Candidate Genetic Risk Factors of Stroke: Results of a Multilocus Genotyping Assay

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Background: Epidemiological studies indicate that genetic factors play a role in the risk of stroke, particularly in younger individuals, but the role of single-nucleotide polymorphisms (SNPs) is controversial. We investigated the possible association of a number of previously tested SNPs with stroke risk.

Methods: We investigated the prevalence of 60 polymorphisms located in 35 genes in 450 white patients who suffered an acute stroke or transient ischemic attack before the age of 60 years and in 817 healthy control individuals by a multilocus PCR-based assay. The controls were randomly selected from attendees of a health service program. Genetic variations were detected by hybridization to nylon strips (Roche Molecular Systems) containing detection oligonucleotides for the SNPs. We used P values of <0.05 for confirmatory analysis of the SNPs in the genes for APOE (allele 4), angiotensin converting enzyme, factor V, prothrombin, and methylenetetrahydrofolate reductase. To account for multiple testing we defined a P value of <0.001 as statistically significant for all exploratory tests. The genes represented in the test panel by more than 1 SNP were also evaluated by haplotype analysis.

Results: Frequencies of all 60 tested SNPs among patients and controls were very similar. No SNPs reached an odds ratio of 2, and no association with stroke risk was statistically significant.

Conclusions: Our results do not indicate a clinically relevant role of any of the investigated SNPs for stroke risk in individuals hospitalized for ischemic stroke/transient ischemic attack before or at 60 years of age. These results are in accordance with previous metaanalyses showing at most a very modest or no significant effect of these SNPs on stroke risk.

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From epidemiological studies it can be concluded that genetic predisposition plays a role in premature stroke (1, 2). The involvement of several genetic factors reported to play a role has not yet been definitely proven, however, and their contributions to the pathomechanisms of stroke are still poorly understood (3–5). Although a large amount of data is available associating single-nucleotide polymorphisms (SNPs)7 in candidate genes with the risk of ischemic stroke, the results for individual polymorphisms and genes are controversial. For only a few genes metaanalyses provide some evidence for a minor contribution to stroke risk, whereas for the majority of polymorphisms the influence on the odds for stroke are unclear (6).

We performed a case-control study in which patients who suffered an acute ischemic cerebrovascular event [ischemic stroke or transient ischemic attack (TIA)] before the age of 60 years were compared with random controls participating in a health service program who were free of a history of vascular events. We evaluated 60 common variations in 35 genes that have been considered as potential genetic risk factors in vascular diseases.

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7 Nonstandard abbreviations: SNP, single-nucleotide polymorphism; TIA, transient ischemic attack; IQR, interquartile range; OR, odds ratio; CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase.
Materials and Methods

Patients and Controls
The study population included 450 consecutive white patients who suffered an acute ischemic stroke (n = 358; 80%) or TIA (n = 92; 20%) before the age of 60 years [168 female, 282 male; median age 52 years; interquartile range (IQR) 45–57 years] who were documented in the Vienna Stroke Registry (7, 8). Diagnoses were established clinically (9), and all patients underwent cranial computed tomography or magnetic resonance imaging. Patient data were carefully documented according to a standardized protocol (7) that included risk factors, medical history (with particular reference to vascular diseases), laboratory investigations, technical investigations, and stroke etiology and severity as measured by validated scales. Excluded from the study were patients with hemorrhagic stroke or sinus thrombosis; patients with rare causes of stroke, e.g., arterial dissection; and patients from whom no written informed consent could be obtained. Reported medication intake among the study patients included antihypertensive medication in 167/450 (37%), antidiabetic medication in 44/450 (10%), lipid-lowering medication in 47/450 (10%), antiplatelet agent in 82/450 (18%), and oral anticoagulant in 11/450 (2%). Control individuals were 817 randomly selected healthy white individuals from the area of Vienna [393 female; 424 male; median age 44 (IQR 37–53) years], all voluntary participants in a healthcare program of the city of Vienna. All control individuals were free of clinically manifest arterial vascular disease and reported no arterial vascular diseases in 1st degree relatives. Medical history, including vascular risk factors and results of laboratory investigations were documented according to a standardized protocol.

The study complied with the Declaration of Helsinki and was approved by the local ethics committee. All patients and controls gave written informed consent to participate in the study.

