Proteomic Changes in Cerebrospinal Fluid of Presymptomatic and Affected Persons Carrying Familial Alzheimer Disease Mutations

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Objective: To identify cerebrospinal fluid (CSF) protein changes in persons who will develop familial Alzheimer disease (FAD) due to PSEN1 and APP mutations, using unbiased proteomics.

Design: We compared proteomic profiles of CSF from individuals with FAD who were mutation carriers (MCs) and related noncarriers (NCs). Abundant proteins were depleted and samples were analyzed using liquid chromatography–electrospray ionization–mass spectrometry on a high-resolution time-of-flight instrument. Tryptic peptides were identified by tandem mass spectrometry. Proteins differing in concentration between the MCs and NCs were identified.

Setting: A tertiary dementia referral center and a proteomic biomarker discovery laboratory.

Participants: Fourteen FAD MCs (mean age, 34.2 years; 10 are asymptomatic, 12 have presenilin-1 [PSEN1] gene mutations, and 2 have amyloid precursor protein [APP] gene mutations) and 5 related NCs (mean age, 37.6 years).

Results: Fifty-six proteins were identified, represented by multiple tryptic peptides showing significant differences between MCs and NCs (46 upregulated and 10 downregulated); 40 of these proteins differed when the analysis was restricted to asymptomatic individuals. Fourteen proteins have been reported in prior proteomic studies in late-onset AD, including amyloid precursor protein, transferrin, αβ-glycoprotein, complement components, afamin precursor, spondin 1, plasminogen, hemopexin, and neuronal pentraxin receptor. Many other proteins were unique to our study, including calcsynin 3, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) 4 glutamate receptor, CD99 antigen, di-N-acetyl-chitobiase, and secreted phosphoprotein 1.

Conclusions: We found much overlap in CSF protein changes between individuals with presymptomatic and symptomatic FAD and those with late-onset AD. Our results are consistent with inflammation and synaptic loss early in FAD and suggest new presymptomatic biomarkers of potential usefulness in drug development.

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Alzheimer disease (AD) is a growing public health problem, but our ability to diagnose and treat it is inadequate. Although biomarkers for AD are known, there is no diagnostic procedure accurate enough to screen for presymptomatic individuals in the general population.1 The AD pathologic process is initiated long before overt dementia manifests. The associated biochemical changes, if better understood, could provide a basis for presymptomatic diagnosis, be outcome measures for disease-modifying interventions, or help to identify novel therapeutic targets.

Research on presymptomatic AD is hampered by difficulties in prospective identification of persons who will develop AD. Familial AD (FAD) due to mutations in the presenilin-1 (PSEN1 [NCBI Entrez Gene 5663]) or amyloid precursor protein (APP [NCBI Entrez Gene 351]) genes enables the study of biomarkers in asymptomatic persons in whom the development of AD can be predicted with essentially 100% certainty. The study of persons at risk for FAD therefore provides insight into biomarkers reflecting common elements of disease pathogenesis.

Cerebrospinal fluid (CSF) offers several advantages as a biological fluid for AD research. One of these advantages is that CSF is in direct continuity with the brain and therefore more directly reflects chemical changes occurring there. Cerebrospinal fluid has a narrower dynamic range of protein concentration than does blood, with fewer dominant high-abundance proteins complicating analysis of less abundant ones. Accordingly, the markers most diagnostic and predictive of AD diagno-
sis to date are measurements of tau and amyloid-β (Aβ) in CSF. Recent application of multiplexed antibody-based screens of proteins provides support for CSF biomarkers beyond tau and Aβ providing additional diagnostic information.3

Advancements in mass spectrometry (MS)–based technology have enabled the identification of proteins differentially expressed between populations. Such unbiased approaches permit the identification of novel molecular changes and have been used to study various illnesses, including AD. Such studies have focused on later stages of the disease, comparing control groups with individuals having a diagnosis of AD. Comparison of 10 or fewer people by 2-dimensional sodium dodecyl sulfate–polyacrylamide gels or surface-enhanced laser desorption/ionization MS found a few changes in AD.1,5 In a larger study, Finehout et al6 used 2-dimensional sodium dodecyl sulfate–polyacrylamide gel to identify 23 proteins that separated groups. More extensive profiling was achieved by Zhang et al,7 who used liquid chromatography–mass spectrometry (LC-MS) with isotope labeling in 17 patients with AD and 16 control participants. The investigators found changes in many proteins, including APP and complement components.

We performed proteomic analyses of CSF using high-resolution LC-MS. This label-free method relies on a novel validated approach to quantification of LC-MS data.8 Individual samples are neither mixed nor extensively manipulated while sample preparation and MS conditions are controlled, ensuring that changes in analyte intensities reflect their concentrations in one sample relative to another. Using this technique, we studied CSF from persons with or at risk for inheriting FAD to identify protein changes during the presymptomatic phase and in established disease.

METHODS

POPULATION

Participants were 19 persons consenting to lumbar puncture from a total of 41 who were taking part in a comprehensive study of FAD in a tertiary dementia referral center. These 19 people were from 10 families with or at risk for FAD due to PSEN1 (n=16) or APP (n=3) mutations (Table 1). The Clinical Dementia Rating scale (CDR)9 was administered by investigators blind to mutation status (except for persons with overt dementia). We compared participants who were mutation carriers (MCs) with those who were related noncarriers (NCs) as the control population. All study procedures were approved by the University of California, Los Angeles, Institutional Review Board, and all participants or their representatives gave informed consent.

