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Protein C and protein S deficiencies: similarities and differences between two brothers playing in the same game

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Abstract

Protein C (PC) and protein S (PS) are vitamin K-dependent glycoproteins that play an important role in the regulation of blood coagulation as natural anticoagulants. PC is activated by thrombin and the resulting activated PC (APC) inactivates membrane-bound activated factor VIII and factor V. The free form of PS is an important cofactor of APC. Deficiencies in these proteins lead to an increased risk of venous thromboembolism; a few reports have also associated these deficiencies with arterial diseases. The degree of risk and the prevalence of PC and PS deficiency among patients with thrombosis and in those in the general population have been examined by several population studies with conflicting results, primarily due to methodological variability. The molecular genetic background of PC and PS deficiencies is heterogeneous. Most of the mutations cause type I deficiency (quantitative disorder). Type II deficiency (dysfunctional molecule) is diagnosed in approximately 5%–15% of cases. The diagnosis of PC and PS deficiencies is challenging; functional tests are influenced by several pre-analytical and analytical factors, and the diagnosis using molecular genetics also has special difficulties. Large gene segment deletions often remain undetected by DNA sequencing methods. The presence of the PS pseudogene makes genetic diagnosis even more complicated.
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Introduction

Venous thromboembolism (deep vein thrombosis and/or pulmonary embolism, VTE) and its consequences occur with high frequency Western societies. VTE is still a major cause of morbidity and mortality during pregnancy and stillbirth, and occurs with a relatively high frequency in young women using oral contraceptives. VTE can be recurrent and may also lead to post-thrombotic syndrome, a chronic disease with disabling pain and ulceration.

VTE is a typical example of common complex diseases; both acquired (environmental) and genetic causes play an important role in the development of the disease (1). Over the last decades, several genetic risk factors for VTE have been identified. Loss of function mutations in different components of the natural anticoagulant system lead to antithrombin III (ATIII), protein C (PC) and protein S (PS) deficiencies, while gain of function mutations in the genes of coagulation factors, such as Factor V Leiden (FVL) and prothrombin 20210A allele are responsible for the majority of inherited thrombophilia. The aim of this review is to give an overview of the physiology of PC and PS, on the molecular basis of their deficiencies and on the laboratory diagnosis of these disorders including the difficulties and challenges in this field.

The role of protein C-protein S system in the regulation of coagulation

PC and PS play important roles in the regulation of blood coagulation as natural anticoagulants (2, 3). PC is activated by thrombin in the presence of thrombomodulin (TM). TM is an endothelial cell surface protein, and upon binding, thrombin becomes a potent activator of PC. Endothelial protein C receptor (EPCR) also is highly important in the activation process; EPCR binds PC through its Gla-domain and presents it to the thrombin-TM complex. Thrombin cleaves the activation peptide domain of PC at Arg169 resulting in a 12-amino acid long activation peptide being released from the N-terminal end of its heavy chain. Activated PC (APC) inactivates membrane-bound active factor VIII (FVIIIa) and factor V (FVa) by cleaving these factors specific arginine residues. FVa is cleaved at Arg506, which is the preferred cleavage site. However, full inactivation of FVa also requires cleavage at Arg306. Cleavage at Arg679 seems unimportant for inactivation of FVa. FVIIIa is cleaved at Arg336 and Arg562. Non-activated forms of FVIII and FV are poor substrates for APC. Esmon and co-workers showed that the membrane phospholipid requirement for the anticoagulant APC complex differs from that of the procoagulant complexes (4). Phosphatidyl ethanolamine, instead of phosphatidyl serine, is required for the binding of the APC complex to the membrane of endothelial cells. APC can also cleave intact FV at Arg506, which makes FV a cofactor of APC in inactivating FVIIIa. The main inhibitor of APC is protein C

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inhibitor, a single chain glycoprotein serine protease inhibitor synthesized in the liver. The inhibitor forms a 1:1 complex with APC and is cleaved at the reactive site (Arg354). APC is also inhibited by α-1 antitrypsin.

In addition to its anticoagulant function, PC plays an important role in cytoprotection, which has been partially clarified in the last decade. The multiple cytoprotective effects of APC is not the subject of this review, it has been reviewed by Mosnier et al. (5).

The free form of PS (described later) is an important cofactor of APC, enhancing its affinity to negative charged phospholipid surfaces. PS is able to displace FXa from its complex with FVa, allowing APC to cleave FVa at Arg506. In the process of FVIIIa inactivation APC activity is synergistically stimulated by PS and the non-activated form of FV, while in the process of FVa inactivation, free PS is the only cofactor of APC. PS also forms a complex with the complement 4b binding protein (C4bBP); this complex lacks APC cofactor activity.

PS also has direct, APC-independent effects (6, 7). PS binds to FVa present on phospholipid vesicles, and inhibits prothrombinase activity by competing with prothrombin for binding to FVa. Binding of PS to FXa has also been demonstrated. APC-independent PS function does not seem to be restricted to the free form; C4bBP-complexed PS has the same activity. A direct interaction of PS with tissue factor pathway inhibitor (TFPI) in the inhibition of FXa was recently reported (8).

