

# Genetic characterization of antithrombin, protein C, and protein S deficiencies in Polish patients

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## KEY WORDS

antithrombin,  
protein C, protein S,  
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## ABSTRACT

**INTRODUCTION** Inherited deficiencies of natural anticoagulants such as antithrombin (AT; gene: *SERPINC1*), protein C (PC; *PROC*), and protein S (PS; *PROS1*), with the prevalence in the general European population of 0.02% to 0.17%, 0.2% to 0.3%, and 0.5%, respectively, are associated with increased risk of thromboembolic events. Only a few case reports of Polish deficient patients with known causal mutations have been published so far.

**OBJECTIVES** The aim of the study was to characterize the frequency of *SERPINC1*, *PROC*, and *PROS1* mutations and their thromboembolic manifestations in patients with AT, PC, or PS deficiencies, inhabiting southern Poland.

**PATIENTS AND METHODS** Ninety unrelated patients (mean [SD] age, 40.1 [13.2] years) with AT (n = 35), PC (n = 28), or PS (n = 27) deficiencies, with a history of venous 73 (81%) or arterial 17 (19%) thromboembolism, were screened for mutations using the Sanger sequencing or multiplex ligation-dependent probe amplification.

**RESULTS** Twenty mutations (29%) described here were new, mostly in the *SERPINC1* and *PROC* genes. Missense mutations accounted for 84% of all mutations in the *PROC* gene and approximately 50% of those in the *SERPINC1* and *PROS1* genes. In all 3 genes, the ratio of nonsense and splice-site mutations was 8% to 31% and 8% to 23%, respectively. The mutation detection rate was 90% for AT or PC when anticoagulant activity was below 70%, while for the *PROS1* gene, the rate reached 80% at the free PS levels below 40%.

**CONCLUSIONS** To our knowledge, this is the largest cohort of Polish patients deficient in natural anticoagulants and evaluated for the causal genetic background. Several new Polish detrimental mutations were detected, mostly in AT- and PC-deficient patients.

**INTRODUCTION** The deficiencies of natural anticoagulants such as antithrombin (AT), protein C (PC), and protein S (PS) are inherited in most of the cases in an autosomal dominant pattern with variable penetrance. In the general European population, they have a frequency of 0.02%

to 0.2%, 0.2% to 0.3%, and 0.5%, respectively.<sup>1-3</sup> Individuals with inherited deficiencies of natural anticoagulants have an increased risk of venous thromboembolism (VTE), a disease whose management is still challenging and requires ongoing research and clinical studies.<sup>4,5</sup>

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A meta-analysis of case-control and cohort studies has shown that AT-deficient individuals have a higher risk of the first VTE (odds ratio [OR], 16.3; 95% CI, 9.9–26.7) than those with PC (OR, 7.1; 95% CI, 3.2–17.5) or PS (OR, 5.4; 95% CI, 2.7–10.7) deficiency.<sup>6</sup> In patients after the first incident of VTE, the prevalence of AT, PC, and PS deficiencies is estimated at 0.5% to 4.9%, 3%, and 2% to 12%, respectively.<sup>2,3,7</sup> Evidence linking natural anticoagulant deficiencies with arterial thromboembolism, mostly ischemic stroke, is conflicting and largely derived from case reports.<sup>2,8,9</sup>

AT is the main endogenous anticoagulant serpin (serine-protease inhibitor) that primarily inactivates thrombin and activated factor (F) X. The gene encoding AT (*SERPINC1*) is located on chromosome 1q25.1 and consists of 7 exons and 6 introns spanning 13,574 bp (ENSG00000117601).<sup>10</sup> PC and PS are vitamin K-dependent glycoproteins. PC is a precursor of the serine protease, activated protein C (APC). Its proteolytic activation by thrombin occurs on the surface of endothelial cells and involves thrombomodulin and endothelial PC receptor (EPCR). In the presence of PS, phospholipids, and calcium, APC inactivates activated FV and FVIII by their cleavage at the specific arginine residues.<sup>11</sup> Plasma PS is in 60% noncovalently complexed with the complement 4b-binding protein (C4BP), while the remaining 40% circulates free.<sup>12</sup> Both forms of PS serve as cofactors of APC but free PS is more potent.<sup>13</sup>

PC is encoded by *PROC*, a gene located on the long arm of chromosome 2q14. *PROC* is approximately 10 842 bp long (ENSG00000115718) and comprises 9 exons separated by 8 introns.<sup>14</sup> The PS gene (*PROS1*) is in turn located near the centromere of chromosome 3q11.2, spans 101058 bp (ENSR00001368680), and consists of 15 exons and 14 introns.<sup>15</sup>

The mutation profiles of all the 3 genes, *SERPINC1*, *PROC*, and *PROS1*, are similar with the highest prevalence for missense mutations (63%–78%), followed by nonsense mutations (7%–11%), splice-site mutations (7%–13%), small deletions (1%–8%), small insertions/duplications (1%–4%), and large deletions (3%–6%).<sup>16</sup>

Thus far, the genetic deficiency of AT has been reported in 18 Polish families from Warsaw.<sup>17</sup> To the best of our knowledge, we report here on the largest cohort of patients from Poland, the country with over 38 million inhabitants, with the deficiency of natural anticoagulants, evaluated for the causal genetic background. Previously, we were the first to publish individual Polish cases genetically confirmed for AT,<sup>18</sup> PC,<sup>19</sup> and PS<sup>20</sup> deficiencies.

**PATIENTS AND METHODS** **Patients** A total of 100 unrelated patients with a personal history of thromboembolic events (n = 89), positive family history (n = 4), or obstetric complications (n = 7) in association with reduced AT, PC, or PS levels were enrolled in the current study between

January 2013 and December 2016. Of the initially enrolled subjects, 10 were ineligible owing to incomplete clinical and laboratory data (n = 7) or patient refusal (n = 3); thus, 90 patients with AT (n = 35), PC (n = 28), and PS (n = 27) deficiencies were available for the purpose of the present study. All patients were inhabitants of southern Poland, mainly the Lesser Poland (Małopolska) region.

