Association of the Thrombomodulin Gene c.1418C>T Polymorphism With Thrombomodulin Levels and With Venous Thrombosis Risk

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Objective—To investigate the association of the THBD c.1418C>T polymorphism, which encodes for the replacement of Ala455 by Val in thrombomodulin (TM), with venous thromboembolism (VTE), plasma soluble TM, and activated protein C levels. In addition, human umbilical vein endothelial cells (HUVEC) isolated from 100 umbilical cords were used to analyze the relation between this polymorphism and THBD mRNA and TM protein expression.

Approach and Results—The THBD c.1418C>T polymorphism was genotyped in 1173 patients with VTE and 1262 control subjects. Levels of soluble TM and activated protein C were measured in 414 patients with VTE (not on oral anticoagulants) and 451 controls. HUVECs were genotyped for the polymorphism and analyzed for THBD mRNA and TM protein expression and for the ability to enhance protein C activation by thrombin. The 1418T allele frequency was lower in patients than in controls (P<0.001), and its presence was associated with a reduced VTE risk, reduced soluble TM levels, and increased circulating activated protein C levels (P<0.001). In cultured HUVEC, the 1418T allele did not influence THBD expression but was associated with increased TM in cell lysates, increased rate of protein C activation, and reduced soluble TM levels in conditioned medium.

Conclusions—The THBD 1418T allele is associated with lower soluble TM, both in plasma and in HUVEC-conditioned medium, and with an increase in functional membrane–bound TM in HUVEC, which could explain the increased activated protein C levels and the reduced VTE risk observed in individuals carrying this allele.

(Key Words: endothelial cells ■ gene expression ■ protein C ■ thrombomodulin ■ venous thrombosis)

Venous thromboembolism (VTE) has a significant hereditary component,1 and some reports have assessed the genetic components of plasma variability in hemostasis-related phenotypes through family-based sampling designs.2,3 Heritabilities range from 0.11 to 0.83. The protein C (PC) anticoagulant pathway plays an important role in regulating thrombin generation and inflammatory reactions. PC circulates in plasma as a zymogen, which is activated on the surface of endothelial cells by the thrombin–thrombomodulin (TM)–endothelial cell PC receptor (EPCR) complex.4 Once activated, PC is a potent anticoagulant and anti-inflammatory protease.5–7

TM is an endothelial cell membrane protein that acts as a cofactor for thrombin in the activation of PC. TM is also independently involved in cytoprotective responses.6,9 Studies in animal models suggest that TM deficiency is associated with a prethrombotic state.10–12 Heterogeneous soluble forms of TM (sTM) circulate in plasma,13 and their levels are increased in several clinical conditions.14 Some of these soluble forms might have anticoagulant activity.15 Normal levels vary according to the assay used for the measurements, ranging from 2.7 to 5.4 ng/mL16 or from 25 to 65 ng/mL.17

Genetic studies have identified many mutations and polymorphisms in the THBD gene, but no clear association with venous or arterial thrombosis has been found.18 A common single-nucleotide polymorphism in the coding region of THBD (c.1418C>T) (rs1042579), which results in the replacement of Ala455 by Val, has been described.19 This dimorphism is located in the TM region responsible for thrombin binding and PC activation, suggesting a potential role in the modulation of thrombosis risk.20

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of TM function. However, its association with venous\textsuperscript{19–23} and arterial thrombosis\textsuperscript{24–26} is not consistent.

In the present study, we investigated the association of this polymorphism with VTE and plasma TM and activated PC (APC) levels. In addition, human umbilical vein endothelial cells (HUVEC) isolated from 100 umbilical cords were used to analyze the relation between this polymorphism and \textit{THBD} mRNA and TM protein expression. We found that the presence of the \textit{THBD} 1418T allele, which encodes for TM Val455, is associated with a decrease in sTM, both in plasma and in HUVEC-conditioned medium, and an increase in functional membrane–bound TM, which could explain the increased levels of circulating APC and the reduced risk of VTE in individuals carrying this allele.

Materials and Methods

Study design, experimental methods, and statistical analyses are described in detail in the online-only Supplement.

Our study included 1173 unrelated white patients with VTE and 1262 unrelated healthy subjects with no history of thromboembolic disease.

