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

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Protein S, which circulates in plasma in both free and bound forms, is an anticoagulant protein that stimulates activated protein C and tissue factor pathway inhibitor. 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, or having type I or type III deficiency 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, tissue factor pathway inhibitor and prothrombin levels than type I deficient individuals. Thrombin generation assays sensitive to the activated protein C- and tissue factor pathway inhibitor-cofactor activities of protein S revealed similar hypercoagulable states in type I and type III protein S-deficient plasma. Twelve *PROS1* mutations and two 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 risk of thrombosis. These findings may, however, 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.

Key words: protein S, Kaplan-Meier analysis, activated protein C (APC), tissue factor pathway inhibitor (TFPI), thrombin generation.

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Introduction

Protein S (PS) is a vitamin K-dependent glycoprotein mainly synthesized in the liver and present in plasma, platelets and endothelial cells.[1] Plasma PS (350 nM) is distributed between two pools: approximately 40% circulates free, while about 60% is bound to the complement regulatory factor C4b-binding protein (C4BP).[2] Only C4BP molecules with a β-chain (C4BP-β$^+$) bind PS and, given the high affinity of this interaction (Kd = 0.1 nM),[2] all C4BP-β$^+$ present in plasma (~200 nM) is complexed with PS.[3]

PS is a potent anticoagulant protein that down-regulates thrombin formation via two mechanisms.[4] On the 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).[6,7] Although free PS is the most active form, recent reports indicate that the PS-C4BP complex also exhibits APC- and TFPI-cofactor activities.[6,8]

Hereditary PS deficiency is a rare coagulation disorder associated with an increased risk of venous thrombosis.[9,10] It usually occurs as a partial (heterozygous) deficiency and segregates as an autosomal dominant trait. Presently, more than 200 mutations impairing PS synthesis and/or function have been described in the PS gene (*PROS1*), and large deletions/duplications within the *PROS1* locus have been identified as a relatively common cause of PS deficiency.[11,12] Based on PS antigen and (APC-cofactor) activity levels, PS 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 PS levels are strongly influenced by age, sex and hormonal status,[13] as well as by several acquired conditions, the diagnosis of PS deficiency states based on PS levels is far from straightforward in practice.

Type I and type III deficiencies account for 95% of cases of PS-deficiency and often occur together in the same family as phenotypic variants of the same genetic defect (mixed type I/III deficiency).[14] The reasons for the different phenotypic expression are poorly understood, but the age-dependent increase in total PS is thought to play a role, as type III PS-deficient family members tend to be older than their type I-deficient relatives.[15] Differently, other families with PS deficiency only express the type III phenotype (pure type III deficiency). This type of PS deficiency is often, but not always,[16] associated with the *PROS1* Ser^{460}→Pro (Heerlen) mutation.[17]

Although PS deficiency and, particularly, low levels of free PS[18,19] are an established risk factor for venous thrombosis, risk estimates differ widely among studies, possibly reflecting the different severity of the underlying molecular defects.[20] Moreover, the few epidemiological studies that distinguish between type I and type III deficiencies are rather contradictory with respect to the risk of thrombosis associated with type III deficiency, which was found to be none,[21] the same as in type I deficiency[22] or intermediate.[23]

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

Design and Methods

Study population

Thirty families with type I and/or type III PS deficiency (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 (n=27) or arterial (n=3) thrombosis. 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.[24] PS deficiency was defined on the basis of free PS levels,[25,26] applying a cut-off of 65%. The type of deficiency was assigned according to total PS levels, using a cut-off of 70%. These cut-offs were based on the variation of total and free PS levels in a population of 140 healthy individuals, irrespective of age and sex. Application of sex-specific cut-offs for total and free PS levels hardly affected the classification of the subjects.

Plasma samples for functional studies and blood cells for DNA extraction could be obtained for 23 of the 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 into 0.129 M sodium citrate (1:9 vol/vol) and platelet-poor plasma was prepared by centrifugation at 2000×g 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 enzyme-linked immunosorbent assays were used to measure the plasma antigen levels of total,[27] free[28] and C4BP-bound PS,[8] as well as full-length TFPI.[29] Prothrombin levels were determined with a chromogenic assay, as previously described.[30] Antithrombin activity levels were determined using the Coamatic® Antithrombin kit (Chromogenix, Mölndal, Sweden). The levels of all factors were expressed as percentages of the levels in normal pooled plasma.

Thrombin generation measurements

Thrombin generation was measured with the Calibrated Automated Thrombogram (CAT) method.[31] Coagulation was initiated with tissue factor (Innovin®, DADE-Behring, Marburg,

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).

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

To probe the activity of the APC/PS system, thrombin generation was initiated with 6.8 pM tissue factor and 30 μM phospholipids in the absence and presence of 5 nM human APC (Kordia Life Sciences, Leiden, The Netherlands), as described elsewhere.[30] The outcome of the assay was expressed as the ratio of the endogenous thrombin potentials (ETP) 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 PS is a major determinant of this assay,[32] the nAPCsr is an indirect measure of the APC-dependent activity of PS. The nAPCsr varies between 0 and 10 and increases as the plasma level of PS (and hence its APC-cofactor activity) decreases.

