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Similar hypercoagulable state and thrombosis risk in type I and type III protein S-deficient individuals from mixed type I/III families

Running title: Type I and type III protein S deficiency

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Background. Protein S, which circulates in plasma in a free and bound form, is an anticoagulant protein that stimulates both activated protein C (APC) and tissue factor pathway inhibitor (TFPI). Hereditary type I protein S deficiency (low total and low free protein S) is a well-established risk factor for venous thrombosis, whereas the thrombosis risk associated with type III deficiency (normal total and low free protein S) has been questioned.

Design and Methods. Kaplan-Meier analysis was performed on 242 individuals from 30 families with protein S deficiency. Subjects were classified as normal, type I deficient or type III deficient according to their total and free protein S levels. Genetic and functional studies were performed in 23 families (132 individuals).

Results. Thrombosis-free survival was not different between type I and type III protein S-deficient individuals. Type III deficient individuals were older and had higher protein S, TFPI and prothrombin levels than type I deficient individuals. Thrombin generation assays sensitive to the APC- and TFPI-cofactor activities of protein S revealed similar hypercoagulable states in type I and type III protein S-deficient plasma. Twelve *PROS1* mutations and 2 large deletions were identified in the genetically characterized families.

Conclusions. Not only type I, but also type III protein S deficiency is associated with a hypercoagulable state and increased thrombosis risk. However, these findings may be restricted to type III deficient individuals from families with mixed type I/III protein S deficiency, as these represented 80% of type III deficient individuals in our cohort.

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Introduction

Protein S (reviewed in ref.[1]) is a vitamin K-dependent glycoprotein mainly synthesized in the liver and present in plasma, platelets and endothelial cells. The mature protein comprises a Gla domain, a thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like domains and a large sex hormone-binding globulin (SHBG)-like domain. Plasma protein S (350 nM) is distributed between two pools: ~40% circulates free and ~60% is bound to the complement regulatory factor C4b-binding protein (C4BP).[2] Only C4BP molecules with a _-chain (C4BP-beta^{+}) bind protein S and, given the high affinity of this interaction (Kd =0.1 nM),[2] all C4BP-beta^{+} present in plasma (~200 nM) is complexed with protein S.[3]

Protein S is a potent anticoagulant protein that down-regulates thrombin formation *via* two mechanisms.[4] On one hand it stimulates the proteolytic inactivation of coagulation factors (F) Va and VIIIa by activated protein C (APC),[5] and on the other hand it enhances the inhibition of FXa by tissue factor pathway inhibitor (TFPI).[6-8] Although free protein S is the most active form, recent reports indicate that the protein S-C4BP complex also exhibits APC- and TFPI-cofactor activities.[6,9]

Hereditary protein S deficiency is a rare coagulation disorder associated with an increased risk of venous thrombosis.[10,11] It usually occurs as a partial (heterozygous) deficiency and segregates as an autosomal dominant trait. Presently, more than 200 mutations that impair protein S synthesis and/or function have been described in the protein S gene (*PROS1*), and large deletions/duplications within the *PROS1* locus have been identified as a relatively common cause of protein S deficiency.[12,13] Based on protein S antigen and (APC-cofactor) activity levels, protein S deficiency is classified as type I (low total and free antigen, reduced activity), type II (normal total and free

antigen, reduced activity) and type III (normal total antigen, reduced free antigen and activity). However, since protein S levels are strongly influenced by age, sex and hormonal status,[14] as well as by several acquired conditions, the diagnosis of protein S deficiency states based on protein S levels is far from straightforward in practice.

Type I and type III deficiencies account for 95% of protein S-deficient patients and often occur together in the same family as phenotypic variants of the same genetic defect (mixed type I/III deficiency).[15] The reasons for the different phenotypic expression are poorly understood, but the age-dependent increase in total protein S is thought to play a role, as type III protein S-deficient family members tend to be older than their type I deficient relatives.[16] Differently, other families with protein S deficiency only express the type III phenotype (pure type III deficiency). This type of protein S deficiency is often, but not always,[17] associated with the *PROS1* $Ser^{460}\rightarrow$Pro (Heerlen) mutation.[18]

Although protein S deficiency, and particularly low levels of free protein S,[19,20] are an established risk factor for venous thrombosis, risk estimates differ widely among studies, possibly reflecting the different severity of the underlying molecular defects.[21] Moreover, the few epidemiological studies that distinguish between type I and type III deficiencies are rather contradictory with respect to the thrombosis risk associated with type III deficiency, which was found to be none,[22] the same as in type I deficiency[23] or intermediate.[24]

To clarify this issue, we have re-evaluated the risk of thrombosis associated with type I and type III protein S-deficiencies by Kaplan-Meier analysis of a large cohort of protein S-deficient families. In support of our findings, we present a detailed characterization of type I and type III protein S-deficient plasma based on the measurement of coagulation factor levels and *ad hoc* thrombin generation assays.

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Design and Methods

Study population

Thirty families with type I and/or type III protein S deficiencies (242 individuals), identified at Padua University Hospital (Italy) between 1996 and 2002, were included in the Kaplan-Meier analysis. Families were ascertained *via* a proband who underwent thrombophilia screening after a first episode of venous thrombosis (deep-vein thrombosis, pulmonary embolism or superficial-vein thrombosis; n=27) or arterial thrombosis (myocardial infarction or stroke; n=3). Family members of each proband were invited to participate, and information on thrombosis history and age at onset of the first thrombotic event was recorded for each participant. Criteria for diagnosing venous thromboembolism have been previously reported.[25,26] Protein S deficiency was defined on the basis of free protein S levels,[27,28] applying a cut-off of 65%. The type of deficiency was assigned according to total protein S levels, using a cut-off of 70%. These cut-offs were based on the variation of total and free protein S levels in a population of 140 healthy individuals, irrespective of age and sex. However, application of sex-specific cut-offs for total and free protein S levels hardly affected the classification of the subjects and did not change the results described in this paper.

Plasma samples for functional studies and blood cells for DNA extraction could be obtained for 23 of the above-mentioned 30 families. Blood samples were collected from probands and consenting family members for a total of 151 subjects. Patients on oral anticoagulant therapy (n=13) and women taking oral contraceptives (n=3) or hormone replacement therapy (n=3) at the time of blood collection were subsequently excluded, leaving 132 individuals available for study (Table 1). All participants

provided informed consent to the study, which was carried out in accordance with the Helsinki protocol.

Blood collection and plasma preparation

Venous blood was drawn in 0.129 M sodium citrate (1:9 vol/vol) and platelet-poor plasma was prepared by centrifugation at 2000 μg for 10 min. Plasma was aliquoted, snap-frozen and stored at -80 °C until use. Buffy coats were stored at -20 °C for later DNA isolation.

