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Scientific and Standardization Committee Communication
Protein S Deficiency: A Database of Mutations - FIRST UPDATE

For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis

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Introduction

A protein S mutation database was collated and published in 1997 by investigators working on the molecular bases of protein S deficiency (1). The intention was to provide an up-to-date source of published and unpublished mutations of the protein S gene, together with structural and functional data allowing mutations to be rapidly located in the protein sequence and to understand the functional defects associated with some of them.

Protein S deficiency is an autosomal dominant trait affecting up to 10% of families with congenital thrombophilia. Since heterozygous subjects belonging to deficient families are at risk for venous thromboembolic disease, it is crucial to establish the diagnosis unequivocally.

However, many issues remain to be clarified in the pathophysiology of protein S deficiency. Around 60% of circulating protein S forms a complex with C4b-binding protein (C4b-BP). While a role for protein S in the protein C/thrombomodulin anticoagulant pathway is now well established, there are many uncertainties relating to the activated protein C independent

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functions of this protein, diagnosis of deficiency states, the expression of its phenotype in deficiency, and regulation of the gene.

The first database comprised 126 mutations postulated to be detrimental and 19 mutations that were considered as neutral polymorphisms. The present database contains the mutations identified in 200 PS-deficient patients, of which 78 are new potentially detrimental mutations and 7 are new polymorphisms (two of which are frequent). In addition, several mechanisms leading to free protein S deficiency have been described since the first database was published and are included in this update.

Many uncertainties remain to be settled, and more information is needed to understand the PS-deficiency phenotype observed in some patients.

Functions and structure of protein S

Protein S is a vitamin K-dependent protein which plays an important role in the regulation of coagulation. It is synthesized by the hepatocytes (2), endothelial cells (3, 4), megakaryocytes (5, 6), human testis Leydig cells (7) and brain (8). Plasma concentration of protein S is approximately 25 mg/L (0.33 µM). The protein functions as a non-enzymatic cofactor to activated protein C (aPC), a serine protease, in the proteolytic degradation of factors Va and VIIIa (9-13).

Protein S increases 10-fold the affinity of aPC for negatively charged phospholipids (10, 11). The two proteins form a putative 1:1 complex on lipid surfaces such as platelets or platelet microparticles (14-18). Protein S has been shown to relocate the active site of membrane-bound aPC (19). A 20-fold enhancement of factor Va cleavage after Arg 306 by aPC has been observed in the presence of PS (13).

In purified systems, protein S exhibits a direct aPC-independent anticoagulant activity by inhibition of prothrombinase activity (20-22) and of factor X activating complex by binding to factor VIII (23). The importance of these properties in physiological anticoagulant mechanisms remains to be demonstrated.

Protein S circulates in human plasma in two forms, free (≈ 40%) and bound to a regulator of the classical complement pathway, C4b-BP (24). Only the free protein S has cofactor activity for aPC (25). C4b-BP is a multimeric protein composed of 6 to 7 identical 70 kD α-chains with or without a 45 kD β-chain (26, 27, for review see 28); its plasma concentration is about 150 mg/L (0.26 µM). Interaction between protein S and C4b-BP is non-covalent and reversible (29-31). Protein S interacts with the β-chain (26), while the α-chains are devoted to interaction with the complement protein C4b (32). Thus, only C4b-BP isoforms containing a β-chain (α7β1 and α6β1=C4b-BP β+), representing 80% of circulating C4b-BP (27), are able to bind protein S. In

the presence of calcium ions, the dissociation constant is approximately $5x10^{-10}$M (29, 30, 33, 34), and all C4b-BP β+ is linked to protein S. In healthy individuals, the concentration of free protein S is largely determined by the concentration of C4b-BP ß+ and corresponds to the molar excess of protein S over C4b-BP β+ (35, 36).

