Evaluation of the PC-1 K121Q and G2906C variants as independent risk factors for ischaemic stroke

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PC-1, polymorphism, stroke, vascular disease, risk factor

Summary
Overexpression of plasma cell membrane glycoprotein-1 (PC-1) inhibits insulin receptor tyrosine kinase activity and thus favours insulin resistance and atherosclerotic vascular disease. Recent findings indicate that the minor variant K121Q in the PC-1 gene confers an increased risk for early myocardial infarction independent of other established risk factors. We hypothesized that genetic variants in PC-1 may also influence the risk for cerebrovascular disease. Aim: Therefore, we assessed the association of the PC-1 K121Q variant in the coding region and a polymorphism (G2906C) in the 3' untranslated region of the PC-1 gene with the risk of stroke. Patients: We analyzed 1014 patients with a history of ischaemic stroke from the Vienna stroke registry and 1001 control individuals without vascular disease. Results, conclusion: Genotype frequencies of both genetic variants were similar in patients and controls in the total study population. By multivariate analysis, no interactions were observed between the PC-1 genotype and established vascular risk factors. However, the PC-1 2906C allele was significantly more frequent in patients who suffered from stroke before the age of 40 years. In these patients the risk for ischaemic stroke was increased fourfold.

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Ischaemic stroke is a multi-factorial disease and a major cause of death and disability in the Western world. Both, epidemiological and animal-based studies suggest that genetic factors are important in the pathogenesis of stroke although acquired risk factors (hypertension, cigarette smoking, diabetes mellitus) account for up to 69% of the total risk. A variety of candidate genes involved in the haemostatic system, controlling the homocysteine metabolism, the angiotensin converting enzyme gene, and the endothelial nitric oxide synthase gene have been associated with the pathogenesis of ischaemic stroke (8, 9, 11). Ischaemic stroke comprises a number of different phenotypes, which may have different genetic profiles. In combination with acquired risk factors such as smoking the presence of one or the combination of several predisposing genes may favour the occurrence of stroke (8).

Recently, we demonstrated that the plasma cell membrane glycoprotein-1 (PC-1) K121Q variant in the coding region of the PC-1 gene is associated with early myocardial infarction (4). PC-1 is a class II transmembrane glycoprotein with extra-
cellular phosphodiesterase and pyrophosphatase activity. Over-expression of PC-1 has been associated with insulin resistance (3, 6, 12). Interestingly, however, insulin resistance is not mediated by hydrolysis of adenosine triphosphate or the generation of adenosine but via direct interaction of the PC-1 molecule with the insulin receptor alpha subunit (7, 10). Several functionally important regions have been identified in PC-1 including an EF-hand domain which binds Ca$^{2+}$ and stabilizes the structure of the molecule, an ATP-binding site, a phosphodiesterase site, a proteolytic cleavage site allowing cells to shed PC-1, and two somatomedin B-like domains (one of them harboring the K121Q variation). The physiological or pathophysiological importance of these domains remains to be determined. However, one may speculate that PC-1 mediated insulin resistance and endothelial dysfunction promote atherogenesis and thrombus formation, events that contribute to the occurrence of ischaemic stroke.

We investigated the association of the PC-1 minor variant K121Q and the 2906 G>C polymorphism with ischaemic stroke in patients from the Vienna Stroke Registry. The cluster of three SNPs at nt 2897, 2906, and 2948 in the 3' UTR of the PC-1 gene was reported (5) to be associated with increased PC-1 mRNA stability, elevated PC-1 protein expression, and insulin resistance.

**Patients and methods**

**Patients**

1014 patients from the Vienna Stroke Registry (2) were included in the study and were compared to 1001 control individuals (median age 46 years, 516 men, 485 women). Controls were unrelated Caucasian participants in an official health care program of the city of Vienna, who came from the same geographic area as the patients. The controls were free of clinically manifest vascular disease, and reported no arterial vascular diseases in first-degree relatives. The study was approved by the ethics committee of the Medical University of Vienna and all patients and controls gave their written informed consent to participation in the study.

