BRIEF REPORT

Thrombomodulin (THBD) gene variants and thrombotic risk in a population-based cohort study

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Abstract

Background: The protein C anticoagulant system plays a key role in maintaining the hemostatic balance. Although several studies have identified thrombomodulin gene (THBD) variants among venous thromboembolism (VTE) patients, the role of THBD in relation to VTE in humans remains to be clarified.

Objectives: This study aimed to determine the thrombotic risk of rare and common THBD variants in a large population-based cohort of middle-aged and older adults.

Patients/Methods: The exome sequence of THBD was analyzed for qualifying variants in 28,794 subjects (born 1923–1950, 60% women), who participated in the Malmö Diet and Cancer study (1991–1996). Patients were followed from baseline until the first event of VTE, death, or 2018. Qualifying variants were defined as loss-of-function or non-benign (PolyPhen-2) missense variants with minor allele frequency <0.1%.

Results: The single common coding variant rs1042579 was not associated with incident VTE. Sixteen rare variants were classified as qualifying and included in collapsing analysis. Seven individuals with VTE compared to 24 individuals without VTE carried one qualifying variant. Cox multivariate regression analysis adjusted for age, sex, body mass index, systolic blood pressure, smoking and alcohol consumption, rs6025, rs1799963, and the top two eigenvectors from a principal components analysis showed a hazard ratio of 3.0 (95% confidence interval 1.4–6.3) for the rare qualifying variants. The distributions of qualifying variants in THBD showed a difference for individuals with and without incident VTE indicating a possible position effect.

Conclusions: Rare qualifying THBD variants were associated with VTE, suggesting that rare variants in THBD contribute to development of VTE.

KEYWORDS
epidemiology, genetics, THBD, thrombomodulin, venous thromboembolism

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1 | INTRODUCTION

Thrombomodulin (TM) was discovered in the 1980s following recognition that the zymogen protein C (PC) was converted by thrombin to activated protein C (APC). The PC anticoagulant system plays a key role in regulating hemostasis. The vitamin-K-dependent zymogen PC is activated on the surface of intact endothelial cells by thrombin that has bound to the membrane protein TM. In contrast, at sites of vascular disruption, the procoagulant effects of thrombin are expressed. Endothelial PC receptor (EPCR) binds PC, and enhances its activation. APC together with its cofactor protein S, in turn, cleaves the cofactors factors Va and VIIIa, which leads to inhibition of the coagulation system.

The TM gene THBD consists of a single exon that encodes a 557-amino-acid residue type 1 transmembrane glycoprotein. Animal model data suggest that THBD mutations are prothrombotic. Although naturally occurring human mutations in TM with decreased expression and/or function have been described, variants in THBD remain to be linked to venous thromboembolism (VTE). Hernandez et al. linked the THBD gene to VTE in a genome-wide association study (GWAS) among African Americans. However, two much larger GWAS have not been able to replicate the results. Although several small studies have identified THBD variants among VTE patients, the role of THBD in relation to VTE in humans remains to be clarified.

Based on the antithrombotic role of TM, we hypothesized that variants within THBD could predispose to VTE. To test this hypothesis, the coding sequence of THBD was analyzed in 28,794 individuals in the large population-based Malmö Diet and Cancer Study (MDC).

2 | METHODS

2.1 | Participants

The MDC is a population-based prospective cohort study from the city of Malmö in the south of Sweden. Sample characteristics, data collection, and clinical definitions for MDC have been described previously. Participants underwent a medical history, physical examination, and laboratory assessment at baseline (1991-1996). The MDC population has only 12% admixture from foreign-born individuals. Among foreign-born individuals only 1% were non-European. A total of 30,446 individuals, men (n = 12,120, born 1923-1945) and women (n = 18,326, born 1923-1950) attended a baseline examination between March 1991 and September 1996. Clinical data and information on DNA were available for 29,387 subjects sampled at baseline. Of these individuals 593 (2.0%; 315 women, 278 men) were affected by VTE between 1970 and baseline and were excluded. The final study population was 28,794 individuals. The study was conducted according to the principles of the Declaration of Helsinki. The Regional Ethics Review Board at Lund University, Lund, Sweden, approved the study (LU 51/90) and all participants provided written informed consent.

