Objective: To use proteomic analysis of cerebrospinal fluid to discover novel proteins and peptides able to differentiate between patients with stable mild cognitive impairment (MCI) and those who will progress to Alzheimer disease (AD).

Design: Baseline cerebrospinal fluid samples from patients with MCI and healthy controls were profiled using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry.

Setting: Memory disorder clinic.

Participants: Patients with MCI (n=113), of whom 56 were cognitively stable and 57 progressed to AD with dementia during a 4- to 6-year follow-up, as well as 28 healthy controls who were followed up for 3 years.

Main Outcome Measure: During follow-up, 57 patients progressed to AD and 56 patients had stable MCI. Cerebrospinal fluid from these 2 groups of patients was compared using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry.

Results: We identified a panel of 17 potential biomarkers that could distinguish between patients with stable MCI and patients with MCI who progressed to AD. We have positively identified and characterized 5 of the potential biomarkers.

Conclusions: Proteomic profiling of cerebrospinal fluid provided a novel panel of 17 potential biomarkers for prediction of MCI progression to AD. The 5 identified biomarkers are relevant to the pathogenesis of AD and could help gain an understanding of the molecular pathways in which they may function.

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The prevalence of dementia doubles every 5 years from the age of 65 years, and approximately 40% of those aged 90 to 95 years are affected. With increasing life expectancy, dementia is a growing socioeconomic and medical problem. Alzheimer disease (AD) is the most common cause of dementia. The neuropathological hallmarks of AD include amyloid plaques (composed of β-amyloid) and neurofibrillary tangles (composed of hyperphosphorylated tau) together with degeneration of the neurons and synapses.

The pathogenic process of AD probably starts decades before clinical onset of the disease. During this preclinical period, there is a gradual neuronal loss. The first symptoms, most often impaired episodic memory, appear at a certain threshold. This clinical phase is often designated as mild cognitive impairment (MCI). Diagnostic criteria for MCI have been suggested, and these criteria are becoming adopted in research-oriented clinical practice. The interest in this group of patients increased when research focused on the discovery of markers for very early stages of AD. Many patients with MCI have incipient AD, whereas others have a stable form of MCI as part of the normal aging process. The conversion rate to AD with clinical dementia is 8% to 15% per year. Identification of patients with MCI who will progress to AD would allow for the appropriate application of disease-modifying treatments at a point where clinical manifestations are limited.

There is a need for biochemical tests that can discriminate MCI progressing to AD from stable MCI. The cerebrospinal fluid (CSF) biomarkers β-amyloid$_{42}$, total tau, and phosphorylated tau have shown high sensitivity for the identification of AD$^6$ and MCI$^7$, and they are used routinely in parts of Europe for research and diagnostic purposes. However, the diagnostic specificity of these biomarkers is not optimal for discrimination of AD from other dementias.$^8$
We have described the discovery of a novel panel of 30 CSF biomarkers in a study of 95 patients with AD and 72 healthy controls using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (A.H.S., J.M., V.N.P., H.D., G.W., L.M., K.B., I. Skoog, MD, PhD, N. Andreasen, PhD, A. Wallin, MD, PhD, unpublished data, January 2006). In our current explorative study, we have compared CSF protein profiles from patients with MCI who progressed to AD, cognitively stable patients with MCI, and control individuals with the aim of investigating the performance of the biomarker panel in the differential diagnosis.

METHODS

PARTICIPANTS

The present study is a substudy of a larger study where patients with MCI from whom CSF was obtained at the initial visit were recruited at Malmo¨ University Hospital, Malmo¨, Sweden.7 At the initial visit, patients underwent physical, neurological, and psychiatric examination, reported their careful clinical history, and underwent functional assessment. Moreover, computed tomography of the brain and cognitive tests were performed. The criteria of probable AD defined by NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association).10 The present study included 56 patients and control individuals with the aim of investigating the performance of the biomarker panel in the differential diagnosis.

The patients were followed up clinically at least until they developed a certain type of dementia or had been cognitively stable for more than 4 years (mean follow-up, 3.2 years; range, 4.0-6.8 years). The patients who received a diagnosis of AD during follow-up were required to meet the Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition criteria of dementia and the criteria of probable AD defined by NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer’s Disease and Related Disorders Association).10 The present study included 56 cognitively stable patients with MCI and 57 patients with MCI who developed AD during follow-up.

Moreover, 28 healthy controls were included, all of whom underwent lumbar puncture at the initial visit. Inclusion criteria incorporated absence of memory complaints or any other cognitive symptoms, preservation of general cognitive functioning, and no active neurological or psychiatric disease. Individuals with other medical conditions, such as diabetes, hypertension, and arthrosis, that did not affect cognition were not included. The controls were followed up over 3 years. Demographic data are shown in Table 1.

The study was conducted according to the provisions of the Helsinki Declaration and approved by the ethics committee of Lund University, Malmo¨. Patients gave informed consent to participate in the study.