To quantify the activity of the TFPI/PS system, thrombin generation was initiated with 1.36 pM tissue factor and 30 μM phospholipids in the absence and presence of neutralizing antibodies against PS (270 μg/mL polyclonal IgG; DAKO, Glostrup, Denmark) or TFPI (64 μg/mL monoclonal IgG1; Sanquin, Amsterdam, The Netherlands), essentially as described previously.[29] To exclude any contribution of the APC-dependent activity of PS, these measurements were conducted in the presence of 100 μg/mL anti-protein C polyclonal antibodies (DAKO). Moreover, corn trypsin inhibitor (Hematologic Technologies, Essex Junction VT, USA) was added to a final concentration of 30 μg/mL to prevent contact activation. The ratio of the thrombin generation peaks obtained in the absence and presence of anti-PS antibody (PS-ratio) is a measure of the TFPI-cofactor activity of PS, 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/PS system as a whole.[29] Both ratios vary between 0 and 1 and increase as the TFPI-cofactor activity of PS 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 polymerase chain reaction-mediated amplification of each exon (including splicing junctions) and of the proximal promoter followed by direct sequencing. Primers and conditions (available on request) were chosen such as to avoid co-amplification of the highly homologous PS pseudogene. 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.

In families without apparent mutation, the possibility of a large deletion was verified by multiplex 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.[30]

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. 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 Student's t-test. Thrombin generation parameters were compared with the non-parametric Mann-Whitney-Wilcoxon U test 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 and forty-two individuals belonging to 30 families with PS deficiency (28 with pure type I deficiency or mixed type I/III deficiency and two with pure type III deficiency) were investigated. Of the 30 probands, 18 presented with type I deficiency and 12 with type III deficiency. Out of 212 family members, 112 had normal PS levels and 100 were PS-deficient (55 type I and 45 type III). Detailed demographic and clinical characteristics of the study population are reported in the *Online Supplementary Appendix*.

The risk of thrombosis associated with PS deficiency was evaluated by Kaplan-Meier analysis. As expected, the cumulative proportion of thrombosis-free individuals was higher ($P<0.001$) in the normal group than in the PS-deficient group (Figure 1A,B). However, no significant difference in thrombosis-free survival was observed between type I and type III PS-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 PS-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 PS Heerlen mutation (see below).

Females were over-represented among PS-deficient sub-

jects, especially in the type I subgroup (P=0.020 *versus* normal subjects). Mean age was not different between normal and PS-deficient individuals, but type III deficient subjects were significantly older than type I deficient subjects (56.0 *versus* 38.3 years, P<0.001). The PS-deficient group was enriched in thrombotic patients (P=0.007 *versus* 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 eight heterozygous carriers in three different families. Twelve individuals belonging to five different families were heterozygous for the prothrombin G20210A mutation.

Plasma factor levels

Plasma levels of PS (total, free and C4BP-bound), prothrombin, antithrombin and full-length TFPI were measured in all subjects and compared between groups (Figure 2). Total PS 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 PS 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 PS 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 *versus* type III). 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 *versus* normal and type I deficient individuals, respectively). This difference persisted after exclusion of carriers of the prothrombin G20210A mutation. 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 elsewhere.[33] 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 PS (total/free: r=0.885; total/bound: r=0.413; free/bound: r=0.434; P<0.001 for all three correlations), total PS and full-length TFPI (r=0.607, P<0.001), and total PS and prothrombin (r=0.371, P<0.001). These correlations remained significant when normal and PS-deficient individuals were analyzed separately.

Activated protein C-cofactor activity of protein S

To quantify the activity of the APC/PS system, thrombin generation was measured at 6.8 pM tissue factor 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 PS-deficient plasma than in normal plasma, both in the absence and presence of APC (*Online Supplementary Table S1*). In the absence of APC (Figure 3A), there was no difference between the ETP 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 ETP of type I and type III deficient individuals were similar and approximately 3.5-fold 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

