Protein Z Gene Polymorphisms, Protein Z Concentrations, and Ischemic Stroke

J. Staton, PhD; M. Sayer, PhD; G.J. Hankey, MD, FRACP, FRCP; V. Cole, BSc; J. Thom, BSc; J.W. Eikelboom, MBBS

Background and Purpose—We aimed to determine whether A-13G or G79A polymorphisms of the protein Z gene that have been reported to be an important determinant of blood concentrations of protein Z are associated with risk of ischemic stroke in a broad range of stroke patients and controls.

Methods—We conducted a case control study of 151 hospital cases of first-ever ischemic stroke and 164 randomly selected community controls. Protein Z genotype was determined for the A-13G promoter polymorphism and the G79A intron F polymorphism, and plasma protein Z concentrations were measured during the first 7 days and at 3 to 6 months after the acute stroke event.

Results—Geometric mean concentrations of protein Z measured within 7 days of acute stroke were significantly higher in cases compared with controls (1.51 μg/mL versus 1.13 μg/mL; P<0.0001). Protein Z concentrations were highest among subjects with the A-13G AA genotype, intermediate among those with the AG genotype, and lowest among those with the GG genotype (1.39 μg/mL versus 1.05 μg/mL versus 0.76 μg/mL; P<0.0001); and highest among those with the G79A GG genotype, intermediate among those with the GA genotype, and lowest among those with the AA genotype (1.47 μg/mL versus 1.13 μg/mL versus 0.66 μg/mL; P<0.0001). The prevalence of A-13G and G79A genotypes was not significantly different between cases of ischemic stroke and controls. However, compared with the G79A GG genotype (reference), the odds of ischemic stroke was progressively lower for the heterozygote GA (odds ratio [OR], 0.83; 95% CI, 0.52 to 1.33) and the homozygote AA genotype (OR, 0.63; 95% CI, 0.20 to 1.98). A pooled analysis showed that compared with the G79A GG genotype (reference), the odds of ischemic stroke was progressively lower for the heterozygote GA (OR, 0.78; 95% CI, 0.57 to 1.07) and the homozygote AA genotype (OR, 0.31; 95% CI, 0.14 to 0.69).

Conclusion—The consistency of the association between protein Z genotypes, blood concentrations of protein Z, and ischemic stroke, determined using 2 different methods that have different sources of bias strengthens the evidence that increased blood concentrations of protein Z concentrations are associated causally with an increased risk of ischemic stroke. (Stroke. 2005;36:1123-1127.)

Key Words: genes ■ protein Z ■ stroke ■ thrombosis
tion\textsuperscript{13,14}), the association between protein Z genotype and ischemic stroke should not be subject to reverse causality bias and should also be largely free from confounding by other determinants of protein Z concentrations in the blood or risk factors for stroke.\textsuperscript{15,16} Therefore, examining the consistency of the association between (1) protein Z polymorphisms and blood concentrations of protein Z, and (2) protein Z polymorphisms and ischemic stroke, may help to clarify whether the association between protein Z and stroke is causal or confounded.

Lichy et al recently reported the A allele of the protein Z G79A polymorphism to be a novel protective genetic marker for stroke risk in 200 young stroke patients (age 50 years or younger) and 199 controls.\textsuperscript{17} Because the A allele also is associated with reduced blood concentrations of protein Z,\textsuperscript{15,17} these findings are consistent with the hypothesis that increased blood concentrations of protein Z concentrations are prothrombotic. However, this remains to be independently confirmed and has not been studied in unselected stroke patients.

We aimed to determine the relationship between (1) protein Z genotype for the common A-13G and G79A polymorphisms, (2) protein Z concentrations in the blood, and (3) ischemic stroke in our group of previously studied consecutive patients admitted to hospital with ischemic stroke and a similar number of age- and sex-matched controls resident in the same community.\textsuperscript{12}

Subjects and Methods
The study was approved by the Institutional Review Board of Royal Perth Hospital and informed consent was provided by all study participants.