Measurement of plasma factor levels

In-house ELISAs were used to measure the plasma antigen levels of total,[29] free[30] and C4BP-bound protein S,[9] as well as full-length TFPI.[31] Prothrombin levels were determined with a chromogenic assay, as previously described.[32] Antithrombin activity levels were determined using the Coamatic® Antithrombin kit (Chromogenix, Mölndal, Sweden). The levels of all factors were expressed as percentage of normal pooled plasma.

Thrombin generation measurements

Thrombin generation was measured with the Calibrated Automated Thrombogram (CAT) method.[33] Coagulation was initiated with tissue factor (TF, Innovin®, DADE-Behring, Marburg, Germany), synthetic phospholipid vesicles (DOPS/DOPC/DOPE, 20/60/20 mol/mol/mol) and $CaCl_2$, and thrombin activity in plasma was monitored continuously with fluorogenic substrate Z-Gly-Gly-Arg-AMC (BACHEM, Bubendorf, Switzerland). Fluorescence was read in a Fluoroskan Ascent® reader (Thermo Labsystems, Helsinki, Finland) and thrombin generation curves were

calculated using the Thrombinoscope™ software (Thrombinoscope, Maastricht, The Netherlands).

To probe the activity of the APC/protein S system, thrombin generation was initiated with 6.8 pM TF and 30 μM phospholipids in the absence and presence of 5 nM human APC (Kordia Life Sciences, Leiden, The Netherlands), as described.[32] The outcome of the assay was expressed as the ratio of the endogenous thrombin potentials (ETPs) obtained in the presence and absence of APC, and normalized against the ETP ratio of normal pooled plasma measured in parallel (normalized APC-sensitivity ratio, nAPCsr). Since protein S is a major determinant of this assay,[34] the nAPCsr is an indirect measure of the APC-dependent activity of protein S. The nAPCsr varies between 0 and 10 and increases as the plasma level of protein S (and hence its APC-cofactor activity) decreases.

To quantify the activity of the TFPI/protein S system, thrombin generation was initiated with 1.36 pM TF and 30 μM phospholipids in the absence and presence of neutralizing antibodies against protein S (270 μg/ml polyclonal IgG; DAKO, Glostrup, Denmark) or TFPI (64 μg/mL monoclonal IgG1; Sanquin, Amsterdam, The Netherlands), essentially as described.[31] To exclude any contribution of the APC-dependent activity of protein S, these measurements were conducted in the presence of 100 μg/ml anti-protein C polyclonal antibodies (DAKO). Moreover, corn trypsin inhibitor (CTI, Hematologic Technologies, Essex Junction VT, USA) was added to a final concentration of 30 μg/mL to prevent contact activation. At the low TF concentration used in this assay, the height of the thrombin peak proved to be a more sensitive indicator of thrombin generation than the ETP and was therefore used as output parameter. The ratio of the thrombin generation peaks obtained in the absence and presence of anti-protein S antibody (PS-ratio) is a measure of the TFPI-cofactor

activity of protein S, while the ratio of the thrombin generation peaks obtained in the absence and presence of anti-TFPI antibody (TFPI-ratio) is a measure of the activity of the TFPI/protein S system as a whole.[31] Both ratios vary between 0 and 1 and increase as the TFPI-cofactor activity of protein S decreases.

Genetic analysis

Buffy coats were available for 110 of the 132 eligible participants. Genomic DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega, Madison WI, USA). *PROS1* mutation screening was performed by PCR-mediated amplification of each exon (including splicing junctions) and >400 bp of the promoter region[35] followed by direct sequencing. PCR primers and conditions (available on request) were chosen such as to avoid co-amplification of the highly homologous protein S pseudogene. Selective amplification of *PROS1* was verified by restriction analysis of the PCR products. Whenever a *PROS1* mutation was identified, all family members were tested for carriership of that mutation by high-resolution agarose gel electrophoresis, restriction analysis or direct sequencing, as detailed in Table 2. The *PROS1* Heerlen genotype was determined by Rsa I-restriction analysis of exon 13.

In families without apparent mutation, the possibility of a large deletion was verified by multiple ligation-dependent probe amplification (MLPA) analysis using the SALSA MLPA P112 *PROS1* kit (MRC-Holland, Leiden, The Netherlands). MLPA reactions were carried out according to the manufacturer's instructions. After the addition of GeneScan™ 600 LIZ® Size Standard and HiDi™ formamide (Applied Biosystems, Foster City CA, USA), MLPA products were denatured at 80 °C for 2 min and separated by capillary electrophoresis on an ABI 3730 DNA analyzer (Applied Biosystems).

Carriership of the FV Leiden and prothrombin G20210A mutations was determined as previously described.[32]

Statistics

Kaplan-Meier analysis. Thrombosis-free survival was analyzed with the Kaplan-Meier method. The age at onset of the first thrombotic event for symptomatic individuals and the age at the time of inclusion in the study for asymptomatic subjects were used to evaluate the thrombosis-free survival time. Thrombosis-free survival was compared between normal, type I protein S-deficient and type III protein S-deficient individuals, with probands included or excluded, and in probands only. The analysis was done including all patients or only patients whose first thrombotic event had been deep-vein thrombosis and/or pulmonary embolism. Differences between the curves were assessed with the log-rank test.

General statistics. Data are reported as mean ± standard deviation (SD), unless otherwise indicated. Plasma factor levels were compared between groups with the Student's t-test. Thrombin generation parameters were compared with the non-parametric Mann-Whitney-Wilcoxon test (U) due to their non-normal distribution in some population subgroups. Correlations were expressed as Pearson coefficients (r). The determinants of thrombin generation parameters were identified by multiple regression analysis and their effects were expressed as unstandardized regression coefficients (B). Statistical analyses were performed with SPSS 14.0 for Windows (SPSS, Chicago IL, USA).

Results

Clinical evaluation

Two-hundred-forty-two individuals belonging to 30 families with protein S deficiency (28 with pure type I deficiency or mixed type I/III deficiency and 2 with pure type III deficiency) were investigated. Of the 30 probands, 18 presented with type I deficiency and 12 with type III deficiency (age at inclusion 46.8±11.9 *vs*. 52.5±15.1 years, P=n.s.). The age at the first thrombotic event was 30±10.4 years (median, 28.5 years) for probands with type I deficiency and 36.6±15.5 years (median, 33.5 years) for probands with type III deficiency, respectively (P=n.s.). Out of 212 family members, 112 had normal protein S levels and 100 were protein S-deficient (55 type I and 45 type III). The age of family members at the time of inclusion was 46.9±20.2 years for normal individuals, 48.5±19.8 years for type I deficient individuals (P=n.s.) and 56.9±15.9 years for type III deficient individuals (P=0.037 *vs.* normal individuals). At the time of inclusion, thrombotic events in family members had occurred in 17/55 (30.9%) type I deficient subjects, 19/45 (42.2%) type III deficient subjects and in 7/112 (6.2%) normal subjects. The mean age at the first thrombotic event was 40.6±16.7 years (median, 42 years) in type I deficient individuals, 42.6±16.7 years (median, 45 years) in type III deficient individuals and 47±23.4 years (median, 41 years) in normal individuals (P=n.s.).

