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Quantification of A β 40 in CSF

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Abstract

Background

Truncated forms and full-length forms of the amyloid-beta 40 (A β 40) are key molecules in the pathogenesis of dementia, and are detectable in CSF. Reliable methods to detect these biomarkers in CSF are of great importance for understanding the disease mechanisms and for diagnostic purposes.

Material and Methods

VU- α -A β 40, a monoclonal antibody (mAb) specifically detecting A β 40, was generated and characterized by solid and fluid phase ELISA, surface plasmon resonance spectroscopy (SPRS), immunoprecipitation (IP), immunohistochemical and Western blot (WB) analysis. In addition, an ELISA with VU- α -A β 40 as catching and 6E10 as detecting mAbs was set up and validated. This ELISA was used to measure A β 40 in CSF of controls (N=27), patients with Alzheimer's disease (AD; N=20), Frontotemporal lobe dementia (FTLD; N=14), noninflammatory (N=15) and inflammatory (N=15) neurological conditions.

Results

VU- α -A β 40 specifically recognizes A β 40 with high affinity ($K_A=1.3 \times 10^9 \text{ M}^{-1}$) and detects A β 40 in AD brain specimens. The developed sandwich ELISA has a detection limit of 0.21ng/mL, a mean recovery of 90%, and an intra- and inter-assay CV of 1.4% and 7.3%. FTLD patients had a lower mean level of A β 40 (8.8(1.9)ng/mL) than controls (12.0(1.7)ng/mL); $p < 0.01$).

Conclusions

VU- α -A β 40 was successfully implemented in an ELISA which enables us to measure A β 40 accurately in human CSF. Clinical validation revealed lower levels of A β 40 in FTLD patients. This finding opens new possibilities for early and differential diagnosis of dementia.

Introduction

The main constituent of senile plaques in brains of patients with Alzheimer's disease (AD) is the amyloid-beta peptide ($A\beta$), a proteolytic cleavage product of the larger, membrane-bound, amyloid precursor protein (APP). $A\beta$ is produced by various cell types and is secreted into the interstitial fluid, ultimately resulting in detectable $A\beta$ levels in blood and cerebrospinal fluid (CSF). There are multiple cleavage sites in the $A\beta$ domain, and various $A\beta$ fragments can be generated. This results in several variations in the N- as well as the C-terminus, but the majority of the peptides present in CSF terminate at residue 40⁸⁷. In a recent preliminary study, we showed that levels of $A\beta$ 40 (the full-length $A\beta$ 1-40 peptide as well as N-terminally truncated forms) in CSF can distinguish patients with Frontotemporal lobe dementia (FTLD) from controls and AD patients⁶¹. The ELISA used to measure $A\beta$ 40 in that study, made use of monoclonal antibody (mAb) 6E10 (Signet, Dedham, MA, USA) as capture mAb which has a high affinity for N-terminal amino acids 3-8 of the amyloid peptide and a polyclonal antibody against the C-terminus of the $A\beta$ peptide at residue 40 was used to detect $A\beta$ 40^{70, 88}. We now set out to develop an assay based on the use of a C-terminal specific anti- $A\beta$ 40 mAb (VU- α - $A\beta$ 40) for capturing in combination with 6E10 to detect bound $A\beta$, as the approach previously used is prone to inadequate measurements of $A\beta$ 40 concentrations. This results from the fact that several amyloid peptides with other C-terminal residues in addition to soluble APP can be captured by 6E10. This may interfere (competition and/or steric hindrance) with $A\beta$ 40, resulting in lower amounts of bound $A\beta$ 40 and, consequently, in underestimated $A\beta$ 40 levels. Coating with a specific mAb against the C-terminus of the $A\beta$ 40 peptide followed by detection with a mAb against the N-terminus of $A\beta$, seems to be the ideal option to measure the truncated form of amyloid 40 peptide ($A\beta$ x-40) and the full peptide of amyloid-beta 1-40 ($A\beta$ 1-40)⁸⁹⁻⁹¹. However, these ELISAs have not been extensively validated for use in CSF measurements. In the present study we describe the production, characterization and the clinical use of VU- α - $A\beta$ 40. Surface plasmon resonance spectroscopy (SPRS), immunohistochemical (IHC), Western blot (WB), immunoprecipitation (IP) and solid and fluid phase ELISAs were performed to characterize this mAb. Subsequently, VU- α - $A\beta$ 40 was implemented as catching antibody in a highly specific ELISA system with 6E10 as detecting mAb and this measurement system was validated to quantify $A\beta$ 40 in human CSF, including the measurement of $A\beta$ 40 in CSF of controls and patients with AD patients, FTLD, noninflammatory and inflammatory neurological diseases.

Material and Methods

Peptides

For WB and IP, synthetic $A\beta$ 1-38, $A\beta$ 1-41 to $A\beta$ 1-49 peptides were obtained from Bachem (Bubendorf, Switzerland) and, $A\beta$ 1-37 and $A\beta$ 1-39 peptides were synthesized by solid-phase methods on an automated peptide synthesizer using Fmoc chemistry according to Janek et al.⁹². For SPRS experiments $A\beta$ 1-37 and $A\beta$ 1-39 were obtained from Anaspec (San Jose, California) and $A\beta$ 1-38 from Bachem (Bubendorf, Switzerland). For WB, IP, SPRS, the standardization of the sandwich ELISA and for monoclonal screening, $A\beta$ 1-40 (Biosource, Nivelles, Belgium) was used. Cys- $A\beta$ 33-40, Cys- $A\beta$ 33-42 and Cys- $A\beta$ 1-17

peptides were produced by Fmoc chemistry and found to be >95% pure as judged from HPLC reversed phase analysis (Dr Hilkmann; NKI-AVL, Amsterdam, the Netherlands). For SPRS and ELISA experiments A β 1-37, A β 1-38, A β 1-39, A β 1-40 and A β 1-42 were HFIP (Hexafluoroisopropanol) dissolved, aliquoted and dried at room temperature (RT) for 2h and subsequently stored at -80°C in polypropylene tubes. In each ELISA a new aliquot of A β 1-40 was used as a standard.

CSF samples

In our laboratory and clinic, CSF samples are handled according to standardized protocols. CSF samples (3-15 mL) were obtained by lumbar puncture between the L3 and L4 or L4 and L5 intervertebral space, and collected in polypropylene tubes. Routine analysis (including total cell, erythrocyte count and total protein determination) was performed on part of the CSF sample within 2h after the lumbar puncture. The remaining CSF was immediately centrifuged at 1,800 x g for 10 min at 4°C, aliquoted into polypropylene tubes of 0.5 mL or 1.0 mL and immediately stored at -80°C until analysis.

