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ORIGINAL ARTICLE

Measurement of Aβ1–42 in cerebrospinal fluid is influenced by matrix effects

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Abstract

Aβ1–42 measurement in CSF is an important biochemical marker for Alzheimer disease (AD). However, our understanding of why this biomarker is predictive and why it is often difficult to measure in a reproducible fashion is still lacking. To study these questions, the concentration of Aβ1–42 in CSF was compared before and after denaturation with 6M guanidine and reverse-phase HPLC. Measurement of the Aβ1–42 after denaturation and reverse-phase HPLC demonstrated that considerably more Aβ1–42 was present in CSF than revealed when assaying non-denatured CSF. A comparison of Aβ1–42 concentrations before and after HPLC in AD CSF with that in normal controls suggested that matrix interference may affect the differentiation between the diagnostic groups. A similar effect was observed with dilutions of crude CSF. Together, these results suggested that at least part of the mechanism by which low Aβ1–42 concentrations in CSF function as a biomarker of AD is related to matrix components which preferentially hide a portion of the Aβ1–42 from detection in AD CSF. In contrast, we show that the association of the *APOE* ε4 allele with lower Aβ1–42 concentrations in CSF is preserved even after denaturation and HPLC. A similar relationship between the presence of the *APOE* ε4 allele and lower concentrations of Aβ1–40 was also apparent, thereby generating similar ratios of Aβ1–42/ Aβ1–40 across the *APOE* genotypes. The results from the present study suggested that Aβ1–42 in CSF functions as a biomarker of AD in tandem with other CSF matrix components that are increased in AD CSF. Further studies are needed to identify which matrix factors (e.g. binding of Aβ to proteins) underlie the increased detection of Aβ1–42 concentrations after denaturation and HPLC. The data also suggested that denaturation and HPLC of CSF may be a useful approach for studies using Aβ1–42 as a pharmacodynamic marker or in other paradigms where measurement of total non-covalently bound Aβ1–42 is required.

Keywords: Aβ peptides, Biomarker, CSF, HPLC, Immunoassay.

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Alzheimer's disease (AD) is a devastating neurological disorder that affects more than 26 million people worldwide (Blennow *et al.* 2006). The causes of the sporadic form of the disease are poorly understood which has been a major impediment to providing effective treatment. However, this climate is changing and new therapies are being tested that are likely to help ameliorate the progression of the disease. As a result, it has become imperative that we develop measurements that can identify patients that are very early in the disease process (Hampel *et al.* 2010). To date, the earliest appearing biomarker in patients developing AD has been decreased Aβ1–42 concentrations in CSF (Jack *et al.* 2010;

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Abbreviations used: AD, Alzheimer Disease; AUC, area under the curve; ROC, receiver operator curve; RP-HPLC, reverse-phase HPLC.

Stomrud *et al.* 2010). Consequently, it was of considerable interest to further understand the nature of this measurement in CSF and how it functions as a biomarker.

Aβ1–42 measurements in CSF have been challenging. Issues with assay performance using multiple immunoassay systems have included non-linearity as CSF samples are diluted (Bjerke *et al.* 2010) and stochastic inter-assay reproducibility across laboratories (Mattsson *et al.* 2011). A contributing factor to these issues may be interference from proteins or other components present in CSF. To examine this possibility, a well-used paradigm using denaturation of biological samples in high concentrations of guanidine hydrochloride and reverse phase-HPLC (RP-HPLC) was employed (Berrettini *et al.* 1986; Morgan *et al.* 1988; Ditto *et al.* 2009). RP-HPLC has been a preferred method for extracting and concentrating peptides for some time because it is both highly reproducible and easily automated.

In addition to challenges with immunoassays, pathophysiological processes have been suggested to affect Aβ1–42 concentrations in human CSF. An important line of experimentation has highlighted that the diminution of Aβ1–42 concentrations in AD CSF may reflect the increase of peptide deposition in brain that produces cerebral amyloid angiopathy or neuritic plaques (e.g. Strozyk *et al.* 2003; Fagan *et al.* 2006; Grimmer *et al.* 2009). The use of Aβ1–42 measurements in CSF that include a denaturation and enrichment step prior to immunoassay may be able to shed additional light on this inverse relationship by determining CSF Aβ1–42 concentrations in the absence of CSF proteins, protein aggregates or other components (matrix) that may influence the assay. In particular, it was of interest to examine if the reduction of CSF proteins prior to assay would enhance the ability of Aβ1–42 assays from CSF to differentiate between AD patients and non-demented controls. Because the presence of the *APOE* ε4 allele has also been shown to produce lower Aβ1–42 concentrations in CSF (e.g. Prince *et al.* 2004), the current enrichment scheme was exploited to evaluate the potential effect of CSF matrix on the measurement in AD patients as a function of different distributions of the ε4 allele.

Methods

AD and control subjects, CSF sampling and biomarker analyses

The study was approved by the ethics committee at Karolinska University Hospital, Huddinge, Stockholm. CSF samples were collected by lumbar puncture through the L3/L4 or L4/L5 interspace. The first 12 mL of CSF was collected in a polypropylene tube, immediately transported to the local laboratory for centrifugation at 2000 *g* at 4°C for 10 min. The supernatant was pipetted off, gently mixed to avoid possible gradient effects, and aliquoted in 2–5 mL portions that were stored at −80°C pending testing.