The diagnosis of deep vein thrombosis (DVT) was established on the basis of a positive finding of color duplex sonography (the visualization of an intraluminal thrombus in the calf, popliteal, femoral, or iliac vein). The diagnosis of pulmonary embolism (PE) was based on the presence of typical symptoms and positive results of computed tomography (CT) pulmonary angiography.

A VTE episode was defined as unprovoked (idiopathic) if the patient had no history of cancer, surgery requiring general anesthesia, major trauma, a plaster cast or hospitalization within the last month, or pregnancy or delivery within the last 3 months. A proximal DVT was defined as thrombosis in the popliteal veins, including the trifurcation, femoral, or iliac veins.

The diagnosis of ischemic stroke was based on clinical symptoms according to the World Health Organization definition ([http://www.who.int/medicines/areas/priority\\_medicines/BP6\\_6Stroke.pdf?ua=1](http://www.who.int/medicines/areas/priority_medicines/BP6_6Stroke.pdf?ua=1)) and brain imaging, usually noncontrast CT. The diagnosis of myocardial infarction was based on the 2012 European Society of Cardiology criteria.<sup>21</sup>

The diagnosis of portal, splenic, and subclavian vein thrombosis was based on positive findings on a color duplex scan. In more complicated cases, CT angiography or magnetic resonance imaging was needed. The diagnosis of central retinal vein occlusion was based on typical clinical symptoms (abrupt unilateral vision deterioration) and typical appearance of the eye fundus.

The diagnosis of femoral and brachial artery thrombosis was established on the basis of clinical symptoms of acute extremity ischemia and visualization of artery stenosis by color duplex scan sonography or by digital subtraction ultrasound. The diagnosis of mesenteric artery thrombosis was based on clinical symptoms of acute intestinal ischemia and positive results of CT angiography. The diagnosis of cerebral venous sinus thrombosis was established by visualizing sinus stenosis on magnetic resonance angiography.

The exclusion criteria were previous or current diagnosis of liver disease, cancer, pregnancy, and blood sampling at acute thrombotic event.

The Ethical Committee of Jagiellonian University Medical College approved the study, and all 90 participants provided their written informed consent.

**Sample collection** Blood samples were drawn from an antecubital vein into tubes containing citrate anticoagulant (9:1 of 0.106 M sodium citrate), centrifuged at 2.500 g at a temperature of

18°C to 22°C for 20 minutes and processed immediately or stored in aliquots at –80°C until analysis. Whole blood samples for DNA isolation were drawn into EDTA-K3 collection tubes and stored in aliquots at –80°C until processing.

**Laboratory tests** The antigen levels and activities of AT, PC, and PS were measured using established commercial assays after temporary withdrawal of vitamin K antagonists (for at least 10 days)<sup>22</sup> or oral novel anticoagulants (for at least 24 hours).<sup>23</sup>

**Antithrombin** AT activity was measured using an assay based on FXa inhibition (INNOVANCE™ ATIII, Siemens Healthcare Diagnostics, Marburg, Germany) or thrombin-inhibition assays (Siemens Healthcare Diagnostics); the reference range for both was from 83% to 118%. AT antigen was determined nephelometrically (Siemens Healthcare Diagnostics; reference range, 0.19–0.31 g/l). AT deficiency was classified as described previously.<sup>10,24</sup>

**Protein C** Plasma PC activity was quantified using a chromogenic assay (HemosIL Protein C Instrumentation Laboratory, Milan, Italy or Berichrom Protein C, Siemens Healthcare Diagnostics), and PC antigen was measured by an enzyme-linked immunosorbent assay (Asserachrom Protein C, Diagnostica Stago, Asnieres, France) (reference range for all, 70%–140%). PC deficiency was classified as described previously.<sup>25</sup>

**Protein S** Free PS levels were measured using an immunoturbidimetric assay (HemosIL Free Protein S; reference range for women, 55%–124%; reference range for men, 74%–146% or INNOVANCE® Free PS Ag, Siemens Healthcare Diagnostic; reference range for women, 60%–114%; reference range for men, 67%–139%). Total PS levels were assessed using Asserachrom Kit (Diagnostica Stago; reference range, 75%–140%). PS deficiency was classified as previously described.<sup>25</sup>

**Genetic analysis** DNA was extracted from whole blood or a buffy coat according to the manufacturer's protocol, using Gene MATRIX Quick Blood DNA Purification Kit (Eurex, Gdańsk, Poland) and stored at –80°C until analysis.

The *SERPINC1* (NM\_000 488.3), *PROC* (NM\_000 312.3), and *PROS1* (NM\_000 313.3) genes were analyzed as previously described.<sup>26–28</sup> For all samples with no causative point mutation identified, screening for large deletion/insertion was performed by multiplex ligation-dependent probe amplification (SALSA MLPA kit for P227 SerpinC1; P265-PROC and P112-A2 PROS1; MRC-Holland, Amsterdam, the Netherlands).

The analysis of a congenital disorder of glycosylation was performed as described previously.<sup>29</sup>

**In silico analysis** Each novel sequence variation was subjected to in silico analyses with a Sorting

Intolerant From Tolerant tool (<http://sift.jcvi.org>) integrated into Alamut Visual v2.3.6 software application (<http://www.interactive-biosoftware.com/alamut-visual/>); a structure-based method, Polymorphism Phenotyping v2 (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph/>); and the splicing prediction module of Alamut Visual, which uses the Human Splicing Finder v3.0 (HSF; <http://www.umd.be/HSF/>) algorithms.

