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# *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 ($K_s$) 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 $K_s$ 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

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## 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 ± 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 $Chi^2$ 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 *SERPINE1* 5G/5G 43 (20.8) | ESUS *SERPINE1* 4G/5G 90 (43.7) | ESUS *SERPINE1* 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 |
| $K_s$, $10^{-9}$ $cm^2$ | 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; $K_s$ — 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 *SERPINE1* 5G/5G 43 (20.9) | ESUS *SERPINE1* 4G/5G 90 (43.7) | ESUS *SERPINE1* 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 |
|---|---|---|---|---|---|---|
| *F5L* c.1691G > A mutation, n (%) | 4 (9.3) | 3 (3.3) | 1 (1.4) | 0.17 | 0.05 | 0.44 |
| *F2* c.20210G > A mutation, n (%) | 1 (2.3) | 2 (2.2) | 4 (5.5) | 0.96 | 0.40 | 0.27 |
| *MTHFR* c.665C > T variant, n (%) | 25 (58.1) | 44 (48.9) | 38 (52.1) | 0.55 | 0.73 | 0.78 |
| *MTHFR* 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 *SERPINE1* carriers (n = 163) | AF-related stroke (n = 25) | CAD-related stroke (n = 21) | Healthy controls (n = 30) | P-value ESUS *SERPINE1* carriers vs AF-related stroke | P-value ESUS *SERPINE1* carriers vs CAD-related stroke | P-value ESUS *SERPINE1* 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) x $10^{-9}$ cm$^2$, 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 $K_s$, 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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## References

1. Ntaios G. Embolic stroke of undetermined source: JACC Review topic of the week. J Am Coll Cardiol. 2020; 75(3): 333–340, doi: 10.1016/j.jacc.2019.11.024, indexed in Pubmed: 31976872.
2. Saver JL. CLINICAL PRACTICE. Cryptogenic stroke. N Engl J Med. 2016; 374(21): 2065–2074, doi: 10.1056/NEJMcp1503946, indexed in Pubmed: 27223148.

3. Zadro R, Herak DC. Inherited prothrombotic risk factors in children with first ischemic stroke. Biochem Med (Zagreb). 2012; 22(3): 298–310, doi: 10.11613/bm.2012.033, indexed in Pubmed: 23092062.
4. Seshadri S, Beiser A, Pikula A, et al. Parental occurrence of stroke and risk of stroke in their children: the Framingham study. Circulation. 2010; 121(11): 1304–1312, doi: 10.1161/CIRCULATIONAHA.109.854240, indexed in Pubmed: 20212282.
5. Jerrard-Dunne P, Cloud G, Hassan A, et al. Evaluating the genetic component of ischemic stroke subtypes: a family history study. Stroke. 2003; 34(6): 1364–1369, doi: 10.1161/01.STR.0000069723.17984.FD, indexed in Pubmed: 12714707.
6. Kelly PJ, Lemmens R, Tsivgoulis G. Inflammation and stroke risk: A new target for prevention. stroke. 2021; 52(8): 2697–2706, doi: 10.1161/STROKEAHA.121.034388, indexed in Pubmed: 34162215.
7. Barthels D, Das H. Current advances in ischemic stroke research and therapies. Biochim Biophys Acta Mol Basis Dis. 2020; 1866(4): 165260, doi: 10.1016/j.bbadis.2018.09.012, indexed in Pubmed: 31699365.
8. Farjat-Pasos JI, Nuche J, Mesnier J, et al. Transcatheter patent foramen ovale closure in stroke patients with Thrombophilia: Current Status and Future Perspectives. J Stroke. 2022; 24(3): 335–344, doi: 10.5853/jos.2022.01697, indexed in Pubmed: 36221936.
9. Omran SS, Lerario MP, Gialdini G, et al. Clinical impact of thrombophilia screening in young adults with ischemic stroke. J Stroke Cerebrovasc Dis. 2019; 28(4): 882–889, doi: 10.1016/j.jstrokecerebrovasdis.2018.12.006, indexed in Pubmed: 30595511.
10. Attia J, Thakkinstian A, Wang Y, et al. The PAI-1 4G/5G gene polymorphism and ischemic stroke: an association study and meta-analysis. J Stroke Cerebrovasc Dis. 2007; 16(4): 173–179, doi: 10.1016/j.jstrokecerebrovasdis.2007.03.002, indexed in Pubmed: 17689414.