Protein S in its mature form is a single-chain glycoprotein of 635 amino acids resulting from post-translational modifications of a 676 amino acid precursor (37-39). It contains three glycosylation sites (Asn 458, 468 and 489) and it is composed of 7 domains with functional or structural roles. The signal peptide (residues -41 to -18), containing a hydrophobic sequence, targets the protein translocation into the rough endoplasmic reticulum; the propeptide (residues -17 to -1) is necessary for carboxylase recognition and γ-carboxylation. These two domains are released by a cleavage reaction before secretion (37-40). The mature N-terminal part of the protein is composed of a GLA-domain (residues 1 to 37) containing 11 γ-carboxyglutamic acids which binds multiple calcium ions. The resulting stabilized structure has a high affinity for negatively charged phospholipid membranes (34, 41, 42). The GLA-domain is followed by a short helical stack (residues 38 to 45) with a relatively high content of aromatic residues. Whereas the above domains are present in all vitamin K-dependent proteins, another domain,the thrombin-sensitive region (residues 46 to 72), is found only in protein S. This domain contains two Cys residues (47 and 72) linked by a disulphide bridge. In this loop, three peptide bonds are sensitive to thrombin proteolysis (43, 44) and it has recently been shown that circulating cleaved protein S is cleaved after Arg 60 (45), a site already described as sensitive to factor Xa cleavage (46). Whatever the enzyme responsible for the *in vivo* cleavage of protein S, the GLA-domain remains linked to the rest of the molecule by the disulphide bond. However, since the GLA domain can no longer adopt the calcium-dependent conformation required for biological activity, protein S cannot bind to phospholipids at physiological calcium ion concentration, and aPC cofactor activity is lost (47, 48). These findings suggest that the thrombin-sensitive loop interacts with aPC and is involved in GLA-domain folding (49). Four epidermal growth factor-like domains (residues 76 to 242) are adjacent to the thrombin-sensitive region. EGF1 contains a ß-hydroxylated Asp residue while the other three contain ß-hydroxylated Asn. The EGF domains contain high-affinity calcium ion binding sites. The carboxy-terminal half of protein S is a large module homologous to sex hormone binding globulin (50-52). It contains two small disulphide loops formed by internal disulphide bonds. This module does not bind steroids, but contains at least two potential interaction sites with C4b-BP: residues 420 to 433 and 583 to 635 (31, 53-56).

Difficulties in crystallizing protein S have hindered studies at the atomic level. However, a theoretical model of GLA-TSR-EGF1 has been constructed by means of computer modelling (57, 58), based on structural homologies with domains of other vitamin K-dependent factors. In the same way, the secondary structure of the SHBG-like domain has been predicted (59). In both

cases, protein structure modelling takes into account the phenotype associated with naturally occurring mutations, or sheds lights on the molecular mechanisms responsible for protein S deficiency.

The protein S genes

The human genome contains two protein S genes, one of which is active (PSα or *PROS1*), the other being a pseudogene (PSβ or *PROS2*) (60-62). Both are located on chromosome 3, at position 3p 11.1-3q 11.2 (63-65) and are within 4 centiMorgans of each other (66).

The *PROS1* gene occupies 80 kb of genomic DNA (60-62). It contains 15 exons and 14 introns containing 6 repetitive "Alu" sequences. The two genes exhibit 97% and 95.4% homology between exons and introns, respectively. The *PROS2* gene lacks exon 1 and contains multiple base changes in the coding part of this gene, resulting in termination codons (corresponding to codons 61, 299, 410 and 522 of the *PROS1* gene) and in a frameshift in exon 10, precluding the presence of an open reading frame; *PROS 2* is therefore a pseudogene, and circulating protein S is exclusively the product of the *PROS1* gene (67, 68).

Three human protein S mRNA species have been isolated (38-40). The major mRNA form is about 4 kb long and contains a 5' untranslated region (112 bp of the 5' part of exon 1), a protein coding region (corresponding to the 3' end of exon 1, exons 2 to 14 and the 5' part of exon 15) and a 3' untranslated region of 1139 bp (39, 60). The regulatory region of the gene is poorly defined (69).

Three frequent polymorphisms have been described in the protein S gene. One is located in the coding region: Pro 626 is encoded either by CCA or by CCG, with frequencies of 0.48 and 0.52 respectively (70) in the Caucasian population. The other two (71) are located in non coding regions: the first is a C to T transition (allelic frequencies of 0.24 and 0.76, respectively) in intron k, 54 nucleotides downstream of exon 11, and the other is a C to A transversion (allelic frequencies of 0.83 and 0.17, respectively) 520 nucleotides downstream of the Stop codon.

