Hereditary multi-infarct dementia of the Swedish type is a novel disorder different from \textit{NOTCH3} causing CADASIL


Several hereditary small vessel diseases (SVDs) of the brain have been reported in recent years. In 1977, Sourander and Wålinder described hereditary multi-infarct dementia (MID) in a Swedish family. In the same year, Stevens and colleagues reported chronic familial vascular encephalopathy in an English family bearing a similar phenotype. These disorders have invariably been suggested to be cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) but their genetic identities remain unknown. We used molecular, radiological and neuropathological methods to characterize these disorders. Direct DNA sequencing unexpectedly confirmed that affected members of the English family carried the R141C mutation in the \textit{NOTCH3} gene diagnostic of CADASIL. However, we did not detect any pathogenic mutations in the entire 8091 bp reading frame of \textit{NOTCH3} or find clear evidence for \textit{NOTCH3} gene linkage in the Swedish DNA. This was consistent with the lack of hyperintense signals in the anterior temporal pole and external capsule in Swedish subjects upon magnetic resonance imaging. We further found no evidence for granular osmiophilic material in skin biopsy or post-mortem brain samples of affected members in the Swedish family. In addition, there was distinct lack of \textit{NOTCH3} N-terminal fragments in the cerebral microvasculature of the Swedish hereditary MID subjects compared to the intense accumulation in the English family afflicted with CADASIL. Several differences in arteriosclerotic changes in both the grey and white matter were also noted between the disorders. The sclerotic index values, density of collagen IV immunoreactivity in the microvasculature and number of perivascular macrophages were greater in the English CADASIL samples compared to those from the Swedish brains. Multiple approaches suggest that the Swedish family with hereditary MID suspected to be CADASIL has a different novel disorder with dissimilar pathological features and belongs to the growing number of genetically uncharacterized familial SVDs.

\textbf{Keywords}: CADASIL; genetics; multi-infarct dementia; \textit{NOTCH3}; vascular dementia

\textbf{Abbreviations}: CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy; EM = electron microscopy; MID = multi-infarct dementia; SI = sclerotic index; SVD = small vessel disease

Introduction
Hereditary forms of neurovascular disease are estimated to comprise only a small proportion (<10%) of the burden of disease but they can cause extreme morbidity and mortality. To date several autosomal dominant conditions and at least one recessive disorder presenting with ischaemic or haemorrhagic strokes have been described (Grand et al., 1988; Jen et al., 1997; Terwindt et al., 1998; Hassan and Markus, 2000; Kalaria, 2001; Ophoff et al., 2001; Yanagawa et al., 2002; Hagel et al., 2004; Kalimo and Kalaria, 2005; Verreault et al., 2006). Mutations in several genes involved in cardiovascular function or the circulatory system causing systemic pathologies and strokes are known but the molecular genetics of those disorders directly affecting cerebral vessels, whether endothelial or vascular smooth muscle structure and function remain virtually unclear. It would be important to understand how products of distinct genes cause cerebral infarction and white matter changes leading to cerebrovascular and post-stroke dementia syndromes.

Previous descriptions of cerebrovascular disease with familial traits included a report on a Swedish family suffering from recurrent strokes and cognitive dysfunction. Sourander and Wålinder (1977) had described it as hereditary multi-infarct dementia (MID). Soon after Stevens et al. (1977) concisely described chronic familial vascular encephalopathy in an English family. The genetic identities of these disorders have remained unclear. In the intervening years, Tournier-Lasserve et al. (1993) described cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy or cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) in several French families suffering from migraine attacks and recurrent strokes. The clinical phenotype among both French (Chabriat et al., 1995) and German (Dichgans et al., 1998) families indicated CADASIL to be a subcortical small vessel disease (SVD) leading to cognitive impairment and progressive dementia. Mutations in the NOTCH3 gene were linked to CADASIL (Joutel et al., 1996). A subsequent study (Joutel et al., 1997) reported strong clustering and stereotyped nature of NOTCH3 mutations in unrelated CADASIL patients. All mutations are restricted to the EGF repeat domain of the extracellular portion of the NOTCH3 cell receptor and particularly concentrated within exons 3–5. In addition to single missense mutations, novel splice site and in-frame deletion mutations (Joutel et al., 2000b; Dichgans et al., 2001) have also been detected. Thus, far >100 NOTCH3 mutations have been recorded (Kalimo et al., 2002; Kalimo and Kalaria, 2005).

The aim of this study was to evaluate whether hereditary MID of the Swedish type (Sourander and Wålinder, 1977) and chronic familial vascular encephalopathy (Stevens et al., 1977) were genetically and pathologically identical and akin to NOTCH3-linked CADASIL or novel disorders.

