Cumulative Effect of Predisposing Genotypes and Their Interaction With Modifiable Factors on the Risk of Ischemic Stroke in Young Adults

Alessandro Pezzini, MD; Mario Grassi, PhD; Elisabetta Del Zotto, MD; Silvana Archetti, PhD; Raffaella Spezi, MD; Veronica Vergani, MD; Deodato Assanelli, MD; Luigi Caimi, MD; Alessandro Padovani, MD, PhD

Background and Purpose—Combinations of multiple predisposing polymorphisms and their interactions with modifiable factors may result in synergistic effects on the risk of ischemic stroke. These mechanisms are more likely to play a relevant role in younger individuals.

Methods—The cumulative effect of the 20210A variant of prothrombin gene, the 1691A variant of factor V gene, the TT677 genotype of the methylenetetrahydrofolate reductase (MTHFR) gene, and the e4-carriership of the apolipoprotein (APOE) gene, as well as their interactions with modifiable predisposing factors, were determined in a series of 163 stroke patients aged younger than 45 years and 158 controls.

Results—Odds ratios (ORs) for stroke were 1.73 (95% confidence interval [CI], 1.20 to 2.51) in subjects with 1 polymorphism and 3.00 (95% CI, 1.43 to 6.30) in those with ≥2. Compared with nonsmokers with none of the studied polymorphisms, ORs for stroke were 1.88 (95% CI, 1.18 to 3.00) and 3.55 (95% CI, 1.43 to 8.98) for nonsmokers with 1 and 2 polymorphisms, respectively, and 3.99 (95% CI, 1.01 to 15.99) and 15.99 (95% CI, 1.01 to 63.3) for smokers. Compared with nonhypertensive subjects bearing no polymorphisms, ORs were 1.91 (95% CI, 1.28 to 2.87) and 3.68 (95% CI, 1.64 to 8.26) for nonhypertensive subjects with 1 and 2 polymorphisms, 3.28 (95% CI, 1.01 to 10.7) and 10.79 (95% CI, 1.01 to 115.4) for hypertensive.

Conclusions—These data suggest a gene–dose effect of the examined prothrombotic and proatherogenic gene variants and a synergistic effect of these polymorphisms and modifiable risk factors in the pathogenesis of cerebral ischemia in young adults. (Stroke. 2005;36:533-539.)

Key Words: genetics ■ risk factors ■ stroke, ischemic

Ischemic stroke is a complex multifactorial disorder whose incidence increases as a function of the number of risk factors, including hypertension, smoking, and diabetes mellitus. However, approximately half of stroke risk is not explained by these predisposing conditions.1 Although classical Mendelian pattern of inheritance can be demonstrated in <1% of cases,2 evidence from twin, family-based,3 and animal studies4 suggests that genetic influences may account for some of this additional risk. Despite such evidence, the search for genes responsible for stroke has yielded inconsistent results to date, with only the gene encoding for phosphodiesterase 4D (PDE4D) recently identified as a putative candidate for cerebral ischemia.5 One possible explanation for such inconsistency is that, like in other complex traits, this inherited susceptibility is supposed to reflect the influence of a wide spectrum of pathogenic alleles playing together in modulating different pathophysiological processes. Single polymorphisms may have a weak effect on the risk of stroke when analyzed individually but their influence may be more pronounced in the presence of a permissive background. Genetic predisposition for cerebral ischemia may result from an additive effect of several genes (a gene–dose effect) or from synergistic co-effects. In addition, an increased risk of stroke may be the result of the interactions of a gene with environmental or behavioral factors. Finally, a single gene may have an age-dependent effect on stroke risk, with a more prominent influence in early-onset disease, because environmental and behavioral factors have not had the time to substantially modify the phenotype.1,6 This implicates that studies investigating the role of the genetic components in the pathogenesis of ischemic stroke might be made more effective by analyzing: (1) the interactive effects of multiple...
candidate polymorphisms; (2) their interactions with conventional predisposing factors; and (3) younger age groups.

