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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 Aβ analyses, 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*.[3] The only known mutation at the APP β-cleavage site, the double-Swedish mutation (APP K670N/M671L),[4] 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 plaques[8–10] 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-

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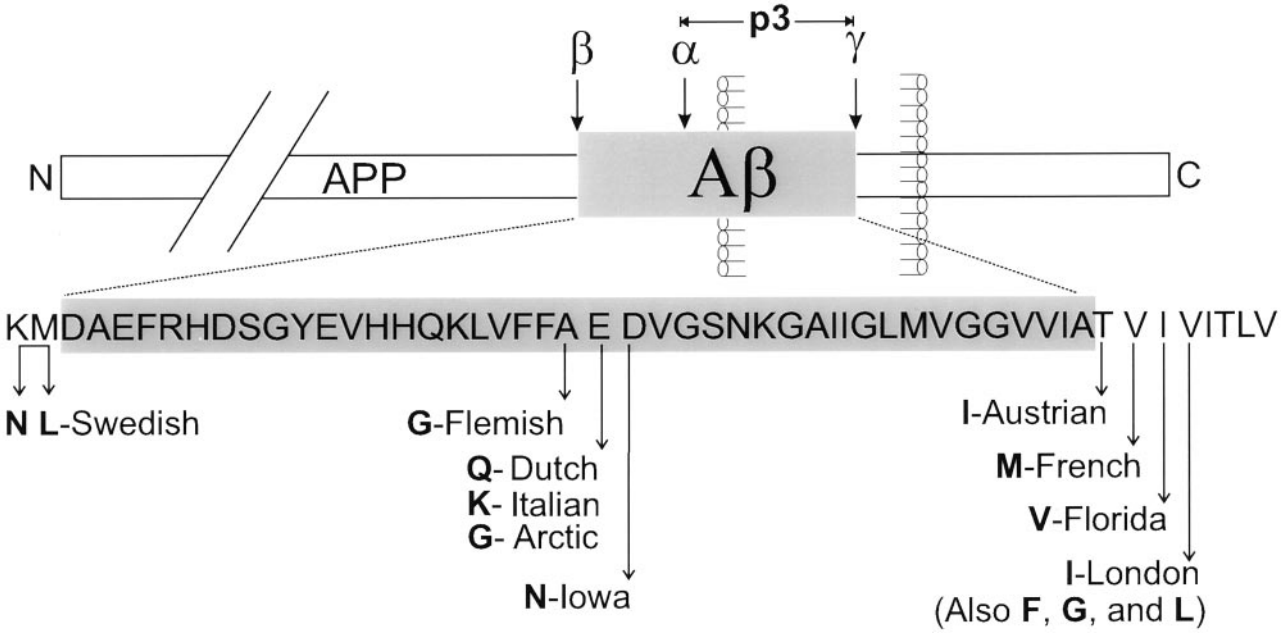


Figure 1. The position of APP mutations in relation to its major cleavage sites and Aβ. Other mutations can be assessed on frequently updated databases (*http://molgen-www.uia.ac.be/admutations* and *http://www.alzforum.org/members/resources/app_mutations/app_table. html*).

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.[1,12] The pathological relevance of SPs in AD pathology is further strengthened by Aβ vaccination strategies in a murine AD model in which their 50% reduction significantly reduces cognitive dysfunction.[13]

Congophilic or ThS(+) amyloid deposits in vessels with primary protein as Aβ (especially Aβ40) is the most common form of CAA.[14] CAA variably occurs in AD,[15] however, it is a predominant feature in diseases linked to APP α-cleavage site mutations as in the Dutch (E693Q),[16,17] Flemish (A692G),[18] Italian (E693K),[19] Arctic (E693G),[20] and Iowa (D694N)[21] mutations. The Dutch mutation carriers suffer from hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D), characterized by recurrent cerebral hemorrhage associated with parenchymal diffuse deposits, but only rarely SP or neurofibrillary tangle formation.[22–26] In a few HCHWA-D patients dementia also occurs, which correlates with the number of amyloid-laden severely stenotic vessels (ALSSVs).[27] However, progressive dementia is a pathological hallmark of Flemish AD (AD/Fl), characterized by SPs with the largest SP cores in AD, and a severe degree of neurofibrillary pathology.[18,26] Recently identified Arctic and Iowa mutations also present with clinical AD that has also been neuropathologically confirmed in one Iowa AD patient.[20,21]

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.[28,29] 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.[30–37] 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 β-secretase homologue, BACE 2.[38,39] In addition, the Flemish homologue fibrillizing slower than wild-type Aβ, forms larger and more stable, neurotoxic aggregates.[33,40,41]

The purpose of this study is twofold: First, never before has FAD associated with α-secretase site-related mutations been systematically analyzed for type of Aβ deposition. Second, because the plaque cores are the largest reported in AD/Fl,[26] 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.

