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Mass spectrometric characterization of brain amyloid beta isoform signatures in familial and sporadic Alzheimer's disease

Erik Portelius,

Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, 431 80 Mölndal, Sweden

Nenad Bogdanovic,

Alzheimer Disease Research Center, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden

Mikael K. Gustavsson,

Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, 431 80 Mölndal, Sweden

Inga Volkman,

Alzheimer Disease Research Center, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden

Gunnar Brinkmalm,

Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, 431 80 Mölndal, Sweden

Henrik Zetterberg,

Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, 431 80 Mölndal, Sweden

Bengt Winblad, and

Alzheimer Disease Research Center, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden

Kaj Blennow

Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, 431 80 Mölndal, Sweden

Erik Portelius: erik.portelius@neuro.gu.se

Abstract

A proposed key event in the pathogenesis of Alzheimer's disease (AD) is the formation of neurotoxic amyloid β ($A\beta$) oligomers and amyloid plaques in specific brain regions that are affected by the disease. The main plaque component is the 42 amino acid isoform of $A\beta$ ($A\beta$ 1-42), which is thought to initiate plaque formation and AD pathogenesis. Numerous isoforms of $A\beta$, e.g., $A\beta$ 1-42, $A\beta$ 1-40 and the 3-pyroglutamate derivate of $A\beta$ 3-42 (pGlu $A\beta$ 3-42), have been detected in the brains of sporadic AD (SAD) and familial AD (FAD) subjects. However, the relative importance of these isoforms in the pathogenesis of AD is not fully understood. Here, we report a detailed study using immunoprecipitation in combination with mass spectrometric analysis to determine the $A\beta$ isoform pattern in the cerebellum, cortex and hippocampus in AD,

including subjects with a mutation in the presenilin (M146V) or amyloid precursor protein (KM670/671NL) genes, SAD subjects and non-demented controls. We show that the dominating A β isoforms in the three different brain regions analyzed from control, SAD, and FAD are A β 1-42, pGluA β 3-42, A β 4-42 and A β 1-40 of which A β 1-42 and A β 4-42 are the dominant isoforms in the hippocampus and the cortex in all groups analyzed, controls included. No prominent differences in A β isoform patterns between FAD and SAD patients were seen, underscoring the similarity in the amyloid pathology of these two disease entities.

Keywords

Alzheimer's disease; Amyloid precursor protein; Brain; Immunoprecipitation; Mass spectrometry

Introduction

According to the amyloid cascade hypothesis, there is an imbalance in the production and clearance of amyloid beta (A β) leading to the formation of neurotoxic oligomers and larger assemblies of A β [15]. In Alzheimer's disease (AD), A β forms amyloid plaques in specific regions in the brain, which has been considered a key element in the pathogenesis of AD [3, 6, 8]. Of all AD cases, the majority have the sporadic form (SAD) of unknown cause, while a minority (accounting for less than 1% of all AD cases) have the familial form (FAD) with an onset before the age of 65 years [17]. Most FAD cases are caused by autosomal dominant mutations in amyloid metabolism-associated genes. To date, more than 170 different AD-causing missense mutations have been identified in the amyloid precursor protein (*APP*) and presenilin 1 and 2 (*PSEN1* and *PSEN2*, respectively) [4, 33].

A β is produced along the amyloidogenic pathway via cleavages of APP by β - and γ -secretase [1]. β -Secretase is an aspartyl protease encoded by the β -site APP-cleaving enzyme 1 (*BACE1*) gene [18, 34, 37, 39] while γ -secretase is a protease complex consisting of at least four essential components of which the homologous presenilin 1 and 2 proteins (*PSEN1* and *PSEN2*, respectively) constitute the active site [5]. Due to its imprecise cleavage preference, γ -secretase directly or indirectly mediates cleavage of A β at multiple sites between amino acids 17 and 42 [31]. This was shown by treating cells with a γ -secretase inhibitor with the result that all isoforms longer than and including A β 1-17 were abolished while the shorter isoforms were expressed at even higher levels. In another pathway, APP is first cleaved by β -secretase followed by α -secretase, thus generating shorter isoforms, e.g., A β 1-15 and A β 1-16 [19, 31]. In yet another non-amyloidogenic pathway, α -secretase cleaves between amino acid 16 and 17 in the A β sequence generating α -sAPP followed by γ -secretase cleavages, generating a fragment called p3 (A β 17-40/42) [7, 11, 12]. This isoform has been isolated from AD brains containing vast deposits of diffuse amyloid plaques and also from patients with Down's syndrome, but it has not been detected in cerebrospinal fluid (CSF) [10, 20, 29].