Protein and gene structure of protein C

PC is a vitamin K-dependent glycoprotein synthesized by the liver as a single chain protein. It exists in the plasma as a precursor of a serine protease at a concentration of 3–5 mg/L (9). Its half-life is short, approximately 8 h in the circulation. The mature 62 kDa protein is composed of a heavy (41 kDa) and a light (21 kDa) chain, these chains are held together by a single disulfide bond between Cys141 and Cys265. The domain structure of PC shows high similarity to other vitamin K-dependent proteins. It has a pre-pro leader sequence (numbered as –42 to –1 by the traditional numbering system, where the first methionine corresponds to –42), which is required for γ-carboxylation of glutamic acid residues in the Gla-domain and for secretion. The mature protein contains a Gla-domain (amino acids 1–37) with the nine glutamic acid residues that are carboxylated during post-translational maturation. A short amphipathic helix (amino acids 38–45) connects the Gla-domain to the first epidermal growth factor (EGF) domain (amino acids 46–91). The second EGF domain (amino acids 92–136) is followed by the activation peptide domain (amino acids 137–184). This region contains the Lys156–Arg157 dipeptide that is released upon maturation, and the cleavage site for thrombin activation (Arg169). The heavy chain of PC contains the activation peptide and the catalytic domain (amino acids 185–419).

The gene for human PC (*PROC*) is located at the 2q13–q14 position and contains nine exons encoding for a 1.7-kb messenger RNA (mRNA) and eight introns (10, 11). All the exon/intron boundaries follow the GT-AG rule. Exon 1 is a non-coding exon and a long intron separates it from the initiator ATG codon; this phenomenon is unique among the vitamin K dependent factors. The PROC contains two Alu repeats in intron 5. The major transcriptional start site is located 1515 base pairs upstream from the initiator ATG codon. Two minor transcription start sites were also recognized at –7 and +13 bp relative to the major start site. The regulation of transcription was extensively studied. Cis-elements within PROC are the HNF-1, HNF-3 and Sp1 binding sites. A strong silencer region and two liver specific enhancer regions have also been described.

Protein and gene structure of protein S

PS is a vitamin K-dependent single-chain 71 kDa glycoprotein. It is synthesized primarily in the liver. However, significant amount of PS are also produced by endothelial cells and megakaryocytes (12). Its plasma concentration is 20–25 mg/L and PS circulates with a half-life of 42 h. Its domain structure is different from other vitamin K dependent proteins. The pre-pro leader sequence (numbered as –41 to –1), the Gla-domain (amino acids 1–36) with 11 glutamic acid residues and a short amphipathic helix (amino acids 37–46) also exists in other vitamin K dependent hemostatic proteins. In addition PS contains unique domains, a thrombin cleavage sensitive loop formed from 24 residues, four EGF domains, and a huge sex hormone domain with the binding site for the complement regulator C4bBP. About two third of PS circulates in complex with C4bBP. In this complex, PS is not a cofactor of APC (13).

The gene for human PS (*PROS1*) is located at position 3q11.2 and contains 15 exons producing a 3.5-kb messenger RNA (mRNA) and 14 introns (10). All exon/intron boundaries follow the AG/GT rule. Exon 1 encodes for the first part of the pre-pro leader sequence, exon 2 codes for the second part of the leader sequence and the Gla-domain. Exon 3 encodes for the short hydrophobic region and exon 4 codes for the thrombin sensitive region. Exons 5–8 encode for the four EGF domains. Exons 9–15 code for the very large C4bBP-binding (sex homone binding) domain. Six Alu repeats are located within the *PROS1* gene. In addition to the active gene, a pseudogene of PS (*PROS2*) has also been discovered. This inactive gene shows 97% homology to the active gene, but lacks exon 1 and contains multiple base changes. The presence of *PROS2* makes the molecular genetic diagnosis of hereditary PS deficiency a difficult task (see later).

Protein C and S deficiencies: epidemiological aspects and clinical symptoms

The first patient with PC deficiency was described by Griffin et al. in 1981 (14). PS deficiency was first reported in 1984 by Comp and Esmon (15). Both cases were associated with

recurrent venous thromboembolism. Since then, a large number of deficient patients have been identified and the underlying genetic defects have been clarified in a number of cases (see later). Soon afterwards it was revealed that PC and PS deficiencies are associated with increased thrombotic risk. The degree of the risk and the prevalence of PC and PS deficiencies among patients with thrombosis and in the general population have been examined in several population studies, with conflicting results (16–29).

The estimated prevalence of PC deficiency in the general population is surprisingly high (0.4%) (18, 19). The prevalence of PS deficiency seems to be less, but data are uncertain, which is due, at least in part, to difficulties in the laboratory diagnosis. In a Scottish study, it was within a range of 0.03%–0.13% (20), while PS deficiency was shown to be more prevalent in a general Japanese population (1%–2%) (21).

Among 163 randomly selected patients with lower extremity venous thrombosis (LEVT), 2%–4% of the patients suffered from PC or PS deficiency (22). Interestingly in the same study, when patients with confirmed cerebral venous sinus thrombosis were examined (n = 163), no PC deficiency was found, while the prevalence of PS deficiency did not differ significantly from that of the LEVT group. In an Italian cohort, the prevalence of PC and PS deficiency among patients with first time VTE was found to be 4.7% and 3.7%, respectively (23). PC or PS deficiency was diagnosed in 3% and 2% of patients with proximal deep vein thrombosis, respectively (n = 920) (24).

According to the results of the Leiden Thrombophilia Study, one of the first case-control studies on this topic, the risk of first VTE in PC deficiency was 3-fold, while in PS deficiency, no significant risk was demonstrated in unselected patients (25). In other studies, a 3–11-fold risk of VTE was demonstrated in PC or PS deficiency. The results depended on the selection of patients, including ethnicity, study design, and the methods for determining PC and PS activity and concentration. The annual incidence of recurrent VTE were 6.0% for PC deficiency and 8.4% for PS deficiency (23, 26). In the large prospective EPCOT study (European Prospective Cohort on Thrombophilia), the risk of first VTE in asymptomatic relatives of patients with confirmed thrombophilia was investigated (n = 575). During the 5.7 years of follow-up, 4.5% of individuals developed VTE. The annual incidence of VTE in PC (0.7%) and PS (0.8%) deficiency was higher than in cases of FVL, and thrombosis developed at a mean age of 40 (27). Putting the results of all epidemiological studies together, one can conclude that the risk of developing thrombosis among individuals with genetic defect in *PROC* or *PROS1* varies considerably in the various studies that have been preformed. In addition to methodological variability, this could be due to gene-gene or gene-environment interactions, many of which have not yet been discovered (30, 31).

