Proteomic Analysis of Alzheimer's Disease Cerebrospinal Fluid from Neuropathologically Diagnosed Subjects

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Abstract: A crucial need exists for reliable Alzheimer's disease (AD) diagnostic and prognostic tests. Given its intimate communication with the brain, the cerebrospinal fluid (CSF) has been surveyed intensively for reliable AD biomarkers. The heterogeneity of AD pathology and the unavoidable difficulties associated with the clinical diagnosis and differentiation of this dementia from other pathologies have confounded biomarker studies in antemortem CSF samples. Using postmortem ventricular CSF (V-CSF) pools, two-dimensional difference gel electrophoresis (2D DIGE) analyses revealed a set of proteins that showed significant differences between neuropathologically-diagnosed AD and elderly non-demented controls (NDC), as well as subjects with non-AD dementias. The 2D DIGE system identified a set of 21 different protein biomarkers. This panel of proteins probably reflects fundamental pathological changes that are divergent from both normal aging and non-AD dementias.

Keywords: Alzheimer's disease, cerebrospinal fluid, proteomics, DIGE, biomarkers

INTRODUCTION

Alzheimer's disease (AD) affects 20 million people worldwide and over 5 million individuals in the USA alone and these numbers are projected to increase due to enhanced longevity. It has been estimated that by the year 2050 the number of AD cases will triple [1]. Therefore, there is a crucial need for a diagnostic test that will detect AD before it causes irreversible disability. Such a test would need to distinguish AD not only from normal elderly non-demented controls (NDC), but also from a number of other, less common diseases that have nearly identical clinical characteristics, but belong to the non-AD dementia (non-ADD) group. Additionally, an ideal test would predict the eventual development of AD, have the sensitivity and specificity for the diagnosis of AD and also provide an index of disease severity, which is useful in assessing the efficacy of prospective therapeutic agents. It is expected that a combination of protein markers will be required to accurately diagnose AD due to the high degree of human variability embodied by the genetic heterogeneity of AD and the intervention of environmental, lifestyle and intrinsic aging factors that dictate an individual's distinctive aging [2]. Thus far, the CSF levels of Aβ42, total tau and phospho-tau 181 have had some success in diagnosing AD [3-8], but these studies have largely been based on clinically defined AD.

We hypothesize that the pathologic changes of AD will be associated with specific ventricular cerebrospinal fluid (V-CSF) protein profiles and that these patterns will be useful for predicting AD risk, diagnosis, prognosis and monitoring of the clinical course of disease. In the present investigation, we examined postmortem V-CSF from neuropathologically diagnosed AD, NDC and non-ADD individuals to investigate a group of potential AD biomarkers. The most important advantage of utilizing postmortem V-CSF is the neuropathological verification of diagnosis. Earlier studies have largely used CSF only from living individuals. However, these studies may be confounded due to the inherent difficulty of diagnosing dementia type based solely on clinical findings [9;10].

Pools of V-CSF from a large number of cases involving the three patient categories under study (AD, NDC and non-ADD), in which each individual was equally represented in terms of total protein content, were prepared and the most abundant proteins separated by antibody affinity chromatography, thus permitting an evaluation of the less plentiful proteins. The pools of V-CSF were analyzed by two-dimensional difference gel electrophoresis (2D DIGE). The separated proteins were submitted to computerized scanning densitometry. Those proteins that significantly differed between two sets of samples were digested with trypsin and the resulting peptides submitted to mass spectrometry for identification.

MATERIALS AND METHODS

Cerebrospinal Fluid Samples

Cerebrospinal fluid samples were collected in the immediate postmortem period by syringe aspiration from the lateral ventricles, centrifuged at 1600 x rpm for 10 min and the

