ORIGINAL ARTICLE

Association of Plasma Clusterin Concentration With Severity, Pathology, and Progression in Alzheimer Disease

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Context: Blood-based analytes may be indicators of pathological processes in Alzheimer disease (AD).

Objective: To identify plasma proteins associated with AD pathology using a combined proteomic and neuroimaging approach.

Design: Discovery-phase proteomics to identify plasma proteins associated with correlates of AD pathology. Confirmation and validation using immunodetection in a replication set and an animal model.

Setting: A multicenter European study (AddNeuroMed) and the Baltimore Longitudinal Study of Aging.

Participants: Patients with AD, subjects with mild cognitive impairment, and healthy controls with standardized clinical assessments and structural neuroimaging.

Main Outcome Measures: Association of plasma proteins with brain atrophy, disease severity, and rate of clinical progression. Extension studies in humans and transgenic mice tested the association between plasma proteins and brain amyloid.

Results: Clusterin/apolipoprotein J was associated with atrophy of the entorhinal cortex, baseline disease severity, and rapid clinical progression in AD. Increased plasma concentration of clusterin was predictive of greater fibrillar amyloid-β burden in the medial temporal lobe. Subjects with AD had increased clusterin messenger RNA in blood, but there was no effect of single-nucleotide polymorphisms in the gene encoding clusterin with gene or protein expression. *APP/PS1* transgenic mice showed increased plasma clusterin, age-dependent increase in brain clusterin, as well as amyloid and clusterin colocalization in plaques.

Conclusions: These results demonstrate an important role of clusterin in the pathogenesis of AD and suggest that alterations in amyloid chaperone proteins may be a biologically relevant peripheral signature of AD.

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PERIPHERAL COMPARTMENTS including blood and cerebrospinal fluid exhibit signals reflecting neuropathological changes in Alzheimer disease (AD).[1,2] In cerebrospinal fluid, these include a decrease in amyloid-β peptide (Aβ) and an increase in total and phosphorylated tau concentrations,[3] reflecting amyloid sequestration as plaques and neurofibrillary degeneration, respectively.[4,5] Similarly, while numerous articles suggest that plasma concentrations of several metabolites and proteins might represent responses to neuropathological changes in AD,[6-11] these findings have not been conclusively replicated.[12] A limitation of such studies may be their reliance upon demonstrating changes between affected and unaffected people, a design of study that might identify secondary changes lacking relevance to core disease biology.

Advances in methods such as proteomics present a further challenge in case-control studies, often generating data showing numerous analytes differentially expressed in AD patients. However, validating these results with alternative methods in independent patient

populations has been difficult.[13,14] These studies also ignore the clinical heterogeneity in disease progression in AD, wherein some patients show rapid cognitive decline, while others remain relatively stable and/or progress slowly.[15,16]

We applied mass spectrometry–based proteomics to discover plasma proteins associated with disease, using brain atrophy in AD as well as rapid clinical progression, rather than binary distinction between case and control. As a proxy measure of in vivo pathology, we used structural neuroimaging of atrophy in the hippocampus and entorhinal cortex (ERC), 2 components of the medial temporal lobe (MTL) that show early pathological changes in AD.[17] For rate of clinical progression, we used both retrospective and prospective measures of cognitive decline. We initially performed 2 independent discovery-phase studies using proteomic analysis of plasma in separate groups of subjects. In the first, we sought proteins that reflect hippocampal atrophy in mild cognitive impairment (MCI) and established AD. In the second, we identified proteins differentially expressed in rapidly progressing AD patients relative to those with a less aggressive disease course. Our aim was to identify plasma proteins common to both paradigms, followed by replication using quantitative immunoassays such as enzyme-linked immunosorbent assay (ELISA) in a large independent cohort of AD, MCI, and control subjects. **Figure 1** illustrates the design of this study.

METHODS

SUBJECTS AND SAMPLES

We used samples from 2 studies: the Alzheimer Research Trust–funded cohort at King's College London (KCL-ART)[7] and the AddNeuroMed study.[18] The KCL-ART study, which began in 2001, includes a cohort of people with AD and MCI[19] and healthy elderly individuals. All subjects are white UK citizens with grandparents born in the United Kingdom and are assessed annually. AddNeuroMed is a cross-European cohort; AD cases are assessed at 3-month intervals in the first year and annually thereafter; MCI and control groups are assessed annually. All subjects are white Europeans recruited from 6 centers in the United Kingdom, France, Italy, Finland, Poland, and Greece. Standardized assessments include demographic and medical information; cognitive assessment, including the Mini-Mental State Examination (MMSE) (both studies; all subjects), Alzheimer Disease Assessment Scale–cognitive subscale (ADAS-cog) (AddNeuroMed only), and Consortium to Establish a Registry for Alzheimer's Disease battery; and scales to assess function, behavior, and global levels of severity, including the Clinical Dementia Rating. Cases with probable AD (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [NINCDS-ADRDA] criteria) and amnestic MCI were identified as previously described[7] and evaluated with a standardized assessment shown to have high diagnostic validity.[20] Cases with amnestic MCI were defined as having subjective memory complaints, Clinical Dementia Rating scores of less than 1, and evidence of objective memory impairment using the Consortium to Establish a Registry for Alzheimer's Disease delayed word list recall (–1.5-SD cutoff). Normal elderly controls, defined as having no evidence of cognitive impairment (MMSE score >28), were recruited systematically from primary care patient lists in the KCL-ART study and from both primary care services and elsewhere in the AddNeuroMed study. Blood samples were collected and stored as previously described.[7,18] In total, we studied 95 and 689 subjects in discovery and validation studies, respectively, with an additional 60 subjects from the Baltimore Longitudinal Study of Aging (eTables 1-4, available at http://www.archgenpsychiatry.com).[21] Ethical approval was obtained in each of the participating countries.

