Dense-Core Senile Plaques in the Flemish Variant of Alzheimer’s Disease Are Vasocentric

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Alzheimer’s disease (AD) is characterized by deposition of β-amyloid (Aβ) in diffuse and senile plaques, and variably in vessels. Mutations in the Aβ-encoding region of the amyloid precursor protein (APP) gene are frequently associated with very severe forms of vascular Aβ deposition, sometimes also accompanied by AD pathology. We earlier described a Flemish APP (A692G) mutation causing a form of early-onset AD with a prominent cerebral amyloid angiopathy and unusually large senile plaque cores. The pathogenic basis of Flemish AD is unknown. By image and mass spectrometric analysis, we demonstrated that in contrast to other familial AD cases with predominant brain Aβ42, Flemish AD patients predominantly deposit Aβ40. On serial histological section analysis we further showed that the neuritic senile plaques in APP692 brains were centered on vessels. Of a total of 2400 senile plaque cores studied from various brain regions from three patients, 68% enclosed a vessel, whereas the remainder were associated with vascular walls. These observations were confirmed by electron microscopy coupled with examination of serial semithin plastic sections, as well as three-dimensional observations by confocal microscopy. Diffuse plaques did not associate with vessels, or with neuritic or inflammatory pathology. Together with earlier in vitro data on APP692, our analyses suggest that the altered biological properties of the Flemish APP and Aβ facilitate progressive Aβ deposition in vascular walls that in addition to causing strokes, initiates formation of dense-core senile plaques in the Flemish variant of AD. (Am J Pathol 2002, 161:507–520)

In Alzheimer’s disease (AD), β-amyloid (Aβ) is deposited in two of the most common types of parenchymal deposits—diffuse and senile plaques (SPs)—and variably in vessels [cerebral amyloid angiopathy (CAA)].1 In the present article, the term “senile plaque” is used to refer to only classic SPs having a central amyloid core (plaque core) surrounded by filamentous bundles and granules of amyloid as well as reactive cells (coronal plaque).2 Aβ is a cleavage product of the amyloid precursor protein (APP), produced by the activity of N-terminal β-secretase and C-terminal γ-secretase (Figure 1). However, the major cleavage of APP is by α-secretase that cleaves the Aβ from within and after the sequential γ-secretase activity, releases an ~3-kd peptide (p3). As yet, all mutations in APP associated with familial (early-onset) forms of AD (FAD) or hereditary diseases characterized by CAA are located around one of the major cleavage sites (http://molgen-www.uia.ac.be/admutations http://www.alzforum.org/members/resources/app_mutations/app_table.html).3 The majority of the FAD-associated mutations in APP lie close to its γ-secretase site, that, similar to FAD-causing mutations in presenilin (PS) 1 and PS2, increase the production of the more amyloidogenic Aβ42 in vitro and in vivo.4 The only known mutation at the APP β-cleavage site, the double-Swedish mutation (APP K670N/M671L),5 increases in vitro both Aβ40 and Aβ42, although in brain parenchyma Aβ42 is predominantly deposited.5 Structural heterogeneity is also noted at the Aβ N-terminus, eg, Aβ residue R5, E11, or L17 (p3), and such N-truncated forms are known to be more fibrillogenic and toxic than full-length Aβ.6,7 Accordingly, N-truncated Aβ42 is proposed to be deposited as early, diffuse plaques8-10 that seed the deposition of more abundantly secreted Aβ40, leading to the formation of SPs.11 Despite the anatomical separation of Aβ deposits and their proposed consequence, viz., intraneuronal accumulation of hyperphosphorylated tau in dystrophic neurites and neurofibril...
lary tangles, neuritic pathology is also predominantly present in the vicinity of SPs and other Thioflavin-S (ThS)-positive (+) amyloid deposits, but not diffuse plaques. The pathological relevance of SPs in AD pathology is further strengthened by 

The mechanisms by which the mutations on the same or adjacent codons cause distinct diseases are not fully understood. Transgenic Dutch and Flemish APP mice showed that mutant APP/Aβ is toxic, however, brain Aβ levels in these mouse models did not exceed the critical level to get deposited. Most of the knowledge on these mutations is thus derived from extensive in vitro modeling. It has been shown that the Dutch mutation increases Aβ beginning at D1, V18, and Y19, accelerates Aβ fibril formation and stability, increases in situ aggregation on cultured cell surfaces, and enhances neurotoxicity to both smooth muscle and endothelial cells. On the other hand, the Flemish mutation also leads to an increased production of Aβ beginning at D1, R5, and E11, proposed to be mediated by a BACE 2. In addition, the Flemish homologue fibrillizing slower than wild-type Aβ, forms larger and more stable, neurotoxic aggregates.

