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Circulating Protein Z Concentration, *PROZ* Variants, and Unexplained Cerebral Infarction in Young and Middle-Aged Adults

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Summary

Protein Z (PZ) is a vitamin K-dependent plasma protein that exhibits both pro- and anticoagulant properties. Both low and high PZ levels have been linked to ischemic stroke. Although PZ-lowering gene variants have been found to be less common in ischemic stroke, the relationship remains unclear. We investigated PZ levels and *PROZ* variants in a multi-ethnic case-control study of unexplained stroke in participants aged 18 to 64. Plasma PZ was measured in cases (≥2 months post-stroke) and controls. PZ polymorphisms G79A (rs3024735) and A13G (2273971) were genotyped. A combined genetic score (0–4 minor alleles) was created assuming additive effects. A total of 715 subjects (1:1.4 cases:controls) were included. Analyses revealed evidence of a non-linear association. After adjusting for demographic and clinical covariates, PZ level >2.5 μg/mL (90^{th} %ile) were significantly associated with cryptogenic stroke (OR 2.41 [95% CI 1.34, 4.34]) as compared with lower levels. Higher genetic score was related to progressively lower levels of PZ, and the presence of 4 minor alleles was associated with lower odds of stroke (adjusted OR 0.26 [95% CI 0.07, 0.96]) versus 0 minor alleles. In this multi-ethnic study of young and middle-aged adults, there was evidence of a non-linear positive association between PZ level and unexplained stroke, with a directionally consistent association for genetic variants related to PZ levels and

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cryptogenic stroke. These findings support elevated PZ levels as a risk factor for cryptogenic stroke.

Keywords

biomarkers; genetic association studies; plasma protein Z; risk factors; stroke

Introduction

Protein Z (PZ), a 62-kD vitamin K-dependent plasma glycoprotein synthesized by the liver, was first identified in bovine plasma in 1977 (1) and isolated from human plasma in 1984 (2). Unlike other vitamin-K dependent proteins, PZ is not a zymogen, and its physiologic function is not well defined. While bovine PZ can promote the assembly of thrombin on phospholipid surfaces, suggesting that the protein has a pro-thrombotic role (3), human PZ lacks this function (3). PZ also acts as a cofactor for the PZ-dependent protease inhibitor (ZPI) to enhance the inhibition of coagulation factor Xa, intimating that its role in humans may be as an anti-thrombotic factor (4, 5).

A potential contribution of PZ to vascular occlusive diseases in humans has been investigated, but clinical studies have yielded contradictory results. An initial report documented low PZ level to be more common in young patients with ischemic stroke, but not venous thromboembolism, as compared with controls (6). These findings were consistent with the prothrombotic phenotype potentiated in mice homozygous for factor V Leiden by knockout of the PZ gene (7), supporting PZ deficiency as a risk factor for cerebral infarction. This clinical study, however, was quickly followed by another that linked higher, not lower, PZ level to increased odds of ischemic stroke of unknown etiology (8). Subsequent studies of PZ have found inverse (9, 10), null (11), or positive associations (12) for PZ level and stroke. Similarly inconsistent findings have been reported for coronary heart disease (13–16), and for venous thrombosis (17–19). Hence, although a meta-analysis reported a significant association between PZ deficiency and increased risk of arterial and venous thrombosis, as well as pregnancy complications, there was marked heterogeneity across studies (14).

Additional studies have examined variation in the human PZ gene, *PROZ*, to determine its role in vascular thrombosis in humans. The G allele of a A13G polymorphism in the *PROZ* promoter, together with the A allele of an intronic G79A polymorphism, have been shown to be associated with decreased PZ levels (20–22). Pooled results from two case-control studies documented a significant association of G79A A allele with ischemic stroke (21, 22), supporting PZ elevation as a risk factor, but this was not reproduced in a subsequent case-control study (23).

Given the unresolved nature of the association, we investigated the relationship of plasma concentration of PZ and two key genetic determinants of such concentration with unexplained cerebral infarction in young and middle-aged adults.

Materials and Methods

Study Participants

The THrombophilia In Cryptogenic stroKe (THICK) Study is a prospectively designed case-control study to investigate prothrombotic determinants of unexplained cerebral infarction (24). Cases consisted of patients 18–64 years old presenting to Weill Cornell Medical Center (WCMC) for evaluation of first-ever ischemic stroke for which a definite or probable etiology could not be established. Stroke was defined as a focal neurological deficit with rapid onset, features consistent with a vascular origin, and duration of at least 24 hours or with imaging evidence of brain infarction compatible with the clinical presentation. A classification of "cryptogenic" stroke was based on modification of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria (25), whereby competing etiologies or insufficient evaluation were grounds for exclusion. Controls included employees and visitors to WCMC, along with residents of surrounding areas, aged 18–64 years without a prior history of stroke recruited through print and electronic advertisements and by word of mouth. Enrollment of cases and controls occurred between October 2002 and September 2012.

Exclusion criteria reflected human subjects' protections and were intended to remove known or potential risk factors for stroke, as well as to insure comparability of cases and controls. These comprised an inability to give informed consent, expected survival <6 months, venous thromboembolism (VTE) in the previous 6 months (except newly detected VTE at the time of stroke presentation), recent acute coronary syndrome (<3 months), chronic liver disease (transaminases >3x upper limit of normal), chronic kidney disease (serum creatinine ≥2.0 mg/dl), pregnancy or <3 months post-partum, systemic malignancy not in remission ≥5 years, chronic inflammatory disease (e.g., collagen vascular disease), human immunodeficiency virus (HIV) infection, and major trauma or surgery in the past 6 months.

