Plasminogen Activator Inhibitor-1 4G/5G Polymorphism and Risk of Stroke

Replicated Findings in Two Nested Case–Control Studies Based on Independent Cohorts

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Background and Purpose—Impaired fibrinolytic function secondary to elevated plasminogen activator inhibitor-1 (PAI-1) levels has been implicated in ischemic stroke. PAI-1 levels are determined by genetic factors and environmental factors, triglyceride levels in particular. The aim of this study was to investigate the common functional 4/5 guanosine (4G/5G) polymorphism in the promoter region of the PAI-1 gene and the risk of stroke.

Methods—A nested case–control study design was applied, using baseline data for 2 independent cohorts obtained at population-based surveys in northern Sweden. In study A, there were 113, and in study B, there were 275 individuals without major concomitant disease at baseline who later experienced a first-ever stroke. Blood samples obtained at baseline were analyzed for potential risk factors, including the 4G/5G polymorphism of the PAI-1 gene.

Results—The 4G allele of the PAI-1 polymorphism was associated with an increased risk of future ischemic stroke in both studies (odds ratio [OR] of 4G homozygosity, 1.87; 95% CI, 1.12 to 3.15 in study A; OR of 4G homozygosity, 1.56; 95% CI, 1.12 to 2.16 in study B). Individuals with the combination of hypertriglyceridemia and 4G homozygosity were at the greatest risk of developing stroke. Multiple logistic regression analysis identified 4G homozygosity, systolic blood pressure, and diabetes as independent predictors of ischemic stroke.

Conclusions—Identical findings in 2 independent studies strongly suggest a true and clinically important association between PAI-1 4G/5G genotype and risk of future ischemic stroke. The observed modification of the genotype effect by triglycerides may be interpreted as a gene–environment interaction.

Key Words: genetics ■ plasminogen activator inhibitor-1 ■ polymorphism ■ stroke

Impaired fibrinolytic function secondary to elevated plasma plasminogen activator inhibitor-1 (PAI-1) activity is associated with coronary heart disease, and PAI-1 levels are consistently elevated in blood samples collected during the acute phase of ischemic stroke. However, when samples are obtained before stroke, the power of PAI-1 levels to predict future stroke may be limited.

Across different populations, plasma levels of PAI-1 antigen are associated with a 4/5 guanosine (4G/5G) polymorphism in the promoter region of the PAI-1 gene. People homozygous for the 4G allele have the highest and 5G homozygotes have the lowest PAI-1 levels. The relationship between 4G/5G polymorphism of the PAI-1 gene and stroke is unclear. It has been reported that the 4G/4G genotype confers an increased risk of stroke, but other investigators have reported the same genotype to be neutral or even protective in terms of stroke risk.

High PAI-1 levels are linked to several components of the insulin resistance syndrome, most notably hypertriglyceridemia. There may be a genetic determinant of the association between PAI-1 and triglycerides, in that a triglyceride response element has been identified in the promoter region of the PAI-1 gene.

There are many reasons why results of association studies in common disorders are difficult to replicate. There may be bias from selected patient populations, inappropriate controls, or misclassification of outcome. Inadequate sample size may give rise to false-negative results, and there may exist differences between populations (eg, allelic heterogeneity). In stroke, with complex interactions between genetics, lifestyle,
and social and environmental factors, it is obvious that genetic factors may have a different impact depending on the population burden of nongenetic stroke risk factors.

In the present study, we tried to rectify some of the problems in previous studies. A population-based nested case–control approach was used (ie, blood samples donated before stroke were analyzed), thereby avoiding problems of patient selection. Fatal as well as nonfatal cases were included after careful validation of clinical diagnoses. Matched controls were selected from the same background population. The findings of 1 stroke population were replicated in an independent stroke population.

The aims of this study were to investigate the role of 4G/5G PAI-1 polymorphism in the causation of stroke and to explore the interaction between 4G/5G genotype and hypertriglyceridemia.