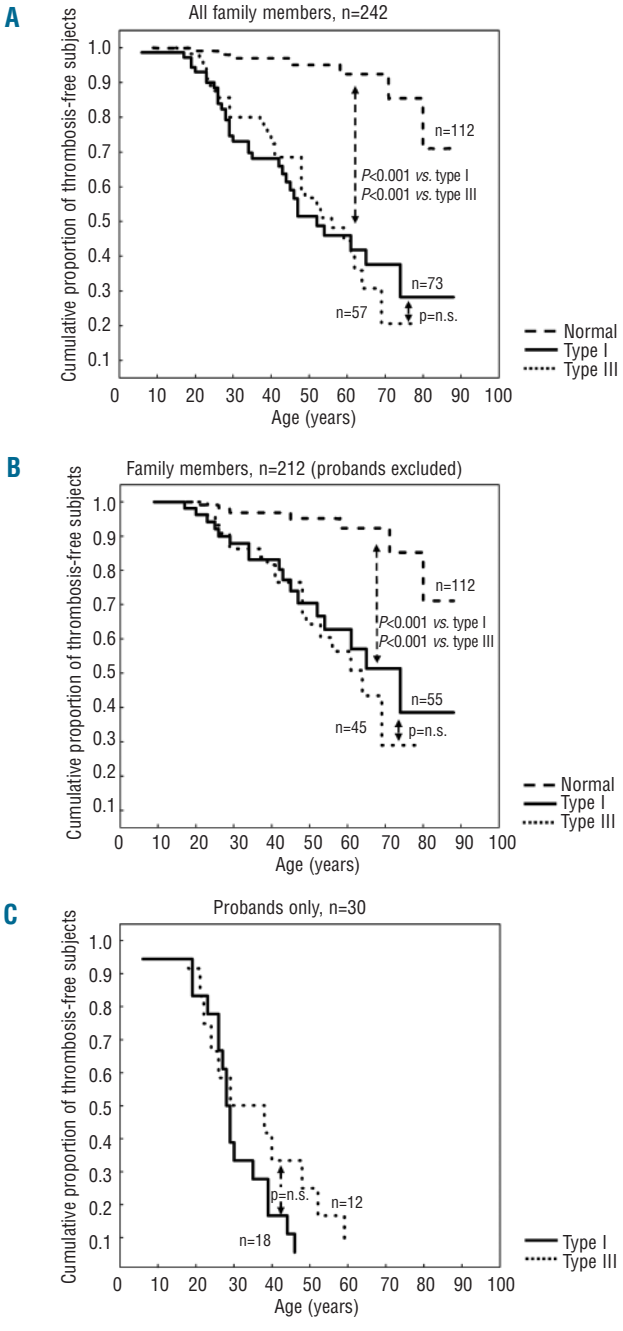

Figure 1. Thrombosis-free survival analysis of PS-deficient individuals. Kaplan-Meier analysis of (A) the whole study cohort (probands and family members); (B) family members after exclusion of probands; and (C) probands only. The dashed lines represent normal individuals, solid and dotted lines represent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

patients had a higher nAPCsr than healthy relatives, both in the type I (6.36±1.38 *versus* 5.77±1.37) and in the type III (5.59±2.04 *versus* 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 coagulation factors and inhibitors on the ETP obtained in the absence and presence of APC were determined by multiple regression analysis in the normal group. Results are reported in the *Online Supplementary Appendix*.

Tissue factor pathway inhibitor-cofactor activity of protein S

The activity of the TFPI/PS system was quantified by measuring thrombin generation at 1.36 pM tissue factor in the absence and presence of neutralizing antibodies against PS (αPS) or TFPI (αTFPI) and by calculating the PS- and TFPI-ratios. FV Leiden carriers were excluded.

Thrombin generation in the absence of antibodies was faster and higher in PS-deficient plasma than in normal plasma. However, this difference largely disappeared in the presence of antibodies, with only the lag time remaining slightly but significantly shorter in PS-deficient plasma (*Online Supplementary Table S2*). Also in this test, a large inter-individual variability was observed. In the absence of antibodies (Figure 4A), the peak height was virtually identical in type I and type III deficient individuals, and higher than in normal individuals ($P \leq 0.011$). Addition of αPS, and even more so addition of αTFPI, caused an increase in thrombin generation, due to the partial (αPS) or complete (αTFPI) inhibition of the TFPI/PS system. In the presence of αPS (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 αTFPI antibody (Figure 4C), normal and type I deficient plasmas yielded similar peak heights, which 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 ($P=0.034$).

The determinants of the peak height of thrombin generation measured in the absence and presence of αPS or αTFPI antibodies, as determined by multiple regression analysis, are reported in the *Online Supplementary Appendix*.

Relationship between the normalized activated protein sensitivity ratio and the tissue factor pathway inhibitor ratio

Multiple regression analysis showed that free PS and full-length TFPI levels were major determinants of both the nAPCsr (B=-0.37/10% PS, $P<0.024$; B=-0.10/10% TFPI, $P=0.007$) and the TFPI-ratio (B=-0.06/10% PS, $P=0.011$; B=-0.02/10% TFPI, $P<0.001$). Accordingly, the nAPCsr and the TFPI-ratio were strongly correlated in the population (r=0.629, $P<0.001$).

PROS1 *mutation screening*

Due to the availability of genomic DNA from at least one PS-deficient family member, *PROS1* mutation screening was possible in 20 of the 23 families. By sequencing the

