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ORIGINAL ARTICLE

The impact of *PROS1* mutation position on thrombotic risk in protein S–deficient patients

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Abstract

Background: Inherited protein S deficiency is a thrombophilic risk factor associated with venous thromboembolism. However, there is not much data on the impact of mutation position on thrombotic risk.

Objectives: The aim of this study was to evaluate the risk of thrombosis due to mutations located in the sex hormone–binding globulin (SHBG)–like region as opposed to the rest of the protein.

Methods: Genetic analysis of *PROS1* was performed in 76 patients with suspected inherited protein S deficiency, and the effect of missense mutations present in the SHBG region on thrombosis risk was analyzed by statistical methods.

Results: We found 30 unique mutations (13 of them novel), of which 17 were missense mutations, in 70 patients. Patients with missense mutations were then divided into 2 groups: the "SHBG-region" mutation group (27 patients) and the "non-SHBG" group (24 patients). The multivariable binary logistic regression analysis showed that mutation position in the SHBG region of protein S is an independent risk factor for thrombosis in deficient patients (OR, 5.17; 95% CI, 1.29-20.65; P = .02). The patients with a mutation in the SHBG-like region also developed a thrombotic event at a younger age compared to the "non-SHBG" group in the Kaplan-Meier analysis (median thrombosis-free survival of 33 vs 47 years, respectively; P = .018).

Conclusion: Our findings show that a missense mutation located in the SHBG-like region may contribute to higher thrombotic risk rather than a missense mutation located elsewhere in the protein. However, as our cohort was relatively small, these findings should be taken with this limitation.

KEYWORDS
protein S deficiency, venous thromboembolism, missense mutation, thrombosis, protein S

Essentials

- Inherited protein S (PS) deficiency is an established risk factor for venous thromboembolism.
- The impact of missense mutations and their location on the risk of thrombosis is not clear.
- Mutations located in a specific region of PS may have a higher impact on thrombotic risk.
- Thus, genetic analysis could contribute to further risk prediction in PS-deficient patients.

1 | INTRODUCTION

Protein S (PS) acts as a cofactor for activated protein C (APC) in proteolytic inactivation of blood coagulation factors (F) Va and VIIIa, thus participating in regulation of thrombin generation through the APC pathway. Protein S was also reported to have direct anticoagulant activity, ie, independent of APC—in the presence of zinc ions, it directly binds to FIXa and inhibits the activation of FX, and it also directly binds FVa and FXa and inhibits their combined activity [1,2]. It also serves as a cofactor for tissue factor pathway inhibitor-α (TFPIα) together with FV or with its splice variant (FV-short) during factor Xa inhibition, in which it enhances TFPI-FXa complex formation [3]. Protein S in plasma can circulate free or bound to the complement regulatory factor complement factor 4b (C4b)–binding protein. Free PS (FPS) accounts for around 40% of total PS and is responsible for most of its cofactor activity [4].

Protein S deficiency can be acquired or hereditary. Inherited PS deficiency is divided into 3 types—type I (quantitative) with low PS activity and FPS and total PS (TPS) levels; type II (qualitative) with low PS activity and normal FPS and TPS levels; and type III (mixed) with low PS activity and FPS levels and normal TPS levels [5]. In some families, different individuals can present with either type I or type III protein S deficiency, suggesting that the same mutation can cause different laboratory patterns [6,7].

The protein S gene (*PROS1*) is located on chromosome 3q11.2. Protein S in its mature form is a single-chain (75 kDa) glycoprotein. The N-terminal gamma-carboxyglutamic (Gla) domain (exon 3), containing 11 Gla residues, interacts with calcium ions to facilitate high-affinity binding to negatively charged phospholipid membranes. The thrombin-sensitive region (exon 4) contains 2 thrombin-sensitive cleavage sites and an FXa-sensitive cleavage site. After proteolysis, the Gla domain remains attached to the remaining PS via the disulphide bridge but loses affinity for the membrane, and thus, PS loses its APC cofactor activity. Four connected epidermal growth factor–like domains (exons 5-8) are important for PS activity as they also facilitate Ca^{2+} binding. The following sex hormone–binding globulin (SHBG)–like region (exons 9-15, Val243-Ser635), comprising 2 laminin G-type domains, hosts several critical functions. The Gla domain is essential for all anticoagulant functions of PS due to the fact that it binds to negatively charged phospholipids. The SHBG-like region has been identified as having a number of important functions, including all of the direct anticoagulant functions of PS (direct binding to FIXa, FVa [aa 621-635], and FXa in the presence of zinc ions and binding to the TFPIα Kunitz-3 domain) and C4BP binding (specifically aa 420-434, 447-460, and 605-615) [8,9]. There are data showing the interaction with tyrosine kinase receptors of the Tyro3, Axl, and Mer family, which is also located within the laminin G-type domains [10,11].

