Hereditary Cerebral Hemorrhage With Amyloidosis Associated With the E693K Mutation of APP

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Objective: To report the clinical, genetic, neuroimaging, and neuropathologic studies of patients with the hereditary cerebral hemorrhage with amyloidosis linked to the APP E693K mutation.

Design: Case series. Clinical details and laboratory results were collected by direct evaluation and previous medical records. DNA analysis was carried out in several affected subjects and healthy individuals. Neuropathologic examination was performed in 2 subjects.

Setting: Southern Lombardy, Italy.

Patients: Individuals with and without amyloidosis in 4 unrelated Italian families (N=37).

Main Outcome Measure: Genotype-phenotype relationship.

Results: The affected individuals presented with recurrent headache and multiple strokes, followed by epilepsy and cognitive decline in most of them. The disease was inherited with an autosomal dominant trait and segregated with the APP E693K mutation. Neuroimaging demonstrated small to large hematomas, subarachnoid bleeding, scars with hemosiderin deposits, small infarcts, and leukoariosis. Amyloid-β immunoreactivity was detected in the wall of leptomeningeal and parenchymal vessels and in the neuropil, whereas phosphorylated tau, neurofibrillar changes, and neuritic plaques were absent.

Conclusions: These findings expand the number of APP mutations linked to hereditary cerebral hemorrhage with amyloidosis, reinforcing the link between this phenotype and codon 693 of APP.

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associated with HCHWA,23 we herein provide an overview of our 4 families to offer more information concerning Aβ-CAA phenotypes.

### METHODS

#### PATIENTS

The study started with the observation of 4 unrelated individuals originating from Southern Lombardy, Italy, who had long had headaches, multiple strokes, epilepsy, mild to severe cognitive decline, and had relatives presenting with similar symptoms. When hospitalized, they underwent a diagnostic protocol that included computed tomography, magnetic resonance imaging, DNA analysis for APP and APOE, and a neuropsychological assessment when possible. The relatives who accepted our invitation underwent the same protocol whether or not they had a history of stroke. Data concerning deceased relatives were included when reliable.

### DNA ANALYSIS

The probands and 16 relatives consented to undergo DNA analysis (Table). The DNA was obtained from lymphocytes in all but 1 case (family A, III-5) whose DNA was isolated from a skin biopsy. APP exons 16 and 17 were analyzed as described by Levy et al,19 and the product of exon 17 amplification was digested by the restriction enzyme MboI. APOE was analyzed as described by Wenham et al,24 and the products of polymerase chain reaction amplification of the region with APOE polymorphic sites were digested by the restriction enzyme CfoI. The APP analysis was extended to 130 healthy subjects selected from unrelated people born in the same region.

### NEUROPATHOLOGIC PROTOCOL

The brain of patient III-3 of family A was studied after formal fixation. Ten micrometer-thick sections, cut from paraffin-embedded blocks of cerebral hemispheres, cerebellum, and brainstem, were stained by hematoxylin-eosin, Nissl, Heidenhain-Woelcke, and Congo red stains and treated with thioflavine S. Immunostaining was carried out by antibodies to Aβ after 98% formic acid (Panβ polyclonal, 1:1000; Aβ40 polyclonal, 1:2000; Aβ42 polyclonal, 1:1000; Biosource), hyperphosphorylated tau (AT8 monoclonal, 1:300, Innogenetics), actin (monoclonal, 1:200, Neomarkers), collagen IV (monoclonal, 1:50, DakoCytomation), ubiquitin polyclonal, 1:500, DakoCytomation), glial fibrillary acidic protein (polyclonal, 1:800, DakoCytomation), and the CR3/43 marker for activated microglia (monoclonal, 1:200, DakoCytomation). Immunolabeling was visualized by the ENVision Plus/horseradish peroxidase system for rabbit and mouse immunoglobulins (DakoCytomation), using 3,3'-diaminobenzidine as a chromogen. Double immunostaining for Aβ40/actin and for Aβ40/collegen IV were revealed by fluorescein isothiocyanate-conjugated antirabbit IgG (1:200, Molecular Probes) and Texas red–conjugated antimouse IgG (1:200, Molecular Probes). Electron microscopy was carried out on cortical specimens that had been washed, postfixed by glutaraldehyde and osmium tetroxide, and embedded in epoxy resin. Thin sections were stained with lead citrate and uranyl acetate. Postembedding immunolabeling for Aβ40 and Aβ42 (1:100 Biosource) was revealed by goat antirabbit serum conjugated to 10-nm gold particles (goat antirabbit serum conjugated to 10-nm gold particles). A biopsy specimen of the frontal cortex of patient III-2 from family C was fixed with Carnoy fixative and embedded in Paraplast (Bio Optica). Seven-micrometer-thick sections were used to investigate Aβ, hyperphosphorylated tau, actin, collagen IV, and ubiquitin.

