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## **Risk factors for first and recurrent venous thrombosis : new insights from old concepts**

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# Chapter 5

## *Protein S levels and the risk of venous thrombosis: results from the MEGA case-control study*

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## ABSTRACT

In thrombophilic families, protein S deficiency is clearly associated with venous thrombosis. We aimed to determine whether the same holds true in a population-based case-control study (n=5317). Subjects were regarded protein S deficient when protein S levels were <2.5<sup>th</sup> percentile of the controls. Free- and total protein S deficiency was not associated with venous thrombosis: free protein S <53 U/dL, odds ratio [OR] 0.82 (95%CI, 0.56-1.21) and total protein S <68 U/dL, OR 0.90 (95%CI, 0.62-1.31). When lower cut-off values were applied, it appeared that subjects at risk of venous thrombosis could be identified at levels <0.10<sup>th</sup> percentile of free protein S (<33 U/dL, OR 5.4; 95% CI, 0.61-48.8). In contrast, even extremely low total protein S levels were not associated with venous thrombosis. *PROS1* was sequenced in 48 subjects with free protein S level <1<sup>st</sup> percentile (<46 U/dL), and copy number variations were investigated in 2718 subjects, including all subjects with protein S (free or total) <2.5<sup>th</sup> percentile. Mutations in *PROS1* were detected in five patients and five controls reinforcing the observation that inherited protein S deficiency is rare in the general population. Protein S testing and *PROS1* testing should not be considered in unselected patients with venous thrombosis.

## INTRODUCTION

Protein S is a vitamin K-dependent glycoprotein encoded by *PROS1* and synthesized mainly in hepatocytes and endothelial cells [1]. Protein S circulates at a plasma concentration of ~350 nM, of which 40% is free and 60% is bound to C4b-binding protein [2]. Protein S is a major anticoagulant protein that assists in the down-regulation of thrombin formation by stimulating the activity of both activated protein C and tissue factor pathway inhibitor [3, 4].

Hereditary protein S deficiency is a haploinsufficiency disorder, associated with an increased risk of venous thrombosis [5]. Protein S deficiency is associated with decreased activated protein C (APC)-cofactor activity and can be classified as type I (decreased levels of both total and free protein S antigen), type II (decreased APC-cofactor activity but total and free protein S antigen levels within their normal ranges), and type III (decreased levels of free protein S antigen levels only) [6]. The genetic basis of protein S deficiency is heterogeneous. Currently more than 200 mutations in *PROS1* have been described, the vast majority missense or nonsense mutations [7-9]. Sequencing exons and splicing junctions of *PROS1* has been successful in the identification of a mutation in only around 50% of families with type I or mixed type I and type III protein S deficiency [8]. Recently, we and others have shown that gross deletions and insertions of *PROS1* are present in around 30% of the point mutation-negative families with protein S deficiency [5, 10, 11].

Most of the insights regarding the association between protein S deficiency and venous thrombosis have been derived from studies of thrombophilic families in which there was evidence for inherited protein S deficiency. This has led to the notion that the risk of venous thrombosis in protein S deficient subjects is five to ten times increased in comparison to non-protein S deficient relatives [5, 12-14]. The clinical relevance for the individual patient is substantial as subjects with familial protein S deficiency have a first venous thrombosis incidence of 0.7% per year [15] and an annual venous recurrence risk of 6-10% [14, 16, 17]. It is not clear to what extent the conclusions from these family studies can be extrapolated to the general population. The co-inheritance of other defects may contribute to or explain this effect in families, as was shown for factor V Leiden, and protein C deficiency [18, 19]. Evidence obtained from relatively small population-based studies (maximum number of participants 327) [19] point towards a much lower risk of thrombosis associated with protein S deficiency. Although some studies reported an up to 2.5 times increased risk, others found no increase at all [19-21].

Due to these uncertainties, we set out to determine in a large population-based case-control study whether low levels of free or total protein S were associated with an increased risk of venous thrombosis. The molecular basis for protein S deficiency was also investigated by analysis of copy number variation of *PROS1* and resequencing of subjects with the lowest levels of protein S in an attempt to support the risk estimates from a genetic viewpoint.

## METHODS

### Subjects

The MEGA case-control study has been described in detail previously [22]. Consecutive patients aged 18 to 70 years with a first episode of deep venous thrombosis or pulmonary embolism between March 1, 1999 and August 31, 2004 were included, to a total of 4956 patients. Age- and sex-matched controls were partners of patients (n=3297) or subjects recruited by random digit dialing (RDD) between January 1, 2002 and December 1, 2004 (n=3000). The participants filled in a questionnaire on potential risk factors for venous thrombosis. For logistic reasons, blood sampling was performed for participants included up to June 2002.

All subjects provided informed consent. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands.

### Blood samples

Patients in the first half of the study, diagnosed before June 1, 2002 were invited, together with their partners as controls, to the anticoagulation clinic for a blood sample at least three months after discontinuation of the oral anticoagulant therapy. In those patients who continued to take oral anticoagulant therapy for more than one year after the event, blood was drawn during therapy. Blood was collected into tubes containing trisodium citrate 0.106 mol/L as previously described [22]. An additional control group was recruited from the population by RDD. DNA was obtained by standard methods and was available for 4485 patients and 4889 control subjects.

Total and free protein S were measured in plasma on a STA-R automated coagulation analyzer (Diagnostica Stago, Asnières, France). Total protein S was measured by an enzyme-linked immunosorbent assay (ELISA, Diagnostica Stago, Asnières, France) and free protein S was measured by an immune-turbidimetric method (Diagnostica Stago, Asnières, France), both accordingly to the manufacturer instructions.

## Cut off points

The range from 2.5th-97.5th percentile of both total and free protein S in control subjects who did not use vitamin K antagonists at time of blood sampling were considered as reference values. We also classified cut off points of protein S into different percentiles (i.e. 97.5-75th percentile as reference group compared with the 50th-75th percentile, 50th-25th percentile, 10th-25th percentile and <10th percentile of the control population), to see if this would change our results. Furthermore, a sensitivity analysis was performed in which very low protein S levels (i.e. less than 0.5th percentile) were compared with the 2.5th-97.5th percentile of (free) protein S. We did not include the 97.5-100th percentile in the reference population as other studies suggest that high levels protein S may be indicative of underlying comorbidity [23].

