RESEARCH ARTICLE

Identification and validation of novel CSF biomarkers for early stages of Alzheimer's disease

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The pathology of Alzheimer's disease (AD) begins years prior to clinical diagnosis. The development of antecedent biomarkers that indicate the presence of AD pathology and predict risk for decline in both cognitively normal and mildly impaired individuals will be useful as effective therapies are developed. While cerebrospinal fluid (CSF) markers such as amyloid-β (Aβ) 42 and tau are useful, additional biomarkers are needed. To identify new markers, we utilized 2-D difference gel electrophoresis (2-D DIGE) of individual CSF samples from subjects with very mild AD *versus* controls after depletion of high-abundant proteins. Protein spots displaying differential abundance between the two groups were identified with MS. A number of candidate biomarkers were identified in 18 gel features. Selected candidates were quantified in a larger clinical set using ELISA. The mean levels of α1-antichymotrypsin (ACT), antithrombin III (ATIII), and zinc-α2-glycoprotein (ZAG) were significantly higher in the mild AD group, and the mean level of carnosinase 1 (CNDP1) was decreased. When these biomarkers are optimally combined, there is a strong trend toward greater specificity and sensitivity based on clinical diagnosis than when used individually. Our findings provide novel biomarker candidates for very mild and mild AD that can be further assessed as antecedent markers and predictors of clinical progression.

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Abbreviations: Aβ, amyloid β; **ACT,** α1-antichymotrypsin; **AD,** Alzheimer's disease; **AGT,** angiotensinogen; **ATIII,** antithrombin III; **AUC,** area under the receiver operating characteristic curve; **CDR,** clinical dementia rating; **CSF,** cerebrospinal fluid; **FDR,** false discovery rate; **GSN,** gelsolin; **LP,** lumbar puncture; **ZAG,** zinc-α2-glycoprotein

1 Introduction

Alzheimer's disease (AD) is the most common cause of dementia [1]. Abundant evidence suggests the existence of a "preclinical" stage of AD that can start 10–20 years before a subject can be diagnosed, where an individual is cognitively normal but is developing extensive pathological changes in the brain, particularly the buildup of amyloid plaques [2, 3]. While there are no proven treatments that delay the onset or prevent the progression of AD, many candidates have this potential. During the development and implementation of

these therapies, it will be important to have biomarkers that can identify individuals with preclinical AD or at the earliest clinical stages in order to target them for therapeutic trials, to predict longitudinal change in cognition, and to monitor therapy.

Cerebrospinal fluid (CSF) is a potentially rich source for AD biomarkers because it is contiguous with the extracellular space of the brain. Thus biochemical changes in the brain are often reflected in CSF. Moreover, lumbar puncture (LP), a standard method of obtaining CSF for biochemical analysis, is straightforward, and with the recent, more widespread use of small caliber and blunt-ended needles, is associated with minimal discomfort and low (1–2%) incidence of post-LP headache in those above the age 60 [4]. At present, a few CSF biomarkers have been identified for AD, the most studied being $A\beta_{42}$ (where Aβ stands for amyloid β), total tau, and phosphorylated tau species [5]. There is usually sizable overlap of biomarker concentrations between AD and control groups, and specificity against other dementias is not perfect [6]. However, low CSF $A\beta_{42}$ has recently been shown to correlate very well with the presence of brain amyloid regardless of clinical status [7], and the ratio of CSF tau/$A\beta_{42}$ predicts (i) conversion of individuals who are cognitively impaired at a very mild level to the next stage of impairment over a 5-year period [8] and (ii) conversion of cognitively normal individuals to very mild impairment over a 3–4-year period [9]. While this is important and useful, given the probability of multiple underlying factors involved in the pathogenesis of late-onset AD, it seems likely that a battery of biomarkers that predicts outcome in cognitively normal individuals as well as progression in those that are very mildly impaired will be more useful than individual biomarkers alone.

The ongoing development of more sensitive and higher-throughput proteomic tools holds significant promise for the discovery of novel biomarkers. Comparative proteomic studies of AD biomarkers in CSF have been conducted using different proteomic strategies [10–17]. Although a list of proteins/peptides is usually generated in such studies, thorough validation of candidate biomarkers in large independent sample sets has not yet been accomplished in most studies. We recently developed a proteomic approach that utilizes depletion of high-abundant proteins, 2-D difference gel electrophoresis (2-D DIGE), and high-resolution MS to analyze the CSF proteome and reliably detect differences in protein abundance between CSF samples from different individuals [18]. In the present study, we have utilized this proteomic approach and assessed unique CSF samples of control subjects with those at the very earliest clinical stages of AD, with a sample selection criteria that maximizes the likelihood of either having AD pathology *versus* being pathology-free. We identified one known as well as new biomarkers for very mild and mild AD and validated potential candidates with ELISA measurements on a much larger, independent sample set.

2 Materials and methods

2.1 Subjects

Research subjects were participants at the Alzheimer's Disease Research Center (ADRC) at the Washington University School of Medicine (WUSM) and were recruited by the ADRC to this study. All subjects gave informed consent to participate in this study and all protocols were approved by the institutional review board for human studies at Washington University. Study investigators were blind to the cognitive status of the participants, which was determined by ADRC clinicians in accordance with standard protocols and criteria, as described previously [19, 20]. Subjects were assessed on clinical grounds to be cognitively normal in accordance with a clinical dementia rating (CDR) of 0 ($n = 55$) or to have very mild (CDR 0.5; $n = 20$) or mild (CDR 1; $n = 19$) dementia that is believed to be caused by AD as described [3]. While the CDR was used to rate the severity of dementia, the diagnosis of AD *versus* other causes of dementia was determined by trained clinicians based on a detailed informant-based history, neurological examination, and assessment of medical records. See Table 1S in the Supporting Information for demographic information on the subjects.

