Lower Contribution of Factor V Leiden or G202104 Mutations to Ischemic Stroke in Patients With Clinical Risk Factors: Pair-Matched Case-Control Study

Davor Eterović, PhD,* Marina Titlić, MD,† Viktor Čulić, MD,‡
Renata Zadro, PhD,§ and Dragan Primorac, MD, PhD**

*Departments of *Physiology and Biophysics and **Medical Diagnostics, Medical School at Split, and Departments of †Neurology and ‡Medicine, University Hospital Split, Split; and §Department of Laboratory Diagnostics, University Hospital Rebro, Zagreb, Croatia*

Summary: It was suggested that factor V Leiden and prothrombin G20210A mutations increase the risk of ischemic stroke only in combination with clinical risk factors of arterial ischemic disease. In these studies the controls were derived from the general population, with fewer clinical risk factors, which might have produced biased results. The factor V Leiden and prothrombin G20210A mutations were examined by polymerase chain reaction technique in 120 ischemic stroke patients and 120 controls younger than 65 years of age. Each patient had his own control, tightly matched in clinical risk factors. The prevalences of factor V Leiden and prothrombin G20210A mutations in patients were 8.3% (P = 0.02) and 7.5% (P = 0.04), respectively, and 2.5% for controls for both mutations. All carriers were single heterozygotes. In patients, but not in controls, the carriers of either mutation were mostly women and with fewer clinical risk factors for arterial ischemic events. In particular, considering both mutations as a single coagulation deficit, their presence increased the likelihood of ischemic stroke (odds ratio [OR] = 3.6; 95% confidence interval [CI] 1.4–9.3), especially among women (OR = 4.6; 95% CI: 1.2–17.8), normotensive persons (OR = 9.2; 95% CI: 1.1–17.8) and those having normal cholesterol (OR = 5.9; 95% CI: 1.6–21.2) and triglyceride serum concentrations (OR = 4.3; 95% CI: 1.5–12.8). In the studied sample of adult North Mediterranean population younger than 65 years the prevalences of factor V Leiden and prothrombin G20210A mutations were greater in patients with ischemic stroke than in matched controls. Unlike in studies with unmatched controls, we observed an apparently negative interaction of these mutations with clinical risk factors.
Key Words: Factor V Leiden—G20210A—Stroke.

Two single-point prothrombotic mutations in the genes coding for coagulation factor V (G1691A, also called factor V Leiden) and factor II (prothrombin G20210A) have been convincingly shown to increase the risk of venous thromboembolic events (1,2). However, it remained less clear whether these relatively common mutations increase the risk of thrombosis in arterial circulation. Hitherto published studies suggest that the presence of factor V Leiden and prothrombin G20210A increase the risk of ischemic stroke only slightly when present alone (3), but rather strongly in combination with hypertension or diabetes mellitus (4).

In all previous studies the controls were apparently healthy persons, unmatched with patients in clinical risk factors of arterial ischemic events. The interaction of clinical with genetic risk factors was assessed by multivariate methods, with post hoc adjustment of confounding variables. However, in order that these statistical methods produce reliable estimates, many prerequisites are required (5), which were usually not met. The problems of overfitting (less

Address correspondence to Prof. dr. Davor Eterović, Department of Physiology and Biophysics, Medical School at Split, Šoltanska 2, 21000 Split, Croatia; e-mail: eterovic@bsb.mefst.hr.

Clinical and Applied Thrombosis/Hemostasis
Vol. 13, No. 2, April 2007 188-193
DOI: 10.1177/1076029606298999

than 10 times as many mutations as there are independent variables) and mutual correlations of predictors (lipids concentrations) were usually encountered, with possible biased estimates. To avoid the possibility of selection bias, we decided to undertake logistically more demanding, fine individual pairing of patients and controls. Therefore, in this study we investigated the role of factor V Leiden and prothrombin G20210A mutations in occurrence of ischemic strokes, by using control subjects who do not differ from patients in clinical risk factors on an individual, pair-matched basis.