The purpose of this study is twofold: First, never before has FAD associated with -secretase site-related mutations been systematically analyzed for type of Aβ deposition. Second, because the plaque cores are the largest reported in AD/Fl, and the biophysical and biochemical studies suggested that the Flemish Aβ is less aggregatable than the wild type, we attempted to identify the underlying structures that might initiate the formation of plaque cores in AD/Fl brains. We first describe here a time-dependent development of neurofibrillary pathology in a recently autopsied APP692 family member from whom a biopsy specimen was also available. Including this patient, we showed in three APP692 patients a predominant Aβ (1-40) content of the SPs, suggesting that AD/Fl is not associated with an increased Aβ42 brain deposition as in other familial AD. Detailed investigations of SPs in AD/Fl revealed that the plaque cores were centered on vessels. Our studies suggest that progressive Aβ40 deposition in vascular walls in AD/Fl not only results in strokes, but also initiates the formation of SPs, accelerating neuronal injury to cause the Flemish variant of AD.

Figure 1. The position of APP mutations in relation to its major cleavage sites and Aβ. Other mutations can be assessed on frequently updated databases (http://molgen-www.uia.ac.be/admutations and http://www.alzforum.org/members/resources/app_mutations/app_table.html).
Patients and Methods

Family 1302

The APP692 (1302) family is a multigeneration Dutch family whose members have presenile dementia and cerebral hemorrhage, inherited in an autosomal-dominant pattern (Figure 2). The clinical phenotypes overlap because hemorrhagic stroke was reported in an offspring of a demented patient, and conversely, progressive dementia has also occurred in an offspring of a stroke patient. Two patients (IV-2 and IV-5) had strokes and were diagnosed with AD in their lifetimes, for instance, individual IV-2 first had dementia at age 41 years and later suffered a hemorrhagic stroke at age 46 years. We earlier studied two patients who had clinical dementia and fulfilled the neuropathological criteria of AD (patients IV-4 and IV-13) (Table 1). Here, we report the neuropathological analysis of an additional member of the family, patient IV-5, who had hemorrhagic stroke at age 42 years. A biopsy taken while evacuating a large hematoma in the left parieto-occipital cortex, revealed CAA and both senile and diffuse plaques, however, neurofibrillary tangles or hyperphosphorylated tau (AT8)-positive neurites were absent. Consistently, before the intracerebral hemorrhage, this patient did not show any signs of cognitive impairment. However, in the course of disease, the patient developed progressive dementia and at age 48 years was diagnosed with dementia indistinguishable from AD. The patient slowly progressed to a vegetative state and died at age 55 years. The brain was fixed in 4% formaldehyde and analyzed.

Histology Including Immunohistochemistry

Examination of the brain of patient IV-5 was performed after a postmortem interval of 5½ hours. The right cerebral hemisphere was fixed by immersion in 10% formalin and embedded in paraffin. Five-μm thick sections were taken from superior frontal gyrus, superior temporal gyrus, superior occipital gyrus, superior parietal lobule, hippocampus and entorhinal cortex, basal ganglia, brain stem, and cervical spinal cord. Sections were examined by routine histopathological methods and also with Thioflavin S (ThS), Congo red, Cresyl violet, periodic acid-Schiff, and Bielschowsky. For Aβ subspecies identification and other immunohistochemistry (Table 2), serial 4- to 5-μm sections were sliced from the hippocampus, superior temporal gyrus, superior frontal gyrus, and cerebellum of the following cases: three AD/Fi patients (IV-4, IV-5, and IV-13), sporadic AD patients (n = 5), AD with PS1 mutations (I143T; n = 5 and A384A; n = 5), and a HCHWA-D patient. For immunohistochemical study of the SPs in three AD/Fi patients, serial 2- to 3-μm-thick sections were sliced from paraffin-embedded blocks of the superior frontal gyrus, superior temporal gyrus, and cerebellum. On an average, 500 sections were sliced from each block. The sections were double immunostained with Aβ antibodies and blood vessel markers [CD31, CD34, smooth muscle actin or collagen type IV (C-IV)] (Table 2). From the two neocortical and one cerebellar region, 300 and 200 SPs, respectively, from each patient (n = 3) were serially

Table 1. Clinical Events and Neuropathological Changes Noted in Three Autopsied Patients of the APP692 Family

