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Identification of genetic aberrations in thrombomodulin gene in patients with recurrent venous thromboembolism

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Running title: *THBD* polymorphisms in recurrent VTE patients

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Abstract:

Introduction: Thrombomodulin (THBD) serves as a cofactor for thrombin-mediated activation of anticoagulant protein C pathway. Genetic aberrations in *THBD* have been studied in arterial and venous thrombosis. However, their role in risk assessment of recurrent venous thromboembolism (VTE) is not well understood. Aim of the present study was to identify the genetic aberrations in *THBD* and their association with the risk of VTE recurrence in a prospective population based study.

Material and methods: We sequenced the entire *THBD* gene, first in selected VTE patients (n=95) by Sanger's sequencing and later validated those polymorphisms with minor allele frequency (MAF) ≥ 5% in whole study population (n=1465 with follow up period 1998-2008) by Taqman PCR.

Results: In total, we identified 8 polymorphisms in *THBD* and 3 polymorphisms with MAF ≥ 5% were further validated. No significant association between *THBD* polymorphisms and risk of VTE recurrence on univariate or multivariate Cox regression analysis was found [Hazard ratios (HR) =0.89 and 95% confidence intervals (CI) =0.62-1.28, HR=1.27, CI=0.88-1.85 and HR=1.15, CI=0.80-1.66 for *THBD* rs1962, rs1042580 and rs3176123 polymorphisms respectively] adjusted with family history, acquired risk factors for VTE, location of DVT and risk of thrombophilia. Sub-analysis on unprovoked first VTE also showed no significant association with the identified *THBD* polymorphisms and risk of VTE recurrence.

Conclusion: Our results show that aberrations in *THBD* gene may not be useful for assessment of VTE recurrence; however, further studies with large sample size are needed to confirm these findings.

Key words: recurrent VTE, multivariate analysis, anticoagulant therapy.

Introduction:

Venous thromboembolism (VTE), that includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is a frequent, potentially lethal disease with an incidence rate of 1-2 cases per 1000 persons-years [1,2]. Patients with first episode of VTE are at increased risk of new events of VTE. The risk of recurrence varies with time after first event, it is higher in first 6-12 months and never becomes zero [3]. Rate of VTE recurrence is increased with time and is reported as 17.5% 24.6% and 30.3% after 2, 5 and 8 years of first diagnosis with primary VTE respectively [4]. Previous studies show that the rate of recurrence is higher in unprovoked VTE (without known acquired risk factors for VTE e.g. older age, trauma, major surgery, immobilization, female hormone therapy, pregnancy) as compared to those patients with provoked VTE [5]. Standard treatment regimen of acute VTE is the use of anticoagulant drugs for several months. Anticoagulant therapy is a "double-edged sword" that is efficient to prevent from recurrent VTE, albeit at the cost of severe bleeding with a fatality rate of 11.3% [6,7]. Despite the number of known risk factors such as sex, residual thrombosis and D dimers level, the probability of VTE recurrence after stopping the anticoagulation treatment could not be predicted precisely [6-8]. From the clinical perspective, therefore it is important to identify new biomarkers which allow early prediction of patients with high and low risk of VTE recurrence for better treatment strategies.

The risk of venous thrombosis is increased when the hemostatic balance between pro- and anti-coagulant forces is shifted in favor of coagulation. When this is caused by an inherited defect, the resulting hypercoagulable state is a lifelong risk factor for thrombosis. Familial and twin studies suggest that the heritability of VTE is as high as 50-60% that could be explained by genetic factors and most of which are still unknown [9,10]. Germain *et al*. suggest that 7% of total genetic variation involved in VTE susceptibility might be explained by the chromosome 20 because it may harbor unidentified genetic variants [11].

Even though a number of genes involved in VTE have been identified, the major part of the heritability for VTE remains unknown. Role of *THBD* in coagulation is well defined in pre-clinical models, i.e., it has an indirect anticoagulant function. Animal model data suggests that transgenic mice with *THBD* mutations (targeted point mutation that substitute the glutamic acid 404 with proline) possess a prothrombic disorder [12]. Another study showed that mice with ablated *THBD* died right after the birth because of the consumptive coagulopathy [13]. *THBD* is localized on chromosome 20 which transcribes a transmembrane protein that is present on the surface of vascular endothelial cells. *THBD* have at least three independent activities involved in anticoagulation pathway: inhibition of thrombin by antithrombin; activation of protein C; and inhibition of thrombin mediated clotting (activation of procoagulant factors V, VIII, XI, XIII and platelets) by altering the substrate specificity [14,15].

First mutation, a missense, in *THBD* gene was identified by Ohlin *et al.* predicting a change in amino acid from Asp468 to Tyr [16]. Clinical studies have shown numerous functional genetic alterations in *THBD* that lead to impaired function of *THBD* in arterial and venous thrombosis [17-20]. Several studies have analyzed the genetic aberrations in *THBD* and their association with risk of primary VTE with controversial results [20-26]. However, there is not much information available on the role of genetic aberrations in *THBD* gene in recurrent VTE. To investigate genetic aberrations related to VTE recurrence, we in this study sequenced the whole *THBD* gene including promotor region -1600 base pairs (bps), exon, 5´ and 3´ untranslated regions (UTRs) in a well-established recurrent VTE cohort. To our knowledge, this is the first study in which the whole *THBD* gene is sequenced in prospective, follow up study of recurrent VTE patients.

