

The influence of genetic and cardiovascular risk factors on the CADASIL phenotype

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Summary

The clinical phenotype in cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL), an autosomal dominant cerebral arteriopathy, is variable, but the reasons for this remain uncertain. Possible factors include the mutation site and the influence of additional modulating factors, which could include both epistatic interactions and interactions with cardiovascular risk factors known to cause sporadic small vessel disease. In a large prospectively recruited cohort of CADASIL subjects we determined relationships between phenotype and mutation site, the apoE genotype and cardiovascular risk factors. In addition to clinical features, disease severity was assessed by MRI lesion volume, measured both semiquantitatively (Scheltens scale) and quantitatively. One hundred and twenty-seven CADASIL cases from 65 families with 17 different mutations were studied. Site of mutation was not associated with the presence or age of onset of stroke, migraine, dementia,

dependency or MRI lesion load. There was no evidence of intrafamilial clustering of particular phenotypes. Amongst subjects with stroke/transient ischaemic attack, smoking at the time of the event was independently associated with earlier age of onset ($P = 0.01$). There were no associations between age of onset or presence of stroke and other cardiovascular risk factors, including homocysteine. Homocysteine levels were higher in migraineurs [mean (SD) 12.8 (5.6) versus 9.8 (3.4) $\mu\text{mol/l}$, $P = 0.02$] and elevated homocysteine was independently associated with an earlier age of onset of migraine ($P = 0.01$). No relationship was found between MRI lesion volume and risk factors, or between apoE genotype and phenotype. Our results show no notch 3 genotype–phenotype correlations. This implies that modulating factors influence phenotype. Smoking appears to increase the risk of stroke, while high homocysteine levels are associated with an increased risk of migraine.

Keywords: CADASIL; genotype–phenotype; homocysteine; risk factors; apolipoprotein E

Abbreviations: apoE = apolipoprotein-E; CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy; EGF = epidermal growth factor; MMSE = Mini-Mental State Examination; OR = odds ratio; PCR = polymerase chain reaction; TIA = transient ischaemic attack

Received February 17, 2004. Revised April 28, 2004. Accepted April 28, 2004. Advanced Access publication June 30, 2004

Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) is an autosomal dominant cerebral arteriopathy caused by mutations in the notch 3 gene (Joutel *et al.*, 1996). Characteristic features include migraine, recurrent subcortical strokes and subcortical dementia. Mutations are highly stereotyped and result in the gain or loss of cysteine residues in epidermal growth factor (EGF)-like domains in the extracellular portion of the transmembrane notch 3 protein. Each EGF-like domain contains three paired cysteine–cysteine bonds. Almost all mutations are simple missense mutations resulting in an unpaired number of

cysteine residues. A few splice site mutations (Joutel *et al.*, 2000) and small in-frame deletions (Dichgans *et al.*, 2000) have also been reported, but these also result in an unpaired number of cysteine residues within the EGF-like domain.

Despite the highly stereotyped nature of the mutations causing CADASIL, marked phenotypic variation has been reported (Chabriat *et al.*, 1995; Dichgans *et al.*, 1998). Some individuals present with stroke in their 20s, while others remain stroke free into their 60s. The age of onset of the subcortical dementia that frequently develops is variable. Migraine is a common manifestation, but many patients remain migraine-free throughout

life. A number of other neurological presentations, including epilepsy and an acute reversible encephalopathy (Schon *et al.*, 2003), have been reported, but these affect a minority and early reports suggest they do not seem to segregate within families or with particular mutations. With the exception of a single report suggesting that a novel C455R mutation located in the predicted ligand-binding domain may be associated with an earlier age of stroke onset (Arboleda-Velasquez *et al.*, 2002), previous studies suggest the site of the notch 3 mutation has little bearing on phenotype. However, there have been limited systematic studies examining genotype–phenotype correlations. The lack of clear genotype–phenotype correlations has led to the suggestion that other factors may modulate the disease process. These could include both environmental and other genetic factors, but the role of either has not yet been explored.

CADASIL is characterized by a systemic arteriopathy, which specifically affects the cerebral small vessels. Damage is particularly severe in smooth muscle cells in the media, although endothelial changes have also been reported. The most attractive disease hypothesis is that these diffuse changes result in reduced cerebral perfusion and inability of the cerebral vessels to autoregulate. This is supported by imaging studies showing both reduced resting perfusion and impaired vasodilatory reserve in response to carbon dioxide (Pfefferkorn *et al.*, 2001) or acetazolamide (Chabriat *et al.*, 2000).

