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Deficiencies of the natural anticoagulants – novel clinical laboratory aspects of thrombophilia testing

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ABSTRACT

Venous thrombosis is a typical common complex disease as acquired and genetic causes play a role in its development. The different „loss of function“ mutations of the natural anticoagulant system lead to antithrombin (AT), protein C (PC) and protein S (PS) deficiencies. Since thrombophilia testing has high cost and it has several methodological issues (analytical, pre-analytical), which makes the interpretation of results difficult, considerations should be made on the indications of testing, on the parameters that are measured and on the best available method to use. The latest guideline on clinical and laboratory management of thrombophilia kept the relatively old laboratory recommendations unchanged. This is partly because of the existence of unresolved problems with the laboratory tests used for diagnosis. Based on the literature and our previous research here we discuss the unresolved problems, the recently raised questions and issues concerning AT, PC and PS laboratory diagnosis and summarize the recent findings in molecular genetic investigations.

INTRODUCTION

Thrombosis is a common pathology underlying atherothrombotic diseases and venous thromboembolism (VTE), which are highly frequent and the major determinants of morbidity and mortality (1). Primary and secondary prevention is key to reducing death and disability from these diseases. VTE is a typical common complex disease as acquired and genetic causes play a role in its development (2). The different „loss of function" mutations of the natural anticoagulant system lead to antithrombin (AT), protein C (PC) and protein S (PS) deficiencies and the "gain of function" mutations known as Factor V Leiden (FVL) resulting in activated PC (APC) resistance and prothrombin 20210A allele (FII20210A) are responsible for the majority of inherited thrombophilia. Further hereditary factors are non-O blood group, elevated factor VIII, IX and XI, certain types of dysfibrinogenaemia and hyperhomocysteinaemia, however except for blood type all of these may have acquired components in their variability. Antiphospholipid syndrome (APS) is an acquired condition (3). The incidence of thrombosis in individuals having inherited thrombophilia is variable; it depends on the particular genotype, the co-existence of other genetic alterations (polymorphisms) and environmental factors (4). Moreover, several so far unidentified genetic factors may contribute to the risk of VTE, as it is suggested by the different genome-wide –association studies, like MARTHA or FARIVE (5).

RECOMMENDATIONS FOR THROMBOPHILIA TESTING-RECENT STATEMENTS

After the discovery of the above-mentioned hereditary risk factors testing for thrombophilia became more and more popular and the number of laboratory requests showed a rising tendency. Since thrombophilia testing has high cost and it has several methodological issues (analytical and pre-analytical ones; mentioned later in detail) which makes the interpretation of results difficult, considerations should be made on the indication of testing, on the parameters that are measured and on the best available method to use. In recent years, several contradictory papers and recommendations have been released by experts, different committees and working groups on the indications for thrombophilia testing and on the laboratory parameters to be determined (6-16). One can conclude from these that thrombophilia testing should be performed in a very carefully selected population in which the test results have a direct impact on the clinical decision either on primary or secondary thrombosis prophylaxis. Thrombophilia testing is not recommended routinely after a provoked VTE according to most of the guidelines, however the definition of "provoked" itself is not always clear in the different papers. There are situations, or conditions in which thrombophilia testing is advisable according to most of the recommendations. These are idiopathic (unprovoked) VTE, especially below the age of 50 years, thrombosis in unusual sites, recurrent VTE, first VTE with strong positive family history, asymptomatic family members of relatives having severe inherited thrombophilia, pregnancy complications or in women taking contraceptive pills, or under hormonal replacement. Thrombophilia testing, although its association with arterial thrombosis is uncertain mainly due to the lack of large population-based studies, may be considered in young patients especially without any well-defined risk factors of arteriosclerosis. A comprehensive review has been published most recently on the clinical aspects of thrombophilia testing, in which the existing guidelines are summarized (17). The major question is to estimate the risk of recurrence after the first VTE, which influences the duration (and perhaps the aggressiveness) of

anticoagulation. The VTE risk for asymptomatic family members of a proband with thrombophilia is the second important issue, when primary prophylaxis is considered in different risk situations. If thrombophilia testing helps to answer these questions its execution is definitely worthwhile.

THE THROMBOPHILIA PANEL

As no single well-standardized and widely accepted method exists for thrombophilia screening a list of investigations should be performed in a patient suspected for thrombophilia. The latest guideline kept the old (2001) laboratory recommendations unchanged (8). This is partly because of the existence of unresolved problems with the laboratory tests used for diagnosis, especially in the case of AT, PC and PS deficiencies.

Investigations for thrombophilia usually include AT, PC and PS assays, tests for APC resistance and/or FVL and the FII20210A. This panel is completed by the laboratory investigations for APS (18). It is advisable to perform the screening tests of coagulation (i.e. prothrombin time, activated partial thromboplastin time, thrombin time) to detect the presence of different anticoagulant drugs, which may interfere with certain laboratory tests. Thrombin time is also useful to screen for fibrinogen abnormalities, like dysfibrinogenaemia. Some authors also recommend testing for elevated FVIII and for APC resistance not due to FVL. Thrombophilia testing should be completed by measurement of plasma homocysteine and blood typing is also advisable (19). Besides taking the correct parameters to be tested into consideration, appropriate timing of investigation is also important (18).

