New functional assays to selectively quantify the activated protein C- and tissue factor pathway inhibitor-cofactor activities of protein S in plasma

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ORIGINAL ARTICLE

New functional assays to selectively quantify the activated protein C- and tissue factor pathway inhibitor-cofactor activities of protein S in plasma

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Essentials

- Protein S is a cofactor of activated protein C (APC) and tissue factor pathway inhibitor (TFPI).
- There are no assays to quantify separate APC and TFPI cofactor activities of protein S in plasma.
- We developed assays to measure the APC- and TFPI-cofactor activities of protein S in plasma.
- The assays were sensitive to protein S deficiency, and not affected by the Factor V Leiden mutation.

Summary. *Background:* Protein S plays an important role in the down-regulation of coagulation as cofactor for activated protein C (APC) and tissue factor pathway inhibitor (TFPI). *Aim:* To develop functional assays to quantify the APC- and TFPI-cofactor activities of protein S in plasma. *Methods:* APC- and TFPI-cofactor activities of protein S in plasma were measured using calibrated automated thrombography in protein S-depleted plasma supplemented with a small amount of sample plasma either in the presence of anti-TFPI antibodies and APC (APC-cofactor activity) or at excess full-length TFPI without APC (TFPI-cofactor activity). Total and free protein S levels in plasma were measured by ELISAs. *Results:* Average APC-cofactor activities of protein S were 113%, 108% and 89% in plasma from normal individuals (n = 15), FV Leiden heterozygotes (n = 14) and FV Leiden homozygotes (n = 7), respectively, whereas the average APC-cofactor activity of protein S in plasma from heterozygous protein S-deficient individuals (n = 21) was significantly lower (55%). Similar trends were observed for the TFPI-cofactor activity of protein S, with averages of 109%, 115% and 124% in plasma from individuals with normal protein S levels and different FV Leiden genotypes, and 64% in plasma from protein S-deficient patients. APC-cofactor activities of protein S correlated significantly with free and total protein S antigen levels, whereas TFPI-cofactor activities correlated less with protein S antigen levels. *Conclusion:* We have developed functional protein S assays that measure both the APC- and TFPI-cofactor activities of protein S in plasma, which are hardly if at all affected by the FV Leiden mutation.

Keywords: activated protein C resistance; blood coagulation; factor V Leiden; protein C; protein S; tissue factor pathway inhibitor.

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Introduction

Protein S is a vitamin K-dependent plasma glycoprotein that is involved in many biological processes, including angiogenesis, apoptosis, inflammation and coagulation [1]. As an anticoagulant, it plays an important role in the down-regulation of coagulation. Protein S acts as non-enzymatic cofactor of two major natural anticoagulants, activated protein C (APC) and tissue factor pathway inhibitor (TFPI) [2]. It acts as a cofactor for APC in the inactivation of factor (F) Va and FVIIIa [3–5] and as a cofactor for TFPI by enhancing the formation of the FXa–TFPI complex and the subsequent inhibition of the TF–FVIIa complex [6–9].

Protein S circulates in blood at a total concentration of 350 nM, of which ~40% (150 nM) is free and ~60% (200 nM) is in complex with C4b-binding protein (C4BP), a regulator of the complement pathway [10,11]. Protein S deficiency is a risk factor for thrombotic diseases, which, however, is more clearly observed in thrombophilic families [12–14] than in the general population [15–17]. Hereditary as well as acquired conditions (e.g. the use of oral contraceptives [18,19]) can cause protein S deficiency. Based on plasma levels of total and free protein S antigen and on the functional activity of protein S, three types of protein S deficiency have been defined: type I is a quantitative deficiency in which total and free protein S antigen levels and activity are low, type II is a qualitative deficiency in which total and free protein S levels are normal but activity is reduced, and type III is characterized by a normal total protein S level but reduced free form and activity. Many mutations in the gene encoding protein S (*PROS1*) have been identified [20]. Homozygous *PROS1* mutations result in severe thrombotic complications and purpura fulminans, whereas heterozygous mutations are compatible with life and are associated with increased risks of venous and arterial thrombosis [21].

The protein S level in plasma can be measured both with enzyme-linked immunosorbent assays (ELISAs) and with functional clotting-based assays. The ELISAs quantify the antigen concentration of free protein S or total protein S, but provide no information on the functional activity of protein S. Functional protein S assays are based on measuring the ability of protein S to prolong the clotting time of plasma in the presence of APC. Unfortunately, functional protein S assays are often influenced by the presence of the factor V (FV) Leiden mutation [22,23]. Moreover, there is no specific functional assay for the TFPI-cofactor activity of protein S. In fact, the previously described assay, based on the measurement of thrombin generation in the absence and presence of neutralizing antibodies against protein S [24], has more determinants than plasma protein S levels/activity alone [25]. The goal of this study is to develop thrombin generation-based assays that enable quantification of both the APC- and TFPI-cofactor activities of protein S in plasma and that are not affected by the presence of the FV Leiden mutation.

Materials and methods

Materials

The fluorogenic substrate I-1140 (Z-Gly-Gly-Arg-7-amino-4-methylcoumarin-HCl) was purchased from Bachem (Bubendorf, Germany). The phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Phospholipid vesicles (DOPS/DOPC/DOPE; 20:60:20; mol/mol/mol) were prepared as described earlier [26].

