



SERPINE1 and *MTHFR* genetic variants in patients with embolic stroke of undetermined source: links with fibrin clot properties

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ABSTRACT

Introduction. The *SERPINE1* c.-820G (4_5), *MTHFR* gene variants, and unfavourably altered fibrin clot features, have been suspected to be associated with embolic stroke of undetermined source (ESUS). We investigated the *SERPINE1* c.-820G (4_5) gene variants alone and coexisting with *MTHFR* c.665C > T and c.1286A > C gene variants in relation to thrombophilic factors and plasma fibrin clot properties in Polish patients with ESUS.

Patients and methods. Unrelated consecutive patients with ESUS (n = 206) were genotyped by TaqMan assay. Thrombophilia screening was performed four weeks or more after a thrombotic event while off oral anticoagulation. Factor VIII (FVIII) activity was determined by a coagulometric assay, while lipoprotein(a) was determined using immunoturbidimetry. We determined fibrin clot permeability (K_a) and clot lysis time (CLT). Apparently healthy individuals without a family history of stroke or venous thromboembolism (n = 30), and patients with a history of atrial fibrillation (n = 25) or carotid artery disease-related stroke (n = 21), served as controls.

Results. Among ESUS patients, the *SERPINE1* c.-820G (4_5) minor allele frequency was 0.57. There were no differences in common factors associated with thrombophilia among ESUS patients regarding *SERPINE1* variants. The overall prevalence of FVIII > 150IU/dL was 26% (n = 53) and elevated FVIII predominated in *SERPINE1* variants carriers (n = 45; 84.9%), including 36 (68%) carriers of *MTHFR* variant. Moreover, 4.3-fold higher Lp(a) levels along with 50% reduced K_a and 46% prolonged CLT were found in patients with mutant *SERPINE1* combined with mutant homozygotes in the *MTHFR* c.665C > T variant compared to the wild type *SERPINE1* combined with mutant homozygotes in the *MTHFR* c.665C > T ($P < 0.001$).

Conclusions. The *SERPINE1* c.-820G (4_5) variants carriers have increased FVIII levels, while the *SERPINE1* c.-820G (4_5) mutant homozygotes coexisting with *MTHFR* c.665C > T have more prothrombotic fibrin clot features and elevated Lp(a). Our study underlines the cumulative effect of genetic risk factors in patients with ESUS that might require specific antithrombotic therapy.

Keywords: *SERPINE1* variants, *MTHFR* variants, embolic stroke of undetermined source, factor VIII, lipoprotein(a), fibrin clot properties

Introduction

Patients with nonlacunar ischaemic strokes with no clear aetiology are described as having embolic stroke of uncertain source (ESUS) [1]. ESUS is an aetiologically diverse group

wherein the stroke can be brought on by a number of different possible thromboembolic causes [1]. Left atrium, left ventricle, and atherosclerotic plaques in the arterial tree supplying the infarct area appear to be the most common of these [1]. Atrial cardiopathy, left ventricular disease, atherosclerotic plaques,

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patent foramen ovale, cardiac valvular disease, and cancer are the main pathologies that may be aetiologically responsible for ESUS [1]. A prothrombotic state, inherited or acquired, has been proposed as contributing to this event occurrence [2, 3]. A positive family history has been shown to be associated with a 2- to 3-fold increased risk of stroke [4, 5]. Identification of genetic factors predisposing to stroke is difficult, since strokes are associated with multiple factors such as heart and blood vessel diseases, chronic inflammation, diabetes and environmental factors i.e. smoking, stress or obesity [6, 7]. Except for standard thrombophilic factors [i.e. factor V Leiden (*F5L*) c.1691G > A mutation, prothrombin (*F2*) c.20210G > A mutation, deficiencies of antithrombin (AT), protein C (PC), and protein S (PS), presence of anti-phospholipid syndrome (APS) and increased factor VIII (FVIII) activity], genetic variants of *SERPINE1* c.-820G (4_5) and the methylenetetrahydrofolate reductase (*MTHFR*; 1p36.22) have also been suspected to be linked with stroke occurrence [8, 9].