Symptoms of PC or PS deficiencies are deep venous thrombosis and/or pulmonary embolism in early adulthood, which is often recurrent. Thrombosis might also develop at unusual sites, such as the proximal extremities and in mesenterial and cerebral veins. Intracardial thrombus was reported in a 2-year-old child having inherited PC deficiency (32), and intracardial multichamber thrombi were identified in a middle aged patient with combined PC and PS deficiency (33). Pregnancy associated thrombosis has also been reported and PS deficiency was also found in the background of late fetal loss (34). In severe PC and PS deficiency when plasma PC or PS concentrations are extremely low, severe thrombosis develops in newborns, frequently in disseminated form, named purpura fulminans (35). Warfarin-induced skin necrosis is a severe complication of PC or PS deficient patients receiving vitamin K antagonist treatment.

In addition to venous thrombosis, patients with PC or PS deficiency can also suffer from thrombotic complications of arterial origin (36). In addition to isolated case reports (37–39), a large cohort of relatives of VTE patients with PC, PS or ATIII deficiency (n = 468) had a higher incidence of arterial thrombosis compared to subjects who were not deficient. The risk of arterial thrombosis was especially high in individuals < 55 years of age; adjusted hazard ratios for PC and PS deficiencies were 6.9 (95% CI, 2.1–22.2) and 4.6 (95% CI, 1.1–18.3), respectively (40). In the ARIC cohort which enrolled more than 13,000 patients with coronary events or ischemic stroke, and had a follow-up time of almost 17 years, low PC concentrations were associated with the development of stroke (41). A Japanese study demonstrated that patients with PC deficiency were 10 years younger at the onset of myocardial or cerebral infarction compared with those without deficiency (16). A meta-analysis of studies involving children with arterial ischemic stroke calculated an odds ratios of 8.46 for PC and 3.20 for PS deficiency (42). These findings suggest the importance of screening for inherited thrombophilia, primarily PC and PS deficiencies in young patients with arterial thrombotic events, especially in those without any other obvious risk factor.

Molecular genetic background of protein C and S deficiency, genotype-phenotype correlations

Protein C deficiency

PC deficiency is classified as type I (quantitative) and type II (qualitative) deficiency. In type I deficiency, PC activity and the antigen concentration are decreased equally, suggesting defective synthesis or secretion of the protein, while in type II deficiency, the activity is decreased without a significant decrease in antigen concentrations. The latter type could be due to abnormalities in substrate, calcium-ion or receptor binding. The inheritance of PC deficiency is not as clear as was first thought. It may show an autosomal recessive or dominant inheritance, often with incomplete penetrance.

The majority of PC deficient patients are heterozygous for the defect, with typical PC activity values between 30% and 65%. Homozygous or compound heterozygous patients often have undetectable PC concentration and/or activity and exhibit life-threatening thrombosis very early in life. The molecular genetic background of PC deficiency is hetero-

geneous. Most of the mutations cause type I deficiency, type II deficiency is diagnosed in approximately 10%–15% of cases. Summary reports of mutations leading to decreased PC concentrations were first described in 1995 (43, 44). To date, approximately 250 causative mutations have been published, which are collected in different databases (http://www.hgmd.cf.ac.uk and http://www.isth.org) (Figure 1). A recently developed mutation database, ProCMD, is an interactive tool that contains phenotypic descriptions with functional and structural data obtained by molecular modeling (47).

Most of the mutations causing type I deficiency are single nucleotide substitutions within the coding region of PROC, leading to amino acid changes (approx. 70%). If performed, molecular modeling studies, in almost all cases, suggested misfolding and instability of the mutant proteins as a consequence. In vitro expression studies were performed in approximately one third of the cases only. Point mutations introducing a stop codon were also reported (approx. 5%). A smaller number of the point mutations (approx. 9% of all mutations) were found at the exon/intron boundaries leading to splicing defects. Most of the small deletions (approx. 8%) or insertions (approx. 4%) introduced frameshifts, resulting in a premature stop codon and truncated protein. Gross deletions were identified in only 1% of cases. Although almost all missense mutations result in an absolute block in secretion, a few mutations allow the protein to be secreted. However, the rate of secretion is much lower when compared to the wild type protein (48). In homozygous patients having such mutations, PC concentrations are low, but higher than 1%.

Diagnosis of type II deficiency is based on the discrepancy between the results of functional testing and antigen measurements (see later for details). Missense mutations are the most frequently reported types; the resulting amino acid change involving the Gla-domain or the pro-peptide result in defective calcium and phospholipid binding (49–57). Mutations in the serine protease domain result in defective protease activity or decreased substrate binding (58–60).

Of interest there are mutations that are enriched in certain populations, suggesting the presence of a founder effect. For example, all Finnish type II protein C deficient cases show a single mutation (p.W380G) (61). The p.R147W mutation within PROC is common in Taiwanese Chinese patients with VTE (62). Five frequent mutations account for almost 50% of all PC deficiencies in patients from Japan (c.1268delG, p.F139V, p.R211W, p.V339M and p.M406I) (63). A common ancestor was identified for probands with the p.R306X mutation in the Dutch population (64). The c.3363insC mutation was introduced by French settlers to North America (65).

