A Common Polymorphism of the Protein Z Gene Is Associated With Protein Z Plasma Levels and With Risk of Cerebral Ischemia in the Young

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Background and Purpose—The vitamin K–dependent protein Z (PZ) has been shown to possess anticoagulant as well as procoagulant properties. Plasma levels of PZ show a broad interindividual variation, but it is unknown to which extent this variation is under genetic control. Recent clinical studies revealed contradictory results on the association of PZ plasma levels and the risk of ischemic stroke.

Methods—We performed a case-control study including 200 patients with cerebral ischemia aged ≤50 years and 199 control subjects from the same South German region. We investigated a possible association of 2 common single nucleotide mutations in the PZ gene with the risk of cerebral ischemia. Furthermore, enzyme-linked immunosorbent assay measurements were done in control subjects without vascular disease to detect a potential association of different genotypes with PZ plasma (antigen) levels.

Results—In patients, the frequency of the A allele of the intron F polymorphism G79A was significantly lower than in controls (15.7% versus 24.4%; odds ratio, 0.58; 95% CI, 0.39 to 0.86; \(P=0.007\); adjusted for age, sex, and conventional risk factors). The G allele of the promoter polymorphism A-13G tended to be less common in patients (4.2% versus 7.0%; adjusted odds ratio, 0.56; 95% CI, 0.28 to 1.13; \(P=0.105\)). In 42 control subjects, the A allele of the intron F polymorphism was associated with lower PZ antigen levels (\(P=0.0032\); Spearman correlation coefficient \(r_s=−0.48\)).

Conclusions—The A allele of an intron F polymorphism of the PZ gene appears to be a novel protective genetic marker for the risk of cerebral ischemia in young adults. In the context of juvenile stroke, high PZ plasma levels may represent a prothrombotic condition. (Stroke. 2004;35:40-45.)

Key Words: cerebral ischemia ■ polymorphism ■ protein Z

Protein Z (PZ) is a member of the family of vitamin K–dependent plasma coagulation factors, which includes procoagulant proteins such as factors II, VII, IX, and X, as well as the anticoagulant protein C.1,2 Two major mechanisms have been described to represent its role in coagulation. First, PZ promotes the assembly of thrombin with phospholipid surfaces, thus enhancing coagulation.3,4 Second, it is responsible for the binding of a specific PZ-dependent protease inhibitor to factor Xa and therefore indirectly acts as a natural anticoagulant.1,2,5

The physiological role of PZ is uncertain. In patients with low PZ levels, a bleeding tendency has been described.4,6,7 In contrast, the combination of PZ deficiency and factor V Leiden results in a strong thrombophilic phenotype in mice and in humans.8 Gris and colleagues9 reported low PZ levels in women with unexplained early fetal loss, suggesting an anticoagulant role of PZ in pregnancy. Thus, PZ seems to play a paradoxical role in thrombosis.2 Furthermore, the presence of PZ in atherosclerotic lesions suggests a role in the development of atherosclerotic disease.10

Five recent clinical case-control studies, mainly in young patients, on PZ levels and the risk of cerebral ischemia yielded contradictory results. All but 1 study investigated plasmatic PZ antigen levels in the postacute phase after ischemia and excluded patients on oral anticoagulation. Whereas Kobelt and colleagues11 described a significant association of high plasma levels of PZ with cryptogenic stroke, Vasse and colleagues12 found decreased levels in juvenile patients with cerebral ischemia. Another study by Heeb et al13 performed in an elderly population also depicted an association of low PZ and risk of stroke, but this finding was restricted to nondiabetic males. The study by Hankey et al14 was the only one that investigated PZ levels in the acute stage of cerebral ischemia. It found increased levels of PZ in
acute stroke, especially of atherosclerotic etiology. Interestingly, this association was no longer detectable in the same population 3 months after the ischemic event, thus indicating that PZ either plays a role in the acute stage of ischemia or is an acute phase reactant. Finally, Lopaciuk et al.\textsuperscript{15} did not detect any association between PZ levels and juvenile stroke in their study.