We also constructed cumulative distribution functions to visualize a possible difference between free and total protein S levels in patients versus controls. For these analyses we excluded patients and controls who used vitamin K antagonists at blood draw, were pregnant or used oral contraceptives and made a distinction between men, women, and between patients that had provoked or unprovoked venous thrombosis.

## Genetic analysis of PROS1

Copy number variations were analyzed by multiplex ligation-dependent probe amplification (MLPA) [24] using the SALSA MLPA KIT P112 *PROS1* (MRC-Holland, Amsterdam, The Netherlands) as previously described [11].

Exons and their flanking regions, and the 5' and 3' prime untranslated regions were re-sequenced using primers designed to avoid the amplification of the highly similar *PROSP*. After amplification, the PCR product was sequenced using an ABI Prism<sup>®</sup> 3730 DNA Analyzer (Applied Biosystems, Carlsbad, California, USA). Primers and PCR conditions are available on request. Results were analyzed using vector NTI<sup>®</sup> software version 10 (Invitrogen, Paisle, UK).

## **Statistical analysis**

With linear regression methods we estimated the effect of vitamin K antagonist use, estrogen use, pregnancy and puerperium on protein S levels. Based on previously reported associations [25], we also estimated the effect of obesity on protein S levels. Mean differences were adjusted for age and sex, as both age and sex influence protein S levels [26], and for each other in a multivariate analysis. Because there is no reason to assume that low levels of protein S are more common among partners of patients than in the general population, both RDD subjects and partners of patients were considered as control subjects and all analyses were unmatched.

Since protein S levels were collected after venous thrombosis, it is conceivable that the event itself influenced protein S levels ('reverse causation'). To analyze the possibility of such a post-hoc phenomenon, we performed a sensitivity analysis: scatter diagrams were constructed with regression lines, wherein protein S level was plotted against time between venous thrombosis and blood sampling (in patients not on vitamin K antagonists).

Odds ratios with 95% confidence intervals (CIs) were calculated, and were adjusted for age and sex by logistic regression methods. Since in the MEGA study cases were patients with a first venous thrombosis who were sampled from a stable, dynamic population, and since partner controls were matched on time to cases and since random controls were selected from the same population as the cases, these odds ratios can be interpreted as relative risks [27]. Subjects who used vitamin K antagonists at the time of the blood draw were excluded when obtaining relative risk estimates. A preplanned sensitivity analysis was performed in which we excluded estrogen users and pregnant women at the time of the blood draw (as estrogen use and pregnancy decrease protein S levels) [28, 29]. To further diminish the influence of estrogen related hormones to protein S levels, we restricted an analysis to men only. In addition, we assessed the risk of free and total protein S deficiency in individuals with a positive family history [30]. Stratified analyses were performed in which the common thrombophilias (i.e. blood group non-O, factor V Leiden and prothrombin G20210A) were compared with normal and low (free) protein S levels. By doing so, one can identify possible interaction or confounding of low free and total protein S levels to the risk of venous thrombosis by the common thrombophilias.

All statistical analyses were performed with SPSS for Windows, release 20.0 (SPSS Inc, Chicago, Ill).



## RESULTS

### General characteristics

Approximately 50% of patients and controls who were included in MEGA were tested for free and total protein S levels. No differences in clinical characteristics between tested participants and non-tested participants was observed, indicating that the tested individuals were representative of the whole of MEGA (Table 1). Of note, only 2331 patients were tested on free protein S while 2377 patients were tested on total protein S. Of controls, 2872 were tested on free protein S (n= 1479 partners and n= 1393 RDD controls) and 2940 on total protein S (n= 1481 partners and n=1459 controls). That some patients or controls were tested on total protein S only and not on free protein S was due to technical failures. Both total and free protein S levels were as expected reduced in vitamin K antagonist users, in pregnant women, and in women using estrogens (Table 2). Protein S levels were higher in overweight/ obese control subjects than in those with normal weight.

### Risk estimates

Subjects with low free protein S levels (<2.5<sup>th</sup> percentile, 53 U/dl) or low total protein S levels (<2.5<sup>th</sup> percentile, < 68 U/dl) were not at increased risk of venous thrombosis as compared with subjects with protein S levels in the 2.5<sup>th</sup>-97.5<sup>th</sup> percentile; odds ratio 0.82 (95% CI, 0.56-1.21) and 0.92 (95% CI, 0.63-1.33), respectively. Acquired conditions such as estrogen use, pregnancy or puerperium may transiently decrease protein S levels, which may have influenced these findings. We therefore repeated the analysis by excluding all women who used estrogens, were pregnant or in puerperium at time of venous thrombosis or at time of blood sampling. This adjustment increased the odds ratios to 1.51 (95% CI, 0.82-2.78) for subjects with low free protein S levels and to 1.34 (95% CI, 0.74-2.44) in subjects with low total protein S levels as compared with subjects with protein S levels in the 2.5<sup>th</sup>-97.5<sup>th</sup> percentile (Table 3). If protein S levels are somehow correlated with lifestyle, patients and partners may have been overmatched. To analyze if this occurred, we added analyses where patients were only compared with RDD controls. Risk estimates for the higher levels of free protein S were somewhat higher than when RDD and partner controls were combined. For the lower (free) protein S levels, however, risk estimates remained close to 1.0.

When we classified subjects into different percentiles (Table 4), again no increases in risks for venous thrombosis were observed for lower levels of free and total protein S.

