5
Genetic factors and stroke

5.1 The ACE-gene polymorphism and risk of stroke

Abstract

Background and objectives - It has been suggested that the D-allele of the ACE-gene may be related to stroke. Limited prospective data exist on this relation. We investigated the relation between the ACE gene polymorphism and stroke and the role of hypertension in this relation in a large population-based cohort among the elderly.
Methods - The study was performed among 5,312 participants of the Rotterdam Study who were free from previous stroke at baseline (1990-1993) and were followed for stroke until January 1, 1999. The relation between ACE genotype and risk of stroke and subtypes of cerebral infarction was assessed using Cox regression. Adjustments were made for age and sex, and additionally for smoking, diabetes mellitus and carotid intima-media thickness.
Results - Overall, the D-allele was not related to the risk of stroke or cerebral infarction. In normotensives the D-allele increased the risk of cerebral infarction (RR 2.01 (95% CI 0.96-4.21)), whereas it decreased the risk in hypertensives (RR 0.58 (95% CI 0.34-1.00)).The interaction between hypertension and the D-allele in relation to cerebral infarction was statistically significant ($p<0.005$). Hypertension was less strongly related to cerebral infarction in carriers of the D-allele as compared to non-carriers (RR 1.31 (95% CI 0.70-2.44) and 7.14 (95% CI 2.33-21.81), respectively).
Conclusion - Overall, the D-allele is not related to the risk of stroke or cerebral infarction. Presence of the D-allele may modify the effect of hypertension, or vice versa, in relation to stroke.

INTRODUCTION

Genetic factors are considered to play a role in the etiology of stroke. They may be independently related to the risk of stroke, or by modulating the effect of risk factors such as hypertension.[1,2] The D-allele of the angiotensin converting enzyme (ACE) gene is reported to be related to elevated ACE levels, possibly leading to hypertension and increased risk of vascular disease.[3-9] The D-allele could also directly effect stroke risk, or through other factors e.g. influence on the endothelium.[10] Studies on the relation between ACE polymorphism and stroke thus far have reported conflicting results.[3-9,11-14] Some studies reported a relationship,[3,4,6,9,11] whereas others did not find any relation between the ACE gene polymorphism and stroke.[5,7,8,12-14] The majority of these studies were case control studies. The only prospective cohort study among 14,916 persons found that the D-allele was not related to the risk of stroke or cerebral infarction in normotensives without diabetes.[13] Among the positive studies, some have reported that the D-allele is particularly related to an increased risk of lacunar infarction,[11,14] but this relation has not yet been explored in a prospective cohort. We investigated the existence of a relationship between the ACE gene polymorphism and risk of stroke and subtypes of cerebral infarction in a large cohort of elderly persons. The further aim was to identify possible underlying mechanisms.

METHODS

Population

The study is part of the Rotterdam Study, a population-based cohort study on chronic and disabling diseases in the elderly. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55 years or more were invited. People living in homes for the elderly were included. Participation rate of those invited for the study was 78% and in total 7,983 subjects participated.[15] The Medical Ethics Committee of Erasmus University Rotterdam approved the study. Written informed consent to retrieve information from treating physicians was obtained from all participants. Baseline measurements were obtained from 1990 to 1993 and consisted of an interview at home and two visits to the research center for physical examination. From the 7,129 subjects who visited the research center,

216 had experienced a previous stroke. Among those without previous stroke, blood samples were taken in 6,846 persons. Missing blood samples were due to logistic reasons. In 1,534 persons no DNA was available or genotyping failed, resulting in a study population of 5,312 persons.