2.2 CSF and plasma collection

CSF (20–35 mL) was collected at 8:00 AM after overnight fasting, as described previously [21]. LPs (L4/L5) were performed by a trained neurologist using a 22-gauge Sprotte spinal needle. CSF samples were free from any blood contamination. Samples were gently inverted to avoid gradient effects, briefly centrifuged at low speed to pellet any cellular elements, and aliquoted (500 μL) into polypropylene tubes before freezing at −80°C. Fasted blood (10–15 mL) was also obtained from each subject just before LP, and plasma was prepared by standard centrifugation techniques. Plasma samples were aliquoted (500 μL) into polypropylene tubes before freezing at −80°C.

2.3 Multiaffinity immunodepletion of abundant CSF proteins

Since albumin, IgG, α1-antitrypsin, IgA, transferrin, and haptoglobin collectively account for ~80% total CSF protein content, we selectively removed these proteins in order to enrich for proteins of lower abundance. An antibody-based multiaffinity removal system (Agilent Technologies, Palo Alto, CA) was employed according to the manufacturer's instructions, as described previously [18].

2.4 2-D DIGE

Twelve CSF samples were analyzed by 2-D DIGE: CDR 1 subjects ($n = 6$) and age-matched CDR 0 controls ($n = 6$).

These samples were selected based on $A\beta_{42}$ concentration, *i.e.*, we selected CDR 1 samples whose $A\beta_{42}$ concentration is less than 500 pg/mL and the converse for CDR 0 samples. Fifty micrograms of protein from each depleted CSF sample was labeled with 400 pmol of one of the three *N*-hydroxysuccinimide cyanine dyes for proteins (GE Healthcare, Piscataway, NJ), mixed with 30 μg of unlabeled protein from the same sample, diluted with rehydration buffer, combined according to experimental design, and equilibrated with IPG strips (24 cm; pH 3–10, nonlinear). The three samples that were equilibrated with each IPG strip consisted of a CDR 0 sample, a CDR 1 sample, and a pooled sample (pooled using an equal volume aliquot of each of the 12 CSF samples) (labeled with 1-(5-carboxypentyl)-1′-propylindocarbocyanine halide *N*-hydroxy-succinimidyl ester (Cy3)) as the internal standard. To avoid possible dye-related bias, half of the CDR 0 samples were labeled with 3-[(4-carboxymethyl)phenylmethyl]-3′-ethyloxacarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy2) and half with 1-(5-carboxypentyl)-1′-methylindodi-carbocyanine halide *N*-hydroxysuccinimidyl ester (Cy5) and the same protocol was used to label the CDR 1 samples. First-dimension IEF was performed at 65.6 kV·h in an Ettan IPGphor system (GE Healthcare). The second dimension was performed on a gradient SDS-PAGE gel (10–20%). The Cy2, Cy3, Cy5-labeled images were acquired on a Typhoon 9400 scanner (GE Healthcare).

2.5 Image analysis

Intragel spot detection and quantification and intergel matching and quantification were performed using differential in-gel analysis (DIA) and biological variation analysis (BVA) modules of DeCyder software v5.01 (GE Healthcare), respectively, as described previously [18]. A subset of protein spots ($n = 514$) was matched across all six gels. Student's *t*-test was performed to compare the average (relative) abundance of a given spot between the two groups (CDR 1 *versus* CDR 0). Spots that had a *p*-value below 0.05 and were well resolved were selected for subsequent mass spectrometric analysis.

2.6 Protein digestion and MS

Gel spots of interest were flagged with the DeCyder software and the *X*- and *Y*-coordinates were used by a robotic spot picker (ProPic; Genomics Solutions, Ann Arbor, MI) to cut and transfer gel features into a 96-well plate for *in situ* gel digestion with trypsin (at a final concentration of 1 μg/μL) using a modification of a previously described method [22]. Briefly, excised gel cylinders (2 mm in diameter) were robotically transferred to 96-well plates (Axygen Scientific, Union City, CA) and overlayered with 100 μL of water. After washing and trypsin digestion (Sigma, St. Louis, MO) in 5 μL of 5 mM triethylammonium bicarbonate buffer (pH 8.0; Sigma), an aliquot (0.5 μL) was removed from each well and placed in a microfuge tube (200 μL) containing 0.5 μL of MALDI Matrix (Agilent Technologies). The tubes were vortexed, spun in a microfuge for 30 s and spotted (1 μL) onto a stainless steel target (192 spot plate) for MALDI-TOF/TOF MS, as previously described [23]. To the remaining gel samples, 12 μL of aqueous ACN/formic acid (1%/1%) (Sigma) was added and the plate was incubated at 37°C for 1 h. The samples (~10 μL) were then transferred to polypropylene auto sampler vials, spun at $10\,000 \times g$ for 15 min in a Sorvall centrifuge equipped with a HB-6 rotor, and stored at −80°C for analysis by LC-MS/MS. LC-MS/MS was performed using a capillary LC (Eksigent, Livermore, CA) interfaced to a nano-LC-linear quadrupole IT Fourier transform ICR mass spectrometer (nano-LC-FT-MS) [24].

Database searching was performed using MASCOT 1.9.05 software (Matrix Science, Oxford, UK) against the NCBI nonredundant database (March 24, 2005). Protein identifications were supported by at least two peptides with MASCOT scores of >40 and accurate measurements of the peptide parent masses within 5 ppm. Protein identifications were confirmed by manual interpretation of the fragmentation spectra with a minimal acceptance criterion of four contiguous b- or y-ions for each peptide sequence.