**Statistical analysis** The distributions of quantitative variables were analyzed by the Shapiro–Wilk test. Normally distributed variables were compared using 1-way analysis of variance (ANOVA) or the *t* test and were presented as mean (SD). Variables deviating from normal distribution were analyzed by the Kruskal–Wallis ANOVA or Mann–Whitney test and were presented as median (interquartile range) if not otherwise indicated. Qualitative parameters were analyzed by the Pearson  $\chi^2$  or 2-tailed Fisher exact test. Pairwise correlations were calculated using the Pearson coefficient on variables approximated to the normal distribution if required. A *P* value of less than 0.05 was considered significant. Statistical calculations were performed using STATISTICA Version 10.0 (StatSoft, Inc., Tulsa, Oklahoma, United States).

**RESULTS** The mean (SD) age of 90 participants with natural anticoagulant deficiencies was 40.1 (13.2) years. Forty-seven patients (52.2%) were male. The mean (SD) age at the first VTE event was 35.6 (12.4) years. Thirty-one patients (34%) experienced a provoked VTE event, mostly despite pharmacological thromboprophylaxis. These patients were younger than those with an unprovoked VTE (mean [SD] age, 32.2 [2.2] years vs 44.3 [1.8] years; *P* = 0.02). Thirty-four patients (38%) had a positive family history of VTE.

Of the 90 patients enrolled, 65 (72%) carried genetic defects. Twenty mutations (29%) detected in the current study have been reported for the first time, according to the comparison with the HGMD® database records. All new mutations were probably damaging and disease-causing as suggested by the in silico analysis.

**Antithrombin deficiency** The main characteristics of the AT-deficient cohort (*n* = 35) are shown in **TABLE 1**. The mean (SD) age was 43.0 (13.9) years, and 20 patients (57%) were male. The mean (SD) age at the first VTE event was 37.1 (14.1) years. The mean (SD) AT activity was 57.8% (9.3%) (less than 75% in all participants), and the median (IQR) AT antigen level was 0.16 g/l (0.15–0.22 g/l). Type I AT deficiency was detected in 19 patients (54.3%) and type II—in 11 patients (31.4%). In the remaining 5 patients (14.3%), the type of AT deficiency could not be determined due to incomplete laboratory data.

Antigen levels correlated with age of the first VTE event (*r* = 0.6; *P* = 0.002). Patients with type I AT deficiency were younger when they had experienced the first VTE event compared with patients

**TABLE 1** Characteristics of patients with antithrombin deficiency (n = 35)

Patient ID	Sex/age	AT activity, %	AT antigen, g/l	Type of AT deficiency	Type of mutation in SERPINC1 gene	Exon number	New/Reported <sup>a</sup>	Clinical manifestation	Age of first thromboembolic event	Number of VTE events	Unprovoked/Provoked	Family history of VTE
1	M/51	39	0.09	I	c.1012G>T p.Glu338* g.12686G>T	Exon 5	New	Portal thrombosis and superior mesenteric artery thrombosis	48	2	1/0	0
2	F/40	62	0.17	I	c.1373_1384del p.Val458_Cys462del insGly g.18468_18479del	Exon 7	New	DVT + PE, stroke	39	2	1/0	1
3	F/31	55	0.13	I	c.9484delC p.Leu317Cysfs*11 g.12622del	Exon 5	New	DVT	27	1	1/0	1
4	F/46	44	0.12	I	c.352G>C p.Ala118Pro g.7770G>C	Exon 2	New	DVT	26	2	0/postpartum period (n = 2)	0
5	M/50	66.1	0.22	II	c.373G>T p.Gly125Cys g.7791G>T	Exon 2	New	DVT + PE	37	2	1/0	0
6	F/20	60	0.13	I	c.625-2A>G p.IVS3-2A>G g.11486A>G	Intron 3	New	DVT, portal vein thrombosis	15	3	0/longhaul travel	1
7	M/27	55	0.15	I	c.1297A>T p.Thr433Ser g.18392A>T	Exon 7	New	DVT	27	2	1/0	0
8	F/61	38	N/D	N/D	c.302C>G p.Ser101Cys g.7720C>G and c.341G>A p.Ser114Asn g.7759G>A	Exon 2 (both)	c.302C>G – new c.341G>A – reported <sup>1</sup>	DVT	22	3	1/0	1
9	F/30	56	0.16	I	c.4384delA p.Lys146delifs*444 g.10394delA	Exon 2	Reported (AT Kraków) <sup>7</sup>	Asymptomatic	N/A	0	N/A	1
10	M/26	53	0.15	I	c.624+1G>T p.IVS3a+1G>T g.10581G>T	Intron 3	Reported (AT Kraków II) <sup>3</sup>	DVT + PE	26	2	0/trauma	0
11	F/44	46	0.15	I	c.683 G>T p.Gly228Val g.11546G>T	Exon 4	Reported (AT Rybnik) <sup>5</sup>	DVT + PE, bilateral superficial venous thrombosis of the lower limbs	22	5	0/cesarean delivery	1
12	M/46	50	0.13	I	Gross deletion of the whole exon 1	Exon 1	Reported (AT Katowice) <sup>5</sup>	DVT + PE	44	2	0/longhaul travel	1
13	F/32	67	0.22	II	c.1157T>C p.Ile386Thr g.14868T>C	Exon 6	Reported <sup>6</sup>	DVT and DVT + PE	18	3	0/longhaul travel	1
14	M/47	60	0.25	IIb	c.391C>T p.Leu131Phe g.7809C>T	Exon 2	Reported (AT Budapest III) <sup>7</sup>	DVT	59	1	0/arthroscopic surgery	0
19	F/73	59.8	0.34	II HBS	c.166C>T p.Arg56Cys g.7584C>T	Exon 2	Reported (AT Rouen IV) <sup>3</sup>	PE	71	1	1/0	0