Female stroke cases were uniformly advised to discontinue contraceptive steroids after the index event as part of standard clinical care. They were therefore off such therapy at the time of convalescent blood testing, whereas female controls receiving such medications did not discontinue them. To minimize potential bias, female controls on contraceptive steroids (n=36) were excluded from the analysis of PZ levels.

Study Procedures

Cases underwent neuroimaging and evaluation of their head and neck vasculature at the discretion of their primary neurologist. Vascular imaging with duplex ultrasound, magnetic resonance angiography, computed tomographic angiography or conventional angiography to exclude vascular pathology responsible or potentially responsible for the index stroke was a requirement for inclusion. All cases also underwent transesophageal echocardiography to exclude a definite or probable cardiac source of embolism. Duplex ultrasound of the lower extremity veins was encouraged in the setting of interatrial shunt detection. Holter or ambulatory ECG monitoring were at the discretion of the primary physician, as was clinical laboratory evaluation.

All participants underwent a standardized medical interview for collection of demographic variables and medical history, supplemented by chart review in cases. Both cases and

controls received transthoracic echocardiography with agitated-saline contrast involving a standardized protocol. Cases and controls also had standard laboratory evaluation consisting of comprehensive metabolic panel, complete blood count, prothrombin and activated partial thromboplastin time, lipid profile, C-reactive protein (CRP) and a standard panel of tests for prothrombotic factors. Such testing occurred a minimum of 2 months after the index stroke in cases, or on the day of their visit for controls. Participants were asked to fast for ≥8 hours prior to phlebotomy. Warfarin was discontinued ≥14 days prior to blood collection; subcutaneous enoxaparin was used as a bridge when necessary, and discontinued 24 hours prior to the phlebotomy visit.

The THICK study was approved by the institutional review board at WCMC. All participants provided informed consent.

Protein Z levels Measurement

PZ antigen levels were measured in citrated plasma specimens frozen at −70°C following collection. Measurements were performed with a commercially available enzyme-linked immunosorbent assay (ELISA, Diagnostica Stago Inc, Parsippany-Troy Hills, New Jersey), usually within 6 to 12 months of specimen collection. This ELISA has a reference range of 0.26–3.76 μg/mL. The intra-assay and inter-assay coefficients of variability are 8% and 5%, respectively.

Protein Z genotype

Two single nucleotide polymorphisms (SNPs) of the *PROZ* gene, G79A (rs3024735) and A13G (rs2273971), were genotyped. According to 1000 Genomes pilot 1 data, G79A and A13G can be considered independent in European (r^2=0.069) and African American populations (r^2=0.067). Analysis of single nucleotide polymorphisms (SNPs) of *PROZ* (rs3024735 and rs2273971) and genotyping of factor V Leiden (G1691A) and the prothrombin mutation (G20210A) were performed in the Molecular Core Laboratory of the Clinical and Translational Science Center of WCMC. Briefly, the genomic DNA was isolated from blood samples, and the concentration and quality of the DNA were determined using Nanodrop2000C (Thermo Scientific, Wilmington, Delaware). The target fragments were amplified by polymerase chain reaction (PCR) using the following primers: *PROZ* (forward primer: GGGTCCTCTG AGCCTTCACCGTTCATTT; reverse primer: CAGGCACAACAGACAGGTAAGCCA GATG), Factor V Leiden (forward primer: GCACAATGTTCCACCA GGTGAGAAG; reverse primer: GCCGCCGTTACCCACTCACTAATAC), and prothrombin G20210A (forward primer: CCCTTTAACAACCGCTGGTATCAAATGG; reverse primer: GCACCAGGTGGTGGATTCTTAAG TCTTCTT). The PCR conditions for *PROZ* were 94 °C for 2 min, then 40 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 1min, and a final cycle of 68 °C for 5min. For factor V Leiden and prothrombin G20210A, the PCR conditions were 94 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1min with a final extension of 68 °C for 7 min. The amplified fragments were purified by agarose gel electrophoresis using a Qiagen gel extraction kit and sequenced at the Life Sciences Core Laboratories Center of Cornell University using both forward and

reverse primers. *P* values for Hardy-Weinberg Equilibrium were ≥ 0.01 in race-specific controls.

Definitions

Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg (at the time of the convalescent visit for cases), or use of anti-hypertensive medications or self-reported hypertension. Diabetes mellitus was defined as non-fasting blood glucose ≥200 mg/dL, fasting blood glucose ≥126 mg/dL or hemoglobin A1c ≥6.5%, or by current use of antidiabetic medication or self-reported diabetes. Hyperlipidemia was defined as LDL cholesterol ≥160 mg/dL, or HDL cholesterol <40 mg/dL in men or <50 mg/dL in women, or total/HDL cholesterol ≥4.0, or history of previous use of lipid-lowering medication. Family history of cardiovascular disease (CVD) was defined as family history of premature coronary artery disease, stroke or VTE (first-degree male relative under age 55, and first-degree female relative under age 65). Migraine with aura was defined by criteria from the International Headache Society (26). Antiphospholipid syndrome was defined according to published criteria (27).

Statistical Analysis

Baseline characteristics of the study cohort were summarized by case/control status using standard descriptive statistics; differences were assessed with Student's t-test or chi-squared test, as appropriate. Proportions of cryptogenic stroke cases and controls with PZ levels exceeding specific percentile cutpoints of the biomarker's distribution among controls were graphically illustrated. Univariable linear regression was performed to assess the association of all demographic and clinical covariates with PZ level in controls. The Locally Weighted Scatterplot Smoothing (LOWESS) method, as a type of nonparametric regression, was performed to graphically represent the relation of PZ level with case vs. control status (28, 29). Logistic regression was used to evaluate the association between PZ level and cryptogenic stroke after adjustment for potential confounders.