Proteins

Corn trypsin inhibitor (CTI) was from Haematologic Technologies (Essex Junction, VT, USA). Recombinant TF (Innovin) was from Siemens Healthcare (Marburg, Germany). mAbs against each Kunitz domain and the C-terminus of human TFPI were purchased from Sanquin (Amsterdam, the Netherlands) and mixed in an equimolar ratio. Antibodies were heat treated for 2 h at 56 °C to remove possible contaminating non-IgG proteins, centrifuged for 2 min at 1500 × *g* and the supernatant was stored at −20 °C. The amount of heat-treated anti-TFPI antibody mixture required to neutralize the anticoagulant activity of TFPI in normal plasma was determined for each new batch of TFPI antibodies and a 2-fold excess was subsequently used in the assay mixtures. Recombinant human full-length TFPI (1–276), produced in a bacterial expression system (*Escherichia coli*), was kindly provided by T. Lindhout (Maastricht University, the Netherlands). APC (Xigris) was a gift from our hospital. TFPI, TF and APC were diluted to the desired concentrations in 25 mM Hepes (pH 7.5), 175 mM NaCl and 5 mg mL^{-1} BSA and stored at −80 °C. Thrombin calibrator was purchased from Thrombinoscope BV (Maastricht, the Netherlands).

Study subjects

All study subjects were enrolled at Padua Academic Hospital (Italy). From a previously described cohort of 23 families with hereditary protein S deficiency [25], we selected 21 individuals with heterozygous type I protein S deficiency (total protein S < 70%, free protein S < 65%) and 36 individuals with normal protein S levels, 21 of whom were carriers of the FV Leiden mutation (14 heterozygotes and seven homozygotes). At the time of blood collection, none of the volunteers was on oral anticoagulant treatment, oral contraceptives or other medication. All participants provided informed consent according to the Declaration of Helsinki.

Blood collection and plasma preparation

Blood was drawn from the study participants in 3.8% sodium citrate and platelet-poor plasma was prepared by centrifuging blood at 2000 × *g* for 10 min. Plasma was aliquoted, snap-frozen and stored at −80 °C until use.

To prepare normal pooled plasma, venous blood from 24 healthy volunteers (13 male and 11 female) was collected in 3.2% sodium citrate. The first milliliters of blood were discarded. The remaining blood was centrifuged at

2000 × *g* for 15 min to separate plasma from blood cells and again at 11 000 × *g* for 5 min to obtain platelet-poor plasma, which was immediately pooled, snap-frozen and stored at −80 °C until use.

Protein S-depleted plasma was purchased from Affinity Biologicals (Ancaster, ON, Canada).

Thrombin generation-based assays for measurement of the APC- and TFPI-cofactor activities of protein S

Calibrated automated thrombography [27] was used to measure thrombin generation in protein S-depleted plasma supplemented with 0, 2, 4, 6, 8 or 10 μL normal pooled plasma (calibration curve) or 6 μL sample plasma to result in a total volume of 80 μL plasma per well. To the plasma were added 1 pM TF, 30 μM phospholipid vesicles (DOPS/DOPC/DOPE; 20:60:20; mol/mol/mol), 40 μg mL^{-1} CTI and 30 nM APC plus an equimolar mixture of mAbs directed against the Kunitz 1, Kunitz 2 and Kunitz 3 domains and the C-terminus of TFPI in the APC-cofactor activity assay; or 1 pM TF, 30 μM phospholipid vesicles (DOPS/DOPC/DOPE; 20:60:20; mol/mol/mol), 40 μg mL^{-1} CTI and 1 nM TFPI in the TFPI-cofactor activity assay. Thrombin generation was initiated with 20 μL of a $CaCl_2$/I-1140 mixture resulting in 16 mM $CaCl_2$ and 300 μM I-1140. All concentrations given are final concentrations in a total volume of 125 μL. Thrombin generation was followed at 37 °C during 60–80 min and quantified with I-1140. The thrombin peak height, endogenous thrombin potential (ETP) and the lag time of thrombin generation were calculated using software obtained from Thrombinoscope BV.

Determination of total and free protein S antigen levels in plasma

Total and free protein S antigen levels in sample plasmas were measured by in-house ELISAs [28,29] and expressed as percentage of the antigen levels present in normal plasma.

Results

Effect of protein S, APC and TFPI on thrombin generation

Thrombin generation was measured in protein S-depleted plasma supplemented with protein S (in the form of normal plasma), APC or TFPI or combinations of these proteins. In the absence of APC and TFPI (Fig. 1A) protein S had no effect on thrombin generation, neither at 1 pM TF (high thrombin generation) nor at 0.1 pM TF (low thrombin generation). In the absence of protein S, both APC (Fig. 1B) and TFPI (Fig. 1C) expressed anticoagulant activity and inhibited thrombin generation at 1 pM TF. When protein S-depleted plasma was supplemented with protein S (i.e. with 10 μL normal plasma) inhibition of thrombin generation by APC and TFPI was much more pronounced, indicating that protein S enhances the anticoagulant activity of both APC (Fig. 1B) and TFPI (Fig. 1C).