Cases
Consecutive patients presenting to Royal Perth Hospital between March 1996 and June 1998 with first-ever ischemic stroke were approached for consent to participate in the study. Stroke was defined as a clinical syndrome characterized by rapidly developing clinical symptoms and/or signs of focal, and at times global, loss of brain function, with symptoms lasting >24 hours or leading to earlier death and with no apparent cause other than that of vascular origin.\textsuperscript{18,19} Ischemic stroke was defined as a stroke with either a normal CT brain scan, CT or MRI brain scan evidence of recent infarct in the clinically relevant area of the brain (performed within 3 weeks of the event), or at autopsy. Patients with cerebral hemorrhage, cerebral venous thrombosis, or liver disease were not included.

Baseline demographic data (age, sex), history of conventional vascular risk factors (hypertension, diabetes, hypercholesterolemia, current smoker), and history of previous vascular events (myocardial infarction, angina, claudication, amputation for peripheral vascular disease) were obtained.

All patients underwent a CT brain scan. Echocardiography and an extracranial duplex ultrasound were performed at the discretion of the clinician. Within 7 days of the acute stroke event, an overnight fasting blood sample was obtained to measure protein Z concentrations and for genetic analysis. Survivors were invited to return for review at 3 to 6 months after the acute event, at which time a second blood sample was taken to measure protein Z concentrations in the convalescent state. Patients using oral anti-coagulants at the time of stroke or subsequently treated with oral anticoagulants were excluded from the study.

Controls
Control subjects were randomly selected from the Western Australian Electoral roll, stratified by 5-year age groups, sex, and postal code. A letter of invitation to participate, together with a stamped and self-addressed envelope, were sent to potential controls. Nonresponders were contacted by telephone. Controls who agreed to participate in the study were required to fast for a minimum of 8 hours before their appointment and were given the option of attending the hospital outpatient clinic or being visited at home by the study nurse.

Baseline demographic data (age, sex), history of conventional vascular risk factors, and history of previous vascular events were obtained for each control. An overnight fasting blood sample was obtained for measurement of protein Z concentrations and genetic analysis. Controls were excluded if they were using oral anticoagulants.

Laboratory Analysis
Blood samples were collected and processed with the use of a standardized protocol and were analyzed in a central core laboratory. Blood samples were collected into evacuated tubes containing a 1:10 volume of 0.109 mol/L 3.8% sodium citrate (Becton Dickson) and centrifuged at 1200g for 10 minutes. The plasma was separated, centrifuged a second time at 1200g for 10 minutes, and stored at −80°C until assayed. Processing and freezing of blood samples was completed within 4 hours of blood collection.

We have previously reported our methods for assaying blood concentrations of protein Z.\textsuperscript{12} Samples were thawed for the first time to perform protein Z assays. Blood concentrations of protein Z antigen were determined with a commercially available enzyme-linked immunosorbent assay manufactured by Diagnostica Stago (France). The intra-assay variation is 8% and the interassay coefficient of variation is 5%. Samples from cases and controls were assayed at random and in duplicate.

DNA was extracted from peripheral blood lymphocytes using published methods.\textsuperscript{20} For detection of the PZ A-13G polymorphism, a 194-bp fragment was amplified and for detection of PZ Intron F G79A polymorphism, a 174-bp fragment was amplified by polymerase chain reaction using biotinylated forward primers. After processing, samples were analyzed using a PSQ HS 96 Pyrosequencer.

Statistical Analyses
Means and proportions were calculated for baseline demographics and vascular risk factors in cases and controls. The significance of any differences between cases and controls was tested with Student’s \( t \) test for means and a \( \chi^2 \) test for proportions.

Because protein Z concentrations were skewed, geometric means were calculated after log transformation of the raw data. The significance of any differences in geometric mean protein Z concentrations between cases and controls and among protein Z genotypes was examined using Student \( t \) test for 2 group comparisons or ANOVA for multiple groups. The significance of any differences in the proportion of protein Z genotype between cases and controls was examined using a \( \chi^2 \) test.