The disease process can result in both acute focal lacunar infarction and more diffuse ischaemic changes, referred to radiologically as leucoaraiosis, and best seen as high signal on T2-weighted MRI. Lacunar stroke is thought to arise from an abrupt disruption of perfusion and/or thrombosis in the perforating end arteries. It could be hypothesized, therefore, that any factor either exacerbating the diffuse arteriopathy or resulting in an increased tendency to thrombosis might be associated with an earlier onset of stroke in patients with CADASIL. The pathology underlying leucoaraiosis includes ischaemic demyelination, neuronal loss and gliosis (Ruchoux and Maurage 1997). It affects the white matter regions supplied by the same perforating vessels affected in lacunar stroke. The neuroimaging changes in leucoaraiosis are first seen in regions most distal to the origin of these perforating vessels, i.e. those in which perfusion pressure is lowest. Thus, it is possible that factors resulting either in exacerbation of the underlying arteriopathy or in increased susceptibility to neuronal or other brain tissue injury could increase this diffuse ischaemic damage.

One explanation for the marked variation in phenotype could be that vascular risk factors exacerbate the disease process and thus modulate the CADASIL phenotype. These could include both conventional risk factors, such as hypertension and diabetes, which are recognized risk factors for non-inherited small vessel disease, and novel risk factors, such as homocysteine. Elevated homocysteine was reported in a small study in patients with CADASIL (Flemming *et al.*, 2001), and has been shown to be a risk factor for cerebral small vessel disease, possibly acting via endothelial dysfunction (Hassan *et al.*, 2004). An alternative factor influencing

disease progression, particularly the extent of leucoaraiosis, could be the apolipoprotein-E (apoE) genotype. Presence of the $\epsilon 4$ allele predisposes towards Alzheimer's disease (Strittmatter *et al.*, 1993) and has been reported to influence the extent of cerebral damage following a number of different brain injuries, including traumatic brain injury and subarachnoid haemorrhage (Niskakangas *et al.*, 2001), and in patients with cerebral amyloid angiopathy (Greenberg *et al.*, 1995).

In this study we examined factors affecting the CADASIL phenotype in a large cohort of CADASIL individuals recruited from Great Britain. We determined whether the site of the notch 3 mutation itself, conventional cardiovascular risk factors, homocysteine and the apoE genotype influenced phenotype. We sought to detect influences both on the extent of visible diffuse cerebral injury, represented by the degree of leucoaraiosis, and on acute ischaemic injury, presenting as a stroke or transient ischaemic attack (TIA). We therefore examined the relationship between putative risk factors and both MRI measures of total leucoaraiosis lesion load, with the presence and age of onset of stroke/TIA, as well as other typical phenotypic presentations.

Methods

Subjects

One hundred and twenty-seven CADASIL cases from 65 families were studied. Individuals were recruited prospectively as part of a British CADASIL prevalence study, and all gave informed consent. The study was approved by the South Thames Multi Region Ethics Committee. Diagnosis was confirmed by direct sequencing of the notch 3 gene (123 cases), by skin biopsies showing the presence of granular osmiophilic material in combination with typical neuroimaging appearances (two cases), and by typical neuroimaging appearances in a family with biopsy-proven CADASIL (two cases).

The following phenotypic features were recorded: presence and age of onset of stroke/TIA; presence and age of onset of migraine; the Mini-Mental State Examination (MMSE) score (Folstein *et al.*, 1975); the presence of dementia, defined either as a previous diagnosis of dementia made by a neurologist/psychiatrist, or an MMSE score <23, which has been found to be consistent with DSM-III-R (Diagnostic and Statistical Manual of Mental Disorders) criteria for dementia (Zaudig, 1992); and a history of acute reversible encephalopathy or primary epilepsy. Functional dependence was measured using the modified Rankin scale, a six-point scoring system which has been validated in the assessment of handicap in stroke patients (van Swieten *et al.*, 1988).

Using a standard questionnaire and examination, the following cardiovascular risk factors were assessed: hypertension, hypercholesterolaemia, smoking status, and diabetes mellitus. Hypertension was defined as an age-adjusted systolic blood pressure >140 mmHg, or diastolic blood pressure >90 mmHg, or the prescribed use of anti-hypertensive medication. Hypercholesterolaemia was defined as an age adjusted non-fasting cholesterol >6 mmol/l or the presence of prescribed statin therapy. Smoking was studied in two ways: ever-smokers at the time of recruitment; and current smokers at the first ischaemic event. In a subgroup of 68 individuals, non-fasting serum homocysteine was measured and hyperhomocysteinaemia defined as >15 μ mol/l.