Material and methods
Families and subjects
A Swedish family presenting with stroke-like episodes, neuropsychiatric symptoms and progressive dementia was initially diagnosed having hereditary MID (Fig. 1A). The index patient (III.1) first presented in 1956 at the age of 32 years. In addition, seven other affected members (III.2, III.3, IV.1, IV.3, IV.4, IV.5, IV.6 and V.1) underwent clinical examination and evaluation for various diagnostic tests in subsequent years. Six unaffected family members were also interviewed at various times to establish the family tree.
Table 1 Demographic details and clinical characteristics of the Swedish MID and English (CADASIL) families

<table>
<thead>
<tr>
<th>Feature</th>
<th>Swedish family with MID (Sourander and Wålinder, 1977)</th>
<th>English (CADASIL) family (Stevens et al., 1977)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of affected members clinically evaluated*</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Gender</td>
<td>6 M, 3 F</td>
<td>3 M, 1 F</td>
</tr>
<tr>
<td>Mean age at onset (years)</td>
<td>34.6 ± 3.4</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Onset age range (years)</td>
<td>29–38</td>
<td>39–57</td>
</tr>
<tr>
<td>Mean age at death (n)</td>
<td>44.3 ± 4.3 (6)‡</td>
<td>60 ± 7 (3)‡</td>
</tr>
<tr>
<td>Age at death range (years)</td>
<td>29.5–50</td>
<td>52–65</td>
</tr>
<tr>
<td>Duration of disease (years)</td>
<td>0.5–15</td>
<td>10–15</td>
</tr>
<tr>
<td>Range of duration of disease (years)</td>
<td>11 ± 2.1 (6)‡</td>
<td>14.6 ± 4.5 (3)‡</td>
</tr>
<tr>
<td>Vascular risk factors</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>No. of stroke episodes</td>
<td>&gt;4</td>
<td>&gt;6</td>
</tr>
<tr>
<td>Migraine attacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White matter abnormalities</td>
<td>Prominent</td>
<td>Prominent</td>
</tr>
<tr>
<td>Bulbar symptoms</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Behavioural symptoms</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Progressive cognitive impairment</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Numbers show mean (±SD) ages and duration in years. (n) Indicates mean (±SD) for number of subjects who died and were assessed. Symbols indicate: +, presence; —, absence. *For the Swedish family subjects included III.1, III.2, III.3, IV.1, IV.3, IV.4, IV.5, IV.6 and V.1, and for the English family II.1, II.2, III.1 and III.5 (per Fig. 1). ‡Includes the normotensive subject who presented at age 29 years and died six months later after a massive intracerebral haemorrhage involving the thalamus and striatum. §Migraine attacks with aura were not noted but three Swedish subjects reported occurrences of unilateral tension headaches.

and clinical phenotype. The principal clinical findings are summarized in Table 1. The age at onset was taken as the first time the individual presented with a stroke-like episode. Autopsies were performed on six affected family members (III.1, III.2, III.3, IV.1, IV.4 and IV.5). Clinical and neuropathological findings in four of these cases were reported previously by Zhang et al. (1994). For the current study, blood samples were collected from only six affected and unaffected individuals who gave consent.

An English family described with chronic familial vascular encephalopathy was discovered approximately a decade later (Fig. 1B) in 1967. Several members of the family underwent clinical examination over a 15 year period. The family tree was established after interviews with unaffected family members over three generations. Demographic and clinical features of affected members examined from this family were compared with those of the Swedish family (Table 1). Brains from three affected members of this family who came to autopsy (II.1, II.2 and III.1) were assessed in parallel with the Swedish family (Table 1). Informed consent was obtained from all the subjects following the guidelines for ethical research approved by the Ethics Committees of the University of Göteborg, Sweden, and the Bristol and Gloucestershire Hospitals NHS Trusts.

**Neuroimaging**

Computerized axial tomography (CT) or magnetic resonance (MR) imaging was performed in affected individuals at the time of clinical evaluation. MR imaging scans from two members of the Swedish family were further evaluated (OA and attending radiologist at Sahlgrenska Hospital) to establish whether changes were characteristic of CADASIL (O’Sullivan et al., 2001).

**NOTCH3 gene PCR and DNA sequencing**

*NOTCH3* gene sequencing was undertaken first to quickly establish whether the disorders were CADASIL (Joutel et al., 1996). Genomic DNA was extracted from whole blood or brain tissue (in some cases) using standard methods. DNA was amplified by the PCR to screen the entire coding region of *NOTCH3* including exon–intron boundaries and splice sites of the 33 exons (Joutel et al., 1996; Joutel et al., 2000b; Low et al., 2006). Larger exons such as exon 24 and 33 with high GC contents were divided into smaller fragments to facilitate easier screening. Special attention was paid to exons 3, 4, 5, 8, 11, 18 and 19 including the clustering region of *NOTCH3*. In addition, DNA from at least 50 patients suspected with CADASIL was screened in tandem as an internal control to reduce any false-negatives. The *NOTCH3* DNA and translated protein sequences were obtained from the Genbank database, Accession numbers NM_000435 and AH006054. Sequence data were used to design two different sets of primers for each exon. The PCR and sequencing analyses was carried independently at two sites (Newcastle and Turku) to ensure the findings. Details of primer sequences, expected PCR product size and conditions for annealing temperatures and PCR cycles are available upon request (R. N. Kalaria and H. Kalimo, unpublished data).