A rare dimorphism of residue 460 (Ser 460→Pro) suppressing a glycosylation site has also been described as a polymorphism (72).

The intron-exon organization of the gene reflects the structural partition of the protein domain. The first 8 exons, probably assembled by exon shuffling, encode structural/functional protein units also found in other vitamin K-dependent coagulation proteins (except for exon IV, coding for the thrombin-sensitive loop) and have been placed upstream of the ancestral gene of a steroid hormone binding protein. The 3' part of exon 1 codes for the signal peptide, exon 2 for the propeptide and the GLA-domain, exon 3 for the helical stack domain, exon 4 for the thrombin-sensitive loop, exons 5 to 8 for four epidermal growth factor-like domains, and exons 9 to 14 and the first 161 bp of exon 15 for the sex hormone-binding globulin-homologous domain.

The cDNA sequences of protein S from cow (37), rabbit (73), mouse (74), rat (75), pig and rhesus monkey (76) have been reported. They share ≈ 59% sequence homology at the amino acid level.

Protein S deficiencies

Hereditary protein S deficiency is an autosomal dominant disorder that is associated with a risk of recurrent venous thrombosis. The first deficiencies were described in 1984 by several groups (77-79) and the thrombophilic tendency of protein S-deficient patients was confirmed by many others (80-88).

Most clinical manifestations are venous thrombosis and pulmonary embolism, but protein S deficiency may also predispose patients to arterial thrombotic disease (88-90). Several reports (91-94) have estimated the risk for thrombosis for subjects with protein S deficiency. Initially, Engesser et al (91) evaluated 12 families with the disorder and found that venous thrombosis occurred in 55% of the protein S deficient patients. Recently, Martinelli et al (93) have found a risk ratio of 8.5 for subjects with protein S deficiency. However, because of the difficulties encountered in the diagnosis of phenotypic protein S deficiency, others have studied the risk associated with protein S deficiency by performing genetic diagnosis. Simmonds et al (92) found that the risk associated with a point mutation causing protein S deficiency was 11.5 within a large (122-member) kindred. More recently, Makris et al (94) found that first-degree relatives with a *PROS-1* defect had a 5.0-fold higher risk of thrombosis than those without mutation in the *PROS-1*.

Few homozygous or compound heterozygous subjects have been reported (95-98). Such a genotype is usually regarded as incompatible with adult life without treatment, bearers developing severe purpura fulminans shortly after birth. However, two recent papers described severe protein S deficiency, one due to compound heterozygosity in a boy who had his first thrombotic episode at the age of 10 years (97), and another (98) due to homozygosity in a man who had had his first thrombotic episode at the age of 20 years; interestingly, one of his siblings, a brother aged 23 with a free protein S level below 1% -- and thus probably homozygous too -- is still asymptomatic.

The prevalence of protein S deficiency in the general population varies between 0.005 (99) and 0.7 to 2.3% (100) whereas it is found in up to 10% of patients with thrombosis (99-104).

While hereditary protein S deficiency is relatively rare, many other circumstances lead to acquired protein S deficiencies, such as oral anticoagulant therapy, oral contraception, liver disease, nephrotic syndrome, disseminated intravascular coagulation, pregnancy (105-111).

A recent study by Liberti et al (112) shows that protein S levels are influenced by gender (women have lower protein S levels than men) and by age (total protein S levels increase with age in women), due to an influence of the hormonal state. Total and free PS levels are also positively correlated with triglyceride and cholesterol levels (113).

Classification of inherited protein S deficiencies

Two classifications of inherited protein S deficiencies, based on the plasma phenotype, have been proposed, one by Comp (114) and the other by Bertina at the ISTH Subcommittee meeting in 1991. Both nomenclatures differentiate three types of protein S deficiency. In both classifications, a parallel reduction in both total and free protein S is designated as type I deficiency which corresponds to typical quantitative deficiency (77, 80, 81, 83). Decreased protein S activity associated with normal levels of total and free protein S, which is characteristic of classical qualitative deficiency, corresponds to type IIb according to Comp and type II according to Bertina (86).