Definitions
Arterial hypertension was defined as either history of arterial hypertension, blood pressure values above 140/90 mmHg (in patients measured 1 week after the qualifying event), or intake of antihypertensive medication. Presence of diabetes mellitus was defined by fasting blood glucose concentrations >6.94 mmol/L, history of diabetes, or treatment with antidiabetic medication. Hyperlipidemia was defined as fasting total serum cholesterol >5.18 mmol/L, a history of hyperlipidemia, or intake of lipid-lowering medication.

Genotyping
Citrated blood was collected by venipuncture and frozen at −20°C within 3–5 h after sampling. DNA was extracted using the Puregene® DNA Isolation Kit (Gentra System Inc). The SNPs analyzed in the study are contained on gene strips developed by Roche Molecular Systems. Selection of SNPs contained on the strips by Roche was based mainly on associations with cardiovascular diseases.

The SNPs (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue4) were genotyped by a multilocus PCR-based assay, essentially as described (10). Briefly, DNA was amplified using 2 cocktails of biotinylated primer pairs targeting genomic fragments ranging from 75 to 375 bp in size. Amplified fragments within the PCR product pools were detected colorimetrically after hybridization to sequence-specific oligonucleotide probes immobilized in a linear array on nylon membranes (11). Probe specificities had previously been confirmed by sequencing or restriction length polymorphism or allele-specific PCR analysis (10). All results were evaluated independently by 2 investigators blinded to phenotype. In addition to the 60 sites in Table 1 in the online Data Supplement, 4 very rare SNPs also contained on the gene strips were genotyped but excluded from further analysis: only 3 carriers of APOB arg3500gln and no carriers of CETP asp442gly, IVS14 G(+1)A, or (+3)Tins were observed in our study population.

Statistical Methods
Continuous data are given as median and IQR (range from the 25th to the 75th percentile). Discrete data are given as counts and percentages. We used χ² tests or, if appropriate, exact tests to compare groups of categorical data. Groups of continuous data were compared by the Mann–Whitney U-test or, as appropriate, Kruskal–Wallis tests.

Associations between the investigated polymorphisms and the risk of stroke were analyzed by means of logistic regression models adjusted for age (in tertiles) and sex. Regression diagnostics and overall model-fit were analyzed according to standard procedures (12).

Results of the logistic regression models are presented as odds ratio (OR) and confidence interval (CI). We used 95% CIs and defined P values of <0.05 for confirmatory analysis of the SNPs in the genes for APOE (allele 4 carriers), factor V, prothrombin, methylenetetrahydrofolate reductase (MTHFR), and angiotensin-converting enzyme (ACE IVS16), for which metaanalyses indicated an association with stroke risk (6). For all exploratory tests we calculated 99.9% CIs for the ORs and defined a P value of <0.001 as statistically significant to account for multiple testing. Calculations were performed using SPSS for Windows (version 10.0, SPSS Inc). Statistical analyses were performed by 2 of the authors (M.S. and P.B.).

The study was powered to detect clinically relevant differences assuming an OR of 2 as the targeted effect. To find this effect by the 2-sided χ² test at the level 0.05 (in the small group of genes or SNPs with previous evidence), the sample sizes of our study provide a power of >80% if the prevalence in the control group is as small as 5% (only the prothrombin variant is less frequent, and for ACE IVS16 and MTHFR 677 C>T even the homozygous geno-
types are more frequent). If the prevalence in the control group is 10%, the power to detect an existing OR of 2 is >90% (e.g., MTHFR). To reach a power of 80% for an OR of 1.5 a prevalence of 19% is necessary. For prevalences lower than that the study is not sufficiently powered to detect small effects.

When applying a significance level of 0.001 for testing the remaining 56 SNPs on the strip, a power of 80% at an OR of 2 can be achieved only for a prevalence of 11% or higher (this is the case for the APOE allele 2, the heterozygous state of 45 SNPs, and the homozygous state of 14 SNPs). For a prevalence of 15% the power already exceeds 90%. For SNPs with prevalences <11% in the heterozygous state the study is not sufficiently powered (in total 8 exploratory SNPs).