11. Yende S, Angus DC, Ding J, et al. Health ABC Study. 4G/5G plasminogen activator inhibitor-1 polymorphisms and haplotypes are associated with pneumonia. Am J Respir Crit Care Med. 2007; 176(11): 1129–1137, doi: 10.1164/rccm.200605-644OC, indexed in Pubmed: 17761618.
12. Crainich P, Jenny NS, Tang Z, et al. Lack of association of the plasminogen activator inhibitor-1 4G/5G promoter polymorphism with cardiovascular disease in the elderly. J Thromb Haemost. 2003; 1(8): 1799–1804, doi: 10.1046/j.1538-7836.2003.00255.x, indexed in Pubmed: 12911596.
13. Panahloo A, Mohamed-Ali V, Lane A, et al. Determinants of plasminogen activator inhibitor 1 activity in treated NIDDM and its relation to a polymorphism in the plasminogen activator inhibitor 1 gene. Diabetes. 1995; 44(1): 37–42, doi: 10.2337/diab.44.1.37, indexed in Pubmed: 7813812.
14. Nikolopoulos GK, Bagos PG, Tsangaris I, et al. The association between plasminogen activator inhibitor type 1 (PAI-1) levels, PAI-1 4G/5G polymorphism, and myocardial infarction: a Mendelian randomization meta-analysis. Clin Chem Lab Med. 2014; 52(7): 937–950, doi: 10.1515/cclm-2013-1124, indexed in Pubmed: 24695040.
15. Klajmon A, Cichoń M, Natorska J, et al. Methylenetetrahydrofolate reductase (MTHFR) gene c.665C>T and c.1286A>C and SERPINE1 -675 4G/5G polymorphisms in Polish patients with venous thromboembolism and cryptogenic ischemic stroke. Pol Arch Intern Med. 2022; 132(2), doi: 10.20452/pamw.16218, indexed in Pubmed: 35226442.
16. Veeranki S, Gandhapudi SK, Tyagi SC. Interactions of hyperhomocysteinemia and T cell immunity in causation of hypertension. Can J Physiol Pharmacol. 2017; 95(3): 239–246, doi: 10.1139/cjpp-2015-0568, indexed in Pubmed: 27398734.
17. De Meyer SF, Andersson T, Baxter B, et al. Clot Summit Group. Analyses of thrombi in acute ischemic stroke: A consensus statement on current knowledge and future directions. Int J Stroke. 2017; 12(6): 606–614, doi: 10.1177/1747493017709671, indexed in Pubmed: 28534706.
18. Undas A, Podolec P, Zawilska K, et al. Altered fibrin clot structure/function in patients with cryptogenic ischemic stroke. Stroke. 2009; 40(4): 1499–1501, doi: 10.1161/STROKEAHA.108.532812, indexed in Pubmed: 19246700.
19. Sacco RL, Kasner SE, Broderick JP, et al. An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke. 2013; 44(7): 2064–2089, doi: 10.1161/STR.0b013e318296aeca, indexed in Pubmed: 23652265.
20. Błażejewska-Hyżorek B, Czernuszenko A, Członkowska A, et al. Wytyczne postępowania w udarze mózgu. Polski Przegląd Neurologiczny. 2019; 15(A): 1–156, doi: 10.5603/PPN.2019.0001.
21. Hart RG, Diener HC, Coutts SB, et al. Cryptogenic Stroke/ESUS International working group. Embolic strokes of undetermined source: the case for a new clinical construct. Lancet Neurol. 2014; 13(4): 429–438, doi: 10.1016/S1474-4422(13)70310-7, indexed in Pubmed: 24646875.
22. Hahn RT, Abraham T, Adams MS, et al. Guidelines for performing a comprehensive transesophageal echocardiographic examination: recommendations from the American Society of Echocardiography and the Society of Cardiovascular Anesthesiologists. J Am Soc Echocardiogr. 2013; 26(9): 921–964, doi: 10.1016/j.echo.2013.07.009, indexed in Pubmed: 23998692.
23. Faraoni D, Levy JH, Albaladejo P, et al. Groupe d'Intérêt en hémostase périopératoire. Updates in the perioperative and emergency management of non-vitamin K antagonist oral anticoagulants. Crit Care. 2015; 19(1): 203, doi: 10.1186/s13054-015-0930-9, indexed in Pubmed: 25925382.