No mutation in the PROC is detectable in 10%–30% of families with PC deficiency (66). This does not necessarily mean that PROC lacks causative mutations in these cases. The larger gene deletions may remain undetectable when using the classical Sanger (i.e., chain termination) sequencing method for detection of mutations. There are known polymorphisms within the *PROC* gene which may affect measured PC activity or antigen concentrations (see later for details). This may be regulated by loci other than PROC. As part of the GAIT project, a genome-wide linkage study was performed to localize genes influencing variations in PC plasma concentrations. A region flanked by microsatellite markers D16S3106 and D16S516 on chromosome 16 (16q22–23) with one candidate gene was identified as a major quantitative trait locus influencing variation in PC concentrations. This gene encodes a quinone reductase, NADPH:menadione oxidoreductase 1 (NQO1), involved in vitamin K metabolism. The association of this locus with other vitamin K dependent factor concentrations was also demonstrated, however, the linkage was not as strong as in the case of PC (67).

Protein S deficiency

Initially three types of PS deficiency were distinguished according to the results of the functional test, and free and total PS antigen concentration measurements. In type I deficiency, a low amount of activity, total and free PS antigen concentrations can be found. In type II deficiency, only the result of the functional test is abnormal, while in type III deficiency, low PS activity is associated with low free PS but normal total PS antigen concentrations. Based on extensive family studies, it was suggested that type I and type III deficiencies are phenotypic variants of the same genetic alteration (68). The molecular background of this finding was that C4BbP and PS could bind to each other with very high affinity. Therefore, in the case of mild PS deficiency, the complexed form of PS is not decreased (69). Later, genetic differences between type I and type III PS deficiency was demonstrated, and no linkage to the *PROS1* locus was found in most of the patients having type III deficiency. In families having the *PROS1* mutation, the phenotype more often shows type I rather than type III deficiency. These findings led to the conclusion that type I PS deficiency is a monogenic disease caused by *PROS1* mutations, while type III PS deficiency is more complex or heterogeneous disorder (70, 71).

The majority of protein S-deficient patients are heterozygous for an inherited defect, and homozygous or compound heterozygous deficiency can cause the same symptoms as in the case of PC deficiency. The molecular genetic background of PS deficiency is also heterogeneous (72). Most of the mutations cause type I deficiency, type II deficiency is diagnosed in approximately 5% of cases. The mutations are listed in the HGMD and in the ISTH databases (http://www.hgmd.cf.ac.uk and http://www.isth.org) (Figure 2). Among the 200 different mutations found to date, missense (approx. 53%) mutations are the most frequent. Approximately 20% of the mutations are small deletions or insertions; non-sense and splice-site mutations are present in approximately 14% and 10% of the cases, respectively. Type II PS deficiency is caused by missense mutations affecting the Gla-domain or EGF4 domain (73). Gross deletions are more frequent than in the case of PC deficiency, they comprise approximately 3% of all cases and are associated with quantitative PS deficiency. However, it is very likely that the number of large

