

Nonoverlapping but synergetic tau and APP pathologies in sporadic Alzheimer's disease

A. Delacourte, PhD; N. Sergeant, PhD; D. Champain, BSc; A. Watzet, BSc; C.-A. Maurage, MD; F. Lebert, MD; F. Pasquier, MD; and J.-P. David, MD

Abstract—Objective: To determine the spatiotemporal mapping of tau pathologies and insoluble pools of A β in aging and sporadic AD, and their contribution to the physiopathologic, clinical, and neuropathologic features. **Methods:** The authors studied 130 patients of various ages and different cognitive status, from nondemented controls (n = 60) to patients with severe definite AD (n = 70) who were followed prospectively. Insoluble A β 42 and 40 species were fully solubilized and quantified in the main neocortical areas, with a new procedure adapted to human brain tissue. Tau pathology staging was determined in 10 different brain areas, using Western blots. **Results:** In AD, there is a constellation of amyloid phenotypes, extending from cases with exclusively aggregated A β 42 to cases with, in addition, large quantities of insoluble A β 40 species. Five other points were observed: 1) There was no spatial and temporal overlap in the distribution of these two insoluble A β species in cortical brain areas. 2) In contrast to solubilized A β 40 aggregates composed essentially of monomers and dimers, solubilized A β 42 was essentially observed as dimers and multimers. 3) A β 42 aggregates were observed at the early stages of tau pathology, whereas the insoluble A β 40 pool was found at the last stages. 4) During the progression of the disease, A β aggregates increase in quantity and heterogeneity, in close parallel to the extension of tau pathology. 5) There was no spatial overlap between A β aggregation that is widespread and heterogeneously distributed in cortical areas and tau pathology that is progressing sequentially, stereotypically, and hierarchically. **Conclusions:** These observations demonstrate that A β 42 aggregation, and not A β 40, is the marker that is close to Alzheimer etiology. It should be the main target for the early biological diagnosis of AD and modeling. Furthermore, the spatial mismatch between amyloid β -precursor protein (APP) and tau pathologies in cortical brain areas demonstrates that neurodegeneration is not a direct consequence of extracellular A β neurotoxicity. Hence, there is a synergetic effect of APP dysfunction, revealed by A β aggregation, on the neuron-to-neuron propagation of tau pathology.

NEUROLOGY 2002;59:398–407

AD is a neurodegenerative disorder characterized by the coexistence of two degenerating processes: amyloidosis and tau pathology. Amyloidosis corresponds to the extracellular aggregation of A β peptides into amyloid plaques.¹ Tau pathology, also named tauopathy, corresponds to the intraneuronal association of tau proteins into abnormal filaments.^{2,3} Amyloidosis is closely related to etiology¹ and tau pathology is strongly correlated to the clinical expression of the disease.^{4–10} Little is known about the relationship between amyloid β -precursor protein (APP) and tau pathologies, which is the missing link in fully understanding AD. Indeed, although the pathway of tau pathology is very precise in the brain of AD patients,^{6,7} amyloidosis seems to be more heterogeneously and randomly distributed.⁸ A β peptides derive from the catabolism of a large transmembrane

glycoprotein precursor (APP). Molecular heterogeneity of APP processing that generates A β peptides results from different types of mutations in familial autosomal dominant AD (FAD), located near the beta or the gamma cleavage sites.¹¹ These different pathogenic mutations can be modeled in transgenic mice.¹² However, AD is nonfamilial in more than 99% of patients, according to a large scale population study.¹³ The formation and turnover of amyloid deposits in the human brain are essentially known through immunohistochemical techniques,¹⁴ because a biochemical quantification is not easy, as these amyloid deposits are extremely insoluble, even in harsh detergents.¹⁵ ELISA immunoassays (EIA) and Western blot techniques were used to quantify soluble and insoluble A β species accessible to this technique.^{16–19} Together, all these studies tend to demonstrate that in the human brain, amyloidosis is observed first as diffuse aggregates of A β 42 peptides, which accumulate progressively as amyloid plaques, followed by the deposition of A β 40 pep-

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the August 13 issue to find the title link for this article.

From Unité Inserm 422 (Drs. Delacourte, Sergeant, and David, and A. Watzet), Lille; and CH&U, EA2691, and ADERMA (Drs. Maurage, Lebert, and Pasquier), Salengro Hospital, Lille, France.

Received June 19, 2001. Accepted in final form May 17, 2002.

Address correspondence and reprint requests to Dr. A. Delacourte, Unité Inserm 422, 1, Place de Verdun, 59045 Lille Cedex, France; e-mail: delacourte@lille.inserm.fr

tides. A microglial cell proteolysis of A β 42 into A β 40 species has also been suggested.²⁰ The latter peptides are also observed in large quantities in the cerebral vessel walls, to constitute amyloid angiopathy, which is found in variable amounts in AD brains.^{14,21} A good correlation between soluble A β and the severity of the disease has been reported, although surprisingly this was not observed for the most insoluble fraction. Indeed, the insoluble pool of A β corresponds to one of the two brain lesions that characterize the disease. Furthermore, the definition of what is an insoluble pool of A β is different according to the studies, in that different extraction buffers were used (guanidinium, urea, urea + sodium dodecyl sulfate [SDS], different concentrations of formic acid) as well as different centrifugation forces to pellet the insoluble material.

We have developed a reproducible biochemical method to analyze at the quantitative and qualitative levels the amyloid deposits in aging and AD. This method is easy to perform, efficient in that it allows a complete solubilization of all A β aggregates, and informative. Quantification of A β 42 and 40 aggregates was performed in four brain regions of 65 cases from our prospective study, and compared to the quantification of tau pathology. This allows a better description of the natural and molecular history of AD and helps to set up strategies for diagnostic and therapeutic approaches. Here we show first that there is a good correlation between the amounts of insoluble A β 42 and the progression of the disease, and second that there is a synergetic interaction between APP and tau pathologies, despite their different spatiotemporal distribution.