2.7 ELISA analyses

$A\beta_{42}$ and total tau concentrations in our CSF samples were analyzed by a commercially available ELISA (Innotest; Innogenetics, Ghent, Belgium) for other purposes previously. Albumin ELISA was performed with an ELISA kit (Bethyl Laboratories, Montgomery, TX). A sandwich ELISA was developed for α1-antichymotrypsin (ACT) measurement: rabbit antihuman ACT antibody (1:1000; DAKO, Carpinteria, CA) was used for capture and a sheep antihuman ACT antibody (1:5000; The binding site, San Diego, CA) was used for detection. ACT purified from human plasma was used as standard (Sigma). For AT III measurement, a sandwich ELISA was developed: rabbit antihuman AT III antibody (1:1000; DAKO) was used for capture and a mouse monoclonal antihuman AT III (clone HYB 230-04, 1:5000; Assay designs, Ann Arbor) was used for detection. Antithrombin III (ATIII) purified from human plasma was used as standard (Sigma). For zinc-α2-glycoprotein (ZAG), a sandwich ELISA was developed: rabbit antihuman ZAG antibody (1:1000; gift from Dr. Iwao Ohkubo, Shiga University of Medical Science, Japan) was used for capture and a mouse monoclonal antihuman ZAG antibody (clone 1D4, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection. ZAG purified from human seminal plasma was used as standard (gift from Dr. Iwao Ohkubo). For gelsolin (GSN) ELISA, CSF samples were directly coated on the plate, followed by incubation with a mouse monoclonal anti-GSN antibody (clone GS-2C4, 1:2000; Sigma). GSN purified from human plasma was used as standard (Sigma). For angiotensinogen

(AGT) measurement, a sandwich ELISA was used: mouse monoclonal anti-AGT antibody (clone F8A2, 1:285; gift from Dr. Claus Oxvig, University of Aarhus, Denmark) was used for capture and a chicken antibody (1:1200; gift from Dr. Claus Oxvig) was used for detection. AGT purified from human plasma was used as standard (Calbiochem, San Diego). For carnosinase I (CNDP1) ELISA, CSF samples were directly coated on the plate, followed by incubation with goat antihuman CNDPI antibody (1:500; R&D systems, Minneapolis, MN).

For all ELISA measurements: (i) biotinylated secondary antibody (1:5000; Jackson ImmunoResearch, West grove, PA) and poly-HRP streptavidin (1:2000; Research Diagnostics, Concord, MA) were used. For color development, TMB super slow or TMB super sensitive (Sigma) were used. (ii) Samples were kept continuously on ice, and assays were performed on sample aliquots after a second thaw after initial freezing. (iii) The levels of a given protein in all CSF samples were measured in the same experiment. (iv) Data from a single experiment are presented here and similar data have been obtained from at least one replicate experiment.

2.8 Statistical analyses

We tested the hypothesis of a normal distribution for levels of ACT, ATIII, ZAG, CNDP1, Aβ42, and tau using the Shapiro–Wilk test. The distributions were log transformed to approximate a normal distribution. Values were then adjusted for age at LP, gender, and the number of *APOE4* alleles. The residuals were tested for association with case-control status using a *t*-test.

The area under the receiver operating characteristic (ROC) curve (AUC) for each trait was calculated using the ROCR package in R (http://www.R-project.org; [25]). We implemented a method proposed by Xiong *et al.* [26] to determine the optimum linear combination of these traits and calculate confidence intervals on the AUC. This method requires a complete dataset; removal of individuals with missing phenotype data resulted in a slight decrease in the sample size ($N = 83$).

2.9 Immunohistochemistry

ACT immunostaining was performed on ethanol-fixed paraffin sections (5 μm thickness) of human brain (frontal cortex) with a rabbit antihuman ACT antibody (1:100; Accurate chemical, Westbury, NY). ATIII immunostaining was performed on formalin-fixed frozen sections (12 μm thickness) of human brain (frontal cortex) using a rabbit antihuman ATIII antibody (1:500; DAKO). The staining was developed chromogenically (DAB). Standard immunohistochemical protocol was employed for the above immunostaining [27]. Thioflavine-S and X-34 staining was performed as described [28].

3 Results

3.1 Sample selection

To identify new CSF biomarkers for mild AD, we compared the CSF proteomes of six mild AD subjects to those of six age-matched cognitively normal subjects. The mild AD subjects were clinically characterized with a CDR score of 1, indicating mild dementia and the controls all had a CDR score of 0, indicating nondemented. To maximize the likelihood of selecting CDR 1 subjects which truly had AD pathology from CDR 0 subjects which did not, selection was based on CSF $A\beta_{42}$ concentrations. A recent study comparing *in vivo* amyloid imaging with CSF levels of various biomarkers showed that $A\beta_{42}$ is an excellent marker of cerebral amyloid deposition, independent of clinical diagnosis [7]. We also decided to analyze these CSF samples individually (as opposed to pooling the samples) to increase the statistical power of our analysis.

3.2 Candidate biomarkers identified

Upon 2-D DIGE analysis, we were able to match 514 protein spots across all six gels. Since a pooled sample was included on each gel, we were able to normalize the relative abundance of the matched spots in all 12 individual CSF samples. Student's *t*-test (using $p<0.05$ to define statistical significance) was performed to examine whether the average (relative) abundance of a given protein spot in the two CDR groups was significantly different. We found 21 spots displaying differential abundance (p-values<0.05) between the two groups. Three spots were excluded due to poor-gel resolution and the remaining 18 were selected for excision, in-gel tryptic digestion, and mass spectrometric identification.

To estimate whether most of these proteins are truly different in abundance as opposed to artifacts, we assessed the false discovery rate (FDR) in this study by using data from our previous study with this method [18] to approximate a "same–same comparison". In that study, the same comparative proteomic approach (depletion – followed by 2-D DIGE – MS/MS) was applied to the analysis of 12 CSF samples, composed of pairs of samples from the same six individuals, obtained 2 wk apart. When we compared the six CSF samples (as a group) obtained at time point 1 to the six samples (as a group) obtained at time point 2, it is similar to a same–same comparison because CSF samples from the same subject should be quite similar. When that comparison was performed, we found 0 spots (out of 306 matched spots) that were differentially expressed between the two groups (using a p-value of 0.05 as cutoff). This result indicates that the false positive rate in our study (when using a p-value of 0.05 as cutoff) is likely to be very low.

