Received: 25 March 2022 | Revised: 9 September 2022 | Accepted: 19 October 2022

DOI: 10.1002/rth2.12842

rpth research & practice in thrombosis & haemostasis

BRIEF REPORT

Genetic variation of the blood coagulation regulator tissue factor pathway inhibitor and venous thromboembolism among middle-aged and older adults: A population-based cohort study

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Funding information
ALF-funding from Region Skåne; Sparbanken Skåne; Vetenskapsrådet

Handling Editor: Dr Neil Zakai

Abstract

Background: Tissue factor is the main initiator of blood coagulation, and tissue factor pathway inhibitor (TFPI) is the primary inhibitor of the initiation of blood coagulation. The genetic variation of *TFPI* and the relation to venous thromboembolism (VTE), that is, venous thrombosis and pulmonary embolism, remains to be clarified. This exome sequencing study aimed to determine the molecular epidemiology of the *TFPI* gene and the relation to VTE in a large population-based cohort of middle-aged and older adults.

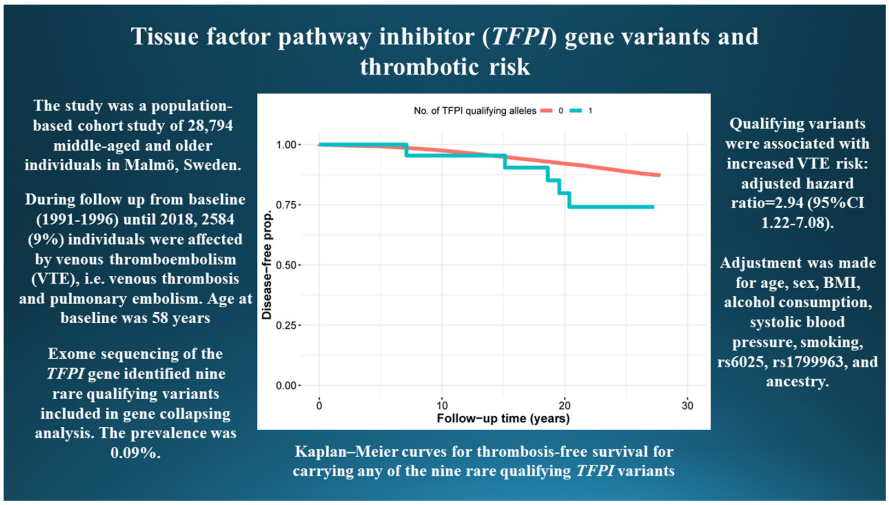
Methods: The exomes of *TFPI* were analyzed for variants in 28,794 subjects without previous VTE (born 1923–1950, 60% women), who participated in the Malmö Diet and Cancer Study (1991–1996). Patients were followed until the first event of VTE, death, or 2018. Qualifying variants were defined as loss-of-function or nonbenign (PolyPhen-2) missense variants with minor allele frequency less than 0.1%.

Results: No common variant was associated with VTE. Nine rare variants (two loss-of-function and seven nonbenign missense) were classified as qualifying and included in collapsing analysis. Prevalence of qualifying variants was 0.09%. Five individuals with VTE compared to 17 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 ancestry showed a hazard ratio of 2.9 (95% CI, 1.2–7.1) for rare qualifying variants.

Conclusion: Rare qualifying *TFPI* variants were associated with VTE, suggesting that rare variants in *TFPI* contribute to the development of VTE. The qualifying *TFPI* gene variants were very rare, suggesting a constrained gene.

A list of the Regeneron Genetics Centers is provided in Appendix A.

KEYWORDS
blood coagulation, genetic variation, molecular epidemiology, thrombophilia, venous thromboembolism



Essentials

- The *TFPI* gene has not been linked to venous thromboembolism (VTE).
- The study was a population-based cohort study of 28,794 middle-aged and older individuals.
- Sequencing of the *TFPI* gene identified nine rare qualifying variants in 0.09% of the population.
- Qualifying variants were associated with an almost 3 times increased risk of VTE.