When we restricted the analysis to subjects who did not change hormone use status between thrombosis and blood draw, i.e., either consistently used hormones at both time points, or did not use hormones at either time point, these odds ratios were 1.12 (95% CI, 0.69-1.81), and 0.83 (95% CI, 0.48-1.44), respectively. In men, the odds ratio for venous thrombosis in those with low free protein S was 2.15 (95% CI, 0.74-6.29). For total protein S levels, this odds ratio was 1.57 (95% CI, 0.70-3.49). Interestingly, subjects with free protein S levels who were >97.5<sup>th</sup> percentile appeared to be at increased risk of venous thrombosis (odds ratio 1.55; 95% CI, 1.14-2.11), which could only partly be attributed to overweight or obesity, as further adjustments of overweight or obesity attenuated this odds ratio only marginally to 1.48 (95% CI 1.07-2.04) (data not shown).

To analyze if our results were sensitive to the cut-off level of free or total protein S to identify inherited protein S deficiency, we subsequently compared decreasing cut-off values of free and total protein S levels, respectively, on the risk of venous thrombosis as compared to the reference group (subjects with protein S levels that were between the 2.5<sup>th</sup>-97.5<sup>th</sup> percentile). Although numbers became small, it appeared that a free protein S cut off level of  $\leq 0.10^{\text{th}}$  (free protein S < 33 U/dL) -  $0.20^{\text{th}}$  (free protein S < 34 U/dL) percentile could identify subjects at high risk of venous thrombosis (Figure 1A). Low, or even extremely low (<  $0.20^{\text{th}}$  percentile or total protein S < 53 U/dL) total protein S levels were not associated with venous thrombosis (Figure 1B). Due to small numbers, the latter analysis (performed in Figure 1) could not be performed in subjects that were not pregnant or were not using hormones.

As shown in the cumulative distribution functions, patients had as compared with controls, slightly higher free protein S levels (mean 100 U/dL in patients versus 94 U/dL in controls) and total protein S levels (mean 107 U/dL in patients versus 105 U/dL in controls) (Figure 2).

Increased risks for venous thrombosis in protein S deficient subjects are mainly reported from thrombophilic family studies. We therefore selected patients with a positive family history and repeated the analysis (Table 5). Again, no effects were apparent. These null findings could also not be explained by confounding of the common thrombophilias or by an interaction between the common thrombophilias and low levels of free and total protein S (Table 6).

Because vitamin K antagonist use was self-reported (and therefore we might have included subjects with low protein S levels that was due to unreported

vitamin K antagonist treatment), we performed an extra sensitivity analysis by looking at the distribution of the vitamin K dependent coagulant factors (i.e. factors II, VII, IX and X) in cases and control subjects that did not report to be on vitamin K antagonist treatment. All factors were normally distributed, and there were no apparent outliers in the lower range, suggesting that we did not include subjects with low protein S levels that was due to vitamin K antagonist treatment.

## Genetic analysis

### Copy number variation

First, we tested samples from 2270 consecutive MEGA subjects (1395 patients and 875 controls). An abnormal MLPA pattern was detected in only one individual and the test was repeated confirming the result. This individual was a female patient of 66 years old who was not using estrogens or vitamin K antagonists at the moment of thrombosis or sample collection. She had an unprovoked first venous thrombosis and her family history was not available. The patient was heterozygous for a complete deletion of *PROS1*. Her total protein S was 64 U/dL (< 2.5<sup>th</sup> percentile of the control group), and her free protein S was 22 U/dL (< 2.5<sup>th</sup> percentile of the control group).

The presence of only one copy number variation in 2270 samples shows that copy number variation of *PROS1* is at most a rare cause of venous thrombosis, and a rare finding in a normal population. To verify this, we zoomed in on potentially protein S deficient subjects by selecting DNA from all subjects with low (i.e. lower than the 2.5<sup>th</sup> percentile of the control group) total or low free protein S. Patients who used vitamin K-antagonists at the time of the blood draw were also included to guarantee that protein S deficient subjects were not missed. In total, 191 new samples were selected (84 patients and 107 controls), in which no new copy number variation of *PROS1* was identified.

## **Sequencing of PROS1**

To further investigate the molecular basis of protein S deficiency, we selected the first 48 subjects with the lowest levels of free protein S. All these subjects were below the 1st percentile from the controls (cutoff < 46 U/dL); of the 48 selected subjects 24 were patients and 24 were controls. For this analysis, patients using vitamin K antagonists were excluded.

Eleven non-synonymous variations were identified in ten subjects (5 patients and 5 controls); one of these subjects (subject 25) is carrier of two different variations. The variations identified included: 1 complete deletion, 1 indel, 7 missense and 1 nucleotide substitution in the regulatory 3'-untranslated region. Table 7 summarizes the results and gives references for variations previously described. Subjects are numbered according to increasing levels of free protein S. *PROS1* coding DNA reference sequence is NM\_000313.3 and protein reference sequence is NP\_000304.2.

Four variations were novel; subject 2 (patient) carried an indel mutation (Phe323fs) in the SHBG-like domain that is predicted to result in a truncated protein; subject 17 (patient) carried a 684C>G (Cys228Trp) substitution in the EGF-domain 3; subject 12 (control) carried a nucleotide substitution in the 3'UTR; and subject 45 (control) carried a 1095T>G (Asn365Lys) substitution in the SHBG-like domain.

A complete deletion of *PROS1* was detected in subject 4 (patient). Subject 6 is a patient who carried a 431C>A (Thr144Asn) substitution in the EGF-domain 1. Protein S Heerlen (Ser501Ala) was detected in two control subjects (subjects 25 and 32), and one carrier of protein S Heerlen (subject 25) carried also a second variation in the SHBG-like domain (13012T>C; Val434Ala). This variation is known as dbSNP (rs6803112) with a reported minor allele frequency (MAF) of 0.097. Subject 43 (control) carried a 698G>A (Arg233Lys) substitution, which is also listed in dbSNP (rs41267007, MAF 0.002). Finally, control subject 44 carried an 814G>A (Gly272Arg) variation in the EGF-domain 4. This variation is listed in dbSNP (rs41267005, MAF 0.002) but no information regarding protein S levels is available.