2.2 | Clinical endpoints

One outcome, VTE, was examined. Events were identified through linkage with the Swedish National Patient Register (SNHDR) and outpatient register. The SNHDR had a 100% coverage for inpatients in Malmö during the whole follow-up time and for outpatients from 2001. VTE was defined based on International Classification of Diseases 7th, 8th, 9th, and 10th revisions (ICD-7 and ICD-10) codes. ICD-7, ICD-8, and ICD-9 were used to identify prevalent cases. Only ICD-9 and ICD-10 were used for incident VTE during follow-up. The diagnosis of VTE in the SNHDR has been shown to have an accuracy of 95%, whereas the overall validity of the SNHDR is 87%. A quality control of 118 patients with VTE in the MDC cohort was performed. In 106 (90%) of cases the diagnosis was correct.

2.3 | Genetic and statistical analysis

Whole exome sequencing (WES) was performed by the Regeneron Genetics Center such that >85% of targeted bases were covered at a read depth of >20X. ANNOVAR was used to aggregate variant annotation, allele frequencies (AF), and in silico predictions of deleteriousness. Cox proportional hazards regression was used to examine the association between genotype and incident VTE. Age, sex, ancestry, factor V Leiden (rs6025), and the rs1799963 prothrombin were included as covariates in the sex- and age-adjusted model. R (version 4.0.0) was used for all statistical analyses. Alcohol abuse defined by official registers has been associated with VTE. To control for possible population stratification, a principal component analysis (PCA) was performed and the two largest principal components were included in the statistical model. PCA was performed as described elsewhere. The reference genomes were obtained from the 1000 Genomes Project server. The PCA was performed with independent
(R² measure of linkage disequilibrium [LD] <0.2) common (minor allele frequency [MAF] ≥5%) autosomal bi-allelic variants that were detected in both the reference genomes and the MDC exomes. To avoid extended LD and high variability regions, such as the major histocompatibility complex (MHC), these regions were omitted from the PCA. The principal components were first obtained from the reference genomes and then projected individuals from the MDC onto the principal-component space via PLINK2.27 The fit of the proportional hazards model was checked visually by plotting the incidence rates over time and by calculating Schoenfeld (partial) residuals.21 No violation against proportional hazards assumption was observed. Possible interactions between gene variants and age, sex, and risk factors (BMI, smoking status, systolic blood pressure, high alcohol consumption) on VTE were explored by introducing interaction terms in the multivariable models. No interactions were observed. The subjects were categorized according to genotype and Kaplan–Meier plots were calculated for VTE. For curve comparisons, the log-rank test was used.

3 | RESULTS AND DISCUSSION

A total of 28,794 individuals from the MDC cohort were available for analysis and out of these 2584 (9%) were affected by a VTE event during follow-up until December 31, 2018. First VTE diagnosis was deep vein thrombosis of the lower extremities in 964 cases, 928 cases had pulmonary embolism, and 692 cases had other causes of VTE. The sum of the follow-up time was 587,992.7 years, corresponding to a VTE incidence rate of 4.4 (95% confidence interval [CI] 4.2–4.6) per 1000 person-years. Resequencing identified a total of 98 THBD variants in the study population, 33 synonymous, 63 missense, and 2 loss-of-function (LoF) variants. All variants detected in the total population were compared for their MAFs in individuals with and without VTE in Figure 1A. Fifty-three out of the 98 variants were detected in single individuals and 5 of the 23 variants lacking an rs-number were found among individuals with incident VTE and 17 among individuals without VTE. There was a single THBD variant with an MAF >1% (rs1042579). For the single common variant, the number of heterozygotes among individuals with and without VTE was 929 versus 9132, and the number of homozygotes was 120 versus 1282. For this variant, a minor overrepresentation (0.3%) in cases was observed, which was not significant (P = .61; Figure 1B). This result is concordant with the results obtained by Lindström et al.11 and Klarin et al.12

Several naturally occurring mutations in THBD with decreased expression and/or function have been described.7 Rs1800578 (p. Pro501Leu) reduces thrombomodulin expression with 23% and rs1800579 (p. Pro495Ser) with 18%. Both variants were detected in the present study, but their frequencies did not differ significantly between individuals with and without VTE (13 vs. 111 individuals for rs1800579, P = .56 and 1 vs. 11 individuals for rs1800578, P = .94). Neither of these two variants were classified as qualified in the present study as they are both benign according to PolyPhen-2.