### RESULTS

#### PEDIGREES AND DNA ANALYSIS

**Figure 1** shows abridged pedigrees of the 4 families; the details concerning living patients and healthy carriers have been omitted for ethical reasons. In 6 symptomatic subjects, APP analysis revealed a G→A mutation at codon 693 predicting the E→K substitution at position 22 of Aβ; this mutation was absent in 4 healthy relatives and the controls. The same analysis revealed 4 healthy carriers among 6 members of the last generation. The results were confirmed by MboI digestion.

The study of APOE genotypes revealed 12, 2, and 1 subjects with ε3/ε3, ε3/ε4, and ε2/ε3 alleles, respec-
tively; E693K was associated with ε3/ε3 (3 affected subjects and 4 healthy carriers), ε2/ε3 (1 affected subject), and ε3/ε4 (1 affected subject) alleles.

FAMILIES AND PATIENTS

Family A

The proband (III-3) underwent neurosurgery at the age of 44 years to drain a hemorrhage in the left temporal region with subarachnoid bleeding. The arteriograms were negative for vascular malformations or tumors, and the patient did not have hypertension or other risk factors for stroke or hemorrhagic disease. He subsequently began having recurrent headaches and focal epilepsy. The bleeding recurred when he was 60 years old and his Mini-Mental State Examination (MMSE) score was 25 of 30; fatal bilateral bleeding occurred 6 months later. Seven more members of the family were affected. The proband’s mother (II-10) died at the age of 56 years after experiencing a sudden headache that evolved into a coma; a similar history was reported for her younger sister (II-14), father (I-3), and an uncle (I-2), who died at the ages of 61, 72, and 63 years, respectively, and for 3 sisters of the proband who died as a result of cerebral hemorrhage at the age of 57 years (2 sisters) and 49 years. Ten other members of the family were healthy.

Family B

The proband (III-1) developed mild memory decline and inappropriate behavior before the age of 60 years, when he started experiencing multiple hemorrhagic strokes. His MMSE score was 20 when he was aged 63 years, and he died at the age of 64 years. Two relatives (II-1 and II-5) had recurrent strokes, epilepsy, and severe cognitive decline; 4 other relatives were healthy.

Family C

At the age of 50 years, the proband (III-2) underwent surgery to drain a large hematoma in the right frontal region, and a biopsy was carried out on that occasion. He developed epilepsy and dementia with 3 recurrences and died at the age of 63 years. His mother (II-1) and grandmother (I-1), who both had a history of recurrent headache and stroke without cognitive decline, died when they were aged 61 years. A maternal uncle aged 82 years (II-2), a brother aged 62 years (III-1), and 2 relatives in the last generation were healthy.

Family D

The proband (II-3) was hypertensive and had had recurrent hemorrhagic strokes from the age of 56 years. Bleed-
ing occurred in the frontal lobes and was followed by focal epilepsy and behavioral problems. His MMSE score was 27 when he was 62 years old. Four relatives had a similar history. The proband's father (I-8), who died at the age of 61 years, and his brother (I-7), who died at the age of 80 years, both had recurrent strokes with cognitive decline. The proband's sister (II-1) experienced multiple strokes from the age of 56 years and was severely demented and bedridden at age 65 years. Another sister (II-4) presented with her first stroke at the age of 50 years and after 3 recurrences in 3 years had an MMSE score of 26. A third sister (II-2), aged 63 years, and a brother (II-5), aged 52 years, were healthy.

**NEURORADIOLOGICAL FINDINGS**

All of the probands showed computed tomography and magnetic resonance imaging evidence of recent and previous intracerebral and subarachnoid bleeding, multiinfarct encephalopathy, and leukoaraiosis. There were 6 (III-3, family A), 3 (III-2, family C), 2 (III-1, family B), and 1 (II-3, family D) intracerebral hematomas detected by computed tomography. There were slightly more hematomas in the left hemisphere (7 vs 5) and the anterior portion of the cerebral hemispheres (4 temporal, 3 frontal, 3 parietal, and 2 occipital lobe). The extended hematomas were associated with subarachnoid bleeding. Magnetic resonance imaging T2*-weighted sequences revealed round shadows of hemosiderin deposits that were uniformly distributed along the cortical ribbon in 3 probands and prevailed over the frontal region in 1; a few additional shadows were detected in the thalamus and cerebellar cortex. T2-weighted and fluid-attenuated inversion recovery sequences showed small focal and large diffuse hyperintensities of the white matter of the cerebral hemispheres, indicating ischemic and hypoxic changes. Most of the focal images crossed the cortical-subcortical junction in the frontal region, whereas diffuse hyperintensity was localized in the deep white matter, particularly around the posterior horns of the ventricles. The cerebellar white matter was involved in 1 patient.