Materials and Methods

Study Population
All subjects had been participants in population-based cardiovascular risk factor surveys in which blood samples and a broad range of interview data were collected. Surveys were conducted in northern Sweden. Screenings within the framework of the World Health Organization Monitoring Trends Determinants in Cardiovascular Disease (WHO MONICA) Project were performed in 1986, 1990, 1994, and 1999. In all, 6952 randomly selected men and women in the age group of 25 to 74 years participated in the MONICA surveys. In parallel, men and women in Västerbotten County were asked to participate in a health survey (the same design as the MONICA population surveys) the year they turned 30, 40, 50, or 60 years of age. During the period January 1, 1985, to September 20, 2000, ~67 500 people participated.

Participants in the MONICA and the Västerbotten surveys consented in writing to donate a blood sample stored at ~80°C at the Northern Sweden Medical Research Bank for future research.

Case Ascertainment and Validation
Since 1985, a population-based stroke registry (ages 25 to 74 years) covering the same area as the risk factor surveys has been kept at the Northern Sweden MONICA Center. Procedures for case ascertainment were described in detail previously. Data on subtype of stroke (ischemic or hemorrhagic) was based on CT scan, and subarachnoidal hemorrhages were excluded. The present studies used a nested case–control design that included all individuals who experienced a first-ever stroke at <75 years of age after having participated in either the MONICA or Västerbotten health surveys. Thus, all patients provided baseline information and donated blood samples before the stroke event, and the population-based stroke registry ensured that all stroke events were prospectively included.

Study A included 157 patients with first-ever stroke events occurring during the period January 1, 1985, to August 31, 1996, and study B, which covered September 1, 1996, to September 20, 2000, included 427 stroke cases. Both studies used the same inclusion and exclusion criteria, and individuals with previous myocardial infarction (n=15 versus 55), a cancer diagnosis (n=13 versus 29), or insufficient amount of blood in the sample at baseline (n=16 versus 68) were excluded. After exclusion, 113 (91 ischemic, 19 hemorrhagic, and 3 unspecified strokes; 71 men and 42 women) and 275 (229 ischemic, 42 hemorrhagic, and 4 unspecified stroke; 157 men and 118 women) cases remained for analysis in the 2 studies, respectively.

Two controls for each case were selected among participants in the same health surveys and matched for sex, age (±2 years), and domicile. Control subjects were excluded under the same criteria as the cases.

The studies were approved by the research ethics committee of Umeå University and the data handling procedures by the National Computer Data Inspection Board.

Risk Factors, Biochemical Analyses, and Genotyping
Baseline data included age, smoking status, height, weight, blood pressure, and presence of diabetes mellitus. A diagnosis of diabetes was self-reported or the results of a glucose tolerance test according to older WHO criteria. Elevated blood pressure was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 or antihypertensive treatment. Fasting glucose and total serum cholesterol were determined at baseline. In study A, serum triglycerides were measured enzymatically, and PAI-1 antigen was determined using an ELISA with kits from Biopool AB (Imulys PAI-1).

DNA was isolated from whole blood or buffy coats. Genotyping of the PAI-1 4G/5G promoter polymorphism was performed using the method described by Margaglione et al in study A. In study B, primers and probes were supplied by Assays-by-Design Service (Applied Biosystems). Polymerase chain reaction was run with the manufacturer-recommended TaqMan protocol with 10 ng of genomic DNA on an Applied Biosystems PRISM 7900 HT Sequence Detector System (Perkin-Elmer). The sequences were VIC probe: VICGTGACCTCCCCACGGT; FAM probe: 6FAMACTCCCACGTGTC; forward primer: GCCAGACAAAGGTGTGTA-CACA; and reverse primer: GCCGCGCTCGATGATAC.

Statistical Methods
Conditional logistic regression analyses, appropriate for this kind of 1:2 matched case–control study design, were used in the main analyses to identify predictors of stroke. Odds ratios (ORs) are presented with corresponding 95% confidence limits. Baseline characteristics were compared between cases and controls using simple conditional logistic regression. To adjust for classical known stroke risk factors, a multiple model was used to test the effect of the genotype. In the complementary analyses (ie, analyses of cases and controls separately) and analyses of subgroups defined by a specific genotype or level of serum triglycerides, where matching no longer applies because of “splitting” of the strata, 2-tailed t tests were used for continuous variables and Pearson’s χ² tests for categorical variables. A P value <0.05 was considered statistically significant. SPSS version 12.0 was used in the analysis.