NEUROIMAGING

Magnetic Resonance Imaging Data Acquisition

In the KCL-ART study, whole-brain coronal 3-dimensional spoiled-gradient recalled images (repetition time=14 milliseconds, echo time=3 milliseconds, $256\times192\times124$ acquisition matrix, 1.5-mm slices) were obtained on a GE Signa 1.5-T neuro-optimized magnetic resonance system. In the AddNeuroMed study, whole-brain sagittal 3-dimensional magnetization-prepared rapid acquisition gradient echo images (repetition time=8.6 milliseconds, echo time=3.8 milliseconds, 256×192 acquisition matrix, 180×1.2–mm slices) were obtained on a 1.5-T magnetic resonance system at each of the 6 centers. Quality control was undertaken using the ADNI Magphan phantom and 2 volunteers who visited each of the centers, ensuring compatibility across the study. Thickness of the ERC was calculated with Freesurfer using a cortical reconstruction technique.[22,23]

^{11}C–Pittsburgh Compound B Positron Emission Tomographic Studies

Dynamic ^{11}C–Pittsburgh Compound B (^{11}C-PiB) positron emission tomographic (PET) studies (37 time frames across 90 minutes) were acquired in 3-dimensional mode on a GE Advance scanner immediately after intravenous bolus injection of approximately 5.55×10^8 Bq (15 mCi) of ^{11}C-PiB. Dynamic images were reconstructed using filtered back projection with a ramp filter (image size, 128×128; pixel size, 2×2 mm; slice thickness, 4.25 mm), yielding a spatial resolution of about 4.5 mm full width at half maximum at the center of the field of view. Parametric images of distribution volume ratios were calculated by simultaneously fitting a reference tissue model using linear regression and spatial constraint with the cerebellum as a reference region.[24,25] The SPM5 program (Statistical Parametric Mapping 5; Wellcome Department of Imaging Neuroscience, London, England) was used to investigate the association between clusterin and medial temporal ^{11}C-PiB retention (significance threshold of $P\leq.05$, with a spatial extent of 25 voxels). Based on a priori hypotheses in light of our results on the association between ERC atrophy and clusterin concentration in AD, a restricted search of the MTL was performed using the regional definition from the WFU PickAtlas.[26]

PROTEOMICS

Two-dimensional gel electrophoresis and liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) were performed as previously described.[7] Gels were analyzed using image analysis software (either Melanie 2-D or Progenesis SameSpots version, 3.0, Nonlinear Dynamics). Protein spots of interest were excised, washed, digested in gel with trypsin, and analyzed by LC-MS-MS.[7] Mass spectral data were processed into peptide peak lists