## DISCUSSION

Conflicting data have been reported regarding venous thrombotic risk in subjects with protein S deficiency within the community [2]. In the present study, subjects with low levels of free protein S had an increased risk of unprovoked venous thrombosis (odds ratio 2.31; 95% CI, 1.06-5.05). However, the prevalence of patients with such levels was low (n=8, or 0.4% of the total patient population), making it unpractical to use free protein S levels to help identify patients at risk in a clinical setting. The present study, confirms the results of a previous population based case-control study (Leiden Thrombophilia Study) [20], where only very low levels of free protein S (i.e. < 0.10<sup>th</sup> percentile or < 33 U/dL), but not of total protein S, were associated with an increased risk for venous thrombosis (odds ratio 5.44; 95% CI, 0.61-48.78). This increased risk at very low free protein S levels corresponds to some extent with findings from family studies of protein S deficiency on venous thrombosis risk (although relative risk estimates in family studies for first venous thrombosis in subjects with very low free protein S levels are even higher than 5) [12, 31], as well as the finding from family studies that showed that the cut off level of free protein S to identify true inherited protein S deficiency lies far below the commonly used normal range [12, 14]. Total protein S levels were not able to identify subjects at risk of venous thrombosis even when these levels were very low. This is in agreement with studies where free protein S was a better indicator of venous thrombosis risk than total protein S [32]. Nevertheless, as shown by its broad confidence interval, this analysis suffers from small sample size (n=7 of the patient population), indicating that even if very low free protein S levels are associated with venous thrombosis, large numbers of patients with venous thrombosis will need to be tested to identify only a few with a true free protein S deficiency. Such a strategy is unlikely to become clinically useful.

Many patients with venous thrombosis who are on anticoagulant treatment at the time of the blood sampling are identified with a protein S deficiency, which is thought to have clinical consequences [17, 33]. However, both our study and previous studies show reduced protein S levels in vitamin K antagonist and oral contraceptive users in the absence of a known true (genetic) deficiency [34, 35]. This reinforces the importance of avoiding the collection of blood for protein S measurement at moments when acquired deficiency can be present.

Molecular analysis of *PROS1* in 48 subjects with very low levels of protein S (below the 1<sup>st</sup> percentile) identified mutations in only ten subjects. This finding suggests that inherited protein S deficiency is rare in the general population and in patients with thrombosis. It is important to note, however,

that our analysis was limited to copy number variations, exons and their flanking regions, and the 5'- and 3'-UT regions. We cannot exclude that genetic variations, for example in introns or in promoter regions not covered in the present experiments, could play a role in protein S deficiency.

Mutations were equally distributed among patients and controls. However, three out of six patients with protein S levels below percentile 0.2 carried mutations that are likely to be detrimental (complete deletion of *PROS1*, 967delTinsGG and Thr144Asn). In contrast, mutations in controls were likely not to be detrimental. Protein S Heerlen (known to be associated with type III protein S deficiency) was detected in two controls. Another control carried a variation previously described as a neutral polymorphism (Arg233Lys) and a final control carried a variation in the 3'-UT region.

*PROS1* deletion was present in only one patient, showing that gross copy number variation of *PROS1* is rare in the general population.

Some aspects of our study need additional comment. First, we have noted that high levels of free protein S were associated with an increased risk of venous thrombosis in our study. Interestingly, high levels of free protein S have been associated with an increased risk of coronary heart disease in another study [36]. Free protein S has been described as a semi-delayed acute phase reactant [19, 37] and protein S levels have been described to be higher, on average, in overweight/obese control subjects than in controls subjects with normal weight [25]. Obesity is associated with a chronic inflammatory status and with increased risk for venous thrombosis [38]. It is possible that high levels of free protein S are a consequence of the former. However, adjustment for overweight and obesity only attenuated the risk of venous thrombosis associated with high levels of free protein S, suggesting that other mechanisms could play a role.

Second, our study is a case-control study in which the blood sample was collected after the thrombotic event. Therefore, we cannot exclude the possibility that differences in plasma levels of protein S in patients and control subjects were the result of the thrombotic event itself. However, the blood draw was performed at least 3 months after the thrombotic event, and it is unlikely that the thrombotic event itself caused persistent abnormalities in protein S levels. Furthermore, no differences in protein S levels were observed between patients in whom blood was drawn 3-6 months after their thrombotic event compared with those in whom the blood draw took place later (e.g. more than 6-9, 9-12 or >12 months).

Third, we only had one measurement of free and total protein S. This may have led to misclassification of protein S deficiency when protein S levels fluctuate due to transient acquired conditions. However, only use of female hormones and vitamin K antagonists are known determinants of protein S

levels. Sensitivity analyses in which oral contraceptives, and pregnant or puerperic women were excluded did not materially affect our risk estimates for total protein S, but did slightly for free protein S. Moreover subjects who were still on vitamin K antagonists at time of blood draw were excluded from analyses where relative risk estimates were determined. Finally, qualitative analysis of protein S was not performed, as the laboratory analysis of protein S activity levels is known to produce spurious false low levels. Therefore, we may have missed individuals with type II protein S deficiency [39].

In summary, low free protein S and low total protein S levels could at best rarely identify subjects at risk for venous thrombosis in a population based study. Only when cut off levels for free protein S were far below the normal range or when unprovoked venous thrombosis was considered as outcome event, we found a 2- to 5-fold increased risk of venous thrombosis, which is far below the relative risk estimates that are obtained for free protein S in family based studies. Protein S testing and subsequent testing on *PROS1* mutations should therefore not be considered in unselected patients with venous thrombosis.

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Table 1. Clinical characteristics

	Patients in MEGA (N=4956)	Patients tested (N=2377)	Controls in MEGA (N=6297)	Controls tested (N=2940)
Male	2274 (46)	1083 (46)	2914 (46)	1393 (47)
Age at enrollment	49 (13)	48 (13)	47 (13)	48 (12)
Vitamin K antagonist use†	294 (6)	288 (12)	34 (0.5)	30 (1.0)
Estrogen use (women)	1384 (54)	739 (58)	934 (29)	412 (27)
Pregnancy/puerperium (women)	183 (7)	94 (7)	87 (3)	29 (2)
Normal weight (BMI 18.5-25 kg/m <sup>2</sup> )	1634 (37)	818 (37)	2805 (49)	1354 (48)
Overweight (BMI 25-30 kg/m <sup>2</sup> )	1834 (42)	965 (43)	2112 (37)	1109 (39)
Obesity (BMI ≥ 30 kg/m <sup>2</sup> )	907 (21)	456 (20)	763 (13)	365 (13)

Continuous variables denoted as mean (SD), categorical data as count (%).