Patients

Twenty-seven controls, 20 AD patients, 14 patients with FTLD, 15 patients with noninflammatory neurological conditions (6 patients with epilepsy, 3 patients with mild head injury, 3 patients with a subarachnoid haemorrhage, 1 patient with a meningioma, 1 patient with a cerebellar ischemic stroke and 1 patient with amyotrophic lateral sclerosis) and 15 patients with inflammatory neurological conditions (7 patients with multiple sclerosis, 2 patients with myelitis transversa, 2 patients with neuroborreliosis, 1 patient with meningoencephalitis and 2 patients with neuromyelitis optica) were included. All patients underwent a standardized clinical investigation including medical history, physical and neurological examination, screening laboratory tests, and MRI. Clinical diagnosis was made by consensus. The diagnosis of AD was made using the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria¹. For FTLD we used the clinical diagnostic criteria of Neary and colleagues⁹³. The control group consisted of subjects who presented at our memory clinic with subjective complaints but who had normal clinical investigations (including normal CSF results for total cell count, erythrocyte count and total protein) and did not have significant cognitive deficits (mean(SD) MMSE score 29(1.1) and normal neuropsychological test). The local ethical review board approved the study protocol and all subjects gave written informed consent.

Statistics

Data were analyzed with the SPSS software package (version 15 for Windows SPSS, Chicago IL). The coefficients of variances (CV) were calculated according to the following formula: mean of (SD of the two measurements) / (mean of the two measurements). Pearson correlation coefficients (r_s) between age and A β 40 levels were determined for the control group. Differences between groups were tested using Chi-squared test and analysis of variance (ANOVA) with post-hoc test Bonferroni tests. Statistical significance was set at $p < 0.05$.

Post mortem brain tissue

A brain specimen of an AD patient (male, 69 years, Braak stage 6-c) and a sample from a control brain (male, 84 years, Braak 1-o) were obtained at autopsy (post-mortem interval 5 and 7 h, respectively; the Netherlands Brain Bank, Amsterdam, The Netherlands). Clinical diagnosis of AD had been made using DSM-III-R criteria and the severity of dementia was evaluated according to the Global Deterioration Scale of Reisberg (GDS)⁹⁴. Neuropathological evaluation was performed on formalin fixed, paraffin-embedded tissue from different sites, including the frontal cortex (F2), temporal pole cortex, parietal cortex (superior and inferior lobule), occipital pole cortex and the hippocampus (essentially CA1 and entorhinal area of the parahippocampal gyrus). The distribution and the density of neurofibrillary tangles was determined in Bodian-stained sections, while senile plaques were stained with the methenamine silver method⁹⁵. Staging of AD was evaluated according to Braak and Braak^{27, 28, 55}.

Establishment of monoclonal antibody VU- α -A β 40

VU- α -A β 40 was generated as follows. The synthetic peptide corresponding to A β 33-40 was conjugated with bovine serum albumin (BSA) through a cysteine residue added at the N-terminus of the peptide. Mice were subcutaneously immunized with 100 μ g of the conjugate in complete Freund's adjuvant (first immunization) and again 3 times with 25 μ g antigen without adjuvant at 2-3 weeks intervals. Three days after the final boost and after testing the mouse for reactivity against Cys-A β 33-40 in ELISA, spleen cells and lymph nodes were removed and fused with mouse myeloma SP2/0 cells using 42% polyethylene glycol 4200 (Merck, Whitehouse Station, NY). Fused cells were cultured in 96-wells culture plates (Costar, Inc., NY) with use of Iscove's Modified Dulbecco's medium (Biowhittaker Europe, Verviers, Belgium) supplemented with 5.2 μ M azaserine, heat-activated fetal bovine serum (Invitrogen, Paisley, UK), hypoxanthine, 0.5ng/mL of human interleukin-6 in a humidified atmosphere (at 37°C; 5% CO₂). The wells were regularly screened with use of a microscope. The hybridoma containing wells were marked and the supernatants of these wells were tested in a solid phase ELISA after approximately 2 weeks (see below). Antibody-producing hybridomas which the culture supernatants reacted positively (Optical Density higher than 1.5) with A β 1-40 and Cys-33-40, and did not react with the other coated peptides (A β 1-42, Cys-A β 33-42 and Cys-A β 1-17) in solid phase ELISA were cloned. After limiting dilution cycles and repeated testing in solid phase ELISAs, one clone designated VU- α -A β 40 was selected as being the best A β 40 C-terminal specific mAb on solid phase ELISA. For large-scale antibody production hybridomas were cultured in 1L growth medium containing 10% heat-inactivated fetal bovine serum and 0.5 ng/ml human interleukin-6 in tissue culture roller bottles (Falcon, Becton Dickinson) for at least 4 weeks. Culture supernatants were concentrated in capillary flow dialyzers (Fresenius, Bad Homburg, Germany) and mAbs were purified by protein A sepharose affinity chromatography (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. The Ig fraction was dialyzed against phosphate buffered saline (PBS i.e. 9 mM Na₂HPO₄, 1mM NaH₂PO₄, 140mM NaCl, pH 7.2-7.4).

Screening of monoclonal antibody VU- α -A β 40: solid phase ELISA

Wells of a flat-bottomed microtiter plate (Costar, Corning Inc., NY) were coated overnight at RT with 100 μ L (80ng/well) of A β 1-40, A β 1-42, Cys-A β 33-40, Cys-A β 33-42 and Cys-A β 1-17 in coating buffer (125mM Na₂CO₃ x 10H₂O, 125 mM NaHCO₃, pH 9.6). The microtiter plates were washed twice using PBS with 0.02% Tween20 and blocked with 200 μ L 2% low fat milk in PBS for 1 h at RT. After five washes with PBS with 0.02% Tween20, different dilutions (1:10; 1:100; 1:1000; 1:10,000; 1:100,000; dilutions in PBS with 0.1% Tween20) of the hybridoma supernatants (90 μ L/well) were incubated at RT and 500rpm for 1 h. Following five washes with PBS with 0.02% Tween20, the plates were incubated with polyclonal goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (DAKO, Cytomation, Glostrup, Denmark) for 1 h at RT and 500 rpm. After washing, the plates were developed with tetramethylbenzidine (TMB) and the reaction was stopped with 1M sulphuric acid.

Determination of the isotype of VU- α -A β 40

The (sub)class of the mAb was determined with a test strip (Hycult Biotechnology b.v., Uden, the Netherlands). In short, the one-step procedure involved the capture of the mouse immunoglobulins by subclass specific rat anti-mouse mAbs which are immobilized on the test strip. Captured mouse immunoglobulins were detected directly by a second rat mAb which is coupled to colloid particles. When reactive, this results in two coloured spots on the test strip, one spot identifying the isotype and one spot identifying the light chain.