The gene encoding for PZ has been characterized,\textsuperscript{16} and several common single nucleotide polymorphisms in the gene were found.\textsuperscript{17} At present, nothing is known about the influence of different genotypes on the widely varying plasma levels of PZ. Investigation of genotypes has the advantage of being independent of various influences such as oral anticoagulation or acute effects early after ischemia.\textsuperscript{18} We therefore performed a case-control study to detect a potential association of common genotypes with juvenile cerebral ischemia, and, in the case of positive findings, to investigate the relation between relevant genotype(s) and PZ plasma levels.

**Subjects and Methods**

We analyzed 200 consecutive patients aged $\leq$50 years who were admitted to our university hospital for acute cerebral ischemia (56 transient ischemic attacks and 144 completed strokes) and 199 control subjects without a history of vascular disease. Control subjects were randomly selected from the population registries of the same region in southwest Germany. Non-German descent and the inability to provide informed consent or to perform the interview were exclusion criteria for both groups. Participation rate of eligible patients was 99.5\%, and that of control subjects was 70.6\% of all persons contacted by mail. All study participants underwent a standardized interview regarding vascular risk factors and family history of juvenile vascular disease. Estimates of conventional risk factors are based on the subject's own awareness of their presence in the standardized interview. In patients, only risk factors that had been diagnosed before the event were acknowledged.

All patients received a full clinical workup, including brain imaging (CT or MRI), ultrasound studies, and search for cardiac sources of embolism. We categorized patients as belonging to 1 of the following 5 etiologic subgroups: (1) large-artery atherosclerosis ($n=37$, defined as stenosis of $\geq50\%$ diameter reduction); (2) cardiac embolism ($n=46$, defined according to the Trial of Org 10172 in Acute Stroke Treatment [TOAST] criteria of cardiac embolism$^{19}$ and additionally including persistent foramen ovale as diagnosed by transthoracal echocardiogram and ultrasound studies); (3) dissection of brain-supplying arteries ($n=37$, as diagnosed by ultrasound and MRI or digital subtraction angiography); (4) cryptogenic embolism ($n=45$); and (5) others ($n=35$, including, eg, cerebral microangiopathy, vasculitis).

The study was approved by the local ethics board, and all subjects gave informed consent.

DNA was isolated from leukocytes with the use of a commercial kit according to the instructions of the supplier (Qiagen). For analysis of the PZ promoter polymorphism A-13G, a 272-bp fragment from the promoter region was amplified by polymerase chain reaction (PCR) with the use of the following oligonucleotides as primers: 5'-GGGTCTCCTGAGCCTTCACCGTTCATTT-3' and 5'-CAGGCACAACAGACAGGTAAGCCAGATG-3'. After incubation for 1 hour at 37°C with HpaII, an isoschizomere of HhaI (MBI Fermentas), the G allele yielded 2 DNA fragments of 157 and 115 bp on a 2% agarose gel after ethidium bromide staining. The A allele was not digestible.

The G79A polymorphism of intron F of the PZ gene was analyzed by amplification of a 320-bp sequence with the use of the primers 5'-TAACACCATAGACAGTCCGATTTCCGC-3' and 5'-ATGAACCTCGGCATTAGAACATGTGTTGAA-3'. Digestion for 1 hour at 37°C with an isoschizomere of HpaI, BstHPI (MBI Fermentas), yielded 2 products of 221 and 99 bp in length in the presence of the A allele, whereas the G allele was not digestible.

The different genotypes of both polymorphisms as detected by PCR and restriction analysis were confirmed in selected subjects by sequencing of the PCR amplificon (ABI-Prism 310, PE Biosystems).

