Mutations in the Hemochromatosis Gene (HFE) and Stroke

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Background and Purpose—Increased serum iron is found to be a risk factor for stroke. Carriers of HFE C282Y and H63D mutations have elevated serum iron levels and may have an increased risk for stroke. We studied the association between HFE gene mutations, carotid atherosclerosis, and stroke.

Methods—We compared the frequency of the HFE C282Y and H63D gene mutations in 202 prevalent and incident cases of stroke with that of 2730 controls from a population-based study, the Rotterdam Study. The influence of HFE mutations on the relationship between hypertension, smoking, and stroke was studied by use of a logistic regression model. In the analyses of hypertension, we used noncarriers and nonhypertensives as reference; in the analysis of smoking, we used noncarriers and those who never smoked as the reference group. Furthermore, we studied the mean intima-media thickness of the common carotid artery in relation to hypertension, smoking, and the HFE genotype in subjects without stroke.

Results—The percentage of both C282Y and H63D carriers in cases (43.7%, n=87) did not differ significantly (P=0.09) from that of controls (37.6%, n=986). The odds ratio for stroke for HFE carriers who also suffered from hypertension was 3.0 (95% CI, 1.9 to 4.6), and for HFE carriers who were also smokers, the odds ratio for stroke was 2.6 (95% CI, 1.4 to 5.0). The mean±SD intima-media thickness of the carotid artery was 0.77±0.14 mm for noncarriers without a history of hypertension or smoking compared with 0.81±0.17 mm for HFE carriers who smoked (P<0.004) and 0.84±0.20 mm for HFE carriers who were hypertensive (P<0.001).

Conclusions—Mutations in the HFE gene were not significantly related to stroke or atherosclerosis in the carotid artery. The HFE gene may modify the relationship between smoking and stroke. (Stroke. 2002;33:2363-2366.)

Key Words: atherosclerosis ■ hemochromatosis ■ intima-media thickness ■ stroke

Studies of the role of mutations in the hemochromatosis (HFE) gene and the risk of atherosclerosis and stroke have yielded controversial results.1–4 Two major mutations are known in the HFE gene, the C282Y and H63D mutations. Although these mutations may determine only minor (<5%) variation, they are associated with a significant lifetime increase in levels of serum iron, ferritin, and transferrin saturation.5,6 This elevation may be clinically relevant, particularly in the elderly, because of lifetime accumulation of iron. Rossi et al7 reported that the C282Y mutation does not influence the formation of plaques or the mean intima-media thickness (IMT) of the carotid artery. However, they found that serum ferritin levels were independently associated with the formation of plaques in the carotid artery of female carriers of the C282Y mutation. Mortality from cerebrovascular disease was found to be significantly related to the C282Y mutation in women heterozygous for the C282Y mutation.3 The association was strongest in women with a history of hypertension and/or smoking, which are important risk factors for stroke. Until now, the influence of H63D on the pathogenesis of atherosclerosis and stroke has been given little attention. We studied the association between the C282Y and H63D mutations in the HFE gene in relation to atherosclerosis and stroke in a population-based sample of elderly people ≥55 years of age.

Methods

Study Population
This study was conducted within the Rotterdam Study, an ongoing population-based cohort study for which all inhabitants ≥55 years of age living in a suburb of Rotterdam, the Netherlands, were invited. The rationale and design of the Rotterdam Study have been described elsewhere.8 Baseline data collection was performed between 1990 and 1993. Written informed consent and permission to retrieve information from medical records were obtained from every participant. The study has been approved by the medical ethics committee of the Erasmus Medical Centre. A total of 7983 subjects participated (response rate, 78%) in the study, which includes individuals from the general population and those living in nursing homes. At baseline interview, information on current medication, alcohol intake, and smoking habits was obtained. People who smoked were asked for the age at which they first smoked, for the duration of interval periods...
without smoking, and for the average daily number of cigarettes smoked. For the purpose of this study, only current smokers (n = 761, 26%) and those who never smoked (n = 792, 27%) were considered. Former smokers (n = 1353, 46%) were excluded for 2 reasons: (1) many patients may have quit smoking after the stroke, and (2) in nonaffected individuals, smoking in the past may have irreversible effects on the process of atherosclerosis. By contrasting never smokers to current smokers, we maximized both this contrast and the statistical power. Former smokers were excluded only in the analysis of smoking.