HAPLOTYPE RECONSTRUCTION
Haplotype frequencies in patients and controls were estimated using the Phase 2.1 software (http://archimedes.well.ox.ac.uk/pise/PHASE-simple.html) (13), which uses a Bayesian approach. Only those haplotypes with estimated frequencies >1% were further evaluated. Deviations from random distribution of haplotypes were calculated by permutation testing within the Phase 2.1 program. Haplotype reconstruction was performed by 1 of the authors (G.E.).

Results
Baseline characteristics of patients and controls are shown in Table 1. Although all patients and controls were ≥60 years old there was a statistically significant age difference. In addition, patients were more often males and had a higher prevalence of vascular risk factors than did controls.

GENOTYPE FREQUENCIES
Most of the investigated polymorphisms were equally frequent in patients and controls, yielding ORs close to 1 (Fig. 1, Table 2 in the online Data Supplement). None of the tested polymorphisms differed between patients and controls with statistical significance <0.001 univariately and adjusted for age and sex. Genotype frequencies in all study participants, stratified by sex, are given in Tables 2 and 3 in the online Data Supplement. Exclusion of the patients with a TIA (n = 92) did not alter the results (data not shown).

Being aware of the considerable difference in age between patients and controls, we performed formal testing for interaction between the age, the target polymorphisms, and the classification as patient or control to account for this potential confounder; no significant effect modification or change of the model fit was observed by log-likelihood ratio tests (all P >0.1), suggesting that the lack of association between these polymorphisms and stroke was not attributable to the younger age of the controls.

HAPLOTYPE FREQUENCIES
Haplotype frequencies of all genes containing at least 2 genotyped polymorphisms were estimated and are given in Table 4 in the online Data Supplement. Because the SEs of all haplotype frequencies were <0.01, these were not indicated separately. We did not find significant differences in overall haplotype frequencies between patients and controls.

Discussion
Epidemiological data suggest an important hereditary component of stroke risk (1, 14). Therefore, the relation between SNPs and ischemic stroke has been examined in many studies. Some of the studies and also some meta-analyses indicate an association between the risk of stroke and certain SNPs (15, 16). No large-scale study, however, has unequivocally identified a role of most of the investigated polymorphisms as a risk factor of stroke (3, 5). In our investigation of 60 SNPs located in 35 genes, we did not find significant associations of specific genotypes or haplotypes with an increased or decreased risk for ischemic cerebrovascular events before the age of 60 years. Many of the polymorphisms included in our study were previously reported to be associated with vascular disease, but in general positive associations were observed in small studies. In the confirmatory analyses of our study none of the 5 polymorphisms reported to be significantly associated with stroke in metaanalyses reached significance at the level 0.05. Notably, the CIs for the association between these 5 SNPs with stroke risk found in our study overlap with those of published metaanalyses (6, 15, 16).

Table 1. Baseline characteristics of patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients, n = 450</th>
<th>Controls, n = 817</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age median, IQR</td>
<td>52 (45–57)</td>
<td>44 (37–53)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female, %</td>
<td>168 (37)</td>
<td>393 (48)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>264 (59)</td>
<td>228 (28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>92 (20)</td>
<td>13 (2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current cigarette smoking, %</td>
<td>241 (54)</td>
<td>230 (28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hyperlipidemia, %</td>
<td>320 (71)</td>
<td>544 (67)</td>
<td>0.098</td>
</tr>
<tr>
<td>Cholesterol [median (IQR)], mmol/L</td>
<td>5.7 (4.9–6.4)</td>
<td>5.6 (4.9–6.3)</td>
<td>0.441</td>
</tr>
<tr>
<td>BMI [median (IQR)]</td>
<td>26.9 (24.2–30.1)</td>
<td>24.6 (22.3–27.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

BMI, body mass index.
Our study was underpowered to reach statistical significance for the genotype distributions found in our populations (sample size calculations for the confirmatory analyses are given in Table 2 in the online Data Supplement). Previous metaanalyses showed ORs well below 2 for these SNPs. Given the limited sample size of our population, we cannot exclude such small effects of these SNPs on stroke risk.