In the genetic analysis, an additive genetic model was assumed for *PROZ* polymorphisms G79A (rs3024735) and A13G (rs2273971). A *PROZ* genetic score (0–4) combining G79A and A13G was created based on an additive assumption. Linear and logistic regressions were performed to examine the adjusted associations of individual polymorphisms and genetic scores with PZ levels and cryptogenic stroke, respectively.

To account for potential confounding, linear and logistic regression models were adjusted for age, sex, race-ethnicity, smoking, hypertension, diabetes, family history of premature CVD, and migraine with aura. Additional adjustment for LDL, HDL, triglycerides, lipid-lowering therapy was also undertaken. In additional analyses, we examined the impact of further adjusting for interatrial shunt on the association of PZ level with cryptogenic stroke, and for interatrial shunt and contraceptive steroids on the association of *PROZ* variants. Owing to the small proportion of participants with missing covariates (<0.8% for any single covariate), these participants were excluded from multivariable adjusted models. We conducted formal tests of interaction by age, sex, and race-ethnicity by examining cross-

product terms. We also tested the interaction of PZ level or the genetic risk score by prothrombotic factors, including factor Leiden V, homocysteine, prothrombin G20210A, antiphospholipid syndrome, and interatrial shunt.

As a sensitivity analysis, the mean PZ levels of participants with normal CRP (<3 mg/L) and high CRP (≥3 mg/L) were compared using Student's t-test and linear regression was performed to examine the association of CRP with PZ level. Furthermore, we excluded post-menopausal women (10) and cases who reported taking contraceptive steroids to examine the difference in results from the main analysis.

To account for population stratification, the genetic analysis was stratified by self-reported race-ethnicity for each individual variant. We tested for evidence of effect modification for each individual polymorphism by race-ethnicity. We next performed meta-analysis using fixed effect and random effect models across major race-ethnic groups (whites, blacks, Hispanics, and Asians) to test for heterogeneity using Q and I^2 tests. In addition, testing for effect modification by age, sex, race-ethnicity, prothrombotic factors and use of contraceptive steroids was also undertaken in the genetic analyses.

We performed power calculations assuming an additive genetic effect, which revealed limited power for race- or ethnicity-specific analyses. Specifically, based on reported minor allele frequencies for G79A and A13G and available sample sizes for each race-ethnic group, the minimally detectable effect sizes per minor allele at a two-sided α=0.05 and power=0.80 were β's of −0.38 to −0.13 for PZ level, and odds ratios of 0.37 to 0.75 for cryptogenic stroke (Quanto V.1.2.4, Los Angeles, CA). Analyses were performed with Stata, version 12 (StataCorp LP, College Station, Texas). All P values were 2-tailed, with statistical significance defined by $P < 0.05$.

Results

Baseline Characteristics

In total, 733 subjects were enrolled in the THICK study. Eighteen subjects were excluded because of missing data on both PZ level and *PROZ* genotyping, which resulted in a total of 715 participants (292 cases and 423 controls) eligible for the current analyses. As shown in Table 1, cases and controls were similar in demographic characteristics and several cardiovascular risk factors, but cases more often had hypertension and migraine with aura. Cases used contraceptive steroids more frequently as compared with controls. There were also no differences in mean PZ and CRP levels between cases and controls. Prothrombotic factors were similar in cases and controls except that cases had higher homocysteine and a higher prevalence of interatrial shunt.

All cases underwent neuroimaging evaluation with computed tomography of the head or magnetic resonance imaging of the brain, with 73.3% having the latter. Neurovascular evaluation was performed in all patients, most commonly consisting of magnetic resonance angiography (60.3%). The index ischemic stroke was hemispheric in 57.5% of cases, involved the brainstem in 11.6% of cases, and both in 2.4% of cases; it affected the anterior circulation in 47.6%, the posterior circulation in 21.9%, and both in 2.1%. Outpatient

monitoring for arrhythmia was performed in 50% of cryptogenic stroke cases, consisting of 24–48-hour Holter monitoring in 44.2% and ≥14-day ambulatory ECG monitoring in 5.8%.

Correlates of PZ Level in Controls

In univariable analysis among controls, Asian ethnicity was associated with lower PZ levels (β=0.41, 95% CI −0.70, −0.13, P=0.005) as compared with non-Hispanic White ethnicity. Family history of premature CVD (β=0.18, P=0.030), HDL (β=0.006, P=0.002), and antiphospholipid syndrome (β=0.96, P=0.028) were associated with higher PZ level. No other demographic and clinical covariates in Table 1 were associated with PZ level in controls.

Protein Z Level and Cryptogenic Stroke

After exclusion of 89 participants who lacked PZ measurements and 36 controls on contraceptive steroids, plasma PZ level was available in 239 cases and 351 controls. Participants without available PZ measurements did not differ from those with measurements in demographic or clinical characteristics, except that participants missing PZ measurement had more migraine with aura (20.2% vs.12.3%, P=0.041). PZ level was normally distributed in controls (Shapiro-Wilk test *P*=0.16). As shown in Figure 1, there were no significant differences in the proportions of cases and controls below or above specific percentile cutpoints for PZ level, with the exception of the 90th percentile (2.5 μg/mL), above which the fraction of cases significantly exceeded that of controls. In turn, the LOWESS smoother curve (Figure 2) shows that the relationship between PZ level and cryptogenic stroke was nonlinear, with an inflection point of increasing stroke risk above the 90th percentile (2.5 μg/mL) of PZ's distribution among controls. Based on this finding, participants were categorized into two groups: high PZ (PZ>2.5 μg/mL, n=54) and normal/low PZ (PZ≤2.5 μg/mL, n=536). After adjustment for age, sex, race-ethnicity, hypertension, diabetes, smoking, family history of premature CVD, and migraine with aura, participants in the high PZ group had >2-fold higher odds of cryptogenic stroke as compared with the normal/low PZ group (OR=2.41, 95% CI 1.34, 4.34, P=0.003). Additional adjustment for LDL, HDL, triglycerides, and lipid-lowering therapy, or for interatrial shunt, did not meaningfully alter the results. There was no statistical evidence of effect modification by demographic variables or prothrombotic factors.