MRI analysis

In 112 individuals in whom original MRI scans were available, lesion load was assessed both by the semiquantitative Scheltens scale, and by quantitative measurement of lesion volume. The Scheltens scale was designed for measuring the load and spatial distribution of subcortical lesions in patients with sporadic vascular white matter disease, and has been shown to have good intra- and inter-observer reliabilities (Scheltens *et al.*, 1993). Predefined regions are scored depending first on size, and then on number of lesions. It was extended to include scores for the anterior temporal lobe, external capsule and corpus callosum, as these regions have all been reported to be frequently affected in CADASIL (Coulthard *et al.*, 2000; Auer *et al.*, 2001; O'Sullivan *et al.*, 2001). All scans were scored on the Scheltens scale by the same consultant neuroradiologist (P.R.).

To derive a quantitative measure of lesion load, hard copies of T2-weighted MRI scans were digitized and converted to a format suitable for analysis using in-house software (© T. R. Barrick, St George's Hospital Medical School, London). White matter lesions, the brain circumference and the lateral ventricles were contoured using commercially available image analysis software Dispunc (© D. L. Plummer, University College London). Brain parenchymal area was calculated as the difference between brain circumferential areas and lateral ventricle areas. Infratentorial structures were not included in the analysis, because of incomplete acquisition on some scans. The most inferior slice for analysis was designated as the first slice to show bilateral anterior temporal lobes as distinct structures. To account for differences in brain size between subjects, total lesion load was expressed as a percentage of brain parenchyma. All scans were analysed by the same person (S. S.). Intra-observer reproducibility was determined on a random sample of 10 scans. The mean of the percentage difference in lesion volume between repeat lesion load estimation by the same observer was 8.74%. To determine within-subjects variation, the mean of the square of SDs of the two scores for each scan was calculated (Bland, 2000). The within-subjects SD, derived from the square root of this value, was 0.65% [95% confidence interval (CI) −1.15% to +2.45%].

Genetic analysis

DNA was extracted from leucocytes using a commercial kit (Nucleon, Amersham Bioscience, Uppsala, Sweden), and polymerase chain reaction (PCR) amplification of notch 3 exons was performed. Primers used have been previously described (Markus *et al.*, 2002). Notch 3 mutations were sequenced directly with an ABI 377 automated sequencer. Analysis of apoE subtypes was undertaken using previously published methods (Hixson and Vernier, 1990). Genomic DNA was amplified by PCR in 10 µl of 75 mmol/l Tris-HCl (pH 9), 0.01% (v/v) Tween, 1.5 mmol/l MgCl₂, 0.25 U Taq polymerase (Abgene, Epsom, UK), 20 mmol/l (NH₄)₂SO₄, 0.1% (w/v) BSA, 0.2 mmol/l each dNTP and each primer at 5 ng/µl. Thirty cycles of PCR were performed at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min with a final extension step of 72°C for 10 min. Products were then digested with 10 U of restriction endonuclease *Hha*I (NEB, Hitchin UK) at 37°C overnight and analysed by 2% low melting point agarose gel electrophoresis (Abgene). Genotype was determined by comparison of the digest pattern to known reference genotypes.

Statistical analysis

All dependent variables were analysed with age as a covariate. In addition, continuous, normally distributed, independent variables were age-adjusted, and these values were used in all analyses and

results. The logarithm of homocysteine level, the reciprocal of cholesterol level and the square root of MRI lesion load were taken to normalize distributions. For statistical analysis of the relationship of phenotype with mutation site, the position of the mutated amino acid in the extracellular portion of the notch 3 receptor was determined. The reciprocal of this was used to obtain a normal distribution. We also compared lesion volume and clinical features between patients with mutations in the predicted ligand binding domain and patients with mutations outside this domain. Patients were categorized as functionally independent or dependent if they had Rankin scores of 0–2 or 3–5 respectively. Univariate logistic regression was used to assess correlations between risk factors and categorical outcomes (e.g. presence of stroke/TIA). Linear regression was used for correlations with continuous dependent variables (MRI scores). Multivariate analyses covarying for gender and vascular risk factors, in addition to age, were carried out when a statistically significant association was found on univariate analysis. To determine the effect of risk factors on the age of onset of clinical outcomes, Kaplan–Meier analysis was used, followed by Cox regression to determine the effect of controlling for other potentially confounding variables.