PARTICIPANTS AND METHODS

Selection of Patients and Controls

In this prospective study the data on 120 white Croatian ischemic stroke patients from Split, Middle Dalmatia (representing a sample of North Mediterranean population), and 120 control persons from the same area were analyzed (77 men and 43 women in both groups). The patients were admitted to the Department of Neurology, Clinical Hospital Split, between January 1, 1999, and May 3, 2003, after being examined by a physician in the emergency admissions or by a general practitioner at their homes. Aside from exclusion criteria, these persons were consecutive patients with acute, first-time ischemic stroke. To reduce the possible confounding effects on the studied association of coagulation deficit and ischemic stroke, the following patients were excluded from the study: those older than 65 years (N = 172), persons with secondary hypercoagulability status (N = 11), those with diabetes mellitus type 1 (N = 14), and those with significant obstruction of carotid arteries (N = 17).

The control persons were without symptoms or history of cerebrovascular disease and unrelated to the patients. They were selected from persons attending regular checkups at an outpatient clinic of occupational medicine (N = 82), and the blood donors (N = 21) and volunteers from the University Hospital Split staff (N = 17). One control person was assigned to each patient who agreed in gender, age (±1 year), family history of cerebrovascular disease, presence of hypertension, hypercholesterolemia, hypertriglyceridemia, diabetes mellitus type 2, cardiac disease (having 4 categories: arrhythmia, history of myocardial infarction, stable angina pectoris, and unstable angina pectoris), habits of smoking (having 3 categories: smoker, past smoker, and nonsmoker) and drinking alcohol (having 5 categories: not at all, only occasionally, every day less than 2 dL of red wine equivalent, every day more than 2 dL of red wine equivalent), and body mass index (having 4 categories: less than 18.5 kg/m^2, 18.5–25 kg/m^2, more than 25 but less than 30 kg/m^2, and more than 30 kg/m^2).

All participants gave their informed consent for the study enrollment and for the DNA analysis. This study was approved by the Ethical Committee of the University Hospital Split.

Clinical Procedures

All stroke patients underwent a detailed clinical assessment that included general physical and neurological tests, an exploration of the medical history, family anamnesis, the evaluation of the clinical risk factors, extended laboratory tests, electrocardiography, Doppler sonography of the carotid and cerebral arteries, and computed tomography and, in some cases magnetic resonance imaging. When appropriate, the subjects underwent detailed cardiological assessment (transthoracic or transoesophageal ultrasound, stress testing, or Holter monitoring) if they had not made such an assessment in the previous 6 months.

Risk Factors Assessment

The presence of clinical risk factors was determined from previous medical records and from data obtained by interview. Hypertension was defined if the person was taking an antihypertensive drug and the repeated measurements exceeded 95 mm Hg in case of diastolic or 160 mm Hg in case of systolic blood pressure. In the absence of previous evidence, diabetes mellitus was defined when the glucose level was greater than 7.8 mmol/L in a fasting state and/or at least equal to 11.1 mmol/L 2 hours after a meal or 75 g of oral glucose. The presence of heart disease was established if it had previously been diagnosed and verified by a cardiologist or during clinical assessment for the purpose of this study.

DNA Analysis

Whole blood for DNA analysis was collected in sodium citrate (0.129 mol/L) or EDTA. Genomic DNA was prepared according to standard procedures using either phenol/chloroform extraction with ethanol precipitation or the salting-out method.

The presence of factor V Leiden and G20210A was determined by the polymerase chain reac-

tion–restriction fragment length polymorphism (PCR-RFLP) method. A 287-bp fragment encompassing nucleotide position 1691 of the factor V gene was amplified with primers according to Zöller et al (6). Following the digestion with MnlI (Stratagene, Austin, TX), the wild-type allele (1691G allele) resulted in 37-bp, 93-bp, and 157-bp fragments, whereas the mutant allele (1691 allele) resulted in 130-bp and 157-bp fragments. Analysis for G20210A was performed according to the method described by Poort et al (2). After the digestion of amplified 345-bp fragments with HindIII (Roche Diagnostics, Mannheim, Germany), the mutation A allele was cleaved in two 23-bp and 322-bp fragments, whereas the wild-type G allele remained undigested. Digested PCR products were separated by electrophoresis on 1.5% agarose gels (Applied Biosystems, Foster City, CA) for factor V Leiden and on Spreadex gels (Guest Elchrom Scientific, Cham, Switzerland) for G20210A. DNA analysis was performed by operators blinded to the results of the clinical assessment.