Assessment of stroke

During the baseline interview a previous stroke was assessed by asking "did you ever suffer from a stroke, diagnosed by a physician?". Medical records of subjects who answered 'yes' were checked in order to verify the diagnosis.[16] A history of TIA was also assessed during the baseline interview. A neurologist reviewed all TIAs.[17] Once subjects enter the Rotterdam Study they are continuously monitored for major events through automated linkage of the study database with the files from general practitioners. Information on vital status is obtained at regular intervals from the municipal authorities in Rotterdam. When an event or death has been reported, additional information is obtained by interviewing the general practitioner and scrutinizing information from hospital discharge records in case of admittance or referral. A neurologist (P.J.K.) reviewed information on all possible strokes. A stroke was classified as definite if the diagnosis was based on typical clinical symptoms and neuro-imaging excluded other diagnoses. A stroke was considered probable in case typical clinical symptoms were present but neuro-imaging was not performed. For fatal strokes, other causes of death, especially cardiac should have been excluded. A stroke was classified as possible if clinical symptoms were less typical and neuro-imaging was not performed, or if a cardiac cause of death could not be excluded in case of a fatal stroke. We only used definite and probable strokes in the analyses. Subclassification in hemorrhagic or ischemic stroke was based on neuro-imaging, which was available for 67.5% of all cases. Cerebral infarctions were considered lacunar if consciousness and higher cerebral function were maintained in the setting of one of the typical lacunar syndromes. CT or MRI usually showed a small (<1.5 cm) infarction in the territories supplied by the perforating branches of major cerebral arteries. For the present study, follow up for stroke was complete for all participants until January 1, 1999.

Assessment of cardiovascular risk factors

At baseline, information on current health status, medication use and medical history was obtained using a computerized questionnaire. Participants were

classified as current, former or never smokers. Sitting blood pressure was measured twice on the right arm with a random-zero sphygmomanometer. We used the average of the two measurements in the analyses. Hypertension was defined as a systolic blood pressure of 140 mm Hg or over, or a diastolic blood pressure of 90 mmHg or over, or current use of antihypertensive drugs for the indication of hypertension. The pulse pressure was calculated as the difference between systolic and diastolic blood pressure. Diabetes mellitus was defined as use of oral blood glucose lowering drugs or insulin or random or post-load serum glucose level higher than 11.0 mmol/L. Participants underwent B-mode ultrasonography of both carotid arteries during the baseline visit to the research center. We measured intima-media thickness of the common carotid artery according to a standard protocol.[18]

Assessment of ACE-gene polymorphism

Peripheral venous blood samples were drawn using standard techniques and genomic DNA was isolated from whole blood. Genotypes for the dinucleotide polymorphism in the ACE gene were identified on the basis of multiplex polymerase chain reaction (PCR) amplification of the respective fragments of intron 16 of the ACE gene according to Lindpaintner et al[19] followed by visualization by electrophoresis. Two independent investigators scored the PCR results. Because in heterozygous status D allele is preferentially amplified, each sample that was found to be DD genotype was subjected to a second PCR amplification with a primer pair that recognizes an insertion specific sequence. No intra-individual variability was found on repeated readings from the same gel.

Data analysis

First, we tested whether the cohort was in Hardy Weinberg equilibrium regarding ACE genotype using analysis of covariance. We used Cox regression to examine the overall relation between ACE polymorphism and risk of stroke and subtypes of cerebral infarction. The ACE polymorphism was analyzed as an additive (DD vs. II and ID vs. II) and as a dominant (DD and ID vs. II) model of inheritance, respectively. Analyses were adjusted for age and sex and additionally for hypertension, smoking, diabetes and carotid intima-media thickness. We performed analyses in strata of hypertension for two reasons. The first reason was to explore any effect modification by hypertension. Secondly, it

allowed us to investigate the relation in a low risk group, namely normotensives. We further investigated whether there was an interaction between hypertension and presence of the D-allele in relation to stroke. To minimize the effect that antihypertensive medication e.g. ACE inhibitors may have on the relationship, we excluded persons who used antihypertensive medication at baseline in the following analyses. All anti-hypertensive medication was excluded since no distinction was made between type of anti-hypertensive drug at baseline. In order to investigate the effects of hypertension and the D-allele separately, we split up the cohort in groups according to presence of hypertension (yes/no) and D-allele (yes/no). We analyzed the risk of stroke and cerebral infarction in the subgroups, taking persons without hypertension and D-allele as reference group. Finally we assessed the relation between hypertension and risk of stroke in strata of the D-allele (present/absent), adjusting for age and sex.

RESULTS

The mean follow-up was 6.1 years and a total of 268 strokes occurred. Subtyping revealed 138 cerebral infarctions, 39 intracerebral hemorrhages and 91 unspecified strokes. The cerebral infarctions were non-lacunar in 102 and lacunar in 36 cases.