The clinical indication for performing the spinal tap in the patients was to exclude neuroinflammatory and neuroinfectious conditions. All of the included samples had normal albumin ratio, IgG index and cell counts according to established reference limits accredited by the Swedish Board for Accreditation and Conformity Assessment.

Samples from three different sets of patients and controls were included in the study (sets A, B and C) (Table 1). Set A patients (n = 20) received a diagnosis of AD using the DSM-IIIR (American Psychiatric Association 1987) and National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (McKhann *et al.* 1984) criteria of dementia and probable AD. The mini-mental state examination score was used as a global measure of functioning (Folstein *et al.* 1975). Inclusion criteria for set A controls (n = 20) were that they should be physically and mentally healthy and not experiencing or exhibiting any cognitive impairment. All controls were thoroughly interviewed about their somatic and mental health

Table 1 Demographics and biochemical characteristics of set A and B patients and controls

	Set A[a]		Set B[b]	
	AD patients (n = 20)	Controls (n = 20)	AD patients (n = 21)	Non-AD patients (n = 23)
Age (years)	68 ± 7.2	73 ± 6.6	72 ± 10	63 ± 12
Gender (m/f)	10/10	12/8	4/17	8/15
MMSE	21 ± 4.6[c]	30 ± 0	NA	NA
T-tau (ng/L)	184 ± 56.3[c]	99 ± 58	189 ± 77.7[c]	65 ± 25
P-tau (ng/L)	40 ± 20[c]	23 ± 10	80 ± 44[c]	29 ± 12
Aβ1–42 (ng/L)	200 ± 38.3[c]	287 ± 87.0	159 ± 45.4[c]	333 ± 106

AD, Alzheimer's disease; MMSE, mini-mental state examination; NA, not available.

[a]Clinically diagnosed AD patients and cognitively healthy controls.

[b]Neurochemically diagnosed AD and non-AD patients.

[c]$p < 0.001$ versus controls.

Values represent mean ± standard deviation.

by a research nurse before inclusion in the study and were cognitively stable over at least 2 years after the initial examination.

Set B samples were from patients (n = 44) who sought medical advice because of cognitive impairment. Very little clinical data were available for these patients. Patients were designated as non-AD (n = 23) or AD (n = 21) according to CSF biomarker levels obtained in clinical routine analyses using INNOTEST ELISAs (Innogenetics, Ghent, Belgium) with verified longitudinal stability and -cutoffs that are 90% specific for AD (Hansson *et al.* 2006): total tau (T-tau) > 350 ng/L and Aβ1–42 < 530 ng/L.

Set C samples came from patients who fulfilled the clinical criteria for AD (American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, third edition, revised). In addition, they were selected to represent different *APOE* ε4 allele carrier groups (Table 2). For the purpose of the present analysis, patients with very high or very low CSF Aβ1–42 concentrations were excluded to make the different carrier groups comparable with regards to measurement of CSF Aβ1–42 concentrations in crude CSF. Some *APOE* ε4 carriers display very low Aβ1–42 concentrations, which may bias the comparison.

CSF T-tau, P-tau and Aβ1–42 data for sets A and B were obtained by analysis of aliquots of CSF from all patients and controls on one occasion using INNO-BIA xMAP technology (INNO-BIA AlzBio3; Innogenetics) as previously described (Olsson *et al.* 2005). CSF data for set C were obtained using INNOTEST ELISAs as previously described (Hulstaert *et al.* 1999; Vanmechelen *et al.* 2000). *APOE* genotyping was performed as previously described (Blennow *et al.* 2000).

Aβ measurements

As part of the process to establish the HPLC separation, the elution time window from HPLC which contained the Aβ1–40 peptide was determined using the Invitrogen β-Amyloid (1–40) ELISA Kit (Invitrogen, Camarillo, CA, USA; Cat. No. 99-0063). The elution position of Aβ1–42 from HPLC was determined with the use of the Wako Human β Amyloid (1–42) ELISA High-Sensitive Kit (Wako Chemicals U.S.A. Inc., Richmond, VA, USA; Cat. No. 296-64401).

Analysis of Aβ1–42 in CSF samples assayed before and after HPLC was carried out using INNO-BIA xMAP technology (INNO-BIA AlzBio3; Innogenetics) according to the manufacturer's instructions. Quantification was carried out using a Bio Plex 100 instrument using Bio Plex Manager Version 5 software. Additional experiments measured Aβ1–42 in unprocessed CSF using both the Wako Human β Amyloid (1–42) ELISA High-Sensitive kit (Wako Chemicals USA Inc., Richmond, VA, USA) and a microplate ELISA employing 6E10 (mouse monoclonal antibody to human Aβ1–16; Covance, Princeton, NJ, USA) as a capture antibody with alkaline phosphatase-conjugated 12F4 (mouse monoclonal antibody specific for the carboxyterminus of Aβ1–42) used for detection.