Epitope mapping VU- α -A β 40: one-dimensional SDS-PAGE and WB analysis

For separation A β peptides, A β -SDS-PAGE/ immunoblot was conducted as described by Wiltfang and colleagues⁹⁶⁻⁹⁸. Gels were run at RT for 2 h at a constant current of 12 mA/gel using the MiniProtean II electrophoresis unit (Bio-Rad Laboratories, Hercules, CA, USA). Immunological detection of A β peptides was conducted as published by Wiltfang et al.⁹⁶⁻⁹⁸. For detection of A β to the PVDF-membranes, the amino-terminal-selective mouse mAb 1E8 (provided by Schering, Berlin, Germany; stock: 0.25mg/mL) and VU- α -A β -40 were (1.08 mg/ml) were diluted 1:300 and 1:500, respectively. Mix 1 (= 60pg/ μ L A β 1-37, 120pg/ μ L A β 1-38, 60pg/ μ L A β 1-39, 240pg/ μ L A β 1-40, 120pg/ μ L A β 1-42) and Mix 2 (100pg/ μ L A β 1-41, 100pg/ μ L A β 1-42, 100pg/ μ L A β 1-43, 100pg/ μ L A β 1-44, 100pg/ μ L A β 1-45, 100pg/ μ L A β 1-46, 100pg/ μ L A β 1-47, 100pg/ μ L A β 1-48, 100pg/ μ L A β 1-49) were used as standards. Membranes were further incubated with a biotinylated anti-mouse polyclonal antibody (Vector Laboratories, Burlingame, CA) and horseradish peroxidase coupled streptavidin (Amersham Pharmacia Biotech, Buckinghamshire, England) for 1 h each. Washing steps were performed in between. Chemiluminescent visualization was performed using ECLPlusTM solution (Amersham Pharmacia).

IP of CSF samples using VU- α -A β 40

Magnetic microparticles (Dynabeads M-280, German Dynal GmbH, Hamburg, Germany) were activated with mAb according to the protocol of the manufacturer (direct IP method). CSF (200 μ L; pool of seven CSF samples) was added to 500 μ L of RIPA detergent buffer (2.5% Nonidet P-40, 1.25% sodium deoxycholate, 0.25SDS, 750mM NaCl, 250mM HEPES, one tablet of Protease Inhibitor Cocktail Complete Mini (Roche, Basel, Switzerland) per 2 mL of RIPA, pH adjusted to 7.4 with NaOH) and 25 μ L of magnetic micro-particles coated

with VU- α -A β 40 (1 μ g VU- α -A β 40/1.68 \times 10⁷ beads) or mAb 1E8 (1 μ g mAb 1E8/1.68 \times 10⁷ beads), 600 μ L H₂O. Samples were incubated under rotation overnight at 4°C. Beads were washed four times with PBS/0.1% bovine serum albumin (BSA), once with 10mM Tris/HCL, pH 7.4. For A β -SDS-PAGE/immunoblot, bound A β peptides were eluted by heating the sample to 95°C for 5 min with 25 μ L buffer containing 0.36M Bitris, 0.16M Bicine, 1% (w/v) SDS, 15 % (w/v) sucrose, and 0.004% (w/v) Bromophenol blue.

Surface plasmon resonance spectroscopy

SPRS experiments were performed using a BIAcore[®] 2000 (Uppsala, Sweden) biosensor instrument. Sensor chips and protein coupling chemicals were purchased from BIAcore[®]. Cys-A β 33-40, Cys-A β 33-42 and Cys-A β 1-17 were dissolved in PBS with 0.1% Tween20 (1 mg/mL) and were coupled to the surface of the sensor flow cell by thiol coupling. A β 1-37, A β 1-38, A β 1-39, A β 1-40, and A β 1-42 (HFIP dried; stored at -80°C in polypropylene tubes) were dissolved separately in 10% μ L DMSO and diluted in sodium acetate (10mM, pH 5.0) to yield end concentrations of 0.1, 1, 0.1, 0.5 and 0.5mg/mL, respectively. These peptides were separately coupled to the surface of the sensor flow cell by amine coupling. Coupling reactions were performed as recommended by the manufacturer (BIAcore[®] 2000, Uppsala, Sweden). Kinetic binding and dissociation measurements were carried out at 25°C in PBS with 0.1% Tween20. Interaction of VU- α -A β 40 (stock 1mg/mL), at various dilutions with the different coatings, was analyzed. This was also performed with 6E10 (Signet, Dedham, MA; stock 1mg/mL). Regeneration of the sensor surface was performed with 40 μ L of 0.1M HCL. The obtained binding and dissociation curves were analysed using the standard 1:1 Langmuir binding model provided within the BIAcore[®] evaluation software. Furthermore, all experiments were carried out in duplicate per chip, and at least two different sensor chips were used to exclude chip-to-chip variations. In addition different antibody batches were tested.

Immunohistochemistry

Tissue sections (5 μ m) were mounted on Superfrost Plus tissue slides (Menzel-Gläser, Germany) and dried overnight at 37°C. For all stainings sections were deparaffinized and subsequently immersed in 0.3% H₂O₂ in methanol for 30 min to quench endogenous peroxidase activity. Formic acid treated sections were incubated with primary antibody overnight at 4°C. VU- α -A β 40 was tested in serial dilutions on sections. The optimal dilution of 1:3200 in PBS with 1% (w/v) bovine serum albumin (BSA, Boehringer Mannheim, Germany) was chosen for further studies. Upon incubation overnight with VU- α -A β 40, the sections were washed and incubated with EnVision-HRP (anti-rabbit/mouse) undiluted solution (DAKO) for 30 min. Colour was developed with 3,3'-diaminobenzidine (EnVision Detection system/HRP, 1:50 dilution; DAKO) as chromogen. As controls, mouse mAb 6E10 (dilution 1:3,200) which detects virtually all forms of A β , and G2-10 (dilution 1:64,000; the genetics company Schlieren, Switzerland⁹⁹), a mAb which recognizes A β with C-terminal 39 and 40 were used. The staining procedure with 6E10 was identical to that with VU- α -A β 40, for G2-10. Sections were pre-incubated with normal rabbit serum (1:50; DAKO) for 10 min, incubated with G2-10 overnight at 4°C after washing with PBS, incubated with rabbit anti-mouse F(ab')₂ (1:500 dilution, DAKO) for 30 min. Subsequently, sections were incubated with streptavidin-biotin horseradish peroxidase complex (streptABComplex/HRP, 1:200,