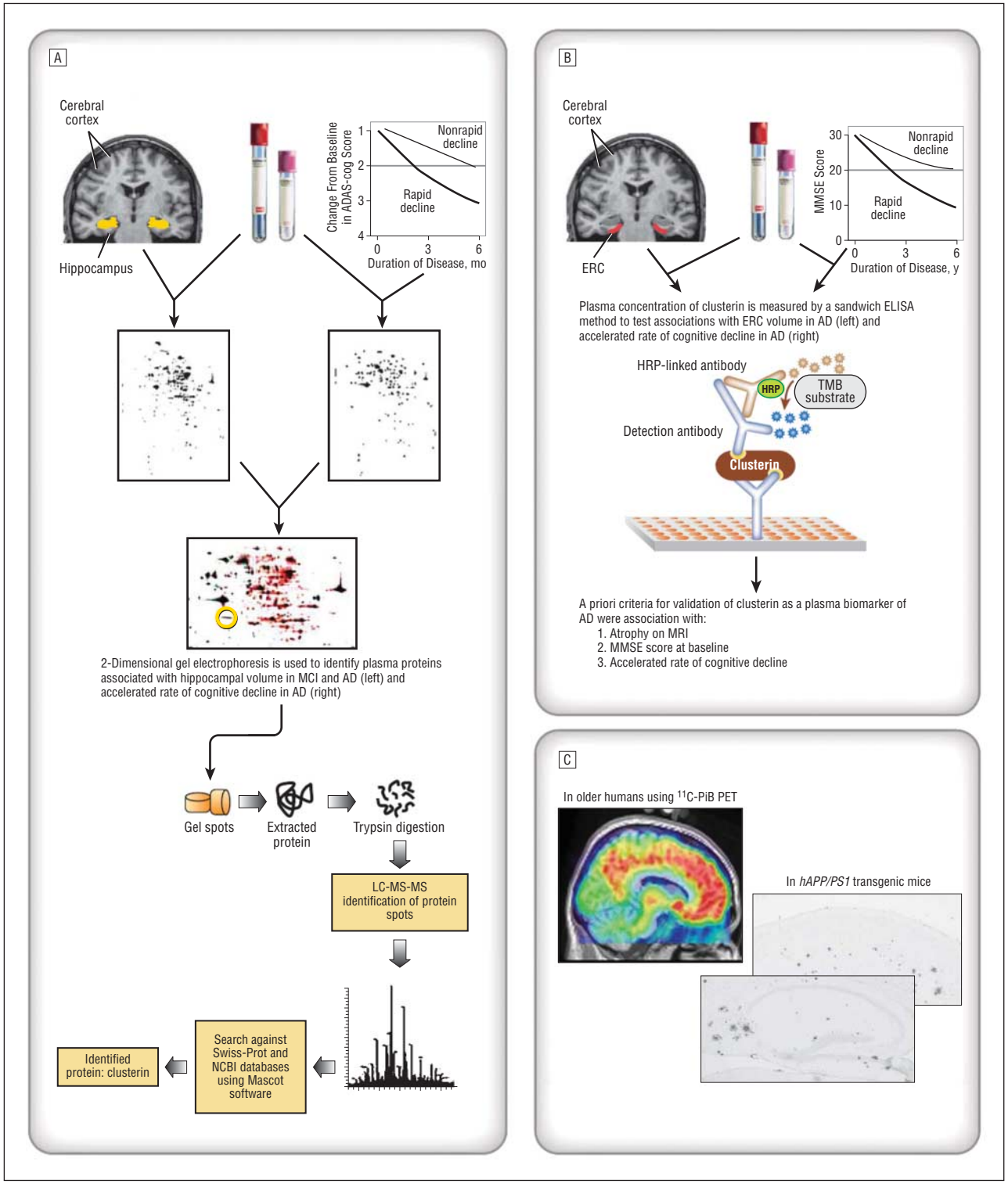


Figure 1. Study design. Schematic diagram of the design of discovery- (A) and validation- (B) phase studies for the identification of blood-based Alzheimer disease (AD) biomarkers associated with both in vivo disease pathology as well as rate of disease progression. C, Association of plasma clusterin concentration with brain amyloid burden was tested in both nondemented older humans and a transgenic mouse model of AD. ADAS-cog indicates Alzheimer Disease Assessment Scale–cognitive subscale; ELISA, enzyme-linked immunosorbent assay; ERC, entorhinal cortex; HRP, horseradish peroxidase; LC-MS-MS, liquid chromatography coupled to tandem mass spectrometry; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MRI, magnetic resonance imaging; NCBI, National Center for Biotechnology Information; TMB, tetramethylbenzidine; and ^{11}C-PiB PET, ^{11}C–Pittsburgh Compound B positron emission tomography.

and searched against the Swiss-Prot Database using Mascot software (Matrix Science, London, England).

For validation experiments, plasma clusterin concentration was assayed by a commercially available ELISA kit (Human Clusterin ELISA, RD194034200R; Biovendor Laboratory Medicine Inc, Modric, Czech Republic). Samples were run in duplicate. Coefficient of variation of the ELISA for all studies overall was 3.5% (baseline data, 3.7%; follow-up data, 3.5%; and BLSA substudy, 3.1%).

GENOMICS

Gene Expression of Clusterin

Approximately 2.5 mL of venous blood was collected into a PAXgene tube for each subject at the baseline visit, processed according to the manufacturer's instructions, and stored at –20°C overnight prior to –80°C storage. RNA was extracted using the PAXgene Blood RNA kit according to the manufacturer's instructions. Samples were assessed for yield using a spectrophotometer and quality using the RNA 6000 Pico Chip on the Agilent Bioanalyser. Samples with an RNA integrity number greater than 7.0 were used for polymerase chain reaction (PCR) assays.

Using the Quantitect Reverse Transcription kit (Qiagen), 500 ng of RNA was reverse transcribed to complementary DNA in a 40-µL reaction and subsequently diluted to 200 µL. Reverse transcriptase–PCR reactions were performed in 384-well plates in the 7900HT Fast Real-Time PCR machine (Applied Biosystems, Foster City, California). The geNORM housekeeping selection kit (Primer Design Ltd, Southampton, England) was used to assay 12 housekeeping genes in a subset of the samples. Using NormFinder software, the 2 most stable genes for normalization were determined to be *SF3A1* and *ATP5B*. Samples were assayed in duplicate, and a standard curve of known copy number was run on each plate for clusterin, *SF3A1*, and *ATP5B*. Data were nonparametric and were therefore log transformed.

Clusterin Genotyping

Tagger software (http://www.broad.mit.edu/mpg/tagger/) identified 7 single-nucleotide polymorphisms (SNPs) (rs9331908, rs11136000, rs867231, rs867230, rs9331888, rs9314349, and rs484377) that captured more than 90% of variation in the clusterin gene. Genotypes were determined using a TaqMan allele-specific assay (Applied Biosystems). The PCR amplifications were performed on an ABI PRISMR 7000 Sequence Detection System (Applied Biosystems). A total of 946 individuals (358 AD subjects, 373 controls, and 215 MCI subjects) were genotyped for the 7 SNPs.

TASTPM Transgenic Mouse Model Experiments

Heterozygote transgenic mice overexpressing hAPP695swe (*Tas10*) and presenilin 1 M146V mutations (*Tpm*) were generated as previously described.[27] Western blot analysis of clusterin was performed in plasma samples at 6 months using an anti–apolipoprotein J mouse polyclonal antibody (Abcam AB349-50; 1:5000). For immunohistochemistry, antigen retrieval was undertaken as described previously.[27,28] Primary antibodies were 1E8 (pan-Aβ), 20G10 (Aβ42; GlaxoSmithKline; 1:1000), and anticlusterin (R&D Systems goat polyclonal AF2747; 1:20 000). Images were captured at ×4 magnification on an Axioscope microscope and analyzed by Image J software to generate percentage Aβ or clusterin load. Animal experiments were conducted according to the Council of Europe guidelines.