Considering the diverse functions of the SHBG-like region of PS, it is possible that mutations located within that region would be more damaging in respect to the disrupted folding and subsequent loss of function of this region. Therefore, the aim of this study is to evaluate the risk of thrombosis due to mutations located in the SHBG-like region, as opposed to the rest of the protein.

2 | METHODS

2.1 | Study design and population characteristics

Our study consisted of 2 parts. First, we determined the *PROS1* mutations in our patients. Next, we conducted a retrospective cohort study to evaluate whether mutation position in the SHBG-like region affects thrombotic risk. The monitored outcomes were occurrence of any thrombotic event and the age of the patient when the patient's first thrombotic event occurred.

Informed consent was obtained from all participants, and the study met the institutional ethics requirements. The families selected for the study population had confirmed low PS activity and/or antigen levels in the index patient and, if possible, at least 1 close relative. Other causes of thrombophilia (antithrombin or protein C deficiency, FV Leiden [FVL], FII20210A polymorphisms, and high FVIII levels) were tested in all patients. The study population includes PS-deficient patients from the Czech Republic from different hospitals and centers with different testing routines. Thus, some of the patients did not have FPS and TPS antigen levels tested, as the primary method for PS deficiency screening in the Czech Republic has, for many years, been solely the PS activity assay. PS deficiency was defined per our laboratory reference range as PS activity levels of <50% for women and 65% for men, free PS levels of <50% for women and <70% for men, and total PS levels of <65%.

When our patients underwent thrombophilia screening and PS deficiency was found, we first repeated the PS activity assay after approximately 3 months to exclude anomalous results or interference of medication. We would then consult the patient's physician to determine any relevant factors that could influence PS activity levels (anticoagulants, recent thrombosis, high FVIII levels, nephrotic syndrome, etc.). Gravidity or hormonal therapy status was noted, and

additional testing was postponed in these patients. With these confounding factors ruled out, FPS and TPS levels were analyzed along with the confirmatory PS activity, and if the abnormal results persisted, the patient was indicated for genetic testing. If a mutation was found, we would then test all available first-degree relatives of the index patient and evaluate the personal and family history of thrombosis. The patient and family history data were obtained through clinical records search. Family members were selected for genetic analysis if they were PS-deficient, regardless of their thrombosis history.

Screening for PS deficiency in the index patients was done based on patient and/or family history of thrombosis (deep vein thrombosis, pulmonary embolism, and thrombosis in atypical locations such as portal, mesenteric, or cerebral vein), first venous thromboembolic event (VTE) before the age of 50, recurrent VTE, and in some cases, pregnancy complications or miscarriages. Deep vein thrombosis and other thrombotic events were documented by duplex ultrasonography and pulmonary embolism by computed tomography angiography. In some cases, PS deficiency was detected in thrombophilia screening done before beginning of oral contraceptive use or due to suspected thrombosis that was, however, later not confirmed.

We indicated 42 index patients for genetic analysis of *PROS1*. Out of the referred families, 43 PS-deficient family members were also indicated. However, sample material for genetic testing was only available from 34 of them, and therefore, in total, 76 PS-deficient patients were analyzed.

2.2 | Protein S plasma levels

PS coagulation activity was measured using STA-Staclot Protein S assay (Diagnostica Stago), which was also used as a screening method for suspected PS deficiency. Free PS antigen was measured by STA Liatest Free Protein S (Diagnostica Stago). PS total antigen was measured by Laurell immunoelectrophoretic assay [12] using polyclonal rabbit anti-human protein S antibodies (A0384, Dako Denmark A/S). All our assays used in this study are verified by external quality assessment (UK NEQAS). Other laboratories contributing to this study have interlaboratory traceability verified (use of IVD/CE reagents and participation in external quality assessment).

2.3 | *PROS1* genetic analysis

Informed consent for genetic analysis was obtained from all patients. Human genomic DNA was isolated from leukocytes from peripheral whole blood [13].

