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## Studies of potential cerebrospinal fluid molecular markers for Alzheimer's disease

There is a need for a reliable, molecular-based *ante mortem* diagnostic test for Alzheimer's disease (AD). In this study, we examined the use of two-dimensional protein electrophoresis for generating molecular barcodes which may be useful for the clinical differentiation of AD patients from normals. We compared cerebrospinal fluid samples taken from AD patients with confirmed *post mortem* pathology to comparable specimens from normal volunteers. Using canonical correlation analysis, a panel of nine molecular markers were identified which segregated diseased cases from normal controls. Using the scaled volume image analysis variable, a principal factor analysis was also used to distinguish normal from AD spinal fluid, based on molecular markers identified using a heuristic clustering algorithm. The use of panels of molecular markers derived from proteomic analysis may offer the best prospect for developing molecular diagnostic tests for complex neurodegenerative disorders such as AD.

**Keywords:** Alzheimer's disease / Cerebrospinal fluid / Proteomics

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### 1 Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative dementing disorder affecting more than 5% of people over the age of 65 and nearly half of the population over 85. Three autosomal dominant disease-causing mutations, one confirmed genetic susceptibility locus and several putative environmental risk factors have been linked to Alzheimer causation, consistent with the model of AD as an age-related, complex genetic disorder. AD is characterized neuropathologically by findings of excessive numbers of amyloid-laden senile plaques and Tau-containing neurofibrillary tangles in specific subcortical, limbic and neocortical association areas of the brain. Widespread synaptic thinning and neuronal loss, inflammatory changes, neurochemical alterations and other pathologic features further distinguish AD from normal aging and other neurodegenerative disorders.

Despite incontrovertible evidence that AD is a distinctive disorder with identifiable neuropathological features, accurate clinical diagnosis of AD remains a challenge. The initial symptoms of AD occur on a continuum with normal age-related memory loss, making AD difficult to identify in its earliest stages. Overlap of the symptoms of

AD with those of other neurodegenerative dementing disorders further complicates its differential diagnosis. Autopsy studies remain the gold standard for confirming an Alzheimer diagnosis. No single *ante mortem* diagnostic test has been developed that permits identification of AD with comparable accuracy to autopsy. The inability to reliably diagnose AD during life has implications beyond the diagnostic domain. Without an objective marker of the onset of AD, administration of available pharmacologic treatment is often delayed for months or years from time of first symptoms. Lack of an objective biological measure for AD onset and progression also limits our ability to assess the effectiveness of potential new therapies in clinical trials. These difficulties have delayed the development of more effective AD treatments and preventatives, and in other ways undermine clinical management of patients with AD.

While significant progress has been made in the identification and characterization of some of the molecules involved in AD's causal pathways, no single laboratory test yet identified permits accurate and reliable *ante mortem* diagnosis. A large number of putative AD diagnostic markers have been identified in past studies of brain, blood, cerebrospinal fluid (CSF), fibroblasts, urine and other tissue products [1]. There is evidence to suggest that certain CSF proteins (e.g., Tau, A $\beta$ 42, and AD7c-NTP) can be useful adjuncts in the clinical diagnosis of AD. Among clinically demented patients, elevated Tau, decreased A $\beta$ 42 and possession of the  $\epsilon$ 4 allele of APOE supports an AD diagnosis. Another proposed AD diagnostic measures the expression of AD7c-NTP in CSF or urine. AD7c is a 41 kDa neural thread associated protein

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**Abbreviations:** AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; SV, scaled volume

that is elevated in some cases of AD compared to normals [2]. Unfortunately, the sensitivity and specificity of any of the above-mentioned markers, either alone or in combination, is insufficient to merit routine clinical use [3].

The failure of past approaches to molecular-based AD diagnosis may be related to AD's inherently multifactorial nature. It has been estimated that only 2% of human diseases are the result of a single gene defect [4]. AD is a complex genetic disorder arising from the interaction of multiple genes and environmental factors. In this context, there may be particular merit to the use of approaches which simultaneously assay multiple biologic markers and their interactions [1]. In this paper we describe the use of a proteomics approach, with the goal of segregating Alzheimer's disease subjects from normals by the study of CSF. We examine the ability of the two-dimensional protein electrophoresis (2-DE) technology to classify samples into appropriate categories using different multivariate statistical methods and using various image analysis variables.