Materials and methods:

Study subjects

VTE patients (n=1465) were selected from Malmö thrombophilia study (MATS), a prospective population based study of consecutive unselected VTE patients performed at Skåne University Hospital. MATS is a well characterized cohort in which VTE patients were included and followed from 1998 until VTE recurrence or death or at the end of the study (December 2008) [27,28]. The inclusion criteria in MATS were: an objective diagnosis of DVT, PE or recurrence performed by duplex ultrasonography, phlebography, computed tomography (CT), lung scintigraphy or magnetic resonance imaging (MRI), age >18 years and patients' ability to communicate in Swedish. For the screening of hospital records of VTE patients, a research nurse was assigned. Rate of consensual participation in MATS was 70%. The remaining 30% VTE patients were excluded because they did not participate in questionnaire, language problems, and presence of other severe diseases and in a few cases, dementia and unwillingness to participate in MATS. Immobilization and cast therapy, hospitalization, surgical intervention, malignancies that were diagnosed previously or at diagnosis of VTE, hormonal therapy, use of contraceptive pills, pregnancy and postpartum period (first 6 weeks after delivery), family history of VTE (history of VTE in first degree relatives), VTE events before inclusion to study, VTE recurrence during follow up period and location of DVT at inclusion were recorded.

Malmö University Hospital standard protocol was used to treat all the VTE patients, i.e. with low molecular weight heparin (LMH) or unfractionated heparin (UFH) and then with warfarin as an oral anticoagulants (OAC). Malmö University Hospital treatment protocol recommends 3-6 months OAC therapy for first-time VTE with consideration of extension of treatment if VTE recurrence occurs. Thrombophilia was defined as presence of the factor II G20210A mutation (rs1799963), factor V Leiden (FVL) mutation (rs6025) or a level below the laboratory

reference range of protein C (<0.7 kilo international unit (kIU)/L), antithrombin (<0.82 kIU/L) or free protein S (women <0.5 kIU/L, men <0.65 kIU/L) in patients without warfarin treatment.

After stopping anticoagulant treatment, follow up period (Mean ± SD, 3.9 ± 2.5) was counted in years until the diagnosis for VTE recurrence or the end of study (December, 2008).

All the participants provided written permission before their inclusion to the study according to the declaration of Helsinki. Ethical approval for this study was obtained from the Lund University ethical committee.

Laboratory methods

DNA extraction and primer designing

Whole blood was used to extract the DNA by using the QiAmp 96 DNA Blood Kit (Qiagen, Hilden, Germany). Online software, Primer-Blast, by National Center for Biotechnology Information (NCBI) was used to design the polymerase chain reaction (PCR) primers for *THBD* gene including promotor region (-1600bp), exon, 5´UTR and 3´UTR [29]. *THBD* gene sequence was obtained from the publicly available NCBI database (NCBI Reference Sequence: NC_000020.11, GI: 568815578). M13 tailed primer sequences (M13 forward (F) = TGTAAAACGACGGCCAGT, M13 reverse (R) = CAGGAAACAGCTATGACC) were added to each PCR primer before primer synthesis to get better sequencing results. To minimize the chances of sequencing errors at the beginning and end point of the amplicon, during the primer designing it was kept in mind that each primer should overlap the amplicon of next primer for at least 100 base pairs. Primers were subjected to PCR for optimization at different temperatures. A total of 10 primer pairs were designed to sequence the above mentioned *THBD* gene regions. Primer sequences (forward and reverse primers) along with their amplicon lengths are provided in Table 1.

Patient selection and *THBD* sequencing

From 1465 VTE patients, initial screening was performed on age at first VTE and sex matched non-recurrent (n=60) and recurrent VTE patients (n=35) samples. Sanger's sequencing was used to sequence the *THBD* gene in selected samples [30]. PCR amplification of *THBD* was performed by using BigDye® Direct Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, for each sample, 2.5µl of deionized water, 5.0µl of big dye direct master mix (MM), 1.5µl of M13 tailed PCR primers (0.8µM) and 1.0µl genomic DNA (5ng/µl) was used for first PCR amplification. Following thermal cycling conditions were used to perform PCR, denaturation for 5 minutes (min) at 95°C for 1 cycle, followed by 35 cycles of 30 seconds (sec) at 94°C, 45 sec at 62°C, and 45 sec at 68°C. The last cycle was performed at 72°C that lasts for 2 min. In second PCR, amplified PCR product from 1st PCR was added with BigDye® sequencing MM (2µl) and BigDye® Direct M13 forward/reverse primers (1.0 µl) to run second PCR with following thermal conditions; start with 37°C for 15 min, 80°C for 2 minutes, 96°C for 1min followed by 25 cycles of 10sec at 96°C, 5 sec at 50°C and 4 min at 60°C. All PCR reactions were conducted in T100 Thermal Cycler (Bio-Rad Laboratories, Marnes-la-Coquette, France). DyeEx® 96 Kit (QIAGEN, Hilden, Germany) was used to purify the PCR product according to the manufacturer's instructions. Purified DNA samples were sent to Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany) for Sanger's sequencing analysis. Lasergene Sequence Analysis Software; DNA Star software (DNASTAR, Madison, WI 53705 USA) was used to align and analyze the Sanger's sequencing data.

Genotyping of selected polymorphisms in whole population was performed by TaqMan® SNP Genotyping Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). For *THBD* polymorphisms, predesigned Taqman genotyping assays were available that

were used according to the manufacturer's instructions (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA).

Briefly, for each sample, a PCR master mix was prepared by adding 2.5µl Taqman master mix, 0.25µl Taqman gene specific assay probes complimentary to wild type and the mutant allele (VIC and FAM probes) and 0.25µl deionized water. A 384 PCR plate was used to run the assay and 3µl of master mix was added to each well followed by addition of 10ng (2µl) genomic DNA. PCR plates were vortexed and centrifuged at 1000 rpm (revolutions per minute) for 30 seconds. BioRad CFX384 real-time PCR (1000 Alfred Nobel Drive Hercules, California 94547 USA) was used for polymorphism analysis with following temperature conditions, 95°C for 10 minutes followed by 40x (92°C for 15 sec, 60°C for 1min). Different alleles of the polymorphisms were determined by BioRad CFX manager software 3.1.