The exons, intron-exon boundaries, and 439 base pairs of the proximal promoter area of *PROS1* (gene ID: 5627, OMIM#176880, reference sequence for genomic DNA: NG009813.1) were examined by direct sequencing as previously described [14]. Primers and conditions were chosen so as to avoid coamplification of the highly homologous PS pseudogene (*PROS2*, *PROSP*), and restriction analysis was performed for each amplification product to ensure that the pseudogene was not present. Apart from the 15 exons in the PS transcriptional variant 2 (reference sequence NM000313.4), we designed primers for the additional exon from the alternatively spliced transcriptional variant 1 (reference sequence NM001314077.2; primer sequences available on request), positioned between exons 1 and 2 of variant 2, so as to prevent overlooking any variations influencing the processing of PS. The additional exon is in further text referred to as "exon 2-variant 1."

The possibility of a large deletion or duplication was verified by multiplex ligation–dependent probe amplification (MLPA) analysis using the SALSA MLPA P112 *PROS1* kit, (MRC; probemix lots: A3-0313, A3-0416, and A4-0318). The assay contains probes for 12 of the 15 exons of *PROS1*. MLPA was done in parallel with direct sequencing in all index patients regardless of mutations found. In family members, gene analysis was restricted to the abnormal region determined in the index patient.

For each novel sequence variation, the likelihood that the mutation could be disease-causing was evaluated by using several prediction software algorithms, such as Polyphen-2 [15], PMut [16], MutationTaster2 [17], PROVEAN [18], SNPs&GO [19], HOPE [20], and VarSome [21]. For splice-site changes prediction, Splice site prediction by Neural Network tool was used [22]. Novel mutations (newly discovered mutations in our patient cohort) were indicated as "disease-causing" when at least 5 of 7 in silico tools used in our study provided this prediction. Other disease-causing mutations were previously described as associated with PS deficiency.

2.4 | Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM Corp) and R version 1.2.5019 (Development for R, RStudio Inc). Continuous variables are expressed as median (IQR), and categorical variables are expressed as number (percentage) of participants within each group. The normality of data distribution was tested using the Kolmogorov-Smirnov test. Since a substantial part of the data did not fit normal distribution, only nonparametric statistical methods were used. The Mann–Whitney U-test was used for continuous variables, and chi-squared test was used for categorical variables. Binary logistic regression was used to assess the risk factors of thrombotic events. Aside from the mutation position (main variable of interest), the variables included in the multivariable model were age at admission, sex, and FVL, as these factors likely affect the outcome. Furthermore, PS activity was considered for inclusion; however, it should be viewed as at least a partial mediator in the relationship between mutation position and thrombotic risk and, therefore, cannot be included in the same model as it is a colinear. Thus, the variables included in the final multivariable model are mutation position, sex, age at admission, and FVL. The variable selection is depicted by a directed acyclic graph (Supplementary Figure). In the multivariable model, there were 3 instances of missing values of PS activity, and in these cases, the median value was inputted in the regression. There were no missing values for categorical variables. The results of multivariable analyses were expressed as odds ratios (ORs) with 95% CIs. For all other analyses,

missing values were ignored (complete case analyses). Kaplan-Meier survival curves and the log-rank test were used to project thrombosis-free intervals for patients with mutations in different parts of PS. "Thrombosis-free interval" herein represents the period from birth to the first VTE event. All reported tests were 2-sided, and a *P* value of <.05 was considered significant.

3 | RESULTS

3.1 | Mutation profile

In our patient cohort, we found a disease-causing mutation in 92% (70 of 76) of PS-deficient patients, consisting of 13 novel and 17 previously reported mutations. The disease-causing mutations include 13 null mutations (1 large deletion, 4 nonsense mutations, 4 splice-site substitutions, 3 small insertions <40 base pairs, and 1 small deletion) and 17 missense mutations, respectively. All found mutations were in heterozygous form; 2 families (3 patients) had 2 different concurrent heterozygous mutations. The prevalence of large deletions in our study was 7% (3 of 42 families).

3.2 | Patient characteristics

The study flowchart is shown in Figure 1. Further supporting data are available in the Supplementary Table 1.