15	M/45	57	0.22	II	HBS	c.166C>T p.Arg56Cys g.7584C>T	Exon 2	Reported (AT Rouen IV) <sup>8</sup>	DVT	44	1	1/0	1 (MI in father at young age)
16	F/40	47–81	N/D	II		c.1317C>A p.Pro407Thr g.18316C>A	Exon 7	Reported (AT Budapest V) <sup>9</sup>	Right subclavian vein thrombosis	37	1	1/0	0
17	M/43	58	0.17	I		c.1154–14G>A p.IVSS–14G>A g.9788G>A	Intron 5	Reported <sup>10</sup>	DVT	39	5	0/longhaul flight	0
18	M/68	66.7	N/D	II		c.1274G>A p.Arg425His g.18369G>A	exon 3	Reported (AT Glasgow) <sup>11</sup>	DVT + PE	52	2	1/0	1
20	M/52	53.9	N/D	II		c.236G>A p.Arg79His g.7654G>A	Exon 2	Reported (AT Rouen I) <sup>12</sup>	Splenic vein thrombosis, left testis ischemia	49	1	1/0	0
21	F/42	66–74	0.20	I		c.89T>A p.Val30Glu g.7507T>A	Exon 2	Reported (AT Dublin) <sup>13</sup>	DVT + PE	42	1	1/0	0
22	M/21	61	0.15	I		c.481C>T p.Arg161* g.10437C>T	Exon 3	Reported <sup>14</sup>	DVT	17	1	1/0	0
23	M/31	55	0.16	I		c.667T>C p.Ser223Pro g.11530T>C	Exon 4	Reported <sup>15</sup>	DVT + PE	31	1	1/0	0
24	M/21	53	0.29	II		c.1246G>C p.Ala416Pro g.18341G>C	Exon 7	Reported <sup>16</sup>	PE	20	1	1/0	0
25	M/53	52	0.16	I		Duplication of exon 6	Exon 6	New	Bilateral femoral artery thrombosis, stroke	17	4	0/strenuous physical exercise	1
26	F/41	48	N/D	N/D		Deletion of exon 2 to 5	Exon 2–5	New	DVT + PE	40	1	1/0	0
27	M/54	53	0.16	I		Transient glycosylation disorder	N/A	N/D	DVT	53	1	1/0	0
28	M/43	42–63	0.15	I		Glycosylation disorders	N/A	p.Arg141His in PMM2 gene <sup>17</sup>	DVT	39	1	1/0	0
29	M/33	65	0.2	II		Glycosylation disorders	N/A	N/D	DVT + PE	33	1	1/0	0
30	F/42	69	N/D	N/D		No mutation	N/A	N/A	DVT	37	3	1/0	0
31	F/58	70.7	0.17	I		No mutation	N/A	N/A	DVT, stroke	52	2	1/0	0
32	M/18	66.2	0.17	I		No mutation	N/A	N/A	DVT + PE	16	1	1/0	0
33	M/56	48.7	N/D	N/D		No mutation	N/A	N/A	DVT + PE	51	1	1/0	0
34	F/57	73–82	0.23	II		No mutation	N/A	N/A	DVT, MI (n = 2)	57	1	1/0	0
35	M/37	72	N/D	N/D		No mutation	N/A	N/A	Central retinal vein occlusion	37	1	1/0	0

<sup>a</sup> References 1–17 are listed in Supplementary material online.

Mutations were given coordinates according to the HUGO recommendations for mutation nomenclature (<http://www.hgvs.org>).

Abbreviations: DVT, deep vein thrombosis; F, female; M, male; MI, myocardial infarction; N/A, not applicable; N/D, not definable; PE, pulmonary embolism

with type II AT deficiency (mean [SD] age, 32.2 [3.2] years vs 45.7 [4.3] years;  $P = 0.02$ ).

**SERPINC1 mutations** Of the 35 patients with AT deficiency, 26 individuals had a mutation in the *SERPINC1* gene, with a mutation detection rate of 74%. Glycosylation disorders with mutation in the phosphomannomutase 2 gene (*PMM2*) were detected in 3 patients.

Missense mutations represented the most frequent causal genetic variant type and were identified in 15 patients (57.7%), followed by nonsense mutations in 4 patients (15.4%), splice-site mutations in 3 patients (11.5%), large deletions in 2 patients (7.8%), and small deletion and large duplication (1 patient [3.8%] each). In half of the patients (50%), mutations were located mainly in exon 2 (9 patients) or exon 7 (4 patients).

Eight (31%) mutations were reported here for the first time (TABLE 1). The whole spectrum of mutations was present in patients with type I AT deficiency, while in patients with type II AT deficiency, only the missense variants were found. Patients with missense mutations tended to have higher AT antigen expression than individuals with other types of causal genetic variants (0.21 g/l vs 0.15 g/l,  $P = 0.06$ ).

**Venous and arterial thrombosis** The most common thrombotic manifestation was isolated DVT ( $n = 14$ ; 40%) or combined with PE ( $n = 13$ ; 37%). Isolated PE was present only in 2 patients. Moreover, we observed patients with portal vein thrombosis ( $n = 2$ ; 6%), subclavian vein thrombosis ( $n = 1$ ; 3%), splenic vein thrombosis and testis ischemia ( $n = 1$ ; 3%), and central retinal vein occlusion ( $n = 1$ ; 3%) (TABLE 1).

Arterial thromboembolic events occurred in 5 patients (14%) and comprised 3 cerebral ischemic strokes, 1 myocardial infarction (with recurrence), and 1 superior mesenteric artery thrombosis combined with portal vein thrombosis. One individual was asymptomatic but had a positive family history.

**Protein C deficiency** The main characteristics of the PC-deficient patients are shown in TABLE 2. The mean (SD) age of the patients was 38.3 (13.4) years. Sixteen patients (57%) were male. The first VTE event occurred at a mean (SD) age of 35.0 (12.6) years.