Analysis of known thrombophilic variants

Taqman allele discrimination assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) was used for DNA mutations analysis in FVL and factor II G20210A as described previously [31]. Protein C levels were analyzed by a chromogenic method using the Berichrom® Protein C reagent (Siemens Healthcare Diagnostics, Upplands Väsby, Sweden) [32]. Latex immunoassay with Coamatic® Protein S-Free (Chromogenix, Haemochrom Diagnostica AB, Gothenburg, Sweden) was used for free Protein S analysis [33]. Antithrombin analysis was performed by thrombin-based method using Berichrom Antithrombin (Siemens Healthcare Diagnostics) [34]. For all analysis, BCS-XP coagulation analyzer (Siemens Healthcare Diagnostics) was used.

Statistical analysis

SPSS version 21 (IBM, Armonk, NY, USA) was used to perform statistical analyses. Continuous variable were compared by Mann-Whitney U test and Dichotomous variables were

compared by Chi-square test or Fisher's exact test where appropriate. Univariate and multivariate Cox regression analyses, after adjusting for family history and location of VTE, mild and severe thrombophilia and acquired risk factors for VTE, were performed using Cox proportional hazards models. Hazard ratios with 95% confidence intervals were calculated for each group of patients. Multivariate Cox regression analyses were performed as sensitivity analyses by including all VTE patients except those who had had thrombotic events before inclusion. Follow up period for sensitivity analyses was calculated from the time of inclusion of patients and was adjusted for duration of anticoagulation treatment, family history, location of VTE, mild and severe thrombophilia and acquired risk factors for VTE. Hardy–Weinberg equilibrium analysis was performed to see the genotypic distribution.

Results

Clinical data of the patients

Of 1465 patients, 154 had thrombotic events before inclusion therefore were excluded from further analysis. For the remaining VTE patients (n=1311), 148 (11.3%) had recurrence during follow up period. Of the patients with recurrent VTE, 40.8% had FVL mutations as compared to 28.5% in non-recurrent VTE (P=0.002). Frequency of family history for VTE was significantly lower in non-recurrent VTE patients (23.5%) as compared to recurrent VTE (32.4%) (P=0.024). Whereas no significant difference in age, gender, BMI, factor II mutations, protein C, protein S and antithrombin deficiency was found between recurrent and non-recurrent VTE patients (P >0.05), Table 2.

Screening *of THBD* genetic aberrations in VTE

During initial screening on age and sex matched samples (n=95), we identified 8 polymorphisms (*THBD* rs3176117 (2%), rs3176130 (1%), rs369909901 (1%), rs41282276 (1%), rs1042579 (22%), rs1042580 (36%), rs1962 (25%) and rs3176123 (22%) in *THBD* gene. Out of them, 4 polymorphisms (*THBD* rs1042579, rs1042580, rs1962 and rs3176123 polymorphisms) were identified with MAF >5% in VTE patients. *THBD* rs1042579 (1418C>T) polymorphism has been previously studied for its association with risk of VTE recurrence in the same cohort [24], the remaining three polymorphisms i.e., *THBD* rs1962 (3646T>C), rs1042580 (2521A>G) and rs3176123 (2729A>C) polymorphisms were analyzed in all MATS samples (n=1311) by TaqMan PCR. No deletions or insertions in *THBD* gene were identified in our cohort.

All the *THBD* polymorphisms have three different genotypic forms, homozygous wild type, heterozygous and homozygous mutated form. In data analysis, all 3 genotypic forms (for each polymorphism) were analyzed separately as well as by combining homozygous mutated and

heterozygous form. As the results were similar after combing the homozygous mutated and heterozygous form, in unprovoked VTE and sensitivity analyses, we only presented the results for combined homozygous mutated and heterozygous forms. The distribution of identified *THBD* polymorphisms was not significantly different between recurrent and not-recurrent VTE (Table 2).

Genotypic distributions in *THBD* polymorphisms did not deviate significantly (P >0.05) in Hardy-Weinberg equilibrium analyses.

THBD polymorphisms and risk of VTE recurrence

Of 1311 VTE patients, those who died, had VTE recurrence during anticoagulant treatment or whom complete information was missing were excluded for the risk analysis of recurrent VTE (n=261). Among remaining 1050 VTE patients, 126 (12%) had recurrent VTE during follow up period. On univariate Cox regression analysis, we found no significant association between risk of VTE recurrence and *THBD* polymorphisms identified in this study and the HRs and CIs were as follows; HR= 0.90, CI=0.63-1.29, HR=1.30, CI=0.90-1.87 and HR=1.14, CI=0.80-1.62 for *THBD* rs1962, rs1042580 and rs3176123 polymorphisms respectively. Similar results were found on multivariate Cox regression analysis (HR= 0.89, CI=0.62-1.28, HR=1.27, CI=0.88-1.85 and HR=1.15, CI=0.80-1.66 for *THBD* rs1962, rs1042580 and rs3176123 polymorphisms respectively) adjusted for acquired risk factors of VTE, mild (heterozygous prothrombin G20210A or FVL) and severe thrombophilia (homozygous carriers of FVL or those patients who had natural anticoagulant deficiencies, e. g. protein C, antithrombin, protein S deficiency and or carriers of multiple abnormalities), family history of VTE and location of DVT. We also analyzed the association between identified polymorphisms and risk of VTE recurrence according to gender and however found no significant associations between identified polymorphism and the risk of VTE recurrence in either sex (Table 3).