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supernatant stored at -80°C until analysis. The total protein concentration of each individual's V-CSF was measured using the Micro BCA protein assay kit (Pierce, Rockford, IL). The average amount of protein in the AD, NDC and non-ADD cases amounted to 0.87 mg/ml (range: 1.79 - 0.52 mg/ml, SD = 0.24), 0.88 mg/ml (range 1.74 – 0.46 mg/ml, SD = 0.33) and 1.20 mg/ml (range: 1.81-0.81, SD = 0.27), respectively. A description of the three populations under investigation is given in Table **1** which includes average age, gender, postmortem interval (PMI), *apolipoprotein E* (*ApoE*) allelic frequency and the neuropathological scores for amyloid plaques, neurofibrillary tangles and Braak stage. The plaque and neurofibrillary tangle (NFT) total scores were derived by adding the scores from 5 different areas of the brain: frontal, parietal, occipital, temporal lobes and hippocampus. Each area is classified as 0 = none, 1 = mild, 2 = moderate or 3 = severe. All cases were carefully selected and only those with a PMI of less than 4 h were included. With respect to the NDC cohort, all individuals had no history of dementia or other neurological conditions and did not meet the neuropathological criteria for AD. In contrast, all subjects in the AD group met the neuropathological criteria as defined by the CERAD ("definite" or "probable") [11] and the NIA-Reagan Institute ("high" or "intermediate" probability of AD) [12]. The AD cases were carefully selected to the extent that none of them had any other neuropathological conditions, such as non-ADD, and likewise none of the non-ADD cases had a concurrent neuropathological diagnosis of AD. The pathologic distribution in the non-ADD cohort was as follows: 4 with progressive supranuclear palsy and dementia, 3 with hippocampal sclerosis dementia, 4 with non-specific sporadic tauopathy and/or argyrophilic grains, 2 with frontotemporal lobar degeneration with ubiquitinated inclusions and progranulin gene mutations, 1 with frontotemporal dementia with tau gene mutation, 1 with normal pressure hydrocephalus, 1 with corticobasal degeneration and 1 with dementia lacking distinctive histology. Due to low number of individuals with diverse non-ADD in our CSF bank, we were compelled to pool the non-ADD specimens into one category in order to have sufficient statistical power. *Apolipoprotein E* ε4 still remains the major risk factor in sporadic AD hence, all 107 individuals involved in the present study were *Apo E* genotyped according to a previously published protocol [13]. The allelic frequency shown in Table **1** demonstrated as expected, an increase of *ApoE* ε4 in the AD population compared to the NDC and non-ADD cohort.

2D-DIGE

Equal amounts of total protein were pooled from each of the 47 AD, 43 NDC and 17 non-ADD cases. Albumin and IgG were selectively removed using an antibody affinity resin kit following the technique described by the manufacturer (GE Healthcare, Piscataway, NJ) and elsewhere [14]. The proteins were concentrated by acetone/trichloroacetic acid (TCA) precipitation. Briefly, samples were mixed with 3 volumes of cold 13.3% TCA in acetone (stored at -20°C) and incubated at -20°C overnight. Samples were centrifuged at 15,000 x *g* for 30 min and the supernatant was removed. The pellet was washed with the same volume of cold acetone, vortex-mixed, and incubated at -20°C for 30 min. Samples were centrifuged at 15,000 x *g* for 15 min and the supernatant was removed. Pellets were allowed to air dry for about 5 min, and then were suspended in 7 M urea, 2 M thiourea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 2% 3-[N,N-Dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate (ASB-14), 15 mM Tris, pH 8.3. A pooled reference sample, loaded on every analytical gel, was created by combining 30 µg from each sample group, and all samples were diluted to 2 mg/ml using the above buffer. Thirty µg of each individual sample group was labeled with 200 pmol minimal Cy3 and Cy5 NHS ester (GE Healthcare, Piscataway, NJ), and the pooled reference sample was labeled in bulk with minimal Cy2 NHS ester (at a ratio of 200 pmol dye:30 µg sample) at 4°C for 30 min. The labeling reaction was quenched with 10 nmol of lysine. Labeled protein extracts were combined and proteins were separated on 18 cm pH 4-7 IPG strips for 32,000 Vhr using the IPGphor (GE Healthcare, Piscataway, NJ) in 7 M urea, 2 M thiourea, 2% CHAPS, 2% ASB-14, 0.5% IPG buffer, 18.2 mM dithiothreitol (DTT) and 0.002% bromophenol blue using active rehydration at 30V. After isoelectric focusing, IPG strips were equilibrated in 6 M

Table 1. Mean Demographics and Neuropathology for Ventricular Cerebrospinal Fluid Pools