To evaluate the risk of thrombosis associated with protein S deficiency, Kaplan-Meier analysis was performed. As expected, the cumulative proportion of thrombosis-free individuals was higher (P<0.001) in the normal group than in the protein S-deficient group (Figure 1A,B). However, no significant difference in thrombosis-free survival was observed between type I and type III protein S-deficient individuals in the

whole cohort (probands and family members, Figure 1A), nor after exclusion of probands (Figure 1B) or in probands only (Figure 1C). Results did not change when survival analysis was restricted to patients whose first thrombotic event had been deep-vein thrombosis and/or pulmonary embolism or when males and females were analyzed separately (data not shown).

Laboratory evaluation: characteristics of the study population

Plasma factor levels and thrombin generation were measured in a random subset of the population used for Kaplan-Meier analysis (23 families). After excluding patients taking oral anticoagulants and women on hormonal therapy, 132 individuals (53 normal, 59 type I deficient and 20 type III deficient) were available for plasma phenotyping (Table 1). Of the 20 type III protein S-deficient individuals, 18 were from families with mixed type I/III deficiency and only two from families with pure type III deficiency. However, two of the type III deficient individuals from a mixed type I/III family turned out to carry the protein S Heerlen mutation (see below).

The female gender was over-represented among protein S-deficient subjects, especially in the type I subgroup (p=0.020 *vs.* normal subjects). Mean age was not different between normal and protein S-deficient individuals, but type III deficient subjects were significantly older than type I deficient subjects (56.0 *vs.* 38.3 years, P<0.001). The protein S-deficient group was enriched in thrombotic patients (P=0.007 *vs.* normal subjects), but the percentage of patients was not different between the type I and type III subgroups.

Genotyping for the FV Leiden mutation identified 8 heterozygous carriers in three different families. Twelve individuals belonging to 5 different families were heterozygous for the prothrombin G20210A mutation.

Plasma factor levels

Plasma levels of protein S (total, free and C4BP-bound), prothrombin, antithrombin and full-length TFPI were measured in all subjects and compared between groups (Figure 2). Total protein S levels (Figure 2A) were higher in type III deficient individuals (79.0±6.3%) than in type I deficient individuals (52.9±9.0%, P<0.001), but still significantly lower than in normal individuals (94.3±17.2%, P<0.001). Similarly, free protein S levels (Figure 2B) were lower in type III deficient individuals (54.7±6.6%) than in normal individuals (89.5±15.4%, P<0.001), but still significantly higher than in type I deficient individuals (40.4±9.6%, P<0.001). C4BP-bound protein S levels (Figure 2C) were not different between normal (119.4±37.3%) and type III deficient individuals (111.2±24.5%), but were significantly reduced in type I deficient individuals (91.7±31.5%, P=0.007 *vs.* type III), in line with a recent report.[36] Prothrombin levels (Figure 2D) did not differ between normal (92.5±18.7%) and type I deficient individuals (88.8±12.0%), but they were significantly elevated in type III deficient individuals (104.1±16.0%, P=0.011 and P=0.001 *vs.* normal and type I deficient individuals, respectively). This difference persisted after exclusion of the prothrombin G20210A mutation carriers. Antithrombin levels (Figure 2E) were similar in all three groups. Finally, full-length TFPI levels (Figure 2F) were not different between normal (122.9±47.7%) and type III deficient individuals (121.3±31.4%), but were significantly reduced in type I deficient individuals (76.9±32.5%, P<0.001) as observed and explained elsewhere.[37] All inter-group differences in plasma factor levels persisted after correction for age and sex.

Plasma levels of coagulation factors and inhibitors were correlated in the study population. The strongest correlations were observed between total, free and bound

protein S (total/free: r=0.885; total/bound: r=0.413; free/bound: r=0.434; P<0.001 for all three correlations), total protein S and full-length TFPI (r=0.607, P<0.001), and total protein S and prothrombin (r=0.371, P<0.001). These correlations remained significant when normal and protein S-deficient individuals were analyzed separately.

APC-cofactor activity of protein S

To quantify the activity of the APC/protein S system, thrombin generation was measured at 6.8 pM TF in the absence and presence of APC, and the nAPCsr was calculated. Since FV Leiden strongly influences this assay, FV Leiden carriers were excluded from the analysis.

Despite a large inter-individual variability, thrombin generation started earlier (shorter lag time) and was higher in protein S-deficient plasma than in normal plasma, both in the absence and presence of APC (Table 1 Supplemental Material). In the absence of APC (Figure 3A), there was no difference between the ETPs of normal and type I deficient individuals, but type III deficient individuals had significantly higher ETP values (P≤0.001). In the presence of APC (Figure 3B), the ETPs of type I and type III deficient individuals were similar and ~3.5 higher than those of normal individuals (P <0.001). As a consequence, the nAPCsr (Figure 3C), was highest in type I deficient plasma, slightly lower in type III deficient plasma (P=0.057) and much lower in normal plasma (P<0.001). Thrombotic patients had a higher nAPCsr than healthy relatives, both in the type I (6.36±1.38 *vs*. 5.77±1.37) and in the type III (5.59±2.04 *vs.* 4.97±1.83) deficiency groups, but these differences did not reach statistical significance.

To interpret these results, the effects of age, sex and the levels of prothrombin, antithrombin, total and free protein S and full-length TFPI on the ETP obtained in the absence and presence of APC were determined by multiple regression analysis in the normal group. Age (B=4.4 nM.min/year, P=0.001) and prothrombin level (B=129.3 nM.min/10%, P<0.001) were both found to be strong determinants of the ETP_{-APC}, which accounts for the higher ETP_{-APC} in type III deficient individuals. Similarly, age (B=6.4 nM.min/year, p<0.001) and prothrombin (B=89.4 nM.min/10%, P<0.001) were positive determinants of the ETP_{+APC}, whereas free protein S (B=-58.6 nM.min/10%, p=0.028) and TFPI (B=-16.5 nM.min/10%, p=0.009) were negative determinants. This explains the absence of a difference in ETP_{+APC} between type I and type III deficient individuals. In fact, type III deficient individuals would be expected to have a lower ETP_{+APC} than type I because of their higher free protein S and TFPI levels, but their concomitant higher age and prothrombin level tend to abolish this difference, making the ETP_{+APC} similar in the two groups.

TFPI-cofactor activity of protein S

The activity of the TFPI/protein S system was quantified by measuring thrombin generation at 1.36 pM TF in the absence and presence of neutralizing antibodies against protein S (alphaPS) or TFPI (alphaTFPI) and by calculating the PS-and TFPI-ratios. Also in this case FV Leiden carriers were excluded.