LABORATORY DIAGNOSIS OF INHERITED AT, PC AND PS DEFICIENCIES

Two reviews are recommended for interested readers, which describe the molecular basis and epidemiology of AT, PC and PS deficiencies and introduction into the laboratory issues (20, 21). In this paper we are going to discuss the unresolved problems, the recently raised questions and issues concerning AT, PC and PS laboratory diagnosis and summarize the recent findings in molecular genetic investigations.

STRUCTURE AND FUNCTION OF ANTITHROMBIN; ANTITHROMBIN DEFICIENCIES

AT is the most important circulating inhibitor of blood coagulation proteases, synthesized by hepatocytes and is a member of the serine protease inhibitor (serpin) superfamily (20). The mature AT molecule is a single-chain 58 kDa glycoprotein with half-life of 2.4 days. The plasma concentration of AT is around 150 mg/L in the circulation. AT contains an N-terminal heparin-binding domain, a carbohydrate rich domain and a COOH-terminal serine protease-binding domain. It has two isoforms that differ only in the extent of glycosylation. The major α isoform, which represents 90-95% of total AT, is N-glycosylated on 4 Asn residues (127, 167, 187 and 224), while the β isoform (5-10%) is not glycosylated at Asn167. This latter isoform has higher affinity to negatively charged glycosaminoglycans, like heparin. The AT encoding gene, *SERPINC1* is located on the chromosome band 1q23-25, has 7 exons and 6 introns. The heparin-binding site of AT is encoded by exon 2 and exon 3. The reactive site, which is located in the carboxy-terminal part of the protein, is encoded by exon 7.

AT primarily inactivates thrombin mediated fibrin clot formation and the generation of thrombin by activated FX (FXa). It is also able

to inhibit activated coagulation factors FXII, FXI and FIX (FXIIa, FXIa and FIXa) in the intrinsic and FVIIa-tissue factor complex in the extrinsic pathway (22).

AT deficiency was first described by Egeberg in 1965 (23) and the first functional AT defect, named as AT Budapest, was reported by Sas et al in 1974 (24). AT deficiency is classified as type I (quantitative) and type II (qualitative) (25). In type II deficiency, the defect may involve the reactive site (II RS), the heparin-binding site (II HBS) or it may exert a pleiotropic effect. Individuals with inherited AT deficiency have a highly increased thrombotic risk and homozygosity in type I deficiency and in most type II deficiencies, with notable exception of type II HBS variants, are incompatible with life (26). The type II HBS deficiency is considered as a lower risk of VTE (27). The mutation profile of *SERPINC1* is highly heterogeneous and the most prevalent mutations are AT Cambridge II (p.Ala416Ser), AT Budapest 3 (ATBp3, p.Leu131Phe) and AT Basel (p.Pro73Leu), which were reported in a number of studies (28-34). AT Cambridge II is frequent in the British population; the mutation has a prevalence around 0.5-2.0% in French, Spanish and German VTE patients. AT Cambridge II, however was not detected in other populations like in Hungary and in Southern China (32, 35). The ATBp3 is a founder mutation in the Hungarian population with prevalence of 86.5% within type II HBS deficiency (32).

METHODOLOGICAL PROBLEMS AND RECENTLY RAISED QUESTIONS IN ANTITHROMBIN DEFICIENCY

A first-line test for the diagnosis of AT deficiency is based on a chromogenic functional assay, in which the inhibition of FIIa or FXa by AT in the presence of heparin is detected by measuring the residual enzyme (FIIa or FXa) activity using their specific chromogenic substrates (20). If the assay is performed in the presence of heparin, which ensures a fast inhibitory effect of AT, the assay is named as heparin-cofactor AT test (hc-anti-FIIa, or hc-anti-FXa AT assay). If heparin is not used in the assay then the so-called progressive AT activity is measured (p-anti FIIa or p-anti-FXa AT assay). Several commercially available reagents can be used for measuring AT activity and the heparin cofactor AT activity assays are dedicated to detect all types of AT deficiency. In case of using hc-anti-FIIa AT activity assay, it is important to choose those with bovine thrombin instead of human thrombin to avoid the influencing effect of heparin cofactor II on AT activity results (36). Human vs. bovine source of enzyme is not a problem in hc-anti-FXa assays, since FXa does not react with heparin cofactor II at all.