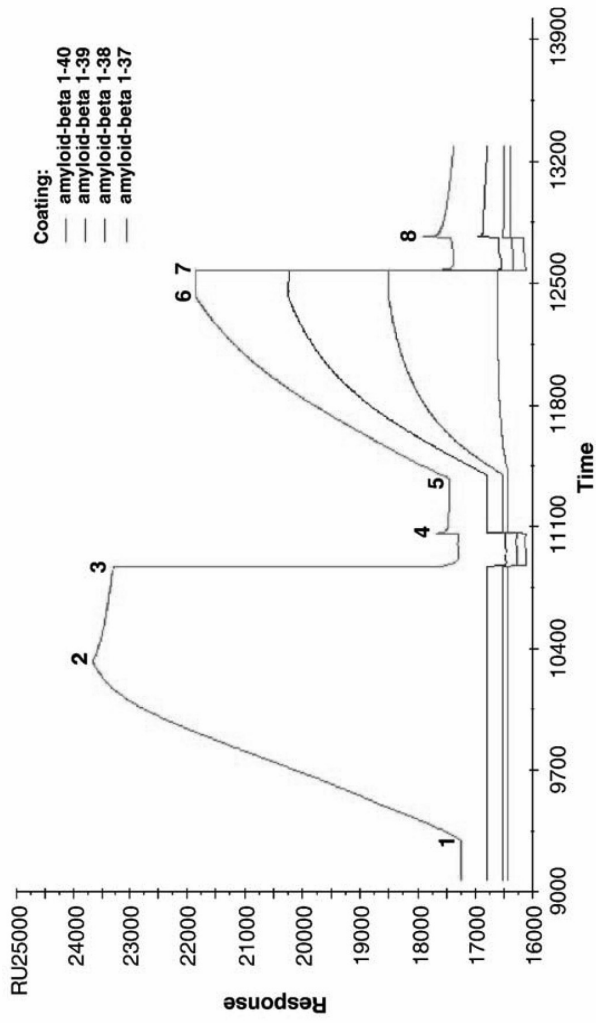


Figure 3

Interaction analysis of VU- α -A β 40 and 6E10 with A β 1-37, A β 1-38, A β 1-39, A β 1-40, A β 1-37, A β 1-38, A β 1-39, and A β 1-40 (HFIP dried; stored at -80°C in polypropylene tubes) were dissolved in 10% DMSO and diluted in Na-acetate (10mM, pH 5.0) to yield end concentrations of 0.1, 1, 0.1 and 0.5mg/mL. These peptides were separately coupled to the surface of the sensor flow cell by amine coupling (flow rate 10 μ L/min; for 7 min). Kinetic binding and dissociation measurements were carried out at 25°C in PBS with 0.1% Tween20. Each lane was coated with one peptide; A β 1-37 (red line), A β 1-38 (green line), A β 1-39 (blue line), A β 1-40 (pink line). 1: in the figure: VU- α -A β 40 (325 μ L; 2.7 μ g/mL; flow rate 19 μ L/min) was injected simultaneously in four lanes. VU- α -A β 40 reacts only with A β 1-40. 2: The VU- α -A β 40 injection was stopped. 3: The sensor surface was regenerated with 40 μ L of 0.1M HCL. 4: The HCL injection was stopped. 5: 6E10 (325 μ L; 2.5 μ g/mL; flow rate 19 μ L/min) was injected, showing an association curve for all four peptides. 6: The 6E10 injection was stopped. 7: The HCL injection was started for regeneration. 8: The HCL injection was stopped.

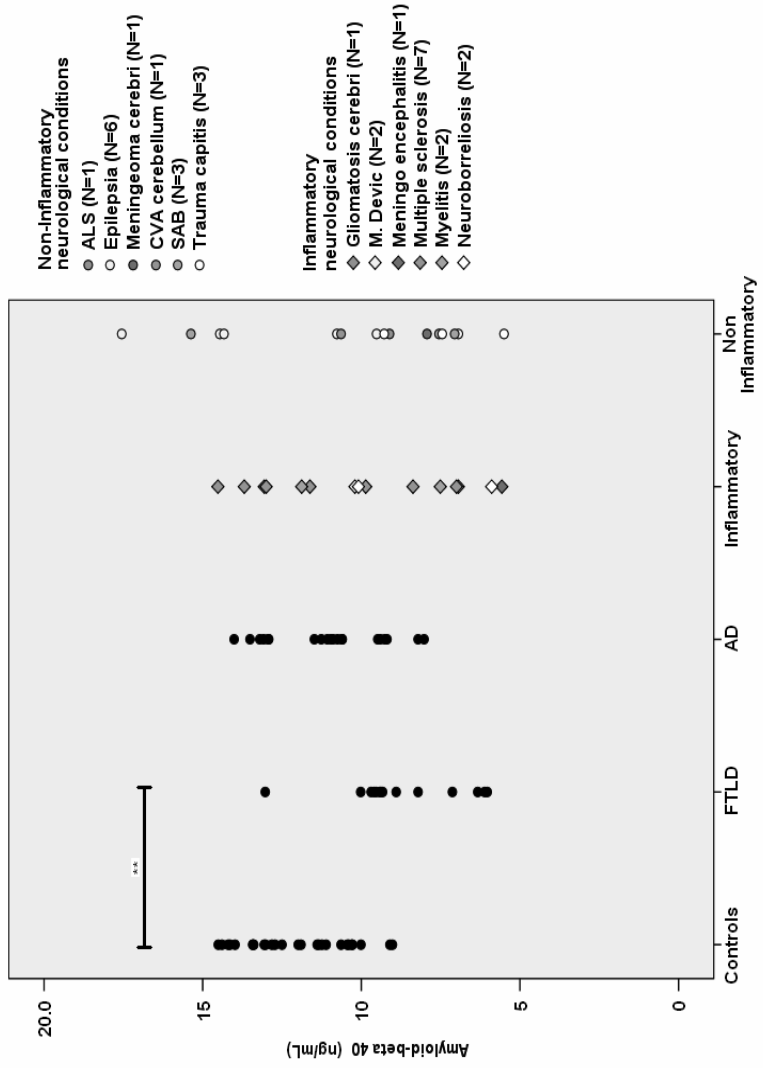


Figure 5
 CSF Aβ40 levels in different groups. The Bonferroni t test was used for the post-hoc analysis and the p-value was set at 0.01. **FTLD versus controls (p<0.05). FTLD=Frontotemporal lobar degeneration, AD=Alzheimer disease, Inflammatory=inflammatory neurological conditions (7 patients with multiple sclerosis, 2 patients with myelitis transversa, 2 patients with neuroborreliosis, 1 patient meningoencephalitis, and 2 patients with Devic's disease), Noninflammatory=noninflammatory neurological conditions (6 patients with epilepsy, 3 patients with a trauma capitis, 3 patients with a subarachnoidal bleeding, 1 patients with a meningeoma, 1 patient with a cerebrovascular accident in the cerebellum and 1 patient with amyotrophic lateral sclerosis).

DAKO) for 60 min. All sections were counterstained with haematoxylin and mounted with DePeX (BDH Laboratories Supplies).