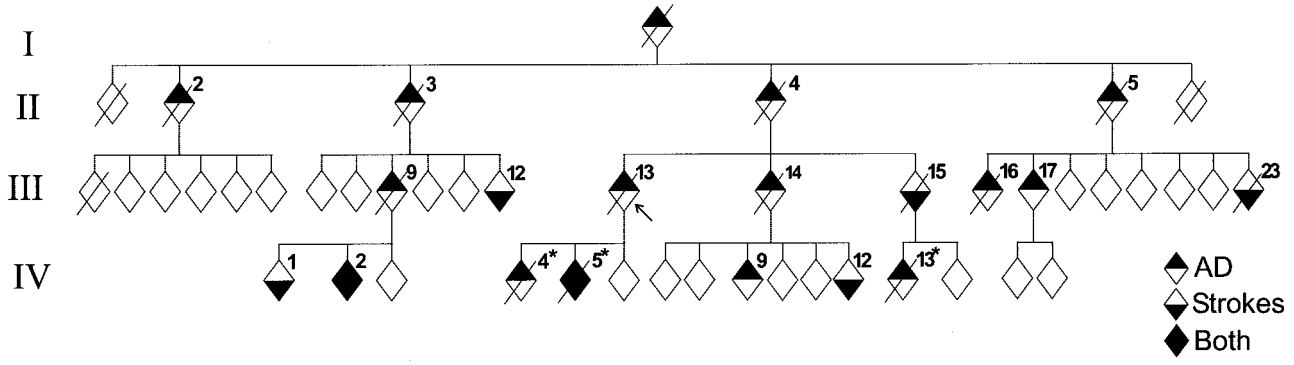


Figure 2. Family 1302 pedigree. The pedigree is disguised for reasons of confidentiality. Roman numbers are the generations and affected individuals are numbered on the upper corner. The proband is indicated with an **arrow** and an **asterisk** indicates individuals in which autopsy is performed. **Upper half-filled symbols** represent patients presenting with AD, and **lower half-filled symbols**, with cerebral hemorrhage. Few patients had strokes and were also diagnosed to have AD (**filled symbols**). Patients IV-4 and IV-13 have been previously confirmed to have AD pathologically.[26] Dementia in individual III-16 was because of unrelated etiology as the mutation was absent here.

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).[18] 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.[42] 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.[42] We earlier studied two patients who had clinical dementia and fulfilled the neuropathological criteria of AD (patients IV-4 and IV-13) (Table 1).[18,26,43] Here, we report the neuropathological analysis of an additional member of the family, patient IV-5, who had hemorrhagic stroke at age 42 years.[18] 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.[42] 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, midbrain with substantia nigra, pons, cerebellum, 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/Fl 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 immunohistological study of the SPs in three AD/Fl 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

APP692 patients	Biopsy	Autopsy	First presentation	AAO (years)	AD (Braak staging)	Severe degree of CAA	ApoE*	Reference
IV-4	−	+	Progressive dementia	49	+ (V/VI)	+	3/4	[26]
IV-5	+	+	Stroke	42	+ (V/VI)	+	3/4	[18] and this report
IV-13	−	+	Progressive dementia	48	+ (V/VI)	+	3/3	[26]

*There is no evidence that *APOE* modifies the disease onset for this family.[79] AAP, age at onset.

Table 2. Antibodies Used in the Characterization of SP in AD/Fl Patients

Antibody	Epitope/marker	Type	Dilution	Antigen retrieval	Reference
4G8	Aβ residues 17–24	IgG_{2b}	1:20,000	Formic acid	Senetek, Maryland Heights, MO
6E10	Aβ N-terminus; recognizes Aβ 5–11	IgG_1	1:10,000	Formic acid	Senetek
6F3D	Aβ N-terminus	IgG_1	1:25	Formic acid	Dako, Glostrup, Denmark
JRF/AβN/11	Aβ N-terminus	IgG_{2a}	1:500	Formic acid	Gift from M. Mercken
JRF/cAb40/6	Aβ40	IgG_{2a}	1:500	Formic acid	Gift from M. Mercken
R209	Aβ40	pAb-rabbit	1:400	Formic acid	Gift from P. Mehta
FCA3340	Aβ40	pAb-rabbit	1:150	Formic acid	Gift from F. Checkler
JRF/cAb42/12	Aβ42	IgG_1	1:500	Formic acid	Gift from M. Mercken
FCA3542	Aβ42	pAb-rabbit	1:250	Formic acid	Gift from F. Checkler
R226	Aβ42	pAb-rabbit	1:400	Formic acid	Gift from P. Mehta
AT8	Abnormally phosphorylated tau	IgG_1	1:20,000	—	Gift from Innogenetics, Zwijraarde, Belgium
Anti-CD31	Endothelium	IgG_1	1:100	Citrate buffer	JC70; Dako
Anti-CD34	Endothelium	IgG_1	1:100	Citrate buffer	QBEnd/10; Dako
Anti-SMA	Smooth muscle cell actin			Citrate buffer	Dako
Anti-collagen type IV	Vascular basement membrane	IgG_1	1:50	Citrate buffer	Dako
Anti-GFAP	Glial fibrillary acidic protein	IgG_1	1:1000	Citrate buffer	Dako
Anti-CD68	Macrophage	IgG_3	1:100	Pronase digestion	Dako
Anti-ubiquitin	Ubiquitin	pAb-rabbit		Citrate buffer	Dako
Anti-C1q complement	Complement cascade	pAb-rabbit	1:100	Citrate buffer	Dako
Anti-HLA-DP,DQ,DR	Complement cascade	IgG_1	1:100	Citrate buffer	Dako
Anti-VEGF	Angiogenesis	pAb-goat	1:100	Citrate buffer	R&D Systems, Abingdon, UK
Anti-bFGF	Angiogenesis	pAb-goat	1:100	Citrate buffer	R&D Systems

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 appearance exemplified (Figures 3 and 4), thus closely resembling such deposits in other familial and sporadic AD patients (Figure 3L).