	Avg Age, years	Gender	Avg PMI, hours	*ApoE* Allelic Frequency	Avg Plaque Total Score*	Avg NFT Total Score*	Median Braak Stage**
NDC n=43	84 (6.0)	20M/23F	2.6 (0.8)	ε2 = 0.07 ε3 = 0.77 ε4 = 0.16	5.8 (4.8)	4.0 (2.3)	III
AD n=47	79 (9.9)	19M/28F	2.6 (0.7)	ε2 = 0.04 ε3 = 0.64 ε4 = 0.32	13.4 (1.1)	14.0 (1.6)	VI
non-ADD n=17	78 (12.5)	12M/5F	2.6 (0.8)	ε2 = 0.06 ε3 = 0.88 ε4 = 0.06	2.2 (3.2)	3.0 (2.2)	II

Avg = average; NDC = non-demented controls; AD = Alzheimer's disease; non-ADD = non-Alzheimer's disease dementia; PMI = postmortem interval; ApoE = apolipoprotein E; NFT = neurofibrillary tangle; M = male; F = females; *Maximum score of 15; **Braak Stage: I-VI; standard deviations are in parenthesis

urea, 2% SDS, 65 mM DTT, 30% glycerol, 50 mM Tris, pH 8.8, and 0.002% bromophenol blue for 15 min at room temperature (RT). IPG strips were then equilibrated in the above buffer, replacing DTT with 135 mM iodoacetamide, for 15 min at RT. Proteins were then separated on 20 x 26 cm 8-15% SDS polyacrylamide gels using the Dalt II (GE Healthcare, Piscataway, NJ). Gels were fixed in 30% ethanol and 7.5% acetic acid overnight at RT. Gels were imaged using the Typhoon 9400 (GE Healthcare, Piscataway, NJ), optimizing the photomultiplier tube voltage for each laser to achieve the broadest dynamic range, and analyzed for significant differences by ANOVA based on the log2 standardized volume using DeCyder 6.5 software (GE Healthcare, Piscataway, NJ). In summary, three analytical gels were completed in total, running 30 µg of pooled reference sample labeled with Cy2, along with two samples (30 µg each), one labeled with Cy3 and the other labeled with Cy5. The final gel experimental design resulted in three analytical gels with two technical replicates (Table **2**).

Gels selected for picking were stained with LavaPurple (Fluorotechnics, Guelph, Ontario) in 100 mM sodium borate, pH 10.5, for 2 hrs at RT, washed in 15% ethanol for 30 min at RT, and acidified in 15% ethanol/7.5% acetic acid for 30 min at RT. Spots were matched across gels by matching the pooled reference sample images to each other using DeCyder 6.5. Quantitation of protein abundance was determined based on pixel intensity using DeCyder software and calculating the pixel intensity volume for each spot. Statistical analyses for each spot were performed using the log2 standardized volume. The standardized volume is calculated by normalizing (ie. dividing) the volume determined for every Cy3 and Cy5 labeled spot to the Cy2 labeled spot volume for each gel (Table **2**). Approximately 1100 spots were matched across all three analytical gels. All three analytical gels were picked using an automated robotic system, the Spot Handling Workstation (GE Healthcare). The pick list was created based on the LavaPurple image. Two mm gel plugs were picked, washed, reduced and alkylated, digested with trypsin, and the resulting peptides were extracted and spotted using the Spot Handling Workstation (GE Healthcare, Piscataway, NJ). Briefly, plugs were washed twice with 50 mM ammonium bicarbonate/50% methanol for 20 min at RT and then with 75% acetonitrile for 20 min at RT and dried at 40°C for 10 min. Plugs were incubated in 10 mM DTT/20 mM ammonium bicarbonate at 37°C for 1 hr. The DTT solution was removed and immediately replaced with 100 mM iodoacetamide/20 mM ammonium bicarbonate and incubated at RT in the dark for 30 min. Plugs were washed as above, and then incubated with 200 ng sequencing grade trypsin (Promega, Madison, WI) at 37°C for 2 hrs. Peptides were extracted twice with 50% acetonitrile/0.1% trifluoroacetic acid for 20 min at RT and concentrated by SpeedVac (Jouan).

Mass Spectrometry

Approximately 25% of the resulting peptides were spotted with partially saturated α-cyano-4-hydroxy-cinnamic acid (Sigma, St. Louis, MO). Mass spectrometry (MS) and MS/MS data were acquired on the 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) using standard acquisition methods. MS spectra were calibrated using two trypsin autolysis peaks (1045.56 and 2211.096 m/z) and MS/MS spectra were calibrated using the instrument default calibration. The most recent version of NCBInr database was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/ entrez/) and was incorporated into a licensed copy of Mascot 1.9.05 (http://www.matrixscience.com/). Mass lists were submitted to the various sub-databases (NCBInr-Mammals, and NCBInr-Homo sapiens) considering fixed cysteine carbamidomethylation and partial methionine oxidation modifications, 1 missed tryptic cleavage, and 25 ppm mass accuracy. Identifications were cross-examined using mass accuracy, molecular weight, and pI.