<table>
<thead>
<tr>
<th>APP692 patients</th>
<th>Biopsy</th>
<th>Autopsy</th>
<th>First presentation</th>
<th>AAO (years)</th>
<th>AD (Braak staging)</th>
<th>Severe degree of CAA</th>
<th>ApoE*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV-4</td>
<td>+</td>
<td>+</td>
<td>Progressive dementia</td>
<td>49</td>
<td>+ (V/VI)</td>
<td>+</td>
<td>3/4</td>
<td>26</td>
</tr>
<tr>
<td>IV-5</td>
<td>−</td>
<td>+</td>
<td>Stroke</td>
<td>42</td>
<td>+ (V/VI)</td>
<td>+</td>
<td>3/4</td>
<td>this report</td>
</tr>
<tr>
<td>IV-13</td>
<td>−</td>
<td>+</td>
<td>Progressive dementia</td>
<td>48</td>
<td>+ (V/VI)</td>
<td>+</td>
<td>3/3</td>
<td>26</td>
</tr>
</tbody>
</table>

*There is no evidence that APOE modifies the disease onset for this family. AAP, age at onset.
Table 2. Antibodies Used in the Characterization of SP in AD/Fl Patients

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope/Marker</th>
<th>Type</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4G8</td>
<td>Aβ residues 17–24, recognizes Aβ 5–11</td>
<td>IgG3, 1:20,000</td>
<td>F oric acid</td>
<td>Senetek, Maryland Heights, MO</td>
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<tr>
<td>6E10</td>
<td>Aβ N-terminus</td>
<td>IgG1, 1:10,000</td>
<td>F oric acid</td>
<td>Senetek</td>
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<td>6F3D</td>
<td>Aβ N-terminus</td>
<td>IgG1, 1:25</td>
<td>F oric acid</td>
<td>Dako, Glostrup, Denmark</td>
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<tr>
<td>JRF/AβN/11</td>
<td>Aβ N-terminus</td>
<td>IgG3, 1:500</td>
<td>F oric acid</td>
<td>Gift from M. Mercken</td>
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<tr>
<td>JRF/cab40/6</td>
<td>Aβ40</td>
<td>IgG3, 1:500</td>
<td>F oric acid</td>
<td>Gift from F. Checkler</td>
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<tr>
<td>R209</td>
<td>Aβ40</td>
<td>IgG3, 1:400</td>
<td>F oric acid</td>
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<tr>
<td>FCA3340</td>
<td>Aβ40</td>
<td>pAb-rabbit, 1:150</td>
<td>F oric acid</td>
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<td>JRF/cab42/12</td>
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<td>IgG1, 1:500</td>
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<td>FCA3542</td>
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<td>pAb-rabbit, 1:250</td>
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<tr>
<td>R226</td>
<td>Aβ42</td>
<td>pAb-rabbit, 1:400</td>
<td>F oric acid</td>
<td>Gift from P. Mehta</td>
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<tr>
<td>ATB</td>
<td>Abnormally phosphorylated tau</td>
<td>IgG1, 1:20,000</td>
<td>—</td>
<td>Gift from Innogenetics, Zwijraarde, Belgium</td>
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<td>Endothelium</td>
<td>IgG1, 1:100</td>
<td>Citrate buffer</td>
<td>JC70; Dako</td>
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<tr>
<td>Anti-CD34</td>
<td>Endothelium</td>
<td>IgG1, 1:100</td>
<td>Citrate buffer</td>
<td>QBEnd/10; Dako</td>
<td></td>
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<tr>
<td>Anti-SMA</td>
<td>Smooth muscle cell actin</td>
<td>IgG1, 1:100</td>
<td>Citrate buffer</td>
<td>Dako</td>
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<td>Pronase digestion</td>
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<tr>
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<td>Ubiquitin</td>
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<td>Citrate buffer</td>
<td>Dako</td>
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<tr>
<td>Anti-C1q complement</td>
<td>Complement cascade</td>
<td>pAb-rabbit, 1:100</td>
<td>Citrate buffer</td>
<td>Dako</td>
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<tr>
<td>Anti-HLA-DP,DQ,DR</td>
<td>Complement cascade</td>
<td>pAb-rabbit, 1:100</td>
<td>Citrate buffer</td>
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<tr>
<td>Anti-VEGF</td>
<td>Angiogenesis</td>
<td>pAb-goat, 1:100</td>
<td>Citrate buffer</td>
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<tr>
<td>Anti-bFGF</td>
<td>Angiogenesis</td>
<td>pAb-goat, 1:100</td>
<td>Citrate buffer</td>
<td>R&amp;D Systems</td>
<td></td>
</tr>
</tbody>
</table>

Imaged by a digital charge-coupled device color camera (Sony Corporation, Tokyo, Japan) connected to a Vidas image analysis frame grabber (Kontron, München, Germany). SPs and other amyloid deposits were serially studied. SPs were so addressed if on any section had the many). SPs and other amyloid deposits were serially immunohistochemical staining to avoid bias of staining for any one particular antibody.