## 2 Materials and methods

### 2.1 Samples

CSF samples were obtained in collaboration with a number of brain banks (listed in acknowledgements). In general, CSF was obtained by lumbar puncture from individuals with probable AD or from normal volunteers. Stage of disease at the time of lumbar puncture was in most cases mild to moderate. The diagnosis of AD was made according to standard clinical criteria [5]. *Post mortem* studies were carried out by neuropathologists at the various institutions contributing CSF to confirm the AD diagnosis [6]. Normals were defined both by the absence of significant cognitive impairment during life and lack of AD or other significant neuropathology at autopsy. Because of the difficulty in obtaining an ideal set of normal CSF controls (including neurological workup, age- and gender-matched controls, *etc.*), we emphasize the preliminary nature of these data.

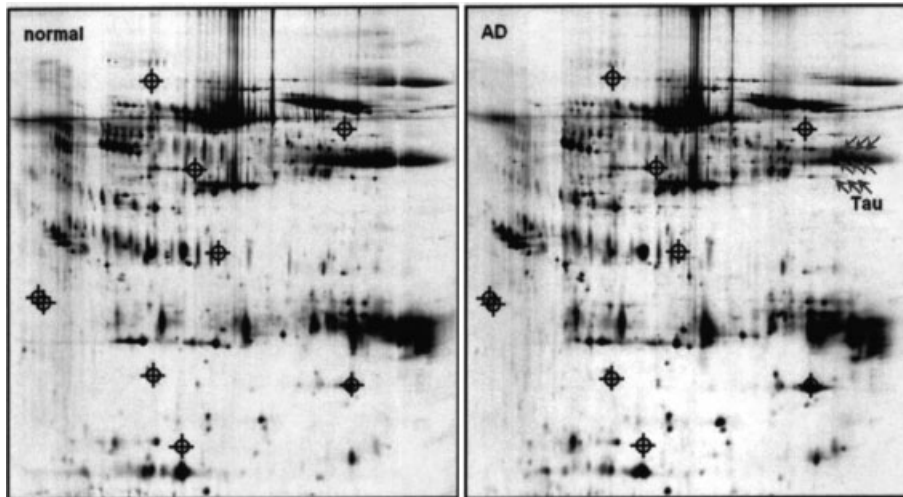
### 2.2 Procedures

The detailed protocols for performing 2-DE and ammoniacal silver staining of CSF proteins which were used in this study have been described elsewhere [5, 6]. Briefly, a 250  $\mu$ L aliquot of CSF was subject to an overnight ice-cold ethanol protein precipitation. The protein pellet was resuspended in a mixture containing 9 M urea, 2% 2-mercaptoethanol, 2% IGEPAL, and 0.8% carrier ampholytes. This sample was then hydrated directly into commercially available pH 3–10 nonlinear immobilized pH gradient