All patients were questioned for known cardiovascular risk factors including diabetes, smoking (>20 cigarettes per day for more than 5 years), hypertension, body mass index (BMI), and family history of cardiovascular disease (4). Arterial hypertension was defined as either history of arterial hypertension, or blood pressure values above 140/90 mmHg in patients measured one week after the qualifying event) or intake of antihypertensive medication. Presence of diabetes mellitus (DM) was defined by fasting blood glucose levels above 125 mg/dl according to American Diabetes Association Criteria (1) or history of DM or treatment with anti-diabetic medication. Hyperlipidaemia was defined as fasting total serum cholesterol >200 mg/dl, or a history of hyperlipidaemia or intake of lipid-lowering medication.

Stroke etiology was classified according to the Banff classification in

- large-vessel disease (ipsilateral carotid stenosis ≥ 70%, presumable local thrombosis of a large intracranial vessel, arterio-arterial embolism from aortic plaques/thrombi),
- small-vessel disease (clinical lacunar syndrome and no lesion or subcortical lesion < 1.5 cm on CT or MRI),
- cardioembolic (high risk source of cardiac embolism) or
- undetermined etiology.

Obesity was considered present in individuals with a BMI >30 kg/m².

**PCR analysis of the PC-1 variants**

DNA analysis was performed after obtaining the patient’s informed consent. DNA
was isolated according to standard procedures. The PC-1 variants were tested by mutagenically separated PCRs essentially as described previously (4). In brief, PC-1 PCR products were generated in 25 μl volumes containing 1.7 U AmpliTaq Gold (Perkin Elmer Cetus, Norwalk, CT), 1.5 mmol/l MgCl₂, 20 mmol/l of each dNTP (Amersham Pharmacia Biotech, Upsala, Sweden), 6.5 pmol PC-1 121K reverse primer, 3.5 pmol PC-1 121Q reverse primer, 6.5 pmol common forward primer, and approximately 50 ng of genomic DNA. Amplifications were performed in an Eppendorf thermocycler (Mastereyzer, Eppendorf AG, Hamburg, Germany). A 10-min denaturation period at 95°C was followed by 33 cycles of 95°C for 45 s, 54°C for 45 s, and 72°C for 45 s. A final extension step of 7 min at 72°C completed the reaction. PCR products were separated on 10% Criterion gels (Bio-Rad, Hercules, CA) for 1 hour at 170 V. After staining with Sybr Green (Molecular probes, Eugene, OR) for 10 min bands were visualized on a UV transilluminator at 306 nm and documented using the Polaroid camera.

For the 3’ UTR SNP cluster (2897G>A, 2906G>C, 2948C>T) two MS PCRs were developed using the following primers: 2906G reverse primer: 5’- gAg gTg TCC gCA gCA CC – 3’ (5 pmol), 2906C reverse primer: 5’- Aag TTG CCC TTT TTG gTC gCg ATT ACg – 3’ (5 pmol), 2006 common forward primer: 5’- gAC TgA gTg TTG TTG TAT CCC CAA – 3’ (10 pmol), 2948T forward primer: 5’- Agg gAA ATA AgC gTA CTC AgC ATA gT – 3’ (5 pmol), 2948C forward primer: 5’- CTT gCg TAC TCA gCg CAg C – 3’ (10 pmol), 2948A forward primer: 5’- ATT gTT CAg AAA TAT TCg ACC AgA gTT ATA ACA – 3’ (5 pmol), 2987G forward primer: 5’- CTg TCg ACC AgA gTT AgC Acg – 3’ (2 pmol), and 2948+2987 common reverse primer 5’- AAg gCA gTT TTA gGT TTA gTg gTg gTg – 3’ (10 pmol) all synthesized by TIB Molbiol, Berlin, Germany. PCRs were performed essentially as described above using 36 cycles and an annealing temperature of 53°C. The 2897G>A and 2948C>T PCRs were combined to one multiplex PCR and performed as a single tube reaction, the 2906 SNP (single nucleotide polymorphism) was analyzed in a separate reaction. For the PC-1 2906 C allele a PCR product with a length of 140 bp, for the PC-1 2906 C allele a product of 150 bp, for the PC-1 2987 G allele a product of 178 bp, for the PC-1 2897 A allele a product of 190 bp, for the PC-1 2987 G allele a product of 125 bp, and for the PC-1 2948 T allele a product of 130 bp were generated. The interpretation and documentation of the results were done by two independent researchers blinded for the clinical variables.