The mean (SD) PC activity was 51.1% (12.9%), and the mean (SD) total PC was 63.4% (25.9%). Thirteen patients (46.4%) had type I PC deficiency, and 2 individuals (7.2%)—type II. In 13 patients (46.4%), the type of PC deficiency could not be determined owing to incomplete laboratory data.

As expected, PC activity was positively correlated with age ( $r = 0.38$ ;  $P < 0.05$ ). Patients who experienced an idiopathic VTE were older than those with a provoked VTE (mean [SD] age, 40.8 [3.4] years vs 30.8 [3.6] years;  $P = 0.05$ ) and had higher PC activity levels (mean [SD], 57.9% [3.5%] vs 42.8% [3.7%];  $P = 0.008$ ).

**PROC mutations** Of the 28 patients with PC deficiency, 25 individuals had the mutation in the *PROC* gene, with a mutation detection rate of 89.3%.

Missense mutations occurred in 21 patients (84%), while nonsense and splice-site mutations in 2 patients each (8% for both). No gross *PROC* gene rearrangements were observed.

The detected mutations were mainly located in exon 9 (9 patients), exon 5 (5 patients), and in exon 7 (4 patients), which comprised 72% of the patients. Eight mutations (32%) have been reported here for the first time (TABLE 2).

**Venous and arterial thrombosis** The most common thrombotic manifestation was isolated DVT ( $n = 10$ ; 36%) or combined with PE ( $n = 9$ ; 32%). Isolated PE was not found. One patient (3.6%) suffered from DVT and superficial vein thrombosis. Among atypical VTE, portal vein thrombosis ( $n = 1$ ; 3.6%) and cerebral venous sinus thrombosis ( $n = 1$ ; 3.6%) were observed. One woman experienced 3 miscarriages (3.6%).

Arterial thrombosis occurred in 5 patients (18%) and included 2 myocardial infarctions, 2 strokes, and brachial arterial embolism. Three subjects were asymptomatic but had a positive family history (TABLE 2).

**Protein S deficiency** The main characteristics of the PS-deficient cohort are shown in TABLE 3. The mean (SD) patient age was 38.2 (13.1) years. Ten patients (37%) were male. The first VTE event occurred at the mean (SD) age of 34.0 (9.4) years.

The mean (SD) free PS levels were 37.0% (14.7%) and the mean (SD) total PS antigen was 62.7% (18.0%). Fourteen patients (52.5%) had type I and 11 patients (40.1%) had type III PS deficiency. In 2 patients (7.4%), the type of PS deficiency could not be determined owing to incomplete laboratory data or variations in total PS levels.

Patients with idiopathic VTE had lower free PS levels than those with provoked VTE (mean [SD], 27.1% [4.0%] vs 41.6% [4.0%];  $P = 0.02$ ).

**PROS1 mutations** Of the 27 patients with *PROS1* deficiency, 14 individuals had the *PROS1* gene mutation with a mutation detection rate of 52%. If the cutoff value for free PS levels was above 40%, the mutation detection rate reached 77%.

Missense mutations were observed in 6 patients (46%); nonsense mutations, in 4 patients (31%); and splice-site mutations, in 3 patients (23%). One deletion of exons 1 to 12 was found. The detected mutations were located across the whole *PROS1* gene. Three mutations (21%) have been reported here for the first time (TABLE 3).

**Venous and arterial thrombosis** The most common thrombotic manifestation was isolated DVT ( $n = 15$ ; 55.7%). DVT combined with PE was observed in 3 patients (11.1%), and isolated PE—in 1 patient (3.7%). One event of cerebral venous

sinus thrombosis (3.7%) and four miscarriages (14.8%) were noted.

Arterial thrombosis was found in 7 patients (26%), including 7 strokes, 2 of which occurred after myocardial infarction (TABLE 3).

**Mutation detection rate** AT activity levels ranged between 38% and 67% in patients with a detected *SERPINC1* gene mutation and glycosylation disorders, and between 49% and 77.5% in subjects without the mutation. In individuals with AT activity above 70%, no mutations were found, while in those with AT activity below 70%, the mutation detection rate was 90%.

Similarly, patients with a mutation in the *PROC* gene showed PC activity between 19% and 67%. In 3 patients without the mutation, PC activity levels ranged from 18% to 53%. For PC activities close to the normal range (70%), the mutation detection rate was above 90%.

Patients with a mutation within the *PROS1* gene had the PS activity levels of 8% to 53%, and those without the mutation—of 37% to 59%. For free PS levels below 40%, the mutation detection rate was 77%, and for free PS levels below 35%, the rate was 100%.

**DISCUSSION** To the best of our knowledge, this is the first large and comprehensive study analyzing the genetic background of deficiencies in natural anticoagulants, AT, PC, and PS, not only in the Polish but also in the Slavic population. Twenty novel mutations were identified, all of which present in the heterozygous state.

The frequency of missense, nonsense, and splice-site mutations was similar for all 3 genes/proteins. Missense mutations accounted for approximately 50% of all the causal variants detected. Large rearrangements were found in the *SERPINC1* and *PROS1* genes. The detected mutations were located mainly in exon 2 and 7 of the *SERPINC1* gene, in exons 9, 7, and 5 of the *PROC* gene, and, with no exon-wise clustering, across the whole *PROS1* gene.

The newly detected p.Glu338\* and the p.Val458\_Cys462del insGly mutations in the *SERPINC1* gene might impair protein synthesis/secretion and are most likely responsible for type I AT deficiency. Both variants were found also in the families of the probands and were associated with VTE events. Similarly, another new variant, deletion of cytosine (c.948delC), may lead to production of truncated protein followed by a frameshift and the appearance of a premature stop codon in the *SERPINC1* gene. In turn, the new p.Ala118Pro and the p.Gly125Cys variants both affect a very highly conserved residues in serpins located at helix B and may thus probably disturb the correct folding of AT. They may lead to the polymerization of the variant AT molecules and formation of disulphide-linked dimers present in the patient's plasma. These dimers may accumulate intracellularly in hepatocytes, affecting the synthesis of a wild-type AT and other inflammatory or

hemostatic proteins, especially in situations associated with increased thrombotic risk, such as pregnancy or infection.<sup>30,31</sup> In the current study, only a patient carrying the p.Ala118Pro variant had 2 DVT events in the postpartum period. The proband's daughter inherited this mutation and so far remains asymptomatic.