RESULTS

Figs. (**1B, 1C** and **1D**) show the 2D DIGE gels of AD, NDC and non-ADD V-CSF pools, respectively. Two replicates of each pooled sample were run, labeling one replicate with Cy3 and one replicate with Cy5, resulting in three analytical gels (Table **2**). The 2D DIGE comparative analysis of the three diagnosis groups revealed 22 spots with statistically significant ($p \geq 0.05$) differences. These spots are shown in (Fig. **1A**) encircled in red and reported in Tables **3** and **4**. The blue encircled spots represent additional spots that were also investigated by MS (Fig. **1A**). The protein identities of the 22 statistically significant spots are shown in Table **3**, which also includes the 1-ANOVA analysis, National Center for Bioinformatics (NCBI) gi accession number, predicted molecular weight (MW), the predicted pI, number of matching peptides, protein MASCOT score and protein MASCOT confidence. With the exception of spots 13, 18 and 20, which show MASCOT scores of 67, 60 an 65, all MASCOT scores were greater than 72 ($p = 0.05$) and had a MASCOT confidence of 99.6-100 (Table **3**). Only one highly confident protein assignment resulted from the MS analyses. As the NCBInr database is not completely non-redundant, many of the searches did return several protein variants of the same

Table 2. Summary of Gel Experimental Design

Gel	Cy2 Labeled	Cy3 Labeled	Cy5 Labeled
1	30 µg pooled reference sample	30 µg NDC	30 µg AD
2	30 µg pooled reference sample	30 µg Non-ADD	30 µg NDC
3	30 µg pooled reference sample	30 µg AD	30 µg Non-ADD

NDC = non-demented control; AD = Alzheimer's disease; non-ADD = non-Alzheimer's disease dementia