Data were missing for some participants in some subgroups

\* There were slightly less patients (n=2331) and controls (n=2872) tested on free protein S due to sampling errors.

† At time of blood draw.

Table 2. Association of protein S with venous thrombotic risk factors in control subjects

	Free protein S			Total protein S		
	Subjects	Mean difference	Mean difference†	Subjects	Mean difference	Mean difference†
	(N)	(U/dL)	(95% CI)	(N)	(U/dL)	(95% CI)
No vitamin K antagonists	2858	92	Reference	2910	102	Reference
Vitamin K antagonists	14	44	-50 (-61 to -39)	30	62	-37 (-48 to -28)
Non-estrogen users (women)	1067	88	Reference	1097	105	Reference
Estrogen users (women)	408	79	-4 (-6 to -1)	412	89	-9 (-12 to -7)
No pregnancy/puerperium (women)	1473	86	Reference	1505	101	Reference
Pregnancy/puerperium (women)	28	70	-6 (-13 to 2)	29	89	1 (-6 to 8)
Normal weight	1332	87	Reference	1354	98	Reference
Overweight	1072	96	6 (4 to 7)	1109	105	5 (4 to 7)
Obesity	358	97	8 (6 to 11)	365	108	8 (6 to 10)

\* Adjusted for age and sex where applicable.

† Adjusted for age, sex (where applicable), and the effects of all other factors in the table.

Table 3. Risk of venous thrombosis for high or low levels of protein S

Total	Patients, n (%)	Controls, n (%)	Odds ratio (95% CI)*	
			Overall	Overall min†
<b>Free protein S antigen</b>				
> 97.5th percentile (> 139 U/dL)	88 (4.3)	83 (2.9)	1.55 (1.14-2.11)	1.81 (1.31-2.50)
97.5th-2.5th percentile (53 - 139 U/dL)	1937 (94)	2705 (95)	Reference	Reference
< 2.5th percentile (< 53 U/dL)	44 (2.1)	70 (2.4)	0.82 (0.56-1.21)	1.51 (0.82-2.78)
<b>Total protein S antigen</b>				
> 97.5th percentile (> 146 U/dL)	42 (2.0)	72 (2.5)	0.82 (0.56-1.21)	1.00 (0.66-1.53)
97.5th-2.5th percentile (68 - 146 U/dL)	1997 (96)	2766 (95)	Reference	Reference
< 2.5th percentile (< 68 U/dL)	50 (2.4)	72 (2.5)	0.92 (0.63-1.33)	1.34 (0.74-2.44)
<b>Unprovoked‡</b>				
<b>Free protein S antigen</b>				
> 97.5th percentile (> 139 U/dL)	33 (6)	83 (2.9)	1.74 (1.13-2.67)	1.72 (1.12-2.65)
97.5th-2.5th percentile (53 - 139 U/dL)	504 (92)	2705 (95)	Reference	Reference
< 2.5th percentile (< 53 U/dL)	8 (1.5)	70 (2.4)	1.77 (0.84-3.71)	2.31 (1.06-5.05)
<b>Total protein S antigen</b>				
> 97.5th percentile (> 146 U/dL)	16 (2.9)	72 (2.5)	1.21 (0.70-2.10)	1.15 (0.66-2.00)
97.5th-2.5th percentile (68 - 146 U/dL)	524 (94)	2766 (95)	Reference	Reference
< 2.5th percentile (< 68 U/dL)	19 (3.4)	72 (2.5)	1.19 (0.55-2.57)	1.59 (0.71-3.58)
<b>Provoked‡</b>				
<b>Free protein S antigen</b>				
> 97.5th percentile (> 139 U/dL)	55 (3.8)	83 (2.9)	1.61 (1.12-2.30)	1.91 (1.30-2.80)
97.5th-2.5th percentile (53 - 139 U/dL)	1390 (94)	2705 (95)	Reference	Reference
< 2.5th percentile (< 53 U/dL)	35 (2.4)	70 (2.4)	0.69 (0.45-1.05)	1.17 (0.53-2.58)
<b>Total protein S antigen</b>				
> 97.5th percentile (> 146 U/dL)	26 (1.7)	72 (2.5)	0.74 (0.47-1.18)	0.95 (0.56-1.59)
97.5th-2.5th percentile (68 - 146 U/dL)	1431 (96)	2766 (95)	Reference	Reference
< 2.5th percentile (< 68 U/dL)	39 (2.6)	72 (2.5)	0.78 (0.52-1.17)	1.14 (0.54-2.43)

\* Adjusted for age and sex

† Estrogen users or pregnancy/puerperium

‡ Minus estrogen users or pregnancy/puerperium. Control group confined to random digit dialing subjects only.

§ Provoked venous thrombosis classified as thrombosis due to trauma, plaster cast use, surgery,

pregnancy/puerperium, or immobilization, within three months prior to the index date,

> 4 hours travelling by air, train, bus, or car in the 8 weeks before the index date,

estrogen/progestagen use at time of the index date, and diagnosis of malignancy within five years