Another new variant is an intronic c.625-2A>G mutation affecting splicing by disturbing the acceptor sequence of intron 3. This mutation is associated in our patient with type I AT deficiency and was also detected in the proband's father, suffering from DVT. In turn, the next novel variant, the p.Thr433Ser mutation, affects a residue which, although not conserved in serpins, is located at the first strand of C  $\beta$ -sheet and may thus affect protein stability.

We also detected 1 compound heterozygote within the *SERPINC1* gene comprising the novel p.Ser69Cys variant and previously described as p.Ser82Asn mutation.<sup>32</sup> The p.Ser69Cys variant affects helix A, potentially influencing protein stability. However, the p.Ser82Asn mutation, responsible for creating a new N-glycosylation site associated with disturbances in peptide folding ultimately leading to no protein secretion,<sup>32</sup> seems to play a more important role. In patients in whom no point mutations were found, 2 new large gene rearrangements were detected: a duplication of exon 6 and a deletion of exons 2 to 5.

The majority of the newly detected *PROC* gene mutations, including p.Cys387Tyr, p.Val434Ala, and p.Leu320Pro, cluster in exon 9, within the region encoding a catalytic domain. These variants may potentially affect binding of the substrate.<sup>33</sup> Moreover, in the missense variant at position 387, cysteine is replaced by tyrosine, which may be important for disulfide bond formation, associated with protein stabilization. In turn, the p.Cys64Tyr variant of the *PROC* gene is located in the  $\gamma$ -carboxylglutamic domain within the PC structure. This domain is implicated in interactions of PC with its cofactors PS and EPCR for inactivation of FVa and FVIIIa.<sup>34</sup> The p.Cys64Tyr variant eliminates cysteine residues, which might result in aberrant protein multimerization or creation of the high-molecular-weight protein complex and impaired protein secretion.<sup>33,35</sup> A similar mutation in the same codon but with a different amino acid change (p.Cys64Gly) has been reported by Kuismanen et al<sup>35</sup> in a family from Finland, with a history of thrombosis and type I PC deficiency. The next new p.Cys175Arg variant within the *PROC* gene is located in the epidermal growth factor (EGF)-2 domain and might affect PC stability.<sup>33</sup>

Another novel mutation, p.Gln226\*, is located in the serine-protease domain and may yield a truncated protein product. It has been found that amino acids at position 225–235 of the PC serine-protease domain play an important role in the interaction between the PC and the thrombin-thrombomodulin complex through a calcium-binding mechanism.<sup>36</sup> Two other missense

**TABLE 2** Characteristics of patients with protein C deficiency (n = 28)

Patient ID	Sex/age	PC activity, %	PC total, %	Type of PC deficiency	Type of mutation in <i>PROC</i> gene	Exon number	New/Reported <sup>a</sup>	Clinical manifestation	Age of first thromboembolic event	Number of VTE events	Unprovoked/Provoked	Family history of VTE
1	M/33	55.4	N/D	N/D	c.191G>A p.Cys64Tyr g.7984G>C	Exon 3	New	DVT	32	1	1/0	0
2	F/60	68	61.5	I	c.536-99C>G p.IVS6-99C>G g.12567C>G	Intron 6	new	DVT	59	1	1/0	1
3	M/31	39	N/D	N/D	c.523T>C p.Cys175Arg g.997T>C	Exon 6	New	DVT	9	2	0/1	0
4	M/59	63.6	N/D	N/D	c.676C>T p.Gln226* g.12806C>T	Exon 7	New	DVT	49	2	1/0	0
5	M/51	56.1	N/D	N/D	c.1160G>A p.Cys387Tyr g.15301G>A	Exon 9	New	DVT	49	1	0/1	1
6	F/41	60.7	N/D	N/D	c.1301T>C p.Val434Ala g.15442T>C	Exon 9	New	Portal vein thrombosis	40	1	1/0	0
7	F/33	56	82.4	II	c.1301T>C p.Val434Ala g.15442T>C	Exon 9	New	DVT	33	2	0/oral contraceptives	1
8	M/30	51.6	N/D	N/D	c.959T>C p.Leu320Pro g.15100T>C	Exon 9	New	DVT + PE	24	1	1/0	0
9	M/29	42	44	I	c.316T>C p.Cys106Arg g.9668T>G	Exon 5	Reported <sup>18</sup>	DVT + PE	29	2	0/trauma	0
10	M/37	51	65.5	I	c.316T>C p.Cys106Arg g.9668T>G	Exon 5	Reported <sup>18</sup>	DVT + PE	37	0	1/0	0
11	F/22	42.4	N/D	N/D	c.316T>C p.Cys106Arg g.9668T>G	Exon 5	Reported <sup>18</sup>	DVT	20	1	0/1	0
12	M/26	48	44.9	I	c.316T>C p.Cys106Arg g.9668T>G	Exon 5	Reported <sup>18</sup>	Asymptomatic	N/A	N/A	N/A	1
13	F/49	41.5	55.7	I	c.632G>A p.Arg211Gln g.12762G>A	Exon 7	Reported <sup>19</sup>	DVT + PE	47	1	0/1	1
14	F/32	64.4	66.3	I	c.169C>T p.Arg57Trp g.7962C>T	Exon 3	Reported <sup>20</sup>	DVT + PE, MI	20	2	0/pregnancy	0
15	M/28	49	46	I	c.325G>C p.Gly109Arg g.9677G>C	Exon 5	Reported <sup>21</sup>	STEMI	28	0	N/A	0
16	F/38	19	N/D	N/D	c.400+2T>C p.IVS5+2T>C g.9754T>C	Intron 5	Reported <sup>22</sup>	DVT + PE, stroke	21	2	0/pregnancy	0
17	M/43	60	64.8	I	c.1015G>A p.Val339Met g.15156G>A	Exon 9	Reported <sup>23</sup>	Internal carotid artery thrombosis, stroke (n = 2)	42	1	1/0	0