Materials and methods. *Patients.* The 60 nondemented and 70 demented patients were from the geriatric department of E. Roux Hospital at Limeil-Brevannes and the Lille CH&U Hospital, France, as described in reference 7. They represent all patients who were hospitalized for various disorders and died at this hospital, excluding those whose family opposed autopsy, or for whom postmortem delay was more than 24 hours. Clinical data were detailed in reference 7. Cognitive status was evaluated using the Mini-Mental State Examination (MMSE) and the Clinical Dementing Rating (CDR) score. Clinical criteria for dementia were based on Diagnostic and Statistical Manual of Mental Disorders, 3rd ed, rev; for AD, National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer’s Disease and Related Disorders Association; for vascular dementia, National Institute of Neurological Disorders and Stroke–Association Internationale pour la Recherche et l’Enseignement en Neurosciences; and for mixed dementia, Hachinski score. Clinical diagnosis was summarized as AD (possible, probable), vascular dementia, mixed dementia (AD with a strong vascular involvement revealed by investigations), or dementia (for patients with an uncertain clinical diagnosis). Clinical data about the patients who were analyzed here in detail for their amyloid content are summarized in table e1 (available online at www.neurology.org). Similarly, neuropathologic data for each patient were reported in the previous study (amyloid

plaques,²² amyloid angiopathy, Braak stages of neurofibrillary degeneration,²² vascular pathology, other lesions), and are summarized in table e1 (available online at www.neurology.org).

Mice. The APP Swedish mouse brain tissue was a generous gift of Dr. M. Staufenbiel.²³ Twenty-four-month-old mice containing the highest amounts of amyloid deposits, as observed at the immunohistochemical level, were studied.

Biochemical studies. Amyloid extraction. A total of 100 mg of brain tissue was homogenized in 1 mL of pure formic acid. Two microliters of the brain homogenate were used for the dot-blotting, or 50 μ L were evaporated under nitrogen, solubilized in 50 μ L of the SDS sample buffer (5% SDS, 20% glycerol, 2% β -mercaptoethanol, 150 mM Tris-HCl pH 6.8), and boiled 10 minutes before electrophoresis. A total of 10 μ L (100 μ g of protein) were loaded per well.

Immunologic probes. Tau pathology was revealed with AD2, a monoclonal antibody (mAb) against paired helical filaments that is directed against phosphorylated Tau proteins²⁴ and quantified according to reference 7. Amyloid plaques and aggregated A β peptides were detected using rabbit polyclonal antisera, named ADA40 and ADA42, generated against synthetic peptides corresponding to the seven last carboxy-terminal amino acids of A β 40 and 42. The specificity of these antibodies was checked by absorption with the corresponding synthetic A β 1-40 and 1-42 (Bachem, Bubendorf, Switzerland), by the specific labeling of these commercial peptides at concentration up to 500 ng, both using dot-blot and electrophoresis. Their immunoreactivity, specificity, and sensitivity were similar to the well-characterized FC3542 and FC3340 antibodies.¹⁴ A β 42 species were also specifically detected using the monoclonal antibody 21F12 that recognized the 42, 43 carboxy-terminal end of A β .²⁵ A β 40 and 42 species were also analyzed using the monoclonal antibody WO2 against the amino acid 4 to 10 of A β .²⁶

Immunoblots. Tau pathology was investigated as already described.⁷ Amyloid pathology was analyzed using electrophoresis adapted to the separation of small peptides.²⁷ After the proteins were blotted on nitrocellulose membranes, the upper part was reacted with AD2 for the estimation of tau pathology. The lower part of the membranes was reacted first with ADA40 for the detection of A β 40 and then, after stripping, with 21F12 or ADA42 for the detection of A β 42 species.

Dot-blot analyses. Amyloid was detected and quantified by dot-blot, using the procedure described in reference 28. The centrifugation step was removed and formic acid homogenates were directly loaded on the polyvinylidene fluoride (PVDF) membrane.

Image analysis of immunoblot and dot-blot quantification. Immunoblots and dot-blots were analyzed using the ImageMaster 1D Elite software (Amersham-Pharmacia, Orsay, France). The quantification is expressed in micrograms per gram of tissue, using A β 1-40 or A β 1-42 synthetic peptides (Bachem) as standards.

Results. *Quantification of A β aggregates.* Human brain amyloid is extremely difficult to dissociate and solubilize. A β species from the human brain tissue were extracted with different lysis buffers: SDS, guanidinium chloride, urea, urea + SDS, and formic acid (FA), at differ-

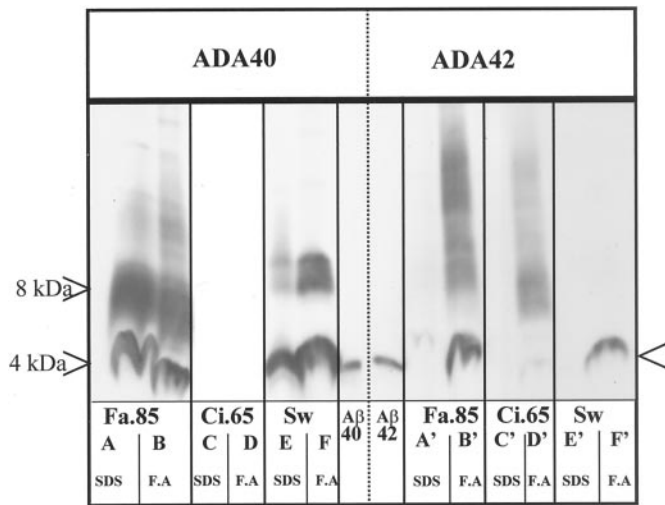


Figure 1. Western blot detection of A β 40 and 42 species in brain homogenates from Alzheimer patients and APP Swedish mice. The brain tissue was homogenized in a sodium dodecyl sulphate (SDS) buffer, either directly or after a formic acid (FA) treatment, and then solubilized A β peptides were resolved by SDS gel electrophoresis. A β 40 and 42 peptides were immunolabeled with specific polyclonal antibodies against A β 40 (ADA40, left part) and A β 42 (ADA42, right part). A total of 12.5 μ g of synthetic A β 40 and 42 were loaded in the central wells. Note that for patient Fa.85, huge amounts of A β 40 monomers and dimers, solubilized in SDS (A) or after FA treatment (B) were detected, whereas A β 42 aggregates, poorly solubilized in SDS (A') but well extracted with FA, were detected as monomers, dimers, and oligomers (B'). The other patient, Ci.65, had exclusively A β 42 deposits, detected only after FA treatment (D') as dimers and mainly multimers. Large amounts of A β 40 were detected in APP^{Sw} mice brain homogenized in SDS buffer (E) as well as after FA treatment (F), and low amounts of A β 42 species (F') were detected after FA extraction.