(IPG) isoelectric focusing gels (Amersham Biosciences) with 8 M urea, 2% CHAPS, 20 mM dithiothreitol, 2% carrier ampholytes and a trace of bromophenol blue. Isoelectric focusing was performed for a total of 64 250 Vh at 20°C using the Multiphor instrument (Amersham Biosciences). The IPG gels were equilibrated in solutions containing dithiothreitol and iodoacetamide before polyacrylamide gel electrophoresis in 10–16%T vertical gradient slab gels (Bio-Rad Laboratories). Proteins were detected using an ammoniacal silver stain and scanned using a Molecular Dynamics laser densitometer. The resulting gel image files were imported into the commercially available Melanie 3 gel analysis software package (GeneBio, Geneva, Switzerland). Spot detection was performed using default parameter values and features were manually edited to remove technical artifacts. The percent integrated optical density (% volume) was calculated for each of the protein spots detected with this procedure. The % volumes of spots of interest and which appeared in duplicate gels were exported into the Unistat 4.53 (Unitstat Ltd., London) software package running on Windows NT 4.0. Multivariate statistical analyses of these data were performed in Unistat 4.53 to generate a multiple discriminant function analysis of the data sets. Additionally, the newly introduced scaled volume variable for ammoniacal silver staining [7] was used to classify AD from normal samples in a principle factor analysis of resulting data. Immunostaining of 2-DE blots was done with a monoclonal antibody against Tau (Sigma T5530). A set of two 2-DE gels were run in parallel. One of the gels was silver-stained as a reference. The other gel was blotted to a PVDF membrane (Millipore Immobilon P) at 150 mA for 6 h. The resulting blot was first stained with Colloidal Gold Total Protein Stain (Bio-Rad Laboratories #170-6527) and washed thoroughly in deionized water. The membrane was blocked in TBS with Tween (TBST) containing 5% dry powdered milk. The primary antibody was used at a 1:250 dilution and a peroxidase-linked secondary goat anti mouse antibody (Sigma #A9917) was used at 1:2500 dilution prior to detection with a SuperSignal West Dura kit (Pierce 34075). The blot was exposed to a chemiluminescence screen and scanned on a Bio-Rad GS525 imaging system. An ELISA kit for CSF A $\beta$ 42 was purchased from Biosource International (Camarillo CA, 88–344). The assay was run according to manufacturer's directions and data was acquired on an ISA SPEX FluoroMax 2 fluorimeter with MicroMax plate reader.

## 3 Results

A typical 2-DE gel of AD CSF is shown in Fig. 1 compared to normal. We obtained a series of 15 *ante mortem* CSF samples from various brain banks and Alzheimer Disease



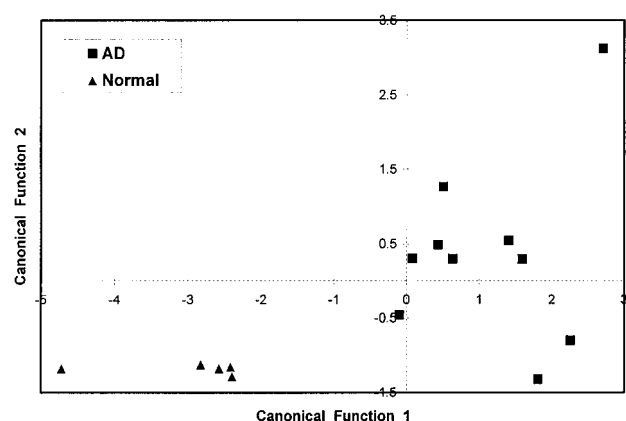
**Figure 1.** Silver-stained 2-DE gel of normal and AD CSF. Proteins are separated by charge and by size. The location of the nine proposed markers for AD and of the Tau immunoreactive spots are shown.

Research Centers in the United States. Ten of these samples were *ante mortem* CSF samples from patients clinically diagnosed with probable AD and later confirmed to have AD based on *post mortem* pathology (7 male, 3 female, mean age 75.4 years). The other five samples were *ante mortem* normal controls (5 male, mean age 42.3 years). Using computer aided image analysis we first identified 9 molecular markers (Fig. 1), based on changes in %vol measurements, which appear potentially useful for segregating the normal and AD populations. We performed a canonical correlation analysis of the % vol parameter for each of the nine spots across the available samples. We used the %vol data, exported from the Melanie software and into the Unistat software, to generate canonical discriminant functions. Eigenvalues and eigenvectors of the system were found using Choleski decomposition. The canonical discriminant functions (or 'barcodes') are able to distinguish normal from AD CSF as shown in Fig. 2 which depicts the relative locations of each of these samples on a plot of the two canonical discriminant functions. There is a clear segregation between all of the normal controls and all of the AD cases.