Statistical Analysis

The differences in prevalences of genetic mutations between patients and pair-matched controls were assessed by the McNemmar test. The odds ratios (OR) and their 95% confidence intervals (CI) of ischemic cerebrovascular insult for the studied mutations were calculated from Miettenan's formula (7). Due to explicit control of confounding variables (clinical risk factors) by pair-matching, this univariate test produced the same results as if the logistic regression was utilized without, however, relying on the limited ability of multivartiate methods in post hoc adjustment of the results. The interaction between variables of clinical profile (listed in Table 1) and presence of either of the studied mutations in predicting the ischemic stroke was tested by logistic regression. If this test produced a P value less than 0.05, the comparison between patients and controls was extended over the particular subgroups of participants. The differences in prevalences of clinical risk factors between subsamples of patients or controls were assessed by χ^2 test or Fisher exact test (binary categorical variables) and Mann-Whitney test (age). For this purpose, the multicategorical variables (heart disease, smoking, alcohol consumption, and obesity), which were used for fine-pairing of cases with controls, were converted to binary variables. All analyses were carried using SPSS 11.0.1 (SPSS Inc, Tulsa, OK, USA) software.

RESULTS

Due to pair-matching the clinical profiles of patients and control persons were identical and typical for patients with cerebrovascular disease, with relatively high prevalence of clinical risk factors for arterial ischemic events, that is, of hypertension, diabetes mellitus, hyperlipoprotenemia, obesity, habits of smoking and drinking alcohol, and positive family history of cerebrovascular disease. There were more men than women and men more frequently had type 2 diabetes mellitus, increased triglyceride level, and smoked or drank alcohol (see Table 1).

Rather strong association between the studied mutations and ischemic stroke emerged: Both mutations were more than 3 times more prevalent in patients than in controls. All mutation carriers were heterozygotes, carrying only one of the mutations (Table 2). In patients, but not in controls, the mutation carriers were mostly confined to women and persons with fewer clinical risk factors for arterial ischemic events (Tables 3 and 4). In accord with this, considering both mutations as one entity, a coagulation deficit, the presence of either one of the two mutations increased the likelihood of ischemic stroke, especially among women, normotensive persons, nondiabetics, and those having normal cholesterol and triglyceride serum concentrations (Table 5).

Thus, in contrast to studies using unmatched controls, we observed stronger association of the inherited coagulation deficits with ischemic stroke, with negative, rather than positive, contribution of the clinical risk factors for adverse arterial events.

DISCUSSION

In the studied sample of North Mediterranean population the prevalences of factor V Leiden and prothrombin G20210A mutations were approximately in the middle of the range of other populations reported thus far. However, their association with cerebrovascular disease appeared stronger than in other studies (3).

The most striking finding of this study is an apparent negative interaction of most of the clinical risk factors and the studied mutations in predicting the cerebrovascular disease. It appeared that in women and persons without clinical risk factors the presence of the studied mutations increased the risk of ischemic stroke more than in men and persons with clinical risk factors.

TABLE 1. The Clinical Risk Factors of 120 Patients With Ischemic Cerebrovascular Insult or 120 Control Persons Having Identical Clinical Risk Factors*

Clinical Risk Factor; Prevalence (%)	Patients or Controls Men and Women (N=120)	Men (N=77)	Women (N=43)
Family history of cerebrovascular disease	52 (48.3)	35 (45.5)	17 (39.5)
Hypertension	67 (55.8)	45 (58.4)	22 (51.2)
Type 2 diabetes mellitus	36 (30)	30 (39)	6 (14)
Hypercholesterolemia	38 (31.7)	22 (32.5)	13 (30.2)
Hypertriglyceridemia	32 (26.7)	25 (32.5)	13 (30.2)
Cardiac disease	44 (36.7)	28 (36.4)	16 (37.2)
Smoking	46 (38.3)	39 (50.6)	7 (16.3)
Alcohol consumption	31 (25.8)	31 (40.3)	0 (0)
Body mass index > 25 kg/m^2	75 (62.5)	47 (61.0)	28 (65.1)
Age in years, median (range)	61 (33–65)	60 (33–65)	62 (34–65)

*For each patient there was one control person who agreed in gender, age, and other clinical risk factors for arterial ischemic events.

TABLE 2. The Relationship Between Genotype and Ischemic Cerebrovascular Disease

Genotype, N (%)	Patients (N=120)	Controls* (N=120)	*P* Value†
Arg-506-Gln mutation, heterozygotes	10 (8.3)	3 (2.5)	.023
G20210A mutation, heterozygotes	9 (7.5)	3 (2.5)	.047
Homozygotes for either mutation or combined defect	0	0	—
No Arg-506-Gln or G20210A mutation	101 (84.2)	114 (95)	—
Arg-506-Gln or G20210A mutation	19 (15.8)	6 (5)	.019

*For each patient with ischemic cerebrovascular insult there was one control person who agreed in gender, age, and other clinical risk factors for arterial ischemic events.