Regression models were used to examine the association between protein Z genotype and ischemic stroke and independent baseline predictors of protein Z concentrations. Data on the association between protein Z genotype and stroke risk were pooled from the study by Lichy et al\textsuperscript{17} and our study using Review Manager Software, version 4.2.7.\textsuperscript{21}

Results
One hundred fifty-one patients with ischemic stroke (100 men, 51 women; mean age, 67.3 years [SD, 11.7 years]) and 164 controls (103 men, 61 women; mean age, 66.1 years [SD, 11.8]) were studied. Sixty-one patients with ischemic stroke agreed to return for follow-up and underwent repeat laboratory testing at 3 to 6 months. All subjects included in our study were white. An additional 22 ischemic stroke cases and
22 controls were excluded because they were not white or did not have DNA available.

**Baseline Characteristics**
There was no significant difference in the age and sex distribution of ischemic stroke cases and controls. The prevalence of hypercholesterolemia was not significantly different among cases compared with controls but other conventional vascular risk factors were significantly more common among cases (Table 1).

**Baseline Predictors of Protein Z Concentrations in the Blood**
The only independent baseline predictors of protein Z concentrations in the overall study cohort were case/control status \((P=0.0001)\) and protein Z genotype \((P=0.0001)\).

**Protein Z Concentrations in Ischemic Stroke Cases and Controls**
Geometric mean concentrations of protein Z measured within 7 days of acute stroke were significantly higher in cases compared with controls \((1.51 \mu g/mL versus 1.13 \mu g/mL; P<0.0001)\), as previously reported (Table 2). Protein Z concentrations measured during the convalescent phase 3 to 6 months after the acute stroke event were substantially lower compared with the acute phase \((1.15 \mu g/mL versus 1.13 \mu g/mL; P=0.07)\) and were similar to concentrations in controls \((1.15 \mu g/mL versus 1.13 \mu g/mL; P=0.85)\) (Table 2).

**Association Between Protein Z Genotype and Blood Concentrations of Protein Z**
All genotypes examined were in Hardy-Weinberg equilibrium.

**Protein Z A-13G**
In the overall study cohort, protein Z concentrations in the blood during the first 7 days after stroke and in controls were highest among those with the A-13G AA genotype, intermediate among those with the AG genotype, and lowest among those with the GG genotype \((1.39 \mu g/mL versus 1.05 \mu g/mL versus 0.76 \mu g/mL; P<0.0001)\) (Table 3). The same pattern was observed when cases and controls were analyzed separately (Table 3).

**Protein Z G79A**
In the overall study cohort, protein Z concentrations in the blood were highest among cases or controls with the G79A GG genotype, intermediate among those with the GA genotype, and lowest among those with the AA genotype \((1.47 \mu g/mL versus 1.13 \mu g/mL versus 0.66 \mu g/mL; P<0.0001)\). The same pattern was observed when cases and controls were analyzed separately.

**Prevalence of Protein Z Genotypes in Ischemic Stroke Cases and Controls**
There were no significant differences in the prevalence of protein Z A-13G \((P=0.08)\) or protein Z G79A \((P=0.58)\) polymorphisms among cases compared with controls (Table 4).

**Pooled Analysis**
When we pooled our results with the only other previous study that has examined the association between protein Z...
polymorphisms and ischemic stroke, the association between the protein Z G79A AA genotype and ischemic stroke was highly statistically significant (OR, 0.31; 95% CI, 0.14 to 0.69; P = 0.004) (Table 4). The association between protein Z A-13G and stroke was not significant (Table 4).

**Discussion**

This is the first study that has examined the association between protein Z polymorphisms, protein Z concentrations, and cerebral vascular events among unselected cases of first-ever ischemic stroke compared with community-based controls.