Table 4. Risk of venous thrombosis for strata of protein S levels

Measurement	Patients, n (%)	Controls, n (%)	Total	
			Overall	Odds ratio (95% CI)*
<b>Free protein S antigen</b>				
97.5th-75th percentile (105 - 139 U/dL)	511 (25.8)	617 (22.2)	Reference	Reference
50th-75th percentile (91 - 105 U/dL)	528 (26.7)	692 (25.0)	0.85 (0.72-1.00)	0.77 (0.64-0.92)
25th-50th percentile (77 - 91 U/dL)	507 (25.6)	738 (26.6)	0.81 (0.69-0.96)	0.70 (0.57-0.85)
10-25th percentile (77 - 66 U/dL)	291 (14.7)	449 (16.1)	0.75 (0.61-0.92)	0.55 (0.43-0.72)
< 10th percentile (< 66 U/dL)	144 (7.2)	279 (10.0)	0.61 (0.45-0.85)	0.53 (0.33-0.83)
<b>Total protein S antigen</b>				
97.5th-75th percentile (115 - 146 U/dL)	498 (24.3)	653 (23.0)	Reference	Reference
50th-75th percentile (101 - 115 U/dL)	573 (28.0)	720 (25.4)	1.02 (0.86-1.19)	0.99 (0.82-1.19)
25th-50th percentile (89 - 101 U/dL)	500 (24.4)	723 (25.5)	0.85 (0.72-1.01)	0.73 (0.60-0.89)
10-25th percentile (78 - 89 U/dL)	296 (14.5)	470 (16.6)	0.79 (0.65-0.97)	0.68 (0.53-0.88)
< 10th percentile (< 78 U/dL)	180 (8.8)	272 (9.6)	0.86 (0.63-1.16)	0.78 (0.51-1.20)

\* Adjusted for age and sex

† Estrogen users or pregnancy/puerperium

**Table 5. Risk of venous thrombosis for high or low levels of protein S, according to family history**

	Patients, n	Controls, n	Odds ratio (95% CI)*	
			Overall	Overall minus†
<b>Negative family history</b>				
<b>Free protein S antigen</b>				
97.5th-2.5th percentile (53 - 139 U/dL)	936	2705	Reference	Reference
< 2.5th percentile (< 53 U/dL)	24	70	0.75 (0.46-1.21)	1.95 (0.93-4.05)
<b>Total protein S antigen</b>				
97.5th-2.5th percentile (68 - 146 U/dL)	964	2766	Reference	Reference
< 2.5th percentile (< 68 U/dL)	21	72	0.65 (0.40-1.07)	1.41 (0.66-3.00)
<b>Positive family history</b>				
<b>Free protein S antigen</b>				
97.5th-2.5th percentile (53 - 139 U/dL)	524	2705	Reference	Reference
< 2.5th percentile (< 53 U/dL)	10	70	0.86 (0.44-1.70)	0.92 (0.27-3.08)
<b>Total protein S antigen</b>				
97.5th-2.5th percentile (68 - 146 U/dL)	540	2766	Reference	Reference
< 2.5th percentile (< 68 U/dL)	12	72	1.00 (0.53-1.86)	1.08 (0.37-3.15)

\* Adjusted for age and sex

† Estrogen users or pregnancy/puerperium



Table 6. Risk of venous thrombosis for low levels of protein S, stratified analysis with common thrombophilias

		Patients, n (%)		Controls, n (%)		Odds ratio (95% CI)*
<b>Free protein S antigen</b>						
<b>Blood group non-O</b>						
97.5th-2.5th percentile (53 - 139 U/dL)	Absent	556 (28.1)	1270 (45.9)	Reference		
< 2.5th percentile (< 53 U/dL)	Absent	13 (0.7)	30 (1.1)	0.93 (0.48-1.79)		
97.5th-2.5th percentile (53 - 139 U/dL)	Present	1378 (69.7)	1429 (51.6)	2.21 (1.95-2.50)		
< 2.5th percentile (< 53 U/dL)	Present	31 (1.6)	39 (1.4)	1.71 (1.05-2.78)		
<b>Total protein S antigen</b>						
<b>Blood group non-O</b>						
97.5th-2.5th percentile (68 - 146 U/dL)	Absent	575 (28.1)	1300 (45.9)	Reference		
< 2.5th percentile (< 68 U/dL)	Absent	13 (0.6)	32 (1.1)	0.86 (0.45-1.65)		
97.5th-2.5th percentile (68 - 146 U/dL)	Present	1418 (69.4)	1459 (51.5)	2.20 (1.95-2.49)		
< 2.5th percentile (< 68 U/dL)	Present	37 (1.8)	40 (1.4)	2.00 (1.27-3.18)		
<b>Free protein S antigen</b>						
<b>Factor V Leiden</b>						
97.5th-2.5th percentile (53 - 139 U/dL)	Absent	1633 (82.5)	2578 (92.9)	Reference		
< 2.5th percentile (< 53 U/dL)	Absent	42 (2.1)	66 (2.4)	0.94 (0.63-1.40)		
97.5th-2.5th percentile (53 - 139 U/dL)	Present	303 (15.3)	127 (4.6)	3.79 (3.05-4.71)		
< 2.5th percentile (< 53 U/dL)	Present	2 (0.1)	4 (0.1)	0.79 (0.14-4.33)		
<b>Total protein S antigen</b>						
<b>Factor V Leiden</b>						
97.5th-2.5th percentile (68 - 146 U/dL)	Absent	1689 (82.6)	2630 (92.7)	Reference		
< 2.5th percentile (< 68 U/dL)	Absent	44 (2.2)	69 (2.4)	0.94 (0.64-1.39)		
97.5th-2.5th percentile (68 - 146 U/dL)	Present	307 (15.0)	136 (4.8)	3.35 (2.86-4.37)		
< 2.5th percentile (< 68 U/dL)	Present	6 (0.3)	3 (0.1)	2.99 (0.75-12.0)		
<b>Free protein S antigen</b>						
<b>Prothrombin G20210A</b>						
97.5th-2.5th percentile (53 - 139 U/dL)	Absent	1843 (93.0)	2658 (95.8)	Reference		
< 2.5th percentile (< 53 U/dL)	Absent	42 (2.2)	69 (2.5)	0.84 (0.57-1.24)		
97.5th-2.5th percentile (53 - 139 U/dL)	Present	94 (4.7)	47 (1.7)	2.87 (2.01-4.09)		
< 2.5th percentile (< 53 U/dL)	Present	1 (0.1)	1 (0.0)	1.46 (0.09-23.4)		
<b>Total protein S antigen</b>						
<b>Prothrombin G20210A</b>						
97.5th-2.5th percentile (68 - 146 U/dL)	Absent	1905 (93.1)	2718 (95.8)	Reference		
< 2.5th percentile (< 68 U/dL)	Absent	48 (2.3)	70 (2.5)	0.93 (0.61-1.35)		
97.5th-2.5th percentile (68 - 146 U/dL)	Present	92 (4.5)	48 (1.7)	2.73 (1.92-3.89)		
< 2.5th percentile (< 68 U/dL)	Present	2 (0.1)	2 (0.1)	1.37 (0.19-9.78)		