Quantification of Aβ1–40 in crude unprocessed or HPLC-purified CSF was accomplished with the use of a microplate ELISA using TSD9S3.2 as a capture antibody (mouse monoclonal antibody to the Aβ40 neoepitope) and horseradish peroxidase-conjugated 26D6 antibody (mouse monoclonal antibody to the N-terminus of Aβ) for detection.

Table 2 Demographics and biochemical characteristics of set C patients according to *APOE* ε4 allele carrier status

	APOE ε4 allele carrier status		
	No	One	Two
Age (years)	80 ± 5.3	77 ± 7.8	75 ± 7.8
Gender (m/f)	11/14	6/11	10/9
MMSE	23 ± 4.6	23 ± 3.0	23 ± 5.3
Aβ1–42 (ng/L)	502 ± 58.6	382 ± 62.7[a]	388 ± 105[a]

MMSE, mini-mental state examination.
[a]p < 0.001 versus *APOE* ε4 non-carriers.
Values represent mean ± standard deviation.

Statistical analysis

Analysis of immunoassay data was carried out using the Origin Pro 8.1 Data Analysis and Graphing Software (OriginLab Corp., Northampton, MA, USA) and Graph Pad Prism version 5 analysis programs (GraphPad Software Inc., La Jolla, CA, USA).

Results

Information on patients and control subjects

Demographics and biochemical characteristics of patients and controls in sets A–C are given in Tables 1 and 2.

Repetitive recovery of Aβ1–42 and Aβ1–40 on RP-HPLC

Consistent with the high utility of RP-HPLC for peptide separations, the data in Table 3 demonstrated that both Aβ1–42 and Aβ1–40 could be recovered from CSF with high reproducibility. The data in the table represents a combination of variability from HPLC and variation in the measurements from immunoassay. The recovery of Aβ1–42 from the HPLC system used here had been determined to be about 84 ± 4% (Ditto *et al.* 2009), which demonstrated sufficient recovery to provide a quantitative method. The percent coefficient of variation (%CV) for repetitive recovery in this set of CSF samples after HPLC and immunoassay was between 4.2 and 9.1%. This was less than 10%, which is the expectation for intra-assay variation in the immunoassay systems employed. The coefficient of variation (%CV) for repetitive recovery of Aβ1–40 was 4.8%, which is consistent with the results from Aβ1–42. Examples of the HPLC separation and the details of the process are described in Figure S1.

Effect of matrix reduction with HPLC or sample dilution on Aβ1–42 as a biomarker

The data shown in Fig. 1a (lanes 1 and 2) demonstrated the separation of an age-matched set of samples from cognitively normal or AD patients based upon apparent concentrations of Aβ1–42 (CSF Set A, Table 1). The separation is characteristic of results from the AlzBio3 assay, showing lower concentrations of Aβ1–42 on average in the AD population. The receiver operator curve (ROC) analysis in panel b demonstrated the separation between AD and control in these

Table 3 HPLC provided high repetitive recovery of Aβ1–42 and Aβ1–40 from CSF

CSF set	Total no. HPLC separations	No. pooled-CSF controls	Mean Aβ1–42 pg/mL	%CV	Mean Aβ1–40 pg/mL	%CV
A	50	5	1294	4.2	ND	_
B	57	8	684	9.1	ND	_
C	76	7	1619	7.7	4785	4.8

All samples were separated on HPLC in a sequential manner. Therefore, it was important to ensure that the integrity of the HPLC separations was maintained across all of the samples being processed within an experiment. To accomplish this, the repetitive recovery of Aβ1–42 and Aβ1–40 was monitored in a control sample of CSF made by pooling CSF from normal subjects. There were three experimental sets of CSF (A, B and C in Tables 1 and 2). Each experimental set of CSF used a different pool of normal CSF to monitor reproducibility. 0.5 mL aliquots of pooled CSF were processed at the same time and in the same manner as the CSF from the study subjects and these quality control samples were evenly distributed throughout the CSF samples in line to undergo HPLC separation. In this manner, a change in the performance of the HPLC that might differentially affect samples across the HPLC sequence would be detected. The %CV (percent coefficient of variation) values for all sets of HPLC quality control samples were less than 10%, indicating that the HPLC-Immunoassay procedure used for these studies was very reproducible. The %CV is a combined error from both HPLC and immunoassay using the INNO-BIA AlzBio3 kit. ND, not determined. The details of the RP-HPLC procedure and instruments used in this study are described in Ditto *et al.* (2009).

unprocessed samples (area under the curve, AUC = 0.75, Fig. 1b, dashed line). Panel a (lanes 3 and 4) shows the same samples assayed after enrichment of the Aβ peptides on HPLC. All samples demonstrated greater concentrations of Aβ1–42 after HPLC extraction, indicating that the CSF samples contained peptide that was occluded from the immunoassay before denaturation and HPLC. Release of this peptide to the assay was greater in the AD samples, thereby causing a loss of differentiation between AD patients and normal subjects as determined by ROC analysis (AUC = 0.49, Fig. 1b, solid line).