The Genius Thermal Cycler with HotStar Taq PCR mastermix kit (Qiagen) was used to screen the exons of *NOTCH3* except for exons 1, 24a, 24b, 33b and 33c in which case the GC2 advantage PCR kit (Clontech) was used in view of the high GC rich content within these exonic regions. At each site all findings were confirmed by either repeating the PCR screening at least twice, including DNA samples from affected and unaffected family members or including analysis of DNA samples from CADASIL subjects with known *NOTCH3* mutations. Entire PCR were run on 1.5% agarose–TBE gel. Amplicons of expected size for each PCR were then extracted and purified with the QIAquick gel extraction kit (Qiagen). DNA concentrations of PCR products measured at UV260/280 were adjusted to about 200 ng per 100 bp sequences and sent for direct DNA sequencing (Department of Molecular Biology, Newcastle University and Genetics Department, University of Turku) employing the same forward and reverse primers previously used for PCR. The sequence data were subjected to BLAST search against GenBank database for verification of changes. Initially, the
amyloid precursor protein (APP) gene was also screened in the Swedish DNA for possible mutations essentially as described previously (Heckmann et al., 2004).

**NOTCH3 linkage analysis**

Limited haplotype analysis was undertaken as a confirmatory measure to rule out the possibility that the NOTCH3 gene was associated with the Swedish MID. CADASIL was originally mapped to a 2 cM interval of NOTCH3 bracketed by D19S199 and D19S226 (Joutel et al., 1996). We therefore selected three microsatellite markers, D19S253, D19S923 and D19S841, located immediately downstream, within and upstream of the gene from the genetic and physical map of the locus region (Fig. 2). The microsatellite markers were amplified by PCR carried out in the Genius Thermal Cycler with HotStar Taq PCR Mastermix kit (Qiagen) according to manufacturer’s instructions. Amplified PCR products were directly used for Genescan analysis on the ABI Prism 1800 (ABI Biosystems). The analysis was repeated on three different occasions. Primer sequences for the microsatellite markers were obtained from the ENSEMBL website, and conditions used for PCR (product size in bp, annealing temperature in °C and number of cycles) were as follows: for D19S841 forward and reverse sequences were 5’ TCC TGA GCT CAG GCA AT 3’ and 5’ CCA AGC TTT GTA GAT GTC 3’ (264 bp; 50°C, 45 cycles), for D19S923 they were 5’ CTA GTC ACT TGT CTT GGA TAC 3’ and 5’ CGG CAG TAA GCC AAG ATT GT 3’ (228 bp; 55°C; 45 cycles), and for D19S253 they were 5’ ATA GAC AGA CAG AGC GAC TG 3’ and 5’ GGG AGT GGA GAT TAC CCC 3’ (228 bp; 55°C; 45 cycles). Forward primers for all three sets of markers were tagged with either Hex or Tet, fluorescent marker.

**Autopsy, tissues and neuropathological analyses**

To evaluate the cerebrovascular pathologies of the Swedish patients against those of the English family, we obtained brain tissue from three affected subjects of the Swedish family (III.2, III.3 and IV.1) and three subjects of the English family (II.1, II.2 and III.1). The mean (ages at death of these individuals were 60 (±7) and 44 (±4) years old (Table 1). At autopsy, brains were cut in the coronal plane, photographed and examined for gross morphological changes. While some of the findings in the Swedish subjects were reported previously (Sourandor and Wålinder, 1977; Zhang et al., 1994) we compared them with those in the English subjects to note apparent differences.

Ten micrometre paraffin embedded tissue sections were cut and stained by conventional histopathological and immunocytochemical methods from the frontal, temporal and parietal lobes, hippocampal formation, basal ganglia, cerebellum and the brainstem. For comparison with the vascular cases we also evaluated in parallel brain tissue sections from a 61-year-old CADASIL (R169C) patient, a 78-year-old male patient with Alzheimer’s disease and five age-matched control subjects without any evidence of neurological disease or neuropathological diagnosis. The ages (and gender) of the controls were 43M, 51M, 51F, 56M and 58M years. These specimens were obtained from the Newcastle Brain Tissue Resource centre at the MRC building, Newcastle General Hospital. The length of fixation of the brain tissues from the patients in the two families and controls was between 1 and 5 months.