Sensitivity Analyses

No significant difference in PZ levels was observed between participants with normal and high CRP levels (2.4 vs. 1.8 mg/L, P=0.464), and CRP was not associated with PZ levels (β=0.005, P=0.06). Consistent with this lack of association, exclusion of participants with CRP ≥3.0 mg/L did not have a meaningful impact on the results. Furthermore, exclusion of post-menopausal women or of cases who reported taking contraceptive steroids did not materially influence the findings.

PROZ genotypes and Cryptogenic Stroke

PROZ G79A genotypes were completed in 290 cases and 423 controls, while A13G genotypes were available in 287 cases and 421 controls. There was no evidence of

interaction by race-ethnicity (all P>0.05), and random-effects meta-analysis of associations of G79A or A13G variants with PZ level and cryptogenic stroke across individual race-ethnic groups (Table 2) showed no evidence of important heterogeneity (all P for heterogeneity>0.05, I^2<50%). We therefore combined data from all race-ethnic groups to examine associations in the entire cohort. These combined analyses demonstrate that heterozygous and homozygous presence of the A allele of the G79A polymorphisms was associated with 0.36 and 0.79 μg/mL lower adjusted PZ level as compared with the GG genotype, respectively, with correspondingly lower adjusted PZ level of 0.34 and 0.84 μg/mL for single and double copies of the G allele of the A13G polymorphism. These analyses also show that minor-allele genotypes for each polymorphism, particularly homozygous genotypes, tended to be associated with lower odds of cerebral infarction, but these associations were not statistically significant (Table 3).

We next combined the two polymorphisms into a PZ genetic score. As detailed in Table 4, an increase in the total number of minor alleles was associated with a progressive decline in PZ level (P trend<0.001). After adjustment for demographic variables and CVD risk factors, participants with the highest genetic score (4 minor alleles) had significantly lower odds of cryptogenic stroke as compared with the lowest genetic score (0 minor alleles). In additional analyses, risk estimates were not influenced by further adjustment for contraceptive steroids and interatrial shunt. There was also no evidence of effect modification by other demographic factors, contraceptive use, prothrombotic factors or interatrial shunt in the genetic analyses.

Discussion

Main Findings

In this prospectively designed multi-ethnic case-control study of young and middle-aged adults, we found a non-linear relationship between PZ levels and cryptogenic ischemic stroke. Specifically, participants with PZ levels above the 90th percentile of the distribution in controls had over two-fold greater odds of cerebral infarction of indeterminate origin as compared with those having lower PZ levels. Moreover, across four major US race-ethnic groups, our study revealed significant associations of minor alleles at two major *PROZ* variants (polymorphisms G79A and A13G, and their combination) with lower plasma PZ levels, and significantly lower adjusted odds of cryptogenic stroke for participants with all 4 minor alleles at these two *PROZ* SNPs as compared with no minor alleles.

Previous Studies

The physiologic role of PZ in humans is not well delineated. Unlike its bovine form, human PZ lacks the structural component shown to support thrombin assembly on phospholipid surfaces, but does retain the capacity to bind ZPI, bolstering the latter's inhibition of factor Xa. Such experimental findings argue for an anti-thrombotic role for human PZ, one that is supported by several clinical studies that have documented an association between PZ deficiency and ischemic stroke, as well as with coronary heart disease, peripheral arterial disease, and venous thrombosis. Yet clinical studies examining plasma PZ levels in relation to vascular thrombosis have been inconsistent. This has been particularly the case for

ischemic stroke, where not just null (11), but contradictory (8, 12), findings have been reported, namely, associations between PZ excess and higher risk of cerebral infarction.

Discrepancies in these results, derived exclusively from case-control studies, have several possible explanations. The inconsistent findings may be attributable to differences in patient populations (young vs. old; prevalence of conventional risk factors), selection of controls (community-based vs. hospital-based), timing of PZ measurement after stroke (acute vs. convalescent phase) or methodological biases. It bears noting in this context that PZ levels varied greatly between studies. PZ was initially reported to have a mean value of 2.9 μg/mL and a SD of 1.0 μg/mL in 455 normal healthy blood donors (30). In subsequent studies, the reported mean value of PZ levels in controls ranged from 1.1 μg/mL (22) to 2.3 μg/mL (6). This may relate to differences in testing methods and laboratory protocols, in addition to differences in study populations and their clinical characteristics. Last, but of no less importance, comparisons of PZ levels between cases and controls in some studies did not adjust, or lacked the ability to properly adjust, for various stroke risk factors that were not well balanced between the groups, making the findings prone to confounding.