Ref	Sex	Age	Genotype	Exon	Reported <sup>24</sup>	DVT	33	2	0/0	1		
18	M/49	61	57.3	I	c.677A>T p.Gln226Leu g.12807A>T	Exon 7	Reported <sup>24</sup>	DVT	33	2	0/0	1
19	F/54	54	N/D	N/D	c.759C>A p.His253Gln g.13766C>A	Exon 8	Reported <sup>25</sup>	DVT + PE	45	1	1/0	0
21	F/65	66.9	N/D	N/D	c.759C>A p.His253Gln g.13766C>A	Exon 8	Reported <sup>25</sup>	Cerebral venous sinus thrombosis	62	1	1/0	0
20	M/41	66.2	N/D	N/D	c.658C>T p.Arg220Trp g.12788C>T	Exon 7	Reported <sup>26</sup>	DVT, superficial vein thrombosis	32	1	1/0	0
22	M/34	57.5	N/D	N/D	c.1042C>T p.Arg348* g.15183C>T	Exon 9	Reported <sup>27</sup>	DVT	32	1	0/1	0
23	F/40	35	43.7	I	c.802T>C p.Tyr268His g.14943T>C	Exon 9	Reported <sup>28</sup>	DVT + PE	40	1	0/surgery	0
24	F/33	55.5	64.9	I	c.925G>A p.Ala309Thr g.15066G>A	Exon 9	Reported <sup>29</sup>	Miscarriages (n = 3)	20	N/A	N/A	0
25	F/25	45	50.3	I	c.935C>T p.Ser312Leu g.15076C>T	Exon 9	Reported <sup>30</sup>	Asymptomatic	N/A	N/A	N/A	1
26	M/50	18.3	148.5	II	No mutation	N/A	N/A	Bilateral DVT + PE	39	3	1/0	0
27	M/41	36.6	N/D	N/D	No mutation	N/A	N/A	DVT + brachial arterial embolism	34	1	1/0	0
28	M/1	53	55.4	I	No mutation	N/A	N/A	Asymptomatic	N/A	N/A	N/A	1

<sup>a</sup> References 18–30 are listed in Supplementary material online.

Mutations were given coordinates according to the HUGO recommendations for mutation nomenclature (<http://www.hgvs.org>).

Abbreviations: see TABLE 1

mutations at the same position, p.Gln226His and p.Gln226Leu, have been described in a newborn Spanish child with purpura fulminans and a Spanish patient screened for thrombophilia after a DVT event, respectively.<sup>37,38</sup> In the current study, the missense variant Gln226Leu in exon 7 was also detected in a 49-year-old male patient with DVT. Interestingly, the p.Cys106Arg variant, previously described by us in a young Polish man with provoked DVT and PE,<sup>39</sup> was detected in the current study in 3 other unrelated patients.

The 2 new mutations in the *PROS1* gene, p.Cys126Gly and the p.Cys241\*, are located in the EGF-like-1 and the EGF-like-3 calcium binding domains, respectively. It has been shown that most missense mutations identified within the EGF domains of PS affect protein stability, and cysteine residues are replaced in half of them.<sup>3</sup> It may be speculated that our novel p.Cys126Gly variant disrupts the protein structure, resulting in protein instability. The second newly identified mutation, p.Cys241\*, might lead to the production of truncated protein. As PS is a principal cofactor of APC, its impaired secretion would limit the degradation of FVa and FVIIIa and enhance the procoagulant conditions.<sup>3,40</sup> The last new variant within the *PROS1* gene, p.Gly489Arg, is located in the laminin-G-like-2 domain, suggested to comprise the C4BP-binding sites within segments 408–434, 447–460, and 583–635.<sup>41</sup> Although this new variant is located outside the reported C4BP-binding sites, changing nonpolar and neutral glycine to polar and basic arginine might be expected to influence the C4BP binding.

In 32% of AT-deficient, 6% of PC-deficient, and 50% of PS-deficient individuals, no mutation in the respective genes was detected in the present study. It is possible that other genes on chromosome 16q23 and 1q32 influence the PC and PS levels.<sup>16,42,43</sup> PC activity can be modulated also by the genetic variants of EPCR<sup>44</sup> and the polymorphisms of the genes involved in the vitamin K-dependent  $\gamma$ -carboxylation reaction may also influence the activities of PC and PS.<sup>45</sup> It should be also noted that for the measurement of AT activity, both the bovine thrombin- and the FXa inhibition-based tests, together with an antigen method, are advisable to be performed in patients with suspected type II AT deficiency.<sup>46</sup> For thrombin-based tests, a bovine thrombin should be preferred because a human thrombin reacts also with the heparin cofactor II, thus leading to overestimation of AT.<sup>47</sup> On the other hand, false negative detection of all types of AT deficiencies with a FXa inhibition-based test, which may overestimate AT activity levels, is also possible.