Over the past decade, the role of different candidate genes in the pathogenesis of ischemic stroke has been examined in numerous association studies. In particular, the G1691A polymorphism of the factor V gene, the G20210A polymorphism of the prothrombin gene, and the C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene are among the most frequently studied. The evidence provided by these analyses is that despite the overall effect of each single genetic marker alone on disease risk is likely to be modest, it might be stronger in younger individuals.7,8 The same age-dependent association with cerebral ischemia has been suggested for the apolipoprotein E (APOE) polymorphisms.9,10 However, most previously published reports have investigated these markers as isolated gene mutations. To date, no studies have examined the cumulative effect of these prothrombotic and proatherogenic polymorphisms and their potential interactions with modifiable risk factors in young adults with ischemic stroke. On the basis of these observations, we performed such analyses in the setting of a hospital-based case-control study of young individuals.

Subjects and Methods

Cases and Controls

Patients with first-ever ischemic stroke occurring before the age of 45 consecutively admitted to our department between January 1997 and December 2002 were invited to participate in a research program for the evaluation of gene–environment interactions in the development of ischemic cerebrovascular disease. From a series of 172 unrelated subjects, 164 were prospectively considered for participation. Unwillingness to undergo genetic analysis explains the exclusion of 8 cases.

A detailed description of the diagnostic work-up has been presented previously.11 To investigate any potential association between the studied polymorphisms and specific subtypes of infarct, cases were divided into 4 major etiologic categories, according to a classification based on the Trial of ORG 10172 in Acute Stroke Treatment criteria, accommodated and validated for the cause of stroke in the young:12 (1) atherosclerotic vasculopathy; cerebral infarction caused by large-vessel atherosclerotic vasculopathy or small-vessel disease; (2) cerebral artery dissection (CAD); (3) cardiac/transcardiac embolism: also including cases with a cardioembolic source in combination with a proven thrombophilic disorder; and (4) other: cerebral infarction that did not meet the criteria for one of the categories outlined.

One hundred sixty-three subjects from the staff members of our hospital with no known history of vascular disease, matched to the cases by sex and age in 3-year bands, were invited to participate in the study as controls. Both cases and controls were white and were from the same geographic area and social status.

Demographic data and history of hypertension, diabetes mellitus, cigarette smoking, and hypercholesterolemia were assessed in each subject. Hypertension was considered present if systolic blood pressure was >160 mm Hg and diastolic pressure was >95 mm Hg in 2 separate measurements after the acute phase, or if the subject was undergoing treatment with antihypertensive drugs before recruitment. The diagnosis of diabetes mellitus was established according to WHO criteria.13 Cigarette smokers were categorized as current smokers or nonsmokers (the latter included former smokers who had quit smoking for at least 6 months before the study). Hypercholesterolemia was considered present if cholesterol serum levels were >220 mg/dL or if the subject was undergoing treatment with cholesterol-lowering drugs. Body mass index (BMI) was also calculated as a measure of relative weight. BMI was weight divided by height squared (kg/m²). Obesity was considered present when BMI was >30.

The study was designed and performed in observance of the ethical principles established by the local Institutional Guidelines on Clinical Investigation. Written informed consent was provided by all study participants.

Genetic Analyses

Venous blood sampling for biochemical determinations took place in the early morning (before 7:00 AM) after overnight fasting in all subjects. In patients, blood samples were obtained 7 to 10 days after the acute event.

Genomic DNA was isolated in all subjects from −20°C frozen samples of EDTA anticoagulated whole blood using a standard DNA extraction. The G1691A mutation in the factor V gene (factor V Leiden) and the G20210A mutation in the prothrombin gene were determined according to a standardized multiplex polymerase chain reaction (PCR) method.14 The C677T MTHFR genotypes were determined according to the method of Frosts et al.15 The APOE genotypes were determined according to the method of Hixson and Vernier.16

Statistical Analysis

Bivariate mean differences, odds ratios (ORs), and 95% confidence intervals (CIs) were estimated for conventional risk factors and polymorphisms. A binary variable was determined for each polymorphism ("yes" or "no" based on the status of "carrier" or "noncarrier" of the FV 1691A allele, the PT 20210A allele, the TT677 MTHFR genotype, and the APOE e4 allele). An individual genetic score (GS) was calculated based on the number of such genetic markers (from 0 to 4) in each subject. None of the study participants bore all 4 polymorphisms undergoing investigation. The only individual with 3 genetic markers was entered in the subgroup with 2. Therefore, the entire study group was stratified into 3 categories corresponding to a GS of 0, 1, and 2, respectively.