Results

Clinical characteristics and risk factor profile

Eight cases identified through family screening were asymptomatic. They had a mean age of 31 (range 20–45) years, and four (50%) were female. The 119 symptomatic patients had a mean age of 48 (range 21–82) years, and 72 (61%) were female. At recruitment, stroke/TIA had occurred in 71 symptomatic patients (60%), migraine in 92 (77%), dementia in 22 (19%), epilepsy in 13 (11%), and acute reversible encephalopathy in 15 (13%).

Frequencies of cardiovascular risk factors were as follows: hypertension, 24/120 (20%); hypercholesterolaemia, 53/119 (45%); current and ex-smoker, 67/126 (52%); diabetes mellitus, 5/127 (4%); hyperhomocysteinaemia, 12/68 (9%). Mean (SD) cholesterol ($n = 115$) was 5.6 (1.4) mmol/l, and mean (SD) homocysteine ($n = 68$) was 12.2 (5.3) µmol/l.

Relationship between mutation site and phenotype

The distribution of notch 3 mutations is shown in Table 1. All were simple missense mutations resulting in the loss or gain of a cysteine residue in one of the EGF-like repeats. After covarying for age, site of mutation was not associated with stroke/TIA [odds ratio (OR) (95% CI) 1.118 (0.926–1.350), $P = 0.25$], migraine [OR 0.985 (0.817–1.188), $P = 0.88$], dementia [OR 0.782 (0.602–1.016), $P = 0.07$], acute encephalopathy [OR 0.938 (0.724–1.215), $P = 0.63$], primary epilepsy [OR 1.008 (0.721–1.409), $P = 0.96$] or Rankin dependency [OR 0.811 (0.623–1.057), $P = 0.12$]. There was no evidence of intrafamilial clustering of any particular phenotype, although power to detect this was limited by the small number of affected individuals recruited in most families.

Site of mutation was not associated with quantitative MRI lesion load [B regression coefficient (95%CI) −0.0003

(−0.075–0.074), $P = 0.99$] or with total Scheltens score [B regression coefficient −0.625 (−1.805–0.554), $P = 0.38$]. Figure 1 shows the quantitative lesion load distribution plotted against age, stratified by affected exon.

Three cases, from a single family, had mutations (C440G) in the predicted ligand-binding domain. The individuals were aged 67, 56 and 37 years and presented with dementia at 66 years, migraine at 14 years and depression at 23 years; none had experienced stroke or TIA. There was a non-significant trend to higher age-adjusted lesion volume in cases with this mutation than in cases with other mutations [mean (SD) 9.27 (SD 2.32)%

Table 1 Mutation spectrum for the 64 families and 123 individuals where a mutation was identified

Affected exon	Nucleotide mutation	Amino acid change	No. of families	No. of individuals
3	T304C	C76R	1	1
	C346T	R90C	5	7
	C406T	R110C	1	1
4	C499T	R141C	12	24
	C535T	R153C	5	11
	C583T	R169C	13	25
	C622T	R182C	9	22
	T625C	C183R	1	1
	A644G	Y189C	1	1
	T658A	C194S	2	3
	C697T	R207C	3	4
5	T829C	C251R	2	5
6	C1072T	R332C	5	9
8	T1396G	C440G	1	3
11	C1897T	R607C	1	2
18	G2935T	G953C	1	1
22	C3769T	R1231C	1	3

All amino acid changes involved gain or loss of a cysteine (C) residue.

versus 6.56 (4.45) %, $P = 0.093$]; individual values are indicated in Fig. 1. Age-adjusted Scheltens score was also slightly higher [61.81 (4.38) versus 53.55 (13.39), $P = 0.058$].

Relationship between vascular risk factors and phenotype

Relationship with clinical features

Stroke/TIA. A history of stroke/TIA was positively associated with age [OR 1.077 (1.040–1.116) per year, $P = 0.0003$]. After covarying for age, current smoking was associated with an increased risk of stroke/TIA [OR 2.280 (0.996–5.222), $P = 0.05$]. Following multivariate analysis, covarying for age, gender, hypertension, hypercholesterolaemia and diabetes, the association was no longer significant [OR 1.961 (0.812–4.739), $P = 0.14$]. After covarying for age, there were no associations between stroke/TIA and gender, hypertension, hypercholesterolaemia, diabetes, ever smoking, serum cholesterol or serum homocysteine (Table 2).

Amongst subjects with stroke/TIA, current smoking at the time of the event was associated with earlier onset of event ($P = 0.004$, Fig. 2). Following adjustment for gender, hypertension, hypercholesterolaemia and diabetes, the association with smoking remained ($P = 0.01$). On Cox regression there were no associations between age of onset of stroke/TIA and hypertension [OR 0.822 (0.470–1.439), $P = 0.49$], hypercholesterolaemia [OR 0.787 (0.482–1.285), $P = 0.34$], diabetes [OR 0.847 (0.205–3.507), $P = 0.82$], serum cholesterol [OR 1.015 (0.958–1.076), per 100/mmol/l, $P = 0.61$] or serum homocysteine [OR 1.002 (0.978–1.026), per 100 × logμmol/l, $P = 0.90$].