ent concentrations. EDTA or EGTA, which improve the solubilization of physiologic A β ,²⁹ did not yield higher extracted amounts of A β peptides. Different anti-A β antibodies, from our laboratory or commercially available, were compared in order to select the best immunologic probes for the detection of solubilized A β aggregates on Western blots or dot-blots. Among the antisera directed against different regions of A β , the most reactive were against the carboxy-terminal part of A β peptides. Our antibodies ADA42 and ADA40 were the most specific and sensitive to detect A β aggregates (estimated sensitivity of less than 1 μ g/g of wet tissue). The use of pure FA was necessary to extract completely A β aggregates, and especially A β 42 species, which were the most insoluble. The quantities of A β extracted with our method were very different from one brain to another brain, and between different areas from the same brain (figure 1).

For Alzheimer patient Fa.85 (see table e1 at www.neurology.org), the Western blot analysis of a SDS brain extract revealed the presence of A β monomers and dimers (A), composed essentially of A β 40 species, as they were detected by ADA40 (A), but not by ADA42 (A') (see figure 1). FA treatment dramatically improved the solubilization

of A β aggregates into monomers, dimers, and multimers (B). A β 42 variants were almost exclusively detected in FA extracts, but not after SDS extraction, and mostly consisted of dimers and multimers (B'). The biochemical profile of A β aggregates was very heterogeneous among Alzheimer patients. For instance, Alzheimer patient Ci.65 had no SDS-soluble A β variants (C, C'). Insoluble A β variants extracted with FA treatment (D, D') were only detected with ADA 42 (D'), and consisted almost exclusively of A β 42 dimers and multimers. No immunodetection of A β species was found in controls of different ages who were selected for their absence of amyloid deposits at the neuropathologic level (not shown). This demonstrates that our method quantifies A β aggregates and not physiologic A β .

A β variants from 24-month-old APP Swedish (APP^{Sw}) transgenic mice with a huge burden of amyloid plaques were quantified on Western blots, using our specific anti A β 40 and A β 42 antibodies (see figure 1). With ADA40, a strong labeling of 4 kDa (monomers) and 8 kDa A β (dimers) bands were detected in SDS extracts, with no smears (E). A similar labeling was observed with FA extracts (F) demonstrating that amyloid aggregates of APP^{Sw} mice are completely SDS soluble and essentially composed of A β 40 monomers and dimers. A β 42 were detected as traces in SDS and weakly in FA extracts, but were in lower amounts than A β 40 species (E', F').

A comparison with other commercially available mAb was performed. In our hands, WO2 was the most sensitive antibody for the detection of both synthetic A β 40 and 42 species, in good agreement with the literature.¹⁹ However, it failed to detect the most insoluble human A β species, while it detected strongly A β products generated by transgenic mice with the Swedish mutation, as shown in figure 2. The absence of detection of a large pool of human insoluble A β material by WO2 is due to the fact that this antibody is directed against the N-terminal part of A β , which is truncated and chemically modified in the most insoluble aggregated A β species. This pool is essentially composed of N-truncated A β 42.^{30,31} The very insoluble A β material labeled by ADA42, but not by WO2, is strongly detected by 21F12, a mAb directed against the carboxy terminal part of A β 42, 43.²⁵

Together, this comparison of immunologic probes led us to use mAb 21F12, a commercially available antibody against A β 42, following ADA40, a polyclonal antibody against A β 40, to detect with a good specificity and sensitivity all A β species on our Western blots (figure 3).

Different patterns of amyloidosis in Alzheimer cases. The different biochemical signatures of insoluble A β were investigated further. To understand better the physiopathologic significance of these different patterns, we quantified A β aggregates and tau pathology in different brain areas, randomly chosen among the 130 cases (60 nondemented, 70 demented patients) of our prospective study⁷ (see figure 3).

Alzheimer patients presented contrasted A β phenotypes. Some cases were characterized by their almost pure and large amounts of insoluble A β 42 variants (see figure 3A', Case Ci.65). Other cases had moderate (see figure 3, Case Fa.85) to large amounts of insoluble A β 40 (see figure 3B', Cases Gs.86, My.74, Pn.70). A second type of heterogeneity was found in the cerebral distribution of aggregated A β variants, which were sometimes in larger quantities