Tissue sections were stained with haematoxylin and eosin (H&E), Luxol Fast Blue and the periodic acid Schiff methods. Immunocytochemical staining was performed on 10 μm sections essentially as described previously using the Vectastain in ABC System (Kalaria and Hedera, 1995). We compared the vascular pathology of the two disorders with various polyclonal and monoclonal antibodies to microvascular markers. These were to the following antigens (source): α-actin (Dako, UK), medin (courtesy of Dr P. Naslund, Karolinska Institute, Sweden), NOTCH3 N-terminal peptides upstream (N1) and downstream (N2) to EGF domain, NOTCH3 C-terminal peptide downstream to PEST sequence (Low et al., 2006), collagen I (Dako), collagen III (Chemicon, USA), collagen IV (Sigma, UK), collagen VI (Chemicon) and glucose transporter 1 (GLUT1; Chemicon) (Kalaria and Hedera, 1995). Sections were also immunostained with antibodies to CD68 (Dako), the APP (22C11, Boehringer Mannheim, Germany) and ubiquitin (Dako). In addition to these markers, we checked tissue sections for amyloid β and neurofibrillary (tau) pathology. Specificity of the end-terminal NOTCH3 antibodies and the others has been well-established previously. Upon immunoblotting, the NOTCH3 antibodies recognized the expected band(s) of proteins in solubilized preparations of NOTCH3 cDNA transfected SH-SYSY cells (Low et al., 2006).

**Electron microscopy on skin biopsy and post-mortem brain tissue**

Skin biopsy taken from one affected member of the Swedish family (IV.3) was prepared for examination by electron microscopy (EM) as described previously (Miao et al., 2004). For comparison, sural nerve and post-mortem brain tissue fixed in 0.2% glutaraldehyde and 5% formaldehyde from a CADASIL case with confirmed R169C NOTCH3 mutation were also similarly examined. In addition, paraffin embedded tissue blocks (2 × 1 × 3 mm) from a brain sample of an affected Swedish subject (III.3) were processed for EM. The blocks were deparaffinized in xylene, hydrated in water...
and then fixed in osmium tetroxide for 1 h. Subsequent to fixation, they were washed twice in 0.1 M phosphate buffer, pH 7.4, dehydrated, immersed in propylene oxide and embedded in agar 100 epoxy resin for 2 days at 60°C. One micrometre sections were cut and stained with 1% toluidine blue. Ultrathin sections were cut at 500–1000 Å on a diamond knife from the area of interest, transferred to a copper grid, stained with uranyl acetate–lead citrate and viewed in a Philips 201 transmission electron microscope.

**Arteriosclerotic changes, quantitative microvascular pathology and in vitro digital imaging**

To determine the degree of small vessel arteriopathic changes in the Swedish and CADASIL samples, we compared the sclerotic index (SI) of arterial vessels (Lammie et al., 1997) <300 μm in the grey and white matter from the frontal lobe sections of both diseases (Miao et al., 2004). Briefly, the outer and inner diameters of 60–80 arterial vessels in cross-section were measured from multiple digital images of H&E stained 10 μm tissue sections and substituted in the formula (SI = 1 – (internal diameter/outer diameter)) to obtain mean SI for the grey and white matter for three cases each of Swedish MID and CADASIL. Where oblong vascular profiles in cross-section were encountered at least two sets of measures across the longest and shortest diameters were taken and averaged to provide one value. SI values below 0.3 were considered as normal as apparent in control subjects without vascular risk (Miao et al., 2004). From the same tissue sections we also quantified the number of perivascular cells in 40–63 microvessels of >100 μm diameter that showed arteriosclerotic changes. Only cells at the abluminal layer (edge of vessel wall) previously verified to be CD68 positive by comparing with adjacent sections were counted. The mean number of perivascular cells in the grey matter and white matter for each of the Swedish and CADASIL groups were determined.

Tissue sections from Swedish and CADASIL cases and controls immunostained with α-actin, collagen IV, GLUT1, NOTCH3 N1 or C2 immunoreactivity were coded and used for quantitative analysis. Digital images of sections stained with the antibody were acquired through a ×10 Zeiss plan objective using Kohler illumination. Images of 10 randomly selected fields of known area within each brain region of interest (grey or white matter) were captured using a JVC KY-F55B three-chip CCD colour video camera. Image capture analysis (Image Pro Plus package, version 4) was used to measure the percentage immunostained area within each field (microvessels including capillaries) which comprised the entire width of the neocortex or equivalent area of white matter as appropriate. A mean value was determined for at least 10 randomly selected fields within each region of interest per section per subject sample. Quantification of the NOTCH3 N1 or C2 immunoreactivity was determined by simply recording the presence or absence of antibody immunoreaction in a total of 100 arterial vessels within the grey and white matter of each case or control. All of the above analyses were performed blind to the diagnosis.

**Statistical analysis**

Standard statistical methods including non-parametric techniques were appropriate for the data analysis using the SPSS v.13 software (Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed to assess the disease effect and where appropriate using Tukey’s test for multiple post hoc pair-wise comparisons. Differences between the groups were compared using the Mann–Whitney U-test. Mean values of SI, per cent α-actin, collagen IV or GLUT1 immunoreactivities were determined after repeated measures for each case so that a matched analysis could be performed to examine disease differences. Standard deviation (SD) is represented by the ± symbol. Statistical significance was considered at a probability (P) value <0.05.