LABORATORY METHODS

Samples (10-12 mL) of CSF were obtained by lumbar puncture, collected in polypropylene tubes, and gently mixed. The samples were centrifuged at 2000g for 10 minutes to remove cells and other insoluble material. Supernatants were frozen in aliquots and stored at –80°C. No sample contained more than 500 erythrocytes/µL to exclude contamination from serum proteins.

After clinical follow-up of the patients was complete, 5 µL of each CSF sample was diluted into 45 µL of binding buffer for each of the ProteinChip Array types (Ciphergen Biosystems, Fremont, Calif). To ensure reproducibility of sample preparation and array analysis, a reference CSF standard was randomly distributed in separate aliquots among the clinical samples and analyzed under the same conditions. Reproducibility was measured by calculating average coefficients of variation for each set of acquisition parameters. All array preparation was performed using a Biomek 2000 robot (Beckman Coulter, Fullerton, Calif) and randomized sample placement. The samples were allowed to bind for 60 minutes at room temperature. Each array was washed 3 times with binding buffer and twice with water. Energy-absorbing molecule application was performed using a modified BioDot AD3200 robot (BioDot, Inc, Irvine, Calif). Two aliquots of 0.75 µL of solution containing 12.5-mg/mL sinapinic acid in 50% acetonitrile with 0.5% trifluoracetic acid were applied with drying in a controlled atmosphere between applications. The arrays were read at 2 different instrument settings to focus on lower and higher masses. Each sample was run in duplicates on separate arrays. All arrays were analyzed using a SELDI-TOF-MS ProteinChip Reader, series PCS4000 (Ciphergen Biosystems). A protein profile was generated in which individual proteins were displayed within spectra as unique peaks based on their mass-charge ratio.

Selected biomarkers were purified using combinations of chromatographic techniques with a range of sorbents (BioSepra; Pall Corp, East Hills, NY) typically followed by sodium decyl sulfate–polyacrylamide gel electrophoresis. Colloidal Blue-stained bands were excised from gels. One quarter of each band was extracted using 50% formic acid, 25% acetonitrile, 15% isopropanol, and 10% water and reanalyzed using the ProteinChip Reader to confirm that the mass of the extracted protein matched the mass of the original biomarker. The remainder of each band was in-gel digested with bovine trypsin. Tryptic digests were analyzed by tandem mass spectrometry using a QSTAR XL mass spectrometer (Applied Biosystems, Foster City, Calif) equipped with a PCI-1000 ProteinChip Interface (Ciphergen Biosystems). A more detailed description and an illustration of methods on purification and identification of CSF biomarkers discovered by SELDI-TOF-MS have been included in a related article.11

Table 1. Clinical Characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Patients, No.</th>
<th>MMSE Score, Mean (SD)</th>
<th>Age, Mean (SD), y</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCI-AD</td>
<td>57</td>
<td>26.8 (1.4)</td>
<td>74.3 (5.8)</td>
</tr>
<tr>
<td>MCI-MCI</td>
<td>56</td>
<td>27.3 (1.9)</td>
<td>64.3 (9.0)</td>
</tr>
<tr>
<td>Healthy control</td>
<td>28</td>
<td>29.0 (1.1)</td>
<td>74.0 (8.4)</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer disease; MCI, mild cognitive impairment; MMSE, Mini-Mental State examination.

BIOINFORMATICS AND STATISTICAL METHODS

ProteinChip profiling spectral data were collected using CiphergenExpress data management software version 3.0 (Ciphergen Biosystems), where data handling and univariate analysis were also performed. All spectra were internally mass calibrated and peak intensities were normalized using total ion current. Spectra were omitted if the normalization coefficient was greater than twice the average. For biomarker selection, the primary comparison was between MCI-AD and MCI-stable. P values for individual peaks across 2 or 3 groups were calculated using Mann-Whitney and Kruskal-Wallis tests, respectively. As a post hoc test after the Kruskal-Wallis test, the Dunnes test was used. For all tests, the level of significance was P < .05.
The intra-assay reproducibility of the discovery method was measured on reference CSF samples and the coefficient of variation was found to be between 14% and 19% (data not shown). No spectra were omitted from the study.

A 2-group comparison between the patients with stable MCI and those who progressed to AD was performed. Furthermore, we performed a 3-group comparison between the patients with stable MCI, patients with MCI who progressed to AD, and healthy controls.

In this study, 17 of the 30 potential markers from the previous study were differentially expressed between the patients with MCI who progressed to AD and those who did not progress (A.H.S., J.M., V.N.P., H.D., G.W., L.M., K.B., I. Skoog, MD, PhD, N. Andreasen, PhD, A. Wallin, MD, PhD, unpublished data, January 2006). All of the 17 proteins were present in all of the samples. Three of the 17 proteins were not significant in the 3-group comparison (Table 2). In addition, 5 of the 17 proteins were positively identified. Figure 1 shows scatter plots for the 4 most significant peaks between MCI-AD and MCI-MCI: ubiquitin, a phosphorylated C-terminal fragment of osteopontin, an unidentified 7944-Da peak, and an unidentified 8641-Da peak. Figure 2 shows representative spectra for ubiquitin for a patient with MCI-MCI, a patient with MCI progressing to AD, and a healthy control subject.