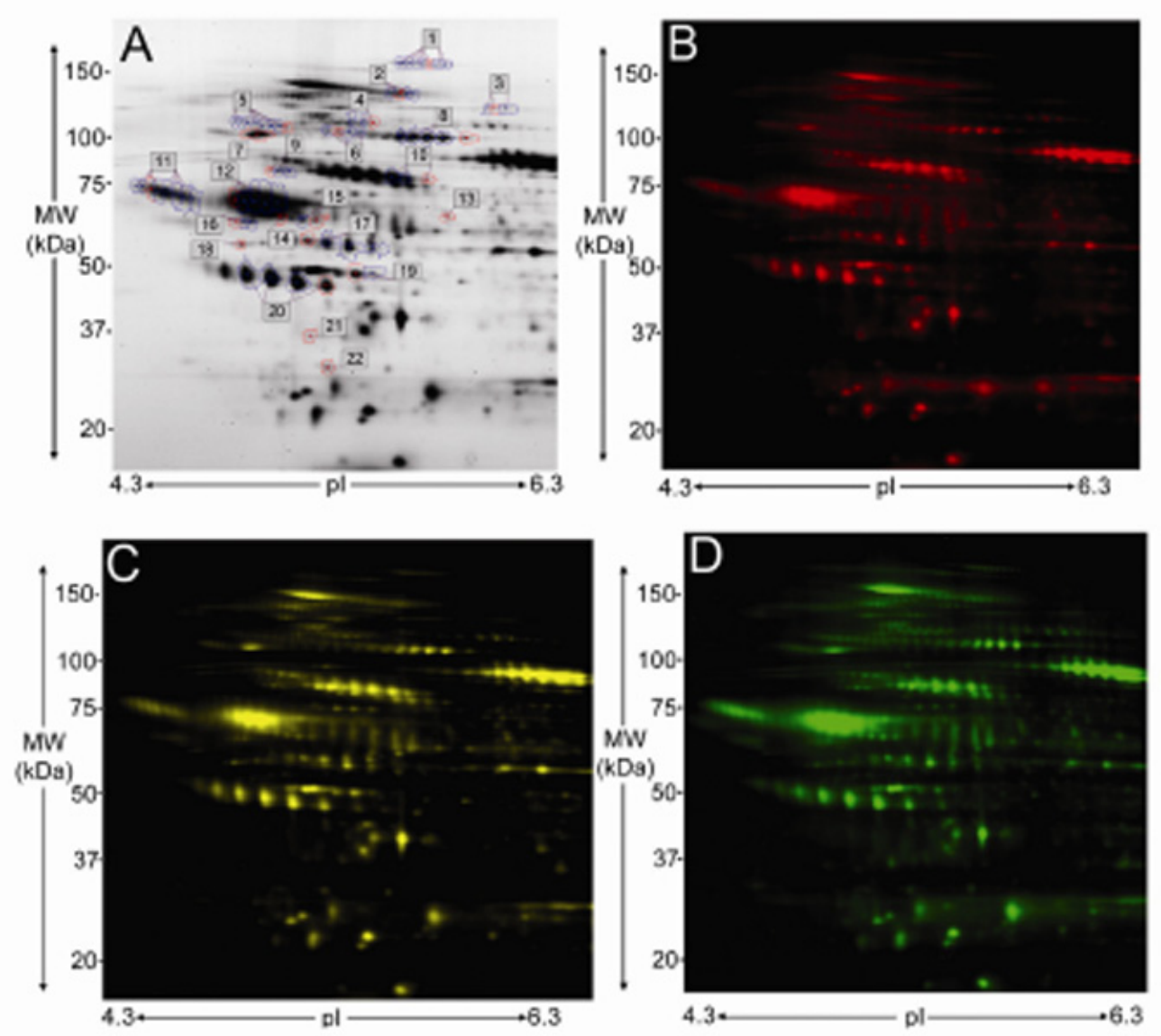

Fig. (1). Cerebrospinal fluid 2D-DIGE gels loaded with 30 µg of total protein. **A)** The CSF master gel showing the proteins that were found to be statistically different and identified by mass spectrometry. The identification numbers refer to those proteins listed in Tables **3** and **4**. **B)** Alzheimer's disease. **C)** Non-demented controls. **D)** Non-Alzheimer's disease dementia.

functional protein. However, the next ranking protein was always below the 95% confidence limit.

The Student's *t*-test and average ratio of intensity are shown in Table **4**. Column I shows 7 proteins were increased and 12 were decreased in the NDC when compared to the non-ADD group, with the biggest difference being in transthyretin (TTR), which was on average 3X higher in the non-ADD gels. Of the 14 spots found to be statistically different when comparing NDC and AD gels (Table **4**, column II), 7 were increased and 7 were decreased in the NDC group. Once again TTR had the most significant change in average ratio (-2.76). As seen in Table **4**, column III, 6 proteins were increased and 11 decreased in AD gels compared to the non-ADD gels. The largest difference in average ratio was α1-antitrypsin which was 2X higher in the non-ADD group. We included the pigment epithelium derived factor (PEDF; spot 17) because in the comparison between NDC/AD the *p* value was borderline (0.07). In addition, a previous biomarker assessment performed in our laboratory [14], in which pools of NDC and AD V-CSF were investigated, two isoforms of PEDF, were significantly increased in the AD pool ($p = 0.027$ and $p = 0.014$).

DISCUSSION

A justification for the use of postmortem V-CSF is the ability to use the neuropathological diagnosis as a gold standard. While existing clinical diagnostic methods have a high sensitivity for AD, about 85%, they have a low specificity, about 45%, as determined by a comprehensive study of results from the National Institute on Aging Alzheimer's disease centers [10]. Furthermore, in many instances the clinical diagnosis of AD is complicated by concurrent pathologies such as vascular dementia, Lewy body disease, Parkinson dementia, progressive supranuclear palsy and other non-AD

Table 3. Mass Spectrometry Protein Identification of 2D DIGE Spots of Interest and Statistical Analyses Using 1-ANOVA Comparing Non-Demented Controls, Non-Alzheimer's Disease Dementia and Alzheimer's Disease Gels