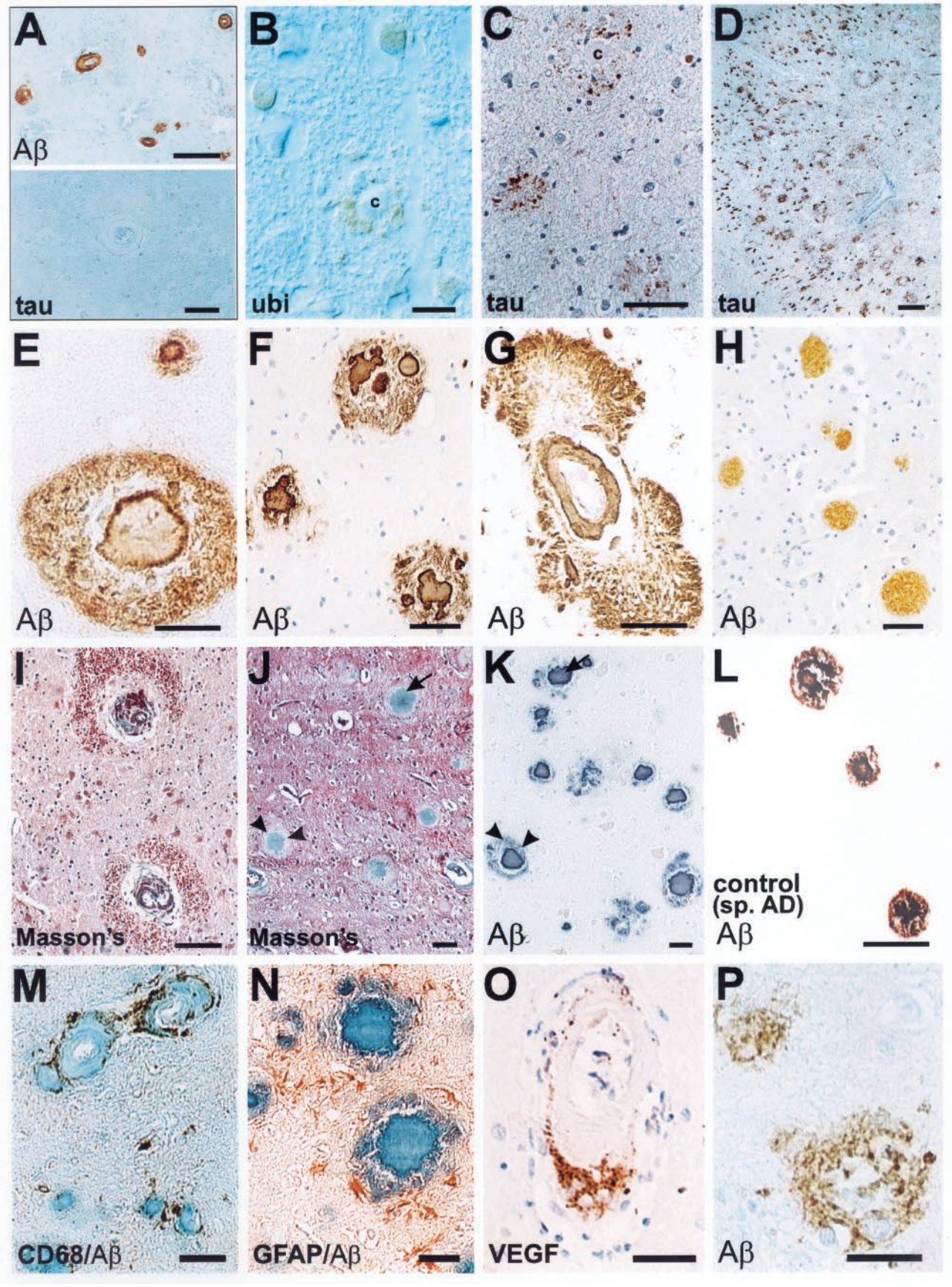
Antigen retrieval for Aβ immunohistochemistry was performed on sections treated in 98% formic acid for 5 minutes at room temperature. All dilutions were made in 0.1 mol/L of phosphate-buffered saline with 0.1% bovine serum albumin. Staining for single antigen was performed using streptavidin-biotin-horseradish peroxidase using chromogen 3′,3′diaminobenzidine (Roche Diagnostics, Vilvoorde, Belgium), described previously.[29] Immunohistochemistry involving detection of more than one antigen was done using species-specific or IgG subtype-specific secondary antibodies, conjugated directly to biotin, horseradish peroxidase, alkaline phosphatase, or galactosidase (Southern Biotechnology, Birmingham, AL). This was followed by color development using one of the following chromogens (acquired from Roche): 3′,3′diaminobenzidine, 3-amino-9-ethylcarbazole, Fast-red, 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium solution, or 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal). Different cycles of primary, secondary, and tertiary antibodies, and of chromogens were used during double-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