Using antibodies specific for Aβ N-terminus (eg, 6E10), and contrasting with reactivity for Aβ17–24 (4G8), distinguished full-length and N-truncated Aβ, as described previously.44 Specificity of Aβ antibodies was examined by dot blotting using 50 ng of synthetic wild-type, Flemish, and Dutch Aβ peptides either as full-length Aβ (Biosource, Nivelles, Belgium) or N-truncated Aβ (12–42) peptide. Although 4G8 recognized the Flemish and Dutch Aβ less avidly compared to the wild type, no difference was observed for any N- or C-terminus Aβ antibody used in this study, or between any full-length wild type, or mutated Aβ40 or Aβ42, and their corresponding N-truncated forms (data not shown).

Fluorescence and High-Resolution Transmission Electron Microscopy

Labeling for confocal laser-scanning microscopy was done on 10- to 50-μm-thick sections incubated overnight.
with 4G8, or mouse Aβ40 or Aβ42 antibody, washed and labeled with an anti-mouse tetramethylrhodamine B isothiocyanate-conjugated antibody (Molecular Probes, Eugene, OR). For multiple labeling, sections were co-incubated overnight with rabbit anti-Aβ40 or anti-Aβ42 and mouse IgG1 against vessel components (varied combinations of CD31, CD34, smooth muscle actin, and or C-IV), washed, and labeled with an anti-mouse tetramethylrhodamine B isothiocyanate conjugate and an anti-rabbit fluorescein isothiocyanate antibody. Images were acquired with a Zeiss CLM-410 (Carl Zeiss NV-SA, Zaventum, Brussels) using either 488 nm line of argon single laser or 632 nm helium-neon double laser for excitation. Three-dimensional reconstructions were made by AnalySIS (Soft Imaging System, Münster, Germany).

Araldite blocks of neocortex, hippocampus, and cerebellum of patients IV-4 and IV-13 were used for electron microscopy. Immersion fixation was achieved with 4% neutral buffered glutaraldehyde followed by 2% buffered osmium tetroxide. Blocks were sectioned with a Reichert Jung microtome (Leica, Wein, Austria) equipped with a section counter, and ribbons of 0.25-μm-thick sections of small regions of interests were collected on copper grids. Sections were contrasted with routine uranyl acetate and lead citrate. Sections were contrasted with routine uranyl acetate and lead citrate. The ultra-thin sections were interspersed with thicker 1-μm sections collected on glass slides for light microscopy.

**Morphometric, Densitometric, and Mass Spectrometric Analyses**

Morphometric and densitometric analyses were done by a self-written software on a Vidas Image Analysis System, described previously.45 Sizes of SPs and CAA (n = 300 each) from various regions of AD/Fl patients were measured and compared by a two-tailed unpaired t-test. Amyloid-laden vessels were ascribed severely stenotic when the ratio of the lumen diameter and the vessel diameter was less than one half.

For Aβ subspecies identification, sections were stained with different Aβ40 and Aβ42 antibodies (Table 2). Semi-interactive quantification was done as described46 and the percentage areas of reactivity in vessels and parenchymal plaques were assessed.

Isolation of Aβ from meningeal vessels in AD/Fl and sporadic AD brains, frozen at −70°C, was performed as described46 with slight modification. Different parenchymal amyloid deposits were carefully extracted with tissue microdissection aided by immunohistochemistry on adjacent sections. Samples were thawed and washed three times in ice-cold Tris-buffered saline and homogenized in a buffer of 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0, containing protease inhibitors (ethylenediaminetetraacetic acid-Na, 2 mmol/L; leupeptin, 10 μmol/L; pepstatin, 1 μmol/L; phenylmethyl sulfonyl fluoride, 1 mmol/L; TLCK, 0.1 mmol/L; TPCK, 0.2 mmol/L). The homogenates were centrifuged at relative centrifugal force (RCF) 100,000 × g for 1 hour and brain tissue pellets were washed three times with ice-cold Tris-buffered saline and then extracted using 1.0 ml of 70% formic acid by sonication and vortexing for 2 hours at 4°C. The formic acid extracts were centrifuged at 100,000 × g for 2 hours and the formic acid layers were collected and stored at −20°C. From these samples, Aβ was immunoprecipitated using 4G8 and protein G Plus/Protein A agarose beads (Oncogene Science, Inc., Cambridge, MA) and analyzed using a matrix-assisted laser-desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE STR Bio-Spectrometry Workstation, PE/PerSeptive Biosystem) described previously.47,48