Figure 1c shows the separation between AD and control CSF for the same samples studied with HPLC in 1A, but only after dilution of the crude CSF. The Wako Human Beta Amyloid (1–42) ELISA Kit, High-Sensitivity [BAN50 capture (Aβ1–16)/BC05 detection (Aβ1–42)] had been used for this analysis. Because of its excellent sensitivity, it allowed analyses of Aβ1–42 using a broad range of dilutions. At a dilution of ×2 to ×4, the area under the ROC curve of approximately 0.74 was similar to the separation seen in the AlzBio3 assay of 0.75 (Fig. 1b). However, there was a loss of differentiation that was dependent upon further dilution. At the highest dilution (×32), there was essentially no differentiation between AD and non-demented controls. This was the same result as observed on the sample set after extraction of the Aβ1–42 using RP-HPLC. The same analysis carried out with Aβ1–42 concentrations determined from an ELISA using antibody 6E10 capture (Aβ3-8) and 12F4 detection (Aβ42-specific), showed no differentiation at any dilution.

Effect of HPLC on a well-differentiated set of AD and control CSF

The loss of differentiation and greater than 200% increase in measured Aβ1–42 peptide after HPLC enrichment was counter to the expectation that differentiation might be improved. Consequently, an additional analysis was performed on another set of CSF from an additional group of AD and cognitively normal subjects (CSF Set B, Table 1). These samples were pre-selected to provide strong separation between AD and cognitively normal subjects based on AlzBio3 immunoassay data using crude CSF. The data in Fig. 2a (lanes 1–4) demonstrated the excellent reproducibility of this separation, which had been repeated in a second laboratory at the time the samples from HPLC were assayed (correlation data is shown as Figure S2). After matrix reduction, the Aβ1–42 concentrations determined on the same assay plate with the non-processed CSF samples again demonstrated a large increase in apparent Aβ1–42 concentration. Additionally, the high degree of separation based on ROC analysis (Fig. 2b) was diminished from 0.97 to 0.87. Interestingly, in about one-quarter of the CSF samples in the AD group, the Aβ1–42 concentration remained relatively low. However, the remainder of the Aβ1–42 concentrations in the AD group now overlapped with the non-demented group after matrix reduction on HPLC. This resulted in a conspicuous loss of differentiation between the disease and non-disease groups.

Effect of *APOE* genotype on Aβ1–42 concentrations after HPLC in AD patients

That a smaller subgroup of AD CSF samples showed only modest increases in Aβ1–42 concentration after RP- HPLC was reminiscent of the effect that the *APOE* genotype can have on CSF levels of Aβ1–42 (Prince *et al.* 2004; Sunderland *et al.* 2004; Peskind *et al.* 2006; Andersson *et al.* 2007; Mosconi *et al.* 2008; Kauwe *et al.* 2009; Kester *et al.* 2009; Vemuri *et al.* 2010). Therefore, it was of interest to determine if the effect of the *APOE* genotype was still apparent after HPLC enrichment of Aβ peptides from CSF.

Figure 3 (lanes 1, 3 and 5) displays the Aβ1–42 concentrations in crude CSF from a third set of CSF samples obtained from AD patients with different *APOE* genotypes (CSF Set C, Table 2). The *APOE* ε4 non-carriers and *APOE* ε4 homozygous patients were separated by an AUC of 0.79 upon ROC analysis, with the *APOE* ε4 homozygous patients demonstrating lower levels of Aβ1–42. After HPLC the same samples demonstrated more than twice the apparent Aβ1–42 concentration than as the crude CSF, even though all samples were assayed on the same assay plate. Furthermore, the separation between the *APOE* ε4 non-carriers and *APOE* ε4 homozygotes had increased from 0.79 to an AUC of 0.88. This suggested that Aβ1–42 measurements from *APOE* ε4 non-carriers were more greatly affected by the matrix proteins in crude CSF. The results from RP-HPLC also demonstrated that the *APOE* ε4 allele has a demonstrable effect on Aβ1–42 concentrations even after denaturation and HPLC enrichment.

To determine if the concentration of Aβ1–40, relative to Aβ1–42, was different across the *APOE* genotype groups, the ratios for Aβ1–42 to Aβ1–40 were compared in HPLC-enriched samples. Fig. 3c shows the results of that comparison. There was no demonstrable difference in the amount of Aβ1–42 present in the sample as a function of the amount of Aβ1–40 based upon *APOE* ε4 allele carrier status.

Additional data for CSF sets B and C is presented in (Figure S3) that describes comparisons of Aβ concentrations in aliquots of the same CSF determined before and after HPLC.