STATISTICAL ANALYSIS

Discovery-phase proteomic data were analyzed by partial least-squares regression using SIMCA-P, version 8.0. Spot data were scaled to unit variance and $\log_{10}$ transformed where appropriate. Observations with greater than 50% missing values were excluded. Partial least-squares discriminant analysis was used to derive a panel of protein spots that discriminated between rapidly and slowly declining AD groups.

Validation-phase protein data were examined using SPSS, version 17. Covariates were chosen in cases in which such variables were significantly different between the groups of interest or in which they were likely to influence the dependent variable. To test associations between plasma clusterin concentration and ERC thickness, partial correlation analysis was performed with age and sex as covariates. In analyzing associations between MMSE score and plasma clusterin concentration, partial correlation was performed with age as a covariate. While testing differences in clusterin concentration between rapidly and nonrapidly declining AD patients, age and sex were not included as covariates because they were not significantly different between the 2 groups. However, duration of disease was significantly different between these groups (retrospective analysis) and was therefore included as a covariate in an analysis of covariance (ANCOVA) model. In the prospective analysis, there was no significant difference in disease duration between rapid and nonrapid decliners, and clusterin concentration between these groups was therefore compared using an independent samples *t* test. Linear regression adjusting for disease status, age, sex, and *APOE* ε4 status was performed to investigate the association between *CLU* SNPs and clusterin plasma levels and to examine the relationship between *CLU* messenger RNA (mRNA) and disease. Image analysis is described in the relevant sections. All other statistical analyses were performed using SPSS, version 17, and are described in the text.

RESULTS

PROTEOMIC IDENTIFICATION OF PLASMA PROTEINS ASSOCIATED WITH HIPPOCAMPAL ATROPHY AND RAPID CLINICAL PROGRESSION IN AD

To identify plasma proteins associated with disease as reflected by cerebral atrophy, we first performed a discovery-phase proteomics experiment using 2-dimensional gel electrophoresis and LC-MS-MS, with hippocampal atrophy as the independent variable. We analyzed samples from 44 subjects from the KCL-ART cohort, representing a continuum of disease (27 individuals with mild to moderate AD and 17 with MCI; eTable 1). Bivariate correlation of integrated optical densities of spots detected by 2-dimensional gel electrophoresis revealed 13 spots that were significantly associated with hippocampal volume ($r \geq \pm 0.35$, $P < .05$). Subsequently, using partial least-squares regression,[29] a method suited to analysis of proteomic data in which colinearity among predictor variables is common, a model with 2 components was fitted to the hippocampal volume data. This was constituted by 8 of the 13 spots which, together, explained 34% of the variance (R2Y, ie, explained variance in the outcome variable) in hippocampal volume. Using LC-MS-MS, we identified these 8 spots as complement C3, γ-fibrinogen, serum albumin, complement factor I, clusterin (in 2 spots), α_1-macroglobulin, and serum amyloid P (**Figure 2**). We then performed a second discovery-phase experiment in an independent set of samples in 51 carefully matched (for age, sex, severity at the time of blood sampling, and cholinesterase inhibitor treatment [all were taking the drug]) AD subjects from the AddNeuroMed cohort who we could divide into fast (n=22) or slow (n=29) progressors based on their annualized rate of cognitive decline (eTable 1). We defined a priori fast de-

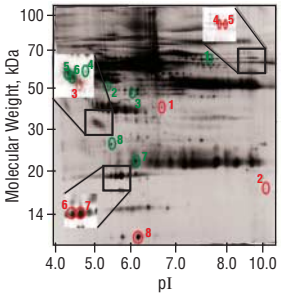
cline as a fall of 2 or more points on the ADAS-cog scale during 6 months. A partial least-squares discriminant analysis model distinguishing the rapidly from the slowly progressing AD groups was constituted by the integrated optical densities of 27 silver-stained 2-dimensional gel electrophoresis spots. Of these, 8 were well defined, discrete, and present in all 51 gels and were identified by LC-MS-MS. These spots contained complement component C4 (in 3 spots), complement C8, clusterin, apolipoprotein A1 (in 2 spots), and transthyretin (Figure 2).

CLUSTERIN AND ATROPHY OF THE ERC, SEVERITY OF COGNITIVE IMPAIRMENT, AND SPEED OF PROGRESSION IN AD

Only 1 protein was common to both discovery-phase studies: clusterin. We therefore sought to confirm this finding in a large cohort of 689 subjects, including 344 from the AddNeuroMed study (119 with AD, 115 with MCI, and 110 controls) and 345 (all with AD) from the KCL-ART cohort (eTable 2). We used atrophy in the ERC as an alternative measure of disease pathology (Figure 1). The 689 validation-phase subjects included the 95 subjects in the discovery phase albeit with entirely different analytical measures in the 2 studies.

Confirming the discovery-phase study, we observed a trend toward association between clusterin concentration and ERC atrophy in the combined AD and MCI cohort (n=219; $R=-0.12$, $P=.06$) after covarying for age and sex. This relationship was driven primarily by a highly significant association between ERC atrophy and clusterin concentration in AD patients (n=113; $R=-0.30$, $P=.001$). We also correlated plasma clusterin concentration with MMSE score—a measure of cognition available in 576 subjects with MCI and AD—and again found a highly significant negative correlation ($r=-0.22$; $P<.001$, age as a covariate).

