Plasma Levels of Protein Z and Antiphospholipid Protein Antibodies in Patients with Ischemic Stroke

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ABSTRACT
The relationship between protein Z (PZ) deficiency and ischemic stroke, was firstly evidenced, but not confirmed by all the epidemiological studies. Furthermore, antiphospholipid (aPL) antibodies was considered as an independent stroke risk factor. We designed the present study to investigate the relation of PZ deficiency and aPL antibodies to the risk of ischemic stroke and the impact of aPL antibodies on risk stratification of patients with PZ deficiency and ischemic stroke. We measured PZ and aPL antibodies levels in 40 patients with cerebral ischemic stroke in addition to 12 healthy control subjects. PZ deficiency at or below the tenth percentile of controls (≤1.13µg/ml) was significantly more frequent in stroke cases compared to controls (P=0.040). The stroke risk was increased in young age cases with PZ deficiency and the estimated Odd's ratio was 0.23 (95% CI 0.06–0.88; P=0.032). There was significant increase of anticardiolipin (aCL) antibodies levels in stroke patients compared to controls P=0.004 and P=0.047 for aCL IgG and IgM respectively. The risk of stroke is not increased when aPL are associated with PZ deficiency (OR 0.54; 95% CI 0.09–3.21; P=0.50). There is significant association between PZ deficiency as well as elevated aCL antibodies levels and ischemic stroke. Furthermore, there is increased risk of ischemic stroke in young patients with PZ deficiency. APL antibodies mainly aCL have no impact on risk stratification of patients with PZ deficiency and ischemic stroke. PZ assay should be considered in the diagnostic work up of young patients with ischemic stroke.

INTRODUCTION
Protein Z (PZ) is a vit K–dependent plasma glycoprotein that forms a Ca²⁺ dependent complex with activated coagulation factor X (FXa) at the phospholipid surface and thereby serves as a cofactor to enhance by more than 1000 times in inhibition of FXa produced by a PZ–dependent protease inhibitor (ZPI)¹,². On this basis, lower PZ levels are hypothesized to be a risk factor for thrombosis. Although PZ null mice have an apparently normal phenotype, thrombotic complications were exacerbated in those with a factor V leiden pedigree³. This is consistent with human data showing that a combination of prothrombotic traits significantly enhances the risk of thrombosis and underscores the multigenic nature thrombophilia⁴.

However, epidemiological studies report conflicting associations between PZ concentrations in the blood and thrombotic phenotype⁵, risk of acute coronary syndrome⁶,⁷ and stroke⁸–¹². Deficiency of PZ has, to date, been associated with arterial ischemic stroke, and early fetal loss⁸,⁹,¹³, although some contrasting data on PZ levels and stroke have also been published¹⁰,¹¹. Moreover, the association of stroke and autoimmune antiphospholipid antibodies (aPL) is well documented and several reports consider aPL, mainly anticardiolipin (aCL), as an independent stroke risk factor¹⁴,¹⁵. APL are mainly directed against phospholipid–binding plasma proteins such as beta 2–glycoprotein I (β₂ GPI), and are known to interfere with phospholipid–dependent hemostatic pathways¹⁶. However, the potential relationship between PZ plasma levels and acquired prothrombotic risk such persistent aPL in cerebral
stroke has only been investigated in few studies\textsuperscript{18,19}. To further clarify the relationship between PZ and ischemic stroke, we performed this study to evaluate the association between low PZ levels as well as aPL antibodies and the risk of cerebral stroke and whether the degree of the risk is increased when aPL antibodies are associated with PZ deficiency.

**SUBJECTS AND METHODS**

**Selection of patients**

We analyzed 40 consecutive consented patients (23 females, 17 males, mean age 53 years) who were admitted to neurology department between March 2006 to April 2007 with ischemic stroke and 12 age-matched healthy consented controls (6 females, 6 males, mean age 46.5 years).

All patients received a full clinical workup, including neurological examination, brain imaging (CT or MRI) and ultrasound studies. Baseline demographic data (age and sex) and history of conventional vascular risk factors (hypertension and diabetes) were obtained.

Plasma samples were obtained to measure PZ concentrations only in the convalescent phase, at least 3 months after acute stroke. Patients using oral anticoagulants at the time of stroke or subsequently treated with oral anticoagulants were excluded from the study.

**Methods**

Blood samples were collected into plastic tubes containing 1:10 volume of (3.8%) tri sodium citrate, centrifuged and the platelet-poor plasmas were used to perform the global coagulation tests, aPL antibodies and PZ assays.

**Estimation of global tests of coagulation:**

Prothrombin time (PT) was estimated according to the method of Quick\textsuperscript{20}, using Thromborel-S Kit (Dade Behring, Marburg G mbH, Germany).