THBD polymorphisms and risk of VTE recurrence in unprovoked VTE patients

We further performed a sub-analysis on patients with unprovoked VTE. Patients with a recorded acquired risk factor for VTE i.e. immobilization or cast therapy within the last month, surgical intervention, use of contraceptives pills, malignancies diagnosed prior to or at diagnosis of the first VTE event, current pregnancy and postpartum period (first 6 weeks after delivery) and female hormone therapy were excluded during this analysis. Unprovoked VTE (n=618) with 81 (13.1%) recurrent VTE patients were selected to investigate the association between *THBD* polymorphisms and risk of VTE recurrence as a whole and according to gender. No significant association was found between *THBD* polymorphisms and risk of VTE recurrence in univariate Cox regression analysis (HR= 0.68, CI= 0.43-1.07, HR=1.43, CI= 0.90-2.26 and HR= 1.25, CI= 0.80-1.95 for *THBD* rs1962, rs1042580 and rs3176123 polymorphisms respectively) (Table 4). On stratification of data according to gender, we found that *THBD* rs1962 polymorphism was associated with risk of VTE recurrence in female patients, however, results did not reach significant level (HR, 0.53, CI=0.27-1.04, P=0.065) on univariate Cox regression analysis (Table 4). On multivariate Cox regression analysis, after adjusting for mild and severe thrombophilia, family history of VTE and location of DVT, *THBD* 1962 (T>C) polymorphism was significantly associated with lower risk of VTE recurrence in female patients (HR=0.41, CI=0.21-0.82, P= 0.012) (Table 4).

We also performed sensitivity analyses on all MATS patients except those who were diagnosed with VTE before inclusion (n=154). Multivariate analyses were performed for remaining patients (n=1311 including 148 recurrent VTE patients) with follow up time from the time of inclusion for this study (adjusted for duration of anticoagulant treatment, mild and severe thrombophilia, location of DVT, family history and acquired risk factors for VTE) and similarly, found no association between any of the identified *THBD* polymorphism and risk of VTE recurrence (supplementary Table 1).

Discussion

In the present study, we have sequenced the whole *THBD* gene (promotor region -1600bps, exon, 5' and 3'UTRs) in a well-defined prospective cohort of recurrent VTE patients to identify the genetic aberrations in *THBD* associated with VTE recurrence. We first performed an initial screening by Sanger's sequencing and in total, identified 8 single nucleotide polymorphisms in *THBD* (rs3176117, rs3176130, rs369909901, rs41282276, rs1042579, rs1042580, rs1962 and rs3176123). For validation, polymorphisms with MAF more than 5% (rs1042580, rs1962 and rs3176123) were analyzed in the whole population.

Our results show that none of these three polymorphisms (rs1042580, rs1962 and rs3176123) were significantly associated with risk of VTE recurrence ($P > 0.05$) in all patients. We could not find any other study showing the role of *THBD* rs1042580, rs1962 and rs3176123 polymorphisms in recurrent VTE, however, these polymorphisms have been analyzed in primary VTE and other cardiovascular diseases. Similar findings for *THBD* rs1042580 polymorphism were reported by Sugiyama *et al*. and Arellano *et al*. showing that this polymorphism was not associated with risk of primary venous thrombosis [25,35]. Furthermore, it has also been shown that *THBD* rs1042580, rs1962 and rs3176123 polymorphisms did not contribute significantly to the risk of cardiovascular events [23]. Another study shows that *THBD* rs1042580 polymorphism alone may not be a risk factor for cardiovascular events, however, in combination with polymorphisms in another gene involving coagulation pathway (*Factor V* gene), may contribute to risk for cardiovascular events [36]. Therefore, there is a possibility that *THBD* rs1042580 polymorphism alone may not play any significant role in risk assessment of recurrent VTE.

THBD rs3176123 and rs1962 polymorphisms have also been studied in primary venous thrombosis. *THBD* rs3176123 polymorphism was significantly associated with higher risk of venous thrombosis in male patients and also with the levels of the soluble THBD (sTHBD) [25].

Furthermore, this polymorphism was also associated with increased mortality after coronary artery bypass surgery [37]. However, in our study, *THBD* rs3176123 polymorphism was not associated with the risk of VTE recurrence. A possible explanation for these results could be that the risk factors associated with primary VTE may not be the same as for the VTE recurrence [8,38].

It remains a challenge to predict the individual risk of VTE recurrence in unprovoked VTE patients. We further investigated the role of *THBD* polymorphisms in unprovoked VTE patients; none of the identified polymorphisms were significantly associated with risk of VTE recurrence in this group of patients either. However, on multivariate Cox regression analysis, *THBD* rs1962 polymorphism was significantly associated with lower risk of VTE recurrence in female patients when adjusted with family history, showing that the family history was a confounding factor. These results show that *THBD* rs1962 polymorphism may be a risk factor for VTE recurrence in female patients with family history of VTE. However, due to the low sample number (Total 320 female patients with 41 having recurrent VTE during follow up) in this analysis; these results should be interpreted with caution. We suggest further studies with larger number of female patients to confirm the association between *THBD* rs1962 polymorphism and risk of recurrent VTE in unprovoked VTE patients.

THBD is an integral part of protein C pathway which plays important role in coagulation inhibition. It is present on the endothelium throughout the vascular system. However, its concentration is higher (approximately 500 nmol/l) in the capillaries as compared with the large vessels (approximately 0.1-0.2 nmol/l). This scarcity of THBD in large vessels is compensated by another receptor called Endothelial Protein C Receptor (EPCR) which binds to protein C and activates the protein C complex [39-41]. Our results show that *THBD* polymorphisms may not have a role in VTE recurrence. It is therefore possible that *THBD* polymorphisms may be important for thrombosis events in microvasculature/capillaries where there is no other receptor

to bind with protein C and activate protein C pathway while in large vessels, the lack of THBD is counterbalanced by EPCR. Thus polymorphisms in *THBD* might be relevant in diseases with microthrombotic mechanisms e.g. Purpura fulminans rather than large vessel thrombosis.