Spot	Protein ID	1-ANOVA (*p*)	NCBI accession (gi)	Predicted MW (Da)	Predicted pI	# of peptides	MASCOT score	MASCOT confidence
1	α2-macroglobulin	0.00038	224053	162,072	5.95	17	85	99.83
2	α2-macroglobulin	0.005	224053	162,072	5.95	17	102	99.99
3	CLIC6 protein	0.0096	34783345	44,538	4.62	8	86	99.86
4	Compl C4-B precursor	0.0054	81175167	194,212	6.73	18	86	99.86
5	Fibulin 1	0.0014	18490682	78,479	5.1	10	82	99.62
6	C4B1	0.0047	40737313	48,007	5.92	11	123	100
7	HSP90AA1 protein	0.018	62914009	74,179	5.08	20	209	100
8	Gelsolin isoform	0.0016	4504165	86,043	5.9	19	182	100
9	Compl 9	0.00061	2258128	61,728	5.42	13	98	99.99
10	Hemopexin	0.0037	11321561	52,385	6.55	12	174	100
11	α1-antichymotrypsin	6.40E-05	1340142	49,986	5.5	15	150	100
12	α1-antitrypsin	0.015	1942629	44,280	5.37	21	206	100
13	β2-glycoprotein	0.0016	6435718	36,666	8.26	5	67	88.35
14	α-tubulin	0.0027	340021	50,804	4.94	8	85	99.94
15	Angiotensinogen	0.00011	62089124	54,029	5.97	8	157	100
16	Tubulin, β 2C	0.0076	119608775	49,250	4.88	14	215	100
17	PEDF	0.073	39725934	46,454	5.97	12	189	100
18	γ enolase	0.0018	182118	44,568	4.94	5	60	78.67
19	Creatine kinase	0.0089	180570	42,876	5.34	11	105	99.99
20	HP protein	0.0021	34785974	25,727	6.06	5	65	82.37
21	TTR dimer	0.004	55669575	12,836	5.33	6	149	100
22	Cathepsin D	0.0082	494296	26,457	5.31	5	116	100

NCBI = National Center for Bioinformatics; MW = molecular weight; Da = Daltons; Compl = complement; HSP = heat shock protein; PEDF = pigment epithelium derived factor; TTR = transthyretin

Table 4. Statistical Analyses of 2D DIGE Ventricular Cerebrospinal Fluid Proteins Using Student's t-Test

Spot	Protein ID	I. NDC/non-ADD		II. NDC/AD		III. AD/non-ADD	
		t-test (*p*)	Avg Ratio	*t*-test (*p*)	Avg Ratio	*t*-test (*p*)	Avg Ratio
1	α2-macroglobulin	0.002	1.21	0.0061	1.14	0.007	1.07
2	α2-macroglobulin	0.021	1.21			0.016	1.28
3	CLIC6 protein	0.021	1.32			0.026	1.35
4	ComplC4-B precursor	0.0041	-1.46			0.045	-1.23
5	Fibulin 1	0.0062	-2.05	0.024	-1.37	0.0053	-1.49
6	C4B1	0.012	-1.36	0.0091	-1.27		
7	HSP90AA1 protein			0.017	1.22	0.031	-1.25
8	Gelsolin isoform	0.0049	-1.25	0.026	1.25	0.0057	-1.55

(Table 4) contd….

Spot	Protein ID	I. NDC/non-ADD		II. NDC/AD		III. AD/non-ADD	
		t-test (*p*)	Avg Ratio	*t*-test (*p*)	Avg Ratio	*t*-test (*p*)	Avg Ratio
9	Compl. 9	0.0026	-1.53			0.0044	-1.53
10	Hemopexin	0.0023	1.35			0.024	1.22
11	α1-antichymotrypsin	0.00084	-1.65	0.011	1.19	0.0005	-1.97
12	α1-antitrypsin	0.037	-1.83			0.021	-2.04
13	β2-glycoprotein	0.0051	-1.67	0.019	-1.36	0.012	-1.23
14	α-tubulin	0.011	-1.42			0.011	-1.47
15	Angiotensinogen	0.0013	-1.45	0.012	-1.04	0.0015	-1.39
16	Tubulin, β 2C	0.0022	-1.8			0.027	-1.69
17	PEDF			0.07	-1.17		
18	γ enolase	0.01	1.22	0.0084	1.26		
19	Creatine kinase	0.016	1.67	0.038	1.24		
20	HP protein			0.0056	-1.17	0.012	1.15
21	TTR dimer	0.015	-3.07	0.0006	-2.76		
22	Cathepsin D	0.018	1.23	0.04	1.12	0.049	1.1