HUVEC from 100 umbilical cords were obtained by collagenase digestion and grown to confluence in T-75 flasks. TM levels were measured in HUVEC-conditioned medium and in cell lysates. For Western blot analysis, cell lysates (1.0 μg per lane) from representative HUVEC carrying the 1418 CC (n=2), CT (n=2), and TT (n=2) genotype were subjected from 4% to 12% SDS-polyacrylamid gel electrophoresis (PAGE) under reducing conditions and transferred to a polyvinylidene difluoride membrane. PC activation on HUVEC was performed by incubating cells with PC and bovine thrombin, in the presence or absence of a rat monoclonal anti-EPCR antibody, followed by the measurement of the amidolytic activity of the APC formed.

Genotyping of the \textit{THBD} c.1418C>T polymorphism was performed by direct sequencing and quantification of mRNA transcripts by real time–quantitative polymerase chain reaction using semiautomatic equipment (Light-Cycler Real-Time PCR Detection System, Roche, Mannheim, Germany).

Results

Clinical Characteristics of the Subjects

The clinical characteristics of the study subjects are presented in Table 1. These features did not significantly differ in the samples from the 3 hospitals (data not shown). About 32% of the patients (375 of 1173) had spontaneous thrombosis, which is defined as thrombosis in the absence of known triggering risk factors (use of contraceptives, pregnancy, surgery, trauma, and immobilization). The prevalence of classical prothrombotic polymorphisms was similar to that described in other series.

\textbf{\textit{THBD} c.1418C>T Polymorphism and VTE Risk}

The 1173 patients and 1262 control subjects were successfully genotyped for the \textit{THBD} c.1418C>T polymorphism. Table 2 shows the genotype distribution. Among the 1262 healthy subjects, the frequency of the 1418T allele was 0.175, which is similar to that reported in previous studies (0.180,\textsuperscript{19} 0.169,\textsuperscript{21} and 0.184\textsuperscript{25}) but lower than in other studies (0.261\textsuperscript{20} and 0.280\textsuperscript{25}). Given that the control subjects were recruited in 3 different geographical areas of Spain, we analyzed the 1418T allele frequency in the 3 control populations. The frequencies of the 1418T allele were 0.184, 0.172, and 0.169 in the control groups from Valencia, Murcia, and Salamanca, respectively, with no significant differences between them. Among the 1173 patients with VTE, the frequency of the 1418T allele was 0.136 (0.140, 0.136, and 0.125 in the group of patients from Valencia, Murcia, and Salamanca, respectively, with no significant differences among them). This is not so different from that observed in a previous study (0.154\textsuperscript{19}) but lower than that reported in other studies (0.213,\textsuperscript{20} 0.196,\textsuperscript{21} and 0.180\textsuperscript{25}).

To identify the associations between genotypes and VTE, we performed logistic regression analyses (Table 2). In a univariate analysis, the presence of the 1418T allele or the TT genotype significantly reduced the risk of VTE. Adjustment for sex, age, and the presence of thrombophilic defects did not significantly modify the odds ratio (OR). When the analyses were performed in the subgroups of patients with rethrombosis (adjusted OR for TT: 0.41, 0.14–1.20) or without rethrombosis (adjusted OR for TT: 0.56, 0.30–1.05), and in the subgroup of patients with provoked risk factors (0.60, 0.26–1.24) or without provoked risk factors (0.48, 0.25–0.92), the ORs did not significantly change.

When both patients and controls were grouped into 2 subgroups, according to whether they were >45 years or not, the protective effect of the 1418T allele was even more pronounced in the younger individuals (adjusted OR for CT and TT: 0.55, 0.43–0.71), whereas the protective effect of the 1418T allele disappeared in those subjects aged >45 years (1.06, 0.81–1.39).

\textbf{sTM Levels and VTE Risk}

To investigate whether sTM levels associated with the risk of VTE, we compared sTM levels in 414 patients with VTE (excluding those undergoing coumarin therapy) and in 451...
sTM was significantly higher in the 414 patients with VTE than in the 451 healthy individuals ($P=0.005$; Table 3). To study whether the sTM plasma levels were influenced by the presence of the THBD 1418T allele, we also calculated these levels according to the genotype, in overall individuals as well as in the subgroups of individuals aged <46 years or >45 years (Table 3). In both patients and controls, sTM levels significantly decreased when the number of 1418T alleles increased ($P<0.001$).