Figure 3. Flemish AD (AD/Fl) pathology. Biopsy analysis of the occipitocortical region of patient IV-5 stained for antibodies against 4G8 and AT8 (**A**) and ubiquitin (**B**). Autopsy analysis of the same patient of the occipitocortical region with AT8 (**C**) and of hippocampus with AT100 (**D**). Micrographs (**E–H**) illustrate the repertoire of ThS(+) plaques in AD/Fl stained with 4G8: a SP with a central plaque core surrounded by a coronal plaque (**E**). Note in the upper half of this illustration a small SP that otherwise resembles dyshoric angiopathy; multicentric SP (**F**); CAA with its perivascular plaque (**G**); and primitive plaques (**H**). **I:** A cortical region to demonstrate a recent hemorrhage associated with affected vessels. **J:** Histochemical stains bound avidly to SP dense core regions (**arrow**) but not coronal plaque (**arrowheads**; whereas with Aβ immunohistochemistry, the edges of the plaque core stained stronger compared to its centers (**K**; compare with **J**). A similar immunohistochemical pattern was observed in sporadic AD brain (**L**). **M:** Association of microglia (CD68, brown) with plaque cores as well as with amyloid-laden severely stenotic vessel (ALSSVs, blue). **N:** Association of astroglia (GFAP, red) with SP (blue). **O:** Vascular endothelial growth factor reactivity in association with an ALSSV. **P:** A neurocentric diffuse plaque. *c*, Plaque core. Scale bars: 50 μm (**A–C**); 100 μm (**D**); 40 μm (**E–P**).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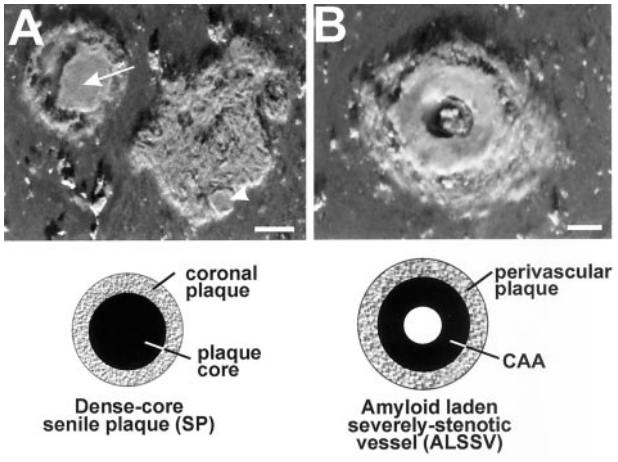

Figure 4. Morphometric image analysis in AD/Fl brains for SPs (**A**) and amyloid-laden severely stenotic vessels (ALSSVs) (**B**). Neither the sizes of CAAs and SPs, nor the percentage areas occupied by perivascular and coronal plaques in CAAs and SPs, respectively, were significantly different ($P = 0.2$). For this analysis, SPs with only one central core were considered (**arrow**) and atypical SPs, eg, with an eccentric core (**arrowhead**) were disregarded. Scale bars, 40 mm.

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 IgG_1 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 tetraoxide. 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, and 20 SPs were analyzed by a Philip CM10 electron microscope (Philip, Eindhoven, The Netherlands) equipped with a goniometric coordinator. 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 described[45] 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 described[46] 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 mm^2). 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(+) plaques were observed. AT8(+) dystrophic neurites and neuropil threads

were also present in varying severity in all regions except cerebellum, where only Ubi(+) dystrophic neurites were observed. Especially in the neocortical and hippocampal regions, both SPs and CAA were equally associated with Ubi(+) and AT8(+) dystrophic neurites in the surrounding parenchyma. Silver impregnation also recognized a large number of neurofibrillary tangles in hippocampus and neocortex. Based on the presence of tangles in all sectors of hippocampus and subiculum as well as a severe involvement of isocortex, the brain was assigned Braak and Braak[49] stage V/VI (Table 1 and Figure 3; A to D).

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 multicentric. 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 elsewhere[2] 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]