Migraine. Homocysteine level was associated with age of onset of migraine [OR 1.024 (1.004–1.044), $P = 0.02$]. This remained after controlling for age, gender and all vascular risk

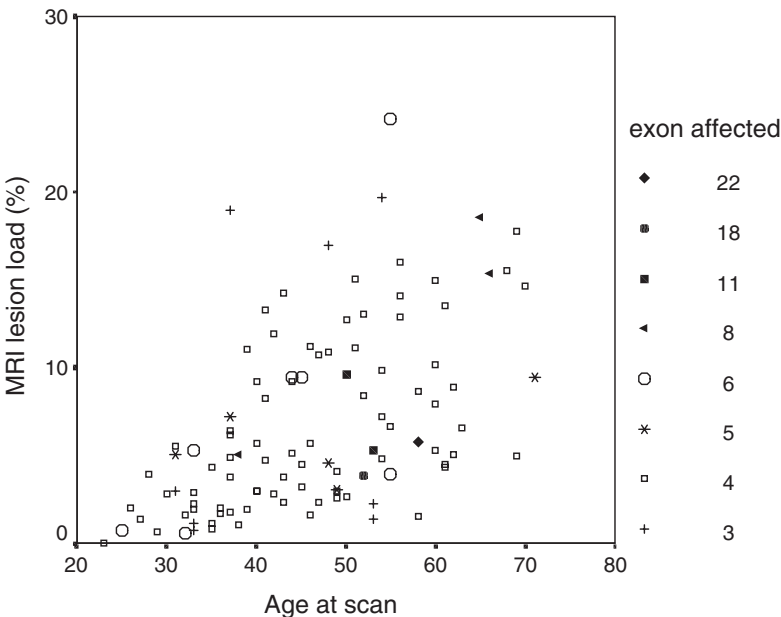
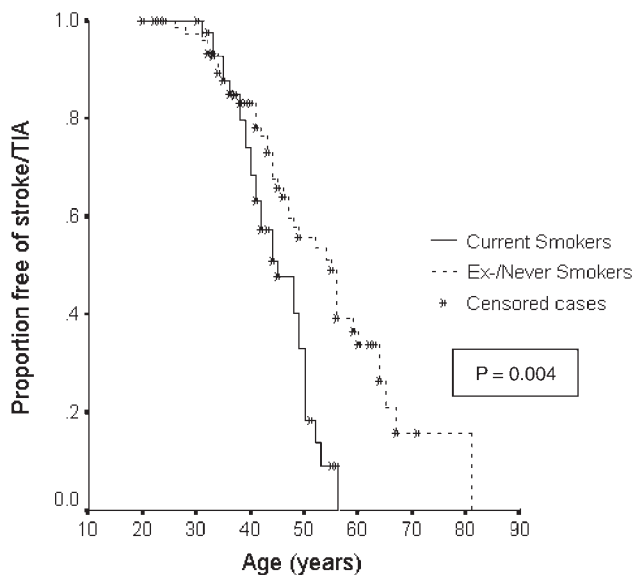


Fig. 1 Quantitative lesion load and age, stratified by affected exon. The three exon 8 mutations are in the ligand-binding domain.

Table 2 Association between vascular risk factors or apoE4 allele and presence of clinical features

	Stroke/TIA	Migraine	Dementia	Rankin dependency
Male gender	1.194 (0.545–2.617)	0.620 (0.282–1.364)	3.163** (1.130–8.854)	1.126 (0.404–3.133)
Hypertension	1.337 (0.467–3.827)	1.687 (0.549–5.182)	0.806 (0.232–2.799)	0.869 (0.246–3.070)
Ever smoking	1.225 (0.566–2.652)	0.939 (0.430–2.054)	1.698 (0.596–4.836)	1.370 (0.479–3.917)
Hypercholesterolaemia	0.603 (0.258–1.410)	1.344 (0.583–3.101)	0.932 (0.341–2.550)	0.694 (0.247–1.946)
Diabetes	0.663 (0.088–4.996)	1.552 (0.167–14.440)	1.703 (0.134–21.696)	1.760 (0.127–24.403)
Serum cholesterol per 100/mmol/l	1.063 (0.959–1.179)	0.970 (0.879–1.071)	0.995 (0.874–1.132)	1.014 (0.889–1.157)
Serum homocysteine per 100 × log µmol/l	1.003 (0.974–1.034)	1.052* (1.008–1.099)	0.999 (0.940–1.061)	1.011 (0.955–1.070)
ApoE4 allele	1.076 (0.362–3.204)	1.071 (0.351–3.263)	1.345 (0.345–5.246)	0.395 (0.068–2.300)