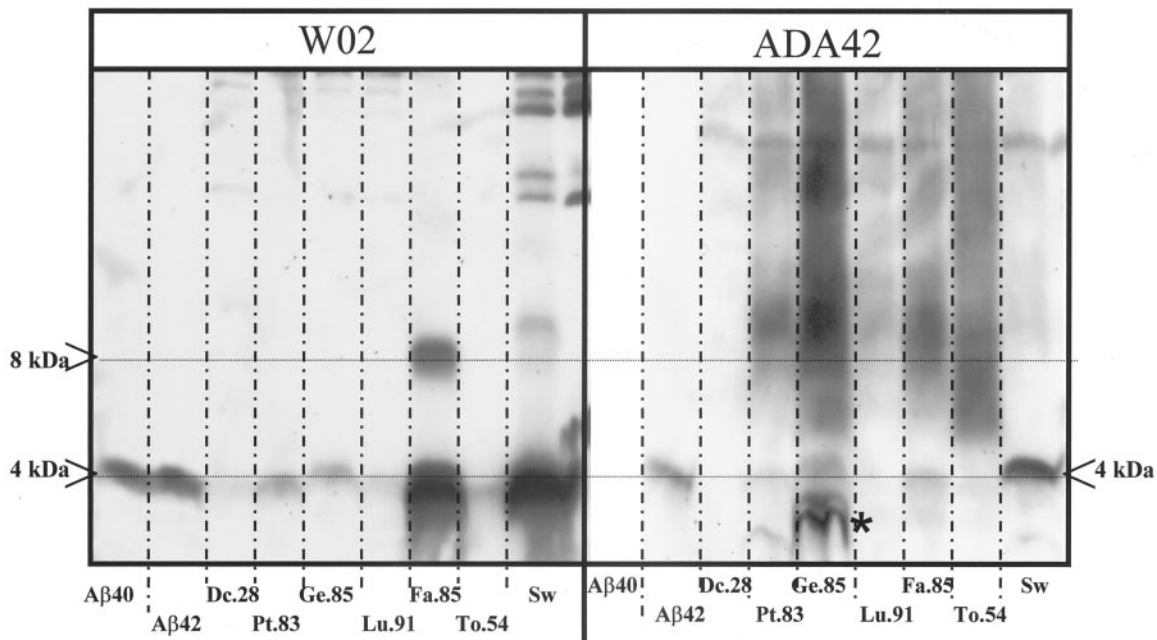


Figure 2. A total of 10 ng of synthetic A β peptides were loaded as internal controls. Fifty micrograms of formic acid extracted proteins from the frontal cortex of different patients were loaded. After transfer, the membrane was reacted and immunostained successively by W02 and ADA42. Note the strong detection of both synthetic A β 40 and 42 peptides and the major detection of 4 kDa A β by W02, whereas ADA42 immunodetected A β multimers and smears in infraclinical and clinical Alzheimer brain extracts (all cases apart from Dc.28, a young control). A β overproduced by APP^{S_w} transgenic mice were better detected by W02. Shorter A β species are detected with ADA42, demonstrating that they are N-terminally truncated (see * in lane Ge.85). Fa.85 is a last stage Alzheimer patient with similar amounts of insoluble A β 40 and 42 (table e1 [at www.neurology.org]; see figure 1). A β 40 monomers and dimers are well detected by W02, whereas the A β 42 multimers are not, but are detected by ADA42. To.54 is a late stage Alzheimer patient with intense neurofibrillary degeneration (see table e1 [at www.neurology.org]) who has essentially insoluble A β 42, with almost no A β 42 monomers and exclusively multimers. The latter are well detected by 21F12, but not by W02.

either in the temporal or the parietal or the occipital cortex (see figure 3, A', B'). In general, the frontal pole contained less A β aggregates, while more insoluble A β 40 variants were found in the occipital region. A third type of heterogeneity was the absence of overlap between A β 40 and 42 aggregates in some cases. For a given brain, the two aggregated A β species mapped differently, as shown for Case Fa.85, where insoluble A β 42 was in larger quantities in the occipital cortex while insoluble A β 40 was found essentially in the frontal and parietal cortex (see figure 3, A', B'). A fourth type of heterogeneity was revealed by the detailed electrophoretic pattern of A β deposits. Interestingly, solubilized A β 42 variants mainly consisted of dimers and multimers (figure 3A') whereas solubilized A β 40 consisted mainly of monomers and dimers (figure 3B'). A fifth type of heterogeneity was observed in the relationship between the distribution of tau and A β pathologies. Insoluble A β was heterogeneously distributed in cortical areas, but frequently found in more important quantities in the occipital cortex. However, the occipital cortex was always the less affected brain area by tau pathology.

We also investigated the biochemical patterns of amyloidosis in nondemented patients. In good agreement with our previous studies,⁷ we found a few aged cases with no trace of A β aggregates, despite the presence of tau pathology (see figure 3A, Cases Bl.93 and Hn.95). Patients up to stage 7 of tau pathology had almost exclusively A β 42 aggregates (see figure 3A, Cases Pt.83, As.85, Ge.85).

Biochemical quantification of amyloid deposits. We quantified A β aggregates by dot-blot and Western blots, using A β 40 and 42 synthetic peptides as standards. Results obtained with both approaches were similar. The dot-blot technique was faster,²⁸ whereas the Western blot approach gave additional qualitative values on the content of A β aggregates, such as the percentage of monomers, dimers, and multimers, as well as more specificity. Values obtained with these methods are reported in table e1 (at www.neurology.org) and figure 4. We analyzed at random at least three cases per Alzheimer stage of tau pathology, as defined in reference 7. With this approach, we found insoluble A β concentration ranging from 25 to 1250 μ g per gram of human brain tissue from Alzheimer patients. Then, the quantification was performed in the four main brain areas of nondemented and Alzheimer patients. Our results demonstrate the strong heterogeneity of A β aggregates, in quantity and quality, with a A β 42/40 ratio that varied from infinite (absence of A β 40) to 0.4. For patients with a tau pathology restricted to the hippocampal area, such as Pt.83 and Ds.59, the amyloid burden was in the range of 2 to 145 μ g of insoluble A β 42 peptide per gram of wet tissue. For these patients, insoluble A β 40 species were not detected.

Using the same approach, we found A β insoluble material in APP^{S_w} mice at a concentration of 750 μ g per gram of brain tissue, which were almost totally SDS soluble and essentially composed of A β 40 peptide aggregates (figure 1).