We also analyzed the distribution of sTM levels in patients and controls according to sex. Among the patients with VTE, sTM was higher in men (4.18, 3.70–4.86) than in women (3.88, 3.39–4.68; $P=0.001$). Also in controls, sTM was higher in men (4.09, 3.42–4.76) than in women (3.72, 3.3–4.23; $P<0.001$). These results are in agreement with those in previous studies.16,28

To assess whether the sTM levels were associated with an increased risk of VTE, we distributed sTM levels into quartiles, as measured in the healthy control group. By taking the first quartile as the reference group, the ORs for the second, third, and fourth quartiles were 1.23 (0.81–1.87), 1.72 (1.16–2.56), and 1.75 (1.18–2.60), respectively, after adjusting for age, sex, and the presence of thrombophilic defects ($P$ for trend <0.001).

Because sTM levels are apparently associated with the THBD c.1418C>T polymorphism, we performed a multivariate analysis to include age, sex, presence of thrombophilic defects, sTM<3.86 ng/mL (50th percentile of the distribution among controls), and the presence of the 1418T allele in the model. The ORs for the presence of the 1418T allele (CT and TT carriers) were 0.75 (0.62–0.90) in the absence and 0.61 (0.45–0.84) in the presence of sTM<3.86 in the model. The OR for sTM<3.86 was 0.65 (0.49–0.86) when the 1418T allele was not present in the model, and 0.61 (0.45–0.84) when the 1418T allele was present, suggesting that both parameters independently protect against VTE.

## Activated PC

Given that one possible mechanism by which the carriers of the 1418TT genotype have a decreased risk of VTE is the presence of increased plasma APC levels, we also determined the plasma levels of APC in 414 patients with VTE (excluding those undergoing coumarin therapy) and 451 healthy individuals in whom sTM was measured (Table 3). As previously reported,29 the level of circulating APC was lower in patients

### Table 2. Genotype and Allele Distribution of THBD Gene c.1418C>T Polymorphism in 1173 Patients and 1262 Control Subjects

<table>
<thead>
<tr>
<th>THBD c.1418C&gt;T Genotype</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>Crude</th>
<th>Adjusted*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>875 (74.6)</td>
<td>857 (67.9)</td>
<td>1†</td>
<td>1†</td>
</tr>
<tr>
<td>CT</td>
<td>277 (23.6)</td>
<td>368 (29.2)</td>
<td>0.74 (0.61–0.88)</td>
<td>0.77 (0.63–0.93)</td>
</tr>
<tr>
<td>TT</td>
<td>21 (1.8)</td>
<td>37 (2.9)</td>
<td>0.56 (0.32–0.96)</td>
<td>0.55 (0.31–0.98)</td>
</tr>
<tr>
<td>CT and TT</td>
<td>298 (25.4)</td>
<td>405 (32.1)</td>
<td>0.72 (0.60–0.86)</td>
<td>0.75 (0.62–0.90)</td>
</tr>
<tr>
<td>T allele</td>
<td>319 (13.6)</td>
<td>442 (17.5)</td>
<td>0.74 (0.63–0.87)</td>
<td>-</td>
</tr>
</tbody>
</table>

CI indicates confidence interval; and OR, odds ratio.
*Adjusted for age, sex, and presence of thrombophilic defects (factor V Leiden, prothrombin 20210A, protein C deficiency, protein S deficiency, and antithrombin deficiency).
†Reference group.

### Table 3. Levels of sTM and APC (Median and 25th–75th Percentile) in 414 Patients (Without Coumarin Therapy) and 451 Controls According to the THBD gene c.1418C>T Polymorphism