The *SERPINE1* c.-820G (4_5) prevalence is known to vary among different ethnic groups, ranging from 0.28 in African Americans to 0.52 in Caucasians [10–12]. However, to the best of our knowledge, there have been no studies on the prevalence of *SERPINE1* c.-820G (4_5) among Polish ESUS patients. The c.-820G (4_5) variant in the *SERPINE1* gene (7q22.1) is related to the presence of a sequence of four or five guanosine nucleotides (4G and 5G, respectively) in the promoter region of the *SERPINE1* gene. Both alleles, 4G and 5G, bind a protein that activates transcription of the gene, but the 4G allele also binds a repressor protein that inhibits transcription. For this reason, the 4G allele has been suspected to be associated with increased production of plasminogen activator inhibitor type-1 (PAI-1) (by 30%) and a roughly doubled risk of thrombosis [10, 13, 14]. The frequencies of the *MTHFR* c.665C > T and c.1286A > C variants in Polish ESUS patients are around 0.32 and 0.34 [15]. The two variants of *MTHFR* gene reduce the *MTHFR* enzyme activity, increasing plasma homocysteine levels up to 70% [9]. Elevated homocysteine levels (> 15 µM) have been linked with thrombosis [16].

According to the Clot Summit Group, intravascular clot composition is an important issue in the strategy of stroke treatment [17]. Unfavourably altered fibrin clot properties assessed *ex vivo*, including formation of denser fibrin clot networks relatively resistant to lysis, have been identified in patients following ischaemic stroke [18]. Therefore, we aimed to evaluate in Polish ESUS patients the frequency of *SERPINE1* c.-820G (4_5), the prevalence of coexisting *SERPINE1* c.-820G (4_5) and *MTHFR* c.665C > T and c.1286A > C variants, and their associations with thrombophilic factors and fibrin clot properties.

Patients and methods

A total of 282 samples were studied. Patients with documented ESUS (n = 206) were recruited for diagnostic work-up

due to a suspicion of inherited thrombophilia. All patients were treated with ASA [monotherapy or ASA + clopidogrel (n = 14, 8.6%)]. Patients with a history of stroke related to atrial fibrillation (AF) (n = 25) or carotid artery disease (CAD) (n = 21), and apparently healthy individuals (n = 30) without a family history of stroke or venous thromboembolism (VTE) served as controls. Patients with ESUS were recruited between 2018 and 2022 at the Centre for Coagulation Disorders, Kraków, Poland. Studied patients were mainly inhabitants of the Lesser Poland (Małopolska) region. Stroke was defined based on clinical symptoms [19, 20]. Stroke diagnosis was considered in every patient with an acute focal neurological deficit unless another neurological diagnosis was made. ESUS was defined based on the prespecified criteria as a nonlacunar brain infarct in the absence of extra- or intracranial atherosclerosis causing ≥ 50% luminal stenosis in arteries supplying the area of ischaemia, major cardioembolic sources, and any other specific cause of stroke [21]. The diagnosis of stroke was confirmed by cranial computed tomography and/or magnetic resonance imaging. Additionally, carotid ultrasound with Doppler imaging, echocardiography, and Holter monitoring was conducted. Patent foramen ovale (PFO) screening was performed using transthoracic echocardiography and multiplane transoesophageal echocardiography (TEE) with intravenous injections of agitated saline while the patient was at rest and with the Valsalva manoeuvre. Three-dimensional TEE and two-dimensional TEE studies were performed with a × 7-2t transducer and a Phillips IE33 system (Philips Healthcare, Andover, MA, USA) according to the American Society of Echocardiography guidelines [22]. All TEE data was reviewed blindly and independently evaluated by two researchers. Hypertension was defined as a history of blood pressure higher than 140/90 mm Hg or the use of antihypertensive drugs. Hypercholesterolemia was stated based on medical records or the use of cholesterol-lowering therapy. Hypertriglyceridemia was diagnosed as the presence of triglycerides > 1.7 mmol/L. All individuals gave written informed consent, and the local ethical committee approved the study.