**Results**

**Pathological Confirmation of AD for Patient IV-5**

Weight of the brain at autopsy was 855 g and showed atrophy of all cortical areas. Old cystic infarcts were present in temporal and occipital cortices bilaterally. Microscopic examination of the right cerebral hemisphere revealed diffuse cortical atrophy and microspongiosis of upper cortical layers. Silver impregnation and Aβ immunohistochemistry revealed a huge load of CAA, SPs with large plaque cores, and diffuse plaques, in hippocampus, parahippocampus, neocortex, basal ganglia, and cerebellum. Amyloid deposits were manually counted on 4G8-stained sections by a Zeiss MC80 microscope equipped with an ocular graticule and a ×10 objective (1.56 mm²). On an average, ≥10 SPs were present in the gray matter fields of all neocortical regions as well as in hippocampus, and ≥4 SPs were present in basal ganglia, substantia nigra, and cerebellum. In other brain regions, including the central gray matter of the spinal cord, at least some forms of ThS(+ plques) were observed. AT8(+) dystrophic neurites and neuropil threads...
AD/Fl Pathology

Core-containing SPs and coreless primitive plaques were the most abundant plaques in the neocortical and limbic areas in the three AD/Fl brains, where they were far more common in the gray, than in the white matter (Figure 3; E to H). SPs were associated with unusually large plaque cores and were also sometimes multieentric. However, a small number of SPs had a relatively smaller plaque core size compared to the surrounding coronal plaque and thus closely resembled SPs commonly described in AD.2,26 SPs were also predominantly present in the granular layer of cerebellum. The primitive plaques as described elsewhere2 were circumscribed clusters of ThS(+) amyloid wisps without a central dense core and also had a striking textural resemblance to the perivascular and coronal plaques.

A huge load of amyloid was also deposited in capillaries and small- to middle-sized arteries often displaying vessel-within-vessel configurations and frequently associated with microvascular changes such as microaneurysms, fibrinoid necrosis, and small hemorrhages (Figure 3I). These microvascular changes have earlier been observed in HCHWA-D patients in which they correlate with the severity of the amyloid angiopathy.50 The perivascular amyloid plaques, however, were different in the AD/Fl and HCHWA-D brains. Whereas in AD/Fl, copious perivascular amyloid deposits were often seen in association with severely affected vessels, such deposits were minimal to absent in HCHWA-D. Even in HCHWA-D patients with one of the most severe degree of CAA, severely stenotic vessels were not shown to be associated with appreciable perivascular plaque pathology.27

Figure 5. Immunohistochemistry to differentiate Aβ40 from Aβ42 (A, C) or of full-length Aβ from N-truncated forms (B, D) in the neocortex of AD/Fl (A, B) and HCHWA-D patients (C, D). Aβ that stained with 4G8, but not with antibodies against the first five residues was interpreted as being N-truncated. Diffuse plaques were solely composed of N-truncated Aβ42 (blue in A and B and red in C and D), whereas SPs and CAs were predominantly composed of full-length Aβ40 (brown in A and B and purple in C and D). E: A higher magnification of the Aβ40 and Aβ42 distribution in SPs in an AD/Fl patient. F: Image analysis for Aβ42 and Aβ42 within SPs and CAs in AD-Fl patients, a single patient of HCHWA-D, two different PS1 mutations, and sporadic AD patients (n = 5 each), by antibodies FCA3340 and FCA3542. Other Aβ C-terminal antibodies offered similar results. Scale bars, 40 μm (A-E).
brains was observed with not only Ub(+) dystrophic neurites, but also AT8(+) dystrophic neurites, along with a prominent glial and inflammatory pathology (Table 2 and Figure 3, D, M, and N). Staining AD/FI brains with angiogenic markers such as vascular endothelial growth factor or basic fibroblast growth factor, a strong reactivity was observed in the vicinity of severely occluded vessels (Figure 3O). Diffuse plaques in AD/FI, similar to those described in other AD as well as in HCHWA-D patients, did not consistently associate with a neuritic, glial, inflammatory, or angiogenic pathology (Figure 3P).

Morphometric Analysis for AD/FI Brains

Perivascular plaques, especially those associated with amyloid-laden severely stenotic vessels (ALSSVs) had a striking resemblance to the coronal plaques. We used image analysis to assess sizes of SPs and ALSSVs, the latter defined as when the vessel lumen diameter/CAA diameter was less than or equal to one half. Both ALSSVs and SPs ranged from a few to 600 μm in diameter and the average diameter (±SD) of ALSSVs was 53.4 μm (±43.3) and not significantly different from that of the plaque cores (51.6 ± 48.8 μm, P = 0.3). Furthermore, comparing the ratios of the perivascular and coronal plaque areas to their respective total ALSSV and SP areas, the perivascular plaques constituted 43.2% (±16.4) of ALSSVs, which was not significantly different from the proportion of coronal deposits constituting SPs (49.2 ± 18.3%; P = 0.2) (Figure 4).