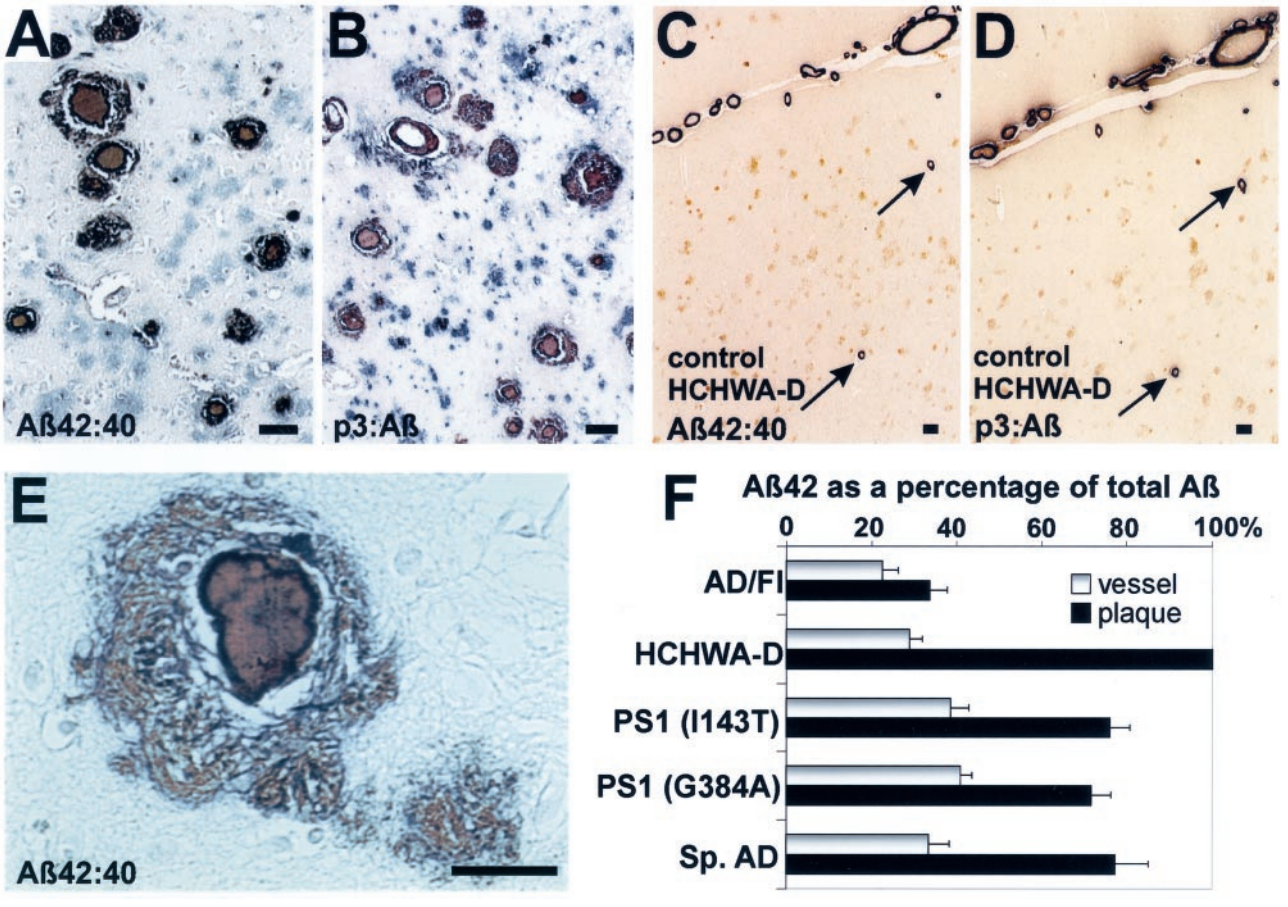

Figure 5. Immunohistochemistry to differentiate Aβ40 from Aβ42 (**A**, **C**) or of full-length Aβ from N-truncated forms (**B**, **D**) in the neocortex of AD/Fl (**A**, **B**) and HCHWA-D patients (**C**, **D**). Aβ that stained with 4G8, but not with antibodies against the first five residues was 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 CAAs 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 Aβ40 and Aβ42 within SPs and CAAs 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**).

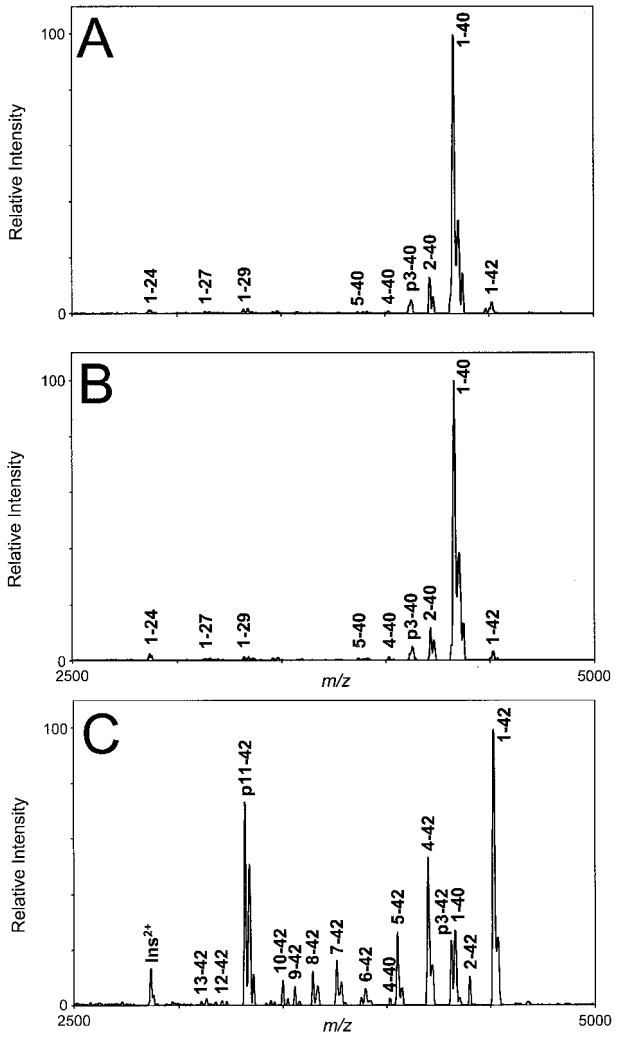

Figure 6. Formic acid extracts of Aβ deposited in brain, immunoprecipitated with 4G8, and analyzed by MALDI-TOF mass spectrometry. From an AD/Fl patient, Aβ extracted from (**A**) SPs and (**B**) CAAs revealed a major peak at Aβ(1-40). **C:** A familial AD (PS1 G384A) brain analysis for a neocortical region enclosing predominantly diffuse plaques. Peaks corresponding to Aβ are labeled with their amino acid sequence numbers. Peaks marked p3-x and p11-x represent peptides beginning with pyroglutamic acid (pyroGlu-3 or pyroGlu-11). Ins2+ peak corresponds to a doubly charged ion of insulin used for mass calibration.

