective Cohort on Thrombophilia (EPCOT). J Thromb Haemost 3: 459–464.

7. Thompson AR (1994) Molecular genetics of hemostatic proteins. In: Colman RR, Marder VJ, Hirsh J, et al., editors. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia: Lippincott. pp. 55–80.

6. Le DT, Bovill EG, Long GL (1994) Molecular mechanisms for familial protein C deficiency and thrombosis in protein C V235Met. J Biol Chem 269: 29032–29038.

5. Egeberg O (1965) Inherited Antithrombin Deficiency Causing Thrombophilia. J Clin Invest 44: 222–226.

4. Lu D, Bovill EG, Long GL (1994) Molecular mechanisms for familial protein C deficiency and thrombosis in protein C V235Met. J Biol Chem 269: 29032–29038.

3. Prandoni P, Lensing AW, Cogo A, Cuppini S, Villalta S, et al. (1996) The long-term clinical course of acute deep vein thrombosis. Ann Intern Med 125: 1–7.

2. Middeldorp S, Briel C, Conard J (1998) Familial thrombophilia. In: Verstraete M, Fuster V, Topol EJ, editors. Cardiovascular thromboscirhombocardiography and thrombomodulation. Philadelphia: Lippincott–Raven. 59–75.

1. Silverstein MD, Heit JA, Mohr DN, Pettersen TM, O’Fallon WM, et al. (1998) Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study. Arch Intern Med 158: 503–509.
57. Wong JH, Dukes J, Levy RE, Sos B, Mason SE, et al. (2008) Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. J Clin Invest 118: 2969–2978.
58. Tripodi A, Mannucci PM (2011) The coagulopathy of chronic liver disease. N Engl J Med 365: 147–156.
59. Tomczak JA, Ando RA, Sobel HG, Bovill EG, Long GL (1994) Genetic analysis of a large kindred exhibiting type I protein C deficiency and associated thrombosis. Thromb Res 74: 243–254.
60. Orfeo T, Brufato N, Nesheim ME, Xu H, Butenas S, et al. (2004) The factor V activation paradox. J Biol Chem 279: 19580–19591.
61. Butenas S, Orfeo T, Gissel MT, Brummel KE, Mann KG (2004) The significance of circulating factor IXa in blood. J Biol Chem 279: 22875–22882.
62. Doyle MF, Mann KG (1990) Multiple active forms of thrombin. IV. Relative activities of meizothrombins. J Biol Chem 265: 10693–10701.
63. Cote HC, Bajzar L, Stevens WK, Samis JA, Morser J, et al. (1997) Functional characterization of recombinant human meizothrombin and Meizothrombin(-desF1). Thrombomodulin-dependent activation of protein C and thrombin-activatable fibrinolysis inhibitor (TAFI), platelet aggregation, antithrombin-III inhibition. J Biol Chem 272: 6194–6200.
64. Brummel KE, Paradis SG, Butenas S, Mann KG (2002) Thrombin functions during tissue factor-induced blood coagulation. Blood 100: 148–152.
65. Mann KG (2011) Thrombin generation in hemorrhage control and vascular occlusion. Circulation 124: 225–235.
66. Almasy L, Blangero J (1998) Multipoint quantitative-trait linkage analysis in general pedigrees. Am J Hum Genet 62: 1196–1211.