More consistent findings, however, have come from studies of genetic variants in *PROZ* with demonstrated associations with circulating PZ level. A German study documented a significantly lower prevalence of the A allele of the G79A intronic polymorphism in young patients with cerebral ischemia as compared with control subjects, which was associated with lower PZ levels measured only in a subset of control participants (21). An Australian case-control study documented lower plasma PZ levels, measured in cases acutely after ischemic stroke, for the minor alleles of the G79A and A13G polymorphisms, but could only demonstrate a significant association of the G79A polymorphism with cerebral ischemia after pooling its data with the German study (22). Such an association for G79A was not replicated by a subsequent Spanish case-control study, which was not able to measure concurrent PZ levels (23). More recently, however, a family-based study of German children with nonvascular stroke or venous thrombosis determined that a haplotype block composed of 3 tagging SNPs in *PROZ* associated with higher circulating PZ level was related to both cerebral infarction and venous thromboembolism (31). Taken together, such genetic findings, which are less prone to confounding by traditional CVD risk factors than phenotypic analyses of PZ levels, provide evidence favoring a relationship between higher PZ concentration and cerebral ischemia.

Support for Elevated PZ Levels and Potential Explanation

The present results, coming from the largest case-control study of cerebral infarction in adults to measure both circulating PZ level and *PROZ* genotypes, and to do so in a sample of mixed race-ethnicity, lend strong support to the premise that elevated PZ levels are associated with ischemic stroke. Our findings are bolstered by measurement of plasma PZ in the convalescent phase of stroke, and by a sample size conducive to evaluating the shape of the relationship between PZ level and cerebral infarction, which permitted detected of a non-linear association. But the concept that higher, not lower, plasma PZ is a risk factor for stroke is reinforced particularly by the directionally consistent finding that *PROZ* variants associated with lower PZ level were related to reduced odds of cryptogenic stroke.

The basis for the observed adverse association between elevated PZ levels and cryptogenic ischemic stroke, and the consistent protective association between *PROZ*-lowering variants and this disorder, is unclear. It has been suggested that higher PZ levels could reflect acquired or genetic reductions in circulating levels of ZPI, which would lead to diminished factor Xa inhibition and increased susceptibility to thrombosis (31). It is not clear in this regard whether the *PROZ* polymorphisms studied here could affect, or be linked to, other polymorphisms that might influence binding of the PZ molecule to ZPI, reducing the latter's anti-thrombotic efficacy. Because we did not measure circulating ZPI levels or evaluate variants in the ZPI gene, we are unable to address these questions, but these possibilities will require investigation in future studies. Beyond PZ's interactions with ZPI, it has lately been demonstrated that apart from its associations with thrombosis, PZ also acts on vascular endothelium to promote angiogenesis (32). It remains uncertain, however, whether and how such vascular effects relate to the higher risk of ischemic stroke shown here for elevated PZ concentration.

Results of the current study should be interpreted with some limitations in mind. First, although cases were classified prospectively and recruited consecutively, and despite inclusion of various covariates in multivariable models, there may be residual confounding because of the study's observational nature. Second, only one-half of cases underwent outpatient arrhythmia monitoring, and a minority received prolonged ambulatory ECG monitoring, reflecting available technology and practice patterns during the enrollment period. Still, it is unlikely that unrecognized paroxysmal atrial fibrillation was an etiologic factor in a meaningful proportion of cases, given their young age and low frequency of traditional cardiovascular risk factors. In particular, none had evidence of low left atrial appendage velocities or spontaneous echo contrast on transesophageal echocardiography. Third, the control group was recruited from employees and visitors to WCMC, and residents of surrounding areas, and is not community-based. This would tend to bias the association toward the null, however, as the control group tended to be enriched with subjects with hyperlipidemia and a family history of premature CVD. Fourth, although the present study included different ethnic groups in proportions that are representative of the broader race-ethnic composition of the US and may be more generalizable to the larger population compare to prior studies, the analyses lacked sufficient power to assess associations in individual race-ethnic groups, and these will require further investigation in larger cohorts.

Conclusions

The present findings provide evidence of a non-linear relationship between circulating PZ levels and cryptogenic ischemic stroke in young and middle-aged adults of multiple race-ethnicities, wherein higher plasma PZ was associated with heightened risk. Analysis of genetic variants related to lower PZ level indicated an inverse association with cryptogenic stroke, a finding that is consistent in direction with the observed phenotypic association, reinforcing its validity. These findings provide impetus for further clinical and mechanistic studies to further define this association and its underlying basis, information that could potentially identify novel approaches for prevention of premature thrombotic events in individuals at risk.