<table>
<thead>
<tr>
<th>c.1418C&gt;T Genotype</th>
<th>All Individuals</th>
<th>CC Carriers</th>
<th>CT Carriers</th>
<th>TT Carriers</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTM, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>4.06 (3.56–4.67)</td>
<td>4.18 (3.70–4.86)</td>
<td>3.77 (3.48–4.11)</td>
<td>3.28 (2.79–3.57)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>(n=414)</td>
<td>(n=319)</td>
<td>(n=86)</td>
<td>(n=9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>3.86 (3.38–4.48)</td>
<td>4.05 (3.51–4.68)</td>
<td>3.68 (3.21–4.26)</td>
<td>2.99 (2.60–3.45)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>(n=451)</td>
<td>(n=293)</td>
<td>(n=138)</td>
<td>(n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005†</td>
<td>0.008†</td>
<td>0.435†</td>
<td>0.517†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>0.98 (0.72–1.22)</td>
<td>0.97 (0.72–1.23)</td>
<td>0.97 (0.72–1.17)</td>
<td>1.23 (0.79–1.41)</td>
<td>0.386*</td>
</tr>
<tr>
<td>(n=414)</td>
<td>(n=319)</td>
<td>(n=86)</td>
<td>(n=9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1.20 (0.98–1.49)</td>
<td>1.19 (0.95–1.47)</td>
<td>1.22 (1.00–1.61)</td>
<td>1.29 (1.13–1.54)</td>
<td>0.191*</td>
</tr>
<tr>
<td>(n=451)</td>
<td>(n=293)</td>
<td>(n=138)</td>
<td>(n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.001†</td>
<td>&lt;0.001†</td>
<td>&lt;0.001†</td>
<td>0.724†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

APC indicates activated protein C; and sTM, soluble thrombomodulin.
*Kruskal–Wallis Test; †Mann-Whitney U test.
with VTE (0.98; 0.72–1.22) than in controls (1.20; 0.98–1.49; P=0.001). We also distributed the APC levels according to the THBD c.1418C>T genotype. In both patients and controls, there was a trend to higher APC levels when the number of 1418T alleles present increased, although (given the low number of subjects carrying the TT genotype) these differences were not significant.

An inverse correlation was observed between the APC and sTM levels among controls (r = −0.161; P=0.001).

**TM and mRNA Levels in HUVEC**

To further investigate whether the THBD c.1418C>T polymorphism is functional, we analyzed the HUVECs derived from 100 human umbilical cords from white newborns. Three cultures were 1418TT, 26 were 1418CT, and 71 were 1418CC. In these HUVECs, we determined the amount of TM in culture medium (sTM) by ELISA and in the cell lysates (membrane-bound TM) by ELISA and Western blot. TM levels in the cell lysate increased (P<0.001) and the level of sTM in the cultured medium decreased (P<0.001) in parallel with the increase of the number of 1418T alleles present (Figure 1). Western blot data confirmed the results obtained by ELISA as follows: the concentration of TM increased when the number of 1418T alleles increased (P<0.001; Figure 2).

We also determined the THBD mRNA levels in 95 HUVECs in which mRNA was available. THBD mRNA levels in the 67 HUVECs carrying the 1418CC genotype (7.5, 4.1–8.9) did not significantly differ from those found in the 26 HUVECs with the 1418CT genotype (6.1, 3.6–6.6) and in the 2 HUVECs with the 1418TT genotype (6.8 and 3.0; P=0.496).

**PC Activation on HUVEC**

PC activation on HUVEC carrying the 1418CC, CT, and TT genotypes was compared (Figure 3). Because 2 haplotypes of the PROCR, H1 and H3, have been associated with the rate of activation of PC in cultured HUVEC30 and with the levels of APC in human plasma,28,31 we selected HUVEC with the same PROCR genotype (H1H2). The rate of PC activation increased in parallel with the increase of the number of 1418T alleles present. Thus, the activation of PC on HUVEC CC was ≈60% of the activation on HUVEC TT. To estimate the amount of PC activation that is attributable to TM in the absence of EPCR, HUVECs were incubated with mAb RCR-379 to block EPCR-dependent PC activation. Incubation with the anti-EPCR antibody drastically reduced PC activation, but residual PC activation still was higher on HUVEC TT than on HUVEC CC (Figure 3).

**Figure 1.** Thrombomodulin (TM) levels in conditioned medium (CM) and cell lysates from human umbilical vein endothelial cells (HUVEC), according to their c.1418C>T genotype. HUVEC were grown from 80% to 100% confluence and exposed for 3 hours to free serum-CM (50 µL/well), as indicated in the online-only Data Supplement. Thereafter, CM was stored at −72 ºC, and cells were lysed with 50 µL/well of lysis buffer. Levels of TM in CM and cell lysates were measured as indicated in the online-only Data Supplement. Data are expressed as mean±SEM; *Kruskal–Wallis test.