Predominant Aβ40 Composition of SPs

To identify the precise Aβ species deposited in brains of AD/FI patients, and also to explore the constitutional similarities of amyloid deposits between AD/FI, HCHWA-D, and PS1 and sporadic AD patients, serial brain sections were stained with antibodies specific for C-terminal Aβ40 or Aβ42, Aβ N-terminus, and the middle portion of Aβ (Aβ17-24; 4G8). Double immunohistochemistry for these antibodies in various combinations revealed a predominant Aβ(1-40) content of CAA and plaque cores, and a comparable reactivity for Aβ(1-40) and N-truncated Aβ42 in the perivascular and coronal plaques. Diffuse plaques in AD/FI were entirely composed of N-truncated Aβ42, again resembling diffuse plaques present in the familial and sporadic AD, as well as in HCHWA-D patients. Percentage areas of plaques stained by Aβ40 and Aβ42 were studied on serial brain sections using an image analysis program. Aβ40 was the predominant amyloid in vessels in AD/FI patients (77%), sporadic AD patients (67%), PS1 mutation carriers (60%), and HCHWA-D patients (71%). Aβ40 also constituted a major fraction of Aβ in parenchymal deposits in AD/FI patients (66%), but only a minor fraction in sporadic and PS1 AD (23% and 26%, respectively) and being completely absent in HCHWA-D patients (Figure 5).

Quantification of the relative amounts of Aβ42 and Aβ40 was done by MALDI-TOF mass spectrometry on Aβ immunoprecipitated from frozen AD/FI brain extracts with
4G8 that does not distinguish between Aβ40 and Aβ42. Full-length Aβ was the major peptide identified in SPs with a 25-fold abundance of Aβ(1-40) greater than Aβ(1-42). Mass spectrometric analysis of amyloid-laden ves-
sels extracted from the brains of Flemish and sporadic AD patients also showed respective 32- and 10-fold higher levels of Aβ(1-40) than Aβ(1-42). However, consistent with earlier observations, 52 analyses of different regions from familial and sporadic AD brains showed that diffuse plaques, but not SPs, were predominantly com-
posed of Aβ(1-42) and Aβ(11-42) (Figure 6).

Vasocentric SPs in the Flemish AD

Confocal laser-scanning microscopy using Aβ40- and Aβ42-specific antibodies showed all Aβ40(+) plaques to be positioned around microvessels (positive for CD31, CD34, smooth muscle actin, or C-IV). Three-dimensional reconstructions further displayed a close relationship of amyloid-laden vessels and plaque cores, and the abrupt development of CAA at their points of branching (Figure 7, A and B). Light microscopic examination of plastic 1-μm-thick serial sections stained with toluidine blue also demonstrated a close relationship of vessels with plaque cores (Figure 7C). Double immunohistochemistry for Aβ and vessel markers revealed the presence of a central or paracentral vessel within 68% of the 2400 SPs studied (Table 3; Figure 8), whereas the remaining were closely associated with the vascular basement membranes of comparatively large-caliber vessels. Most of the primitive plaques enclosed a plaque core or an amyloid-laden vessel in serial section analysis.

Serial ultra-thin section examination revealed a radial arrangement of Aβ fibrils projecting from the basement membrane into the surrounding neuropil as has been classically described for dyshoric angiopathy.53 With larger vessels, this phenomenon was often limited to only a part of the vessel and core-like compact structures seemed to evolve from the vascular basement mem-

branes (Figure 9). The gruel of these larger amyloid deposits or also amyloid deposited abluminally was amorphous, or haphazardly arranged in loose bundles, whereas the amyloid at core-periphery was radially ar-
ranged in filamentous bundles. As interpreted from light microscopic studies, the compact amyloid at the center bound more avidly to histochemical stains, whereas the peripheral radial spicules bound more intensely to Aβ antibody; the latter could be because of a relative accessibility of Aβ epitopes.

The noncongophilic diffuse plaques did not have any consistent relationship with vessels, although small amy-
lroid-laden vessels were sometimes noted in these plaque deposits, as has also been observed earlier.26 Many of these diffuse plaques were also associated with neurons (Figure 3P).