Sandwich ELISA determination of A β 40 in CSF

A β 40 levels were measured using VU- α -A β 40 as coating antibody and biotinylated 6E10 as detecting antibody in a double antibody sandwich ELISA. Briefly, 100 μ L of mAb VU- α -A β 40 (1.08 μ g/mL) diluted in coating buffer (125mM Na₂CO₃ x 10H₂O, 125mM NaHCO₃, pH 9.6) were used to coat flat-bottomed 96-well microtiter plates (Costar, Inc, NY) and incubated overnight at RT. After washing plates once with PBS, plates were blocked for 2 h with 300 μ L of 3% BSA in PBS (to avoid nonspecific binding). All subsequent incubations were at RT on a shaker at 500 rpm. For use as a standard sample, an aliquot of 10 μ g/vial HFIP dried A β 1-40, 100 μ L of 70% formic acid was added and was mixed for ten min RT. Next, 900 μ L sample diluent (20mM Tris-HCL pH 7.5/0.1% Tween20/ 0.1% BSA) was added to a final concentration of 10 μ g/mL. Standards were further diluted in sample diluent using 25 ng/mL as highest standard. Ten microliters of diluted standards or CSF were preincubated for 2.5 h with 240 μ L of detection antibody (biotinylated 6E10 diluted 1:10,000 in sample diluent with 0.2% normal mouse serum (DAKO) in wells of polypropylene microtiter plates. After washing the blocked plates three times with wash solution (20 mM Tris-HCl pH 7.5 / 0.05% Tween20), 100 μ L of preincubated standards or CSF samples were added in duplo in the coated wells and incubated for 1 h at 500rpm. After 3 washes, all wells were incubated with 100 μ L of Streptavidin/poly-HRP conjugate (diluted 1:10,000 in sample diluent with 0.2% normal mouse serum; Sanquin, Amsterdam, The Netherlands) for 20 min at 500rpm. After four washes, the plate was developed with 100 μ L TMB for approximately 5 min and the reaction was stopped with 100 μ L 1M sulphuric acid. The absorbance was read at 450nm within 5 min.

Results

Establishment and characterization of mAb VU- α -A β 40

VU- α -A β 40 was generated after immunization of a mouse with a BSA-Cys-A β 33-40 conjugate. Spleen and lymph node cells were isolated and fused with mouse SP2/0 myeloma cells. Limiting dilutions were used to select and subclone the antibody producing hybridoma cells. VU- α -A β 40 was identified as IgG1 kappa subclass and to react with the C-terminal residue 40 of the amyloid peptide only. This clone was selected by solid phase ELISA experiments in which A β 1-40, Cys-A β 1-17, A β 1-42, Cys-A β 33-42 and Cys-A β 33-40 were used as coating peptides and a positive reaction was only seen with A β 1-40 and Cys-A β 33-40. This was in line with Western blot experiments which revealed that VU- α -A β 40 is very specific and sensitive for the last C-terminal amino acid of synthetic A β 1-40. In addition, no cross-reactivity was seen with other A β forms (A β 1-37 to A β 1-39 and A β 1-41 to A β 1-49) (Figure 1a) and APP (data not shown). IP experiments showed also that VU- α -A β 40 binds only synthetic and human CSF A β 1-40 (figures 1b and 2). These findings were confirmed by SPRS experiments which showed a strong affinity of VU- α -A β 40 for synthetic human A β 1-40 ($K_A=1.3 \times 10^9$ M⁻¹ and $K_D=7.7 \times 10^{-10}$ M; Figure 3, see Chapter 7) and for Cys-A β 33-40 ($K_A=2.8 \times 10^{10}$ M⁻¹ and $K_D=3.57 \times 10^{-11}$ M). No cross-reactivity with synthetic human A β 1-37, A β 1-38 and A β 1-39 (Figure 3) and Cys-A β 1-17, Cys-A β 33-42 and A β 1-42 was seen. To

further characterize VU- α -A β 40, the staining pattern of VU- α -A β 40, when used for immunohistochemistry on brain tissue of one AD patient and one control, was determined (Figure 6, see Chapter 7). For comparison, the antibodies 6E10 (recognizing virtually all forms of A β) and G2-10 were used to immunostain serial sections from the temporal cortex. Immunohistochemical staining with VU- α -A β 40 revealed positivity of compact and classical amyloid plaques which were comparable with the immunostainings performed with G2-10. Furthermore, vascular amyloid deposition could be observed. Besides staining of amyloid in plaques and in the vascular walls, no additional immunohistochemical positivity could be seen. The staining performed with G2-10 was comparable with the staining performed with VU- α -A β 40, confirming that VU- α -A β 40 recognize A β 1-40 (Figure 6, see Chapter 7).

Sandwich ELISA properties

Figure 4 shows the calibration curve for A β 1-40 (doubling dilutions with highest standard of 25ng/mL). The detection limit is 0.21ng/mL (optical density=0.182). This was calculated by taking the mean absorption of 18 zero standards [mean optical density 0.167(0.005)] plus three times the standard deviation. To show parallelism, three fractional dilutions of two CSF samples were plotted together with the calibration curve (Figure 4). The fractional dilutions were within the working range of the assay and consisted of first dilution: 20 μ L CSF in 230 μ L sample diluent; second dilution: 10 μ L CSF in 240 μ L sample diluent; third dilution: 4 μ L CSF in 246 μ L sample diluent. Additionally, the linearity of the assay was confirmed (mean r^2 of the two different CSF samples=0.997) by plotting the A β 40 levels of the fractional dilutions against the dilution factor.

- Recovery: Analytical recovery was studied by adding different amounts of synthetic A β 1-40 (between 1 and 10ng/mL) to CSF. Concentrations determined (with the different amounts added) were calculated and compared to the theoretical concentration. The recovery was between 83% and 96% (mean 90%; N=6)

- Stability of A β 40 in human CSF: The effect of repeated freeze/thaw cycles was investigated by thawing two CSF samples for five times. A β 40 was measured each time that the sample was thawed (Table 1). The measurement of the second thawed cycles revealed a CSF A β 40 decrease of 32% (CSF 1) and 17% (CSF 2), indicating that A β 40 has to be measured in CSF samples with a maximum of one freeze/thaw cycle.

- Precision: The intra-assay coefficient of variation (CV) was 1.4%. The CSF measurements were performed twice in one assay and for each the duplo coefficient of variation (CV) was calculated. The intra-assay CV was calculated by taking the mean of the duplo CV of CSF samples (N=634; mean of CSF A β 40=9.4ng/mL; with a range of 1.8-21.9ng/mL). The inter-assay variation was calculated by use of three different CSF pools which were stored at -80°C in polypropylene tubes. The mean value of the first CSF pool is 5.6(0.7) ng/mL with an inter-assay CV of 11.9% (N= 25). The mean value of the second CSF pool is 5.8(0.3) ng/mL with a CV of 5.0% (N= 25), and the mean value of the third CSF pool is 11.1(0.5)ng/mL with a CV of 5.1% (N= 10). The mean inter-assay CV is 7.3%.

- Reference range for A β 40: A β 40 was measured in CSF of 27 control subjects. The mean value was 12.0 ng/mL with a SD of 1.7 ng/mL (Figure 5). The range was from 9.0 to 14.5ng/mL. No significant correlation between age (49-83 years) and CSF A β 40 level (r_s = -0.31; p =0.11) was observed.