We further observed a different binding pattern of SP subcomponents for histochemical and immunohistochemical reagents (Figure 3, J and K). The histochemical dyes such as Masson's trichrome bound less avidly to the coronal, perivascular, and the primitive plaques, but intensely to the plaque cores, especially to their centermost regions. By contrast, the most peripheral regions of the plaque cores, and also the coronal, perivascular, and primitive plaques, stained strongly with Aβ immunohistochemistry. Such Aβ reactivity pattern is not uncommon for SPs in sporadic or other familial AD.

We studied the association of different plaques in AD/Fl brains with hyperphosphorylated tau (AT8) and reactive glial pathology by double immunohistochemistry. As described earlier,[25,51] CAA in HCHWA-D was associated with only Ubi(+) dystrophic neurites. By contrast, an equal association of both SPs and CAA in AD/Fl brains was observed with not only Ubi(+) 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/Fl 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/Fl, similar to those described in other AD as well as in HCHWA-D patients,[2,23] did not consistently associate with a neuritic, glial, inflammatory, or angiogenic pathology (Figure 3P).

Morphometric Analysis for AD/Fl 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 ($\pm$SD) of ALSSVs was 53.4 μm ($\pm$43.3) and not significantly different from that of the plaque cores (51.6 $\pm$ 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% ($\pm$16.4) of ALSSVs, which was not significantly different from the proportion of coronal deposits constituting SPs (49.2 $\pm$ 18.3%; $P = 0.2$) (Figure 4).

Predominant Aβ40 Composition of SPs

To identify the precise Aβ species deposited in brains of AD/Fl patients, and also to explore the constitutional similarities of amyloid deposits between AD/Fl, 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/Fl 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.[5,8] 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/Fl 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/Fl 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/Fl brain extracts with

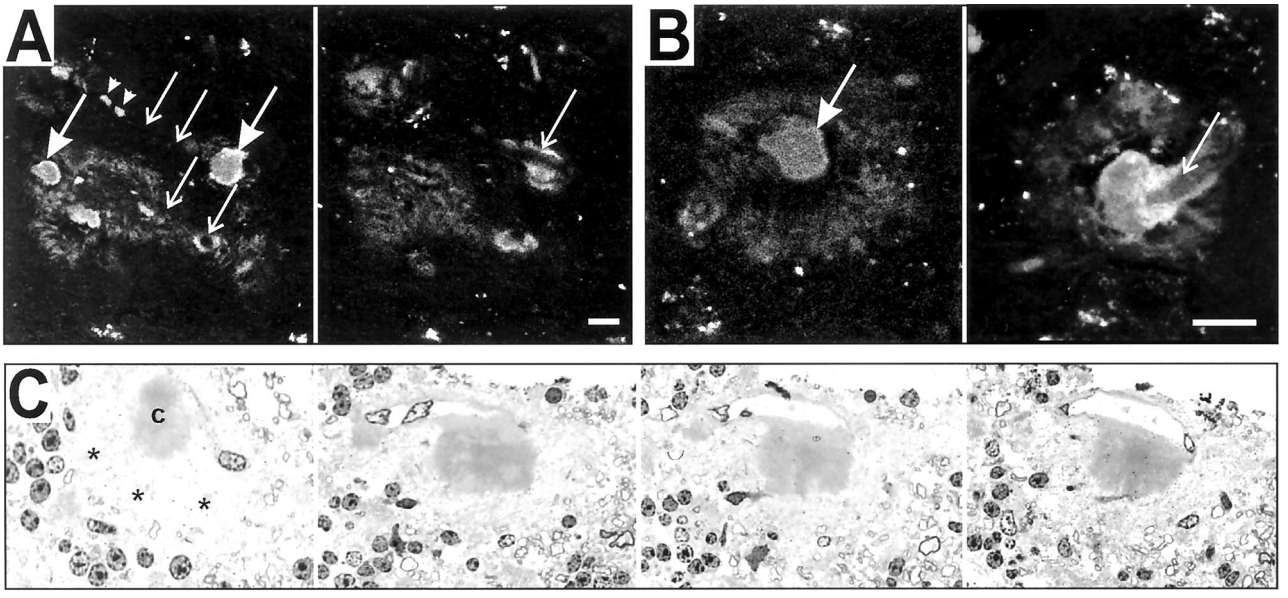

Figure 7. Confocal microscopic (**A**, **B**), and serial semi-thin plastic sections study (**C**). **A** and **B:** Neocortical regions demonstrating continuation of SP (**closed arrows**) into vessels (**open arrows**). Note that plaque cores develop at points of vessel branching (**arrowheads** in **A**). **C:** An example of SPs analyzed in the granular layer of cerebellum showing a vascular link. *c*, Plaque core; *, coronal region. Scale bars, 100 μm.