Using SELDI-TOF-MS analysis of CSF from MCI cases and nondemented controls, we have discovered a panel of putative biomarkers for the prediction of progression from MCI to AD. Of the 17 proteins, 4 were down-regulated and 13 were up-regulated in the MCI-AD group.

The finding of both up- and down-regulated proteins in the MCI-AD group also suggests that there were no systematic errors, such as differences in total protein levels or blood contamination between the groups. To our knowledge, this is the first comprehensive proteomics study of CSF from patients with MCI.

In this study, the patients with stable MCI were approximately 10 years younger than both the patients with MCI-AD and the healthy controls. Confirmatory experiments with age-matched samples should be performed to address this issue.

We detected increased levels of C3a anaphylatoxin des-Arg and C4a anaphylatoxin des-Arg in the CSF of patients with MCI progressing to AD. C3a and C4a are part of the complement system implicated in the inflammatory processes of AD.12 β-Amyloid directly activates the complement cascade by binding to C1q, which can produce the anaphylactic peptides C3a, C4a, and C5a.13 C3a anaphylatoxin des-Arg has also been found to be upregulated in patients with AD in a previous study by our group (A.H.S., J.M., V.N.P., H.D., G.W., L.M., K.B., I. Skoog, MD, PhD, N. Andreasen, PhD, A. Wallin, MD, PhD, unpublished data, January 2006).

We also found increased levels of ubiquitin in the CSF of patients with MCI progressing to AD. This is in agreement with previous studies on AD using enzyme-linked immunosorbent assay methods, which found increased CSF levels of both free14 and conjugated15 ubiquitin. Ubiquitin is known to be involved in targeting proteins for degradation.16 In the brain, ubiquitin is covalently associated with the insoluble neurofibrillary material of neurofibrillary tangles and senile plaques.17

Furthermore, we described the discovery of a candidate cytokine-related biomarker increased in the CSF of patients with MCI progressing to AD, namely, a...
Figure 1. Scatter plots for the 8569-Da ubiquitin peak (A), a phosphorylated osteopontin C-terminal fragment (B), a 7944-Da peak (C), and an 8641-Da peak (D). MCI-MCI indicates patients with stable mild cognitive impairment; MCI-AD, patients with mild cognitive impairment who progressed to Alzheimer disease.

Figure 2. Representative spectra for a patient with stable mild cognitive impairment (MCI-MCI), a patient with MCI who progressed to Alzheimer disease (MCI-AD), and a healthy control for ubiquitin (highlighted in box).
phosphorylated C-terminal fragment of osteopontin. Osteopontin is a pleiotropic integrin-binding protein and proinflammatory cytokine with functions in cell-mediated immunity, inflammation, tissue repair, and cell survival. It has been identified as the most prominent cytokine-encoding gene expressed within multiple sclerosis lesions.\(^{18}\)

\(\beta_2\)-Microglobulin constitutes the small constant component of the class I major histocompatibility complex, and its presence in biological fluids represents the balance between membrane protein turnover and elimination.\(^{19}\) Partially folded \(\beta_2\)-microglobulin is a key intermediate in the generation of amyloid fibrils in vitro.\(^{20}\) \(\beta_2\)-Microglobulin has been found to be elevated in a previous SELDI study.\(^{21}\)

The CSF biomarkers \(\beta\)-amyloid\(_{42}\), total tau, and phosphorylated tau have shown high sensitivity for the identification of MCI,\(^{7}\) but we believe that the potential biomarkers could add further confidence to the diagnosis.

Using a proteomic approach, we have discovered a unique panel of proteins in CSF that may shed light into the pathophysiological processes of MCI and have the potential to identify the patients with MCI who will progress to AD. The panel of potential biomarkers found in this study needs to be confirmed in larger cohorts of clinical samples to establish their true diagnostic value. This panel has great potential for diagnosis and the selection of patients who will benefit from emerging disease-modifying treatments.

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Correspondence: Anja H. Simonsen, MSc, Ciphergen Biosystems, Symbion Suite 253, Fruebjergvej 3, 2100 Copenhagen O, Denmark (asimonsen@ciphergen.com).

Author Contributions: Dr Blennow had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Simonsen, McGuire, Hansson, Davies, Waldemar, Minthon, and Blennow. Acquisition of data: Simonsen, McGuire, and Hansson. Analysis and interpretation of data: Simonsen, McGuire, Zetterberg, Podust, Waldemar, and Blennow. Drafting of the manuscript: Simonsen. Critical revision of the manuscript for important intellectual content: Simonsen, McGuire, Hansson, Zetterberg, Podust, Davies, Waldemar, Minthon, and Blennow. Statistical analysis: Simonsen, McGuire, and Zetterberg. Obtained funding: Blennow. Administrative, technical, and material support: Simonsen, Zetterberg, Podust, and Minthon. Study supervision: Davies, Waldemar, and Blennow.

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Additional Information: The raw data will be available for potential collaborators interested in joint data mining.

REFERENCES