According to the latest results of the external quality control surveys both hc-anti-FIIa and hc-anti-FXa assays are used in equal number by the different laboratories. In the latest UK NEQAS program 119 and 184 laboratories used hc-anti-FIIa and hc-anti-FXa assays, respectively and in the ECAT program 154 and 145 laboratories reported results by using hc-anti-FIIa and hc-anti-FXa assays, respectively. Among hc-anti-FIIa assays, Siemens Berichrom AT and Diagnostica Stago Stachrom AT are the most popular ones. Among hc-anti-FXa assays Siemens Innovance AT, Werfen HemosIL (liquid) AT and Chromogenix Coamatic AT tests are performed by most of the laboratories. All these kits have very similar performance within the reference interval (i.e. in the case of non-AT deficient samples). It is of great importance, however, to realize that despite the numerous functional assays available on the market, no single one appears to be able to recognize all defects (37). FXa-based assays in general are less sensitive to detect AT deficiencies caused by certain mutations around the reactive site, like in the case of AT Cambridge II (28,29). On the contrary, it was demonstrated

by some studies that hc-anti-FXa assays had higher sensitivity to type II HBS AT deficiency (37, 38). In our study Siemens Berichrom AT test was inferior to Siemens Innovance AT and Labexpert AT H+P assays. The latter two tests, which are based on hc-anti-FXa methodology, gave practically identical results with all AT deficient patients (n=37) (39). By the investigation of the highest number of patients with the type II HBS ATBp3 (n=102), we confirmed that the hc-anti-FXa assay (Innovance AT) was the method of choice in type II HBS AT deficiency (32). In the study of Puurunen et al. patients with AT Basel (n=88) were also successfully detected by Innovance AT reagent (34). It is interesting that there are big differences in the sensitivity even among hc-anti-FXa assays to type II HBS AT deficiency (37, 40). According to the results of these studies the HemosIL AT and the Coamatic AT reagents were less sensitive, while Innovance AT

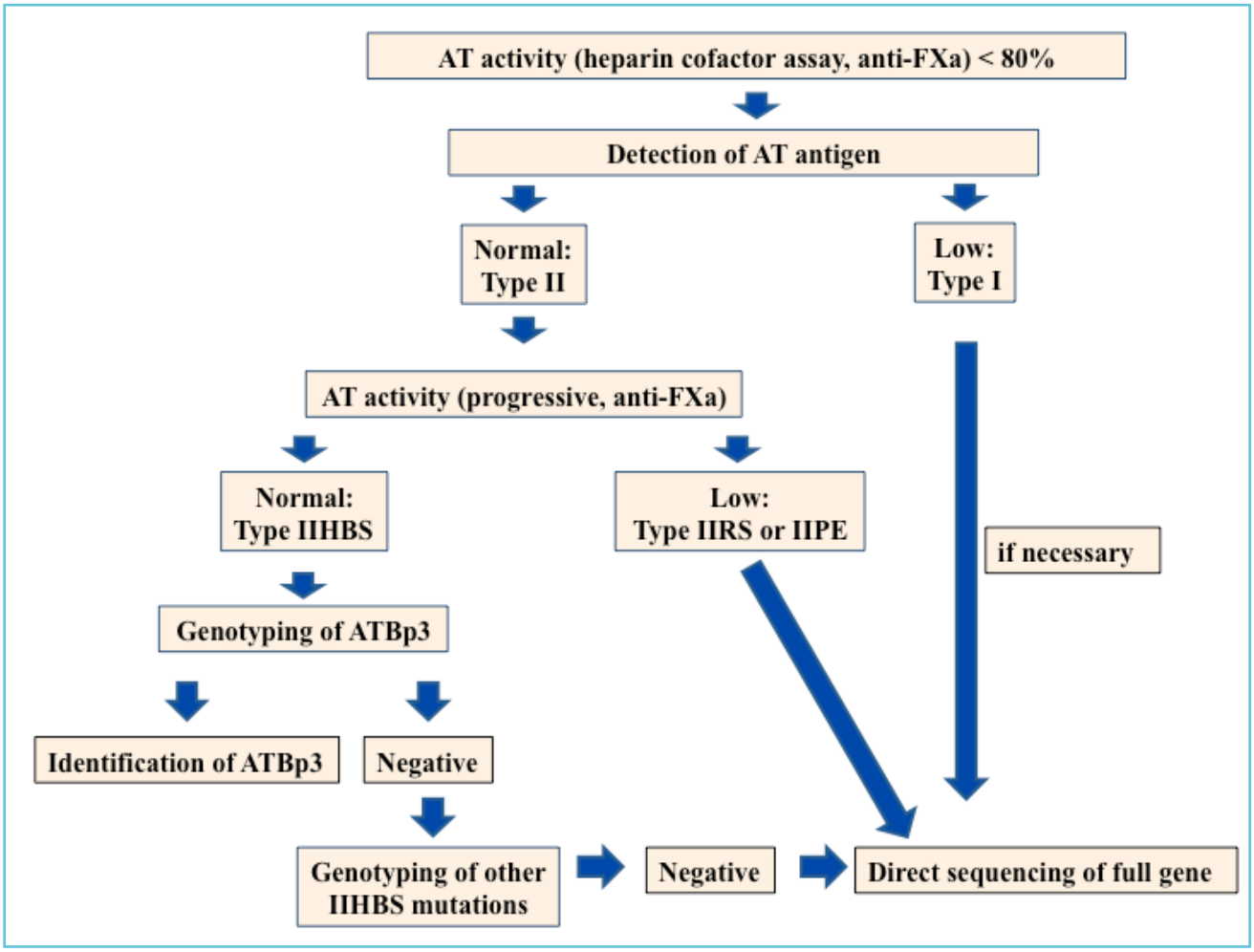
Table 1 Standard characteristics of the different commercial heparin cofactor anti-FXa AT activity assays