1 | INTRODUCTION

Tissue factor (TF) is the main initiator of blood coagulation, and tissue factor pathway inhibitor (TFPI) is the primary inhibitor of the initiation of blood coagulation.[1,2] The initiation of coagulation due to exposure of TF, the extrinsic pathway, is the main mechanism by which coagulation is initiated in vivo in response to trauma.[1,2] TF is a membrane protein abundantly present in cells surrounding the vascular bed.[1,2] It binds both zymogen and activated forms of factor VII (factor VIIa). The TFPI inhibits the reactions involving TF and factor VIIa.[1,2] A lack of TFPI is probably not compatible with life since no homozygote deficiency states have been described in humans.[2] This theory is suggested by the lethal phenotype found in TFPI knockout mice.[2,3] These mice exhibit uncontrolled activation of coagulation with consumption of coagulation factors.[2,3] In mice, low TFPI levels are associated with thrombosis.[4]

The *TFPI* gene spans approximately 90 kb on chromosome 2 (q32.1) and consists of 10 exons and nine introns.[1] Polymorphisms and mutations in the human *TFPI* gene have been identified.[5–13] *TFPI* gene variants have been associated with venous thromboembolism (VTE), that is, venous thrombosis or pulmonary embolism (PE), but studies have not been consistent.[5–13] Two large genome-wide association studies have not found any associations with common *TFPI* gene variants and VTE.[14,15] Moreover, whole-exome sequencing (WES) of 393 individuals with unprovoked VTE and 6114 controls could not link the *TFPI* gene to VTE.[16] The reason why the *TFPI* gene (which has very important anticoagulant functions) has not been linked to VTE is unclear.

Based on the important antithrombotic role of TFPI, we hypothesized that variants within the *TFPI* gene that alter gene expression and/or impair anticoagulant function could predispose to VTE. This exome sequence study aimed to determine the genetic variation in the *TFPI* gene and a possible thrombotic risk in a large population-based study. We therefore analyzed the exome sequence and molecular epidemiology in 28,794 individuals in the large Malmö Diet and Cancer Cohort (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.[17,18] Sample characteristics, data collection, and clinical definitions for MDC have been described previously.[17,18] 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 informed written consent.

2.2 | Clinical end points

One outcome, VTE, was examined. Events were identified through linkage with the Swedish National Patient Registry (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 the *International Classification of Diseases*, Seventh, Eighth, Ninth, and Tenth Revisions (ICD-7, ICD-8, ICD-9, and ICD-10) codes in the SNHDR. ICD-7, ICD-8, and ICD-9 were used to identify prevalent cases in the SNHDR according to Manderstedt et al.[18] (ICD codes in appendix). All different VTE manifestations were included because they share familial susceptibility in the Swedish population reviewed by Zöller et al.[19] Moreover, segregation analysis indicates that major genes are important.[20] Included VTE manifestations were PE, deep venous thrombosis (DVT) of the legs, superficial venous thrombosis, migrating thrombophlebitis, portal vein thrombosis, vena cava thrombosis, cerebral venous thrombosis, Budd–Chiari syndrome, renal vein thrombosis, and thrombosis of unspecified vein.[18–20] Only ICD-9 and ICD-10 were used for identification of incident VTE cases in the SNDHR during follow-up. The diagnosis of DVT and PE in the SNHDR has been shown to have an accuracy of 95%,[21] whereas the positive predictive value of the SNHDR is generally 85%–95%.[22] A quality control of 118 patients with DVT and PE in the MDC was performed.[17] In 106 (90%) of cases, the diagnosis was correct.[17] Patients with VTE in Malmö University Hospital and in Sweden in general usually are diagnosed with objective methods.[17,18,21]