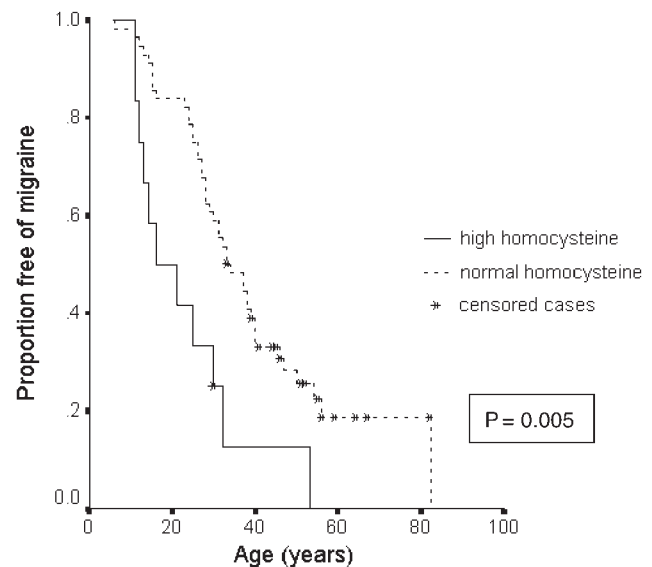
Odds ratios and 95% confidence intervals are given. * $P = 0.02$; ** $P = 0.03$.

**Fig. 2** Kaplan–Meier plot showing the difference in ages of onset of stroke/TIA stratified according to smoking status at the time of stroke/TIA.

factors [OR 1.027 (1.006–1.048), $P = 0.01$]. A Kaplan–Meier plot for migraine age of onset, comparing patients with hyperhomocysteinaemia with normal homocysteine levels, is shown in Fig. 3. Amongst the 68 subjects in whom homocysteine levels were performed, age adjusted levels were higher in migraineurs compared with non-migraineurs [mean (SD) homocysteine 12.8 (5.6) and 9.8 (3.4) µmol/l respectively, $P = 0.02$ (t test on log-transformed data)].

A history of migraine was not associated with age [OR 1.001 (0.970–1.032), $P = 0.98$]. After covarying for age, a history of migraine was associated with homocysteine level [OR 1.052 (1.008–1.099) per 100 × log µmol/l, $P = 0.02$]. After covarying for age, gender and all vascular risk factors, the association was no longer significant [OR 1.047 (0.997–1.099), $P = 0.066$]. A history of migraine was not associated with gender, hypertension, hypercholesterolaemia, diabetes, ever smoking or serum cholesterol (Table 2).

Dementia. A diagnosis of dementia was positively associated with age [OR 1.082 (1.035–1.130), $P = 0.0004$]. After

**Fig. 3** Kaplan–Meier plot showing the difference in ages of onset of migraine between individuals with high (>15 µmol/l) and normal serum homocysteine levels.

covarying for age, there was also a relationship with male gender [OR 3.163 (1.130–8.854), $P = 0.03$]. There were no associations with hypertension, hypercholesterolaemia, diabetes, ever smoking, cholesterol or homocysteine (Table 2).

Disability. Rankin score was associated with age at disability assessment [OR 1.092 (1.044–1.143), $P = 0.0001$]. After covarying for age, Rankin dependency was not associated with gender, hypertension, hypercholesterolaemia, ever smoking, serum cholesterol or homocysteine (Table 2).

Relationship with MRI parameters

Quantitative MRI lesion load positively correlated with age [B regression coefficient 0.049 (0.035–0.062), $P < 0.00001$]. After covarying for age, lesion load was not associated with gender, hypertension, hypercholesterolaemia, diabetes, ever smoking, serum cholesterol or homocysteine (Table 3).