	Substrate source	Incubation time	Heparin concentration	Chromogenic substrate	Sample predilution	Dilution buffer	Final dilution of sample
Siemens Innovance® Antithrombin	Human FXa	180-190 s	1500 U/L	Z-D-Leu-Gly-Arg-ANBA-methylamide-acetate	1:4	Tris/HCl pH 8.0	1:20
HemosIL® Liquid Antithrombin	Bovine FXa	100-140 s	3000 U/L	S-2765 (N-α-Z-D-Arg-Gly-Arg-pNA•2HCl)	1:40	0.15 M Sodium Chloride	1:120
Chromogenix Coamatic® Antithrombin	Bovine FXa	100-140 s	5000 U/L	S-2765 (N-α-Cbo-D-Arg-Gly-Arg-pNA·2HCl)	1:121	0.15 M Sodium Chloride	1:484
Hyphen Biophen Antithrombin	Bovine FXa	60 s	(ready to use)	SXa-11 (Suc-Ile-Glu-(γPip)Gly-Arg-pNA, HCl)	1:20	0.15 M Sodium Chloride	1:170
Labexpert Antithrombin H+P	Bovine FXa	60 s	1 USP units/mL	BIOPHEN CS-11 [Suc-Ile-Gly-(γPip)Gly-Arg-pNA, HCl]	1:50	50 mmol/L pH 8.4 Tris-HCl	1:150

showed practically 100% sensitivity. This suggests that not only the source of enzyme (FIIa or FXa) is responsible for the difference in performance of AT functional tests. Table 1 demonstrates the assay characteristics of the most commonly used hc-anti-FXa AT assays. All but one reagent contains bovine FXa as enzyme in the reaction. The different tests differ in plasma dilution, in the added heparin concentration, in the source of chromogenic substrate and also in the incubation time. It can be concluded that clarification of the situation concerning heparin cofactor AT functional assays warrants future research to establish improved recommendation for AT testing.

Progressive AT assay is based on the same principle as heparin cofactor assays, but it is performed in the absence of heparin on less diluted plasma samples usually with prolonged incubation time. It was demonstrated in several clinical samples that comparison of p-anti-FXa and hc-anti-FXa activities is a useful tool in the diagnosis of type II HBS AT deficiency since in this type hc-anti-FXa decreases, while p-anti-FXa remains

Figure 1 **Laboratory diagnostic algorithm in antithrombin deficiency used in the authors' laboratory**



AT, antithrombin; type II HBS, heparin-binding site AT deficiency; type II RS, reactive site AT deficiency; type II PE, AT deficiency with pleiotropic effects; ATBp3, AT Budapest 3 deficiency caused by p.Leu131Phe mutation

normal, or shows only a slight decrease. The ratio of p-anti-FXa and hc-anti-FXa therefore is well above the upper limit of the reference interval in heterozygous type II HBS patients and even higher in homozygotes (41). The reagent developed in our laboratory (Labexpert AT H+P) is able to measure both hc-anti-FXa and p-anti-FXa AT activities and reference interval has been determined according to CLSI guidelines for both. A diagnostic algorithm that is followed in our laboratory is shown in Figure 1.

MOLECULAR GENETIC DIAGNOSIS IN AT DEFICIENCY

Molecular genetic testing is a useful diagnostic tool for confirming inherited AT deficiencies especially for patients with borderline activities (31). Moreover, genetic analysis helps to distinguish among the different AT deficiency subtypes that has of great importance from the point of view of clinical patient management. The mutation detection rate in the case of *SERPINC1* in general is quite high, more than 80%. Current evidence shows that not all *SERPINC1* mutations causing AT deficiency lead to decrease in AT activity in the commercially available functional assays (42). Genetic analysis has been therefore recently suggested to be included in clinical practice when screening for AT deficiency in individuals experiencing unprovoked thrombotic diseases, even if the hc-AT activity is above 80% (43).

In *SERPINC1* 215 causative mutations were found before 2010; since then 61 mutations were reported in HGMD database (Table 2). It is to be noted, however, that several novel variants have not been reported in the HGMD, yet (Table 3) (44-50).