\* Adjusted for age and sex

Table 7. Molecular analysis of PROS1 in subjects with free protein S level below 1<sup>st</sup> percentile, as determined in MEGA controls

Subject number*	Venous thrombosis	fPS (U/dL)	tPS (U/dL)	Sequence change†	Predicted protein change‡	Reference	Comments
1	NO	16	123	-	-	-	-
2	YES	18	63	967delTinsGG	Phe323fs	-	NOVEL
3	YES	18	68	-	-	-	-
4	YES	22	64	(?-133)_(+1_?)del	Complete deletion	5,10,11	-
5	YES	30	74	-	-	-	-
6	YES	30	58	431C>A	Thr144Asn	40	Associated with quantitative deficiency of protein S
7	NO	31	54	-	-	-	-
8	NO	32	50	-	-	-	-
9	YES	33	78	-	-	-	-
10	YES	33	88	-	-	-	-
11	NO	34	63	-	-	-	-
12	NO	34	90	*370G>A	-	-	3'UTR. NOVEL
13	NO	34	66	-	-	-	-
14	YES	34	53	-	-	-	-
15	NO	34	99	-	-	-	-
16	YES	36	63	-	-	-	-
17	YES	37	66	684C>G	Cys228Ttp	-	NOVEL
18	NO	37	57	-	-	-	-
19	YES	38	60	-	-	-	-
20	NO	39	96	-	-	-	-
21	YES	39	81	-	-	-	-
22	NO	39	65	-	-	-	-
23	YES	40	70	-	-	-	-
24	YES	40	58	-	-	-	-
25	NO	40	74	1301T>C	Val434Ala	-	rs6803112
25	NO	40	74	1501T>G	Ser501Ala	23	Protein S Heerlen
26	YES	40	76	-	-	-	-
27	NO	40	81	-	-	-	-

Subject number*	Venous thrombosis	fPS (U/dL)	tPS (U/dL)	Sequence change†	Predicted protein change‡	Reference	Comments
28	NO	40	57	-	-	-	-
29	NO	41	76	-	-	-	-
30	YES	41	61	-	-	-	-
31	YES	41	53	-	-	-	-
32	NO	41	81	1501T>G	Ser501Ala	23	Protein S Heerlen
33	NO	41	82	-	-	-	-
34	YES	42	69	-	-	-	-
35	YES	42	81	-	-	-	-
36	NO	42	59	-	-	-	-
37	YES	42	43	-	-	-	-
38	NO	42	96	-	-	-	-
39	YES	43	74	-	-	-	-
40	YES	43	77	-	-	-	-
41	NO	43	62	-	-	-	-
42	NO	43	132	-	-	-	-
43	NO	43	60	698G>A	Arg233Lys	7,41,42	rs41267007 Probably neutral polymorphism
44	YES	43	60	814G>A	Gly272Arg	-	rs41267005
45	NO	44	61	1095T>G	Asn365Lys	-	NOVEL
46	NO	44	63	-	-	-	-
47	NO	45	88	-	-	-	-
48	YES	45	77	-	-	-	-

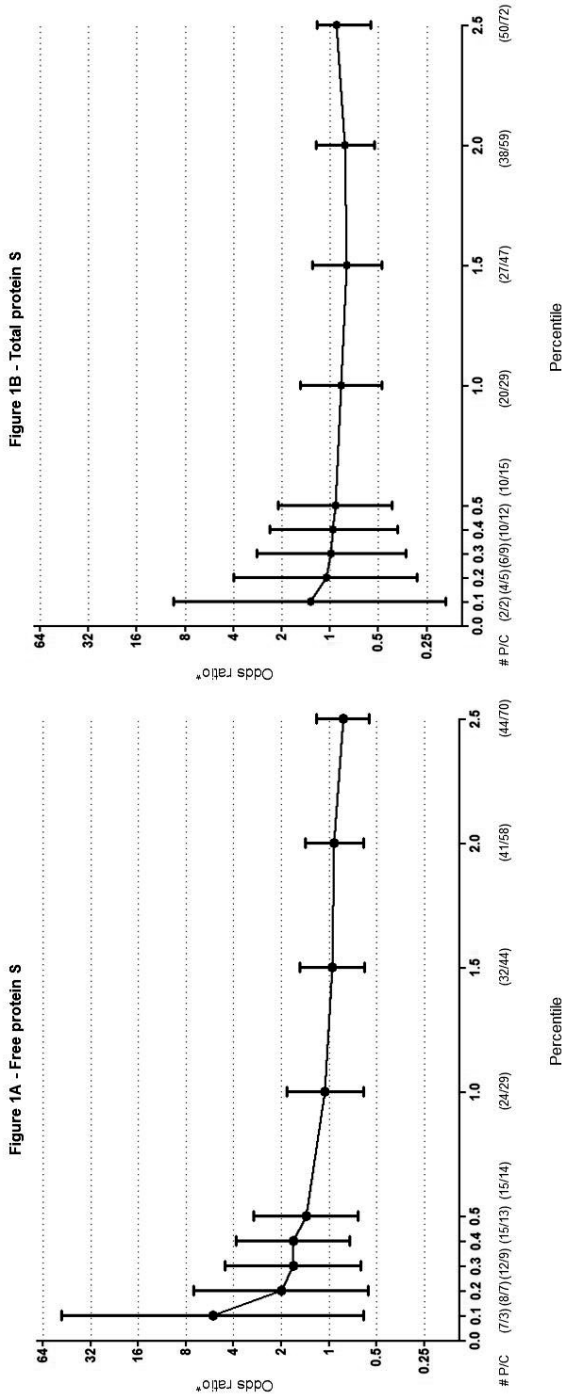
Mutations are described accordingly to the Human Genome Variation Society.<sup>43</sup>

fPS indicates free protein S; tPS indicates total protein S; MAF indicates minor allele frequency.