In the exploratory part of our study, applying a significance level of 0.001 gave a power of 80% at an OR of 2 for any SNP with a prevalence of 11% or higher (this is the case for the APOE 2 allele, the heterozygous state of 45
SNPs, and the homozygous state of 14 SNPs). For a prevalence of 15% (44/55 in the heterozygous and 12/55 in the homozygous state) the power exceeded 90%. For SNPs with lower frequencies (8 SNPs in the heterozygous state) the power was lower depending on the exposition rates of the polymorphism in the control group. For sake of completeness, however, we included in the statistical calculation all results obtained in the study. The observed effect sizes were close to 1.0 for all tested SNPs, a result indicating that there were no systematic differences between patients and controls in the genetic background, i.e., through selection bias. Our study was not powered to reach statistical significance for small differences between patients and controls observed in our population, e.g., for the APOC(-482) polymorphism with a prevalence of carriers of the T-allele of 53% vs 51% (OR 1.17) a sample size of 21 308 in each population would be necessary to reach statistical significance at the 0.001 level; for the CETP_405 polymorphism with a prevalence of VV carriers of 5% vs 10% (OR 2.1) a sample size of 946 in each population would be necessary.

Polymorphisms in the gene for 5-lipoxygenase activating protein were recently reported to be associated with stroke risk (17, 18), but because we began our study before this genetic variant was identified, analysis of this SNP is not included.

Three quarters of strokes happen after the age of 60 years. Age is the most important nonmodifiable risk factor of stroke. In patients who suffer a stroke at a younger age than the majority of stroke patients, genetic influences are probably more important than in older patients (2, 19). We therefore analyzed the role of SNPs in individuals younger than 60 years.

A recent metaanalysis of studies of ischemic stroke patients, including studies with older patients, addressed several of the variations that we evaluated: factor V arg506gln (FVL), MTHFR 677C/T, factor II 20210G/A, ACE insertion/deletion (I/D), apolipoprotein E, glycoprotein IIIa leu33pro, endothelial nitric oxide synthase glu298asp, plasminogen activator inhibitor-1 4G/5G, factor II (-323)ins10 (A1/A2), and lipoprotein lipase asn291ser. The authors concluded that the 4 most studied factors (FVL, MTHFR 677 C/T, F 2 20210 G/A, ACE I/D) were significantly associated with stroke (6). The effects were modest, however, with ORs of 1.24 for MTHFR, 1.33 for FVL, 1.44 for prothrombin G20210A, and 1.21 for ACE I/D.

The results obtained in our large sample of patients with ischemic cerebrovascular events before the age of 60 years show that none of the investigated polymorphisms reaches an OR >2.0 or has a strong and clinically relevant impact on stroke risk in this age group. To correct for the testing of multiple genes in one investigation we introduced stringent criteria for statistical significance (P =<0.001), except for those SNPs for which previous metaanalyses indicated a significant association with stroke. Polymorphisms associated with stroke risk with ORs <2.0 or those that contribute to stroke risk only in combination with external risk factors may have been missed. Our study is in accord with previously published data demonstrating at most a very modest effect of several SNPs on the risk of stroke.

Our findings underline the fact that stroke is a polygenic disorder. The effects of gene–gene as well as gene–environment interactions on the predisposition to cerebrovascular disease are still under investigation. Indeed, such interactions have recently been described (20). In our own studies performed on the same patient population, we found an effect of the SCNN1A trp493arg polymorphism on stroke risk in relation to age and sex (21). We also observed that the effect of factor V Leiden as well as the 20210 G>A prothrombin mutation on stroke risk is related to sex and smoking status (22). In the present study we did not evaluate interactions between single genotypes and sex, age, or external factors.

Our control population was randomly included although it was not entirely population based and was self selected. Individuals were recruited in different parts of Vienna and during different times of the year. Through the information obtained regarding history of vascular diseases, we identified and excluded as controls members of families with cerebrovascular disease in 1st degree relatives. Although this selection process might have led to a certain degree of overselection, the genetic similarities between patients and controls indicated that overselection did not occur.

Our study provides no evidence for a strong (i.e., OR >2) effect of the investigated SNPs on stroke risk before 60 years of age in the investigated population. Our study was underpowered to reach significance for smaller effects, however, because they were also reported for some SNPs in previous metaanalyses. The possibility that 1 or more of the investigated polymorphisms influences stroke risk under certain conditions is not precluded, however. Detailed analyses of our data may reveal hitherto undetected gene–environment interactions for 1 or more of these polymorphisms.

In conclusion, our analysis of 60 candidate gene polymorphisms showed no significant association (OR =2.0) with the risk of stroke or TIA in a large population of patients with ischemic cerebrovascular events before the age of 60 years.

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References