Interestingly, in the current study, no mutations were found in patients with AT activity above 70%. This is in contrast to a study by Caspers et al,<sup>16</sup> conducted in German patients, in whom the mutation detection rate remained at a level of about 70% for AT activities up to 75%.<sup>16</sup> It seems that in our population, a genetic analysis of the *SERPIC1* gene should be performed in patients

**TABLE 3** Characteristics of patients with protein S deficiency (n = 27)

Patient ID	Sex/age	Free PS, %	Total PS, %	Type of PS deficiency	Type of mutation	Exon number	New/Reported <sup>a</sup>	Clinical manifestation	Age of first thromboembolic event	Number of VTE events	Unprovoked/Provoked	Family history of VTE
1	F/33	10–49	N/D	N/D	c.376T>G p.Cys126Gly g.72977T>G	Exon 6	New	DVT, stroke	23	2	0/trauma	1
2	F/28	23	53.6	I	c.723T>A p.Cys241* g.78283T>A	Exon 8	New	DVT	27	1	0/cesarean section	1
3	F/46	35	68.1	III	c.1465G>C p.Gly489Arg g.94336G>C	Exon 13	New	Stroke	45	1	1/0	1
4	M/42	8	65.08	I	c.1020C>A p.Tyr340* g.86023C>T	Exon 11	Reported <sup>31</sup>	DVT	42	1	1/0	1
5	F/17	15	60.21	I	dup GA-Tyr131Aspfs*3	Exon 6	Reported <sup>31</sup>	DVT	17	1	1/0	1
6	M/50	53	110.5	III	c.1501T>C p.Ser501Pro Heerlen g.99785T>C	Exon 14	Reported <sup>32</sup>	DVT	38	4	1/0	1
7	M/45	38	81.6	III	c.1501T>C p.Ser501Pro Heerlen g.99785T>C	Exon 14	Reported <sup>33</sup>	DVT, MI, stroke	42	3	0/arthrosopy	1
8	M/21	35	37.1	I	c.1351C>T p.Arg451* g.94222C>T	Exon 13	Reported <sup>34</sup>	DVT+PE	21	2	0/trauma	1
9	F/22	40	68.9	III	c.903C>G p.Tyr301* g.82453C>G	Exon 10	Reported <sup>35</sup>	DVT	22	1	0/oral contraception	1
10	M/69	25	37.4	I	c.1155+5G>A p.IVS10+5G>A g.86163G>A	Intron 11	Reported <sup>34</sup>	DVT+PE	40	2	1/0	1
11	M/34	19.6	28.6	I	c.1168G>A p.Glu390Lys g.92599G>A	Exon 12	Reported <sup>36</sup>	Bilateral DVT + PE	27	2	1/0	0
12	F/47	18	68.2	III	c.557G>A p.Cys186Tyr g.73263G>A	Exon 7	Reported <sup>37</sup>	Stroke	46	1	1/0	0
13	M/42	16	41.8	I	c.965+4A>G p.IVS9+4A>G g.82519A>G	Intron 10	Reported <sup>38</sup>	PE	40	1	1/0	0
14	M/25	18.9	40	I	Deletion of exon 1 to 12	Exon 1–12	Reported <sup>39</sup>	DVT	24	2	1/0	1
15	M/38	46	62.5	I	No mutation	N/A	N/A	DVT	35	1	0/strenuous physical exercise	0
16	F/28	42	77.3	I	No mutation	N/A	N/A	DVT	28	1	0/pregnancy	0
17	F/53	38	62.1	I	No mutation	N/A	N/A	DVT, stroke	44	2	0/pregnancy	1
18	F/39	53	36.7	I	No mutation	N/A	N/A	DVT	32	1	0/pregnancy	0
19	F/26	59	82.6	III	No mutation	N/A	N/A	DVT	26	1	0/surgery	0
20	M/66	50	72.2	III	No mutation	N/A	N/A	DVT	49	1	1/0	0
21	F/41	40	79.3	III	No mutation	N/A	N/A	DVT, miscarriages (n = 3)	38	1	1/0	0

22	F/34	54.7	60.4	I	No mutation	N/A	N/A	Cerebral venous sinus thrombosis, stroke	33	1	0	0
23	F/39	37	74	III	No mutation	N/A	N/A	Miscarriages (n = 2)	37	0	N/A	0
24	F/33	52	67.3	III	No mutation	N/A	N/A	Miscarriage	N/A	0	N/A	1
25	F/33	49.4	58.7	I	No mutation	N/A	N/A	Miscarriage	N/A	0	N/A	1
26	F/25	51	57.9–77.3	N/D	No mutation	N/A	N/A	Stroke	26	0	N/A	1
27	F/56	53	67.7	III	No mutation	N/A	N/A	MI, stroke	53	0	N/A	0

**a** References 31–39 are listed in Supplementary material online.

Mutations were given coordinates according to the HUGO nomenclature (<http://www.hgvs.org>).

Abbreviations: see [TABLE 1](#)

with AT activity below 70%, where the mutation detection rate was 90%. However, due to a small sample size, this finding should be interpreted with caution. On the other hand, the study on the German population showed that the genetic analysis of the *PROC* gene should be performed in patients with PC activities below 70%, which worked fine also for our patients.<sup>16</sup> Similarly, in our PS-deficient patients, no mutations were detected above 53% of PS activity, which is in line with the study by Caspers et al.<sup>16</sup>

The major study limitation was a relatively small sample size; however, we analyzed all eligible patients suspected of these types of thrombophilia in our center. For the new mutations, functional studies should be performed in addition to in silico predictions to prove their detrimental effect.

In conclusion, we report here the largest cohort of Polish Slavic patients deficient in natural anticoagulants, whom we evaluated for the causal genetic background. Several new Polish detrimental mutations were detected mostly in patients with AT and PC deficiencies.

**Supplementary material online** Supplementary material is available with the online version of the article at [www.pamw.pl](http://www.pamw.pl).

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**Contribution statement** EW contributed to the study design, interpreted the data, and wrote the manuscript. JC and MA-G performed the laboratory analysis and interpreted the data. WS, MC-L, KZ, and JM recruited patients and interpreted the data. TI performed the laboratory analysis. DPP and AB interpreted the data. AU conceived the study, recruited patients, and gave the final approval.

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