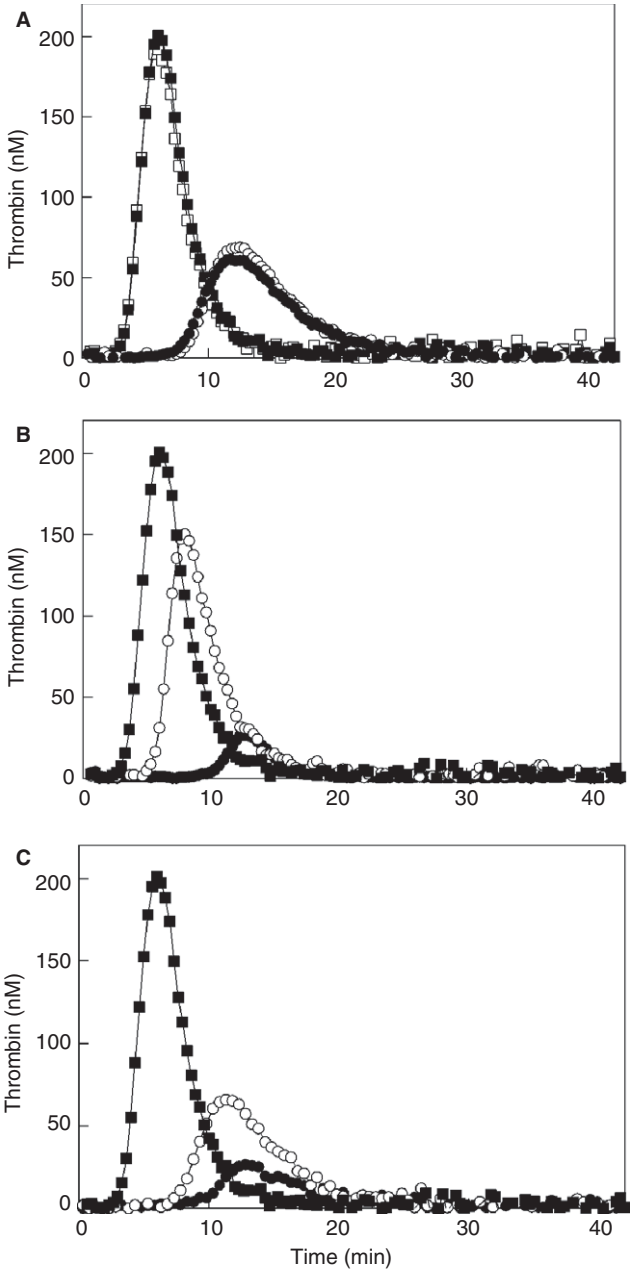

Fig. 1. The effect of normal plasma (protein S) on thrombin generation determined in protein S-depleted plasma in the absence and presence of APC or TFPI. (A) Thrombin generation in protein S-depleted plasma triggered with 1 pM TF without (■) or with 10 μL normal plasma (□) or triggered with 0.1 pM TF without (●) or with 10 μL normal plasma (○). (B) Thrombin generation in protein S-depleted plasma triggered with 1 pM TF in the absence of APC and normal plasma (■) or in the presence of 30 nM APC (○) or of 30 nM APC and 10 μL normal plasma (●). (C) Thrombin generation in protein S-depleted plasma triggered with 1 pM TF in the absence of TFPI and normal plasma (■) or in the presence of 1 nM TFPI (○) or of 1 nM TFPI and 10 μL normal plasma (●). Further experimental details are presented in the Methods section. APC, activated protein C; TFPI, tissue factor pathway inhibitor, TF, tissue factor.

Functional assay to quantify the APC-cofactor activity of protein S in plasma

In order to generate a calibration curve that enables quantification of the APC-cofactor activity of protein S in plasma, a normal plasma titration was performed in protein S-depleted plasma supplemented with CTI to prevent contact activation. Because the APC-cofactor activity of protein S should be the only determinant of the protein S activity in the assay, anti-TFPI antibodies were added to plasma to eliminate the contribution of the TFPI-cofactor activity of protein S to the inhibition of thrombin generation. Before recalcification, 1 pM TF, 30 μM phospholipid vesicles and 30 nM APC (final concentrations in the well) were added to the plasma and thrombin generation was initiated with FluCa (a mixture of $CaCl_2$ and the fluorogenic substrate I-1140). A high APC concentration was chosen in order to make the assay sensitive to low amounts of protein S.

In the presence of APC both the peak height and the endogenous thrombin potential (ETP) strongly decreased and the lag time of thrombin generation increased at increasing amounts of normal plasma (Fig. 2A). A linear relation was observed between the amount of normal plasma and the logarithmic values of the lag time of thrombin generation, the thrombin peak height and the ETP (Fig. 2B). The logarithm of the peak height showed the largest change upon variation of the amount of normal plasma in protein S-depleted plasma. Hence, this parameter was used to quantify the APC-cofactor activity of protein S in plasma from patient populations (see below).

Functional assay to quantify the TFPI-cofactor activity of protein S in plasma

This assay is based on the observation that protein S, in addition to being a cofactor of APC, also enhances the anticoagulant activity of TFPI in plasma and in model systems [6]. Because protein S-immunodepletion of plasma also removes TFPI, resulting in plasma with no or a very low amount of TFPI [30], the protein S-depleted plasma used in the TFPI-cofactor assay was supplemented with a high concentration of TFPI (1 nM TFPI, final concentration) to overcome influences of the variable TFPI concentrations in sample plasma. A normal plasma titration was performed in TFPI-supplemented protein S-depleted plasma in the presence of CTI. Before recalcification, 1 pM TF and 30 μM phospholipid vesicles (final concentrations) were added to the plasma mixture and thrombin generation was initiated with FluCa.

Normal plasma (protein S) had no effect on thrombin generation in protein S-depleted plasma that did not contain TFPI (i.e. supplemented with an excess of neutralizing TFPI antibodies) (Fig. 1A). However, upon increasing the amount of normal plasma in protein S-depleted plasma supplemented with 1 nM TFPI, both the thrombin peak height and the ETP gradually decreased, whereas the lag time of thrombin generation increased with the normal plasma concentration (Fig. 3A,B).

As in the APC-cofactor assay, the thrombin generation parameter that changed most in the TFPI-cofactor assay upon variation of the amount of normal plasma in protein S-depleted plasma was the logarithm of the peak height (Fig. 3B). Hence this parameter was used to quantify the TFPI-cofactor activity of protein S in plasma from patient populations (see below).