**Results**

**Clinical features**

Upon screening the DNA from the English family as the first step, we identified the common mutation R141C in exon 4 of NOTCH3 gene in all three affected members. The disorder in the English family with chronic familial vascular encephalopathy (Stevens et al., 1977) was henceforth designated as CADASIL. Comparison of the demographic and clinical features of the Swedish MID subjects with the CADASIL subjects in the English family revealed some differences (Table 1). The mean ages at onset were 35 and 48 years in the two families. The mean ages at death of subjects who came to autopsy were 44 years for the Swedish and 60 years for the English family members. The duration of disease was ~11 and 14 years. The mean age at death in the Swedish subjects was likely skewed by one subject who died 6 months after presentation at age 29 years (Table 1). While recurrent strokes were very common among both families migraine episodes were not apparent as described in CADASIL. Only three affected subjects in the Swedish family reported having mild to severe headaches, which did not appear entirely characteristic of CADASIL. They were unilateral, usually non-pulsating but preceded by light phenomena and relieved with aspirin or paracetamol. Interestingly, depressive illness and behaviour symptoms including mood changes were evident in both the families. There was also evidence of moderate to severe cognitive impairment in affected members of both families.

**Neuroimaging**

To establish if affected members in the Swedish family exhibited similar cerebral changes upon MRI as do CADASIL patients, we examined head MR images of affected members in the Swedish family. Affected members revealed mild cerebral atrophy with white matter hyperintensities in subcortical regions as well as multiple lesions in the basal ganglia and thalamus (Fig. 3). However, upon further scrutiny the images were not entirely consistent with abnormalities characteristic of CADASIL, particularly white matter hyperintensities in the anterior temporal pole (Fig. 3). There were no apparent changes in the external capsule or corpus callosum. Thus, upon MRI there were clear differences in the abnormalities between Swedish MID and typical CADASIL.

**NOTCH3 gene screening in the Swedish family**

A definitive diagnosis of CADASIL was dependent on sequencing the entire 8091 bp of NOTCH3 exonic sequences
rather than limiting to the 23 exons encoding the EGF domains. Extensive and repeated screening of the gene in at least four affected members showed no pathogenic mutations in any of the exons. Sequencing of some PCR amplicons, however, revealed common silent polymorphisms not involving an amino acid change (data not shown). These polymorphisms were found within exons 3, 4 and 16 and were detected in different members of the family as well as other non-related individuals with no specific pattern of change. Therefore, these polymorphisms were a random occurrence. The possibility of false-negative results was reduced by using DNA from other patients suspected to be suffering from CADASIL as additional internal controls. We also ruled out mutations in the APP gene by direct sequencing. We found no apparent sequence variation in any of the four affected compared to all the unaffected siblings or cousins in the fourth generation and two disease free individuals from the third generation.

**NOTCH3 locus is not associated with the disorder affecting the Swedish family**

A full-scale linkage-based analysis could not be undertaken due to availability of limited DNA samples from the family. However, the haplotype analysis with albeit a few samples clearly showed an absence of a shared allele in the D19S923 marker even though there were shared alleles of the two other markers for the three affected members (Fig. 1A). Similarly, no distinctive haplotype could be attributed to only the diseased members of the family as similar haplotypes were also found to occur in the unaffected individuals. The most distinctive clue for non-linkage was from the haplotype of individual IV.6 who is the first cousin of IV.3 and aunt of V.1 where no shared alleles for D19S923 marker were noted between these three affected members. Since the D19S923 marker is located within the NOTCH3 locus itself, it is highly improbable that there would be any independent cross-over event during independent meiosis that did not affect NOTCH3. Taken together, the genetic analysis rejected the possibility of NOTCH3 causing CADASIL as the primary disorder affecting this family.

**Pathological findings**

**EM of skin biopsy and brain tissue**

EM examination of several sections of a skin sample from an affected Swedish family member (IV.3) showed degenerative smooth muscle cells with irregular contours, thickened basement membrane and increased collagen fibres but no evidence for the presence of granular osmiophilic material (GOM) or other granular material in association with the muscle cells (Fig. 4C and D). Similarly, examination of at least 30 vessels in brain tissue from another Swedish subject...
(III.3) revealed no GOM deposits. In contrast, we observed distinct GOM deposits juxtaposed to smooth muscle cells of small and medium sized arteries upon EM of peripheral nerve and brain tissue from a CADASIL case with a known R169C mutation (Fig. 4).

