

## Blood Coagulation, Fibrinolysis and Cellular Haemostasis

# The plasminogen activator inhibitor (PAI-I) 4G/5G promoter polymorphism and PAI-I levels in ischemic stroke

## A case-control study

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### Summary

High levels of plasminogen activator inhibitor type I (PAI-I) have been implicated as a risk factor for cardiovascular disease, but its precise role remains controversial. The 4G allele of the PAI-I 4G/5G promoter polymorphism is associated with higher levels of PAI-I. We studied the relationship between ischemic stroke and the PAI-I 4G/5G polymorphism and PAI-I antigen levels. We performed a case-control study among patients aged 18–75 years with first ischemic stroke, confirmed by CT. All patients were screened for cardiovascular risk factors, cardiac disorders and large vessel disease. We excluded patients with a definite non-atherosclerotic cause of the stroke and patients using oral anticoagulants. Population-controls were age- and sex-matched, without a history of stroke, and of the Caucasian

race. Venous blood samples were taken for PAI-I 4G/5G polymorphism and PAI-I level one week after stroke. We included 124 patients and 125 controls. Mean age was 56 yrs (range 18 to 75 yrs). Sixty one patients (50%) and 58 (47%) controls were heterozygous for the PAI-I 4G/5G polymorphism. The homozygous 4G/4G genotype was found in 33 patients (27%) and in 36 controls (29%). The odds ratio of ischemic stroke associated with 4G-carriers versus 5G/5G homozygotes was 1.0 (95% CI: 0.6–1.8). The relative risk of ischemic stroke associated with the level of PAI-I in the upper quartile was 0.73 (95% CI: 0.4 to 1.4). Neither the PAI-I 4G/5G polymorphism nor the PAI-I antigen level is a strong risk factor for ischemic stroke.

### Keywords

PAI-I 4G/5G polymorphism, ischemic stroke, case-control, PAI-I level

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## Introduction

Increased levels of the plasminogen activator inhibitor-1 (PAI-1) have been reported in patients with myocardial infarction and stroke (1–3). Whether these increased levels are as a result of a genetic variation of the PAI-1 gene, and whether they increase the risk of ischemic stroke is not yet clear.

PAI-1 is a glycoprotein with a molecular weight of approximately 50 KD that serves as the major physiological inhibitor of tissue plasminogen activator (t-PA), thereby attenuating fibrinolysis (4, 5). The human PAI-1 gene is located on chromosome 7 and several polymorphisms within this gene are associated with PAI-1 level in plasma (6, 7). A common polymorphism, located in the promoter region, consists of a single guanosine insertion/deletion variation (4G or 5G), resulting in two alleles

containing either 4 or 5 guanines in a row. In the general population, an association has been found between the 4G/5G polymorphism and the PAI-1 level, with individuals homozygous for the 4G allele having a significantly higher PAI-1 concentration in plasma (7). Thus, the 4G allele could increase the risk of ischemic stroke through its association with high PAI-1 levels, that inhibit endogenous fibrinolysis.

The association of PAI-1 level and the 4G/5G polymorphism has been more extensively studied in myocardial infarction than in stroke. A recent meta-analysis of nine studies showed a significant 20% increased risk of myocardial infarction for the 4G/4G genotype (8). In stroke patients, studies have yielded conflicting results. Several case-control studies failed to find an association, but they often considered selected patients, such as women, patients with TIA and minor stroke or even hemorrhagic

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stroke (2, 9, 10). Furthermore, most studies reported on genotype only, instead of genotype and phenotype simultaneously. Recently, the 4G allele was reported to be protective for ischemic stroke in the elderly (11). In the same age group in women, the allele has been associated with a reduced mortality from stroke (12). In contrast, in another case-control study, the 4G/4G genotype was found to be an independent risk factor for atherothrombotic stroke (13). Finally, a recent and large prospective follow-up study could not support an association between the PAI-1 polymorphism and stroke in the elderly (14).

To clarify whether a higher risk of stroke as a result of elevated PAI-1 levels is genetically determined, it would be of interest to study the PAI-1 polymorphism and its phenotypic expression simultaneously in a well defined population. We therefore designed a case-control to investigate the possible association between 4G/5G PAI-1 gene promoter polymorphism and the occurrence of ischemic stroke and secondly, to study the role of PAI-1 level.