**Figure 2.** Western blot analysis of human umbilical vein endothelial cells (HUVEC) thrombomodulin (TM) expression according to the THBD gene c.1418C>T polymorphism. A, Cell lysates were prepared as indicated in the online-only Data Supplement. One microgram of representative lysates from 2 CC, 2 CT, and 2 TT HUVECs was electrophoresed on a 4% to 12% gradient SDS-polyacrylamide gel, electroblotted onto polyvinydene difluoride membranes, and stained with anti-TM and anti β-actin antibodies. B, TM protein in arbitrary optical units (AU) expressed as means±SD of 2 different HUVECs of each genotype. Values were normalized by β-actin and taking the 1418CC genotype as 100, P<0.001 (Kruskal–Wallis test).

**Figure 3.** The rate of protein C activation increases with the increase in the number of THBD 1418T alleles present. Human umbilical vein endothelial cells (HUVEC) from 2 CC, 2 CT, and 2 TT genotype were incubated with protein C and thrombin at 37°C for half-an-hour as described in the online-only Data Supplement. The amount of activated protein C (APC) formed/2×10⁴ cells is plotted against the genotype indicated. The results represent the mean±SD of 2 separate experiments performed in duplicate. Solid bars represent protein C activation in the absence of anti-endothelial cell protein C receptor (EPCR) mAb, RCR-379. Open bars represent protein C activation in the presence of anti-EPCR mAb, RCR-379. In the absence of cells, the rate of protein C activation was ≈0.2 nmol/L APC.
Discussion
In the present study, we observed that carriers of the 1418T allele in the THBD gene show a lower risk of VTE than noncarriers. We also found that the risk of VTE increased at increasing levels of plasma sTM, and that the presence of the 1418T allele and low sTM levels was independently associated with a decrease in the risk of VTE. Furthermore, we showed that HUVEC carrying the 1418T allele had significantly higher membrane-bound TM and lower sTM levels in culture medium than those not carrying, whereas the THBD mRNA level was similar in carriers and noncarriers. Finally, we showed that cellular TM activity, defined as the thrombin-mediated PC activation on intact HUVEC, was also higher in TT cells than in CC cells.

Several studies have investigated the association between the THBD c.1418C>T polymorphism and venous and arterial thrombosis, with conflicting results. Some studies did not find an association between the polymorphism and the risk of VTE, although 1 study observed a lower risk in blacks carrying the 1418T allele. One study observed a higher risk in the presence of the 1418T allele. Regarding the risk of arterial thrombosis, 1 study found a protective effect for the T allele, whereas another observed an increased risk associated with the presence of the T allele. Wu et al reported that the 1418T allele increased the risk of coronary heart disease in blacks, but not in whites. An explanation for these discrepancies may lie in differences in study designs and patient populations, and in small sample sizes. In fact, the sample size in the majority of the previous reports ranged from 18 to 302 patients with VTE, whereas in most studies the age of patients and controls was quite higher than in our study. To further explore the possibility that age could explain the observed differences, we analyzed the association of the c.1418C>T polymorphism with the risk of VTE after classifying patients and controls in 2 subgroups using a cutoff point of 45 years. The adjusted OR for CT and TT was 0.55 (0.43–0.71) in individuals aged ≤45 years and 1.06 (0.81–1.39) in individuals aged >45 years. This might indicate that the protective effect of the 1418T allele against VTE is mainly manifested in younger people. Further studies are needed to confirm and analyze this age-related effect in more detail.

The mechanism by which the 1418T allele protects against VTE is not clear. One explanation could be that the Val455 form has higher cofactor activity for PC activation. However, recombinant Val455 and Ala455, produced by Cos-1 cells, were found equally active in PC activation. Another explanation could be that the 1418T allele is associated with increased membrane-bound TM, resulting in increased PC activation. In fact, the present study shows that the 1418T allele is associated with increased TM in cell lysates and with decreased sTM levels in both plasma and HUVEC-cultured medium. This study also shows that the increased amount of TM in the cell lysates is associated with increased PC activation, which would result in the subsequent increase in circulating APC, as observed in the present study in the plasma of both control subjects and patients with VTE, and a decrease in the risk of VTE. Because there were no significant differences in THBD mRNA levels according to the c.1418C>T polymorphism in HUVEC, together these results suggest that the membrane-bound TM carrying the 455Val (1418T allele) might be more stable and less prone to shedding. Soluble TM represents cleaved forms of membrane-bound TM with loss of part of the serine–threonine rich region, the transmembrane domain, and the cytoplasmic tail. The 455Val residue is located not far from the presumed cleavage site and may induce a protection from TM cleavage by proteases.