Protein identifications of the 18 spots are shown in Table 1. We were able to successfully determine the protein identity of 16 out of the 18 spots. MS data for these spots are included in the Supporting Information (Table 2S). For some spots, multiple proteins have been identified within one

Table 1. List of proteins that were identified through 2-D DIGE and MS analysis to have differential abundance in mild AD and control CSF samples

Spot	Protein	Change in AD	*p*-value
1	ATIII	I	0.028
2	ATIII	I	0.023
3	ATIII AGT	I	0.033
4	ACT	I	0.031
5	CNDP1 Secretogranin III Kininogen	D	0.018
6	ACT	I	0.031
7	ATIII	I	0.022
8	ACT	I	0.0091
9	No ID	I	0.0041
10	ACT	I	0.015
11	ACT Secretogranin III	I	0.011
12	ACT Secretogranin III Kininogen	I	0.0013
13	AGT Kinongen	I	0.0005
14	ZAG	I	0.027
15	ZAG Chromagranin B	I	0.014
16	GSN	D	0.028
17	No ID	D	0.048
18	Neuronal pentraxin Prostaglandin D2 synthase β Trace Secretogranin III	D	0.043

Column 1 is the coded protein spot ID. Column 2 is the protein name. Column 3 shows whether these proteins are increased (I) or decreased (D) in the CDR 1 group (compared to the CDR 0 group). Column 4 is the *p*-value of the comparison (Student's *t*-test).

spot, most likely due to comigration of certain proteins. In addition, some proteins were identified in multiple proteins spots, including ACT and ATIII, likely reflecting the existence of different protein isoforms (*e.g.*, PTMs). A total of 11 proteins were identified as candidate biomarkers for mild AD. Levels of GSN and CNDP1 were found to be lower in the mild AD group compared to the CDR 0 group whereas levels of the other candidate markers were higher in the CDR 1 group compared to controls. The relative difference in abundance between the averages of the two groups observed in 2-D DIGE is moderate, most within ± two-fold.

3.3 Validation of candidate biomarkers with ELISA

In order to confirm our findings with another method and also to be more quantitative, we assayed the levels of candidate biomarkers first in the original 12 CSF samples (CDR 0 ($n = 6$), CDR1 ($n = 6$)) using ELISA. Based on the availability of antibodies, we first assessed the following candidates: ACT, ATIII, ZAG, CNDP1, GSN, and AGT. We then assayed a larger test set of CSF samples of cognitively normal subjects (CDR 0, $n = 49$) *versus* subjects with very mild (CDR 0.5, $n = 19$) or mild dementia (CDR 1, $n = 13$) judged to be due to AD. The goal was to determine whether our findings could be validated in a larger, independent sample set. This level of validation is very important and has not been done before in previous AD proteomic biomarker studies to the best of our knowledge. For all the candidate biomarkers evaluated, when we assayed the larger sample set, we always observed the same trend as when we assayed the 12 original samples. Therefore, values from the 12 original samples were combined with the test sample set in the subsequent graphs. In addition, since we observed no differences between the CDR 0.5 and CDR 1 groups, these groups were combined to form the mild AD group and were then compared to the CDR 0 group. ELISA data that display CDR 0.5 and CDR 1 separately can be found in the Supporting Information (Fig 1S). For reference, we have also included the concentrations of $A\beta_{42}$ and total tau (which have been obtained previously to this study) in these CSF samples (Fig. 1).

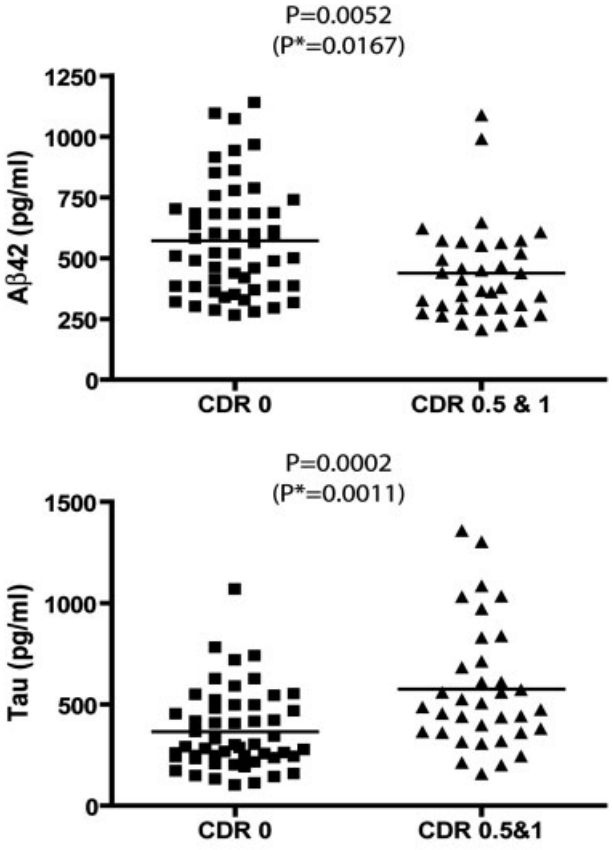

Figure 1. Mean levels of CSF $A\beta_{42}$ are decreased and levels of total tau are increased in very mild AD *versus* control subjects. CDR 0 equals no cognitive impairment, CDR 0.5 represents very mild dementia, and CDR 1 represents mild dementia due to AD. *P*-values calculated using the raw data (*p*) and those calculated using the log-transformed and adjusted dataset (*p**) are also displayed.