Two blood pressure measurements were taken with a random-zero sphygmomanometer with the subject in sitting position, and the average of these 2 measurements was taken. Hypertension was defined as systolic blood pressure $\geq 160$ mm Hg or diastolic blood pressure $\geq 95$ mm Hg on 2 consecutive measurements or current use of blood pressure–lowering drugs for indication of hypertension.

Assessment of Stroke and Atherosclerosis at the Carotid Artery
During the interview at baseline, a previous stroke was assessed by asking the question, “Did you ever suffer from a stroke diagnosed by a physician?” Medical records of subjects who answered “yes” were checked, and a previous stroke was considered to have occurred if confirmed by medical records.2 Once subjects enter the Rotterdam Study, they are continuously monitored for major events through automated linkage with files from the general practitioners. When an event or death was reported, additional information was obtained by interviewing the general practitioner and by scrutinizing information from hospital discharge records in case of admittance or referral. Information from reports on all possible strokes was reviewed by 2 research physicians and a neurologist (P.J.K.) who classified the stroke as definite, probable, or nonstroke. The stroke was definite if the diagnosis was based on typical clinical symptoms and neuroimaging excluding other diagnoses. The stroke was considered probable when typical clinical symptoms were present but neuroimaging was not performed. For fatal strokes, other causes of death, especially cardiac, should have been excluded. Because a mixture of multiple genetic and environmental factors may determine stroke at late age, the present study focused on early stroke (age at onset $\leq 75$ years). In total, 202 stroke cases (110 stroke cases at baseline, 92 newly identified cases by January 1, 1998) were considered. The controls consisted of a group of 2730 subjects (age $\leq 75$ years) without any history of stroke and selected randomly from the total cohort.

The IMT and presence of atherosclerotic plaques of the common carotid artery were assessed with ultrasound.10 For each subject, the mean IMT (left plus right divided by 2) was taken as the measure of wall thickness of the distal common carotid artery.

**Laboratory Procedures**

Blood samples were collected from all subjects by venepuncture and kept frozen until analysis. Genomic DNA was extracted from frozen buffy coat with the salting out procedure. DNA fragments were amplified by polymerase chain reaction and genotyped by use of oligonucleotide primers as described elsewhere.11

**Statistical Analysis**

Given the small number of patients studied (n = 202), there were only 1 homozygote for the C282Y and 2 homozygotes for the H63D mutation. We therefore pooled the homozygotes and heterozygotes in the analysis. To increase the statistical power of the study, data of incident and prevalent cases were pooled, resulting in a series of 202 patients. Based on this number of patients and the 2730 control subjects, the statistical power was 80% to find a 2-fold increase in frequency of C282Y heterozygosity (percentage carriers in control subjects, 12.2%). For H63D (percentage carriers in control subjects, 26.5%), the power was $>80\%$ to find a 1.5-fold increase in frequency. Earlier, we studied the relation of HFE to iron parameter in a subset of 335 subjects based on genotype (74 wild type, 73 H63D heterozygotes, 71 C282Y heterozygotes, 60 H63D homozygotes, 51 compound heterozygotes, and 6 C282Y homozygotes).

**Table 1. Baseline Characteristics of the Study Population**

<table>
<thead>
<tr>
<th></th>
<th>Stroke Patients (n=202)</th>
<th>Control Subjects (n=2730)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean $\pm$ SD age, y</td>
<td>69.3 $\pm$ 6.5</td>
<td>65.1 $\pm$ 5.41</td>
</tr>
<tr>
<td>Men, n %</td>
<td>114 (56.4)</td>
<td>1323 (48.5)*</td>
</tr>
<tr>
<td>Hypertensives, n (%)</td>
<td>128 (64.0)</td>
<td>1004 (38.2)†</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>62 (57.4)</td>
<td>699 (48.4)*</td>
</tr>
<tr>
<td>Past</td>
<td>94 (30.7)</td>
<td>1259 (46.6)*</td>
</tr>
<tr>
<td>Never</td>
<td>46 (22.8)</td>
<td>746 (27.6)</td>
</tr>
<tr>
<td>Mean $\pm$ SD IMT, mm (SD)</td>
<td>0.873 $\pm$ 0.218</td>
<td>0.771 $\pm$ 0.144</td>
</tr>
<tr>
<td>HFE carriers, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C282Y</td>
<td>28 (14.1)</td>
<td>323 (12.2)</td>
</tr>
<tr>
<td>H63D</td>
<td>62 (31.2)</td>
<td>707 (26.5)</td>
</tr>
<tr>
<td>HFE</td>
<td>87 (43.7)</td>
<td>986 (37.6)</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.005.