3.3 | *PROS1* mutation position groups

The patients with missense mutations (null mutations were excluded) were divided into 2 groups: the "SHBG-region" mutation group (27 patients with 5 different missense mutations in exons 9-15) and the

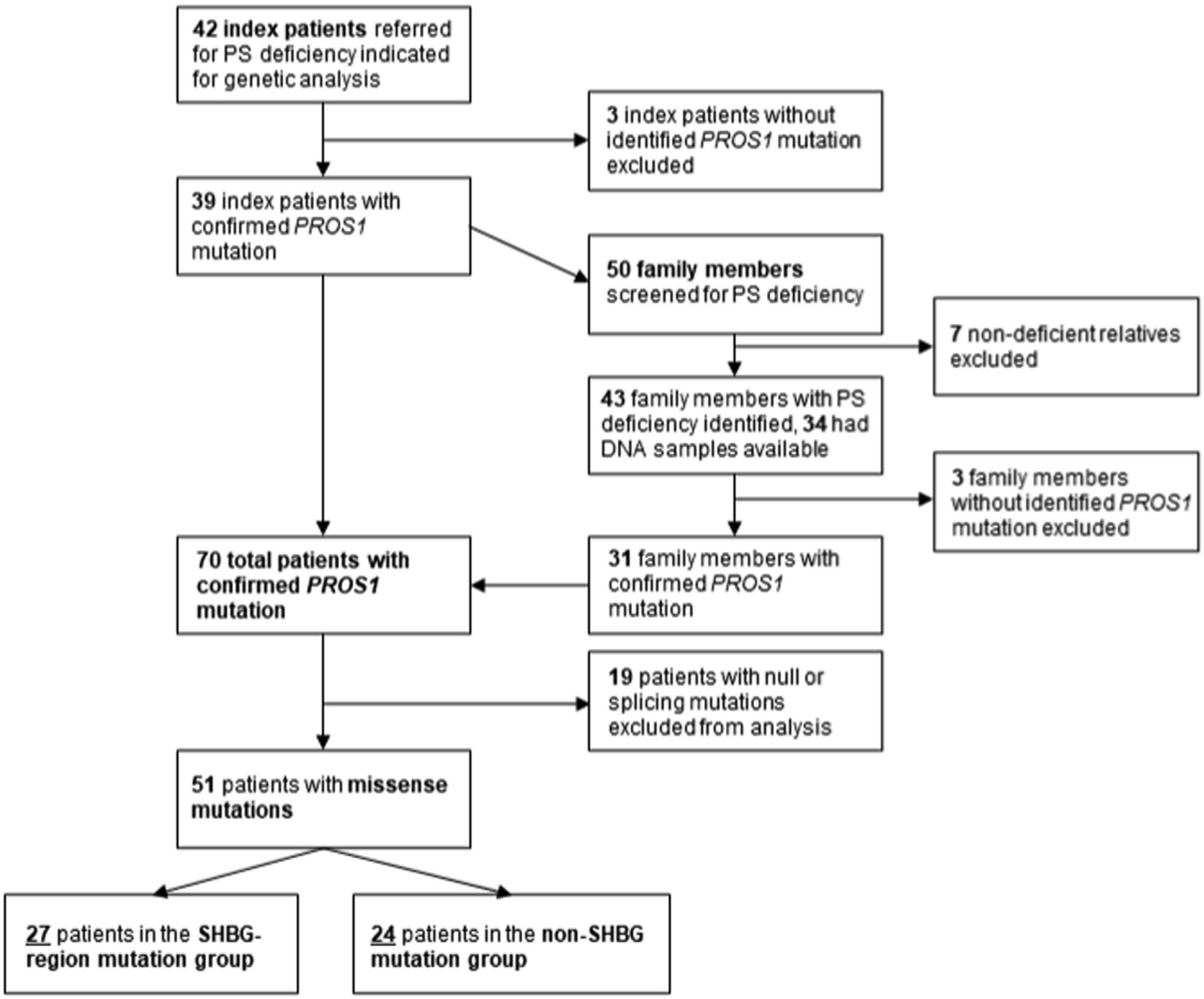

FIGURE 1 Summary flowchart of the study population showing patient selection and analysis process. PS, protein S; SHBG, sex hormone–binding globulin.

TABLE 1 Basic characteristics of the mutation groups.