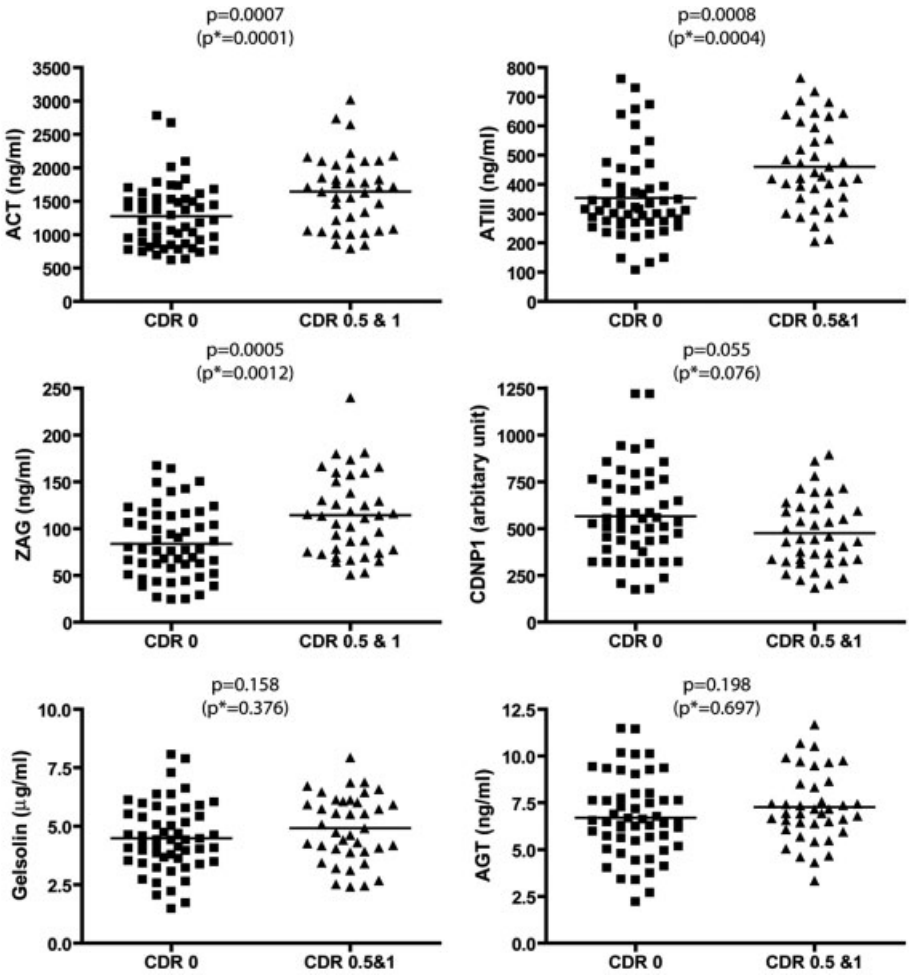

Figure 2. Levels of selected candidate biomarkers in a large CSF sample set as assayed by ELISA. *P*-values calculated using the raw data (*p*) and those calculated using the log-transformed and adjusted dataset (*p**) are displayed. ACT, ATIII, and ZAG are significantly increased in AD (CDR 0.5 and 1) *versus* control (CDR 0) samples.

As shown in Fig. 2, the CSF levels of ACT, ATIII, and ZAG are significantly increased in the very mild/mild AD group, confirming the 2D-DIGE findings. There is a trend for a decrease in CNDP1 levels in the very mild/mild AD group (Fig 2), though this was not quite statistically significant ($p = 0.055$). In contrast to the 2-D DIGE findings, we did not confirm a difference in levels of GSN or AGT between the two groups (Fig. 2). After log transformation (to approximate a normal distribution), we examined our datasets for any possible interactions between CSF concentrations of candidate biomarkers (including $A\beta_{42}$ and total tau) and age/gender/number of *APOE4* alleles. We found a significant correlation between the number of *APOE4* alleles and CSF levels of $A\beta_{42}$, total tau, and AGT (data not shown). There is also a positive correlation between age and CSF levels of ACT and GSN (data not shown). In addition, we observed an interaction between gender and CSF ZAG concentrations, with males having higher levels than females (data not shown). Our initial observations based on the raw concentration data are not qualitatively different from those using the log-transformed and adjusted traits. We included the adjusted *p*-values in Fig. 2 (designated by *p**) for comparison. Since the differences in levels of GSN or AGT between the two groups were not confirmed, we did not include these two candidates in subsequent analyses.

We next sought to assess whether similar differences exist in plasma as were found in CSF. Levels of ACT, ATIII, and ZAG in paired plasma samples were not significantly different between the very mild/mild AD and control groups (Fig. 3). There was also no significant correlation between the CSF and plasma levels of ACT or ATIII, but there was a weak correlation between the CSF and plasma levels of ZAG (Fig. 2S in the Supporting Information).

We also examined the relationships among the CSF concentrations of the candidate biomarkers (together with $A\beta_{42}$ and tau) (Table 2). None of the newly identified candidate biomarkers correlate with $A\beta_{42}$ though ACT, CNDP1, and ZAG moderately correlate with tau. Interestingly, there are very strong, highly significant correlations between ACT, ATIII, and ZAG (but not CNDP1) (Table 2). To preliminarily assess the potential use of these candidate biomarkers in differentiating subjects with very mild/mild AD from controls, we applied a method proposed by Xiong *et al.* [26] to determine the optimum linear combination of these bio-

Table 2. Pearson correlation coefficients for the normalized, adjusted biomarkers

	ATIII	CNDP1	ACT	ZAG	Aβ42	Tau
ATIII	1					
CNDP1	−0.007 (0.95)	1				
ACT	0.73 (<0.0001)	0.030 (0.78)	1			
ZAG	0.66 (<0.0001)	0.026 (0.81)	0.71 (<0.0001)	1		
Aβ42	0.018 (0.87)	0.090 (0.40)	−0.056 (0.61)	0.024 (0.83)	1	
Tau	0.092 (0.40)	0.32 (0.003)	0.36 (0.0009)	0.32 (0.0024)	−0.065 (0.55)	1

Pearson correlation coefficients were derived using log-transformed data that were also adjusted for interacting factors (*i.e.*, age, gender, or the number of *APOE4* allele). *P*-values are displayed in the parentheses.