2.3 | Genetic and statistical analysis

Whole-exome sequencing was performed by the Regeneron Genetics Center (Tarrytown, NY),[23] such that more than 85% of targeted bases were covered at a read depth of greater than 20X. ANNOVAR was used to aggregate variant annotation, allele frequencies, and in silico predictions of deleteriousness.[24] Cox proportional hazards regression was used to examine the association between genotype and incident VTE. Age, sex, and ancestry were adjusted for in Model 1. The two most common strong genetic risk factors, rs6025 and rs1799963, were added in Model 2. To further reduce for statistical noise body mass index (BMI), smoking status, blood pressure (systolic), and high alcohol consumption (women, more than 30 g/day; men, more than 40 g/day), that is, potential cardiovascular risk factors, were added in the multivariate Model 3.[17,18] R version 4.0.0 R Foundation for Statistical Computing) was used for all statistical analyses. Alcohol problems have been associated with VTE.[21] To control for possible population stratification, a principal component analysis (PCA) on common variants was performed, and the two largest principal components were included in the statistical model. PCA was performed as described.[25] The reference genomes were obtained from the 1000 Genomes Project server.[25] The PCA was performed with independent (R^2 measure of linkage disequilibrium [LD] less than 0.2) common (minor allele frequency [MAF] 5% or greater) autosomal biallelic 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, 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.[26] The fit of the proportional hazards model was checked visually by plotting the incidence rates over time and by calculating Schoenfeld (partial) residuals.[17,18] No violation against proportional hazards assumption was 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 of these, 2584 (9%) were affected by a VTE event during follow-up until December 31, 2018. First VTE diagnosis was DVT of the lower extremities in 964 cases, 928 cases had PE, and 692 cases had other causes of VTE (thrombosis of unspecified vein, n = 529; superficial thrombophlebitis, n = 101; portal vein thrombosis, n = 30; vena cava thrombosis, n = 17; cerebral venous thrombosis, n = 7; migrating thrombophlebitis, n = 4; renal vein thrombosis, n = 3; Budd–Chiari syndrome, n = 1). The sum of the follow-up time was 587,992 years, corresponding to a VTE incidence rate of 4.4 (95% CI, 4.2–4.6) per 1000 person-years. Resequencing identified a total of 41 *TFPI* gene variants in the study population, 14 synonymous, 25 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 and C. Fifteen of the 41 variants were detected in single individuals, and 2 of the 10 variants lacking an rs number were found among individuals with incident VTE and 10 among individuals without VTE. There was one *TFPI* gene variant with a MAF greater than 1% (rs5940). For this p.Val292Met variant, no significant overrepresentation in cases was