Numerous isoforms of A β have previously been detected in the brains of SAD and FAD subjects [35]. However, the relative importance of these truncated isoforms in the pathogenesis of AD is not fully understood. Different studies of brain A β report that the most abundant isoform is either A β 1-42 or A β 1-40 while others have reported the 3-pyroglutamate derivate of A β 3-42 (pGluA β 3-42) as one the most abundant isoforms [16, 24, 26, 27]. These divergent results may be due to that the different studies are studying different brain regions, different ages of the subjects and different stages of disease. Further, different protocols and techniques are used for analysing the different A β isoforms.

To date, no detailed mass spectrometric survey of the different A β isoforms present in different brain regions of SAD and FAD subjects has been performed. Proteomic studies using targeted approaches and mass spectrometry have previously led to the design of several new Alzheimer-related protein and peptide panels [40]. Here, we report the results of a study using immunoprecipitation (IP) in combination with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) or nanoflow liquid chromatography (LC) electrospray (ESI) high resolution tandem mass spectrometry (MS/MS) to determine the A β isoform pattern in the cerebellum, cortex and hippocampus of FAD subjects harboring a mutation in the *PSEN1* (M146V) or *APP* (KM670/671NL) gene, SAD subjects and non-demented controls.

Materials and methods

Patient characteristics

The brain samples from AD patients obtained from Huddinge Brain Bank at Krolinska Institute, Stockholm, fulfilled the clinical NINCDS criteria for probable AD [23] and met the neuropathological CERAD criteria for definitive AD [25]. Neuropathological controls were cases who had died from acute cardiac or malignant disease, without history of dementia, psychiatric or neurological diseases. The *PSEN1* M146V mutation carriers had a history of AD traced through four generations with an early age at onset (mid-late 30 s) of the disease and a rapid progression [14]. The Swedish APP double mutation (KM670/671NL) induces increased cleavage by β -secretase to generate more A β 1-40 and A β 1-42 [13, 21]. Detailed patient characteristics are given in Table 1. The study was conducted in accordance with the provisions of the Helsinki declaration and approved by the regional ethics committee.

Brain tissue

Brain tissue (cortex, hippocampus and cerebellum) from SAD ($n = 5$), APP670/671 (KM670/671NL, $n = 5$) and *PSEN1* (M146V, $n = 2$) subjects and non-demented controls ($n = 5$) (Table 1) was homogenized on ice in Tris-buffered saline containing complete protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) as described previously [30]. Briefly, formic acid (FA) was added (final concentration 70%) followed by further homogenization, sonication and centrifugation (30,000g, 1 h, +4°C). The supernatant was collected and dried in a vacuum centrifuge. The dried fraction was dissolved in 70% FA followed by centrifugation (30,000g, 1 h, +4°C). Before IP, the supernatant was neutralized using 0.5 M Tris.

Immunoprecipitation

The IP using the KingFisher magnetic particle processor (Thermo Scientific, Waltman, MA, USA) was performed as described earlier with some modifications [32]. Briefly, an aliquot (8 μ L, 1 mg/mL) of the A β specific antibodies 6E10 and 4G8 (epitope 4–9 and epitope 18–22, respectively, Signet Laboratories, Inc., Dedham, MA, USA) was separately added to 50 μ L Dynabeads M-280 (Dyna[®]) sheep anti-mouse according to the manufacturer's product description. The washed beads with bound antibody (50 μ L 6E10 and 50 μ L 4G8) were combined and used for immunoprecipitation of the neutralized FA fraction to which Tween-20 (Bio-Rad Laboratories Inc., end concentration 0.025%) was added and incubated.