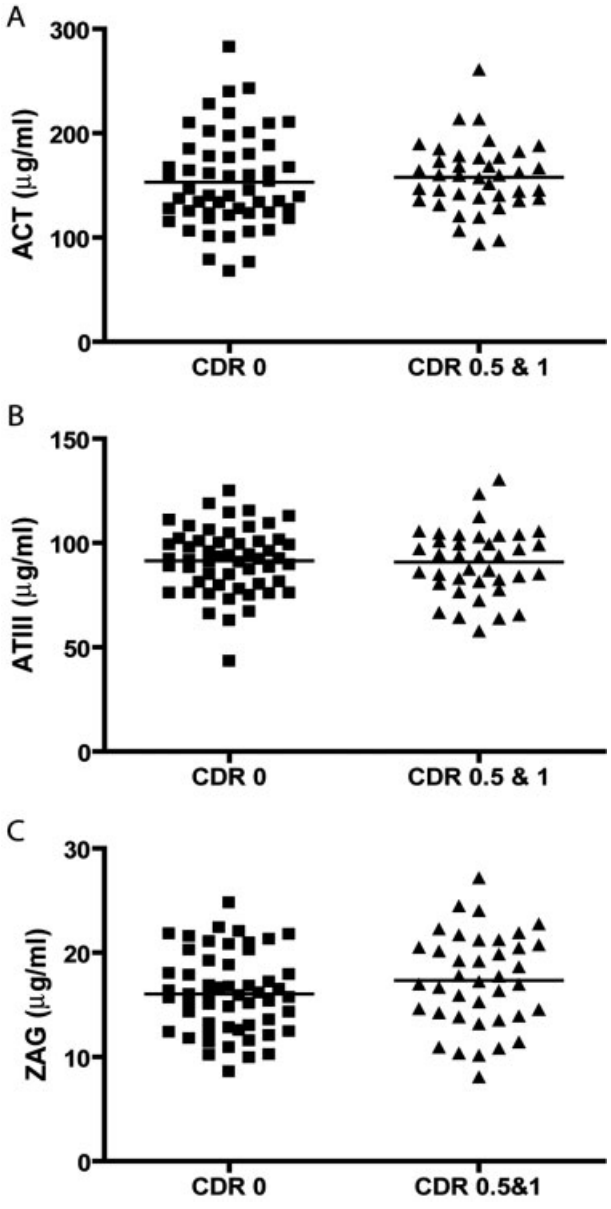

Figure 3. Levels of ACT, ATIII, and ZAG are not significantly different in plasma between AD (CDR 0.5 and 1) *versus* control (CDR 0) samples.

markers and calculated the confidence intervals on the AUC. We found that individual candidates by themselves are very comparable to those of $A\beta_{42}$ and total tau in differentiating very mild/mild AD from controls (Table 3, Fig. 4). Importantly, when all biomarkers are optimally combined, this results in a higher AUC and sensitivity than for any single biomarker.

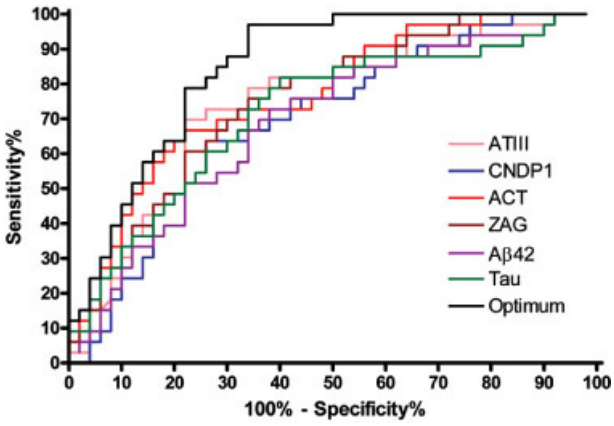

Figure 4. ROC curve for the normalized and adjusted CSF concentrations of each biomarker candidate and the optimum linear combination (optimum) combining data from all biomarkers.

Table 3. Comparison of the AUC of the optimum linear combination (optimum) to that of each individual biomarker

Marker	AUC (SE)	Sensitivity at 80% specificity	*p*-value *vs.* optimum
ATIII	0.75 (0.10)	0.48	0.29
CNDP1	0.65 (0.12)	0.24	0.099
ACT	0.75 (0.11)	0.3	0.32
ZAG	0.72 (0.10)	0.42	0.21
Aβ42	0.64 (0.12)	0.36	0.085
Tau	0.68 (0.11)	0.36	0.14
Optimum	0.88 (0.07)	0.7	–

Presented in this table are area under the receiver operating curve, sensitivity at 80% specificity, and the *p*-value of the *t*-test comparing each trait to the optimum linear combination of these traits.