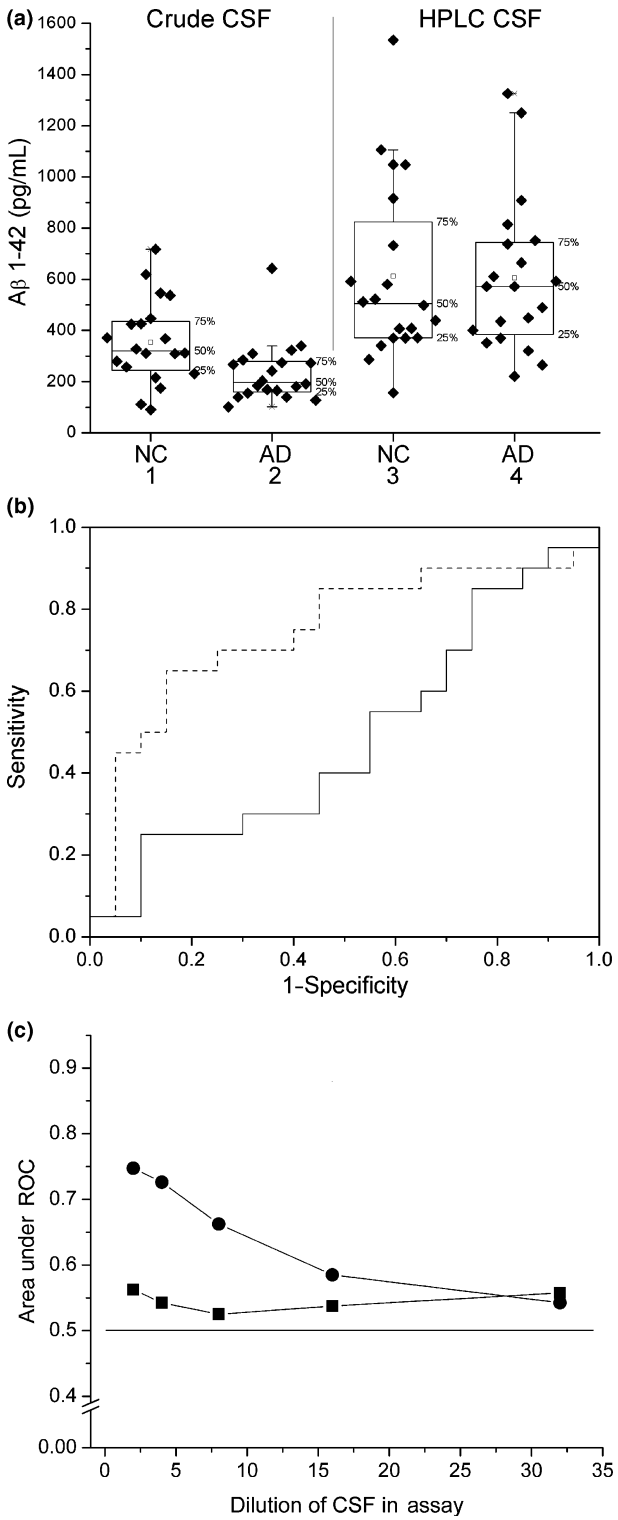

Fig. 1 Effect of Aβ 1-42 enrichment on differentiation between AD and controls. (a) Aβ1–42 measured using the AlzBio3 assay system. Lane 1, Aβ1–42 concentrations in non-demented individuals (NC) assayed as crude CSF; lane 2, Aβ1–42 concentrations in patients with probable AD assayed as crude CSF; lane 3, Aβ1–42 determined from additional aliquots of the samples shown in lane 1 after the Aβ1–42 had been enriched on RP-HPLC; lane 4, Aβ1–42 concentrations in CSF from additional aliquots of the samples shown in lane 2 after enrichment on RP-HPLC. (b) Analysis using area under the curve (AUC) from receiver operator curves (ROC) showing differentiation between AD and controls based on Aβ1–42 measurements after RP-HPLC (solid line, AUC = 0.49) and on crude CSF (dashed line, AUC = 0.75). The difference between the AD and control groups was greatly diminished after removal of CSF proteins by RP-HPLC. (c) ROC analysis of the differentiation between AD and control groups at different dilutions of the crude CSF in the assay. Aliquots of the CSF samples analyzed in panel a were measured in additional Aβ1–42 immunoassays. Solid circle, differentiation by AUC as a function of CSF dilution in the Wako Human Beta Amyloid (1–42) ELISA Kit; solid square, effect of CSF dilution in the 6E10/12F4 Aβ1–42 ELISA.

Discussion

The RP-HPLC enrichment employed in the present study demonstrated that substantially more than one-half of the Aβ1–42 in many crude CSF samples is not detected in traditional immunoassays (e.g. INNO-BIA AlzBio3) because of interference by sample constituents (matrix). Interestingly,