Age at onset of disease in FAD tends to be consistent within, but can vary among, families.10,11 Therefore, to compare participants relative to the expected onset of clinical disease, their ages relative to the median age of disease diagnosis in their families were calculated (adjusted age).

Of the 19 participants, 14 were MCs and 5 were NCs (Table 1). The mean ages were 34.2 and 37.6 years, respectively. Mean adjusted age for MCs was 11 years younger than the family-specific age of dementia diagnosis. Among the 14 MCs, 10 were asymptomatic (CDR, 0), 2 were mildly symptomatic but did not have dementia (CDR, 0.5), 1 had mild dementia (CDR, 1; S212Y PSEN1 mutation), and 1 had moderate dementia (CDR, 2; A431E PSEN1 mutation). Eleven of the 14 MCs were women. Of the 10 asymptomatic MCs, 8 carried PSEN1 mutations (7 with A431E and 1 with L235V) and 2 carried APP mutations (V717I). Of the 2 MCs with CDR scores of 0.5, one carried the A431E mutation and the other carried the L235V PSEN1 mutation. Of the 5 NCs, 3 were women and 2 were men.

GENETIC TESTING

Extraction of DNA and genotyping of apolipoprotein E were performed using standard techniques. The presence of the A431E and L235V substitutions in PSEN1 were assessed using restriction fragment length polymorphism analyses. The participant carrying the S212Y PSEN1 mutation was evaluated using a commercial test (Athena Diagnostics) in which the open reading frame of the coding region of the PSEN1 gene was sequenced. The presence of the V717I substitution in APP was assessed with direct sequencing.

CSF ANALYSES

Cerebrospinal fluid was collected at various times of the day within the same week as clinical assessments. The fluid was aspirated and placed in polystyrene tubes on ice. Within 2 hours,

Table 1. Participant Characteristics and Mean CSF Levels of Aβ42, t-Tau, and p-Tau181

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NCs (n=5)</th>
<th>Symptomatic FAD MCs (n=4)</th>
<th>P Valuea</th>
<th>Presymptomatic FAD MCs (n=10)</th>
<th>P Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (range), y</strong></td>
<td>37.6 (29 to 55)</td>
<td>43.5 (37 to 55)</td>
<td>.11</td>
<td>30.5 (23 to 43)</td>
<td>.13</td>
</tr>
<tr>
<td><strong>Adjusted age (range), yc</strong></td>
<td>−5.8 (−18 to −18)</td>
<td>0.3 (−12 to 12)</td>
<td>.41</td>
<td>−15.5 (−22 to −5)</td>
<td>.13</td>
</tr>
<tr>
<td><strong>Female sex, No. (%)</strong></td>
<td>3 (60)</td>
<td>3 (75)</td>
<td>.60</td>
<td>8 (80)</td>
<td>.41</td>
</tr>
<tr>
<td><strong>Genetic alteration in family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSEN1 A431E (n=3)</td>
<td>PSEN1 A431E (n=2)</td>
<td>PSEN1 A431E (n=7)</td>
<td>PSEN1 A431E (n=7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP V717I (n=1)</td>
<td>PSEN1 L235V (n=1)</td>
<td>PSEN1 L235V (n=1)</td>
<td>APP V717I (n=2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CSF t-tau levels, mean (SD), pg/mL</strong></td>
<td>618.4 (100.1)</td>
<td>277.1 (162.9)</td>
<td>.03</td>
<td>387.6 (130.1)</td>
<td>.004</td>
</tr>
<tr>
<td><strong>CSF p-tau levels, mean (SD), pg/mL</strong></td>
<td>14.0 (69.9)</td>
<td>14.0 (69.9)</td>
<td>.02</td>
<td>14.3 (58.6)</td>
<td>.001</td>
</tr>
<tr>
<td><strong>CSF p-tau181 levels, mean (SD), pg/mL</strong></td>
<td>24.6 (9.2)</td>
<td>67.0 (29.0)</td>
<td>.03</td>
<td>71.2 (31.8)</td>
<td>.007</td>
</tr>
</tbody>
</table>

Abbreviations: Aβ, beta-amyloid; CSF, cerebrospinal fluid; FAD, familial Alzheimer disease; MCs, mutation carriers; NCs, noncarriers.

a P value for Mann-Whitney tests and Fisher exact tests comparing symptomatic MCs (Clinical Dementia Rating [CDR], >0) and all NCs.
b P value for Mann-Whitney tests and Fisher exact tests comparing presymptomatic (CDR, 0) MCs and NCs.
c Adjusted age indicates the ages of participants relative to the median age of dementia diagnosis within their family.
CSF was centrifuged and aliquotted into 0.5-mL siliconized polypropylene tubes. A protease inhibitor mixture containing aprotenin, 1 µg/mL, and sodium azide, 0.05%, was added, and the samples were centrifuged at 1200g for 15 minutes and frozen to −80°C. The CSF was analyzed for Aβ42, t-tau, and p-tau181 levels, using multiplex assays (INNO-BIA AlzBio3; Innogenetics) on standardized technology (xMAP; Luminex Corp). Centrifugation was repeated at 16 000g for 15 minutes at 4°C to remove debris after thawing. The CSF was then subjected to MS analysis for profiling and protein identification at a proteomic biomarker discovery laboratory. Abundant proteins were substantially depleted by an antibody-based affinity resin column (model MARS Hu-14; Agilent Technologies) to increase the effective dynamic range, the total number of tracked proteins, and accuracy of the measurements. The proteins depleted were albumin, IgG, IgA, IgM, transferrin, fibrinogen, apolipoprotein A-I, apolipoprotein A-II, haptoglobin, α1-antitrypsin, α1-acid glycoprotein, α2-macroglobulin, complement C3, and transthyretin.