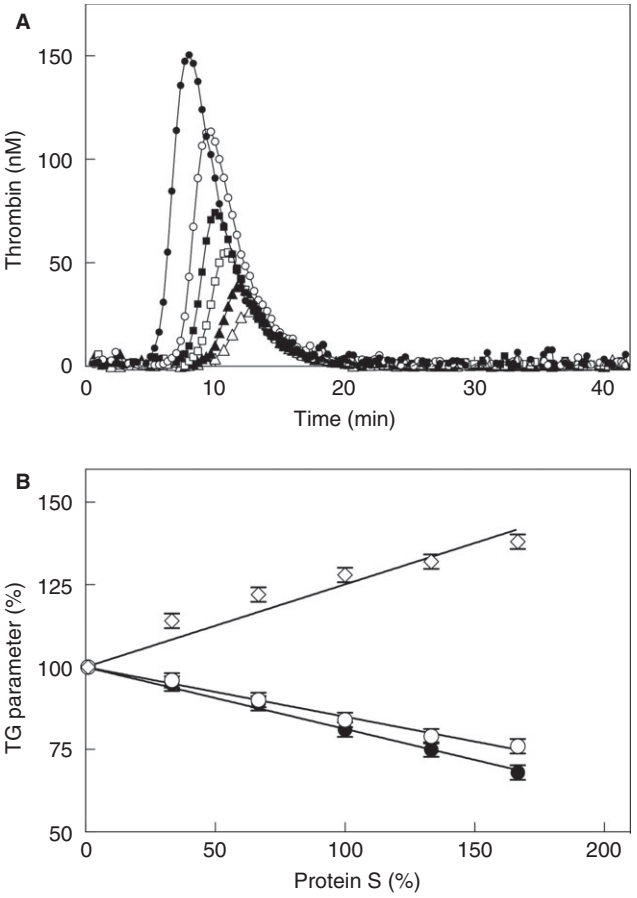

Fig. 2. (A) Thrombin generation curves determined in protein S-depleted plasma triggered with 1 pM TF in the presence of 30 nM APC and anti-TFPI antibodies and 0 μL (●), 2 μL (○), 4 μL (■), 6 μL (□), 8 μL (▲) or 10 μL (△) normal plasma. (B) Logarithmic values of the lag time (◊), ETP (○) and peak height (●) plotted as function of the amount of protein S (normal plasma) in protein S-depleted plasma (total plasma volume was kept constant at 80 μL). Because the APC-cofactor activity of protein S of study subjects was determined with 6 μL plasma, the protein S concentrations in the calibration curves (B) were expressed as % protein S taking 6 μL normal plasma as 100%. The calibration curves shown are the average of eight calibration curves determined on four different days. Error bars indicate standard error of the mean, which for all data points was < 2.5%. Further experimental details are presented in the Methods section. TF, tissue factor; APC, activated protein C; TFPI, tissue factor pathway inhibitor; ETP, endogenous thrombin potential; TG, thrombin generation.

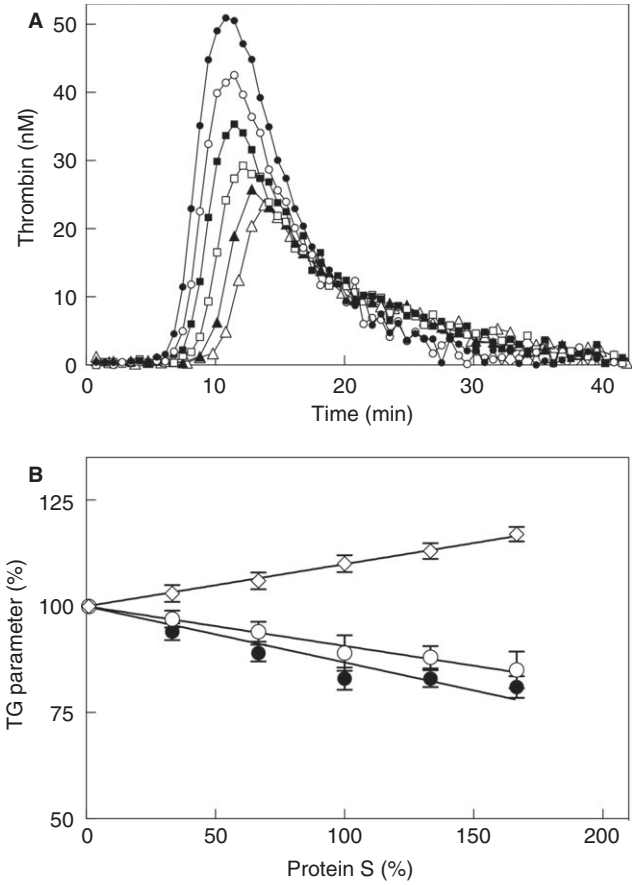

Fig. 3. (A) Thrombin generation curves determined in protein S-depleted plasma supplemented with 1 nM TFPI and triggered with 1 pM TF in the presence of 0 μL (●), 2 μL (○), 4 μL (■), 6 μL (□), 8 μL (▲) or 10 μL (△) normal plasma. (B) Logarithmic values of the lag time (◊), ETP (○) and peak height (●) plotted as function of the amount of protein S (normal plasma) in protein S-depleted plasma (total plasma volume was kept constant at 80 μL). Because the TFPI-cofactor activity of protein S of study subjects was determined with 6 μL plasma, the protein S concentrations in the calibration curves (B) were expressed as % protein S taking 6 μL normal plasma as 100%. Error bars indicate standard error of the mean. Further experimental details are presented in the Methods section. TFPI, tissue factor pathway inhibitor; TF, tissue factor; ETP, endogenous thrombin potential; TG, thrombin generation.