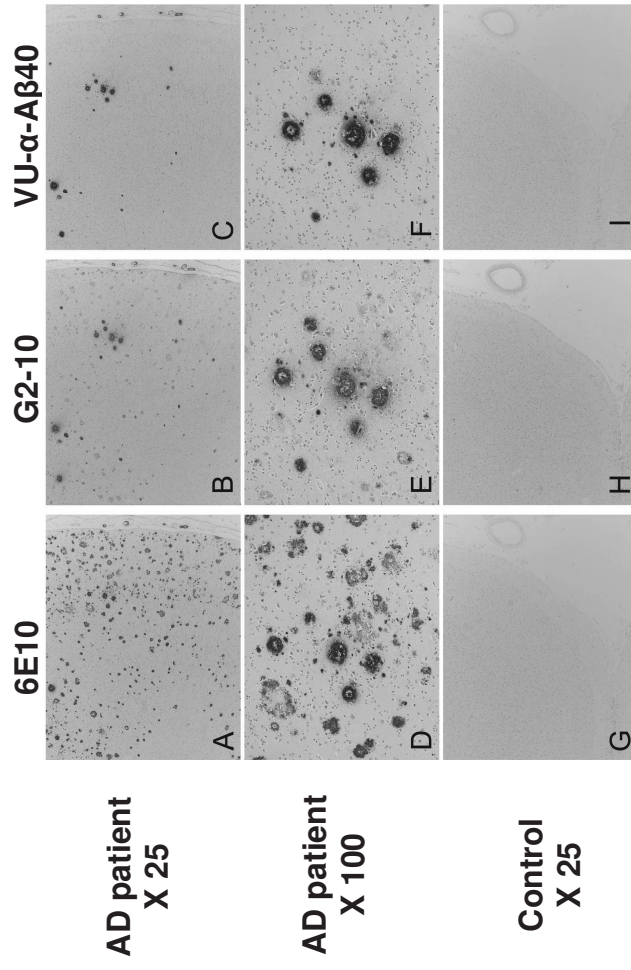


Figure 6

The staining performed with 6E10 (monoclonal antibody which has a high affinity for N-terminal amino acids 3-8 of the A β peptide; Signet, Dedham, MA) revealed different types of amyloid plaques in the temporal cortex of the AD patient. The staining performed with VU- α -A β 40 revealed only diffuse, compact and classical A β plaques in the temporal cortex of an AD case. Further vascular amyloid deposition could be observed. Besides staining of amyloid in plaques and in the vascular walls, no other immunohistochemical detection could be seen. The staining performed with G2-10 (monoclonal antibody which recognizes A β with C-terminal residue 40 and A β with C-terminal residue 39 (Jensen et al. 2000); the Genetics Company, Schlieren, Switzerland) was comparable with the staining performed with VU- α -A β 40, confirming that VU- α -A β 40 recognizes A β 1-40. A, D, G=stainings with 6E10; B, E, H=stainings with G2-10; C, F, I=stainings VU- α -A β 40. AD=Alzheimer Disease patient.

- Clinical validation: The clinical characteristics and levels of CSF A β 40 by diagnostic groups are shown in Table 2 and Figure 6. Significant group differences were seen for age and CSF A β 40 levels (both $p < 0.01$). Additionally, post hoc tests revealed lower A β 40 levels in FTLD patients [8.8ng/mL (1.9ng/mL)] compared to controls [12.0ng/mL (1.7ng/mL)]; $p < 0.01$; Figure 5, see Chapter 7).

Discussion

In the present study we describe the synthesis of a mouse mAb that upon testing with use of various techniques appeared to recognize the last amino acid of the A β 40 C-terminus with high specificity and sensitivity. This antibody was used to develop a sandwich ELISA for the detection of A β 40 in human CSF with the goal to use this test as a diagnostic tool. Besides characterization of the mAbs used in an ELISA, a complete validation of an ELISA is needed before it can be used in the clinic, which includes the determination of a detection limit, recovery, stability, precision and parallelism⁸⁶. Additionally, for a reference range, an ELISA has to be used to measure control samples and these results have to be compared with measurements of patients with different pathologies. Till now, four different ELISA systems, composed of mAbs, have been described for the measurement of A β 40 in human CSF^{89, 90, 99, 100}. In previous studies, two sandwich ELISAs claim to measure A β 40 in human CSF^{99, 100}. However, in these sandwich ELISAs, mAbs specific for the N-terminus of A β were used for capturing.

Table 1

No of times thawed	CSF 1 A β 40 (ng/mL)	CSF 2 A β 40 (ng/mL)
1	9.4	7.3
2	6.8	5.9
3	6.5	6.3
4	6.3	6.2
5	6.0	6.0

Five freezing/thawed cycles were performed using two CSF samples. Each time that the samples were thawed, A β 40 was measured. The measurement of the second thawed cycles revealed a CSF A β 40 decrease of 32% (CSF 1) and 17% (CSF 2), indicating that A β 40 has to be measured in CSF samples with one freezing/thawing cycle. However, levels of CSF A β 40 remained stable during the other cycles after the second freezing/thawing cycle.

As a result not only A β 40 will be bound by the coating mAbs but also other A β peptides with different C-terminal ends and maybe also soluble APP¹⁰¹. These bound peptides could potentially interfere in the sandwich ELISA and could lead to misinterpretation of the exact concentration of A β 40.