Sixteen variants were classified as qualifying and included in collapsing analysis using the following selection criteria, LoF or non-benign (PolyPhen-2) missense variants with MAF <0.1% (Table 1). Seven individuals with VTE compared to 24 individuals without VTE carried one qualifying variant. No homozygotes were observed nor was any individual with more than one qualifying variant observed. The total prevalence of these variants in the population was 0.1%. The thrombosis-free survival curve using Kaplan–Meier analysis of the rare qualifying variants is presented in Figure 2. Hazard ratios (HRs) for incident VTE as well as incidence rates (IR) and incidence rate ratios (IRR) are summarized for the rare qualifying variants in Table 2. The Cox multivariate regression analysis showed a fully adjusted HR of 3.0 (95% CI 1.4–6.3) for the rare qualifying variants (Table 2). The qualifying variants in VTE patients were all located in the first part of the gene, before amino acid 313 out of 575 (Table 1 and Figure 1C). The 31 individuals analyzed above with LoF or non-benign (PolyPhen-2) missense variants with MAF <0.1% showed an
odds ratio (OR) of 3.2, whereas the benign missense variants with MAF <0.1% were present in many more individuals (17 individuals with VTE and 119 without VTE) but showed a lower OR of 1.6. This indicates that the classification algorithm works well in identifying an absolute majority of the qualifying variants, although some of the variants classified as benign may also contribute to VTE in this population. To investigate whether cancer influenced the risk associated with carrying a \( THBD \) variant, hazard ratio was calculated including only those without malignancy before VTE (HR = 3.0, 95% CI 1.1–8.1).

Although several small studies have identified \( THBD \) variants among VTE patients,\(^7,9,10,13-20\) the \( THBD \) remains to be linked to VTE in humans.\(^8,11,12,20\) The \( THBD \) gene is the only major gene involved in the PC anticoagulant system that has not been linked formally to VTE in humans (in contrast to \( PROC \), \( PROS1 \), \( F5 \), and \( PROCR \)). The present study confirms that common variants in \( THBD \) are not associated with VTE.\(^13,14\) However, rare qualifying variants, that is, missense variants and LoF variants, might be associated with VTE. The effect size in the present study (HR = 3.0, 95% CI 1.4–6.3) is similar to rs6025 and the rs1799963 variant.\(^8,11,12\) The present study involved middle-aged and older patients and the effect size might be higher among younger VTE patients. Due to the rareness of qualifying \( THBD \) gene variants, a very large study population is necessary to obtain a genome-wide significant \( P \)-value in gene collapsing studies. This illustrates one problem with agnostic analysis of whole exome studies. Still,

### TABLE 1: Description of qualifying \( THBD \) variants, defined as loss-of-function or non-benign missense variants according to PolyPhen-2 with minor allele frequency <0.1%

| Sequence position (GRCh38) | Alleles | Existing variation | Amino acid | PolyPhen−2 | No. of individuals |
|---------------------------|---------|-------------------|------------|------------|-------------------|
|                           |         |                   |            |            | VTE no VTE       |
| 20:23047808               | A>C     | rs755593080       | p. Val566Gly | Possibly damaging | 0          | 1          |
| 20:23047817               | A>T     | rs1210868104      | p. Leu563Gln | Probably damaging | 0          | 1          |
| 20:23047959               | C>G     | rs138861385       | p. Gly516Arg | Probably damaging | 0          | 1          |
| 20:23048174               | T>C     | rs375671812       | p. Glu444Gly | Probably damaging | 0          | 2          |
| 20:23048373               | A>C     | –                 | p. Cys378Gly | Probably damaging | 0          | 1          |
| 20:23048529               | C>A     | rs538228511       | p. Val326Leu | Possibly damaging | 0          | 6          |
| 20:23048568               | C>G     | –                 | p. Gly313Arg | Probably damaging | 1          | 0          |
| 20:23048574               | C>A     | COSV65703156      | p. Glu311Ter | NA         | 1          | 1          |
| 20:23048783               | C>T     | rs1458658302      | p. Gly241Asp | Probably damaging | 0          | 1          |
| 20:23048921               | G>A     | rs755591112       | p. Thr195Ile | Probably damaging | 1          | 4          |
| 20:23049014               | C>A     | –                 | p. Gly164Val | Probably damaging | 0          | 1          |
| 20:23049098               | A>C     | rs550522588       | p. Leu136Trp | Probably damaging | 1          | 1          |
| 20:23049183               | A>T     | rs1384827446      | p. Phe108Ile | Probably damaging | 1          | 0          |
| 20:23049188               | C>T     | rs764904057       | p. Arg106His | Probably damaging | 1          | 3          |
| 20:23049244               | C>T     | –                 | p. Trp87Ter  | NA         | 1          | 0          |
| 20:23049501               | G>A     | rs1175683983      | p. Leu2Phe  | Probably damaging | 0          | 1          |