The depleted samples were analyzed with a 2-dimensional liquid chromatographic separation approach (DeepLook analysis); Biomarker Discovery Sciences, PPD, Inc). Proteins were denatured, disulfide bonds were reduced, and sulfhydryl groups were carboxymethylated before digestion by modified trypsin. Four fractions of peptides per sample were obtained by off-line (fraction collected) strong cation-exchange chromatography. The tryptic peptides were then profiled. Individual molecules were tracked across samples, and their differential expression was quantified by liquid chromatography–electrospray ionization mass spectrometry on a high-resolution (R > 5000, where R indicates resolving power, per the International Union of Pure and Applied Chemistry) time-of-flight (TOF) instrument with an online reverse-phase capillary liquid chromatography using a 1-hour solvent gradient of increasing acetonitrile in an aqueous solution with formic acid, 0.1%.

IDENTIFICATION OF PEPTIDES AND PROTEINS

For peptide identification, MS-MS spectra were acquired under identical chromatographic conditions as for the profiling, using a linear ion-trap mass spectrometer (model LTQ; Thermo Scientific). Peptide identifications for MS-MS spectra were made, and a high-confidence protein list was created from them (ByOnic and ConByne programs; Palo Alto Research Center). The ByOnic identification searches were performed using a decoy database in which equal numbers of proteins were included but their sequences were reversed. The appearance of decoy sequences among the identifications was used to determine an objective false discovery rate and retain only high-quality identifications. Ions observed in the 2-dimensional LC-MS analysis of the individual samples were linked to MS-MS identifications, using accurate mass and chromatographic retention time, in a computation that included postacquisition nonparametric mass calibration. A differential quantification method was used to compare peptide and protein levels among samples, as detailed by Wang et al,11 which relies on the changes in analyte intensities directly reflecting their concentrations in one sample relative to another.

STATISTICAL METHODS

We performed statistical analyses, comparing MCs with NCs for each tryptic peptide’s molecular ion tracked by MS, based on t tests or Wilcoxon rank sum 2-sample statistics depending on the outcome of a Shapiro-Wilk test for normality. Two analyses were performed for each ion: analysis 1 compared all MCs with NCs and analysis 2 compared all asymptomatic (CDR, 0) MCs with NCs. These analyses were performed in R, version 2.7.2 (http://www.r-project.org), and later. No corrections for multiple comparisons were made.

Two protein lists were generated from these statistical comparisons using heuristic protein-level assessment based on tryptic peptides. For a protein to be included in a list, there had to be more than 1 peptide showing a statistically significant change (P < .05), substantial agreement between peptides regarding the direction of change, at least 8% of the tracked molecular ions for a given protein had to reach statistical significance, and at least 2 molecular ions with P < .05.

Cerebrospinal fluid levels of Aβ42, t-tau, and p-tau181 were compared between groups by Mann-Whitney tests using commercial software (PASW 18.0 IBM SPSS).

PATHWAY ANALYSIS

We performed pathway analysis in which networks, canonical pathways, and functional processes for the proteins were identified from analysis 1 (Ingenuity Pathways Analysis; Ingenuity Systems).

RESULTS

TARGETED ASSAYS OF Aβ42, t-TAU, AND p-TAU181

Mean CSF Aβ42 levels were lower and t-tau and p-tau181 levels were higher among symptomatic and presymptomatic MCs relative to NCs (Table 1).

PROTEOMICS RESULTS

A total of 49 261 molecular ions were tracked and quantified. Median coefficients of variation (CVs) within the MC and NC groups were 21% to 22%. A total of 16 940 ions were linked to 7407 peptide sequences corresponding to 600 proteins, 585 of which had 2 or more peptides reported. Although there were not many ions with a low P value relative to the number of ions tracked, the consistent results between multiple peptides representing these proteins revealed clear changes in many. From analysis 1, a condensed list of 56 proteins with consistent evidence of differences in concentration between all MCs and NCs was produced (Table 2). Although most of the proteins were novel, one-fourth (14 of 56) have been shown to have differential levels in patients with AD. These include APP, transferrin, α1β-glycoprotein, complement components, afamin precursor, spondin 1, and plasminogen.

Twelve of the 14 proteins have shown changes in the same direction in at least 2 of these studies. In analysis 2, only presymptomatic MCs were compared with those who were NCs. Among the 56 proteins from the list from analysis 1, 41 were also found to be different comparing presymptomatic MCs and NCs, and 38 were not selected in the condensed list for analysis 1 (Table 3). At least 2 of these, neuronal pentraxin receptor and hemopexin, have been shown to be elevated in the CSF of patients with AD.