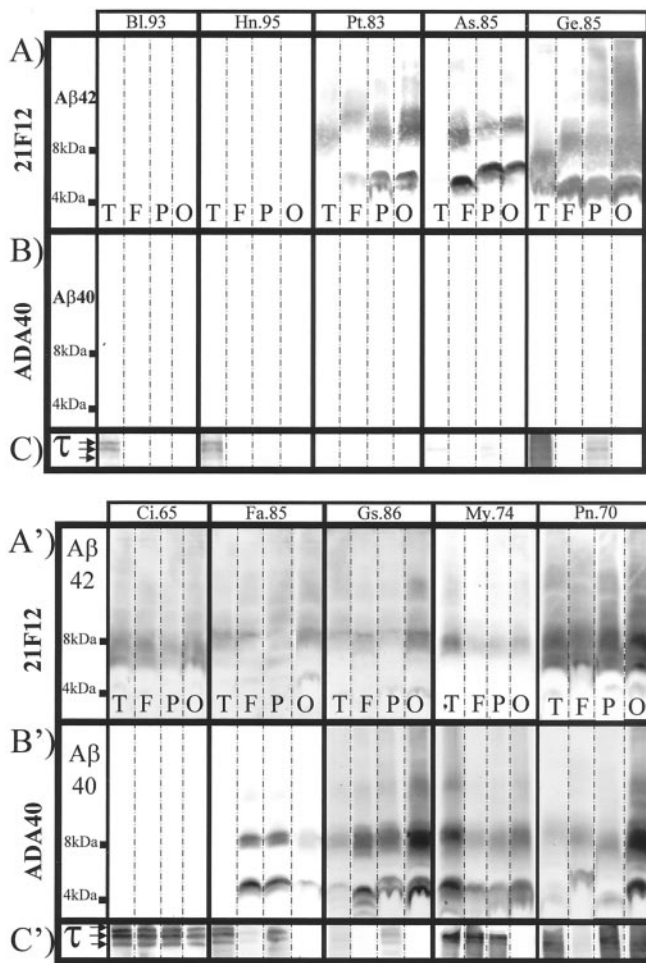


Figure 3. Western blot analysis of A β deposition (A, A', B, B') and tau pathology (C, C') in the different brains areas of nondemented and Alzheimer patients. Top, aging and infraclinical AD; bottom, definite AD. The specific immunolabeling with A β 42 (A, A'), A β 40 (B, B'), and tau (C, C') antibodies is presented. The temporal (T), frontal (F), parietal (P), and occipital (O) cortices were studied. A, A', B, B': A β 42 (E), 40 (F), and tau (G) were quantified in different brain areas from nondemented and demented patients. We used successively the monoclonal 21F12 antibody, because of its very high sensitivity and specificity toward A β 42 species, and then the polyclonal ADA40 antibody. C, C': Tau pathology revealed with AD2 antibody. Tau detection was performed on the same membrane (upper part). Pathologic tau proteins correspond to the main triplet of immunodetected band (tau 60, 64 and 69 kDa). A–C: Some representative nondemented cases are presented here: two cases with tau pathology (C) and no A β aggregates (Bl.93 and Hn.95); Case Pt.83, Case As.85, and Case Ge.85 at stages 1, 3, and 6 of tau pathology, respectively, and with various amounts of aggregated A β 42 species, but no A β 40 aggregates. Note that some cases with tau pathology had no A β aggregates. Case Pt.83 had tau pathology in the entorhinal formation (not shown). A'–C': A β 42 aggregates were found in large quantities in all brain areas of Alzheimer patients. Patient Ci.65 had exclusively A β 42 aggregates, as no trace or trace amounts of A β 40 were detected in the four brain regions. Patients Gs.86 and Fa.85 had huge aggregates of A β 40 peptides. A β 40 was in larger quantities in the occipital area of

Spatiotemporal analysis of A β deposits in AD. In our prospective study,⁷ we demonstrated that the clinical features were well correlated with the extension of tau pathology in association polymodal cortical areas. For the present study, it was interesting to compare the clinical and neuropathologic features of these patients with tau pathology as well as with the different biochemical phenotypes of amyloid deposition and their concentrations.

For Alzheimer demented patients, we always found severe tau pathology (stage 7 to stage 10) well correlated with a huge and widespread A β burden. As shown in table e1 (available online at www.neurology.org), the presence of A β 40 aggregates is a landmark of the late stages of AD. Also, the most affected AD cases, with an early onset, a rapid cognitive degradation, and a huge neurofibrillary degeneration process, were cases with large amounts of A β 42, and rare or absent A β 40 aggregates (Cases To.54, Fu.63, Ci.65, Cu.70).

When all biochemical data were collected, it was striking to observe the excellent parallelism between the increase of the disease process, tau pathology, and the increase of A β 42 aggregates (table e1, figure 4). However, at the level of specific brain areas, there was no overlap between the mapping of tau and APP degenerating processes. It was interesting to observe brain areas such as the occipital cortex with enormous A β aggregates but with absolutely no trace of tau pathology in this region (see figure 3A', Case Gs.86, occipital). Conversely, brain areas with more tau pathology were not those that had larger amounts of A β aggregates (see figure 3A', Fa.85, temporal cortex). Moreover, each time we were able to detect A β aggregates, we found a tau pathology, at least in the entorhinal region. The opposite was not true because we found cases with tau pathology up to stage 6 and no trace of A β aggregates (see figure 3, A through C).

Discussion. Our objective was to describe the natural and molecular history of AD as well as to develop a molecular basis for the definite diagnosis of AD. Indeed, at present, the definite diagnosis of AD, and the distinction from other neurologic disorders, is still difficult, even when working directly on the brain tissue: this is demonstrated by the fact that tau pathology has been recently reintroduced in the criteria for the definite diagnosis of AD,³² after a long absence, to fit now with Alois Alzheimer's first and sound description. This is also demonstrated by the fact that the 1997 consensus report recommends a

Gs.86 whereas it was essentially found in the frontal and parietal cortex of Patient Fa.85. A β 40 aggregates were essentially solubilized as A β monomers and dimers whereas A β 42 aggregates were dissociated and solubilized as A β dimers and multimers. They were abundant in all brain areas of Patients Ci.65 and Cu.70. For Patient Gs.86, at stage 9 and mildly affected, the temporal and parietal cortex were moderately affected by tau pathology, but not the frontal and occipital cortex. Note that the region with more A β 40 and 42 deposits, the occipital cortex, is not affected by tau pathology. In the same way, tau pathology and amyloid deposits do not overlap in the brain areas of Patient Fa.85.