Scheltens score positively correlated with age [B regression coefficient 0.916 (0.709–1.123), $P < 0.00001$]. After covarying for age, there was no association between Scheltens score and

Table 3 Relationships of vascular risk factors and apoE4 allele with MRI lesion load, measured quantitatively and using the semiquantitative Scheltens scale

	Lesion volume	Scheltens score
Male gender	-0.164 (-0.487 to 0.159)	2.694 (-2.349 to 7.737)
Hypertension	0.149 (-0.283 to 0.581)	-0.789 (-7.41 to 5.84)
Ever-smoking	-0.243 (-0.560 to 0.074)	0.857 (-5.17 to 6.88)
Hypercholesterolaemia	-0.027 (-0.307 to 0.362)	0.767 (-4.43 to 5.97)
Diabetes	0.276 (-0.708 to 1.260)	-1.85 (-17.38 to 13.67)
Serum cholesterol per 100/mmol/l	0.001 (-0.41 to 0.43)	-0.301 (-0.946 to 0.344)
Serum homocysteine per 100 × log µmmol/l	-0.006 (-0.019 to 0.007)	-0.116 (-0.291 to 0.059)
ApoE4 allele	-0.308 (-0.740 to 0.128)	-3.037 (-9.638 to 3.563)

B regression coefficients and 95% confidence intervals are shown. No associations were significant.

gender, hypertension, hypercholesterolaemia, ever smoking, serum cholesterol or homocysteine (Table 3).

Relationship between apoE genotype and phenotype

Amongst the 117 individuals in whom successful genotyping was performed, the apoE genotype frequencies were: $\epsilon 2/\epsilon 3$ 7.7%, $\epsilon 2/\epsilon 4$ 1.7%, $\epsilon 3/\epsilon 3$ 76.1%, $\epsilon 3/\epsilon 4$ 13.7%, $\epsilon 4/\epsilon 4$ 0.9%. After covarying for age, there was no association between the presence of at least one $\epsilon 4$ allele and dementia, stroke/TIA or Rankin dependency (Table 2). There was no association between possession of an $\epsilon 4$ allele and quantitative MRI lesion load or Scheltens score (Table 3), and mean MRI scores did not significantly differ for subjects with ($n = 17$) or without ($n = 85$) an $\epsilon 4$ allele (lesion load, 6.14 versus 7.25%, $P = 0.163$; Scheltens score, 52 versus 54, $P = 0.370$).

Discussion

Our results, from a large prospectively recruited series of CADASIL patients from a single country, found no relationship between the mutation site and either clinical phenotype or the extent of white matter damage on MRI. Furthermore, there was no evidence of clustering of particular phenotypes within families. This is consistent with previous smaller studies (Dichgans *et al.*, 1998) and suggests that the marked phenotypic variability seen in the disease is likely to be due to additional modulating factors. These could include both epistatic interactions with other genes and interactions with environmental risk factors. It has recently been suggested, by study of a single family, that mutations in the recombination signal binding protein J Kappa, ligand-binding domain might be associated with more severe disease (Arboleda-Velasquez *et al.*, 2002). These mutations in EGFR10 and 11 are predicted to be required for ligand binding by homology to the *Drosophila melanogaster* notch receptor, and functional studies have reported that such a mutation (C428S) exhibited a significant reduction in Jagged1-induced transcriptional activity of a RBP/JK-responsive luciferase reporter, relative to wild-type notch 3, via loss of jagged binding activity (Joutel *et al.*, 2004) In contrast, other mutations (e.g. R90C and C212S) located in the

mutational hotspot EGF-repeats 2–5 retained the ability to bind Jagged 1 and were associated with apparently normal levels of signalling activity. In our study there were only three patients from one family with a mutation in this domain. There was a trend to increased age-adjusted lesion volume, measured both quantitatively and with the Scheltens scale, but the numbers are too small for firm conclusions. However, such ligand-domain mutations account for only a small minority of CADASIL mutations and could not account for most of the phenotypic variation found in the disease.

Conventional cardiovascular risk factors, such as hypertension and smoking, which are associated with an increased risk of sporadic small vessel disease, could potentially exacerbate the damage to the small cerebral vessels caused by the notch 3 mutation. Such a gene–environment interaction could manifest itself as an increased tendency to acute ischaemia (lacunar stroke) and/or more extensive chronic white matter damage seen as leucoaraiosis. Therefore, we examined associations between risk factors and both clinical phenotype and age of onset of stroke, and the extent of leucoaraiosis on MRI. We used two estimates of MRI lesion volume. First, we used the Scheltens score, a semiquantitative scale specifically designed for evaluation of the extent of white matter ischaemic changes (Scheltens *et al.*, 1993). Secondly, we measured lesion volume from MRI scans using an image analysis programme.