We then compared clusterin levels in rapidly declining AD patients relative to slow decliners using both retrospective and prospective measures of decline relative to the time of blood sampling (Figure 1 and eTable 2). Retrospective decline was estimated from the duration of disease and the MMSE score at the point of blood sampling, allowing the annualized fall in MMSE score to be calculated. We used MMSE score, as the ADAS-cog score was not available in all subjects, and defined fast decline as a fall of 2 points or more during a 1-year period relative to the time of blood sampling. Prospective decline was directly measured as the fall in MMSE score 1 year after blood sampling. We observed a significant increase in clusterin concentration in AD patients with accelerated cognitive decline prior to blood sampling (n=344; ANCOVA, $t_{341}=3.40$, $P<.001$, duration of disease as covariate) (**Figure 3**A) and an increase in clusterin concentration in AD patients with faster cognitive decline subsequent to blood sampling (n=237; independent samples t test, $P=.01$) (Figure 3B). Cox proportional regression analysis showed that higher plasma clusterin concentration was associated with a greater risk of rapid cognitive decline 1 year after blood sampling (Figure 3C). We then performed an analysis of variance (age and sex as covariates) between AD, MCI, and control groups in the entire sample to test for differences in plasma clusterin concentration. There were no significant differences: AD, 82.4 ng/mL (SD, 25.6 ng/mL; n=336); MCI, 77.6 ng/mL (SD, 22.5 ng/mL; n=222); and control subjects, 82.2 ng/mL (SD, 23.8 ng/mL; n=385). Finally, we compared differences in plasma clusterin concentration between *APOE* ε4 carriers and noncarriers (independent samples t test) in the combined cohort of AD, MCI, and control subjects and did not find any significant difference.



Protein	Accession No.
1, Complement C3	P01024
2, γ-Fibrinogen	P02679
3, Serum albumin	P02768
4, Complement factor I	P05156
5, Clusterin	P10909
6, Clusterin	P10909
7, Serum amyloid P	P02743
8, α_1-Macroglobulin	P02760

Protein	Accession No.
1, Complement C4a	P0C0L4
2, Complement component C8	P07360
3, Clusterin	P10909
4, Complement C4a	P0C0L4
5, Complement C4a	P0C0L4
6, Apolipoprotein A1	P02647
7, Apolipoprotein A1	P02647
8, Transthyretin	P02766

Figure 2. Gel-based proteomic discovery-phase studies. Proteomic identification of plasma proteins associated with hippocampal volume in subjects with Alzheimer disease (AD) and mild cognitive impairment (MCI) and those associated with fast AD progression (bottom panel). A representative 2-dimensional gel electrophoresis gel is shown with spots outlined in green denoting proteins associated with hippocampal volume in AD and MCI and those in red highlighting proteins associated with fast AD progression.

CLUSTERIN AND FIBRILLAR Aβ BURDEN IN THE ERC IN NONDEMENTED OLDER INDIVIDUALS

Because high clusterin levels are associated with brain atrophy and a more rapid rate of cognitive decline in AD patients, we hypothesized that increased clusterin concentration might be an antecedent marker of pathology in otherwise normal older individuals. We tested this hypothesis in participants of the Baltimore Longitudinal Study of Aging who had stored samples of plasma and underwent PET imaging of fibrillar Aβ burden with ^{11}C-PiB (n=60; eTable 3). Although all participants were nondemented at the time of the PiB-PET study, a range of in vivo amyloid burden is observed in cognitively normal individuals[30] and increased amyloid deposition may represent the earliest phase of AD pathology in these subjects. Measuring plasma clusterin concentration from samples collected 10 years before the PiB-PET studies, we investigated associations between clusterin concentration and subsequent development of in vivo fibrillar amyloid burden.

We conducted a directed search of significant associations between clusterin and MTL PiB values using the MTL region defined by the WFU PickAtlas[26] and the SPM5 multiple regression module, adjusting for age and sex. These results indicated that higher antecedent clusterin concentrations were associated with greater PiB retention in bilateral ERC; it was higher on the right (right ERC, $P=.009$; and left ERC, $P=.03$) (**Figure 4**A). This suggests that