### Statistical analysis

For statistical analysis we used the SPSS 10.0 software package (SPSS, Chicago, USA). Continuous data are presented as the median and the interquartile range (IQR, range from the 25th to the 75th percentile). Discrete data are given as counts and percentages. Multivariate logistic regression analysis was applied to assess the association of the PC-1 genotype and the group classification adjusting for potentially confounding variables and to test for interactions. Regression diagnostics were performed according to standard recommendations: The logit assumption was checked for continuous variables, an analysis of residuals was performed, global goodness of fit testing was performed using the Hosmer Lemeshow test. Interactions were evaluated using multiplicative interaction terms and log likelihood ratio chi square tests. Interactions were considered present if they resulted in a p-value < 0.1. Results of the logistic regression models are given as odds ratio (OR) and the 95% confidence interval (95% CI). A two sided p-value <0.05 was considered statistically significant.

### Results and discussion

In this case control study we investigated associations of the PC-1 K121Q variant and the SNP cluster at nt 2897G>A, 2906G>C, 2948C>T in the 3’ UTR of the PC-1 gene with ischaemic stroke in patients from the Vienna Stroke Registry. We had recently found that the minor PC-1 K121Q variant (PC-1 121Q) predisposes to cardiovascular disease leading to myocardial infarction (MI) at young age. From these results we concluded that PC-1 may be considered an independent risk factor for early MI (4). It is known that the critical PC-1 121Q allele is associated with insulin resistance and endothelial dysfunction (6, 12). We hypothesized that the PC-1 K121Q variant might also influence the risk for ischaemic stroke. In addition, it has been shown that PC-1 gene expression is affected by a cluster of three SNPs in the 3’ UTR. These SNPs have been reported to be associated with increased PC-1 mRNA stability, elevated PC-1 protein expression, and insulin resistance (5). We studied the association of these genetic variants in the 3’ region of PC-1 with the risk of stroke. For 250 individuals all three SNPs were determined. We found that these polymorphisms are in strong linkage disequilibrium (only two individuals were homozygous for 2906 G/G and heterozygous for 2897 G/A and 2948 C/T, data not shown). Therefore, we selected one representative SNP for the remaining patients and controls, and chose the SNP 2906 G>C.

Genotype frequencies were in Hardy-Weinberg equilibrium in all groups. The frequencies of PC-1 121KK, 121KQ and 121QQ were not different in patients (n =

<table>
<thead>
<tr>
<th>PC-1</th>
<th>total study population</th>
<th>controls (n = 1001)</th>
<th>patients (n = 1014)</th>
<th>p-value*</th>
<th>patients (n = 287)</th>
<th>controls (n = 52)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>121 Q</td>
<td></td>
<td>230 (23.0%)</td>
<td>246 (24.3%)</td>
<td>0.498</td>
<td>67 (23.3%)</td>
<td>13 (25.0%)</td>
<td>0.796</td>
</tr>
<tr>
<td>121 KK</td>
<td></td>
<td>771 (77.0%)</td>
<td>768 (75.7%)</td>
<td></td>
<td>220 (76.7%)</td>
<td>39 (75.0%)</td>
<td></td>
</tr>
<tr>
<td>2906 C</td>
<td></td>
<td>46 (4.6%)</td>
<td>46 (4.5%)</td>
<td>0.966</td>
<td>8 (2.8%)</td>
<td>5 (9.6%)</td>
<td>0.018</td>
</tr>
<tr>
<td>2906 G</td>
<td></td>
<td>955 (95.4%)</td>
<td>967 (95.5%)</td>
<td></td>
<td>279 (97.2%)</td>
<td>47 (90.4%)</td>
<td></td>
</tr>
</tbody>
</table>