Results

Baseline Characteristics
For the 2 studies, Table 1 shows baseline characteristics of individuals who subsequently had a first-ever stroke and of matched controls who remained free of cardiovascular disease during follow-up. In the 2 studies, stroke occurred on average 30 and 59 months, respectively, after participation in the cardiovascular risk factor survey. Cases had higher systolic and diastolic blood pressures and higher fasting plasma glucose than controls in both studies. More cases than controls had a diagnosis of diabetes. In study B, with more statistical power, body mass index (BMI) and serum cholesterol were significantly higher in cases, and current smoking was more prevalent. Serum triglyceride levels and PAI-1 levels were measured only in study A and showed elevated serum triglycerides in stroke patients.

PAI-1 4G/5G Promoter Genotype
Genotyping for the PAI-1 4G/5G promoter polymorphism was performed in 329 of the 339 participants in study A and in 809 of the 824 subjects in study B. Genotyping was not possible because of technical problems in 10 (2 cases and 8
controls) and 15 subjects (8 cases and 7 controls), respectively. The genotype distributions were in Hardy–Weinberg equilibrium and are shown in Table 2. 4G homozygosity was more common among stroke cases, the difference reaching statistical significance in study A ($P = 0.018$) but not in study B ($P = 0.061$). When cases were subgrouped according to stroke subtype, the overrepresentation of 4G homozygotes in the ischemic phenotype group was greater and found in both studies ($P = 0.018$ and $P = 0.008$, respectively). Patients with hemorrhagic stroke did not show any overt difference in PAI-1 genotype distribution compared with controls, but the statistical power to detect a difference was low.

Smoking habits, blood pressure, serum lipid and lipoprotein concentrations, and plasma glucose level did not differ according to PAI-1 genotype in either cases or controls (data not shown).

The OR of 4G/4G genotype for ischemic stroke was 1.87 (95% CI, 1.12 to 3.15) in study A. This was replicated in study B (OR, 1.56; 95% CI, 1.12 to 2.16). Elevated blood pressure and diabetes also conferred increased risk of ischemic stroke in both studies. A small but significant rise in stroke risk was seen for BMI and serum cholesterol in study B. A multiple conditional logistic regression model, including 4G homozygosity, elevated blood pressure, diabetes, and current smoking applied to the 2 studies found these variables, apart from smoking, to be associated independently with increased risk of future ischemic stroke (Table 3).

**Interaction Between PAI-1 Genotype and Serum Triglycerides**

Because in vivo and in vitro studies have suggested an interaction between triglycerides and PAI-1 4G/5G genotype,
we investigated whether such an interaction could be of importance for the occurrence of stroke in study A. When subjects were subgrouped according to serum triglyceride level (cut-off of 1.7 mmol/L), the association of the PAI-1 4G allele with stroke was found to be confined to hypertriglyceridermic individuals ($\chi^2 = 6.789; 2 \text{ df}; P = 0.034$). Conversely, serum triglycerides discriminated between cases and controls in 4G homozygotes (OR, 2.50 [95% CI, 1.13 to 5.53]; $P = 0.022$) but not in heterozygotes or 5G homozygotes (OR, 1.33 [95% CI, 0.72 to 2.46]; $P = 0.36$).

In study A, we then applied a conditional regression model including an interaction factor of triglycerides and genotype as predictor of ischemic stroke. After adjustment for elevated blood pressure, diabetes, and smoking, this factor was found to be associated with ischemic stroke ($P = 0.036$). The excess risk was confined to individuals with the combination triglyceride $\geq 1.7 \text{ mmol/L} \times 4G/4G$ (OR, 3.58 [95% CI, 1.27 to 10.09]; $P = 0.016$).

PAI-1 antigen was measured in study A. There was an association between PAI-1 genotype and plasma PAI-1 antigen levels ($40.3 \pm 21 \mu g/L$ in 4G homozygotes, $37.0 \pm 19$ in heterozygotes, and $33.3 \pm 20$ in 5G homozygotes, cases and controls analyzed together), with a significant difference between 4G and 5G homozygotes ($P = 0.038$).