Table 3. Proportion of SPs Enclosing a Central or Paracentral Vessel Recognizable by Vessel Markers on Serial Section Analysis

<table>
<thead>
<tr>
<th>APP692 patients</th>
<th>Superior temporal cortex (n = 300, each)</th>
<th>Superior frontal cortex (n = 300, each)</th>
<th>Cerebellum (n = 200, each)</th>
<th>Total (n = 2400)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-4</td>
<td>212 (70.7%)</td>
<td>199 (66.3%)</td>
<td>169 (84.5%)</td>
<td>1626 (67.8%)</td>
</tr>
<tr>
<td>III-5</td>
<td>166 (55.3%)</td>
<td>176 (58.7%)</td>
<td>164 (82.0%)</td>
<td></td>
</tr>
<tr>
<td>III-13</td>
<td>204 (68.0%)</td>
<td>190 (63.3%)</td>
<td>146 (73.0%)</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

In this study, we first demonstrate that irrespective of the initial clinical presentations of stroke or progressive dementia, the end-stage neuropathology of APP692 patients is remarkably similar. In all these patients, unusually large SP cores and a severe degree of CAA are associated with severe neurofibrillary pathology in neocortical and limbic regions (reference 26 and this report). We next showed a predominant Aβ40 content of SPs in AD/FI brains by image and MALDI-TOF mass spectrometric analyses. These data are in sharp contrast to other familial and sporadic AD brains, where predominantly Aβ42 is deposited. In recently identified Iowa AD patients, appreciable amounts of Aβ40 were also noted in SPs in one patient, however, it remains to be studied whether Aβ40 similarly constitutes predominant amyloid deposit in these brains as well. This increase in deposited Aβ40 in patients with mutations near the α-secretase site might be in part because of an alteration of APP processing distinct from those caused

Figure 8. Serial section study by double immunohistochemistry with Aβ antibody 4G8 (blue) and a combination of endothelial cell marker CD31 and CD34 (brown), in superior frontal cortex (A), superior temporal cortex (B–C), and cerebellum (D), from patients IV-4 (A, B), IV-13 (C), and IV-5 (D). Almost all dense-core SPs shown here are associated with vessels, with indication that SPs might occur at the points of vascular branching (arrowheads). Scale bars, 40 μm.
by β- or γ-secretase site APP mutations. For instance, it has been shown that in contrast to the increased in vitro Aβ42/Aβ40 noted for the γ-secretase cleavage site-related APP mutations or mutations in PS,3,54 Flemish APP transfectants in CHO-K1 and H4 cells do not alter the relative levels of Aβ(1-40) and Aβ(1-42).55 This actually holds true for all N-truncated Aβ forms secreted from HEK-293 Flemish transfectants and analyzed by MALDI-TOF mass spectrometry (S Kumar-Singh, R Wang, C De Jonghe, and C Van Broeckhoven; unpublished results). Instead, the effect of the Flemish mutation is observed on Aβ N-terminus processing, in which relative to the wild type, an increase in Aβ(2-fold) and in Aβ N-truncated at F19 and F20 occurs.38 This has been proposed to be partly because of an increased BACE2 activity.39 In brains of AD/Fl patients, however, we identified only Aβ(1-40). To confirm that a minor proportion Aβ(1-42) and N-truncated forms of Aβ in AD/Fl brains was not because of their added aggregative property,6 we showed in the same set of experiments a predominance of highly fibrillogenic Aβ(1-42) and Aβ(1-42) in mutant PS1 brains, data consistent with a recent report.52 Thus, an unaltered Flemish APP processing at the Aβ C-terminus, leading to a normally occurring nine times higher proportion of Aβ40 correlates well with classical AD/Fl pathology in the form of a severe degree of CAA and large SP cores—the only deposits known to comprise predominantly Aβ40 in AD.12,15,26

The observed dimensional, morphological, and constitutional similarities between amyloid-laden vessels and SPs in AD/Fl further suggested that these Aβ deposits represent a spectrum of the same etiopathogenic process. Scholtz56 first observed that plaque cores were intimately related to material permeating from vessels. Many groups since then either suggested a vascular origin of SPs and/or diffuse plaques,57–63 or proved the contrary.64–68 (Figure 10). In this study, we convincingly demonstrated that at least in one form of AD, SPs but not diffuse plaques are centered on vessels. A proportion of amyloid cores enclosing a vessel refutes a coincidental relationship, for instance, the likelihood of endothelial cell proliferation into any established plaque cores is remote, taken the compactness of such structures and the toxic nature of amyloid on potential budding endothelial cells. In other words, 68% of SPs so addressed were ALSSVs examined paracentrally to their existing lumen. The remaining 32% were associated with vascular walls and further suggested that one of the components of vascular basement membrane seed the formation of these SPs.