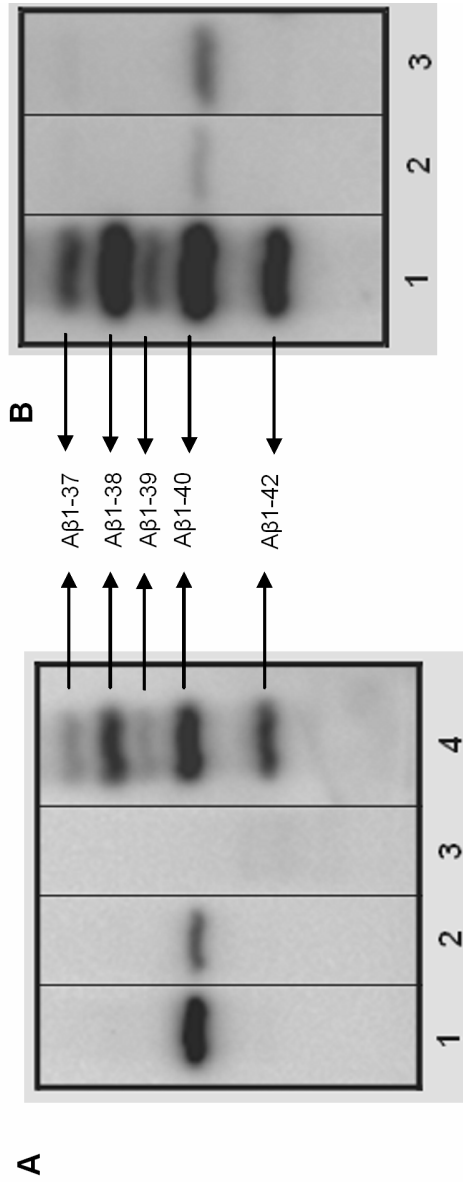


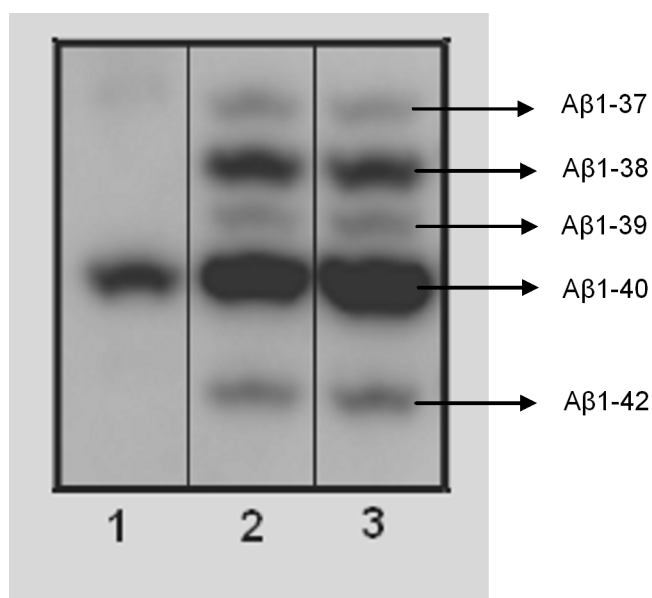
Figure 1a

VU- α -A β 40 detects only A β 1-40 from a mixture of synthetic A β forms on Western blot. Lane 1: 10 μ L of Mix 1; protein content: 2.4ng A β 1-40; 1.2ng A β 1-42, 1.2ng A β 1-38, 0.6ng A β 1-39, 0.6ng A β 1-37. Lane 2: 10 μ L of Mix 1 diluted 1:2; protein content 1.2ng of A β 1-40; 0.6ng of A β 1-42, 0.6ng of A β 1-38, 0.3ng of A β 1-39, 0.3ng of A β 1-37. Lane 3: 10 μ L of Mix 2; protein content 1.0ng of A β 1-41, 1.0ng of A β 1-42, 1.0ng of A β 1-43, 1.0ng of A β 1-44, 1.0ng of A β 1-45, 1.0ng of A β 1-46, 1.0ng of A β 1-47, 1.0ng of A β 1-48, 1.0ng of A β 1-49. Lane 4: 10 μ L of Mix 1 diluted 1:20; protein content 120pg of A β 1-40; 60pg of A β 1-42, 60pg of A β 1-38, 30pg of A β 1-39, 30pg of A β 1-37. Lane 1 to 3 is immunostained with VU- α -A β 40 (1:500 of stock solution). Lane 4 is immunostained with 1E8 (1:300 of stock solution), used as control.

Figure 1b

Immunoprecipitation of synthetic A β 1-40 with VU- α -A β 40 and immunostained with 1E8, showing that VU- α -A β 40 captured only A β 1-40. Lane 1: 10 μ L of Mix 1 diluted 1:20; protein content 120pg of A β 1-40; 60pg of A β 1-42, 60pg of A β 1-38, 30pg of A β 1-39, 30pg of A β 1-37. Lane 2: 5 μ L of Mix 1 (protein content 1.2ng of A β 1-40; 0.6ng of A β 1-42, 0.6ng of A β 1-38, 0.3ng of A β 1-39, 0.3ng of A β 1-37) diluted with 100 μ L RIPA detergent buffer, 400 μ L H₂O and 25 μ L VU- α -A β 40 beads and incubated at 4 $^{\circ}$ C for one night. After washing, bound A β was eluted with 25 μ L sample buffer for electrophoresis. 5 μ L of the IP was diluted (1:2) and applied to gel. Lane 3: The same procedure as for lane 2, except that 5 μ L of an undiluted sample was applied.

Figure 2



IP of Aβ1-40 with VU-α-Aβ40 in CSF and immunostained with 1E8, showing that VU-α-Aβ40 captured only Aβ1-40 in CSF. Lane 1: IP of CSF with VU-α-Aβ40 and immunostained with 1E8. 100μL RIPA detergent buffer, 200μL H₂O and 25μL VU-α-Aβ40 beads were added to 200μL CSF-pool and immunoprecipitated overnight at 4 °C. After washing, bound Aβ was eluted from the beads with 25μL sample buffer. 10μL of IP is shown in lane 1. Lane 2: 500μL of Aβ1-40 depleted IP solution (with VU-α-Aβ40) was added to 25μL 1E8 beads and immunoprecipitated overnight at 4 °C. After washing, bound Aβ was eluted with 25μL sample buffer. 5μL of the IP is diluted 1:4 and shown (immunostained with 1E8) in lane 2. Lane 3: IP of CSF with 1E8 and immunostained with 1E8. 100μL RIPA detergent buffer, 200μL H₂O and 25μL 1E8 beads were added to 200μL CSF-pool and immunoprecipitated overnight at 4 °C. After washing, bound Aβ was eluted with 25μL sample buffer. 5μL of the IP is diluted 1:4 and shown in lane 3.

Furthermore, the reports on the validation of these two ELISAs are not complete, making it difficult to compare these methods with ours. The W0-2/G2-10 ELISA described by Jensen et al.⁹⁹ showed a concentration range from 20 pM to 250 pM Aβ40 and an intra- and inter-assay variation of 2-3% and 10%, respectively. Although the sensitivity of this assay is good, questions about specificity remain, since, whereas the detecting mAb G2-10 does not recognize APP, it in addition to Aβ1-40 also recognizes Aβ with C-terminal residue 39 in epitope mapping experiments⁹⁹. This set of mAbs (W0-2/G2-10) has been used to measure Aβ40 levels in human CSF, showing no difference between different patient groups^{62, 69, 101}. The ELISA, described by Tamaoka et al.¹⁰² showed a recovery of 37% for insoluble forms and 100% for soluble forms of Aβ40. The detection limit was beyond the concentration of 0.16 fmol/well and the intra- and inter-assay CV's were less than 10%. The Ban50/BA27