Table 2 Distribution of different mutations within the genes for antithrombin, protein C and protein S

Mutation types	*SERPINC1*	*PROC*	*PROS1*
Missense/nonsense	155 (56%)	231 (74.5%)	171 (64%)
Splicing	17 (6%)	25 (8%)	27 (10%)
Regulatory	0 (0%)	12 (4%)	3 (1%)
Small deletions	52 (19%)	24 (8%)	28 (10%)
Small insertions	23 (8%)	13 (4%)	15 (6%)
Small indels	2 (0.8%)	3 (1%)	4 (1.5%)
Gross deletions	24 (9%)	2 (0.5%)	14 (5%)
Gross insertions/ duplications	1 (0.4%)	0 (0%)	4 (1.5%)
Complex rearragements	2 (0.8%)	0 (0%)	2 (1%)
Total	**276**	**310**	**268**

According to HGMD database (The Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/search.php*), accessed on 14th December 2015.*

Table 3 Novel mutations in *SERPINC1* published in the last five years and not indicated in the HGMD database

	Nucleotide exchange	Amino acid exchange	Type of deficiency	References
Missense/ nonsense	c.133 C>T	p.Arg45Trp	I†	Caspers (2012)
	c.134 G>A	p.Arg45Gln [1]	I	Deng (2013)
	c.335 C>G	p.Pro112Arg [2]	I	Maruyama (2013)
	c.342 T>G	p.Ser114Arg [1]	I	Deng (2013)
	c.347 C>T	p.Ser116Phe	I†	Caspers (2012)
	c.452 T>G	p.Ile151Ser	I†	Caspers (2012)
	c.455 A>C	p.His152Pro	I†	Caspers (2012)
	c.458 T>A	p.Phe153Tyr	NA	Zeng (2015)
	c.464 T>G	p.Phe155Cys	I	Ding (2013)
	c.491 G>A	p.Arg164Gln	NA	Zeng (2015)
	c.539 G>A	p.Gly180Glu	I†	Caspers (2012)
	c.569 A>C	p.Tyr190Ser	I†	Caspers (2012)
	c.569 A>G	p.Tyr190Cys	I†	Caspers (2012)
	c.598 G>C	p.Ala200Pro	NA	Zeng (2015)
	c.883 G>A	p.Val295Met	NA	Zeng (2015)
	c.886 G>C	p.Ala296Pro	I†	Caspers (2012)
	c.899 A>G	p.Gln300Arg	I†	Caspers (2012)
	c.934 A>G	p.Thr312Ala	IIRS	Bhakuni (2015)
	c.938 T>C	p.Met313Thr	NA	Zeng (2015)
	c.1114 C>T	p.Leu372Phe	I	Ding (2013)
	c.1121 A>G	p.Asp374Gly	IIRS	Castaldo (2012)
	c.1307 C>G	p.Ala436Gly	I†	Caspers (2012)
	c.178 A>T	p.Lys60X	I†	Caspers (2012)
	c.203 C>G	p.Ser68X	I	Ding (2013)
	c.1016 G>A	p.Tyr339X	I	Ding (2013)
	c.1024 G>T	p.Glu342X	I†	Caspers (2012)
	c.1394 A>C	p.X465Sext28*X	I	Castaldo (2012)

Splicing	c.408 +1 G>A	-	I†	Caspers (2012)
	c.409 -10 G>A	-	I†	Caspers (2012)
	c.624 +1 G>T	-	I†	Caspers (2012)
	c.1219 -1 G>A	-	I	Castaldo (2012)
Small deletions	c.86_87delinsCT	p.Cys29Ser	NA	Zeng (2015)
	c.173del	p.Pro58ArgfsX3	I	Castaldo (2012)
	c.412_417del	p.Phe138-139Lysdel	I†	Caspers (2012)
	c.457_459del	p.Phe154del	I†	Caspers (2012)
	c.462_464del	p.Phe155fs	I†	Caspers (2012)
	c.490del	p.Arg164GlufsX8	I	Nadir (2015)
	c.614del	p.Leu205fs	I†	Caspers (2012)
	c.712_719del	p.Asn240fsX1	I†	Caspers (2012)
	c.1332_1333del	p.Ile444MetfsX19	II	Castaldo (2012)
	c.1390_1393del	p.X465MetfsX13	I	Castaldo (2012)
Small insertions	c.1172dupG	p.Asp392fs	I†	Caspers (2012)
	c.1340_1341insA	p.Pro448SerfsX16	IIHBS	Bhakuni (2015)
Gross deletions	c.243_263del	p.80-86del	I	Castaldo (2012)
	Exon 6-7	-	I	Caspers (2012)
Large in-frame insertion/ deletion	c.625_630del_30ins	p.Glu241_ Leu242del_241_243ins_ Val_Leu_Val_Leu_Val_ Asn_Thr_Arg_Thr_Ser [3]	IIHBS	Martínez-Martínez (2012)
	c.1066_1083del	p.Arg356_Phe361del [4]	I	Zeng (2015)

These data were collected from publications available on NCBI-PubMed (indexed by MEDLINE) database.
Nucleotide and amino acid numbering follows the HGVS nomenclature.
NA, non applicable (i.e. AT functional assay showed normal result)

† These mutations seem to lead to type I deficiency, however they were not confirmed. In vitro expression studies indicated:
[1] decreased AT secretion and heparin affinity
[2] impaired secretion and intracellular degradation
[3] impaired heparin affinity and the mutation transforms the structure of serpin
[4] represented impaired secretion and reduced functional activity