\*Numbers are accordingly to increasing levels of free protein S. †PROS1 coding DNA reference sequence is NM\_000313.3.

‡Protein reference sequence is NP\_000304.2.

Figure 1. Risk of venous thrombosis for low free and total protein S levels



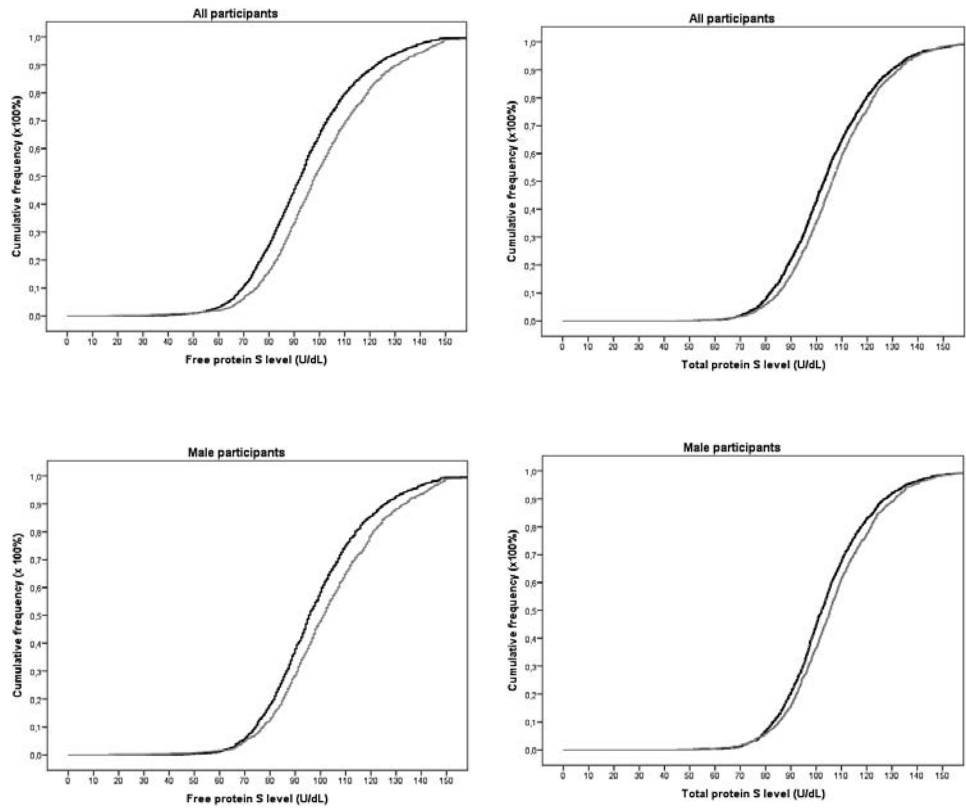
All odds ratios are compared to free or total protein S levels between 2.5th-97.5th percentile

I denotes 95% confidence interval.

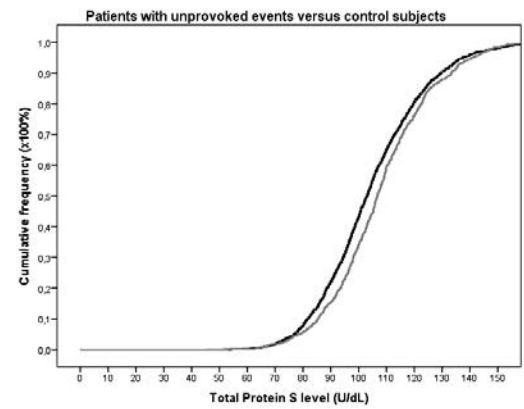
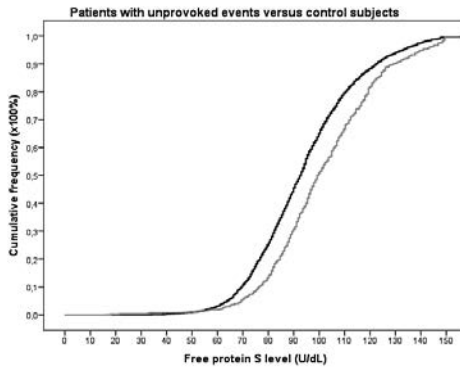
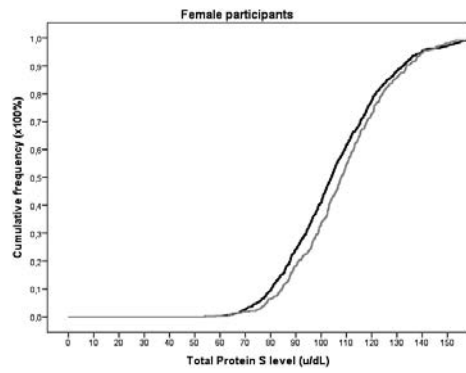
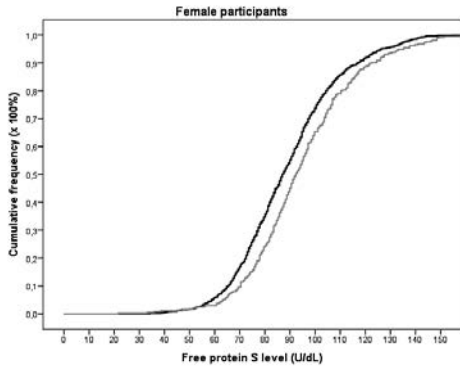
\* Adjusted for age and sex.

# P denotes number of patients and C number of controls per percentile

**Figure 2. Cumulative distribution functions of free and total protein S levels in patients and controls**



*Protein S levels and the risk of venous thrombosis:  
results from the MEGA case-control study*



Grey lines indicate patients, black lines controls.



*Part* ***II***

*Risk factors for recurrent  
venous thrombosis*