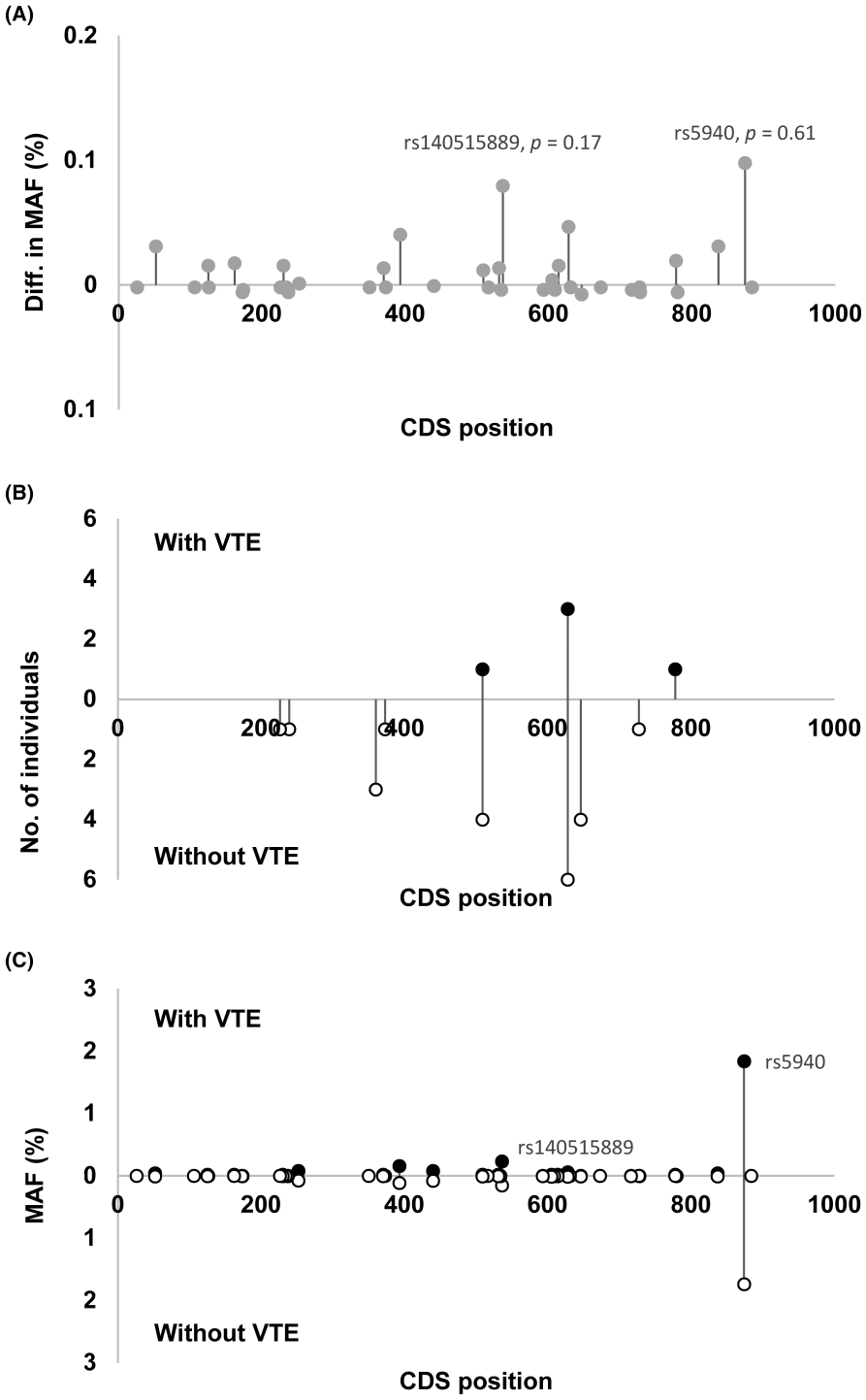


FIGURE 1 (A) The difference in minor allele frequencies (MAFs) between individuals with and without incident venous thromboembolism (VTE). (B) The distribution and number of qualifying variants in individuals with and without incident VTE. (C) MAF and coding sequence positions for all detected *TFPI* variants in individuals with and without incident VTE. CDS, coding sequence.

observed ($p = 0.61$; Figure 1A). Nor was there any significant association with rs140515889 p.Pro179Leu ($p = 0.17$; Figure 1A).

Nine variants were classified as qualifying and included in collapsing analysis using the following selection criteria: LoF or nonbenign (PolyPhen-2) missense variants with MAF less than 0.1% (Table 1; Figure 1B). Five individuals with VTE compared to 21 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 0.09%. 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 are summarized for the rare qualifying variants in Table 2. The Cox multivariate regression analysis showed a fully adjusted HR of 2.9 (95% CI, 1.2–7.1) for the rare qualifying variants (Table 2).