References

1. Prowse CV, Esnouf MP. The isolation of a new warfarin-sensitive protein from bovine plasma. Biochem Soc Trans. 1977; 5(1):255–6. [PubMed: 892175]
2. Broze GJ Jr, Miletich JP. Human Protein Z. J Clin Invest. 1984; 73(4):933–8. [PubMed: 6707212]
3. Hogg PJ, Stenflo J. Interaction of vitamin K-dependent protein Z with thrombin. Consequences for the amidolytic activity of thrombin and the interaction of thrombin with phospholipid vesicles. J Biol Chem. 1991; 266(17):10953–8. [PubMed: 2040612]
4. Han X, Fiehler R, Broze GJ Jr. Isolation of a protein Z-dependent plasma protease inhibitor. Proc Natl Acad Sci U S A. 1998; 95(16):9250–5. [PubMed: 9689066]
5. Han X, Fiehler R, Broze GJ Jr. Characterization of the protein Z-dependent protease inhibitor. Blood. 2000; 96(9):3049–55. [PubMed: 11049983]
6. Vasse M, Guegan-Massardier E, Borg JY, Woimant F, Soria C. Frequency of protein Z deficiency in patients with ischaemic stroke. Lancet. 2001; 357(9260):933–4. [PubMed: 11289354]
7. Yin ZF, Huang ZF, Cui J, Fiehler R, Lasky N, Ginsburg D, et al. Prothrombotic phenotype of protein Z deficiency. Proc Natl Acad Sci U S A. 2000; 97(12):6734–8. [PubMed: 10829076]
8. Kobelt K, Biasiutti FD, Mattle HP, Lammle B, Wuillemin WA. Protein Z in ischaemic stroke. Br J Haematol. 2001; 114(1):169–73. [PubMed: 11472363]
9. Heeb MJ, Paganini-Hill A, Griffin JH, Fisher M. Low protein Z levels and risk of ischemic stroke: differences by diabetic status and gender. Blood Cells Mol Dis. 2002; 29(2):139–44. [PubMed: 12490280]
10. Heeb MJ, Fisher M, Paganini-Hill A. Association of low protein Z levels with ischemic stroke in young women. Thromb Haemost. 2007; 97(3):495–6. [PubMed: 17334520]
11. Lopaciuk S, Bykowska K, Kwiecinski H, Czlonkowska A, Kuczynska-Zardzewialy A. Protein Z in young survivors of ischemic stroke. Thromb Haemost. 2002; 88(3):536. [PubMed: 12353088]
12. McQuillan AM, Eikelboom JW, Hankey GJ, Baker R, Thom J, Staton J, et al. Protein Z in ischemic stroke and its etiologic subtypes. Stroke. 2003; 34(10):2415–9. [PubMed: 12970515]
13. Fedi S, Sofi F, Brogi D, Tellini I, Cesari F, Sestini I, et al. Low protein Z plasma levels are independently associated with acute coronary syndromes. Thromb Haemost. 2003; 90(6):1173–8. [PubMed: 14652653]
14. Sofi F, Cesari F, Abbate R, Gensini GF, Broze G Jr, Fedi S. A meta-analysis of potential risks of low levels of protein Z for diseases related to vascular thrombosis. Thromb Haemost. 2010; 103(4):749–56. [PubMed: 20076855]
15. Morange PE, Juhan-Vague I. Protein Z plasma levels are not associated with the risk of coronary heart disease: the PRIME Study. J Thromb Haemost. 2004; 2(11):2050–1. [PubMed: 15550045]
16. Refaai MA, Ahn C, Lu L, Wu K, Broze GJ Jr. Protein Z and ZPI levels and cardiovascular events. J Thromb Haemost. 2006; 4(7):1628–9. [PubMed: 16839367]
17. Al-Shanqeeti A, van Hylckama Vlieg A, Berntorp E, Rosendaal FR, Broze GJ Jr. Protein Z and protein Z-dependent protease inhibitor. Determinants of levels and risk of venous thrombosis. Thromb Haemost. 2005; 93(3):411–3. [PubMed: 15735788]
18. Martinelli I, Razzari C, Biguzzi E, Bucciarelli P, Mannucci PM. Low levels of protein Z and the risk of venous thromboembolism. J Thromb Haemost. 2005; 3(12):2817–9. [PubMed: 16359525]
19. Santacroce R, Sarno M, Cappucci F, Sessa F, Colaizzo D, Brancaccio V, et al. Low protein Z levels and risk of occurrence of deep vein thrombosis. J Thromb Haemost. 2006; 4(11):2417–22. [PubMed: 16938126]
20. Santacroce R, Cappucci F, Di Perna P, Sessa F, Margaglione M. Protein Z gene polymorphisms are associated with protein Z plasma levels. J Thromb Haemost. 2004; 2(7):1197–9. [PubMed: 15219213]
21. Lichy C, Kropp S, Dong-Si T, Genius J, Dolan T, Hampe T, et al. A common polymorphism of the protein Z gene is associated with protein Z plasma levels and with risk of cerebral ischemia in the young. Stroke. 2004; 35(1):40–5. [PubMed: 14671240]
22. Staton J, Sayer M, Hankey GJ, Cole V, Thom J, Eikelboom JW. Protein Z gene polymorphisms, protein Z concentrations, and ischemic stroke. Stroke. 2005; 36(6):1123–7. [PubMed: 15879328]

23. Obach V, Munoz X, Sala N, Garcia de Frutos P, Chamorro A. Intronic c.573 + 79G>A polymorphism of protein Z gene in haemorrhagic and ischaemic stroke. Thromb Haemost. 2006; 95(6):1040–1. [PubMed: 16732388]

24. Beheshtian A, Shitole SG, Segal AZ, Leifer D, Tracy RP, Rader DJ, et al. Lipoprotein(a) level, apolipoprotein(a) size, and risk of unexplained ischemic stroke in young and middle-aged adults. Atherosclerosis. 2016 in press.

25. Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke. 1993; 24(1):35–41. [PubMed: 7678184]

26. Headache Classification Committee of the International Headache Society. Classification and diagnostic criteria for headache disorders, cranial neuralgias and facial pain. Cephalalgia: an international journal of headache. 1988; 8(Suppl 7):1–96.

27. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006; 4(2):295–306. [PubMed: 16420554]

28. WS, Cleveland RM. Graphical Perception: Theory, Experimentation, and Application to the Development of Graphical Methods. Journal of the American Statistical Association. 1984; 79(387):531–54.