We found no association between conventional risk factors, including hypertension, cholesterol and smoking, and either MRI measure of the extent of leucoaraiosis. In contrast, there was an association between current smoking and age of onset of lacunar stroke, current smokers suffering stroke at an earlier age. This suggests that smoking may predispose to episodes of acute ischaemia, resulting in occlusion of a perforating artery. Potential mechanisms could include induction of a prothrombotic state (Hung *et al.*, 1995; Hioki *et al.*, 2001) or impaired vasomotor function, which has been demonstrated following single cigarette smoking in both animals and man (Silvestrini *et al.*, 1996; Iida *et al.*, 1998). No other risk factors were associated with the presence or age of onset of stroke.

Despite the consistent finding that homocysteine levels are elevated in patients with sporadic stroke (Hankey and Eikelboom, 2001) and small vessel disease in particular (Bertsch *et al.*, 2001; Hassan *et al.*, 2004), we found no

association between homocysteine level and the presence or age of onset of stroke, or the extent of MRI lesion load. In contrast, there was a highly significant association between elevated levels and migraine. As in other CADASIL cohorts, the great majority of cases of migraine were migraine with aura. There are a number of potential mechanisms by which elevated homocysteine could predispose to this symptom. It could act by exacerbating the vascular injury which presumably leads to both migraine and stroke. However, the lack of association with stroke or extent of ischaemic damage would argue against this. Alternatively, there is evidence that homocysteine increases susceptibility to oxidative injury and excitotoxicity, either by activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Lipton *et al.*, 1997), or through DNA damage, p53 activation and subsequent mitochondrial dysfunction (Kruman *et al.*, 2000). All of these mechanisms have been suggested as potential mediators in migraine (Tepper *et al.*, 2001). There is some evidence for an association between migraine and homocysteine in the general population. Higher frequencies of the C677T polymorphism of the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene, which is associated with higher homocysteine levels, have been found in patients with migraine compared with controls, and particularly in patients with aura (Kowa *et al.*, 2000; Kara *et al.*, 2003). Serum homocysteine levels were not, however, measured in these studies, and a recent study failed to find elevated homocysteine levels in migraineurs compared with controls. (Hering-Hanit *et al.*, 2001)

The $\epsilon 4$ allele of the apoE genotype has been found to modulate the extent of brain injury damage in a variety of vascular and non-vascular brain injuries, including Alzheimer's disease (Strittmatter *et al.*, 1993), subarachnoid haemorrhage (Niskakangas *et al.*, 2001) and amyloid angiopathy (Greenberg *et al.*, 1995). We have hypothesized that it might also result in more extensive brain injury in response to chronic ischaemia in CADASIL patients. However, no association was found between the $\epsilon 4$ allele and the extent of leucoaraiosis on MRI, or with any other features of the disease, including dementia.

There was an unexpected relationship between male gender and the presence of dementia. Some reports have shown gender to be a risk factor for Alzheimer's dementia. After controlling for age, women were at greater risk of developing Alzheimer's dementia (Andersen *et al.*, 1999; Molero *et al.*, 2001). Data in vascular dementia have been more varied, but a higher risk has been reported in men (Ruitenberg *et al.*, 2001; Di Carlo *et al.*, 2002). This may reflect the influence of male gender as a risk factor for stroke and therefore for vascular dementia (Meyer *et al.*, 2000). This is unlikely to be the explanation in our population, because stroke/TIA risk was not associated with gender.

In summary, this study confirms the lack of strong genotype-phenotype relationships in CADASIL. It is possible that weaker associations may emerge when larger populations are recruited. It is the first study to examine the effect of potential gene-environment interactions and provides some

evidence that conventional cardiovascular risk factors may play a modest role in modulating the phenotype in patients with CADASIL. These results need confirming in prospective CADASIL populations. However, they may have important implications for the management of patients with the disease. The finding emphasizes the importance of smoking cessation. Homocysteine levels can be lowered with folic acid and B vitamins (van Guldener and Stehouwer, 2001). Whether this will result in a reduction in the frequency of migraine needs to be determined in therapeutic trials.

Although this is one of the largest series of CADASIL patients published to date, our findings need replicating in larger populations. Larger studies will require international collaboration, but similarly sized CADASIL cohorts have now been identified in a number of countries. Such studies will provide further understanding of the reasons for the marked phenotypic heterogeneity found in CADASIL, and may also provide more fundamental information about the molecular and other processes resulting in brain damage in response to chronic white matter ischaemia.

Acknowledgements

We wish to thank Dr Roswell Martin for help with clinical data collection and genotyping, and Dr Yabin Dong and Kelly Gormley for assistance with genotyping. CADASIL case identification was assisted by the British Neurological Surveillance Unit. This work was supported by the Harrison Lectureship from the Atkinson Morley Neuroscience Research Foundation.

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