Thrombin generation in the absence of antibodies was faster and higher in protein S-deficient plasma than in normal plasma (Table 2 Supplemental Material). However, this difference largely disappeared in the presence of antibodies, only the lag time remaining slightly but significantly shorter in protein S-deficient plasma (Table 2 Supplemental Material). Also in this test, a large inter-individual variability was

observed. In the absence of antibodies (Figure 4A), the peak height was virtually identical between type I and type III deficient individuals, and higher than in normal individuals ($p \leq 0.011$). Addition of alphaPS, and even more so addition of alphaTFPI, caused an increase in thrombin generation, due to the partial (alphaPS) or complete (alphaTFPI) inhibition of the TFPI/protein S system. In the presence of alphaPS (Figure 4B), the peak height was similar between normal and type III deficient individuals, while type I deficient individuals had slightly higher thrombin peaks, possibly due to their lower TFPI levels. In the presence of the alphaTFPI antibody (Figure 4C), normal and type I deficient plasmas yielded similar peak heights, which however were lower than in type III deficient plasma (P=0.022 and P=0.010, respectively). The PS-ratio (Figure 4D) and TFPI-ratio (Figure 4E) increased progressively from normal to type III and type I deficient plasma. However, the difference between type I and III deficient individuals was only significant for the TFPI-ratio (P=0.034).

Multiple regression analysis revealed that the peak height in the absence of antibodies was dependent not only on free protein S (B=-13.4 nM/10%, P=0.021) and TFPI (B=-5.7 nM/10%, P<0.001), but also on age (B=0.8 nM/year, P=0.035) and prothrombin (B=8.3 nM/10%, P=0.023), thereby accounting for the equally elevated peaks in type I and type III deficient individuals. The higher $peak_{+\_PS}$ of type I deficient individuals was attributable their lower TFPI levels (B=-6.8 nM/10%, P=0.002), whereas the higher age (B=1.1 nM/year, P<0.001) and prothrombin level (B=17.5 nM/10%, P<0.001) of type III deficient individuals were responsible for their elevated $peak_{+\_TFPI}$.

Relationship between nAPCsr and TFPI-ratio

The nAPCsr and the TFPI-ratio reflect the activities of the APC/protein S and TFPI/protein S systems, respectively. Both were elevated in protein S-deficient individuals, especially in those with type I deficiency, in line with the respective protein S levels.

Multiple regression analysis showed that free protein S and full-length TFPI levels were major determinants of both the nAPCsr (B=-0.37/10% protein S, P<0.024; B=-0.10/10% TFPI, P=0.007) and the TFPI-ratio (B=-0.06/10% protein S, P=0.011; B=-0.02/10% TFPI, P<0.001). Accordingly, the nAPCsr and the TFPI-ratio were highly correlated in the population (r=0.629, P<0.001). The correlation between nAPCsr and TFPI-ratio was also present within the normal (r=0.663, P<0.001) and type I deficiency (r=0.510, p<0.001) groups, as well as within the type III deficiency group (r=0.501, p=0.034) after exclusion of a single outlier.

***PROS1* mutation screening**

Due to the availability of genomic DNA from at least one protein S-deficient family member, *PROS1* mutation screening was possible in 20 of the 23 families. By sequencing the coding region (including splicing junctions) and the proximal promoter, a *PROS1* mutation was identified in 14 families (Table 2). In the six families where no mutation was detected, MLPA analysis was performed to check for the presence of major rearrangements of the *PROS1* gene. Two large deletions, spanning exons 2-15 and 9-11, respectively, were identified in two different families (Figure 5).

When the genetic screening was extended to family members, the respective mutation or deletion was identified in 56/58 (96.5%) individuals classified as protein S-deficient on the basis of protein S levels and in 2/30 (6.7%) of individuals classified as

normal. The *PROS1* Heerlen mutation was present in 3 related individuals, two with type III deficiency and one with protein S levels within the normal range. All mutations were in the heterozygous state. In all mixed type I/III families (except in the family with the Heerlen mutation), the same mutation accounted for type I as well as type III deficient members.

Interestingly, protein S-deficient individuals from families with a *PROS1* mutation or deletion had lower protein S levels and higher nAPCsr, PS-ratio and TFPI-ratio than protein S-deficient individuals from families without an identifiable *PROS1* defect (data not shown). Thrombosis-free survival was not different between protein S-deficient individuals with and without an identifiable *PROS1* defect, but it was significantly reduced in carriers of a *PROS1* mutation or deletion as compared to non-carriers (log-rank, p=0.039).

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Discussion

Although the thrombotic predisposition associated with protein S deficiency was recognized as early as 1984,[10,11] accurate risk estimates have been hindered by the difficulty of correctly diagnosing protein S deficiency. Currently, type I protein S deficiency is generally considered a risk factor for venous thrombosis, while conflicting results have been reported for type III deficiency.[22-24]

In the present study we have re-evaluated the thrombosis risk associated with type I and type III protein S deficiencies by Kaplan-Meier analysis of a large cohort of protein S-deficient families. Our data indicate that hereditary type III protein S deficiency is a risk factor for venous thrombosis and that it confers a similar risk as type I protein S deficiency (Figure 1). Although type III deficient individuals were older than type I deficient individuals and advancing age is also a risk factor for thrombosis, the age at onset of thrombosis was not different between the two groups.

To account for this finding, genetic and functional studies were performed in 23 families with type I and/or type III protein S deficiency (Table 1). Genetic screening was possible in 20 families and yielded 12 *PROS1* mutations (3 novel) in 14 families (Table 2). Moreover, large *PROS1* deletions were present in two families (Figure 5), in line with the recent report that major rearrangements of the *PROS1* gene are relatively common among mutation-negative protein S-deficient individuals.[13] When family members were tested for carriership of the mutation found in the respective proband, an excellent co-segregation between *PROS1* genotype and protein S levels was observed. This not only confirms the causal role of the identified mutations, but also indicates that the free protein S level cut-off used to define protein S deficiency is appropriate and reliable.[28] Remarkably, the only two mutation-positive individuals who were classified

as normal on the basis of their protein S levels carried the Thr^{103}→Asn mutation, which has been associated with qualitative (type II) protein S deficiency in another study.[38] However, the nAPCsr and TFPI-ratio of our two carriers were within the respective normal ranges.

For functional studies, the population was divided in three groups (normal, type I deficient and type III deficient) based on the levels of total and free protein S. In line with other reports,[16,17] type III protein S-deficient individuals were older than their normal and type I deficient relatives. Moreover, they had higher protein S (total, free and bound), prothrombin and TFPI levels than type I deficient individuals (Figure 2). Interestingly, a recent study reported that type III deficient individuals have elevated protein C levels as well.[17] Since the levels of all these factors increase with age,[34] our data support the hypothesis that type III deficient individuals from families with mixed type I/III deficiency start out as type I deficient, but become type III deficient later in life because of the age-related increase in total protein S levels.[16] If this is the case, not only protein S, protein C and prothrombin, but also other age-dependent coagulation factors might be increased in type III deficient plasma. Differently, type I (but not type III) deficient individuals had markedly reduced TFPI levels, in line with the observation that the levels of protein S and TFPI are highly correlated in plasma.[37,39] These additional abnormalities in plasma factor levels may synergize with the low protein S levels to aggravate the hypercoagulable state associated with type I and type III protein S deficiencies and underscore the importance of global phenotyping tests to evaluate the associated thrombosis risk.