Genetic analyses

Whole blood samples were drawn into K3-EDTA collection tubes and stored in aliquots at -80 °C until processing. DNA was isolated from whole blood using a Gene Proof Pathogen Free DNA Isolation Kit (Gene Proof as., Brno, Czech Republic) according to the manufacturer's protocol. The major genetic coagulation factors such as *F5L* c.1691G > A (rs6025) and *F2* c.20210G > A (rs1799963) were genotyped using TaqMan® SNP genotyping assays (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA); assay IDs: *F5L* (rs6025: C__11975250_10), and *F2* (rs1799963: C__8726802_20) on admission. The studied *MTHFR* c.665C > T and *MTHFR* c.1286A > C variants were genotyped using TaqMan SNP

assays (Applied Biosystems); assay IDs for the *MTHFR* rs1801133 and *MTHFR* rs1801131, C__1202883_20 and C__850486_20, respectively). The *SERPINE1* c.-820G (4_5) variant was genotyped using Gene Proof PAI-1 Genotyping PCR Kit (rs1799889) (Gene Proof as.). The results were analysed using StepOne™ Software (Applied Biosystems).

Laboratory tests

Blood samples were drawn from an antecubital vein into tubes containing citrate anticoagulant (9:1 of 0.106 M sodium citrate). Thrombophilia screening was performed four weeks or more after a thrombotic event while off oral anticoagulation. To perform thrombophilia screening, including FVIII level, antiplatelet therapy was interrupted for 5–7 days before the visit to the outpatient clinic. During this visit, blood was also taken to perform additional laboratory assessment, such as fibrin clot phenotype. All AF patients were treated with direct oral anticoagulants (DOACs), according to appropriate guidelines. Patients were asked to take the last dose of DOAC 24 hours before the visit to the outpatient clinic. DOAC blood levels assessed at enrollment were < 30 ng/ml in all patients on DOACs, which is considered to be negligible, even to perform surgical procedures [23]. Basic thrombophilic factors determined involved *F5L*, *F2* mutation, deficiencies of AT, PC, PS, presence of APS and the FVIII activity. Laboratory tests were performed 1–6 months following stroke, and deficiencies were confirmed at least one month apart, on two separate occasions [24, 25].

Plasma FVIII activity was determined by coagulometric assay using a deficient plasma (Siemens Healthcare Diagnostics, Erlangen, Germany) and levels of 150 IU/dL or more were considered elevated [26]. PAI-1 antigen levels were measured using a Zymutest PAI-1 antigen test (Hyphen BioMed, Neuville-Sur-Oise, France). Lipoprotein(a) [Lp(a)] levels were determined using a Tina-quant® Lp(a) Gen. 2 assay (Roche Diagnostics, Mannheim, Germany).

Fibrin clot permeation (K_s) was determined using a pressure-driven system as previously described [27]. Briefly, K_s was calculated based on the equation: $K_s = Q \times L \times \eta / t \times A \times \Delta p$, where Q is the flow rate in time t , L is the length of a fibrin gel, η is the viscosity of liquid (in poise), A is the cross-sectional area (in cm^2), and Δp is the differential pressure (in dyne/cm^2).

To assess the efficiency of fibrinolysis, clot lysis time (CLT) was measured using the protocol devised by Pieters et al. [28]. Briefly, citrated plasma was added to 15 mM calcium chloride, 0.5 U/mL human thrombin (Merck), 15 μM phospholipid vesicles (Rossix, Mölndal, Sweden) and 20 ng/mL recombinant tPA (rtPA; Boehringer Ingelheim, Boehringer, Germany). The turbidity of the mixture was measured at 405 nm at 37°C. CLT was defined as the time from the midpoint of the

clear-to-maximum-turbid transition, which represents clot formation, to the midpoint of the maximum-turbid-to-clear transition (representing the lysis of the clot).