†Assessed by McNemmar test of matched pairs.

TABLE 3. The Association Between Arg-506-Gln or G20210A Mutation and Clinical Risk Factors in Patients With Ischemic Cerebrovascular Insult

Clinical Risk Factor, Prevalence (%)	Arg-506-Gln or G20210A Mutation Yes (N=19)	No (N=101)	*P* Value*
Male gender	8 (42)	69 (68)	.05
Family history of cerebrovascular disease	11 (58)	41 (41)	.25
Hypertension	11 (58)	56 (55)	.96
Type 2 diabetes mellitus	1 (5.3)	35 (35)	.02
Hypercholesterolemia	4 (21)	34 (34)	.41
Hypertriglyceridemia	1 (5.3)	31 (31)	.04
Cardiac disease	6 (32)	38 (38)	.80
Smoking	5 (26)	41 (41)	.36
Alcohol consumption	3 (16)	28 (28)	.42
Body mass index > 25 kg/m^2	12 (63)	63 (63)	.85
Age in years, median (range)	58 (34–65)	62 (33–65)	.68

*Assessed by χ^2 or Fisher exact test (binary categorical variables) and by Mann-Whitney test in case of age.

TABLE 4. The Association Between Arg-506-Gln or G20210A Mutation and Clinical Risk Factors in Persons Without Cerebrovascular Disease*

Clinical Risk Factor, Prevalence (%)	Arg-506-Gln or G20210A Mutation		*P* Value†
	Yes (N=6)	No (N=114)	
Male gender	3 (50)	74 (65)	.76
Family history of cerebrovascular disease	3 (50)	49 (43)	.93
Hypertension	5 (83)	62 (54)	.34
Type 2 diabetes mellitus	1 (17)	35 (21)	.78
Hypercholesterolemia	3 (50)	35 (31)	.59
Hypertriglyceridemia	1 (17)	31 (27)	.92
Cardiac disease	1 (17)	43 (38)	.54
Smoking	2 (33)	44 (39)	.86
Alcohol consumption	0 (0)	31 (27)	.32
Body mass index > 25 kg/m^2	5 (83)	71 (62)	.54
Age in years, median (range)	55 (34–64)	62 (33–65)	.11

*For each patient with ischemic cerebrovascular insult there was one control person who agreed in gender, age, and other clinical risk factors for arterial ischemic events.

†Assessed by χ^2 or Fisher exact test (binary categorical variables) and by Mann-Whitney test in case of age.

TABLE 5. The Odds Ratios (95% confidence intervals)† for Ischemic Cerebrovascular Insult Due to Presence of Either Arg-506-Gln or G20210A Mutation in Participants' Subgroups*

	Clinical Risk Factor for Arterial Ischemic Disease	
	Present	Absent
Male gender	2.9 (0.7–17.8)	4.6 (1.2–17.8)
Hypertension	2.4 (0.8–7.4)	9.2 (1.1–17.8)
Type 2 diabetes mellitus	1 (0.06–16.6)	4.3 (1.5–12.2)
Hypercholesterolemia	1.4 (0.3–6.6)	5.9 (1.6–21.2)
Hypertriglycerdemia	1 (0.06–16.7)	4.3 (1.5–12.8)

†The odds ratios and their 95% confidence interval were calculated by Miettenan's formula.

*The subgroups considered are those elements of clinical profile that significantly interacted with the studied mutations in predicting the ischemic stroke, ie, $P < .05$, as assessed by logistic regression.

The role of gender in mediating the genetic coagulation abnormalities is in concordance with the meta-analysis (3). Although men are at greater risk of developing ischemic stroke than women (due to differences in fibrinolytic activity, presumably mediated by sex hormones [8,9]), it appears that in women the protective role of estrogens in developing ischemic stroke is abolished by inherited prothrombotic mutations.

However, our other findings may appear as obscure ones, and in contrast with the study of Szolnoki et al (4), which reported that factor V Leiden increases the susceptibility to ischemic stroke only in combination with hypertension or diabetes mellitus.