Activated partial thromboplastin time (APTT) was estimated according to the method reported by Procter and Rapaport\textsuperscript{21}, using Pathromtin-SL Kit (Dade Behring, Marburg G mbH, Germany).

Fibrinogen assay was done according to the method of Clauss\textsuperscript{22}, using Multifibren U Kit (Dade Behring, Marburg G mbH, Germany).

**Antiphospholipid antibodies assays**

Lupus anticoagulant (LA) activity was identified according to the revised criteria of the subcommittee for standardization of lupus anticoagulants\textsuperscript{23}. ACL IgG & IgM antibodies were measured by ELISA technique using Orgentec Kit (BL Diagnostica, Germany). Titres greater than 20 units were required for a diagnosis of antiphospholipid syndrome (APS)\textsuperscript{24}.

**Protein Z assay**

PZ plasma concentrations were measured by ELISA technique using Zymutest Kit (Hyphen Biomed, France).

**Statistical analysis of data**

Statistical analysis of data was carried out using SPSS (statistical package for social science) computer program version 10. Kolmogrov Smirnof test was used to evaluate the distribution of data. Student's t-test was used to compare between parametric quantitative variables of two groups. Mann–Whitney U test was used to compare between non-parametric quantitative variables of two groups. Chi-Squared test was used to compare between categorical variables. Mantel–Haenszel test was used to estimate the odds ratio. P<0.05 was considered significant.

**RESULTS**

Table (1) Reveals that, as regards the age and sex distribution of ischemic stroke cases and controls, there was no significant difference between them. Also, the global tests of coagulation including PT, APTT and fibrinogen concentration, showed no significant difference among stroke cases compared to controls.

Table (2) shows that, PZ levels equal or below the 10th percentile (1.13ug/ml) of normal values distribution in controls was selected as a cut off value of protein Z deficiency. Accordingly, we demonstrated PZ deficiency in 40% of stroke cases compared to 8.3% in controls. This difference was statistically significance (P=0.040).
Table (3) shows that, the odds ratio for stroke was increased among cases with PZ deficiency compared to controls, which was non significant (OR 7.33; 95% CI 0.86 - 62.5; P=0.068).

From Table (4) it is clear that, protein Z deficiency (≤ 1.13 ug/ml) was significantly associated with stroke in young age cases (OR 0.23 ; 95% CI 0.06 – 0.88 ; P = 0.032) but not in relation to gender (OR 1.08; 95% CI 0.30 – 3.91; P = 0.896) and conventional risk factors including diabetes (OR 0.64; 95% CI 0.17–2.41; P=0.506) and hypertension (OR 2.0; 95% CI 0.55 – 7.31; P=0.295).

Table (5) shows that, there was no significant difference in the mean value of PTT–LA in stroke cases compared to controls (P=0.223), while there was significant increase in the median value of aCL antibodies IgG (P = 0.004) and IgM (P=0.047) in stroke cases compared to controls.

Table (6) reveals that, APS were found in 12.5% of stroke cases with PZ deficiency with no significant increase in the relative risk of cerebral stroke (OR 0.54 ; 95% CI 0.09 – 3.21 ; P = 0.501).

Table (1): Baseline demographics and global tests of coagulation in stroke cases and controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 12)</th>
<th>Cases (n = 40)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), mean±SD</td>
<td>46.50±17.51</td>
<td>53.05±15.86</td>
<td>0.226</td>
</tr>
<tr>
<td>Gender (male / female)</td>
<td>6/6</td>
<td>17/23</td>
<td>0.646</td>
</tr>
<tr>
<td>Prothrombin time (INR)</td>
<td>1.06±0.07</td>
<td>1.13±0.14</td>
<td>0.119</td>
</tr>
<tr>
<td>APTT (Sec)</td>
<td>31.41±1.92</td>
<td>31.43±3.05</td>
<td>0.982</td>
</tr>
<tr>
<td>Fibrinogen conc. (mg/dl)</td>
<td>501.91±50.33</td>
<td>504.85±97.55</td>
<td>0.921</td>
</tr>
</tbody>
</table>

Table (2): Frequency of protein Z deficiency in stroke cases and controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 12)</th>
<th>Cases (n = 40)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Z (≤ 1.13 ug/ml)</td>
<td>1 (8.3%)</td>
<td>16 (40%)</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Table (3): Odds ratio for stroke associated with protein Z deficiency.

<table>
<thead>
<tr>
<th>Protein Z (≤ 1.13 ug/ml)</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke cases</td>
<td>7.33</td>
<td>0.86 – 62.5</td>
<td>0.068</td>
</tr>
</tbody>
</table>

Table (4): Odds ratios for stroke associated with protein Z deficiency in relation to demographics and conventional risk factors.