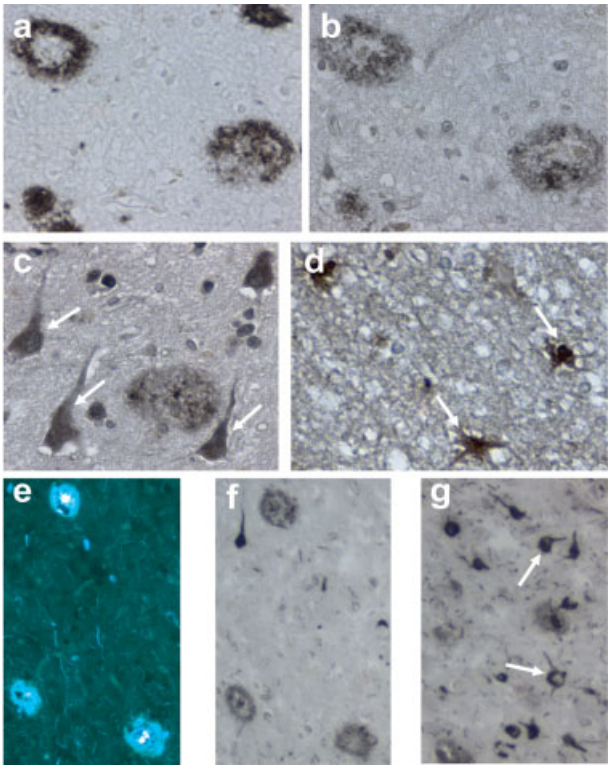

Figure 5. ACT and ATIII are associated with AD pathology in human brain (frontal cortex). Immunostaining for Aβ (a) colocalizes with immunostaining for ACT (b) in human AD frontal cortex as shown in adjacent paraffin sections. ACT is also present in neurons (c, arrows) and astrocytes (d, arrows). Immunostaining for ATIII (f) is colocalized with fibrillar Aβ-containing plaques (e) in human AD brain, shown by X34 staining and ATIII imunostaining of adjacent frozen sections. ATIII is also present in NFTs (g, arrows). Photomicrographs in a–d are at 200× and e–g are at 100×.

3.4 Cellular localization of ACT and ATIII

We then examined the cellular localization of some of the candidate biomarkers in human and transgenic AD mouse brain. We found that, consistent with previous reports [29, 30], ACT is colocalized with amyloid plaques in human AD brain and is also present in neurons and astrocytes (Fig. 5). We also found that antibodies to ATIII label amyloid plaques and neurofibrillary tangles (NFT) in human AD brain (Fig. 5), in accordance with a previous report [31].

4 Discussion

In this study, we identified candidate biomarkers for mild AD by analyzing a small group of CSF samples (mild AD *versus* control) with a comparative proteomic approach. Importantly, selected biomarkers were validated with ELISA using a larger sample set. Among them, we have identified ACT, which has been identified previously in other reports. We have also identified novel biomarkers such as ATIII, ZAG, and CNDP1, whose roles as AD biomarkers in CSF have not been previously assessed and validated. To the best of our knowledge, this is the first proteomic study on AD biomarkers that not only discovered candidate protein biomarkers, but also validated these candidates in a much larger and independent sample set with a sensitive and quantitative method (*i.e.*, ELISA) in multiple, well-characterized individuals. Thus, this proof-of-principle study demonstrates that this proteomic approach can be used to reliably identify AD biomarkers in CSF. Furthermore, this panel of CSF markers along with $A\beta_{42}$, tau, and p-tau may be useful for improving diagnostic accuracy and in future studies should be assessed as antecedent biomarkers.

The proteomic approach we took included multiaffinity depletion, 2-D DIGE, and nano-LC-FT-MS. The sensitive, high-resolution mass spectrometric analysis resulted in the identification of 16 out of the 18 gel features that showed significant differential abundances between the two CDR groups. The overall result was the generation of reliable data, which is demonstrated by the low FDR in our study. FDR is defined as the number of expected false predictions divided by the number of total predictions, which in our study is 21. The number of expected false predictions was estimated using a previous set of CSF proteomic data from our group (generated using the same approach and performed in the same laboratory by the same people). We approximated a "same–same" comparison by comparing a set of six CSF samples from six individuals to a second set of six CSF samples from the same individuals, taken 2 wk apart. Any differentially expressed proteins found in such a comparison would be false positives because in the ideal scenario, there should be no differences detected in a same–same comparison. Interestingly, we found no protein spots (0 out of 306 matched spots) that were significantly different between the two groups. With a close-to-zero FDR when the *p*-value cutoff is set at 0.05, we could even potentially increase the *p*-value cutoff to maximize the number of biomarker candidates discovered while still maintaining a low FDR.

We were able to validate four out of the six selected biomarker candidates using ELISA with a much larger characterized sample set. Although protein spots that correspond to GSN and AGT display differential abundance in 2-D DIGE, we could not detect a difference between the mild AD and control group *via* ELISA. One possible explanation is the existence of different isoforms for a single protein. GSN has two isoforms "a" and "b" which differ only by a stretch of 24 amino acids at the N-terminus. We did not detect the unique peptides for the two isoforms in the MS data and the ELISA is not isoform specific. AGT is known to have different isoforms due to glycosylation [32]. If what we have observed with 2-D DIGE only reflects differences in certain isoforms, it might not result in a difference in total protein level measured by ELISA. Thus, to the extent that AD is associated with changes in the level of specific protein isoforms, ELISA for total protein levels might not detect such changes.

One of our validated candidate biomarkers, ACT, has been previously proposed as a possible biomarker for AD. We observed an increase in CSF ACT level in the AD group, in agreement with a number of previous studies [33–36]. There was neither a significant change in ACT levels in plasma in the dementia group nor a correlation between the CSF and plasma levels of this protein. This is similar to some previous findings [33, 36–39] but not others [33, 34, 36, 40, 41]. We also measured the ACT index (a calculated quotient using the ACT and albumin content of CSF and plasma to detect intrathecal ACT synthesis) and found that 90% of the samples have an index above 1, with an average index of 2.4. These results indicate independent within-CNS production as the main source of ACT in CSF. Brain ACT is produced primarily by astroglial cells and has been shown to be associated with amyloid plaques [29]. One can propose a scenario to define ACTs role in AD pathogenesis [34]. ACT is increasingly produced by reactive astrocytes in response to large amounts of the proinflammatory cytokine interleukin-1 secreted by reactive microglia in the AD brain [42]. Once in the Aβ-rich brain neuropil, ACT may bind to Aβ to promote the formation of fibrils or prevent their disassembly. In contrast to ACT, other validated candidates (*i.e.*, ATIII, ZAG, and CNDP1) have not been well studied in AD and, therefore, represent novel potential biomarkers. ATIII, like ACT, is a serine protease inhibitor. In the present and another study [31], ATIII was shown to localize to both amyloid plaques and NFT in AD brain, suggesting potential involvement in AD pathogenesis. ATIII has not previously been reported to be increased in AD CSF. It is interesting to note that ACT and ATIII that are found elevated compared to controls are secreted by reactive astrocytes [43, 44], suggesting that the inflammation occurring in AD is a very early event. It is not clear why ZAG and CNDP1 are altered in AD CSF or their potential roles in AD pathogenesis. ZAG is a soluble glycoprotein and present in a variety of body fluids [45]. The biological functions of ZAG are largely unknown. It is overexpressed in certain malignant tumors such that it may serve as a cancer marker [46]. One 2-D gel-based proteomic study identified ZAG as one of the proteins that was increased in AD CSF [14] though this was not validated in a large sample set. CNDP1 hydrolyzes carnosine (β-alanlyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine), both of which are believed to be neuroprotective [47]. Decreased serum levels of CNDP1 have been found in patients with Parkinson's disease, multiple sclerosis and in patients after a cerebrovascular accident [48]. To the best of our knowledge, association of CNDP1 with AD has not been reported.