## Methods

### Study design

We performed a case-control study with prospective inclusion of the participants. Cases were consecutively recruited patients with first ever incidence of acute ischemic stroke, admitted to the Department of Neurology of a university hospital, between January 1999 and December 2001. This university hospital is an area hospital serving an urban population, and has no specific selection criteria for the admission of stroke patients. However, young patients are referred more frequently to this center than to non-academic centers in the regions. Fifty percent of the urban population is foreign and originally stems from Caribbean or Mediterranean areas. We used population controls, i.e. partners, friends or neighbours of the patients. They were age- and sex matched, of the same race, without a history of stroke and unrelated to the patient.

### Inclusion and exclusion criteria

Patients, controls and their parents should be born in Northern Europe and be of the Caucasian race, because substantial differences in the prevalence of genetically determined coagulation disorders have been reported between people from different geographical regions or race (15). Patients with a definite non-atherosclerotic cause for stroke, such as a mechanical heart valve, endocarditis, DIC or carotid dissection, were excluded because we considered these conditions as a likely and sufficient cause of the stroke. Other exclusion criteria were age over 75 years and the use of oral anticoagulants.

### Definitions and measurements

Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia with symptoms lasting more than 24 hours. Patients with TIA (symptoms lasting less than 24 hours) were included only if the neurological deficit in the acute phase was observed by a neurologist. In all patients, a CT of the brain was made within three days from onset of symptoms to confirm the diagnosis of ischemic stroke and to rule out hemorrhagic stroke or other uncommon causes. Clinical stroke

subtypes were classified according to the OCSF criteria, modified by the results of CT (16). Etiologic stroke types were classified according to the TOAST criteria (17). Stroke severity was assessed by means of the Barthel index (18).

### Blood samples and procedures

One week after stroke, venous blood samples were taken under strictly standardized conditions. The patient was fasting, with no exposure to tobacco or alcohol for at least 8 hours and the drawing of blood took place following 15 minutes of rest. Blood was drawn using the vacutainer system and collected in CTAD-tubes, containing citrate and platelet stabilizing agents (Beckton Dickinson, Plymouth, UK). Blood was centrifuged for 20 min at 4°C and plasma was stored using small aliquots at -80°C until use. PAI-1 level, cholesterol and glyco-Hb were determined in the blood samples. PAI-1 antigen levels were measured using a commercially available ELISA (TintElize®PAI-1, Biopool, Umea, Sweden) and were expressed in ng/ml. Genomic DNA was isolated from the white cell fraction of citrated blood, according to the high-salt concentration standard procedure (19). The PAI-1 4G/5G gene polymorphism was detected by allele-specific PCR amplification (20).

In patients as well as in controls, we collected detailed information about cardiovascular risk factors, such as smoking habit, hyperlipidemia, hypertension, diabetes, use of oral contraceptives, patient medical history and family medical history. Patients were screened for cardiac abnormalities by means of standard twelve lead ECG examination. A cardiologist was consulted in female patients aged 55 years or less, in male patients aged 45 years or less, and in patients with ECG abnormalities or a clinical suspicion of cardiac disease. In these patients, 24-hour ECG monitoring, transthoracic and/or transesophageal echocardiography was carried out. Screening for large vessel disease included duplex ultrasound or, if indicated, angiography of the carotid or vertebral arteries.

### Statistical aspects

The relationship between the 4G/5G PAI-1 gene polymorphism and ischemic stroke was expressed as an odds ratio with a 95% confidence interval. The PAI-1 levels were divided into quartiles and the relationship between PAI-1 level and ischemic stroke was estimated by odds ratio for the highest quartile versus the lower three quartiles. Multiple logistic regression analysis was used to adjust for possible confounders, such as smoking and hypertension, as these vascular risk-factors are also known to affect PAI-1 level. Furthermore, we assessed the relationship between genotype and stroke severity by relating genotype to a dichotomized Barthel score (0–19 vs. 20 points) (18).

## Results

During the study period 1034 patients with acute stroke were admitted to our hospital. Twenty percent of the patients had an intracerebral hemorrhage, 13% died within a few days as a result of the stroke, 28% had a recurrent ischemic stroke. Of the remaining patients, 38% were of Caribbean or Mediterranean origin and were therefore not included. One patient was excluded later, after she was found to have DIC, induced by a pancreatic malignancy.