Statistical analysis

Continuous variables were expressed as mean \pm standard deviation or median and interquartile range (IQR), whereas categorical variables were expressed as number (percentage), as appropriate. Categorical variables were analysed by a Pearson's χ^2 test or a Fisher's exact test. Equality of variances was assessed using a Levene's test. Normality was assessed by a Shapiro-Wilk test. Differences between groups were compared using a Student's or a Welch's t-test depending on the equality of variances for normally distributed variables. A U Mann-Whitney test was used for non-normally distributed variables. Multiple group comparisons were performed using analysis of variance (ANOVA) or a Kruskal-Wallis test. Based on a previous report showing associations between *MTHFR* variants and clot permeability in VTE patients [29], this study was powered to have a 90% chance of detecting a 20% difference in K_s using a P-value of 0.01. At least 19 individuals in each group were required to detect such a difference. The exact (post-hoc) power of the test ranged between 91% and 97% for this key fibrin measure in ESUS *SERPINE1* carriers, AF-related stroke or CAD-related stroke when compared to controls. A P-value < 0.05 was considered statistically significant. Statistical analysis was performed using StatSoft Statistica 13.3 (TIBCO, Palo Alto, CA, USA).

Results

Among unrelated ESUS patients, the *SERPINE1* c.-820G (4_5) minor allele frequency was 0.57 (Tab. 1, 2). The minor allele frequency in *MTHFR* c.665C > T and c.1286A > C was 0.33 and 0.30, respectively. There was no deviation from the Hardy-Weinberg equilibrium regarding the studied genetic variants ($P > 0.05$). As shown in Table 1 and Table 2, the *SERPINE1* c.-820G (4_5) minor allele carriers and non-carriers were similar in terms of demographic features and thrombophilia risk factors.

In the *SERPINE1* c.-820G (4_5) heterozygotes compared to wild type and mutant homozygotes, we found 33% higher fibrinogen levels, although in all three groups fibrinogen levels were within the reference range (Tab. 1). The *SERPINE1* c.-820G (4_5) heterozygotes had 15%, while mutant homozygotes had 12% higher FVIII levels than did the wild type homozygotes (Tab. 1). However, mutant type *SERPINE1* homozygotes vs. heterozygotes had similar FVIII levels ($P = 0.69$). Interestingly, patients with ESUS did not differ regarding FVIII levels compared to AF- and CAD-related stroke patients, and all patients following stroke had higher FVIII levels than did the healthy controls (Tab. 3).

Table 1. Characteristics of patients with embolic stroke of undetermined source based on *SERPINE1* c.-820G (4_5) genotype