Abbreviation: VTE, venous thromboembolism.
animal studies have shown that homozygosity resulting in complete deficiency of thrombomodulin is not compatible with life. Thus, a negative evolutionary pressure together with a small gene size compared to $\text{PROC}$, $\text{PROS1}$, and $\text{SERPINC1}$ contributes to a low frequency of potential qualifying variants in the $\text{THBD}$ gene. Still, the $\text{THBD}$ gene has important biological functions and animal studies in which no demands for genome-wide significance levels exist suggest that deficiency of thrombomodulin leads to a hypercoagulable state. Considered together, the results of previous experimental studies are in line with the present study indicating a role of rare $\text{THBD}$ variants in VTE. A limitation is that the diagnosis of VTE was based on ICD codes from the inpatient and outpatient registers and that the outpatient register only exist from 2001. However, the latter is most likely a non-differential bias regarding VTE risk. Violation against proportional hazards assumption over time was observed. A strength is the high coverage of VTE diagnosis in the Malmö registers and that VTE diagnosis is confirmed by objective methods in Malmö and Sweden, and the high quality of Swedish registers. A validation of 118 patients with incident VTE in the MDC cohort confirms this (90% accuracy). Moreover, the high VTE incidence rate of 4.4 (95% CI 4.2–4.6) per 1000 person-years is similar to a study from Gothenburg in Sweden with a VTE incidence of 387 per 100,000 observation-years.

Another limitation is lack of information about anticoagulant and aspirin treatment, but we adjusted for potential cardiovascular risk factors. In conclusion, exome sequencing of the $\text{THBD}$ gene suggests that qualifying rare missense and LoF variants are associated with VTE among middle-aged and old individuals. Thus, the present large population-based genetic epidemiological study suggests that $\text{THBD}$ is linked to VTE, just as are the other major genes of the PC anticoagulant system. This work was supported by a grant awarded to Dr. Bengt Zöller by ALF-funding from Region Skåne, Sparbanken Skåne, and by the Swedish Research Council.

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### Author Contributions

E.M., C.H., and B.Z. conceived and designed the study, analyzed and interpreted data, drafted the manuscript, and gave final approval of the submitted manuscript. All authors interpreted data, critically revised the manuscript for important intellectual content, and gave final approval of the submitted manuscript.

### Conflict of Interest

None.

### Table 2

| Participants | VTE | Age at VTE event | Crude IR | Crude IRR | Allele frequencies of $\text{THBD}$ gene variants | Age, sex, ancestry, $\text{rs6025}$, and $\text{rs1799963}$ adjusted HR | Multivariable HR |
|-------------|-----|------------------|----------|-----------|-----------------------------------------------|-----------------|-----------------|
| Complete cohort | 28,794 | 2584 | 73.7 (8.6) | 4.4 (4.2–4.6) | 1 | 1 | 1 |
| Model with qualifying variant (i.e., rare non-benign missense or Loss-of-Function variant) | Reference no qualifying variant | 28,763 | 2577 | 73.7 (8.6) | 4.4 (4.2–4.6) | 1 | 1 | 1 |
| 1 qualifying variant | 31 | 7 | 70.6 (10.5) | 10.4 (4.2–21.5) | 2.4 (1.1–5.0) | 2.8 (1.3–5.9) | 0.0069 | 3.0 (1.4–6.3) | 0.0039 |

Note: Qualifying variant—rare (minor allele frequency <0.1% in gnomAD or any of its regional subpopulations or the present population) non-benign (according to PolyPhen-2) missense or loss-of-function variant.

Abbreviations: CI, confidence interval; HR, hazard ratio; IR, incidence rates; IRR, incidence rate ratios; SD, standard deviation; VTE, venous thromboembolism.

### Table 2 Notes

- Qualifying variant—rare (minor allele frequency <0.1% in gnomAD or any of its regional subpopulations or the present population) non-benign (according to PolyPhen-2) missense or loss-of-function variant.

- IRs and IRRs are also presented. Prevalent cases of VTE were excluded.
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