**Gross examination and histopathology**

Cerebral hemispheres of brains from the Swedish family revealed necrotic lesions in the subcortical structures and the pons. The white matter was reduced and there was secondary dilatation of the ventricles. Similar changes with no clear distinction in laterality or size distribution were seen in coronal slices from affected subjects of the English cases with CADASIL. Whereas lacunes, microinfarcts and degrees of perivascular changes in the subcortical structures including the thalamus were evident in both disorders, lesions in the neocortex were rare (data not shown). There was moderate to severe arteriopathy, hyalinization, fibrosis and perivascular necrosis in all the cases of both disorders (Fig. 5). Several vessels in vicinity of infarcts were completely stenosed. Fragmentation of the elastic lamina and perivascular polymorphonuclear leucocytes and macrophages were common in both disorders (Fig. 5A and B). Loss of arterial smooth muscle cells was prominent in the white matter compared to grey in both hereditary MID and the CADASIL cases. We determined the SI values of arterial microvessels (<300 μm) in the three Swedish and CADASIL cases. Mean (±SD) values of SI for the grey and white matter for the Swedish cases were 0.54 (±0.13) and 0.62 (±0.12) \( (P = 0.001, \text{Mann–Whitney } U\text{-test}) \). Similarly, values for the grey and white matter for the CADASIL cases were 0.60 (±0.13) and 0.64 (±0.14) \( (P = 0.09) \). Further analysis indicated significant differences between the Swedish and CADASIL cases in the grey matter (0.54 versus 0.60; \( P = 0.008 \)) but not in the white matter in the grey matter (0.62 versus 0.64; \( P = 0.243 \)). Thus, white matter tended to exhibit a higher degree of sclerosis and the grey matter in the Swedish cases showed significantly less sclerosis compared to that in CADASIL.

**Immunocytochemical findings**

α-Actin immunostaining of small vessels in both disorders showed scattered deposits within vessel walls representing disrupted smooth muscle cells that could not be distinguished readily between the disorders (Fig. 5C and D). The white matter was consistently more affected. This was reflected in the mean per cent α-actin immunoreactivity determined by in vitro digital imaging. Also consistent with the SI values, α-actin immunoreactivity was reduced in both the Swedish MID (0.34%) and CADASIL (0.25%) compared to the controls (0.42%, ANOVA, \( P = 0.04 \)). Immunoreactivity to medin, an arterial muscle cell marker, similarly revealed severe disarray and depletion of the wall cell contents (Fig. 5E and F). There were no apparent qualitative differences in immunostaining for collagen I, III or VI between the two disorders although overall there appeared a greater number of stained vessels in the CADASIL cases. Strong collagen IV immunoreactivity was particularly evident in the medial layers of many vessels (Fig. 5G and H). Quantitative immunocytochemical imaging showed that total collagen IV immunoreactivity in the grey matter (frontal) was significantly increased in CADASIL cases.
compared to the Swedish MID cases and controls. The percent collagen IV immunostaining in CADASIL was 5.26 compared to 3.17 in Swedish and 3.73 in controls (ANOVA, \( P = 0.01 \) and post hoc Tukey’s, \( P = 0.03 \)). These basement membrane changes between the CADASIL and Swedish MID cases were not age (at death) dependent as there were no differences in collagen IV immunoreactivities between the Swedish cases and controls.

Sections stained for the cerebral endothelium with GLUT1 antiserum showed abnormalities in form of ‘blebs’ in the neocortex of Swedish cases but this was not readily evident in the CADASIL cases. Quantitative immunocytochemistry indicated total GLUT1 immunoreactivity to be significantly increased in the Swedish cases compared to controls (2.5% versus 1.3%, \( P = 0.017 \)). This observation was also supported by increased GLUT1: collagen IV ratios (basic elements of the microvasculature) in the Swedish samples (0.79%) compared to controls (0.37%; \( P = 0.04 \)) and to a lesser degree in the CADASIL cases (0.39%; \( P = 0.08 \)). We also undertook quantification of perivascular cells (mostly CD68+ve microglia) in the grey and white matter of the two groups (Fig. 5I and J). While the mean number (±SD) of perivascular cells was not different between the grey and white matter in either the Swedish or CADASIL cases we noted significantly increased perivascular cells in both white (6.4 ± 3.7 versus 4.4 ± 4.1; \( P = 0.001 \)) and grey (7.7 ± 6.3 versus 3.2 ± 3.5; \( P < 0.001 \); Mann–Whitney U-test) matter in CADASIL compared to the Swedish samples. In addition, we noted diffuse ubiquitin immunoreactivity within vessel walls, especially in the absence of smooth muscle, to be comparable in both disorders (data not shown). None of the cases exhibited amyloid \( \beta \) plaques or neurofibrillary tangles which are diagnostic of Alzheimer’s disease.