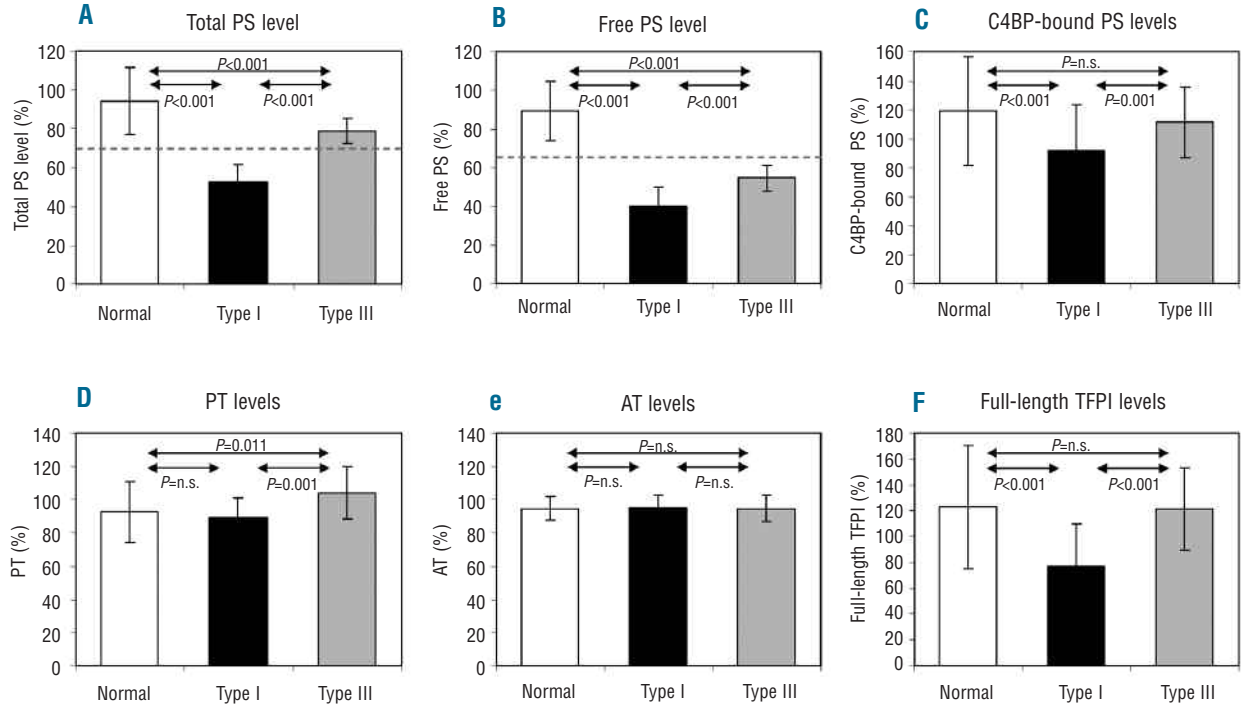

Figure 2. Plasma levels of coagulation factors and inhibitors. Mean plasma levels of total PS (A), free PS (B), C4BP-bound PS (C), prothrombin (D), antithrombin (E) and full-length TFPI (F) in normal individuals (white bars) and in individuals with type I (black bars) or type III (gray bars) PS deficiency.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

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 (1st EGF domain)	21,42	Mfe I-restriction
PD 84	III	919 A→G	Exon 8	Missense	Asn[217]→Ser (4th 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 Schmidel et al.;[46] del, deletion; ins, insertion; NMD, nonsense-mediated decay; *mutagenic primer needed to introduce restriction site.*

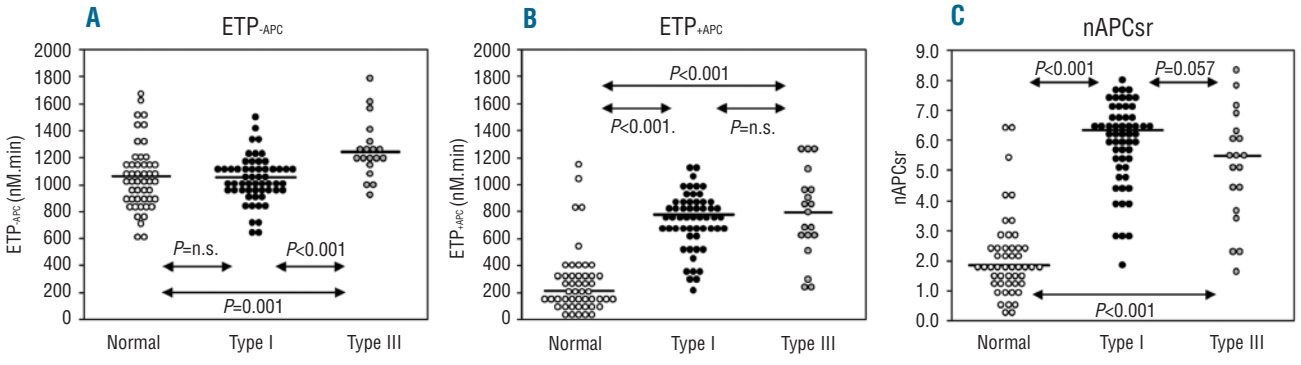

Figure 3. Thrombin generation in the absence and presence of APC. Thrombin generation was measured at 6.8 pM tissue factor in the absence and presence of 5 nM APC. FV Leiden carriers were excluded. (A) ETP measured in the absence of APC; (B) ETP measured in the presence of APC; (C) nAPCsr. White circles,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