PATHWAY ANALYSIS

A pathway analysis was performed on the 56-protein list generated from analysis 1. The first level examined the functions linked to these proteins in the literature. Many
### Table 2. The List of 56 Proteins Different Between FAD MCs and NCs in the CSF 2D Proteomic Study

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein Name</th>
<th>Trend</th>
<th>Fold Change</th>
<th>Effect Size</th>
<th>Changed in Presymptomatic Participants?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP_001124.1</td>
<td>Afamin precursor</td>
<td>Up</td>
<td>1.30</td>
<td>0.85</td>
<td>+</td>
</tr>
<tr>
<td>NP_56971.2</td>
<td>α₁I-Types collagen isoform 3 precursor</td>
<td>Down</td>
<td>−1.13</td>
<td>−1.38</td>
<td>+</td>
</tr>
<tr>
<td>NP_57060.2</td>
<td>α₂G-Glycoprotein precursor</td>
<td>Up</td>
<td>1.35</td>
<td>1.07</td>
<td>+</td>
</tr>
<tr>
<td>NP_00005.2</td>
<td>α₂-Macroglobulin precursor</td>
<td>Up</td>
<td>1.23</td>
<td>1.28</td>
<td>−</td>
</tr>
<tr>
<td>NP_00135750.1</td>
<td>Amyloid β(A4) precursorlike protein 2 isoform 4</td>
<td>Up</td>
<td>1.19</td>
<td>0.85</td>
<td>−</td>
</tr>
<tr>
<td>NP_00129603.1</td>
<td>Amyloid β A4 protein isoform g</td>
<td>Up</td>
<td>1.23</td>
<td>1.34</td>
<td>+</td>
</tr>
<tr>
<td>NP_001636.1</td>
<td>Apolipoprotein C-I precursor</td>
<td>Up</td>
<td>1.52</td>
<td>1.11</td>
<td>−</td>
</tr>
<tr>
<td>NP_000051.1</td>
<td>Biotinidase precursor</td>
<td>Up</td>
<td>1.59</td>
<td>1.24</td>
<td>+</td>
</tr>
<tr>
<td>NP_055207.1</td>
<td>Brain neuron cytoplasmic protein 1</td>
<td>Down</td>
<td>−1.34</td>
<td>−1.76</td>
<td>+</td>
</tr>
<tr>
<td>NP_055332.2</td>
<td>Calcytine 3</td>
<td>Down</td>
<td>−1.32</td>
<td>−0.91</td>
<td>−</td>
</tr>
<tr>
<td>NP_066203.1</td>
<td>Cathepsin L1 preproprotein</td>
<td>Up</td>
<td>1.35</td>
<td>1.17</td>
<td>−</td>
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<tr>
<td>NP_001116370.1</td>
<td>CD99 antigen isoform b precursor</td>
<td>Up</td>
<td>1.35</td>
<td>0.99</td>
<td>+</td>
</tr>
<tr>
<td>NP_000087.1</td>
<td>Ceruloplasmin precursor</td>
<td>Up</td>
<td>1.41</td>
<td>0.98</td>
<td>+</td>
</tr>
<tr>
<td>NP_004379.1</td>
<td>Di-α-acetyloyl-chitobiase</td>
<td>Up</td>
<td>1.30</td>
<td>1.12</td>
<td>+</td>
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<tr>
<td>NP_00139375.1</td>
<td>Complement component 2 isoform 2 preproprotein</td>
<td>Up</td>
<td>1.49</td>
<td>1.48</td>
<td>+</td>
</tr>
<tr>
<td>NP_009224.2</td>
<td>Complement component 4A preproprotein</td>
<td>Up</td>
<td>1.32</td>
<td>0.98</td>
<td>−</td>
</tr>
<tr>
<td>NP_001002093.3</td>
<td>Complement component 4B preproprotein</td>
<td>Up</td>
<td>1.56</td>
<td>0.97</td>
<td>+</td>
</tr>
<tr>
<td>NP_001726.2</td>
<td>Complement component 5 preproprotein</td>
<td>Up</td>
<td>1.51</td>
<td>1.03</td>
<td>+</td>
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<tr>
<td>NP_000056.2</td>
<td>Complement component 6 preproprotein</td>
<td>Up</td>
<td>1.36</td>
<td>1.00</td>
<td>+</td>
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<tr>
<td>NP_000057.1</td>
<td>Complement component 8, β polypeptide preproprotein</td>
<td>Up</td>
<td>1.55</td>
<td>1.34</td>
<td>+</td>
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<tr>
<td>NP_001701.2</td>
<td>Complement factor B preproprotein</td>
<td>Up</td>
<td>1.40</td>
<td>1.28</td>
<td>+</td>
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<tr>
<td>NP_001966.1</td>
<td>Enolase 2</td>
<td>Up</td>
<td>1.28</td>
<td>1.35</td>
<td>+</td>
</tr>
<tr>
<td>NP_001121089.1</td>
<td>Fructose-bisphosphate aldolase A</td>
<td>Up</td>
<td>1.39</td>
<td>1.19</td>
<td>+</td>
</tr>
<tr>
<td>NP_001106283.1</td>
<td>Glutamate receptor, ionotrophic, AMPA 4</td>
<td>Up</td>
<td>1.27</td>
<td>0.78</td>
<td>+</td>
</tr>
<tr>
<td>NP_001138648.1</td>
<td>Hypothetical protein LOC729956 (Homo sapiens)</td>
<td>Down</td>
<td>−1.18</td>
<td>−1.05</td>
<td>−</td>
</tr>
<tr>
<td>NP_002169.1</td>
<td>Insulinlike growth factor binding protein 6</td>
<td>Down</td>
<td>−1.55</td>
<td>−1.99</td>
<td>+</td>
</tr>
<tr>
<td>NP_002206.2</td>
<td>Inter-α (globulin) inhibitor H1</td>
<td>Up</td>
<td>1.64</td>
<td>1.10</td>
<td>+</td>