Genotype /Variable	ESUS <i>SERPINE1</i> 5G/5G 43 (20.8)	ESUS <i>SERPINE1</i> 4G/5G 90 (43.7)	ESUS <i>SERPINE1</i> 4G/4G 73 (35.2)	Overall P-value	P-value 5G/5G vs. 4G/5G	P-value 5G/5G vs. 4G/4G	P-value 4G/5G vs. 4G/4G
Age, years	46.0 ± 10.4	48.0 ± 10.7	46.0 ± 11.3	0.68	0.93	0.48	0.45
Women, n (%)	24 (55.8)	56 (62.2)	46 (63.0)	0.92	0.76	0.70	0.93
Comorbidities							
PFO, n (%)	8 (18.6)	14 (15.6)	22 (30.1)	0.069	0.71	0.29	0.08
Cancer, n (%)	1 (2.3)	2 (2.2)	0	-	0.97	0.19	0.21
Hypertension, n (%)	16 (37.2)	27 (30.0)	23 (31.5)	0.84	0.56	0.50	0.88
Hypercholesterolemia, n (%)	19 (44.2)	35 (38.9)	21 (28.8)	0.20	0.71	0.25	0.34
Hypertriglyceridemia, n (%)	2 (4.7)	2 (2.2)	0	-	0.59	0.14	0.50
Diabetes mellitus, n (%)	1 (2.3)	1 (1.1)	2 (2.7)	0.75	0.60	0.90	0.45
Laboratory investigations							
Fibrinogen, g/L	2.7 [2.5–3.0]	3.6 [3.2–3.8]	2.7 [2.5–3.1]	0.001	0.002	0.26	0.010
Total homocysteine, μM	10.5 [9.3–13.7]	11.1 [9.4–13.3]	11.2 [8.7–13.8]	0.41	0.91	0.36	0.16
Factor VIII, IU/dL	120.0 [105.6–135.4.0]	138.0 [119.0.0–155.0]	134.0 [123.1–159.0]	0.026	0.032	0.015	0.69
Protein C, %	119.0 [102.0–129.0]	119.3 [106.0–131.0]	124.8 [102.8–137.8]	0.59	0.27	0.48	0.80
Protein S, %	99.0 [82.4–118.2]	98.5 [83.0–114.0]	100.0 [85.5–111.4]	0.91	0.96	0.78	0.68
Antithrombin, %	99.0 [92.0–109.0]	101.0 [94.0–107.0]	101.0 [93.0–106.0]	0.98	0.85	0.85	0.99
PAI-1 Ag, ng/mL	7.57 [4.4–19.3]	8.8 [6.2–22.5]	9.9 [5.9–16.3]	0.61	0.33	0.52	0.68
Lp(a), mg/dL	10.9 [4.3–39.1]	8.9 [4.2–24.6]	8.7 [4.0–41.4]	0.59	0.35	0.41	0.84
K _v , 10 ⁻⁹ cm ²	4.98 [4.2–5.9]	5.13 [3.8–6.1]	5.18 [3.5–6.0]	0.59	0.64	0.82	0.84
CLT, min.	91.0 [78.5–116.0]	104.5 [81.5–126.5]	99.5 [93.5–121.1]	0.57	0.41	0.33	0.83

CLT — clot lysis time; ESUS — embolic stroke of undetermined source; K_v — clot permeability; Lp(a) — lipoprotein (a); PAI-1 Ag — plasminogen activator inhibitor type 1 antigen; PFO — patent foramen ovale. Data shown as mean ± standard deviation, median (interquartile range) or number (percentage)

Table 2. Factors associated with thrombophilia among patients with embolic stroke of undetermined source

Genotype /Variable	ESUS <i>SERPINE1</i> 5G/5G 43 (20.9)	ESUS <i>SERPINE1</i> 4G/5G 90 (43.7)	ESUS <i>SERPINE1</i> 4G/4G 73 (35.4)	P-value 5G/5G vs. 4G/5G	P-value 5G/5G vs. 4G/4G	P-value 4G/5G vs. 4G/4G
<i>F5L</i> c.1691G > A mutation, n (%)	4 (9.3)	3 (3.3)	1 (1.4)	0.17	0.05	0.44
<i>F2</i> c.20210G > A mutation, n (%)	1 (2.3)	2 (2.2)	4 (5.5)	0.96	0.40	0.27
<i>MTHFR</i> c.665C > T variant, n (%)	25 (58.1)	44 (48.9)	38 (52.1)	0.55	0.73	0.78
<i>MTHFR</i> c.1286A > C variant, n (%)	20 (46.5)	45 (50.0)	30 (41.1)	0.85	0.72	0.51
Protein C deficiency, n (%)	3 (7.0)	4 (4.4)	7 (9.6)	0.55	0.61	0.20
Protein S deficiency, n (%)	2 (4.7)	5 (5.6)	6 (8.2)	0.82	0.41	0.44
Antithrombin deficiency, n (%)	2 (4.7)	7 (7.8)	1 (1.4)	0.47	0.61	0.16
Antiphospholipid syndrome, n (%)	8 (18.6)	17 (18.9)	9 (12.3)	0.99	0.35	0.27
FVIII > 150IU/dL, n (%)	8 (18.6)	22 (24.4)	23 (31.5)	0.50	0.24	0.51
Hyperhomocysteinemia, n (%)	7 (16.3)	12 (13.3)	15 (20.5)	0.51	0.61	0.15
Lp(a) > 50 mg/dL, n (%)	4 (9.3)	7 (7.8)	4 (5.5)	0.78	0.47	0.59
Lp(a) > 30 mg/dL, n (%)	11 (25.6)	17 (18.9)	19 (26.0)	0.42	0.78	0.21