<table>
<thead>
<tr>
<th>Protein Z (≤ 1.13 ug/ml)</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (≤ 50 y)</td>
<td>0.23</td>
<td>0.06 – 0.88</td>
<td>0.032</td>
</tr>
<tr>
<td>Gender (female / male)</td>
<td>1.08</td>
<td>0.30 – 3.91</td>
<td>0.896</td>
</tr>
</tbody>
</table>
Table (5): Antiphospholipid antibodies in stroke cases and controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 12)</th>
<th>Cases (n = 40)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTT – LA (Sec)</strong></td>
<td>32.17±1.7</td>
<td>33.3±3.03</td>
<td>0.223</td>
</tr>
<tr>
<td><strong>aCL – IgG (GPL/ml)</strong></td>
<td>4.10 (1.8 – 8.1)</td>
<td>6.30 (2.0 – 70.4)</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>aCL – IgM (MPL/ml)</strong></td>
<td>4.45 (1.5 – 6.1)</td>
<td>5.55 (1.5 – 21.0)</td>
<td>0.047</td>
</tr>
</tbody>
</table>

Table (6): Frequency of antiphospholipid antibody syndrome among stroke cases with protein Z deficiency.

<table>
<thead>
<tr>
<th></th>
<th>PZ (&gt; 1.13 ug/ml) (n =24)</th>
<th>PZ (≤ 1.13 ug/ml) (n = 16)</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>APS</strong></td>
<td>5 (20.8%)</td>
<td>2 (12.5%)</td>
<td>0.54</td>
<td>0.09 - 3.21</td>
<td>0.501</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Several recent clinical studies on PZ levels and the risk of cerebral ischemia yielded contradictory results. Some studies suggest that reduced blood concentrations of PZ increase stroke risk, whereas others suggest no associations or that increased concentrations increase stroke risk. However, one report found that patients presenting with factor V leiden and low PZ levels show earlier onset and higher frequency of thromboembolic events than do patients with only FV leiden. Thus, we designed this study to evaluate the association between low PZ levels and the risk of cerebral stroke and whether the degree of risk is increased when acquired prothrombotic risk such as persistent aPL are associated with PZ deficiency.

In the present study, the frequency of PZ deficiency [at or below the 10th percentile of controls (≤ 1.13 ug/ml)] was significantly greater in stroke cases (40%) compared to controls (8.3%) (P=0.040). The risk of stroke was increased among cases with PZ deficiency compared to controls which was non significant (OR 7.33; 95% CI 0.86 – 62.5; P=0.068). PZ deficiency was significantly associated with stroke in younger age cases (OR 0.23; 95% CI 0.06 – 0.88; P=0.032) but not in relation to gender (OR 1.08 ; 95% CI 0.30 – 3.91; P = 0.896) and conventional risk factors including diabetes (OR 0.64; 95% CI 0.17 – 2.41; P=0.506) and hypertension (OR 2.0; 95% CI 0.55 – 7.31; P=0.295).

These results are consistent with those reported by Heeb et al., who reported significantly lower PZ values in a group of 154 patients (mean age 57 years) with ischemic stroke compared to healthy controls (p=0.0003). They further showed that low PZ were significantly associated with increased risk of ischemic stroke, particularly in younger male patients and in the absence of the known risk factors of diabetes and hypertension. However, stroke risk was higher in subjects with PZ levels at or below the fifteenth percentile of controls (≤1.46 ug/ml) with odds ratio 2.6 (95% CI 1.5 – 4.3 ; P=0.0003) and this is in contrast to ours, probably due to the small number of subjects included in our study.

Moreover, these results coincide with those of the French study by Vasse et al., who reported a significant (P<0.01) association between ischemic stroke and PZ deficiency (<1.0 ug/ml plasma; < 44% of the mean in control subjects). There is increase in the relative risk of stroke by four fold and this difference possibly due to their patients differed from ours in that they were European and their mean age was 33 years, and non had hypertension or dyslipidemia.
Thus, the association between PZ deficiency and ischemic stroke appears to be stronger in younger patients and in patients who don’t have conventional vascular risk factors as suggested by Tran and Eikelboom. However, it remains unclear whether the link between PZ levels and stroke is confounded or causal or whether blood levels of PZ are altered as a consequence of the acute stroke event.

In this study, we have analyzed the antiphospholipid profile in ischemic stroke patients to further determine the potential relationship between PZ deficiency and acquired prothrombotic risk factors and its effect on the risk of cerebral stroke. Our results showed significant increase in the median value of aCL antibodies IgG (P=0.004) and IgM (P=0.047) in stroke cases compared to controls with no significant difference in the mean value of PTT-LA (P=0.223). These results are in accordance with many reports who have found that the most frequently cited aPL is immunoglobulin (IgG) aCL, which, when present increase the risk of stroke.