Factor	All participants (*n* = 51)	SHBG group (*n* = 27)	Non-SHBG group (*n* = 24)	*P* value
Age (y), median (IQR)	32 (21-47)	32 (22-48)	33 (17-46)	.93
Female sex, *n* (%)	36 (70.6)	17 (63)	19 (72.9)	.21
FV Leiden, *n* (%)	13 (25.5)	12 (44.4)	1 (4.2)	.001
Thrombotic event, *n* (%)	29 (56.9)	20 (74.1)	9 (37.5)	.008
Age at first thrombosis (y), median (IQR)	31 (20–40.5)	26 (19–38.5)	33 (31.5–46)	.03

F, factor; SHBG, sex hormone–binding globulin.

"non-SHBG" mutation group (24 patients with 8 different mutations in exons 1-8). Basic characteristics of the mutation groups are summarized in Table 1. The age of admission and sex did not significantly differ between "SHBG-region" and "non-SHBG" groups. The mean PS activity level was significantly lower in the "SHBG-region" group than in the "non-SHBG" group, and the same trend was observed in FPS (P = .005 for PS activity and P = .001 for FPS, Figure 2). On the other hand, TPS levels did not show any significant difference (P = .30, Figure 2).

3.4 | *PROS1* mutation position and thrombosis risk

Next, the patients were divided into 2 groups according to whether they had a history of thrombotic event, and the basic characteristics are shown in Table 2. There was no difference in age at admission and sex between the groups. Patients with thrombotic events had higher rates of mutations in the "SHBG-region" group (69 vs 31.8%; P = .008). Interestingly, the rates of thrombotic events in patients with FVL