NDC = non-demented controls; non-ADD = non-Alzheimer's disease dementia; AD = Alzheimer's disease; Avg = average; Compl = complement; HSP = heat shock protein; PEDF = pigment epithelium derived factor; TTR = transthyretin

dementias. Another advantage is that the V-CSF is very seldom contaminated by blood. However, it must be recognized that the diagnostic value of V-CSF biomarkers needs to be correlated with measurements obtained from living individuals using lumbar CSF. In a small proteomics study, quantitative deviations between ventricular and lumbar CSF were observed [15]. Although the authors attributed the differences to PMI, these disparities may also be due to protein gradient differences that exist between the two anatomical sites (lumbar versus V-CSF). Consideration should be given to the fact that some proteins that are generated by the brain have higher concentrations in the V-CSF. On the other hand, those proteins derived from the blood are more concentrated in the lumbar CSF [16]. In the best of the situations, antemortem CSF collection of clinically diagnosed individuals should be followed by postmortem neuropathological confirmation. These conditions are difficult to achieve given that, in most cases, relatively few individuals are autopsied. Other considerations for CSF biomarker studies include long PMI, which should be avoided, due to protein degradation. Additionally, lumbar Aβ CSF protein values are unreliable to a certain degree because of fluctuations that occur at different times of the day as recently demonstrated by Bateman and colleagues [17]. An important caveat in dealing with lumbar or V-CSF is the long duration of AD and its presymptomatic period, which altogether can last for several decades. It is likely that different protein profiles would be obtained at different disease stages if there is not a selective stratification of patients, an issue that may confound the utility of the results. In an ideal study, lumbar CSF could be taken at different time points along the disease course to monitor how protein profiles change during AD progression, but problems with patient attrition, the slow rate of death and limited funding make this difficult to accomplish. Notwithstanding these disadvantages, the CSF remains the most promising venue to discover a set of reliable biomarkers, since it is in close contact with the brain and is a natural basin for some interstitial brain molecules.

Our failure to detect increases in CSF tau (reviewed in [3;8]) could be due to postmortem changes in the phosphorylation of these molecules. In rat models, the kinetics of phosphorylation/dephosphorylation of tau in the immediate postmortem results in a net loss of phosphate groups, being a relatively fast and site specific event [18]. In a second report [19], tau levels remained steady up to 8 h postmortem, although the electrophoretic mobility of some tau isoforms was modified. Similar cytoskeletal alterations were observed in fresh postmortem and surgically removed tissues.

Another limitation of biomarker studies is the use of small numbers of cases due to the expense and technical difficulties of proteomic experiments. Instead of performing 107 individual 2D DIGE tests, we decided to investigate pools of V-CSF obtained from our rapid autopsy program with short PMI. Our strategy was to individually measure the amount of V-CSF total protein and then to pool individual aliquots, with an equal protein representation from each case, with the aim of finding the most prominent deviations shared by a cohort of demented individuals and compare them with an equally represented NDC group. We are aware that such studies are preliminary and need to be confirmed or rejected by individual assessments using immunoassay approaches.

Presently, the literature on AD CSF biomarkers is extensive with more than 500 publications recorded by the National Library of Medicine (USA) as of September 2008. Consequently, it is beyond the scope of this paper to discuss this vast body of information. However, it is noteworthy that most of the proteins identified in our study have already been implicated, directly or indirectly, in AD pathophysiology and/or Aβ metabolism or have been found altered in the CSF of AD patients. These include α-2-macroglobulin [20;21], α1-antichymotrypsin [22-24], α1-antitrypsin [24-27], complement proteins [28], heat shock proteins [29], fibulin-1 [30], gelsolin [31;32], hemopexin [14], tubulin [33;34], PEDF [14;35], TTR [14;36;37], cathepsin D [14;38-40], enolase [41-43] and creatine kinase [44]. In addition, in a previous pilot CSF proteomic study from our laboratory using 2D-gel electrophoresis [14], 3 out of 5 proteins identified in that study were confirmed in the present investigation by DIGE (PEDF, TTR, cathepsin D). This panel of proteins is likely reflective of fundamental pathological pathways in the AD brain that are divergent from normal aging or non-ADD. Additional work with a larger number of cases assessed individually, with quantitative reliable methods, such as ELISA, is needed to determine if the present array of CSF proteins would provide the sensitivity and specificity, useful for the accurate diagnosis of sporadic AD.

In conclusion, our results indicate that protein separation techniques based on physicochemical principles are promising in providing significant differences between AD, NDC and non-AD dementias.

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