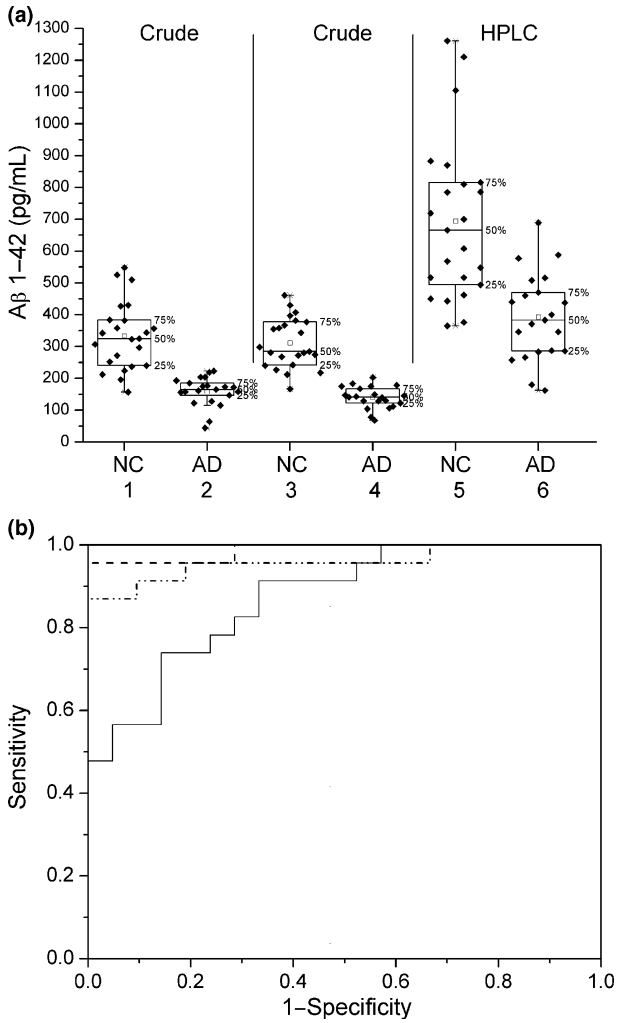

Fig. 2 Effect of RP-HPLC enrichment of Aβ1–42 on differentiation between AD and controls when separation based on assay of crude CSF is robust. The sample set was selected based on strong differentiation when unprocessed CSF was assayed. Lanes 1, 3 and 5 are measurements of the samples in the non-demented control group (NC) and lanes 2, 4 and 6 are from the AD group. Lanes 1 and 2 were assayed as unprocessed CSF in laboratory 1. Lanes 3 and 4 were parallel aliquots assayed as unprocessed CSF in laboratory 2. Lanes 5 and 6 were assayed for Aβ1–42 after denaturation and separation on RP-HPLC. (b) ROC analysis of the differentiation between AD patients and non-demented controls based on Aβ1–42 concentrations in crude or RP-HPLC enriched CSF. Dash-dot line, samples analyzed as unprocessed CSF in laboratory 1 (AUC 0.96, lanes 1 and 2 from panel a); dashed line, re-assay of unprocessed samples in laboratory 2 (AUC 0.99, lanes 3 and 4 from panel a); solid line, same CSF as in 1 to 4 (a) analyzed after RP-HPLC (AUC 0.87, lanes 5 and 6 from panel a). Separation of CSF proteins from Aβ1–42 in the assay diminished the segregation of the groups.

there was a trend that the differentiation between AD and non-demented subjects was better when this interference was present. However, in the current study the magnitude of this effect was different between the two patient groups, possibly due in part to a difference in abundance of *APOE* ε4 alleles in the groups. These results suggested that differential occlusion of Aβ1–42 from the immunoassay signal appears to influence the discriminatory power of this AD biomarker. They also suggested that an improved understanding of how matrix impacts the diagnostic performance of the Aβ1–42 measurement could direct improvements to the assay that would allow for better monitoring of the biochemical pathophysiology of the disease.

A similar effect on the differentiation between AD and control groups also occurred when matrix interference was decreased by increasing the dilution of the CSF in buffer prior to assay. The separation between the AD and normal control groups were essentially the same whether assayed at low dilution of crude CSF in the AlzBio3 immunoassay system or at a modest dilution in the Wako Aβ42 ELISA (AUC $\approx$ 0.75). After increased dilution of the sample in the Wako assay system, differentiation diminished in a manner similar to the result from the HPLC purified CSF samples in the AlzBio3 assay. Interestingly, the second ELISA (6E10/12F4) failed to show any separation between the disease and normal groups at any dilution suggesting that this assay may be less sensitive to interference by CSF constituents. In all, the results from the different immunoassays demonstrated that the choice of immunoassay and the preparation of the sample to be assayed can affect the performance of Aβ1–42 as a diagnostic biomarker.

The data presented in this study are consistent with a considerable literature that describes a high utility for Aβ1–42 measurements as a biomarker for AD when performed on crude CSF (e.g. Blennow *et al.* 2010; Jack *et al.* 2010). It is important to understand why this is true and how this measurement functions to design the best assay strategy for developing a robust diagnostic measurement. Based on the results from the present study, one reason appears to be that CSF from AD patients contains more sequestered Aβ1–42 which escapes detection under traditional immunoassay conditions.