The beads/FA fraction was transferred to a KingFisher magnetic particle processor (polypropylene tubes, Thermo Scientific) for automatic washing and elution of the A β peptides. The collected supernatant was dried in a vacuum centrifuge and redissolved in 5 μ L 0.1% FA in 20% acetonitrile. The A β peptides were analyzed using MALDI-TOFMS and nanoflow LC-MS/MS. All solvents used were of HPLC quality. Control experiments

included IP with only beads (pre-coated with sheep anti mouse IgG from the manufacturer) and beads with coupled IgG from mouse serum.

Mass spectrometry

MALDI-TOFMS measurements were performed using an AUTOFLEX instrument (Bruker Daltonics, Bremen, Germany) operating in linear or reflector mode. Each spectrum represents an average of 1,500 shots acquired 75 at a time. The MALDI samples were prepared with the seed layer method as described previously [29] with α -cyano-4-hydroxycinnamic acid (CHCA) used as matrix. The sequence of A β peptides best matching the molecular mass obtained with MALDI-TOFMS was evaluated with an in-house developed software (PeptideMassCalculator).

The A β levels are expressed as MS signal/mg tissue, calculated from the original brain weight (40–250 mg) measured before homogenization. A MS signal/mg tissue <100 is called small, 100–500 is called minor, 500–1,000 is called intermediate and a MS signal/mg tissue >1,000 is called major.

LC-MS/MS was conducted by nanoflow liquid chromatography coupled to electrospray ionization Fourier transform ion cyclotron resonance tandem mass spectrometry (LC-ESI-FTICR-MS/MS) with an Ettan MDLC (GE Healthcare, Uppsala, Sweden) coupled to an LTQ-FT Ultra (ThermoFisher Scientific, Bremen, Germany), a hybrid linear quadrupole ion trap-Fourier transform ion cyclotron resonance mass spectrometer equipped with a 7 T magnet.

Quantification of neuropathology

Sample processing and plaque counts on all the brain tissues used in the present study were performed as described previously in detail [2]. In short, brain tissue was processed within 24–48 h of death. The right hemisphere was fixed in buffered 4% formaldehyde for 4 weeks. Blocks were embedded with paraffin, and stained using the Bielschowsky method. The number of plaques was counted within superficial layers 1–3 and deep layers 4–6 for 11 cortical areas and for 12 subcortical structures using the Olympus Video Stereological Analysis System (BICO, Copenhagen) and the GRID v2.0 software (Interactivision ApS, Silkeborg). Plaque counts are given as number of plaques per square millimeter. In the cortex neuritic plaques, while in the cerebellum and basal ganglia diffuse plaques were counted. List of the regions used is described elsewhere [2].

Results

In general, the most abundant isoforms throughout all different brain regions analyzed were A β 1-40, A β 1-42, A β 4-42 and pGluA β 3-42. See Fig. 1a–c for representative mass spectra displaying the A β isoform pattern from the cortex, hippocampus and cerebellum from a FAD subject.

Control brains

In the cerebellum, only one out of five subjects displayed four minor peaks corresponding to A β (Fig. 2). In the hippocampus, another subject displayed two major peaks corresponding to A β 1-42 and A β 4-42 and four small peaks corresponding to A β 2-42, A β 3-42, pGluA β 3-42 and A β 5-42. The major isoforms detected in the cortex were A β 1-42 and A β 4-42, constituting ~80% of all A β isoforms detected (Fig. 6a). In one of the subjects, no peaks corresponding to A β isoforms were detected. Notably, A β 1-40 was not detected in the cortex, hippocampus or cerebellum.

SAD brains

All SAD subjects, except one, displayed various A β isoforms in all different brain regions analyzed (Fig. 3). In the cerebellum, all of the A β isoforms detected were minor except A β 1-42 and A β 4-42, which were small in three of the subjects. In the hippocampus, A β 4-42 and A β 1-42 were intermediate while A β 1-40 and pGluA β 3-42 were minor (Fig. 6b). In the cortex, A β 4-42 and A β 1-42 constituted ~60% of all A β isoforms detected while A β 1-40, constituting ~18% of all A β isoforms detected, were intermediate and pGluA β 3-42 was minor (Fig. 6b). In the three brain regions analyzed, the MS signal/mg tissue of A β 4-42 and A β 1-42 was higher compared to A β 1-40 (Fig. 6b).