A third type of deficiency, type IIa according to Comp and type III according to Bertina, is characterized by normal concentrations of total protein S but low levels of free protein S (78, 82, 87, 115, 116). Theoretically, this kind of deficiency may result from abnormal interaction with C4b-BP due to a variant of protein S itself, an abnormality of C4b-BP, or by an interfering additional plasma component (leading to an imbalance between C4b-BP ß+ and protein S concentrations).

Since the first database was published, several studies have reported the coexistence of the type I and type III phenotype within the same family, leading the authors to postulate that the two deficiencies are phenotypic variants of the same genetic disease (117).

A first explanation for this apparent discrepancy was provided by the study from Simmonds and colleagues (92), who showed that the same mutation (the Gly 295 to Val substitution) was associated with a type I deficiency phenotype in younger subjects of the kindred, whereas older subjects had a type III deficiency phenotype. This switch from type I to type III was due to aging. Indeed, total protein S antigen levels increase with age, leading to the onset of the type III phenotype in patients who initially have the type I phenotype.

It should be noted that this does not apply to all mutations: in several families the mutation is associated with the type III phenotype in all carriers, regardless of age.

Besides the effect of aging, genetic status should also be taken into account. Espinosa-Parrilla et al (118) identified a subject homozygous for the Arg 520 to Gly mutation, with a total protein S level of 36% (i.e. a type I deficiency phenotype). Three of his relatives, all heterozygous for the mutation, had total protein S levels between 71 and 99%, with free protein S

levels below the normal range, i.e. the type III deficiency phenotype. Similarly, the Ser 460 to Pro substitution is associated with a type III deficiency phenotype in heterozygous subjects but a type I deficiency phenotype in homozygous subjects (119, and Gandrille et al, unpublished observation).

The mutation itself may be involved in the observed phenotype. A very recent study by Espinosa-Parilla et al (120) revealed a correlation between *in vitro* secretion of recombinant protein S mimicking mutations identified in deficient subjects and the phenotype observed in the patients. The missense mutations that markedly inhibited *in vitro* secretion were associated with a type I phenotype. Other mutations cutting secretion by approximately one-half were associated with a type III or a less pronounced type I phenotype. Rezende et al performed a similar study studying exclusively mutations associated with a variable phenotype (types I/III). Some defects completely abrogated protein S expression, whereas others did express, albeit at a reduced level. This suggests that each mutation may have an individual effect on phenotype and that broad generalisation will not be possible (S. Rezende, Personal Communication).

Thus, evidence is accumulating that the type III deficiency phenotype is due to various mechanisms resulting not only from the nature of the mutation itself but also from the patient's genetic and physiological status.

The classification proposed by Bertina will be used throughout this paper.

Structure of the database

All detrimental mutations are listed in Table I, which is divided into 9 columns.

The order of entry corresponds to the position of the mutation from 5' to 3' extremities of the *PROS1* gene. The codons are numbered according to Schmidel et al (60).

The pedigree numbering comprises the telephone code of the country where the *propositus* was born followed by a number corresponding to the laboratory acronym attributed by the individual contributors to the database.

The nucleotide sequence of the gene has not been completed since publication of the first database. We thus use the same numbering system as that adopted for the first version.

The position of mutations is indicated as follows:

1/ nucleotide substitutions : in exons, they are indicated by the mutated codon number followed by the three nucleotides of the normal codon followed by the three nucleotides of the mutated codon. In introns, the position of the mutation is designated by the intron involved, designated by letters from "a" to "l" according to Schmidel et al (60), followed by the nucleotide change (normal nucleotide→mutated nucleotide), followed by the exon after or before which the

mutation occurs, followed by the position of the mutated nucleotide from this exon. Mutations occurring on nucleotides from the 5' flanking sequence of an exon are numbered as negative ("-") and mutations occurring on nucleotides from the 3' flanking sequence of an exon as positive ("+").

2/ insertions and deletions are designated by "ins" and "del", respectively. If the deleted/inserted sequence is short (less than 10 nucleotides), the entire nucleotide sequence is indicated. If the inserted/deleted sequence is 10 nucleotides or more, the number of inserted/deleted nucleotides is indicated.