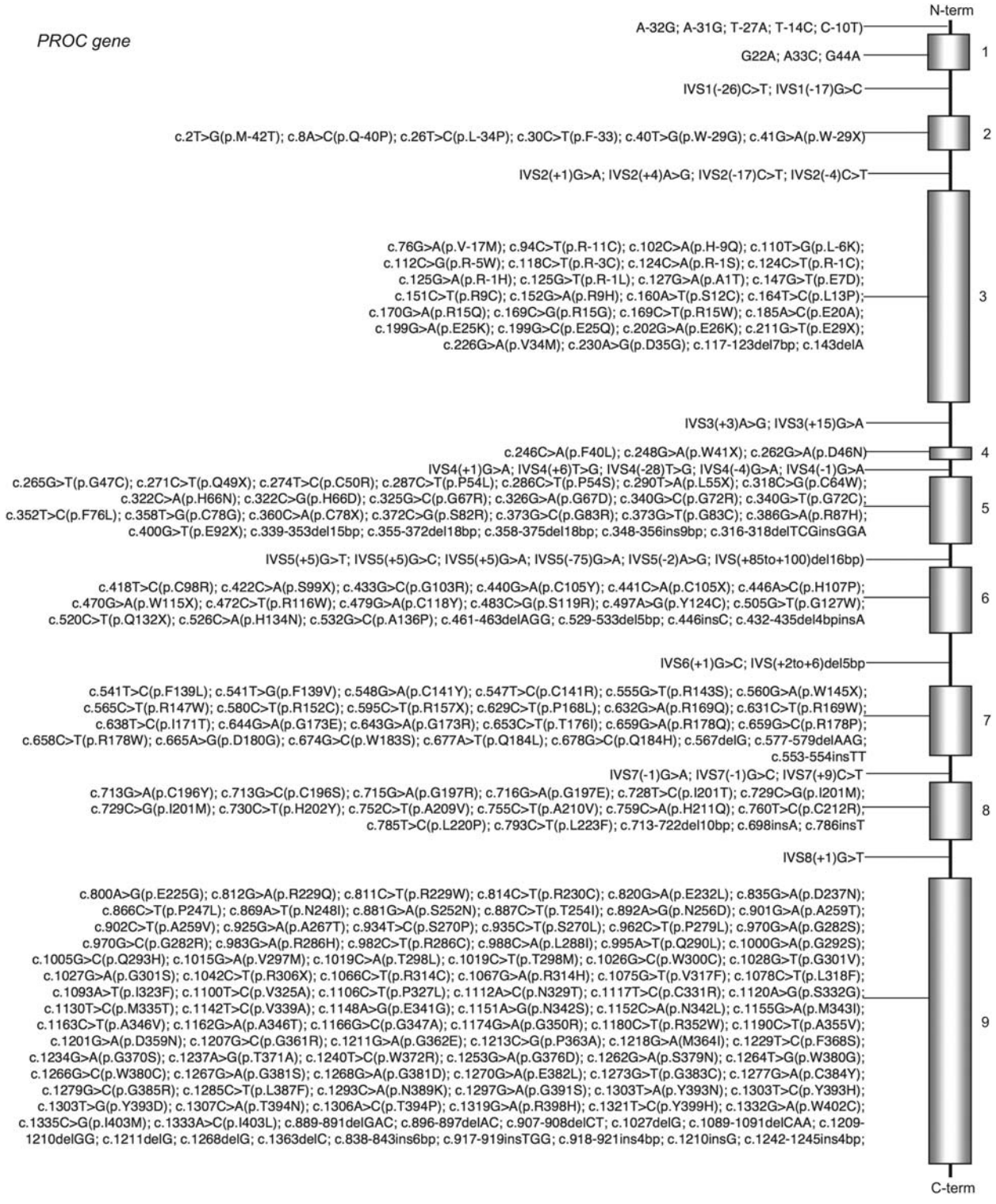

Figure 1 Distribution of causative mutations published to date within the *PROC* gene according to the HGMD (www.hgmd.cf.ac.uk) database.

Nucleotide numbering in exon 1 and nearby is given relative to the first nucleotide of the non-coding exon 1. Nucleotide numbering in the coding region is given relative to the first nucleic acid of the initiator ATG codon. Amino acids are numbered according to the mature protein, where the first methionine is numbered as –42. The literature also mentions two gross deletions: one includes the entire gene, the other includes exons 1–9 (45, 46) (not shown in the Figure.).

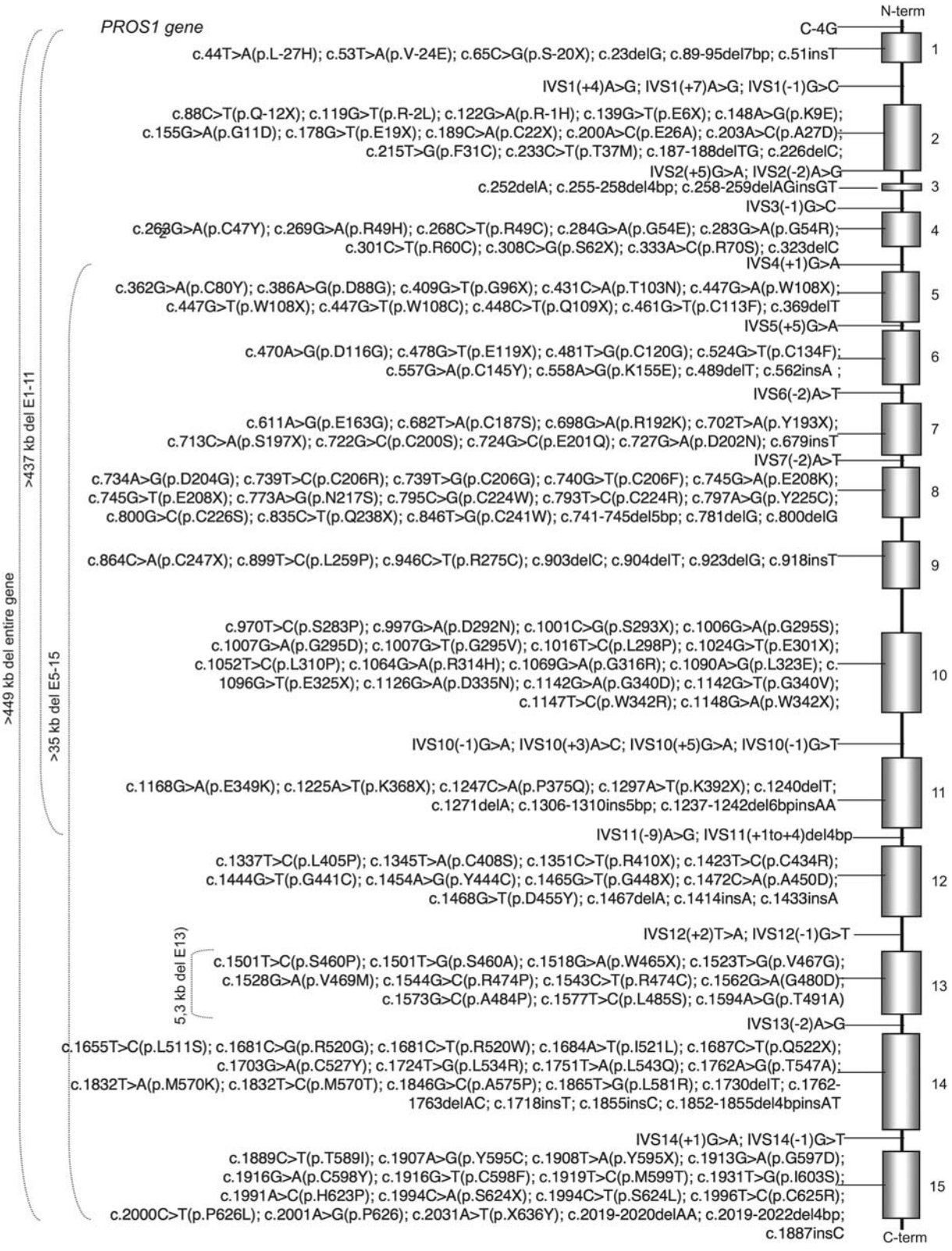

Figure 2 Distribution of causative mutations published to date within the *PROS1* gene according to the HGMD (www.hgmd.cf.ac.uk) database.