ESUS — embolic stroke of undetermined source; F2 — factor 2; F5L — factor V; FVIII — factor VIII; Lp(a) — lipoprotein (a); MTHFR — methylenetetrahydrofolate reductase. Data shown as mean ± standard deviation, median (interquartile range) or number (percentage)

Table 3. Comparison of laboratory parameters associated with prothrombotic fibrin clot phenotype in patients with stroke of different aetiology and healthy controls

Study Group /Variable	ESUS <i>SERPINE1</i> carriers (n = 163)	AF-related stroke (n = 25)	CAD-related stroke (n = 21)	Healthy controls (n = 30)	P-value ESUS <i>SERPINE1</i> carriers vs. AF-related stroke	P-value ESUS <i>SERPINE1</i> carriers vs. CAD-related stroke	P-value ESUS <i>SERPINE1</i> carriers vs. healthy controls
Age, years	46.08 ± 11.00	66.60 ± 10.63	66.00 ± 7.23	65.00 ± 3.50	< 0.01	< 0.01	< 0.01
Women, n (%)	102 (62.6)	13 (52.0)	12 (57.1)	17 (56.7)	0.31	0.63	0.54
Fibrinogen, g/L	3.12 [2.50–3.59]	3.01 [2.45–4.04]	4.46 [3.66–5.16]*	2.92 [2.53–3.38]	0.89	< 0.01	0.76
CLT, min	102.3 [83.5–125]	106 [93–111]*	103 [94–113]*	90 [79–101]	0.51	0.14	< 0.01
K _s , 10 ⁻⁹ cm ²	5.16 [3.54–6.06]	4.44 [3.55–5.35]*	5.30 [4.39–6.50]*	7.3 [6.88–8.35]	0.38	0.21	< 0.01
PAI-1 Ag, ng/mL	10.0 [6.49–15.96]	14.20 [11.81–17.47]	9.48 [7.33–13.76]	9.38 [6.36–12.97]	0.53	0.41	0.46
Factor VIII, IU/dL	134.0 [113.0–157.0]	138.0 [125.0–185.0]*	134.5 [92–164]*	108 [86–142]	0.58	0.24	0.036
Lp(a), mg/dL	8.87 [4.20–34.92]	9.11 [4.72–19.23]	8.30 [4.12–17.13]	7.61 [3.23–14.11]	0.46	0.19	0.33

AF — atrial fibrillation; CAD — carotid artery disease; CLT — clot lysis time; ESUS — embolic stroke of undetermined source; K_s — clot permeability; Lp(a) — lipoprotein (a); PAI-1 Ag — plasminogen activator inhibitor type 1 antigen. Data shown as mean ± standard deviation, median (interquartile range) or number (percentage); *P < 0.05 compared to controls

In the ESUS patients, the overall prevalence of FVIII levels > 150 IU/dL was 26% (n = 53). Of them, 23 (43.3%) individuals were mutant homozygotes, 22 (41.5%) were heterozygotes, and 8 (15.1%) were wild type homozygotes in the *SERPINE1* c.-820G (4_5) variant. In the subgroup of patients with FVIII levels > 150 IU/dL, as many as 36 (67.9%) individuals were carriers of *SERPINE1* c.-820G (4_5) minor allele and either of the *MTHFR* variants. Among patients with FVIII levels > 150 IU/dL, carriers of *SERPINE1* c.-820G (4_5) minor allele combined with *MTHFR* c.665C > T or *MTHFR* c.1286A > C variant had similar FVIII levels (P > 0.05, data not shown). There were no differences with regard to the frequency of reported comorbidities between *SERPINE1* c.-820G (4_5) genotypes (Tab. 1).