Similar to the findings of Whitfield et al.,6 this study showed that the relation of the 2 mutations in the heterozygote state was similar for both mutations (serum iron, 17.1 $\mu$mol/L for C282Y versus 17.3 $\mu$mol/L for H63D; serum ferritin, 189.9 $\mu$g/L for C282Y versus 172.2 $\mu$g/L for H63D; serum transferrin saturation, 29.5% for C282Y versus 28.5% for H63D). The presence of either mutation was significantly associated with serum iron (P<0.001), ferritin (P<0.003), and transferrin saturation (P<0.001). The 2 mutations were pooled, yielding a frequency of 37.6% in control subjects. This further improved the statistical power for our analysis of interaction with smoking and hypertension.

The $\chi^2$ statistic was used to compare categorical variables and the 2-sample $t$ test to study normally distributed and continuous variables. The carrier frequencies for the C282Y and H63D mutations were estimated by counting genes and calculating sample proportions. We used logistic regression methods to estimate the odds ratios (ORs) for stroke, with 95% CIs adjusted for age and sex. Effect modification of the relation between smoking, hypertension, and stroke by HFE was explored by stratifying the data into 4 categories. For hypertension, the first category consisted of non-HFE carriers and nonhypertensives (reference group); the second category was non-HFE carriers with a history of hypertension; the third category was made up of HFE carriers with no history of hypertension; and the last category was HFE carriers with a history of hypertension. For smoking, the first category consisted of noncarriers who never smoked; the second category was noncarriers who smoked; the third category was HFE carriers who never smoked; and the fourth category was made up of carriers who were smokers. Interaction was evaluated according to an additive model and using the synergy index.12,13 which is defined as the ratio of the relative risk of both measurements indicating severe outcome minus 1 divided by the sum of the risk of each exposure minus 2. A synergy index of 1 indicates no synergy. Furthermore, the significance level of the interaction was evaluated by adding a product term of the 2 factors studied in the regression model.

**Results**

Table 1 shows the baseline characteristics of the study population. Mean age of cases (69.3 years) was significantly (P<0.001) different from that of controls (65.1 years). There were significantly more men, hypertensives, and smokers among the cases compared with controls (P<0.05). The percentage of both C282Y and H63D carriers in cases (43.7%, n=87) did not differ significantly (P=0.09) from that of controls (37.6%, n=986).
The effect of HFE on the relation between stroke, hypertension, and smoking is shown in Table 2. Hypertension was significantly associated with stroke in the absence of the HFE mutations (adjusted OR, 2.3; 95% CI, 1.5 to 3.4). By themselves, the mutations in the HFE gene showed only a weak association with stroke (OR, 1.3; 95% CI, 0.8 to 2.2). Patients with hypertension who were also carriers of the HFE mutations showed a significant relationship with stroke (adjusted OR, 3.0; 95% CI, 1.9 to 4.6). The synergy index was 1.25, whereas the probability value for the interaction was 0.36. Neither smoking nor HFE mutations were significantly associated with stroke if the other factor was not present. But in those subjects who smoked and were HFE carriers, a significant relationship was observed (OR, 2.6; 95% CI, 1.4 to 5.0). The synergy index was 2.67, and the probability value for the interaction was 0.18. We obtained similar findings when comparing cases of stroke separately (data not shown).

Table 3 shows the effect of HFE mutations and its interaction with hypertension and smoking on the mean IMT. There was no significant difference in the mean IMT between HFE carriers (0.761 mm; SD, 0.136 mm) and noncarriers (0.774 mm; SD, 0.151 mm). In the absence of smoking or hypertension, the HFE mutations were not significantly associated with IMT. Hypertension was associated with a significant increase in mean IMT ($P=0.001$) in both the presence and absence of the HFE mutations. Among hypertensives, there was a minor difference in the mean IMT between those with and without an HFE mutation (0.005, $P=0.12$). Smoking was also significantly associated with an increased mean IMT in the presence ($P<0.004$) or absence ($P=0.01$) of HFE mutations. In smokers, the difference in the mean IMT between those with and without an HFE mutation was 0.011 ($P=0.06$), suggesting an additive effect of smoking and HFE.

**Discussion**

In our study, the C282Y and H63D mutations were not significantly associated with stroke or carotid atherosclerosis by themselves. The classic risk factors for stroke—hypertension and smoking—were associated with the disease in the absence of HFE mutations. However, the presence of HFE mutations modified this association, particularly the relation between smoking and stroke.