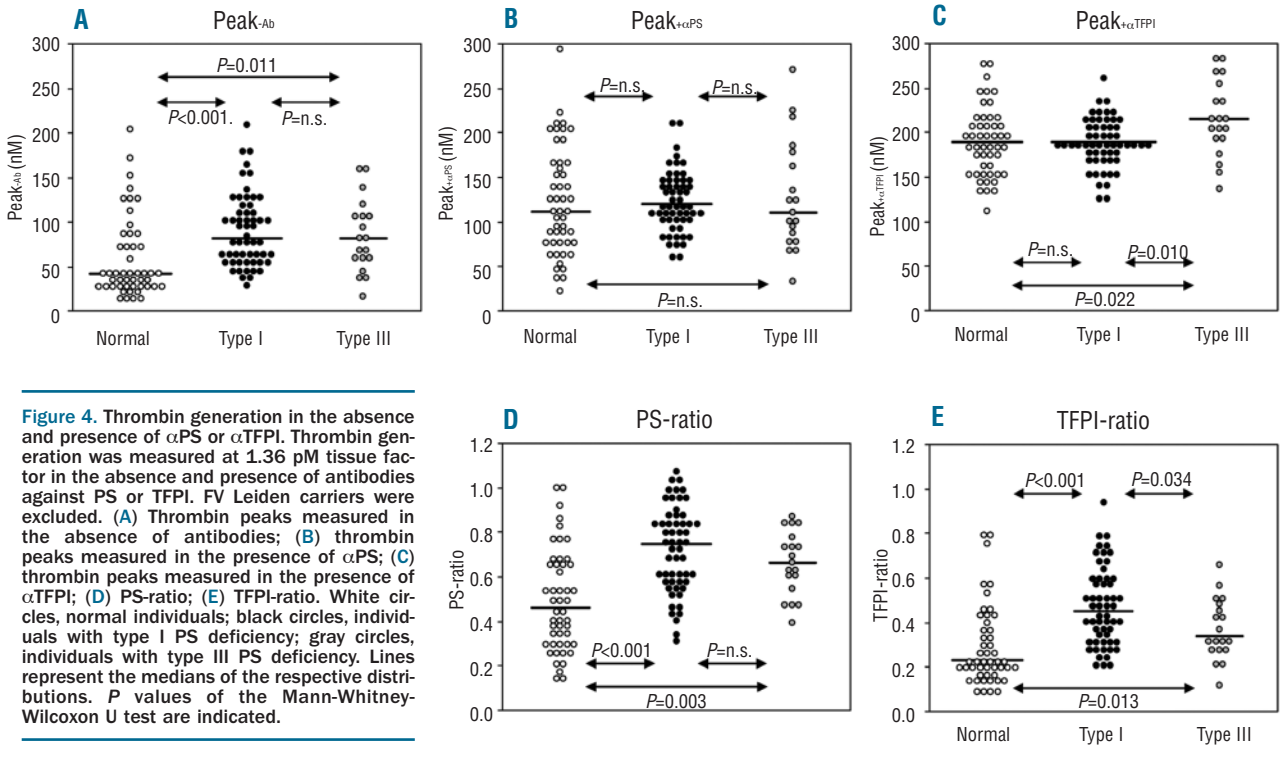

Figure 4. Thrombin generation in the absence and presence of αPS or αTFPI. Thrombin generation was measured at 1.36 pM tissue factor in the absence and presence of antibodies against PS or TFPI. FV Leiden carriers were excluded. (A) Thrombin peaks measured in the absence of antibodies; (B) thrombin peaks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

coding region (including splicing junctions) and the proximal promoter, a *PROS1* mutation was identified in 14 families (Table 2). In the six families in which 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 exons 9-11 were identified in two different families (Figure 5).

When genetic screening was extended to family members, the respective mutation or deletion was identified in 56/58 (96.5%) individuals classified as PS-deficient on the basis of PS levels and in 2/30 (6.7%) individuals classified as normal. The *PROS1* Heerlen mutation was present in three related individuals, two with type III deficiency and one with PS levels within the normal range. All mutations were in the heterozygous state. In all families with mixed type I/III deficiency (except in the family with the Heerlen mutation), the same mutation accounted for type I as well as type III deficient members.

Interestingly, PS-deficient individuals from families with a *PROS1* mutation or deletion had lower PS levels and higher nAPCsr, PS-ratio and TFPI-ratio than PS-deficient individuals from families without an identifiable genetic defect (*data not shown*). Thrombosis-free survival was not different between PS-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).

Discussion

Although the thrombotic predisposition associated with PS deficiency was recognized as early as 1984,[9,10] accurate risk estimates have been hindered by the difficulty of correctly diagnosing PS deficiency. Currently, type I PS deficiency is generally considered a risk factor for venous thrombosis, while conflicting results have been reported for type III deficiency.[21-23]

In the present study we re-evaluated the thrombosis risk associated with type I and type III PS deficiencies by Kaplan-Meier analysis of a large cohort of PS-deficient families. Our data indicate that hereditary type III PS deficiency is a risk factor for venous thrombosis and that it confers a similar risk as type I PS 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 PS 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). When family members were tested for carriership of the genetic defect found in the respective proband, an excellent co-segregation