**Discussion**

In these 2 prospective nested case–control studies, the 4G allele of the PAI-1 4G/5G promoter polymorphism was associated with an increased risk of future ischemic stroke. The single base pair 4G/5G polymorphism in the PAI-1 promoter is common $^7$ and has been shown to influence the level of transcription of the PAI-1 gene in vitro in endothelial cells. $^9$ The 4G allele, having a higher transcriptional activity than the 5G allele, has been associated with enhanced plasma PAI-1 activity. $^{19,20}$ Apart from impaired fibrinolytic capacity attributable to high levels of circulating PAI-1, the local tissue effects are not fully understood, but PAI-1 seems to influence processes of smooth muscle cell proliferation, plaque, and matrix remodeling in the direction of promoting as well as protecting against atherothrombosis. $^{21}$

Our findings that the PAI-1 4G allele is linked to increased risk of ischemic stroke only in hypertriglyceridermic individuals and that serum triglycerides is a predictor of future stroke only in 4G homozygotes are consistent with in vivo and in vitro studies. Hypertriglyceridermia is associated with increased plasma PAI-1 levels, $^{22}$ and the interaction of triglycerides with plasma PAI-1 activity is dependent on the 4G/5G polymorphism. $^{20}$ This is caused by a very-low-density lipoprotein–inducible transcription factor binding to a site in the PAI-1 promoter overlapping the 4G/5G polymorphic site. $^{13}$ Thus, part of the risk of stroke attributed to hypertriglyceridermia might be mediated by genotype-specific influences on PAI-1. It may seem surprising that PAI-1 genotype was a stronger predictor of future stroke than plasma PAI-1 antigen, but these levels are known to be influenced by several other metabolic, inflammatory, and vascular factors, $^{23}$ and this may reduce the capacity of PAI-1 antigen measurements to predict stroke. $^{24}$

Although an association between polymorphism of the PAI-1 gene and stroke has been shown previously, $^8$ most studies have failed to confirm a relationship, $^4,9–12$ and indeed, a meta-analysis has shown the 5G/5G PAI-1 genotype to be associated with increased risk of stroke. $^{25}$

There are several possible explanations for the discrepant results, the most obvious being differences in study population and design. An advantage of the nested case–control design is the prospective property with data collection before the stroke event. This eliminates the risks of recall bias, missing information in fatal cases, and changes in exposure in nongenetic variables caused by the occurrence of stroke itself. The weakness of this approach is that lifestyle and social factors may change during the follow-up. The relatively short median interval from participation in the risk factor surveys and the stroke event (30 and 59 months in the 2 studies) make this less of a problem.

The need for replication of associations before evidence is declared as convincing is increasingly recognized in clinical genetic studies. $^{14,26}$ If nonsystematic biases exist in an initial study, they are unlikely to be the same when the study is replicated. $^{14}$ One of the strengths of the present work is that the findings of 1 study population was replicated in a second population, with the same hypothesis being tested and both studies showing that 4G/5G promoter polymorphism predicted future risk of ischemic stroke. The 2 populations were independent in the sense that they were separated in time and predefined before any analyses were performed. However, they were performed in the same geographical area, which leaves a possibility that the findings are specific to this population. We calculate the population attributable risk of 4G homozygosity to be $\approx 17\%$.

In summary, the present study suggests that PAI-1 genotype influences the risk of future stroke and that knowledge of an individuals PAI-1 4G/5G genotype may be more predictive than a single measurement of the plasma level of PAI-1. The excess risk in carriers of the 4G allele is modified by triglyceride levels.

**TABLE 3. ORs for Ischemic Stroke; Multiple Conditional Logistic Regression**

<table>
<thead>
<tr>
<th></th>
<th>Study A</th>
<th></th>
<th>Study B</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P Value</td>
<td>OR (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td>4G/4G</td>
<td>1.79 (1.01–3.19)</td>
<td>0.048</td>
<td>1.60 (1.12–2.29)</td>
<td>0.010</td>
</tr>
<tr>
<td>Elevated blood pressure</td>
<td>2.58 (1.31–5.08)</td>
<td>0.006</td>
<td>2.43 (1.65–3.58)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5.26 (1.88–14.72)</td>
<td>0.002</td>
<td>2.83 (1.43–5.61)</td>
<td>0.003</td>
</tr>
<tr>
<td>Current smoking</td>
<td>0.99 (0.48–2.05)</td>
<td>0.98</td>
<td>1.39 (0.88–2.19)</td>
<td>0.16</td>
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Acknowledgments

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