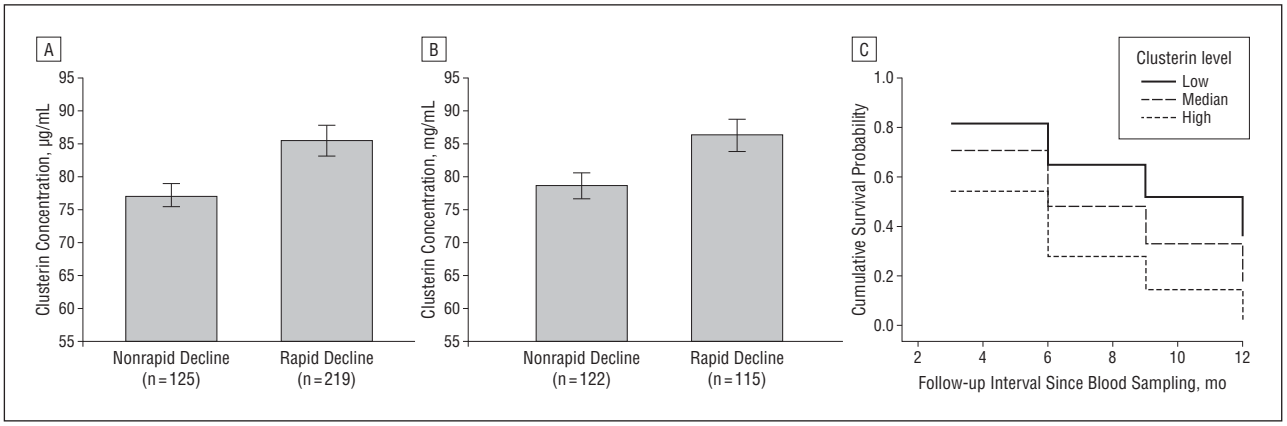


Figure 3. Increased concentration of plasma clusterin and rate of clinical progression in Alzheimer disease (AD). Patients with AD with a rapid progression rate, measured prior to blood sampling (A) and 1 year after blood sampling (B) have significantly increased clusterin concentration relative to slow progressors. C, High levels of clusterin are associated with a significantly greater risk of accelerated cognitive decline subsequent to blood sampling. Patients with AD (n=204) were assigned a prognostic index derived as their plasma clusterin concentration multiplied by its corresponding regression coefficient (β) in a Cox proportional regression analysis. C, Cumulative hazard functions for the effect of the prognostic factor (ie, plasma clusterin concentration) on the survival probability, ie, maintaining a nonaggressive clinical course (decline in Mini-Mental State Examination score ≤2 points per year). The cumulative survival functions represent estimated survival probabilities for 3 representative AD patients with the lowest (5.87 ng/mL), median (76.84 ng/mL), and highest plasma clusterin (159 ng/mL) concentrations showing that an AD patient with the highest clusterin concentration has the lowest probability of maintaining a nonaggressive clinical course 1 year after sampling. The reported hazard ratio for a 10-ng/mL rise in plasma clusterin concentration for risk of becoming a rapid AD decliner was 1.071 (95% confidence interval; 1-1.147; $P=.05$).