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<tr>
<td>NP_001137435.1</td>
<td>L1 cell adhesion molecule isoform 3 precursor</td>
<td>Up</td>
<td>1.16</td>
<td>1.00</td>
<td>+</td>
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<tr>
<td>NP_20823.3</td>
<td>Laminin, β2 precursor</td>
<td>Up</td>
<td>1.90</td>
<td>0.87</td>
<td>−</td>
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<tr>
<td>NP_000419.1</td>
<td>Latent transforming growth factor β-binding protein 2</td>
<td>Up</td>
<td>1.26</td>
<td>0.82</td>
<td>−</td>
</tr>
<tr>
<td>NP_443204.1</td>
<td>Leucine-rich α2-glycoprotein 1</td>
<td>Up</td>
<td>1.35</td>
<td>0.99</td>
<td>+</td>
</tr>
<tr>
<td>NP_006491.2</td>
<td>Melanoma cell adhesion molecule</td>
<td>Down</td>
<td>−1.19</td>
<td>−1.12</td>
<td>+</td>
</tr>
<tr>
<td>NP_002490.2</td>
<td>Neogenin homologue 1</td>
<td>Up</td>
<td>1.29</td>
<td>1.39</td>
<td>−</td>
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<tr>
<td>NP_007669.2</td>
<td>Neuronal growth regulator 1</td>
<td>Up</td>
<td>1.16</td>
<td>0.85</td>
<td>+</td>
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<td>NP_148935.1</td>
<td>Osteoglycin preproprotein</td>
<td>Down</td>
<td>−1.14</td>
<td>−1.08</td>
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<td>NP_443122.3</td>
<td>Peptidoglycan recognition protein 2 precursor</td>
<td>Up</td>
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<td>1.02</td>
<td>+</td>
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<tr>
<td>NP_000910.2</td>
<td>Peptidyglycine α-amidating monoxygenase</td>
<td>Up</td>
<td>1.34</td>
<td>1.11</td>
<td>−</td>
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<tr>
<td>NP_000292.1</td>
<td>Plasminogen</td>
<td>Up</td>
<td>1.50</td>
<td>1.18</td>
<td>+</td>
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<tr>
<td>NP_002846.3</td>
<td>Poliovirus receptor–related 1 isoform 1</td>
<td>Up</td>
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<td>1.32</td>
<td>+</td>
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<tr>
<td>NP_005304.3</td>
<td>Protein disulfide-isomerase A3 precursor</td>
<td>Down</td>
<td>−1.37</td>
<td>−1.38</td>
<td>+</td>
</tr>
<tr>
<td>NP_001035802.1</td>
<td>Protein tyrosine phosphatase, receptor type, D isoform 5 precursor</td>
<td>Down</td>
<td>−1.21</td>
<td>−1.20</td>
<td>+</td>
</tr>
<tr>
<td>NP_002837.1</td>
<td>Protein tyrosine phosphatase, receptor type, N precursor</td>
<td>Up</td>
<td>1.27</td>
<td>1.05</td>
<td>+</td>
</tr>
<tr>
<td>NP_003380.1</td>
<td>Protein-1-isoaspartate (D-aspartate)</td>
<td>Up</td>
<td>1.31</td>
<td>0.99</td>
<td>−</td>
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<tr>
<td>NP_000087.1</td>
<td>Protein-α-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<td>1.11</td>
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<td>NP_002846.3</td>
<td>Poliovirus receptor–related 1</td>
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<td>1.23</td>
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<td>+</td>
</tr>
<tr>
<td>NP_002846.3</td>
<td>Poliovirus receptor–related 1 isoform 1</td>
<td>Up</td>
<td>1.23</td>
<td>1.32</td>
<td>+</td>
</tr>
<tr>
<td>NP_005304.3</td>
<td>Protein disulfide-isomerase A3 precursor</td>
<td>Down</td>
<td>−1.37</td>
<td>−1.38</td>
<td>+</td>
</tr>
<tr>
<td>NP_001035802.1</td>
<td>Protein tyrosine phosphatase, receptor type, D isoform 5 precursor</td>
<td>Down</td>
<td>−1.21</td>
<td>−1.20</td>
<td>+</td>
</tr>
<tr>
<td>NP_002837.1</td>
<td>Protein tyrosine phosphatase, receptor type, N precursor</td>
<td>Up</td>
<td>1.27</td>
<td>1.05</td>
<td>+</td>
</tr>
<tr>
<td>NP_003380.1</td>
<td>Protein-1-isoaspartate (D-aspartate)</td>
<td>Up</td>
<td>1.31</td>
<td>0.99</td>
<td>−</td>
</tr>
<tr>
<td>NP_000087.1</td>
<td>Protein-α-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
<td>Up</td>
<td>1.31</td>
<td>1.11</td>
<td>−</td>
</tr>
</tbody>
</table>

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CSF, cerebrospinal fluid; 2D, 2-dimensional; NCs, noncarriers; +, change in presymptomatic (Clinical Dementia Rating [CDR] score, 0) familial Alzheimer disease (FAD) mutation carriers (MCs) as well as all the group of all MCs; −, no change in presymptomatic FAD MCs.