Alternatively, the observed association between the THBD c.1418C>T polymorphism and the risk of VTE could be attributed to another polymorphism in tight linkage disequilibrium with c.1418C>T. In fact, we previously observed complete linkage disequilibrium (r²=0.98) between the c.1418C>T and c.2729A>C (rs3176123) polymorphisms, and a moderate linkage disequilibrium between the c.1418C>T and c.–1208-1209delTT (r²=0.27).

As to the levels of sTM, our results indicate that increased levels are associated with higher VTE risk, and this association is, at least in part, independent of the THBD c.1418C>T polymorphism. Increased sTM levels have also been reported in patients with recurrent VTE. In contrast, 1 report did not find association between sTM and risk of VTE.

In conclusion, this work provides new data on the age-dependent association between the presence of the 1418T allele in the THBD gene and a lower VTE risk. Our results suggest that the 1418T allele in the THBD gene may produce a more stable TM that is protected from shedding, which will result in higher PC activation rate and a lower risk of VTE.

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Disclosures
None.

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Significance
Thrombomodulin (TM) is an important co-factor for the activation of protein C by thrombin on the endothelial cell membrane. In animal models, TM deficiency has been associated with thrombosis risk. Several variants of the human THBD gene have been reported, but their association with thrombosis is not conclusive. One of these is the THBD c.1418C>T polymorphism. In the present study, we demonstrated that in cultured human umbilical vein endothelial cells the THBD 1418T allele is associated with increased levels of functionally active membrane–bound TM and lower levels of TM in the human umbilical vein endothelial cells-conditioned medium. In concordance, individuals with the THBD 1418T allele showed lower plasma levels of soluble TM, higher plasma levels of circulating activated protein C, and a reduced risk of venous thromboembolism. Together, these data support the view that the THBD c.1418C>T polymorphism is functional and that the 1418T allele protects from venous thromboembolism. Identification of new genetic variants associated with thrombosis will contribute to future strategies to estimate the thrombosis risk of an individual.
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Supplementary Methods:

Patients and controls. Our study included 1173 unrelated Caucasian patients with VTE. Patients with an objectively confirmed episode of VTE who consecutively entered the anticoagulation clinics from 3 Spanish hospitals in a time window of 2-7 years were enrolled. Objective diagnoses were made by clinical probability, D-dimer levels, compression ultrasonography, ventilation perfusion lung scan and, when necessary, phlebography or pulmonary angiography. Those patients with known malignant disorders were excluded. The control group included 1262 unrelated healthy subjects with no history of thromboembolic disease. Controls were randomly and prospectively selected to match cases by age, gender and geographic distribution.

All subjects gave their informed consent to enter the study, which was approved by the Ethics Committees of each participating institution, and was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Blood collection. Blood was collected at least 6 month after the acute event. For the thrombophilic study and the determination of sTM, blood was collected in vacuum tubes containing 0.129 M trisodium citrate. For APC determination, blood was collected as indicated before. Blood tubes were centrifuged at 1500 x g for 30 min at 4 °C. Plasma was frozen and stored at -72 °C. Tubes containing K3EDTA were used for DNA studies.

Plasma thrombomodulin and APC measurement. Levels of plasma sTM were measured with the Imubind Thrombomodulin ELISA kit (American Diagnostica, Stamford, CT). The intra- and inter-assay coefficients of variation were 4.5% and 5.8%, respectively, and the lower limit of detection was 0.4 ng/ml. TM in the HUVEC-CM and in the cell lysates was measured using the Asserachrom TM kit (Diagnostica Stago, Asnières-sur-Seine, France). The intra- and inter-assay coefficients of variation were 3.6% and 5.1%, respectively, and the lower limit of detection was 1.0 ng/ml. Circulating APC was determined as previously reported.