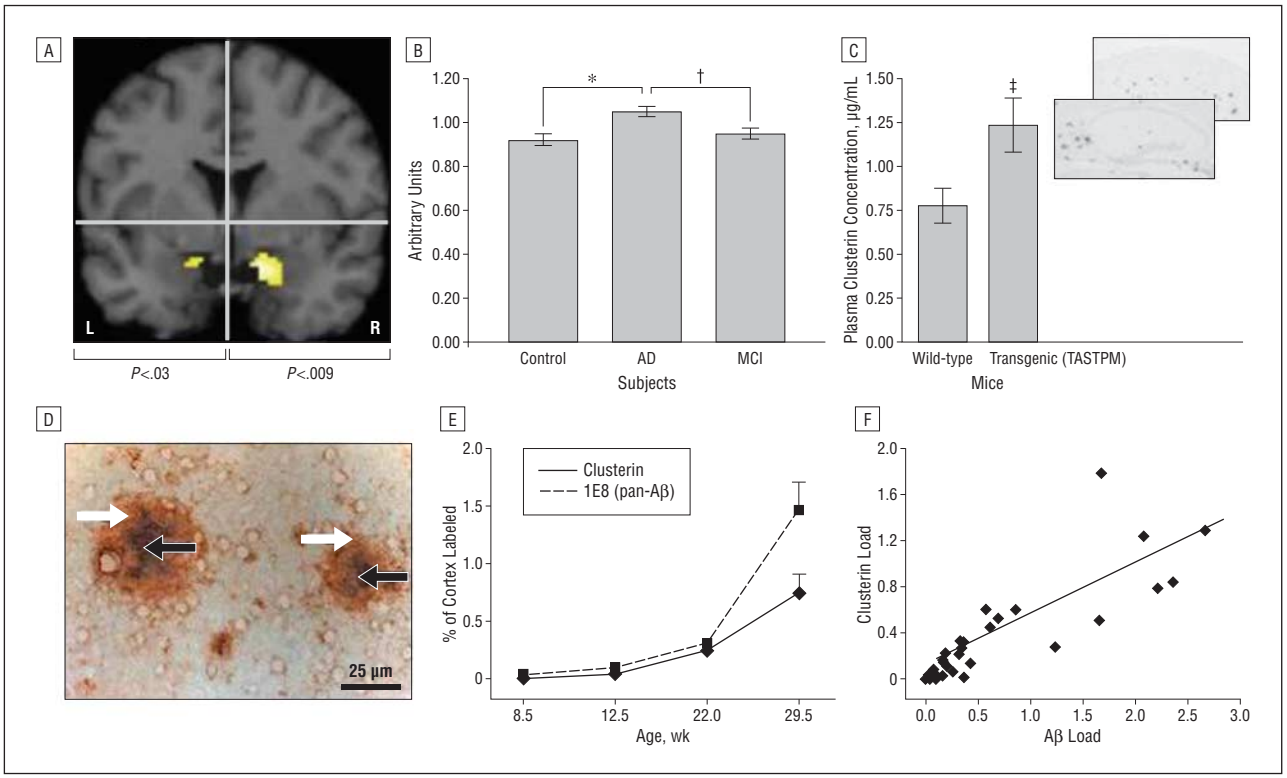


Figure 4. Clusterin expression and amyloid pathology. A, Clusterin is an antecedent biomarker of in vivo fibrillar amyloid-β (Aβ) burden in the entorhinal cortex in nondemented older individuals (n=60). SPM analysis shows correlation between plasma clusterin concentration and ^{11}C–Pittsburgh Compound B (^{11}C-PiB) uptake controlling for age and sex ($P<.05$, uncorrected). Highlighted areas denote regions in the entorhinal cortex of both hemispheres that show significant association with plasma clusterin concentration 10 years prior to the PiB positron emission tomographic scans. B, Gene expression of clusterin is altered in Alzheimer disease (AD). Clusterin messenger RNA levels are significantly elevated in blood cells from AD patients (n=182) relative to healthy controls (n=179, *$P<.001$) and subjects with mild cognitive impairment (MCI) (n=207, †$P=.008$) after correcting for age. C, Transgenic TASTPM mice (n=10) overexpressing both human *APP* and *PS1* genes have significantly higher plasma concentration of clusterin relative to wild-type litter mates (n=10) at 6 months of age ($P=.02$). Inset shows hippocampal and cortical amyloid plaques in a 6-month-old TASTPM mouse stained by a monoclonal antibody against Aβ1-42. Wild-type mice show no amyloid pathology at this age (not shown). ‡Statistically significant. D, Representative photomicrograph of cortical amyloid plaques in a 6-month-old TASTPM mouse. A close association is observed between Aβ within amyloid plaques (black arrows indicate monoclonal antibody to Aβ42; gray-black labeling, diaminobenzidine) and clusterin (white arrows indicate polyclonal antibody; brown-labeled with Novared). Colors have been slightly enhanced digitally for illustrative purposes. E, TASTPM mice show age-dependent increases in cortical Aβ and clusterin load as determined by quantitative image analysis of immunohistochemical labeling. F, TASTPM mice demonstrate a highly significant ($P<.001$) correlation between Aβ and clusterin load (n=39, male and female mice, 8-30 weeks of age). B, C, and E, Error bars indicate standard error.

increased plasma concentration of clusterin, even in nondemented older individuals, predicts a greater extent of fibrillar amyloid burden in the ERC, the same region where we have also demonstrated robust association with atrophy in subjects with MCI and AD.

GENE EXPRESSION OF CLUSTERIN IS ALTERED IN AD

To investigate the mechanisms underlying the associations between plasma concentration of clusterin and both imaging measures of atrophy and accelerated clinical progression, we measured clusterin mRNA levels in blood cells from AD patients (n=182), MCI subjects (n=179), and controls (n=207) (eTable 4). Diagnosis had a significant effect on clusterin gene expression (ANCOVA, $df=2$, $P<.001$, age as a covariate). Pairwise comparisons between the 3 groups showed significantly higher clusterin gene expression in AD patients than in MCI and control subjects ($P=.008$ and $P<.001$, respectively, Bonferroni adjustment for multiple comparisons) (Figure 4B). Sex and presence of the *APOE* ε4 allele did not have a significant effect on clusterin mRNA levels. We did not observe a significant association between clusterin mRNA in blood cells and plasma concentration of clusterin protein nor did we find a correlation between plasma mRNA levels and either MMSE score or rate of decline in MMSE score within groups or with atrophy on neuroimaging.

LACK OF EFFECT OF VARIATION IN THE CLUSTERIN GENE ON PERIPHERAL CLUSTERIN EXPRESSION

We did not observe significant effects of the 7 clusterin gene SNPs on either clusterin mRNA expression in blood cells or plasma concentration of clusterin (eTable 5 and eTable 6). The SNPs analyzed included those reported on in the recent large Genome-Wide Association Studies to be associated with risk of sporadic AD.[31,32]

PLASMA CONCENTRATION OF CLUSTERIN IN TRANSGENIC MICE WITH PLAQUE PATHOLOGY

To extend our findings on the association of clusterin with brain amyloid deposition, we examined its plasma concentration in a transgenic mouse model of AD. TASTPM mice overexpress the hAPP695swe and presenilin 1 M146V mutations, resulting in overproduction of human amyloid precursor protein,[27] and mimic various hallmarks of AD including amyloid plaques as well as cognitive and behavioral deficits.[27,28] In light of our magnetic resonance imaging data in AD patients and PiB-PET results in nondemented older individuals, we hypothesized that plasma clusterin concentration in transgenic TASTPM mice would be higher than wild-type controls. As predicted, we observed a significantly greater plasma concentration of clusterin ($P=.02$, independent samples *t* test) in 6-month-old transgenic TASTPM mice (n=10) relative to wild-type litter mates (n=10) (Figure 4C). Previous studies have established both marked cerebral Aβ deposits as well as cognitive deficits in TASTPM mice at this age relative to wild-type litter mates.[27,28]

BRAIN CLUSTERIN AND AMYLOID IN A TRANSGENIC MOUSE MODEL OF AD

Using double-labeling immunohistochemistry, we demonstrated that cortical plaques in TASTPM mice contained both Aβ and clusterin (Figure 4D). Finally, we established the close association between Aβ and clusterin by showing that both cortical Aβ burden and clusterin deposition increase with age in TASTPM mice (n=9-11) (Figure 4E) and that there is a highly significant correlation ($F_{1,37}=107.57$, $P<.001$, adjusted $R^2=0.737$) between cortical Aβ and clusterin load (Figure 4F).

COMMENT

We have combined a novel proteomic and neuroimaging approach to establish that plasma concentration of clusterin is associated with in vivo pathology, disease severity, and clinical progression in patients with AD. The primary outcomes in our discovery-phase studies were association with both atrophy of the MTL and the rate of progression of cognitive decline. In the discovery phase, we used hippocampal atrophy derived from manual tracing of the hippocampal formation from magnetic resonance imaging, and in the much larger validation phase, from automated regional analysis of the ERC, an adjacent region of the MTL and the site of earliest pathology in AD.