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 vessels 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 composed 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 membranes (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 arranged 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 amyloid-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

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

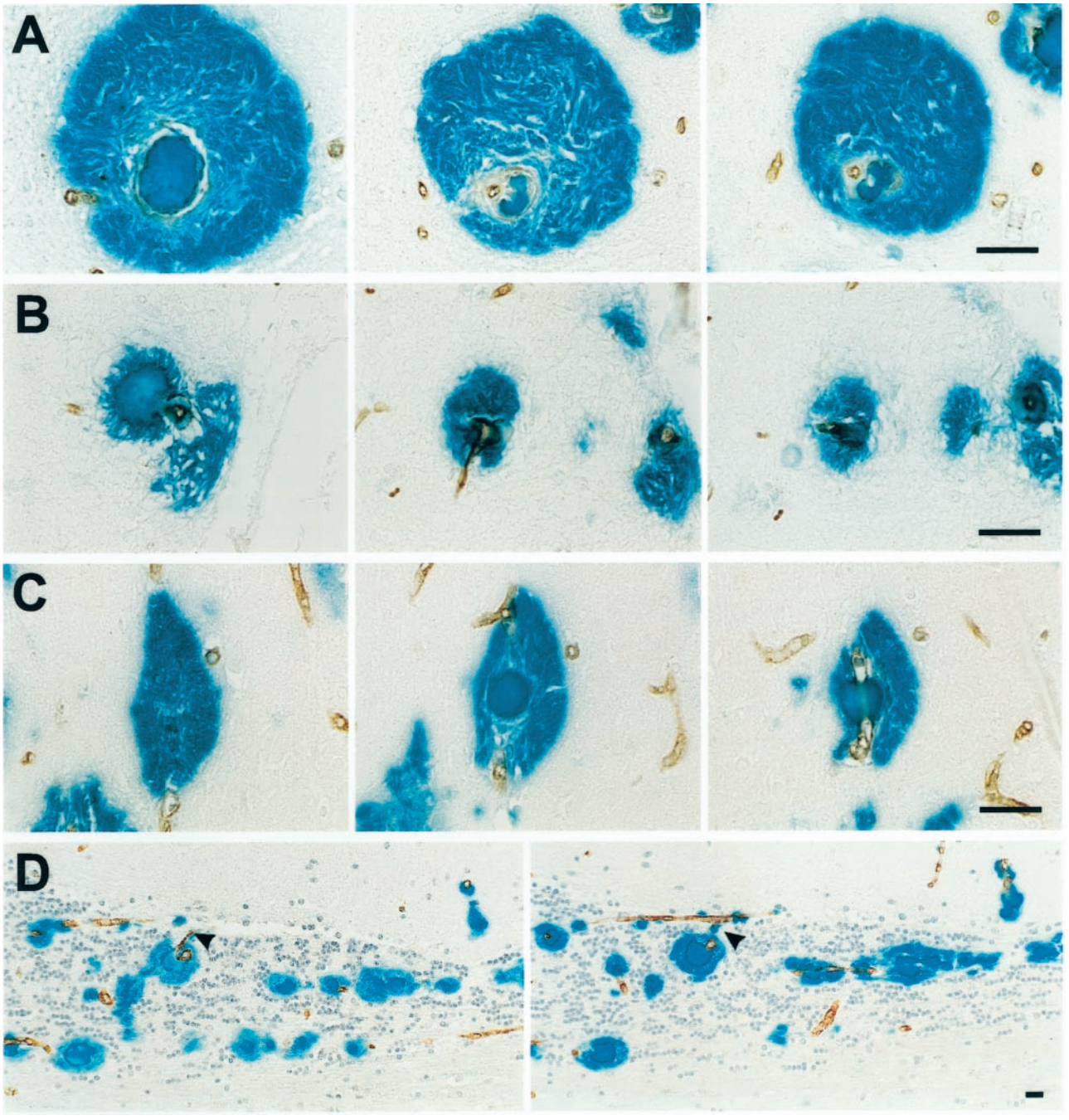

Figure 8. Serial section study by double immunohistochemistry with Aβ antibody 4G8 (blue) and a combination of endothelial cell marker CD31 and CD34 (brown), in superior frontal cortex (**A**), superior temporal cortex (**B–C**), and cerebellum (**D**), from patients IV-4 (**A, B**), IV-13 (**C**), and 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.

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.[3] In recently identified Iowa AD patients, appreciable amounts of Aβ40 were also noted in SPs in one patient,[21] 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