Measurement of the APC-cofactor activity of protein S in protein S-deficient individuals and normal controls

The APC-cofactor assay was applied to plasma samples from 21 type I protein S-deficient individuals and 36 healthy individuals with normal protein S levels, of whom 14 were heterozygous and seven were homozygous carriers of the FV Leiden mutation. To perform the assay, a small volume (6 μL) of sample plasma was mixed with 74 μL of protein S-depleted plasma and the APC-cofactor activity of protein S was determined in duplicate as described above. The average peak heights of thrombin generation curves were calculated using the Thrombinoscope software and the APC-cofactor activity of protein S in the plasma samples was expressed as percentage of the amount of protein S present in normal plasma using a calibration curve of the log of the peak height as function of the normal plasma concentration determined on the same microtiter plate (Fig. 2B). An amount of 6 μL sample plasma was chosen, which, as judged from the calibration curve (Fig. 2A), allows optimal quantification of protein S in plasma samples that either have lower or higher protein S activities than present in normal plasma. With respect to the accuracy of the quantification, the intra-assay variation (% CV) of the thrombin peak height and the log of the peak height in the APC-cofactor assay at 7.5 μL normal plasma were 6% and 1.8%, respectively. The inter-assay variation of the protein S concentration determined in a normal plasma sample read on a calibration curve determined on the same microtiter plate was 3%.

Plasma from protein S-deficient patients had a low protein S activity, whereas the FV Leiden carriers and controls had normal protein S activities (Fig. 4A, Table 1). Interestingly, there was no overlap between the protein S-deficient patients and the controls, neither with nor without the FV Leiden mutation. However, FV Leiden homozygotes showed a trend toward lower protein S activities.

Measurement of the TFPI-cofactor activity of protein S in protein S-deficient individuals and normal controls

The TFPI-cofactor activity of protein S was measured in the same plasma collection. To this end, 6 μL plasma from protein S-deficient individuals or from normal controls was mixed with 74 μL of protein S-depleted plasma and the TFPI-cofactor activity of the protein S was determined in duplicate as described above. As in the APC-cofactor assay, an amount of 6 μL patient plasma was chosen, because on the basis of the calibration curve (Fig. 3B) this allows optimal quantification of both lower and higher protein S activities than present in normal plasma. The intra-assay variation (% CV) of the peak height and the log of the peak height in the TFPI-cofactor assay performed with 7.5 μL normal plasma were 11.2% and 3.4%, respectively. The TFPI-cofactor activity of protein S in the plasma samples was expressed as percentage of the activity of protein S present in normal plasma using a calibration curve of the log of the peak height as function of the normal plasma concentration determined on the same microtiter plate (Fig. 3B). The inter-assay variation of the protein S concentration determined in a normal plasma sample that was read on a calibration curve was 9.6%.

Plasma from PS-deficient individuals had a significantly lower TFPI-cofactor activity than the plasma from normal individuals and from FV Leiden carriers (Fig. 4B, Table 1). However, there was some overlap between the protein S-deficient individuals and those with normal protein S levels.

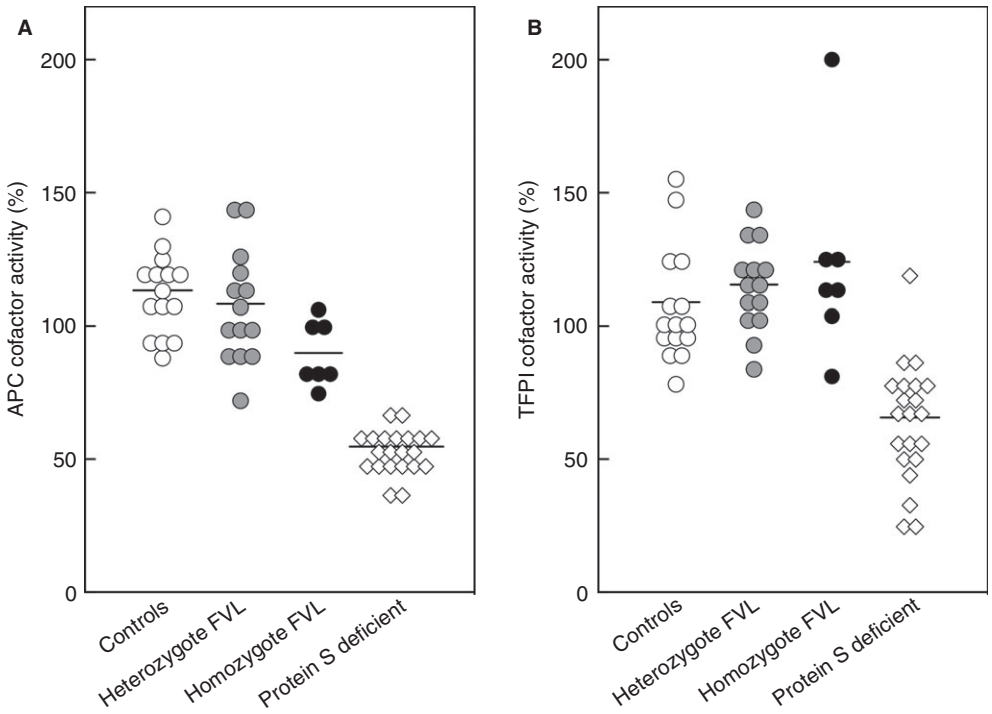

Fig. 4. (A) APC-cofactor activity of protein S in plasma from protein S-deficient individuals (◊), controls without the factor V Leiden mutation (○) or heterozygous (●) or homozygous carriers of the FV Leiden mutation (●). APC-cofactor activities of protein S are given as % of that present in normal pooled plasma, which was taken as 100%. (B) TFPI-cofactor activity of protein S in plasma from protein S-deficient individuals (◊), controls without the FV Leiden mutation (○) or heterozygous (●) or homozygous carriers of the FV Leiden mutation (●). TFPI-cofactor activities of protein S are given as % of those present in normal pooled plasma, which was taken as 100%. APC, activated protein C; TFPI, tissue factor pathway inhibitor.