**NOTCH3 immunolocalization**

In addition to GOM accumulation, NOTCH3 ectodomain deposition in the microvasculature was previously reported as a distinct characteristic (96% sensitivity and 100% specificity) of CADASIL (Joutel et al., 2001). Immunocytochemical staining of brain sections with antibodies to NOTCH3 ectodomain showed intense accumulation of the NOTCH3 N-terminal fragment within walls of the microvasculature in both grey and white matter of CADASIL cases from the English family but virtually absent in the Swedish samples (Fig. 6). N1 antibodies immunostained 81 (±1)% of the microvessels in the CADASIL cases but there were no vessels with distinct staining in any of the Swedish samples. N2 antibody also showed lack of staining in Swedish MID but was similar to that of N1 in CADASIL cases (data not shown). Specificity of the lack of immunostaining in Swedish cases was further demonstrated by the presence of similar levels of C2 immunostaining in the controls, CADASIL and Swedish cases (82, 87 and 80%). Double-immunostained sections with NOTCH3 N1 and \( \alpha \)-actin antibodies similarly revealed disrupted smooth muscle cells but no NOTCH3 ectodomain reactivity in the Swedish cases (Fig. 6E). Brain sections from age-matched controls and the Alzheimer’s disease patient, the different CADASIL subject served as negative and positive controls (data not shown). Thus, in addition to the subtle differences in the microvascular pathology there was distinct lack of immunoreactivity to the NOTCH3 N-terminal specific antibodies in the Swedish samples.

**Discussion**

We suggest that despite previous suspicions the original Swedish family described by the eponym hereditary MID (Sourander and Wålinder, 1977) is not NOTCH3 gene causing CADASIL. While several of the clinical, radiological and pathological features in the brain closely resemble CADASIL (Zhang et al., 1994) and those of the first English family (Stevens et al., 1977), our evidence including absence of temporal pole hypersignals on MRI (O’Sullivan et al., 2001) and most pertinently genetic evidence indicates the Swedish disorder is a novel SVD. We failed to detect a single
mutation within the entire exon coding region of NOTCH3 in affected members of the Swedish family. Although the possibility of a large frame deletion mutation could not be excluded based on the PCR screening method used, any possible splice site missense mutations or any novel missense mutation in other domain of the receptor would still have been detected. However, linkage analysis using three affected and two non-affected members of the Swedish family showed absence of shared allele or haplotype within the three markers of the NOTCH3 locus. This indicated that the NOTCH3 gene locus is not linked to the disorder affecting this Swedish family.

Whether a change in the regulatory elements of NOTCH3 gene is responsible for MID in the Swedish family is worthy of consideration. There are no reports on polymorphisms or mutations affecting the regulation of NOTCH3 expression but the limited RT–PCR in the Swedish MID samples indicated that at least the RNA expression level was not different between affected and non-affected family members. While we cannot entirely exclude NOTCH3 allelic variation based on our genetic data the clinical, radiological and pathological findings strongly imply otherwise.

It is further possible that the Swedish MID is caused by mutations in the receptor ligands JAGGED and DELTA (Gridley, 2003). While there are no known mutations or polymorphisms in JAGGED or DELTA that resemble the phenotype described, several reports indicate that all mutations in JAGGED result in the Alagille syndrome. The syndrome is characterized by developmental abnormalities of the liver, heart, eye, skeleton and patients typically present with neonatal jaundice and cholestasis, which results from a paucity of intrahepatic bile ducts (Gridley, 2003). There is a remote possibility that the DELTA gene is responsible for the Swedish MID.

Until the DNA from the Swedish patients could be exhaustively screened using current genetic analysis, it was readily plausible that based on the clinical phenotype the Swedish cases were suspected to be CADASIL. CADASIL patients express a varied clinical phenotype (Joutel et al., 1994, 1995) and several similarities in neuropathological features such that a misdiagnosis may occur. Phenotypic variation in CADASIL has previously been mistaken for several disorders including multiple sclerosis, cerebral vasculitis, viral encephalitis, Binswanger’s disease, leucoencephalopathy of undetermined cause and Alzheimer’s disease. This study also emphasizes the import of genetic screening for diagnosis of suspected CADASIL cases (Peters et al., 2005) and successful management of the patient.

Our pathological findings indicated distinct absence of GOM in the skin and the cerebral microvasculature, and NOTCH3 N-terminal peptide accumulation in cerebral microvessels of affected Swedish subjects. While the absence of GOM deposits in itself may not be used for the definitive diagnosis of CADASIL (Ruchoux et al., 1994, 1995) it was previously suggested that accumulation of NOTCH3 N-terminal fragments within the vessels wall is highly specific for CADASIL (Joutel et al., 2000a, 2001). It is possible we may have missed blood vessels containing GOM in the Swedish skin specimen but unlikely that the abundant microvessels in brain tissue examined in parallel were missed if they exhibited GOM. On the other hand, cases examined in parallel from the English family described with chronic familial vascular encephalopathy (Stevens et al., 1977) were consistent with the diagnosis of CADASIL in every manner including the relatively common mutation in exon 4 of NOTCH3.