There are several rare mutations/polymorphisms in *THBD* that were reported previously in other diseases (e.g. Asp468Tyr, Ala25Thr, del791-801 etc.) but not identified in our cohort [16,19,21,22]. The reasons for this discrepancy could be lower frequency of those polymorphisms, different diseases and different ethnic populations involved in previous studies [42,43].

MAFs for *THBD* rs3176117, rs3176130, rs369909901 and rs41282276 polymorphisms were found to be very low (1% for each polymorphism except rs3176117 with 2% MAF) and therefore were not further analyzed in this study. *THBD* rs3176117, rs369909901 and rs41282276 polymorphisms were not studied previously in any other disease; most probably due to very low MAFs (0.4%, 0.06% and 0.08% respectively as are reported in NCBI) [44]. *THBD* rs3176130 polymorphism was studied previously in only one study on acute respiratory distress syndrome; however, it was not associated with the risk of disease [45].

One of the potential limitations of our study is that we could not analyze *THBD* rs3176117, rs3176130, rs369909901 and rs41282276 polymorphisms due to lower MAFs.

In conclusion, for the first time, whole *THBD* gene has been sequenced in recurrent VTE patients. We found 8 polymorphisms in *THBD* gene in Swedish population. None of these polymorphisms was found to be significantly associated with risk of VTE recurrence. Our results indicate that *THBD* polymorphisms may not be a risk factor for VTE recurrence although larger studies may be needed to rule out *THBD* polymorphisms as useful predictors for recurrent VTE.

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Authors' contribution:

KS, BZ, JS and AAM conceived and designed the study; AAM and AA performed the experiments; AA, KS, BZ, PJS, JS and AAM performed the data analysis and interpretation; and AA, KS, BZ, PJS, JS and AAM drafted and revised the article, and approved the final version.

Conflict of Interest

Authors declare no conflict of interest

Table 1: Forward and reverse primers for *THBD* promotor, Exon, 5´ UTR and 3´UTR along with their amplicon length.

***THBD* primer**		**Primer sequence**	**Amplicon length**	**Polymorphisms**
Primer 1	Forward	CGACCCCCTGATTCAGCCTA	668	rs3176117
	Reverse	CCCCCATAATGGTGAGAGGC		rs3176130
Primer 2	Forward	GGACTCTGTGCTCCTACACC	834	rs369909901
	Reverse	CCCGAAGACAGGATCGCAAG		
Primer 3	Forward	GATGGGGTCTGGCGTTGG	688	
	Reverse	GGACGTTCGGGAAAAGGAAG		
Primer 4	Forward	TCGTCTTGTTACAGGGGTGC	781	
	Reverse	AGCTGGTGTTGTTGTCTCCC		
Primer 5	Forward	CCTAATGACAGTGCGCTCCT	826	
	Reverse	GGGCTCCAGTATGCAGTCAT		
Primer 6	Forward	GAGCACTTCTGCGTTCCCAA	697	rs1042579
	Reverse	TCGCGATGGAGATGCCTATG		
Primer 7	Forward	CACATTGGCACCGACTGTGA	656	
	Reverse	TAGTCATCCCTAGCCCACGA		
Primer 8	Forward	TGGACCACTGGGCAATGATG	821	rs41282276
	Reverse	TCCTGGGAGGTGTTTGTCTC		
Primer 9	Forward	ACCTGTGCCTGACCCTACTT	651	rs1042580
	Reverse	TTCTTGTTCAGGGGCCACAT		rs3176123
Primer 10	Forward	GATCCTGGAGGATGCCCAAT	999	rs1962
	Reverse	GACACACAGCTGGGATTCG		

M13 tail sequence was added to all primers before synthesis.

Table 2: Characteristics of studied population including the distribution of *THBD* genotypes stratified by recurrent and non-recurrent status.

Parameters	Mean (±SD) or n(%)		Total n (%)	¶P-value
	Non recurrent VTE n (%)	Recurrent VTE n (%)		
Age a inclusion				
Years (Mean±SD)	62.9±17.5	61.3±15.3	62.7±17.3	0.087*
Gender				
Male	565 (48.6)	78 (52.7)	643 (49.0)	0.383
Female	598 (51.4)	70 (47.3)	668 (51.0)	
BMI				
Mean±SD	26.6±4.7	27.4±5.1	26.6±4.8	0.110*
PE				
PE	343 (29.5)	45 (30.4)	388 (29.6)	0.848
No PE	820 (70.5)	103 (69.6)	923 (70.4)	
DVT+PE				
DVT	736(68.2)	98 (68.5)	834 (68.2)	0.323
PE	277 (25.7)	32 (22.4)	309 (25.3)	
DVT+PE	66 (6.1)	13 (9.1)	79 (6.5)	
Malignancy				
Yes	140 (12.1)	13 (8.8)	153 (11.7)	0.278
No	1020 (87.9)	135 (91.2)	1155 (88.3)	
Protein C deficiency				
Yes	16 (1.6)	0 (0.0)	16 (1.4)	0.242
No	1009 (98.4)	1136 (100.0)	1145 (98.6)	
Protein S deficiency				
Yes	20 (2.0)	1 (0.7)	21 (1.8)	0.499
No	998 (98.0)	135 (99.3)	1133 (98.2)	
Factor V mutations				
Yes	330 (28.5)	60 (40.8)	390 (29.9)	0.002
No	829 (71.5)	87 (59.2)	916 (69.9)	
Factor II mutations				
Yes	39 (3.9)	9 (7.0)	48 (4.2)	0.104
No	969 (96.1)	120 (93.0)	1089 (95.8)	
Antithrombin deficiency				
Yes	12 (1.2)	1 (0.7)	13 (1.1)	0.726
No	1013 (98.8)	135 (99.3)	1148 (98.9)	
Family history				
Yes	269 (23.5)	47 (32.4)	316 (24.5)	0.024
No	875 (76.5)	98 (67.6)	973 (75.5)	
rs1962				
TT	647 (56.1)	86 (58.5)	733 (56.4)	0.827
TC	432 (37.5)	53 (36.1)	485 (37.3)	
CC	74 (6.4)	8 (5.4)	82 (6.3)	
TC and CC	506 (43.9)	61 (41.5)	567 (43.6)	0.597
rs1042580				
AA	484 (42.1)	53 (36.1)	537 (41.4)	
AG	523 (45.4)	71 (48.3)	594 (45.8)	0.317
GG	144 (12.5)	23 (15.6)	167 (12.9)	
AG and GG	667 (57.9)	94 (63.9)	761 (58.6)	0.182
rs3176123				
AA	696 (60.8)	86 (58.9)	782 (60.6)	0.549
AC	399 (34.8)	56 (38.4)	455 (35.2)	
CC	50 (4.4)	4 (2.7)	54 (4.2)	
AC and CC	449 (39.2)	60 (41.1)	509 (39.4)	0.719