Although studies have identified *TFPI* variants among VTE patients that even may affect TFPI plasma concentration,[6–13,26] the *TFPI* gene remains to be linked consistently to VTE and thrombophilia in humans.[6–13] The present study confirms that common variants in *TFPI* are not associated with VTE.[6–13] Rare qualifying variants, that is, nonbenign

TABLE 1 Description of rare qualifying *TFPI* variants in the Malmö Diet and Cancer Study, defined as loss-of-function or nonbenign missense variants according to PolyPhen-2 with minor allele frequency <0.1% according to gnomAD

Location (GRCh38)	Consequence	Codon	Protein position	aa	Exon/intron	PolyPhen-2	ACMG	Heterozygotes		rsID
								VTE	No VTE	
2:187467783	Missense	Aaa/Caa	260	K/Q	Exon 7	Possibly damaging	US	1[a]	0	rs747717498
2:187467834	Missense	Cgc/Tgc	243	R/C	Exon 7	Probably damaging	US	0	1	rs1055198806
2:187467915	Missense	Tgg/Cgg	216	W/R	Exon 7	Probably damaging	US	0	4	rs746779363
2:187484124	Missense	Gaa/Caa	210	E/Q	Exon 6	Possibly damaging	US/P	3[a]	6	rs775447533
2:187484837	Missense	gAa/gGa	170	E/G	Exon 5	Probably damaging	LB	1[a]	4	rs769566808
2:187484973	Missense	Tgc/Cgc	125	C/R	Exon 5	Probably damaging	US/LP	0	1	–
2:187484989	Splice acceptor	–	–	–	Intron 4	NA	P	0	3	–
2:187488377	Splice_acceptor	–	–	–	Intron 3	NA	LP	0	1	–
2:187496974	Missense	Act/Gct	76	T/A	Exon 3	Possibly damaging	P	0	1	–

Note: There was no linkage disequilibrium between any of the variants.

Abbreviations: aa, amino acid; ACMG, American College of Medical Genetics and Genomics; LB, likely benign; LP, likely pathogenic; NA, not applicable; P, pathogenic; US, uncertain significance; US/LP, uncertain significance with minor pathogenic evidence; US/P, uncertain significance with some pathogenic evidence; VTE, venous thromboembolism.

[a]All the 5 affected heterozygous carriers of a rare qualifying *TFPI* variant were women. One had superficial thrombophlebitis (rs747717498), two had deep venous thrombosis of the legs (both rs775447533), and two had thrombosis of unspecified vein (rs775447533 and rs769566808). One individual (rs775447533) was heterozygous for the rs1799963 variant. No affected individual with a qualifying *TFPI* variant carried the rs6025 variant or any disease-causing variants in the *PROS1*, *PROC*, or *SERPINC1* genes. Individuals carrying the rs775447533 were unrelated.

FIGURE 2 Kaplan–Meier curves for thrombosis-free survival for individuals carrying any of nine qualifying *TFPI* variants (defined as loss-of-function or non-benign missense variants according to PolyPhen-2 with minor allele frequency <0.1%)