Table 1

Baseline characteristics of the study population.

	Cohort (n=5312)
Age	69.3 (9.2)
Sex (% female)	61.7%
SBP (mm Hg)	139.1 (22.4)
DBP (mm Hg)	73.7 (11.6)
Diabetes	9.9%
Smoking (% current)	21.9%
Common carotid IMT (mm)	0.80 (0.16)

SBP: Systolic blood pressure, DBP: Diastolic blood pressure, IMT: Intima-media thickness. Values represent means (SD).

Table 1 shows baseline characteristics of the cohort. The genotype frequencies in the cohort were in Hardy-Weinberg equilibrium ($p>0.05$) (table 2). Overall, the D-allele was not related to the risk of stroke or subtypes of cerebral infarction (table 3).

Table 2

Frequencies of the ACE D/I polymorphism.

Genotype						
Outcome,	Stroke		Cerebral infarction		Cohort	
P value for HWE	P=0.65		P=0.49		P=0.87	
	number	frequency	number	frequency	number	frequency
DD	75	0.28	34	0.25	1484	0.28
ID	137	0.51	73	0.53	2653	0.50
II	56	0.21	31	0.22	1175	0.22
All	268	1.00	138	1.00	5312	1.00

HWE: Hardy Weinberg Equilibrium.

Adjustment for cardiovascular risk factors including hypertension did not change the results. When we stratified for hypertension, presence of the D-allele was also not related to the risk of stroke in normotensives. However, presence of the D-allele in normotensives nearly significantly doubled risk of cerebral infarction and significantly more than doubled the risk of non-lacunar infarction, as compared to II individuals (RR 2.01 (95% CI 0.96-4.21) and 2.51 (95% CI 1.00-6.34), respectively) (table 3). The risk estimates remained largely similar, but lost statistical significance with adjustment for carotid intima-media thickness (RR 2.17 (95% CI 0.93-5.08) and 2.60 (95% CI 0.93-7.30), respectively). We found no relation between the D-allele and risk of lacunar infarction in normotensives. In hypertensive persons, presence of the D-allele seemed to have a protective effect on the risk of cerebral infarction (RR 0.57 (95% CI 0.34-0.96)) and in particular on non-lacunar infarction (RR 0.50 (95% CI 0.27-0.92)). When we tested for interaction between hypertension and presence of the D-allele, the interaction term was significant for cerebral infarction ($P<0.005$) and non-lacunar infarction ($P<0.005$), but not for stroke ($P=0.08$).

Table 5 shows that the effect of having both hypertension and the D-allele did not increase the risk more, as compared to only having hypertension. The risks did not change after adjustment for diabetes, smoking and carotid IMT.

Table 3

Relative risk of stroke and cerebral infarction in relation to ACE polymorphism, adjusted for age and sex.

ACE polymorphism	No. at risk	Stroke		Cerebral infarction		Non-lacunar infarction		Lacunar infarction	
		Cases	RR (95% CI)	Cases	RR (95% CI)	Cases	RR (95% CI)	Cases	RR (95% CI)
II (reference)	1175	56	1.00	31	1.00	23	1.00	8	1.00
Additive model									
ID	2653	137	1.02 (0.75-1.39)	73	1.00 (0.66-1.52)	55	1.01 (0.62-1.64)	18	0.97 (0.42-2.24)
DD	1484	75	0.98 (0.70-1.39)	34	0.84 (0.52-1.37)	24	0.79 (0.45-1.41)	10	0.97 (0.38-2.76)
Dominant model									
ID/DD	4137	212	1.01 (0.75-1.35)	107	0.94 (0.63-1.40)	79	0.93 (0.58-1.48)	28	0.97 (0.44-2.13)

Table 4

Relative risk of stroke and cerebral infarction in relation to ACE polymorphism in strata of hypertension, adjusted for age and sex.