Hippocampal atrophy is an early event in the pathogenesis of AD, is associated with an increased risk of conversion from MCI to AD, and may even precede the development of cognitive decline.[33,34] Cerebrospinal fluid levels of phosphorylated tau correlate with hippocampal volume, indicating that this measure reflects an integral feature of AD pathology.[35] Moreover, decreased hippocampal volume in AD patients is associated with neuronal loss, confirming its validity as a marker of neurodegeneration.[35] A second independent outcome variable in the discovery-phase studies was rate of cognitive decline, derived as a measure of decrease in the ADAS-cog scores during a 6-month interval in AD patients. Using this measure, we dichotomized AD patients as fast and slow decliners, an approach previously shown to predict long-term prognosis in AD.[36]

Only clusterin was associated both with hippocampal atrophy in AD and MCI subjects and with fast progressing, or more aggressive, AD. Evidence from human cerebrospinal fluid, postmortem brain, and transgenic animal models suggests a plausible link between clusterin and AD pathology.[37-40] We therefore sought to confirm the association of clusterin with AD pathology, severity, and progression in a much larger validation-phase study.

We confirmed highly significant associations of plasma clusterin concentration with atrophy of the ERC ($P=.001$), MMSE score ($P<.001$), and rate of progression in AD ($P<.001$). We also demonstrated a significantly greater risk of subsequent accelerated cognitive decline associated with increased concentration of clusterin in patients with AD and, in normal individuals, with subse-

quent deposition of fibrillar Aβ in the ERC. Our finding of raised plasma clusterin concentration 10 years before fibrillar Aβ deposition in the brain in normal elderly individuals suggests that clusterin is raised very early, possibly as an etiopathological event, and is not simply a reaction to other pathology in AD. The observation that clusterin mRNA is significantly increased in blood cells in AD suggests that the observed changes in protein levels reflect changes in expression in disease and not, for example, altered turnover. However, the increase in clusterin mRNA in AD patients does not correlate directly with plasma clusterin concentration, suggesting that the primary sources of plasma clusterin that we find predictive of more aggressive disease are organs other than blood cells such as the liver or possibly even the brain. In the course of this study, 2 groups, including one in which we participated, reported from genome-wide studies that polymorphic variation in *CLU*, which encodes clusterin, was associated with AD.[31,32] One possible mechanism for this association would be for the SNPs associated with disease to be modifiers of gene expression. To investigate this, we determined the effect of variations in the clusterin gene on both peripheral mRNA levels and plasma concentration of clusterin protein, including the principal variant associated with disease and 6 other SNPs determined to cover most of the variation in the gene. We did not find significant effects of these SNPs on either peripheral mRNA levels or plasma clusterin concentration, suggesting that our observed association of clusterin protein and mRNA with AD-related pathological processes is independent of genetic variation in the clusterin gene. Our findings raise the possibility of 2 possibly linked mechanisms whereby both altered expression and some other factor in the gene linked to the disease-associated SNPs are active in moderating disease pathology. However, we cannot exclude an effect of genetic variation not examined in this study on clusterin expression or a small effect of *CLU* variation, below the power of our study to detect, on expression. Nonetheless, the finding of association with both genetic variants and, as we now report, gene and protein expression adds considerable weight to the importance of clusterin to AD pathogenesis. It is interesting that we observe clusterin in 2 closely related but distinct spots in the discovery-phase 2-dimensional gel electrophoresis studies. Proteins are components of multiple spots on 2-dimensional gel electrophoresis because of changes in posttranslational modification, complex formation, and splicing changes resulting in different isoforms. It is possible that some of these variations might be associated with disease processes in addition to the overall amount of protein as measured in the validation-phase study. Finally, we confirmed a previous report of significantly higher plasma concentration of clusterin in TASTPM mice overexpressing *APP/PS1* mutations,[41] and we also show that clusterin is closely associated with cortical amyloid plaques, showing an age-dependent concomitant increase with brain amyloid burden.

Previous studies suggest that clusterin belongs to a family of extracellular chaperones that regulate amyloid formation and clearance.[42] In vitro experiments show that clusterin regulates amyloid formation in a biphasic manner with low clusterin to substrate ratios enhancing and higher ratios inhibiting amyloid formation, respectively.[43] In mice, in vivo binding of Aβ to clusterin enhances its clearance and efflux through the blood-brain barrier.[44] However, previous studies reporting differences in cerebrospinal fluid clusterin concentration between AD patients and controls have been inconclusive.[39,40] Our findings may have implications for the discovery and characterization of other amyloid chaperone proteins in blood linked to AD pathogenesis. In this context, α_2-macroglobulin has recently been characterized as an amyloid chaperone that inhibits fibril formation.[45,46] In a previous proteomic analysis of plasma, we reported the differential expression of α_2-macroglobulin in AD patients and have also found associations between the plasma concentration of α_2-macroglobulin and hippocampal metabolite abnormalities in AD.[7,47] In this previous study,[7] in addition to α_2-macroglobulin, we also identified components of the complement pathway associated with AD. In the discovery phase of the current study, we note many of the same proteins and also that clusterin may itself play a role in complement activation, suggesting that further examination of this pathway may be useful to identify markers associated with AD.[7]

In summary, we have used a novel proteomic-neuroimaging discovery paradigm in which the primary end points were well-established measures of pathology in the MTL and rate of disease progression. We identified clusterin as a plasma protein associated with disease pathology, severity, and progression in AD. Although these findings do not support the clinical utility of plasma clusterin concentration as a stand-alone biomarker for AD, they reveal a robust peripheral signature of this amyloid chaperone protein that is responsive to key features of disease pathology. Our findings clearly implicate clusterin, but there may well be other proteins in plasma related to the disease process, and indeed our previous studies and those of others suggest this is the case. These results may have wider implications for the identification of other amyloid chaperone proteins in plasma, both as putative AD biomarkers as well as drug targets of disease-modifying treatments.

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