To estimate the effect of the GS on the risk of cerebral ischemia, 2 logistic regression models were fit in which the independent variable GS was considered a continuous variable (trend logistic regression model) or a categorical variable (heterogeneity logistic regression model), respectively. To compare such models and select the most appropriate, difference in chi² goodness-of-fit tests and the Akaike information criterion (Akaike information criterion = −2 × model log-likelihood + 2 × number of model parameters) were computed.17 Data provided support for the trend logistic regression model with respect to the heterogeneity logistic regression model in all 2-model comparisons (data not shown). The 6×2 table approach18 was used to estimate the additive interaction between the GS and conventional risk factors. Nonsmokers carrying no polymorphism (GS = 0) were the reference category and were compared with nonsmokers with 1 polymorphism (GS = 1), nonsmokers with 2 polymorphisms (GS = 2), smokers with no polymorphism, smokers with 1 polymorphism, and smokers with 2 polymorphisms. The same 6×2 table approach was also used to explore the interaction between the GS and hypertension. The modeling strategies included assessment of interaction with adjustment for covariates (sex, age, smoking habit, hypertension, hypercholesterolemia) by logistic regression. Diabetes mellitus was not entered into the multiple regression equations because of the low frequency of this condition in the present series. The Rothman synergy (S) measure was also computed.19 The S index is the ratio of the observed effect with joint exposure divided by the effect predicted for joint exposure assuming additivity of the effects. No additive interaction corresponds to S = 1, whereas S > 1 (S < 1) can be interpreted as measure of relative increase (decrease) in the effect among those exposed to both factors. To test the effect of the procoagulant genotypes on stroke risk, separate analyses were also performed after exclusion of the proatherosclerotic APOE polymorphisms from the GS computation. Because of the low prevalence of subjects carrying 2 or more polymorphisms, additive interactions were estimated by a 4×2 table approach including a GS = 0 for subjects carrying no genetic markers and a GS = 1 for...
apoE a significant increase of stroke risk was associated with the 20210A gene variant between cases and controls. In contrast, the distributions of the FV 1691A gene variant or the PT 20210A gene variant were more often smokers, and had higher levels of cholesterol compared with controls.

Atherosclerotic vasculopathy was the presumed cause of infarct in 38 cases (23.3%), CAD in 22 cases (13.5%), and other causes in the remaining 51 (31.3%) cases.

Genotyping
The genotype distributions among cases and controls are presented in Table 1. Stroke cases more often had hypertension, were more often smokers, and had higher levels of cholesterol compared with controls.

Characteristics of the Study Group
Because DNA could not be amplified in 6 subjects (1 case and 5 controls), data from 163 cases and 158 controls were entered into final analysis. Demographic characteristics and prevalence of selected risk factors of the participants are presented in Table 1. Stroke cases more often had hypertension, were more often smokers, and had higher levels of cholesterol compared with controls.