**NEUROPATHOLOGICAL STUDY**

Gross examination of the brain of patient III-3 (family A) revealed surgical tracks in the left temporal region, small hemorrhages, and yellow scars in the cerebral cortex and recent bleeding with blood that had drained into the subarachnoid space and ventricles. Microscopically, there were many small vessels with thickened and/or split walls due to a hyaline material that were visible after hematoxylin-eosin staining (Figure 2A), congophilic and fluorescent after thioflavine S (Figure 3C), and immunoreactive for Aβ and Aβ40 (Figure 2B-D, Figure 3A, and Figure 4C). Most of the abnormal vessels were in the leptomeninges, in the cerebral and cerebellar cortex, and in the white matter close to the cortex. In appropriate sections, a few such vessels could be followed into the centrum ovale (Figure 4C). Amyloid-β and Aβ40 were also detectable in cortical capillaries and leptomeningeal vessels that looked normal on hematoxylin-eosin staining (Figure 3F). There was much less Aβ42 vascular immunoreactivity and it involved cortical capillaries (Figure 5B), whereas only an average of 1 in every 50 cortical arterioles contained some Aβ42 labeling in the outer and inner layers of the wall (Figure 5A). Immunoelectron microscopy showed a network of Aβ40 fibrils, which were denser close to the endothelium; further out, fibrils were mixed with osmiophilic debris (Figure 3B).

Double Aβ40/actin and Aβ40/collagen IV staining showed that Aβ immunoreactivity was associated with severe structural changes in the vessel walls. Most of the arterioles loaded with Aβ40 showed actin loss (Figure 3D),

![Figure 2. Hematoxylin-eosin and immunoreactivity to amyloid-β peptide (Aβ) (Panp antibody). A, Hematoxylin-eosin showed thickened vessel wall with reduction of the lumen. Amyloid-β deposition occurred in the wall of leptomeningeal (B) and parenchymal vessels (C) as well as in clusters of capillaries (D). E, Panp antibody also labeled numerous diffuse preamyloid deposits in the neuropil. B, C, D, and E are the same magnification.](https://www.archneurol.com/content/67/8/990/fig2)
whereas collagen IV was split into 2 or 3 concentric layers enveloping Aβ (Figure 3E). Antibodies to collagen IV, ubiquitin, and glial fibrillary acidic protein and epitopes of activated microglia revealed ubiquitinated profiles, microglial cells, and astrocytic feet joining the outer layer of collagen and a network of astrocytic processes growing into the perivascular neuropil.

Amyloid-β immunoreactivity was present in the neuropil of the gray structures (particularly the cerebral cortex and striatum), where it formed smoky, noncongophilic deposits recognized by anti-Aβ42 but not anti-Aβ40 antibodies (Figures 2E and 5C). Immunoelectron microscopy with anti-Aβ42 antibodies showed that the material decorated by gold particles was poorly osmiophilic and had a cotton-wool–like structure with very few embedded fibrils. In adjacent sections treated for ubiquitin, glial fibrillary acidic protein, activated microglia or hyperphosphorylated tau, the Aβ42 deposits harbored minute profiles that were positive for ubiquitin, but negative for hyperphosphorylated tau; they also showed a delicate network of astrocytic processes in the absence of microglial cells. Searches for neurofibrillary tangles, neuropil threads, or neuritic plaques were consistently unsuccessful at the neocortical level, but isolated neurites and neuropil threads were detected in the entorhinal cortex.

Secondary lesions included fresh blood in the subarachnoid space, tissue, and ventricles; hemorrhagic scars and hemosiderin deposits; and infarcts and edema of the white matter. Most of the hemorrhagic scars were found in the cerebral cortex. The burden of such scars and small intraparenchymal hemorrhages was comparable with that of ischemic lesions. In semiserial sections, there was no clear evidence of ischemic changes being adjacent to hemorrhagic lesions. The infarcts were small and lay across the corticosubcortical junction (Figure 4D); most of them were localized in the frontal and temporal lobes and could merge into larger lesions. Some vessels with secondary changes such as aneurysm-like enlargements, perivascular cuffings of macrophages and histiocytes, or necrosis of the vessel wall were detectable close to the infarcts and hemorrhagic scars. Hematoxylin-eosin and Heidenhain-Woelke staining revealed the white matter cuffing of the occipital horns to be pale and edematous (Figure 4F).