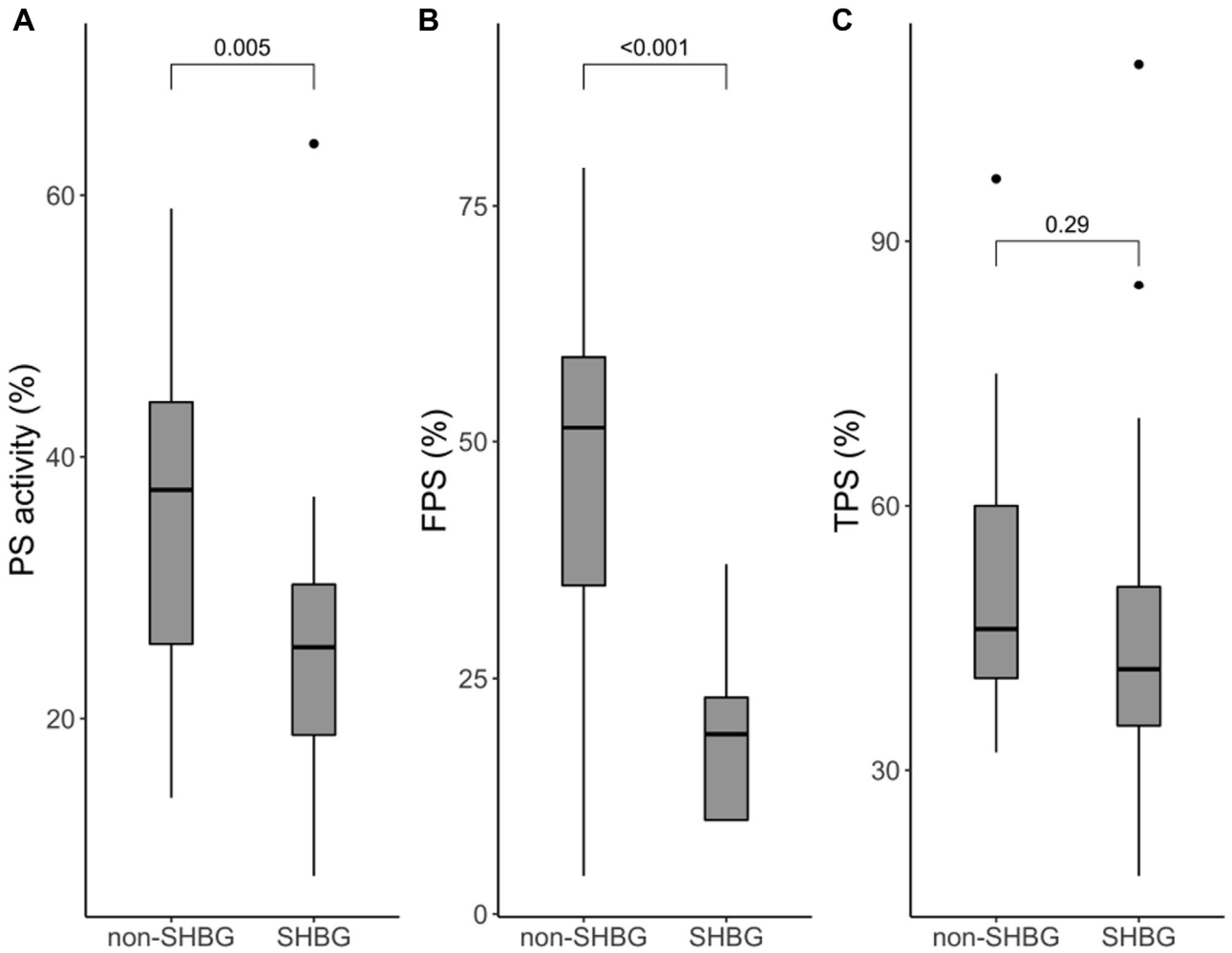

FIGURE 2 Results of comparison of PS levels in 2 groups of missense mutations: "SHBG-region" group (27 patients) and "non-SHBG" group (24 patients). A. PS activity: *n* = 51. B. Free PS (FPS): *n* = 32 (12 patients in "SHBG-region" group and 20 in "non-SHBG" group). C. Total PS (TPS): *n* = 31 (7 type III patients were excluded [3 and 4, respectively]; counted were 11 patients in "SHBG-region" group and 19 in "non-SHBG" group). PS, protein S; SHBG, sex hormone–binding globulin.

TABLE 2 Basic characteristics of the groups of patients with and without thrombotic events.

Factor	Thrombosis + (*n* = 29)	Thrombosis − (*n* = 22)	*P* value
Age (y), median (IQR)	35 (22-46)	29.5 (16-48.3)	.36
Female sex, *n* (%)	20 (69)	16 (72.7)	.77
FV Leiden, *n* (%)	9 (31)	4 (18.2)	.30
SHBG+, *n* (%)	20 (69)	7 (31.8)	.008

F, factor; SHBG, sex hormone–binding globulin.

mutations did not significantly differ (31% vs 18.2%; *P* = .30) even though FVL was found significantly more in patients in the "SHBG-region" mutation group (44.4% vs 4.2%, *P* = .001).

It is also important to note that the families from which multiple participants were enrolled had overall similar rates of family members affected by thrombosis with no 1 family being the majority driving force for thrombotic outcomes (Supplementary Table 2).

To evaluate the baseline impact of PS levels on thrombotic risk in patients with missense mutations (independent of mutation position), we compared them in patients with and without history of a thrombotic event. We found that patients with a history of thrombotic events showed a significant decrease in PS activity (*P* = .02) and FPS (type II patients excluded, *P* < .001) but not TPS (type II and III patients excluded; *P* = .07; Table 3).

To assess the association of mutation position with thrombotic risk, we performed a multivariable binary logistic regression analysis. FVL was included because this polymorphism is an established risk factor for thrombosis [23] and was present in 13 of 51 studied patients in heterozygous form, consisting of 12 patients in the "SHBG-region" group and 1 in the "non-SHBG" group. Since the representation of FVL in the mutation groups was uneven, the model was adjusted for FVL. The analysis showed that mutation position in the "SHBG-region" mutation group (as opposed to the "non-SHBG" group) is an independent risk factor for thrombotic events in PS-deficient patients (Table 4).

Additionally, several sensitivity analyses were performed. To explore whether the skewed distribution of FVL carriers in the 2 patient groups could have affected the results, we have excluded all FVL carriers and the multivariable model was adjusted for age at admission and sex. In this model, the mutation position was still independently associated with risk of thrombotic event (Supplementary Table 3). Next, to assess if the inclusion of index cases in the study affected the results, we performed another sensitivity analysis on only family members and excluded all index patients, regardless of whether they experienced a thrombotic event or not. In this sensitivity analysis, it was found that the mutation position was independently associated with the risk of thrombotic event even after the index patients were excluded (Supplementary Table 3). Similarly, the PS levels were lower in patients with mutation in the "SHBG-region" than in those with mutation in the "non-SHBG" region when index patients were removed (median, 25 [IQR, 19-31] vs 38 [IQR, 28-44]; *P* = .02).

Lastly, Kaplan-Meier method was used to compare the thrombosis-free period in both patient mutation groups. We found that patients in the "SHBG-region" group had shorter thrombosis-free survival (median thrombosis-free survival of 33 vs 47 years, *P* = .02; Figure 3). Furthermore, the median age of the first thrombotic event in the "SHBG-region" group was 26 years, compared with 33 years in the "non-SHBG" group (*P* = .04).