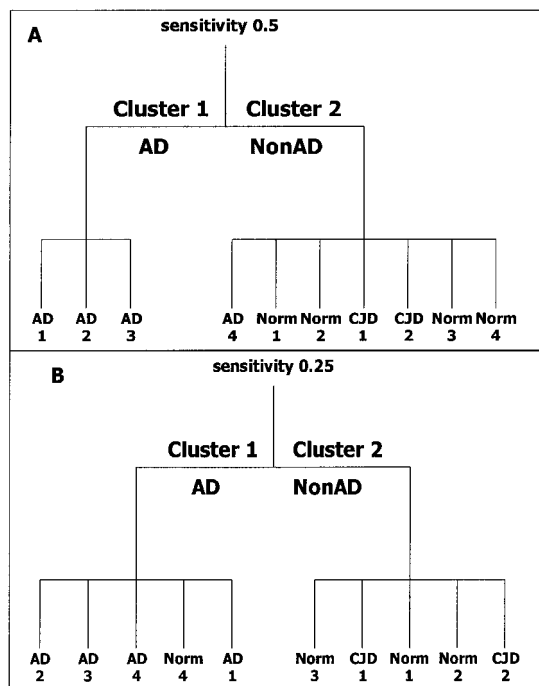
We also measured the quantity of CSF A $\beta$ 42 and of CSF Tau for these samples. A $\beta$ 42 was measured by ELISA and Tau was measured by %vol based on immunostaining against all isoforms of Tau (Fig. 1). For these samples, we measured levels of A $\beta$ 42 to be  $376 \pm 97$  pg/mL for normal controls and  $358 \pm 94$  pg/mL for AD samples. The main utility in the A $\beta$ 42 CSF marker is the ability to distinguish AD from other neurological disorders present in the differential diagnosis of AD. In this study, we are limited to an analysis of normal from AD without consideration of the ability to distinguish neurologic controls. Our data is consistent with prior observations that A $\beta$ 42 levels are somewhat reduced in AD patients when compared to normal

controls [8]. Motter *et al.* [9] described the use of a cut-off of  $>505$  pg/mL as indicative of an absence of AD. Using this criterion, they observe a sensitivity of 100% for a population of  $n=89$  subjects. If we apply the same criterion to this data, we also calculate a sensitivity of 100%.

The location of Tau was found by using a monoclonal antibody to Western stain against total Tau. Tau can be hyperphosphorylated resulting in the appearance of many charge and molecular weight isoforms as measured by 2-DE. The amount of Tau was measured using % vol and was calculated to be  $48 \pm 22$  for normals and ranged between 34 and 191 for AD CSF with an average of 187 (relative units). Consistent with the known utility and limitations of the Tau marker, we observe that Tau expression is elevated in AD patients compared to normal controls. The Tau antibody did not highlight any of the nine putative molecular markers for AD.

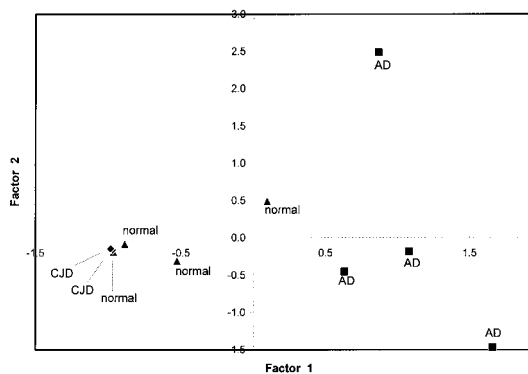


**Figure 2.** Canonical function analysis of AD versus normal using nine molecular markers.



**Figure 3.** Heuristic clustering of AD versus normal using spot groups identified by Student's *t*-test. (A) Clustering using Melanie default parameters, sensitivity parameter = 0.5; (B) clustering using sensitivity parameter = 0.25.

In a second analysis, we took a subset of eight of the patient samples and performed a very different multivariate statistical analysis of the same 2-DE data and including two gels from cases of Creutzfeldt-Jakob disease (CJD) as neurologic controls. Using the Melanie software and these samples (four normal, four AD, two CJD), we used the Student's *t*-test function to identify spots, present on all gels, and which have a >95% probability of having differential expression between normal and AD based on %vol measurements. The two groups defined were AD and non-AD samples. The software identified 46 spot groups with this characteristic and a heuristic clustering of the gel images, using default Melanie parameters, is shown in Fig. 3A. Based on this clustering which segregated three of the four AD cases on one branch and all other samples on the non-AD branch, the software identified only 2 of the 46 spot groups as being characteristic of this clustering pattern. The sensitivity parameter in Melanie was initially set at 0.5 as a default value and when this value was lowered to 0.25, a different clustering was observed as shown in Fig. 3B. Here, the clustering results in all four AD cases plus one normal case clustered onto the AD branch and all other gels on the non-AD branch and the algorithm identified 12 spot groups as being characteristic for this clustering pattern and there was no overlap between these 12 spot groups



**Figure 4.** Principal factor analysis of SV data for AD versus normal and neurologic control samples.

and the 2 spot groups identified in the previous clustering. We used the data for all 14 of these spot groups as the basis for a principal factor analysis using the scaled volume and not as the primary means of segregating AD and non-AD CSF directly.