To investigate a potential functional role of gene polymorphisms on the expression of PZ, we measured PZ antigen in the plasma of 42 control subjects. These subjects were randomly selected with respect to their genotype among those without conventional risk factors to avoid potential confounding effects of risk factors on PZ plasma levels. The aim of the selection was to investigate approximately the same number of heterozygotes and homozygotes for the G allele. This group included 9 homozygotes for the A allele, 16 heterozygotes, and 17 homozygotes for the G allele of the G79A intron polymorphism. Venous blood was drawn between 4 and 8 PM to avoid the daytime-dependent variations known for other coagulation proteins. A commercially available enzyme-linked immunoassay kit was used (DiagnosticaStago). All samples were measured twice, and mean values were used for further analysis. Variation of all measurements was $<10\%$.

We further performed testing for the presence of factor V Leiden and of the prothrombin G20210A mutation in all patients and control subjects by combined PCR and restriction fragment length analysis as described earlier.\textsuperscript{20}

The statistical software package SAS release 6.12 (SAS Institute, Inc) was used for statistical analysis. Odds ratios (ORs) and 95\% CIs were computed with the use of multivariate conditional logistic regression analyses. Because of differences between patients and control subjects in age and in sex, we performed an age- (by 5-year steps) and sex-stratified analysis of PZ genotype frequencies. The following dichotomous variables were included in the multivariate analysis: arterial hypertension, high cholesterol, and family history of stroke or myocardial infarction. Test for trend was performed by scoring the included categories and entering this ordinal variable into the regression analysis.

Differences of measured PZ levels in the subgroups with different PZ intron genotypes were analyzed by nonparametric Kruskal-Wallis test. Correlations were expressed by the Spearman correlation coefficient. To analyze the effects of sex and age on these differences, multivariate linear regression was performed.

Tests for allelic associations between the 2 loci were performed with the log-likelihood ratio statistic (EH program by Jürg Ott; http://linkage.rockefeller.edu/ott/eh.htm).

**Results**

Table 1 depicts demographic data and conventional vascular risk factors in both groups. Patients were significantly older than controls. Smoking was significantly less prevalent in patients than in controls (23\% versus 31.2\%; $P=0.029$), whereas hypertension, hypercholesterolemia, and diabetes mellitus (which was very rare in controls) were more common in patients.

Table 2 shows the prevalence of PZ genotypes in patients and controls. All tested loci were in Hardy-Weinberg equilibrium. There was a significant allelic association between the 2 loci in the control population ($P=0.004$) but not among cases ($P=0.156$). Estimated haplotype frequencies were significantly different in cases and controls ($P=0.012$).

The frequency of the A allele (mutant) of the intron F polymorphism was significantly lower in patients than in controls (15.7\% versus 24.4\%; OR, 0.57; 95\% CI, 0.4 to 0.81, after adjustment for age and sex; OR, 0.58; 95\% CI, 0.39 to 0.86; $P=0.007$, after additional adjustment for hypertension, hypercholesterolemia, and positive family history). We also found a comparable negative association of the G allele (mutant) of the promoter polymorphism with cerebral ische-
mi; however, this failed to achieve statistical significance because of the lower prevalence of this mutation (4.2\% versus 7.0\%; OR, 0.59; 95\% CI, 0.3 to 1.1; adjusted OR, 0.56; 95\% CI, 0.28 to 1.13; \(P=0.105\)).

Additionally, the association of individual PZ genotypes with ischemia was investigated (Table 2). Homozygosity for the A allele of the intron F polymorphism significantly decreased the risk of cerebral ischemia (OR, 0.2; 95\% CI, 0.1 to 0.4; \(P=0.029\)).

### TABLE 1. Demographic Data of Study Population and Prevalence of Conventional Vascular Risk Factors and of Established Thrombophilic Conditions