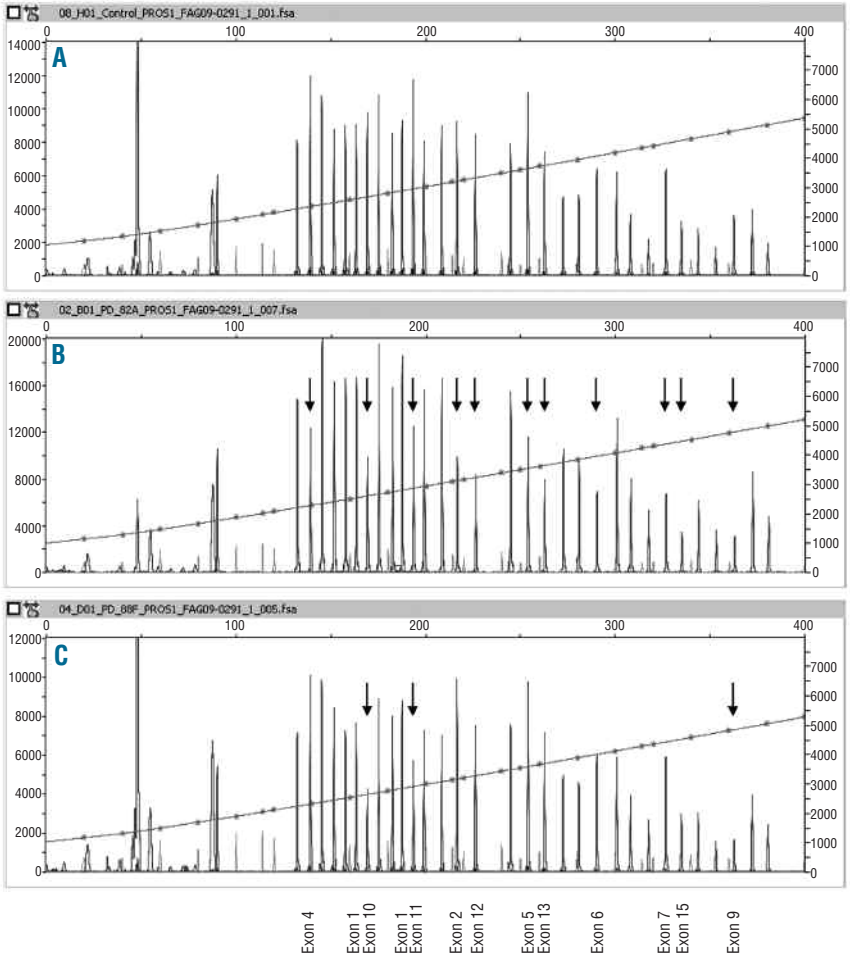

Figure 5. Detection of gross *PROS1* deletions by MLPA analysis. MLPA electropherograms obtained in a normal control (A) and in two unrelated PS-deficient individuals with a partial *PROS1* deletion (B, C). The peaks corresponding to *PROS1* exons are labeled at the bottom. The unlabeled peaks correspond 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.

between *PROS1* genotype and PS levels was observed. This not only confirms the causal role of the identified mutations, but also indicates that the free PS level cut-off used to define PS deficiency is appropriate and reliable.[26]

For functional studies, the population was divided into three groups (normal, type I deficient and type III deficient) based on the levels of total and free PS. In line with other reports,[15,16] type III PS-deficient individuals were older than their normal and type I deficient relatives. Moreover, they had higher PS (total, free and bound), prothrombin and TFPI levels than type I deficient individuals (Figure 2). Interestingly, a recent study found that type III deficient individuals have elevated protein C levels as well.[16] Since the levels of all these factors increase with age,[32] our data support the hypothesis that type III deficient individuals from mixed type I/III families start out as type I deficient, but become type III deficient later in life because of the age-related increase in total PS levels.[15] If this is the case, not only PS, 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 notion that the levels of PS and TFPI in plasma are strongly correlated.[33,34] These additional abnormalities in plasma factor levels may synergize with the low PS levels to aggravate the hypercoagulable state associated with type I and type III PS deficiencies and underscore the importance of global phenotyping tests to evaluate the associated thrombosis risk.

Despite the expectation (based on PS 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 PS 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 $peak_{+\alpha TFPI}$ (Figure 4). Multiple regression analysis pointed to age and prothrombin, in addition to free PS, as being the major determinants of elevated thrombin generation in type III deficient plasma. While high prothrombin levels are known to cause APC-resistance,[30] and to interfere with plasma assays that probe the APC-independent (TFPI-dependent) anticoagulant activity of PS,[35] age might act by elevating the levels of several coagulation factors.[32]

As the nAPCsr is a marker of the risk of venous thrombosis,[36] the data presented in Figure 3C support the outcome of the Kaplan-Meier analysis and confirm that type I and type III PS deficiencies confer similar risks of thrombosis. This finding is in line with that from a previous study,[22] but contrasts with data from other studies in which the risk of thrombosis associated with type III PS deficiency was found to be half that associated with type I deficiency[23] or not elevated at all.[21] A possible explanation for these discrepancies may be the population selection criteria, as it has been reported that type III deficient individuals from families with pure type III deficiency are not at risk of thrombosis, while those from mixed type I/type III deficient families are.[23] Also, no risk is generally attributed to the *PROS1* Heerlen mutation.[17] In this respect, it is important to point out that in our study type III deficiency was defined solely on the basis of PS levels, irrespective of mutational status. Due to the small sample size, no distinction was made between type III deficient individuals originating from families with pure type III deficiency and mixed type I/III deficiency, although most belonged to mixed type I/III deficient families, and only two carried *PROS1* Heerlen. Since type III PS deficiency is a heterogeneous category, a difference in thrombosis risk between type III deficient individuals from families with pure type III deficiency and those with mixed type I/III deficiency cannot be excluded and should be verified in a larger cohort of type III deficient individuals. In 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 PS-deficient individuals belonging to families with mixed type I/III deficiency (nAPCsr 3.65 and 4.42 *versus* 5.29±1.93; PS-ratio 0.39 and 0.49 *versus* 0.69±0.13), underscoring the importance of family studies to distinguish pure type III from mixed type I/III PS deficiency.