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proteins are involved in cellular-mediated immune response and cell signaling functions and have been associated with inflammatory and neurologic diseases. The next level of analysis investigated whether functional cellular networks in the literature could be identified that link the proteins. The more nodes of a particular network that are represented by candidate proteins, the more likely this network is relevant to the biological question being investigated. Our analysis revealed 3 networks, with the first 2 having substantial numbers of proteins identified from the present study. Network 1 is related to antigen presentation, cell-mediated immune response, and humoral immune response (Figure 1). This network contains 29 proteins, 25 of which were upregulated and 4 of which were downregulated in MCs. Network 2 is associated with metabolic disease, renal disease, urologic disease, and molecular transport and contains 15 proteins (12 upregulated and 3 downregulated in MCs) (Figure 2).

In this CSF proteomic study of persons inheriting FAD mutations, most of whom were presymptomatic, we identified known and novel candidate biomarkers. Differences were found in 56 CSF proteins between the MC and NC cohorts based on univariate analysis. The fold changes among the 56 proteins were relatively small, ranging from 1.18 to 1.90 (mean, 1.33). The differences in CSF Aβ42, t-tau, and p-tau181 found in MCs were large and consistent with reported fold changes for these traditional markers in persons with mild cognitive impairment and AD of later onset. In this study, the changes were detected 10 or more years before diagnosis. Many more of the 56 proteins are upregulated (n=46) than downregulated (n=10) in MCs. The finding of both upregulated and downregulated proteins suggests that our results are not due to nonspecific brain degeneration.

### Table 3. The List of 38 Proteins Different Between Presymptomatic (CDR, 0) FAD MC and NC Cohorts Not Found When Affected MCs Were Included