The precise pathogenesis of SVD in either CADASIL or the Swedish cases remains largely unknown. The loss of arterial smooth muscle cells in CADASIL linked to mutations in NOTCH3 has been proposed to be due to a gain of toxic function (Donahue and Kosik, 2004). Whether gain of toxic function also occurs in hereditary MID of the Swedish type is unclear. However, it is conceivable that the onset of the smooth muscle cell degeneration in the Swedish disorder occurs downstream of the effect of NOTCH3 receptor signal activation (Low et al., 2006). This would be consistent with the absence of mutations and lack of ectodomain accumulation in the vasculature as is characteristic of CADASIL. Although severe arteriopathy was manifest in both diseases we observed subtle differences in the composition of the microvascular components including vascular smooth muscle, endothelium, basement membrane and perivascular infiltrates in the two disorders. The increased SI values and collagen IV immunoreactivity in CADASIL compared to the Swedish disorder suggests different mechanisms may be occurring in the breakdown/remodelling of the basement membrane that could be attributed to reduced activity of the matrix metalloproteinasises (Liu and Rosenberg, 2005) or increased endothelial secretion of the collagens. Sourander and Wålinder (1977) had originally suggested that the pathogenesis of hereditary MID could be attributed to either an autoimmune process or metabolic disturbance of the vascular connective tissues. We previously reported that an immune response, implicated by complement factor B reactivity in the CSF (Urluo et al., 2000), was involved in CADASIL. This is supported by the finding of complement 3 (or factor B) reactivity in the brain microvasculature of CADASIL patients but not those with the Swedish disorder (W. C. Low and R. N. Kalaria, unpublished data). That different immunopathogenic mechanism appear to be involved in CADASIL and Swedish hereditary MID is also suggested by the fewer perivascular cells in the Swedish disorder.

CADASIL is clearly the most common form of familial SVD. Other hereditary SVDs have been identified including hereditary endotheliopathy retinopathy nephropathy and stroke or HERNS (Jen et al., 1997), cerebroretinal vasculopathy (Ophoff et al., 2001), hereditary vascular retinopathy (Terwindt et al., 1998; Ophoff et al., 2001), cerebral autosomal recessive arteriopathy with subcortical infarcts and leucoencephalopathy or CARASIL (Yanagawa et al., 2002), hereditary infantile hemiparesis, retinal arteriolar

---

**Novel hereditary small vessel disease**

*Brain (2007), 130, 357–367* 365
tortuosity and leucoencephalopathy (Vahedi et al., 2003). However, the clinical features in the Swedish family are not consistent with any of the characteristics of these disorders suggesting the hereditary MID is another autosomal dominantly inherited SVD. Recessive transmission, retinal abnormalities or infantile hemiparesis were not described in any of our Swedish family members. More recently, Chabriat and colleagues (Verreault et al., 2006) have reported a novel autosomal dominant with incomplete penetrant SVD of the brain characterized by motor hemiplegia, memory deficits, executive dysfunction and white matter lesions upon MRI in the general absence of vascular risk factors. It is premature to suggest that this SVD is the same as the Swedish disorder we describe.

In summary, collective evidence bearing particularly on genetic and pathological features suggests hereditary MID of the Swedish type is not CADASIL in contrast to the disorder in the English family fittingly described as chronic familial vascular encephalopathy. We propose that it is a novel form of hereditary SVD leading to vascular dementia. Another gene appears responsible for the disorder that displays certain phenotypic features and disease manifestation resembling NOTCH3 causing CADASIL. Interestingly, these may belong to the group of several SVDs recently reported in European and Japanese families (Santa et al., 2003; Hagel et al., 2004; Tomimoto et al., 2006) (R. N. Kalaria, W. C. Low and T. Mizuno, unpublished data). Until further genetic identity of the original hereditary MID (Sourander and Wålander, 1977) is revealed we suggest this eponym describes another distinct SVD of the brain.

Acknowledgements
We are very grateful to the families and patients for their co-operation in the investigation of this study. We thank Prof Kate Bushby, Natalie Cookson, Alison Gibson and Dr Bo Lange for initial help in screening DNA samples and linkage analyses. We also thank Nithia Angamuthu, Yumi Yamamoto, Janet Slade and Arthur Oakley for help with the immunocytochemical analysis and image reproduction. We are indebted to Drs K. Takahashi and T. Tabira (NILS, Japan) for providing the antisera to Notch peptides. Our work was supported by grants from the Medical Research Council (UK), Alzheimer’s Research Trust (UK) and EU (QLT-1999) and the CADASIL Trust (UK) for a fellowship to W.C.L. and the Academy of Finland, Sigrid Juselius Foundation, Päiviikki ja Sakari Sohlberg’s foundation, EVO research Funds of Helsinki University Hospital and of Turku University and City Hospitals.

References


Donahue CP, Kosik KS. Distribution pattern of Notch3 mutations suggests a gain-of-function mechanism for CADASIL. Genomics 2004; 83: 59–65.


Kalaria RN. Advances in molecular genetics and pathology of cerebrovascular disorders. Trends Neurosci 2001; 24: 392–400.


Downloaded from http://brain.oxfordjournals.org/ by guest on May 1, 2016
Novel hereditary small vessel disease