Despite the expectation (based on protein S and TFPI levels) that type I deficient plasma would be more procoagulant than type III deficient plasma, thrombin generation assays sensitive to the APC- and TFPI-cofactor activities of protein S

detected only a minor difference between type I and type III deficient plasmas (Figures 3C and 4D,E). Remarkably, the slightly lower nAPCsr of type III deficient individuals as compared to type I deficient individuals was not due to a lower ETP_{+APC}, but to a higher ETP_{-APC} (Figure 3). Similarly, their lower TFPI-ratio was not due to a lower $peak_{-Ab}$, but to a higher $peak_{+\_TFPI}$ (Figure 4). Multiple regression analysis pointed at age and prothrombin, in addition to free protein S, as the major determinants of elevated thrombin generation in type III deficient plasma. While high prothrombin levels are known to cause APC-resistance,[32] and to interfere with plasma assays that probe the APC-independent (TFPI-dependent) anticoagulant activity of protein S,[40] age might act by elevating the levels of several coagulation factors.[34] Since both the nAPCsr and the TFPI-ratio reflect protein S as well as TFPI levels, they were highly correlated in the population.

As the nAPCsr is a marker for the risk of venous thrombosis,[41] the data presented in Figure 3C support the outcome of the Kaplan-Meier analysis and confirm that type I and type III protein S deficiencies confer similar thrombosis risks. This finding is in line with a previous study,[23] but contrasts with other studies where the risk of thrombosis associated with type III protein S deficiency was found to be half than in type I deficiency[24] or not elevated at all.[22] A possible explanation for these discrepancies may be the population selection criteria, as it has been reported that type III deficient individuals from pure type III deficient families are not at risk for thrombosis, while those from mixed type I/type III deficient families are.[24] Also, no risk is generally attributed to the *PROS1* Heerlen mutation.[18] In this respect, it is important to point out that in our study type III deficiency was defined solely on the basis of protein S levels, irrespective of mutational status. Due to the small sample size, no distinction was made between type III deficient individuals originating from pure

type III and mixed type I/III deficient families, although most belonged to mixed type I/III deficient families, and only 2 carried *PROS1* Heerlen. Since type III protein S deficiency is a heterogeneous category, a difference in thrombosis risk between type III deficient individuals from pure type III and mixed type I/III families cannot be excluded and should be verified in a larger cohort of type III deficient individuals. As a matter of fact, the nAPCsr and PS-ratio of the two carriers of the Heerlen mutation in this study were lower than those of the 16 type III protein S-deficient individuals belonging to mixed type I/III families (nAPCsr 3.65 and 4.42 *vs.* 5.29±1.93; PS-ratio 0.39 and 0.49 *vs.* 0.69±0.13), which underscores the importance of family studies to distinguish pure type III from mixed type I/III protein S deficiency.

In summary, we have shown that type III protein S deficient individuals (at least those belonging to mixed type I/III deficient families) experience a similar risk of venous thrombosis as type I deficient individuals. This conclusion is based not only on Kaplan-Meier analyses, but also on a detailed characterization of the hypercoagulable states associated with type I and type III deficiencies, including the levels of several plasma factors, the APC-cofactor activity of protein S and, for the first time, the TFPI-cofactor activity of protein S. Although type I deficient individuals had lower protein S and TFPI levels than type III deficient individuals, the latter had higher levels of prothrombin and possibly other age-dependent coagulation factors, resulting in an almost equivalent impairment of both anticoagulant functions of protein S. These findings may have implications for the counseling and management of type III protein S-deficient patients.

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Authorship and Disclosures

Study design: PS, EC, JR; patient selection and enrolment: PS, DT, LS; experiments: EC, LM, SG, CR; data analysis (clinical part): PS, DT; data analysis (experimental part): EC, JR, LM, TH; manuscript writing: EC, PS; critical review of the manuscript: JR, PS, TH.

None of the authors has a conflict of interest.

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Figure 1. Thrombosis-free survival analysis of protein S-deficient individuals. Kaplan-Meier analysis of A) the whole study cohort (probands and family members); B) family members after exclusion of probands; and C) probands only. Dashed line represents normal individuals, solid and dotted lines represent type I and type III protein S-deficient individuals, respectively. Differences between the curves were evaluated with the log-rank test.

Figure 2. Plasma levels of coagulation factors and inhibitors. Mean plasma levels of total protein S (A), free protein S (B), C4BP-bound protein S (C), prothrombin (D), antithrombin (E) and full-length TFPI (F) in normal individuals (white bars) and in individuals with type I (black bars) or type III (grey bars) protein S deficiency. Error bars represent standard deviations. The dotted lines mark the cut-offs for total and free protein S levels. P-values of Student's t are indicated.

Figure 3. Thrombin generation in the absence and presence of APC. Thrombin generation was measured at 6.8 pM TF in the absence and presence of 5 nM APC. FV Leiden carriers were excluded. A) ETPs measured in the absence of APC; B) ETPs measured in the presence of APC; C) nAPCsr. White circles, normal individuals; black circles, individuals with type I protein S deficiency; grey circles, individuals with type III protein S deficiency. Lines represent the medians of the respective distributions. P-values of the Mann-Whitney-Wilcoxon U are indicated.

Figure 4. Thrombin generation in the absence and presence of alphaPS or alphaTFPI. Thrombin generation was measured at 1.36 pM TF in the absence and presence of antibodies against protein S or TFPI. FV Leiden carriers were excluded. A) Thrombin peaks measured in the absence of antibodies; B) thrombin peaks measured in the presence of alphaPS; C) thrombin peaks measured in the presence of alphaTFPI; D) PS-ratio; E) TFPI-ratio. White circles, normal individuals; black circles, individuals with type I protein S deficiency; grey circles, individuals with type III protein S deficiency. Lines represent the medians of the respective distributions. P-values of the Mann-Whitney-Wilcoxon U are indicated.

Figure 5. Detection of gross *PROS1* deletions by MLPA analysis. MLPA electropherograms obtained in a normal control (A) and in two unrelated protein S-deficient individuals with a partial *PROS1* deletion (B,C). The peaks corresponding to *PROS1* exons are labeled at the bottom. The unlabeled peaks correspond to control gene regions. A marked reduction of the height of a *PROS1*-specific peak (arrows) relative to the control gene peaks indicates a deletion of one copy of that particular *PROS1* exon. The small peaks represent the size standard.

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Figure 1.