<table>
<thead>
<tr>
<th></th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>OR Crude (95% CI)</th>
<th>OR Adjusted* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=200</td>
<td>n=199</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age, mean±SD</strong></td>
<td>40.1±7.8</td>
<td>35.1±9.1</td>
<td>(-0.0001)</td>
<td>(-0.001)</td>
</tr>
<tr>
<td><strong>Female sex</strong></td>
<td>77 (38.5)</td>
<td>111 (55.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>66 (33)</td>
<td>13 (6.5)</td>
<td>OR 7.0 (3.7–13.2)</td>
<td>OR 5.5 (2.9–10.5)</td>
</tr>
<tr>
<td>Diabetes mellitus†</td>
<td>18 (9)</td>
<td>2 (1)</td>
<td>OR 9.8 (2.2–42.6)</td>
<td>OR 6.1 (1.4–27.2)</td>
</tr>
<tr>
<td>High cholesterol (or medical treatment)</td>
<td>45 (22.5)</td>
<td>19 (9.5)</td>
<td>OR 2.9 (1.6–5.2)</td>
<td>OR 2.5 (1.4–4.6)</td>
</tr>
<tr>
<td>Current smoking‡</td>
<td>46 (23)</td>
<td>62 (31.2)</td>
<td>OR 0.7 (0.5–1.1)</td>
<td>OR 0.6 (0.4–1.0)</td>
</tr>
<tr>
<td>Positive family history§</td>
<td>21 (10.5)</td>
<td>32 (16.1)</td>
<td>OR 0.6 (0.4–1.2)</td>
<td>OR 0.6 (0.3–1.1)</td>
</tr>
<tr>
<td>Factor V Leiden</td>
<td></td>
<td>11 (5.5)</td>
<td>15 (7.5)</td>
<td>OR 0.8 (0.3–1.75)</td>
</tr>
<tr>
<td>Prothrombin G20210A mutation</td>
<td></td>
<td>5 (2.5)</td>
<td>5 (2.5)</td>
<td>OR 1.3 (0.3–4.8)</td>
</tr>
</tbody>
</table>

*Stratified by age in 5-year categories and by sex.
†Not stratified by sex because of no male controls with diabetes; not included into multivariate analysis because of extremely low prevalence in controls.
‡Despite statistical significance, not included into multivariate analysis because likely due to systematic bias.
§Family history was looked upon as positive if at least 1 first-degree relative was reported with stroke or myocardial infarction before the age of 50 years.
¶Neither subjects homozygous for Factor V Leiden or for the prothrombin mutation nor carriers of combined heterozygous mutations were detected.

### TABLE 2. Analysis of PZ Intron F G79A and Promoter A-13G Polymorphism Genotypes in Total Patients and Control Groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>OR* Crude (95% CI)</th>
<th>OR† Adjusted (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein Z intron G79A polymorphism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>140 (70)</td>
<td>118 (59.3)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>At least 1 A allele</td>
<td>60 (30)</td>
<td>81 (40.7)</td>
<td>0.6 (0.4–0.9)</td>
<td>0.6 (0.4–0.95)</td>
</tr>
<tr>
<td><strong>Protein Z promoter A-13G polymorphism‡</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>184 (92)</td>
<td>173 (86.9)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>At least 1 G allele</td>
<td>16 (8)</td>
<td>26 (13.1)</td>
<td>0.6 (0.3–1.2)</td>
<td>0.5 (0.2–1.1)</td>
</tr>
</tbody>
</table>

*Stratified by age and sex.
†Stratified by age and sex and additionally adjusted for hypertension, high cholesterol, and family history.
‡Separate analysis for heterozygosity and homozygosity for the PZ promoter polymorphism was not performed with respect to the very low number of subjects homozygous for the G allele (n=1 in patients and n=2 in control group, respectively).
Presence of at least 1 A allele (heterozygous or homozygous) also showed a significant negative association with ischemia (OR, 0.6; 95% CI, 0.4 to 0.9). The risk of stroke was significantly associated with the number of A alleles (P \leq 0.009, test for trend). All associations were consistent after adjustment for hypertension, hypercholesterolemia, and positive family history (Table 2). Additional adjustment for the confounders smoking and diabetes mellitus did not substantially change the results (eg, for homozygosity for the A allele of the intron F polymorphism: OR, 0.2; 95% CI, 0.05 to 0.7 instead of OR, 0.2; 95% CI, 0.05 to 0.8; or, for presence of at least 1 A allele of the intron F polymorphism: OR, 0.6; 95% CI, 0.4 to 0.93 instead of OR, 0.6; 95% CI, 0.4 to 0.95).