In exons, insertions and deletions are entered by indicating the unchanged nucleotide(s) of the normal codon followed by the nature of the frameshift (ins or del) and the inserted/deleted nucleotide(s) sequence, or by the size of the inserted/deleted sequence between brackets. The amino acid deduced from the (new) codon at the position of the frameshift is indicated.

Insertions and deletions within introns are indicated by the intron involved, followed by the nature of the frameshift (ins or del) and the inserted/deleted nucleotide(s) sequence or the size of the inserted/deleted sequence between brackets. This is followed by the exon number after or before which the frameshift occurs, and the number of the nucleotide located immediately upstream of the first nucleotide in the inserted/deleted sequence.

Unless otherwise indicated, the plasma levels of total and free protein S, and protein S activity, are given for the *propositus*. As regards the free antigen level, determinations were done directly on untreated plasma, except when PEG is mentioned, indicating that free protein S levels were determined after precipitation of the C4b-BP bound fraction by PEG.

The cosegregation column indicates the size of the family studied. Two numbers are given: x and y, where x is the number of heterozygotes and/or homozygotes in the pedigree in which the mutation has been identified, and y the total number of family members tested (i.e. normal subjects and homo/heterozygotes).

The Comments column gives specific data such as the qualitative deficiency phenotype, rare genetic status (homozygosity or compound heterozygosity) of the propositus, absence of symptoms in the propositus, the presence (mutated mRNA+) or absence (mutated mRNA -) of detectable levels of mutated mRNA, the position of a new stop codon in the case of frameshift mutations, mutations occurring on a splice site, and exon skipping.

Finally, the last column indicates references to the mutations listed.

Methods used to search for *PROS1* mutations

The main difficulty in studying the protein S active gene *PROS1* is to discriminate *PROS 1* from the pseudogene *PROS2* . One way to avoid confusion with *PROS2* is to analyze the

mRNA after cDNA synthesis, as mRNA is the product of the *PROS1* gene only (67, 68). This is, at least in principle, less time-consuming than studying the 15 exons separately.

Since protein S is also synthesized by megakaryocytes, platelets contain residual mRNA and have been used as a source of mRNA for mutation screening. The RT-PCR screening strategy can rapidly identify mutations responsible for type II and type III mutations (121, 122). However, allelic exclusion is frequent in protein S-deficient patients with type I (121-123) and most gene defects leading to this phenotype (by interfering with mRNA synthesis and/or processing) remain undetectable. Thus, the best way to detect mutations responsible for type I deficiency is the time-consuming amplification of each exon using *PROS1*-specific primers.

Mutations in the protein S gene

The protein S database of mutations comprises 203 entries corresponding to 131 different mutations postulated to be detrimental (Table I). 31 entries corresponding to rare polymorphisms are listed in Table II. These mutations are distributed throughout the protein sequence.

❑ Detrimental mutations

The existence of two genes with close sequence of homology suggests the possibility of gene rearrangments. However, no recombinations between *PROS1* and *PROS2* have been described.

The types of mutations are shown in Table III. Missense mutations account for 47% of all the mutants. The second column gives the number of unique events, some mutations being identified in several apparently unrelated subjects.

Only 8 of the patients were homozygotes or compound heterozygotes (see Table IV). Table V summarizes biological and clinical data on these patients. The other 192 subjects had a phenotype compatible with heterozygous status, except for patient PS-43-4 who had an extremely low total protein S antigen level suggesting compound heterozygosity for the 633 del AA mutation and another, unidentified mutation located in a non coding part of the gene.

An attempt has been made to classify patients according to the type of deficiency, when the latter could be established. However, as a single mutation can be associated with both the type I and type III phenotypes, we now classify patients in two categories: those with a quantitative deficiency (including patients with Type I and Type III deficiency phenotypes), and

patients with a qualitative (type II) deficiency. Table VI lists the mutations potentially associated with both the type I and type III phenotypes in a given family.

Quantitative deficiency (type I and III) was defined on the basis of protein S levels determined using three assays, or at least two assays evaluating total and free protein S antigen. Qualitative deficiency (type II) was assessed only when the three assays were performed.

Using the above criteria, 146 patients could be classified: 94% had a quantitative deficiency and 6% had a qualitative deficiency. One subject was compound heterozygous, with two different mutations responsible for a combined quantitative and qualitative deficiency phenotype (PS-33-108). The type of the protein S deficiency in 46 patients could not be determined, either because one of the plasma assays was missing or because the subject was on oral anticoagulant therapy.