That the interference in the assay contributes to the diagnostic power suggests a few mechanisms. One mechanism may involve the absorption of Aβ1–42 to CSF proteins that are more abundant in CSF from AD patients. A number of proteins have been reported to be elevated in some AD CSF. A recent proteomics study using the Rules Based Medicine Human DiscoveryMAP assay (Myriad RBM, Austin, TX, USA; 151 analyte panel; Hu *et al.* 2010) described at least 17 such proteins that are elevated in AD CSF, but it is not known if they bind Aβ1–42 sufficiently or if their concentrations are high enough to interfere. Specific proteins that are found in CSF have been reported to bind Aβ1–42 including apolipoprotein J (Nuutinen *et al.* 2009), apolipoprotein E (Bell *et al.* 2007), transthyretin (Costa *et al.* 2008), gelsolin (Chauhan *et al.* 1999) and β-trace protein

(Kanekiyo *et al.* 2007). Unfortunately, the lack of information describing the affinity of Aβ1–42 binding to these proteins in CSF and the potential complexity of Aβ1–42 binding to multiple proteins within a sample has made modeling this process difficult. Future studies are needed to examine the nature and extent of Aβ1–42 peptide that is in complex with other CSF proteins. In particular it will be important to identify complexes in CSF that are tightly bound because this could reflect functional complexes produced in the brain parenchyma that persist in lumbar CSF. CSF proteins associated with Aβ1–42 in this manner may also be useful biomarkers.

Another mechanism could include Aβ1–42 oligomeric complexes which have been demonstrated to be elevated in the CSF of AD patients (Stenh *et al.* 2005; Fukumoto *et al.* 2010). These could differentially mask some portion of the Aβ1–42 peptide from assay resulting in lower estimates of total Aβ1–42 in crude AD CSF. Estimating the fraction of Aβ1–42 that exists in oligomers has been challenging because there are limited assays available for measuring them and it has not yet been feasible to directly relate measures of oligomeric Aβ1–42 peptide to monomeric Aβ1–42 peptide. Although the above possibilities are not exhaustive or exclusive, they represent parts of the measurement that may contribute to the diagnostic power of the assay. Any of these Aβ-masking mechanisms could be contributors to the basis of Aβ1–42 as a biomarker in CSF. Interestingly, the extent to which Aβ1–42 measurements are affected by matrix is also influenced by *APOE* genotype, which itself is an important prognosticator of the disease. However, the way in which *APOE* affects the disease is not completely understood. In any case, it can be expected that the utility of the Aβ1–42 measurement as a biomarker of AD will be improved as we gain a better understanding of the factors that affect this assay.

The trend to see proportionately more Aβ1–42 being occluded from assay in AD CSF was surprising in light of the