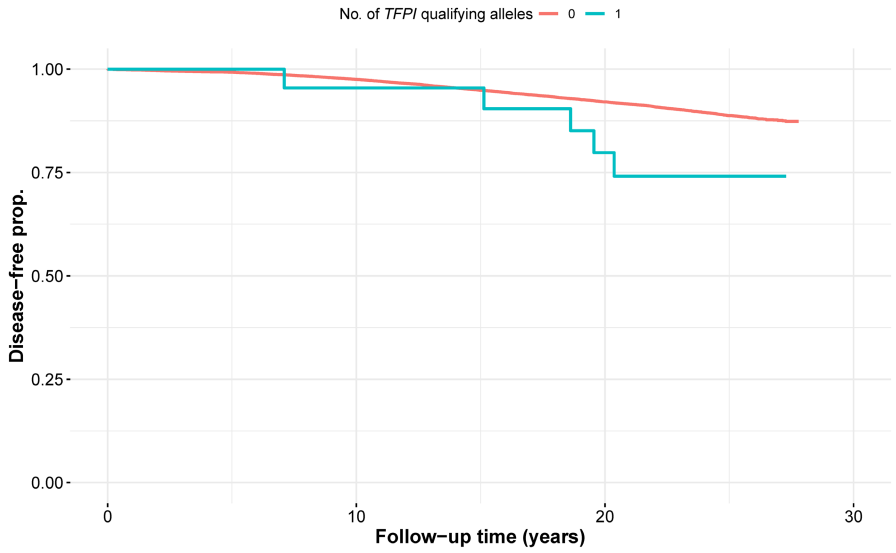


TABLE 2 HRs for incident VTE

	N VTE cases, *n* (%)	N noncases, *n* (%)	HR (95% CI), *p* value		
			Model 1	Model 2	Model 3
No *TFPI* variant	2579 (99.8)	26,189 (99.9)	1	1	1
1 *TFPI* variant	5 (0.2)	21 (0.1)	2.65 (1.11–6.40), 0.029	2.70 (1.12–6.50), 0.026	2.94 (1.22–7.08), 0.016

Note: Prevalent cases of VTE were excluded. Model 1 was adjusted for age, sex, and ancestry. Model 2 was adjusted for age, sex, rs6025, rs1799963, and ancestry. Model 3 was adjusted for age, sex, BMI, alcohol consumption, systolic blood pressure, smoking, rs6025, rs1799963, and ancestry.

Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio; VTE, venous thromboembolism.

missense variants (PolyPhen-2) and LoF variants, were associated with VTE. This is line with the finding that low levels TFPI increase the risk of venous thrombosis in the Leiden Thrombophilia Study and in the Longitudinal Investigation of Thromboembolism Etiology.[27,28] However, these variants were very rare (0.09%), and a large study size is necessary to be able to have enough statistical power to show this

with genome-wide significance for WES studies (2.5×10^{-6}). The rareness of these potentially damaging variants explains why the *TFPI* gene has not been linked to VTE. According to data from gnomAD, *TFPI* has a probability of being LoF intolerant score of 0.248 (https://gnomad.broadinstitute.org/) and observed/expected LoF metric of 0.25 (90% CI, 0.12–0.56).[29] The gene also exhibits constraints for missense variants with observed/expected 0.71 (90% CI, 0.61–0.83).

Experimental *TFPI* knockout mice studies indicate a lethal phenotype not compatible with life.[2–4] These mice exhibit uncontrolled activation of coagulation with consumption of coagulation factors.[3,4] We found no homozygote or compound heterozygote for qualifying *TFPI* gene variants in accordance with these animal studies.[2–4] A low TFPI level in mice is associated with thrombosis,[2–4] which is line with an adjusted HR of 2.9 found in the present study. In fact, in the present population-based cohort study of middle-aged and older individuals, the HR of 2.9 should be compared to 1.8 of rs6025 heterozygotes, 1.6 for rs1799963 heterozygotes, and 1.6 for high-risk variants of the *SERPINC1*, *PROC*, and *PROS1* genes.[18] This suggests that qualifying *TFPI* gene variants are at least as strong VTE risk factors as classical thrombophilia.[18]

A limitation is that the diagnosis of VTE was based on ICD codes from the inpatient and outpatient registers and that the outpatients register only existed from 2001. Thus, we do not have the exact diagnostic information for the individual patients. However, the latter is most likely a nondifferential bias regarding VTE risk. No violation against proportional hazards assumption over time was observed. A strength of our study is the high coverage of VTE diagnosis in the SNDHR since 1970 in Malmö and that VTE diagnosis is usually confirmed by objective methods in Malmö and throughout Sweden, as well as the high quality of SNDHR.[22,23] A validation of 118 patients with incident VTE in the MDC cohort confirms this (90% accuracy).[17] 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 3.87 per 1000 person-years.[30] A limitation is also lack of information about anticoagulant and aspirin treatment, but we adjusted for potential cardiovascular risk factors. We had no information about provoked and unprovoked VTE. A further limitation is the lack of a replication study. Still, the present study is based on 28,794 exomes and gives a good overview of the rare genetic variation of the *TFPI* gene.