APP670/671 brains

In the cortex, most of the subjects displayed major or intermediate peaks corresponding to A β 4-40, A β 4-42, A β 1-40 and A β 1-42, of which the dominant A β isoforms were A β 4-42 and A β 1-40, constituting ~54% of all A β isoforms detected (Fig. 4). The hippocampus from two of the subjects was not available, but the A β isoform pattern from the remaining three showed distinct peaks corresponding to A β 4-42, A β 1-40, A β 1-42 and pGluA β 3-42. A β 4-42 was the most abundant isoform in the hippocampus while A β 1-40 was the most abundant isoform in the cerebellum (Fig. 6c). A β 1-42 and pGluA β 3-42 were equally abundant in the hippocampus and cortex. Interestingly, one of the subjects (subject 12, see Fig. 4) also had peaks corresponding to A β 1-39, A β 1-38 and A β 1-37 in all brain regions. These isoforms were not detected in any other subjects.

PSEN1 brains

The hippocampi were not available from the *PSEN1* M146V subjects. The remaining brain regions displayed numerous A β isoforms with A β 1-42, pGluA β 3-42 A β 4-42 and A β 1-40 as the dominant isoforms (Fig. 5). A β 4-42 was the dominant isoform in the cortex constituting ~48% of all A β isoforms detected. This isoform was ~9 times more abundant compared to A β 1-40 and ~3 times more abundant than A β 1-42 (Fig. 6d). In the cerebellum, A β 1-40 was the most abundant isoform, ~9 times more abundant compared to A β 1-42 and ~4 times more abundant compared to A β 4-42.

Correlation of A β isoform results with plaque counts

In general, the detected A β isoform signals corresponded well to neuropathological plaque counts. The one control with major A β peaks in the hippocampus also had high plaque counts in this brain region (subject 4, Fig. 2). Another control with no detectable A β in the mass spectra had no plaques in the brain (subject 5, Fig. 2). With regards to the AD brains, patients with many plaques all had major or intermediate A β peaks in the mass spectra of the corresponding brain region (Figs. 3, 4, 5).

Discussion

Here, we describe a detailed study of A β and its isoforms in different brain regions from different SAD, FAD and control subjects. Using 70% FA in the extraction protocol, we extracted most isoforms of A β present in the brain tissue. Overall, for all diagnoses, controls included, the cortex contained the most A β isoforms followed by hippocampus and cerebellum. This is in agreement with the view that plaque pathology begins with A β deposits in the neocortex, extending to the hippocampus and finally to the cerebellum [36]. A β deposition was frequently detected in the cortices of the controls, but was rarely seen in the cerebellum and hippocampus. Previous studies have shown that some elderly people with no AD symptoms have A β deposits [22, 26], which is in agreement with the present study.

In the cortex of APP670/671 subjects, A β 1-40 was the most abundant isoform (accounting for ~27% of all A β isoforms detected), whereas it was the third most abundant isoform in the cortex from SAD subjects and absent in the cortex from control subjects. Of the two PSEN1 subjects, one did not have any A β 1-40. Thus, A β 1-40 retention in the brain might be an AD-specific phenomenon rarely seen in controls.

The major A β isoforms detected in the different brain regions analyzed from control, SAD, and FAD subjects were A β 1-42, pGluA β 3-42, A β 4-42 and A β 1-40 with the exception that A β 1-40 was not detected in the brain regions from controls. These isoforms also displayed an additional peak, which had the mass of the peptide plus 16 Da, corresponding to the oxidized form. In the cortex, most of the SAD and FAD patients also displayed minor or small peaks corresponding to pGluA β 11-42 and pGluA β 3-40 while pGluA β 3-40 was absent in the cortex from control subjects. Studies have suggested that pGluA β 3-42 has a higher aggregation propensity and shows increased toxicity compared to A β 1-42 and that pGluA β appears in early stages of AD. Further, it has also been suggested that A β 4-42 has faster aggregation kinetics than the intact A β 1-42 and that N-terminal deletions generally enhance aggregation of A β into neurotoxic, β -sheet fibrils in a manner that may initiate and/or nucleate the pathological deposition of A β [28]. Even though there are suggestions (e.g., neprilysin and plasmin), the enzymes responsible for the N-terminal cleavages remain to be verified [1]. Yet another minor peak detected in the hippocampus and cortex from most of the patients corresponded to the mass of A β 1-43. This isoform has recently been identified to be presented in plaque cored sodium dodecyl sulfate preparations from both SAD as well as FAD cases [38].