ACE polymorphism	No at risk	Stroke		Cerebral infarction		Non-lacunar infarction		Lacunar infarction	
		Cases	RR (95% CI)	Cases	RR (95% CI)	Cases	RR (95% CI)	Cases	RR (95% CI)
Non-hypertensives									
II (reference)	776	22	1.00	8	1.00	5	1.00	3	1.00
Additive model									
ID	1696	69	1.35 (0.84-2.18)	40	2.20 (1.03-4.69)	32	2.78 (1.08-7.15)	8	1.20 (0.32-4.53)
DD	944	37	1.26 (0.74-2.14)	17	1.68 (0.72-3.87)	13	2.03 (0.72-5.69)	4	1.06 (0.24-4.76)
Dominant model									
ID/DD	2640	106	1.31 (0.83-2.09)	57	2.01 (0.96-4.21)	45	2.51 (1.00-6.34)	12	1.15 (0.32-4.08)
Hypertensives									
II (reference)	349	30	1.00	20	1.00	15	1.00	5	1.00
Additive model									
ID	889	67	0.80 (0.52-1.24)	32	0.59 (0.34-1.04)	22	0.54 (0.28-1.05)	10	0.75 (0.26-2.20)
DD	498	35	0.76 (0.47-1.24)	15	0.52 (0.27-1.02)	9	0.42 (0.18-0.95)	6	0.83 (0.25-2.74)
Dominant model									
ID/DD	1387	102	0.79 (0.53-1.19)	47	0.58 (0.34-1.00)	31	0.50 (0.27-0.92)	16	0.78 (0.29-2.13)

When we analyzed the relation between hypertension and stroke in strata of ACE genotypes we found that hypertension increased the risk of stroke and cerebral infarction in carriers of the D-allele (RR 1.69 (95% CI 1.14-2.52) and 1.31 (0.70-2.44), respectively). The corresponding risks in II individuals were 2.69 (95% 1.12-6.46) and 7.14 (95% CI 2.33-21.81), respectively.

Table 5

Relative risk of stroke and cerebral infarction in relation to presence of hypertension and D-allele, adjusted for age and sex.

	No. at risk	Pulse pressure (SD)	No. at risk	Stroke		Cerebral infarction	
				No. of cases	RR (95% CI)	No. of cases	RR (95% CI)
Ht– D–	682	59.8 (13.8)	682	15	1.00 (reference)	6	1.00 (reference)
Ht+ D–	112	85.6 (5.7)	112	8	2.64 (1.12-6.24)	7	6.67 (2.23-19.96)
Ht– D+	2340	59.5 (6.3)	2340	89	1.60 (0.92-2.76)	48	2.24 (0.96-5.23)
Ht+ D+	422	87.7 (5.9)	422	35	2.72 (1.48-5.00)	13	2.96 (1.12-7.84)

RR: relative risk; Ht+: hypertension present; Ht–: hypertension absent; D+: D-allele present; D–: D-allele absent; Pulse pressure in mm Hg.

DISCUSSION

In our prospective population-based study among 5,312 elderly persons we found that overall, the D-allele was not related to the risk of stroke or cerebral infarction. The D-allele increased the risk of cerebral infarction in normotensives and decreased the risk in hypertensives. We further found a significant interaction between hypertension and presence of the D-allele in relation to cerebral infarction. Before we interpret our results, some methodological issues need to be addressed.

We may have misclassified some strokes or ACE genotypes. We took extra care to avoid misclassification in genotyping. A second PCR has been performed whenever scoring of two investigators were not consistent. Also to avoid mistyping of ID to DD genotype another PCR with an insertion specific primer has been done. However, since classification of stroke was performed blinded to information on ACE genotype and vice versa, misclassification, if any, would have led to an underestimation of the relations that we have found.

The Physician's Health Study reported that there was no relation between the ACE polymorphism and risk of stroke or cerebral infarction.[13] In that study,

348 strokes occurred during 12 years of follow-up. In men without hypertension and diabetes, presence of the D-allele was related to a 1.58-fold (95% CI 0.88-2.82) increased risk of cerebral infarction. In contrast, the present study was based on both men and women and we analyzed subtypes of cerebral infarction. We found no relation between the D-allele and lacunar infarction, which contrasts with studies that did find such a relation. Those studies had a cross-sectional design and were based on limited numbers of cases.[11,14,20] A major pitfall of cross-sectional studies is selection bias, since only stroke survivors are included. This may have distorted the results.