STRUCTURE AND FUNCTION OF PROTEIN *C* AND *S*; PROTEIN *C* AND *S* DEFICIENCIES

Protein C and S are Vitamin-K-dependent plasma glycoproteins with molecular masses of about 62 and 71 kDa, respectively (21). Plasma concentrations and half-lives of PC and PS are 3-5 mg/L and 20-25 mg/L, and 8h and 42h, respectively. The domain structure of PC and PS is highly homologous to other vitamin K-dependent coagulation factors (pre-pro leader sequence, Gla-domain and epidermal growth factor like (EGF) domains). PC is a two-chain protein in its mature form and it contains an activation peptide domain and the serine protease domain, which is responsible for its anticoagulant effect. PS is a single-chain molecule having a thrombin-sensitive region (TSR) and a C-terminal region homologous to the sex-hormone-binding globulin (SHBG-like domain). The gene encoding PC (*PROC*) is positioned on chromosome 2q13-q14 and contains nine exons, eight of which encode the protein and the 1.7-kb messenger RNA (mRNA) and 8 introns (51). The human PS gene, *PROS1*, is located on chromosome 3q11.2, where it spans 80 kb of genomic DNA and contains 14 introns and 15 exons. In addition to the active gene, a transcriptionally inactive pseudo gene (*PROS2*) is located on chromosome 3. It shows 97% homology to the active gene. This makes the molecular genetic investigations of PS deficiency difficult (please see below).

The zymogen PC is activated by the thrombin-thrombomodulin (TM) complex on the surface of endothelial cells, and binding to endothelial protein C receptor (EPCR) further increases its activation rate (4). Activated PC (APC) inactivates membrane bound activated factor V (FVa) and activated factor VIII (FVIIIa). The free form, approximately 40% of total PS, which is not bound to its natural binding protein (C4bBP), acts as a cofactor for APC. It is to be noted that PS also has APC independent anticoagulation effects that are not investigated in routine laboratories (52, 53). Both APC and PS have roles in a variety of physiological processes distinct from hemostasis. APC has a direct cytoprotective nature (54). PS is involved in cell proliferation/survival, apoptosis, regulation of inflammatory cytokine release, atherosclerosis, vasculogenesis and cancer development (55). This issue, although very interesting, is beyond the scope of this review.

In type I PC deficiency, which is more common (75-80%), both the activity and antigen concentration of PC is decreased, which suggests defective protein synthesis and/or secretion. In type II deficiency, normal amount of dysfunctional protein is synthesized, and the functional defect can be due to abnormalities in substrate, calcium-ion or receptor binding (56). Type I PS deficiency is associated with a decrease in the total PS antigen and free PS antigen, and hence a decrease in APC cofactor activity; type II is a qualitative deficiency, characterized by a normal total and free PS antigen level but a decrease in the APC cofactor activity; and in type III deficiency there is a normal total PS antigen but a decrease in free PS antigen and in APC cofactor activity. Several reports have proposed that types I and III deficiencies may be phenotypic variants of the same genetic disease (57).

METHODOLOGICAL PROBLEMS AND RECENTLY RAISED QUESTIONS IN PROTEIN C AND S DEFICIENCY

For routine screening and classification of PC and PS deficiencies two types of assays are available, functional tests and antigen assays (21). In the diagnosis of PC deficiency, first, a functional test should be performed and in case of abnormal results, a PC antigen is measured. The ratio of PC activity to PC antigen is then calculated, which can distinguish type I from type II deficiency (58). PC activity can be measured in

plasma by either a clotting-based assay (mostly based on APTT measurement) or chromogenic (amidolytic)-based assay, and PC antigen is usually measured by ELISA. In the diagnosis of PS deficiency, the clotting-based PS activity assays are designed to measure APC-dependent anticoagulant activity. PT-, APTT- or FXa-based assays are commercially available. No chromogenic functional assay is available. Free PS antigen is considered as the "functional" anticoagulant fraction of PS although it is not a true measure of activity. Free PS antigen is determined using ELISA, or immunoturbidimetry. Total PS assays measure both the free and bound fractions of PS by immunological methods. The principles of these assays are detailed elsewhere (21).

Functional assays of PC and PS have several advantages and disadvantages. Clotting-based assays of PC can detect all aberrations regarding the activation, activity, cofactor-and phospholipid (PL) binding, while chromogenic PC assay can analyze only the core function (i.e. activation and activity) of the protein and can not detect defects in cofactor binding, PL surface binding, and receptor binding (59). Due to this phenomenon some cases with mutations affecting regions, which are responsible for these interactions (PC deficiency type IIb) are not detected by the chromogenic functional assay and remain undiagnosed. Chromogenic assays are subjected to interference from haemolysis, icterus and lipemia. Results of clotting tests of PC and PS are influenced by a lot of factors that have an effect on clotting time (i.e. lupus anticoagulant, anticoagulant drugs including direct FXa or thrombin inhibitors and high FVIII level). Presence of APC resistance (FVL) is a special issue. In patients having FVL mutation the PC and PS activity values measured by the clotting assays (despite diluting the patient's plasma in PC or PS deficient plasma wild type for FVL and using wild type FVa as substrate) are falsely decreased. Since antigenic assays are not influenced by APC resistance and give normal result, FVL patients may be misdiagnosed as type II PC or PS deficiency (60-62). Antigenic assays may be subjects of interferences generally seen in immunological methods (e.g. rheuma factor).