Large deletions

Many teams have attempted to find large deletion/insertions of the *PROS1* gene by Southern blot analysis (68, 121, 157-161). As shown in Table III, only 3 families out of 93 with quantitative deficiencies had large deletions (n=2). The first is a 5.3-kb deletion including exon 13 and was detected in two families (158). The second occurs between exons 7 and 12 of the *PROS1* gene in a type I family (159, 160).

Nucleotide substitutions

22.2% of nucleotide substitutions identified in *propositi* were nonsense mutations, 59.3% were missense mutations, 17.3% were splice site mutations, and 1.2% abolished the natural stop codon.

A total of 101 different single base-pair substitutions have been entered in the database (77% of the unique events, 80% of the mutants). Only 25 nucleotide substitutions (5.3% of the unique events, 12.3% of the mutants) occur in CpG dinucleotides and are C→T or G→A transitions compatible with the model of methylation-mediated deamination (162); this is very low compared to the proportion (32%) in mutations responsible for protein C deficiency (163). The distribution of mutations does not correlate with the distribution of CpG in the *PROS1* gene.

As CpG dinucleotides are hypermutable, some mutations may have originated independently. This is the case of the Arg 410→Stop mutation in patient PS-33-068. His mutated allele did not bear the rare polymorphism located in the 3'-untranslated part of the gene found in three other patients (PS-33-067, PS-33-069 and PS-33-071) with the same mutation. In patient PS-33-068, the mutation appears to have originated independently, while a founder effect could

explain the combined presence of the mutation and the polymorphism in the other three patients. Such a founder effect was recently proved by the use of microsatellite markers in Danish patients bearing the same mutation (Arg 410 → Stop) (151).

Only 7 different nucleotide substitutions are known to be responsible for qualitative (type II) deficiency. Five are missense mutations (Arg -2 → Leu, Arg -1 → His, Lys 9 → Glu, Thr 103 → Asn, Lys 155 → Glu), 3 being located in the propeptide or the GLA domain. It is noteworthy that mutations occurring in corresponding positions of the propeptides of factor IX and protein C are also associated with qualitative deficiencies (164, 165). The plasma phenotype of the patient with the Thr 103 → Asn mutation supports the role of this amino acid in the interaction with aPC (76). None of the patients with Arg -2 → Leu, Arg -1 → His, Lys 9 → Glu and Thr 103 → Asn mutations bear the factor V Arg 506 to Gln mutation. The mutated factor V molecule, present in aPC-resistant plasma, has been shown to affect coagulation-based functional assays for protein S (166-168).

Two splice site mutations were also associated with the type II phenotype (Bernardi, personal communication, 137). The first (intron g, A → T, exon 8 -2) activated a cryptic splice site, leading to a deletion of two amino acids from the protein (Ile-Asp 203-204) . The other (137) resulted in two alternative splice transcripts, lacking either exon 5 or both exons 5 and 6. An EGF1-lacking protein S species, corresponding to the exon 5-lacking transcript, was detected in the patient's plasma.

Frameshift mutations

36 *propositi* (17,7%) bore 27 different insertions/deletions smaller than 7 nucleotides. The plasma phenotype of all *propositi* whose protein S levels were established using the three plasma assays revealed a type I deficiency phenotype. Some of these mutations can be explained by mechanisms outlined in (169). Two deletions (in codons 82 and 633) overlapped with quasi-symmetric elements. Inverted repeats flanked an insertion of C in codon 78, whereas insertions in codons 186 and 565 introduced an axis of internal symmetry in the sequence.

Splice site mutations

28 propositi (13,8%) bore 13 different mutations occurring in the intron splice site, and 2 missense mutations (45-46 Leu-Val → Leu-Phe; 46, Val → Leu) affected the splice site. All are nucleotide substitutions, except two that were four- and eight-bp deletions, respectively (ivs k,

exon 11, del +1 to +4 ; ivs n, exon 15, del -28 to -21). These mutations are mostly associated with quantitative deficiency, but two were associated with qualitative deficiency, as indicated above.