A β 17-40/42 (p3) was not detected in any of the brain regions analyzed. Earlier reports have shown its presence in AD brains containing vast deposits of diffuse amyloid plaques and also from patients with Down's syndrome [10, 20]. One can argue that this study did not analyze diffuse amyloid plaques and/or the soluble fractions of the brain homogenate. However, p3 is a hydrophobic peptide that should be present in plaques extracted using FA. Importantly, we have previously shown that synthetic p3 can be retrieved from CSF upon spiking and that it can be analyzed using the MS method employed in the present work [29]. It has previously been suggested that the p3 fragment actually might be A β 11-42 [9], which is closer in mass to 3 kDa (3,334 Da and 3,153 Da for A β 11-42 and A β 11-40, respectively) than the weight of p3 (2,578 Da and 2,394 Da for A β 17-42 and A β 17-40, respectively). Notably, many studies that have detected and analyzed the p3 fragment have used antibodies reactive to epitopes within the amino acid sequence 17–42 of A β followed of tryptic digestion. However, if the antibody is not end-specific for the N-terminus of p3, it also recognizes A β 11-42. Tryptic digestion of this peptide will generate a A β 17-28 fragment, thus the N-terminus will start after the proposed α -secretase cleavage site. Altogether, the data presented here suggest that p3, if existing at all, is a minor A β isoform.

We recently identified a set of 18N- and C-terminally truncated A β peptides in CSF along with additionally 11 APP/A β peptides starting N-terminally of the β -secretase site using IP-MS. These shorter isoforms, e.g., A β 1-16/17, were not detected in this study. This might be due to that either they are not present in the brain due to their more hydrophilic nature or the method used is not sensitive enough for detecting these shorter isoforms.

It should be noted that the ratio between the different isoforms detected in the mass spectrum cannot be interpreted as a direct reflection of their absolute or relative abundance in the brain since the ionization efficiency might be different for the different isoforms and since different isoforms are more hydrophobic and less soluble than others. Further, the different A β isoforms may differ in protein interaction capacity, including affinity to

antibodies, and active or passive transport from brain tissue into CSF. Altogether, there are several non-quantitative aspects of IP-MS.

The brain tissue was processed within 24–48 h of death. The different lag times could potentially introduce artefacts in the mass spectrometric analysis such as proteolytic degradation and/or oxidation of the isoforms. However, oxidation of the isoforms can be monitored in the mass spectrometric analysis; the mass of the peptide plus 16 Da and the degree of oxidation of methionine were not different between the different subjects. Further, no new A β isoforms were detected when comparing the A β isoform pattern from brains which had been processed within a short period of time or 48 h. However, degradation can still be a factor to have in account.

The identification of the different isoforms is based on the average mass of the peak detected which is matched to the calculated theoretical mass and some of the isoforms might be false positive. However, all isoforms presented showed high mass accuracy and corresponded well to the calculated peptide masses (100 parts per million deviation or less). Further, the identities of the A β 1-42, A β 1-40, A β 4-42 and A β 4-40 peaks were confirmed using LC-ESI-FTICR-MS/MS (data not shown).

In conclusion, we here show that the dominating A β isoforms in AD brains are A β 4-42 and A β 1-40 and A β 1-42. These peptides are also frequently detected in the cortex of controls. Their presence in hippocampus and cerebellum is, however, firmly associated with AD. An AD-like brain A β isoform pattern was seen in one of the five controls. No prominent differences in A β isoform patterns between FAD and SAD patients were seen, underscoring the similarity in the amyloid pathology of these two disease entities.

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Fig. 1.