29. Austin PC, Steyerberg EW. Graphical assessment of internal and external calibration of logistic regression models by using loess smoothers. Stat Med. 2014; 33(3):517–35. [PubMed: 24002997]

30. Miletich JP, Broze GJ Jr. Human plasma protein Z antigen: range in normal subjects and effect of warfarin therapy. Blood. 1987; 69(6):1580–6. [PubMed: 3580568]

31. Nowak-Gottl U, Frohlich B, Thedieck S, Huge A, Stoll M. Association of the protein Z ATG haplotype with symptomatic nonvascular stroke or thromboembolism in white children: a family-based cohort study. Blood. 2009; 113(10):2336–41. [PubMed: 19050305]

32. Butschkau A, Wagner NM, Genz B, Vollmar B. Protein z exerts pro-angiogenic effects and upregulates CXCR4. PLoS One. 2014; 9(12):e113554. [PubMed: 25474349]

What is known about this topic?

- Protein Z (PZ) is a vitamin K-dependent plasma protein
- The relationship between PZ level and ischemic stroke remains unclear

What does this paper add?

- PZ level>2.5 μg/mL was associated with higher, and a PZ-lowering *PROZ* score with lower, odds of cryptogenic stroke
- These consistent findings for PZ level and *PROZ* variants support elevated PZ as a risk factor for ischemic stroke

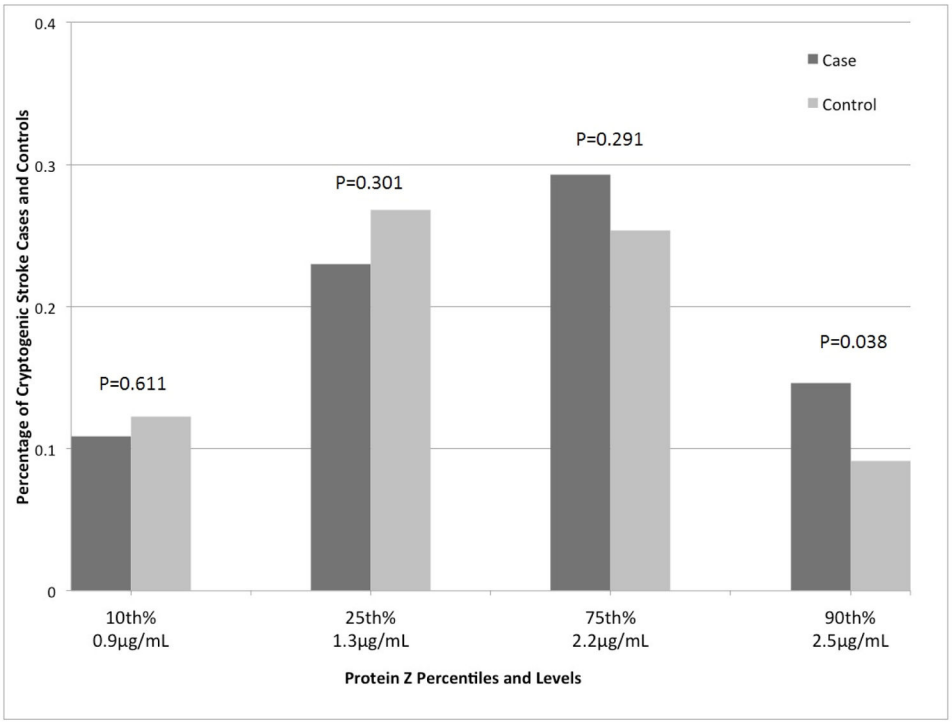

Figure 1.

Percentage of cryptogenic stroke cases or stroke-free controls with protein Z levels below or above specific percentile cutpoints based on the distribution of protein Z levels in controls.

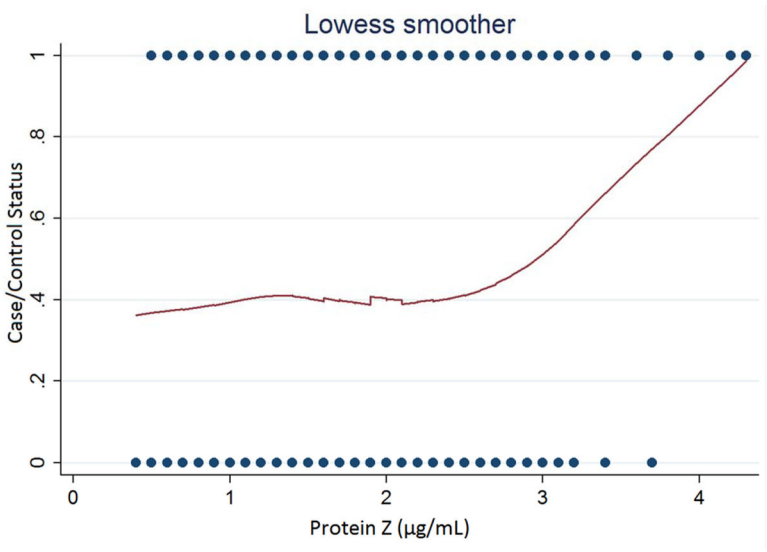

Figure 2.

Relationship between protein Z levels and cryptogenic stroke using the locally weighted scatterplot smoothing (LOWESS) method. For Case/Control Status on the Y-axis, 1=Case and 0=Control.