In the study of atherosclerosis of the carotid artery, a major risk factor for stroke, there was no evidence for an effect of HFE on atherosclerosis, nor was there evidence for modification of the relationship between hypertension and stroke. Although HFE was not significantly associated with IMT in the absence of smoking, there was evidence for an additive effect of smoking and HFE.

Our data are based on a mixture of prevalent and incident cases. The use of prevalent patients imposes limitations in that patients may change (smoking) habits after the stroke and risk factors studied may be related to survival. We aimed to overcome this problem here by studying atherosclerosis of the carotid artery, a major risk factor for stroke, as a proxy for early disease pathology. However, because atherosclerosis of the carotid artery and stroke are only partly equivalent, our data remain to be confirmed, preferably in a series of incident cases. In particular, the study of an interaction with smoking needs replication in larger patient series. Although the synergy index was high (2.67), the test for interaction was not statistically significant ($P=0.18$). The a priori statistical power to find an interaction was low on the basis of the 202 patients studied. In an observational study, misclassification is difficult to exclude, but such mishaps are not likely to be related to the genotype of the person and are therefore less of a problem in genetic studies than in studies of environmental factors. The main advantage of our study is its population-based design, which is less susceptible to selection bias. However, a point of concern with regard to the generalizability of our data is that only whites were studied, limiting extrapolation to other ethnic groups. Also, the difference in...
effects of H63D and C282Y found across studies in whites urges caution in the generalization of findings.

Our findings are compatible with those of Roest et al., who found that in female carriers of the C282Y mutation, mortality for cerebrovascular disease was 2.4 times increased. Roest et al found a strong effect modification by hypertension and smoking. In our study, the ORs for stroke in HFE carriers who were also hypertensives was 3.0 (95% CI, 1.9 to 4.6), and for HFE carriers who were also smokers, the OR for stroke was 2.6 (95% CI, 1.4 to 5.0). The evidence for effect modification of hypertension, smoking, and HFE reported by Roest et al was only partially confirmed in our study. The synergy index for hypertension and HFE was modestly increased (1.25), and the probability value for an interaction was not significant ($P=0.36$). For smoking, however, the synergy was 2.67 times increased, and the probability value for interaction was low (0.18) given the sample size, suggesting that the effect of smoking and HFE combined was more than additive based on the multiplication of risks. This observation concerned only the outcome of stroke. When IMT was studied as a risk factor for stroke, the effects of HFE and smoking were additive. The finding of an additive effect of smoking and HFE on IMT suggests that smoking and HFE are both involved in stroke in a common pathway, ie, atherosclerosis. This observation is compatible with the high iron concentration found in human atherosclerotic lesions. Although it is still a matter of controversy in the literature as to whether HFE mutations are associated with coronary heart diseases, experimental studies have shown that iron overload contributes to atherosclerosis. It has also been reported that iron overload increases the risk of cardiovascular diseases. The mechanism through which the HFE gene may modify the risk of stroke is not known. Increased blood iron concentration may lead to an increase in blood viscosity, which may result in thrombosis. We and others have indeed found that carriers of HFE mutations do have significantly increased levels of iron. Although not replicated in all studies, at least in our population, we found that C282Y and H63D were significantly associated with serum iron, ferritin, and transferrin saturation. However, because we have data on serum iron, ferritin, and transferrin in only a very limited subsample of this population, we cannot verify this hypothesis in the statistical analysis.

The modification of the relationship between smoking and HFE in relation to stroke and the additive effect of the 2 factors on atherosclerosis suggest another possible mechanism. Smoking and HFE mutations may both result in increased oxidative stress and thus cause damage to the vessel walls. Adding the oxidative effect of smoking to that of high iron levels in HFE carriers may increase the risk of atherosclerosis according to an additive model. Interestingly, with regard to the risk of stroke, our data suggest that the effect of these 2 risk factors may be more than additive. Further research is needed on the role of HFE mutations in stroke.

Our data suggest that the C282Y and H63D mutations by themselves are not strongly related to stroke or atherosclerosis. In the presence of smoking, these mutations increase the risk of carotid atherosclerosis and stroke in carriers of HFE mutations.

Acknowledgments

This study was supported by the Netherlands Organization for Scientific Research (NWO), the NESTOR Stimulation Program for Geriatric Research in the Netherlands (Ministry of Health and Ministry of Education), and the Municipality of Rotterdam. We thank J. Veerge and W. Luijten for HFE genotyping and Dr H.J.M. Smeets for providing reference samples.

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Stroke. 2002;33:2363-2366
doi: 10.1161/01.STR.0000029779.37078.C7

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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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