Aside from the population differences, the possible explanation for these divergent findings is the differences in the study designs. In contrast to others, we have used the exact, individual pairing of cases and controls with respect to the presence and intensity of clinical risk factors for ischemic stroke. This is evidently advantageous over using controls from the general population, with much lower presence of clinical risk factors. In this way any excess in the prevalence of the studied mutations in the patient group speaks directly to its association with ischemic stroke, and we do not have to rely on the limited ability of multifactorial methods to screen the confounding effects (7). This is also advantageous when

studying patient subgroups. Hypertensive persons from the general population, for example, differ from typical hypertensive stroke patients, having more likely one or more clinical risk factors in addition to hypertension. This can also be the source of a bias when studying interactions of genetic polymorphisms with clinical risk factors using controls from the general population.

Consider, for example, a group of hypertensive persons having the clinical risk profile typical for patients with ischemic stroke (our control persons). Such persons are likely to take one or more medications to treat hypertension or some other disease. Some of these drugs (acetylsalicylate, warfarin, ADP antagonists) have proven indirect anticoagulative effect. The use of such a drug may, in turn, prevent against ischemic stroke mediated by an inherited gene polymorphism with a trend toward a hypercoagulable state. In this way, a hypertensive person using an anticoagulative drug may be at a lower risk of developing coagulative-precipitated ischemic stroke than the normotensive person not using such or a similar drug. This can explain why hypertension and the studied mutations were more frequently found in control persons than in cases of ischemic stroke in our study. If we used controls from the general population, as Szolnoki et al (4), the quite different results could be obtained. The fact that hypertension and diabetes mellitus are more frequent in patients with small-vessel disease than in patients with large brain infarcts (8), in contrast to prothrombotic mutations, which more often give rise to large brain infarcts (9), may also be associated with the use of drugs that affect blood coagulation. Evidently, in investigating the interaction of clinical risk factors with genetically determined coagulation disturbances, the use of drugs with potential effect on the coagulatory cascade is frequent and should be accounted for. In previous studies this problem was left unrecognized. We also did not systematically address this issue, since we have not anticipated all of our findings.

In conclusion, in the studied sample of adult North Mediterranean population younger than 65 years, the presence of factor V Leiden and prothrombin G20210A mutations was associated with first-time ischemic stroke, especially in women and in patients without clinical risk factors. Further studies are needed to enlighten the possible role of medication used in affecting the risk of stroke mediated by inherited prothrombotic mutations.

REFERENCES

1. Swensson PJ, Dahlback B. Resistance to activated protein C as a basis for venous thrombosis. *N Engl J Med* 1994;330:517.
2. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996;88:3698.
3. Kim RJ, Becker RC. Association between factor V Leiden, prothrombin G20210A and methylenetetrahydrofolate reductase C677T mutations a events of the arterial circulatory system: A meta-analysis of published studies. *Am Heart J* 2003;146:948.
4. Szolnoki Z, Somogyvári F, Kondacs A, et al. Evaluation of the modifying effects of unfavourable genotypes on classical clinical risk factors for ischaemic stroke. *J Neurol Neurosurg Psychiatry* 2003;74:1615.
5. Concato J, Feinstein AR, Holford TR. The risk of determining risk with multivariate models. *Ann Intern Med* 1993;118:201.
6. Zöller B, Svensson PJ, He X, Dahlbäck B. Identification of the same factor V gene mutation in 47 out of 50 thrombosis: Prone families with inherited resistance to activated protein C. *J Clin Invest* 1994;94:2521.
7. Kirkwood BR. Essentials of Medical Statistics. Oxford: Blackwell Scientific Publications; 1988.
8. Hassan A, Markus HS. Genetics and ischemic stroke. *Brain* 2000;123:1784.
9. Szolnoki Z, Somogyvári F, Kondacs A, Szabó M, Fodor L. Evaluation of the roles of the Leiden mutation and ACE/I/D polymorphism in subtypes of ischaemic stroke. *J Neurol* 2001;248:756.
10. MacCallum PK, Cooper JA, Howarth DJ, Meade TW, Miller GJ. Sex differences in the determinants of fibrinolytic activity. *Thromb Haemost* 1998;79:587.
11. Gebara OC, Mittleman MA, Walsh BW, et al. Fibrinolytic potential is significantly increased by oestrogen treatment in postmenopausal women with mild dyslipidaemia. *Heart* 1998;80:235.