Figure 9. Serial section study by electron microscopy. Examination of ultra-thin serial sections revealed a close link of SPs with vessels exemplified here in two series. A: Plaque P2 on follow-up was shown to have an eccentric vessel within the plaque core. Similarly plaque P1 was also shown in other sections to enclose a vessel within (not shown). B: Amyloid-free vessel followed serially was linked to an amyloid deposit (identified on semi-thin sections as SPs). Note the continuity of vascular basal lamina around compact amyloid (arrow). Scale bars, 20 μm.
But not Dutch, affects a string of amino acids (A/\text{model based on observations that the Flemish mutation,\text{ suggestion that vascular A/\text{FH252}} have also suggested that the aggregates formed by\text{alternatively, these might also represent A/\beta occcluding smaller vascular branches because CAA is shown to initiate at points of vascular branching.}\text{Interestingly, some of the aspects of Flemish AD patholology have been reproduced in vitro. It has been shown that Flemish A/\beta although aggregating slower than the wild type, however, progress to form exceptionally large and insoluble amorphous aggregates.}\text{Recent studies have also suggested that the aggregates formed by Flemish A/\beta are as neurotoxic as those formed by the wild-type A/\beta.}\text{An increase in the number and size of vascular Flemish A/\beta deposits is also supported by a model based on observations that the Flemish mutation, but not Dutch, affects a string of amino acids (A/\beta17-21) that govern the A/\beta nucleation-dependent polymerization process.}\text{However, the evolution of vascular A/\beta deposits in AD/FI brains to form SPs remains elusive and so is the precise mechanism of formation of CAA. The original suggestion that CAA forms entirely from vascular smooth muscle cells, remains disputed. An alternate suggestion that vascular A/\beta is derived from parenchymal sources draining with interstitial fluid along the periarterial pathways is supported by studies on transgenic mice in which neuron-derived A/\beta is sufficient to cause CAA.}\text{Formation of large SPs in AD/FI can thus be most convincingly explained by an increased neuronal secretion of Flemish A/\beta with slower aggregation kinetics, facilitating its extensive permeation along the interstitial fluid to form not only vascular deposits, but extensive perivascular/coronal deposits as well.}\text{Notwithstanding the role of CAA in causing neural toxicity through the formation of SPs, if one accepts a primary parenchymal amyloid pathology to instigate a secondary neuritic pathology, then severely affected amyloidotic vessels should also be directly capable of causing neuritic pathology and therefore progressive dementia. In a striking illustration, progressive senile dementia has also been described in two patients with APOE 4/4 genotype in the complete absence of amyloid plaques, but in the presence of a severe degree of CAA associated with both perivascular amyloid plaques and neurofibrillary pathology. Also, it was recently demonstrated that a severe degree of CAA and an increased number and size of SPs strongly correlates with mutations in PS1 after codon 200. In some of these mutations where CAA is prominent, both CAA and SPs are equally associated with all pathological hallmarks of AD.}\text{In this light, an equal association in AD/FI brains of SP and CAA with neurofibrillary, gliotic, and inflammatory pathology was not surprising. However, the precise reason for a complete absence of neurofibrillary degeneration in association with ThS(+) CAA in HCHWA-D patients remains elusive. It could either be because of the specific A/\beta mutation or the relative absence of perivascular amyloid noted in HCHWA-D vascular amyloidosis. If the latter is relevant, it might suggest that development of perivascular/coronal plaques could be a critical factor that temporally governs the development of neuronal toxicity and therefore clinical dementia. A time-dependent association of parenchymal amyloid to instigate a neuritic pathology occurs in patient IV-5. This patient showed a complete absence of neurofibrillary pathology at the time of biopsy despite the presence of abundant SP cores (associated sometimes with small-sized coronal plaques) and CAA,}\\text{however, on autopsy elicited a full-blown neuritic pathology in all neocortical and limbic regions analyzed.}\text{Besides a direct A/\beta induced toxicity, CAA is also known to cause cerebral hypoperfusion in AD. In disease in which CAA is prominent, vascular hypoperfusion could be sufficiently severe to impact on the final dementia phenotype. For instance, progressive dementia in a minority of HCHWA-D patients has been correlated with the most severe degree of CAA.}\\text{White matter lesions present in young APP692 presymptomatic carriers as well as a hypoxic neoangiogenic response as we show in this study, suggests a third mechanism by which CAA might cause progressive dementia syndrome in AD/FI patients.}\text{Acknowledgments}\text{We thank Drs. M. Maat-Schieman and R. A. C. Roos for the HCHWA-D specimen; Dr. Frédéric Checler for FCA3340 and FCA3542 antibodies; Dr. M. Mercken for antibodies JRF/A/\betaN/11, JRF/cAb40/6, and JRF/cAb42/12; Dr. P. Mehta for R209 and R226 antibodies; Dr. C. Labeur for A/\beta 12-42 wild-type, Flemish, and Dutch peptides; and Mr. A. Van Daele for writing software for the image analysis.}\text{References}\text{1. 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