Isolation of HUVEC. HUVEC from 100 umbilical cords were obtained by collagenase digestion and grown to confluence in T-75 flasks precoated with endothelial cell attachment factor (Sigma-Aldrich Co, Germany), in medium M199 1X (+) Earle’s, 2mM L-glutamine, 25 mM HEPES, L-aminoacids (Gibco, Life Technologies, Paisley, UK), supplemented with 20% fetal bovine serum (FBS) (Cultek, Madrid, Spain), 1% endothelial cell growth factor (ECGF) (Sigma-Aldrich Co, Germany), 1 mM sodium pyruvate (Gibco), 50 U/ml penicillin, and 50 µg/ml streptomycin sulphate (Gibco) in an atmosphere of 95% air-5% CO₂. Confluent endothelial cell monolayers were harvested from the culture flasks with 0.25% trypsin/EDTA solution/0.02% PBS (Biochrom, Cambridge, UK). Cells were used within two passages. Cells to be used for total RNA isolation were stored at –72 °C after adding 5 volumes of RNAlater™ (Ambion, Austin, TX).

Thrombomodulin measurements in HUVEC. For TM determination in HUVEC-CM and cell lysates, cells were plated in 96-well culture plates, at a density of approximately
20x10^3 cells/well, and grown to reach 80-100% confluence in the aforementioned CM. Confluent subcultures of HUVEC were incubated for 3 hours with 50 µl of CS-C medium without serum for endothelial cell lines (Sigma-Aldrich Co), supplemented with 2% FBS, 1% ECGF, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin sulphate. After the incubation, the conditioned medium was stored at -72 ºC and cells were washed three times with PBS. For the measurement of TM in cell lysates, washed cells were stored overnight at -80ºC and then treated with 50 µl of lysis buffer pH 7.4 per well (20 mM Tris, 6 mM NaCl, 1% Triton X-100, 5 mM EDTA and 1 mM phenylmethylsulphonyl fluoride), during 2 hours with constant shaking, at 4 ºC.

**Western blot detection of thrombomodulin.** For Western blot analysis, cell lysates (1.0 µg per lane) from representative HUVEC carrying the CC (N=2), CT (N=2) and TT (N=2) genotypes were subjected to 4-12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and transferred to a PVDF membrane. The membrane was blocked for 1 h at room temperature with 1% BSA in Tris-buffer solution containing 0.05% Tween 20 and then incubated for 2 h with a primary antibody in the same buffer at room temperature. The primary detection antibody used was a rabbit monoclonal anti-TM antibody (1/100) (Abcam, Cambridge, UK). A monoclonal anti-beta-actin antibody (1/500) (Sigma-Aldrich Co, Missouri, USA) was used as loading control of the blots. Then, the bands were visualized using an acid phosphatase conjugated secondary antibody and the nitro-blue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Sigma) substrate system. Finally, the bands were digitalized using an image analyser (DNR Bio-Imaging Systems, Israel) and quantified by the Gel Capture (v.4.30) and the TotalLab TL-100 (v.2008) software.

**Protein C activation on HUVEC.** Confluent HUVEC in 96-well plates were washed two times with 50 mM Tris-HCl, pH 7.5, 2 mM CaCl_2_, 0.6 mM MgCl_2_, 100 mM NaCl, 1% BSA (buffer A). The activation of human PC was initiated by the addition of 100 nM human PC and 2 U/ml bovine thrombin (final concentrations) in a total volume of 50 µl. After 30 min at 37 ºC, the reactions were stopped by addition of 50 µl of hirudin (50 U/ml). 75-µl aliquots of the supernatants were transferred into a 96-well microplate and amidolytic activity of APC was determined by adding 15 µl of 8 mM S-2366 in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl. The concentration of APC was determined by comparison to a standard curve of amidolytic activity vs. APC concentration constructed with fully activated PC. Under the conditions used in this study, <10% of the PC was activated during the assay. All measurements were performed in duplicate. Where indicated, 50 µg/ml of rat monoclonal anti-EPCR antibody RCR-379 (Abcam, Cambridge, UK) was added to the cells 15 min prior to the addition of PC and thrombin, after which cells were washed. This antibody blocks the ability of EPCR to enhance PC activation by TM.