Figure 3. Lesions of the vessel wall immunoreactive to amyloid-β 40 (Aβ40) and related changes. A, The abnormal material in the vessel wall was intensely immunoreactive for anti-Aβ40. Immunoelectron microscopy demonstrated the fibrillary composition of vascular Aβ40 deposition (B) that was fluorescent after thioflavine S (C). The vessel wall showed actin depletion (D) (actin green, Aβ40 red) and splitting of basement membrane (E) (anti–collagen IV). F, Cortical capillaries were decorated by anti-Aβ40. A, D, E, and F are the same magnification.

This study of 20 symptomatic subjects (8 males and 12 females) and relatives in 4 families showed that the APP E693K mutation was associated with a disease transmitted as a dominant trait. The disease was clinically characterized by headache and stroke occurring once or more from age 53 years (range, 44–60 years, n = 14) to 63 years (49–80 years, n = 17) and by seizures and cognitive decline occurring after stroke in most instances. The combined clinical, neuroimaging, and neuropathology data suggested that there was a relationship between headache, multiple strokes, and small to large subarachnoid and intracerebral hemorrhages; between recurrent bleed-
and CAA with actin replaced by Aβ and collagen; between seizures and scars with hemosiderin deposits; and between cognitive decline and multiple vascular lesions. These findings make the phenotype of our patients quite close to that of HCHWA–Dutch type (HCHWA-D), but leave some points for discussion.

In some patients with HCHWA-D, and in one of ours as well, cognitive decline preceded stroke or progressed in its absence. Accordingly, it was regarded as the issue of neurotoxicity of the mutant Aβ. This is supported by the fact that the neuropil in HCHWA is targeted by nonfibrillary Aβ, which could interfere with cell function in the absence of cytoskeletal changes involving tau protein. This mechanism was regarded as the cause of cognitive decline in a Japanese family carrying the E693K mutation on the grounds that neuroimaging ruled out cerebrovascular lesions and in vitro studies have shown that the mutant Aβ could raise synaptic signaling. However, it is quite likely that multiple strokes causing circuitry damage large enough to affect cognition play a role in most patients with HCHWA. In fact, the patient with APP G705C was not the only one with dementia in the absence of Aβ immunoreactivity in the neuropil. All but 1 of the patients with the E693K mutation were cognitively disabled (9 of 18 patients who had reliable information in this regard) and most HCHWA-D patients had had a stroke long before the onset of cognitive decline.

Furthermore, the pathologic spectrum in HCHWA includes chronic edema of the periventricular white matter recognized by magnetic resonance imaging as leukoaraiosis. In HCHWA-D, the role of leukoaraiosis in cognitive decline has been questioned since the decline of many patients could better be correlated with aging, but there is increasing evidence that leukoaraiosis contributes to cognitive decline in healthy elderly people and patients with recurrent cerebral hemorrhage. Leukoaraiosis is also regarded as a risk factor of global deterioration following the involvement of long association fiber pathways. Pathologically, it has long been recognized that CAA is quite often associated with periventricular edema, which could indicate the narrowing of long perforating arteries (Figure 4C) as a cause of reduced blood flow and chronic hypoxia with tissue acidosis and edema in the deep white matter. Chronic hypoxia at the cortical level might also explain the association between dementia and the narrowing of vessels supplying the frontal cortex that has been found in patients with HCHWA-D in the absence of infarcts and scars. However, capillaries (and not only arterioles) might be involved in this process, as capillary occlusions with blood flow impairment and chronic tissue hypoxia have been observed in a mouse model of HCHWA. Additional microvascular changes, such as microaneurysms, inflammatory perivascular cuffings, intramural infiltrates, and necrosis of the vessel wall that have been found in HCHWA-D patients, participate in this process and might be influenced by hypertension. In our normotensive patient who was assessed neuropathologically, most such changes were found in the tissue around the ischemic and hemorrhagic lesions, which also suggests a relationship of such changes with the breakdown process.