Nucleotide numbering is given relative to the first nucleic acid of the initiator ATG codon. Amino acids are numbered according to the mature protein, where the first methionine is numbered –41.

deletions in PROS1 is even higher, but often remains undetected. In the PROSIT study, mutations in PROS1 were found only in 70% of probands with PS deficiency (17). However, using DNA sequencing methods, large deletions or gene segment duplications may remain undetected (74, 75). Moreover, there are mutations affecting the transcription regulatory sequences at the 5' of the gene (76, 77). Most recently, the first case of PS deficiency due to chromosome translocation has been reported. The diagnosis was established by painting fluorescence in situ hybridization (78). Although a founder effect is not confirmed, it is to be noted that PS Tokushima (p.K155E) shows a high prevalence in the Japanese population.

Polymorphisms in the protein C and S gene

It has been reported that individuals with the homozygous C/G/T haplotype at the nucleotide positions -1654 (rs1799808), -1641 (rs1799809), and -1476 (rs1799810) in the promoter region of the PROC had lower plasma PC concentrations compared to individuals with the T/A/A homozygous haplotype (79). The -1654/-1641 CC/GG genotype was associated with a slightly increased risk of thrombosis (OR, 1.39, 95% CI, 1.04–1.87) (80). In a large population-based case-control study, individuals having this genotype had the lowest plasma PC concentration, and the highest risk for venous thrombosis (OR, 1.27, 95% CI, 1.09–1.48) compared with individuals having the TT/AA genotype (81). In contrast to these findings, variation at the PROC structural locus did not influence plasma PC concentrations in the GAIT project, but the chromosome region 16q22-23 was found to be a major determinant (67). Polymorphisms in genes involved in the vitamin K dependent γ-carboxylation of PC and PS may also be responsible for the inter-individual variation in the plasma concentrations of these proteins in the general population (82).

PS Heerlen (p.S460P) results in the loss of N-glycosylation at Asn458. The concentration of free PS in the plasma of carriers was slightly lower than that of non-carriers and was considered to be a type III PS deficiency. However, the risk of thrombosis conferred by this mutation is a matter of debate. PS Heerlen displayed reduced anticoagulant activity as cofactor to APC in plasma based assays, as well as when using a FVIIIa degradation system. In a purified system using recombinant proteins, PS Heerlen was a good cofactor of APC in the degradation of normal FVa, but became a poor cofactor in the degradation of FVa carrying the Leiden mutation (83). This suggested a synergistic contribution between FV Leiden and PS Heerlen that increases the risk of thrombosis. However, this hypothesis was not confirmed by another study. The cause of the decrease in free PS concentrations associated with PS Heerlen has not been clarified, but most likely is a consequence of increased clearance (84).

A transition of adenine to guanine transition at nt 2148 (p.P626P, silent) and an A to C substitution at nt 2698 have been suggested to decrease PS concentration in healthy individuals (85). However, this finding was not confirmed in another study (86). No decrease in the secretion of p.P626P variant was demonstrated in an in vitro expression system; this variant was not a risk factor for VTE and did not modify the risk of patients with causative mutations (87).

Laboratory tests of protein C deficiency

Two different types of assays are available for the diagnosis and classification of PC deficiency, functional tests and antigen assays. For screening, a functional test should be performed, and if the results are abnormal, the antigen assay can distinguish between type I and type II deficiencies, with concentrations of antigen being normal in the latter.

There are two different methods for determination of PC activity (Figure 3A). In both assays, PC present in patient plasma is activated by the venom of Agkistrodon contortrix, now commercially available under the trade name Protac. The advantage of Protac is its insensitivity to plasma protease inhibitors. Also, it can be added directly to plasma. Protac activated PC can be measured either using a chromogenic assay or a clotting assay. In chromogenic assays, paranitroaniline (pNA) is cleaved-off from a small synthetic peptide by APC. Peptide bound pNA does not absorb light at 405 nm, while the liberated chromogenic compound has an intense color at this wavelength. The spectrophotometric measurement can be either end-point or kinetic, with the latter being preferred. The higher the APC activity, the more intense the increase in absorbance during the test.

The rationale of the clotting time based assays is the fact that if APC degrades its natural substrates FVa and FVIIIa, it leads to clotting time prolongation. Determination of clotting time can be based on the prothrombin time, activated thromboplastin time (APTT) or Russell viper venom time (RVV), and there is a linear relationship between PC activity and clotting time.

There are numerous advantages and disadvantages of both functional assays (88) (Table 1). Clotting tests are influenced by several pre-analytical or analytical variables. Despite predilution of the sample with PC deficient plasma, in the presence of lupus anticoagulant, heparin or direct thrombin (or factor Xa) inhibitor which prolongs the clotting time, falsely increased PC activity can be measured (89). Interference by heparin (up to 1–2 U/mL) is eliminated by adding a heparin-neutralizing substance, e.g., hexadimethrine bromide (polybrene) to the reagent. The interference caused by lupus anticoagulant is more pronounced in tests using APTT as activator (90). In the case of increased Factor VIII, the opposite effect might be seen; shortening the clotting time (APTT) will lead to falsely decreased PC activity (91). The most important problem with the clotting method is the influence of the FV Leiden mutation. In patients having this mutation, falsely low PC activity could be detected despite pre-dilution of patient plasma with PC deficient plasma containing wild type FV. If PC antigen concentrations are normal, antigen measurements are not influenced by the mutation; such patients are easily misdiagnosed as having type II PC deficiency (92, 93).