In summary, we have shown that type III PS-deficient individuals from families with mixed type I/III deficiency have a similar risk of venous thrombosis as type I deficient individuals. This conclusion is based not only on Kaplan-Meier analysis, 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 PS and, for the first time, the TFPI-cofactor activity of PS. Although type I deficient individuals had lower PS 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 PS. These findings may have implications for the counseling and management of patients with type III PS deficiency.

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.

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References

1. Rezende SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. Blood. 2004;103(4):1192-201.
2. Dahlbäck B. The tale of protein S and C4b-binding protein, a story of affection. Thromb Haemost. 2007;98(1):90-6.
3. Griffin JH, Gruber A, Fernández JA. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. Blood. 1992;79(12):3203-11.
4. Castoldi E, Hackeng TM. Regulation of coagulation by protein S. Curr Opin

Hematol. 2008;15(5):529-36.
5. Walker FJ. Protein S and the regulation of activated protein C. Semin Thromb Hemost. 1984;10(2):131-8.
6. Hackeng TM, Seré KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. Proc Natl Acad Sci USA. 2006; 103(9):3106-11.
7. Ndonwi M, Broze G Jr. Protein S enhances the TFPI inhibition of factor Xa but not its inhibition of factor VIIa/tissue factor. J Thromb Haemost. 2008;6(6):1044-6.
8. Maurissen LF, Thomassen MC, Nicolaes GA, Dahlbäck B, Tans G, Rosing J, Hackeng TM. Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation. Blood. 2008;111(6):3034-41.
9. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med. 1984;311(24):1525-8.
10. Schwarz HP, Fischer M, Hopmeier P, Batard MA, Griffin JH. Plasma protein S deficiency in familial thrombotic disease. Blood. 1984;64(6):1297-300.
11. García de Frutos P, Fuentes-Prior P, Hurtado B, Sala N. Molecular basis of protein S deficiency. Thromb Haemost. 2007;98(3):543-56.
12. Pintao MC, Garcia AA, Borgel D, Alhenc-Gelas M, Spek CA, de Visser MC, et al. Gross deletions/duplications in PROS1 are relatively common in point mutation-negative hereditary protein S deficiency. Hum Genet. 2009;126(3):449-56.
13. Liberti G, Bertina RM, Rosendaal FR. Hormonal state rather than age influences cut-off values of protein S: reevaluation of the thrombotic risk associated with protein S deficiency. Thromb Haemost. 1999;82(3):1093-6.
14. Zöller B, García de Frutos P, Dahlbäck B. Evaluation of the relationship between protein S and C4b-binding protein isoforms in hereditary protein S deficiency demonstrating type I and type III deficiencies to be phenotypic variants of the same genetic disease. Blood. 1995;85(12):3524-31.
15. Simmonds RE, Zöller B, Ireland H, Thompson E, García de Frutos P, Dahlbäck B, Lane DA. Genetic and phenotypic analysis of a large (122-member) protein S-deficient kindred provides an explanation for the familial coexistence of type I and type III plasma phenotypes. Blood. 1997;89(12): 4364-70.
16. ten Kate MK, Platteel M, Mulder R, Terpstra P, Nicolaes GA, Reitsma PH, et al. PROS1 analysis in 87 pedigrees with hereditary protein S deficiency demonstrates striking genotype-phenotype associations. Hum Mutat. 2008;29(7):939-47.
17. Bertina RM, Ploos van Amstel HK, van Wijngaarden A, Coenen J, Leemhuis MP, Deutz-Terlouw PP, et al. Heerlen polymorphism of protein S, an immunologic polymorphism due to dimorphism of residue 460. Blood. 1990;76(3):538-48.
18. Faioni EM, Valsecchi C, Palla A, Taioli E, Razzari C, Mannucci PM. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78(5):1343-6.
19. Borgel D, Reny JL, Fischelis D, Gandrille S, Emmerich J, Fiessinger JN, Aiach M. Cleaved protein S (PS), total PS, free PS, and activated protein C cofactor activity as risk factors for venous thromboembolism. Clin Chem. 2003;49(4):575-80.
20. Biguzzi E, Razzari C, Lane DA, Castaman G, Cappellari A, Bucciarelli P, et al. Molecular diversity and thrombotic risk in protein S deficiency: the PROSIT study. Hum Mutat. 2005;25(3):259-69.
21. Libourel EJ, Bank I, Veeger NJ, Hamulyák K, Middeldorp S, Prins MH, et al. Protein S type III deficiency is no risk factor for venous and arterial thromboembolism in 168 thrombophilic families: a retrospective study. Blood Coagul Fibrinolysis. 2005; 16(2):135-40.
22. Mateo J, Oliver A, Borrell M, Sala N, Fontcuberta J. Increased risk of venous thrombosis in carriers of natural anticoagulant deficiencies. Results of the family studies of the Spanish Multicenter Study on Thrombophilia (EMET study). Blood Coagul Fibrinolysis. 1998;9(1):71-8.
23. Brouwer JL, Veeger NJ, van der Schaaf W, Kluin-Nelemans HC, van der Meer J. Difference in absolute risk of venous and arterial thrombosis between familial protein S deficiency type I and type III. Results from a family cohort study to assess the clinical impact of a laboratory test-based classification. Br J Haematol. 2005;128(5): 703-10.