Table 1 presents the demographic and clinical characteristics of stroke patients and controls.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Stroke Patients (n=163)</th>
<th>Control Subjects (n=158)</th>
<th>Crude OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male)</td>
<td>84 (51.5)</td>
<td>85 (53.8)</td>
<td>1.01</td>
<td>0.65–1.56</td>
</tr>
<tr>
<td>Current smokers</td>
<td>77 (47.2)</td>
<td>40 (25.3)</td>
<td>2.77</td>
<td>1.73–4.44</td>
</tr>
<tr>
<td>Hypertension</td>
<td>27 (16.5)</td>
<td>10 (6.3)</td>
<td>3.05</td>
<td>1.42–6.54</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>5 (3.0)</td>
<td>5 (3.2)</td>
<td>1.00</td>
<td>0.28–3.54</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>46 (28.2)</td>
<td>31 (19.6)</td>
<td>1.68</td>
<td>1.00–2.83</td>
</tr>
<tr>
<td>Obesity</td>
<td>14 (8.8)</td>
<td>10 (6.3)</td>
<td>1.46</td>
<td>0.62–3.40</td>
</tr>
<tr>
<td>Age (y)</td>
<td>35.0±7.5</td>
<td>34.8±6.1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Body Mass Index, Kg/m²</td>
<td>24.0±4.3</td>
<td>23.9±3.6</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Interaction Analysis
Table 4 and Figure summarize the results of the analyses undertaken to find potential interactions between genetic background and conventional risk factors. When compared with the reference category of nonhypertensive individuals carrying none of the studied polymorphisms, the presence of 1 polymorphism resulted in an almost 2-fold increased risk of stroke (OR, 1.88; 95% CI, 1.18 to 3.00), whereas the presence of 2 polymorphisms increased the risk >3-fold (OR, 3.55; 95% CI, 1.40 to 8.98) among nonhypertensives. Current smoking was associated with an almost 4-fold increase in the risk of stroke when in combination with 1 polymorphism (OR, 3.99; 95% CI, 1.18 to 13.3), and with the APOE e4 carrier status (OR, 2.01; 95% CI, 1.28 to 3.27) and with the TT677 MTHFR genotype when compared with the CC genotype (OR, 1.91; 95% CI, 1.28 to 2.87) and with the TT677 MTHFR genotype and the APOE e4 carriers were more, although not significantly, represented in the categories of CAD (7 of 22 [31.8%] versus 27 of 125 [21.6%]), respectively.

A trend toward a significant positive interaction was observed when the GS hypertension effect was analyzed in the same interaction model (Table 4). Compared with the reference category of nonhypertensive individuals carrying none of the studied polymorphisms, the risk of stroke was increased almost 2-fold among nonhypertensive subjects with 1 polymorphism (OR, 1.91; 95% CI, 1.28 to 2.87) and >3-fold among nonhypertensive individuals carrying 1 polymorphism (OR, 3.68; 95% CI, 1.64 to 8.26). The presence of hypertension resulted in a 3-fold increase of risk when combined with 1 polymorphism (OR, 3.28; 95% CI, 1.01 to

Subjects carrying at least 1 genetic marker. Analyses were conducted with the SPSS (version 11.1) software package.
10.7) and in >10-fold increase of risk when combined with 2 polymorphisms (OR, 10.79; 95% CI, 1.01 to 115.4).

A significant GS-smoking interaction was also observed after exclusion of the APOE polymorphisms from the GS computation (OR, 1.72; 95% CI, 0.87 to 3.39, when non-smokers with at least 1 of the 3 genetic markers were compared with nonsmokers carrying none of the studied polymorphisms; OR, 4.56; 95% CI, 1.87 to 11.1, for current smokers with at least 1 of the 3 genetic markers). Again, a trend toward a significant GS effect among subjects with increased arterial blood pressure compared with those without was detected (OR, 1.81; 95% CI, 1.02 to 3.20, when nonhypertensive with at least 1 of the 3 genetic markers were compared with nonhypertensive carrying none of the studied polymorphisms; OR, 2.51; 95% CI, 0.44 to 14.1, for hypertensive with at least 1 of the 3 genetic markers).

**Discussion**

Our results suggest a gene dose effect of the studied polymorphisms on the risk of cerebral ischemia in young adults and a potential interaction of these genetic factors with conventional predisposing conditions, prompting us to hy-
pothesize a synergistic combination on the risk of stroke. In particular, an increased risk of disease was associated with the presence of 1 of the genetic markers and was even more pronounced in subjects with \(H_1\), particularly in the subgroup of individuals who were current smokers or hypertensive.