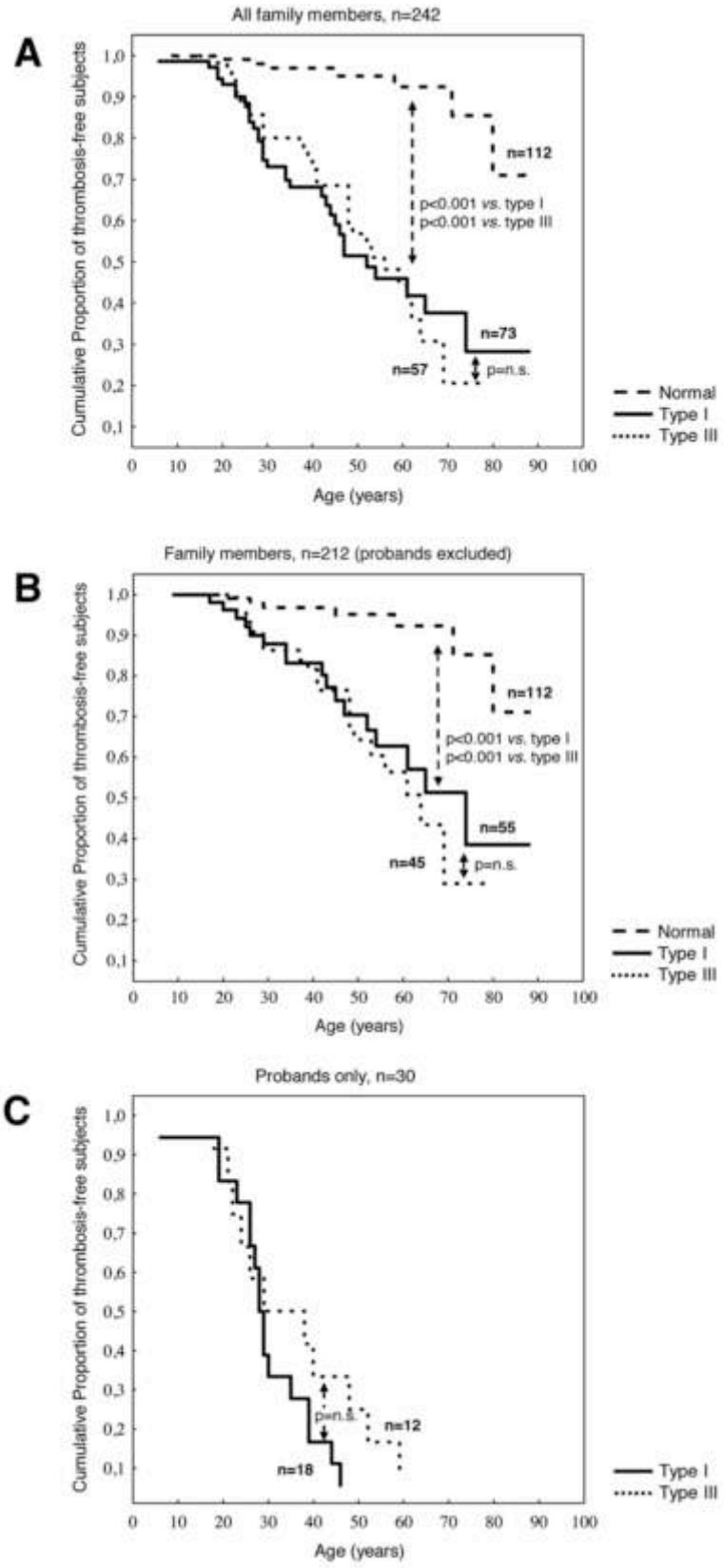

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Figure 2.

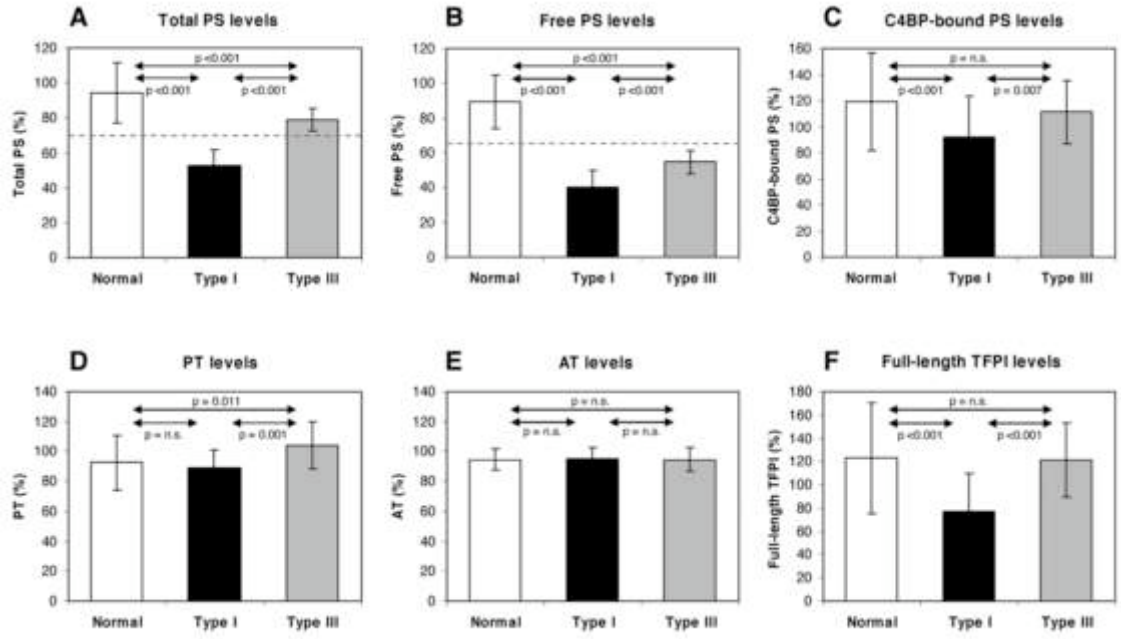

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Figure 3.

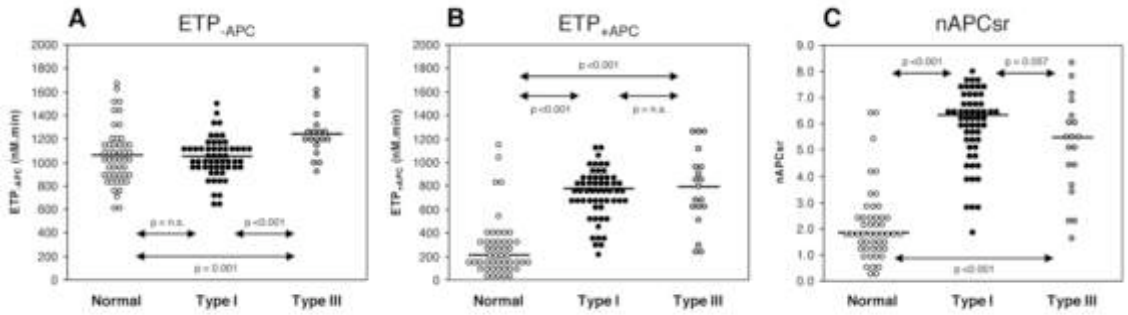

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Figure 4.