**Genotyping of the THBD c.1418C>T polymorphism.** Genomic DNA was isolated from plasma and HUVEC using the Wizard Genomic DNA purification kit (Promega, Madison, WI), following the manufacturer’s instructions. The THBD c.1418C>T polymorphism (rs1042579) was analyzed by direct sequencing with the ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA), using the following set of primers: forward 5´-GTGGCTTCGAGTGCCACTGC-3´ and reverse 5´-CGCACTTTGTACTCCATCTTGCCCTG-3´. The reaction mixture contained 3 µl of 7 ng/µl DNA, 10 µl of 5X colorless Go Taq®Flexi buffer (Promega), 3 µl of 25 mM MgCl_2_, 1 µl of dNTPs (10 mM/each), 0.3 µl of 10 pmol/µl forward primer, 0.135 µl of
1485 ng/µl reverse primer, 0.25 µl of 5 U/µl Taq DNA polymerase (Promega) and 40.27 µl of dH₂O. The reaction mixture was incubated at 95°C for 4 minutes, followed by 33 cycles of 95°C for 45 s, 66°C for 45 s and 72°C for 45 s, with a final extension of 4 min at 72 °C. THBD gene numbering is according to GenBank Accession ID NM_000361.

**mRNA isolation and real-time quantitative PCR analysis.** Total RNA was isolated from HUVEC using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, and genomic DNA was digested using 1 U/µl of DNase I Amp grade (Invitrogen), according to the manufacturer’s instructions. RNA concentration was measured at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). First strand cDNA was synthesized from the reverse transcription of 1 µg of total RNA in a final reaction volume of 20 µl using the SuperScript III RT and Oligo(dT)₁₂₋₁₈ (Invitrogen). Samples were incubated at 65°C for 5 minutes, and cooled for 1 minute on ice. Then, the reaction mixture was incubated with 4 µl 5X First-Strand Buffer, 1 µl DTT 0.1 M, 1 µl RNaseOUT (40 U/µl) (Invitrogen, Paisley, UK) and 1 µl SuperScript III RT (200 U/µl) at 50°C for 30-60 minutes. Reverse transcriptase was inactivated by heating for 15 minutes at 70°C then cooling for 5 minutes on ice. Finally, 1 µl RNase H (2 U/µl) (Invitrogen) was added, and the mixture was incubated at 37°C for 20 minutes. cDNA was stored at -20°C.

Quantification of mRNA transcripts was performed following the previously reported protocol.³ The primer sequences were: forward 5´-TAA CGA AGA CAC AGA CTG CGA TT-3´ and reverse 5´-CTA GCC CAC GAG GTC AAG GT -3´. After testing several housekeeping genes, the results were normalized using the TATA-binding protein (TBP) transcripts as best control. RT-qPCR was carried out using a semi-automatic equipment (Light-Cycler Real-Time PCR Detection System, Roche, Mannheim, Germany). Each 15 µl reaction contained 2 µl of 1/10 diluted cDNA, 1.5 µl of 10X LightCycler® FastStart DNA Master Mix, 1.5 µl of 25 mM MgCl₂, 0.188 µl of 2 U/µl UNG (only for THBD qPCR), 1.5 µl of 10 µM THBD primer mix and 0.75 µl TBP primers mix (6 pmol/µl each) and 7.1 µl of nuclease-free H₂O. The amplification reaction for the THBD gene was initially incubated at 40°C for 10 minutes, followed by 95°C for 10 min, and 40 cycles of 5 seconds at 95°C, 10 seconds at 60°C, and 5 seconds at 72°C.

**Statistical analysis.** The linkage disequilibrium between polymorphisms was analyzed with the HaploView program. Other statistical analyses were conducted using the SPSS for Windows release 11.5 statistical software (SPSS Inc., Chicago, IL). Data are reported as medians and interquartile ranges (25th - 75th percentiles) or means±SEM. Allele frequencies were calculated by gene counting. The Chi-squared test was used to compare percentages. Parameter levels were compared with the Mann-Whitney U-test or the Kruskal-Wallis One ANOVA test. Correlations were assessed by the Spearman test. Logistic regression analysis was done to identify the associations between genotypes and VTE risk. Multivariate analysis was performed using multiple logistic regression by including all the significant covariates in a single step. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from the logistic model. Any differences with a 2-tailed $P$ value of <0.05 were considered statistically significant.

**Supplementary References:**