❑ Rare polymorphisms

Table II lists the three frequent polymorphisms and 18 different sequence variations that are probably rare polymorphisms as they occurred in subjects with normal protein S levels (Pro 35→Leu; Arg 192→Lys or Thr 477→Met), did not change the encoded amino acid (Leu -30→Leu; Pro 35→Pro; Ile 303→Ile or Gly 418→Gly), or did not co-segregate with the protein S deficiency (5'UT T→C exon 1 -286; intron a, A→G, exon 1 +7; intron a, del ATT, exon 2 -67; intron b, G→A, exon 2 +5; intron g, G→A, exon 8 -20, Arg 49→His, Thr57→Ser, Met 344→Val and Ile 518→Met). Some of these polymorphisms were identified in patients bearing the same detrimental mutation (Ile 303→Ile identified in two patients with Arg -2→Leu mutation; 3'-UT, T→G, exon 15 +18 identified in four patients bearing an Arg 410→Stop mutation), indicating that a founder effect is probably responsible for these repeat mutations.

❑ The Ser 460 to Pro mutation

The Ser 460 to Pro mutation was first identified in two compound heterozygous subjects belonging to type I protein S-deficient families (72). Ser 460 is part of a consensus sequence and belongs to a potential glycosylation site of protein S, and its replacement by a Pro residue precludes glycosylation at this site, resulting in a circulating protein S with a lower MW than normal protein S. Since the frequency of the protein S Heerlen allele in a population of subjects with unexplained thrombosis (0.67%) did not differ significantly from that observed in the general population (0.52%) (and because the mutation did not affect plasma levels of total protein S, and the mutated protein S had normal cofactor activity for activated protein C), this mutation was thought to be a polymorphism.

However, in two series of 118 French and 46 Spanish protein S-deficient families (134, 170-172), the protein S Heerlen allele was observed in 26 (22%) and 12 (26%) protein S-deficient families, respectively, while the frequencies of this allele in the normal population of these two countries were 0.9 and 0.66% of individuals analyzed, respectively. In addition, linkage disequilibrium was observed (134,170) between the Pro 460 allele and type III protein S deficiency (24 of 63 type III-deficient patients bore the Heerlen polymorphism, versus 2 of 47 type I-deficient patients, the difference being statistically significant) (134, 170). In heterozygous

subjects, total protein S levels remained within the normal range but were significantly lower than in normal subjects (119, 171), and free protein S levels were decreased (59±12%) (119, 171). Homozygous subjects (119) had a clear type I deficiency. Thus, it is not clear whether this mutation is a polymorphism or a deleterious mutation.

However, whereas in some families the free protein S deficiency co-segregates with the Ser 460 to Pro mutation, no co-segregation between the plasma phenotype and the protein S mutation can be found in others, suggesting that other genetic factor(s) may contribute to the protein S deficiency in conjunction with the protein S Heerlen allele. Involvement of the PROS1 gene (172) or of the C4b-BP ß-chain gene (172, 173) has been excluded in several families.

The thrombotic risk associated with this mutation remains to be evaluated. In a series of 26 symptomatic patients bearing the Ser 460→Pro mutation, 42% had another genetic abnormality such as the factor V Arg 506 to Gln mutation, a protein C gene mutation, another protein S gene mutation (134), or the prothrombin 20210 G/A mutation (119).

Protein S deficiency and factor V Leiden (Arg 506→Gln mutation)

Unlike antithrombin III and protein C deficiency, protein S deficiency was not a significant risk factor for thrombosis in a large case-control study (101) possibly because of the low prevalence of protein S deficiency in that particular population. Indeed, previous family-based, as opposed to population-based, studies have clearly shown that protein S deficiency is a risk factor for thrombosis (174). It is now agreed that thrombophilia can be the result of interacting genetic defects. Thus, the co-existence of other genetic factors with the protein S gene mutations, such as the factor V Arg 506 to Gln mutation, may explain the highly variable penetrance of this defect (175-180). It is interesting to notice that the factor V Leiden mutation has been found as an additional genetic risk factor in 22 to 35% (179, 180) of thrombosis-prone protein S-deficient families, versus only 14 to 19% (81, 182) of thrombosis-prone protein C-deficient families.

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