Table 1 Protein S activities and antigen concentrations in normal and heterozygous protein S-deficient subjects and in carriers of the FV Leiden mutation

Population	APC-cofactor activity Mean (95% CI)	TFPI-cofactor activity Mean (95% CI)	Protein S total Mean (95% CI)	Protein S free Mean (95% CI)
Normal protein S (n = 36)	106.8 (100.6–112.9.)	114.4 (107.7–122.1)	102.7 (98.0–107.5)	102.1 (97.3–107.0)
No FVL (n = 15)	113.2 (105.8–120.7)	108.9 (98.0–119.7)	98.7 (92.6–105.1)	97.7 (90.7–104.7)
Heterozygote FVL (n = 14)	108.3 (97.1–119.4)	115.5 (106.8–124.1)	105.3 (96.9–113.7)	109.7 (101.6–117.7)
Homozygote FVL (n = 7)	89.9 (80.9–98.9)	124.0 (96.6–151.6)	106.3 (94.6–118.0)	94.0 (87.7–100.3)
Protein S deficient (n = 21)	54.8 (51.3–58.2)	65.7 (56.2–75.3)	51.0 (47.8–54.1)	39.2 (34.8–43.5)

FVL, FV Leiden; APC, activated protein C; TFPI, tissue factor pathway inhibitor. Within the normal protein S population; sub-populations with different FVL status are indicated.

Determination of total and free levels of protein S by ELISA

The antigen levels of total and free protein S were measured with ELISAs in plasma from the protein S-deficient individuals and from the normal controls with or without FV Leiden. Total protein S (Fig. 5A, Table 1) and free protein S levels (Fig. 5B, Table 1) were significantly lower in individuals with heterozygous type I protein S deficiency than in normal controls.

Correlations between APC- and TFPI-cofactor activities of protein S and antigen levels of total and free protein S

The correlations between the functional activities of protein S and the total and free antigen concentrations of protein S in plasma are summarized in Fig. 6(A–F) and Table 2. In the whole study population there appeared to be good correlations (solid lines) between the APC- and TFPI-cofactor activities of protein S (Fig. 6A), between antigen levels of total and free protein S (Fig. 6B), and between the APC-cofactor activity of protein S and total (Fig. 6C) and free (Fig. 6D) protein S antigen levels. The correlations between the TFPI-cofactor activity of protein S and the antigen levels of free and total protein S were considerably lower (Fig. 6E,F). Correlations between protein S antigen levels and functional protein S activities were much less when the protein S-deficient individuals and the controls with normal protein S levels were analyzed separately (Fig. 6A–F, dashed lines). The Pearson correlation coefficients (r) obtained from Fig. 6(A–F) are

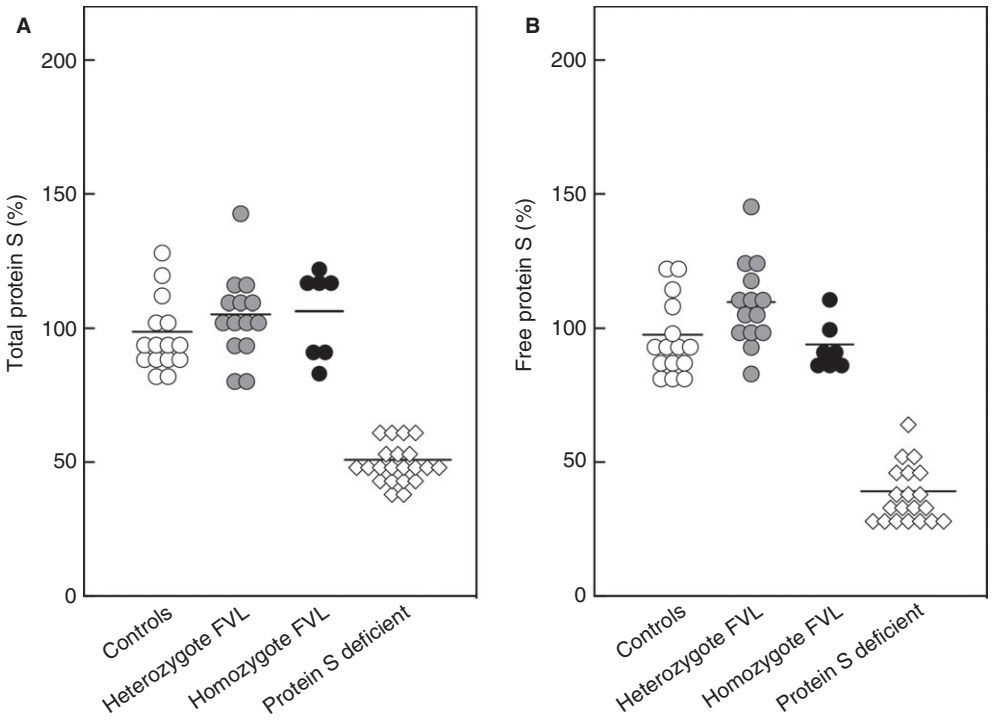

Fig. 5. Protein S antigen levels in plasma from protein S-deficient individuals and from controls. Antigen levels of total (A) and free protein S (B) were determined in plasma from protein S-deficient individuals and controls with normal protein S levels with ELISAs described in the Methods section. Protein S-deficient individuals (◊), controls without the factor V Leiden mutation (○) or heterozygous (●) or homozygous carriers of the FV Leiden mutation (●).

summarized in Table 2 and confirm the conclusions presented above.

Discussion

Protein S plays a fundamental role in down-regulating coagulation by enhancing the anticoagulant activities of APC and TFPI [2]. Accordingly, low plasma levels of protein S are associated with an increased risk of venous thrombosis [21].

Determination of the protein S concentration in plasma is mainly based on ELISAs that quantify the antigen levels of total protein S and free protein S [31]. Existing functional assays of protein S measure the effect of protein S on the prolongation of the clotting time of plasma by APC. However, functional protein S assays give unreliable protein S activity in APC-resistant plasma (e.g. in plasma from carriers of the FV Leiden mutation) [22,23] and can be affected by other plasma factors such as high variability of TFPI levels.