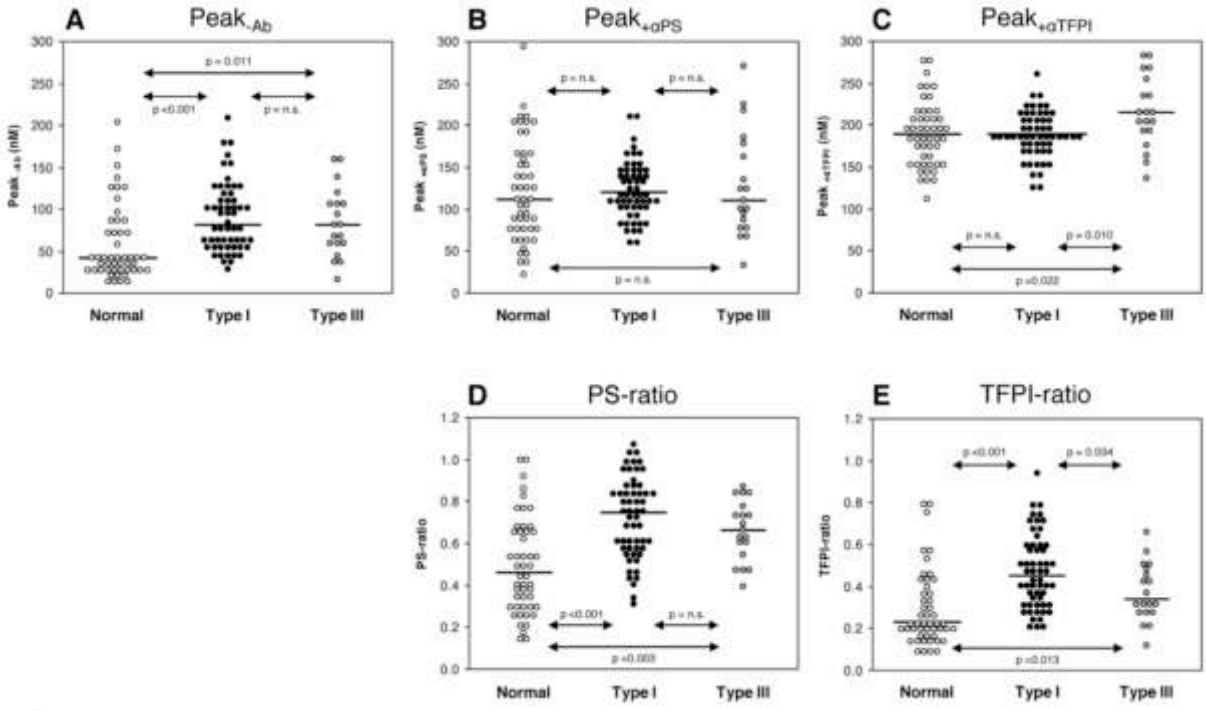

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Figure 5.

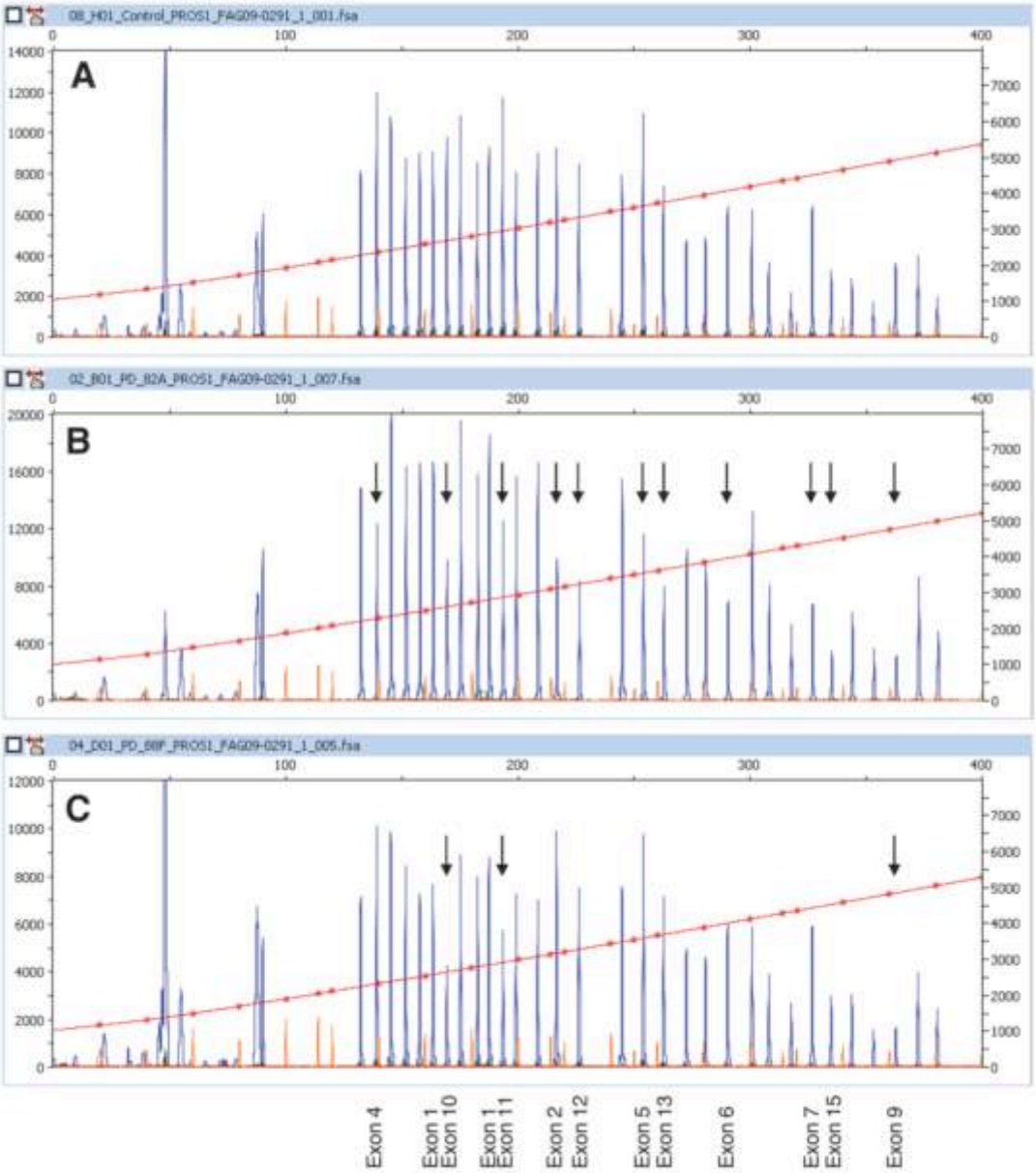

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Table 1. Demographic characteristics of the study population

	N	Sex (M/F)	Age (years)	VTE N (%)
Normal	53	31/22	38.4 ± 20.0	4 (7.5%)
PS-deficient	79	31/48	42.7 ± 19.0	24 (30.4%)
Type I	59	22/37	38.3 ± 17.9	18 (30.5%)
Type III	20	9/11	56.0 ± 16.0	6 (30.0%)

PS, protein S; VTE, venous thromboembolism (superficial vein thrombosis, deep vein thrombosis and/or pulmonary embolism).