4 | DISCUSSION

In our study, we were able to identify a disease-causing mutation in 92% (70 of 76) of PS-deficient patients. Our analyses of the data from the deficient patients show that a missense mutation located in the SHBG-like region may contribute to higher thrombotic risk in our patients rather than a missense mutation located elsewhere in the protein. The group with mutations in the SHBG-like region also displayed shorter thrombosis-free survival.

4.1 | Impact of mutation position on protein S

The association of PS deficiency and low FPS levels with higher thrombotic risk has been previously described [24,25]. However, the impact of various *PROS1* missense mutations on thrombotic risk is poorly understood. Furthermore, there are cases in, for example, antithrombin (*SERPINC1*) missense mutations, in which there is a significant impact on thrombotic risk due to the location of the mutation [26]. Therefore, in this study, we sought to assess the influence of mutations located in the SHBG-like region on thrombotic risk as opposed to the rest of the protein.

We have shown that patients with mutations in the SHBG-like region present with lower PS activity and FPS levels than patients with mutations in other regions. There may be additional biological detrimental roles of mutations in the SHBG-like region outside of APC

TABLE 3 Comparison of PS levels in patients with and without history of thrombosis. Free PS values of type II and total PS values of type II and III–deficient patients were excluded.

Factor	Thrombosis + (*n* = 29)	Thrombosis − (*n* = 22)	*P* value
PS activity, median (IQR)	26 (19-31)	36.5 (27.5-43.25)	.02
Free PS, median (IQR)	22 (10-29)	51 (37-55)	<.001
Total PS, median (IQR)—type III patients excluded	39 (35-47)	45 (42-52.25)	.07

PS, protein S; SHBG, sex hormone–binding globulin.

TABLE 4 Results of logistic regression analysis of selected factors and their association with thrombotic risk.

	Univariable regression		Multivariable regression	
Factor	OR (95% CI)	*P* value	OR (95% CI)	*P* value
FVL	2.03 (0.53, 7.73)	.3	0.88 (0.17, 4.43)	.88
Age at admission (y)	1.01 (0.975, 1.05)	.59	1.01 (0.97, 1.05)	.58
Mutation position	4.76 (1.44, 15.7)	.01	5.17 (1.29, 20.65)	.02
Sex (female)	0.83 (0.25, 2.84)	.77	1.12 (0.29, 4.32)	.86
PS activity	0.94 (0.89, 0.99)	.03		

FVL, factor V Leiden; OR, odds ratio; PS, protein S.

cofactor activity, possibly lowering TFPIα-cofactor activity of PS as well, which may be the explanation for our findings. This study may provide valuable insight given the recent focus on the TFPIα-cofactor activity. Both major inhibitory pathways (APC and TFPI) use the SHBG-like region of PS in their functions of regulating thrombin generation; however, for the TFPIα pathway, this region is critical [27]. This may be further elucidated by using a specific TFPIα-cofactor activity assay when it becomes commercially available [28]. However, the lower PS levels in the "SHBG-region" mutation group may also cause lower free TFPIα levels, as these proteins circulate in complex, which can also contribute to the thrombosis risk [29].

Besides the simple synthesis reduction, there may be other reasons for the lower PS levels in the "SHBG-region" mutation group. First, as the C4BP binding site is located in the SHBG-like region of PS, the reduction in FPS could be attributed to decreased C4BP binding and, consequently, lower half-life [30]. This would lead to an overall reduction in PS activity as well. On this note, the FPS assay is based on the principle of binding a monoclonal antibody to the region where PS-C4BP binding is located, differentiating the bound and free portion of PS. This binding could then be disrupted by mutations in the SHBG-like region due to possible structural differences. Since this interaction has not been examined in this study, we cannot rule out assay-based interference.

Second, while all studied patients were heterozygotes, their PS activity levels are far below the reference range, even in patients with type II deficiency. This would suggest either problems in expression that lead to reduced synthesis of the healthy protein product (wild-type allele) or some other unknown interactions. The further reduction in PS activity, besides low FPS, could be caused by a disruption of structure by the mutation, which would then influence the anticoagulant activities of PS and its interaction with APC, TFPIα, and FV. The abnormal protein, when expressed, may thus be inactive in the classic PS activity assay based on APC pathway.