With regard to PZ genotypes, no difference was found between patients with transient ischemic attack and those with completed stroke (data not shown).

Table 3 depicts the prevalence of intron F genotypes in different subgroups. With respect to the marked difference between dissection and other etiologies, we performed a post hoc analysis that compared controls and all patients with other etiologies without dissection. In the group of patients without dissection, the adjusted OR for presence of at least 1 A allele of the intron F polymorphism was 0.4 (95% CI, 0.2 to 0.7; P=0.0009).

In healthy carriers of the A allele of the intron F polymorphism, the measured PZ plasma levels were lower than in carriers of the G allele (Figure). Accordingly, the phenotype difference between the 3 genotype groups was statistically significant (P=0.0032). Spearman coefficient for the existing correlation was r_s = -0.48. A similar correlation was seen when we stratified for age and sex. In a multivariate linear regression model, age and sex were not significantly different in genotype groups (P=0.25 and P=0.8, respectively).

The prevalence of factor V Leiden and of the prothrombin G20210A mutation was not significantly different in patients and control groups (Table 1). In the controls, the prevalence of both mutations was similar to that described earlier for healthy white populations.21

### Table 3. Analysis of PZ Intron F G79A Polymorphism Genotypes in Different Etiological Subgroups

<table>
<thead>
<tr>
<th>Protein Z Intron G79A Genotype</th>
<th>n (%)</th>
<th>OR * Crude (95% CI)</th>
<th>OR† Adjusted (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>118 (59.3)</td>
<td>81 (40.7)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cryptogenic embolism (n=45)</td>
<td>36 (80)</td>
<td>9 (20)</td>
<td>0.3 (0.2–0.7)</td>
<td>0.2 (0.1–0.4)</td>
</tr>
<tr>
<td>Macroangiopathy (n=37)</td>
<td>29 (78)</td>
<td>8 (22)</td>
<td>0.4 (0.2–0.9)</td>
<td>0.4 (0.1–1.0)</td>
</tr>
<tr>
<td>Cardiac embolism (n=46)</td>
<td>33 (72)</td>
<td>13 (28)</td>
<td>0.5 (0.2–1.0)</td>
<td>0.4 (0.2–0.8)</td>
</tr>
<tr>
<td>Dissection (n=37)</td>
<td>19 (51)</td>
<td>18 (49)</td>
<td>1.4 (0.7–2.9)</td>
<td>1.4 (0.7–3.0)</td>
</tr>
<tr>
<td>All etiologies without dissection (n=163)</td>
<td>121 (74.2)</td>
<td>42 (25.8)</td>
<td>0.5 (0.3–0.8)</td>
<td>0.4 (0.2–0.7)</td>
</tr>
</tbody>
</table>

*Stratified by age and sex.
†Stratified by age and sex and additionally adjusted for hypertension, cholesterol, and family history.

### Discussion

Our study demonstrates a significant association between the PZ gene and the risk of cerebral ischemia in young adults. This association was independent of age and sex as well as conventional risk factors. The prevalence of both polymorphisms in our control group was not significantly different from those found in another healthy European population.17 We also showed a significant negative allele dose-dependent correlation between the more common of the 2 polymorphisms under investigation and PZ antigen levels. This effect was independent of age and sex. Therefore, our results (1) indicate a strong genetic control of plasma PZ levels and (2) indirectly indicate a higher risk of juvenile stroke in subjects with high PZ plasma levels.

The methodology of our study was limited to the detection of associations between genotypes and the risk of stroke and between genotypes and PZ levels. A direct regulatory role of the intron polymorphism on plasma protein levels seems unlikely but is possible. More likely, the linkage disequilibrium between intron and promoter polymorphisms as well as linkage with other, unknown mutations of the PZ gene may explain this finding.