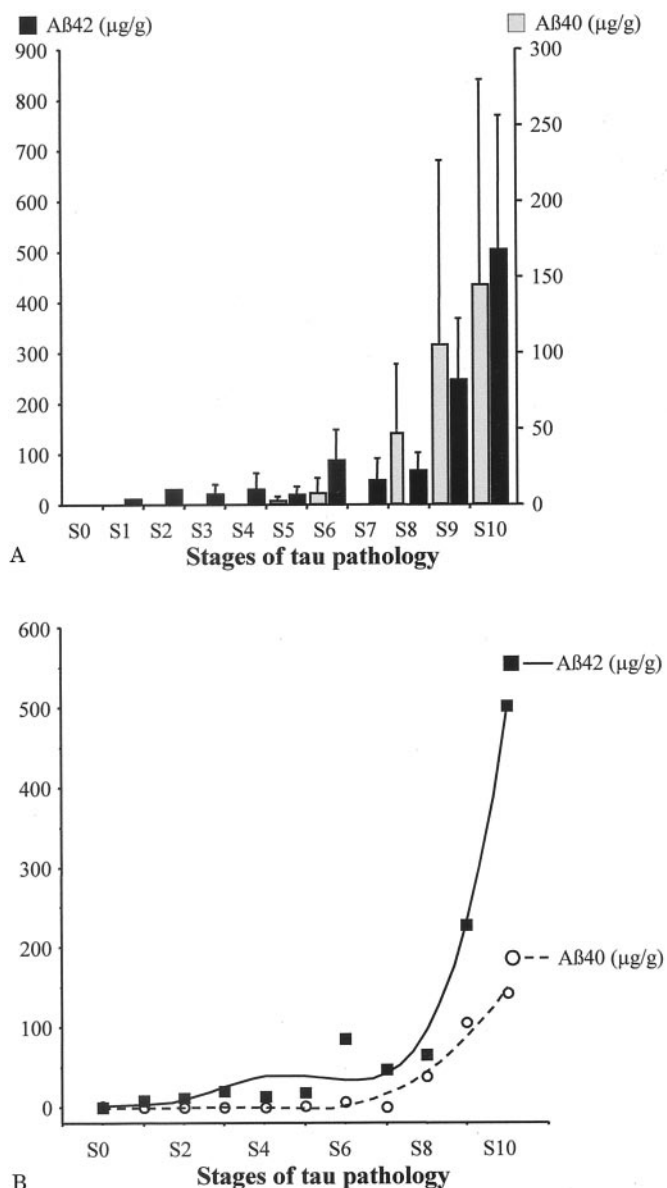


Figure 4. Average amounts of Aβ aggregates in the brain tissue of nondemented and AD patients correlated with tau pathology staging. The amount of Aβ 40 (gray bars) and Aβ 42 (black bars) are expressed as average amounts of the four brain region analyzed (temporal, frontal, parietal, and occipital region) in 48 patients distributed among the 10 stages of tau pathology (x-axis). The average amounts were calculated only for patients with Aβ aggregates. (A) The average amounts are in μg/g wet tissue, the left y-axis corresponds to Aβ42 amounts, and the right y-axis corresponds to Aβ 40 amounts. The standard deviation is also indicated. (B) Polynomial regression between Aβ aggregates and tau pathology staging. Polynomial regression of Aβ 40 (round circle) and Aβ 42 (black square) were both significant ($r_{A\beta 40} = 0.984$, $r_{A\beta 42} = 0.963$, $p < 0.0001$).

probabilistic analysis. This is also obvious when we see that the molecular markers of neurodegenerative disorders, namely amyloid pathology, tau pathology, and synuclein, are overlapping markers of many differ-

ent diseases. For example, tau pathology is observed in more than 24 neurodegenerative disorders.³ Also, amyloid pathology is frequently observed in Lewy body dementia,^{33,34} a disease with a synucleopathy.³⁵ Aβ deposits are also basic components of amyloid angiopathy, a pathologic entity distinct from AD.²¹ Therefore, a reliable quantification of brain lesions is necessary, and only biochemical analyses can fulfill the work rapidly, precisely, and with a good interlaboratory reproducibility. The usefulness of such a procedure is that it could bring a quantitative and qualitative aspect to the diagnosis, as required by the consensus criteria,³² which is difficult to obtain with an immunohistochemical approach. Brain banks could use it to type precisely the extent of the degenerating processes in the frozen tissue that will be used for the search of markers or for the description of the pathologic process. A molecular basis for the diagnosis at the CNS level should also open the gates for a biological diagnosis in the blood or the CSF. A biochemical approach should also provide a possibility for a definite antemortem diagnosis, based on the quantification of pathologic tau and Aβ in a stereotaxic biopsy. This controversial approach is not yet an issue, but could become one if an efficient anti-Alzheimer drug is discovered.

Aβ quantification was performed on the postmortem brain tissue of 70 patients from our prospective study. Western blot or dot-blot techniques gave similar values. The dot-blot technique is more efficient than EIA to quantify total Aβ because it enables one to quantify whole Aβ aggregated species (from the most soluble to the most insoluble). One important feature of this technique is that we were able to load directly the antigen dissolved in pure FA on the PVDF membrane. This methodology avoids the possible pitfalls due to Aβ reaggregation that would inevitably happen if the FA-treated tissue is then neutralized or treated in a less denaturant buffer. This strong tendency to reaggregation is demonstrated by the presence of smears of insoluble material on Western blots, when FA-treated material is removed and dissolved in a SDS buffer. These smears that are often in large quantities cannot be processed by ELISA.¹⁹ However, the dot-blot technique necessitates working with anti-Aβ antibodies that do not react with other higher molecular weight APP catabolic products, a property that is rarely found.¹⁹ The Western blot technique gives additional information such as the molecular weight of the detected Aβ species as well as their distribution into monomers, oligomers, and multimers, and guarantees the specificity of the analytical approach of insoluble Aβ pools.

Therefore, with our methodology, it has been possible to develop a large scale study for the quantitative and qualitative analyses of Aβ aggregation in the gradient of pathology of our series of 130 cases, from normal aging to severe AD, via infraclinical stages of AD. Furthermore, it has been possible to study the temporal distribution of Aβ aggregates in four brain areas (temporal, frontal, parietal, occipi-

tal), which summarizes the precise and sequential degenerating process revealed by tau pathology. Also, all our biochemical data were compared to clinical and neuropathologic data.

Different biochemical phenotypes of amyloidosis were found in nonfamilial Alzheimer cases. The detection and quantification of amyloid deposits show the extensive heterogeneity and the different patterns of A β aggregation in the brain of Alzheimer patients. For some cases, the A β burden was almost exclusively composed of insoluble A β 42 species. These cases had generally an early onset and a rapid evolution, as indicated in the clinical data. A β 42 deposits were also exclusively found in cases that are at the infraclinical stage of AD. ADA42 and ADA40 had similar sensitivities, suggesting that A β 42 aggregation is the first event of amyloidosis. Conversely, some cases at the last stages of tau pathology were strongly affected by A β 40 aggregates. The profile of A β 40 consisted of well resolved monomers and dimers, partially soluble in SDS extracts, in contrast to highly SDS-insoluble A β 42 aggregates that consisted of dimers and multimers. Cases with A β 40 frequently had amyloid angiopathy, but this was not always the case, showing that the production of A β 40 can be partially independent of amyloid angiopathy. In general, we found that A β 42 burden was mainly found in temporal and parietal cortices, whereas A β 40 deposits were mostly in the occipital areas. There is no clear explanation for the heterogeneous A β 40 phenotypes. One explanation could be that A β 40 species derive from a carboxy-terminal proteolytic degradation of A β 42 species, which is lately and heterogeneously activated in brain areas and among individuals. For example, this activity could be triggered by microglial cells that are known to phagocyte amyloid debris, as already suggested.²⁰

A β 42 aggregates were exclusively found at the infraclinical stages of AD, showing that they are the exclusive biochemical phenotype associated with the early development of Alzheimer amyloidosis. A β 42 aggregates were also exclusively found in the most severe Alzheimer cases, showing again the close relationship with Alzheimer pathology.