DNA was not enough for genotyping in 11 samples for *THBD* rs1962 and 13 for rs1042580 and 20 samples for rs3176123, BMI, body mass index, DVT, deep vein thrombosis; PE, pulmonary embolism; P-value, Chi square test until unless indicated, *Mann-Whitney U test, ¶comparing non-recurrent with recurrent VTE.

Table 3: Uni- and multivariate analyses of *THBD* rs1962, rs1042580 and rs3176123 polymorphisms in recurrent VTE patients.

	All patients				**Men**				**Women**			
	Univariate	**P**	**Multivariate**	**P***	**Univariate**	**P**	**Multivariate**	**P***	**Univariate**	**P**	**Multivariate**	**P***
Genotypes	HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)	
rs1962												
TT	Reference		Reference		Reference		Reference		Reference		Reference	
TC	0.90 (0.62-1.30)	0.565	0.89 (0.61-1.30)	0.544	1.05 (0.63-1.75)	0.857	1.08 (0.63-1.83)	0.781	0.77 (0.45-1.34)	0.357	0.67 (0.38-1.17)	0.157
CC	0.94 (0.46-1.96)	0.874	0.92 (0.44-1.91)	0.813	0.83 (0.30-2.33)	0.721	0.84 (0.30-2.38)	0.742	1.13 (0.40-3.16)	0.820	0.94 (0.33-2.65)	0.903
TC and CC	0.90 (0.63-1.29)	0.573	0.89 (0.62-1.28)	0.539	1.01 (0.62-1.65)	0.968	1.04 (0.62-1.72)	0.894	0.82 (0.49-1.37)	0.44	0.70 (0.41-1.19)	0.190
rs1042580												
AA	Reference		Reference		Reference		Reference		Reference		Reference	
AG	1.24 (0.84-1.82)	0.283	1.23 (0.83-1.82)	0.303	1.10 (0.64-1.87)	0.740	1.08 (0.62-1.87)	0.797	1.37 (0.79-2.39)	0.263	1.40 (0.80-2.45)	0.240
GG	1.52 (0.90-2.58)	0.116	1.44 (0.83-2.49)	0.193	1.58 (0.75-3.32)	0.226	1.59 (0.73-3.48)	0.244	1.48 (0.70-3.14)	0.300	1.34 (0.61-2.92)	0.462
AG and GG	1.30 (0.90-1.87)	0.163	1.27(0.88-1.85)	0.203	1.18 (0.71-1.96)	0.520	1.16 (0.69-1.96)	0.577	1.40 (0.83-2.36)	0.208	1.38 (0.82-2.35)	0.228
rs3176123												
AA	Reference		Reference		Reference		Reference		Reference		Reference	
AC	1.18 (0.82-1.70)	0.358	1.21 (0.84-1.76)	0.312	1.04 (0.62-1.74)	0.875	0.98 (0.57-1.66)	0.925	1.35 (0.80-2.26)	0.259	1.58 (0.93-2.68)	0.090
CC	0.77 (0.28-2.11)	0.613	0.74 (0.27-2.04)	0.562	1.00 (0.24-4.14)	0.998	1.11 (0.27-4.65)	0.882	0.64 (0.15-2.67)	0.541	0.57 (0.14-2.39)	0.442
AC and CC	1.14 (0.80-1.62)	0.471	1.15 (0.80-1.66)	0.440	1.04 (0.63-1.71)	0.882	0.98 (0.59-1.65)	0.955	1.24 (0.75-2.06)	0.395	1.40 (0.83-2.35)	0.205

P*=adjusted for acquired risk factors, family history of VTE, mild and severe thrombophilia and location of VTE

Table 4: Uni- and multivariate analyses of *THBD* rs1962, rs1042580 and rs3176123 polymorphisms in unprovoked recurrent VTE patients.