24. Simioni P, Sanson BJ, Prandoni P, Tormene D, Friederich PW, Girolami B, et al. Incidence of venous thromboembolism in families with inherited thrombophilia. Thromb Haemost. 1999;81(2):198-202.
25. Simmonds RE, Ireland H, Lane DA, Zöller B, García de Frutos P, Dahlbäck B. Clarification of the risk for venous thrombosis associated with hereditary protein S deficiency by investigation of a large kindred with a characterized gene defect. Ann Intern Med. 1998;128(1):8-14.
26. Makris M, Leach M, Beauchamp NJ, Daly ME, Cooper PC, Hampton KK, et al. Genetic analysis, phenotypic diagnosis, and risk of venous thrombosis in families with inherited deficiencies of protein S. Blood. 2000;95(6):1935-41.
27. Seré KM, Janssen MP, Willems GM, Tans G, Rosing J, Hackeng TM. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. Biochemistry. 2001;40(30):8852-60.
28. Koenen RR, Christella M, Thomassen LG, Tans G, Rosing J, Hackeng TM. Effect of oral contraceptives on the anticoagulant activity of protein S in plasma. Thromb Haemost. 2005;93(5):853-9.
29. Maurissen LF, Castoldi E, Simioni P, Rosing J, Hackeng TM. Thrombin generation-based assays to measure the activity of the TFPI-protein S pathway in plasma from normal and protein S-deficient individuals. J Thromb Haemost. 2010;8(4):750-8.
30. Castoldi E, Simioni P, Tormene D, Thomassen MC, Spiezia L, Gavasso S, Rosing J. Differential effects of high prothrombin levels on thrombin generation depending on the cause of the hyperprothrombinemia. J Thromb Haemost. 2007; 5(5):971-9.
31. Hemker HC, Giesen P, AlDieri R, Regnault V, de Smed E, Wagenvoord R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. Pathophysiol Haemost Thromb. 2002;32(5-6):249-53.
32. Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyák K, Ten Cate H, Rosing J. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. J Thromb Haemost. 2008;6(1):125-31.
33. Castoldi E, Simioni P, Tormene D, Rosing J, Hackeng TM. Hereditary and acquired protein S deficiencies are associated with low TFPI levels in plasma. J Thromb Haemost. 2010;8(2):294-300.
34. Dahm AE, Sandset PM, Rosendaal FR. The association between protein S levels and anticoagulant activity of tissue factor pathway inhibitor type 1. J Thromb Haemost. 2008;6(2):393-5.
35. Koenen RR, Tans G, van Oerle R, Hamulyák K, Rosing J, Hackeng TM. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. Blood. 2003;102(5): 1686-92.
36. Tans G, van Hylckama Vlieg A, Thomassen MC, Curvers J, Bertina RM, Rosing J, Rosendaal FR. Activated protein C resistance determined with a thrombin generation-based test predicts for venous thrombosis in men and women. Br J Haematol. 2003;122(3):465-70.
37. Gandrille S, Borgel D, Eschwege-Gufflet V, Aillaud M, Dreyfus M, Matheron C, et al. Identification of 15 different candidate causal point mutations and three polymorphisms in 19 patients with protein S deficiency using a scanning method for the analysis of the protein S active gene. Blood. 1995;85(1):130-8.
38. Simmonds RE, Ireland H, Kunz G, Lane DA. Identification of 19 protein S gene mutations in patients with phenotypic protein S deficiency and thrombosis. Protein S Study Group. Blood. 1996;88(11):4195-204.
39. Marchetti G, Legnani C, Patracchini P, Gemmati D, Ferrati M, Palareti G, et al. Study of a protein S gene polymorphism at DNA and mRNA level in a family with symptomatic protein S deficiency. Br J Haematol. 1993;85(1):173-5.
40. Formstone CJ, Wacey AI, Berg LP, Rahman S, Bevan D, Rowley M, et al. Detection and characterization of seven novel protein S (PROS) gene lesions: evaluation of reverse transcript-polymerase chain reaction as a mutation screening strategy. Blood. 1995;86(7):2632-41.
41. Rezende SM, Lane DA, Zöller B, Mille-Baker B, Laffan M, Dalhbäck B, Simmonds RE. Genetic and phenotypic variability between families with hereditary protein S deficiency. Thromb Haemost. 2002;87(2): 258-65.
42. Beauchamp NJ, Dykes AC, Parikh N, Campbell Tait R, Daly ME. The prevalence of, and molecular defects underlying, inherited protein S deficiency in the general population. Br J Haematol. 2004;125(5):647-54.
43. Espinosa-Parrilla Y, Morell M, Souto JC, Tirado I, Fontcuberta J, Estivill X, Sala N. Protein S gene analysis reveals the presence of a cosegregating mutation in most pedigrees with type I but not type III PS deficiency. Hum Mutat. 1999;14(1):30-9.
44. Beauchamp NJ, Daly ME, Cooper PC, Makris M, Preston FE, Peake IR. Molecular basis of protein S deficiency in three families also showing independent inheritance of factor V leiden. Blood. 1996;88(5):1700-7.
45. Hermida J, Faioni EM, Mannucci PM. Poor relationship between phenotypes of protein S deficiency and mutations in the protein S alpha gene. Thromb Haemost. 1999;82(6):1634-8.
46. Schmidel DK, Tatro AV, Phelps LG, Tomczak JA, Long GL. Organization of the human protein S genes. Biochemistry. 1990;29(34):7845-52.