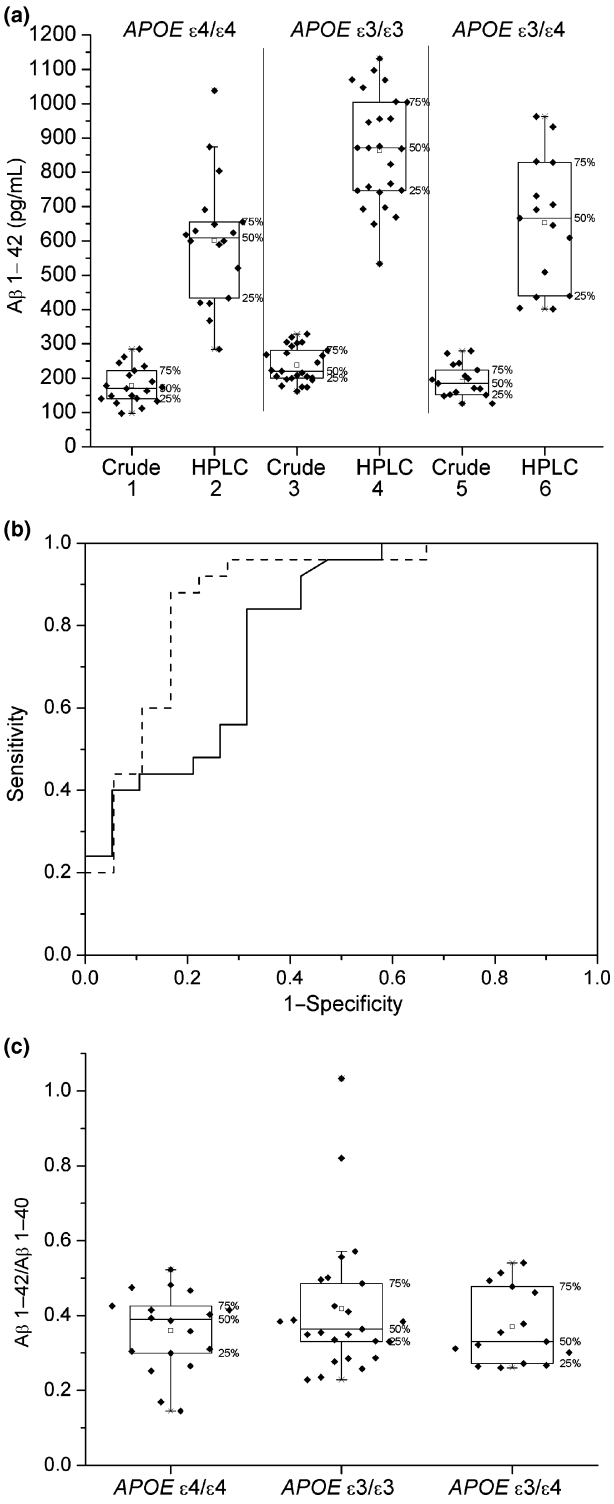

Fig. 3 Effect of *APOE* genotype on Aβ1–42 measurement in CSF from AD patients before and after RP-HPLC. (a) 1 = lanes 1, 3 and 5 are measurements on crude CSF and lanes 2, 4 and 6 are parallel CSF aliquots assayed after denaturation and HPLC. Lanes 1 and 2 = *APOE* ε4:ε4, lanes 3 and 4 = *APOE* ε3:ε3 and lanes 5 and 6 = *APOE* ε3:ε4. All samples had been assayed within the same plates after combining the reagents from two AlzBio3 kits. Considerably more Aβ1–42 is revealed after RP-HPLC enrichment. (b) Analyses of differentiation between *APOE* ε4 homozygotes and *APOE* ε3 homozygotes using area under the curve (AUC) from receiver operator curves (ROC) before and after HPLC enrichment of Aβ1–42. Solid line, Aβ1–42 measured in crude CSF (AUC = 0.79, lanes 1 and 3 in panel a); dashed line, Aβ1–42 measured in HPLC-processed CSF (AUC = 0.88, lanes 2 and 4 in panel a). The *APOE* ε3 homozygotes were affected more by Aβ1–42 enrichment than the *APOE* ε4 homozygotes. (c) Comparison of Aβ1–42 and Aβ1–40 ratio as a function of *APOE* genotype. Aβ1–42 and Aβ1–40 measurements were performed on RP-HPLC enriched peptide. Lane 1, Aβ1–42/Aβ1–40 in *APOE* ε4:ε4 patients; lane 2, Aβ1–42/Aβ1–40 in *APOE* ε3:ε3 patients; lane 3, Aβ1–42/Aβ1–40 in *APOE* ε3:ε4 patients. Aβ1–40 appeared proportionately lower in *APOE* ε4 carriers resulting in a similar ratio across genotypes.

inverse correlation between amyloid neuropathology and Aβ1–42 concentrations in crude CSF (e.g. Strozyk *et al.* 2003; Grimmer *et al.* 2009; Fagan *et al.* 2006). This may indicate that the Aβ1–42 which escapes detection in crude CSF is somehow related to the pathological complexes formed in the brain. Future studies will be needed to better understand the relationship between AD pathology and matrix affects on Aβ1–42 measurements.

The *APOE* genotype is known to affect Aβ1–42 concentrations when measured in crude CSF (Prince *et al.* 2004; Sunderland *et al.* 2004; Peskind *et al.* 2006; Andersson *et al.* 2007; Mosconi *et al.* 2008; Kauwe *et al.* 2009; Kester *et al.* 2009; Vemuri *et al.* 2010). The present study demonstrated that this relationship was conserved in samples assayed after RP-HPLC enrichment of the Aβ1–42 peptide. As is the case for Aβ1–42 in crude CSF, the Aβ1–42 concentrations after HPLC declined in the order *APOE* ε4 non-carriers > ε4 heterozygotes > ε4 homozygotes. Interestingly, the separation between the *APOE* ε4 homozyotes and *APOE* ε4 non-carriers increased after enrichment of the Aβ1–42 on HPLC. This result highlighted that the *APOE* genotype has a robust effect on Aβ1–42 concentrations in CSF and that the *APOE* ε4 non-carriers were more affected by the CSF matrix constituents. However, lower Aβ1–42 concentrations in the *APOE* ε4 carriers were not unique to this Aβ peptide because Aβ1–40 was also decreased. The ratio of Aβ1–42 to Aβ1–40 was consistent across all genotypes. This suggested that the cause for the reduction of Aβ1–42 in CSF by expression of the ε4 isoform of APOE is closely related to that which causes the reduction of Aβ1–40. Additionally, this further highlights the need to separate patients by *APOE* genotype when assessing the utility of Aβ1–42 as a biomarker. Future studies are needed to understand why *APOE* influences Aβ peptide production and if the CSF matrix is different in *APOE* ε4 patients. Studies to identify which protein or other CSF constituents are responsible for this difference may yield biomarkers that work in conjunction with Aβ. It may also provide avenues for developing new CSF biomarkers for early AD that are associated with the deposition of amyloid. Adding such biomarkers to the diagnostic test could improve sensitivity and selectivity of the biomarker.

That the matrix components in CSF add to the discriminatory power of this biomarker is advantageous. However, the same interference may also impact the reproducibility of the measurement across different immunoassays or laboratories in a manner that is not easily controlled. The identity of the interfering molecules and their affinities for different Aβ isoforms are unknown, making it difficult to purposefully design procedures that can standardize their effect on the measurement. Careful and consistent handling of samples can help deliver reproducible analyses; however, this may not be attainable across multiple laboratories. Additionally, it is difficult to ascertain if the detection of Aβ1–42 is impeded because of binding to CSF matrix constituents or to labware such as assay tubes. Consequently, it is possible that prior denaturation and HPLC of Aβ1–42 before assay will provide a more robust and consistent pharmacodynamic marker for following the inhibition of peptide production. Such a possibility needs to be examined in future studies.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Reverse-phase separation of proteins in CSF.

Figure S2. Correlation of repeated measurements using the Inno-Bia AlzBio3 assay for Aβ1–42.

Figure S3. Comparison of Aβ concentrations determined in crude CSF and after HPLC.

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