We found a significant relation between the D-allele and non-lacunar infarction in normotensive persons. The majority of non-lacunar infarctions are caused by large artery disease. One possible explanation is that carotid atherosclerosis is the intermediate factor in this relationship, since the D-allele is considered to be related to atherosclerosis.[8] However, the relation remained largely similar after adjustment for carotid IMT, which undermines this explanation. Alternatively, the D-allele may influence the vascular system by inducing hypertension, influencing endothelial function or regulation of smooth muscle cell proliferation.[10,13,21]

In contrast to the normotensives, the D-allele was not related to an increased risk of stroke in hypertensive persons. Several mechanisms may explain this finding. First, hypertensive carriers of the D-allele may be more susceptible for coronary heart disease which may have resulted in a selective non-response in the study, leading to less stroke cases in this group. Second, in hypertensives, the II genotype is possibly related to an increased arterial stiffness. Benetos et al reported a relation between II genotype and arterial stiffness in hypertensive, but not in normotensive persons.[22] Hypertensive persons with the II genotype may be exposed to chronically low levels of angiotensin converting enzyme, which may upregulate the angiotensin II type 1 receptor, or be related to insulin resistence.[22,23] Both upregulation of the angiotensin II receptor and insulin resistance are reported to increase arterial stiffness.[22] The combination of hypertension and increased stiffness in hypertensive persons with the II genotype may explain why they not at lower risk than persons carrying the D-allele. However, this explanation is still controversial and it needs further study, as our data showed no difference in pulse-pressure between the ACE genotypes in strata of hypertension.

In summary, presence of the D-allele overall is not related to an increased risk of stroke. Presence of the D-allele may modify the effect of hypertension or vice versa. Further studies are needed to verify our findings and to investigate whether assessment of ACE genotype is helpful in selecting people who may benefit most from preventive therapy, such as use of ACE inhibitors.

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5.2 Mutations in the hemochromatosis gene (HFE) and stroke

Abstract

Background and objectives - 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 cases (onset before the age of 75 years) of stroke to that of 2,730 controls from a population-based study, the Rotterdam Study. The relationship between *HFE* mutations and stroke and its influence on the relation between hypertension, smoking and stroke were studied using a logistic regression model adjusted for age and sex. We computed the mean intima-media thickness of the common carotid artery and evaluated the effect of hypertension and smoking by *HFE* genotype.
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 ratios for stroke (95% CI) for *HFE* carriers who also suffered from hypertension was 3.0 (1.9-4.6) and for those *HFE* carriers who were also smokers, the odds ratio for stroke was 2.6 (1.4-5.0). The mean (SD) intima-media thickness of the carotid artery was 0.77 (0.14) for non carriers without a history of hypertension or smoking compared to 0.81 (0.17) for *HFE* carriers who were smokers (P<0.004) and 0.84 (0.20) for *HFE* carriers who were hypertensives (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.

Introduction

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, i.e. the C282Y and the H63D mutation. These mutations determine serum iron, ferritin and transferrin saturation.[5,6] Rossi et al, (2000)[7] reported that the C282Y mutation did 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 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.

Up until now, the influence of the 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 aged 55 years and over.

Methods

Study population

This study was conducted within the Rotterdam Study, an ongoing population-based cohort study for which all inhabitants aged 55 years or over, 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 Center. A total of 7,983 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 first smoking, 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, whereas past smokers (n=1,353, 46%) were excluded.

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

Assessment of stroke

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.[9] 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 had been 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 two research physicians and a neurologist (PJK) who classified the stroke as definite, probable or non-stroke. The stroke was definite if the diagnosis was based on typical clinical symptoms and neuro-imaging excluded other diagnoses. The stroke was considered probable in case typical clinical symptoms were present but neuro-imaging was not performed. For fatal strokes, other causes of death, especially cardiac, should have been excluded. Since 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). In total 202 stroke cases (110 prevalent, 92 incident cases) were considered. The controls consisted of a group of 2,730 subjects (aged $\leq$75 years) without any history of stroke and selected randomly from the total cohort.