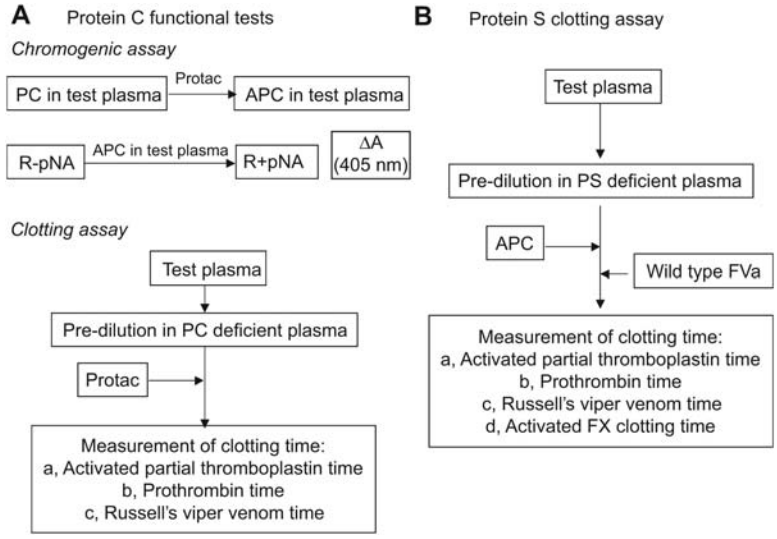

Figure 3 Schematic presentations of protein C and protein S functional assays.
PC, protein C; PS, protein S; APC, activated protein C; R-pNA, chromogenic substrate containing oligopeptide (R) and p-nitroaniline (pNA); ΔA, difference in absorbance.

Chromogenic assays are not sensitive to high FVIII, lupus anticoagulant or FV Leiden. They show lower inter-laboratory and intra-laboratory variation (94). There are two major limitations in the chromogenic assays; the chromogenic peptide substrates do not have exclusive specificity for APC and may overestimate PC in the presence of other proteolytic enzymes, such as plasmin, kallicrein, and thrombin which also cleave the chromogenic substrate(s). The second problem is that chromogenic assays are insensitive to a certain type of qualitative PC deficiency. When the mutation affects

Table 1 The most important difficulties in the diagnosis of protein C and protein S deficiency.

Analytical/methodical problems	Protein C	Protein S
Functional clotting assays		
Lupus anticoagulant	Overestimation	
High heparin concentration	Overestimation	
Direct thrombin (or FXa) inhibitor	Overestimation	
High FVIII level (usually >250%)	Underestimation	
FV Leiden mutation	Underestimation	
Functional amidolytic assay		
Presence of enzymes cleaving the chromogenic substrate	Overestimation	NA
Mutations result in altered γ-carboxylation, or phospholipid binding	Normal result despite genetic defect	NA
Problems with molecular genetic diagnosis	Large gene segment alterations are not diagnosed by the DNA sequencing method	
	NA	Presence of the pseudogene (PROSP) causes difficulties
Physiological conditions which influence PC-PS levels		
Pregnancy	Significant elevation in the first 22 weeks of pregnancy	Decreased
Oral anticoncipients, hormonal replacement therapy	–	Decreased
Infants	Levels are significantly lower than adult values at birth and infancy	
Age	–	Increases with age
Gender	–	Lower in women
Acquired deficiency		
Vitamin K antagonist therapy	Decreased	
Hepatic disease	Decreased	
Consumption (DIC, VTE)	Decreased	
Presence of autoantibodies (SLE, varicella, malignancies, sepsis, HIV)	Decreased	

the Gla-domain or the propeptide, the functional clotting test gives a low value for activity, while the amidolytic (chromogenic) assay shows normal result. Mutations in the propeptide affecting the γ-carboxylation process, substitution of a Gla residue, or mutations influencing phospholipid binding decrease PC activity as measured using the clotting test, while amidolytic activity remains unaltered (49–54). In general, PC deficiency caused by mutations in the serine protease domain can be diagnosed using both clotting and amidolytic assays. However, some mutations in this region (p.R229Q and p.S252N) result in abnormalities that can only be demonstrated with the clotting assay. It is estimated that approximately 5% of patients with type II PC deficiency have normal amidolytic activity, and the deficiency is detectable only by clotting assays.

Initially PC antigen was measured using electroimmunoassay. However, this assay is now considered obsolete and no longer routinely used. Most laboratories perform commercially available ELISA testing using pairs of monoclonal or polyclonal antibodies against PC.

Laboratory tests of protein S deficiency

Three types of assays are available for the determination of plasma PS: the functional PS activity assay, free and total PS antigen determinations. The test for PS activity measures the effect of PS as a cofactor on the degradation of FVa and FVIIIa by APC. Such clotting tests are the function of free PS concentrations; they are not influenced by PS in complex with C4bBP (Figure 3B). Commercially available tests use thromboplastin, APTT, RVV or FXa for initiation of coagulation. APC is added to patient or control plasma that has been pre-diluted with PS deficient plasma. Following this, the clotting time test is performed. The effect of APC on clotting time, i.e., the extent that the clotting time is prolonged, depends on the content of the cofactor PS in the plasma to be investigated. PS activity assays have a number of limitations (Table 1). First, in patients having FV Leiden mutation, significantly lower PS activity is measured, the results often overlap with the range for true PS deficiency. Such situations may lead to an incorrect diagnosis of type II PS deficiency (95). In the majority of assays, purified FVa is added to the test. This step decreases somewhat the interference from FV Leiden, but does not eliminate the problem completely. Different kits give highly variable results; they have different cut-off values and most of them are very sensitive to reagent handling. Pre-analytical variables are similar to those described for PC activity measurements; high FVIII may lead to underestimation, while the presence of lupus anticoagulant may lead to overestimation of PS activity. Plasma samples are sensitive to repeated freezing and thawing; in vitro activation of FVII may shorten the clotting time in assays using thromboplastin as activator, resulting in underestimation of PS activity.

Commercially available PS activity assays give the correct diagnosis of PS deficiency in 97% of patients having PROS1 mutations (96). However, the specificity of the functional tests is low due to the above-mentioned interfering factors that might lead to inappropriate interpretations of test results, resulting in a false-positive diagnosis. For this reason, some authors do not recommend the use of PS activity assays for diagnosis of PS deficiency, and instead favor free PS antigen (see below) determinations (97, 98). However, by omitting the functional assay, type II deficient patients would remain undiagnosed. Therefore, it has been suggested that both activity and free antigen assays be perfomed from the same sample (99).