The mutations found in the "SHBG-region" patient group are relatively diverse. The missense mutations (p.W383R, p.L446R, p.R515C, p.A525T, p.T617I, p.G621D, and p.C639Y) affect highly conserved residues in which the substitution would result in disruption of the

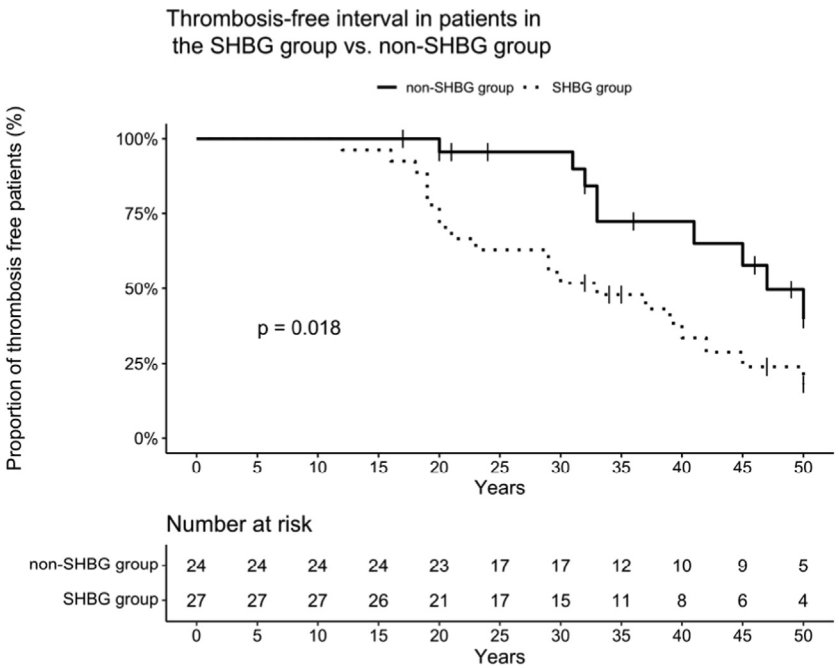

FIGURE 3 Thrombosis-free interval in patients in the "SHBG group" (*n* = 27) vs "non-SHBG group" (*n* = 24). SHBG, sex hormone–binding globulin.

secondary structure. Since there were similar rates of thrombosis in individual families affected with mutation in SHBG-like region, we assume that the observed higher risk of thrombosis is not caused by 1 specific mutation but the overall effect of mutations in this specific region.

Interestingly, even though FVL was found significantly more in patients with mutations in the SHBG-like region, the rates of thrombotic events in patients with FVL mutations did not significantly differ from the rest.

Among the limitations of our study is the relatively small study cohort stemming from rarity of the observed disease. Furthermore, due to the recruitment for this study, many of the participants have shared ancestry, and therefore, the outcomes are not fully independent as outcomes from members of the same family are likely correlated. Therefore, we have attempted to account for the kinship of the individuals by employing a mixed effects model. Unfortunately, the model could not be adjusted for family status as each family has a mutation specifically in either the SHBG-like region or non-SHBG–like region, and the mutation position is thus closely correlated with family status. Furthermore, we have included index patients into the analysis of association of mutation position and risk of thrombosis in order to increase the sample size of the study. Although not all index patients were identified due to a history of a thrombotic event, this could have been a potential source of bias, nonetheless. However, several sensitivity analyses were performed, including an analysis in which index patients were removed, and these analyses further support the conclusions of the study. Lastly, we have not done any experiments to verify if the mutated proteins undergo expression and secretion or if they show any activity in the assay.

5 | CONCLUSION

In conclusion, our analyses suggest that mutations in the SHBG-like region may have a larger impact on thrombotic risk in PS-deficient patients than those in other domains, as it is independently associated with risk of thrombosis, which is experienced at a younger age. Genetic analysis is routinely performed to confirm hereditary origin of PS deficiency and could have additional clinical value in its contribution to risk prediction in deficient patients.

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AUTHOR CONTRIBUTIONS

T.F. performed the genetic analysis, protein S measurements, and statistical analysis. M.M. provided majority of the patient data and clinical insight. V.G. and Z.K.-K. supplied additional patient data. D.P. provided expertise on genetic analysis. T.F. drafted the manuscript that was further revised by D.P., F.M., and I.H. I.H. designed and supervised the overall project and approved the final version of the manuscript. All authors have read and approved the final version of the paper.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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