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Table 2. *PROS1* mutations identified in families with protein S deficiency

Family	Def. type	Mutation	Location	Mutation type	Predicted consequence	Reference	Detection
PD 85 PD 92 PD 99	I I I	346 A->C	Exon 2	Missense	Glu^{26} ->Ala (Gla domain)	[21,42,43]	Fnu4H I-restriction
PD 81	I	577 C-> A	Exon 5	Missense	Thr^{103} ->Asn (1^{st} EGF domain)	[21,42]	Mfe I-restriction
PD 84	III	919 A-> G	Exon 8	Missense	Asn^{217} ->Ser (4^{th} EGF domain)	[44-46]	Tsp509 I-restriction
PD 94	I	941 C-> A	Exon 8	Nonsense	Cys^{224} ->Stop	Novel mutation	Sfc I-restriction*
PD 93	I/III	1010 C-> A	Exon 9	Nonsense	Cys^{247} ->Stop	[21]	Sequencing
PD 83	I	1048 delC	Exon 9	Frame-shift	NMD/premature termination	[21]	Sequencing
PD 89	I/III	1300 ins(15 nt)	Exon 10	In-frame ins	5-amino acid insertion (or splicing?)	Novel mutation	Electrophoresis
PD 87	I/III	-3 T-> G	Intron 11	Splicing	NMD/premature termination	[47]	Sequencing
PD 95	I III	1823 A-> G 1647 T-> C	Exon 14 Exon 13	Missense Missense	Ile^{518} ->Met (SHBG domain) Ser^{460}->Pro (SHBG domain)	Polymorphism?[21,48] Heerlen mutation[18]	Sequencing Rsa I-restriction
PD 90	I/III	1874 delA	Exon 14	Frame-shift	NMD/premature termination	Novel mutation	Tsp509 I-restriction
PD 91 PD 97	I/III I/III	2140 C-> T	Exon 15	Missense	Ser^{624} ->Leu (SHBG domain)	[49,50]	BstX I-restriction

cDNA numbering according to Schmiedel et al. 1990.[51]

del, deletion; ins, insertion; NMD, nonsense-mediated decay; *mutagenic primer needed to introduce restriction site.

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SUPPLEMENTAL MATERIAL

Table 1 Supplemental Material. Thrombin generation parameters (median and interquartile range) at 6.8 pM TF ± APC

		-APC			+APC			
	N*	**Lag time (min)**	**Peak (nM)**	**ETP (nM.min)**	**Lag time (min)**	**Peak (nM)**	**ETP (nM.min)**	**nAPCsr**
Normal	**49**	**2.55** 2.25 – 3.03	**295** 246 – 337	**1065** 890 – 1183	**2.69** 2.50 – 3.22	**50.4** 33.0 – 79.8	**211** 143 – 347	**1.84** 1.33 – 2.59
PS-def.	**75**	**2.36** 2.15 – 2.47	**330** 308 – 357	**1114** 992 – 1227	**2.50** 2.25 – 2.69	**207** 161 – 253	**784** 650 – 902	**6.14** 4.90 – 6.82
p (U)		<0.001	0.003	0.207	0.001	<0.001	<0.001	<0.001
Type I	**56**	**2.36** 2.15 – 2.47	**322** 303 – 341	**1053** 963 – 1150	**2.54** 2.25 – 2.88	**213** 170 – 249	**778** 673 - 877	**6.34** 5.16 – 6.96
Type III	**19**	**2.25** 2.02 – 2.55	**359** 321 – 387	**1241** 1141 - 1314	**2.35** 2.25 – 2.58	**191** 161 – 270	**795** 623 - 979	**5.51** 3.65 – 6.30
p (U)		0.678	0.005	<0.001	0.394	0.697	0.784	0.057

* FV Leiden carriers were excluded.

ETP, endogenous thrombin potential. U, Mann-Whitney-Wilcoxon test.

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Table 2 Supplemental Material. Thrombin generation parameters (median and interquartile range) at 1.36 pM TF ± _PS or _TFPI

		No antibodies			+_PS			+_TFPI				
	N*	**Lag time (min)**	**Peak (nM)**	**ETP (nM.min)**	**Lag time (min)**	**Peak (nM)**	**ETP (nM.min)**	**Lag time (min)**	**Peak (nM)**	**ETP (nM.min)**	**PS-ratio**	**TFPI-ra**
ormal	**49**	**3.82**	**42.5**	**528**	**3.08**	**112**	**582**	**2.73**	**189**	**589**	**0.47**	**0.23**
		3.05 – 5.12	31.5 – 81.7	398 – 654	2.77 – 3.66	77.0 - 167	510 – 694	2.59 – 3.15	158 – 215	529 – 662	0.31 – 0.67	0.18 – 0.
S-def.	**75**	**2.98**	**81.3**	**576**	**2.77**	**121**	**549**	**2.63**	**193**	**605**	**0.73**	**0.42**
		2.70 – 3.33	59.4 – 115	521 – 662	2.51 – 2.98	95.1 – 150	514 – 619	2.33 – 2.94	177 – 220	521 – 670	0.57 – 0.85	0.31 – 0.
(U)		<0.001	<0.001	0.019	<0.001	0.541	0.297	0.018	0.347	0.884	<0.001	<0.001
ype I	**56**	**2.98**	**81.9**	**568**	**2.77**	**121**	**538**	**2.63**	**189**	**586**	**0.75**	**0.45**
		2.70 – 3.36	58.7 – 118	512 – 658	2.51 – 3.00	105 – 147	493 – 602	2.45 – 2.94	174 – 210	515 - 627	0.58 – 0.87	0.33 – 0.
ype III	**19**	**3.20**	**81.3**	**621**	**2.77**	**111**	**576**	**2.50**	**215**	**664**	**0.67**	**0.34**
		2.63 – 3.27	59.4 – 110	571 - 742	2.51 – 2.81	80.7 – 178	541 - 821	2.33 – 2.63	193 – 255	635 - 810	0.54 – 0.78	0.29 – 0.
(U)		0.826	0.559	0.050	0.859	0.683	0.007	0.127	0.010	<0.001	0.192	0.034

* FV Leiden carriers were excluded.

ETP, endogenous thrombin potential. U, Mann-Whitney-Wilcoxon test.