While no correlation has been found between structural changes and APOE polymorphisms in HCHWA-D,

![Figure 4](https://www.archneurol.com/article-graphics/992.png)

**Figure 4.** Secondary lesions. Intracortical microhematomas (A) and round reduced signal shadows in axial T2*-weighted gradient-echo magnetic resonance imaging (MRI) sequences (B) owing to hemosiderin deposits. C, Distribution of amyloid angiopathy involving leptomeningeal vessels and short and long penetrating arteries. D, Small ischemic lesion crossing the corticocortical junction. E, Subcortical hyperintensities in axial T2*-weighted gradient-echo MRI sequences revealing multiple infarcts and posthemorrhagic scars. Perventricular edema (F) and leukoaraiosis in fluid-attenuated inversion recovery MRI sequences (G). D and F are the same magnification.
our patients with the E693K mutation and their related healthy carriers (n = 15) had shown a large prevalence of the ε3 allele over the ε4 and ε2 alleles (27:2:1). Likewise, the 3 affected individuals of the APP G705C family were homozygous for the ε3 allele. These results were unexpected given the role of ε4 and ε2 in modulating the deposition of Aβ in the neuropil and vessel walls in AD and CAA: ε4 should favor vascular over parenchymal deposition of Aβ, whereas ε2, which is highly represented in cerebral hemorrhage due to amyloid angiopathy, should promote vascular changes, such as the concentric splitting of the vessel wall, that increase the risk of bleeding.43 However, in patients with APP E693K and G705C, splitting of the vessel wall occurred regardless of the ε2 allele. These discrepancies might be explained on the grounds that the APP mutations associated with HCHWA overwhelm the effect of APOE polymorphisms, likely because these mutations, which predict substitutions clustered around the central hydrophobic core of Aβ, induce charge modifications44; decrease the clearance of the mutant species,45 which are highly fibrillogenic46 and self-aggregating;47 and increase the production of Aβ40 targeted to the vessel walls.48 This last point is crucial in explaining the severity of CAA in HCHWA, as it has been found that CAA pathology was severe and the Aβ40:Aβ42 ratio increased in a transgenic mouse model of HCHWA overexpressing the mutant E693Q form of APP.49 As this ratio is lowered by additional genetic engineering, the distribution of Aβ immunoreactivity may switch from the vessel walls to the neuropil.48 The pathogenic relevance of this observation can be more clearly understood by considering that mutant E22Q and E22K Aβ were proved toxic to vascular smooth muscle cells and endothelia in vitro.47-49,50

On the other hand, the absence of neuritic plaques and tangles in the neocortex of HCHWA patients is still unexplained. These lesions would be justified by the strong neurotoxicity of Aβ species mutated at position 22,51 the amount of Aβ42 immunoreactivity in the gray structures,52 and the age of most patients. We have long speculated whether their absence may be related to the ε4 allele being underrepresented in HCHWA families, but the distribution of APOE polymorphisms in carriers of the APP E693G mutation responsible for the Arctic dementia definitely argues against this hypothesis because these patients, who presented with the clinical and pathological AD phenotype with severe CAA but no bleeding, showed a large prevalence of the ε3 allele over the ε4 and ε2 alleles (17:3:2, n = 11) (Hans Basun, MD, PhD, Karolinska Institutet, Huddinge, Sweden, e-mail communication, May 2009).

Our findings are in keeping with the idea that the angiotoxic effect of Aβ may be independent of the neurotoxic effect that progresses to neuritic plaques and tangles. The APP E693Q, E693K, L705V, A692G, E693G, D694N, and A713T phenotypes share severe CAA and Aβ42 immunoreactivity in the neuropil, but the first 3 differ from the others in the absence of neuritic plaques and tangles. This implies that the determinism of the amyloid cascade theory, which rigidly links Aβ overloading in the neuropil with cytoskeletal pathology, should be attenuated by adopting the hypothesis of a factor (other than APOE polymorphisms) that modulates the vulnerability of neurons to the toxic effect of Aβ.

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REFERENCES


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**Announcement**

**Trial Registration Required.** As a member of the International Committee of Medical Journal Editors (ICMJE), *Archives of Neurology* will require, as a condition of consideration for publication, registration of all trials in a public trials registry (such as http://ClinicalTrials.gov). Trials must be registered at or before the onset of patient enrollment. The trial registration number should be supplied at the time of submission.

For details about this new policy, and for information on how the ICMJE defines a clinical trial, see the editorials by DeAngelis et al in the September 8, 2004 (2004;292:1363-1364) and June 15, 2005 (2005;293:2927-2929) issues of *JAMA*. Also see the Instructions to Authors on our Web site: www.archneurol.com.