Our results demonstrate that both protein Z A-13G and G79A are independent determinants of protein Z concentrations in the blood and suggest that the A allele at intron F position 79 is associated with lower concentrations of protein Z in the blood and exerts a protective effect against ischemic stroke. The consistency of the data for the association between G79A genotype, protein Z concentrations, and ischemic stroke strengthens the evidence that increased blood concentrations of protein Z are associated with increased stroke risk and are consistent with the hypothesis that elevated protein Z concentrations are prothrombotic. A protective effect of protein Z A-13G G allele was not confirmed despite being associated with significantly lower protein Z concentrations, but this is most likely because of lack of statistical power.

Our study does not clarify the mechanism of the association between protein Z genotype and stroke, and it remains possible that the protein Z polymorphisms studied are simply markers of an enhanced acute phase response in patients with an acute atherothrombotic vascular event. The acute phase reactant, C-reactive protein, is also elevated during the first 7 days after acute ischemic stroke but, unlike protein Z, remains significantly elevated at 3 to 6 months.

Our findings are in accordance with those of Lichy et al who reported a significant association between protein Z G79A genotypes, reduced protein Z concentrations, and ischemic stroke or transient ischemic attack in young German stroke patients. Lichy et al did not measure protein Z concentrations in stroke patients but reported significantly lower protein Z concentrations among 42 healthy controls with the GA and AA genotypes compared with the GG genotype for the G79A polymorphism. Our findings are also consistent with the study by Santacroce et al who found a significant association between G79A genotype and protein Z concentrations among healthy Italian subjects.

The data reported in this study cannot explain the apparently conflicting results of previous studies examining the

**TABLE 3. Association Between Protein Z Polymorphisms and Protein Z Concentrations Measured Within 7 Days of Acute Ischemic Stroke and in Controls**

<table>
<thead>
<tr>
<th>Protein Z Genotype</th>
<th>Cases (n=151)</th>
<th>Controls (n=164)</th>
<th>Combined (n=315)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-13G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (AA)</td>
<td>1.60 (1.46, 1.75)</td>
<td>1.24 (1.14, 1.34)</td>
<td>1.39 (1.31, 1.48)</td>
</tr>
<tr>
<td>Heterozygote (AG)</td>
<td>1.30 (1.11, 1.52)</td>
<td>0.79 (0.68, 0.93)</td>
<td>1.05 (0.93, 1.19)</td>
</tr>
<tr>
<td>Homozygote (GG)</td>
<td>*</td>
<td>0.76 (0.45, 1.31)</td>
<td>0.76 (0.45, 1.31)</td>
</tr>
<tr>
<td>P</td>
<td>0.02</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G79A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (GG)</td>
<td>1.72 (1.57, 1.88)</td>
<td>1.25 (1.14, 1.38)</td>
<td>1.47 (1.37, 1.58)</td>
</tr>
<tr>
<td>Heterozygote (GA)</td>
<td>1.27 (1.12, 1.45)</td>
<td>1.02 (0.91, 1.14)</td>
<td>1.13 (1.03, 1.23)</td>
</tr>
<tr>
<td>Homozygote (AA)</td>
<td>0.59 (0.42, 0.82)</td>
<td>0.71 (0.50, 1.00)</td>
<td>0.66 (0.52, 0.85)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>0.0007</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*There were no cases with A-13G GG genotype.