For the measurement of total PS concentrations, ELISA is the most frequently used method. Measurement of free PS antigen was originally performed from the supernatant following precipitation of C4bBP-bound PS by polyethylene glycol. This method was time consuming and poorly reproducible. Later, monoclonal antibodies against the C4bBP binding domain of PS were produced and measurement of free PS antigen became easier and faster using ELISA assays. Further development led to the introduction of latex enhanced immunoassays (LIA) which were easily adapted to automated coagulometers (100). In the latest ECAT exercise, 77% of laboratories used a LIA method for free PS antigen measurements. In addition to monoclonal antibodies specific for free PS, a ligand binding assay has also been developed. In this assay, C4bBP is used to capture free PS from the plasma (101). Assays for the free form of PS are preferred over total PS determinations since this has higher positive predictive value for PS deficiency (68, 69). In the latest exercise of the European Concerted Action on Thrombosis (ECAT) thrombophilia testing program, only 89 laboratories reported total PS antigen results and 225 laboratories performed free PS antigen measurements.

Conditions affecting protein C and protein S levels; acquired deficiencies

Adult reference intervals for PC and PS are wide, and there may be overlap between values seen in healthy individuals and deficient patients (102). In infants, both PC and PS concentrations are lower than adult values. In a healthy full term infant, PC activity is 35% (17%–53%), PS activity is 36% (24%–48%); these reach the lower limit of the adult reference interval by 1 year of age. The PC concentration may remain below the adult reference interval until adolescence (103–106). It should also be noted that PS concentrations are influenced by age and gender; lower results are obtained in women compared with men, and PS values increase with age. PS concentrations may decrease markedly during pregnancy, to a mean level of 46%, and to a lesser extent in individuals using oral contraceptives or on hormone replacement therapy (107, 108). PS measurements in pregnant women can only be used as a test for exclusion, decreased PS concentrations cannot be considered as being deficient. During the first 22 weeks of pregnancy, PC concentrations show a significant increase. It has been postulated that this increase may play a role in maintaining early pregnancy by regulating both coagulation and inflammation (107).

Treatment of patients with vitamin K antagonist (VKA) therapy influences plasma PC and PS activity and antigen concentrations. Activities of PC and PS are markedly decreased and, depending on the assay, antigen may also be lower. Using typical therapeutic doses of VKA, PC antigen and activity decreases to approximately 50% and 25%, respectively (109). Patients should not receive VKA therapy for at least 10 days prior to testing, they should be switched to low molecular weight heparin therapy until collecting the sample for PC and PS measurements. As the half-life of PC is much more shorter than that of PS, it decreases faster following the initiation of VKA therapy and recovers more rapidly following discontinuation. Abnormal γ-carboxylation due to vitamin K deficiency also results in decreased PC and PS activity and antigen concentrations.

PC and PS deficiency can develop with DIC, severe infection, sepsis and acute excessive thrombosis due to consumption (110, 111). Decreased synthesis of PC and PS can be a consequence of liver disease or immaturity of the liver in preterm infants. Autoimmune syndromes can also be associated with acquired PC and PS deficiency due to the presence of autoantibodies. Postvaricella purpura fulminans is a rare complication in children caused by acquired PC or PS deficiency (112). PS deficiency has also been described in patients with AIDS (113, 114). Therapy with L-asparaginase may lead to decreased PC concentrations by decreasing its synthesis in the liver. Patients having nephrotic syndrome may also exhibit low PS concentrations.

Molecular genetic diagnosis of protein C and protein S deficiencies

Since both PC and PS deficiencies may be acquired. Prior to suggesting a genetic defect, all the possible acquired conditions must be excluded. Equivocal cases require confirming the presence of a true inherited deficiency using mutation analysis. As multiple sites of mutation have already been described in *PROC* and *PROS1* genes, and since no so-called hot spot could be identified within these genes, DNA sequencing is the most reliable method for establishing a genetic diagnosis.

The molecular genetic diagnosis of PS deficiency represents a particularly difficult situation. The presence of the PS pseudogene makes the genetic diagnosis of PS deficiency rather complicated; careful design of primers are required to eliminate the amplification of pseudogene fragments. In a high number of individuals with PS deficiency, no mutation was found when using DNA sequencing. This discrepancy is due to larger gene alterations that are not diagnosed by this method (66, 75, 115). A recently developed and commercially available method for demonstrating large gene segment deletions or duplications is the multiplex ligation-dependent probe amplification (MLPA) method. Re-analysis of DNA samples from ''mutation negative'' PS deficient patients using this method has revealed large deletions or duplications in a number of cases (74). The presence of such larger gene alterations should be confirmed by other methods, such as quantitative PCR or long PCR.

Concluding remarks

The diagnosis of PC and PS deficiency is not an easy task; the functional tests are influenced by several pre-analytical and analytical factors, and the molecular genetic diagnosis is also challenging. In the case of PC, the use of the chromogenic or the clotting functional test as screening tests is a matter of debate. A clinical guideline most recently issued by UK-based medical experts recommends the chromogenic PC assay as being the preferred test (116). In our experience, only the use of both chromogenic and clotting tests could cover the full range of PC deficiencies and reduce the problems arising from interfering conditions. In the diagnosis of PS deficiency, if a PS activity assay is used for initial screening, low results should be further investigated using a immunoreactive assay for free PS. Acquired deficiencies should be considered and looked for when establishing the diagnosis. In all cases, repeat testing is crucial for establishing the diagnosis. If the results of functional and antigenic assays do not confirm the diagnosis unequivocally, genetic testing is indicated.

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