	All patients (unprovoked VTE)				**Men (unprovoked VTE)**				**Women (unprovoked VTE)**			
	Univariate	**P**	**Multivariate**	**P***	**Univariate**	**P**	**Multivariate**	**P***	**Univariate**	**P**	**Multivariate**	**P***
Genotypes	HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)	
rs1962												
TT	Reference		Reference		Reference		Reference		Reference		Reference	
CT and CC	0.68 (0.43-1.07)	0.096	0.66 (0.42-1.06)	0.083	0.84 (0.45-1.57)	0.584	0.90 (0.48-1.70)	0.756	0.53 (0.27-1.04)	0.065	0.41 (0.21-0.82)	**0.012**
rs1042580												
AA	Reference		Reference		Reference		Reference		Reference		Reference	
AG and GG	1.43 (0.90-2.26)	0.132	1.37 (0.86-2.20)	0.187	1.49 (0.78-2.87)	0.231	1.37 (0.70-2.66)	0.354	1.34 (0.69-2.58)	0.384	1.42 (0.72-2.78)	0.308
rs3176123												
AA	Reference		Reference		Reference		Reference		Reference		Reference	
AC and CC	1.25 (0.80-1.95)	0.322	1.29 (0.82-2.03)	0.275	0.99 (0.53-1.86)	0.977	0.93 (0.49-1.78)	0.824	1.65 (0.88-3.09)	0.121	1.82 (0.95-3.48)	0.071

P*=adjusted for family history of VTE, mild and severe thrombophilia and location of VTE.

References:

1. Heit JA. The epidemiology of venous thromboembolism in the community. *Arteriosclerosis, thrombosis, and vascular biology.* 2008;28(3):370-372.
2. White RH. The epidemiology of venous thromboembolism. *Circulation.* 2003;107(23 Suppl 1):I4-8.
3. Mason C. Venous thromboembolism: a chronic illness. *The Journal of cardiovascular nursing.* 2009;24(6 Suppl):S4-7.
4. Prandoni P, Lensing AW, Cogo A, et al. The long-term clinical course of acute deep venous thrombosis. *Annals of internal medicine.* 1996;125(1):1-7.
5. Iorio A, Kearon C, Filippucci E, et al. Risk of recurrence after a first episode of symptomatic venous thromboembolism provoked by a transient risk factor: a systematic review. *Archives of internal medicine.* 2010;170(19):1710-1716.
6. Kyrle PA, Eichinger S. Deep vein thrombosis. *Lancet (London, England).* 2005;365(9465):1163-1174.
7. Carrier M, Le Gal G, Wells PS, Rodger MA. Systematic review: case-fatality rates of recurrent venous thromboembolism and major bleeding events among patients treated for venous thromboembolism. *Annals of internal medicine.* 2010;152(9):578-589.
8. Kyrle PA, Rosendaal FR, Eichinger S. Risk assessment for recurrent venous thrombosis. *Lancet (London, England).* 2010;376(9757):2032-2039.
9. Heit JA, Phelps MA, Ward SA, Slusser JP, Petterson TM, De Andrade M. Familial segregation of venous thromboembolism. *Journal of thrombosis and haemostasis : JTH.* 2004;2(5):731-736.
10. Larsen TB, Sorensen HT, Skytthe A, Johnsen SP, Vaupel JW, Christensen K. Major genetic susceptibility for venous thromboembolism in men: a study of Danish twins. *Epidemiology (Cambridge, Mass.).* 2003;14(3):328-332.

11. Germain M, Saut N, Greliche N, et al. Genetics of venous thrombosis: insights from a new genome wide association study. *PloS one.* 2011;6(9):e25581.
12. Weiler-Guettler H, Christie PD, Beeler DL, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. *The Journal of clinical investigation.* 1998;101(9):1983-1991.
13. Isermann B, Hendrickson SB, Zogg M, et al. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. *The Journal of clinical investigation.* 2001;108(4):537-546.
14. Wen DZ, Dittman WA, Ye RD, Deaven LL, Majerus PW, Sadler JE. Human thrombomodulin: complete cDNA sequence and chromosome localization of the gene. *Biochemistry.* 1987;26(14):4350-4357.
15. Weiler H, Isermann BH. Thrombomodulin. *Journal of thrombosis and haemostasis : JTH.* 2003;1(7):1515-1524.
16. Ohlin AK, Marlar RA. The first mutation identified in the thrombomodulin gene in a 45-year-old man presenting with thromboembolic disease. *Blood.* 1995;85(2):330-336.
17. Norlund L, Holm J, Zoller B, Ohlin AK. A common thrombomodulin amino acid dimorphism is associated with myocardial infarction. *Thrombosis and haemostasis.* 1997;77(2):248-251.
18. Li YH, Chen CH, Yeh PS, et al. Functional mutation in the promoter region of thrombomodulin gene in relation to carotid atherosclerosis. *Atherosclerosis.* 2001;154(3):713-719.
19. Kunz G, Ohlin AK, Adami A, Zoller B, Svensson P, Lane DA. Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. *Blood.* 2002;99(10):3646-3653.

20. Navarro S, Medina P, Bonet E, et al. Association of the thrombomodulin gene c.1418C>T polymorphism with thrombomodulin levels and with venous thrombosis risk. *Arteriosclerosis, thrombosis, and vascular biology.* 2013;33(6):1435-1440.

21. van der Velden PA, Krommenhoek-Van Es T, Allaart CF, Bertina RM, Reitsma PH. A frequent thrombomodulin amino acid dimorphism is not associated with thrombophilia. *Thrombosis and haemostasis.* 1991;65(5):511-513.

22. Faioni EM, Franchi F, Castaman G, Biguzzi E, Rodeghiero F. Mutations in the thrombomodulin gene are rare in patients with severe thrombophilia. *British journal of haematology.* 2002;118(2):595-599.

23. Auro K, Komulainen K, Alanne M, et al. Thrombomodulin gene polymorphisms and haplotypes and the risk of cardiovascular events: a prospective follow-up study. *Arteriosclerosis, thrombosis, and vascular biology.* 2006;26(4):942-947.

24. Ahmad A, Sundquist K, Zoller B, Svensson PJ, Sundquist J, Memon AA. Thrombomodulin gene c.1418C>T polymorphism and risk of recurrent venous thromboembolism. *Journal of thrombosis and thrombolysis.* 2016;42(1):135-141.

25. Sugiyama S, Hirota H, Kimura R, et al. Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population. *Thrombosis research.* 2007;119(1):35-43.