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![Graph showing PZ antigen plasma levels in carriers of different genotypes of the PZ intron F G79A polymorphism.](image-url)
A strength of our study was the almost complete study participation of patients. This fact might be due to the keen interest of usually well-informed young patients in helping to provide a better understanding of juvenile cerebral ischemia in the future. Furthermore, the control group of our study was population based and also had a relatively high participation rate. It therefore might claim to be representative of the genetic background in our region of southwest Germany. However, there was a statistically significant difference in age and sex between patients and control subjects. For this reason, statistical adjustment for age and sex was done and revealed substantially unchanged results from the crude analyses, suggesting a minor role of these factors. Surprisingly, our study found a slightly significant negative association of current smoking with the risk of ischemia. This may partly reflect a bias caused by the interview used, which inquired about only current but not former smoking. In patients with previous ischemic events or with multiple risk factors, such knowledge may have led to cessation of smoking in several cases. Furthermore, many other studies found smoking to be only a weak and hardly significant risk factor for juvenile cerebral ischemia, and our finding in a restricted study population may also be considered accidental. We therefore decided not to include smoking as a variable in the multivariate model of analysis. However, if smoking (as well as diabetes mellitus, which was extremely rare in controls) had been included, this would not have changed the estimated risk of ischemia dependent on PZ genotype.

In a typical population of elderly stroke patients, the high prevalence of conventional vascular risk factors is likely to mask the influence of relatively weak factors such as genetically determined thrombophilic conditions. For instance, the presence of the prothrombin G20210A mutation has inconsistently been described to represent a risk factor in juvenile stroke but not in elderly patients. Therefore, we chose to investigate a highly selected population of juvenile patients for the potential impact of PZ genotypes in cerebrovascular ischemia. Although we did not find an increased prevalence of factor V Leiden or the prothrombin mutation, the association found with PZ genotypes was relatively strong and statistically highly significant, thus indicating a crucial role of this factor in cerebrovascular ischemia.

One should be aware of the heterogeneity of stroke etiologies compared with ischemic heart disease, especially in juvenile patients. Therefore, we divided our patient group into etiologic subgroups using criteria different from those developed for elderly stroke patients. Even if our study was not statistically powered to test this issue, our study showed marked differences between etiologic subgroups with respect to PZ genotype distribution. There was no association of genotype with the large subgroup of dissections. Thus, a post hoc analysis excluding patients with dissection showed a significant and even more pronounced negative association of the intron F A allele with juvenile cerebral ischemia. Since PZ is a plasminogen activator (co)factor, it may be hypothesized that its impact on thromboembolic cerebral ischemia is higher than that in patients with arterial dissection. However, in addition to impairment of hemodynamics, local thrombosis at the injured endothelial site can lead to the complication of cerebral ischemia in arterial dissection as well.

Our findings are in accordance with the study of Kobelt and colleagues and with the known role of PZ as an enhancer of thrombin activity. However, Broze described a much higher relevance of PZ as a mediator of factor X inhibition in humans compared with its procoagulant role. Furthermore, the study by Vasse et al also showed a higher risk of stroke in subjects with low PZ. Our study cannot directly clarify the pathophysiological role of PZ in ischemic disease. However, our consistent and significant findings point toward a protective role of genetically determined low PZ plasma levels in juvenile cerebral ischemia.

In view of the contradictory data available thus far, the role of PZ certainly deserves further investigation in larger study populations. However, interference with multiple exogenous factors considerably limits the investigation of plasma PZ (antigen) levels in the context of cerebral ischemia. Reactive changes after ischemia do not permit reliable measurement in the acute phase, whereas analysis in the subacute phase is restricted to patients who do not receive anticoagulant therapy. One approach to avoiding these biases, ie, the performance of large, prospective trials, is costly and difficult to achieve. Therefore, an invariable genetic marker of individual PZ levels appears to be a promising tool for larger studies of the role of PZ in the pathogenesis of cerebral ischemia.

Acknowledgment

We are indebted to F. Litfin for technical assistance with the performance of factor V Leiden and PT G20210A mutation analysis.

References


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