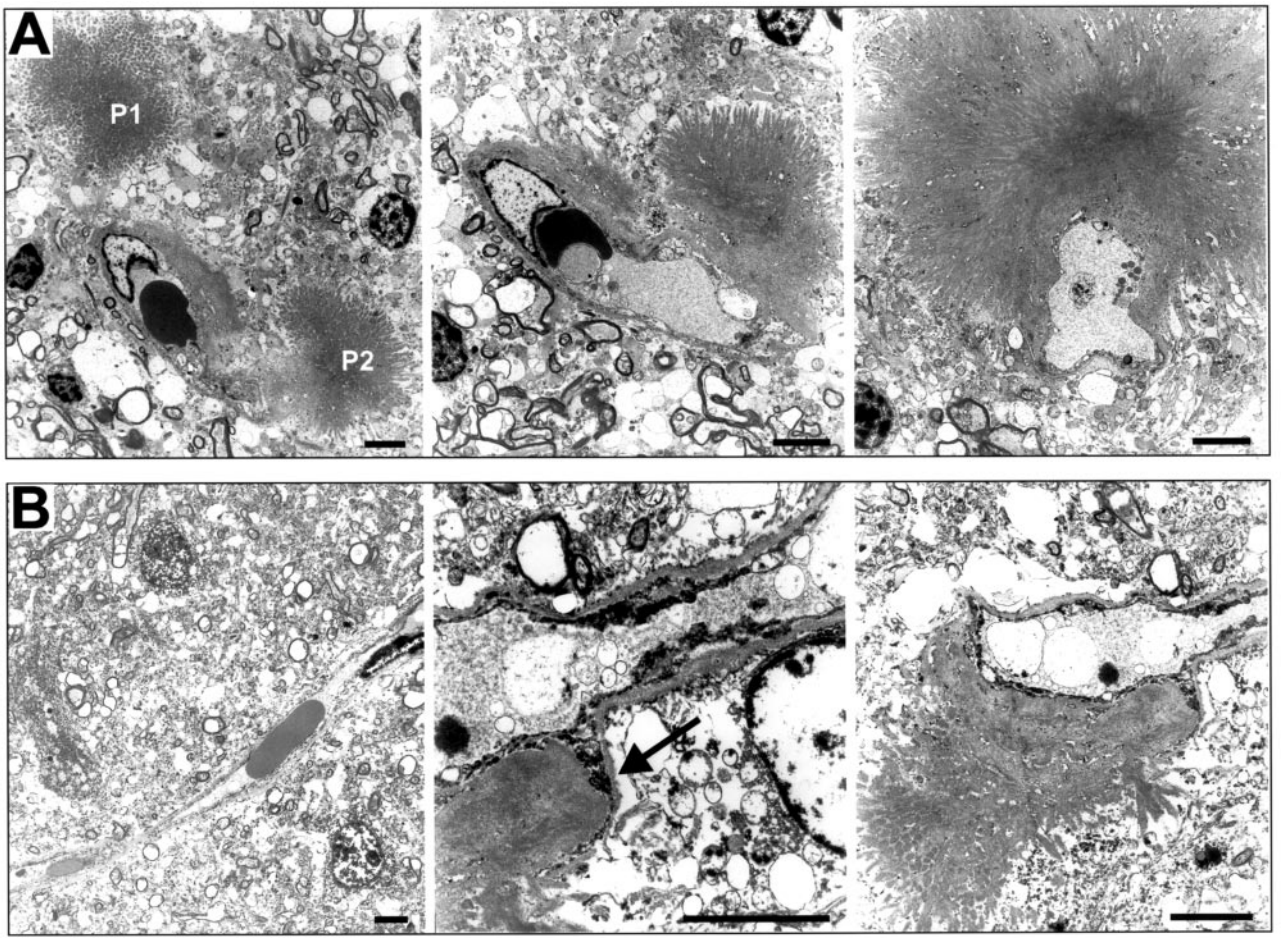

Figure 9. Serial section study by electron microscopy. Examination of ultra-thin serial sections revealed a close link of SPs with vessels exemplified here in two series. **A:** Plaque P2 on follow-up was shown to have an eccentric vessel within the plaque core. Similarly plaque P1 was also shown in 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.

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(11-42) 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. Scholtz[56] 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 co-incidental 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.

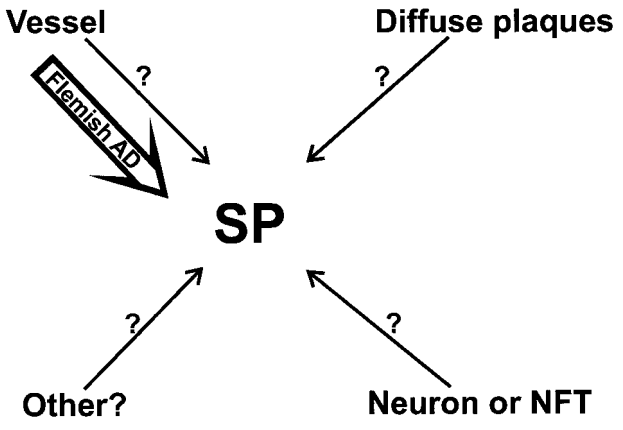

Figure 10. Multiple pathways involved in the formation of SPs. Although in AD/Fl amyloid-laden vessels give rise to these plaques, their contribution to other familial or sporadic AD is unknown.

Alternatively, these might also represent Aβ occluding smaller vascular branches because CAA is shown to initiate at points of vascular branching.[69]

Interestingly, some of the aspects of Flemish AD pathology have been reproduced *in vitro*. It has been shown that Flemish Aβ although aggregating slower than the wild type, however, progress to form exceptionally large and insoluble amorphous aggregates.[40] Recent studies have also suggested that the aggregates formed by Flemish Aβ are as neurotoxic as those formed by the wild-type Aβ.[41] An increase in the number and size of vascular Flemish Aβ deposits is also supported by a model based on observations that the Flemish mutation, but not Dutch, affects a string of amino acids (Aβ17-21) that govern the Aβ nucleation-dependent polymerization process.[19,70] However, the evolution of vascular Aβ deposits in AD/Fl 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,[71] remains disputed.[72] An alternate suggestion that vascular Aβ is derived from parenchymal sources draining with interstitial fluid along the periarterial pathways[72] is supported by studies on transgenic mice in which neuron-derived Aβ is sufficient to cause CAA.[73] Formation of large SPs in AD/Fl can thus be most convincingly explained by an increased neuronal secretion of Flemish Aβ 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.

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.[74] 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.[75] In some of these mutations where CAA is prominent, both CAA and SPs are equally associated with all pathological hallmarks of AD.[76] In this light, an equal association in AD/Fl 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 Aβ 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[77] 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,[18] however, on autopsy elicited a full-blown neuritic pathology in all neocortical and limbic regions analyzed.

Besides a direct Aβ induced toxicity, CAA is also known to cause cerebral hypoperfusion in AD.[78] 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.[27] White matter lesions present in young APP692 presymptomatic carriers[42] 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/Fl patients.

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