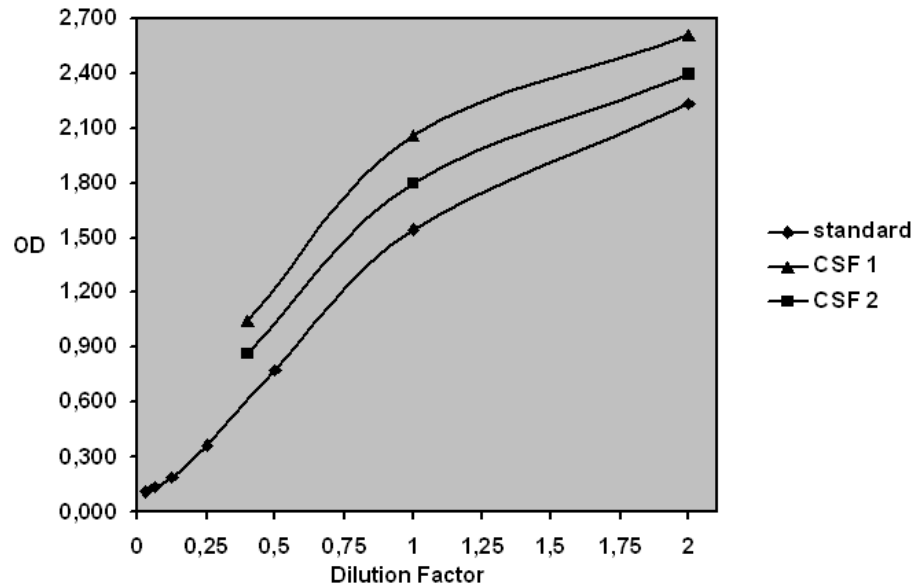
ELISA was used to measure levels of A β 40 in CSF, showing no difference between controls and AD patients^{66, 100}. In other studies sandwich ELISAs, which claim to measure A β 1-40 in human body fluids, were reported⁸⁹⁻⁹¹. These ELISAs use a different strategy compared to the two ELISAs described above. In these ELISAs mAbs against the C-terminal at residue 40 were used for capture. Horikoshi et al.⁸⁹ used 1A10 as capture mAb and 82E1 for detection. 1A10 showed virtually no cross-reactivity (<1.8%) with A β 1-42, which is in line with their WB study. Monoclonal antibody 82E1 was shown not to cross-react with APP, and to recognize A β 1-5, leaving open the possibility that 82E1 not only recognizes the first amino acid of the A β peptide but also N-truncated forms. This test had a good linearity ($r^2=0.98$) and a detection limit as low as 1.5 pg/mL, and was used to measure A β 40 in CSF samples⁸⁹, showing no difference between AD and controls. Cirrito et al.⁹¹ and Citron et al.⁹⁰ described a sandwich ELISA using 2G3 as capturing mAb against the C-terminal of A β at residue 40, and 3D6 for detection. 2G3 has less than 0.4% of cross-reactivity with A β 1-42 and 3D6 recognizes only the first N-terminal amino acids of the amyloid peptide⁸⁸. Although an extensive validation for measuring A β 40 in human CSF is lacking, clinical samples have been measured using this sandwich ELISA¹⁰³. In our study A β 40 was measured in the CSF of controls, AD patients, FTLD patients and patients with noninflammatory and inflammatory neurological conditions, showing lower CSF A β 40 levels in FTLD patients compared to controls. Since we have used a new ELISA, a comparison with our previous study is quite difficult⁶¹. The application of different carboxyterminal- and/or aminoterminal-specific anti-A β antibodies (as coating or as detecting antibodies) with distinct affinities, specificities and sensitivities may yield different results. However, the here above mentioned finding opens new possibilities for early and differential diagnosis in dementia patients, but it has to be confirmed in a larger clinical study.

Table 2

	Controls	FTLD	AD	Inflamm.	Noninflamm.	p-value
N	27	14	20	15	15	
Sex F (%)	12 (44)	6 (43)	12 (60)	7 (47)	6 (40)	0.8
Age	61 (10)	59 (8)	64 (7)	46 (13)	58 (7)	<0.01 ^b
Aβ40 (ng/mL)	12.0 (1.7)	8.8 (1.9)	11.0 (1.8)	9.9 (3.0)	10.2 (3.6)	<0.01 ^a

A β 40 measured in CSF of several subjects with VU- α - A β 40 as capture antibody and 6E10 as detecting antibody. Biomarker levels are shown as mean (SD). To analyze the differences within the three groups we used Chi-square in % and ANOVA in mean (SD). The Bonferroni t test was used for the post-hoc analysis and the p-value was set at 0.05. Significant Post-Hoc results: ^a FTLD versus controls; ^b Inflammatory versus AD, FTLD, noninflammatory and controls. FTLD=Frontotemporal lobar degeneration, AD=Alzheimer's Disease, Inflamm.=inflammatory neurological conditions (7 patients with multiple sclerosis, 2 patients with myelitis transversa, 2 patients with neuroborreliosis, 1 patient with meningoencephalitis and 2 patients with neuromyelitis optica), Noninflamm.=noninflammatory neurological conditions (6 patients with epilepsy, 3 patients with mild head injury, 3 patients with a subarachnoidal haemorrhage, 1 patients with a meningioma, 1 patient with a cerebellar ischemic stroke and 1 patient with amyotrophic lateral sclerosis).

Figure 4



A representative calibration curve of A β 1-40 with the sandwich ELISA VU- α -A β 40/ 6E10 with a lower detection limit of 0.21ng/mL [mean optical density=0.182 (0.005)]. (This was calculated by taking the mean absorption of 18 zero blanco standards plus three times the standard deviation.). 25ng/mL of A β 1-40 was used as highest standard for the calibration curve and was used for doubling dilutions (dilution factor 1=25.0ng/mL (optical density 2.233); dilution factor 0.5=12.5ng/mL (optical density 1.543); dilution factor 0.25=6.25 ng/mL (optical density 0.773); dilution factor 0.125=3.13 ng/mL (optical density 0.368); dilution factor 0.0625=1.56ng/mL (optical density 0.192); dilution factor 0.03125=0.78ng/mL (optical density 0.132); dilution factor 0.015625=0.39 ng/mL (optical density 0.109); dilution factor 0=0ng/mL (optical density 0.092). To show parallelism⁸⁶, three different fractional dilutions of two CSF samples were prepared (which fall evenly across the working range of the assay). The first dilution consisted of 20 μ L CSF sample in 23 μ L sample diluent. The second dilution consisted of 10 μ L CSF sample in 240 μ L sample diluent. And the third dilution consisted of 4 μ L CSF sample in 246 μ L sample diluent. CSF1: dilution factor 1=36.4ng/mL (optical density 2.602); dilution factor 0.5=20.0ng/mL (optical density 2.056); dilution factor 0.2=8.3ng/mL (optical density 1.044). CSF2: dilution factor 1=30.0ng/mL (optical density 2.393); dilution factor 0.5=15.4ng/mL (optical density 1.794); dilution factor 0.2=6.9ng/mL (optical density 0.860).

Ideally, A β 40 is measured with a specific mAb against the C-terminus of the amyloid peptide at residue 40 as capture antibody and a mAb against the N-terminal residue as detecting antibody. Here we developed a specific ELISA system with VU- α -A β 40, a new produced capturing antibody produced in our department. Additionally, we validated the method for accurate measurement of A β 40 in human CSF of controls and patients.