The intima-media thickness (IMT) and the presence of atherosclerotic plaques of the common carotid artery (CCA) were assessed with ultrasound.[10] For each subject, the mean intima media thickness ((left + right)/2) was taken as

measure of wall thickness of the distal common carotid artery. Both CCAs were evaluated for the presence of atherosclerotic plaques. Both presence of plaques and increased CCA intima-media wall thickness were considered to be indicators of generalized atherosclerosis.

Laboratory procedures

From all the subjects, blood samples were collected by venepuncture and kept frozen until analysis. Genomic DNA was extracted from frozen buffy coat using the salting out procedure. Fragments of DNA were amplified by the Polymerase Chain Reaction (PCR) and genotyped using oligonucleotides primers as described elsewhere.[11]

Statistical Analysis

The chi-square statistic was used to compare categorical variables and the two sample t test to study normally distributed and continuous variables. The carrier frequencies for the C282Y and H63D mutations were estimated by counting gene and calculating sample proportions. As heterozygosity for C282Y and H63D were found to have a similar effect on serum iron levels in our population (submitted for publication elsewhere), we pooled the data on both mutations. We used logistic regression methods to estimate the odds ratios for stroke with 95% confidence interval adjusted for age and sex. Effect modification of the relation between smoking, hypertension and stroke by *HFE* was explored by stratifying the data by absence of both risk factors (reference group), presence of any risk factor or both factors simultaneously. Interaction was evaluated according to an additive model and using the synergy index 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 interaction.

Results

Table 1 shows the baseline characteristics of the study population. Mean age of cases (69.3 years) was significantly ($P<0.001$) different to that of controls (65.1 years). There were significantly more men, hypertensives and smokers among the cases compared to the controls ($P=0.03$). When pooling the two mutations,

among cases, 43.7% were *HFE* carriers compared to 37.6% of controls but this difference was not significant (P=0.09).

Table 1

Baseline characteristics of the study population.

	Stroke (n=202)	Controls (n=2730)
Mean age (years)	69.3 (6.5)	65.1 (5.4)†
Men (%)	114 (56.4)	1323 (48.5)
Hypertensives (%)	128 (64.0)	1004 (38.2)†
Smoking		
Current (%)	62 (57.4)	699 (48.4)
Past (%)	94 (30.7)	1259 (46.6)
Never (%)	46 (22.8)	746 (27.6)
Mean IMT in mm (SD)	0.873 (0.218)	0.771 (0.144)
HFE carriers		
C282Y (%)	28 (14.1)	323 (12.2)
H63D (%)	62 (31.2)	707 (26.5)
HFE (%)	87 (43.7)	986 (37.6)

Values are means (SD) or number (%) based on all available data.
† P<0.05.

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 odds ratio: 2.3, 95% CI 1.5-3.4). By themselves, mutations in the *HFE* gene show only a weak association with stroke (odds ratio: 1.3, 95% CI: 0.8-2.2). Patients with hypertension who were also carriers of *HFE* mutations showed a significant relationship with stroke (adjusted odds ratio: 3.0, 95% CI: 1.9-4.6). The synergy index was 1.25. Neither smoking nor *HFE* mutations were significantly associated with stroke if the other factor was not present. But in those subjects who smoked and who were also *HFE* carriers, there was a significant relationship observed (odds ratio: 2.6, 95% CI: 1.4-5.0). The synergy index was 2.67. We obtained similar findings when comparing men and women and when we analyzed prevalent and incident cases of stroke separately (data not shown).

Table 2

Interaction between HFE C282Y and H63D mutations, hypertension, smoking and stroke.