According to the experiences in the international external quality control programs there is no consensus among the laboratories as to which functional assays are better for detecting PC and PS activity. Several assays (both chromogenic and clotting based for PC) are commercially available with different sensitivity, specificity and significant variability. Table 4 demonstrates the most frequently used methods in the routine diagnosis of PC and PS deficiencies as it is indicated in two ECAT surveys. Concerning PC functional assays, chromogenic methods that show lower between-laboratory variability are used by the majority of participants (approximately 75%). In the latest NEQAS survey n=290 vs. n=14 laboratories preferred the chromogenic method. PC antigen is not measured routinely by a large number of laboratories. The ratio of laboratories measuring PS activity vs. free PS antigen is approximately 0.7 in the ECAT and 0.24 (!) in the NEQAS program, that reflects the unresolved methodological problems in PS functional assays. Total PS antigen is measured only by a minority of the laboratories. It is well demonstrated in the table that presence of FVL influences the results of clotting-based PC and PS assays, the lowest values are obtained by Siemens reagents. PS activity in general is markedly lower than free PS antigen. Baron et al analyzed North American Specialized Coagulation Laboratory Association (NASCOLA) PC deficiency testing data from six surveys conducted in 2009 and 2010 (63). They demonstrated that performance of the assays showed considerable variety.

Based on the above-mentioned problems there are controversies in the different recommendations

Table 4 Most commonly used assays for PC/PS determination and their results in the latest surveys of ECAT Foundation

Type of assays *n*		Plasma of a patient with a heterozygous PS deficiency			Abnormal Coagulation Control Plasma			Normal Coagulation Control Plasma			Plasma of a patient with a heterozygous Factor V Leiden mutation		
		assigned value	*CV (%)*	*n*	*assigned value*	*CV (%)*	*n*	*assigned value*	*CV (%)*	*n*	*assigned value*	*CV (%)*	*n*
Protein C	**Chromogenic activity**	**267**	**89**	**5.2**	**267**	**20**	**14.9**	**255**	**98**	**5.0**	**255**	**109**	**5.1**
	Chromogenix Coamatic Protein C	25	89	5.2	25	18	20.9	27	97	6.8	27	109	6.4
	Hyphen Biomed Biophen Protein C	16	89	4.1	16	21	9.0	14	97	4.6	14	110	4.2
	I.L. HemosIL Protein C	71	88	4.5	71	17	9.4	62	95	3.4	62	107	4.1
	Siemens Berichrom Protein C	94	91	5.7	94	21	10.1	96	98	5.5	96	110	5.5
	Diagnostica Stago Stachrom Protein C	59	90	5.3	59	21	7.3	54	100	3.3	54	108	4.2
	Clotting activity	**86**	**90**	**10.0**	**84**	**18**	**30.4**	**77**	**105**	**10.9**	**77**	**98**	**15.9**
	I.L. HemosIL Proclot C	13	94	8.2	13	12	40.7	14	105	9.4	14	92	10.6
	Siemens Prot C Reagent (coagulometric)	20	86	6.0	20	25	16.2	18	98	5.6	18	85	9.2
	Diagnostica Stago Staclot Protein C	42	92	9.1	42	16	12.7	35	112	10.0	35	109	12.4
	Antigen (Enzyme Immuno Assays)	**70**	**81**	**9.8**	**71**	**20**	**13.7**	**67**	**96**	**11.9**	**67**	**99**	**9.8**
	BioMerieux Vidas Protein C	13	76	8.3	13	21	ND	13	89	5.5	13	96	2.9
	Diagnostica Stago Asserachrom PC	28	82	8.9	28	20	13.5	28	98	11.6	29	100	10.5

Protein S	**Activity**	**155**	**34**	**12.7**	**155**	**26**	**19.2**	**148**	**76**	**11.2**	**148**	**61**	**19.8**
	I.L HemosIL ProS	28	37	8.9	28	19	20.2	28	74	8.3	28	62	12.6
	Siemens Protein S Ac	40	35	14.8	40	25	13.1	40	68	10.2	40	45	19.4
	Diagnostica Stago Staclot Protein S	80	33	9.9	80	28	10.6	72	80	6.8	72	68	8.3
	Free PS antigen Latex Immuno Assays	**220**	**37**	**10.8**	**220**	**31**	**7.2**	**211**	**90**	**6.4**	**211**	**80**	**6.7**
	Coamatic Free PS/I.L. Hemosil Free PS	93	35	11.4	93	31	7.8	92	91	6.7	92	81	8.6
	Siemens Innovance Free Prot. S antigen	53	40	5.5	53	30	5.8	47	91	3.7	47	80	4.4
	Diagnostica Stago Liatest Free Protein S	71	37	8.3	71	30	7.3	67	87	6.0	67	80	5.7
	Enzyme Immuno Assays	**38**	**38**	**22.6**	**38**	**29**	**15.7**	**37**	**85**	**12.1**	**36**	**78**	**9.6**
	Diagnostica Stago Asserachrom Free PS	24	35	13.1	24	27	9.1	24	83	6.6	23	76	6.7