24. Wypasek E, Corral J, Alhenc-Gelas M, et al. Genetic characterization of antithrombin, protein C, and protein S deficiencies in Polish patients. Pol Arch Intern Med. 2017; 127(7-8): 512–523, doi: 10.20452/pamw.4045, indexed in Pubmed: 28607330.
25. Gajek A, Natorska J, Wypasek E, et al. Determinants of elevated factor VIII in patients screened for thrombophilia. Thromb Res. 2020; 188: 28–30, doi: 10.1016/j.thromres.2020.01.027, indexed in Pubmed: 32044504.
26. Kamphuisen PW, Eikenboom JC, Bertina RM. Elevated factor VIII levels and the risk of thrombosis. Arterioscler Thromb Vasc Biol. 2001; 21(5): 731–738, doi: 10.1161/01.atv.21.5.731, indexed in Pubmed: 11348867.
27. Ząbczyk M, Natorska J, Janion-Sadowska A, et al. Prothrombotic fibrin clot properties associated with NETs formation characterize acute pulmonary embolism patients with higher mortality risk. Sci Rep. 2020; 10(1): 11433, doi: 10.1038/s41598-020-68375-7, indexed in Pubmed: 32651425.
28. Pieters M, Philippou H, Undas A, et al. Subcommittee on factor XIII and fibrinogen, and the subcommittee on fibrinolysis. An international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: communication from the SSC of the ISTH. J Thromb Haemost. 2018; 16(5): 1007–1012, doi: 10.1111/jth.14002, indexed in Pubmed: 29658191.
29. Klajmon A, Głowacki R, Piechocka J, et al. Plasma thiol levels and methylenetetrahydrofolate reductase gene c.665C > T and c.1286A > C variants affect fibrin clot properties in Polish venous

thromboembolic patients. Mol Genet Metab. 2023; 139(3): 107623, doi: 10.1016/j.ymgme.2023.107623, indexed in Pubmed: 37302269.
30. Jood K, Ladenvall P, Tjärnlund-Wolf A, et al. Fibrinolytic gene polymorphism and ischemic stroke. Stroke. 2005; 36(10): 2077–2081, doi: 10.1161/01.STR.0000183617.54752.69, indexed in Pubmed: 16179568.
31. van Goor ML, Gómez García E, Leebeek F, et al. The plasminogen activator inhibitor (PAI-1) 4G/5G promoter polymorphism and PAI-1 levels in ischemic stroke. A case-control study. Thromb Haemost. 2005; 93(1): 92–96, doi: 10.1160/TH04-09-0560, indexed in Pubmed: 15630497.
32. Karttunen V, Alfthan G, Hiltunen L, et al. Risk factors for cryptogenic ischaemic stroke. Eur J Neurol. 2002; 9(6): 625–632, doi: 10.1046/j.1468-1331.2002.00464.x, indexed in Pubmed: 12453078.
33. Sabater-Lleal M, Huffman JE, de Vries PS, et al. INVENT Consortium; MEGASTROKE Consortium of the international stroke genetics consortium (ISGC). Genome-wide association transethnic meta-analyses identifies novel associations regulating coagulation factor VIII and von Willebrand factor plasma levels. circulation. 2019; 139(5): 620–635, doi: 10.1161/CIRCULATIONAHA.118.034532, indexed in Pubmed: 30586737.
34. Siegler JE, Samai A, Albright KC, et al. Factoring in Factor VIII With Acute Ischemic Stroke. Clin Appl Thromb Hemost. 2015; 21(7): 597–602, doi: 10.1177/1076029615571630, indexed in Pubmed: 25669199.
35. Lasek-Bal A, Puz P, Kazibutowska Z. Elevated factor VIII level and stroke in patients without traditional risk factors associated with cardiovascular diseases. Neuropsychiatr Dis Treat. 2013; 9: 847–852, doi: 10.2147/NDT.S43461, indexed in Pubmed: 23807850.
36. Isordia-Salas I, Leaños-Miranda A, Sainz IM, et al. Association of the plasminogen activator inhibitor-1 gene 4G/5G polymorphism with ST elevation acute myocardial infarction in young patients. Rev Esp Cardiol. 2009; 62(4): 365–372, doi: 10.1016/s1885-5857(09)71663-9, indexed in Pubmed: 19401121.