*calculated using the chi-square test

Tab. 2 PC-1 genotypes

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No association between stroke etiology and PC-1 genotype was observed (Tab. 3), nor were relevant interactions found between stroke etiology, PC-1 genotype, and conventional risk factors (data not shown). In a multivariate logistic regression model including both variants and male sex, age, smoking, hyperlipidaemia, hypertension, diabetes and obesity the PC-1 2906C allele showed relevant interactions with age.

**PC-1 2906C and age**

Interestingly, in individuals who suffered their first ischaemic stroke before the age of 40, the PC-1 2906C allele was significantly more frequent (5/52 individuals, 9.6%) compared to controls (8/287 individuals, 2.8%) resulting in an increased odds of 4.1 (95%CI: 1.1–13.7, p = 0.017). This association remained unchanged after adjustment for sex, hypertension, obesity, smoking, hyperlipidaemia, and diabetes. Thus, the PC-1 2906C allele could represent a new independent risk factor for ischaemic stroke in young patients. Notably, in this group of patients besides the PC-1 2906C allele only smoking and diabetes remained statistically significant contributors to the risk of stroke in a multivariate logistic regression model (p < 0.01 and 0.04, respectively). In the other age groups no significant association of the PC-1 2906C allele with stroke was observed.

Up to date, no detailed results on PC-1 function in different vessel types are available. Since in the pathogenesis of MI mainly intermediate vessels and in ischaemic stroke large vessels are involved, we speculate that the difference in the impact of the 2906G>C and the K121Q variants of the PC-1 gene on vascular physiology might depend on other yet unidentified factors such as differences in tissue gene/protein expression patterns which may determine the availability of PC-1 interaction partners in different vessel types. Additionally, the K121Q mutation is located in the somatomedin B-like region (a potential protein-protein interaction site) of PC-1 suggesting that the mutant protein might interact with vascular proteins in a different way than the normal protein. The 2906G>C variant in the 3’UTR, is thought to stabilize PC-1 mRNA leading to a higher abundance of the PC-1 protein and consequently to enhanced association of PC-1 with the insulin receptor alpha chain and to insulin resistance.

Concluding from our data it appears that neither the PC-1 121Q nor the 2906C allele represent independent risk factors for ischaemic stroke in unselected patients.

In individuals with stroke at an age younger than 40 years published data suggest that familial aggregation of stroke is more frequent (13) and genetic factors seem to play a more important role in the development of stroke (8) than in patients with later-onset. In these patients the PC-1 2906C allele seems to be associated with a 4.1 fold increased risk for suffering ischaemic stroke. However, our findings have yet to be proven in an independent confirmatory study before the PC-1 2906C variant can be considered an independent risk factor for ischaemic stroke.

**Limitations**

We are aware that the influence of polymorphisms in a single gene on a complex disease such as stroke may be influenced by patient selection, ethnic background or sample size among others. Our patient population represents a cross-sectional sample of survivors of cerebrovascular events. Patients who died within 24 h, were not included in our study. Therefore, our findings can only be applied to the survivors of an acute cerebrovascular event. In addition, cases and controls differed in the prevalence of traditional vascular risk factors (e.g. hypertension, hyperlipidaemia, diabetes mellitus). Controls were also younger, and we cannot exclude that some still healthy individuals might develop a vascular event in the future. However, we only observed an association between stroke and PC-1 2906C in patients younger than 40 years. For these patients the control group did not differ in age. However, there was a significant difference in the number of smokers, which may have influenced the results.

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**References**


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