We did not observe differences in Lp(a) levels between patients following any type of stroke compared to healthy controls (Tab. 3). However, 4.3-fold higher Lp(a) levels were found in patients with mutant *SERPINE1* c.-820G (4_5) combined with mutant homozygotes in the *MTHFR* c.665C > T variant carriers compared to the wild type *SERPINE1* c.-820G (4_5) combined with mutant homozygotes in the *MTHFR* c.665C > T [46.2 (21.5–94.6) vs. 10.8 (4.3–18.0 mg/dL); P < 0.001]. A similar association was not observed for the *MTHFR* c.1286A > C variant.

Fibrin clot properties, including K_s and CLT, did not differ between mutant *SERPINE1* c.-820G (4_5) and wild type patients. However, K_s was roughly halved in patients with mutant *SERPINE1* c.-820G (4_5) combined with mutant *MTHFR* c.665C > T variant compared to wild type *SERPINE1* c.-820G (4_5) combined with the mutant *MTHFR* c.665C > T variant [3.33 (2.86–4.10) vs. 4.98 (4.15–5.09) × 10⁻⁹ cm², P < 0.001]. The same held true for CLT [111 (98–143) vs. 76 (64–85) min, p < 0.001], which was 46% prolonged

in mutant *SERPINE1* c.-820G (4_5) combined with mutant *MTHFR* c.665C > T variant.

Of note, the ESUS carriers of the *SERPINE1* c.-820G (4_5) did not differ in K_s and CLT from AF- or CAD-related stroke patients (Tab. 3). However, the three groups of patients with a history of stroke had reduced K_s and prolonged CLT compared to healthy controls (Tab. 3).

Discussion

This study is the first to report a high prevalence of the *SERPINE1* c.-820G (4_5) variant in Polish ESUS patients. We have also shown for the first time associations between *SERPINE1* variants and increased FVIII levels. Moreover, ESUS patients with the variants of *SERPINE1* c.-820G (4_5) and *MTHFR* c.665C > T were characterised by elevated Lp(a) and more prothrombotic fibrin clot phenotype compared to patients with the wild type *SERPINE1* c.-820G (4_5) and mutant *MTHFR* c.665C > T.

The frequency of *SERPINE1* 4G allele in Polish patients with ESUS was slightly higher than that reported in patients with cryptogenic and ischaemic stroke from Sweden and in ischaemic stroke patients from the Netherlands (all 0.53) [30, 31], and it was similar to the values observed in white stroke patients from the USA (0.58) [13]. Our data constitutes a valuable confirmation of previous reports.

We found that FVIII levels in ESUS *SERPINE1* c.-820G (4_5) carriers were higher compared to healthy controls but similar to patients following AF- and CAD-related stroke. These observations need further research. However, it has been shown that elevated FVIII levels are associated with both acquired [32] and genetic factors [33]. Siegler et al. [34] suggested that

elevated FVIII levels are associated with an increased risk of ischaemic stroke [34]. Moreover, elevated FVIII has been linked to strokes of different origin [35]. We observed higher FVIII levels in the *SERPINE1* c.-820G (4_5) heterozygotes and mutant homozygotes compared to the *SERPINE1* 5G/5G homozygotes, together with 33% higher fibrinogen levels in *SERPINE1* c.-820G (4_5) heterozygotes compared to wild type or mutant homozygotes. A similar observation was reported by Isordia-Salas et al. [36], however, in all carriers of the *SERPINE1* 4G allele. Moreover, a causal association between both FVIII and fibrinogen levels has been reported in patients with cardiovascular disease or venous thrombosis [37, 38], which may indicate that in patients following stroke, higher levels of fibrinogen and FVIII reflect prothrombotic tendencies.