37. Kamphuisen PW, Eikenboom JC, Vos HL, et al. . Increased levels of factor VIII and fibrinogen in patients with venous thrombosis are not caused by acute phase reactions. Thromb Haemost. 1999; 81(5): 680–683, doi: 10.1055%2Fs-0037-1614553, indexed in Pubmed: 10365736.
38. Tracy RP, Bovill EG, Yanez D, et al. Fibrinogen and factor VIII, but not factor VII, are associated with measures of subclinical cardiovascular disease in the elderly. Results from The Cardiovascular Health Study. Arterioscler Thromb Vasc Biol. 1995; 15(9): 1269–1279, doi: 10.1161/01.atv.15.9.1269, indexed in Pubmed: 7670938.
39. Pera J, Undas A, Topor-Madry R, et al. Fibrin clot properties in acute stroke: what differs cerebral hemorrhage from cerebral ischemia? Stroke. 2012; 43(5): 1412–1414, doi: 10.1161/STROKEAHA.111.646729, indexed in Pubmed: 22343641.
40. Franchini M, Martinelli I, Mannucci PM. Uncertain thrombophilia markers. Thromb Haemost. 2016; 115(1): 25–30, doi: 10.1160/TH15-06-0478, indexed in Pubmed: 26271270.
41. Kelly PJ, Rosand J, Kistler JP, et al. Homocysteine, MTHFR 677C-->T polymorphism, and risk of ischemic stroke: results of a meta-analysis. Neurology. 2002; 59(4): 529–536, doi: 10.1212/wnl.59.4.529, indexed in Pubmed: 12196644.
42. Undas A. prothrombotic fibrin clot phenotype in patients with deep vein thrombosis and pulmonary embolism: A new risk factor for recurrence. Biomed Res Int. 2017; 2017: 8196256, doi: 10.1155/2017/8196256, indexed in Pubmed: 28740853.
43. Nave AH, Lange KS, Leonards CO, et al. Lipoprotein (a) as a risk factor for ischemic stroke: a meta-analysis. Atherosclerosis. 2015; 242(2): 496–503, doi: 10.1016/j.atherosclerosis.2015.08.021, indexed in Pubmed: 26298741.
44. Harpel PC, Gordon BR, Parker TS. Plasmin catalyzes binding of lipoprotein (a) to immobilized fibrinogen and fibrin. Proc Natl Acad Sci U S A. 1989; 86(10): 3847–3851, doi: 10.1073/pnas.86.10.3847, indexed in Pubmed: 2524834.
45. Hancock MA, Boffa MB, Marcovina SM, et al. Inhibition of plasminogen activation by lipoprotein(a): critical domains in apolipoprotein(a) and mechanism of inhibition on fibrin and degraded fibrin surfaces. J Biol Chem. 2003; 278(26): 23260–23269, doi: 10.1074/jbc.M302780200, indexed in Pubmed: 12697748.
46. Szegedi I, Nagy A, Székely EG, et al. PAI-1 5G/5G genotype is an independent risk of intracranial hemorrhage in post-lysis stroke patients. Ann Clin Transl Neurol. 2019; 6(11): 2240–2250, doi: 10.1002/acn3.50923, indexed in Pubmed: 31637872.
47. Doggen C, Bertina RM, Cats V, et al. The 4G/5G Polymorphism in the plasminogen activator inhibitor-1 gene is not associated with myocardial infarction. thrombosis and haemostasis. 2017; 82(07): 115–120, doi: 10.1055/s-0037-1614639.
48. Stegnar M, Uhrin P, Peternel P, et al. The 4G/5G sequence polymorphism in the promoter of plasminogen activator inhibitor-1 (pai-1) gene: relationship to plasma pai-1 level in venous thromboembolism. thrombosis and haemostasis. 2017; 79(05): 975–979, doi: 10.1055/s-0037-1615105.
49. Ye S, Green FR, Scarabin PY, et al. The 4G/5G genetic polymorphism in the promoter of the plasminogen activator inhibitor-1 (pai-1) gene is associated with differences in plasma pai-1 activity but not with risk of myocardial infarction in the ectim study. thrombosis and haemostasis. 2018; 74(03): 837–841, doi: 10.1055/s-0038-1649833.