These findings are consistent with previous studies that indicate the importance of the cumulative effect of several polymorphisms in combination with other cardiovascular risk factors on the occurrence of myocardial infarction in young adults, and as with recent observations from series of ischemic stroke patients not stratified by age, and focus on a relevant biological interaction between modifiable exposures and genotype. In this regard, although an association between the \(APOE\) allele as well as the TT677 \(MTHFR\) genotype and ischemic stroke was observed, we believe that the direct independent effect of these genetic variants on the risk of cerebral ischemia at a young age may be limited, and it may be more prominent in combination with additional factors. This concept of “context dependency” of genetic factors on stroke risk is generally accepted. However, the effect of such interactions has been scarcely investigated in the specific category of young adults so far. Because genetic–environmental interactions might vary throughout life, specific genotype–environment combinations may determine different possible phenotypes at different points in time. Genetic factors might be more expressed at a young age, because a family history of stroke is more frequent, and environmental risk factors are less expressed, and, when present, have acted for a shorter time. Furthermore, the mechanisms leading to stroke at a young age are different than in the elderly. As a consequence, the results of our study can apply to young individuals and may not be generalized to other age groups. Despite this, the recent observations of Szolnoki et al in a series of older stroke patients indirectly support our findings and suggest that the interaction between these genetic variants and modifiable factors may have similar influence on stroke risk in different periods of life. Our findings do not exclude the alternative possibility that single genes, involved in other pathophysiological mechanisms, can make a substantial contribution to predicting the risk of even sporadic cases, or that each of the genetic markers we studied can have a more relevant influence on specific pathogenic subtypes of cerebral ischemia, as opposed to others. Rather, it supports the notion that genetic factors may act cumulatively to determine stroke phenotype.

Some limitations of the present analysis should be pointed out. The relatively small sample size of the study group might increase the likelihood of spurious associations. Because of the large confidence intervals, the results of our interaction analysis may be statistically unstable and require confirmation in further studies involving larger number of subjects. Furthermore, because the group of controls was recruited among hospital employees, we cannot theoretically rule out the possibility of a biased case-control matching, because of population stratification. However, because the frequency of mutations in our subgroup of controls is similar to that found in other groups of healthy individuals from the same geographic area, we believe that such a potential bias did not play a part in our analysis.

In conclusion, our findings provide a further insight on the complex genotype–phenotype relationships involved in stroke pathogenesis and put emphasis on the possibility to

| TABLE 3. Prevalence and Trend Odds Ratios of Genetic Markers Among Cases and Controls |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Stroke patients | Controls        | Trend OR        | 95% CI          |
|                  | (n=163)         | (n=158)         |                 |                 |
| GS with apolipoprotein E polymorphisms |                 |                 |                 |                 |
| 0                | 89 (54.6)       | 113 (71.6)      | 1               |                 |
| 1                | 60 (36.8)       | 37 (23.4)       | 1.73            | 1.20–2.51       |
| 2*               | 14 (8.6)        | 8 (5.0)         | 3.00            | 1.43–6.30       |
| GS without apolipoprotein E polymorphisms |                 |                 |                 |                 |
| 0                | 118 (72.4)      | 128 (81.0)      | 1               |                 |
| 1                | 41 (25.1)       | 29 (18.4)       | 1.63            | 1.01–2.64       |
| 2                | 4 (2.5)         | 1 (0.6)         | 2.66            | 1.02–6.99       |

*Including one subject with GS=3.

Genetic score (GS) for smoking interaction and GS for hypertension interaction based on the trend logistic regression model.
better-identify “high-risk” young individuals for more effective risk factors modification. The observation that a cumulative effect of multiple genotypes and an interaction between specific genetic and environmental agents may contribute to the emergence of a premature cerebral event extends previous findings obtained in series of older patients, suggests the relative effect of multiple genotypes and an interaction between arterial hypertension should be recommended in patients with young age, and might eventually result in new and individualized prognostic and therapeutic measures. In this regard, based on the results of the present study, a more efficient anistomising counseling and more aggressive treatment of arterial hypertension should be recommended in patients with a predisposing genetic background.

Acknowledgments

We gratefully acknowledge Dr Mauro Magoni and Dr Angelo Costa, Clinica Neurologica, Università degli Studi di Brescia, for critical reading of the manuscript, and Michela Cossandi and Selene Romele, III Laboratorio di Analisi, Biotecnologie, Università degli Studi di Brescia, for technical assistance with genotyping. We also express our gratitude to all individuals who participated in the study.

References


Cumulative Effect of Predisposing Genotypes and Their Interaction With Modifiable Factors on the Risk of Ischemic Stroke in Young Adults

Alessandro Pezzini, Mario Grassi, Elisabetta Del Zotto, Silvana Archetti, Raffaella Spezi, Veronica Vergani, Deodato Assanelli, Luigi Caimi and Alessandro Padovani

*Stroke*. 2005;36:533-539; originally published online February 3, 2005; doi: 10.1161/01.STR.0000155741.31499.c2

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/36/3/533

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/