We have previously shown the ability of the scaled volume (SV) image analysis variable to more quantitatively predict changes in protein amount based on ammoniacal silver stain [7]. Here, we calculated the SV for each of these 14 spot groups. Because of the possibility of experimental error or technical artifacts, we arbitrarily set a minimum standard of a fourfold change in SV as significant (spots that change in SV staining intensity by less than a factor of four are considered not significant changes). Using this criteria, 10 of the 14 spot groups identified in the heuristic clustering are considered significant. A principal factor analysis of this data was performed using the Unistat software package as shown in Fig. 4. Figure 4 clearly depicts the ability of the principal factor analysis to segregate the normal from the AD cases. Factor 1 in this analysis accounts for 71.6% of the variance while factor 2 accounts for 21.4% of the variance. Thus, this combined approach is able to account for 93.0% of the variance in this sample set which suggests that a multivariate approach to molecular diagnosis using a proteomics strategy may be more effective than any approach based on a single factor or single test.

#### 4 Discussion

We have demonstrated the potential utility of the proteomics approach to classify *ante mortem* AD CSF as distinct from *ante mortem* normal CSF, using a panel of molecular markers defined by use of various multivariate statistical methods. Thus far, we have yet to characterize any of the proteins by comparison with on-line databases or by mass spectrometry. Limitations in the quantity of protein

and in the ability to purify these markers from CSF may be overcome by the application of improved technologies for *de novo* sequencing or by purification from *post mortem* CSF if the markers are present in ventricular *post mortem* CSF.

Here, we collected data using CSF samples from AD and normals. While in this preliminary study, the samples are not controlled ideally in terms of age, we note that a review of the published literature does not identify any known changes in protein expression in CSF that occur with age as measured by this technique. However, there are weakly associated changes in prealbumin, albumin, immunoglobulin G and  $\alpha$ -2-macroglobulin probably related to blood-brain-barrier breakdown [12]. Age-related changes are identified in other neurologic diseases, such as Guillain-Barre syndrome [13], for which we do not have CSF. Using these sample, we first identified possible molecular markers for AD by a combination of visual inspection and Melanie-aided analysis which identified 9 molecular markers. A canonical function analysis is able to segregate AD from normals. The canonical function analysis, which is useful to reduce the dimensionality of data, does not account for variance within the predictor variables (the % vol measurements of individual spots) and thus we performed a second analysis using a principal factor analysis based on the SV image analysis variable. To reduce the computation time, we began with a Student's *t*-test (in Melanie) to reduce the number of candidate molecular markers before performing a data-driven heuristic clustering with different sensitivity parameters. Based on this analysis, we identified a subset of ten spots (not overlapping with the nine molecular markers used in the canonical correlation analysis) which are able to segregate normal from AD CSF in a principal factor analysis. Although established differential diagnostic markers were not among those identified, this is not unexpected given the more defined goal of distinguishing AD from normal pursued in the present study. Our findings concerning using Tau and A $\beta$ 42 CSF levels based on 2-DE are consistent with other published observations.

Both of the multivariate statistical approaches have merits for the identification of possible molecular markers for AD and here we only demonstrate that multiple approaches may be useful. The proteomics paradigm simultaneously measures the expression of a large number of CSF proteins, which could permit the development of a multiple marker test. This is more appropriate for providing information on molecular aspects of this complex disease and potentially may simplify and/or supplement the serial Bayesian diagnostic approach employed currently. These data suggest the possibility of using multiple markers to distinguish AD from normal, and speak to

the potential use of proteomics in developing differential diagnostic tests for AD. We are currently working to characterize these markers and to study the effectiveness of this approach in a larger subject population that includes a variety of neurologic controls.

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