On the other hand, in our study ESUS patients did not differ with regard to fibrin clot properties between the *SERPINE1* c.-820G (4_5) genotypes, but all genotypes were characterised by prothrombotic fibrin clot phenotype, similarly to AF- or CAD- related strokes, compared to healthy individuals. Considering previous data, which showed that patients with acute stroke as well as those with a history of stroke had unfavourably altered fibrin clot properties [39], our observation may suggest that the influence of the *SERPINE1* c.-820G (4_5) variant on fibrin features may not be strong enough to be observed in patients with a history of ESUS. Further studies are needed to elucidate whether *SERPINE1* c.-820G (4_5) carriers following ESUS might benefit from long-term anticoagulant treatment.

Divergent results regarding the associations of *MTHFR* and *SERPINE1* genetic variants with ischaemic stroke have been reported [10, 30, 40, 41]. To the best of our knowledge, the current study is the first to show the impact of *MTHFR* and *SERPINE1* variants coexistence on fibrin clot phenotype in Polish ESUS patients. The combination of *SERPINE1* c.-820G (4_5) heterozygous allele and mutant homozygotes in *MTHFR* c.665C > T was associated with 50% reduced K_a, prolonged CLT, and elevated Lp(a) levels. Previously, in patients with a history of ischaemic stroke, associations between prothrombotic fibrin clot phenotype and increased Lp(a) and PAI-1 levels were observed [42]. Elevated Lp(a) has been identified as an independent risk factor for ischaemic stroke [43]. Lp(a) is a low-density lipoprotein (LDL)-like particle attached to the apolipoprotein(a) (Apo(a)) chain, highly homologous with plasminogen. As an inactive homologue of plasminogen, Lp(a) inhibits fibrinolysis [44]. Moreover, Hancock et al. [45] have demonstrated that Apo(a)/Lp(a) quaternary complex inhibits plasminogen activation. Markedly prolonged CLT in the subgroup of patients with elevated Lp(a) levels may indicate that Lp(a), at least in part, contributes to a prothrombotic state in ESUS patients. Our findings may also suggest a synergistic effect of factors such as FVIII, Lp(a), or homocysteine on fibrin clot phenotype in the subgroup of patients homozygous for

SERPINE1 c.-820G (4_5) combined with mutant homozygotes in the *MTHFR* c.665C > T variant.

In our study, the *SERPINE1* c.-820G (4_5) was not associated with plasma increased PAI-1 levels, which is in line with the data provided by Jood et al. and Szegedi et al. [30, 46] who studied patients with ischaemic stroke. On the other hand, van Goor et al. [30] showed slightly higher PAI-1 levels in ischaemic stroke subjects with 4G/4G genotype compared to 4G/5G and 5G/5G *SERPINE1* c.-820G (4_5) genotypes. However, it has been suggested that higher PAI-1 levels in 4G/4G homozygotes may be associated with previous VTE or myocardial infarction [47–49].

This study has some limitations. Firstly, the studied group was relatively small and the associations between genetic variants and fibrin clot properties need to be studied in a larger group of patients. Secondly, all fibrin clot characteristics were performed only once, while some changes in assessed parameters could be observed over time. Thirdly, we assessed only PAI-1 antigen levels but not PAI-1 activity, although, as reported by Szegedi et al. [46], PAI-1 antigen shows less fluctuation than activity.

In conclusion, we have established that the frequency of *SERPINE1* gene variant in Polish patients with ESUS is similar to that observed in Caucasian Americans. The *SERPINE1* c.-820G (4_5) carriers have increased FVIII levels and the *SERPINE1* c.-820G (4_5) mutant homozygotes in combination with *MTHFR* c.665 C > T variant have more prothrombotic plasma fibrin clot features accompanied by increased Lp(a).

Our study showed the cumulative effect of *SERPINE1* c.-820G (4_5) and *MTHFR* c.665C > T variants, suggesting that these patients might benefit from long-term anticoagulant therapy, although further studies are needed to confirm our findings.

Article information

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