The degree of the changes of these validated biomarker candidates (in AD CSF) is moderate (within 30%), with overlaps between the AD and control group. However, the magnitude of the difference in levels of ACT, ATIII, and ZAG is comparable to that of $A\beta_{42}$, currently one of the best CSF biomarkers of AD that correlates very well with the presence or absence of amyloid in the brain [7]. Importantly, changes in these biomarkers are already evident in the very mildly demented CDR 0.5 group (Fig. 1S, Supporting Information), suggesting the suitability of these candidates for the early detection of AD. Due to the existence of preclinical AD in cognitively normal individuals, the CDR 0 group is likely heterogeneous, containing subjects with and without AD pathology in the brain. Indeed, a recent study from our group demonstrated the presence of brain amyloid and low levels of CSF $A\beta_{42}$ in a subset of cognitively normal individuals [7]. Such preclinical AD pathology might in part account for the overlap between AD and control group in terms of biomarker concentrations and will be the subject of future studies.

Statistical analyses demonstrate that the biomarker candidates we have identified have diagnostic power/accuracy comparable to that of $A\beta_{42}$ and total tau. When all biomarkers are combined, we can achieve a greater AUC and sensitivity. Although the difference is not statistically significant in our study (including 50 CDR 0 subjects and 33 mild AD subjects), this trend may become significant when larger sample sets are assessed. In recently published CSF proteomic study [49], Finehout *et al.* analyzed antemortem CSF proteins from a test cohort of subjects (34 AD and 34 non-AD subjects that included normal individuals and those with other neurological diseases) with 2-D gels and MS. They identified a panel of 23 protein spots that differentiated AD and non-AD samples with high sensitivity and specificity and then validated this biomarker panel using another independent cohort of CSF from 18 AD and 10 non-AD subjects with similar methods. Curiously, there is almost no overlap between the candidate biomarkers identified in the Finehout *et al.* and our study. Why is this the case? There are some similarities between the studies in that we both utilized 2-D gels combined with MS to compare antemortem CSF in AD *versus* control subjects. However, there are many differences between our studies. One major difference is that the Finehout *et al.* study used a random forest multivariate statistical method to use the percentage volume data from all of the spots on their gels together and assessed groups of spots to compare between CSF samples. This method generates a group of spots which when the data is pooled together was used to differentiate AD from controls to the greatest extent possible. Out of the 23 spots that together were found useful to best differentiate between groups with this method, many of the individual spots identified by Finehout *et al.* are not different in their abundance when comparing AD *versus* control samples in their study as well as in other studies in the literature. In contrast, in our study, we only assessed protein spots on our 2-D gels that were significantly different in abundance on an individual basis between AD and control samples. All of the spots that were significantly different between the AD and control test set were then further assessed and validated in a much larger sample set with another quantitative methodology (ELISA). There are also other differences between our study and that of Finehout *et*

al. First, the Finehout *et al.* study utilized CSF from AD subjects at different clinical stages of the disease (numbers from each stage are not listed in their paper). Our discovery phase utilized age-matched controls (CDR 0) with normal CSF $A\beta_{42}$ and mild AD cases (CDR 1) with low CSF $A\beta_{42}$ levels. Second, we used an immunoaffinity column to deplete the most highly abundant proteins from CSF prior to 2-D gel analysis. In the Finehout *et al.* study, some candidate biomakers identified (*e.g.*, albumin, Ig, α1-antrypsin) are abundant proteins that we depleted. Third, the Finehout *et al.* study utilized conventional 2-D gels and we used 2-D DIGE. Fourth, more than a third of the protein spots in the Finehout *et al.* study were identified by comparing gel images to previously published 2-DE CSF maps. We identified all spots of interest with MS. It is likely that all of the issues mentioned here led to nonoverlapping results between out studies. Both the Finehout *et al.* and our results suggest there may be new biomarkers that when analyzed and measured in different ways will be useful for either diagnosis or prognosis in AD. However, as in any biomarker study, a critical issue that is required to truly evaluate the ultimate usefulness of such tests will be to determine if other groups can replicate the results with a much larger number of subjects. In terms of finding reliable differences in the level of specific proteins in AD *versus* control CSF, this has only been done to date with $A\beta_{42}$, tau, and forms of phospho-tau.

To follow up on the biomarker candidates identified in our study, their specificity against other neurological diseases needs to be addressed using CSF samples from patients with other neurological diseases. Studies using even larger sample sets should be done to assess their usefulness in diagnosis when combined with existing biomarkers or imaging tools. Follow-up with cognitively normal subjects should be done to assess whether these biomarkers can be used to predict the conversion from normal cognition to dementia. In addition, future studies to determine if these markers predict rate of progression in subjects that are very mildly demented will also be important.

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