26. Heit JA, Petterson TM, Owen WG, Burke JP, M DEA, Melton LJ, 3rd. Thrombomodulin gene polymorphisms or haplotypes as potential risk factors for venous thromboembolism: a population-based case-control study. *Journal of thrombosis and haemostasis : JTH.* 2005;3(4):710-717.

27. Ahmad A, Sundquist K, Zoller B, et al. Identification of polymorphisms in Apolipoprotein M gene and their relationship with risk of recurrent venous thromboembolism. *Thrombosis and haemostasis.* 2016;116(3).

28. Isma N, Svensson PJ, Gottsater A, Lindblad B. Prospective analysis of risk factors and distribution of venous thromboembolism in the population-based Malmo Thrombophilia Study (MATS). *Thrombosis research.* 2009;124(6):663-666.

29. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics.* 2012;13:134.

30. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America.* 1977;74(12):5463-5467.

31. Sveinsdottir SV, Saemundsson Y, Isma N, Gottsater A, Svensson PJ. Evaluation of recurrent venous thromboembolism in patients with Factor V Leiden mutation in heterozygous form. *Thrombosis research.* 2012;130(3):467-471.

32. Francis RB, Jr., Seyfert U. Rapid amidolytic assay of protein C in whole plasma using an activator from the venom of Agkistrodon contortrix. *Am J Clin Pathol.* 1987;87(5):619-625.

33. Giri TK, Hillarp A, Hardig Y, Zoller B, Dahlback B. A new direct, fast and quantitative enzyme-linked ligandsorbent assay for measurement of free protein S antigen. *Thrombosis and haemostasis.* 1998;79(4):767-772.

34. Odegard OR, Lie M, Abildgaard U. Heparin cofactor activity measured with an amidolytic method. *Thrombosis research.* 1975;6(4):287-294.

35. Arellano AR, Bezemer ID, Tong CH, et al. Gene variants associated with venous thrombosis: confirmation in the MEGA study. *Journal of thrombosis and haemostasis : JTH.* 2010;8(5):1132-1134.

36. Auro K, Alanne M, Kristiansson K, et al. Combined effects of thrombosis pathway gene variants predict cardiovascular events. *PLoS genetics.* 2007;3(7):e120.

37. Lobato RL, White WD, Mathew JP, et al. Thrombomodulin gene variants are associated with increased mortality after coronary artery bypass surgery in replicated analyses. *Circulation.* 2011;124(11 Suppl):S143-148.

38. Zhu T, Martinez I, Emmerich J. Venous thromboembolism: risk factors for recurrence. *Arteriosclerosis, thrombosis, and vascular biology.* 2009;29(3):298-310.

39. Laszik Z, Mitro A, Taylor FB, Jr., Ferrell G, Esmon CT. Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. *Circulation.* 1997;96(10):3633-3640.

40. Esmon C. The protein C pathway. *Critical care medicine.* 2000;28(9 Suppl):S44-48.

41. Esmon CT. The normal role of Activated Protein C in maintaining homeostasis and its relevance to critical illness. *Critical care (London, England).* 2001;5(2):S7-12.

42. Xie HG, Stein CM, Kim RB, et al. Frequency of functionally important beta-2 adrenoceptor polymorphisms varies markedly among African-American, Caucasian and Chinese individuals. *Pharmacogenetics.* 1999;9(4):511-516.

43. Faioni EM, Merati G, Peyvandi F, Bettini PM, Mannucci PM. The G1456 to T mutation in the thrombomodulin gene is not frequent in patients with venous thrombosis. *Blood.* 1997;89(4):1467.

44. Sherry ST, Ward MH, Kholodov M, et al. dbSNP: the NCBI database of genetic variation. *Nucleic acids research.* 2001;29(1):308-311.

45. Sapru A, Calfee CS, Liu KD, et al. Plasma soluble thrombomodulin levels are associated with mortality in the acute respiratory distress syndrome. *Intensive care medicine.* 2015;41(3):470-478.

Supplementary Table 1. Multivariate analyses of *THBD* polymorphisms in recurrent VTE patients with follow up from time of inclusion for this study and adjusting for duration of warfarin treatment.

	All patients				**Men**				**Women**			
	Model 1: adjusted	**P**	**Model 2: adjusted**	**P***	**Model 1: adjusted**	**P**	**Model 2: adjusted**	**P***	**Model 1: adjusted**	**P**	**Model 2: adjusted**	**P***
Genotypes	HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)	
rs1962												
TT	Reference		Reference		Reference		Reference		Reference		Reference	
CT and CC	0.88 (0.62-1.24)	0.466	0.88 (0.62-1.26)	0.479	1.05 (0.65-1.69)	0.852	1.07 (0.65-1.76)	0.787	0.73 (0.44-1.21)	0.219	0.65 (0.39-1.10)	0.109
rs1042580												
AA	Reference		Reference		Reference		Reference		Reference		Reference	
AG and GG	1.30 (0.91-1.86)	0.147	1.28 (0.89-1.84)	0.182	1.11 (0.68-1.82)	0.671	1.09 (0.66-1.82)	0.738	1.54 (0.92-2.58)	0.105	1.51 (0.89-2.55)	0.124
rs3176123												
AA	Reference		Reference		Reference		Reference		Reference		Reference	
AC and CC	1.19 (0.84-1.69)	0.315	1.21 (0.85-1.72)	0.293	1.03 (0.63-1.68)	0.907	0.98 (0.59-1.63)	0.948	1.37 (0.84-2.24)	0.206	1.54 (0.93-2.55)	0.094

Model 1= Adjusted for duration of anticoagulant treatment, Model 2= Adjusted for acquired risk factors and family history of VTE, duration of anticoagulant treatment, mild and severe thrombophilia and location of DVT.