**Table 1: Baseline characteristics.**

	Cases (n=124)	Controls (n=125)
<b>Demographics</b>		
Age, yrs (SD)	56 (12)	56 (12)
Female sex	58 (47%)	59 (47%)
<b>Index event</b>		
Stroke : TIA	115 : 9	NA
<b>Risk factors</b>		
Smoking	61 (49%)	37 (30%)
Hypertension	60 (48%)	24 (19%)
Diabetes	18 (14%)	5 (4%)
Hypercholesterolemia	78 (63%)	84 (67%)
Oral contraceptives	11 (19%)	8 (14%)
Family history of cardiovascular disease	75 (61%)	56 (45%)
Previous DVT	6 (5%)	0 (0%)

The final study population consisted therefore of 124 patients and 125 controls. In 123 patients and 123 controls, blood samples were available for detection of the PAI-1 polymorphism and PAI-1 level.

### Population characteristics

Table 1 shows the baseline demographics and vascular risk factors in patients and controls. Patients were more likely to be smokers and more often had hypertension, diabetes or previous cardiovascular disease than controls.

In half of the cases no cause for the stroke was found according to the TOAST classification. Eleven patients (9%) suffered from large vessel disease and 46 (37%) from small vessel disease. Four patients (3%) had a probable cardiac source of embolism. In two young patients (2%), the evaluation was not complete because both refused echocardiography.

### Genotype distribution

The distribution of PAI-1 genotypes in patients was: 4G/4G, 33 (27%), 4G/5G, 61 (49%) and 5G/5G 29 (24%). When compared with the matched controls, whose genotype distribution was 4G/4G, 36 (29%), 4G/5G, 58 (47%) and 5G/5G 29 (24%), there were no significant differences. The allele frequencies in patients were 0.52 for 4G and 0.48 for 5G, compared with 0.53 for 4G and 0.47 for 5G in controls. The genotype frequencies in controls were in Hardy-Weinberg equilibrium. The odds ratio (OR) of ischemic stroke associated with the 4G-allele versus 5G/5G-homozygotes was 1.0 (95% CI: 0.6–1.8). There was no difference in relative risk of ischemic stroke between heterozygous and homozygous 4G-genotype (Table 2).

### PAI-I levels

The mean PAI-1 level was lower in patients than in controls, 22.9 ng/ml vs. 25.1 ng/ml respectively, but this difference was not statistically significant (student's *t*  $p > 0.22$ ). We divided the PAI-1 level in quartiles and the relative risk for stroke in the highest

**Table 2: Frequencies of 4G/5G PAI-I gene polymorphism among patients and controls and the odds ratio (with 95% confidence interval) of ischemic stroke associated with the PAI-I gene polymorphism.**

	Cases (n=123)	Controls (n=123)	OR (95%CI)
5G/5G	29	29	reference
4G/5G	61	58	1.1 (0.6–2.0)
4G/4G	33	36	0.9 (0.5–1.8)
Frequency of 5G allele	0.48	0.47	
Frequency of 4G allele	0.52	0.53	

**Table 3: Mean PAI-I level (ng/ml) and standard deviation (ng/ml) according to genotype in controls and in patients one week after the stroke. n=number of patients or controls, SD=standard deviation.**

Genotype	Controls			Patients		
	mean	SD	n	mean	SD	n
4G/4G	28.3	16.8	35	25.3	17.5	31
4G/5G	25.6	14.6	57	21.5	12.5	58
5G/5G	20.3	12.7	28	22.7	10.4	28

quartile versus the lower three quartiles was 0.74 (95% CI: 0.4–1.4). In patients as well as in controls, the highest PAI-1 levels were associated with the 4G/4G genotype (Table 3).

### Subgroups

We estimated the relative risk of stroke associated with the PAI-1 promoter gene polymorphism in several patient subgroups. There were no statistically significant differences in the distribution of genotypes according to gender, smoking habits, hypertension, diabetes or stroke type. There was no relationship between stroke severity and genotype (data not shown).

In controls, the mean PAI-1 level was higher in subjects with diabetes (38.1 vs. 24.5,  $p < 0.04$ ). In patients, we found no differences in PAI-1 level in those with or without diabetes (24.5 vs. 22.6,  $p > 0.59$ ). Similarly, the mean PAI-1 level was significantly higher in controls with hyperlipidemia (27.3 vs. 20.7,  $p < 0.02$ ) than in those without, whereas in patients with or without hyperlipidemia no differences were found in PAI-1 levels (24.6 vs. 20.1,  $p = 0.08$ ).

## Discussion

### Internal validity

The strength of our study is the prospectively consecutive inclusion of patients by a neurologist. Furthermore, all patients underwent neuro-imaging to rule out hemorrhage. We were able to include population controls, thus avoiding the biases induced by "hospital controls". We collected detailed information about cardiovascular risk factors, medical history and family history from patients, as well as controls. All subjects participating were of

Caucasian race and allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied a representative group.