In conclusion, exome sequencing of the *TFPI* indicates that rare qualifying missense and LoF variants were associated with VTE among middle-aged and older individuals. However, these qualifying variants were very rare, and a large population size is necessary for confirmation of this at a genome-wide significance level.

AUTHOR CONTRIBUTIONS

EM, CH, and BZ 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. Whole-exome sequencing was performed by the Regeneron Genetics Center (see the Regeneron Genetics Center Banner Author List and Contribution Statements below). EM, CH, and BZ are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

ACKNOWLEDGMENTS

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.

RELATIONSHIP DISCLOSURE

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How to cite this article: Manderstedt E, Lind-Halldén C, Halldén C, et al. Genetic variation of the blood coagulation regulator tissue factor pathway inhibitor and venous thromboembolism among middle-aged and older adults: A population-based cohort study. *Res Pract Thromb Haemost*. 2022;6:e12842. doi:10.1002/rth2.12842

APPENDIX A

Regeneron Genetics Center Banner Author List and Contribution Statements

All authors/contributors are listed in alphabetical order. *RGC Management and Leadership Team*: Goncalo Abecasis, PhD; Aris Baras, MD; Michael Cantor, MD; Giovanni Coppola, MD; Aris Economides, PhD; Luca A. Lotta, MD, PhD; John D. Overton, PhD; Jeffrey G. Reid, PhD; Alan Shuldiner, MD. Contribution: All authors contributed to securing funding, study design, and oversight. All authors reviewed the final version of the manuscript. *Sequencing and Lab Operations:* Christina Beechert; Caitlin Forsythe, MS; Erin D. Fuller; Zhenhua Gu, MS; Michael Lattari; Alexander Lopez, MS; John D. Overton, PhD; Thomas D. Schleicher, MS; Maria Sotiropoulos Padilla, MS; Louis Widom; Sarah E. Wolf, MS; Manasi Pradhan, MS; Kia Manoochehri; Ricardo H. Ulloa. Contribution: CB, CF, AL, and JDO performed and are responsible for sample genotyping. CB, CF, EDF, ML, MSP, LW, SEW, AL, and JDO performed and are responsible for exome sequencing. TDS, ZG, AL, and JDO conceived and are responsible for laboratory automation. MP, KM, RU, and JDO are responsible for sample tracking and the library information management system. *Genome Informatics:* Xiaodong Bai, PhD; Suganthi Balasubramanian, PhD; Andrew Blumenfeld; Boris Boutkov, PhD; Gisu Eom; Lukas Habegger, PhD; Alicia Hawes, BS; Shareef Khalid, Olga Krasheninina, MS; Rouel Lanche; Adam J. Mansfield, BA; Evan K. Maxwell, PhD; Mrunali Nafde; Sean O'Keeffe, MS; Max Orelus; Razvan Panea, PhD; Tommy Polanco, BA; Ayesha Rasool, MS; Jeffrey G. Reid, PhD; William Salerno, PhD; Jeffrey C. Staples, PhD. Contribution: XB, AH, OK, AM, SO, RP, TP, AR, WS, and JGR performed and are responsible for the compute logistics, analysis, and infrastructure needed to produce exome and genotype data. GE, MO, MN, and JGR provided compute infrastructure development and operational support. SB, SK, and JGR provide variant and gene annotations and their functional interpretation of variants. EM, JS, RL, BB, AB, LH, JGR conceived and are responsible for creating, developing, and deploying analysis platforms and computational methods for analyzing genomic data. *Research Program Management:* Marcus B. Jones, PhD; Jason Mighty, PhD; Lyndon J. Mitnaul, PhD. Contribution: All authors contributed to the management and coordination of all research activities, planning, and execution. All authors contributed to the review process for the final version of the manuscript.