We have developed thrombin generation-based assays to separately measure the APC- and TFPI-cofactor activities of protein S in plasma. These assays are hardly if at all affected by the presence of the FV Leiden mutation or other influences from individual plasma backgrounds. The assay for the quantification of the APC-cofactor activity of protein S has been designed to be highly sensitive to protein S by the addition of a high concentration of APC (30 nM). To ensure that the APC-cofactor activity of protein S is the only determinant of the activity of protein S in this assay, neutralizing antibodies were used against TFPI. In the assay in which the TFPI-cofactor activity of protein S was determined, protein S-depleted plasma was supplemented with an excess of full-length TFPI (1 nM) to reduce the effect of variable TFPI levels in different batches of protein S-depleted plasma and in patient plasma and to make the assay exclusively dependent on the TFPI-cofactor activity of protein S. In both assays, thrombin generation was initiated with a low trigger (1 pM TF), which is optimal for detection of both the APC- and TFPI-cofactor activities of protein S, because it is well established that the activity of anticoagulant proteins/pathways is particularly observed when coagulation is initiated with a low TF concentration [26,32]. As a recommendation, a TF titration in protein S-depleted plasma is a good strategy to determine the optimal TF concentration for quantification of the APC- and TFPI-cofactor activities of protein S. We observed that optimal sensitivity for protein S was obtained at a TF concentration that yielded a peak height of thrombin generation between 35 and 45 nM at 1 nM TFPI (TFPI-cofactor assay) and a peak height between 100 and 200 nM at 30 nM APC in the presence of TFPI antibodies in the APC-cofactor assay (data not shown).

A unique and strong feature of the assays is the small volume of donor plasma that is required to perform the

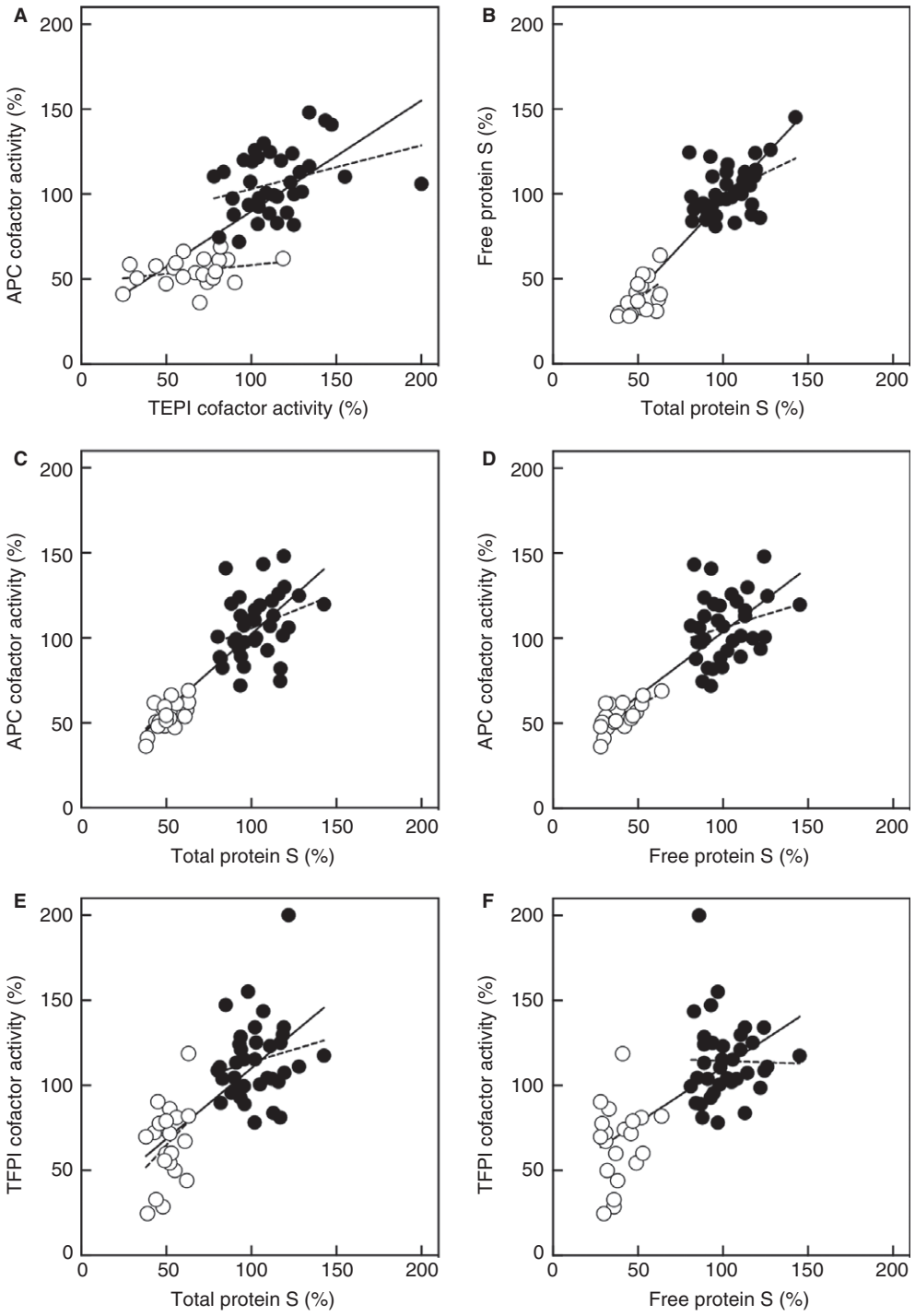

Fig. 6. Correlations between functional activities and antigen levels of protein S. Correlations between APC- and TFPI-cofactor activities (A), total and free antigen levels of protein S (B), APC-cofactor activity of protein S and the antigen levels of total (C) and free protein S (D) and between the TFPI-cofactor activity of protein S and the antigen levels of total (E) and free protein S (F) in protein S-deficient individuals (○) and controls with normal protein S levels (●). The solid lines represent correlations in the total population and the dashed lines represent correlations in the populations of protein S-deficient individuals and in the controls. APC, activated protein C; TFPI, tissue factor pathway inhibitor.

assay. In addition, dilution of a small volume of sample plasma in protein S-depleted plasma eliminates the influences of other variables such as increased FVIII levels and the presence of the FV Leiden mutation, which can interfere with the outcome of the assays and result in an underestimation of the activity of protein S in plasma.