**TABLE 4. Association Between Protein Z Polymorphisms and Ischemic Stroke**

<table>
<thead>
<tr>
<th>Protein Z Genotype</th>
<th>Cases</th>
<th>Controls</th>
<th>OR for Ischemic Stroke (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-13G, N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (AA)</td>
<td>111 (73.5)</td>
<td>130 (79.3)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>Heterozygote (AG)</td>
<td>40 (26.5)</td>
<td>31 (18.9)</td>
<td>1.51 (0.89, 2.58)</td>
</tr>
<tr>
<td>Homozygote (GG)</td>
<td>0 (0)</td>
<td>3 (1.8)</td>
<td>0.17 (0.01, 3.27)</td>
</tr>
<tr>
<td>At least 1 G allele</td>
<td>40 (26.5)</td>
<td>34 (20.7)</td>
<td>1.38 (0.82, 2.33)</td>
</tr>
<tr>
<td>G79A, N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (GG)</td>
<td>97 (64.2)</td>
<td>97 (59.2)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>Heterozygote (GA)</td>
<td>49 (32.5)</td>
<td>59 (36.0)</td>
<td>0.83 (0.52, 1.33)</td>
</tr>
<tr>
<td>Homozygote (AA)</td>
<td>5 (3.3)</td>
<td>8 (4.9)</td>
<td>0.63 (0.20, 1.98)</td>
</tr>
<tr>
<td>At least 1 A allele</td>
<td>54 (35.8)</td>
<td>67 (40.9)</td>
<td>0.81 (0.51, 1.27)</td>
</tr>
<tr>
<td>*Pooled data: A-13G, N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type (AA)</td>
<td>295 (84.0)</td>
<td>303 (83.5)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>At least one G allele</td>
<td>56 (16.0)</td>
<td>60 (16.5)</td>
<td>0.92 (0.39, 2.18)</td>
</tr>
<tr>
<td>*Pooled data: G79A, N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type (GG)</td>
<td>237 (67.5)</td>
<td>215 (59.2)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>Heterozygote (GA)</td>
<td>106 (30.2)</td>
<td>124 (31.2)</td>
<td>0.78 (0.57, 1.07)</td>
</tr>
<tr>
<td>Homozygote (AA)</td>
<td>8 (2.3)</td>
<td>24 (6.6)</td>
<td>0.31 (0.14, 0.69)</td>
</tr>
<tr>
<td>At least 1 A allele</td>
<td>114 (32.5)</td>
<td>148 (40.8)</td>
<td>0.70 (0.52, 0.95)</td>
</tr>
</tbody>
</table>

*Adjusted for conventional cardiovascular risk factors.
†Pooled with data from Lichy et al.‡P for trend 0.002.
‡§P for trend 0.002.
association between protein Z concentrations in the blood and cardiovascular disease, including stroke. The same antigenic assay for protein Z was used in all the studies. However, previous studies were performed primarily in younger stroke patients and in different ethnic populations. Because genetic factors may play a disproportionately greater role in the cause and pathogenesis of stroke in selected younger patients and in certain ethnic populations and are also important determinants of protein Z concentrations in the blood, genetic differences may account for some of the disagreement between the studies.

A strength of our study was that we included a broad range of patients with stroke who were compared with randomly selected community based controls, matched for age, sex, and postal code. In addition, we measured both protein Z genotype and functional concentrations of protein Z in the blood. Because these 2 measures have different sources of potential error, their consistency supports an association between increased blood concentrations of protein Z and ischemic stroke.

Our study also has limitations. First, although the association between genotype and ischemic stroke is less prone to reverse causality or confounding than the association between protein Z concentrations and stroke, confounding cannot be eliminated by this approach. The mechanism of the association between protein Z genotype and protein Z concentrations or ischemic stroke has not been established and there may be linkage disequilibrium with polymorphic variants of loci that predispose to conventional or novel risk factors for stroke. Second, our study was underpowered to reliably detect an association between individual protein Z genotypes and ischemic stroke or etiologic subtypes of ischemic stroke. Consequently, we used data pooling to confirm an independent association between protein Z genotypes associated with lower protein Z concentrations in the blood and ischemic stroke, an approach that is subject to selection and publication bias and the limitations of the included studies. Third, we decided to study the 2 polymorphisms described in our article because they are both common and have been reported to be important determinants of protein Z concentrations, thus allowing us to further clarify the association between protein Z and stroke. However, we did not determine linkage disequilibrium between the 2 polymorphisms and did not study the numerous other protein Z polymorphisms that have also been described.

In conclusion, our results are consistent with the hypothesis that elevated protein Z concentrations are prothrombotic. Further advances in understanding of the underlying mechanisms of the association between protein Z and thrombosis may provide new insights into the cause, pathogenesis, or prognosis of acute ischemic stroke and could potentially yield new therapeutic strategies in the future.

Acknowledgments

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References

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