HFE and hypertension

HFE carrier	Hypertensive	Stroke	Controls	Odds ratios (95% CI)	
				Unadjusted	Adjusted*
No	No	41 (21.0)	968 (39.5)	1.0 Reference	
No	Yes	71 (36.4)	601 (24.5)	2.8 (1.9-4.2)	2.3 (1.5-3.4)†
Yes	No	30 (15.4)	552 (22.5)	1.3 (0.8-2.1)	1.3 (0.8-2.2)
Yes	Yes	53 (27.2)	332 (13.5)	3.8 (2.5-5.8)	3.0 (1.9-4.6)†

HFE and smoking

HFE carrier	Smoker	Cases	Controls	Odds ratios (95% CI)	
				Unadjusted	Adjusted*
No	No	24 (22.9)	422 (31.4)	1.0 Reference	
No	Yes	29 (27.6)	435 (32.4)	1.2 (0.7-2.1)	1.3 (0.7-2.3)
Yes	No	22 (21.0)	261 (19.4)	1.5 (0.8-2.7)	1.3 (0.7-2.4)
Yes	Yes	30 (28.6)	224 (16.7)	2.4 (1.3-4.1)	2.6 (1.4-5.0)†

Values are number of individuals (percentage).

* Adjusted for age and sex

† P<0.05.

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 and non carriers. Hypertension was associated with a significant increase in mean IMT (P=0.001) both in the presence and absence of *HFE* mutations. Smoking was also significantly associated with an increased mean IMT in the presence (P<0.004) or absence (P=0.01) of *HFE* mutations. The association was strongest in mutation carriers. The effect of smoking and *HFE* on IMT was additive.

Table 3

Interaction between HFE C282Y and H63D mutations, hypertension, smoking and intima-media thickness.

HFE and hypertension			
HFE carrier	Hypertensive	Mean intima-media thickness (SD)	P-value
No	No (n=904)	0.761 (0.136)	Reference
No	Yes (n=671)	0.831 (0.163)	<0.001
Yes	No (n=530)	0.774 (0.151)	0.10
Yes	Yes (n=386)	0.836 (0.190)	<0.001
HFE and smoking			
HFE carrier	Smoker	Mean intima-media thickness (SD)	P-value
No	No (n=458)	0.771 (0.143)	Reference
No	Yes (n=419)	0.796 (0.150)	0.01
Yes	No (n=280)	0.784 (0.151)	0.24
Yes	Yes (n=226)	0.807 (0.173)	0.004

DISCUSSION

In our study, the C282Y and H63D mutations were not significantly associated with stroke or carotid atherosclerosis by themselves. However, the presence of *HFE* mutations modified the association between hypertension, smoking and stroke.

Our data are based on a mixture of prevalent and incident cases and remain to be confirmed, preferably using a set of incident cases. The findings on stroke and IMT show an additive effect of smoking and *HFE*, suggesting that *HFE* is involved in atherosclerosis, a major risk factor for stroke. The main advantage of our study is its population-based design.

Our findings are compatible with those of Roest et al, (1999)[3] who found that in women carriers of the C282Y mutation, mortality for cerebrovascular disease was 2.4 times increased. Roest et al, (1999)[3] found a strong effect modification by hypertension and smoking. In our study, the odds ratios for stroke (95% CI) in *HFE* carriers who were also hypertensives was 3.0 (1.9-4.6) and for those *HFE* carriers who were also smokers, the odds ratio for stroke was 2.6 (1.4-5.0). The mean IMT was not different between *HFE* carriers and non

carriers nor was this modified by hypertension or smoking. There have been various hypotheses as to why mutations in the *HFE* gene may be a risk factor for stroke. Among the most powerful ideas is the iron hypothesis which stipulates that iron depletion decreases and iron overload increases the risk of cardiovascular diseases.[12] High iron concentrations have been found in human atherosclerotic lesions[13] and it has been experimentally observed that iron overload contributes to atherogenesis.[14] Increased blood iron concentration may lead to an increase in the viscosity of blood, which may result in thrombosis. We and others have indeed found that carriers of *HFE* mutations do have significantly increased levels of iron.[5,6,15] Since we have data on serum iron, ferritin and transferrin in only a very limited sub-sample 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 opens another possible mechanism. Smoking and *HFE* mutations may both result in increased oxidation and thus cause damage to the vessel walls. Since *HFE* mutations are associated with iron overload, the effect of *HFE* mutation on the risk of stroke might be through excess of iron in carriers of the *HFE* mutations. 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, the effect of these two risk factors may be more than additive, i.e. these risk factors may interact with regard to the outcome of stroke.

In conclusion, 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.

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