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein Name</th>
<th>Trend</th>
<th>Fold Change</th>
<th>Effect Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP_002070.1</td>
<td>Aspartate aminotransferase 1</td>
<td>Up</td>
<td>1.33</td>
<td>1.79</td>
</tr>
<tr>
<td>NP_002071.2</td>
<td>Aspartate aminotransferase 2 precursor</td>
<td>Up</td>
<td>1.24</td>
<td>1.83</td>
</tr>
<tr>
<td>NP_000713.2</td>
<td>Calcium channel, voltage-dependent, α2/delta subunit 1</td>
<td>Up</td>
<td>1.30</td>
<td>1.30</td>
</tr>
<tr>
<td>NP_002405.1</td>
<td>CD99 antigen isofrom a precursor</td>
<td>Up</td>
<td>1.36</td>
<td>1.32</td>
</tr>
<tr>
<td>NP_054660.1</td>
<td>Cell recognition molecule Caspr2 precursor</td>
<td>Up</td>
<td>1.29</td>
<td>0.96</td>
</tr>
<tr>
<td>NP_004377.2</td>
<td>Chondroitin sulfate proteoglycan 5</td>
<td>Up</td>
<td>1.26</td>
<td>1.39</td>
</tr>
<tr>
<td>NP_000497.1</td>
<td>Coagulation factor II preproprotein</td>
<td>Up</td>
<td>1.33</td>
<td>1.05</td>
</tr>
<tr>
<td>NP_000542.1</td>
<td>Complement component 2 isofrom 1 preproprotein</td>
<td>Up</td>
<td>1.41</td>
<td>1.47</td>
</tr>
<tr>
<td>NP_000552.1</td>
<td>Complement component 3 preproprotein</td>
<td>Up</td>
<td>1.33</td>
<td>1.08</td>
</tr>
<tr>
<td>NP_733746.1</td>
<td>Cytosolic sialic acid 9-O-acetyleraserase homologue</td>
<td>Up</td>
<td>1.39</td>
<td>1.35</td>
</tr>
<tr>
<td>NP_002014.2</td>
<td>Fibromodulin precursor</td>
<td>Down</td>
<td>−1.60</td>
<td>−1.63</td>
</tr>
<tr>
<td>NP_001987.2</td>
<td>Fibulin 1 isofrom C preproprotein</td>
<td>Down</td>
<td>−1.35</td>
<td>−1.30</td>
</tr>
<tr>
<td>NP_000604.1</td>
<td>Hemopexin</td>
<td>Up</td>
<td>1.42</td>
<td>1.09</td>
</tr>
<tr>
<td>NP_000590.1</td>
<td>Insulinlike growth factor binding protein 5</td>
<td>Up</td>
<td>1.28</td>
<td>1.07</td>
</tr>
<tr>
<td>NP_001001.851.1</td>
<td>Inter-α trypsin inhibitor heavy chain precursor 5 isofrom 3</td>
<td>Up</td>
<td>1.39</td>
<td>1.08</td>
</tr>
<tr>
<td>NP_001095886.1</td>
<td>Kininogen 1 isofrom 1</td>
<td>Up</td>
<td>1.48</td>
<td>1.05</td>
</tr>
<tr>
<td>NP_01008701.1</td>
<td>Latrophilin 1 isofrom 1 preproprotein</td>
<td>Down</td>
<td>−1.17</td>
<td>−1.46</td>
</tr>
<tr>
<td>NP_005568.2</td>
<td>Liprotein Lp(a) preprotein</td>
<td>Up</td>
<td>1.65</td>
<td>1.17</td>
</tr>
<tr>
<td>NP_079032.2</td>
<td>Multimerin 2 preproprotein</td>
<td>Up</td>
<td>1.34</td>
<td>1.49</td>
</tr>
<tr>
<td>NP_002505.1</td>
<td>Nephroblastoma overexpressed precursor</td>
<td>Up</td>
<td>1.60</td>
<td>1.41</td>
</tr>
<tr>
<td>NP_620060.1</td>
<td>Neurexin 2 isofrom α2 preproprotein</td>
<td>Down</td>
<td>−1.21</td>
<td>−1.31</td>
</tr>
<tr>
<td>NP_877421.2</td>
<td>Neuroblastoma, suppression of tumorigenicity 1 isofrom 1</td>
<td>Down</td>
<td>−1.27</td>
<td>−1.61</td>
</tr>
<tr>
<td>NP_002514.1</td>
<td>Neuronal pentraxin II</td>
<td>Up</td>
<td>1.27</td>
<td>1.07</td>
</tr>
<tr>
<td>NP_055108.2</td>
<td>Neuronal pentraxin receptor</td>
<td>Up</td>
<td>1.26</td>
<td>1.21</td>
</tr>
<tr>
<td>NP_00116224.1</td>
<td>Neuroserpin precursor</td>
<td>Up</td>
<td>1.18</td>
<td>1.18</td>
</tr>
<tr>
<td>NP_775733.3</td>
<td>Paplin</td>
<td>Up</td>
<td>1.26</td>
<td>1.80</td>
</tr>
<tr>
<td>NP_000883.2</td>
<td>Plasma kalikrein B1 preproprotein</td>
<td>Up</td>
<td>1.38</td>
<td>1.21</td>
</tr>
<tr>
<td>NP_065138.2</td>
<td>Plexin domain containing 1 preproprotein</td>
<td>Up</td>
<td>1.37</td>
<td>1.03</td>
</tr>
<tr>
<td>XP_001715435.1</td>
<td>PREDICTED: hypothetical protein</td>
<td>Up</td>
<td>1.36</td>
<td>1.20</td>
</tr>
<tr>
<td>NP_001035519.1</td>
<td>Protocadherin 17 preproprotein (Homo sapiens)</td>
<td>Up</td>
<td>1.22</td>
<td>1.19</td>
</tr>
<tr>
<td>Q9Y4C0.4</td>
<td>Neuroxin-3-α</td>
<td>Up</td>
<td>1.41</td>
<td>1.13</td>
</tr>
<tr>
<td>NP_006206.2</td>
<td>Serine (or cysteine) proteinase inhibitor, clade A, member 4</td>
<td>Up</td>
<td>1.40</td>
<td>1.62</td>
</tr>
<tr>
<td>NP_443142.1</td>
<td>Sirt and trk-like 1 protein</td>
<td>Down</td>
<td>−1.24</td>
<td>−1.16</td>
</tr>
<tr>
<td>NP_003246.1</td>
<td>TIMP metalloproteinase inhibitor 2 preproprotein</td>
<td>Down</td>
<td>−1.14</td>
<td>−1.46</td>
</tr>
<tr>
<td>NP_006455.2</td>
<td>trans-Golgi network protein 2</td>
<td>Down</td>
<td>−1.23</td>
<td>−1.58</td>
</tr>
<tr>
<td>NP_001129174.1</td>
<td>Tyrosine 3/tryptophan 5-monooxygenase activation protein, zeta polypeptide</td>
<td>Up</td>
<td>1.34</td>
<td>1.51</td>
</tr>
<tr>
<td>NP_061828.1</td>
<td>Ubiquitin B preproprotein</td>
<td>Up</td>
<td>1.25</td>
<td>1.40</td>
</tr>
<tr>
<td>NP_006867.1</td>
<td>UDP-GlcNAcβ:Gal β-1,3-N-acetylgalcosaminyltransferase 1</td>
<td>Up</td>
<td>1.19</td>
<td>1.18</td>
</tr>
</tbody>
</table>

Abbreviations: CDR, Clinical Dementia Rating; FAD, familial Alzheimer disease; MC, mutation carrier; NC, noncarrier; TIMP, tissue metalloproteinase inhibitor; UDP, uridine diphosphate.