APL antibodies are responsible for defining the antiphospholipid antibody syndrome (APS), a disorder characterized by the presence of aPL (including LA and/or medium or high titre IgG or IgM aCL), with arterial and venous thromboembolic events, recurrent pregnancy loss, or thrombocytopenia. And according to the Sapporo criteria (APS is present in patients with 1 clinical and 1 laboratory criterion), our investigations have revealed APS in 7 stroke patients (17.5%) with elevated aCL only. These findings are supported by Galli et al., who reported that the presence of IgG aCL antibodies at medium to high titres, either alone or in various combinations with other tests, is clinically useful to establish the patient’s risk of thrombosis in the APS.

Furthermore, we evaluated the frequency of APS among stroke cases with PZ deficiency and we have found that APS were present in 12.5% of stroke cases with PZ deficiency with no significant increase in the relative risk of cerebral stroke (OR 0.54; 95% CI 0.09 – 3.21; P=0.501). These findings are in contrast with forastiero et al., who reported that the prevalence of low PZ levels (below the 5th percentile of controls) was significantly greater in the definite APS, but not in the non-APS group, compared with the normal group and concomitant PZ deficiency increased by approximately sevenfold the risk of arterial thrombosis in aPL patients. They found that B2 GPI modestly delayed the factor Xa inactivation by PZ/ZPI and most isolated aPL-IgGs were found to further increase the inhibitory potential of B2 GPI on PZ/ZPI activity thus probably increasing the thrombotic risk.

However, our findings coincide with Steffano et al., who observed that the decrease of PZ was mainly found in aPL patients having LA activity with or without positive aCL but not in patients with aCL alone, and this observation is supported by Galli et al., who reported that the association between antiphospholipid antibodies and thrombosis is stronger with LA than with aCL.

In conclusion, our data indicate that there is a significant association between PZ deficiency as well as elevated anticardiolipin antibodies levels and ischemic stroke. Also, there is increased risk of ischemic stroke in young patients with PZ deficiency. However, the risk of thrombosis is not increased.

When aPL mainly aCL are associated with PZ deficiency. Further work is necessary to explain our findings.

REFERENCES

7. Morange PE, Juhan-Vague I: On behalf of the PRIME study Group Protein Z plasma levels are not associated with the risk of coronary heart
المتخصصة العربي

مستويات بروتين زد والأجسام المضادة للفوسفوليبيدات في بلازما المصابين بالاختشاء المخيخ

لقد أظهرت معظم الدراسات الوبائية وجود علاقة بين نقص بروتين زد والاختشاء المخيخ، ولكنها لا تزال علاقة غير مؤكدة، وذلك بالإضافة للأقسام المعادن للفوسفوليبيدات جامعية خطورة مستقبل لمثل هذا المرض. تهدف هذه الدراسة إلى إظهار العلاقة بين نقص بروتين زد ومرض الاختشاء المخيخ وكذلك العلاقة التبادلية بينها وبين الأجسام المضادة للفوسفوليبيدات كعامل مهم في نشوء هذا المرض. أجريت الدراسة على مجموعة من مرضى الاختشاء المخيخ (40 مريض) بالإضافة إلى مجموعة من الأصحاء (12 شخص) كمجموعة ضابطة. وتم قياس مستويات بروتين زد والأجسام المضادة للفوسفوليبيدات في بلازما كل من المصابين والمجموعة الضابطة. أظهرت نتائج الدراسة وجود نقص ذو دالة إحصائية في مستوى بروتين زد في المصابين بالاختشاء المخيخ مقارنة بالمجموعة الضابطة. ووجد أن خطر الإصابة بالاختشاء المخيخ يزيد في المرضى صغار السن الذين يعانون نقصًا في مستوى بروتين زد. وقد لوحظ وجود ارتفاع ذو دالة إحصائية في مستوى الأجسام المضادة للفوسفوليبيدات في المصابين بالاختشاء المخيخ مقارنة بالمجموعة الضابطة. وقد أظهرت الدراسة أن خطر الإصابة بالاختشاء المخيخ لا يزيد عندما تكون الأجسام المضادة للفوسفوليبيدات مصاحبة لنقص بروتين زد في المرضى. خلصت الدراسة إلى أن هناك علاقة ذات دالة إحصائية بين نقص بروتين زد وارتفاع الأجسام المضادة للفوسفوليبيدات والإصابة بمرض الاختشاء المخيخ، كما وجد أن خطر الإصابة بالاختشاء المخيخ يزداد في المرضى صغار السن الذين يعانون نقصًا في مستوى بروتين زد، وفي حين وجد أن الأجسام المضادة للفوسفوليبيدات تحديدا الأجسام المضادة للفوسفوليبيدات ليس لها دور في زيادة خطر الإصابة بالاختشاء المخيخ في المرضى الذين يعانون نقصًا في مستوى بروتين زد.

وتوصى الدراسة باعتبار قياس مستوى بروتين زد من التحاليل التشخيصية في المرضى صغار السن المصابين بالاختشاء المخيخ.