The thrombin generation-based APC- and TFPI-cofactor assays were validated in plasma from 21 protein

Table 2 Correlations between APC- and TFPI-cofactor activities of protein S and antigen levels of total and free protein S

	Pearson correlation coefficient		
	All	Normal	Protein S deficient
TFPI- vs. APC-cofactor activity	0.721*	0.320	0.286
Protein S total vs. protein S free	0.906*	0.478*	0.538*
APC-cofactor activity vs. protein S total	0.845*	0.317	0.634*
APC-cofactor activity vs. protein S free	0.839*	0.240	0.652*
TFPI-cofactor activity vs. protein S total	0.711*	0.187	0.350
TFPI-cofactor activity vs. protein S free	0.660*	−0.021	0.184

*$P < 0.05$. APC, activated protein C; TFPI, tissue factor pathway inhibitor.

S-deficient individuals and from 36 individuals with normal protein S levels, including 14 FV Leiden heterozygotes and seven FV Leiden homozygotes. In both the APC- and TFPI-cofactor assays protein S-deficient individuals had significantly lower protein S activities than the controls with normal antigen levels of protein S. In the TFPI-cofactor assay, some overlap was observed between the protein S-deficient individuals and study subjects with normal protein S antigen levels. In the APC-cofactor assay there was no overlap between the two populations, which shows that this assay is 100% specific and 100% sensitive for type I protein S deficiency as defined by antigen levels of total protein S below 70% and free protein S below 65%. In the TFPI-cofactor assay no differences were observed between the protein S activities in individuals without FV Leiden and heterozygous and homozygous FV Leiden carriers (Table 1). However, in the APC-cofactor assay FV Leiden homozygotes had lower protein S activities than normal individuals and FV Leiden heterozygotes. This may be either because of the lower free protein S levels in the homozygous FV Leiden carriers (Table 1) or the small amount of FV Leiden (~8%) present in the assay mixture, which may result in somewhat higher thrombin generation and hence in a slight underestimation of the APC-cofactor activity of protein S. Remarkably, the results of the TFPI-cofactor activity assay showed hardly any correlation with the protein S-ratio previously determined in the same samples by measuring thrombin generation in the absence and presence of neutralizing antibodies against protein S without pre-dilution in protein S-depleted plasma [24]. This may be because the latter assay is sensitive to other plasma variables in addition to protein S, including TFPI and prothrombin levels [25].

The results obtained with the thrombin generation-based functional assays were mutually compared and correlated with the antigen levels of total and free protein S (Table 2). In the whole population there appeared to be good correlations between the APC- and TFPI-cofactor activities of protein S and between the APC-cofactor activity of protein S and the antigen levels of total and free protein S. The TFPI-cofactor activity of protein S showed a weaker correlation with the antigen concentrations of total and free protein S. Correlations between functional protein S activities and antigen levels of total and free protein S appeared to be much lower or even absent when the analysis was restricted to the populations of protein S-deficient individuals and normal controls, respectively. Because correlations between two parameters are hard to prove if there is little variation in the values of these parameters, this may not be surprising because the variation of the protein S levels within the separate populations is much less than within the total population. If present, correlations were more pronounced, particularly in the APC-cofactor assay, in the protein S-deficient population than in the normal controls. This can be explained by the observation that the absolute changes in the thrombin generation parameters were larger at low than at high protein S concentrations and were also more pronounced in the APC-cofactor assay than in the TFPI-cofactor assay, together resulting in a more reliable quantification of functional activities in protein S-deficient patients, which makes it easier to establish correlations, if present.

Finally, we would like to point out that results of functional assays do not necessarily have to correlate with antigen concentrations determined with an ELISA because ELISAs do not provide information on the actual activity of a protein. For instance, type II protein S deficiency (normal antigen, reduced activity) will not show up in an ELISA but will result in low protein S activity in functional assays. Because different domains of protein S have been reported to be involved in the enhancement of the anticoagulant activities of APC [33–35] and TFPI [36,37], type II protein S-deficient patients may have mutations that selectively impair one of the two cofactor functions of protein S and hence may have differential effects on the APC- and TFPI-cofactor activities. In this respect, it is of interest to mention that two individuals (Fig. 4B) had TFPI-cofactor activities of protein S that were 2-fold higher than the APC-cofactor activity of protein S and protein S antigen levels. One patient was a homozygous carrier of FV Leiden and the other was protein S deficient. We are currently investigating why protein S of these individuals has a high TFPI-cofactor activity.

In conclusion, we have developed thrombin generation-based assays that enable quantification of the APC- and TFPI-cofactor activities of protein S in plasma. We have shown that both the TFPI- and APC-cofactor activities of protein S are impaired in hereditary protein S deficiency. It will be interesting to also analyze populations with acquired protein S deficiency, such as that occurring during hormonal changes in women (pregnancy and use

of oral contraceptives), and to validate the clinical use of these assays in case–control studies of venous thrombosis.

Addendum

N. A. Alshaikh performed the experiments, analyzed the data, and wrote the manuscript. J. Rosing supervised the project, designed the experiments, analyzed data, and revised the manuscript. S. Thomassen performed experiments and revised the manuscript. E. Castoldi critically revised the manuscript. P. Simioni carried out the plasma collection from protein S-deficient patients and controls and approved the manuscript. T. M. Hackeng was responsible for conception and design of the study and revised the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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