*Direction of change (trend), magnitude of change, and effect size are indicated.*
Figure 1. Network 1 (antigen presentation, cell-mediated immune response, and humoral immune response) generated by pathway analysis of the hot list. This network is displayed as nodes (genes or gene products) and edges. “Acts on” and “inhibits” edges may also include a binding event. Lines indicate biological relationships between nodes. Blue proteins are downregulated in individuals who are mutation carriers, and red proteins are upregulated. Uncolored proteins were not found to be different in our study but are linked by pathway analyses. Fx indicates function.
Fourteen proteins found to be differentially expressed in previous studies were also seen in our study, indicating that identifiable molecular changes occur 10 years before diagnosis. Transthyretin, albumin, and apolipoprotein A1 may also change prior to the appearance of symptoms but were among the abundant proteins depleted from our samples. There was sufficient residual 2-macroglobulin and complement component 3 after depletion to identify it as differentially expressed, consistent with previous findings. Multiple complement components were elevated, consistent with previous studies indicating an early role of inflammation in AD. Some of the proteins showing the most robust differences between MCs and NCs (CD99 antigen isoform b, di-N-acetyl chitobiase, and secreted phosphoprotein 1 isoform b) were unique, and their relationship to AD pathogenesis is unclear.

Numerous peptides derived from APP were detected and quantified. The median concentration change observed in these peptides is 23% upward for the MC cohort. The normal functions of APP are not clear but may be related to neurite growth, synaptogenesis, and synaptic plasticity. The APP peptides from the soluble extracellular region were consistently elevated. Our finding of elevated CSF APP before diagnosis in the study participants with FAD mutations is consistent with models in which Aβ42 aggregates induce a positive feedback cascade that elevates APP production through BACE (beta-site APP cleaving enzyme) activity. Both the current and previously reported data in the same population determined with use of immunoassays found reduced levels of Aβ42 in the CSF of the MCs, a finding thought to represent selective deposition of Aβ42 in the brain. The short Aβ peptides were likely removed in the proteomic processing used in this study, which uses a 5-kDa filter cutoff prior to tryptic digestion of these proteins.

Secreted phosphoprotein 1 (ie, osteopontin), a cytokine expressed by activated T cells, was elevated in the CSF of MCs. Osteopontin has been found to be elevated in the pyramidal cells in the brains of persons with AD and in transgenic mice; in addition, there is a single report of it being elevated in the CSF of persons with AD. Osteopontin is also elevated in multiple sclerosis, where it might serve as a therapeutic target. A similar role might be considered in AD.
Superoxide dismutase 3 was decreased in the CSF of the FAD MC group. The activity of superoxide dismutase has been found to be decreased in the CSF of persons with AD. Diminished superoxide dismutase activity could contribute to oxidative stress and therefore propagate disease pathologic characteristics. However, consistent with a proteomic study of CSF in postmortem AD cases, we found elevated levels of hemopexin, a protein that binds heme, in presymptomatic MCs, thus preventing its pro-oxidant and proinflammatory effects.

We also observed changes in several proteins related to excitatory synapses. Neuronal pentraxin 2, neuronal pentraxin receptor, and the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) 4 glutamate receptor were found to be elevated in presymptomatic MCs. Neuronal pentraxin 2 is a secreted protein homologous to acute-phase reactants and therefore an indicator of inflammation. Neuronal pentraxin receptor was found to be elevated in proteomic studies of CSF in patients with AD but NP1 and neuronal pentraxin receptor were decreased in another such study. Neuronal pentraxin receptor is a transmembrane protein expressed on neurons and glia that binds AMPA receptors and plays a role in synaptogenesis. Elevation of these proteins in CSF could represent synapse turnover or loss occurring early in the course of the disease. Calsyntenin 3, a postsynaptic membrane protein with putative Ca2+-binding capacity associated with γ-aminobutyric acid–ergic neurons, was decreased in the CSF of MCs in our study. The calstytins have been linked to APP transport.

A potential criticism of this study is the apparent lack of control for multiple comparisons. In the present study, P values were computed at the ion levels, whereas the tables are protein-level summaries of peptide results and consistent changes across peptides representing a single protein were required to make the list. Using the current methods in which hundreds of peptides were profiled, no false-positives were found at the protein level in an anonymized comparison of proteomics groups in which identical samples were spiked with differing levels of 12 proteins.

There are many advantages to the study of persons with FAD mutations. Discovery of biomarkers for late-onset neurodegenerative diseases that develop over decades is confounded by the fact that disease-associated markers may also change with normal aging. In addition, some healthy control participants in their 70s and 80s may be presymptomatic for neurodegenerative diseases diagnosed after the study. Another advantage of studying persons at risk for FAD is that the population is more homogeneous, enabling smaller cohort sizes, and that control participants and those who are presymptomatic carriers are younger, with fewer age-dependent neurologic and somatic changes. There are, however, potential limitations in the generalizability of findings in FAD to late-onset AD. It is thought that the pathogenesis of FAD is driven by a relative overproduction of Aβ42, whereas late-onset AD may be due to decreased degradation of Aβ. A previous investigation in this population revealed a unique pattern of APP-derived peptides in carriers of the A431E PSEN1 mutation that was not present in CSF from persons with late-onset AD.

In summary, although the statistical power of the present study was limited by small numbers, many novel proteomic differences were found in addition to others consistent with prior studies. We identified candidate biomarkers associated with mutation status that suggest changes occurring a decade before clinical dementia, including increases in APP, increases in inflammatory markers, and changes in proteins related to synaptic plasticity. These measures may be useful in diagnosis, patient stratification, and monitoring of response to therapy. Replication in a larger FAD population, longitudinal analysis of the same individuals, and comparison with changes in mild cognitive impairment and AD of late onset would provide further validation of our findings.

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