Since the primary aim of the study was to investigate the influence of a genetic feature, the 4G allele of the PAI-1 polymorphism, on the risk of stroke, confounding by other variables is unlikely. Although the phenotypic expression, i.e. PAI-1 level, may vary, it is unlikely that genotype is affected by confounding variables or by the occurrence of ischemic stroke. As a consequence of its design, our study is limited to nonfatal cases of stroke. If the 4G allele was associated with fatal cases of stroke, we would underestimate the true risk for ischemic stroke associated with the 4G allele.

PAI-1 levels in patients were not increased in comparison with controls. The blood samples in patients and controls were collected according to ECAT procedures (21) and taken under strictly standardized conditions, i.e. while fasting, with no exposure to alcohol or nicotine and after 15 minutes of rest. By so doing, we were able to exclude the confounding influence on the PAI-1 level by smoking, diabetes or physical exercise. Therefore, we believe that the measurements of the PAI-1 level and the lack of difference in PAI-1 level between patients and controls are valid. These results are in agreement with one other case-control study (22). In some other studies that reported on PAI-1 level and stroke, non-fasting blood samples were taken (1, 2, 11). This might explain the higher PAI-1 levels in patients, especially since cardiovascular risk factors that increase the PAI-1 activity like smoking, diabetes and hyperlipidemia were more prevalent in the patient group. Furthermore, in these studies tubes containing citrate were used instead of CTAD-tubes containing platelet stabilizing agents. This might further explain the differences in PAI-1 level. Another explanation for the differences in PAI-1 level might be the different timing of the blood sampling, i.e. during the acute phase or not.

### External validity

A limitation of our study is its size. One could argue that our study is not large enough to exclude an association between ischemic stroke and the PAI-1 4G/5G polymorphism. The OR in our study was 1.0 with a narrow 95% confidence interval of 0.6 to 1.8. Our study indicates that even if the polymorphism is a risk factor, it cannot be a strong one. This observation is in agreement with several other case-control studies that also found no evidence for an increased stroke risk in the presence of the polymorphism (2, 14, 22, 23). Our results are inconsistent with a recent prospective study that described a protective effect against stroke

for the 4G allele in the elderly (11). In that study, the population was not in Hardy-Weinberg equilibrium, which may suggest selection bias. Other studies which reported the 4G allele to be protective for stroke also concerned subgroups of patients, like young women (10) or patients with minor stroke (9). On the other hand, one other case control study concluded that the 4G allele is a significant risk factor in a selected patient group of atherothrombotic stroke (13). Patient selection might partly explain the heterogeneous results of the studies concerning the 4G/5G polymorphism of the PAI-1 gene and stroke.

In our study, the highest PAI-1 levels were associated with 4G allele in patients as well as controls, which is in agreement with the accepted relation between PAI-1 level and the 4G allele (15, 20). Elevated plasma PAI-1 level is a core feature in the insulin resistance syndrome (IRS). This syndrome is defined as a cluster of abnormalities, which includes obesity, glucose intolerance and dyslipidemia and which is strongly related with low grade inflammation (24). In our control population, PAI-1 levels were higher in those with diabetes and hyperlipidemia. An explanation for this might be that these cardiovascular risk factors are better treated in the patient group. However, only a small percentage of the patients suffered from diabetes and there was no difference in the prevalence of hyperlipidemia between patients and controls. This is not enough to explain why the PAI-1 level was not increased in our patients.

The relationship between elevated PAI-1 levels and MI seems to be more straightforward than the relationship between PAI-1 and ischemic stroke. An explanation for this could be the difference in pathophysiological mechanism between MI and ischemic stroke. In MI patients, rupture of atherosclerotic plaques is considered the most important underlying mechanism. Atherosclerotic plaques are associated with elevated PAI-1 levels (25). In stroke patients, small vessel disease and thromboembolism without significant large vessel disease are more common causes of cerebral ischemia than carotid and aortic arch atherosclerosis (17). This may, at least partially, explain the lack of association.

In conclusion, our results indicate that PAI-1 promoter gene polymorphism is not a risk factor for ischemic stroke. Although our results confirm the association between plasma PAI-1 level and genotype, they do not support the hypothesis that increased PAI-1 levels are an important risk factor for ischemic stroke.

Future studies should focus on other promoter gene polymorphisms and their phenotypic expression to further examine to what extent genetically determined coagulation disorders contribute to the occurrence of ischemic stroke.

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