The relationship between amyloidosis and tau pathology was studied. The correlation between amyloid deposits and tau pathology is weak at the level of their spatial distribution in brain areas, but strong if we compare the stages of tau pathology versus the average amount of A β 42 aggregates in the neocortical areas. Indeed, we demonstrated with our biochemical approach, and in excellent agreement with numerous neuropathologic studies,^{4,6,8-10} that the pathway of tau pathology is precise, sequential, predictable, stereotypical, and hierarchical. Ten stages of tau pathology were defined, according to the 10 brain areas that are successively affected.⁷ Here, our precise typing and quantification of amyloid deposits shows that the A β burden is diffuse, widespread, and extremely heterogeneous, also in

good agreement with neuropathologic studies.⁸ It is interesting to note that each time we detected A β 42 cortical aggregates, we found a tau pathology, at least in the entorhinal region. But the opposite was not true, as a few rare cases had not a trace of amyloid but tau pathology up to stage 6. The absence of detection of A β aggregates in the brain of certain control patients should be considered as a function of two parameters: the definition of what is a soluble or insoluble A β pool and the sensitivity of the technique.

There are numerous pools of A β in the human brain of Alzheimer patients. First, A β is intracellular, resulting from a cleavage of APP that occurs in nerve cells and in glial cells. Then A β is extracellular and found in the parenchyma. The logical steps for a formation of A β fibrils suggest that there is a dimerization of A β , then a formation of oligomers and multimers. In parallel, there is a transformation from soluble dimers to stable dimers, a loss of helical structures at the expense of beta sheet structures to form fibrils. Furthermore, in the human brain, there is also a chemical transformation of A β species, with a truncation at the N and C-carboxy terminal endings of A β , and also biochemical modifications: tyrosine cross-linking, pyroglutamate, oxidation.^{30,31} At the end of the cycle, it is also possible that the degradation of amyloid by glial and microglial cells releases soluble degradation products of A β . Therefore, the quantification of A β pools is complex. Soluble pools can be quantified using the sensitive ELISA methods. The quantification of soluble A β pools is more precisely related to the production of A β and its neurotoxicity as a soluble agent.¹⁶⁻¹⁹ It is well demonstrated that soluble A β pools are increased in FAD (demonstration in experimental models as well as in the peripheral fluids and tissues of FAD patients). Interestingly, several articles have reported a close relationship between the amounts of soluble A β pools and the severity of the disease in sporadic AD.^{16,18,19}

The analysis of insoluble A β pools is related to the physiopathologic process of aggregation, and its relationship to a possible neurotoxicity as an insoluble agent. In the current work, we demonstrate that the quantification of insoluble A β pools necessitates a complete solubilization with pure FA, a Western blot approach, and the use of precise immunologic probes. ELISA methods should be adapted to quantify stable A β complex,¹⁹ which represents over 40% of total A β species in the human brain.³⁰ The major technical difficulty is the reaggregation of extracted A β when physiologic buffers are used for the immunologic detection. Other technical difficulties are reported in reference 19. Even using the Western blot approach, the amounts of insoluble A β pools can be underestimated using buffers that are not stringent enough or probes that do not detect chemically modified A β pools. Together, this explains why we found 10 to 50 times more insoluble A β than the amounts reported in the literature.^{17,18,29}

Another important point to address the physiopa-

thology of insoluble A β pools is the sensitivity of the technique. This is an essential point for nondemented cases with tau pathology in the entorhinal formation and with no A β aggregates. Using mAb 21F12, which was, in our hands, the most sensitive and specific tool for the detection of insoluble A β species, we were able to detect with a good reproducibility insoluble A β amounts at a concentration of 1 μ g per gram of tissue. This concentration is 1000 times lower than the one detected in some Alzheimer brains. However, we cannot exclude the presence of an abnormal concentration of soluble A β pools for cases with tau pathology and no A β aggregates, as suggested by reports using the ELISA approach.

Together, our data presented here reinforce the idea that nonfamilial autosomal AD is an amyloid (neurotoxic) or APP (loss of trophic activities) burden that will extend tau pathology from the temporal cortex to the association brain areas.³⁶ The data are in favor of a synergy of tau and APP pathologies. They show that tau pathology can be initiated before and likely independently of amyloidosis or APP dysfunctions, up to stage 6 of tau pathology. Our data also show that both neurodegenerative processes evolve systematically in parallel after stage 6, and that APP pathology amplifies tau pathology. Indeed, the progression of tau pathology in the association cortical areas is only observed when there is a simultaneous amyloid burden. Therefore, our study demonstrates that tau and APP pathologies work together to produce nonfamilial AD, and that tau pathology cannot be considered as a late secondary event of nonfamilial AD pathology, as frequently suggested. In that respect, the recent mice models proposed^{37,38} fit well with the human physiopathology³⁶ and as described here, because they demonstrate a synergy between APP and tau pathology. Furthermore, it is interesting to note that in one model, the amyloid burden is acting through neuronal connections and not by a direct contact to nerve cells, as observed here in human brains.³⁷

The quantification of A β and tau pathologies in the different brain areas allowed us to refine the criteria for a biochemical diagnosis of AD. We found a good correlation among clinical data, amyloidosis, and tau pathology, if a notion of threshold is added. Indeed, brain lesions at low levels, observed at the beginning of the pathology, do not impede cognitive functions. Neuroplasticity is certainly a significant factor that will modify the threshold of clinical manifestations, almost independently of the physiopathologic process.^{7,39}

Our study shows that all clinically defined probable AD cases had huge amounts of amyloid deposits and widespread tau pathology, in good agreement with the recent neuropathologic criteria.³² As mentioned previously, the polymodal association areas are the most informative brain areas for an accurate definite diagnosis. The hippocampal area, early and frequently affected by tau pathology and amyloidosis in nondemented patients, is only informative to

stage the infraclinical stages of AD. In agreement with our previous reports, this technique of amyloid quantification is able to define the criteria to establish a biochemical diagnosis of AD (CEBDAD) and shows that it is possible, with only biochemical means, to distinguish between normal aging and infraclinical stages of AD on one hand, and between infraclinical and clinical AD on the other.