The table summarizes the most frequently used (n>10 laboratories provided results) commercially available assays for PC/PS according to the data provided in ECAT survey 2015-3 and 2014-4. Since CV was not calculated if less than 10 laboratories sent results obtained by a certain method, these methods are not described here. Total PS antigen is measured by the minority of the laboratories therefore no data is shown here. ND: not determined

for diagnosis of PC and PS deficiencies. Most of the guidelines recommend the use of chromogenic PC assays because these assays are less subjected to interference and more specific than clotting based assays (8, 58). Furthermore, chromogenic assays seemed to be cost-effective (64). In the contrary, clotting based assays are preferred by others since they ensure the diagnosis even in type IIb PC deficiency (65). Since the prevalence of IIb PC deficiency is not known and may show differences in different populations the ratio of undiagnosed cases remains uncertain by using chromogenic assay alone. For example, the p.Asn44Ile (c.131 C>T) causative mutation in the Gla-domain of PC was detected only by clotting assays, moreover there was a great discrepancy among the PC activity values of the different clotting assays (66, 67).

Recommendations concerning PS deficiency are also heterogeneous, free PS antigen is considered as superior over clotting functional assay, however, type II PS deficiency is misdiagnosed by the exclusive usage of it. The Plasma Coagulation Inhibitor Subcommittee of the International Society of Thrombosis and Hemostasis (ISTH) is now working on exploring the background of the discrepancies in PS activity results obtained by the different reagents. Until the development of a reliable PS functional assay that is free from FVL (and other) interferences the PS activity assay alone is definitely not suitable for the laboratory diagnosis of PS

deficiency and the investigation of free PS antigen and sometimes, molecular genetic tests are also suggested.

A Japanese study group developed a novel assay system for precise simultaneous determination of total PS activity and total PS antigen level, allowing PS specific activity (ratio of total PS activity to total PS antigen level) to be calculated (68). In this assay, first PS in the patient's plasma and C4bBP are dissociated by high dilution and adding liposome A that has high affinity to PS. PS is then activated by APC in the presence of PL and calcium and human FVa as substrate is added. The degradation of FVa is followed by a chromogenic reaction in which bovine FXa, prothrombin and S-2238, the chromogenic substrate of thrombin is added in the presence of PL and the change in absorbance is detected at 405 nm. To measure total PS antigen levels, first purified C4bBP is mixed with free PS in plasma and the concentration of total PS is then measured using a latex agglutination method. This assay showed good performance in detecting type II PS deficiency caused by PS Tokushima, p.Lys155Glu, a frequent mutation in the Japanese population. Interference of factors that make the results of commercial PS clotting assays difficult to interpret, especially FVL, however has not been evaluated with this reagent, yet.

MOLECULAR GENETIC DIAGNOSIS IN PC AND PS DEFICIENCY

Molecular genetic analysis of PC and PS deficiencies is also seemed to be useful like in the case of AT deficiency, but testing of patients with PC levels above 70% and with PS levels above 55% may not be indicated (31). The mutation detection rate by Sanger sequencing in the cases of *PROC* (69%) and *PROS1* (43%) is rather low. It is important to mention, that in the case of PS deficiency the presence of *PROS2* requires careful primer design to avoid amplification of pseudogene fragments. As second line test multiplex ligation-dependent probe amplification (MLPA) is suggested to detect large rearrangements and increase the mutation detection rate.

In *PROC* and *PROS1* 299 and 147 causative mutations were found before 2010; since then 11 mutations within *PROC* and 24 mutations within *PROS1* were reported in HGMD database (Table 2). It should be mentioned that several novel variants have not been reported in the HGMD, yet (e.g. ref. 69-72).

CONCLUSIONS

The majority of recommendations concerning AT/PC/PS deficiencies are based on low-quality evidence or on experts' opinions because they belong to the group of rare diseases (17) and it is impossible to conduct large clinical trials to explore the impact of diagnosis and treatment (prevention) in VTE patients, in asymptomatic affected individuals and in patients with arterial thrombosis. As in all rare diseases the impact of well-documented case reports and high quality research on genotype-phenotype associations and structural-functional studies is highly important in these diseases. More research is warranted in method development concerning well-established risk factors, like AT, PC and PS deficiencies and in clarifying so far unknown functional aspects of them. Due to the heterogeneous background of thrombosis and the different gene-gene, gene-environment interactions, population-based guidelines maybe helpful for thrombophilia testing regarding the patients' selection, the parameters to be tested and the correct methodology.

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