Table 1

Baseline Characteristics* of Participants

	Cases (n=292)	Controls (n=423)	*P* Value
Age, years	43.4±11.2	42.9±11.4	0.556
Women, %	49.0	51.3	0.541
Race-ethnicity			0.949
Non Hispanic white, %	63.0	63.6	
Non-Hispanic black, %	12.7	11.1	
Hispanic, %	16.1	17.5	
Asian, %	5.8	5.2	
Other, %	2.4	2.6	
Body mass index, kg/m^2	26.6±5.8	26.2±5.1	0.355
Hypertension, %	23.0	12.3	<0.001
Diabetes mellitus, %	7.2	4.0	0.063
Hyperlipidemia, %	31.2	33.6	0.500
Current smoker, %	14.1	8.5	0.055
Family history of premature cardiovascular disease, %	15.4	14.7	0.790
Contraceptive use†, %	14.4	8.5	0.014
Menopause†, %	6.8	2.8	0.141
Migraine with aura, %	20.6	8.5	<0.001
C-reactive protein, mg/L	2.4±14.7	1.8±4.0	0.464
Protein Z, μg/mL	1.8±0.7	1.7±0.6	0.215
Prothrombotic factors			
G20210A, %	1.3	3.4	0.257
Factor V Leiden, %	5.0	5.1	0.929
Homocysteine, μmol/L	10.0±2.8	9.5±2.8	0.049
Antiphospholipid syndrome, %	0	0.5	0.239
Interatrial shunt, %	58.6	23.4	<0.001

*Given as mean±standard deviation for continuous variables.

†Percentage in women only

Table 2

Association of *PROZ* G79A and A13G Variants with Protein Z Level and Cryptogenic Stroke in Major Race-ethnic Groups.

Race-ethnicity	N	Minor Allele Frequency	Comparison	Association with Protein Z		Association with Stroke	
				Adjusted β* (95% CI)	*P*	Adjusted OR† (95% CI)	*P*
G79A							
White	453	21.8%	GA vs. GG	−0.36 (−0.49, −0.23)	<0.001	1.10 (0.71, 1.68)	0.673
			AA vs. GG	−0.74 (−1.02, −0.46)	<0.001	1.15 (0.44, 2.99)	0.772
Black	83	8.5%	GA vs. GG	−0.41 (−1.01, 0.19)	0.177	0.65 (0.11, 3.87)	0.632
			AA vs. GG	0.04 (−1.41, 1.49)	0.953	NC	NC
Hispanic	120	18.9%	GA vs. GG	−0.43 (−0.70, −0.16)	0.002	1.66 (0.64, 4.29)	0.295
			AA vs. GG	−1.07 (−1.62, −0.52)	<0.001	0.55 (0.05, 5.58)	0.615
Asian	39	68.2%	GA vs. GG	0.04 (−0.69, 0.77)	0.910	0.92 (0.09, 9.77)	0.944
			AA vs. GG	−0.63 (−1.37, 0.10)	0.087	0.10 (0.01, 1.21)	0.070
A13G							
White	447	8.8%	AG vs. AA	−0.47 (−0.63, −0.30)	<0.001	0.80 (0.46, 1.41)	0.438
			GG vs. AA	−1.23 (−2.45, −0.01)	0.049	NC	NC
Black	84	30.9%	AG vs. AA	−0.13 (−0.47, 0.22)	0.458	0.64 (0.22, 1.84)	0.406
			GG vs. AA	−0.34 (−1.12, 0.43)	0.379	1.32 (0.12, 15.14)	0.822
Hispanic	120	25.7%	AG vs. AA	−0.27 (−0.53, −0.00)	0.049	1.50 (0.62, 3.67)	0.370
			GG vs. AA	−0.88 (−1.35, −0.42)	<0.001	0.37 (0.04, 3.43)	0.383
Asian	39	65.9%	AG vs. AA	−1.09 (−2.44, 0.26)	0.109	1.38 (0.05, 41.60)	0.854
			GG vs. AA	−1.67 (−3.01, −0.32)	0.018	0.14 (0.00, 5.26)	0.290

CI: confidence interval; NC: not computable; OR: odds ratio

*Association of *PROZ* G79A or A13G variants with protein Z level using multivariable linear regression adjusting for age, sex, hypertension, diabetes, smoking, family history of thrombosis, and migraine with aura.

†Association of *PROZ* G79A or A13G variants with protein Z level using multivariable logistic regression adjusting for age, sex, hypertension, diabetes, smoking, family history of thrombosis, and migraine with aura.

Table 3

Associations of *PROZ* Variants with Protein Z Level and Cryptogenic Stroke

Variant	Protein Z Level		Cryptogenic Stroke	
	Adjusted β* (95% CI)	*P*	Adjusted OR* (95% CI)	*P*
G79A				
GA vs. GG	−0.36(−0.48, −0.25)	<0.001	1.09(0.76, 1.56)	0.642
AA vs. GG	−0.79(−1.02. −0.57)	<0.001	0.64(0.31, 1.34)	0.236
A13G				
AG vs. AA	−0.34(−0.46, −0.21)	<0.001	0.92(0.61, 1.37)	0.670
GG vs. AA	−0.84(−1.13, −0.55)	<0.001	0.37(0.13, 1.01)	0.053

CI=confidence interval; NC=not computable; OR=odds ratio.

*Linear or logistic regression adjusting for age, sex, race-ethnicity, hypertension, diabetes, smoking, family history of thrombosis, and migraine with aura.

Table 4

Association of *PROZ* Score with Protein Z Level and Cryptogenic Stroke

Score	0 n=359	1 n=202	2 n=110	3 n=15	4 n=20
Protein Z Level					
Adjusted * β (95% CI)	Reference	−0.34(−0.45, −0.22)	−0.58(−0.73, −0.43)	−0.57(−0.92, −0.22)	−1.17(−1.52, −0.82)
Cryptogenic Stroke					
Adjusted * OR (95% CI)	Reference	1.13(0.78, 1.64)	0.82(0.50, 1.34)	1.09(0.36, 3.20)	0.26(0.07, 0.96)

CI=confidence interval; OR=odds ratio.

* Linear or logistic regression adjusting for age, sex, race-ethnicity, hypertension, diabetes, smoking, family history of thrombosis, and migraine with aura.