Clinical AD. All of our clinically and then neuropathologically diagnosed cases with AD had significant tau pathology in the frontal pole and the parietal cortex (stage 7 to 10) and the presence of A β 42 aggregates above 50 μ g/g of wet tissue in these cortical areas. Therefore, these biochemical criteria are necessary and sufficient to demonstrate that an Alzheimer process is present that could explain the cognitive deficit.

Infraclinical AD. All nondemented patients, or those with a mild cognitive impairment, with insoluble A β 42 at a concentration of 10 μ g/g of tissue in a polymodal association area, such as the frontal pole or the parietal cortex, and a tau pathology in the hippocampal area can be reasonably considered as patients at the infraclinical stage of AD. The choice of 10 μ g is arbitrary and corresponds to a sizable detection of insoluble A β , 10 times higher than the threshold of sensitivity of the technique. At this concentration, there is no doubt that the amyloidogenic process of AD has started. Staging of tau pathology is necessary to define the extent of the neurodegenerating process. Tau pathology can be asymptomatic up to stage 6, and rarely but possibly up to stages 7 or even 8.

Normal aging. All nondemented patients can be considered as pure 'controls,' as far as tau and APP pathologies are concerned, if tau pathology is absent in all cortical areas, including the entorhinal area, and if there is no trace of A β 42 aggregates in neocortical areas. If the patients are older than 75 years, tau pathology, very discrete or moderate, is likely to be found in the entorhinal and hippocampal areas (stages 1 to 3), due to aging or a pathologic process that remains to be determined. But these aged nondemented patients can be considered as controls if they have no detectable A β 42 aggregates (arbitrary value of 10 μ g/g of tissue).

Here we show that A β 42 aggregates is the main and sufficient variable to demonstrate and quantify amyloidosis. Our CEBDAD criteria can be used first for the staging of AD, in complement to the neuropathologic one. Our method gives a rapid and reliable quantification of amyloid deposition and tau pathology. This method can be used to further characterize Alzheimer pathology of the frozen tissue from brain banks or from animal models. This approach is likely to be extremely valuable, as the brain tissue is the source for the search of biochemical (proteomic) or molecular markers (differential display, DNA chips). The CEBDAD should also be useful for the search of markers for the biological diagnosis, in the context of a prospective study.

Amyloid patterns led us to define their significance for a biological diagnosis of AD. The data presented here show the great variety of insoluble A β patterns in AD patients, from pure A β 42 to huge A β 40 peptide accumulations. The ratio A β 42/40 is varying from infinite to 0.4 according to the cases studied. This heterogeneity is likely to be mirrored by the A β peptides that are released in the CSF or the blood. It could explain the relative lack of specificity observed for the biological tests based on the ratio A β 40/42.⁴⁰ Indeed, at the early stages of AD, we demonstrate here that all amyloid deposits are in the A β 42 form. Biological parameters dealing with A β 40 could bring a lack of specificity, due to the extreme heterogeneity of A β 40 deposits as well as to the late appearance of insoluble A β 40 in the course of the disease. Therefore, we recommend testing exclusively A β 42 for the early diagnosis of AD.

Amyloid and tau patterns reveal some aspects of the etiology of AD. The quantification of A β in the different brain areas also demonstrates that the spreading pathway of tau pathology remains constant, whatever the cortical distribution of A β aggregates. We conclude that if there is a toxicity of amyloid, this one is acting through neuron to neuron connections, and not by a direct neurotoxicity of A β toward surrounding neuronal cell bodies.³⁶ In that perspective, our results suggest more a loss of APP function as a neurotrophic factor than an amyloid toxicity as the cause of AD, A β 42 being clearly an early marker of Alzheimer pathology, but not necessarily the etiologic factor. A dysfunction of APP carboxy-terminal fragments, that generate A β , are likely to be upstream markers closer to Alzheimer etiology.⁴¹

A β patterns described here can be used for setting up experimental models. Indeed, conversely to references 18 and 19, we also demonstrate herein that A β aggregates, and mainly A β 42 species, are well correlated to tau pathology and to the severity of the pathology. The amyloid substance from transgenic mice with mutated APP gene was quantified in comparison to human brains. Our biochemical approach shows that, unexpectedly, APP^{Sw} mice that are known to produce a fivefold increase of physiologic A β 40 and a 14-fold increase of A β 42⁴² are essentially composed of A β 40 aggregates, as described here, that were easy to dissociate and solubilize in SDS buffers. Our results on these transgenic mice are similar to those obtained using HPLC means,³⁰ showing that human A β aggregates are by far more strongly cohesive. However, our quantification of noncohesive amyloid in mouse brain fits well with previous studies.²⁹ Together, we observe that A β aggregates from APP^{Sw} mice are similar in quantities from those of the human brain, but with different physical and biochemical properties. In that perspective, APP^{Ld} mice could be more relevant to Alzheimer physiopathology, as they produce essentially A β 42 deposits,^{43,44} a feature of the first steps of AD. The values of A β 42 aggregates observed in the human brain could be used for cellular and animal models of

A β 42 toxicity. At last, our quantitative approach was validated by comparison with the neuropathologic one, and led us to refine the criteria to establish a biochemical diagnosis of Alzheimer's disease (CEBDAD).

Acknowledgment

The authors thank Innogenetics (E. VanMechelen) for the gift of 21F12 monoclonal antibody and Dr. M. Staufenbiel, from Novartis, Basel, Switzerland, for the gift of transgenic mouse brain tissues.

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