Representative mass spectra from a sporadic AD patient displaying the A β isoform patterns from three different brain regions; the cerebellum, hippocampus and cortex. 1 A β 4-40 [M+2H]²⁺, 2 A β 4-42 [M+2H]²⁺, 3 A β 1-40 [M+2H]²⁺ and 4 A β 1-42 [M+2H]²⁺. Asterisk represents an unidentified peak

Region		Cerebellum					Hippocampus					Cortex				
Subject ID		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Plaque count (lesions/m ²)		0	0	0	0	0	0	0	1	2	0	1	0	1	4	0
Sequence	Mass (Da)															
pGlu 11-42	3319.0															
11-42	3337.0															
9-40	3372.9															
10-42	3500.1															
9-42	3557.2															
8-42	3644.3															
7-42	3759.4															
5-40	3868.4															
4-40	4015.6															
2-38	4017.5															
5-42	4052.7															
1-37	4075.6															
pGlu 3-40	4126.7															
1-38	4132.6															
3-40	4144.7															
4-42	4199.9															
1-39	4231.8															
pGlu 3-42	4311.0															
3-42	4329.0															
1-40	4330.9															
2-42	4400.1															
1-42	4515.1															
1-43	4616.3															

Fig. 2.

Chart displaying the distribution of A β isoforms in the cerebellum, hippocampus and cortex of control subjects. A *light blue box* a small peak, a *blue box* minor peak, a *dark blue/white box* an intermediate peak and a *dark blue/red box* a major peak. Plaque count = lesions/m²

Fig. 3. Chart displaying the distribution of Aβ isoforms in the cerebellum, hippocampus and cortex of SAD patients. A light blue box a small peak, a blue box minor peak, a dark blue/white box an intermediate peak and a dark blue/red box a major peak. Plaque count = lesions/m²

Fig. 4. Chart displaying the distribution of Aβ isoforms in the cerebellum, hippocampus and cortex of APP670/671 patients. A light blue box a small peak, a blue box minor peak, a dark blue/white box an intermediate peak and a dark blue/red box a major peak. Plaque count = lesions/m²

Region		Cerebellum		Cortex	
Subject ID		16	17	16	17
Plaque count (lesions/m ²)		87	78	74	86
Sequense	Mass (Da)				
pGlu 11-42	3319.0				
11-42	3337.0				
9-40	3372.9				
10-42	3500.1				
9-42	3557.2				
8-42	3644.3				
7-42	3759.4				
5-40	3868.4				
4-40	4015.6				
2-38	4017.5				
5-42	4052.7				
1-37	4075.6				
pGlu 3-40	4126.7				
1-38	4132.6				
3-40	4144.7				
4-42	4199.9				
1-39	4231.8				
pGlu 3-42	4311.0				
3-42	4329.0				
1-40	4330.9				
2-42	4400.1				
1-42	4515.1				
1-43	4616.3				

Fig. 5.

Chart displaying the distribution of A β isoforms in the cerebellum, hippocampus and cortex of PSEN1 patients. A *light blue box* a small peak, a *blue box* minor peak, a *dark blue/white box* an intermediate peak and a *dark blue/red box* a major peak. Plaque count = lesions/m²

Fig. 6.

The MS signal/mg tissue is displayed for **a** controls, **b** SAD, **c** APP670/671 and **d** PSEN1 patients. The *error bars* represent one standard of the mean. It should be noted that the ratio between the different isoforms detected in the mass spectrum cannot be interpreted as a direct reflection of their absolute or relative abundance in the brain since the ionization efficiency might be different for the different isoforms and since different isoforms are more hydrophobic and less soluble than others

Table 1

Subject characteristics

Subject ID	Diagnosis	Mutation	Age	Sex	PM	Plaque count		
						Cerebellum	Hippocampus	Cortex
1	Control	NA	80	F	7	0	0	1
2	Control	NA	64	F	5	0	0	0
3	Control	NA	81	F	25	0	1	1
4	Control	NA	81	F	3	0	2	4
5	Control	NA	80	M	13	0	0	0
6	AD	NA	78	F	12	0.5	16	29
7	AD	NA	70	F	12	0.5	20	32
8	AD	NA	80	M	12	1	24	35
9	AD	NA	83	F	4	0	5	9
10	AD	NA	79	F	7	0	5	10
11	FAD	APP670/671	68	M	<24	6	27	35
12	FAD	APP670/671	66	M	<24	2	28	28
13	FAD	APP670/671	62	M	40	12	14	56
14	FAD	APP670/671	56	M	<24	3	24	36
15	FAD	APP670/671	62	F	<24	24	14	35
16	FAD	PSEN1 M146 V	48	F	?	46	87	74
17	FAD	PSEN1 M146 V	50	F	?	34	78	86

NA not applicable, PM postmortem, time in hours